Asian Journal of Research in Biochemistry

11(1): 30-42, 2022; Article no.AJRB.92573
ISSN: 2582-0516

Biochemical and Thermodynamic Characterization of β amylase from *Dioscorea alata L.*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI:10.9734/AJRB/2022/v11i1208

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/92573

Original Research Article

Received 03 August 2022
Accepted 05 October 2022
Published 25 October 2022

ABSTRACT

β-amylase (E.C.3.2.1.2) is a starch hydrolyzing enzyme fondly used in foods, pharmaceuticals, and brewing industries to convert starch into maltose. The aim of this study was to determine the physicochemical, kinetic, and thermodynamic properties, as well as the potential industrial use of β-amylase from *Dioscorea alata L*. Studying the enzyme stability with Arrhenius methods, showed that the enzyme was stable at a temperature range of 20–50^{0}C, and had good pH stability by retaining over 50 % of its initial activity over a wide range of pH from 4 – 8 and kinetic stability by increasing the half-life of the enzyme. The activation energy (Ea) for catalysis by water yam β-amylase at 25^{0}C was 6.45kcal/mol. The activation energy (Ea), half-life, free energy change ($\Delta G^{\ddagger}$), enthalpy change ($\Delta H^{\ddagger}$), and entropy change ($\Delta S^{\ddagger}$) for inactivation at optimum temperature (40^{0}C) and pH 5 were 13.92 kcal/mol, 41.25 min, 20.89 kcal/mol, 13.30 kcal/mol and -24.25 cal/mol/K respectively. Km and Vmax values were reduced from 2.25 to 2.13mg/ml and 2.95 to 1.48 µmol/min/ml respectively. The optimum pH shifted from 5 to 6, while the Optimum temperature increased from 40 to 50^{0}C after immobilization. Enzyme retained up to 67 % activity after 5 cycles. The enzyme would be of importance in manufacturing companies based on the kinetics and application features reported in this study since it is a cheap and readily available source.

Keywords: Dioscorea alata; β-Amylase; thermo-stability; half-life; immobilization; industrial application.

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1. INTRODUCTION

α-1,4-glucanbonds from the non-reducing ends of starch and other carbohydrate polymers are attacked by β-amylase, which converts them to maltose units. Maltose is a two-glucose disaccharide with numerous applications in the food and pharmaceutical industries [1- 4].

Amylases are found in a wide range of microbes, plants, and mammals, and have found use in a variety of industries, including starch liquefaction. [5-7]. Although chemical methods can be used to liquefy starch, enzymatic hydrolysis can be done under mild circumstances, avoiding the harsh conditions required by chemical treatments. Furthermore, unlike chemical procedures, the enzymatic approach did not result in water pollution [8].

The β-amylase reaction is important in the formation of maltose and the creation of fermented foods and alcoholic beverages [9]. β-amylase is used as a biocatalyst in a variety of systems, including packed bed reactors and fluidized-bed reactors.

In the tropics, yam is a staple food with nutritional value. It is one of the most widely cultivated and eaten plants. About 75-84 percent of the dry weight of yam is starch [10]. This might be used to determine the expression of numerous enzymes in the crop's subcellular regions, catalysing the conversion of starch into its many derivatives. It could be a suitable source for industrial starch hydrolyzing enzymes like β-amylase, which are in great demand. Although neglected compared to the popular type of *Dioscorea rotundata*, water yam (*Dioscorea alata*) is known for its bulkiness, high moisture, and starch content [11].

Dioscorea alata is one of the six economically important yam species, although it is underutilized in Nigeria for main food products due to traditional prejudice, which fails to identify the species' distinctive qualitative traits and agronomic adaptability.

The purpose of this research was to look at the kinetics of chemical reactions, isothermal conditions, and the behaviour of phase transition processes using classical kinetic models. The major benefit was to look at the mechanism of interaction between kinetic models and activation energies, both of which are responsible for the process response, in relation to temperature changes [12]. We can circumvent issues with free enzyme activity by immobilizing enzymes on solid supports. It has the ability to improve enzyme activity, selectivity, and stability. It also permits the biocatalyst to be reused as long as the enzyme is active in a continuous process, lowering process costs, particularly in industrial applications [13-17]. The immobilization procedure could improve enzyme activity and solubility in reaction fluids [16-18].

