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CELLULASE PRODUCTION BY WILD STRAINS OF *ASPERGILLUS NIGER*, *PENICILLIUM CHRYSOGENUM* AND *TRICHODERMA HARZIANUM* GROWN ON WASTE CELLULOSIC MATERIALS.

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ABSTRACT

Waste cellulosic materials (corncob, sawdust and sugarcane pulp) and crystalline cellulose induced cellulase production in wild strains of *Aspergillus niger, Penicillium chrysogenum* and *Trichoderma harzianum* isolated from a wood-waste dump in Lagos, Nigeria. Cellulose-supplemented media gave the maximum cellulase activity of 0.54, 0.67 and 0.39 units mg Protein^{-1} for *A. niger*, *P. chrysogenum* and *T. harzianum* respectively. The maximum enzyme activity for *A. niger* was obtained at 36 hours of cultivation, while *P. chrysogenum* and *T. harzianum* gave their optimal enzyme activities at 12 and 60 hours respectively. Of the three cellulosic wastes, best enzyme activity was obtained with sawdust. Maximum enzyme activity of 0.30, 0.24 and 0.20 units mg Protein^{-1} respectively was obtained with *A. niger*, *P. chrysogenum* and *T. harzianum* at 144 hours of cultivation using the substrate. *A. niger* gave the highest enzyme activity with any of the three cellulosic materials followed by *P. chrysogenum*. It thus appears that the use of sawdust presents the best option for low-cost commercial production of cellulase using *A. niger* and *P. chrysogenum* as discussed herewith.

Keywords: Cellulolytic fungi, Cellulase activity, Low-cost enzymes, Corncob, Sawdust, Sugarcane Pulp.

INTRODUCTION

Cellulosic biomass constitutes the most abundant organic molecules on earth and is continually replenished by carbon dioxide fixation via photosynthesis (Fan *et al.,* 1987). All cellulosic materials, including the agro-industrial wastes can be converted into commercially important products such as ethanol, methane, glucose syrups and single cell proteins (Ryu and Mandel, 1980; Wu and Lee, 1997; Solomon *et al.,* 1999). Bioconversion, particularly enzymatic hydrolysis, of these cellulosic materials into simple sugars, has been a subject of intensive research (Smiths *et al.*, 1996). The development of an industrial process for cellulose bioconversion would help alleviate shortages in food and animal feeds and also reduce the problems of urban waste disposal and overdependence on fossil fuels (Kumakura, 1997). Successful utilization of these renewable resources is dependent on the development of an economically viable process which would include the production of cellulases required for the enzymatic hydrolysis of cellulosic materials (Fan *et al.*, 1987; Smiths *et al.*, 1996).

Cellulase, a group of hydrolytic enzymes which hydrolyze the -glycosidic bonds of native cellulose and related cellooligosaccharides, is the key enzyme of potential use for industrial saccharification of cellulosic materials into simple sugars. Cellulase production was found to be the most expensive step, accounting for about 40% of the total cost, during the production of ethanol from cellulosic biomass (Spano *et al*, 1987). Cellulase production by different cellulolytic microfungi using various waste cellulosic materials is being vigorously studied for cost reduction strategies (Abu *et al.*, 2000; Ojumu *et al.,* 2003). Although a large number of microorganisms (fungi, bacteria and actinomycetes) are capable of degrading cellulose, only a few of them produce significant quantities of cell-free enzyme fractions capable of complete hydrolysis of cellulose in vitro (Berry and Paterson, 1990). Among the cellulolytic mircofungi, the genera *Trichoderma* and *Aspergillus* are notable cellulase producers (de Vries and Viser, 2001). Cellulase preparations from species such as *T. viride* and *A. niger* and of several species

of *Penicillium* have also been purified and studied extensively (Wood and McCrae, 1986).

In this study, we examined the relative potentials of common waste cellulosic materials - corncob, sawdust and sugarcane pulp (Bagasse) - as microbial substrate for cellulase production using wild strains of *A. niger, P. chrysogenum* and *T. harzianum* isolated from wood waste dump in Lagos, Nigeria and identified as described previously (Nwodo-Chinedu *et al*., 2005). Crystalline cellulose was used for comparative purposes to assess the relative effect of the various cellulosic wastes on cellulase production by the cultures of these macrofungi.

