REGULATION OF EXPRESSION OF MULTIPLE β- GLUCOSIDASES OF *ASPERGILLUS TERREUS* AND THEIR PURIFICATION AND CHARACTERIZATION

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This study reports the regulation and purification of β-glucosidases from a thermotolerant *Aspergillus terreus* AN_1 strain, previously reported for efficient deinking of composite paper waste. The differential expression of four β-glucosidase isoforms, in response to carbon sources in production medium, was studied by electrophoretically resolving proteins by polyacrylamide gel electrophoresis analysis (PAGE) and developing zymograms using methylum-belliferyl β-D glucoside as substrate. Three β-glucosidases (βGI, βGII & βGIII) were purified using chromatographic techniques. SDS-PAGE revealed the respective molecular masses of βGI, βGII, and βGIII, as 29, 43, and 98 KDa, and isoelectric point (pI) to be 2.8, 3.7, and 3.0. The β-glucosidases exhibited diverse pH and temperature optima as well as stability. β-Glucosidase I (βGI) specifically recognized pNP-β-glucopyranoside (pNPG) as a substrate, whereas, β-glucosidase II (βGII) and III (βGIII) also showed activities against cellobiose and salicin. In contrast to βGII and βGIII, the activity of βGI was positively influenced in the presence of hexoses/pentoses and alcohols. K_m and V_{max} for hydrolysis of pNPG by βGI, βGII, andβGIII were found to be 14.2 mM and 166.9 µmol $^{-1}$mg protein $^{-1}$, 4.37 mM, and 34.7 µmol $^{-1}$mg proteins $^{-}$1, and 11.1 mM and 378.7µ mol $^{-1}$ mg protein $^{-1}$, respectively.

Keywords: Aspergillus terreus; β-glucosidases; Multiplicity; Differential expression; Regulation; Purification and characterization

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INTRODUCTION

β-glucosidases constitute a critical component of cellulase systems (Caughlan and Ljunghai 1988), catalyzing the final step in cellulose hydrolysis. For most bioconversion process, endo (1,4)-β-D glucan hydrolases (E.C. 3.2.1.4) and exo (1,4)-β-D glucan cellobiohydrolases (E.C.3.2.1.91) catalyze the hydrolysis of cellulose to produce cellobiose and other short cello oligosaccharides, which are ultimately hydrolyzed to glucose by β-glucosidases (E.C. 3.2.1.21). Complete degradation of cellulose requires synergistic action of all three types of enzymes. β-glucosidase is generally responsible for the regulation of the whole cellulolytic process and is considered to be the rate-limiting factor during enzymatic hydrolysis of cellobiose, as both endoglucanase and exoglucanase activities are often inhibited by cellobiose (Harhangi et al. 2002). Thus, β-glucosidase not only produces glucose from cellobiose, but it also reduces cellobiose inhibition, allowing endoglucanase and exoglucanase enzymes to function more efficiently. In addition, some β-glucosidases display transglycosylation activity (Xie *et*

al. 2004) and are thought to be involved in formation of positional isomers that act as inducers.

The interest in β-glucosidases has been rejuvenated in the last few years owing to its role in various biotechnological processes including production of fuel ethanol from cellulosic agricultural residues (Bothast and Saha 1997). It is well understood that a cellulase enzyme preparation for bioconversion of cellulose to ethanol should have appreciably high levels of efficient β-glucosidases (Kim *et al.* 2007). In addition, β-glucosidases are useful in the flavor industry as it is the key enzyme in the enzymatic release of aromatic compounds from glucosidic precursors present in fruits and fermenting products (Guegen *et al.* 1996). A recent U.S. patent 5,454,389 reports that a crude cellulase preparation with high ratio of β-glucosidase to filter paper activity shows improved efficiency of deinking of paper (Yang *et al.* 1999). The role of β-glucosidases in catalyzing different transglycosylation reactions for producing functional foods as well as pharmaceutically important neoglycoproteins is also being envisaged (Eneyaskaya *et al.* 2003).

Fungal strains are known to be efficient producers of β-glucosidases (Lynd *et al.* 2000). There are number of reports on production of β-glucosidase from yeast (*S. cerevisiae, Pichia etchellshii* and mesophillic fungi *(Trichoderma harzianum* and *Aspergillus* sp). Recent reports suggest that thermophilic fungi (*Chaetomium thermophilum, Humicola insolens, Sporotrichum thermophile* and *Melanocarpus sp., Myceliophthora* sp.). (Iwashita *et al.* 1998; Van Rensburg 1998; Oh *et al.* 1999; Kaur *et al.* 2007; Badhan *et al.* 2007; Sonia *et al.* 2008) and hydrocarbon utilizing novel fungi *Cladosporium resinae* are also good sources of novel β-glucosidases (Maheshwari *et al.* 2000; Parry *et al.* 2001; Yun *et al.* 2001). In few of these studies fungal multiplicity of β-glucosidase has also been reported. However, studies on the regulation of differential expression of these multiple β-glucosidases, and characterizing them on the basis of being preferentially hydrolytic/ transglycosylating in action has not been carried out to the best of our knowledge. The understanding about the regulation would be important in designing culture conditions for overproducing desired kind of isoforms. This study reports the regulation of expression of the multiple β-glucosidase from a thermotolerant fungus *A. terreus*, as well as purification and characterization of three isoforms of β-glucosidases to establish them as diverse and distinct.

EXPERIMENTAL

Culture

A thermotolerant fungal strain isolated from composting soil of the Jammu region (India) and identified as *Aspergillus terreus* (AN_1) was employed in this study (Soni et al. 2008). The fungus was grown and maintained on yeast potato soluble starch (YPSs) of the following composition (%; w/v), starch 1.5, yeast extract 0.4, KH_2PO_4 0.2, K_2HPO_4 0.23, $MgSO_4.7H_2O$ 0.05, citric acid 0.057, and agar 2.0. The pH of medium was adjusted to 7.0. The fungus was cultured at 45 ^oC for 7 days and stored at 4 ^oC.

