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Full Length Article

Effect of Metal Ions on Kinetics and Thermostability of α-Amylase Isolated from *Aspergillus oryzae*

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Abstract

Alpha amylase produced from *Aspergillus oryzae* after thermal treatment has been rarely investigated. The purpose of current research was to study the effect of metals on the activity and stability of α-amylase. Supplementation of Ca^{2+} and Co^{2+} improved the enzyme activity up to 2 folds, while rest of the metals did not affect the enzyme activity. The Michaelis-Menten constants (V_{max}, K_m and V_{max}/K_m) for soluble starch hydrolysis by α-apo-amylase were 65 U min^{-1} mg^{-1}, 2.01% (w/v) and 32.26 Umg^{-1}, respectively. The thermodynamics constants (ΔH^*, ΔG^* and ΔS^*) of irreversible thermal inactivation for Ca^{+2} bonded amylase were 154.49 kJ mol^{-1}, 106.09 kJ mol^{-1} and -147 J mol^{-1} k^{-1}, respectively. The Ca^{2+} ions made the α-amylase about 53 folds more stable as compared to the control. The thermostability of α-amylase was also increased after thermal treatment and become more heat tolerant which is necessary requirement for α-amylase to perform ideal result in different industries.

Keywords: Enzyme production; *Apergillus oryzae*; Kinetic parameters; Submerged culture; Metal ions; Thermostability

Introduction

Aspergillus oryzae is a probiotic filamentous fungus, widely used to produce a variety of proteins known for beneficial effects in pharmaceutical, beverages and conventional food industries (Lene *et al.*, 2000; Bozic *et al.*, 2011). *Aspergillus* sp. has gained much attention in modern biotechnology due to easy availability, proteins production, higher productivity and suitability for genetic manipulations. Therefore, different species of the genus *Aspergillus* such as *A. niger*, *A. oryzae*, *A. flavus*, *A. tamarie*, *A. fumigatus* and *A. kawachii* are being frequently used for the production of α-amylase, protease and glucoamylase etc. (Nagamine *et al.*, 2003; Ramachandran *et al.*, 2004; Rasooli *et al.*, 2008; Bakri *et al.*, 2009).

Amylases (EC 3.2.1.1) correspond about 25–33% of the total enzymes used for starch hydrolysis in various industries to produce maltose, glucose and a variety of alpha-limit dextrin containing α1-6 bond and a combination of malto oligosaccharides (Elayaraja *et al.*, 2011; Haq *et al.*, 2012; Shah *et al.*, 2014). Amylases are classified into exo amylases (β-amylase, glucoamylase), endo amylases (α-amylases) and debranching enzymes (isoamylase, pullulanase). Most of α-amylases need a metallic ion as cofactor, thus named as metalloenzyme. Mostly they need calcium ions for optimal function, prolonged stability and structural integrity. The α-amylase family is roughly categorized in two groups, one of which hydrolyses the starch while other modifies it. The enzymatic hydrolysis is favoured in starch processing industry used for acid hydrolysis due to many different benefits including stability of the generated products, the reaction specificity, removal of neutralization steps and minimum requirements for energy (Sathyanarayana *et al.*, 2005).

At industrial scale enzymes may be obtained from plant, animals and microbes, but the concentration of enzyme obtained from first two sources is limited while, starch processing industries require higher amounts of α-amylase. Hence, microbial sources have gained much attention for enzyme production in abundant quantities to meet the necessary industrial requirements. Furthermore, microbial enzymes are also quite useful due to enhanced features (Hussain *et al*., 2013). Different species of the genus *Aspergillus*, such as *A. niger* and *A. oryzae* are being used for the production of alpha amylase. Owing to rising demand for these enzymes transgenic strains have become focus of a number of industries. The enzyme produced by these mutant strains has higher stability to survive under

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extreme pH and temperature fluctuations at industrial level (Umbreen *et al*., 2013). Furthermore, it has been demonstrated that such strains require more dipositive ion as $Ca^{+2}$for thermostability (Niaz *et al*., 2010). Therefore, present study deals with the determination of metalo nature and kinetic mechanism of activation/inhibition of α-amylase from transgenic strain of *A. oryzae* (*Oryzae* mutant) induced by addition of calcium and cobalt ions.

