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Biofuels and Biodiesel

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Biofuels and Biodiesel

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Dedication

This book is dedicated to my father (Baba) Professor C.R. Basu

Preface

The world population is 7.8 billion and has been increasing significantly. It is undeniable that the energy demand will be significantly higher than the supply due to increased population density and the high need for energy. Nonrenewable fossil fuels are a principal source of anthropogenic (human-made) greenhouse gases. Biofuel is a type of fuel obtained from plant, animal, or algal biomass. Biodiesel refers specifically to diesel fuel synthesized through the transesterification of oils. Biofuel and biodiesel are viable alternatives to petroleum-based fuels.

Biofuels are sustainable and can lessen the level of greenhouse gases. This book explores a wide range of topics in the fields of principle, production, molecular aspects, and sustainability of biofuel and biodiesel. A total of 20 chapters are divided into four sections, namely, (1) Biofuel Production, (2) Biodiesel Production, (3) Molecular Genetics and Biotechnology of Biofuel and Biodiesel Production, and (4) Economics and Sustainability of Biofuel Production. The first section (Biofuel Production) contains chapters discussing biobutanol production, biomass pretreatment, algal biofuel, life cycle assessment of biofuel, and quantification of biofuels and other fermentation metabolites. The second section (Biodiesel Production) contains chapters on the topics of the biodiesel production process, biodiesel production from sorghum, and screening oil seeds for biodiesel production. The third section of the book (Molecular Genetics and Biotechnology of Biofuel and Biodiesel Production) is a collection of chapters covering various molecular aspects of biofuel and biodiesel production, including RNAi-based gene silencing in sugarcane for biofuel production, the molecular diversity of biofuel crops, genetic engineering of *Trichoderma* spp. for production of plant cell wall-degrading enzymes for biofuels production, isolation and PCR amplification of DNA from cellulolytic bacteria, production of steryl glucosidase enzymes in microorganisms for biodiesel production, production of biocatalyst enzyme lipase in yeast cells for biofuel production, use of artificial small RNAs for gene regulation in cyanobacteria, use of sorghum as a biofuel crop, and genetic transformation of biofuel tree *Paulownia elongata*. The final section in the book (Economics and Sustainability of Biofuel Production) contains two chapters on the sustainability of sorghum and pearl millet for biofuel production and biofuels and sustainability issues. Due to the diverse topics covered in this book, the book will be useful for researchers, students, and enthusiasts in the field of biofuel and biodiesel. This book is a true international collaborative project. Researchers from Nigeria, India, the USA, Brazil, Austria, China, Argentina, France, Zimbabwe, The Netherlands, Thailand, Singapore, and Egypt contributed to this book. I want to thank all contributors for their valuable contributions wholeheartedly.

Los Angeles, CA ***Chhandak Basu***

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Part I

Biofuel Production



Chapter 1

Overview of Current Developments in Biobutanol Production Methods and Future Perspectives

J. Iyyappan, B. Bharathiraja, A. Vaishnavi, and S. Prathiba

Abstract

Renewable biobutanol production is receiving more attention toward substituting fossil-based nonrenewable fuels. Biobutanol is recognized as the top most biofuel with extraordinary properties as compared with gasoline. The demand for biobutanol production is increasing enormously due to application in various industries as chemical substituent. Biobutanol production technology has attracted many researchers toward implementation of replacing cost-effective substrate and easy method to recover from the fermentation broth. Sugarcane bagasse, algal biomass, crude glycerol, and lignocellulosic biomass are potential cost-effective substrates which could replace consistent glucose-based substrates. The advantages and limitations of these substrates have been discussed in this chapter. Moreover, finding the integrated biobutanol recovery methods is an important factor parameter in production of biobutanol. This chapter also concentrated on possibilities and drawbacks of obtainable integrated biobutanol recovery methods. Thus, successful process involving cost-effective substrate and biobutanol recovery methods could help to implementation of biobutanol production industry. Overall, this chapter has endeavored to increase the viability of industrial production of biobutanol.

Key words Biobutanol, Microorganisms, Substrates, Cofermentation, Product recovery

1 Introduction

Recently, biofuel production through fermentation has received greater attention due to world environment problems and exhaustion of nonrenewable fossil-based fuels. Biofuels generally belong to renewable resources and have the capacity to compete with fossil-based fuels in terms of efficiency and compatibility [1]. At present, world energy consumption particularly transportation is mainly dependent on the nonrenewable fossil-based fuels and instantaneously it is estimated that energy consumption will get increased with 60% of growth in the year of 2030 [2]. Enormous demand in the energy sector cannot be fulfilled by limited source of fossil-based fuels. Consequently, biobased fuels could satisfy the growing need of energy supply. Among the various biofuels,

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biobutanol is recognized as the top most biofuel with extraordinary properties as compared with gasoline. Importantly, biobutanol can be used in various sectors like food industry and pharmaceutical and chemical industry [3].

Acetone–butanol–ethanol (ABE) fermentation is the major route for the production of biobutanol through fermentation using microorganisms. At present, industrial production of butanol is accomplished by chemical synthesis using petrochemically derived propylene as raw material. Hydroformylation of propylene results in the formation of butyraldehyde and n-butanol is produced by the reaction of hydrogenation of butyraldehyde [3]. Many researchers and industrialist are currently focusing on the biobased production of butanol using cost-effective substrates. Since, employment of petrochemically derived raw materials leads to environment-related problems. Thus, wide opportunity exists for the production of biobutanol using cost-effective substrates. This chapter aims to discuss about current technologies available on the proper selection of substrates and process conditions for developments in biobutanol production.

2 Physio-Chemical Properties of Butanol

Butanol is a four-carbon alcohol and having the molecular formula of C_4H_9OH. The boiling point of butanol is 118 °C, which is higher than the boiling point of ethanol 78 °C. Butanol is less corrosive in nature and less evaporative when compared with ethanol [4, 5]. Particularly, butanol is having low volatility and high energy content. The ability of butanol to adsorb the moisture is very less [6–8]. The air fuel ratio of butanol is 11.2 and butanol can be blended with petrol with increased ratio when compared with ethanol. Butanol has the energy content which is very close to fossil-based fuel of petrol [5, 9]. The oxygen content present in the butanol is about 21.5% and the flash point of butanol is 35 °C. The specific heat and viscosity of butanol at 20 °C are 2.63 kJ/kg. K and 3.6 CP, respectively. Further, the freezing point and latent heat of butanol are about −88.9 °C and 582 kJ/kg. The solubility of butanol in water at 25 °C is about 73 g/L [10–13]. Importantly, butanol can be used alone in our current engines systems without any modifications of the engine systems and can also be blended with other fuels like gasoline, hydrogen, methanol, ethanol, and isopropanol [14]. It ensures that butanol has top most superior position among the various alternative renewable fuels.

3 Methods of Butanol Production

Butanol can be produced by using both chemical synthesis and biochemical synthesis. The growing demand for butanol leads to make increasing attention toward achievement of butanol production by means of both chemical route and biological route [3]. Butanol production through fermentation still has many disadvantages over chemical method of butanol production. In order to implementation of biobutanol production, the challenges behind the process technologies have to be rectified in future. Searching competent microorganism, alternative low cost substrates and less effort of the production methods has to be concentrated more on the production of butanol using fermentation. When compared with the biological route for the production of butanol, chemical route has simplest method by involving single step [3].

3.1 Chemical Synthesis

Ethanol is the feedstock for the production of butanol using chemical method. Dehydrogenation, aldol condensation, and hydrogenation are the three sequential reactions involved in the production of butanol from ethanol [15, 16]. Initially, ethanol is converted into acetaldehydes by hydrogen removal phenomenon due to dehydrogenation. This step is an industrially important process since dehydrogenation of ethanol could lead to produce variety of petrochemicals and chemicals such as ethyl acetate. The next step is conversion of acetaldehydes into β-hydroxyketone or β-hydroxyaldehyde through aldol condensation reaction. Carbonyl compound reacts with the enolate or enol compounds and results in the carbon-carbon bond formation. The final step is hydrogenation resulting in the formation of butanol. Catalyst involvement is an essential stage for the chemical synthesis of butanol. Recently, researchers are concentrating on the development of catalyst system for the production of butanol by using chemical methods. Mg and Al mixed oxides [17], hydroxyapatite [18], and MgO [16] are some of the catalysts used for the production of butanol from ethanol. The direct conversion of ethanol from butanol has many benefits over biological production of butanol. Even though currently industrial production of butanol is well established by chemical rout, handling of chemicals could lead to arise environmental-related issues.

3.2 Biochemical Synthesis

Biochemical synthesis of butanol contains several steps. Anaerobic fermentation of carbohydrates by *Clostridia* species is well documented [19, 20]. The formation of biobutanol mainly involves Acetone Butanol Ethanol (ABE) fermentation. There are two stages in ABE fermentation like acidogenesis and solventogenesis. Initially, Embden–Meyerhof pathway (EMP) results in the

degradation of carbohydrate into pyruvate. At this stage, two moles of pyruvate are generated from one mole of carbohydrate. Further, the generated pyruvate is converted into CO_2 and acetyl-CoA. Further, acetyl-CoA is converted into acetoacetate, butyryl coA, and acetaldehyde. Butanol is formed by consecutive reactions of butyryl coA into butyraldehyde. During the acidogenesis, organic acids are formed and require the condition of pH > 5 in the fermentation medium. At the end of acidogenesis, formed organic acids are converted into solvents such as butanol, acetone, and ethanol, and it is known as solventogenesis [19, 20]. The key enzymes involved in the formation of butanol due to ABE fermentations are alcohol dehydrogenase, CoA–acylating aldehyde dehydrogenase, 2-ketoacid decarboxylase, pyruvate dehydrogenase, butyryl–CoA dehydrogenase, and crotonase [21].

4 Butanol-Producing Microorganisms

Biological production of butanol is specific to several *Clostridia* species involving *Clostridium acetobutylicum*, *Clostridium saccharobutylicum*, *Clostridium thermocellum*, *Clostridium beijerinckii*, *Clostridium saccharoperbutylacetonicum*, *Clostridium aurantibutyricum*, *Clostridium pasteurianum*, *Clostridium sporogenes*, and *Clostridium cadaveris*. Among these, *Clostridium acetobutylicum* is considered as a vital species for biobutanol production. Table 1 summarizes the important microorganism used for biobutanol production.

Clostridium acetobutylicum is a heterofermentative, rod-shaped, spore-forming gram-positive bacterium, nonpathogenic species, and mostly anaerobic in nature [22]. *Clostridium acetobutylicum* has been used for large-scale production of solvents from starch-related substrates. During the stationary phase of the organism, organic acids are completely converted into organic solvents. Usually, *C. acetobutylicum* involves two phases comprising acidogenic and solventogenic phases. *C. acetobutylicum* produced about 2.7 g/L of butanol [23]. During the exponential phase acidogenic part takes place, where acid-forming pathways get activated. In this phase pH reduced to 5. During solventogenic phases, organic acid gets converted into acetone, butanol, and ethanol.

Clostridium beijerinckii has the capacity to utilize sugars like hexose and pentose [24]. *C. beijerinckii* is a mesophilic, saccharolytic, and rod-shaped bacteria. It possesses flagella for its locomotion. *C. beijerinckii* has the ability in fermenting starch. During the growth cycle, the morphology changes slightly. Solventogenesis and endospore formation has been restricted by the final products [25].

Table 1
List of microorganisms used for biobutanol production

S. No	Microorganism	Reference
1.	*Clostridium acetobutylicum*	[23]
2.	*Clostridium beijerinckii*	[24]
3.	*Escherichia coli*	[26]
4.	*Cyanobacteria*	[28]
5.	*Clostridium tyrobutyricum*	[33]
6.	*Klebsiella pneumoniae*	[29]
7.	*Saccharomyces cerevisiae*	[31]
8.	*Pseudomonas putida*	[35]

Altering the genetic material of a particular microorganism by a natural recombination is called genetically modified organisms. The mutant generated plays an essential role in butanol production. Apart from *Clostridial* species, several other mutants play a vital role in biobutanol production. *Escherichia coli* is a predominant anaerobic gram-negative, rod-shaped bacteria with lateral flagella. In addition to flagella, the proteinaceous structure called pili is also produced for attachment. The outer membrane of the cell is superiorly made of lipopolysaccharide layer. *E. coli* can be generally retrieved from specimens on selective media. High production of biobutanol is accomplished by *E. coli* [26]. *E coli* can be used for aerobic and anaerobic fermentation of butanol. Generally, *E. coli* has the doubling time of around 20 min.

The biofuel can be obtained by *Cyanobacteria* organisms which is a free-living photosynthetic bacterium. Isobutyraldehyde, isobutanol, and ethylene can be produced with the help of cyanobacteria. *Cyanobacteria* is also known as cyanophyta. Cyanobacteria need not require expensive nutrients. *Cyanobacteria* cells can be grown fast when compared to other cells. *Cyanobacteria* are easily compliant [27]. Cyanobacteria involves in the transformation of carbon-di-oxide to biofuels. Cyanobacteria are unicellular. Cyanobacteria are commonly called as Blue green algae. Cyanobacteria have the ability to consume carbon dioxide and solar energy. Butanol-producing efficiency can be promoted by Co-A dependent biochemical pathway involved in cyanobacteria. The recent advances in genomic sequences ensure cyanobacteria to be involved in metabolic engineering [28]. *Klebsiella pneumoniae* had been employed for the biobutanol production and it is the gram-negative, rod--shaped bacterium. *Klebsiella pneumoniae* consumes carbon source when it is grown on glycerol substrate [29]. *Klebsiella pneumoniae* produced about 1030 mg/L of butanol. Glycerol can be metabolized by *Klebsiella pneumonia* [30].

Yeasts like *Saccharomyces cerevisiae* have been widely used for the production of biobutanol. *Saccharomyces cerevisiae* can be genetically manipulated to obtain metabolites; *S. cerevisiae* is a unicellular organism. *Saccharomyces cerevisiae* is notably used in food industries. *Saccharomyces cerevisiae* can withstand high concentrations of n-butanol [31]. *Saccharomyces cerevisiae* produced about 2.1 mg\L of butanol [32]. Genetically engineered *Saccharomyces cerevisiae* could become a prominent microorganism in producing biobutanol.

Clostridium tyrobutyricum is an anaerobic, gram-positive, spore-forming and rod-shaped bacteria. The butanol production ability is more due to the absence of acetone-forming genes. Biosynthesis pathway of *C. tyrobutyricum* is not related with autolysis of cell and its spores [33]. *Clostridium tyrobutyricum* produced biobutanol with the yield of around 4.645 mg/L [34]. Apart from these species, *Pseudomonas* plays a major role in butanol production. *Pseudomonas* is a gram-negative bacterium. Pseudomonas comes under the family of *Pseudomonadaceae*. *Pseudomonas putida* can stand up to high butanol concentrations [35]. *Pseudomonas putida* is extensively used in bioremediation sites. Terminal oxidation pathway was primarily followed by *Pseudomonas butanovora* [36].

Recently, genetically engineered microalgae are used extensively in biofuel production. In saline water, various microalgal can be grown, which in turn produces biodiesel involving methane, ethanol, and butanol. Microalgal are independent of the fertility of the soil. Microalgal has the tremendous capacity in absorbing carbon dioxide from atmosphere [37]. Microalgal growth rate is considerably high and it consumes wastewater for efficient biofuel production. Microalgae are unicellular in nature. Microalgal requires simple nutrients for its growth. Greater lipid productivity is observed in microalgae. Proteins, lipids, and carbohydrates are the major constituents of microalgae. Microalgal is eminently used as an antioxidant. Microalgal can be commercially used in pharmaceutical and food industries due to the presence of small proportion of chlorophyll, carotenoids [38].

5 Methods for Selection of Potential Substrates for Biobutanol Production

Selection of cost-effective substrate for the production of biobutanol is essential in order to implementation of biobutanol-producing industry. Figure 1 indicates the utilization of several feedstocks(substrates) from agricultural residues and marine ecosystem especially seaweeds in the biobutanol production. Certain important potential substrates have been discussed in detail in this subchapter.

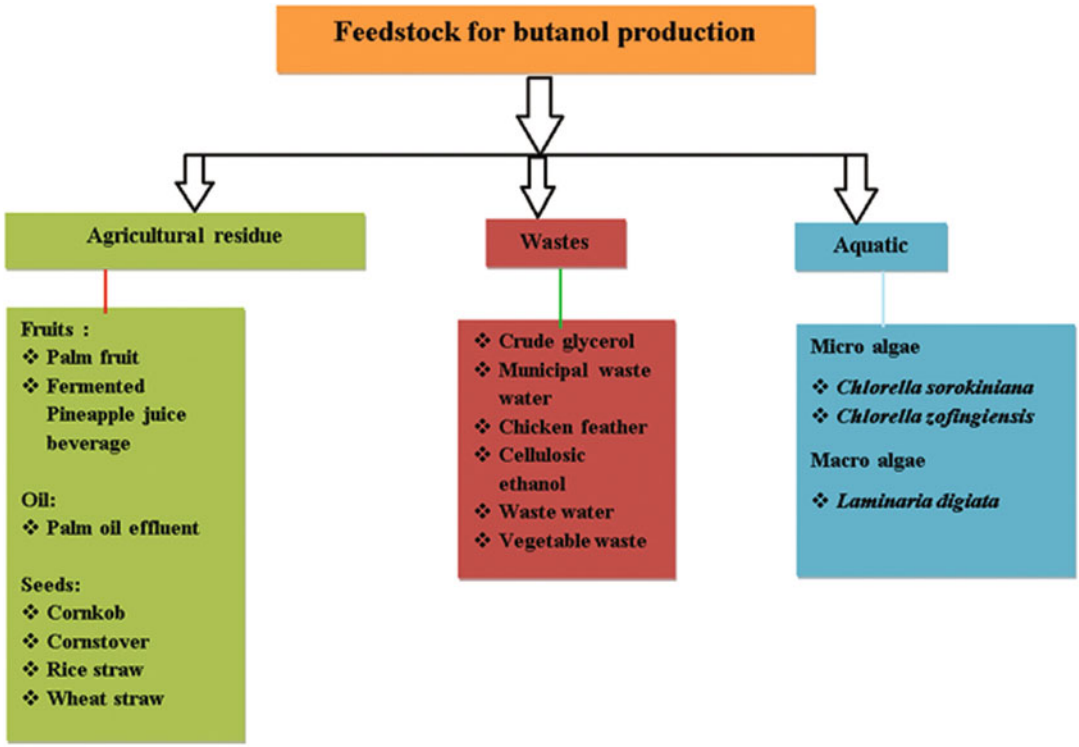


Fig. 1 Various feedstock used for production of biobutanol

5.1 Sugarcane Bagasse

Sugarcane bagasse, a fibrous residue obtained after extraction process of sugarcane juice, is one among the most effectively utilizing agro-industrial waste. Various products and process are being reported on its usage. One of the remarkable applications is in the production of biofuel (butanol). The composition of bagasse approximately contains 50% of cellulose and 25% of each of hemicellulose. Chemically, bagasse contains 50% of α-cellulose, 30% of pentosans, and 2.4% of ash. Bagasse pretreatment improves digestibility, enlargement of inner surface area of the substrate, and microbial attack [39]. Establishment of second-generation biorefinery in the view of utilizing agricultural residue in producing acetone, butanol, and ethanol using non-detoxified sugarcane bagasse with appreciable amount of glucose and xylose as a substrate obtained from sugarcane mill in Brazil by utilizing *C. acetobutylicum* DSM6228 was an initiative. He then pretreated the wet bagasse with sulfuric acid and adjusted the pH to 5 for wet and solid fraction using ammonium hydroxide following various process bagasse was separated into SBH-1, SBH-2 (sugarcane bagasse hydrolysate-1,2), respectively. The yield of butanol, ethanol, and acetone was more when using SBH-2 as a substrate without detoxification [40]. Utilizing sugarcane bagasse with other strains in future can increase the potential for scaling up in the biobutanol production by ABE fermentation.

5.2 Algal Biomass as Substrate

Various biomass from different sources such as agricultural and aquatic is efficiently utilized as a raw material for the production of biodiesel, biogas, bio-oil, and biofuels which include butanol and ethanol. Plants that grow on land cannot effectively capture sunlight; in addition, switchgrass, a fast-growing terrestrial plant, can convert more solar energy. On the other hand, reports showed that the aquatic plants micro- and macroalgae (seaweeds) have great efficiency in photosynthesis. Carbon sources are found in the form of carbohydrates such as starch, glucose, sugar, and other polysaccharides. Algal lipids also comprised sugars, fatty acids, and glycerol [41]. Usually *Clostridium* species are restricted for butanol production; newly, algal species had caused the curiosity among the researchers in the production of biobutanol as a substrate. The main advantage of using algal species is summarized in Table 2.

Batch experiment with microalgae *Chlorella sorokiniana* CY1 biodiesel residue using *C. acetobutylicum* were examined to produce biobutanol. Optimized substrate conditions were reported additionally added glucose as a co-substrate and butanol production was evaluated. The residue was pretreated with methanol and microwaved. By following various steps, the pretreated slurry was used as substrate for butanol production. Gas chromatography, HPLC, and flame ionization detector were used to analyze acetone, butanol, and ethanol. Finally, the butanol yield of 3.86 g/L was reported [41]. Thus, butanol production by microalgae was possible but more research work has to be done in future for high yield of butanol.

In another study, Hou and his colleagues reported for the first time the enzymatic hydrolysate of macroalgae *Laminaria digitata* as a substrate in producing butanol due to the presence of main structural components such as alginate and cellulose and main energy storage compounds are laminarian and mannitol. *L. digitata* was collected from coast of Danish North Sea. He used freeze-dried culture of *C. beijerinckii* DSM6422 as an

Table 2
Advantages of algal species for butanol production

S. No	Characteristics	Remarks
1.	Growth rate	Higher which leads to the production of large amount of biofuel production in a short duration
2.	Land and water requirement	Very less
	Tolerance to gaseous environment	Highly tolerable in CO_2 gas stream, which helps in CO_2 mitigation
3.	Maintenance cost	More effective than conventional farming

inoculum; anaerobic conditions are favored. This work showed a high butanol yield and ABE molar ratio [42]. Moreover, the utilization of locally available seaweeds as a substrate needs to be investigated in detail to increase productivity of butanol.

The biomass concentration is very low in the microbial culture due to the limit of light penetration, in combination with the small size of algal cells that makes the harvest of algal biomass very costly. The large water content of harvested algal biomass also means its drying would be an energy-consuming process. The higher capital costs and the rather intensive care required by a microalgal farming facility comparative a conventional agricultural farm is another factor that impedes the commercial implementation of biofuel from microalgal strategy are the disadvantages of using microalgae as a substrate. These problems are expected to overcome or minimized by technology development [43].

Onay (2020) reported the effect of Indole-3-Acetic acid (IAA) and Hydrogen Peroxide of microalgae using *Chlorella zofingiensis CCALA 944*. The later was used as an oxidizing agent in the pretreatment step to form free radicals in a flat photobioreactor. This algae has high kinetic parameters under autotrophic, heterotrophic, and mixotrophic conditions that had been reported in previous works. In this study he used *C. acetobutylicum* strain. He cultivated microalgae at various concentration of municipal wastewater. The usefulness of wastewater for the growth of microalgae was studied by him; in addition to this the algae was cultivated at various concentrations of IAA (plant derivative hormone). He analyzed that cell number, absorbance, and dry weight increased at higher concentrations of IAA. During pretreatment at 4% conc. of Hydrogen peroxide he found maximum carbohydrate in algae which is more suitable in butanol production. Finally, he concluded that maximum biomass content and butanol yield is higher at 25% municipal wastewater, IAA 80 μM, and 4% hydrogen peroxide in a flat photobioreactor [44]. Usage of algae in butanol production can be still investigated in detail in future.

5.3 Crude Glycerol

Crude glycerol is a secondary product obtained in biodiesel industry and can be considered as a cheaper carbon source than others for biofuel production. It is produced through the trans-esterification of triglycerides such as rapeseed, soya, and sunflower oil. It has low economic value due to low glycerol content and presence of various contaminants. Crude glycerol from biodiesel industry contains 25% carbon and elements like Na, Ca, K, Mg, P, and S [45].

Kaushal et al. [46] addressed the issue of high cost of substrate, so he demonstrated the cost-effective strategy by replacing all the major components with cheaper sources such as glucose with lignocellulosic biomass such as rice straw hydrolysate, sugarcane bagasse, and nut husk from local farms; glycerol with crude glycerol; and nitrogen with dry yeast. Since, the acetone is not used as a

fuel, they worked with a non-acetone-forming *C. sporogenes* NCIM2918 strain. He followed dual substrate strategy. The biomass was pretreated with alkaline NaOH solution. The biomass composition is analyzed both quantitatively and qualitatively. Then the fermentation was carried out with batch with and without continuous gas stripping. Fed batch coupled with continuous gas stripping had higher productivity. He reported that this was the first fermentation carried out with fed batch with cheaper source [46]. More works related to biobutanol production from crude glycerol has to be investigated in future.

5.4 Lignocellulosic Biomass

The most abundant material that can serve as a main source of biobutanol production is the lignocellulosic material. It represents the best option as feedstock for biofuel production due to high yield, output/input energy ratio, and availability and can be easily affordable. Maize, wheat, rice, and sugarcane are the four major agricultural crops that yield more lignocellulosic material. This material mainly contains lignocelluloses and small quantities of cellulose, lignin, hemicelluloses, and extractives [47]. The potential substrates such as switch grass, rice straw, poplar, wheat straw, and barley straw are available at low cost [48]. Chicken feathers and the wheat straw are the most convenient material used by Branska and his colleagues which in combination they processed under mild condition. This is more advantageous due to the utilization of waste streams that are locally available, minimized inhibitor formation, and no detoxification that generate wastewater. Their work also offers a solution for the local waste management.

Saekhow et al. [49] was the first to report the utilization of cassava stem hydrolysate for ABE fermentation *Clostridium* species. Cassava stem was pretreated to get highest sugar concentration with NaOH before enzymatic hydrolase of amylase and cellulose. They concluded that cassava stem has capability for the cost-effective fermentation and yield of butanol [49]. Biochar is a carbon-rich material obtained from the carbonization, pyrolysis, or gasification of lignocellulosic biomass, animal waste, and solid wastes [50]. pH buffering capacity, alkalinity, cation exchange capacity, rich micro and macro elements. The most important application of Biochar is in anaerobic digestion. Thus, the usage of biochar could be an economical strategy for ABE fermentation [50].

Rice straw is one of the cheapest and useless lignocellulosic material among all agricultural wastes which can be properly utilized for biobutanol production [51]. Globally rice is considered as major agricultural crop. Cellulose, hemicelluloses, and lignin are main constituents present in the lignocellulosic material. Cellulose is the main constituent present in rice straw. The components present in rice straw are (%w/w): cellulose 32–43%, hemicelluloses 19–25%, lignin 5–12%, ash 18.8%, and extractives 10–12%

Table 3
Different potential substrates used for the production of biobutanol

S. No	Substrate	Butanol (g/L)	Microorganism	References
1.	Sugarcane bagasse	9.1	*C. acetobutylicum* DSM6228	[40]
2.	Lignocellulosic hydrolysate	4.5–5.3	*C. saccharobutylicum* DSM13864	[66]
3.	Palm oil effluent	0.9	*C. saccharoperbutylacetonicum* N1–4	[67]
4.	Chicken feather and wheat hydrolysis	4.6	*E. coli* DSM 6897	[48]
5.	Microalgae *Chlorella sorokiniana* CY1	3.86	*C. acetobutylicum* ATCC 824	[41]
6.	Cane sugar factory wastewater (CSFW)	0.3	*C. beijerinckii* CG1	[68]
7.	Cellulosic ethanol Pilot plant wastewater (CFPW)	0.6	*C. beijerinckii* CG1	[68]
8.	Corn waste (scale up)	11.92	*C. beijerinckii*	[69]
9.	Rice straw hydrolysate and crude glycerol	21.5	*C. sporogenes* NCIM2918	[46]
10.	Fermented pineapple juice beverage	13.3	*C. acetobutylicum* DSM 792	[70]

[52]. Due to the increasing demand of biobutanol, the use of rice straw will be the challenging area of research in future aspects. Concise of potential substrate utilized by the microorganisms for the butanol production is depicted in Table 3.

5.5 Co-fermentation

Co-fermentation is a term related to usage of two different substrates for specific purpose. Even though cost-effective carbon sources had been employed for the production of biobutanol using *Clostridia* species, involvement of other necessary nutrients required for metabolic process, like nitrogen source, minerals, and vitamins, will reflect directly in terms of cost for the production [53]. Hence replacing the compounds that are required for the cell growth and metabolic activity by cost-effective renewable residues could decrease cost involved for the production of butanol.

Mechmech et al. [53] reported that two different substrates containing rich source of nitrogen and carbohydrate were mixed together and butanol production was achieved by using *Clostridium acetobutylicum* ATCC 842 species. Canadian alfalfa juice contained notable amount of nitrogen and was used with the hardwood hydrolysate for the production of biobutanol. But this study had negative effect in terms of inhibitors present in the fermentation medium [53]. Similar studies like usage of

co-fermentation of two different substrates which are less expensive and agro-industrial waste residue should be investigated in future for the production of biobutanol.

In another study, Cao et al. [54] reported that corn steep liquor was exposed as nitrogen source for the production of biobutanol using *Clostridium tyrobutyricum Δcat1::adhE2*. Notable amount of biobutanol (16.5 g/L) was produced when co-fermentation of paper mill sludge and corn steep liquor was employed. The major disadvantage of this study is pretreatment required for the paper mill sludge, since employment of enzymatic pretreatment methods could lead to increase the production cost [54]. Thus, cost-effective pretreatment methods or without employment of pretreatment methods should be investigated while performing co-fermentation strategy.

6 Current Developments in Biobutanol Production with Integrated Product Recovery

Various stages involved in butanol fermentation process are represented in Fig. 2, where integrated product recovery was discussed briefly. In situ product recovery has been employed to overcome the low production of ABE. Liquid-liquid extraction, pervaporation, adsorption, gas stripping, extraction, and reverse osmosis are a wide variety of techniques which are demonstrated to reduce the butanol inhibition. These techniques are simple and it does not cause any damage to culture cells. Moreover, these techniques are cost effective. Butanol efficiency is relatively increased by these techniques.

Gas stripping technique essentially separates the butanol. Gas stripping do not require higher energy. About 500 mL of fermentation broth was taken in a flask. Gas is allowed to sparge into the spinner flask with the help of the impellers and the flask containing about 10 g/L of butanol and 1.6 L/min of flow rate is maintained. The condenser collects the gas and the spinner flask contains the condensate where the required flask is submerged into the ice-cold water bath of about low temperature. Gas stripping rate, cell density, and butanol concentration were considerably found by withdrawing the liquid samples from the flask [55]. When the liquid solutions reach the maximum temperature, gas stripping technique commences. Liquid samples were usually stored at a cooling temperature.

Fermentation can be prominently carried out in a two-stage condensate. FBB (Fibrous bed bioreactor) equipment is literally connected to the two condensers. The condensers, flask was autoclaved for about 45 min. The nitrogen gas was allowed to sparge into the complete setup to secure anaerobic surroundings. At a rate of 1.5 L\min the air was allowed to sparge into the ABE solutions. At low temperature, the condenser is sustained deliberately and the

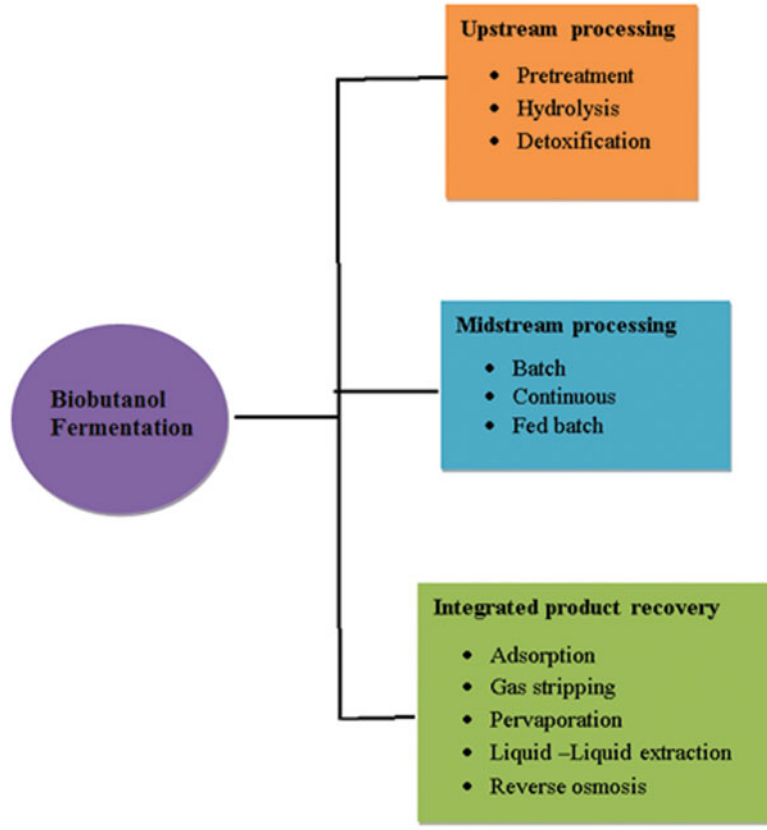


Fig. 2 Biobutanol fermentation process

condensate is completely submerged into cold water bath. The initial stage of butanol is removed and passed on to the second stage condensate gas stripping tank. To achieve perfect purification, second stage condensate is relatively integrated with the first stage condensate. By skipping the gas stripping technique, the total fermentation period takes around 53 h, while performing a gas stripping technique the fermentation period is considerably reduced to about 48 h. Initially during the organic phase absence of acid is observed and after integrating with the first phase the concentration of butanol is tremendously high. It is observed that in order to initiate the gas stripping technique, concentration of butanol should be 5 g/L. The end product of the butanol is about 400 g/L [56].

Adsorption is a productive separation technique. Adsorption integrates the substance or a compound to its surface of the solid. Adsorbent plays a major role in adsorption. Activated carbon, Molecular Sieve Zeolites, silica gel, activated alumina, and polymeric adsorbents are the various types. Adsorption technique is cost effective and relatively considered to have high affinity [57]. In his study the activated carbon has been used as an adsorbent.

Continuous adsorption process is carried out with the activated carbon and also studied about acetone, butanol, and acetic acid. Desorption process is carried out, where ABE solvents are completely adsorbed and the dissolved solids are removed using filter paper. Desorption is further enhanced by raising the temperature. For the recovery of butanol adsorbents were initially heated and further raised to a high temperature. Batch fermentation was also performed with clostridium acetobutylicum and activated carbon. With the help of the syringe, the liquid solvents were removed which is used to determine the cell density. Fed batch fermentation is also performed in this process. ABE solvents are fed into the bioreactor and the activated carbon. Butanol adsorption and desorption were performed in the heating oven coupled to the cold trap. Large amount of butanol gets adsorbed in the cold trap. With the help of immobilized cells, further fermentation was carried out. During this process, productivity is considerably high and total time period for the fermentation process is low. This study reveals butanol production of about 54.6 g/L at the time period of about 0.45 g/h. Purification of biodiesel has been enhanced by the adsorption technique.

Liquid-liquid extraction inhibits the level of butanol toxicity. The cells are not affected during liquid-liquid extraction technique. Phase changes take place when the ABE solvents are subjected to liquid-liquid extraction [58, 59]. Liquid-Liquid extraction can be executed inside the fermenter. Ionic liquids can be used as a substitute instead of other liquid solvents in the liquid-liquid extraction [60]. Vapor pressure of ionic liquids is insignificant. At low pressure, residual products get extracted from the ionic liquids. This study reveals that by varying the charges of the ions, recovery of butanol is achieved. He carried out his study with numerous imidazole-related alkyl chains. Ionic liquid properties were mostly corresponded to the solvent chemical properties. High-performance liquid chromatography estimates the ionic liquid. The butanol and ionic liquid mixture are taken to estimate the distribution coefficient. Gas chromatography plays a vital role in regulating the butanol concentration. In liquid-liquid extractions ionic liquid solubility and solvent solubility are significant. Ionic liquid solubility and water rely on temperature. Polarity plays a predominant role in butanol recovery. Efficiency, selectivity, and distribution coefficient linearly get increased. Before performing the liquid-liquid extraction process, anions must be subjected to hydrolysis because some of the products cause harm to microorganisms. Around 74% of butanol was extracted [60].

Membrane systems are employed in the pervaporation technique. Pervaporation technique is extensively used to separate the liquid mixtures. Generally, pervaporation technique does not require higher energy. Unwanted materials can be easily extracted from the ABE solvents. Usually pervaporation membrane is

nonporous in nature. Pervaporation technique does not cause any damage to microorganisms. Suitable membrane must be selected for the operation [61]. There are two other modes of pervaporation which include thermo pervaporation and the sweeping gas pervaporation. Pervaporation technique thereby tremendously increases the yield of biobutanol. Pervaporation technique is cost effective. Solvent phase and the vapor phase can be separated. Higher selectivity is observed in the pervaporation membrane. PDMS (Polydimethylsiloxane) silicone-based membrane can be employed for the butanol recovery. Pervaporation technique has greater selectivity and flexibility [61].

Sarabia et al. [62] had studied about the ionic liquids within the membrane. He used polymer inclusion membrane for his study. Butanol and membrane liquid were used for the construction of polymeric membrane. Fourier transform infrared spectroscopy were used to determine the interactions. Scanning electron microscope was used to determine the physical properties. Linear membrane with a pervaporation cell was taken and the solvents were allowed to pass through the membrane and the vacuum pressure is maintained and applied on to the opposite side. Permeate tank stored the liquid. Solvents and the permeate were withdrawn from tank separately. Pervaporation separation index were used to estimate the pervaporation effect. Mass transfer resistances had been evaluated in this study. Additionally, it was observed that due to the greater affinity of ionic liquids, there is a reduction in the resistance of the membrane [62]. Permeation flux considerably raised and the butanol isolation is not so effective in this case. Butanol is separated from the ABE solvents with the usage of permeate flux, PSI values.

Wu et al. [63] studied the butanol recovery using the PDMS membrane which was completely submerged into the TEOS solution with the addition of the catalyst. The membrane was covered with the ceramic layer. Determination of permeation flux and separation factor was also carried out. Batch fermentation was also performed in this study. With the minimum amount of butanol contained in the batch fermentation process, butanol was allowed to pass through the pervaporation membrane. Separation factor had relatively reduced in the ABE solvents. Separation factor of butanol was around 15. It is reported that microorganisms present in the broth could cause damage to the membrane. Toxicity of butanol depends on the solvent. About 64.6 g of ABE was obtained. Around 5 g/L must be present initially to initiate the process [63]. Low pH was maintained to increase the productivity. About 46% of solvent was removed using the membrane. Around 0.303 g/L of solvent yield was obtained. Table 4 shows the succinct of in situ product recovery.

Table 4
In situ product recovery techniques for biobutanol production

S. No	Techniques	Butanol recovery	Microorganisms	References
1.	Gas stripping	400 g/L	*Clostridium acetobutylicum* JB 200	[55]
2.	Adsorption	54.6 g/L	*Clostridium beijerinckii* CC 101	[56]
3.	Liquid-liquid extraction	70%	–	[60]
4.	Pervaporation	0.303 g/L	*Clostridium acetobutylicum* XY 16	[59]

Semipermeable membrane is used in the process of reverse osmosis. Reverse osmosis is used in extracting the greater butanol from the ABE solvents. Greater pressure is applied to remove water from the solvents. Porous membrane is applicable for the reverse osmosis technique. By using ultrafiltration process, solids are extracted from the solvents and nanofiltration technique can also be demonstrated [64, 65]. It is observed that permeate flux is considerably lesser and about 90% of butanol was also extracted.

7 Conclusion

This chapter concentrated on the noteworthy efforts to increase production of biobutanol. Various cost-effective substrates could be utilized for successful biobutanol production. Important microorganisms and their attraction toward process strategy has been discussed in this chapter. Although sugar-based substrates have involved attention toward biobutanol production, sugarcane bagasse, algal biomass, crude glycerol, and lignocellulosic biomass are more attractive and highly abundance. Significant effort has to be performed in order to reduce the encumbrance related to product inhibition phenomenon.

References

1. Xue C, Zhao XQ, Liu CG et al (2013) Prospective and development of butanol as an advanced biofuel. Biotechnol Adv 31:1575–1584
2. Bengelsdorf BS, Montoya J, Set L (2013) Butanol fermentation. Environ Technol 34 (13–16):1691–1710. https://doi.org/10.1080/09593330.2013.827746
3. Ndaba B, Chiyanzu I, Marx S (2015) n-Butanol derived from biochemical and chemical routes: a review. Biotechnol Rep 8:1–9
4. Morone A, Pandey RA (2014) Lignocellulosic biobutanol production: gridlocks and potential remedies. Renew Sust Energ Rev 37:21–35
5. Sarathy S, Vranckx S, Yasunanga K (2012) A comprehensive chemical kinetic combustion model for the four butanol isomers. Combust Flame 159:2028–2055
6. Durre P (2007) Biobutanol: an attractive biofuel. Biotechnol J 2:1525–1534
7. Lee SY, Park JH, Jang SH et al (2008) Fermentative butanol production by clostridia. Biotechnol Bioeng 101:209–228

8. Nigam PS, Singh A (2011) Production of liquid biofuels from renewable resources. Prog Energy Combust Sci 37:52–68
9. Fernandez JC, Arnal JM, Gomez J et al (2012) A comparison of higher alcohols/diesel fuel blends in a diesel engine. Appl Energy 95:267–275
10. Liu KM, Li YT, Yang J et al (2018) Comprehensive study of key operating parameters on combustion characteristics of butanol gasoline blends in a high speed SI engine. Appl Energy 212:13–32
11. Feng RH, Fu JQ, Yang J et al (2015) Combustion and emissions study on motorcycle engine fueled with butanol-gasoline blend. Renew Energy 81:113–122
12. Ibrahim A (2016) Performance and combustion characteristics of a diesel engine fueled by butanol-biodiesel-diesel blends. Appl Therm Eng 103:651–659
13. Shahsavan M, Mack JH (2018) Numerical study of a boosted HCCI engine fueled with bio-butanol and isobutanol. Energy Convers Manag 157:28–40
14. Zhen X, Wang Y, Liu D (2020) Bio-butanol as a new generation of clean alternative fuel for SI (sparkignition) and CI (compression ignition) engines. Renew Energy 147:2494–2521
15. Santacesaria E, Carotenuto G, Tesser R et al (2012) Ethanol dehydrogenation to ethyl acetate by using copper and copper chromite catalysts. Chem Eng J 179:209–220
16. Juben N, James C, Dumesi A (2007) An overview of dehydration, aldol-condensation and hydrogenation processes for production of liquid alkanes from biomass derived carbohydrates. Catal Today 123:59–70
17. Carvalho DL, Avillez RR, Rodrigues MT et al (2012) Mg and Al mixed oxides and the synthesis of n-butanol from ethanol. Appl Catal 416:96–100
18. Scalbert J, Starzyk FT, Jacquot R et al (2014) Ethanol condensation to butanol at high temperatures over a basic heterogeneous catalyst: how relevant is acetaldehyde self-aldolization. J Catal 311:28–32
19. Huang H, Liu H, Gan Y (2010) Genetic modification of critical enzymes and involved genes in butanol biosynthesis from biomass. Biotechnol Adv 28:651–657
20. Ezeji T, Milne C, Price ND et al (2010) Achievements and perspectives to overcome the poor solvent resistance in acetone and butanol-producing microorganisms. Appl Microbiol Biotechnol 85:1697–1712
21. Jiang Y, Liu J, Jiang W et al (2015) Current status and prospects of industrial bio-production of n-butanol in China. Biotechnol Adv 33:1493–1501
22. Majidian P, Tabatabaei M, Zeinolabedini M et al (2017) Metabolic engineering of microorganisms for biofuel production. Renew Sustain Energy Rev 82:3863. https://doi.org/10.1016/j.rser.2017.10.085
23. Nilsson RLK, Helmerius J (2015) Biobutanol production by *Clostridium acetobutylicum* using xylose recovered from birch Kraft black liquor. Bioresour Technol 176:71–79
24. Qureshi N, Saha BC, Cotta MA (2007) Butanol production from wheat straw hydrolysate using *Clostridium beijerinckii*. Bioprocess Biosystems Eng 30:419–427. https://doi.org/10.1007/s00449-007-0137-9
25. Liyanage H, Young M, Kashket ER (2000) Butanol tolerance of clostridium beijerinckii NCIMB 8052 associated with down-regulation of gldA by antisense RNA. J Mol Microbiol Biotechnol 2(1):87–93
26. Atsumi S, Cann AF, Connor MR et al (2008) Metabolic engineering of *Escherichia coli* for n-butanol production. Metab Eng 10:305–311
27. Machado IMP, Atsumi S (2012) Cyanobacterial biofuel production. J Biotechnol 162:50–56
28. El L, Liao JC (2011) Metabolic engineering of cyanobacteria for 1-butanol production from carbon dioxide. Metab Eng 13:353–363
29. Wang M, Fan L, Tan T (2014) 1-Butanol production from glycerol by engineered. *Klebsiella pneumonia*. RSC Adv 4:57791–57798
30. Wang M, Liu L, Fan L et al (2017) CRISPR based system for enhancing 1-butanol production inengineered *Klebsiella pneumoniae*. Process Biochem 56:139–146
31. Steen EJ, Chan R, Prasad N et al (2008) Metabolic engineering of *Saccharomyces cerevisiae* for the production of n-butanol. Microb Cell Factories 7:36
32. Krivoruchko A, Amatriain CS, Chen Y et al (2013) Improving biobutanol production in engineered Saccharomyces cerevisiae by manipulation of acetyl-CoA metabolism. J Ind Microbiol Biotechnol 40:1051–1056
33. Yu M, Du Y, Jiang W et al (2011) Effects of different replicons in conjugative plasmids on transformation efficiency, plasmid stability, gene expression and n-butanol biosynthesis in *Clostridium tyrobutyricum*. Appl Microbiol Biotechnol 93(2):881–889. https://doi.org/10.1007/s00253-011-3736-y
34. Yu M, Zhang Y, Tang IC et al (2011) Metabolic engineering of Clostridium tyrobutyricum for n-butanol production. Metabol Eng 13:373–382

35. Ruhl J, Schmid A, Blank LM (2009) Selected *Pseudomonas putida* strains able to grow in the presence of high butanol concentrations. Appl Environ Microbiol 75:4653–4656
36. Vangnai AS, Arp DJ (2001) An inducible 1-butanol dehydrogenase, a quinohaemoprotein, is involved in the oxidation of butane by '*Pseudomonas butanovora*'. Microbiology 147:745–756
37. Stephens E, Ross IL, Mussgnug JH et al (2010) Future prospects of microalgal biofuel production systems—a review. Trends Plant Sci 15(10):554–564. https://doi.org/10.1016/j.tplants.2010.06.003
38. Spolaore P, Cassan CJ, Duran E et al (2006) Commercial applications of microalgae. J Biosci Bioeng 101:87–96
39. Pandey A, Soccol CR, Nigam P, Soccol VT (2000) Biotechnological potential of agro-industrial residues. I: sugarcane bagasse. Bioresour Technol 74:69–80
40. Gomes A, Rodrigues MI, Passos DF et al (2019) Acetone–butanol–ethanol fermentation from sugarcane bagasse hydrolysates: utilization of C5 and C6 sugars. Electron J Biotechnol 42:16–22
41. Cheng HH, Whang LM, Chan KC et al (2015) Biological butanol production from microalgae-based biodiesel residues by *Clostridium acetobutylicum*. Bioresour Technol 184:379–385. https://doi.org/10.1016/j.biortech.2014.11.017
42. Hou X, From N, Angelidaki I et al (2017) Butanol fermentation of the brown seaweed Laminaria digitata by Clostridium beijerinckii DSM-6422. Bioresour Technol 238:16–21
43. Li Y, Horsman M, Wu N et al (2008) Biocatalysts and bioreactor design. Biotechnol Prog 24:815–820
44. Onay M (2020) The effects of indole-3-acetic acid and hydrogen peroxide on Chlorella zofingiensis CCALA 944 for bio-butanol production. Fuel 273:117795
45. Kumar LR, Yellapu SK, Tyagi RD et al (2019) A review on variation in crude glycerol composition, bio-valorization of crude and purified glycerol as carbon source for lipid production. Bioresour Technol 293:122155
46. Kaushal M, Ahlawat S, Makut BB et al (2019) Dual substrate fermentation strategy utilizing rice straw hydrolysate and crude glycerol for liquid biofuel production by Clostridium sporogenes NCIM 2918. Biomass Bioenergy 127:105257
47. Saini JK, Saini R, Tewari L (2015) Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments. 3 Biotech 5:337–353. https://doi.org/10.1007/s13205-014-0246-5
48. Branska B, Fortova L, Dvorakov M et al (2020) Chicken feather and wheat straw hydrolysate for direct utilization in biobutanol production. Renew Energy 145:1941–1948
49. Saekhow B, Chookamlang S, Na-u-dom A et al (2020) Enzymatic hydrolysis of cassava stems for butanol production of isolated Clostridium sp. Energy Rep 6:196–201
50. Sun X, Atiyeh HK, Adesanya Y et al (2019) Feasibility of using biochar as buffer and mineral nutrients replacement for acetone-butanol-ethanol production from non-detoxified switchgrass hydrolysate. Bioresour Technol 298:122569. https://doi.org/10.1016/j.biortech.2019.122569
51. Moradi F, Amiri H, Zad SS et al (2013) Improvement of acetone, butanol and ethanol production from rice straw by acid and alkaline pretreatments. Fuel 112:8–13
52. Malik K, Tokkas J, Anand RC et al (2015) Pretreated rice straw as an improved fodder for ruminants—an overview. J Appl Nat Sci 7 (1):514–520
53. Mechmech F, Marinova M, Chadjaa H et al (2016) Co-fermentation of alfalfa juice and hardwood hydrolysate for butanol production in combined biorefinery systems. Ind Crop Prod 89:29–33
54. Cao X, Chen Z, Liang L et al (2019) Covalorization of paper mill sludge and corn steep liquor for enhanced n-butanol production with *Clostridium tyrobutyricum Δcat1::adhE2*. Bioresour Technol 296:122347. https://doi.org/10.1016/j.biortech.2019.122347
55. Xue C, Zhao J, Liu F et al (2013) Two-stage in situ gas stripping for enhanced butanol fermentation and energy-saving product recovery. Bioresour Technol 135:396–402
56. Xue C, Du GQ, Sun JX et al (2014) Characterization of gas stripping and its integration with acetone–butanol–ethanol fermentation for high-efficient butanol production and recovery. Biochem Eng J 83:55–61
57. Xue C, Liu F, Xu M et al (2016) Butanol production in acetone-butanol-ethanol fermentation with in situ product recovery by adsorption. Bioresour Technol 219:158–168
58. Bonilla PJ, Wang Y (2017) *In-situ* biobutanol recovery from clostridial fermentations: a critical review. Crit Rev Biotechnol 38:469–482. https://doi.org/10.1080/07388551.2017.1376308
59. Outram V, Lalander CA, Jonathan GM (2017) Applied in situ product recovery in ABE

fermentation. Biotechnol Prog 33:563–579. https://doi.org/10.1002/btpr.2446

60. Ha SH, Mai NL, Koo YM (2010) Butanol recovery from aqueous solution into ionic liquids by liquid–liquid extraction. Process Biochem 45:1899–1903
61. Kujawska A, Kujawski J, Bryjak M et al (2015) ABE fermentation products recovery methods—a review. Renew Sustain Energy Rev 48:648–661
62. Sarabia CA, Revuelta DG, Fallanza M et al (2020) Polymer inclusion membranes containing ionic liquids for the recovery of n-butanol from ABE solutions by pervaporation. Sep Purif Technol 248:117101
63. Wu H, Chen XP, Liu GP et al (2012) Acetone–-butanol–ethanol (ABE) fermentation using Clostridium acetobutylicum XY16 and in situ recovery by PDMS/ceramic composite membrane. Bioprocess Biosyst Eng 35:1057–1065. https://doi.org/10.1007/s00449-012-0721-5
64. Abdehagh N, Tezel FH, Thibault J (2014) Separation techniques in butanol production: challenges and developments. Biomass Bioenergy 60:222–246
65. Li H, Luo W, Gu Q et al (2013) Acetone, butanol, and ethanol production from cane molasses using Clostridium beijerinckii mutant obtained by combined low-energy ion beam implantation and N-methyl-N-nitro-N-nitrosoguanidine induction. Bioresour Technol 137:254–260)
66. Magalhães BL, Grassi MCB, Pereira GAG et al (2018) Improved n-butanol production from lignocellulosic hydrolysate by *Clostridium* strain screening and culture-medium optimization. Biomass Bioenergy 108:157–166
67. Al-Shorgani NKN, Shukor H, Abdeshahian P et al (2015) Process optimization of butanol production by *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC13564) using palm oil mill effluent in acetone–butanol–ethanol fermentation. Biocatal Agricul Biotechnol 4:244–249. https://doi.org/10.1016/j.bcab.2015.02.004
68. Comwien J, Boonvithaya N, Chulaluksananukul W et al (2015) Direct production of butanol and ethanol from cane sugar factory wastewater and cellulosic ethanol pilot plant wastewater by Clostridium beijerinckii CG1. Energy Procedia 79:556–561
69. Zhang J, Jia B (2018) Enhanced butanol production using Clostridium beijerinckii SE-2 from the waste of corn processing. Biomass Bioenergy 115:260–266
70. Sanguanchaipaiwong V, Leksawasdi N (2018) Butanol production by *Clostridium beijerinckii* from Pineapple waste juice. Energy Procedia 153:231–236



Chapter 2

Biomass Pretreatment via Hydrodynamic Cavitation Process

Ramesh Desikan, Sivakumar Uthandi, and Kiruthika Thangavelu

Abstract

There are three essential steps involved in bioethanol production from lignocellulosic biomass feedstocks. They are pretreatment, hydrolysis, and fermentation process. Among them, biomass pretreatment is an expensive and energy-intensive process used to remove the lignin and make the feedstock amenable for bioethanol production. The hydrodynamic cavitation can also be used for biomass pretreatment process. In order to improve the effectiveness of biomass pretreatment, a combination of any two methods of physical, chemical, and biological pretreatment can be used. A combination of the hydrodynamic cavitation pretreatment of biomass with the chemical or biochemical catalyst can be performed better than the individual pretreatment method. In this chapter, a protocol is describes the biomass pretreatment via a combined hydrodynamic cavitation with biocatalyst process.

Key words Biomass pretreatment, Hydrodynamic cavitation, Combined pretreatment

1 Introduction

The lignocellulosic biomass (LCB) feedstocks comprise 75% of carbohydrates (40–50% of cellulose, 25–30% of hemicellulose) and 25% of lignin [1]. To use the LCB feedstocks efficiently for bioethanol production, polysaccharides in the feedstocks must be released and also make more cellulose accessibility by breaking the lignin–carbohydrate complex based barrier [2]. It can be achieved through only the biomass pretreatment process. Presently, any of the physical, chemical, biological, and combined methods can be used to pretreat the LCB feedstocks [3, 4]. Each pretreatment method has its own merits and demerits in the pretreatment process. Overall, the pretreatment process is contributing a significant share in bioethanol production cost.

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Bioethanol production from LCB is not commercialized due to uneconomical production cost [4, 5]. The production cost is higher due to the biomass pretreatment process, and it is ineffective in lignin removal. Furthermore, several researchers are working to reduce pretreatment processing costs, which will help LCB based bioethanol industries run successfully and at a commercial scale [6–14]. A combinational pretreatment can be performed better than that of individual biomass pretreatment and several combined methods tested for their pretreatment effectiveness. Recently, the hydrodynamic cavitation process is used for biomass pretreatment [15–17]. The LCB biomass is subjected to the cavitation process, which made violent collapsing of vapor bubbles at millions of sites in the working liquid and reactant materials. This leads to a sudden rise and fall of temperature and pressure within the cavity in a few microseconds [18].

Based on the particle size and place of LCB materials, the hydrodynamic cavitation based pretreatment can be achieved in two ways. One method involves placing LCB feedstocks in the cavitation zone for the entire reaction period [17]. Other method deals with biomass slurry supplied continuously through the cavitation zone for the entire reaction period [16]. The first method prefers big size biomass particles to keep in a wire mesh type container in the cavitation zone and only working fluid (mostly water/chemical solution) will be circulated in the reactor. Another method uses fine-sized biomass particles (<212 μm) to prepare biomass slurry and it will be circulated to cavitation zone through holes of the orifice plate. In order to cavitation, we can use a venturi tube or orifice plate in the reactor. During the pretreatment process, decomposition of water molecules causes free radical formation, such as hydroxyl radicals leading to turbulence action of working fluid in the cavitation zone, which eventually helps in rupturing the lignin barrier. The chemical/biocatalyst added in the process would help the reaction speed [16]. In this chapter, the protocol briefly describes a combined biocatalyst and hydrodynamic cavitation pretreatment used to pretreat the biomass slurry.

2 Materials

2.1 Biomass Powder Preparation

1. The corncob biomass is dried to remove the moisture, and then the size reduced using different size reduction machinery such as shredder, pin mill, and grinder.
2. In order to collect the biomass sample, the biomass powder is passed through ASTM 70 sieve. The particle size of the biomass powder used for this experiment should be <212 μm.

2.2 Biomass Slurry Preparation

2.2.1 Biocatalyst

The biomass slurry is prepared by mixing appropriate quantities of biomass powder and a biocatalyst (laccase enzyme) in an acetate buffer pH 4.5. For example, 200 g of biomass and 6.5 U g^{-1} of the enzyme to be added to 4000 mL of acetate buffer to make biomass slurry for 5% biomass loading and 6.5 U g^{-1} of the enzyme [5].

2.2.2 Chemical Catalyst

The biomass slurry will be prepared by mixing appropriate quantities of biomass powder and a chemical catalyst in the water to maintain fixed biomass loading rate and chemical concentration in the experiment.

3 Methods

3.1 Hydrodynamic Cavitation Reactor Configuration

1. The hydrodynamic cavitation (HC) reactor consists of circulation tank, orifice plate with flange arrangement, centrifugal pump, electric motor, connecting pipes (suction pipe and main pipeline), and control gate valves (Fig. 1).
2. For pressure drop measurement, one pressure gauge can be fixed downstream and other at the upstream side of the orifice plate.

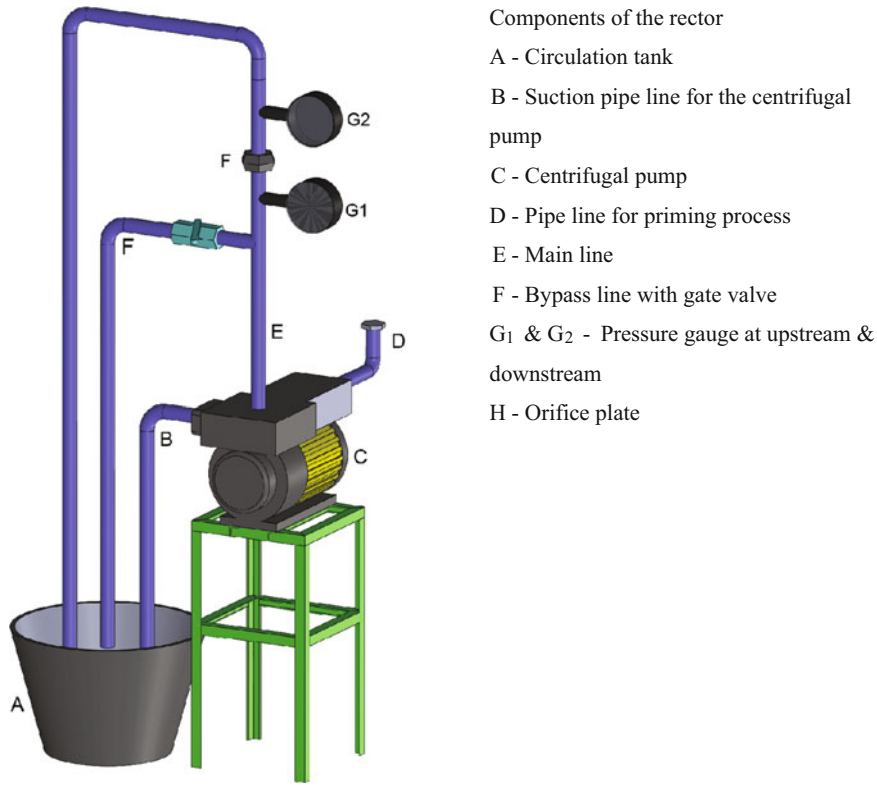


Fig. 1 Hydrodynamic cavitation reactor for pretreating the lignocellulosic biomass feedstocks

3. One end of the suction pipe is connected to the inlet of the centrifugal pump, and another end is put in the circulation tank.
4. The main pipeline was divided into three sub-pipelines. The first sub-pipeline is for doing priming operation. The second line is acting as a bypass line for regulating the flow rate of biomass slurry. The third sub-pipeline is used to fit the orifice plate, which is connected with the centrifugal pump.
5. For more exposure to biomass in the cavitation zone, the working fluid (biomass slurry) was circulated in a closed-loop arrangement. This operation will help the working fluid to several passes through the orifice plate to the tank by circulation. The circulation of the working fluid is achieved by operating the centrifugal pump.
6. The control gate valve can regulate the flow rate of biomass slurry in the HC reactor setup.

3.2 The Operating Procedure Used for Biomass Pretreatment in HC Reactor

3.2.1 Before Starting the HC Reactor

1. Check all components of HC reactor are in good conditions.
2. Run the reactor with water for 10 min to test the leakage in the pipelines, and this step will also help in the removal of residues of the previous run.

3.2.2 Operating Procedure for HC Reactor

1. In the first step, the quantity of the water should be filled up to 80 percent of capacity of circulation tank used in the HC reactor.
2. For the initial start-up of the reactor, the gate valve in the pipeline is in an open position.
3. Before starting the centrifugal pump, the priming operation should be done for the easy water circulation in the HC reactor.
4. The centrifugal pump is turned "ON" to run.
5. The water is continuously supplied circulated in a closed-loop.
6. The gases present in the pipelines and working liquid may lead to instability conditions in the reactor. Hence, the reactor should run for 3 min to remove the gases present in the pipelines.
7. The inlet pressure can be adjusted to the recommended operating pressure by regulating the gate valve fitted in the bypass line.
8. After 5 min operation of the reactor, a known quantity of biomass powder can be added to the water to make biomass slurry as described in the Materials section. The quantity of

biomass used in slurry preparation is based on the biomass loading rate and chemical concentration used in the experiment.

9. The biomass slurry can be continuously supplied from the circulation tank to circulate via an orifice plate of the HC reactor, which subjects the biomass exposed to the cavitation zone several times in the reaction time. In the cavitation zone, biomass will undergo chemical and physical transformations due to the aggressive collapse of micro-bubbles and their hot-spots formations. Record the time, once the experiment is started.
10. HC reactor is continuously operated for 60 min. The pretreated biomass samples can be drawn at regular intervals for assessing the effectiveness of the pretreatment performance in terms of lignin reduction, hemicellulose reduction, and cellulose increase.
11. Switch "OFF" centrifugal pump to stop the pretreatment process.
12. For compositional analysis of pretreated biomass, the biomass slurry from the HC reactor can be collected after each run, and the filter cloth is used to separate the hydrolysate and pretreated biomass samples.
13. In order to neutralize (pH 7.0), the pretreated biomass washed twice with distilled water, and the drying of samples was done in a hot air oven at 45 °C. National Renewable Energy Laboratory procedure is adopted for biomass compositional analysis of both raw and pretreated biomass samples [7, 19].
14. Based on the results of biomass compositional analysis, the percentage of lignin reduction and hemicellulose reduction, the percentage of cellulose increase of biomass samples after pretreatment can be calculated with the help of the following equations.

$$\text{Percentage of lignin reduction} = \frac{\text{Lignin in raw biomass} - \text{lignin in pretreated biomass}}{\text{Lignin in the raw biomass}} \times 100$$

$$\text{Percentage of hemicellulose reduction} = \frac{\text{Hemicellulose in the raw biomass} - \text{hemicellulose in pretreated biomass}}{\text{Hemicellulose in the raw biomass}} \times 100$$

$$\text{Percentage of cellulose increase} = \frac{\text{Cellulose in pretreated biomass} - \text{cellulose in raw biomass}}{\text{Cellulose in pretreated biomass}} \times 100$$

3.2.3 Before Stopping the HC Reactor

1. The HC reactor is rerun for 10 min with pure water to remove the biomass particles in the pipelines. This step will help in the removal of the adhering biomass in the pipeline.
2. Drain the water in the circulation tank. Now, the reactor is ready for the next experiment.
3. This procedure can be repeated for the next run.

4 Notes

4.1 Drying of Biomass Materials

The raw biomass materials are spread on the floor to dry at ambient conditions for 24 h, which will prevent the structural/compositional changes in biomass.

4.2 Priming Operation

There is no foot valve connected to a suction pipe of a centrifugal pump of the HC reactor. So, the pump should be started after a priming operation. The priming means water is filled in the suction pipeline and impeller section to remove the gases present in the system, which will help the pump to run smoothly.

4.3 Acetate Buffer for Biomass Slurry Preparation with Biocatalyst

1. Take a quantity of 800 mL distilled water in a suitable container.
2. Add 7.721 g of sodium acetate to the solution.
3. Add 0.353 g of acetic acid to the solution.
4. Adjust solution to final desired pH (4.5) using HCl or NaOH.
5. Add distilled water until volume is 1 L.
6. The commercial laccase enzyme was stored in acetate buffer of pH 4.5 at 4 °C.

5 Conclusion

A combination of the hydrodynamic cavitation with the biochemical catalyst is a viable technology for pretreating the lignocellulosic biomass feedstocks. The combined effects of catalyst and free radicals can improve the lignin reduction in biomass feedstocks, and the pretreatment process takes place under ambient conditions. Therefore, this method requires lesser energy inputs as compared to other energy-intensive pretreatment methods.

References

1. Baruah J, Nath BK, Sharma R, Kumar S, Deka RC, Baruah DC, Kalita E (2018) Recent trends in the pretreatment of lignocellulosic biomass for value-added products. Front Energy Res 6:141
2. Saini JK, Saini R, Tewari L (2015) Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments. 3 Biotech 5:337–353
3. Wyman CE, Hinman ND (1990) Ethanol: fundamentals of production from renewable feedstocks and use as transportation fuel. Appl Biochem Biotechnol 24:735–775
4. Agbor VB, Cicek N, Sparling R, Berlin A, Levin DB (2011) Biomass pretreatment: fundamentals toward application. Biotechnol Adv 29:675–685
5. Sathitsuksanoh N, George A, Zhang Y, Percival H (2013) New lignocellulose pretreatments using cellulose solvents: a review. J Chem Technol Biotechnol 88(2):169–180
6. Ramesh D, Kiruthika T, Sivakumar U (2019) Hydrodynamic cavitation: a promising technology for biomass pretreatment. Int J Environ Sci Nat Res 19:84–88
7. Rosales-Calderon O, Arantes V (2019) A review on commercial-scale high-value products that can be produced alongside cellulosic ethanol. Biotechnol Biofuels 12(1):240
8. Kim JS, Lee YY, Torget RW (2001) Cellulose hydrolysis under extremely low sulfuric acid and high-temperature conditions. Appl Biochem Biotechnol 91–93:331–340
9. Jacobsen S, Wyman CE (2000) Cellulose and hemicellulose hydrolysis models for application to current and novel pretreatment process. Appl Biochem Biotechnol 84–86:81–96
10. Kim TH, Kim JS, Sunwoo C, Lee YY (2003) Pretreatment of corn stover by aqueous ammonia. Bioresour Technol 90:39–47
11. Yoo CG, Nghiem NP, Hicks KB, Kim TH (2011) Pretreatment of corn stover using low-moisture anhydrous ammonia (LMAA) process. Bioresour Technol 102:10028–10034
12. Choi WI, Park JY, Lee JP, Oh YK, Park YC, Kim S, Park JM, Kim CH, Lee JS (2013) Optimization of NaOH-catalyzed steam pretreatment of empty fruit bunch. Biotechnol Biofuels 6:170
13. Timung R, Mohan M, Chilukoti B, Sasmal S, Banerjee T, Goud VV (2015) Optimization of dilute acid and hot water pretreatment of different lignocellulosic biomass: a comparative study. Biomass Bioenergy 81:9–18
14. Liu ZH, Chen HZ (2015) Xylose production from corn stover biomass by steam explosion combined with enzymatic digestibility. Bioresour Technol 193:345–356
15. Hilares RT, de Almeida GF, Ahmed MA, Antunes FA, da Silva SS, Han JI, dos Santos JC (2017) Hydrodynamic cavitation as an efficient pretreatment method for lignocellulosic biomass: a parametric study. Bioresour Technol 235:301–308
16. Thangavelu K, Desikan R, Taran OP, Uthandi S (2018) Delignification of corncob via combined hydrodynamic cavitation and enzymatic pretreatment: process optimization by response surface methodology. Biotechnol Biofuels 11(1):203
17. Nakashima K, Ebi Y, Shibasaki-Kitakawa N, Soyama H, Yonemoto T (2016) Hydrodynamic cavitation reactor for efficient pretreatment of lignocellulosic biomass. Ind Eng Chem Res 55(7):1866–1871
18. Pandit AB, Pinjari DV (2010) Cavitation milling of natural cellulose to nanofibrils. Ultrason Sonochem 17:845–852
19. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker DLAP (2008) Determination of structural carbohydrates and lignin in biomass. Laboratory analytical procedure 1617(1):1–16



Chapter 3

Algae: Biomass to Biofuel

Vineet Kumar Soni, R. Krishnapriya, and Rakesh Kumar Sharma

Abstract

Worldwide demand for ethanol alternative fuel has been emerging day by day owing to the rapid population growth and industrialization. Culturing microalgae as an alternative feedstock is anticipated to be a potentially significant approach for sustainable bioethanol biofuel production. Microalgae are abundant in nature, which grow at faster rates with a capability of storing high lipid and starch/cellulose contents inside their cells. This process offers several environmental advantages, including the effective utilization of land, good CO2 sequestration without entering into "food against fuel" dispute. This chapter focuses on the methods and processes used for the production of bioethanol biofuels from algae. Thus, it also covers significant achievements in the research and developments on algae bioethanol production, mainly including pretreatment, hydrolysis, and fermentation of algae biomass. The processes of producing biodiesel, biogas, and hydrogen have also been discussed.

Key words Microalgae, Biofuel, Bioethanol, Biomass, Biogas, Biohydrogen

1 Introduction

Rising concerns over the ever-growing energy demand and limited availability of fossil fuels led to the search for alternative sources [1, 2]. The use of these fossil fuels increases greenhouse gas (GHG) in the environment and relates to climate change. In this prospect, algae are foreseen as promising renewable feedstock [3–9]. This chapter focuses on the methods and processes to produce biofuel from algae and simultaneously reviews significant achievements in this field. Over the first (edible starch, sugar, animal fats, and vegetable oils) and second-generation (cellulose, hemicellulose, or lignin) biomass feedstocks, third-generation, which mainly based on micro- and macroalgae, have several advantages. Marine algae, which make up about 80% of the world's living organisms, cover many energy sources, such as sugars and lipids. These are simple photosynthetic, eukaryotic organisms and have a rapid growth rate. Unlike microalgae, which are unicellular, the macroalgae are multicellular and possess plant-like features [10, 11]. Some forms of microalgae have air bladders that help them to float on water

surfaces. Some types of algae are of long blades, some types are of branched and others are leafy and forms mats [6]. Algae are eukaryotic organisms, which grow rapidly in mass by photosynthesis. The cultivation of algae is very simple as it does not require freshwater bodies and can tolerate and adapt to adverse environmental conditions [9]. Algae can be efficiently grown in brackish coastal water, seawater, and wastewater by utilizing the water and fertilizers. The land areas which are inappropriate for the agricultural crop can be effectively used to cultivate algae [12, 13]. In comparison with other terrestrial plants, algae consist of 70–90% of water, quite high protein content, and variable levels of carbohydrates. Another remarkable benefit of algal biomass is that it does not compete with common food resources.

Algae can produce valuable by-products such as lipids (which can be processed into biodiesel), carbohydrates (which can be processed into ethanol), proteins (which can be used for consumption), and other useful materials [14–19]. The fuels produced from algae are carbon neutral or proved to be carbon-capturing than releasing [20]; therefore, the practice of utilizing lignocellulosic biomass from higher plants for energy requirements can be ideally replaced by microalgal biomass [21]. However, the production of microalgal biomass is comparatively expensive than lignocellulosic plant biomass, which can possibly be dealt with through genetic and metabolic engineering. The average value of the maximum specific growth rate of microalgae is about ten times higher than higher plants [9]. Furthermore, algae are photosynthetically more active for the reason being composed of individual cells that absorb all the required components including nutrients directly from the surroundings [8]. More interestingly, CO_2 can be absorbed by algae in the form of bicarbonate unlike higher plants, which absorb it through pores [8]. The ease of harvesting algae biomass due to its short life cycles beats the plant biomass, which remains grounded much longer. The steps of producing algae and its processing to access fuel and other valuable products are shown in Fig. 1.

Macroalgae or seaweeds are commonly used for food, and extraction of useful substances such as agars, alginates, and carrageenans. However, macroalgae offer no real advantage in terms of productivity, which is comparable to terrestrial plants. For example, the annual dry biomass productivity of sugarcane is ~10 kg m^{-2} compared to ~13.1 kg m^{-2} for the cultivated brown macroalgae [23].

2 Classification of Algae

All algae contain green chlorophyll, which is concealed by specific photosynthetic pigments that provide distinct color to them. Based on the type of chlorophyll present, algae are classified into different

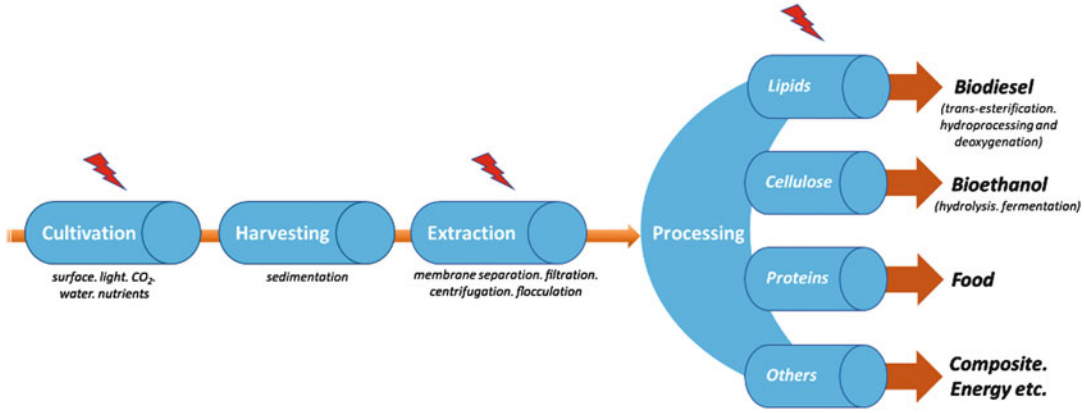


Fig. 1 Major processes in algae production and utilization (modified from ref. [22])

groups [24]: (i) brown algae (*Phaeophyceae)*, which consist of the large kelps; (ii) green algae (*Chlorophyceae*), which include sea lettuce; (iii) red algae (*Rhodophyceae*); (iv) blue-green algae *(Cyanophyta)*; and (v) golden algae (*Chrysophyceae*). Among these, green algae have the evolutionary and biochemical affinity with higher plants [25]. Brown seaweed is the single largest macroalgae resource and is one of the most likely candidates for energy processing. Depending on the size and morphology, algae are classified into two main categories, namely macroalgae and microalgae. Due to the lipid content, microscopic algae are capable of floating in waters and are called *phytoplankton*. While macroscopic algae can attach to rocks and are called seaweeds. Scientists have identified more than 30,000 species of microalgae, and they are broadly circulated in lakes, sea, and other freshwater bodies. Microalgae are widely researched as a fuel due to their high photosynthetic efficiency, and due to its ability to produce lipids. On the other hand, macroalgae do not generally contain lipids but they are rich in natural sugars and carbohydrates, and therefore fermented to produce biogas or alcohol-based fuels.

3 Cultivation of Algae

To develop a suitable cultivation process, an understanding of each species and their characteristic life history is needed. Macroalgae (seaweed) and microalgae have different cultivation process. Since the small microalgae have the size of a few micrometers (μm), they are usually cultivated in a specifically dedicated area such as on land or floating on water. However, macroalgae can be grown directly in the open sea. The cultivation can be done in different ways varying from small laboratory-scale production using a small fermenter/illuminated photobioreactor to the box-type water tank

[26–29]. For a laboratory scale, the fermenter system uses either an external or external irradiation system by a fluorescent lamp. Continuous illumination photobioreactor has many advantages like it can be heat sterilized under pressure and can minimize the contamination. Typically, the seeds grown to plantlet size in greenhouses are attached to some rope like substrates and transplanted to coastal farms for growing into harvestable size. Mass cultivation of algae can be done in either open pond system or closed systems (photobioreactors-PBRs) [30–32]. Open pond systems include natural waters, such as lakes, lagoons, and ponds. The artificial ponds or containers include shallow ponds or containers. Even though these are very cost-effective and easy to set up and operate, there are certain significant limitations. This includes the difficulty in controlling the process, poor diffusion of CO_2 to the atmosphere, poor absorption of light, and availability of large land area and these systems are prone to environmental changes, etc. Contamination of the system by the invasion of heterotrophic algae as well as other bacteria also experienced significant problems for its commercial production. To avoid this problem, researchers have introduced a genetic modification of the algae for industrial applications [33]. For this, different molecular methods have been developed to synthesize as well as clone genes and thereby transform or transfer them into living organisms. Most of the microalgae species have undergone modifications to produce genetically modified organisms (GMOs) or genetically enhanced organisms (GEOs). The technique of this genetic modification is known as recombinant DNA technology, which develops different organisms with modified or new traits [34, 35]. In a closed system, the typical growth requirements are internally maintained. It facilitates well control of the culture environment by allowing ideal growth conditions of CO_2, H_2O, pH, light, etc. There are two major types of enclosed PBRs, tubular and plate type, respectively. The most suitable PBR is the tubular one that has a high surface area, low shear forces, low cost, high efficiency of CO_2 use efficiency, and the ability to use sunlight effectively [36]. Here, the solar collector tubes generally consist of plastic/glass, and continuous circulation of the culture is maintained by a pump as well as an air-lifting method.

4 Factors Affecting the Algae Growth

Several factors that influence the algae growth are the intensity of incident light, culture temperature, the density of algal biomass, homogeneity of culture mixing, presence of nutrients (nitrogen, phosphorus, sulfur, silica, and iron), etc. [37, 38].

5 Algae Biomass as a Potential Source of Biofuel

Considering the fact that almost 80% of the energy consumed for domestic purposes across the world is derived from fossil fuels (kerosene, diesel, gasoline, etc.) [39], there is an urgent need for alternative sources to satisfy the growing demand as a part of expanding civilization. To address this serious issue, there is a need to discover renewable carbon energy sources from biomass. Furthermore, the large-scale production of biofuel provides sustainable benefits to the environment, society, and economy, as the combustion of fossil fuel results in greenhouse gas emissions and thus the global warming. Bioethanol and biodiesel are the most commercially available forms of renewable energies from starch and oil yielding crops, respectively. However, the production of ethanol from cellulosic material of corn and sugarcane is yet to reach commercial value. Besides, the first- (fatty acid esters) and second-generation (deoxygenated hydrocarbons) biodiesel are derived from naturally occurring vegetable oils or animal fat [40, 41]. In recent years, there is still an urge to find new sustainable sources without competing with agriculture. Henceforth, algae seem more promising as an alternative carbon energy source [42]. In particular, microalgae are a good source with a promising amount of biomass as well as high oil content suitable for biodiesel production [21]. Comparatively, microalgae have high productivity with rapid growth by 5–20 times higher CO_2 fixation than plants and good potential of utilizing solar energy (around 10% of total absorbed energy) during photosynthesis [30]. Microalgae have no competition with other food crops as it can grow in several conditions. Furthermore, microalgae biomass can be extracted and processed more conveniently for cleaner production of biofuel than petroleum fuel [43]. Also, the residual biomass after the extraction of cellulose and lipid contents can be used to produce high-value products such as biogas. Similarly, seaweeds or macroalgae have higher biomass productivity than planktonic species and terrestrial plants, and therefore can be considered as an alternative energy source [23]. In addition, algae secrete some premium metabolites with mixed bioactivities, derived from essential acids, carbohydrates, pigments, fertilizers, and nutraceuticals. The sequestering of greenhouse gas to yield lipids is very promising besides their possible use as metal bio-indicators and pest control. Remarkably, the choice of algae class being cultivated and advances in metabolic engineering can support the production of one or more high-value products [7, 44].

The biofuel from microalgae can be classified into the following types based on their chemical composition and applications:

1. Algae biomass: direct combustion, thermochemical upgrading to syngas, biohydrogen, bio-oil and char, and biogas through anaerobic digestion
2. Algal carbohydrates/sugars: for bioethanol production via fermentation
3. Algal crude oil (lipids): for combustion or chemical processing to produce biofuels such as diesel, biodiesel, jet fuel, and gasoline
4. Biogas and biohydrogen

5.1 Direct Processing of Biomass

In a nutrition-rich medium with no starvation period, the microalgae comprise (by dry mass) 30–50% protein, 20–40% carbohydrate, and 8–15% lipids [45]. Wherein, the energy-rich lipid content can be increased by deploying the special conditions, particularly nitrogen starvation during algae cultivation. The enthalpy of combustion of dry algal biomass is generally in the range of 16.9–21 MJ kg^{-1} [46]. Though the algal biomass can be burnt directly in a boiler or together with coal in power plants, the process of converting them into more valuable and clean energy substances is highly desirable [47]. Therefore, thermochemical processes such as gasification, pyrolysis, and hydrothermal liquefaction are in practice to transform biomass into various products as potential fuels. However, the practicability of reported processes is dubious in view of the high water content in algal biomass [8]. As we know, pyrolysis thermally decomposes the dry biomass to gaseous, liquid, and solid products under anaerobic conditions [48–50]. Wherein, the fast pyrolysis of lignocellulosic biomass, performed at temperatures of 450–600 °C, is suitable to produce fuels such as bio-oil, syngas, and char. On the other hand, gasification is usually performed at 800–1000 °C of dry biomass to produce syngas (CO, CO_2, and H_2) by inducing partial oxidation [51, 52]. Another advanced treatment, hydrothermal liquefaction, requires relatively low temperature, i.e., 300–400 °C, but in the presence of a catalyst and hydrogen at 4–20 MPa pressure process to majorly produce bio-oil along with gas and char [53].

5.2 Biomass to Crude Oil and Its Processing

The process of extracting biomass from harvested algae culture is followed by chemical and/or enzymatic treatments to obtain the products for cleaner energy. Besides, the seaweeds collected often have the presence of stones, sand, litter, adhering epifauna, and other forms of debris that should be removed before further processing [54]. The degree of processing always depends on the mode of culture and its use. The general preprocessing involves mainly three steps: removal of foreign particles by washing, milling, and then dewatering. The main steps of producing biofuels are described in the following sections.

5.2.1 Extraction

Biomass can be separated from the culture medium by filtration, centrifugation, or flocculation (colloidal separation) [55]. Alum, ferric chloride, and chitosan are the chemical flocculants that are generally used in the flocculation step. Due to the use of these chemicals, the flocculation process is not cost-effective for large operations. The separation of lipid contents from the biomass residue is another challenging process. When algae are dried, it retains the oil content. Crushing of biomass with an oil press can give the oil. The commercial manufacturers make use of a method based on combining mechanical pressing and solvent extraction. In the enzymatic extraction process, the degradation of algal cell walls occurs by enzymatic action. Other methods are osmotic shock and ultrasonic extraction technique, in which the sudden reduction in osmotic pressure ruptures the cell wall and allowed to release of the lipid contents into a specific solvent. In solvent extraction process, firstly, the algae were ground with mortar and pestle and subsequently dried in the incubator to remove the water content. To extract the oil content, dried biomass is kept for 24 h in a mixture of hexane and ether. The solvents that are commonly used for chemical extraction are hexane and toluene. Hexane is most appropriate to extract oil from dried microalgal biomass as well as from microalgal paste [56]. The use of hexane for solvent extraction has the disadvantage of additional processing methods such as distillation to remove the excess and residual solvent. To address this, recently, a switchable solvent was synthesized from amides and moderately hydrophilic molecule, which can be easily removed from the extracted oil with the help of carbonated water [57–59].

5.2.2 Bioethanol Production

Bioethanol production from algae requires four main steps comprising pretreatment, hydrolysis, fermentation, and distillation, as shown in the block diagram of Fig. 2.

Among these different processes, pretreatment is the most important step, because it directly influences the efficiency of overall bioconversion. The significance of the pretreatment step is to disturb the structure of cellulose and to make them more accessible to the enzymes during the fermentation process [60]. It is understood that the effective pretreatment can terminate physical barriers in the cell wall and greatly improve the enzymatic hydrolysis rates, fermentation, and other process variables. The process separates the cellulose, hemicellulose, and lignin by the enzyme-catalyzed hydrolysis. Afterward, the sugars are fermented with the use of specific ethanol-producing microorganisms. As a final point, the produced ethanol is purified by distillation. Various physical, physicochemical, chemical, and biological processes are used for the pretreatment of algal materials, wherein each pretreatment process is subdivided.

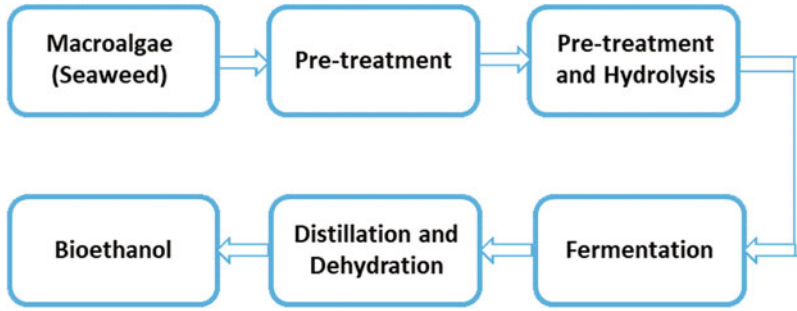


Fig. 2 Steps to produce bioethanol from macroalgae

Physical Pretreatment

Comminution: Size Reduction of Biomass

The mechanical process of pretreatment is used to reduce the particle size of seaweeds by methods like ball milling, chipping, and grinding. The resultant small particles exhibit high surface area, which is desirable for effective processing in next steps such as fermentation.

Microwave/γ-Ray Irradiation

These irradiation methods often give a high yield of sugars from algae biomass. When combined with acid treatment, the irradiation technique enhances the saccharification process to yield bioethanol. During the γ irradiation, the cell walls of algae break, and the resulting concentration of reducing sugar in hydrolysates can be increased effectively. Another efficient method is the microwave irradiation. This method appears to be the easiest method, where the samples are subjected to microwave irradiation, and further any of the treatment step is not required [61]. Here, the internal heat is generated inside the biomass and the aqueous medium by the microwave radiation through the mechanisms of dipolar polarization and ionic conduction. This action induces vibrations in the polar bonds of molecules, and the electrons try to align in the applied electric field. As the applied field oscillates, the dipole or ion field attempts to realign itself with the alternating electric field, and the energy is lost in the form of heat through molecular friction and dielectric loss. Thus, effective homogenous heating of the sample occurs [62, 63].

Ultrasonication

This process utilizes the sound energy of high frequencies to break particles and agglomerates by cavitation, the expansion, and the implosion of bubbles. When ultrasonic waves are allowed to pass through the liquid medium, a large number of microbubbles form. These microbubbles can grow and finally collapse in a very short time of a few microseconds. As a result, alternating low-pressure and high-pressure waves are produced in liquids, which leads to the formation of small vacuum bubbles, and finally the violent collapse of these formed bubbles. This enables the breakdown of algae biomass into tiny particles with a very high surface area, which

enhances the enzymatic reaction process [64]. In this situation, the microalgal cells experience a force that drives them into the planes of pressure nodes when exposed to ultrasonic standing waves. When ultrasonication is turned off, the aggregated cells settle rapidly due to the gravitational force [30, 65]. The ultrasound method can be further modified as a solvent-free method to extract lipids from fresh microalgae cells [66]. This solvent-free extraction technique is sustainable and cost-effective since there is no requirement of solvent removal from the medium. Furthermore, this process eliminates the chance of contamination.

Biological Pretreatment

In biological pretreatments, cell degradation of algae was done by purified enzymes, enzymatic cocktails (either a mixture of purified enzymes or extracts produced by hydrolytic microorganisms), and to the use of cultures of microorganisms with hydrolytic activities [67]. Most of the pretreatment methods need costly instruments and high energy input for biomass conversion. Whereas, the biological treatment makes use of various types of rot fungi which is very safe and environmentally friendly, and has high selectivity and product recovery. The rot fungi at lower temperatures are often used and found to be a safe and environmentally friendly method [68]. Microorganisms such as brown-, white-, and soft-rot fungi are often used to degrade lignin and hemicellulose biomass materials. Brown rots fungi mostly attack the cellulose content, while white as well as soft rots attack both cellulose and lignin. Biological pretreatment in combination with other pretreatment technologies has also been studied for algae biomass. Although the biological pretreatment has the advantage of using very low energy and mild environmental conditions, the rates of hydrolysis of these processes are comparatively very low with other pretreatment methods.

Physicochemical Pretreatment

This method is commonly used for the pretreatment of lignocellulosic biomass materials [69], in which the biomass is treated with water under high pressure and temperature around 15 MPa and 150 °C. The water molecules can penetrate the biomass, and thereby hydrate cellulose and remove hemicellulose and part of lignin. Consequently, the hydrolysis efficiency of the algae increases. The main advantage of this technique is the cost-effectiveness, as it does not need any added chemicals.

Hydrothermal Pretreatment

Steam Explosion

Here the small biomass particles are heated rapidly at high-pressure saturated steam for a certain time. When the pressure is suddenly reduced, the biomass particle experiences an explosive decompression. The steam penetrates the cell wall, instigating hemicellulose breakdown, and lignin transformation due to high temperature, and thus enhance the process of cellulose hydrolysis [70]. Usually, the steam explosion starts at a temperature of 160–260 °C and a pressure at 0.69–4.83 MPa for several seconds to a few minutes

before the material is exposed to any atmospheric pressure. The chemicals like H_2SO_4 or SO_2 are added into the steam to effectively improve the conversion efficiency of polysaccharides to monomeric sugars. With autohydrolysis and explosive depressurization size distribution, shapes, and chemical composition of the biomass feedstock are altered. The most significant factors which affect the steam explosion are temperature (T) and residence time (t). Also, the combined effect of both temperature and time affect the conversion process. On the other hand, the method is not suitable for algal biomass conversion because of its high cost.

Supercritical Carbon Dioxide

The process is similar to the steam explosion, but it does not cause any degradation of sugars, and therefore considered as an environmentally benign technology [71]. The CO_2 is nontoxic, nonflammable, inexpensive, and can be readily available as a by-product of many industrial processes. Supercritical fluids have properties of both gas and liquid. Above the critical point, the fluid exhibits gas-like viscosity and liquid-like density. Since the CO_2 is gas at ambient pressure, the separation of the targeted product is very easy. In addition, the depressurization can be done step by step, allowing a fractionating of the extracted compounds based on their solubility variation with density. Thus, it can effectively penetrate the small pores of algae biomass residue and can hold more fluid. Approximately 30 diverse algae species have been successfully studied by supercritical-CO_2 pretreatment [72]. It usually adds acidity to the wet biomass, which again enhances the pretreatment process.

Chemical Pretreatment

Alkaline Pretreatment

The alkaline pretreatment makes use of bases, such as Na, K, Ca, and NH_4OH, in the process. During this process, the lignin content from the biomass is removed and the reactivity of polysaccharides is enhanced. This can also remove acetyl and other uronic acid substitutions on hemicellulose [73]. The method has the advantage of using lower reaction temperatures and pressure, but the damage of cellulose content is a major concern, which results in low ethanol yields. When NH_4OH is used, the process has the advantages of lower moisture content, less sugar degradation products due to moderate operational conditions, almost complete recovery of solid material, and high cellulose digestibility for enzymatic fermentation. However, the major concern of this process is the high cost of ammonia and the need for efficient recycling technologies.

Acid Hydrolysis

Acid hydrolysis is the commonly accepted chemical pretreatment method, wherein the concentrated (H_2SO_4 and HCl), as well as dilute, acids are used [72]. The hydrolysis reaction takes place at temperatures as low as (40 °C) with 30–72% concentrated acids, and up to 100% of sugar yields can be obtained at such low temperatures. Conversely, the use of concentrated acid is not

desirable for large-scale reactions because of the toxicity and corrosive nature of acids. The use of such strong acid requires acid-resistant bath materials to avoid corrosion. Furthermore, the recovery of these acid materials and the related recycling steps make the process expensive. Mainly, dilute acids were used extensively for the hydrolysis process. Among different acids used (such as H_2SO_4, HCl, H_3PO_4, and $C_4H_4O_4$), dilute H_2SO_4 is widely accepted for algae biomass treatment [73]. The parameters which directly influence the acid treatments involve the quantity of biomass feed, the concentration of acid, temperature, and the treatment time. Notably, the processing temperature plays a crucial role in bioethanol production. The formation of certain by-products that are responsible for the inhibition of reaction and pH neutralization of the medium causes major disadvantages in the acid pretreatments.

Enzymatic Hydrolysis

In this process, complex carbohydrates are converted into simple monomers with the help of enzymes [74]. The process can be done in mild environmental conditions at a pH of 4.5–5 and temperatures of 40–50 °C and is not associated with any of the corrosion problems. Cellulosic hydrolysis is catalyzed by a class of enzymes called cellulase produced by bacteria or fungi. Cellulase degrades the cellulose and converts into sugars that can be fermented by yeasts or bacteria to ethanol. The action of cellulolytic enzymes occurs through three steps of adsorption, biodegradation, and desorption. Particularly, *Trichoderma* is the most successfully used microbial strain. Temperature, pH, biomass concentration, and the amount of enzyme loading are the main factors that directly influence the enzymatic hydrolysis.

Fermentation

Fermentation is the biological process whereby sugars are converted to bioethanol with the help of fermenting microorganisms. During this, fermentable sugars, such as glucose, fructose, maltose, and rhamnose, are used as substrates by microorganisms and convert them to ethanol and CO_2 [75]. The selection of this process mainly depends on the composition of biomass feedstocks. The fermentation of biomass to ethanol involves mainly two reactions. The first reaction is the hydrolysis of organic material to simple sugars. This step involves the use of high temperatures, acids, or enzymes. The second reaction is the fermentation of sugars to ethanol. The fermentation step is doing with the help of some microorganisms, which will utilize all sugars, glucose, xylose, mannose, galactose, and arabinose. The best-proved microorganisms for ethanol production from hexoses are the yeast *Saccharomyces cerevisiae* and the bacterium *Zymomonas mobilis*. Enzymatic hydrolysis and fermentation process can be accomplished using different strategies: Separate enzymatic hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and simultaneous saccharification and co-fermentation (SSCF).

Fungal enzymes to convert lignocellulose to sugars require temperatures in the range of 45–60 °C with hydrolysis time 3 days or longer. Separate hydrolysis and fermentation (SHF) can be run to let each of the processes to take place at the ideal temperature. Accordingly, this method can be applied to large-scale applications [76]. This involves the use of low-cost chemicals, short processing time, and straightforward equipment systems. In SHF, the pretreated algae biomass is hydrolyzed to glucose and successively fermented to ethanol as separate units. The only shortcoming of this method is the released sugars during the fermentation inhibit the activity of enzyme cellulases.

SSF is a single process, which combines both the fermentation and saccharification. In this process, the enzyme cellulase and the microorganism are introduced into the same process unit, and the fermenting microorganism consumes the glucose immediately. The yield of ethanol using the SSF process is comparatively higher than the SHF. The advantages of this method are the high bioethanol yield with the use of the low quantity of enzyme, reduced foreign contamination, less inhibitory effects, and low cost, but the disadvantage is that neither hydrolysis nor fermentation can be performed under optimal conditions [77]. SSCF represents the hydrolysis and co-fermentation of both hexose and pentose sugars in one reaction vessel [78].

The fermentation of the hexose sugar can be achieved easily using a variety of yeast and bacteria, whereas the fermentation of pentose sugar is complicated. The genetically engineered microorganisms are used for the fermentation process.

Distillation

To extract oil from algae, several efficient methods including mechanical systems, enzymatic, osmotic, supercritical, and ultrasonic extraction techniques are used. However, these methods are not capable enough to offer long-term solutions due to the excessive use of solvents. Extra care must be taken to design the extraction system to eliminate vapor exposure and direct contact. This makes the extraction process highly expensive. Most of the plants use simple distillation or fractional distillation to intensify the extracted oil [79]. Herein, the biomass is treated with the solvent *n*-hexane to extract the remaining oil after the pretreatment, which can be separated using any of the distillation units, as stated earlier. Another advanced distillation technique is reactive distillation, which combines the chemical reactions and product separation in a single unit [80]. This has the advantage of continuous removal of the product formed and avoids the conversion limitation of equilibrium [81]. Since there is no need for separation units, the time and cost of the production can be lowered considerably. As this distillation process integrates both reaction and separation in a single unit, the method can provide high conversion, selectivity, and flexibility.

5.2.3 Biodiesel Production

In comparison, microalgae have lipid content higher than macroalgae, which could be increased further by nitrogen starvation during microalgae cultivation. Consequently, the agronomy of microalgae is centered on producing biodiesel. The solubility of lipid contents including triglycerides, hydrocarbons, sterols, and some carotenoids in organic solvents (such as toluene, ether, and chloroform) is useful in the production of diesel, kerosene, and gasoline-equivalent biofuel from algal biomass. The solvent-based extraction process avoids the expensive and time-consuming drying process for the recovery of crude algae oil [82, 83]. The process of producing first-generation biodiesel is easier, wherein, the transesterification reaction of triglycerides with alcohol (i.e., methanol, ethanol) is carried out to produce methyl/ethyl esters [84, 85]. These fatty acid esters can be blended with conventional diesel for application in existing diesel engines. However, recent advancements in the process of making biodiesel target the production of oxygen-free biodiesel, i.e., diesel-like hydrocarbons. This process requires deoxygenation catalysts, usually heterogeneous (supported transition metal) catalysts in the presence of variable amounts of hydrogen [86]. Indeed, significant developments have been made in the recent past for the efficient and selective deoxygenation of triglycerides utilizing noble and non-noble metal-based catalysts [87, 88]. Further, the isomerization and hydrocracking of resulting molecules in situ or as an additional step yield gasoline and jet fuel-like hydrocarbons [89]. The process of producing biodiesel is shown in Fig. 3.

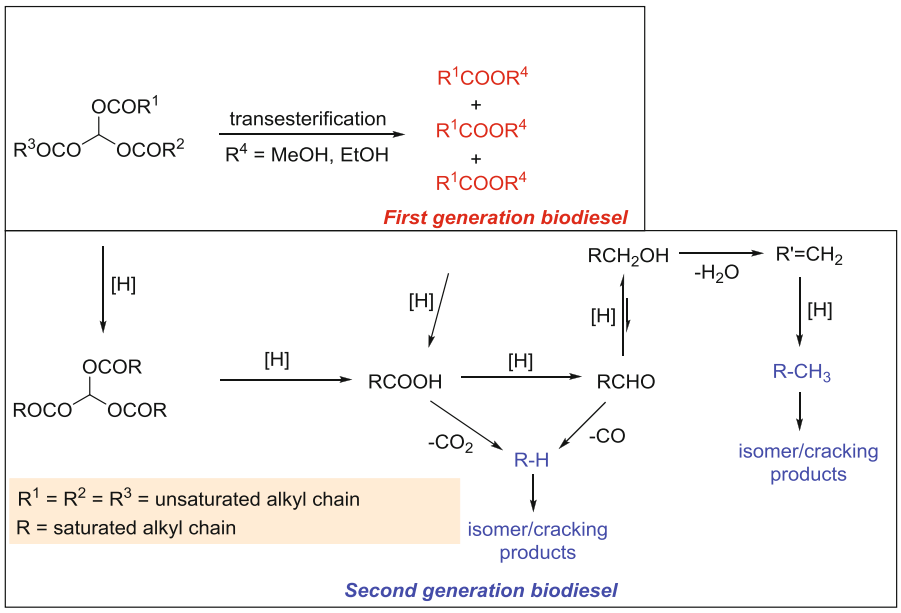


Fig. 3 Production of biodiesel from algae-derived triglycerides (modified from ref. [90])

Compared with the fatty acid ester-based biodiesel, the second-generation biodiesel possesses a higher cetane number, lower cloud point, and higher oxidation stability, and therefore can be fed directly to the current diesel engines. The exploitation of suitable targets with low oxygen content such as vegetable oils, nonedible oils, and microalgae oil is more convenient and affordable. The hydrodeoxygenation selectivity (HDO) to yield C_N hydrocarbons over decarboxylation/decarbonylation processes is highly desirable to obtain increased mass yields and high energy value [90–92]. The reactions under hydrogen modest environments are distinguished; however, some amount of H_2 is required for hydrogenolysis of triglyceride bonds, which avoids oligomer formation or catalyst deactivation [93, 94]. The use of organic solvents causes environmental concerns and requires additional separation processes. Therefore, solvent-free deoxygenation is highly desirable [90]. In alternative approaches, where hydrothermal deoxygenation (rather limited to free fatty acids) is realized, the incomplete conversion, nonselective deoxygenation, and formation of oligomerized products as well as sintering or phase transition of metallic phases in heterogeneous catalysts are major concerns [95–100].

5.2.4 Biogas

The production of biogas from algae biomass is an expensive deal; therefore, only the oil-extracted spent biomass is utilized for the anaerobic digestion process [101]. The exact composition of biogas subjects to the biomass feed, and usually consists of methane (50–75%) and carbon dioxide (25–50%), wherein the presence of trace amounts of hydrogen sulfide and ammonia cannot be discarded [102]. The process of anaerobic digestion benefits from recovering and recycling of N and P containing inorganic nutrients. Diverse wastes can be anaerobically digested using mixed microbial cultures. In principle, the concentrated whole microalgal biomass paste may be anaerobically digested instead of the oil-extracted spent biomass. Notably, the process of anaerobic digestion of total lipid-based biomass produces molecules with higher energy compared to the sequence of biodiesel extraction and microbial digestion [103]. The process of producing biogas requires different microorganisms at different stages. Firstly, the acidogenic bacteria hydrolyze the wet biomass to degrade the proteins, carbohydrates, and fats to amino acids, sugars, and fatty acids, respectively. Other byproducts are produced, including sulfide, carbon dioxide, and ammonia. Next, the acetogenesis bacteria convert volatile fatty acids and alcohols to hydrogen and carbon dioxide. Finally, the process of methanogenesis is realized using methanogen bacteria, which converts hydrogen, carbon dioxide, acetic acid, and water into methane and carbon dioxide [8]. The anaerobic digestion process requires mesophilic (operation temperature 30–38 °C) or thermophilic digesters (at 50–60 °C). Since the mesophilic

digesters are appropriate for algal biomass, the hydraulic retention time in a digester is typically 15–30 days at a pH of 6.8–7.2 to make the most of biogas. On the other hand, the biomass of algae grown in seawater may contain some inhibitor ions or molecules to interfere in the digestion process [102].