Regulation of β- glucosidase in *A. terreus*

The induction of β-glucosidase was studied in a medium containing KH_2PO_4, 0.4%, corn steep liquor 0.85%, and $(NH_4)_2SO_4$ 1.3%, along with 1 % (w/v) of monosaccharides (glucose and fructose), polysaccharides (Avicel®, solka floc, CMC, and cellobiose), and lignocelluloses (rice straw, wheat straw, wheat bran, corn cob, paper, and bagasse) as carbon source. The synergistic / negative effects of different substrates (fructose, glucose, cellobiose, sucrose, and mannitol (@1 % w/v), glycerol and ethanol (@1 % v/v) to the inducer corn cob containing medium were studied. The flasks were inoculated with 2 ml of spore suspension ($1x10^7$spores ml^{-1}) of 5 days old culture of *A. terreus* and incubated at 45°C /120 rpm for 120 h in an orbital shaker. The sampling was carried out at 12 h intervals up to 120 h, the contents were centrifuged (8000 x g for 10 min), and the supernatants were assayed for β-glucosidase activity.

Assay of β-Glucosidase

β-glucosidases were assayed using *p*-nitrophenyl-β-D-glucopyranoside (pNPG) as substrate by the micro titer plate method (Parry *et al.* 2001). A reaction mixture of 100 µl containing 25 µl of enzyme, 25 µl of pNPG (10 mM) as substrate, and sodium acetate buffer (50 mM, pH 5.0) was incubated at 50 °C for 30 min. The reaction was terminated by addition of 100 µl of NaOH-glycine buffer (0.4 M, pH 10.8), and the developed yellow color was read at 405 nm using an ELISA Reader (MULTIKAN; Labsystem). The amount of *p*-nitrophenol released was quantified using the pNP standard. One unit of glucosidase activity was expressed as the amount of enzyme required to release 1 µ mole of pNP per minute under assay conditions.

Native Polyacrylamide Gel Electrophoresis and Zymogram

Crude enzyme preparations (protein 70 µg) were fractionated by native polyacrylamide gel electrophoresis (PAGE) using 10 % acrylamide gel with 4% stacking gel (Soni et al. 2008). The β-glucosidase activity in gels was detected by developing each zymogram against 10 mM 4-methylumbelleferyl-β-D glucoside (Sigma) as substrate prepared in sodium citrate buffer (50 mM, pH 6.0). Upon completion of electrophoreses, the gel was immersed in substrate solution for 45 min at 50 °C in the dark. The β-glucosidase bands in the gel were detected under UV light using Gel Documentation system (Gene Genius, Cambridge, UK).

Production and Purification of β-Glucosidases

For the preparation of inoculum, the culture was grown in 250 ml Erlenmeyer flasks containing 50 ml of glucose broth of the following composition (%; w/v); glucose 1.5, yeast extract 0.4, K_2HPO_4 0.2, and $MgSO_4$ 0.1. The pH of the medium was adjusted to 7.0, and flasks were incubated in an orbital shaker (120 rpm) at 45 °C for 24 h.

Solid state fermentation was carried out in Erlenmeyer flasks (250 ml) containing 5 g rice straw (ground to particle size 2-7mm) as sole carbon source and 15 ml of basal medium with the following components: KH_2PO_4 0.4 %, corn steep liquor 0.85 %, and $(NH_4)_2SO_4$ 1.3 %, (pH 7.0). After sterilization, the medium was inoculated with 2 ml of actively growing culture of *A. terreus* and incubated at 45 °C for 5 days. The enzyme was harvested by adding 50 ml of sodium citrate buffer (50 mM, pH 6.0) to the flasks and

kept at 45 ^{o}C for 1 h under mild shaking. The resultant slurry was filtered through muslin cloth and centrifuged at 8000xg for 10 min. The filtrate was concentrated using an Amicon ultrafiltration cell fitted with a PM-10 membrane (10 kDa cut off) and used for purification of β-glucosidases.

The concentrated sample was centrifuged (10,000 x g for 20 min.) and loaded onto a DEAE-sepharose (fast flow) column (24 cm x 2.6 cm; Pharmacia) pre-equilibrated with 20mM Tris HCl buffer (pH 7.0). The column was eluted first with the above buffer (2 bed volume), followed by a linear gradient of 1.0 M NaCl in 50 mM Tris HCl buffer (pH 7.0) at a flow rate of 1 ml min^{-1}. Two active peaks (one during isocratic and one during NaCl gradient elution) were obtained. All fractions (10 ml each) were analyzed for β-glucosidase activity. Fractions corresponding to a β-glucosidase peak obtained during NaCl gradient elution were pooled, concentrated equilibrated with 1.7 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer (pH 7.0), and applied onto a phenyl sepharose 6FF (Amersham) hydrophobic interaction column (5 ml), equilibrated with the same buffer. The column was eluted with a linear gradient of $(NH_4)_2SO_4$ (1.7 M to 0 M) in 50 mM phosphate buffer (pH 7.0) at a flow rate of 1 ml/min. Three peaks of β-glucosidase were obtained. Peak I obtained during gradient processing was found to be purified. Peaks II and III were further pooled, desalted, and concentrated using an Amicon ultrafiltration cell fitted with a PM-membrane (10 kDa cut off). Both peaks were further purified by using a Sephacryl HR 200 (SIGMA) gel filtration column (1.8 x 90cm). The column was eluted with 0.15 M NaCl in Tris HCl buffer (20 mM, pH 7.0) at a flow rate of 12 ml/h. The fractions corresponding to the β-glucosidases were pooled and stored at -20 ^{o}C for subsequent characterization. Purified enzymes were assayed for β-glucosidase activity using pNPG as substrate.

