



Plant Waste Hydrolysis by Extracellular Enzymes of *Aspergillus niger* and *Penicillium chrysogenum*: Effect of Ammonia Pretreatment

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ABSTRACT

Aspergillus niger (ANL301) and *Penicillium chrysogenum* (PCL 501) cultured in basal media with cellulose as sole carbon source yielded extracellular enzymes which partially hydrolyzed sawdust and sugarcane pulp into simple sugars. Pre-treatment of sawdust by ammonium hydroxide steeping increased the yield of simple sugars. The reducing sugars released from the pretreated sawdust by the crude enzymes of *A. niger* (ANL301) and *P. chrysogenum* (PCL 501) were 3.58% and 7.02% of the total hydrolysable sugars respectively. This is in contrast to the 0.92% and 1.02% of the total hydrolysable sugars released respectively by the enzymes of *A. niger* (ANL301) and *P. chrysogenum* (PCL 501) from the non-pretreated sawdust. Enzymatic hydrolysis of sugarcane pulp by the crude enzymes was not significantly affected by ammonia pre-treatment. Reducing sugars released from non-pretreated sugarcane pulp by the crude enzymes of *A. niger* (ANL301) and *P. chrysogenum* (PCL 501) were respectively 4.17% and 5.08% of the total hydrolysable sugars.

Keywords: *Aspergillus niger*, *Penicillium chrysogenum*, extracellular enzymes, sawdust, sugarcane pulp, ammonia pre-treatment, glucose yield.

INTRODUCTION

Cellulose is the predominant component of plant wastes. It occurs naturally in plant cell walls as elementary fibrils closely associated with other structural polysaccharides, particularly hemicelluloses, and a structural non-carbohydrate, lignin. A lignocellulose typically contains by weight 35–50% cellulose, 20–35% hemicellulose, and 5–30% lignin (Klein and Snodgrass, 1993; Lynd *et al.*, 2002). Of the three plant cell wall components, lignin is the most recalcitrant to degradation (Howard *et al.*, 2003). The usefulness of cellulose and other cell wall polysaccharides is dependent upon their hydrolysis into simple sugars (Zhang

and Lynd, 2004). The potential importance of the hydrolysis is widely recognized in the context of conversion of the residual plant biomass considered as ‘wastes’ into various value-added products such as biofuels, chemicals, cheap carbon and energy sources for fermentation, improved animal feeds and human nutrients (Belewu and Afolabi, 2000; Solomon *et al.*, 1999; Wu and Lee, 1997). Conversion of these wastes into such economic products could transform them into a valuable resource, alleviate food and energy shortages, and reduce pollution-load (Kumakura, 1997).

Alkaline and acid hydrolytic methods have been

used to degrade lignocelluloses. Such chemical processes usually involve extreme conditions of pH and high temperatures which could promote glucose degradation into non-specific by-products. There is also the issue of environmental concerns over the disposal of the spent acid or alkali. Unlike the chemical methods, enzymatic hydrolysis operates under very mild conditions and does not produce the undesirable products. It is also environmentally friendly. Three types of cellulases, namely, endoglucanases, exoglucanases and β -glucosidases, and hemicellulases, particularly xylanases, are required for the hydrolysis of lignocelluloses (Khan, 1980). A major limitation of the enzymatic hydrolysis is the poor yield of glucose. Efforts at improving the yield have in recent times centered on the search for new strains of plant cell wall hydrolyzing organisms with novel enzyme properties and pre-treatment of the lignocelluloses to eliminate or reduce the lignin component.

The aim of the present study was to investigate the enzymatic hydrolysis of two plant wastes, sawdust and sugarcane pulp, by extracellular enzymes of *A. niger* (ANL301) and *P. chrysogenum* (PCL501) with emphasis on the effect of ammonia pre-treatment. The strains of *A. niger* and *P. chrysogenum* were isolated from a wood-waste dump in Lagos, Nigeria (Nwodo-Chinedu *et al.*, 2005). The organisms have been shown to grow effectively in basal media supplemented with sawdust or sugarcane (Nwodo-Chinedu *et al.*, 2007 a) and to produce cellulases (Chinedu *et al.*, 2008 a; Nwodo-Chinedu *et al.*, 2007 b) and xylanases (Okafor *et al.*, 2007a, b) in media containing agro-wastes as sole carbon sources.

MATERIALS AND METHODS

Chemicals

All reagents were of analytical grade. Potato Dextrose agar and crystalline cellulose (Avicel) were obtained from Merck, Germany. Carboxymethyl-Cellulose (CM52) was obtained from Whatman Ltd, England. All other reagents were obtained from Sigma Chemicals Co. Ltd, England.

