▼ Journal of Research (Science), Bahauddin Zakariya University, Multan, Pakistan.
Vol. 19, No. 1, January 2008, pp. 43-51 ISSN 1021-1012

Article presented in 6th International and 16th National Chemistry Conference
"Quality of Life and Chemical Sciences" (April 6-8, 2006)

KINETIC STUDIES OF β-GLUCOSIDASE PRODUCED FROM *ASPERGILLUS NIGER* NRRL 567

M. Ishfaq Ghori, M. A. Malana, Kashif Younis Butt
Dept. of Chemistry, Bahauddin Zakariya University, Multan, Pakistan
M. Javed Iqbal
Dept. of Chemistry, Quaid-i-Azam University, Islamabad, Pakistan
Amer Jamil
Dept. of Chemistry, University of Agriculture, Faisalabad, Pakistan
email: amerjamil@yahoo.com

Abstract
β-glucosidase was produced from *Aspergillus niger* NRRL 567 grown on 2% corn stover under continuous shaking for 96 hours. Maximal enzyme activity was obtained at pH 3.5 and temperature 30 ^{o}C. Kinetic investigations revealed that according to Lineweaver-Burk plot, Woolf Augustisson-Hofstee plot and Hanes-Woolf plot, Bgl had K_M 0.45, 0.39 and 0.40% and Vmax 1.01, 0.93 and 0.95 μmol $mL^{-1}min^{-1}$., respectively. Energy of activation E_a for the enzyme was found to be 19.0 $kJK^{-1}mol^{-1}$, whereas rate of reaction was increased to 13.3 after every 10 ^{o}C rise in temperature. Conclusively, small K_M value of the enzyme showed a large affinity of the enzyme with substrate. Therefore, the enzyme was found good catalytic agent for the bioconversion of waste material into glucose and biomass protein.

Keywords: *Aspergillus niger* NRRL 567, β-glucosidase, energy of activation, enzyme, rate of reaction.

INTRODUCTION
Cellulose is the most abundant and renewable biomass in the biosphere Yoshihiko and Takahisa [2002] produced as a result of photosynthesis Sakka *et al.* [2000]. Economical production of cellulose is a key for successful utilization of cellulosic materials as renewable carbon sources. Huge amount of lignocellulosic material is disposed off each year as waste [Srinivasan *et al.* 1984] which is a global concern for environment. Cellulose in the form of cereal, grain residue, stalks, husks, bagasse and sawdust is produced by photosynthesis. These agricultural wastes are either thrown away or burnt which cause pollution in the environment. These agricultural wastes predominantly consist of cellulose which is a polymer of ß-D-glucose units. Cellulose is hydrolysed by cellulases to yield glucose as the final product. The cellulases are also used in the manufacturing of useful products such as ethanol production [Rajoka *et al.* 1998] which is valuable in many respects. Cellulase complex mainly consist of three types of enzyme systems viz. Endo-1,4-β-D-glucanase or carboxymethyl cellulase (CMCase)

(E.C.3.2.1.4), Exo-1,4-β-D-glucanase or cellubiohydrolase or avicelase (E.C.3.2.1.91) and ß-glucosidase or cellobiosidase (E.C.3.2.21). These enzymes work synergistically to hydrolyze cellulose into glucose. The hydrolysis action is completed by ß-glucosidase that changes cellubiose into glucose.

Efforts are being made for producing celluloses from agricultural wastes [Shewale and Sadana 1978, Garg and Neelakanton 1981, Chahal *et al.* 1985, Gadgil *et al.* 1995]. Rigorous controls are necessary for the fermentation of agro-industrial wastes and by products to achieve culture conditions for optimum production of fungal celluloses which vary from species to species. The reaction conditions need to be optimized for maximal production of enzyme from indigenous sources. Determination of different kinetic parameters is of utmost importance before the enzymes can be utilized in industry. In the present paper we report kinetic parameters of β-glycosidase from *Aspergillus niger* NRRL 577.

MATERIALS AND METHODS

GROWTH CONDITIONS

Culture of *Aspergillus niger* NRRL 567 procured as a gift from Agricultural Research Service Culture Collection, Northern Regional Research Lab. U.S. Department of Agriculture, Illinois, U.S.A. was transferred to sporulation medium (Corn Stover, Dextrose, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, KH_2PO_4, Urea, Agar as 4.0, 2.0, 0.005, 0.005, 0.3 and 2.0% respectively, pH 4) and incubated at 30°C ± 2 for two weeks.