2. MATERIALS AND METHODS

2.1 Materials

Water yam was obtained from a local vendor Ketu, Lagos, and identified by a Taxonomist from the University of Lagos, Nigeria. 8-anilino-1-napthalene sulfonic acid (ANS), Trizma base, sodium chloride, and spectroscopic grade ethanol were purchased from Sigma-Aldrich Fine Chemicals, St. Louis, MO, USA. BSA standard and Bradford reagent kit were products of Bio-Rad, Palo Alto, CA, USA. All other chemicals were commercial products of reagent/analytical grade.

2.2 β-Amylase Assay Method

The DNSA method was used to determine β-amylase activity. The reaction mixture contained 100 µl of 1 % soluble starch in 50 mM sodium acetate buffer (pH 5) and 100 µl of the enzyme. After 3 min of incubation at 25 °C with 200 µl of 3,5-DNSA color reagent, the reaction was stopped. The mixture was boiled for 5 min before being cooled in ice and diluted with 1000 µl of distilled water. A UV-visible spectrophotometer was used to measure absorbance at 540 nm.

The enzyme activity was measured in micromoles of maltose released per minute per milliliter, which is the amount of reducing sugar released by the enzyme during the reaction [19].

2.3 β-amylase Immobilization on Sodium Alginate Beads

The beads were made according to Sachin and Sandeep, 2012 with slight modifications [20].4g of sodium alginate was dissolved in 100 ml acetate buffer to make a 4 % solution. 10 ml of 3.4 mg of the enzyme was mixed with 10 ml of 4 % sodium alginate solution. The beads were formed by dripping the polymer solution from a height of approximately 20 cm into an excess of

stirred 0.1 M $CaCl_2$ solution with a syringe and needle at room temperature in the calcium solution, left to cure for 30 min, filtered, washed thoroughly with distilled water several times, dried with filter paper, and exposed to open-air for 1hour before use.

$$\text{Immobilization yield \%} = \frac{activity\ of\ immobilized\ enzyme}{A-B} X100 \quad (1)$$

WhereA= activity of free enzyme added,
B= activity of theremaining enzyme in washed water

2.4 The Effect of Substate Concentration on Enzyme Activity

To determine the kinetic constants Km, Vmax, and Kcat, 100 µL of purified β-amylase was incubated with 100 µL of 50 mM sodium acetate buffer pH 5 at various concentrations of soluble starch solution ranging from 0 to 10mg/ml for 3 min at 25 °C. This was also done with amylose, amylopectin, and glycogen. The immobilized enzyme was treated in the same way, with 1 g incubated. A Lineweaver-Burk plot was used to calculate the values of Km, Vmax, and Kcat.

2.5 Effect of pH on Enzyme Activity and Stability

Glycine-HCl pH3, sodium acetate (pH 4-5), phosphate (pH 6-7), and Tris-HCl pH 8 were used to measure β-amylase activity at different pH levels. The β-amylase assay was used to carry out the reaction. The maximum activity was set to 100 %, and relative activity was plotted against various pH values.

The immobilized enzyme was treated in the same way. Purified β-amylase's stability was determined by pre-incubating the enzyme at 25°C with 50 mM of the above-mentioned buffers, followed by a determination of residual activities at different time intervals. The initial value was set to 100 %, and relative activity was plotted against various time intervals.

2.6 Effect of Temperature on Enzyme Activity and Thermodynamic characterization

In a regulated water bath,100 µl of1% soluble starch was mixed with 100 µl of the appropriately diluted enzyme was incubated for 3 min at different temperatures ranging from 20^0C to 50^0C. The assay was performed in accordance with standard procedures.

