Int J Pharm Bio Sci 2012 July; 3(3): (B) 407 - 413

Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

PARTIAL PURIFICATION AND THERMODYNAMIC ANALYSIS OF THERMOSTABLE α-AMYLASE FROM *BACILLUS CEREUS* MTCC 1305.

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ABSTRACT

This study investigated the ability of *Bacillus cereus* MTCC-1305 to produce alpha amylase and its kinetics, thermodynamic characterization using submerged fermentation. The enzyme was then purified by acetone precipitation and anionic-exchange chromatographic technique. Effects of pH, substrate concentration, and temperature on enzymatic reaction were studied after partial purification of enzyme solution. Thermal stability was investigated at 90˚C. The half-life period of the enzyme was obtained as 7.42 min at 90˚C. Inhibition kinetic study was investigated using 0.05 M $CuSO_4$ solution as an inhibitor. Maximum velocities of reaction were found to be 0.13g/l min (without inhibitor) and 0.061 g/l min (with inhibitor). K_m and K_I values were obtained to be 6.49 g/l and 0.3526 g/l respectively from Line-weaver Burke plot. Thermodynamic variables such as enthalpy change of activation ΔH^*, entropy change of activation ΔS^* were obtained from effect of temperature on enzyme activity study using transition state theory

KEYWORDS: Thermodynamic characterization, submerged fermentation, *Bacillus cereus*, α amylase.

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INTRODUCTION

Alpha amylases (endo-1, 4 α -D-glucan glucohydrolase, E.C. 3.2.1.1) are extra cellular enzymes that break down the internal α-1, 4 linkages in starch to form glucose, maltodextrins and maltose. Industrially important enzyme α- amylase plays a vital role in starch liquefaction, brewing and food industries[1-5]. *Bacillus sp.,* such as*, B. subtilis, B. amyloliquefaciens, B. stearothermophilu* and *B. licheniformis* are well known potential producers of α amylase[1,6]. Babu and Satyanaraya had investigated α-amylase production by a thermophilic *Bacillus coagulans*[7]. Ikram-Ul-Haq et al. explained about kinetics and thermodynamic charaterisation of α-amylase from *Bacillus licheniformis*[8]. The effect of the calcium ion concentration on BMW-amylase and kinetic and thermodynamic properties were studied by Marzieh Ghollasi et al.[9]

The separation and purification α-amylase from the fermented broth is much essential for thermo stability and characterization [10]. Alpha amylase enzyme is separated and purified by centrifugation, precipitation (ammonium sulphate fractionation, acetone precipitation) ion exchange chromatography and hydrophobic interaction chromatography [3, 11]. For any enzyme process, it is essential to investigate kinetics and to understand the mechanism. Thermodynamic parameters are very important to understand enzyme reaction kinetics[12]. Thermodynamics study offers an exhaustive mechanism for most of bio chemical reactions [12].

Due to greatest applications of alpha amylase, it is essential to optimize the enzyme activity and characterize the kinetic parameters such as v_m, K_m and thermodynamic parameters since poor enzyme constancy will interfere with productivity. The knowledge of above parameters is so important to design the enzymatic reactors. The purpose of this work is to study the kinetics and thermodynamics using transition state theory[13] on *Bacillus cereus* α-amylase activity and to optimize the processing conditions for enzymatic reactions.

MATERIALS AND METHODS

(i) Cell cultivation

The Bacillus cereus strain was obtained from Microbial Type Cell culture (MTCC). It was introduced to growth medium (Beef extract: 1g/l, yeast extract: 2g/l, peptone: 5g/l, NaCl: 5g/l and agar: 15g/l). *B. cereus* cultivation was adequate for 24 hours in the already mentioned growth medium at 28 ˚C. 5% V/V of this inoculum was added to 150 ml sterilized production medium (glucose: 6g/l, ammonium sulfate: 10 g/l, potassium dihydrogen phosphate: 4g/l, magnesium sulfate: 0.5 g/l, calcium chloride: 0.02 g/l) in 250 ml Erlenmeyer flask which was then kept in a rotary shaker at 150 rpm and 30 ˚C. The enzyme concentration was estimated by Lowrys assay[14]. The enzyme assay was performed by DNS method [15].

