

Xylanase Production of *Aspergillus niger* and *Penicillium chrysogenum* from Ammonia Pretreated Cellulosic Waste

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Abstract: Effect of ammonia pretreatment of cellulosic wastes on xylanase production was studied using two microfungi, *Aspergillus niger* ANL301 and *Penicillium chrysogenum* PCL501. Xylanase activity of culture supernatants of the two microfungi, fermented in basal media containing as sole carbon source pretreated and non-pretreated wastes (sawdust of *Mitragyna ciliata*, sugarcane pulp and wheat bran), was measured at 24 h intervals for 120 h. Ammonia pretreatment of the cellulosic wastes enhanced xylanase production by the organisms, inferred from the activity of extracellular xylanase enzyme (endo- β -xylanase: EC 3.2.1.8). Pretreatment of sawdust increased the optimal specific xylanase activities of *A. niger* ANL301 and *P. chrysogenum* PCL501 by 40.2 and 192.7%, respectively. An increase of 72.9 and 63.5% in optimum activity was obtained for *A. niger* ANL301 and *P. chrysogenum* PCL501 respectively by ammonia pretreatment of sugarcane pulp. Pretreatment of wheat bran gave a marginal increase of 3.3% in the optimum xylanase activity of *A. niger* ANL301 and 143.4% activity increase for *P. chrysogenum* PCL501. The present results show that ammonia steeping of the agro-wastes significantly improved xylanase production by the microfungi. The pretreatment method is a cost-effective means for producing xylanases from cellulosic wastes.

Key words: Cellulosic wastes, ammonia pretreatment, xylanase production, Endo- β -xylanase, *Aspergillus niger* ANL301, *Penicillium chrysogenum* PCL501

INTRODUCTION

The structural polymers of the plant cell-wall which constitute the major components of lignocellulosic biomass are important renewable carbon sources available in great quantities on earth (Andren *et al.*, 1975). This underscores the growing interest in their transformation and optimal utilization by man (Howard *et al.*, 2003). Of great importance is the production of industrial enzymes such as cellulases, xylanases and pectinases from these materials using micro-organisms and their bioconversion via enzymatic hydrolysis (Abu *et al.*, 2000; Raji *et al.*, 1998).

Hydrolysis of lignocellulosic biomass is complicated due to the presence of non-glucan components, particularly, lignin. Removal of lignin is believed to have a significant effect on observed rates of enzymatic hydrolysis such that some form of pretreatment to increase amenability to enzymatic hydrolysis is included in most process concepts for bioconversion of lignocelluloses (Lynd *et al.*, 2002). A number of pretreatment methods such as hammer and ball milling, high-energy

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radiation, steaming, alkali or acid wet oxidation as well as biological treatments have been advanced to breakdown lignin structure and cleave lignin from the cellulose and hemicellulose components of the plant cell wall (Lynd *et al.*, 2002; Shipnei *et al.*, 1995; Sun and Cheng, 2002). One important pretreatment method for biomass conversion is the ammonia pretreatment process involving the steeping of biomass in 2.9 M NH₄OH. The method has been reported to remove about 80-90% lignin along with almost all the acetate from cellulosic residues (Cao *et al.*, 1996) and also to reduce the content of toxic compounds, such as acetic acid and phenolic compounds, created in xylitol fermentation from rice straw hydrolysate by *Candida tropicalis* As 2.1776 (Deng *et al.*, 2007). In a comparative study of cellulase production from sorghum bran and wheat bran using different pretreatment methods, a strain of *Aspergillus niger* (SL.1) gave the best cellulase activity through the combination of milling and ammonia steeping process (Abu *et al.*, 2000). Pretreatment of rice straw pulp with Titanium (IV) oxide (TiO₂) has been reported to improve xylanase production by *Streptomyces* sp. (Rifaat *et al.*, 2005). However, not much is known about the effect of ammonia pretreatment of lignocelluloses on xylanase production.

