ORIGINAL SCIENTIFIC PAPER

Purification and Biochemical Characterization of pH Tolerant and Acid Stable α-amylase from *Aspergillus oryzae* JGI 21 Isolated from Soil

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Summary

This paper describes the purification and characterization of a novel acid stable and pH tolerant α-amylase from a Aspergillusoryzae JGI 21 isolated from Mangalore. The enzyme displayed a molecular weight of 22 kDa and it was stable over a broad range of acidic and alkaline pH with maximum activity and stability at 6.5. The optimum temperature of enzyme stability was found to be around 24+/-2◦C. The purification of α-amylase by ammonium sulphate precipitation and ion-exchange chromatography resulted in 23.56 fold increase in its activity (100.38 U/mg protein). Considering its promising properties, this enzyme can find potential applications in the food industry as well as in laundry detergents.

Key words: *Aspergillus oryzae JGI 21, acidophilic α-amylase, pH stability, purification, solid-state fermentation*

Introduction

α-Amylases (E.C. 3.2.1.1.) are starch-degrading enzymes that catalyze the hydrolysis of internal α-1, 4-O- glycosidic bonds in polysaccharides with the retention of α-anomeric configuration in the products. Amylases have been reported to occur in microorganisms, although they are also found in plants and animals.α-amylase is a keyenzyme in the production of starch derivatives and also widely used in food, textile, paper, detergent, clinical, pharmaceutical and other industrial fields (Bhat 2000; Fogarty and Kelly, 1990; Kandra, 2003; Rozell, 1999). The enzymatic hydrolysis is preferred to acid hydrolysis in starch processing industry due to a number of advantages such as specificity of the reaction, stability of the generated products, lower energy requirements and elimination of neutralization steps (Cha *et al*., 1998). Due to the increasing demand for these enzymes in various industries, there is enormous interest in developing enzymes with better properties such as raw starch degrading amylases suitable for industrial applications and their cost effective production techniques. It has so far beenreported that fungi and yeast, such as Aspergillus sp., *Rhizopussp*. and *Cryptococcus* sp., are good producers ofraw starch-digesting amylases, capable of acting at low pH andhigh temperatures (Hayashida and Teramoto,1986; Liu and Xu, 2008; Shih and Labbe 1995).

The present study, reports on the purification and characterizationof an acidicand pH stable α-amylase produced by *Aspergillus oryzae* which was isolated from the soil from a paddy fieldin rural Mangalore. The properties of the enzyme revealed that it can be considered a novel α-amylase.

MATERIALS AND METHODS

Isolation of Fungi

Several species of *Aspergillus* were isolated from different seeds like, paddy, ragi, Bengal gram, green gram, blackgram, wheat, sesame and Kabulchana from various locations by Standard Blotter Method (Baki and Anderson, 1973). The seeds were placed on a moist filter paper disc (9 cm diameter) in the Petridishes. After incubation at room temperature (24+/-2° C), the Petridishes were observed for the growth of different fungi. Fungi were also isolated from soil collected from various locations by serial dilution method.

Screening and Identification of the Isolate

Primary screening was done by starch agar plate method. Plates containing modified Starch agar, containing 2% starch as the sole source of carbon were prepared and spot inoculated with the isolates. The plates were incubated for 4-5 days. The starch hydrolysis test was performed by adding few drops of freshly prepared iodine into the plate after the incubation period. Blue black color was observed due to the formation of starch-iodine complex and a zone of clearance was observed around the cultures which were producing amylase.

Microscopy

The isolated fungi were identified with the help of the Microbiologist Prof. SB Sullia (Adjunct Professor, Jain University), based on colony characteristics and also by lactophenol cotton blue staining and observation under a Trinocular Research Microscope (Labomed, India). Pure cultures were deposited in the Culture Collection Centre of Jain University.

Substrates used

Wheat bran was procured from Bangalore local market. The substrate was dried and grounded into coarse powder with a blender. Production media contained 5 g of solid substrate and 8 mL of sterile distilled water was used to adjust the moisture content in 250 mL Erlenmeyer flasks (Ajayi and Fagade, 2003). Fermentation was carried out for four days at room

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temperature.For production of enzymes in solid-state fermentation (SSF), 1 mL of fungal spore suspension was used as the inoculum.

