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ORIGINAL PAPER

Production and Characterization of α-Amylase from *Aspergillus niger* JGI 24 Isolated in Bangalore

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Abstract

Five fungal isolates were screened for the production of α-amylase using both solid-state and submerged fermentations. The best amylase producer among them, *Aspergillus niger* JGI 24, was selected for enzyme production by solid-state fermentation (SSF) on wheat bran. Different carbon and nitrogen supplements were used to enhance enzyme production and maximum amount of enzyme was obtained when SSF was carried out with soluble starch and beef extract (1% each) as supplements. Further attempts to enhance enzyme production by UV induced mutagenesis were carried out. Survival rate decreased with increase in duration of UV exposure. Partial purification of the enzyme using ammonium sulphate fractionation resulted in 1.49 fold increase in the enzyme activity. The enzyme showed a molecular weight of 43 kDa by SDS-PAGE. Metal ions Ca^{2+} and Co^{2+} increased the enzyme activity. The enzyme was optimally active at 30°C and pH 9.5.

Key words: *Aspergillus niger* JGI 24, α-amylase, mineral salt medium, solid-state fermentation, wheat bran

Introduction

Recent discoveries on the use of microorganisms as sources of industrially relevant enzymes have led to an increased interest in the application of microbial enzymes in various industrial processes. Amylases catalyze the hydrolysis of glycosidic linkages of starch components and glycogen molecules. They are important enzymes employed in the starch processing industries for hydrolysis of starch into simple sugars (Alva *et al.*, 2007). Amylases are widely distributed in plants, animals and microorganisms which show varying action patterns depending on the source (Pandey *et al.*, 2000, Saboury, 2002). However, amylases from microbial sources, especially fungi (*Aspergillus* spp.), have gained much attention because of the availability and high productivity of fungi, which are also amenable to genetic manipulation. Fungi are involved in a variety of industries ranging from food, chemical, detergent, textile and paper industries (Moreira *et al.*, 1999, Moreira *et al.*, 2001, Kathiresan and Manivannan, 2006).

From the 1940s, industries have focused on the production of fungal enzymes and secondary metabolites through fungal cultivation under submerged conditions, which allow automation of process parameters necessary for optimum growth. An alternative method for fungal cultivation is solid state fermentation (SSF), where the fungus is grown on a moist solid substrate. For specific applications, SSF offers improved yields and product spectra compared to submerged fermentation (Moreira *et al.*, 2001, Pandey, 2003). This procedure has been employed to produce amylases. The hyphal mode of growth and good tolerance to low water activity and high osmotic conditions make fungi most efficient for bioconversion of solid substrates (Raimbault, 1998).

Present study was taken up to isolate fungi from air, soil and different seeds, and to screen them for α-amylase production and selection of best isolate. The selected strain was studied for optimization of growth conditions for production of α-amylase. α-amylase from the selected strain was partially purified and characterized.

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Experimental

Materials and Methods

Isolation of fungi. The fungi were isolated by 3 methods: (1) Blotter method in which several species of *Aspergillus* were isolated from different seeds (paddy, ragi, bengal gram, greengram, blackgram, wheat, blackgram, sesame, kabul chana, cowpea, tur dal, beans, cluster beans, urad dal, masur dal) from various locations by Standard Blotter Method (Baki and Anderson, 1973) onto Czapek-Dox agar plates supplemented with streptomycin. They were identified on the basis of morphological characteristics, maintained in slants (Potato dextrose agar) and stored at 4°C. (2) Air – by exposure plate method in which fungi were isolated by settle plate technique from different locations (*viz*., laboratory, three different hotels, and drains of Bangalore) as per Aneja (2002). (3) Soil and potato – by serial dilution method in which isolates of fungi were obtained by baiting and serial dilution as per Abe *et al.* (1988).

Screening of isolates. Primary screening of isolates was carried out by starch agar plate method (Aneja, 2002).

Substrate. Wheat bran procured from Bangalore local market served as the substrate. The substrate was dried and ground into coarse powder with a blender.

