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Full Length Research Paper

Metabolic engineering and thermodynamic characterization of an extracellular β-glucosidase produced by *Aspergillus niger*

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The production of an extracellular β-glucosidase by *Aspergillus niger* NRRL 599 was optimized using submerged fermentation technique. Effect of different media, different carbon sources, initial pH of the fermentation medium, temperature, incubation period and inoculum size on the production of β-glucosidase enzyme was investigated. *A. niger* NRRL 599 produced maximum extracellular β-glucosidase (4.48 U/mg) in Eggins and Pugh medium with 1% wheat bran (w/v) at pH 5.5 inoculated with 4% conidial suspension after 96 h of incubation at 30°C. Purified β-glucosidase gave K_m and V_{max} values of 3.11 mM and 20.83 U/mg respectively for pNPG hydrolysis. The enzyme was optimally active at pH 4.8 and at temperature of 60°C. Thermodynamic parameters, E_a, ΔH and ΔS were found to be 52.17 KJ/mol, 49.90 J/mol.K and -71.69 KJ/mol, respectively. The pKa_1 and pKa_2 of ionizable groups of active site residues involved in V_{max} were calculated to be 4.1 and 6.0 respectively.

Key words: β-Glucosidase, *Aspergillus niger*, kinetics, thermodynamics.

INTRODUCTION

β-Glucosidase (EC 3.2.1.21) catalyzes the hydrolysis of β-1,4 glucosidic bonds in disaccharides, oligosaccharides, alkyl and aryl β-D-glucosides by attacking on the non-reducing terminal and releases β-D glucose (Iwashita et al., 1999; Wallecha and Mishra, 2003). β-Glucosidase is produced by humans, insects, plants, bacteria, fungi and yeast (Villena et al., 2007). This enzyme has been exploited in a variety of biotechnological and biochemical industries. Moreover, β-glucosidase component plays a pivotal role in the final step of cellulose degradation (Seidle and Huber, 2005). β-Glucosidase is generally responsible for the regulation of the whole cellulolytic process and is a rate-limiting factor during enzymatic hydrolysis of cellulose, as both endoglucanase and exoglucanase activities are often inhibited by cellobiose. Thus, β-glucosidase not only produces glucose from cellobiose, but also reduces cellobiose inhibition, allowing endoglucanase and exoglucanase enzymes to function more efficiently (Kaur et al., 2007). Supplementing β-glucosidase in commercial cellulase preparations has been reported to result in increased rate of cellulose hydrolysis and ethanol production (Wallecha and Mishra, 2003).

Aspergillus niger is by far the most efficient producer of β-glucosidase among the microorganisms investigated. It is generally recognized as safe by the Food and Drug Administration (Tailor and Richardson, 1979). β-Glucosidase can be produced both by solid-state and submerged fermentation techniques (Christakopoulos et al., 1995; Iembo et al., 2002; Fawzi, 2003; Garcia-Kirchner et al., 2005; Daroit et al., 2007; Leite et al., 2007). Sub-

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Abbreviations: CMC, Carboxymethylcellulose; **FPLC,** fast protein liquid chromatography; **pNPG,** *p*-nitrophenyl-β-D-glucopyranoside.

merged fermentation offers several advantages, among them a possibility to automate many operations, the ease with which various parameters can be monitored (by periodic sampling of broth) and controlled if necessary by the addition of further nutrients or reagents and easier removal of heat generated during the microbial growth in a large scale reactor (Jakubikova et al., 2006). Extracellular production of β-glucosidase using submerged fermentation technique had been investigated by different researchers. Gunata and Vallier (1999) studied the production of extracellular β-glucosidase from *A. niger* and *Aspergillus oryzae* using submerged fermentation technique. Jager et al. (2001) investigated the extracellular production of β-glucosidase from different *Aspergillus* strains using submerged fermentation and Garcia-Kirchner et al. (2005) studied the effect of media composition and growth conditions on the production of β-glucosidase by *A. niger* C-6 under submerged culture.

This study was initiated with the aim to exploit *A. niger* NRRL 599 for maximum yield of β-glucosidase and the subsequent characterization of β-glucosidase.

MATERIALS AND METHODS

Organism

A. niger NRRL 599 was obtained from the stock culture of Institute of Industrial Biotechnology, GC University, Lahore. The culture was maintained on 4% potato dextrose agar medium (pH: 5.5). The slants were incubated at 30°C for maximum growth and stored at 4°C. Sub-culturing was carried out every 15 days.

Inoculum

The conidial suspension was used as an inoculum. The number of conidia was counted with the help of haemacytometer slide bridge (Neubauer improved, Precicder HBG, Germany). Ten milliliters of sterilized distilled water was transferred to each slant having profused conidial growth on its surface. The sterilized inoculating needle was used to break the clumps of conidia. The tube was shaken vigorously to make a homogenous mixture of conidial suspension.