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. The optical density (OD) of the spore suspension wasmeasured at 660 nm. One milliliter of the spore suspension showing 0.8 OD which contains $7x10^5$ spores/ml was used as the inoculum. Wheat bran was used as the solid-substrate for the growth of *A. oryzae* JGI 21.

Optimization of Culture Conditions for Enzyme Production

Effect of Temperature and pH

The growth of *Aspergillus oryzae* JG1 21 was followed at several different temperatures (room temperature, 37, 40, and 50°C) and in addition in media with different pH adjustments (5.5, 6.5, 7.5, 8.5, 9.5 and 10) at 24 ± 2°C in order to determine the most suitable conditions for α-amylase production.

Enzyme Extraction and Assay

22 ml of phosphate buffer (pH 6.5, 0.1 M) was added to the culture flasks and mixed well in a rotary shaker (200 rpm) at room temperature (24 ± 2°C) for 30 min. The mixture was filtered through muslin cloth and centrifuged at 10,000rpm for 10 min at 4ºC. Collected supernatant, after centrifugation, were used for determination of amylase activity.

Estimation of α-amylase activity was carried out according to the dinitro salicylic acid (DNS) method of Miller (1959).Each of the enzyme samples was assayed by adding 0.5 ml of the enzyme solution to 0.5 ml of phosphate buffer (pH 6.5) into which 1% starch substrate was added. The solution incubated at 37°C for 10 min and the reaction mixture was stopped by adding 5 ml of DNS reagent. It was heated at 100ºC for 15 min and cooled 2.5 ml of distilled water was added and allowed to cool down to room temperature for 10 min. The absorbance was read at 540 nm with the help of a colorimeter against glucose as the standard. One unit of enzyme activity is defined as the amount of amylase which releases 1µmole of reducing sugar as glucose per minute (U/ml/min), under the assay conditions. The experiments were carried out in triplicates and standard error was calculated.

Protein Estimation

The amount of Protein content in the enzyme extracts were 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 the experiments were carried out in triplicates and the standard error was calculated.

Enzyme Characterization

Amylase Purification by Ammonium sulphate precipitation

Ammonium sulfate was added to the crude culture supernatant to 20% saturation. The mixture was kept overnight at 4°C on magnetic stirrer. After centrifugation, the supernatant was assayed for presence of the enzyme, and this assay indicated that the enzyme did not precipitate in 20% saturation of ammonium salt. As a result, the concentration of salt was increased from 20% to 40% and subsequently to 60% and 80%. In the 60% saturation, the supernatant did not contain any measurable amount of the enzyme, indicating that it had precipitated. The precipitates were dissolved in a minimum volume of 0.1M phosphate buffer (pH 6.5), and dialyzed overnight against 0.05M phosphate buffer (Gomori, 1955).

Ion exchange chromatography

The dialyzed enzyme preparation was applied to a DEAE cellulose (supplied by Bangalore Genei) column (3 cm×10 cm) previously equilibrated with 50mM phosphate buffer (pH 6.5). After washing through all the unbound proteins, the column was eluted using the same buffer containing 0.1–0.8M NaCl at a flow rate of 1ml/min. The fractions containing protein (absorbance 280 nm) were pooled and tested for enzyme activity, and the active fractions for enzyme assays were concentrated, and then dialyzed.

SDS-PAGE analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in order to determine the purity and molecular weight of the α-amylase (Laemmli, 1970). A 6.5% (w/v) stacking gel and a 15% (w/v) separating gel were employed. The enzyme sample (50 µg) was mixed thoroughly with 1% sodium dodecyl sulphate (SDS), 5% 2-mercaptoethanol and 0.001% bromophenol blue (Laemmli, 1970). A drop of glycerol was added and 20 µl of the sample was loaded on 15% Polyacrylamide gel andelectrophoresed at 25°C for 2,5h. The gels were stained for proteins with 2,5% Coomassie brilliant blue. The bands were scanned in Elico spectrophotometer at 620 nm. Molecular weights were determined by comparing the irmobilities with those of the marker proteins. 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.