Inoculum preparation. Spore suspension was prepared by mixing seven loopful of fungal spores in 10 ml of sterile distilled water. A uniform spore suspension was obtained by mixing vigorously, which was measured for absorbance under white light. One milliliter of spore suspension showing OD 0.8 ($\sim 7 \times 10^5$ spores/ml) was used as inoculum.

Medium composition. Submerged fermentation: wheat bran (100 g) was boiled and filtered, to which a mineral salt composition of $ZnSO_4 \times 7H_2O$ (6.2 mg), $FeSO_4 \times 7H_2O$ (6.8 mg) and $CuSO_4 \times 7H_2O$ (0.8 mg) was added and volume was made up to one liter with distilled water. pH was adjusted to 4.5. This is the Mineral Salt Medium (MSM). One milliliter of spore suspension was used as inoculum. Fermentation was carried out for four days.

Solid substrate fermentation: production medium contained 5 g of wheat bran and 8 ml of mineral salt medium (MSM), to adjust the moisture content from 43 to 81% in 250 ml Erlenmeyer flasks. Fermentation was carried out for four days (96 h). One milliliter of fungal spore suspension was used as inoculum for Erlenmeyer flasks of 250 ml capacity.

Optimization of culture conditions for enzyme production. Effect of temperature, pH, carbon sources and nitrogen sources: present study was carried out at different temperatures (22, 37 and 40°C) and pH (4.5, 6.0, 7.5 and 9). Different sources of carbon (1% each of glucose, maltose, sucrose and soluble starch) and nitrogen (1% each of beef extract, meat extract, casein and urea) were used to determine their effect on amylase production.

Enzyme extraction. From solid substrate fermentation – 22 ml of 0.1 M phosphate buffer (pH 6.5) was added to the culture flasks and mixed well for 30 min in a rotary shaker (150 rpm) at room temperature (22 ± 2^oC) for 30 min. The mixture was filtered through cheesecloth and centrifuged at 8,000 rpm for 15 min. Supernatant was filtered through Whatmann no 1 filter paper and filtrate was used as enzyme source.

From submerged fermentation – after 96 h, the fermented broth was centrifuged at 8,000 rpm for 15 min. Supernatant was filtered through Whatman no 1 filter paper and filtrate was used as the enzyme source.

Enzyme assay. Amylase activity was estimated by the analysis of reducing sugar released during hydrolysis of 1% (w/v) starch in 0.1 M phosphate buffer, pH 6.5, at 25°C for 20 min by the dinitro-salicylic acid (DNS) method (Miller, 1959). One unit of amylase activity was defined as the amount of enzyme that releases 1 mmol of reducing sugar as glucose per min under assay conditions. Enzyme activity is expressed as the specific activity, which is represented as U/mg of protein. The experiments were carried out in triplicates and standard deviations were calculated.

Protein estimation. Protein content of the enzyme extracts was estimated by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard. Enzyme activity is expressed as specific activity, which is equivalent to U/mg protein. All experiments were carried out in triplicates and the standard deviations were calculated.

Mutagenesis. Five test tubes with fungal spore suspension (with one control kept under dark conditions) were exposed to UV radiation (2600 Å) for varying time periods of 5, 10, 15 and 20 min. They were kept in dark (12 h duration) for stabilization of thymine-thymine (T-T) dimmers after exposure to UV radiation. The fungal spore suspensions were then used as inoculum for enzyme production by solid substrate fermentations. After four days of fermentation, enzymes were extracted and their activity assayed. 0.1 ml of the UV-treated fungal spore suspensions was then inoculated onto petri dishes containing 25 ml of Czapek-Dox medium. The above fungal preparations were used for the analysis of percentage survival of the organisms after UV irradiation (Saha, and Bhattacharyya, 1990)

Enzyme characterization. Amylase purification and molecular mass determination – amylase was purified by ammonium sulphate (40%) precipitation method (Young *et al.*, 1995). The precipitate obtained by centrifugation was dissolved in 0.1 M phosphate

buffer (pH 7) and dialyzed overnight against 0.01 M phosphate buffer. The experiment was carried out under low temperature (4°C) to prevent enzyme denaturation. The samples thus obtained were subjected to polyacrylamide gel electrophoresis (PAGE) for molecular weight determination. Phosphorylase *b* (97 kDa), albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa) and lysozyme (14.3 kDa) were used as molecular mass markers.

