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RESEARCH ARTICLE

Purification and characterization of β-glucosidase from *Melanocarpus* sp. MTCC 3922

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Abbreviations:
IEF: Isoelectric focusing
MTCC: Microbial Type Culture Collection
PBE: Poly-buffer exchanger
pNPG: p-nitrophenyl-β-D-glicopyronoside
SSF: Solid-state fermentation
YpSS: Yeast potato soluble starch agar

This study reports the purification and characterization of β-glucosidase from a newly isolated thermophilic fungus, *Melanocarpus* sp. Microbial Type Culture Collection (MTCC) 3922. The molecular weight of β-glucosidase was determined to be ~ 92 and 102 kDa with SDS PAGE and gel filtration, respectively, and p*I* of ~ 4.1. It was optimally active at 60ºC and pH 6.0, though was stable at 50ºC and pH 5.0 - 6.0. The presence of DTT, mercaptoethanol and metal ions such as Na^+, K^+, Ca^{2+}, $Mg^{2+}$and Zn^{2+} positively influenced the activity of β-glucosidase but the activity was inhibited in the presence of $CuSO_4$. β-Glucosidase recognized pNP- β-glucopyranoside (pNPG) as the preferred substrate, and showed very low affinity for pNP- β-D-cellobioside. K_m and V_{max} for the hydrolysis of pNPG by β-glucosidase was calculated as 3.3 mM and 43.68 µmolmin^{-1}mg

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protein^{-1}, respectively and k_{cat} was quantified as 4 x 10^3 min^{-1}. β-Glucosidase activity was enhanced appreciably in the presence of alcohols (methanol and ethanol) moreover, purified β-glucosidase showed putative transglycosylation activity that was positively catalyzed in presence of methanol as an acceptor molecule.

Cellulose, which constitutes the highest proportion of municipal and plant wastes, represents a major source of renewable energy and raw materials. Therefore, the utilization of cellulosic wastes to produce energy is potentially of great importance. Cellulases bring about the hydrolysis of cellulose, a homo-polymer of β-1,4 linked glucose units that comprises of amorphous and crystalline regions, by synergistic action of its constituent enzymes. These enzymes include; a) β-1,4-endoglucanase (1,4-β-D-glucan 4-glucanohydrolase; EC 3.2.1.4, cellulase), which cleaves internal β-1,4-glycosidic bonds, b) cellobiohydrolase (1,4-β-D-glucan cellobiohydrolase; EC 3.2.1.91, cellulase 1,4-β-cellobiosidase), an exo-acting enzyme which releases cellobiose from reducing and non reducing ends of cellulose and c) β-glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21, cellulase 1,4-β-glucosidase) that hydrolyzes cellobiose to glucose (Bhat and Bhat, 1997). 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 (Harhangi et al. 2002). Thus, β-glucosidase not only produces glucose from cellobiose, but also reduces cellobiose inhibition, allowing endoglucanase and exoglucanase enzymes to function more efficiently.

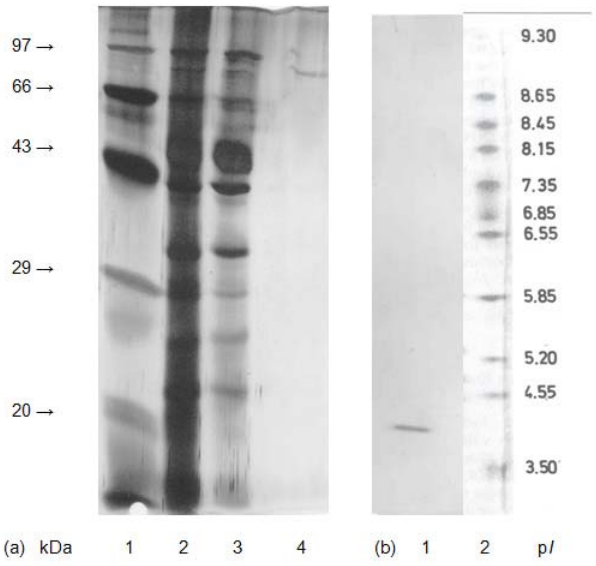

Figure 1. Analysis of purified β-glucosidase from *Melanocarpus* sp. MTCC 3922. (a) SDS-PAGE of purified Lane 1, standard protein markers in the order of increasing molecular mass: Soyabean trypsin inhibitor (20 kDa); Carbonic anhydrase (29 kDa); Ovalbumin (43 kDa); Bovine serum albumin (66 kDa); Phosphorylase b (97.4 kDa); Lane 2, crude protein; Lane 3, protein from pool 1 of DEAE-Sepharose column; Lane 4, protein of purified β-glucosidase after PBE-94 column. **(b) Iso-electric focusing of purified β-glucosidase, Lane 1, protein of purified β-glucosidase after PBE-94 column and Lane 2 standard p*I* markers.**