For the production of β-glycosidase the culture was grown in enzyme production medium with the composition as given above except that agar was omitted. After 96 hours, the filtrate after filtration of the harvested sample through Whatman filter paper No.1 was subjected to centrifugation (4000 rpm, 10 min) for the further removal of undissolved matter and impurities. The resulting spore free filtrate was assayed for β-glycosidase.

ß-GLYCOSIDASE ASSAY

The assay was based on principle that β-glycosidase hydrolyzes salicin and releases free glucose units. The free glucose units after reaction with dinitrosalicylic acid produce colored complexes which are determined by spectrophotometer at 550nm [Gadgil *et al.* 1995]. One unit of enzyme activity (IU per mL) was defined as the amount of glucose equivalents released in µmole by one mL of original enzyme extract per minute.

pH AND TEMPERATURE FOR MAXIMAL ENZYME PRODUCTION

To optimize the pH for maximal production of the enzyme, the culture was grown in growth medium containing different pH i.e., from 3.0 to 4.5 and incubated on orbital shaker at 120 rpm for 96 hours at 30 °C. Maximum production of the enzyme was found at pH 3.5.

To optimize the growth temperature for maximal production of the enzyme the culture was grown on different temperature i.e. 30, 35, 40 and 45 °C under the same conditions as discussed above. 30 °C was found to be optimum temperature for the maximum production of β-glycosidase.

DETERMINATION OF V_{MAX} AND K_M

β-glucosidase produced by *Aspergillus niger* NRRL 567 was assayed in 0.5 *M* acetate buffer of pH 5.0 with variable amounts (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mL) of 1% salicin as substrate. The data obtained were plotted according to Lineweaver Burk, Woolf-Augustinsson-Hofstee, and Hanes-Woolf plots as described by Price and Dwek [1986].

OPTIMUM TEMPERATURE

Optimum temperature of the enzyme was determined as described by Sanyal *et al.* [1988]. Enzyme solution (1 mg mL^{-1}) in 0.5 *M* acetate buffer was assayed at different temperature (10 to 70 °C with 10°C intervals) for the enzyme activity as described by Rajoka and Malik [1986].

ACTIVATION ENERGY (E_a)

Activation energy of the β-glucosidase was determined by using the data for optimum temperature as under:

Ea = Slope x R, Where R = gas constant, Slope = $\Delta H/R$

RISE IN REACTION RATE PER 10 °C RISE IN TEMPERATURE (Q_{10})

The value of activation energy was used to calculate the rise in reaction rate for every 10 °C increase in temperature with the help of following formula:

$$Q_{10} = \frac{E_a}{K}\left(\frac{1}{T_2} - \frac{1}{T_1}\right)$$

RESULTS AND DISCUSSION

OPTIMUM PH AND TEMPERATURE FOR MAXIMAL PRODUCTION OF β-GLUCOSIDASE

pH and temperature were optimized to get maximal protein yield of the enzyme. After 96 hours of incubation period with continuous shaking maximum activity for ß-glucosidase was obtained at pH 3.5 and temperature 30 °C (Figs. 1 and 2, respectively). The activities decreased after increasing pH and temperature. Statistical analysis showed that data had significant ($P<0.01$) difference in the production of the enzyme activity by varying the temperature and pH.

Optimal pH and temperature are very important for growth of microorganisms. Our results for both the conditions are in accord with the reports from literature. *Arachniotus* sp. gave maximum β-glucosidase (1.12 IU mL^{-1}) activity at pH 4 and 32°C [Asghar *et al*. 1999]. The results of Skory and Freer [1996] had contradiction with our results. They got maximum β-glucosidase activity at pH 6 to 6.5, temperature 35°C.

KINETIC STUDY OF β-GLUCOSIDASE

K_M AND V_{MAX}

The dependence of the reaction rate on the concentration of different enzyme substrates was calculated using linear transformation of Michaelis-Menton equation; Lineweaver-Burk Plot (1/V vs 1/[S], Woolf-Augustinsson-Hofstee Plot (V vs V/[S]) and Hanes-Woolf Plot ([S] vs [S]/V).