Enzyme activity was estimated in the purified enzyme samples and protein content estimated by the method of Lowry *et al.* (1951). Purified enzymes were subjected to activity staining. Procedures for sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) and peptide mapping (Andrews, 1990) were described by Hames (1990). Nondenaturing gel electrophoresis (PAGE) for activity staining was performed in a 7.5% polyacrylamide gel containing 0.5 M Tris-HCl (pH 9.1) at 4°C. To identify the location of amylase activity (clear zones on a blue background), the gels were incubated for 1 h at 30°C in 2% soluble starch in 0.2 M phosphate buffer (pH 6.5) and stained with an acidic iodine solution (0.2% I_2 and 2% KI in 0.2N HCl). Molecular weight determination by SDS PAGE was determined with a mixture of standards from Bangalore Genei Ltd., catalog no PMWM 105979 (Shih and Labbe, 1995).

Factors affecting enzyme activity. Temperature: optimum temperature for enzyme activity was determined by incubating the enzyme substrate reaction mixture at different temperatures for 20 minutes and then assaying the enzyme activity.

Thermostability of amylase: thermal stability of α-amylase was determined by incubating the enzyme without the substrate fractions at various temperatures between 30 and 60°C for 1 h. At 10 min intervals, aliquots of 1 ml of the incubated enzyme were assayed for activity.

pH: the effect of pH on amylase activity was determined by incubating the reaction mixture with different buffers of 0.1 molarity (pH 3.5 – Citrate buffer; pH 5.5, 6.8, and 7.5 – Phosphate buffer; pH 9.5 – Tris-HCl buffer).

Effect of metal ions: enzyme assays were performed using 10 mM concentration of various metal ions (Na^+, Mg^{2+}, Ca^{2+}, Co^{2+}). The chloride salts of these metal ions were used (NaCl, $MgCl_2$, $CaCl_2$, $CoCl_2$). The amylase activity was measured at pH 6.8 and room temperature (22 ± 2^oC) in the presence of these metal ions at 10 mM concentrations. The relative activity of the enzyme was compared with the activity obtained using 0.1 M phosphate buffer.

Results and Discussion

Strain selection. Different fungal isolates were tested for amylase production by starch hydrolysis test. When starch agar medium is point inoculated with the organism and subsequently flooded with iodine solution, production of amylase is indicated by the zone of clearance around the microbial growth. On the basis of the area of clearance, five out of twenty fungal isolates were selected for further studies on α-amylase production.

Enzyme production. Comparison of SSF and SmF – of the five fungi subjected to solid state and submerged fermentations, *A. niger* isolate JGI 24 was found to be the best amylase producer with values 74 and 58.06 IU/mg protein for solid-state and submerged fermentations respectively. This value is much higher than that reported by Haq *et al.* (2005) for their isolate of *A. niger* GCB-34. So, this potential strain was selected for further optimization of culture conditions.

Effect of pH – for the selected fungal strain, *A. niger* isolate JGI 24, (Fig. 1), best enzyme production was observed at pH 7.5 by solid-state fermentation.