Fermentation

β-Glucosidase fermentation was carried out using submerged technique in 250 ml Erlenmeyer flasks containing 25 ml of Eggins and Pugh (1962) medium consisting of: $(NH_4)_2SO_4$ (0.5 g/l), KCl (1.0 g/l), KH_2PO_4 (0.5 g/l), $MgSO_4.7H_2O$ (0.2 g/l), L-asparagine (0.5 g/l), $CaCl_2$ (0.1 g/l), yeast extract (0.5 g/l) with 1% carbon source (w/v) at pH 5.5 inoculated with 4% conidial suspension. The flasks were incubated in an orbital shaking incubator (SANYO, Gallenkamp, PLC, UK) at 30°C (200 rpm) for 96 h. After the incubation, the fermented broth was centrifuged at 6,000 rpm for 10 min and the supernatant was used for analytical purposes.

Optimization of fermentation parameters

Eight different types of fermentation media, (M-I to M-VIII) already reported by Eggins and Pugh (1962), Mandel and Reese (1960), Romanelli et al. (1975), Coutts and Smith (1976), Saddler (1982), Macris and Panayatou (1989), Almin et al. (1975) and Riou et al. (1998) respectively, were screened for the production of β-glucosidase. Different carbon sources, for example, carboxymethyl cellulose (CMC), cellulose powder, lactose, maltose, sucrose, wheat bran and cellobiose (1%, w/v) were added to fermentation medium to test their effect on enzyme production. Time course study for the production of β-glucosidase was carried up to 144 h. Effect of different incubation temperature (25 to 35°C), initial pH of the fermentation medium (4.0 to 7.0) and conidial inoculum (1 to 5%) was also investigated on the production of β-glucosidase. All the experiments were carried out in triplicate.

Analysis

β-Glucosidase activity was estimated according to the method used by Rajoka and Malik (1997). "One unit of β-glucosidase activity is defined as the amount of enzyme required to release 1.0 µmol of p-nitrophenol per min under the assay conditions". Total protein was estimated according to the method of Bradford (1976) using bovine serum albumin as standard.

Purification of β-glucosidase

Crude β-glucosidase was purified to homogeneity by using ammonium sulfate precipitation and fast protein liquid chromatography (FPLC) by using RESOURSE S (AMERSHAM BIOSCIENCES, USA). Solid ammonium sulfate was slowly added to crude enzyme to 80% saturation at 0°C to precipitate the β-glucosidase with stirring at an interval of 5 min. The pellet was collected by centrifugation at 12000 ×*g* for 20 min (4°C) to recover precipitated proteins. The pellet was dissolved in 3.0 ml of phosphate buffer (pH: 7.2) and dialyzed against 0.05 M sodium phosphate buffer (pH: 7.5) by constant stirring for 24 h and periodic change of buffer. Dialysis membrane with a molecular weight cut-off of 12000 to 14000 was used. The dialyzed sample, filtered through 0.4 µm Millipore filter, was loaded on an anion exchange column RESOURCE-S (6.0 ml, 16 x 30 mm; AMERSHAM BIOSCIENCES, USA) equilibrated with 50 mM sodium phosphate buffer (pH: 7.5) with a flow rate of 1.0 ml/min. The protein was eluted with a linear gradient of NaCl (0 to 1 M) in the buffer. The elution profile was examined at 280 nm. The fractions were collected and assayed for enzyme activity.

Enzyme characterization

The purified enzyme was characterized as a function of temperature and pH. The optimal temperature for enzyme activity was determined over a temperature range of 20 to 65°C using the standard assay conditions. To determine the optimum pH for the enzyme, β-glucosidase activity was measured over a pH range of 3.0 to 7.0 using the standard assay conditions.

