KINETICS AND THERMODYNAMIC STUDIES OF ALPHA AMYLASE FROM *BACILLUS LICHENIFORMIS* MUTANT

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Abstract

The present investigation deals with the purification and characterization of enzyme α-amylase from a mutant strain of *Bacillus licheniformis* EMS-6. A laboratory scale stirred fermentor of 7.5 L capacity was used for the enzyme production under optimal conditions. The enzyme was purified up to homogeneity level by Ammonium sulphate and ion-exchange chromatography using a fast protein liquid chromatography (FPLC) system. The specific activity of the enzyme increased 4-5 times while the yield was found to be 40.4%. The purification fold by RESOURCE-S was recorded to be 3.58. The molecular weight was found to be 55 KDa. In the present research work, the V_{max} (2778 U/mg/min) and K_m (8.3mg/ml) of α-amylase were derived from the Lineweaver Burke plot. Thermodynamic parameters for soluble starch hydrolysis, E_a, ΔH, ΔS and ΔG of α-amylase from *B. licheniformis* EMS-6 were found to be 25.14 KJ/mol, 22.53 KJ/mole, -110.95J/mole/K and 36968 J/mole, respectively. The enzyme was stable over a pH range of 4.5-9.0 and gave pH optimum of 7.0. The pKa_1 and pKa_2 of ionizable groups of active site controlling V_{max}, determined by Dixon plot, were 6.0 and 7.5, respectively.

Introduction

Alpha amylase (EC 3.2.1.1), hydrolyzes the internal α-1, 4 linkages in starch in a random fashion leading to the formation of soluble maltodextrins, maltose, and glucose. This enzyme is extensively used in starch liquefaction, brewing, food, paper, textile and pharmaceutical industries (Rasiah & Rehm, 2009; Rajagopalan & Krishnan, 2008; Gangadharan *et al*., 2008; Thippeswamy *et al*., 2006; Akpan *et al*., 2004). Highly active enzyme is generally required for the conversion of starch into oligosaccharides. So, it is worthwhile to select a potent strain of microorganism for enzyme production. *Bacillus* species such as *B. amyloliquefaciens, B. subtilis*, *B. licheniformis B. stearothermophilus* are known as potent producers (Rasiah & Rehm, 2009; Gangadharan *et al*., 2006). The amylolytic bacterial cultures normally grow at pH ranging from 4.5-10.5 while enzyme activity remains optimal at 5.5-8.0 (Goyal *et al*., 1995; Takasaki *at al*., 1994). The optimal temperature for enzyme activity has been reported from 40-60ºC; while in the presence of Ca^{+2} ions, it was increased to about 75ºC. Enzyme production, purification and characterization are a growing field of biotechnology. The purification of α-amylase from the fermented broth is essential for stability and characterization (Declan *et al*., 1997). The enzyme has been purified by Ammonium sulphate fractionation, acetone precipitation, ion exchange chromatography and hydrophobic interaction chromatography (Najafi *et al*., 2005; Swain & Ray, 2007; Gangadharan *et al*., 2008). Thermodynamics and activation parameters provide a detailed mechanism for many chemical and biological reactions (Tanaka & Hoshino, 2003). It is necessary to have enzyme kinetic information for any enzymatic process. Poor enzyme stability under standard conditions of pH, temperature and pH inhibition affects the end product yield (Riaz *et al*., 2007).

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In this research work, we report the purification, kinetic and thermodynamic studies of a novel α-amylase from a mutant strain of *Bacillus licheniformis* EMS-6. The mutant was previously obtained after treating with ethyl methane sulphonate (EMS) using antimetabolite 2-deoxy-D-glucose.

Materials and Methods

Microorganism and enzyme production: Bacterial strain (*Bacillus licheniformis* EMS-6), obtained from the available stock culture of IIB, was maintained on nutrient agar medium (g/l: nutrient broth 8.0, agar 20, pH 7.0). Stirred fermentor studies were carried out in a 7.5 L glass fermentor (Bioflow 110, New Brunswick Scientific, USA), with 5.0 L working volume provided with two impellers and six blades. The fermentation medium which was already optimized was prepared by dissolving following (g/l) soluble starch 10, nutrient broth 8.0, $(NH_4)_2SO_4$ 2.25, $(NH_4)_2HPO_4$ 1.0, NaCl 0.85, $MgSO_4.7H_2O$ 0.5, $CaCl_2$ 0.1 in phosphate buffer (0.02 M) pH 7.0. The vegetative inoculum (24 h old, 2.45×10^7 CFU) at 8.0% level was used to inoculate 5 L of sterile fermentation medium under aseptic conditions. The incubation temperature was kept at 37°C, while the aeration and agitation rates were maintained at 1.0 vvm and 200 rpm, respectively. The air was sterilized by passing through a membrane filter (0.45 μm). Sterilized silicone oil (10%) was used to control foaming. Periodically harvested fermented broth was centrifuged (at $6000 \times g$ for 10 min) and supernatant was analysed for α-amylase activity and biomass.

