



Use of Fibrous Feedstock for Fungal Cellulase Production

¹Raji, A. I. and ²Balogun, M. A.

¹Department of Biological Sciences, University of Ilorin, Ilorin, Nigeria.

²Department of Food Technology, Federal Polytechnic, Offa, Kwara State, Nigeria

ABSTRACT: Two fungal species (*Aspergillus niger* CS9 and *Penicillium digitatum* YF50) with cellulase producing ability were isolated from compost soil and yam flour respectively. The optimization of cultural conditions for the production of fungal cellulase by these organisms using readily available fibrous feedstock (corn cob and sugar cane fibre) was carried out. An optimum pH of 4.0 and temperature of 30°C was observed for corn cob medium and pH 3.5 and 35°C was observed for sugar cane fibre medium. Groundnut cake (1%) supported better cellulase production than the other nitrogen sources tested. The result of this study shows the potential of these fibrous wastes as substrate for cellulase production.

KEY WORDS: Corn cob, sugar cane, cellulase.

*Corresponding Author.

INTRODUCTION

Corn cob and sugar cane fibre are agricultural wastes obtained from corn and sugar cane. An increase in the production of these agricultural crops led to an increase in the generation of their waste, which is usually not properly disposed, thus leading to their pollution of the environment (Bukoye, 2001). Bioconversion, particularly enzymatic hydrolysis of these wastes to useful products has proved to be of great potential. However, the problem encountered is that lignocellulosic materials (of which these wastes belong to) are composed of resistant crystalline structured lignin, which forms a physical barrier and limits the sites available for enzymatic attack (Katzen, *et al.*, 1995). Hence, these materials have to be pretreated for easy conversion to glucose (Layokun, *et al.*, 1990). Such pretreatments open up the cellulose structure and remove interaction between glucose chains (Kleman-Leyer, *et al.*, 1996).

Cellulose, which is the principal constituent of the cell wall of most terrestrial plants is an unbranched, linear homopolymer of up to 14,000 glucose units linked by β -1, 4 glucosidic bonds, that forms rigid fibres through intra- and inter- molecular hydrogen bonds (Leuschner and Antranikian, 1995). The length of a cellulose molecule is determined by the number of glucan units in the polymer, expressed as $(C_6H_{10}O_5)_n$ where 'n' is the degree of polymerization (DP) (Katzen, *et al.*, 1995).

Cellulose fibrils, which are bond together by an amorphous matrix, are highly insoluble and inelastic, and because of their molecular configuration, have a tensile strength comparable to that of steel (Yamanaka *et al.*, 1989).

Despite an enormous world wide utilization of natural cellulosic sources, there are still abundant quantities of cellulose containing raw materials or waste products that are not exploited or which could be used more efficiently (Okafor, 1987). The main problem in this respect is the development of processes that are economically profitable.

The conversion of cellulose microfibrils require that cellulolytic microorganisms employ an array of specialized enzymes to convert cellulose to soluble oligomers and ultimately to glucose (Kleman-Leyer, *et al.*, 1996). This explains the fact that a single enzyme does not mediate cellulase activity in vivo rather, it is a complex of several different enzymes which act in concert (Lamed and Bayer, 1988; Gbekeloluwa and Moo-young, 1991). However, with the present knowledge of cellulolytic

enzyme systems, it is still hard to make generalization valid for all type of microorganisms (Leuschner and Antranikian, 1995).

Although cellulase is produced by a number of microorganisms, only a few appear to be good sources for high yield of the enzyme in the culture medium. Fungi such as *Trichoderma*, *Aspergillus* and *Penicillium* species are among the organisms that have been reported to have good cellulolytic activity (Das *et al.*, 1997; Addis *et al.*, 1998), while a few bacteria such as *Clostridium cellulolyticum*, *Sporocytophaga* and *Cytophaga* species also demonstrate cellulolytic activities (Desvaux *et al.*, 2000).

In the light of these, this work was aimed at studying the potentiality of corn cob and sugar cane waste product as substrates for fungal cellulase production using *Aspergillus niger* CS9 and *Penicillium digitatum* YF50 isolated from soil and yam flour respectively. The cultural conditions for the production of this enzyme were also optimized. The success of this therefore, will ensure the conversion of these hitherto waste agricultural residues to useful product.

