Characterization of α-amylase purified from Thermophilic *Bacillus spp.*

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توصيف انزيم الفا – اميليز المنقى من بكتريا *Thermophilic Bacillus spp.*

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الخلاصة

دُرسَت بعض‘ صفات انزيم الفا – اميليز المنقى من بكتريا *Thermophilic bacillus* باستعمال كبريتاتَ الأمونيوم والتبادل الايوني . أظهرت نتائج الترشيح الهلامي أن الوزن الجزيئي للانزيم المنقى 51285 دالتون ، الرقم الهيدروجيني الأمثل لفعالية الانزيم هو عند pH = 9 كما أظهر الانزيم ثباتاً بمدى من الارقام الهيدروجينية تراوحت بين ٧ – ٩ . وكانت درجة الحرارة المثلى لفعالية الانزيم هي ٧٠ مْ واظهر الانزيم ثباتا حراريا بين ٤٠ – ٧٠ مْ لمدة ١٥ دقيقة. بلغت قيمة طاقة التنشيط لتحويل المادة الاســـاس (النشا) الى نواتج ٩.٥٦ كيلو سعرة / مول ، أما معدلات قيم ثابت ميكالس (km) ومعدل قيم السرعة القصوى للانزيم كانت ٠.٧٢ % ، ١٠.٤ ملي مولر / دقيقة على التوالي . اظهر كل من اثيلين ثنائي الامين ثلاثي حامض الخليك و ازايد الصوديوم دورا مثبطا لفعالية الانزيم وعلى العكس من ذلك كان لكلوريد الكالسيوم دورا منشطا للانزيم إذ أدى إلى زيادة فعالية الانزيم بنسبة ١٤٥ % و زيادة ثباته الحراري من ٧٠ الى ٩٠ مْ لمدة ١٠ دقائق، وذلك عند اضافة تركيز ٠.٣ ملي مولر من كلوريد الكالسيوم الى وسط التفاعل.

ABSTRUCT

Partial characterization of α-amylase purified from *Thermophilic Bacillus spp.* were studied by using ammonium sulfate and ion-exchange. The results of gel-filtration were demonstrated that the molecular weight of purified enzyme was 5128° Dalton. The optimum pH for enzyme's activity was pH = 9 and the enzyme showed stability at pH in the range pH 9 – 7. The optimum temperature for the enzyme's activity was 70 Ċ and the enzyme showed thermo stability between 40 Ċ to 70 Ċ to 15 minutes. The value of activation energy to convert the substrate (starch) into products reached to 9.56 kcal /mol. The averages value of Mechalis constant (km) and maximum velocity of enzyme (Vmax) were 0.72%, 10.4 mM/min respectively. The EDTA and NaN_3 inhibits α-amylase activity while the calcium chloride increases the enzyme activity and thermostability 145%, 70 to 90 Ċ for 10 minutes respectively, when adding 0.3 mM calcium chloride to the reaction solution.

Introduction

Amylases are enzymes responsible for the hydrolysis of disaccharides, trisaccharide and polysaccharides into: glucose, maltose and dextrin occupying nearly 80% of the enzymes which used in food technology. Starch is a biopolymer consists of glucose residues connect into large macromolecules. Liner amylose, where they are bound almost exclusively through α-1, 6-glycosidic bonds and amylopectin, where α-1, 6-glycosidic bonds form about 5% connections, that cause significant branching of this polymer (1). Industrial use of starch often requires its depolymerization, which could be done chemically or enzymatically. Traditional chemical hydrolysis has been prevailed by enzymatic treatment because of the following advantages: better controlling of physical and chemical properties, fewer side reactions and causing unwanted changes like browning (2). Alpha-amylase, β-amylase and glucoamylase from different origins are use in industry; they can hydrolysis α-1,4-glycosidic bonds while glucoamylase, isoamylase and pullulanase hydrolysis amylopectin branches. Alpha-amylase (EC: 3.2.1.1), (α-1, 4- glucanglu--cano hydrolase) catalyzes the cleavage of α-1, 4-D-glycosidic linkages in polysaccharides (3, 4). Alpha-amylase is one of the most important industrial enzymes obtained from microbial, plant and animal sources. *Bacillus licheniformis* α-amylase is a liquefying bacterial enzyme widely used in industry and is the only heat and p^H stable enzyme which shows substantial activity in an alkaline p^H range at high temperature (5, 6). This enzyme have several applications: baking, detergents, leather tanning, sweetener food, produce fuel ethanol from starch materials, produce cold-soluble laundry starches, paper coating, depolymerized starch products like glucose or fructose syrup and fruits juice and act as predigesting substance in children food (7, 8, 9).

