HYPER-PRODUCTION OF β-GLUCOSIDASE AND β-XYLOSIDASE BY *ASPERGILLUS NIGER* NCIM 1207 IN XYLAN-CONTAINING MEDIA

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Aspergillus niger NCIM 1207 produced significantly high levels of β-glucosidase and β-xylosidase activities in submerged fermentation. Cellulose induced only β-glucosidase, while xylan induced both β-glucosidase and β-xylosidase activities. Both the enzymes of this strain were found to undergo catabolite repression in the presence of high concentrations of glucose and glycerol. The sudden drop in pH of the fermentation medium below 3.5 caused the inactivation of enzymes when the fungus was grown in glycerol-containing media at lower temperatures. The growth of the organism at 36 ^{o}C led to an increase in pH of the fermentation above 6.0 that affected β-xylosidase activity significantly. Highest levels of β-glucosidase ((19 IU mL^{-1} or 633 IU g^{-1} of substrate) and β-xylosidase (18.7 IU/mL^{-1} or 620 IU g^{-1} of substrate) activities were detected when *A. niger* was grown at 30 ^{o}C for first five days followed by further incubation at 36 ^{o}C. Such a process of growing the organism at lower temperatures (growth phase) followed by producing the enzymes at higher temperatures (production phase) in case of fungal systems has not been reported so far. The zymogram staining of the β-glucosidase demonstrated that *A. niger* produced only single species of β-glucosidase. We feel that *A. niger* NCIM 1207 is a potential candidate to produce both β-glucosidase and β-xylosidase in high amounts that can be used to supplement commercial cellulase preparations.

Key words: Aspergillus niger NCIM 1207; β-glucosidase production; β-xylosidase production

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INTRODUCTION

β-Glucosidases and β-xylosidases are the critical components of a total cellulolytic complex that catalyzes the final step in cellulose and hemicellulose components in lignocellulosic biomass. The endoglucanases and exoglucanases hydrolyze cellulose to cellobiose and cellooligosaccharides, which are finally converted to glucose by β-glucosidases. Both endo- and exoglucanases are inhibited by cellobiose, and hence it is necessary to degrade cellobiose to achieve complete cellulose degradation. Complete degradation of cellulose requires the synergistic action of all the enzymes in the cellulase complex. β-glucosidase produces glucose from cellobiose, reducing the cellobiose inhibition, which allows the endoglucanase and exoglucanase enzymes to function efficiently. In addition, β-glucosidases are useful in the flavor industry, since they release

aromatic compounds from glycoside precursors present in fruits and fermenting products (Guegen et al. 1996). Fungal strains are known to be efficient β-glucosidase producers; for instance *Trichoderma* and *Aspergillus* sp. Thermophilic fungi (*Chaetomium thermophilum, Humicola insolens, Sporotrichum thermophile* (Kaur et al. 2007; Badhan et al. 2007; Sonia et al. 2008) are good sources of novel β-glucosidases.

β-xylosidases are necessary for the complete hydrolysis of xylans. Endoxylanases hydrolyze β-1,4-linkages in insoluble xylans to produce soluble xylooligosaccharides. β-xylosidases cleave alkyl- and aryl- glycosides, xylobiose, and xylooligosaccharides to xylose. These enzymes are employed in wine making because they hydrolyze bitter compounds present in grape juice during extraction and liberate aroma from grapes during wine making (Manzanares et al. 1999). Filamentous fungi such as *Aspergillus niger, Aspergillus awamori, Trichoderma reesei, Talaromyces emersonii* are known to be efficient producers of β-xylosidases.

Earlier we have reported hyperproduction of β-glucosidase (Gokhale et al. 1984) and β-xylosidase (Gokhale et al. 1986) by *Aspergillus niger* NCIM 1207. Mutants of these strains showing different levels of endoglucanase, xylanase, and β-glucosidase activities were reported (Gokhale et al. 1988). Cellulases of *A. niger* NCIM 1207 were found to undergo catabolite repression in presence of glucose and glycerol accompanied by sudden drop in pH of the fermentation medium below 2.0. This sudden drop in pH caused inactivation of the cellulase enzymes (Gokhale et al. 1991). The pH inactivation was reversed by addition of urea in the growth medium, which helps to maintain the pH between 4.0 and 5.0 in the fermentation medium (Gokhale et al. 1992). It was observed that β-glucosidase activity was dependent on the temperature profile used for growth and enzyme production. Tangnu et al. (1981) used two different temperatures for growth and β-glucosidase production by *T. reesei* Rut C-30, but they found that maintaining the temperature at 25 ^{o}C throughout the fermentation appeared optimal. We report here the production of β-glucosidase and β-xylosidase enzymes by *A. niger* NCIM 1207 in xylan-containing media supplemented with glycerol and urea. In addition, it is also shown that growth of the strain first at 25 ^{o}C followed by incubation at 37 ^{o}C resulted in maximum production of both β-glucosidase and β-xylosidase enzymes.