MATERIALS AND METHODS

Isolation and identification of fungi

One gram of sample (soil or yam flour) was collected into sterile universal bottles containing 99ml of sterile distilled water and shaken thoroughly by hand to dissolve the materials and disperse the clumps. The mixture was allowed to settle and the supernatant decanted. This served as stock solution. Serial dilutions ranging from 10^{-1} to 10^{-6} of the stock was prepared and 0.1ml of each dilution was spread on potato dextrose agar (PDA) plate containing 1% streptomycin (to inhibit bacterial growth) using sterile spreaders. The inoculated plates were incubated at 28°C for 48hr.

After incubation, representative fungal colonies were picked from each plate and purified on sterile PDA plates. Pure isolates were stored on PDA slants in the refrigerator at 4°C prior to use.

Isolated fungal colonies were identified according to the method of McGinnins, (1980) based on their macroscopic and microscopic properties.

Substrate Pretreatment

Corn cob and sugar cane fibres were pretreated using the method of Raji, *et al.* (1998) with some modifications. The substrates were washed to remove dirt and dried in the oven at 70°C to constant weight. The dried corn cob and sugar cane fibres were chopped into smaller pieces which were autoclaved for one hour at 121°C at 15psi with 5% NaOH (20ml/g of substrate). The autoclaved materials were filtered through muslin cloth and neutralized with dilute 0.1M HCl. The treated substrates, which were finally washed with distilled water and dried at 70°C were blended with a domestic blender (PHILIPS BLENDER HR 2815).

Fermentation media

The fermentation media used were Mineral salt sugar cane (MSS) medium containing KH_2PO_4 , 10g; $(\text{NH}_4)_2\text{SO}_4$, 10.5g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3g; CaCl_2 , 0.5g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.013g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.004g; Yeast extract, 0.5g; distilled water, 1litre and 40g of pretreated sugar cane fibre) and Mineral salt corn cob (MSCC) medium containing KH_2PO_4 , 10g; $(\text{NH}_4)_2\text{SO}_4$, 10.5g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3g; CaCl_2 , 0.5g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.013g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.004g; Yeast extract, 0.5g distilled water, 1litre and 4% of pretreated corn cob.

Inoculum Development

The fermentation inoculum was developed by transferring a loopful of the respective organism from the PDA slant into 50ml of glucose-yeast extract medium (yeast extract, 0.05% and glucose 4%). This was incubated at 30°C in an orbital shaker (Gallenkhamp, England) at 100 rev/min for 72 hours.

Substrate inoculation

Five milliliter (5ml) of the developed inoculum of each organism (*Aspergillus niger* CS9 and *Penicillium digitatum* YF50) was transferred into 150ml of each of the fermentation medium (Mineral Salt Sugar cane (MSS) medium and Mineral Salt Corn cob (MSCC) medium).

The inoculated media were incubated at 30°C in an orbital shaker (GALLENKHAMP, ENGLAND) at 100rev/min for 96hrs. The effect of pH, substrate concentration, temperature and nitrogen sources were tested, to optimize conditions for the production of this enzyme.

Determination of optimal conditions for cellulase production

Effect of pH

pH of the fermentation media were adjusted to various values ranging from 2.5 to 8.0 with 0.1N NaOH or 0.1N HCl. The pH was determined using a pH meter (PHILLIPS PW 9418).

Effect of substrate concentration

Different concentrations of pretreated corn cob and sugar cane fibre ranging from 1.0% to 8.0% were used in the fermentation media

Effect of temperature

The fermentation media were incubated at different temperatures ranging from 25°C to 45°C in the incubator.

Effect of nitrogen sources

The fermentation media were supplemented with one percent (1%) organic nitrogen source (groundnut cake, fermented locust bean seed, yeast extract and peptone) and inorganic nitrogen source (urea, ammonium chloride, sodium nitrite and potassium nitrate). The nitrogen sources were added to the medium prior to sterilization.

