

Cellulase Production by Wild-type *Aspergillus niger*, *Penicillium chrysogenum* and *Trichoderma harzianum* Using Waste Cellulosic Materials

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Abstract

Waste cellulosic materials (corn cob, 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 h of cultivation, while *P. chrysogenum* and *T. harzianum* gave their maximum enzyme activities at 12 and 60 h respectively. For the cellulosic wastes, highest enzyme activity was obtained with sawdust where *A. niger*, *P. chrysogenum* and *T. harzianum* gave the maximum enzyme activity of 0.30, 0.24 and 0.20 units mg Protein⁻¹ respectively after 144 h of cultivation. *A. niger* recorded 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: Cellulosic wastes, Cellulolytic fungi, Cellulase activity, Industrial enzymes, Bioconversion.

Introduction

Cellulosic biomass constitutes the most abundant organic molecules on earth and is continually replenished by carbon dioxide fixation via photosynthesis^[1]. 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^[2, 3, 4]. Bioconversion, particularly enzymatic hydrolysis, of these cellulosic materials into simple sugars, has been a subject of intensive research^[5]. 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^[6]. 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^[1].

Cellulase, a group of hydrolytic enzymes which hydrolyze the β -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^[7]. Cellulase production from various waste cellulosic materials using different cellulolytic microfungi is being vigorously studied for cost reduction strategies^[8, 9]. 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^[10]. Among the cellulolytic microfungi, the genera *Trichoderma* and *Aspergillus* are notable cellulase producers^[10]. Cellulase preparations from species such as *T. viride* and *A. niger* and of several species of *Penicillium* have also been purified and studied extensively^[11].

In this study, we examined the relative potentials of common waste cellulosic materials - corn cob, 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^[12]. 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 microfungi.

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 officinarum*) 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. Fine powder obtained by passing the ground materials through a sieve (about 0.5mm pore size) was used as microbial substrates.

Other Substrates and Chemicals:

Crystalline cellulose (Avicel) and Potato Dextrose Agar (PDA) were obtained from Merck, 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 *Aspergillus niger*, *Penicillium chrysogenum*, and *Trichoderma harzianum* were isolated from wood-waste dump in Lagos, Nigeria and identified by the Department of Botany and Microbiology, University of Lagos as described previously^[12]. The organisms were maintained on PDA slant at 4°C. The organisms 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): NaNO₃, 3.0g; KCl, 0.5g; MgSO₄·7H₂O, 0.5g; KH₂PO₄, 1.0g; FeSO₄·7H₂O, 0.01 g; with 1.0 % crystalline cellulose (or 0.5 % corncob, sawdust or sugarcane pulp) as sole carbon source. Each liter of the respective media contained 1.0ml of a supplement of trace metal containing 0.1% (w/v) ZnSO₄ and 0.05% (w/v) Cu SO₄. 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 (EC 3. 2. 1. 4) activity was assayed by a modification of the reducing-sugar method described by^[13] 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 by the enzyme was measured as glucose equivalent using dinitrosalicylic acid reagent^[14]. 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

Fig 1 shows changes in cellulase activities of *A. niger*, *P. chrysogenum* and *T. harzianum* in cellulose-containing media assayed during incubation for 72 hours. Highest enzyme activity was obtained from the culture broth of *P. chrysogenum* after 60 h incubation with an enzyme activity of 0.67 Units mg protein⁻¹. Enzyme activity of *A. niger* was maximum at 36 h with an activity of 0.54 units mg protein⁻¹ while that of *T. harzianum* was maximum at 12 hours of incubation, with a value of 39 units mg protein⁻¹. The enzyme activity profiles show two busts with *A. niger* and *P. chrysogenum* during the 72 hours incubation period (Fig. 1). With *A. niger*, the major enzyme activity (0.54 units mg protein⁻¹) was obtained at 36 hours whereas the minor activity was at 12 hours. The minor activity for *P. chrysogenum* (0.44 units mg protein⁻¹) occurred at 36 hours, with a major activity 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 the pure crystalline cellulose was the substrate (Table 1). Fig. 2 (a – c) show the graph of 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 activity from *A. niger* broth. The highest cellulase activity of 0.30 unit mg protein⁻¹ was obtained with sawdust after 144 hours of cultivation. The maximum cellulase activity value for sugarcane pulp and corncob were 0.26 and 0.21 unit mg protein⁻¹ respectively (Fig. 2a).