Thermo Stability and pH stability of Amylase

The thermal stability of the enzyme was determined by incubating the partially purified enzyme without the substrate fractions at various temperatures between 30 to 60 ºC for 1 h. At 10 min. intervals, aliquots of 0.1 mL of the incubated enzyme were assayed for activity. The enzyme activity was determined before incubating at various temperatures and this activity was considered as the control activity of the enzyme. For checking the pH stability, equal volumes of the enzyme and the buffer of different pH (5.0-9.0) were incubated at room temperature overnight. The effect of pH on amylase enzyme

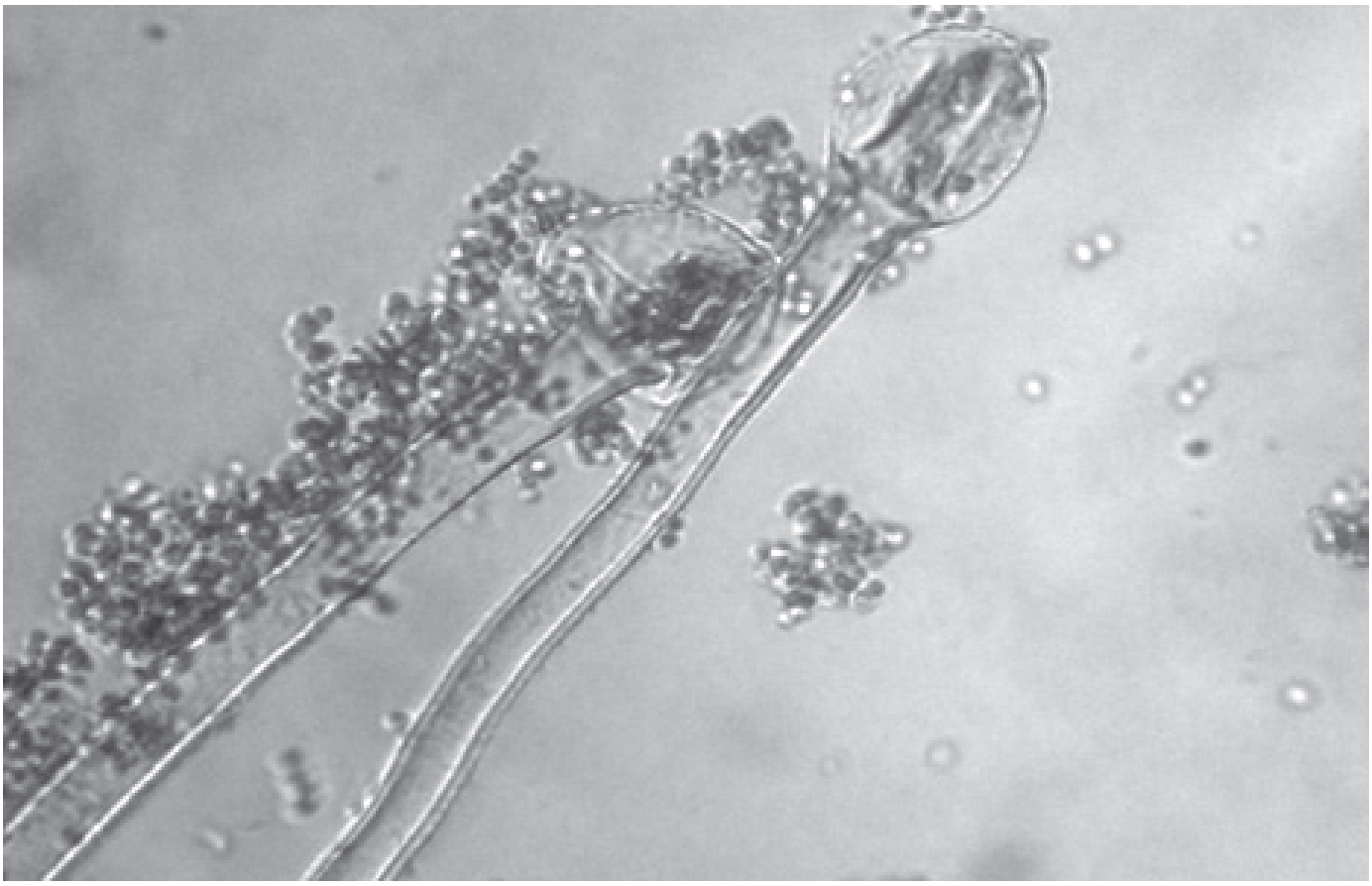

Figure 1. *Aspergillus oryzae JGI 21*

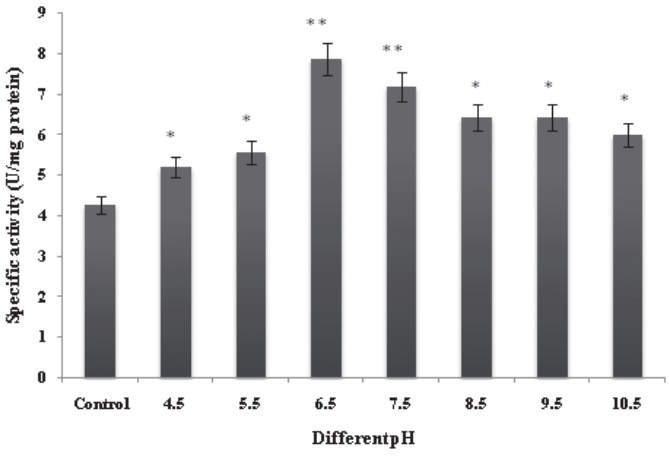

Figure 2. *Effect of pH on α- amylase production by A. oryzae JGI 21.*p<0.05 compared with control which had a pH of 7.0. **indicate highly significant with p<0.001.*

was determined by checking the residual enzyme activity using the above enzyme. The enzyme activity was measured before overnight incubation of the enzyme with buffers of different pH and was considered as the enzyme activity of the control.

Statistical analysis

All experiments were carried out in triplicates. The results were calculated as mean ± standard error (SE) values. Statistical significance was calculated using one-way analysis of variance (ANOVA) to test the null hypothesis. Duncans multiple range test (DMRT) was done to compare that the sample means were significantly different from eachother at a significant level of $P < 0.05$.

RESULTS

Different fungi and bacteria that were isolated from different sources were tested for the production of amylase by the starch hydrolysis test. On the basis of the area of clearance, one fungus was isolated and identified as *Aspergillus oryzae* JGI 21(**Fig.1**).

