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DIFFERENTIAL EFFECT OF ADDITIVES ON THERMOSTABILITY OF β-GLUCOSIDASE FROM *ASPERGILLUS NIGER* S1

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ABSTRACT
Growth of *Aspergillus niger* S1 on 2% wheat bran for 14 days produced 5973 IU/L of thermostable *â*-glucosidase under submerged culture conditions. The fungi also produced enoglucanase (1678IU/L) in the medium. The cellulases were stable at initial pH and temperature of 4 and 25°C respectively. The endoglucanase showed half -life of 15 min at 72°C and 30 min at 59°C. The *â*-glucosidase was thermostable with half-life for 90 min and 30 min at 70°C and 80°C but only for 15 min at 90°C. The addition of various additives increased the thermostability of these enzymes appreciably. Addition of 30% sorbitol and 50% glycerol increased the half-life of *â*-glucosidase to 120 and 100 min respectivly at 90°C. Sorbitol (20%), glycerol (30%) and PEG (1%) also offered protection against thermal inactivation.

INTRODUCTION

Enzymes are inherently labile and their operational stability is of great importance for any bioprocess. Temperature rising in bioreaction media produce opposite effects on enzymes leading to increased molecular collision followed by enzyme disruption and denaturation. In recent years interest in thermostable enzymes has increased dramatically as the resistance to thermal inactivation has become a desirable property of enzymes used in many industrial applications. These enzymes are used at high temperature for efficient hydrolysis of substrates and can be effectively recycled (Liu *et al*., 2011)

Cellulolytic hydrolytic enzymes are produced by many types of micro-organisms (Singh *et al*., 2010; Baig *et al*., 2003). Filamentous fungi have been widely studied, and the genus *Aspergillus* has displayed a great capacity to produce cellulases. The hydrolytic enzyme *â*-glucosidase work synergistically with endoglucanase and exoglucanases to degrade cellulose. The *â*-glucosidase allow the cellulolytic enzymes to function more efficiently by producing glucose from cellobiose and thus reducing cellobiose inhibition (Saha *et al*., 1994). *â*-glucosidase obtained from various sources are widely used for many applications, such as the enzymatic saccharification of cellulosic materials, the liberation of flavor compounds in fruit juices and wines, and the release of phenolic compounds with antioxidant activity from fruit and vegetable residues (Liu *et al*., 2012). The increased need for a considerable *â*-glucosidase activity, especially in the enzymatic saccharification of cellulose for bio energy, has strongly stimulated the study of *â*-glucosidase.The stability of the enzymes can be increased by chemical modification, cross linking, protein engineering and immobilization (Gouda *et al*., 2003). Engineering the reaction media to change the microenvironments provides a simple but practical means of increasing stability of enzymes (Naika *et al*., 2009). Thermostability and operational stability of enzymes can be made by addition of salts, polyols (polyethylene glycol, glycerol), sugars (sorbitol, xylitol, sucrose) and other modulators (Ray *et al*., 1993).The selection of appropriate additive depends upon the nature of enzymes.

Low cost of enzyme production improves the economics, as the cost of enzyme constitutes a major part of the total cost of hydrolysis (Baig *et al*., 2003). So it is desirable to minimize the cost of enzyme extraction and thermostabilization. Enzyme purification is very tedious process and economically not suited to industry. Our efforts therefore will be concentrated in improving thermostability of crude form of enzymes.

Though thermostable *â* -glucosidase has various applications in industry, very few reports have been encountered for enhancing its thermostabilization. The present paper reports some of characteristics of thermostable *â* -glucosidase produced by *Aspergillus niger* S1and improving its stability by selection of suitable additives in an aqueous solution. reen pea.

MATERIALS AND METHODS

Liquid state fermentation

Aspergillus niger S1 was inoculated in Erlenmeyer flasks (250 ml) containing sterilized Czapek enzyme production medium with 1-3% of wheat bran as sole carbon source. The broth

culture was incubated at 25ºC up to 21 days. The supernatants, collected after centrifugation of contents, were used for assaying cellulase. All assays were performed in triplicates unless or otherwise specified.