MATERIALS AND METHODS

Waste Cellulosic Materials

The cellulosic material used as microbial substrates were prepared as follows: Sawdust of *abora* wood (*Mitragyna ciliata*) was collected from Okobaba saw-mills, Ebute-Metta, Lagos, Nigeria. Mature Sugarcane (*Saccharum offinarum*) and fresh maize (*Zea mays*) were purchased from Oshodi market in Lagos, Nigeria. Fibrous pulp of the sugarcane was obtained by crushing and washing the pulp repeatedly in water to remove all residual sugars. Corncob was obtained by removing the maize grains. The materials were cut into small pieces and sun-dried for 3-5 days to reduce the moisture content and make them easier to grind. Grinding was done using Marlex, Excella Mixer Grinder (Mumbai, India). Fine powder obtained by passing the ground materials through a sieve (about 0.5mm pore size) was used as microbial substrates.

Composition of the Cellulosic Wastes

The standard method of the Association of Official Analytical Chemists (A. O. A. C) was used to determine the proximate composition of the waste materials (AOAC, 1990).

Other Substrates and Chemicals

Crystalline cellulose (Avicel) and Potato Dextrose Agar (PDA) were obtained from Merck, Darmstadt, Germany; Carboxymethyl-Cellulose (CM52) was obtained from Whatman Ltd, England. All other chemicals and reagents used were obtained from Sigma Chemicals Co. Ltd, England and were of analytical grade.

Organisms

The strains of *Aspergillius niger, Penicillium chrysogenum* and *Trichoderma harzianum* obtained from wood-waste dump in Lagos, Nigeria and characterized as described previously (Nwodo-Chinedu *et al*, 2005). The organisms, maintained on PDA slant at 4°C, were sub-cultured on PDA plates and incubated at 30°C for 3 -5 days to obtain the inocula used in this study.

Cultivation and Cellulase Production

The organisms were grown on synthetic media containing (in 1 liter of distilled water): $NaNO_3$, 3.0g; KCI, 0.5g; $MgSO_4.7H_2O$, 0.5g; KH_2PO_4, 1.0g; $FeSO_4.\ 7H_2O$, 0.01 g; with 1.0 % crystalline cellulose (or 0.5 % corncob, sawdust or sugarcane pulp) as sole carbon source. One (1) liter of supplemented media and the pH was adjusted to 5.6. Conical flasks (250 ml) containing 100 ml of the respective media were autoclaved at 121°C for 15 minutes, cooled and inoculated with 10 discs of 5.0 millimeter diameter of the 3-day culture of the organisms from PDA plates using a sterile cork borer. The cultures were incubated in the dark at 25°C with shakings. Cells were harvested at 12-hour intervals for cellulose-containing media and at 48-hour intervals in media containing corncob, sawdust or sugarcane pulp by centrifugation at 6000 x g for 15 minutes at 4°C using ultra centrifuge (Superspeed RC-B, USA). The cell-free culture supernatants were used as source of crude extracellular enzyme.

Cellulase Assay

Cellulase enzyme (Endo- -1, 4-Glucanase, EC 3. 2. 1. 4) activity was assayed by a modification of the reducing-sugar method described by Khan (1980) using carboxymethyl-cellulose (CMC) as substrate. The reaction mixture contained 2 ml of 1.0% (w/v) CMC in 0.1M solution of sodium acetate buffer, pH 5.0, and 2.0 ml of the cell-free culture supernatant. The mixture was incubated at 37°C with shaking for 30 minutes. The reducing sugar released was measured as glucose equivalent

using dinitrosalicyclic acid reagent (Miller, 1959). A unit of activity was defined as the amount of enzyme required to liberate 1µmol of glucose per minute under the assay conditions.

Protein Assay

The Protein content of the crude enzyme preparations was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard.

RESULTS

The proximate composition of the cellulosic materials is shown in Table 1. High crude fiber contents of 38.8 ± 2.6%, 61.0 ± 3.4% and 46.1 ± 2.9% was obtained for corncob, sawdust and sugarcane pulp respectively. Generally, the waste materials contained low levels of moisture, ash, fat and crude protein. Figure 1 shows the changes in specific cellulase activities of *A. niger, P. Chrysogenum* and *T. harzianum* in cellulose-containing media assayed during incubation for 72 hours. Highest specific enzyme activity of 0.67 Units mg protein^{-1} was obtained after 60 hours of incubation from the culture broth of *P. chrysogenum*. Specific enzyme activity of *A. niger* was maximum at 36 hours with an activity of 0.54 units mg protein^{-1} whereas maximum value of 0.39 units mg protein^{-1} was obtained for *T. harzianum* at 12 hours of incubation. The enzyme activity profiles show two busts with *A. niger* and *P. chrysogenum* during the 72 hours incubation period (Figure 1).