Assay for Cellulase Activity

Cellulase activity was determined colorimetrically by measuring the increase in reducing group due to the hydrolysis of carboxymethylcellulose (CMC) substrate (Ali *et al.*, 1991). Cultured samples were centrifuged at 13,000rpm for 5 minutes to precipitate the mycelia and the supernatant used as crude enzyme extract. The supernatant (enzyme solution) was diluted with 0.1M phosphate buffer (pH 7.4) and 0.5ml dispensed into test tubes containing 1.0ml of 4.0% (w/v) CMC. The reaction mixture containing 0.5ml of the enzyme solution and 1.0ml of 4% CMC was incubated at 37°C for 30 minutes and the reaction stopped by adding 1.5ml dinitrosalicylic acid reagent (dinitrosalicylic acid 1%; phenol 0.2%; sodium sulphate 0.5% and sodium hydroxide 1%). The mixture was heated in boiling water bath for 15 minutes and then cooled under running tap water at ambient temperature. The absorbance of the resulting orange-coloured solution was read at 650nm using a spectrophotometer (JENWEY 6405 UV/visible).

One unit of cellulase activity was defined as the amount of enzyme that will release 10µg of reducing group as glucose per milliliter of digest in 30 minutes under the specified conditions.

RESULTS

Both Corn cob and sugar cane media supported good cellulase production by both organisms. An optimum corn cob concentration of 4% and 2% was recorded for *A. niger* CS9 and *P. digitatum* YF50 respectively while the highest cellulase activity was recorded with 5% and 3% pretreated sugar cane fibre for *A. niger* CS9 and *P. digitatum* YF50 respectively (Figure 1). An optimum pH of 4.0 was observed for both organisms in corn cob medium and pH 3.5 recorded for both organisms in sugar cane medium (Figure 2). Enzyme production was best at 30°C in corn cob medium and 35°C in sugar cane medium for both organisms (Figure 3).

Groundnut cake (1%) and peptone (1%), which are organic nitrogen sources supported better cellulase

production compared to the inorganic sources generally. Groundnut was the best nitrogen source for *A. niger* CS9 in corn cob and sugar cane media with an activity of 201 units/ml and 124 units/ml respectively. For *P. digitatum* YF50, optimum cellulase yield of 180 units/ml was supported by groundnut cake in corn cob medium while in sugar cane medium peptone supported the highest cellulase yield of 171 units/ml (Table 1).

Generally, corn cob medium supported higher cellulase production than sugar cane medium in all the conditions tested.

