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Cellulase Production by *Trichoderma longi*, *Aspergillus niger* and *Saccharomyces cerevisae* Cultured on Plantain Peel

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Abstract: In this study, three fungi: *Trichoderma longibrachiatum*, *Aspergillus niger* and *Saccharomyces cerevisiae* were cultured on plantain peel, a cellulosic waste. The waste was dried, pre-treated with alkali and steam, re-dried and then blended. The powdered waste was then used as substrate in shake-flasks which contained Mineral Salts Medium (MSM) and inoculi of the three test fungi. Fermentations were initially carried out in flasks containing the MSM, waste substrate and the inoculum at pH 5.0, 1% substrate concentration, 10% inoculum size and cultured on a rotary shaker at 29±1°C for 5 days to verify cellulase production by the organisms from the waste substrates, then for 7 or 9 days while varying different fermentation parameters. Cellulase activity and amount of glucose produced by the three test organisms from the waste substrate was determined and compared. Glucose production was optimized by varying the fermentation parameters: Time, pH, Substrate concentration, Inoculum size and Temperature. The results obtained from the fermentations showed that *Trichoderma longibrachiatum* produced the highest amount of glucose among the cultures tested (1.64 mg mL^{-1}). This was produced from plantain peel at pH 5.0 and temperature of 45°C on day 7 of fermentation. The highest amount of glucose produced by *Aspergillus niger* from plantain peel was 1.18 mg mL^{-1} at pH 4.5 and temperature of 45°C on day 7 of fermentation. The highest amount of glucose produced by *Saccharomyces cerevisiae* was 1.00 mg mL^{-1} at pH 3.5 and temperature of 45°C on day 5 of fermentation.

Key words: Cellulase, plantain wastes, *Trichoderma longibrachiatum*, *Saccharomyces cerevisiae*, *Aspergillus niger*

INTRODUCTION

Processing of fruits produces two types of wastes, a solid waste of peel/skin, seeds, stones and a liquid waste of juice and wash waters. In some fruits, the discarded portion can be very high, for example, in banana up to 20% (Umoh, 1998). Therefore, there is often a serious problem of waste disposal which can lead to problems with flies and rats around the processing room, if not correctly dealt with.

Cellulose is a potentially valuable resource for fiber, fuel and feed. Investigation into ability of microbes to degrade native cellulose so far revealed that ouly a few fungi possesses the ability to degrade native cellulose. A majority of microbes can however, degrade modified cellulose. The crystallinity and lignification limit the accessibility and susceptibility of cellulose to cellulolytic enzymes and other hydrolytic agents (Caritas and Humphrey, 2006).

Bananas and plantains are monocotyledonous plants of the genus *Musa* family Musaceae and order Zingiberales. Plantains are designated *M. paradisica* L. Bananas, they are large herbaceous, perennial plants which consist of branched underground stems or rhizomes with abundant roots, several lateral buds and erect leafy trunk which eventually bears bunches. Fruits of bananas and

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plantains are formed partheno-carpically and their seeds are so rare or virtually absent as a result of very high degree of sterility in both pollen and ovules (Osagie and Eka, 1998). Plantains are concentrated within the high forest zones in various locations in the southern and central parts of Nigeria. A large portion of agricultural, industrial and urban wastes consist of polysaccharides which could be converted into valuable fermentable soluble sugars for the production of chemicals (Brown *et al*., 1987). Modification of cellulose can lead to a much more rapid production of reducing sugars by enzymes. Enzymatic conversion of cellulose to food, fuel and chemical feedstock is a well established process. However, high cost of cellulase production has hindered the use of this enzyme in industry. The source of raw material is available and generally free of cost. This could be converted into fermentable sugars. Cellulase production from different agro wastes has been reported using a variety of microorganisms. These include banana peel, millet, maize straw, guinea corn, rice husk and orange peel using *Saccharomyces cerevisae* and *Aspergillus niger* (Baig *et al*., 2004; Milala *et al*., 2005; Omojasola *et al*., 2008).

