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Stability of A-amylase from Germinating Breadfruit Seeds (*Treculia africana*)

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Abstract: Amylase was extracted from the seeds of *Treculia Africana* in a 0.2M sodium acetate buffer (pH 5.5) and partially purified using a two-step purification technique of ammonium sulphate precipitation and dialysis. The enzyme exhibited maximum activity at 40°C with thermal stability up to 55°C at pH of 6.0. The activation energies (Ea) of the enzyme for the inactivation process calculated from Arrhenius plot when starch from breadfruit seeds and cassava tubers were used as substrates were 526KJ/mol and 607KJ/mol respectively. Gibb's free energy (ΔG) and enthalpy (ΔH) value were found to be in the range of 44752-57075 KJ/mol and 44752-51066KJ/mol when starch from breadfruit seeds and cassava tubers were used as substrates respectively at different temperatures. ΔS and $t_{1/2}$ was calculated as -156KJ/mol/K and 0.693min^{-1} respectively when starch from breadfruit seeds and cassava tubers were used as substrates. The enzyme has affinity for the indigenous starch more than starch from cassava tubers and can be applied in biotechnology since high activation energy as recorded in this report suggests higher stability indicating that this enzyme could be suitable for industrial applications.

Key words: Amylase · Thermal Stability · Metal Ions · Hydrolysis · Breadfruit

INTRODUCTION

α-amylase (EC 3.2.1.1) is calcium containing enzyme which catalyses the cleavage of α-D-(1, 4) glycosidic bonds in starch and related carbohydrates, producing glucose, maltose, maltotriose and dextrins [1]. α- amylases can be found in plants, microorganisms and higher organisms where they play a major role in carbohydrate metabolism and show varying hydrolytic potential depending on the source [2, 3]. α-amylases have been produced at commercial level from fungal sources and different plant sources such as barley, millets, wheat, sorghum and maize [4].

The demand for α-amylase have been on the increase and have found wide applications in areas such as detergents, bread making, production of glucose and fructose syrup, textile industries and production of fuel ethanol among others. Thermal stability represents the capability of an enzyme molecule to resist thermal unfolding in the absence of a substrate whereas thermophilicity is the ability of an enzyme to work at elevated temperatures in the presence of substrate [5]. High temperatures often cause inactivation of enzymes as a result of protein denaturation. The way enzymes respond to temperature is fundamental to many areas of biotechnology [6]. Enzyme thermostability encompasses thermodynamic and kinetic stabilities [7]. Thermodynamic data on enzyme catalyzed reactions play an important role in the prediction of the extent of reaction and the position of equilibrium for any process in which these reactions occur. It is also needed in biotechnology when one needs to optimize product yields and to calculate the energy requirements of a given reaction. Calcium is essential for the activity and stability of α-amylases [8].

Despite the variety of α-amylases from different plant sources, no work has been done on α –amylases from African Breadfruit seeds. African breadfruit seeds have carbohydrate content of 72% [9] with about 40% carbohydrate as starch [10]. In this research work, we report the stability of α-amylase from germinating breadfruit seeds which could be exploited for several industrial applications.

MATERIALS AND METHODS

Materials: 3, 5- dinitrosalicylic acid (DNS) was a product of Sigma chemical company, USA. Bovine serum albumin (BSA) (Bio Rad. Laboratories, India), Folin-Ciocalteau

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(E- merk Ltd India). Other chemicals used were of analytical grade and were freshly prepared unless otherwise stated.

Collection of Breadfruit Seed: Matured breadfruit seeds were collected from Eha-Alumona, in Nsukka Local Government Area of Enugu State, Nigeria

Methods

Processing of Breadfruit Seeds: The seeds were processed from the pulpy fruit heads in flowing water with sand, sponge and were identified by Mr. Ozioko, Fabian of the Department of Botany, University of Nigeria, Nsukka as *Treculia africana*.

