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PARTIAL PURIFICATION AND CHARACTERIZATION OF α-AMYLASE FROM HYPERACTIVE MUTANTS OF *ASPERGILLUS NIGER* FOR ECONOMIC ENZYME PRODUCTION AND STARCH HYDROLYSIS

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ABSTRACT

Amylase is an enzyme that hydrolyzes starch for the production of maltose and glucose. In present study, the enzyme was isolated and partially purified to homogeneity by ammonium sulfate fractionation. The protein element drifted as a 20 kDa polypeptide on 10% SDS–PAGE in wild as well as in mutants. Kinetic evaluation of enzyme from wild and mutants revealed maximum activity at pH 4.5. The pKa_1 and pKa_2 of acidic and basic limbs of active site residues of native were 3.8 and 6.65 whereas 3.6 and 6.8 by An-UV-5.6 and 3.4 and 7.2 by An-Ch-4.7, respectively. The optimum temperature of enzyme was 60 °C and temperature quotient was 1.0010, 1.0012 and 1.0011 of *A. niger* FCBP–198, An-UV-5.6 and An-Ch-4.7, respectively. E_a of An-UV-5.6 was 50.38 kJ mol^{-1} that was significantly higher than required by native and chemically modified α–amylases i.e., 43.14 and 44.06 kJ mol^{-1}, respectively. The enzyme was highly active at 50 °C while the activity sharply declined after 10 min at 60 to 80 °C by all the test strains. The values of Michaelis–Menten constant K_m and V_{max} revealed by parental, An-UV-5.6 and An-Ch-4.7 were 0.476, 0.25, 0.21 and 5.0, 5.26, 5.56, respectively.

Keywords: α-amylase enzyme, *Aspergillus niger* FCBP–198, An-UV-5.6, An-Ch-4.7.

INTRODUCTION

As the amylase group of enzymes is of inordinate worth because of its widespread applications in therapeutic and experimental sector, it requires high purity amylases (Pandey *et al*., 2000). There are number of methods by which extraction and separation can be achieved and these methods have been detailed and assessed by a number of workers (Wu *et al*., 2006; Mertens and Skory, 2007), although data on the application of these methods to the filamentous fungi is scarce as compared to the data on bacteria and yeasts.

Earlier the purification of α–amylase from fungi was confined to a few species (Khoo *et al*., 1994; AbouZeid, 1997). Nevertheless, Amirul *et al*. (1996) extracted α –glucosidase, α –amylase and two types of glucoamylase from *Aspergillus niger* grown on a liquid medium having raw tapioca starch as the carbon source. In other studies Nagy *et al*. (2001) reported that β–galactosidase from *Penicillium crysogenum* Thom is a multimeric enzyme of about 270 kDa composed of monomers with a molecular mass of 66 kDa after purification. Quang *et al.* (2002) worked on amylolytic enzymes (α–amylase and glucoamylase) from *Thermomyces lanuginosus* (Tsiklinsky) Miehe ATCC 34626 and sanitized these enzymes to electrophoretic homogeneity. The molecular mass of purified α–amylase and glucoamylase were 61 and 75 kDa, respectively. Sumitra *et al.* (2004) carried out solid–state fermentation for the production of α–amylase using *Aspergillus oryzae* (Ahlburg) Cohn. Partial purification of the enzyme using ammonium sulfate fractionation resulted in 2.4 times intensification in the activity. The enzyme showed molecular weight of 68 KDa by SDS–PAGE. Thermostable enzymes are presently under investigation for the improvement in industrial processes of starch saccharification. In this study, the characterization and physiological and kinetic properties of partially purified α–amylase from indigenous *A. niger* FCBP–198 and its hyperactive mutants are reported.

MATERIALS AND METHODS

Enzyme Isolation and Partial Purification

The selected isolates (*A. niger* FCBP–198 and its mutants An-UV-5.6 and An-Ch-4.7) were cultured in conical flasks containing 300 mL of the enzyme production medium (soluble starch 15 gL^{-1}, potato starch 15 gL^{-1}, lactose 10 gL^{-1}, $(NH_4)_2 SO_4$ 5 gL^{-1}, $CaCl_2$ 2 gL^{-1}, NaCl 2 gL^{-1}) on a rotary shaker at 200 rpm and 30 ± 2 °C. After 5 days cultivation, cells were impassive by centrifugation at 7000 rpm for 15 min at 25 °C and extra cellular α–amylase in the supernatant was then filtered through a sterilized 0.2 µm Millipore filter to get cell free filtrate (CFF). The crude enzyme supernatant/cell free filtrate was chilled and subjected to further studies. All successive steps were carried

out at 0 °C. CFF was partially purified by 20–100% ultra pure grade ammonium sulfate (W/V) percentage saturations and steadily stirred for 10–15 min The aqueous phase was then preserved overnight at 4 °C. The protein fraction was precipitated out by binding with ammonium sulfate (McCleary and Harrington, 1988; Najafi *et al*., 2005). The precipitates were collected by centrifugation at 7000 rpm for 20 min at 4 °C. The solid pellet so obtained was dissolved in distilled and deionized water and dialyzed against distilled water at room temperature for 24 hours. Then diffusion bioassay was executed to spy the α–amylase enzyme activity.

