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PARTIAL PURIFICATION AND PROPERTIES OF AMYLOGLUCOSIDASE FROM *FUSARIUM SOLANI*

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ABSTRACT

Fusarium solani, a mesophilic fungus, was grown for 4 days at 35°C, pH 5 under solid state growth conditions using wheat bran for the production of amyloglucosidase.The specific activity of crude enzyme was 23.08 units mg^{-1}.The enzyme was purified using fractional precipitation, anion-exchange and gel filtration chromatography. The recovery of amyloglucosidase after gel filtration was 35.65 % with 6.32 fold increase in specific activity. Maximum activity of the enzyme was observed at 40°C and pH 5.0. The K_m value for soluble starch was 0.4 mg ml^{-1}. Amyloglucosidase from *F.solani* was thermally stable as it possessed a half-life of about 30 min at 56°C

Key Words: Amyloglucosidase; *Fusarium solani*; glucoamylase; Michaelis constant; solid state fermentation; thermal stability

INTRODUCTION

Fungal carbohydrases have a wide range of applications in food industry, in addition to their role in the ecological recycling of cellulose, hemicellulosic and starchy biomass materials. Of these extracellular enzymes, starch degrading amylases represent the largest potential for industrial use. Several starch-degrading enzymes are of commercial importance, such as α-amylase, glucoamylase, isoamylase and pullulanase (Marlida *et al.*, 2000).

Amyloglucosidase (EC 3.2.1.3, glucoamylase, 1, 4 α-D-glucan glucohydrolase) is an important industrial enzyme that hydrolyses 1, 4-linked α-D-glucosyl residues successively from the non-reducing end of oligo- and polysaccharide chains with the release of D- glucose. The most important application of amyloglucosidase is the production of high glucose syrup, which is used to produce crystalline glucose and high fructose syrup (Fogarty, 1983).Moreover; it is used in the production of different antibiotics, amino acids, ethanol and organic edible acid production processes (Nigam and Singh, 1995)

The primary sequences of several filamentous fungal, yeast and bacterial glucoamylases have been reported (Coutinho and Reilly, 1994). Structure-function relationship studies indicate the functional independence of catalytic and starch binding domains and structure similarities in microbial glucoamylases (Coutinho and Reilly, 1994). Specific residues were shown to be crucial for directing protein conformational changes, substrate binding, thermal stability and/or catalytic activity (Sierks *et al.*,1993). Nevertheless, the industrial use of amyloglucosidase is impaired by the limited glucose yields. The slow hydrolysis of α-(1,6)-glucosidic bonds in starch and formation of condensation products may be the reasons for low yields (Nikolov *et al.*,1989).The poor stability of enzymes under standard conditions and end product inhibition of enzyme activity also affect glucose yields (Campos and Flex, 1995). The possibility of solving the problems of starch industry encourages the search for a superior amyloglucosidase having better activity and thermostability.*Fusarium solani* is a novel filamentous fungus in the sense that there is hardly any report of this enzyme from it. Therefore, the present investigation described the isolation, purification and characterization of amyloglucosidase from this fungus.

MATERIALS AND METHODS

Organism and Amyloglucosidase Production

Pure culture of *Fusarium solani* was obtained from National Fungal Culture Collection of Pakistan (NFCCP), Department of Plant Pathology, University of Agriculture, Faisalabad.The culture was maintained on potato dextrose agar (PDA) slants. The fungus was grown on wheat bran having 70 % initial moisture content having pH 5.0 for 4 days. At the end of fermentation, the enzyme was extracted with distilled by squeezing the mixture through muslin cloth. The crude extract was then centrifuged at 10,000 x *g* at 4°C for 30 min and the clear supernatant was used as the enzyme source.

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Protein Estimation

Total proteins were estimated by Bradford micro assay (Bradford, 1976) using bovine serum albumin (BSA) as the standard (Fig. 1).

Amyloglucosidase Assay

Amyloglucosidase assay was carried out as described by Iqbal *et al.*, (2003). Appropriately diluted enzyme was reacted with 1 % soluble starch solution in 50 mM MES buffer (pH 5.5) at 40 °C for 40 min. The reaction was then stopped by boiling water for 5 min, and then was cooled on ice. The released glucose was measured using a glucose oxidase method. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmole of glucose equivalent ml^{-1} min^{-1} at pH 5.5 and 40°C".