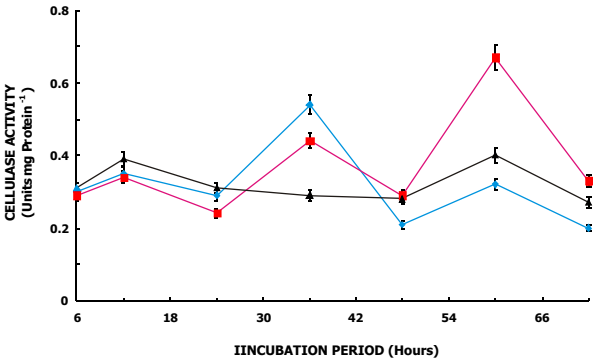

Figure 1: Specific Cellulase Activities of *A. Niger* (♦), *P. chrysogenum* (■) and *T. harzianum* (▲), grown in Cellulose-containing Media and Incubated for 72 hour.

The major enzyme activity peak for *A. niger* (0.54 units mg protein^{-1}) was obtained at 36 hours whereas the minor activity peak was at 12 hours. The minor activity peak for *P. chrysogenum* (0.44 units mg protein^{-1}) occurred at 36 hours, with a major activity peak of 0.67 units mg protein^{-1} occurring at 60 hours.

All the organisms showed much higher specific cellulase activity when grown on pure crystalline cellulose than on any of the waste cellulosic materials. However, more proteins were produced with the cellulosic materials than when pure crystalline cellulose was the substrate (Table 1).

Figure 2 (a c) show the graph of specific cellulase activities of *A. niger* , *P. chrysogenum* and *T. harzianum* grown on the three cellulosic materials (corncob, sawdust and sugarcane pulp) for a period of 192 hours. Figure 2a shows the cellulase

Table 1: Proximate Composition of the Waste Cellulosic Materials (Corncob, Sawdust and Sugarcane pulp)

Cellulosic waste	Moisture (%)	Ash (%)	Fat (%)	Crude Protein (%)	Crude Fiber (%)	Carbohydrate (%)
Corncob	2.7 ± 0.4	3.5 ± 0.2	2.4 ± 0.3	3.0 ± 0.2	38.8 ± 2.6	49.8 ± 3.1
Sawdust	6.1 ± 0.6	1.1 ± 0.1	6.4 ± 0.4	3.2 ± 0.2	61.0 ± 3.4	22.3 ± 1.8
Sugarcane pulp	4.3 ± 0.5	1.5 ± 0.1	5.2 ± 0.5	4.3 ± 0.4	46.1 ± 2.9	38.6 ± 2.4

Table 2: Extracellular Protein Released by *A. niger, P. chrysogenum* and *T. harzianum* Incubated with Different Cellulosic Materials

Organisms	Average extracellular protein released (µg mL^{-1})			
	Cellulose	Corncob	Sawdust	Sugarcane Pulp
A. niger	153 ± 30	495 ± 65	473 ± 144	333 ± 70
P. chrysogenum	135 ± 25	418 ± 80	358 ± 45	258 ± 35
T. harzianum	128 ± 23	408 ± 130	385 ± 50	353 ± 60

activity from *A. niger* broth. The highest specific cellulase activity of 0.30 unit mg protein^{-1} was obtained with sawdust after 144 hours of cultivation. The maximum value for sugarcane pulp and corncob were 0.26 and 0.21 unit mg protein^{-1} respectively (Fig. 2a).

The specific cellulase activity of *P. chrysogenum* culture on corncob, sawdust and sugarcane pulp for 192 hours is shown in Fig. 2b. Sawdust and sugarcane pulp had similar cellulase activities between 96 to 144 hours. Highest cellulase activity of 0.24 unit mg protein^{-1} was obtained at 144 hours with sawdust as substrate while the enzyme value of 0.23 unit mg protein^{-1} was obtained with sugarcane pulp at 96 hours. Maximum value of 0.17 unit mg protein^{-1} was obtained for corncob 144 hours of cultivation. The cellulase activity obtained from the broth of *T. harzianum* is shown in Fig. 2c. Highest cellulase activity (0.20 unit mg protein^{-1}) was obtained with sawdust after incubation for 144 hours. The other two cellulosic materials (sawdust and sugarcane pulp) did not induce cellulase production in this organism.

