PRODUCTION OF FERMENTABLE SUGAR FROM LOCALLY SOURCED LIGNOCELLULOSIC BIOMASS BY *ASPERGILLUS NIGER* ISOLATE

*SALIU, Bolanle Kudirat, SANI, Alhassan & OKE, Mushafau, A.
Department of Microbiology, University of Ilorin, Ilorin. Nigeria.
*E-mail: bks3264@gmail.com, saliu.bk@unilorin.edu.ng
Phone No: +234-802-309-3294, +234-705-767-4167

Abstract

Switchgrass, sunflower stalks, sugarcane baggase, corn cob, corn stover and groundnut shell were pulverised to powder and pre-treated differently with dilute sulphuric acid and sodium hydroxide in preparation for enzymatic conversion to fermentable sugar. Aspergillus niger was isolated from rotten wood, tree bark and soil mixed with sawdust; and screened for cellulase activity using the agar diffusion method with carboxyl methyl cellulose as the substrate. A total of 27 isolates which produced zone of clearance ranging from 4.4 mm to 33.6 mm were obtained. The pre-treated materials were hydrolysed in a submerged fermentation using the A. niger isolate that produced the highest cellulase activity. Acid pre-treatment was found to be more effective in terms of yield of fermentable sugar. Among the lignocellulosic materials, switchgrass pre-treated with acid, produced the highest sugar yield of 1.80±0.05 mg ml^{-1} while the lowest yield of 0.38±0.05 mg ml^{-1} was obtained from alkali pre-treated groundnut shell.

Keywords: Lignocellulose, pre-treament, *Aspergillus niger*, fermentable sugar

Introduction

Lignocelluloses are major forms of biomass that are not only renewable but also sustainable because they do not serve as food for humans and livestock (Binod *et al*., 2011). Lignocelluloses are composed of sugar rich cellulose and hemicelluloses held together by lignin which makes its breakdown difficult. Physical, physico-chemical, chemical and biological treatments have been applied variously to achieve this. A combination of processes has been applied to efficiently and economically convert lignocelluloses to fermentable sugars. Besides biofuels, several organic acids, including lactic, citric, acetic, and succinic acids, can be produced from the products of lignocelluloses (Mussatto and Teixeira, 2010).

One of the major challenges of utilizing lignocelluloses as bio-energy is the cost of producing cellulases and hemicellulases that are responsible for its breakdown to fermentable sugar (Zhang *et al.,* 2006). A reduction in cost is achievable if all materials including the cellulosic microorganisms are locally available. *Aspergillus niger* is one of the fungi that have been studied extensively and is been used for the industrial production of cellulases, the enzyme that hydrolyse cellulose in lignocelluloses to fermentable sugar (Czajkowska *et al*., 1988; Cherry and Fidantsef, 2003; Chandra *et al*., 2007; Narasimha *et al*., 2005). *A. niger* is ubiquitous and grow very well on minimal nutrient (Onions, 1981). Different strains of the fungus have been reported to produce varying yield of fermentable sugar from lignollulosic materials (Coral *et al.,* 2002; Acharya *et al*., 2008; Sridevi *et al.,* 2008). In view of this, a large number of isolates are often screened for cellulase activity in order to select for high productivity.

In addition, the nature of the lignocellulosic material also determines the yield of sugar produced (Mohammed, 2012). Switchgrass, Sunflower stalk, Sugarcane bagasse, Corn cob and stover, as well as *G*roundnut shell reportedly have high cellulose and hemicelluloses content (Solomon,

1999; Ohgren *et al.,* 2005; Mussatto and Teixeira, 2010), and therefore have potentials as feedstock in the production of fermentable sugars. Moreover, these lignocellulosic materials are locally available and in abundance. The prospect of producing high yield of fermentable sugar which can be used for the production of biofuel or other products such as organic acids, from the aforementioned lignocellulosic materials, using the fungus, *A. niger* was the focus of this study.

Materials and Methods

Collection of Samples: Whole corn plants were harvested from one of the farms of the Agronomy Department, Faculty of Agriculture, University of Ilorin, Kwara State, Nigeria after obtaining due permission from the authority. Corn cobs were separated after removing the grains leaving the corn stover. Stems of switch grass and Sunflower plants were collected within the campus of the University of Ilorin. Sugar cane was purchased from a local vendor, cut into small pieces of about 10 to 15 grams each, and the bagasse collected after chewing the cane to squeeze out the juice. Fresh groundnut was purchased from a local retailer at Ipata market in Ilorin. All the materials were thoroughly washed and soaked in distilled water to remove residual sugar and other minerals. The materials were rinsed severally with distilled water, after which they were sundried to constant weight and pulverised using a mechanical milling machine (Vyas *et al.*, 2005).