The aim of this study is partial characterization of purified α-amylase from thermophillic *Bacillus spp.*

Materials and Methods:

Determination of α-amylase activity:

Purified α-amylase activity was determined by measuring the amount of reducing sugar released during incubation with soluble starch (10). The reaction mixture containing 0.1 ml of purified enzyme, 0.9 ml of 0.5% soluble starch pH 8 incubated at 60 Ċ for 10 min. after this time the reaction was stopped by adding 1 ml of 3,5-dinitrosalsalic acid (DNSA) reagent. The absorbance was measured in a spectrophotometer at 540 nm. One unit of enzyme activity was defined as the amount of the enzyme that librated one micromole of reducing sugar (maltose) per minute under the experimental conditions.

Characterization of the purified enzyme:

The molecular weight of purified enzyme was determined by gel-filtration chromatography using Sephacryl S-200 column (70 × 1.9) cm. the void volume (Vo) of column was determined by loading blue dextran-2000 (2×10^6 Dalton)

solution (5mg/ml) through the column, the elution volume (Ve) of the standard proteins were determined by loaded its solutions on the column, the proteins were eluted from the column with 0.2 M sodium phosphate buffer at a flow rate 30 ml/hr. The purified α-amylase solution was loaded on the column then it was eluted from the column with the same buffer and same flow rate. Standard proteins include: trypsin, ovalbumin, ovaltransferase and lysozyme. The effect of pH and temperature on the purified α-amylase activity and stability were examined. The effect of the pH on the α-amylase activity was determined by using substrate solution with deferent values from pH 3 to 11; all of the assays were performed at 60 ̊C. To determine the influence of temperature on the enzymatic activity, samples were incubated at temperature from 40 ̊C to 100 ̊C for 10 min and the enzymatic activity was measured. The influence of the pH on the enzyme stability was tested in the following way: Samples were incubated for 30 min at various pH values from 3 to 11 at 60 ̊C and then they cooled in an ice bath. Substrate was added to measure the residual enzyme activity. The activation energy of purified enzyme to convert substrate into products was determined according to (11) method, by using different temperatures in the range (40 – 90) ̊C. Thermostability was investigated after incubation of the samples at different temperatures in the range (40 – 90) ̊C, for 15 min, then they cooled in an ice bath and residual enzymatic activity was measured. The kinetics constant values of purified enzyme were determined by using different concentration in the range 0.1 – 1 % from soluble starch as substrate that prepared in 0.02 M sodium phosphate buffer at pH 9 according to (12) method. Using linweaver-bark reciprocal plot: the effect of various substances (EDTA, NaN_3 and $CaCl_2$) on α-amylase activity was examined after incubation of purified enzyme with these substances in various concentrations at 70 ̊C for 10 min, then they cooled in ice and α-amylase activity were tested.

Results and Discussion:

The purified enzyme had an apparent molecular mass 51285 Da. (Fig. 1) determined by gel-filtration which aggress well with molecular weight of purified α-amylase from hyperthermophilic bacterium (13) was estimate to be approximately 50000 Da. Another α-amylase from the *Bacillus licheniformis* was purified and it's molecular weight was estimated to be approximately 57700 Da. (6).