EXPERIMENTAL

Chemicals

Cellulose-123 was obtained from Carl Scheicher and Schull Co. Dassel, FRG. Solka Flok SW 40 was from Brown Co, Berin. *p*-Nitrophenyl-β–D-glucopyranoside (*p*NPG) *p*-Nitrophenyl-β–D xylopyranoside (*p*NPX) and Oat spelt xylan were obtained from Sigma Chemical Company, USA. All the other chemicals were of analytical grade.

Microbial Strains and Enzyme Production by Submerged Fermentation

Aspergillus niger NCIM 1207 was obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India. It was maintained on Potato Dextrose Agar (PDA) slopes and sub-cultured once in every two months. The Aspergillus minimal medium (AMM) for enzyme production contained

(g L^{-1}) $NaNO_3$ 0.5; $MgSO_4.7H_2O$ 0.5; KCl 0.5; KH_2PO_4 2.0; yeast extract 1.0, and bacto-peptone 5.0. Enzyme production was performed in 100 mL Erlenmeyer flasks with 75 mL of the Aspergillus minimal medium (AMM) with cellulose or xylan as carbon source. The medium was inoculated with the spore suspension (1 mL) containing 10^7 spores from 7 day old culture grown on PDA slope and incubated at 30 ^oC on a rotary shaker (150 rpm) for 14 days. The mycelium was separated by filtration, and the culture filtrate was used as a source of extracellular enzyme.

Native Polyacrylamide Gel Electrophoresis (Native PAGE) and Zymogram

Native PAGE was performed as described by Laemmli (1970) at pH 8.3 using 10% acrylamide as resolving gel with 5% stacking gel. Aliquots of 10 µL were loaded into sample wells and electrophoresed at a constant voltage of 150 volt for 2 h. The β-glucosidase activity in gels was detected by developing zymogram against 10 mM 4-methyl umbelliferyl-β-D-glucoside (Sigma) as a substrate prepared in 50 mM sodium citrate buffer, pH 4.5 (Van Tilbeurgh, 1988). After electrophoresis, the gel was immersed in substrate solution for 45 min at 50 ^oC in the dark. The β-glucosidase bands in the gel were detected under UV light using gel documentation system (Syngene).

Analytical

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) activity was estimated as reported earlier (Gokhale et al. 1984) using *p*NPG as substrate. The total 1 mL of reaction mixture consisted of 0.9 mL of pNPG (1mg mL^{-1}) and 0.1 mL of suitably diluted enzyme. The reaction was initiated by the addition of enzyme followed by incubation at 65 ^oC for 30 min. The *p*-nitrophenol liberated was measured at 410 nm, after developing the color with 2% sodium carbonate. β-Xylosidase (β-D-xylan xylohydrolase, EC 3.2.1.37) activity was estimated by the same method as above using *p*NPX (1mg mL^{-1}) as a substrate. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmole of *p*-nitrophenol from the substrate. Protein was estimated according to Bradford's method (1976) with bovine serum albumin as standard.

RESULTS AND DISCUSSION

Production of β-glucosidase and β-xylosidase on Cellulose and Xylan-Containing Media Supplemented with Different Additives

A. niger NCIM 1207 was grown in AMM containing cellulose and xylan, and it was found that cellulose induced only β-glucosidase and little amount of β-xylosidase, while xylan induced both the activities in equal amounts (Table 1). Further studies were performed using xylan as substrate. The effect of glucose and glycerol on the production of both the enzymes was studied. The results showed no repression of enzyme production at 1% glucose. Glucose at higher concentration and glycerol at all concentrations suppressed both the enzymes activities (data not shown). The growth of *A. niger* in a medium containing higher concentrations of glucose or glycerol caused the pH to suddenly drop below 3.0. This sudden drop could be responsible for the inactivation of the enzymes. Similar observations were made earlier in the case of β-glucosidase produc-

tion by *A. niger* NCIM 1207 in cellulose-containing media (Gokhale et al 1991). The supplementation of urea was shown to maintain the pH of the fermentation medium between 3.0 and 4.0 and to protect β-glucosidase from pH inactivation (Gokhale et al. 1992). To investigate whether the addition of urea to glycerol-containing medium helps to maintain the pH of the medium, the culture was grown in a medium containing xylan (3%) in combination with glycerol (2.5%) and urea (0.5%). It is clear from the results that activities of both β-glucosidase and β-xylosidase were increased (Table 1).