Isolation of Microorganisms: The fungi used in this study were isolated from samples collected from rotten wood and bark of *Eucalyptus* tree in University of Ilorin; and soil samples from the floor of a wood processing factory at Tanke area of Ilorin town. The wood samples were crushed using mortar and pestle. One gram each of the wood and soil samples were poured into separate test tubes containing 9ml of sterile distilled water and thoroughly shaken to properly dislodge the microorganisms. In a serial dilution technique, 0.1 ml each of 10^{-5} and 10^{-6} diluents were used to inoculate Potato Dextrose Agar (PDA) plates by spread plate method. Plates were incubated at 28 ± 2^{o}C for 72 h. Isolates whose colonial morphology were found to be similar to that of *Aspergillus niger* as described by Onions (1981) were transferred to fresh PDA plates to obtain pure cultures. The isolates were further characterised by cellular morphology and maintained on PDA slants at 4^{o}C.

Screening of Isolates for Cellulase Production: All *Aspergillus niger* isolates were screened for cellulolytic activity by the agar diffusion method described by Hankin and Anagnostakis (1977). Plates containing Potato Dextrose Agar incorporated with 0.5% Carboxyl Methyl Cellulose were inoculated with fungal isolates from 72 hours old culture by streaking once across the middle of the agar medium. Plates were incubated at 28 ± 2^{o}C until visible growth was observed. Cellulolytic isolates were detected after growth, by flooding the plates with 1% congo red solution for 15 minutes. The dye was drained and plates were flooded with 1N sodium chloride solution for another 15 minutes. Clearance around growth of isolate represents cellulase activity. The clearance zone was measured across the growth of organism; the area covered by the organism was also measured at five different locations.

Pretreatment of Lignocellulosic Materials: The lignocellulosic samples were pre-treated with acid and alkali as described by Vyas *et al.* (2005). Powdered samples (50 g each) were measured separately into a set of 500 ml Erlenmeyer flasks containing 250 ml of 0.25N sodium hydroxide solution and another set containing 250 ml hydrochloric acid (0.25N) for alkali and acid pre-treatments respectively. This was brought to boil on a Bunsen flame and left in suspension for 24 hours after which it was washed several times with distilled water. The pH of

the water was monitored until pH 7.0 was attained. The residue was drained through muslin cloth; air dried for 6 hours; and dried again in electric oven at 65^{o}C to constant weight. The dried powder was stored in sealed polythene bags at room temperature (28 ± 2^{o}C).

Enzymatic Hydrolysis

(a) Inoculum Development: Spores of *A. niger* from 7 days old slant culture was harvested by rinsing with 5 ml sterile distilled water into Mandels mineral salt medium (Mandels *et al*., 1974), containing 0.2 % (w/v) carboxyl methyl cellulose (CMC), in 250 ml Erlenmeyer flask and incubated on a gyratory shaker (150 rpm) at room temperature (28 ± 2^{o}C) for 72 h. After the incubation period, spores were counted by using haemocytometer and confirmed by plate count method using PDA.

(b) Fermentation: Mandels mineral salt medium (100 ml each), containing 10 g of the pre-treated cellulosic samples in separate 250 ml Erlenmeyer flasks were inoculated with 5 ml of inoculums culture containing approximately 3.6 x 10^6 spores/ml. Flasks were incubated at 28 ± 2°C on a gyratory shaker (150 rpm) for 10 days and culture fluid were separated by filtering through Whatman no 1 filter paper. The fluid were clarified in a table top high speed refrigerated centrifuge H1850R at 13,000 x g for 20 min at 4°C.

Assays for Reducing Sugar: The amount of sugar in the hydrolysate was determined following the DNSA method of Miller (1959). Dinitro-salicylic acid (DNSA) reagent (1 ml) was added to an aliquot (1 ml) of the hydrolysate in a test tube and properly mixed. The mixture was boiled for 5 min and cooled under running tap water. Five mililitres of 40 % Rochelle salt solution was added to the mixture and absorbance was read in a Genesys-20 Thermo Scientific Spectrophotometer at 540 nm. Amount of reducing sugar was read off glucose standard curve and expressed as mg ml^{-1}.

