

# Biochemical Characterization of purified $\beta$ -amylase from *Dioscorea alata* (water yam)

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## Research

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## Biochemical Characterization of purified $\beta$ -amylase from *Dioscorea alata* (water yam)

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### ABSTRACT

Water yam (*Dioscorea alata*)  $\beta$ -amylase was isolated and purified to apparent homogeneity. The enzyme was found to be monomeric with a molecular weight of 30.1 kDa based on SDS-PAGE and 31.0 kDa based on non-denaturation gel filtration. The enzyme readily hydrolyzed soluble starch, amylose, amylopectin and glycogen. The enzyme was stable over a wide range of pHs (4 - 8). The enzyme has a  $K_m$  of 2.24 mg/ml for soluble starch. Activation energy ( $E_a$ ) for catalysis by  $\beta$ -amylase at 25 °C was 6.45 kcal/mol.  $\beta$ -amylase contains high content of both acidic and hydrophobic amino acid but low in arginine and histidine. The activation energy ( $E_a$ ), half-life, free energy change ( $\Delta G^\ddagger$ ), enthalpy change ( $\Delta H^\ddagger$ ), and entropy change ( $\Delta S^\ddagger$ ) for inactivation were 13.92 kcal/mol, 41.25 min, 20.89 kcal/mol, 13.30 kcal/mol and -24.25 cal/mol/K, respectively. The binding profile of  $\beta$ -amylase with epicatechin, quercetin, rutin and gallic acid were all spontaneous with stoichiometric ratio of 1:1. Hydrophobic bonding played a major role in stabilizing the  $\beta$ -amylase-ligand complex. Sodium alginate immobilization of the enzyme improved the optimum temperature from 40 to 50 °C and changed the optimum pH from 5.0 to 6.0 with retainment up to 67 % activity after 5 cycles of usage. The enzyme would be of importance in manufacturing company based on the kinetics and other parameters reported in this study.

**KEY WORDS:**  $\beta$ -amylase, kinetics, thermo-stability, immobilization, industrial application

### INTRODUCTION

$\beta$ -amylase (EC 3.2.1.2) is an exo-amylase acting on starch with the successive removal of maltose molecules from the non-reducing ends of the glucose polymers with inversion of the anomeric configuration [Derde et al. 2012]. It is an enzyme widely distributed in most living organisms, playing an important role in starch liquefaction process and in starch conversion technology both of biotechnological importance and of scientific and industrial interest. Amylases are one of the most important industrial enzymes with a wide variety of applications in various industrial fields such as food, textile, paper, detergent, and baking industries. It is also used in the production of glucose and fructose syrups, ethanol fuel, fruit juices, alcoholic beverages and sweeteners and as a digestive aid and spot remover in dry cleaning [Saini et al. 2017; Amid et al. 2014].

$\beta$ -amylase occurs widely in higher plants and also in micro-organisms [Nanmori et al. 1993]. It has been well studied in a number of plants such as sweet potatoes [Cochrane et al. 1991] malted barley [Evans et al. 1997]. However, most of the microbial  $\beta$ -amylases produced, have been found not to be active and thermostable enough to replace the indigenous plant enzymes from agrarian sources such as barley, soybean, cassava [Fossi et al. 2009]. Starch degrading enzymes are distributed widely in the

microbes, plants and animals. They degrade starch and related polymers to yield products characteristic of individual amylolytic enzymes. However, some reports show that  $\beta$ -amylase from higher plants and from micro-organisms are neither active nor stable at temperature

above 60°C [Fogarty and Kelly 1990]. It is therefore important to source for a  $\beta$ -amylase that can withstand the stress of operating condition in the industries, getting a beta-amylase that can produce desirable physicochemical properties.

Yam, *Dioscorea* genus is a staple food of cultural, economic and nutritional importance in the tropics. It produces edible starchy storage tubers [Pervez et al. 2014] and is definitely one of the mostly cultivated and consumed species. Most significantly, yam comprises about 75-84% dry weight starch [Moura et al. 2016] which could actually serve as measure of expression of various enzymes in sub-cellular locations of the crop responsible for catalysis of its starch into respective products. Yam could be good source to meet high demand of industrial starch hydrolyzing enzymes such as  $\beta$ -amylase. Meanwhile, there have been studies on  $\beta$ -amylase from sweet potatoes [Sikander et al. 2014; VanDamme et al. 2001], *Dioscorea dumetorum* tuber [Pervez et al. 2014], however there has been no reported works on water yam  $\beta$ -amylase. Hence, the study was sought to characterize purified  $\beta$ -amylase isolated from *Dioscorea alata* and explore its biochemical characteristics.

### MATERIALS AND METHODS

Ammonium sulfate, amylose, amylopectin, epicatechin, 3,5-dinitrosalicylic acid (DNSA) were products of Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Acrylamide, bis-acrylamide, low molecular weight protein marker, bovine serum albumin and Bradford reagent were products of BioRad (Hercules, California, USA). All chemicals used were of analytical reagent grade. Freshly harvested and infection free water yam

was obtained from Ketu, Lagos and authenticated by Prof. Olowokudejo of botany department, University of Lagos, Nigeria.

#### **Preparation of crude extract**

Enzyme extraction was carried out according to the method described [Chiba 2006] with slight modification. Water yam of 600 g was cut into pieces, mixed with equal volume of cold water and homogenize with blender, the resulting slurry was filtered with cheese cloth, cold centrifuged at 4000 X g for 1 hour to remove the debris, and the resulting supernatant was adjusted to pH 5 by adding same volume of 50 mM acetate buffer pH 5. The obtained solution was mixed with equal volume of 0.8mM dodecyl trimethyl ammonium bromide and cold centrifuged at 20 000 X g for 1 hour at 4°C. The supernatant was used as crude enzyme solution for subsequent purification steps.

#### **Acid treatment**

The crude enzyme (1500 ml) was adjusted to pH 3.6 with ice cold 1 N HCl. This was allowed to stand for 10 minutes to selectively denature  $\alpha$ -amylase. The acidified enzyme was readjusted to pH 5 with cold 3 % NH<sub>4</sub>OH solution [ Matsui et al. 1977].

#### **Ammonium Sulphate Fractionation**

The supernatant was fractionated by ammonium sulfate precipitation (40 - 80% saturation). The precipitate was collected by centrifugation at 10 000 X g for 30 minutes and dissolved in 20 ml of 50 mM acetate buffer pH 5. This solution was dialyzed overnight and extensively against 50 mM acetate buffer pH 5.

#### **Ion Exchange Chromatography on DEAE-Sephadex A<sub>50</sub>**

The dialyzed sample (30ml) was loaded onto a DEAE-Sephadex A<sub>50</sub> column (2.5 cm x 20cm), previously equilibrated with over 1000 ml acetate buffer (50 mM, pH 5). The column was washed with the same buffer at 25 ml/hour until the absorbance at 280 nm of the fractions reached zero. The adsorbed proteins were eluted with linear gradient of 0–1 M NaCl in 50 mM acetate buffer pH 5. Fractions of 5 ml each were collected. The fractions with  $\beta$ -amylase activity were pooled and concentrated with 10 kDa Amicon Membrane.

#### **$\beta$ -Amylase Assay Method and Protein Determination**

The  $\beta$ -amylase activity was determined by the DNSA method [Zhang et al. 2006]. The reaction mixture consisted of 100  $\mu$ L of 1 % soluble starch in 50 mM sodium acetate buffer, pH 5 and the enzyme (100  $\mu$ L). The reaction was stopped after 3 min of incubation at 25°C with the addition of 200  $\mu$ L of DNSA colour reagent. The mixture was boiled for 5 minutes, cooled in ice and thereafter diluted with 1000  $\mu$ L of distilled water. Absorbance was measured at 540 nm using UV-visible spectrophotometer against reagent blank containing no enzyme. The enzyme activity was expressed in micromoles of maltose released per minute per milliliter of enzyme.

The presence of protein was monitored during purification and routinely by measuring absorbance at 280 nm. The protein content was estimated using bovine serum albumin (BSA) as the standard protein by Bradford method.

#### **Electrophoresis**

SDS-PAGE was performed on a 12 % (w/v) polyacrylamide gel using Tris-glycine buffer system to determine the homogeneity and the molecular mass of  $\beta$ -amylase. The proteins were stained with 0.05 % Coomassie Blue R-250, and the excess dye was washed out using destaining solution.

#### **Size Exclusion Chromatography**

The molecular weight of the native enzyme was determined by gel filtration in a 1.5 cm X 96 cm Sephadex G-100 column, using 25 mM acetate buffer, pH 5.0. The standard proteins used in calibrating the column were ovalbumin (Mr 45,000), bovine serum albumin (Mr 68,000), aldolase (Mr 158,000) and cytochrome C (Mr 12,400).

#### **Kinetic Studies**

The kinetic values for the pure enzyme were determined by incubating 100  $\mu$ L of purified  $\beta$ -amylase with 100  $\mu$ L 50 mM sodium acetate buffer pH 5 at various concentrations of soluble starch solution, ranging from 0 to 10 mg/ml at 25°C for 3min. Same was done for other substrates and the immobilized enzyme where 1 g was incubated. The values of  $K_m$ ,  $V_{max}$  and  $K_{cat}$  were determined from a Lineweaver-Burk plot.

#### **Effect of pH on enzyme activity and stability**

$\beta$ -amylase activity was determined at various pH values using 50 mM activity of different buffers: Glycine- HCl pH 3, sodium acetate (pH4 -5), phosphate (pH 6 -7), and Tris-HCl pH8. The reaction was carried out using the  $\beta$ -amylase assay procedure. The maximum activity was taken as 100 % and relative activity was plotted against different pH values.

#### **Effect of Temperature on Enzyme Activity and Stability**

A mixture of 100  $\mu$ L of 1 % soluble starch and 100  $\mu$ L of the appropriately diluted enzyme was incubated at different temperatures ranging from 20°C to 50°C for 3minutes in a regulated water bath. The assay was carried out according to the standard assay procedure. The effect of temperature on the stability of the enzyme was determined by incubating the enzyme solution at various temperatures (20°C-50°C) without substrate for 1 hour. At interval of 10 minutes, aliquot was withdrawn and assayed for  $\beta$ -amylase residual activity.

#### **MALDI-TOF MS**

Spots of  $\beta$ -amylase were excised and placed in Eppendorf tubes. They were washed with autoclaved distilled water, destained (50 mM ammonium bicarbonate, 50 % v/v acetonitrile) and dehydrated with 70 % (v/v) acetonitrile. The samples were digested with 1 L (9ng/L) trypsin solution (25 mM ammonium bicarbonate, 10 % v/v acetonitrile) for

24 h at 37°C, 1 L of sample was thoroughly mixed with 1 L of a saturated matrix solution (recrystallized acyano-4-hydroxycinnamic acid in 50 % v/v acetonitrile, 0.5 % v/v TFA). 1µl of sample was loaded onto the sample plate and left to dry at room temperature. Mass spectra were recorded using an Applied Biosystem MALDI-TOF instrument.

### Effect of Metals on 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<sub>2</sub>, KCl, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, ZnSO<sub>4</sub>, MgSO<sub>4</sub>, FeSO<sub>4</sub>, CuSO<sub>4</sub> and HgCl. The final concentration of metallic chloride in the reaction mixture was 0.1M. Residual activity was measured at 10 min interval. The blank used contained the buffer solution with the metal ion under consideration without the enzyme. The control consisted of the enzyme and the substrate without any chloride. Effect of metallic chlorides on the half-life of the enzyme was determined at a final concentration of 100mM. The half-life was determined from the relationship:

$$t_{1/2} = \frac{\ln 2}{k_d} \quad 1$$

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

$$V = V_0 e^{-k_d t} \quad 2$$

Where  $V_0$  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:

$$\ln V = \ln V_0 - k_d t \quad 3$$

### Measurement of Activation Energy

β-amylase was assayed as described in the assay method at various temperatures ranging from 20°C to 40°C. Data of  $\ln k$  was plotted against the absolute temperature. Activation energy for catalysis of the enzyme was calculated from the slope of the plot and thermodynamic data were calculated by rearranging Eyring's absolute equation derived from transition state theory [Kolawole et al. 2006].

The first-order rate constants for denaturation  $K_d$  of the enzyme at different temperatures were determined from semi-logarithmic plots. The first-order rate constants ( $K_d$ ) were plotted and analyzed [Kolawole et al. 2006]. Thermodynamic data were calculated by rearranging the Eyring absolute rate equation:

$$\text{slope} = -\frac{E_a}{R} \quad 4$$

Where,  $R$  is universal gas constant (1.987 calK<sup>-1</sup> mol<sup>-1</sup>)

And  $E_a$  is the Activation energy

$$K_d = \left(\frac{K_B T}{h}\right) e^{(-\Delta H^*/RT)} e^{(\Delta S^*/R)} \quad 5$$

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

$$\Delta H^* = E_a - RT \quad 6$$

Where  $R$  (gas constant) = 1.987cal/degreemol<sup>-1</sup>

$$\Delta G^* = -RT \left(\frac{\ln K_d h}{K_B T}\right) \quad 7$$

$$\Delta S^* = \left(\frac{\Delta H^* - \Delta G^*}{T}\right) \quad 8$$

### Amino acid composition and identification

Extraction and instrumentation were carried out by following the modified method in [AOAC 2006; Danka et al. 2012]. 1 ml of sample was defatted by extracting the sample with 10 ml of petroleum ether three times to achieve the complete hydrolysis of the sample for the maximum recovery of the amino acid. The sample was mixed with 30 ml of 1 M KOH and incubated for 48 hours at 110°C. The hydrolysed sample was neutralized to a pH range 2.5-5. The solution was purified by cation-exchange solid phase extraction. The purified amino acids were derivatized with ethyl chloroformate, the derivatized amino acids were made up to 1 ml in a vial for Gas Chromatography.

### Thermal Inactivation

Thermal inactivation of the β-amylase was determined by incubating the enzyme in 25 mM sodium acetate buffer pH 5 buffers at 40°C for 60 minutes. Aliquots (100 µL) were withdrawn at different times, cooled on ice and residual activity of the enzyme was investigated. Stability of the enzyme against heat was studied by incubating definite concentration of enzyme in the presence of 10 mM Fructose, glucose, galactose, and maltose at 40°C for 60 minutes. Then the β-amylase of each sample was assayed as usual. Magnitudes of  $k_{inact}$  against thermal condition can be obtained from the equation:

$$\ln A/A_0 = -k_{inact} t \quad 9$$

The inactivation rate constant ( $k_{inact}$ ) value is the best parameter describing the effect of osmolytes on the kinetic stability of the enzyme.  $A$  and  $A_0$  are the enzyme activity at each time and the initial time, respectively.

### Fluorescence quenching

Fluorescence spectra were obtained using a Hitachi F4500 luminescence spectrophotometer. An excitation wavelength of 280 nm with a bandwidth of 5nm was applied while the emission spectra recorded between 300 and 500 nm using a protein concentration of 0.41 µM of the enzyme. All fluorescence spectra were corrected for background scattering as measured with pure buffer. The experiment was performed at 25°C. Fluorescence quenching was monitored over a concentration range of 0–125 µM rutin, quercetin, gallic acid and epicatechin added to the enzyme solution separately. Stern Volmer constant,  $K_{sv}$  was calculated using the equation

$$\frac{F_0}{F} = 1 + K_{sv}[Q] = 1 + K_q \tau_0 [Q] \quad 10$$

Where,  $F_0$  and  $F$  are fluorescence intensities before and after the addition of the quencher respectively.  $K_{SV}$  is the Stern–Volmer quenching constant,  $[Q]$  is the concentration of the quencher,  $K_q$  is the quenching rate constant of biomolecule and it is equal to  $k_{sv}/\tau_0$ .  $\tau_0$  is the average lifetime of the biomolecule without quencher ( $\tau_0=10^{-8}s$ ). The binding constant,  $K_a$ , the dissociation constant,  $K_d$  and the number of binding sites were also determined [Wang et al. 2007; Kolawole et al. 2018].

