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Submerged Cultivation of *Aspergillus niger* on Pretreated Sugarcane Bagasse

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Abstract: In the present study *Aspergillus niger* was employed for the production of cell wall degrading enzyme in submerged fermentation using Mendel and Weber media and sugarcane bagasse as a substrate. Sugarcane bagasse was treated with various concentrations (0.5-10% w/v) of Na_2SO_4, Na_2SO_3, H_2O_2 (1-5% v/v) and H_2O_2 + 2% NaOH. Among all these pretreatments, H_2O_2 + 2% NaOH found to be best which favored enzyme production as compared to control (untreated bagasse). Highest enzyme activities of CMCase 26.4 IU/ml, FPase 15.7 IU/ml with 1% w/v Na_2SO_3 treated bagasse and Xylanase 65.8 IU/ml on 2% v/v H_2O_2 + 2% NaOH, respectively after 72hr of harvestation period.

Key words: Sugarcane Bagasse · Pretreatment · *Aspergillus niger* · Submerged fermentation

INTRODUCTION

The most abundant polymer available on earth is cellulose. About 10^{12} metric tons cellulose produced each year by plants [1] and degraded by cellulases produced by variety of fungi and bacteria [2]. Cellulases are of prime importance due to its wide range of applications. Major areas of application includes food, animal feed, textile, fuel, chemical industries, paper and pulp industry, waste management, medical/pharmaceutical industry, protoplast production, genetic engineering and pollution treatment [2-5]. Among all the fungus, *A. niger* is being used in food industry for the production of different enzymes like á-amylase, cellulases, lactase, amyloglucosidase, invertase, pectinases and proteases that advocates its safety regarding food applications [6,7].

Cellulases from various sources have shown their distinctive features as they carry their specific pH optima, substrate specificity, thermostability, solubility and amino acid composition. Most of the cellulases have optimum pH of 4 and 5 and temperature of 40-50°C [8,9].

Xylanases (E.C.2.8.1.8) is a group of hemicellulolytic enzymes which are required for the hydrolyisis of ß1, 4-xylans present in lignocellulosic materials [10]. Xylanases are the microbial enzymes that have aroused great interest recently due to their potential application in many industrial processes viz; production of hydrolysates from agro-industrial wastes [10, 11,] nutritional improvement of lignocellulosic feed stuff [12], clarification of juices and wines [13] and biobleaching of craft pulp in paper industry [14].

There are various reports on production of enzymes, ethanol, single-cell protein (SCP), etc., using raw bagasse or treated-bagasse in submerged fermentation. Generally basidiomycetes have been employed for the production of extra-cellular cellulases (exo-glucanase, endo-glucanase and b-glucosidase) and ligninases [15]. The present study was conducted to check the production of enzyme from various pretreated sugarcane bagasse by *Aspergillus niger* in submerged production.

MATERIAL AND METHODS

Lignocellulosic Biomass: Sugar cane bagasse procured from Shakar Gunj Sugar mills (Pvt.) Limited, Jhang Road, Faisalabad, Pakistan. Used as a source of lignocellulosic biomass. The biomass was washed and dried to remove the unwanted particles and then milled into powdered form (2mm) with hammer beater mill.

Pretreatment of Biomass: Sugarcane bagasse samples (10g) were soaked in different concentration of solutions like Na_2SO_4 (0.5-10% w/v), Na_2SO_3 (0.5-10% w/v) H_2O_2

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(1-5% v/v) and H_2O_2 + 2%NaOH ranging from 1-5% (v/v) at the ratio of 1: 10 (solid: liquid) for 2hr at room temperature [16,17]. After that the samples were heated at 121°C for 15 min at 15lb psi. Then samples were filtered and solid residues were washed up to neutrality.

Preparation of Spore Suspension: The slants of five days old cultures were wetted by adding 10 ml of sterilized distilled water. The spores were scratched by sterile wire loop to break clumps and obtain homogeneous spore suspension. One milliliter of spore suspension containing 1 x 10^8 spores was used as inoculum.