In the present study, the effect of ammonia pretreatment of sawdust of *Mitragyna ciliata*, sugarcane pulp and wheat bran, on xylanase production was investigated using strains of *Aspergillus niger* (ANL301) and *Penicillium chrysogenum* (PCL501). The microfungi which were isolated from decomposing wood-wastes in Lagos, Nigeria (Nwodo-Chinedu *et al.*, 2005) grow effectively in mineral salt medium supplemented with sawdust or sugarcane as sole carbon sources (Nwodo-Chinedu *et al.*, 2007a) and produce cellulases (Nwodo-Chinedu *et al.*, 2005, 2007b) and xylanases (Okafor *et al.*, 2007) when cultured in media containing agrowastes as sole carbon sources. The effect of pretreatment of cellulosic wastes on xylanase enzyme production from *the microfungi* was investigated in submerged fermentation. Data obtained show that ammonia steeping of the lignocellulosic materials significantly enhanced the production of the xylanase enzymes, inferred from endo- β -xylanase (EC 3.2.1.8) activity of the culture supernatants.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents were of analytical grade. Potato Dextrose agar was obtained from Merck, Germany. All other chemicals and reagents were obtained from Sigma Chemicals Co. Ltd, England.

Cellulosic Waste Materials

Sawdust of Abora wood (*Mitragyna ciliata*) was collected from Okobaba Saw-mills, Ebute-Metta, Lagos, Nigeria. Mature Sugarcane (*Saccharum officinarum*) and dry 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. Wheat bran was obtained from Mushin market in Lagos, Nigeria. All the materials were identified in the Department of Botany and Microbiology, University of Lagos, Akoka-Yaba, Lagos, Nigeria. The samples were dried in the oven at 80°C for 2 h, ground with Marlex Exceller Grinder (Mumbai, India) and passed through a sieve (about 0.5 mm pore size) to obtain the respective fine powder (non-pretreated samples) used for the study.

Pretreatment of Cellulosic Materials

The cellulosic wastes were pretreated by ammonia steeping as described by Cao *et al.* (1996). Twenty grams of the powdered cellulosic waste (non-pretreated sample) was mixed with 100 mL of 2.9 M NH₄OH solution in a 250 mL Erlenmeyer flask and placed on an orbital shaker at 30°C for

24 h. After filtering, the residue was washed repeatedly with distilled water to remove excess ammonia and dried to a constant weight in an oven at 80°C.

Organisms

The strains of *Aspergillus niger* ANL301 and *Penicillium chrysogenum* PCL501 were isolated from wood-wastes in Lagos, Nigeria and identified as previously described (Nwodo-Chinedu *et al.*, 2005) and maintained at 4°C on Potato Dextrose Agar (PDA) slants. The organisms were separately cultivated on fresh, sterile PDA plates and incubated at 30°C to obtain the inocula used for the study.

Media Preparations and Enzyme Production

The media contained (per liter 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 a carbon source (Sawdust, Sugarcane pulps and Wheat bran), 10.0 g. One liter of the media was supplemented with 1.0 mL of trace metal solution containing (per liter of distilled water) ZnSO₄, 1.0 g and CuSO₄·5H₂O, 0.5 g. The pH of each media was adjusted to 5.6. Then, 100 mL of the respective liquid medium was placed in 250 mL Erlenmeyer flask and sterilize by autoclaving at 121°C for 15 minutes. This was cooled and inoculated with 10 discs of 5.0 mm diameter of the organism from 3-day PDA culture plates using a sterile cork borer. The cultures were incubated at 30°C with continuous agitation using Griffin flask shaker (100 Osc min⁻¹) and harvested in triplicates at 24 h intervals by centrifugation at 1000 X g over a period of 120 h. The supernatants were the crude extracellular enzyme source.

Protein Assay

Protein contents of the culture supernatants were assayed by the folin ciocalteu method of Lowry *et al.* (1951) using Bovine Serum Albumin (BSA) as standard.

Xylanase Assay

A modification of the reducing sugar method described by Khan (1980), was used to for the assay of xylanase (EC 3.2.1.8) activity. Oat spelt xylan (Fluka) was used as enzyme substrate. The reaction mixture contained 0.5 mL of 0.1% (w/v) substrate in 0.1 M sodium acetate buffer (pH 5.0) and 0.1 mL of cell-free culture supernatant. The mixture was incubated at 40°C in water bath with shaking for 30 min. The reducing sugar released was measured using 3,5-dinitrosalicylic acid (Miller, 1959) and xylose as standard. The colour was developed by boiling in water bath for 5 minutes. Absorbance was read at 540 nm using spectrophotometer (Spectronic Genesys TMS, USA). One unit of activity was defined as amount of enzyme required to liberate 1 µmol of xylose per minute under the assay conditions.

