African Journal of Biotechnology Vol. 5 (8), pp. 609-614, 18 April 2006
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2006 Academic Journals

Full Length Research Paper

An innovative approach for hyperproduction of cellulolytic and hemicellulolytic enzymes by consortium of *Aspergillus niger* MSK-7 and *Trichoderma viride* MSK-10

Ikram-ul-Haq, Muhammad Mohsin Javed* and Tehmina Saleem Khan

Institute of Industrial Biotechnology, G C University, Lahore, Pakistan

Accepted 17 January, 2006

The present work describes the production of cellulolytic enzymes (CMC-ase, FP-ase, β-glucosidase) and hemicellulolytic enzyme (xylanase) along with total extracellular protein by *Aspergillus niger* and *Trichoderma viride* using submerged fermentation. Among seven different kinds of experiments, secretion rate of protein and enzymes was investigated by mono- and co-cultures of *A. niger* and *T. viride* using wheat bran as substrate with the supply of Eggins and Pugh salt medium. Co-culture of *A. niger* and *T. viride* (when both were mixed together simultaneously) gave 30-50% higher production of total protein (0.58 mg/ml) and enzymes; CMC-ase (2.79 U/ml/min), FP-ase (1.75 U/ml/min) and xylanase (189.7 U/ml/min) than mono-cultures and all other combinations. Biosynthesis of β-glucosidase was found higher i.e., 4.66 U/ml/min in co-culture of *A. niger* and *T. viride* (when *T. viride* was mixed with 24 hours old culture of *A. niger*). Simultaneous co-culture of *A. niger* and *T. viride* was further optimized with fermentation rate, different carbon sources, incubation temperature and different pH of fermentation media.

Key words: Strain compatibility, co-culture, mono-culture, fermentation.

INTRODUCTION

Enzymes are among the most important bio-products and are being utilized in a large number of processes in the areas of industrial, environmental and food biotechnology. Moreover, current developments in biotechnology are yielding new applications for enzymes (Pandey et al., 2004). Filamentous fungi are preferred for commercially important enzymes production, because the level of the enzymes produced by these cultures is higher than those obtained from yeast and bacteria (Bakri et al., 2003).

Aspergillus niger and *Trichoderma viride* are the most important and safe microorganisms for industrial use (Camacho and Aguilar, 2003; Seyis and Aksoz, 2003). These had been in use already for many decades to produce extracellular enzymes (Schuster et al., 2002) with homologous and heterologous proteins due to high capacity of their protein secretion machinery (Iwashita, 2002; Pandey et al., 2004).

Microbial consortium consisting of two or more different microorganisms is known to be largely responsible for many biotransformations in natural environment. Mixed culture fermentations are widely used in biotechnology for many processes including the production of antibiotics, enzymes, fermented food, composting, dairy fermentation, bioconversion of apple distillery and domestic wastewater sludge (Alam et al., 2001).

MATERIALS AND METHODS

Cultural strains

Fungal strains; *Aspergillus niger* MSK-7 and *Trichoderma viride* MSK-10 belonging to phylum Ascomycota were used in the present study, obtained from the stock cultures of Institute of Industrial Biotechnology, Govt. College University, Lahore and were stored in

*Corresponding authors E-mail: mmj_bot@yahoo.com.

culture tubes containing mineral salt agar media (g/L; 1.0 KH_2PO_4, 0.5 KCl, 0.5 $(NH_4)_2SO_4$, 0.2 $MgSO_4$. $7H_2O$, 0.1 $CaCl_2$. $2H_2O$, 0.5 L-asparginine, 0.5 yeast extract with 1.8% agar) as described by Eggins and pugh (1962) at 4±1°C.

Fermentation technique

Fungal growth and the production of enzymes were carried out in 250 ml Erlenmeyer conical flasks containing 25 ml of Eggins and pugh medium with 1.0% (w/v) wheat bran at 5.0 pH using *A. niger* and *T. viride* inoculum (conidial inoculum with 2.50×10^7 conidia/ml). The flasks were incubated at 200 rpm on an orbital shaking incubator (SANYO, Gallenkamp, PLC, UK) at 30±1°C for 72 h. The fermented broth was centrifuged at 8000×g for 10 min and suspension was used for analytical purposes.