Figure 3 is a graph of specific cellulase activities of the three organisms, *A. niger, P. chrysogenum* and *T.*

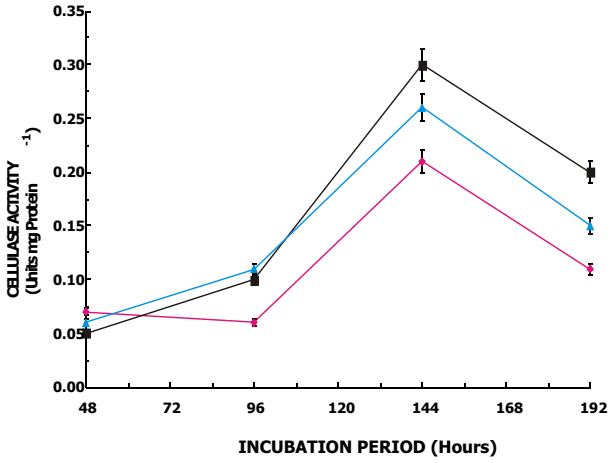

Figure 2a: Specific Cellulase Activity of *A. niger* Incubated on Sugarcane pulp (▲), Corncob (◆), and Sawdust (■) for 192 Hours.

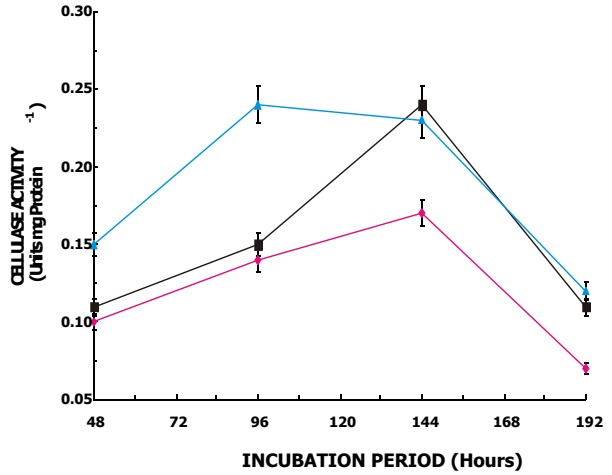

Figure 2b: Specific Cellulase Activity of *P. chrysogenum* Incubated on Sugarcane pulp (▲), Corncob (◆) and Sawdust (■) for 192 Hours.

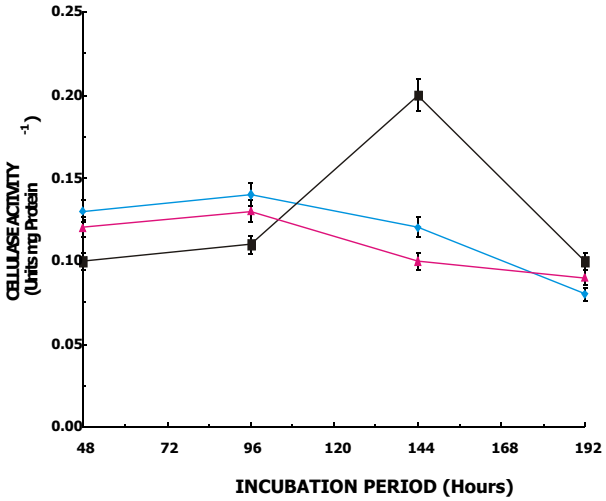

Figure 2c: Specific Cellulase Activity of *T. harzianum* Incubated on Sugarcane pulp (▲) Corncob (◆) and Sawdust (■) for 192 Hours.

