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# Production and Characterization of Thermal Acid Amylase from *Aspergillus aculeatus* DBF9

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## ABSTRACT

Amylase was produced through submerged fermentation of *Aspergillus aculeatus* DBF9. Initial media pH of 6.0, incubation time of 48 h and an incubation temperature of 30°C were found optimum for amylase production. The enzyme was partially purified through acetone precipitation. Amylase activity was found highest at 50°C and at pH 5.5. The enzyme was stable within a temperature range of 30-40°C and pH 5.0-7.0. Among different metal ions studied, $Cu^{+2}$ showed maximum induction (47%) of enzyme activity. Significant inhibitory effect of EDTA (82%) on amylase activity was noticed. From SDS-PAGE and native protein gel electrophoresis only one type of amylase was found from *Aspergillus aculeatus* DBF9.

**Keywords:** amylase, *Aspergillus aculeatus*, characterization, filamentous fungi

## INTRODUCTION

The first enzyme produced industrially was an amylase from fungal source in 1894, which was used for the treatment of digestive disorders (Pandey *et al.* 2000). In the world enzyme market amylase is the second important enzyme (Ben *et al.* 2008) after proteases. Amylase is a hydrolysable enzyme and used by several plants, animals and microorganisms for different purposes (Peixoto *et al.* 2003). This enzyme is widely used in medicinal products, starch saccharification, manufacture of different foods, baking, brewing, detergent, textile, paper, and distilling industry (Ramachandran *et al.* 2004b). Due to its industrial importance several researchers tried to produce amylase from different sources. At present a lot of literature gathered in this enzyme research. Among all the sources microbial amylases are considered as best due to its different advantages like time savings, cost savings, etc. (Sivaramakrishnan *et al.* 2006). Amylase production is possible throughout the year using cheap starch-containing raw materials. Microbes generally produce large amount of amylase in a short period of time. Although several reported bacteria produces large amount of amylase but special interest given to filamentous fungi as they degrade starch more efficiently. From industrial point of view some limiting factors are considered critical for amylase production and these are poor stability, limited production, slow rate of starch degradation, and feedback inhibition by end product, etc (Pandey *et al*. 2000; Sivaramakrishnan *et al.* 2006). For solving such problems scientists already applied recombinant DNA technology in different organisms (Niu *et al.* 2009). But the promising way is to find new attractive wild microorganisms able to produce good quality amylase.

*Aspergillus* spp. are widely used in different industrial enzyme production. *Aspergillus aculeatus* DBF9 already reported for intracellular and extracellular tannase production (Banerjee *et al*. 2001). To date this strain was not applied for any other enzyme production. Here, for the first time we highlight the production and characterization of partially purified thermo stable acid amylase from *Aspergillus aculeatus* DBF9.

## MATERIALS AND METHODS

### Microorganism and production media

*Aspergillus aculeatus* DBF9 used in this work was previously isolated (Banerjee *et al.* 2001). The cultures were grown on modified Czepek's Dox broth (Thom and Raper 1945) containing 1% starch in place of sucrose at 30°C for 48 h. The organism was maintained and stored on this modified Czepek's Dox slants at 30 and 4°C, respectively.

### Effect of incubation time on amylase production

To find out the optimum incubation time period for amylase production, organism was grown for 120 h at 30°C, pH 5.5. Amylase produced was estimated at different time interval.

### Effect of initial media pH and incubation temperature on amylase production

To find out the optimum pH of the media for amylase production, the test organism was grown in different pH 4.0 to 8.0 at 30°C for 48 h. Optimum incubation temperature for amylase production was estimated after growing the organism at different incubation temperatures (20 to 35°C) at pH 5.5 for 48 h.

### Partial purification

Culture filtrate was used as the source of crude enzyme. A double volume of acetone was slowly mixed with crude enzyme at 4°C and kept for 24 h. Proteins that were precipitated are separated by centrifugation at 10,000 rpm at 4°C for 10 min. The separated proteins were dissolved in 0.5 ml of 0.1 M acetate buffer (pH 5.0) and stored at 4°C for further analysis.

