Characterization of thermo-tolerant and acid/alkali tolerant β-glucosidase from bacterial isolate M+

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This study presents isolation and characterization of β-glucosidase (BG) from a bacterial isolate. Enzyme showed maximum activity at 50°C (2200 IU/l), however, relatively good activity (1600-1900 IU/l) was maintained even at higher temperature (60-80°C). Enzyme was thoroughly stable at 50-80°C for 30 min to 1 h and retained activity (75-100%), indicating its substantial thermo-tolerant nature. Maximum activity of BG was found at pH 6 (2200 IU/l), but significantly good activity was also observed at pH 5 (1800 IU/l), 7 (2000 IU/l), 8 (1900 IU/l) and 9 (1500 IU/l). Pb²⁺ caused considerable increase in enzyme activity (47%), while Zn²⁺ caused a little reduction (7%). SDS-PAGE showed a single band of BG (mol wt, 24 kDa).

Keywords: Acid/alkali tolerant, Characterization, β-Glucosidase, Purification, Thermo-tolerant

Introduction
Cellulose is most abundant on earth constituting about half of the carbonaceous compounds in terrestrial biomass. Endo and exo-glucanases act synergistically and promote solubilization of crystalline cellulose into soluble sugars, while β-glucosidase (BG) completes hydrolysis by converting cellobiose and cellobio-oligosaccharides into glucose. BG acts upon β-1,4 bonds linking two glucose or glucose substituted molecules (disaccharide-cellobiose). Studies have been carried out on the role and mechanism of action of cellulase components.

Biotechnical applications of BGs include converting phytoestrogen glucosides in fruits and vegetables to aglycone moieties, detoxification of cassava, aroma enhancement, and removing bitter compounds from citrus fruit juices or unripe olives. BG from Aspergillus kawachii has been implicated in flavor formation of sweet potato shochu. There is an increasing demand for the development of a thermostable BG for application in the conversion of cellulose to glucose for subsequent production of fuel ethanol. BGs are important in the formation of floral tea aroma and development of resistance to pathogens and herbivores in tea plants (Camellia sinensis) by catalyzing cleavage of defensive glucosides, such as cyanogenic glucosides. BG catalyzes transglycosylation reactions, which improve aroma of wines. In flavor industry, BGs are key enzymes in enzymatic release of aromatic compounds from glucosidic precursors present in fruits and fermentating products. BGs are more effective and specific than acid hydrolysis process for liberating terpenol (volatile alcohols) from terpenylglucosides, such transglycosidically bound volatiles also have interest in the food, cosmetic and tobacco industries.

This study presents purification and characterization of highly thermostable and acid/alkalitolerant β-glucosidase from a bacterial isolate M+.

Materials and Methods
Chemicals and Microorganisms
All chemicals, media and media components used were of analytical grade, obtained from Sigma Chemicals Ltd., USA; HiMedia Laboratories Ltd.; Ranbaxy Fine Chemicals Ltd. India; Qualigens Fine Chemicals Ltd. India, and Merck and Co. Inc., USA.

Bacterial strains (10) were isolated from decomposing rice straw samples, collected from rice mills in R S Pura, Jammu, India. Sample (5-10 g) was inoculated into nutrient broth (pH 8-11) and incubated at 45°C for 24-48 h. Enriched samples were plated on nutrient agar
and isolated colonies were purified further on nutrient agar. All cultures were maintained in culture collection of Fermentation Biotechnology Laboratory of Department of Biotechnology, University of Jammu, Jammu. Bacterial strains were streaked on CYPE-agar (carboxymethyl cellulose 1.0%, peptone 0.5%, yeast extract 0.5%, K$_2$HPO$_4$ 0.1%, MgSO$_4$ 0.02% and NaCl 0.5%; pH 9-10) and plates were incubated at 37°C for 24 h. Then, bacterial colonies were subjected to congo red staining for determining their cellulase producing ability$^{10}$.