Purification of Amyloglucosidase

Amyloglucosidase was precipitated from the culture supernatant using ammonium sulfate (65 % w/v final concentration).It was kept at 4 °C over night. After 24 h the resulting precipitate was collected by centrifugation at 10,000 x *g* for 30 min. The protein was dissolved in minimum quantity of water and dialyzed extensively against three changes of distilled water to remove the salts. The dialyzed sample was applied on DEAE-cellulose column equilibrated with Tris/HCl buffer pH 7.5.The linear gradient of NaCl (0 - 0.5M) in 20 mM Tris/HCl pH 7.5 was used as elution buffer.3 ml size fractions were collected .The active fractions were pooled and dialyzed against distilled water. The sample from DEAE- cellulose column was then applied to Sephadex G-100 column previously equilibrated with acetate buffer pH 5.0 and eluted with the same buffer as described by Deutscher (1990).2 ml size fractions were collected and active fractions were pooled.

Effect of Temperature

The effect of temperature on the activity of amyloglucosidase was determined by incubating the enzyme at temperatures from 20 to 75°C at pH 5.5 as described earlier (Siddiqui *et al.*, 1997).The assay methodology was same as described above.

Effect of pH

The effect of pH on the activity of amyloglucosidase was determined over a 3.0 to 8.0 pH range with 0.1 M citrate-phosphate buffer at 40°C as described by Stamford *et al.* (2002).

Effect of Substrate

Amyloglucosidase from *Fusarium solani* was assayed in the reaction mixtures containing variable amounts of soluble starch [0.025-0.15 % (w/v)] at pH 5.5.The data were plotted according to Lineweaver-Burk to determine the values of kinetic constants as described by Siddiqui *et al.*(2000).

Thermostability

In order to determine thermal stability the enzyme was incubated at different temperatures (30-75°C) for 5 min at pH 5.5. At the end of incubation the enzyme was cooled on ice bath for 30 min and finally assayed at 40°C as described above. Total thermal denaturation of the enzyme was determined by incubating the enzyme in 50 mM MES monohydrate buffer pH 5.5 at 56°C.Time course aliquot were withdrawn at different time intervals, cooled in ice bath for 30 min and finally assayed at 40°C for enzyme activity (Siddiqui *et al.*, 1997).The data were fitted to first order plots and analyzed as described by Montes *et al.* (1995).

RESULTS AND DISCUSSION

The production, purification and characterization of extracellular amyloglucosidase by *Fusarium solani* was studied in solid state fermentation (SSF). Solid state fermentation has numerous advantages over submerged fermentation (SmF), including superior productivity, simple technique, low capital investment, low energy requirement and less waste water output, better product recovery and lack of foam build up (Lonsane and Ramesh, 1990; Babu and Satyanarayana, 1995; Pandey, 1995). There is no report regarding the production, purification and characterization of amyloglucosidase from this strain. *Fusarium solani*, a mesophilic filamentous fungus was used in the present study for the production of amyloglucosidase using wheat bran under solid fermentation (SSF) conditions.

Crude dialyzed enzyme was partially purified after subjecting it to ammonium sulphate, anion-exchange chromatography and gel filtration. The specific activity of crude enzyme was 23.08 U mg^{-1}The onset of

amyloglucosidase precipitation occurred at 30 %, while complete precipitation was observed at 65 % of ammonium sulfate at 0 °C (**Fig. 2**).The % recovery of amyloglucosidase was 84.56 after fractional precipitation (Table 1).The glucoamylases from *Monascus purpureus* (El-Sayed *et al.*, 2000) and *Arachniotus citrinus* (Niaz *et al.*, 2004) have been found to be precipitated at 50 % and 75 % respectively.

Partially purified enzyme after ammonium sulfate precipitation was loaded to anion-exchange column. Purification of amyloglucosidase after anion-exchange column was 2.72 fold and 53.47 % recovery. Purified amyloglucosidase, after anion-exchange chromatography was further purified by applying on gel filtration column. The % recovery of enzyme after gel filtration was 35.65 while its purification became 6.32 fold with respect to crude dialyzed enzyme. The results of purification are summarized in Table 1