Materials and Methods

All the chemicals used were of analytical grade and obtained from Sigma Chemical Company, USA. Soluble starch was purchased from Rafhan Maize Products (Pvt) Ltd, Faisalabad. The transgenic strain of *A. oryzae* (oryzae mutant) was obtained from IBD, NIBGE Faisalabad, culture was maintained on potato-dextrose agar medium (PDA) and preserved at 4°C (Rashid and Saddique, 1998).

Enzyme Production and Harvesting

For production of enzyme soluble starch (1% w/v) was added in 45 mL of Vogel's medium in 250 mL elnermeyer flask and was set at pH 5.0. About 7 glass beads were added in each flask after washing with water to rupture the mycelium. All flasks were capped with cotton, wrapped with aluminium foil and autoclaved at 121°C for 20 min and 1.05 kg/cm^2 pressure. The flasks were inoculated with the spores of *A. oryzae* and incubated on orbital shaker (orbital shaker incubator TI-OSI-HR) at 120 ± 5 rpm, 30°C for 72 h. After 72 h of incubation, α-amylase was harvested, filtered and centrifuged at 10,000 rpm at 4°C for 20 min. Finally the obtained culture was concentrated by freeze drying (Lypholizer Alpha1-5).

Enzyme Assay

The amylase activity was determined as described by (Huma *et al.,* 2012) using 1% soluble starch as a substrate and amount of released product was estimated by using di-nitro-salicylic acid (DNS) method. The reaction mixture (2100 µL) contained 1 mL of sodium acetate buffer (50 m*M*, pH 5), 1 mL of soluble starch (1% W/V) and 100 µL of enzyme extract. The reaction tubes were incubated at 45ºC for 30 min, after incubation the test tubes were dipped in boiling water for 5 min. The reaction was quenched rapidly by adding 2 mL of DNS solution and volume was made up to 4.1 mL, and then boiled in water bath for 10 min. The tubes were cooled in ice bath and the absorbance was measured accurately by spectrophotometer at 550 nm. The amount of maltose released was determined by maltose standard curve.

The enzyme activity was determined using the formula mentioned below:

$$\text{U mL}^{-1}\text{ min}^{-1} = \frac{\Delta A_{550}\text{ of sample} \times \text{Maltose standard factor (1.833µmol)} \times \text{Total reaction mixture (2.1 mL)}}{\text{Enzyme (0.1 mL)} \times \text{Time (30 min)} \times \text{Reaction mixture for colour development (1 mL)}}$$

One unit of alpha amylase activity was defined as the amount of enzyme required to release reducing sugars 1 µmole of maltose per minute at pH 5.0 at 45°C.

Bradford method was used to determine the total protein content in the solution (Bradford, 1976) using bovine serum albumin (BSA) as standard.

Optimization and Characterization of α-amylase from *A. oryzae*

Crude α-amylase obtained from the mutant strain of *A. oryzae* was partially purified by ammonium sulphate precipitation. Total proteins and alpha amylase activity were determined before and after dialysis of ammonium sulfate precipitation (Riaz *et al*., 2012). Enzyme activity of α-amylase produced by *A. oryzae* and purified was further analyzed for effect of different metals ($CaCl_2$, $MgCl_2$, $FeCl_2$, $MnCl_2$, $CoCl_2$, $AlCl_3$) in concentration range of 1–8 m*M* (Niaz *et al*., 2010). Moreover, apo-α-amylase was assayed in the presence of varied concentrations of $CaCl_2$ (6.0–7.0 m*M*) and $CoCl_2$ (6.0–7.0 m*M*) with temperature range 40–60°C and 34–55°C, respectively (Rashid and Saddique, 1998). The optimum pH of the alpha amylase isolated from *A. oryzae* was determined by measuring activity at different temperatures ranging from (35–50°C) against various pH ranging buffers from 3.8–10.4. The enzyme activity was determined in the presence of $CaCl_2$ (6.0 m*M*) and $CoCl_2$ (6.0 m*M*) (Dixon and Webb, 1979).

Kinetic Characterization of Enzyme

The activation energy for starch hydrolysis was determined by plotting the data of temperature optimum according to Arrhenius as described (Rashid and Saddique, 1998; Siddiqui *et al.,* 2000). Effect of calcium and cobalt ions on Michaelis-Menten kinetic constants (K_m, V_{max}, K_{cat}) were determined by using different concentrations of soluble starch as a substrate (0.025–0.6% w/v), while keeping the metal and enzyme concentrations constant. Line weaver Burk plot was used to determine the Michaelis-Menten constant (Rashid and Saddique, 1998; Siddiqui *et al.,* 2000). The enzyme was incubated at 45°C with 6 m*M* $CaCl_2$ and $CoCl_2$ in a separate test tube for 30 min. Irreversible thermal inactivation of metal treated α-amylase enzyme was estimated by incubating the enzyme at different temperatures (45, 48, 51, 57, 62, 65°C). Time course aliquots were withdrawn, cooled on ice for at least 30 min and then assayed for α-amylase activity at 45°C. The data was fitted to first order plots and analyzed (Umbreen *et al*., 2013).

