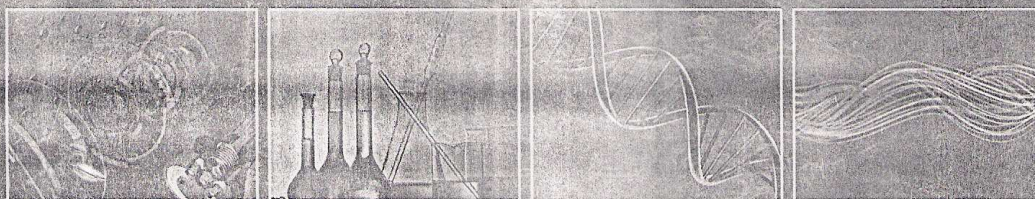




**"BIOTECHNOLOGY AS A TOOL FOR PEACE
AND NATIONAL DEVELOPMENT"**



Proceedings of the
25th Annual
Conference
of
**THE BIOTECHNOLOGY SOCIETY
OF NIGERIA**

Held at The National Open University of Nigeria, Abuja Liaison Office.
NOUN-BSN Abuja 2012
August 26th to 31st, 2012

Edited by
V. B. Ogumela, Momoluwa O. Olaniyi, A. Ogunsipe
and Jane-Frances O. Agbu

BIOENGINEERING

PROCEDURE FOR *IN VITRO* SEED STERILIZATION, GERMINATION AND ASEPTIC SEEDLING ESTABLISHMENT OF *Zehneria capillacea* (Schumacher) C. Jeffrey 94

ACCLIMATIZATION OF *IN VITRO* RESCUED EMBRYOS OF *Hevea brasiliensis* MUEL. ARG. 98

ENHANCING THE EXISTING COFFEE GENETIC RESOURCES IN NIGERIA 103

MOLECULAR MARKERS FOR MAPPING AND TAGGING GENES CONTROLLING STAY-GREEN IN PEARL MILLET 106

IITA BIOSCIENCE CENTER: A PLATFORM FOR AGRI-INNOVATION AND DISCOVERY IN AFRICA 109

GENETIC VARIABILITY OF TILAPIA IN DIFFERENT WATER BODIES USING RAPD MARKERS 116

INVESTIGATION OF PLASMID PROFILE OF *SALMONELLA* SPECIES ISOLATED FROM CLINICAL SOURCES IN PARTS OF EDO STATE, NIGERIA 120

EXPRESSION OF AN ENGINEERED CYSTEINE PROTEINASE INHIBITOR (OC-IDD86) FOR NEMATODE RESISTANCE IN TRANSGENIC PLANTAIN: HYPOTHESIS – MOLECULAR IDENTIFICATION AND MORPHOMETRIC CHARACTERIZATION OF HATCHERY BRED *Clarias batrachus* LIN, *Clarias gariepinus* BUR AND THEIR HYBRIDS 124

NEW INDUSTRIAL AND NUTRITIONAL PRODUCTS SEARCH: THE PLACE OF PROTEOMIC ANALYSIS OF FABACEAE SEEDS 134

COMPARISON OF DNA EXTRACTION FROM FRESH AND FROZEN FISH SAMPLES USING DIFFERENT EXTRACTION PROTOCOLS 140

FOOD AND INDUSTRIAL BIOTECHNOLOGY

COMPARATIVE CULTIVATION OF MUSHROOM (*Pleurotus florida*) ON CORNCOB, WOOD SHRED AND OLD NEWSPAPER BIOMASS PYROLYSIS PROCESSES AND APPLICATIONS 146

✓ AMYLASE PRODUCTION BY *PENICILIUM CHRYSOGENUM* IN A SUBMERGED FERMENTATION OF CASSAVA POWDER 161

EFFECTS OF ALKALINE STEEP-TREATMENTS ON THE MALTING PROPERTIES OF A LOCAL CULTIVAR OF NIGERIA MILLET-MALT (*Pennisetum maiwa*) 168

EFFECTS OF DIFFERENT CONCENTRATIONS OF EXOGENOUS-ALPHA AMYLASE PRODUCED FROM *Bacillus licheniformis* ON THE REDUCING SUGAR CONTENT AND VISCOSITY OF WORTS OBTAINED FROM THREE SORGHUM MALT CULTIVARS 171