### Amylase assay

The amylase activity was determined by modified method of Bernfeld (1955) and liberated reducing sugars were estimated by dinitrosalicylic acid (DNS) method (Miller 1959). The activity of α-amylase was assayed after incubating 0.3 ml enzyme with 0.5 ml soluble starch (1%, w/v) prepared in 0.05 M phosphate buffer,

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pH 5.0. After incubation at 50°C for 10 min the reaction was stopped and the reducing sugars released were assayed colorimetrically after addition of 1 ml of 3-5-dinitrosalicylic acid (Merck, India) reagent. An enzyme unit is defined as the amount of enzyme releasing 1 µmole of glucose from the substrate in 1 min at 50°C.

### Effect of pH on activity and stability of α-amylase

Effect of pH on the activity of α-amylase was measured after incubating 0.3 mL of enzyme and 0.5 ml of buffers, adjusted to pH of 5.0 to 8.0, containing soluble starch (1%). Stability of the enzyme at different pH values was also studied by incubating the enzyme at various pH values ranging from 5.0-8.0 for 24 h and then estimating the residual activity.

### Effect of temperature on activity and stability of α-amylase

The effect of temperature on the enzyme activity was determined by performing the standard assay procedure as mentioned earlier for 10 min at pH 5.5 within a temperature range of 30-70°C. Thermostability was determined by incubation of crude enzyme at temperatures ranging from 30-70°C for 2 h in a constant-temperature water bath. After treatment the residual enzyme activities were assayed as mentioned earlier.

### Effect of metal ions

The effect of different metal ions on amylase activity was determined by the addition of the corresponding ion at a final concentration of 1 mM to the reaction mixture, and assayed under standard conditions (Igarashi *et al.* 1988). The enzyme assay was carried out in the presence of $Ca^{2+}$, $Mg^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Hg^{2+}$ and $Cu^{2+}$. The effect of EDTA was also studied on amylase activity.

### Detection of amylase in native gel electrophoresis and SDS-PAGE

The enzyme was detected by native gel electrophoresis and SDS-PAGE. SDS-Polyacrylamide gel electrophoresis (12%) of partially purified alpha amylase was performed in accordance with the procedure of Laemmli *et al*. (1970). Alpha amylase activity was localized by running the enzyme in a native-PAGE using the method followed by Ramachandran *et al*. (2004a). The gel was immersed in soluble starch (1%) for 1 h at 25°C. The gel was then transferred and stained with iodine solution (0.05 g iodine and 0.5 g KI in 100 ml of distilled water) for 5 min. Protein molecular weight markers were used as standard (Genei, India).

### Experimental design and statistical analyses

Each experiment was done thrice and the average values were taken for analysis.

## RESULTS AND DISCUSSION

*A. aculeatus* DBF9 previously used for intracellular and extracellular tannase production (Banerjee *et al*. 2001). Here we have optimized amylase production from *A. aculeatus* DBF9 and characterized the partially purified enzyme.

Microbial enzyme synthesis is highly dependable to the pH of culture media. Amylase production by *A. aculeatus* DBF9 was studied at various initial media pH (4.0-8.0) and the results are presented in **Fig. 1**. Significant enzyme production by the organism was observed in pH range of 5.5 to 7.0. It was also observed that *A. aculeatus* DBF9 needs acidic pH for its amylase synthesis and in basic environment it was unable to synthesize amylase or the enzyme becomes unstable. The initial media pH for optimum amylase synthesis by *A. aculeatus* DBF9 was 6.0, whereas yeast strain BA3 (Fossi *et al.* 2005) and *Aspergillus ochraceus* (Nahas and Waldemarin 2002) have been reported to have their optimal media pH 5.0 for amylase synthesis.

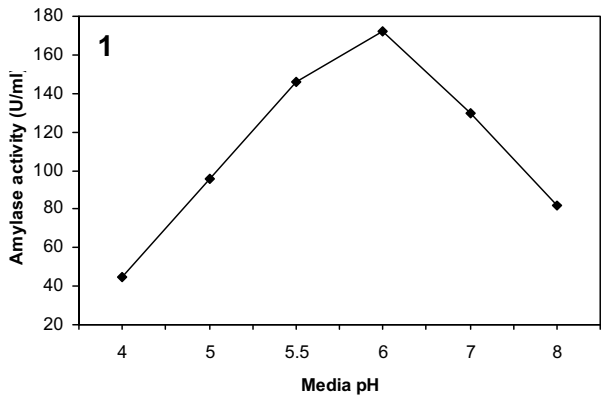
Enzyme production by *A. aculeatus* DBF9 was studied at various incubation temperatures (20-35°C) and result is presented in **Fig 2**. Among the different temperatures tried, the maximum enzyme production was observed at 30°C. With further increase in temperature amylase synthesis was found to decrease. Earlier *Aspergillus ochraceus* was reported to produce maximum amylase at 30°C (Nahas and Waldemarin 2002).