Processing of Starch: Cassava starch was processed using the method described by Corbishley and Miller [11] with the following modifications. Freshly harvested cassava tubers were peeled washed clean and grated. The grated cassava (1.2 kg) was soaked in 4 L of distilled water for an hour after which it was sieved (3 times) with muslin cloth. This was allowed to stand for 4 hrs, the supernatant was then decanted. The isolated wet starch was sun dried and packaged in plastic air tight container, labeled and kept in a cool, dry place.

Breadfruit starch was processed using the method described by Agboola *et al.* [12] with the following modifications. The seeds were dehulled to recover the kernel. The kernels were sun dried and ground to fine flour. 300g of the flour was suspended in 3L of distilled water for 24 hrs. The suspended breadfruit seed flour was sieved using muslin cloth and was allowed to sediment for 4 hrs at room temperature. The supernatant was decanted off and the starch washed with 3L of distilled water twice and finally allowed to stand for 4 hrs. The supernatant was then decanted. The resulting wet starch was sun dried and packaged in an air tight container and stored at room temperature.

Determination of Enzyme Activity with Days of Germination: 5, 7, 9, 11, 13, 15, 18 and 20 days germinated breadfruit seeds as well as un-imbibed seeds (Day zero) were decoated separately and the seeds (80g each), were homogenized for 10 min with 160ml of cold 0.2M sodium acetate buffer (pH 5.5) using Philips electric blending machine (R2001) as described by Afiukwa *et al.* [13]. The homogenate was filtered using Whatman No 1 filter paper and centrifuged for 10 min at 5000 rpm. The supernatant was collected and used as the crude extract while the pellets were discarded. Amylase activity was determined throughout the period of germination at pH 5.5 and 9.0 since preliminary study on the ungerminated seeds gave high activity at this pH values.

Purification of Enzyme: The crude α-amylase (300ml) was initially brought to 20% ammonium sulphate $[(NH_4)_2SO_4]$ saturation and allowed to stand for 12 hrs at 4°C. This was centrifuged at 15000 rpm for 10 min. The supernatant was made up to 50% ammonium sulphate saturation and allowed to stand for 12 hrs at 4°C. The precipitate was recovered by centrifugation at 15000 rpm for 10 min. This was dissolved in 0.2M sodium acetate buffer (pH 5.5) to a final volume of 20ml and dialyzed for 12 hrs against the same buffer. The dialysate was used as the partially purified α-amylase.

Assay of Amylase Activity: Amylase activity was assayed in duplicate using the method described by Miller [14]. Each of the test tube contained 0.1ml of 1% starch solution (Prepared by dissolving 1g of starch in 40ml of distilled water which was added to 50ml of boiling water of which the final volume was 100ml after cooling), 2.3 ml of 0.2M sodium acetate buffer pH 5.5and 0.1ml of the enzyme. This was incubated at room temperature for 30 min after which 0.5ml of DNS (3, 5-dinitrosalicylic acid) was added. The test tubes were immersed in boiling water bath for 10 min. The test tubes were allowed to cool at room temperature and absorbance taken against the substrate blank at 540nm using UV- VIS (JENWAY 6405). Amylase activity was estimated from a standard glucose curve. One unit of enzyme activity was defined as the amount of amylase that releases 1 μmole of reducing sugar as glucose per min under the assay conditions.

Protein Concentration: Protein concentration was determined as described by Lowry *et al.* [15].

Glucose Standard Curve: This was prepared as described by Miler *et al*. [14] using 5mM glucose solution.

Effect of Heat on Amylase Activity: The temperature dependence of amylase activity was determined using the method described by Varalakshmi *et al*. [16] with the following modifications. The enzyme was incubated for 30 min in the absence of the substrate at different temperatures (25-70°C) at 5°C interval. Aliquot (0.1ml) were withdrawn at appropriate intervals of time and assayed for amylase activity as described in the assay section.

Thermal denaturation of amylase was carried out at different time intervals (0-180 min) at different temperatures (25-70 °C) at 5°C intervals in the absence of the substrate and activity determined using the assay method above. The log of percentage enzyme activity versus time of incubation was plotted at each temperature and apparent rate constants (k) were obtained from the slope of the straight line. Using the Arrhenius equation, the lnk versus 1/T was plotted. The straight line obtained has a slope of Ea/-2.303R [17] where R is the universal gas constant ($8.314JK^{-1}mol^{-1}$) and T is temperature in kelvin (K).