#### **$\beta$ -amylase immobilization on sodium alginate beads**

The sodium alginate bead was prepared according to [Wang et al. 2007] with slight modification. 3 g of sodium alginate was dissolved in 100 ml acetate buffer to make a 3 % solution. 10ml of 3.4 mg of enzyme was mixed with 10 ml of 3 % sodium alginate solution. The beads were formed by dripping the polymer solution from a height of approximately 20 cm into an excess (100 ml) of stirred 0.1M  $CaCl_2$  solution with a syringe and a needle at room temperature. The beads in the calcium solution were left to cure for 30minutes, filtered, and washed thoroughly with distilled water 3 times, dried with filter paper and exposed to open air for 1hour before use [Sachin and Sandeep 2012; Rajagopalan and Krishnan 2008].

#### **Determination of Binding constant and the number of binding sites**

Investigation of the binding constant of the ligand on the protein and number of binding sites was achieved using the Scatchard analysis:

$$\log\left(\frac{F_0 - F}{F}\right) = \log K_a + n \log [Q] \quad 11$$

$K_a$  is the binding/quenching constant of interaction between the quencher and the protein,  $n$  is the number of binding sites. The value of  $K_a$  and  $n$  were obtained from the plot of  $\log (F_0-F)/F$  versus  $\log [Q]$ .

The thermodynamic analysis of the binding mode was analyzed from the Van't Hoff equation at different temperature range of 15 to 40°C.

$$\Delta G = -RT \ln K_a \quad 12$$

$$\Delta G = \Delta H - T\Delta S \quad 13$$

Where  $R$  is the gas constant,  $T$  is the experimental temperature and  $K_a$  is the binding constants at corresponding  $T$  and  $G$  is the Gibbs free energy.

#### **Determination of Dissociation constant**

## **RESULTS AND DISCUSSION**

The results obtained in this study confirmed the presence of  $\beta$ -amylase in water yam. The purification steps employed in this work were adequate for the purification of the  $\beta$ -amylase from the water yam to homogeneity with 5.53 purification fold but appreciable yield of 24.1 % (Table 1). The homogeneity shown by single band after SDS-PAGE

The dissociation constant of the ligand on the protein was ascertained by using the Hanes-Woolf plot from the equations below:

$$EI \rightleftharpoons E + I \quad 14$$

Where,  $E$ ,  $I$ , and  $EI$  represent the enzyme, inhibitor, and the enzyme – inhibitor complex respectively. In conditions where the enzyme concentration  $[E]$  is negligible compare to the inhibitor  $[I]$  concentrations it can be written as:

$$[EI] = [E]_t I / (K_d + [I]) \quad 15$$

Where  $K_d$  is the dissociation constant of the  $EI$  complex and  $[E]_t$  is the total enzyme concentration.

Dissociation constant  $K_d$  can be calculated from the Equation below

$$\Delta F = \frac{\Delta F_{max}}{K_d + [I]} [I] \quad 16$$

By considering that  $\Delta F$  is proportional to  $EI$ , it can be rewritten in a linear form

$$\frac{I}{\Delta F} = K_d / \Delta F_{max} + I / \Delta F_{max} \quad 17$$

Where  $\Delta F_{max}$  is the maximum decrease fluorescence observed when the enzyme is saturated by epicatechin.

#### **Application of $\beta$ -amylase**

Wash efficiency of starch stains was studied in the presence of Mama gold and Rana detergents and  $\beta$ -amylase [Longo et al. 1992]. White cotton cloth pieces stained with starch solution (1 %) were placed at 80°C for 30 min to assume the firm binding of stains to the material support. Washing performance was tested by varying the cleaner, as water, water + detergent, water + enzyme, water + detergent (7 mg/mL) + enzyme. Stained cotton cloth piece was incubated in the presence of the corresponding cleaner mixture on a shaker for 30min at 50°C. Obtained solution was collected for each mixture to measure the concentration of reducing sugars released from starch. The efficiency of starch removal by the washing process was expressed as the following equation [Kolawole et al. 2011]:

$$\text{Efficiency \%} = \frac{100 * A * 0.9}{B} \quad 18$$

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.

proved that purification steps employed in this work were adequate for  $\beta$ -amylase from water yam. The molecular mass of 30.1 kDa obtained by SDS-PAGE (Fig. 1) is comparable with the apparent relative molecular weight of 31.1 kDa by gel filtration on Sephadex G100 under non-denaturing condition and showed that it is monomeric. One form of  $\beta$ -amylase obtained in this study is in accordance with various works reported by these authors from different sources; *Bacillus subtilis* [Sikander et al. 2014],

Cereal [Matsui et al. 1977], Finger millet [Kolawole et al. 2011], Chinese yam [Chiba 2006] Soybean [Ajele 1997] and Sweet potato [Chandrika et al. 2013]. In contrast homotetramers have been reported from sweet potato and bindweed [VanDamme et al. 2001; Chandrika et al. 2013] while existence of five forms of  $\beta$ -amylase in ungerminated and germinated rice seeds had previously been reported by [Matsui 1977]. The estimated molecular weight of the enzyme was the same order of magnitude as values that had been reported for  $\beta$ -amylase from *Bacillus subtilis* Isolated from Kolanut Weevil (39.4 kDa) [Femi-Ola 2013], *Bacillus subtilis*  $\beta$ -amylase isolated from peels of Cassava barley (34.67 kDa) [Adeyanju et al. 2012], higher than 6.5kDa reported for sweet potato [Manisha et al. 2016] and 24kDa for *Pergularia tomentosa* [ImenLahmar et al. 2018], but lower than that of Chinese yam tuber (56kDa) [Chiba 2006], Glycine max seed (57kDa) [Chandrika et al. 2013] hedge bindweed (55 kDa) [Vandamme et al. 2001] and soybean (57 kDa) [Ajele 1997] and millet (58 kDa) [Yamasaki 2003].

$\beta$ -amylase is enriched in both acidic and hydrophobic amino acids but low in arginine and histidine. The 28 amino acid after tryptic digestion is in agreement with the values of MALDI-TOF MS (Fig. 2) which gave a more accurate molecular weight for  $\beta$ -amylase from water yam than the values obtained for SDS-PAGE and size exclusion chromatography.  $\beta$ -amylase could not be identified as the peptide fingerprint did not match any available peptide mass on protein data base.

The Michaelis-Menten constant,  $K_m$  value of 2.24 mg/ml (Fig. 3) and 2.13mg/ml were obtained for free and immobilized (Fig. 4) enzyme respectively in this study using soluble starch as substrate were similar to that of finger millet [Eke and Oguntimehin 1992] and rice [Matsui 1977]  $\beta$ -amylase with the  $k_m$  values of 2.1 mg/ml and 3 mg/ml respectively while obtained higher  $K_m$  value of 5.89 mg/ml for *Digitaria exilis*  $\beta$ -amylase [Kolawole 2006]. The lower  $K_m$  value obtained in this study revealed that water yam  $\beta$ -amylase has high affinity for the substrate, soluble starch which makes it distinct from previous studies. The assay method revealed that starch, amylose and amylopectin are better substrates for the purified enzyme than glycogen. This might indicate that starch, amylose and amylopectin are the physiological substrates for the enzyme (Table 2,3). The results revealed that gelatin immobilized  $\beta$ -amylase formed irreversible and stable aldimine bonds that led the immobilized enzyme to acquire higher temperature optima, increased tolerance to higher pH range and could be reused for starch hydrolysis. All these criteria, could, therefore be successfully utilized in continuous production of maltose.

It appears that the enzyme has specificity for divalent metallic chlorides (10 mM concentration) compared to monovalent and trivalent metallic chlorides, which might possibly prolong the initial phase of reversible conformation change. This result agrees with the result obtained by [Ray et al. 1994] that divalent metals increased the thermostability of  $\beta$ -amylase from *Bacillus megaterium* B<sub>6</sub>, but heavy metals brought about the accelerated thermo

inactivation (Table 4). It seems that  $\beta$ -amylases from different sources have their metal specificity for increased thermostability. Metal binding is related to differences between metal with respect to co-ordination geometries and influence the metal preferences [Vieille et al 2001]. The mode of action of these metals in improving the thermostability and prolong the initial phase of reversible conformational change is still a mystery.

The effect of pH on the activity of  $\beta$ -amylase is illustrated in Fig 5. The optimum activity was obtained at pH 5 for free form of  $\beta$ -amylase while the immobilized form was pH 6. Meanwhile, the free form of the enzyme was very active between pH of 4, and 6–8 while the immobilized form showed drastic decline in optimal activity (Fig. 6). The pH shifting from 5 to 6 upon immobilization may be due to diffusion limitations altering the hydrogen ion concentration in the immobilized enzyme vicinity and/or to the presence of secondary interactions between the enzyme and the polymeric matrix [Rajagopalan and Krishnan 2008]. In contrast, most reported  $\beta$ -amylase lack significant activity at the extreme acidic pH [Eke and Oguntimehin 1992]. The fact that the  $\beta$ -amylase was active over a wide pH range may imply its usefulness in the processes that require wide pH range especially shifting of pH from acidic to alkaline region. The optimum pH 5 obtained in this study is consistent with the report that most plant  $\beta$ -amylases have their pH optima in the acidic region while microbial  $\beta$ -amylases have theirs in the alkaline region. However, the following authors reported  $\beta$ -amylase from various sources to be optimal at different pH: Adeyanju et al (2012) reported pH 4.5 for soybean  $\beta$ -amylase, [Ajele 1997] obtained pH 4.6-6 for sweet potato  $\beta$ -amylase and [Matsui (1977) gave report of 5.5 - 6.5 for rice  $\beta$ -amylase while the optimum pH of 6–7 was reported for *Aspergillus carbonarium*  $\beta$ -amylase [Ajele 1997]. Yam  $\beta$ -amylase showed the same features with these reported works which signify relationship in their catalysis.

The enzyme exhibited maximum stability at alkaline pH of 7 and 8 with about 80 % residual activity (Table 5) while at acidic region 4, 5 and 6 percentage remaining activities of 65 %, 70 % and 73 %, respectively, were obtained after incubating for 4 hours (Fig. 7). Amylase from ruminal particle associated microorganism reported by [Martinez et al. 2002] exhibited stability at acidic region while Chiba (2006), Kolawole et al. (2011) and Kolawole (2006) reported alkaline stable  $\beta$ -amylase from Chinese yam tuber, *Digitaria exilis*, and *Eleusine coracana*. VanDamme (2001) asserted that other regulatory part of the enzyme aside the catalytic site might responsible for stability of  $\beta$ -amylase at alkaline pH. Water yam  $\beta$ -amylase demonstrated substantial residual activity as it retained over 60 and 70 % of its initial activity at 60°C and 50 °C after 60 min incubation (Fig. 8) might suggest its employability in food and beverage industries to convert starch into maltose where high interest has been placed on the thermostability and thermo-activity of the enzyme because of high temperature operating conditions [Swamy et al.1994].

The result of this study has shown that the free energy of activation of  $\beta$ -amylase in (Table 6) is related to reaction rate [Piszkiewicz 1977]. The low free energy of activation data is quite instructive. It shows that the rate of reaction catalyzed by  $\beta$ -amylase is fast under the activation temperature range. The activation energy of inactivation was reported to be 13.92 kcal/mol (Table 7).

The addition of  $Zn^{2+}$  and  $Mg^{2+}$  brought about slight increase in both the enzyme half-life and  $\Delta G^\ddagger$  of thermal denaturation and decrease in  $\Delta S^\ddagger$ . Other metals decreased both the half-life and the  $\Delta G^\ddagger$ , but increased the  $\Delta S^\ddagger$  of thermal denaturation (Table 8). The addition of metal ions did not affect the  $\Delta H^\ddagger$  of denaturation. Thermal denaturation in the presence of metal ions did not affect the non-covalent 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 [Pal and Chakrabarti 1999]. It seems that  $\beta$ -amylases from different sources have their metal specificity for increase in thermo-activity and thermostability. Metal binding is related to differences between metal with respect to coordination geometries and influence the metal preferences [Vieille et al. 2001]. The mode of action of these metals in improving the activity, thermostability and prolonging the initial phase of reversible conformational change is still a mystery. There is possibility of the metal increasing the chemical potential of the protein and thereby favoring the folded state over the unfolded state [Ray et al. 1994].

Apparent pK values of 5 (general base) and 7 (general acid) obtained suggest the presence of imidazole side chain of histidine residue and the thiol group of cysteine residue as being involved in the catalytic mechanism of the  $\beta$ -amylase. The X-ray crystallographic work by [Mikami et al. 1994] on soyabean  $\beta$ -amylase confirmed Glu 186 and Glu 380 as catalytic residue. It was also reported that the nonreducing end (Glc I) is hydrogen bonded to four amino acid residues of Asp 101, Asp 53, His 93 and Arg 420 in the catalytic site. It was further suggested that Asp 101 might play a key role in the catalytic mechanism of soyabean  $\beta$ -amylase. These amino acid residues may be distinct from the amino acids of the water yam  $\beta$ -amylase. It could be speculated that the role of imidazole group of histidine was to electrostatically stabilize the positively charged oxonium ion intermediate formed during catalysis (Fig. 9).

The fluorescence spectra of the interaction of  $\beta$ -amylase with epicatechin is shown in Fig 10. The Stern-Volmer  $K_{sv}$  values have been used as an indicator of conformational flexibility [Schein 1990]. However,  $K_{sv}$  parameters obtained showed highest flexibility with Gallic acid (Table 10). The interaction between  $\beta$ -amylase and Epicatechin was studied by fluorescence spectroscopy (Table 11). Fluorescence experiments results revealed that the fluorescence of  $\beta$ -amylase was quenched through static quenching process (Fig. 11). The thermodynamic parameters according to the Van't Hoff equation show that

the reaction was exothermic, spontaneous and electrostatic bond played a major role (table 12). The  $K_a$  value of Epicatechin-  $\beta$ -amylase interaction at 25°C of  $1.18 \times 10^3 \text{ L mol}^{-1}$  was not different from expected values of  $10^4$  to  $10^3 \text{ L mol}^{-1}$  of non-specific organic ligands-protein interaction using fluorescence spectroscopic methods [Bourassa et al. 2013]. The thermodynamic parameters, Enthalpy changes ( $\Delta H$ ) and Entropy change ( $\Delta S$ ) of the binding reaction provides main evidence for the binding mode. The thermodynamic parameter associated with various individual kinds of interaction that may take place in protein association process, which can be easily concluded as: (a)  $\Delta H > 0$  and  $\Delta S > 0$ , hydrophobic force; (b)  $\Delta H < 0$  and  $\Delta S < 0$ , van der Waals force and hydrogen bond; (c)  $\Delta H < 0$  and  $\Delta S > 0$ , electrostatic interactions. The enthalpy change ( $\Delta H$ ) and entropy ( $\Delta S$ ) of reaction, was calculated from the slope of the plot of  $\ln K_a$  versus  $1/T$  ( $T$ , absolute temperature). These forces combine with conformational changes and solvent rearrangement account for the signs and magnitude of the protein-ligand binding as well binding stability [Arroyo-Maya et al. 2016]. The binding constant ( $K_a$ ) of the ligand on the protein ( $\beta$ -amylase) and number of binding sites ( $n$ ) was explored and the linear regression plots were shown in Table 13, at 298K, the binding constant shows a high binding affinity for the enzyme-ligand complex and the number of binding sites for the complex is approximately 1.