Table 1. Production of β-glucosidase and β-xylosidase Activities on Cellulose and Xylan as Carbon Sources

Aspergillus minimal medium (AMM) supplemented with	pH	Enzyme activity	
		β-glucosidase IU/mL	β-xylosidase IU/mL
1%Glucose	3.0	1.2	0.2
2% Cellulose 123	3.0	3.2	0.3
2%Cellulose +1% Glucose+1% Urea	7.0	4.8	0.6
1% Xylan	3.0	2.2	2.3
2% Xylan	3.1	3.0	3.2
3% Xylan	3.2	5.0	3.4
3% Xylan + 1% glucose	3.2	6.1	4.2
3% Xylan + 2% glucose	2.8	4.1	3.8
3% Xylan + 5% glucose	2.0	0.2	0.1
3% Xylan + 1% glycerol	3.0	1.3	1.4
3% Xylan + 2% glycerol	2.7	1.0	1.1
3% Xylan + 3% glycerol	2.7	1.0	0.9
3% Xylan + 1% glucose +1% urea	3.4	8.4	7.8
3% Xylan + 0.5% urea +2% glycerol	3.4	12.2	9.8
3% Xylan + 0.5% urea +2.5% glycerol	3.4	13.6	13.3
3% Xylan + 0.5% urea +3.0% glycerol	3.5	12.0	5.7

The enzyme activities were calculated after 14 days of incubation. The values are the average of three independent experiments with 4-6% standard deviation.

Effect of Temperature on Enzyme Production

To study the effect of temperature on enzyme production, the organism was grown at three different temperatures, viz. 25, 30, and 36 ^{o}C. In addition, the organism was also grown at 30 ^{o}C for the first five days followed by incubation at 36 ^{o}C for a further 8 days. Highest levels of β-glucosidase ((19.0 IU mL^{-1} or 633 IU g^{-1} of substrate) and β-xylosidase (18.7 IU mL^{-1} or 620 IU g^{-1} of substrate) activities were detected when *A. niger* was grown at 30 ^{o}C for the first five days followed by further incubation at 36 ^{o}C.

Table 2. Effect of Temperature on Production of β-glucosidase and β-xylosidase by *Aspergillus niger* NCIM 1207

Temperature	Enzyme activity (IU/mL)		pH	Protein mg/mL
	β-glucosidase	β-xylosidase		
25°C ± 1.0	7.6	7.6	3.8	0.124
30°C ± 1.0	13.1	13.3	3.8	0.168
36°C ± 1.0	8.8	0.01	7.7	0.259
30°C ± 1.0 (0-5 days) 36°C ± 1.0 (6-14 days)	19.0	18.7	4.5	0.205

The fungus was grown in AMM medium containing 3% xylan, 0.5% urea, and 2.5% glycerol with shaking at 150 rpm. The enzyme activities were calculated after 14 days of incubation at various temperatures. The values are the average of three independent experiments with 5% variation.

The profiles of enzyme production at different temperatures are given in Figs. 1 through 3. The growth of fungus at 30 °C helped to maintain the pH between 3.5 and 4.5 throughout the period of fermentation (Fig. 1). This supports our earlier observation that the pH must be maintained between 3.5 and 4.5 to obtain maximum enzyme production. It was also observed that growth of *A. niger* at 36 °C caused the pH of the medium to rise above 7.0, which specifically affected β-xylosidase activity more than β-glucosidase (Fig. 2). This observation is well supported by the pH inactivation profile of β-xylosidase activity (Fig. 3).