(ii) Partial purification of Enzyme solution

The crude sample obtained from the medium was added with equal volume of 0.5 M acetone for precipitation [16]. The fermentation medium was centrifuged at 12000×g for 20 min at 4 ˚C and the pellet was collected. The pellet was taken and dissolved in sufficient volume of 0.02 M phosphate buffer at pH 6.0. This dissolved sample was introduced into anion-exchange chromatography Q Hitrap 1ml column (Akta Prime Plus System, GE, Sweden). The column was equilibrated with start buffer 5mM KH_2PO_4 and K_2HPO_4, pH 8 and sample was again adjusted to pH 8 with start buffer solutions and about 0.4ml was injected through injection port. The column was washed with same buffer. The bound protein was recovered using about ten column volumes of elution buffer 5mM NaCl, pH 8. Then the fraction was collected when UV spectrum showed peaks. Then the fractions were used for further analysis.

(iii) Enzyme Kinetic studies

Effect of initial pH on enzyme activity was investigated at different initial pH. Effect of substrate concentration on enzymatic reaction was studied with and without inhibitor. 0.05 M $CuSO_4$ was used as an inhibitor. Effect of temperature on enzyme activity was studied between the temperatures 30 ˚C and 90 ˚C. The thermal stability of the enzyme was checked by introducing the enzyme solution to 90 ˚C for varying time durations before an enzyme reaction.

RESULT AND DISCUSSION

1. Fermentation and partial purification of enzyme

Table 1
Partial purification steps for amylase

Steps	Enzyme activity(U/ml)	Enzyme concentration(mg/ml)	Specific activity(U/mg)	% yield	Purification fold
Crude sample from fermentation	1.022	0.71	1.4394	100	1
Acetone precipitation	1.124	0.104	10.8076	13.3	7.5
Anion exchange chromatography	1.95	0.04	48.75	2.9	33

Fermentation was carried out in Bioflow110, New Brunswick Scientific, USA, with a working volume of 1.5L and provided with two impellers with 6 blades-disc type turbine in each one. The aeration and agitation were maintained as1vvm and 150rpm respectively. Then crude sample after fermentation was subjected to acetone precipitation and anion exchange chromatography. Ikram-Ul-Haq et al. observed that 27.64% yield of pure alpha amylase using ammonium sulfate precipitation followed by anionic exchange chromatography from *Bacillus licheniformis*[8]. Maximum enzyme activity was found to be at pH 7 at 30° C (figure 1). Terui et al. was reported that optimum pH for amylase activity by B.subtilus was 6.8 [17]. Henna Anto et al. reported that amylase enzyme was optimum under pH 5 at 55°C from Bacillus cereus[18].

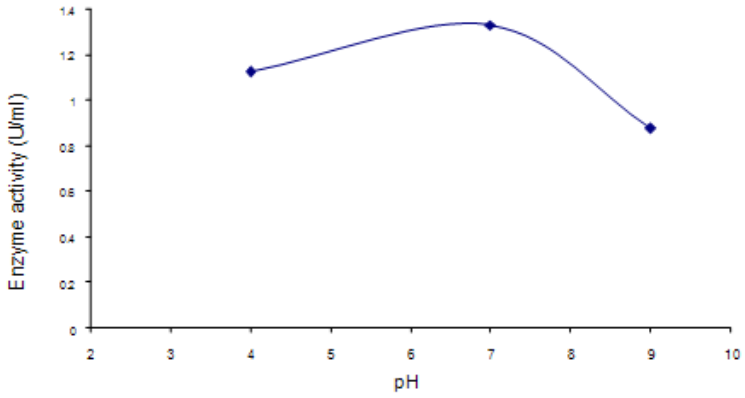
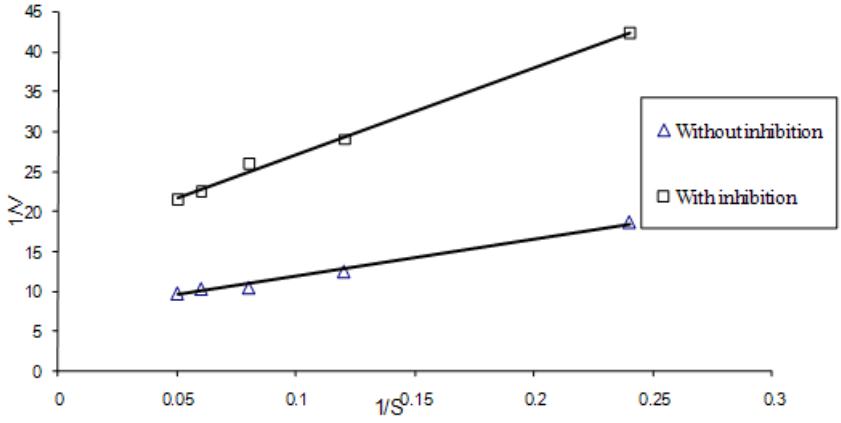