Enzyme Production: *Aspergillus niger* was grown on medium as described by Mandles and Weber [18]. The media contained (per liter of distilled water): Urea 0.3 g, $(NH_4)_2SO_4$ 1.4 g, KH_2PO_4 2.0 g, $CaCl_2$ 0.3 g, $MgSO_4$-$7H_2O$ 0.3 g, protease peptone 1.0 g, $FeSO_4$-$7H_2O$ 5.0 mg, $MnSO_4$-$7H_2O$ 1.6 mg, $ZnSO_4$-$7H_2O$ 1.4 mg, $CoCl_2$ 2.0 mg. The pH of media was adjusted to 5.0 ± 0.2. Then, 25 ml of the liquid medium was placed in 250 ml Erlenmeyer flask and sterilized at 121°C for 15 min. After sterilization, the media was allowed to cooled and inoculated with 0.5 ml of spore suspension of *Aspergillus niger* containing approximately 10^8 spores per milliliter. The inoculated flasks were incubated at 30±1°C for 96 hrs with the agitation speed of 120rpm. After termination of fermentation period the culture filtrate was centrifuged at 8000 rpm for 10 min at 4°C to remove unwanted particles and spores. The supernatants obtained after centrifugation were used as the crude extracellular enzyme source.

Estimation of CMCase: 500 µL of the enzyme sample along with 500 µL of 1% (w/v) CMC in 50 mM acetate buffer pH 5 was incubated, in a water bath at 50°C, for 30 min. After incubation 1.5 mL of DNS was added and boiled for 5 minutes and absorbance was taken spectrophotometrically at 550nm. The reducing ends liberated were then measured with DNS [19]. One unit of CMCase activity was defined as the amount of enzyme that required to release one micromole of glucose per minute under assay conditions.

Estimation of Fpase: 500 µL of culture filtrate was added to test tube containing Whatman No.1 filter paper strip (1x 6 cm) incubated at 50°C for 30 min. After that 1.5 ml DNS were added to test tube and boiled for 5 minutes and absorbance was taken spectrophotometrically at 550nm. The reducing ends liberated were then measured with DNS [20]. One unit of enzyme activity was defined as the amount of enzyme that required to release one micromole of glucose per minute under assay conditions.

Estimation of Xylanase: Xylanase activity in the culture filtrate was measured by adding 0.5 ml of 1% birch wood xylan prepared in 0.05 M sodium citrate buffer pH (5.0) and 0.5 ml of appropriately diluted enzyme were incubated at 50°C for 30 min. The reaction was terminated by adding 1.5ml of DNS [20]. After this the test tubes containing reaction mixtures were boiled for 5 minutes and absorbance was taken spectrophotometrically at 550nm. One unit (U) of xylanase was defined as the amount of enzyme releasing 1 micromole of xylose per minute under the assays conditions.

Total Protein Determination: Total protein in the culture filtrate was determined by the method as described by Lowery [21] using BSA as standard.

Glucose Determination: Glucose produced in the culture filtrate was determined by Miller's [20] method.

RESULTS AND DISCUSSION

In the present study *Aspergillus niger* was grown on variously treated sugarcane bagasse in submerged fermentation. During growth in submerged fermentation various secondary metabolites like CMCase, FPase and Xylanases were estimated in culture filtrate. Total proteins and reducing sugars as glucose equilent produced by *Aspergillus niger* was also measured in the culture filtrate. In the whole study sugarcane bagasse was used as substrate which was pretreated with different concentrations of Na_2SO_4, Na_2SO_3, H_2O_2 and H_2O_2 + 2% NaOH. The main purpose of the study was to check the effect of pretreatment on enzyme production from bagasse. Pretreatment is process in which biomass was easily accessible for microbial attack.

Figure 1 represents the data showing enzyme production from variously concentrations of Na_2SO_4 treated sugarcane bagasse. Results indicated that highest activities of CMCase (19.2 IU/ml) was observed at 7% Na_2SO_4 treated bagasse, FPase activity of 10.7 IU/ml at 4% Na_2SO_4 treated bagasse and highest xylanase activity of 46.7 IU/ml was observed with 2.5% Na_2SO_4 treated bagasse in submerged fermentation. Highest protein production and glucose was observed at 0.73 mg/ml at 5.5% Na_2SO_4 treated bagasse and 0.066mg/ml at 6% Na_2SO_4 treated bagasse respectively. Lowest glucose production was noted at 2% Na_2SO_4 treated bagasse and protein 0.54mg/ml at 4 and 5% Na_2SO_4 treated bagasse.