β-Glucosidase Production, Assay and Total Protein Estimation

Overnight grown culture of bacteria was inoculated in 250 ml of CYPE broth contained in Erlenmeyer flask (500 ml) and submerged fermentation was effected under shaking conditions (200 rpm) at 45°C (Innova, New Brunswick, USA). Crude enzyme was obtained by centrifugation of a suitable volume of fermentation broth at 10,000 x g for 5 min at 4°C (Sigma, 3K 30), and supernatant was considered equivalent to crude enzyme and was used for assaying BG activity at different time intervals.

Assay for BG activity was done using p-nitrophenyl-β-D-glucopyranoside (1 mM) as substrate. Enzyme assay reaction mixture [750 µl of substrate (1 mM), 500 µl enzyme and 250 µl of acetate buffer (50 mM, pH 5.5)] was incubated for 30 min at 50°C, and then reaction was stopped by adding 1.5 ml of sodium carbonate (0.5 M). Contents were cooled to room temperature and colour developed was measured at 410 nm using UV-V spectrophotometer (Lambda 35, Perkin-Elmer), and translated to µ mol of p-nitrophenol using a standard graph prepared under same conditions. Unit of BG is expressed as the amount of enzyme required to release 1 µ mol of p-nitrophenol per min under above assay conditions. Protein content in supernatant was estimated$^{11}$ using bovine serum albumin (BSA) fraction V as standard.

Partial Purification of β-Glucosidase

Crude enzyme preparation obtained after cultivation of organism under submerged fermentation in shake flask (200 rpm, 45°C) was subjected to ammonium sulfate precipitation to obtain different saturation levels (20-100%). Precipitated fractions were dissolved in small quantity of acetate buffer (50 mM, pH 5.5), and examined for protein content and BG activity. The fractions that showed significantly high activity were pooled and dialyzed. For dialysis, enzyme preparation was filled in dialysis bag and suspended in same buffer, in which ammonium sulfate precipitated enzyme was dissolved. Dialysis was affected for 12 h and buffer outside the dialysis bag was replaced with fresh buffer after every 3 h. Then, dialyzed preparation was used for characterization of BG for some properties.

Influence of Temperature and pH on β-Glucosidase Activity

Crude enzyme preparation was assayed for BG activity at different temperatures (30-100°C). For determining effect of pH on enzyme activity, pH of assay mixture was varied (3-10) using citrate, acetate, phosphate, tris and carbonate-bicarbonate buffers (50 mM).

Thermostability and pH Stability of β-Glucosidase

Thermostability of BG was determined by pre-incubating enzyme preparation at 40-80°C for different time periods, and then assaying residual activity. For determining pH stability, enzyme was pre-incubated with buffers of varying pH (5-9) for different time periods, and residual activity was analyzed.

Effect of Detergents, Metal Ions and EDTA on β-Glucosidase Activity

Partly purified enzyme was used to study the effect of various additives like metal ions (Zn$^{2+}$, Pb$^{2+}$, Hg$^{2+}$), chelating agent (EDTA) and detergents (SDS, Tween-20) on activity by including them in enzyme assay mixture at a final concentration of 10 mM.