Optimization of fermentation environment in shake flask

Four different carbon sources e.g., xylan, carboxymethyl cellulose (CMC), cellulose powder, rice bran and wheat bran (1.0%, w/v) were chosen as test substrates on the basis of literature data and availability. Incubating the experimental flasks at 25, 30, 35 and 40°C, the growth temperature was optimized for best production of enzymes. The effect of different initial pH (3.0-8.0) of fermentation media on the production rate was also investigated. Time course production profile for the enzymes was also studied up to 168 h. All the parameters were run in triplicates.

Process flow

Strains of *Aspergillus niger* and *Trichoderma viride* were used with five different combinations, namely; simultaneous mix culturing of *A. niger* and *T. viride* (A+T), mixing of *T. viride* in 24 and 48 h old culture of *A. niger* (24A+T and 48A+T) and mixing of *A. niger* in 24 and 48 h old culture of *T. viride* (24T+A and 48T+A) with their mono-cultures to make a comparison.

Analytical methods

Carboxymethyl cellulase (CMC-ase) activity was determined by the method of Wood and Bhat (1988). Bradford (1976) method was followed for total protein (enzymic and residual protein). Filter paper-cellulase (FP-ase) activity was determined by the method of Mandels and Sternberg (1976) and β-glucosidase activity was estimated according to the method used by Rajoka and Malik (1996). The reducing sugar released in case of CMC-ase, FP-ase and xylanase was measured by standard dinitrosalicylic acid method (Miller, 1959).

Statistical analysis

Significance difference among replicates was presented as Duncan's multiple range tests in the form of probability (*p*) values by finding out the treatment effects using Costat cs620W.exe (Computer software) after Snedecor and Cochrane (1980).

RESULTS AND DISCUSSION

In the mono-cultures, it was observed that enzyme production profile with respect to the active enzyme's units of *A. niger* was xylanase (154.56 U/ml/min) > β-glucosidase (4.47 U/ml/min) > CMC-ase (1.98 U/ml/min) and FP-ase (1.05 U/ml/min) and that of *T. viride* was

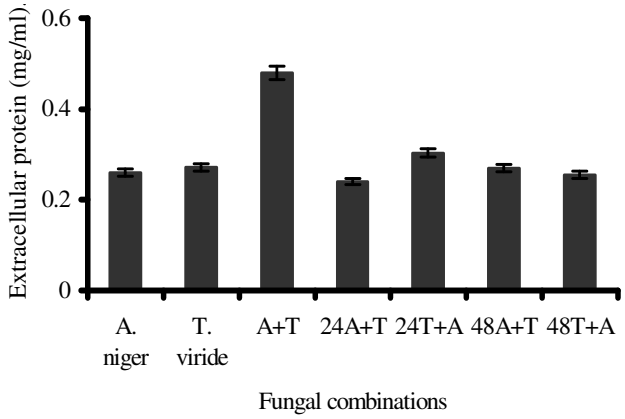

Figure 1. Total protein of the fermented broth obtained from mono-cultures and different combinations of *A. niger* and *T. viride* incubation at 30˚C for 72 h at pH 5.0 using wheat bran as carbon source.

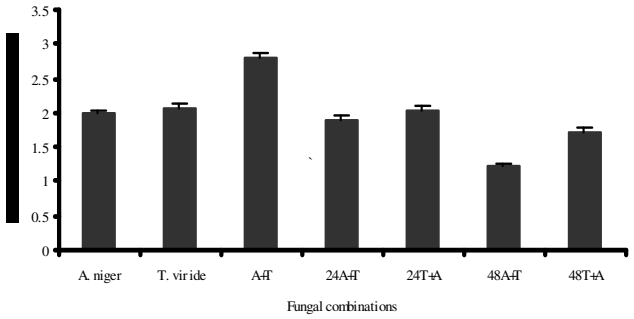

Figure 2. CMC-ase activity of the fermented broth obtained from monocultures and different combinations of *A. niger* and *T. viride* incubation at 30ºC for 72 h at pH 5.0 using wheat bran as carbonsource.