5.2.5 Biohydrogen

The production of hydrogen can be realized directly from live microalgae. Several green algae yield hydrogen under anaerobic conditions, following photo- and non-photochemical pathways [104–106]. In this regard, *Chlamydomonas reinhardtii* is the most commonly used freshwater algae, which is also adaptable to seawater medium. The photochemical pathway either gets electrons through oxidation of water or glycolytic breakdown of internal substrates (such as starch) to convert H^+ to hydrogen. Another non-photochemical pathway involves the fermentation of endogenous substrates. The hydrogen production requires an oxygen-free environment, as these processes involve hydrogenases that suffer from a permanent inactivation by oxygen. Currently, the continuous production of hydrogen is not possible. The genetic and metabolic engineering of algae bargain hopes for practical hydrogen production.

5.3 Major Limitations in the Commercialization of Biofuel Production

The dense dark-green commercial algal cultures are less productive due to the lack of deep penetration of light required for photosynthesis. Therefore, the biomass concentration of culture is 0.5–5.0 g/L [101]. The practice to provide artificial light is not cost-effective, and thus delimits the commercial production of algae biofuel. Further, the excess of produced oxygen in a closed culture system has a detrimental effect on productivity. Besides, the cultivation in open ponds provides a better solution to address this problem; however, the high density of algae culture still prevents the desorption of oxygen by trapping a considerable amount of oxygen. In addition, the photooxidation of algae biomass due to the presence of excess oxygen and sunlight together is highly undesirable. The ideal temperature for photosynthesis is another concern, where 25–30 °C temperature is considered suitable for photosynthesis, whereas a somewhat lower temperature is good for biomass growth. In open ponds, the evaporation of water has a cooling effect to some extent; however, the temperature adjustments are required in closed cultures [103]. The high growth of algae due to efficient photosynthesis requires a high amount of carbon dioxide in addition to nutrients. Accordingly, the lower quantity of dissolved CO_2 in seawater than freshwater is another important issue. Similarly, the amounts of dissolved nitrates and phosphates must be supplied in significant excess, as the bioavailability of these ions depends on the presence of complex-forming metal ions in the medium [3]. Moreover, the bacterial activity on converting nitrate into nitrous oxide is also a factor.

The large-scale production of microalgae in specially designed large photobioreactors to reduce the cost of algal biomass is an expensive deal with operational difficulties [27]. The use of electrically driven paddlewheel in open channel ponds is the most widely used technology for the commercial production of algal biomass [46, 107, 108].

6 Concluding Remarks

The high productivity and sustainable processing of algae biomass have recently attracted considerable attention to produce renewable biofuels; however, the challenges of large-scale production, cost-effective and easy processing of biomass remain as major hurdles in the commercialization of algae-derived biofuels. To explore the real potential of algae biomass production, more focused long-term research is required. In this prospect, the selection of high lipid producing strains while considering their compatibility in local conditions to induce large-scale production of algae biomass is crucial. Simultaneously, the extraction of some bioactive molecules from algal stain will add to the cost-effective production of microalgae. Furthermore, the greater possibilities of genetic and metabolic engineering of microalgae cultures as compared with terrestrial plants are significant. The cultivation of algae in the presence of appropriate microorganisms and direct production of biofuel by microbial action can be a deciding factor in some ground-breaking developments in this field.

References

1. Vertès AA, Qureshi N, Blaschek HP, Yukawa H (2010) Biomass to biofuels—strategies for global industries, vol 84. John Wiley & Sons Ltd, Hoboken, pp 1819–1822. https://doi.org/10.1002/cite.201290099
2. Agarwal AK, Agarwal RA, Gupta T, Gurjar BR (2017) Biofuels: technology, challenges and prospects. Springer Nature Singapore Pte Ltd, Singapore. https://doi.org/10.1007/978-981-10-3791-7
3. Chisti Y (2008) Biodiesel from microalgae beats bioethanol. Trends Biotechnol 26:126–131. https://doi.org/10.1016/j.tibtech.2007.12.002
4. Branyikova I, Marsalkova B, Doucha J, Branyik T, Bisova K, Zachleder V, Vitova M (2011) Microalgae—novel highly efficient starch producers. Biotechnol Bioeng 108:766–776. https://doi.org/10.1002/bit.23016
5. Borowitzka MA, Moheimani NR (2013) Open pond culture systems. In: Algae for biofuels and energy, pp 133–152
6. Pandey A, Lee D-J, Chisti Y, Soccol CR (2014) Preface. In: Biofuels from algae. Elsevier, Amsterdam, pp xi–xii
7. Faizal Bux YC (2016) Algae biotechnology: products and processes. Springer Nature Singapore Pte Ltd., Singapore
8. Lee DJ, Pandey A, Chang J-S, Chisti Y, Soccol C (2018) Biomass, biofuels, biochemicals. Elsevier, Amsterdam
9. Chisti Y (2010) Fuels from microalgae. Biofuels 1:233–235
10. Hussian A (2018) The role of microalgae in renewable energy production: Challenges and opportunities. In Türkoglu, M, Önal, U, Ismen, A (eds) Marine Ecology-Biotic and Abiotic Interactions. Intech Open, London, UK, 257–283. https://doi.org/10.5772/intechopen.73573
11. Leite GB, Abdelaziz AE, Hallenbeck PC (2013) Algal biofuels: challenges and opportunities. Bioresour Technol 145:134–141. https://doi.org/10.1016/j.biortech.2013.02.007

12. Sharma YC, Singh B, Korstad J (2011) A critical review on recent methods used for economically viable and eco-friendly development of microalgae as a potential feedstock for synthesis of biodiesel. Green Chem 13:2993–3006. https://doi.org/10.1039/c1gc15535k
13. Li K, Liu S, Liu X (2014) An overview of algae bioethanol production. Int J Energy Res 38:965–977. https://doi.org/10.1002/er.3164
14. Jayathilakan K, Sultana K, Radhakrishna K, Bawa AS (2012) Utilization of byproducts and waste materials from meat, poultry and fish processing industries: a review. J Food Sci Technol 49:278–293. https://doi.org/10.1007/s13197-011-0290-7
15. Baldev E, Mubarakali D, Saravanakumar K, Arutselvan C, Alharbi NS, Alharbi SA, Sivasubramanian V, Thajuddin N (2018) Unveiling algal cultivation using raceway ponds for biodiesel production and its quality assessment. Renew Energy 123:486–498. https://doi.org/10.1016/j.renene.2018.02.032
16. Banerjee C, Ghosh S, Sen G, Mishra S, Shukla P, Bandopadhyay R (2014) Study of algal biomass harvesting through cationic cassia gum, a natural plant based biopolymer. Bioresour Technol 151:6–11. https://doi.org/10.1016/j.biortech.2013.10.035
17. Barros AI, Gonçalves AL, Simões M, Pires JCM (2015) Harvesting techniques applied to microalgae: a review. Renew Sust Energ Rev 41:1489–1500. https://doi.org/10.1016/j.rser.2014.09.037
18. Brennan L, Owende P (2010) Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products. Renew Sust Energ Rev 14:557–577. https://doi.org/10.1016/j.rser.2009.10.009
19. Cesário MT, da Fonseca MMR, Marques MM, de Almeida MCMD (2018) Marine algal carbohydrates as carbon sources for the production of biochemicals and biomaterials. Biotechnol Adv 36:798–817. https://doi.org/10.1016/j.biotechadv.2018.02.006
20. Sayre R (2010) Microalgae: the potential for carbon capture. Bioscience 60:722–727. https://doi.org/10.1525/bio.2010.60.9.9
21. Goh BHH, Ong HC, Cheah MY, Chen W-H, Yu KL, Mahlia TMI (2019) Sustainability of direct biodiesel synthesis from microalgae biomass: a critical review. Renew Sust Energ Rev 107:59–74. https://doi.org/10.1016/j.rser.2019.02.012
22. Chew KW, Yap JY, Show PL, Suan NH, Juan JC, Ling TC, Lee DJ, Chang JH (2017) Microalgae biorefinery: high value products perspectives. Bioresour Technol 229:53–62. https://doi.org/10.1016/j.biortech.2017.01.006
23. Milledge J, Smith B, Dyer P, Harvey P (2014) Macroalgae-derived biofuel: a review of methods of energy extraction from seaweed biomass. Energies 7:7194–7222. https://doi.org/10.3390/en7117194
24. https://www.biologyonline.com/dictionary/algae
25. Cyanophyta Naselli-Flores L, Barone R (2009) Green algae. In: Likens GE (ed) Encyclopedia of Inland waters. Academic Press, New York, pp 166–173. https://doi.org/10.1016/B978-012370626-3.00134-4
26. Molina GE, Fernández FGA, García Camacho F, Chisti Y (1999) Photobioreactors: light regime, mass transfer, and scaleup. J Biotechnol 70:231–247. https://doi.org/10.1016/S0168-1656(99)00078-4
27. Miron ASN, Gomez AC, Camacho FGA, Grima EM, Chisti Y (1999) Comparative evaluation of compact photobioreactors for large-scale monoculture of microalgae. J Biotechnol 70:249–270
28. Carvalho AP, Meireles LA, Malcata FX (2006) Microalgal reactors: a review of enclosed system designs and performances. Biotechnol Prog 22:1490–1506. https://doi.org/10.1021/bp060065r
29. Acién FG, Molina E, Reis A, Torzillo G, Zittelli GC, Sepúlveda C, Masojídek J (2017) 1—Photobioreactors for the production of microalgae. In: Gonzalez-Fernandez C, Muñoz R (eds) Microalgae-based biofuels and bioproducts. Woodhead Publishing, Kindlington, pp 1–44
30. Suali E, Sarbatly R (2012) Conversion of microalgae to biofuel. Renew Sust Energ Rev 16:4316–4342. https://doi.org/10.1016/j.rser.2012.03.047
31. Terry KL, Raymond LP (1985) System design for the autotrophic production of microalgae. Enzym Microb Technol 7:474–487. https://doi.org/10.1016/0141-0229(85)90148-6
32. Sompech K, Chisti Y, Srinophakun T (2012) Design of raceway ponds for producing microalgae. Biofuels 3:387–397. https://doi.org/10.4155/bfs.12.39
33. Molina Grima E, Belarbi EH, Acién Fernández FG, Robles Medina A, Chisti Y (2003) Recovery of microalgal biomass and metabolites: process options and economics.

Biotechnol Adv 20:491–515. https://doi.org/10.1016/S0734-9750(02)00050-2

34. Kuiper HA, Kleter GA, Noteborn HPJM, Kok EJ (2001) Assessment of the food safety issues related to genetically modified foods. Plant J 27:503–528. https://doi.org/10.1046/j.1365-313X.2001.01119.x
35. Letourneau DK, Robinson GS, Hagen JA (2003) Bt crops: predicting effects of escaped transgenes on the fitness of wild plants and their herbivores. Environ Biosaf Res 2:219–246. https://doi.org/10.1051/ebr:2003014
36. Chiu S-Y, Kao C-Y, Chen C-H, Kuan T-C, Ong S-C, Lin C-S (2008) Reduction of CO_2 by a high-density culture of Chlorella sp. in a semicontinuous photobioreactor. Bioresour Technol 99:3389–3396. https://doi.org/10.1016/j.biortech.2007.08.013
37. Gao K, Yu H, Brown MT (2007) Solar PAR and UV radiation affects the physiology and morphology of the cyanobacterium Anabaena sp. PCC 7120. J Photochem Photobiol B Biol 89:117–124. https://doi.org/10.1016/j.jphotobiol.2007.09.006
38. Doucha J, Lívanský K (2009) Outdoor open thin-layer microalgal photobioreactor: potential productivity. J Appl Phycol 21:111–117. https://doi.org/10.1007/s10811-008-9336-2
39. In 2011, fossil fuels accounted for approximately 82 percent of the world's primary energy use, but this is expected to fall to 78 percent by 2040 (from EIA, Monthly Review, 2011)
40. Demirbas A, Karslioglu S (2007) Biodiesel production facilities from vegetable oils and animal fats. Energy Sources Part A Recovery Utilization Environmental Effects 29:133–141. https://doi.org/10.1080/009083190951320
41. Alptekin E, Canakci M, Sanli H (2014) Biodiesel production from vegetable oil and waste animal fats in a pilot plant. Waste Manag 34:2146–2154. https://doi.org/10.1016/j.wasman.2014.07.019
42. Suparmaniam U, Lam MK, Uemura Y, Lim JW, Lee KT, Shuit SH (2019) Insights into the microalgae cultivation technology and harvesting process for biofuel production: a review. Renew Sust Energ Rev 115:109361. https://doi.org/10.1016/j.rser.2019.109361
43. Raheem A, Prinsen P, Vuppaladadiyam AK, Zhao M, Luque R (2018) A review on sustainable microalgae based biofuel and bioenergy production: recent developments. J Clean Prod 181:42–59. https://doi.org/10.1016/j.jclepro.2018.01.125
44. Lü J, Sheahan C, Fu P (2011) Metabolic engineering of algae for fourth generation biofuels production. Energy Environ Sci 4:2451–2466. https://doi.org/10.1039/C0EE00593B
45. de Farias Silva CE, Bertucco A (2016) Bioethanol from microalgae and cyanobacteria: a review and technological outlook. Process Biochem 51:1833–1842. https://doi.org/10.1016/j.procbio.2016.02.016
46. Chisti Y (2013) Raceways-based production of algal crude oil. Green 3:195
47. Azizi K, Keshavarz Moraveji M, Abedini Najafabadi H (2018) A review on bio-fuel production from microalgal biomass by using pyrolysis method. Renew Sust Energ Rev 82:3046–3059. https://doi.org/10.1016/j.rser.2017.10.033
48. Yang C, Li R, Zhang B, Qiu Q, Wang B, Yang H, Ding Y, Wang C (2019) Pyrolysis of microalgae: a critical review. Fuel Process Technol 186:53–72. https://doi.org/10.1016/j.fuproc.2018.12.012
49. Lee J, Kwon EE, Park Y-K (2019) Recent advances in the catalytic pyrolysis of microalgae. Catal Today 355:263–271. https://doi.org/10.1016/j.cattod.2019.03.010
50. Dry ME (2002) The Fischer–Tropsch process: 1950–2000. Catal Today 71:227–241. https://doi.org/10.1016/S0920-5861(01)00453-9
51. Duman G, Uddin MA, Yanik J (2014) Hydrogen production from algal biomass via steam gasification. Bioresour Technol 166:24–30. https://doi.org/10.1016/j.biortech.2014.04.096
52. Díaz-Rey MR, Cortés-Reyes M, Herrera C, Larrubia MA, Amadeo N, Laborde M, Alemany LJ (2015) Hydrogen-rich gas production from algae-biomass by low temperature catalytic gasification. Catal Today 257:177–184. https://doi.org/10.1016/j.cattod.2014.04.035
53. Chen Y, Wu Y, Hua D, Li C, Harold MP, Wang J, Yang M (2015) Thermochemical conversion of low-lipid microalgae for the production of liquid fuels: challenges and opportunities. RSC Adv 5:18673–18701. https://doi.org/10.1039/C4RA13359E
54. Rachna B, Renu D (2014) Algae as biofuel. Biofuels 5:607–631. https://doi.org/10.1080/17597269.2014.1003701
55. Fasaei F, Bitter JH, Slegers PM, van Boxtel AJB (2018) Techno-economic evaluation of

microalgae harvesting and dewatering systems. Algal Res 31:347–362. https://doi.org/10.1016/j.algal.2017.11.038

56. Halim R, Gladman B, Danquah MK, Webley PA (2011) Oil extraction from microalgae for biodiesel production. Bioresour Technol 102:178–185. https://doi.org/10.1016/j.biortech.2010.06.136
57. Samorì C, López Barreiro D, Vet R, Pezzolesi L, Brilman DWF, Galletti P, Tagliavini E (2013) Effective lipid extraction from algae cultures using switchable solvents. Green Chem 15:353–356. https://doi.org/10.1039/C2GC36730K
58. Jeevan Kumar SP, Vijay Kumar G, Dash A, Scholz P, Banerjee R (2017) Sustainable green solvents and techniques for lipid extraction from microalgae: a review. Algal Res 21:138–147. https://doi.org/10.1016/j.algal.2016.11.014
59. Cicci A, Sed G, Jessop PG, Bravi M (2018) Circular extraction: an innovative use of switchable solvents for the biomass biorefinery. Green Chem 20:3908–3911. https://doi.org/10.1039/C8GC01731J
60. Onumaegbu C, Mooney J, Alaswad A, Olabi AG (2018) Pre-treatment methods for production of biofuel from microalgae biomass. Renew Sust Energ Rev 93:16–26. https://doi.org/10.1016/j.rser.2018.04.015
61. Dai Y-M, Chen K-T, Chen C-C (2014) Study of the microwave lipid extraction from microalgae for biodiesel production. Chem Eng J 250:267–273. https://doi.org/10.1016/j.cej.2014.04.031
62. Wahidin S, Idris A, Shaleh SRM (2014) Rapid biodiesel production using wet microalgae via microwave irradiation. Energy Convers Manag 84:227–233. https://doi.org/10.1016/j.enconman.2014.04.034
63. El Sherbiny SA, Refaat AA, El Sheltawy ST (2010) Production of biodiesel using the microwave technique. J Adv Res 1:309–314. https://doi.org/10.1016/j.jare.2010.07.003
64. Bosma R, van Spronsen WA, Tramper J, Wijffels RH (2003) Ultrasound, a new separation technique to harvest microalgae. J Appl Phycol 15:143–153. https://doi.org/10.1023/A:1023807011027
65. Cravotto G, Boffa L, Mantegna S, Perego P, Avogadro M, Cintas P (2008) Improved extraction of vegetable oils under high-intensity ultrasound and/or microwaves. Ultrason Sonochem 15:898–902. https://doi.org/10.1016/j.ultsonch.2007.10.009
66. Adam F, Abert-Vian M, Peltier G, Chemat F (2012) "Solvent-free" ultrasound-assisted extraction of lipids from fresh microalgae cells: a green, clean and scalable process. Bioresour Technol 114:457–465. https://doi.org/10.1016/j.biortech.2012.02.096
67. Carrillo-Reyes J, Barragán-Trinidad M, Buitrón G (2016) Biological pretreatments of microalgal biomass for gaseous biofuel production and the potential use of rumen microorganisms: a review. Algal Res 18:341–351. https://doi.org/10.1016/j.algal.2016.07.004
68. Kumar P, Barrett DM, Delwiche MJ, Stroeve P (2009) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind Eng Chem Res 48:3713–3729. https://doi.org/10.1021/ie801542g
69. Ximenes E, Farinas CS, Kim Y, Ladisch MR (2017) Hydrothermal pretreatment of lignocellulosic biomass for bioethanol production. In: Ruiz H, Hedegaard TM, Trajano H (eds) Hydrothermal processing in biorefineries. Springer, Cham
70. Lorente E, Farriol X, Salvadó J (2015) Steam explosion as a fractionation step in biofuel production from microalgae. Fuel Process Technol 131:93–98. https://doi.org/10.1016/j.fuproc.2014.11.009
71. Lorenzen J, Igl N, Tippelt M, Stege A, Qoura F, Sohling U, Brück T (2017) Extraction of microalgae derived lipids with supercritical carbon dioxide in an industrial relevant pilot plant. Bioprocess Biosyst Eng 40:911–918. https://doi.org/10.1007/s00449-017-1755-5
72. Hernández D, Riaño B, Coca M, García-González MC (2015) Saccharification of carbohydrates in microalgal biomass by physical, chemical and enzymatic pre-treatments as a previous step for bioethanol production. Chem Eng J 262:939–945. https://doi.org/10.1016/j.cej.2014.10.049
73. Saha BC, Iten LB, Cotta MA, Wu YV (2005) Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Process Biochem 40:3693–3700. https://doi.org/10.1016/j.procbio.2005.04.006
74. Yang B, Dai Z, Ding S-Y, Wyman CE (2011) Enzymatic hydrolysis of cellulosic biomass. Biofuels 2:421–449. https://doi.org/10.4155/bfs.11.116
75. Khan MI, Shin JH, Kim JD (2018) The promising future of microalgae: current

status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. Microb Cell Factories 17:36. https://doi.org/10.1186/s12934-018-0879-x

76. Teter SA, Sutton KB, Emme B (2014) 7—Enzymatic processes and enzyme development in biorefining. In: Waldron K (ed) Advances in biorefineries. Woodhead Publishing, Cambridge, pp 199–233
77. Ravanal MC, Camus C,, Buschmann AH, Gimpel J, Olivera-Nappa Á, Salazar O, Lienqueo ME (2019) Chapter 4—Production of bioethanol from brown algae. In: Hosseini M (ed) Advances in feedstock conversion technologies for alternative fuels and bioproducts. Woodhead Publishing, Sawston, pp 69–88
78. Liu ZH, Chen HZ (2016) Simultaneous saccharification and co-fermentation for improving the xylose utilization of steam exploded corn stover at high solid loading. Bioresour Technol 201:15–26. https://doi.org/10.1016/j.biortech.2015.11.023
79. Silva C, Soliman E, Cameron G, Fabiano LA, Seider WD, Dunlop EH, Coaldrake AK (2014) Commercial-scale biodiesel production from algae. Ind Eng Chem Res 53:5311–5324. https://doi.org/10.1021/ie403273b
80. Mondal B, Jana AK (2019) Techno-economic feasibility of reactive distillation for biodiesel production from algal oil: comparing with a conventional multiunit system. Ind Eng Chem Res 58:12028–12040. https://doi.org/10.1021/acs.iecr.9b00347
81. Mittelbach M (2012) 6—Advances in biodiesel catalysts and processing technologies. In: Luque R, Melero JA (eds) Advances in biodiesel production. Woodhead Publishing, Oxford, pp 133–153
82. Chatsungnoen T, Chisti Y (2016) Oil production by six microalgae: impact of flocculants and drying on oil recovery from the biomass. J Appl Phycol 28:2697–2705. https://doi.org/10.1007/s10811-016-0823-6
83. Chatsungnoen T, Chisti Y (2016) Optimization of oil extraction from Nannochloropsis salina biomass paste. Algal Res 15:100–109. https://doi.org/10.1016/j.algal.2016.02.015
84. Nematian T, Barati M (2019) Nanobiocatalytic processes for producing biodiesel from algae. In Rai, M Avinash PI(eds) Sustainable Bioenergy. Elsevier, 299–326. https://doi.org/10.1016/B978-0-12-817654-2.00011-3
85. Park J-Y, Park MS, Lee Y-C, Yang J-W (2015) Advances in direct transesterification of algal oils from wet biomass. Bioresour Technol 184:267–275. https://doi.org/10.1016/j.biortech.2014.10.089
86. Zhao C, Brück T, Lercher JA (2013) Catalytic deoxygenation of microalgae oil to green hydrocarbons. Green Chem 15:1720–1739. https://doi.org/10.1039/C3GC40558C
87. Ooi XY, Gao W, Ong HC, Lee HV, Juan JC, Chen WH, Lee KT (2019) Overview on catalytic deoxygenation for biofuel synthesis using metal oxide supported catalysts. Renew Sust Energ Rev 112:834–852. https://doi.org/10.1016/j.rser.2019.06.031
88. Oi LE, Choo M-Y, Lee HV, Rahman NA, Juan JC (2019) Chapter 9—Mesoporous and other types of catalysts for conversion of non-edible oil to biogasoline via deoxygenation. In: Rai M, Ingle AP (eds) Sustainable bioenergy. Elsevier, Amsterdam, pp 257–281
89. Bwapwa JK, Anandraj A, Trois C (2018) Microalgae processing for jet fuel production. Biofuels Bioprod Biorefin 12:522–535. https://doi.org/10.1002/bbb.1878
90. Soni VK, Sharma PR, Choudhary G, Pandey S, Sharma RK (2017) Ni/co-natural clay as green catalysts for microalgae oil to diesel-grade hydrocarbons conversion. ACS Sustain Chem Eng 5:5351–5359. https://doi.org/10.1021/acssuschemeng.7b00659
91. Peng B, Yao Y, Zhao C, Lercher JA (2012) Towards quantitative conversion of microalgae oil to diesel-range alkanes with bifunctional catalysts. Angew Chem Int Ed 51:2072–2075. https://doi.org/10.1002/anie.201106243
92. Song W, Zhao C, Lercher J (2013) Importance of size and distribution of Ni nanoparticles for the hydrodeoxygenation of microalgae oil. Chemistry 19:9833–9842. https://doi.org/10.1002/chem.201301005
93. Rogers KA, Zheng Y (2016) Selective deoxygenation of biomass-derived bio-oils within hydrogen-modest environments: a review and new insights. Chem Sus Chem 9:1750–1772. https://doi.org/10.1002/cssc.201600144
94. Lee K, Kim MY, Choi M (2018) Effects of fatty acid structures on ketonization selectivity and catalyst deactivation. ACS Sustain Chem Eng 6:13035–13044. https://doi.org/10.1021/acssuschemeng.8b02576
95. Peterson AA, Vogel F, Lachance RP, Fröling M, Antal JMJ, Tester JW (2008) Thermochemical biofuel production in hydrothermal media: a review of sub- and supercritical water technologies. Energy Environ Sci 1:32–65. https://doi.org/10.1039/B810100K

96. Fu J, Lu X, Savage PE (2010) Catalytic hydrothermal deoxygenation of palmitic acid. Energy Environ Sci 3:311–317. https://doi.org/10.1039/B923198F
97. Fu J, Lu X, Savage PE (2011) Hydrothermal decarboxylation and hydrogenation of fatty acids over Pt/C. Chem Sus Chem. 4:481–486. https://doi.org/10.1002/cssc.201000370
98. Kim D, Vardon DR, Murali D, Sharma BK, Strathmann TJ (2016) Valorization of waste lipids through hydrothermal catalytic conversion to liquid hydrocarbon fuels with in situ hydrogen production. ACS Sustain Chem Eng 4:1775–1784. https://doi.org/10.1021/acssuschemeng.5b01768
99. Miao C, Marin-Flores O, Dong T, Gao D, Wang Y, Garcia-Pérez M, Chen S (2018) Hydrothermal catalytic deoxygenation of fatty acid and bio-oil with in situ H2. ACS Sustain Chem Eng 6:4521–4530. https://doi.org/10.1021/acssuschemeng.7b02226
100. Jin M, Choi M (2019) Hydrothermal deoxygenation of triglycerides over carbon-supported bimetallic PtRe catalysts without an external hydrogen source. Mol Catal 474:110419. https://doi.org/10.1016/j.mcat.2019.110419
101. Chisti Y (2013) Constraints to commercialization of algal fuels. J Biotechnol 167:201–214. https://doi.org/10.1016/j.jbiotec.2013.07.020
102. Kwietniewska E, Tys J (2014) Process characteristics, inhibition factors and methane yields of anaerobic digestion process, with particular focus on microalgal biomass fermentation. Renew Sust Energ Rev 34:491–500. https://doi.org/10.1016/j.rser.2014.03.041
103. Montingelli ME, Tedesco S, Olabi AG (2015) Biogas production from algal biomass: a review. Renew Sust Energ Rev 43:961–972. https://doi.org/10.1016/j.rser.2014.11.052
104. Eroglu E, Melis A (2016) Microalgal hydrogen production research. Int J Hydrog Energy 41:12772–12798. https://doi.org/10.1016/j.ijhydene.2016.05.115
105. Ghirardi ML (2015) Implementation of photobiological H2 production: the O_2 sensitivity of hydrogenases. Photosynth Res 125:383–393. https://doi.org/10.1007/s11120-015-0158-1
106. Ghirardi ML, Zhang L, Lee JW, Flynn T, Seibert M, Greenbaum E, Melis A (2000) Microalgae: a green source of renewable H_2. Trends Biotechnol 18:506–511. https://doi.org/10.1016/S0167-7799(00)01511-0
107. Fulbright SP, Robbins-Pianka A, Berg-Lyons-D, Knight R, Reardon KF, Chisholm ST (2018) Bacterial community changes in an industrial algae production system. Algal Res 31:147–156. https://doi.org/10.1016/j.algal.2017.09.010
108. Rogers JN, Rosenberg JN, Guzman BJ, Oh VH, Mimbela LE, Ghassemi A, Betenbaugh MJ, Oyler GA, Donohue MD (2014) A critical analysis of paddlewheel-driven raceway ponds for algal biofuel production at commercial scales. Algal Res 4:76–88. https://doi.org/10.1016/j.algal.2013.11.007



Chapter 4

Life Cycle Assessment of Biofuels

L. Reijnders

Abstract

Life cycle assessment (LCA) assesses the environmental burdens or impacts of products from cradle to grave. It is also possible to assess such burdens or impacts for parts of the life cycle. A brief overview is given of LCA methodology. A number of choices have to be made in the goal and scope definition, inventory analysis, and impact assessment stages of life cycle assessments. Such choices can have substantial impacts on LCA outcomes. There are uncertainties in outcomes linked to inventory data and modeling. In the case that future biofuels and production processes are studied, assessment outcomes are characterized by relatively large uncertainties. Choices and uncertainties should be considered in the interpretation stage of life cycle assessments. Methodologies applied to several important environmentally relevant aspects of biofuel life cycles are discussed. These aspects are: emissions of substances impacting climate, depletion of virtually nonrenewable abiotic resources, primary energy demand, and water footprint. LCA can be useful in identifying life cycle stages and processes that are major contributors to environmental burdens, for determining the energetic return on energy invested in biofuels, for the identification of environmental trade-offs, and for comparing the life cycle environmental burdens of products.

Key words Life cycle assessment, Biofuels, Functional unit, System boundaries, Valuation, Inventory, Characterization, Environmental burdens, Environmental impacts, Sensitivity analysis, Uncertainty

1 Introduction

Life cycle assessment (LCA) is widely used to assess the environmental burdens or impacts of products from cradle to grave. LCA can be applied to current and future products and production processes.

Methodological LCA manuals exceed the length of chapters in this book by an order of magnitude [1]. So, it was decided to briefly present LCA methodology with illustrative examples relating to biofuels and with references for more information. Subheading 1 of this chapter gives a methodological overview of life cycle assessment. Subheading 2 deals with the methodologies applied to the assessment of several important environmentally relevant aspects of biofuel life cycles. Emissions of substances impacting climate,

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depletion of virtually nonrenewable abiotic resources, primary energy demand, and the water footprint are considered. In Subheading 3 applications of LCA to biofuels that can be considered useful will be briefly outlined.

2 Brief Methodological Overview of Life Cycle Assessment

Life cycle assessment has been developed to establish the environmental burdens or impacts of products from cradle to grave. According to the International Organisation of Standardisation (ISO) [2] full life cycle assessments are divided in four stages:

- *Goal and scope definition*
- *Inventory analysis*
- *Impact assessment*
- *Interpretation*

In the *goal and scope definition* stage, the object, aim, and scope of life cycle assessment are established. The object is commonly defined in terms of a functional unit: e.g., the amount of biofuel able to generate 1 MJ of energy (in short: 1 MJ biofuel). When a functional unit of 1 MJ biofuel is chosen, the impact thereof on the environment will be marginal. When the functional unit is large, because for instance the aim of the LCA is to evaluate a large expansion of biofuel production, the effect can be non-marginal, which necessitates a different characterization approach in the *impact assessment* stage [3, 4]. In this chapter only functional units with a marginal impact on the environment will be considered.

As environmental burdens of energetic inputs, agricultural product yields, soil characteristics, and environmental impacts show substantial geographical variation [5–9], the location (s) relevant to the biofuel life cycle should preferentially be specified.

Regarding the definition of aim: it might, e.g., be established that the LCA should be focussed on identifying parts of the life cycle with relatively large environmental burdens or impacts ("hotspots"). This, in turn, may be the basis for efforts aimed at the reduction of such burdens or impacts, for instance by applying alternative technologies. Also, it might be established that the LCA should be comparative, e.g., comparing the life cycle environmental burden of ethanol derived from cane sugar with the life cycle environmental burden of fossil petrol.

In the *goal and scope definition* stage system boundaries are determined. These boundaries establish what should, and what should not, be included in the assessment. One of the matters to be decided in this context is the time horizon for impacts. A variety of

time horizons (20, 50, 100, 1000 years and infinite) are used in LCA [1, 10]. As to the system boundaries regarding inputs it should be decided whether the infrastructure needed for production and distribution of inputs should be included or excluded. Also, there is the matter of including or excluding indirect effects of production. For instance, it has been noted that the demand for food is inelastic, and that using food crops for biofuel production can lead to the indirect effect of additional acreage elsewhere devoted to food production, and thereby to land use change [11, 12]. If the focus is on forestry products, it should be decided whether the system boundary regards the forestry stand, a region, or a country [13]. Differences in system boundaries can lead to substantial differences in LCA results, and in actual practice system boundaries used for peer-reviewed biofuel LCAs vary widely [14–17].

Another important matter to be decided in the *goal and scope definition* stage is the valuation of production outputs (product outputs and non-product outputs), inputs, and avoided processes.

Regarding product outputs, allocation must be practiced for processes that have more than one output. For instance, in biodiesel production glycerol and meal can be co-product outputs [18]. In ethanol production from corn, corn oil, gluten feed, gluten meal, and dried distillers' grains can be co-product outputs [19]. In the production of biodiesel and corn ethanol there are also non-product outputs. How should the life cycle environmental burden be allocated to (or distributed over) these outputs? Allocation in practice usually regards product outputs, under the tacit assumption that allocation to non-product outputs should be zero. Allocation to product outputs can be done on a financial basis (e.g., product prices or production costs), on a physical basis (e.g., mass, energy content, exergy content), or on the basis of substitution (also called: system expansion). In the latter case the environmental burden of a similar output is used. For example, fossil fuel-based glycerol (derived from epichlorohydrin) has been substituted for the co-product glycerol in LCAs regarding biodiesel production [17]. There is no agreement about the best way to allocate environmental burdens to product outputs.

As to choices in the valuation of non-product outputs one might, e.g., consider the emission of the greenhouse gas CO_2 originating in the combustion of biofuels. In biofuel LCAs this emission is often valued at a zero environmental burden [17]. An argument used in favor of this valuation is that this emission will be sequestered by the generation of new biomass. However, in the real world the generation of new biomass in part serves the sequestration of CO_2 emitted by the combustion of fossil fuels and there is no guarantee that sequestration of CO_2 emitted by the combustion of biofuels will be indefinite [20]. Thus, there is also a case to consider CO_2 originating in the combustion of biofuels as having

an above-zero environmental burden. There is no agreement about the best way to valuate CO_2 originating in the combustion of biofuels.

Regarding the valuation of inputs, there is the possibility that they originate in multi-output processes. For instance, fossil fuels used in biofuel logistics often originate in multi-output oil refineries. Here again, allocation applies that can be done on a financial basis, on a physical basis, or on the basis of substitution. When inputs in biofuel production are non-product outputs, there also choices regarding valuation. In this context one may, for example, consider the example of fossil fuel-derived CO_2 that is used as an input in microalgal biodiesel production. The environmental burden of production allocated to such CO_2 is often zero because it is considered to be a waste. One may feel, however, that in this case CO_2 should not be considered as a waste, but rather as a co-product as it usefully enhances the growth of microalgae. Also, one may argue that input CO_2 is actually sequestered in microalgal growth and should thus be treated in terms of an emission credit (a negative emission of CO_2). There is no agreement as to the best valuation of fossil fuel-derived CO_2 that is used as an input in microalgal biodiesel production.

Choices regarding the valuation of avoided processes may be illustrated by the example of wastewater input in the production of microalgae for biodiesel production. It has been argued that by doing so, conventional wastewater treatment becomes an avoided process [20]. This would imply a credit for the input of energy in conventional wastewater treatment. However, it is not clear whether wastewater treatment by microalgae performs as well as conventional state-of-the-art wastewater treatment [20]. Furthermore, the quality of the co-product algal meal may be negatively impacted by using wastewater in biodiesel production [21].

Different ways to valuate inputs, outputs, and avoided processes can lead to substantially different outcomes of life cycle assessment [15, 16]. Peer-reviewed biofuel LCAs vary widely in the choices made as to these valuations [17].

In the *inventory analysis* stage, the necessary data for all environmentally relevant processes, avoided processes, inputs, and outputs (including non-product outputs) are compiled and a set of quantified environmentally relevant interventions (*see* Box 1) per functional unit is generated. Data used in inventory analysis may have different origins. Data may be taken from databases covering many countries, single countries, or regions [1, 5, 8, 22–24]. Quality assurance for such databases has been reviewed by Ciroth et al. [23]. Data may also be taken from scientific literature or may be estimates, for instance based on extrapolations of laboratory data. Data used in the *inventory analysis* stage of life cycle assessment are uncertain [25, 26]. When future technologies and biofuels that are as yet not commercially produced are studied, the uncertainty of data used in *inventory analysis* is relatively large [27].

In the *impact assessment* stage data gathered at the inventory stage can be converted into environmental burdens or impacts. A range of interventions may be considered in the *impact assessment* stage (*see* Box 1).

Box 1: Interventions That May Be Considered in Life Cycle Assessment of Biofuels [17, 35, 49, 59–62]

Interventions that may be considered in life cycle assessment of biofuels
Consumption of abiotic resources (e.g., fossil fuels, ores) leading to resource depletion
Cumulative primary energy demand
Cumulative exergy demand
Water footprint (freshwater consumption)
Land use and land use change affecting ecosystems and carbon stocks
Greenhouse gas emissions that affect climate
Other interventions that may affect climate (e.g., due to change in albedo or evaporation of water)
Emissions of acidifying substances affecting soils (terrestrial acidification) and/or fresh water (aquatic acidification)
Nutrient emissions affecting water and/or soils (eutrophication)
Emissions that may lead to ozone layer depletion
Emissions that may give rise to photo-oxidant formation
Emissions of human toxics (substances that are hazardous to human health)
Emissions of substances that may lead to ecotoxicity
Emissions that may lead to nuisance (e.g., of noise, odor)
Emissions of waste heat that may affect ecosystems

Though the range of interventions that may be studied in LCA is wide, in actual practice biofuel LCAs often consider only a few of the interventions indicated in Box 1 [17].

LCA as defined by the International Organisation of Standardisation [2] is not time- or place-dependent. However, actual impacts may have time and/or place-dependency. The importance of place-dependency has been relatively well analyzed [1, 6, 24, 28, 29]. For instance, Kounina et al. [6], modeling human intake fractions of hazardous substances emitted to fresh water, did show that geographical variations led to variations in intake fractions by humans of up to 15 orders of magnitude. Similarly, the impact on ecosystems and humans of freshwater consumption depends on the absence or presence of pre-existing local water scarcity or -stress

and, when there is water scarcity, the severity thereof [1, 22, 30]. Crespo-Mendes et al. [31] analyzing terrestrial acidification did show that the assumption that acidifying deposition can only decrease biodiversity [1] is incorrect for large parts of Brazil, where soils are alkaline and where acidifying deposition can increase biodiversity.

By now *impact assessment* can be adapted to include location-specificity. A variety of LCA methods have been published that have this adaptation [1, 28, 29, 32].

Temporal aspects can also be important. For instance, after harvesting in forestry, regenerating the carbon stock of the stand may take many years [13]. This leads to substantially different global warming potentials of forestry products, such as biofuels, depending on the temporal system boundary [13]. The balance (emission minus sequestration) of the greenhouse gases CO_2, CH_4, N_2O for oil palm plantations, a major source of biodiesel, varies by plantation age [33]. Releases of C, N, and P from agricultural land to the environment show substantial temporal variations linked to variable climatic factors, such as precipitation, and variable agricultural practices, such as fertilizer applications and crop rotations. Multi-year modeling has been developed to deal with temporal changes [7, 9, 13].

The first stage of *impact assessment* is characterization. In characterization, interventions with similar impacts are aggregated on the basis of a common unit of the impact category. For instance, regarding the impact on climate of greenhouse gases that become well mixed in the atmosphere, all emissions of such "well-mixed greenhouse gases" (CO_2, CH_4, N_2O, and a number of halogenated organic compounds) should be included in characterization [1, 34, 35]. The common unit is in this case: kg CO_2 equivalent (CO_{2eq}). Also, land use and land use change affecting ecosystem carbon stocks, which in turn impact carbonaceous greenhouse gas concentrations in the atmosphere, should be included [34–37]. Including land use change requires a choice regarding amortization of the change in the ecosystem carbon stock, that is the period over which the change in carbon stock should be linearly distributed [36]. A choice common in LCAs regarding biofuels is a period of 20 years [36], but one may also argue in favor of other periods (e.g., the time that the new land use is expected to continue). Regarding characterization of well-mixed greenhouse gases time frames for the impact on climate may be 20, 50, 100, or 1000 years [1, 10]. Two types of results of applying characterization to emissions of well-mixed greenhouse gases may be considered: the global warming potential (abbreviated: GWP) or the global temperature change potential (abbreviated: GTP). The GWP integrates the radiative forcing within a time frame. The GTP is the change in global mean surface temperature at a chosen point in time after the intervention (commonly 20 or 100 years). Relative GWPs and

Table 1
Relative global warming potentials (GWPs) and global temperature change potentials (GTPs) for kg of the well-mixed greenhouse gases CO_2, CH_4, and N_2O [1, 40, 57, 58]

Greenhouse gas	GWP for 20 years	GWP for 100 years	GWP for 1000 years	GTP for 20 years	GTP for 100 years
CO_2	1	1	1	1	1
CH_4	83–85	28–36	4.8	66–69	4–7
N_2O	264	265–298	78.8	277	234

Table 2
Examples of common characterization units and outcomes of characterization for impact categories [1, 60]

Impact category	Common characterization unit	Common characterization units are used for the calculation of:
Human toxicity	kg 1,4-dichlorobenzene equivalent	Human toxicity potential
Photochemical oxidant formation	kg NOx equivalent kg non-methane volatile organic compounds	Photochemical oxidant formation potential
Fine particulate matter formation	kg PM 10 (particulate matter with diameter <10 μm) equivalent kg PM 2.5 (particulate matter with diameter <2.5 μm) equivalent	Particulate matter formation potential
Terrestrial acidification	kg SO_2 equivalent	Terrestrial acidification potential
Freshwater eutrophication	kg P equivalent	Freshwater eutrophication potential
Water footprint	m^3 fresh water	Water consumption potential

GTPs of CO_2, CH_4, and N_2O in CO_2 equivalents for a variety of time frames are in Table 1. Calculating such relative GWPs and GTPs requires modeling, and differences in the models used may lead to different results: hence the ranges shown in Table 1. In biofuel LCAs global warming potentials are often used [17].

For other impacts than climate change due to emissions of well-mixed greenhouse gases there are also common units of the impact category. Table 2 presents a number of these common units for such impacts. For example, the major terrestrial acidifiers in biofuel LCAs are: SO_2, NO_x, and NH_3 [38]. As shown in Table 2, for terrestrial acidification kg SO_2 equivalents are used as common unit.

Many LCAs for biofuels end with quantifications on the basis of common units for the impact category: environmental burdens. Others continue by converting these burdens into impacts.

Examples of regionalized models that may be used for this purpose are LC-IMPACT [32], ReCiPe 2016 [1], and IMPACT World + [28].

In the case of climate change due to the emission of well-mixed greenhouse gases, the global warming potential is the basis for calculating radiative forcing, which in turn can be used to calculate increases in global temperature. The increase in global mean temperature may be calculated but, because increases in temperature vary geographically, regionalized models also take the geographical distribution of temperature change into account. This distribution is important for the next step in impact assessment: for instance, finding the consequences of temperature change for human health and/or ecosystems. LC-IMPACT [32], ReCiPe 2016 [1], and IMPACT World + [28] contain modeling for this step.

In the case of terrestrial acidification, three consecutive regionalized models can be used for impact assessment after the quantification of emissions in kg SO_2 equivalents: the first to establish the geographical distribution of acidifying deposition, the second to determine the location-specific pH change of soils due to acidifying deposition, and the third to establish the impact of soil pH changes on terrestrial plants and thereby on terrestrial ecosystems [1].

It is possible to further aggregate impacts for a range of impact categories presented in Box 1 into categories such as: damage to human health, damage to ecosystems (or ecosystem quality), damage to resources (also: resource depletion, resource scarcity), and damage to resources and ecosystem services [1, 28, 29]. In IMPACT World + [28], for instance, damage to human health (measured in terms of DALYs: disability-adjusted loss of life years) combines the impacts of the categories, climate change, water availability, fine particulate matter formation, emission of substances hazardous to human health (toxics), emission of substances that leads to ozone layer depletion, and emissions leading to photo-oxidant formation.

The uncertainty of impacts obtained by using models such as LC-IMPACT [32], ReCiPe 2016 [1], and IMPACT World + [28] tends to be relatively large if compared with the uncertainty in quantifications of the environmental burdens on the basis of common units for the impact category [1].

In the *interpretation* stage the validity of results is evaluated. Uncertainty analysis considering uncertainties in inventories and modeling belongs to this stage. For analysis of uncertainties in inventories Monte Carlo simulations are often chosen [26, 39]. Sensitivity analysis may be used to analyze the impacts of choices made in the previous stages. Often, published life cycle assessments of biofuels lack adequate uncertainty and sensitivity analyses [17]. Also, in the *interpretation* stage conclusions are drawn from the study. Such conclusions may for instance regard priorities for reduction of the environmental burden.

3 Methodologies Applied to Environmental Aspects of Biofuel Life Cycles

In this section the methods applied to the following important environmentally relevant aspects of biofuel life cycles will be discussed: emissions of substances impacting climate, primary energy demand, water footprint, and depletion of virtually nonrenewable abiotic resources.

3.1 The Emission of Substances Impacting Climate

The emission of well-mixed greenhouse gases CO_2, CH_4, N_2O is often included in biofuel LCAs [17]. The time horizon chosen for the impact of these gases in biofuel LCAs tends to be 100 years [17]. There are also substances that are emitted during biofuel life cycles that are not well mixed in the atmosphere, but which can significantly impact climate [34, 40]. These include aerosols and their precursors and substances that affect atmospheric ozone [17, 34, 40]. The inclusion thereof in LCAs regarding biofuels is reported to be very rare [17].

In the case of specific biofuel life cycles (e.g., regarding ethanol from corn grown in the US Midwest) there tend to be substantial differences in the published global warming potentials of well-mixed greenhouse gas emissions. Choices made, such as the inclusion or exclusion of changes in ecosystem carbon stocks and of indirect effects linked to cropping for biofuel production, are important determinants of such differences in results. As to biofuels derived from starch, sugars or oils present in food crops, including indirect effects and changes in ecosystem carbon stocks may lead to a life cycle emission of CO_2 equivalents that exceeds the corresponding emission linked to fossil fuel life cycles [41]. Also, the inclusion or exclusion of indirect N_2O emissions of cropping for biofuels, due to the conversion of volatilized NH_3 and leached nitrate, may substantially affect published global warming potentials of biofuel life cycles [42].

In the case that there is as yet no commercial biofuel production the differences between published LCA results for the emission of well-mixed greenhouse gases linked to specific biofuels can be very large. An example thereof regards autotrophic microalgal biodiesel. Peer-reviewed studies of well-mixed greenhouse gas emissions for full microalgal biodiesel life cycles, and for cradle-to-pump and cradle-to-factory-gate parts of such life cycles, range from −95.7 g to +534 g CO_{2eq} CO MJ^{-1} biodiesel, whereas the full cradle-to-grave emission for conventional fossil diesel is estimated to be (+)72–96 g CO_{2eq} MJ^{-1} [16, 27]. The negative emissions for microalgal biodiesel originate in the use of credits, e.g., for avoided wastewater treatment or the sequestration of fossil fuel-derived CO_2 in growing microalgae.

3.2 Primary Energy Demand

Primary energy demand is important, because in the case of (bio)-fuel production the output of energy should preferentially much exceed the primary energy input. In this context the energetic return on energy invested (EROI) can be determined. This is the quotient of the energy generated by combustion of a fuel and the cradle-to-factory-gate input of primary energy. Inputs in production (e.g., fertilizers for crop-derived biofuels) are valued in terms of the primary energy needed for their cradle-to-factory-gate production and transport (embodied energy). Determining the EROI of biofuels can be a useful application of life cycle assessment. EROIs in the range 5–8 have been proposed to guide the energetically responsible substitution of fossil fuels by biofuels [35, 43]. EROIs for specific biofuels published in peer reviewed scientific literature vary widely. A very wide range of EROIs—0.01–3.35—was found by Ketzer et al. [44] reviewing the literature on autotrophic microalgal biodiesel. Reijnders [20] reviewed peer-reviewed studies on EROIs for liquid and solid terrestrial biofuels. It was found that for refined liquid biofuels such as ethanol and biodiesel the EROIs were often <5. An exception is ethanol derived from Brazilian cane sugar, which has an EROI >5 [20]. Solid biofuels may have EROIs up to 20 [20].

3.3 Water Footprint

As two-thirds of mankind is expected to live in areas with freshwater scarcity or -stress by 2025 [20], freshwater consumption in biofuel life cycles is an important matter. Hoekstra [45] defined the water footprint as a freshwater consumption volume. According to Hoekstra [45] there are three components of this freshwater consumption: green, blue, and gray water. Green water regards consumption of precipitation such as rainwater. Blue water concerns the consumption of ground- and surface water. Gray water is the volume of water required to dilute the load of pollutants to meet existing water quality standards. As water quality standards are political constructs that lack consistency and because the standards used in practice only refer to N- and P- compounds, there is a case to consider only the consumption blue and green water [20]. Available studies calculating the combined green and blue water volumes for the life cycles of 1 MJ biofuel and for the corresponding amounts of energy for the life cycles of fossil fuels have been reviewed [20]. It was found that the combined green and blue water footprints of the biofuels biodiesel and bioethanol are at least a factor 10^3 larger than the corresponding footprints for fossil fuels.

The International Standards Organisation (ISO) [46] defined the water footprint as covering a set of impact categories regarding both seawater and fresh water. A comprehensive footprint as defined by the International Standards Organisation [46] includes, besides freshwater scarcity or freshwater availability, impacts on marine and freshwater systems such as acidification, eutrophication, ecotoxicity, thermal, and radioactive water pollution [28]. In the

context of life cycle assessments such comprehensiveness runs the risk of double-counting as emissions of heat and acidifying, eutrophying and radioactive substances are also interventions that can be separately considered in impact assessment [47]. The ISO standard [46] also allows for studying one aspect of the water footprint, e.g., freshwater scarcity. Freshwater scarcity was defined by ISO [46] as: water consumption that approaches or exceeds the natural regeneration capacity of a water body.

The freshwater consumption volume linked to a functional unit is, as pointed out in Subheading 1, not the only determinant of the environmental impact on humans and ecosystems. This impact should rather be defined in terms of water scarcity or -stress, and the consequences thereof for human health and ecosystems. Several studies have been made of the impacts of freshwater consumption on regional water basins when water scarcity might develop or might already occur [22, 30, 48]. Impacts of increased freshwater scarcity on human health due to increased malnutrition among the poor, impacts on terrestrial ecosystems in terms of potential reduction of vegetation and plant diversity, and impacts of rivers in terms of disappearing fish have been modeled for application in LCA [1].

3.4 Depletion of Virtually Nonrenewable Abiotic Resources

Fossil fuels used in biofuel life cycles are derived from abiotic natural resources such as mineral oil-, natural gas- and coal deposits that originate in slow geological processes. These are often called nonrenewable resources. As geological processes continue, the designation nonrenewable is, strictly taken, not correct. For this reason, and as the current consumption rate is very much larger than the rate of resource formation, the designation virtually nonrenewable has been coined. In LCAs depletion of fossil fuel resources may be an impact category, with kg mineral oil equivalent as common characterization unit [49]. The designation virtually nonrenewable is also used for ores that have been formed in slow geological processes.

In biofuel life cycles ores and fossil fuels are often important. This raises the matter of depleting such resources due to biofuel production. Examples of virtually nonrenewable resources in biofuel LCAs are phosphate ores (the source of synthetic phosphate fertilizers widely used in agriculture), fossil fuels (that can be used for powering irrigation, agricultural machinery, biofuel production plants and biofuel logistics), and ore-derived copper (e.g., used in biofuel production plants and in vehicles for biofuel transport). Sonderegger et al. [50] and Berger et al. [51] have addressed methodologies to assess virtually nonrenewable abiotic resources in life cycle assessment. Twenty-seven available methods were identified [50, 51]. Available methods can be allocated to four main categories, respectively focussing on: resource depletion, thermodynamic accounting, future efforts (to compensate for depletion), and near-term supply risk [50]. In part, these methods regard

physical aspects of resource depletion, such as cumulative exergy extraction, reduction of resource grades, or the ratio of resource extraction to estimated ultimately recoverable resources [50]. Methods used can also show economic effects of resource depletion, such as surplus cost, future welfare loss, or part of the economic value of extracted abiotic resources needed to maintain in the future the benefit obtained from these resources [1, 50]. The method focussing on the ratio of abiotic resource extraction to estimated ultimate resources may seem attractive. It has a common characterization unit (antimony (Sb) equivalent), which allows for combining of the depletion of such resources as, e.g., phosphate ore, copper ore, and fossil fuels into one abiotic resources depletion potential [52]. A main problem of this method is, however, that ultimately recoverable resources can be very uncertain [51]. For instance, in the case of copper ore estimates of ultimately recoverable resources vary by more than a factor 27 [53, 54]. Economic methods can use common monetary units (e.g., $) for depleting a variety of resources, but suffer from the problem that future economic considerations and the financial performance of future extraction technologies are uncertain [50]. Thermodynamic methods do not convincingly link the consumption of virtually nonrenewable resources to changes in their availability [50]. There is as yet no agreement about the best way to assess depletion of mineral resources in LCA [51].

4 For Which Purposes Are Biofuel LCAs Useful?

It appears from the previous sections that life cycle assessment covering the full environmental impacts of biofuels is a work in progress. There is no consensus over the best ways to assess environmental impacts and the impacts that are results of current assessments often show a high degree of uncertainty.

There are, however, applications of biofuel LCAs that can be considered useful. Establishing the energetic return on energy investment can be, as pointed out in Subheading 2, a useful application. There are also other applications that can be useful. These include:

– *The identification of stages* (e.g., *cultivation of crops, harvesting of microalgae) and processes* (e.g., *centrifugation) that are major contributors to selected life cycle environmental burdens (within the chosen system boundaries)*. This can be done by identifying contributions of stages and processes to selected environmental burdens such as life cycle green and blue water consumption or terrestrial acidification potential (cf. Box 1 and Table 2). This in turn may be conducive to the prioritization of efforts to reduce specified environmental burdens of product life cycles.

- *Identifying environmental trade-offs.* It has been pointed out in Subheading 2 that forestry-derived solid biofuels may do relatively well regarding the energetic return on energy invested (EROI). However, studies dealing with the use of forestry-derived solid fuels as alternative for natural gas suggest that solid biofuels originating in forestry do less well than natural gas as to life cycle eutrophication potential, acidification potential, ecotoxicity potential and blue and green freshwater consumption [20, 55, 56]. Studies of environmental trade-offs are, for instance, also available for food crops used in biofuel production and for liquid biofuels derived from such crops [7, 30].
- *Comparing the life cycle environmental burdens of products.* LCAs can serve comparisons between the life cycle environmental burdens of products providing energy services. This may help in making choices between such products. In doing so, the choices made, the implications thereof as assessed by sensitivity analysis, and the certainty with which conclusions can be drawn from such comparisons should be clearly stated (also [26]). Meeting this requirement has been reported to be rare in comparative biofuel LCAs [17].

References

1. Huijbregts MAJ, Steinman ZJN, Elshout PMF (2016) ReCiPe 2016. RIVM Report 2016-0104. RIVM, Bilthoven, the Netherlands (191 pp). www.rivm.nl
2. International Organisation for Standardisation (2006) ISO 140140 and 14044 environmental management-life cycle assessment-principles and framework. Switzerland, Geneva
3. Boulay A-M, Benini L, Sala S (2020) Marginal and non-marginal approaches in characterization: how context and scale affect the selection of an adequate characterization model. The AWARE model example. Int J Life Cycle Assess. https://doi.org/10.1007/s11367-019-01680-0
4. Forin S, Berger M, Finkbeiner M (2020) Comment to 'Marginal and non-marginal approaches in characterization: how context and scale affect the selection of an adequate characterization model. The AWARE model example'. Int J Life Cycle Assess 25:663–666. https://doi.org/10.1007/s11367-019-01726-3
5. Yang Y, Tao M, Sun S (2018) Geographic variability of agriculture requires sector-specific uncertainty characterization. Int J Life Cycle Assess 23:1581–1589
6. Kounina A, Magni M, Henderson AD et al (2019) Global spatial analysis of toxic emissions to freshwater: operationalization for LCA. Int J Life Cycle Assess 24:501–517
7. Lee EK, Zhang X, Adler PR et al (2020) Spatially and temporally explicit life cycle global warming, eutrophication, and acidification impacts from corn production in the US Midwest. J Clean Prod 242:118465. (11 pp)
8. Patouillard L, Collet P, Lesage P et al (2019) Prioritizing regionalization efforts in life cycle assessment through global sensitivity analysis: a sector meta-analysis based on ecoinvent v3. Int J Life Cycle Assess 24:2238–2254
9. Romeiko XX, Lee EK, Sorunmu Y (2020) Spatially and temporally explicit life cycle assessment impacts of soybean production in the US Midwest. Environ Sci Technol 54:4758. https://doi.org/10.1021/acs.est.9b06874
10. Hauschild MZ, Goedkoop M, Guinée J et al (2013) Identifying best existing practice for characterization modelling in life cycle impact assessment. Int J Life Cycle Assess 18:683–697
11. Chen R, Qin Z, Han J et al (2018) Life cycle energy and greenhouse gas emission effects of biodiesel in the United States with induced land use change impacts. Bioresour Technol 251:249–258
12. Searchinger T, Heimlich R, Houghton RA et al (2008) Use of US croplands for biofuels

increases greenhouse gases through emissions from land use change. Science 319:1238–1240
13. Penaloza D, Royne F, Sandin G et al (2019) The influence of system boundaries and baseline on the climate impact assessment of forest products. Int J Life Cycle Assess 24:160–176
14. Ahlgren S, Björklund A, Ekman A et al (2015) Review of methodological choices in LCA of biorefinery systems—key issues and recommendations. Biofuels Bioprod Biorefin 9:606–619
15. Gerbrandt K, Chu PL, Simmonds A et al (2016) Life cycle assessment of lignocellulosic ethanol: a review of key factors and methods affecting calculated GHG emissions and energy use. Curr Opinion Biotechnol 38:63–70
16. Reijnders L (2017) Greenhouse gas balances of microalgal biofuels. Recent Adv Renew Energy 1:354–364
17. Agostini A, Giuntoli J, Marelli L et al (2010) Flaws in the interpretation phase of bioenergy LCA fuel the debate and mislead policymakers. Int J Life Cycle Assess 25:17–35
18. Reijnders L, Huijbregts MAJ (2009) Biofuels for road transport: a seed to wheel perspective. Springer, London
19. Ortiz-Reyes E, Anex RP (2010) Life cycle impacts on non-cellulosic fermentable carbohydrates for the production of biofuels and chemicals. Int J Life Cycle Assess 25:548–563
20. Reijnders L (2019) Biofuels, water footprints and green perspectives. In: Maurice P (ed) Encyclopedia of water: science, technology, and society. Wiley, Hoboken, NJ, pp 2391–2400. https://doi.org/10.1002/9871119300762.wsts0099
21. Hess D, Quinn J (2018) Impact on inorganic contaminants om microalgal biofuel production through multiple conversion pathways. Biomass Bioenergy 11:237–245
22. Andrade EP, de Araujo Nunes AB, de Freitas AK et al (2020) Water scarcity in Brazil: part 1—regionalization of the AWARE model characterization factors. Int J life Cycle Assess. https://doi.org/10.1007/s11367-019-01643-5
23. Ciroth A, Foster C, Hildenbrand J et al (2020) Life cycle inventory dataset review criteria—a new proposal. Int J Life Cycle Assess 25:483–494
24. Fischknecht R, Pfister S, Bunsen J et al (2019) Regionalization in LCA: current concepts, software and databases. Int J Life Cycle Asses 24:364–369
25. Bamber N, Turner I, Arulnathan V (2020) Comparing sources and analysis of uncertainty in consequential and attributional life cycle assessment: review of current practice and recommendations. Int J Life Cycle Assess 25:168–180
26. Heijungs R, Guinnée JB, Beltrán AM et al (2019) Everything is relative and nothing is certain. Toward a theory and practice of comparative probabilistic LCA. Int J Life Cycle Assess 24:1573–1579
27. Tu Q, Eckelman M, Zimmerman J (2017) Meta-analysis and harmonization of life cycle assessment studies for algae biofuels. Environ Sci Technol 51:9418–9432
28. Bulle C, Margni M, Patouillard L et al (2019) IMPACT World +: a globally regionalized life cycle impact assessment method. Int J Life Cycle Assess 24:1653–1674
29. Mutel C, Liao X, Patouillard L et al (2019) Overview and recommendations for regionalized life cycle impact assessment. Int J Life Cycle Asses 24:856–865
30. Emmengger MF, Pfister S, Koehler A et al (2011) Taking into account water use impacts in the LCA of biofuels: an Argentinean case study. Int J Life Cycle Assess 6:869–877
31. Crespo-Mendes N, Laurent A, Hauschild MZ (2019) Effect factors of terrestrial acidification in Brazil for use in life cycle impact assessment. Int J Life Cycle Assess 24:1105–1117
32. Verones F, Hellweg S, Huijbregts MAJ (2010) LC-IMPACT: overall framework. Trondheim (Norway). www.lc-impact.eu. Accessed 5 Apr 2020
33. Meijide A, de la Rua C, Guillaume T et al (2020) Measured greenhouse gas budget emission savings from palm oil biodiesel. Nat Commun 11:1089
34. Murphy DM, Ravishankara AR (2018) Trends and patterns in the contributions to cumulative radiative forcing from different regions of the world. Proc Natl Acad Sci U S A 115:13192–13197
35. Reijnders L (2015) Life cycle assessment of greenhouse gas emissions—Chapter 2-2. In: Chen WY et al (eds) Handbook of climate change mitigation and adaptation. Springer, New York. (31 pp). https://doi.org/10.1007/978-1-4614-6431-0_2-2
36. de Bikuna KS, Hamelin L, Hauschild MZ et al (2018) A comparison of land use change accounting methods; seeking common grounds for key modelling choices in biodiesel assessments. J Clean Prod 127:52–61
37. Bessou C, Tailleur A, Godard C et al (2020) Accounting for soil organic carbon role in land use contribution to climate change in agricultural LCA: which methods? Which impacts? Int J Life Cycle Assess 25(7):1217–1230. https://doi.org/10.1007/s11367-019-01713-8

38. Roy PO, Huijbregts M, Deschenes L (2012) Spatially-differentiated atmospheric source-receptor relationships for nitrogen oxides, sulfur oxides and ammonia emissions at the global scale for life cycle impact assessment. Atmosph Environ 62:74–81
39. Heijungs R (2020) On the number of Monte Carlo runs in comparative probabilistic LCA. Int J Life Cycle Assess 25:394–402
40. Myrhe G, Shindell D, Bréon F-M et al (2013) Anthropogenic and natural radiative forcing. In: Climate change 2013: the physical science basis. Cambridge University Press, Cambridge, pp 659–730
41. Searchinger T, Heimlich R. (2015) Avoiding bioenergy competition for food crops and land. World Resources Institute. www.worldresourcesreport.org
42. Helias A (2019) At the boundary between anthropogenic and environmental systems: the neglected emission of indirect nitrous oxide. Int J Life Cycle Assess 24:412–418
43. Chisti Y (2013) The problem with algal fuels. Biotechnol Bioeng 110:2319–2328
44. Ketzer F, Skarka J, Rösch C (2018) Critical review of microalgae LCA studies for bioenergy production. Bioenergy Res 11:95–105
45. Hoekstra AY (2011) The water footprint manual. Earthscan. London. ISBN 978-1-84971-279-8
46. International Standardisation Organisation (2014) Standard 14046. Environmental management, water footprint—principles, requirements and guidelines. International Standardisation Organisation, Geneva
47. Pradinaud C, Nunez M, Roux P et al (2019) The issue of considering water quality in life cycle assessment of water use. Int J Life Cycle Assess 24:590–603
48. Nunez M, Pfister S, Vargas M et al (2015) Spatial and temporal specific characterization factors for water use impact assessment in Spain. Int J Life Cycle Assess 20:128–138
49. Collotta M, Champagne P, Mabee W et al (2018) Waste water and waste CO_2 for sustainable biofuels from microalgae. Algal Res 29:12–21
50. Sonderegger T, Berger M, Alvarenga R et al (2020) Mineral resources in life cycle impact assessment part: a review. Int J Life Cycle Assess 25:784–797. https://doi.org/10.1007/s11367.020.01736-6
51. Berger M, Sonderegger T, Alvarenga R et al (2020) Mineral resources in life cycle assessment: part II—recommendations on application-dependent use of existing methods and future method development needs. Int J Life Cycle Assess 25:798–813. https://doi.org/10.1007/s11376-020-1737-5
52. van Oers L, Guinée JB, Heijungs R (2020) Abiotic resource depletion potentials (ADPs) for elements revisited updating ultimate reserve estimates and introducing time series for production data. Int J Life Cycle Assess 25:294–308
53. Kesler SE, Wilkinson BH (2008) Earth's copper resources estimated from tectonic diffusion of porphyry copper deposits. Geology 36:255–258
54. Sverdrup HU, Olafsdottir AH, Ragnarsdottir KV (2020) On the long-term sustainability of copper, zinc and lead supply using a system dynamics model. Resourc Conserv Recycl 4:100007. https://doi.org/10.1016/j.rcrx.2019.100007
55. Havukainen J, Nguyen MT, Väisänen S et al (2018) Life cycle assessment of small-scale combined heat and power plant: environmental impacts of different forest biofuels and replacing district heat produced from natural gas. J Clean Prod 172:837–848
56. Maier JM, Sowlati T, Salazar J (2019) Life cycle assessment of forest-based biomass for bioenergy: a case study in British Columbia, Canada. Resourc Conserv Recycl 146:598–609
57. Etminan M, Myrhe G, Highwood EJ (2016) Radiative forcing of carbon dioxide, methane and nitrous oxide: a significant revision of methane radiative forcing. Geophys Res Lett 45:12814–12823
58. Munoz I, Schmidt JH (2016) Methane oxidation, biogenic carbon, and the IPCC's emission metrics. Proposal for a consistent greenhouse-gas accounting. Int J Life Cycle Assess 21:1069–1075
59. Taelman SE, de Meester S, Roef LJ et al (2013) The environmental sustainability of microalgae as a feed for aquaculture: a life cycle perspective. Bioresour Technol 150:513–522
60. Colotta M, Busi L, Champagne P et al (2016) Evaluating microalgae-to-energy systems: different approaches to life cycle assessment (LCA) studies. Bofuels Bioprod Bioref 10:883–895
61. Reijnders L (2018) Biofuels from microalgae: biodiesel. In: Jacob-Lopes E et al (eds) Energy from microalgae: green energy and technology. Springer, Cham, pp 171–180
62. Ubando AT, Rivera DRT, Chen W (2019) A comprehensive review of life cycle assessment (LCA) of microalgal and lignocellulosic bioenergy products from thermochemical processes. Bioresour Technol 291:121837. (16 pp)



Chapter 5

Quantification of Branched-Chain Alcohol-Based Biofuels and Other Fermentation Metabolites via High-Performance Liquid Chromatography

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Abstract

As the consequences of climate change become apparent, metabolic engineers and synthetic biologists are exploring sustainable sources for transportation fuels. The design and engineering of microorganisms to produce bio-gasoline and other biofuels from renewable feedstocks can significantly reduce dependence on fossil fuels as well as lower the emissions of greenhouse gases. A significant amount of research over the past two decades has led to the development of microbial strains for the production of advanced fuel compounds. Crucial to these efforts are robust methods to quantify the amount of the biofuel compound being produced as well as the other metabolites that might be present during fermentation. Here, we provide a protocol for the quantification of branched-chain alcohols, specifically isobutanol and isopropanol, using high-performance liquid chromatography (HPLC).

Key words High-performance liquid chromatography (HPLC), Biofuels, Isobutanol, Biofuel quantification

1 Introduction

Microbial production of high-energy fuels via environmentally sustainable and economically efficient bioprocesses has emerged as an attractive alternative to the traditional production of transportation fuels [1–4]. While the first-generation biofuel ethanol has played an important role for the transition to bio-based fuels, ethanol is not an ideal gasoline replacement due to its poor physicochemical properties (low energy density, high hygroscopicity and high vapor pressure) [5]. In contrast, branched-chain alcohols, such as isopropanol, isobutanol, and isoamyl alcohol (3-methyl-1-butanol), have superior energy density and are more compatible with the existing distribution and storage infrastructures. In short, branched-chain alcohols are ideal gasoline substitutes for high-performance petrol engines.

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A robust and accurate method for separation, identification, and quantification of isobutanol and other branched-chain alcohols in fermentation media is critical to the development of high-performing microbial strains. Two predominant methods exist for quantifying microbial production of these branched-chain and higher alcohols: gas chromatography (GC) and high-performance liquid chromatography (HPLC) [6, 7]. We routinely use the latter as it also allows quantification of other metabolites that are generated during fermentation as well as the starting carbon substrate glucose, giving us a more complete fermentation profile of the strain that can aid in further strain improvements [8]. Here, we outline a protocol for quantifying isobutanol and other branched-chain alcohols such as isopropanol and isopentanol by using high-performance liquid chromatography (HPLC). Our protocol also enables simultaneous quantification of several other cellular metabolites such as ethanol, glycerol, pyruvate, and the substrate glucose.

2 Materials

2.1 Yeast Growth

1. 1.7 mL snap-cap microcentrifuge tubes, polypropylene (Corning Costar, cat. no. CLS3620).
2. Bottle-top vacuum filters (0.22 μm pore size, polyethersulfone membrane filter, 70 mm neck diameter, funnel capacity 500 mL, Corning, cat. no. CLS431117).
3. Pyrex round bottles with GL45 screw cap (sterile, 1000 mL, Corning, cat. no. 1395-1L).
4. Haraeus Pico 17 microcentrifuge (with 24 × 1.5/2.0 mL rotor with ClickSeal biocontainment lid, Thermo Fisher Scientific, cat. no. 75002410).
5. Falcon 50 mL conical centrifuge tubes (Corning, polypropylene, with CentriStar cap, cat. no. CLS430828).
6. Non-baffled Pyrex Erlenmeyer flasks (sterile, 250 mL, Corning, cat. no. 4980-250) with cotton wool plugs.
7. SmartSpec Plus spectrophotometer for UV/visible absorbance measurements from 200 to 800 nm (Bio-rad, cat. no. 170-2525).
8. Semi-microvolume disposable cuvettes (Bio-rad, polystyrene, cat. no. 2239955).
9. −20 °C freezer for sample storage.

2.2 Detection of Isobutanol and Other Fermentation Metabolites

1. Clear glass wide opening screw thread vials (2 mL, Clear Glass, 12 mm diameter, screw thread, Thermo Fisher Scientific cat. no. C4010-1).

2. 1.7 mL snap-cap microcentrifuge tubes, polypropylene (Corning Costar, cat. no. CLS3620).
3. Assembled Target Caps with PTFE/silicone septa for screw-cap vials (Thermo Fisher Scientific cat. no. C4010-60AY).
4. Haraeus Pico 17 microcentrifuge (with 24 × 1.5/2.0 mL rotor with ClickSeal biocontainment lid, Thermo Fisher Scientific, cat. no. 75002410).
5. Puradisk nylon membrane syringe filter (0.2 μm pore size, 13 mm neck diameter, Whatman, cat. no. WHA67901304).
6. Disposable syringes (1 mL, sterile, without needle, Nipro, reference code SY3-1SC-EC).
7. Bottle-top vacuum filters (0.22 μm pore size, polyethersulfone membrane filter, 70 mm neck diameter, funnel capacity 500 mL, Corning, cat. no. CLS431117).
8. Glass or Pyrex round medium storage bottles with GL45 screw cap, sterile, 1000 mL (Corning, cat. no. 1395-500).
9. UltiMate 3000 HPLC instrument (Thermo Fisher Scientific, UltiMate 3000 HPLC Series with UltiMate 3000 series DAD detector and Shodex RI-101 RID detector) (Fig. 1).
10. Aminex HPX-87H ion-exchange column (Biorad, 300 × 7.8 mm, 9 μm particle size, cat. no. 1250140).

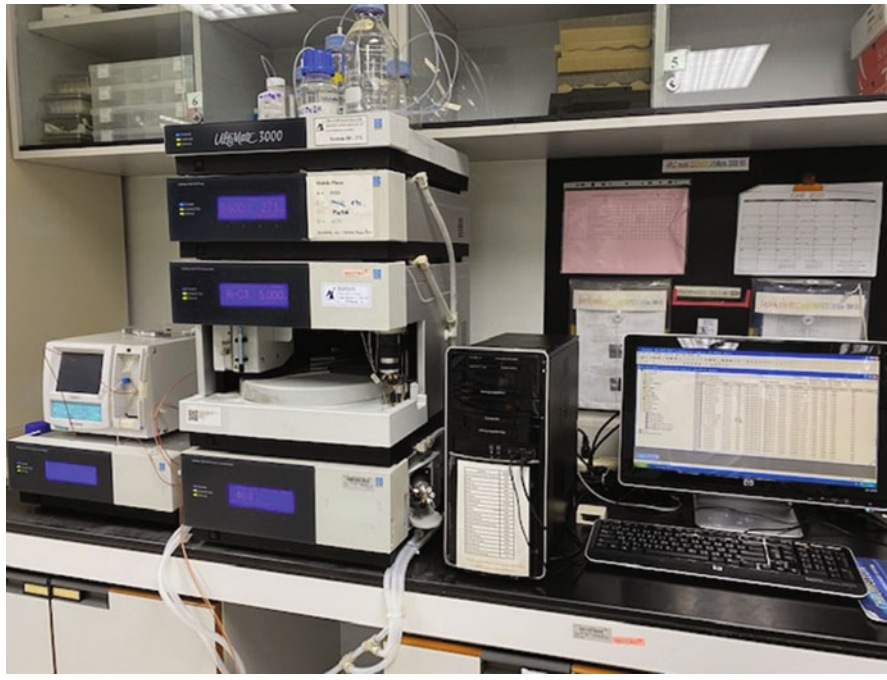


Fig. 1 UltiMate 3000 HPLC instrument (Thermo Fisher Scientific, UltiMate 3000 HPLC Series with UltiMate 3000 series DAD detector and Shodex RI-101 RID detector)

11. Cation H refill cartridge guard column (Biorad, 30 × 4.6 mm, cat. no. 1250129).
12. Guard column holder (Biorad, for 30 × 4.6 mm cartridge, cat. no. 1250131).
13. Chromeleon chromatography data system software package for data acquisition and data processing (Thermo Fisher Scientific, cat. no. CHROMELEON7).

2.3 Reagent Setup

Sterile YNB without amino acid solution (5×)

Dissolve 34 g of yeast nitrogen base without amino acid powder in 400 mL of milli-Q water in a 500 mL medium bottle. Mix with a stir bar on a stir plate until the YNB is fully dissolved. Add milli-Q water to a final volume of 500 mL. Sterilize the solution by filtration into a sterile 500 mL glass medium bottle using a bottle-top 0.22-μm filter. Store the solution at 4 °C.

Sterile 20% (vol/vol) glycerol solution

Add 100 mL of glycerol to a 500 mL medium storage bottle, and then add 400 mL of milli-Q water to the bottle. Autoclave the solution and store at 4 °C.

Sterile 40% (wt/vol) glucose solution

Dissolve 200 g of glucose in 300 mL milli-Q water in a 500 mL medium storage bottle. Add milli-Q water to a final volume of 400 mL. Autoclave the solution and store at 4 °C.

Sterile 1M **K_2HPO_4**

Dissolve 69.68 g of K_2HPO_4 in 300 mL of milli-Q water in a 500 mL medium storage bottle. Add milli-Q water to a final volume of 400 mL. Autoclave the solution and store at 25 °C.

Sterile 1 M KH_2PO_4

Dissolve 54.44 g of KH_2PO_4 in 300 mL of milli-Q water in a 500 mL medium storage bottle. Add milli-Q water to a final volume of 400 mL. Autoclave the solution and store at 25 °C.

Sterile D-biotin solution (500×; 0.2 g/L)

Dissolve 10 mg of D-biotin in 40 mL milli-Q water in a 50 mL Falcon tube. Add a small amount of 1 N NaOH until the D-biotin completely dissolves. Add milli-Q water to a final volume of 50 mL. Sterilize the solution using a 0.22-μM Nylon filter and store at 4 °C.

Sterile L-histidine solution (200×; 4 g/L)

Dissolve 200 mg of L-histidine in 40 mL milli-Q water in a 50 mL Falcon tube. Add milli-Q water to a final volume of 50 mL. Sterilize the solution using a 0.22-μM Nylon filter and store at 4 °C.

Sterile thiamine-hydrochloride solution (20 g/L)

Dissolve 1 g of thiamine-hydrochloride in 40 mL milli-Q water in a 50 mL Falcon tube. Add milli-Q water to a final volume of 50 mL. Sterilize the solution using a 0.22-μM Nylon filter and store at 4 °C.

Pre-production medium (MGYH with 2% glycerol, pH 6)

To make 1000 mL of the pre-production medium, add 100 mL of 20% (vol/vol) sterile glycerol solution, 200 mL of sterile YNB without amino acid solution (5×), 13.2 mL of sterile 1 M K_2HPO_4, 86.8 mL of sterile 1 M KH_2PO_4, 5 mL of sterile L-Histidine solution, 2 mL of sterile D-Biotin solution, and 6.5 mL of sterile thiamine-hydrochloride solution in a sterile 1000-mL glass medium bottle. Add sterile milli-Q water to a final volume of 1000 mL. This can be stored at 4 °C for at least 1 month.

Production medium (MGYH with 10% glucose)

This medium contains 10% glucose (wt/vol) to enable strong expression of the isobutanol pathway genes under the control of the constitutive P_{GAP} promoter. To make 1000 mL of production medium, add 250 mL of 40% (wt/vol) sterile glucose solution, 200 mL of sterile YNB without amino acid solution (5×), 13.2 mL of sterile 1 M K_2HPO_4, 86.8 mL of sterile 1 M KH_2PO_4, 5 mL of sterile L-Histidine solution, 2 mL of sterile D-Biotin solution, and 6.5 mL of sterile thiamine-hydrochloride solution in a sterile 1000 mL glass medium bottle. Add sterile milli-Q water to a final volume of 1000 mL. This can be stored at 4 °C for at least 1 month.

HPLC mobile phase (5 mM H_2SO_4)

Slowly add 2.805 mL of the 95% w/w H_2SO_4 to 250 mL milli-Q water. Adjust the final volume of solution to 1000 mL with milli-Q water. Filter using the bottle-top vacuum filter into a 1000 mL glass medium bottle.

Needle wash solvent (10% methanol (vol/vol))

Prepare 10% methanol by mixing 10 mL of methanol with 90 mL of milli-Q water. Filter using the bottle-top vacuum filter into a 100 mL glass medium bottle.

1× Standard stock solutions

Prepare a 1× standard stock solution that contains 7.5 g/L isobutanol, 7.5 g/L isopropanol, 15 g/L ethanol, 1 g/L glycerol, 2 g/L acetate, and 0.5 g/L pyruvate. For glucose, which has a similar retention time to that of pyruvate, prepare a separate 1× stock solution at 100 g/L.

3 Methods

3.1 Preparation of Pre-production and Production Cultures (See Note 1)

1. Pipette 5 mL of sterile pre-production medium to a sterile 50 mL Corning tube.
2. Inoculate the medium with a single *P. pastoris* colony from a selective plate. Three to five individual colonies from each strain to be tested should be selected. Additionally, we recommend including a control strain (e.g., strain lacking the isobutanol pathway genes integrated into the genome or a laboratory strain).
3. Incubate the culture tubes at 30 °C with shaking at 250 r.p.m. for 16 h.
4. Measure the OD_{600} of the overnight cultures before proceeding to the next step. This can be done by removing 50 μL of the overnight culture and diluting it 20 folds with water in a plastic cuvette. Measure the OD_{600} by using a spectrophotometer. The OD_{600} of the cultures should be about 3–6.
5. Pipette 50 mL of production medium to a sterile 250 mL Erlenmeyer flask closed with a cotton wool plug.
6. Inoculate the flask with the pre-production culture so that the starting OD_{600} is 0.05.
7. Incubate the culture tubes at 30 °C with shaking at 250 r.p.m. for 72 h. We recommend sampling the culture at least every 24 h for measurement of isobutanol, glucose, ethanol, glycerol, pyruvate, acetate, and biomass (OD_{600}).

3.2 Procedure for Sampling for Isobutanol Analysis

1. Transfer 1 mL of the culture to a sterile 1.7 mL microcentrifuge tube. Make sure the culture is well suspended by swirling the flask a few times prior to sampling.
2. Determine the OD_{600} of the culture by removing 50 μL and diluting it 20 times with water in a plastic cuvette. Cultures at later time points may need to be diluted even further (40 times) depending on the linear range of the spectrophotometer used.
3. Centrifuge the collected samples in a benchtop microcentrifuge at 1000 × *g* for 5 min at 4 °C.
4. Use a 1-mL sterile syringe to withdraw as much of the supernatant as possible (about 800 μL) without perturbing the cell pellet. Note: The cell pellet can be used for total RNA extraction if a gene expression analysis is required.
5. Filter the withdrawn sample through a sterile 0.45 μm Nylon filter directly into an HPLC glass vial. The samples can be stored at −20 °C prior to HPLC analysis.

3.3 Quantification and Data Analysis

1. Serially dilute the 1× standard solution in HPLC vials. Add 500 μL of the 1× stock to 500 μL of milli-Q water to give a 0.5× standard. Serially dilute 200 μL of this 0.5× standard in 800 μL of milli-Q water 3 times to obtain a set of standards at 0.1×, 0.02×, and 0.004×.
2. Prepare a blank sample by adding 1000 μL milli-Q water into an HPLC vial.
3. Set up the HPLC with the following settings. For the isocratic LC program, use 5 mM H_2SO_4 as the solvent at a flow rate of 0.68 mL/min for 30 min (*see* **Notes 2** and **3**). Set the column oven temperature at 60 °C. The injection volume is 10 μL (*see* **Note 4**).
4. Start up and equilibrate the HPLC column by purging the HPLC system as well as the RI detector with the mobile phase. Turn on the UV lamp ($\lambda = 210$ nm) before sample analysis for a minimum of 30 min to ensure a stable baseline for quantitation (*see* **Note 5**). If the HPLC column is connected to the HPLC system, open the purge valve to prevent the deterioration and overload of the equipment due to high pressure. After the purge, equilibrate HPLC column by flushing the system with the mobile phase at the flow rate of 0.68 mL/min (the same condition used for sample analysis). Prime the needle wash and purge 10% methanol into the autosampler if available. A needle wash step is required before and after each sample injection.
5. When the HPLC system is equilibrated, reset (or "zero") the baseline for RI detector and UV detector prior to each sample injection. Initiate the HPLC analysis by injecting in order: (i) the blank sample, (ii) the standard samples, and (iii) the experiment samples (*see* **Note 6**).
6. Determine the chromatographic peak areas for isobutanol, ethanol, isopropanol, glycerol, glucose, pyruvate, and acetate using the Chromeleon software (Fig. 2). Use the Refractive Index (RI) spectra for isobutanol, ethanol, isopropanol, glycerol, and glucose peaks. Use the UV spectra for acetate and pyruvate peaks. Make sure that the same integration parameters are used for the entire set of samples. Retention times for the compounds are as follows: pyruvate, 7.89 min; glucose, 8.08 min; glycerol, 11.80 min; acetate, 13.22 min; ethanol, 18.92 min; isopropanol, 20.73 min; isobutanol, 27.85 min.
7. To generate a calibration curve for each compound, plot the peak area for that compound against its corresponding concentrations. Obtain the linear equation by linear regression analysis. The r^2 value should be greater than 0.97.

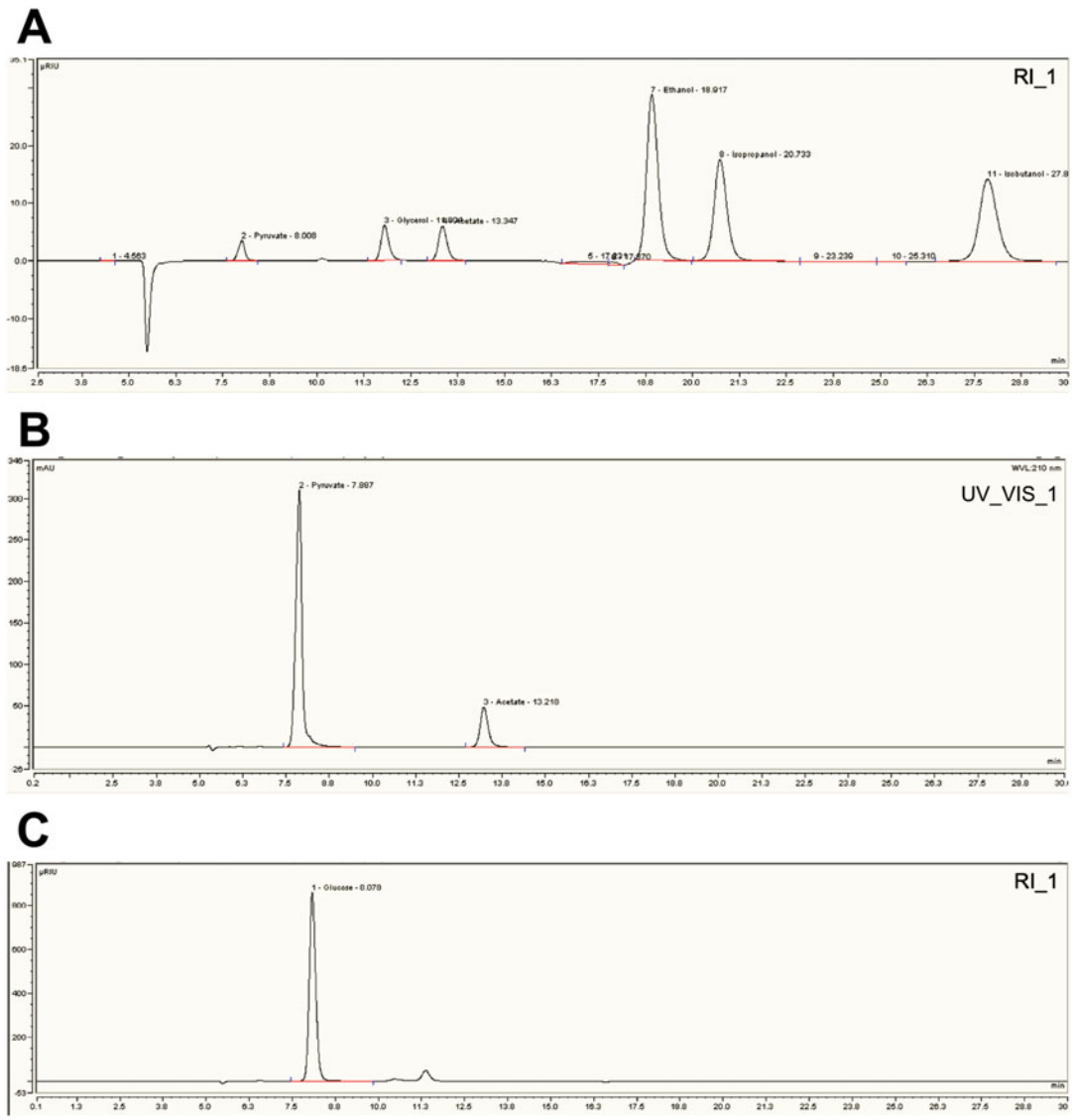


Fig. 2 Representative HPLC chromatograms. (**a**) Refractive index (RI) spectra of 0.1× standard solution. (**b**) UV spectra of 0.1× standard solution. (**c**). RI spectra of 0.1× glucose standard solution

8. To quantify the concentrations of each compound, integrate the peak at the expected retention time for that compound and apply the linear equation obtained from the calibration curve.

4 Notes

1. Yeast inoculation and sampling should be performed in a sterile environment (e.g., in a laminar flow hood) to minimize risks of contamination.

2. The HPLC program described in this protocol is optimized for the production of isobutanol. However, if the detection of isoamyl alcohol (3-methyl-1-butanol) is also required, we recommend extending the HPLC run time to 45 min.
3. To protect the Aminex HPLC column, we recommend using the Cation H refill guard column, which prevents particulate matter and aggressive reagents from getting into the HPLC column.
4. Measurement of glucose concentrations at earlier time-points may require diluting the samples by 10 folds to obtain concentrations that are within the linear range of the calibration curve (0.4–50 g/L).
5. Simultaneous determination of sugar, isobutanol, ethanol, glycerol, pyruvate, and acetate in a single sample injection could be carried out by connecting UV and RI detectors in series.
6. When sample analysis is finished, remove the column and clean the HPLC system as well as detectors by flushing the unit out with the mobile phase, then milli-Q water and store the system in 100% methanol to mitigate bacterial growth when the HPLC system is running idle.

Acknowledgments

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References

1. Liao JC, Mi L, Pontrelli S, Luo S (2016) Fuelling the future: microbial engineering for the production of sustainable biofuels. Nat Rev Microbiol 14:288–304
2. Keasling JD (2010) Manufacturing molecules through metabolic engineering. Science 330:1355–1358
3. Kung Y, Runguphan W, Keasling JD (2012) From fields to fuels: recent advances in the microbial production of biofuels. ACS Synth Biol 1:498–513. https://doi.org/10.1021/sb300074k
4. Peralta-Yahya PP, Zhang F, Del Cardayre SB, Keasling JD (2012) Microbial engineering for the production of advanced biofuels. Nature 488:320–328
5. Lan EI, Liao JC (2013) Microbial synthesis of n-butanol, isobutanol, and other higher alcohols from diverse resources. Bioresour Technol 135:339–349. https://doi.org/10.1016/j.biortech.2012.09.104
6. Atsumi S, Hanai T, Liao JC (2008) Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. Nature 451:86–89. https://doi.org/10.1038/nature06450
7. Avalos JL, Fink GR, Stephanopoulos G (2013) Compartmentalization of metabolic pathways in yeast mitochondria improves production of branched chain alcohols. Nat Biotechnol 31:335–341. https://doi.org/10.1038/nbt.2509.Compartmentalization
8. Siripong W, Wolf P, Kusumoputri TP et al (2018) Metabolic engineering of Pichia pastoris for production of isobutanol and isobutyl acetate. Biotechnol Biofuels 11:1. https://doi.org/10.1186/s13068-017-1003-x



Chapter 6

Jatropha curcas, a Novel Crop for Developing the Marginal Lands

Waleed Fouad Abobatta

Abstract

Jatropha curcas L. has more attention from researchers and policymakers as an inexpensive source for produce biofuel to reduce environmental pollution by fossil fuel in the next decades without competing for lands and freshwater currently used for food production. Jatropha is a perennial deciduous, succulent oilseed shrub, belonging to family *Euphorbiaceae*. It is native to Central and South America. It is a multipurpose shrub, each part of the plant can be used for various purposes, Jatropha produces flowers throughout the year and enables multiple harvests, while, in arid and semi-arid regions it is harvesting twice time per year.

Jatropha is a drought-tolerant plant that could be growing under malnutrition conditions, and in different climatic conditions; therefore, it is proper plant for developing marginal lands and rural areas.

Due to the growing demand for biofuel, jatropha cultivation has received more attention to providing seeds. While, there are various aspects of using jatropha include use as a traditional medicine for treating skin ailments, as a hedge for protecting other crops, to reduce soil degradation, combating desertification, and deforestation, also, jatropha cultivation protects the environment through using wastewater in irrigation and reduce sequester carbon dioxide.

Conventional propagation of Jatropha propagated by seeds or stem cutting which is a more satisfactory technique to produce high-yielding seedlings, while, tissue culture method used in propagation but on small scale.

Jatropha curcas L. contains mixtures of numerous active substances in all parts of the plant, which are used as biopesticides, larvicides, fungicide, and nematicide; also extracts are used as pesticides for whiteflies and termites.

Jatropha crude oil is used for industrial purposes like manufacturing candles, soaps, varnishes, and as a lubricant; also press seedcake is used to produce biogas and organic fertilizers. Jatropha propagated by seeds or stem cutting which is more applicable techniques to produce high-yielding seedlings, also, tissue culture method used in propagation but on small scale for scientific work.

Key words *Jatropha curcas*, Biofuel, Biopesticides, Environment, Marginal lands, Wastewater

1 Introduction

As the world looking to sustainable development and the provision of adequate food to the growing world population, which will reach to 9 billion in 2050 and 12 billion in 2100 [1], the main challenge

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faces the world is the limitations of freshwater and arable land resources, however, there is a wide area of marginal land in different regions of the world, for example in Egypt, more than 90% of the total land area is marginal desert land [2], also, there is a huge quantity of wastewater considered a valuable source for planting energy crop and forestry. Currently, there is a competition between energy crops and main crops on land and water use [3]. An overview of *Jatropha curcas L.* planting is the possibility and consequences of using marginal land in different regions to produce economic products like biofuel.

Jatropha curcas L. is a deciduous oilseed shrub, belonging to family *Euphorbiaceae*; jatropha is native to Central and South America [4]. *Jatropha curcas* is known with different names in different world regions like "the physic nut," "goat nuts," "(pinhão-manso)" in Brazil, "Barbados nut," "purging nut," "nettle spurge," "Purgiemuss," "Pignon d'lnde," "Jarak pagar," "sabuu dam," "xiao tong zi," "nanyo aburagiri," "جتروفا" or just Jatropha [5].

Jatropha curcas L. is a multipurpose shrub, each organ of plant parts can be used for various purposes, Jatropha subjected to various studies in numerous countries over the world, to explore more benefits of jatropha, currently, produce biofuel considered the main use of jatropha to avoid use edible crops to produce [6], jatropha planting to reduce soil degradation, combat desertification, and deforestation [7], planting as hedges for protecting other crops from roaming animal, traditional medicine for people and as veterinary medicine from ancient time, Jatropha soap is valued as a medicinal soap for treating skin ailments [8], woody stem and branches used in rural housing, leaves extracts used as pesticides, and produce compost from press seedcake, crude seed oil used for lighting, as a cooking fuel instead of kerosene or other fossil fuel [9], also, seed oil used in for industrial purposes includes manufacturing candles, soaps, varnishes, crude oil used as lubrication, and seeds press cake use to produce biogas [10].

J. curcas one of the main biofuel crops worldwide, from the last decades there more attention to the cultivation of J. curcas in different tropical and temperate regions as a source for renewable energy and other various benefits [11]. Jatropha has great yield potential and can be grown well under stress conditions, and represent a new key to development of marginal lands; it is claimed to be drought tolerant and can be grown under salinity conditions [12]. Jatropha plant needs proper agricultural practice including irrigation, fertilizing, and pest control, but less than other crops [13]. The crop yield of seeds is affected by planting destination, malnutrition, and insects; therefore, planting jatropha by seedlings instead of sowing seed directly in the soil, with proper planting

spaces, adding proper nutrients is the key to achieving high seed yield from Jatropha, particularly after harvesting the first yield in May.

Jatropha gets more attention from the last decades as a nonedible and cheap source for biodiesel to reduce environmental pollution by fossil fuel [14]. *Jatropha curcas L.* represents a novel opportunity to develop marginal desert land and reduce the hazards of wastewater [15].

2 Why We Need Jatropha?

From the beginning of this century, there is more attention to looking alternative sources for energy, particularly renewable energy; *J. curcas* has more interest from decision-makers and businessmen worldwide since 2004/2005 approximately and there is more attention to jatropha as an economic source for biofuel production [14]. Jatropha has good potential to produce diesel engines as well as for medicinal and insecticidal purposes [16]. There is a valuable oil in seeds reach up 30–40% from their size, this valuable oil considered the most proper vegetable oil to produce biodiesel in the next decades [17], therefore, establishing new jatropha plantation in marginal lands will develop these areas and provides a cheap source for renewable energy [18].

3 Jatropha Around the World

Jatropha curcas L. considered the most proper crop to produce biofuel in the next decades, the main advantage of jatropha that it is not competing with food crops, due to its ability to grow well in marginal lands, degraded lands and irrigated with wastewater, also, Jatropha protect soil from degradation and reduce pollution hazards of wastewater [19], in addition, Jatropha adapted to aridity conditions and produce economic crop seeds in arid and semi-arid environments [20], also, Jatropha is planted in tropical regions as a hedge to protect other crops from the roaming animal like cattle, sheep and goats [21].

Under current climatic conditions and expectations changes in climate globally, Jatropha cultivation should be the main fuel crop in different areas including tropical and hot temperate and temperate areas like Africa (e.g., Zambia, Tanzania, Mozambique) [21], Senegal [22], MENA region (e.g., Egypt) [17], South America (e.g., Argentina, Paraguay, Nicaragua) [23], South and East Asia like in Philippine [24], and (Northern India, Nepal, and China) [25] which expected to be main areas for Jatropha cultivation in the next decades.

4 Importance of Jatropha

In the current era, Jatropha is considered a keener solution for various problems like increasing emission of CO_2, reducing fossil fuel, soil degradation, and increasing wastewater quantities; therefore, Jatropha is considered a promising crop for developing rural communities, particularly in marginal lands, due to its peculiar properties like drought tolerance, higher oil content than other energy crops, fast growth, two times harvesting in the year, and easy propagation [26]. There are different uses for *Jatropha curcas L.*; nowadays, the major purpose for planting jatropha to produce biodiesel is because the seeds of jatropha has higher oil content than other energy oil crops, and could produce biogas and organic fertilizers from the waste of oil extraction [10].

Jatropha is a proper crop for developing marginal lands and rehabilitating degraded lands, It's growing under harsh conditions such as drought, low nutrient supply, and salinity, whereas, other plants can't grow [27], also, there is a positive effect for the cultivation of jatropha on marginal lands for the local community and protect soil from erosion and degradation [18].

5 Advantage of Jatropha

There are many advantages for jatropha cultivation: it is prober for reclamation marginal lands, grows well under environmental stress, improves income for local communities, reaches quick to maturation, and also used to improve the climate in arid areas and reduce CO_2 ratio.

1. Reduce sequester carbon dioxide [28].
2. Competing desertification and protecting the environment from the hazards of wastewater [19].
3. Rapid growth and quick maturation [economic yield after 2–3 years] [29].
4. Long productivity period [35–50 years] [5].
5. Easy propagation [30].
6. Produce biogas and organic fertilizers from press seedcake [10].
7. Combating rural poverty [9].

For all the above advantages and applications, *J. curcas* should be a favorite plant for producing biodiesel and preferably growing in marginal lands; therefore, jatropha as an economic crop could be a novel key to the sustainable development of poor countries under growing global demand for biofuel in the near [31].

6 Main Aspects of Using Jatropha

1. Produce biofuels from seed oil [15].
2. Rehabilitate degraded lands that are not proper for food production [32].
3. Jatropha is considered a source for improved livelihoods in poor areas [33].
4. Used as hedges to protect other crops, like in Malaysia the jatropha shrubs are used as a belt around oil palm areas from grazing animals [34].
5. Prevent soil erosion [19].
6. Improving marginal soil characters that are not proper for producing other crops [18].
7. Creating livelihood opportunities and improving socioeconomic for poor people in marginal lands [35].
8. Crude oil used for lighting and cooking in rural areas [26].
9. Seed coat powder used for purification of wastewater from heavy metals.

Therefore, jatropha as economic crops is considered a novel key to the sustainable development of poor regions under growing global demand for biofuel in the near future [31].

For all these aspects (Fig. 1) jatropha is considered the most appropriate crop to produce biodiesel, protect the environment, and improve socioeconomic status in rural regions.

7 Botanical

Jatropha (*Jatropha curcas L.*) belongs to family Euphorbiaceous and its genus include about 175 different species [7]; most species are spread mostly in America and Africa, while some species are

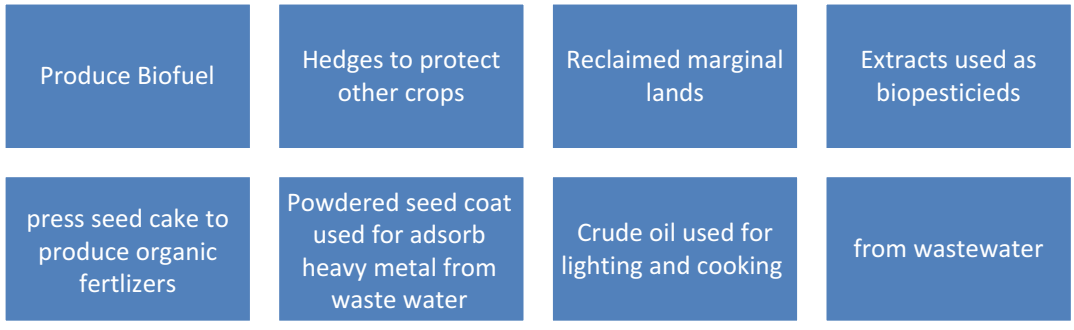


Fig. 1 Some aspects of using jatropha



Fig. 2 Jatropha trees in Egyptian desert 2018

found in Arabian region and a dozen species in India [36, 37]. Jatropha name is derived from the Greek words "jatros" meaning "physician," and "trophe" meaning "nutrition"; *Jatropha curcas* is a vigorous, perennial deciduous, succulent shrub, it is a C3 shrub, jatropha large shrub or small tree attains to 3–5 m (Fig. 2). It grows up to 8–10 m under appropriate conditions, jatropha produces oil-rich seeds (about 30–40%) size/size) [38].

[Image showing jatropha trees in Egyptian desert 2018].

Botanical classification of *Jatropha curcas*:

Division: Spermatophyta

Class: Dicotyledonae

Family: Euphorbiaceae

Genus: Jatropha

Specie: Curcas

Botanical name: *Jatropha curcas L.*

Jatropha has its native distributional range in Mexico, Central America, and part of South America near from the equator, but nowadays jatropha planting is in different conditions like temperate climate and in arid and semi-arid areas; also jatropha is considered as pest-tolerant plant and is grown under malnutrition conditions.

There are various names for Jatropha in different regions worldwide like "purging nut" or "physic nut" in English; "(Tubang-bakod)"; "goat nuts"; "(pinhão-manso)" in Portuguese; "Barbados nut"; "nettle spurge"; "nettle spurge"; "bagbherenda" in Hindi; "Purgiemuss" in Germany; "Pignon d'lnde" in French; "Jarak pagar"; "sabuu dam" in Thai; "xiao tong zi" in Chinese; "nanyo aburagiri" in Japanase; "جتروفا "in Arabic; or just Jatropha.

7.1 Leaves

Leaves of jatropha are about 6 cm in length and 15 cm in width approximately; it is a green or pale-green and consist of 3–7 lobes.

7.2 Root System

Jatropha root system is influenced by propagation techniques; plants from directly sowing by seeds in soil has a thick primary tap root and little lateral roots, while plants from vegetative propagation have secondary roots only [39].

Jatropha roots could stretch in the soil about 100 cm during the first 2 years of cultivation and up to 150 cm after 5 years approximately to extract adequate water quantity [40].

Rooting patterns are significantly influenced by the propagation method. Plants originating from seeds and directly sown into the soil normally develop a rooting system with a thick primary tap root and four lateral roots, and with abundant and straight secondary roots [41], whereas plants propagated by cuttings only develop secondary roots [42].

7.3 Flowering

Jatropha is monoecious and flowers are unisexual; the plant produces individual male and female flowers in a compound dichasium cyme pattern. The inflorescence is complex and forms in the leaf axial at the terminal of branches; it can either be simple with six individual cymes or be more complicated with up to ten individual cymes [43].

Climate conditions effect on flower formation and time of flowering, dry weather promote flower bud formation, while excessive irrigation increase vegetative buds formation; therefore, flowering occurred after dry and dormancy period [44].

Jatropha flowering depends on proper temperature between 22 and 25 °C [45, 46]; there is no effect of day length on flowering, so under arid and semi-arid conditions flowering occurs in different time of the year [41], while its flowering one time yearly in tropical regions [45].