Statistical Analysis: All data were subjected to analysis of variance and the sample means tested for significant differences using the Duncan multiple intervals and T tests. This was carried out using the statistical package SPSS 15.0.

Results and Discussion

A total of 27 isolates of *Aspergillus niger* were obtained from the various samples tested. Five were isolated from the scrapings of the bark of *Eucalyptus* tree; nine from rotten wood; and the rest were from the soil of a wood processing factory (Table 1). All the isolates were found to be cellulolytic producing zone of clearance ranging from 4.4 mm to 33.6 mm across fungal growth on CMC loaded PDA plates (Table 1). This compared favourably with the cellulolytic *A. niger* used in our earlier work for bio-ethanol production (Saliu and Sani, 2012). The occurrence of cellulolytic fungi on rotten wood and tree bark is an indication that they may be responsible for the degradation of the wood structure. The soil is the greatest reservoir of microorganisms including fungi and *A. niger* is one of the commonest and ubiquitous fungi that occur in most environments being able to utilize a wide range of nutrients due to its ability to secrete a large number of digestive enzymes (Onions *et al.*, 1981). Khalid *et al.* (2006) reported highest frequency for *A. niger* among forty two fungal species isolated from soil samples. The high occurrence of cellulolytic *A. niger* in the soil of wood processing factory is also consequential. Isolation of cellulolytic fungi from soil and other sources have been severally reported (Khalid *et al.*, 2006; Peciulyte, 2007). Three fungal isolates from mangrove leaves and mangrove wood litters were reported to be positive for alkaline cellulases under plate assay (Ravindran *et al.*,

2010). Results from the current study also support the hypothesis that microorganisms for enzyme production be assayed in the environments where the substrates are available as suggested by Fossi *et al.* (2005).

Aspergillus niger (MOT), isolated from rotten wood produced the highest activity as zone of clearance and was used to hydrolyse acid and alkali pre-treated cellulosic samples. A comparative yield of sugar from the hydrolysis is presented in Table 2. Overall, highest yield was obtained from acid pre-treated switch-grass followed by alkali and acid pre-treated corn stover respectively. Schmer *et al.* (2008) reported a high net energy yield and economic feasibility for switchgrass as cellulosic bio-energy crops. Except for corn stover, the acid pre-treated cellulosic samples yielded higher sugar concentration compared to the alkali pre-treated. Yield from alkali pre-treated groundnut shell; sugarcane baggase and sunflower stem were very low compared to others. This may be due to the high lignin content (Mussatto and Teixeira, 2010), which may not have been completely dissolved by the chemical treatments since pre-treatment is said to have significant implications on the configuration and efficiency of other processes in the breakdown of lignocelluloses (Mabee *et al.*, 2006). Moreover, it has been reported that acid and alkali pre-treatments are not universally successful with all types of biomass (Mosier, *et al.*, 2005).

A comparative analysis showed significant difference ($p < 0.05$) between the sugar yield from the alkali and acid pre-treated corn stover, corn cob and groundnut shell; and no significance for those of switchgrass, sugarcane baggase and sunflower stem. Pre-treatment with acid produced a higher yield of sugar after the hydrolysis, compared to the use of alkali. Acid pre-treatment method is an age long and effective method. Dilute acid has been used to solubilise hemicelluloses to yield pentose sugar (Corredor, 2008). However, concentrated acid disrupts the hydrogen bonding between cellulose chains, converting it to a completely amorphous state and making it susceptible to hydrolysis (Binod *et al.*, 2011).

This study has demonstrated that highly cellulotic *A. niger* can be found in abundance in association with wood and soil mixed with pieces of wood; and can be used for the bioconversion of lignocellulosic materials to fermentable sugars. Also, the importance of pre-treatment of lignocelluloses before hydrolysis is established. Finally, acid pre-treatment was shown to produce more fermentable sugar compared to the use of alkali. The prospect of bioethanol from lignocellulosic materials in Nigeria is bright as materials and technology are available locally. Optimization of the pre-treatment and fermentation process may however be required to further improve productivity.