Determination of Protein

The protein in the fractions was determined by reading the absorbance at 280 nm using a Shimadzu 1240 spectrophotometer, as well as the Bradford method at 595 nm (Bradford 1976).

Characterization of β-Glucosidases

SDS Page and IEF

The homogeneity and molecular mass of β-glucosidases were determined by SDS-PAGE. This was performed using 10 % acrylamide gel (Laemmali 1970). The M_r (relative molecular mass) of β-glucosidases was determined using the plot of log M_r of standard protein markers (Bangalore Genei, India) versus their relative mobility.

Isoelectric focusing (IEF) was performed according to the instructions provided by Novex, using 5% acrylamide gel containing 2.4% broad pH range (3-10.0) ampholine carrier ampholyte in a Mini-protein II system (Biorad). Ethanolamine (0.4% v/v) and sulphuric acid (0.2%, v/v) were used as cathodic and anodic electrolyte solutions, respectively (Bhat and Wood 1989). Isoelectric focusing was carried out for 1 h each at constant voltage 100 and 200, followed with 500V for 30 min. The gel was silver stained, and pI of β-glucosidases was determined using plots of relative mobility of standard protein pI markers (FLUKA) versus their pI.

Temperature and pH stability

The temperature profile of purified β-glucosidases was obtained by determining the activity against pNPG between 30 and 80 ^{o}C. The optimal pH was determined by measuring the activity between pH 3.0 and 10.0, using 50 mM acetate (pH 3.0-5.0), phosphate (pH 6.0 – 8.0), and glycine NaOH (pH 9.0 – 10.0) buffers at 50 ^{o}C. For determination of temperature and pH stability, the enzyme aliquots were pre-incubated at different temperatures, and pH values for 0-240 min and assayed for β-glucosidase activity using pNPG as substrate, thereafter.

Effect of metal ions and other reagents

Purified β-glucosidase aliquots were incubated in 10 mM of $MnSO_4$, $CaCl_2$, $MgSO_4$, EDTA, NaCl, KCl, $CuSO_4$, $ZnSO_4$, DTT, and mercaptoethanol for 30 min at room temperature. The activity was then determined using pNPG as substrate.

Substrate Specificity

Substrate specificity of β-glucosidase was determined against pNP-β-gluco-pyranoside, pNP-α-D-glucopyranoside, pNP-β-galactopyranoside, ONP-β-D galactopyr-anoside, ONP-β-D-xylopyranoside, pNP-β-D-xylopyranoside, and pNP cellobioside (3Mm) as substrates (SIGMA) using the method described for β-glucosidase assay. The activities against polysaccharides (CMC low viscosity), CMC (high viscosity), solka floc, avicel, birch wood xylan) were determined by incubating the reaction mixture (1 ml) containing 100 µl enzyme, 400 µl citrate buffer (50mM, pH 6.0), and 500 µl of respective substrate (1 % w/v) at 50 ^{o}C for 1 h. The reducing sugars released were quantified using DNS reagent. The activities on cellobiose and salicin (3 mM) were estimated by assaying the amount of released glucose using GOD-POD method (Lin et al. 1999). The apparent K_m, V_{max} and K_{cat} for β-glucosidase were determined against pNPG, using a Lineweaver Burk plot.

Effect of mono / disaccharides and alcohols

The effect of mono/disaccharides (1 mg/ml) and alcohols (methanol, ethanol and propan-2-ol @ 20 % v/v) on β-glucosidase activity was studied using pNPG as a substrate.

RESULTS

Regulation of β-Glucosidases

The results in Fig. 1 show the observed levels of β-glucosidase in shake flask cultures. Fructose and cellobiose were found to be good inducers of β-glucosidase activity (6.93 U/ml and 6.75 U/ml) when compared to glucose, polysaccharides (Avicel®, solka floc, and CMC) and lignocellulosic substrates, except corn cobs, which was found to be the best source for production of β-glucosidase (8.85 U/ml) activity. Paper waste (5.25 U/ml) also supported appreciable levels of activity.

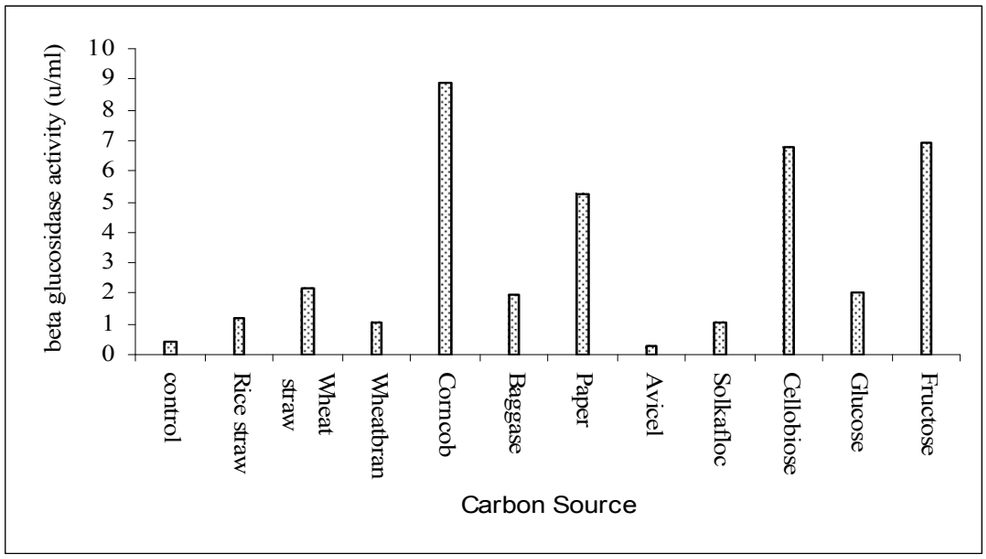

Figure 1. Enzyme activity pattern of extracellular protein from *A. terreus* grown on different carbon sources under shake culture. The values are the mean of three replicates.