Flowers formed individually and terminally, with pale green color and unisexual [37], the majority is male flowers, while female flower lower than male one, Normally the ratio of male to female flowers widely varies from 27:1 to 108 (male flowers): 1 (female flowers) and female flowers are larger than the male flowers; however, hot summer induce to produces more female flowers; also, appropriate irrigation improve flowering pattern, number of flowers, and fruit sets; the male flowers open earlier and for a long duration (8–11 days), while female flowers only open for 3–4 days which promotes cross-pollination [47].

7.4 Pollination

Jatropha depends on insects to complete cross-pollination; different insects like ants, bees, and flies are attracted to the huge number of flowers arranged in groups within inflorescences [48].



Fig. 3 Jatropha fruits at different maturity stages [50]

7.5 Fruits

Jatropha flowering several times during the growing season which enables produce multiple crops annually, generally it is two times per year; each inflorescence produces a few numbers of fruits, mostly about three seeds [44] due to low fertility of female flowers set [49], and differential ripening time of fruits on the same inflorescence [47]; therefore, there are mature fruits found at the upper terminal of each inflorescence, and green fruits found in the middle of inflorescence in the same time (Fig. 3).

The flower and fruit development need about 3 months till the maturity stage; therefore, jatropha could have many cyclical fruiting peaks from two to four cycles depending on environment conditions and cultivation practices.

Image field showing jatropha fruits at different maturity stages [50].

7.6 Seed Yield

Currently, Jatropha is grown their valuable oil, which represents 40% of seed weight approximately under proper agricultural practice, however, seed content of oil could be reduced to 18.4% under malnutrition [41].

There are different planting space used for jatropha cultivation depending on climate conditions; in tropical and semitropical regions planting space could be 2 × 2 m, while under irrigation conditions planting could be 2.5 × 2.5, 3 × 3, and 4 × 2 m depending on soil fertility [51].

Jatropha starts flowering after the first year of plantation and remains productive life over 30 years and could reach 50 years depending on the environment and good nutrition [52].

There is great variation in seed yield productivity from area to another due to environmental conditions and agriculture practice [26, 53] reported that annual seed yield per mature tree ranged

from 0.2 to 2.0 kg; jatropha seeds are moderate size, its weight is about 0.72 g, the averagely of kg contain about 1375 seeds [14].

There are different elements that affect the yield of jatropha include environmental factors like the cool temperature during flowering and fruit and set period [44], genetics, and, agricultural practices that include proper available water quantity both of rainfall, or supplemental irrigation, soil fertility, adequate nutrition particularly nitrogen and phosphorus,. . .etc [54].

7.6.1 Main Factors Affect Seed Yield of Jatropha

1. Soil type and fertility [55].
2. Adequate irrigation [26].
3. Source of plant materials [56].
4. Planting space [51, 57].
5. Tree age [58].
6. Nutritional status [13].
7. Pests and disease [8].

As mentioned above, there are various factors that affect jatropha seed yield, in addition to proper management factors like (correct pruning, adequate fertilization, and efficient Pest control), which considered the key for achieving great seed yield from Jatropha., in general, the average of seed yield ranged from 0.4 to 12 tons per hectare depending on management practices [59].

On another side, environmental conditions affect jatropha yield, seed size, weight, and oil content more than genetic factors [60], in the arid area the average yield of jatropha in marginal lands reach about 2–3 ton/ha [41, 61], however, adequate irrigation and proper management practice could increase seed yield to 5 tons/ha with [23], while, Jongschaap et al. [44] reported that Jatropha seed yield could reach up to 8 tons/ha yearly under proper cultivation practice.

Seeds would retain their vitality for up to 12 months for planting purpose, or oil extraction when stored in a dry and well-ventilated atmosphere.

7.7 Harvesting

In humidity climate jatropha has fruits, flowers throughout the year, while in arid and semi-arid regions jatropha harvesting is two times yearly.

Jatropha seeds reach maturity when fruit color changes to yellow and brown; usually these occurs after 90 days of flowering, but the fruit reaches maturity in different times; therefore, it needs continuous picking; however, under shortage in harvest labor some farmers pick fruits in weekly cycles from the trees and on the ground around the tree during maturity season [52]. Henning [62] estimated labor work average for harvesting as 24 kg each workday, while the average of the workday rises to 50 kg per labor in India (NOVOC).

Due to the multiple flowering of the jatropha during the growing season, there is a difference in the dates of ripening of the fruits, which requires weekly harvesting and labor-intensive.

8 Agricultural Practices

Jatropha like any crops requires most favorable agricultural practices to produce luxury seed. Jatropha grow rapidly after cultivation and could reach up to 1 m in the first season; therefore, it is very important to use the proper agricultural practice to improve jatropha growth, enhancing the quality of seed yield, and avoid competition with weeds.

8.1 Pruning

Generally, economic flowering and yielding from the second years of planting [63]; therefore, top-cutting is a very important factor affecting seed yield production, the main roles of pruning are to make balancing between vegetative and reproductive growth, through controls the excessive vegetative growth and induce laterals growth to increase seed yield, in general, preferable pruning during the dormant period to form a robust canopy with low height and wide-spreading which increase flowering, seed yield, and facilitate harvesting [8].

There is no accurate information on the ideal pruning system of jatropha in various climate conditions and at different ages [64].

There is the relation between the number of inflorescence per plant and the number of braches on the plant. Samsam [24] reported that 10–12 branches per tree gave the highest seed yield than other numbers of branches of *J. curcas*.

There are various benefits for the pruning in the dormancy period:

(i) Increase branching and the number of tip-borne inflorescences.
(ii) Form a wide low-canopy that is easier to harvest.

The pruning height and number of branches per plant affect vegetative growth and seed yield [24, 65]; also it is suggested that rejuvenation of old trees after 10 years by cutting down stumps to 45 cm increase seed yield; the regrowth will be faster and the tree starts bearing fruits after 1 year [63].

8.2 Fertilization

Generally, jatropha needs average quantities of nutrients compared to other crops, while proper nitrogen and phosphorous fertilization increase the yield and the oil content in the seeds, also, Jatropha growing in marginal lands under low nutritional content [23] before growing season and after first harvesting, also, use foliar application of micronutrients improves growth and total seed yield. Suriharn et al. [64] reported that using 312.5 kg per hectare

of NPK fertilizers is considered an ideal dose for obtaining optimum seed yield of *Jatropha curcas*, while the overdose of fertilizers reduced yield; also, fertilizer rate depends on soil type and soil fertility.

There are various factors affecting jatropha nutrient requirement including soil types, soil fertility, moisture, tree ages, pruning techniques, and pest and weed control. In general, the previous literature emphasized that adequate fertilizing is essential to increase seed yield.

Jatropha needs average quantities of nutrients compared to other crops, while proper nitrogen and phosphorous fertilization increase the yield and the oil content in the seeds, particularly in marginal lands under low nutritional content mainly during flowering and fruit set to produce economic seed yield [26, 44].

Jatropha requires annual fertilizing with little quantity of phosphorus, potassium, calcium, magnesium, and sulfur to achieve significant growth and satisfy seed yield [27].

From the other side, Garg, et al. [14] found that in Indian wastelands optimum levels of fertilizers increase total yield [8]. However, the overdose of fertilizers reduced seed yield, while until now there is insufficient data on jatropha response to proper fertilizer in different growing areas [12].

The optimum levels of chemical fertilizers vary according to different factors like:

1. Soil fertility.
2. Age of the tree.
3. The vigor of the tree.
4. Fruit growth stage.

Also, inoculation jatropha farms with Mycorrhizal fungi increase the absorption of phosphorus and micronutrients [44], enhancing germination ratio, and increase nutrients uptake.

More research requires determining optimal nutrition for jatropha in different planting area, particularly in marginal sandy soils which are expected to be the main areas to planting jatropha in the near future.

8.3 Irrigation and Water Requirements

According to the Food and Agriculture Organization of the United Nations [FAO], the average of water requirement for arable land in arid and semi-arid areas should be 12,000 m^3/h/year at least for keeping this land away from degradation and loss, because of the hot climate that increases water evaporation rate from the soil surface [1]. However, cultivation jatropha in wastelands could be the proper solution for protecting lands from more degradation [66].

Till now the literature surveys have not determined the actual water requirement of jatropha; however, there is little known on water use and water use efficiency of *J. curcas* as an economic crop

in different areas around the world [29]. In general, jatropha water requirements depend on climatic conditions, soil type, tree age, and fruit growth stage.

Jatropha can be grown in a wide range of rainfall or supplemental irrigation from 300 to 3000 mm; in some area jatropha need about 500–600 mm yearly, while water requirements could reach up to 1500 mm/year respectively [8, 25, 67]. Jatropha is highly adaptable with warm temperatures and aridity conditions; also, jatropha is considered a drought-tolerant plant; therefore, it is growing well in arid and semi-arid areas with supplemental irrigation to produce economic seed yield.

Dagar et al. [68] reported that under irrigation with saline water Jatropha growing vegetative only.

Cultivation of jatropha considered a key solution to protect the environment from the hazards of wastewater and use it as a productive component through the production of biodiesel in marginal lands which strengthens the local rural community and improves the local economy and reduces the environment pollution by utilizes wastewater [19].

In arid and semi-arid regions jatropha could use more water quantity to have vigor growth and produce higher seed yield [40]. However, excessive water particularly in tropical areas increase vegetative growth and may reduce final seed yield due to negative effects on flowering and fruit set.

8.4 Weeds Control

There is a competition between weeds and jatropha plants for water and nutrients, also, pests used weeds as a host during pest control, so, weed control during dormancy and regularly during the growing season is very important, also, in arid and semi-arid regions weeds could use weeds after cutting as a mulch to reduce water evaporation from the soil [63].

8.5 Pests and Diseases

However, it's not resistant to infection of pest and pathogens [69], and despite whole plant toxicity, it is expected that increase pests and diseases infestation which affects significantly on jatropha seed productivity, therefore, expected to widely spread pest and disease in different cultivation areas, particularly with climate change conditions [70].

Previous reports clear that there are notably damage from pests, diseases, and viruses affect jatropha production in different countries as follow:

8.5.1 Diseases

Jatropha is exposed to attack by various pests and pathogens, particularly under water-logging conditions, like wilt diseases [71] black rot, collar rot, root rot [72], mosaic virus [73, 74], and vascular disease which could kill the whole plant, therefore, application of fungicides before new growth cycle suppressed fungal disease and protect root system [22].

8.5.2 Pests

There are different pests that infest jatropha and affect growth and seed production, including bugs [75], *Pachycoris torridus* in Nicaragua [76], leaf miner *(Stomphastis thraustica)* [77], the leaf and stem miner *Pempelia morosalis*, and the shield-backed bug *(Calidea panaethiopica)* which reduce seed yield; also, the larvae of the moth *Pempelia morosalis* is considered serious pests which demolish the flowers and young fruits [70].

8.6 Biological Control

Previous research indicated that biological control could be effective for control pests in jatropha farm, for instance Pseudotelenomus pachycoris is effective as egg parasitoids of Pachycoris klugii [76], furthermore, using the dipteran parasitoid to control larva of both Pempelia morosalis and Oncocera faecella, has promising results [70, 78].

9 Propagation Methods

Jatropha may be propagated by different methods, including vegetatively (stem cuttings and tissue culture) or by seeds [37]. Selection of planting materials is very important for establishing a successive farm. In general, it is preferable to use cutting stem from healthy trees that have high seed yield for many years growing under the same conditions (soil, watering, and climate).

9.1 Vegetative Propagation

Vegetative propagation produces healthy seedlings that produce seed yield earlier than seedy plants also, increases farmers profits [42]. Stem cutting considered the commercial vegetative propagation technique use for jatropha multiplication. Furthermore, micro-propagation can be the most effective technique to produce high-yielding seedlings with high seed yield and proper oil ratio. On the other hand, tissue culture techniques used for produce limited quantity of seedlings due to leakage in varieties with proper characters, so it's use for determined purpose such as scientific work.

9.1.1 Stem Cutting

It is considered a traditional and promising method for multiplication; it is becoming an effective method for propagating of Jatropha [30, 39].

Advantage of cutting stem propagation:

1. Propagation by cutting is more efficient and applicable.
2. Preserving unique trees with high seed yield and proper oil ratio.
3. Produce true-to-type clones.
4. It enables the production of high-yielding seedlings.

Gaikwad [30] reported that, using high yield trees as a source for plant materials produce healthy seedlings for the new plantation, also, it is preferable to use cutting with proper diameter more than 30 mm to produce new plant could reach earlier to higher yield than seedy plants [41], therefore, use of stem cuttings taken from carefully selected trees with higher yield potential provide the continued production of high-yielding trees. On the other hand, the difficulty of obtaining sufficient cuttings is a consideration due to a shortage of breeding programs to produce high yield trees.

9.1.2 Tissue Culture

Producing plantlets from tissue culture is being available, and producing protocols have been developed, but because jatropha is a latex-producing plant, it represents the major problem to produce seedlings on a large scale; till now there are no reports about seedlings production on commercial scale by tissue culture techniques; it is just used for research purposes.

9.2 Seed Propagation Method

It is the easier and cheapest method; seeds could sow directly in the field of pre-cultivation in the nursery.

9.3 Directly Sowing in the Field

Seeds could be sown directly in the soil; it is an easier method for planting jatropha, but the defect of this method is low germination rate (less than 50%) which needs replanting and increase establishment costs [41].

9.3.1 Sowing in the Nursery

Seeds planting in raised beds or containers to provide proper conditions to improve germination and survival rates through control various conditions like moisture, shade, nutrition, and control weeds, pests, and diseases, also, using planting container with proper characters particularly aeration and enough deep to avoid unduly restricting taproot growth.

The negative point of seedy propagation is the short viability period of seeds and higher genetic heterozygosity which produces a non-harmony seedling variant in growth and seed yield productivity.

9.3.2 Seed Viability

Germination of jatropha seeds as other oil seeds is affected with different storage conditions, storage period, and seeds moisture content; the seed viability depends on both storage conditions and quality of packaging [79]; also, fruit maturity affects seed viability, seeds from yellow fruits have higher germination ratio than seeds from brown fruits [80]; in general, jatropha seeds lose their viability after 5 months if stored in an ambient room condition [81], or also seeds could storage under cool conditions for a longer period [82].

10 Climate Conditions

Also, due to the continuous population increase, which will reach 9 billion in 2050 and about 12 billion in 2100, there is more interest in cultivating marginal lands with economic crops such as jatropha to produce biofuel from non- edible crops.

10.1 Climate

Due to adverse effects of climate change on growth and productivity of various crops, under this conditions, jatropha cultivation could be a part of an effective strategy to guarantee adequate crop production, produce biofuel, and reduce adverse effects of climate change particularly in fragile soil, through enhance marginal lands characters and using wastewater for irrigation [19, 28, 40].

Jatropha grown in various climatic regions ranging from up to 30° North and 35° South latitude, also, it is growing in a wide range of temperatures, whereas, proper temperature from 22 °C to 25 °C for enhancing flowering and increase seed yield, Jatropha is highly adaptable with warm temperatures and aridity conditions; therefore it is grown better in arid and semiarid regions [46].

10.2 Soil

Due to the shortage of arable lands due to increasing demands of lands for different purposes like urban and industries, etc., there is more attention for marginal lands to planting some appropriate plants to provide agricultural products and improve local community income.

Jatropha could grow in different soils conditions, it is native in tropical conditions, so it could grow in clay soil (with good drainage system) and sandy soils in arid and semiarid areas; however, heavy soil affects growth and spreading of root system [27]; also, jatropha grows in arable lands and marginal lands.

Jatropha requires well-drained alkaline soils; therefore, the aerated sand and loamy soil within pH 6.0–8.0 is considered the best soil for jatropha cultivation, particularly in the first 50 cm depth [63], while heavy clay soils are not much proper for jatropha growth and should be avoided, particularly where drainage is impaired; also jatropha cannot grow under waterlogged conditions.

Cultivation of jatropha as a perennial plant in dry climates is improving soil health and reducing soil disturbance through increase organic matter in the soil yearly; also, jatropha has the ability to survive under malnutrition conditions in dry soils where no other economic crops can grow; also, roots of jatropha could grow and extend inside rock crevices; on the other hand, survival ability does not mean that high seed yield can be obtained under these harsh environments, but in this case, jatropha planting protect soil from more degradation.

Therefore, marginal lands could be the proper soil for jatropha cultivation to avoid competing with main crops for food production and other crops in arable lands.

Jatropha is a proper plant to develop marginal soils, produce valuable oil use to produce biodiesel, and protect these soils from further degradation [66].

11 Phytochemicals Properties of Jatropha

Jatropha as a member of the Euphorbiaceae family contains highly toxic compounds, so the whole plant is toxic and seeds nonedible. *J. curcas* is used as medicinal plants in traditional medicines from ancient time [83]. Jatropha plant contains mixtures of numerous active substance compounds in all parts of the whole plant; it could be toxic for various species, from microorganisms to big animals; also, all organ extracts have antimicrobial activities for different pests and pathogens, particularly bark and seed extracts which has high efficiency in killing larva of Diptera flies (*Bactrocera cucurbitae* and *Bactrocera zonata*); also, jatropha extractions are used as biopesticides, insecticidal, and rodenticidal [84, 85].

11.1 Jatropha as Biopesticides

Jatropha has numerous bioactive compounds particularly in leaves and seeds, it could use to produce biopesticides, particularly leaves and seeds which considered a reach source of bioactive ingredients, seed oil is highly toxic for various insects and microorganisms, Aravinda et al. [86] reported that seed extraction use as pest control for *Helicoverpa armigera* and adult white fly (*Bemicia tabaci*) also, leaves extraction used as a nematicide, while pressed seedcake considered a cheap source for produce pesticides.

11.2 Antifungal Properties

The extract of jatropha press seedcake contained phorbol esters which have antifungal effects for numerous pathogens and could be used as fungicides for numerous agricultural pathogens, including *Fusarium oxysporum*, *Pythium aphanidermatum, Lasiodiplodia theobromae, Curvularia lunata, Fusarium semitectum, Colletotrichum capsici,* and *Colletotrichum gloeosporiodes* [87, 88].

11.3 Pesticidal, Insecticidal, and Larvicidal Activity

The insecticidal properties of *Jatropha curcas* and their extractions are well documented [41]; it affects the insects of various families, and inhibits the growth of several species like *Helicoverpa armigera* (Lepidoptera) and *Sitophilus zeamais (*Coleoptera) [89, 90]; *Culex quinquefasciatus* (Diptera) [91]; *Aedes aegypti* and *Culex quinquefasciatus* (Diptera: Cluicidae) [92]; also, jatropha extract is used for the control of vector mosquitoes [93], and against the termite (*Coptotermes vastator*) [94].

12 Medicinal Uses

All parts of *Jatropha curcas* plant are of medicinal value, bark, latex, leaves, and seeds; jatropha is used from ancient time as an effective medicine for human and animals in different parts of the world; currently, jatropha has a potential candidate for new pharmaceuticals.

Extracts of different parts of jatropha play an essential role in treating bacterial and fungal diseases. It is molluscicidal and is used in insecticidal properties. There are various reports showing the role of jatropha extracts as antitumor and antimicrobial activities [84]. Afzal et al. [88] clear showed that the latex of *J. curcas* has possessed antibacterial activity against *Staphylococcus aureus* (*S. aureus*). Extracts of leaves, stem, and roots particularly showed systemic and efficiency anti-inflammatory effects in curing acute carrageenan-induced rat paw edema [95].

Acharya and Rai [96] cleared that seeds are effective against cholera and dysentery diseases. It is used in treating skin diseases and rheumatism; also, seed oil is useful for medical purposes particularly against bacterial and fungal diseases [85].

13 Jatropha and Marginal Lands

Marginal lands could be due to natural reasons or due to inappropriate soil and water management; there are huge areas of marginal lands in arid and semiarid areas all over the world; in Egypt, more than 90% of the Egyptian lands are marginal soil which could be used for producing biofuel from jatropha seeds through utilizing wastewater [2]; also, there is about 50 million hectares (ha), or 16% of the Indian land area classified as wasteland [14].

Developing marginal lands is necessary to provide socioeconomic benefits for small farmers or landless peoples [97].

Using marginal lands for producing biofuel from nonedible plants like jatropha is a good solution to avoid debate about the food versus fuel; also, the establishment of jatropha plantations on marginal lands affect ecosystems and enhance soil properties, and improve local communities' livelihoods.

13.1 The Advantage of Utilizing Jatropha in Marginal Lands Includes

1. Produce biofuel without competing with food crops [11].
2. Improving energy security [98].
3. Provide new job opportunities.
4. Improving the economy for local communities [99].

13.2 Jatropha and Rural Region Development

Currently, jatropha has more attention as a key tool to developing marginal lands in the rural area, due to their advantage and aspects that include fighting poverty ratio (harvesting laborers) [100], protecting other crops (planting as hedges) from roaming animals, also, Jatropha oil used for soap production, seed oil used for lighting and cooking, and as a fuel in special diesel engines which provide a local energy source for the local communities in rural areas [55].

14 Conclusion

There is a global trend to planting jatropha for biofuel production and compete desertification particularly in developing countries. Jatropha cultivation in rural areas can improve agricultural output without competing for currently available soil and water resources. Jatropha represents an important crop for developing countries that looking for new energy sources like India, Brazil, Egypt, and Tanzania, jatropha represent novel resources of biofuel production particularly there are wide areas of marginal lands in these countries.

Jatropha as a multipurpose shrub used for various purposes include biofuel production, protecting other crops, reducing soil degradation, and deforestation; also, jatropha protects the environment through using wastewater in irrigation and reducing sequester carbon dioxide; besides that jatropha extract is used as biopesticides for fungus and whiteflies, and also as nematicide, while pressed seedcake is used to produce biogas and organic fertilizers.

References

1. Faostat (2017). http://www.faostate.fao.org/
2. El-Nahrawy, D.M.A. (2011) Country pasture profiles, Egypt. http://www.fao.org/ag/AGP/AGPC/doc/Counprof/Egypt/Egypt.html
3. Gamal-Fakhry A, Azazz NAE, Abdel-Monem A, Mohamedin A (2016) Use of waste water and treated water for jatropha curcas cultivation and the possibility of oil seed use as a biofuel. Minia J Agric Res Develop 36(2):245–269
4. Annarao S, Sidhu O, Roy R, Tuli R, Khetrapal C (2008) Lipid profiling of developing Jatropha curcas L. seeds using 1H NMR spectroscopy. Bioresource Technol 99:9032–9035
5. Siang CC (2009) Jatropha curcas L.: development for a new oil crop for biofuel, IEEJ: February 2009 online (in 15 September 2019). http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.651.6466&rep=rep1&type=pdf
6. Deng X, Fang Z, Liu Y, Yu C (2011) Production of biodiesel from Jatropha oil catalyzed by nanosized solid basic catalyst. Energy 36:777–784
7. Agamuthu P, Abioye O, Aziz A (2010) Phytoremediation of soil contaminated with used lubricating oil using Jatropha curcas. J Hazard Mater 179:891–894
8. Achten WMJ, Verchot L, Franken YJ, Mathijs E, Singh VP, Aerts R, Muys B (2008) Jatropha bio-diesel production and use. Biomass Bioenergy 32(12):1063–1084
9. Saverys S, Toussaint A, Defrise L, van Rattinghe K, Baudoin JP, Terren M, Jacquet

de Haveskercke P, Mergeai G (2008) Possible contributions of Jatropha curcas L. to rural poverty alleviation in Senegal: vision and facts. Tropicultura 26(2):125–128

10. Staubmann R, Foidl G, Foidl N, Gubitz GM, Lafferty RM, Valencia AVM (1997) Production of biogas from J. curcas seeds press cake. In: Gubitz GM, Mittelbach M, Trabi MM (eds) Biofuels and industrial products from Jatropha curcas, proceedings from the symposium "Jatropha 97", Nicaragua, February 23–27. Dbv-Verlag, Graz, pp 123–131
11. Achten W, Almeida J, Fobelets V, Bolle E, Mathijs E, Singh V (2010) Life cycle assessment of Jatropha biodiesel as transportation fuel in rural India. Appl Energy 87:3652–3660
12. Yin L, Hu TX, Liu YA, Yao SF, Ma J, Liu WT, He C (2010) Effect of drought on photosynthetic characteristics and growth of Jatropha curcas seedlings under different nitrogen levels. Ying Yong Sheng Tai Xue Bao 21:569–576
13. Behera SK, Srivastava P, Tripathi R, Singh JP, Singh N (2010) Evaluation of plant performance of Jatropha curcas L. under different agro-practices for optimizing biomass—a case study. Biomass Bioenergy 34:30–41
14. Garg KK, Karlberg L, Wani SP, Berndes G (2011) Jatropha production on wastelands in India: opportunities and trade-offs for soil and water management at the watershed scale. Biofuels Bioproducts Biorefining 5 (4):410–430. https://doi.org/10.1002/bbb.312
15. Koh MY, Ghazi TID (2011) Review of biodiesel production from Jatropha curcas L. oil. Renew Sust Energ Rev 15:2240–2251
16. FACT Foundation: Hand book on Jatropha curcas first draft. 2006
17. Abobatta WF (2019) Jatropha curcas: an overview. J Adv Agric 10:1650–1656. https://doi.org/10.24297/jaa.v10i0.8145
18. Francis G, Edinger R, Beckerm K, A. (2005) Concept for simultaneous wasteland reclamation, fuel production, and socio-economic development in degraded areas in India: need, potential and perspectives of Jatropha plantations. Nat Res Forum 29:12–24
19. Alabadi LAS, Alawsy WSA, Khaeim HM, AL-Hadithy, A. H. (2018) Utilization of treated wastewater in irrigation and growth of Jatropha plant to protect the environment from pollution and competing desertification. Plant Arch 18(2):2429–2434
20. Basha S, Francis G, Makkar H, Becker K, Sujatha MA (2009) Comparative study of biochemical traits and molecular markers for assessment of genetic relationships between Jatropha curcas L. germplasm from different countries. Plant Sci 176:812–823
21. Ribeiro D, Matavel N (2009) Jatropha! A socio-economic pitfall for Mozambique. Justica Ambental (JA) and Uniao Nacional de Camponess (UNAC), Mozambique
22. Terren M, Mignon J, Declerck C, Jijakli H, Savery S, de Haveskercke PJ, Winandy S, Mergeai G (2012) Principal disease and insect pests of Jatropha curcas L. in the lower valley of the Senegal River. Tropicultura 30 (4):222–229
23. Foidl N, Foidl G, Sanchez M, Mittelbach M, Hackel S (1996) Jatropha curcas L. as a source for the production of biofuel in Nicaragua. Bioresour Technol 58:77–82
24. Samsam CL (2013) Pruning techniques for Jatropha curcas L. to increase seed yield production. MMSU Sci Technol J 3(1):59–68
25. Trabucco A, Achten WMJ, Bowe C, Aerts R, Orshoven JV, Norgrove L, Muys B (2010) Global mapping of Jatropha curcas yield based on response of fitness to present and future climate. GCB Bioenergy 2:139–151
26. Henning R (2009) The Jatropha book, the Jatropha system, an integrated approach of rural development, Weissenberg, Germany. http://www.jatropha.de/
27. Lim S, Teong LK (2010) Recent trends, opportunities and challenges of biodiesel in Malaysia: an overview. Renew Sust Energ Rev 14(3):938–954
28. Becker, K., Wulfmeyer, V., Berger, T. and Munch, V. (2013). Carbon farming in hot, dry coastal areas: an option for climate change mitigation. Published in Earth System Dynamics. Published by Copernicus Publication on behalf of the European Geosciences Union
29. Rao AVRK, Wani SP, Singh P, Srinivas K, Rao CS (2012) Water requirement and use by Jatropha curcas in a semi-arid tropical location. Biomass Bioenergy 39:175–181
30. Gaikwad RS (2011) Vegetative propagation of Jatropha species by stem cuttings. Curr Bot 2 (1):39–40
31. Arnold JG, Fohrer N (2005) SWAT2000: current capabilities and research opportunities in applied watershed modeling. Hydrol Process 19:563–572
32. Biswas PK, Pohit S, Kumar R (2010) Biodiesel from Jatropa: can India meet the 20% blending target? Energy Policy 38:1477–1484

33. Sreedevi TK, Wani SP, Srinivasa Rao CH, Chaliganti R, Reddy RL (2009) Jatropha and Pongamia rainfed plantations on wastelands in India for improved livelihoods and protecting environment, 6th international biofuels conference, March 4–5
34. Kalam MA, Ahamed JU, Masjuki HH (2012) Land availability of Jatropha production in Malaysia. Renew Sust Energ Rev 16:3999–4007
35. Wani SP, Osman M, D'Silva E, Sreedevi TK (2006) Improved livelihoods and environmental protection through biodiesel plantations in Asia. Asian Biotechnol Develop Rev 8(2):11–29
36. Dehgan B, Webster GL (1979) Morphology and infrageneric relationships of the genus Jatropha (Euphorbiaceae). Univ Calif Publ Bot 74:1–73. + 33 plates
37. Ratha Krishnan P, Paramathma M (2009) Potentials and Jatropha species wealth of India. Curr Sci 97:1000–1004
38. Ariza-Montobbio P, Lele S (2010) Jatropha plantations for biodiesel in Tamil Nadu, India: viability, livelihood trade-offs, and latent conflict. Ecol Econ 70(2):189–195
39. Severino SL, de Lourdes Silva de Lima R, Bezerra Leão A, Esberard de Macêdo Beltrão N (2007) Root system characteristics of Jatropha curcas plants propagated through five methods. Expert seminar on Jatropha curcas L. agronomy and genetics. 26–28 March 2007, Wageningen, the Netherlands, Published by FACT
40. Kesava Rao AVR, Wani SP, Singh P, Srinivas K, Srinivasa Rao C (2012) Water requirement and use by Jatropha curcas in a semi-arid tropical location. Biomass Bioenergy 39:175–181
41. Heller J. (1996) Physic nut. Jatropha curcas L in: international plant genetic resources institute (IPGRI), promoting the conservation and use of under utilised and neglected crops (prom underused crops). 1996;1:1–66
42. Gopale KD, Zunjarrao RS (2011) Effect of auxin, length of stem cutting, substrate, and seasonal variations on Jatropha curcas L.: a biodiesel plant. Bioscience Discovery 02 (1):76–81
43. Dasumiati M, Triadiati T, Hartana A (2017) Sex types in flowering of Jatropha curcas. Biodiversitas 18(1):442–446. https://doi.org/10.13057/biodiv/d180158
44. Jongschaap REE, Corre WJ, Bindraban PS, Brandenburg WA (2007) Claims and facts on Jatropha curcas L. Plant Research International B.V, Wageningen
45. Raju AJS, Ezradanam V (2002) Pollination ecology and fruiting behavior in a monoecious species, Jatropha curcas L. (Euphorbiaceae). Curr Sci 83:1395–1398
46. Jamaluddin SHB (2008) Breeding Horticultural Crops@ Mardi, 1st edn. Malaysian Agricultural Research and Development Institute, Malaysia, ISBN-10: 9679365263, p 549
47. Noor Alam N-C, Abdullah TL, Abdullah N-A, P. (2011) Flowering and fruit set under Malaysian climate of Jatropha curcas L. Am J Agric Biol Sci 6(1):142–147
48. Pradhan R, Naik SN, Bhatnagar N, Vijay VK (2009) Moisture-dependent physical properties of jatropha fruit. Ind Crop Prod 29 (2–3):341–347. https://doi.org/10.1016/j.indcrop.2008.07.002
49. Bhattacharya A, Datta K, Kumar SD (2005) Floral biology, floral resource constraints and pollination limitation in Jatropha curcas L. Pakistan J Biol Sci 8:456–460. https://doi.org/10.3923/pjbs.2005.456.460
50. Firdaus IM, Psyquay NA, Ghizan SA, Maznah I (2010) Anthesis and flower visitors in curculigo latifolia dryand. J Biol Life Sci 1:13–15
51. Jones N, Miller JH (1992) Jatropha curcas: a multipurpose species for problematic sites. World Bank, Washington DC, USA, ASTAG Technical Papers—Land Resources. 1: 12
52. Cardoso PMR, Dias L-A d-S, de Resende MDV, de Freitas RG, Corrêa TR, Muniz DR, Zaidan LR (2018) Genetic evaluation and selection in Jatropha curcas L. Crop Breed Appl Biotechnol 18:192–199. https://doi.org/10.1590/1984-70332018v18n2a27
53. Henning RK (2004) Integrated rural development by utilization of Jatropha curcas as raw material and as renewable energy. Rothkreuz II, D-83138, Weissensberg
54. Patolia JS, Ghosh A, CHikara J, Chaudhary DR, Parmar DR, Bhuva HM (2007) Response of Jatropha curcas grown on wasteland to N and P fertilization. Proceedings of the FACT Seminar on Jatropha curcas L. Agronomy and Genetics, March 26–28, 2007, The Netherlands, pp 1–10
55. Jingura RM, Matengaifa R, Musademba D, Musiyiwa K (2011) Characterization of land types and agro-ecological conditions for production of Jatropha as a feedstock for biofuels in Zimbabwe. Biomass Bioenergy 35:2080–2086
56. Divakara BN, Upadhyaya HD, Wani SP, Laxmipathi G (2010) Biology and genetic improvement of Jatropha curcas L.: A review. Appl Energy 87:732–742

57. Montobbio PA, Lele S (2010) Jatropha plantations for biodiesel in Tamil Nadu, India: viability, livelihood trade-offs, and latent conflict. Ecol Econ 70:189–195
58. Carels N (2009) Chapter 2 Jatropha Curcas: a review. Adv Bot Res 50:39–86
59. Openshaw K (2000) A review of Jatropha curcas: an oil plant of unfulfilled promise. Biomass Bioenergy 19(1):1–15
60. Kaushik N, Kumar K, Kumar S, Kaushik N, Roy S (2007) Genetic variability and divergence studies in seed traits and oil content of Jatropha (Jatropha curcas L.) accessions. Biomass Bioenergy 31:497–502
61. Tiwari AK, Kumar A, Raheman H (2007) Biodiesel production from Jatropha (Jatropha curcas) with high free fatty acids: an optimized process. Biomass Bioenergy 31:569–575
62. Henning RK (2008) Jatropha Curcas L. in Africa. An evaluation. Global Facilitation Unit for Underutilized Species (GFUUS), Weissensberg
63. Gour VK (2006) Production practices including post-harvest management of Jatropha curcas. Proceedings of the Biodiesel Conference Toward Energy Independence-Focus of Jatropha, June 9–10, Hyderabad, India, New Delhi, Rashtrapati Bhawan, pp 223–251
64. Suriharn B, Sanitchon J, Songsri P, Kesmala T (2011) Effects of pruning levels and fertilizer rates on yield of physic nut (Jatropha curcas L.). Asian J Plant Sci 10:52–59. https://doi.org/10.3923/ajps.2011.52.59
65. Cutler KD (2000) The why and when of pruning. The New England Gardener's Book of Lists (2000). 1000 Washington Avenue, Brooklyn, NY 11225
66. Achten WMJ, Maes WH, Aerts R, Verchot L, Trabucco A, Mathijs E, Singh VP, Muys B (2010b) Jatropha: from global hype to local opportunity. J Arid Environ 74:164–165
67. Da Schio B (2010) Jatropha curcas L., a potential bioenergy crop, on field research in Belize. M.Sc. dissertation, Padua University, Italy and Wageningen University and Research Centre, Plant Research International, the Netherlands
68. Dagar JC, Tomar OS, Kumar Y, Bhagwan H, Yadev RK, Tyagi K (2006) Performance of some under-explored crops under saline irrigation in a semiarid climate in Northwest India. Land Degrad Develop 17:285–299
69. Shanker C, Dhyani SK (2006) Insect pests of Jatropha curcas L. and the potential for their management. Curr Sci 91(2):162–163
70. Otieno B, Mwangi L (2009) Jatropha under attack. A host of insect pests and diseases has been reported in Kenya. MITI 4:28–31
71. Hu HR, Sun YC, Chen F, Sun Q (2009) Pathogen identification of Jatropha curcas L. wilt disease and screening of its fungicides. J Sichuan Univ 46(6):1823–1827
72. Rao CS, Kumari MP, Wani SP, Marimuthu S (2011) Occurrence of black rot in Jatropha curcas L. plantations in India caused by Botryosphaeria dothidea. Curr Sci 100 (10):1547–1549
73. Gao SQ, Qu J, Chua NH, Ye J (2010) A new strain of Indian cassava mosaic virus causes a mosaic disease in the biodiesel crop Jatropha curcas. Arch Virol 155(4):607–612
74. Narayana DSA, Shankarappa KS, Govindappa MR, Prameela HA, Rao MRG, Rangaswamy KT (2006) Natural occurrence of Jatropha mosaic virus disease in India. Curr Sci 91 (5):584–586
75. Grimm C (1999) Evaluation of damage to physic nut (Jatropha curcas) by true bugs. Entomol Exp Appl 92(2):127–136
76. Rodrigues SR, de Oliveira HN, dos Santos WT, Abot AR (2011) Biological aspects and damage of Pachycoris torridus on physic nut plants. Bragantia 70(2):356–360
77. Sajap AS (2010) First report of a field outbreak of a leaf miner, Stomphastis thraustica meyrick (Lepidoptera: Gracillariidae) on bioenergy crop Jatropha curcas L. (euphorbiaceae) in Malaysia. Serangga 15(1–2):65–71
78. Xiao CL, Zhou JH, Guo HX, Liu YG, Xiao YB, Feng B (2010) Biological characteristics of Oncocera faecella infesting Jatropha curcas and screening of pesticides. Chin Bull Entomol 47(4):773–778
79. Pinto Junior AS, Guimarães VF, Dranski JAL, Steiner F, Malavasi MM, Malavasi UC (2012) Storage of Jatropha seeds in different packages and environments. Brazil J Seeds 34:636–643
80. Santoso BB, Budianto A, Aryana IM (2012) Seed viability of Jatropha curcas in different fruit maturity stages after storage. Nusantara Biosci 4:113–117
81. Vieira HD, Dardengo AO, de Oliveira MTR, Berbert PA, Deminicis BB (2017) Evaluation of storage potential of Jatropha curcas L. Seeds Am J Plant Sci 8:3013–3023. https://doi.org/10.4236/ajps.2017.812204
82. Pereira MD, Martins Filho S, Laviola BG (2012) Accelerated aging of Jatropha seeds. Trop Agric Res 42:119–123

83. Srinivasan D, Nathan S, Sures T, Lakshmana PP (2001) Antimicrobial activity of certain Indian medicinal plants used in folkloric medicine. J Ethnopharmacol 74:217–220
84. Sillma R, Daneshwar P, Rajesh J (2016) Jatropha curcas L: phytochemical, antimicrobial and larvicidal properties. Asian Pac J Trop Biomed 6(10):858–865. https://doi.org/10.1016/j.apjtb.2016.01.019
85. Omoregie EH, Folashade KO (2013) Broad spectrum antimicrobial activity of extracts of Jatropha curcas. J Appl Pharm Sci 3 (4):083–087
86. Aravinda A, Muralidhara Rao D, Devanna N (2009) Bioefficacy of Jatropha curcas L. (Euphorbiaceae) on Helicoverpa armigera Hude. and Bemisia tabaci Genn. Curt Biotica 2:429–438
87. Solsoloy AD (1995) Pesticidal efficacy of the formulated physic nut, Jatropha curcas L. oil on pests of selected field crops. Philip J Sci 124:59–74
88. Afzal M, Kazmi I, Khan R, Singh R, Chauhan M, Bisht T (2012) Bryophyllum pinnatum: a review. Int J Res Biol Sci 2 (4):143–149
89. Solsoloy AD (1993) Insecticidal efficacy of the formulated product and aqueous extract from physic nut, Jatropha curcas L. seed kernel oil on cotton insect pests. Philip J Sci 6:28–34
90. Sauerwein M, Sporer F, Wink M (1993) Insect-toxicity of phorbol esters from Jatropha curcas seed oil. Planta Med 59:A686
91. Karmegam N, Sakthivadivel M, Anuradha V, Thilagavathy D (1997) Indigenous plant extracts as larvicidal agents against Culex quinquefasciatus. Bioresour Technol 59:137–140
92. Rahuman AA, Gopalakrishnan G, Venkatesan P, Geetha K (2008) Larvicidal activity of some Euphorbiaceae plant extracts against Aedes aegypti and Culex quinquefasciatus (Diptera: Cluicidae). Parasitol Res 102:867–873
93. Sakthivadivel M, Daniel T (2008) Evaluation of certain insecticidal plants for the control of vector mosquitoes viz. Culex quinquefasciatus, Anopheles stephensi and Aedes aegypti. Appl Entomol Zool 43:57–63
94. Acda MN (2009) Toxicity, tunneling and feeding behavior of the termite, Coptotermes vastator, in sand treated with oil of the Physic nut, J curcas. J Insect Sci 6:1–8
95. Nayak BS, Patel KN (2010) Anti-inflammatory screening of Jatropha curcas root, stem, and leaf in albino rats. Rom J Biol Plant Biol 55:9–13
96. Acharya D, Rai MK (2012) Traditional knowledge for curing various ailments among Gonds and Bharias of Pathlkot valley, M.P. www.selfgrowthy.com/articles /acharya12.html.2012
97. Mmopelwa G, Kgathi DL, Kashe K, Chanda R (2017) Economic sustainability of Jatropha cultivation for biodiesel production: lessons from Southern Africa. J Fundam Renewable Energy 7:244. https://doi.org/10.4172/2090-4541.1000244
98. Wendimu MA (2017) Jatropha potential on marginal land in Ethiopia: reality or myth? www.ifro.ku.dk/english/publications/foi_series/working_papers/
99. Phalan B (2009) The social and environmental impacts of biofuels in Asia: an overview. Appl Energy 86:21–S29
100. Benge M (2006) Assessment of the potential of Jatropha curcas (biodiesel tree), for energy production and other uses in developing countries. http://www.ascension-publishing.com/BIZ/jatropha.pdf. Accessed 26 Sep 2019

Part II

Biodiesel Production



Chapter 7

Protocol for Biodiesel Production by Base-Catalyzed Transesterification Method

David A. Animasaun, Mubarak O. Ameen, and Moshood A. Belewu

Abstract

The importance of biodiesel and its production cannot be overemphasized; biodiesel has assumed a very prominent position in the energy development of both the developed and developing nations. This is due probably to climate change and the fear of the depletion of the fossil fuel. Biodiesel being not only clean fuel but also obtained from renewable sources is believed to be a better alternative to the traditional petrodiesel. Thus, development geared toward the production and utilization of biodiesel will go a long way in conserving the ecosystem as well as serve as a source of income. This chapter therefore itemizes the protocol for production of biodiesel from plant material using base-catalyst transesterification reaction method.

Key words Alternative fuel, Biofuel, Petrodiesel, Sodium methoxide

1 Introduction

Energy is one of the most fundamental requirements for human existence and activities. The need for energy is increasing continuously due to increased industrialization and population growth. Petroleum has been serving the world to meet its need of energy consumption for many decades, but human dependence on fossil fuels as the major source of energy could cause a major deficit in future. Global fluctuation in fossil fuel price, its effects on global climate (global warming), environmental pollution and degradation, and continuous increase in global energy demand are major reasons for seeking alternative, cheap, and clean energy sources. Beside the desire to address the menace of global warming and pollution, there is need to develop fuel substitutes that are renewable and sustainable [1–4]. It is therefore imperative to shift attention toward alternative fuel that could guarantee energy security, safe environment, and improved socioeconomic indices. Furthermore, increase in oil prices, depletion of oil reserves, and the

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attendant problems associated with fossil energy sources such as petroleum, coal, and natural gas has made biofuel and biodiesel a viable and popular alternative fuels and renewable sources of energy [3–5].

Over the last few years biofuel and biodiesel have gained importance as an alternative fuel for engines. Biofuels and biodiesel production rose to 105 billion liters in 2010, which signified 17% more than production in 2009. Biofuels accounted for 2.7% of the world fuel for road transportation in the same year with over 90% produced in the United State of America and Brazil, while European Union accounted for over half of the biodiesel produced in the year [6]. In the following years, there was increment in the production rate with more nations joining the league of countries for production of clean, cheap, and environmentally friendly energies. According to the International Energy Agency, biofuels may be used for a quarter transportation fuel need by the year 2050.

1.1 Biodiesel

Biodiesel is the name for a variety of ester-based oxygenated fuels from renewable biological sources. Chemically, biodiesel is defined as the monoalkyl esters of long-chain fatty acids derived from renewable biolipids (*see* Fig. 1). Typically, it is produced through the reaction of a vegetable oil or animal fat with methanol or ethanol in the presence of a catalyst to yield methyl or ethyl esters (biodiesel) and glycerin (*see* Fig. 2). Biodiesel is similar to petroleum diesel in many aspects of its chemical and physical properties [7–10].

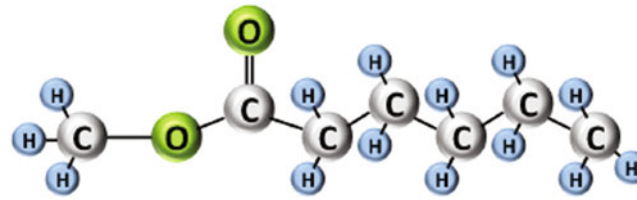


Fig. 1 Chemical structure of a fatty acid methyl ester (biodiesel)

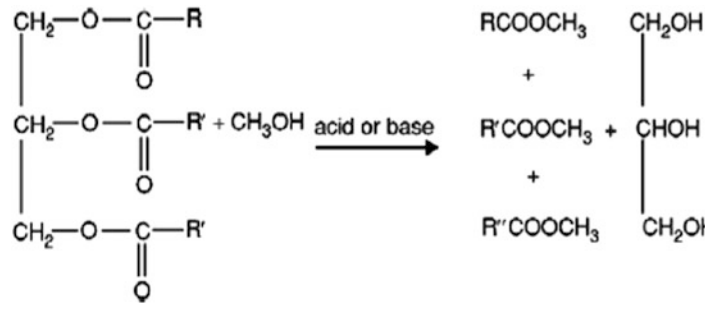


Fig. 2 Transesterification reaction

2 Materials

Vegetable oil from plant sources is the best starting material for production of biodiesel because the conversion of pure triglyceride to fatty acid methyl ester is high and the reaction time is relatively short [11–13]. However, due to high demand of vegetable oils for food, biodiesel production from spent oil is fast gaining acceptance [14–17]. The use of spent oil has comparative advantage of reducing pollution and it is cheap.

2.1 Biological Raw Materials

1. Crop seeds rich in oil, these include soybean, oil palm, sunflower, peanut, flax, safflower, castor seed, tung, cotton, jojoba, Jatropha, avocado, etc.
2. Other sources of biodiesel raw material include microalgae.

2.2 Chemicals and Reagents

1. Alkali hydroxide (Sodium hydroxide (NaOH) or potassium hydroxide (KOH)).
2. Alcohol (methanol or ethanol).
3. Distilled water.
4. *n*-Haxane.

2.3 Equipment and Apparatus

Basic equipment and apparatus required are:

Pipette, thermometer, conical flask, beaker, separating funnel, retort stand, measuring cylinder, oven, hydrometer, burette, magnetic stirrer, water bath, digital weighing balance, stop watch, and electric hot plate.

2.4 Transesterification of Oils

The current technology for biodiesel production is the transesterification of oils with alcohol to give biodiesel as main product and glycerin as by-product. Our routine technique for production of biodiesel is catalytic transesterification method which involve heating oil with an alcohol and using a catalyst (*see* Fig. 3a, b).

3 Methods

Transesterification is widely used for the transformation of triglyceride into fatty acid methyl ester. The manufacturing process is based on the transesterification of triglycerides by alcohols to fatty acid methyl esters, with glycerol as a by-product. The base catalyzed production of biodiesel generally has the following processes.

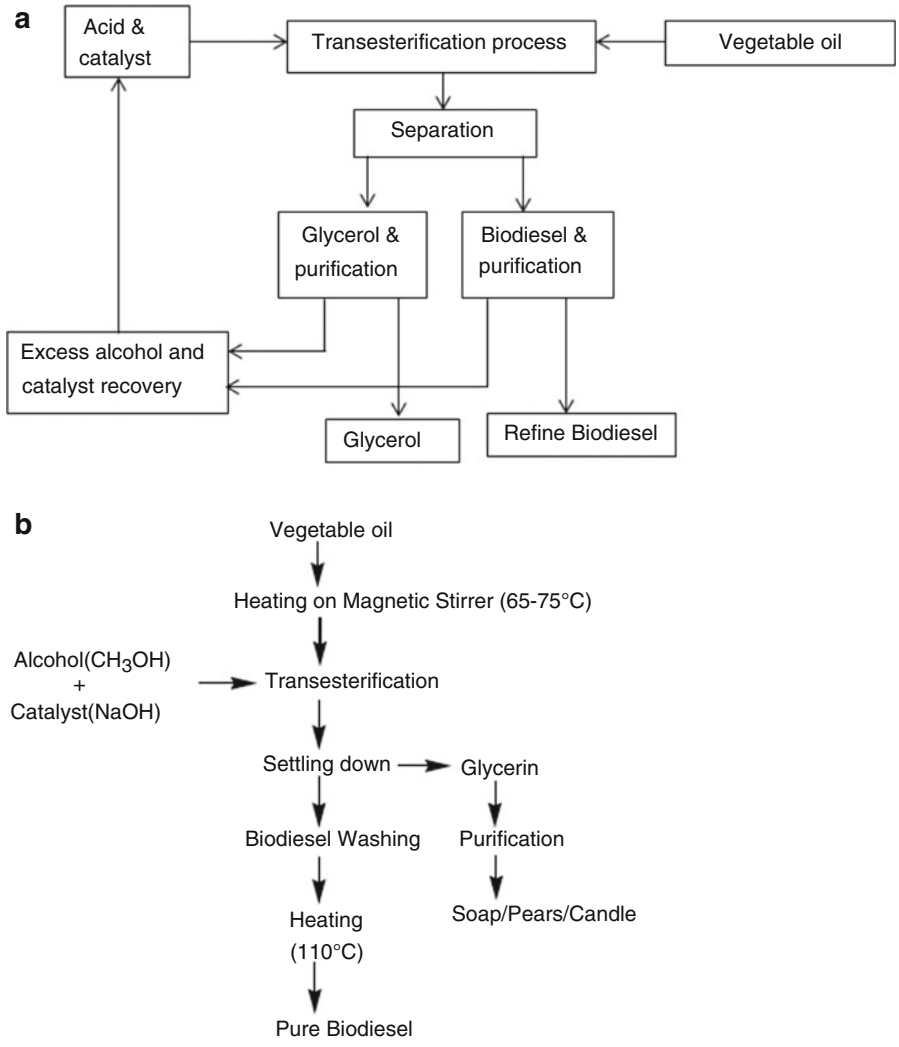


Fig. 3 Transesterification process (**a**) Schematic diagram of the process of biodiesel production from vegetable oils. (**b**) The steps and stages of transesterification of vegetable oil for biodiesel production

3.1 Treatment of the Seeds and Oil Extraction

The prerequisite for oil extraction is seed preparation

3.1.1 Feedstock Preparation

1. Grade the seeds and remove all unwanted materials.
2. Remove the fruit layers to expose the kernels or seeds (*see* **Note 1**).
3. Dry the seeds to reduce moisture content. Drying could be done in the oven at 60–80 °C till constant weight is obtained.

3.1.2 Mechanical Oil Extraction Method

1. For high oil yield, mechanical press oil extraction is employed.
2. Either an engine driven screw press or manual ram press can be used (*see* **Note 2**).

3. Place the seeds in the expeller machine.
4. Squeeze under high pressure in a single step.

3.1.3 Solvent Extraction Method

Solvent extraction can either be cold or hot extraction. Hot extraction employ the use of Soxhlet extractor (*see* **Note 3**).

In the case of cold extraction, the following steps can be followed

1. Place a known amount of the seed in a jar.
2. Add enough solvent (*n*-hexane, petroleum ether, diethyl ether, etc.) to percolate the seed.
3. Depending on availability, shake the mixture or leave to percolate for 24 h.
4. Collect the extract and repeat the percolating process until maximum oil extraction, i.e., the solvent remain colorless.
5. Combine the extract and concentrate it to recover the solvent by simple distillation.

   Hot solvent extraction by Soxhlet extractor.
6. Place a known amount of the seeds in a muslin cloth.
7. Put the muslin cloth with the weighed seed in the thimble of the Soxhlet extractor.
8. Set up the extractor as shown in Fig. 4.
9. Run the extraction for at least 4–6 h.
10. Concentrate the extract and recover the solvent (n-hexane, petroleum ether, diethyl ether, etc.) by simple distillation.

3.2 Determination of Percentage Oil Yield

1. Determine the percentage oil yield as:

$$\%\ \text{Oil yield} = \frac{\text{volume of oil yield}}{\text{volume of raw material}} \times 100$$

3.3 Determinations of the Physicochemical Properties of the Starting Oil

3.3.1 Moisture Content

1. Weigh and record the mass of oil as w_1.
2. Dry the weighted oil in the oven at 120 °C for 20 min and take the weight as w_2.
3. Determine the percentage moisture content in the biodiesel as:

$$\%\text{Moisture content} = \frac{w_1 - w_2}{w_1} \times 100$$

3.3.2 Specific Gravity

Use density bottle to determine the specific gravity of the oil as follows:

1. Weigh a clean dry density bottle (usually a 25 mL capacity bottle) and record the value (w_0).
2. Fill the bottle with oil, cork seal with a stopper and then retake the weight of the bottle and the oil (w_1).
3. Remove the oil from the bottle, wash and dry the bottle, then fill with water and cork to seal.



Fig. 4 Soxhlet extraction setup

4. Weigh the bottle and the water in it and record as (w_2).
5. Determine the specific gravity as:

$$\text{Specific gravity} = \frac{w_1 - w_0}{w_2 - w_0}$$

3.3.3 *Boiling Point*

1. Pour some quantity of biodiesel into a beaker.
2. Place the beaker on a hot plate with a thermometer inserted into it. Heat the oil to boiling.
3. Take the temperature at boiling.

3.3.4 *Viscosity*

1. Filter the biodiesel using 0.1 mm sieve or muslin cloth to remove solid impurities and dust.
2. Charge a capillary viscometer with the biodiesel by inverting the tube's thinner arm into the liquid sample and suction force was drawn up to the upper timing mark of the viscometer.

3. Return the viscometer to vertical position.
4. Place the instrument (viscometer) on a holder and insert to a pre-warm water bath at 30 °C for about 20 min.
5. Apply suction slightly to the thinner arm to draw in some sample. Let the sample drawn be a little above the upper timing mark.
6. Record the kinematic viscosity by timing the flow of the biodiesel from the upper timing to lower timing mark.

3.3.5 Free Fatty Acid

1. Dissolve 2 g of the biodiesel oil in 50 mL of the neutral solvent in 250 mL beaker.
2. Add 2–3 drops of phenolphthalein indicator.
3. Titrate against 0.1 M sodium hydroxide. Agitate the mixture to ensure proper mixing.
4. Stop the titration at end point, i.e., when a pink coloration which is stable for about 10 min appears. Record the titter value as the free fatty acid value.

3.3.6 Iodine Value

1. Weigh 0.5 g of the oil into a 250 mL conical flask with cork seal.
2. Add 15 mL chloroform, stir the mixture. Then add 25 mL of iodine solution to the mixture.
3. Place the bottle in the dark for about 30 min, then add 20 mL of 15% potassium iodide (KI) solution.
4. Titrate the solution against 0.1 M sodium thiosulfate solution till the yellow color of the iodine become quite faint and almost disappear. Add 2 mL 1% starch indicator and continue the titration.
5. At total disappearance of color, plug the bottle with a stopper and shake thoroughly so that the organic solvent layer mix with water layer.
6. After titration, record the titer value as the iodine value (*see* **Note 4**).

3.3.7 Saponification Value

1. Filter the oil to remove impurities (*see* **Note 5**).
2. Weigh 5 g of the oil sample into a flask and add 5 mL of alcoholic KOH.
3. Fix a reflux condenser to the flask and allow to boil slowly for an hour, then allow to cool.
4. With distilled water, rinse down the condenser inside and remove the condenser.
5. With two drops of indicator, titrate the solution against 0.5 M HCl until the pink coloration disappears and colorless precipitate is obtained. The titer value is recorded as the saponification value.

3.3.8 pH

1. Pour little oil into a clean beaker.
2. Insert the pH meter electrode into the oil to take the reading (*see* **Note 6**).
3. Record the pH value.

3.3.9 Peroxide Value

1. Weigh 1 g of the oil into dry boiling tube.
2. Add 1 g of potassium iodide (powder) and 2 mL of solvent to the oil in the boiling tube. Place the boiling tube on high heat for about 30 s.
3. Remove the boiling tube from heat, transfer the content into a flask, then add 20 mL of 5% potassium iodide (*see* **Note 7**).
4. Titrate 0.002 M of $Na_2S_2O_3$ with the solution until the yellow precipitate almost disappears, then add 0.5 mL starch solution, mix properly, and continue the titration until precipitate disappeared.

3.4 Steps Involved in Biodiesel Production

1. Reduction of the fatty acid in vegetable oil.
2. Transesterification process.

3.4.1 Reduction of the Fatty Acid in the Vegetable Oil

1. Pour the extracted oil into a beaker and heat to 60–75 °C on an electric hot plate.
2. Heat a mixture of concentrated H_2SO_4 (1% w/w) and methanol/ethanol (30% v/v) separately to 50 °C.
3. Add the heated mixture of the acid and ethanol to the heating oil in the beaker.
4. Stir the mixture using magnetic stirrer for 1 h and then allow to cool for 2 h.

3.4.2 Transesterification

Stepwise procedure of production:

1. Pour about 20 mL of the plant oil into a 500 mL capacity conical flask and heat the oil to 50 °C.
2. In a flask connected to Soxhlet apparatus, pour some ethanol/methanol and place on heat to purify the alcohol.
3. Weigh 0.5 g of sodium hydroxide pellets. Then prepare a solution of sodium methoxide in a 500 mL beaker using 0.5 g to get 0.5% catalyst concentration of sodium hydroxide.
4. Alternatively, prepare a solution of potassium methoxide using 0.50 g of potassium pellets in 128 mL of methanol (molar ratio of 1:6).
5. Stir the solution thoroughly to completely dissolve the hydroxide pellet.
6. Keep the solution at 60 °C in the oven for 5 min.

7. Pour the solution into the pre-warmed vegetable oil (close the reaction system firmly to prevent the loss of alcohol to the atmosphere by evaporation) connect to condenser and place on a hot plate, warm and stir vigorously using magnetic stirrer for about 1 h.
8. Keep the reaction mixture near the boiling point of the alcohol (64.7 °C for methanol and 78.4 °C for ethanol) to speed up the reaction. Excess alcohol is normally used to ensure total conversion of the oil to its esters as there is no problem of recovering of the alcohol for later use after recycling.
9. Pour the mixture into a separating funnel fixed on a beaker and allow to cool overnight for proper settling of the fractions. The biodiesel will form the upper layer, while the lower layer will comprise glycerol and soap.

3.5 Separating the Reaction Products

After the reaction is completed, there will be glycerol and biodiesel formation. Both have a significant amount of the excess alcohol used in the reaction which needs to be recovered.

1. The reacted mixture may require neutralization at this step if the basic media that is caused by alkali hydroxide occurred.
2. The glycerol phase is much denser than biodiesel phase, making biodiesel to float.
3. The two products can be separated by gravity using settling vessel or separating funnel.
4. The glycerol is drawn off at the bottom of the settling vessel or separating funnel.
5. And biodiesel is drawn off at the top.

   In some cases, a centrifuge is used to separate the two materials faster by screening both phases.

3.6 Purification of the Biodiesel

After the glycerol and biodiesel phases have been separated, excess alcohol in each phase is removed by:

3.6.1 Alcohol Removal from the Biodiesel

(a) Flash evaporation process by placing the biodiesel or the glycerol on low heat to evaporate excess alcohol or/and

(b) By extractive distillation.

On the other hand, the alcohol is removed and the mixture neutralized before the glycerol and esters have been separated to prevent the effect of basic media inside the reactor. The recovered alcohol can be reused as raw material.

3.6.2 Biodiesel Washing

1. Excess alkali can be neutralized by adding acetic acid.
2. Washing the biodiesel (*see* **Note 8**) with water maintained at 40–50 °C.

3. Wash by spraying warm water (40–50 °C) over the biodiesel; precautions should be taken to avoid soap formation (repeat this step until the clear water is seen beneath the biodiesel in the separating funnel).
4. After washing process the biodiesel may contain some traces of water, heat the biodiesel to 110 °C to remove any water molecule in it.
5. The quantity of the biodiesel produced should be measured and recorded.

4 Notes

1. Separate the seeds from the fruits by removing the outer layers of the fruits. Dehisce fruits can be open along the splitting plane to release the seeds while non-dehisce fruits can be cracked either manually or by mechanical mean to release the seeds. The separated kernel and seeds may be sieved, cleaned, and stored at room temperature. Prepare the seeds or kernels in such a way to obtain optimum oil yield. Usually, moisture content of 15% is considered.
2. Engine driven screw press is more effective and can extract between 68% and 80% of the seed oil; the ram presses oil yield is usually lower (60–65%).
3. Although chemical extraction method may be costlier than mechanical method, it is more efficient and higher oil yield can be achieved. Factors such as nature of solvent, particle size, agitation speed of the mixture, and temperature affect the oil yield by solvent extraction method. Other solvents that could be used include petroleum ether, ethanol, carbon tetrachloride, and isopropyl alcohol. Select extraction solvent base on the properties of the solvent.
4. Carry out blank titration with 5 mL of chloroform and equal proportion of iodine. Allow the precipitate to dissolve for equal period of time as the analyzed sample.
5. Filter the oil using filter paper or muslin cloth to remove impurities such as dust and crumps of seed coat.
6. Prior to taking the pH reading, test the probe reading in standard buffers (pH 4.2, 7, and 8.5) to ensure correct calibration of the probe. Take the oil pH reading in replicates and use the mean values for reliability.
7. Wash the tube twice with 25 mL distilled water and collect the water in the flask after each wash.
8. Wash about 4–6 times. After transesterification the upper ester layer may contain traces of NaOH, methanol, and glycerol.

Since the remaining unreacted methanol in the biodiesel has safety risks and can corrode engine components, the residual catalyst (NaOH or KOH) can damage engine components, and glycerol in the biodiesel can reduce fuel lubricity and cause injector coking and other deposits and should therefore be removed by washing. In some cases washing may not be necessary, depending on the quality of biodiesel produced.

References

1. Prafulla DP, Veera Gnaneswar G, Harvind KR, Tapaswy M, Shuguang D (2012) Biodiesel production from waste cooking oil using sulfuric acid and microwave irradiation processes. J Environ Prot 3:107–113
2. Krishnamurthy R, Animasaun DA, Ingalhali RS, Rumani AD (2014) A preliminary attempt of ethanol production from fig (*Ficus carica*) and Date (*Phoenix dactylifera)* fruits using *Saccharomyces cerevisiaes.* Int J Pure Appl Biosci 2 (2):2014
3. Belewu MA, Ameen MO, Krishnamurthy R (2018) Green chemistry for low carbon emission and healthy environment. Chem Res J 3 (1):28–33
4. Ogunwole OA (2012) Production of biodiesel from jatropha oil (*Curcas* oil). Res J Chem Sci 2(11):30–33
5. Garlapati VK, Kant R, Kumari A, Mahapatra P, Das P, Banerjee R (2013) Lipase mediated transesterification of *Simarouba glauca* oil: a new feedstock for biodiesel production. Sustainable Chem Process 1(11):1–6
6. Kurevija T, Kukulji N (2011) Algae biofuels as a possible alternative to environmentally doubtful conventional methods of biodiesel production. In: Gokcekus H (ed) Survival and sustainability, environmental earth sciences. Springer, Berlin, pp 499–510
7. Adebayo GB, Ameen OM, Abass LT (2011) Physico-chemical properties of biodiesel produced from *Jatropha curcas* oil and fossil diesel. J Microbiol Biotech Res 1(1):12–16
8. Demirbas A (2008) Biodiesel a realistic fuel alternative for diesel engines. Springer, London
9. Soon LB, Rus AZM, Hasan S (2013) Continuous biodiesel production using ultrasound clamp on tubular reactor. Int J Automotive Mech Eng 8:1391–1402
10. Vashist D, Ahmad M (2014b) Statistical analysis of diesel engine performance for castor and jatropha biodiesel-blended fuel. Int J Automotive Mech Eng 10:2155–2169
11. Ameen OM, Adekola FA, Adebayo GB, Adekola OF, Belewu MA, Rahman SG (2014) Conversion of *Jatropha curcas* oil to biodiesel using potash from cocoa pod husk (CPH) and palm kernel frond (PKF) as catalyst. Nigerian J Pure Appl Sci 27:2515–2524
12. Belewu MA, Adekola FA, Adebayo GB, Ameen OM, Muhammed NO, Olaniyan AN, Adekola OF, Musa AK (2010) Physico-chemical characteristics of oil and biodiesel from Nigerian and Indian *Jatropha curcas* seeds. Int J Biol Chem Sci 4(2):524–529
13. Nwosu FO, Ameen OM, Salami AA, Adebayo SA (2014) Optimum process conditions for the production of biodiesel using *Jatropha curcas* oil. Int J Phytofuels Allied Sci 3(1):53–76
14. Refaat AA (2010) Different techniques for the production of biodiesel from waste vegetable oil. Int J Environ Sci Tech 7(1):183–213
15. Math MC, Kumar SP, Chetty SV (2010) Technologies for biodiesel production from used cooking oil—a review. Energy Sustain Dev 14 (4):339–345
16. Gude VG, Grant GE (2013) Biodiesel from waste cooking oils via direct sonication. Appl Energy 109:135–144
17. Elkady MF, Zaatout A, Balbaa O (2015) Production of biodiesel from waste vegetable oil via KM micromixer. J Chem 2015:630168. https://doi.org/10.1155/2015/630168



Chapter 8

Organosolv Pretreatment of Sorghum Stalks Using Glycerol

Shereena P. Joy and Chandraraj Krishnan

Abstract

Glycerol is a promising low-cost solvent for biomass pretreatment since a large amount of glycerol is generated as a by-product in the biodiesel industry. Pretreatment is a method of disintegration of the recalcitrant structure of biomass to enhance the accessibility of cellulose and hemicelluloses to enzymes for complete saccharification. During pretreatment, glycerol breaks the lignin carbohydrate complex and selectively solubilizes lignin. Thus, the glycerol pretreatment improves the accessibility of cellulose to cellulases leading to higher sugar yields. The glycerol pretreatment is carried out at high temperature (>190 °C) to disintegrate the structure of biomass. The glycerol pretreatment in the presence of acid or base catalyst such as H_2SO_4 or NaOH results in lower pretreatment temperature and higher glucan hydrolysis. This chapter describes the methodology to carry out glycerol pretreatment of sorghum biomass with or without acid/alkali as catalyst and the basic calculations to determine the efficiency of the pretreatment.

Key words Organosolv pretreatment, Glycerol pretreatment, Sorghum biomass, Lignocellulose, Acidic glycerol, Alkaline glycerol

1 Introduction

The continuous rise in population and urbanization has led to increased energy demand, which are mainly met by fossil based resources. Burning of the fossil fuels emits large amounts of GHG to the atmosphere, which causes global warming and climate change [1]. To mitigate the emission of GHG, alternative clean energy produced from renewable resources has attracted great importance. The combustion of liquid fossil fuels like gasoline and diesel for road transportation is the major source of atmospheric GHG [2]. Bioethanol produced from renewable plant materials like sugars, grains, and stalks is considered as a clean, renewable, and sustainable biofuel to partly replace gasoline. Currently ethanol is used as ethanol-petrol-blend (EPB), where the proportional ethanol concentration varies from 5% to as high as

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85% [3]. Hence ethanol significantly reduces CO_2 emission from fossil fuels and provides energy security to countries importing crude oil [4, 5]. First-generation bioethanol produced from cane sugar and starchy grains is currently blended with gasoline worldwide. However, these sugar sources have conflict with the production and supply of food. The "food vs. fuel" conflict can be avoided by using nonedible sources such as lignocelluloses.

Lignocelluloses are composed of >70% polysaccharides comprising cellulose and hemicelluloses, which can be utilized for the production of ethanol through microbial fermentation. The lignocellulose also contains lignin, a phenolic polymer, associated strongly with the cellulosic polysaccharides. The complex carbohydrate-lignin structure makes the feedstock highly recalcitrant to degradation by biocatalysts [6]. Hence, the biocatalytic route of converting lignocellulose into ethanol requires an appropriate pretreatment step to facilitate the degradation of cellulose and hemicelluloses to fermentable sugars. The pretreatment of biomass disintegrates the rigid structure of biomass to expose the cellulosic fibers for enzymatic hydrolysis to fermentable sugars [7]. There are several pretreatment methods using different reagents such as acid, alkali, steam, solvents, and ionic liquids. Each method of pretreatment has merits and limitations based on various factors like versatility, efficiency, inhibitors formation, equipment corrosion, environmental impact, etc. [8]. Hence, there are opportunities to further improve pretreatment methods to improve the enzymatic conversion of cellulose and hemicellulose to fermentable sugars without generating inhibitors and effluent [9].

Organosolv pretreatment is a process of removal of lignin from lignocellulosic biomass using organic solvents. It has been developed as an environmental friendly process alternative to the conventional Kraft and sulfite pulping process [10]. Organosolv pretreatment using organic solvent is an attractive method, since the solvent can be recycled leading to low levels of both effluent and inhibitors. The organic solvents break down the complex structure of biomass by removing lignin without much degradation of cellulose. Besides, high-quality lignin is obtained, which can be converted into high-value chemicals [10]. Most of the organosolv pretreatments employ low boiling point solvents like ethanol, butanol, acetone, etc. These solvents are highly flammable and volatile, which require high-pressure reactors making commercial process capital intensive [11]. Whereas the high boiling point solvent like glycerol can be used at atmospheric pressure using simple reactors. Besides, glycerol is nontoxic and is generated as a low-cost by-product in the biodiesel industry [12].

A flowchart of pretreatment of biomass using glycerol resulting in fractionation of lignin and cellulose is shown in Fig. 1. The glycerol is recovered from the effluent by distillation and recycled.

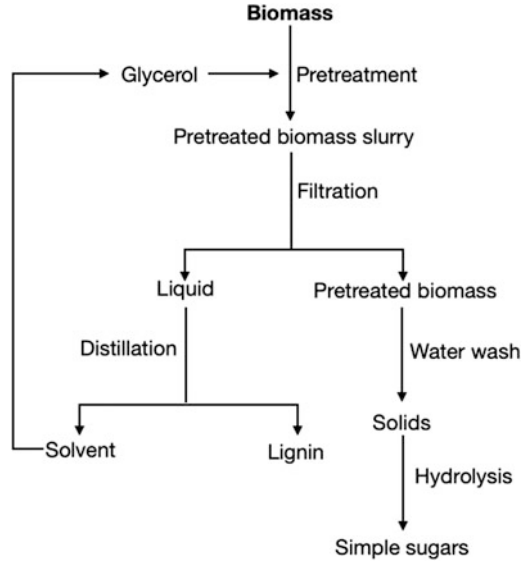


Fig. 1 Flowchart of organosolv pretreatment of biomass using glycerol. The pretreatment solubilizes lignin in the biomass and results in cellulose-rich solid fraction with high enzymatic digestibility, which is converted into simple sugars by enzymes. The liquid stream containing glycerol and lignin is distilled to recover glycerol for repeated use in the pretreatment. The residual lignin is used for its conversion into other useful products

Glycerol pretreatment selectively removes lignin with a small fraction of xylan, which results in the formation of larger pores. As glycerol is highly polar, it effectively penetrates into the biomass during pretreatment. At higher temperature, autoionization of water forms hydronium ions which break the ether bond in lignin, and then glycerol solubilizes the lignin fragments [13]. Glycerol also reduces the particle size of the biomass helping in easier accessibility and digestibility of cellulose by cellulases [14]. Glycerol pretreatment can reduce the surface tension and thus assists the easier penetration of chemical into the biomass and diffusion of the degradation products into the liquor. This results in uniform distribution of reaction within the biomass [15]. Glycerol can also be used with acid or base catalysts such as H_2SO_4 or NaOH to reduce the pretreatment temperature and enhance the pretreatment efficiency [16]. Hence, glycerol is a promising organic solvent for pretreatment of biomass for higher cellulose digestibility. This chapter describes the glycerol pretreatment of sorghum stalks and evaluation of its efficiency based on digestibility and structural changes of sorghum stalks.

2 Materials

2.1 Sorghum Stalks and Preprocessing

1. Sorghum stalks (harvested after 125 days) were obtained from The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), India.
2. Biomass milling unit was used to reduce the particle size of sorghum stalks. For lab scale experiments, a blender can be used to reduce the particle size.
3. Stainless steel sieves (Based on Tyler standard and USA standard ASTM E11) of appropriate mesh size were used to obtain biomass particles of different sizes.

2.2 Solvents and Chemicals for Pretreatment

1. Pure glycerol (99%), industrial grade glycerol (95%), and crude glycerol (70–85%) from the biodiesel industry (*see* **Note 1**).
2. Acids (sulfuric acid (98%) or hydrochloric acid (35%)).
3. Alkali (KOH or NaOH).

2.3 Pretreatment Reactor

Stainless steel reactor equipped with temperature sensor, pressure sensor, and stirrer (Fig. 2) (*see* **Note 5**). A heating mantle to fit the reactor size.

2.4 Analysis of Pretreated Biomass

The efficiency of pretreatment conditions is determined by analyzing the pretreated biomass by change in composition and enzymatic hydrolysis. The chemicals, enzymes, and equipment required for analysis are given below.

1. Sodium citrate buffer (0.05 M, pH 4.8): Prepare 0.1 M stock solution of trisodium citrate (weigh 2.58 g trisodium citrate and dissolve in 100 mL of dissolved water). Prepare 0.1 M stock solution of citric acid (weigh 1.92 g of citric acid and dissolve in 100 mL of distilled water). Mix 27 mL of 0.1 M trisodium citrate solution and 23 mL of 0.1 M citric acid solution in a beaker and adjust the pH to 4.8 using NaOH (*see* **Note 2**). Make up the solution to 100 mL with distilled water (*see* **Note 3**).

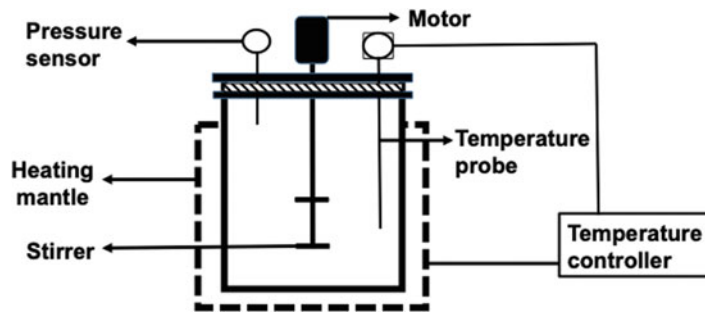


Fig. 2 A simplified representative diagram of a stainless steel reactor for glycerol pretreatment

2. Prepare stock solution of antibiotics, cyclohexamide (10 mg/mL) and chloramphenicol (10 mg/mL) in distilled water.
3. Any commercial cellulase with known Filter Paper Cellulase (FPase) is used. The following cellulases can be purchased from Sigma Aldrich, Novozyme, Genencore and Megazyme. Cellic CTec 2, Accellarase, Celluclast. The cellulases can be mixed with β-glucosidase like Novozyme 188 and xylanases like Cellic HTec2 and other commercial xylanases. Store all enzymes at 4 °C (*see* **Note 4**).
4. Sulfuric acid (72% (w/w)).
5. Pressure tubes (Sigma).
6. Hot air oven: Temperature range of ambient to 200 °C.
7. Muffle furnace: Temperature range of ambient to 1000 °C.
8. Sulfuric acid (5 mM): Take 500 mL of MilliQ water in a glass cylinder. Add 272 μL of concentrated sulfuric acid (98%) and make up the volume to 1 L.
9. Sample filtration unit. Filtering module with 13 mm 0.2 μ filter paper.

3 Methods

A schematic flowchart of glycerol-pretreatment of sorghum stalks with the pre-processing and post-pretreatment analysis is given below (*see* Fig. 3). The methods of each step are described below.

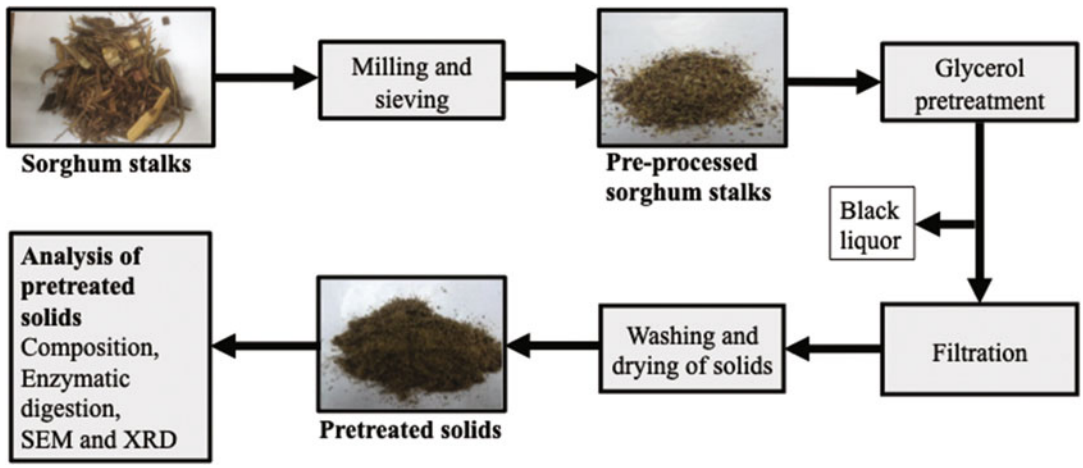


Fig. 3 Schematic flow diagram of glycerol pretreatment of sorghum stalks with preprocessing and post-pretreatment analysis

3.1 Preparation of Biomass

1. Wash the sorghum biomass with tap water to remove dirt and other water-soluble compounds like free sugars.
2. After washing, air-dry or oven-dry the biomass at 60 °C till the moisture content is about 10%. Measure the moisture content using a moisture analyzer or method of drying in oven until constant weight.
3. Mill the biomass and pass through a sieve of 1 mm mesh size (*see* **Note 6**). Refer the conversion chart for mesh size to particle size to choose appropriate sieves.
4. Store the sieved biomass in air-tight bags at room temperature until pretreatment.

3.2 Pretreatment of Biomass

3.2.1 Glycerol Pretreatment

1. Mix the preprocessed biomass at a solids loading of 5% (w/w) by adding 10 g of biomass in 200 g of glycerol (70–80%) (*see* **Note 7**).
2. Transfer the biomass-glycerol slurry to the stainless steel reactor and close the reactor tightly with the lid.
3. Place the reactor on the heating mantle and rise the reactor temperature to 190 °C. The temperature could be varied between 190 °C and 230 °C. Maintain the reactor temperature for 60 min residence time. The residence period could vary from 1 to 4 h (*see* **Note 8**).
4. Switch off the reactor and let the reactor temperature come down to <50 °C.
5. Transfer the pretreated materials to the filtration unit. Filter the pretreated slurry by vacuum filtration to separate the black liquor from the pretreated solids.
6. Wash the pretreated solids as per Subheading 3.2.3.

3.2.2 Acidic/Alkaline Glycerol Pretreatment

Glycerol pretreatment is normally carried out at higher temperature of >190 °C for longer periods which makes the process energy intensive. The addition of acid/alkali to glycerol can reduce the pretreatment temperature and improve the efficiency.

1. Weigh 5 g of preprocessed biomass and mix the biomass in 200 g of glycerol blended with either acid (HCl (0.1–1.2% w/w) or H_2SO_4 (0.9–1.1%w/w)) or alkali NaOH (2–5.6% w/v) or KOH (2–7% w/w).
2. Transfer the biomass-glycerol reaction mixture into the stainless steel reactor and tightly close with the lid.
3. Heat the reactor using heating mantle to 120 °C and hold the reactor temperature for 60 min residence time (*see* **Note 8**).
4. After the reaction is complete, let the reactor temperature come down to <50 °C.

5. Filter the pretreated slurry using vacuum filtration to separate the black liquor from the pretreated solids.
6. Wash the pretreated solids as per Subheading 4.3.

3.2.3 Washing of Pretreated Biomass

1. Wash the filtered pretreated solids with 200 mL of aqueous glycerol (1:1 ratio of glycerol to water) or 1% w/v of sodium hydroxide solution to remove lignin deposits from the pretreated biomass.
2. The first wash is followed by water washing till the biomass reaches pH 7.
3. Air-dry the washed pretreated biomass and store in ziplock bags for further use (*see* **Note 11**).

3.3 Enzymatic Digestibility of Pretreated Biomass

Saccharification of the glycerol-pretreated biomass provides insights into the effectiveness of the pretreatment process. Enzymatic hydrolysis efficiency could be considered as the major parameter to select the pretreatment conditions. Figure 4 shows the representative improvement in the efficiency of enzymatic hydrolysis of sorghum stalks after glycerol pretreatment. The overall efficiency of the pretreatment is not only based on the enzymatic saccharification, but the recovery of glucan should also be high. The glucan recovery is determined by compositional analysis as described in the following section.

1. Take 0.2 g dry weight (dwt) of pretreated solids and add 9.6 mL of 0.05 M sodium citrate buffer (pH 4.8) to obtain 2% solids loading in a 15 mL glass vial (*see* **Note 9** and **10** for actual buffer volume).

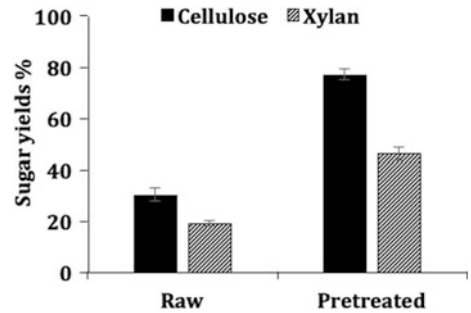


Fig. 4 Effect of glycerol pretreatment on enzymatic digestibility of sorghum stalks. Sorghum stalks were ground and sieved using 1 mm mesh size screen and pretreated with 60% glycerol at 190 °C for 60 min at 5% solids loading in a stainless steel reactor. The pretreated biomass at 2% (dwt basis) solids loading was hydrolyzed using Cellic CTec2 cellulase at an enzyme loading of 10 FPU/g dwt solids for 72 h. The yields of glucose and xylose formed from cellulose and xylan, respectively, by enzymatic hydrolysis indicating the efficiency of pretreatment were determined

2. Add each of antibiotics cyclohexamide and chloramphenicol to a final concentration of 50 μg/mL to prevent microbial contamination during hydrolysis.
3. Add 100 μL of cellulase from the working stock to obtain a final cellulase activity of 10 FPU/g dwt of pretreated solids in the vial.
4. Incubate the hydrolysis mixture at 50 °C in an orbital shaker and set the agitation at 150 rpm.
5. After 72 h of hydrolysis, filter the hydrolyzed slurry and measure the concentration of glucose, xylose, and arabinose in the clear hydrolyzate using HPLC fitted with Aminex HPX-87H column and 5 mM sulfuric acid as the eluent at 0.6 mL/min flow rate and at 60 °C column temperature.

3.4 Composition of Pretreated Biomass

The analysis of change in the composition of pretreated biomass is necessary to optimize the conditions for maximum recovery of glucan. It is important that after each pretreatment the composition analysis is carried out. The glucan recovery and the enzymatic digestibility should be high (>90%) for choosing efficient pretreatment conditions.

1. Acid hydrolyze the sorghum biomass as per NREL standard protocol (*see* ref. [17]).
2. Dilute the liquid fraction containing sugars with milliQ water and filter through 0.2 μ membrane using filtration unit into 1.5 mL glass vials.
3. Analyze the sample using HPLC fitted with Aminex HPX-87H column and 5 mM sulfuric acid as the eluent at 0.6 mL/min flow rate and at 60 °C column temperature.
4. Measure the acid-soluble lignin in the liquid fraction and acid-insoluble lignin in the solids fraction.

3.5 Structural Analysis of Pretreated Biomass

The changes in surface morphology and crystallinity index of the biomass after the glycerol pretreatment are direct evidence for effective pretreatment. Scanning electron microscopy (SEM) and X-ray diffraction (XRD) are used to determine the surface morphology and the crystallinity index of the biomass. The representative SEM pictures and XRD patterns of untreated and glycerol-pretreated sorghum stalks are shown in Figs. 5 and 6. The crystallinity of the biomass is determined using crystallinity index (CrI). The crystallinity index is calculated using Eq. (1).

$$\mathrm{CrI}(\%) = \frac{(I_{002} - I_{\mathrm{am}}) \times 100}{I_{002}} \tag{1}$$

where I_{002} is intensity of the diffraction from the 002 plane at $2\theta = 22.4^{\circ}$ and I_{am} is intensity of amorphous region at $2\theta = 18.4^{\circ}$.

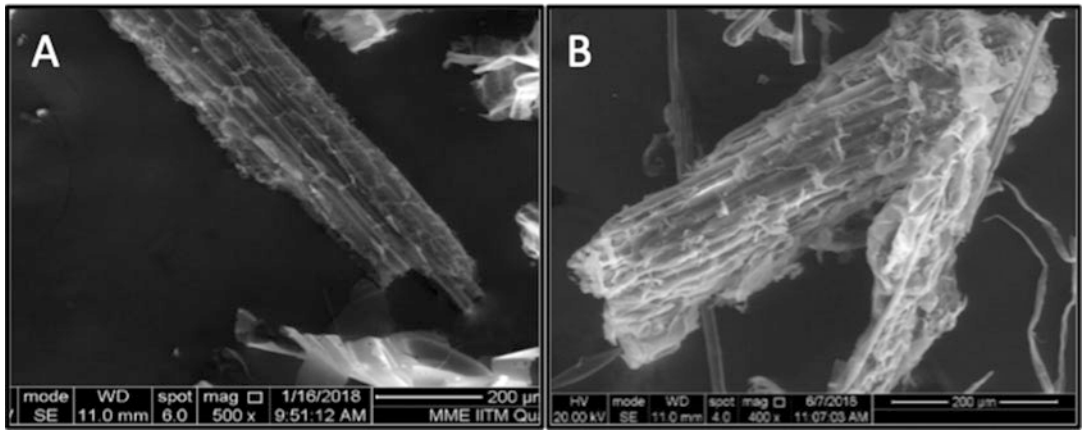


Fig. 5 SEM image of untreated and glycerol pretreated sorghum stalks. Sorghum stalks were ground and sieved through 1 mm mesh size screen and pretreated with 60% glycerol at 190 °C for 60 min at 5% solids loading in a stainless steel reactor. The SEM images of untreated and glycerol pretreated sorghum stalks were obtained using Quanta 400 (**a**) untreated sorghum stalks (500×); (**b**) glycerol pretreated sorghum stalks

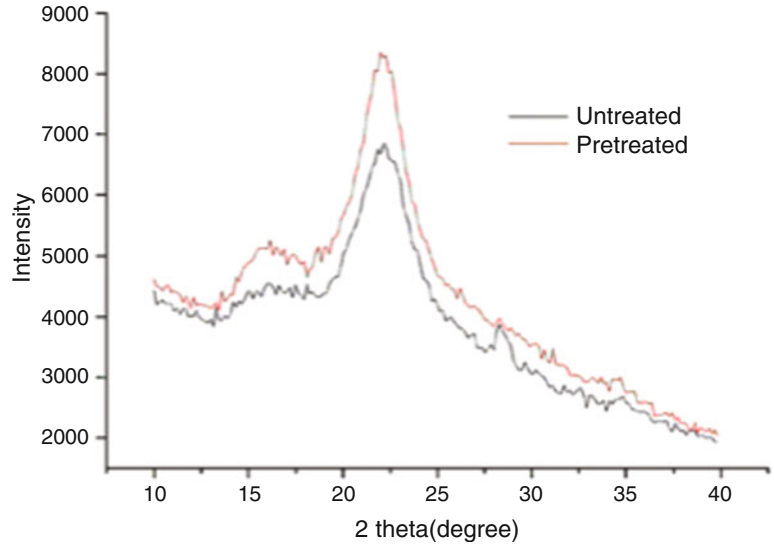


Fig. 6 XRD patterns of untreated and glycerol pretreated sorghum stalks. Sorghum stalks were ground and sieved through 1 mm mesh size screen and pretreated with 60% glycerol at 190 °C for 60 min at 5% solids loading in a stainless steel reactor. X-Ray diffraction patterns of untreated and glycerol pretreated sorghum biomass were obtained using D8 ADVANCE diffractometer. (**a**) Untreated sorghum stalks; (**b**) glycerol pretreated sorghum stalks

4 Conclusion

Organosolv pretreatment using glycerol has several benefits over other solvents. Due to its high boiling point, the reaction can be carried out at atmospheric conditions without any need for high-pressure reactors. As glycerol is a major by-product of biodiesel industry, it is a cheaper solvent for the pretreatment. Glycerol selectively removes significant amount of lignin with complete recovery of cellulose. Glycerol pretreatment can be carried out with acid or alkali as catalyst to lower the pretreatment severity and improve the hydrolysis of the biomass. The methodology to carry out glycerol pretreatment with or without catalyst followed by enzymatic hydrolysis is described in this chapter.

5 Notes

1. Crude glycerol from biodiesel industry could be used for the glycerol pretreatment process, which would reduce the process cost and also help the biodiesel industry by utilizing its major by-product. When the crude glycerol is used in the pretreatment, it tends to result in lower delignification of biomass as compared to pure glycerol. This is due to the pitch deposits on the biomass formed by the lipophilic substances from crude glycerol which interferes with the reaction rate, thus acting as barrier for the lignin removal. These pitch deposits are difficult to remove during washing and survives the pretreatment process. Thus, it is suggested that lipophilic components from crude glycerol be removed before using it for pretreatment in case it is interfering in lignin removal and the enzymatic hydrolysis.
2. 5 N NaOH is used to adjust the pH in order to avoid change in the concentration of sodium citrate buffer.
3. Prepare sodium citrate buffer fresh for every hydrolysis reaction in order to avoid contamination issues.
4. In some cases, xylanases are added along with cellulase enzymes as the xylanase activity in cellulase enzyme cocktail will not be sufficient to hydrolyze xylan effectively.
5. The stainless steel reactor material of construction (MOC) would depend on the nature of chemicals being used. When acid is used, SS 316L material should be used; otherwise lower grade steel, SS304 is used as the MOC.
6. The lower particle size of the biomass helps in ease of operation. The particle size of the ground biomass is generally preferred to be <1 mm for providing more surface area for chemicals to penetrate into the biomass, after pretreatment

the size would further reduce and it is observed that lower size improves the enzymatic hydrolysis reaction (*see* ref. [18]).

7. The biomass loading during pretreatment would depend on the density and the particle size of the feedstock. The bulk density of biomass varies with different feedstock, wheat straw is having a low density of 18 kg/m^3 whereas corn cob has a bulk density of 250 kg/m^3 (*see* ref. [19]). In case of lower density, the volume occupied in the reactor would be more leaving little headspace and also it would limit the heat and mass transfer. Hence, for biomass with lower density, low-solids loading is preferred.
8. Pretreatment temperature and time vary with biomass and hence these are optimized for higher sugar yield with maximum recovery. The retention time and pretreatment temperature has an inverse relationship, higher temperature requires lower retention time whereas lower temperature requires longer retention time.
9. The pretreated solids are used either as wet or dry substrate for enzymatic hydrolysis. The drying of the pretreated solids should be done at ambient or lower temperature, as higher temperature may collapse the pores making it difficult for the cellulase enzyme to reach the substrate (*see* ref. [22]). Most of the literature reported use of dry solids for hydrolysis reaction but in some cases wet solids have shown higher hydrolysis yield than dried biomass (*see* ref. [11]).
10. Measure the wet weight of solids obtained after washing the pretreated biomass and estimate the moisture content. Add appropriate amount of buffer to the hydrolysis mixture to a final volume of 10 mL considering the water already present in biomass as moisture.
11. Low-solids loading for enzymatic hydrolysis is carried for screening the pretreatment conditions required for maximum sugar release (*see* ref. [20]). Once the pretreatment process is optimized, solids loading is increased and the effect of high solids is studied. High-solids loading is preferable for obtaining higher ethanol titer in fermentation, for reducing the distillation cost. But the operation of high-solids loading in batch mode is difficult due to the high viscous nature of slurry. Hence, fed batch strategy is employed, where solids are added in subsequent time intervals (*see* ref. [21]). For example, to carry out 20% solids loading enzymatic hydrolysis, initially the reaction is started with 10% solids in which mixing is not an issue, followed by the addition of 5% solids in next two time periods. The time of addition of solids depend on the liquefaction of pretreated biomass by cellulases. The total cellulase is added at start of the hydrolysis.

6 Calculations

The recovery of total solids, cellulose, xylan, and lignin after glycerol pretreatment is calculated using the Eqs. (2)–(5). The yields of glucose and xylose after the enzymatic hydrolysis of the pretreated solids are calculated using the Eqs. (7) and (8).

$$\text{Solids recovery (\%)} = \frac{\text{Weight of solids obtained after pretreatment (gdwt)} \times 100}{\text{Weight of biomass loaded to reactor (gdwt)}} \quad (2)$$

$$\text{Cellulose recovery (\%)} = \frac{\text{Cellulose content in the pretreated solids (mg/gdwt)} \times 100}{\text{Cellulose content in the untreated biomass (mg/gdwt)}} \quad (3)$$

$$\text{Xylan recovery (\%)} = \frac{\text{Xylan content in the pretreated solids (mg/gdwt)} \times 100}{\text{Xylan content in the untreated biomass (mg/gdwt)}} \quad (4)$$

$$\text{Lignin recovery (\%)} = \frac{\text{Lignin content in the pretreated solids (mg/gdwt)} \times 100}{\text{Lignin content in the untreated biomass (mg/gdwt)}} \quad (5)$$

$$\text{Delignification (\%)} = 100 - \text{Lignin recovery (\%)} \quad (6)$$

$$\text{Glucose yield (\%)} = \frac{\text{Amount of glucose in the hydrolyzate (mg)} \times 100}{\text{Cellulose content in pretreated solids } \left(\frac{\text{mg}}{\text{g}}\text{dwt}\right) \times \text{Weight of pretreted solids loaded (mgdwt)} \times 1.11} \quad (7)$$

$$\text{Xylose yield (\%)} = \frac{\text{Amount of xylose in the hydrolyzate (mg)} \times 100}{\text{Xylan content in pretreated solids } \left(\frac{\text{mg}}{\text{g}}\text{dwt}\right) \times \text{Weight of pretreted solids loaded (mgdwt)} \times 1.136} \quad (8)$$

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References

1. Odeh NA, Cockerill TT (2008) Life cycle GHG assessment of fossil fuel power plants with carbon capture and storage. Energy Policy 36(1):367–380
2. Singh A, Gangopadhyay S, Nanda P, Bhattacharya S, Sharma C, Bhan C (2008) Trends of greenhouse gas emissions from the road transport sector in India. Sci Total Environ 390(1):124–131
3. Baeyens J, Kang Q, Appels L, Dewil R, Lv Y, Tan T (2015) Challenges and opportunities in improving the production of bio-ethanol. Prog Energy Combust Sci 47:60–88
4. Di Donato P, Finore I, Poli A, Nicolaus B, Lama L (2019) The production of second generation bioethanol: the biotechnology potential of thermophilic bacteria. J Clean Prod 233:1410–1417
5. Robak K, Balcerek M (2018) Review of second generation bioethanol production from residual biomass. Food Technol Biotechnol 56 (2):174
6. Li M, Pu Y, Ragauskas AJ (2016) Current understanding of the correlation of lignin structure with biomass recalcitrance. Front Chem 4:45

7. Bernier-Oviedo DJ, Rincón-Moreno JA, Solanilla-Duqué JF, Muñoz-Hernández JA, Váquiro-Herrera HA (2018) Comparison of two pretreatments methods to produce second-generation bioethanol resulting from sugarcane bagasse. Ind Crop Prod 122:414–421
8. Kumar P, Barrett DM, Delwiche MJ, Stroeve P (2009) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind Eng Chem Res 48 (8):3713–3729
9. Singh J, Suhag M, Dhaka A (2015) Augmented digestion of lignocellulose by steam explosion, acid and alkaline pretreatment methods: a review. Carbohydr Polym 117:624–631
10. Zhang K, Pei Z, Wang D (2016a) Organic solvent pretreatment of lignocellulosic biomass for biofuels and biochemicals: a review. Bioresour Technol 199:21–33
11. Sun F, Chen H (2008) Organosolv pretreatment by crude glycerol from oleochemicals industry for enzymatic hydrolysis of wheat straw. Bioresour Technol 99(13):5474–5479
12. Ebrahimi M, Villaflores OB, Ordono EE, Caparanga AR (2017) Effects of acidified aqueous glycerol and glycerol carbonate pretreatment of rice husk on the enzymatic digestibility, structural characteristics, and bioethanol production. Bioresour Technol 228:264–271
13. Ling TP, Hassan O, Badri K, Maskat MY, Mustapha WAW (2013) Sugar recovery of enzymatic hydrolysed oil palm empty fruit bunch fiber by chemical pretreatment. Cellulose 20 (6):3191–3203
14. Zhang W, Sathitsuksanoh N, Barone JR, Renneckar S (2016b) Enhanced enzymatic saccharification of pretreated biomass using glycerol thermal processing (GTP). Bioresour Technol 199:148–154
15. Demirbas A (2010) Direct and alkaline glycerol liquefaction of hazelnut shell. Energy Sources Part A 32(8):689–696
16. Martin C, Puls J, Saake B, Schreiber A (2011) Effect of glycerol pretreatment on component recovery and enzymatic hydrolysis of sugarcane bagasse. Cellul Chem Technol 45(7):487
17. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D (2008) Determination of structural carbohydrates and lignin in biomass. Lab Anal Proc 1617:1–16
18. Khullar E, Dien BS, Rausch KD, Tumbleson ME, Singh V (2013) Effect of particle size on enzymatic hydrolysis of pretreated Miscanthus. Ind Crop Prod 44:11–17
19. Clarke S, Preto F (2011) Biomass densification for energy production. Ontario Ministry of Agriculture, Food and Rural Affairs, Guelph, Ontario
20. Shereena P. Joy, Ashok Kumar A, Gorthy S, Jaganathan J, Kunappareddy A, Gaddameedi A, Chandraraj K (2021) Variations in structure and saccharification efficiency of biomass of different sorghum varieties subjected to aqueous ammonia and glycerol pretreatments. Ind Crop Prod 159:11307221
21. Kanak Raj, Chandraraj K (2019) Improved high solid loading enzymatic hydrolysis of low-temperature aqueous ammonia soaked sugarcane bagasse using laccase-mediator system and high concentration ethanol production. Ind Crop Prod 131:32–40
22. Pandey A, Negi S, Binod P, Larroche C (2014) Pretreatment of biomass: processes and technologies. Academic Press, Cambridge



Chapter 9

Seed Viability Test: A Semi-Throughput Method to Screen Oilseeds for Biodiesel Production

T. Eevera, D. Ramesh, M. Djanaguiraman, and R. Umarani

Abstract

Biofuels offer a solution to combat climate change and secure energy surplus. Among the various sources for biodiesel production, the seed of oilseed crops is primary raw material. The biodiesel industry demands a supply of good quality oil so that it can reduce processing costs and price of biodiesel. In general, the oils with low free fatty acid contents are considered as good quality oil and for which the quality of the oil should be analyzed in seed lot for biodiesel production. Currently, oil quality testing is done to the oil extracted from seed lot using sophisticated instruments. However, the analysis of oil seed lot is not tested, which forms the basic materials for oil extraction. Therefore, analysis of the quality of raw materials (seed lot in this case) is mandatory for assessing oil quality.

Till now, there is no standard method available for screening the quality of the oilseed before biodiesel production. The free fatty acid (FFA) content of the oil is the critical factor for biodiesel production. In seeds, the FFA content of oil is directly associated with seed viability. In this chapter, an easy-to-perform protocol is presented for determining the seed viability potential, which is having a significant positive association with FFA content of the seed.

Key words Oilseeds, Transesterification, Biodiesel production, Screening, Seed viability

1 Introduction

Biodiesel can be produced from vegetable oils or animal fats. Generally, the transesterification method is employed to produce biodiesel, which involves a reaction between catalyst, alcohol, and oil/fat at a particular temperature and time. The seeds of different oilseed crops are used as potential biodiesel feedstocks. Oilseed crops production is season oriented, and seed oil yield may vary with species, and agroclimatic conditions.

The oilseed crop will be harvested at field maturity, and the seeds will be dried under sun or in a mechanical drier to attain optimum moisture content of 8–10% [1]. Drying of seeds to optimum moisture content is a crucial step since it can influence the oil recovery percentage as well as the shelf life of the oil. The oil which

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is going to be used for biodiesel production should be free from plant debris, lower moisture content, and low FFA content to ensure economic biodiesel yield [2]. The FFA content of the oil is considered as an important parameter for determining the appropriate method of biodiesel extraction from the oil seeds [3]. The commercial biodiesel industries are currently employing alkaline catalyst-based transesterification method for biodiesel extraction. The conversion of higher FFA content of oil via alkaline catalyst-based transesterification process can result in lower yield of biodiesel and increased processing cost [4].

The quality of oil extracted may be affected due to injured/immature/poorly stored oilseed crop seeds, since they are mainly responsible for the increase in the FFA content of oil present in the oilseed during storage. An opportunity to test the quality of oilseeds based on FFA-linked biological characteristics has immense scope to screen the source material before oil extraction. Existing oil quality analysis is done by using the oil samples extracted from oilseeds. Presently, no method is employed for screening of oilseeds for biodiesel production. In this chapter, a simple and rapid screening protocol to assess the oilseed quality is presented. The quality of the oilseed crop seed is directly associated with biodiesel production.

2 Materials

1. Oilseeds.
2. Watch glasses.
3. Petri dishes.
4. Beakers (100–250 mL capacity).
5. Single-edge razor blade.
6. Needles.
7. Forceps.
8. Magnifying devices.
9. Medicine dropper.
10. Dispensing bottle.
11. Oven or incubator.

Chemicals

1. 2,3,5-Triphenyl Tetrazolium Chloride.
2. KH_2PO_4.
3. NaH_2PO_4.

2.1 Preparation of Seed Sample and Solution

2.1.1 Conditioning and Preparing of Oilseeds: Exposure of Tissues for Staining

The seed of oilseed crops is taken, and using a needle and forceps the pericarp (seed coat) is carefully removed to facilitate the quick permeation of 2,3,5-triphenyl tetrazolium chloride (TCC) solution into the seed tissues to ensure quick staining of viable tissues.

2.1.2 Preparation of Solution

2,3,5-Triphenyl tetrazolium chloride solution with a pH of 6.0–7.5 was prepared with distilled water. The concentration of 2,3,5-triphenyl tetrazolium chloride will vary between 0.1 and 1.0% depending on crop species. If the pH of the solution is more than 7.5, the intensity of staining will be very high. However, if the pH is less than 6.0, it will lead to poor staining, irrespective of the viability status of the seed. Therefore, the 2,3,5-triphenyl tetrazolium chloride solution is prepared by using the buffer solution to maintain the pH.

1. Solution A is prepared by dissolving 9.078 g KH_2PO_4 in 1000 mL of distilled water.
2. Solution B is prepared by dissolving 11.876 g NaH_2PO_4 in 1000 mL of distilled water.
3. Solution A and B are mixed in the ratio of 2:3.
4. 0.2 g of 2,3,5-triphenyl tetrazolium chloride is mixed in 100 mL of buffer solution to prepare 0.2% solution.

3 Methods

Seed sampling

The first step is to draw a sample that is "representative" of the entire seed lot. The quantity of seeds tested for its quality attributes is very less compared to the size of the seed lot which is intended to represent. Hence, the seed sampling procedure has to be implemented with the utmost care.