Protein Estimation and Molecular Mass Determination

Protein estimation was made according to Bradford method (Bradford, 1976) with crystalline BSA as standard at a concentration of 1 mg mL^{-1} in distilled water.

For the estimation of protein, different samples prepared were monitored on spectrophotometer at 595 nm wavelength. The molecular weight of the partially purified enzyme of isolated strains was resoluted by 10% Sodium Dodecyl Sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) method (Najafi *et al*., 2005). A protein marker of molecular weight ranging from 11–170 KDa was used for localizing the desired purified protein. The gel was run at 120 Volts for 14 hours at room temperature. At the same time a native gel electrophoresis was performed without SDS to obtain proteins in their native state after the separation. Afterwards, gel was stained with Coomasie Blue (1%) for 1 hour on shaker and then de-stained in 10% acetic acid to remove methanol for 2 hours.

Entire gel columns from native gel were separately placed on 1% soluble starch agar petri plates which were incubated at 30 ± 2 °C for 48 hours. Subsequently, each petri plate was flooded with iodine solution. A transparent zone was visible against the dark–blue background of the starch–iodide complex, indicating that the starch has been hydrolyzed by the enzyme in that portion of gel.

Enzyme Characterization

The enzyme was characterized by studying the effect of different biochemical factors on enzymatic relations as described by El–Safey and Ammar (2004).

Effect of pH

The purified enzyme was incubated at different pH values viz., 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 using 2 buffer solutions: citrate buffer (0.05 M, pH 3.5 to 7.0) and tris buffer (0.1 M tris HCl, pH 7.5 to 9.0). Reaction mixture was incubated for 30 min and the activity of purified enzyme was measured to determine pK_a's of active site residues (Dixon and Webb, 1979).

Optimum Temperature, Activation Energy and Temperature Quotient

Optimum temperature and activation energy (*E*a) of partially purified α–amylase enzyme were determined by incubating enzyme with 1% soluble starch at different temperatures viz., 30, 40, 50, 60, 70, 80, 90 °C, respectively, in 0.05 M citrate buffer for 20 min at pH 4.5. Activation energy (*E*a) was estimated by constructing Arrhenius plot (Riaz *et al*., 2007). The effect of temperature on the rate of reaction was articulated as temperature quotient (Q_{10}), which demonstrates the boost in reaction rate with every 10 °C rise in the temperature. Q_{10} was calculated by using the equation of Dixon and Webb (1979):

$Q_{10} = \text{antilog}_e [E \times 10/RT^2]$

where $E = Ea$ = activation energy.

Thermostability of Purified Enzyme

Thermal stability of native and modified α–amylase, in the absence of substrate, was determined by keeping the enzyme in 0.05 M citrate buffer (pH 4.5), at a temperature range of 50 to 80 °C. After the time course, aliquots were cooled in ice bath and assayed under standard conditions. The data was fitted to first–order plot and evaluated statistically.

Effect of Substrate Concentration

To assess the effect of different substrate concentrations on enzyme, varied soluble starch concentrations (w/v) from 0.005 to 0.075% were incubated with fixed amount of enzyme. The purified enzyme was assayed at standard assay conditions and Lineweaver–Burk plots were applied as described by Siddiqui *et al*. (2000).

RESULTS

The partial purification of the active enzyme from cell free supernatant of cells and other constituents of the medium was carried out by ammonium sulfate percentage saturation method. The complete precipitation was resulted at 70% saturation of ammonium sulfate at 0 °C. Likewise, the maximal activity of partially purified protein fraction (enzyme) was noticed at 70% ammonium sulfate saturation in pellet. All the test strains exhibited almost

similar pattern of precipitation. The subsequent data on partial purification of α–amylase enzyme, from cultures of wild type and its mutants is summarized in Table 1. Approximately 4–fold purification with specific activity values, as high as 0.708, 1.025 and 1.102 Units mg^{-1} of protein were acquired by wild, UV and chemical mutant strains, respectively.