Kinetic and thermodynamic studies

The kinetic constants, Michaelis constant (K_m), and maximum

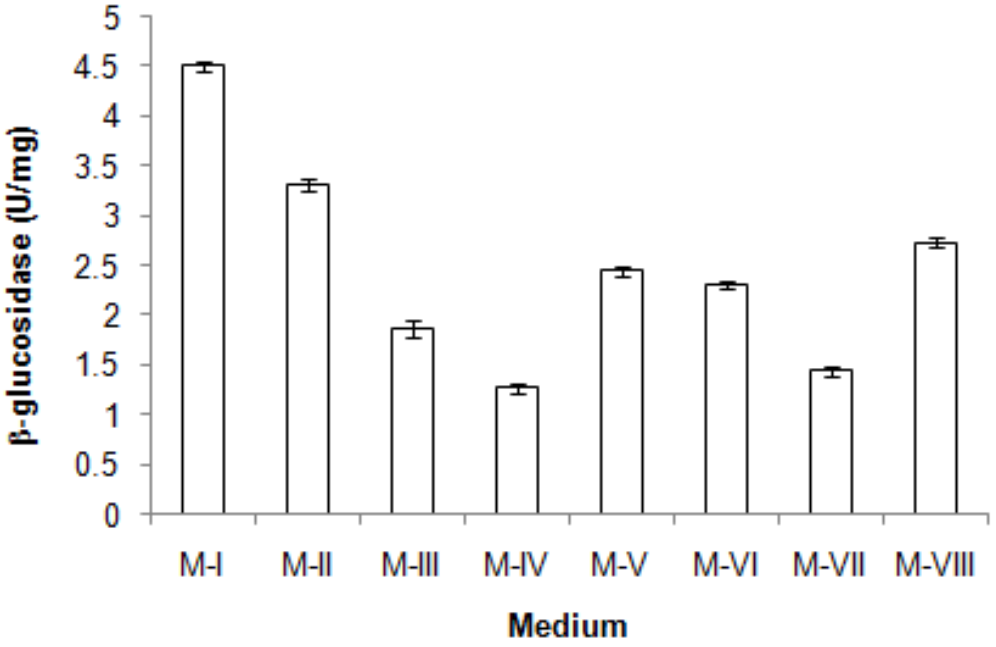

Figure 1. Screening of fermentation media for β-glucosidase production by *Aspergillus niger* NRRL 599 in shake flasks. ± indicates the standard deviation among the three parallel replicates. Incubation at 30ºC for 96 h at pH 5.5 using wheat bran as carbon source.

velocity (V_{max}), were evaluated by determining the β-glucosidase activity at 60°C against different *p*-nitrophenyl-β-D-glucopyranoside (pNPG) concentrations ranging from 1-15mM and keeping the enzyme concentration constant. K_m and V_{max} were calculated from Lineweaver-Burk double reciprocal plot using Equation 1 (Lineweaver and Burk, 1934):

$$1/v = (1/V_{max}) + (K_m/V_{max}) \cdot 1/[S] \quad (1)$$

Thermodynamic constants, activation energy (E_a), enthalpy of activation (ΔH) and entropy of activation (ΔS), were calculated from Arrhenius plot using Equations 2 to 4 after Siddiqui et al. (1997a):

$$E_a = - \text{slope } (R) \quad (2)$$

$$\Delta H = E_a - RT$$

$$\ln(V_{max}/T) = \ln(K_B/h) + \Delta S/R - (\Delta H/R)1/T \quad (3)$$

where slope= ΔH/R and intercept= $\ln(K_B/h) + \Delta S/R$

$$\Delta S = R\,[\text{intercept} - \ln(K_B/h)] \quad (4)$$

Where, T, K_B, h, R, E_a, ΔH and ΔS are absolute temperature (K), Boltzmann constant (1.38×10^{-23} J/K), Planck's constant (6.626×10^{-34} Js), Gas constant (8.314 J/K.mol), activation energy, enthalpy of activation and entropy of activation, respectively. pK_{a1} and pK_{a2} values of ionizable groups of active site residues were calculated using Dixon plot (Dixon and Webb, 1979) (Figure 10).

Statistical analysis

Treatment effects were compared after Snedecor and Cochrane (1980) using computer software Costat, cs6204W.exe. Significant difference among replicates has been presented as Duncan's multiple range tests in the form of probability (*p*) values.

RESULTS AND DISCUSSION

The composition of fermentation media has a marked influence on enzyme production. Eight different type of fermentation media were tested for the production of β-glucosidase. M-I medium reported by Eggins and Pugh (1962) was found to be the best medium for the production of β-glucosidase (Figure 1). In M-I, the presence of L-asparagine and $(NH_4)_2SO_4$ in addition to other essential components might have resulted in the increased production of β-glucosidase. Other media gave relatively less amount of enzyme as they might be lacking in any of the components essential for the growth of fungi and enzyme production. Grajek (1987) also reported $(NH_4)_2SO_4$ as the best nitrogen source for the production