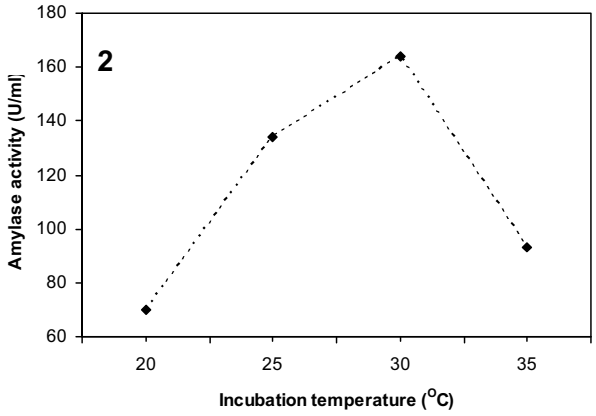
Amylase production by *A. aculeatus* DBF9 was recorded at different incubation time (24-120 h). Maximum amylase was synthesized at 48 h (**Fig. 3**). It was also noticed that the enzyme was stable in culture medium for a long time. Earlier maximum amylase production by *Penicillium* sp. was also reported after 48 h of incubation (Gouda and Elbahloul 2008).

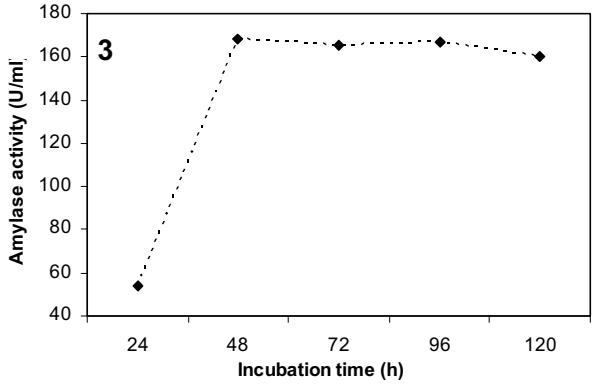
Extracellular amylase produced by *A. aculeatus* DBF9 in liquid submerged fermentation was partially purified through acetone precipitation and different properties of the enzyme was studied. The effect of pH on the enzyme activity depends on the nature of amino acids at the active side which undergo protonation or deprotonation (Dixon and Webbe 1996). The optimum pH for amylase activity was found 5.5 (**Fig. 4**) in *A. aculeatus* DBF9. A decline in the reaction velocity was recorded on either side of pH 5.5. Earlier such type of result was also reported with the amylase from *A. tamari* (Moreira *et al.* 1999). Amylase from *A. aculeatus* DBF9 was stable (above 90%) over a broad range of pH, between 4.0-7.0 (**Fig. 4**). It was reported (Michelin *et al.* 2008) that amylase produced by *Paecilomyces variotii* was stable in alkaline pH. This isolated amylase from A. *aculeatus* was stable in acidic pH and showed its maximum catalytic activity in acidic range, hence characterized as acid amylase. Previously Prakasham *et al*. (2007) reported enhanced production of acid amylase from *Aspergillus awamori*.