β-Glucosidase from *Aspergillus* supplemented to *Trichoderma reesei* cellulase preparations, plays an important role for cellulose saccharification on an industrial scale (Reczey et al. 1998). In recent years, interest in β-glucosidase has gained momentum owing to their ability to catalyze transglycosylation reactions. These types of reactions have great importance in wine industry because of its ability to improve the aroma of wines. The glycosylated precursor such as terpenes (Caldini et al. 1994) is found in mango, passion fruits and grapes (Sarry and Gunata, 2004) and β-glucosidases are more effective and specific than acid hydrolysis process for liberating terpenol (*i.e.*, volatile alcohols) from terpenylglucoside. These glycosidically bound volatiles also have interest in the food, cosmetic and tobacco industries (Jerković and Mastelić, 2004). *Saccharomyces cerevisiae* cannot utilize cellulosic materials; therefore for the direct conversion of cellulose to ethanol various cellulase and β-glucosidase genes have been expressed in *S. cerevisiae* (Van Rensburg et al. 1998). A recent U.S. patent 5,454,389 reports that a crude cellulase having high ratio of β-glucosidase activity to filter paper units provide improved efficiency of deinking (Yang et al. 1999).

Although, there are number of reports on the production of β-glucosidase from yeast (*S. cerevisiae, Pichia etchellsii)* and mesophilic fungi (*Trichoderma harzianum* and *Aspergillus* sp.). Recent reports suggest that thermophilic fungi (*Thermoascus aurantiacus*, *Chaetomium thermophile, Humicola insolens*, *Sporotrichum thermophile*) and hydrocarbon utilizing novel fungus *Cladosporium resinae* are also good sources of β-glucosidase (Pandey and Mishra, 1997; Iwashita et al. 1998; Van Rensburg et al. 1998; Oh et al. 1999; Maheshwari et al. 2000; Parry et al. 2001; Yun et al. 2001). Recently, we have reported endoglucanase and β-glucosidase production from a rare thermophilic fungus *Melanocarpus* sp. Microbial Type Culture Collection (MTCC) 3922 (Kaur et al. 2006), a fungus initially reported to be devoid of cellulases (Maheshwari and Kamalam, 1985). This study for the first time reports the purification and characterization of β-glucosidase from *Melanocarpus* sp. MTCC 3922.

MATERIALS AND METHODS

Culture

A thermophilic fungus isolated from composting soil and identified as *Melanocarpus* sp. MTCC 3922 was employed in this study. The fungus was grown and maintained on yeast potato soluble starch agar (YpSS) of 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

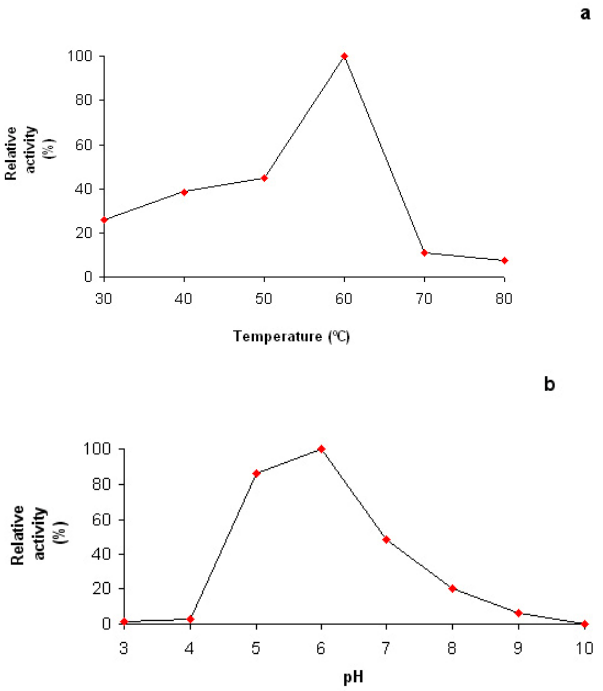

Figure 2. Optimal temperature (a) and pH (b) of β-glucosidase from *Melanocarpus* sp. MTCC 3922.

acid, 0.057 and agar, 2.0. The pH of medium was adjusted to 7.0. The fungus was cultured at 45ºC for 7 days and stored at 4ºC.

Production of β-glucosidase

For the preparation of inoculum, the culture was grown in 500 ml Erlenmeyer flask containing 100 ml glucose-urea medium of the following composition (% w/v); glucose, 1.0; yeast extract, 1.0; KH_2PO_4,0.6; K_2HPO_4, 0.04; $MgSO_4.7H_2O$, 0.05; urea, 0.05. During shake flask culturing few glass beads (1 cm in diameter) were added to the flasks to avoid clumping of the mycelium so as to attain homogeneous growth. The pH of the medium was adjusted to 6.0 and flasks were incubated in an orbital shaker (120 rpm) at 45ºC for 72 hrs.