The change in 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 h with sawdust as substrate while the enzyme value of 0.23 unit mg protein⁻¹ with sugarcane pulp was obtained at 96 hours. Maximum value of 0.17 unit mg protein⁻¹ was obtained for corncob 144 h 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 did not induce cellulase production in this organism.

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

Organisms	Average extracellular protein released ($\mu\text{g ml}^{-1}$)			
	Cellulose	Corn cob	Saw dust	Sugar cane Pulp
<i>A. niger</i>	153 \pm 30	495 \pm 65	473 \pm 144	333 \pm 70
<i>P. chrysogenum</i>	135 \pm 25	418 \pm 80	358 \pm 45	258 \pm 35
<i>T. harzianum</i>	128 \pm 23	408 \pm 130	385 \pm 50	353 \pm 60

TABLE 1: Extracellular protein released by *A. niger*, *P. chrysogenum* and *T. harzianum* incubated with different cellulosic materials.

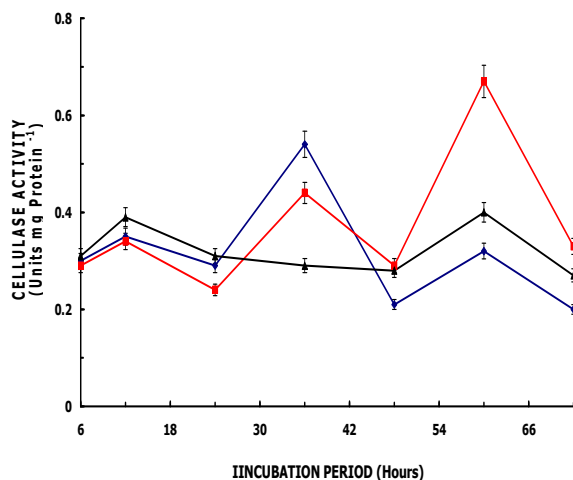


Fig. 1: Cellulase activities of *A. niger* (■), *P. chrysogenum* (■) and *T. harzianum* (●), incubated in cellulose-containing media for 72 hours.

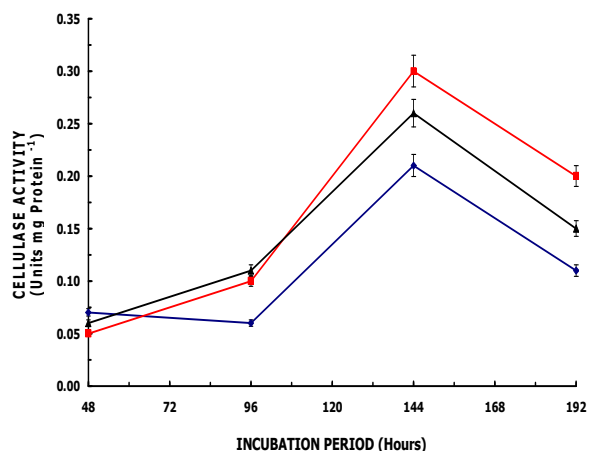


Fig. 2a: Cellulase activity of *A. niger* incubated on sugarcane pulp ▲, corncob ◆, and sawdust ■ for 192 hours.

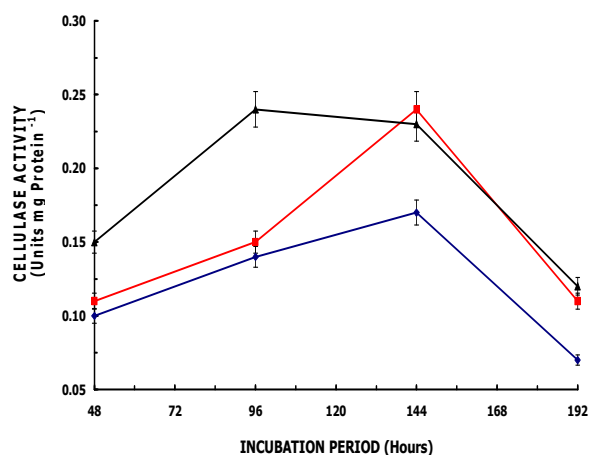


Fig. 2b: Cellulase activity of *P. chrysogenum* incubated on sugarcane pulp ▲, corncob ◆, and sawdust ■ for 192 hours.

