

Purification and Characterization of Extracellular α -amylase from *Aspergillus niger* K8

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ASPERGILLUS NIGER K8 was selected from twenty-two fungal isolates for α -Amylase production. The crude enzyme was partially purified and precipitated by salting out with ammonium sulphate. The fraction salted out with 60% ammonium sulphate saturation showed the highest specific α -amylase activity with 5.42 folds of purification than that of free extract. The optimum temperature for α -amylase enzyme activity was at 60°C. The enzyme was very stable at 60°C for 1 hr and retained more than 80% of its original activity. The optimum specific activity for *A. niger* K8 was in a narrow range of pH 5.0-6.0. Alpha amylase enzyme was stable at all pH tested. However, it retains more than 75% of its original activity for 1 h at pH value 5.5.

Keywords: *Aspergillus niger*, α -amylase, Precipitation, Characterization, Thermal stability, pH stability.

α -Amylases are one among the largest selling industrial enzymes that find use in a wide variety of industrial applications such as production of ethanol, high fructose corn syrup, detergents, desizing of textiles, modified starches, hydrolysis of oil-field drilling fluids and paper recycling (Richardson *et al.*, 2002). Amylases are produced by a wide spectrum of organisms, although each source produces biochemical phenotype that significantly differ in parameters like pH and temperature optima as well as metal ion requirements. Amylases have specific applications in the food and pharmaceutical industries, which require higher purity of the enzymes (Pandey *et al.*, 2000).

α - Amylases 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. Most of the α -amylases are metalloenzymes, which require calcium ions (Ca²⁺) for their activity, structural integrity and stability. They belong to family 13 (GH-13) of the glycoside hydrolase group of enzymes (Bordbar *et al.*, 2005). Amylases have a wide variety of applications ranging from conversion of starch to sugar syrups, to the production of cyclodextrins for the pharmaceutical industry (Van der Maarel *et al.*, 2002).

α - Amylases are ubiquitous enzymes produced by plants, animals and microbes, where they play a dominant role in carbohydrate metabolism. However, Amylases from plant and microbial sources have been employed for centuries as food additives. Filamentous fungi have been widely used for the production of amylases for centuries. As these moulds are known to be prolific producers of extracellular proteins, they are widely exploited for the production of different enzymes including α -amylase. Fungi belonging to the genus *Aspergillus* have been most commonly employed for the production of α -amylase. Detailed literature is available on various microbial sources for the production of amylases (Pandey *et al.*, 2000).

The main focus of this paper is to partial purifying alpha amylase and enhance the specific activity and stability of α -amylase from a fungal source.

Material and Methods

1. Culture media

Agar plates "A" of *Czapek-Dox medium* (Oxoid, 1982) was used in purification and maintenance of isolates. Furthermore, this medium can be used as a basal medium but without the addition of agar and sucrose. This medium contained (g/l): NaNO₃, 3; K₂H PO₄, 1; MgSO₄. 7H₂O, 0.5; KCl, 0.5; FeSO₄.7H₂O, 0.01; agar, 15; sucrose, 20 at initial pH of 6. The fungal isolates were grown for seven days at 35 °C then stored at 4°C.

Agar plates "B" of Modified Czapek-Dox (MCD). It was used as a primary isolation medium. Only 1% (w/v) of the starch was used as the sole carbon source. It was used for screening fungi capable of producing starch digesting enzymes. It was used also as a production medium but without addition of agar.

2. Isolation and identification of α -amylase producing fungi

Samples from some agricultural materials (Mandarin; M, Feyxoa; F, Potato; K, Apple; A, Lemon; L and Tomato; T) were collected from different regions of Azerbaijan.

Isolation of fungi from each of the blemished fruits and vegetables was carried out using the method of (Amusa *et al.*, 2002; Baiyewu *et al.*, 2007). Segments (3-5 cm) of tissues from the margins of the rotted fruits or vegetables were cut with a sterile scalpel and placed on the previously prepared agar plate 'B' and incubated at 35 °C for seven days for isolation of mesophilic fungi.

The plates were daily examined for *Aspergillus* sp. colonies developed on each plate. Strains capable of producing alpha amylase were screened by allowing them to grow on Agar plate "B". After 7 days of incubation at 35°C, plates were stained with Gram's iodine solution. Fungus produced the largest halo-zone was considered as the most promising strain. Identification was based on morphological and microscopic features as described by (Raper and Fennell, 1965).