Ion exchange Chromatography, SDS-PAGE and Molecular Weight of β-Glucosidase

Enzyme preparation obtained after ammonium sulfate precipitation was subjected to ion-exchange chromatography using DEAE-Cellulose column (1 cm x 20 cm). Dialedyzed enzyme preparation was loaded on the column, which was pre-equilibrated with acetate buffer (50 mM, pH 5.5). Column was washed first with equilibration buffer, and then bound proteins were eluted using linear gradient of 0.1-1.0 M NaCl (in acetate buffer) at a flow rate of 1 ml per min. Fractions (2.5 ml each) were assayed for BG activity, and those showing significantly high activity were pooled, and used for SDS-PAGE analysis$^{12}$. Resolving gel consisted of 12.5% polyacrylamide in Tris-HCl (1.5 M, pH 8.8), while stacking gel consisted of 4.5% polyacrylamide in Tris-HCl (1.0 M, pH 6.8).
Results and Discussion
Cellulolytic Bacteria and Fermentation for Enzyme Production
All bacterial strains were analyzed for cellulolytic activity by congo red staining method. Strains (5) that showed cellulase producing ability were subjected to fermentation in shake flask in CYPE broth. Optimum BG activity of M-5 (920 IU/l) and M+ (760 IU/l) reached after 48 h of fermentation (Fig. 1). In case of M-5, activity decreased rapidly after 48 h while in case of M+, activity increased (960 IU/l) after 72 h of fermentation. M+ was selected for further studies. There are many reports of BG production by bacterial species isolated from various sources\(^\text{13}\). Optimum time for maximum enzyme production by different bacterial spp. is quite variable. *Candida peltata* produced maximum BG after 96 h of fermentation\(^\text{14}\), while *Thermoascus aurianticus* did so after 48 h\(^\text{8}\). Maximum BG production from *Melanocarpus* sp. occurred after 72 h of fermentation\(^\text{13}\), which is in agreement with this study. Maximum enzyme production stage of organism largely depends upon the type of microbial strains and their genetic make up, and on cultural and environmental conditions employed during growth of organism\(^\text{10,15}\).

Partial Purification of β-Glucosidase
Ammonium sulfate (AS) precipitation of crude enzyme resulted in maximum activity at 20-40% AS saturation. Precipitated protein was dialyzed and used for studying some properties of enzyme. AS precipitation has widely been used for protein/enzyme purification. AS at saturation levels of 40-60% has been used for cellulases or xylanases purification from different microbial sources\(^\text{10,15,16}\). AS precipitation at 35-53% saturation level has been used for BG purification from *Paenibacillus* sp. and *Bacillus polymyxa*\(^\text{4,6}\). However, BG from *A. kawachii* precipitated at 80% saturation level of AS\(^\text{5}\).

Effect of Temperature on Enzyme Activity
BG showed maximum activity at 50°C (2200 IU/l), however, significantly high activity (Fig. 2) was maintained even at higher temperatures of 60-80°C (1600-1900 IU/l). Activity at still higher temperatures (90-100°C) got decreased but presence of significant activity at such higher temperatures indicates thermostable nature of enzyme. BGs from different microorganisms have temperature optima of 35-50°C\(^\text{4,17}\). However, BGs from fungus *Aureobasidium pullulans* have temperature optima of 75°C\(^\text{6}\). *Pyrococcus furiosus* based BG showed optimum temperature of 102-105°C for maximum activity reflecting highly thermostable nature of enzyme\(^\text{18}\).

BG was found thoroughly stable (activity, 75-100%) at 50-80°C for 30 min to 1 h (Fig. 3). However, activity decreased slightly at 40°C (residual activity, 67-71%) but sharply at 80°C (residual activity, 45-63%) after 1 h of pre-incubation. BG from *Melanocarpus* sp. was stable at 50°C\(^\text{13}\), while that from *Sinorhizobium kostiense* was unstable at 50°C and above\(^\text{19}\). *Paenibacillus* sp. based BG was stable at 25°C for more than 1 h but lost its stability at 40°C after only 5 min\(^\text{4}\). In contrast, BG from *Thermoascus aurianticus* had temperature stability up to 70°C, however, lost activity after 80°C\(^\text{8}\). *Aspergillus oryzae* based BG was highly stable up to 45°C but was almost inactivated at 60°C and above\(^\text{20}\).
Effect of pH on Enzyme Activity

Although, BG showed maximum activity at pH 6 (2200 IU/l), but significantly high activity was observed at pH 5 (1800 IU/l), 7 (2000 IU/l), 8 (1900 IU/l) and 9 (1500 IU/l) indicating broad range pH stability of enzyme (Fig. 4). However, at very high acidic (pH 3-4) and alkaline pH (10) activity decreased drastically. Presence of activity over broad range of pH indicates potential suitability of enzyme for wide range of industrial processes, which generally are accomplished at pH extremes.