Enzyme production

Effect of pH

According to obtained results the selected fungal strain, *A. oryzae* JG1 showedthe highest enzyme production of 7.885 U/mg at pH 6.5. At pH 4.5 and 5.5 also the enzyme production was highercompared tothe controls. At the alkaline pH of 8.5 and 9.5, the amylase activity was lower than the one determi-

Table 1. *Purification of α-amylase from A. oryzae JGI 21*

Step	Volume (ml)	Total Activity (Units/ml)	Total Protein (mg/ml)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Crude extract	100	1226	160	4.26	100	1
$(NH_4)_2 SO_4$	70	175	11.2	31.13	14.27	7.16
DEAE celluloseIon exchange chromatography	1	426.66	4.25	100.38	34.8	23.56

nedat pH6.5 but these values were found to be higher than that observed by the control group.These differences were found to be statistically significant (P<0.05). The enzyme production was observed at both alkaline pH and acidic pH with less variations (**Fig.2**).

Effect of Temperature

A. oryzae JGI 21 when incubated at different temperatures, room temperature (24 ± 2°C) was found to be the favourable temperature for enzyme production. The specific activity was found to be 8.09 U/mg (Fig.3). The enzyme production in solid state is greatly affected by the temperature, and it was found that the enzyme production decreased with increase in temperature and there was no enzyme production at 50 °C. The influence of temperature on amylase production is related to the growth of the organism. Hence, the optimum temperature depends on whether the culture is mesophilic or thermophilic. Among the fungi, most amylase production studies have been done with mesophilic fungi within the temperature range of 25–37 °C (Francis et al., 2003;Ramesh and Lonsane, 1991).