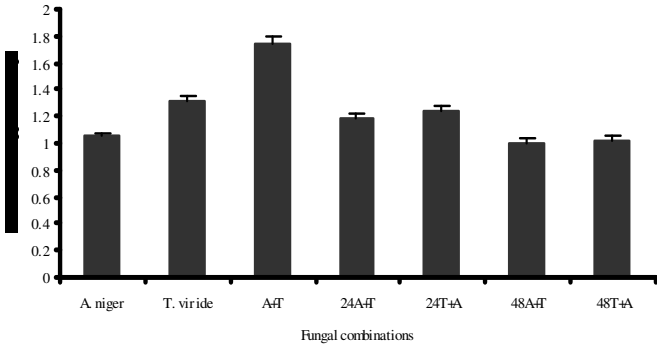

Figure 3. FP-ase activity of the fermented broth obtained from mono-cultures and different combinations of *A. niger* and *T. viride* incubation at 30ºC for 72 h at pH 5.0 using wheat bran as carbon source.

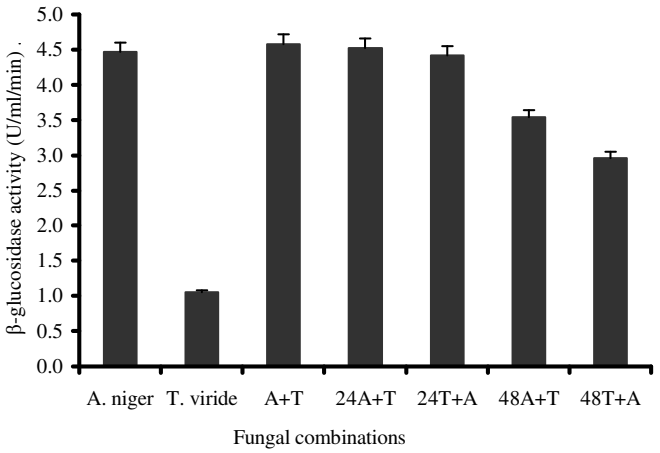

Figure 4. β-glucosidase activity of the fermented broth obtained from mono-cultures and different combinations of *A. niger* and *T. viride* incubation at 30ºC for 72 h at pH 5.0 using wheat bran as carbon source.

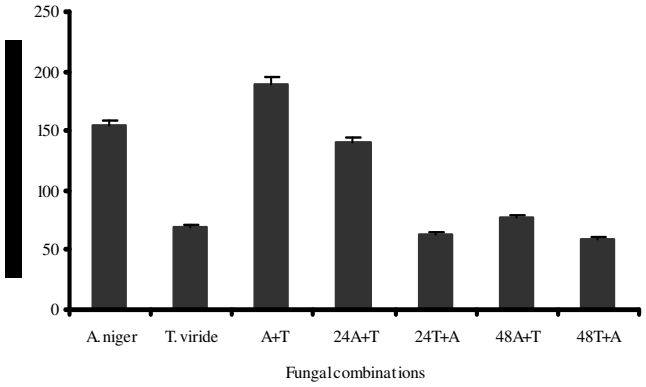

Figure 5. Xylanase activity of the fermented broth obtained from mono-cultures and different combinations of *A. niger* and *T. viride* incubation at 30ºC for 72 h at pH 5.0 using wheat bran as carbon source.

xylanase (68.6 U/ml/min) > CMC-ase (2.06 U/ml/min) > FP-ase (1.315 U/ml/min) and β-glucosidase (1.05 U/ml/min). *A. niger* produced more xylanase and β-glucosidase than *T. viride* (Figures 1 to 5) but *T. viride* produced better CMC-ase and FP-ase than *A. niger*. Tamas et al. (2003) also reported similar findings.