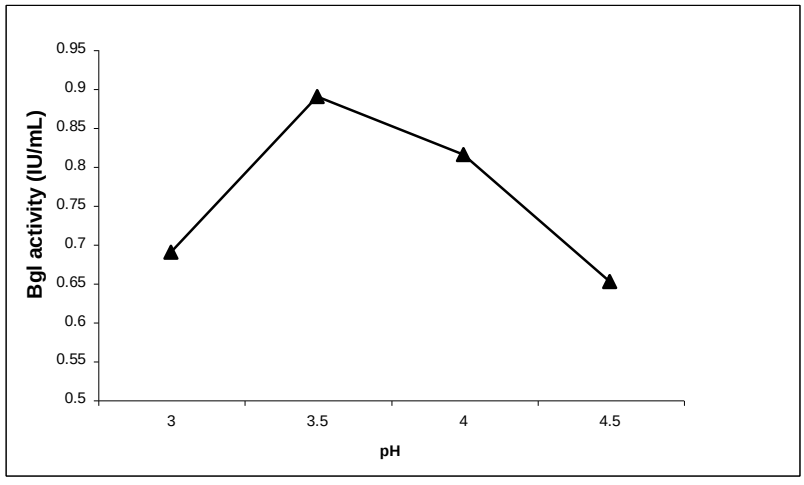

Fig. 1: Productivity of β-glucosidase by *Aspergillus niger* NRRL 567 at different pH values.

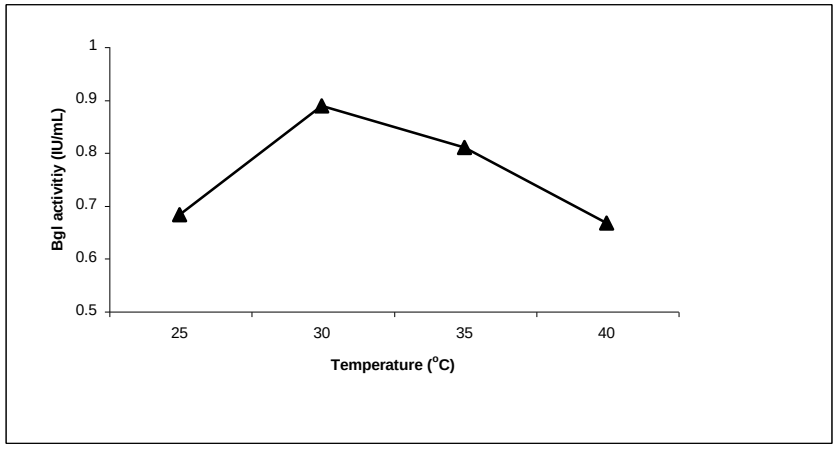

Fig. 2: Productivity of β-glucosidase by *Aspergillus niger* NRRL 567 at different temperatures.

Salicine was used as a substrate for β-glycosidase. By using Lineweaver-Burk plot (1/V vs 1/S), the K_m value was 0.4512% and V_{max} value was 1.011µmol mL^{-1} min^{-1} (Fig. 3). The K_m constants and V_{max} values calculated with the other two linear transformations were as follows:

For Hanes Woolf plot ([S] vs [S]/V), the K_m value found to be 0.4026% and V_{max} 0.9494µmol $mL^{-1}min^{-1}$ (Fig. 4). For Woolf-Augustinsson Hofstee plot (V/[S] vs V), the K_m value was 0.3914% and V_{max} was 0.9308µmol $mL^{-1}min^{-1}$ (Fig. 5)

The results are close to those obtained by Schmid and Woundrey [1987] who reported Km value 0.5% for ß-glycosidase of *Trichoderma reesei* strain QM 9414. The results are contrary to as described by Evan [1985] who reported Km value 0.276 m*M* in colorless vesicular. Li and Calza (1991) reported K_m 2.5 m*M* and V_{max} 5.02 IU mg^{-1} for β-glycosidase using pNPG as substrate. Roy *et al.* [1981] produced β-glycosidase from a culture broth of *M. thermophila* ATCC 48104 grown on crystalline cellulose and reported that Km of enzyme was 1.6

m*M* for p-nitrophenyl-β-D-glucoside. Siddiqui *et al.* [1997a] produced β-glucosidase from *Cellulomonas biazotea* NIAB 442 and reported that K_m and V_{max} values for pNPG were 4.25 m*M* and 1.526 μ mg^{-1} protein respectively. Our results indicate small K_m values for the enzyme. This demonstrates high affinity of the enzyme with its substrate.