DRILLING MUD WASTES MANAGEMENT IN NIGERIAN OIL INDUSTRY: THE ROLE OF

AMYLASE PRODUCTION BY *PENICILLIUM CHRYSOGENUM* IN A SUBMERGED FERMENTATION OF CASSAVA POWDER

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ABSTRACT

Penicillium chrysogenum was grown on pure soluble starch and cassava powder as sole carbon sources in a submerged fermentation. Amylase production of *P. Chrysogenum* in the cultures was assessed. Maximum amylase activity was obtained at the early stationary phase (after 72hrs of fermentation). The organism was found to be substrate specific: higher amylase activities was obtained when the organism was grown in mineral salt medium supplemented with cassava powder than when pure soluble starch was used as the sole carbon source. Amylase activities increased as the cassava powder concentration increased up to 3%. *Penicillial* amylase had optimum activities at temperatures between 50 °C and 60 °C and pH between 5 and 6. The maximum hydrolytic period of the enzyme on cassava powder was 90mins, beyond which there was a repression on amylase activities.

Keywords: *Penicillium chrysogenum*, Cassava powder, Submerged fermentation, *Penicillial* amylase, Optimum activities

INTRODUCTION

The expensive chemical hydrolysis and the ineffective heating method of hydrolysis have necessitated the use of microbial amylase. Amylase-producing microorganisms have been isolated from the soil. They include bacteria such as *Bacillus subtilis* (Uguru et al., 1997); Actinomycetes e.g. *Thermoactinomyces thalophilus* (Uguru et al., 1997) and Fungi including *Aspergillus* species (Sani et al., 1992; *Penicillium* spp (Balkan and Ertan, 2005); *Rhizopus* spp (Ayogu and Amadi, 2010;) and *Saccharomyces cerevisiae* (Ohno et al., 1992).

Fungal amylases are mostly employed in the hydrolytic industries due to their thermostability (Sharma and Shukla, 2008) and their ability to survive in a low-pH environment (Inui et al., 1965;) which is usually a characteristic of most of the cassava sampling sites.

Amylase enzymes have been employed in processes like, digestion of complex polysaccharides such as starch and glycogen into sugars, syrups and dextrin. These hydrolysates are used in a range of manufactured foods and beverages, and as carbon sources in fermentations for the production of alcohol both as a fuel and in the brewing industry (O'Rourke, 1996).

Cassava is largely cultivated in the Tropics (Nigeria being the world largest producer) and characterised by

high postharvest losses. It needs to be processed almost immediately after harvest, as the root is highly perishable and enzymatic processes accelerate deterioration within 1-2 days after harvest (IITA, 2005).

In this study therefore, the production of amylase from *P. chrysogenum* on cassava powder was compared with soluble starch with a view to obtaining a cheaper industrial carbon source for high amylase yield.

Experimental Sample Treatment

Tubers of newly harvested cassava (*Mannihot esculenta*) were obtained from Concordial Global Alliance (COGA) Farms, Bode Sadu Ilorin. 5 kg of the cassava tubers were peeled and washed in clean water. These were cut into chips and oven-dried at 90 °C for 1 hr. The dried chips were milled into fine cassava flour and stored in sterile containers prior to use.

Isolation of fungal amylase producers

Soil samples were collected from cassava processing fields and cassava dumping sites at "Oja titun", Ilorin, Nigeria. One gram of each soil samples, collected as above, was mixed with 10 ml of 0.1% (w/v) peptone water in a test tube by vortex to break clumps. 1 ml of the suspension from the interface of the column of sedimented material and supernatant fluid was serially diluted to 10^3 , and 0.1 ml of the dilution was plated on (BDH). Two percent PenStrept antibiotics were introduced to prevent bacterial growth. The agar plates were incubated at 27 °C for 72 hrs. The primary criterion for the selection of amylase-producing fungal isolates was the formation of clear zone around a fungal colony in a PDA-Starch (2%) medium flooded with iodine solution (0.3% [w/v] I_2 in 3% [w/v] KI). Identification of isolates was made using the scheme of Mc Ginnins (1980).

Production of crude amylase and culture conditions

Amylase synthesis in liquid cultures was studied in 250 ml Erlenmeyer flasks, each containing 50 ml of mineral salt medium with the following composition in (g/l): Na_2HPO_4 (1.0), $MgSO_4 \cdot 7H_2O$ (0.5), KCl (0.5), $FeSO_4 \cdot 7H_2O$ (0.001) $NaNO_3$ (2.0), and Cassava powder-Soluble starch (20-40). The medium pH was adjusted 7.0 before sterilization at 121 °C for 15 mins. Spores of a 48 hr-old pure cultures of the fungal isolates were harvested by a slightly modified method of Akinsoye and Akinyanju (1989) and Sani *et al.*, (1992). Fermentation was carried out at 27 °C $\pm$ 2 at 120 rpm for 5 days. The following variables were monitored daily: pH, starch disappearance and amylase production. The supernatant obtained after sunction filtration through Whatman No. 1 filter paper was employed for enzyme activity.