The effect of temperature on enzyme stability was studied by incubating the enzyme solution at different temperatures without substrate for 1 hour. An aliquot was taken every 10 min and tested for β-amylase residual activity. The ln k data was plotted against the reciprocal of absolute temperature. Semi-logarithmic plots were used to calculate and analyze the first-order rate constants for denaturation K_d of the enzyme at various temperatures [21]. Thermodynamic parameters were calculated by rearranging the Eyring absolute rate equation:

$$slope = -{}^{E_a}/_{R} \quad (2)$$

Where Ea is the Activation energy
R (gas constant) = 1.987 cal/degreemol^{-1}

$$\Delta H^* = E_a - RT \quad (3)$$

$$\Delta G^* = -RT\left(\frac{\ln K_d h}{K_B T}\right) \quad (4)$$

$$\Delta S^* = \frac{(\Delta H^* - \Delta G^*)}{T} \quad (5)$$

$$K_d = \left(\frac{K_B T}{h}\right) e^{\left(-\Delta H^*/RT\right)} e^{\left(\Delta S^*/R\right)} \quad (6)$$

where *h* (Planck's constant) = 1.584×10^{-34} cal/k,
K_B=*Boltzman* constant,
[*R/N*] = 3.298×10^{-24}cal S,
N (Avogadro's no.) = 6.02×10^{23} mol^{-1}.

2.7 Thermal Inactivation

Thermal inactivation of purified β-amylase was determined by incubating the enzyme for 60 min in 50 mM sodium acetate buffer pH 5 buffers at 40°C. Aliquots (100 µl) were taken at different times, cooled on ice, and the residual enzyme activity was measured.

The heat stability of the enzyme was investigated by incubating it at 40 °C for 60 min in the presence of 10 mM Fructose, Glucose, Galactose, and Maltose. Each sample's β-amylase level was measured [22]. The following equation was used to calculate the magnitudes of K_{inact} versus thermal condition:

$$\ln {}^{A}/_{A_0} = -K_{ianct}\, t \quad (7)$$

A and A_0 are the enzyme activity at each time and the initial time, respectively

2.8 Effect of Metals on the Enzyme Activity and Stability

The enzyme was incubated in 50 mM acetate buffer pH 5 in the presence of each of the following salts: NaCl, $CaCl_2$, KCl, $(NH_4)_2SO_4$, $ZnSO_4$, $MgSO_4$, $FeSO_4$, $CuSO_4$,and HgCl. The enzyme activity was measured according to the standard assay. The effect of metals on stability was determined by incubating the enzyme in the presence of various metals for one hour at 40^0C. Residual activity was measured at 10 min intervals. The blank contained the buffer solution with the metal ion under consideration without the enzyme. The control consisted of the enzyme and the substrate without any metals.

The effect of metals on the half-life of the enzyme was determined at a final concentration of 100 mM. The half-life was determined by the relationship:

$$t_{1/2} = \frac{\ln 2}{K_d} \quad (8)$$

Where k_d is the deactivation rate constant that can be derived from:

$$V = V_0 e^{-K_d t} \quad (9)$$

Where Vo is the initial enzyme activity while V is the enzyme activity at time t of incubation; and $-k_d$ can be obtained from the plot of:

$$\ln V = \ln V_0 - K_d t \quad (10)$$

The D-value (decimal reduction time) is the time (min) to reduce 90 % of the enzyme activity and is given by the following equation

$$D = \frac{\ln 10}{Kd} \quad (11)$$

2.9 Application of β-amylase

Wash efficiency of starch stains was studied in the presence of Mama gold and Rana detergents [23]. White cotton cloth pieces stained with a 1 % starch solution were baked at 80 °C for 30 min to ensure a firm binding of stains to the material support.

The cleaning performance was evaluated by varying the cleaner, which included water, water + detergent, water + enzyme, and water + detergent (7 mg/mL) + enzyme.

A stained cotton cloth piece was shaken for 30 min at 50 ^{0}C in the presence of the corresponding cleaner mixture. Each mixture obtained solution was collected in order to determine the concentration of reducing sugars released from starch.