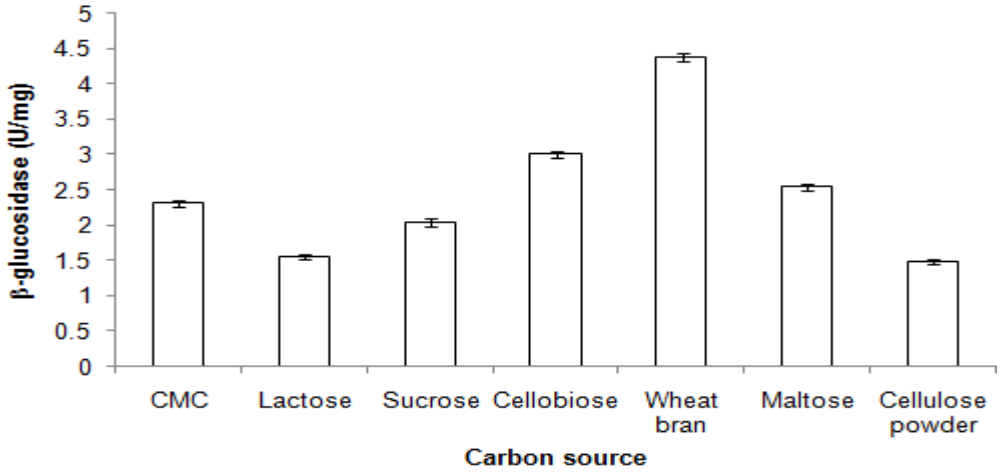

Figure 2. Effect of different carbon sources on β-glucosidase production by *Aspergillus niger* NRRL 599 in shake flasks. ± indicates the standard deviation among the three parallel replicates. Incubation at 30ºC for 96 h at pH 5.5 in M-I medium.

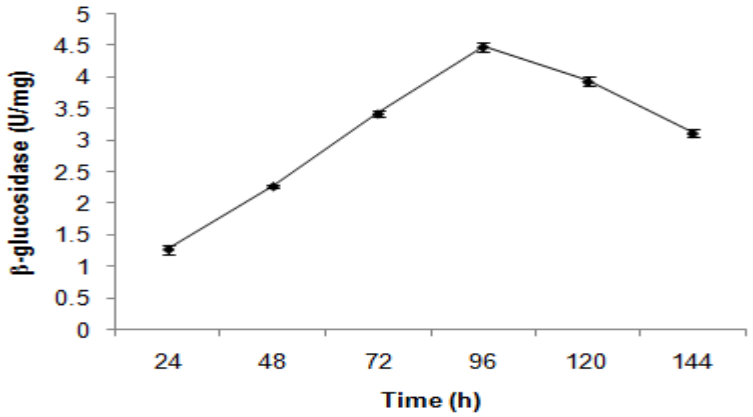

Figure 3. Effect of incubation time on β-glucosidase production by *Aspergillus niger* NRRL 599 in shake flasks. ± indicates the standard deviation among the three parallel replicates. Incubation at 30ºC and pH 5.5 using wheat bran as carbon source in M-I medium.

of β-glucosidase from *Sporotrichum (Chrysosporium) thermophile*.

β-Glucosidase is an inducive enzyme (Busto et al., 1995; Daroit et al., 2007). In order to get maximum production, wheat bran used in Eggins and Pugh medium was replaced with different carbon sources but no positive change was observed with other carbon sources (Figure 2). It is reported that cellobiose as a carbon source is the best for the induction of β-glucosidase (Busto et al., 1995) but in this study CMC, cellulose powder, lactose, maltose, sucrose and cellobiose gave less β-glucosidase production than wheat bran. Wheat bran contain plenty of cello-oligosaccharides that results in the increased activity of extracellular β-glucosidase (Sun et al., 2008). Jager et al. (2001) and Rajoka et al. (2006) found wheat bran as the best substrate for the production of β-glucosidase from different *Aspergillus* strains using solid state fermentation.

The optimization of time course for the production of β-glucosidase by *Aspergillus niger* NRRL 599 was investigated up to 144 h after inoculation. Maximum β-glucosidase was obtained after 96 h of fermentation (Figure 3). However, further increase in the incubation time reduced the enzyme production. It might be due to

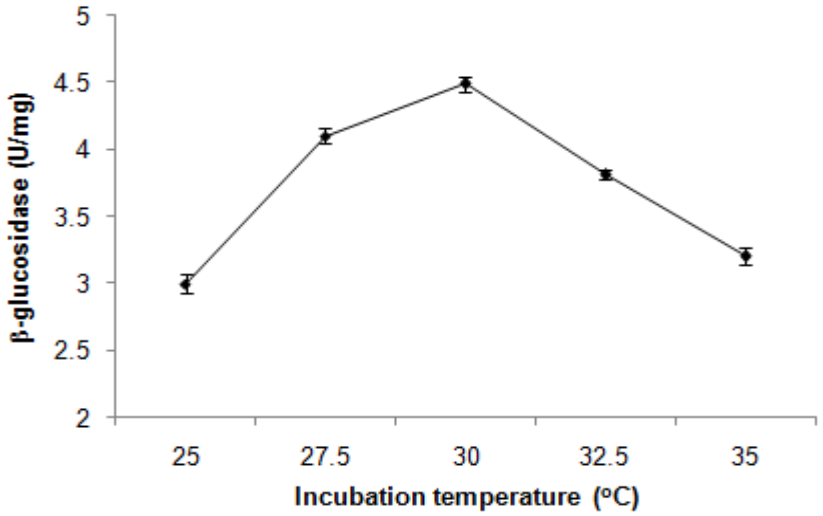

Figure 4. Effect of incubation temperature on β-glucosidase production by *Aspergillus niger* NRRL 599 in shake flasks. ± indicates the standard deviation among the three parallel replicates. Incubation at pH 5.5 for 96 h using wheat bran as carbon source in M-I medium.