Enthalpy of inactivation (ΔH) was calculated according to the equation

$$\Delta H = E\alpha - RT \quad (1)$$

The values for free energy of inactivation (ΔG) at different temperatures were obtained from equation (2).

$$\Delta G = -Rtln(k.h/Kb.T) \quad (2)$$

where h is the Planks constant ($6.62607x10^{-34}m^2kgs^{-1}$) and Kb is the Boltzmann constant (1.3807 x10-23J/K).

Enthropy of inactivation (ΔS) was calculated from equation (3)

$$\Delta S = (\Delta H - \Delta G) - T \quad (3)$$

The half-life, $t_{1/2}$i.e. time required for 50% reduction of initial activity at any given temperature was determined with equation (4).

$$t_1/2 = ln2/k \quad (4)$$

Effect of Metal Ions on the Enzyme Activity: This was carried out using the method described by Varalakshmi *et al.* [16] with the following modifications. The partially purified enzyme was incubated for 30 min in the absence and presence of different concentrations of metal ions (0.1, 0.5 and 1.0M) added as chloride salts. The enzyme activity was assayed as described in the assay section.

RESULTS AND DISCUSSION

Processing of Starch: Starch from cassava tubers gave a yield of 34.74%. This is high compared to the 32, 18.32 and 24% obtained by [18-20] respectively. A percentage yield of 17.4% starch was obtained from breadfruit seeds. [21, 22] have reported a low yield of 14.26 and 15.4% respectively. [24] also reported a yield of 18.05% for starch from breadfruit seeds.

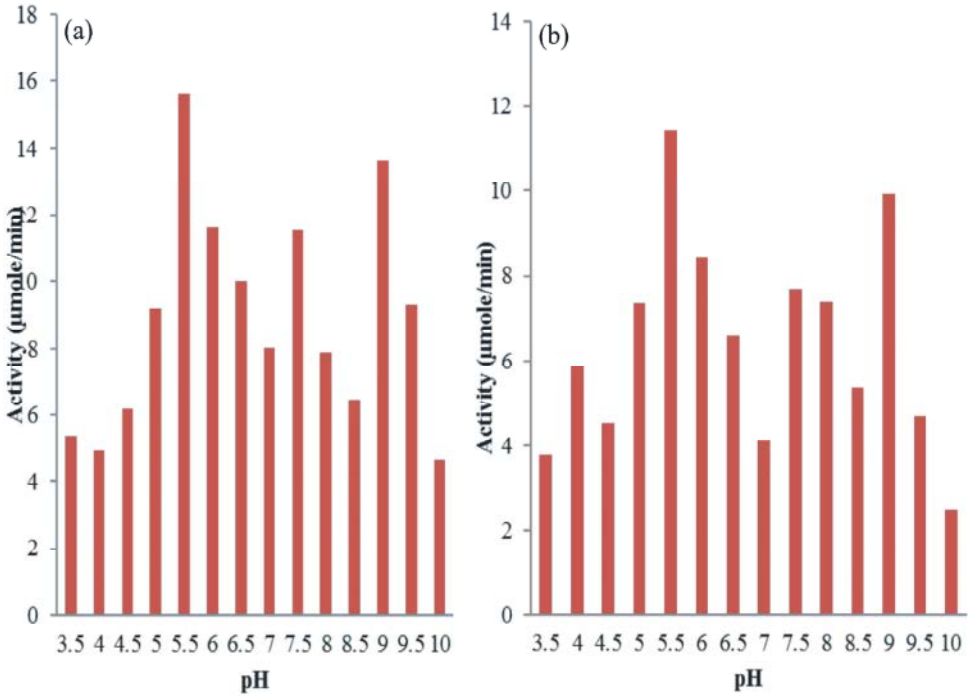

Fig. 1: Activity of α-amylase from breadfruit seeds at different pH before imbibitions using starch from (a) breadfruit seeds (b) cassava tubers as substrates