Enzyme assay: The enzyme assay was performed after Rick & Stegbauer (1974). The enzyme extract (0.5 ml) was transferred to a test tube containing 0.5 ml of 1.0% soluble starch solution. The mixture was incubated at 60ºC for 10 min. Then 1.0 ml of dinitrosalicylic acid reagent (DNS) was added to each test tube. The tubes were placed in boiling water for 5 min and cooled at room temperature. The contents of tubes were diluted up to 10 ml with distilled water. The absorbance was determined at 546 nm using a spectrophotometer and converted to mg of maltose from the standard. One unit is equivalent to that amount of enzyme, which catalyse the hydrolysis of soluble starch into 1.0 mg maltose hydrate per minute under standard assay conditions. Total protein content was estimated by the Bradford method using bovine serum albumin as the standard (Bradford, 1976).

Enzyme purification: Crude α-amylase was purified to homogeneity using Ammonium sulfate precipitation and fast protein liquid chromatography (FPLC) using RESOURSE S (Amersham Biosciences, USA). Solid Ammonium sulfate was slowly added to crude enzyme to 80% saturation at 0°C to precipitate the α-amylase with stirring at an interval of 5 min. The pellet was collected by centrifugation at $12000 \times g$ for 20 min (4ºC) to recover precipitated proteins. The pellet was dissolved in 3.0 ml of phosphate buffer (pH 7.2) and dialyzed against 0.05 M Sodium phosphate buffer (pH 7.5) by constant stirring for 24 h and periodic change of buffer. Visking dialysis membrane with a molecular weight of 12000-14000 Da was used. The dialyzed sample was filtered through 0.4 μm Millipore filter and loaded on an anion exchange column RESOURCE-S (6.0ml, 16 mm x 30 mm; *Amersham Biosciences*) equilibrated with 50 mM Sodium phosphate buffer (pH 7.5) with a flow rate of 1.0 ml/min. The protein was eluted with a linear gradient of NaCl (0 to 1 M) in the buffer. The elution profile was examined at 280 nm. The fractions were collected and assayed for enzyme activity.

Molecular mass determination: Molecular mass was determined by SDS PAGE (12% acrylamide gel) using Bio-Rad miniprotean II electrophoresis unit (Laemmli, 1970). The protein marker ladder ranged 10-200 kDa (*Fermentas*) was used.

Kinetics and thermodynamics of starch hydrolysis: The kinetic constants (V_{max}, K_m, K_{cat}) were derived from Lineweaver Burke plot using equation 1 & 2. From the straight line equation 3, ΔH^* was calculated as slope and $\ln (K_B/h) + \Delta S^*/R$ as intercept on Y-axis from Arrhenius plot (Copeland, 2000).

$$1/v = (1/V_{max}) + (K_m/V_{max}) \cdot 1/[S] \qquad (1)$$

$$K_{cat} = V_{max}/[e] \qquad (2)$$

where [e] = Molar conc. of enzyme

$$\ln (V_{max}/T) = \ln (K_B/h) + \Delta S^*/R - \Delta H^*/RT \qquad (3)$$

where, T, K_B, h, R, ΔH^* and ΔS^* are absolute temperature (K), Boltzmann constant (1.38×10^{-23} J/K), Planck's constant (6.626×10^{-34} Js), Gas constant (8.314 J K^{-1} mol^{-1}), enthalpy of activation and entropy of activation, respectively.

$$\Delta G = \Delta H - T\Delta S \qquad (4)$$

where ΔG is change in free energy of the system at constant temperature

Dixon plot was used to calculate pK_{a1} and pK_{a2} values of ionizable groups of active site residues (Rajoka *et al.*, 2005).

Results and Discussion

Purification of alpha amylase: The biosynthesis of α-amylase from *B. licheniformis* EMS-6 was carried out in 7.5 L glass fermentor (Bioflow 110, New Brunswick Scientific, USA). The purification of α-amylase is summarized in Table 1. The enzyme produced was purified up to homogeneity level by three steps purification. Ammonium sulfate precipitation was employed for the partial purification of the enzyme. Maximum enzyme activity was obtained in the 80% fraction with a high yield of 44.53% and specific activity of 1374. The collected fraction was dialyzed against 0.05 M Sodium phosphate buffer (pH 7.5) and subjected to FPLC using RESOURSE-S column (Fig. 1). The purification fold was recorded to be 3.58 (Table 1). The molecular characterization of α-amylase was conducted with SDS-PAGE after purification of the enzyme by ion-exchange chromatography. It was estimated that molecular weight is 55 kDa (Fig. 2). In a similar study, Duy & Fitter (2005) and Rao *et al.*, (2002) reported molecular weight of raw starch digesting α-amylase from *B. lichenifromis* is 58.274 and 58 kDa, respectively.

Table 1. Overall purification summary of α-amylase from *B. licheniformis* EMS-6.