Many researchers have studied the effect of agro waste pre-treatment by alkali or steam (Baig *et al*., 2004). However, many physical, chemical and microbial pre-treatment methods for enhancing bioconversion of cellulosic materials have been reported by Caritas and Humphrey (2006). Pretreatment of cellulose opens the structure and removes secondary interaction between glucose chains (Tang *et al*., 1996). Cellulase production depends on the type of substrate, pretreatment and strain of microorganisms used (Caritas and Humphrey, 2006). Cellulose fibres absorb water readily and swell, but the swelling is limited to amorphous regions of the fibre as it is counteracted by the crystalline regions. A swelling, in excess of that obtained by more hydration, can be obtained by suitable mechanical or physical treatment such as stream treatment, milling and ultrasonic treatment. Also, mineral acids and alkali in high concentrations will induce swelling of the whole fibre, since these agents are able to break the hydrogen-bond network and are able to penetrate into the crystalline areas. For effective hydrolysis of cellulose by cellulase in vitro, a pretreatment to cause swelling is frequently necessary. The number of glucosidic bonds available for enzyme action will thus, to a large extent depends on the degree of swelling of the cellulose (Jostein and Jonny, 1980).

Nigeria can usefully utilize the over-abundant cellulolytic wastes and agricultural wastes to produce alcohol which can be exported to gain foreign reserve. Thus, also controlling the enviroumental pollution currently experienced all over the country by these useful agricultural and domestic wastes.

The objective of the study is to use plantain peel a readily available agrowaste, as a substrate for the production of cellulase an industrial enzyme. The parameters of the fermentation will be varied in an attempt to optimize cellulase production.

MATERIALS AND METHODS

Microorganisms and Waste Substrates

This study was carried out in 2007 in the Microbiology Laboratory of the Department of Microbiology, University of Ilorin, Nigeria. The organisms used for this study were isolated from three sources: *Aspergillus niger* was isolated from Rotten Wood (RW) picked up on the premises of Unilorin permanent site, *Saccharomyces cerevisiae* was isolated from Palm Wine (PW) bought from a palm wine tapper at Offa garage in Ilorin and *Trichoderma longibrachiatum* was collected from the Faculty of Agriculture, University of Ilorin. The microorganisms were identified in the Microbiology Laboratory, University of Ilorin.

The plantains were washed with water to remove dirt. They were peeled and the peel sun-dried before drying in the oven at 70°C.

The Plantain Peels (PP) were pounded into smaller pieces using a mortar and pestle. The pounded pieces of the samples were autoclaved for 1 h at 121°C with 5% (w/v) NaOH (20 mL g^{-1} of substrate)

in separate conical flasks for delignification. The autoclaved materials were filtered through muslin cloth. The samples were then washed thoroughly with water and neutralized with 1 M HCl. They were finally washed with distilled water and dried at 70°C. The treated samples were then ground in a blender (Moulinex-model 242) for 10 min.

Screening and Identification of Microorganisms

Plate screening of the isolated organisms for cellulase production was carried out according to the method of Brown *et al.* (1987). A point of inoculation the spores of each organism was grown on PDA supplemented with 2% (w/v) carboxymethylcellulose (CMC). The plates were incubated at 29±1°C for 48 h after which they were stained with Congo red stain for 15 min. Excess dye was removed by washing with 1 M NaCl and the plates were fixed with 1 NHCl. The production of extracellular cellulase by the organisms was indicated by a zone of clearance around the colony.

The microorganisms were identified in Microbiology Laboratory in the University of Ilorin according to Chaturvedi (2001). All organisms were maintained on PDA slants. A spore suspension from 3 to 4 day old 10 mL PDA slants of each culture in 10 mL sterile distilled water was made. Mineral salts glucose medium was prepared and approximately 2.8×10^6 spores/cells of each culture were inoculated into 500 mL flasks containing 100 mL of medium each. The spores/cells were counted using a Neubauer counting chamber. The flasks were incubated for 24 h at 29±1°C on a Gallenkamp (England) rotary shaker at 250 rpm to develop the inoculum. Mary Mandels' Mineral salts solution was used for the fermentation and it was prepared as described by Jeffries (1996). Culture conditions involved autoclaving the fermenting media containing 10 g L^{-1} of waste substrate at pH 5.0 and inoculating with pure suspension of germinated spores of *Trichoderma longibrachiatum*, *Aspergillus niger* and *Saccharomyces cerevisiae*.

Enzyme Assays

The three selected fungi were cultivated using the submerged culture technique. All organisms were maintained on PDA slants. A spore suspension from 3 to 4 day old 10 mL PDA slants of each culture in 10 mL of distilled water was made. The fermentation media used was Mary Mandels' mineral salts solution and it was used along with different carbon and nitrogen sources.

Mineral salts glucose medium was prepared and approximately 2.8×10^6 spores/cells of each culture were inoculated into 500 mL flasks containing 100 mL of medium each. The spores/cells were counted using a Neubauer counting chamber. The flasks were incubated for 24 h at 29±1°C on a Gallenkamp (England) rotary shaker at 250 rpm to develop the inoculum.