The  $\Delta F$  at  $\lambda_{ex}$  was 280 nm with increasing concentrations of epicatechin indicates that the fluorescence of  $\beta$ -amylase due to its tryptophan residues was quenched by epicatechin. The thermodynamic parameters ( $\Delta G$ ) of the binding of  $\beta$ -amylase with epicatechin were slightly affected by a change in temperature. High temperatures result in lower diffusion coefficients. The result shows a high binding affinity for the enzyme-ligand complex and this implies that the rate at which the ligand will bind to the enzyme is higher than the rate of dissociation; this indicates that  $\beta$ -amylase metabolizes epicatechin (fig. 12). The values of enthalpy change ( $\Delta H$ ) and entropy change ( $\Delta S$ ) calculated indicated that electrostatic interactions were the dominant intermolecular forces in stabilizing the complex. The impact on  $\beta$ -amylase by epicatechin is predicted to result in functional changes which could affect  $\beta$ -amylase normal functions after the bioflavonoid interaction.

The activity of immobilized enzyme was evaluated using a repeated batch process in order to observe its reuse (Fig. 13). After 5 cycles, it still retained 67% of its initial activity. The immobilized enzyme may have been protected from thermal denaturation. This indicates that sodium alginate is an excellent material which can be used for  $\beta$ -amylase immobilization. It was found that immobilized  $\beta$ -amylase improved the washing efficiency of detergents and removed starch stains from clothes which are difficult to remove under normal washing conditions. Thus, the immobilized  $\beta$ -amylase could be considered as a potential candidate for use as a cleaning additive in detergents in order to facilitate the removal of starch stains. Due to this

promising feature, it may find applications in laundry detergents (Fig. 14).

The amino acid composition of beta-amylase was displayed in table 14.  $\beta$ -amylase from water yam was enriched in both acidic and hydrophobic amino acid but low in arginine and histidine.

### CONCLUSION

Additional improvements on enzymatic properties, with regard to industrial applications, could be attempted by genetic engineering to hopefully improve the enzyme function and stability.

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**Author's contribution:** this is to declare that this is a collaborated project by the authors. Authors EOAT and AIS supervised the project, analysis of the project done by RO

and KAO. Project carried out by FMA, RO and KAO. FMA wrote the manuscript. All authors read and approved the final manuscript

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**Ethics approval and consent to participate:** all the authors have read and agreed with the ethics for publishing the manuscript

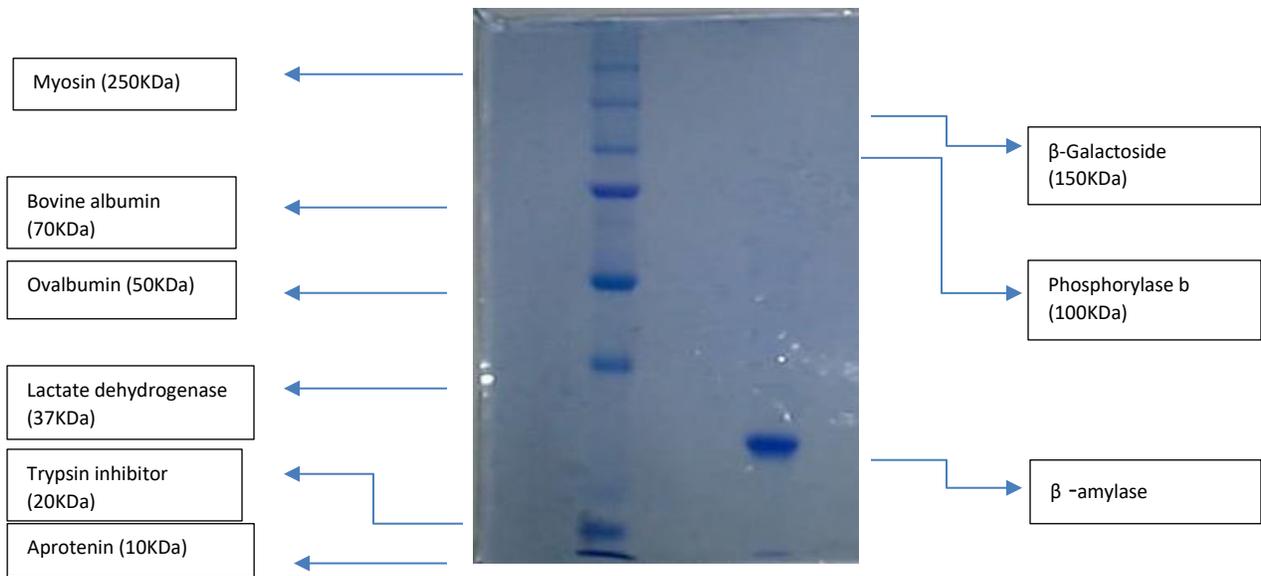
**Consent for publication:** the authors approve the consent for publishing the manuscript

**Competing of interest:** Authors declare that there is no competing interest

### RESULTS

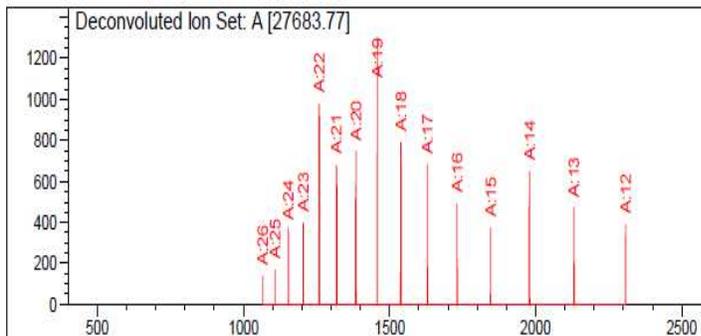
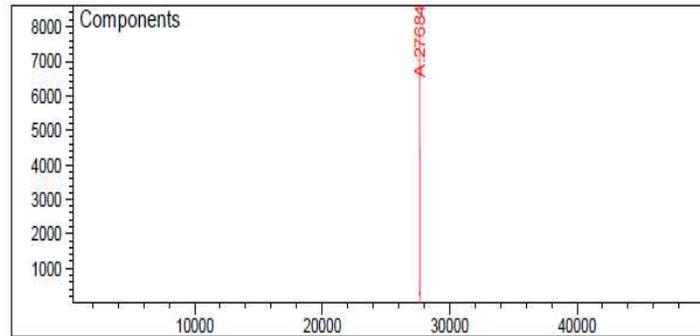
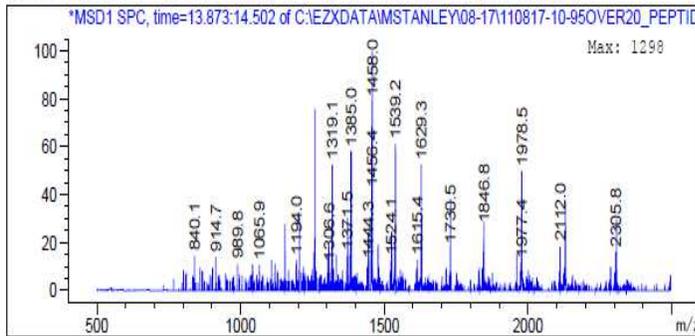
**Table 1: Summary of the purification of  $\beta$ -amylase from *D. alata***

|                                      | Total activity (Units) | Total Protein(mg) | Specific activity ( $\mu\text{mol}/\text{min}/\text{mg}$ ) | Fold | Yield % |
|--------------------------------------|------------------------|-------------------|--|------|---------|
| Crude                                | 10115                  | 6180              | 1.64   | 1    | 100     |
| Acid treatment                       | 9900                   | 5280              | 1.88   | 1.14 | 97.9    |
| 40-80 % $(\text{NH}_4)_2\text{SO}_4$ | 232.2                  | 63                | 3.69   | 1.96 | 2.35    |
| Ion exchange on DEAE $A_{50}$        | 55.9                   | 2.74              | 20.40  | 5.53 | 24.1    |



**Fig. 1 Electrophoretogram of purified water yam on SDS-PAGE in 10% gel using sodium acetate buffer pH 5. Markers are Myosin(250KDa),  $\beta$ - Galactoside (150KDa), Phosphorylase b (100KDa), Bovine albumin (70KDa), Ovalbumin (50KDa), Lactate dehydrogenase (37KDa), Trypsin inhibitor (20KDa), and Aprotinin (10KDa).**

Deconvolution of Spectrum # 1 @ 13.873 - 14.502 min

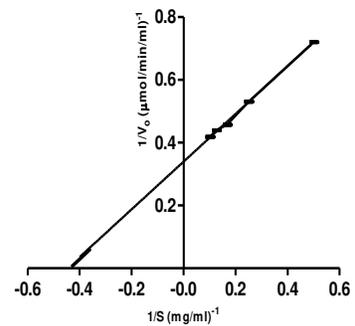


| Component | Molecular Weight | Absolute Abundance | Relative Abundance |
|-----------|------------------|--------------------|--------------------|
| A         | 27683.77         | 7711               | 100.00             |

**Fig.2 Peptide map of  $\beta$ -amylase**

**Table 2 kinetic parameters of various substrates**

| Substrates  | $K_m$ (mg/ml) | $V_{max}$ ( $\mu\text{mol}/\text{min}/n$ ) | $V_{max}/K_m$ | $K_{cat}$ ( $S^{-1}$ ) | $K_{cat}/K_m$ |
|-------------|---------------|--|---------------|------------------------|---------------|
| Starch      | 2.24          | 2.94                                       | 1.31          | 317.5                  | 141.1         |
| Amylose     | 2.56          | 2.60                                       | 1.02          | 279.6                  | 109.2         |
| Amylopectin | 2.53          | 3.18                                       | 1.26          | 341.9                  | 135.2         |
| Glycogen    | 4.27          | 3.74                                       | 0.88          | 402.2                  | 94.2          |

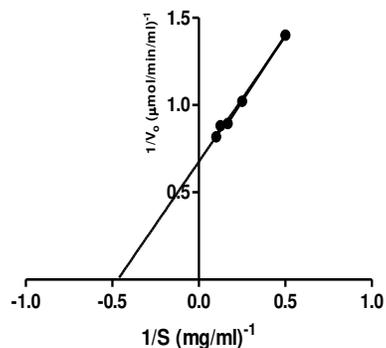


**Fig. 3 Lineweaver-Burk double reciprocal plot.**

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/ml.

**Table 3 Effects of various substrate on  $\beta$ -amylase**

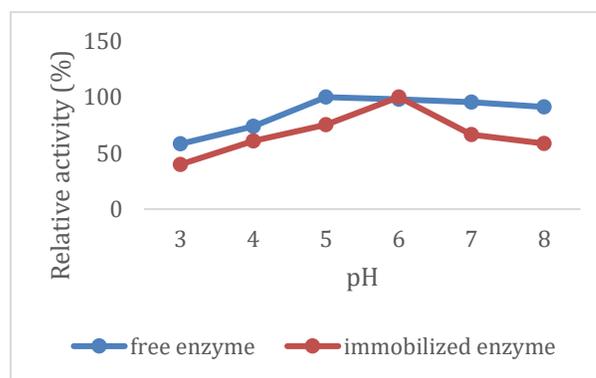
| SUBSTRATE      | RELATIVE ACTIVITY (%) |
|----------------|-----------------------|
| Soluble starch | 100                   |
| Amylose        | 91                    |
| Amylopectin    | 87                    |
| Glycogen       | 40                    |
| Maltose        | 0                     |
| Sucrose        | 0                     |
| Fructose       | 0                     |
| Lactose        | 0                     |
| Galactose      | 0                     |



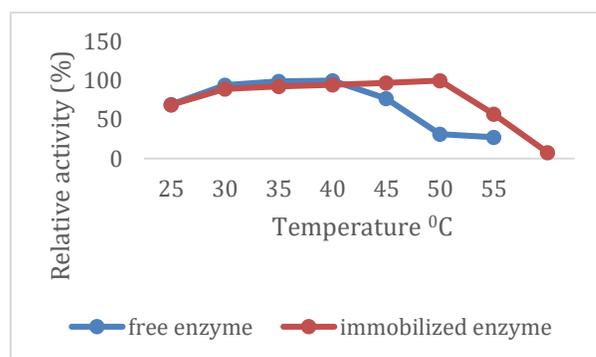
**Fig.4 Effect of Substrate Concentration on Immobilized  $\beta$ -amylase**

**Table 4 Effect of metal ions on  $\beta$ -amylase**

| SUBSTRATE                    | RELATIVE ACTIVITY (%) |
|------------------------------|-----------------------|
| None                         | 100                   |
| Ca <sup>2+</sup>             | 110                   |
| Mg <sup>2+</sup>             | 122                   |
| Zn <sup>2+</sup>             | 120                   |
| Fe <sup>2+</sup>             | 31                    |
| Pb <sup>2+</sup>             | 22                    |
| Na <sup>+</sup>              | 100                   |
| K <sup>+</sup>               | 108                   |
| Hg <sup>+</sup>              | 14                    |
| Iodoacetate                  | 72                    |
| Urea                         | 91                    |
| Fe <sup>3+</sup>             | 89                    |
| Cu <sup>2+</sup>             | 51                    |
| EDTA                         | 81                    |
| Sn <sup>2+</sup>             | 28                    |
| NH <sub>4</sub> <sup>+</sup> | 78                    |
| Hg <sup>+</sup>              | 14                    |



**Fig. 5 Effect of pH on free and immobilized enzyme**



**Fig.6 Effect of Temperature on free and immobilized enzyme**

**Table 5 Thermodynamic parameters for catalysis of water yam  $\beta$ -amylase**

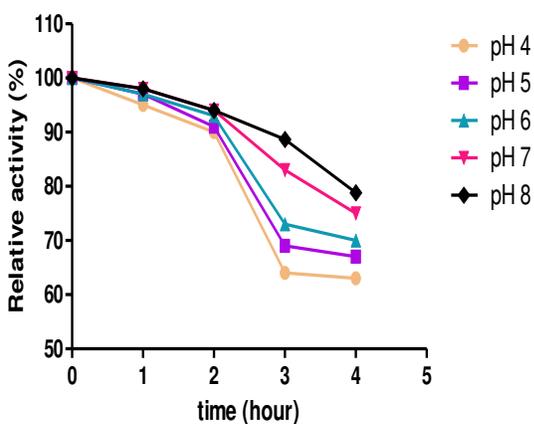
| Temperature (K) | $\Delta G^\ddagger$ kcal/mol | $\Delta H^\ddagger$ kcal/mol | $\Delta S^\ddagger$ cal/mol/k |
|-----------------|------------------------------|------------------------------|-------------------------------|
| 293             | 12.86                        | 5.87                         | -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 6.48kcal/mol

**Table 6 Thermodynamic parameters for thermal inactivation of yam - $\beta$ -amylase at various pH**

| pH | Ea<br>Kcal/mol | Half-life<br>(min) | $\Delta G^\ddagger$<br>kcal/mol | $\Delta H^\ddagger$<br>kcal/mol | $\Delta S^\ddagger$<br>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                           |

Ea is 13.92 kcal/mo

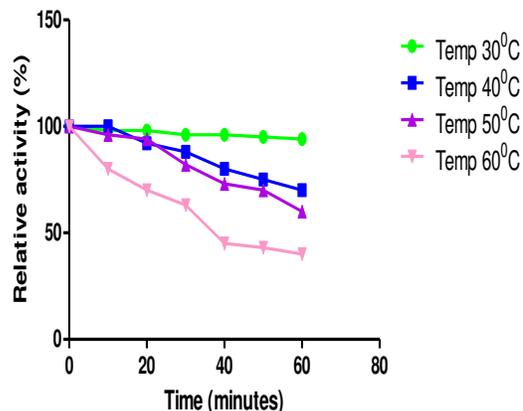


**Fig.7 Effect of pH on stability of the  $\beta$ -amylase.**

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.