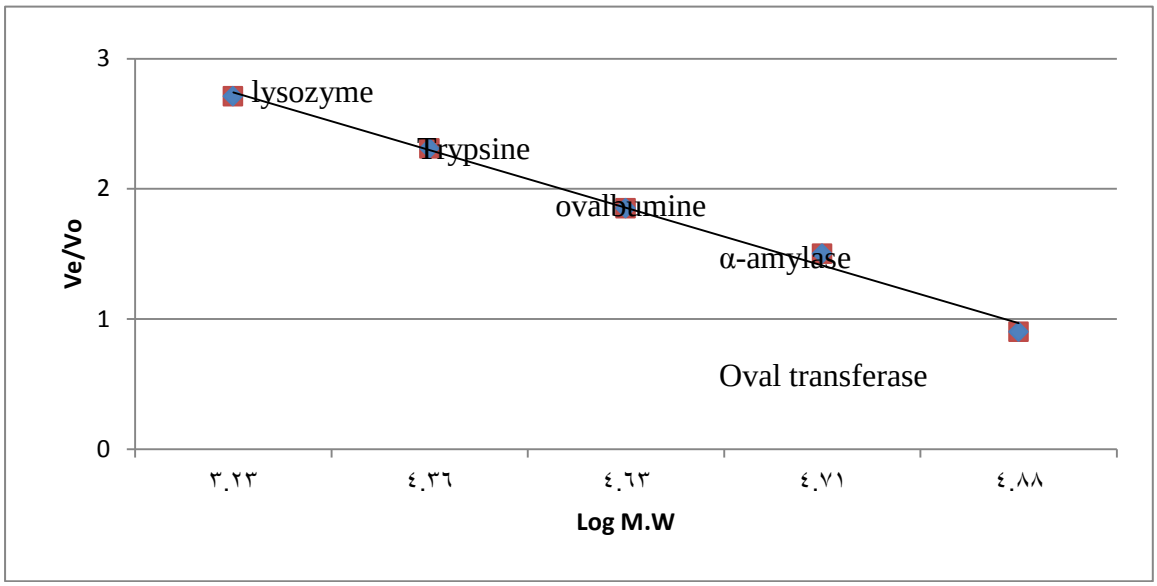

Figure (1) Molecular weight of purified α-amylase using gel filtration method

The purified enzyme was active in the range of pH from 7.5 to 10 (Fig. 2) maximal activity was observed at pH 9 indicating that this enzyme might be the alkaline α-amylase.

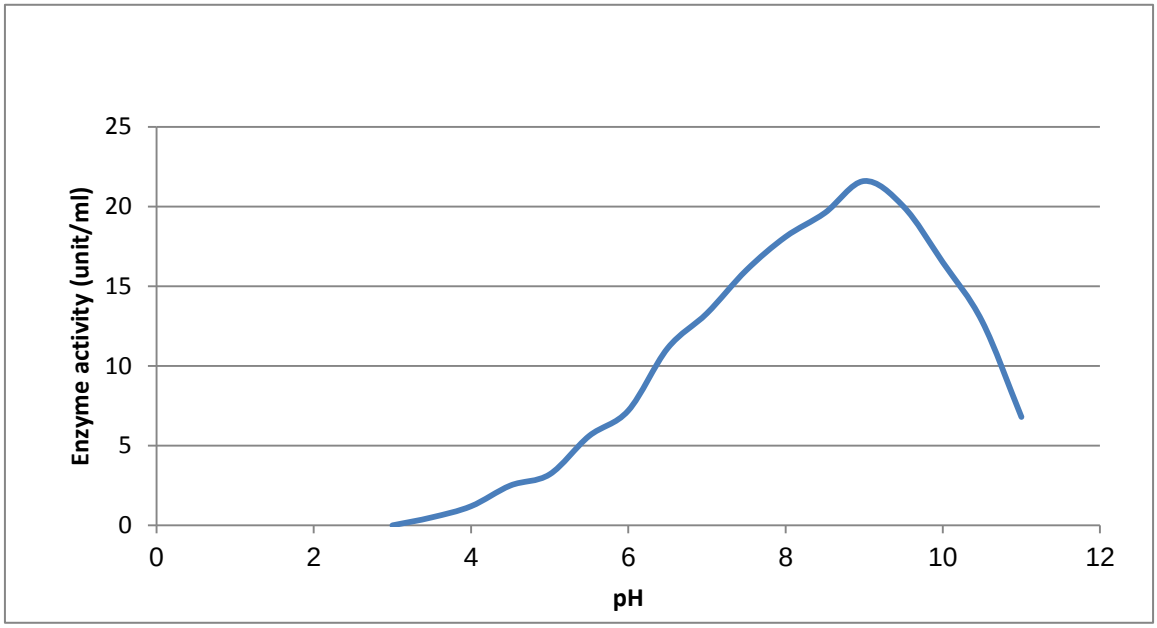

Figure (2) Determination of optimum pH corresponding to highest activity of purified α-amylase

An optimum pH of 9 of α-amylase was observed in α-amylase produced by *B. licheniformis* (14). The optimum pH was found to be 10.5 in α-amylase produced by *Bacillus spp.* (15). The α-amylase activity with the relative activity of more than 95% was found in the pH range 7 to 9 (Fig. 3). The enzyme lost practically 85% , 75 % of its maximum activity when treated at pH values 4,11 respectively. The enzyme was more sensible to pH changes in the reaction medium.