Solid-state fermentation (SSF) was carried out in 500 ml Erlenmeyer flasks that contained 10.0 g of rice straw as carbon source and 35 ml of basal salt medium of following compositions (% w/v): urea, 1.5; KH_2PO_4, 0.12; $MgSO_4.7H_2O$, 0.05; $CaCl_2$ $2H_2O$, 0.01 and (1% v/v) trace element solution (% w/v): $(NH_4)_2SO_4$, 0.2; KCl, 0.5; $CaCl_2$, 0.1; $MgSO_4$, 0.5; $ZnSO_4$, 0.01 and $CuSO_4$, 0.005. The basal medium was supplemented with Tween 20 (0.1% v/v). The initial moisture content of the medium was adjusted to 80%. Prior to sterilization, the initial pH of the medium was adjusted to 6.0. The sterile production medium was inoculated with 4 ml of the culture grown on glucose-urea medium and incubated in a water-saturated atmosphere at 45ºC for 7 days. After incubation, 100 ml sodium citrate buffer (50 mM, pH 6.0) was added to flasks, and these flasks were kept at 50ºC for 1 hr under mild stirring. The fermented slurry was filtered through muslin cloth and centrifuged at 11000 X g for 15 min. The filtrate was concentrated using Amicon cell fitted with a PM-10 membrane (10 kDa cut off) and used for β-glucosidase purification.

Purification of β-glucosidase

Ion-exchange chromatography (DEAE Sepharose). The concentrated sample was centrifuged (10,000 x g for 20 min) and loaded on DEAE-Sepharose (FF) column (24 x 2.6 cm, Pharmacia), equilibrated with sodium acetate buffer (20 mM, pH 5.0). The column was eluted first with equilibration buffer (2 bed volumes) followed by linear gradient of 0-1.0 M NaCl in acetate buffer (20 mM, pH 5.0) at a flow rate of 1 ml min^{-1}. The fractions were assayed for β-glucosidase and active fractions were pooled, desalted and concentrated using Amicon ultra-filtration cell fitted with a PM-10 membrane (Millipore).

Poly-buffer exchanger (PBE 94). β-Glucosidase was further purified using column (10 x 0.75 cm) packed with poly-buffer exchanger (PBE-94; Pharmacia Biotech). The column was pre-equilibrated with sodium acetate buffer (20 mM, pH 5.0). The sample applied was eluted with sodium

Table 1. Summary of purification of β-glucosidase from *Melanocarpus* sp. MTCC 3922.

Purification step	Total activity (units)	Total protein (mg)	Specific activity (µmol min^{-1} mg protein $^{-1}$)	Fold purification	Yield (% recovery)
Culture filtrate	4895.0	1975.0	2.47	-	100
Ultra- filtration	4104.0	1445.2	2.8	1.13	83.84
DEAE-Sepharose	1476.0	197.3	7.48	3.03	30.15
PBE-94	778.0	77.47	10.04	4.06	15.89

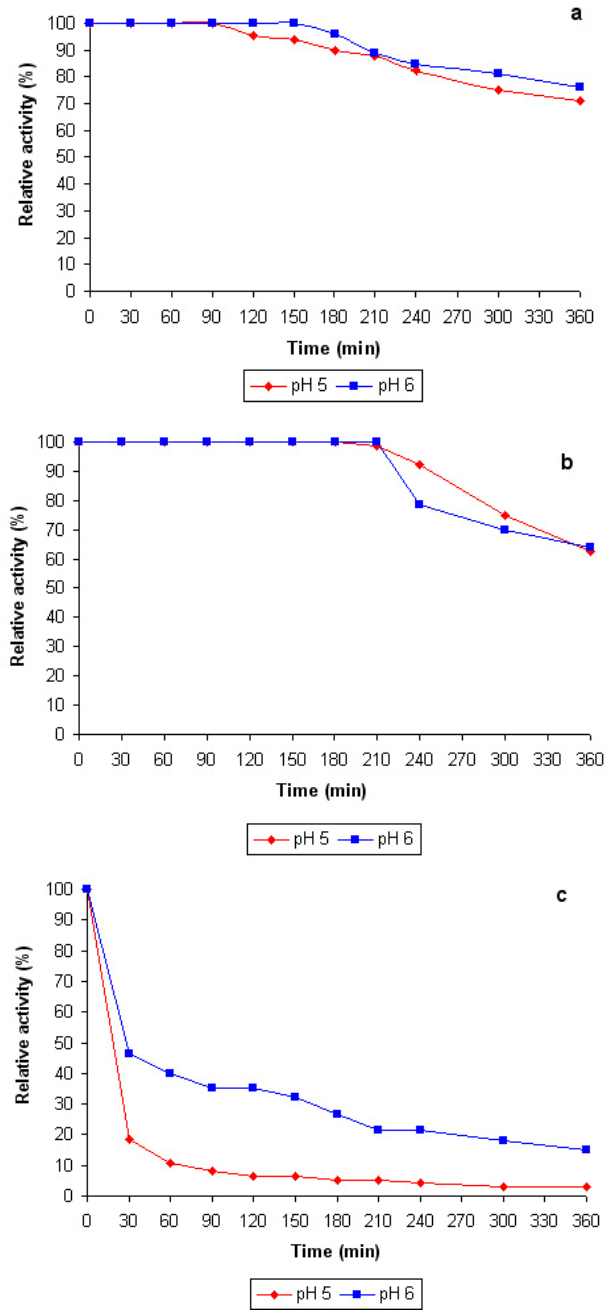

Figure 3. Stability of β-glucosidase from *Melanocarpus* sp. MTCC 3922 at pH 5.0 and 6.0at different temperatures: (a) Temperature 40ºC as a function of time. (b) Temperature 50ºC as a function of time. (c) Temperature 60ºC as a function of time.

