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Purification and characterization of amyloglucosidase produced by a mutant strain of *Aspergillus Niger*

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Abstract

Amyloglucosidases, are the enzymes which releases glucose by hydrolyzing starch and oligosachharides as it hydrolyzes ∝-1, 4 and ∝-1, 6 linkages of the saccharides formed by the action of other amylases on starch and has great importance in the starch industries. The present study is concerned with the partial purification of amyloglucosidase produced by the mutant strain of *Aspergillusniger* using ammonium sulphate precipitation method and characterization of the enzyme. The maximum activity of amyloglucosidase was achieved after 60 min of incubation when maintained at pH 4.75 and temperature of 60°C with 5% starch concentration.

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Introduction

Glucoamylases, also known as amyloglucosidases, are the enzymes which releases glucose by hydrolyzing starch and oligosachharides as it hydrolyzes ∝-1, 4 and ∝-1, 6 linkages of the saccharides formed by the action of other amylases on starch. Glucoamylase has also the ability to hydrolyze terminal non-reducing ∝-1, 4-gluco pyranose (Kelly *et al.*, 1983). Glucose is produced by the action of amyloglucosidase on starch (Ford, 1999; Reilly, 1999) which is used in various food industries (Polakovic and Bryjak, 2004; Dile r*et al.*, 2015) and commercial production of amyloglucosidase. *Aspergillus niger* has a very major role (Pandey and Radhakrishan, 1993; Arassaratnam *et al.*, 1997; Khalaj *et al.*, 2001 Haq *et al.*, 2002; Omemo *et al.*, 2005; Spier *et al.*, 2006; Costa *et al.*, 2007). Ammonium sulphate precipitation has been used for the partial purification of amyloglucosidase. It was reported that maximum glucoamylase activity was obtained in the 40-60% fractionation (Selva *et al.*, 1996).

Partially purified enzyme has also been analyzed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) to determine the molecular weight of amyloglucosidase. The molecular weight of amyloglucosidase from *A. niger* was 63 kDa (Fogarty and Benson, 1983). Amyloglucosidase is very sensitive to temperature and pH (Arassaratnam *et al.*, 1994). The activity of the enzyme increased with increase in the incubation period and found to be optimum after 60 min (Manera *et al.*, 2008).

Starch protect glucoamylase from heat inactivation and this effect was dependent on the concentration of starch and at 5% starch concentration, the enzyme was completely stable at higher temperature (Hyun and Zeikas, 1985). The main objectives of present study were to partially purify amyloglucosidase and then optimize the condition to get maximum activity of the enzyme.

Materials and methods

Microorganism

For the present investigation a mutated strain of *Aspergillus niger* (BT) was used.

Composition of medium

The culture media used for the production of amyloglucosidase by *A. niger* wasg/L **(**Starch, 20.0; glucose, 10.0; $(NH_4)_2SO_4$, 4.0; $MgSO_4.7H_2O$, 2.0; $CaCl_2.2H_2O$, 2.0; KH_2PO_4, 1.50; K_2HPO_4, 0.1; Distilled water, 1000 ml; pH 5.0).

Enzyme assay

The assay of amyloglucasidase was carried out according to the method reported by Malik *et al.* (2011). One ml of the diluted enzyme extract was added to 1.0 ml of 5% soluble starch solution prepared in acetate buffer (pH 4.8).

Partial purification of amyloglucosidase

Separation of fungus from the fermented broth: Separation of fungal mass from fermentation medium, was done by using centrifugation technique. The culture medium was centrifuged at 10,000 rpm for 30 min at 4°C.

Ammonium sulphate precipitation: The fermented broth contained crude preparation of amyloglucosidase. It was subjected to ammonium sulphate fractionation. Saturation between 20-80% was achieved. The precipitates of different fractions were resuspended separately in minimum amount of Tris-Cl buffer (pH 8.0) in different test tubes.

Dialysis

The resuspended precipitates were dialyzed against 50mM Tris-Cl buffer (pH 8.0) by putting them in a visking dialysis membrane with a molecular weight cut of (MWCO) 12000 – 14000. The assay of amyloglucasidase was carried out according to the method as reported by Malik *et al.*, (2011a). The protein content of the dialyzed sample was assayed by Bradford method. (Bradford, 1976).

Charaterization of enzyme

For maximum amyloglucosidase activity optimum pH, temperature, time and concentration of starch were investigated.

Effect of pH:

The pH of the enzyme-substrate complex has a great effect on the activity of amyloglucosidase. The pH of the enzyme-substrate complex was varied from 3.5-5.5.