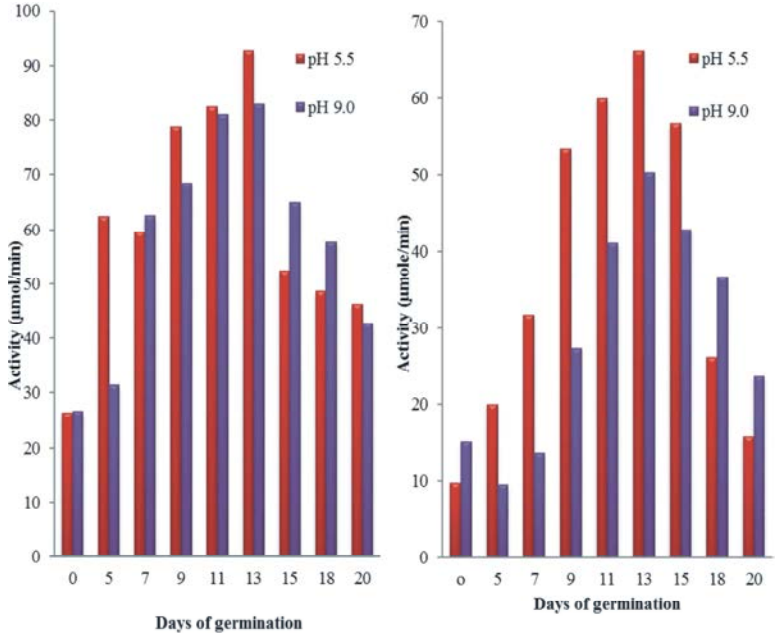

Fig. 2: Determination of α-amylase activity during the germination of breadfruit seeds (*Treculiaafricana*) using starch from (a) breadfruit seeds (b) cassava tubers.

The moderately high breadfruit starch yield of 17.14% from this study is of significant importance in domestic and industrial food utilization. The difference in yield obtained here might be as a result of difference in processing methods. However, variation in the starch content of breadfruit seeds may depend on the maturity, variety and differences in climatic and agronomic conditions. The starch sources could be an important source of industrial starch based on their yields.

Germination of Breadfruit Seed: Germination started 7 days after imbibitions and was completed in 20 days. [25] observed that breadfruit seeds started germinating on the 10^{th} day.

Purification of the Amylase from Breadfruit Seeds: Precipitations with solid ammonium sulphate between 10% - 100% were tested to find the proper saturation point. As a result, amylase activity of the precipitate at 50% ammonium sulphate saturation was found to give the best precipitation and was used in subsequent extraction process. This precipitated out most of the proteins and improved amylase purification. The precipitated protein was then dialyzed. The result of the purification profile is shown on Table 1. The result shows that the enzyme was purified 2.23 fold with a specific activity of 100.40U/mg proteins after ammonium sulphate saturation. The enzyme was then dialyzed and shows 2.38 fold enzyme purification with a specific activity of 107.09U/mg protein. [26], reported that an extra- cellular α-amylase produced by *Penicillium chrysogenum* in solid state fermentation gave 6.2- fold purification after dialysis. The result of the ammonium sulphate precipitation of α-amylase produced by the strain *Penicillium cameberti* PL21 showed that the enzyme precipitated at 60% ammonium sulphate and when subjected to dialysis resulted in 16.3-fold purification [26]. Sidkey *et al.* [27] reported 60% ammonium sulphate precipitation and a purification fold of 10.9 after dialysis against sucrose. El –Safey and Ammar [28] also purified α-amylase using ammonium sulphate precipitation and dialysis against sucrose resulted in 6.20 purification fold.