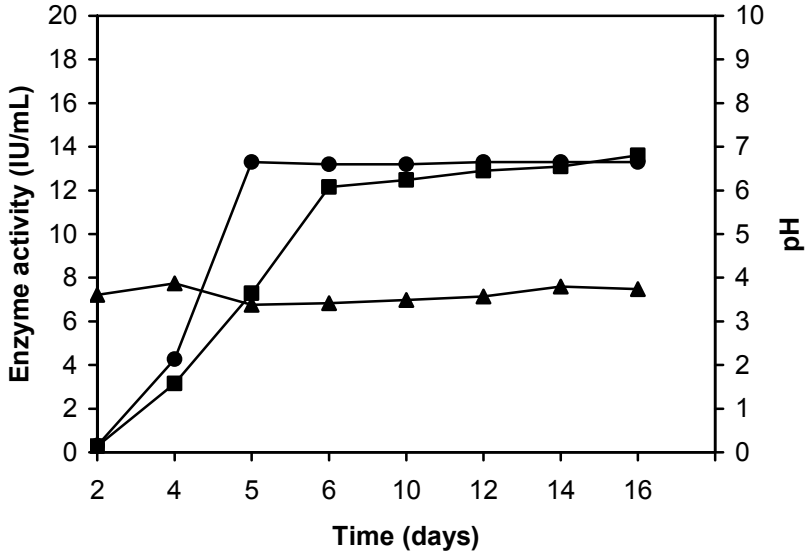

Fig. 1. Production of β-glucosidase (■), β-xylosidase (●) and pH profile (▲) in *Aspergillus* minimal medium supplemented with 3% xylan, 0.5% urea and 2.5% glycerol at 30 °C

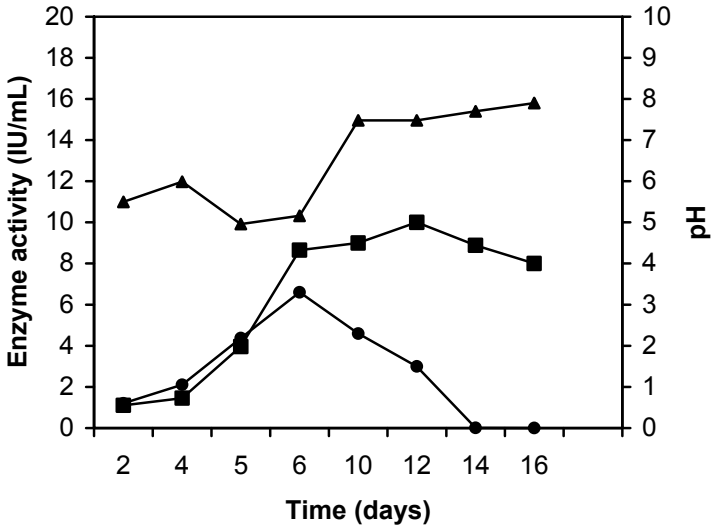

Fig. 2. Production of β-glucosidase (■), β-xylosidase (●) and pH profile (▲) in *Aspergillus* minimal medium supplemented with 3% xylan, 0.5% urea and 2.5% glycerol at 36 °C

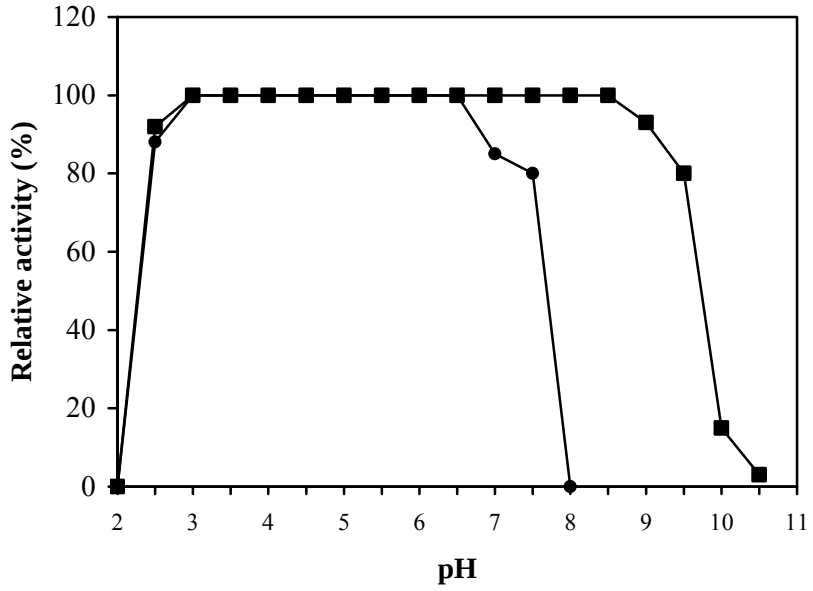

Fig. 3. Effect of pH on stability of xylan induced β-glucosidase and β-xylosidase after 24h incubation at room temperature. β-glucosidase (■), β-xylosidase (●)