Effect of temperature:
The temperature of the enzyme-substrate complex was varied from 25-65°C.

Effect of time of incubation:
The time of incubation for amyloglucosidase activity was varied from 10-60 min in the reaction mixture.

Effect of different concentration of starch:
The concentration of the starch in the enzyme-substrate complex was varied from 1-7% to obtain maximum activity of amyloglucosidase.

Sodium dodecyl sulphate polyacrylamide gel
Electrophoresis (SDS-page):
Laemmli SDS-PAGE system has four components. These are electrode buffer, the sample, the upper stacking gel and lower resolving gel. The two gels i.e., stacking gel and resolving gel, were cast with different porosities and pH.

Electrophoresis:
The electrophoresis cell was placed in a vertical position. The upper and the lower reservoirs were filled with the electrode buffer (Running buffer). The glycerol in the samples provided the density required for the sample to sink towards the bottom of the well. The Bromophenol Blue tracking dye was used so that the sample could be seen during loading. Mixture of marker proteins (Biorad precision plus protein catalog No 161-0363) with known molecular weight was also loaded as standard. The range of the marker proteins were from 20-250 kDa. The leads were attached to the unit and were connected to the power supply.

The electrophoresis was carried out at voltage of 30 Amp for stacking gel and 100 Amp for reservoir gel. The process was completed in 4-5 h. When the electrophoresis completed, the gel assembly was removed and glass plates were separated and finally the spacers were removed.

Gel staining:
The gel was stained with solution of Coomassie Brilliant Blue G- 250 at room temperature for 30 minutes.

Destaining:
The gel was destained using a destaining solution. The stained background was removed and the protein bands became clearly visible.

Results and discussion
Partial purification of amyloglucosidase by ammonium sulphate precipitation. It was observed that the ammonium sulphate fractions from 20-30% did not show any amyloglucosidase activity.

The enzyme started to precipitate at 40% ammonium suphate fraction. Maximum amyloglucosidase activity (21000 U/ml/min) was otained at 70% saturation as shown in table No.2, after which it again decreased and at 90% saturation no pellet was obtained.

The concentration of any salt necessary to cause precipitation of a particular enzyme is related to the number and distribution of charges on the surface of the protein (Deutscher, 1990., Roe, 2001).

Table 1. Formulation of gels for SDS-PAGE.

Solutions	Resolving gels 12 %	Stacking gels 5 %
30% Acrylamide/Bisacrylamide	4.0 ml	0.670 ml
0.5 M Tris pH 6.8	-	1.25 ml
1.5 M Tris pH 8.8	2.5 ml	-
10%SDS	0.1 ml	50 µl
APS	0.1 ml	50 µl
TEMED	20 µl	10 µl
Distilled water	3.28 ml	3.075 ml

Initially, the addition of solid ammonium sulphate precipitated unwanted proteins and then the desired enzyme was precipitated which is salting out of proteins (Singh, 2007).

In the present study enzyme precipitation occurred in the range of saturation level 40-80%. Maximum precipitation took place at 70% and the results are in line with the result of Selva *et al.* (1996).

Table 2. Partial purification of amyloglucosidase produced by *A. niger* strain using ammonium sulphate fractionation.

Sr. No.	Ammonium sulphate (%)	Amyloglucosidase activity (units/L)	Protein (mg/L)	Specific activity (U/mg/L)	Recovery (%)	Purification fold
	Crude enzyme	25000	131	190.83	100	1
1	20	-	114	-	-	-
2	30	-	112	-	-	-
3	40	11500	106	108.49	46	0.56
4	50	14900	101	147.52	59.6	0.77
5	60	18750	94	199.46	75	1.04
7	70	21000	87	241.38	84	1.26
8	80	6100	64	95.31	24.4	0.49
9	90	-	-	-	-	-

Molecular weight determination

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was run to determine the molecular weight of amyloglucosidase. The band of the partially purified amyloglucosidase appeared in between the marker proteins having molecular weight 50 kDa and 75 kDa which shows that amyloglucosidase have the molecular weight of 63- 65 kDa approximately (Fig.1). This observation is in accordance to the findings of Fogarty and Benson (1983), they reported the molecular weight of amyloglucosidase from *A. niger* was 63 kDa.