Expression analysis of β-glucosidases produced by *A. terreus* in the presence of different substrates was studied by developing zymograms (Fig. 2a).

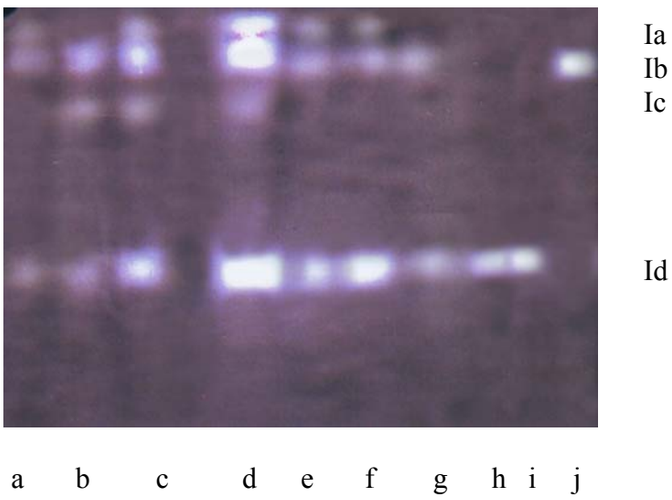

Figure 2 a. Zymogram showing effect of different carbon sources on expression of multiple β-glucosidases by *A. terreus*. Lanes from left to right: (a) Rice straw; (b) Wheat bran; (c) wheat straw; (d) Corn cob; (e) Bagasse; (f) Paper; (g) Solka floc; (h) Glucose; (i) Fructose; (j) Cellobiose, under submerged condition.

The activity gel revealed that four electrophoretically distinct isoforms of β-glucosidases designated as Ia, Ib, Ic, and Id were expressed in the presence of corn cob and wheat straw. However, isoform Ib in the presence of cellobiose, and isoform Id were the sole expressed β-glucosidases in the presence of monosaccharide (glucose and fructose). In the presence of rice straw, paper waste, and baggase, isoforms Ia, Ib, and Id were expressed, whereas, in the case of wheat bran and solka floc, expression of isoform Ia was not observed. The result in Fig. 2b shows that during solid state fermentation (SSF) isoform Ib was not expressed on any of the complex polysaccharide. In the presence of rice straw and wheat straw at a maximum three isoforms (Ia, Ic, and Id) were expressed. The β-glucosidase activities in the culture extracts of rice straw and wheat straw were observed as 470 and 333 (U/g substrate), respectively. On the other hand, corn cob, which was found to support maximal expression of β-glucosidase in shake flask culture, expressed isoforms Ia and Ic and produced β-glucosidase activity of 252 (U/g substrate). Bagasse, however, showed expression of isoforms Ic and Id with β-glucosidase activity of 81 (U/g substrate). This shows that the differential expression is not only limited to complexity of carbon source but also culture conditions employed for growth of the micro-organism.

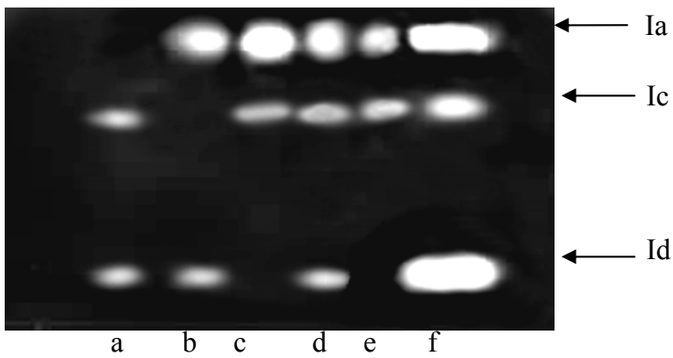

Figure 2 b: Zymogram showing effect of different carbon sources on expression of multiple β-glucosidases by *A. terreus*. Lanes from left to right: (a) bagasse; (b) paper; (c) wheat bran; (d) wheat straw; (e) corn cob; (f) rice straw, under solid state condition.

Furthermore, it was observed that production of β-glucosidases in *A. terreus* was weakly repressed when glucose, fructose, or mannitol was supplemented to medium containing corn cob. However, expression was unaffected by other supplements – sucrose, cellobiose, and glycerol (Fig. 3). The production profile revealed that the culture of *A. terreus* achieved a maximal level of β-glucosidase in a medium containing corn cob after 120 h (Fig. 4). No activity was detected up to 12 h of culturing. The expression of β-glucosidase activity was observed at 24 h and remained steady until 48 h, followed by a steady increase until 120 h. The inset of Fig. 4 shows that initially isoform Ib is expressed between 24 and 48 h of incubation (lane b and c). At 72 h, expression of isoforms Ib and Id was observed, followed by expression of all the four isoforms, thereafter. The level of expression of isoform Id was maximal, as evident from the band intensity.