Table 1. Purification of amyloglucosidase from *Fusarium solani.*

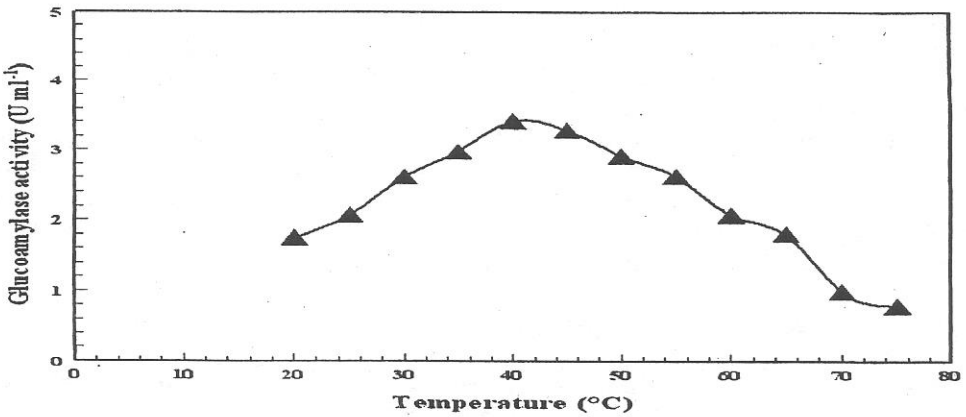
Treatment	Total Units	Total protein (mg)	Specific activity (U mg^{-1})	Purification factor	% recovery
Crude	5030	218	23.08	1.00	100
$(NH_4)_2 SO_4$ precipitation	4253	98	43.05	1.86	84.56
Anion-exchange chromatography	2689	42	62.99	2.72	53.47
Gel filtration	1793	12	145.79	6. 32	35.65

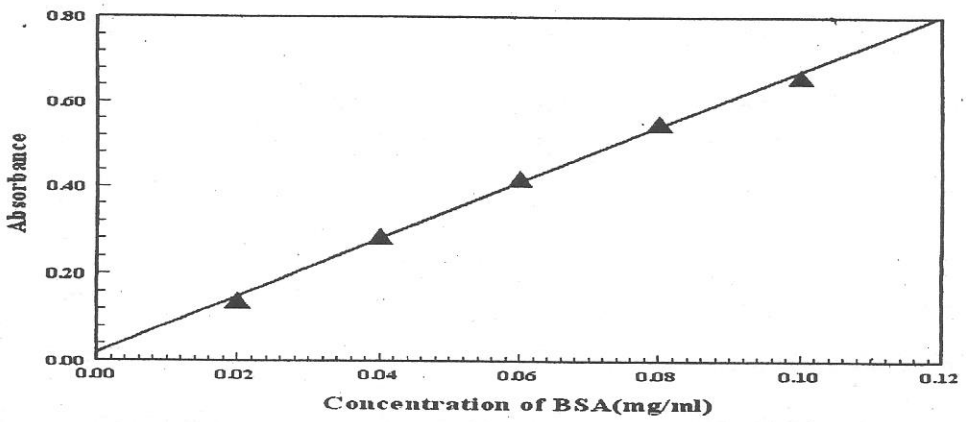
Where, all values were after dialysis against distilled water.