Cellulosic materials

Sawdust of Abora wood (*Mitragyna ciliata*) was collected from Okobaba Saw-mill, Ebute-Metta, Lagos, Nigeria, while mature sugarcane stems (*Saccharum officinarum*) were purchased from Oshodi market, Lagos, Nigeria. The sugarcane pulp was cut into small pieces, crushed in a mortar

and squeezed to remove liquid content. The fibrous pulp was then soaked overnight and washed thoroughly in distilled water until no trace of simple sugar was detected. Samples of sawdust and sugarcane pulp were dried in the oven at 80°C for 2 hours, milled with Marlex exceller grinder (Mumbai, India) and passed through a sieve of about 0.5 mm pore size. The fine powder was used for subsequent analysis (Nwodo-Chinedu *et al.*, 2007a).

Ammonia Pre-treatment

Samples of sawdust and sugarcane pulp were pre-treated by steeping in 2.9M ammonium hydroxide (NH₄OH) solution using the modified ammonia steeping method of Cao *et al.*, (1996). Twenty percent (w/v) of each sample in the NH₄OH solution was incubated at room temperature (28°C) for 24 hours. The NH₄OH solution was removed by decanting and filtration through muslin cloth. The pre-treated materials were washed repeatedly with distilled water to remove residual ammonium hydroxide. After decanting and filtration, the residues were dried to constant weight at 80°C in the oven.

Composition of the cellulosic materials

The proximate compositions of the pre-treated and non-pre-treated samples were determined by the official method of the Association of Official Analytical Chemists (A. O. A. C, 1990) as follows: moisture (section 926.08 and 925.09), protein (section 955.04C and 979.09), fat (section 922.06 and 954.02), ash (section 923.03) and crude fiber (section 962.09).

Fungal strains:

The strains of *Aspergillus niger* (ANL301) and *Penicillium chrysogenum* PCL501 were isolated from wood-wastes in Lagos, Nigeria and identified as described previously (Nwodo-Chinedu *et al.*, 2005). Each organism was maintained at 4°C on Potato Dextrose Agar (PDA) slants.

Cultivation and Enzyme Production:

The extracellular enzymes were produced through submerged fermentation. The organism was grown on basal medium containing (per litre of distilled water): NaNO₃, 3.0 g; KCl, 0.5 g; KH₂PO₄, 1.0 g; MnSO₄ · 7H₂O, 0.5 g; FeSO₄ · 7H₂O, 0.01 g; and 10.0 g cellulose. One litre of the media was supplemented with 1.0 ml of trace solution containing (per litre of distilled water) ZnSO₄, 1.0 g and

CuSO₄ · 5H₂O, 0.5 g. The pH of each media was adjusted to 5.6 using 0.1M HCl. Conical flasks (250 ml) containing 100 ml of respective media were autoclaved at 121°C for 15 minutes, cooled and inoculated with 1.0 ml of spore suspension in 0.1% Tween80 (2- 4 X 10⁶ spores per ml) of the pure fungal isolate. The cultures were incubated for 72 hours with continuous agitation at 100 Osc/min using Griffin flask shaker. Cells were harvested by centrifugation at 6000 x g for 15 minutes at 4°C using ultra centrifuge (Superspeed RC-B, USA). The cell-free culture supernatant was used as source of crude extracellular enzyme.

Protein estimation:

Protein content of the culture supernatant was determined by the Folin Ciocalteu method described by Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard.

1, 4-β- Endoglucanase (EC 3. 2. 1. 4) assay:

A modification of the reducing sugar method described by Khan (1980) was used for the assay of 1, 4-β-Endoglucanase (EC 3. 2. 1. 4) activity as follows: Carboxymethyl-cellulose (CMC) was used as enzyme substrate. The reaction mixture contained 2.0 ml of 0.1% (w/v) CMC in 0.1M sodium acetate buffer (pH 5.0) and 2.0 ml of cell-free culture supernatant. The mixture was incubated at 40°C in water bath with shaking for 30 minutes. The released reducing sugar was assayed by Miller's method (1959) and expressed in glucose equivalent. A unit of activity was defined as amount of enzyme required to liberate 1 μmol of glucose per minute under the assay conditions.

β-Glucosidase (EC 3. 2. 1. 21) Assay:

β-Glucosidase activity was assayed using a modification of Hagerdel method as described by Workman and Day (1982) with p-nitrophenol-β-glucopyranoside (p-NPG) as substrate. A unit of β-glucosidase activity was defined as amount of enzyme required to liberate 1 μmol of nitrophenol per minute under the assay conditions (Workman and Day, 1982).

Xylanase (EC 3. 2. 1. 8) Assay

Xylanase activity was assayed by the modification of the reducing sugar method described by Khan (1980) using β-D-xylan as enzyme substrate. A unit of activity was defined as amount of enzyme required to liberate 1 μmol of xylose per minute under the assay conditions.