The results obtained from pure cultures of *A. niger* and *T. viride* were compared to that obtained from five kinds of mixed cultures (A+T, 24A+T, 24T+A, 48A+T and 48T+A) to evaluate the production of total protein and enzymes like CMC-ase, FP-ase, β-glucosidase and xylanase (Figures 1 to 5). It was observed that 24A+T (0.72 mg/ml protein, 1.89, 1.19, 4.52 and 139.5 U/ml/min for CMC-ase, FP-ase, β-glucosidase and xylanase, respectively) and 24T+A (0.303 mg/ml protein, 2.03, 1.24, 4.42 and 62.9 U/ml/min CMC-ase, FP-ase, β-glucosidase and xylanase, respectively) combinations were better as compared to the combinations 48A+T (0.24 mg/ml protein, 1.62, 1.00, 3.54 and 76.73 U/ml/min CMC-ase, FP-ase, β-glucosidase and xylanase, respectively) and 48T+A (0.255 mg/ml protein, 1.73, 1.02, 2.69 and 59.93 U/ml/min CMC-ase, FP-ase, β-glucosidase and xylanase, respectively). In combinations 24A+T and 24T+A, the production of total protein and enzymes was 10% higher than the pure cultures of first organisms but in the combinations 48A+T and 48T+A there was no significant rise in production as compared to the pure culture of first inoculated organism. In the combinations 24A+T and 24T+A, the first organism was less established than in 48A+T and 48T+A thus, the environment was not suitable for the incoming fungi to grow in the later case. It might be due to that *T. viride* and *A. niger* have antagonism effect to the growth of other fungi (Benitez et al., 1998). It was also observed that the total protein and all the enzymes produced by simultaneous co-cultures were 30-50% higher with 2.79 (CMC-ase), 1.75 (FP-ase), 4.58 (β-glucosidase) and 189.7 (xylanase) U/ml/min with 0.48 mg/ml (total protein) than all other culture designs. Due˜nna et al. (1995) and Gutierrez-Correa et al. (1999) also obtained same kind of results.

Optimization of incubation time for higher production rate

The optimization of time course for fermentation of simultaneous co-culture of *A. niger* and *T. viride* was also investigated from 0.0 to 168 hours (Figure 8). The maximum production rate was observed at 72-96 h after inoculation with high rate of protein, CMC-ase, FP-ase, β-glucosidase and xylanase (0.61 mg/ml, 2.90, 1.76, 4.67 and 195.3 U/ml/min, respectively). However, further increase in the incubation time, reduced the enzymes production. It might be due to the depletion of macro- and micronutrients in the fermentation medium with the lapse in time, which stressed the fungal physiology resulting in the inactivation of secretary machinery of the enzymes (Nochure et al., 1993). In addition, the substances were initially more susceptible, making a rapid rise in enzymes biosynthesis. But with the prolongation of cultural time, the susceptible portions were completely hydrolyzed by microorganisms, which inhibited the enzyme secretion pathways.

Induction by different carbon sources

The nature of carbon sources also takes important part in the production of cellulases and xylanase. A total of five carbon sources i.e., carboxymethyl cellulose (CMC), cellulose powder, xylan, wheat bran and rice bran were optimized. Among them, wheat bran was proved to be the best for β-glucosidase and xylanase. When agricultural by-products, other than wheat bran were used, the production of cellulase was reduced (Figure 6).