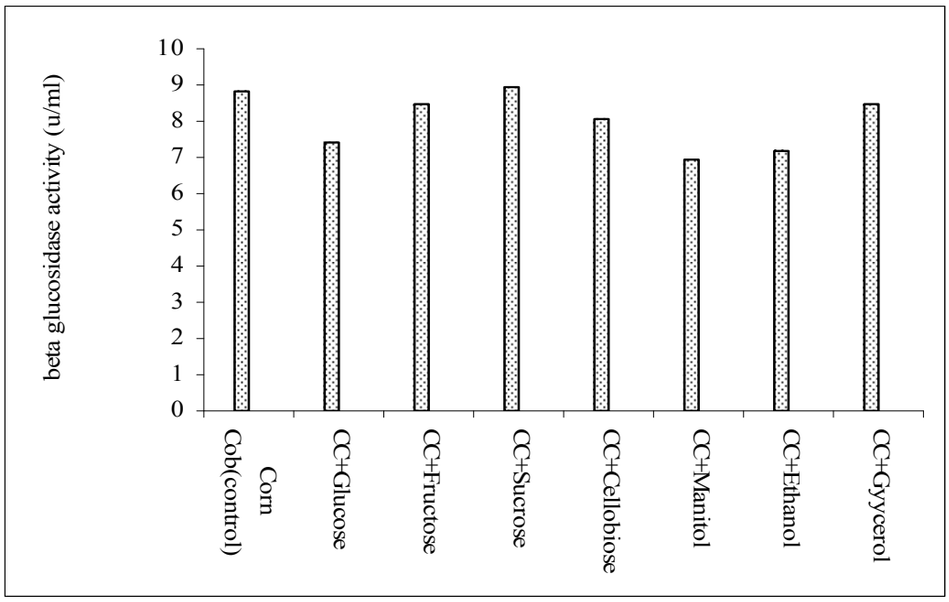

Figure 3. Effect of addition of different additives @1% (w/v) to corn cob (CC) 1% (w/v) containing production medium on the production of β-glucosidase by *A. terreus* after 120 h of incubation

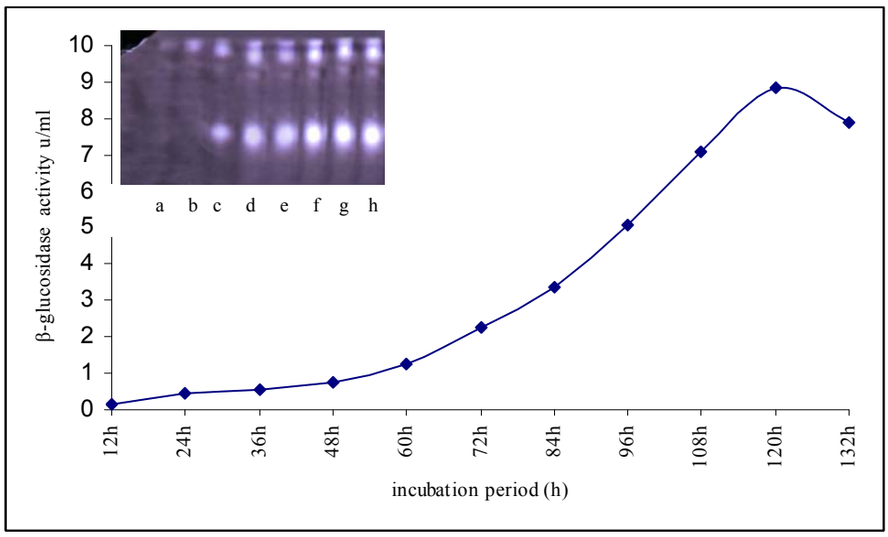

Figure 4. Profile of β-glucosidase production by *A. terreus* grown on corn cob containing medium. Inset zymogram shows the sequential expression of β-glucosidase isoforms after 36 h (a), 48 h (b), 60h (c), 72h (d), 84 h (e), 96 h (f), 108 (g), and 120h.

Purification of β-Glucosidases

For purification, *A. terreus* was grown in an optimized medium containing rice straw as the carbon source produced maximal β-glucosidase (650 U/g substrate) activity under solid state fermentation (SSF). The purification of three β-glucosidases (βGI, βGII, and βGIII) was carried out as summarized in Table 1.

Table 1. Summary of Purification of β-Glucosidases from *A. terreus*

Steps	Total activity (IU)	Total protein (mg)	Specific activity (μ mol min^{-1} mg protein $^{-1}$)	Purification folds	Enzyme recovery (%)
Crude filtrate	3000	1200.4	2.4	-	100
Ultra filtration	1226.4	80	15.3	6.3	40.8
DEAE-sepharose β-GI	825	26.6	31.0	12.9	27.5
HIC	275.9	1.5	183.9	76.6	9.19
β-GII					
HIC	500	12	41.6	17.3	16.6
Gel filtration	256	4	64	26.6	8.5
β-GIII					
HIC	112.5	5	22.5	9.3	3.75
Gel filtration	15.35	0.05	307	127.9	0.51

The ion exchange chromatography on DEAE-Sepharose yielded a single peak of β-glucosidases, which was further fractionated on a phenyl sepharose hydrophobic interaction column (HIC). HIC yielded three peaks. No contamination of other protein was found in peak I (βGI). The purified enzyme βGI exhibited specific activity of 183.9 μmol min^{-1} mg $protein^{-1}$ with purification fold and yield of 76.6 and 9.19 %, respectively. The fractions corresponding to peaks II and III were found to be contaminated with endoglucanase. Fractionation of these respective peaks on gel filtration column separated β-glucosidases from endoglucanase, and final purification of βG II and βG III was achieved. The purified βGII and βGIII exhibited 64 and 307 μ mol min^{-1}mg protein $^{-1}$ specific activities, corresponding to 26.6 and 127.9 folds purification with 8.5 and 0.51 % yield, respectively. β-glucosidases I, II and III were apparently homogeneous on SDS-PAGE with molecular weight (M_r) of ~29, 43, and 98 kDa, respectively (Fig. 5a). The purity was further confirmed by IEF, which showed single band with pI value of ~2.5, 3.7 and 3.5, respectively (Fig. 5b).