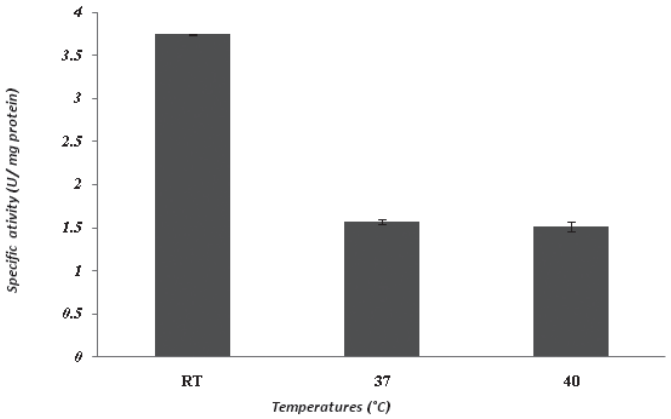

Figure 3. *Effect of temperature on α- amylase production by A. oryzae JGI 21.*

Enzyme Characterization

Ammonium sulphate precipitation

Partial purification of α-amylase was carried out by addition of ammonium sulphate to different saturation levels.When the precipitated proteins at various saturation points were checked for amylase activity, the highest activity was observed at 40-60% of $(NH_4)_2SO_4$ (31.13 U/mg) (**Fig.4**). Earlier studies conducted on ammonium sulphate fractionation of α-amylase indicated that 0-50% saturated precipitate contained higher α-amylase activity than 50-100% saturated precipitate (De Silva, 1997).Sidkeyet al (2011), reported that α-amylase isolated from A. flavus when purified by ammonium sulphate precipitation, 40-60% saturation gave the highest enzyme activity.

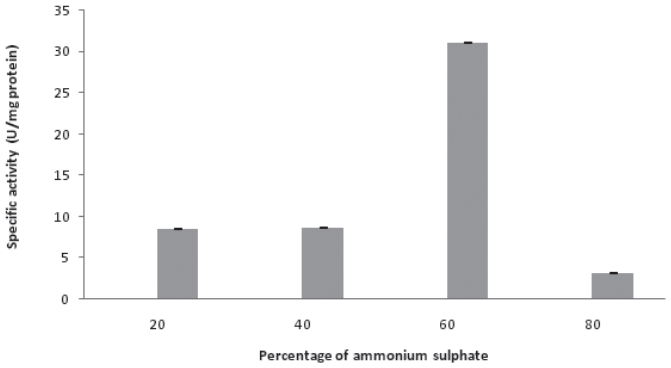

Figure 4. *Purification of α-amylasefrom A.oryzae JGI 21 by ammonium sulphate precipitation*

DEAE-cellulose anion column chromatography

The anion exchange chromatography of DEAE cellulose is presented in **Figure 5**, and the results of the purification are summarized in Table I. The amylase activity was in protein peaks 1 to 10 that were in 6th, 11th, 12th, 13th, and 15th with the highest amylase activity measured in fractions 6th and 13th (426.66 U/ml). The purified enzyme exhibited 34.8% of total initial activity, which corresponded to a 23.56- fold increase in specific activity when compared to crude culture filtrate. Purification fold of the purified enzyme was calculated by comparing its specific activity with that of the crude enzyme. The specific activity of the partially purified enzyme was 100.38 U/mg protein whereas, the specific activity of the crude enzyme

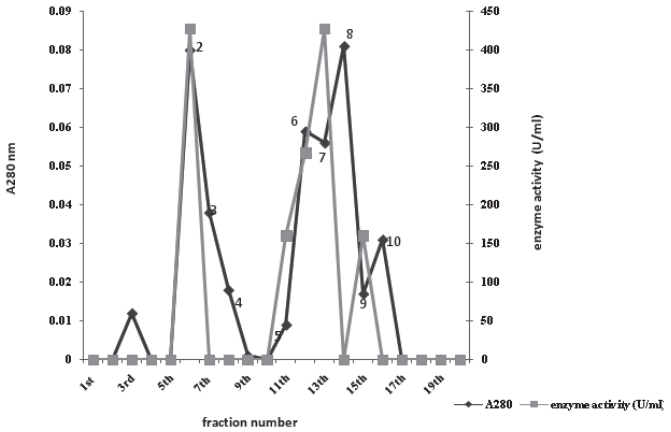

Figure 5. *"Elution profile of α-amylase isolated from A. oryzae JG1 21 obtained by DEAE cellulose anion exchange chromatography".*

was only 4.26 U/mg protein.As shown in **Figure 6**, the purified enzyme demonstrated an apparent single protein band on SDS-PAGE (**Fig. 6**). The molecular weight of single protein band was estimated to be 22 kDa from its mobility relative to those of standard proteins on SDS-PAGE.