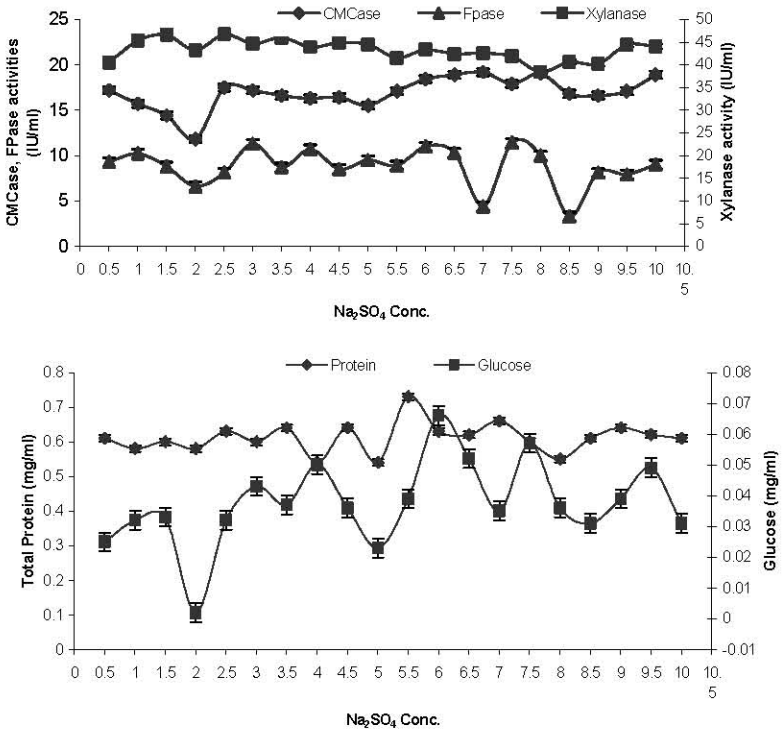

Fig. 1: Xylanase, CMCase FPase activities and protein and glucose production from *Aspergillus niger* on various concentrations of Na_2SO_4 treated sugarcane bagasse in submerged fermentation at 30°C with agitation speed of 120rpm. The error bars represent the standard deviation of the means based on three replicates.

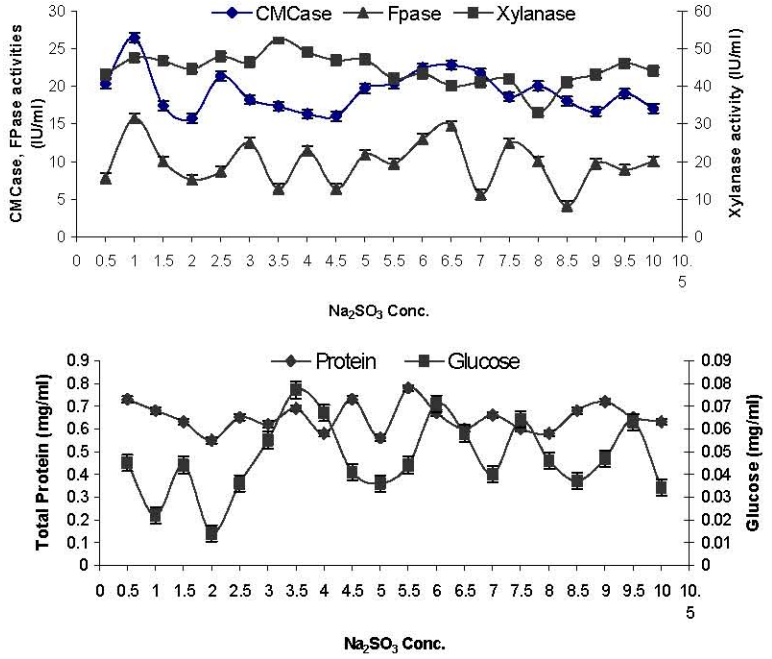

Fig. 2: Xylanase, CMCase FPase activities and protein and glucose production from *Aspergillus niger* on various concentrations of Na_2SO_3treated sugarcane bagasse in submerged fermentation at 30°C with agitation speed of 120rpm. The error bars represent the standard deviation of the means based on three replicates.