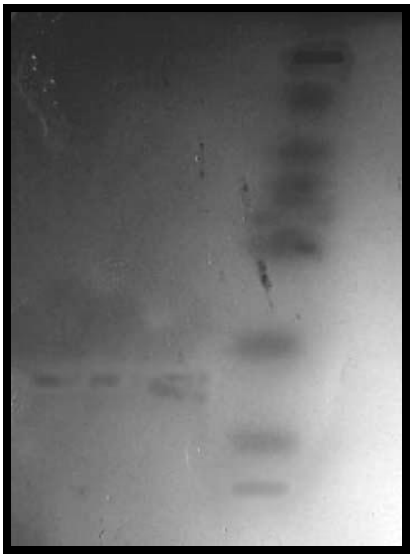

Fig. 1. 10% SDS–PAGE for the determination of molecular weights of α–amylase enzyme of *A. niger* FCBP–198, An-UV-5.6 and An-Ch-4.7.

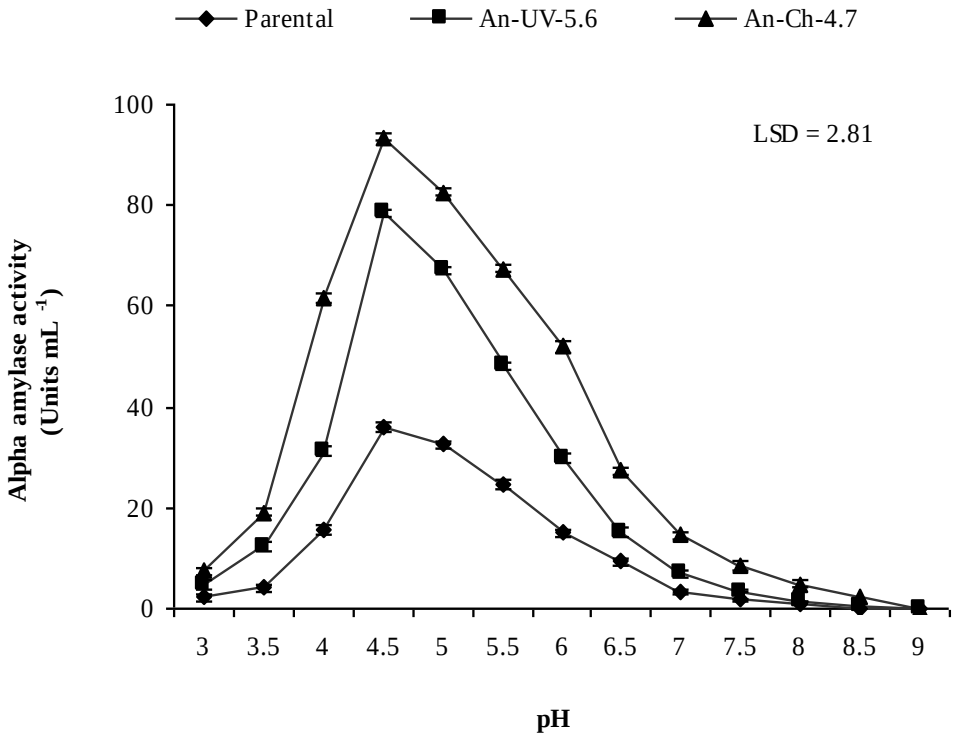

Fig. 2. Effect of pH on activity of partially purified α–amylase enzyme from wild strain of *A. niger* FCBP–198 and mutants An-UV-5.6 and An-Ch-4.7.

Vertical bars indicate standard error of means of three replicates. LSD at P≤0.05; Substrate: 1% soluble starch; Temperature of reaction mixture: 60 °C.

Table 1. Purification of α–amylase enzyme from *Aspergillus niger* FCBP–198 and its mutants An-UV-5.6 and An-Ch-4.7.

Strains	Steps	Total Protein (mg)	Total Units (U)	Specific Activity (U mg^{-1})	Purification factor
A. niger FCBP–198	Crude Enzyme	394.13	61.71	0.156	1.00
	$(NH_4)_2SO_4$ Precipitation	81.66	57.85	0.708	4.53
An-UV-5.6	Crude Enzyme	397.85	99.59	0.250	1.00
	$(NH_4)_2SO_4$ Precipitation	84.32	86.47	1.025	4.1
An-Ch-4.7	Crude Enzyme	417	110.94	0.266	1.00
	$(NH_4)_2SO_4$ Precipitation	85.08	93.77	1.102	4.14

The evaluation of molecular mass of the partially purified α–amylase enzyme of *A. niger* FCBP–198 and its mutants revealed that molecular masses of α–amylase were almost same i.e., 20 kDa which indicated that α–amylase enzyme is mono–meric in nature. Activity staining of native gel also showed a single band with the same mobility as that of the protein band. For the confirmation of presence of α–amylase enzyme in partially purified protein fraction, the bands of each gel column were sliced and placed on agar plates and verified through activity staining. The clearing of zones analogous to the band validated the presence of α–amylase enzyme activity in isolated protein fraction (Fig 1).