Optimum pH of BGs from different microbial species has been found to be quite variable. BG from \textit{S. kostiense} showed optimum pH of 6.0\textsuperscript{19} but A. \textit{niger} based BG was found active at optimum pH of 3.0\textsuperscript{20}. BG from \textit{Paenibacillus} sp. showed activity over a broad pH range (5.5-10.9)\textsuperscript{4}. However, contrary to present results, BG from \textit{T. aurantiacus} exhibited activity over a relatively narrow pH range (3.8-5.0)\textsuperscript{8}.

pH stability of BG was determined by incubating enzyme at different pH (5-9) for 30 min to 1 h, and then determining residual activity. BG was thoroughly stable at pH 5-9 for 30 min and retained 77-100% of activity (Fig. 5). However, 1 h pre-incubation of enzyme caused considerable loss of activity at pH 9 (reduction by 62%) and moderate loss of activity at pH 7-8 (reduction by 31-37%), but activity at pH 5-6 was maintained at very high level (77-95%). Thus, enzyme has got sufficient stability over acidic as well as alkaline pH indicating potential commercial importance of enzyme.

Effect of Additives on Enzyme Activity

Pb\textsuperscript{2+} caused considerable increase in activity (47%) while Hg\textsuperscript{2+} did so slightly (5%), and Zn\textsuperscript{2+} caused a little reduction (7%) in enzyme activity (Fig. 6). Though, EDTA also caused activity reduction but only to a moderate extent (21%). Detergents also lead to low to moderate reduction in enzyme activity (31-47%).
Various inhibitory ions, detergents or EDTA though caused activity reduction but maintenance of significantly high activity in presence of these inhibitors indicates industrial significance of enzyme. EDTA is reported to be an inhibitor of Paenibacillus sp. based BG\(^4\). However, Orpinomyces sp. based BG remained uninfluenced by EDTA but got inhibited by SDS; divalent cations (Zn\(^{2+}\) and Hg\(^{2+}\)) inhibited BG while Pb\(^{2+}\) had positive effect\(^2\)\(^1\). Activity of BG from S. kostiense got suppressed by Hg\(^{2+}\) and SDS\(^1\).

**Ion exchange Chromatography**

AS purified enzyme was further purified by ion exchange chromatography using DEAE-Cellulose. Ion exchange chromatography resulted in purification of enzyme by 1.2 folds (specific activity of 9.72 in fraction vs specific activity of 8.1 in the original preparation). This preparation was used for SDS-PAGE analysis. BG purification has been attained to varying levels by different researchers. A. pullulans based BG was purified by ion exchange chromatography to 12.9 folds\(^6\). BG from B. polymyxa was purified by 10.2 folds\(^2\), while that from Melanocarpus sp. could be purified by 3.03 folds\(^1\).

**SDS-PAGE Analysis and Molecular Weight Determination**

Purified enzyme preparation was subjected to SDS-PAGE analysis and showed only single band corresponding to molecular weight of approx. 24 kDa (Fig. 7). Orpinomyces sp. based BG was found to have molecular weight of 85 kDa\(^2\). BGs from Candida peltata and Daldinia eschscholzii had molecular weights of 43 kDa and 64 kDa respectively\(^1\)\(^4\),\(^1\)\(^7\). Molecular weight of Melanocarpus sp. based BG was 92 kDa\(^1\), while, A. oryzae based BG showed molecular weight of 43 kDa\(^2\)\(^9\).

**Conclusions**

Bacterial isolate M+ has capability to produce thermostolerant and acid/alkalitolerant β-glucosidase, which may have potential industrial significance. Further work on cost effective means for production of enzyme
and more detailed characterization of enzyme must be initiated.

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