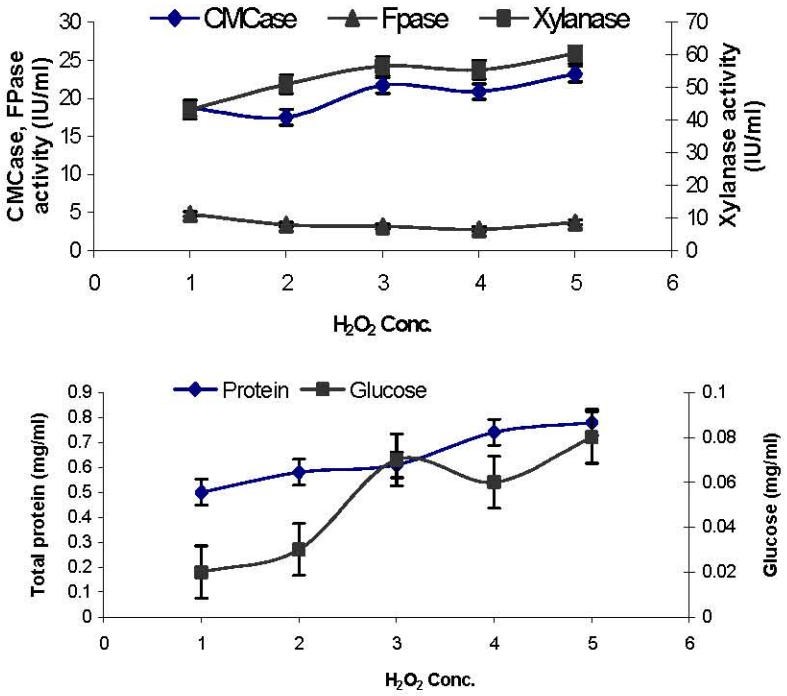

Fig. 3: Xylanase, CMCase FPase activities and protein and glucose production from *Aspergillus niger* on various concentrations of H_2O_2 treated sugarcane bagasse in submerged fermentation at 30°C with agitation speed of 120rpm. The error bars represent the standard deviation of the means based on three replicates.

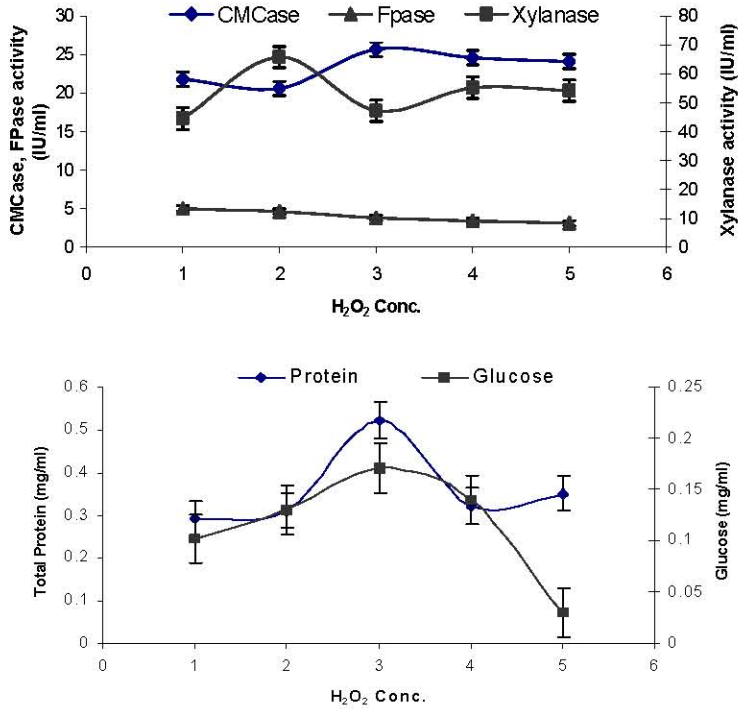

Fig. 4: Xylanase, CMCase FPase activities and protein and glucose production from *Aspergillus niger* on various concentrations of H_2O_2 + 2% NaOH treated sugarcane bagasse in submerged fermentation at 30°C with agitation speed of 120rpm. The error bars represent the standard deviation of the means based on three replicates.