Enzyme assays

Activity of cellulase was assayed by reported method (Ray *et al.*, 1993). For endoglucanase activity, suitably diluted enzyme solution was incubated with 1% carboxy methyl cellulose (CMC) and 0.5M citrate buffer (pH 4.8) in a total volume of 2 ml at 50ºC for 30 min. β-glucosidase activity was measured with 0.05-0.1 ml of enzyme solution in a reaction mixture of 2 ml containing 1 mL of 1% cellobiose and 0.5M citrate buffer (pH 4.8). The reaction mixture was incubated for 15 min at 50ºC. The liberated sugars in the above assays were estimated as described by Nelson (1944) and the activity was expressed in International Unit (IU). One international unit of cellulase is defined as the amount of enzyme that released one micromole of reducing sugar per minute under the assay conditions (pH 4.8, 50ºC).

Optimization of culture condition

Optimum culture conditions including incubation period (7, 11, 14, 17 days), carbon concentration (1-3%), pH (4 - 6.5), temperature (20, 25, 30, 35ºC) and nitrogen source (ammonium chloride, sodium nitrate, ammonium nitrate, ammonium sulphate, urea and peptone) for maximum growth of *A. niger* and cellulase activity were worked out.

Thermostability of enzymes was determined by measuring the residual activity after exposing the enzyme preparation to various temperatures (40-95ºC for â-glucosidase and 40-80ºC for endo glucanase) for 15 minutes and then cooled in an ice-bath. The temperature (Tm) at which the residual activity was reduced to 50% of the original activity was determined.

Effect of additives on β-glucosidase thermostability

Different additives glycerol (30% & 50%), sorbitol (20% & 30%), sucrose (20% &30%), sodium azide (0.001%) and 1% polyethylene glycol (PEG) were added to crude enzyme solution at the beginning of pre incubation, in their respective final concentration. The thermal stability of β-glucosidase in the presence of polyols was investigated for varying periods of time in a temperature controlled water bath. The enzyme solution along with polyols was placed in a pre-warmed tube at the specified temperature, and aliquots were withdrawn at 30 min time intervals, ice-cooled and residual activity assayed. The incubation period ($T_{1/2}$) at which the residual activity was half of original activity was determined by plotting percent residual activity Vs exposure time. The incubation was carried out in sealed vials to prevent change of volume of the sample and hence, the enzyme concentration due to evaporation.

RESULTS AND DISCUSSION

During enzymatic hydrolysis, â-glucosidase converts cellobiose to reducing sugars. Among different incubation periods, *Aspergillus niger* S1 a soil isolate, produced higher â-glucosidase (2453IUL^{-1}) at 14 days of inoculation (Fig 1A). The enzyme production was reduced by 30% at 17 days of incubation. The endoglucanase production was increased with incubation period and highest level was achieved at 17

Table 1: Effect of different nitrogen sources on cellulase activity

Nitrogen source	Enzyme activity (IU/L) â –Glucosidase	Endoglucanase
NH_4Cl	2320^{b}	450^{d}
$NaNO_3$	1970^{c}	867^{b}
NH_4NO_3	1297^{d}	913^{a}
$(NH_4)_2SO_4$	5393^{a}	508^{c}
Urea	1180^{e}	365^{e}
Peptone	2222^{b}	870^{b}

^{a,b,c,d,e} values bearing different superscripts in a column differ significantly (P < 0.05