Thermodynamics of Thermal Inactivation of α-amylase

The first order rate constant for irreversible thermal denaturation (K_d) of alpha amylase and the activation energy for denaturation (E_d) were determined using the Arrhenius plot. The thermodynamic parameters for thermostability were calculated by rearranging the Eyring's absolute rate

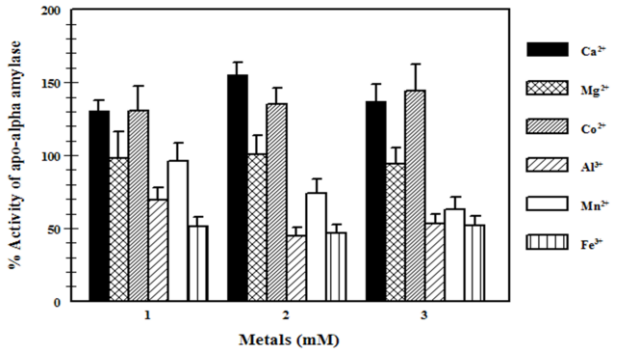

Fig. 1: Effect of metals on activity of α-amylases from *A. oryzae*. The presented data is an average values ± SD of three experiments

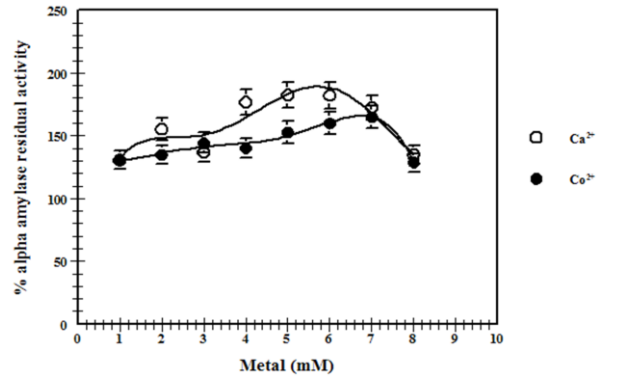

Fig. 2: Effect of various concentrations of calcium and cobalt on activity of α-amylases from *A. oryzae*. The presented data is an average values ± SD of three experiments

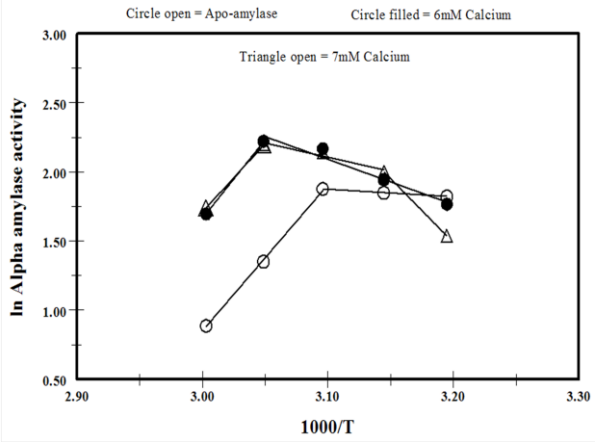

Fig. 3: Effect of $CaCl_2$ on energy of activation for soluble starch hydrolysis by *A. oryzae* α-amylase

equation derived from the transition state theory (Eyring and Stearn, 1939).

Statistical Analysis

All the analysis was made in triplicate and data was analyzed statistically. The mean and standard deviation of the values were calculated using SPSS (Version 17, Chicago, SPSS, Inc) and Slide Write Plus (Version 7.0.1, Advance Graphic Software, Inc.) was used to draw graphs.