Results in the figure 2 described the enzymes production with Na_2SO_3 (Na2SO3 2 & 3 in subscript form) treated bagasse. Maximum CMCase (26.4 IU/ml) and FPase (15.7 IU/ml) production was observed at 1% Na_2SO_3 treated bagasse and maximum Xylanase yield (52.6 IU/ml) was obtained with 3.5% Na_2SO_3 treated bagasse. Protein content of 0.78mg/ml was also observed at 5.5% Na_2SO_3 treated bagasse which was highest among all the experiments conducted with Na_2SO_3 treated bagasse. Glucose content of 0.077mg/ml was also estimated at 3.5% Na_2SO_3 treated bagasse.

In another experiments sugarcane bagasse was treated with various concentrations of $H_2O_2$and its combination with 2% NaOH. When enzyme production was checked on H_2O_2treated bagasse, highest enzyme activities was observed as compared to Na_2SO_4 and Na_2SO_3 treated bagasse. Highest xylanase and CMCase activity of 60.3 IU/ml and 23.2 IU/ml was obtained with 5% v/v H_2O_2treated bagasse and FPase activity of 4.8 IU/ml was obtained with 1% v/v H_2O_2 treated bagasse. Maximum protein (0.78mg/ml) and glucose (0.08mg/ml) was observed with 5% v/v H_2O_2treated bagasse as shown in figure 3.

Figure 4 represented the enzyme production on various concentrations of H_2O_2 + 2% NaOH treated bagasse. Results showed that maximum CMCase activity of 25.7IU/ml was observed at 3% v/v H_2O_2 + 2% NaOH treated bagasse and maximum xylanase activity of 65.8IU/ml with 2% v/v H_2O_2 + 2% NaOH treated bagasse and FPase activity of 5.0 IU/ml was observed with 1% v/v H_2O_2 + 2% NaOH treated bagasse. Secretion of protein (0.52 mg/ml) and glucose (0.17 mg/ml) obtained with 3% v/v H_2O_2 + 2% NaOH treated bagasse which was highest in this set of experiment.

Sridevi *et al*. [22] worked on cellulase production by *Aspergillus niger* using natural and pretreated substrates. They reported that 2fold increase in CMCase and FPase production was observed with pretreatment of sugarcane bagasse with 1% NaOH and 1% H_2O_2. Gamarra *et al.* [23] studied on endoglucanase and xylanase production from *Aspergillus niger* and reported that after 72hr of fermentation period highest yield of endoglucanase (1854 U/L) and xylanase activity of 5051 U/L with protein secretion of 0.3 g/L in submerged fermentation. Ojumu *et al*. [24] produced cellulase from *Aspergillus flavus linn* isolate using 1% NaOH treated bagasse and reporting highest enzyme production within twelfth hour of fermentation period. The increase or decrease in enzyme production after pretreatment might be due to the production of some water soluble aromatic products which inhibit the cellulytic action of enzymes produced during pretreatment [25,26]. Tao *et al*. [27] studied on enzymes of *Aspergillus glaucus* using sugarcane bagasse as substrate and reported that maximum CMCase, FPase, Xylanase activity was found at fourth, sixth and third day of fermentation period respectively. Pretreatment of saw dust with 2N NaOH gave the highest cellulase activity of 0.1813 IU/ml using *Aspergillus niger* in submerged fermentation [28]. In cellulase production, nature of substrate is very important which affect the induction of enzyme production in fermentation [29]. In various fermentation processes the CMCase activity was higher than FPase [30] which was in line with the present work. Highest cellulase activity depends upon the nature of substrate, fungus and different cultural conditions [24, 31]. Milagres *et al.* [32] reported that the production of xylanase was inducible by bagasse using a local fungal isolate. Adsul *et al.* [33] pretreated bagasse samples with $NaClO_2$ and enhanced level of xylanase and β–glucosidase production by *P.janthinellum* NCIM1171 and *T.viride* NCIM 1051 in submerged fermentation. Bharathiraja and Jayamuthunagai [34] treated wood with solution of 4%NaOH and reported maximum cellulase activity of 6 IFPU/ml during seventh day of fermentation period.