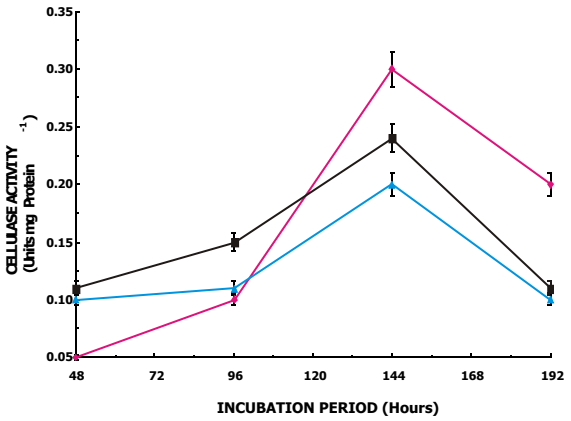

Figure 3: Specific Cellulase Activities of *A. Niger* (◆), *P. chrysogenum* (■), and *T. harzianum* (▲), grown on Sawdust-containing Media and Incubated for 192 Hours.

harzianum grown on sawdust-containing media for 192 hours. Typically, *A. niger* produced the highest amount of cellulase at 144 hours followed by *P. chrysogenum*.

DISCUSSION

The organisms have different periods for optimal cellulase yield. The time was shorter when incubated on pure cellulose compared to the cellulosic waste materials. The time also varies with the different organisms. For instance, it was shorter for *A. niger* (36 hours) compared to *P. chrysogenum* and *T. harzianum* (60 hours) when cultured on cellulose (Figure 1).

P. chrysogenum was the best organism for cellulase production using pure cellulose as carbon source. On the other hand, T. *harzianum* gave the least cellulase activity compared to the other two fungi. Depression in cellulase activities after the initial increase which occurred between 24 hours and 48 hours was observed for all the organisms when cultured on cellulose. This is as expected for enzymatic reactions that may be prone to post-reaction accumulation of hydrolytic by-products (Howell, 1978). The pattern of production of cellulase when the organisms were grown on waste cellulosic materials offers interesting observations. Firstly, peak activity occurred much later, after 72 hours, for all the organisms and on all the three substrates. Secondly, *A. niger,* rather than *P. chrysogenum*, was the best cellulase producer using the waste cellulosic materials. Thirdly, sawdust seems to be the only substrate capable of inducing cellulase production for *T. harzianum*.

Sawdust appears to be the best of the three cellulosic materials for cellulase production for all the organisms. This was followed by sugarcane pulp (for *A. niger* and *P. chrysogenum*). A similar result was obtained for *A. flavus* Linn isolate fermented on sawdust, bagasse (sugarcane pulp) and corncob where sawdust gave the highest enzyme yield followed by bagasse (Ojomu *et al.,* 2003). This may also be as a result of adaptation; since the organisms were isolated from wood-wastes (Nwodo-chinedu et *al*, 2005). For *P chrysogenum,* peak cellulase activity obtained with sugarcane pulp was very close to that of sawdust but at a shorter time (Figure 2b).

In terms of protein yield, higher values were obtained in media containing the waste cellulosic materials compared to pure crystalline cellulose (Table 2). The high protein released in the cellulosic materials suggests the presence of other proteins (beside the cellulase enzyme) which may include other cell-wall hydrolyzing enzymes. Secondary plant cell-wall such as the cellulosic materials contains other polymers, particularly hemicelluloses and lignin, in addition to cellulose. This accounts for the high crude fiber contents of the cellulosic materials (Table 1). The presence of the polymers could induce the production of other degrading enzymes such as hemicellulases and ligninases (Howell, 1978). Fungi such as *Aspergillus species* are known to produce many plant cell-wall hydrolyzing enzymes (de Vries and Viser, 2001). Hemicellulases particularly xylanase is required for the hydrolysis of natural cellulose (Khan, 1980). Therefore, the presence of these cell-wall hydrolyzing enzymes would enhance the solubilization of cellulosic materials. Higher cellulase activities could be obtained by pretreatment of the cellulosic materials to reduce the association of cellulose with other plant cell-wall polymers (Kanosh *et al*, 1987; Solomon e*t al*, 1999; Abu *et al*, 2000).

In conclusion, the wild strains of *A. niger, P. chrysogenum* and *T. harzianum* are capable of producing cellulases from sawdust, sugarcane pulp and corncob. The best enzyme activity in the three cellulosic materials was obtained with *A. niger*. of the three cellulosic materials, sawdust gave the highest yield of the enzyme. Sawdust, the by-product of saw-mill and carpentry operations, is therefore the most suitable low-cost substrate for cellulase production using the organisms. The waste cellulosic material is potentially useful for commercial cellulase production. Its use will undoubtedly result in production of cheaper cellulase for the transformation of the huge waste cellulosic materials available in our environment.

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