3.1 Seed Sampling Procedure

Details of procedure that need to be followed for taking the seed sample are given below [5].

1. Several individual samples (oilseeds) have to be drawn from various containers, or different parts of the bulk are called primary samples.
2. All the primary samples are pooled or combined in a suitable container to arrive at composite sample.

3. The size of the composite sample should be suitably or appropriately reduced; the reduced sample size should be submitted to the seed testing laboratory for viability assessment.
4. Under laboratory conditions, the submitted sample will be reduced with the help of mixing and dividing instruments, and finally the working sample will be arrived. The working sample of oilseed crop seeds shall contain at least 2500 seeds or prescribed weight as per the ISTA [5] standards.
5. As per the physical purity analysis guidelines of the ISTA [5], working sample should be segregated into pure seed, other crop seed, and inert matter.
6. Among the three fractions, 400 numbers of seeds will be randomly taken from pure seed fraction to assess the suitability of oilseed lot for biodiesel production based on seed viability analysis.

3.2 Screening Test

Testing seeds for germination by conventional method takes a longer time, but often the viability of seeds is known within a day or two by using the tetrazolium test [6].

3.2.1 Principle

All living cells in seed "respire" depending on their vigor level. During respiration, the hydrogen transfer or energy transfer system is controlled by a group of enzymes called "dehydrogenases" present in the cells of seeds. The activity of this enzyme is higher in vigorous tissues compared to the less vigorous tissues. A colorless indicator dye or staining solution (2,3,5-Triphenyl Tetrazolium Chloride) in which seeds are allowed to imbibe, interact with the reduction processes during respiration, and accept one "H" from the living cells by hydrogenation. As a result of hydrogenation processes, the 2-3-5, triphenyl tetrazolium chloride is reduced into a stable, nondiffusible substance called 2-3-5, triphenyl tetrazolium formazan. Based on the intensity and pattern of staining, the seeds can be classified into viable or nonviable.

3.2.2 Methodology

The sequential steps involved in this test are (Fig. 1):

1. Conditioning and preparing the seed for staining.
2. Exposure of tissues for staining.
3. Preparation of solutions.
4. Staining of the seeds.
5. Assessment or evaluation of stained seeds.

3.2.3 Procedure for Staining and Assessment of Seed Viability

1. A quantity of 400 seeds is taken from the working sample.
2. The collected seeds are preconditioned by soaking in water for 1 h.
3. The seed coat is removed from the seed after preconditioning.

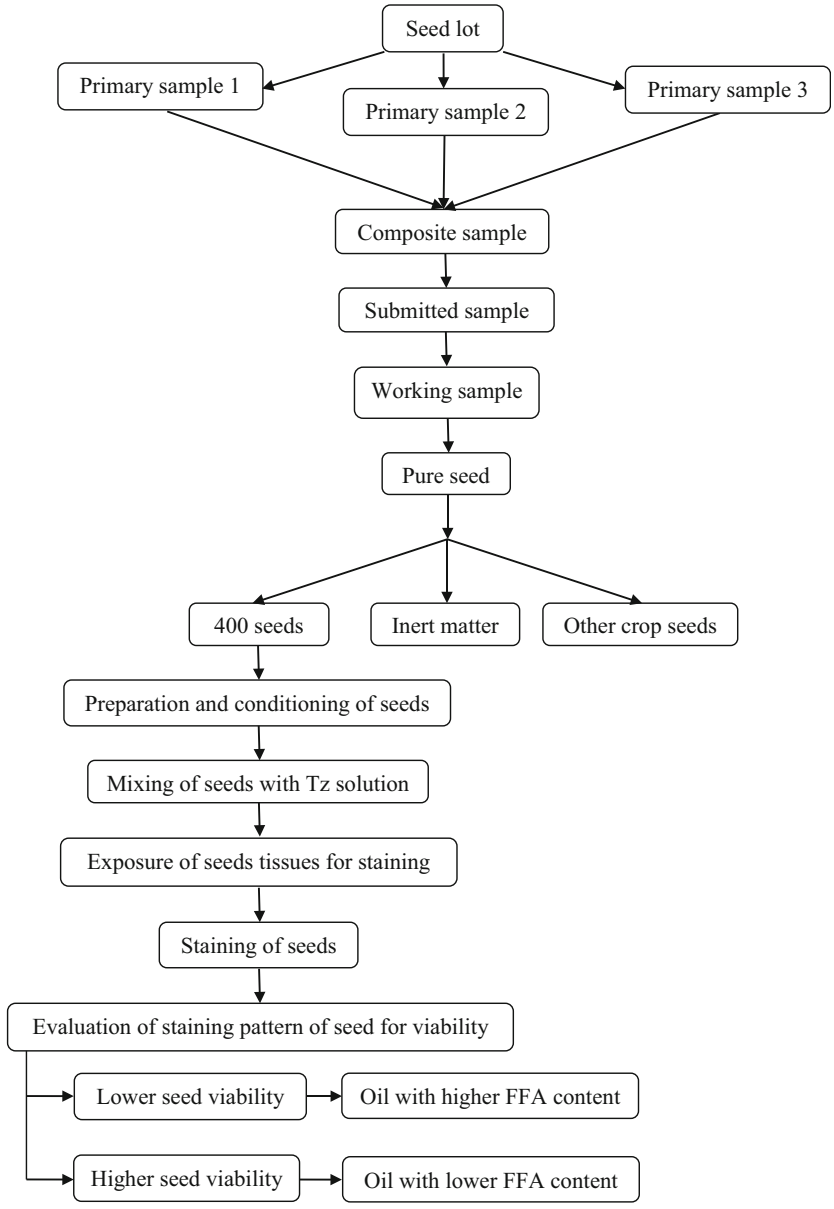


Fig. 1 Assessment of biodiesel feedstock based on oilseed viability

4. The naked seeds are placed in a Petri dish, and an adequate quantity of 0.2% tetrazolium chloride solution is added to immerse them.
5. The seed sample is incubated in darkness for 4 h.
6. Decant the 2-3-5, triphenyl tetrazolium chloride solution after incubation (4 h), and naked seeds are washed thoroughly in distilled water.
7. The staining pattern of cotyledons is observed, and seeds are segregated into viable and nonviable seeds.

8. Calculate the viability percentage of seeds based on viable and nonviable seeds

$$\text{sSeed viability, \%} = \frac{\text{Number of viable seeds}}{\text{Total number of seeds taken for screening}} \times 100$$

9. Higher viability percentage of the seeds indicates that higher oil quality with low free fatty acid content is present in the seeds and vice versa. In other words, based on the intensity and staining pattern on oilseeds, the oilseeds can be divided into suitable or not suitable for alkali-based transesterification for biodiesel production.
10. The following rating scale can be used for identifying seed lot suitable for biodiesel production.

Intensity and staining pattern on oilseeds	Results
More to the moderate staining pattern	Well suitable for the alkali transesterification process
Less or no staining at all	Not suitable for the alkali transesterification process

11. In conclusion, the higher viability of oilseeds is well suited for the single-step alkali transesterification process.

4 Oilseed Sampling

4.1 Sampling Intensity

The following sampling procedure [5] can be adopted for oilseeds stored in the standard size of bags or other containers

<5 containers	Sample each container, but with at least five primary samples
6–30 containers	Sample 5 containers or 1 in every 3 containers
31–400 containers	Sample 10 containers or 1 in every 5 containers
400 or more containers	Sample 80 containers or 1 in every 7 containers

The following procedures can be followed when seeds are stored in bulk in bins or heaps or processing streams:

<500 kg	At least five individual samples
501–3000 kg	One sample for each 300 kg, but not less than five primary samples
3001–20,000 kg	One sample for each 500 kg, but not less than ten primary samples

When the seeds are in small containers such as tins, cartons, or packets, a 100 kg weight of seeds is taken as one basic unit, and the small containers are combined to form a sampling unit. This sampling unit should be less than 100 kg (e.g., 20 containers of 5 kg; 33 containers of 3 kg; 100 containers of 1 kg). For sampling, each unit is considered as "one container," and the sampling intensity prescribed as in the case of containers is applied.

4.1.1 Mixing and Dividing of Seed Samples

Mixing and dividing of seed samples are done in order to obtain the uniformly blended quantity of representative sample as well as to obtain the required quantity of submitted and working samples. Methods used for mixing and dividing the seed samples in the laboratory are mechanical dividers, e.g., conical or soil or modified halving method and hand halving method.

4.1.2 Mechanical Mixing and Dividing

This method is suitable for seeds that are free-flowing and non-chaffy. The apparatus divides the sample into two halves, which are again pooled, and the process is repeated until satisfied that the mixing and dividing are done properly. Depending upon the seed, the process is repeated until obtaining the required quantity of seed samples is obtained.

4.1.3 Conical or Boerner Divider

It is the most commonly used divider, which is working on the principle of gravity. The divider consists of a seed hopper, a valve, and a conical structure. Inside the conical structure, there is a series of baffles with alternate channels and space which directs the seeds into two spouts. The width of the space and channels are the same. The series of baffles are arranged in a spiral manner in such a way that they direct the seeds inward and downward toward the spout. The channels are leading to one spout while the space leading to an opposite spout to divide the seed samples. This divider is not suitable for chaffy seeds, and checking for cleanliness is difficult.

4.1.4 Soil Divider

It has channels or ducts arranged in a straight manner, a hopper, two receiving pans, and a pouring pan. Channels are about 13 mm wide, and the total number of channels is 18. The alternate channels are leading to one spout in one direction and the other to the spout in the opposite direction. This divider is suitable for large size seeds.

4.1.5 Modified Halving Method

This consists of a tray with a number of cubical cups fitted inside. The cups will be open at the top, and every alternate one will not have the bottom. After preliminary mixing, the seeds are evenly poured as that of the random cup method. When the grid, along with the cup, is lifted, approximately half the quantity will be retained in the cups leaving the remaining half in the tray. This process is done until the required quantity of the working sample is obtained.

4.1.6 Hand Halving Method

In the absence of any of the methods mentioned above, this method can be followed. In this method, the sample is divided into four quarters, and the two of the opposite quarters are rejected, and the other half is again pooled, and the processes are repeated till the desired quantity of the working sample is obtained.

4.1.7 Physical Purity Analysis

Physical purity is one of the basic quality characters, and it denotes the cleanliness of the seed, and this is the first step in seed quality evaluation. In purity analysis, the working samples of the seed lot have to be cleaned into various components as detailed below.

4.2 Components of Purity Analysis

Based on the recommendation of the purity working group of ISTA after 1985, the components which are verified in the purity analysis are the pure seed, other crop seeds, and inert matter.

4.2.1 Pure Seed

It shall refer to the species of kind/species stated by the sample sender or found to predominate in the working sample. In addition to mature and undamaged seed, it includes immature seeds, shriveled seeds, pre-germinated seeds, and even diseased seeds provided they can be identified as that of the species under consideration.

4.2.2 Half Seed Rule

A broken seed, which is more than half of the original size, is counted as the pure seed. Seeds with the seed coat entirely removed are regarded as inert matter.

4.2.3 Other Crop Seeds

Seeds and seed units of any plant other than that of oilseeds are regarded as other crop seeds. Weed seeds are also included as other seeds. The half seed rule is applicable for other crop seeds also.

4.2.4 Inert Matter

It includes seed-like structures, other plant parts, nematode galls, sclerotic bodies, sand, stone, and other related substances, glumes, lemma, and palea. A seed that is less than half of the original size of the seeds and/or other crop seeds are regarded as inert matter. Seeds without seed coat are also grouped as inert matter.

5 For Staining Conditions

Temperature influences the staining reaction. For every 5–6 °C increase in temperature will reduce the duration for about one half of the original time required for complete staining. The recommended temperature for effective staining is 30 °C. However, the temperature must be between 20 and 45 °C.

5.1 Correlation Between Seed Viability and FFA of Oil

The oil quality and oil recovery depend on the conditions of the oilseeds. In other words, oilseeds with higher seed viability will yield an excellent quality of oil (in terms of FFA content) that is required for biodiesel production. However, the viability of seed

depends on moisture content, seed coat hardness, and percent damage. Kinman and Ibert [7] and Vinoth Kumar et al. [8] reported that the FFA content of the oil extracted from low viability or dead seeds were found higher and vice versa. The seed viability could be measured using the 2-3-5, triphenyl tetrazolium chloride protocol (mentioned earlier). Based on the intensity and staining pattern on oilseeds, the samples can be classified into viable or nonviable seeds.

To confirm and cross-validation of the above scientific finding a correlation between seed viability and FFA of the oil should be performed. For the estimation of the FFA content of oil, the procedure given in the AOCS Protocols Ca 5a-40 can be used [9]. The seed viability is the primary determinant of the biochemical property of oil available in the oilseeds [8]. The relationship between the percentage of seed viability and the FFA content of oilseed could be useful for assessment of the suitability of oilseeds for biodiesel production using results of either seed viability or FFA content. The correlation equation can be employed to predict the FFA content of oil using the viability of oilseeds and also for verification purposes.

6 Conclusion

The critical quality parameter of the oil prescribed for testing the oil quality before biodiesel production is free fatty acid content. The content of free fatty acid is in turn directly proportional to the seed viability potential. In this chapter, an easy-to-perform protocol is presented for determining the seed viability potential, which is having a significant positive association with free fatty acid content of seed. High quality of raw material is quintessential for ensuring high biodiesel production. In order to quickly estimate the quality of the seed lot, screening of seed viability through tetrazolium test and correlation with FFA content of the seed is very useful. Obtaining "representative" seed sample forms the basis for achieving reproducible test results. Hence, it is recommended that prescribed procedure should be followed for drawing "representative" seed sample and the seeds should be subjected to tetrazolium test or "quick viability test." The higher seed viability percentage forms the quality criteria for selecting the oil seed lot for subsequent production of biodiesel.

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References

1. Agarwal RL (2011) Seed technology, 2nd edn. Oxford & IBH Publishing Co Pvt.Ltd., New Delhi
2. Zahan KA, Kano M (2018) Biodiesel production from palm oil, its by-products, and mill effluent: a review. Energies 11:2132
3. Zhang Y, Dubé MA, McLean DD, Kates M (2003) Biodiesel production from waste cooking oil: 2. Economic assessment and sensitivity analysis. Bioresour Technol 90:229–240
4. Ma F, Hanna MA (1999) Biodiesel production: a review. Bioresour Technol 70:1–15
5. ISTA (2013) International rules for seed testing. In: Fast analytical procedure, 12th edn. ISTA, Basserdorg, CH
6. Lakon G (1942) Topographischer Nachweis der Keimfähigkeit der Getreidefrüchte durch Tetrazoliumsalze. Ber Deutsch Bot Ges 60:434–444
7. Kinman ML, Ibert ER (1956) Seed germination as an index of the potential free fatty acid content of sesame oil. JAOCS 33:637–639
8. Vinoth Kumar M, Eevera T, Ramesh D, Umarani R (2019) Influence of groundnut seed viability on biodiesel feedstock quality. Ind Crop Prod 140:111697. https://doi.org/10.1016/j.indcrop.2019.111697
9. American Oil Chemists' Society (2017) AOCS official method Ca 5a-40: free fatty acids (revised 2017). AOCS, Champaign, IL

Part III

Molecular Genetics and Biotechnology of Biofuel and Biodiesel Production



Chapter 10

RNAi-Based Gene Silencing in Sugarcane for Production of Biofuel

Naveenarani Murugan, Chakravarthi Mohan, and Baskaran Kannan

Abstract

RNA interference (RNAi) is an innate cellular mechanism triggered by a double-stranded RNA (dsRNA) molecule causing selective inhibition of gene expression. Here, we demonstrated the RNAi technology for gene silencing in sugarcane for biofuel production. This chapter describes an efficient model system that established to target the caffeic acid O-methyltransferase (COMT) gene and the RNAi construct is designed and delivered through *Agrobacterium* mediated stable sugarcane transformation. Also, the approach for an analysis of resulting putative transgenic plants for a targeted RNAi mediated gene silencing is described.

Key words RNA interference, Sugarcane, Gene silencing, Biofuel, Caffeic acid O-methyltransferase (COMT), Agrobacterium, Transgenic sugarcane

1 Introduction

Sugarcane (*Saccharum spp.* hybrids), a very efficient perennial C4 plant with a highly polyploid and complex genome, is considered a top-notch candidate for biomass production all over the world [1]. Sugarcane is a high biomass producing plant and is necessary to improve sugarcane biomass productivity, making them more suitable for lignocellulosic ethanol production. The major barrier that hinders the use of energy crops biomass for biofuel production is the recalcitrance of the plant cell wall [2]. The manipulation of bioenergy crops with reduced lignin content or modifying cellulose and lignin biosynthesis is a prime strategy to reduce biomass recalcitrance and boost the ethanol production. Therefore, RNAi mediated genetic manipulation of lignin biosynthetic gene is a successful strategy to improve saccharification of lignocellulosic biomass [3–5]. RNA interference (RNAi) is a gene silencing phenomenon which involves sequence-specific gene regulation triggered by double-stranded RNA (dsRNA), resulting in a degradation of target gene transcripts. This phenomenon of

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RNAi gene regulation involves small noncoding RNAs called small interfering RNA (siRNA) and microRNA (miRNA) that are the cleavage products of dsRNA [6]. In plants, RNAi has been extensively applied to accomplish desirable traits by manipulating the gene expression [7]. Recently, sugarcane was genetically manipulated by targeting the lignin biosynthetic pathway genes, which have been successfully demonstrated for biofuel production, i.e., caffeic acid O-methyltransferase (COMT), caffeoyl-CoA *O*-methyltransferase (*CCoAOMT*), ferulate 5-hydroxylase (*F5H*) [3, 5, 8, 9], 4-coumarate:coenzyme A ligase (4CL) gene [5], and BAHD acyl-coenzyme A (CoA) transferase [10]. The present protocol facilitates RNAi-based gene silencing in sugarcane which involves: (a) selection of target region(s) within the target gene, (b) generating a RNAi construct with hairpin cassette (target gene cloned in a sense and antisense orientation flanking a spacer or intron), (c) genetic transformation of sugarcane and screening of transgenic lines, and (d) evaluation of traits of interest for desired quality.

2 Materials

2.1 Bacterial Strains and Plasmids

1. *Escherichia coli* strain DH5α.
2. *Agrobacterium tumefaciens* strain EHA105/LBA4404.
3. pGEM®-T Easy cloning vector (Promega, Mannheim, Germany).
4. Intermediate Vector pRNAi-GG.
5. Binary vector pRI101 AN (Takara Bio, Inc.).

2.2 Reagents for Molecular Cloning

1. RNeasy plant mini kit (QIAGEN, Germany).
2. cDNA synthesis kit (Invitrogen, USA).
3. Primers (Gene and vector specific primers).
4. Platinum Taq DNA polymerases High Fidelity (HF) kit (Thermo Fisher Scientific, USA) and PCR thermal cycler.
5. QIAquick PCR Purification kit (QIAGEN, Germany).
6. Restriction enzymes and corresponding 10× buffer (Thermo Fisher Scientific, USA).
7. T4 DNA ligase and 10× T4 DNA ligase buffer (Thermo Fisher Scientific, USA).
8. LB medium and plates (*see* Subheading 2.4.2.1) containing 100 μg/mL ampicillin (for pGEM) or 100 μg/mL kanamycin (for pRI101 AN) for *E.coli* cultures.
9. YEP medium and plates (*see* Subheading 2.4.2.2) containing 50 μg/mL rifampicin and 100 μg/mL kanamycin for *Agrobacterium* cultures.

10. QIAprep Spin Plasmid miniprep kit (QIAGEN, Germany).
11. Temperature-controlled incubators equipped with a rotary shaker (37 °C and 28 °C).
12. NanoDrop 2000/c Spectrophotometer (Thermo Fisher Scientific, USA).
13. Agarose gel electrophoresis unit.
14. Agarose.
15. Calcium chloride.
16. Ethidium bromide.
17. 50× TAE buffer.
18. 6× Gel loading buffer.
19. QuantiTect SYBR Green PCR kit (QIAGEN, Germany).

2.3 Materials for Transgenic Sugarcane Development and Screening

1. Bunsen burner, forceps, scalpel.
2. Petri dishes with lid.
3. Glass bottles with a lid.
4. Pipettes and disposable autoclaved tips (0.2–10, 20–200, 100–1000 and 1000–5000 μL).
5. Filter paper (autoclaved).
6. Refrigerated centrifuge.
7. Sterile screw-cap polypropylene centrifuge tubes (round bottom 50 mL and conical bottom of 15 and 50 mL).
8. Erlenmeyer flasks (250 mL).
9. Sterile polypropylene cryotubes (1.5 mL).
10. Sterile double-distilled water.
11. Murashige and Skoog (MS) medium (HiMedia).

2.4 Solutions and Nutrient Media

2.4.1 Solutions

1. Calcium chloride (20 mM): Dissolve 294.04 mg in 100 mL of distilled water, autoclave, and store at 4 °C.
2. Hygromycin (50 mg/L): Dissolve in distilled water, filter-sterilize, and store at 4 °C.
3. Rifampicin (50 mg/L): Dissolve in dimethylsulfoxide (DMSO), filter-sterilize, and store at –20 °C.
4. Kanamycin (50 mg/L): Dissolve in distilled water, filter-sterilize, and store at 4 °C.
5. Acetosyringone (1 M): 196.2 g/L, dissolve in DMSO and store at –20 °C.
6. 2, 4-Dichlorophenoxyacetic acid (2,4-D, 1 mM): 221 mg/L, dissolve in a few drops of 50% ethanol by heating gently, make up to the final volume with hot distilled water, filter-sterilize, and store at 4 °C.

7. Cefotaxime (250 mg/mL): Dissolve 1 g of powder in 4 mL of double-distilled water. Sterilize by filtration and store at −20 °C.
8. 6-Benzylaminopurine (6-BAP, 1 mM): 0.225 g/L, dissolve in a few drops of 1 M NaOH, then make final volume in distilled water, filter-sterilize, and store at 4 °C.
9. Naphthalene acetic acid (NAA) 1 mg/mL: Dissolve well with some drops of 1 N NaOH, make up the volume to 1 mL with distilled water, filter-sterilize, and store at 4 °C.
10. Kinetin (1 mg/mL): Dissolve well with some drops of 1 N NaOH, make up the volume to 1 mL with distilled water, filter-sterilize, and store at 4 °C.
11. Ethanol (70%): Mix 700 mL of 99.9% ethanol and 300 mL of distilled water, store at RT.
12. 50× TAE: Tris (base) 2 M (242 g), EDTA (disodium salt) 0.05 M (18.6 g). Adjust to pH 8.0 with glacial acetic acid (~57 mL) and make volume up to 1 L.
13. Ethidium bromide: 0.5 μg mL^{-1}, dissolved in water.
14. 6× Loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol in water.

2.4.2 Nutrient Media

Medium for *E. coli* Transformation

LB Medium (Luria—Bertani broth): Yeast extract 5.0 g/L, tryptone 10.0 g/L, Sodium chloride 5.0 g/L. Dissolve in distilled water and make up the volume to 1 liter. Adjust the pH to 7.0 with 1 N NaOH. Autoclave and store at room temperature. To make the solidified LB medium, 1.5% (w/v) agar is added.

Medium for *A. tumefaciens* Transformation

YEP Medium (Luria—Bertani broth): 10.0 g/L yeast extract, 10.0 g/L peptone, 5.0 g/L sodium chloride. The pH is adjusted to 7.0, the solution is autoclaved and cooled, and then respective antibiotics are added. To make the solidified medium, add 1.5% (w/v) agar.

Media for Transgenic Sugarcane Development

1. MS Medium (M0): MS medium + 50 g/L sucrose, pH 5.8.
2. Infection medium (M1): 1/2M0 + 200 μmol/L acetosyringone + 15 g/L dextrose, pH 5.8.
3. Co-cultivation medium (M2): M0 + 3.0 mg/L 2.4-D + 200 μmol/L acetosyringone + 3 g/L phytagel, pH 5.8.
4. MS Cef Medium (M3): M0 + 3.0 mg/L 2.4-D + 0.5 mg/L Cefotaxime + 3 g/L phytagel, pH 5.8.
5. Selection medium (M4): M0 + 3.0 mg/L 2.4-D + 30 mg/L hygromycin + 0.5 mg/L Cefotaxime + 3 g/L phytagel, pH 5.8.

6. Regeneration medium (M5): M0 + 1 mg/L Kinetin + 0.5 mg/L NAA + 30 mg/L hygromycin + 3 g/L phytagel, pH 5.8.
7. Rooting medium (M6): White's medium [11] + 5 mg/L hygromycin, pH 5.8.

3 Methods

3.1 Isolation of Gene-of-Interest from Sugarcane

Select a target gene of interest; for example, here we are describing *COMT* gene (*Saccharum officinarum*, AJ231133) as a gene-of-interest. The sequence of this gene is available in the public domain. Design a forward primer and reverse primer to amplify the target gene. Isolate total RNA from top leaves of 3–8-month-old sugarcane variety using the RNeasy Plant Mini Kit. Verify the integrity of RNA by running 1 μg of total RNA on 1% (w/v) agarose gel and the concentration and purity of the extracted RNA is analyzed with the NanoDrop 2000/c spectrophotometer. Using 2 μg of total RNA, synthesize complimentary DNA (cDNA) using an Oligo-dT18 primer (the primer hybridizes to the poly(A) tail of mRNA) and Thermo Scientific Revert Aid Reverse Transcriptase. Amplify the *COMT* gene using the High fidelity (HF) Taq (*see* **Note 1**), under the following conditions: 35 cycles of 94 °C for 30s, 56 °C for 45 s and 72 °C for 120 s. Purify and clone the PCR product into the pGEM®-T Easy vector (*see* **Note 2**), a TA cloning vector, and sequence 2–5 independent clones of pCOMT to confirm the successful cloning of target gene.

3.2 RNAi Target Selection and Vector Construction

To design a siRNA, an mRNA or cDNA sequence of the gene of interest (Subheading 3.1) is required and reference mRNA sequence (can be retrieved from the NCBI database) for the target gene is preferred because it represents the most reliable mRNA sequence for the gene. Select a siRNA sequence with significant homology to the target sequence, which is highly recommended to avoid off-target effects. The selection of a silencing target region should be an important factor for determining the specificity of RNAi. It is highly recommended that in plants, using ~300–700 bp of dsRNAs possess a greater success of silencing [12, 13]. Therefore, a well-characterized potential target is very crucial for increased success rate.

1. Select an RNAi target sequence based on sugarcane's closely related genome sequences of maize (*Zea mays*) and sorghum (*Sorghum bicolor*). Perform multiple sequence alignment using ClustalW (*see* **Note 3**) to identify a highly conserved homologous gene sequence of *COMT* from the available genome resources like sugarcane EST.
2. Design and synthesize a customized pair of specific PCR primers targeting the most conserved region, the primer set is also used to introduce BsaI (both 3′ and 5′ of sense and antisense

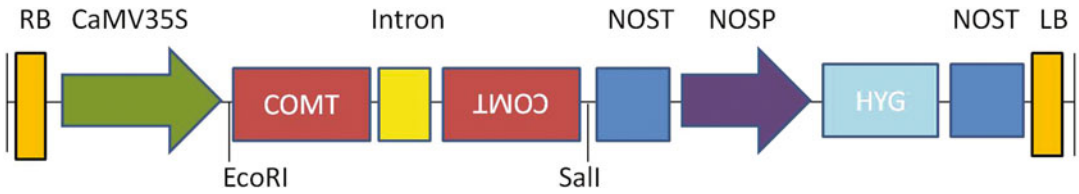


Fig. 1 Map of pRI101 AN–COMT RNAi vector. *RB* right border, *CaMV35S* cauliflower mosaic virus promoter, *NOST* NOS terminator, *35S* CaMV 35S promoter, *NOSP* NOS promoter, *Hyg* hpt gene coding for hygromycin resistance, *LB* left border. BsaI, EcoRI, SalI are restriction enzymes used for the cloning of fragments

strand), EcoRI (5′ end of sense strand) and SalI (3′ end of antisense strand) and overhanging nucleotides (*see* **Note 4**) to enable sub-cloning of hairpin-structure under regulatory sequences (Fig. 1).

3. For constructing an RNAi silencing cassette, amplify RNAi target region by using a primer set 1 from the pCOMT plasmid (*see* Subheading 3.1) as a template.
4. Set up the PCR reaction:

(a)	Reverse primer (10 mM)	0.5 μL
(b)	Forward primer (10 mM)	0.5 μL
(c)	10× buffer	2.0 μL
(d)	dNTPs (10 mM)	1.0 μL
(e)	HF Taq	0.2 μL
(f)	DNA	100 ng
(g)	Water up to	20 μL

5. Run the PCR using the following program (*see* **Note 5**):

(a)	94 °C	5 min	
(b) (c) (d)	94 °C 55 °C 72 °C	1 min 1 min 1 min	35 cycles
(e)	72 °C	8 min	
(f)	10 °C	end	

6. Separate the PCR product in 1% agarose gel. Excise and purify the target fragment by using a gel extraction kit according to the manufacturer's recommendations and quantify 1 μL of the gel product in Nanodrop 2000/c spectrophotometer (*see* **Note 6**).

7. Ligate the fragments into 5′ and 3′ arms of the intron in sense and antisense direction allowing the formation of self-complementary intron-hairpin structures along the pRNAi-GG vector (Fig. 1). Clone and sequence few independent colonies (as described in Subheading 3.1).
8. Double-digest both donor (pRNAi-GG) and acceptor plasmids (pRI101 AN) independently with EcoRI/SalI Fastdigest enzymes (*see* **Note 7**).
   (a) For binary vector pRI101 AN prepare the following reaction mix:
      (i) 2 μg of pRI101 AN.
      (ii) 5 μL of 10× Fastdigest Green buffer (supplied with restriction enzymes).
      (iii) 1 μL of Fastdigest EcoRI.
      (iv) 1 μL of Fastdigest SalI.
      (v) Distilled, deionized H_2O to 50 μL.
      (vi) Incubate the digestion reaction at 37 °C for 1 h.
   (b) For pRNAi-GG, prepare the following reaction mixes:
      (i) 2 μg of pRNAi-GG.
      (ii) 5 μL of 10× Fastdigest Green buffer (supplied with restriction enzymes).
      (iii) 1 μL of Fastdigest EcoRI.
      (iv) 1 μL of Fastdigest SalI.
      (v) Distilled, deionized H_2O to 50 μL.
      (vi) Incubate the digestion reaction at 37 °C for 1 h.
      (vii) After digestion, immediately stop the reaction by inactivating the enzyme activity by incubating the samples at 65 °C for 20 min (*see* **Note 8**).
9. After digestion, purify the restriction digested products with QIAquick Gel Extraction Kit according to the manufacturer's recommendations.
10. Run a 1 μL aliquot of each purified digested product to check the concentration and size of the expected fragments in 0.8% agarose gel prepared with 1× TAE buffer at 100 V for 45 min.
11. Ligate pRI101 AN with pRNAi-GG using the following ligation reaction:

(a) Purified pRI101 AN	1 μL (100 ng/μL)
(b) Purified pRNAi-GG	3 μL (300 ng/μL)
(c) 10× T4 DNA ligase buffer	2 μL
(d) T4 DNA ligase (5 U/μL)	1 μL
(e) Distilled, deionized H_2O to	20 μL
(f) Incubate the ligation reaction for 1 h at room temperature or 4 °C overnight. Freeze it or proceed with transformation.	

12. Transform 5 μL of ligation product into competent *E. coli* cells (*see* **Note 9**) using standard protocols [14] and plate 100–150 μL on LB plates containing 100 mg/L kanamycin stock, incubate at 37 °C overnight (*see* **Note 10**).
13. Pick up to 10 colonies and inoculate to 5 mL LB medium with 100 mg/L kanamycin stock for plasmid verification. Shake overnight at 150 rpm in 37 °C.
14. Isolate plasmid DNA using a miniprep kit and verify the silencing-hairpin construct (pRI101 AN–COMT; Fig. 1) by double digestion with restriction enzymes (EcoRI/SacI) as described in **step 8**. Run 0.8% agarose gel of digestion reaction as described for **step 10**.
15. In addition, confirm the RNAi cassette by using the primers designed on the promoter sequence as forward and COMT sense fragment as a reverse primer binding site, follow the PCR steps as mentioned in **step 4**.
16. Sequence 2–5 plasmids to confirm correct integration of the expression cassettes.
17. Initiate another set of overnight liquid LB culture at 37 °C with 150 rpm and mix 900 μL of sequence confirmed culture with 100 μL 100% (v/v) glycerol and store at –80 °C for future use.

3.3 Introduction of RNAi construct into A. tumefaciens

The choice of *Agrobacterium* transformation methods, e.g., chemical transformation vs. electroporation, is solely at the discretion of the laboratory. Here we are describing the chemical competent cells used for transformation by freeze-thaw procedure as follows.

1. Preparation of chemically competent *Agrobacterium* cells.
   (a) Grow a colony of *A. tumefaciens* EHA105 in 5 mL YEP liquid media by incubating at 20 °C overnight with shaking (~250 rpm).
   (b) Transfer the cells to 50 mL YEP liquid media and incubate for 3–4 h with shaking until OD600 = 0.5–1.0.
   (c) Chill the culture on ice for 10 min, then centrifuge at 6000 × *g* for 5 min at 4 °C, and discard the supernatant.
   (d) Rinse the cell pellet with 10 mL of 20 mM ice-cold $CaCl_2$, resuspend the cells gently on ice and spin again at 6000 × *g* for 5 min at 4 °C (*see* **Note 11**).
   (e) Add 1 mL of 20 mM ice-cold $CaCl_2$ and resuspend the cells gently on ice.
   (f) Take an aliquot of 0.1 mL of the competent cells into a sterile prechilled microcentrifuge tubes (*see* **Note 12**).
2. Add 1–3 μg of pRI101 AN–COMT plasmid DNA mixture (Subheading 3.2, **step 14**) to the 0.1 mL of competent cells, mix gently, and thoroughly, and then freeze in liquid nitrogen or dry ice-EtOH slurry (*see* **Note 12**).

3. Heat shock the cells in a 37 °C water bath for 5 min. Add 900 μL YEP liquid media and incubate at 28 °C for 3 h with 250 rpm shaking; then spin at 3000 rpm for 3 min and spread all cells on YEP plate containing 50 mg/L kanamycin and 20 mg/L rifampicin antibiotics, if appropriate, and incubate for 2–3 days at 28 °C.
4. Confirm the positive colonies by a colony PCR method. If desired, plasmid can be isolated and used for verification by restriction analysis and DNA sequencing (*see* **Note 13**).
5. The agrobacterium colonies are ready to be used for plant transformation.

3.4 Generation of Transgenic Plants

Agrobacterium mediated sugarcane transformation using *A. tumefaciens* strain EHA105 carrying pRI101 AN–COMT plasmid as described below:

1. Collect apical meristematic tissues from 6- to 10-month-old, field-grown sugarcane plants (cv. *Saccharum officinarum*). Remove leaf sheath, outermost leaves, and wipe with 70% ethanol before transferring to sterile laminar airflow cabinet.
2. Wipe again with 70% ethanol, remove 1–2 outer layers, and cut immature leaf whorl cross-sections of approximately 2-mm thickness under aseptic conditions with a scalpel from the 1- to 10-cm region above the apical node (*see* **Note 14**).
3. Transfer 6–8 leaf whorl discs with top-side of discs facing the culture medium in 90 × 20-mm polystyrene petri dishes containing M2 medium (Fig. 2a). Incubate the leaf disc at 27 ± 2 °C in the dark for 2 days.
4. On the day of leaf disc culture initiation, inoculate the agrobacterium colonies containing pRI101 AN–COMT from the glycerol stock (Subheading 3.3, **step 5**), in 10 mL YEP medium containing 50 μg/mL kanamycin and 50 μg/mL rifampicin antibiotics. Incubate the culture at 28 °C on an orbital shaker at 150 rpm, overnight.
5. Next day, transfer 1 mL of the pre-inoculum to a sterile 250 mL Erlenmeyer flask containing 50 mL YEP medium supplemented with selective antibiotics, and incubate with shaking at 28 °C on an orbital shaker at 150 rpm, to the OD600 0.5–1.
6. Transfer the bacterial culture to sterile 50 mL conical tubes, centrifuge for 10 min at 6000 rpm, at room temperature and discard the supernatant.
7. Suspend the pellet gently in 25 mL of sugarcane infection medium M1 (*see* media composition in Subheading 2.4.2.3) and transfer the culture to a sterile 250-mL Erlenmeyer flask and incubate the culture for 1 h at 28 °C with shaking at 150 rpm, in the light.

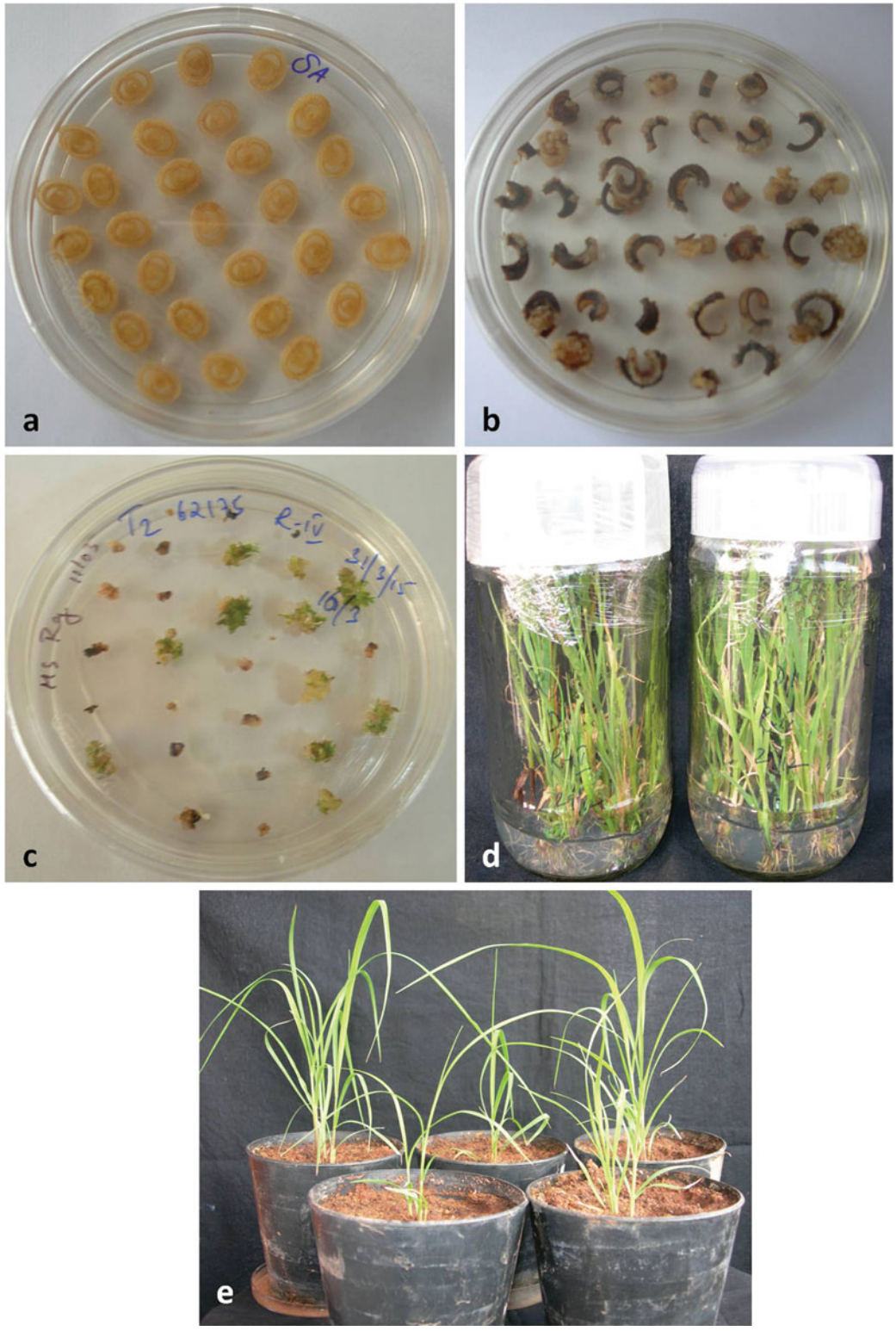


Fig. 2 Different stages of Agrobacterium mediated sugarcane transformation. (**a**) Sugarcane leaf whorls for co-cultivation, (**b**) embryogenic calli emerging on the selection medium, (**c**) regeneration of putative sugarcane lines, (**d**) rooted transgenic sugarcane lines, (**e**) transgenic sugarcane lines acclimatized in soil

8. Transfer the leaf discs from **step 3**, mix gently, and incubate in the dark for 1 h at 28 °C with gentle shaking.
9. Take out Erlenmeyer flask and infiltrate culture by applying vacuum for 2 min (perform in the laminar airflow cabinet) and incubate in the dark for 1 h without shaking.
10. Discard the agrobacterium culture, blot dry the leaf whorl discs on sterile Whatman filter paper No. 1 and transfer the agro-inoculated leaf whorls into sterile petri dishes containing co-cultivation medium M3. Incubate the leaf whorls for 3 days at 22 °C in the dark (*see* **Note 15**).
11. Transfer the agro-inoculated leaf whorls on to petri dishes containing M3 solid medium. Incubate for 15 days at 25 °C in the dark (*see* **Note 16**).
12. After 15 days in the dark, transfer agro-inoculated leaf whorls (starts to form a small callus) to petri dishes containing MS media with selection agent M4 (1st selection) and incubate at 27 ± 2 °C in the dark.
13. Subculture every 12–15 days up to 6 rounds of selection onto fresh M4 medium and keep at 27 ± 2 °C in the dark. Remove the dying tissues, which turns brown/black in each selection process (*see* **Note 17**).
14. After six selection process, subculture the callus onto M5 medium for regeneration, and keep the petri dishes in a growth chamber at 26 ± 1 °C under a 16/8-h light/dark photoperiod at 150 m^{-2} s^{-1} LED light illumination.
15. Transfer the regenerated putative transgenic plants onto M6 sugarcane rooting medium. Keep it in a growth chamber at 26 ± 1 °C under a 16/8-h light/dark photoperiod at 150 m^{-2} s^{-1} LED light illumination until the desired rooting for acclimatization.
16. Transfer the regenerated plants to soil/plant substrate/vermiculite mixture (3:1:0.5 ratio), and keep them in a growth chamber at 26 ± 2 °C, under a 16/8-h light/dark photoperiod and 60–65% relative humidity.
17. Transfer the regenerated plants to a greenhouse and grow up to 5–6-month-old plants and proceed with molecular characterization. *See* Figure 2, which depicts the pictorial representation of different stages of sugarcane transformation

3.5 Screening of Transgenic Plants for Transgene Integration

After successful generation of putative transgenic sugarcane, confirm the integration of transgene into the genome. This screening procedure includes PCR analysis, DNA sequencing of PCR positive events, and quantitative RT-PCR.

3.5.1 PCR Analysis

1. Extract the genomic DNA from putative transgenic lines (leaves) using the standard CTAB method [15] and use 50–100 ng of DNA as a template for PCR amplification.
2. Confirm the presence of COMT RNAi cassette in the genomic DNA by PCR using gene-specific primer, which is specifically designed to amplify the fragment spanning the promoter region (forward primer) to the sense fragment of the COMT gene (as mentioned in Subheading 3.2, **step 15**) and confirm them by sequencing.

3.5.2 Quantitative Real-Time RT-PCR for Quantification of Target Gene Expression

1. Collect leaves from the third internode section of COMT-RNAi putative transgenic and non-transformed sugarcane (wild-type) plants.
2. Isolate the total RNA and synthesize the cDNA as described in Subheading 3.1. Use COMT specific primers to amplify the target region as mentioned above in Subheading 3.1. Perform all these steps on ice.
3. Run three duplicated qRT-PCR reactions for each pair of primers using cDNA as templates and non-template control separately, as follows:

(a)	Template:cDNA	10^4 to 10^5 copies or 1:10 diluted cDNA.
(b)	2× SYBR Green mix	10 μL
(c)	5 μM Forward primer	0.8 μL
(d)	5 μM Reverse primer	0.8 μL
(e)	Distilled H_2O upto	20 μL

4. Use following quantitative real-time PCR conditions: 95 °C for 3 min denaturation, 40 cycles at 95 °C for 10 s and 58 °C for 45 s, melt curve between 55 °C and 95 °C (*see* **Note 18**).
5. Verify the amplicon specificity by melt curve analysis and run the PCR product in 2.5% agarose gel electrophoresis.
6. Perform statistical analysis to determine the level of transgene expression in the transgenic plants as compared to wild type.

4 Notes

1. High Fidelity (HF) Taq polymerases have proofreading ($3' \rightarrow 5'$ exonuclease) activity that allows the enzyme to synthesize DNA strands from complex templates with high fidelity under an error rate 1.6×10^{-6}, which is lower than Taq polymerase. However, the use of HF enzymes results in blunt end product. Therefore, the PCR product amplified using HF

Taq requires A-tailing for use in a TA cloning vector. Moreover, Taq polymerase enzyme without 3′ → 5′ proofreading activity generates single adenine overhang at the 3′ end and results in sticky ends with an error rate of 1–20 × 10^5, but it can be used to ligate directly into the plasmid with thymine 3′ overhangs (TA cloning vectors). The Platinum® *Taq* DNA Polymerase, High Fidelity enzyme used here is provided by a mixture of Platinum® *Taq* DNA Polymerase and the proofreading (3′ → 5′ exonuclease activity) enzyme that can generate overhangs which is more convenient for cloning process after gel or PCR purification.

2. The pGEM®-T Easy Vector is linearized TA cloning vector with a single 3′-terminal thymidine at both ends. The T-overhangs at the insertion site greatly improve the efficiency of the vector and providing a compatible overhang for PCR products generated by non-proofreading thermostable polymerases. Thermostable DNA polymerases with proofreading activity generate blunt-end fragments and overhang can be added by A-tailing procedure as per the manufacturer's instructions before ligation.
3. ClustalW, Clustal Omega, MUSCLE, MSA, and T-Coffee are few freely available online tools to perform multiple sequence alignments.
4. Addition of overhanging nucleotides at the 5′ end of the primers is an important consideration when adding restriction sites to a primer. Most enzymes require 2–5 nonspecific extra bases at the 5′ end of the primer, which is a random sequence to ensure efficient DNA cleavage by the restriction enzyme. In siRNA molecule along with the target sequence, bearing 2-nt overhang at 3′ ends increases the binding affinity, siRNA's potency, and the specificity. However, it also prevents miRNA-like off-target effects and largely enhances the silencing efficiency.
5. The annealing temperature (typically varies between 50–65 °C) is related to the melting temperature (Tm) of the primers which can be determined for each primer pair used in PCR reaction. The *selection of annealing temperature* is the most critical step for optimizing the efficiency and specificity of the PCR reaction. The rule of thumb is to use 3–5 °C lower than the Tm of the primers used.
6. Ethidium bromide is a mutagenic agent and needs to be handled with care or other DNA staining agents like SYBR Green can be used as an alternative for staining DNA (that are claimed to be safer than ethidium bromide). Wear gloves at all times and be careful in handling and disposal of ethidium bromide contaminated gloves and pipette tips.

7. While doing a double digestion with two different enzymes simultaneously, check on the compatibility of buffer that works for both enzymes. The enzyme should always be added at last and should be kept at –20 °C until used.
8. Temperatures required for the heat inactivation of enzymes are variable, for some cases 80 °C heat inactivation is needed. Check on the inactivation temperature requirement for each enzyme.
9. When using –80 °C stored competent cells, thaw the cells until just ice crystals disappear in ice before adding plasmid DNA. It is also very important to keep the cells at 4 °C throughout the procedure. The cells, and any bottles or solutions that they come in contact with, must be prechilled to 4 °C.
10. Obtain *E. coli* competent cells and thaw them in ice. Add the ligation mixture and heat treat the cells at 42 °C for 90 s and then return immediately to the ice. After 2 min, add LB up to 1000 μL and place the cells at 37 °C for 1 h on a shaker with 250 rpm.
11. Autoclave the $CaCl_2$ solution and store at 4 °C (not more than 2 months old), before making the competent cells. All glassware should be detergent free. Presence of detergent reduces competency of cells.
12. Run a control transformation to determine transformation efficiency before using a batch of cells for experimental transformations. Freeze any extra aliquots in liquid nitrogen or dry ice-EtOH slurry and store at –80 °C for future use.
13. For isolation of binary plasmid from Agrobacterium, inoculate a single colony in YEP liquid media with 50 μg/mL kanamycin and 50 μg/mL rifampicin antibiotics, overnight incubate at 28 °C, with 150 rpm shaking, and then use Plasmid Miniprep kit to isolate the plasmid DNA with minor modifications: add 10 μL of freshly prepared lysozyme solution (100 mg/mL), into the cell suspension buffer followed by incubation for 5 min at room temperature.
14. Work inside the laminar airflow cabinet without lights. Light enhances the phenolic oxidation process, which reduces the efficiency of successful transformation.
15. Blot dry the excess bacterial culture, and maintain the temperature not exceeding 22 °C. Excess bacteria in culture medium contribute to the overgrowth of Agrobacterium, which kills the plant tissue by peroxidation.
16. Maintain the plant tissues in cefotaxime medium, which controls the overgrowth of *A. tumefaciens.*
17. Subculturing the plates every 12–15 days increases the efficiency of transformation under respective antibiotic selection

agents and reduces the oxidation of tissues. During the successive subcultures, non-transformed/escaped callus would be eliminated.

18. Optimize the PCR conditions with specific concentrations of primers as well as template by performing PCR reactions in a thermo-cycler before performing the quantitative real-time PCR test.

References

1. Kandel R, Yang X, Song J, Wang J (2018) Potentials, challenges, and genetic and genomic resources for sugarcane biomass improvement. Front Plant Sci 9:151. https://doi.org/10.3389/fpls.2018.00151
2. Ahorsu R, Medina F, Constantí M (2018) Significance and challenges of biomass as a suitable feedstock for bioenergy and biochemical production: a review. Energies 11:3366. https://doi.org/10.3390/en11123366
3. Jung JH, Fouad WM, Vermerris W, Gallo M, Altpeter F (2012) RNAi suppression of lignin biosynthesis in sugarcane reduces recalcitrance for biofuel production from lignocellulosic biomass. Plant Biotechnol J 10:1067–1076. https://doi.org/10.1111/j.1467-7652.2012.00734.x
4. Jung JH, Kannan B, Dermawan H, Moxley GW, Altpeter F (2016) Precision breeding for RNAi suppression of a major 4-coumarate: coenzyme A ligase gene improves cell wall saccharification from field grown sugarcane. Plant Mol Biol 92:505–517. https://doi.org/10.1007/s11103-016-0527-y
5. Jung JH, Altpeter F (2016) TALEN mediated targeted mutagenesis of the caffeic acid O-methyltransferase in highly polyploid sugarcane improves cell wall composition for production of bioethanol. Plant Mol Biol 92:131–142. https://doi.org/10.1007/s11103-016-0499-y
6. Li Z, Rana TM (2012) Molecular mechanisms of RNA-triggered gene silencing machineries. Acc Chem Res 45:1122–1131. https://doi.org/10.1021/ar200253u
7. Kamthan A, Chaudhuri A, Kamthan M, Datta A (2015) Small RNAs in plants: recent development and application for crop improvement. Front Plant Sci 6:208. https://doi.org/10.3389/fpls.2015.00208
8. Jung JH, Vermerris W, Gallo M, Fedenko JR, Erickson JE, Altpeter F (2013) RNA interference suppression of lignin biosynthesis increases fermentable sugar yields for biofuel production from field-grown sugarcane. Plant Biotechnol J 11:709–716. https://doi.org/10.1111/pbi.12061
9. Bewg WP, Poovaiah C, Lan W, Ralph J, Coleman HD (2016) RNAi downregulation of three key lignin genes in sugarcane improves glucose release without reduction in sugar production. Biotechnol Biofuels 9:270. https://doi.org/10.1186/s13068-016-0683-y
10. de Souza WR, Pacheco TF, Duarte KE, Sampaio BL, de Oliveira Molinari PA, Martins PK et al (2019) Silencing of a BAHD acyltransferase in sugarcane increases biomass digestibility. Biotechnol Biofuels 12:111. https://doi.org/10.1186/s13068-019-1450-7
11. White PR (1943) Handbook of plant tissue culture. The Ronald Press, New York
12. Preuss S, Pikaard CS (2003) Targeted gene silencing in plants using RNA interference. In: David E (ed) RNA interference (RNAi): nuts & bolts of siRNA technology. Sigma-Aldrich, Saint Louis, USA, pp 23–36
13. March JC, Bentley WE (2007) Methods for gene silencing with RNAi. In: Murhammer DW (ed) Baculovirus and insect cell expression protocols. Humana Press, New York, pp 427–433
14. Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4326. https://doi.org/10.1093/nar/8.19.4321
15. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York



Chapter 11

Assessment of Molecular Diversity in Biofuel Crops

Sriram Parameswaran, Nalini Eswaran, and T. Sudhakar Johnson

Abstract

Sustainable biofuel sources require the new sources of biofuel crops that can be developed into scalable plantation to meet the growing energy demands. Diverse supply sources of bioenergy plantations (edible, nonedible, and perennial grasses) will enable de-risking impact on geography and climate change that humans are likely to face in future. Use of phenotypic descriptors alone does not provide a deep insight into plantation population dynamics and molecular diversity of a biofuel crop. We provide protocols and methods to rapidly assess population parameters for emerging biofuel crops using genomics. This article has an application focus on next-generation sequencing to assess biofuel crop diversity. Use of these methods can accelerate germplasm assessment to accelerate population development and creation of sustainable biofuel plantations.

Key words Biofuel crop, Next-generation sequencing, Molecular diversity, Population diversity, Climate change impact, Soil microbiome

1 Introduction

Demand for sources of bio-energy plantations and creation of sustainable green energy sources that can replace carbon fossil fuel sources are central consideration in today's applied industrial biofuel research. In the last three decades several oil crops have been assessed for their suitability to develop sustainable biodiesel feedstock and plant-based fermentable bioethanol-based feedstock requires the establishment of biofuel crop plantations [1–3]. Most assessment to date has focused on phenotypic characteristics and compatibility of oil or fermentable sugar source to meet industrial conversion processes [2–4]. Besides the edible feedstock such as such as sunflower, rapeseed, canola (oil seeds), and sugarcane, corn (lignocellulosic fermentable sugars), emerging biofuel crops such as Castor, *Jatropha*, and *Pongamia* (nonedible oil seeds) have been identified as sources for plant-based biofuels [2, 4–6]. Biofuel crops with new plant have propagation challenges and pilot plantation is based on clonal tissue culture expansions or plantations developed from a narrow population base [7–10].

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To meet a sustainable long-term plantation, depending on tree species or shrub biofuel plantations ensures a diverse population base. Planning and evaluation of plantation crop population diversity is key to mitigate environmental and biotic stress imposed by changing environment yet sustaining the plantation [4, 10–12]. Conventional selection approaches rely on key phenotypic traits such as seed numbers, oil yield, plant architecture, and so on. Phenotype-based selection criteria are valuable in defining the economic yield and traits essential for harvesting [13–15]. A cross-breeding population diversity can sustain over several decades [13, 16].

Genomic tools especially next-generation sequencing (NGS) provide a rapid and direct method to assess molecular diversity [17–19]. We provide an NGS-based framework to assess the diversity of less characterized emerging biofuel crops (Fig. 1). Use of these tools can be a powerful complement to classical phenotypic selection approaches to create a sustainable biofuel crop plantation. Here, in this chapter we provide broad application tools (protocols) to carry out NGS sequencing and soil microbiota sequencing. The reason for soil microbiota sequencing is to understand soil microbe population diversity and optimize fertilizer use.

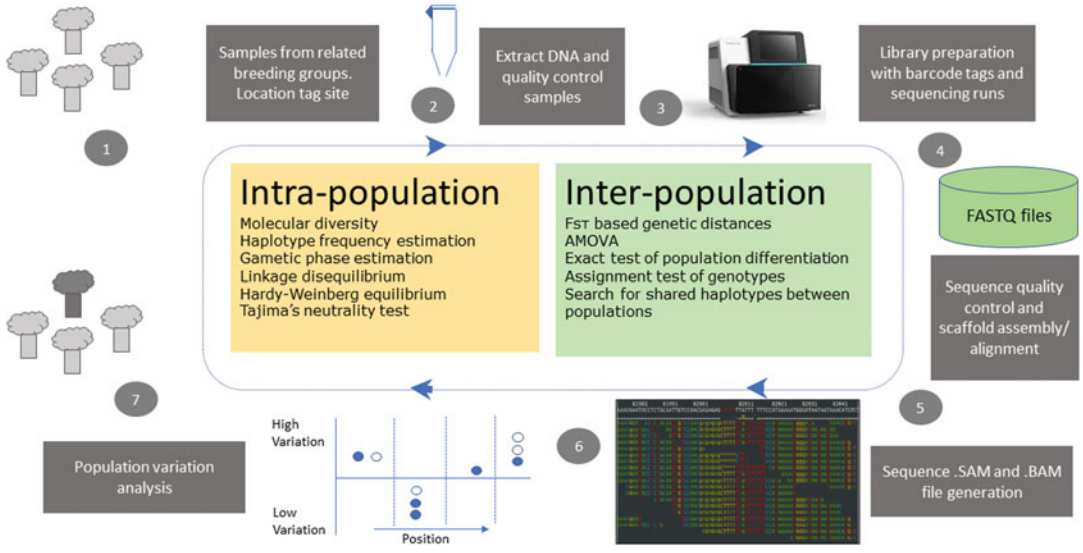


Fig. 1 Schematic of the molecular variation analysis workflow for a biofuel crop. The plants can either be from one location or across locations. The assessment of molecular variation though NGS enables rapid and direct assessment of breeding diversity and population parameters to improve the breeding selection and manage a crop diversity for a sustainable biofuel plantation

2 Concept and Theory

Next-generation sequencing has provided rapid and cost-effective methods to enable discovery of sequence variations [20, 21]. Sequencing with next-generation technologies is based on conversion of the extracted DNA into an amplicon library. Often during the conversion to this step, unique synthetic sequence tags called barcodes or indexes are added to the libraries. Sequencing in two current dominant NGS methods avoids cloning and plasmid recovery steps required in the Sanger dye terminator chemistry, dramatically reducing time and cost of data acquisition. Illumina-based methods cluster generation on flow cells that are sequenced using the reversible dye terminator technology. In the ion sequencing chemistry, the amplicons are amplified on beads called ISP (Ion spherical particles) following enrichment by emulsion PCR. Sequencing signal are measured using pH shifts on ISFET (Ion specific Field Effect Transistor) chip, on released proton, during base incorporation on template, loaded on the beads [20–22].

Tree plantation crops typically are highly cross-pollinated that display high heterozygosity and long generation time [14–16, 18]. Few biofuel tree crops genomes have recently been sequenced. However, it still lacks the ready availability of breeding tools. Genomics protocols can be applied to directly read the DNA or transcript from these crops, to characterize trait specific molecular diversity [17–19]. Broadly molecular diversity of a biofuel crop population can be assessed using DNA-based methods, or RNA-sequencing of a tissue-specific transcriptome [17, 19]. Investigation of changes in the soil microbiota using NGS technologies using the 18S rDNA and 16SrDNA sequence to assess the metagenome can be applied to optimize fertilizer use and monitor microbial diversity [23, 24].

3 Methods

1. DNA-based method is more suitable, with easier handling and storage of genomic DNA. DNA-based method can be analytically challenging when the genome of the crop is large, repetitive, or the crop displays extensive ploidy. We present two recipes to assess molecular diversity with genomic DNA samples.
   (a) Amplicon based (*see* more details of general target capture strategies [25]).
   (b) Ecological analysis of soil microbiome.

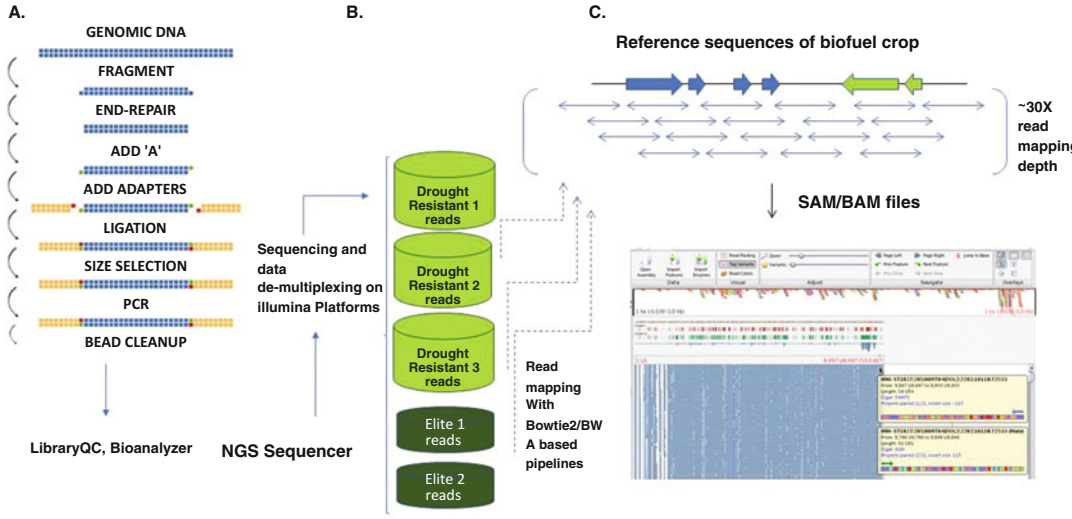


Fig. 2 Overview of the amplicon-based NGS variant analysis workflow. (**a**) DNA library preparation steps for amplicon sequencing. (**b**, **c**) Schematic of the workflow for read alignment and variant calling post mapping with short read alignment program BWA or Bowtie2

2. RNA extraction is technically more demanding compared to genomic DNA extraction and requires specialize care to handle, to avoid degradation. RNA-based assessment of molecular diversity offers advantages in scoring variation in expressed transcripts and often offers the ability to investigate coding variation and identify allelic variants of expressed traits.

A high-level workflow of key steps and experimental consideration to apply NGS-based workflows for crop population diversity and soil microbial assessment in the context of biofuel has been outlined (Fig. 2).

4 Materials

Prepare all solution with molecular biology grade nuclease free water (Cat No. AM9932, Thermo Fisher Scientific). Ensure the working area is cleaned with hypochlorite (0.5% v/v, Sigma) or RNA-Zap (Cat No. AM9780 Thermo Fisher Scientific). Use nucleic acid free nitrile gloves (Cat No. Z423254 Sigma-Aldrich) and barrier tips (Cat No. TF-200-R-S Axygen) to prevent library cross contamination. Availability and access to a standard molecular biology facility, NGS sequencing core and availability of instruments to assess nucleic acid quality (NanoDrop™ 2000/2000c, Qubit™ 4 Fluorometer ThermoFisher Scientific; 2200 TapeStation/2100 Bioanalyzer Agilent) is core requirement. The data analysis workflow can be performed in a Linux workstation with

at least 256GB RAM, 2Tb Disk space. The software for analysis is open source code or free downloads from respective instrument manufacturers.

5 Methods

1. **Sample preparation, nucleic acid extraction, and quality assessment**
   (a) For DNA or RNA from tissue samples, 500 mg to 1 g fresh tissue (preferable from growing tissue such as young leaves or developing fruits) should be snap frozen in liquid nitrogen. Wrap the tissue in fresh aluminum foil or plastic vials. Ensure the vials have a bleed hole or puncture to prevent explosion due to pressure build up. Transfer collected tissue from field transfer to ultradeep freezer (−80 °C) without thawing cycle. If access to liquid nitrogen or transport is limited, samples may be collected in *RNALater* (Cat No. AM7020, RNAlater™ Stabilization Solution, Thermo Fisher Scientific, USA) (*see* **Note 1**).
   (b) DNA extraction should be done by pulverizing the frozen tissue in liquid nitrogen with a bead mill (Cat No.85300TissueLyser II, Bead mill, Qiagen GmBH). CTAB-based DNA extraction of plant tissue and purification of genomic DNA using silica milk/silica resin columns is recommended. Commercial plant extraction kits—Sigma. Qiagen are suitable for most extractions. *See* detail for specific instruction in Cat No. 69104 DNeasy Plant Mini Kit, Qiagen GmBH; Cat No. G2N10-1KT GenElute™ Plant Genomic DNA Miniprep Kit, Sigma-Aldrich Co. LLC (*see* **Note 2**). For recalcitrant sample refer to DNA extraction modifications [26].
   (c) Three to 5 g fresh soil can be collected in 50 mL vials (Cat No. 339652ThermoFisher Scientific, USA). Store the soil sample in −80 °C till further extraction. DNA can be extracted from soil with DNeasyPowerSoil Kit (Cat No. 12888, Qiagen GmBH). Extracted DNA can be stored at −20 °C (*see* **Note 3**). The quantity and quality of extracted nucleic acid can be assessed using agarose gel electrophoresis and/or Qubit fluorometer (ThermoFisher Scientific, USA).
   (d) RNA extraction with commercial GITC or modified Hot Borate method is recommended (*see* **Note 4**). RNA extraction can be done with commercial kits such (Cat No. 74904 RNeasy Plant Mini Kit, Qiagen, GmBH) (*see* **Note 4**). DNAaseI treatment of RNA is recommended.

Store extracted RNA at −80 °C. Quantity of DNA and RNA is assessed using benchtop fluorometer Qubit 4 (*see* **Note 5**). Integrity of DNA is assessed by gel electrophoresis on 2% agarose gel. RNA integrity can be measured using Tapestation/BioAnalyzer (Agilent, USA) (*see* **Note 5**).

2. **NGS library preparation and sequence data generation for molecular diversity assessment**

   (a) **DNA-based methods**. *See* Fig. 2

   - Targeted amplicon approach [24] (*see* **Note 6**). PCR Amplicons for multiple targeted loci are designed to target genomic regions. PCR amplicon are designed using Primer 3 [28–30] (*see* **Note 7**). Amplicon from each locus are assessed for quality and quantity using agarose gel electrophoresis.
   - Equimolar ratios of PCR amplicon are pooled. Molar value of DNA can be determined using Qubit 4 (ThermoFisher Scientific, USA). Two hundred ng of pooled amplicon from each plant sample is sheared to 300–400 bp (S220 Focused-ultrasonicators, Covaris USA) OR enzymatically fragmented and converted into sequencing libraries (*see* detailed protocol in Cat No. A43602024 Collibri™ PCR-free ES DNA Library Prep Kit, ThermoFisher Scientific, USA) (*see* **Note 8**).
   - For microbiome assessment dilute the sample preparation of the soil nucleic acid to 10 ng (*see* **Note 3**). Amplify the 16S rDNA with the V3-V4 primers and 18S rDNA ITS sequence. For details in primer and variation in amplicon for microbiome analysis *see* **Note 9**. Refer [31–33] for selection of amplicon to assess the diversity of prokaryotic or eukaryotic microbiota.
   - The amplicon for each sample are analyzed using E-Gel (ThermoFisher Scientific), quantified with Qubit. Equimolar ratio of libraries is pooled and stored at −20 °C, until loaded to the sequencing flow cells. For bacterial analysis V3–V4 amplicon are read to 100–150 K reads OR 30,000 to 40,000 reads on 600 cycle chemistry (Cat No. MS-102-3003, Illumina MiSeq V3 600 cycle kit, IIlumina, USA) (*see* **Note 9**).

   (b) **RNA transcript library**

   - Take 200 ng to 1 μg of total RNA from target tissue (*see* **Note 10**). Poly A transcripts are captured and converted to directional RNA libraries (Cat No. A38994024, *see* details in Collibri™ Stranded

RNA Library Prep Kit for Illumina™ Systems, ThermoFisher Scientific, USA). Each sample library has unique barcode index.

- Sequence library quality assessment and evaluation of library concentration (*see* **Note 10**). Prior to sequencing, NGS libraries are quantified on Qubit 4, Size distribution of library fragments analyzed on Agilent 2200 Tape-station. Library size range for amplicon and RNA-seq should be ~450–550 bp, while the metagenome libraries have a range ~550–700 bp. NGS Libraries are quantified using qPCR (Cat No. A38524500, Collibri™ Library Quantification Kit, ThermoFisher Scientific, USA). Equimolar ratio of libraries is pooled and stored at −20 °C (*see* **Note 11**). For transcript analysis 10–25 million reads per sample are adequate for analysis of expression and molecular diversity of the population.

For sequence data acquisition multiplex pools of libraries are loaded and run as per the version and sequencer type and selected chemistry (*see* **Note 6**).

3. **Data analysis and inference of molecular diversity**

(a) Following sequencing, the per cycle base call files, index file, and run information logs are stored either in local server or transferred to Network Storage (*see* **Note 12**). Demultiplexing of sequence is performed using BCL2FastQ (*see* **Note 12**). Raw read from demultiplexing are assessed for sequence counts per index.

(b) Quality assessment and read filtering and clipping of sequencing and library adapter is performed [34]. The sequence quality and quantity of R1 and R2 files are assessed pre and post clipping and trimming using FastQC [34]. Data with sequence pHRED quality >Q30 is used in the data analysis (*see* **Note 12**).

(c) **Amplicon-targeted sequencing**

- The target loci reference sequence files for the alignment of reads are set up. The variant calling using GATK is detailed in [35]. Short reads are aligned to the reference sequence (*see* **Note 13**), and the single-nucleotide variation (SNP), short-insertion and deletion (InDels) are parsed for downstream analysis of molecular variation (Figs. 3 and 4).

(d) **Metagenome analysis**

- Processed read post demultiplexing are joined using Pair-end joining with PandaSeq [36]. The quality of the reads is assessed and sequences with >80% pHRED

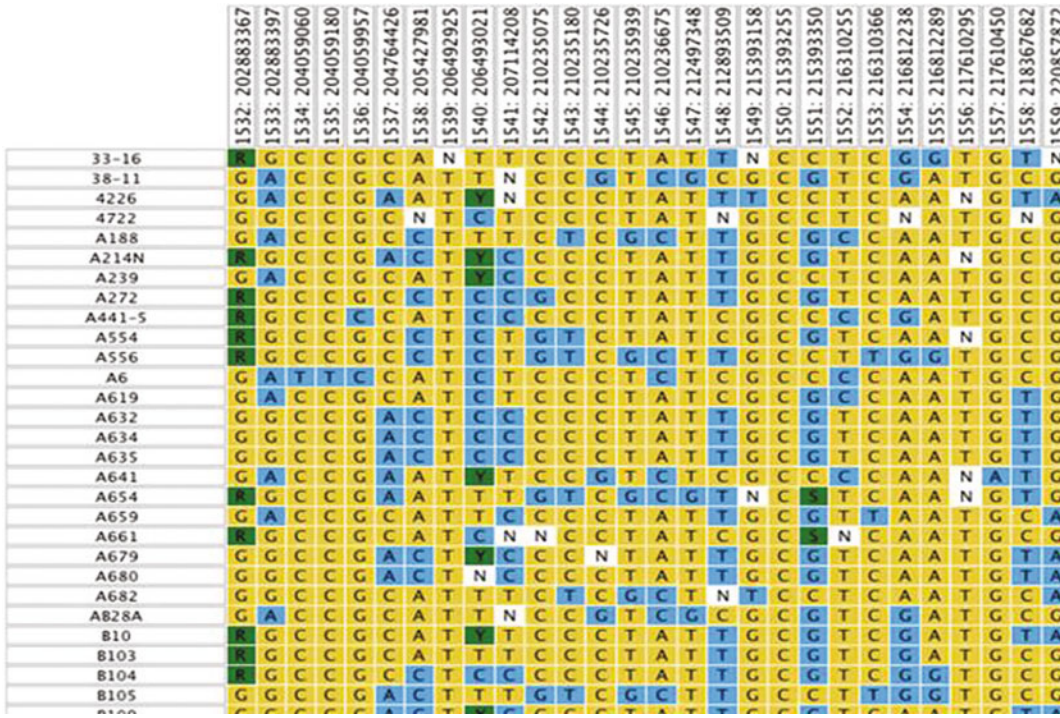


Fig. 3 Illustrative representation of a sample-wise variant position file. This file has been processed from NGS variant analysis workflow, and filtered for altered variants. The output of this data matrix can be load for molecular population analysis

Q > 30 are considered in the analysis. The metagenome analysis is performed by matching to RDB using QIIMEpipeline [37]. The analysis and visualization of data is supported in R using ggplot2 [38].

(e) **RNA-based method**

- Sample-specific reads are processed with the Kallistopipeline [39]. Expression and SNP allelic changes can be used for eQTL analysis to measure variance on expression of allelic forms for selected pathways critical to biofuel crop productivity or response to variations [40, 41].

(f) **Down-stream analysis of variance information**

Analysis of various molecular diversity parameters can be performed using Arlequin [42], Structure [43], and Phylip (*see* **Note 14**) (Fig. 4).

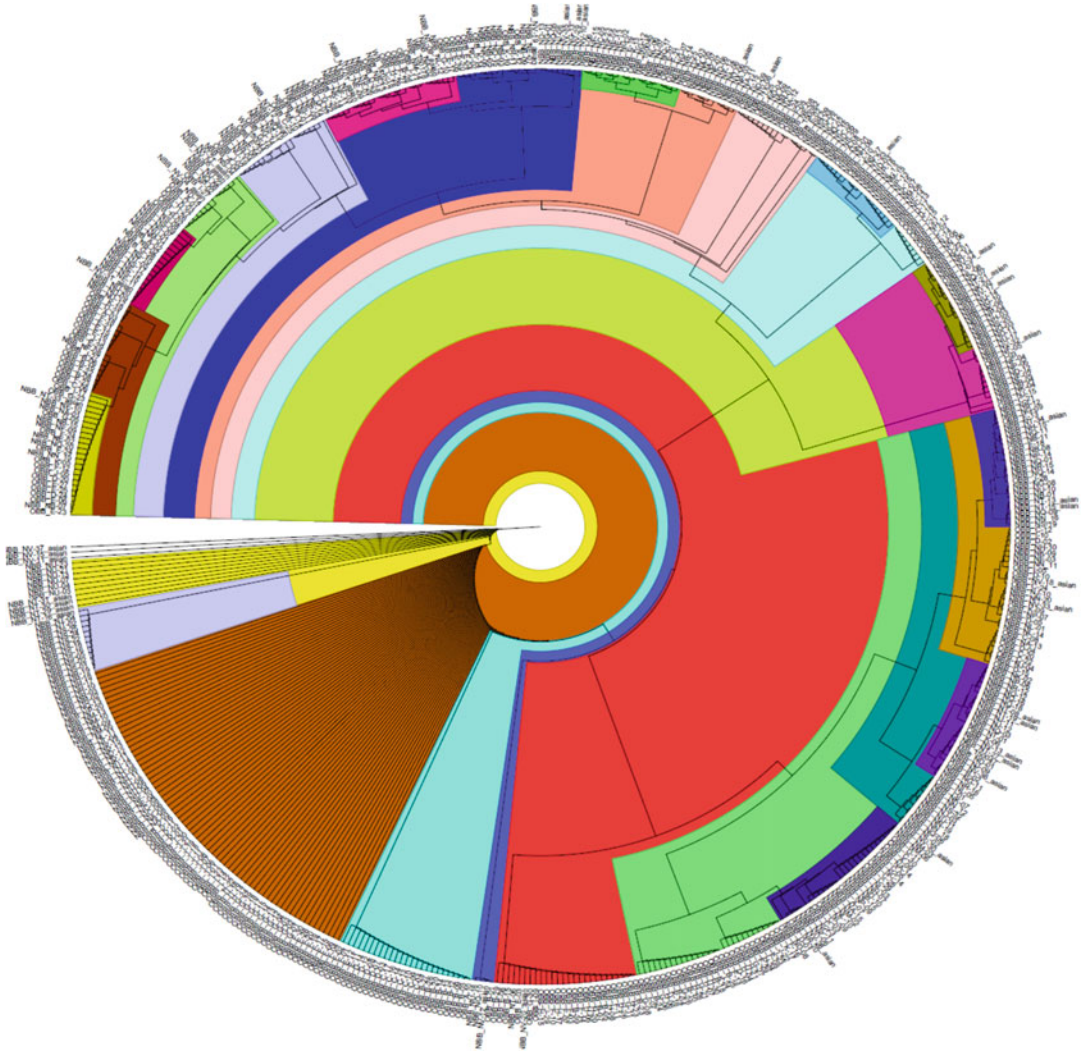


Fig. 4 Illustrative representation of a sample-wise population structure analysis of population variant. Similar color represents related population structure

6 Notes

1. Sample for RNA*later* should be cut into small slivers on a clean tile, pre-wiped with RNA-Zap/0.5%v/hypochlorite solution and then fully immersed in the RNA*later* reagent. Waxy tissue and woody tissue may not preserve well in reagent.
2. Certain extracts of plant DNA contain polyphenolics.
3. These can interfere with ligation and PCR reaction needed for library conversion. Polyvinylpyrrolidone/and or additional wash with ice-cold 70% ethanol and assessing nuclease digestion on an aliquot of nucleic acid should be considered to ensure compatibility with downstream steps.

4. Humic acid found in composting soil is an inhibitor of polymerase chain reaction. To assess the suitability of the DNA preparation the sample can be tested for nuclease sensitivity to restriction endonuclease or diluted 50–100-fold to ~20 ng and assessed for PCR using 16S primers.
5. Lithium chloride extraction methods of RNA are NOT recommended. Presence of lithium chloride in RNA preparation is not compatible with cDNA synthesis enzymes and may lead to inhibition or reduced cDNA conversion efficiency. Ideal tissue for RNA extraction is developing fruits, for oil seed-based biofuel crops. *See* the protocol from Wan and Wilkins [27] and Healey et al. [26] for extraction from recalcitrant tissues.
6. Spectrophotometric methods for DNA and RNA assessment lead to errors and final assessment of nucleic acid should be based on fluorometric measures. RNA extracted from chlorophyll-rich tissue such as leaves yield four peaks on bioanalyzer. RIN (RNA integrity) assessment will require manual scoring prior to library conversion.
7. The summary of recipe is for the Illumina 150 base paired-end sequencing using dual indexes on a benchtop sequencing platform such as Illumina NextSeq550 or Illumina MiSeq. The overall sequencing strategy may be adapted to other sequencing platform by modifying library kit and adapting sequence analysis workflows to a specific platform.
8. PCR condition needs to be optimized, *see* more details [28, 29]. PCR amplification-based method using targeted sequencing offers an advantage to work with genomic DNA 10–30 ng but requires prior sequence information, *see* more details in [25].
9. *See* index selection details for the Illumina Nextera Dual index adapters (https://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/miseq/indexed-sequencing-overview-guide-15057455-04.pdf; https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/experiment-design/index-adapters-pooling-guide-1000000041074-05.pdf). Plan index, the combination to ensure the i5 and i7 indexes are from unique series. The system of indexes supports combinatorial indexing for four distinct sample series.
10. Primer for V3–V4 and 18S fungal ITS regions,V3–V4 primers compatible with Illumina Nextera Dual indexes are Forward Primer 5′- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGAAGTAAAAGTCGTAACAAGG-3′; Reverse Primer

5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGA CAG GCTGCGTTCTTCATCGATGC-3′. *See* 16S metagenomics sequencing library preparation. Illumina Technical note number 15044223. (https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16smetagenomic-library-prep-guide-15044223-b.pdf). For details of ITS sequences refer to [32].

11. Low RNA integrity samples, <7.0 may not provide reproducible data. Recent protocols supported by FFPE compatible kit enable greater reproducibility. RIN for samples should be matched to ensure reproducible data.
12. NGS library quality assessment will need review for each type of sequencer and flow-cell. Specifications presented are suitable for Illumina NextSeq550 High-output flow cells, run on a 300-cycle chemistry. While pooling of NGS libraries key considerations should be checked to avoid potential index clashes that render the sequencing data unusable for analysis. The pooling should factor in the molarity of the library. Larger fragments preferentially underperform in a sequencing run. To ensure adequate yield, it will be advisable to pool libraries of similar size range.
13. Per cycle base call file, index file and conversion to FastQ files can be accessed using instrument manufacturer software and protocols. *See* https://support.illumina.com/content/dam/illumina-support/documents/documentation/software_documentation/bcl2fastq/bcl2fastq_letterbooklet_15038058brpmi.pdf. For further information on the FastQ file look up details in the URL https://support.illumina.com/bulletins/2016/04/fastq-files-explained.html?langsel=/us/
14. Reference alignment pipeline for analysis of short read from NGS use several approaches. Key aligners are based on the Burrow-wheel transform—BWA-MEM, Bowtie-2. *See* the reference [34] for the current GATK pipeline for variant calling. For details in the set-up of read *see* SAM tools and information on GATK https://www.broadinstitute.org/partnerships/education/broade/best-practices-variant-calling-gatk-1
15. For setting up data analysis in Arlequin and STRUCTURE data processing and transformation of the variant call format will be needed. Re-coding the data matrix and handling will be required that can be performed with PERL scripts or spreadsheets. Please refer to version specific instruction manuals for these software.

References

1. Das D, Varanasi JL (2019) Fundamentals of biofuel production processes. CRC Press, Boca Raton
2. Pandey A (ed) (2009) Handbook of plant-based biofuels. CRC Press, Boca Raton
3. Pandey A, Larroche C, Gnansounou E et al (eds) (2019) Biofuels: alternative feedstocks and conversion processes for the production of liquid and gaseous biofuels. Academic Press, Cambridge
4. Solomon BD (2010) Biofuels and sustainability. Ann N Y Acad Sci 1185:119–134
5. Schubert C (2006) Can biofuels finally take center stage? Nat Biotech 24(7):777–784
6. Miladinović D, Vollmann J, Molinero-Ruiz L, Torres M (2019) Advances in oil crops research classical and new approaches to achieve sustainable productivity. Front Plant Sci 10:791. https://doi.org/10.3389/fpls.2019.00791
7. Madan NS, Arockiasamy S, Narasimham JV, Patil M et al (2019) Anther culture for the production of haploid and doubled haploids *in Jatropha curcas* L. and its hybrids. Plant Cell Tissue Org Cult 138(1):181–192
8. Rosado TB, Laviola BG, Faria DA, Pappas MR, Bhering LL, Quirino B, Grattapaglia B (2010) Molecular markers reveal limited genetic diversity in a large germplasm collection of the biofuel crop L. in Brazil. Crop Sci 50:2372–2382
9. Fu Q, Tao YB, Xu ZF (2019) Genetic transformation and Transgenics of *Jatropha curcas*, a biofuel plant. In: Mulpuri S, Carels N, Bahadur B (eds) Jatropha, challenges for a new energy crop. Springer, Singapore, pp 79–93
10. Di Tomaso JM, Barney JN, Fox AM (2007) Biofuel feedstocks: the risk of future invasions. *All U.S. Government Documents (Utah Regional Depository)*. Paper 79
11. Taheripoura F, Hertela TW, Ramankutty N (2019) Market-mediated responses confound policies to limit deforestation from oil palm expansion in Malaysia and Indonesia. PNAS 116(38):19193–19199
12. Rajora OP, Mosseler M (2001) Challenges and opportunities for conservation of forest genetic resources. Euphytica 118:197–212
13. Abdelgadir HA, Johnson SD, Staden J (2009) Pollinator effectiveness, breeding system, and tests for inbreeding depression in the biofuel seed crop, *Jatropha curcas.* J Horticultural Sci Biotech 84:319–324
14. Muranty H, Jorge V, Bastien C, Lepoittevin C et al (2014) Potential for marker-assisted selection for forest tree breeding: lessons from 20 years of MAS in crops. Tree Genet Genomes 10(6):1491–1510
15. Laviola BG, Rodrigues EV, Ribeiro LP, Silva LA, de Azevedo Peixoto L, Bhering LL (2019) Strategies in the genetic breeding of *Jatropha curcas* for biofuel production in Brazil. In: Mulpuri S, Carels N, Bahadur B (eds) Jatropha, challenges for a new energy crop. Springer, Singapore, pp 45–62
16. Ru S, Main D, Evans K, Peace C (2015) Current applications, challenges, and perspectives of marker-assisted seedling selection in Rosaceae tree fruit breeding. Tree Genet Genomes 11:8
17. Xia W, Luo T, Dou Y, Zhang W et al (2019) Identification and validation of candidate genes involved in fatty acid content in oil palm by genome-wide association analysis. Front Plant Sci 10:1263
18. Allwright MR, Taylor G (2015) Molecular breeding for improved second generation bioenergy crops. Trends Plant Sci 21(1):43–54
19. Vandepitte K, Valdés-Rodríquez OA, Sánchez-Sánchez O, De Kort H, Martinez-Herrera J et al (2019) High SNP diversity in the non-toxic indigenous *Jatropha curcas* germplasm widens the potential of this upcoming major biofuel crop species. Ann Bot 124 (4):645–652
20. Xu J (ed) (2014) Next-generation sequencing: current technologies and applications. Caister Academic Press, Norfolk
21. Mitra R, Gill R, Datta S, Datta S (2014) Statistical analyses of next generation sequencing data: an overview. In: Datta S, Nettleton D (eds) Statistical analysis of next generation sequencing data. Frontiers in probability and the statistical sciences. Springer, Cham, pp 1–24
22. Quail MA, Smith M, Coupland P et al (2012) A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. BMC Genomics 13:341
23. Toit A (2019) Core leaf taxa of biofuel crops. Nat Rev Microbiol 17:647. https://doi.org/10.1038/s41579-019-0277-3
24. Mielczarek M, Szyda JJ (2016) Appl Gene 57:71. https://doi.org/10.1007/s13353-015-0292-7
25. Mamanova L, Coffey AJ, Scott CE, Kozarewa I et al (2010) Target-enrichment strategies for next generation sequencing. Nat Methods 7 (2):111–118

26. Healey A, Furtado A, Cooper T et al (2014) Protocol: a simple method for extracting next-generation sequencing quality genomic DNA from recalcitrant plant species. Plant Methods 10:21. https://doi.org/10.1186/1746-4811-10-21
27. Wan CY, Wilkins TA (1994) A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (Gossypium hirsutum L.). Anal Biochem 223(1):7–12
28. MacDonald RJ, Swift GH, Przybyla AE, Chirgwin JM (1987) Isolation of RNA using guanidinium salts. Meth Enzym 152:219–226
29. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012) Primer3-new capabilities and interfaces. Nucleic Acids Res 40(15):115
30. Kumar A, Chordia N (2015) In silico PCR primer designing and validation. In: Basu C (ed) PCR primer design. Methods in molecular biology, vol 1275. Humana Press, New York, NY
31. Bragg L, Tyson GW (2014) Metagenomics using next-generation sequencing. Methods Mol Biol 1096:183–201. https://doi.org/10.1007/978-1-62703-712-9_15
32. De Filippis F, Laiola M, Blaiotta G, Ercolinia D (2017) Different amplicon targets for sequencing based studies of fungal diversity. Appl Environ Microbiol 83(17):00905-17
33. Paulsen IT, Holmes AJ (eds) (2014) Environmental microbiology: methods and protocols, Methods in molecular biology, vol 1096, 2nd edn. Humana Press, New York. https://doi.org/10.1007/987-1-62703-712-9
34. Patel RK, Jain M (2012) NGS QC toolkit: a toolkit for quality control of next generation sequencing data. PLoS One 7(2):e30619. https://doi.org/10.1371/journal.pone.0030619
35. Geraldine A, Van der Auwera Mauricio O, Christopher C et al (2013) From FastQ data to high-confidence variant calls: the genome analysis toolkit best practices pipeline. Curr Protoc Bioinform 11 (1110):11.10.1–11.10.33
36. Masella AP, Bartram AK, Truszkowski JM et al (2012) PANDAseq: paired-end assembler for illumina sequences. BMC Bioinformatics 13:31. https://doi.org/10.1186/1471-2105-13-31
37. Kuczynski J, Stombaugh J, Walters WA et al (2011) Using QIIME to analyze 16S rRNA gene sequences from Microbial Communities. Curr Protoc Bioinform 36:10.7.1–10.7.20
38. Wickham H (2016) ggplot2: elegant graphics for data analysis. Springer International Publishing, Cham
39. Bray N, Pimentel H, Melsted P et al (2016) Near-optimal probabilistic RNA-seq quantification. Nat Biotechnol 34:525–527. https://doi.org/10.1038/nbt.3519
40. Liu P, Wang CM, Li L et al (2011) Mapping QTLs for oil traits and eQTLs for oleosin genes in *Jatropha*. BMC Plant Biol 11:132. https://doi.org/10.1186/1471-2229-11-132
41. Ye J, Hong Y, Qu J, Wang C (2013) Improvement of *J. curcas* oil by genetic transformation. In: Bahadur B, Sujatha M, Carels N (eds) Jatropha, challenges for a new energy crop. Springer, New York, pp 547–562
42. Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour 10:564–567
43. Hubisz MA, Falush D, Stephens M, Pritchard JK (2009) Inferring weak population structure with the assistance of sample group information. Mol Ecol Resour 9:1322–1332



Chapter 12

Genetic Transformation of *Trichoderma* spp.

Feng Cai, Christian P. Kubicek, and Irina S. Druzhinina

Abstract

The production of biofuels from plant biomass is dependent on the availability of enzymes that can hydrolyze the plant cell wall polysaccharides to their monosaccharides. These enzyme mixtures are formed by microorganisms but their native compositions and properties are often not ideal for application. Genetic engineering of these microorganisms is therefore necessary, in which introduction of DNA is an essential precondition. The filamentous fungus *Trichoderma reesei*—the main producer of plant-cell-wall-degrading enzymes for biofuels and other industries—has been subjected to intensive genetic engineering toward this goal and has become one of the iconic examples of the successful genetic improvement of fungi. However, the genetic manipulation of other enzyme-producing *Trichoderma* species is frequently less efficient and, therefore, rarely managed. In this chapter, we therefore describe the two potent methods of *Trichoderma* transformation mediated by either (a) polyethylene glycol (PEG) or (b) *Agrobacterium*. The methods are optimized for *T. reesei* but can also be applied for such transformation-resilient species as *T. harzianum* and *T. guizhouense*, which are putative upcoming alternatives for *T. reesei* in this field. The protocols are simple, do not require extensive training or special equipment, and can be further adjusted for *T. reesei* mutants with particular properties.

Key words *Agrobacterium tumefaciens*, Cellulolytic fungus, Protoplasts, Plant cell wall degrading enzymes, *Rhizobium radiobacter*, RUT-C30, *Trichoderma guizhouense*, *Trichoderma harzianum*, *Trichoderma reesei*, Vector

1 Introduction

The filamentous ascomycete *Trichoderma reesei* (Hypocreales) is the main industrial producer of lignocellulose degrading enzymes (commonly termed "cellulases and hemicellulases") that are used for the hydrolysis of plant-derived polysaccharides to their monomers—a key step in the production of biofuels [1–3]. Methods for its genetic manipulation have been known for over 40 years, and many strategies for genetic improvement and the respective vectors used have been published since then [4–9]. The genome sequences of the parental strain *T. reesei* QM 6a for industrial production and a pedigree of its mutants with altered abilities to secrete cellulases and hemicellulases have been obtained, annotated [10–18], and

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mapped on its seven chromosomes [15, 19, 20]. This has opened the door to the efficient improvement of *T. reesei's* enzyme production by genetic engineering. More recently, several other common *Trichoderma* spp. such as *T. harzianum* and *T. guizhouense* [21, 22] have also been described as potent cellulase and hemicellulase producers. This and the superior plant-beneficial properties of the latter species, their efficiency as bieffectors in biofertilizers and biofungicides, and their common occurrence in a broad range of habitats led to the sequencing of their genomes [23, 24] and fuelled a series of functional genetic investigations [25–27].

The introduction of foreign DNA into fungal genomes (artificial horizontal gene transfer frequently termed "transformation") is the key step in their genetic manipulation. Several techniques have been applied to *Trichoderma* to serve this purpose (reviewed by Malmierca et al. [6]), including polyethylene glycol (PEG)-mediated transformation of protoplasts [28–30], biolistic transformation [31], *Agrobacterium*-mediated transformation [32], electroporation [33], and transformation by the use of shock waves [34][1].

In this chapter, we provide efficient and detailed protocols for the two most frequently used methods: PEG-mediated transformation of protoplasts (Fig. 1) and *Agrobacterium*-mediated transformation (*see* **Note 1**). Their advantages lie in the fact that they involve exclusively microbiological procedures that can be performed in any laboratory with basic equipment for aseptic work, do not bear the risk of damaging cells, and require only general molecular biological instrumentation. In our laboratory, PEG-mediated protoplast transformation is used as the primary method for the efficient production of transgenic *Trichoderma* spp. strains (*T. reesei*, *T. harzianum*, and *T. guizhouense*). The *Agrobacterium*-mediated transformation is recommended as an alternative protocol to the transformation of oligosporic strains or mutants with altered cell-wall biosynthesis (such as *T. reesei* RUT-C30 [35]), or with other metabolic abnormalities preventing the protoplasting of protoplast regeneration (*see* **Note 1**).

2 Materials

2.1 Laboratory Environment

The protocols described below can be performed in a standard microbiological and molecular biological laboratory equipped with the necessary instruments (not listed) for aseptic work, microbial preservation, and preparation of microbial cultivation media. All reagents and cultivation media should be prepared using sterile plastic disposables or chemically cleaned glassware. All solutions

[1] Only the pioneering publications are cited here.

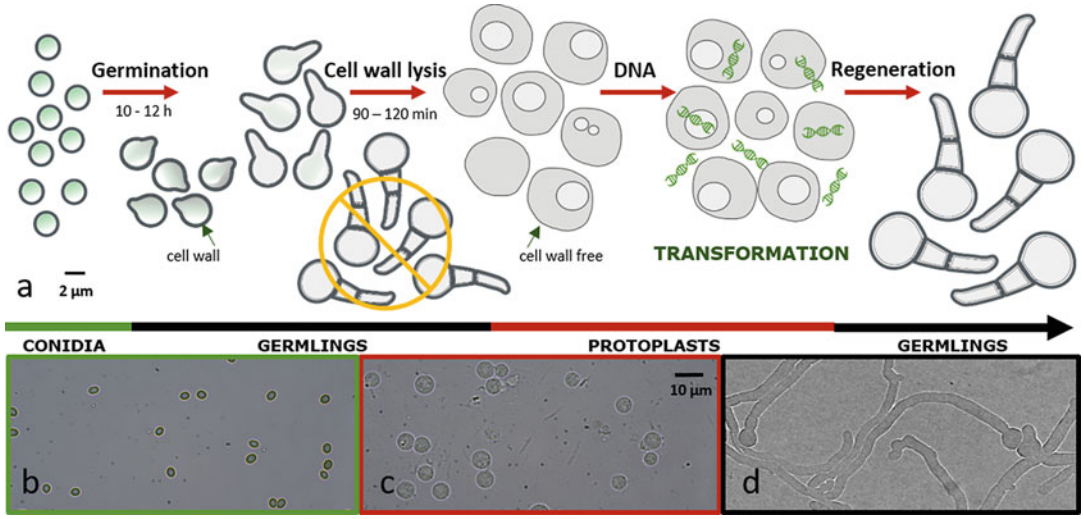


Fig. 1 Polyethylene glycol (PEG)-mediated protoplast transformation. (**a**) Workflow chart of how protoplast transformation is performed from germinating conidia to regenerated transformants. Overgrown germlings highlighted with a yellow Ø symbol will result into a low efficiency for protoplast preparation. (**b**) Conidia of *Trichoderma reesei*. (**c**) Protoplast cells prepared from the conidia shown in panel (**b**). (**d**) Germlings of transformants

should be made with reagents of analytical grade (ASC Reagents grade [36]) in ultrapure water treated by the Milli-Q® Ultrapure Water System.

Unless indicated otherwise, all reagents and solutions that do not contain microorganisms can be stored at room temperature.

2.2 Microorganisms and Biosafety

1. One of the *Trichoderma reesei* or other *Trichoderma* spp. (*T. harzianum*, and *T. guizhouense*) strains (*see* **Note 2**) that is supposed to be transformed.
2. (for Subheading 3.2) Transgenic *Agrobacterium tumefaciens* EHA105 (*see* **Note 3**).

Trichoderma spp. and *A. tumefaciens* are usually classified as microorganisms of a Biosafety Level 1 (BSL1) by the most internationally recognized collections of microorganisms (*see* **Note 4**).

2.3 Instruments

1. Two microbiological growth incubators, including at least one with an illumination option.
2. (for Subheading 3.1) Hemocytometer.
3. Spectrophotometer such as a microplate reader, with the possibility to measure O.D.$_{600}$ nm.
4. Temperature-controlled rotary shaker.
5. (for Subheading 3.1) Refrigerated centrifuge.
6. (for Subheading 3.1) Light microscope.

2.4 Disposables and Glassware

1. Centrifuge tubes (Falcon, Thermo Fisher Scientific, USA) of 1.5, 15, and 50 mL.
2. Nylon net filters (pore diameter of <50 μm, Millipore, USA) or custom-made 1.5 mL Eppendorf tube filter filled with glass wool (PYREX™ Glass Wool, Thermo Fisher Scientific, USA).
3. Round cellophane membranes, 8.5 cm in diameter (Zöllner-Wiethoff GmbH, Germany).
4. Microbiological needles for aseptic work.
5. Drigalski spatula.
6. Filter paper strips (Whatman No. 1 filter paper, Whatman, UK).
7. (for Subheading 3.2) Filter paper round sheets, 8.5 cm in diameter (Whatman No. 1 filter paper, Whatman, UK).
8. Petri dishes (9 cm in diameter) unless specified otherwise.
9. Wide mouthed Erlenmeyer shake flasks, 50 mL.
10. Ice box.

2.5 Chemicals and Solutions

1. Ultrapure water treated by the Milli-Q® Ultrapure Water System.
2. (for Subheading 3.1) Donor DNA for transformation, dissolved in ultrapure water (either a custom-made plasmid or a PCR product).
3. (for Subheading 3.1) Protoplast washing buffer (PWB): 0.1 M KH_2PO_4, pH 5.6, containing 1.2 M sorbitol.
4. (for Subheading 3.1) Protoplast lysing buffer (PLB): 0.1 M KH_2PO_4, pH 5.6, containing 1.2 M sorbitol and 7.5–10 mg mL^{-1} Glucanex® (Sigma L1412, "lysing enzymes from *Trichoderma harzianum*"; *see* **Note 5**).
5. (for Subheading 3.1) Protoplast buffer (PB): 0.01 M Tris–HCl, pH 7.5, containing 1 M sorbitol and 50 mM $CaCl_2$.
6. (for Subheading 3.1) PEG buffer (PEGB): 0.01 M Tris–HCl, pH 7.5, containing 50 mM $CaCl_2$ and 250 g L^{-1} polyethylene glycol (PEG) 6000 (Sigma, USA).
7. An antifungal compound (selection markers): hygromycin B, geneticin (G418), phleomycin, or the respective compounds can be used for the selection of auxotroph (*see* **Note 6**).
8. (for Subheading 3.2) Antibacterial compounds: kanamycin, rifampicin, and cefotaxime (*see* **Note 7**).

2.6 Microbial Cultivation Media

1. PDA: Petri dishes containing potato dextrose agar, 2% (w/v) of agar (PDA).
2. (for Subheading 3.1) PDA-cellophane: Petri dishes containing PDA covered with sterile cellophane disks.

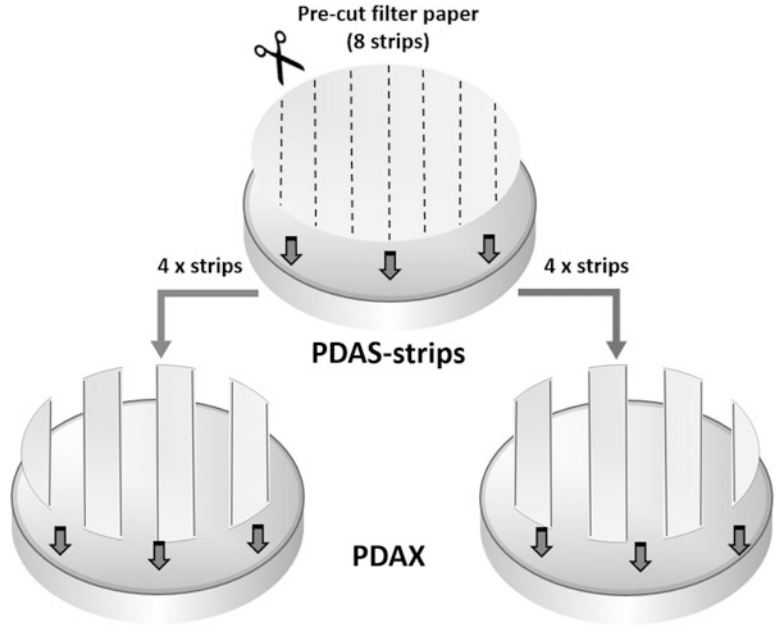


Fig. 2 Schematic diagram of **steps 22–25** in Subheading 3.1. The filter paper (diameter ca. 8.5 cm) is precut into eight pieces of strips with each ca. 1 cm wide before lie on the surface of a PDAS plate (**step 22**, Subheading 3.1). Four of the filter paper strips are transferred on to a 9 cm PDAX plate (containing the designated antifungal compound used for selection), thereby allowing a distance of 1 cm from each other (**step 25**, Subheading 3.1)

3. (for Subheading 3.1) PDAS: PDA containing 1 M sucrose.
4. (for Subheading 3.1) PDAS-strips: Petri dishes containing PDAS with strips of sterile filter paper located on the surface of agar as shown in Fig. 2.
5. PDAX: Petri dishes containing PDA supplemented with the designated antifungal compound.
6. (for Subheading 3.2) LB medium: 10 g L^{-1} trypton, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, pH 7.5, 50 μg mL^{-1} kanamycin, and 34 μg mL^{-1} rifampicin.
7. (for Subheading 3.2) YEPAA: 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 5 g L^{-1} trypton, 1 g L^{-1} yeast extract, 5 g L^{-1} beef extract, and 5 g L^{-1} sucrose, pH 7.2, supplemented with 2% (w/v) of agar, 50 μg mL^{-1} kanamycin, and 34 μg mL^{-1} rifampicin.
8. (for Subheading 3.2) Induction medium (IM): 10 mM KH_2PO_4, 10 mM K_2HPO_4, 10 mM NaCl, 0.6 g L^{-1} $MgSO_4 \cdot 7H_2O$, 10 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 0.5 g L^{-1} NH_4NO_3, 5 g L^{-1} glycerol, 2 g L^{-1} glucose, 1 mg L^{-1} $FeSO_4$, 40 mM 2-(*N*-Morpholino)ethansulfonic acid (MES, Sigma, USA), 200 μM acetosyringone (AS, Sigma, USA), and 0.5 mg L^{-1} each of $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $MnSO_4 \cdot H_2O$, $NaMoO_4 \cdot 7H_2O$, and H_3BO_3.

9. (for Subheading 3.2) Induction agar medium: IM supplemented with 2% (w/v) of agar.
10. (for Subheading 3.2) Selective medium (SM): PDA supplemented with 300 μg mL^{-1} cefotaxime [37] and the appropriate antifungal compound.

3 Methods

3.1 PEG-Mediated Protoplast Transformation

The protocol described below is optimized for *T. reesei* and other species that produce green conidiospores. Species-specific alterations are specified in **Notes** sections.

1. Inoculate strains on several (*see* **Note 2**) Petri dishes containing 20 mL PDA.
2. Incubate the cultures at 28 °C with alternate 12 h period of illumination until green conidia are formed (Fig. 3a) (*see* **Note 2**).

   Collect conidia from each Petri dish by flushing the agar surface with 10 mL sterilized ultrapure water. Oligosporic strains can require gentle scratching of the culture surface with a sterile Drigalski spatula.