The highest levels of β-glucosidase ((19.0 IU mL^{-1} or 633 IU g^{-1} of substrate) and β-xylosidase (18.7 IU mL^{-1} or 620 IU g^{-1} of substrate) activities were detected when *A. niger* was grown at 30 °C for the first five days followed by further incubation at 36 °C. The profile of the pH changes demonstrated that no inactivation of enzymes occurred, since the pH of the medium fluctuated between 3.5 and 4.5 (Fig. 4). In addition, the growth of *A. niger* at two temperatures resulted in increased protein production in the broth as compared to growth at 25 and 30 °C (Fig. 5). Though highest amount of protein was produced at 36 °C, both the enzymes were inactivated due to increase in pH of the fermentation medium. Thus the results suggested that *A. niger* NCIM 1207 could be grown at 30 °C for the first five days (growth phase) followed by incubation at higher temperature, 36 °C, (production phase) to achieve the highest enzyme activities. A zymogram for β-glucosidase was developed by using methyl umbelliferyl glucoside as substrate, which revealed the presence of only one species expressed in presence of both cellulose and xylan indicating that *A. niger* NCIM 1207 produced only one form of β-glucosidase (Fig. 6). Such a procedure for growing the organism at lower temperatures followed by producing the enzymes at higher temperatures in case of fungal systems has not been reported so far.

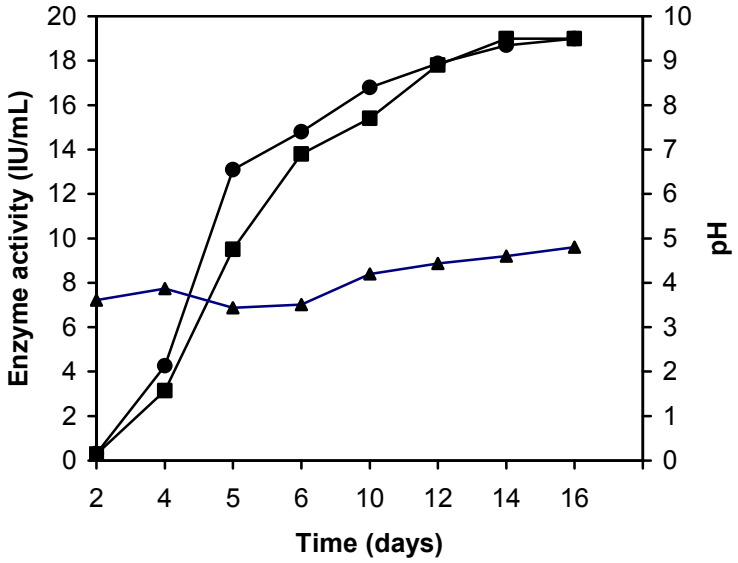

Fig. 4. Production of β-glucosidase (■), β-xylosidase (●) and pH profile (▲) in *Aspergillus* minimal medium supplemented with 3% xylan, 0.5% urea and 2.5% glycerol at 30 °C (days 0 to 5) and 36 °C (days 6 to 16).

It has been reported that *A. niger* KK2 produces β-glucosidase (100 IU g^{-1}) and β-xylosidase (193 IU g^{-1}) activities in 6 days when grown on rice straw in solid state fermentation (Kang et al. 2004). *Trichoderma atroviride* TUB F-1663 produced only β-glucosidase (7.6 IU g^{-1}) when grown on steam pretreated spruce under submerged conditions (Kovacs et al. 2009). Vu et al. (2009) subjected *Aspergillus* sp. to two rounds of repeated γ-irradiation of Co^{60} treatment and four rounds of treatment with N-methyl-N'-nitro-N-nitrosoguanidine.