3. Enzyme production medium

Initial pH of the enzyme production medium was adjusted to 7.0. with 1N NaOH. The medium was sterilized, allowed to cool, and inoculated with 7 days old fungal suspension containing 10^5 spores/ml (Metwally, 1998). This was followed by incubation at temperature (35°C) for 7 days on a rotatory shaker at 150 rpm.

At the end of fermentation process, culture filtrates were centrifuged at 5000rpm for 20 min in a cooling centrifuge. The clear supernatant was considered as the source of crude enzymes.

Partial purification of amylase enzyme

Partial purification of amylase enzyme was achieved by ammonium sulphate precipitation. 100 ml of free extract was saturated with ammonium sulphate up to 90%. The content was incubated over night and centrifuged at 5000 rpm for 20min. Each fraction pellets were immediately dissolved in a minimum volume (5 ml) of 0.1 M citrate phosphate buffer (pH 5.0). The dissolved fractional precipitations were tested for both amylase activity and protein contents.

5. Assay for α -amylase

The method used involved estimating the amount of reducing sugar produced by the activity of each enzyme on buffered starch. α -amylase was assayed as reported by (DeMoraes *et al.*, 1999). The substrate for assay was 0.5 ml of 0.5% soluble starch, buffered with 0.2 ml of 0.1 M sodium acetate (pH 5.6). Crude enzyme extract (0.3 ml) was added to the mixture, mixed and incubated at 40 °C for 30 min in a water-bath. DNSA (3,5-dinitrosalicylic acid) colorimetric method used by (Miller, 1959) was thereafter employed for estimation of reducing sugars produced. One ml of DNSA solution was added to the mixture and boiled for 5 min. Four ml of distilled water was introduced after cooling before absorbance is read at 540 nm in "SPECORD 250 PLUS-223G1020" spectrophotometer. Blank that consisted of 0.3 ml distilled water, 0.5 ml of 0.5% soluble starch, 0.2 ml of buffer was subjected to similar treatments.

The clear supernatant (Crude enzyme) was used for estimation of alpha amylase enzyme activity that was expressed in number of units. One unit of enzyme was defined as the amount of enzyme (protein) in milligram required for hydrolysis of starch to produce a 1 μ mol of reducing sugar (maltose) per minute under the assay conditions. The specific activity was defined as number of units per gram protein (Gupta *et al.*, 2010).

6. Protein estimation

The protein concentration was estimated by measurement of 600 nm absorbance (Lowry *et al.*, 1951).

7. Enzyme characteristics

7.1. Optimum temperature

Temperature optima of α -amylase enzyme were determined by assaying activity in the temperature range of 20–80°C. The temperature at which maximum activity was observed was taken as 100% and relative activities (at different temperatures) calculated.

7.2. Thermal inactivation kinetics

Kinetics of thermal inactivation of α -amylase enzyme was studied at different temperatures in the range of 55–70°C. Enzyme samples (in 0.1 M Citrate phosphate buffer, pH 5.0) were incubated at the test temperature and aliquots withdrawn at appropriate time intervals. Relative activities at the test conditions were calculated. Activity of the unincubated enzyme was taken as 100%.

7.3. Optimum pH

pH optima of α -amylase enzyme were tested by assaying activity in the pH range of 2.0–8.0. Citrate phosphate buffer (0.1 M) was used. Relative activities at the test pH were calculated assuming the maximum activity observed as 100% (during the experiment).

7.4. pH stability

The pH stability was studied for the purified α -amylase by incubation of the enzyme in 0.1 M Citrate phosphate buffer, pH range 4.0–6.0 for a series of time intervals at 60°C, finally the residual activity was determined under assay conditions.

Results and Discussion

Under the experimental conditions, *Aspergillus* sp. was represented by eight isolates. These isolates were screened for their ability to produce α -amylase. Each colony was checked for halo-forming zone whose diameter was measured after Gram's iodine solution was added to each plate to test the presence or absence of starch around it.

The results showed that all tested *Aspergillus* sp. isolates survived well on the experiment modified medium and produced considerable amylase enzyme. The amylolytic activity was varied among the different fungal isolates. The mesophilic fungal isolate K8 was exhibited the maximal α -amylase production (4.6 cm width of halo zone; 10.38 U/mg protein). This isolate was characterized and identified on the basis of morphological and microscopical features. The fungal colony appeared were picked up on Modified Czapek-Dox medium containing 0.05% yeast extract, purified using single spore technique and identified by consulting fungal identification keys.

This isolate K8 was found belong to *A. niger* group, so it was given the name and the code number (*Aspergillus niger* K8). Therefore, this strain was selected and maintained for using at the advanced experiments.