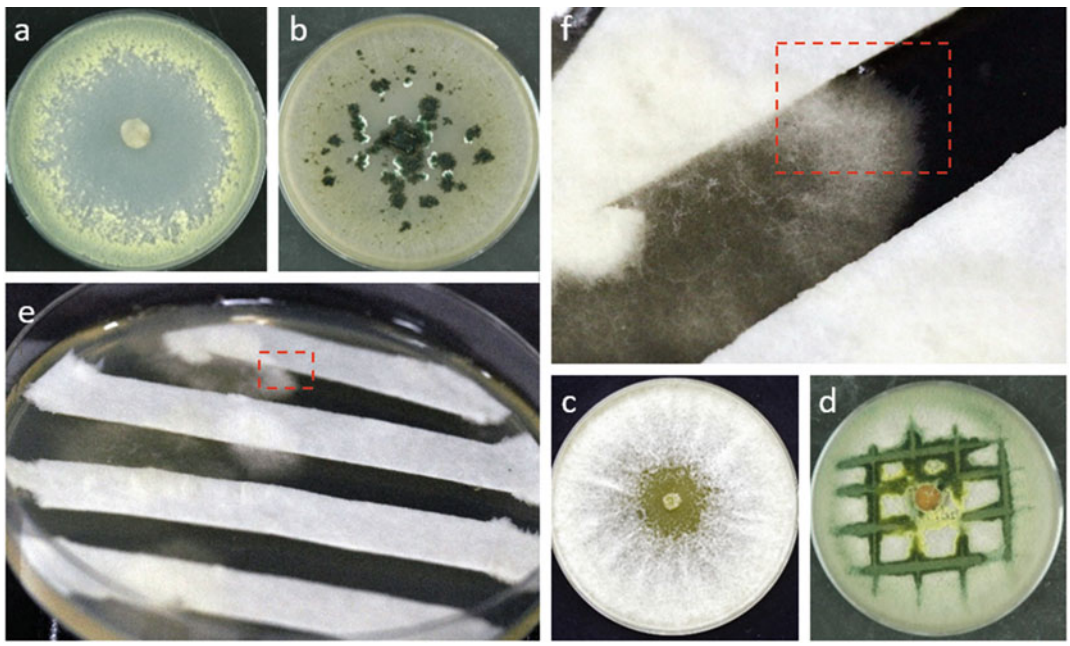


Fig. 3 Selected images of *Trichoderma* colonies grown in different conditions for different purposes. (**a**) A conidiating colony suitable for spore collection. (**b**) An example colony of a *Trichoderma* mutant with altered cell-wall biosynthesis. (**c**, **d**) Conidiation in an oligosporic strain (**c**) can be achieved by mechanical injury and illumination (**d**). (**e**, **f**) Regenerated *Trichoderma* colonies framed grown on a PDAX plate

3. Remove hyphal debris by filtering the conidial suspension through a sterilized nylon net filter or a custom-made tube filter filled with glass wool.
4. Use hemocytometer to adjust conidia concentration to about 10^8 conidia per mL.
5. Spread 200 μL of the suspension on a Petri dish with PDA-cellophane medium.
6. Repeat **step 5** four times to obtain five Petri dishes.
7. Incubate the Parafilm-sealed Petri dishes at 25 °C in darkness for 10–12 h (*see* **Note 7**).
8. Collect the germlings by washing the surface of the cellophane sheet with 4 mL of protoplast lysing buffer (PLB) and transfer the suspension to a 50 mL centrifuge tube.
9. Repeat **step 8** with the remaining Petri dishes and transfer the suspension to the same 50 mL centrifuge tube. Add PLB to a total volume of 20 mL.
10. Incubate the resulting germling suspensions in a rotary shaker at 90 rpm at 28 °C in darkness for 60 min (*see* **Note 7**).
11. Monitor protoplast formation every 30 min after incubation for 60 min using a light microscope (Fig. 1b, c) (*see* **Note 7**).
12. Collect protoplasts by centrifugation at 1200 × *g* and 4 °C for 10 min.
13. Carefully discard the supernatant and gently resuspend the protoplast pellet in 1 mL protoplast buffer (PB).
14. (optional) Use a hemocytometer to verify that the protoplast concentration is $>10^7$ per mL (*see* **Note 7**).
15. Gently pipet the suspension in 0.2 mL aliquots to 1.5 mL Eppendorf tubes for storing; if used on the same day, protoplasts can be kept on ice for up to 8 h. Alternatively, protoplasts can be stored at −80 °C for up to 6 months.
16. Use one of the protoplast stocks (in 1.5 mL Eppendorf tubes) from **step 15** (Subheading 3.1) to genetically transform protoplasts by adding ≥1 μg of vector DNA solution (*see* **Note 8**). Supplement the suspension with 50 μL of PEG buffer (PEGB) and mix gently by pipetting.
17. Incubate the mixture on ice for 20 min.
18. Add 2 mL of PEGB and gently mix.
19. Incubate the mixture at room temperature for 5 min.
20. Add 3 mL of PB and mix gently.
21. Incubate the mixture at 25 °C for 5 min.
22. Gently spread 1 mL of the protoplast suspension onto a Petri dish containing PDAS-strips (Fig. 2).

23. Repeat **step 22** four times to obtain five Petri dishes.
24. Incubate the protoplasts in Parafilm-sealed Petri dishes at 28 °C in darkness for 16 h.
25. Transfer the filter paper strips on other Petri dishes containing PDAX (*see* **Note 9**), thereby allowing distance of 1 cm between each two strips (shown in Fig. 2).
26. Incubate the cultures on Petri dishes at 28 °C in darkness for 24–48 h until *Trichoderma* colonies become visible to the naked eye (Fig. 3e, f).
27. Use the microbiological needle to transfer the <2 mm^2 fragment of the fungal growing colony to a 6 cm PDAX Petri dish and incubate for 48 h.
28. Repeat **step 27** twice. Thereafter, transformants can be considered to be mitotically stable and can be used for further confirmation.

3.2 *Agrobacterium*-Mediated Transformation

1. Prepare the culture of transgenic *A. tumefaciens* strain EHA105 (*see* **Note 3**) by streaking it onto a Petri dish containing 20 mL of YEPAA medium, and incubate it at 28 °C for 36 h.
2. Transfer (visually) a single bacterial colony into 10 mL of LB medium in a 50 mL Erlenmeyer flask.
3. Incubate at 28 °C and 200 rpm on a rotary shaker for 16 h.
4. Collect the bacteria by centrifugation at 7000 × *g* for 5 min at 25 °C.
5. Resuspend the bacterial pellet in a volume of induction medium (IM) to yield an optical density of $O.D._{600} = 0.15–0.3$, determined using a spectrophotometer.
6. Prepare a negative control (*see* **Note 10**).
7. Incubate the bacterial suspension in 50 mL Erlenmeyer flasks in a rotary shaker at 28 °C and 200 rpm for 6 h to obtain a final $O.D._{600} = 0.30–0.45$.
8. Concentrate the culture of *A. tumefaciens* to half volume.
9. Prepare a *Trichoderma* sp. conidial suspension as specified in **steps 1–4** (Subheading 3.1) (for non-conidiating cultures *see* **Note 2**).
10. Use a 1.5 mL Eppendorf tube to mix 200 μL of the *Trichoderma* sp. conidia suspension with 200 μL of the *A. tumefaciens* cell suspension from **step 8**.
11. Spread the suspension onto a Petri dish containing 20 mL of IM agar over a sterilized Whatman filter paper, precut into strips as shown in Fig. 2.

12. Incubate the Petri dishes at 25 °C in darkness for 48 h (*see* **Note 11**).
13. Transfer the filter paper strips onto a Petri dish with selective medium (SM) (*see* **Note 6**).
14. Incubate the Petri dishes at 28 °C in darkness for 48 h.
15. Transfer the filter paper strips onto another Petri dish containing SM as described in **step 25** (Subheading 3.1) and incubate the Petri dishes for 48 h or longer until the *Trichoderma* colonies become visible (Fig. 3e, f).
16. Test the mitotic stability of the transformants as described in **steps 27** and **28** (Subheading 3.1).

4 Notes

1. The detailed description of molecular biological principles exploited by the two transformation methods is beyond the scope of this chapter. Briefly, PEG-mediated transformation of fungal protoplasts—single cells from which the cell wall had been enzymatically removed—is based on the uptake of foreign DNA in the presence of Ca^{2+} (required for the opening of plasma membrane channels) and polyethylene glycol (PEG) (needed for the formation of molecular bridges between foreign DNA and recipient cells and increased membrane permeability) (for reviews, *see* [38, 39]). *Agrobacterium tumefaciens* (syn. *Rhizobium radiobacter*, Rhizobiales [40]) is a pathogenic Gram-negative Alpha-proteobacterium that infects plants or fungi by inserting DNA via a plasmid (the T-plasmid) into the nucleus of the host cell. In plants, it forces abnormal growth of tissues (galls) used by the bacterium [41]. Although frequently called "transformation", this process is more correctly described as "trans-conjugation". Transfer of the plasmid from the bacterium into the host cell is induced by plant metabolites that accumulate in wounded tissue, such as acetosyringone (AS). The use of this process for genetic transformation was pioneered in 1990 by plant molecular biologists for the introduction of foreign DNA into plant cells (reviewed by Gelvin [42]). To this end, they altered the T-plasmid to prevent gall formation and engineered it such that a DNA fragment could be placed between two direct repeats of 25 bp (the left and right borders of the transfer of T-DNA) and, thus, be transformed into the plant. In 1998, it was shown that *A. tumefaciens*-mediated "transformation" can also be used for DNA transfer into filamentous fungi [43, 44]; since then, it has become one of the most widely used fungal transformation systems (for a review, *see* [45, 46]).

2. Conidiating wild-type strains of any *Trichoderma* spp. (Fig. 3) are suitable for both protocols if sufficient genetic or genomic information is available. In the absence of conidia, in certain stress conditions, some strains can produce abundant hyaline (colorless) chlamydospores that may be suitable for protoplasting (further optimization required). Two or three Petri dishes with conidiating colonies are usually sufficient for both protocols (Fig. 3a). Subheading 3.1 is usually not efficient for strains that have reduced or abandoned conidiation or impaired cell wall biosynthesis (Fig. 3b). In such cases, Subheading 3.2 may become more suitable for a case-specific optimization.

   To obtain an efficient amount of conidia, cultivation time should be optimized for each strain, e.g., 7 days for *T. reesei* QM 6a and 5 days of incubation for *T. harzianum* CBS 226.95 and *T. guizhouense* NJAU 4742. Some strains of *Trichoderma* are highly photosensitive (*T. guizhouense* NJAU 4742 or *T. atroviride* IMI 206040 [47]). In this case, the alternate 12 h period illumination with white light (15 w) will speed up the formation of conidia. However, illumination has the minor effect of the conidiation of such strains as *T. reesei* QM 6a and *T. harzianum* CBS 226.95. Conidiation in the oligosporic mutants or strains that have impaired metabolism can be stimulated by mechanical injury, starvation, and/or illumination [48]. For this purpose, the fungus can be cultivated on a minimal medium, scratched by a cold sterile scalpel, and illuminated. Starvation can also be achieved by extended incubation time. Different strains will have individual responses to these stressors and their combinations (Fig. 3c, d).

3. This chapter does not provide a protocol describing the preparation of transgenic *Agrobacterium tumefaciens*. Refer to [49].

4. *T. reesei* and *A. tumefaciens* are usually classified as microorganisms of a Biosafety Level 1 (BSL1) by the American Type Culture Collection (ATCC) or the CBS-KNAW culture collection based on an assessment of the potential risk using the respective formal guidelines. It implies that such microorganisms are not known to cause diseases in immunocompetent adult humans. In particular, *T. reesei* QM 6a and its derived mutants have the FDA's GRAS (Generally Regarded as Safe) status and, therefore, these fungi are used in the food industry for the production of enzymes [50]. However, some strains of such species as *T. longibrachiatum* and *T. harzianum* sensu lato [51, 52] are assigned to a Biosafety Level 2 (BLS2), assuming potential moderate hazards to laboratorians and the environment. It should be considered that several *Trichoderma* spp. are referred to as infrequent but emerging pathogens of immunocompromised humans [53] and, therefore, should be handled following the appropriate standards of microbiological safety.

Fortunately, none of these species have been employed for the production of cellulolytic enzymes so far. For *A. tumefaciens*, Plant Biosafety Level 1 (BSL-1P) is recommended for all experiments with transgenic strains that have limited or no hazardous potential.

5. The optimal amount of lysing enzyme may require preliminary optimization for every individual *Trichoderma* spp. strain and especially for mutant strains with altered spore morphology. Optimal results can usually be obtained by manipulating the concentration of the lytic enzyme between 7.5 and 10 mg mL^{-1} and changing the incubation time in PLB between 90 and 120 min. For example, at 7.5 mg mL^{-1} of Glucanex, 90 min of lysing is an efficient set up for *T. reesei* QM 6a, whereas the protoplasts of *T. harzianum* CBS 226.95 and *T. guizhouense* NJAU 4742 can be obtained after 120 min of lysing. If protoplasting does not yield successful results or if the conidiation is impaired (Fig. 3b), the optimization of the *Agrobacterium*-mediated transformation protocol is suggested (*see* Subheading 3.2).
6. Antibiotic selection markers suitable for *Trichoderma* spp. are reviewed in Malmierca et al. [6]. Briefly, the *hph*, *neo*, *bar*, and *ble* cassettes assist the transformants with resistance to the fungicides hygromycin B [54], geneticin [55], phosphinothricin [4], and phleomycin [55], respectively. The *amdS* and *Hxk1* cassettes, respectively, assist the transformants with the ability to utilize acetamide [56] and mannitol [57] if the parent strain is not able to use these nitrogen/carbon sources. Additionally, 5-fluorotic acid can be applied to the parent strains (usually mutants) with *pyr4* gene deficiency. In this case, uridine should be supplemented in the selection medium to support the uridine prototrophy [4].
7. The incubation time allowing for the germination of conidia at this step is the key starting point for *Trichoderma* protoplast preparation. Usually, 10 h of incubation for *T. reesei* QM 6a and 12 h for *T. harzianum* CBS 226.95 and *T. guizhouense* NJAU 4742 are recommended. Ideally, a concentration of 10^7–10^8 cells per mL of protoplasts can be obtained following the above procedure. The morphology of protoplast cells (6–8 μm) had ca. 3- to 4-fold larger size than the un-lysed conidia (ca. 2–3 μm) and less (green) color. Additionally, the shape of the protoplasts is usually spherical, while the original conidia range from oval (in *T. reesei*) to globose depending on the species and strain. A typical image of protoplasts distinguished from the original conidia can be seen in Fig. 1c.
8. A DNA concentration of >100 ng μL^{-1} is recommended to reduce the volume of the DNA solution to <10 μL. In our experience, the use of linearized vectors will increase the

transformation efficiency. In the case of homologous recombination (e.g., for gene replacement/deletion), about 10% of the positive transformants can be subsequently confirmed as harboring the expected correct construction with the targeted gene removed. In the case of the random insertion of donor DNA to a *Trichoderma* genome, a ratio of ≥80% of positive mutants can be expected.

9. The use of a selection marker is based on resistance to this antibiotic that is strongly species-dependent, and no general prediction can be made (*see* **Note 6**). We routinely apply 100 μg mL^{-1} of hygromycin B or 100 μg mL^{-1} of geneticin for *T. reesei* QM6a, whereas we apply 200 μg mL^{-1} of hygromycin B (Thermo Fisher Scientific, USA) or 300 μg mL^{-1} of geneticin (Thermo Fisher Scientific, USA) for *T. harzianum* CBS 226.95 and *T. guizhouense* NJAU 4742.
10. To prepare a negative control, include an *A. tumefaciens* culture in which acetosyringone (AS), the virulence inducer, has not been added. This sample will serve as confirmation that the transformation of the fungal conidia is dependent on the T-DNA transfer.
11. The optimal temperature for growth of the *A. tumefaciens* strain EHA105 in transformation is 22–25 °C.

Acknowledgments

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References

1. Payne CM, Knott BC, Mayes HB, Hansson H, Himmel ME, Sandgren M, Stahlberg J, Beckham GT (2015) Fungal cellulases. Chem Rev 115(3):1308–1448. https://doi.org/10.1021/cr500351c
2. Gupta VK, Kubicek CP, Berrin JG, Wilson DW, Couturier M, Berlin A, Filho EXF, Ezeji T (2016) Fungal enzymes for bio-products from sustainable and waste biomass. Trends Biochem Sci 41(7):633–645. https://doi.org/10.1016/j.tibs.2016.04.006
3. Kubicek CP, Kubicek EM (2016) Enzymatic deconstruction of plant biomass by fungal enzymes. Curr Opin Chem Biol 35:51–57. https://doi.org/10.1016/j.cbpa.2016.08.028
4. Steiger MG, Vitikainen M, Uskonen P, Brunner K, Adam G, Pakula T, Penttila M, Saloheimo M, Mach RL, Mach-Aigner AR (2011) Transformation system for *Hypocrea jecorina* (*Trichoderma reesei*) that favors homologous integration and employs reusable bidirectionally selectable markers. Appl Environ Microbiol 77(1):114–121. https://doi.org/10.1128/AEM.02100-10

5. Peterson R, Nevalainen H (2012) *Trichoderma reesei* RUT-C30—thirty years of strain improvement. Microbiology 158 (Pt 1):58–68. https://doi.org/10.1099/mic.0.054031-0
6. Malmierca MG, Cardoza RE, Gutiérrez S (2015) *Trichoderma* transformation methods. In: van den Berg MA, Maruthachalam K (eds) Genetic transformation systems in fungi, vol 1. Springer International Publishing, Cham, pp 41–48. https://doi.org/10.1007/978-3-319-10142-2_3
7. Bischof RH, Ramoni J, Seiboth B (2016) Cellulases and beyond: the first 70 years of the enzyme producer *Trichoderma reesei*. Microb Cell Factories 15(1):106. https://doi.org/10.1186/s12934-016-0507-6
8. Gupta VK, Steindorff AS, de Paula RG, Silva-Rocha R, Mach-Aigner AR, Mach RL, Silva RN (2016) The post-genomic era of *Trichoderma reesei*: what's next? Trends Biotechnol 34(12):970–982. https://doi.org/10.1016/j.tibtech.2016.06.003
9. Druzhinina IS, Kubicek CP (2017) Genetic engineering of Trichoderma reesei cellulases and their production. Microb Biotechnol 10 (6):1485–1499. https://doi.org/10.1111/1751-7915.12726
10. Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, Chapman J, Chertkov O, Coutinho PM, Cullen D, Danchin EG, Grigoriev IV, Harris P, Jackson M, Kubicek CP, Han CS, Ho I, Larrondo LF, de Leon AL, Magnuson JK, Merino S, Misra M, Nelson B, Putnam N, Robbertse B, Salamov AA, Schmoll M, Terry A, Thayer N, Westerholm-Parvinen A, Schoch CL, Yao J, Barabote R, Nelson MA, Detter C, Bruce D, Kuske CR, Xie G, Richardson P, Rokhsar DS, Lucas SM, Rubin EM, Dunn-Coleman N, Ward M, Brettin TS (2008) Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). Nat Biotechnol 26 (5):553–560. https://doi.org/10.1038/nbt1403
11. Le Crom S, Schackwitz W, Pennacchio L, Magnuson JK, Culley DE, Collett JR, Martin J, Druzhinina IS, Mathis H, Monot F, Seiboth B, Cherry B, Rey M, Berka R, Kubicek CP, Baker SE, Margeot A (2009) Tracking the roots of cellulase hyperproduction by the fungus *Trichoderma reesei* using massively parallel DNA sequencing. Proc Natl Acad Sci U S A 106(38):16151–16156. https://doi.org/10.1073/pnas.0905848106
12. Vitikainen M, Arvas M, Pakula T, Oja M, Penttila M, Saloheimo M (2010) Array comparative genomic hybridization analysis of *Trichoderma reesei* strains with enhanced cellulase production properties. BMC Genomics 11:441. https://doi.org/10.1186/1471-2164-11-441
13. Lichius A, Bidard F, Buchholz F, Le Crom S, Martin J, Schackwitz W, Austerlitz T, Grigoriev IV, Baker SE, Margeot A, Seiboth B, Kubicek CP (2015) Genome sequencing of the *Trichoderma reesei* QM9136 mutant identifies a truncation of the transcriptional regulator XYR1 as the cause for its cellulase-negative phenotype. BMC Genomics 16:326. https://doi.org/10.1186/s12864-015-1526-0
14. Ivanova C, Ramoni J, Aouam T, Frischmann A, Seiboth B, Baker SE, Le Crom S, Lemoine S, Margeot A, Bidard F (2017) Genome sequencing and transcriptome analysis of *Trichoderma reesei* QM9978 strain reveals a distal chromosome translocation to be responsible for loss of *vib1* expression and loss of cellulase induction. Biotechnol Biofuels 10:209. https://doi.org/10.1186/s13068-017-0897-7
15. Li WC, Huang CH, Chen CL, Chuang YC, Tung SY, Wang TF (2017) *Trichoderma reesei* complete genome sequence, repeat-induced point mutation, and partitioning of CAZyme gene clusters. Biotechnol Biofuels 10:170. https://doi.org/10.1186/s13068-017-0825-x
16. Jourdier E, Baudry L, Poggi-Parodi D, Vicq Y, Koszul R, Margeot A, Marbouty M, Bidard F (2017) Proximity ligation scaffolding and comparison of two *Trichoderma reesei* strains genomes. Biotechnol Biofuels 10:151. https://doi.org/10.1186/s13068-017-0837-6
17. Zou Z, Zhao Y, Zhang T, Xu J, He A, Deng Y (2018) Efficient isolation and characterization of a cellulase hyperproducing mutant strain of *Trichoderma reesei*. J Microbiol Biotechnol 28 (9):1473–1481. https://doi.org/10.4014/jmb.1805.05009
18. Liu P, Lin A, Zhang G, Zhang J, Chen Y, Shen T, Zhao J, Wei D, Wang W (2019) Enhancement of cellulase production in *Trichoderma reesei* RUT-C30 by comparative genomic screening. Microb Cell Factories 18(1):81. https://doi.org/10.1186/s12934-019-1131-z
19. Marie-Nelly H, Marbouty M, Cournac A, Flot JF, Liti G, Parodi DP, Syan S, Guillen N, Margeot A, Zimmer C, Koszul R (2014) High-quality genome (re)assembly using chromosomal contact data. Nat Commun 5:5695. https://doi.org/10.1038/ncomms6695
20. Druzhinina IS, Kopchinskiy AG, Kubicek EM, Kubicek CP (2016) A complete annotation of the chromosomes of the cellulase producer

Trichoderma reesei provides insights in gene clusters, their expression and reveals genes required for fitness. Biotechnol Biofuels 9:75. https://doi.org/10.1186/s13068-016-0488-z

21. Ferreira Filho JA, Horta MAC, Beloti LL, Dos Santos CA, de Souza AP (2017) Carbohydrate-active enzymes in *Trichoderma harzianum*: a bioinformatic analysis bioprospecting for key enzymes for the biofuels industry. BMC Genomics 18(1):779. https://doi.org/10.1186/s12864-017-4181-9
22. Grujic M, Dojnov B, Potocnik I, Atanasova L, Duduk B, Srebotnik E, Druzhinina IS, Kubicek CP, Vujcic Z (2019) Superior cellulolytic activity of *Trichoderma guizhouense* on raw wheat straw. World J Microbiol Biotechnol 35 (12):194. https://doi.org/10.1007/s11274-019-2774-y
23. Druzhinina IS, Chenthamara K, Zhang J, Atanasova L, Yang DQ, Miao YZ, Rahimi MJ, Grujic M, Cai F, Pourmehdi S, Abu Salim K, Pretzer C, Kopchinskiy AG, Henrissat B, Kuo A, Hundley H, Wang M, Aerts A, Salamov A, Lipzen A, LaButti K, Barry K, Grigoriev IV, Shen QR, Kubicek CP (2018) Massive lateral transfer of genes encoding plant cell wall-degrading enzymes to the mycoparasitic fungus *Trichoderma* from its plant-associated hosts. PLoS Genet 14(4):e1007322. https://doi.org/10.1371/journal.pgen.1007322
24. Kubicek CP, Steindorff AS, Chenthamara K, Manganiello G, Henrissat B, Zhang J, Cai F, Kopchinskiy AG, Kubicek EM, Kuo A, Baroncelli R, Sarrocco S, Noronha EF, Vannacci G, Shen Q, Grigoriev IV, Druzhinina IS (2019) Evolution and comparative genomics of the most common *Trichoderma* species. BMC Genomics 20(1):485. https://doi.org/10.1186/s12864-019-5680-7
25. Zhang J, Bayram Akcapinar G, Atanasova L, Rahimi MJ, Przylucka A, Yang D, Kubicek CP, Zhang R, Shen Q, Druzhinina IS (2016) The neutral metallopeptidase NMP1 of *Trichoderma guizhouense* is required for mycotrophy and self-defence. Environ Microbiol 18 (2):580–597. https://doi.org/10.1111/1462-2920.12966
26. Zhang J, Miao Y, Rahimi MJ, Zhu H, Steindorff A, Schiessler S, Cai F, Pang G, Chenthamara K, Xu Y, Kubicek CP, Shen Q, Druzhinina IS (2019) Guttation capsules containing hydrogen peroxide: an evolutionarily conserved NADPH oxidase gains a role in wars between related fungi. Environ Microbiol 21(8):2644–2658. https://doi.org/10.1111/1462-2920.14575
27. Gao R, Ding M, Jiang S, Zhao Z, Chenthamara K, Shen Q, Cai F, Druzhinina IS (2020) The evolutionary and functional paradox of cerato-platanins in fungi. Appl Environ Microbiol. https://doi.org/10.1128/AEM.00696-20
28. Penttilä M, Nevalainen H, Rättö M, Salminen E, Knowles J (1987) A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. Gene 61 (2):155–164. https://doi.org/10.1016/0378-1119(87)90110-7
29. Gruber F, Visser J, Kubicek CP, de Graaff LH (1990) The development of a heterologous transformation system for the cellulolytic fungus *Trichoderma reesei* based on a pyrG-negative mutant strain. Curr Genet 18 (1):71–76. https://doi.org/10.1007/BF00321118
30. Smith JL, Bayliss FT, Ward M (1991) Sequence of the cloned *pyr4* gene of *Trichoderma reesei* and its use as a homologous selectable marker for transformation. Curr Genet 19(1):27–33. https://doi.org/10.1007/BF00362084
31. Lorito M, Hayes CK, Di Pietro A, Harman GE (1993) Biolistic transformation of *Trichoderma harzianum* and *Gliocladium virens* using plasmid and genomic DNA. Curr Genet 24 (4):349–356. https://doi.org/10.1007/BF00336788
32. Zeilinger S (2004) Gene disruption in *Trichoderma atroviride* via *Agrobacterium*-mediated transformation. Curr Genet 45(1):54–60. https://doi.org/10.1007/s00294-003-0454-8
33. Schuster A, Bruno KS, Collett JR, Baker SE, Seiboth B, Kubicek CP, Schmoll M (2012) A versatile toolkit for high throughput functional genomics with *Trichoderma reesei*. Biotechnol Biofuels 5(1):1. https://doi.org/10.1186/1754-6834-5-1
34. Magana-Ortiz D, Coconi-Linares N, Ortiz-Vazquez E, Fernandez F, Loske AM, Gomez-Lim MA (2013) A novel and highly efficient method for genetic transformation of fungi employing shock waves. Fungal Genet Biol 56:9–16. https://doi.org/10.1016/j.fgb.2013.03.008
35. Eveleigh DE, Montenecourt BS (1979) Increasing yields of extracellular enzymes. Adv Appl Microbiol 25:57–74. https://doi.org/10.1016/s0065-2164(08)70146-1
36. Reagents, Buffers, and Indicators (2017) ACS Reagent Chemicals. Am Chem Soc. https://doi.org/10.1021/acsreagents.3001
37. Ogawa Y, Mii M (2004) Screening for highly active β-lactam antibiotics against *Agrobacterium tumefaciens*. Arch Microbiol 181 (4):331–336. https://doi.org/10.1007/s00203-004-0650-z

38. Fincham JR (1989) Transformation in fungi. Microbiol Rev 53(1):148–170
39. Li D, Tang Y, Lin J, Cai W (2017) Methods for genetic transformation of filamentous fungi. Microb Cell Factories 16(1):168. https://doi.org/10.1186/s12934-017-0785-7
40. Flores-Felix JD, Menendez E, Peix A, Garcia-Fraile P, Velazquez E (2020) History and current taxonomic status of genus *Agrobacterium*. Syst Appl Microbiol 43(1):126046. https://doi.org/10.1016/j.syapm.2019.126046
41. Barton IS, Fuqua C, Platt TG (2018) Ecological and evolutionary dynamics of a model facultative pathogen: *Agrobacterium* and crown gall disease of plants. Environ Microbiol 20 (1):16–29. https://doi.org/10.1111/1462-2920.13976
42. Gelvin SB (2003) *Agrobacterium*-mediated plant transformation: the biology behind the "gene-jockeying" tool. Microbiol Mol Biol Rev 67(1):16–37, table of contents. https://doi.org/10.1128/mmbr.67.1.16-37.2003
43. Dunn-Coleman N, Wang H (1998) *Agrobacterium* T-DNA: a silver bullet for filamentous fungi? Nat Biotechnol 16(9):817–818. https://doi.org/10.1038/nbt0998-817
44. de Groot MJ, Bundock P, Hooykaas PJ, Beijersbergen AG (1998) *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nat Biotechnol 16(9):839–842. https://doi.org/10.1038/nbt0998-839
45. Michielse CB, Hooykaas PJ, van den Hondel CA, Ram AF (2005) *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. Curr Genet 48(1):1–17. https://doi.org/10.1007/s00294-005-0578-0
46. Idnurm A, Bailey AM, Cairns TC, Elliott CE, Foster GD, Ianiri G, Jeon J (2017) A silver bullet in a golden age of functional genomics: the impact of *Agrobacterium*-mediated transformation of fungi. Fungal Biol Biotechnol 4:6. https://doi.org/10.1186/s40694-017-0035-0
47. Friedl MA, Schmoll M, Kubicek CP, Druzhinina IS (2008) Photostimulation of *Hypocrea atroviridis* growth occurs due to a cross-talk of carbon metabolism, blue light receptors and response to oxidative stress. Microbiology 154(Pt 4):1229–1241. https://doi.org/10.1099/mic.0.2007/014175-0
48. Carreras-Villasenor N, Sanchez-Arreguin JA, Herrera-Estrella AH (2012) *Trichoderma*: sensing the environment for survival and dispersal. Microbiology 158(Pt 1):3–16. https://doi.org/10.1099/mic.0.052688-0
49. Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res 2 (4):208–218. https://doi.org/10.1007/BF01977351
50. Kunamneni A, Plou FJ, Alcalde M, Ballesteros A (2014) Chapter 24: *Trichoderma* enzymes for food industries. In: Gupta VK, Schmoll M, Herrera-Estrella A, Upadhyay RS, Druzhinina I, Tuohy MG (eds) Biotechnology and biology of *Trichoderma*. Elsevier, Amsterdam, pp 339–344. https://doi.org/10.1016/B978-0-444-59576-8.00024-2
51. Druzhinina IS, Kubicek CP, Komon-Zelazowska M, Mulaw TB, Bissett J (2010) The *Trichoderma harzianum* demon: complex speciation history resulting in coexistence of hypothetical biological species, recent agamospecies and numerous relict lineages. BMC Evol Biol 10:94. https://doi.org/10.1186/1471-2148-10-94
52. Chaverri P, Branco-Rocha F, Jaklitsch W, Gazis R, Degenkolb T, Samuels GJ (2015) Systematics of the *Trichoderma harzianum* species complex and the re-identification of commercial biocontrol strains. Mycologia 107 (3):558–590. https://doi.org/10.3852/14-147
53. Sandoval-Denis M, Sutton DA, Cano-Lira JF, Gené J, Fothergill AW, Wiederhold NP, Guarro J (2014) Phylogeny of the clinically relevant species of the emerging fungus *Trichoderma* and their antifungal susceptibilities. J Clin Microbiol 52(6):2112–2125. https://doi.org/10.1128/JCM.00429-14
54. Blochlinger K, Diggelmann H (1984) Hygromycin B phosphotransferase as a selectable marker for DNA transfer experiments with higher eucaryotic cells. Mol Cell Biol 4 (12):2929. https://doi.org/10.1128/MCB.4.12.2929
55. Hua J, Meyer JD, Lodge JK (2000) Development of positive selectable markers for the fungal pathogen *Cryptococcus neoformans*. Clin Diagn Lab Immunol 7(1):125–128. https://doi.org/10.1128/cdli.7.1.125-128.2000
56. Solis-Escalante D, Kuijpers NGA, Nadine B, Bolat I, Bosman L, Pronk JT, Daran J-M, Pascale D-L (2013) *amdSYM*, a new dominant recyclable marker cassette for *Saccharomyces cerevisiae*. FEMS Yeast Res 13(1):126–139. https://doi.org/10.1111/1567-1364.12024
57. Guangtao Z, Seiboth B, Wen C, Yaohua Z, Xian L, Wang T (2010) A novel carbon source-dependent genetic transformation system for the versatile cell factory *Hypocrea jecorina* (anamorph *Trichoderma reesei*). FEMS Microbiol Lett 303(1):26–32. https://doi.org/10.1111/j.1574-6968.2009.01851.x

Chapter 13

Purification and Amplification of DNA from Cellulolytic Bacteria: Application for Biogas Production from Crop Residues

Reckson Kamusoko, Raphael M. Jingura, Wilson Parawira, and Zedias Chikwambi

Abstract

Polymerase chain reaction (PCR) is a popular molecular tool for detection of bacteria. PCR allows millions of copies of a target segment of DNA to be produced. The DNA is extracted from overnight grown cultures of pure bacterial isolates using either the organo-solvent method or a commercial DNA extraction kit. The quality and purity of the DNA is determined by performing gel electrophoresis on 0.8% agarose gel. The DNA is amplified by performing PCR assay. Bands of approximately 1.5 kb in size are obtained from the amplified products of DNA. The PCR products run on 1.5% agarose gel are visualized with UV light and imaged by gel documentation system. This chapter outlines the protocol for isolation and amplification of DNA from cellulolytic bacteria. Cellulolytic bacteria are considered a potential source of cellulases for pretreatment of crop residues during biogas production. PCR is considered a very powerful, sensitive, specific, fast, and reliable tool in molecular detection and diagnostics.

Key words Amplification, Agarose gel, Bacteria, Electrophoresis, Polymerase chain reaction

1 Introduction

Traditional methods used for detection of novel cellulolytic bacteria strains rely mainly on enrichment growth techniques. These methods support the growth of nonfastidious bacteria and are not ideal for slow-growing bacteria [1]. The main limitation of traditional methods is lack of growth of certain bacteria species on synthetic media, thereby underestimating bacterial diversity with new biochemical applications [1, 2]. According to Pham and Kim [3] and Nguyen et al. [4], about 1% of the total bacterial species have been cultivated using traditional approaches. Growth of bacteria under routine laboratory environment is hindered by nonfulfillment of natural growth conditions and unavailability of information on factors affecting growth [1, 4].

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Over the last decade, advances in molecular biology have led to the advent of modern multipurpose approaches in medical and biological research. These new frontiers of technology rely mostly on the development of polymerase chain reaction (PCR). PCR is a very powerful, sensitive, and reliable tool that allows the amplification of a small quantity of DNA fragments or target sequences to produce a large amount of specific DNA segments called amplicons [5–8]. It is an in vitro downstream process that utilizes purified DNA of high quality as a template for replication [9]. Conventionally, genomic DNA (gDNA) is isolated from bacteria using organosolvent extraction technique. However, this method is slow and mainly effective for rod-shaped Gram-positive bacteria such as *Bacillus subtilis*. Rapid and convenient methods for instance DNA extraction kits are now widely applied at commercial scale [9]. PCR has wide applications such as DNA cloning for sequencing; DNA-based phylogeny or functional analysis of genes; diagnosis of hereditary diseases; DNA finger printing; and detection and diagnosis of infectious diseases [10].

Nowadays, the study of bacterial phylogeny and genus/species classification is largely based on amplification and analysis of the 16S ribosomal rRNA (rRNA) gene fragments [7, 11]. The 16S rRNA gene is around 1.5 kb in size [12, 13]. It is made up of highly conserved and hypervariable regions that are genus- or species-specific [13]. The 16S rRNA gene is ubiquitously present among prokaryotes [14]; often existing as a multigene family or operons [15]; and its function has not changed over time suggesting that random sequence changes are a more accurate measure of evolution and it is large enough for bioinformatics purposes [15]. Furthermore, the 16S rRNA gene provides an inexpensive, scalable, reproducible, and simple technique as compared to other current methods for detection of bacteria [16].

A summary for performing PCR amplification of the 16S rRNA gene prior to sequence analyses is shown on Fig. 1. The variable sequences of 16S rRNA gene are amplified by PCR primers targeting the conserved regions [18]. A standard PCR is a three-step (denaturation, annealing, and extension) cycling process. PCR amplification is followed by sequencing of the PCR product and comparison of nucleotide sequences with known sequences stored in database or genebank [14]. The assembly and comparison of 16S rRNA sequences requires easy, simple, and technically feasible bioinformatics [16]. The BLAST algorithm with similarity threshold of 98.65% is used to compare the16S rRNA sequences with potential phylogenetic neighbors [16]. In this chapter, we demonstrate that genomic DNA of cellulolytic bacteria can be purified and amplified by PCR prior to detection via sequencing of the 16S rRNA gene segments. The method has been previously used by several authors [19–23]. Cellulolytic bacteria are a potential source of cellulases for

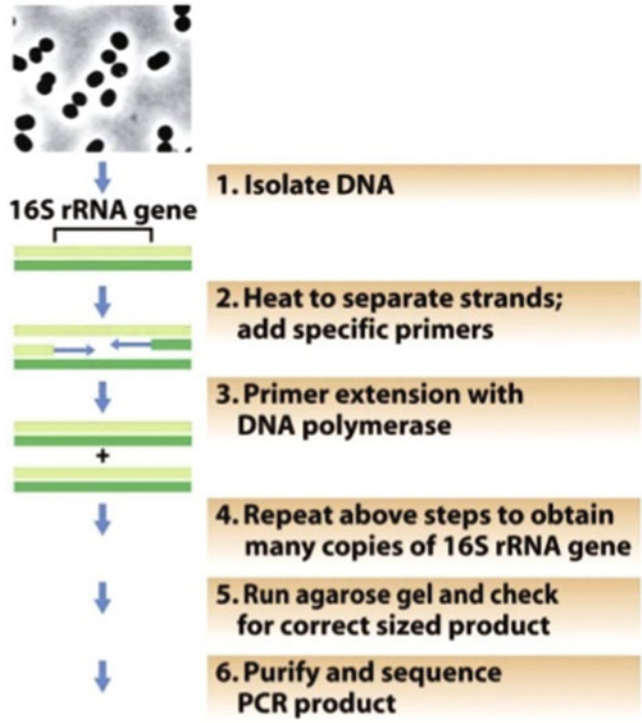


Fig. 1 Diagrammatic presentation of PCR amplification of the 16S rRNA gene for sequencing [17]

pretreatment of crop residues to enhance biogas production. Bacteria genera including *Cellulomonas, Pseudomonas, Micrococcus*, and *Bacillus* are active cellulase-producers [24, 25].

1.1 Cellulolytic Bacteria in Biogas Production

Crop residues are the most predominant agricultural biomass on earth. Their cell walls are mainly composed of polymers of cellulose, hemicelluloses, and lignin [26, 27]. Cellulose is the main source of fermentable sugars in lignocellulosic substrates. It has high potential to be converted into useful biofuel products such as biogas and bioethanol. However, the complex association of cellulose with other polysaccharide components makes crop residues resistant to microbial degradation [24, 26]. Appropriate pretreatment methods can destroy lignocellulosic matrix structure of crop residues, thus rendering cellulose amenable to enzymatic attack. Cellulolytic microorganisms can produce cellulase enzymes that decompose cellulose. Bacterial and fungal isolates from diverse habitats possess cellulase-degrading activities [25]. Cellulolytic bacteria have been isolated from multifarious habitats such as sewage, agricultural wastes, soil, hot springs, and guts of ruminants and insects [28, 29]. Genera include *Bacillus*, *Clostridium*, *Cellulomonas*, *Micrococcus*, *Alteromonas*, *Acetivibrio, Pseudomonas*, and *Bacteroides* [25, 30]. Table 1 shows the taxonomic diversity of bacteria

Table 1
Bacteria involved in cellulolytic activities

Genus	Species
Acidothermus	*A. cellulolyticus*
Bacillus	*Bacillus* sp., *B. megaterium*, *B. amyloliquefaciens, B. subtilis* and *Anoxybacillus flavithermus*
Clostridium	*C. thermocellum, C. acitobutylicum* and *C. cellulovorans*
Pseudomonas	*P. cellulose*
Rhodothermus	*R. marinus*
Cellulomonas	*C. fim* and *C. uda*

Source: [24, 25]

with capability to degrade cellulose. Further exploration is essential to acquire more novel cellulolytic strains with pretreatment hyperactivity.

Cellulase is a complex mixture of three different hydrolytic enzymes: cellobiohydrolase, endo β-glucanase and β-glucanase. These enzymes interact in a synergistic way to degrade lignocellulosic biomass into its constituent glucose, cellobiose, and other oligosaccharides units [24, 31]. Generally, cellulases act as biocatalysts for the transformation of cellulosic material into fermentable sugars [32, 33]. Glucose is an intermediary substrate that can be converted via anaerobic digestion or fermentation into multiple value-added products such as biogas and bioethanol [33].

1.2 Methods to Detect Cellulolytic Bacteria

Traditional phenotypic techniques are commonly used for primary identification of novel cellulolytic bacteria from diverse ecological sources. They are based on phenotypic characteristics including morphological, physiological, and biochemical features [6, 34]. The majority of phenotypic variables are not sensitive enough for strain differentiation. Moreover, phenotypic analysis is a very tedious exercise in identification of bacteria [6]. Traditional methods involve enrichment of bacteria in media. However, not all bacteria are able to grow in media. Current focus has been placed on searching for fast and unambiguous culture-independent tools to detect cellulolytic bacteria.

Nowadays, traditional methods have been accelerated, simplified, and automated due to the advancement in technology [6, 34]. Advances in molecular biology have led to the advent of new approaches for identification and characterization of bacteria taxonomy. These new frontiers of technology rely mostly on 16S rRNA gene sequencing, PCR, and other PCR-related techniques [6, 35]. The popularity of these methods has been derived from their high sensitivity and reproducibility. 16S rRNA gene

Table 2
Comparison of different methods to isolate genomic DNA from cellulolytic bacteria

Method	Parameter						
	Cost	Yield of DNA	Purity of DNA	Time efficiency	Technical requirements	Sensitivity	Toxic compounds
Commercial kits	High	Very high	Very high	Rapid	Simple	High	None
Phenol-chloroform	Moderate	Low	Low	Slow	Simple-complex	Low	Phenol, chloroform
Boiling	Low	High	Low	Rapid	Very simple	Low	None

Source: [38, 40, 41]

sequencing is touted the most accurate method and declared the "gold standard" for detection of bacteria up to species level [35]. The method is the most sensitive, very rapid, highly specific, reliable, reproducible, and allows the detection of bacterial strains that cannot be easily cultured under laboratory conditions [35, 36].

The 16S rRNA gene sequencing is based on PCR amplification and sequencing of DNA. PCR amplification requires sufficient yield of sample DNA of high purity [37]. Extracted DNA should be free from proteins, RNA, and PCR inhibitors [38]. DNA extraction from cellulolytic bacteria can be done using various cell lysis methods such as chemical, mechanical, enzymatic, and heat [39]. The methods that have been commonly used to extract genomic DNA from cellulolytic bacteria include commercial kits, the phenol-chloroform method, and the boiling method [37]. Table 2 provides a comparison of disparate methods to purify DNA from cellulolytic bacteria. It is important to select the most appropriate method to identify cellulolytic bacteria. An ideal DNA extraction protocol should be sensitive, rapid, easy to use, minimize use of specialized equipment, pose minimum risk to users, avoid cross-contamination of samples, and elute DNA that is highly pure [42].

2 Purification and Amplification of Genomic DNA from Cellulolytic Bacteria

2.1 Materials

Make use of deionized or distilled water and reagents of the highest grade to prepare all solutions. Sterilization by autoclaving is highly recommended for most operations. Some of the chemical reagents that are handled in this protocol are hazardous upon exposure. Therefore, it is essential to attain knowledge on the health and safety of handling them. Make sure to wear protective clothing when preparing solutions.

2.1.1 Agarose Gel

1. 0.5 M EDTA stock solution: Weigh 93.05 g of EDTA disodium salt (MW = 372.24 g mol^{-1}). Transfer EDTA to 500 mL graduated cylinder or beaker and dissolve by adding 400 mL of deionized water. Adjust to pH 8.0 with sodium hydroxide (NaOH) pellets (*see* **Note 1**). Add more deionized water to a final volume of 500 mL. Heat in an autoclave to sterilize the solution (*see* **Note 2**).
2. 50× TAE buffer: Dissolve 242 g of Tris-base (MW = 121.14 g mol^{-1}) by adding 700 mL of deionized water in 1 L cylinder. Add 57.1 mL of 100% glacial acid (or acetic acid) and 100 mL of prepared 0.5 M EDTA (pH 8.0). Top up the solution to final volume of 1 L. The pH is not adjusted and should be around 8.5. Store the prepared stock solution at room temperature.
3. 1× TAE buffer: Add 20 mL of 50× TAE buffer to a 1 L cylinder and adjust to a final working volume of 1 L using deionized water. The final concentration in running gel consists of 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA.
4. Agarose gel: Prepare 0.8% agarose gel by heating 0.8 g of agarose gel in 100 mL 1× TAE buffer in a microwave (~30 s^{-1} min full power) until completely dissolved (*see* **Note 3**). The gel must be allowed to cool so that it is comfortable to handle (*see* **Note 4**). Add 1 μL of GelRed or Gel Green Nucleic Acid stain (*see* **Note 5**). Prepare 1.5% agarose gel by dissolving 1.5 g of agarose gel in 100 mL 1× TAE buffer.

2.1.2 Bacterial Genomic DNA Extraction

1. Glycerol stocks of pure cellulolytic bacterial isolates: Prepare by mixing 500 μL of an overnight grown culture (incubated at 37 °C) with 500 μL of 60% sterile glycerol in microcentrifuge tubes and preserve at −80 °C for long-term use.
2. ZR Zymo Fungal/Bacteria DNA MiniPrep™ kit or equivalent: 50 ZR BashingBead Lysis Tubes (0.1 and 0.5 mm), 40 mL Lysis Solution, 100 mL Fungal/Bacterial DNA Binding Buffer, 15 mL DNA Pre-Wash Buffer, 10 mL DNA Elution Buffer, 50 Zymo-Spin™ Spin Filters (orange tops), 50 Zymo-Spin ™ IIC Columns, 150 Collection Tubes (*see* **Note 6**). Preserve the kit at room temperature.

2.1.3 Polymerase Chain Reaction

All chemical reagents for PCR are ready-to-use.

1. Template DNA: Ultrapure gDNA at a concentration of 50 ng. Store at −20 °C to −80 °C for long-term use.
2. 6× DNA Loading Dye or equivalent: 10 mM Tris–HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, and 60 mM EDTA. Store at 4 °C up to 12 months or at −20 °C for longer periods.

3. O'GeneRuler 1 kb Plus DNA ladder (0.1 μg μL^{-1}) or equivalent: 15 chromatographic-purified individual DNA base pairs (20,000, 10,000, 7000, **5000**, 4000, 3000, 2000, **1500**, 1000, 700, **500**, 400, 300, 200, 75) (*see* **Note 7**). Store at 4 °C up to 12 months or at −20 °C for longer periods.
4. DreamTaq™ Hot Start Master Mix (2×) or equivalent: DreamTaq™ Hot Start DNA Polymerase, DreamTaq™ buffer, magnesium, and dNTPs. Store at −5 °C to −30 °C.
5. Primers: 63F (5′-CAGGCCTAACACATGCAAGTC—3′ forward primer) and 1387R (5′-CGGCGGWGTGTACAAGGC—3′ reverse primer) [43] or equivalent.
6. Magnesium chloride: 25 mM $MgCl_2$. Store at 2–8 °C.
7. Nuclease-free water: Store at 15–25 °C.
8. 0.2 mm thin-walled PCR tubes.

3 Methods

Make sure you are well prepared when setting up PCR experiments. Put all reagents required for PCR work in a bucket loaded with fresh ice. Allow the reagents to defrost completely before starting a reaction. Reagents must be kept on ice up to completion of the experiment. Prevent contamination of reaction mixture or reagents by wearing protective gloves. A negative control, and if possible a positive control, is needed [8]. Prepare DNA samples, set PCR mixtures, carry out thermal cycling, and visualize PCR products in different areas.

3.1 DNA Extraction from Pure Bacterial Isolates

Extract total gDNA from glycerol stocks of bacteria using ZR Zymo Fungal/Bacteria DNA MiniPrep™ kit (Zymo Research, USA) according to manufacturer's instructions. The fundamental principles included in all DNA extraction protocols are summarized in Fig. 2. The schematic procedure of the ZR Zymo Fungal/Bacteria DNA MiniPrep™ kit is as follows:

1. Grow overnight pure culture of the target bacteria in nutrient broth by incubating at 37 °C.
2. Transfer 1.5 mL of each culture to a microcentrifuge tube and centrifuge at 18,928 × *g* for 2 min to obtain a pellet (*see* **Note 8**).
3. Decant the supernatant in each tube followed by draining onto tissue paper.
4. Resuspend the pellets in 200 μL of sterile water and add 750 μL of Lysis Solution to each tube (*see* **Note 9**). Carefully mix the contents of the tube by repeated pipetting.

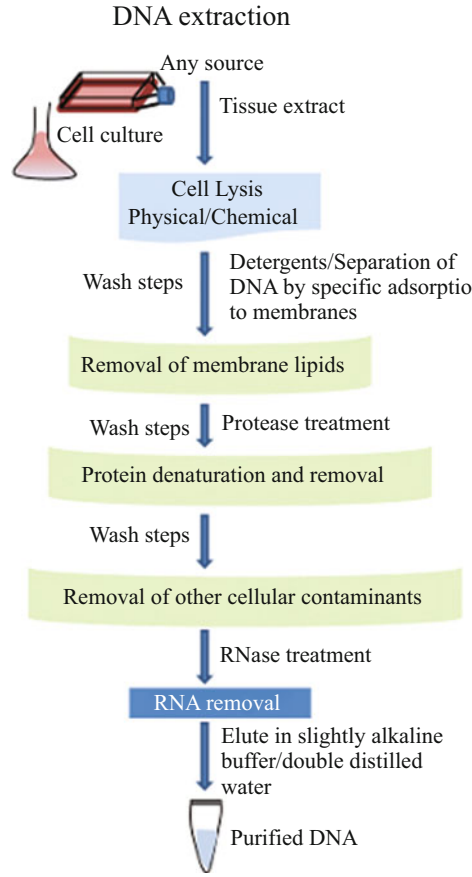


Fig. 2 Basic principles included in all DNA extraction protocols [44]

5. Transfer the mixture to ZR BashingBead™ Lysis Tubes. Mix the contents of the tubes by vortexing at maximum speed for 5 min followed by centrifugation at 11,200 × *g* for 1 min.
6. Transfer 400 μL of the supernatant from each tube to a Zymo-Spin™ IV Spin Filter in a collection tube and centrifuge at 5,488 × *g* for 1 min (*see* **Note 10**).
7. Add 1200 μL Fungal/Bacterial DNA Binding Buffer to the filtrate in each collection tube (*see* **Note 11**).
8. Transfer 800 μL of the resultant mixture from each collection tube to a Zymo-Spin™ IIC Spin Column in a collection tube and centrifuge at 11,200 × *g* for 1 min (*see* **Note 12**).
9. Discard the through-flow from the collection tube and repeat the previous step.
10. Add 200 μL DNA Pre-Wash Buffer to the Zymo-Spin™ IIC Spin Column in each new collection tube preceded by centrifugation at 11,200 × *g* for 1 min (*see* **Note 13**).

11. Add 500 μL of DNA Wash Buffer to each Zymo-Spin™ IIC Spin Column and centrifuge again at 11,200 × *g* for 1 min (*see* **Note 14**).
12. Transfer each Spin™ IIC Spin Column to a clean 1.5 mL microcentrifuge tube and directly add 100 μL of DNA Elution Buffer to the column matrix followed by centrifugation at 11,200 × *g* for 30 s to elute DNA.
13. Ultrapure DNA is stored at −20 °C to −80 °C. The DNA is now ready for PCR experiments.

3.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis is one of the alternatives to quickly determine the concentration of gDNA. Set the agarose gel electrophoresis apparatus according to Lee et al. [45]:

1. Place a gel tray into casting apparatus, with a desired comb to create wells.
2. Pour the molten 0.8% agarose gel into the mold. Allow the gel to solidify at room temperature and remove the comb.
3. Place the gel in gel mold to gel box with 1× TAE buffer.
4. Adjust the power supply to 100 V.
5. Add sufficient running buffer to completely submerge the gel and make sure the maximum fill line is not exceeded. Place the lid to the gel box.
6. Connect the terminals of the gel box to power supply and turn on power to check if the gel box and power supply are functioning.
7. Turn off the power and detach the lid.
8. Add 2 μL-drop of 6× loading dye to 10 μL of each gDNA sample to be separated. Load 1 μL of O'GeneRuler 1 kb Plus DNA ladder along with gDNA samples.
9. Replace the lid to the gel box and make sure the electrode leads are connected to the correct slots in the power supply (*see* **Note 15**).
10. Switch on the power. Run the gel at 100 V for 60–90 min until the dye has migrated to a distance of 10–12 cm (*see* **Note 16**).

3.3 Visualizing Separated Genomic DNA Bands

1. Disconnect the leads when electrophoresis has completed and detach the lid of the gel box.
2. Take off the gel tray from the gel box and place it on paper towel to remove excess buffer from the gel.
3. Remove the gel from the gel tray and visualize it with a suitable UV transilluminator and gel documentation system (*see* **Note 17**). DNA fragments should be visualized as orange fluorescence bands. Take an image of the gel (Fig. 3).
4. Follow the institutional biosafety regulations for correct disposal of the gel and buffer.

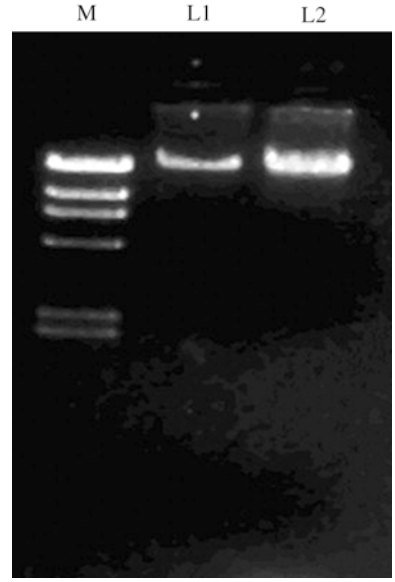


Fig. 3 A picture of bacteria genomic DNA on 0.8% agarose gel, visualized with UV light and imaged by gel documentation system. *Lane M* shows a 1 kb DNA marker; *Lanes 1* and *2* shows two bands of genomic DNA of bacteria isolates (Andreou cited by [9])

3.4 Polymerase Chain Reaction

Prepare PCR reaction mixtures in a lamina flow cabinet with a UV lamp installed. The volumes of reagents for PCR reaction mixture may differ as they are affected by the final concentrations of stock reagents. Prepare a 25 μL reaction mixture as follows (*see* **Note 18**):

1. Calculate the volumes and concentrations of all the reagents that will be added to the reaction mixture for PCR amplification of bacteria gDNA (Table 3).
2. Add 8.5 μL of nuclease-free water to each 0.2 mm thin-walled PCR tube.
3. Thaw, vortex gently, and briefly centrifuge the DreamTaq™ Hot Start Master Mix (2×).
4. Aliquot 12.5 μL of DreamTaq™ Hot Start Master Mix (2×).
5. Add 1 μL of 10 μM primer pair [25].
6. Pipette 2 μL of template bacteria DNA.
7. Pipette all reagents excluding template DNA in a separate PCR tube as a negative control. Recompense the remaining volume by adding 9.5 μL of nuclease-free water.
8. Place reaction mixtures in a thermo cycler.
9. Program the thermo cycler and perform the PCR reaction (Table 4) (*see* **Note 19**).

Table 3
PCR reaction mixture for amplification of bacteria genomic DNA

Reagent	Final concentration	Volume (μL)
Nuclease-free water	–	8.5
DreamTaq™ Hot Start Master Mix	2×	12.5
Forward primer	10 μM	1
Reverse primer	10 μM	1
Template DNA	50 ng	2
Total volume	–	25

Table 4
A standard three-step PCR cycling

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	95	1–3 min	1
Denaturation Annealing Extension[a]	95 T_m 72	30 s 30 s 1 min	25–40
Final extension	72	5–15 min	1

[a]The recommended extension time for PCR products up to 2 kb is 1 min. Beyond 2 kb, the time extends by 1 min/kb

10. Once the PCR reaction is completed, the PCR products are detected by running on 1.5% agarose gel at 100 V for 90 min and visualized with UV light and gel documentation system (*see* Subheadings 3.2 and 3.3) (*see* **Note 20**). Take a photograph of the gel (Fig. 4).
11. Excise the required 1.5 kb DNA amplicons from the gel. Store at −20 °C to −80 °C until the DNA is recovered and purified from the gel prior to sequencing.
12. Follow the institutional biosafety regulations for correct disposal of the gel and buffer.

4 Notes

1. EDTA will not dissolve completely until pH is adjusted to 8.0. The carboxyl groups of EDTA cannot dissociate at low pH. Hence, adding NaOH raises the pH causing more carboxyl groups to dissociate and release more protons into the solution.

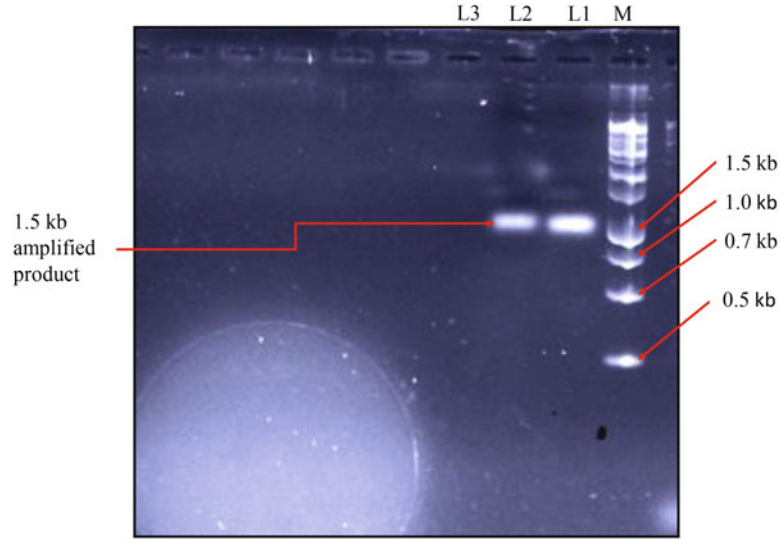


Fig. 4 An image of 16S rRNA PCR amplicons on 1.5% agarose gel. The PCR products were visualized with UV light and imaged by gel documentation system. *Lane M* shows a 1 kb molecular weight marker. *Lanes 1* and *2* show two bands of 1.5 kb PCR amplicons of bacteria isolates. *Lane 3* is a no-template negative control

2. Do not operate an autoclave until you are trained how to do so safely. Familiarize yourself with the healthy and safety issues associated with the use of an autoclave before operating it.
3. Undissolved "chunks" of agarose will impede electrical separation of nucleic acids through the gel. The use of a microwave requires that the lid of glass bottle containing agarose be loosened to avoid explosion.
4. Always wear protective gloves when handling hot agarose solution because it can cause severe burns. Do not allow the agarose gel to solidify before pouring into a gel electrophoresis tank.
5. GelRed or GelGreen Nucleic Acid stain is potentially dangerous as it is suspected to be carcinogenic. The stain must be handled with caution. Always wear latex gloves when handling these chemicals and gels or buffers containing these chemicals.
6. Some reagents contained in the kit are irritants; therefore it is encouraged to wear protective gloves and eye protection during DNA extraction. The integrity of the kit is guaranteed for a period of 1 year from the date of purchase. The reagents are regularly tested on lot-to-lot basis to make sure they provide optimal performance and reliability.
7. O'GeneRuler 1 kb Plus DNA ladder contains three reference bands (5000, 1500, and 100 bp) for easy orientation.

8. Centrifugation at this stage helps to move DNA precipitate to the bottom of the tube and allow it to adhere there so that the supernatant can be decanted without losing the extract.
9. Lysis Solution is a buffer that is used to break cells open during DNA extraction.
10. Centrifugation allows the solution containing DNA to move through the filter.
11. Ensure optimal performance of DNA Binding Buffer by adding beta-mercaptoethanol to 0.5% (v/v) or 500 μL per 100 mL.
12. Centrifugation separates cell debris from DNA-containing supernatant so that the supernatant can be discarded and DNA can be precipitated out of it.
13. DNA Pre-Wash Buffer removes protein and degraded RNA residues on the membrane. If precipitation has occurred in the DNA Pre-Wash Buffer during transportation, completely resuspend the buffer by incubation at 30–37 °C for 30 min and mix by inversion.
14. DNA Wash Buffer removes salt residues on the membrane.
15. If the electrode leads are not properly connected, the dye and DNA samples will migrate in the wrong direction, i.e., to the negatively charged cathode.
16. Exercise extreme caution because the electric current in a gel electrophoresis tank is very dangerous. Do not disconnect the lid or touch the buffer when power is switched on. The surface where the gel is being run must be completely dry.
17. UV light is extremely dangerous. Protect your eyes, face, and hands by wearing a UV blocking face shield and gloves.
18. Prepare the reaction mixture in an ice bucket to prevent nuclease activity and primer dimerization. The reaction mixture can be optimized by adding different volumes of 25 mM $MgCl_2$ if the desired results are not obtained.
19. A wrong annealing temperature leads to nonspecific binding or random binding.
20. The absence of a band in the negative control indicates the lack of reagent contamination.

References

1. Chaudhary DK, Khulan A, Kim J (2019) Development of a novel cultivation technique for uncultured soil bacteria. Sci Rep 9:6666. https://doi.org/10.1038/s41598-019-43182-x
2. Jo J, Kennedy EA, Kong HH (2016) Bacterial 16S ribosomal RNA gene sequencing in cutaneous research. J Invest Dermatol 136(3): e23–e27
3. Pham VHT, Kim J (2016) Improvement for isolation of soil bacteria by using common culture media. J Pure Appl Microbio 10 (1):49–59. https://www.researchgate.net/publication/301348448_Improvement_for_

Isolation_of_Soil_Bacteria_by_Using_Common_Culture_Media
4. Nguyen TM, Seo C, Ji M et al (2018) Effective soil extraction method for cultivating previously uncultured soil bacteria. Appl Environ Microbiol 84(24):e01145-18. https://doi.org/10.1101/322735
5. Woo PC, Lau SK, Teng JL et al (2008) Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. Clin Microbiol Infect 14(10):908–934
6. Bisen PS, Debnath M, Prasad GBKS (2012) Microbes: concepts and applications, 1st edn. John Wiley & Sons, Hoboken, NJ
7. Wang X, Jordan IK, Mayer LW (2015) A phylogenetic perspective on molecular epidemiology. In: Tang Y, Sails A (eds) Molecular medical microbiology, 2nd edn. Academic Press, Cambridge
8. Lorenz TC (2012) Polymerase chain reaction: basic protocols plus troubleshooting and optimization strategies. J Vis Exp 63:e3998. https://doi.org/10.3791/3998
9. Andreou L (2013) Preparation of genomic DNA from bacteria. In: Lorsch J (ed) Methods in enzymology, vol 529. Elsevier, Amsterdam
10. Usman AD, Ali S (2009) An overview of the application of polymerase chain reaction (PCR) in the diagnosis of bacterial infections. Bayero J Pure Appl Sci 2(1):109–114
11. Geevarghese A, Baskaradoss JK, Al Dosari AA (2012) Application of 16S rRNA in identifying oral microflora—a review of literature. World Appl Sci J 17(10):1303–1307
12. Pazo-Bayon MA, Pardo I, Ferrer S, Moreno-Arribas MV (2009) Molecular approaches for the identification and characterisation of oenological lactic acid bacteria. Afr J Biotechnol 8 (17):3995–4001
13. Barb JJ, Oler AJ, Kim H et al (2016) Development of an analysis pipeline characterising multiple hypervariable regions of 16S rRNA using mock samples. PLoS One 11(2):e0148047. https://doi.org/10.1371/journal.pone.0148047
14. Jenkins C, Ling CL, Ciesielczuk HL et al (2012) Detection and identification of bacteria in clinical samples by 16s rRNA gene sequencing: comparison of two different approaches in clinical practice. J Med Microbiol 61:483–488
15. Janda JM, Abbot SL (2007) 16S rRNA gene sequencing for bacteria identification in the diagnostic laboratory: pluses, perils and pitfalls. J Clin Microbiol 45(9):2761–2764
16. Kim M, Chun J (2014) 16S rRNA gene-based identification of *Bacteria* and *Archaea* using the EzTaxon Server. In: Goodfellow M, Sutcliffe I, Chun J (eds) Methods in microbiology, vol 41. Elsevier, Amsterdam
17. Madigan MT, Bender KS, Buckely DH et al (2006) Brock biology of microorganisms, 15th edn. Pearson Prentice Hall, Upper Saddle River, NJ
18. Relman DA (1999) The search for unrecognised pathogens. Science 284:1308–1310
19. Boye K, Høgdall E, Borre M (1999) Identification of bacteria using two degenerate 16S rDNA sequencing primers. Microbiol Res 154 (1):23–26
20. Chan OC, Yang X, Fu Y et al (2006) 16S rRNA gene analyses of bacterial community structures in the soils of evergreen broad-leaved forests in south-west China. FEMS Microbiol Ecol 58(2):247–259
21. Barghouthi SA (2011) A universal method for the identification of bacteria based on general PCR primers. Indian J Microbiol 51 (4):430–444
22. Benga L, Benten WPM, Engelhardt E et al (2014) 16S ribosomal DNA sequence-based identification of bacteria in laboratory rodents: a practical approach in laboratory animal bacteriology diagnostics. Lab Anim 48(4):305–312
23. Chen L, Cai Y, Zhou G et al (2014) Rapid Sanger sequencing of the 16S rRNA gene for identification of some common pathogens. PLoS One 9(2):e88886. https://doi.org/10.1371/journal.pone.0088886
24. Singhania RR (2009) Cellulolytic enzymes. In: Singhnee Nigam P, Pandey A (eds) Biotechnology for agro-industrial residues utilisation. Springer, New York
25. Hussain AA, Abdel-Salam MS, Abo-Ghali HH et al (2017) Optimization and molecular identification of novel cellulose degrading bacteria isolated from Egyptian environment. J Genet Eng Biotechnol 15:77–85
26. Sambusiti C (2013) Physical, chemical and biological pre-treatment to enhance biogas production from lignocellulosic substrates. Dissertation, Politecnico Di Milano
27. Sun L (2015) Biogas production from lignocellulosic: materials microbial community structure. Dissertation, Swedish University of Agricultural Sciences
28. Martin-Ryals AD (2012) Evaluation of the potential for improving anaerobic digestion cellulosic waste via routine bio-augmentation and alkaline pre-treatment. Dissertation, University of Illinois

29. Pinheiro GL, Correa RF, Cunha RS et al (2015) Isolation of aerobic cultivable cellulolytic bacteria from different regions of the gastrointestinal tract of giant land snail *Achatina fulica*. Front Microbiol. https://doi.org/10.3389/fmicb.2015.00860
30. Seo JK, Park TS, Kwon IH et al (2013) Characterisation of cellulolytic and xylanolytic enzymes of *Bacillus licherniformis* JK7 isolated from the rumen of a native Korean goat. Asian Austral J Anim 26(1):50–58
31. Dashtban M, Maki M, Leung KT et al (2010) Cellulase activities in biomass conversion: measurement methods and comparison. Crit Rev Biotechnol 30(4):302–309
32. Akhtar N, Aanchal GD et al (2014) Biodiversity of cellulase producing bacteria and their applications. Cellul Chem Technol 50 (9–10):983–995
33. Deb Dutta S, Tarafder M, Islam R et al (2018) Characterisation of cellulolytic enzymes of *Fusarium* soil isolates. Biocatal Agric Biotechnol 14:279–285. https://doi.org/10.1016/j.bcab.2018.03.011
34. Li Q, Chen X, Jiang Y et al (2015) Cultural, physiological and biochemical identification of Actinobacteria. In: Dhanasekaran D (ed) Actinobacteria—basics and biotechnological Applications. InTech, New York
35. Buszewski B, Rogowska A, Pomastowski P et al (2017) Identification of microorganisms by modern analytical techniques. J AOAC Int 100(6):1607–1623
36. Franco-Duarte R, Cernakova L, Kadam S et al (2019) Advances in chemical and biological methods to identify microorganisms - from past to present. Microorganisms 7(5):130. https://doi.org/10.3390/microorganisms7050130
37. Zhang Q, Dai M, Liu Y et al (2014) Comparison of different methods for isolation of bacterial DNA from retail oyster tissues. J Microb Biochem Technol 6:212–215
38. Abbas HF, Al Musawi IHN (2016) Evaluation three methods of the extraction and purification of bacterial DNA of Gram positive and Gram negative bacteria. World J Exp Biosci 4:62–65
39. Vesty A, Biswas K, Taylor MW et al (2017) Evaluating the impact of DNA extraction method on the representation of human oral bacterial and fungal communities. PLoS One 12(1):e0169877. https://doi.org/10.1371/journal.pone.0169877
40. Wright MH, Adelskov J, Greene AC (2017) Bacterial DNA extraction using individual enzymes and phenol/chloroform separation. J Microbiol Biol Educ 18(2):18.2.48. https://doi.org/10.1128/jmbe.v18i2.1348
41. Ahmed OB, Asghar AH, Elhassan MM (2017) Comparison of three DNA extraction methods forpolymerase chain reaction (PCR) analysis of bacterial genomic DNA. Afr J Microbiol Res 8 (6):598–602
42. Chacon-Cortes D, Griffiths LR (2014) Methods for extracting genomic DNA from whole blood samples: current perspectives. J Biorepos Sci Appl Med 2:1–9
43. Marchesi JR, Sato T, Weightman AJ et al (1998) Design and evaluation of bacteria-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environ Microbiol 64(2):795–799
44. Dhaliwal A (2014) DNA extraction and purification. Labome 3:191. https://doi.org/10.13070/mm.en.3.191
45. Lee PY, Costumbrado J, Hsu CY et al (2012) Agarose gel electrophoresis for the separation of DNA fragments. J Vis Exp 62:e3923. https://doi.org/10.3791/3923



Chapter 14

Cloning and Production of Thermostable Enzymes for the Hydrolysis of Steryl Glucosides in Biodiesel

Andrés Aguirre, Salvador Peiru, Rodolfo Rasia, María Eugenia Castelli, and Hugo G. Menzella

Abstract

Vegetable oil-derived biodiesels have a major quality problem due to the presence of precipitates formed by steryl glucosides, which clog filters and injectors of diesel engines. An efficient, scalable, and cost-effective method to hydrolyze steryl glucosides using thermostable enzymes has been developed. Here, methods to discover, express in recombinant microorganisms and manufacture enzymes with SGase activity, as well as methods to treat biodiesel with such enzymes, and to measure the content of steryl glucosides in biodiesel samples are presented.

Key words Biodiesel, Steryl glucosides, *Thermococcus litoralis*, Thermostable β-glucosidases, Bioprocess development

1 Introduction

There is continuous demand for affordable and sustainable alternatives to petroleum-derived fuels. An environmentally friendly alternative is vegetable oil-derived biodiesel, which consists of methyl esters obtained from the transesterification of oil with short-chain alcohols. First developed in the 90s from rapeseed oil, the production nowadays mostly uses soybean and palm oil, although other feedstocks like sunflower and coconut, and used frying oil are employed depending on the local availability and market prices [1]. Thus, biodiesel is not only renewable but theoretically a limitless source of fuel. Additionally, biodiesel generates a lower amount of particulate residues and less toxic emissions than petroleum-based fuels, because of the lower content of sulfur and aromatic compounds in the exhausted gas emissions [2, 3]. According to the world bioenergy association, compulsory or voluntary biodiesel mandates are set in more than 60 countries [4, 5]. Driven by these and other policies that facilitate the use of renewable

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energy around the world, the biodiesel production is continuously growing, reaching in 2019 a global production of 38.8 million metric tons [6]. Today, biodiesel is mostly used for vehicles in blends with petroleum-based diesel fuels, because they can be consumed by standard fuel-burning engines [7, 8].

A major quality parameter of biodiesel is the content of insoluble material, which causes operational problems by clogging the engine filters and injectors. Thus, the amount of solids present in biodiesel cannot exceed the limits determined by the norms of the American National Standards Institute and European Standard for Biodiesel [9, 10]. The sediments found in biodiesel are linked to the presence of steryl glucosides (SG), compounds naturally present in the vegetable oils used as a feedstock [9–12]. SG and acylated SG (ASG) present in plant tissues are co-extracted with the oil fraction, where they represent the main fraction of unsaponifiable components [13]. In ASG, the 6-position of the sugar moiety is esterified with a long-chain fatty acid. Upon alkaline treatment, the ester bond between the fatty acid and the glucose residue is hydrolyzed. As a result, the amount of SG found in biodiesel is higher than the quantity originally contained in the oil feedstock [10]. The solubility of SG in biodiesel is much lower than that of ASG and a white precipitate is formed [1]. At low temperatures, SG molecules also promote the aggregation of saturated monoacyl glycerides and other compounds, which further reduce the biodiesel fluidity and increase the clogging effect. Aside from the problems caused in engines, SG crystals can precipitate shortly after the transesterification process, blocking pipes and filters in the manufacturing facility, reducing the productivity of the plants, and increasing the production costs [14, 15].

For a given feedstock, the content of SG found in the resultant biodiesel depends on the methods used for the vegetable oil extraction and transesterification conditions [16]. In soybean oil-based biodiesel, the amount of SG varies from 25 to 300 ppm [17]. A biodiesel with a high content of SG appears hazy, and is often rejected by the buyers, since it does not meet the quality standards determined by the filterability tests [18].

The selective removal of SG is required to produce high-quality biodiesel and therefore to facilitate the adoption of this renewable fuel by the users. So far, the only method available to fully remove SG from biodiesel is distillation. The process consumes large amounts of energy, reducing the net energy gain and the cost efficiency of the biodiesel production [19]. A second problem is that distillation destroys natural antioxidants present in oil, affecting the biodiesel stability. Other attempts to eliminate SG from biodiesel including filtering through diatomaceous earth, cold soak filtration, centrifugation, and use of magnesium silicate as an adsorbent require additional removal steps, and are similarly expensive and time-consuming [20].

Enzyme-based refining methods are being rapidly adopted and represent one of the most powerful green chemistry tools, since they require mild working conditions and save energy and water [21]. Many enzymatic processes are today more cost effective than methods based on traditional chemistry, since the specificity of enzymes prevents side reactions and therefore provides higher conversion rates [22]. For example, enzymatic synthesis of biodiesel is commercially available. In the oil industry, a variety of enzymes are today used for soybean oil refining at industrial scale, and provide up to 2.5% of extra oil compared to chemical-based methods [23–28]. Thus, replacing an energy-intensive process like distillation by an enzymatic method to remove SG could make a green fuel even "greener."

We hypothesized that an enzyme with SGase activity added to biodiesel would hydrolyze SG to yield sterols and glucose. In such a case, the released glucose would be water-extracted during the washing step typically used after the transesterification process. Since the generated sterols are soluble in biodiesel, precipitates would no longer be formed. Based on these assumptions and observations, a program to discover enzymes capable of hydrolyzing SG was undertaken by our laboratory.

In 2014, Aguirre and coworkers reported the discovery of the first enzyme with SGase activity (Fig. 1) [2]. During the following years, methods to discover, express in recombinant microorganisms and manufacture enzymes with SGase activity, as well as methods to treat biodiesel with such enzymes and to measure the content of SG in biodiesel samples were developed [3, 7, 8, 14]. All the procedures have been extensively validated at laboratory and industrial scale. In this chapter, we provide a detailed description of these methods and the necessary materials to reproduce them.

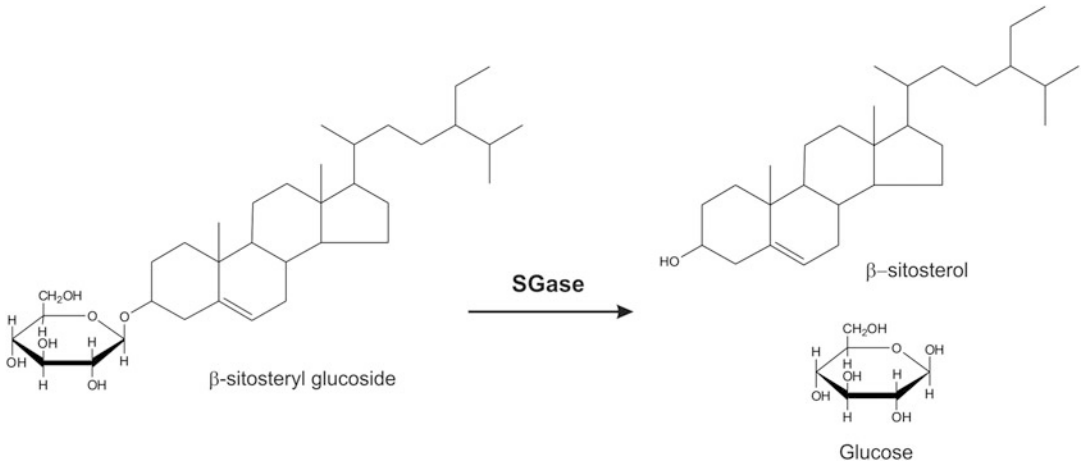


Fig. 1 Schematic representation of the SGase catalyzed reaction, showing the hydrolysis of β-sitosteryl glucoside, the most abundant SG in soybean oil

2 Materials

2.1 Biological and Chemical Materials

1. BL21 AI (DE3) and BL21 (DE3) strains/pET28a system (Novagen).
2. pKCN151 (pET28a:: Tl SGase, freely available upon request, [3]).
3. pGRO7 plasmid for groES-groEL overexpression (Takara).
4. NdeI and EcoRI restriction enzymes (Promega).
5. Ni^{2+}-NTA Agarose resin (Invitrogen).
6. LB agar and Liquid LB medium.
7. Lysis buffer: 50 mM phosphate buffer pH 6.8, 150 mM NaCl, and 1% Triton X-100.
8. Kanamycin (50 mg/mL) and Chloramphenicol (20 mg/mL) stock solutions.
9. HM culture medium [29]: Glycerol 10 g/L, Na_2HPO_4 4 g/L, KH_2PO_4 20.2 g/L, $(NH_4)_2$ HPO_4 3 g/L, K(HO) 3.25 g/L, $MgSO_4.7H_2O$ 1.25 g/L, Yeast Extract 0.5 g/L and 3 mL/L of trace element solution containing (in g/L): $FeCl_3$ 36, $ZnSO_4.7H_2O$ 4.9, $CuSO_4.5H_2O$ 3, MnSO4.$5H_2O$ 10, $Na_2B4O_7.10H_2O$ 0.5, $CaCl_2.2H_2O$ 37.5, $NaMoO_4.2H_2O$ 1, $CoCl_2.6H_2O$ 3, $AlKSO_4.12H_2O$ 2.4; dissolved in 5 M HCl.
10. Nutrient Solution: Glycerol 80% w/v, $MgSO_4.7H_2O$ 20 g/L.
11. Antifoam 204 (Sigma).
12. Standard of SG containing β-sitosteryl glucoside, campesteryl glucoside, and stigmasteryl glucoside (Matreya LLC, 98% pure).
13. SG quantification enzyme mixture: Hexokinase 0.2 units/mL, Glucose-6-phosphate dehydrogenase 0.1 units/mL, 1 mM ATP, 100 μM NADP from Sigma.

2.2 Equipment

1. Orbital Shaker.
2. Bioreactor with pH, temperature, and dissolved oxygen control systems.
3. Centrifuge for cell separation.
4. Ultrafiltration system with 10 kDa MWCO cartridges.
5. Spectrophotometer-Fluorescence microplate reader.
6. Sonicator or French press to disrupt *E. coli* cells.
7. Magnetic stirrer with hot plate temperature control.
8. Peristaltic pump with Viton tubing.

9. 1 L reactor equipped with temperature and agitation control (alternatively, an Erlenmeyer with a hotplate magnetic stirrer can be used).
10. Benchtop centrifuge.

3 Methods

The method described below was used to identify more than ten enzymes with SGase activity. Considering the relaxed specify of type III β-glycosidases family of enzymes found in our experiments and by others for gingenosides [30], the same method may be suitable to find enzymes for the hydrolysis of glucosides carrying other aglycones by setting the appropriate constraints.

3.1 Discovery of Thermostable Enzymes with SGase Activity

SGs are a family of compounds characterized by a sugar moiety attached to the 3 β-hydroxy group of a sterol. Our search for SGases aimed to find out enzymes to hydrolyze the most abundant SG in vegetable oil-derived biodiesel: β-sitosteryl, campesteryl, and stigmasteryl glucosides. In addition, thermophilic enzymes with an optimal temperature near 65 °C are preferred, since the insertion of the enzymatic treatment step in large-scale plants does not require major modifications in the plant (*see* Subheading 4).

The family of thermophilic type III β-glycosidases, comprising enzymes that recognize the glucose moiety and display a relaxed specificity for the aglycone portion of the molecule, is a rich source of SGases. For example, LacS from *Sulfolobus solfataricus* and WP_004069094.1 from *Thermococcus litoralis* were enzymes meeting both criteria and further identified as SGases. A search for new thermostable enzymes with SGase activity can be performed among homologs to these enzymes in thermophilic enzymes databases and the selected digital sequences tested as indicated below.

3.1.1 Cloning and Expression of Putative SGases

1. Select a pool of protein sequences following the abovementioned criteria, and redesign the encoding DNA sequences to optimize the codon usage for expression in *E. coli* using the OptimumGeneTM algorithm (GenScript). The resulting sequences should be synthesized adding an NdeI restriction site overlapping the ATG start codon and an EcoRI site downstream the stop codon. If present, both restriction sites must be erased from the redesigned gene sequence by using an alternative synonymous codon overlapping the restriction site, to allow the insertion of the ORF into the expression vector.
2. Insert the synthetic gene into the NdeI and EcoRI sites of the T7-based pET28a expression plasmid to obtain an N-terminal fusion to a His_6 tag.

3. Transform the expression plasmids into the *E. coli* BL21(DE3) strain for expression tests.
4. Pick three transformant colonies from each plate and grow overnight at 37 °C in liquid LB medium with 50 mg/L of kanamycin for plasmid maintenance.
5. Dilute overnight cultures 1:100 in 100 mL of fresh LB medium, grow in 1 L Erlenmeyer flasks with agitation to an OD_{600} of 0.5 and add IPTG to a final concentration of 0.5 mM.
6. Continue the incubation for 6 h at 22 °C with agitation and harvest the cells.
7. Suspend the cell pellet in cold 50 mM phosphate buffer pH 6.8, 150 mM NaCl, and 1% Triton X-100, and disrupt by sonication.
8. Clarify crude extracts by centrifugation during 30 min at 15,000 × *g* and analyze 10 μL of the soluble and insoluble fractions of the cell lysates by SDS–PAGE. If the target protein is expressed mostly as insoluble aggregates, repeat **steps 3**–7 co-transforming the expression strain with plasmid pGro7. This plasmid is designed to express chaperones GroES-GroEL in the presence of 1 mg/mL of L-Arabinose and proved to increase solubility of *T. litoralis* SGase and related enzymes [7].
9. Purify the enzymes by affinity chromatography, using Ni^{2+}-NTA Agarose resin following the manufacturer instructions. Process the eluted fractions containing the corresponding recombinant enzyme by diafiltration using a UF hollow fiber cartridge to obtain a final protein concentration of 1 mg/mL in ammonium acetate buffer pH 5.5.

3.1.2 SGase Activity Test

1. Prepare a 5 mg/mL stock solution in 3:1 THF:H_2O of a commercial mixture of SGs.
2. Add 60 μL of the SGs stock solution (final SG concentration: 100 ppm) to 3.0 mL of 50 mM phosphate buffer pH 6 in a 5 mL screw capped vial, in the presence of 0.1% Triton X-100 to facilitate SGs dissolution. Start the reaction by the addition of 14 μg of enzyme/mL buffer and incubate at 70 °C for 2 h.
3. Stop the reaction by chilling the reaction tubes on ice and measure the hydrolysis of SGs using the fluorescence-based method described in the Subheading 3.2 protocol.

3.2 Fluorometric Assay for Quantification of Steryl Glucosides in Biodiesel

Quantification of SGs is achieved using *T. litoralis* SGase β-glycosidase to treat the biodiesel sample, followed by glucose quantification. The protocol is performed in two steps. First, the sample is treated with the enzyme in a mixed water:biodiesel system. After treatment and complete hydrolysis, the glucose released

from the SG is quantified in a second step by a coupled reaction of with hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6PDH).

In this reaction, one mole of NADPH is produced per mole of glucose present in the sample. In order to achieve a high sensitivity, the fluorescence of NADPH at 460 nm upon excitation at 360 nm is measured and its concentration in the sample is calculated based on a calibration curve performed in the same conditions. Whereas the method can be set up using a standard fluorometer, the use of a microplate reader allows for the simultaneous acquisition of the data from the sample and the calibration curve.

3.2.1 Biodiesel Treatment

1. Take a 10 mL biodiesel sample.
2. Add 15 μg of *T. litoralis* SGase in 1.5 mL of 100 mM citrate buffer, pH 5.5.
3. Agitate the mixture thoroughly (400 rpm) with a magnetic stirrer at 65 °C, in order to maximize the water:biodiesel interface, for 3 h. The glucose released is partitioned in the water phase.

3.2.2 Glucose Quantification

1. Prepare reference glucose samples with 0, 7.5, 15, 30, 60, and 120 μM glucose in water.
2. Centrifuge the treated sample 1′ at 1,000 × *g* to facilitate phase separation.
3. Dispense 65 μL of water into the microplate wells. Six wells are needed for the calibration curve and 3 wells to quantify each biodiesel sample.
4. Dilute in each well 5 μL of the water phases of each biodiesel sample to be measured (triplicates) and the same volume of glucose reference sample.
5. Add 130 μL of the SG quantification enzyme mixture to the wells. Incubate at room temperature for 10 min in the dark for the reaction to complete.
6. Measure fluorescence at 455 nm with excitation 335 nm on the wells on a microplate reader.

3.2.3 SG Content Calculation

1. Graph the obtained fluorescence intensity vs. glucose concentration for the reference samples. The graph should be linear. Obtain the slope and the zero intercept by linear regression.
2. Using the parameters obtained for the calibration curve, calculate the glucose concentration on each of the biodiesel samples.
3. The concentration of SG in the original biodiesel sample can be calculated with the following formula:

SG (ppm) = [Glucose](mol/L)*(1.5/10)*577 (g SG/mol)*1000 (mg/g)

$$\mathrm{SG(ppm)} = \frac{[\mathrm{Glucose}](\mathrm{M})\cdot 1.5}{10}\cdot 577(\mathrm{gSG/mol}) * 1000\left(\frac{\mathrm{mg}}{\mathrm{g}}\right)$$

3.3 Production of SGase Enzymes in Fed Batch Fermentations

The following protocol provides instructions to produce *T. litoralis* SGase at 1000 L scale, but similar results are routinely obtained in the laboratory at 2 L scale using a volume reduction factor of 500.

3.3.1 Stock Culture

1. Transform by electroporation the *E. coli* BL21AI with plasmids pKCN151 and pGro7 and select in LB agar plates containing 50 mg/L Kanamycin and 20 mg/L Chloramphenicol.
2. Grow a colony of the recombinant clone on 1 L of LB medium at 37 °C in a 5 L shake flask until cell density reaches an $OD_{600} = 1$, add sterile glycerol up to 30% w/v, and store 50 mL aliquots in tubes at −80 °C.

3.3.2 Seed Culture Preparation

1. Thaw a 50 mL stock culture tube and transfer to a 5 L flask containing 1 L of LB medium supplemented with 10% w/v glycerol, 50 mg/L Kanamycin, and 20 mg/L Chloramphenicol. Grow the culture until cell density reaches an $OD_{600} = 4$.
2. Transfer to a 15 L bioreactor containing 10 L of HM medium. Grow for 10 h at 35 °C maintaining dissolved oxygen above 30% and pH at 7 by the addition of 28% $NH_4(OH)$.

3.3.3 Fermentation Process

1. Transfer 10 L of the seed culture to a 1000 L bioreactor containing 700 L of HM medium and grow at 35 °C until glycerol exhaustion.
2. Initiate an exponential feeding of a Nutrient Solution at a rate sufficient to maintain the specific grow rate at a value of $0.25 \pm 0.02\ h^{-1}$.
3. When OD_{600} reaches a value of 80, add 0.4 g/L L-arabinose and feed with nutrient solution at a constant rate of 25 ± 1 L/h for 10 h. Test activity in the culture supernantant as decribed (*see* **Note 1**)
4. Keep dissolved oxygen concentration above 30% of saturation throughout the entire process by enriching the air stream with pure oxygen when necessary.

   Maintain pH at 6.9–7 by the addition of 28% NH_4OH. SGase activity is measured every 1 h.

3.3.4 Enzyme Recovery

1. Stop the feeding pump and set the bioreactor agitation rate at 60 rpm.
2. Add 8 kg of lauryl alcohol ethoxylate—7 MOL to the fermentation broth and open the jacket steam valve until the vessel temperature reaches 80 °C. After 15 min, open the refrigeration valve to cool the broth to 30 °C, separate the solids using a disk stack centrifuge at 5000 × *g*.

3. Concentrate the clarified liquid (800 L) by ultrafiltration using a 10 kDa hollow fiber cartridge to a final volume of 120 L.
4. Replace the broth by diafiltration adding 600 L of 20 mM citrate buffer pH 6.5 at the necessary rate to keep the volume constant at 120 L. Polish the resulting solution with a 0.2 μm filter and save in clean plastic containers. The preparation can be stored at room temperature for at least 24 months.

3.4 Enzymatic Treatment of Biodiesel with SGase Enzymes

We describe here an efficient process for the enzymatic removal of SG from commercial crude biodiesel produced from vegetable oils, in order to improve the quality of this fuel in a cost-effective manner. The process is described at a 1 kg scale, but it was validated from scales spanning from 3 g to 30 tons. The treated biodiesel complies with the most stringent filtration tests with values undistinguishable from those obtained for distilled biodiesel [3].

3.4.1 SGase Treatment

1. Immediately before sampling biodiesel, mix the biofuel container by shaking or stirring in order to homogenize it.
2. Transfer a defined quantity of biodiesel (by weight) to the reactor.
3. Set reactor temperature at 65 °C.
4. Add 75 mL of 20 mM phosphate buffer pH 6.75 and 20 mM NaCl per kg of biodiesel.
5. Set the reactor stirrer at a speed high enough to obtain a visually homogeneous water/biodiesel emulsion (but not much higher).
6. Add 7 mg of SGase enzyme/kg biodiesel. Incubate for 3 h maintaining the agitation.
7. Take samples (1 mL) every 1 h. Centrifuge for 2 min at 13,000 × *g* on a benchtop centrifuge. Use the aqueous (lower) phase for the fluorescent quantification assay as described in Subheading 3.2.
8. Biodiesel drying.
   (a) Turn reactor speed and temperature off, and wait 10–15 min until the two phases are completely separated. Transfer the upper phase (biodiesel) to a clean 1-L tempered glass lab bottle using glass pipette and a peristaltic pump with Viton tubing.
9. Dry biodiesel by carefully placing the glass bottle over a hot plate magnetic stirrer and incubate for 1 h at 110 °C with agitation.
10. Turn temperature off, and keep the bottle at room temperature. Figure 2 shows the aspect of biodiesel treated with *T. litoralis* SGase and a control treated with the buffer with no enzymes. The quality of the treated biodiesel can be futher



Fig. 2 Biodiesel treated with *T. litoralis* SGase (left) and negative control (right)

asseses as described (*see* **Notes 2** and **3**). If the obained results are poor, repeat the cycle at different working temperature (*see* **Note 4**)

4 Notes

1. Quick assay to monitor enzyme activity during the fermentation:

   Preincubate 465 μL of SGase buffer (sodium citrate 25 mM pH 6.1, 20 mM NaCl, 0.1% Triton X-100), with 10 μL of the appropriate dilution (1/30 to 1/500 in SGase buffer) of the enzyme-containing sample for 5 min at 80 °C. Add 25 μL of 20 mM *p*-nitrophenyl-β-D-glucopyranoside (pNPG), and incubate at 80 °C for 5 min. The reaction is stopped by adding 1 mL of cold 200 mM sodium carbonate. The activity of SGase is estimated spectrophotometrically by reading the absorbance of the liberated *p*-nitrophenol at 405 nm ($\varepsilon = 18,700$). One unit (U) is defined as the amount of enzyme required for the hydrolysis of 1 μmol pNPG/min, under the assay conditions.

2. The impact of eliminating SG from biodiesel can be verified by assaying quality tests to treated samples in parallel with control ones, such as the Total Contamination test, the Cold Soak Filtration Test, the Filter Blocking Tendency test, and the Cold Soak Filter Blocking Tendency test, according to the procedures described in the EN 12662, ASTM D7501, ASTM D2068, and CAN/CGSB-3.524-2011 norms, respectively.

3. Depending on the amount of natural antioxidant molecules present in the oil used as a feedstock, a variable degree of biodiesel oxidation has been detected in a few cases. We found out that this oxidation can be avoided by drying the biodiesel at 70 °C and extending the incubation time.
4. We search for enzymes with optimum temperature near 65 °C because the biodiesel is washed at this temperature after the transesterification step, avoiding in this way a heating or cooling step which might add an extra cost at industrial scale. Thus, when possible we focus the search looking for genes in microorganisms with optimal growth temperature close to 65 °C. Obviously, in some cases enzymes with optimum temperature far from 65 °C possess a residual activity sufficient to complete the SG hydrolysis. For example, the optimal temperature for LacS from *S. solfataricus* is near 100 °C, but the SGase specific activity at 65 °C is one of the highest we ever found.

References

1. Lacoste F et al (2009) Quantification of free and esterified steryl glucosides in vegetable oils and biodiesel. Eur J Lipid Sci Technol 111 (8):822–828
2. Aguirre A et al (2014) Enzymatic hydrolysis of steryl glucosides, major contaminants of vegetable oil-derived biodiesel. Appl Microbiol Biotechnol 98(9):4033–4040
3. Peiru S et al (2015) An industrial scale process for the enzymatic removal of steryl glucosides from biodiesel. Biotechnol Biofuels 8:223
4. Jim L (2019) The Digest's Biofuels Mandates Around the World 2020
5. Aguirre A et al (2018) The production, properties, and applications of thermostable steryl glucosidases. World J Microbiol Biotechnol 34(3):40
6. OECD/FAO, Agricultural outlook 2019–2028. 2019: Food and Agriculture Organization of the United Nations OECD Publishing
7. Eberhardt F et al (2017) Strain engineering and process optimization for enhancing the production of a thermostable steryl glucosidase in Escherichia coli. J Ind Microbiol Biotechnol 44(1):141–147
8. Eberhardt F et al (2018) Pilot-scale process development for low-cost production of a thermostable biodiesel refining enzyme in Escherichia coli. Bioprocess Biosyst Eng:1–10
9. Pfalzgraf L et al (2007) The effect of minor components on cloud point and filterability. Inform Supplement Biorenewable Resources 4:17–21
10. Van Hoed V et al (2008) Identification and occurrence of steryl glucosides in palm and soy biodiesel. J Am Oil Chem Soc 85:701–709
11. Bondioli P, Cortesi N, Mariani C (2008) Identification and quantification of steryl glucosides in biodiesel. Eur J Lipid Sci Technol 110 (2):120–126
12. Moreau RA, Scott KM, Haas MJ (2008) The identification and quantification of steryl glucosides in precipitates from commercial biodiesel. J Am Oil Chem Soc 85:761–770
13. Sugawara T, Miyazawa T (1999) Separation and determination of glycolipids from edible plant sources by high-performance liquid chromatography and evaporative light-scattering detection. Lipids 34(11):1231–1237
14. Menzella H, Peiru S, Vetcher L (2012) Enzymatic removal of steryl glycosides. PCT/US2013/031769
15. Soe JB (2010) Method. WO 2010004423 A2
16. Lee I Process of producing biodiesel and biodiesel produced therefrom. World patent application 2007/076163
17. Ringwald SC (2007) Biodiesel characterization in the QC environment. The 98th AOCS annual meeting abstracts. AOCS Press, Urbana, p 15
18. Srinophakun PSMSATP (2017) The response surface optimization of steryl glucosides removal in palm biodiesel using silica adsorption. Fuel 191:1–9

19. Tang H et al (2010) Comparing process efficiency in reducing steryl glucosides in biodiesel. J Am Oil Chem Soc 87(87):337–345
20. Brask J, Nielsen R (2010) Enzymatic removal of steryl glycosides in fatty acid alkyl esters. WO2010102952 A1
21. Menzella HG et al (2010) Using chemobiosynthesis and synthetic mini-polyketide synthases to produce pharmaceutical intermediates in Escherichia coli. Appl Environ Microbiol 76(15):5221–5227
22. Rodriguez E, Menzella HG, Gramajo H (2009) Heterologous production of polyketides in bacteria. Methods Enzymol 459:339–365
23. Elena C et al (2014) Expression of codon optimized genes in microbial systems: current industrial applications and perspectives. Front Microbiol 5:21
24. Elena C et al (2016) Pichia pastoris engineering for the production of a modified phospholipase C. Process Biochem 51(12):1935–1944
25. Ravasi P et al (2015) High-level production of Bacillus cereus phospholipase C in Corynebacterium glutamicum. J Biotechnol 216:142–148
26. Cerminati S et al (2017) Development of a highly efficient oil degumming process using a novel phosphatidylinositol-specific phospholipase C enzyme. Appl Microbiol Biotechnol 101(11):4471–4479
27. Cerminati S et al (2019) Industrial uses of phospholipases: current state and future applications. Appl Microbiol Biotechnol 103 (6):2571–2582
28. Cerminati S et al (2018) The betagammacrystallin domain of Lysinibacillus sphaericus phosphatidylinositol phospholipase C plays a central role in protein stability. Appl Microbiol Biotechnol 102(16):6997–7005
29. Menzella HG, Gramajo HC (2004) Recombinant protein production in high cell density cultures of Escherichia coli with galactose as a gratuitous inducer. Biotechnol Prog 20 (4):1263–1266
30. Park CS et al (2010) Biotransformation of ginsenosides by hydrolyzing the sugar moieties of ginsenosides using microbial glycosidases. Appl Microbiol Biotechnol 87(1):9–19



Chapter 15

Recombinant Protein Production and Purification Using Eukaryotic Cell Factories

Asmaa Missoum

Abstract

Cloning proteins enables their production and characterization for further studies. This requires inserting the gene of the studied protein to be inserted in a vector, which then will be transformed to the host cell used as "factory." Consequently, the "biomass" of host cells will be produced using bioreactors. Here we describe the production of *Rhizomucor miehei* lipase (RML) by cloning the corresponding genes in the yeast *Pichia pastoris*. This enzyme is used as a biocatalyst for biofuel production. The successfully produced recombinant proteins are then purified using ion exchange chromatography.

Key words *Pichia pastoris*, Cloning, Recombinant protein, *Rhizomucor miehei*, Lipase, Fermentation, Ion exchange chromatography, SDS-PAGE

1 Introduction

A "recombinant" protein refers to a protein that is produced by a cell whose genetic material has been modified by genetic recombination. The gene encoding the protein of interest is introduced into the genome of the producer species (bacteria, yeast, cultured mammalian cells, etc.) [1]. Recombinant proteins can be purified and used for therapeutic, industrial, or even research activities. This has provided important breakthroughs in various biotechnological applications [2]. However, the choice of the microbial expression system crucially depends on the nature of the produced protein. Nevertheless, new engineering strategies were employed to solve issues such as incompatibility between microbial translation machineries and eukaryotic protein architecture [3]. Yeast systems are easy to manipulate genetically, require cost-effective media, and provide posttranslational modifications. For instance, *Pichia pastoris* can achieve high cell densities, provide reduced protein glycosylation, and secrete properly folded proteins, simplifying their recovery [4].

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Here we demonstrate that *Rhizomucor miehei* lipase (RML) can be efficiently produced using a recombinant *P. pastoris* strain X33. This enzyme catalyzes the biotransformation of fatty acids or acyl glycerides from microalgae oil into biodiesel [5, 6] (Fig. 1). A similar procedure has been used to produce and purify *Leptosphaeria maculans* avirulence proteins, in order to study their three-dimensional structure and elaborate on their infectious mechanism [7]. Combining protocols used in this research project with those concerning *Rml* gene and plasmid construction [5, 8] would optimize for better lipase production.

Thus, the obtained recombinant lipases can be purified using ultrafiltration and ion exchange chromatography, and then analyzed via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) [9]. Subsequently, many researchers have also attained similar results and in a quantitative manner.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Culture Media

1. Yeast extract Peptone Dextrose medium (**YPD**) composition for 1 L: 1% yeast extract, 2% peptone, 2% dextrose (glucose), 2% agar, and 100 μg/mL Zeocin.
2. Yeast extract Peptone Dextrose medium (**YPDS**) composition for 1 L: 1% yeast extract, 2% peptone, 2% dextrose (glucose), 1 M sorbitol, 2% agar, and 100 μg/mL Zeocin.
3. Dissolve the following in 900 mL of water: 10 g yeast extract, 20 g of peptone, and 182.2 g of **sorbitol** in case of **YPDS**.
4. Include 20 g of agar if making YPD slants or plates.
5. Autoclave for 20 min on liquid cycle.
6. Cool solution to ~60 °C and add 100 mL of 10× Dextrose. Add 1.0 mL of 100 mg/mL Zeocin, if desired (*see* **Note 1**).

2.2 Construction of Expression Vector

1. *Escherichia coli TOP10* (Invitrogen, USA).
2. Yeast expression vector pPICZαA (Invitrogen, USA) (Fig. 2).
3. Lipase encoding gene *rml* gene from *R. miehei* (GenBank accession number A02536.1) [10].
4. PCR machine (thermocycler) and *Taq*-polymerase.
5. DNA restriction enzymes and T_4 DNA ligase (2.5 units/μL).
6. Electroporator or water bath (42 °C).