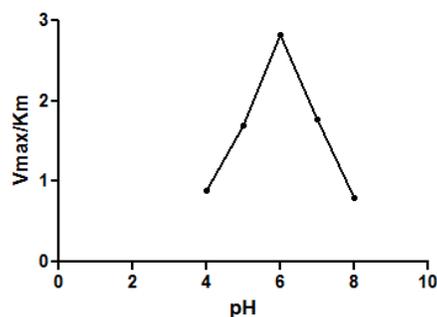
**Table 7 Thermodynamic parameters for thermal inactivation of water yam  $\beta$ -amylase at various temperature.**

| Temperature<br>(K) | Half-life<br>(min) | $\Delta G^\ddagger$<br>(kcal/mol) | $\Delta H^\ddagger$<br>(kcal/mol) | $\Delta S^\ddagger$<br>(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                             |

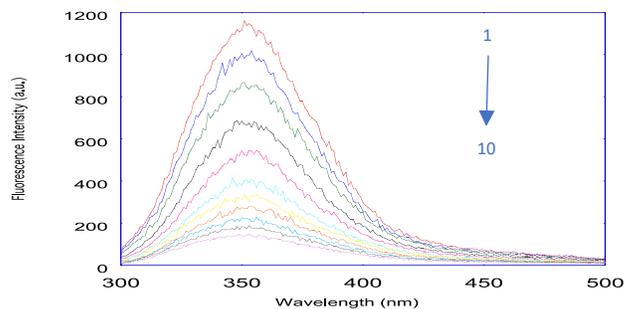


**Fig 8 Effect of temperature on enzyme stability**

100  $\mu$ l aliquot Withdrawn at an interval of 10min, and assay carried out according to standard procedure. The residual activities were expressed relative to the maximum activity at 0min which was taken as 100%.



**Fig. 9 Graphical illustration of logarithm of  $V_{max}/K_m$  vs pH suggesting the amino acid participating in catalysis.**



**Fig. 10 Fluorescence spectra of epicatechin.**

Emission spectra of  $\beta$ -amylase (0.274mg/ml) in the presence of various concentration of Epicatechin. Lane 1-10: from 0.0 to 125 $\mu$ M; first curve shows the emission spectrum of  $\beta$ -amylase only. (T ) 298 K,  $\lambda_{exc}$  348nm

**Table 8** Thermodynamic parameters for metals on thermal inactivation of water yam  $\beta$ -amylase

| 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 <sup>+</sup>              | 41.25           | 20.89                        | 13.33                        | -24.25                        |
| Ca <sup>2+</sup>             | 40.29           | 20.88                        | 13.33                        | -24.19                        |
| Zn <sup>2+</sup>             | 42.78           | 20.92                        | 13.33                        | -24.35                        |
| Mg <sup>2+</sup>             | 43.31           | 20.93                        | 13.33                        | -24.38                        |
| NH <sub>4</sub> <sup>+</sup> | 40.54           | 20.88                        | 13.33                        | -24.22                        |

**Table 9** The effect of Osmolytes on the kinetic stability

| Osmolytes (10mM) | K <sub>inact</sub> (S <sup>-1</sup> ) | $\Delta G$ (Kcal/mol) |
|------------------|---------------------------------------|-----------------------|
| Control          | 6.17 X 10 <sup>-5</sup>               | 20.76                 |
| Glucose          | 3.80 X 10 <sup>-5</sup>               | 21.04                 |
| Maltose          | 5.39 X 10 <sup>-5</sup>               | 20.84                 |
| Fructose         | 5.10 X 10 <sup>-5</sup>               | 20.87                 |
| Galactose        | 7.10 X 10 <sup>-5</sup>               | 19.37                 |

**Table 10** Constant value of K<sub>sv</sub> and K<sub>q</sub> of interaction between  $\beta$ -amylase and Quercetin, Rutin and Gallic acid at 25°C

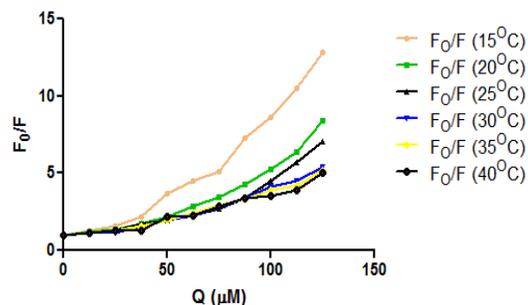
| Flavonoids  | K <sub>sv</sub> (M <sup>-1</sup> ) | K <sub>q</sub> (MS)     |
|-------------|------------------------------------|-------------------------|
| Quercetin   | 5.00 X 10 <sup>4</sup>             | 5.00 X 10 <sup>12</sup> |
| Rutin       | 2.39 X 10 <sup>5</sup>             | 2.39 X 10 <sup>13</sup> |
| Gallic acid | 1.88 X 10 <sup>6</sup>             | 1.88 X 10 <sup>14</sup> |

**Table 11** Stern–Volmer quenching constant (K<sub>sv</sub>) and (K<sub>q</sub>) of the interaction of Epicatechin with  $\beta$ -amylase at six temperatures.

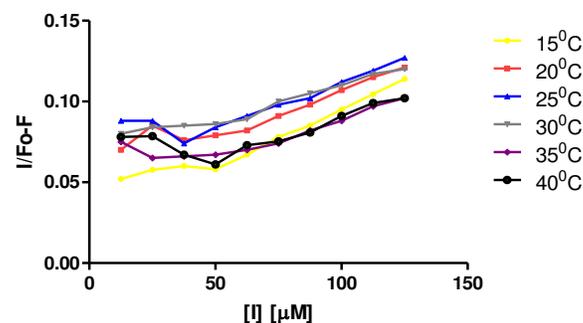
| Temp (K) | K <sub>sv</sub> (x10 <sup>4</sup> M <sup>-1</sup> ) | K <sub>q</sub> X 10 <sup>12</sup> MS |
|----------|---|--------------------------------------|
| 288      | 7.62  | 7.62                                 |
| 293      | 4.40  | 4.40                                 |
| 298      | 3.57  | 3.57                                 |
| 303      | 2.92  | 2.92                                 |
| 308      | 2.80  | 2.80                                 |
| 313      | 2.64  | 2.64                                 |

K<sub>q</sub> = Quenching rate constant of biomolecules

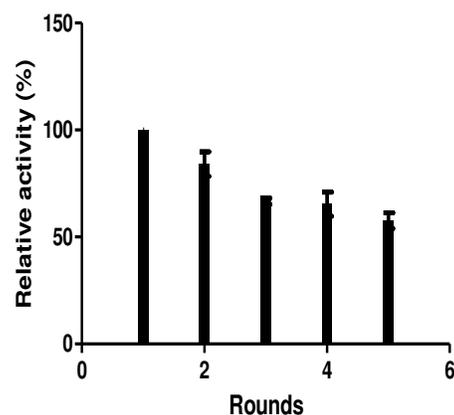
K<sub>sv</sub> = Stern–Volmer quenching constant



**Fig 11** The Classical Stern–Volmer plots of  $\beta$ - amylase by Epicatechin at 288 K–313K to determine the quenching constant (K<sub>sv</sub> and K<sub>q</sub>). [ $\beta$ - amylase] = 0.230mM, pH 5 in Acetate buffer 50mM,  $\lambda_{ex}$ =280n.



**Fig 12** Intensity of fluorophore and its dissociative properties by modified Stern–Volmer plots for the determination of the dissociation constant (kd) of  $\beta$ -amylase by Epicatechin at different temperatures 288K–313K



**Fig. 13** Reusability of  $\beta$ -amylase

Table 12: The temperature dependence on thermodynamic parameters of Epicatechin-  $\beta$ -amylase system at 15-40°C by Van't Hoff plot.

| Temp (K) | $\Delta H$<br>(KJ/mol) | $\Delta S$ (J/mol/k) | $\Delta G$<br>(KJ/mol) |
|----------|------------------------|----------------------|------------------------|
| 288      |                        |                      | -17.39                 |
| 293      |                        |                      | -17.47                 |
| 298      | -12.81                 | 15.91                | -17.55                 |
| 303      |                        |                      | -17.63                 |
| 308      |                        |                      | -17.71                 |
| 313      |                        |                      | -17.79                 |

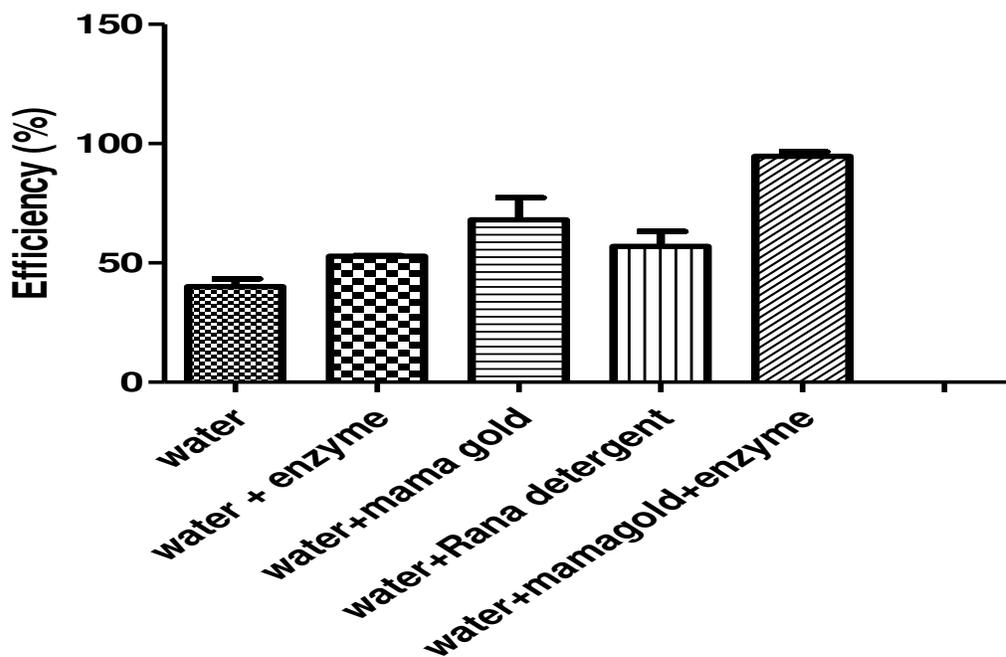


Fig. 14 Efficiency of starch stain removal by  $\beta$ -amylase

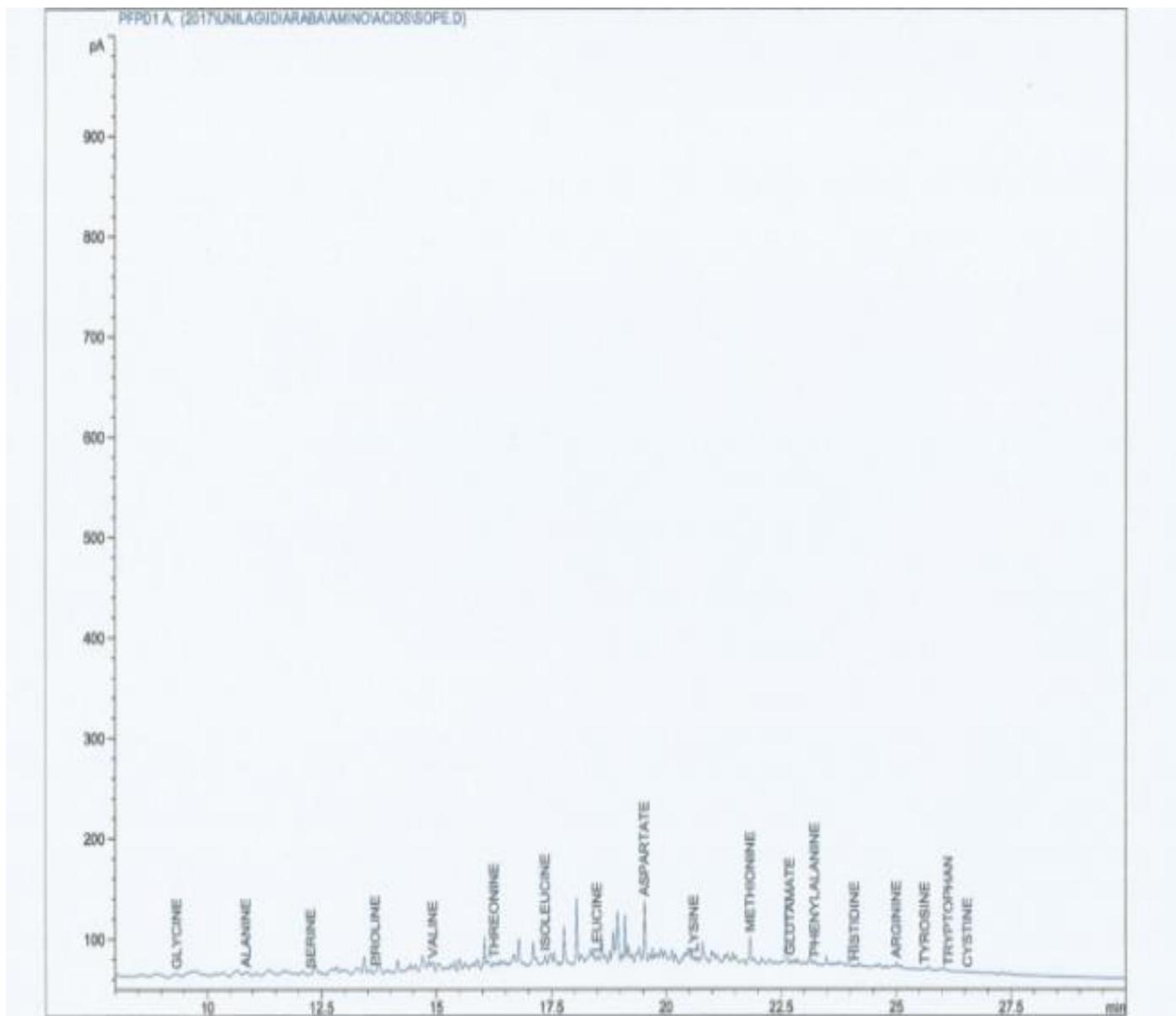
$$\% = \frac{\text{activity of immobilized enzyme}}{A-B} \times 100$$

19

Where, A= activity of free enzyme added,  
B= activity of remaining enzyme in wash water

**Table 13 Binding Parameters of  $\beta$ -amylase by epicatechin**

| Temperature (K) | Binding constant $K_a \times 10^3$ ( $\mu\text{M}$ ) | n           | $K_d \times 10^{-6}$ | $\Delta H$ (KJ/mol) | $\Delta S$ (J/mol/k) | $\Delta G$ (KJ/mol) |
|-----------------|--|-------------|----------------------|---------------------|----------------------|---------------------|
| 288             | 1.48   | 172 ± 0.07  | 69.34                |                     |                      | -17.39              |
| 293             | 1.24   | 1.66 ± 0.08 | 145.4                |                     |                      | -17.47              |
| 298             | 1.18   | 1.61 ± 0.10 | 178.8                | -12.81              | 15.91                | -17.55              |
| 303             | 1.11   | 1.61 ± 0.04 | 188.0                |                     |                      | -17.63              |
| 308             | 1.06   | 1.61 ± 0.04 | 190.2                |                     |                      | -17.71              |
| 313             | 0.92   | 1.64 ± 0.05 | 238.9                |                     |                      | -17.79              |