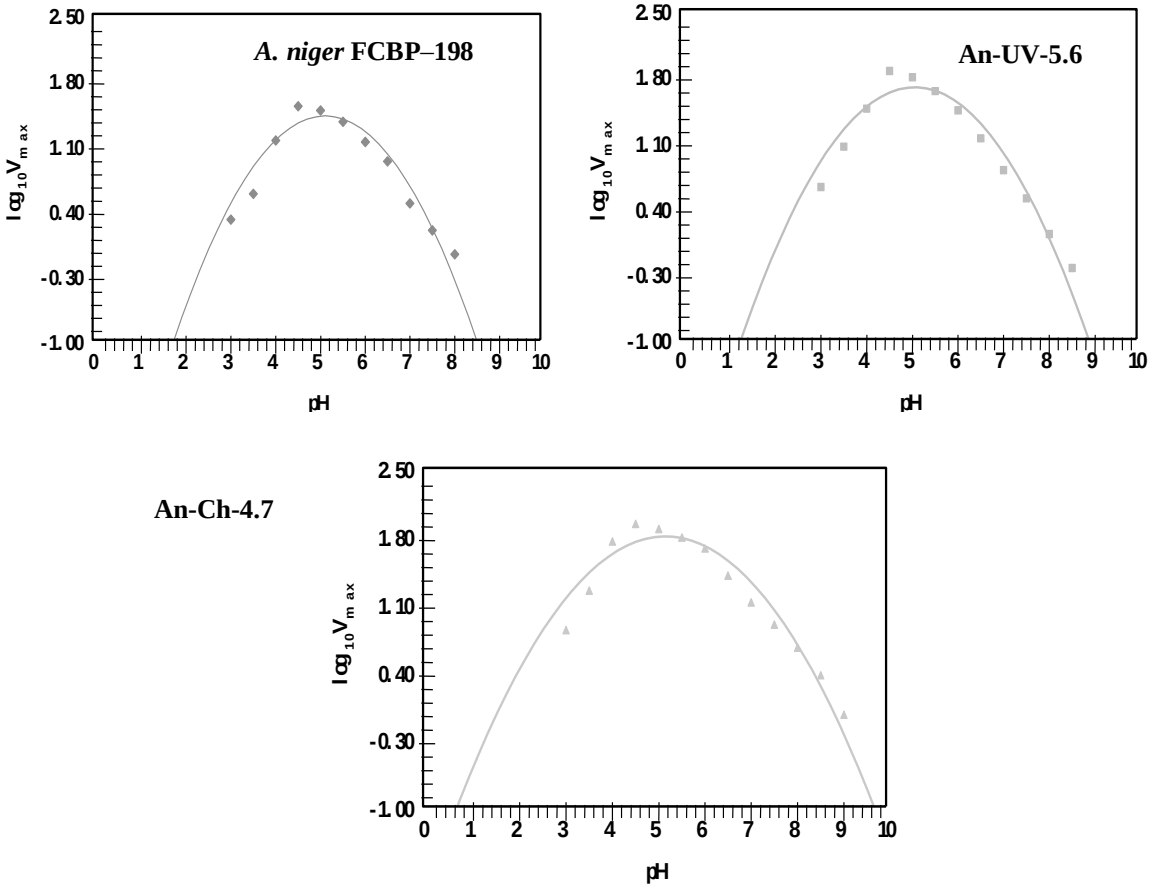

Fig. 3. Dixon plot of α–amylase for the determination of pKa values of active site residues of native and mutants that control V_{max}.