acetate buffer (20 mM, pH 5.0) by 3 bed volumes and followed by linear gradient of 0-1.0 M NaCl in acetate buffer (20 mM, pH 5.0) at a flow rate of 0.2 ml min^{-1}. The fractions were collected using automated liquid chromatography system (ÄKTA Pharmacia Biotech). Purified fractions containing β-glucosidase were pooled for biochemical assays.

Assay of β-glucosidase

β-Glucosidase was assayed using p-nitrophenyl- β-D-glucopyranoside (pNPG) by micro titer plate method as described (Parry et al. 2001). A reaction mixture (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 50ºC for 30 min, the reaction was terminated by addition of 100 µl of NaOH-glycine buffer (0.4 M, pH 10.8). The developed yellow colour was read at 405 nm using ELISA Reader (MULTISKAN; Labsystems). The amount of p-nitrophenol released was quantified using the pNP standard. One unit of β-glucosidase activity was expressed as the amount of enzyme required to release 1 µmole of pNP per minute under the assay conditions. However, during purification 50 µl of enzyme was used for assay.

Determination of protein

The protein was determined by protein dye binding method of Bradford (1976). Absorbance at 280 nm was used to monitor the protein content in the column fractions.

Characterization of β-glucosidase

Gel Filtration, SDS-PAGE and IEF. The homogeneity and molecular mass of β-glucosidase was determined by gel filtration and SDS-PAGE. The gel filtration of purified β-glucosidase (10 µg) and standard protein markers (Bangalore GENEI, India) comprising of β-galactosidase (116 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa) was carried out on a Sephacryl HR 200 column (1.8 x 90 cm). The column was equilibrated with sodium acetate buffer (50 mM, pH 5.0), containing 10 mM NaCl and eluted at a flow rate of 12 mlh^{-1}. The fractions (2.5 ml) were assayed for β-glucosidase and protein. SDS-PAGE was performed using acrylamide gel (10%) by the method of Laemmli (Laemmli, 1970) using Bio-Rad mini-protean II electrophoresis unit. The protein markers were run along side of the sample (10 µg protein sample was used) and protein bands were visualized by silver staining method. The M_r of β-glucosidase was determined using the plot of log M_r of the standard protein markers versus their elution volume (gel filtration) and relative mobility (SDS-PAGE).

Isoelectric focusing (IEF) was performed according to the instructions provided by Novex, using 5% acrylamide gel containing 2.4% broad pH range (3.5-10.0) ampholine carrier ampholyte. The cathode buffer contained lysine 2.9% (w/v) and arginine 3.5% (w/v), whereas, phosphoric acid (10 mM) was used as anode buffer. Isoelectric focusing was carried out for 1 hr each at constant voltage of 100 V and 200 V followed with 500 V for 30 min. The gel was fixed in TCA (10% w/v) for 2 hrs and silver staining was performed. The p*I* of β-glucosidase was determined using the plot of relative mobility of standard protein p*I* markers (FLUKA) versus their p*I*.

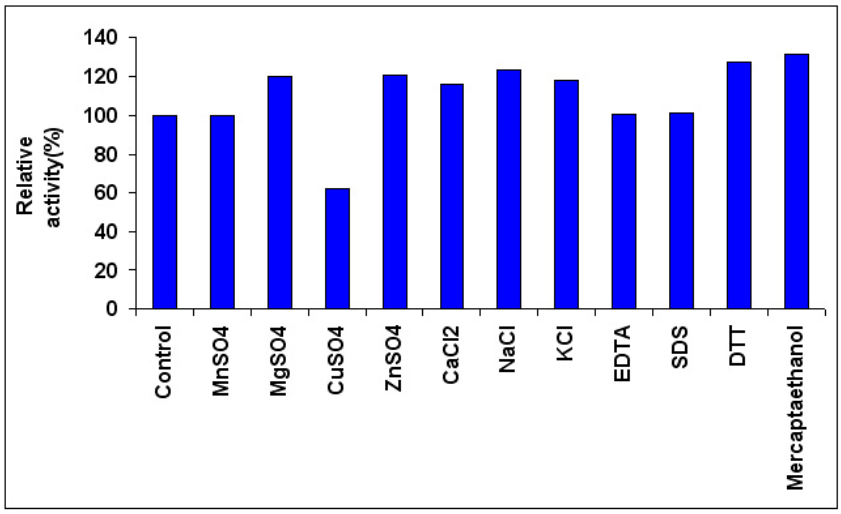

Figure 4. Effect of metal ions and other chemical reagents (10 mM each) on β-glucosidase activity from *Melanocarpus* sp. MTCC 3922. The experiment was carried out in triplicates and experimental error was in the range of 1-5%.