**Fig. 15 Chromatogram of amino composition of  $\beta$ -amylase**

**Table 14 Amino acid composition of  $\beta$ -amylase**

| Amino acid    | Amount g/100g protein      | Mole fraction         | Molar ratio | Molar composition   |
|---------------|----------------------------|-----------------------|-------------|---------------------|
| Glycine       | 4.705                      | 0.063                 | 19.45       | 20                  |
| Alanine       | 4.957                      | 0.056                 | 17.25       | 17                  |
| Serine        | 5.311                      | 0.051                 | 15.81       | 16                  |
| Proline       | 5.742                      | 0.050                 | 15.50       | 16                  |
| Valine        | 6.626                      | 0.057                 | 17.67       | 18                  |
| Threonine     | 5.129                      | 0.043                 | 13.33       | 13                  |
| Isoleucine    | 4.804                      | 0.037                 | 11.47       | 12                  |
| Leucine       | 7.823                      | 0.060                 | 18.60       | 19                  |
| Aspartate     | 10.207                     | 0.077                 | 23.87       | 24                  |
| Lysine        | 8.752                      | 0.060                 | 18.60       | 19                  |
| Methionine    | 2.013                      | 0.013                 | 4.03        | 4                   |
| Glutamate     | 9.559                      | 0.065                 | 20.15       | 20                  |
| Phenylalanine | 4.013                      | 0.024                 | 7.44        | 7                   |
| Histidine     | 1.953                      | 0.013                 | 4.03        | 4                   |
| Arginine      | 4.701                      | 0.027                 | 8.37        | 8                   |
| Tyrosine      | 6.898                      | 0.038                 | 11.78       | 12                  |
| Tryptophan    | 7.371X10 <sup>-1</sup>     | 3.61X10 <sup>-3</sup> | 1.12        | 1                   |
| Cystine       | 9.163X10 <sup>-1</sup>     | 3.81X10 <sup>-3</sup> | 1.18        | 1                   |
|               | <b>94.11g/100g protein</b> | <b>0.74142</b>        |             | <b>231 residues</b> |

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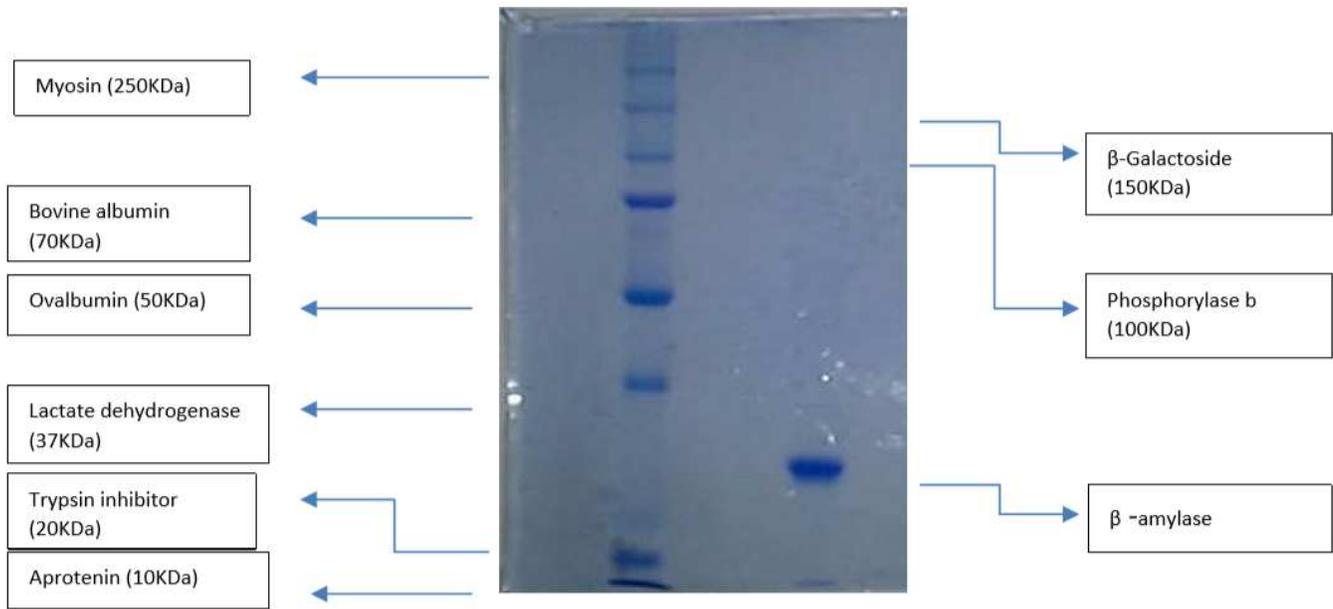
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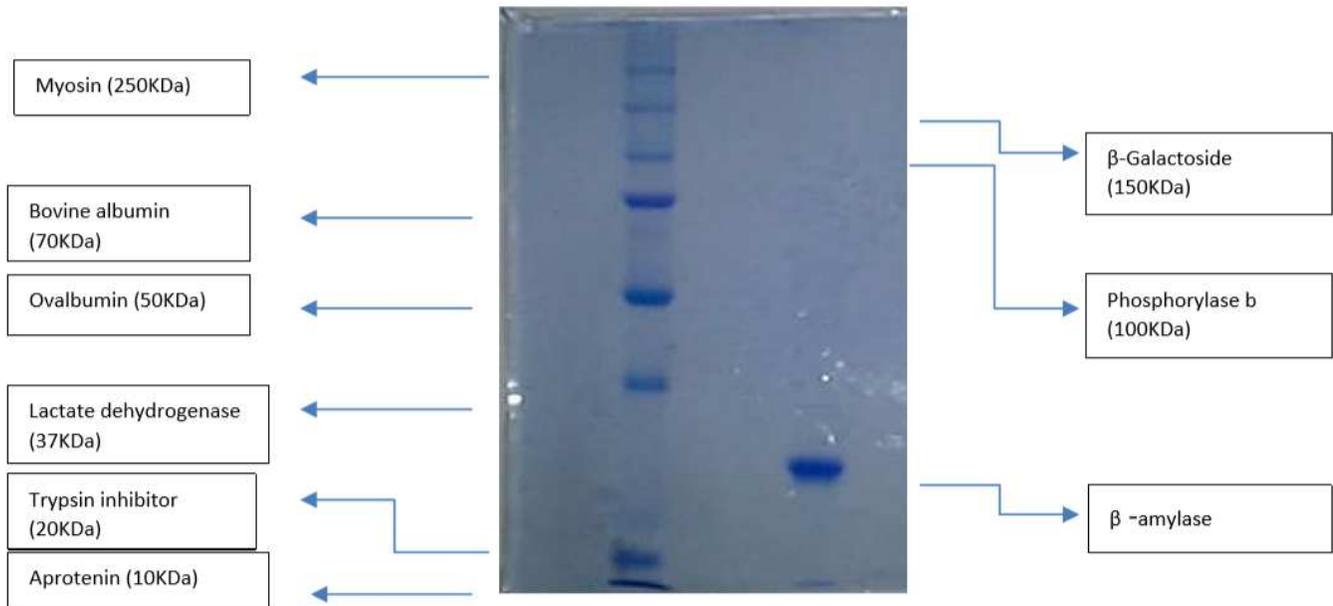
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# Figures



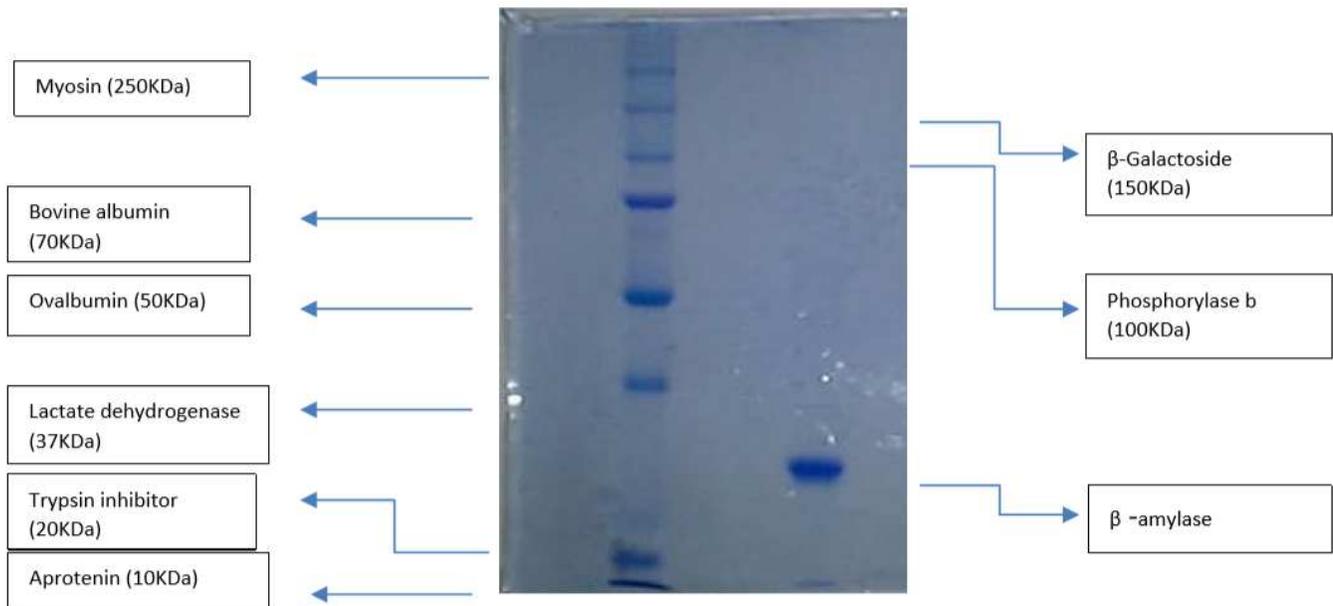
**Figure 1**

Electrophoretogram of purified water yam on SDS-PAGE in 10% gel using sodium acetate buffer pH 5. Markers are Myosin(250KDa),  $\beta$ - Galactoside (150KDa), Phosphorylase b (100KDa), Bovine albumin (70KDa), Ovalbumin (50KDa), Lactate dehydrogenase (37KDa), Trypsin inhibitor (20KDa), and Aprotinin (10KDa).



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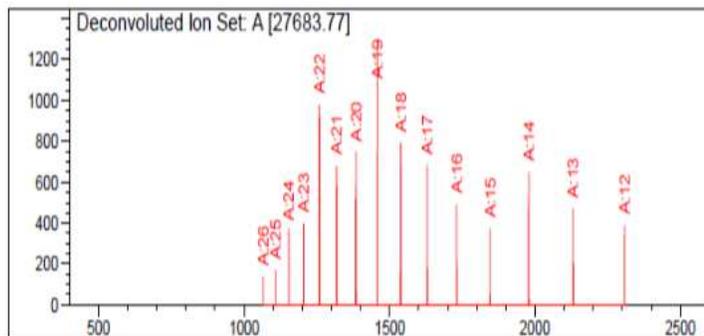
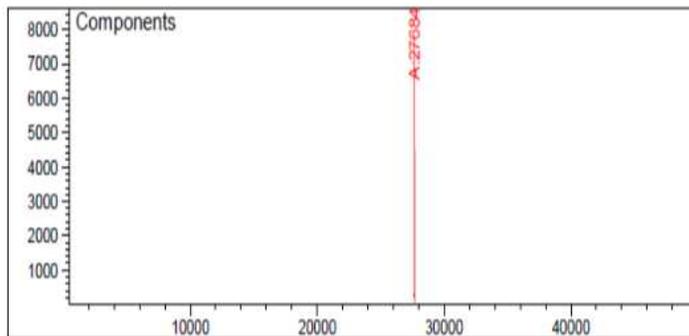
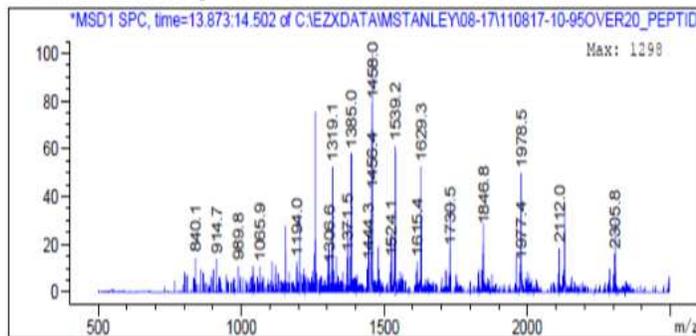
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Deconvolution of Spectrum # 1 @ 13.873 - 14.502 min

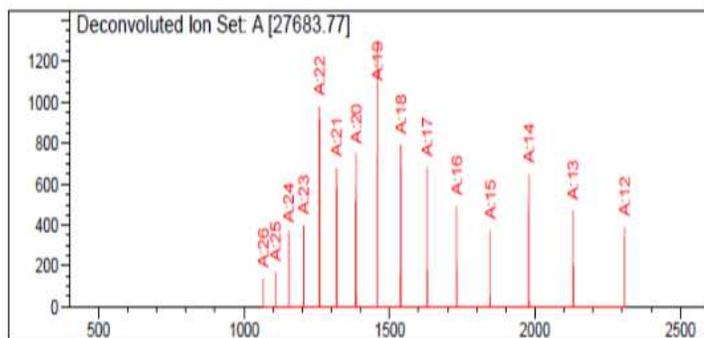
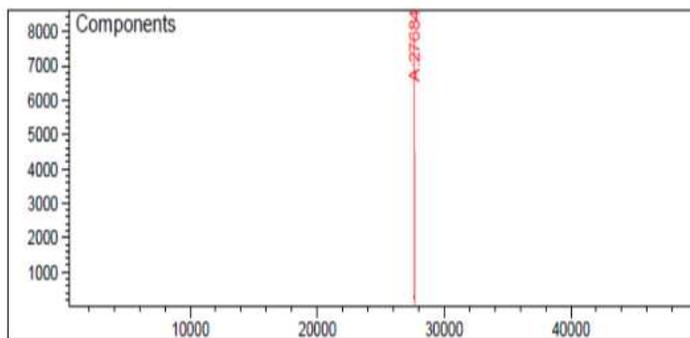
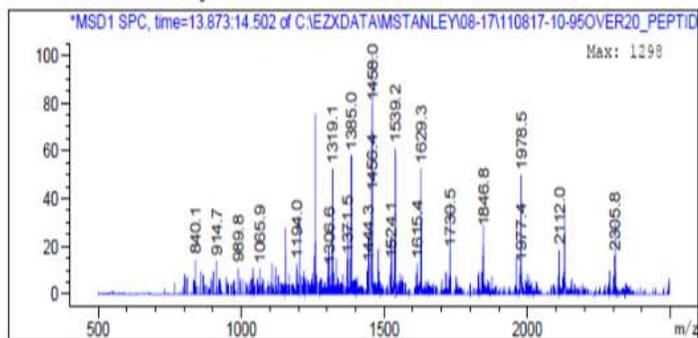


| Component | Molecular Weight | Absolute Abundance | Relative Abundance |
|-----------|------------------|--------------------|--------------------|
| A         | 27683.77         | 7711               | 100.00             |