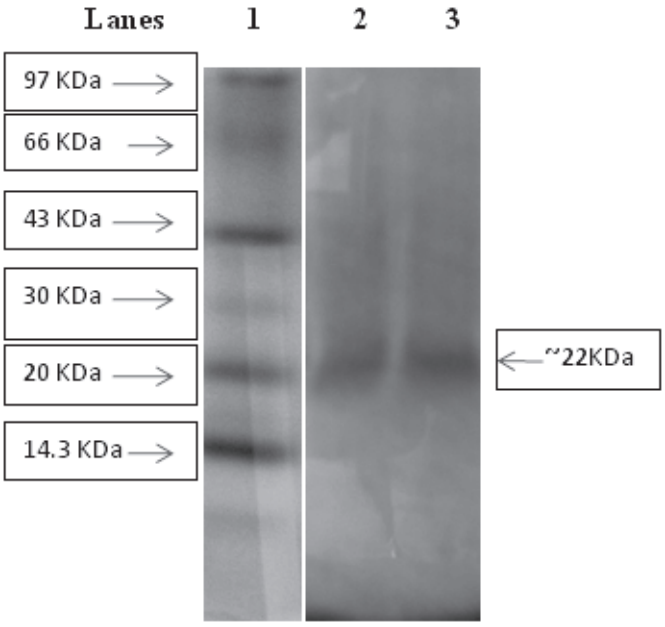

Figure 6. *SDS- PAGE of partially purified α- amylase from A. oryzae JGI 21.*
Lane: 1 - Molecular mass marker.
Lane: 2 - Enzyme purified by NH_4SO_4 precipitaion.
Lane: 3 - Enzyme purified by DEAE ion-exchange chromatography.

Effect of Temperature on Enzyme Stability

The enzyme showed thehighest stability atroom temperatureretaining 60% of its original activity when incubated for 60 min., followed by 37º C (**Fig.7**).The least stability was at 60ºC, retaining <50% of its original activity after 60 min. Similar results were reported from our earlier studies (Alva et al., 2007; Varalakshmi et al., 2009).

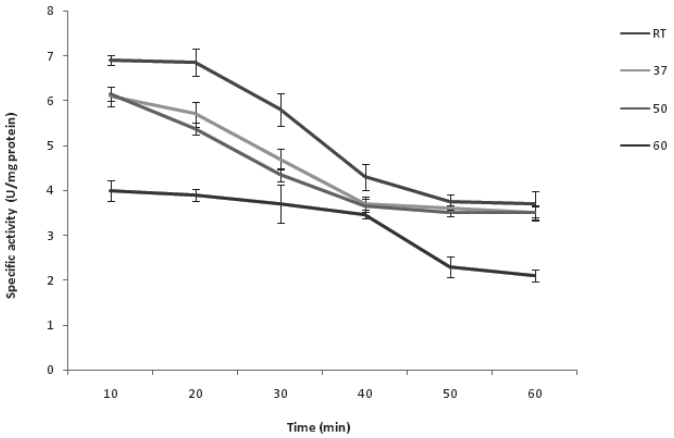

Figure 7. *Effect of temperature on the on the activity of partially purified α- amylase from A. oryzae JGI 21.*

Effect of pH on Enzyme Stability

Different buffers of pH ranging from 4.5, 5.5, 6.5, 7.5, 8.5 and 9.5 were used to study their effect on enzyme stability. The enzyme was incubated in various buffers of different pH for 24 hours. Enzyme incubated with pH 6.5 showed the maximum residual specific activity of (6.24 U/mg of protein) in comparison to other pH (**Fig. 8**). The enzyme showed significant stability towards both alkaline and acidic pH.

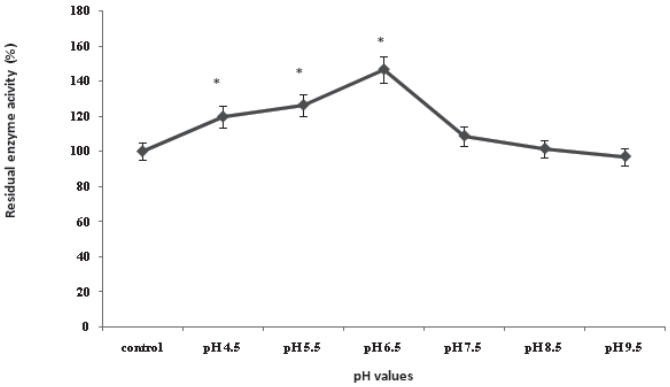

Figure 8. *Effect of pH on the stability of α- amylase from A. oryzae JGI 21. Control pH=7.0. *p <0.05 when compared with control.*