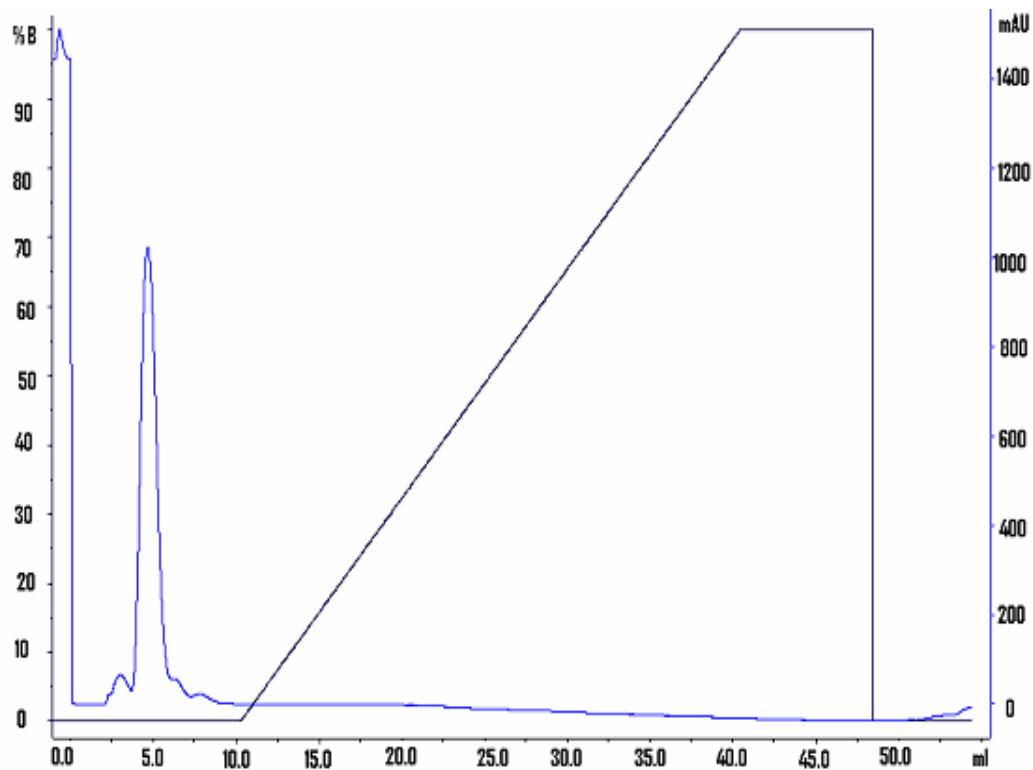| Sample	Volume (ml)	Total units	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	180	21600	28.54	756.83	**100**	1.0
(NH₄)₂SO₄ precipitation	3.0	9620	7.04	1366	**44.53**	1.815
Ion-exchange chromatography (RESOURCE Q)	5.0	7250	3.55	2042	**33.56**	2.8
Ion-exchange chromatography (RESOURCE S)	2.0	5970	2.24	2665	**27.64**	3.58

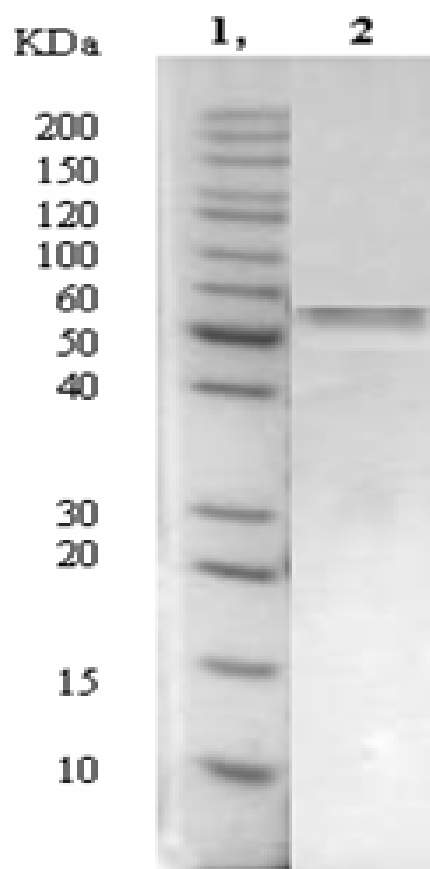Fig. 1. Purification of α-amylase using FPLC using RESOURSE-S column.

Fig. 2. SDS-Page analysis of purified α-amylase from *B. licheniformis* EMS-6. Lane 1: Protein marker (Fermentos SM 0671) and Lane 2: Purified *B. licheniformis* α-amylase.

Kinetic constants of starch hydrolysis: The kinetic constants (V_{max}, k_m, k_{cat}, and k_{cat}/k_m) for α-amylase from *B. licheniformis* EMS-6 were determined by incubating fixed amount of enzyme with varied concentrations of soluble starch as a substrate (0.1 to 1.2%). The enzyme followed the Michealis Menten kinetics of catalysis. The K_m and V_{max} values of different enzymes are difficult to compare as they depend on the substrate used and the reaction conditions. In present research work, the V_{max} and K_m of α-amylase were derived from the Lineweaver Burke plot and found to be 2778 U/mg/min and 8.3mg/ml, respectively (Fig. 3). Low values of Km indicate high affinity of the enzyme for the substrate (Hamilton *et al.*, 1998). Gangadharan *et al.*, (2008) reported K_m and V_{max} values for soluble starch to be 4.11 mg/min and 3.076 mg at 50°C, respectively, for α-amylase from *B. amyloliquefaciens*. For α-amylase from *Lactobacillus manihotivorans*, K_m and V_{max} values were 3.44 mg/ml and 0.45 mg hydrolyzed starch/ml/min at 50°C, respectively (Goyal *et al.*, 2005). The immobilized alpha-amylase from *Bacillus licheniformis* showed Michaelis-Menten enzyme kinetics exerting a V_{max} of about 506 U/mg of bead protein with a K_m of about 5 µM, consistent with that of free alpha-amylase (Rasiah & Rehm, 2009).