Assay of Amylase

Amylase activity was assayed according to the method of Bernfeld (1955). The activity of one unit (U) of amylase is defined as that amount of enzyme which liberates 1 μ mol of reducing sugar (with glucose as standard) per min under the assay conditions.

Effect of period of incubation *Penicillium chrysogenum* was cultivated in mineral salt medium for five days during which amylase activity was assayed daily in three substrate concentrations (2%, 3% and 4%). Starch disappearance and pH were also recorded.

Effect of medium composition

The production medium was assayed for amylase activity when supplemented with and without cassava

powder. Two percent (w/v) concentrations of both cassava powder and soluble starch were (respectively) used for the assay of amylase activity on the 4th day of incubation.

Physicochemical properties

Crude amylase from the fermentation medium was employed to determine the effects of substrate concentrations, period, temperature and pH of incubation on amylase activity.

Cassava powder at concentrations 2, 3 and 4% (w/v) were dissolved each in 100 ml of sterile distilled water to make 'Dissolved Cassava Medium'. This was prepared by a slight modification of the method by Uguru *et al.*, (1997). By this method, the required concentration (2-4%) of cassava powder was dissolved in 100 ml of sterile distilled water, and gelatinised at 100 °C. The medium was buffered at pH 6.0 with Na₂HPO₄ and NaH₂PO₄. Crude amylase was added to each reaction mixture in ratio (1:10) and these were incubated for 60 mins at 27 °C. Amylase activity was determined as described.

Specified concentrations of the dissolved cassava medium were hydrolysed with the crude amylase (1 : 10 of the medium). This was left to stand at varying incubation periods: 30, 60, 90 and 120 mins) to determine the maximum hydrolytic period of the crude amylase.

Dissolved cassava medium at 3% concentration was prepared and equilibrated for 10 mins at specified temperature ranges from 30 – 100°C at 10 °C increment intervals. Amylase activity was assayed at each temperature after 30mins of incubation.

For effect of pH, cassava medium at 3% concentrations and equilibrated for 60mins each at specified pH ranges from 3-10 at 1 unit increment were used. Amylase activity was assayed as described.

Experiments were carried out three times and means of data presented.

Results and Discussion

Microbial profiles of soil samples obtained from cassava sampling sites

Bacteria and fungi spp were isolated from the soil at the sampling sites. Three fungal species were characterised and identified as *Rhizopus stolonifer*, *Penicillium chrysogenum* and *Saccharomyces cerevisiae*. The absence of some common known amylase-producing fungi such as *Aspergillus* species suggests that environmental conditions, usually nutrients and water availability affect the number and types of the soil organisms.

Amylase synthesis by the fungal isolates in mineral salt medium supplemented with only soluble starch

The fungal isolates were compared for amylase synthesis in mineral salt medium supplemented with 2% soluble starch. After 4 days of incubation, *Penicillium chrysogenum* produced the highest amylase activity (52.50U), more than *R. stolonifer* 50U (Fig. 1). This was the basis for selection of *Penicillium chrysogenum*.

Effect of incubation duration on amylase synthesis

The effect of the period of incubation on amylase synthesis by *P. chrysogenum* in medium supplemented with different concentrations (2-4%) of soluble starch is shown in Figures 2. The highest amylase activity was recorded on the 4th day of incubation at all medium concentrations, with 2% starch solution giving the highest amylase activity (52.5U).