The crude enzyme preparation from culture fluid of *A. niger* K8 was partially purified and precipitated by salting out with ammonium sulphate. The enzyme activity in a variety of ammonium sulphate saturation can be seen in Fig. 1.

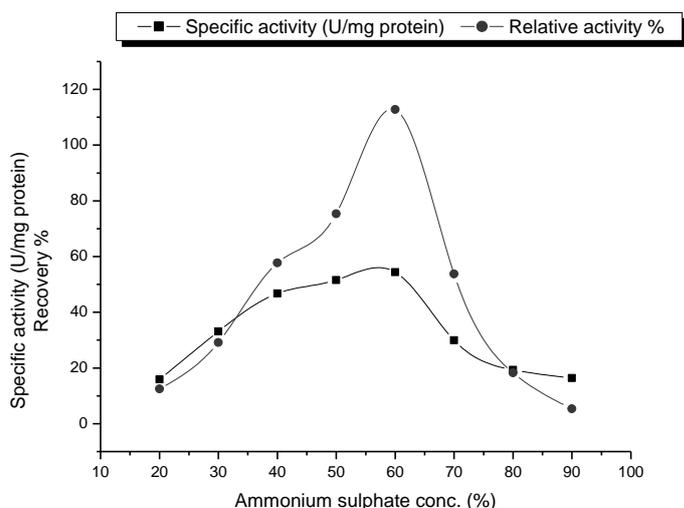


Fig. 1. *Aspergillus niger* K8 α -amylase activity after precipitation by Ammonium sulphate.

Ammonium sulphate fractions in between 20-90% furnished a specific alpha amylase activity of tested organism higher than that of cell free extract to indicative a positive purification in those fractions. The fraction salted out with 60% ammonium sulphate saturation showed the highest specific α -amylase activity of *A. niger* K8 reaching 54.87 U/mg protein with 5.42 folds of purification than that of culture fluid. The same fraction showed also the highest recovered as well as high protein content of tested organism. Therefore, it was selected for further studies.

The present findings are in accordance with Yandri *et al.* (2010) who mentioned that α -amylase possess the highest specific activity at fraction of 40-60%. Also, Prakash *et al.* (2011) found that among the three fractions (0-30, 30-80 and 80-100%) selected for the study, protein precipitated in the range of 30-80% saturation of ammonium sulphate. Moreover, alpha amylase enzyme was purified using 60% ammonium sulfate precipitation (Sidkey *et al.*, 2011). Maximum amylase activity was obtained in the 80% fraction with a high specific activity (Haq *et al.*, 2010).

The results indicated that specific alpha amylase activity increased parallel to the increasing in reaction temperatures (Fig. 2) reaching a maximum value at 60°C (58.29 U/mg protein). Further increase of reaction temperature resulted in gradual decrease in specific activity.

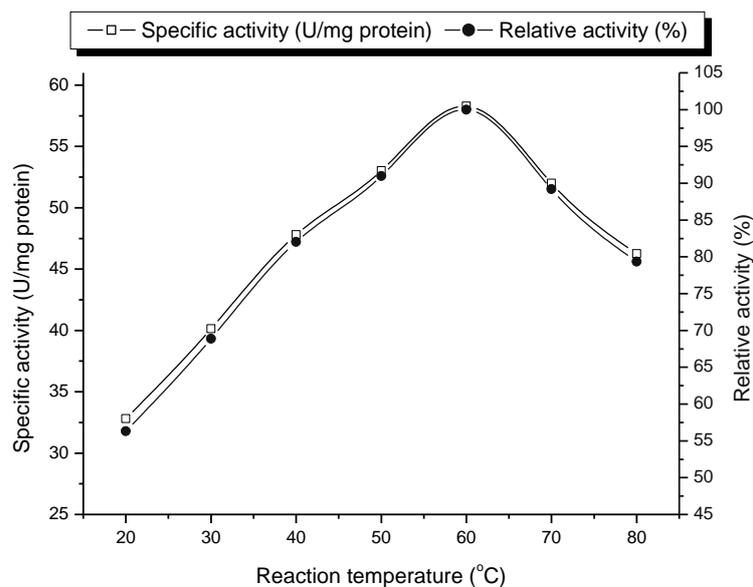


Fig. 2. Effect of reaction temperatures on the partial purified α -amylase produced by *A. niger* K8. The maximum enzyme activity obtained was taken as 100% and residual activities at different temperatures calculated.