The waste substrates represent the carbon sources in the fermentation media. These were combined with M1 to give Mineral Salts Glucose Medium (MSGM) and Mineral Salts Plantain Peel Medium (MSPPM).

All the media mentioned above were prepared separately and dispensed in conical flasks. They were sterilized in the autoclave at 121°C for 15 min.

The final pH was adjusted to 5.0 using a pH meter (Denver Instrument, Model 20 pH/Conductivity meter).

The pH of the fermenting media containing the waste substrates at a level of 10 g L^{-1} was adjusted to 5.0. The suspension of germinated spores was inoculated at a level of 10% (v/v) into the production medium contained in flasks. These were incubated at 29±1°C on a shaker at 100 rpm. Glucose production in the medium was measured on 5th day of fermentation (Srivastava *et al*., 1987; Jeffries, 1996).

Cellulase activity was determined colorimetrically by measuring the increase in reducing groups by the hydrolysis of a carboxymethylcellulose (CMC) substrate (Jeffries, 1996). The procedure followed the 0.5 mL assay described by Jeffries (1996).

Samples were withdrawn from the culture at 2 day intervals over a period of 7-9 days and the supernatant that resulted following centrifugation at 3,000 rpm for 15 min to remove solids, were assayed for total reductant sugar measurement using DNSA method of Miller (1959). Enzyme solutions were diluted in 0.05 M citrate buffer, pH 4.8. The enzyme diluted in buffer and 1% CMC (0.5 mL each) was mixed well and incubated for 30 min at 50°C. Three milliliters of the DNSA was added and the tubes were placed in boiling water bath for 5 min. The tubes were cooled and the reducing sugar, glucose was determined (Jeffries, 1996). The sample, enzyme blank, glucose standard and control were boiled together and absorbance was read at 540 nm using a spectrophotometer (CamSpecM105). A control (substrate and buffer) otherwise called spectro zero, was used to set the spectrophotometer at zero absorbance. During the course of the experiments, the absorbance of the sample tube, corrected by subtraction of the enzyme blank was translated into glucose during the reaction using a glucose standard. The linear glucose standard was used to translate the absorbance values of the sample tubes into glucose i.e., mg glucose produced during the reaction. For a 30 min assay, 1 mg of glucose equals 0.185 unit $\left(\frac{1}{30\times 0.18}\right)$.

Optimization experiments were carried out and each of the organisms were grown on each of the substrates and hydrolyzed under conditions that produced maximal activity of the enzyme from all the previous experiments. In accord with the Internatioual Union of Biochemistry, one enzyme unit equals 1 μM (μ) of substrate hydrolyzed per minute.

Determination of Optimal Conditions for Enzyme Production

Effect of Varying Time

Cellulase activity was measured at regular intervals while fermentation was observed at 29±1°C for a period of 9 days and the period of maximnm enzyme production was determined. Samples were withdrawn on days 0, 1, 3, 5, 7 and 9.

Effect of Varying pH

The pH of the fermentation media were adjusted to various values ranging from 2.0-6.0 with 0.1 N NaOH or 0.1 N HCl. The pH was determined using the pH meter (Denver Instrument, Model 20 pH/Conductivity meter).

Effect of Varying Substrate Concentration

Different concentrations of the waste substrate (plantain peel), ranging from 1.0 to 5.0% were used in the fermentation media.

Effect of Varying Temperature

The fermentation was carried out at different temperatures ranging from 30 to 45°C.

Effect of Varying Inoculum Size

The fermentations were carried with varying amounts of inocula. The inoculnm sizes were varied from 2 to 10%.

RESULTS AND DISCUSSION

Plantain peel can yield glucose by the activities of cellulolytic organisms. The initial fermentation was carried out using 10% substrate concentration, pH 5.0, 10% inoculnm size, temperature 29±°C, for 5 days. *Aspergillus niger* produced the highest amount of glucose 0.86±0.06 mg mL^{-1}; followed by *T. longibrachiatum* which produced 0.80±0.04 mg mL^{-1} and the least amount by *S. cerevisae* which produced 0.72±0.03 mg mL^{-1} of glucose.