The washing process's efficiency in removing starch was expressed as the following equation [24]:

$$\text{Efficiency } \% = \frac{100 * A * 0.95}{B} \quad (12)$$

where A is the amount of maltose released (g/mL) during the wash procedure and B is the amount of starch (g/mL) used for staining the cotton cloth piece.

2.10 Reusability of Immobilized β-amylase

Enzyme reusability was checked by carrying out an enzyme assay a couple of times. At the end of each cycle, the derivative was washed with distilled water and activity buffer, a new substrate solution was added to start a new round of reaction. The initial and remaining activities were assayed at pH 5, 25 ^{0}C (initial activity at these conditions is regarded as 100 % [25].

2.11 Statistical Analysis

All experiments were carried out in triplicates, unless otherwise stated. The results were analyzed using Graph pad prism (version 5.0) and Kaleida Graph 4.5.4 synergy software. They were expressed as mean ± standard deviation. $P< 0.05$ was considered as level of significance.

3. RESULTS AND DISCUSSION

β-amylase hydrolyzes starch, amylose, and glycogen but has no effect on maltose, sucrose, fructose, lactose, or galactose (Table 1). The optimal pH after immobilization increased from 5 to 6 (Fig. 1), while the optimal temperature increased from 40 °C to 50 °C (Fig. 2). Fig. 3 depicts the Lineweaver-Burk plot for soluble and immobilized enzyme with soluble starch. The Km and Vmax values for free enzyme were 2.25 mg/ml and 2.95 µmol/min/ml, respectively, while the values for immobilized enzyme were 2.13 mg/ml and 1.48 µmol/min/ml. For soluble starch, amylose, amylopectin, and glycogen, purified β-amylase from water yam exhibited typical Michaelis-Menten kinetics. The purified enzyme with soluble starch had a Kcat of 317.5 s^{-1} (Table 2), which is higher than 137.93 min^{-1} obtained from fenugreek β-amylase by Srivastava *et al.*,

2014 [26]. Fig. 4 depicts the Lineweaver-Burk plot with and without α-cyclo-dextrin as an inhibitor. The Ki and Kii concentrations were 2.70 mg/ml and 3.33 mg/ml, respectively.Fig. 5 depicts the Lineweaver-Burk plot of β-amylase from water yam in the presence and absence of maltose as an inhibitor. Vi and Vii values were 2.089 and 2.42 µmol/ min/ml, respectively.

Maltose had a non-competitive inhibition pattern on the enzyme, whereas α-cyclodextrin had a competitive pattern, which is consistent with Srivastava *et al*., 2014 [26].

The enzyme was most stable at alkaline pH levels of 7 and 8, with approximately 80 % residual activity, while at acidic pH levels of 4, 5, and 6, remaining activities of 65, 70, and 73 percent, respectively, were obtained after incubating for 4 hours (Fig 6). Acidic stable β-amylase from a luminal particle-associated microorganism was reported [27], whereas Kolawole *et al.*, [22], Chiba and Kuwashima, [28], and Kolawole *et al.*, [29] reported alkaline stable β-amylase from Chinese yam tuber, *Digitaria exilis*, and *Eleusine coracana*. Van Damme *et al*., (2001) [30] proposed that, in addition to the catalytic site, other regulatory parts of the enzyme may be responsible for the stability of β-amylase at alkaline pH.

After 60 min of incubation, water yam β-amylase retained over 70 % of its initial activity at 50°C (Fig. 7). This could indicate its potential use in the food and beverage industries to convert starch to maltose, where thermostability and thermo-activity of the enzyme are important due to high temperature operating conditions [31].

The K_{inact} value of the inactivation rate constant is the best measure for describing the effect of osmolytes on the enzyme's kinetic stability. The thermo-inactivation data in Table 3 revealed that glucose increased the kinetic stability of β-amylase. It protected the enzyme against heat inactivation by stabilizing it. Sugars are known to protect proteins from losing their function [32,33], chemical [34], and thermal denaturation [35].