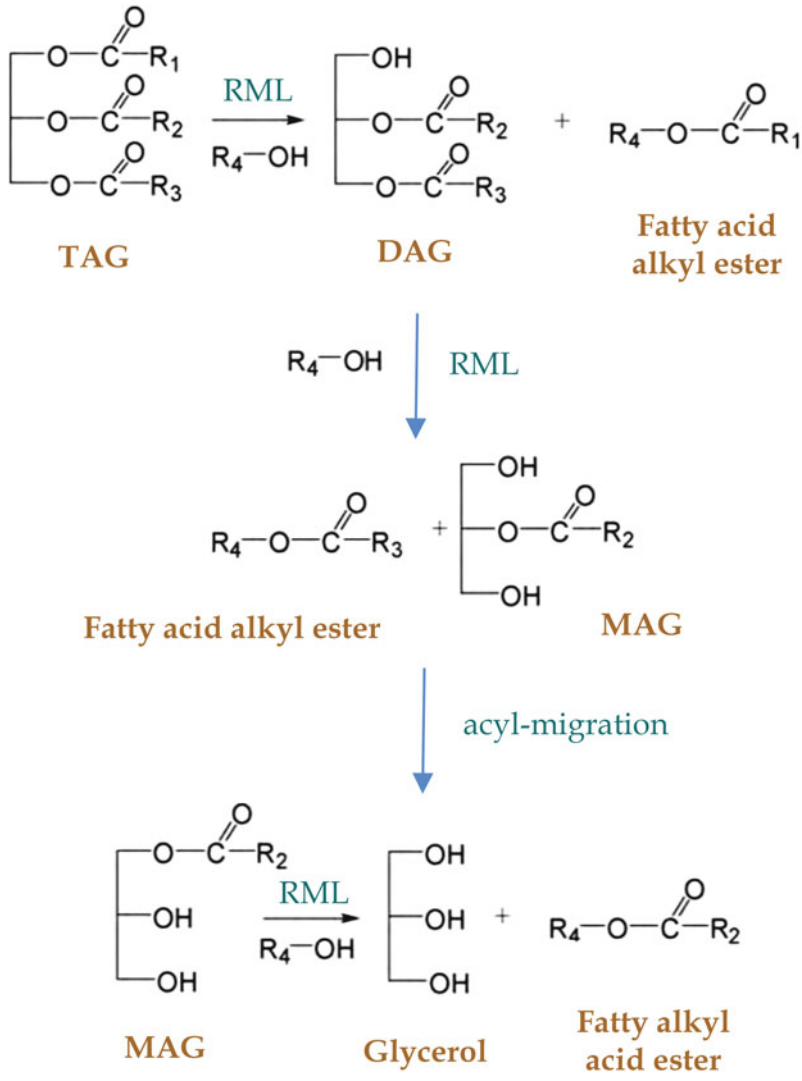


Fig. 1 Schematic transesterification processes catalyzed by RML, where tri-acyl glycerol (TAG), di-acyl glycerol (DAG), and mono-acyl glycerol (MAG) are converted to fatty acid alkyl esters such as FAME (fatty acid methyl ester). These are the major components of microalgae oil, which is one of the most important renewable resources of the environment-friendly biofuel (reproduced from refs. 5, 6)

7. Low Salt LB (Luria-Bertani) Medium with 25 μg/mL Zeocin (1% Tryptone, 0.5% Yeast Extract, 0.5% NaCl, pH 7.5):
   (a) For 1 L, dissolve the following in 950 mL deionized water: 10 g tryptone, 5 g yeast extract, and 5 g $NaCl_2$.
   (b) Adjust the pH of the solution to 7.5 with 1 N NaOH and bring the volume up to 1 L.
   (c) Autoclave for 20 min at 15 lb./sq. in and 121 °C. Let cool to ~55 °C and add desired antibiotics at this point. Add Zeocin to 25 μg/mL final concentration.
   (d) Store medium at 4 °C and in the dark.

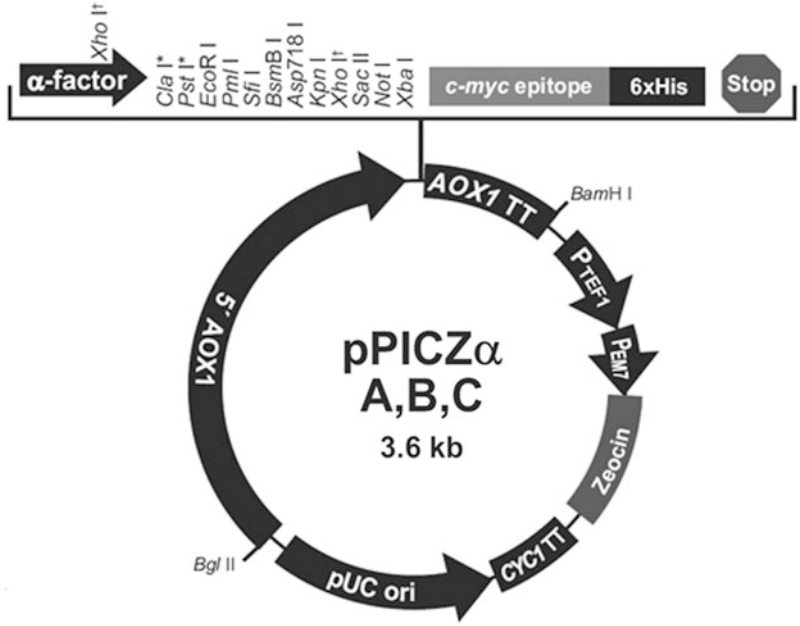


Fig. 2 Schematic drawing of lipase expression plasmids adapted from Invitrogen (USA). Some of the features in pPICZαA include: 5′ *AOXI* promoter region, α-factor signal sequence, multiple cloning site, *c-myc* epitope, polyhistidine (6×his) tag, 3′ *AOXI* priming site, 5′ *AOXI* transcription termination region, and zeocin resistance gene for selection. The AOX1 promoter allows methanol-inducible, high-level expression in *Pichia* (reproduced from Invitrogen's *pPICZα A, B, and C* Manual)

8. Low salt LB plates with 25 μg/mL Zeocin:
   (a) Make Low Salt LB Medium above and add 15 g/L agar before autoclaving.
   (b) Autoclave for 20 min at 15 lb./sq. in.
   (c) Let cool to ~55 °C and add desired antibiotics at this point. Add Zeocin to 25 μg/mL final concentration.
   (d) Pour into 10 cm petri plates. Let the plates harden, then invert, and store at 4 °C and in the dark. Plates containing Zeocin are stable for 1–2 weeks.
9. 37 °C rotary shaking incubator.
10. DNA extraction and purification kit (Invitrogen, USA).
11. Digestion enzymes: *SacI*, *PmeI*, or *BstXI*.
12. EDTA (1 mM).
13. 3 M sodium acetate.
14. Phenol/chloroform.
15. 80% and 100% ethanol.

2.3 Transformation of P. pastoris by Electroporation

1. *Pichia pastoris X33* (Invitrogen, USA).
2. 5–10 μg pure pPICZαA ligated with lipase gene (*see* **Note 2**).
3. YPD Medium.
4. 30 °C incubator.
5. 50-mL conical polypropylene tubes.
6. 1 L cold (4 °C) sterile water (place on ice the day of the experiment).
7. 25 mL cold (4 °C) sterile 1 M sorbitol (place on ice the day of the experiment).
8. Electroporation device and 0.2 cm cuvettes.
9. YPDS plates containing 100 μg/mL Zeocin (*see* Subheading 2.1).

2.4 Screening Target Transformants

1. Minimal Dextrose (MD) agar plates (1.34% YNB, 4×10^{-5}% biotin, 2% dextrose):
   (a) Autoclave 800 mL of water for 20 min on liquid cycle.
   (b) Cool to about 60 °C and then add: 100 mL of 10× YNB, 2 mL of 500× Biotin, and 100 mL of 10× Dextrose.
   (c) Add 15 g agar to the water in **step (a)** and mix well. Then, pour the plates immediately. MD stores well for several months at 4 °C.
2. Minimal Methanol (MM) agar plates (1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol):
   (a) Autoclave 800 mL of water for 20 min on liquid cycle.
   (b) Cool to 60 °C and add: 100 mL of 10× YNB, 2 mL of 500× Biotin, and 100 mL of 10× Methanol.
   (c) Add 15 g agar to the water in **step (a)** and mix well. Then, pour the plates immediately. MM stores well for several months at 4 °C.
3. Sterile toothpicks and Scoring Templates.
4. Streak out the strains GS115 Albumin (Mut^S) and pPICZ (Mut^+) on an MD plate as controls for Mut^+ and Mut^S growth.

2.5 Expression of Recombinant Lipase

1. 100% methanol, Yeast Nitrogen Base (YNB), Glycerol (GY).
2. Sterile cheesecloth or gauze.
3. Buffered Glycerol-complex Medium (**BMGY**) composition for 1 L: 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol.
4. Buffered Methanol-complex Medium (**BMMY**) composition for 1 L: 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol.

5. Dissolve 10 g of yeast extract, 20 g peptone in 700 mL water.
6. Autoclave 20 min on liquid cycle.
7. Cool to room temperature, then add the following and mix well: 100 mL 1 M potassium phosphate buffer pH 6.0, 100 mL 10× YNB, 2 mL 500× Biotin, 100 mL 10× GY 4.
8. For BMMY, add 100 mL 10× Methanol instead of glycerol.
9. Store media at 4 °C. The shelf life of this solution is approximately 2 months.

2.6 Protein Purification

1. Filter of 0.45 μM membrane.
2. NaOH, HCl, and sterile distilled water.
3. 3 M Imidazole (*see* **Note 3**).
4. Ion exchange chromatography column (ProBond resin, 5 mL).
5. For 200 mL of 5× Purification Buffer (250 mM NaH_2PO_4, pH 8.0 and 2.5 M NaCl): add 7 g of NaH_2PO_4 and 29.2 g NaCl to 180 mL of deionized water. Then, mix well and adjust the pH with NaOH to pH 8.0. Bring the final volume to 200 mL with deionized water.
6. For 30 mL of Binding Buffer (with 10 mM imidazole): combine 30 mL of 1× Purification Buffer and 100 μL of 3 M Imidazole at pH 6.0. Mix well and adjust pH to 8.0 with NaOH or HCl (*see* **Note 4**).
7. For 50 mL of Wash Buffer (with 20 mM imidazole): combine 50 mL of 1× Purification Buffer and 335 μL of 3 M Imidazole at pH 6.0. Mix well and adjust pH to 8.0 with NaOH or HCl.
8. For 15 mL of Elution Buffer (with 250 mM imidazole): combine 13.75 mL of 1× Purification Buffer and 1.25 mL of 3 M Imidazole at pH 6.0. Mix well and adjust pH to 8.0 with NaOH or HCl (*see* **Note 5**).

2.7 SDS-PAGE Analysis

1. Sample 5× loading buffer [250 mM Tris–HCl, pH 6.8, 10% (w/v) SDS, 0.5% (w/v) BPB, 50% (v/v) glycerin, 5% (w/v) β-mercaptoethanol].
2. Coomassie Brilliant Blue stain.
3. Polyacrylamide gel powder, deionized water.
4. Electrophoresis apparatus.

3 Methods

Carry out all procedures at room temperature unless otherwise specified and in sterile conditions.

3.1 Construction of Expression Vector

1. Design the expression vector (Fig. 2). Then, prepare a set of primers corresponding to the sequences at 5′ and 3′ ends of the selected lipase coding region with suitable enzyme restriction sites [11].
2. Amplify the coding region of lipase by PCR.
3. Ligate the PCR product with the expression vector after restriction digestion of the PCR product.
4. Transform the cloned vector into *E. coli* competent cells by electroporation (2.5 kV, 129 ohms, 5.0 ms) or chemical activation (heat shock at 42 °C for 45 s using $CaCl_2$-treated competent cells) [11].
5. Plate 10 and 100 μL of the transformation mix on Low Salt LB medium with 25 μg/mL Zeocin.
6. Incubate overnight at 37 °C.
7. Select 10 Zeocin-resistant transformants and inoculate into 2 mL Low Salt LB medium with 25 μg/mL Zeocin. Grow overnight at 37 °C with shaking. Remember also to isolate control strains for background protein expression in *Pichia*.
8. Isolate plasmid DNA by miniprep for restriction analysis and sequencing (Invitrogen, USA).
9. For safekeeping, make a glycerol stock of purified clone (*see* **Note 6**).
10. Once you have purified plasmid DNA, proceed to preparing transforming DNA:
    (a) Digest ~5–10 μg of plasmid DNA with one of the restriction these enzymes: *Sac I* (209 bp), *Pme I* (414 bp), and *BstX I* (707 bp) (*see* **Note 7**).
    (b) If the vector is completely linearized, heat inactivate or add EDTA to stop the reaction, phenol/chloroform extract once, and ethanol precipitate using 1/10 volume 3 M sodium acetate and 2.5 volumes of 100% ethanol.
    (c) Centrifuge the solution to pellet the DNA, wash the pellet with 80% ethanol, air-dry, and resuspend in 10 μL sterile, deionized water. Use immediately or store at −20 °C.

3.2 Preparation of Competent Cells

1. Grow 5 mL of your *P. pastoris* strain *X33* in YPD in a 50 mL conical tube at 28 °C and 200 rpm overnight.
2. Inoculate 500 mL of fresh medium in a 2 L flask with 0.1–0.5 mL of the overnight culture. Grow overnight again to an $OD_{600} = 1.3–1.5$.
3. Centrifuge the cells at 2000 × *g* for 5 min at 4 °C. Resuspend the pellet with 500 mL of ice-cold (0 °C), sterile water.
4. Centrifuge the cells as in **step 3**, then resuspend the pellet with 250 mL of ice-cold (0 °C), sterile water.

5. Centrifuge the cells as in **step 3**, then resuspend the pellet in 20 mL of ice-cold (0 °C) 1 M sorbitol.
6. Centrifuge the cells as in **step 3**, then resuspend the pellet in 1 mL of ice-cold (0 °C) 1 M sorbitol for a final volume of approximately 1.5 mL (*see* **Note 8**).

3.3 Transformation of P. pastoris

1. Mix 80 μL of the cells from **step 6** (*see* Subheading 3.1) with 5–10 μg of linearized plasmid DNA (in 5–10 μL sterile water). Then, transfer them to an ice-cold 0.2 cm electroporation cuvette (For circular DNA, use 50–100 μg).
2. Incubate the cuvette with the cells on ice for 5 min.
3. Pulse the cells for 5 ms and at 1.5–2 kV.
4. Immediately add 1 mL of ice-cold 1 M sorbitol to the cuvette. Transfer the cuvette contents to a sterile 15-mL tube and incubate at 30 °C without shaking for 1–2 h.
5. Spread 10, 25, 50, 100, and 200 μL each on separate, labeled YPDS plates containing 100 μg/mL Zeocin. Plating at low cell densities favors efficient Zeocin selection (*see* **Note 9**).
6. Incubate plates from at 28 °C for 48–72 h (or even for 10 days) until colonies form.
7. Pick 10–20 colonies and purify (streak for single colonies) on fresh YPD or YPDS plates containing 100 μg/mL Zeocin.

3.4 Screening and Analyzing Target Transformants

1. To select putative multi-copy recombinants, plate the transformation mix on increasing concentrations of Zeocin:
   (a) Prepare YPDS plates containing 500, 1000, and 2000 μg/mL Zeocin.
   (b) Plate 100–200 μL of the transformation mix on each plate and incubate at 28–30 °C for 48 h.
2. Select 6–10 of Zeocin-resistant *Pichia* transformants and test for the Mut phenotype (*see* **Note 10**) as the following:
   (a) Using a sterile toothpick, pick one colony and streak or patch one Zeocin-resistant transformant in a regular pattern on an MM plate first, and then an MD plate.
   (b) Using a new toothpick for each transformant, continue patching 10 transformants (1 plate).
   (c) To differentiate Mut^+ from Mut^S, make one patch for each of the controls (GS115/Mut^S Albumin and pPICZ Mut^+) onto the MD and MM plates.
   (d) Incubate the plates at 30 °C for 2 days or longer.
   (e) After incubation, score the plates. Mut^+ strains will grow normally on both plates, while Mut^S strains will grow normally on the MD plate but show little or no growth on the MM plate.

3. You may test for expression of the protein using SDS-PAGE analysis, western blot, or/and functional assay (*see* next sections).
4. You may also analyze for the presence of insert and copy number by performing real-time PCR (*see* **Note 11**).

3.5 Expression of Recombinant Lipase

1. Using a single colony, inoculate 25 mL of **BMGY** medium in a 250 mL baffled flask. Grow at 29 °C in a shaking incubator (under constant agitation at 200–220 rpm) until the culture reaches an OD_{600} = 3–5 (log-phase growth, approximately 18–20 h).
2. Harvest the cells by centrifuging at 1500–3000 × *g* for 5 min at room temperature. Decant the supernatant and resuspend the cell pellet to an OD_{600} of 1.0 in 50 mL **BMMY** medium to induce expression (can modify quantity to approximately 100–200 mL).
3. Place the culture in a 1 L baffled flask. Cover the flask with two layers of sterile gauze or cheesecloth and return it to the incubator to continue growth.
4. Add 100% methanol to a final concentration of 1% methanol every 24 h to maintain induction. Be sure to check the volume of the culture and add methanol accordingly. Evaporation may reduce the culture volume.
5. At each of the times indicated below, transfer 1 mL of the expression culture to a 1.5 mL microcentrifuge tube.

   Centrifuge the samples at maximum speed in a tabletop microcentrifuge at room temperature for 2–3 min. Time points (hours): 0, 6, 12, 24 (1 day), 36, 48 (2 days), 60, 72 (3 days), 84, and 96 (4 days) (*see* **Note 12**).
6. Transfer the supernatant to a separate tube. Freeze the supernatant and the cell pellets quickly in a dry ice/alcohol bath, and store them at −80 °C until ready to assay.

3.6 Protein Purification

1. Filter the supernatant via a 0.45 μM membrane (*see* **Note 13**).
2. Combine 1 mL (2–3 mg/mL total protein) of the lysate with 7 mL Binding Buffer (*see* **Note 14**).
3. Take a pre-equilibrated Ni^{2+}-charged 5 mL column and resuspend the resin in 4 mL of the diluted lysate from **step 2**.
4. Seal the column and batch-bind by rocking gently at room temperature for 10 min. This is to keep the resin suspended in the lysate solution.
5. Let the resin settle by gravity or low speed centrifugation (800 × *g*) and carefully remove the supernatant. Save the supernatant at 4 °C to check for unbound protein by SDS-PAGE analysis.

6. Repeat **steps 2** through **4** with the remaining 4 mL of diluted lysate.
7. Wash with 4 mL Wash Buffer. Settle the resin by gravity or low speed centrifugation (800 × *g*), and carefully aspirate the supernatant. Save supernatant at 4 °C for SDS-PAGE analysis. Repeat this step three more times.
8. Clamp the column in a vertical position and snap off the cap on the lower end. Elute the protein with 4 mL Elution Buffer. Collect fractions for SDS-PAGE analysis (*see* **Note 15**).
9. Store the eluted fractions at 4 °C. If −20 °C storage is required, add glycerol to the fractions. For long-term storage, add protease inhibitors to the fractions.
10. Analyze the supernatants and the cell pellets for protein expression by Coomassie-stained SDS-PAGE (*see* Subheading 3.6) and western blot (*see* **Note 16**), or functional assay (*see* **Note 17**).

3.7 Protein Analysis by SDS-PAGE

1. Include different samples as controls (*see* **Note 18**).
2. Thaw supernatants and place on ice.
3. Mix 50 µL of the supernatant with 50 µL of SDS-PAGE Gel 5× sample loading buffer. Then, boil 10 min.
4. Prepare 5% stacking and 12% resolving polyacrylamide gel.
5. Then load 10–30 µL of sample onto the gel. You may store the remaining sample at −20 °C for western blots, if necessary. You may store the supernatants at −80 °C for further analysis.
6. Run the gel at 100 V until the dye reaches the bottom of the gel.
7. Stain gel with Analyze Coomassie solution and analyze or transfer the gel to the nitrocellulose membrane for western blotting (if necessary).
8. If you do not see any protein by Coomassie or by western blot, then concentrate the supernatant five- to tenfold and analyze samples again by western blot.

4 Notes

1. Liquid medium without Zeocin can be stored at room temperature. Medium containing Zeocin should be stored at +4 °C in the dark. YPD slants or plates are stored at +4 °C. The shelf life of medium is several months. Medium containing Zeocin has a shelf life of 1–2 weeks.
2. For transforming with circular DNA, you will need 50–100 µg plasmid DNA. If you have constructed multimers in pPICZα, you will not be able to linearize the plasmid.

3. Imidazole is included in the Native Wash and Elution Buffers to minimize the binding of untagged, contaminating proteins and increase the purity of the target protein with fewer wash steps. Moreover, if the level of contaminating proteins is high, you may add imidazole to the Native Binding Buffer. If the protein does not bind well under these conditions, you can lower or eliminate the imidazole in the buffers and increase the number of wash and elution steps.
4. To prepare 100 mL 1× Purification Buffer, combine: 80 mL of sterile distilled water and 20 mL of 5× Purification Buffer. Mix well and adjust pH to 8.0 with NaOH or HCl.
5. The composition of binding and elution buffers could be optimized as the following: Binding Buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4), Elution Buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4) [12].
6. Once you have cloned and sequenced your insert, generate enough plasmid DNA to transform *Pichia* (5–10 μg of each plasmid per each transformation).
7. Each enzyme cuts one time in the 5′ AOX1 region to linearize the pPICZα. Choose the enzyme that does not cut within your gene. Moreover, it is recommended to check a small aliquot of the digest by agarose gel electrophoresis for complete linearization.
8. It is advised to keep the cells on ice and use immediately the same day. However, if cells are needed to be stored, take aliquots of 100 μL and freeze rapidly at −80 °C.
9. Negative control for transformation: Plate 100 μL of strain X33 on YePDS agar + Zeocin 100 mg/L, and without Zeocin.
10. Strain X33 contains a native AOX1 gene. Thus, Zeocin-resistant transformants will be Mut^+ as transformation of X-33 with linearized constructs favor single crossover recombination at the AOX1 locus [13].

    However, there is a chance that recombination will occur in the 3'AOX1 region, which disrupts the wild-type AOX1 gene and creats Mut^S transformants. Testing on MD and MM plates will allow to confirm the Mut^+ phenotype. Furthermore, it is recommended to purify Zeocin-resistant transformants before carrying out the Mut phenotype test to ensure isolation of a pure clonal isolates. This is done by streaking for single colonies on YPD [13].
11. The absolute quantification of target gene copies can be performed by quantitative PCR using SYBR Green I Mix (Roche, Mannheim, Germany) [10].

12. These samples will be used to analyze expression levels and to determine the optimal time postinduction to harvest.
13. Depending on the type of protein purified and quantity, you may first centrifuge the culture broth at 10,000 × *g* and 4 °C for 10–15 min. In some cases, concentrating the collected supernatant approximately 10 times by ultrafiltration through 10 kDa cutoff membrane could be employed as well.
14. The concentration of the protein can be quantified by various methods such as Bradford assay using bovine serum albumin as standard or using commercial kits, e.g., BCA protein kit.
15. Various studies report that deglycosylated *R. miehei* lipase have improved activity than the glycosylated one [10, 12]. Therefore, after this step, it is advised to treat the purified enzyme with Endo Hf (New England Biolabs; Beverly, Massachusetts, United States) as per the manufacturer's instructions. The molecular weights of the enzyme before deglycosylation and after deglycosylation can be determined by SDS polyacrylamide gel electrophoresis [12].
16. **SDS-PAGE** separates protein based on molecular weight, while **western blotting** is done to detect the protein of interest using specific antibodies. Thus, in this protocol, the extracellular enzyme can be detected using Monoclonal mouse-anti-His–Tag antibody as the primary antibody and goat anti-mouse IgG antibody conjugated to alkaline phosphatase as the secondary antibody. BCIP/NBT Chromogenic reagent kit (Tiangen) can be utilized for color reaction according to the manufacturer's instructions [14, 15].
17. The lipase activity in the culture filtrate can be determined in various ways. NaOH titration method is widely used to carry out lipase hydrolysis using olive oil emulsion as a substrate [16, 17]. Other assays include the hydrolysis of *p*-nitrophenyl laurate (*p*-NPL) into lauric acid and *p*-nitrophenol [18].
18. Include the following samples as controls on your SDS-PAGE:
    (a) Appropriate molecular weight standards.
    (b) A sample of your protein as a standard (if available).
    (c) Glycosylated and deglycosylated proteins.
    (d) A sample of X-33 with the parent plasmid transformed into it. This shows the background of native *Pichia* proteins that are present intracellularly. Inclusion of this sample will help you differentiate your protein from background if you express it intracellularly.
    (e) Analyze the pPICZ and Albumin controls also as they should indicate any problems with the media or expression conditions.

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References

1. Wingfield PT (2015) Overview of the purification of recombinant proteins. Curr Protoc Protein Sci 80:6.1.1–6.1.35. https://doi.org/10.1002/0471140864.ps0601s80
2. Andersen DC, Krummen L (2002) Recombinant protein expression for therapeutic applications. Curr Opin Biotechnol 13(2):117–123. https://doi.org/10.1016/s0958-1669(02)00300-2
3. Kim K, Choe D, Lee DH, Cho BK (2020) Engineering biology to construct microbial chassis for the production of difficult-to-express proteins. Int J Mol Sci 21(3):990. https://doi.org/10.3390/ijms21030990
4. Tripathi NK, Shrivastava A (2019) Recent developments in bioprocessing of recombinant proteins: expression hosts and process development. Front Bioeng Biotechnol 7:420. https://doi.org/10.3389/fbioe.2019.00420
5. Rodrigues RC, Fernandez-Lafuente R (2010) Lipase from *Rhizomucor miehei* as a biocatalyst in fats and oils modification. J Mol Catal B Enzym 66(1–2):15–32
6. Firdaus MY, Guo Z, Fedosov SN (2016) Development of kinetic model for biodiesel production using liquid lipase as a biocatalyst, esterification step. Biochem Eng J 105:52–61
7. Blondeau K, Blaise F, Graille M, Kale SD, Linglin J, Ollivier B, Labarde A, Lazar N, Daverdin G, Balesdent MH, Choi DH, Tyler BM, Rouxel T, van Tilbeurgh H, Fudal I (2015) Crystal structure of the effector AvrLm4-7 of Leptosphaeria maculans reveals insights into its translocation into plant cells and recognition by resistance proteins. Plant J 83(4):610–624. https://doi.org/10.1111/tpj.12913
8. Yan J, Zheng X, Du L, Li S (2014) Integrated lipase production and in situ biodiesel synthesis in a recombinant Pichia pastoris yeast: an efficient dual biocatalytic system composed of cell free enzymes and whole cell catalysts. Biotechnol Biofuels 7(1):55. https://doi.org/10.1186/1754-6834-7-55
9. Hu H, Gao J, He J, Yu B, Zheng P, Huang Z, Mao X, Yu J, Han G, Chen D (2013) Codon optimization significantly improves the expression level of a keratinase gene in *Pichia pastoris*. PLoS One 8(3):e58393. https://doi.org/10.1371/journal.pone.0058393
10. Huang J, Xia J, Yang Z, Guan F, Cui D, Guan G, Jiang W, Li Y (2014) Improved production of a recombinant *Rhizomucor miehei* lipase expressed in *Pichia pastoris* and its application for conversion of microalgae oil to biodiesel. Biotechnol Biofuels 7:111. https://doi.org/10.1186/1754-6834-7-111
11. Barredo J (2010) Microbial enzymes and biotransformations. Humana Press, Totowa, NJ
12. Liu Y, Xie W, Yu H (2014) Enhanced activity of *Rhizomucor miehei* lipase by deglycosylation of its propeptide in Pichia pastoris. Curr Microbiol 68(2):186–191. https://doi.org/10.1007/s00284-013-0460-0
13. Krainer FW, Dietzsch C, Hajek T, Herwig C, Spadiut O, Glieder A (2012) Recombinant protein expression in Pichia pastoris strains with an engineered methanol utilization pathway. Microb Cell Factories 11:22. https://doi.org/10.1186/1475-2859-11-22
14. Wang JR, Li YY, Xu SD, Li P, Liu JS, Liu DN (2013) High-level expression of pro-form lipase from *Rhizopus oryzae* in Pichia pastoris and its purification and characterization. Int J Mol Sci 15(1):203–217. https://doi.org/10.3390/ijms15010203
15. Huang J, Yang Z, Zhu R, Qian X, Wang Y, Li Y, Li J (2018) Efficient heterologous expression of an alkaline lipase and its application in hydrolytic production of free astaxanthin. Biotechnol Biofuels 11:181. https://doi.org/10.1186/s13068-018-1180-2
16. Huang J, Wang Q, Bu W, Chen L, Yang Z, Zheng W, Li Y, Li J (2019) Different construction strategies affected on the physiology of Pichia pastoris strains highly expressed lipase by transcriptional analysis of key genes. Bioengineered 10(1):150–161. https://doi.org/10.1080/21655979.2019.1614422
17. Huang J, Zhao Q, Chen L, Zhang C, Bu W, Zhang X, Zhang K, Yang Z (2020) Improved production of recombinant *Rhizomucor miehei*

lipase by coexpressing protein folding chaperones in *Pichia pastoris*, which triggered ER stress. Bioengineered 11(1):375–385. https://doi.org/10.1080/21655979.2020.1738127

18. Wongwatanapaiboon J, Malilas W, Ruangchainikom C, Thummadetsak G, Chulalaksananukul S, Marty A, Chulalaksananukul W (2016) Overexpression of *Fusarium solani* lipase in *Pichia pastoris* and its application in lipid degradation. Biotechnol Biotechnol Equip 30(5):885–893. https://doi.org/10.1080/13102818.2016.1202779



Chapter 16

Designing and Constructing Artificial Small RNAs for Gene Regulation and Carbon Flux Redirection in Photosynthetic Cyanobacteria

Shubin Li, Tao Sun, Lei Chen, and Weiwen Zhang

Abstract

Photosynthetic cyanobacteria are not only model organisms for studying photosynthesis and biological cycling of carbon in biosphere but also potential "green microbial factories" to produce renewable fuels and chemicals, due to their capability to utilizing solar energy and CO_2. Therefore, strategies for gene regulation and carbon flux redirection are important for both fundamental research and metabolic engineering of cyanobacteria. To address the challenges, regulatory tools based on artificial small RNAs have been developed with satisfactory effects for single or multiple gene(s) regulation in various cyanobacterial species. When combined with the promoters of varying gradient strength and the inducible switches developed in recent years, it is now feasible to realize precise gene regulation in photosynthetic cyanobacteria for producing fuels and chemicals. Here in this chapter, we provide a detailed introduction of the design principles and constructing methods of the artificial sRNA tools to achieve accurate inducible regulation of cyanobacterial gene(s).

Key words Cyanobacteria, Small RNA, Gene regulation, Carbon flux redirection, Synthetic biology

1 Introduction

Overconsumption of fossil fuel leads to serious energy crisis and environmental pollution. One of the most serious consequences is the massive emission of the greenhouse gas carbon dioxide. In 2019, global carbon dioxide emissions have been increased to 33 billion tons/year and the trend is continuing, which will further aggravate global warming and ultimately lead to serious changes in the global climate, which promotes the recent research and development efforts on green fuels and chemicals using "microbial cell factory" [1, 2]. Among them, "photosynthetic cell factories" based on cyanobacteria and microalgae have attracted significant

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intention as great chassis for their ability of conversing carbon dioxide and water to fuels and chemicals using solar energy at ten times the efficiency of terrestrial higher plants. Meanwhile, cyanobacteria and microalgae can fix a large amount of carbon dioxide in the air, which can effectively mitigate the greenhouse effects on earth. Cyanobacteria are considered to be ideal chassis due to their relatively simple structure and mature genetic operations [3–5]. Up to now, more than 50 types of fuels and chemicals have been successfully synthesized in engineered cyanobacteria [6, 7], including biofuels like butanol [8] and ethanol [9], platform chemicals like 3-hydroxypropionic acid [10] as well as natural antioxidant like astaxanthin [11] in various cyanobacterial chassis, including *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) [12], *Synechococcus elongatus* PCC 7942 (hereafter *Synechococcus* 7942) [13], *Synechococcus* sp. PCC 7002 (hereafter *Synechococcus* 7002) [14], and *Synechococcus elongatus* UTEX 2973 (hereafter *Synechococcus* 2973) [15].

To achieve production for endogenous or heterogeneous fuels and chemicals at high efficiency, it is always necessary to realize the accurate control of the gene expression in competitive pathways [6, 7]. In many cases, totally blocking these pathways via gene knockout is not applicable especially when they are essential to support the cell survival and growth [16]. For example, in order to increase the production of downstream products derived from precursor malonyl-CoA, inhibition of the fatty acid biosynthetic pathway can be effective to increase the intracellular content of malonyl-CoA; however, over-inhibition or total blockage of fatty acid biosynthetic pathway affects cell growth and survival negatively [10, 17]. For another example, inhibition of the glycogen synthesis can effectively direct the carbon flow toward the synthesis of many target products and increase their yields in cyanobacteria, but this will make cyanobacteria unable to accumulate glycogen as an energy reserve to cope with nutritional deficiencies such as darkness and nitrogen deficiency, which will seriously affect the survival of cyanobacteria in the absence of nutrition [18]. To address this issue, the gene regulatory tool can be used to adjust the corresponding pathway preciously to the appropriate level, so that more carbon flux is redirected to the synthesis of the target products, while no negative impact on the cell growth and survival is created [19]. Small RNA (sRNA) regulatory tools based on artificial sRNA molecules have showed promising applications due to its modularity, tunable base-pair complementation and *trans*-acting ability. More importantly, expression of artificial sRNAs exerts almost no metabolic burden on host cells [20, 21]. In a recent study, Na et al. established the artificial sRNA regulatory systems based on a *E. coli* natural system which consists of chaperone protein Hfq and synthetic sRNAs, in which synthetic sRNAs were composed of a scaffold sequence with binding ability to Hfq and an antisense sequence of 20–30 nucleotides binding to the target

mRNA (Fig. 1) [22]. The artificial sRNA regulatory systems were then applied in engineered *E. coli* to inhibit UDP-*N*-acetylmuramoylalanyl-D-glutamate 2,6-diaminopimelate ligase (*murE*) in the competitive pathway of the lysine of cadaverine synthesis pathway, resulting in increased production of cadaverine by 55%. The tool was later successfully applied in *E. coli*, *Clostridium acetobutylicum, Corynebacterium glutamicum,* and cyanobacteria, resulting in increased yields of cadaverine, butanol, and glutamate [22–24], respectively. Sun et al. (2018) introduced the artificial sRNA regulatory system into *Synechocystis* 6803, achieving the enhanced production of malonyl-CoA by 41% via controlling expression of genes *fabH*, *fabF,* and *fabD* essential to the synthesis of fatty acids [17]. Similarly, application of the tool also achieved the significant inhibition of target gene(s) in *Synechococcus* 2973, suggesting its general application in cyanobacteria [25].

In many cases, the inhibition of the competitive pathway of the target product will inhibit the growth and survival of cells due to the important or even essential function of the competitive pathway. For example, fatty acid synthesis is the main competitive pathway for the downstream products of malonyl-CoA like 3-hydroxypropionic acid in cyanobacteria, and its inhibition will have a negative impact on cell growth and survival [17]. In order to make better use of "microbial cell factories" to produce fuels and chemical, it is crucial to achieve precise and controllable regulation of metabolic pathways so that the fine balance between product synthesis and cell growth and survival can be maintained [19, 26–28]. In a recent study, Sun et al. (2018) used theophylline-inducible riboswitch to control the expression of chaperone protein Hfq in *Synechocystis* 6803, realizing the inducible inhibition of fatty acid synthesis pathway, which could avoid the negative effect on the growth if fatty acid synthesis pathway was inhibited during the growth process [17]. In another study, Li et al. (2018) combined theophylline-inducible riboswitch with T7 RNA polymerase to achieve simultaneous control of sRNA and chaperone protein Hfq, reducing leakage and expanding the controllable range of suppression in *Synechococcus* 2973 [25].

To achieve precise and controllable control of metabolic pathways, in general, an abundant library of promoters of different strength and a library of induction systems with broad induction range are necessary [29–31]. Early researches have led to characterization of a series of promoters with different strengths like P_{psbA2} [32], P_{psbA3} [33], P_{cpc560} [34], and a library of induction systems with broad induction range in cyanobacteria like anhydrotetracycline induction system [35], theophylline induction system [25], metabolite induction system [36], and ion induction system [37]. For detailed information about promoters in cyanobacteria, readers may also refer to several excellent reviews published earlier [38–41]. Combination of these promoters and induction systems with artificial sRNA tools will greatly facilitate the control of the

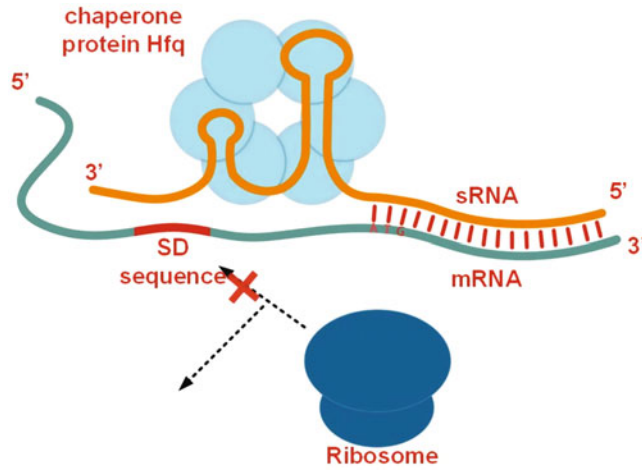


Fig. 1 Schematic diagram of the structure of artificial sRNA tool based on chaperone protein Hfq

metabolic flow in cells and help achieve high-efficiency "photosynthetic cyanobacteria cell factories" for producing fuels and chemicals.

In this chapter, we presented the detailed protocols related to the design principles, construction, and transformation methods of using reporter genes to test promoters with various strength and inducible systems, as well as their combination with the artificial sRNA tool to achieve accurate regulation of gene expression and carbon flux redirection in *Synechocystis* 6803 and *Synechococcus* 2973, which are crucial for the high-efficiency production of fuels and chemicals.

2 Materials

2.1 Cultivation of E. coli *and Cyanobacteria*

2.1.1 Instruments for Cultivation

Constant temperature incubator; illumination constant temperature incubator; constant temperature shaker; illumination constant temperature shaker; ultraclean workbench; Microplate reader; or UV-Vis spectrophotometer.

2.1.2 BG11 Culture Medium for Cyanobacteria

0.04 g/L K_2HPO_4. Prepared as 1000× stock solution; 0.075 g/L $MgSO_4{\cdot}7H_2O$. Prepared as 1000× stock solution; 0.006 g/L Citric acid. Prepared as 1000× stock solution; 0.001 g/L EDTA (disodium salt). Prepared as 1000× stock solution; 0.02 g/L Na_2CO_3. Prepared as 1000× stock solution; 1 mL/L Trace metal mix A5. Prepared as 1000× stock solution composed of 2.86 g/L H_3BO_3, 1.81 g/L $MnCl_2{\cdot}4H_2O$, 0.222 g/L $ZnSO_4{\cdot}7H_2O$, 0.39 g/L $NaMoO_4{\cdot}2H_2O$, 0.079 g/L $CuSO_4{\cdot}5H_2O$ and 49.4 mg/L $Co(NO_3)_2{\cdot}6H_2O$; 0.036 g/L $CaCl_2{\cdot}2H_2O$. Prepared as 1000× stock solution; 0.006 g/L Ferric ammonium citrate.

Prepared as 1000× stock solution; 1.5 g/L $NaNO_3$; 1 g/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); 5 g/L $Na_2S_2O_3$: Adding only when preparing solid medium; 15 g/L agar powder: Adding only when preparing solid medium.

2.1.3 Luria-Bertani (LB) Broth for E. coli

10 g/L tryptone; 2 5 g/L yeast extract; 10 g/L NaCl; 15 g/L agar powder: Add only when preparing solid medium.

2.1.4 BG11 Agar Plate Containing 5% (v/v) LB Broth for Conjugation of Cyanobacteria

Ingredients required for BG11 agar plate; 0.25 g/L yeast extract; 0.5 g/L NaCl; 0.5 g/L tryptone.

2.2 Transformation of E. coli *and Cyanobacteria*

2.2.1 Instruments for Transformation

All instruments listed in 2.1.1; –80 °C Ultralow temperature freezer; Constant temperature water bath; Ice box; Low temperature centrifuge.

2.2.2 Transformation of E. coli

0.1 M $CaCl_2{\cdot}2H_2O$ in ddH_2O; 0.1 M $CaCl_2{\cdot}2H_2O$ in ddH_2O containing 15% (v/v) glycerol; LB broth; LB agar plate containing appropriate antibiotic(s) (i.e., 50 μg/mL chloramphenicol, 50 μg/mL kanamycin, 100 μg/mL spectinomycin, and/or 100 μg/mL ampicillin).

2.2.3 Transformation of Cyanobacteria

BG11 medium; LB broth; BG11 agar plate containing 5% (v/v) LB broth; BG11 agar plate containing appropriate antibiotic(s) (i.e., 20 μg/mL chloramphenicol, 20 μg/mL kanamycin, and/or 20 μg/mL spectinomycin).

2.3 Plasmid Construction

2.3.1 Instruments for Plasmids Construction

PCR thermal cyclers; Oscillation constant temperature metal bath; Low-speed desktop centrifuge.

2.3.2 Amplification of DNA Fragments

Commercial PCR enzymes; the buffer corresponding to the enzyme used; 100 mM dNTP mix (25 mM each); primers; template for PCR product (genome, plasmids, or other PCR product).

2.3.3 Purification of PCR Product

Commercial PCR purification kit.

2.3.4 Golden Gate Assembly

DNA fragments or plasmids; Thermo Scientific™ T4 DNA Ligase buffer (10×); Thermo Scientific™ FastDigest *Bsa*I, *Bsm*BI, *Sap*I, *Aar*I, *Bbs*I, etc.; Thermo Scientific™ T4 DNA ligase.

2.3.5 DNA digestion by restriction enzyme

Thermo Scientific™ FastDigest enzyme; Thermo Scientific™ FastDigest Buffer; PCR product or plasmid to be digested.

2.3.6 5′ End Phosphorylation of PCR Products

Thermo Scientific™ T4 Polynucleotide Kinase; Thermo Scientific™ 10× reaction buffer A; PCR product; 100 mM ATP.

2.3.7 Ligation of DNA Fragments

Thermo Scientific™ T4 DNA Ligase (5 U/μL); PCR products or plasmid to be ligated; Thermo Scientific™ T4 DNA Ligase buffer (10×); 50% (m/v) PEG4000 solution. Add only when blunt ends exist in the DNA fragments of the ligation reaction.

2.4 Functional Validation of Promoters and Artificial sRNA Tools

2.4.1 Instruments for Validation

PCR thermal cyclers; Oscillation constant temperature metal bath; Low-speed low temperature desktop centrifuge; Ultraclean workbench; Real-time PCR instruments; Microplate reader; or UV-Vis spectrophotometer.

2.4.2 Reverse Transcription of RNA to cDNA

Commercial bacterial RNA extraction kit; Commercial reverse transcription kit; Random primers.

2.4.3 qRT-PCR

Commercial qRT-PCR kit; Primers for qRT-PCR.

2.4.4 Detection of β-Galactosidase Activity

Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 40 mM β-mercaptoethanol); 0.1% (m/v) SDS; Chloroform; 4 g/L ortho-nitrophenyl-beta-d-galactopyranoside (ONPG;); 1 M Na_2CO_3.

3 Methods

3.1 Plasmids Construction and Transformation into E. coli

Since the integrated plasmids and the free plasmids used in cyanobacteria require many functional elements, the traditional construction method based on restriction enzyme digestion and DNA ligase is inefficient, time-consuming, and may leave some unwanted scars.

3.1.1 Plasmids Construction

Golden Gate Assembly

1. Draw a map according to the function to be achieved by the plasmid.
2. Screen for one or more of the fewest recognition sites of restriction enzymes that can be used for Golden Gate Assembly (*Bsa*I, *Bsm*BI, *Sap*I, *Aar*I, *Bbs*I, etc.) in the map.
3. Delete or mutate the site recognized by selected restriction enzyme according to the synonymous codons of amino acids and the function of noncoding regions.

4. Design primers using Golden Gate Assembly primer design website like https://goldengate.neb.com/. The primers annealing temperature should be above 55 °C, the difference between the two primer annealing temperatures should be less than 3 °C, and the protection base should be 3–6 bp.
5. Use PCR to amplify each fragment for Golden Gate Assembly.
6. Carry out Golden Gate Assembly reaction after purifying each PCR product. Take FastDigest restriction enzyme and T4 DNA ligase produced by Thermo Scientific™ as example. In a reaction system with total volume of 20 μL, add 10× T4 DNA ligase Buffer 2 μL, FastDigest enzyme 1 μL, T4 DNA ligase 1 μL, Equimolar mixture of DNA fragments (10 ng per 1000 bp DNA) and nuclease-free water to 20 μL.
7. Put the PCR tube loaded with the reaction system into the PCR thermal cyclers and cycle it 20–30 times in a manner of reacting at 37 °C for 3 min and reacting at 22 °Cs for 3 min per cycle.
8. Transform the reaction product into *E. coli* competent cells.

Traditional Construction Methods

In some special cases, such as endonuclease recognition site required for the Golden Gate Assembly which cannot be deleted exists in the plasmid backbone or insert sequence, the Golden Gate Assembly is not applicable. At this time, it is necessary to adopt the traditional method of digestion and ligation or blunt end ligation. The method of digestion and ligation uses restriction enzymes to digest plasmids and inserts sequence obtained by PCR to produce sticky ends that can complement each other, and then uses T4 DNA ligase to complete the nicks to form a new plasmid. The method of blunt end ligation divided into ligating of insert sequence with the backbone of plasmid and self-ligating of backbone of plasmid. The ligation of insert sequence with the backbone needs the plasmid backbone and the insert sequence obtained by PCR, phosphorylate the 5′ end of the insertion, and then ligate the plasmid backbone and insertion by T4 DNA ligase. The self-ligation of the vector only needs the plasmid backbone obtained by PCR and phosphorylate its 5′ end and ligate itself by T4 DNA ligase.

Digestion

The method here is using Thermo Scientific ™ FastDigest restriction enzyme.

1. Determine the concentration of plasmids or PCR product used for digestion by measuring absorbance or gel electrophoresis.
2. Add an appropriate amount of purified PCR product (~0.2 μg) or plasmids (~1 μg) for digestion in the reaction system.
3. Add 2 μL 10× FastDigest buffer.
4. Add 1 μL of each required restriction enzyme.

5. Make up to 20 μL with nuclease-free water.
6. Incubate at 37 °C in a heat block or constant temperature metal bath for 20 min.
7. Purify the resulting product by PCR purification kit.

5′ End Phosphorylation of PCR Products

The method here is using Thermo Scientific™ T4 Polynucleotide Kinase.

1. Add 15 μL purified PCR product for 5′ end phosphorylation.
2. Add 2 μL 10× reaction buffer A.
3. Add 2 μL 100 mM ATP.
4. Add 1 μL T4 Polynucleotide Kinase.
5. Incubate at 37 °C in a heat block or constant temperature metal bath for 30 min.
6. Purify the resulting product using PCR purification kit.

Ligation

The method here is using Thermo Scientific ™ T4 DNA Ligase (5 U/μL).

1. Determine the concentration of digested plasmids or PCR product, plasmid backbone for ligation obtained by PCR and 5′ phosphorylated PCR products used for ligation by measuring absorbance or gel electrophoresis.
2. Add 50 ng digested plasmids or plasmid backbone for ligation obtained by PCR.
3. (a) For sticky end ligation, add digested PCR product three times to the number of moles of DNA added in **step 2**;
   (b) For blunt end ligation, add 5′ phosphorylated PCR products ten times to the number of moles of DNA added in **step 2**.
4. Add 1 μL T4 DNA Ligase buffer (10×).
5. Add 1 μL 50% PEG4000 for blunt end ligation only.
6. Add 0.5 μL T4 DNA Ligase.
7. Make up to 10 μL with nuclease-free water.
8. Incubate at 22 °C in a heat block or constant temperature metal bath for 20 min (60 min for blunt end ligation).
9. Transform the ligation product into *E. coli* competent.

3.1.2 Transformation of E. coli *and Verification of Transformants*

1. Take the prepared or purchased *E. coli* DH5α or DH10B competent cells prepared by the $CaCl_2$ method [42] from the –80 °C freezer, and melt on ice.
2. After the competent cells are melted, add the above reaction product to 100 μL of competent cells and transfer the reaction solution to a prechilled 1.5 mL Eppendorf tube.

3. After 30 min of ice bath, 42 °C water bath for 60–90 s, quickly insert on the ice and re-ice bath for 2–3 min. Then add 500 mL of LB medium to each Eppendorf tube and incubate for 60 min in a 37 °C shaker.
4. Centrifuge the Eppendorf tube at 2,500 × *g* for 5 min, discard most of the medium, resuspend the bacteria with the remaining liquid, spread it on LB solid medium containing appropriate antibiotics, and put it in a 37 °C incubator upside down for overnight cultivation.
5. Use PCR to verify which single colony is positive.
6. Transfer positive clones to 10 mL LB medium containing appropriate antibiotics, and extract plasmid after overnight culture.
7. Verify the extracted plasmid by digestion and sequencing.

3.2 Construction of Basic Plasmids for Artificial sRNA Tools and Verification of Promoters and Induction Systems

The primers used in this step are shown in Table 1.

3.2.1 Construction of Universal Plasmids for Artificial sRNA Tool

1. Amplify the plasmid backbone of pBR322 using primers PBR322-m-F1, PBR322-m-R1 and the fragment where the restriction enzyme *Bsm*BI and *Bsa*I recognition sites were mutated by primers PBR322-m-F2, PBR322-m-R2.
2. Two fragments obtained above were assembled with restriction enzyme *Bsa*I by Golden Gate Assembly to obtain pBR322m.
3. Amplify *hfq* gene sequence from *E. coli* MG1655 genome using primers HFQ-F, HFQ-R and then ligate it with pUC57 plasmid digested by restriction enzyme *Eco*RV by blunt-end ligation to get the plasmid pUC57-hfq.
4. Mutate the recognition site of the restriction enzyme BsmBI on gene *hfq* in plasmid pUC57-hfq by amplifying its backbone using primers HFQM-F HFQM-R and ligate itself by blunt-end ligation to get the plasmid pUC57-hfqm.
5. Amplify the plasmid backbone sequence from plasmid pBR322m by primers PBR322-F, PBR322-R, the NSI upstream and downstream homology arm from *Synechococcus* 2973 using primers NSI-US-F, NSI-US-R; NSI-DS-F, NSI-DS-R, the *slr2030-slr2031* upstream and downstream homology arm from *Synechocystis* 6803 by primers 2030-F, 2030-R; 2031-F, 2031-R, chaperone protein Hfq coding sequence from pUC57-hfqm by primers HFQ-F, HFQ-R, T_{rbcl} terminator from *Synechocystis* 6803 by primers RBCL-F, RBCL-R and a spectinomycin-resistant cassette (hereafter Spe^R) from pCP3031 [17] by primers SPE-F, SPE-R.
6. Use SPE-R, MICC-RBCL-F as primers and T_{rbcl} terminator and Spe^R as a template for fusion PCR to get the fusion product

Table 1
Primers used for construction of artificial sRNA tool universal plasmid

Primers	Sequences (5′ to 3′)
PBR322-m-F1	GGCTACGGTCTCCACCCACGCTCACCGGCTCCA
PBR322-m-R1	GGCTACGGTCTCACCGTTCCGGGAGCTGCATGTGTCAGAG
PBR322-m-F2	GGCTACGGTCTCAACGGTCACAGCTTGTCTGTAAG
PBR322-m-R2	GGCTACGGTCTCCGGGTCGCGCGGTATCATTGCAGCACTG
PBR322-F	GGCTACCGTCTCTATGGAAGCCGGCGGCACC
PBR322-R	GGCTACCGTCTCAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATG TATCCGCTCATGAG
NSI-US-F	GGCTACCGTCTCAGGGTTCCGCGGGATGGTGCCCGGTTCTG
NSI-US-R	GGCTACCGTCTCAGCAAATAATCGAGTCCCTGCTCGTCAC
SPE-R	GGCTACCGTCTCATTGCCGACTACCTTGGTG
MICC-RBCL-F	GGCTACCGTCTCGCTTAGCCATGGAGACCGGTCTCCTTTCTGTTGGGCCA TTGCATTGCCACTGATTTTCCAACATATAAAAAGACAAGCCCGAACAGTCG TCCGGGCTTTTTTTACCGGTGTTTGGATTGTCGG
HFQ-F	GGCTACCGTCTCGTAAGGGGCAATCTTTACAAG
RBCL-HFQ-R	GGCTACCGTCTCCTCTTCCTGCTCCAGAAGCGCTGTCGAAGTTGAACATC
NSI-DS-F	GGCTACCGTCTCCAAGATGTCGCGGGCATTAG
NSI-DS-R	GGCTACCGTCTCTCCATTCAGGTCGAGGTGGACGAGGACAAAATTGCC TTTG
SPE-F	TTACTGATGTTCAACTTCGACAGCCGCACACCGTGGAAACGGATG
RBCL-R	GCTGTCGAAGTTGAACATCAGTAA
RBCL-F	ACCGGTGTTTGGATTGTCGG
HFQ-R	CCGACAATCCAAACACCGGTTTATTCGGTTTCTTCGCTGTCC
HFQm-R	CGACGGGACAACAGTAGAAA
HFQm-F	CGCCCGGTTTCTCATCACAG
2031-F	GGCTACCGTCTCCAAGAAAAACCCCGTTAATTTTTCT
2031-R	GGCTACCGTCTCTCCATTCAGGTCGAGGTGGACTCAAAATGGGGGTGTTA TAACAG
2030-F	GGCTACCGTCTCAGGGTTCCGCGGCTTTGCAAAAGGAATTTGC
2030-R	GGCTACCGTCTCAGCAAATAATTAGCAATGGCGGGAGACAGCCA

Spe^{R}-T_{rbcI}-*micC* in which the *micC* scaffold was introduced by primer MICC-RBCL-F.

7. Use the fusion PCR to ligate the chaperone protein Hfq coding sequence and T_{rbcI} terminator obtained in the previous step using primers HFQ-F, RBCL-HFQ-R to get the fusion product Hfq-T_{rbcI}.
8. Assemble the pBR322m plasmid backbone sequence, *slr2030-slr2031* or NSI upstream and downstream homology arms, *Spe*^{R}-T_{rbcI}-*micC* and Hfq-T_{rbcI} to obtain universal plasmids pSR3031 and pSRNSI by Golden Gate Assembly using restriction enzyme *Bsm*BI, respectively.

The schematic diagram of the universal plasmid map is shown below (Fig. 2).

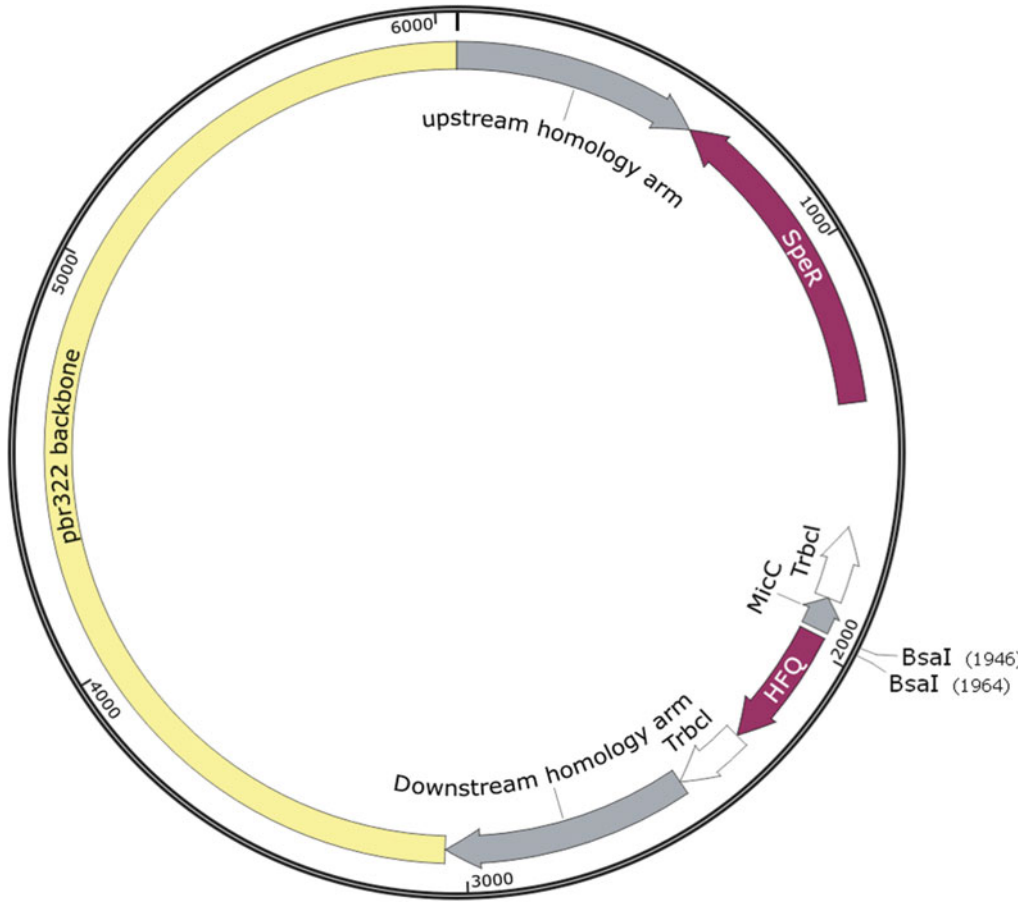


Fig. 2 The schematic diagram of the universal plasmids pSR3031 and pSRNSI for construction of artificial sRNA tool. When the upstream and downstream homology arms are the upstream and downstream homology arms of slr2030–slr2031, the map represents pSR3031. When the upstream and downstream homology arms are the upstream and downstream homology arms of NSI, the spectrum represents pSRNSI

Table 2
Primers used for construction of reporter gene lacZ positive control plasmid and universal plasmid

Primers	Sequences (5′ to 3′)
UNI-RE-F	GGCTACCGTCTCGTTGGATTGTCGGAGTTGTACTCG
UNI-RE-R	GGCTACCGTCTCCCTCTACAGGTCGCACACCGTGGAAACGG
CPC560-F	GGCTACCGTCTCCAGAGAAGAGTCCCTGAATATC
CPC560-R	GGCTACCGTCTCTTCTCCTACTTGACTTTATGAG
LACZ-F	GGCTACCGTCTCTGAGATTAATTCAATGACCATGATTACGGATTC
LACZ-R	GGCTACCGTCTCGCCAAACACCGGTTTATTTTTGACACCAGACC
LACZ-m-R1	ATCACGGAAAATGCCGCTCATCCGCCACAT
LACZ-m-F1	GTCTCGTTGCTGCATAAACC
LACZ-m-R2	TTCTCCAGGTAGCGAAAGCCAT
LACZ-M-F2	ACGCGCCCGCTGATCCTTTGC
BSA-LACZ-F	GGTCTCCATGACCATGATTACGGATTCACTGG
BSA-SPE-F	GGTCTCCCGCACACCGTGGAAACGGATGAAGGCACG

3.2.2 Construction of Reporter Gene lacZ *Positive Control Plasmid*

The primers used in this and the next step are shown in Table 2.

1. Amplify *lacZ* gene sequence from *E. coli* MG1655 genome using primers LACZ-F LACZ-R and then ligate it with pUC57 plasmid digested with restriction enzyme *Eco*RV by blunt-end ligation to get the plasmid pUC57-lacZ.
2. Mutate the recognition site of the restriction enzyme *Bsm*BI on gene *lacZ* in plasmid pUC57-lacZ by amplifying the backbone and repaired insert sequence of pUC57-lacZ using primers LACZ-m-R1, LACZ-M-F2 and LACZ-m-F1, LACZ-m-R2 and ligating them by blunt-end ligation to get the plasmid pUC57-lacZm.
3. Amplify the plasmid backbone sequence from universal plasmid pSR3031 or pSRNSI by primers UNI-RE-F, UNI-RE-R, amplify the mutated *lacZ* gene form plasmid pUC57-lacZm using primers LACZ-F LACZ-R and amplify the promoter P_{cpc560} from *Synechocystis* 6803 by primers by primers CPC560-F CPC560-R.
4. Assemble the pSR3031 and pSRNSI plasmids backbone sequence, promoter P_{cpc560} and *lacZ* gene to obtain reporter gene *lacZ* positive control plasmid pCP3031-lacZ and pCPNSI-lacZ by Golden Gate Assembly using restriction enzyme *Bsm*BI, respectively.

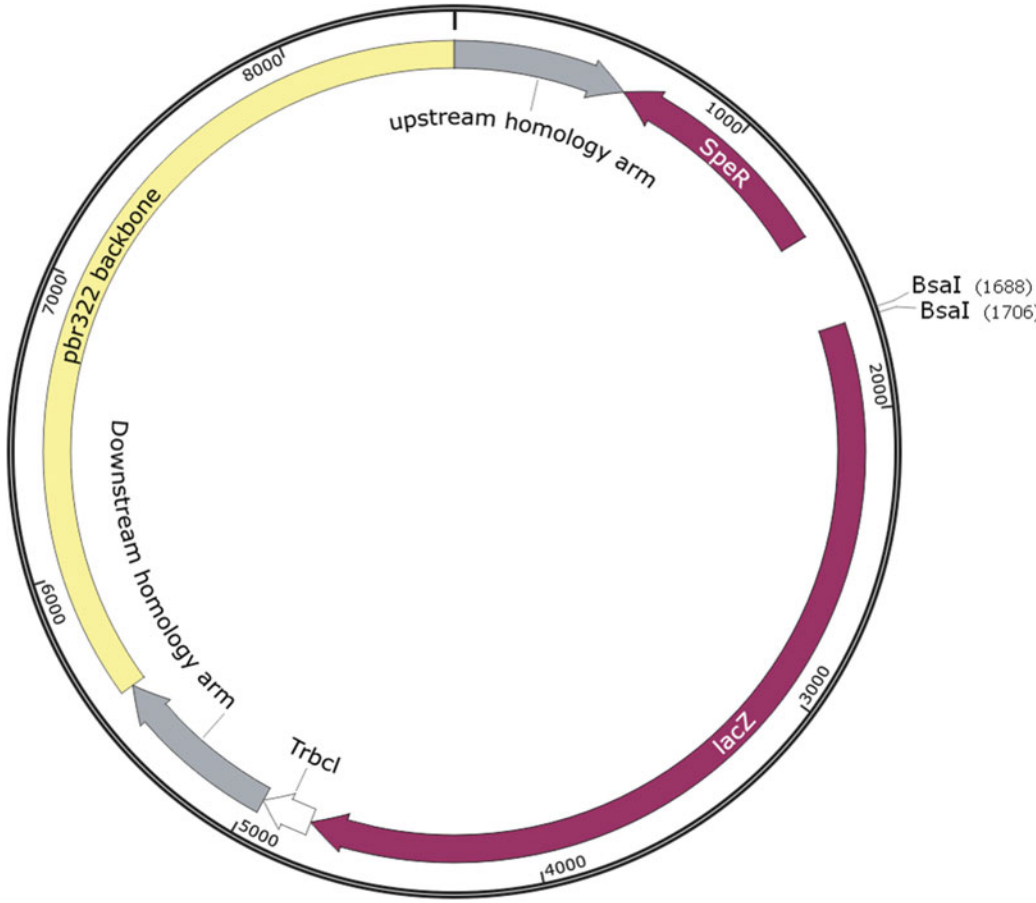


Fig. 3 The schematic diagram of the universal plasmid pLAC3031 and pLACNSI for promoter verification. When the upstream and downstream homology arms are the upstream and downstream homology arms of pLAC3031, the map represents pSR3031. When the upstream and downstream homology arms are the upstream and downstream homology arms of NSI, the spectrum represents pLACNSI

3.2.3 Construction of Reporter Gene lacZ *Universal Plasmid to Verify the Strength of Promoters and Induction Systems*

Amplify the pCP3031-lacZ and pCPNSI-lacZ plasmids backbone sequence using primers BSA-LACZ-F, BSA-SPE-F and then ligate itself by blunt-end ligation to get the plasmid pLAC3031 and pLACNSI. The schematic diagram of the universal plasmids is shown below (Fig. 3). The recognition site of the restriction enzyme *Bsa*I remains in the universal plasmids for subsequent insertion into the promoter or induction system Universal plasmids can also be used as negative control plasmids.

3.3 Use the Reporter Gene System to Verify the Strength of Promoters and Induction Systems

3.3.1 Insert a Promoter or Induction System into a Universal Plasmid

1. Determine the sequence of the promoters and induction systems. For the promoters and induction systems to be verified for controlling the transcription of sRNA, only the transcription intensity needs to be compared. The original RBS sequence needs to be deleted and replaced with a uniform RBS sequence (such as 5′-TTTTGTTTAACTTTAAGAAGGA GATATACAT-3′) to avoid the effect of RBS intensity on the expression intensity of the reporter gene.
2. Determine whether the recognition site of restriction enzyme *Bsa*I exists in the sequences of promoters or induction systems. According to different situations, follow-up different operations:
   (a) If there is no recognition site of restriction enzyme *Bsa*I in these sequences, use the Golden Gate Assembly method to assemble the plasmid.
   (b) If there is any recognition site of restriction enzyme *Bsa*I in the promoters or induction systems, then design the method that uses other restriction enzyme similar to *Bsa*I to digest promoters or induction systems to generate sticky ends that can be complementary to the plasmid digested by *Bsa*I, and ligate them.
   (c) If there is no restriction enzyme generating complementary sticky end of plasmids digested by *Bsa*I, then design the method of blunt-end ligation.

3.3.2 Verify the Gene Expression Intensity of Reporter and Compare the Intensity of Promoters or Induction Performance of the Induction Systems

1. Transform the constructed plasmid to verify the strength of the promoters or the induction performance of the induction systems into the corresponding cyanobacteria.
2. After verification and enrichment of the transformants, they should be cultivated for several generations until genetically stable.
3. Grow the *Synechococcus* 2973 and *Synechocystis* 6803 to exponential phase (volume*$OD_{750\ nm}$ = 1). When verifying the induction performance of the induction systems, the corresponding inducer must be added and the culture without the inducer is used as control. Then centrifuge the cells and resuspend using 1 mL Z Buffer.
4. Add 50 μL 0.1% SDS, 50 μL chloroform, and 200 μL ortho-nitrophenyl-beta-d-galactopyranoside (ONPG; 4 g/L) to the mixture.
5. The reaction solution was incubated at 30 °C at 750 rpm in thermomixer for proper time (3 min for strains preforming high β-galactosidase activity or 1 h for the others).
6. Add 500 μL 1 M Na_2CO_3 to stop the reaction.

7. Centrifuge the solution for 2 min at 14,000 × *g* and measure the absorbance of supernatant at $OD_{420\ nm}$. The result of their corresponding transformants transferred into the negative control plasmid as blank control.
8. Calculate β-galactosidase activity using the Miller value (Miller = 1000 × $OD_{420\ nm}$/(total volume of the cell culture × reaction time × $OD_{750\ nm}$ of the cell)) and compare the intensity of promoters or induction performance of the induction systems.

3.4 Design and Construction of Artificial sRNA Tool

3.4.1 Design Principles of sRNA Target-Binding Sequences

For genes with known Shine-Dalgarno (SD) sequences, the reverse complementary sequence of 20–30 base sequences starting from the SD sequences can be selected as sRNA target-binding sequences.

For genes without known SD sequences, the reverse complementary sequence of 20–30 base sequences starting from the start codon of the protein coding sequence can be directly selected as sRNA target-binding sequences.

The optimal sequence length is 24 bases.

The binding energy of sRNA target-binding sequences and target sequences should be greater than 30 kcal/mol to ensure the binding of sRNA and target mRNA.

1. Get the coding sequence of the gene to be suppressed.
2. Take the 24 base reverse complementary sequence of the coding sequence from the start codon.
3. Confirm binding energy with software like UNAfold.
4. When the binding energy is too low, the binding sequence can be adjusted backward to meet the binding energy.

3.4.2 Selection of Promoters

The artificial sRNA tool is composed of two parts: the target-binding sequence used to bind to the target mRNA and the chaperone protein Hfq assisting the binding. In order to achieve the suppression of the target genes, both parts need to be expressed in engineered cyanobacteria.

1. According to the intensity of the expression of the gene to be suppressed and the level of the need to be suppressed, choose a promoter of suitable strength for transcription of RNA.
2. Truncate the bases after the transcription start site (TSS) of the promoter, so that the promoter region is directly connected to the artificial sRNA, to avoid the effect on artificial sRNA binding target gene by 5′ extra sequence after transcription. The position of the TSS region of the promoter can be referred to the original literature that reported the promoter. Among these, the information of endogenous promoters of

Synechocystis 6803 and *Synechococcus* 2973 can refer to the results of differential RNA-sequencing (dRNA-Seq) studies [43, 44].

3. Select the promoter that controls transcription and translation of chaperone protein Hfq mainly according to the number of genes to be simultaneously suppressed. For the suppression of a single gene, a medium-strength promoter can be selected, and for the simultaneous suppression of multiple genes, a strong promoter should be used.
4. Transfer the constructed plasmids into engineered cyanobacteria and analyze the results of suppression by qRT-PCR or metabolite analysis.
5. Based on the results of the analysis, reselect the promoters that can achieve the predetermined goal.

3.4.3 Construction of Plasmids for Transformation of Artificial sRNA Tool

The restriction enzyme *Bsa*I recognition site left on the above universal plasmid is suitable for the construction of single gene suppression sRNA tools.

Construction of Plasmid for Single Specific Gene Suppression by Artificial sRNA Tool

1. Determine the sequence of the sRNA targeting the mRNA to be suppressed and the promoters that controls the expression of sRNA and chaperone protein Hfq.
2. Determine whether the recognition site of restriction enzyme *Bsa*I exists in the sequences of sRNA and promoters. And design and construct according to the methods described above in Subheading 3.3.1.

Construction of Plasmid for Multiple Specific Gene Suppression by Artificial sRNA Tool

1. Construct plasmids for each single gene suppression by artificial sRNA tool.
2. Determine whether there is a *Bsa*I recognition site in each sequence, and then design and construct according to the methods described above in Subheading 3.3.1.

Construction of Inducible Artificial sRNA Tool

The construction of the inducible artificial sRNA tools is similar to the construction of artificial sRNA tools described above. The main difference is that the functional modules required for induction are needed to be introduced. Here we introduce the construction method using theophylline-induced T7 RNA polymerase system targeting *nbla* encoding a protein involved in the phycobilisome degradation under nutrient-starvation condition as an example. In this system, theophylline-induced riboswitch is used to control the expression of T7 RNA polymerase, and then T7 RNA polymerase simultaneously controls the expression of sRNA and chaperone protein Hfq to achieve inducible suppression of the target gene. The specific construction process is as follows. The primers used are shown in Table 3.

Table 3
Primers used for construction of T7 RNA polymerase-based inducible artificial sRNA tool

Primers	Sequences (5′ to 3′)
TRC-THO-F	TGAGCTGTTGACAATTAATCATCC
T7RNAP-F	CCCTGCTAAGGAGGCAACAAGATGAACACGATTAACATCGCTAA
TRC-THO-R	CTTGTTGCCTCCTTAGCAGGG
T0-T7RNAP-R	TGGACTCACAAAGAAAAAACGCCCGGTGTGCAAGACCGAGCGTTC TGAACAATTACGCGAACGCGAAGTCCGACTCT
sRNA-T7-THO-F	GGCTACGGTCTCCGAAAATGCTCCCCCCTCTCCCCGACTTTCCTATAG TGAGTCGTATTAATGAGCTGTTGACAATTAATCATCC
T0-T7-RBS-R	GGCTACGGTCTCTCCATATGTATATCTCCTTCTTAAAGTTAAACAAAACCTA TAGTGAGTCGTATTATGGACTCACAAAGAAAAAAC

1. Obtain sequence of theophylline-induced T7 RNA polymerase
   Amplify T7 RNA polymerase gene sequence from *E. coli* BL21(DE3) genome using primers T7RNAP-F, T0-T7RNAP-R which introduce T0 terminator at the same time, amplify promoter $P_{trc\text{-}thoE*}$containing theophylline-induced riboswitch from pBA3031-HM(the*) [17] using primers TRC-THO-F, TRC-THO-R, fuse them by fusion PCR using primers TRC-THO-F, T0-T7RNAP-R, and the ligate the fusion PCR product with pUC57 plasmid digested with restriction enzyme *Eco*RV by blunt-end ligation to get the plasmid pUC57-tho-T7RNAP.
2. Construction of inducible sRNA regulation tool controlled by theophylline
   Assemble the pSRNSI plasmid and tho-T7RNAP amplified using primer sRNA-T7-THO-F which introduces sRNA targeting *nbla* and T7 promoter at the same time, T0-T7-RBS-R which introduces T7 promoter and RBS for translation of Hfq at the same time to obtain inducible sRNA regulation tool controlled by theophylline targeting gene *nbla* by Golden Gate Assembly using restriction enzyme *Bsa*I. Then transform the constructed plasmid into *Synechococcus* 2973. The schematic diagram of the universal plasmid map is shown below (Fig. 4).

3.4.4 Verification of Artificial sRNA Tool

For the verification of artificial sRNA tool, qRT-PCR is commonly used.

1. Cultivate cyanobacteria transformants in flask for about 48 h to mid-log phase ($OD_{750\ nm} = 0.4–0.8$).
2. Collect the appropriate amount of culture (volume*$OD_{750\ nm} = 5$) by cryogenic centrifugation (7000 × *g* for 5 min, 4 °C).

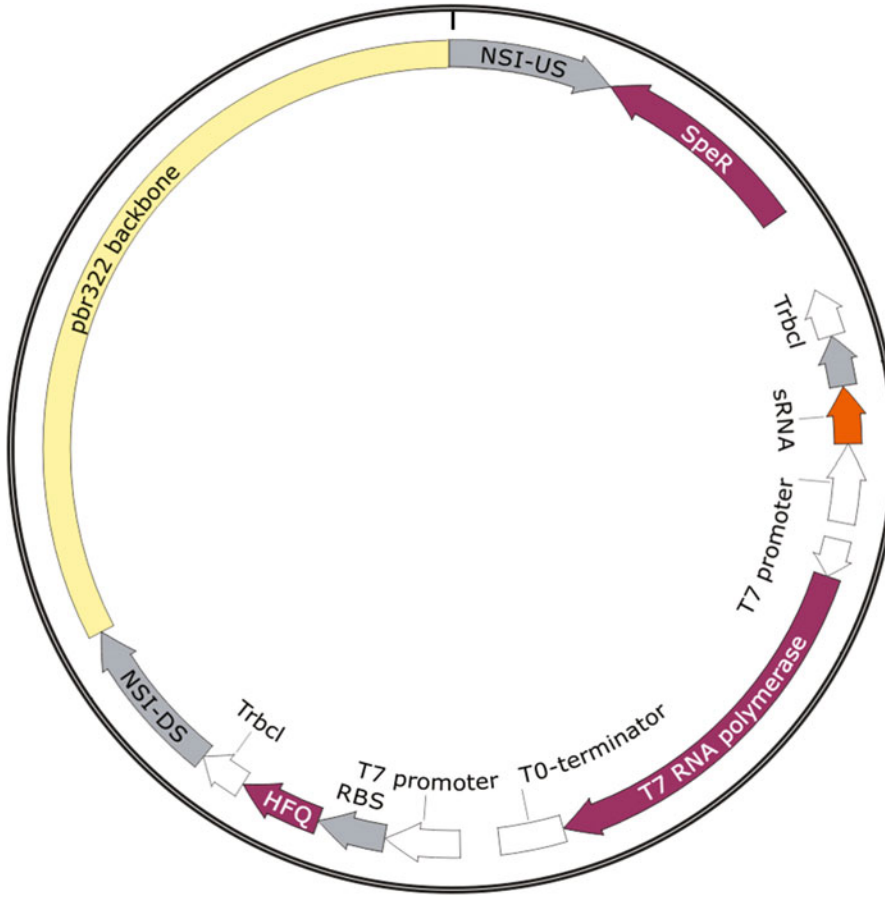


Fig. 4 The schematic diagram of T7 RNA polymerase-based inducible artificial sRNA tool

3. Freeze the cyanobacteria by liquid nitrogen immediately.
4. Extract total RNA by commercial bacterial RNA extraction kit like Zymo Research Direct-zol RNA Miniprep Kits according to the corresponding manual.
5. Reverse transcription of total RNA into cDNA using random primers using reverse transcription kit.
6. Dilute the prepared cDNA 100 times as template for subsequent qRT-PCR.
7. Use the software or website recommended by the selected qRT-PCR instrument or qRT-PCR kit (for example, https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to design primers for target genes and internal reference genes. The length of the PCR product is set at 100–200 bp. The annealing temperature of the primer is set at 60 °C. The internal reference gene in cyanobacteria generally uses 16 s rRNA [45] or *rnpB* gene encoding RNase P subunit B [46].

8. Use the designed primers and diluted template to prepare the PCR reaction according to the instructions of the qRT-PCR kit.
9. Carry out the PCR reaction in qRT-PCR instrument.
10. Apply the corresponding software to analyze the expression of target gene by the $2^{-\Delta\Delta CT}$ method [47].

3.5 Cultivation and Transformation of Cyanobacteria

3.5.1 Preparation of Culture Medium for Cyanobacteria

Add components listed in Subheading 2.1.2 to 850 mL of distilled water except $CaCl_2$ and ferric ammonium citrate, then make up 1000 mL with distilled water. After autoclaving (15 min at 121 °C) and cooling (for solid medium, before solidification), add 1 mL autoclaved $CaCl_2$ stock solution and 1 mL autoclaved ferric ammonium citrate stock solution. Add 20 mL of culture medium to each 100 mL flask or add 20 mL of solid medium to the 9 cm cell culture plate.

3.5.2 Cultivation Condition of Cyanobacteria

Wild type (WT) of *Synechocystis* 6803, WT of *Synechococcus* 2973 and constructed strains were cultivated in BG11 medium (pH 7.5) under a light intensity of approximately 50 μmol photons m^{-2} s^{-1} for *Synechocystis* 6803 or 500 μmol photons m^{-2} s^{-1} for *Synechococcus* 2973 in an illuminating shaking incubator at 200 rpm or on BG11 agar plate in incubator under a light intensity of approximately 50 μmol photons m^{-2} s^{-1} or 200 μmol photons m^{-2} s^{-1}, respectively. The cultivation temperature of *Synechocystis* 6803 is 30 °C, while the temperature of *Synechococcus* 2973 is 37 °C. To maintain the stability of constructed strains, appropriate antibiotic (s) were added when necessary (i.e., 20 μg/mL chloramphenicol, 20 μg/mL spectinomycin, or/and 20 μg/mL kanamycin).

3.5.3 Enrichments of Cyanobacteria

The single colony of cyanobacteria activated or transformed on the plate needs to be enriched in order to be subcultivated due to the small amount of cell. Culture the colonies on the BG11 solid medium to approximately 2 mm in diameter. Then use sterile tips, inoculation rings or toothpicks to pick single colonies and draw line on new BG11 solid medium. When a large amount of cyanobacteria grows on the line pick up all visible cyanobacteria with a sterile inoculation loop and transfer them to 5 mL of BG11 medium in a 100 mL flask and incubate for 1–3 days in a light shaker. When the medium turns into a clear green, supplement the BG11 medium in the flask to 20 mL and continue to cultivate.

3.5.4 Subcultivation of Cyanobacteria

1. Cultivate cyanobacteria in flask to mid-log phase ($OD_{750\ nm} = 0.4–0.8$) and use it as a seed culture.
2. In an autoclaved flask, add BG11 medium and seed culture solution according to the initial $OD_{750\ nm}$ of 0.1 and the final volume of 20 mL.
3. Cultivate in an appropriate culture environment.

3.5.5 Transformation of Cyanobacteria

Conjugation of cyanobacteria was performed according to the method reported previously with some modifications [48].

Conjugation of Cyanobacteria

1. *E. coli* HB101 harboring pRL443 and pRL623 and *E. coli* HB101 harboring target plasmid were cultivated overnight. Then transfer the *E. coli* into fresh LB medium with appropriate antibiotic(s) at 1:50 ratio.
2. When cells grew to exponential phase ($OD_{600} \approx 0.5$), 2 mL of each *E. coli* strain was washed twice by fresh (LB) medium to remove all the antibiotics, then resuspended in 0.1 mL of the LB medium, mixed together, and incubated for 30 min.
3. 1 mL of the cyanobacterial culture at exponential phase ($OD_{750} = 0.4–0.8$) was centrifuged and resuspended in 0.2 mL BG11 medium for each conjugation, two more washes were conducted using strain culture medium contains antibiotics.
4. The sample was then mixed with the *E. coli* suspension mentioned above and incubated for 30 min. The mixtures were spread on sterile filters (0.45 μm pore size) coated on the BG11 agar plates containing 5% (v/v) LB broth.
5. After incubated at suitable condition for 24 h, the filter was transferred onto a new BG11 agar plates with appreciate antibiotic(s) and incubated at suitable condition. Clones would be seen after 1–2 weeks.
6. After enrichment of cyanobacteria, extract the genome for PCR verification.

Natural Transformation of Cyanobacteria

This method is improved according to the previous method [49].

1. Prepare linear recombinant DNA fragments by PCR.
2. Collect 1 mL of the exponentially growing cyanobacteria ($OD_{750} \approx 1$) culture (~3×10^8 cells) by centrifugation and resuspend in 50 μL BG11 with the 500 ng purified PCR product.
3. Incubate the culture at 37 °C for 5 h under light of 50 μmol photons m^{-2} s^{-1} and shake it every 2.5 h.
4. Spread the mixture on BG11 agar plates with appreciable antibiotic(s) and incubated at suitable condition. Clones would be seen after 1–2 weeks.
5. After enrichment of cyanobacteria, extract the genome for PCR verification.

4 Notes

1. $CaCl_2$ and ferric ammonium citrate in the components of BG11 need to be autoclaved separately, and then added after the other components of the autoclaved medium are cooled to avoid precipitation.
2. Both *Synechococcus* 2973 and *Synechocystis* 6803 can be transformed by conjugation. Only *Synechocystis* 6803 can also be transformed by natural transformation, although the transformation efficiency of the conjugation is relatively higher.
3. The reagents used for plasmid construction in this article are all based on Thermo Scientific™ products; readers can adjust the use according to the actual situation.
4. The transformation after the blunt-end ligation should use the highest efficiency competent cells to improve the transformation efficiency.
5. This artificial sRNA tool targets mRNA of the target gene which has different expression intensity due to the control of different promoters. In order to achieve the same level of control for different target genes, it may be necessary to try different strength promoters to control the expression of artificial sRNA tools.
6. Due to the differences in terms of the intracellular environment of different cyanobacteria, the same promoters could have different expression strengths, so to achieve the same level of control, one may need to redefine a suitable promoter to control the expression of artificial sRNA tools.
7. Also due to the difference in terms of the expression level of different genes, to achieve precise inducible regulation of multiple genes at the same time, it may be necessary to introduce multiple induction systems, or change the strength of the sRNA promoters by point mutation or other methods, or to adjust the position and length of the target gene binding sequences.

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References

1. Clark JH, Luque R, Matharu AS (2012) Green chemistry, biofuels, and biorefinery. Annu Rev Chem Biomol Eng 3(1):183–207
2. Wirth TE, Boyden C, Podesta JD (2003) The future of energy policy. Foreign Affairs 82 (4):132–155
3. Blanchard JL, Lynch M (2000) Organellar genes: why do they end up in the nucleus? Trends Genet 16(7):315–320
4. Dismukes GC, Carrieri D, Bennette N, Ananyev GM, Posewitz MC (2008) Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. Curr Opin Biotechnol 19(3):235–240. https://doi.org/10.1016/j.copbio.2008.05.007
5. Gupta RS (2010) Molecular signatures for the main phyla of photosynthetic bacteria and their subgroups. Photosynth Res 104 (2–3):357–372. https://doi.org/10.1007/s11120-010-9553-9
6. Gao X, Sun T, Pei G, Chen L, Zhang W (2016) Cyanobacterial chassis engineering for enhancing production of biofuels and chemicals. Appl Microbiol Biotechnol 100(8):3401–3413. https://doi.org/10.1007/s00253-016-7374-2
7. Dexter J, Fu P (2009) Metabolic engineering of cyanobacteria for ethanol production. Energy Environ Sci 2(8):857–864
8. Lan EI, Liao JC (2012) ATP drives direct photosynthetic production of 1-butanol in cyanobacteria. Proc Natl Acad Sci U S A 109 (16):6018–6023. https://doi.org/10.1073/pnas.1200074109
9. Halfmann C, Gu L, Gibbons W, Zhou R (2014) Genetically engineering cyanobacteria to convert CO_2, water, and light into the long-chain hydrocarbon farnesene. Appl Microbiol Biotechnol 98(23):9869–9877
10. Wang Y, Sun T, Gao X, Shi M, Wu L, Chen L, Zhang W (2016) Biosynthesis of platform chemical 3-hydroxypropionic acid (3-HP) directly from CO_2 in cyanobacterium *Synechocystis* sp. PCC 6803. Metab Eng 34:60–70. https://doi.org/10.1016/j.ymben.2015.10.008
11. Hasunuma T, Takaki A, Matsuda M, Kato Y, Vavricka CJ, Kondo A (2019) Single-stage astaxanthin production enhances the nonmevalonate pathway and photosynthetic central metabolism in *Synechococcus* sp. PCC 7002. ACS Synth Biol 8(12):2701–2709. https://doi.org/10.1021/acssynbio.9b00280
12. Yoshikawa K, Toya Y, Shimizu H (2017) Metabolic engineering of *Synechocystis* sp. PCC 6803 for enhanced ethanol production based on flux balance analysis. Bioprocess Biosyst Eng 40(5):791–796. https://doi.org/10.1007/s00449-017-1744-8
13. Hirokawa Y, Matsuo S, Hamada H, Matsuda F, Hanai T (2017) Metabolic engineering of *Synechococcus elongatus* PCC 7942 for improvement of 1,3-propanediol and glycerol production based on in silico simulation of metabolic flux distribution. Microb Cell Factories 16(1):212. https://doi.org/10.1186/s12934-017-0824-4
14. Zhang S, Liu Y, Bryant DA (2015) Metabolic engineering of *Synechococcus* sp. PCC 7002 to produce poly-3-hydroxybutyrate and poly-3-hydroxybutyrate-co-4-hydroxybutyrate. Metab Eng 32:174–183. https://doi.org/10.1016/j.ymben.2015.10.001
15. Song K, Tan X, Liang Y, Lu X (2016) The potential of *Synechococcus elongatus* UTEX 2973 for sugar feedstock production. Appl Microbiol Biotechnol 100(18):7865–7875. https://doi.org/10.1007/s00253-016-7510-z
16. Wang B, Wang Z, Wang D, Zhang B, Ong S-G, Li M, Yu W, Wang Y (2019) krCRISPR: an easy and efficient strategy for generating conditional knockout of essential genes in cells. J Biol Eng 13(1):35. https://doi.org/10.1186/s13036-019-0150-y
17. Sun T, Li S, Song X, Pei G, Diao J, Cui J, Shi M, Chen L, Zhang W (2018) Re-direction of carbon flux to key precursor malonyl-CoA via artificial small RNAs in photosynthetic *Synechocystis* sp. PCC 6803. Biotechnol Biof 11:26. https://doi.org/10.1186/s13068-018-1032-0
18. Gründel M, Scheunemann R, Lockau W, Zilliges Y (2012) Impaired glycogen synthesis causes metabolic overflow reactions and affects stress responses in the cyanobacterium *Synechocystis* sp. PCC 6803. Microbiology 158 (Pt 12):3032–3043. https://doi.org/10.1099/mic.0.062950-0
19. Kent R, Dixon N (2020) Contemporary tools for regulating gene expression in bacteria. Trends Biotechnol 38(3):316–333. https://doi.org/10.1016/j.tibtech.2019.09.007
20. Gaida SM, Al-Hinai MA, Indurthi DC, Nicolaou SA, Papoutsakis ET (2013) Synthetic tolerance: three noncoding small RNAs, DsrA, ArcZ and RprA, acting supra-additively against acid stress. Nucleic Acids Res 41 (18):8726–8737. https://doi.org/10.1093/nar/gkt651

21. Nakashima N, Tamura T (2009) Conditional gene silencing of multiple genes with antisense RNAs and generation of a mutator strain of Escherichia coli. Nucleic Acids Res 37(15): e103–e103. https://doi.org/10.1093/nar/gkp498
22. Na D, Yoo SM, Chung H, Park H, Park JH, Lee SY (2013) Metabolic engineering of *Escherichia coli* using synthetic small regulatory RNAs. Nat Biotechnol 31(2):170–174. https://doi.org/10.1038/nbt.2461
23. Cho C, Lee SY (2017) Efficient gene knockdown in *Clostridium acetobutylicum* by synthetic small regulatory RNAs. Biotechnol Bioeng 114(2):374–383. https://doi.org/10.1002/bit.26077
24. Sun D, Chen J, Wang Y, Li M, Rao D, Guo Y, Chen N, Zheng P, Sun J, Ma Y (2019) Metabolic engineering of *Corynebacterium glutamicum* by synthetic small regulatory RNAs. J Ind Microbiol Biotechnol 46(2):203–208. https://doi.org/10.1007/s10295-018-02128-4
25. Li S, Sun T, Xu C, Chen L, Zhang W (2018) Development and optimization of genetic toolboxes for a fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973. Metab Eng 48:163–174. https://doi.org/10.1016/j.ymben.2018.06.002
26. Gangopadhyay SA, Cox KJ, Manna D, Lim D, Maji B, Zhou Q, Choudhary A (2019) Precision control of CRISPR-Cas9 using small molecules and light. Biochemistry 58 (4):234–244. https://doi.org/10.1021/acs.biochem.8b01202
27. McNerney MP, Watstein DM, Styczynski MP (2015) Precision metabolic engineering: The design of responsive, selective, and controllable metabolic systems. Metab Eng 31:123–131. https://doi.org/10.1016/j.ymben.2015.06.011
28. Ewald J, Bartl M, Kaleta C (2017) Deciphering the regulation of metabolism with dynamic optimization: an overview of recent advances. Biochem Soc Trans 45(4):1035–1043
29. Markley AL, Begemann MB, Clarke RE, Gordon GC, Pfleger BF (2015) Synthetic biology toolbox for controlling gene expression in the cyanobacterium *Synechococcus* sp. strain PCC 7002. ACS Synth Biol 4(5):595–603. https://doi.org/10.1021/sb500260k
30. Alagesan S, Hanko EKR, Malys N, Ehsaan M, Winzer K, Minton NP (2018) Functional genetic elements for controlling gene expression in *Cupriavidus necator* H16. Appl Environ Microbiol 84(19):e00878–e00818. https://doi.org/10.1128/AEM.00878-18
31. Moser F, Espah Borujeni A, Ghodasara AN, Cameron E, Park Y, Voigt CA (2018) Dynamic control of endogenous metabolism with combinatorial logic circuits. Mol Syst Biol 14(11): e8605. https://doi.org/10.15252/msb.20188605
32. Mohamed A, Eriksson J, Osiewacz HD, Jansson C (1993) Differential expression of thepsbAgenes in the cyanobacterium *Synechocystis* 6803. Mol Gen Genet MGG 238 (1-2):161–168
33. Mulo P, Sicora C, Aro EM (2009) Cyanobacterial psbA gene family: optimization of oxygenic photosynthesis. Cell Mol Life Sci 66 (23):3697–3710
34. Zhou J, Zhang H, Meng H, Zhu Y, Bao G, Zhang Y, Li Y, Ma Y (2014) Discovery of a super-strong promoter enables efficient production of heterologous proteins in cyanobacteria. Sci Rep 4:4500
35. Huang H-H, Lindblad P (2013) Wide-dynamic-range promoters engineered for cyanobacteria. J Biol Eng 7(1):10–10. https://doi.org/10.1186/1754-1611-7-10
36. Babele PK, Kumar J, Chaturvedi V (2019) Proteomic de-regulation in cyanobacteria in response to abiotic stresses. Front Microbiol 10:1315–1315. https://doi.org/10.3389/fmicb.2019.01315
37. Zhou Y, Sun T, Chen Z, Song X, Chen L, Zhang W (2019) Development of a new biocontainment strategy in model cyanobacterium *Synechococcus* Strains. ACS Synth Biol 8 (11):2576–2584. https://doi.org/10.1021/acssynbio.9b00282
38. Sun T, Li S, Song X, Diao J, Chen L, Zhang W (2018) Toolboxes for cyanobacteria: recent advances and future direction. Biotechnol Adv 36(4):1293–1307. https://doi.org/10.1016/j.biotechadv.2018.04.007
39. Wang B, Wang J, Zhang W, Meldrum DR (2012) Application of synthetic biology in cyanobacteria and algae. Front Microbiol 3:344–344. https://doi.org/10.3389/fmicb.2012.00344
40. Berla BM, Saha R, Immethun CM, Maranas CD, Moon TS, Pakrasi HB (2013) Synthetic biology of cyanobacteria: unique challenges and opportunities. Front Microbiol 4:246–246. https://doi.org/10.3389/fmicb.2013.00246
41. Heidorn T, Camsund D, Huang H-H, Lindberg P, Oliveira P, Stensjö K, Lindblad P (2011) Chapter 24: Synthetic biology in cyanobacteria: engineering and analyzing novel functions. In: Voigt C (ed) Methods in Enzymology, vol 497. Academic Press, Cambridge,

pp 539–579. https://doi.org/10.1016/B978-0-12-385075-1.00024-X

42. Russell DW, Sambrook J (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor, New York
43. Tan X, Hou S, Song K, Georg J, Klähn S, Lu X, Hess WR (2018) The primary transcriptome of the fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973. Biotechnol Biof 11:218. https://doi.org/10.1186/s13068-018-1215-8
44. Kopf M, Klähn S, Scholz I, Matthiessen JK, Hess WR, Voß B (2014) Comparative analysis of the primary transcriptome of *Synechocystis* sp. PCC 6803. DNA Res 21(5):527–539. https://doi.org/10.1093/dnares/dsu018
45. Sun T, Pei G, Wang J, Chen L, Zhang W (2017) A novel small RNA CoaR regulates coenzyme A biosynthesis and tolerance of *Synechocystis* sp. PCC6803 to 1-butanol possibly via promoter-directed transcriptional silencing. Biotechnol Biof 10:42–42. https://doi.org/10.1186/s13068-017-0727-y
46. Chen L, Wu L, Wang J, Zhang W (2014) Butanol tolerance regulated by a two-component response regulator Slr1037 in photosynthetic *Synechocystis sp.* PCC 6803. Biotechnol Biof 7:89. https://doi.org/10.1186/1754-6834-7-89
47. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) method. Methods 25(4):402–408. https://doi.org/10.1006/meth.2001.1262
48. Elhai J, Wolk CP (1988) Conjugal transfer of DNA to cyanobacteria. Methods Enzymol 167:747–754
49. Barten R, Lill H (1995) DNA-uptake in the naturally competent cyanobacterium, *Synechocystis sp.* PCC 6803. FEMS Microbiol Lett 129 (1):83–87. https://doi.org/10.1111/j.1574-6968.1995.tb07561.x



Chapter 17

Sorghum as Biofuel Crop: Interdisciplinary Methods to Enhance Productivity (Botany, Genetics, Breeding, Seed Technology, and Bioengineering)

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Abstract

Sorghum is a versatile crop cultivated since time immemorial. It fulfills the basic needs of mankind in the contest of food, feed, fodder, nutrition, and pharmaceutical uses. Now it adds one more paramount importance as a second-generation biofuel. It offers ethanol from grain, stem (sweet sorghum), and biomass (lignocellulose), and the previous one is discouraging because of food versus fuel conflict. However sorghum lignocellulosic biofuel are gaining momentum in order to conserve nature from depleting first-generation fuel. This chapter describes interdisciplinary approaches/methods involving understanding the genetics of biofuel traits, formulating suitable breeding strategies and seed enhancement techniques to achieve higher productivity in marginal lands in order to avoid food vs. fuel conflict, and finally realization of bioethanol by involving bioengineering process. Many reviews, worldwide researches, and policy papers accepted that sorghum has tremendous potential to be used as a crop of biofuel production.

Key words Sorghum, Biofuel, Sweet sorghum, Lignocellulose, Ethanol, *bmr*, Pedigree breeding, Biofuel syndrome

1 Botany, Genetics, and Breeding

1.1 Nomenclature

Linnaeus was the first to classify Sorghum under the genus *Holcus* (1753) but Moench created a separate genus Sorghum. The name *Holus sorghum* (L.) was replaced as *Sorghum vulgare* by Person and further the name was changed to the present name *Sorghum bicolor* (L.) *Moench* by Clayton.

1.2 Sorghum as Biofuel Crop

Sorghum is a versatile crop and has potential to use as food, fodder, fuel and also fiber crop. The natural characteristics of sorghum crops are highly suitable to use them as efficient biofuel crops compared with other crops. Brazil was the pioneer country to initiate biofuel production from sweet sorghum and currently they have 400 ethanol mills and sweet sorghum cultivation in 1.8

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million hectares [1]. The rate of fossil fuel depletion is massive and the consumption of fossil fuel needs to be declined. The risk of fuel shortage in the near future can be solved by using naturally available sources to produce energy, and biofuel from sorghum is an appropriate choice. The usage of biofuel will reduce GreenHouse Gas emission and conserve fossil fuel.

- The rate of photosynthesis is high because of its C_4 nature and so results in increased biomass production.
- Sorghum is a short-day plant and grown in tropical, subtropical, arid, and semiarid regions where the temperature is relatively high and so suitable for almost all countries.
- The input requirement of the crop is low and grown well even in marginal lands with low fertility.
- The crop is tall with thick stem and accumulates sugar in the stalk but the ratio differs based on genotype [2].
- It is a hardy crop and well adapted to various abiotic stress conditions such as drought, salinity, and water logging.
- The water requirement is very low compared with maize, sugarcane, sugar beet, and other biofuel crops. The crop is referred to as "the camel among crops" due to its drought resistance characteristics [1].
- Sugar mills can be converted to Sorghum ethanol producing units and thereby eliminating the need to construct a separate processing unit.
- The grain yield is comparatively low yet the ethanol production is high.
- Lignocellulose biomass is high in sweet sorghum and recovery of ethanol is worthy.
- According to the report of the United States Department of Agriculture (USDA), the sweet sorghum produces 12–16 Btu (British thermal units) for each unit utilized. (https://www.uky.edu/Ag/CCD/introsheets/sorghumbiofuel.pdf).

1.3 Sorghum Origin and Domestication

The lack of archaeological proof related to Sorghum cultivation has made it challenging to track the ancient past of Sorghum [3]. The words like "Dohan" in Hebrew text and "Darri or Durra" in Turkish means millet but not exactly "Sorghum" and so the literature lack typical evidence to prove sorghum cultivation [4]. On the basis of diversity observed, Vavilov placed Sorghum in African Center of Origin and India as the secondary center of origin [5]. But Murdock suggested Sudanic complex is the region where sorghum might be originated. A different view in patterns of origin was presented by Harlan as center and non-center. Sorghum comes under non-center pattern of origin because limitless variability was

observed around its region of origin [6]. According to Snowden [7], race durra and bicolor originated from wild race aethiopicum, race *guinea* from wild race arundinaceum, and race kafir from wild race verticilliflorum.

The Guinea Corn (*Var.guineense*) was noticed in Central Sudan and so Murdock [8] has specified people of western Sudan "Negroes" were the first to cultivate sorghum during the year 5000–4000 BC. Based on the view it is assumed that first domestication of sorghum happened in the area stretching along the River Niger. Mann [9] concluded that sorghum was first domesticated in Northeast Africa.

1.4 Alternate Hypothesis: Origin of Sorghum Cultivation

A trait gained more importance during domestication was absence or presence of abscission zone. The mutant devoid of abscission zone that prevents shattering was selected because it has positive correlation with yield. The progenitor of modern sorghum was race *virgatum* (desert grass) and race arundinaceum (forest grass) by De wet et al. [5]. But Snowden [7] suggests that race arundinaceum may not be a direct ancestor.

Based on Sahara Pump theory, during the wet interpluvial period there may be cultivation of four wild races in Sahara. Intercrossing between Races aethiopicum and verticilliflorum exist in Savanna and also with arundinaceum during wet inter pluvial period [10]. Stemler believes that domestication of sorghum happens late because of the nonexistence of tools to harvest whole panicles and only after the invention of paleolithic stone tool domestication happens. Domestication may happen at different places at different times. As wild sorghum is seen in Sahara highlands and so domestication happens along Fertile Crescent of Sahara. Harlan [6] interprets that race bicolor to be most primitive in domestication. Snowden [7], De wet, and Hukaby [11] suggest race guinea was the result of introgression between race arundinaceum and primitive bicolor but race guinea evolved without introgression as by Harlan and Stembler [11]. Doggett concluded that race durra evolved in Ethiopia and distributed to India and Arab states.

1.5 Distribution

The distribution of Sorghum is restricted to the old world and after colonization of Europeans it reached Australia [12]. The cultivation Sorghum is predominant in Asia and African countries. The statistics of the Food and Agricultural Organization of the United Nation states that the USA stands first in production (11.5 million metric tons) and followed by India (7.5 million metric tons), Nigeria (7.4 million metric tons), and Mexico (6.1 million metric tons).

- *Sorghum bicolor* sub sp. *halepense*—distributed in Mediterranean region and includes mostly weedy species. Two distinct forms based on phenotype were noticed and named as Mediterranean

ecotype (small plant with narrow leaves) and Tropical ecotype (large plant with broad leaves). The Mediterranean ecotype is distributed across Asia Minor with chromosome number of $2n = 40$ and Tropical ecotype widely in Southern India and South East Asia ($2n = 20$).

- *Sorghum bicolor* sub sp. *bicolor*—The members were cultivated sorghum, related weeds, and semi wild relatives and were predominant in Africa.
- *Sorghum bicolour* var. *arundinaceum*—consists of weedy sorghum and distributed over Tropical and Sub—Tropical West Africa.
- *Sorghum bicolour* var. *aethiopicum*—distributed in Nigeria, Somalia, and Nile Valley and freely intercross with var. *arundinaceum*.
- *Sorghum bicolour* var. *verticilliflorum*—seen in the South African Coast and distribution overlaps with var. *aethiopicum* and var. *arundinaceum*. They intercross readily in all possible combinations.
- Race guinea—cultivated in areas that receives 1000 mm rainfall annually and intercross with race kafir results in a complex that is noticed in eastern Africa. This particular race is noticed in India and Burma.
- Race kafir—An African cultivar complex and prominent in various parts of Africa that includes Northern Nigeria, Ghana, etc.,
- Race durra—Predominant in Arabia and Asia Minor. The phenotypic study gives assumptions that durra is the selection of Kafir.
- Race bicolor—Noticed in various parts of Asia, Africa and grown mostly in India, South East Asia. They are developed out of var aethiopicum selection.

The migration and trading are the prime factors that distributed Sorghum from African subcontinent to other parts of the world.

1.6 Classification and Systematic Position

The Sorghum was classified into 31 cultivated species and 17 wild species by Snowden [7]. The classification is further refined by Garber and Doggett. The classification is represented in the following flow chart (Fig. 1).