Figure 1.
Effect of pH on enzyme activity

2. Enzyme Kinetic parameters determination

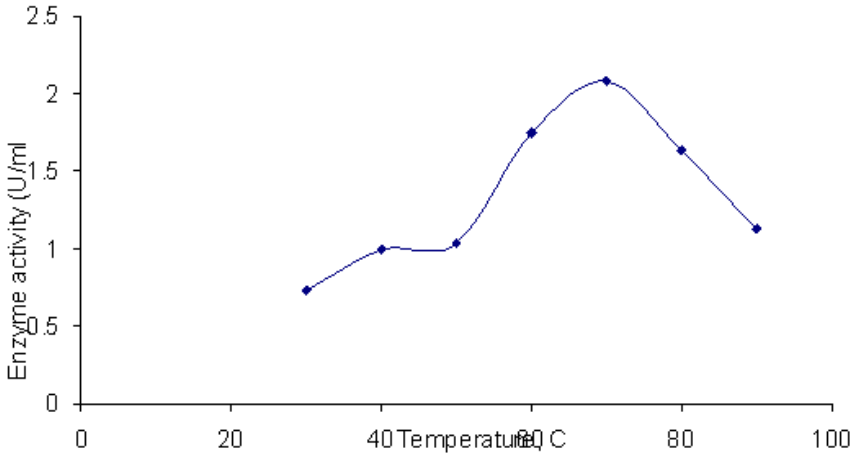
Figure 2
Line-Weaver plot for Enzyme Activity with Inhibitor and without Inhibitor



The Line-Weaver plot for the starch hydrolysis is shown in figure 2. The kinetic parameters such as v_{max}, K_m for α-amylase were estimated by varying substrate concentration from 0 to 20 g/L. In the present work, v_m values were found to be 0.13g/l min (without inhibitor) and 0.061 g/l min (with inhibition). K_m and K_I were found to be 6.49 g/l and 0.3526 g/l respectively at 30 ˚C. Low value of K_m indicates that affinity of the enzyme to the substrate is high[19]. Ikram-ul-haq et al. have reported that v_m and K_m values for α-amylase to be 2778 U/mg and 8.3mg/ml respectively from *Bacillus licheniformis* EMS-6 [8]. Gangadharan *et al*. obtained that K_m and V_{max} values for amylase from *B. amyloliquefaciens* using soluble starch to be 4.11 g/l and 3.076 g/l min at 50°C, respectively[3]. Another report shows that kinetic constants K_m and V_{max} values were 3.44 mg/ml and 0.45 mg hydrolyzed starch/ml min at 50 °C for α-amylase from *Lactobacillus manihotivorans* respectively[20].

3. Thermodynamics approach for Starch Hydrolysis

Figure 3
Effect of Temperature on Enzyme Activity



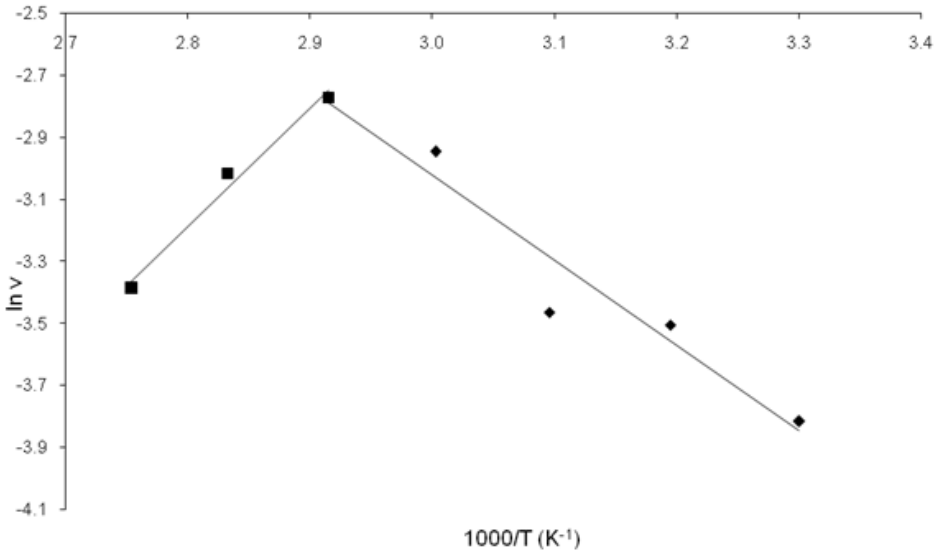
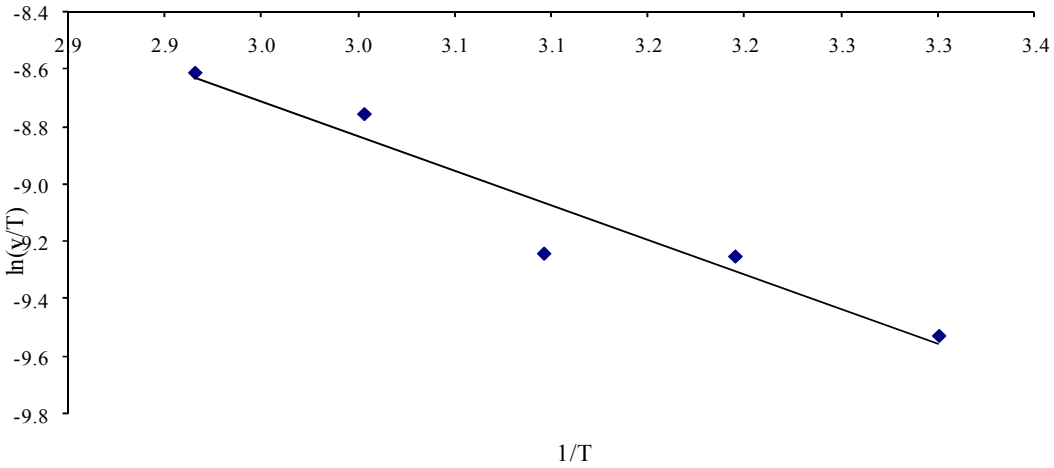