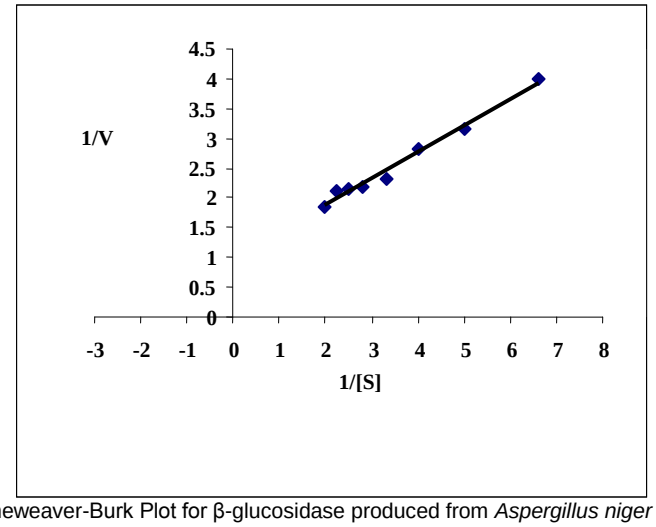

Fig. 3: Lineweaver-Burk Plot for β-glucosidase produced from *Aspergillus niger* NRRL 567.

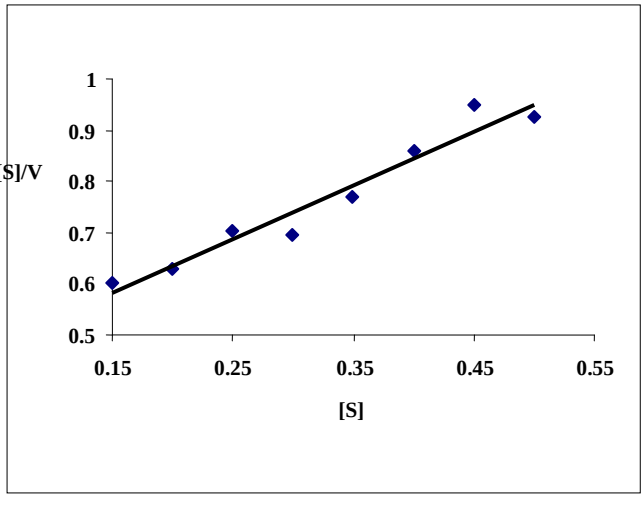

Fig. 4: Hanes-Woolf Plot for β-glucosidase produced from *Aspergillus niger* NRRL 567.

OPTIMUM TEMPERATURE

The experiments with varying temperature viz 10, 20, 30, 40, 50, 60 and 70°C were performed to find out the optimum temperature for the enzyme. It was observed that optimum temperature for the enzyme was 30 °C as shown in Fig. 6. At 20°C the activity was decreased activity. The activity again decreased up to 50 °C consecutively, but at 60 °C again it showed increasing trend which is a

point of great surprise. Such an increase in activity at the higher temperature may be due to the presence of different catalytic domains in the enzyme that range from 1 to 5 [Ahsan *et al.* 1997].

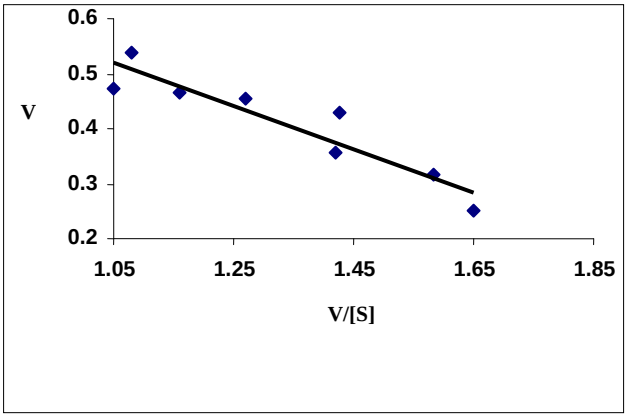

Fig. 5: Woolf-Augustisson Hofstee Plot for β-glucosidase produced from *Aspergillus niger* NRRL 567

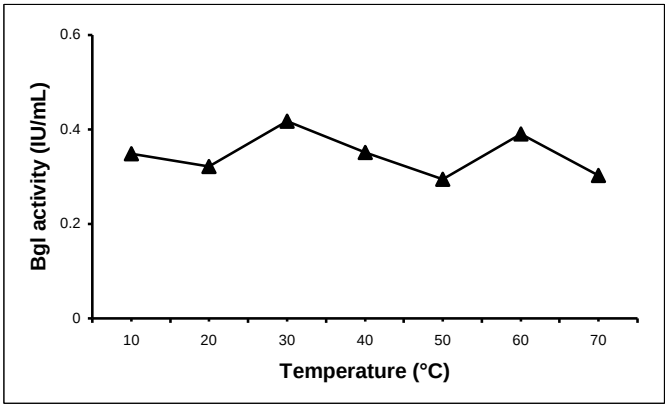

Fig. 6: Effect of temperature on the activity of β-glucosidase.