Results of the present study concluded that pretreatment of biomass significantly favored the fungal growth, but enzyme production was comparatively better than untreated bagasse. Among the various pretreated sugarcane bagasse with Na_2SO_4 (0.5-10% w/v), Na_2SO_3 (0.5-10% w/v) H_2O_2 (1-5% v/v) and H_2O_2 + 2%NaOH ranging from 1-5% (v/v), treatment of with H_2O_2 + 2% NaOH improved enzyme production yielding 65.8 IU/ml Xylanase activity and 25.7 IU/ml of CMCase activity. The results of this study indicated that the strain was a good xylanase and CMCase producer as compared to FPase activity.

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REFERENCES

1. Stryer, L., 1988. Biochemistry, 3rd edn. Freeman, New York.
2. Beguin, P. and J.P. Anbert, 1993. The biological degradation of cellulose. FEMS Microbiol. Rev., 13: 25-58.

3. Coughlan, M.P., 1985. Cellulases: Production properties and applications. Biochem. Soc. Trans., 13: 405-406.
4. Mandels, M., 1985. Applications of cellulases. Biochem. Soc. Trans., 13: 414-415.
5. Tarek, A.A.M. and A.T. Nagwa, 2007. Optimization of cellulase and β-glucosidase induction by sugarbeet pathogen *Sclerotium rolfsii*. Afr. J. Biotechnol., 6(8): 1048-1054.
6. Immanuel, G., C.M.A. Bhagavath, P.L Raj, P. Esakkirajand and A. Palavesam, 2007. Production and partial purification of cellulase by *Aspergillus niger* and *A. fumigatus* fermented in coir waste and sawdust. Internet J. Microbiol., 3(1):
7. Maciel, G.M., L.P.S. Vandenberghe, C. Windson, I. Haminiuk, R.C. Fendrich, B.E.D. Bianca, T.Q.D. Brandalize, A. Pandey and C.R. Soccol, 2008. Xylanase production by *Aspergillus niger* lpb 326 in solid-state fermentation using statistical experimental designs. Food Technol. Biotechnol., 46(2): 183-189.
8. Bhat, M.K., 2000. Cellulases and related enzymes in biotechnology. Biotechnol. Advan.,18: 355-383.
9. Parry, N.J., D.E. Beever, E. Owen, I. Vandnberghe, J.V. Beeumen and M. Bhat, 2002. Biochemical characterization and mechanism of action of a thermostable ß-glucosidase purified from *Thermoascus aurantiacus*. Biochem. J., 353: 117-127.
10. Kheng, P.P. and I.C. Omar, 2005. Xylanase production by a local fungal isolate, *Aspergillus niger* USM AI 1 via solid state fermentation using palm kernel cake (PKC) as substrate. Songklanakarin J. Sci. Technol., 27: 325-336.
11. Gessesse, A. and B.A. Gashe, 1997. Production of alkaline xylanase by an alkaliphilic *Bacillus* sp. isolated from an alkaline soda lake. J. Appl. Microbiol., 83: 402-406.
12. Wallace, R.J., N.M. Wallace, V.L. Nsereko and G.F. Hartnell, 2001. Influence of supplementary fibrolytic enzymes on the fermentation of corn and grass silage by mixed ruminal microorganisms *in vitro*. J. Anim. Sci., 79: 1905-1916.
13. Gable, M. and G. Zacchi, 2002. A review of the production of ethanol from softwood. Appl. Biochem. Biotechnol., 59: 618-628.
14. Yinbo, Q., G. Peizi, W. Dong, Z. Xin and Xiao, 1996. Production, charaterisation and application of the cellulase free xylanase from *Aspergillus niger*. Appl. Biochem. Biotechnol., 57/58: 375-381.
15. Pandey, A., C.R. Soccol, P. Nigam and V.T. Soccol, 2000. Biotechnological potential of agro-industrial residues. I: sugarcane Bagasse. Biores. Technol., 74: 69-80.