Prieto *et al.* (1995) mentioned that a new alpha-amylase from the extracellular culture of the yeast *Lipomyces kononenkoae* CBS 5608 has been purified to homogeneity. The temperature for optimal enzyme activity was 70°C. However, Yadav and Prakash (2009) found that the optimum temperature of the enzyme was found to be $50 \pm 2^\circ\text{C}$. Further increase in temperature resulted in irreversible thermal inactivation of the enzyme. Moreover, Yandri *et al.* (2010) stated that the optimum temperature of purified alpha amylase was 60°C.

The temperature effect over amylase stability was determined by measuring the residual activity after 10, 20, 30, 40, 50 and 60 min of preincubation (in the absence of substrate) at temperatures of 55, 60, 65 and 70°C. The enzyme was very stable at 60 °C for 1 hr and retained more than 80% of its original activity (Fig. 3). However, the enzyme was retained more than 70% after 1 hr at 55, 65 and 70°C. Thus, the α -amylase seemed to be resistance to temperature, a favourable characteristics in industrial processes.

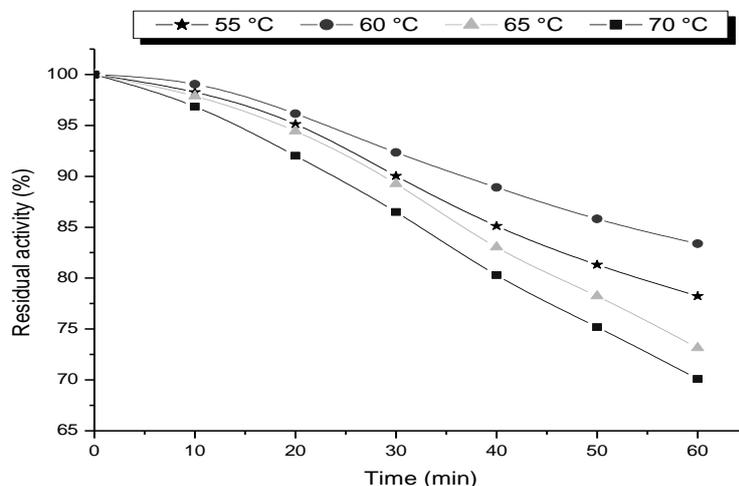


Fig. 3. Thermal inactivation kinetics of partial purified α -amylase produced by *A. niger* K8. The unincubated enzyme activity was taken as 100% for calculating the residual activities.

Due *et al.* (2008) mentioned that α -Amylase exhibited the maximum enzyme stability at 55°C. However, Varalakshmi *et al.* (2012) stated that when amylase enzyme was subjected to temperature stability studies, it was found that the enzyme was not thermostable. At 30°C, 40°C and 50°C, the enzyme activity decreased with time. At 60°C the enzyme showed the least stability retaining negligible activity.

The pH effect over amylase stability was determined by measuring the residual activity at various pH values in the range 2.0-8.0 (Fig. 4.) using citrate phosphate buffer. It was revealed that the activity of *A. niger* K8 was pH-dependent. The optimum specific activity for *A. niger* K8 was in a narrow range of pH 5.0-6.0. Thus, this range was selected for further experiments.

The pH stability of purified alpha amylase was determined by measuring the residual activity (Fig.5) after 10, 20, 30, 40, 50 and 60 min of preincubation (in the absence of substrate) at 60 °C using citrate phosphate buffer with pH values (4.0, 5.0, 5.5 and 6.0).

From pH stability profile, it was verified that the enzyme was stable at all pH tested. However, the actual optimum pH value was 5.5. Since, it retains more than 75% of its original activity for 1 hr. Also, amylase enzyme stability retained 73.51%, 67.24% and 65.52% at pH 5.0, 6.0 and 4.0., respectively. It is known that, α -Amylase enzyme was stable between pH 5.6-6.6. While, it was rapidly inactivated at pH below 5.6 and above 6.6. Also, it was found that optimum pH of purified alpha amylase enzyme was 6.0 (Due *et al.*, 2008; Yandri *et al.*, 2010).