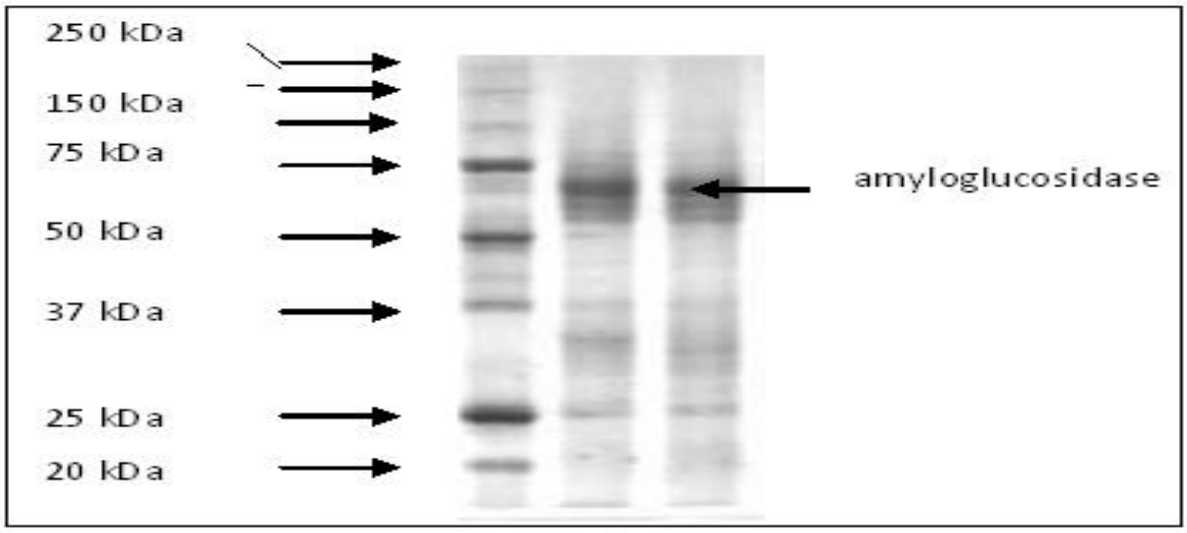

Fig. 1. SDS-PAGE of partially purified amyloglucosidase along with marker proteins.

Characterization of amyloglucosidase

Effect of pH

Fig. 2 shows the effect of pH on amyloglucosidase activity. The enzyme activity was found to be maximum at pH 4.75 (25.29 U/ml/min). When pH of reaction mixture was increased or decreased from 4.75, the activity of enzyme decreased significantly. Amyloglucosidase is very sensitive to temperature and pH (Arassaratnam *et al.*, 1994). Similar results were reported (Preda *et al.*, 1996; Fogarty and Benson, 1983). Any change in pH degenerates the structure of enzyme that lead towards the reduction of activity of amyloglucosidase.

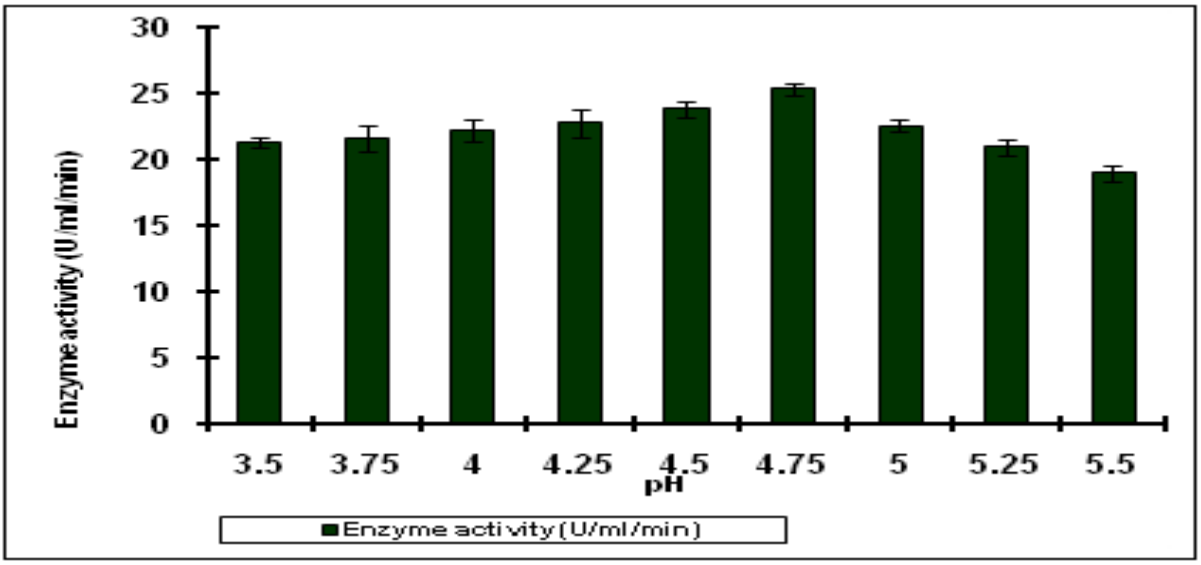

Fig. 2. Effect of pH on enzyme activity.

