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Partial Purification and Characterization of β-glucosidase from *Monascus sanguineus*

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ABSTRACT

The aim of the present work was to study *the production and characterization of β-glucosidase from Monascus sanguineus. Agro-waste residues were screened to obtain the maximum yield of enzyme. Jack fruit seed was the best substrate for enzyme production. Studies on the optimization of pH and temperature showed acidic pH favorable for enzymatic activity, whereas the optimum temperature was 60*°C. *Enzyme kinetics studies with different concentration of pNPG showed the calculated value of K_m approximately 0.89 mM with the non-linear regression and 0.98 mM with the linear regression techniques. The enzyme was predominantly inhibited by KCl (69.8%) and moderately inhibited by $CaCl_2$ (14.8%). Studies on the sensitivity for glucose showed that after 100 mM concentration of glucose, inhibition in pNPG hydrolysis took place. The molecular weight of the protein was estimated as 116 and 66 kDa with SDS- PAGE and zymography was carried out to verify the specific activity.*

Key words: Zymography, agro-waste, kinetics, *Monascus sanguineus*, pNPG

INTRODUCTION

β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21, BGL) is an enzyme, which catalyzes the hydrolytic cleavage of β-glycosidic linkage between two glycone residues or between glucose and an alkyl or aryl aglycone. BGL has great importance in variety of physiological as well as biotechnological processes. Its activity is mainly dependent on the nature of the glycone or aglycone moiety of the substrate. For instance, in plants, it is known to play the role in phytohormone activation, chemical defense against pests, lignifications, etc. It is also used for β-glucan synthesis during cell wall development and cell wall degradation in the endosperm during germination. As far as the mammalians are concerned, the human acid β-glucosidase, commonly known as glucocerebrosidase, plays an important role in the degradation of glucosylceramide in the lysosome (Turan and Zheng 2005). Almost all the living organisms from bacteria to higher living kingdoms are known to contain β-glucosidase enzyme. BGL is having several applications such as the enzymatic saccharification of cellulosic materials, liberation of flavor compounds in fruit juices and wines, etc. It also helps in the release of phenolic compounds with antioxidant activity from fruit and vegetable residues (Dongyang et al. 2012). In view of its efficiency to catalyze the transglycosylation reactions, several studies have focused on β-glucosidase research. These reactions are involved to improve the wine aroma and this property is of immense importance to the wine industry. Terpenes are a glycosylated precursor (Caldini et al. 1994), which is generally found in mango, grapes and passion fruits (Sarry and Gunata 2004). BGL and terpenes are more efficient than acid hydrolysis process for the liberation of terpenol

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from terpenylglucoside (Kaur et al. 2007). This volatile alcohol has enormous importance in food, cosmetics and tobacco industry (Jerkovic and Mastelic 2004). The filamentous fungus *Monascus* is used for centuries as a source of colorants in traditional foods. *Monascus* sp. is a subject of constant studies, mainly due to the growing interest for natural pigments for usage in food industry. Although other metabolic products from *Monascus* species, such as alcohols, organic acids, antimicrobial agents and substances with therapeutic activity have been described, little information about enzymes from *Monascus* is available (Daroit et al. 2007).

The aim of the present work was to study the production and characterization of β-glucosidase enzyme from a *Monascus* sp.

MATERIAL AND METHODS

Culture

Pomegranate was used to isolate the strain of *Monascus*, which was identified as *Monascus sanguineus*. The strain was maintained on Potato Dextrose Agar (PDA) medium and incubated at 28-30°C for seven days. It was preserved at 4°C, and sub-cultured once every four weeks (Dikshit and Tallapragada 2013).

Inoculum preparation

The spores were scraped off from the seven day old culture plate and dissolved in 0.90% saline water to produce a spore suspension. The spore suspension was used as inoculum.

Screening of substrates for β - glucosidase enzyme production

Different agro waste residues were chosen as solid substrates for the screening of BGL viz. wheat bran, coconut residue, tamarind seed and jack fruit seed. These substrates were purchased from a local market of Bangalore, India. Five gram of the substrate was placed in a 250 mL conical flask to which Mandels and Weber (1969) basal media salt solution was added. After cooling, these substrates were inoculated with 10% of the seed culture of *M. sanguineus* and incubated at 30°C for 10 days.

Extraction of enzyme

Enzyme was extracted by adding 50 mL citrate buffer (0.05 M, pH 4.8) in each flask and kept on rotary shaker at 150 rpm for 24 h. Solution was filtered through Whatman filter-paper and centrifuged at 5000×g at 4°C for 10 min. This filtrate was used as crude enzyme.