Effect of Heat on Amylase Activity: Amylase from germinating breadfruit seeds is stable between 25-55°C for 30 min (Figure 4). [27-28] reported that amylase from *hyperthermophilic Bacillus strain* HUTBS71 retained 100% of its activity at 50 - 60°C for 1hr of incubation. Thermostability for 1hr at 80°C has been reported for amylase from *Bacillus substilis* JS-2004 [29]. *Bacillus licheniformis* CUMC305 amylase was heat stable after 4hr incubation at 100°C [30]. In a similar way, Bacillus *species* ANT6 amylase was stable after overnight (85%) and 24hr (55%) incubation at 100°C [31]. Carvalho *et al.* [32] reported that amylase from *thermophilic Bacillus specie* was stable for 1hr at temperature ranges of 40-50°C.

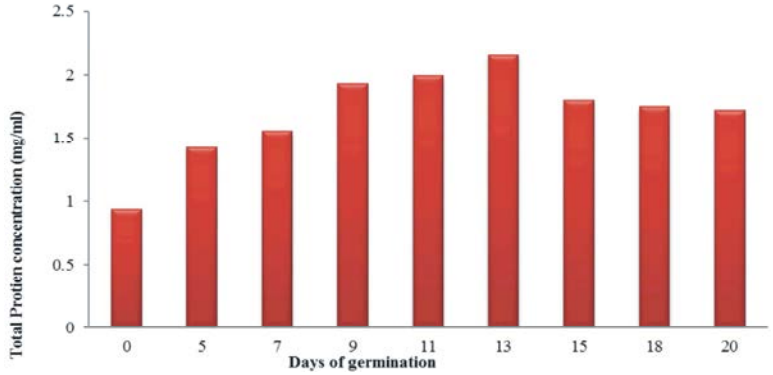

Fig. 3: Determination of day of maximum protein production after germination of breadfruit seeds (*Treculiaafricana)*

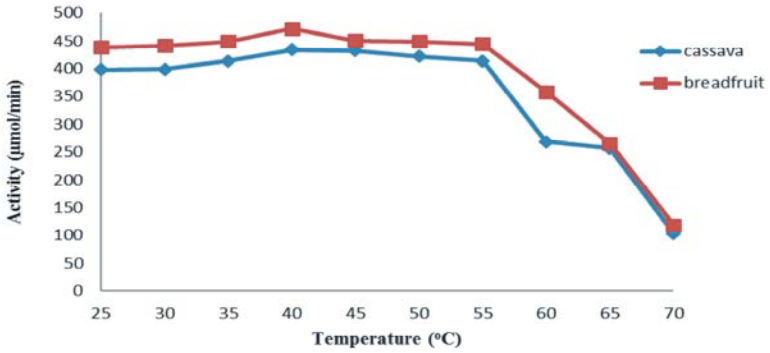

Fig. 4: Effect of heat on the activity of α-amylasefrom germinated breadfruit seeds