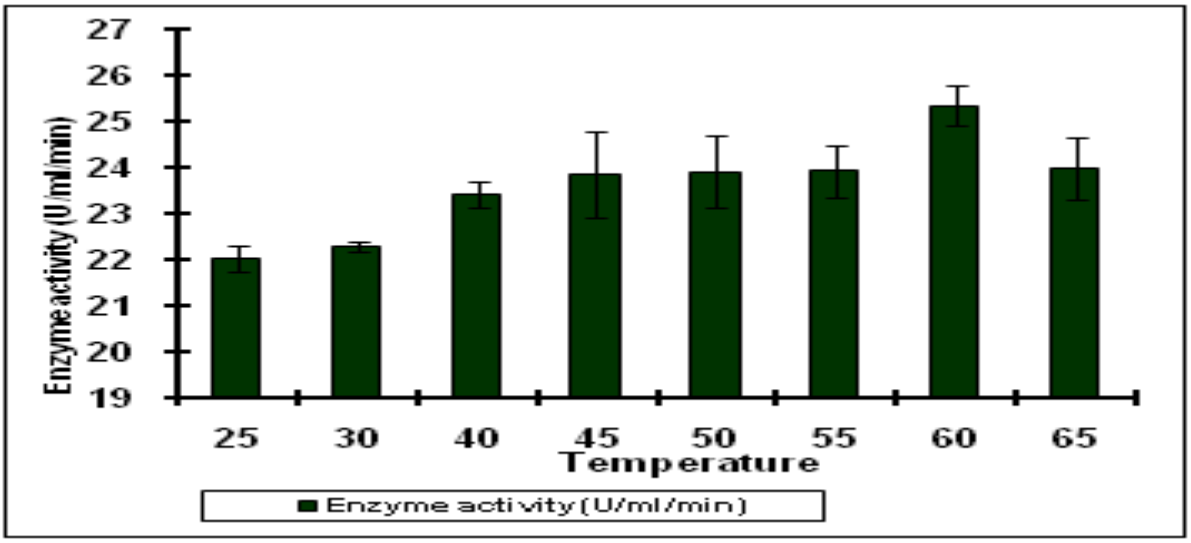

Fig. 3. Effect of temperature on enzyme activity.

Effect of temperature

The effect of incubation temperature of the reaction mixture on the activity of amyloglucosidase was investigated as shown in Fig. 3. The enzyme activity was found to be maximum (25.35 U/ml/min) at 60°C. Further increase in the incubation temperature resulted decreased in the activity of amyloglucosidase.. With increase in the temperature, the denaturing of enzyme started. Similar reports were given by many other researchers (Preda *et al.,* 1996; Tsekovak *et al.,* 1999). The enzyme lost its activity at 65°C. These results are in line with many reports (Selva *et al*., 1996; Omemo *et al*., 2008 and Manera *et al.,* 2008).

Rate of amyloglucosidase activity

The time profile for amyloglucosidae activity was carried out. (Fig.4). The activity of enzyme was increased with increase in the incubation period and found optimum (25.20 U/ml/min) after 60 min. Further increase in the incubation period have no significant effect on the activity of amyloglucosidase and denaturing of enzyme started. The results of the present study are in accordance to findings of Manera *et al.* (2008).

Effect of concentration of starch

The effect of different concentrations of starch on the enzyme activity is shown in Fig. 5.