Water viscosity and surface tension are also affected by osmolytes [36]. Protein dynamics and structure, particularly protein flexibility, can be affected by environmental parameters such as pH, viscosity, ionic strength, temperature, prosthetic groups, solvent composition, and so on. As a result, the specific nature of interactions that determine osmolyte-mediated protein stability is yet unknown.

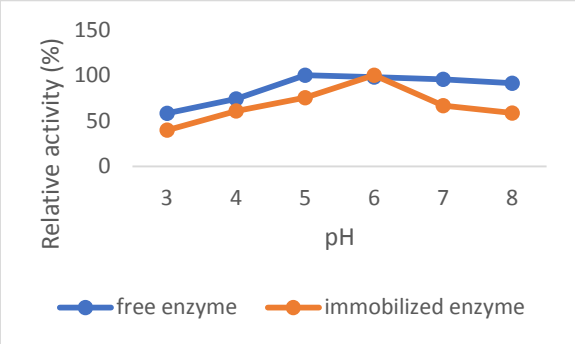

Fig. 1. Effect of pH on both the free and immobilized enzyme

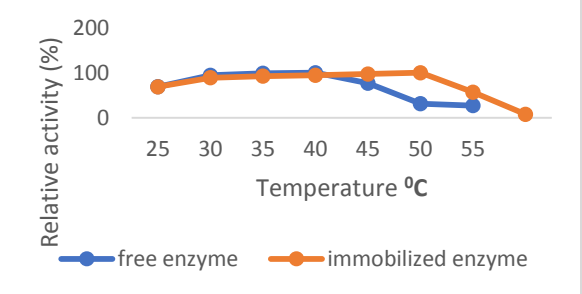

Fig. 2. Effect of Temperature on free and immobilized enzyme

Overall, protein stability is determined by a delicate balance of favorable and unfavorable interactions between native and denatured protein states and co-solvent molecules [37].

The stabilizing impact is thus dependent on the type of protein as well as the co-solvent molecules, and it may not be possible to generalize the effect. Table 4 shows that the activation energy of 6.48 kcal/mol was similar to that of pea epicotyls (6.28 kcal/mol). peanuts (4.5 kcal/mol) [38], and fenugreek [39]. It is related to the reaction rate. At high temperatures, the thermal unfolding of proteins is induced by a large rise in entropy change, which lowers the Gibbs free energy change of the unfolding transition [40]. Ca^{2+}, Mg^{2+}, and Zn^{2+} were added to boost the activity of β-amylase. This is consistent with the findings of Kolawole *et al*. (2011) [22], who found that Ca^{2+} boosted the activity of β-amylase from *E. coracana*, while Cu^{2+} and EDTA decreased activity. Metal binding is linked to variations in metal coordination geometries and influences metal selection [41] (Table 5). The findings show that the thermostability of purified water yam β-amylase is pH dependent, implying that the high stability of water yam β-amylase at alkaline pH at high temperatures and the use of specific metals could imply a high industrial utility potential for the production of various high conversion maltose syrups from raw and liquefied starch (Table 6). Metal specificity appears to be present in β-amylases from several sources for increased thermo-activity and thermostability. The idea of the metal enhancing the chemical potential of the protein and so favoring the folded form over the unfolded state was suggested by Ray *et al*., (1994) [42].

Table 1. Effects of various substrates on β-amylase

Substrates	Relative activity (%)
Soluble starch	100
Amylose	91
Amylopectin	87
Glycogen	40
Maltose	0
Sucrose	0
Fructose	0
Lactose	0
Galactose	0

The main factors that determine an enzyme's stability are its half-life, decimal reduction time (D), and first-order rate constants (K_d) (Table 7). The results show that as the temperature increased, the $t_{1/2}$ and D-value decreased as the K_d increased, indicating that the enzyme is less stable at higher temperatures [43,44].