Temperature and pH optima and stability. The temperature profile of purified β-glucosidase was obtained by determining the activity on pNPG between 30 and 80ºC. The optimal pH was determined by measuring the activity between pH 3.0 and 10.0 (50 mM) using acetate buffer (pH 3.0-5.0), phosphate buffer (pH 6.0-8.0), and glycine-NaOH (pH 9.0-10.0) at 50ºC. For the determination of temperature and pH stability, the enzyme was pre-incubated in the temperature range of 50ºC and 60ºC for 0-6 hrs, or in the initial pH range 5.0-6.0 at 50ºC and 60ºC, respectively, and assayed for β-glucosidase activity using pNPG as substrate.

Effect of metal ions and other reagents. β-Glucosidase was incubated in 10 mM (final concentration) of $MnSO_4$, $CaCl_2$, $MgSO_4$, EDTA, NaCl, KCl, $CuSO_4$, SDS, $ZnSO_4$, DTT, and mercaptoethanol for 30 min at room temperature and the activities were determined thereafter using pNPG as substrate.

Substrate specificity. Substrate specificity of β-glucosidase was determined by using pNP- β-glucopyranoside, pNP-α-D-glucopyranoside, pNP-β-galactopyranoside, oNP-β-D-galactopyranoside, oNP-β-D-xylopyranoside, pNP- β-D-xylopyranoside and pNP-cellobioside, (3 mM) as substrates. A reaction mixture (150 µl) containing 50 µl substrate, 50 µl of purified enzyme and acetate buffer (50 mM, pH 5.0) was incubated at 50ºC for 1 hr. The reaction was terminated by addition of 150 µl glycine-NaOH buffer (0.4M, pH 10.8). The colour developed was read at 405 nm using ELISA Reader (MULTISKAN; Labsystems). The activities on polysaccharides (CMC (low viscosity), CMC (high viscosity), solka floc, birch wood xylan, laminarin and avicel) were determined by incubating reaction mixture (1 ml) containing 100 µl enzyme, 400 µl citrate buffer (50 mM, pH 5.0) and 500 µl of respective substrate (1% w/v) at 50ºC for 1 hr. 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 Lineweaver Burk plot.

Effect of mono/disaccharides on β-glucosidase. The effect of mono/disaccharides (1 mg/ml) on β-glucosidase activity was studied using pNPG as a substrate.

Effect of alcohols. The effect of methanol, ethanol and propan-2-ol (0-100% v/v) on the hydrolysis of pNPG were studied using the pNPG assay.

Table 2. Relative activities towards aryl substrates, cellobiose and salicin on β-glucosidase from *Melanocarpus* sp. MTCC 3922.

Substrate (3 mM)	Relative activity (%)
pNP-β-glucopyranoside	100
pNP-cellobioside	4.69
Cellobiose	24.33
Salicin	4.37

Activities were determined by measuring glucose as described in materials and methods.

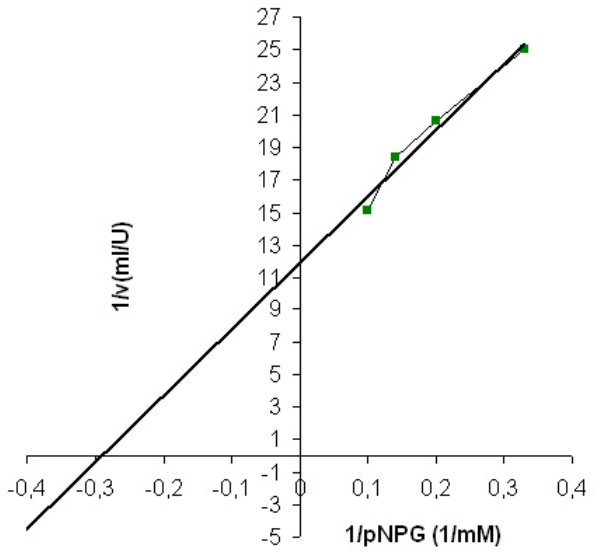

Figure 5. Effect of various concentrations of pNPG on β-glucosidase from *Melanocarpus* sp. MTCC 3922 (Lineweaver-Burk plot). The amount of protein used for the study was 1.99 µg/ml.

Transglycosylation. A reaction mixture (150 µl) comprising of purified β-glucosidase enzyme (1 µg), different concentrations (20-100% v/v) of methanol (50 µl) and acetate buffer (50 mM, pH 5.0) was prepared. The reaction was initiated by adding 50 µl of pNPG (10 mM) and incubating the contents at 50ºC for 60 min, the reaction was terminated by boiling the sample for 10 min. To demonstrate the transglycosylation, 25 µl of the aliquot from the above reaction was mixed with 0.54 µg of purified endoglucanase (40 kDa) from *Melanocarpus* sp. MTCC 3922, used as revealing enzyme, and acetate buffer (50 mM, pH 5.0) to make final volume to 100 µl. The reaction was carried out at 50ºC for 60 min. To the controls (boiled transglycosylation product) no endoglucanase was added. The reaction was terminated by adding 100 µl of NaOH-glycine buffer (0.4 M, pH 10.8). The developed yellow colour was read at 405 nm using ELISA Reader (MULTISKAN; Labsystems).