1.7 Sorghum Taxonomy [12]

Kingdom—Plantae
Sub-kingdom—Tracheobionta
Superdivision—Spermatophyta
Division—Magnoliophyta
Class—Liliopsida
Sub-class—Commelinidae

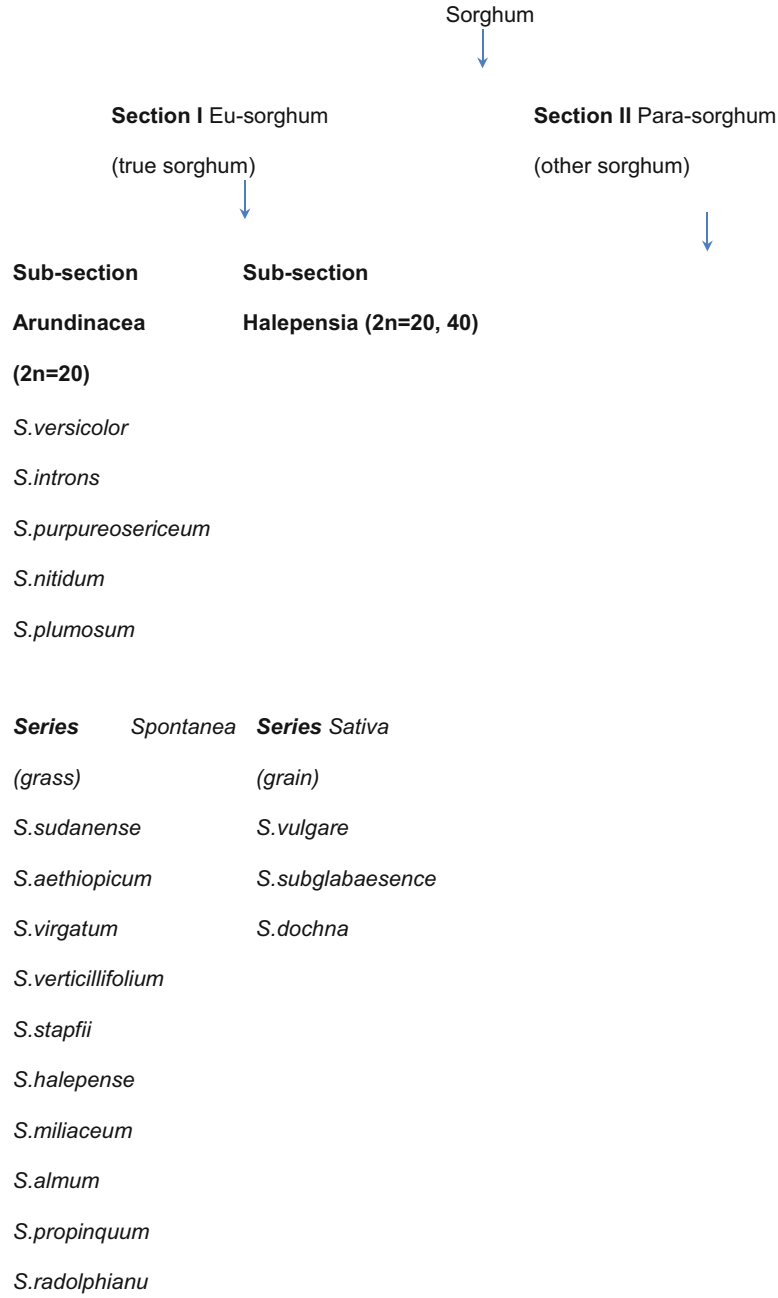


Fig. 1 Classification of sorghum races [13]

Order—Cyperales
Family—Poaceae (grass)
Tribe—Andropogoneae
Sub-tribe—Sorghinae
Genus—Sorghum
Species—*Sorghum bicolor*

Sub-species—*Sorghum bicolor* ssp.*arundinaceum*—common wild sorghum

Sub-species—*Sorghum bicolor* ssp. *bicolor*—grain sorghum

Sub-species—*Sorghum bicolor* ssp. *drummondii*—Sudan grass

1.8 Races of Sorghum and Its Characteristics

Five races of sorghum are classified based on panicle nature, glume coverage, shape, and color of the grain [9]. Characteristic feature of each race is given in Table 1.

Other intermate races were Guinea-bicolor, Guinea-kafir, Caudatum-bicolor, Guinea-durra, Kafir-bicolor, Kafir-caudatum, Durra-bicolor, Durra-caudatum, Guinea-caudatum, and Kafir-durra [13].

1.9 Biomass Production

Sorghum is a hardy crop and well adapted to tropical and subtropical conditions. Therefore, they are cultivated in nearly 110 countries in the world. The biomass production is high due to enhanced photosynthesis rate. The biomass of 58.3–80.5 tons of fresh stems/ha has been cultivated in a semiarid zone with sugary juice of 12,000–20,000 L/ha [14]. The sugar get stored in the parenchymatous cell of the stalk and the ratio of reducing sugar is more in sweet sorghum juice compared with sugarcane juice. The side-by-side comparison of nitrogen consumption to produce equal levels of ethanol reveals that sweet sorghum consumes relatively less nitrogen. The comparison between sugarcane and sweet sorghum is represented in Table 2.

1.10 Genetics of Biofuel Syndrome

A group of traits responsible for biofuel production is called biofuel syndrome [15]. In order to enhance the biofuel efficiency of crop plants, the genetics of related traits must be understood. Central dogma of life is the process by which trait expression in biological organisms is realized. Sorghum harbors a number of useful traits for the human kind, namely high yield, biotic and abiotic stress tolerance, quality of products, and industry oriented traits and bioethanol yield. In sweet sorghum about 858 QTLs are related to yield, low lignin, high biomass, ethanol yield, and stress tolerance. [16]. The following is a list of genetics of biofuel syndrome traits (Table 3).

1.11 Importance of Mutant bmr Trait

The lignocellulosic feedstock consists of cellulose, hemicellulose, and also lignin. The lignin possesses negative impact on ethanol production. The breakdown of cellulose and hemi-cellulose was retarded by lignin and also produce toxin substances during pretreatment which resist the growth of microorganism. Hence, a sorghum mutant which possess reduced lignin content called bmr (Brown midrib) mutant can be used as a solution to eliminate this problem [25].

Table 1
Detailed features of sorghum races

Races	Characters
Bicolor	• Medium size open panicle • Thick glumes with obscure nerves • Depressed and hairy tips of lower glumes • Elliptical small grain • Grain enclosed and tightly clasped by the glumes • Short pedicle • Highly pigmented glumes, seed and plant parts • Fairly low yielding, medium height but tending to tiller profusely
Guinea	• Panicles long, loose, glabrous and pendulous at maturity • Sessile spikelets opening when mature exposing the grain • Glumes involute, opening widely, hairy and awns • Grains small to medium and biconvex • Grain color light or only slightly pigmented • Plants medium to tall and low yielding
Caudatum	• Panicles medium large oblong, dense to slightly open, hairy with stout peduncle and rigid primary branches • Sessile spikelets obovate to elliptical • Grains are turtle backed • Grain chalky white or pigmented, most with a recessive spreader gene • Plants medium to tall and generally high yielding
Kafir	• Panicles erect, elongate, mostly semi compact and cylindrical • Branches and sessile spikelets hairy but glumes at maturity almost glossy • Glumes much shorter than grain • Grain is broadly elliptical, flattened or biconvex • Plants medium height and high yielding
Durra	• Panicles stiff, dense and compact, ovate to oblong shaped or rhomboidal and covered with dense velvety pubescence • Peduncle often recurved but occasionally erect • Panicle branches short, semi-erect and hairy • Pedicellate spikelets large and persistent • Glumes lightly pigmented • Grain generally medium to large, biconvex, broad top and wedge shaped base • Plants medium to tall and good quality

1.12 Breeding Methods

Most of the biofuel traits exhibit additive gene action and few are non-dominant and epistasis and hence following breeding methods are highly suitable to develop superior varieties and hybrids.

1. Pedigree breeding (Recombination)
2. Heterosis breeding (Hybrid breeding)
3. Population improvement (Half sib and full sib)
4. Marker Assisted Backcross Breeding (MABB)
5. Tissue culture and Genetics transformation methods (Biotechnology)

Table 2
Comparison between sugarcane and sweet sorghum [14]

Character	Sweet sorghum	Sugarcane
Sugar concentration in the juice (as ° Brix)	10.5–20.7	16–20
Sucrose (%)	69–74	70–88
Proportion of sugar present as reducing sugars (%)	5–19	4–8
Starch (%)	0.4–5.3	0.001–0.05
pH	4.9–5.5	5.2–5.4
Organic acids (%)	NA	1.5–5.5
Aconitic acid (%)	3.6–4.8	1.0–2.1
Protein (%)	0.9–1.3	0.5–0.6

Table 3
Genetics of sorghum biofuels traits

Sl. No	Traits	Genetics	References
1.	Sugar content	Dominant/additive-2 QTLs	[17]
2.	Stem sugar, composition, grain yield	QTLs with non pleiotropic effect	[18]
3.	Brix (glucose-6-phosphate homology)	Chromosome 1	[19]
4.	Sugar related traits	Quantitative traits	[20]
5.	Sugar yield	Quantitative trait and vary with the genotype, environment, and genotype-by environment effects	[18, 19]
6.	Main culm height	QTLs	[21]
7.	Culm Length, width and number	QTLs	[22]
8.	Number of nodes	QTLs	[23]
9.	And stem diameter	QTLs	[20, 23]
10.	Plant height	Sorghum chromosomes 9 and 6	[19]
11.	Leaf architecture	84 QTLs	[16]
12.	Root morphology	22 QTLs	[16]
13.	Brown midrib (low lignin content)	*bmr* mutants *bmr2*, *bmr6*, and *bmr12* and *bmr19*	[24]

1.13 Genetic Resources Suitable for High Biomass

Anil Kumar and his colleagues [26] conducted a study to identify high biomass sorghum suitable for cultivation in India. A total of 65 biomass sorghum genotypes bred from International Crops Research Institute for the semiarid Tropics (ICRISAT), Indian Institute of Millet Research (IIMR) and National Agriculture Research System (NARS) and Tamil Nadu Agricultural University (TNAU), Coimbatore. The biomass composition of IS 25302 reveals numerically higher cellulose and hemicellulose content than ICSV 25333, though, the dry biomass recorded has marginal differences for IS 25302 (18.9 t ha^{-1}) and ICSV 25333 (19.2 t ha^{-1}). The entries like IS 16529, IS 22879, IS 27246, IS 13762, IS 25301, and ICSV 25333 were best suited for Tamil Nadu and Telangana, ICSSH 28, IS 17349, and ICSV 25333 were best suited for Madhya Pradesh, Gujarat, and Telangana in terms of fresh biomass yield and dry biomass yield. The top ten entries which are stable and showing best performance in terms of fresh biomass yield, dry biomass yield, and grain yield were ICSV 25333, IS 13762, CSH22SS, IS 25302, IS25301, IS 27246, IS 16529, DHBM2, ICSSH 28, and IS17349. Thus, the lines like ICSV 25333, IS 25302, IS22868, IS 13762, and IS 16529 are stable performers both in terms of biomass accumulation and economically viable for ethanol production.

Sorghum recombinant inbred lines (RIL) 236 were for lignocellulosic biomass performance and determined hemicellulose, cellulose, and lignin concentrations through detergent fiber analysis (DFA). The results showed that RIL carrying the brown midrib mutation had significantly high theoretical glucose recovery (>200 g kg^{-1}). Those carrying both mutations had high theoretical ethanol yield (>400 L ton^{-1}) and high theoretical ethanol production (>14,500 L ha^{-1}). Lignin concentration was determined as the most reliable predictor ($R^2 = 0.67$) for glucose recovery. Lignin and stem sugar concentrations ($R^2 = 0.46$ and 0.35, respectively) were good predictors for ethanol yield. Stover yield traits ($R^2 = 0.89$) were most important determinants for ethanol production [27].

1.14 Institutes for Conservation of Sorghum Germplasm

1.14.1 International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India (https:/www.icrisat.org) [28]

The Gene bank of International Crops Research Institute for Semi-Arid Tropics maintains **41,816** Sorghum collections (Table 4). In order to avoid duplication, a true representative core and mini-core was created. The core collection of sorghum includes 2247 accessions and the mini-core collection of sorghum possesses 242 accessions. The Western and Central African country possess great diversity for various traits and exclusive collection from that area is 8020.

Table 4
The sorghum accessions were characterized and classified as follows

Races	Number of collections
Bicolor	163
Guinea	2702
Caudatum	1735
Kafir	8
Durra	694
Guinea—bicolor	171
Caudatum—bicolor	364
Durra—bicolor	174
Guinea—caudatum	726
Guinea—durra	21
Kafir—caudatum	5
Durra—caudatum	858

1.14.2 Indian Institute of Millet Research (IIMR), Hyderabad, India (www.millets.res.in) [29]

The diversity of various millets are conserved at IIMR and number of sorghum accessions maintained were 6823 which includes collections received under CRAB by the National Bureau of Plant Genetic Resources (NBPGR), 58 accessions are received from ICRISAT and 106 accessions are indigenous collections by IIMR.

1.14.3 National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India (www.nbpgr.ernet.in) [30]

Plant genetic diversity is of fundamental importance in the continuity of a species as it provides the necessary adaptation to the prevailing biotic and abiotic environmental conditions and the base collections of sorghum maintained was 26,118.

1.14.4 The Ramiah Gene Bank, Tamil Nadu Agricultural University, Coimbator (www.tnau.ac.in) [31]

The seeds of various agricultural crops were maintained and the number of sorghum collections was 2429.

2 Seed Technological Intervention for Higher Productivity in Sorghum

2.1 Pre-sowing Seed Treatment

Sorghum is cultivated as dryland crops, seed hardening is recommended to increase productivity by overcoming the moisture stress for sustained production under dryland. Moisture stress during initial establishment affects the crop stand and so the productivity. Sorghum seeds hardened with 2% potassium dihydrogen phosphate increased the yield under dryland situation and increased the yield

up to 10% [32]. Pre-sowing hardening of seeds is one of the methods which results in modifying the physiological and biochemical nature of seeds so as to get the characters that are favorable for drought tolerance [33]. It is also the extensive physiological reorganization induced by the dehydration process. During the hardening process a number of physicochemical changes occur modifying the protoplasmic characters and increasing physiological activity of the embryo and associated structures. Sorghum seeds hardened with aqueous solution of botanicals performed significantly better than Control [34].

2.1.1 Principle

When the dry seeds are soaked, the seed imbibes water and expands. This permits the initial process of germination but the further cell elongation and radicle emergence are prevented by shade and sun drying them to original moisture content.

2.1.2 The Physiological Basis for Seed Hardening

The influence results of the pre-sowing hardening treatment under drought condition is due to the following physiological changes that occurred in the seed on seed treatment.

- Greater hydration of colloids.
- Higher viscosity and elasticity of protoplasm.
- Increase in the physiological activity of the embryo and associated structures.
- Increase in bound water content.
- Increase in intensity of photosynthesis.
- Increase in hydrophilic colloids and decrease in hydrophilic colloids.
- Increase in temperature requirement for protein coagulation.
- More intensive respiration.
- Lower water deficit.
- Increase in water balance of plant.
- More efficient root system.

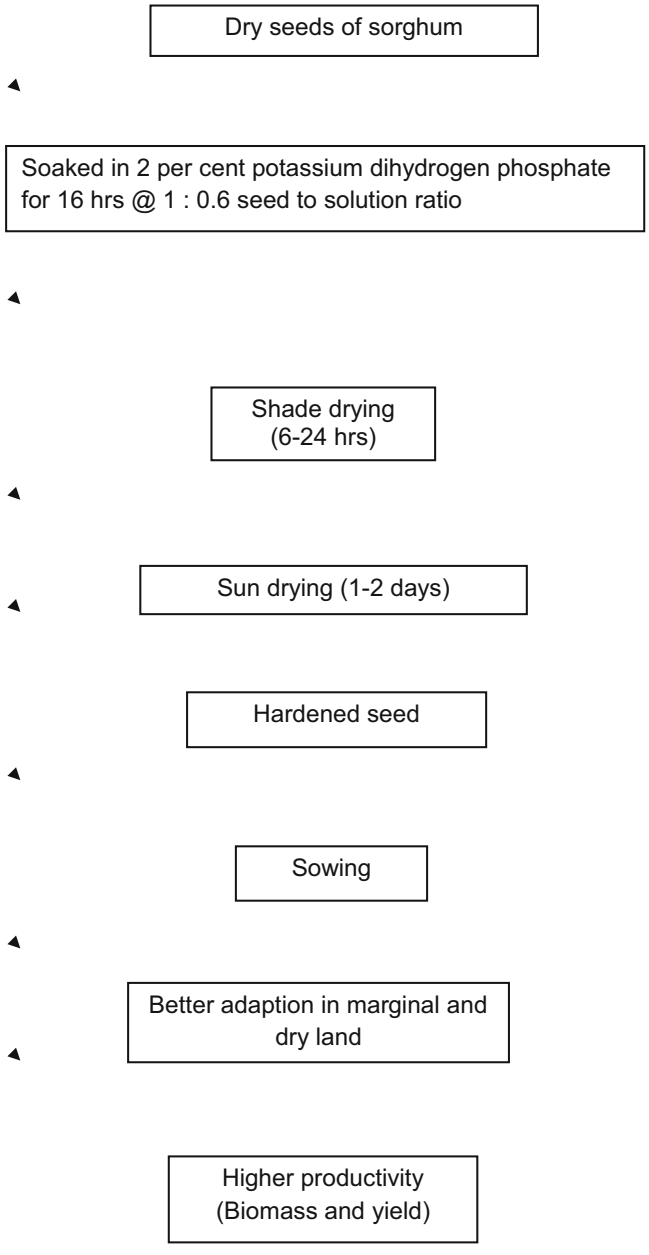
2.1.3 Beneficial Effects of Seed Hardening

- Accelerate rapid germination and growth rate of seedlings.
- Hardened plants recover much more quickly from wilting than those from untreated plants.
- Induces resistance to salinity as well as to drought condition.
- Seeds withstand higher temperature (80–105 °C) for prolonged periods (24–28 h) without loss of viability.
- Flowering is slightly accelerated.
- Early emergence enables seedling to compete more effectively with weeds.
- Hardened seeds perform better and result in more yields up to 10–15%.

2.1.4 Methodology

Seeds are soaked in 2% potassium dihydrogen phosphate solution at seed to solution ratio of 1:0.6 for 16 h. When the moisture content of seed rises to 30–35%, i.e., when the seeds are ready to put forth radicle the soaking is stopped and the seeds are dried to original moisture content of 10.12%. While drying to avoid mechanical injury, it should be first dried under shade for 6–24 h and should be followed by sun drying for 1–2 days to reduce the moisture content.

2.1.5 Flow Chart of Sorghum Seed Hardening



3 Ethanol Production Process

The renewable biomass present in this world is lignocelluloses and consists of cellulose, hemicellulose, and lignin. The prime source for ethanol production is cellulose and hemicellulose. The overexploitation of natural fossil fuels and GHG emissions creating threats had alerted the people to focus on biofuels. The biofuel (Ethanol) are produced from many crops yet Sweet sorghum play unique role because of its own characteristics suitable to use them effectively as biofuel crop.

The ethanol is produced from grains, sugar juice of sweet sorghum stalk, and from feedstock. The utilization of grain and sweet sorghum for ethanol production was not highly acceptable because it may cause negative impact on food security, and therefore feed stock of sorghum can be used to produce ethanol without compromising food [35].

3.1 Basic Steps in Ethanol Production

3.1.1 Pretreatment

The step is carried out to make the cellulose more permeable to breakage by hydrolysis. The polymer of sugar is made more reactive and liable by various pretreatment techniques (heat and combination of chemicals) [36].

Cellulosic bioethanol is produced from lignocellulosic biomass by pretreatment, hydrolysis, and fermentation. Some of the most promising pretreatment methods require the application of chemicals such as acids, alkali, salts, oxidants, and solvents. Thus, advances in research have enabled the development and integration of chemical-based pretreatment into proprietary ethanol production technologies in several pilot and demonstration plants globally, with potential to scale-up to commercial levels. Pretreatment involves the use of physical processes (e.g., size reduction, steaming/boiling, ultrasonication, and popping), chemical methods (e.g., acids, bases, salts, and solvents), physicochemical processes (e.g., liquid hot water and ammonium fiber explosion), biological methods (e.g., white-rot/brown-rot fungi and bacteria), and several combinations thereof to fractionate the lignocellulose into its components [37].

3.1.2 Hydrolysis

The enzymatic reaction in which pretreated cellulose is converted from complex sugar to simple sugar. The enzyme is produced by microorganism such as fungi (Yeast) and reduces the complex nature of sugar [36].

Acid Hydrolysis

There are two basic types of acid processes: dilute acid and concentrated acid, each with variations. Dilute acid processes are conducted under high temperature and pressure, and have reaction times in the range of seconds or minutes, which facilitates continuous processing. Most dilute acid processes are limited to a sugar

recovery efficiency of around 50%. For rapid continuous processes, in order to allow adequate acid penetration, feedstocks must also be reduced in size so that the maximum particle dimension is in the range of a few millimeters.

The concentrated acid process uses relatively mild temperatures and the only pressures involved are usually only those created by pumping materials from vessel to vessel. The low temperatures and pressures minimize the degradation of sugars. To recover the sugars, the hydrolyzed material in the first reactor is soaked in water and drained several times. The primary advantage of the concentrated process is the high sugar recovery efficiency, which can be on the order of over 90% of both hemicellulose and cellulose sugars.

Using some assumed cellulose conversion and fermentation efficiencies, ethanol yields from glucose can be calculated for corn Stover (the above-ground part of the corn plant less the ears) as shown in Tables 5 and 6.

Enzymatic Hydrolysis

Enzymes are naturally occurring plant proteins that cause certain chemical reactions to occur. For enzymatic processes to be effective, some kind of pretreatment process is thus needed to break the crystalline structure of the lignocellulose and remove the lignin to

Table 5
Ethanol yields from glucose

Dry stover	1 tonne (1000 kg)
Cellulose content	0.45
Cellulose conversion and recovery efficiency	0.76
Ethanol stoichiometric yield	0.51
Glucose fermentation efficiency	0.75
Yield from glucose	131 kg ethanol = 151 L (40 gallons)

Table 6
Ethanol yields from xylose

Dry stover	1 tonne (1000 kg)
Hemicellulose content	0.29
Hemicellulose conversion and recovery efficiency	0.90
Ethanol stoichiometric yield	0.51
Xylose fermentation efficiency	0.50
Yield from xylose	66 kg ethanol = 76 L (20 gallons)

expose the cellulose and hemicellulose molecules. Depending on the biomass material, either physical or chemical pretreatment methods may be used. Physical methods may use high temperature and pressure, milling, radiation, or freezing—all of which require high-energy consumption. The chemical method uses a solvent to break apart and dissolve the crystalline structure. Due to the tough crystalline structure, the enzymes currently available require several days to achieve good results. Since long process times tie up reactor vessels for long periods, these vessels have to either be quite large or many of them must be used. But, their efficiency is quite high and their byproduct production can be controlled; their mild process conditions do not require expensive materials of construction, and their process energy requirements are relatively low [38].

3.1.3 Fermentation

The metabolic process in which simple sugar gets converted into organic subtract (alcohol). However, 100% conversion is not possible and only 50–60% are converted to ethanol.

3.1.4 Thermochemical Processes

There are two ethanol production processes that currently employ thermochemical reactions in their processes. The first system is actually a hybrid thermochemical and biological system. A microorganism that is capable of converting the synthesis gas is introduced into the fermenters under specific process conditions to cause fermentation to ethanol. The second thermochemical ethanol production process does not use any microorganisms. In this process, biomass materials are first thermochemically gasified and the synthesis gas passes through a reactor containing catalysts, which cause the gas to be converted into ethanol. Ethanol yields up to 50% have been obtained using synthesis gas-to-ethanol processes. Some processes that first produce methanol and then use catalytic shifts to produce ethanol have obtained ethanol yields in the range of 80%. Unfortunately, like the other processes, finding a cost-effective all-thermochemical process has been difficult.

3.2 Inhibitory Compounds and Their Impact on Microorganisms

Many inhibitors are generated during the pretreatment of lignocellulosic biomass, which can have a negative influence on ethanol production. Inhibitors consist of different compounds, mainly furan derivatives such as 5-hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde (furfural), weak organic acids (formic, acetic and levulinic acids) and phenolic compounds. Phenolic compounds interfere with the function and integrity of cell membranes [39]. Acetic acid is often found in hydrolysates and comes from acetyl side-chain groups in hemicellulose [40]. The removal of inhibitory compounds thus increases the price of second-generation bioethanol. It is possible that ethanologenic microorganisms may become accustomed to living with inhibitor compounds during preconditioning at specific inhibitor concentrations.

3.3 Methods of Ethanol Extraction from Sorghum

Ethanol is being extracted from sink (grain), Stem (Juicy stem), and entire plant as raw materials. Following are methods/procedure for extraction of ethanol from respective plant parts

Sorghum			
Steps	1. Grain to Ethanol	2. Stem (Sweet sorghum) to Ethanol	3. Biomass Waste (Lignocelluloses) to Ethanol
1.	Cultivation under high input condition	Cultivation under high input condition	Cultivation at marginal lands
2.	Harvesting the ear heads at biological maturity	Harvesting the stem at appropriate stage (dough)	Harvest the entire biomass
3.	Threshing	Transportation to crushing mills (immediately)	Drying the biomass
4.	Drying the grains	De-trashing	Mill as fine powder
5.	Cleaning	Cleaning the stem	Sieving
6.	Wet milling	Milling	Pretreatment
7.	Gelatinization	Filtration	Cellulose and lignin
8.	Liquefaction	Concentration	Hydrolysis
9.	Fermentation	Fermentation	Fermentation
10.	Distillation	Distillation	Distillation
11.	Dehydration	Dehydration	Dehydration
12.	Ethanol	Ethanol	Ethanol

3.4 Pilot Plant Study

A trial was conducted at a sugar factory at Karnataka with 356 tons of sweet sorghum stalk. The efficiency was 50% which yields 39 L ethanol/ton of crushed stalks [41]. Currently, India was focusing on ethanol production. The policies and awareness need to be created to enhance the effective utilization of renewable energy and conserve nature.

References

1. Umakanth A, Kumar AA, Vermerris W, Tonapi V (2019) Sweet sorghum for the biofuel industry. In: Breeding sorghum for diverse end uses. Elsevier, Amsterdam, pp 255–270
2. Briand C, Geleta S, Kratochvil R (2018) Sweet sorghum (Sorghum bicolor [L.] Moench) a potential biofuel feedstock: analysis of cultivar performance in the Mid-Atlantic. Renew Energy *129*:328–333
3. Doggett H (1970) Sorghum. Longmans Green, London, p 403

4. De Wet J, Harlan J (1971) The origin and domestication of Sorghum bicolor. Econ Bot 25(2):128–135
5. Vavilov NI (1951) The origin, variation, immunity and breeding of cultivated plants (Vol. 72): LWW. Harlan, J. R. (1971). Agricultural origins: centers and noncenters. Science 174(4008):468–474
6. Snowden J (1936) The cultivated races of sorghum: Allard and Sons
7. Murdock G P (1959) Africa, its peoples and their culture history
8. Mann JA, Kimber CT, Station TAE, Miller FR (1983) The origin and early cultivation of sorghums in Africa: Texas agricultural experiment station. A & M University System, Texas
9. de Wet JM, Huckabay J (1967) The origin of Sorghum bicolor. II. Distribution and domestication. Evolution 21(4):787–802
10. Harlan JR, Stemler A (1976) The races of sorghum in Africa. Origin Afr Plant Domest 1976:465–478
11. De Wet J (1978) Special paper: systematics and evolution of sorghum sect. Sorghum (Gramineae). Am J Bot 65(4):477–484
12. Hariprasanna K, Patil JV (2015) Sorghum: origin, classification, biology and improvement. In: Sorghum molecular breeding. Springer, New York, pp 3–20
13. Bonin C, Lal R (2012) Bioethanol potentials and life-cycle assessments of biofuel feedstocks. Crit Rev Plant Sci 31(4):271–289
14. Lingle SE (2010) Opportunities and challenges of sweet sorghum as a feedstock for biofuel. In: Sustainability of the sugar and sugar—ethanol industries. ACS Publications, Washington, DC, pp 177–188
15. Anami SE, Zhang LM, Xia Y, Zhang YM, Liu ZQ, Jing HC (2015) Sweet sorghum ideotypes: genetic improvement of stress tolerance. Food Energ Secur 4(1):3–24
16. Amami SE, Zhang LM, Xia Y, Zhang YM, Liu ZQ, Jing HC (2015) Sweet sorghum ideotypes: genetic improvement of the biofuel syndrome. Food Energy Secur 4(3):159–177
17. Yun-long B, Seiji Y, Maiko I, Hong-wei C (2006) QTLs for sugar content of stalk in sweet sorghum (*Sorghum bicolor* L. Moench). Agric Sci China 5(10):736–744
18. Murray SC, Sharma A, Rooney WL, Klein PE, Mullet JE, Mitchell SE, Kresovich S (2008) Genetic improvement of sorghum as a biofuel feedstock: I. QTL for stem sugar and grain nonstructural carbohydrates. Crop Sci 48 (6):2165–2179
19. Murray SC, Rooney WL, Hamblin MT, Mitchell SE, Kresovich S (2009) Sweet sorghum genetic diversity and association mapping for brix and height. Plant Genome 2(1):48–62
20. Shiringani AL, Frisch M, Friedt W (2010) Genetic mapping of QTLs for sugar related traits in a RIL population of *Sorghum bicolor* L. Moench. Theor Appl Genet 121 (2):323–336
21. Hart GE, Schertz KF, Peng Y, Syed NH (2001) Genetic mapping of *Sorghum bicolor* L. Moench QTLs that control variation in tillering and other morphological characters. Theor Appl Genet 103:1232–1242
22. Takai T, Yonemaru J, Kaidai H, Kasuga S (2012) Quantitative trait locus analysis for days-to-heading and morphological traits in an RIL population derived from an extremely late flowering F1 hybrid of sorghum. Euphytica 187:411–420
23. Zou G, Zhai G, Feng Q, Yan S, Wang A, Zhao Q, Shao J, Zhang Z, Zou J, Han B et al (2012) Identification of QTLs for eight agronomically important traits using an ultra-high-density map based on SNPs generated from high-throughput sequencing in sorghum under contrasting photoperiods. J Exp Bot 63 (15):5451–5462
24. Sattler SE, Saballos A, Xin Z, Funnell-Harris DL, Vermerris W, Pedersen JF (2014) Characterization of novel Sorghum brown midrib mutants from an EMS-mutagenized population. G3 (Bethesda, MD) *4*(11):2115–2124. https://doi.org/10.1534/g3.114.014001
25. Saballos A (2008) Development and utilization of sorghum as a bioenergy crop. In: Genetic improvement of bioenergy crops. Springer, New York, pp 211–248
26. Kumar G, Vinutha KS, Shrivastava D, Jain S, Syed B, Gami B, Marimuthu S, Yuvraj A, Yadava H, Srivastava S, Yadagiri K, Ansodariya V, Prasuna P, Vishwanath J, Anand S, Rathore A, Umakanth A, Pinnamaneni SR (2018) Identification of ideal locations and stable high biomass sorghum genotypes in semiarid tropics. Sugar Tech 20:323–335. https://doi.org/10.1007/s12355-017-0584-9
27. Rivera-Burgos L, Volenec J, Ejeta G (2019) Biomass and bioenergy potential of brown midrib sweet sorghum germplasm. Front Plant Sci. https://doi.org/10.3389/fpls.2019.01142
28. https://www.icrisat.org
29. www.millets.res.in

30. www.nbpgr.ernet.in
31. www.tnau.ac.in
32. Menaka C, Vanangamudi K (2009) Biochemical and physiological changes of hardened and pelleted seeds of sorghum. J Ecobiol 24 (4):251–255
33. Henckel PA (1964) Physiology of Plants under drought. Annu Rev Plant Physiol 15:363–386
34. Jegathambal R (1996) Pre sowing seed treatment to augment productivity of sorghum cv. Co 26 under rainfed agriculture. Ph.D. Thesis, Tamil Nadu Agricultural University, Coimbatore.
35. Supriya Mathur A, Umakanth A, Tonapi V, Sharma R (2017) Sweet sorghum as biofuel feedstock: recent advances and available resources. Biotechnol Biof 10(146):1–19
36. Mosier N, Wyman C, Dale B, Elander R, Lee Y, Holtzapple M, Ladisch M (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 96 (6):673–686
37. Edem CB, Moses M (2013) Chemical pretreatment methods for the production of cellulosic ethanol: technologies and innovations. Int J Chem Eng 2013:719607
38. Robak K, Balcerek M (2018) Review of second generation bioethanol production from residual biomass. Food Technol Biotechnol 56 (2):174–187. https://doi.org/10.17113/ftb.56.02.18.5428
39. Palmqvist E, Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of inhibition. Bioresour Technol 74(1):25–33
40. Dien BS, Cotta MA, Jeffries TW (2003) Bacteria engineered for fuel ethanol production: current status. Appl Microbiol Biotechnol 63 (3):258–266
41. Hunsigi G, Yekkeli N, Kongawad B (2010) Sweet stalk sorghum: an alternative sugar crop for ethanol production. Sugar Tech 12 (1):79–80



Chapter 18

Optimization of Micropropagation and Genetic Transformation Protocols for *Paulownia elongata*: A Short Rotation Fast Growing Bioenergy Tree

Richa Bajaj, Lani Irvin, Brajesh N. Vaidya, Lubana Shahin, and Nirmal Joshee

Abstract

Various steps of micropropagation include selection of suitable explant, establishment of adventitious shoot induction cultures, proliferation, rooting, and acclimatization of the resulting plantlets. A systematic protocol is provided for the micropropagation and *Agrobacterium tumefaciens*-mediated genetic transformation of a fast growing, multipurpose tree, *Paulownia elongata*. Our studies show that optimum shoot induction is on half leaf with petiole explant on MS medium supplemented with 25 μM thidiazuron and 10 μM indole-3 acetic acid. Micropropagation protocols provided here are applicable to explants collected from the primed in vitro raised seedlings on MS medium containing 2.5 μM 6-benzylaminopurine (BAP) or actively growing shoots collected from greenhouse or field growing plants. We also discuss a possible role of "Python" script guided protocol optimization for higher and consistent multiplication of shoots that can be very helpful for scaled up production in commercial settings. To facilitate future plant improvement and gene editing possibilities, an *A. tumefaciens* based genetic transformation protocol and molecular identification of transgenic plants using Polymerase Chain Reaction (PCR) and Reverse Transcriptase-PCR (RT-PCR) techniques have also been optimized.

Key words Acclimation, Explant, Genetic transformation, Heat map, Organogenesis

1 Introduction

Paulownia species are fast growing semi-hardwood trees comprising nine species and a few natural hybrids, native to China and nearby countries [1]. The wood is light but strong, and is traditionally used to make musical instruments [2]. Wood flour of *Paulownia elongata* has been used to develop a series of biocomposites that are strong and environmentally sound products with acceptable engineering properties [3–5]. *Paulownia* trees can be grown for substantial financial rewards in the form of honey, biochar, biocomposites, short rotation biomass for bioenergy, animal feed, and timber production [3–9].

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Paulownia trees can be produced using seed, shoot, and root cuttings employing conventional vegetative propagation methods but plant tissue culture has many benefits [10]. These methods are economically favorable but are prone to diseases, thereby hindering planting success. Micropropagation techniques are useful for producing disease free, higher quality clonal plant material in large quantity. Earlier reports on the micropropagation of *Paulownia* species have predominantly used shoot tip and nodal explants. We present a protocol that is based on the use of entire leaf or half leaf with petiole as explant that achieves high number of adventitious shoots. We also used programming language "Python" to analyze multiplication numbers in all the replicates in each treatment to understand large variability that exists in in vitro micropropagation. The heat maps generated using "Python" script may provide an alternate method of comparison for shoot multiplication in all the explants in various treatments at the same time. This can further assist in tracing back the superior donor stock cultures providing explants with higher shoot multiplication efficiency and the suitable culture environment for raising stock plants.

2 Materials

Tissue culture facilities: balance, analytical balance, magnetic stirrer with heating function, pH meter, refrigerators at 4 °C and −20 °C, autoclave, laminar air flow cabinet, bacticinerator or glass bead sterilizer to sterilize the stainless steel instruments, stainless steel scalpels and forceps, electronic pipette, 0.22 μM syringe filters, syringes, climatic chamber or culture room maintained at 26 °C, beakers (100, 500, and 1000 mL), pipette or graduated cylinders (100 and 1000 mL), parafilm, and 70% ethanol, magnetic stir bars, baby food jars, magenta boxes and test tubes (*see* **Note 1**).

2.1 Plant Tissue Culture Medium and Growth Regulator Stocks

1. Distilled water.
2. Full strength MS basal medium (*see* **Note 2**).
3. Stock solutions for 6-Benzylaminopurine (BAP), thidiazuron (TDZ), indole-3-acetic acid (IAA), and indole-3-butyric acid (IBA). Plant growth regulator stocks are prepared at 1 mM concentration.
4. Agar Agar.
5. 1 M HCl and NaOH for adjusting pH.

2.2 Disinfection of the Explants

1. Running tap water.
2. Disinfection solution Clorox (2.5% active ingredient).
3. Sterile distilled water.

4. Sterile beakers (100 and 500 mL).
5. Proliferation medium contained in test tubes (15 mL medium in each test tube).

2.3 Explant Excision and In Vitro Culture

1. Paper towels moistened with distilled water and placed in glass Petri dishes (15 cm in diameter). Wrap the Petri dish with aluminum foil and autoclave.
2. Sterile distilled water.
3. Plant material.
4. Semisolid MS medium supplemented with TDZ and IAA, 3% maltose, and 0.7% agar in petri dishes (90 mm).

2.4 Plantlet Acclimatization

1. Tap water.
2. Free-draining potting mix (peat moss, perlite, polystyrene granules, 2:2:1).
3. Fungicide.
4. Tray covered with transparent plastic film/lid.

2.5 Genetic Transformation

1. Luria-Bertani (LB) medium.
2. Temperature controlled rotary shaker.
3. Acetosyringone.
4. *Agrobacterium tumefaciens* EHA105 transformed with binary vector plasmid pq35SGR.
5. Antibiotics-Carbenicillin, Cefotaxime, Rifampicin, and Kanamycin.
6. Agarose gel electrophoresis setup.
7. Tris–Boric acid–Acetic acid (TBA) buffer.
8. Fluorescent microscope to check GFP expression.
9. Loading dye (6×) (Promega, Madison, WI, USA).
10. "Forward" and "Reverse" primer pair for GFP gene
11. One Taq One-Step RT-PCR Kit.
12. TRIzol® reagent.
13. Gel documentation unit.
14. UV-Vis Spectrophotometer.

3 Methods

Figure 1 sums up in vitro response during five stages of *Paulownia* micropropagation. Following is a description of methods used for each step.

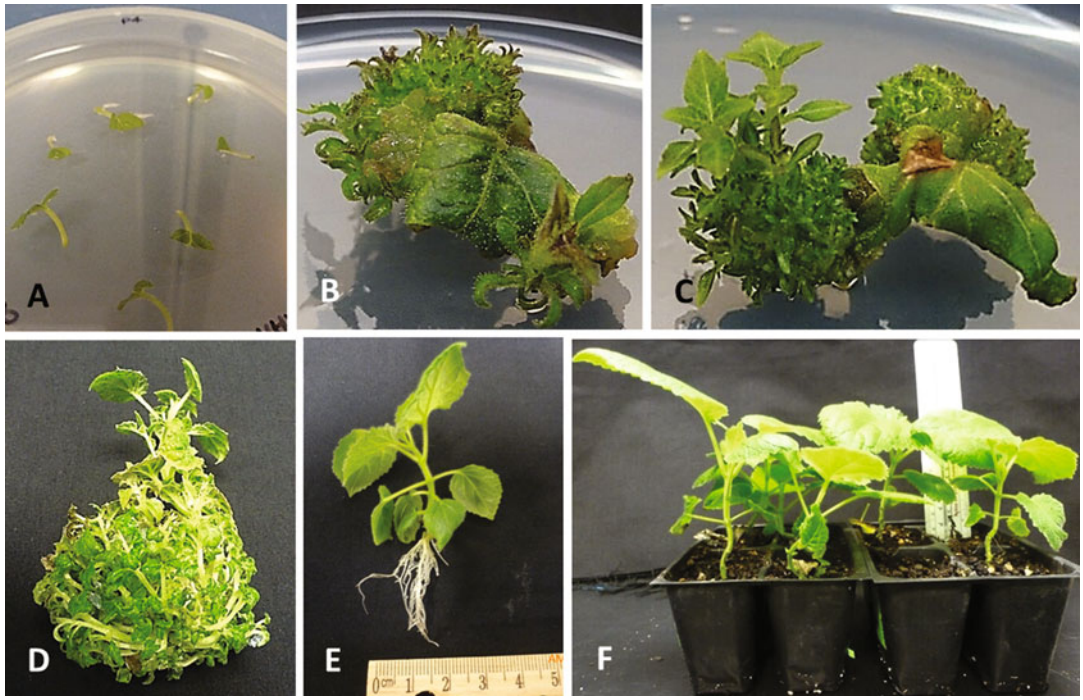


Fig. 1 In vitro micropropagation of *Paulownia elongata*. (**a**) Half leaf with petiole explant in shoot induction medium (SIM), (**b**) petiole with half leaf explant MS + 25 μM TDZ + 5 μM IAA + sucrose 30 g/L, (**c**) petiole with half leaf explant B5 + 25 TDZ + 5 IAA + maltose 30 g/L, (**d**) entire leaf explant on MS + 25 μM TDZ + 10 μM IAA + maltose 30 g/L, and (**e**) a healthy rooted plant for acclimatization, (**f**) acclimated plants in the greenhouse

3.1 Seed Sterilization and Germination

1. Seeds of *P. elongata* were collected from *Paulownia* trees in demonstration plot at Fort Valley State University, Georgia, USA.
2. Wash seeds for 30 min in tap water with few drops of detergent Tween-20 (Fisher Scientific, NH, USA) and 0.1% (v/v) Fungigone (PlantMedia, OH, USA).
3. Rinse with tap water three times and then with 70% ethanol for 1 min.
4. Rinse again with tap water three times and then transfer to laminar flow hood.
5. In laminar hood, wash seeds with 20% commercial bleach CLOROX® and then rinse three times with autoclaved distilled water.
6. Place the sterilized seeds in petri dishes with 1 mL sterile distilled water.
7. Seal the petri dishes with Parafilm® (American National Can™, CT, USA) and place them for germination under fluorescent white lights at 26 °C with 16 h photoperiod.

3.2 In Vitro Donor Plant Culture and Priming with BAP

1. Observe *P. elongata* seeds for germination as they begin to germinate within a week in the presence of sterile water.
2. Prepare MS media [11] (M519, PhytoTechnology Laboratories, KS, USA), supplemented with 30 g L^{-1} sucrose, 2.5 μM BAP (*see* **Notes 3** and **4**), and 7 g L^{-1} agar and pH adjusted at 5.8.
3. Sterilize media by autoclaving at 121 °C for 20 min (1.01 kPa steam pressure) and move it to laminar flow hood. Dispense 40 mL medium in each baby food jar and let the media set overnight.
4. Transfer 3 to 4, 2-week-old seedlings, to the medium prepared in **step 3** and allow to grow for 4–6 weeks.
5. After 4–6 weeks, use vigorously growing in vitro plants for harvesting explants for micropropagation and genetic transformation experiments (*see* **Note 5**).

3.3 Explant Establishment and Shoot Bud Induction

1. Prepare half leaf with petiole explants for multiple shoot induction (Fig. 1) (*see* **Note 6**). Number of regenerated shoots per explant will be evaluated at this point through destructive counting.
2. Prepare shoot induction media with basal MS supplemented with TDZ (25 μM) and IAA (10 μM), supplemented with 30 g L^{-1} maltose as a carbon source (*see* **Notes 3**, **7**, and **8**).
3. Incubate cultures at 26 °C with 16 h photoperiod for a period of 3 weeks. In 2–3 weeks, adventitious shoots are seen all over the explants (Fig. 1b, c). Look at an isolated clump with multiple shoots in Fig. 1d.
4. Weekly morphological changes and adventitious shoot induction is seen in scanning electron microscopic pictures (Fig. 2).

3.4 Shoot Elongation and Rooting

1. Transfer shoot-forming explants for elongation to MS basal medium for 7 weeks. Carry out destructive counting to evaluate the total number of shoots produced per half leaf with petiole explant. For destructive count use shoots with at least two leaves.
2. Transfer elongated shoots (1–5 node long) to MS medium with 5 μM IBA (*see* **Notes 3** and **9**) for rooting. Root induction will be visible within first week and well-rooted plants will be ready for acclimation in 2 weeks (Fig. 1e) (*see* **Note 10**).

3.5 Acclimatization and Transfer to the Greenhouse

1. After 3 weeks, transfer plants with long and multiple roots to trays with sterile potting mix moistened with autoclaved water and a tightly fitting transparent cover.
2. Place trays in an incubator (ThermoFisher, MS, USA) for 14 days under 12 h photoperiod at 26 °C. After 4 days,

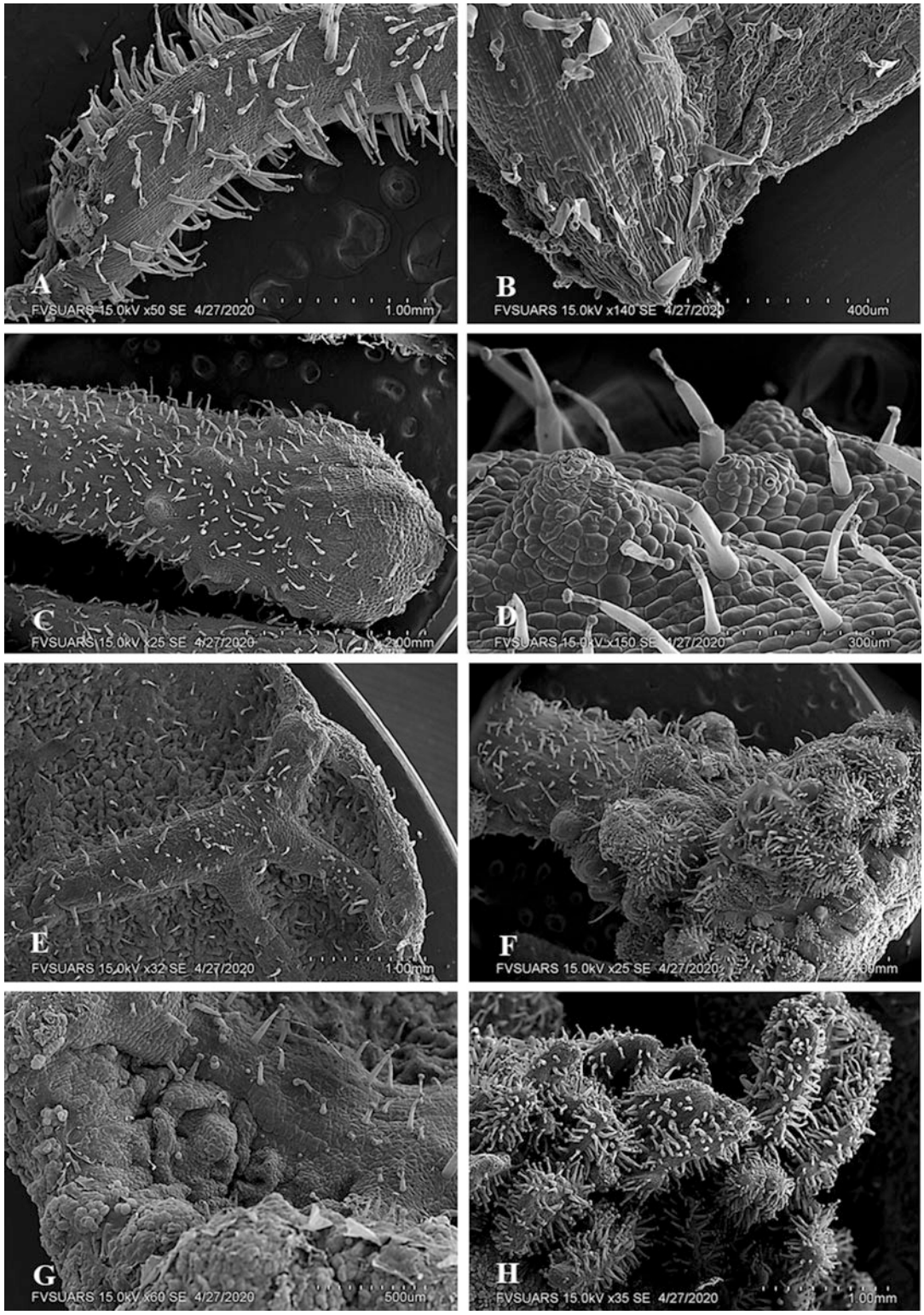


Fig. 2 Chronology of shoot bud induction in half leaf with petiole explants of *P. elongata*. (**a**, **b**) Control explants after 5-day incubation on MS basal medium. (**a**) Presents the petiole and (**b**) shows cut end of the leaf, (**c**)

gradually crack open the lid to reduce humidity for remaining duration and check for active root and shoot growth (*see* **Note 11**).

3. Transfer actively growing hardened plants to the green house (Fig. 1f).

4 Heat Map Analysis of Data

Tree species are usually less amenable to in vitro manipulations for micropropagation. This recalcitrance depends on factors like plant species, explant selection, season, environment of in vitro raised donor plant, media formulation, and use of plant growth regulators. For a commercial crop, consistency of results is paramount and in the current research where in vitro raised plants were used to obtain explants to initiate micropropagation, data analysis was carried out using Python 3.6 to write a Python script to convert the experimental data set into a heat map. When the script was run, the excel file was read into a data frame and was normalized by filling up the treatments. Then the explant types were extracted and each explant type was converted into a heat map (Fig. 3). To make this point, we have presented shoot number per explant data for two types of explants (entire leaf and half leaf with petiole) on various shoot induction media formulations. In each treatment, shoot number in each explant can be seen and compared on a color code basis.

5 *Agrobacterium tumefaciens* EHA105 Mediated Genetic Transformation

1. Introduce the binary vector into *A. tumefaciens* strain EHA105 [12] by the freeze-thaw method of [13] (*see* **Note 12**).
2. Grow the *Agrobacterium* culture overnight in 20 mL LB containing 20 mg L^{-1} rifampicin (Sigma-Aldrich, MO) and 50 mg L^{-1} kanamycin (Sigma-Aldrich, MO) on a rotary shaker (170 rpm) at 28 °C. Bacteria codes for rifampicin and plasmid pq35SGR contributes with kanamycin resistance.
3. Centrifuge overnight cultures of *Agrobacterium*-pq35SGR (OD_{600} = 0.6–0.8) at 3000 rpm for 15 min, resuspend the pellet in 20 mL liquid MS medium, and place on a shaker (100 rpm) at 28 °C for an additional 1 h prior to co-cultivation.

Fig. 2 (continued) petiole base after 5 day incubation on shoot induction medium, (**d**) close-up of petiole surface showing areas of shoot bud formation, (**e**) cut end of the leaf after 5 day incubation showing callus formation, (**f**) adventitious shoot buds at the base of petiole after 10 days of incubation on shoot induction medium, (**g**) shoot bud formation in 10-day-old cultures at the cut end of leaf, (**h**) shoot bud proliferation after 15 day incubation on the shoot induction medium

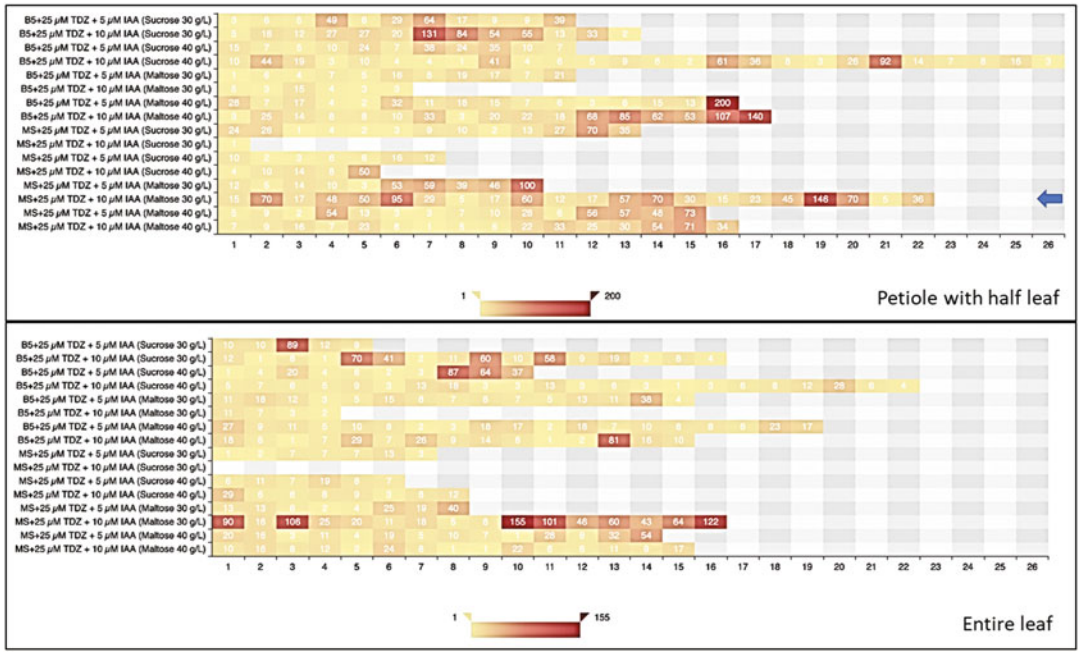


Fig. 3 Heat maps for entire leaf and half leaf with petiole explant to show optimization of micropropagation protocol. Arrow indicates one of the most suitable shoot induction medium on the basis of explant survival and average number of shoots per explant

4. Use half leaf with petiole explants for transformation with modifications.
5. Wound the explants by scratching and then submerge in bacterial suspension with 100 μM acetosyringone for 30 min [14].
6. For agro-infection, bacterial density at OD_{600} of 0.6–0.8 was ideal.
7. Co-cultivate explants for 72 h at 27 °C in dark. Check explants for transient green fluorescent protein (GFP) expression using Olympus BX 43 microscope equipped with epifluorescence illumination and a camera (Olympus Inc., PA, USA).
8. Rinse the explants with liquid induction medium containing 250 mg L^{-1} carbenicillin and cefotaxime (Phytotechnology Lab, KS) for 5 min and place explants on semisolid MS medium supplemented with 250 mg L^{-1} carbenicillin and 150 mg L^{-1} cefotaxime (Phytotechnology Lab, KS) for 60 days.
9. If bacterial growth persists, quarantine the explants in MS medium containing antibiotics until cultures are free of bacterial growth. Transfer the bacteria-free cultures to regeneration medium (MS + 25 μM TDZ, 10 μM IAA, and 30 g L^{-1} maltose) including 20 mg L^{-1} kanamycin.

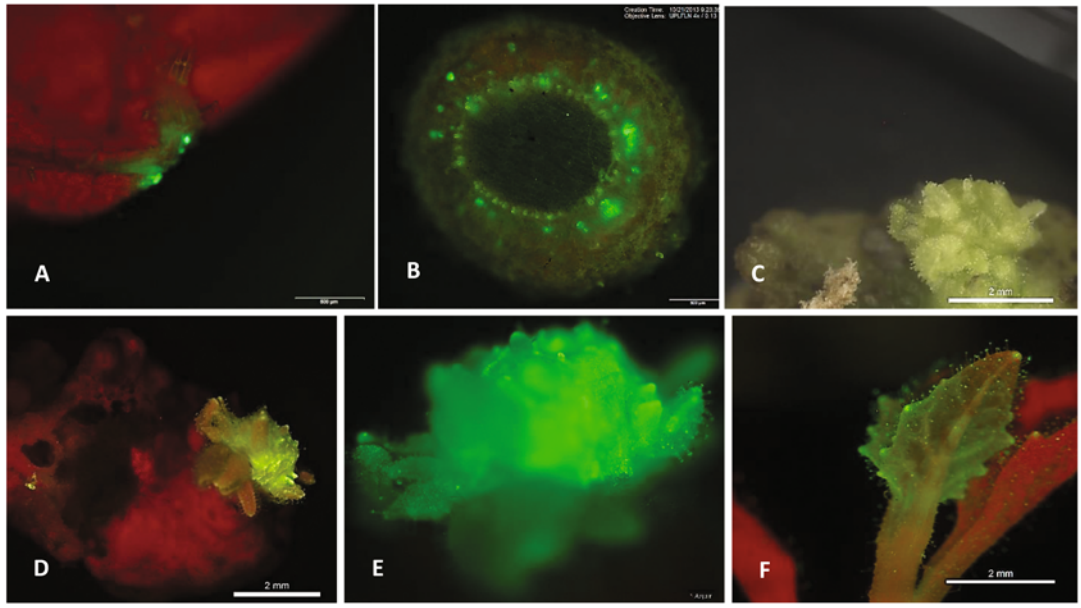


Fig. 4 Successful transformation of *Paulownia elongata* explants with *Agrobacterium tumefaciens* EHA105. (**a**) Agro-infection of leaf explant, (**b**) infection of internode or petiole explant, (**c**) shoot multiplication in a co-cultivated explant, (**d**) same as "C" under UV light to show GFP expression in regenerated shoots, (**e**) shoot bud cluster at a higher magnification, and (**f**) elongating GFP positive shoot

10. Maintain cultures on regeneration medium for 5–7 weeks and frequently screen for bacterial growth and shoot induction. Once transgenic shoots are identified, allow explants to elongate and root, harden and transfer to a greenhouse. The entire process of generating a transgenic shoot has been presented in Fig. 4a–f.

5.1 Molecular Identification of Putative Transgenic Plants

5.1.1 Polymerase Chain Reaction (PCR)

1. Isolate total genomic DNA from young leaves (100 mg) of non-transformed and transgenic plants [15].
2. Resuspend DNA pellet in 50 μL of sterile nuclease-free water and carry out DNA quantification using UV-Vis Spectrophotometer (DeNovix Inc., DE, USA).
3. Amplify enhanced green fluorescent protein (eGFP) gene using forward primer EG-51 (ATGGTGAGCAAGGGCGAG GAGCTGT) and reverse primer EG-32 (CTTGTA CAGCTCGTCCATGCCGAGA) using thermal cycler (BioRAD, CA, USA).
4. Polymerase Chain Reaction (PCR) cycle conditions: initial cycle at 94 °C for 4 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 45 s, 72 °C for 45 s and a final extension cycle at 72 °C for 45 s.

5.1.2 Reverse transcription—PCR [14]

1. Use leaf tissue (100 mg) from non-transgenic and transgenic plants of *P. elongata* for RNA extraction along with 1 mL of TRIzol® reagent (Ambion/Life Technologies™, MA USA).
2. Perform RT-PCR with 1 μg of RNA using One Taq One-Step RT-PCR Kit (New England Biolabs Inc., MA). Suspend RNA pellet in RNase-free water and incubate in water bath at 60 °C for 15 min. Carry out RNA quantification using UV-Vis Spectrophotometer (DeNovix Inc., DE, USA) and store at −80 °C.
3. Perform reverse transcription in PCR tubes at 48 °C for 30 min, followed by initial denaturation at 94 °C for 1 min, and 40 cycles of denaturation at 94 °C for 15 s, annealing at 65 °C for 30 s, and extension at 68 °C for 1 min. Final extension at 68 °C for 5 min.
4. Negative control contains the same amount of RNA from the leaf of wild-type *P. elongata* in the reaction but devoid of enzyme.
5. Use same primers for RT-PCR that were used for PCR amplification of genomic DNA.
6. Analyze PCR and RT-PCR products using 1.0% agarose gel.

   Load samples and markers and run gel for 90 min at 75 V. Observe gels in gel documentation unit (ProteinSimple, CA, USA). Agarose gel electrophoresis results reveal presence/absence of GFP transcript in wild-type and transgenic plants derived through PCR and RT PCR amplifications (Fig. 5a, b).

   The entire process of *P. elongata* micropropagation and genetic transformation has been summarized in Fig. 6.

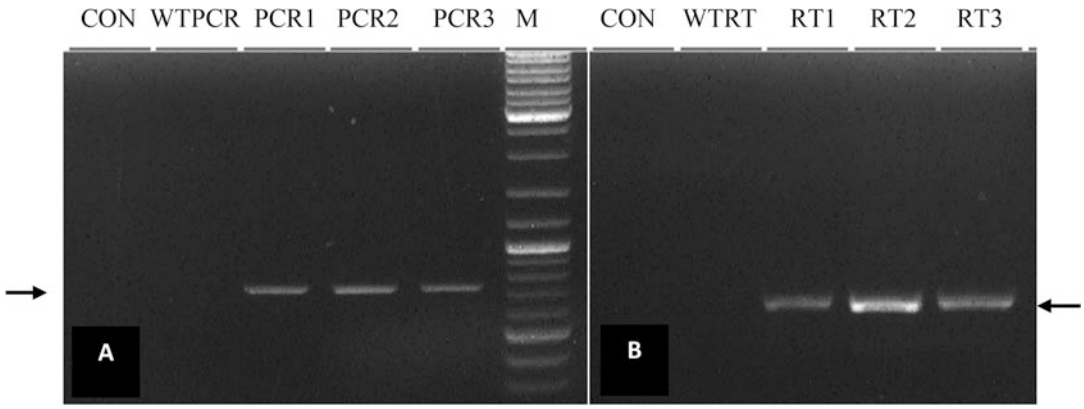


Fig. 5 Molecular testing of *Paulownia elongata* shoots. (**a**) PCR results for GFP, (**b**) RT-PCR results. Control: No DNA or RNA template; WTPCR: Genomic DNA present but no enzyme (negative control); WTRT: RNA present but no enzyme (negative control); PCR 1–3: Transgenic plants, RT 1–3: Transgenic plants; and M: Marker. Arrows indicate amplified GFP gene

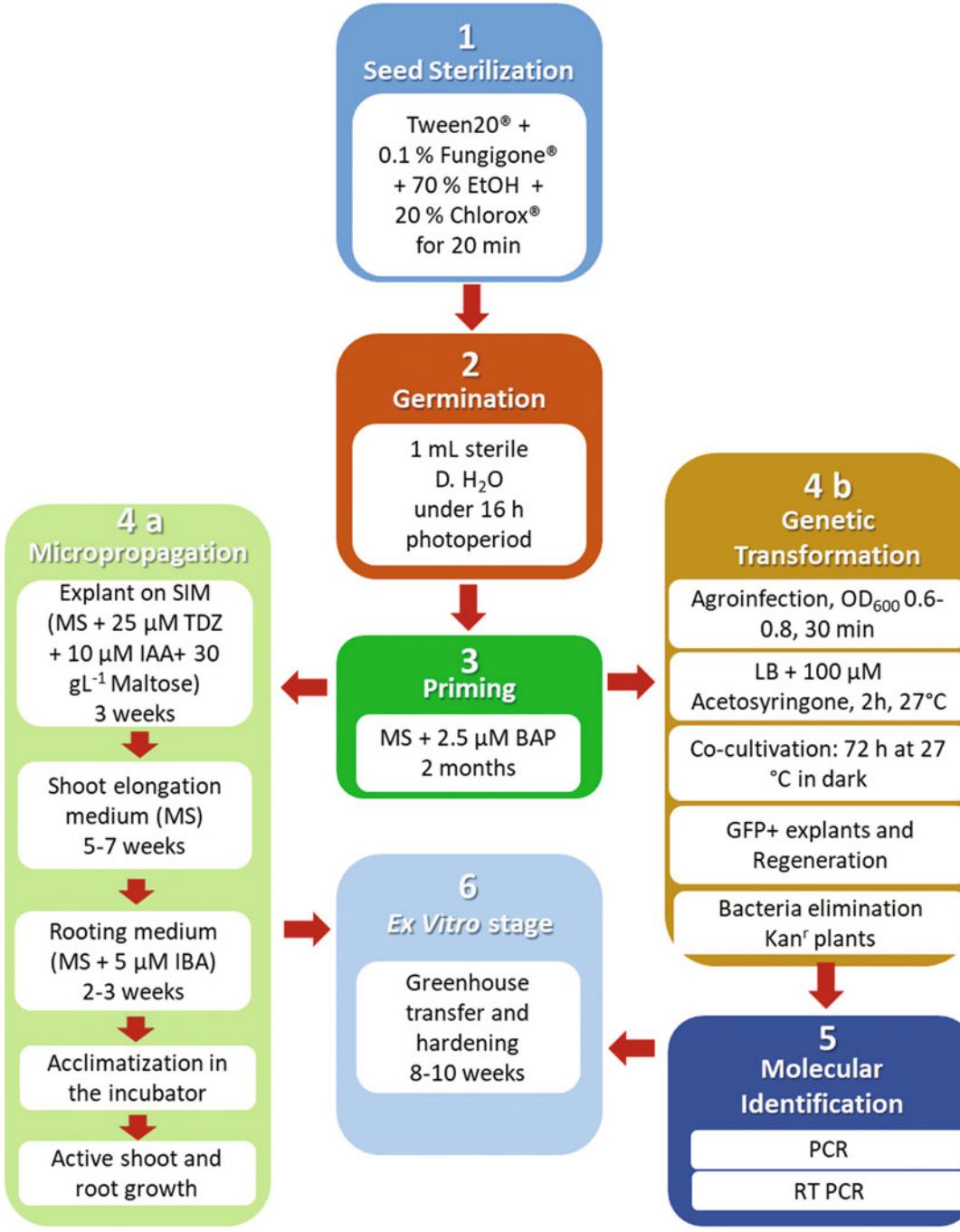


Fig. 6 In vitro propagation and genetic transformation of *Paulownia elongata*

6 Conclusions

Paulownia species provide several environmental and economic benefits. In vitro protocols described here provide clarity on explant and medium selection, choice of plant growth regulators and their concentration and carbon sources for *P. elongata* micropropagation. The heat map exhibits that more uniform response was obtained with higher number of explant survival in the treatment using MS medium supplemented with 25 μM TDZ, 10 μM IAA, and 30 g L^{-1} maltose with higher shoot number reaching up to 146 in an explant. As per heat maps, we can see that for entire leaf and petiole with half leaf explants, MS with 25 μM TDZ, 10 μM IAA, and 30 g L^{-1} maltose provided the best results. The heat map exhibits differences in terms of establishment and survival of explants in shoot induction medium (SIM), general response spread over replicates, and number of shoots produced. Heat maps generated through Python can be useful to optimize micropropagation in a commercial setup identifying ideal stock material to harvest explants and initiate cultures. Heat maps have an advantage over graphical data representation as they show the results of each explant (replicate) in the heat map. Careful analysis of heat maps may help determine the maximum potential of a particular treatment and explant combination so that one can trace back and record the growing conditions, physiology, and genetics of the donor plant of for the best results. This can aid the commercial tissue culture industries as it can help them maximize the potential of the plant and produce higher number of shoots. Using heat maps as a tool for data analysis helps us understand the biological significance of results. Also, through heat maps we can see consistency in shoot numbers (high or low), thereby providing us a scope for improvement. Trees are rather difficult to micropropagate and transform. For the future improvement of the tree, a simple *Agrobacterium tumefaciens* mediated protocol has been included that is supported by micropropagation protocols and can be translated to other trees.

7 Notes

1. Media and glassware sterilization was done using autoclave at 121 °C, 15 psi and 30 min.
2. Media pH is adjusted to 5.8–6.0 before autoclaving.
3. Stocks of all plant growth regulators used in media preparation were prepared at 1 mM concentration. Thidiazuron (Caisson Laboratries, UT, USA), IBA (Sigma Chemical Corporation, MO, USA), BAP (Sigma Chemical Corporation, MO, USA), and IAA (Sigma Chemical Corporation, MO, USA) stocks

were prepared by dissolving it in 1 N NaOH and then making up the required volume by distilled water. Growth regulators, antibiotics, and stock solutions are stored at 4 °C.

4. Stock solution of BAP: Dissolve 0.0225 g (22.5 mg) BAP powder in a few drops of 1 N NaOH and add distilled water to make up the volume to 100 mL. Store at 4 °C.
5. Within 2 weeks, about 80% seed germination is obtained upon seed transfer to semisolid MS medium, devoid of plant growth regulators.
6. On the basis of our micropropagation experiments, half leaf with petiole explant and MS medium supplemented with TDZ (25 μM), IAA (10 μM), and 30 g L^{-1} maltose was identified as optimum treatment for regeneration in genetic transformation experiments.
7. Stock solution of TDZ: Dissolve 0.022 g (22 mg) TDZ powder in a few drops of 1 N NaOH and then use distilled water to make up the volume to 100 mL. Store at 4 °C.
8. Stock solution of IAA: Dissolve 0.017 g (17 mg) IAA powder in a few drops of 1 N NaOH and then use distilled water to make up the volume to 100 mL. Adjust pH of the stock solution to 5.8–6.0. Filter sterilize IAA solution using filter sterilization assembly (filter pore size 0.22 μM) and store at 4 °C.
9. Stock solution of IBA: Dissolve the 0.020 g IBA powder in a few drops of 1 N NaOH and gradually add distilled water, while stirring the solution, to make up the volume to 100 mL. Adjust the pH of the stock solution to 5.8–6.0 and sterilize it using filter sterilization assembly (filter pore size 0.22 μM) and store at 4 °C.
10. Though the elongated shoots can root well in MS basal medium, IBA-supplemented rooting medium helps in early and strong rooting system.
11. Alternatively, acclimatization can be done in the greenhouse, by covering the pots with a plastic cover. Relative humidity is reduced by gradually removing the lid after a week.
12. *Agrobacterium* was transformed with a plasmid pq35SGR (a binary plasmid harboring the β-glucuronidase and green fluorescent protein/neomycin phosphotransferase II fusion reporter gene) to transform *P. elongata* explants. Bacterial culture plates (LB or 2 xYT) with or without antibiotics were streaked using frozen glycerol stock of *A. tumefaciens* EHA105 stored at −80 °C in LB medium. Bacterial suspension cultures were initiated in 35 mL of LB medium with or without 30 mg L^{-1} of kanamycin and 20 mg L^{-1} of rifampicin by adding 50 μL of vigorously growing overnight culture of respective bacterial strain. The culture flask was incubated on shaker at 27 °C for 48 h.

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References

1. Bing L, Yun-Hong T, Liu S, Olmstead R, Min D-Z, Chen Z-D, Joshee N, Vaidya B, Chung R, Li B (2019) Phylogenetic relationships of *Cyrtandromoea* and *Wightia* revisited: a new tribe in Phrymaceae and a new family in Lamiales. J Syst Evol 58(1):1–17. https://doi.org/10.1111/jse.12513
2. El-Showk S, El-Showk N (2003) The *Paulownia* tree: an alternative for sustainable forestry. The Farm, 1–8. http://www.cropdevelopment.org/docs/PaulowniaBrochure_print.pdf
3. Tisserat B, Joshee N, Mahapatra AK, Selling GW, Finkenstadt VL (2013) Physical and mechanical properties of extruded poly(lactic acid)-based *Paulownia elongata* bio-composites. Ind Crop Prod 44:88–96. https://doi.org/10.1016/j.indcrop.2012.10.030
4. Tisserat B, Reifschneider L, Joshee N, Finkenstadt VL (2013) Evaluation of *Paulownia elongata* wood polyethylene composites. J Thermoplastic Comp Mat 28(9):1301–1320. https://doi.org/10.1177/0892705713505856
5. Tisserat B, Reifschneider L, Joshee N, Finkenstadt VL (2013) Properties of high density polyethylene—*Paulownia* wood flour composites via injection molding. BioRes 8 (3):4440–4458
6. Stewart WM, Vaidya BN, Mahapatra AK, Terrill TH, Joshee N (2018) Potential use of multipurpose *Paulownia elongata* tree as an animal feed resource. Am J Plant Sci 9:1212–1227
7. Vaughn SF, Dinelli FD, Tisserat B, Joshee N, Vaughan MM, Peterson SC (2015) Creeping bentgrass growth in sand-based root zones with or without biochar. Sci Hortic 197:592–596. https://doi.org/10.1016/j.scienta.2015.10.021
8. Vaughn SF, Kenar JA, Tisserat B, Jackson MA, Joshee N, Vaidya BN, Peterson SC (2017) Chemical and physical properties of *Paulownia elongata* biochar modified with oxidants for horticultural applications. Ind Crop Prod 97:260–267. https://doi.org/10.1016/j.indcrop.2016.12.017
9. Yadav NK, Vaidya BN, Henderson K, Lee JF, Stewart WM, Dhekney SA, Joshee N (2013) A review of *Paulownia* biotechnology: a short-rotation, fast growing multipurpose bioenergy tree. Am J Plant Sci 4:2070–2082. https://doi.org/10.4236/ajps.2013.411259
10. Bajaj R (2018) Optimization of in vitro propagation and genetic transformation protocols in *Paulownia elongata*. M.S. Diss., College of Agric., Family Sci., and Technol., Fort Valley State Univ, Fort Valley, GA, p 65
11. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 15 (3):473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x
12. Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res 2:208–218
13. Burrow MD, Chlan CA, Sen P, Murai N (1990) High frequency generation of transgenic tobacco plants after modified leaf disk cocultivation with *Agrobacterium tumefaciens*. Plant Mol Biol Report 8:24–139
14. Vaidya BN, Jackson CL, Perry ZD, Dhekney SA, Joshee N (2016) *Agrobacterium*-mediated transformation of thin cell layer explants of *Scutellaria ocmulgee* Small: a rare plant with anti-tumor properties. Plant Cell Tissue Org Cult 127(1):57–69. https://doi.org/10.1007/s11240-016-1029-y
15. Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4325

Part IV

Economics and Sustainability of Biofuel Production



Chapter 19

Economics of Biofuel Production: A Case of Sorghum and Pearl Millet in India

M. Gopinath Reddy and B. Suresh Reddy

Abstract

Reduction of fossil fuels at an alarming rate has attracted increasing attention to blending biofuels worldwide. India's energy demand is expected to grow at an annual rate of 4–5 times over the next couple of decades. With self-sufficiency levels in crude oil becoming a distant dream, there is growing interest to look out for alternative fuels and the biofuels are an important option for policy makers in India. In this context, this paper reviews the experiences in India in the last two decades with respect to biofuel cultivation and its impact on land use, environment, and the livelihoods of rural communities. The objective of this paper is to assess the economics of biofuel production using Sorghum and Pearl millet feedstocks in India using a Life Cycle Analysis (LCA) approach. Baseline study was conducted during the year 2013 in the Madhya Pradesh state of India covering five districts and 333 sample farmers to understand the farmers perception about the various issues related to the production of biofuels using Indian staple food crops Sorghum and Pearl millet. Empirical data from the multi-locational trials conducted during the years 2014–2015 and 2015–2016 in farmers' fields was used to conduct the LCA analysis. Sorghum and Pearl millet feedstocks which are rain-fed crops are considered for bioethanol production with different pretreatment methods. Net Energy Ratio (NER), Net Energy Balance (NEB), Net Carbon Balance (NCB), and % Carbon reduction were some of the key parameters used for analysis and the results are evaluated based on the environmental impacts through the Life Cycle Assessment at 5% blending. Findings reveal that, dilute alkali pretreatment process is most energy intensive due to consumption of alkali consumption. Whereas dilute acid pretreatment has higher conversion efficiency than the other pretreatment processes which is due to higher glucan and xylan conversion efficiencies.

The study concludes that Sorghum feedstock is more energy intensive than Pearl millet feedstock due to higher water requirement and yield. Biofuels, either conventional or advanced, should not been couraged without a comprehensive outlook on the overall impact that will ultimately have on the society, environment, or on the countries' energy security. Efforts should be made toward encouragement of research and development in the field as well as in formulating a comprehensive and effective biofuel policy for India.

Key words Biofuels, Sorghum, Pearl millet, Energy security, Sustainability, Clean energy, Food security, Fodder security, India, Life cycle analysis

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1 Introduction

Depletion of fossil fuels at an alarming rate has attracted increasing attention to blending biofuels worldwide. India's energy demand is growing at an annual rate of 4.2% with highest demand growth of +129% in 2017 [1]. Depletion of fossil fuels at an alarming rate coupled with ever-growing challenges due to anthropogenic induced climate change has attracted increasing attention to blending biofuels worldwide. According to the International Energy Agency (IEA), India will become the largest single source of global oil demand growth after 2020 [2]. Hence, India needs energy security along with environmental sustainability so that the eco-capacity of the conserved and environmental uncertainty arising from events such as climate change is mitigated. Of the total primary energy supplied to Indian economy in 2016, as much as 75% was from commercial fuels while 25% was from noncommercial fuels. Out of the total commercial energy, coal constitutes 56.76%, followed by oil (29.28%), natural gas (6.2%), and carbon-free hydro, nuclear, and other new renewable resources (7.4%) [2]. Despite coal being the country's major resource endowment, the major source of India's energy insecurity is the heavy and growing dependence on oil imports. Of late, there have been sharp rising trends in crude oil prices coupled with volatility. India's transportation fuel requirements are unique as it consumes almost six to seven times more diesel fuel than gasoline, whereas in the rest of the world, almost all the other countries use more gasoline than diesel fuel. Biofuel is a nonpolluting, locally available, accessible, and reliable fuel obtained from renewable sources. It is seen by many as a "clean" form of energy as the amount of CO_2 released, when it is burned, is generally equivalent to the amount of CO_2 captured during the growth of the crop that produced it. Since biofuels can be produced from a diverse set of crops, each country can also adopt its local/regional/country-specific strategy in order to achieve comparative advantage. Globally liquid fuels are produced from plantations, agricultural residues, weeds and organic urban and industrial wastes. Despite their appeal as an alternative to fossil fuels, biofuels are also subject of considerable controversy. Biofuel production is not considered truly carbon-neutral because the stages of production need nonrenewable energy while transporting and processing. In India, the National Policy on Biofuels [3] has an ambitious target of mainstreaming the use of biofuels-bioethanol and biodiesel by 20% blending with Petrol and High Speed Diesel (HSD) by 2017. The policy centers around the plantations and production of Jatropha (Jatropha curcas) on wastelands for the achievement of this target. However the target could not be achieved and even today in 2020 only 5% blending could be achieved. With self-sufficiency levels in crude

oil becoming a distant dream, there is growing interest to look out for alternative fuels and the biofuels are an important option for policy makers in India [4].

The literature review projects a mixed picture about the economic, environmental, and social viability of biofuels. Except for the experiences related to jatropha, no literature is available with reference to biofuel production from staple food crops in India. Experiences from Europe and other South American countries, however, provide learning opportunities with regard to policy, technology barriers especially in terms of conversion, problems associated with trade linkages, and most importantly long-run economic viability. A strong synergy of rationales such as the prospect of reduction in external dependence, better environment, and creation of additional employment opportunities make a strong case for promotion of biofuels in India. However, reviews [4–8] suggest that it is difficult to achieve all of the objectives simultaneously and it would be a demanding task to couple capacity expansion with environmentally substantial production, while at the same time limiting biofuel burden on the state budgets. The outlook for biofuels is also highly sensitive to possible changes in government subsidies and blending mandates, which remain the main stimulus for biofuels use. Over the past year, much uncertainty has developed about how biofuel policies in several key markets will evolve [2]. The important barriers for successful implementation of biofuels come from the farmers—the chief stakeholders, and given the fact that India's majority livelihoods are linked and relinked to agriculture, caution must be exercised in promoting biofuel production from food-based crops.

2 Objectives of the Study

CESS has looked into the socioeconomic and Life Cycle Analysis of biofuel production through cultivation of Jowar and Bajra feedstocks in India with the following objectives.

2.1 Objectives of the Study

1. To understand the existing scenario with reference to two biofuel crops, Jowar (Sorghum bicolor) and Pearl millet (Peenisettum glaucum), in the state of Madhya Pradesh.
2. To understand the socioeconomic aspects of sample farmers.
3. To assess the economics of Jowar and Pearl millet crop cultivation of the sample farmers.
4. To examine the drivers and barriers in cultivation of biofuel crops.
5. To understand the awareness levels of sample farmers regarding biofuel cultivation and its impact of food and fodder security.

6. To conduct Life Cycle Analysis of Sorghum and Bajra feedstocks for biofuel production.
7. To contribute to the overall policy discourse on biofuels cultivation in India.

3 Research Methodology

The state of Madhya Pradesh in India has been selected for the field study. The state of Madhya Pradesh is home to a total population of 72.6 million (2011 Census) and a schedule tribe population of 15.3 million (highest in the country). Schedule tribes constitute 21.1% of the total population of M.P. (India—8.6%). Madhya Pradesh ranks 20th in Human Development Index in India (2008–2009) with a literacy rate of 70.6 (as against India's 74.02%). Similarly, ST literacy rate in Madhya Pradesh state constitutes 50.6% as against all India ST literacy rate of 58.96%. The total geographical area of the state works out to 307.56 lakh ha with a recorded forest area is 95,221 Sq km, constituting 31% of the geographical area of the state and 12.44% of the forest area of the country. Madhya Pradesh state consists of 50 districts, out of which 21 are tribal districts. Jhabua, Dhar, and Mandla districts account for more than 50% of tribal population, while Kharghone, Seoni and Chindwara, Sidhi and Shadol districts for 30–50% population of tribal population. There are 46 tribal communities inhabiting Madhya Pradesh, out of which six communities, i.e., Bhils, Gonds, Kol, Korku, Sahariya, and Baiga constitute 92.2% of the state ST population. There are 12 tribal communities with a population below 1000, out of which 3 are special primitive groups. Bhil is the most populous tribal group (2011 census) with a population of 4,618,068 (37.7%), followed by Gonds (35.6%). Over the last decade the tribal population has increased by 3.1 million. Major livelihood sources of tribals include farming, livestock rearing, casual labor, migration, and collection of minor forest produce such as tendu (Diospyros melanoxylon) leaves, fodder, and fuel wood.

Our study has undertaken the baseline study during the year May 2013–2014 and empirical studies with sample farmers of multi-locational trials during the year 2014–2015 Kharif season (June to Oct) and 2015–2016 Kharif season. These different rounds of studies have primarily focused on the socioeconomic, ecological, food security, and livelihood dimensions of biofuel production through the food crops such as sorghum and Pearl millet.

3.1 Study Area, Sample, and Data Analysis

Gwalior, Khargone, Dewas, Morena, and Bhind districts hosting large areas of Sorghum and Pearl millet were selected for the study. A total of ten villages (consist of more than 15,000 farmers of which approx. 70% farmers cultivate biofuel crops) were selected

from five districts where the trials of high biomass feedstocks were conducted by Work Package I partners of the project (who are responsible for the development of high biomass feedstocks). For baseline survey stratified proportionate random sampling was used covering 333 farmers belonging to different size classes in ten villages. Similarly, in 2014–2015 kharif season, field survey was done with all the 78 farmers in whose fields multi-locational trials of high biomass varieties of Jowar and Bajra were sown. For the year 2015–2016 Kharif all the 83 farmers, in whose fields MLTs were conducted, were selected for the data collection. The study used both qualitative and quantitative methods. Personal interviews were conducted with a structured interview schedule. The study used an ex post facto research design which is a systematic empirical inquiry in which the researcher does not have direct control or influence over the variables because their manifestations have already occurred. Participatory research tool Focused Group Discussions (FGDs) helped to understand the livelihoods, food and fodder security issues of biofuels. Secondary data on land use, fertilizer use, and demographic features of the district were collected from the survey reports by the Directorate of Census, Madhya Pradesh, Fertiliser News, and Ministry of Agriculture. The data analysis was basically conducted in two ways. One was comparing between the various size classes of large, medium, and small farmers. The results of the study are discussed at two levels: one at the household level and the other at the plot level. The data gathered was analyzed using different statistical tools. Averages, frequency, and percentages were used to analyze the various information related to jowar and bajra cultivation.

4 Findings of the Study

4.1 Socioeconomic Analysis: Findings of Baseline Survey

The socioeconomic features, age group, literacy level, livestock population, market distance, farming experience, social participation, caste composition, landholding, net income and borrowings, awareness on biofuels cultivation, use of jowar crop for biofuel production and its impact on food and fodder are some of the important issues discussed so as to get insight into the issues of jowar crop cultivation for biofuel production. Among the total (333) sampled households, 58% belonged to other Backward Communities[1] (OBCs) followed by Other Castes (OCs) 17.40%, SC[2]s 15.3% and STs 9.33%. Out of the total sampled farmers, 29.10% were not literate, followed by upper primary (23.10%), and tenth

[1] **Backward communities** can be defined as a social category which consists of all the socially, educationally, economically and politically backward groups, castes, and tribes.

[2] **Scheduled Castes** (SCs) and **Scheduled Tribes** (STs) are officially designated groups of historically disadvantaged people in India.

class (15.90%). This could be due to lack of proper educational infrastructure in these villages. Majority (63.1%) of the sampled households in the study area live in joint family system which is quite contrary to the emergence of nuclear family system in other parts of India. This will enable the better availability of family labor in farming in general and biofuel production in particular. Primary occupation in the study area was farming, followed by agricultural casual labor. Similarly, livestock was predominantly secondary occupation for many sample households. One of the major objectives of the project was to utilize the existing wastelands in Madhya Pradesh to cultivate high biomass producing jowar and bajra varieties. Nearly 1.2 million ha [3] of culturable waste is present Madhya Pradesh. The fertility level of these soils is very low to support the cultivation of high biomass producing varieties which are generally input-intensive. Moreover, at the national level there is a huge difference in the areas reported under waste land by different agencies. Given the lack of clarity on the exact area of waste land available in India, the argument for promoting sorghum and pearl millet production in these waste lands in future is a questionable proposition. Majority (48.6%) of the sample plots are interestingly having average soil quality as perceived by farmers and 25.3% of the sampled plots are of good quality. This has implication for high biomass jowar cultivation as soil fertility will directly affect crop yield. Borewell is the major source of irrigation (46.4%) for sampled households, followed by rainfall (27.3%), and canal irrigation. Gross cropping area of various crops in Madhya Pradesh clearly indicates that jowar and bajra occupy 2.1% and 0.8%, respectively. However, the study sites of the baseline survey have considerable area under sorghum and pearl millet, and interestingly soya bean did not spread in the study villages. Jowar (around 35%) accounts for the largest share of crop that is being cultivated among the sampled households, followed by bajra (11.5%). This is due to the fodder requirement of the region due to its strong milk economy. Varietal diversity exists in the case of both jowar and bajra crops. High-yielding varieties occupy a major area in case of both crops. During kharif 2013–2014, the major area of the sample households was under High-Yielding Varieties (HYV) jowar (253.12 acres), followed by maize (112.75 acres) and cotton (106 acres). Interestingly, the height of some of the traditional sorghum varieties grown by farmers were of at least 12 feet and the price it fetches in the open market is Rs. 2500/quintal. Farmers perceive that traditional white sorghum fetches a better market price than other sorghum varieties. It is observed that most of the sample households own buffaloes followed by cows and bullocks. It is evident from the baseline study that among the total sampled farmers, the majority (58.6%) had not taken any loan, and those who accessed loans, the primary purpose of loan is observed to be for the purchase of agricultural inputs (21.9%) followed by 6.9% for consumption purpose and irrigation (6%).

Traditional and high-yielding varieties of jowar were yielding well, doing well in the year 2012–2013, as compared with hybrid jowar. During the years of lesser rainfall the hybrids do not perform well. Moreover, hybrid jowar attracts certain pests and diseases, thereby affecting the yield and income. Baseline survey findings clearly indicated that the high biomass jowar and bajra varieties being promoted in the project should be more fetching than the existing cultivars of these staple food crops; they should also have a comparative advantage simultaneously with other crops such as the soya bean, wheat, and mustard. Otherwise the farmers might not be inclined to adopt these varieties for biofuel production.

Farmers' perception regarding biofuels and their cultivation was also assessed in the present study. Nearly 92% of the sample households did not have any awareness about the biofuels and more so about the production of biofuels from agricultural crops such as jowar and bajra. As the probing got deeper, it was interesting to observe that 38.44% of the households agreed that diversion of jowar and bajra for biofuel cultivation will result in shortage of food grains while 61.56% did not perceive a reduction in the food supply. Similarly, majority of the households (51.96%) believed that the use of jowar and bajra crops for biofuel production will affect the fodder security of their animals.

4.2 Multi-locational Trials (MLTs) of High Biomass Varieties

MLTs were conducted during the year 2014–2015 Kharif and 2015–2016 Kharif. Two surveys of these MLTs were conducted with an objective of addressing the suitability of High Biomass Varieties (HBVs) of Jowar and Bajra feedstocks with regard to crop economics, socioeconomic dynamics, and potential up-scaling issues. Data of 2015 and 2016 MLT field trials shows that there is inconsistency in the varieties being used in trials vis-à-vis the varieties that are being used in treatment analysis. Major findings of the 2014–2015 Kharif trials was that the average income (includes both grain and fodder yield) was relatively lower for the HBV varieties promoted in the project as compared to the ones being cultivated in the previous year (base line survey period). The value of Jowar dry fodder changed from village to village. However, it generally ranged between Rs. 1–2/kg. In the case of Bajra crop fodder, there was a wide difference observed during 2015–2016 Kharif and it varied between Rs. 1/kg in Bijoli to Rs. 5/kg in Nahardonki of Gwalior region. The cost of fodder has implications for biofuel production as it is this material that is used as raw material. The lower the fodder price, the more economical will be the biofuel production from these crops. From last 2 years (i.e., from 2014–2015), there is huge increase in market price (went up to Rs. 4500/quintals in 2016) of traditional Safed Jowar variety due to its utility for some industrial purpose. When it comes to biomass yield, 2015-ICFPM-1 of Bajra crop and ICSSH-28 and ICSV 93046 were performing much better than existing varieties in 2015–2016 Kharif.

5 Drivers and Barriers for Bioethanol Production in India

5.1 Drivers

There are several reasons for which biofuel production in general and ethanol production in particular are being encouraged not only in India but also across the world. First is the notion of **energy security.** Energy security is a catch all term to mean increased reliance on domestically produced fuels so as to be insulated from the high volatility of oil prices by switching to biofuels. Secondly, if a good market for ethanol is developed, growing ethanol crops such as corn or sugarcane more extensively will be profitable and result in higher revenues, making farmers well off, thus contributing to **rural development**. It also contributes to job creation and acts as a support to the agricultural economy. Thirdly and finally, **environmental sustainability** is also an important driver in the production of biofuel crops. Biomass fuels such as ethanol are seen as better than fossil fuels for two reasons: (a) they are renewable and hence contribute to sustainable development and (b) they are seen as a means of reducing GHG emissions.

5.2 Barriers

The National Policy on Biofuels mandates a 20% blending of ethanol in petrol by the year 2017. The Ethanol Blending Programme (EBP) can be a reality only when there is an adequate supply of ethanol. One of the main reasons for the lack of adequate supply of ethanol is due to a deficiency in growing biofuel crops in India. According to the National Policy on Biofuels, biofuels have to be produced without compromising the food security. It is based solely on non-food feedstocks to be raised on degraded or wastelands that are not suited to agriculture, thus avoiding a possible conflict of fuel vs. food security. Due to this constraint, India has not been able to look beyond crops other than sugarcane for the production of ethanol.

Another problem is the term wastelands itself. Those lands that are declared as wastelands by the Government in reality are probably used by poor farmers for grazing their livestock or for growing food crops for their sustenance. So allotting these lands for biofuel crops may lead to dispossessing poor farmers of their lands. Another problem with growing biofuel crops, especially sugarcane in India, may actually be a bane instead of a boon. Production of sugarcane on a large scale results in the consumption of a significant amount of water. With water already being scarce in many parts of the nation, biofuel crops may actually be a bane. Other barriers in the non-realization of bioethanol blending in petrol in general include the battle between the alcohol sector, medicinal sector, and fuel sector for ethanol.

6 Life Cycle Analysis of Jowar and Bajra Feedstocks

6.1 Life Cycle Assessment Methodology

"Cradle to Grave" approach (holistic approach that links the information it collects from analysis to product development) of Life Cycle Assessment designed an excel-based model that was used to assess the renewability potential of the bioethanol feedstocks. This model was designed based on the ISO 14040-Life Cycle Assessment Standard [9] to design the system boundaries for a functional unit of 1 ton/Day (TPD) of dry biomass feedstock plants. These system boundaries included feedstock farming, transportation of feedstock, ethanol production, ethanol transportation, ethanol blending, and fuel combustion. Along with the baseline survey data (of 2013) collected for feedstock with 333 farmers covering five districts of Madhya Pradesh state of India, empirical data from the 66 multi-locational trials of sorghum and pearl millet conducted during the years 2014–2015 and 2015–2016 in farmers fields were used to conduct the LCA analysis. Data from secondary sources were also used to conduct the analysis.

Life Cycle Assessment as per the ISO-14040 has multiple steps. They are (a) to define the goal and scope of the work, (b) inventory analysis, (c) impact assessment which demands clear distinction and definition of all the unit processes and their impact separately, and (d) interpretation phase which validates the study and provides concluding remarks for the product under study.

6.2 Approach

To carry out a LCA for sorghum and pearl millet stalks as feedstock and identifying their viability for biofuel production is the main goal of this approach. As per the LCA methodology, it is important to design a system boundary. The first step to any process design is to develop a set of process flow diagrams (PFDs). Figure 1 contains the PFDs used for this study. A typical ethanol production requires separate saccharification of pentose and hexose sugars. In this study, simultaneous saccharification and co-fermentation are considered. Farming data was collected from the field surveys conducted by the authors and the data from agro-India. High Biomass Variety (HBV) was considered for this study and the data on these hybrids were taken from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) and Indian Institute of Millet Research. In the present model analysis was carried out for both rain-fed and irrigated crops. Lignocellulosic feedstock considered in this study are a high biomass variety crops which yield high fodder and are grown specifically for meeting bioethanol blending target. This feedstock gives fodder which is considered as an additional market value gained and hence it is important to holistic approach that links the information it collects from analysis to product development and analyze its life cycle inventory from the farming stage for a better comparison and allocation. Certain inventory input values

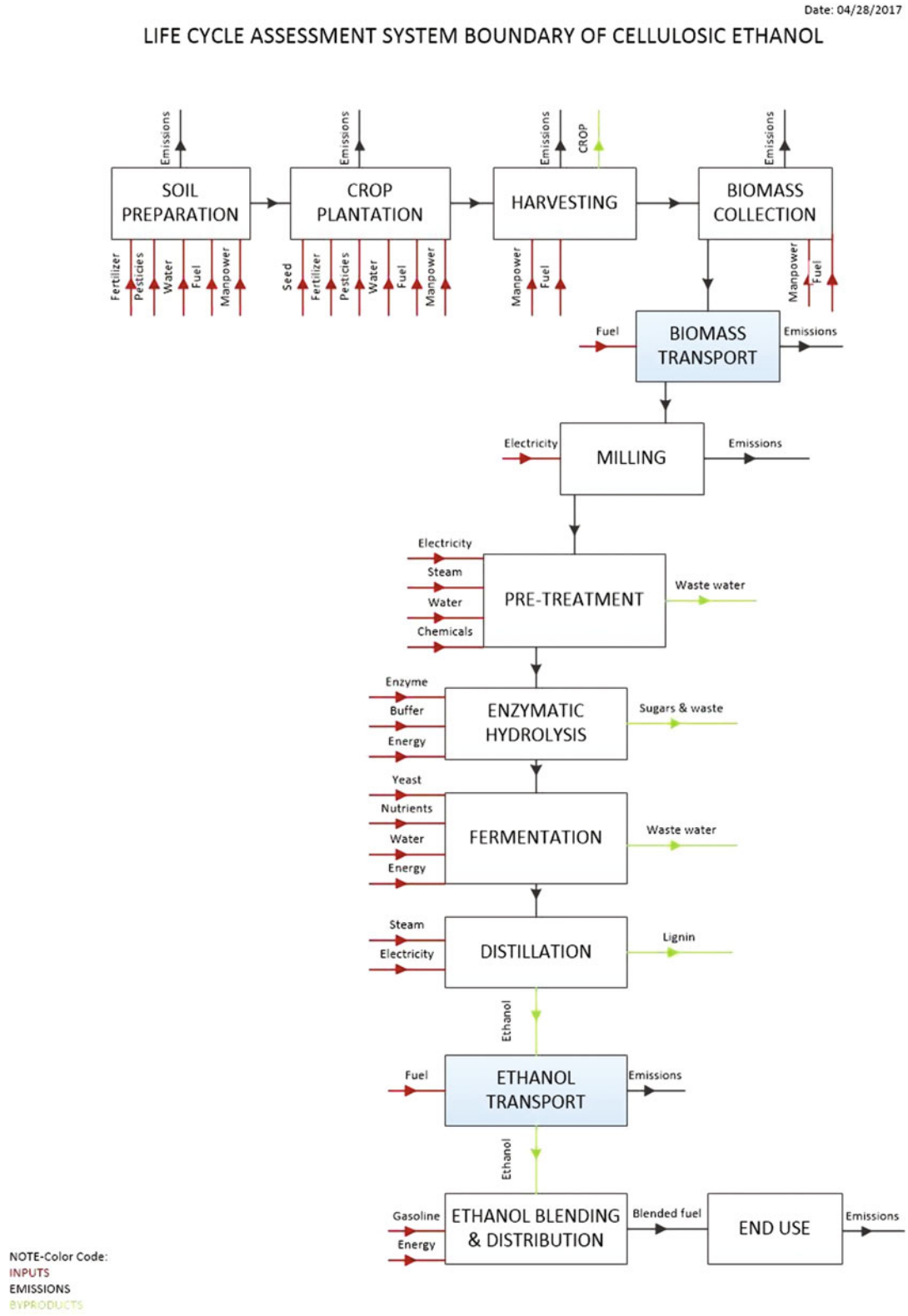


Fig. 1 System boundary

were either calculated for Madhya Pradesh region or collected from secondary data sources. The model considers mass and energy balances associated within the processes.

6.3 Process Overview

Processes included in this study and their significance is explained in this section:

- Farming: The feedstock is cultivated, harvested, and dried in this process.
- Feedstock handling and storage: The feedstock is handled and stored at the farm and transported using a tractor or truck.
- Size reduction: Size reduction is through a knife mill.
- Pretreatment: This process is carried out to separate lignin from the cellulose and hemicellulose.
- Simultaneous Saccharification and co-fermentation: Hydrolysis and fermentation is carried out to convert the glucose and xylose to ethanol.
- Distillation: Steam distillation is employed to purify ethanol.
- Product purification: the product ethanol is purified using a second distillation column to refine the ethanol and then the ethanol is denatured.
- Product storage: Ethanol produced is directly shipped to the nearest blending stations instead of storing.
- Wastewater treatment: Water is treated and the lignin is separated from the wastewater.
- Lignin Combustion: Lignin combustion to produce process steam and excess steam to produce electricity.
- Ethanol transportation to the blending station.
- Ethanol blending at the blending station.
- Ethanol blended fuel combustion.

Process overview is indicated in the process flow diagram shown in Fig. 1.

6.4 Life Cycle Assessment System

Life Cycle Assessment is an analysis to assess the impacts associated with all the stages of the product formation from the raw materials.

6.4.1 Farming and Transportation of the Feedstock

Farming includes soil preparation, sowing, cultivation, and harvesting. Soil preparation involves ploughing and leveling which can also be mechanized. Water will be provided for the crop based on the irrigation requirement and number of irrigations. Crop duration is 120 days and on attaining maturity the crop is harvested by cutting down the stem and allowing the remaining plant to fertilise the soil.

Basis: As per ISO standards, Functional Unit = 1 Tonne/Day (TPD) of dry biomass feedstock.

Table 1
Feedstock or biomass yield and land requirement

Feedstock	Dry fodder yield (kg/acre)	Land requirement (ha)
Sorghum stalk	4000	0.10
Pearl millet Stalk	2000	0.20

Table 2
Water requirement

Feedstock	Water depth (mm)	One irrigation water requirements (kL/ha)	No of irrigations	Water requirement (kL)
Sorghum stalk	550	5500	1–2	550
Pearl millet Stalk	300	3000	1–2	300

A depth of water (mm) requirement is converted to mm^3/ha

6.4.2 Land Requirement

Table 1 indicates the land required for growing feedstock for a plant capacity of one Tonne/day (TPD). It is calculated based on the dry biomass yield [10].

Equation 1

$$\text{Land requirement(hectare)} = \frac{\text{Plant capacity(TPD)}}{\text{Dry biomass yield}\left(\frac{\text{Tonne}}{\text{hectare}}\right)}$$

6.4.3 Water Requirement

Sorghum and Pearl millet are usually rain-fed crops. To estimate the impact caused if the crop was irrigated, water requirement becomes an important parameter. Water requirements for crops are given in terms of depth of irrigation and number of irrigations (*see* Table 2). Average values for kharif and rabi crops are considered for irrigation depths [11]. The crop is irrigated at the flower primordial initiation and flowering stages, i.e., 70–80 days after cultivation.

Equation (2): water requirement

$$\text{Water requirement(kL)} = \text{water depth(mm)} \times \text{Area(ha)} \times \text{No of irrigations}$$

6.4.4 Chemicals Required

Chemical inventory was based on the empirical data collected by CESS from farmers of Madhya Pradesh state (*see* Table 3). Fertilizer and pesticide usage for sorghum and pearl millet crops was based on farmer's survey in Madhya Pradesh.

Table 3
Fertilizer/pesticides requirement for Sorghum and Pearl millet crops

Feedstock	Urea (kg/acre)	Di-ammonium phosphate (DAP) (kg/acre)	Potash (kg/acre)	Complex (kg/acre)	Herbicides (L/acre)	Pesticides (L/acre)
Sorghum stalk	71.3	60.1	41.3	117.8	1.0	1.5
Pearl millet Stalk	45.2	20.0	6.0	0.0	1.0	1.5

Area converted from bigha to acre. 1 acre = 2.49 bigha (Easy Calculations.com 2017)

Table 4
Power consumption for irrigation

Feedstock	Power consumption (kWh)
Sorghum	52.71
Pearl millet	57.47

6.4.5 Diesel Requirement

Land preparation is a one crop time activity where the ploughing is done well in early showers. It is done using a cart or a tractor. By a stakeholder interaction with a farmer, diesel requirement for land preparation is 4–7 L/acre.

6.4.6 Electricity Requirement

3 HP electric pump for irrigation is used in case of water drawn from bore well (*see* Table 4). In case of rain-fed crops, electricity requirement become nil in case of irrigation purpose. This pump has a Total Dynamic Head (TDH) of 20–22 m [12]

Equation (3): Electricity requirement

$$\text{Electricity requirement(kWh)} = \text{water requirement}\left(\frac{\text{L}}{\text{ha}}\right) \times \text{power consumption}\left(\frac{\text{kWh}}{\text{L}}\right) \times \text{water requirement}\left(\frac{\text{L}}{\text{ha}}\right) \times \text{power consumption}\left(\frac{\text{kWh}}{\text{L}}\right) \times \text{Area(ha)}$$

Equation (4): Power of an electric pump

$$\text{Power consumption of a pump} = \frac{2.725 \times \text{TDH}}{\eta}$$

where $\eta = 0.63$

1 Mega L of water to lift to 1 m of height uses 2.725 kilowatt-hours (kWh) of electricity [13].

Table 5
Seed requirement for feedstock

Feedstock	Seed requirement (kg)
Sorghum	1.01
Pearl millet	1.42

Table 6
Labor requirement for farming

Feedstock	Labor requirement (Man-hour)
Sorghum	29.2
Pearl millet	73.0

6.4.7 Seed Requirement Based on the literature, seed requirements are 10 and 7 kg/ha for Sorghum and Pearl millet, respectively [14, 15]. Seed requirement for feedstock sorghum and pearl millet is seen in Table 5.

6.4.8 Labor Requirement Labor requirement in Madhya Pradesh is 36.13 and 45.08 Man-days/ha for Sorghum and Pearl millet feedstock respectively [16, 17]. Assumption of 8 working h is considered per day (*see* Table 6).

6.4.9 Transportation of Feedstock A certain underlying assumptions for making this feedstock viable and to avoid the risk due to expensive transportation, farm to plant distance is 50 km as suggested by CESS. Transportation of the feedstock is usually by a 40 HP tractor from the farm to the plant. Through farmer's survey, an average carrying capacity of a 40 HP tractor is 5 ton which consumes 4 km/L of diesel in loaded condition and 7 km/L in unloaded condition. The feedstock can be collected and transported once in 5 days to divide the transportation inventory.