16. Gharpuray, M.M., Y.H. Lee and L.T. Fan, 1983. Structural modification of lignocellulosics by treatment to enhance enzymatic hydrolysis. Biotechnol. Bioengin., 25: 157-172.
17. Solomon, B.O., B. Amigun, E. Betikue, T.V. Ojumu and S.K. Layokun, 1999. optimization of cellulose production by *Aspergillous flavus* Linn isolates NSPR 101 grown on bagass. JNSChE., 16: 61-68.
18. Mandels, M. and J. Weber, 1969. The production of cellulases. Advan. Chem. Ser., 95: 391-414.
19. Wood, T.M. and K.M. Bhat, 1988. Methods for measuring cellulase activities. Methods Enzymol Biomass Part A Cellulose Hemicellulose, 160: 87-112.
20. Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars, Anal. Chem. 31: 426-428.
21. Lowry, O.H., A.F. Roser and R. Randall, 1951. Protein measurement with folin phenol reagent. J. Biol. Chem., 242: 265-275.
22. Sridevi, A., G. Narasimha and B.R. Reddy, 2009. Production of Cellulase by Aspergillus niger on natural and pretreated lignocellulosic wastes. The Int. J. Microbiol., 7(1).
23. Gamarra, N.N., G.K. Villena and M. Gutierrez-Correa, 2010. Cellulase production by *Aspergillus niger* in biofilm, solid state and submerged fermentation. Appl. Microbiol. Biotechnol., 87: 545-551.
24. Ojumu, T.V., B.O. Solomon, E. Betiku, S.K. Layokun and B. Amigun, 2003. Cellulase Production by *Aspergillus flavus Linn* Isolate NSPR 101 fermented in sawdust, bagasse and corncob. Afri. J. Biotechnol., 2(6): 150-152.
25. Hattaka, A.I., 1983. Biological pretreatment of lignocellulose. Appl. Microbiol. Biotechnol., 18: 350-357.
26. Ali, S., A. Sayed, R.T. Sarker and R. Alam, 1991. Factors affecting cellulase production by *Aspergillus terreus*. World J. Microbiol. Biotechnol., 7: 62-66
27. Tao, Y.M., X.Z. Zhu, J.Z. Huang, S.J. Ma, X.B. Wu, M.N. Long and Q.X. Chen, 2010. Purification and properties of endoglucanase from a sugarcane bagasse hydrolyzing strain, *Aspergillus glaucus* XC9. J. Agric. Food Chem., 58: 6126-6130.
28. Acharya, P.B., D.K. Acharya and H.A. Modi, 2008. Optimization for cellulase production by *Aspergillus niger* using saw dust as substrate. Afr. J. Biotechnol., 7(22): 4147-4152.
29. Kang, S.W., Y.S. Park, J.S. Lee, S.I. Hong and S.W. Kim, 2004. Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass, Biores. Technol., 91: 153-156.

30. Pandey, A., P. Selvakumar, C.R. Soccol and P. Nigam, 1999. Solid State Cultivation for the Production of Industrial Enzymes. Current Sci., 77: 149-162.
31. Alam, M.Z., N. Muhammad and M.E. Mahmat, 2005. Production of cellulase from oil palm biomass as substrate by solid state bioconversion. Amm. J. Appl. Sci., 2(2): 569-572.
32. Milagres, A.M.F., L.S. Lacis and R.A. Prade, 1993. Characterization of xylanase production by a local isolate of *Penicillium janthinellum*. Enzy. Microb. Technol., 15: 248-253.
33. Adsul, MG., J.E. Ghule, R. Singh, H. Shaikh, K.B. Bastawde, D.V. Gokhale and A.J. Varma, 2004. Polysaccharides from Bagasse: applications in cellulase and xylanase production. Carbohyd. Polymers., 57: 67-72.
34. Bharathiraja, B. and J. Jayamuthunagai, 2008. Production and kienetics of cellulase enzyme from saw dust hydrolyzate using *Trichoderma reesei* 992 6a. Advanced Biotech March 2008.