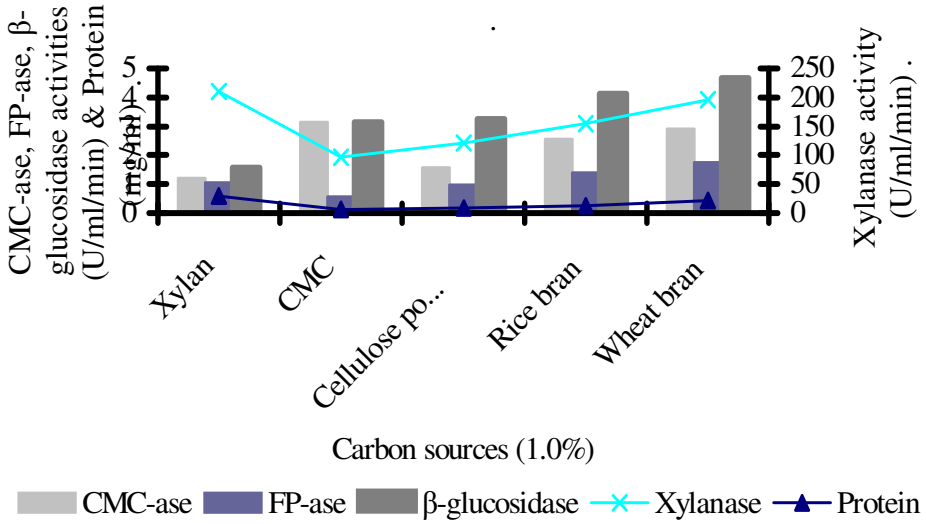

Figure 6. Effect of different carbon sources on the rate of enzymes production by simultaneous co-culture of *A. niger* and *T. viride* (A+T).

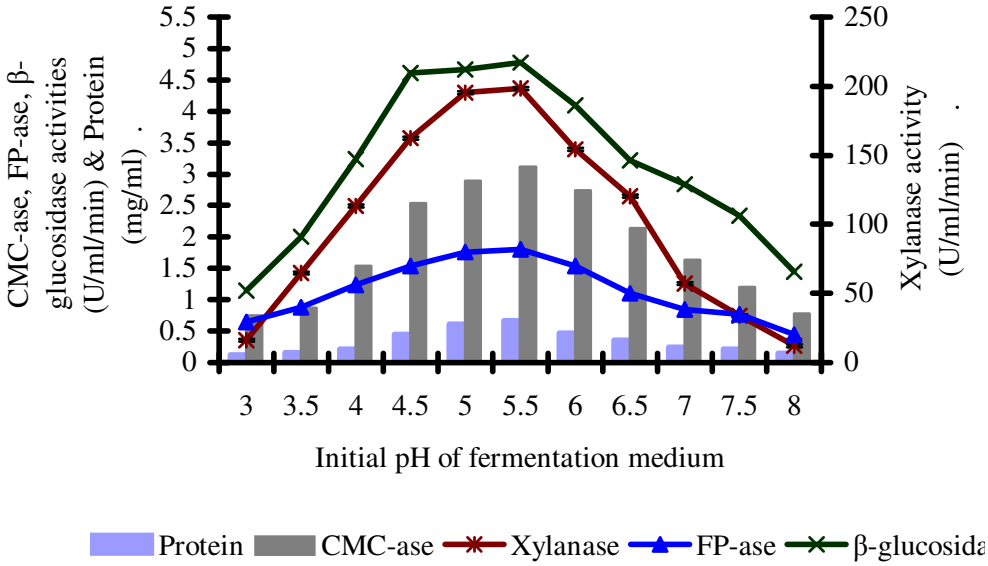

Figure 7. Effect of initial pH of the fermentation medium on the rate of enzymes production by simultaneous co-culture of *A. niger* and *T. viride* (A+T).

It might be due to that wheat bran provided adequate amount of nutrients (proteins 1.32%, carbohydrates 69.0%, fats 1.9%, fiber 2.6%, ash 1.8%, Ca 0.05%, Mg 0.17%, P 0.35%, K 0.45%, S 0.12% and various amino acids) to the microorganism. These nutrients were essential for the growth of fungal cultures and subsequent enzyme production (Nochure et al., 1993).

Effect of different incubation temperatures

Incubation temperature plays an important role in the metabolic activities of microorganism. In the present study, a temperature range starting from 25 to 40°C was investigated (Figure 9) and among these, 30°C was optimised for the best growth of *A. niger* and *T. viride* and subsequent enzyme production. Any change, either increase or decrease in temperature resulted in the gradual decrease in protein production. A higher temperature (above 30°C) alters the cell membrane composition and stimulates protein catabolism, thus, causes the cell death.