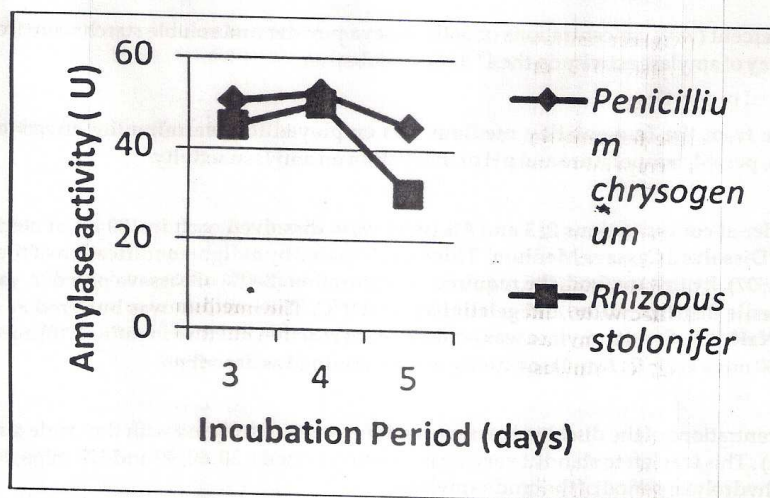


Figure 1. Time course and Amylase synthesis by fungal Isolates in mineral salts medium supplemented with (2%) soluble starch
Note: Data are means of three experiments

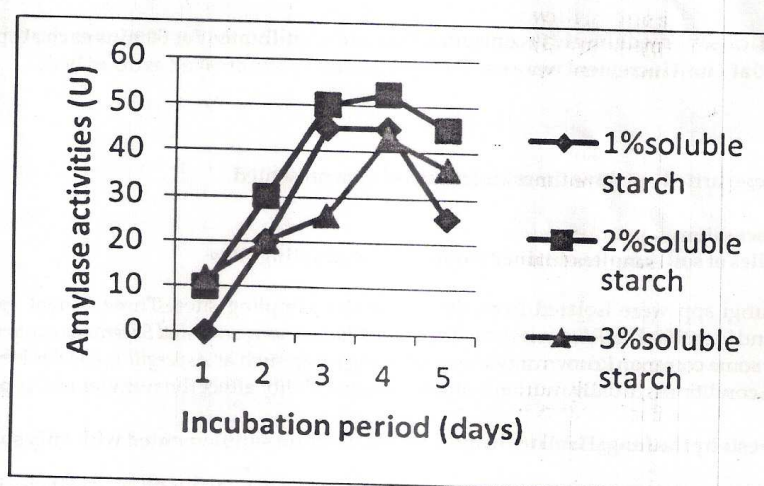


Figure 2. Effect of duration of incubation on amylase production by *P. chrysogenum* in mineral salt medium supplemented with soluble starch. pH 7 and $27^{\circ}\text{C} \pm 2$
Note: Data are means of three experiments

Effect of medium composition on amylase synthesis

As shown in figure 3, there were appreciable levels of amylase activity, when *P. chrysogenum* was grown in mineral salt medium supplemented with different carbon sources, than on mineral salt alone.

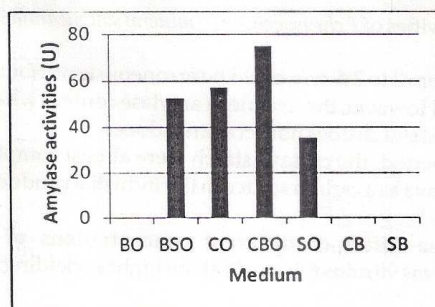


Figure 3. Effect of substrate composition on amylase synthesis BO=Basal medium + organism BSO=Basal medium + soluble starch + organism CO=Cassava starch + organism CBO=Cassava starch + Basal medium + organism SO=Starch + organism CB=Cassava starch + Basal medium_organism SB=Soluble Starch + Basal medium_organism. pH = 7 Temp: $27^{\circ}\text{C} \pm 2$.

This point to the fact that carbon sources are essential for amylase synthesis. When the grown in medium containing carbon substrates alone without mineral salts, very little amylase activities were observed. This implied that, for amylase synthesis to occur in these organisms to an appreciable level, a carbon source as well as mineral salts are essential. Similar observations were made by Sani *et al.* (1992). There was high significant difference in the rate of amylase activities when the organism was grown in mineral salts medium supplemented with cassava starch as carbon source than when pure soluble starch was used. Ray (2004) opined that cassava's fibrous residue constitutes high starch content (63%) and an organic nature-rich nutrient; and can serve as an important substrate for the production of amylase enzyme. This implies that, the ability of cassava to stimulate increased production of amylase in the medium may be due to the presence of other non-starch carbohydrates, proteins, amino acids and cofactors (Uguru *et al.*, 1997), in contrast to the more defined medium containing soluble starch.