The addition of Zn^{2+} and Mg^{2+} resulted in a slight increase in both the enzyme half-life and the ΔG# of thermal denaturation, as well as a decrease in ΔS#. Other metals decreased both the half-life and the ΔG, but increased the ΔS# of thermal denaturation (Table 8). The presence of metal ions had no effect on the ΔH# of denaturation. The non-covalent bonds were unaffected by thermal denaturation in the presence of metal ions bonding but the opening of the structure by the change in $\Delta S^{\ddagger}$. It shows that $\Delta S^{\ddagger}$ contributes to the $\Delta G^{\ddagger}$ denaturation but not the $\Delta H^{\ddagger}$ in the presence of all the metals. Thermal unfolding of proteins at high temperatures is caused by a strong increase of the entropy change that lowers Gibbs free energy change of the unfolding transition [41].

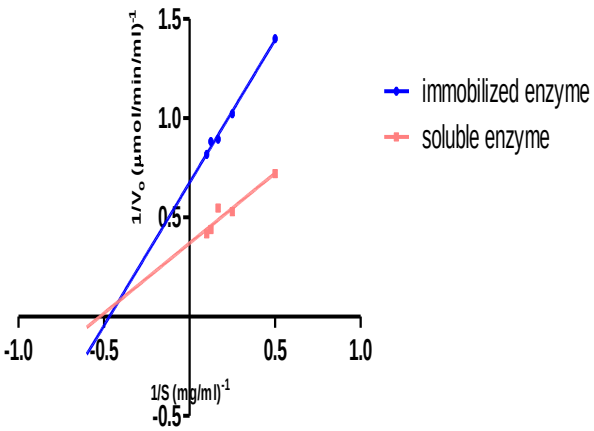

Fig. 3. Effect of concentration on soluble and immobilized enzyme

Table 2. Kinetic parameters of β-amylase with various substrates

Substrates	Starch	Amy-lose	Amylopectin	Glycogen
Km (mg/ml)	2.24	2.56	2.53	4.27
Vmax (µmol/min/ml)	2.94	2.60	3.18	3.74
Vmax/Km	1.31	1.02	1.26	0.88
Kcat (S^{-1})	317.5	279.6	341.9	402.2
Kcat/Km	141.1	109.2	135.2	94.2

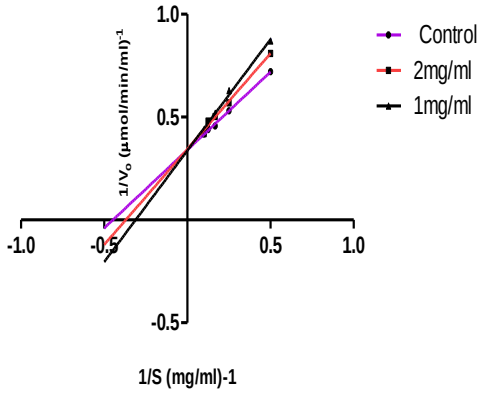

Fig. 4. Lineweaver-Burk plot of β-amylase from water yam in the presence and absence of α-cyclodextrin as inhibitor

The activity was assayed in 50 mM acetate buffer pH 5 at 25°C using DNSA method. The substrate concentration range was 0–10mg/ml. $p < 0.05$

Table 3. The effect of Osmolytes on the kinetic stabilities of the enzyme

Osmolytes (10 mM)	Kinact (S^{-1})	ΔG 25 ^{0}C (Kcal/mol)
Control	6.17×10^{-5}	20.76
Glucose	3.80×10^{-5}	21.04
Maltose	5.39×10^{-5}	20.84
Fructose	5.10×10^{-5}	20.87
Galactose	7.10×10^{-5}	19.37

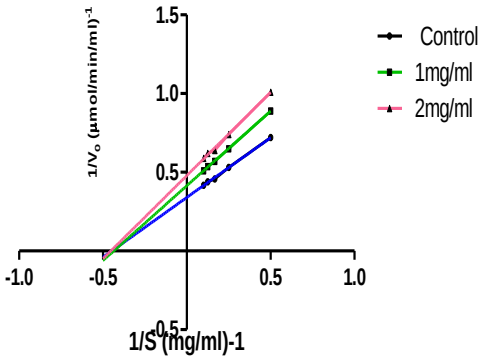

Fig. 5. Lineweaver-Burk plot of β-amylase from water yam in the presence and absence of maltose as an inhibitor

The activity was assayed in 50 mM acetate buffer pH 5 at 25 °C using DNSA method. The substrate concentration range was 0–10 mg.