Partial purification of crude enzyme

Partial purification of BGL was carried out by ammonium sulphate precipitation, followed by dialysis. Cell free extract (250 mL) was saturated with ammonium sulphate up to 80%. The content was incubated over-night and centrifuged at 6000×g for 25 min. Supernatant was collected and saturated up to 90% with ammonium sulphate. Once again the content was centrifuged with above said conditions. Collected pellets were dissolved in phosphate buffer (20 mM, pH 6.5) and transferred in a dialysis bag and immersed in phosphate buffer at 4°C for 24 h. Buffer was changed several times in order to achieve proper purification (Ashwini et al. 2011).

β-glucosidase activity assay

Activity of the BGL from crude as well as partially purified enzyme was determined using 5 mM, 4-Nitrophenyl, β-D-glucopyranoside (pNPG) as substrate. Reaction mixture contained 0.5 mL of diluted enzyme sample (either crude or partially purified), 0.5 mL of 10 mM pNPG and 1.0 mL of citrate buffer (0.05 M, pH 4.8). It was incubated at 40°C for 15 min. This reaction was terminated with 2.0 mL of cold 0.2 M Na_2CO_3. The activity was observed by the liberation of p-nitro-phenol and was estimated in a spectrophotometer by reading the absorbance at 410 nm. One unit of enzyme activity was defined as the amount of enzyme required for the hydrolysis of one μmole of pNP per minute under assay condition and was expressed in units/gram dry substrate (U/gds) (Daroit et al. 2007).

Determination of optimum pH and temperature for enzyme assay

Effect of different range of pH on BGL activity was determined by using 0.05 M concentration of citrate buffer (pH of 3.0, 4.0, 5.0 and 6.0), sodium phosphate (pH 7.0 and 8.0) and Tris-HCl (pH 9.0). Assay was performed as described above. For optimum temperature, the reaction mixture was incubated at different range of temperatures (30, 40, 50, 60, 70, and 80°C) (Daroit et al. 2008).

Protein concentration

Protein concentration was estimated according to Lowry et al. (1951) method using bovine serum albumin as standard.

Poly-acryl amide gel electrophoresis and zymography

Poly-Acryl amide Gel Electrophoresis (PAGE) of the partial purified enzyme was performed according to Laemmli (1970) using 12% acryl amide gel in the presence of sodium dodecyl sulphate and 2-mercaptoethanol. Concentrated protein (1.0 mg/mL) was used for electrophoresis. Staining of the band was done with coomassie brilliant blue, R-250 (CBB). To obtain zymograph, PAGE was performed according to above procedure. After the electrophoresis, the gel was incubated with 20 mM of pNPG solution in citrate buffer at 50°C for 10 min to observe yellow band.

Kinetic parameters for β-glucosidase

Different concentrations of pNPG (0-25 mM) were used to estimate the kinetic parameters, K_m and V_{max} using double reciprocal Lineweaver–Burk plot. Michaelis-Menten equation was used to fit the data for the kinetic constant in non-linear manner and curve fitting was done by using MATLAB software (Daroit et al. 2008).

Effect of activators/ inhibitors on β-glucosidase activity

Different activators/inhibitors in the concentration of 5 mM were used to investigate the enzyme activity. NaCl, sodium dodecyl sulphate (SDS), $CaCl_2$, ethylene diamine tetraacetic acid (EDTA) and KCl were used for enzyme assay. Glucose inhibition was carried out with different concentration of glucose (100-500 mM). Enzyme was pre-incubated with above said compounds for 15 min. After incubation, the activity was observed with above mentioned standard protocols (Moloud et al. 2013).

RESULTS AND DISCUSSION

Screening of substrates for solid state fermentation

Among four substrates tested, maximum enzyme yield was observed with jack-fruit seed and minimum with tamarind seed (Fig. 1). The jack-fruit seeds are rich source of carbohydrates, proteins and good source of fiber and vitamin A. Wheat bran is rich in vitamin B. Coconut oil cake is also rich source of mineral and protein, which can promote fungal growth and yield of secondary metabolites. Tamarind seed is a polysachcharide and contains three major sugars viz. glucose, galactose, and xylose in molar ratio of 3:1:2. Glucose being a major constituent in tamarind seeds, the BGL activity shown was relatively less. There has been an increased exploitation of organic residues from various sectors of agriculture and industries over the past few decades. Crop residues such as bran, husk, bagasse, and fruit seeds have been utilized as potential raw materials in bioprocesses as they provide an excellent substratum for the growth of microorganism, supplying the essential nutrients (Pandey and Soccol 1998, 2000).