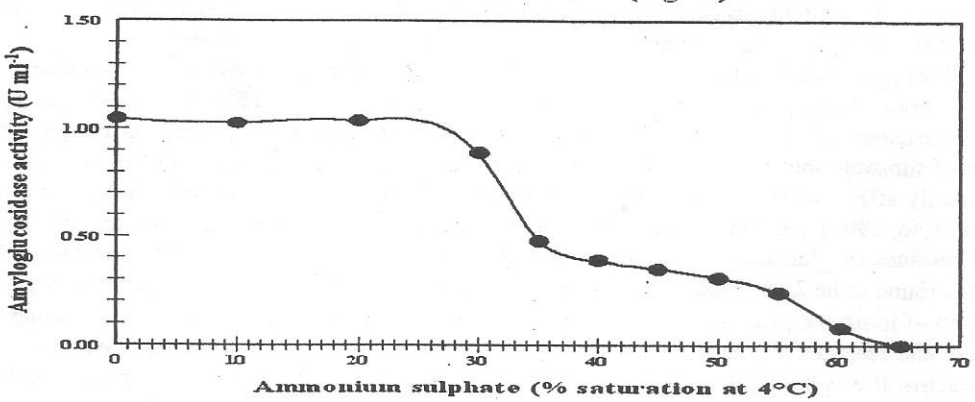
The optimum temperature of amyloglucosidase from *F. solani* toward soluble starch was found to be 40°C (**Fig. 3**). Temperature optima of 40- 45 °C have been reported for glucoamylase from *Aureobasidium pullulans* (Fortina *et al.*, 1993) and *Aspergillus niger* ATCC 1015 (Abou-Zeid, 1999). *Fusarium solani* amyloglucosidase incubated at 40°C in different buffers is active between pH 3.0 and 5.5, with highest activity at pH 4.5(**Fig. 4**).The change in pH affects the ionization of essential active site amino acid residues, which are involved in substrate binding and catalysis i.e. breakdown of substrate into products. The ionization of these residues may cause distortion of active site cleft and hence indirectly affect the enzyme activity. Similar types of pH optima are reported from *Aspergillus niger* ATCC 1015 (Abou-Zeid, 1999) and *Arachniotus* citrinus (Niaz *et al.*, 2004)The effect of pH on the ionization of essential amino acid residues of glucoamylase involved in ES*-complex formation were also determined. The pKa_1 of glucoamylase was found to be 2.45, while pKa_2 was 6.25.The ionization of non-essential residues affects the exact determination of p*K*a of ionizable groups of active site residues (Horton *et al.*, 1996).Therefore, p*K*a values of ionizable groups of active site residues of glucoamylase from *F.solani* were compared with the reported values of amino acid present in proteins. It was found that α-carboxyl may be involved in proton donation during catalysis and histidine might be present in the active site as proton receiver.

The K_m value determined from Lineweaver-Burk plots was 0.4 mg ml^{-1} (**Fig. 5**) at 40°C and pH 5.5. K_m values of 3.5 and 10.0 mg ml^{-1} for soluble starch have been reported for glucoamylases from *Aspergillus niger* –NCIM (Selvakumar *et al.*, 1996) and *Acremoniun* sp (Marlida *et al.*, 2000).

Thermostability is the ability of enzyme to resist against thermal unfolding in the absence of substrates, while thermophilicity is the capability of enzymes to work at elevated temperatures in the presence of substrate (Georis *et al.*, 2000). The thermal denaturation of enzymes is accompanied by the disruption of non-covalent linkages, including hydrophobic interactions with concomitant increase in the enthalpy of activation. The opening up of enzyme structure is accompanied by an increase in disorder, randomness or entropy of activation (Vieille and Zeikus, 1996). The protein melting temperature(T_m) of amyloglucoamylase from *Fusarium solani* was 67 °C. A melting temperature of 65 °C has been reported for glucoamylase from *Arachniotus* sp. (Iqbal *et al.*, 2003). The amyloglucosidase from *F.* solani was thermally stable at 50°C with a half- life of 74.53 min.However, at 56 °C it was not thermally stable and displayed a half life of 30 min (**Fig. 7**). Glucoamylase from *Arachniotus citrinus* (Niaz *et al.*, 2004) have been reported to be thermally very unstable because after heating only 7 min at 55°C, more than 50% of its activity was lost.







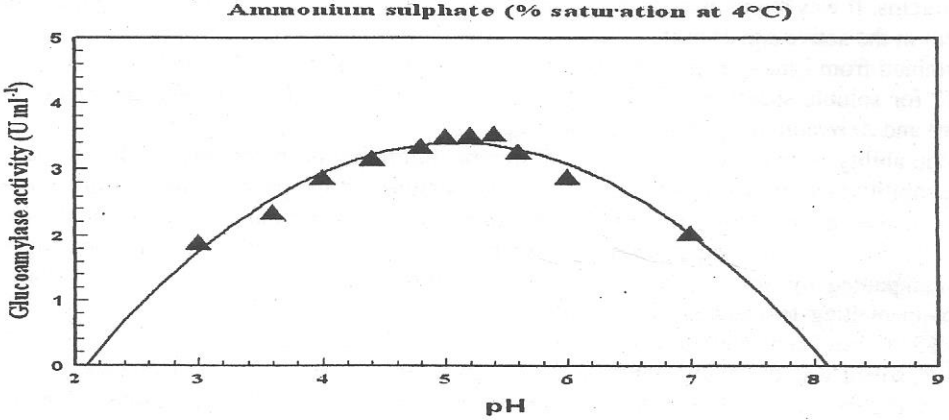


Fig. 4. Effect of pH on amyloglucosidase activity.