Table 4. Thermodynamic parameters for catalysis of water yam β-amylase

Temperature (K)	$\Delta G^{\ddagger}$ kcal/mol	$\Delta H^{\ddagger}$ kcal/mol	$\Delta S^{\ddagger}$ cal/mol/k
293	12.86	5.87	-23.46
298	12.84	5.86	-23.42
303	12.82	5.85	-23.39
308	12.80	5.84	-23.35
313	12.78	5.83	-23.32

Ea of activation is 6.48 kcal/mol

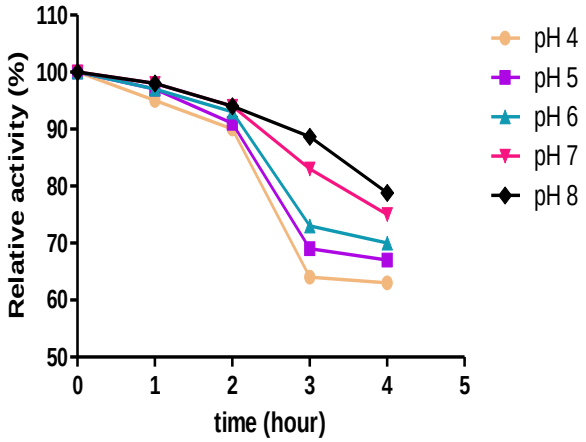

Fig. 6. Effect of pH on stability of the β-amylase

The enzyme was prepared by mixing the purified enzyme with two volumes of the buffer solution of pH 4 -8. Incubated at room temperature and the residual activity assayed hourly.

Table 5. Effects of metal ions on β-amylase activity

Substrates	Relative activity (%)
None	100
Ca^{2+}	110
Mg^{2+}	122
Zn^{2+}	120
Fe^{2+}	31
Pb^{2+}	22
Na^{+}	100
K^{+}	108
Hg^{+}	14
Iodoacetate	72
Urea	91
Fe^{3+}	89
Cu^{2+}	51
EDTA	81
Sn^{2+}	28
NH_4^{+}	78
Hg^{+}	14

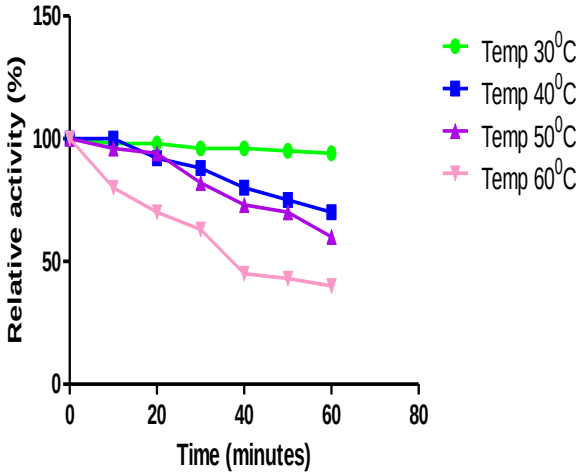

Fig. 7. Effect of temperature on enzyme stability

The residual activities were expressed relative to the maximum activity at 0 min which was taken as 100 %.

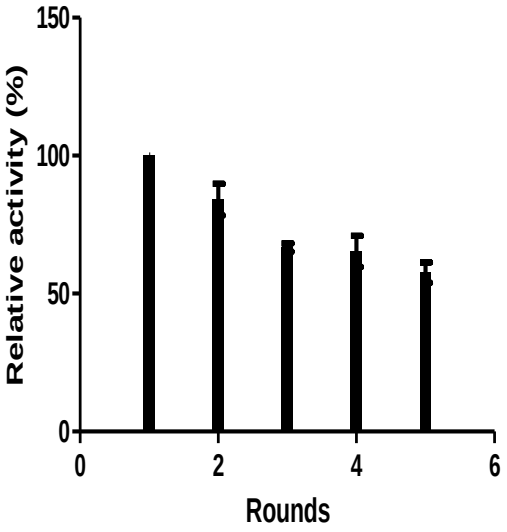

Fig. 8. Reusability of β-amylase

Table 6. Thermodynamic parameters for thermal inactivation of yam β-amylase at various pH at 40°C