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. 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 (Haq et al., 2006). Tsao et al. (2000) also obtained maximum β-glucosidase production from *A. niger* after 96 h of fermentation while Garcia-Kirchner et al. (2005) obtained maximum β-glucosidase titer from *A. niger* within 72 h of fermentation.

Effect of different incubation temperature (25 to 35°C) on the production of β-glucosidase was evaluated. Maximum β-glucosidase was obtained at 30°C (Figure 4). Further increase or decrease in temperature resulted in gradual decrease in enzyme production. At lower temperature, the transport of nutrients in the cells is hindered and lower product yields are attained, while at high temperature, the maintenance energy requirement increases owing to inactivation of proteins of the metabolic pathway and less product formation occurs (Rajoka et al., 2004). Gunata and Vallier (1999) also obtained maximum production of β-glucosidase from *A. niger* and *Aspergillus oryzae* at incubation temperature of 30°C.

The initial pH of the medium has great influence on cell membrane function, cell morphology and structure, the uptake of various nutrients, and metabolite biosynthesis (Li et al., 2008). Effect of different initial pH (4.0 to 7.0) of the fermentation medium was investigated for the production of β-glucosidase. Optimum β-glucosidase production was obtained at a pH of 5.5 (Figure 5). Most of the filamentous fungi prefer acidic pH for maximum growth and subsequently for high production of enzyme. This might be due to the fact that alkaline pH has inhibitory effect on the growth of the fungus and enzyme production. In addition, cultivation of fungi at an unfavorable pH value may result in reduced enzyme production by reducing accessibility of the substrate (Bakri et al., 2008). Garcia-Kirchner et al. (2005) also optimized pH 5.5 for the production of β-glucosidase from *A. niger*. In contrast, Abdel-Fattah et al. (1997) obtained maximum β-glucosidase activity from *A. niger* at a pH of 4.0.

The effect of different size of conidial inoculum (1.0, 2.0, 3.0, 4.0, and 5.0%) on β-glucosidase production by *A. niger* NRRL 599 in shake flasks for 48 to 120 h was investigated. Maximum β-glucosidase was obtained after 96 h of fermentation with 4% conidial inoculum (Figure 6). Increase or decrease in the size of conidial inoculum resulted in the decreased production of the enzyme. Low inoculum levels might be inadequate for enzyme production while the higher inoculum level might result in rapid depletion of nutrients in the media and reduce the enzyme yield. Also, higher cell density would lead to the production of inhibitory metabolites that may be interfering with the enzyme production (Niladevi and Prema, 2008). In contrast, Abdel-Naby et al. (1999) obtained maximum β-glucosidase activity from *A. niger* with 2%

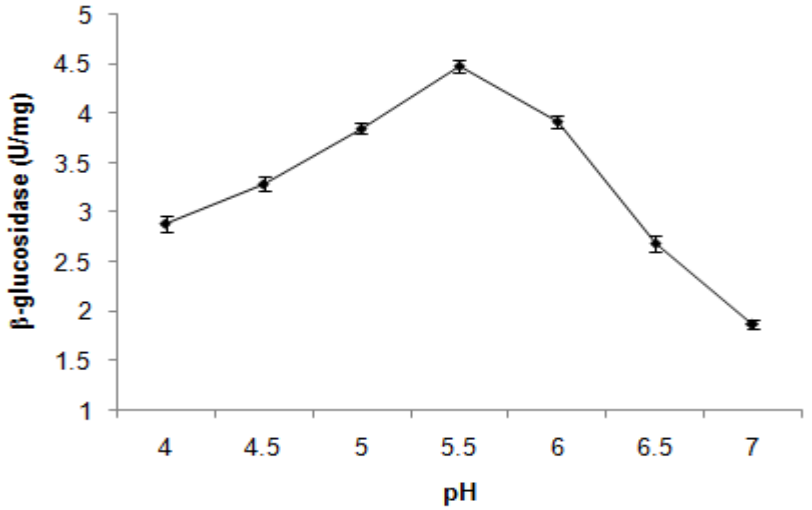

Figure 5. Effect of initial pH on β-glucosidase production by *Aspergillus niger* NRRL 599 in shake flasks. ± indicates the standard deviation among the three parallel replicates. Incubation at 30ºC for 96 h using wheat bran as carbon source in M-I medium.