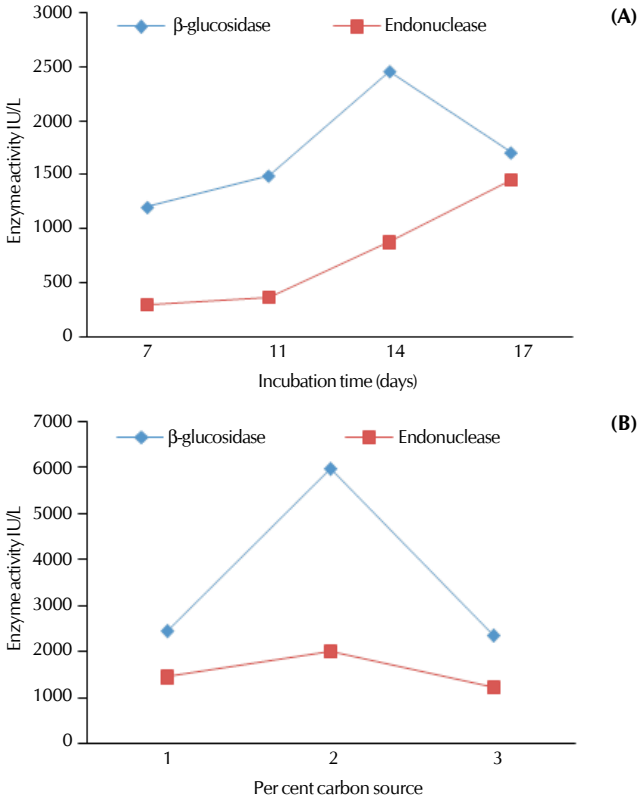

Figure 1: Effect of incubation time (A) and percent carbon source (B) on β-glucosidase and endoglucanase activity at incubation temperature 25ºC and pH 4.0

days. At the optimized incubation time, â- glucosidase and endoglucanase produced maximally with 2% wheat bran as carbon source (5973 IUL^{-1} and 2006 IUL^{-1} respectively, Fig 1B). A recent paper also showed maximum cellulase production with rice bran and wheat bran using *Aspergillus fungus* (Das *et al.*, 2013). Both organic (peptone and urea) and inorganic (NH_4Cl, $NaNO_3$, NH_4NO_3 and $(NH_4)_2SO_4$) nitrogen sources were evaluated for â- glucosidase production. Ammonium sulphate (5393 IUL^{-1}) was the most suitable nitrogen source among all nitrogen sources for â- glucosidase. Ammonium nitrate was the best nitrogen source for maximum endoglucanase production. Out of two organic nitrogen sources, peptone was the most appropriate nitrogen source for â- glucosidase (2222 IUL^{-1}) and endoglucanase (870 IU L^{-1}) production (Table 1). The both enzyme showed maximum production at incubation temperature 25ºC (Fig 1A) and pH 4.0 (Fig 1B). The optimum temperature for maximum enzyme

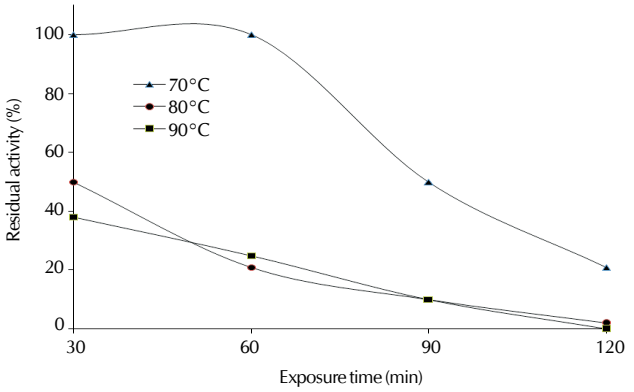

Figure 2: Effect of pre-incubation at 70°C, 80°C, 90°C for different intervals of time on residual activity of β-glucosidase