RESULTS AND DISCUSSION

The summary of the effect of ammonia pretreatment of cellulosic wastes on xylanase activity of *A. niger* (ANL301) and *P. chrysogenum* (PCL501) is presented in Table 1. Over 100% increases in optimal specific xylanase activity were obtained with *P. chrysogenum* PCL501 by ammonia pretreatment of sawdust and wheat bran. Ammonia pretreatment of wheat bran, however, produced only an insignificant 3.3% increase in the optimum specific xylanase activity of *A. niger* ANL301.

Figure 1-3 show the specific xylanase (EC 3.2.1.8) activity values of culture supernatants of *A. niger* ANL301 and *P. chrysogenum* PCL501 fermented in basal media containing as sole carbon source ammonia pretreated and non-pretreated wastes (sawdust of *Mitragyna ciliata*, sugarcane pulp and wheat bran). The specific xylanase activities of culture supernatants of the strains of *A. niger* and *P. chrysogenum* incubated in liquid media containing pretreated and non-pretreated sawdust is shown

Table 1: Effect of Ammonia pretreatment of cellulosic wastes on xylanase activity of *A. niger* (ANL301) and *P. chrysogenum* (PCL501)

Organisms	Cellulosic wastes	Optimum xylanase activity (Units mg protein ⁻¹)		Activity increase (%)
		Non-pretreated	Pretreated	
<i>A. niger</i>	Sawdust	1.74	2.44	40.20
	Sugarcane pulp	2.21	3.82	72.90
	Wheat bran	8.30	8.57	3.30
<i>P. chrysogenum</i>	Sawdust	0.96	2.81	192.70
	Sugarcane pulp	3.70	6.05	63.50
	Wheat bran	2.12	5.16	143.40

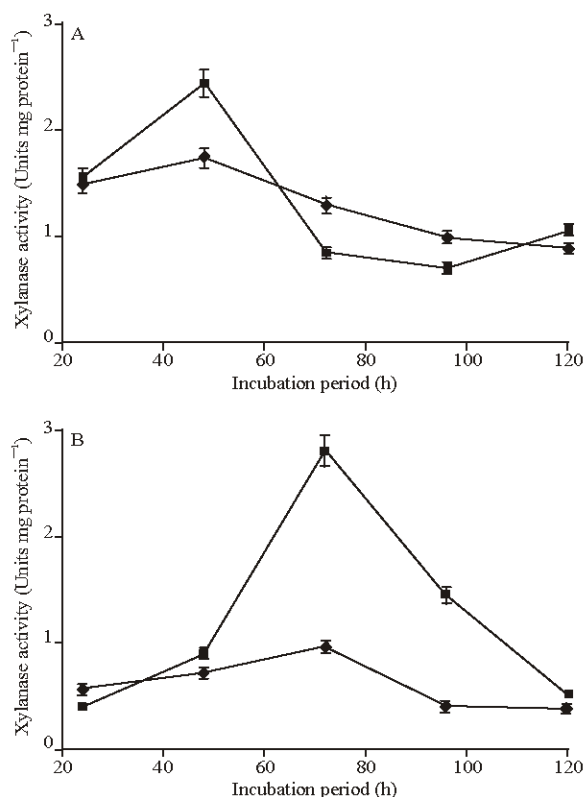


Fig. 1: Specific xylanase activities of the culture supernatants of (A) *Aspergillus niger* ANL301 and (B) *Penicillium chrysogenum* PCL501 incubated for 24-120 h at 30°C in liquid media containing non-pretreated (♦) and pretreated (■) sawdust

in Figure 1. Maximum specific activity values of 2.44 and 1.74 units mg protein⁻¹ respectively were obtained at 48 h of incubation for the pretreated and non-pretreated sawdust using the strain of *A. niger*. The peak specific xylanase activity values obtained with *P. chrysogenum* were 2.81 and 0.96 units mg protein⁻¹ respectively at 72 h for the pretreated and non-pretreated sawdust. *A. niger* gave peak specific xylanase values of 3.82 and 2.21 units mg protein⁻¹ respectively at 48 h for the pretreated and non-pretreated sugarcane pulp, whereas *P. chrysogenum* yielded 6.05 and 3.70 units mg protein⁻¹ respectively at 72 h of incubation (Fig. 2). Peak specific xylanase activity values of 8.57 and 8.30 units mg protein⁻¹ respectively were obtained for the pretreated and