Fig. 1. Standard curve for protein estimation; Fig. 2. Ammonium sulphate precipitation of amyloglucosidase
Fig. 3. Effect of temperature on amyloglucosidase activity; Fig. 4. Effect of pH on amyloglucosidase activity.

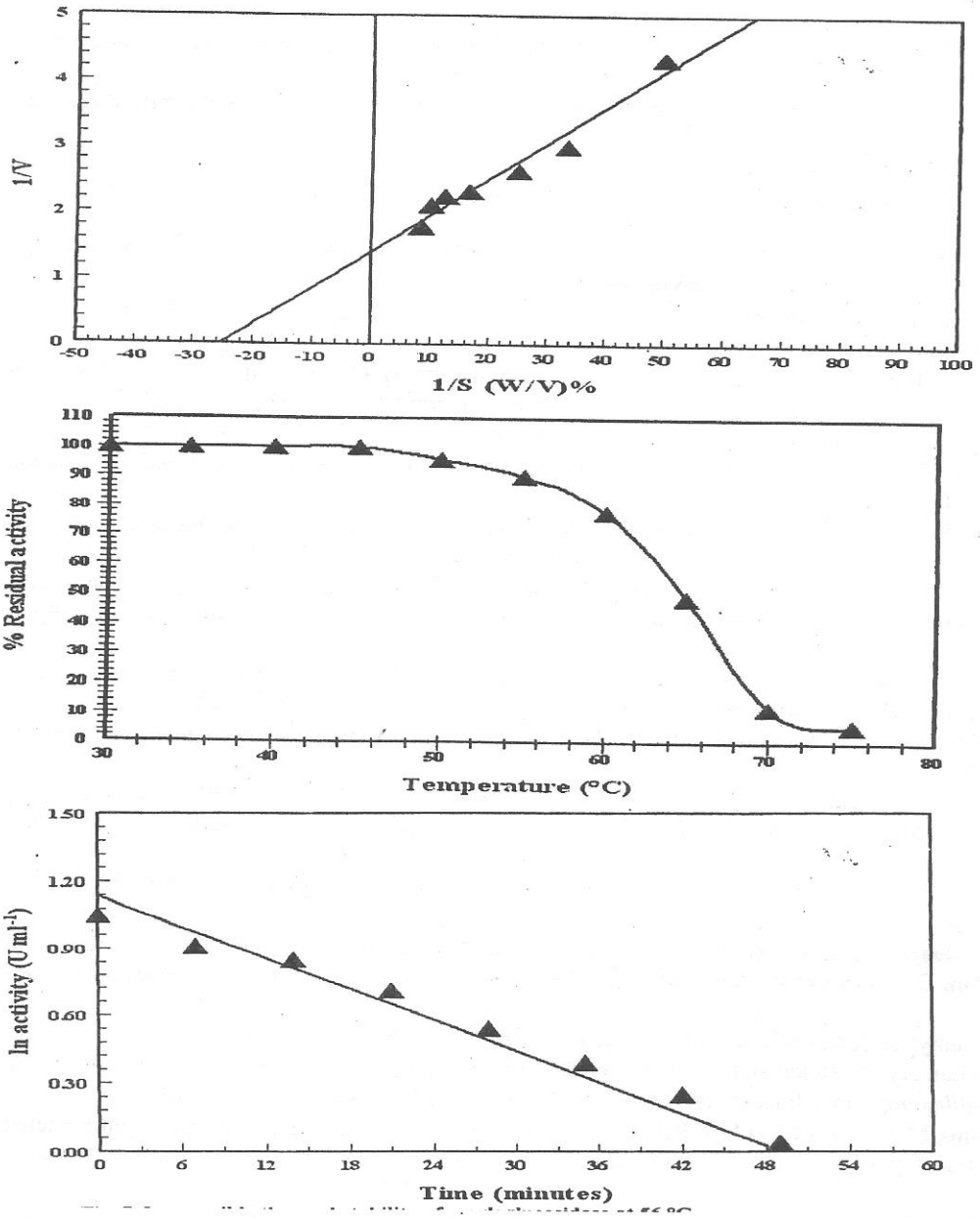


Fig. 5. Double reciprocal plot for the determination of Michaelis Menton constant for soluble starch hydrolysis.
Fig. 6. Thermal stability (%) of amyloglucosidase at different temperatures.
Fig .7. Irreversible thermal stability of amyloglucosidase at 56 °C.

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