All experiments were performed in triplicate and results are given as mean value. The standard error ranged between 1-5%.

RESULTS

Culture

A new strain of *Melanocarpus* sp. MTCC 3922, isolated from the composting soil, grew profusely as white, cottony hyphal mass at 45ºC and touched the lid of plate within 2-3 days of incubation. Due to the lack of sporulation and mating type for bringing about the ascospore formation, (MTCC), Chandigarh, identified this true thermophilic heterothallic ascomycete as *Melanocarpus* sp. MTCC 3922.

Purification of β-glucosidase

An extracellular β-glucosidase from *Melanocarpus* sp. was purified by ion exchange chromatography using DEAE-Sepharose (weak anion exchanger) and PBE 94 (Strong ion exchanger) columns. During DEAE-Sepharose column chromatography, the major β-glucosidase peak was eluted with 0.4 MNaCl gradient; this peak also contained contaminating endoglucanase activities. Finally on PBE 94 column, β-glucosidase peak was separated from endoglucanase at 0.5 M NaCl gradient. The results of purification procedure are summarized in Table 1. The purified enzyme preparation exhibited a specific activity of 10.04 µmol min^{-1}mg $protein^{-1}$, while purification fold and yield were 4.06 and 15.89%, respectively.

Purified β-glucosidase from *Melanocarpus* sp. was homogeneous as judged by SDS-PAGE, gel filtration, and IEF-PAGE. The molecular weight of β-glucosidase, estimated by gel filtration and SDS-PAGE, was 102 and 92 kDa, respectively. Further homogeneity was confirmed by IEF that showed single band of β-glucosidase with p*I* value of ~ 4.1 (Figure 1a and Figure 1b, respectively).

Temperature, pH optima and stability

β-Glucosidase was optimally active at 60ºC and at pH 6.0 (Figure 2a). Above 70ºC, the enzyme showed less than 25% of its optimal activity. However, maximum activity was observed at pH 6.0 at 50ºC (Figure 2b) and less than 10% relative activity was observed at pH 3.0-4.0 and 9.0-10.0. β-Glucosidase was stable (Figure 3) at 50ºC and pH 5.0 and 6.0. The enzyme retained 100% of its original activity at pH 5.0 and 6.0 for 200 min; whereas, 64.0% relative activity was retained at pH 5.0 and 6.0 after 360 min of incubation at 50ºC. The enzyme was less stable at 60ºC and pH 5.0 and lost more than 80.0% activity after 30 min. While at pH 6.0, approximately 55.0% relative activity was lost after 30 min.

Effect of metal ions and other chemical reagents on β-glucosidase

β-Glucosidase showed enhanced activity in presence of reducing agents, DTT and mercaptoethanol (Figure 4). The presence of monovalent and divalent metal ionsNa^{+}, K^{+}, Ca^{2+}, Mg^{2+} and Zn^{2+} also positively influenced the activity of β-glucosidase. The presence of EDTA and SDS did not inhibit the enzyme activity, whereas, $CuSO_4$ inhibited the enzyme activity up to 38.0%.

Substrate specificity of β-glucosidase activity

The action of purified β-glucosidase was tested over different substrates with α and β configurations. The results summarized in Table 2 show that β-glucosidase was maximally active against pNPG. β-Glucosidase activity on

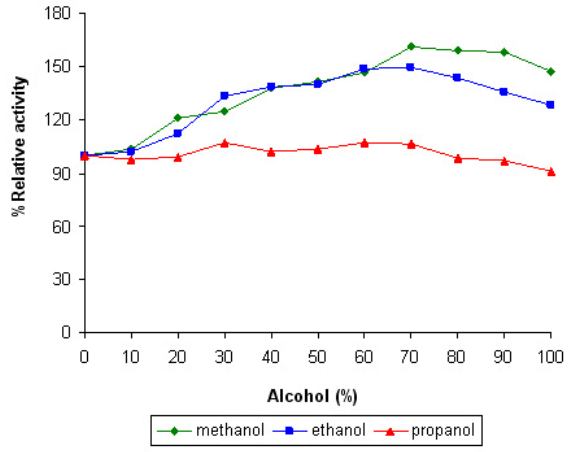

Figure 6. Effect of different % (v/v) of methanol, ethanol, and propan-2-ol (Δ) on the β-glucosidase activity of *Melanocarpus* sp. MTCC 3922.

pNP- β-D-cellobioside was only 4.69% of that on pNPG. No activity was observed in the presence of the nitro group at the *ortho* position in oNP-β-D-galactopyranoside, oNP-β-D-xylopyranoside, as well as pNP-β-D-galactopyranoside and pNP-xylopyranoside. β-Glucosidase recognized cellobiose and salicin as substrate, however, enzyme was inactive against CMC (low and high viscosity), Avicel, Solka floc, laminarin and birchwood xylan.