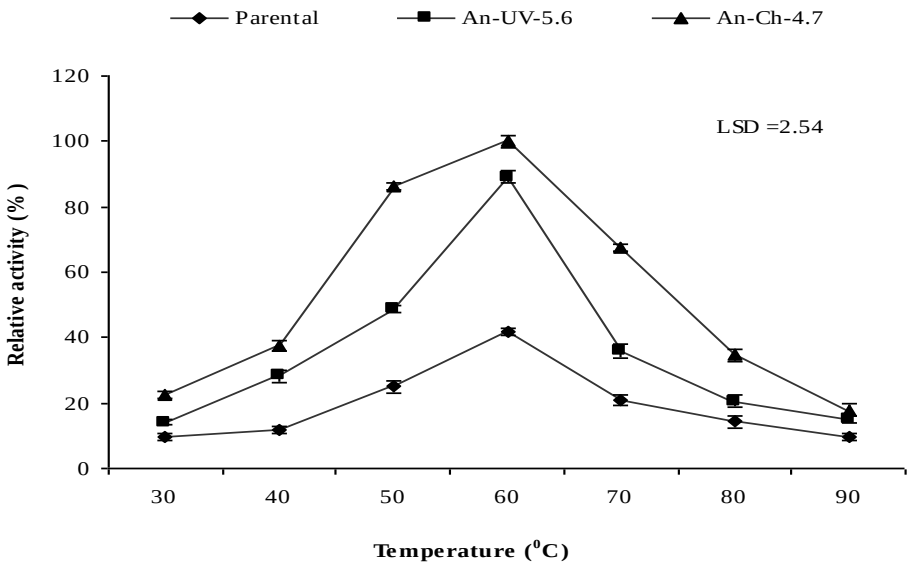

Fig. 4. Effect of temperature on activity of partially purified α–amylase enzyme from wild strain of *A. niger* FCBP–198 and mutants An-UV-5.6 and An-Ch-4.7.
Vertical bars indicate standard error of means of three replicates. LSD at P≤0.05. Substrate: 1% soluble starch, pH: 4.5.

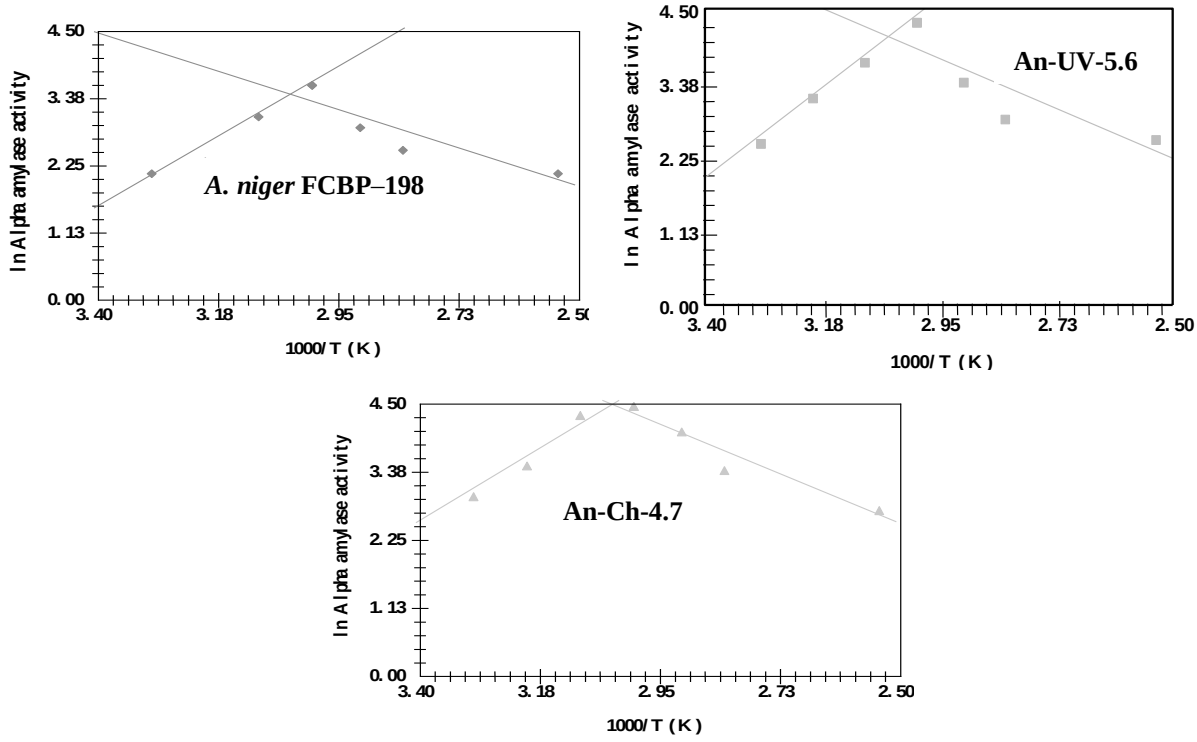

Fig. 5. Arrhenius plot to calculate activation energy of α-amylase enzyme produced from wild and mutant strains of *A. niger* FCBP–198.

Properties and Kinetic Studies of Partially Purified Enzyme

Effect of pH: The enzyme being very sensitive to pH, therefore, the selection of optimum pH is very essential for the enzyme production. The α–amylase activity was maximal at pH 4.5 to 5.0, however substantial activity was also detected in the pH regimes ranging from 3.5 to 7.0 (Fig. 2). The pKa_1 and pKa_2 of acidic and basic limbs of the active site residues that control V_{max} of native demonstrated by Dixon plot were 3.8 and 6.65, respectively.

However, slight alteration in conformation of active site residues of the enzyme was evidenced in mutant strains. The mutant derived α–amylase enzyme demonstrated pKa_1 and pKa_2 of 3.6 and 6.8 for An-UV-5.6 and 3.4 and 7.2 for An-Ch-4.7 strains, respectively (Fig. 3).