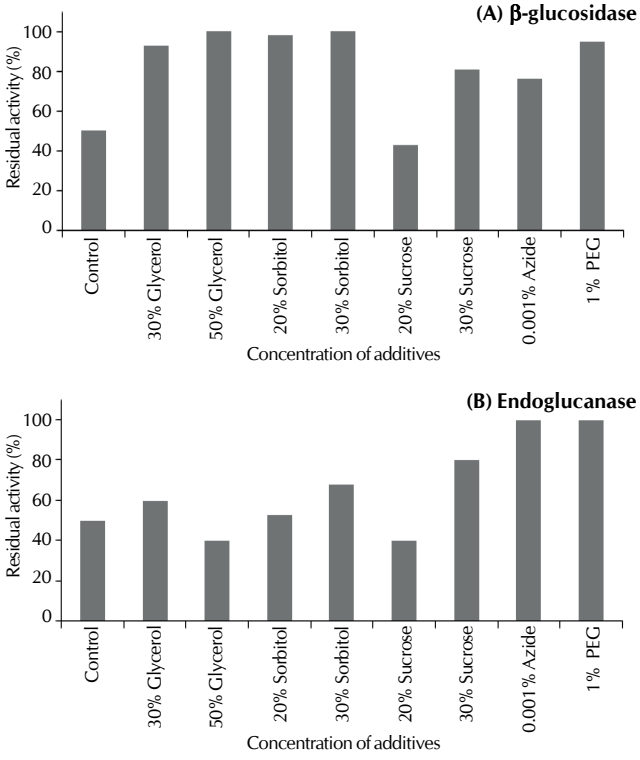

Figure 3: Effect of different additives on percent residual activity of β-glucosidase (A) endoglucanase (B) enzymes after exposure to respective Tm.

activity of both enzymes was 50°C. Chauve *et al*, (2010) however reported 60°C as optimum temperature for *â*- glucosidase activity.

Using all optimized conditions thermostability of enzymes produced by *A niger* S1 was studied. *â* –glucosidases, produced by *A niger* S1was more thermostable than endoglucanase. At 15 min pre-incubation period, *â* –glucosidases exhibited T_m of 90°C compared to 72°C for endoglucanase. To characterize further, *â* –glucosidases was pre-incubated at different temperatures for different intervals of time (Fig. 2). The enzyme retained 100% activity at 70°C

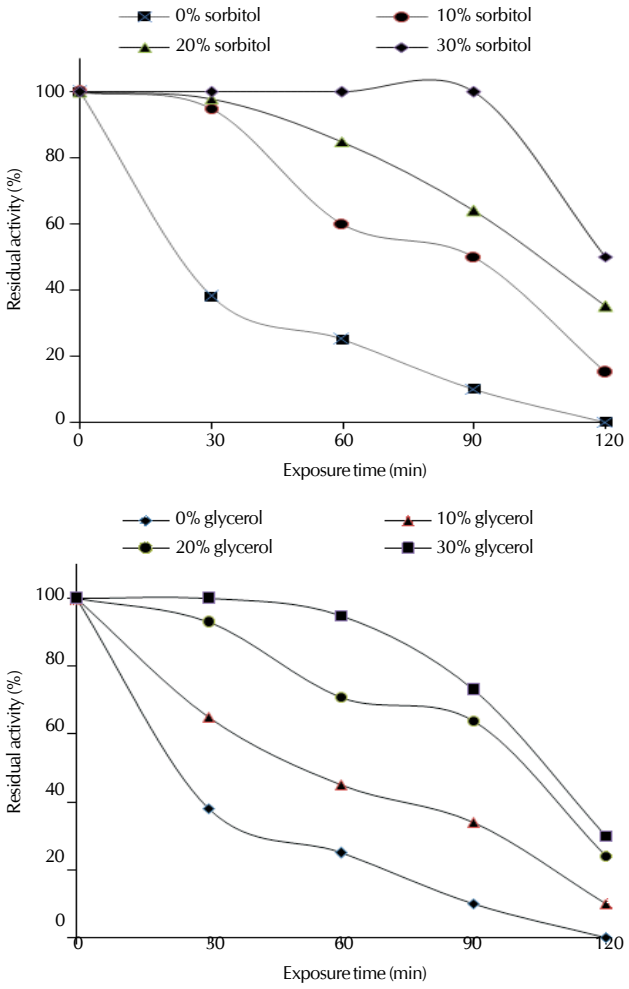

Figure 4: Thermal stability of β-glucosidase at 90°C in the presence of 0,10,20,30% sorbitol (A) and 0,10,20,30% glycerol (B) for different time intervals. At the end of incubation period the enzyme was cooled for 10 min and percent residual activity was determined.

for 60 min but the activity decreased rapidly afterwards and showed a $t_{1/2}$ of 89 min at this temperature. The activity was further decreased with increase of reaction temperature. A 30 min exposure at 90°C reduced residual activity to 40% of original activity. A thermostable *â* -glucosidase from *Aspergillus fumigatus* was purified and characterized by Liu *et al*. (2012). To improve the thermostability of enzymes at higher temperature, the effect of polyols additives such sorbitol (20%, 30%), glycerol (30%, 50%), sucrose (20%,30%), sodium azide (0.001%) and poly ethylene glycol (1%) was studied.