Results

Effect of Metals on α-amylase Activity

Six different metals ($CaCl_2$, $CoCl_2$, $MnCl_2$, $FeCl_2$, $AlCl_3$ and $MgCl_2$) were used in different concentrations (1, 2 and 3 m*M*) to evaluate their effect on α-amylase activity. Results showed (Fig. 1) that $CaCl_2$ and $CoCl_2$ significantly ($p \leq 0.05$) activated the enzyme at all concentrations, while $MnCl_2$, $FeCl_2$, and $AlCl_3$ showed the inhibiting effect on the enzyme activity at all concentration. Maximum activity of 155% and 150% was observed with $CaCl_2$ (2 m*M* concentration) and $CoCl_2$ (3 m*M* concentration) respectively, while $FeCl_2$ and $AlCl_3$ inhibited the amylase activity at all concentrations (Fig. 1). Therefore, further characterization was carried out using different concentrations of $CaCl_2$ and $CoCl_2$ only. Various concentrations of calcium and cobalt (1–8 m*M*) were used to study their effect on pH and temperature optimum, kinetics of soluble starch hydrolysis and irreversible thermal stability of enzyme. Ca^{2+} showed the maximum activity (180%) at 6 m*M* concentration and Co^{2+} gave maximum activity (170%) at 7 m*M* concentration (Fig. 2).

Effect of Metals on Optimum Temperature and Activation Energy

The apo-α-amylase was assayed in the presence of different $CaCl_2$ (6–7 m*M*) concentrations with temperature range of 40–60°C. Effect of metals on temperature and activation energy (E_a) for soluble starch hydrolysis by α-amylase was determined by Arrhenius plot (Fig. 3). The activation energy of apo-α-amylase determined by Arrhenius plot was 4.58 kJ mol^{-1} at optimum temperature for the formation of enzyme substrate–complex. Furthermore, activation energy (E_a) of enzyme coupled with metals at optimum temperature (55°C) for calcium using 6.0 m*M* concentration was calculated to be 27.2 kJ mol^{-1} and for Co^{+2}, at optimum temperature (50°C) E_a was 15.68 kJ mol^{-1}.

Effect of Metals on pH Optimum

The α-amylase activity was determined in the presence of different pH values ranging from 3.8−10.4 at various temperatures. Apo-α-amylase from *A. oryzae* showed optimum pH 5.0 and enzyme coupled with $CaCl_2$ and $CoCl_2$ showed optimum pH of 7.5 and 5.6, respectively (Fig. 4 and 5). The pK_{a1} and pK_{a2} of active site ionizable

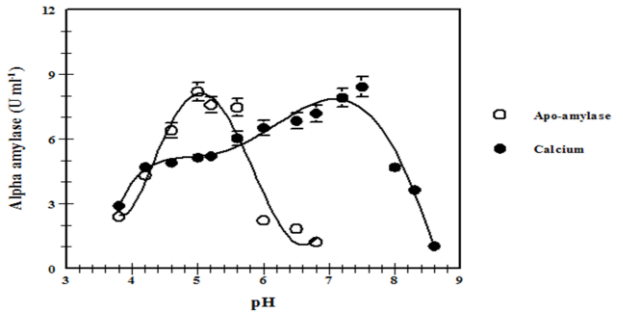

Fig. 4: Effect of $CaCl_2$ (6.0 m*M*) on optimum pH of α-amylase from *A. oryzae*. The presented data is an average values ± SD of three experiments

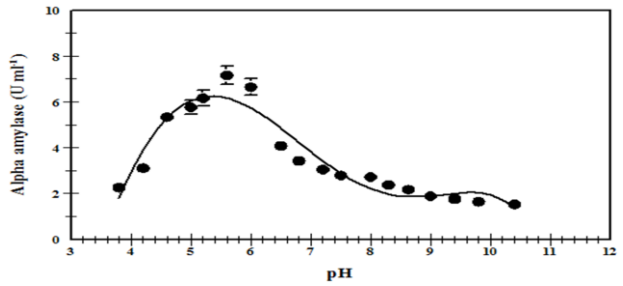

Fig. 5: Effect of $CoCl_2$ (6.0 m*M*) on optimum pH of α-amylase from *A. oryzae*. The presented data is an average values ± SD of three experiments

groups were determined by applying Dixon plot. The pKa_1 and pKa_2 of ionizable groups of apo α-amylase were 4.7 and 5.6, respectively, while calcium and cobalt ions bound α-amylase showed pKa_1 and pKa_2 of 5.4 & 6.9 and 4.95 & 6.75, respectively (Fig. 6 and 7).