The turnover number k_{cat} corresponds to the maximum number of substrate molecules converted to product per active site per unit of time. The ratio k_{cat}/k_m often referred to as the 'specificity constant' is a useful index for comparing the relative rates of enzyme acting on alternative, competing substrates (Eisentha *et al.*, 2007). Therefore, the ratio of k_{cat}/k_m values is an estimation of enzyme specificity only in the sense of discrimination between two competing substrates, as demonstrated by Fersht (1999). The common strategy used to maintain sustainable activity is to produce enzyme with enhanced catalytic efficiency (k_{cat}/k_m). For extracellular enzymes that work at saturating substrate concentrations, adaptation consists mainly of increasing k_{cat} (Amico *et al.*, 2002). Alternatively, for secreted enzymes or intracellular enzymes that could face low substrate concentrations, a decrease in k_m (Michaelis–Menten constant) providing a higher substrate affinity could be useful. In present studies, k_{cat} = 152.8/min and specificity constant (k_{cat}/k_m) was 184.09 (Fig. 3). However, for α-amylase from *Pseudoalteromonas haloplanktis* the values of k_{cat}, k_m and k_{cat}/k_m, using 3.5 mM 4-nitrophenyl-α-D-maltoheptaoside-4,6-O-ethylidene as substrate, were 697 s^{-1}, 234 µM and 2.98 $s^{-1}µM^{-1}$, respectively (Amico *et al.*, 2002) whereas, Tanaka & Hoshino (2003) reported k_{cat} for α-amylase from *B. amyloliquefaciens* 2.26 ×10^3 s^{-1}.

Temperature optima, activation energy and thermodynamics of starch hydrolysis: Thermodynamics and activation parameters provide a detailed mechanism for many chemical and biological reactions (Tanaka & Hoshino, 2003). The optimum temperature range of the purified α-amylase from *B. licheniformis* EMS-6 for hydrolysis of soluble starch was found to be 60- 70ºC. There was decline in V_{max} beyond this range according to Arrhenius plot (Fig. 5). This decline is due to structural unfolding transition at high temperature (Duy & Fitter, 2005). The activation energy (E_a) calculated from Arrhenius plots for α-amylase from *B. licheniformis* EMS-6 was 25.14 KJ/mol in present study (Fig. 5). However, Duy & Fitter (2005) reported E_a around 363.7 kJmol^{-1} at 70ºC for *B. licheniformis* α-amylase. Whereas, a comparable large E_a value was reported for *Aspergillus oryzae* α-amylase (317.9 kJmol^{-1}) seems to be counterbalanced by a relatively large entropic contribution, which leads to its rather low thermo stability.
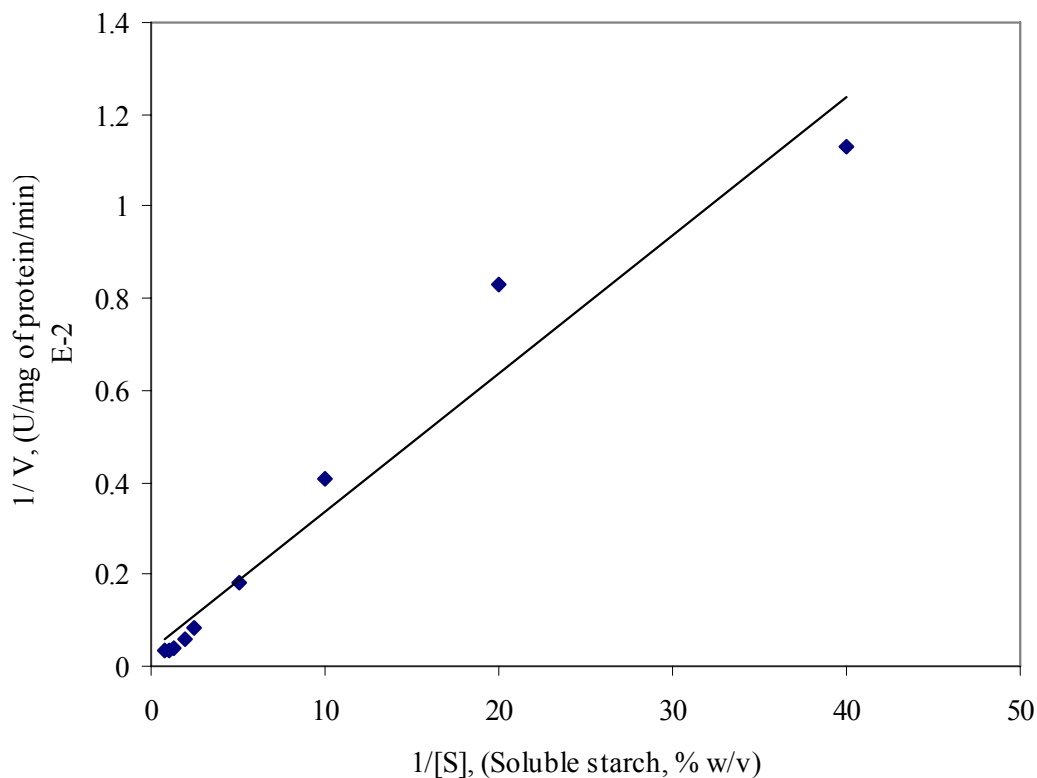
Fig. 3. Lineweaver-Burke double reciprocal plot of α-amylase from *B. licheniformis* EMS-6. All the samples were assayed in 0.02 M phosphate buffer, pH 7.0, at 60ºC containing variable amount of starch. The intercept on the y-axis corresponds to 1/Vmax and the intercept on x-axis to -1/Km. Data presented is an average of values ± S.D. of n=3 experiments.