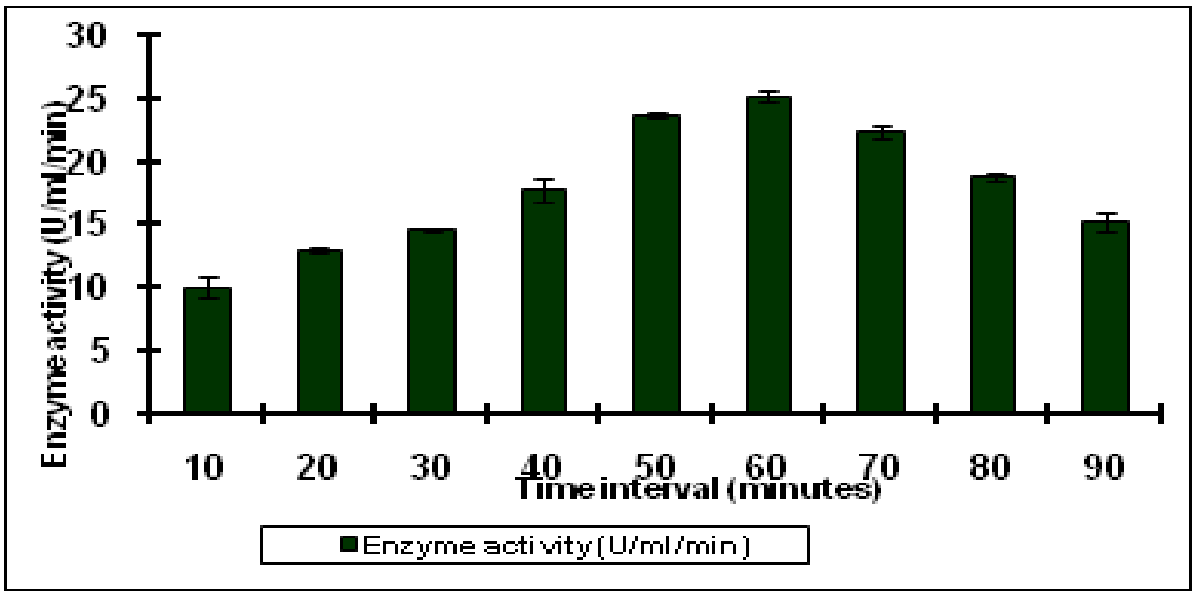

Fig. 4. Effect of time on enzyme activity.

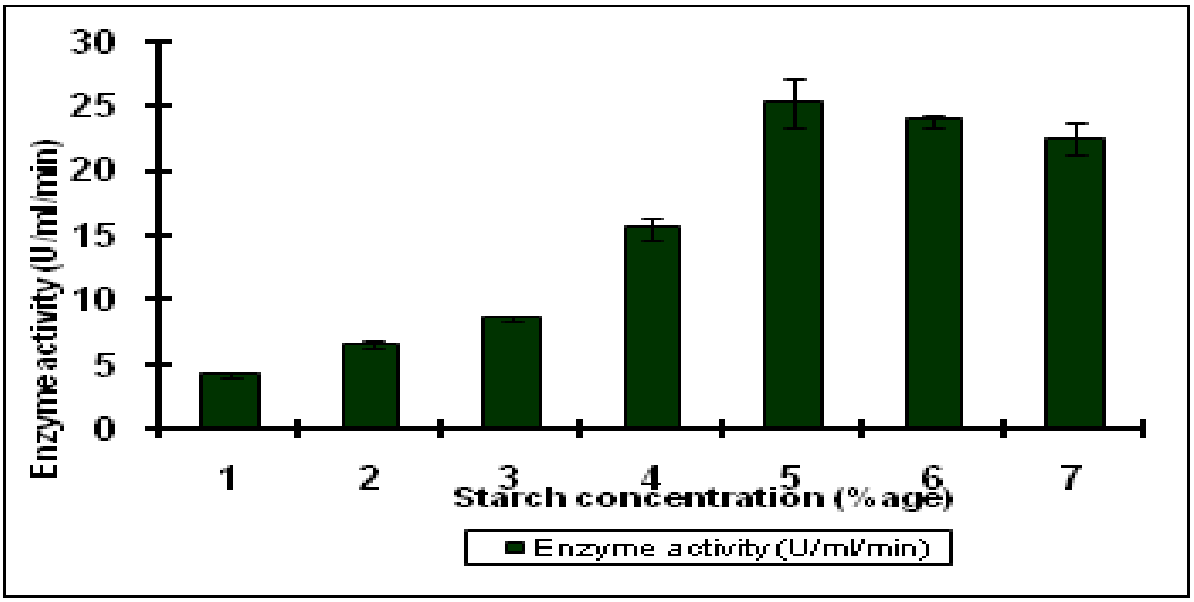

Fig. 5. Effect of concentration of starch on enzyme activity.

The enzyme showed its maximum activity at 5% starch concentration i.e., 25.18 U/ml/min. Further increase in starch concentration decreased the enzyme activity. It was due to the reason that starch protected glucoamylase from heat inactivation and this effect was dependent on the concentration of starch and at 5% starch concentration, the enzyme was completely stable at higher temperature (Hyun and Zeikas, 1985). An inhibitory effect of starch above a critical concentration was observed.

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