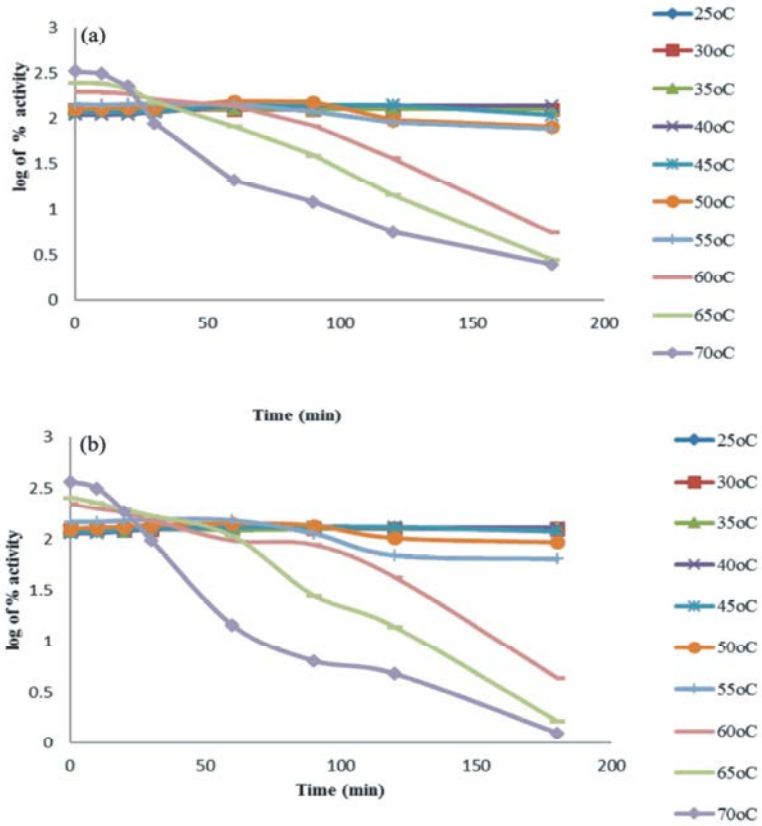

Fig. 5: Log of% activity of amylase using starch from (a) breadfruit seeds (b) cassava tubers as substrate

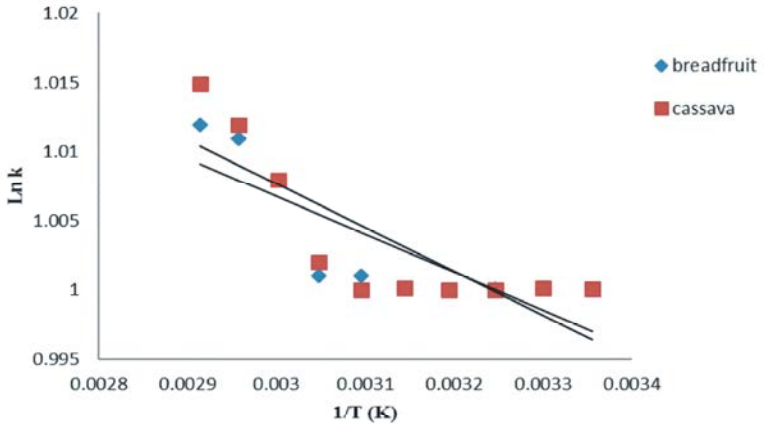

Fig. 6: Arrhenius plot of amylase inactivation

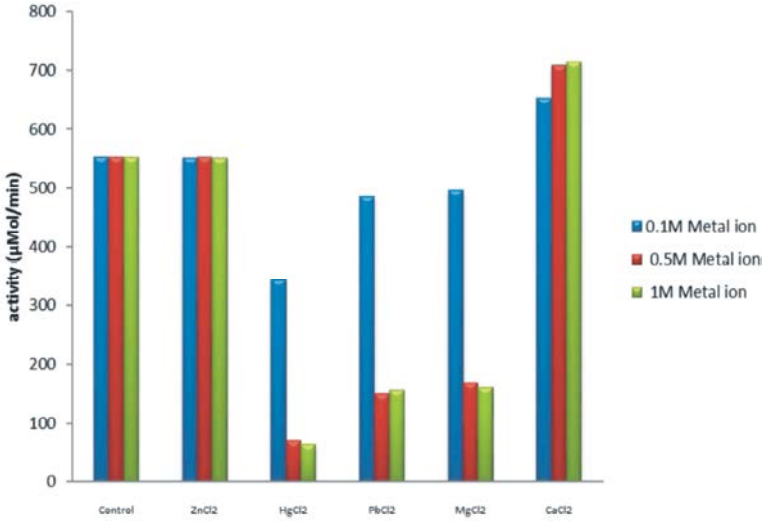

Fig. 7: Effect of metal ion on the activity of amylase

Table 1: Purification table of amylase from germinating breadfruit seeds

Enzyme Sample	Volume (ml)	Activity (U/ml)	Total Activity (U)	Protein (mg/ml)	Total Protein (mg)	Specific Activity (U/mg protein)	Yield (%)	Purification factor
Crude	300.00	93.03	27909.89	2.07	620.70	44.97	100.00	1.00
Precipitation [$(NH_4)_2SO_4$]	20.00	223.69	4473.93	2.23	44.56	100.40	41.59	2.23
Dialysis	22.00	262.37	5772.23	2.34	53.90	107.09	35.48	2.38