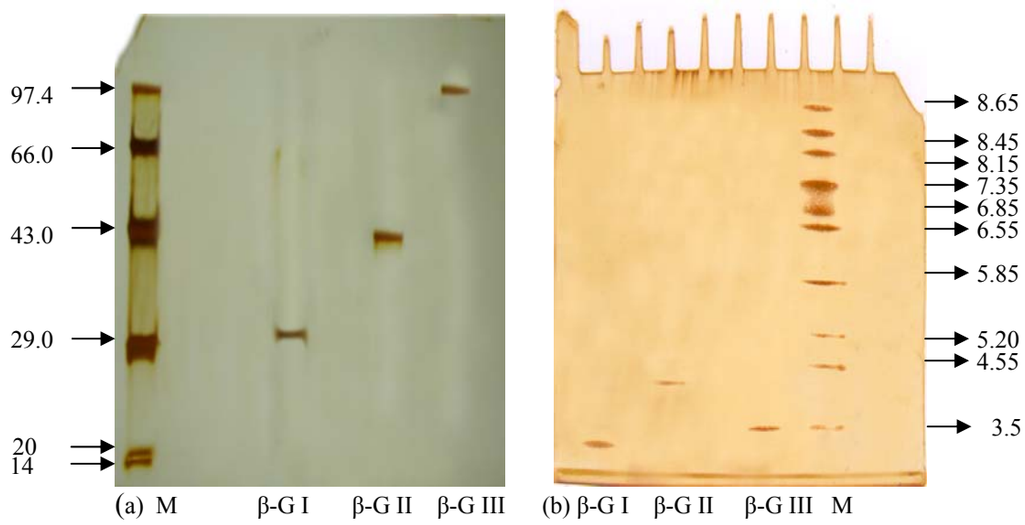

Figure 5. SDS-PAGE (a) of purified isoforms of β-glucosidases from *A. terreus* (AN1). Lane M standard protein markers of increasing molecular mass: Soyabean trypsin inhibitor (20KDa); Bovine Serum albumin (43 KDa); Bovine serum albumin (66 KDa); Phosphorylase (97.4 KDa), Lane 1, purified β-G I; Lane 2, purified β-G II; Lane 3, purified β-G III. Isoelectric focusing (b) of purified isoforms of β-glucosidases Lane 1 purified β-G I; Lane 2, β-G II; Lane 3, β-G III; Lane M standard *pI* markers (Pharmacia)

Temperature, pH Optima and Stability

The results in Fig. 6 (a and b) showed that βGI and βGII were optimally active at temperature 60 ^{o}C, and βG III exhibited optimal activity at 70 ^{o}C. The optimal pH for activity of the isoforms II and III was 5.0. The isoform βGI, however, was active over a broad range of pH (5.0-10.0) and temperature range of 50-90 ^{o}C. βGII was active under acidic pH (2.0 - 6.0) that decreased appreciably, thereafter. However, βGIII activity was observed in a very narrow range.

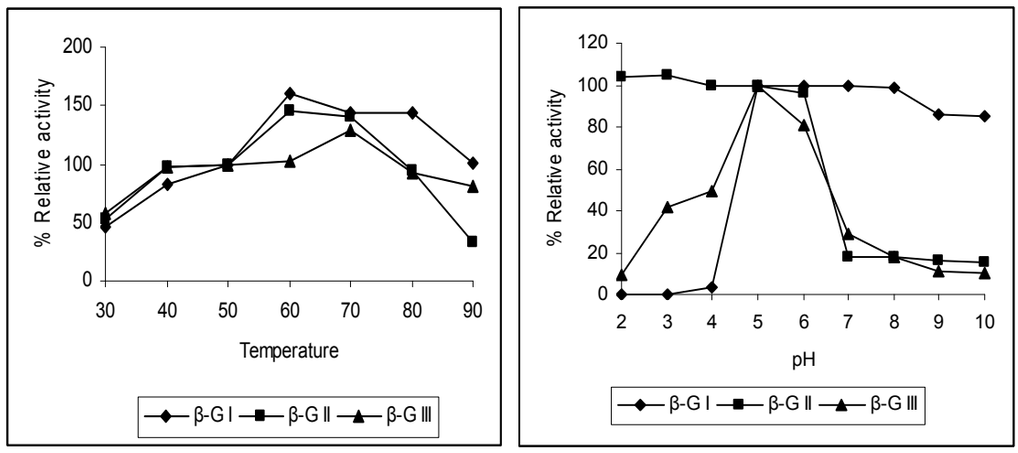

Figure 6. Temperature (a) and pH (b) optima curves of purified β-glucosidase (β-G) I, II, and III from *A. terreus*

The stability of βGI, βGII, and βGIII was studied for 4 h within their near optimal pH range of (5-7), (3-5), and (4-6) at temperatures of 50°C to 70°C, respectively. It was observed that βGI was appreciably stable within the temperature range of 50-70°C for 4 h between pH 5-7. βG II retained almost complete activity at pH 3.0, 4.0, and 5.0 when incubated for 180 min at 50 °C. However, almost complete loss in activity was observed at temperatures of 60-70 °C after 30 min. The isoform βGIII retained complete activity at pH 5.0, whereas, 55.14 % at pH 4.0 and 77 % at pH 6.0 was observed after 240 min of incubation at 50 °C. However at 60 °C, appreciable losses in activity were observed after 30 min of incubation (data not shown).

Effect of Metal Ions and Chemicals

The effect of metal ions and chemicals on enzyme activity of three isoforms of β-glucosidases was investigated (Fig. 7). The presence of the reducing agent mercapto-ethanol enhanced the activity of βGIII, while 82% and 19% decreases were observed in βGI and βGII activities, respectively. It was further observed that the presence of EDTA and DTT did not affect the activity of βGIII; however, in their presence, respective inhibition of βGI (72 % and 73 %) and βGII (17 % and 12 %) was observed. The presence of monovalent and divalent metal ions K^+, Na^+, Fe^{2+}, Mn^{2+}, Zn^{2+}, and Mg^{2+} positively influenced the activity of βGIII, but inhibition of βGI activity by 60, 12, 52, 56, 42, and 15 % and that of βGII activity by 15, 27, 2, 10, 25.7, and 26 %, respectively, was observed. The activity of all three isoforms βGI, II, and III was inhibited by 70, 22, and 57.5 %, respectively, in the presence of Cu^{2+}.