Earlier studies by Clementi *et al.* (1980) suggested that certain constituents of amylolytic products of starch, glucan, a variant of glucose, containing α -1,4 or α -1,6- linkages could induce amylase synthesis. This phenomenon is well known for many amylolytic enzymes of bacterial and fungal origin (Forgaty and Kelly, 1980). Thus, the high level of enzyme synthesis accompanying growth on cassava starch may be due to the continual release of inducer molecules, as a result of action of amylase produced by the organisms. This is in agreement with available reports in which the supply of inducer over an extended period has been found to be important in the production of various carbohydrates by fungi (Rees *et al.*, 1969).

Effect of different concentrations of cassava starch on amylase synthesis

The effect of different concentrations of cassava starch as a substrate in mineral salt medium on amylase synthesis by *P. Chrysogenum* is shown in Figure 4.

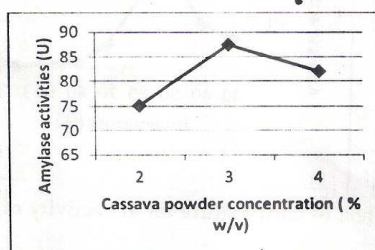


Figure 4. Relative amylase activities of *P.chrysogenum* in mineral salt medium supplemented with cassava at pH7 and $27 \pm 2^\circ\text{C}$.

Increase in amylase activity from 2 to 3% w/v could be erroneously attributed to the inductive power of the starch (Lachmund *et al.*, 1993). However, the decline in amylase content when concentration is increased up to 4%, is a strong indication that starch does not act as an inducer.

At the end of the incubation period, the cassava starch were almost completely exhausted in the medium. This suggest suitability of cassava as a carbon source in the industrial production of glucose.

Properties of crude amylase Effect of different concentrations of cassava on amylase activity
Maximum hydrolytic period was 90 mins (Figure 5) at the highest yielding concentrations of cassava starch medium.

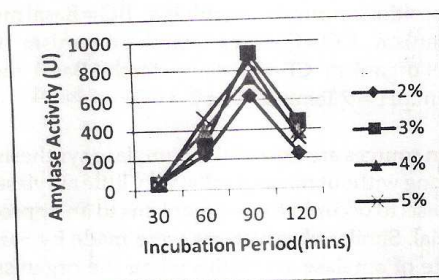


Figure 5: Effect of hydrolytic period on amylase activity

This concentration agrees with the reports of previous work by Omemu *et al.* (2005) but in discord with Balkan and Ertan (2005) who cultivated *Penicillium chrysogenum* on liquid medium containing maltose (2%) as the sole carbon source. The low amylase activity after 1 hr of incubation (Figure 5) may strongly be due to the presence of fungal spores in the crude amylase which tends to consume the available simple sugar and then use them for their growth.

The optimum temperature of activity at 50°C (Figure 6) was quite high and comparable with other fungal (Bhumbhhamon, 1986 and Sharma and Shukla, 2008) amylase; while the optimal pH of 5 (Figure 7) was within the range of the values reported for *P. chrysogenum* amylase by Balkan and Ertan (2005). This study is in corcord with proposition by Uguru *et al.* (1997) that the uptimum temperature of an enzyme may not be related to the growth temperature of the organism.

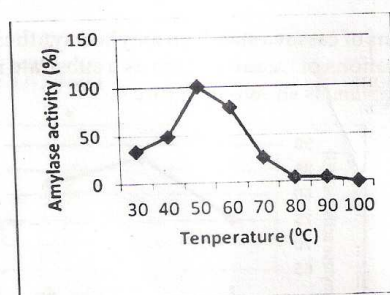


Figure 6. Effect of temperature on the activity of crude amylase*.

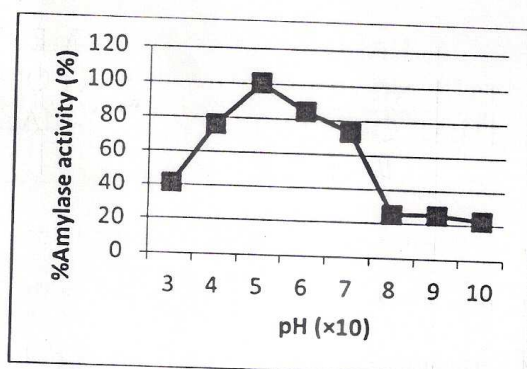


Figure 7: Effect of pH on the activity of crude amylase*

* Percentage (%) activity is expressed relative to the highest amylase activity (920 U) of the crude amylase @ 27°C.

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