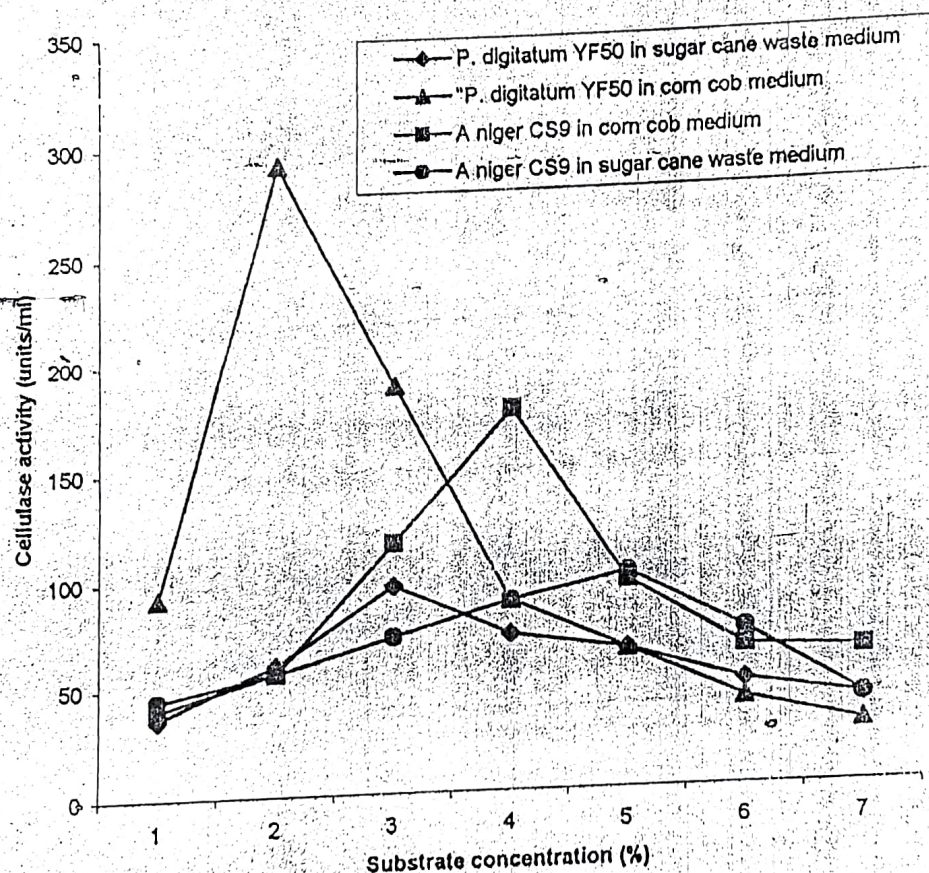


Figure 1. Effect of substrate concentration on cellulase production by *Aspergillus niger* CS9 and *Penicillium digitatum* YF50

DISCUSSION

This study was aimed at determining the potential use of corn cob and waste from sugar cane as possible substrates for fungal cellulase production using *A. niger* CS9 and *P. digitatum* YF50 isolated from the environment.

The increment in the level of cellulase produced by the two organisms, as the substrate concentration increases up to the optimum could be an indication that cellulase production by these organisms is substrate concentration dependent.

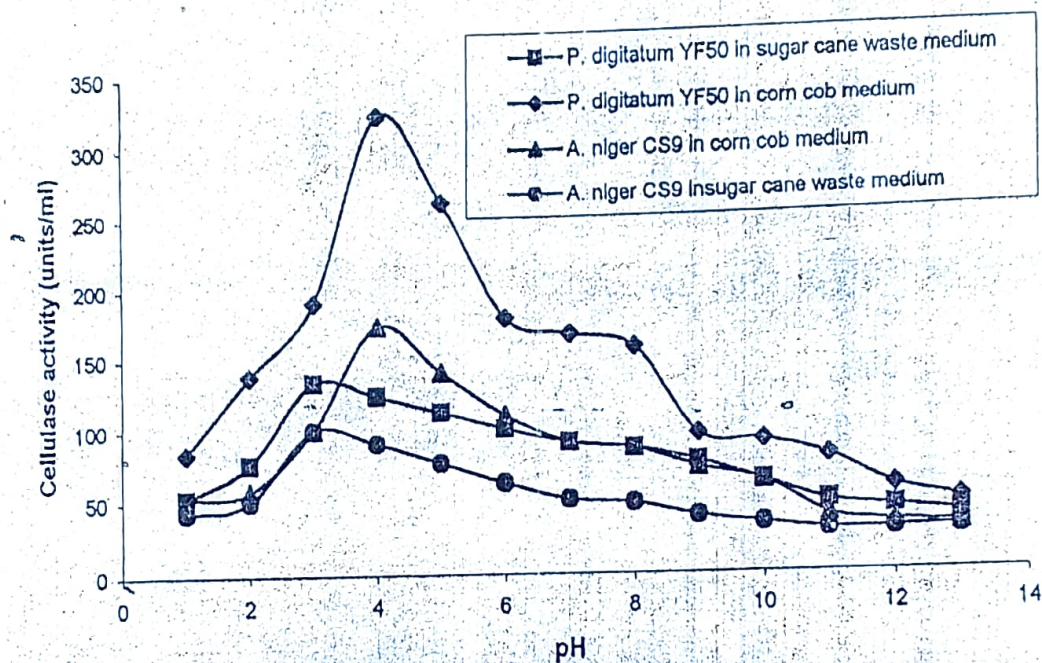


Figure 2. Effect of pH on cellulase production by *Aspergillus niger* CS9 and *Penicillium digitatum* YF50