Effect of Metals on Substrate Hydrolysis

The α-amylase was assayed at various substrate concentrations at 50°C and pH 5.0. Double reciprocal plot (Line-Weaver Burk plot) was applied to determine V_{max} and Km. The V_{max} and K_m of Apo α-amylase was 65 U min^{-1} mg^{-1} protein and 2.0% (w/v), respectively. Calcium ions activated the α-amylase of *A. oryzae* by increasing V_{max} at all $CaCl_2$ concentrations (Fig. 8). Maximum activation was observed at 6.0 m*M* $CaCl_2$ and V_{max} at this concentration was 662 U min^{-1} mg^{-1}. The value of K_m decreased at this concentration 10.0% (w/v). While in case of Cobalt ions, V_{max} and K_m decreased at 6.0 m*M* concentration (Fig. 9). V_{max} and K_m values for $CoCl_2$ were 246 Umin^{-1} mg^{-1} and 6.67% (w/v). For $CaCl_2$ at 6.0 m*M* concentration, the specificity constant (V_{max}/K_m) for soluble starch hydrolysis was 66.23 while in the case of $CoCl_2$ and apo-enzyme, the (V_{max}/K_m) values was 39.61 and 32.26, respectively.

Effect of Metals on Irreversible Thermal Stability

The apo-α-amylase from *A. oryzae* and metal treated

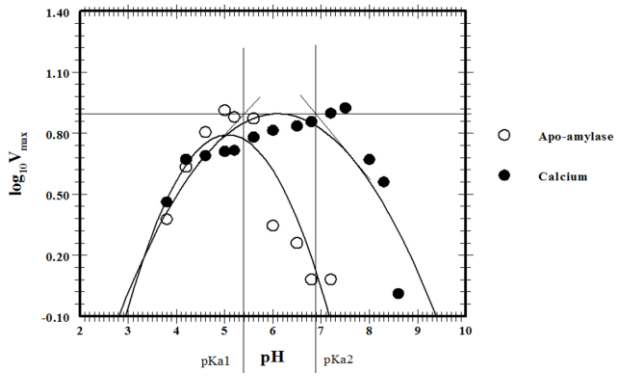

Fig. 6: Dixon plot for the determination of effect of $CaCl_2$ (6.0 m*M*) on p*K*a values of active site residues of α-amylase from *A. oryzae*

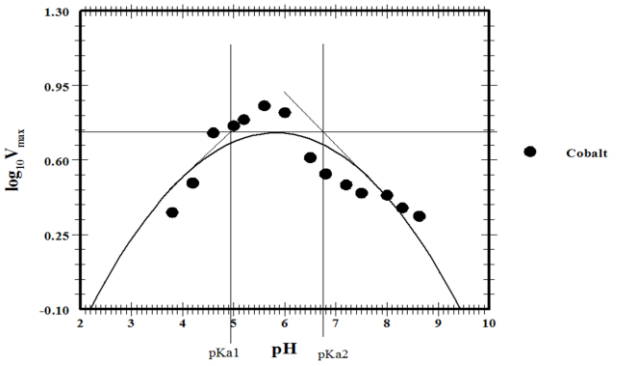

Fig. 7: Dixon plot for the determination of effect of $CoCl_2$ (6.0 m*M*) on p*K*a values of active site residues of α-amylase from *A. oryzae*

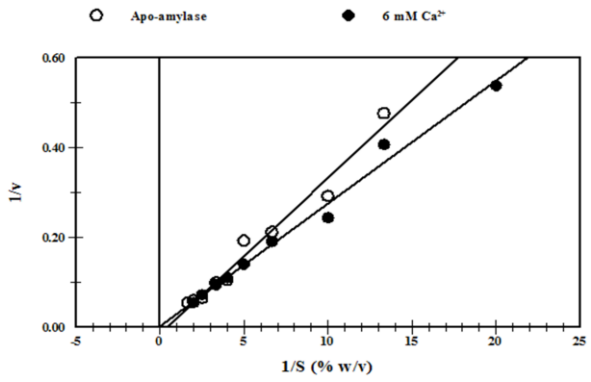

Fig. 8: Double reciprocal plot for the determination of effect of $CaCl_2$ (6.0 m*M*) on kinetic constants (V_{max} & K_m) for soluble starch hydrolysis by α-amylase of *A. oryzae*

enzyme was heated at different temperatures ranging from 45-57°C for 75 min. Aliquots were taken at regular time intervals 0 (control), 5, 10, 15, 20, 30, 40, 50, 60 and 75 min. Pseudo first order plots were applied to determine the rate of irreversible thermal inactivation (Fig. 10, 11,

Table 1: Kinetics and thermodynamics of irreversible thermostability of apo-α-amylase from *A. oryzae*