Figure 2

Peptide map of  $\beta$ -amylase

Deconvolution of Spectrum # 1 @ 13.873 - 14.502 min

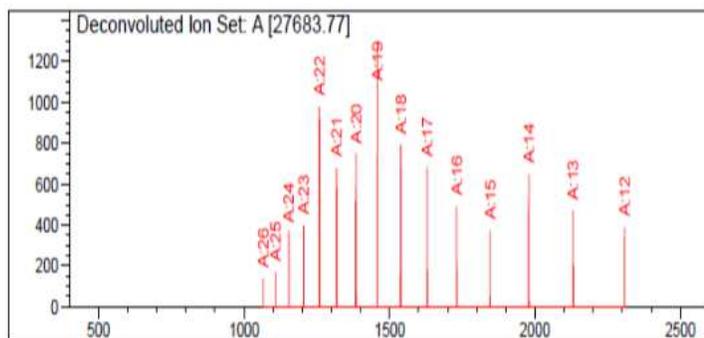
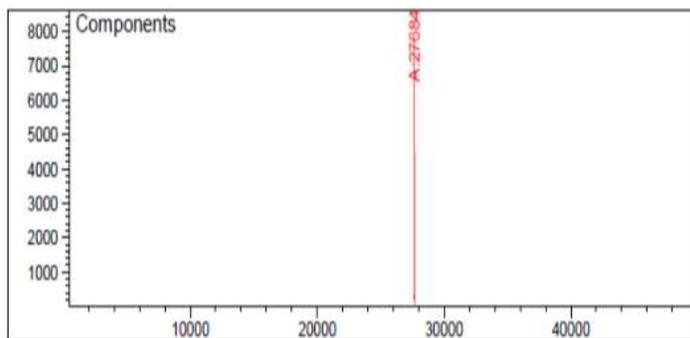
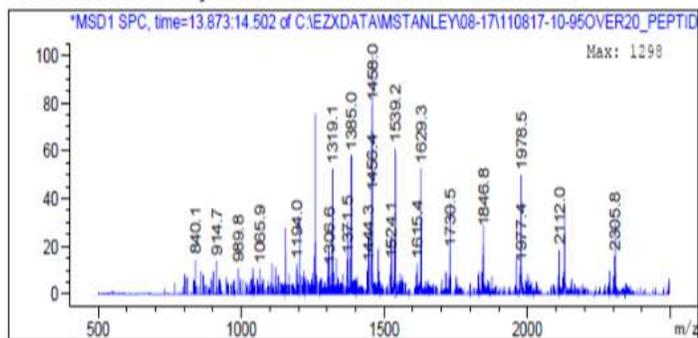


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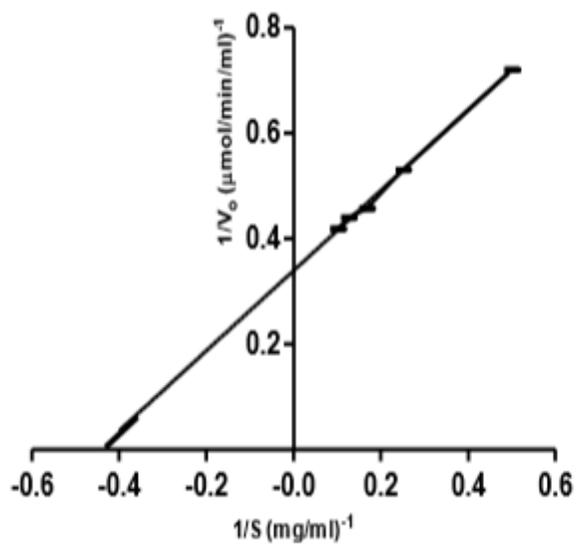


Figure 3

Lineweaver-Burk double reciprocal plot. 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/ml.

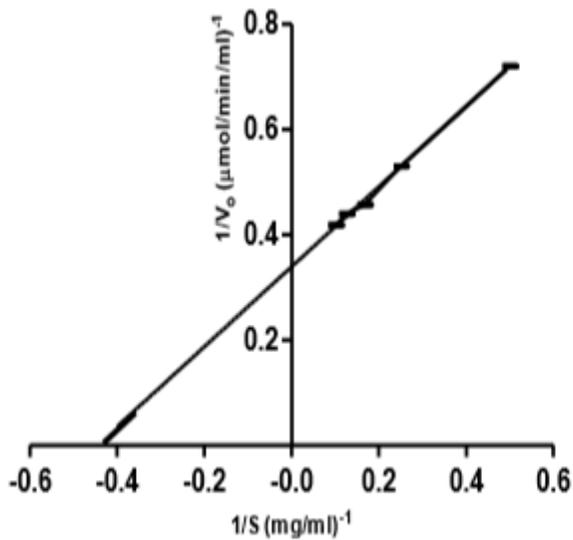


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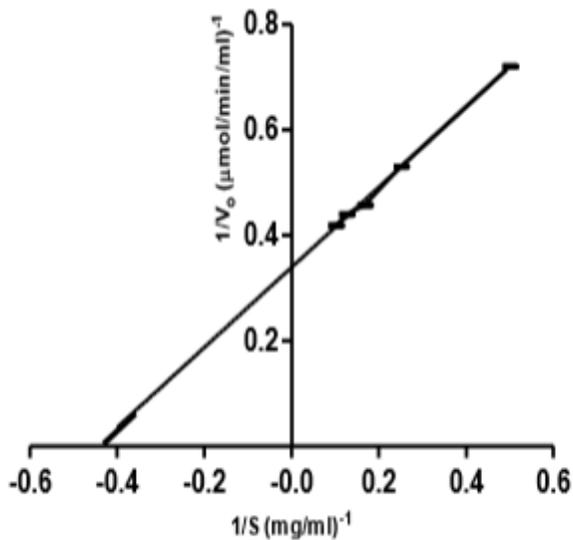


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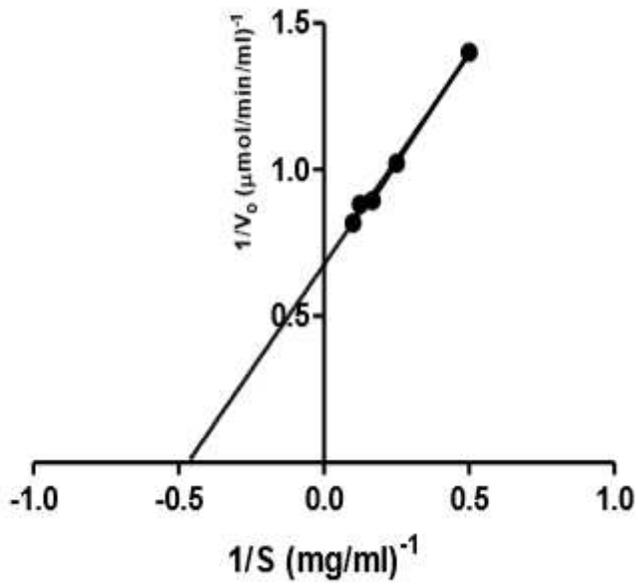


Figure 4

Effect of Substrate Concentration on Immobilized  $\beta$ -amylase

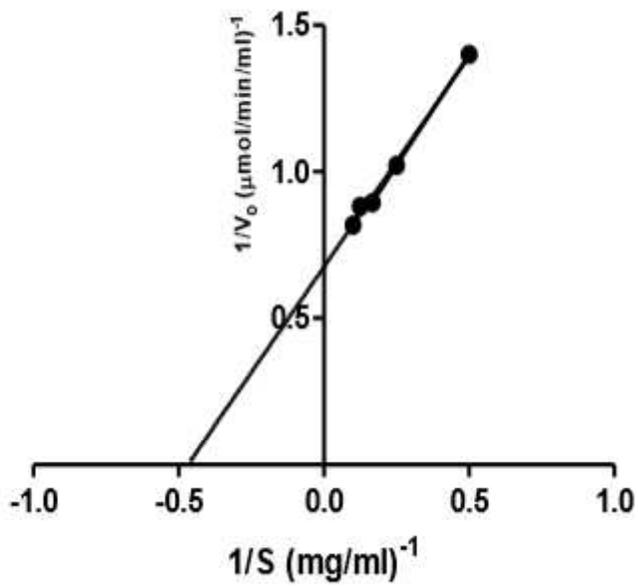


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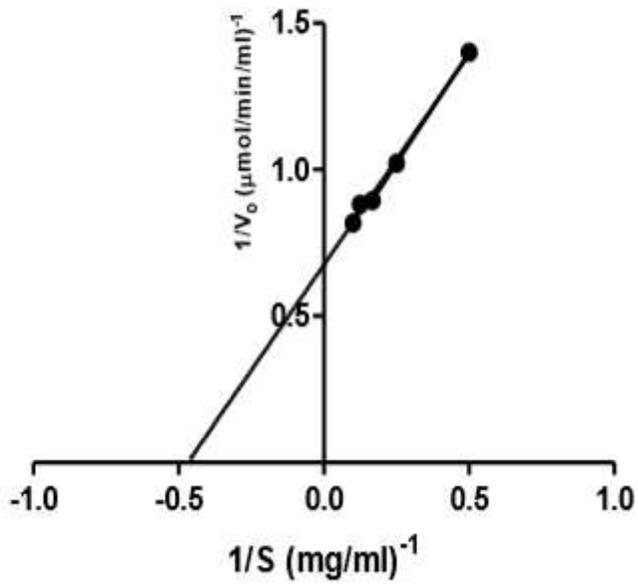


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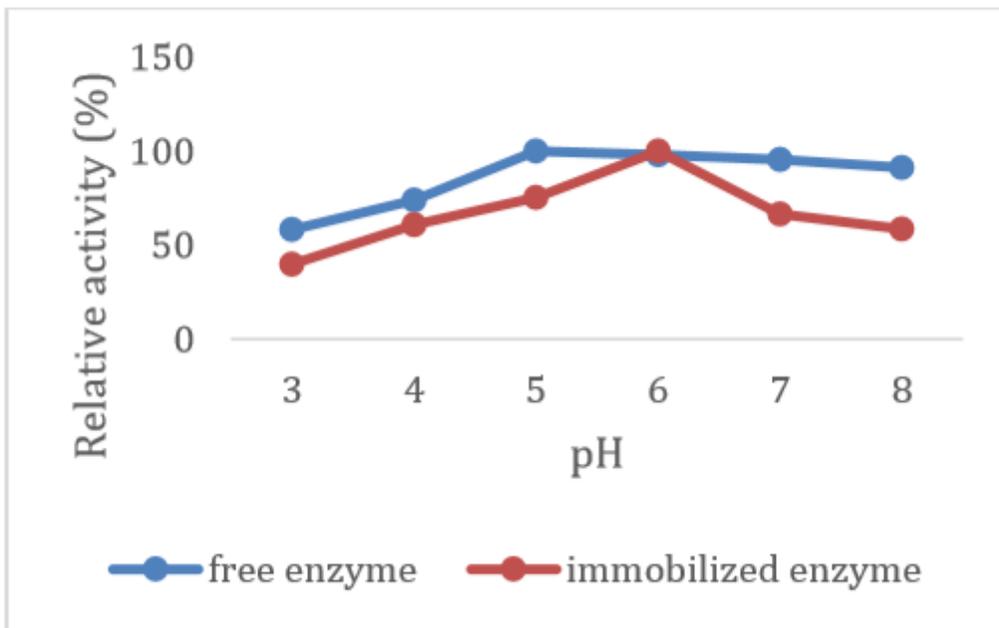


Figure 5

Effect of pH on free and immobilized enzyme

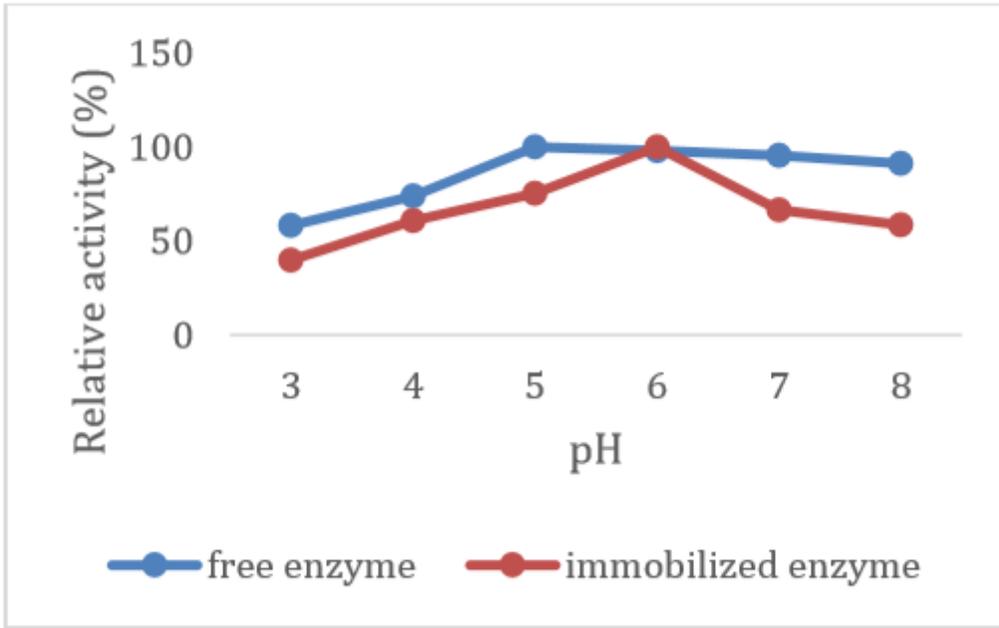


Figure 5

Effect of pH on free and immobilized enzyme

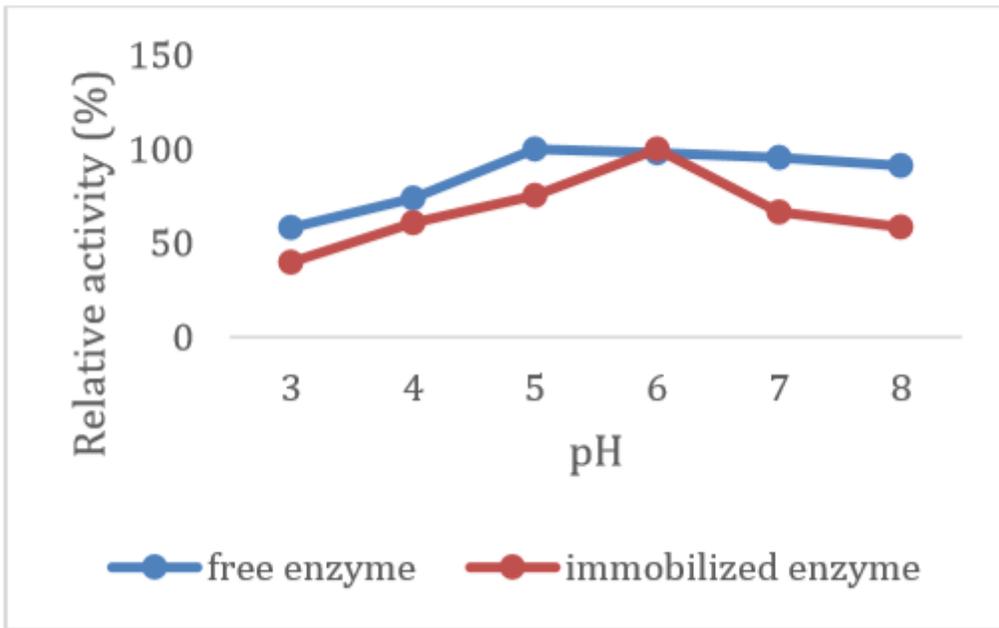


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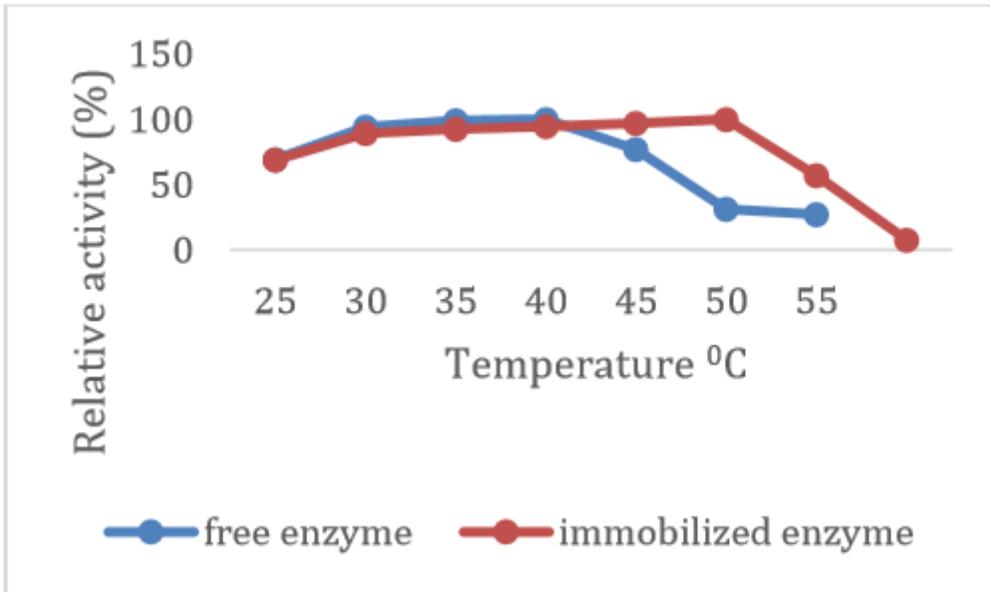