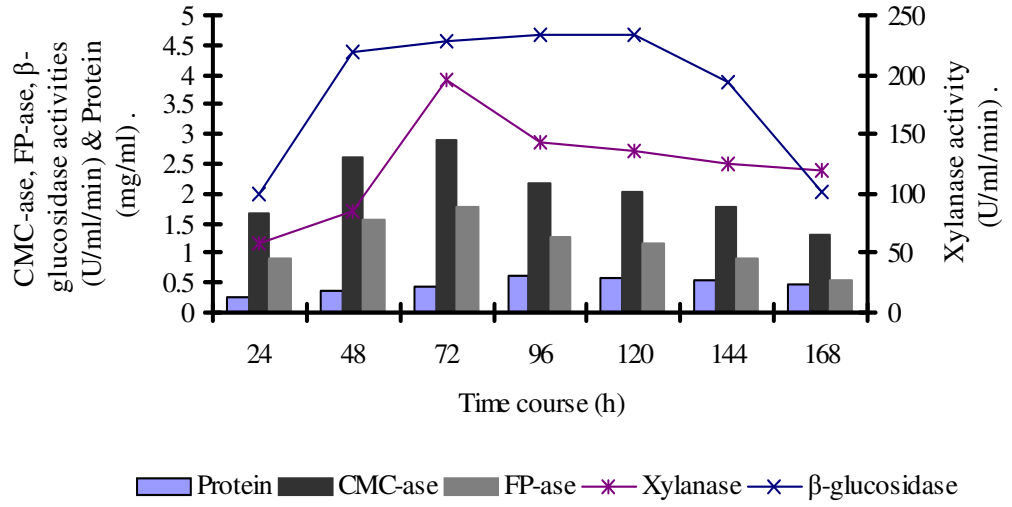

Figure 8. Effect of incubation time on the rate of enzymes production by simultaneous co-culture of *A. niger* and *T. viride* (A+T).

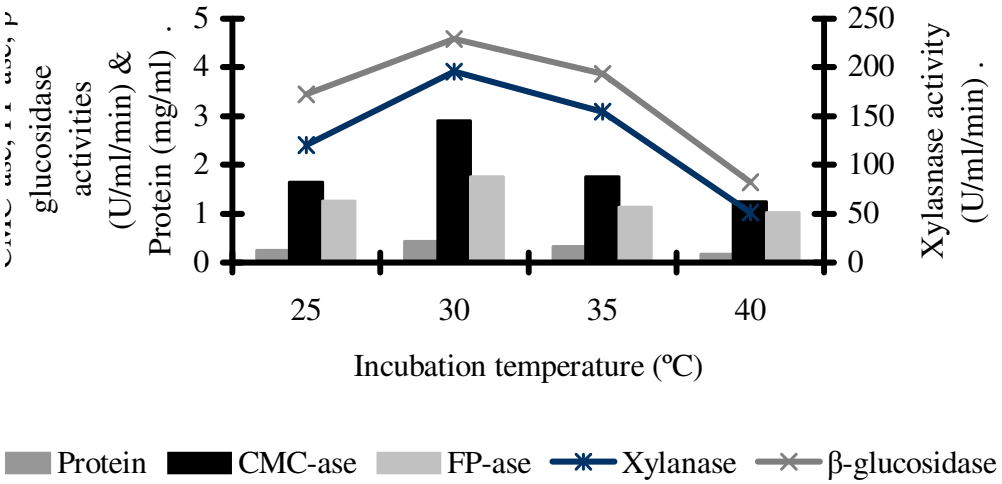

Figure 9. Effect of incubation temperature on the rate of enzymes production by simultaneous co-culture of *A. niger* and *T. viride* (A+T).

Production profile at different pH

Initial pH has a direct effect on the uptake of mineral nutrients, which are present in the fermentation medium. So, the effect of different pH (3.0-8.0) of fermentation medium on the enzymes production was also investigated (Figure 7) with simultaneous co-culturing of *A. niger* and *T. viride*. High acidic and high basic pH, both showed negative effects, but a medium with low acidic pH i.e., 5.5 was ideal for total protein and enzymes production after 72 hours of incubation. The production of the CMCase, FP-ase, β-glucosidase and xylaqnase was found to be optimal at pH 5.5 while, total protein was maximal at 6.0. It might be due to the fact that fungal cultures require slightly acidic pH for their growth and enzyme biosynthesis (Haltrich et al., 1996).