Enzymatic Hydrolysis:

Enzymatic hydrolysis of pre-treated and non-pre-treated cellulosic waste materials by the crude extracellular enzymes of *A. niger* and *P. chrysogenum* was carried out for 1 hour. Two milliliters (2.0 ml) of 0.1% (w/v) of the respective wastes in 0.1M sodium acetate buffer (pH 5.0) and 2.0 ml of cell-free culture supernatant were incubated at 40°C in water bath with shaking for 1 hour.

Determination of Total and Reducing Sugars:

Anthrone method (Morris, 1948) was used to determine the total sugar content of the cellulosic materials while the reducing sugar was measured using 3, 5-dinitrosalicylic acid (Miller, 1959). The total sugar content of the cellulosic waste materials and the reducing sugars obtained by the enzymatic hydrolysis were used to calculate the percentage of the total sugars hydrolyzed by the crude enzymes.

RESULTS

The proximate composition of the pre-treated and non-pre-treated waste cellulosic materials (sawdust and sugarcane pulp) are shown in Table 1. The ash contents were 1.1% and 1.8% respectively for sawdust and sugarcane pulp. Petroleum ether extracts gave 6.4% and 5.2% for sawdust and sugarcane pulp respectively while the protein contents were 3.2% and 4.3% for sawdust and sugarcane pulp respectively. The percentage crude fiber was 61.0% and 46.1% for sawdust and sugarcane pulp respectively. The composition of the pre-treated sawdust and sugarcane pulp did not differ markedly from that of the non-pre-treated equivalents except for the fat extracts where lower values were obtained for the pre-treated samples. The crude fiber content of pre-treated sawdust was significantly lower than that of non-pre-treated sample.

The specific enzyme activities of the 3-day cultures of *A. niger* and *P. chrysogenum* in cellulose-containing basal media are shown in Table 2. Crude extracellular proteins of *A. niger* yielded 0.54 ± 0.02, 9.30 ± 0.60, and 4.42 ± 0.30 units mg protein⁻¹ of 1, 4- β-endoglucanase, β-glucosidase and xylanase activity respectively. The culture of *P. chrysogenum* produced crude protein with 0.67 ± 0.03, 19.94 ± 1.30 and 8.50 ± 0.50 units mg protein⁻¹ of 1, 4- β-endoglucanase, β-glucosidase and xylanase activity respectively. Table 3 shows the total and reducing sugars obtained by the enzymatic hydrolysis of the waste materials with the extracellular enzymes of *A. niger* and *P. chry-*

sogenum. Pre-treatment by ammonium hydroxide hydrolysis improved the enzymatic hydrolysis of sawdust by the extracellular enzymes of *A. niger* and *P. chrysogenum*. The percentage reducing sugar released by hydrolysis with crude enzymes of *A. niger* rose from 0.92% (for the non-pre-treated sawdust) to 3.76 % for the pre-treated sawdust. This represents an over 4-fold increase! For

crude enzymes of *P. chrysogenum*, the percentage reducing sugar released increased from 1.02 % (for non-pre-treated sawdust) to 7.03 % (for the pre-treated sawdust), representing about a 7-fold increase! There was no significant effect of the pre-treatment on enzymatic hydrolysis of sugarcane pulp

Table 1: Proximate composition of the non-pre treated and pre treated sawdust and sugarcane pulp.

Component	Non-pre-treated samples		Pre-treated samples	
	Sawdust	Sugarcane Pulp	Sawdust	Sugarcane Pulp
% Moisture	6.1 ± 0.6	4.3 ± 0.5	11.5 ± 1.3	2.8 ± 0.3
% Ash	1.1 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.6 ± 0.3
% Fat	6.4 ± 0.4	5.2 ± 0.5	3.8 ± 0.2	3.6 ± 0.2
% Protein	3.2 ± 0.2	4.3 ± 0.4	2.7 ± 0.2	5.3 ± 0.4
% Crude fiber	61.0 ± 3.4	46.1 ± 2.9	51.8 ± 2.2	46.2 ± 2.3
% Carbohydrate	22.3 ± 1.8	38.6 ± 2.4	28.8 ± 1.6	41.1 ± 2.2

Table 2: Specific activities the crude extracellular enzymes of *A. niger* (ANL301) and *P. chrysogenum* (PCL501).

Enzyme	Substrate	Standard	Specific Activity (unit/ mg protein)	
			<i>A. niger</i>	<i>P. chrysogenum</i>
Endoglucanase (EC 3. 2. 1. 4)	CMC	Glucose	0.54 ± 0.02	0.67 ± 0.03
β-Glucosidase (EC 3. 2. 1. 21)	p - NPG	p-nitrophenol	9.30 ± 0.60	19.94 ± 1.30
Xylanase (EC 3. 2. 1. 8)	Xylan	Xylose	4.42 ± 0.30	8.50 ± 0.50

Table 3: Total hydrolysable sugars and reducing sugars obtained by enzymatic hydrolysis of the cellulosic materials.