Figure 6

Effect of Temperature on free and immobilized enzyme

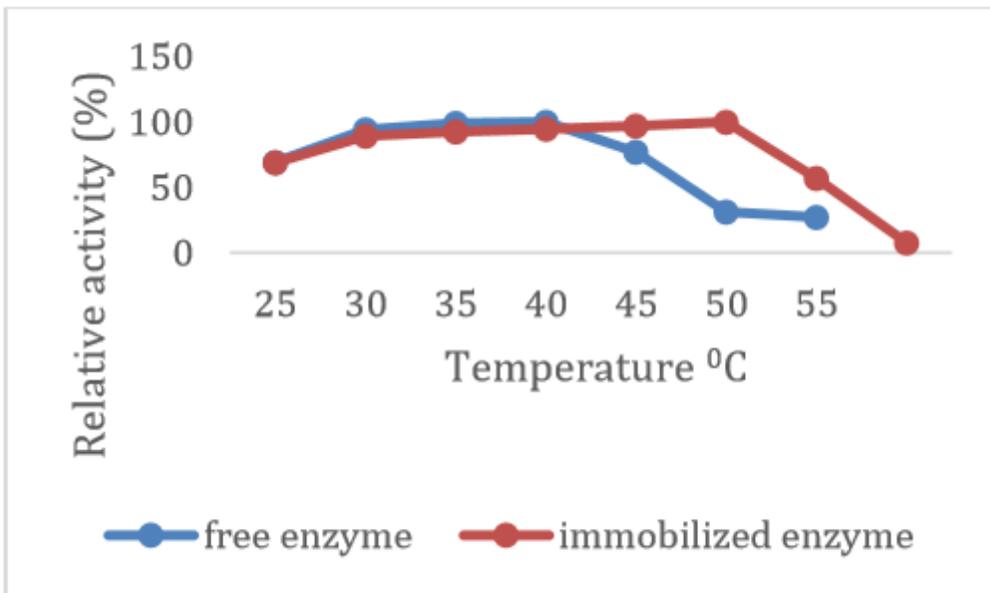


Figure 6

Effect of Temperature on free and immobilized enzyme

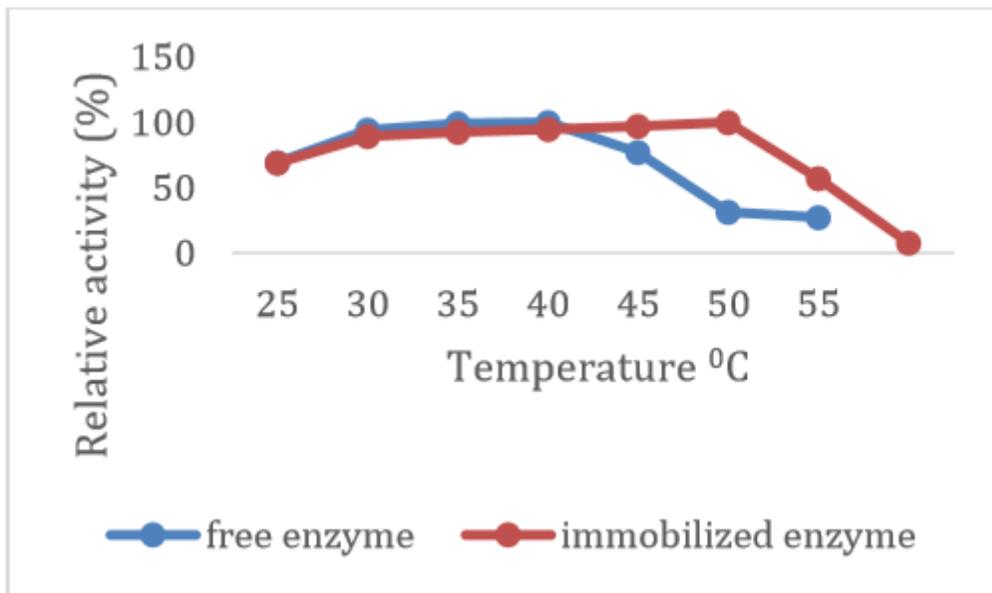


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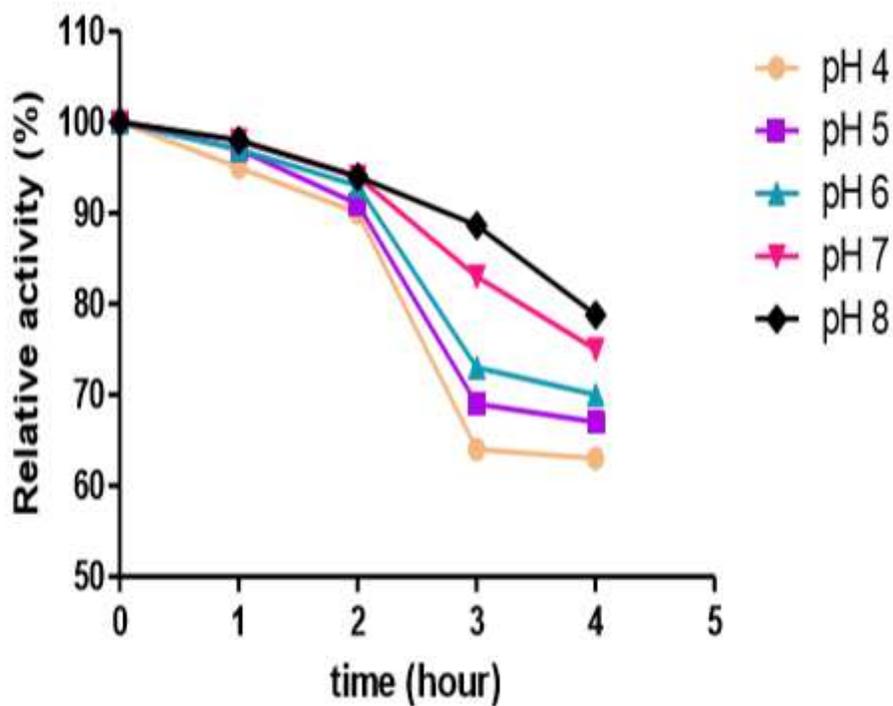


Figure 7

Effect of pH on stability of the  $\beta$ -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.

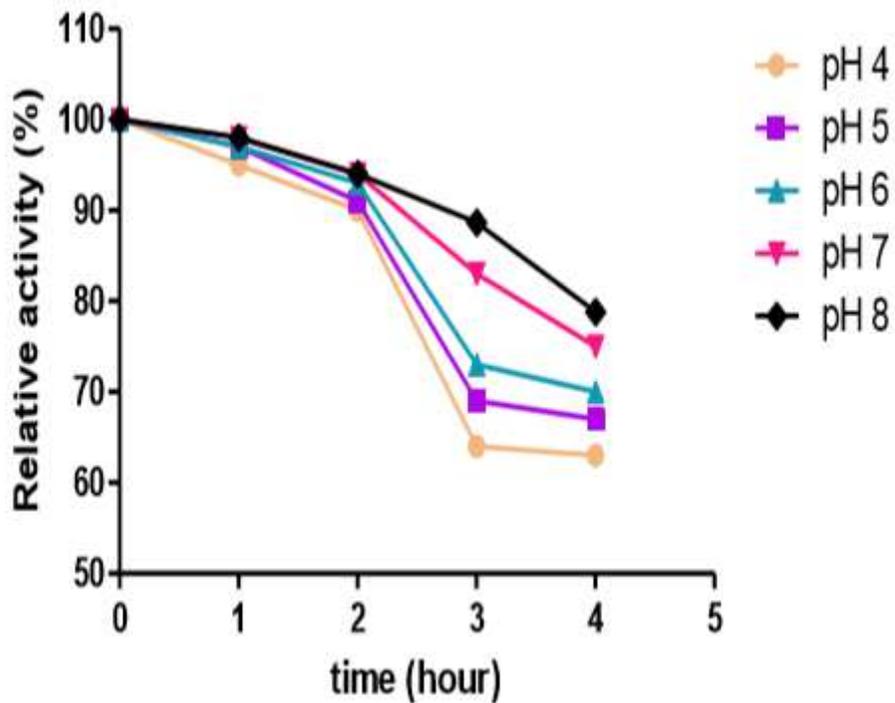


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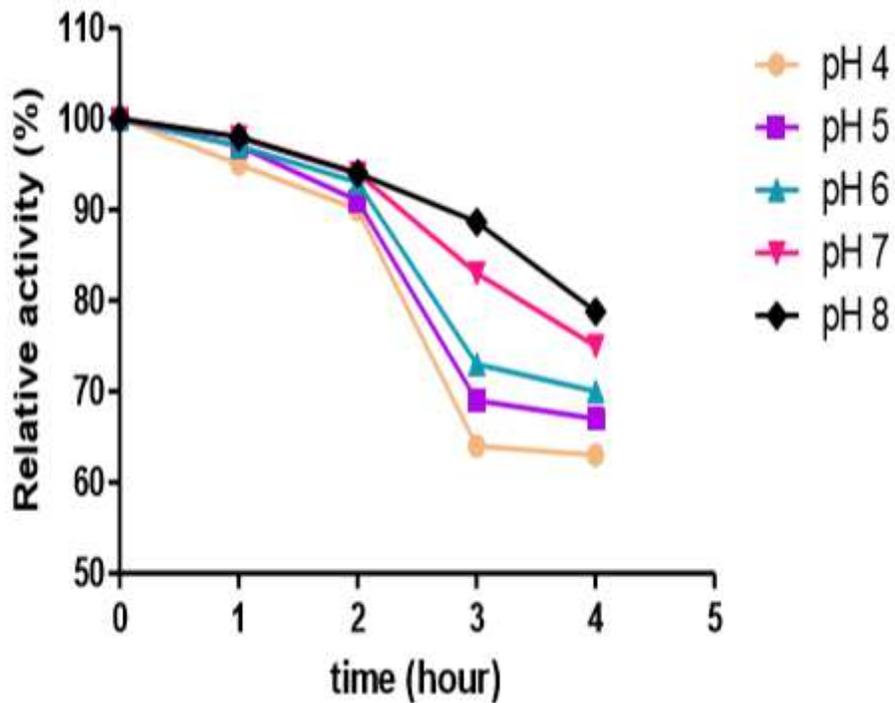


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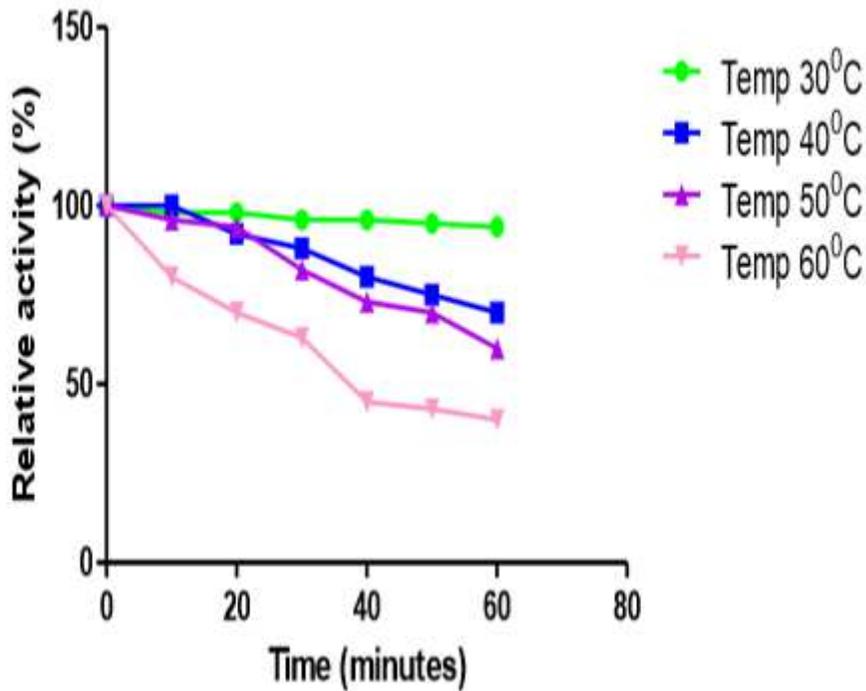


Figure 8

Effect of temperature on enzyme stability 100  $\mu$ l aliquot Withdrawn at an interval of 10min, and assay carried out according to standard procedure. The residual activities were expressed relative to the maximum activity at 0min which was taken as 100%.

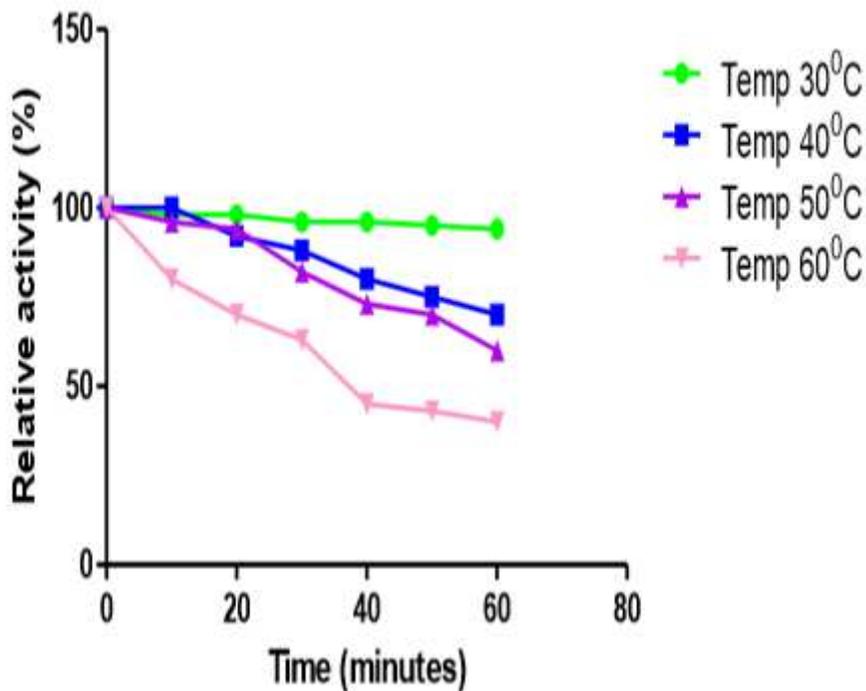


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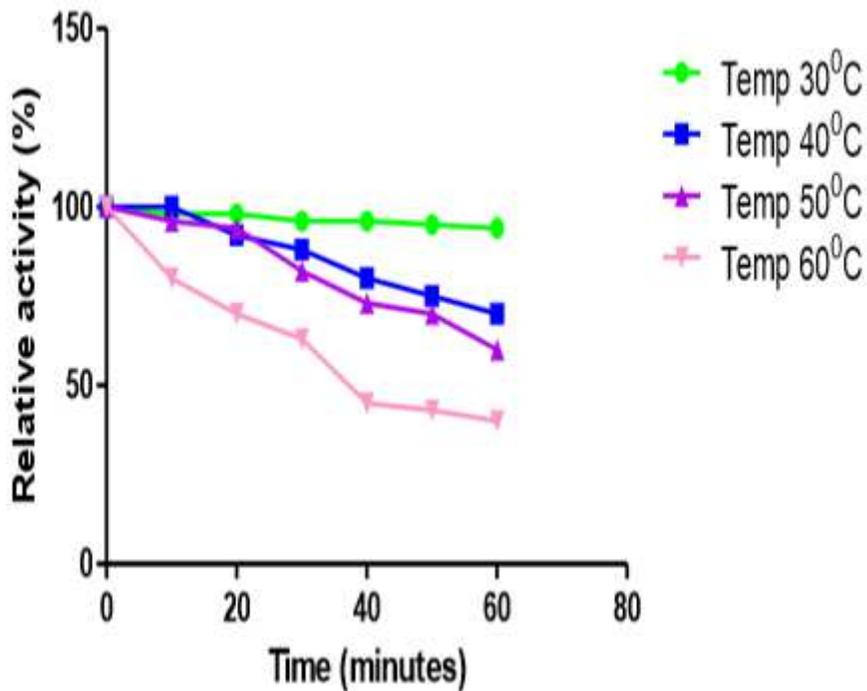