Equation (5): Fuel consumption for transportation

$$\text{Fuel consumed} = \left(C_{\text{p}} \times \text{distance}\right)/\left(C_{\text{t}} \times \text{mileage}\right)$$

where C_{p} is the capacity of the plant (TPD)
C_{t} is the capacity of the vehicle (Tonnes)
Distance in km
Mileage in km/L

Table 7
Feedstock composition

Component	Sorghum stalk (%)	Pearl millet stalk (%)
Cellulose	35.9	41.0
Hemicellulose	26.0	20.9
Lignin	7.5	18.3
Ash	0.7	6.0
Moisture	15.0	10.0
Others	14.9	3.9

7 Ethanol Production

Dry biomass feedstock is fed to a knife mill where the straw is debaled (opening of compressed bale) and size reduced to 10 mm. The crushing capacity of a knife mill is 360 kg/h with the power consumption of 10.53 kWh/Tonne [18]. The crushed biomass feedstock is sent to the pretreatment reactor through a level ground conveyor of 100 TPH capacity (power capacity of 3.75 hp) connecting 300 ft. distance [19]. The composition of dry Sorghum and Pearl millet stalk is in Table 7.

Production of cellulosic ethanol via biological conversion consists of three critical steps:

- Pretreatment of biomass
- Hydrolysis of sugar polymers to sugar monomers and
- Fermentation of sugar monomers to ethanol

7.1 Pretreatment

The recalcitrant and heterogeneous structure of the biomass poses a fundamental challenge to depolymerization of cellulose during enzymatic hydrolysis process. Enzyme accessibility is restricted by the lignin and hemicellulose which makes enzyme irreversibly bind to lignin thus slowing down the process [20]. Pretreatment methods are aimed at enhancing the susceptibility of lingo-cellulosic biomass to enzymes, thereby degrading the hemicellulose and lignin. Cellulose in the form of glucan and hemicellulose in the form of xylans and converted to pentose and hexose sugars in this model for ethanol production. Pretreatment of both Sorghum and Pearl millet feedstocks are explained in this section.

The following are the major pretreatment techniques used in agro-based feedstocks:

- Dilute acid
- Steam explosion

- Hot water
- Dilute alkali
- Alkali hydrogen peroxide

7.1.1 Dilute Acid (DA)

The biomass consisting of cellulose, hemicellulose, and lignin is treated with 1% w/w sulfuric acid at 180 °C and 11 bar in a reactor for 15 min. Sulfuric acid acts as a catalyst. High and low pressure steam is fed into the reactor for maintaining the water in liquid stage. At this stage, some of the cellulose is hydrolyzed to glucose. A fraction of lignin is converted to soluble lignin. However, most of the hemicellulose gets hydrolyzed in this process [20].

7.1.2 Steam Explosion

Biomass is heated at high pressure of 15 bar at temperature of 180°C for 15 min and is flashed into a tank where the rapid expansion of steam causes rupture of the biomass structure. This process is highly effective on feedstock with large particle sizes, thereby reducing the energy requirement for size reduction [20].

7.1.3 Hot Water

In an auto catalyzed hot water pretreatment process, acetic acid released from hemicellulose and self-ionization of water at elevated temperatures act as dilute acid for breaking the cellulose and hemicellulose sugars. Thus, feedstock undergoes hydrolysis [20].

7.1.4 Dilute Alkali

In this process, alkali swells the cellulose, thereby increasing the surface area promoting the separation of carbohydrates from lignin. This treatment also removes acetyl groups of hemicellulose resulting in higher lignin removal during dilute alkali pretreatment compared to other pretreatments [20].

7.1.5 Alkali Hydrogen Peroxide

It is an advanced method developed by the Indian Institute of Chemical Technology (IICT)-Hyderabad and Indian Institute of Technology (IIT)-Delhi has a Work Package-2. This process is effective for higher digestibility and prevents the formation of inhibitors. 1% (w/w) of alkali and hydrogen peroxide is added to the biomass feedstock at 550 °C and atmospheric pressure for 4 h. This pretreatment method eliminates the formation of furfural and hydroxymethy furfural (HMF) which inhibits the hydrolysis.

The reaction conditions of all the pretreatment processes carried out in this study are in indicated in Table 8.

Under the above reaction conditions, the following reaction stoichiometry is attained where hydroxy methyl furfural (HMF) and furfural are fermentation inhibitors.

$$\text{Glucan} + 0.111\,H_2O \rightarrow 1.111\,\text{Glucose}$$

$$\text{Xylans} + 0.136\,H_2O \rightarrow 1.136\,\text{Glucose}$$

$$\text{Lignin} \rightarrow \text{Soluble Lignin}$$

Table 8
Pretreatment conditions

Conditions	Units	Dilute acid	Steam explosion	Hot water	Dilute alkali	Alkali hydrogen peroxide
Temperature	°C	180	180	180	180	55
Pressure	Bar	11	11	11	11	1.0135
Residence time	Min	15	15	15	15	240
Solid loading	%	20	30	20	20	20
Acid/alkali loading	% w/w	1	0	0	1	1

Table 9
Pretreatment conversion

	Dilute acid (%)	Steam explosion (%)	Hot water (%)	Dilute alkali (%)	Alkaline hydrogen peroxide (%)
Cellulose to glucose	13.04	5.00	0.43	0.29	50.00
Xylan to xylose	60.26	70.00	70.00	0.72	22.50
Lignin to soluble lignin	5.00	5.00	5.00	25.00	15.00
Xylose to furfural	5.00	15.00	2.50	0.01	0.00
Glucose to HMF	5.00	15.00	2.50	0.01	0.00

$$\text{Xylose} \rightarrow 0.64\,\text{Furfural} + 0.36\,H_2O$$

$$\text{Glucose} \rightarrow 0.7\,\text{HMF} + 0.3\,H_2O$$

A summary of the pretreatment reaction conversion is mentioned in Table 9 [20].

7.2 Simultaneous Saccharification and Co-fermentation

The pretreated biomass was hydrolyzed using the enzymes cellulase and hemicellulase for cellulose and hemicellulose, respectively. The enzyme loading of 15 Filter Paper Unit/gram (FPU/g) of cellulose was considered for dilute acid, steam explosion, dilute alkali, and hot water treatment (*see* Table 10). Cellulose and hemicellulose hydrolysis can be done by Simultaneous Saccharification and Co-Fermentation (SSCoF) reactor which is proven successful in a pilot-scale study [21–23].

Glucose is hydrolyzed by Saccharomyces Cerevisiae. *Zymomonas mobilis* is an organism tested for co-fermentation of glucose and xylose [24]. Reaction conditions, conversions, and inventories are considered by work done by Deepak Kumar [20].

Table 10
Reaction conditions

Parameters	Units	Dilute acid	Steam explosion	Hot water	Dilute alkali	Alkali hydrogen peroxide
Temperature	°C	35	35	35	35	55
Enzyme loading	FPU/g cellulose	15	15	15	15	15 FPU/g biomass
Time	Days	5	5	5	5	0.25

Table 11
Conversion of ethanol from feedstock

Conversion	Dilute acid treatment (%)	Steam explosion (%)	Hot water (%)	Dilute alkali (%)	Alkaline hydrogen peroxide (%)
Cellulose to Glucose	79.0	70.0	78.5	84.8	67.5
Xylan to xylose	80.0	80.0	80.0	80.0	64.1
Glucose to ethanol	95.0	95.0	95.0	95.0	80.0
Glucose to CO_2	95.0	95.0	95.0	95.0	80.0
Xylose to ethanol	70.0	70.0	70.0	70.0	80.0
Xylose to CO_2	70.0	70.0	70.0	70.0	80.0

Under the above reaction conditions, the following reaction stoichiometry is attained.

$$\text{Glucan} + 0.111\,H_2O \rightarrow 1.111\,\text{Glucose}$$

$$\text{Xylans} + 0.136\,H_2O \rightarrow 1.136\,\text{Glucose}$$

$$\text{Glucose} \rightarrow 0.489\,CO_2 + 0.511\,\text{Ethanol}$$

$$\text{Xylose} \rightarrow 0.489\,CO_2 + 0.511\,\text{Ethanol}$$

Based on the stoichiometric balances, following conversions of ethanol under different pretreatment conditions is shown in Table 11 and their inventory is in Tables 12, 13, and 14 for Sorghum and Pearl millet stalks, respectively.

The Life Cycle inventories are shown below. Chilled water, cooling water are used to dissipate heat during all the post heating process operations. Chemically treated water is used in processes for feedstock processing, dilutions, and cleaning operations. DAP is used as a nutrient source for Z. Mobilis growth [5].

Table 12
Ethanol yield from different pretreatment and feedstocks

Ethanol yield	Dilute acid treatment (%)	Steam explosion (%)	Hot water (%)	Dilute alkali (%)	Alkaline hydrogen peroxide (%)
Sorghum stalk	25.26	22.90	25.00	24.88	24.89
pearl millet stalk	25.62	27.86	25.26	25.55	25.89

Ethanol yield is calculated as output ethanol (kg) to that of the input dry biomass feedstock (kg)

Table 13
Life cycle inventory for sorghum stalk-ethanol production

Inventory	Dilute acid	Steam explosion	Hot water	Dilute alkali	Alkaline hydrogen peroxide
Electricity[a]	179.29	168.35	164.80	164.00	175.47
Steam	1892.17	1169.76	1904.67	1835.54	1836.0
Steam (High pressure)	134.47	179.96	133.10	132.46	132.5
Cooling water	160,159.29	116,523.16	180,642.23	157,850.48	157,886.5
Chilled water	233.72	258.33	266.21	264.92	265.0
Chemically treated water	28,417.84	28,718.62	28,278.43	28,081.93	28,088.3
Water	1920.99	1257.71	1901.50	1892.31	1892.7
Sulfuric acid	65.95	0.00	0.00	15.45	15.5
Ca Hydroxide	32.02	0.00	0.00	0.00	0.00
DAP	0.32	0.29	0.32	0.32	0.32
Cellulase	83.88	87.66	95.71	95.25	95.27
Yeast	0.96	1.16	0.95	0.95	0.97
Sodium hydroxide	0.00	0.00	0.00	49.52	49.53
Gasoline	2.56	2.32	2.54	2.52	2.52
Hydrogen peroxide	0.00	0.00	0.00	0.00	49.53

All units in kg
[a]Units in kWh

7.3 Distillation and Steam Production

Followed by the SSCoF process, the slurry is stored in a beer well for 4 h to allow settling. Ethanol is distilled using two distillation columns D1 and D2. The ethanol vapors from the first column are enriched in the second distillation column. In the process of

Table 14
Life Cycle Inventory for Pearl millet stalk-ethanol production

Inventory	Dilute Acid	Steam explosion	Hot water	Dilute alkali	Alkaline hydrogen peroxide
Electricity[a]	181.86	204.79	166.49	168.40	182.07
Steam	1919.30	1422.96	1924.19	1884.83	1909.9
Steam (High pressure)	136.40	218.92	134.47	136.02	137.8
Cooling water	162,455.52	141,745.27	182,494.03	162,089.01	164,246.2
Chilled water	237.07	314.25	268.94	272.04	275.7
Chemically treated water	28,825.27	34,934.93	28,568.32	28,835.97	29,219.7
Water	1948.53	1529.95	1920.99	1943.12	1969.0
Sulfuric acid	66.90	0.00	0.00	15.87	16.1
Ca hydroxide	32.48	0.00	0.00	0.00	0.00
DAP	0.32	0.35	0.32	0.32	0.33
Cellulase	85.09	106.63	96.69	97.80	99.11
Yeast	0.97	1.41	0.96	0.97	0.33
Sodium hydroxide	0.00	0.00	0.00	50.85	51.52
Gasoline	2.60	2.82	2.56	2.59	2.63
Hydrogen peroxide	0.00	0.00	0.00	0.00	51.52
Ethanol produced	324.76	353.09	320.16	323.85	103.75

All units in kg
[a]Units in kWh

enriching, an azeotrope of ethanol and water is formed in the distillation column D1 is separated using a molecular sieve to produce anhydrous ethanol which upon denaturing is ready for blending with gasoline.

The bottom effluent from the first distillation D1 column has lignin and non-fermentables. The bottom effluent is passed through pneumapress that further separates solids and liquids. Lignin rich solids are combusted in Fluidized Bed Combustor (FBC) for steam generation. A liquid rich stream containing water is evaporated, the evaporated condensate is recycled as process recycle water and the concentrate is sent to FBC for combustion. Steam produced from lignin fraction is more than the steam requirement of the plant. The excess steam is utilized for power generation which can be sold to the grid. The underlying assumption is that 1 kL of ethanol gives 15.6 m^3 of spent wash; 1 m^3 of spent wash gives 35 m^3 of biogas; 1 m^3 of biogas gives 2.5 kWh of

Table 15
Ethanol production and by-product from Sorghum feedstock

Product	Dilute acid	Steam explosion	Hot water	Dilute alkali	Alkaline hydrogen peroxide
Ethanol (L)	320.16	290.26	316.92	315.39	315.46
Electricity (kWh)	437.03	396.21	432.59	430.50	430.60

Table 16
Ethanol production and by-product from Pearl millet feedstock

Product	Dilute acid	Steam explosion	Hot water	Dilute alkali	Alkaline hydrogen peroxide
Ethanol (L)	324.76	353.09	320.16	323.85	328.16
Electricity (kWh)	443.29	481.97	437.03	442.06	447.94

electricity and the same is calculated for different pretreatments for Sorghum and Pearl millet feedstocks in Tables 15 and 16, respectively.

8 Ethanol Transportation

Bioethanol transportation from plant to blending station is by diesel driven tankers of 40 HP. The distance from the plant to the blending and distribution station is 100 km. Truck capacity is 18,000 L with loaded and unloaded mileage of 6 and 7 km/L [6, 25].

9 Blending

Blending of E5, E10, or E15 can be carried out in the model. However, 5% blending of ethanol with gasoline by volume is considered under this report.

10 Combustion

Combustion of the ethanol-blended fuel in comparison to pure gasoline in an IC engine is considered in this study.

11 Results

The results are evaluated based on the environmental impacts through the Life Cycle Assessment at 5% blending. Net Energy Ratio (NER), Net Energy Balance (NEB), Net Carbon Balance (NCB), and % Carbon reduction are essential in analyzing LCA. These parameters are calculated as below.

11.1 Net Energy Ratio

It is the ratio of energy returned on energy invested. A fuel under study is considered renewable only if the NER is greater than one [26].

$$\text{Net Energy Ratio} = \frac{\text{Energy Output from ethanol production}}{\text{Energy Input for ethanol production}}$$

11.2 Net Energy Balance

It is the difference between the output energy to that of the input energy [26].

$$\text{Net Energy Balance} = \text{Energy Output} - \text{Energy Input}$$

11.3 Net Energy Balance/kL of Bioethanol

It is the net energy output/kL of bioethanol produced [26].

$$\text{Net Energy Balance per kilo litre of bioethanol}\left(\frac{\text{GJ}}{\text{kl}}\right) = \frac{\text{Net Energy Balance}}{\text{Quantity of ethanol}}$$

11.4 Net Carbon Balance

It is the difference between the output and input carbon emissions [26].

$$\text{Net Carbon Balance} = \text{Output Carbon emissions} - \text{Input Carbon Emissions}$$

11.5 Net Carbon Balance/kL of Bioethanol

It is the net carbon emission output/kL of bioethanol produced [26].

$$\text{Net Carbon Balance per kilo litre of bioethanol}\left(\frac{\text{tCO}_2\text{e}}{\text{kl}}\right) = \frac{\text{Net Carbon Balance}}{\text{Quantity of ethanol}}$$

11.6 % Carbon Emission Reduction

It is the net quantity of Greenhouse Gas emissions avoided by using biofuel when compared to the use of fossil fuel [26].

Table 17
Energy renewability indicators of Sorghum feedstock-ethanol

	Net energy ratio		Net energy balance (GJPY)		Net energy balance/kL of ethanol (GJ/kL)	
Pretreatment	Rain fed	Irrigated	Rain fed	Irrigated	Rain fed	Irrigated
Dilute acid	2.629	2.492	1853.5	1790.9	17.5	17.0
Steam explosion	2.332	2.213	1549.1	1486.5	16.2	15.5
Hot water	2.421	2.303	1738.1	1675.5	16.6	16.0
Dilute alkali	1.908	1.834	1402.6	1340.0	13.5	12.9
Alkaline hydrogen peroxide	1.855	1.785	1358.9	1296.3	13.1	12.5

Table 18
Energy renewability indicators of Pearl millet feedstock-ethanol

Pre-treatment	Net energy ratio		Net energy balance (GJPY)		Net energy balance/kL of ethanol (GJ/kL)	
	Rain fed	Irrigated	Rain fed	Irrigated	Rain fed	Irrigated
Dilute acid	2.469	2.339	1805.4	1737.1	16.8	16.2
Steam explosion	2.374	1841.3	1909.6	1841.3	16.4	15.8
Hot water	2.279	2.167	1679.1	1610.9	15.9	15.2
Dilute alkali	1.829	1.757	1371.7	1303.5	12.8	12.2
Alkaline hydrogen peroxide	1.788	1.720	1351.8	1283.5	12.5	11.9

$$\text{Percent carbon reduction} = \left(\frac{\left(1 - \frac{\text{Total Carbon Input}}{\text{Quantity of Ethanol produced}}\right)}{\text{Emission factor of petrol}} \right)\%$$

NER, NEB, NCB without allocation for Sorghum and Pearl millet feedstocks are estimated and presented in Tables 17, 18, 19, and 20, respectively.

Sorghum and Pearl millet feedstock is a rain-fed crop and hence rain-fed Sorghum feedstock is considered for bioethanol production with dilute acid pretreatment in inferring the study as follows:

- The net energy ratio of bioethanol production from Sorghum stalk by dilute acid pretreatment is 2.629, which implies that the total energy output for the production of bioethanol is 2.629 times that of the input energy consumed during its production.
- The net energy balance of 1853.5 GJPY is the delivered energy after subtracting the energy required for the production of ethanol.

Table 19
Environmental impact of Sorghum feedstock-ethanol

	Net carbon balance (tCO_2e/year)		Net carbon balance/kL of bioethanol (tCO_2e/kL)		% Carbon reduction	
Pretreatment	**Rain fed**	**Irrigated**	**Rain fed**	**Irrigated**	**Rain fed**	**Irrigated**
Dilute acid	−80.62	−98.41	−0.76	−0.94	−42.45	−49.80
Steam explosion	−98.88	−117.66	−1.03	−1.23	−53.57	−61.68
Hot water	−90.07	−108.86	−0.86	−1.04	−46.51	−53.93
Dilute alkali	−111.09	−129.88	−1.07	−1.25	−55.03	−62.48
Alkaline hydrogen peroxide	−129.71	−148.49	−1.25	−1.43	−62.40	−69.86

Table 20
Environmental impact of Pearl millet feedstock-ethanol

	Net carbon balance (tCO_2e/year)		Net carbon balance/kL of bioethanol (tCO_2e/kL)		% Carbon reduction	
Pretreatment	**Rain fed**	**Irrigated**	**Rain fed**	**Irrigated**	**Rain fed**	**Irrigated**
Dilute acid	−103.15	−123.63	−0.96	−1.17	−50.69	−58.59
Steam explosion	−102.28	−122.76	−0.88	−1.05	−47.19	−54.45
Hot water	−133.15	−133.63	−1.05	−1.26	−55.17	−63.18
Dilute alkali	−133.00	−153.48	−1.24	−1.44	−62.34	−70.26
Alkaline hydrogen peroxide	−151.24	−171.72	−1.40	−1.59	−68.63	−76.44

- 17.5 GJ/kL of ethanol is the net energy balance/kL of ethanol produced implying that the extra energy delivered/kL of ethanol produced.
- The net carbon balance/kL bioethanol is −0.76 tCO_2e which means that for every kL of bioethanol 0.76 tCO_2e of emissions would be reduced.
- The % carbon emission reduction by using bioethanol with respect to the use of petrol is −42.45%, thus signifying that bioethanol is a carbon-negative fuel. This inference has occurred due to the fact that the energy consumed in the farming and chemicals have played a significant role in increasing the carbon emissions. These emissions can be reduced significantly by reducing the inventory like water and acid/alkali used.

Rain-fed Pearl millet feedstock for bioethanol production with dilute acid pretreatment infers the following:

- The net energy ratio of bioethanol production from Sorghum stalk by dilute acid pretreatment is 2.47, which implies that the total energy output for the production of bioethanol is 2.47 times that of the input energy consumed during its production.
- The net energy balance of 1805.4 GJPY is the delivered energy after subtracting the energy required for the production of ethanol.
- 16.8 GJ/kL of ethanol is the net energy balance/kL of ethanol produced implying that the extra energy delivered/kL of ethanol produced.
- The net carbon balance/kL bioethanol is -0.96 tCO_2e which means that for every kL of bioethanol 0.96 tCO_2e of emissions would be reduced.
- The % carbon emission reduction by using bioethanol with respect to the use of petrol is -50.69%, thus signifying that bioethanol is a carbon-negative fuel. This inference has occurred due to the fact that the energy consumed in the farming and chemicals have played a significant role in increasing the carbon emissions. Pearl millet feedstock would be more promising if the inventory was reduced either in the form of chemical consumption or water consumption.

The above inferences indicate that Pearl millet feedstock is less energy intensive than Sorghum feedstock in terms of NER and NEB. The technology needs further modification aiming at reducing the chemical consumption and thereby reducing the overall emissions.

12 Comparative Analysis of Second-Generation Biofuels to First-Generation Biofuel

A comparison of the Net Energy Ratio of first-generation molasses feedstock with second-generation rice straw is in Table 21 [25].

As seen in the Table 21 first-generation molasses and second-generation rice straw have higher percent carbon reduction potential in comparison to current study. The LCA system boundary designed is subjected to the individual study and hence not comparative. The energy invested in the life cycle assessment is a boundary set by the assessor and is not a standard tool to compare the renewability of one feedstock with respect to another.

13 Allocation Approach

The allocation approach is given to the life cycle assessment in order to decide the exact impact of these inventories in the environment. For instance, if the stalk is utilized instead of the grain which is the

Table 21
Comparison of first-generation and second-generation feedstocks

Feedstock	Net energy ratio	% Carbon reduction
Molasses	4.57	75
Rice straw	3.32	68
Sorghum straw	2.629	−42.45
Pearl millet Straw	2.469	−50.69

Table 22
Mass, Energy, and market price allocation

Process	Without allocation (%)	Mass allocation (%)	Energy allocation (%)	Market price allocation (%)
Farming	100	70	70	18
Transportation	100	100	100	100
Production of ethanol	100	85	85	90
Transportation	100	100	100	100
Blending	100	100	100	100

major output of a crop, major burden is allocated to the major producers in terms of the grain nutrient equivalent or energy equivalent. The burden in the form of allocation is distributed in Table 22.

In the farming stage, the bioethanol feedstock yield accounts to 70% of the total plantation and costs 18% of the price invested in cultivating the crops. Ethanol production considered at the standalone distillery, all of the process utilities are accounted (85% allocation) as a major constituent and the rest 15% offered for lignin and biogas as by-products not allocated in the mass and energy allocations. All the other stages of ethanol production do not have any by-products which deliver a market value for distributing the burden and reducing the overall burden of this process.

Incorporating the allocation, the results in the NER vary to a certain extent and the same are explained for Sorghum and Pearl millet stalk with dilute acid treatment as in Tables 23 and 24.

Table 23
Allocation for dilute acid treated Sorghum stalk ethanol production

Process	Without allocation	Mass allocation	Energy allocation	Market price allocation
Farming	545.24	381.7	381.7	98.1
Transportation	10.01	10.01	10.01	10.0
Production of ethanol	642.40	546.04	546.04	578.2
Transportation	1.64	1.64	1.64	1.6
Blending	1.31	1.31	1.31	1.3
Total energy	1200.61	940.68	940.68	689.3
NER	2.49	3.18	3.18	4.3

All units except NER are in GJPY

Table 24
Allocation for dilute acid treated Pearl millet stalk ethanol production

	Without allocation	Mass allocation	Energy allocation	Market price allocation
Farming	632.71	442.9	442.9	113.9
Transportation	10.01	10.01	10.01	10.0
Production of ethanol	651.59	553.85	553.85	586.4
Transportation	1.67	1.67	1.67	1.7
Blending	1.33	1.33	1.33	1.3
Total energy	1297.30	1009.75	1009.75	713.3
NER	2.34	3.01	3.01	4.3

All units except NER are in GJPY

14 Conclusions

Achieving energy security in the country through alternate methods is an important area being focused upon by the Indian policymakers. However, any attempt to promote the use of major staple food crops such as jowar and bajra for biofuel production has a long-lasting impact on the food, fodder, and nutritional security of millions of people and livestock in India. Empirical evidence from baseline survey and MLTs has clearly indicated that existing varieties cultivated by farmers were much better in terms of overall yield

(grain and fodder) as compared with the High Biomass Varieties promoted by the SLABs project. Farmers clearly perceived the threat to fodder security due to diversion of jowar and bajra straw to biofuel production. Cultivation of high biomass jowar and bajra varieties on a large scale could pose a serious threat to the existing rich diversity in these crops. Hence, even for trying out these crops at the research level, it is essential to have a dialogue with the farmers of drylands where these two crops are predominantly grown. The voice of small and marginal farmers and women should be heard before moving further to utilize these crops for biofuel production. More importantly, we should learn from our earlier experiences of jatropha cultivation [7, 8]. The production of feedstocks for biofuels would put additional pressure on agricultural resources such as land and water.

LCA analysis revealed that though the sorghum and pearl millet are rain-fed crops, farming contributes to near 30% of the energy input in the ethanol production cycle. The highest contributors of GHG emissions are pesticides and fertilizers. Reduction in the use of pesticides and fertilizers has a good impact on energy savings. Sorghum feedstock is more energy intensive than pearl millet feedstock due to low grain and fodder yield. Ethanol production from second-generation biomass in terms of pretreatment processes is very energy-intensive because the biomass is separately pretreated. Dilute acid pretreatment has higher conversion efficiency than the other pretreatment processes. Alkali hydrogen peroxide process is more promising with comparative yields. Reduction of process water by 70–80% can reduce the environmental impact significantly. Second-generation ethanol production can be combined with first-generation ethanol plants/distilleries to reduce the energy consumption in the distillation column. In first-generation ethanol production, the energy used for cultivation and farming are not accounted as ethanol produced from the by-products. Whereas, in the second generation, the additional burden comes at the farming stage which can be negotiated in the ethanol production stage by intensifying the process inventories by better enzymes and advanced processes.

Biofuels, either conventional or advanced should not be blindly encouraged without a comprehensive outlook on the overall impact that will ultimately have on the society, environment, or on the countries' energy security. Efforts should be made toward the encouragement of research and development in the field as well as in formulating a comprehensive and effective biofuel policy for India.

15 Note

Indo-US Bilateral Joint Clean Energy Research and Cellulosic Biofuels Systems (SALBS) was initiated in the USA and India with multiple partners in Consortium in each country. The Consortium was led by the University of Florida (UF) in America and the Indian Institute of Chemical Technology (IICT) in India. The Centre for Economic and Social Studies (CESS) was associated with the work package component of Sustainability, Marketing, and Policy, and looked into the socioeconomic and Life Cycle Analysis of biofuel production through cultivation of Jowar and Bajra feedstocks in India.

References

1. Bharat Petroelum (2017) BP statistical review of world energy. BP. https://www.bp.com/content/dam/bp/en/corporate/pdf/energy-economics/statistical-review-2017/bp-statistical-review-of-world-energy-2017-full-report.pdf
2. IEA (2016) Renewable energy outlook. World Energy Outlook Chapter 6:197–232
3. GOI (2009) National policy on biofuels—Ministry of New and Renewable Energy, Government of India, 2009
4. Reddy MG, Reddy SB, Padakandla SR (2014) Reduction of GHG emissions and attainment of energy security through sustainable production of biofuels: Is it a viable option? A review of experiences, Working paper No. 136. Centre for Economic and Social Studies, Hyderabad
5. Davis R (2013) Process design and economics for the conversion of lignocellulosic biomass to hydrocarbons. NREL, Corkery. https://www.nrel.gov/docs/fy14osti/60223.pdf
6. Soam S (2015) Life cycle assessment of fuel ethanol from sugarcane molasses in northern and western India and its impact on Indian biofuel programme. Energy 83:307–315. https://doi.org/10.1016/j.energy.2015.02.025
7. Montobbio PA, Lele S (2010) Jatropha plantations for biodiesel in Tamil Nadu, India: viability, livelihood trade-offs and latent conflict. Ecol Econ 70:189–195
8. Singhal R, Sengupta R (2012) Energy security and biodiesel—implications for land use and food security. Econ Polit Wkly 46:66–73
9. International Organisation of Standards (2006) International Standard-ISO 14040. Switzerland. http://www.pqm-online.com/assets/files/lib/std/iso_14040-2006.pdf
10. Stakeholder (2015) Survey (C. f. Studies, Interviewer)
11. Gangaiah DB (2012) Agronomy—kharif crops. Indian Agricultural Research Institute, New Delhi. http://nsdl.niscair.res.in/jspui/bitstream/123456789/527/1/Millets%20%28Sorghum%2C%20Pearl%20Millet%2C%20Finger%20Millet%29%20-%20%20Formatted.pdf. Accessed 19 Jun 2017
12. Pumpkart (2017) Open well pump. Pumpkart: https://www.pumpkart.com/point-three-phase-open-well-pump-pohm-37-3hp
13. Foley DJ (2015) Cottoninfo. www.cottoninfo.net.au. http://www.cottoninfo.com.au/sites/default/files/documents/Fundamentals%20EnergyFS_A_3a.pdf. Accessed June 2017
14. TNAU Agritech Portal (2017) TNAU Agritech portal. Agriculture: TNAU Agritech portal. http://agritech.tnau.ac.in/agriculture/millets_sorghum.html. Accessed 19 June 2017
15. Agropedia (2017) Seed rate in Paerl Millet. agropedia.iitk.in. http://agropedia.iitk.ac.in/content/seed-rate-pearl-millet. Accessed 19 July 2017
16. Jaiswal A (2009) Economics of production and value addition to Soybean in Madhya Pradesh. University of Agricultural Sciences, Dharwad. http://krishikosh.egranth.ac.in/bitstream/1/83384/1/th9969.pdf
17. Deshmukh D (2010) Costs, returns and profitability of pearl millet production. Int J Comm Bus Manag 3:95–99. http://www.researchjournal.co.in/upload/assignments/3_95-99.pdf

18. Bitra VS (2009) Direct measures of mechanical energy for knife mill size reduction of switchgrass, wheat straw, and corn stover. Bioresour Technol 100:6578–6585. http://biomassprocessing.org/Publications/1-Articles/Bioresource%20Technology%202009%20Knife%20mill%20energy%20Bitra%20et%20al.pdf
19. Engineering Tool Box (2017). www.EngineeringToolBox.com. Engineering tool box. http://www.engineeringtoolbox.com/conveyor-power-load-d_1560.html. Accessed 14 July 2017
20. Kumar D (2011) Impact of pretreatment and downstream processing technologies on economics and energy in cellulosic ethanol production. Biotechnol Biofuels 4:27. https://doi.org/10.1186/1754-6834-4-27
21. Kim J-S, Cho YK, Jeong G-T, Kim S-K (2012) Optimization of saccharification and ethanol production by simultaneous saccharification and fermentation (SSF) from seaweed, Saccharina japonica. Bioprocess Biosyst Eng 35 (1–2):11–18. https://doi.org/10.1007/s00449-011-0611-2
22. Kádár Z, Szengyel Z, Réczey K (2004) Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol. Ind Crop Prod 20:103–110
23. Pothiraj C, Arun A, Eyini M (2015) Simultaneous saccharification and fermentation of cassava waste for ethanol production. Biof Res J 2:196–202
24. Mokomele T, Callanan LH, Clarke KG (2005) Ethanol production from xylose and glucose by Zymomonas mobilis for the development of a membrane bioreactor. http://www.crses.sun.ac.za/files/services/conferences/annual-student-symposium-2012/22_November/5_Mokomele.pdf
25. Hi-Tech Services (2017) Diesel tanker. http://www.hitechspecialpurposevehicles.com/diesel-tanker.html
26. Confederation of Indian Industry (2010) Estimation of energy and carbon balance of biofuels in India. Confederation of Indian Industry. https://www.mycii.in/KmResourceApplication/46884.EnergyCarbonBalanceofBiofuelsinIndia.pdf



Chapter 20

Biofuels and Sustainability

N. Eswaran, S. Parameswaran, and T. S. Johnson

Abstract

Energy security and climate change have cascading effects on the world's burgeoning population in terms of food security, environment, and sustainability. Due to depletion of fossil fuels and undesirable changes of climatic conditions, increase in air and water pollution, mankind started exploring alternate and sustainable means of meeting growing energy needs. One of the options is to use renewable sources of fuel–biofuel. In this chapter the authors have reviewed and presented sustainability impact on production of biofuels. Authors further reviewed state-of-the-art gene editing technologies toward improvement of biofuel crops. The authors recommend a phased transition from first-generation biofuel, and an acceleration toward use of technology to drive adoption of second-generation biofuels. Key aspects of technology and application of resource management models will enable these crops to bridge the global energy demand before we can completely transition to a more sustainable biofuel economy.

Key words Biodiesel, Biofuels, CRISPR/Cas-9, Microalgae, Sustainability

1 Background

Energy security and climate change have cascading effects on the world's burgeoning population in terms of food security, environment, and sustainability. Increase in demand for petrol and diesel as transportation fuels is bound to occur. The world will face depletion of non-renewable natural resources, along with undesirable changes in climatic conditions such as global warming and increase in air and water pollution. Increased GHG emissions due to burning of fossil fuels will lead to undesirable changes in air quality and water [1]. In the worst-case scenario the accumulated CO_2 and other GHGs might pollute water bodies. These developments have led mankind to explore sustainable methods to produce renewable energy to meet the growing future energy demand. Biofuels are the most sought sources of renewable energy, and attracted the attention of researchers as a promising sustainable energy source [1–3]. Biofuels are derived from solid biomass through chemical and biological processes.

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Biofuels are fuels derived from plant sources [4]. For the purpose of this article, authors have considered biodiesel, bioethanol, and other lignocellulose-based fuels under biofuel throughout the article. First-generation biofuels are sourced from edible feedstock such as soybeans, corn oil, palm oil, sunflower, safflower, rapeseed, and coconut, peanut, or from animal fat. Currently, 95% of the world's demand for biodiesel is met from edible oil sources [5]. Top contributors of biodiesel sources are rapeseed (84%), sunflower oil (13%), palm oil (1%), soybean oil, and others (2%) [5]. The fatty acid composition of edible oils from different plant seeds differs. The physical and chemical properties of biodiesel are very similar to that of diesel, enabling use in diesel engines with little modification. Bioethanol is produced by fermentation of lingo-cellulosic stock. In the United States bioethanol is derived mainly from corn starch, in Brazil from sugarcane, in EU from sugar beet and wheat, in China from corn oil, and in India from sugarcane [6]. In the Europe, biodiesel production is mainly from rape seed oil, Malaysia and Indonesia rely on palm oil, while India, Pakistan, African, South-east Asian countries have explored *Jatropha* and *Pongamia* as biofuel crops [6].

First-generation biofuels have sustainability challenges, and research focus leads to biofuels obtained from alternate feedstock beyond edible crops. The "second-generation biofuels" are derived from a wide range of feedstocks, such as nonedible plant oils, lignocellulosic feedstock, secondary agriculture waste, and waste materials. Nonedible plants widely identified as suitable biofuel feedstock are Jatropha (*Jatropha curcas*), castor bean (*Ricinus communis*) karanja (*Pongamia pinnata*), Jojoba (*Simmondsia chinensis*), mahua (*Madhuca indica*), sugarcane (*Saccharum officinarum*), neem (*Azadirachta indica*), polanga (*Calophyllum inophyllum*) [7, 8]. Different types of lignocellulosic feedstock used for biofuels production include waste from food crops, wheat straw and corn husks, secondary agricultural waste, wood chips, and sugarcane bagasse. Third-generation biofuels are mainly microbial sources, and are a promising alternative beyond second-generation biofuels. The microorganisms are different types of algae, yeast, fungi, and bacteria. Extensive recent work on third-generation biofuels has been given impetus as nonedible oil, high oil content with rapid biomass production (Ref. [3] and references cited thereof). Algal-derived biofuels are in the forefront as a major source due to high oil (20–80%) that is convertible to fuel equivalents such as kerosene oil as well as biodiesel [9]. Microalgae biomass is of high research priority due to easy cultivation, rapid growth, and high lipid contents. From an environmental angle, algae are viewed as safer alternative to engineered crops for biodiesel production [9].

2 Sustainability Impacts of Biofuels on the Environment

Biofuels are a promising source of renewable bioenergy and helps to reduce our dependence on petroleum and other fossil fuels. Biofuel production has increased in the past decade and is expected to significantly increase in developing nations such as Brazil, India, and China [6]. In 2018, biofuels contributed to 3% of the world's fuel for road transport. Efforts to expand biofuels to more than a quarter of the transportation fuel requirement by 2050 are underway (https://en.wikipedia.org/wiki/Biofuel). Biofuel production and use can have numerous social, economical, and environmental impacts. Different biofuels have different impacts that are dependent on factors such as feedstock used, feedstock production, and biofuel extraction process [6]. The major economic, social, and environmental impacts which could be advantageous and disadvantageous are elaborated in the following section.

2.1 Food Security

Food crops have been commonly used in first-generation biofuels feedstock. They consume a large share of arable land, labor, and natural resources to produce the biomass. The opportunity cost to direct threat to food security needs to be considered when edible crop use is diverted for biofuels production. The threat can be a direct food-fuel competition when a food crop is directly used for biofuels production, and it can be indirect when the feedstock production for biofuels is competing with food production for inputs such as land, labor, water, and fertilizers. The food–biofuel competition can lead to the spike in food prices due to high fossil fuel prices, globalized trade, etc. [10]. The increase in food prices can affect the complete well-being of humans, especially of the poor at the household level such as security, health, and good life [6, 10]. An additional aspect of first-generation biofuel production is that feedstock cultivation requires the application of phosphorus (P) fertilizers [11]. Since phosphorus is needed for food production, biofuel production can severely affect the global food production and its security which can lead to increase in food prices and have a negative impact on future welfare [11]. Second-generation biofuels, which are derived from nonedible feedstock, secondary agricultural residues, and wastes such as animal fat and waste cooking oil, are expected to buffer the impact on food prices and global food supply. However, the food security issue cannot be entirely resolved with development and promotion of second-generation biofuels, even if they use nonedible feedstock because of indirect land-use changes and because of the potentially huge market demand for renewable energy in comparison to agriculture which increases demand for land, labor, and resources [12]. Yield decline in crops over a period of time will lead to increase in grain prices. Sometimes yield loss occurs due to adverse weather

conditions and/or biotic factors (http://www.etipbioenergy.eu/sustainability/food-vs-fuel-debate). The price hike also could be due to higher agriculture input prices and increase in access to food availability to poorer nations. In addition, experts have also pointed out that increased biofuel production has also a role in hike in food prices, shifting land away from food production triggering competition for land use [13].

2.2 Biodiversity Loss

Large-scale cultivation of monocultures can be particularly harmful to biodiversity [10]. Large-scale cultivation biofuel crops will have direct link to habitat destruction, invasive alien species, pollution, and climate change [10]. Of these, habitat destruction is said to be the greatest threat to biodiversity [6, 10]. Palm oil is having wide demand in the world market. Malaysia is one of leading countries producing palm oil in large quantity. Sensing the demand for biodiesel, Malaysian government has supported large-scale cultivation of oil palm. As a result, rampant destruction, logging, and burning of tropical rain forests occurred to make way for oil palm cultivation thus creating environmental imbalance [14]. Rampant use of chemicals and fertilizers also leads to pollution of water bodies. Moreover, palm oil wastage also created water pollution [14]. Another threat is the growth of non-native feedstock which can become invasive species and have negative impacts on the ecosystem and ecosystem components such as soil regeneration, carbon sequestration, natural chemical cycles, pollination, and protection against flood [15]. The feedstock that have become invasive are Jatropha in countries such as Australia and Africa and hence in some parts of Africa *Jatropha* cultivation has been banned [6, 10]. Soto et al. [16] have reported that several farmers in the state of Chiapas, Mexico, who initially took up cultivation of *Jatropha,* discontinued later because of lack of profitability of the crop commercialization and the presence of pests and diseases that infect *Jatropha* plants which in turn leads to loss in yields.

2.3 Air Pollution

Atmospheric pollutants emitted during biofuel's life cycle production to combustion such as particulate matter (PM), nitrogen oxides (NO_x), sulfur dioxide (SO_2), ammonia (NH_3), carbon monoxide (CO), volatile organic compounds (VOC), and ozone (O_3) impact human health [6, 17]. These pollutants are emitted during biomass production (agricultural phase and agricultural burning), harvest, transport and biomass conversion to biofuels in biorefineries. For example, pollutants emitted during sugarcane burning are PM2.5 and PM10, polycyclic aromatic hydrocarbons, and NO_x [6, 17]. Major health risks from the short-range production and dispersal of pollutants are implied for high-density populated cities compared to rural areas [17]. The air quality life cycle assessment (LCA) of these air pollutants is different from the LCA of GHG emissions [6, 17]. In a counter argument, use of biofuels may have

a positive effect on air quality. The city of São Paulo, Brazil, motor vehicles shift to use of ethanol, leading to the use of gradual phaseout of older, more polluting, and less energy-efficient vehicles [10].

2.4 Water Requirement and Pollution

First-generation biofuels consume large amounts of water, and high direct water use during feedstock production [6]. Increased risk of water scarcity in countries facing water shortage is a real concern, and evaluating biofuels type based on its water consumption pattern and feedstock conversion to oil is needed. First-generation biofuels are considered more water consumers throughout their growth phase. However, different biofuels have different water usage requirements depending on the type of feedstock, geographical location, and agricultural practices. For example, *Jatropha* demands high water requirements in regions where intense irrigation practices are observed, such as India. But in countries like Africa, it is considered a conservative water user compared to natural vegetation. Biofuel expansion might result in excessive water usage which might result in added pressure on water resources in arid and semi-arid regions [6]. Evaluation of impact on water pollution, through fertilizer, agrochemicals, and industrial effluent from biofuel production and processing are critical factors from a public health hazard ecosystem function [6, 10]. Biofuel production from microalgae minimizes the threat to water use. Microalgae culture is feasible in fresh and marine waters, municipal waste waters, recycled and treated effluent, industrial waste waters and animal waste waters as long as there are adequate amounts of nutrients and microelement needed to support microalgae growth.

2.5 Cost

Economic feasibility of biodiesel is directly linked to the availability of low-cost feedstock. Key challenge with large-scale biodiesel production in comparison to petroleum is the high costs associated with biodiesel production. First-generation biofuels feedstock from edible oils has a high exposure (nearly 70–95% of the total biofuel production cost) to raw materials. Waste cooking oils that are not suitable for human or animal consumption can mitigate the cost of material for biofuel conversion, but have lower transesterification yield, with additional esterification reaction prior to economic biofuel extraction process [18]. Current costs associated with microalgal biomass is more due to technology and capital needed relative to crops cultivation. Centrifugal harvesting (mechanical), chemical flocculation, b, prior to biofuel extraction. Oil recovery technology from microalgae is in industrial process development. Present methods yield ~30% extraction from algal biomass, while 70% oils is sequestered in the algal biomass making the process economically challenging. Algal biomass, when applied to

alternative use with biofuel in nutrient feedstock (animal, fish, etc.), pharmaceutical ingredients, cosmetics, toiletries, and fragrance products, makes biofuel production from algal biomass more commercially attractive [19].

2.6 Rural Development

Cultivation of biofuel crops and transfer of manufacturing processes can enable rural development, by employment generation and opportunities for additional income generation. Increase in biofuel production is expected to increase jobs associated with feedstock production, feedstock supply, and skilling of personnel for oil extraction. Small-scale biofuel projects (e.g., *Jatropha* oil in Mali for rural electrification) have contributed positively to the energy security of remote rural areas [6, 10]. Biofuel production will augment income generation and contribute to poverty alleviation by jobs expansion in the allied sectors.

2.7 Impact on Land

Competition for land used for the biofuel feedstock in a biodiverse habitat is a major debate. Replacement of biodiverse habitats with monocultures feedstock production reduces available land and imbalance in ecosystems and potential loss biodiversity. For example, with increased worldwide biodiesel demand, Malaysian palm oil policy supports large-scale cultivation of oil palm. This has led to huge environmental imbalance with rampant tropical forest destruction, logging, and burning to make way for oil palm cultivation [14]. It has created huge environmental imbalance. Moreover, palm oil wastage also created water pollution that affected aquatic biodiversity. The other pollutants such as palm oil mill effluents, fertilizers, and pesticides have also impacted environment. Policy development and implementation should factor environment while seeing higher production. Common biofuel feedstocks ranked from low to high soil erosion hazards are cassava, soybean, sugar cane, sorghum, corn, sugar beet, winter wheat, oil palm, and winter rapeseed [10]. While first-generation biofuels impact soil erosion negatively, *Jatropha* feedstock, for example, can stabilize soil and reduce soil erosion if grown on degraded lands [6]. Agricultural residues such as corn stover, wheat straw, and rice straw are bulk residues from food processing. Use of these sources for energy generation is key for renewable energy. Crop by-product utilization ensures sustainable practice and minimizes food fuel and land competition. Agricultural residues generation is dependent on cultivation practice; excessive erosion and removal of little or no residue may prevent the soil from drying up and negatively affecting the land for the next planting season [20]. The main advantages of algal biofuels are the high yield per acre which is up to 10 times higher than with other biofuels and the fact that algae do not compete for arable land.

2.8 GHG Emissions

Greenhouse gas (GHG) emissions such as carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) are emitted during whole life cycle of the biofuels. The nature of GHG emissions differ from biofuel feed stock and manufacturing process. The choice of the feedstock crop used has an impact due to the CO_2 fluxes associated with tillage and the N_2O fluxes associated with nitrogen fertilizer use. Hence there are cases where the GHG emissions are much lower than conventional fossil fuels, and there have been several occasions where the GHG emissions can be very high. The main advantages of the biodiesels produced from nonedible oils as compared to fossil fuel are lower particulate matter, carbon monoxide, hydrocarbons, nitrogen oxides and smoke emission, higher compression ratio associated with higher injection pressure, and higher diffusion combustion [21]. The biofuels produced from microalgae are considered carbon neutral. The burning process of biofuel produces CO_2 which is in turn utilized by the algae in biomass cultivation. This helps in reducing GHG emissions [19]. Microalgae can provide several different types of renewable biofuels such as methane, biodiesel, and biohydrogen. Biohydrogen that can be developed from both microalgae and cyanobacteria has the advantage of being a completely environmentally clean fuel. But the presence of oxygen tends to generate lower yields of hydrogen, so genetic engineering will be necessary to maximize hydrogen production while adjusting the hydrogenase's tolerance to oxygen [22].

3 Land-Use Impact in Life Cycle Assessment (LCA)

Life cycle assessment (LCA) is defined as the compilation and evaluation of the inputs, outputs, and potential environmental impacts of a product system throughout life cycle from the extraction of raw materials through production and use to waste management [23]. LCA is currently the most widely used method to assess the environmental sustainability of biofuels and critical in policy-making purposes [24]. An LCA study is spread over four separate but overlapping phases, viz. (A) goal and scope definition, (B) inventory analysis, (C) impact assessment, and (D) interpretation. In impact assessment, relevant impact categories are energy balance, global warming potential, and land-use impact. Land-use impact is the land-use changes (LUC) that arise due to land competition with food production. Impact on biodiversity and impacts on quality of the soil and in turn the land for subsequent crops. Land-use impact focuses on the impact of the new use of land for production of energy crops against a reference system such as natural vegetation of the land [25]. Hence the displacement of food crops by the energy crops triggers the LUC mechanisms. There are two types of LUCs: direct land-use changes

and indirect land-use changes. Direct land-use changes take place when there is introduction of a new cropping system in a site where this form of cultivation has not taken place before. The emissions caused by the conversion process can be directly linked to the biofuel load and thus be allocated to the specific carbon balance of that biofuel. Indirect land use occurs when land that was formerly used for the cultivation of food, feed, or fiber is now used for biomass production, shifting the original land use to an alternative area that may have a high carbon stock resulting in significant greenhouse gas emissions from land conversion. This also causes a reduction of the area available for food and feed production and therefore leads to a reduction of food and feed supply on the world market. Consequently, food prices will increase if the demand for food persists. The increase in price will create a need to convert formerly unused area for food production [24, 26]. The quantification of GHG emissions also depends on whether the effects from land use change (LUC) have been considered or not. If the effects from LUC are considered, then the GHG emissions due to biofuels will be very high and harmful to the environment. Studies that have considered such LUC effects have shown that carbon debt is generated due to soil carbon loss that releases significant amounts of GHG, and spans several decades [6, 10]. The LUC not only impacts the availability of land but also affects the quality of soil. The concept of soil quality is linked to the ability of soil to function effectively in a variety of roles. Because of its impact on human health and environment quality, degradation of soil quality due to land-use changes is a major global concern. Hence soil quality status due to LUC should be considered while evaluating the LCA of biodiesel production from any biofuel crop [26].

Although LCA is standardized, its application to biofuels often is inconclusive, with high variability and uncertainty of output results. The analysis can be significantly improved by multiparameter analysis on variability, and by quantifying the influence of variable impacting the result of the model. Some LCA do not consider LUC for estimating GHG emissions and some include it. Jingura et al. [25] have reported the consolidated analysis of 10 LCA conducted for *Jatropha* biodiesel production including nine from Asia and one from Africa. The analysis was based on energy balance, GHG balance, land-use impact, and other environmental impacts such as acidification and eutrophication. *Jatropha* cultivation can be carried out through conversion of lands such as existing cropland, abandoned agricultural land, natural vegetation, degraded natural vegetation, and wasteland. Hence depending on which land is used the land-use impact will vary. Land-use impact considers ecosystem structural quality (ESQ) and ecosystem functional quality (EFQ). Changing wasteland into *Jatropha* plantation leads to increase in ESQ because *Jatropha* plantations have higher storage capacity in terms of biomass, structure, and biodiversity than wasteland and a

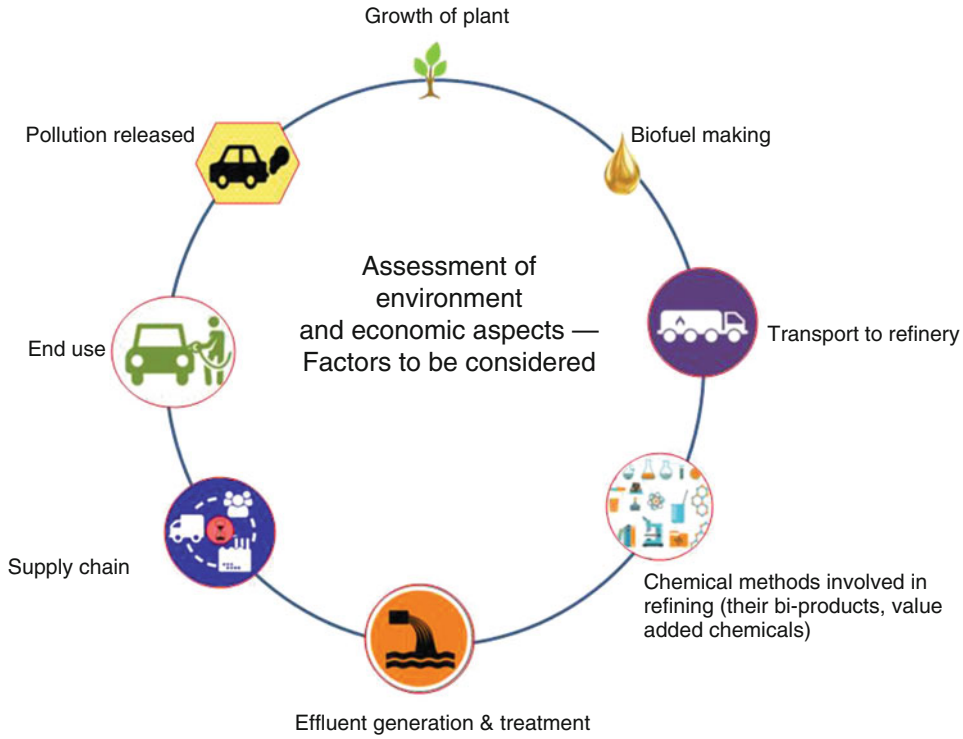


Fig. 1 Factors to be considered during assessment of environment and economic aspects of biofuel

decrease in EFQ due to less control over water, material, and nutrient fluxes than wasteland. But the overall analysis shows that *Jatropha* is suitable for cultivation on wastelands due to the competition between food and biofuel crops. *Jatropha* is also said to have a positive effect on land use since *Jatropha* can grow on marginal land [25]. Factors to be considered during assessment of environment and economic aspects are represented in Fig. 1.

4 Involvement of Genomics for Biofuel Sustainability

In the last few decades, new plant breeding strategies have been applied to improve oil yield, biomass yield, tolerance to biotic and abiotic stress. Biodiesel derived from edible oil crops has direct competition for food vs. fuel, while nonedible crops have disadvantages such as increased cultivation cost, low oil yield, and unsuitable oil composition. The traditional breeding strategies for crop improvement require time that is not economically practical [27]. Recent sequencing and multi-omics technologies along with genetic engineering could be a key link to address the gap in global energy crisis [28]. Genomic technologies have been scaling up in throughput, cost of generating sequence data has been diminishing, time taken to obtain sequence data is reducing, and quality of

data is improving [29]. The state-of-the-art technologies enable the direct determination of population scale genomic information in a rapid and cost-effective manner [30, 31]. Genomic technologies accelerate discovery of sequence diversity and enable lower cost development for molecular markers for breeding [32]. Genome sequencing can rapidly provide information on express sequence tags (ESTs) [33], and can be readily applied to breeding population to develop genetic linkage maps [30, 32], identify quantitative trait loci (QTL), and develop defined molecular markers (such as SNP, SSR, ISSR) for breeding and population development [30, 32]. Genomic tools enable detailed profiling of stress physiology and understand the genome-wide gene expression changes, with sensitive measurements, with minimal sequence information. NGS tools such as de novo transcriptomics and differential gene expression analysis with RNA-seq provide powerful capabilities to understand oil-biosynthetic pathways [34–36].

The information can be applied with advanced genetic engineering tools to target genetic modification in the biofuel crops and enhance desired traits [34]. The application of such technologies in oil plants is at their initial stages to enhance oil content or improved oil composition [37]. Most of the GM oil crops have been evaluated either at laboratory or greenhouse levels, and it is not clear how they would respond under field conditions [2]. In this section we have reviewed contemporary crop improvement strategies such as CRISPR-Cas9 technologies. The basis for including genomic technologies is that the classical technologies are proving to be time-consuming. Progress has been made in first-generation biofuels using the classical breeding tools, whereas developments in molecular marker technologies have been applied to yield improvement in second-generation biofuels. Toward understanding pathway engineering the transcriptomics approach is being evaluated. These methods can enable third-generation biofuel systems and applied to understand pathways and modify them. These technologies are still at development phase that genomics can accelerate. The objective of applying genomic technologies is to make biofuel production a sustainable option that can offer benefit to environment and end user. The readers are requested to consult various review articles published on the traditional plant breeding methods and genetic engineering methods.

5 Crop Improvement Using CRISPR/Cas9 Gene Editing System

Currently new genetic engineering techniques are being tried out for crop improvement which uses site-specific nucleases (SSNs). These are enzymes which lead to single- or double-stranded DNA breaks which can be used for targeted mutagenesis. There are three such SSN-based genome editing which are currently in use, namely

the zinc finger nuclease (ZFN), transcription-like effector nucleases (TALENs), and, the recently developed, clustered regularly interspaced short palindromic repeats that are associated with the RNA-guided Cas9 double-stranded DNA-binding protein (CRISPR/Cas9) [38]. In this review we will be discussing the use of CRISPR/Cas9 as the gene editing system for improvement of crops which are used for biofuel production. The RNA-guided genome editing technology is constantly advancing including options for various genetic manipulations like generating knockouts, making precise modifications, multiplex genome engineering, and activation and repression of target genes. These systems rely on sequence-specific nucleases (SSNs) to produce double-strand breaks (DSBs) at specific locations within the genome. DSB is repaired by the cell using a mechanism of non-homologous end joining or homologous repair. CRISPR/Cas9 has been used in several biofuel crops for many agronomic traits such as improving crop yield, oil yield, increasing resistance against plant diseases, and for tolerance against several abiotic stresses such as salinity and drought [39, 40].

With the aim to validate CRISPR/Cas9 system as a tool for editing both endogenous and exogenous genes, to create gene knockouts, for targeted mutagenesis, to create specific mutations at multiple loci, the use of multiple guide RNAs for complex genome editing, especially the deletion of large DNA fragments and to get highly efficient and stable mutations which are reproducible over generations, many studies have been reported in several edible and nonedible biofuel crops such as soybean [41–44], maize [45–48], cotton [49–52], Jatropha [53], oilseed rape [54, 55], oil palm [56], and barley [57]. List of biofuel crops in which CRISPR/Cas9 system has been validated is given in Table 1. Several studies have described applications of CRISPR systems for the enhancement and development of important agronomic traits in crops. CRISPR/Cas9 method of gene editing has been adopted in nearly 20 crop species (including few biofuel cops) so far [39, 40, 58] for various traits including yield improvement, flowering, fatty acid composition, plant disease resistance, and for abiotic stress such as drought and salinity. List of biofuel crops in which CRISPR/Cas9 system has been used for gene editing for different agronomic traits is given in Table 2. CRISPR/Cas9-based studies in a few of these biofuel crops, which are useful for biofuel sustainability, will be discussed in this review.

5.1 *Maize (Zea mays)*

Shi et al. [59] utilized CRISPR/Cas9 genome editing to create new allelic variants of ARGOS8 gene in maize. The auxin-regulated gene involved in organ size (ARGOS) gene families are negative regulators of the ethylene response and modulate ethylene signal transduction. It has already been shown in the past that overexpression of this gene reduced ethylene sensitivity and improved grain

Table 1
List of biofuel crops in which CRISPR/Cas9 system has been validated

Sr. No	Crop	Target gene	Aim	Transformation method	References
1.	*Glycine max* (Soybean)	GmFT2a (Glyma16g26660) and GmFT5a (Glyma16g04830) flowering genes	Use of multiple guide RNAs for complex genome editing, especially the deletion of large DNA fragments	*Agrobacterium*-mediated transformation	[44]
2.	*Glycine max* (Soybean)	Green fluorescent protein (GFP), glucosyl-transferase (Glyma07g14530), Glyma01g38150, Glyma11g07220, miR1514 and miR1509	CRISPR/Cas9 system can be used for knockout and different mutations in genes	*Agrobacterium*-mediated transformation	[43]
3.	*Glycine max* (Soybean)	GmFEI2 and GmSHR	CRISPR/Cas9 system is a powerful tool for editing both endogenous and exogenous genes in soybean hairy roots	*Agrobacterium*-mediated transformation	[41]
4.	*Glycine max* (Soybean)	*ALS1*Acetolactate synthase	To use *Streptococcus pyogenes* Cas9-guide RNA (gRNA) for targeted mutagenesis, gene integration, and gene editing in soybean	Particle bombardment	[42]
5.	*Zea mays* (Maize)	*Zmzb7*IspH protein	CRISPR/Cas9 system is a robust and efficient tool for genome modification in both euchromatic and heterochromatic regions	*Agrobacterium*-mediated transformation	[48]
6.	*Zea mays* (Maize)	PSY1	CRISPR-Cas9 is an effective tool for the targeted gene mutagenesis in maize	*Agrobacterium*-mediated transformation	[47]
7.	*Zea mays* (Maize)	*MADS* (GRMZM2G059102), *MYBR* (GRMZM2G091201) *AP2* (GRMZM2G050851) *RPL* (GRMZM2G024838),	The utility of the tRNA-processing system-based strategy as an efficient multiplex genome editing tool	*Agrobacterium*-mediated transformation	[46]

(continued)

Table 1
(continued)

Sr. No	Crop	Target gene	Aim	Transformation method	References
		PPR (GRMZM2G087226)			
8.	*Zea mays* (Maize)	Liguleless1 (LIG1) gene, male fertility genes (Ms26 and Ms45), and acetolactate synthase (ALS) genes (ALS1 and ALS2)	Targeted mutagenesis, precise gene editing, and site-specific gene insertion	Biolistic transformation	[45]
9.	*Gossypium hirsutum* (Cotton)	Cloroplastos alterados 1 (*GhCLA1*) andvacuolar HC-pyrophosphatase (GhVP)	Targeted mutagenesis of cotton genome	*Agrobacterium*-mediated transformation	[51]
10.	*Gossypium hirsutum* (Cotton)	*GhPDS*, *GhCLA1*, and *GhEF1*	Fast and efficient method to validate the functionality of sgRNAs in cotton	*Agrobacterium*-mediated transformation	[50]
11.	*Gossypium hirsutum* (Cotton)	Green fluorescent protein (GFP)	To understand the efficacy of the CRISPR/Cas9 system to target a gene	*Agrobacterium*-mediated transformation	[49]
12.	*Gossypium hirsutum* (Cotton)	*GhMYB25-like A* and *GhMYB25-like D*	Targeted mutagenesis	*Agrobacterium*-mediated transformation	[52]
13.	*Jatropha curcas (Jatropha)*	Fatty acid, protein and toxin Biosynthesis genes	Gene editing approaches in Jatropha	*Agrobacterium*-mediated transformation	[53]
14.	*Brassica napus* (Oilseed rape)	BnSPL3-A5/BnSPL3-A4/BnSPL3-C3/ BnSPL3-C4/BnSPL3	Simultaneous mutagenesis of multiple gene homologs	*Agrobacterium*-mediated transformation	[55]
15.	*Brassica napus* (Oilseed rape)	BnaRGA, BnaDA2, and BnaFUL gene families	Specific mutations at multiple loci	*Agrobacterium*-mediated transformation	[54]
16.	*Elaeis guineensis* (Oil Palm)	Isoflavone reductase (IFR) and methallothionine-like protein (MT)	CRISPR-Cas9 is an effective tool for genome editing in oil palm	*Agrobacterium*-mediated transformation	[56]
17.	Barley	–	Gene knockouts in barley using CRISPR	*Agrobacterium*-mediated transformation	[57]

Table 2
CRISPR/Cas9-based genome editing in edible and non-edible biofuel crops

Sr. No.	Crop	Transformation method	Target gene	Target trait	References
1.	*Zea mays* (Maize)	*Agrobacterium*-mediated transformation	*ZmIPK1A*, *ZmIPK*, and *ZmMRP4*	Phytic acid synthesis	[61]
2.	*Zea mays* (Maize)	Biolistic-mediated transformation	*ARGOS8*	Increased grain yield under drought stress	[59]
3.	*Glycine max* (Soybean)	*Agrobacterium*-mediated transformation	*GmPDS11* and *GmPDS18*	Carotenoid biosynthesis	[87]
4.	*Glycine max* (Soybean)	*Agrobacterium rhizogenes*-mediated hairy root transformation	Rj4	Cloning and characterization of Rj4 gene that inhibits nodulation by many strains of *Bradyrhizobium elkanii*	[72]
5.	*Glycine max* (Soybean)	Polyethylene glycol (PEG)-mediated protoplast transformations	*Avr4/6*	Functional genomics in *Phytophthora sojae* for control of the pathogen in Soybean	[73]
6.	*Glycine max* (Soybean)	*Agrobacterium*-mediated transformation	*GmFT2a*	Delayed flowering time	[74]
7.	*Glycine max* (Soybean)	*Agrobacterium*-mediated cotyledon node system.	Fatty acid sesaturase 2 *GmFAD2*	Enhancement of fatty acid composition	[69]
8.	*Glycine max* (Soybean)	Agrobacterium-mediated transformation	Omega-6 desaturase 2 (*FAD2-2)*	Enhancement of fatty acid composition	[70]
9.	*Glycine max* (Soybean)	*Agrobacterium*-mediated transformation	SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors of the SPL9 family (*GmSPL9a*, *GmSPL9b, GmSPL9c*, and *GmSPL9d*)	Altering plant architecture	[75]

(continued)

Table 2 (continued)

Sr. No.	Crop	Transformation method	Target gene	Target trait	References
10.	*Camelina sativa*	*Agrobacterium* floral dip transformation	*FAD2* Fatty Acid Desaturase 2	Enhancement of fatty acid composition	[64]
11.	*Camelina sativa*	*Agrobacterium* floral dip transformation	Delta -12- Desaturase 2 (*CsFAD2-1*, *CsFAD2-2*, *CsFAD2-3*)	Reduced levels of polyunsaturated fatty acids and increased accumulation of oleic acid in the oil	[65]
12.	*Camelina sativa*	*Agrobacterium*-mediated transformation	CRUCIFERIN C (*CsCRUC-G1*, *CsCRUC-G2*, and *CsC*) *RUC-G3*;	Altering seed protein profile	[67]
13.	*Camelina sativa*	*Agrobacterium*-mediated floral vacuum infiltration method	*CsDGAT1* and *CsPDAT1*	Alter seed oil production to produce unusual lipids	[66]
14.	*Jatropha curcas* (Jatropha)	*Agrobacterium*-mediated transformation	Cytokinin metabolic genes *JcCYP735A*	Understanding cytokinin genes in Jatropha and their involvement in growth	[68]
15.	*Gossypium hirsutum* (Cotton)	*Agrobacterium*-mediated transformation	Gh14-3-3d	Resistance against Verticillium dahlia infestation	[62]
16.	*Gossypium hirsutum* (Cotton)	*Agrobacterium*-mediated transformation	CLCuD-associated begomoviruses (CABs)	Resistance against Cotton leaf curl disease (ClCuD)	[63]

yield under drought stress conditions [60]. Here the researchers employed CRISPR/Cas9 system and created variants of the ARGOS8 (ARGOS8-v1 and ARGOS8-v2) gene which were used for creating hybrids and evaluated in the field in many locations. The results showed that the hybrids showed improved yield under drought conditions. This proves the usefulness of CRISPR/Cas9 system which can be used to induce drought resistance in maize and can also be extended to other crops [59]. Seventy-five percent of maize seed phosphorus is made up of phytic acid which is anti-nutritional and an environmental pollutant. In another study, Liang et al. [61] have reported targeted knockout of genes (*ZmIPK1A*, *ZmIPK*, and *ZmMRP4*) involved in phytic acid synthesis encoding

enzymes that catalyze three steps in the phytic acid biosynthetic pathway in maize. Using the CRISPR.Cas9 system, the researchers could successfully create mutations at the target site in transgenic maize which can be used to reduce the phytic acid levels.

5.2 Cotton (Gossypium hirsutum)

Cotton verticillium wilt, called "cotton cancer" (pathogen damage by *Verticillium dahlia*) is a destructive disease, annually leading to severe economic losses. Resistance to *Verticillium dahliae* infestation was reported through gene editing of *Gh14-3-3d* gene. By using the CRISPR/Cas9 system, mutations were created which could be stably transmitted to the next generation. The resulting plants showed a high resistance and can be used as a germplasm to breed disease-resistant cotton cultivars [62]. Cotton leaf curl disease (ClCuD) is another major disease affecting cotton cultivation in India, Pakistan, and Africa leading to huge economic losses. This is induced by begomoviruses (family *Geminiviridae*). All CLCuD-associated begomoviruses (CABs) induce typical symptoms of leaf curling (upward and downward), vein swelling, venation, and stunted growth. Iqbal et al. [63] using the CRISPR/Cas9 system proposed a multiplex type of sgRNA which could be designed to allow simultaneous editing of multiple begomoviruses in just one go instead of a single species. They proposed multiplex sgRNA cassette targeting intergenic region (IR) and Rep of the CABs. To minimize the change of resistance, breaking the IR region is targeted since this region is conserved among all members of the plant virus family geminiviridae. The above-proposed theory will be first confirmed in the model plant *N. benthamiana* and then will be tested in cotton [63].

5.3 Camelina (Camelina sativa)

Camelina Sativa, an allohexaploid, is an oil seed crop and has gained attention as a biofuel crop due to its adaptable nature to grow in regions with limited rainfall and soil fertility. However, its wider-scale application as a biofuel crop is yet to be pursued due to its high content of polyunsaturated fatty acids, particularly linolenic acid (18:3), which accounts for 30–40% of seed oil from most *Camelina* cultivars. This makes the *Camelina* oil susceptible to oxidation and its food products more prone to rancidity. To address this issue, Jiang et al. [64] using the CRISPR/Cas9 system created mutations in the *FAD2* (Fatty acid desaturase 2) gene in *C. sativa*. *FAD2* is the gene involved in the synthesis of polyunsaturated fatty acids such as linoleic acid. The mutations were stable and were inherited in subsequent generations. The seeds obtained had an increase in oleic acid from 16% to over 50%. The increase was associated with concomitant decrease in the polyunsaturated fatty acids, linoleic acid (i.e., a decrease from 16% to less than 4%) and linolenic acid (a decrease from 35% to less than 10%). A similar study was carried out by Morineau et al. [65] where heritable mutations were obtained in three isologous *CsFAD2* genes

(*CsFAD2-1*, *CsFAD2-2*, *CsFAD2-3*) using CRISPR/Cas9 system. The results obtained were similar to Jiang et al. [64] where an increase in oleic acid from around 10–62% is associated with a decrease in linoleic and linolenic acid. Such studies help in the enhancement of the fatty acid composition in *C. sativa*, which will be useful in biofuel production. In order to address seed lipid oil composition in *C. sativa*, Aznar-Moreno and Durette [66] using CRISPR/Cas9 system introduced mutations in three *CsDGAT1* or *CsPDAT1* homeologous genes important for triacylglycerol (TAG) synthesis in developing seeds. Analysis of lipid oil composition revealed that many lines produced reduced oil and altered fatty acid composition. This proved that endogenous genes involved in biosynthetic pathway can be altered for production of unusual lipids [66]. Utilization of camelina oil in feed, biofuel, or industrial feedstock applications requires increased value of the coproduct after oil extraction such as seed meal coproduct to increase the underlying economic feasibility of production. However, the low value of the seed meal discourages profitability. The nutritional quality of the seed meal depends on the presence of specific seed storage proteins and their amino acid composition [67]. Hence in a study using the CRISPR/Cas9 gene editing technology, three homeologous genes encoding the seed storage protein CRUCIFERIN C (*CsCRUC*) have been targeted to create mutations. The results indicated significant changes in the seed amino acid content with an increase in the levels of alanine, cysteine, and proline, and decrease in the levels of isoleucine, tyrosine, and valine. The *CsCRUC* knockout seeds showed significant alteration in the fatty acid profile, but the total oil content remained unchanged. This will help in making the seed meal more nutritious for animal feed in turn making production of *Camelina* more profitable for biofuel production since its coproducts will also be effectively used [67].

5.4 *Jatropha* (Jatropha curcas)

Cytokinins (CKs), which are known to be involved in many biological processes such as flower bud development, floral sex determination, and seed yield, are directly regulated by isopentenyl transferase (IPT), cytochrome P450 monooxygenase, family 735, subfamily A (*CYP735A*), and cytokinin oxidase/dehydrogenase (CKX). Endogenous CKs containing N6-(2-isopentenyl)-adenine (iP), trans-zeatin (tZ), cis-zeatin (cZ), dihydrozeatin (DZ), and their conjugates are known as isoprenoid CKs. The major derivatives are generally iP- and tZ-type CKs. However, the roles of CK biosynthesis genes IPTs and CYP735A and catabolism gene CKXs in *Jatropha* are not clear. Using the CRISPRCas9 system in *J. curcasJccyp735a* knockout mutants were obtained. *Jccyp735a* transgenic plants showed significant decrease in tZ, tZR, cZ, and cZR levels and significant increase in iP and iPR levels. The mutant plants also showed severely retarded growth and were

approximately a quarter the height of the wild-type plants. These results will be useful to conduct more detailed and elaborate functional studies of cytokinin metabolic genes and improving agronomic traits of *Jatropha* by genetic engineering of cytokinin metabolism [68].

5.5 *Soybean* (Glycine max)

Soybean oil is used extensively in the food industry for cooking, baking, and frying, but it is also utilized for biodiesel production and industrial applications. Oleic acid present in soybean seed oil is monounsaturated, and linoleic and linoleinic acids are polyunsaturated fatty acids. Presence of higher ratio of polyunsaturated fatty acids is known to be harmful and can lead to unstability of soybean oil. Do et al. [69] using CRISPR/Cas9 system created mutations in the Fatty Acid Desaturase 2 (*GmFAD2*) gene in soybean. The enzyme helps in the conversion of oleic acid to linoleic acid. The fatty acid profile of the CRISPR-edited plants showed a dramatic increase in oleic acid to over 80% and a decrease in linoleic acid to 1.3–1.7%. A similar study was carried out by Amin et al. [70] where they reported that CRISPR-edited plants carrying mutations in the *FAD2* gene showed a significant increase in oleic acid to 65% and a drop in linoleic acid to 16%. Increase in the monounsaturated fatty acid component in seeds improves stability of soybean oil quality. For biodiesel production unsaturated fatty acid are desirable since they have good temperature properties and helps in maintaining the oil in liquid form. But monounsaturated is preferred over polyunsaturated since too much polyunsaturated fatty acids can form polymers under heat which can block the fuel system of a vehicle. Hence reducing linoleic and linolenic acids and increasing oleic acid will be useful in biodiesel production in soybean [71].

Rj4 is a dominant gene in soybeans (*Glycine max*) that restricts nodulation by many strains of *Bradyrhizobium elkanii* and *Bradyrhizobium japonicum*. *B. elkanii* is a weak symbiotic partner of soybeans because of its low nitrogen-fixation efficiency. Further, the strains also produce a compound called rhizobitoxine which induces chlorosis in the host plant. Thus cultivars of soybean harboring the *Rj4* allele are desirable for growing in areas where the *B. elkanii* is dominant, and the *Rj4* gene will restrict the nodulation. Hence to understand the molecular basis of the *Rj4*-mediated nodulation restriction for improvement of soybean cultivars, Tang et al. [72] mapped the *Rj4* locus on soybean chromosome 1 and also validated the gene using complementation tests and CRISPR/Cas9-based gene knockout experiments. Another plant pathogen called *Phytophthora sojae*, which is an oomycete pathogen, causes "damping off" of soybean seedlings as well as stem and root rot of the plants. This causes a huge damage to the soybean crop. To address this issue, Fang and Tyler [73] used CRISPR to disrupt the pathogen virulence gene (Avr4/6) in *Phytophthora sojae*. They also

validated the contribution of *Avr4/6* to recognition by soybean *R* gene loci, *Rps*4 and *Rps*6. These results provide new pathways for better control of this pathogen.

Flowering has considerable effects on the life cycle of soybean, which is an indication of the transition from vegetative growth to reproductive growth. Hence to understand the functions and molecular mechanisms of photoperiod responses, Cai et al. [74] created CRISPR knockout of the soybean flowering time gene, *GmFT2*, which was stably heritable in the subsequent T2 generation, with homozygous *GmFT2a* mutants exhibiting late flowering under both long-day and short-day conditions. Such studies will help in improving soybean breeding. Plant architecture influences grain yield in soybean, but the knowledge on understanding plant architecture to increase grain yield is very limited. The parameters that are related to high-yield cultivars are stem growth habit, node number, plant height, internode length, branch number, leaf size, and shape. In order to have more understanding on alteration of plant architecture for higher yield, Bao et al. [75] using CRISPR/Cas9 system created mutants of four genes (*GmSPL9a*, *GmSPL9b*, *GmSPL9c*, and *GmSPL9d*) encoding SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors of the SPL9 family in soybean. In T4 generation, higher-order mutant plants carrying various combinations of mutations showed increased node number on the main stem, and branch number consequently increased total node number per plants at different levels. These results provide more knowledge on the regulation of plant architecture that can be useful in future to improve yield in soybean [75].

6 Use of CRISPR Gene Editing Tool in Cyanobacteria and Microalgae

Microalgae and cyanobacteria have emerged as the third-generation biofuel feedstock and production of other commercially important biomolecules, and thus genetic engineering of these organisms is gaining rapid attention, and efforts have been channelized into using genetic engineering techniques. However, gene targeting has been difficult in the past for these organisms since they are not recombinogenic. With the advent of newly developed CRISPR system which uses nucleases, it has been possible to introduce mutations and breaks in the gene of interest [76]. Recently the CRISPR system has been successfully validated and tested in different strains of cyanobacteria such as *Synechococcus elongatus* UTEX 2973, *Synechocystis* 6803, and *Anabaena* 7120 [77, 78] and in different species of microalgae such as *Chlamydomonas reinhardtii*, *P. tricornutum*, and *Nannochloropsis oceanic* [79–82]. List of CRISPR studies conducted in microalgae and cyanobacteria is given in Table 3. The abovementioned species and strains

Table 3
List of CRISPR studies conducted in cyanobacteria and microalgae *(after* [76])

Sr. No.	Organism	Gene	Aim	References
	Cyanobacteria			
1.	*Synechococcus elongatus* UTEX 2973	*nblA*	Genome editing using CRISPR/Cas9-based	[77]
2.	*Synechococcus elongatus* PCC 7942	*glgc*/Gm^R gene	Increase of succinate production	[83]
3.	*Synechococcus* UTEX 2973	*psbA1*, *nblA*	Genome editing using CRISPR/Cpf1-based nucleases	[78]
4.	*Synechocystis* 6803 *Anabaena* 7120	*nblA*, *nifH*	Genome editing using CRISPR/Cpf1-based nucleases	[78]
	Microalgae			
1.	*C. reinhardtii* CC503	*FKB12* gene	First application of CRISPR/Cas9 in microalgae	[79]
2.	*P. tricornutum* CCMP2561	CpSRP54 gene/*Shble*	First application of CRISPR/Cas9 in diatoms	[80]
3.	*C. reinhardtii* CC-124	*MAA7*, *CpSRP43* and *ChlM*	Targeted gene knockout and knock-in via NHEJ in *Chlamydomonas*	[81]
4.	*C. reinhardtii* CC-4349	*ZEP* and *CpFTSY*	Production of two-gene knockout mutant leading to improved photosynthetic productivity	[86]
5.	*C. reinhardtii* CC-400	*PEPC1* and *RFP*	To enhance production of lipids in *Chlamydomonas*	[85]
6.	*N. oceanica* IMET1	Nitrate reductase gene/*HygR*	Targeted gene knockout in *Nannochloropsis*	[82]
7.	*N. gaditana* CCMP1894	ZnCys TF BSD	Doubled lipid production by knockout and attenuation of ZnCys in *Nannochloropsis*	[84]

are industrially important ones used for biofuel production. Hence CRISPR can help us in producing superior industrial species and strains for better and faster biofuel production.

Apart from just validating, there are a few studies conducted in cyanobacteria and microalgae where using CRISPR system a useful trait has been targeted for better understanding and improvement of the strain. Li et al. [83] explored the possibility of engineering cyanobacteria *S. elongatus* PCC 7942 by CRISPR/Cas9-assisted simultaneous *glgc* knockout and *gltA/ppc* knock-in, which

increased the succinate production by 11-fold as compared to wild-type cells. This result shows that the manipulation and understanding of metabolic pathways in cyanobacteria can be easily studied for improving the strain. Ajjawi et al. [84] identified 20 transcription factors as putative negative regulators of lipid production by using RNA-seq analysis of *Nannochlorposis gaditana* during nitrogen deprivation. Further using the CRISPR system, they created mutagenesis of 18 out of the 20 transcription factors. Knocking out of a homolog of fungal Zn (II) $_{2Cys6}$ encoding gene can lead to the conversion of carbon into lipids. The lipid production increased from 20% to 40–55% without affecting the growth conditions [84]. A similar study was also carried out by Kao et al. [85] where the researchers have successfully reported the increase in lipid production in microalgae, *C. reinhardtii*. The gene phosphoenol pyruvate carboxylase (*CrPEPC1*) encodes proteins that are essential for controlling the carbon flux that enters the TCA cycle and plays a crucial role in carbon partitioning of substrates in competition with lipid synthesis. Using CRISPR system, the *CrPEPC1* gene was downregulated in *C. reinhardtii*. The modified strains showed lower chlorophyll color but increased biomass concentration and lipid accumulation rate [85]. Microalgae are versatile organisms capable of converting CO_2, H_2O, and sunlight into fuel and other value added commercially important chemicals for domestic and industrial consumption. Thus improving the photosynthetic productivity and biomass is very important for industrial applications. To address this issue, Baek et al. [86] using CRISPR/Cas9 system created *CpFTSϒ* (signal recognition particle receptor protein, chloroplast) and *ZEP* (zeaxanthin epoxidase) a double gene knockout in *C. reinhardtii* and the resultant double mutant strain produced zeaxanthin and showed improved photosynthetic productivity. The truncated antenna size of the double mutant showed a higher productivity per chlorophyll (Chl) in high light (HL) conditions. Furthermore, growth of the double mutant line under HL conditions was dramatically better than that of the wild type and the single gene *ZEP* knockout mutant. This shows the double mutant strain could lead to higher biomass accumulation under HL growth conditions with greater photosynthetic activity [86]. Such studies in cyanobacteria and microalgae will expand the way to improve the yield, titer, and productivity of biofuels from these organisms.

7 Conclusion

If biofuels have to be sustainable, one has to keep in mind the three Es, viz., economy, energy, and environment. Large-scale operations—be it cultivation, manufacturing, and refinery—have to be sustainable. National policy-makers have to consider conservation

of forest (or land) and carbon generation while framing policies for large-scale cultivation. Alternate measures such as promotion of use of secondary agriculture waste and perennial grasses as feedstock play vital role in making biofuels a sustainable option. Switch to biofuel from petrochemical energy sources had several technological and socioeconomic complexities. In summary, we have provided an argument in favor of a composite strategy to manage the transition to a sustainable biofuel fuel-based economy. Use of a single policy or model looks inadequate to address all the challenges related to a sustainable biofuel strategy. Third-generation biofuel, with genetic engineering and industrial process, currently has the most attractive long-term prospects to meet sustainable biofuel goals. However, in the interim use of rational allocation of land use for second-generation biofuel crops is a key element to drive biofuel adoption and meet current demand. We strongly recommend the use of genomic technology to accelerate crop development of second-generation biofuel sources, while technical hurdles that resolve microalgal production of biofuels and extraction is established. Second-generation biofuel crops such as *Jatropha*, *Pongamia*, and Castors can be enhanced for field performance and genetic gain for oil production. Genomic tools such as gene editing, molecular markers, and molecular breeding should be a key parallel focus to develop plant-based biofuel crops. Given the heavy negative environmental and socioeconomic cost associated with competing demand of first-generation biofuel sources, a strong policy push to dis-incentivize the use of food crops for biofuels should be considered.

Overall, we recommend a phased transition from first-generation biofuel, and an acceleration toward use of technology to drive adoption of second-generation biofuels. Key aspects of technology and application of resource management models will enable these crops to bridge the global energy demand, before we can completely transition to a more sustainable microalgae-based biofuel economy.

References

1. Ndimba BK, Ndimba RJ, Johnson TS, Waditee-Sirisattha R, Baba M, Sirisattha S et al (2013) Biofuels as a sustainable energy source: an update of the applications of proteomics in bioenergy crops and algae. J Proteomics 93:234–244
2. Jouzani SG, Sharafi R, Soheilivand S (2018) Fueling the future; plant genetic engineering for sustainable biodiesel production. Biofuel Res J 5:829–845
3. Hegde K, Chandra N, Sarma SJ, Brar SK, Veeranki VD (2015) Genetic engineering strategies for enhanced biodiesel production. Mol Biotechnol 57:606–624
4. Sustainable biofuels: prospects and challenges, Royal Society, Policy Document 01/08, January 2008
5. Keneni YG, Marchetti JM (2017) Oil extraction from plant seeds for biodiesel production. AIMS Energy 5(2):316–340
6. Gasparatos A, Stromberg P, Takeuchi K (2013) Sustainability impacts of first-generation biofuels. Animal Frontiers 3:12–26

7. Mukherjee P, Varshney A, Johnson TS, Jha TB (2011) *Jatropha curcas*: a review on biotechnological status and challenges. Plant Biotechnol Rep 5:197. https://doi.org/10.1007/s11816-011-0175-2
8. Eswaran N, Johnson TS (2012) Approaches to genetic engineering of *Jatropha curcas* for fatty acid synthesis and abiotic stress tolerance. In: Tuteja N, Gill SS, Tubercio AF, Tuteja R (eds) Improving crop resistance to abiotic stress, vol 2. Wiley-Blackwell, Wiley-VCH Verlag GmbH & Co., Germany, pp 1285–1300. (Chapter 49)
9. Khan S, Siddique R, Sajjad W, Nabi G, Hayat KM, Duan P, Yao L (2017) Biodisel production from algae to overcome the energy crisis. Hayyati J Biosci 24:163–167
10. Stromberg PM, Gasparatos A, Lee JSH, Garcia-Ulloa J, Koh LP, Takeuchi K (2010) Impacts of liquid biofuels on ecosystem services and biodiversity. Institute of Advanced Studies—United Nations University, Japan. UNU-IAS Policy Report
11. Hein L, Leemans R (2012) The impact of first-generation biofuels on the depletion of the global phosphorus reserve. AMBIO 41:341–349. https://doi.org/10.1007/s13280-012-0253-x
12. Elbehri A, Segerstedt A, Liu P (2013) Biofuels and the sustainability challenge: a global assessment of sustainability issues, trends and policies for biofuels and related feedstocks. Food And Agriculture Organization of The United Nations, Rome
13. Lee H, Clark WC, Devereaux C (2008) Biofuels and sustainable development, report of an executive session on the grand challenges of a sustainability transition" San Servolo Island, Venice, Italy: May 19–20, 2008. CID Working Paper No. 174. Joint Center for International Development and Belfer Center for Science and International Affairs Working Paper, Cambridge, MA: Harvard University, July 2008
14. Masjuki H, Kalam A (2013) An overview of biofuel as a renewable energy source: development and challenges. Procedia Eng 56:39–53
15. Viesturs D, Melece L (2014) Advantages and disadvantages of biofuels: observations in Latvia. Eng Rural Dev 13:210–215
16. Soto I, Ellison C, Kenis M, Diaz B, Muys B, Mathijs E (2018) Why do farmers abandon Jatropha cultivation? The case of Chiapas, Mexico. Energy Sustain Dev 42:77–86. https://doi.org/10.1016/j.esd.2017.10.004
17. Hess P, Johnston M, Brown-Steiner B, Holloway T, Andrade JB, Artaxo P (2009) Air quality issues associated with biofuel production and use. In: Howarth RW, Bringezu S (eds) Biofuels: environmental consequences and interactions with changing land use. Proceedings of the Scientific Committee on Problems of the Environment (SCOPE) International Biofuels Project Rapid Assessment, 22–25 September 2008, pp. 169–194
18. Raqeeb MA, Bhargavi R (2015) Biodiesel production from waste cooking oil. J Chem Pharm Res 7(12):670–681
19. Alam F, Mobin S, Chowdhury H (2015) Third generation biofuel from algae. Procedia Eng 105:763–768
20. Mohammed NI, Kabbashi N, Alade A (2018) Significance of agricultural residues in sustainable biofuel development. In: Aladjadjiyan A (ed) Agricultural waste and residues. IntechOpen. https://doi.org/10.5772/intechopen.78374
21. Bankovic-Ilic IB, Stamenkovic OS, Veljkovic VB (2012) Biodiesel production from non-edible plant oils. Renew Sust Energy Rev 16:3621–3647
22. Davidson S (2008) Sustainable bioenergy: Genomics and biofuels development. Nature Education 1(1):175
23. Curran MA (2013) Life cycle assessment: a review of the methodology and its application to sustainability. Curr Opin Chem Eng 2:1–5
24. Aoun WB, Gabrielle N, Gagnepain B (2013) The importance of land use change in the environmental balance of biofuels. Oilseeds Fats Crops Lipids 20:1–12. https://doi.org/10.1051/ocl/2013027
25. Jingura RM, Kamusoko R (2016) Evaluation of life-cycle assessment of Jatropha biodiesel. Energy Sources, Part B: Economics, Planning, and Policy 11(5):396–403. https://doi.org/10.1080/15567249.2011.637546
26. Barnabè D, Bucchi R, Rispoli A, Chiavetta C, Porta PL, Bianchi CL, Pirola C, Boffito DC, Carvoli G (2013) Land use change impacts of biofuels: a methodology to evaluate biofuel sustainability. In: Zhen Fang (ed) Biofuels—economy, environment and sustainability, IntechOpen. doi: https://doi.org/10.5772/52255
27. Tester M, Langridge P (2010) Breeding technologies to increase crop production in a changing world. Science 327:818–822. https://doi.org/10.1126/science.1183700
28. Allwright MR, Taylor G (2016) Molecular breeding for improved second generation bioenergy crops. Trends Plant Sci 21:43–54
29. Levy SE, Myers RM (2016) Advancements in next generation sequencing. Annu Rev Genomics Hum Genet 17:95–115

30. Rosado TB, Laviola BG, Faria DA, Pappas MR, Bhering LL, Quirino B, Grattapaglia D (2010) Molecular markers reveal limited genetic diversity in a large germ plasm collection of the biofuel crop *Jatropha curcas* L. in Brazil. Crop Sci 50:2372–2382
31. Chouvarine P, Cooksey AM, McCarthy FM, Ray DA, Baldwin BS, Burgess SC, Peterson DG (2012) Transcriptome-based differentiation of closely-related *Miscanthus* lines. PLoS One 7:e29850. https://doi.org/10.1371/journal.pone.0029850
32. Lee M, Xia JH, Zou Z, Ye J, Rahmadsyah AY, Jin J et al (2015) A consensus linkage map of oil palm and a major QTL for stem height. Sci Rep 5:8232. https://doi.org/10.1038/srep08232
33. Eswaran N, Parameswaran S, Anantharaman B, Kumar GRK, Sathram B, Johnson TS (2012) Generation of an expressed sequence tag (EST) library from salt-stressed roots of *Jatropha curcas* for identification of abiotic stress-responsive genes. Plant Bio 14:428–437
34. Singh R, Low E-TL, Ooi LC-L, Ong-Abdullah M, Ting N-C, Nagappan J et al (2013) The oil palm SHELL gene controls oil yield and encodes a homologue of SEEDSTICK. Nature 500:340–344. https://doi.org/10.1038/nature12356
35. Jin J, Sun Y, Qu J, Syah R, Lim C-H, Alfiko Y et al (2017) Transcriptome and functional analysis reveals hybrid vigor for oil biosynthesis in oil palm. Nature Sci Rep 7(1):439
36. Bourgis F, Kilaru A, Cao X, Ngando-Ebongue GF, Drira N, Ohlrogge JB, Arondel V (2011) Comparative transcriptome and metabolite analysis of oil palm and date palm mesocarp that differ dramatically in carbon partitioning. Proc Natl Acad Sci U S A 108:12527–12532
37. Sreeharsha RV, Mudalkar S, Singha KT, Reddy AR (2016) Unravelling molecular mechanisms from floral initiation to lipid biosynthesis in a promising biofuel tree species, *Pongamia pinnata* using transcriptome analysis. Sci Rep 6:34315
38. Kah-Yung BL, Yee-Han C, Abdullah WMANW, Swee-Hua EL, Kok-Song L (2018) The CRISPR/Cas9 system for crop improvement: progress and prospects. In: Çiftçi YÖ (ed) Next generation plant breeding. IntechOpen. https://doi.org/10.5772/intechopen.75024
39. Jaganathan D, Ramasamy K, Sellamuthu G, Jayabalan S, Venkataraman G (2018) CRISPR for crop improvement: an update review. Front Plant Sci 9:985. https://doi.org/10.3389/fpls.2018.00985
40. Arora L, Narula A (2017) Gene editing and crop improvement using CRISPR-Cas9 system. Front Plant Sci 8:1932. https://doi.org/10.3389/fpls.2017.01932
41. Cai Y, Chen L, Liu X, Sun S, Wu C, Jiang B et al (2015) CRISPR/Cas9-Mediated genome editing in soybean hairy roots. PLoS ONE 10 (8):e0136064. https://doi.org/10.1371/journal.pone.0136064
42. Li Z, Liu Z, Xing A, Moon BP, Koellhoffer JP, Huang L (2015) Cas9-guide RNA directed genome editing in soybean. Plant Physiol 169:960–970. https://doi.org/10.1104/pp.15.00783
43. Jacobs TB, LaFayette PR, Schmitz RJ, Parrott WA (2015) Targeted genome modifications in soybean with CRISPR/Cas9. BMC Biotechnol 15:16
44. Cai Y, Chen L, Sun S, Wu C, Yao W, Jiang B, Han T, Hou W (2018) CRISPR/Cas9-mediated deletion of large genomic fragments in soybean. Int J Mol Sci. 19(12):3835
45. Svitashev S, Young JK, Schwartz C, Gao H, Falco SC, Cigan AM (2016) Targeted mutagenesis, precise gene editing, and site-specific gene insertion in maize using Cas9 and guide RNA. Plant Physiol 169:931–945. https://doi.org/10.1104/pp.15.00793
46. Qi W, Zhu T, Tian Z, Li C, Zhang W, Song R (2016) High-efficiency CRISPR/Cas9 multiplex gene editing using the glycine tRNA-processing system-based strategy in maize. BMC Biotechnol 16:58. https://doi.org/10.1186/s12896-016-0289-2
47. Zhu J, Song N, Sun S, Yang W, Zhao H, Song W et al (2016) Efficiency and inheritance of targeted mutagenesis in maize using CRISPR-Cas9. J Genet Genomics 43:25–36. https://doi.org/10.1016/j.jgg.2015.10.006
48. Feng C, Yuan J, Wang R, Liu Y, Birchler JA, Han F (2016) Efficient targeted genome modification in maize using CRISPR/Cas9 system. J Genet Genomics 43(1):37–43
49. Janga MR, Campbell LM, Rathore KS (2017) CRISPR/Cas9-mediated targeted mutagenesis in upland cotton (*Gossypium hirsutum* L.). Plant Mol Biol 94:349–360. https://doi.org/10.1007/s11103-017-0599-3
50. Gao W, Long L, Tian X, Xu F, Liu J, Singh PK, Botella JR, Song C (2017) Genome editing in cotton with the CRISPR/Cas9 system. Front. Plant Sci 8:1364. https://doi.org/10.3389/fpls.2017.01364
51. Chen X, Lu X, Shu N, Wang S, Wang J, Wang D, Guo L, Ye W (2017) Targeted mutagenesis in cotton (*Gossypium hirsutum* L.)

using the CRISPR/Cas9 system. Sci Rep 7:44304. https://doi.org/10.1038/srep44304

52. Li C, Unver T, Zhang B (2017) A high-efficiency CRISPR/Cas9 system for targeted mutagenesis in Cotton (*Gossypium hirsutum* L.). Sci Rep 7:43902. https://doi.org/10.1038/srep43902
53. Maghuly F, Laimer M, Freudhofmaier M, Parrott W (2018) Establishment of CRISPR/CAS9-mediated gene editing approaches in *Jatropha curcas*. In: Paper presented at the FAO/IAEA International Symposium on Plant Mutation Breeding and Biotechnology; Vienna (Austria), 27–31 August, 2018
54. Yang H, Wu JJ, Tang T, Liu KD, Dai C (2017) CRISPR/Cas9-mediated genome editing efficiently creates specific mutations at multiple loci using one sgRNA in *Brassica napus*. Sci Rep 7:1–13. https://doi.org/10.1038/s41598-017-07871-9
55. Li C, Hao M, Wang W, Wang H, Chen F, Chu W, Zhang B, Mei D, Cheng H, Hu Q (2018) An efficient CRISPR/Cas9 platform for rapidly generating simultaneous mutagenesis of multiple gene homoeologs in allotetraploid oilseed rape. Front Plant Sci 9:442
56. Budiani A, Putranto RA, Riyadi I, Sumaryono, Minarsih H, Faizah R (2018) IOP Conf Ser: Earth Environ Sci 183:012003
57. Lawrenson T, Harwood WA (2019) Creating targeted gene knockouts in barley using CRISPR/Cas9 in Barley. In: Harwood WA (ed) Methods and protocols. Springer, New York, pp 217–232
58. Ricroch A, Clairand P, Harwood W (2017) Use of CRISPR systems in plant genome editing: toward new opportunities in agriculture. Emerg Top Life Sci 1:169–182. https://doi.org/10.1042/etls20170085
59. Shi J, Gao H, Wang H, Lafitte HR, Archibald RL, Yang M et al (2017) ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield underfield drought stress conditions. Plant Biotechnol J 16:299–311. https://doi.org/10.1111/pbi.12603
60. Shi J, Habben JE, Archibald RL, Drummond BJ, Chamberlin MA, Williams RW, Lafitte HR et al (2015) Overexpression of ARGOS genes modifies plant sensitivity to ethylene, leading to improved drought tolerance in both Arabidopsis and maize. Plant Physiol 169:266–282
61. Liang Z, Zhang K, Chen K, Gao C (2014) Targeted mutagenesis in *Zea mays* using TALENs and the CRISPR/Cas system. J Genet Genomics 41:63–68. https://doi.org/10.1016/j.jgg.2013.12.001
62. Zhang Z, Ge X, Luo X, Wang P, Fan Q, Hu G et al (2018) Simultaneous editing of two copies of Gh14-3-3d confers enhanced transgene-clean plantdefense against *Verticillium dahliae* in allotetraploid upland cotton. Front Plant Sci:842. https://doi.org/10.3389/fpls.2018.00842
63. Iqbal Z, Sattar MN, Shafiq M (2016) CRISPR/Cas9: a tool to circumscribe cotton leaf curl disease. Front Plant Sci 7:475. https://doi.org/10.3389/fpls.2016.00475
64. Jiang WZ, Henry IM, Lynagh PG, Comai L, Cahoon EB, Weeks DP (2017) Significant enhancement of fatty acid composition in seeds of the allohexaploid, *Camelina sativa*, using CRISPR/Cas9 gene editing. Plant Biotechnol J 15:648–657. https://doi.org/10.1111/pbi.12663
65. Morineau C, Bellec Y, Tellier F, Gissot L, Kelemen Z, Nogue F, Faure JD (2017) Selective gene dosage by CRISPR-Cas9 genome editing in hexaploid *Camelina sativa*. Plant Biotechnol J 15:729–739. https://doi.org/10.1111/pbi.12671
66. Aznar-Moreno JA, Durrett TP (2017) Simultaneous targeting of multiple gene homeologs to alter seed oil production in *Camelina sativa*. Plant Cell Physiol 58:1260–1267. https://doi.org/10.1093/pcp/pcx058
67. Lyzenga WJ, Harrington M, Bekkaoui D, Wigness M, Hegedus DD, Rozwadowski KL (2019) CRISPR/Cas9 editing of three CRUCIFERIN C homoeologues alters the seed protein profile in *Camelina sativa*. BMC Plant Biol 19(1):292. https://doi.org/10.1186/s12870-019-1873-0
68. Cai L, Zhang L, Fu Q et al (2018) Identification and expression analysis of cytokinin metabolic genes IPTs, CYP735A and CKXs in the biofuel plant *Jatropha curcas*. Peer J 6:4812. https://doi.org/10.7717/peerj.4812
69. Do PT, Nguyen CX, Bui HT, Tran LTN, Stacey G, Gillman JD, Zhang ZJ, Stacey MG (2019) Demonstration of highly efficient dual gRNA CRISPR/Cas9 editing of the homeologous *GmFAD2-1A* and *GmFAD2-1B* genes to yield a high oleic, low linoleic and α-linolenic acid phenotype in soybean. BMC Plant Biol. 19(1):311. https://doi.org/10.1186/s12870-019-1906-8
70. Amin N, Ahmad N, Wu N, Pu X, Ma T, Du Y, Bo X, Wang N, Sharif R, Wang P (2019) CRISPR-Cas9 mediated targeted disruption of *FAD2–2* microsomal omega-6 desaturase in soybean (*Glycine max.L*). BMC Biotechnol 19:9

71. Islam AKMA, Primandari SRP, Yaakob Z (2018) Non-edible vegetable oils as renewable resources for biodiesel production: South-East Asia perspective. In: Nageswara-Rao M, Soneji JR (eds) Advances in biofuels and bioenergy. IntechOpen. https://doi.org/10.5772/intechopen.73304
72. Tang F, Yang S, Liu J, Zhu H (2016) Rj4, a gene controlling nodulation specificity in soybeans, encodes a thaumatin-like protein but not the one previously reported. Plant Physiol 170:26–32. https://doi.org/10.1104/pp.15.01661
73. Fang Y, Tyler BM (2016) Efficient disruption and replacement of an effector gene in the Oomycete *Phytophthora sojae* using CRISPR/Cas9. Mol Plant Pathol 17:127–139. https://doi.org/10.1111/mpp.12318
74. Cai Y, Chen L, Liu X, Guo C, Sun S, Wu C et al (2018b) CRISPR/Cas9-mediated targeted mutagenesis of *GmFT2a* delays flowering time in soybean. Plant Biotechnol J 16:176–185. https://doi.org/10.1111/pbi.12758
75. Bao A, Chen H, Chen L, Chen S, Hao Q, Guo W, Qiu D, Shan Z, Yang Z, Yuan S et al (2019) CRISPR/Cas9-mediated targeted mutagenesis of *GmSPL9* genes alters plant architecture in soybean. BMC Plant Biol 19:131
76. Jeon S, Lim J, Lee H, Shin S, Kang N, Park YI, Oh H-M, Jeong W-J, Jeong B, Chang YK (2017) Current status and perspectives of genome editing technology for microalgae. Biotechnol Biofuels 10:267
77. Wendt KE, Ungerer J, Cobb RE, Zhao H, Pakrasi HB (2016) CRISPR/Cas9 mediated targeted mutagenesis of the fast growing cyanobacterium *Synechococcus elongatus* UTEX 2973. Microb Cell Fact 15(1):115
78. Ungerer J, Pakrasi HB (2016) *Cpf1* is a versatile tool for CRISPR genome editing across diverse species of cyanobacteria. Sci Rep 6:39681
79. Jiang W, Brueggeman AJ, Horken KM, Plucinak TM, Weeks DP (2014) Successful transient expression of Cas9 and single guide RNA genes in *Chlamydomonas reinhardtii*. Eukaryot Cell 13(11):1465–1469
80. Nymark M, Sharma AK, Sparstad T, Bones AM, Winge PA (2016) CRISPR/Cas9 system adapted for gene editing in marine algae. Sci Rep 6:24951
81. Shin SE, Lim JM, Koh HG, Kim EK, Kang NK, Jeon S, Kwon S, Shin WS, Lee B, Hwangbo K et al (2016) CRISPR/Cas9-induced knockout and knock-in mutations in *Chlamydomonas reinhardtii*. Sci Rep 6:27810
82. Wang Q, Lu Y, Xin Y, Wei L, Huang S, Xu J (2016) Genome editing of model oleaginous microalgae *Nannochloropsis* spp. by CRISPR/Cas9. Plant J 88(6):1071–1081
83. Li H, Shen CR, Huang CH, Sung LY, Wu MY, Hu YC (2016) CRISPR–Cas9 for the genome engineering of cyanobacteria and succinate production. Metab Eng 38:293–302
84. Ajjawi I, Verruto J, Aqui M, Soriaga LB, Coppersmith J, Kwok K, Peach L, Orchard E, Kalb R, Xu W et al (2017) Lipid production in *Nannochloropsis gaditana* is doubled by decreasing expression of a single transcriptional regulator. Nat Biotechnol 35(7):647–652
85. Kao PH, Ng IS (2017) CRISPRi mediated phosphoenolpyruvate carboxylase regulation to enhance the production of lipid in *Chlamydomonas reinhardtii*. Bioresour Technol 245 (Pt B):1527–1537
86. Baek K, Kim DH, Jeong J, Sim SJ, Melis A, Kim JS, Jin E, Bae S (2016) DNA-free two--gene knockout in *Chlamydomonas reinhardtii* via CRISPR–Cas9 ribonucleoproteins. Sci Rep 6:30620
87. Du H, Zeng X, Zhao M, Cui X, Wang Q, Yang H et al (2016) Efficient targeted mutagenesis in soybean by TALENs and CRISPR/Cas9. J Biotechnol 217:90–97. https://doi.org/10.1016/j.jbiotec.2015.11.005

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