Table 1: Effect of varying time on glucose production by test fungi using plantain peel

	Glucose produced (mg mL^{-1}) (days)					
Test fungi	0	1	3	5	7	9
T. longibrachiatum	0	0.24±0.04	0.38±0.04	0.80±0.10	1.22±0.03	0.48±0.04
A. niger	0	0.16±0.03	0.54±0.02	0.86±0.05	0.82±0.04	0.46±0.03
S. cerevisiae	0	0.36±0.03	0.54±0.06	0.72±0.04	0.50±0.05	0.36±0.05

Values are presented as Mean±SD (n = 3)

Table 2: Effect of pH on the production of glucose by test fungi using plantain peel

	pH								
Test fungi	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0
T. longibrachiatum	0.312^{b}	0.318^{b}	0.376^{b}	0.388^{b}	0.580^{f}	0.612^{f}	0.528^{e}	0.416^{e}	0.484^{c}
A. niger	0.324^{b}	0.308^{b}	0.496	0.488	0.492	0.556	0.476	0.580	0.516
S. cerevisae	0.296	0.304	0.368	0.480^{b}	0.472^{b}	0.444	0.424	0.408	0.340

Substrate concentration: 1%, Temp: 29±1°C, inoculum size: 10%, Values are presented as mean, All groups are compared to each other at $p<\alpha=0.05$, Values with different superscripts are statistically different

Table 3: Effect of substrate concentration on the production of glucose by test fungi using plantain peel

	Substrate concentration (%)				
Test fungi	1	2	3	4	5
T. longibrachiatum	0.528±0.06	0.632±0.09	0.764±0.06	0.740±0.08	0.680±0.06
A. niger	0.476±0.11	0.512±0.12	0.468±0.06	0.528±0.09	0.542±0.08
S. cerevisiae	0.424±0.13	0.604±0.08	0.612±0.07	0.608±0.10	0.580±0.02

pH 5.0; Temp 29±1°C; Inoculum size 10%, Values are presented as Mean±SD (n = 3), All groups are compared to each other at $p<\alpha=0.05$, Values with different superscripts are statistically different

Optimal glucose production from *T. longibrachiatum* was on day 7 and 5 for *A. niger* and *S. cerevisiae* (Table 1). Hydrolysis rates decline with time due to depletion of the more amorphous substrates, product inhibition and enzyme inactivation (Ghose, 1987). Caritas and Humphrey (2006) and Narasimha *et al*. (2006) also gave similar time course reports of maximum glucose yield on 5th day of fermentation using *A. niger*.

Effect of pH on glucose production from the waste substrate by the three fungi reveal that optimum amounts of glucose were produced at pH 4.5 for *T. longibrachiatum* and *A. niger* and pH 3.5 for *S. cerevisae* (Table 2). This supports the findings of Lee *et al*. (2002), who reported that CMCase, Avicelase and FPase activities exhibit a pH optimum of approximately 4, while the pH optimum of β-glucosidase was between pH 5 and 6.

The effect of substrate concentration on glucose production indicates that further increase in cellulose concentration beyond the level that gave the optimum glucose did not result in proportionate increase in glucose yield (Table 3). Haapela *et al*. (1995) and Jeffries (1996) reported that maximum endoglucanase activity was recovered on the medium with cellulose at 10 g L^{-1}. Mandels and Reese (1959) also reported that maximal yields of cellulase were obtained on 1% substrate (cellulose, lactose, cellobiose and glucose) using *T. viride* and *Myrothecium verrucia*. However, this study finds substrate concentration of 30 g L^{-1} gave the highest amount of glucose from *T. longibrachiatum* (Table 3). Since the substrates contain different minerals apart from carbon which may serve as nutrient supplements, increase in substrate concentration leads to increase in these nutrients which may adversely affect the cell concentration. The increase in glucose production until the optimum that was obtained was due to the availability of cellulose in the medium; while a decrease in production beyond optimum concentration is explained to be as a result of an inhibitory effect of accumulated cellobiose and cellodextrins of low degree of polymerization to the growth medium. It might also be due to the specific binding of the enzymes with the substrates (Wang *et al*., 2006). Low glucose production after optimum very probably highlights sugar depletion from the substrates into the medium (Brien and Craig, 1996).