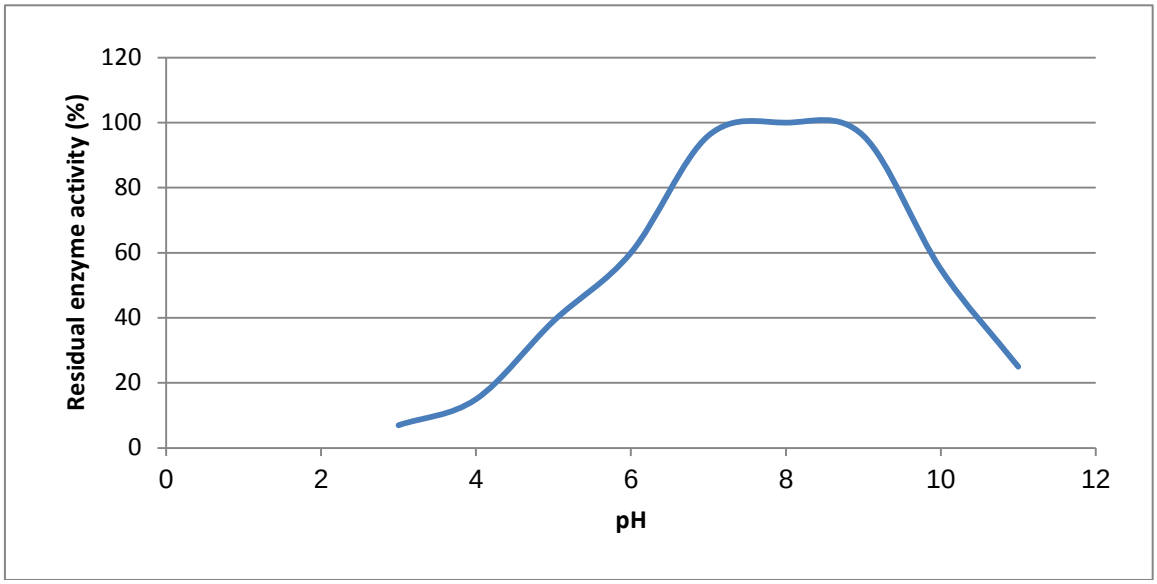

Figure (3) α-amylase stability at a wide pH range. Enzyme activity was calculated as percent of residual activity

The pH stability of α-amylase produce from *Bacillus spp.*(16) and *B.stearothermophillus* (17) were found to be in the ranges of 8 – 9 and 6 – 11 with the relative activity more than 93 % and 95 % respectively. The optimum temperature for α-amylase activity was assayed at different temperatures from 40 to 100 C̊ using soluble starch as a substrate dissolved in sodium phosphate buffer pH 9. The α-amylase activity was active at 50 C̊ to 80 C̊ but inactive in the range of 85 to 100. The optimum temperature of α-amylase was 70 C̊ (Fig. 4).