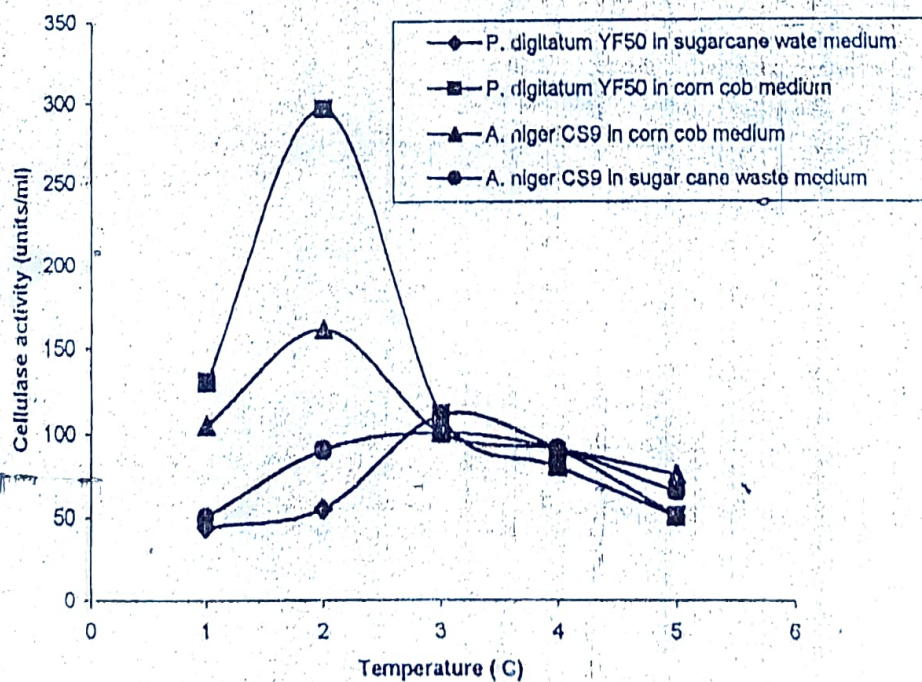


Figure 3. Effect of temperature on cellulase production by *Aspergillus niger* CS9 and *Penicillium digitatum* YF50

The decrease in cellulase production beyond optimum substrate concentration may be due to end product inhibition and/or due to the inhibitory effect of accumulated cellobiose and cellobioses of low degree of polymerization on the growth of the organisms (Gbckeloluwa and Moo-Young, 1991).

The optimal pH for cellulase production by the organisms, which is between 3.5 and 4.0 is in agreement with the reports of other workers (Desphande *et al.*, 1998; Raji and Okeniyi, 1998). The instability of the enzyme at very low or high pH values is because they are proteins, which are generally denatured at the extremes of pH (Steiner *et al.*, 1994).

Table 1. Effect of Nitrogen sources on cellulase production

Nitrogen sources	Cellulose activity (units/ml)			
	<i>Aspergillus niger</i> CS9		<i>Penicillium digitatum</i> YF50	
	MSSC	MSSW	MSSC	MSSW
Groundnut cake (1%)	201	124	180	102
Locust bean seed (1%)	63	41	60	46
Yeast extract (1%)	78	62	65	88
Peptone (1%)	120	98	110	171
Urea (1%)	84	85	46	104
Ammonium chloride (1%)	63.5	61	112	82
Sodium nitrite (1%)	27	37	42	35
Potassium nitrate (1%)	51	42	78	60

MSSC = Mineral salt corn cob medium; MSSW = Mineral salt sugarcane waste medium

The higher yield of the enzyme by groundnut cake (1%) and peptone (1%), which are organic nitrogen sources, as compared to the inorganic sources agrees with the works of Ali *et al.* (1991) and Raji *et al.* (1998). The observation that groundnut cake supported higher cellulase production is important because of the low cost and ready availability of this material which when incorporated into the fermentation medium could lower the cost of enzyme production. However, in an earlier work by Okagbue and Raji (2000), groundnut cake did not support good amylase yield from *Bacillus* species.

The relatively high cellulase activity from corn cob and sugar cane media indicated that they are potential substrates for the production of microbial cellulase. Their utilization as raw materials will definitely reduce the environmental pollution caused by their presence and a possible reduction in the cost of enzyme production.

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