Temp (°C)	Temp (K)	K_d (min^{-1})	t½ (min)	ΔH^* (kJ mol^{-1})	ΔG^* (kJmol^{-1})	ΔS^* (Jmol^{-1}K^{-1})
45	318	0.01439	48	95.22	100.09	-15
48	321	0.01116	62	95.19	101.74	-20
51	324	0.005289	131	95.17	104.72	-29
57	330	0.04677	15	95.12	100.73	-17

K_d (first order rate constant of denaturation) was determined from Fig. 10
t½ (half-life) = 0.693/K_d
$\Delta H^* = Ea_{(d)}$ –RT. $Ea_{(d)}$ of apo-amylase was 97.86 kJ mol^{-1} and was calculated from Fig. 13
ΔG^* = –RT In ($K_d h/k_b$.T)
$\Delta S^* = (\Delta H^* - \Delta G^*)/T$

Table 2: Effect of $CoCl_2$ on kinetics and thermodynamics of irreversible thermostability of α-amylase from *A. oryzae*

Temp (°C)	Temp (K)	K_d (min^{-1})	t½ (min)	ΔH^* (kJ mol^{-1})	ΔG^* (kJmol^{-1})	ΔS^* (Jmol^{-1}K^{-1})
45	318	0.005161	134	59.47	102.8	-136
48	321	0.01046	66	59.45	101.91	-132
51	324	0.00709	98	59.42	103.94	-137
57	330	0.01445	48	59.37	103.96	-135

K_d (first order rate constant of denaturation) was determined from Fig. 12
t½ (half-life) = 0.693/K_d
$\Delta H^* = Ea_{(d)}$ –RT. $Ea_{(d)}$ of Co^{2+} bound amylase was 62.11 kJ mol^{-1} and was calculated from Fig.4.13
ΔG^* = –RT In ($K_d h/k_b$.T) ; $\Delta S^* = (\Delta H^* - \Delta G^*)/T$

Table 3: Effect of $CaCl_2$ on kinetics and thermodynamics of irreversible thermostability of α-amylase from *A. oryzae*

Temp (°C)	Temp (K)	K_d (min^{-1})	t½ (min)	ΔH^* (kJ mol^{-1})	ΔG^* (kJmol^{-1})	ΔS^* (Jmol^{-1}K^{-1})
45	318	0.00041	1691	154.59	109.49	142
48	321	0.006679	104	154.56	103.11	160
51	324	0.000099	7001	154.54	115.44	121
57	330	0.006647	104	154.49	106.09	147

K_d (first order rate constant of denaturation) was determined from Fig 11.
t½ (half-life) = 0.693/K_d
$\Delta H^* = Ea_{(d)}$ –RT. $Ea_{(d)}$ of Ca^{2+} bound amylase was 157.23 kJ mol^{-1} and was calculated from Fig. 13
ΔG^* = –RT In ($K_d h/k_b$.T); $\Delta S^* = (\Delta H^* - \Delta G^*)/T$

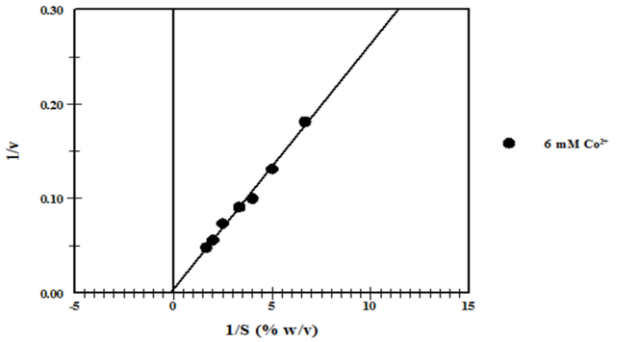

Fig. 9: Double reciprocal plot for the determination of effect of $CoCl_2$ (6.0 m*M*) on kinetic constants (V_{max} & K_m) for soluble starch hydrolysis by α-amylase of *A. oryzae*