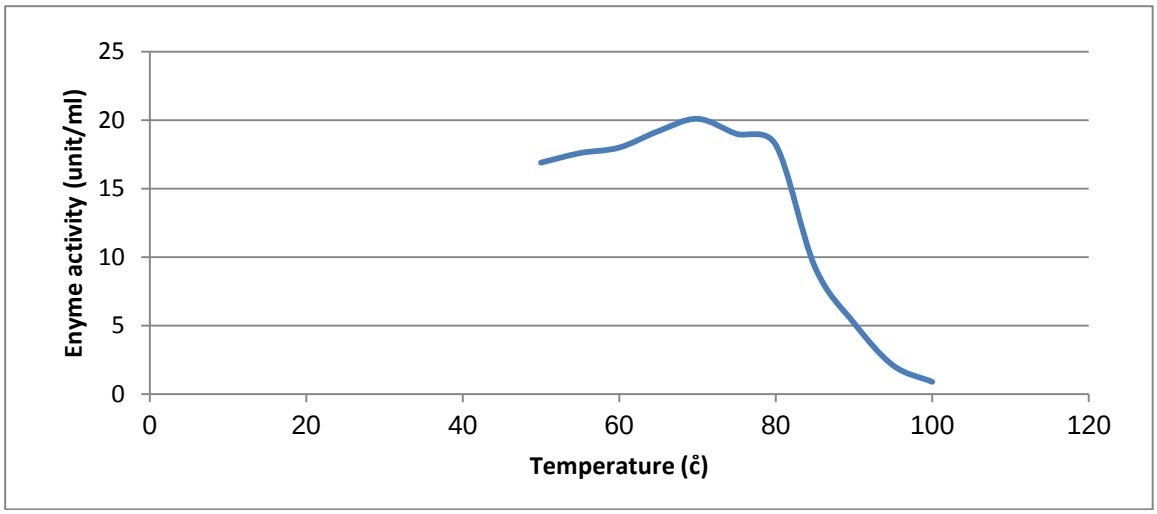

Figure (4) Determination of optimum temperature corresponding to highest enzyme activity

Show *et.al.* (18) reported that the optimum temperature of α-amylase purified from *Thermus* spp. was at 70 C̊. optimum temperature to purified α-amylase by hyperthermophilic bacterium was at 70 C̊ (13). The purified α-amylase solution was incubated at various temperatures 40 to 100 C̊, for 15 min to determine thermalstability. The results were indicated that within 15 min the α-amylase activity with the relative activity of more than 99% was found at 40 – 70 C̊ (fig. 5).

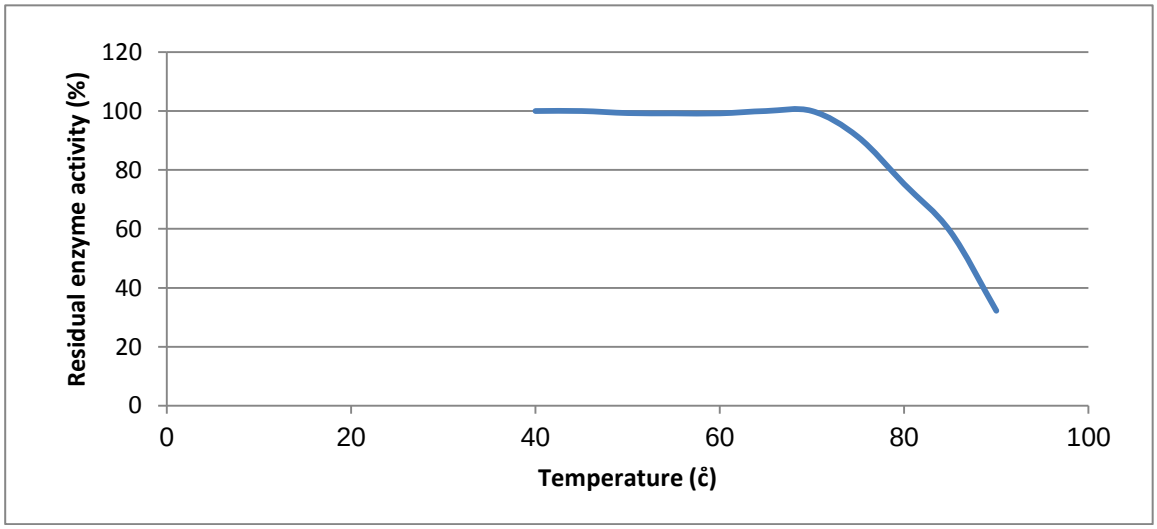

Figure (5) α-amylase thermostability at a range of temperatures. (20-100 C^{o})

The other research also found same trend. For example a relative activity of α-amylase produced by *B. flavothermus* were reported to be 100% after incubation the enzyme at a temperature range from 55 – 60 Ċ (19). The kinetic studies showed that the Mechealis constant value (km) of the purified enzyme using starch as substrate was 0.72 % while maximum velocity (Vmax) value was estimated to be 10.4 mM/min/ ml as shown in (Fig. 6).