pH	Ea (Kcal/mol)	Half-life (min)	$\Delta G^{\ddagger}$ kcal/mol	$\Delta H^{\ddagger}$ kcal/mol	$\Delta S^{\ddagger}$ cal/mol/k
5	13.92	41.25	20.89	13.33	-24.25
6	14.41	41.01	20.89	13.79	-22.68
7	16.00	41.46	20.90	15.38	-17.64
8	16.24	42.00	20.90	15.62	-16.87

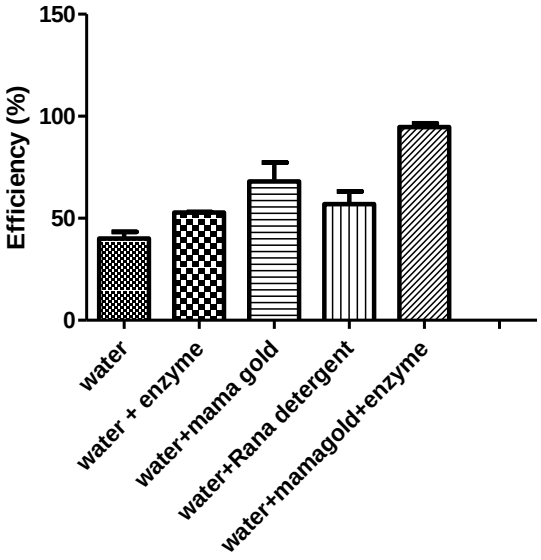

Fig. 9. Efficiency of starch stain removal by β-amylase

Enzyme retained up to 67 % activity after 5 cycles in reusability test after immobilization (Fig. 8), while β-amylase presence made starch removal more efficient as shown in Fig. 9.

Table 7. Thermodynamic parameters for thermal inactivation of water yam β-amylase at various temperature

Temperature (K)	Half-life (min)	$\Delta G^{\ddagger}$ kcal/mol	$\Delta H^{\ddagger}$ kcal/mol	$\Delta S^{\ddagger}$ cal/mol/k
293	41.25	20.89	13.33	-24.25
298	31.50	21.07	13.29	-24.47
303	22.35	21.18	13.28	-24.46
308	16.50	21.32	13.27	-24.45
313	10.34	21.35	13.26	-24.29

Ea is 13.92 kcal/mol

Table 8. Thermodynamic parameters for metals on thermal inactivation of water yam β-amylase at 40 ˚C and pH 5

Metallic Chloride (10mM)	Half-life (min)	$\Delta G^{\ddagger}$ kcal/mol	$\Delta H^{\ddagger}$ kcal/mol	$\Delta S^{\ddagger}$ cal/mol/k
Control	41.25	20.89	13.33	-24.25
Na^{+}	41.25	20.89	13.33	-24.25
Ca^{2+}	40.29	20.88	13.33	-24.19
Zn^{2+}	42.78	20.92	13.33	-24.35
Mg^{2+}	43.31	20.93	13.33	-24.38
NH_4^{+}	40.54	20.88	13.33	-24.22

Ea is 13.92 kcal/mol

4. CONCLUSION

The fact that the β-amylase was active over a wide pH range may imply that the enzyme will be useful in processes that are subjected to wide pH change from the acidic to alkaline range. The enzyme can be employed in food and beverage industries to convert starch into maltose where high value has been placed on the thermostability and thermo-activity of the enzyme.

The high stability of water yam β-amylase at alkaline pH, high temperatures and the use of some specific metals could imply high industrial utility potential for the production of various high

conversion maltose syrups from raw and liquefied starch. The low free energy of activation offers low cost in terms of energy requirements.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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