Effect of Temperature: The assessments on stimulus of temperature on α–amylase enzyme activity of the purified enzyme exhibited a progressive increase with the increase in temperature from 30 °C to 60 °C. Above 60 °C, the activity of enzyme was found to be declined substantially (Fig. 4). The enzyme's temperature quotient was 1.0010, 1.0012 and 1.0011 for *A. niger* FCBP–198, An-UV-5.6 and An-Ch-4.7 strains, respectively.

Activation energy (E_a) profiles for catalysis of substrate by parental strain *A. niger* FCBP–198 and its modified forms were resolved by applying Arrhenius plot (Fig. 5). The plots displayed a biphasic pattern both for native and mutant derivatives. However, the mutant strain An-UV-5.6 exhibited the E_a of 50.38 kJ mol^{-1} that was significantly higher as compared to activation energy modes displayed by protein of native (*A. niger* FCBP–198) as well as chemically modified α–amylases (43.14 and 44.06 kJ mol^{-1}, respectively).

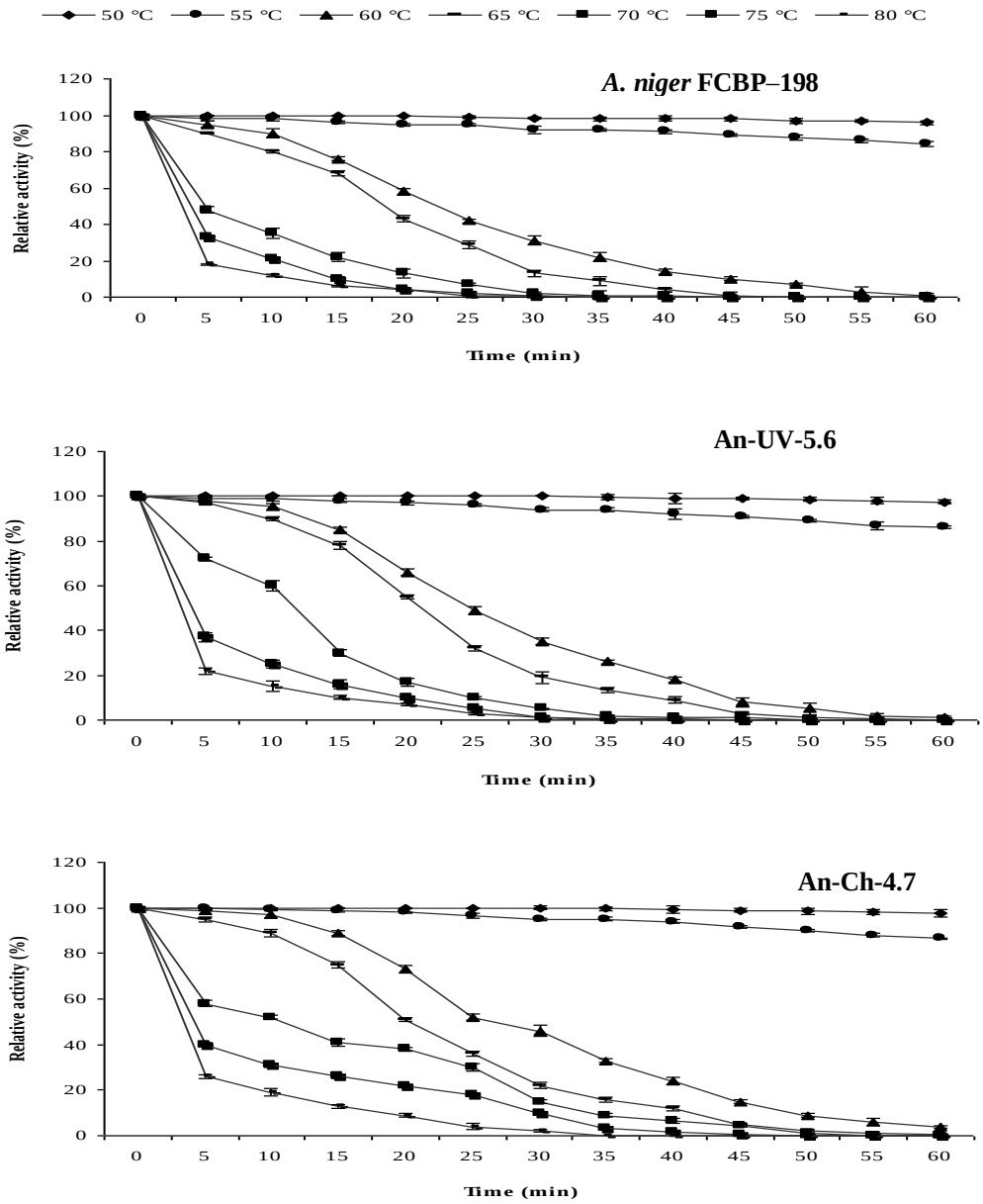

Fig. 6. Plot for irreversible thermostability of partially purified α–amylase enzyme of wild strain of *A. niger* FCBP–198 and mutants An-UV-5.6 and An-Ch-4.7.