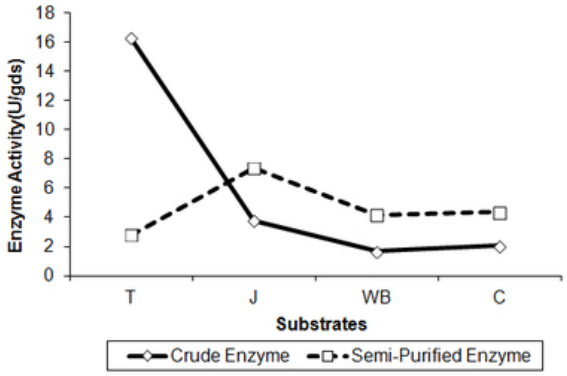

Figure 1 - Screening of substrates for β-glucosidase enzyme (depicting the crude enzyme and the semi-purified enzyme; y-axis showing the enzyme activity in Units/gram dry substrate and x-axis various substrates viz. T- Tamarind seed J- jack-fruit seed, W- Wheat bran, C- Coconut oil cake).

Partial purification, molecular weight and zymography of enzyme

The enzyme present in the crude extract was purified by ammonium sulfate precipitation, which resulted 2.6-fold purification and this further increased up to 3.9-fold with dialysis (Table 1). Partially purified enzyme was associated with other molecules depicting two major bands as revealed from SDS-PAGE. It has been reported that *Monascus* pigments react with amino acids present in the medium to form hydro soluble pigments (Dufosse et al. 2005; Daroit et al. 2008). The molecular weight of the BGL from *M. sanguineus* was calculated by plotting a graph between linear logarithms of relative molecular mass versus the Rf value. Calculated molecular masses of obtained bands were 116 and 66 kDa. The zymography revealed a single yellow band, conforming BGL activity from *M. sanguineus* (Fig. 2).

Table 1 - Purification Summary for β-glucosidase.

Purification Step	Activity (U)	Protein (mg/mL)	Specific activity (U/ mg protein)	Purification factor
Crude extract	3.8	5.1	0.745098	1
Ammonium sulphate precipitation	7.25	3.7	1.959459	2.6
Dialysis	6.1	0.8	7.625	3.9

Figure 2 - Zymogram for β-glucosidase.

Several authors have reported different molecular mass for the BGL from different organisms; for instance, approximately 120 and 66 kDa molecular mass of the protein was observed for the BGL from *Penicillium funiculosum* NCL1. Similarly, *P. purpurogenum* KJS506 and *P. occitanis* had shown the molecular mass of 120, 89.6 and 98 kDa, respectively (Bhiri et al. 2008; Jeya et al. 2010; Ramani et al. 2012). BGL from *A. terreus* NRRL 265 exhibited molecular mass of 116 kDa (Elshafei et al. 2011). Hongzhi et al. (2013) reported a BGL of 126.0 kDa from *P. simplicissimum* identified by 12% SDS- PAGE. As far as *Monascus* sp. is concerned, very few attempts have been made for enzymatic studies. Daroit et al. (2008) had reported high molecular mass of protein (than 100 kDa) for a BGL from *M. purpureus* and single yellow fluorescent band with partially purified sample, when subjected to zymography.

Optimization of enzyme activity with temperature and pH

β-glucosidase activity was observed at 40, 50, 60, 70 and 80°C. The results showed that the BGL activity increased from 40 to 60°C after which decrease in activity was observed (Fig. 3A). Temperature is an important factor for enzymatic activity. Activity of enzyme at higher temperature range is an advantageous factor for the saccharification of biomass and can also prevent contamination to allow the reaction to proceed at higher range of temperature. According to Daroit et al. (2008), increase in activity was observed with increase in incubation temperature. However, when the incubation temperature reached 80°C, the enzymatic activity diminished. As far as pH is concerned, the plot obtained followed the expected bell-shaped curve and the maximum activity was observed at acidic pH range (Fig. 3B). Lucas et al. (2000) had reported optimum BGL activity from *Chalara paradoxa* at the range of pH 4.0-5.0 whereas Daroit et al. (2008) had reported optimum BGL activity at pH 5.5 (acidic) from *M. purpureus.* A BGL from *A. foetidus* also has been reported optimally active at acidic range of pH (Hang and Woodams, 1994).