DISCUSSION

The influence of temperature on amylase production is related to the growth of the organism. Hence, the optimum temperature depends on whether the culture is mesophilic or thermophilic. Among the fungi, most amylase production studies have been done with mesophilic fungi within the temperature range of 25–37 °C (Francis et al., 2003; Ramachandranet al., 2004). Our isolate of *A. oryzae* JGI 21,when incubated at different temperatures, enzyme production was found to be highest at room temperature (24-28°C) with an enzyme activity of 8.09 U/mg. Hayashida and Teramoto (1986), reported a raw starch degrading α-amylase produced by *Aspergillusficuum* at 30°C. The enzyme extracted from our selected fungal strainwas, interestingly, highly active and stable over a wide range of pH (4.5-9.5) with the optimum pH at 6.5. Our isolate showed highest enzyme production at pH 6.5 (7.885 U/mg) followed by 7.5, 8.5, 9.5 and 5.5. At all these acidic and alkaline ranges tested, the enzyme activity was found to be higher than that of the controls which had a pH value of 7.0. Earlier studies have revealed that fungi required slightly acidic pH and bacteria required neutral pH for optimum growth. pH is known to affect the synthesis and secretion of α-amylase just like its stability (Sydkey *et al.*, 2011). Fungi of *Aspergillusspp*. such as *A. oryzae, A. ficuumand A. niger* were found to give significant yields of α-amylase at pH=5.0–6.0. Most of the thermostable α-amylases reported in literature show maximal activity at pH 6.5-7.0 in 0.5-1% starch solution. These alkaline α-amylases exhibit a sharp drop in activity in acidic medium (Ramesh and Lonsane, 1991). Importance should be given to resourceful α-amylases which exhibit more than one pH optimum, with one on either side of pH 7. In our study, the enzyme production was observed at both alkaline pH and acidic pH. This makes this strain suitable for commercial production of α-amylase in industries over a wide range of pH (4.5-9.5). This amylase

also has exhibited maximum stability at pH 6.5 with a residual enzyme activity of 6.24 U/mg protein after 24 hrs of incubation with a buffer of pH 6.5 followed by 5.5, 7.5, 8.5 & 9.5. Even at the alkaline pH of 9.5, the residual enzyme activity was ~80% of that of the controls. So our enzyme has exhibited considerable pH stability at all the tested pH ranges. This propertyis well suited for industrial applications. Sudo *et al* (1994), reported the pH stability of *Aspergillus kawachii* as 2-6.5 and an optimum pH stability at pH 5.0.

The enzyme showed highest stability at RT (24-28°C) retaining 70% of its original activity after 60 min incubation, followed by 37º C retaining 60% of its activity after 60 min and least stability at 60ºC, retaining only 30% ofits original activity after 60 min. Similar results were reported from our earlier studies (Varalakshmi et al., 2009). The purified alpha amylase from *A. oryzae* JGI 21 had a molecular weight of approximately 22kDa and the purification of alpha amylase was confirmed by the appearance of a single band after SDS-PAGE. The activity and specific activity of the purified enzyme was found to be 426.66 U/ml and 100.38 U/mg respectively with 23.56 fold purity and 34.8% yield. Mohammed *et al* (2011), reported that the purity of the enzyme from *Bacillus cereus* Ms6 after DEAE cellulose chromatography was more than 27 fold, when compared to the crude extract of enzyme. Afifiet al (2008), reported that α-amylase from *Penicilliumolsonii* is purified by gel filtration and ion chromatography with 25.14 fold purification and specific activity of 23.43 mg/ml of protein.

CONCLUSION

In this study, the purification and characterization of α-amylase from *Aspergillus oryzae* JGI 21 has been reported. It could be purified to homogeneity by comparatively simpler purification steps to result in 23 fold purification and 34.8% yield with a high final specific activity of 100.38 U/mg protein. The α-amylase produced by *A. oryzae* JGI 21 is active and stable at low pH. Furthermore, it is active at a wide pH range. These two features of the α-amylases are very important in starch industry which is lacking in the majority of reported α-amylases up to now. Since the α-amylase of this strain has a broad pH range of activity and high pH stability, it can be a suitable candidate to be used as an additive for detergent industries. Due to the importance of our finding, further enzymatic studies, structural analysis and determination of gene sequence of this α-amylase can be carried out in order to understand the mechanism of its activity at low pH.

ACKNOWLEDGEMENT

The authors are grateful to the management of Jain Group of Institutions for the facilities provided to carry out the work.

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