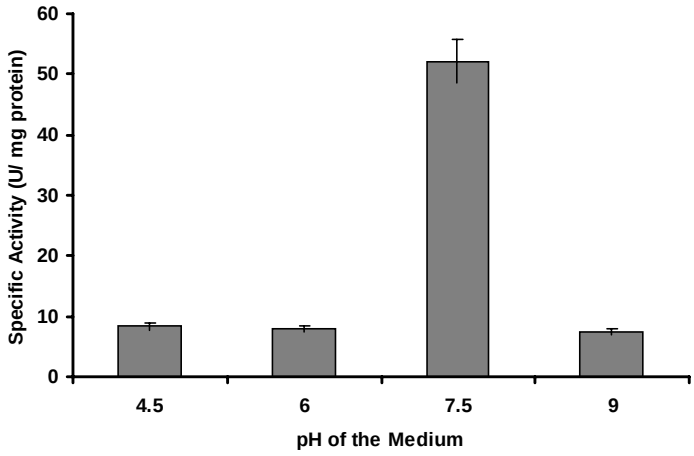

Fig. 1. The effect of pH on fungal amylase production

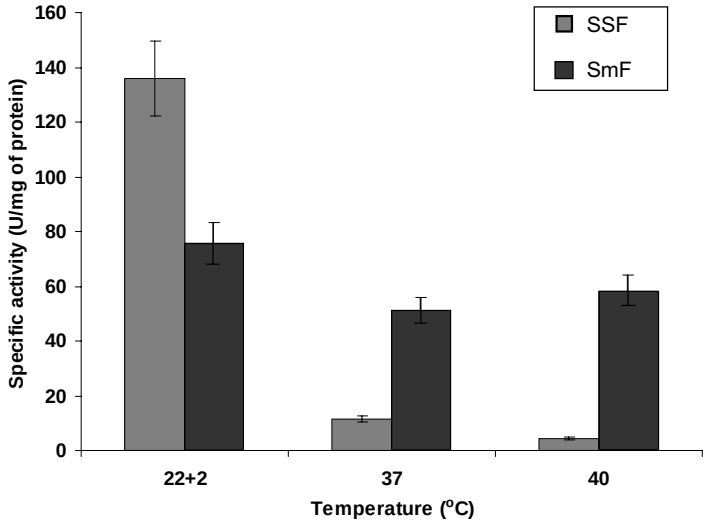

Fig. 2. The effect of temperature on fungal amylase production

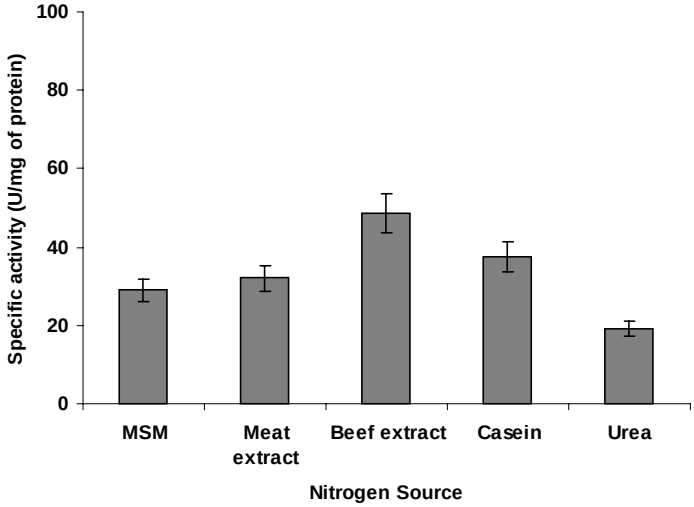

Fig. 3. The effect of nitrogen source on fungal amylase production

Effect of temperature – when incubated at different temperatures, room temperature (22 ± 2ºC) was found to be the best for enzyme production both in solid and submerged fermentations (Fig. 2). Kathiresan and Manivannan (2006), reported 30°C to be the best for enzyme production by *Penicillium fellutanum*. Enzyme production in solid state is greatly affected by temperature, whereas under submerged condition, no significant changes were observed.

Effect of nitrogen source – addition of organic nitrogen sources such as casein, meat extract and beef extract to the medium resulted in a considerable increase in the production of α-amylase compared to control (27.75 U/mg protein). Media supplemented with beef extract showed maximum amylase activity compared to meat extract and casein. Addition of urea to the medium resulted in reduced enzyme production (Fig. 3).

Effect of carbon source – glucose and sucrose supplementation resulted in the repression of enzyme production. Similar results of catabolite repression of enzyme production by glucose, has been reported by Nandakumar *et al.* (1999) for *A. niger* CFTRI 1105 and Alva *et al.* (2007) for *Aspergillus* sp. JGI 12. Our studies showed that soluble starch (40.43 U/mg of protein) and maltose (33.34 U/mg of protein), significantly increased enzyme production when compared to the control (Fig. 4).

Effect of UV-induced mutation – studies on enzyme production by *A. niger* JGI 24 showed that increased time of UV exposure resulted in strains with decreased α-amylase production. On 20 min exposure to UV, enzyme activity was found to be zero.