12 and 13). The half-life ($t_{1/2}$) and thermodynamic parameters of irreversible thermal stability (ΔH^*, ΔG^* and ΔS^*) of α-amylase were calculated as described (Siddiqui *et al.*, 2000) and are presented in Table 1, 2 and 3. The energy of activation for irreversible thermal inactivation ($Ea_{(d)}$) of apo, Ca^{+2}, Co^{+2} bound was 97.86, 157.23, 62.11 kJ mol^{-1}, respectively, which was determined by Arrhenius plot.Co^{+2} bound enzyme showed least value of $Ea_{(d)}$. The binding of the metals ions with α-amylase resulted in increased half life. Ca^{2+} showed increase in half life as compared to the apo-enzyme. While the Co^{2+} showed decrease in half-life as compared to the apo and calcium binding enzyme. The half-life of apo, Ca^{2+}, Co^{2+} bound α-amylase at 51°C was 131, 7001, and 98 min, respectively. The ΔG*, ΔH* and ΔS* of apo α-amylase, Ca^{2+}, Co^{2+} bound α-amylase at 51°C were (104.72 kJ mol^{-1}, 95.17 kJ mol^{-1}, -29 J mol^{-1} k^{-1}), (115.44 kJ mol^{-1}, 154.54 kJ mol^{-1}, 121 Jmol^{-1}K^{-1}), (103.94 kJ mol^{-1}, 59.42 kJ mol^{-1}, -137 Jmol^{-1} k^{-1}), respectively.

Discussion

Amylases are called metaloenzymes and have at least one cation as necessary component for enzyme activity. Selection of cation is important for survival of enzyme during industrial applications at high temperature and pH fluctuation (Zohra *et al.*, 2014). Therefore, effect of various metals ($CaCl_2$, $CoCl_2$, $MnCl_2$, $FeCl_2$, $AlCl_3$ and $MgCl_2$) was evaluated on α-amylase activity. Among all metals Ca^{2+} and Co^{2+} increased the enzyme activity while rest of the metals inhibited α-amylase activity at all concentrations. In accordance with the present study, another research

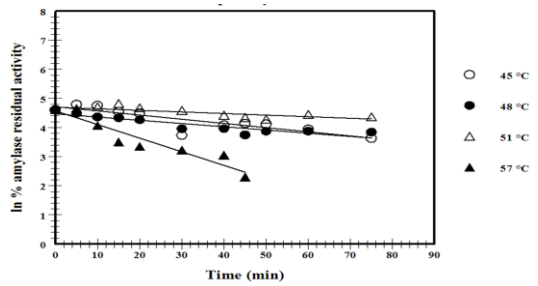

Fig. 10: Pseudo first order plots for irreversible thermal stability of *A. oryzae* apo-α-amylase

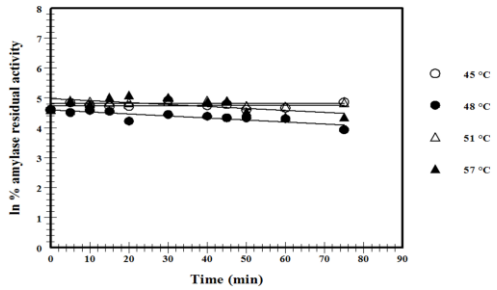

Fig. 11: Pseudo first order plots for the effect of Ca^{2+}on irreversible thermal stability of *A. oryzae* α-amylase

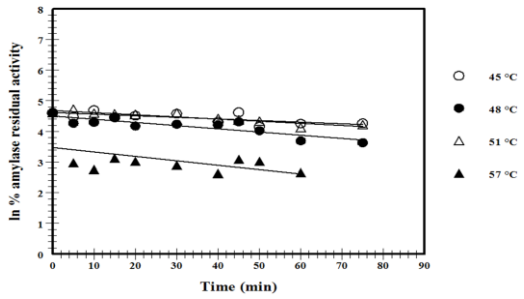

Fig. 12: Pseudo first order plots for the effect of Co^{2+}on irreversible thermal stability of *A. oryzae* α-amylase

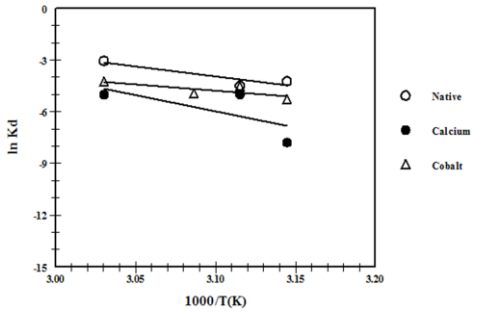

Fig. 13: Arrhenius plots for the determination of $Ea_{(d)}$ of irreversible thermal inactivation of *A. oryzae* α-amylase