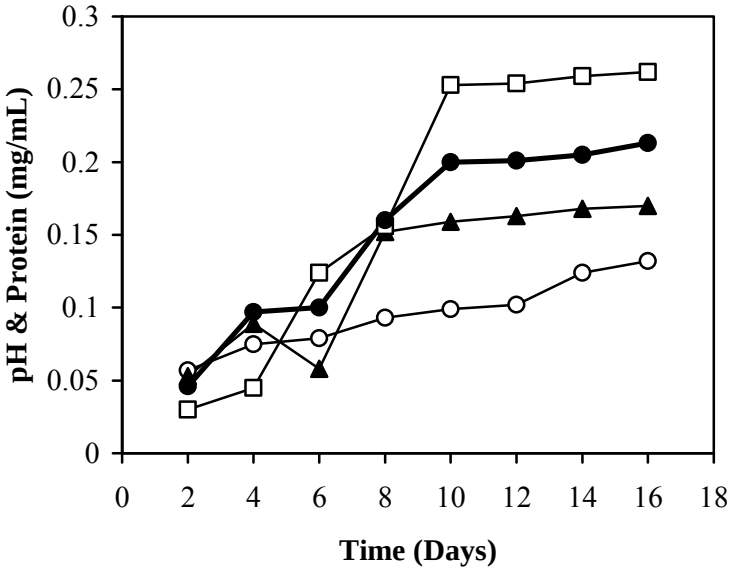

Fig. 5. Protein profile at different temperatures during production of β-glucosidase and β-xylosidase using *Aspergillus niger* in AMM supplemented with 3% xylan, 0.5% urea and 2.5% glycerol. 25 °C (○); 30 °C (▲); 36 °C (□); 30 °C and 36 °C (●)

Fig. 6. Zymogram showing the expression of single species of β-glucosidase in *A. niger* 1207 grown on medium supplemented with cellulose and xylan
Lane 1: Cellulose induced β-Glucosidase (30 °C)
Lane 2: Xylan induced β-Glucosidase (30 °C)
Lane 3: Xylan induced β-Glucosidase (30 °C & 36 °C)

The best mutant designated as *Aspergillus* sp. XTG-4 was selected, and it produced 27.12 IU mL^{-1} of β-glucosidase activity on wheat bran. Thermophilic strains such as *Thermomyces lanuginosus* produce both β-glucosidase and β-xylosidase (Sonia et al. 2005), but the levels of β-xylosidase are low. Bokhari et al (2010) reported the

production of β-xylosidase by a newly isolated mutant of *Humicola lanuginosa*, M7D. It produced maximum β-xylosidase (728 IU g^{-1} substrate) when grown on Vogel's medium with xylan as carbon source. The same mutant produces remarkably high β-glucosidase activity (17.93 IU mL^{-1}) during growth on corncobs-containing medium at 45 ^{o}C (Bokhari et al. 2008). Our strain *A. niger* NCIM 1207 produced significant levels of both β-glucosidase (18.6 IU mL^{-1}) and β-xylosidase (19.0 IU mL^{-1}) when grown on xylan-containing media. This is the first report on production of both of these enzymes in high amounts using xylan as substrate. This enzyme preparation could be efficiently used to supplement commercial cellulase preparations from *Trichoderma reesei* that are deficient in β-glucosidase and β-xylosidase.

Very recently, Qing et al (2010) showed for the first time that xylooligomers were far more inhibitory to cellulase than glucose, cellobiose, and xylose, thereby reducing the cellulose hydrolysis. These results suggest that hemicellulose removal from lignocellulosic materials prior to enzymatic hydrolysis is necessary to achieve higher saccharification. The results also reinforce the importance of β-xylosidase activities in cellulase and β-glucosidase enzyme preparations to hydrolyze hemicellulose to xylose, which is less inhibitory. The supplementation of commercial cellulases with such enzyme complex may also help to reduce enzyme doses needed to achieve complete hydrolysis of cellulose. We feel that *A. niger* NCIM 1207 is a potential candidate to produce both β-glucosidase and β-xylosidase in high amounts that can be used to supplement commercial cellulase preparations.

CONCLUSIONS

Aspergillus niger NCIM 1207 produced high levels of both β-glucosidase and β-xylosidase when grown on xylan-containing medium supplemented with glycerol and urea. Though the optimum temperature for enzymes production was 30 ^{o}C, the growth of the organism at 30 ^{o}C for the first five days followed by further incubation at 36 ^{o}C enhanced the production of both the enzymes.

The pH of the fermentation medium appears to play an important role in the production of enzymes. This enzyme preparation can be used for supplementation of commercial cellulases that are deficient in β-glucosidase and β-xylosidase enzymes to achieve complete degradation of cellulosic materials.