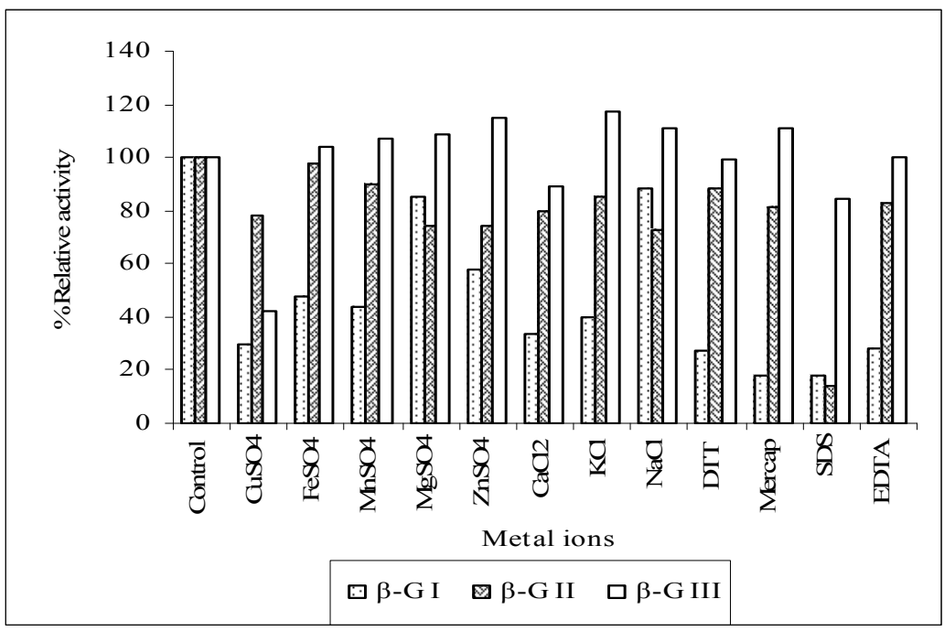

Figure 7. Effect of metal ions and chemicals on activity of β-glucosidase β G I, II, and III

Substrate Specificity of β-Glucosidases

The action of purified β-glucosidases was tested against different substrates with α and β configuration. The results summarized in Table 2 showed that all three isoforms were active against pNPG. No activity was observed against ortho or para nitrophenyl substituted β-D-galactopyronaside and xylopyranoside. The isoforms βG II and βG III recognized cellobiose and salicin as substrate; however, βGI did not catalyze the hydrolysis of either of these. Moreover, none of the three isoforms showed any activity against CMC (low and high viscosity), Avicel®, solka floc, and birch wood xylan.

Table 2. Relative Activities of β-Glucosidase from *A. terreus* (AN_1) towards pNPG, Cellobiose, and Salicin

	Absolute activity U/mg (Relative activity %)		
Substrate	β-G I	β-G II	β-G III
pNPG	5.7 (100)	5.4 (100)	1.08 (100)
Cellobiose	-	1.4 (27.6)	2.0 (20.5)
Salicin	-	2.0 (38.7)	0.27 (25.8)

No activity was detected against pNp-α-D-glucpyronaside, pNp-β-D-glucopyronaside, pNp-β-galactopyronaside, oNp-β-D-galactopyronaside, oNp-β-D-xylopyronaside, pNp-β-D-xylopyronaside, pNp-cellobioside and CMC (low and high viscosity), Avicel, solka floc, and birch wood xylan.

Effect of Monosaccharides and Disaccharides on β-Glucosidases Activity

The effect of different mono and disaccharides on the β glucosidase activities of the three isoforms was studied. The results in Table 3 show that activity of βGI was positively influenced in presence of mono and disaccharides. The activities of βGII and βGIII, however, were inhibited in presence of glucose, cellobiose and salicin.

Table 3. Effect of Various Mono- or Disaccharides on the Activity of β-Glucosidase I, II, and III of *A. terreus*

	Absolute activity Umg^{-1} (Relative activity %)		
Substrate	β-G I	β-G II	β-G III
Control	5.62 (100)	5.4 (100)	1.08 (100)
Monosaccharides			
Glucose	8.3 (147.0)	3.3 (60.7)	0.563 (51.0)
Xylose	10.5 (186.0)	6.0 (110.4)	1.12 (103.0)
Arabinose	9.0 (161.0)	5.9 (109.3)	1.13 (102.0)
Galactose	9.0 (161.0)	6.0 (111.6)	1.11 (101.0)
Fructose	11.0 (196.0)	6.8 (125.7)	1.10 (104.0)
Disaccharides			
Sucrose	8.5 (144.0)	5.9 (108.5)	1.10 (101.0)
Cellobiose	10.7 (190.0)	3.3 (60.5)	0.431 (39.0)
Salicin	7.62 (129.0)	2.3 (43.0)	1.08 (50.0)

Different mono & disaccharides were added @ 0.1% (w/v) to control containing pNPG as substrate.

Effect of Alcohols on Activity of β-Glucosidases

βGII and βGIII activities were inhibited in presence of 20 % (v/v) of methanol, ethanol, and butanol (Fig. 8). However, the activity of βGI was positively influenced in their presence.