Survival rate. Fungal survival rate decreased with increase in duration of exposure to UV light. Five minutes of UV exposure resulted in 70% sur-

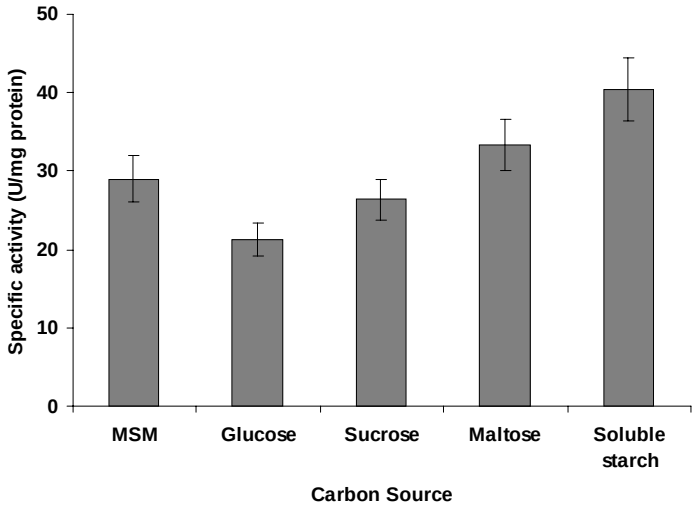

Fig. 4. The effect of carbon source on fungal amylase production

vival and 20 min exposure resulted in the survival of only 20% of the fungi.

Enzyme characterization. Enzyme extracted from the fungus was subjected to characterization studies like the effect of pH, temperature and metal ions. The enzyme was partially purified by ammonium sulphate precipitation, dialised and subjected to SDS-PAGE for molecular weight determination (Fig. 5). The enzymes were run on native gel and amylase activity staining was carried out. From the gel, it could be predicted that the molecular weight of fungal amylase was ~43 kDa.

Ammonium sulphate precipitation and partial purification – fungal amylases were partially purified by ammonium sulphate precipitation. The purity of the enzyme was 1.49 fold greater than the crude enzyme.

Effect of temperature – the enzyme showed optimum activity at 30°C and as temperature increased from 30–70, the activity of the enzyme showed a declining trend showing its least activity at 70°C (Fig. 6).

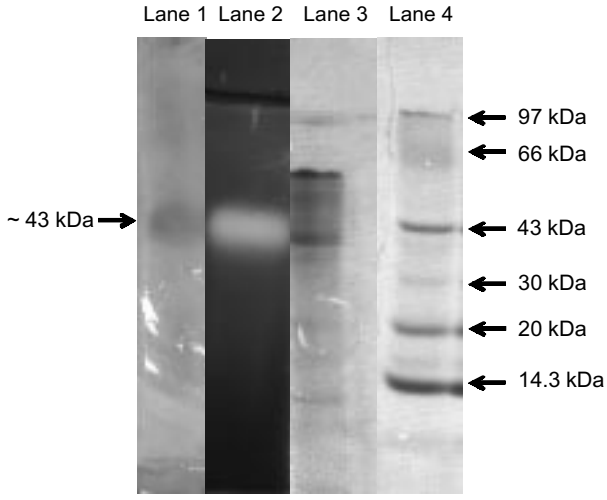

Fig. 5. SDS PAGE of the partially purified fungal amylase. Amylase was visualized by coomassie brilliant blue staining (lane 1 and 3) and by activity staining (lane 2), lane 4, molecular mass markers.