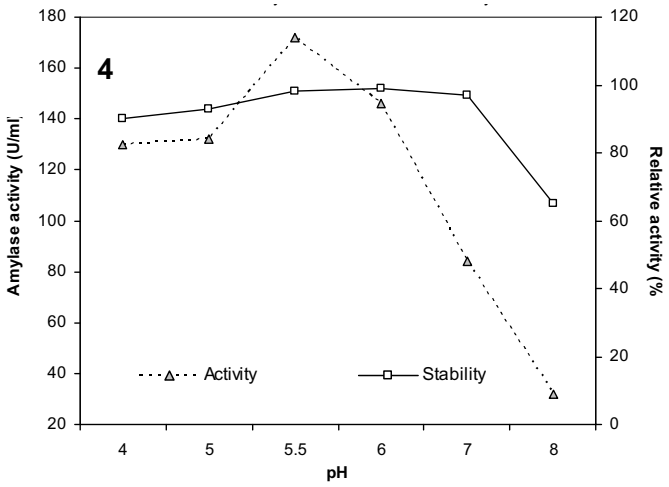
It was noticed that there was an increase in amylase activity with the increase in temperature up to 50°C (**Fig. 5**). With increase in incubation temperature above 50°C amylase activity was decreased. With increase in temperature, the kinetic energy of the substrate and enzyme molecules also increases which affects the reaction rate. The number of collisions per unit time of amylase activity and its substrate, starch increases, resulting in a higher activity with the continuous increases in the temperature level. When the optimum level of temperature obtained, the energy of the molecules increased throughout the process, but the chemical potential energy increases enough, some of the weak bonds determining the three dimensional structure of the active enzymes break leading to thermal denaturation of enzyme causing its inactivation (Natarajan and Rajendran 2009). In our experiment it has been found that amylase from *A. aculeatus* DBF9 showed highest hydrolyzing effect at 50°C (**Fig. 5**). Enzymes with high temperature optima are preferred for industrial applications (Vieille *et al.* 1996). Previously optimum amylase activity at 60°C was reported from amylase produced by thermophilic fungus *Scytalidium thermophilum* (Aquino *et al.* 2003). The enzyme remains stable at a temperature range between 30 to 40°C, above which the stability declined (**Fig. 6**). This enzyme stability trend is comparable to the behavior of amylase from *A. terreus* NA-170 mutant (Ghosh *et al.* 1991), *Rhizopus niveus* and *A. niger* (Pazur *et al.* 1990).

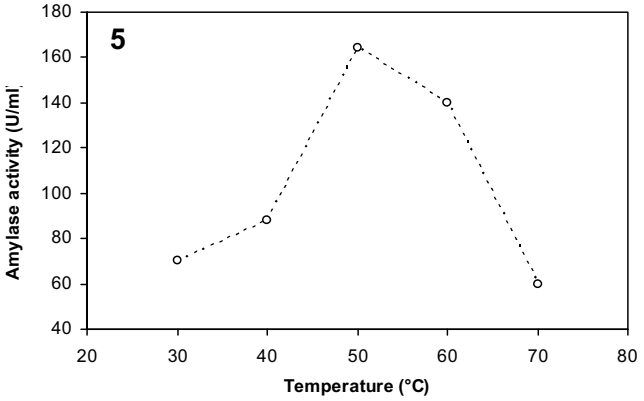
Study on the effect of metal ions is a very simple technique but plays a great role in enzyme research, as it produce significant information about general structure of that enzyme and also produces important information for achieving maximal catalytic efficacy in related industries. In the present study there was a strong positive effect of metal ions on amylase activity (**Fig. 7**). All the tested metal ions showed induction of amylase activity in comparison with control. Among the tested metal ions $Cu^{+2}$ and $Mn^{+2}$ showed about 47 and 22% amylase activity induction respectively. Earlier Odibo *et al.* (2006) and Aquino *et al.* (2003) reported strong negative effect on amylase activity by $Hg^{+2}$ and $Cu^{+2}$, whereas, strong increases in amylase activity was noticed in presence of $Na^{+}$ and $Ca^{+2}$ respectively. Effect of metal chelator, EDTA on amylase activity was also