Table 4: Effect of inoculums size on the production of glucose by test fungi using plantain peel

	Inoculum size (%)				
Test fungi	2	4	6	8	10
T. longibrachiatum	0.202±0.03	0.312±0.02	0.404±0.06	0.428±0.04	0.264±0.06
A. niger	0.268±0.05	0.396±0.07	0.548±0.08	0.480±0.06	0.476±0.03
S. cerevisiae	0.496±0.08	0.496±0.06	0.500±0.08	0.500±0.11	0.424±0.07

Temperature: 29±1°C, pH: 5.0, Substrate concentration: 1%, Values are presented as Mean±SD (n = 3), All groups are compared to each other at $p<\alpha=0.05$

Table 5: Effect of temperature on the production of glucose by test fungi using plantain peel

	Temperature (°C)			
Test fungi	30	35	40	45
T. longibrachiatum	0.528±0.06^{b}	0.504±0.04^{b}	0.632±0.07^{c}	0.776±0.09^{c}
A. niger	0.476±0.04^{b}	0.404±0.08^{a}	0.536±0.05^{b}	0.628±0.04^{c}
S. cerevisae	0.424±0.02	0.452±0.09^{a}	0.544±0.03^{a}	0.552±0.03^{a}

Values are presented as Mean±SD (n = 3), All groups are compared to each other at $p<\alpha=0.05$, Values with different superscripts are statistically different

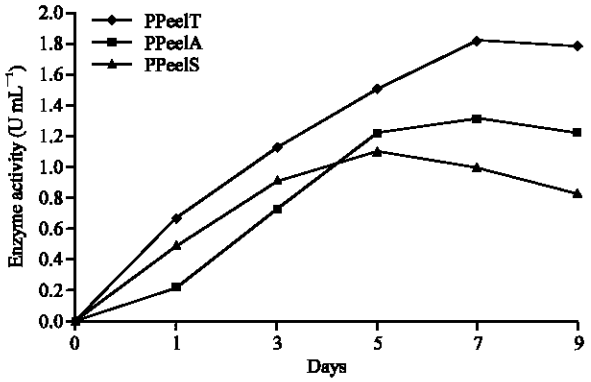

Fig. 1: Activity against cellulase of *T. longibrachiatum*, *A. niger* and *S. cerevisiae* cellulase on Plantain Peel

Effect of inoculum size on glucose production show that decreasing amounts of glucose resulted from inoculum sizes above 8% for all the test fungi (Table 4). This decrease in glucose production with further increase in inoculum might be due to clumping of cells which could have reduced sugar and oxygen uptake rate and also, enzyme release (Srivastava *et al*., 1987).

Highest amounts of glucose were produced at fermentation temperatures of 45°C for all the test organisms used (Table 5). The optimum temperature for the synthesis of enzymes for saccharification of agrowaste in all cases to enzymatic hydrolysis can be attributed to lignin content of the material. Pretreatment of lignocellulosic material enhances enzyme activity and maximum saccharification was achieved within the range 30-45°C coinciding with the characteristics of mesophiles (Baig *et al*., 2004). Optimum glucose production from plantain peel using *T. longibrachiatum* was at pH 4.5, 3% substrate concentration, 8% inoculum size at 45°C on day 7. Optimum glucose from plantain peel using *A. niger* was produced at pH 5.5, 5% substrate concentration, 8% inoculum size at 45°C on day 5. While optimum glucose from the plantain peel using *S. cerevisiae* was produced at pH 3.5, 3% substrate concentration, 8% inoculum size, 45°C on day 5.

These optimal conditions were combined in single fermentations for each organism and cellulase activity was measured (Fig. 1). Glucose yields from these combined optimal conditions were higher than when standard conditions were used for *T. longibrachiatum* 1.036±0.09 mg mL^{-1}.

However, glucose production of *A. niger* and *S. cerevisae* dropped to 0.706±0.06 mg mL^{-1} and 0.654±0.04 mg mL^{-1}, respectively. Cellulase activity from plantain peel was 1.82 U mL^{-1} when hydrolyzed by *T. longibrachiatum*, 1.31 U mL^{-1} when hydrolyzed by *A. niger* and 1.11 U mL^{-1} when hydrolyzed by *S. cerevisiae*. Cellulase activity of *S. cerevisiae* was lowest. The exo-β-1, 3-glucanases produced by *S. cerevisiae* yield glucose as the end product, whereas endo-β-1, 3-glucanase releases a mixture of oligosaccharides with glucose as the minor product. Because β-1, 3-glucan is the main structural polysaccharide responsible for the strength and rigidity of the yeast cell wall, β-1, 3-glucanases have been suggested to play a role in important morphogenetic processes involving the controlled autolysis of β-1, 3 glucan. During vegetative growth, several endo- and exo-1, 3-β-glucanases are synthesized, some of which are secreted ouly to remain entrapped in the cell wall whereas others are released to the surrounding medium (Lee *et al*., 2002).

In conclusion, this study revealed that plantain peel, which is a domestic and industrial agro-waste, can serve as substrate to produce large amounts of cellulase enzymes when hydrolyzed by cellulolytic microorganisms; instead of being thrown away and left to rot and pollute the environment.

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