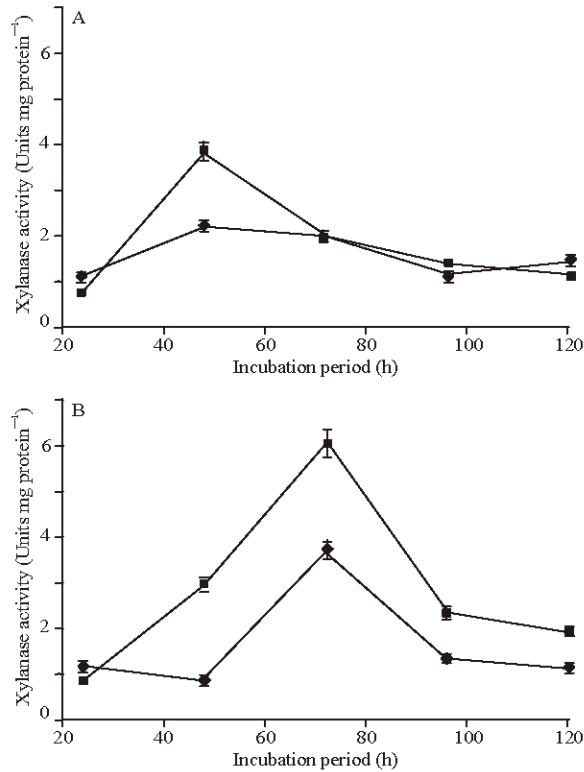


Fig. 2: Specific xylanase activities of the culture supernatants of (A) *Aspergillus niger* ANL301 and (B) *Penicillium chrysogenum* PCL501 incubated for 24-120 h at 30°C in liquid media containing non-pretreated (◆) and pretreated (■) sugarcane pulp

non-pretreated wheat bran at 96 h using *A. niger* and while specific xylanase activity values of 2.12 and 5.16 units mg protein⁻¹ respectively were obtained at 72 h using *P. chrysogenum* (Fig. 3).

Ammonia pretreatment of the cellulosic wastes stimulated higher xylanase activity in the culture supernatants of the two microfungi compared to that from the non-pretreated wastes. This is in line with reports of increased cellulolytic and xylanolytic activities due to pretreatment of lignocelluloses (Abu *et al.*, 2000; Rifaat *et al.*, 2005). The degree of enhancement of xylanase production by ammonia steeping appears to be influenced by the organism involved as well as the type of cellulosic material used. For instance, ammonia pretreatment of sawdust improved xylanase activity of *P. chrysogenum* PCL501 by as much as 192.7%, whereas only an increase of 40.2% was obtained with *A. niger* ANL301. Xylanase production by *P. chrysogenum* PCL501 seems to be much more enhanced by ammonia pretreatment compared to *A. niger* ANL301.

The results also gave an indication on substrate suitability for xylanase production by the microfungi. Of the three cellulosic wastes investigated, wheat bran is the best substrate for xylanase production by *A. niger* ANL301 while sugarcane pulp proved to be the best substrate for *P. chrysogenum* PCL501. Incidentally, ammonia steeping seems to have the least effect on the xylanase production by the organisms when the best substrate was used. For example, ammonia steeping showed the least effect (3.3% increase) on the xylanase activity of *A. niger* ANL301 when wheat bran was used as substrate. Similarly, the enhancement of xylanase activity of *P. chrysogenum* PCL501 by ammonia steeping was lowest (63.5%) with sugarcane pulp. However, in spite of the little effect ammonia pretreatment of wheat bran had on xylanase production by *A. niger* ANL301, the optimal