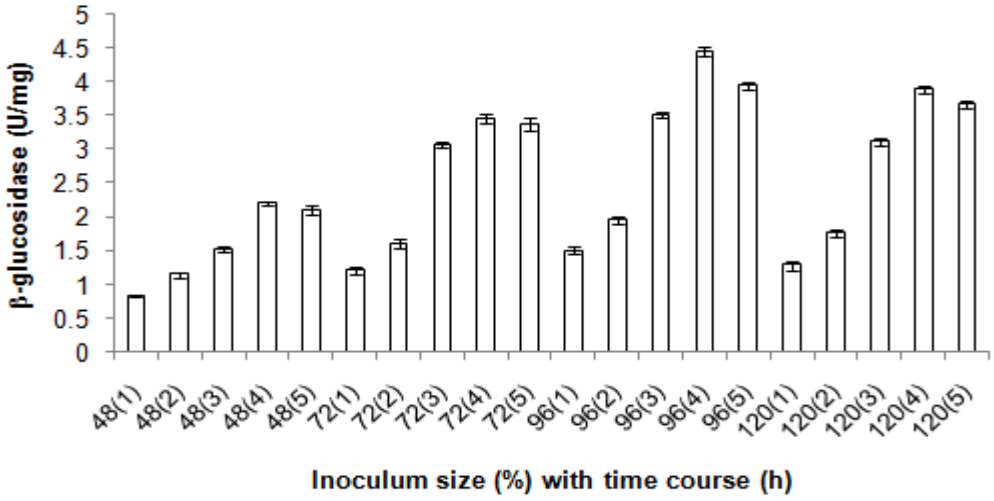

Figure 6. Effect of inoculum size on β-glucosidase production by *Aspergillus niger* NRRL 599 in shake flasks. ± indicates the standard deviation among the three parallel replicates. Incubation at 30ºC for 96 h at pH 5.5 using wheat bran as carbon source in M-I medium.

conidial inoculum.

Enzyme characterization

The enzyme was purified using ammonium sulfate precipitation and FPLC with a purification fold of 4.36. The purified enzyme was then used for enzyme characterization.

The kinetic constants, K_m and V_{max}, for β-glucosidase from *A. niger* were determined at 60℃ using pNPG as a substrate and the data was analyzed using Lineweaver-Burk double reciprocal plot (Figure 7). β-Glucosidase exhibited Michaelis Menten type kinetics and K_m and V_{max}

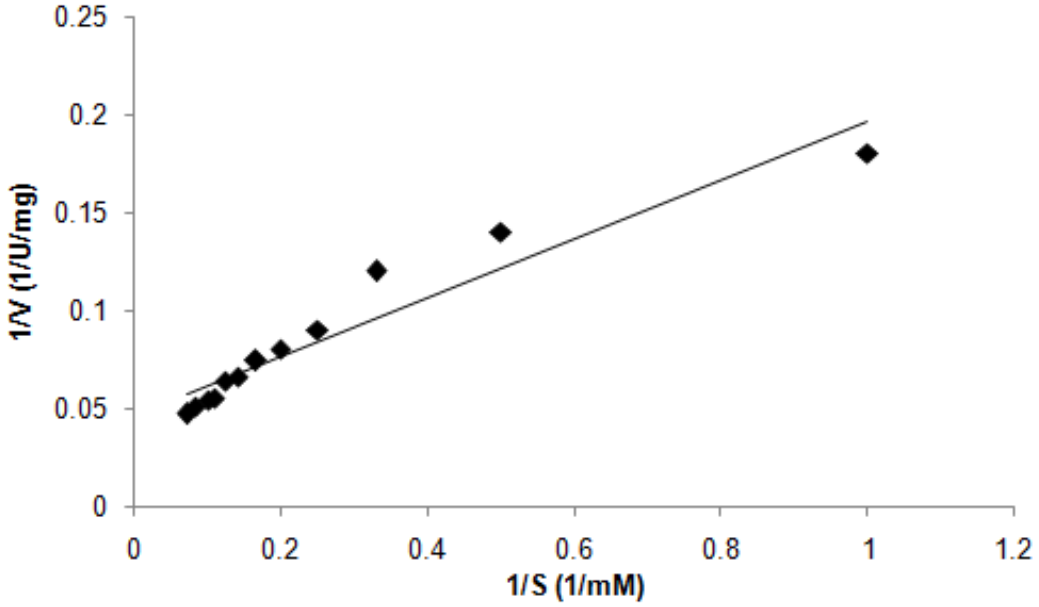

Figure 7. Lineweaver-Burk double reciprocal plot of β-glucosidase from *Aspergillus niger* NRRL 599. Error bars of standard deviation among the three parallel replicates were too small to be visible.

were found to be 3.11 mM and 20.83 U/mg respectively. K_m is a measure of affinity of an enzyme for a substrate and low values of K_m indicate high affinity of the enzyme for the substrate (Hamilton et al., 1998). Seidle et al. (2004) calculated K_m and V_{max} of 1 mM and 40 U/mg respectively for pNPG hydrolysis with a β-glucosidase from *A. niger*. K_m and V_{max} values of 3.3 mM and 43.68 µmol/min. mg respectively were calculated for pNPG hydrolysis by β-glucosidase from the *Melanocarpus* sp. by Kaur et al. (2007). The K_m and V_{max} values for pNPG hydrolysis for *Humicola insolens* β-glucosidase were calculated to be 0.16 mM and 18.1 U/mg respectively (Souza et al., 2010).