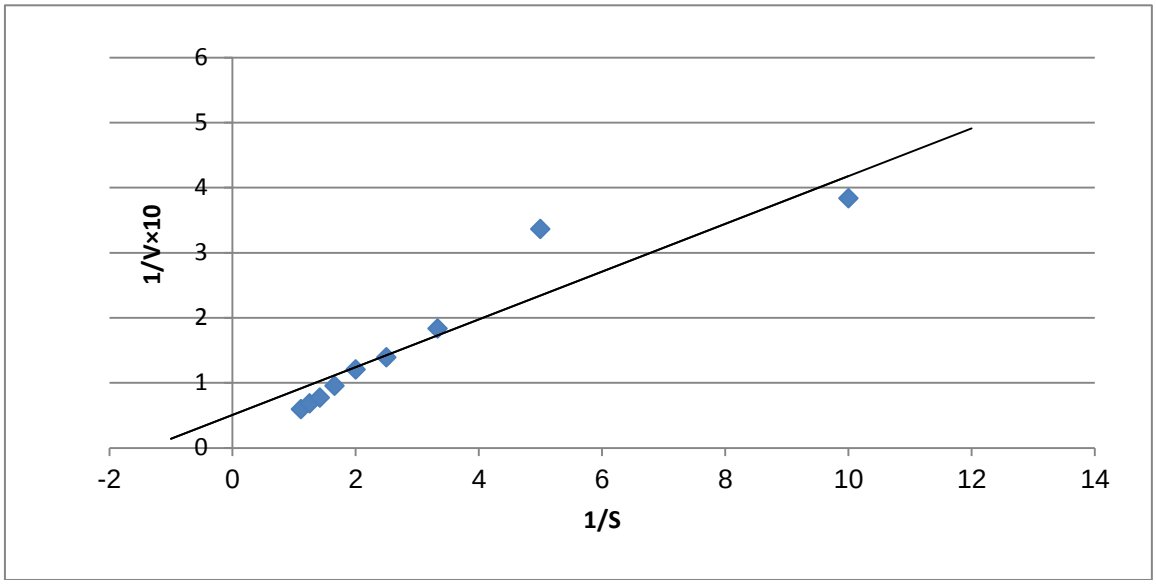

Figure (6) determination of Mechealis constant value (km) and maximum velocity (Vmax) using Linweaver method

The values of km and Vmax to produce α-amylase by *Thermococcus profundus* were 0.23, 0.47, 0.13, 0.05 mM and 9.4, 9.4, 12.2, 9.6 mM per min respectively. With using soluble starch, amylose, amylopectin and glycogen as a substrates respectively (20). The α-amylase activity was measured at pH 9 and 70 Ċ in the presence of some various chemical substances. Addition of EDTA and NaN_3 resulted in inhibition. The α-amylase activity was enhanced with addition of $CaCl_2$ (0.3 mM) resulting in the relative activity of 145% (Fig. 7) also the enzyme thermostability increased from 70 to 90 Ċ (Fig. 8). suggesting that Ca^{++} ions had a capability to protect enzyme against denaturation (13, 8). The enzyme was completely inhibited by 0.9 mM EDTA and 1 mM NaN_3, results shown in (Fig. 9) these substances acting as chelating agents, so it may result in depriving metal ions from active site.