Vertical bars indicate standard error of means of three replicates.; Substrate: 1% soluble starch, pH: 4.5.

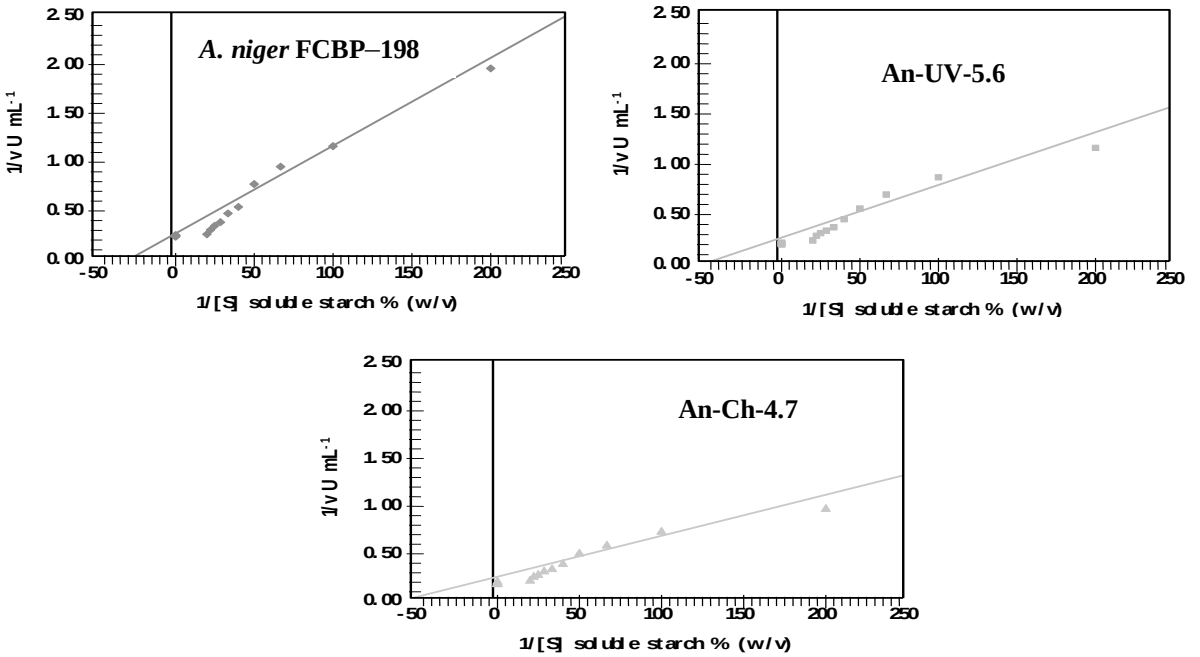

Fig. 7. Lineweaver–Burk plot of partially purified α–amylase enzyme from *A. niger* FCBP–198 and mutants An-UV-5.6 and An-Ch-4.7.

Effect on Thermostability: Thermostability is the aptitude of enzyme to resist thermal unfolding in the absence of substrate. The pseudo–first order plot was applied to deduce the extent of thermal inactivation (Fig. 6). The enzyme extracted from native as well as mutant strains exhibited 100% relative activity at 50 °C while the activity was partially lost after 15 min or above at 55 °C. A sharp decline was detected from 60 to 80 °C after 10 min of incubation. Approximately 50% of enzyme activity of all the test strains was lost within 10 min from 70 to 75 °C.

Effect of Substrate Concentration: The level of enzyme activity is markedly dependent upon the concentration of the starch. The enzyme was assessed at different substrate concentrations and Lineweaver–Burk plot was constructed for calculating the Michaelis Menten constant (V_{max}, K_m) (Fig. 7). The results showed that the α–amylase enzyme of An-UV-5.6 and An-Ch-4.7 mutants exhibited more activity (V_{max} – 5.26 and 5.56 Units mL^{-1}, respectively) as compared to parental strain, *A. niger* FCBP–198 (5.0 Units mL^{-1}). The parental strain displayed K_m value of 0.476 mg mL^{-1} being significantly higher than An-UV-5.6 and An-Ch-4.7 (0.25 and 0.21 mg mL^{-1}, respectively). It confirmed that the enzyme produced by mutants was more substrate specific than the parental type.