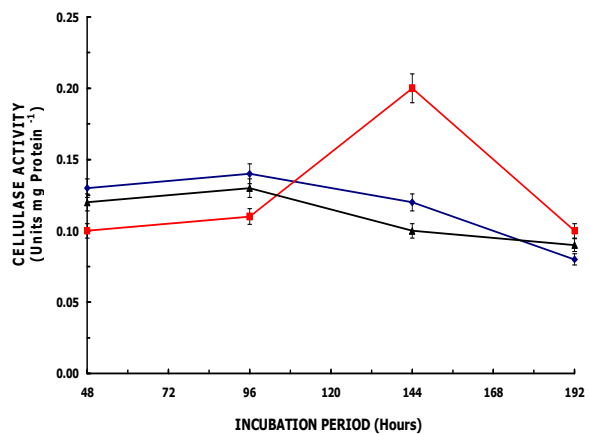


Fig. 2c: Cellulase activity of *T. harzianum* incubated on sugarcane pulp ▲, corncob ◆, and sawdust ■ for 192 hours.

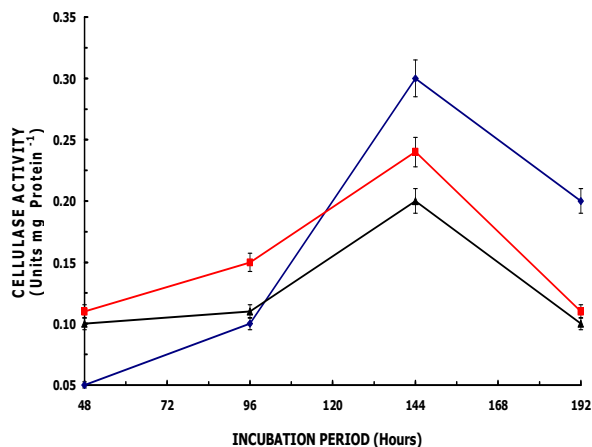


Fig. 3: Cellulase activities of *A. niger* (◆), *P. chrysogenum* (■), and *T. harzianum* (▲), grown on sawdust-containing media and incubated for 192 hours.

Discussion

The organisms have different period for optimal cellulase yield. The time was shorter when incubated on pure cellulose compared to the cellulosic waste materials (Figures 1 and 2). 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 produced the highest amount of cellulase enzyme with pure cellulose as carbon source. On the other hand, *T. harzianum* recorded 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 byproducts [16]. The pattern of production of cellulase when the organisms were grown on waste cellulosic materials offers interesting observations. Firstly, peak activity occurred much later at 144 hours for all the organisms and on all the three substrates. Secondly, *A. niger*, rather than *P. chrysogenum*, was performed better as cellulase producer using the waste cellulosic materials. Thirdly, only sawdust seems to be the only substrate capable of inducing cellulase production for *T. harzianum*.

Sawdust appears to be the most suitable of the three cellulosic materials for cellulase production, for all the organisms, followed by sugarcane pulp (for *A. niger* and *P. chrysogenum*). A similar result was obtained when *A. flavus* (Linn Isolate) was fermented on sawdust, bagasse (sugarcane pulp) and corncob where sawdust gave the highest enzyme yield followed by bagasse [9]. This may also be as a result of adaptation; since the organisms were isolated from wood-wastes [12]. For *P. chrysogenum*, sugarcane pulp yielded cellulase activity very close to that of sawdust but at a shorter time (96 hours) (Figure 2b).

In terms of protein yield, higher values were obtained in media containing the waste cellulosic materials compared to pure crystalline cellulose (Table 1). 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 materials contain other polymers, particularly hemicelluloses and lignin, in addition to cellulose which could induce the production of other degrading enzymes such as hemicellulases [16]. Fungi such as *Aspergillus species* are known to produce many plant cell-wall hydrolyzing enzymes [10]. Hemicelluloses particularly xylanase is required for the hydrolysis of natural cellulose [13]. 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 [4, 8, 17].

In conclusion, the wild strains of *A. niger*, *P. chrysogenum* and *T. harzianum* are capable of producing cellulases from sawdust, sugarcane pulp and corncob. Of the three cellulosic materials, sawdust recorded the highest yield of the enzyme for the three organisms. Sawdust is therefore the most suitable low-cost substrate for cellulase production using the organisms. The waste cellulosic material is a 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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