Inhibition

The effect of different mono and disaccharides were studied in presence of pNPG. The results in Table 3 revealed that in the presence of glucose, ~ 47.0% decrease in hydrolysis of pNPG was observed. However, low level of inhibition was observed in presence of xylose, galactose and sucrose.

Kinetics

K_m and V_{max} for the hydrolysis of pNPG by β-glucosidase was determined using 0-10 mM substrate and analyzed the data from Lineweaver-Burk plots (Figure 5). From this plot the values of K_m and V_{max} were calculated as 3.3 mM and 43.68 :molmin^{-1}mg protein^{-1}, respectively for pNPG. The k_{cat} towards pNPG was 4 x 10^3 min^{-1}.

Effects of alcohols

The results in Figure 6 showed that short chain length alcohols, methanol and ethanol at a final concentration of 70% (v/v) increased the activity of β-glucosidase by 1.5 folds, while 70% (v/v) propan-2-ol has no effect on the enzyme activity but propan-2-ol between 80 and 100% (v/v) resulted in the inhibition of the enzyme activity.

Transglycosylation

The results in Figure 7 showed that in the presence of 20% (v/v) methanol, low transglycosylation activity was observed but as the methanol level was increased to 60% (v/v) there was a steady increase in transglycosylation activity. Further increase in methanol concentration resulted in decrease in activity.

DISCUSSION

The thermophilic fungus, *Melanocarpus* sp. MTCC 3922, isolated from composting soil and used in the present study, is a rare fungus which produced very high amount of β-glucosidase (132.4 U/g of substrate) when rice straw was used as carbon source under solid-state fermentation. This is first report on the purification and characterization of β-glucosidase from *Melanocarpus* sp. MTCC 3922. Previously, we have shown that *Melanocarpus* sp. expressed only one isoform of β-glucosidase with acidic p*I* in the presence of rice straw, when crude filtrate was resolved on IEF gels (Kaur et al. 2006). The enzyme was purified to homogeneity with specific activity of 10.04 μmol min^{-1}mg protein^{-1} and yield (%) 15.89. The molecular weight of the native β-glucosidase estimated by gel filtration was 102 kDa, and by SDS-PAGE analysis was about 92 kDa suggesting that the enzyme is a monomer.

Table 3. Effect of various mono- or di-saccharides on β-glucosidase activity of *Melanocarpus* sp. MTCC 3922.

Substrate [a]	Relative activity (%)
Control	100
Monosaccharides	
Glucose	53.34
Arabinose	72.36
Xylose	75.31
Galactose	76.44
Fructose	73.49
Disaccharides	
Sucrose	76.55
Cellobiose	70.00
Salicin	72.36

[a] Various monosaccharides and disaccharides were added @ 1mg/ml to the control containing pNPG as substrate.

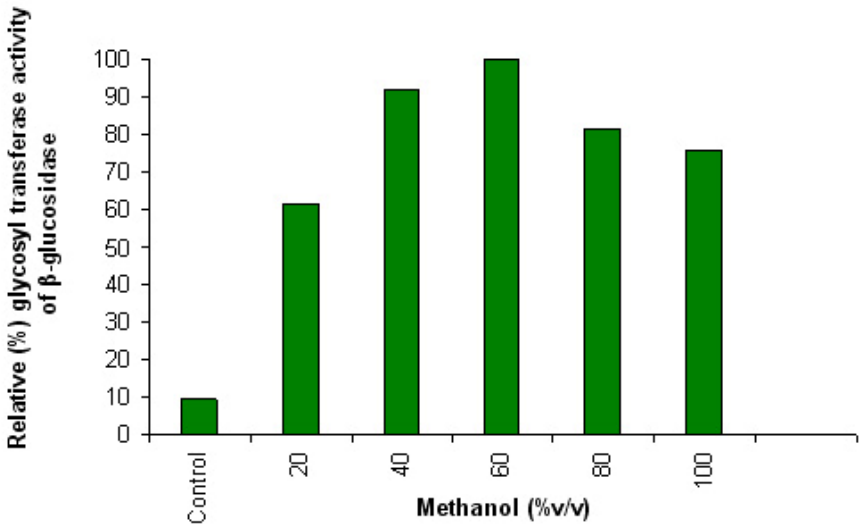

Figure 7. Effect of different % (v/v) of methanol on the glycosyl-transferase activity of β-glucosidase of *Melanocarpus* sp. MTCC 3922.

This property is shared with *Trichoderma harzianum* and *Acremonium persicinum* having single protein of molecular weight of 75 kDa and 128 kDa, respectively (Pitson et al. 1997; Yun et al. 2001). But it was different from β-glucosidase of *Pichia etchellsii* and *Thermoascus aurantiacus*, which had high molecular weights and oligomeric nature (Pandey and Mishra, 1997; Parry et al. 2001). Furthermore, the purity of enzyme was confirmed by isoelectric focusing which showed a single protein band. The purified enzyme has acidic p*I,* which has also been observed in *A. niger* and *Fusarium oxysporum* (p*I* 3.2, 3.8, respectively) (Christakopoulos et al. 1994; Yan and Lin, 1997).