work showed that different metals Fe^{3+}, Cu^{2+}, Zn^{2+} and Al^{3+} strongly inhibit the α-amylase while Ca^{2+} stimulate the enzyme activity (Shafie *et al.*, 2010). Furthermore, various concentrations of Ca^{2+} and Co^{2+} enhanced the enzyme activity to a certain level, after which a fall in activity was observed. Similarly, a significant stimulatory effect in enzyme activity was observed on addition of Co^{2+} with α-amylase by Prakash *et al*. (2011), whereas Afifi *et al*. (2008) did not observe any significant effect of this metal on enzyme activity. Moreover, in a study conducted by Zohra *et al*. (2014) it was declared that Ca^{2+} has stimulatory effect on α-amylase activity. Temperature has a significant role at industrial processes so stability of enzyme was checked under different temperature conditions on addition of Ca^{2+} and Co^{2+}. The activation energy of apo-α-amylase determined by Arrhenius plot for the formation of enzyme substrate–complex was less than that of $Ca^{2+}$and Co^{2+}. Contrary to the present study, Ahmed *et al*. (2008) observed the temperature optima range between 30-40°C for the alpha amylase produced from *A. niger*. Similarly activation energy (E_a) of free enzyme was 2.37 Kcal mol^{-1} which was higher than the immobilized enzyme by covalent binding or by ionic binding 1.05 and 1.59 $Kcalmol^{-1}$, respectively (Ahmed *et al*., 2008). The difference in optimum temperature might be attributed to the strain difference and mutated strain used in present study may have different temperature requirements.

The pH of enzyme has enormous role in survival during industrial processes and most of native enzymes lose activity at higher pH values (Umbreen *et al*., 2013). α-amylase from *A. oryzae* showed lower optimum pH as compared to enzyme coupled with $CaCl_2$ and $CoCl_2$. So, the enzyme activity was observed to increase by coupling the enzyme with $CaCl_2$ and $CoCl_2$. In accordance with the present study, the production of extracellular thermostable α-amylase from moderate thermophilic *Bacillus* strain, isolated from fresh sheep's milk showed maximal activity at pH 6.5 and stability was improved in the presence of Ca^{2+} ion (Konsula and Liakopoulou, 2004). Furthermore it was observed that calcium ions activated the α-amylase of *A. oryzae* and V_{max} was increased at all $CaCl_2$ concentrations, while Cobalt ion resulted in decreased activity. Similarly, study conducted on the purification and characterization of alpha amylase from a bacterial strain of *Bacillus licheniformis* EMS-6 showed that enzyme was stable at the pH range 4.5-9.0 and optimum pH 7.0 (Haq *et al*., 2002).

It has been approved that metals binding with enzymes increases the protein stability. Moreover, the folded proteins are more stable by binding of the metals ions which is coordinated by donating of the lone pair from nitrogen and oxygen atoms. The present study showed that the enzyme was stable at optimum temperature but at high temperature the enzyme is denatured. Thermal denaturation of enzymes results in dissociation of subunit dissociation with an increased enthalpy (Umbreen *et al*., 2013) ultimately leading to increased entropy of activation. Increase in Gibb's free energy (ΔG^*) on addition of metals specially calcium ions showed that enzyme became more stable under high temperature as required by industrial processing. Furthermore negative value of enthalpy on addition of

cobalt ion predicts less disorder in structure of enzyme so showing higher stability. In accordance with the present study Haq *et al*. (2010) observed that thermodynamic parameters Ea, ΔS, ΔG and ΔH for soluble starch hydrolysis of α-amylase from *B. licheniformis* EMS-6 also increased.

Conclusion

In this research work, the effect of metals on kinetics of α-amylase isolated from *A. oryzae* was evaluated. $CaCl_2$ activated the enzyme at all concentrations (1−8 mM) while other metals inhibited the enzyme activity. Ca^{2+} ions improved the temperature optimum with 5°C increase as compared to the Co^{2+} and apo-enzyme. $E_{(a)}$ of Ca^{2+} 27.2 k J mol^{-1}. Ca^{2+} ions shifted the pH optimum toward basic site. Moreover the pka values confirm that the binding of Ca^{2+} withα-amylase change the micro environment of the active site. Ca^{2+} showed the 10 fold activation of α-amylase because the V_{max} value was increased by 10 folds. The affinity of soluble starch to bind with α-amylase seriously affected in the presence of ions. The value of K_m was 5 fold increased. The specificity constant (V_{max}/K_m) confirmed that the Ca^{2+} ions increased the specificity of soluble starch as compared to the apo-enzyme. The Ca^{2+} ions made the α-amylase about 53 folds more stable as compared to the control (apo-enzyme).

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