DISCUSSION

In fungi, detailed studies on α–amylase purification have largely been limited to a few species of fungi (Khoo *et al*., 1994; AbouZeid 1997; El-Safey and Ammar, 2004; Riaz *et al*., 2007). In present exploration, the enzyme was isolated and partially purified to homogeneity by ammonium sulfate fractionation. In SDS–PAGE analysis of the partially purified protein, isolated from wild and its mutants, presence of a protein of 20 kDa was encountered that seemed to involve in hydrolyzing activity as indicated by bioactivity assay of native gel. Similarly, purification and characterization of α–amylase by bacterium, *Bacillus licheniformis* CUMC305 followed by bioactivity staining was performed by (Krishnan and Chandra, 1983). They documented that the purified enzyme was homogenous with molecular weight of 28 kDa showing a single band of hydrolyzed starch on petri plate corresponding to the band pattern on the stained gel. Earlier, an extra cellular α–amylase from *Thermus* sp. was highly purified by affinity absorption on starch granules. SDS–PAGE showed a single band with molecular weight of 59 kDa. A clear–zone–forming band specified the manifestation of the active enzyme. The optimum pH and temperature of the enzyme was 5.5–6.5 and 70 °C, respectively (Shaw *et al*., 1995). The analysis of enzyme for molecular mass and amylolytic activity is in line with earlier findings in several investigations (Lin *et al*., 1998; Coral and Colak, 2000; Nagy *et al*., 2001; Coral *et al*., 2002; Saxena *et al*., 2007).

Importance in thermostable amylases has extremely improved, since resistance to thermal inactivation has become a desirable property in many industrial applications (Kumar and Satyanarayana, 2004; Voutilainen *et al*.,

2007). Hence thermophilic or thermostable organisms are of special interest to acquire thermostable enzymes. For this purpose; efficient species and strains are being developed and improved continuously by scientists through chemical modification and mutation (Chen and Stites, 2001; Khajeh *et al.*, 2001; Riaz *et al.*, 2007). Presently, the enzymatic properties of α–amylase from *A. niger* FCBP–198 and its mutants revealed optimal temperature of 60 °C and temperature quotient for the enzyme was 1.0010, 1.0012 and 1.0011 of *A. niger* FCBP–198, An-UV-5.6 and An-Ch-4.7 strains, respectively. The energy of activation (E_a) of An-UV-5.6 was 50.38 kJ mol^{-1} which was found to be significantly higher than required by parental (43.14 kJ mol^{-1}) and chemically modified α–amylases (44.06 kJ mol^{-1}). The enzyme was highly active at 50 °C while all the test strains displayed a sharp decline in activity after 10 min from 60 to 80 °C. These are the properties which are considered the most important for industrial starch liquefaction. In some earlier investigations by Khoo *et al*. (1994) and Chakraborty *et al*. (2000) the optimum temperature for purified α–amylase activity have been recorded at 55 °C and 50 °C, respectively. Likewise, Odibo and Ulbrich–Hofmann (2001) concluded that the optimum temperature for stable enzyme activity was 60 °C for α-amylase and 70 °C for glucoamylase.

The α–amylase activity of partially purified enzyme extracted from native as well as mutants was maximal at pH 4.5 with pKa_1 and pKa_2 of acidic and basic limbs of active site residues 3.8 and 6.65, 3.6 and 6.8 and 3.4 and 7.2, respectively. Likewise, the values of Michaelis–Menten constant K_m and V_{max} exhibited by *A. niger* FCBP-198, An-UV-5.6 and An-Ch-4.7 were 0.476, 0.25, 0.21 and 5.0, 5.26, 5.56, respectively. Similar to the present study, amylase enzyme isolated from fungal sources has been purified and characterized by many workers (Vandersall *et al*., 1995; Mohapatra *et al.*, 1998). Thermostable enzymes have been explored for the betterment of industrial processes of starch saccharification. A novel glucoamylase was purified from the *Fusarium solani* on a fast protein liquid chromatographic system (FPLC). The retrieval of enzyme after FPLC was 31.8% with 26.2–fold increase in specific activity having a molecular mass of 40 kDa by SDS–PAGE and 41 kDa by gel filtration. The glucoamylase exhibited optimum activity at pH 4.5. The K_{cat} and K_m were 441/min and 1.9 mg/ml, respectively, for soluble starch at specificity constant (K_{cat}/K_m) of 232. The enzyme was thermally stable at 50 °C and retained 79% activity after 60 min at this temperature (Bhatti *et al.*, 2007).

It is manifested from the present study that both activity and stability of α–amylase enzyme can be enhanced by mutagenesis. Mutation besides improving enzyme activity also enhanced the thermostability as well as V_{max} values of enzyme, highlighting increase in catalytic efficiency of the enzyme. Consequent decline in K_m values accounts for better affinity of mutant derived enzymes to bind with substrate to form a product. The augmented enzyme activity and thermostability are important characteristics that render enzyme from these mutants more useful in starch processing industry.

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