The effect of different temperature (20 to 65°C) was also investigated on the activity of β-glucosidase. Maximum β-glucosidase activity was obtained at 60°C. Further increase in temperature resulted in decreased β-glucosidase activity. Kimura et al. (1999) and Kaur et al. (2007) also obtained maximum β-glucosidase activity at 60°C from *Aspergillus sojae* and *Melanocarpus* sp. respectively. However, Christakopoulos et al. (1995) and Jeya et al. (2010) obtained maximum β-glucosidase activity at 65°C from *Fusarium oxysporum* and *Penicillium Purpurogenum* respectively. Thermodynamic studies of β-glucosidase were carried out using Arrhenius plot and activation energy (E_a), change in enthalpy (ΔH) and change in entropy (ΔS) were calculated to be 52.17 KJ/mol, 49.90 KJ/mol and -71.69 J/mol.K respectively (Figures 8 and 9). Low values of enthalpy and negative values of entropy indicate the formation of a more efficient and ordered transition state complex between enzyme and substrate (Akolkar and Desai, 2010). Kvesitadze et al. (1990) reported ΔH and ΔS of 125 KJ/mol and 65 J/mol.K respectively for β-glucosidase of *Aspergillus wentii.*

The activity of the purified β-glucosidase was examined over a pH range of 3.0 to 7.0 at 60°C using sodium acetate buffer. Maximum β-glucosidase activity was achieved at pH 4.8. Further increase or decrease in pH resulted in decrease in activity. Pericin and Jarak (1995) obtained maximum β-glucosidase activity from *Diaporthe* (*Phomopsis*) *helianthi* at pH 4.8. In contrast, Leite et al. (2007) obtained maximum activity of β-glucosidase from *Thermoascus aurantiacus* at pH 4.5 while Hang and Woodams (1994), Karnchanatat et al. (2007), Dhake and Patil (2005) and Zanoelo et al. (2004) obtained maximum β-glucosidase activity from *Aspergillus Foetidus, Daldinia eschscholzii, Penicillium purpurogenum* and *Scytalidum thermophilum* at pH 4.6, 5.0, 5.5 and 6.5 respectively. The change in pH affects the ionization of essential active site amino acid residues, which are involved in substrate binding and catalysis, that is, breakdown of substrate into products. Some ionizable residues may be located on the periphery of the active site, commonly known as non-essential residues. The ionization of these residues may cause distortion of active site cleft and hence indirectly

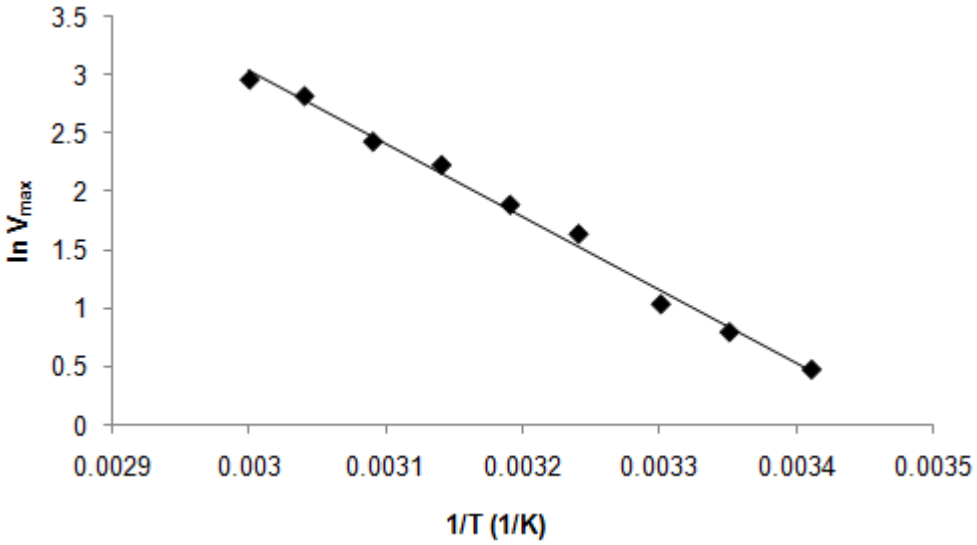

Figure 8. Arrhenius plot for the determination of activation energy (E_a) and enthalpy of activation (ΔH) of β-glucosidase from *Aspergillus niger* NRRL 599. Error bars of standard deviation among the three parallel replicates were too small to be visible.