Effect of additives on enzyme activity

Effect of additives was studied initially at respective T_m, 90°C for *â* –glucosidase and 72°C for endoglucanase. All additives had a positive effect on *â*-glucosidase stability (Fig 3A) but the thermo stabilizing effect was highest with 30% sorbitol and 50% glycerol. With these additives *â*-glucosidase retained 100% activity at 90°C whereas 50% activity was lost with the native enzyme at the same temperature. Sorbitol was also thermo stabilizing polyol in xylanase for *A. niger* DFR5 (Pal and Khanum 2010). The additives, 30% glycerol, 20% sorbitol

and 1% PEG, showed 93-95% retention of original activity at the same incubation temperature. Thus decreasing the concentration of sorbitol and glycerol showed negligible effects on thermo stabilization at 90ºC, thereby protecting the enzyme from denaturation.

For endoglucanase, effect of additives on thermal stabilization is reported in Fig 3B. Maximum stabilization (100%) of activity was observed with 0.001% azide and 1% PEG. With 20% sucrose, 1.6 fold increases in activity was observed over control. A previous study on endoglucanase showed positive effects on thermostability with 40% sucrose (Naika *et al*., 2009).Thermal protection with 30% sorbitol was enhanced only 26% over control. The stabilizing effect of additives is therefore not an absolute effect valid for all enzymes, but it depends on the nature of enzyme, on its hydrophilic and hydrophobic character and on the degree of interaction with additives (Singh *et al*., 2000). Addition of 2 M glycine enhanced half-life of other cell wall degrading enzyme xylanase from 8 to 22 min at 80ºC from *Thermomonospora* sp (George *et al*, 2001).

Low molecular weight polyols (e.g. glycerol) and sugar alcohols (e.g. sorbitol) are useful for stabilizing enzymes by repressing microbial growth due to the reduction in the water activity, and by the formation of protective shells which prevent unfolding processes (Liu *et al*, 2012). A number of research workers correlated the thermostabilizing effect of polyols with their molecular size and thus with the number of hydroxyl groups per polyol molecule (Pal and Khenum,, 2010; George *et al*., 2001). In case of *â*-glucosidase effect of length of polyhydric alcohol molecule is not clearly observed but increasing the concentration of polyols improved the retention of residual activity. The phenomenon of protein stabilization by additives may be explained by changes in the microenvironment of the enzyme, which result in a more rigid conformation of the enzyme structure. Probably, the benefit of polyols is related to the effect they promote by increasing the degree of organization of water molecules, which in turn, intensify the hydrophobic interactions among nonpolar groups. Interaction of glycerol and sorbitol with the active site of the enzyme is another factor in enzyme stabilization (Maharaj *et al*., 2011).

Though sorbitol and glycerol have similar protective effects at shorter high temperature exposure time, the effect at longer periods was compared. A plot of percent residual activity versus exposure time for different concentrations of additives was drawn (Fig 4). Under similar conditions 30% sorbitol seemed to be more protective than 50% glycerol. Though both additives confirmed their thermostabilizing effect but 30% sorbitol was more effective stabilizing agent at longer period of exposure. A 100% retention of activity was observed with 30% sorbitol for 90 min at 90ºC (Fig 4A) but only 73% activity was retained with 50% glycerol (Fig 4B). The 30% sorbitol treated enzyme showed t1/2 of 120 min as compared to 100 min with 50% glycerol at 90ºC. Thus these additives though show similar protective effect at low incubation time and temperature but differ at higher level.

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