CONCLUSION

Simultaneous mix culturing of *A. niger* and *T. viride* proved to be an excellent source for the enzymes production. In the present study, co-cultures produced the amount of extracellular enzymes 30-50% higher than that of monocultures. This might be due to the range of diversity in microbes. Mixed culture combinations have the ability to utilize the substrates as energy sources better than highly versatile pure cultures.

ACKNOWLEDGEMENT

Higher Education Commission of Pakistan financially supported this work.

REFERENCES

Alam MZ, Fakhru'l-Razi SA, Abd-aziz A, Idris (2001). Bioconversion of wastewater sludge by immobilized microbial treatment. In proc, International Water Association (IWA) Conf. on Water and Waste water Management for Developing Countries, Kauala Lampur, Malaysia. pp. 344-353.

Bakri YP, Jacques P, Thonart (2003). Xylanase production by *Penicillium canescens* 10-10c in solid-state fermentation. Appl. Biochem. Biotechnol. 108(1-3): 737-748.

Bradford NM (1976). A rapid and sensitive method for quantitation of microorganism qualities of protein utilizing the principle of protein dye binding analysis. Biochemistry 72: 248-254.

Camacho NA, Aguilar OG (2003). Production, purification and characterization of a low molecular mass xylanase from *Aspergillus sp*. and its application in baking. Appl. Biochem. Biotechnol. 104(3): 159-172.

Due˜nna R, Tengerdy DRP, Gutierrez-Correa M (1995). Cellulase production by mixed fungi in solid-substrate fermentation of bagasse. World J. Microbiol. Biotechnol. 11: 1133-1137.

Eggins H, Pugh PJF (1962). Isolation of cellulose decomposing fungi from soil. Nature 193: 94-95.

Gutierrez-Correa M, Portal L, Moreno P, Tengerdy R (1999). Mixed culture solid substrate fermentation of *Trichoderma reesie* with *Aspergillus niger* on sugar cane bagasse. Biores. Technol. 68: 173-178.

Haltrich D, Nidetzky B, Kulbe KD, Steiner W. Zupancic S (1996). Biores. Technol. 58: 137-161.

Iwashita K (2002). Recent studies of protein secretion by filamentous fungi. J. Biosci. and Bioeng. 94(6): 530-535.

Mandels M, Sternberg D (1976). Recent advances in cellulase technology. J. Ferment. Technol. 54(4): 267-286.

Miller GL (1959). Use of dinitrosalicylic acid reagent for the determination of reducing sugars. J. Anal. Chem. 31: 426-428.

Nochure SV, Roberts MF, Demain AI (1993). True cellulase production by *Clostridium thermocellum* grown on different carbon sources. Biotech. Lett. 15: 641-646.

Pandey A, Selvakumar P, Soccol CR, Nigam P (2004). Solid-state fermentation for the production of industrial enzymes. 1-22.

Rajoka MI, Malik KA (1996). Cellulase production by *Cellulomonas biazotea* cultured in media containing different cellulosic substrates. Biores. Technol. 59: 21-27.

Schuster E, Dunn-Coleman N,. Frisvad JC, Van Dijck PWM (2002). On the safety of *Aspergillus niger*: A Review. Appl. Microbiol. Biotechnol. 59: 426-435.

Seyis I, Aksoz N (2003). Determination of some physiological factors affecting xylanase production from *Trichoderma harzianum* 1073 $D_{3.}$

Snedecor GW, Cochrane WG (1980). Statistical methods. 7th edition. Ames, Iowa: Iowa state university press. ISBN 0-81381560-6.

Tamas J, Kozma K Szengyel Z, Reczey K (2003). Production of β-glucosidase in mixed culture of *Aspergillus* BKMF-1305 and *Trichoderma reesei* RUTC-30. Food Technol. Biotechnol. 41(1): 49-53.

Wood TM, Bhat KM (1988). Methods for measuring cellullase activities. Academic Press 160: 87-112.