Table 1: Cellulase activity of various isolates of *Aspergillus niger* determined as zone of clearance on potato dextrose agar substituted with carboxyl methyl cellulose

ISOLATE	SOURCE[a]	ZONE OF CLEARANCE[b] (mm)
MOT	RTWD	33.6±1.28
SLY	TRBK	25.8±0.38
MML	RTWD	20.2±0.08
BRM	RTWD	24.8±0.74
ABG	TRBK	25.6±0.41
GWA	RTWD	32.2±0.59
TLO	RTWD	26.2±0.70
BID	RTWD	20.6±0.13
ROC	TRBK	4.4±0.13
BMA	RTWD	8.8±0.19
MOT1	TRBK	5.4±0.05
GWA1	RTWD	5.0±0.15
MML1	RTWD	5.6±0.16
ABG1	TRBK	4.6±0.28
AB1	SWPF	12.5±0.56
CD1	SWPF	13.7±048
CD3	SWPF	14.2±1.12
CD4	SWPF	8.0±0.75
CD5	SWPF	27.0±0.46
CD6	SWPF	14.0±0.88
SD3	SWPF	14.6±0.26
SD4	SWPF	14.1±0.45
SD5	SWPF	30.0±0.38
TK1	SWPF	6.0±0.22
TK5	SWPF	24.1±0.78
UB1	SWPF	13.2±0.34
UB2	SWPF	14.4±0.62

[a] Samples from which the cellulolytic *A. niger* were isolated; TRBK = bark of Eucalyptus tree; RTWD = rotten wood; SWPF = soil of wood processing factory: [b] Each value represents a mean of five determinations ± standard deviation.

Table 2: Comparative yield of fermentable sugar from alkaline and acid pre-treated lignocellulosic materials hydrolysed using *A. niger* isolate MOT

Substrate	Concentration of Sugar (mg ml^{-1})[d]	
	Alkaline pre-treated	Acid pre-treated
Corn stover	$1.70\pm0.10^{1,e}$	$1.56\pm0.11^{2,f}$
Switch grass	$1.68\pm0.75^{1,g}$	$1.80\pm0.05^{1,g}$
Corn cob	$0.75\pm0.07^{2,h}$	$1.23\pm0.05^{2,j}$
Groundnut shell	$0.38\pm0.05^{2,k}$	$1.45\pm0.27^{2,m}$
Sugarcane bagasse	$0.61\pm0.02^{2,n}$	$0.80\pm0.13^{3,n}$
Sunflower	$0.70\pm0.08^{2,p}$	$0.71\pm0.07^{3,p}$

Each value represents a mean of three determinations ± standard deviation. [d] Means were compared among different substrates using Duncan multiple intervals test; and between two pre-treatment methods used i.e. alkali and acid pre- treatment, using the paired samples T-test: Means with different superscript along the same column$^{(1-3)}$ and along the same row$^{(e-p)}$, are significantly different for substrate types and pre-treatment methods respectively ($p < 0.05$).

References

Acharya, P. B., Acharya, D. K. & Modi, H. A. (2008). Optimization for cellulase production by *Aspergillus niger* using sawdust as substrate*. African Journal of Biotechnology,* 7(22), 4147 – 4152.

Chandra, S. M., Viswanath, B. & Reddy, B. R. (2007). Cellulolytic enzymes on lignocellulosic substrates in solid state fermentation by *Aspergillus niger Indian Journal Microbiology* 47(4), 323–328.

Cherry, J. R. & Fidantsef, A. L. (2003). Directed evolution of industrial enzymes: An update. *Current Opinion in Biotechnology*, 14,438–443.

Coral, G. K., Arikan, B., Umaldi, M. N. & Venmez, H. G. (2002). Some properties of crude carboxyl methyl cellulase of *Aspergillus niger* Z10 wild type strain. *Turkish Journal of Biology,* 26, 209-213.

Corredor, D. Y. (2008). *Pretreatment and enzymatic hydrolysis of lignocellulosic biomass.* Dissertation for Doctor of Philosophy, Department of Biological & Agricultural Engineering, Kansas State University.

Czajkowska, D., Hornecka, D. & Ilnicka-Olejniczak, O. (1988). Biosynthesis of cellulolytic enzymes by Aspergillus niger A.n. 33 and its selectants *Acta Biotechnologica,* 8(4), 349–355.

Fossi, B. T., Tavea, F. & Ndjonenkeu, R. (2005). Production and partial characterization of a themostable amylase from ascomycetes yeast strain isolated from starchy soils. *African Journal of Biotechnology,* 4(1), 14-18.