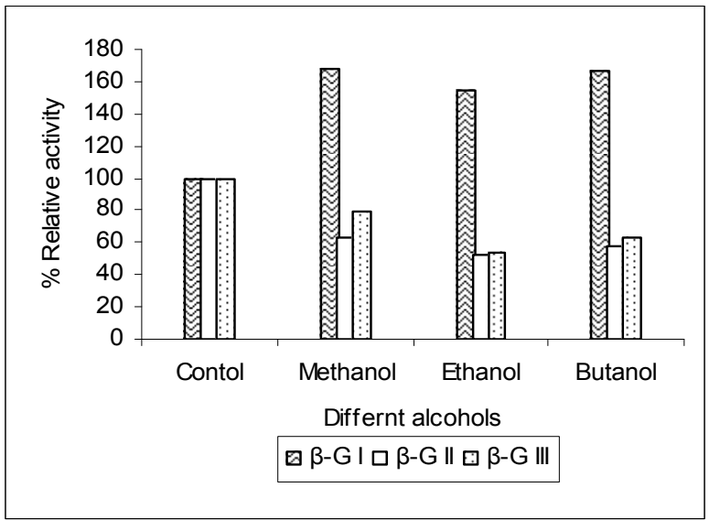

Figure 8. Effect of methanol, ethanol, and butanol added at 20% (v/v) on the activities of β-glucosidase βG-I, II, and III

Kinetics

The isoform βGII exhibited relatively lower K_m values (4.34 mM) as compared to βGI and βGIII (14.2 & 11.1 mM), respectively. Isoform βGIII showed higher V_{max} (378.7 μmol m^{-1}), was catalytically most efficient, and also exhibited the highest K_{cat} as well as K_{cat} / K_m values, followed by βGI and βGII, respectively (Table 4).

Table 4. Kinetic Constants of β-Glucosidase (β-G) I, II, and III

β-glucosidases	K_m(mM)	V_{max}(μ mol^{-1} mg $protein^{-1}$	K_{cat}(min^{-1})	K_{cat}/K_m (mM^{-1} min^{-1})
β-G I	14.2	166.9	$3.2x\ 10^4$	0.2×10^4
β-G II	4.34	34.7	0.3×10^3	0.86×10^3
β-G III	11.1	378.7	7.4×10^5	0.6×10^5

DISCUSSION

Strains of Aspergillus are known to be good producers of β-glucosidases. Several strains, namely *A. kawachii, A. niger*, *A. oryzae* are known produce high levels of β-glucosidases. *A. terreus* isolate used in the present study has been found to be a prolific

producer of β-glucosidase (Soni *et al.* 2008) and produced high levels of enzyme in the presence of rice straw under SSF and corn cobs under shake flask culture. Surprisingly, glucose and fructose also supported appreciable levels of β-glucosidase production by *A. terreus*. We have previously reported fructose to be a good inducer of endoglucanases in *Melanocarpus* sp. (Kaur *et al.* 2007); however, no study reports a positive role of fructose in induction of β-glucosidases. *A. terreus*, like other previously reported strains of *A. tubigenesis* and *A. kawachii*, was found to produce multiple β-glucosidases. We established the production and differential expression of multiple isoforms of β-glucosidases by developing zymograms using methylumbelliferyl β-D glucoside as substrate. Similar gel activity assay based on methylumbelliferyl β-D glucoside has been employed for developing a proteome strategy to discover novel β-glucosidases from *A. fumigatus* (Kim *et al.* 2007). The study showed a higher number of expressed β-glucosidases on complex substrates as compared to fructose/glucose/cellobiose, where only a single isoform was expressed. The observed differential expression can be attributed to structural heterogeneity of the cellulosic based substrates (Morag *et al.* 1990; Saraswat and Bisaria 1997; Badhan *et al.* 2004) in addition to culture conditions (Badhan *et al.* 2004).

The purification and characterization of three β-glucosidase isoforms, βGI, βGII, and βGIII revealed them to be distinct, based on their M_r, pI, and substrate specificity, etc. (Decker *et al.* 2001). In *A. kawachii* all three isoforms of β-glucosidase (two extra cellular and one intracellular), however, were reported to be of similar characters, except for minor differences in the molecular weight that were observed, owing to N glycosylation, and therefore were suggested to be post-translationally modified products of the same gene (Iwashita *et al.* 1998). On the other hand, all four β-glucosidases purified from *Aspergillus tubingensis* were distinct with respect to their physicochemical characteristics, substrate specificity as well as glucose inhibition and acid tolerance properties (Decker *et al.* 2001). Resistance to glucose inhibition and acid tolerance, in addition to heat stability, are positive attributes of β-glucosidases for developing effective bioconversion of cellulose to ethanol technologies (Decker *et al.* 2001; Kim *et al.* 2007).

We determined that all isoforms were fairly stable at high temperatures; moreover, βGI was resistant to inhibition by hexoses / pentoses and furthermore positively influenced the activity of βGI isoform. Similarly βGII isoform was acid tolerant with high activity observed in an acidic pH range. Decker and coworkers (2001) have previously identified β-glucosidase III and IV from *A. tubingensis* as glucose-tolerant isoforms, while isoforms I and II were inhibited in the presence of glucose. The studies on the effect of metal ions and other potential inhibitors revealed that isoform βGI from *A. terreus* apparently contained thiol and sulphydryl group in its active catalytic site, as it was inhibited by metal ions such as cu^{2+}, Zn^{2+} and reducing agents such as DTT and mercaptoethanol (Riou *et al.* 1998). On the other hand, these chemicals did not affect the activity of isoforms II and III and even enhanced their activity to a certain extent. Similar results have been observed previously in β-glucosidases of *Orpinomyces* sp., and *Bacteroides succinogenes* (Chen *et al.* 1994; Forsberg *et al.* 1982).

Of the three isoforms purified in the study, βGI showed absolute specificity for pNPG and was classified as an aryl β-glucosidase (which hydrolyze exclusively aryl β-glucoside), whereas, the isoforms βGII and βGIII can be classified as broad specificity β-

glucosidases (which hydrolyze both aryl β-glucoside and cello-oligosaccharides) (Saha and Bothast 1996).

In the present study we were able to show presence of four functionally distinct isoforms of β-glucosidases, of which three were purified. One of the previously purified β-glucosidases from *A. terreus* seems to be catalytically similar to isoform βGIII; however, no mention of the molecular weight and pI for the previously purified β-glucosidase has been made (Workman and Day 1982). We have isolated and purified previously unreported β-glucosidases from *A. terreus*, and our studies on regulation give an important insight for designing strategies for producing a particular type of isoform that may be of immense use from a biotechnological viewpoint.

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