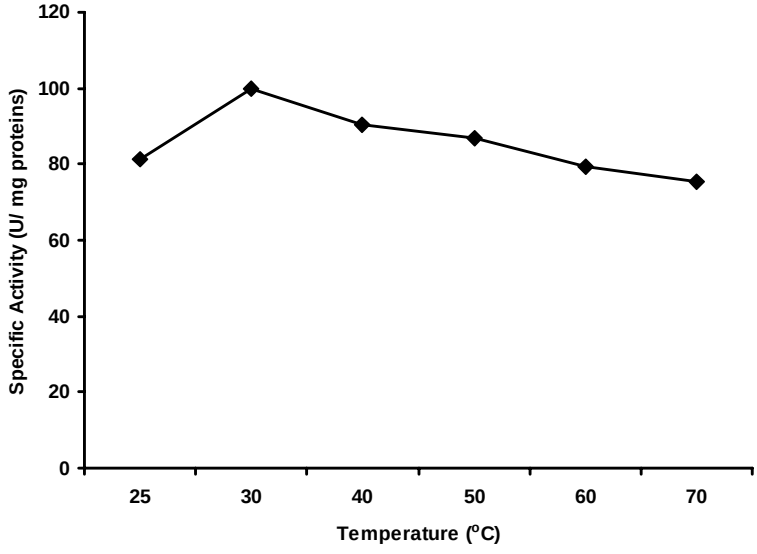

Fig. 6. The effect of temperature on amylase activity

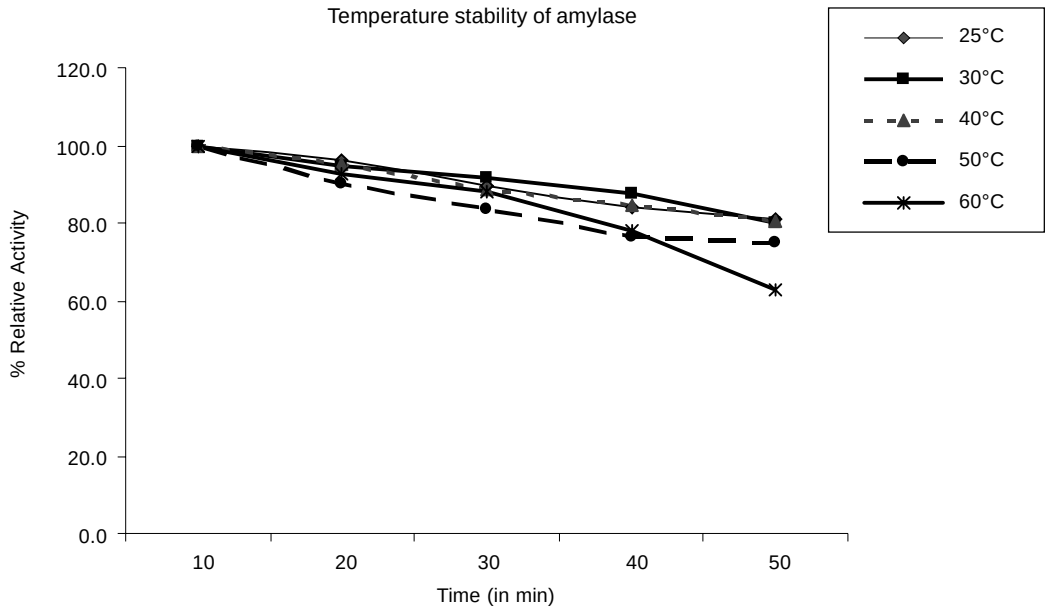

Fig. 7. The effect of temperature on fungal amylase stability

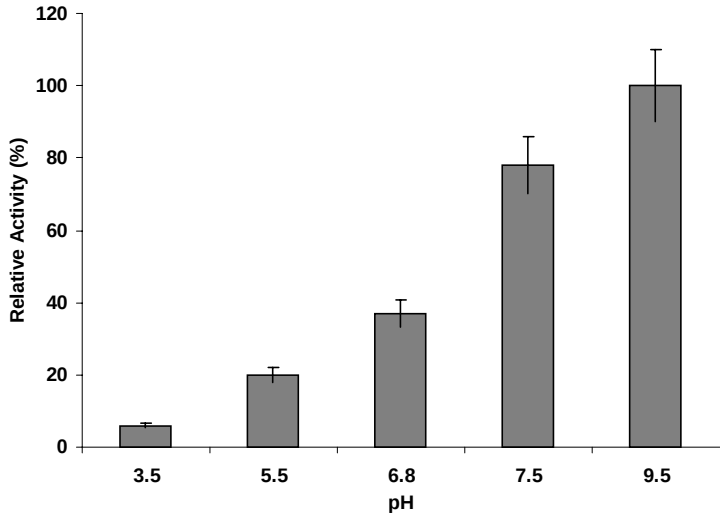

Fig. 8. The effect of pH on fungal amylase activity

Enzyme stability – the enzyme showed highest stability at 30°C and the least stability at 60°C retaining only 60% of its original activity at 30°C (Fig. 7). Similar results were reported from our earlier studies (Alva *et al.*, 2007) on *Aspergillus* sp. JGI 12.