Our results are in close agreement with Rajoka and Malik [1984] who reported temperature optima of different enzymes from *C. biozota* mainly between 30-50°C. The enzymes retained 85-100% activity at 30°C and retained 100% original activity upto 50°C. All enzymes showed a tendency to lose activity above 50 °C and to a great extent at 60 °C. Similar results have also been reported by Honda *et al.* [1988] who observed that ß-glycosidase showed maximum enzymatic activity at 37 °C in *E. coli*. Our results are different to those of Esen [1992] and Iqbal *et al.* [1989] who reported 50-55°C as optimum temperature for ß-glucosidase from Hansenia spora vineae. The differences in findings are attributable to difference in the species of enzyme origin. Esen [1992] stated that the enzyme was stable at 4 °C, but lost its activity above 55 °C completely which is contrary to our work, because enzyme produced by *Aspergillus niger* NRRL

567 showed regain in activity at 60 °C. Activity at 60 °C is supported by Roy *et al.* [1991] who reported 60 °C as optimum temperature of β-glucosidase from *Mycelliophora thermophila.* Another reason may be that the one catalytic site is active at one temperature while other at another temperature and it may be due to folding or unfolding of 3 dimensional structure of the polypeptide.

ENERGY OF ACTIVATION (E_a)

Energy of activation (E_a) for β-glycosidase was 19.00 kJ K^{-1} mol^{-1} as calculated with the help of Arrhenius plot [Atkin 1995]:

$$E_a = -\text{Slope} \times R,$$

where R = gas constant = 8.314 JK^{-1} mol^{-1} and Slope = $-E_a/R$ The slope was found to be –2.286. It was observed that at 30 °C the β-glycosidase had maximum catalysis in the conversion of salicin into glucose by using the activation energy (E_a) mentioned above. After this temperature the enzyme started becoming denatured and showed less activity towards the conversion of substrate into product. The small amount of activation energy indicates a good relationship between the enzyme and the substrate. It was concluded that kinetically the β-glycosidase of *Aspergillus niger* 567 was favorably good for the conversion of cellulose into glucose. Our results are almost similar to those given by Sanyal *et al.* [1988] who have reported the energy of activation of β-glycosidase from *Aspergillus japonicus* to be 34.276 kJ mol^{-1}.

REACTION RATE PER 10 °C RISE IN TEMPERATURE (Q_{10})

Increase in reaction rate for every 10 °C rise in temperature was calculated for the enzyme with the help of activation energy by using the following formula:

$$\ln Q_{10} = \frac{Ea}{R} \text{ x } (\frac{1}{T_1} - \frac{1}{T_2}) \text{ [Atkin 1995]}$$

T_1 = 20°C, T_2 = 30°C, R = gas constant

The Q_{10} value obtained for β-glycosidase was 1.33. This value shows that there was, an average, 1.37 times increase in the reaction rate of the enzyme when the temperature was increased from 20 °C to 30 °C. Lower Q_{10} values demonstrate high catalysis as a distinctive feature of enzyme catalysis is that the Q_{10} of a catalyzed reaction is lower as compared to the same reaction uncatalyzed.

References

Ahsan, M., Maksumoto, M., Karita, S., Kimura, T., Sakka, K. and Ohmiya, K. (**1997**) “Purification and characterization of the family J catalytic domain derived from the *Clostridium thermocellum* endoglucanse cell”, *J. Biosci. Biotechnol. biochem*, **61**(3), 427-431.

Asghar, M. (**1998**) “A study of cellulases and protein biomass produced by *Arachniotus sp*.”, Ph.D. Thesis, Department of Chemistry, University of Agriculture, Faisalabad, Pakistan.

Atkins, P.W. (**1995**) “The Elements of Physical Chemistry”, Oxford University Press, Oxford, pp. 253, 255.

Chahal, D.S. (**1986**) "A new approach in solid state fermentation for cellulose production", In: *Biotechnology and Renewable Energy*, Elsevier, Applied Science Publishers, London, pp.57-69.