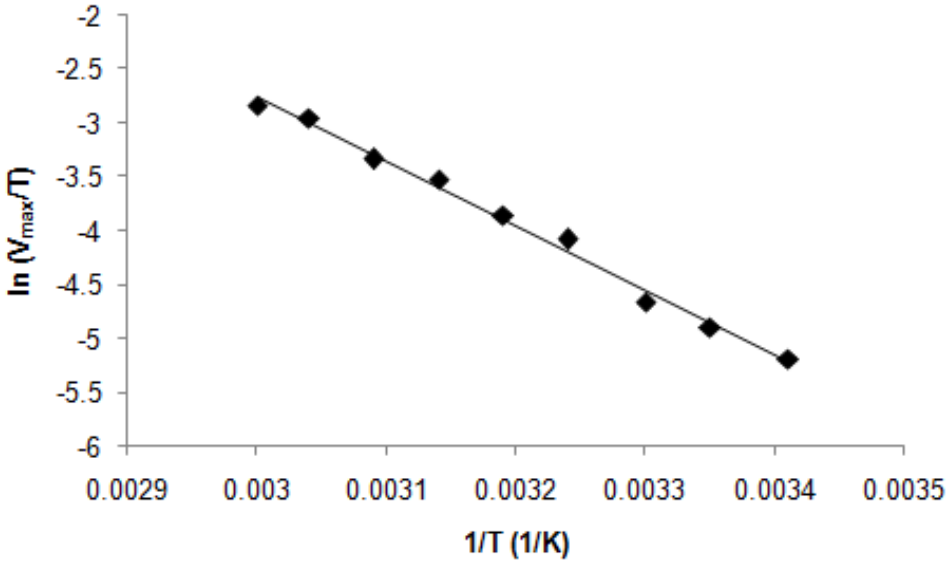

Figure 9. Arrhenius plot for the determination of entropy of activation (ΔS) of β-glucosidase from *Aspergillus niger* NRRL 599. Error bars of standard deviation among the three parallel replicates were too small to be visible.

affect the enzyme activity (Amin et al., 2010). Dixon analysis was carried out to evaluate pKa of ionizable groups of active site amino acid residues at 60°C which were involved in V_{max} (Fig 10). pKa_1 and pKa_2 of β-glucosidase were found to 4.1 and 6.0 respectively. Siddiqui et al. (1997b) reported pKa_1 and pKa_2 values of 5.5 and 7.9 respectively for β-glucosidase of *Cellulomonas biazotea.* pKa_1 and pKa_2 of 3.3 and 6.9 respectively were reported for β-glucosidase of *Schizophyllum commune* by Clarke (1990).

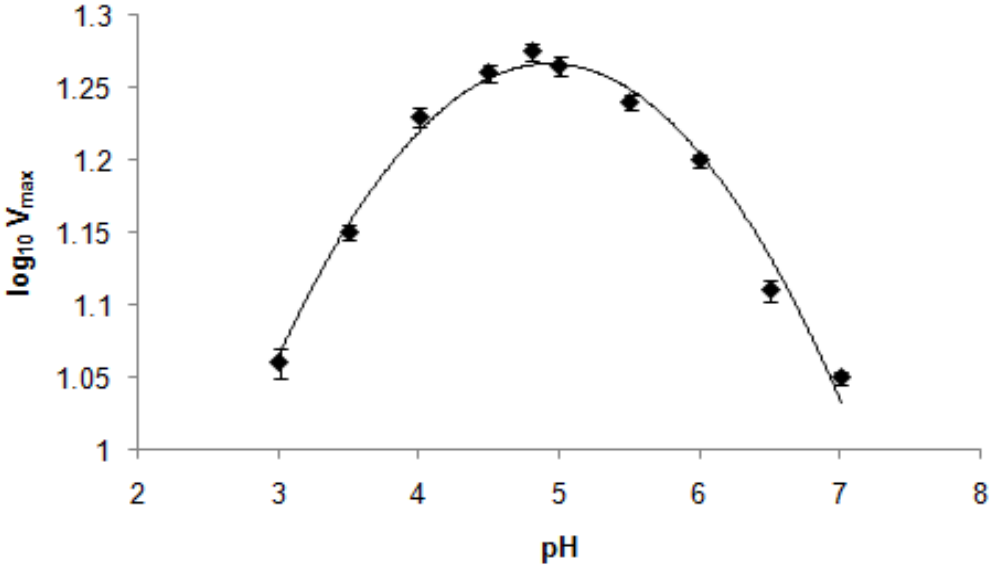

Figure 10. Dixon plot of β-glucosidase from *Aspergillus niger* NRRL 599 at 60°C for the determination of pKa of active site residues that control the V_{max}. ± Indicates the standard deviation among the three parallel replicates.

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