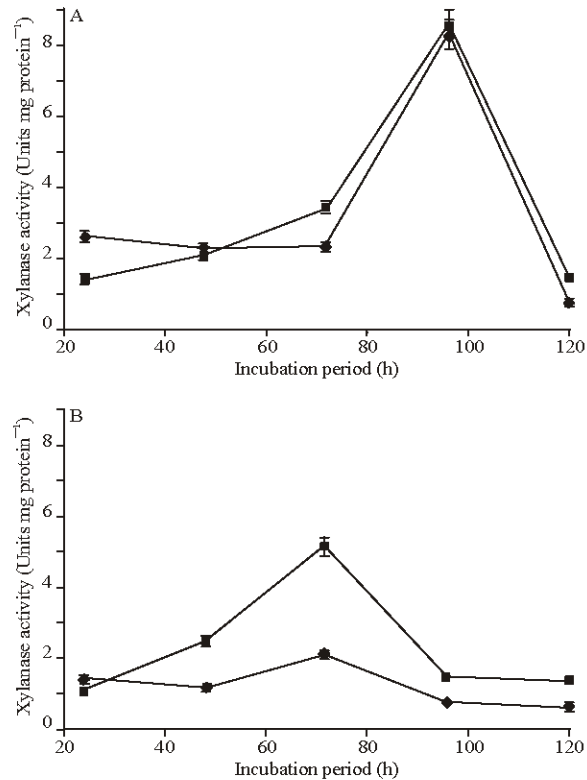


Fig. 3: Specific xylanase activities of the cell-free culture filtrates of (A) *Aspergillus niger* ANL301 and (B) *Penicillium chrysogenum* PCL501 incubated for 24-120 h at 30°C in liquid media containing non-pretreated (◆) and pretreated (■) wheat bran

xylanolytic activity obtained with pretreated wheat bran (8.57 units mg protein⁻¹) was over 3 times and 2 times respectively the values obtained with pretreated sawdust and sugarcane pulp. Thus, wheat bran is a better substrate for xylanase induction in *A. niger* ANL301. For *P. chrysogenum* PCL501, pretreated sugarcane pulp gave the best xylanase activity (6.05 units mg protein⁻¹). This is over 2 times the value obtained with pretreated sawdust and about 1.2 times that obtained with pretreated wheat bran. For both organisms, sawdust gave the least yield of the xylanase enzyme.

Lignin is the most recalcitrant of the major compounds found in the lignocellulosic complex of the plant cell-wall. Experimental results have suggested that alkaline treatment and ammonia steeping of ligninocelluloses involves the cleavage of lignin from the cellulose and hemicellulose component of the plant cell wall, (Shipnei *et al.*, 1995; Cao *et al.*, 1996). The removal of lignin from lignocellulose complexes exposes the cellulose and hemicellulose fractions which can induce the production of xylanases by the organisms. Therefore, the higher levels of the xylanase enzymes imply the availability of more xylan and/or cellulose fractions in the medium. The more the lignin removed, the greater the amount of cellulose/ hemicellulose released for enzyme induction. Several studies have shown that xylanases are co-induced in response to xylan or natural substrates containing hemicellulose or even by pure cellulose (Ganju *et al.*, 1989). Xylanases are commonly induced together with cellulases. Ammonia steeping has been identified as the best pretreatment method for cellulase production (Abu *et al.*, 2000). The organisms are also known to produce high levels of cellulases (Nwodo-Chinedu *et al.*, 2005, 2007b).

Ammonia steeping of the cellulosic wastes also enhances the cultural condition for biomass growth due to the presence of residual nitrogen associated with ammonia (McDonald *et al.*, 1994). This will culminate into better growth of the organism and correspondingly, a higher yield of the hydrolytic enzymes required for the breakdown of the cell wall carbohydrates into simple sugars. Ammonia can be recovered under vacuum at below 60°C with a possible recovery yield of 98% (Cao *et al.*, 1996). Thus, it can be recovered and reused over and over again, thereby ensuring the economic viability of the pretreatment process. In addition, generation of offensive byproducts due to the pretreatment process could be minimized through ammonia removal.

In conclusion, ammonia pretreatment of sawdust of *mitragyna ciliata*, sugarcane pulp and wheat bran significantly enhanced xylanase production by *A. niger* ANL301 and *P. chrysogenum* PCL 501, inferred from endo- β -xylanase (EC 3.2.1.8) activity of the culture supernatants. The effect of ammonia pretreatment on xylanase production appears to depend on the lignocellulosic substrate used and also on the organism employed. Wheat bran and sugarcane pulp are promising substrates for xylanase production using *A. niger* ANL301 and *P. chrysogenum* PCL501. Pretreatment of agro-wastes by ammonia steeping will increase xylanase production and thereby reduce the production cost of the enzyme.

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