Esen, A. (**1992**) "Purification and practical characterization of maize (*Zea may* L.) β-glucosidase", *Plant Physiol.*, **98**(1), 174-182.

Evans, C.S. (**1985**) "Properties of β-gluconsidase from the wood rotting fungus, Cariolus veskolor", *Appl. Microbiol. Biotechnol.*, **22**, 128-131.

Gadgil, N.J., Dagnawala, H.F., Chakrabarti, T. and Khana, P. (**1995**) "Enhanced cellulase production by a mutant of *T. ressei*", *Enzymes and Microbial. Tech.*, **17**, 942-946.

Garg, S.K. and Neelakanton (**1981**) "Effect of culture factors con cellulase activity and protein production by *Aspergillus terrus*", *Biotechnol. Bioeng.*, **24**, 737-742.

Honda, H.T., Saito, Slijima and Kobayashi, T. (**1988**) "Molecular cloning and expression of β-glucoside gene from ruminococeus albus in *E. coli*", *Enzyme Microb. Technol.*, **10**, 559-562.

Iqbal, M.I., Rajoka, M.I. and Malik, K.A. (**1989**) "Production of thermostable β-glucosidase by a mesophilic fungus *Aspergillus niger* NIAB 280", *Proceedings of International Symposium on Biotechnology for Energy*, Dec. 16-21, pp. 157-165.

Li, X. and Calza, R.E. (**1991**) "Purification and characterization for an extra cellular β-glucosidase from the rumen fungus *Neocallimastix frontalis* EB 188", *Enzyme and Microbiol. Technol.*, **13**(8), 622-628.

Price, N.C. and Dwek, R.A. (**1986**) "The kinetics of enzyme catalyzed reactions", In: *Principles and Problems in Physical Chemistry for Biochemists*, 2nd edn. Clarendon Press, London.

Rajoka, M.I. and Malik, K.A. (**1986**) "Comparison of different strains of cellulomonas for production of cellulolytic and xyllano lytic enzymes from biomass produced on the saline lands", *Biotech-nol*., **8**(10), 753-756.

Rajoka, M.I. and Malik. K.A. (**1984**) "Isolation and characterization of cellulytic bacteria", 5th Internat. Cong. on culture collection, Thailand, Nov. 25-Dec.1.

Rajoka, M.I., Bashir, A., Hussain, S.R.S. and Malik. K.A. (**1998**) "ϒ-ray induced mutagenesis of *Cellulomonas biozotea* for improved production of cellulases", *Folia Microbiol.*, **43**(1), 15-22.

Roy, S.K., Raha, S.K., Sadhukhan, R.K. and Chakrabarty, S.L.. (**1991**) "Purification and characterization of β-glucosidase of myceliophthora thermophila", *World J. Microbiol. Biotechnol.*, **7**(6), 613-618.

Sakka, K., Kimura, T., Karita, S., Ohmiya, K. (**2000**) "Molecular breeding of cellulolytic microbes, plants and animals for biomass utilization", *J. Biosci. Bioeng*., **90**, 227-233.

Sanyal, A., Kundu, R.K., Dube, S. and Dube, D.K. (**1988**) "Extracellular cellulolytic system of *Aspergillus* Japonicus 2, purification and characterization of inducible extracellular β-glucosidase", *Enzyme Microb.*, **10**, 91-99.

Shewale, J.C. and Sadana, J.C. (**1978**) Cellulase and β-glucosidase production", *Can. J. Microbiol*., **24,** 1204-16.

Siddiqui, K.S., Rashid, M.H., Ghauri, T.M., Durrani, I.S. and Rajoka, M.I. (**1997**) "Short commonication: Purification and characterization of an intracellular β-glucosidase from *cellulomonas biazotea*", *World J. Microbiol and Biotech*., **13**, 245-247.

Skory, C.C. and Freer, S.N. (**1996**) "Properties of an intracellular β-glucosidase purified fro the cellulose fermenting yeast candia wickerhamii", *Appl. Microbiol. Biotechnol.*, **46**(4), 353-359.

Srinivasan, M.C., Vartak, H.G., Powar, V.K., Rele, M.V. and Bastawele, K.B. (**1984**) "Lignocellulose Biotechnology: Recent advances and technology prospects", *Biotech. Lett.*, **6,** 715.

Yoshihiko, A. and Takahisa, K. (**2002**) "New insights into cellulose degradation by cellulases and related enzymes", *Trends in Glycosci. and Glycotechnol.*, **14**, 27-34.