Organism	Substrate/ Nature		Total Sugar (mg/ ml)	Reducing Sugar (mg/ ml)	Percentage To- tal Sugar Hy- drolyzed
<i>A. niger</i>	Sawdust	NP	9.8 ± 0.6	0.09 ± 0.02	0.92%
		PT	10.1 ± 0.8	0.38 ± 0.06	3.76%
	Sugarcane pulp	NP	13.2 ± 1.2	0.55 ± 0.08	4.17%
		PT	11.9 ± 0.6	0.53 ± 0.04	4.45%
<i>P. chrysogenum</i>	Sawdust	NP	9.8 ± 0.6	0.10 ± 0.02	1.02%
		PT	10.1 ± 0.8	0.71 ± 0.12	7.03%
	Sugarcane pulp	NP	13.2 ± 1.2	0.67 ± 0.08	5.08%
		PT	11.9 ± 0.6	0.64 ± 0.06	5.38%

[NP = Non-pre-treated; PT = Pre-treated].

DISCUSSION

Proximate analysis of the cellulosic materials used in this study showed they contain high levels of crude fiber (61.0% for sawdust and 46.1 for sugarcane pulp). Ammonia pre-treatment reduced the crude fiber content to 51.8%. This implies that they are largely indigestible by the alimentary enzymes of man (Lehninger, 1982). The crude enzyme preparations of *A. niger* and *P. chrysogenum* were found to hydrolyze the cellulosic wastes (sawdust and sugarcane pulp). Reducing sugars obtained from the enzymatic hydrolysis of sawdust ranged between 0.92% of total hydrolysable sugars for non-pre-treated sawdust and 7.03% for the pre-treated sugarcane pulp. Pre-treatment of sawdust by ammonium hydroxide greatly increased the amount reducing sugars obtained from the hydrolysis. The increases in reducing sugars obtained by enzymatic hydrolysis of sawdust as a result of the pre treatment were 4 folds (from 0.92 to 3.48 mg/ml) and 7 folds (from 1.02 to 7.03 mg/ml) with the crude enzymes of *A. niger* and *P. chrysogenum* respectively. Sawdust (wood) is known to contain high level of lignin (Grant and Long, 1981). Pre-treatment of such plant matter by ammonia steeping or alkali is known to remove or reduce the lignin content thereby improving the enzymatic hydrolysis of the polysaccharides (Howard *et al.*, 2003; MacMillian, 1994). The crude fiber content of non-pre-treated sawdust was

61.0 ± 3.4 as against 51.8 ± 2.2 for the pre-treated sample. This shows that pre-treatment with ammonium hydroxide steeping reduces the fiber content of sawdust. Sawdust (wood) is known to contain high level of lignin (Grant and Long, 1981). The removal of lignin by the pre-treatment may be responsible for the reduction of the fiber content. In contrast, the crude fiber contents of pre-treated and non pre-treated sugarcane pulp do not differ significantly. This implies that sugarcane pulp contains little or no lignin. The reducing sugar obtained from enzymatic hydrolysis of pre-treated sugarcane pulp did not differ from that of the non-pre-treated waste probably for its content of little or no lignin. This is expected given that cellulose and xylan are the principal polysaccharides found in such plant materials; more so since crude extracellular enzymes of both *A. niger* (ANL301) and *P. chrysogenum* (501) show high cellulase (Chinedu *et al.*, 2008a; Nwodo-Chinedu *et al.*, 2007 a) and xylanase activities (Chinedu *et al.*, 2008b; Okafor *et al.*, 2007 a, b). Thin Layer Chromatography (TLC) of the hydrolysates from enzymatic hydrolysis of pre-treated sawdust and sugarcane pulp by the crude enzymes revealed the presence of glucose, xylose and cellobiose as major hydrolytic products. Ammonia pre-treatment is also known to increase the production of cellulases by *A. niger* SL 1 (Abu *et al.*, 2000) and xylanase

production by *A. niger* ANL301 and *P. chrysogenum* PCL501 (Chinedu *et al.*, 2008 b). In conclusion, *A. niger* (ANL301) and *P. chrysogenum* (PCL501) produce hydrolytic enzymes capable of hydrolyzing sawdust and sugarcane pulp into simple sugars. Ammonia pre-treatment

substantially increased the enzymatic hydrolysis of sawdust but appears to have no effect on the hydrolysis of sugarcane pulp. About 7.0% hydrolysis of pre-treated sawdust was achieved with crude enzymes of *P. chrysogenum* (PCL501).

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