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REFERENCES CITED

Badhan, A. K., Chadda, B. S., Kaur, J., Saini, H. S., and Bhat, M. K. (2007). "Production of multiple xylanolytic and cellulolytic enzymes by thermophilic fungus *Myceliophthora* sp. IMI 387099," *Bioresour. Technol.* 98, 504-510.

Bokhari, S. A. I., Latif, F., and Rajoka, M. I. (2008). "Kinetics of high level of β-glucosidase production by 2-deoxyglucose resistant mutant of *Humicola lanuginose* in submerged fermentation," *Braz. J. Microbiol.* 39, 724-733.

Bokhari, S. A. I., Latif, F., Akhtar, M. W., and Rajoka. M. I. (2010). "Characterization of β-xylosidase produced by a mutant derivative of *Humicola lanuginose* in solid state fermentation," *Ann. Microbiol.* 60, 21-29.

Bradford, M. M. (1976). "Rapid and sensitive method for the quantification of protein utilizing the principal of protein-dye binding," *Anal. Biochem.* 72, 248-254.

Gokhale, D.V., Puntambekar, U. S., Vyas, A. K., Patil, S. G., and Deobagkar, D. N. (1984). "Hyper-production of β-glucosidase by *Aspergillus* sp.," *Biotechnol. Lett.* 6, 719-722.

Gokhale, D. V., Puntambekar, U. S., and Deobagkar, D. N. (1986). "Xylanase and β-xylosidase production by *Aspergillus niger* NCIM 1207," *Biotechnol. Lett.* 8, 37-38.

Gokhale, D. V., Puntambekar, U. S., Deobagkar, D. N., and Peberdy, J. F. (1988). "Production of cellulolytic enzymes by mutants of *A. niger* NCIM 1207," *Enzyme Microb. Technol.* 10, 442-445.

Gokhale, D. V., Patil, S. G., and Bastawde, K. B. (1991). "Optimization of cellulase production by *Aspergillus niger* NCIM 1207," *Appl. Biochem. Biotechnol.* 30, 99-109.

Gokhale, D. V., Patil, S. G., and Bastawde, K. B. (1992). "Protection of *Aspergillus niger* cellulases by urea during growth on glucose or glycerol supplemented media," *Appl. Biochem. Biotechnol.* 37, 11-17.

Guegen, Y. P., Chemardin, G., Janbon, A., Arnaud, A., and Galzy, P. (1996). "A very efficient glucosidase catalyst for the hydrolysis of flavor precursors of wines and fruit juices," *J. Agric. Food. Chem.* 44, 2336-2340.

Kang, S. W., Park, Y. S., Lee, J. S., Hong, S. I., and Kim, S. W. (2004). "Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass," *Bioresour. Technol*. 91, 153-157.

Kaur, J., Chadha, B. S., Kumar, B. A., Kaur, G. S., and Saini, H. S. (2007). "Purification and characterization of beta-glucosidase from *Melanocarpus* sp. MTCC 3922," *Electronic J. Biotechnol.* 10, 260-270.

Kovacs, S., Macrelli, S., Szakacs, G., and Zacchi, G. (2009). "Enzymatic hydrolysis of steam-pretreated lignocellulosic materials with Trichoderma atroviride enzymes produced in-house," *Biotechnol. Fuels* 2, 1-11.

Manzanares, P., Ramon, D., and Querol, A. (1999). "Screening of non-*Saccharomyces* wine yeasts for production of β-xylosidase activity," *Int. J. Food. Microbiol.* 46, 105-112.

Qing, Q., Yang, B., and Wyman, C. E. (2010). "Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes," *Bioresour. Technol.* 101, 9624-9630.

Sonia, K. G., Chadha, B. S., and Saini, H. S. (2005). "Sorghum straw for xylanase hyperproduction by *Thermomyces lanuginosus* (D2W3) under solid state fermentation," *Bioresour. Technol.* 96, 1561-1569.

Tangnu, S. K., Blanch, H. W., and Wilke, C. R. (1981). "Enhanced production of cellulase, hemicellulose abd β-glucosidase by *Trichoderma reesei* (Rut C-30)," *Biotechnol. Bioeng*. 23(8), 1837-1849.

Van Tilbeurgh, H., and Claeyssens, M. (1985). "Detection and differentiation of cellulase components using low molecular mass fluorogenic substrates," *FEBS Letts*, 187, 283-288.

Vu, V.-H., Tuan, A. H., and Kim, K. (2009). "Fungal strain improvement for cellulase production using repeated and sequential mutagenesis," *Microbiology* 37, 267-271.

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