Figure 4
Arrhenius plot

Figure 5
Transition state theory plot



In this work, the optimum temperature for α-amylase from *B.cereus* was found to be 70° C (figure 3). For hydrolysis of soluble starch, the optimum temperature range for purified α-amylase from *B. licheniformis* EMS-6 was reported to be 60 – 70 ºC [8].

Activation energy was determined to be 22.806 kJ/mole (figure 4). Ikram-Ul-Haq et al. reported that for soluble starch hydrolysis, activation energy for α -amylase from mutant was found to be 25.14 KJ/mole [8]. Duy and Fitter observed that activation energy for α-amylase (B. licheniformis) was about 363.7 kJ/mol at 70ºC for and 317.9 kJ/mol for α-amylase (Aspergillus oryzae)[21]. Low value of activation energy obtained in this study indicates high thermal stability of the enzyme[22].

In this present work, ΔG^*, ΔH^* and ΔS^* were found out (figure 5) to be 92.149 kJ/mole, 20.119 kJ/mole and -210 J/mole K. Lower values of enthalpy change are more efficient which are responsible formation of transition

state between the enzyme-substrate[22]. Ikram-Ul-Haq et al. reported that the free energy change of an enzyme reaction at isothermal condition (ΔG^*), change in enthalpy of activation (ΔH^*) and change in entropy of activation (ΔS^*) for binding of α-amylase isolated from *B.licheniformis* EMS-6 were 36968 J/mole, 22.53 KJ/mole and -110.95J/mole/K respectively[8]. Tanaka and Hoshino observed that enthalpy change of activation (ΔH^*) and entropy change of activation (ΔS^*) for α-amylase isolated from *B. amyloliquefaciens* were 29.3 KJ/mole and -82.6 J/mole/K, respectively[12].

In this work, thermal stability of α-amylase from *B. cereus* was found to be 7.42 min at 90˚C. This also confirms good thermal stability of the enzyme. Thermal stability of α-amylase was 3.5 hrs at 64 °C[23]. Konsoula et al. observed that α-amylase enzyme extract retained 96% activity when incubated at 60°C for 2 hours[24].

CONCLUSION

The present work, production of α-amylase enzyme from the *Bacillus cereus* MTCC-1305 was investigated in submerged fermentation. Partial purification of α-amylase was investigated in acetone precipitation and anionic exchange chromatography. Maximum enzyme activity was achieved at pH 7. v_{max} values were found to be 0.13g/l min (without inhibitor) and 0.061 g/l min (with inhibition). K_m and K_I were found to be 6.49 g/l and 0.3526 g/l respectively at 30 ˚C. Optimum temperature for α-amylase from *B.cereus* was found to be 70°C. Activation energy was determined to be 22.806 kJ/mole using arhenius plot. ΔG^*, ΔH^* and ΔS^* were found out to be 92.149 kJ/mole, 20.119 kJ/mole and -210 J/mole K. Thermal stability of α-amylase from *B. cereus* was found to be 7.42 min at 90˚C.

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