Effect of pH – different buffers of pH 3.5, 5.5, 6.8, 7.5 and 9.5 were used to study the effect of pH on enzyme activity, of which pH 9.5 resulted in maximum enzyme activity of 75 U/mg of protein (Fig. 8). This property of the enzyme can be exploited for its industrial application, *i.e.*, in detergents. Others have reported acidic pH optima for amylases from *A. niger* (Harnandez *et al*., 2006; Mitidieri *et al*., 2006; Uguru *et al*., 1997). Bacterial amylases having alkaline pH optima were reported by different workers (Fogarty and Kelly, 1979; Yamamoto *et al.*, 1972).

Effect of metal ions – after analysis of enzyme activity to be carried in the presence of different metal ions, it was found that enzyme activities were enhanced by calcium on the contrary to the results of Reyed (2007), where calcium slightly inhibited the amylase activity. Asgher *et al.* (2007) reported that Ca^{2+} had no effect on enzyme activity. Magnesium and sodium ions inhibited the enzyme activity (Fig. 9). Similar observation that sodium inhibited enzyme activity was made by Reyed (2007).

Conclusions. Isolation of fungi from seeds, soil and air and their rapid screening by plating on starch agar plates, led to the finding of five fungal strains capable of amylase production. These strains were compared for amylase activity in solid state and submerged fermentations, and a strain of fungus identi-

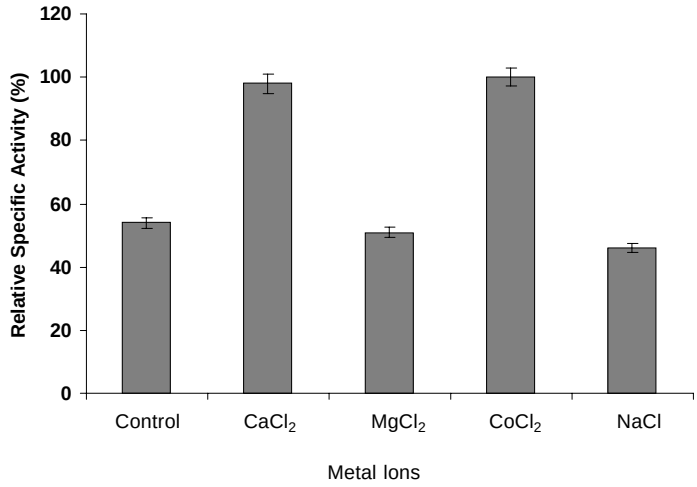

Fig. 9. The effect of metal ions on fungal amylase activity

fied as *A. niger* isolate JGI 24 was found to be the best producer of the enzyme.

The enzyme activity of *A. niger* isolate JGI 24 was also found to be higher than that reported by earlier workers on different *A. niger* isolates (Haq *et al.*, 2005).

This strain was subjected to further studies. The enzyme was very sensitive to pH. Therefore, the selection of optimum pH is essential for the production of α- and gluco-amylases. It was observed that pH 7.5 was best for enzyme production by solid state fermentation and pH 9.5 was better for enzyme activity.

Room temperature (22 ± 2°C) was found to be the best for enzyme production. The optimum temperature for enzyme activity was 30°C. The enzyme was stable at 30°C even after 60 min of incubation. As the temperature increased to 50 and 60°C, the stability of the enzyme was affected as indicated by a lower enzyme activity.

The molecular weight of the enzyme was found to be ~43 kDa.

Thus, the strain of *A. niger* isolate JGI 24 was found to be the best producer of α-amylase. The alkaline pH optima make it suitable for industrial production. The strain is maintained in the laboratory for further exploration regarding industrial applications.

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