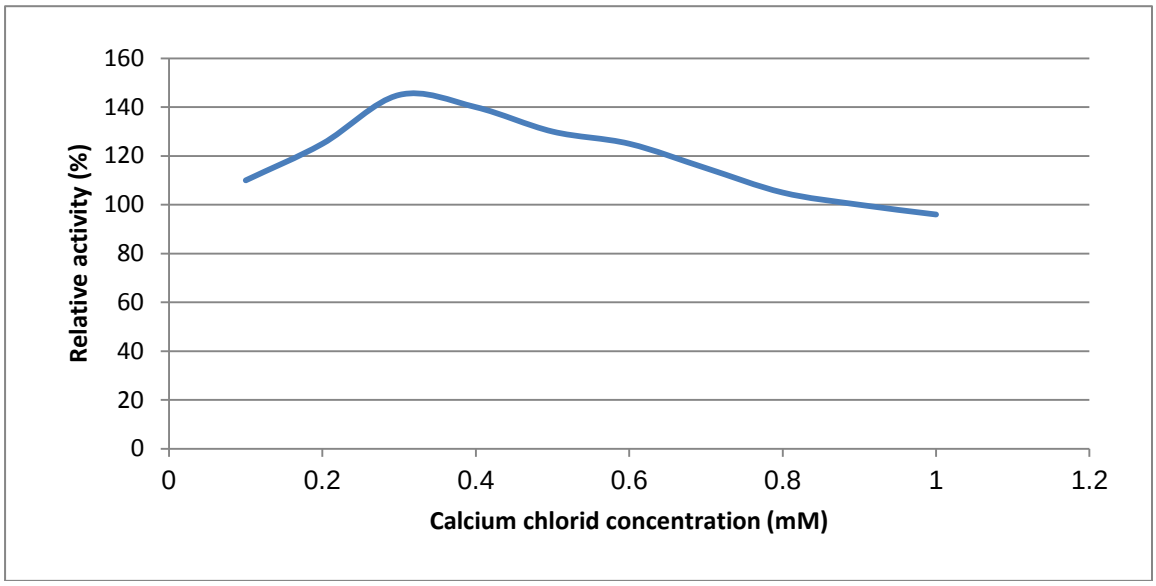

Figure (7) Effect of Calcium chloride concentration on α-amylase activity, calculated as relative activity.

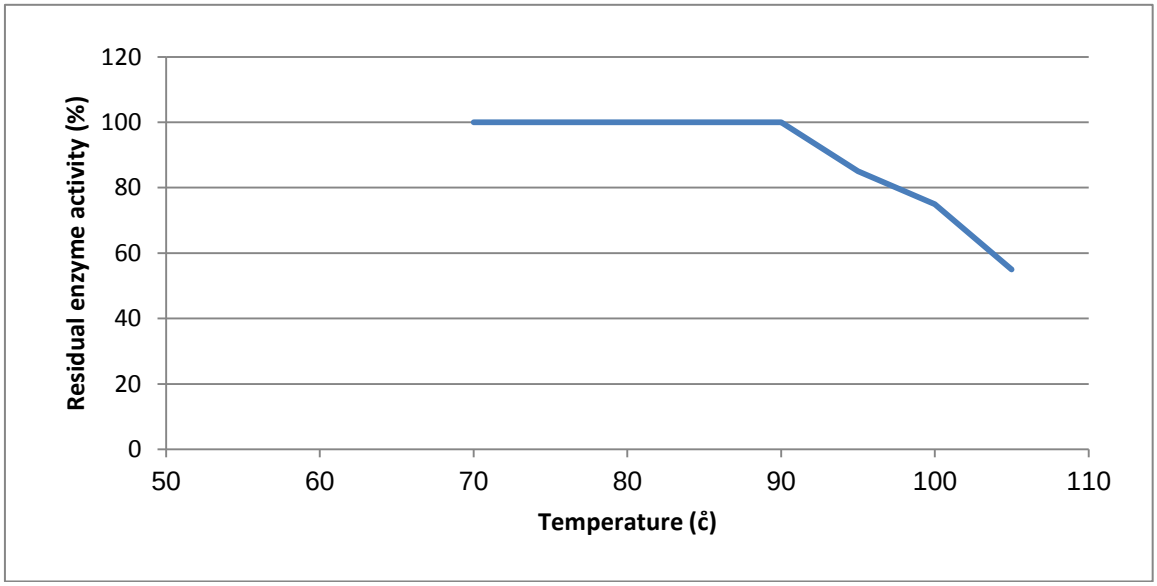

Figure (8) Effect of Calcium chloride concentration on thermostability of α-amylase. Enzyme activity was calculated as residual activity.

The Ca^{++} ions are involved in α-amylase catalysis and the stability of this enzyme is normally increased by the addition of small amounts of calcium.