Hankin, L. & Anagnostakis, S. (1977). Solid media containing carboxyl methyl cellulose to detect CM cellulase activity of microorganisms. *Journal General Microbiololgy* 98: 109–15.

Khalid, M., Yang, W., Kishwar, N., Rajput, Z. I., Abdullah, G. & Arijo, A. G. (2006). Study of cellulolytic soil fungi and two nova species and new medium. *Journal of Zhejiang University of Science,* 7(6), 459 - 466.

Mabee, W. E., Saddler, J. N., Nielsen, C., Nielsen, L. H. & Jensen, E. S. (2006). Renewable-based fuels for transport. In: Renewable energy for power and transport. *Riso Energy Report,* 47–50.

Mandels, M., Atontz, L, & Nystrom, J. (1974). Enzymatic hydrolysis of waste cellulose. *Biotechnology Bioengineering Symposium,* 16,1471–93.

Miller, G. L. (1959). Use of dinitrosalicylic acid for the determination of reducing sugar. *Analytical Chemistry,* 31, 426 – 428.

Mohammed, A. H. (2012). *Conversion of lignocellulosic material into fermentable sugars.* Ph.D Dissertation, University of Berlin, Berlin.

Mosier, N., Wyman, C., Dale, B., Elander, R., Holtzapple, Y. Y. L. M., & Ladisch, M. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology,* 96,673–686.

Mussatto, S. I. & Teixeira, J. A. (2010). Lignocellulose as raw material in fermentation processes. In A. Mendez-Villa (Ed.) *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology,* 897 – 907.

Narasimha, G, Sridevi, A., Buddolla, V., Chandra, S. M., & Reddy, R. B. (2006). Nutrient effects on production of cellulolytic enzymes by Aspergillus niger. *African Journal of Biotechnology,* 5(5), 472-476.

Öhgren, K., Galbe, M., & Zacchi, G. (2005). Optimisation of steam pre-treatment of SO2-impregnated corn stover for fuel ethanol production. *Applied Biochemistry and Biotechnology,* 124, 1055–1167.

Onions, A. H. S., Allsopp, D. & Eggins, H. O. W. (1981). *Aspergillus.* In Smith's Introduction to industrial mycology. 7th Edn, Edward Arnold Publishers.

Binod, P., Janu, K. U., Sindhu, R. & Pandey, A. (2011). *Hydrolysis of lignocellulosic biomass for bioethanol production*. In Ashok, P., Christian, L., Steven, C. R., Claude-Gilles, D., & Edgard, G. (Ed.) Biofuels, Burlington: Academic Press 229-250.

Peciulyte, D. (2007). Isolation of cellulolytic fungi from waste paper gradual recycling materials. *EKOLOGIJA,* 53(4), 11–18.

Ravindran, C. Naveenan, T. & Varatharajan, G. (2010). Optimisation of alkaline cellulase production from marine derived fungi, *Chaetomium* sp., using agricultural and industrial wastes as substrates Author version. *Bot. Mar,* 53(3), 275-282.

Saliu, B. K. & Sani, A. (2012). Bioethanol potentials of corn cob hydrolysed using cellulases of *Aspergillus niger* and *Penicillium decumbens. EXCLI Journal,* 11, 468 – 479.

Schmer, M. R., Vogel, K. P., Mitchell, R. B. & Perrin, R. K. (2008). *Net energy of cellulosic ethanol from switchgrass.* Proceedings of the National Academy of Sciences, 105 (2), 464-469.

Solomon, B. O., Amigun, B., Betiku, E., Ojumu, T. V. & Layokun, S. K. (1999). Optimization of cellulase production by *Aspergillus flavus* linn isolate NSPR 101 grown on Bagasse. *Analytical chemistry,* 16, 61-68.

Sridevi, A., Narasimha, G. & Reddy, R. B. (2008). Production of cellulase by *Aspergillus niger* on natural and pretreated lignocellulosic wastes. *The Internet Journal of Microbiology,* 7(1). Retrieved from http://ispub.com/IJMB/7/1/9302

Vyas, A., Vyas, D. & Vyas, K. M. (2005). Production and optimization of cellulases on pretreated groundnut shell by *Aspergillus terreus* AV49. *Journal of Scientific and Industrial Research,* 64, 281-286.

Zhang, P. H., Himmel, M. E. & Mielenz, J. R. (2006). Outlook for cellulase improvement: Screening and selection strategies. *Biotechnology Advances,* 24, 452 – 481.