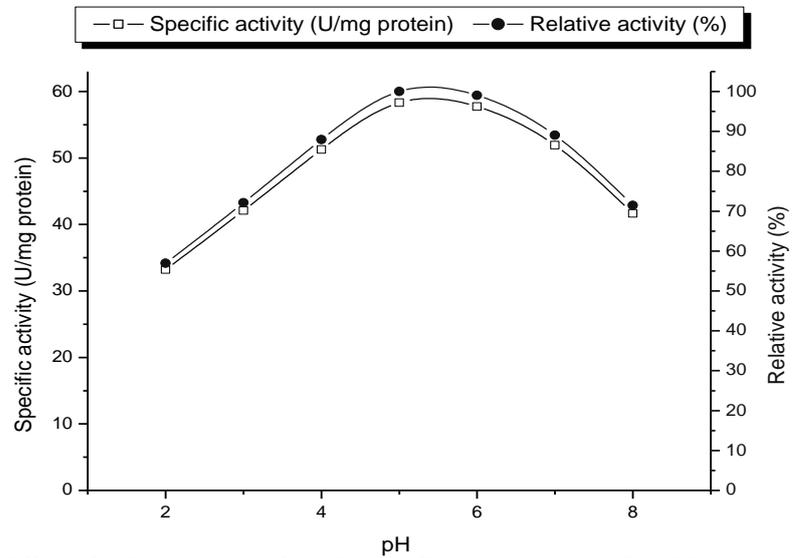


Fig. 4. Effect of pH on activity of partial purified α -amylase produced by *A. niger* K8. The maximum enzyme activity obtained was taken as 100% and residual activities at different temperatures calculated.

pH 5.5 resulted in maximum enzyme activity. There was a 3.25 fold in enzyme activity after incubating the enzyme with a buffer at pH 5 (Varalakshmi *et al.*, 2012).

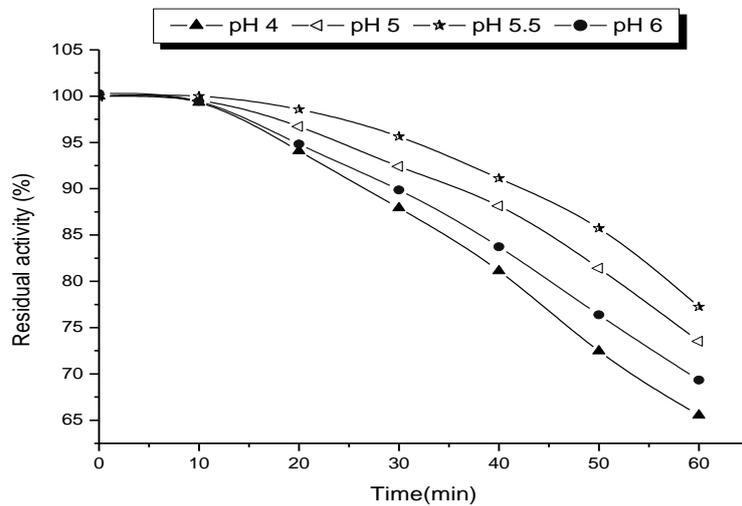


Fig. 5. pH stability of purified α -amylase produced by *A. niger* K8. The unincubated enzyme activity was taken as 100% for calculating the residual activities.

Conclusions

The following conclusions may be drawn from the present work.

1. *A.niger* can be commonly employed for the production of α -amylase.
2. The partial precipitation of alpha amylase by ammonium sulphate saturation significantly increase specific enzyme activity.
3. The optimum temperature for α -amylase enzyme activity was at 60 °C and at this temperature enzyme retained more than 80% of its original activity.
4. α -amylase was pH-dependent with pH optima in a narrow range of pH 5.0-6.0.
5. α -amylase enzyme was stable at all pH tested. However, the actual optimum pH value was 5.5 with more than 75% of its original activity for 1 hr.

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تنقية وخواص ألفا أميليز من فطرة الأسبرجلس نيجر عزلة رقم 8

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تم إختيار عزلة من الفطرة أسبر-جلس نيجر رقم 8 من بين 22 عزلة لإختبار قدرتها على إنتاج إنزيم ألفا أميليز. وقد تم تنقية الإنزيم الخام جزئيا وترسيبية باستخدام التلميح بكبريتات الأمونيوم المشبعة بنسبة 60% إلى الحصول على نسبة من نشاط الإنزيم المتخصص بنسبة 5,42 ضعف للنقاوة من ذلك المقطف من المستخلص الحر. كما كانت درجة الحرارة المثلى لنشاط الإنزيم ألفا أميليز عند درجة 60°م وكان الإنزيم مستقر جدا فى نشاطه عند 60°م لمدة ساعة. كما إحتفظ بأكثر من 80% من نشاطه الأصلي. وقد وجد أن النشاط الأمثل للفطر أسبرجلس نيجر للعزلة 8 كان فى مدى ضيق من الحموضة حيث تراوحت بين 5 - 6. كما وجد أيضا أن الإنزيم كان ثابتا عند كل درجات الحموضة المختبرة. وعلى أية حال وجد أن الإنزيم يحتفظ بأكثر من 75% من نشاطه الأصلي لمدة ساعة على درجة حموضة 5,5.