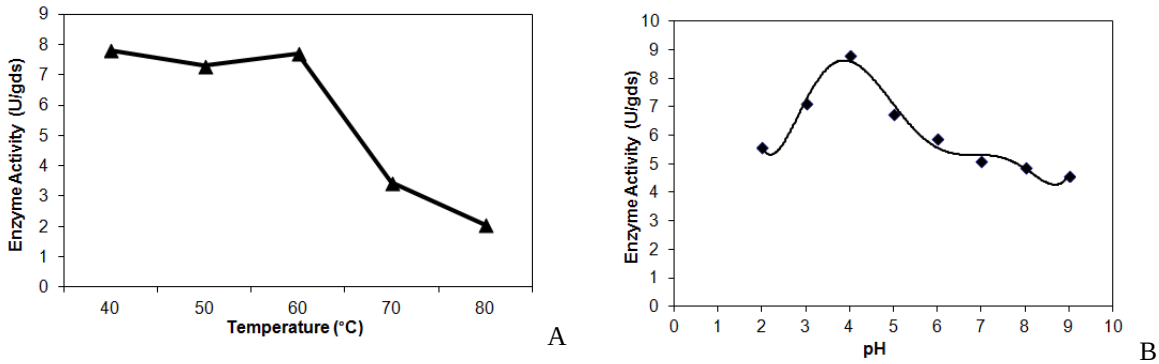

Figure 3 – (A) β-glucosidase activity at varying range of temperature (y-axis showing the enzyme activity in Units/gram dry substrate and x-axis the temperature in °C). (B) - β-glucosidase activity at varying range of pH (y-axis showing the enzyme activity in Units/gram dry substrate and x-axis the pH).

Kinetic constants of β-glucosidase

K_m and V_{max} (kinetic constants) of the BGL were estimated by non-linear regression technique utilizing Michaelis-Menten method. For this, the concentration of the substrate (pNPG) was plotted against the enzyme activity (V). The curve followed the Michaelis-Menten kinetics trend, which in-turn was used to calculate the value of the kinetic constant K_m and V_{max} (Fig. 4). The maximum value of the enzymatic activity (V_{max}) was approximately 7.56 U/mg protein as extrapolated by the Michaelis-Menten graph.

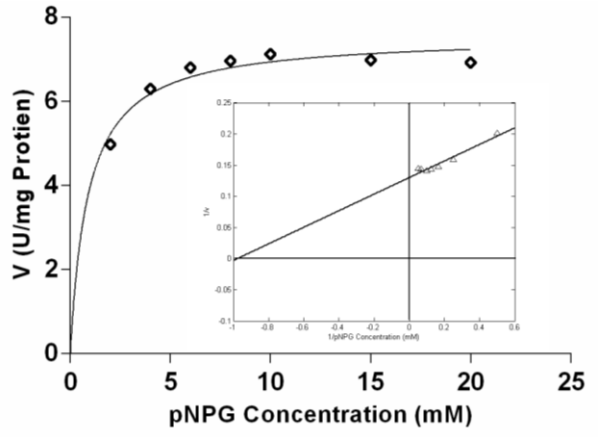

Figure 4 - The non- linear regression analysis of β-glucosidase activity. Inset: Lineweaver-Burk plot (1/V vs 1[S]) for pNPG hydrolysis.

As per the theory, the value of K_m is the substrate concentration required to attain half of the maximum enzyme velocity. The value of K_m, thus obtained was 0.89 mM. Smaller K_m value is a representative of powerful affinity towards the substrate. Similarly, to obtain these values and to further endorse the results, the Lineweaver-Burk plot was attempted (Fig. 4 inset). This calls for the inverse of the substrate concentration on the x-axis and the inverse of the enzymatic activity on the y-axis. The equation can be expressed as:

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

A linear fit is obtained between these two variables. The equation thus obtained can be expressed as

$$\frac{1}{V} = 0.133 \times \frac{1}{[S]} + 0.13 \quad (2)$$

From Eq. (1), it is evident that the intercept at the x axis gives the value of $-1/K_m$ and the intercept at y-axis gives the value of $1/V_{max}$. Hence, the value of V_{max} obtained was 7.52 U/mg protein and the value of K_m was 0.98 mM (Table 2). As the reciprocals distort the experimental errors, this double reciprocal plot does not really follow the assumptions made for linear regression. Still, the results corroborated with the results obtained from the Michaelis-Menten kinetics plot. Figure 4 showed that the hydrolysis of pNPG had not truly obeyed Michaelis-Menten kinetics and some inhibition was observed at higher concentration of the substrate. Daroit et al. (2008) had also reported inhibition in the hydrolysis of pNPG at higher concentration from *M. purpureus*.