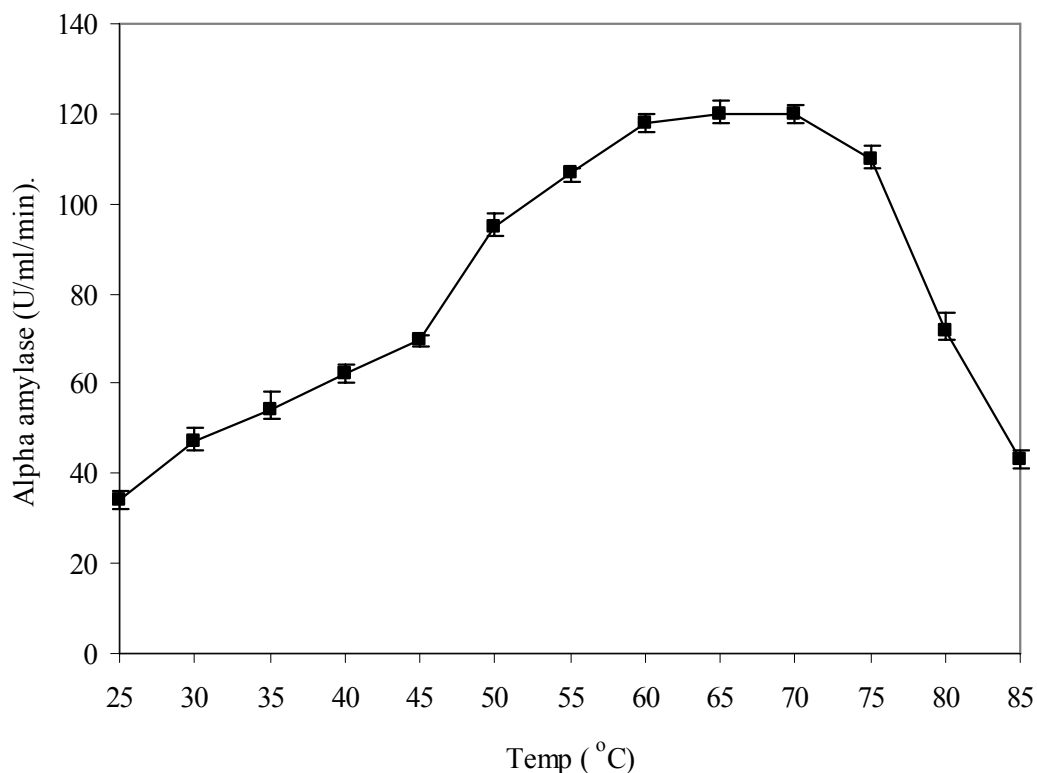Fig. 4. Effect of temperature on the activity of α-amylase from *B. licheniformis* EMS-6. Data presented is an average of values ± S.D. of n=3 experiments.
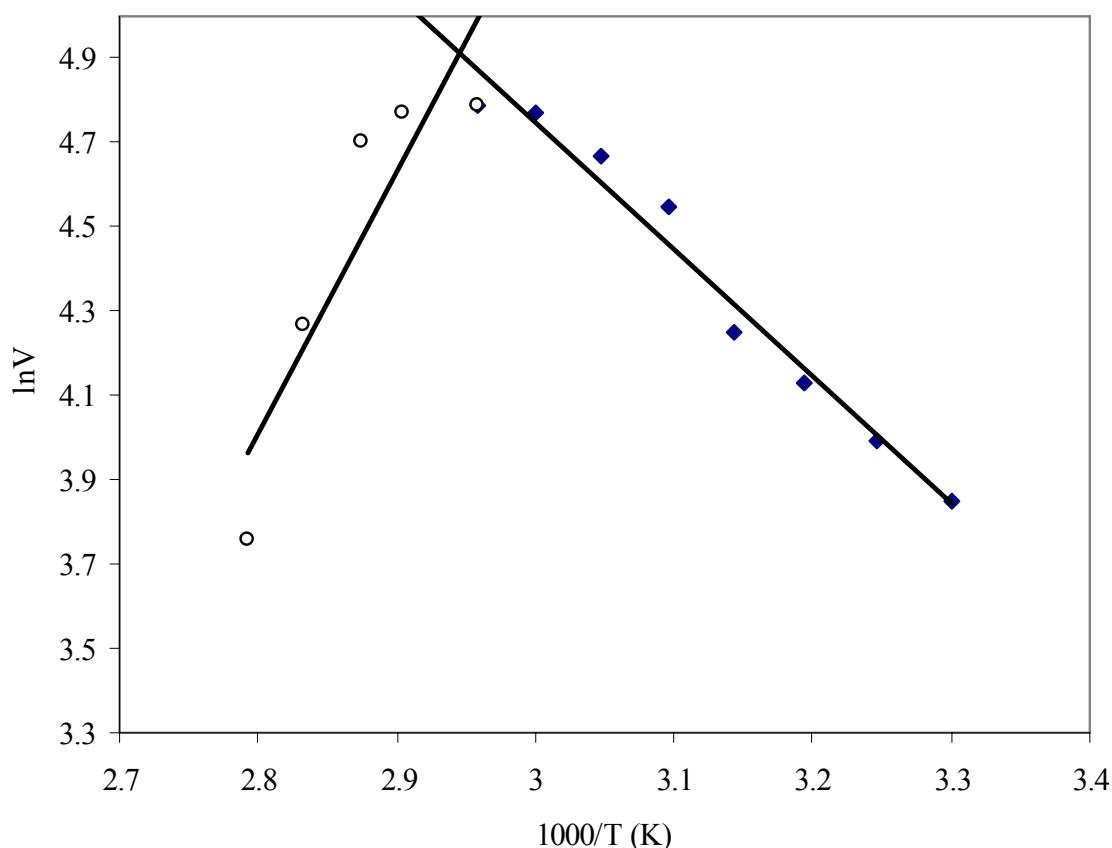
Fig. 5. Arrhenius plot for the determination of activation energy (E_a) for the hydrolysis of soluble starch. Ea = -slope x R (Plot: lnV vs 1/T), where R (gas constant) 8.314 JK^{-1}. Data presented is an average of values ± S.D. of n=3 experiments.