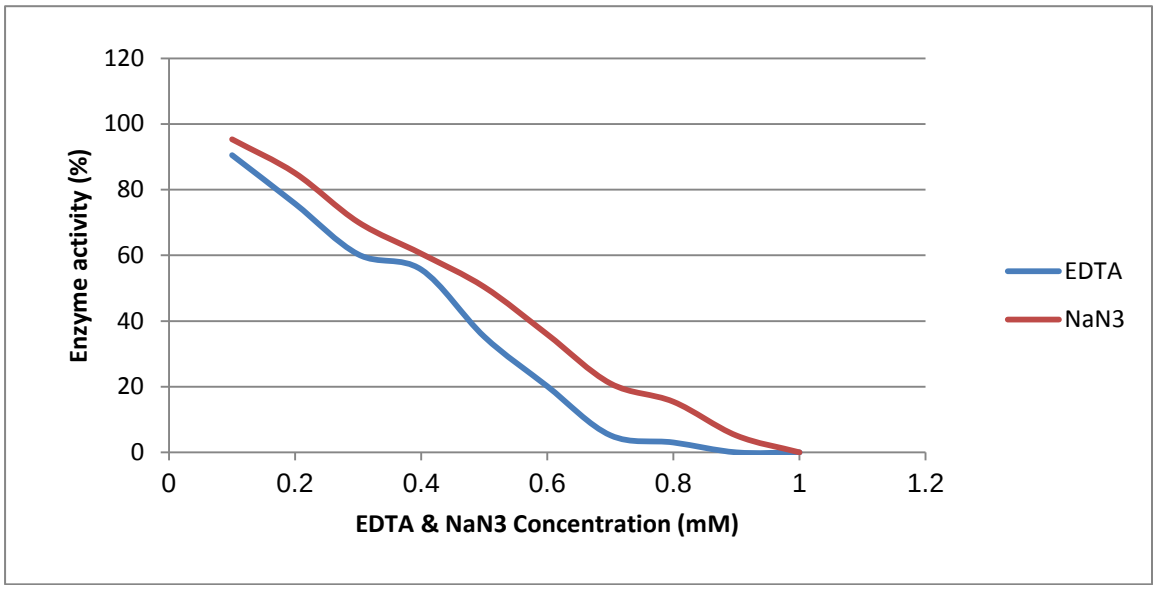

Figure (9) Effect of EDTA and NaN_3 concentration on α-amylase activity. Enzyme activity was calculated as residual activity.

The activation energy of the purified enzyme for conversion of the substrate products was 9.56 kcal / mol (Fig. 10). Schumann *et.al*. (21) reported that the activation energy of α-amylase produced by *Thermotoga maritima* was 14 kcal / mol. The activation energy of α-amylase produced by *Thermococcus profundus* for conversion of the substrate to products was estimated to be 60 – 98 kcal / mo (20).

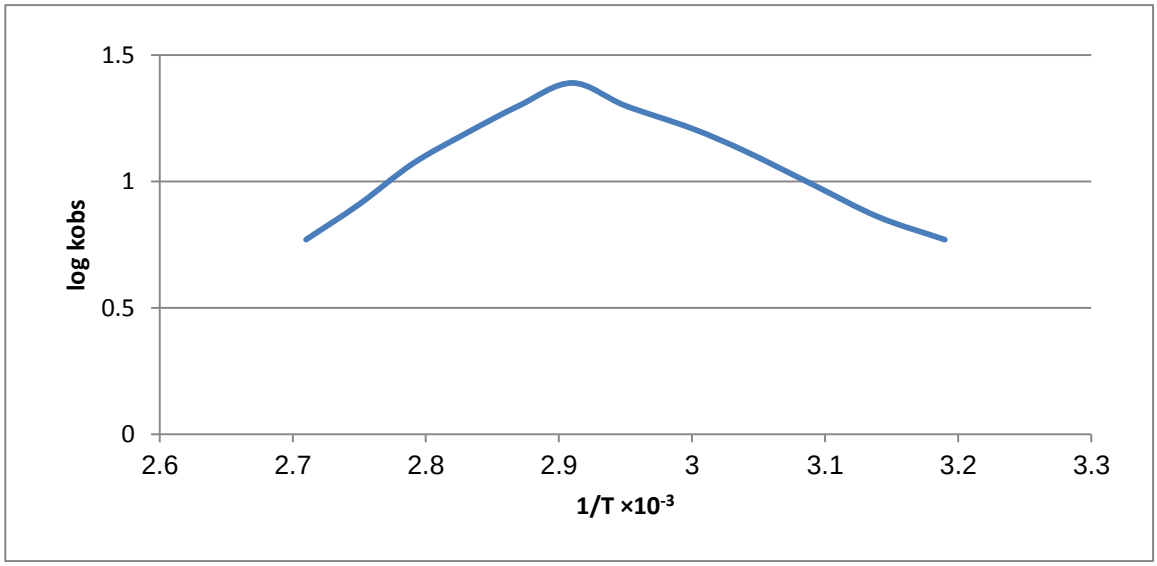

Figure (10) The activation energy of α-amylase purified from thermophilic Bacillus ssp.

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