Table 2 - Kinetic parameters for β-glucosidase from *Monascus sanguineus.*

From Non-Linear Regression			From Linear Regression		
K_m (mM)	V_{max} (U/mg protein)	V_{max} / K_m	K_m (mM)	V_{max} (U/mg protein)	V_{max} / K_m
0.89	7.56	8.49	0.98	7.52	7.68

Influence of chemical reagents on β-glucosidase activity

Studies on the influence of various chemical reagents on the BGL showed reduced activities by all the experimented reagents. Minimum inhibition was observed with $CaCl_2$ (14.8%) and maximum with KCl (69.9%) (Fig. 5). Certain enzymes, apart from a requirement of a coenzyme also need a metal ion for complete activity. Removal of metal ion often reduces the enzymatic activity or even results in a total loss of enzymatic activity. Metals are the common inorganic modifiers. Besides accelerating the rate of enzyme- catalyzed reactions, it can also inhibit the rate of reaction (Jain et al. 2008).

Inhibition of pNPG hydrolysis at different concentration of glucose

Tolerance of glucose is an important factor since it determines the suitability of enzyme for biomass hydrolysis. *M. sanguineus* BGL tolerated the glucose concentration up to certain level and thereafter inhibition in enzymatic activity was observed (Fig. 6). Several authors have reported that glucose acts as an inhibitor for the BGL activity. Daroit et al. (2008) has reported almost 58% reduction in pNPG hydrolysis from *M.*

purpureus with glucose. Glucose acts as a competitive inhibitor and is commonly observed in microbial BGL with a K_i value ranging from 0.5-14 mM (Yan et al. 1998; Yun et al. 2001). Gao et al. (2012) had reported glucose inhibition for BGL activity by *F. proliferatum* and they concluded that it might be due to the availability of variable special residuals on active site of BGL. The changes of the special residuals cause difference in extent of bonding to glucose as these residuals are not only the binding site of glucose but also the binding site for the substrate. This, in turn, makes variation in the degree of tolerance to glucose. However the mechanism of BGL tolerance to glucose is still ambiguous.

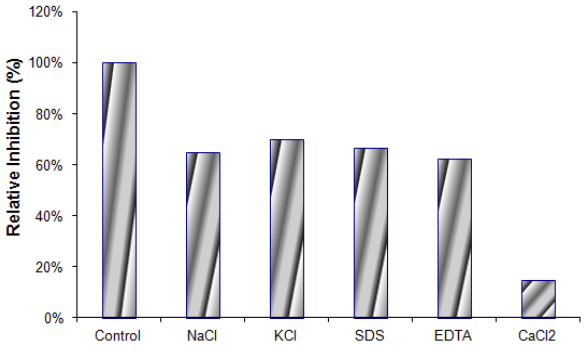

Figure 5 - Influence of chemical reagents on β-glucosidase activity (y-axis showing the percentage relative inhibition and x-axis various substrates).

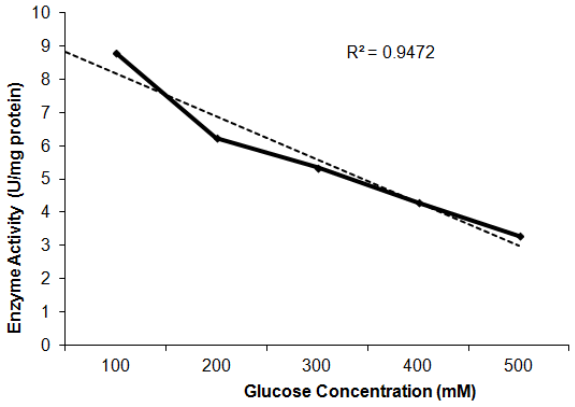

Figure 6 - Inhibition of pNPG hydrolysis at different concentration of glucose (y-axis showing the enzyme activity in Units/gram dry substrate and x-axis the glucose concentration in mM).

CONCLUSIONS

Among the various substrates, jackfruit seed was the best substrate for the production of BGL. The maximum enzyme activity was in acidic range of pH and at 60°C. Since *Monascus* strains are considered as safe organisms and widely used as food microorganisms, BGL produced by *M. sanguineus* could be used for releasing of terpenes and other aromatic compounds from wine and also for the liberation of antioxidant compounds from fruits and vegetables.

This *Monascus* strain needs more exploitation for better yield of enzyme, pigments and other secondary metabolites, which can be used for industrial applications.

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