The free energy of the system at constant temperature (ΔG), enthalpy of activation (ΔH) and entropy of activation (ΔS) for primary binding of α-amylase from *B. licheniformis* EMS-6 were calculated 36968 J/mole, 22.53 KJ/mole and -110.95J/mole/K, respectively (Fig. 6). The lower enthalpy values of enzyme, more efficient are the formation of transition state or activated complex between enzyme-substrate (Riaz *et al.*, 2007). Tanaka & Hoshino (2003) reported ΔH and ΔS of *B. amyloliquefaciens* (α-amylase 29.3 KJ/mole and - 82.6 J/mole/K, respectively.

The hydrolysis of soluble starch by purified α-amylase was worked out at pH ranging from 4.0 to 9.0 at 60ºC. The optimum enzyme activity was found in pH range of 6.5 to 7.5 with maximum activity at pH 7.0 (Fig. 7). The enzyme activity decreased below and above optimum pH range. The pk_{a1} and pk_{a2} values of acid and basic limbs of the active site residues controlling the V_{max}, determined by Dixon plot, were 6.0 and 7.5, respectively. Lee *et al.*, (2006) reported the higher activity of α-amylase from *Bacillus licheniformis* mutant in pH range 4.0-8.0. It was shown that the proton dissociation constants on the acidic and alkaline sides (pKa1 and pKa2) were shifted to more acidic and basic values, respectively by the mutation.
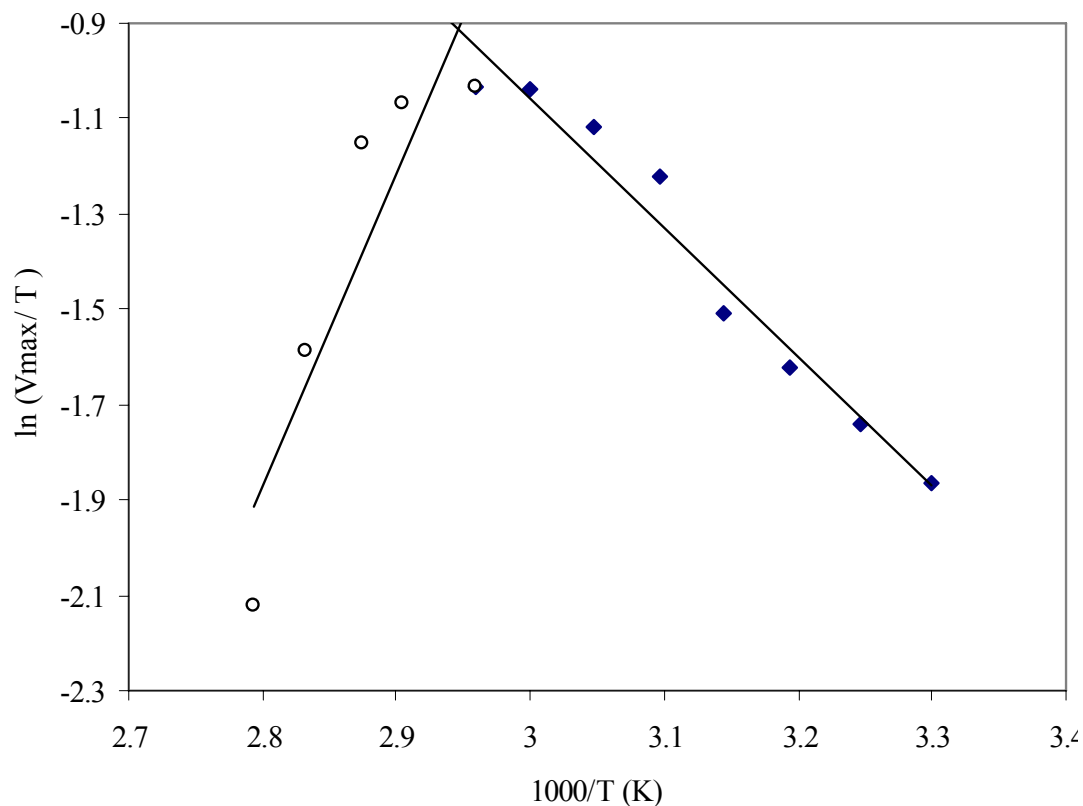
Acknowledgements