The maximum activity for the enzyme was observed at pH 6.0 and 60ºC, which is similar to β-glucosidase purified from *Thermomyces lanuginosus* (Lin et al. 1999). The optimal conditions for most β-glucosidase from other fungus (Bhat et al. 1993; Alconada and Martinez, 1996; Wei et al. 1996) are near pH 6.0 and from 45-65ºC. β-Glucosidase from *Melanocarpus* sp. was not active in the extreme pH of either 3 or 10. The pH (5-6) and the thermostability at 50ºC for 240 min were with in the range of most of fungal β-glucosidases (Bhat et al. 1993; Wei et al. 1996). An increase in β-glucosidase activity in presence of DTT and mercaptoethanol indicated the absence of thiol groups at catalytic sites.

β-Glucosidase from *Melanocarpus* sp. was preferentially active against pNP-β-glucopyranoside when compared to cellobiose. Enari and Niku-Paavola (1987) classified β-glucosidases into three major groups according to their substrate specificity; (1) aryl β-glucosidases with a strong affinity for aryl β-glucosides; (2) cellobiases, which only hydrolyze oligosaccharides (including cellobiose); and (3) β-glucosidases that are active with both type of substrates. Our results showed that β-glucosidase purified from *Melanocarpus* sp. MTCC 3922 was active against both aryl β-glucosides and cellobiose therefore it can be concluded that β-glucosidase from *Melanocarpus* sp. belong to group 3.

Plant et al. (1988) suggested that the preference of β-glucosidases for aryl glycosides is due to the high electrophilicity of the aglycone moiety, which enhances the stability of the *ortho* or *para* nitrophenoxide anion generated during the first step of catalysis. β-Glucosidase from *Melanocarpus* sp. showed broad specificity towards diglycosides. β-Glucosidase from *Melanocarpus* sp. was found to hydrolyze cellobiose to a greater and salicin to lesser extent indicating no steric hindrance with this compound as also observed in *Humicola grisea* (Takashima et al.1996) and *Candida peltata* (Saha and Bothast, 1996). Kinetic study revealed that β-glucosidase from *Melanocarpus* sp. has lower value of K_m (3.3 mM) for pNPG than *Cellulomonas biazotea* (4.25 mM) and *Aspergillus niger* CCRC 31494 (21.7 mM) thus indicating higher affinity of enzyme for pNPG substrate (Siddiqui et al. 1997; Yan and Lin, 1997). β-Glucosidase from *Melanocarpus* sp. was free of endoglucanase, avicelase, xylanase, β-D-galactosidase and arabinofuranosidase activity thus possessed high specificity, though, multiple-functionality is common among other β-glucosidases that shows galactosidase and xylosidase activities (Lin et al. 1999).

Presence of methanol and ethanol had a positive influence on the hydrolytic activity of β-glucosidase. In the presence of methanol and ethanol, an increase in enzyme activity was observed, though, the activity decreased with the

longer alcohol chains. Activation by alcohol has been earlier observed for β-glucosidase from *Thermoascus aurantiacus* (Parry et al. 2001), *Aspergillus oryzea* (Riou et al. 1998), *Fusarium oxysporum* (Christakopoulos et al. 1994). β-Glucosidase from *Melanocarpus* sp. showed high transglycosylation activity in the presence of methanol. Transglycosylation was determined following the concept of using secondary reactions of a primary reaction product to induce a recordable signal (Mayer et al. 2001). The pNPG was used as donor and methanol as an acceptor. The primary reaction was catalyzed using purified β-glucosidase enzyme. To monitor the transglycosidase activity, purified endoglucanase (molecular weight ~ 40kDa) was used as a revealing enzyme. The observations indicated the transglycosylation in the presence of methanol by β-glucosidase. Although purified endoglucanase did not cleave pNPG, but enzyme hydrolyzed the polysaccharide formed by transglycosylation and released yellow nitro-phenol as signal indicating putative transglycosylation activity (Mayer et al. 2001). Previously, we have shown purified endoglucanase from *Melanocarpus* sp. exhibiting processive activity against crystalline cellulose and filter paper (Kaur et al. 2007). Presence of methanol resulted in higher levels of transglycosylation. Parry et al. (2001) have previously shown that methanol enhances the glycosyl-transferase activity of β-glucosidase in thermophilic ascomycete *Thermoascus aurantiacus*. Similarly Matsumura et al. (1999) also reported direct transglycosylation of xylan and octanol to octyl β-D-xylobioside by purified xylanase of *Aureobasidium pullulans*.

Expectedly, β-glucosidase from *Melanocarpus* sp. exhibited synergistic interaction with endoglucanase to increase the efficiency of glucose production from cellulose by converting rice straw to glucose (data not shown) indicating utility of β-glucosidase in enzymatic hydrolysis of cellulosics for subsequent production of ethanol fuel. The observed high activity of β-glucosidase from *Melanocarpus* sp. on rice straw also makes it a promising candidate for application in bioconversions as well as catalysis of novel compounds through transglycosylation reactions.

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