Table 2: Thermodynamic parameters

Temperature (°C)	Ea	ΔH	ΔG	ΔS	Half life
25	526.5464	-1951.03	44752.14	-156.722	0.693133
30	526.5464	-1992.6	45461.05	-156.613	0.693119
35	526.5464	-2034.17	46169.27	-156.505	0.693106
40	526.5464	-2075.74	46877.02	-156.399	0.693147
45	526.5464	-2117.31	47583.69	-156.292	0.693078
50	526.5464	-2158.88	48287.55	-156.181	0.692455
55	526.5464	-2200.45	48993.14	-156.078	0.692455
60	526.5464	-2242.02	49678.81	-155.918	0.687646
65	526.5464	-2283.59	50374.51	-155.793	0.685606
70	526.5464	-2325.16	51075	-155.686	0.684928

Table 3: Thermodynamic parameters of amylase inactivation when cassava starch was used as substrate

Temperature (°C)	Ea	ΔH	ΔG	ΔS	Half life
25	607.3473	-1870.22	44752.09	-156.451	0.693119
30	607.3473	-1911.79	45460.92	-156.346	0.693085
35	607.3473	-1953.36	46169.42	-156.243	0.693147
40	607.3473	-1994.93	46877.02	-156.14	0.693147
45	607.3473	-2036.5	47583.71	-156.038	0.693085
50	607.3473	-2078.07	48290.23	-155.939	0.693147
55	607.3473	2119.64	48990.42	-155.823	0.691764
60	607.3473	-2161.21	49678.81	-155.676	0.687646
65	607.3473	-2202.78	50371.73	-155.546	0.684928
70	607.3473	-2244.35	51066.56	-155.425	0.682904

Thermal Denaturation Kinetics of Amylase: The activation energy (Ea) of the enzyme was calculated as 526 KJ/mol and 607KJ/mol respectively (Tables 2 and 3). reported activation energy (Ea) for denaturation of purified amylase from hyperthermophilic *Bacillus strain* HUTBS71 calculated from the slope of Arrhenius plots to be 2.53KJ/mol. The activation energy (Ea) calculated from Arrhenius plot for α –amylase from B. lichiniformis EMS-6 was 25.14KJ/mol [33]. Duy and Filter [34] reported activation energy (Ea) of 363.7KJ/mol for Bacillus lichiniformis α-amylase. High activation energies as recorded in this report could suggest high stability indicating that this enzyme might be suitable for industrial applications. Gibb's free energy (ΔG) and enthalpy (ΔH) value were found to be in the range of 44752-57075 KJ/mol and 44752-51066KJ/mol when starch from breadfruit seeds and cassava tubers were used as substrates respectively at different temperatures. ΔS and $t_{1/2}$ was calculated as 156KJ/mol/K and 0.693min^{-1} respectively when starch from breadfruit seeds and cassava tubers were used as substrates (tables 2 and 3). 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 Bacillus lichiniformis EMS-6 were calculated as 36968KJ/mol, 22.53KJ/mol and -110.95KJ/mol/K respectively [33]. Tanaka and Hoshino (2005) reported (ΔH) and (ΔS) of Bacillus amyloliquefaciens α-amylase as 29.3KJ/mol and -82.6J/mol/K respectively.

Effect of Metal Ions: It was found from this research that the enzyme activity was enhanced by 0.1M calcium ion. Magnesium, mercury and lead ions inhibited the amylase activity even at 0.1M while zinc was observed as not having any effect on the enzyme activity (Figure 6). This result is contrary to the results of Reyeed [35] where calcium slightly inhibited the amylase activity when compared to the control. Asgher *et al*. [29] reported that calcium ion has no effect on amylase activity. It was observed by Rezaei *et al*. [36] that magnesium ion inhibited the activity of amylase which is contrary to the report by Srinivasa *et al*. [37]. The result of this research also supports the research made by Tapan *et al*. [38] that zinc ion has no effect on amylase activity. Its relatively dependence on calcium ions for its activity and stability may offer a good prospect for its application in industries for bread making, production of glucose and fructose syrup, production of fuel ethanol from starch among others.

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