Fig. 6. Arrhenius plot on enthalpy of activation (ΔH) and entropy of activation (ΔS) of α-amylase activity using V_{max} values at different temperatures. ΔH* = slope and ln (K_B/h)+ ΔS*/R = intercept on Y-axis. Data presented is an average of values ± S.D. of n=3 experiments.
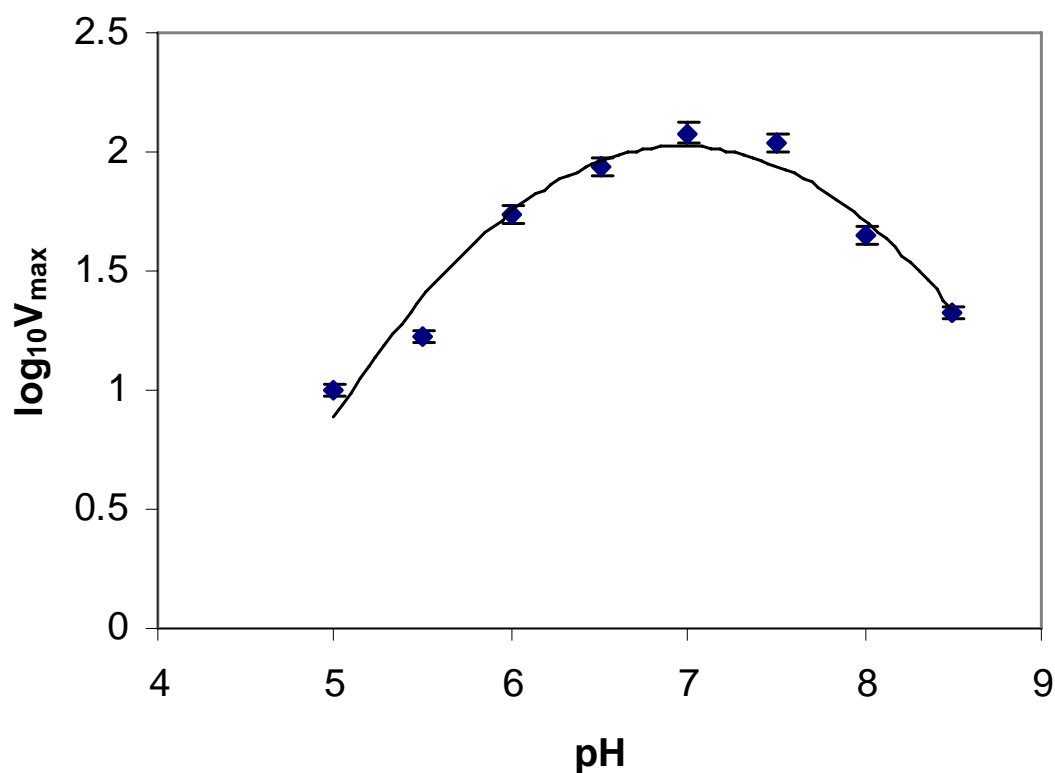

Fig. 7. Dixon plot of α-amylase from *B. licheniformis* EMS-6 at 60ºC for the determination of pK_as of active site residues that control V_{max}. Data presented is an average of values ± S.D. of n=3 experiments.

References

Akpan, T., Y.S. Kawak and T. Kudo. 2004. Production and stabilization of alpha amylase preparation from rice bran solid medium. *World J Microbiol Biotechnol.*, 20:47-50.