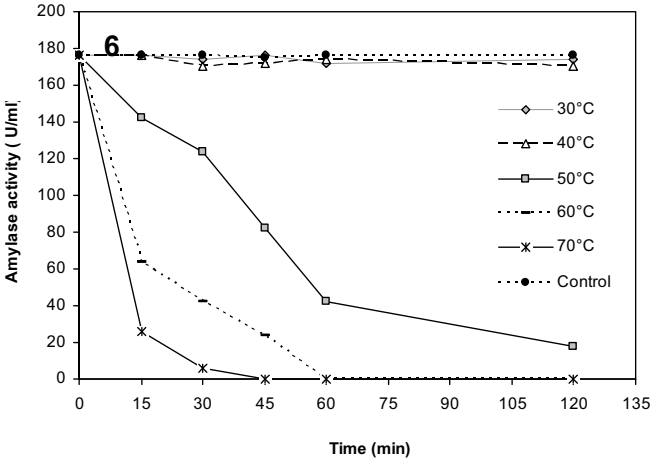


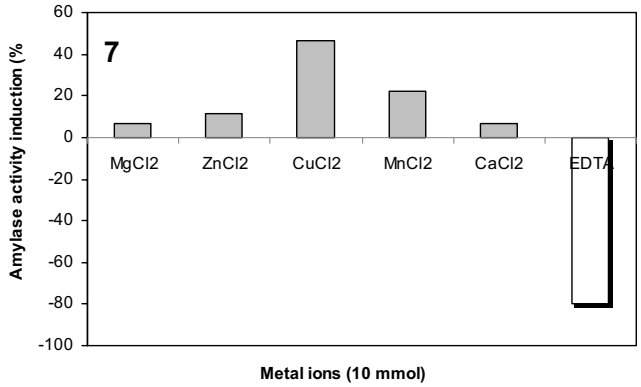



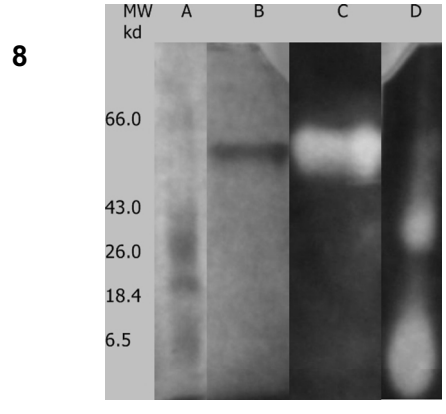




**Fig. 1 Effect of initial media pH on amylase production by *Aspergillus aculeatus* DBF9.**
**Fig. 2 Effect of different incubation temperature on amylase production.**
**Fig. 3 Effect of incubation time on amylase production.**
**Fig. 4 Effect of pH on activity and stability of partially purified amylase.**
**Fig. 5 Effect of incubation temperature on activity of partially purified amylase.**
**Fig. 6 Effect of temperature on stability of partially purified amylase.**
**Fig. 7 Effect of different metal ions and EDTA on activity of partially purified amylase.**
**Fig. 8 Native gel electrophoresis and SDS-PAGE of partially purified amylase.** (**A**) Molecular weight marker proteins; (**B**) amylase from *A. aculeatus* DBF9 (SDS); (**C**) amylase from *A. aculeatus* DBF9 (native); (**D**) commercial amylase from Hi-Media (native).

assessed and it was noticed that there was significant inhibitory effect of EDTA (**Fig. 7**). About 80% inhibition of amylase activity was noticed in presence of EDTA. Previously Kim *et al.* (1995) reported a *Bacillus* sp. that produces amylase which is activated in the presence of 1 mM $Ca^{2+}$, $Mg^{2+}$ $Cu^{2+}$, $Co^{2+}$, or $Ag^{+}$ by 120 to 154% but, the addition of $Zn^{2+}$ or $Fe^{2+}$ does not significantly affect the enzyme activity. Ben *et al.* (2008) reported, metal ions such as $Cu^{2+}$, $Mn^{2+}$, $Ba^{2+}$ decreased the amylase activity and a slightly positive effect was found with $Ca^{2+}$, $Zn^{2+}$, and $Mg^{2+}$ on amylase of *S. sclerotiorum*. Li *et al*. (2007) stated that amylase from marine yeast *Aureobasidium pullulans* was activated by $Ca^{+2}$, $Cu^{+2}$, $Ba^{+2}$, $Na^{+}$, $Mg^{+2}$, $Co^{+2}$. However, when the amylase was incubated with 1 mM EDTA, the enzyme activity dropped to 18% of its initial activity (82% inhibition).

Native gel electrophoresis of alpha amylase showed only one pale yellow band (molecular weight about 60 kd) in the dark colored gel, which confirmed the enzyme activity (**Fig. 8**) and presence of only one type of amylase. SDS-PAGE of partially purified enzyme also showed the presence of only one type of protein. The molecular mass of some previously reported fungal amylases are *Sclerotinia sclerotiorum* 72 kDa (Martel *et al.* 2002), *Aspergillus* sp. 56 kDa, *A. oryzae* 50 kDa, *A. fumigatus* 65 kDa, and *A. flavus* 75 kDa (Khoo *et al.* 1994). Previously, Ramachandran *et al.* (2004a) reported about the activity staining of amylase when they were studied on the α-amylase produced in the solid state fermentation using *A. oryzae*.

## CONCLUSION

In this communication we have reported the production and characterization of thermo stable acid amylase from *Aspergillus aculeatus* DBF9. The organism produces a large amount of α-amylase in a short period of time, so it can be utilized for large scale production of this enzyme.

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