Amico, S.D., P. Claverie, T. Collins, D. Georlette, E. Gratia, A. Hoyoux, M.A. Meuwis, G. Feller and C. Gerday. 2002. Molecular basis of cold adaptation. *Phil. Trans. R. Soc. Lond. B.,* 357: 917-925.

Bradford, M.M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Annal Biochem.*, 72: 48-254.

Copeland, R.A. 2000. *Enzymes: A Practical Introduction to Structure, Mechanism and Data Analysis*. 2nd ed. Wiley-VCH, USA

Declan, J.B., J.K. Catherine and M.F. Wilham. 1997. Purification and characterization of alpha-amylase of *Bacillus flavothermus. Enz. Microbial. Technol.,* 20: 340-343.

Duy, C. and J. Fitter. 2005. Thermostability of Irreversible Unfolding-Amylases Analyzed by Unfolding Kinetics. *J. Biological Chem.,* 280(45): 37360-37365.

Eisenthal, R., M.J. Danson and D.W. Hough. 2007. Catalytic efficiency and k_{cat}/K_M: a useful comparator? *Trends in Biotechnol.*, 25(6): 247-249.

Fersht, A. 1999. *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding*. W.H. Freeman and Co.

Gangadharan, D., S. Sivaramakrishnan, K.M. Nampoothiri and A. Pandey. 2006. a-Amylase Production by *B. amyloliquefaciens, Food Technol. Biotechnol.,* 44(2): 269-274.

Gangadharan, D., K.M. Nampoothiri, S. Sivaramakrishnan and A. Pandey. 2008. Biochemical Characterization of Raw-starch-digesting Alpha Amylase Purified from *Bacillus amyloliquefaciens. Appl. Biochem. Biotechnol.,* DOI 10.1007/s12010-008-8347-4.

Goyal, N., G.S. Sindhu, S.T. Chakraburti and J.K. Gupta. 1995. Thermostability of alpha amylase produced by *Bacillus sp*., E2-a thermophilic mutant. *J. Microbiol. Biotechnol.,* 11: 593-594.

Hamilton, L.M., C.T. Kelly and W.M. Fogarty. 1998. Carbohydrate Research, 314: 251-257.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227(5259): 680-685.

Lee, S., H. Oneda, M. Minoda, A. Tanaka and K. Inouye. 2006. Comparison of starch hydrolysis activity and thermal stability of two *Bacillus licheniformis* alpha-amylases and insights into engineering alpha-amylase variants active under acidic conditions. *J. Biochem.*, 139(6): 997-1005.

Najafi, M.F, D.N. Deobagkar and D.D. Deobagkar. 2005. Purification and characterization of an extracellular alpha-amylase from *Bacillus subtilis* AX20. *Protein Expr. Purif.*, 41(2): 349-54.

Rajagopalan, G. and C. Krishnan. 2008. Alpha-amylase production from catabolite derepressed *Bacillus subtilis* KCC103 utilizing sugarcane bagasse hydrolysate. *Biores. Technol.*, 99: 3044-3050.

Rajoka, M.I., I.S. Durrani and A.M Khalid. 2005. Kinetics studies of native and mutated intracellular β-glucosidases from *Cellulomonas biazotea. Protein and Peptide Letter*, 12: 283-288.

Rao, M.D., A. Purnima, D.V. Ramesh and C. Ayyanna. 2002. Urification of α-amylase from *Bacillus licheniformis* by chromatofocusing and gel filtration chromatography. *World J. Microbiol. Biotechnol.,* 38(6):547-550.

Rasiah I.A. and B.H. Rehm. 2009. One-step production of immobilized alpha-amylase in recombinant *Escherichia coli. Appl. Environ. Microbiol.*, 75(7): 2012-6.

Riaz, M., R. Perveen, M.R. Javed, H. Nadeem and M.H. Rashi. 2007. Kinetics and thermodynamic properties of noval glucoamylase from *Humicola* sp. *Enzyme and Microbial Technol.,* 41: 558-564.

Rick, W. and H.P. Stegbauer. 1974. Alpha amylase measurement of reducing groups. In: *Methods of Enzymetic Analysis*, (Ed.): H.V. Bergmeyer. 2nd Ed, Vol 2, Academic Press.

Swain, M.R. and R.C. Ray. 2007. Alpha-amylase production by *Bacillus subtilis* CM3 in solid state fermentation using cassava fibrous residue. *J. Basic Microbiol.,* 47: 417-425.

Takasaki, Y., S. Furutani, S. Hayashi and K. Imada. 1994. Acid-stable and thermostable alpha-amylase from *Bacillus lichemiformis. J. Ferment Bioeng*., 77: 94-96.

Tanaka, A. and E. Hoshino. 2003. Secondary calcium binding parameter of *Bacillus amyloliquefaciens* α-amylase obtained from inhibition kinetics. *J. Biosciences and bioengineering*, 96(3): 262-268.

Thippeswamy, S., K. Girigowda and V.H. Mulimani. 2006. Isolation and identification of alpha-amylase producing *Bacillus* sp., from dhal industry waste. *Ind. J. Biochem. Biophys.,* 43: 295-298.