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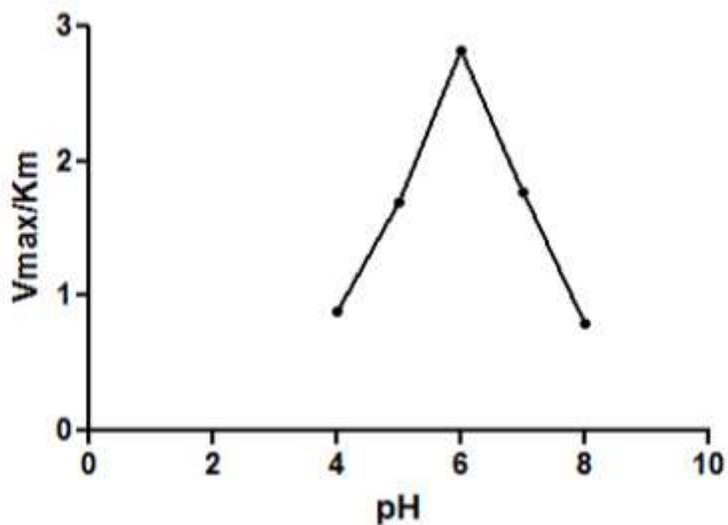


Figure 9

Graphical illustration of logarithm of  $V_{max}/K_m$  vs pH suggesting the amino acid participating in catalysis.

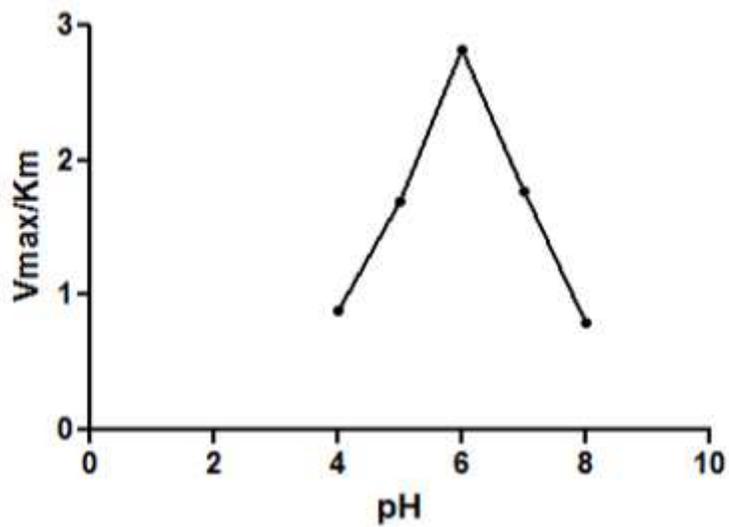


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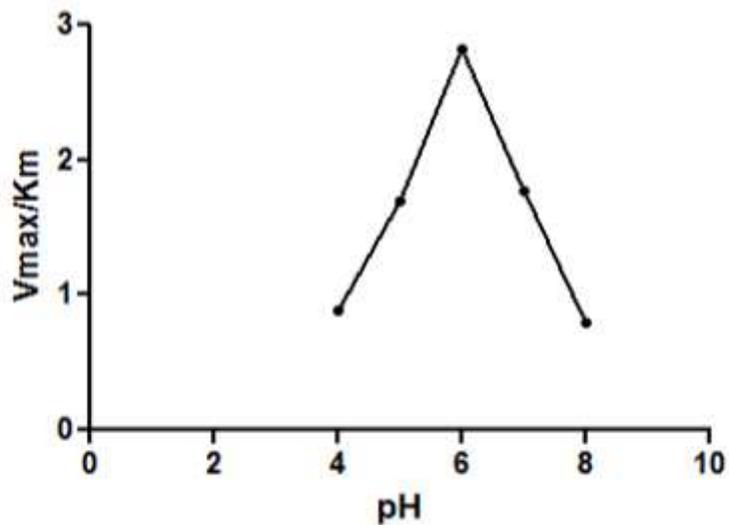
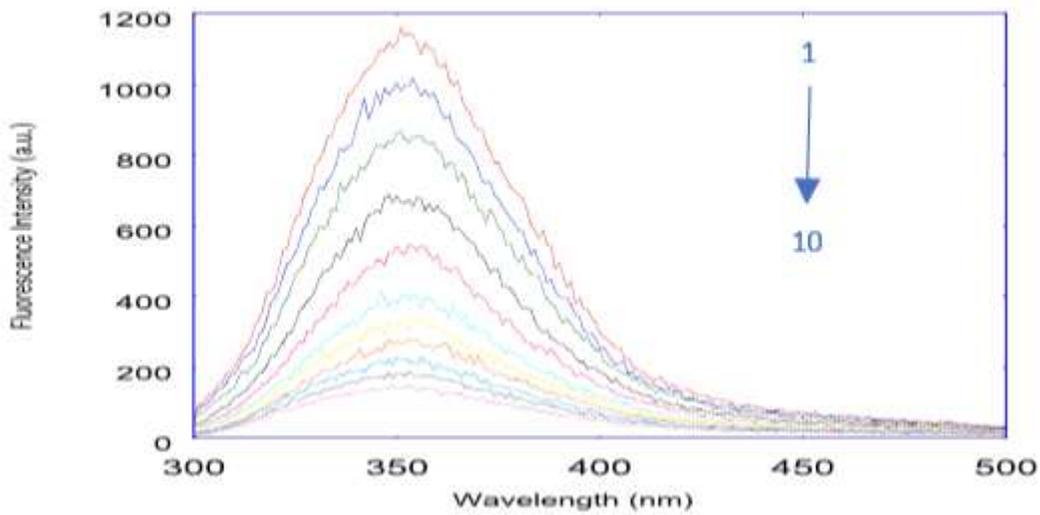


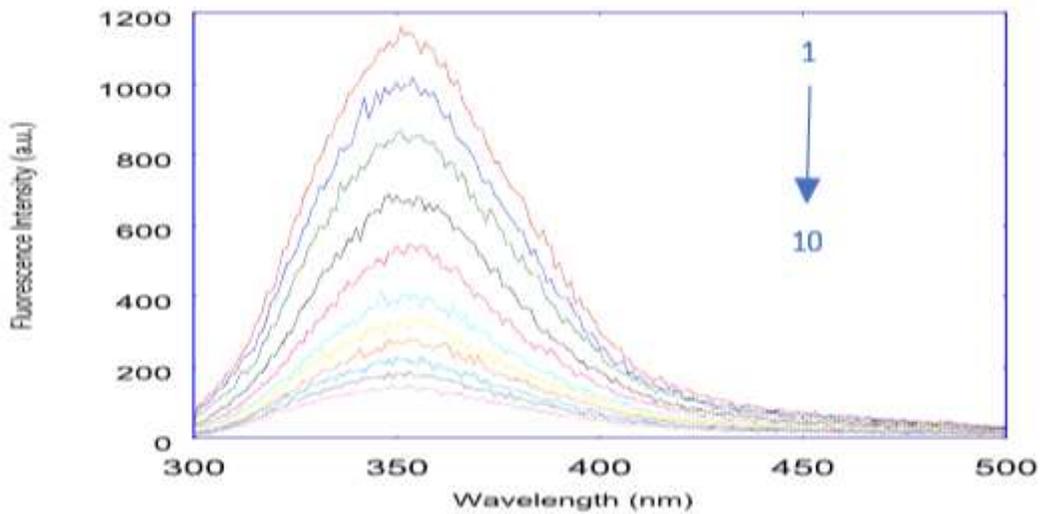
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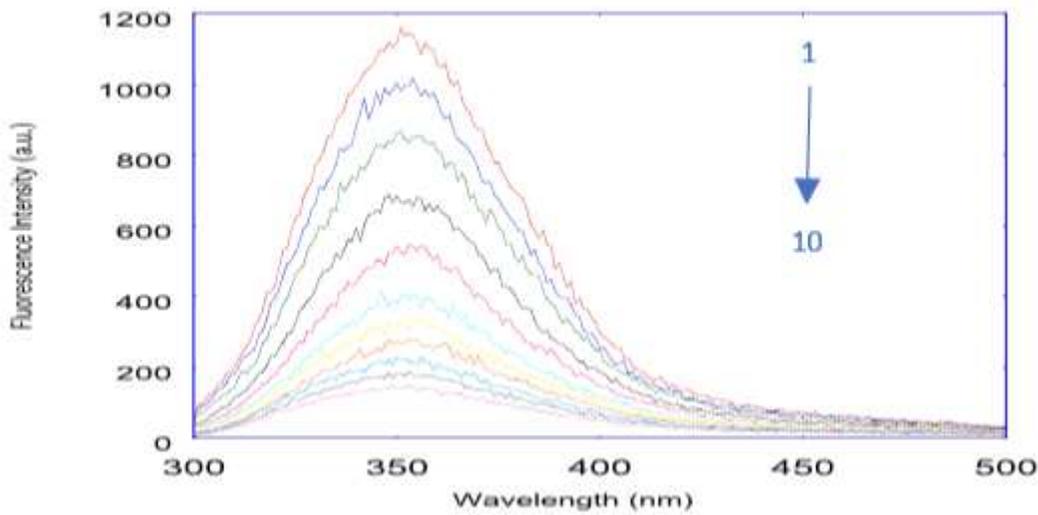
**Figure 10**

Fluorescence spectra of epicatechin. Emission spectra of  $\beta$ -amylase (0.274mg/ml) in the presence of various concentration of Epicatechin. Lane 1-10: from 0.0 to 125 $\mu$ M; first curve shows the emission spectrum of  $\beta$ -amylase only. (T ) 298 K,  $\lambda_{ex}$  348nm



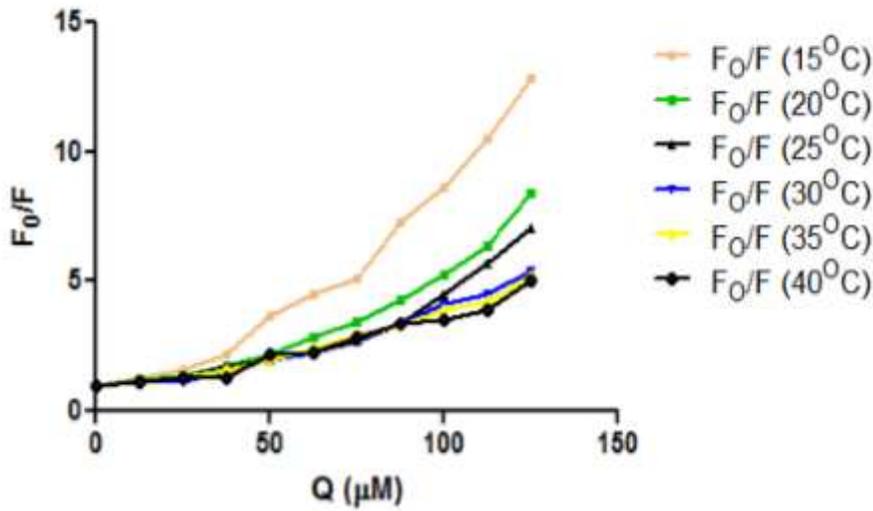
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**Figure 11**

The Classical Stern–Volmer plots of  $\beta$ - amylase by Epicatechin at 288 K-313K to determine the quenching constant (KSV and Kq). [ $\beta$ - amylase] = 0.230mM, pH 5 in Acetate buffer 50mM,  $\lambda_{ex}$ =280n.

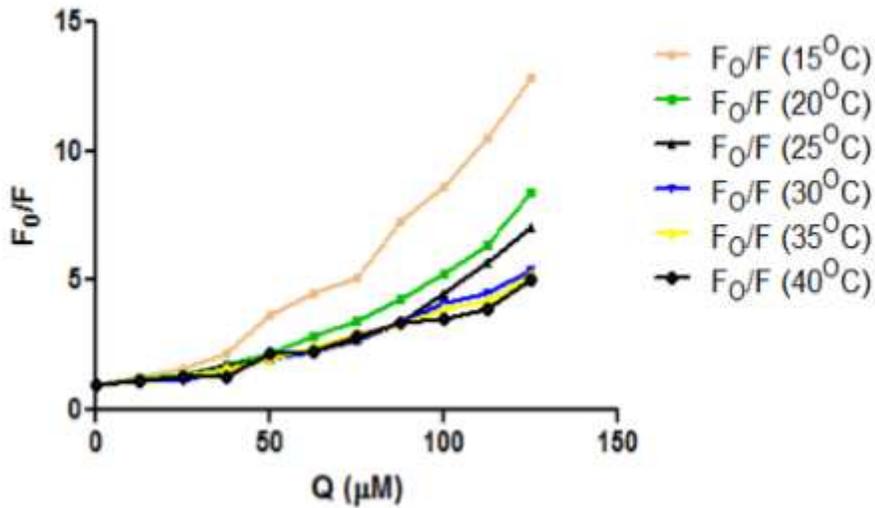


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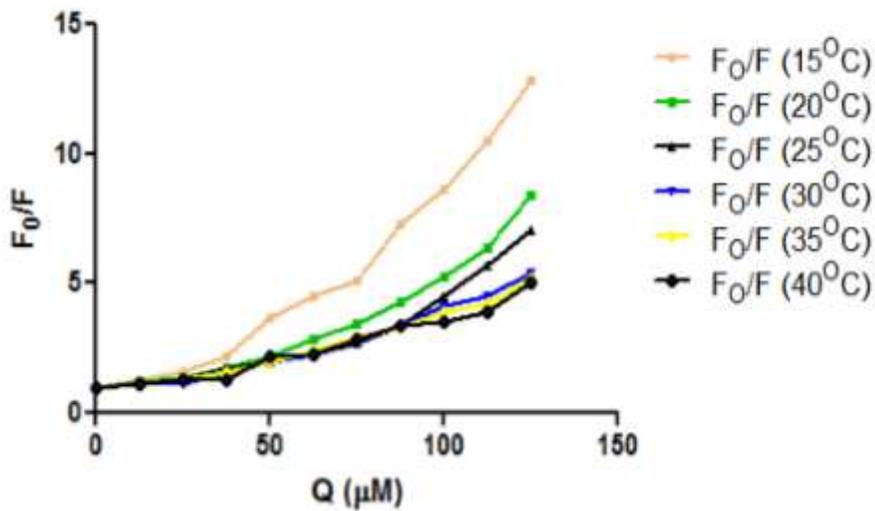


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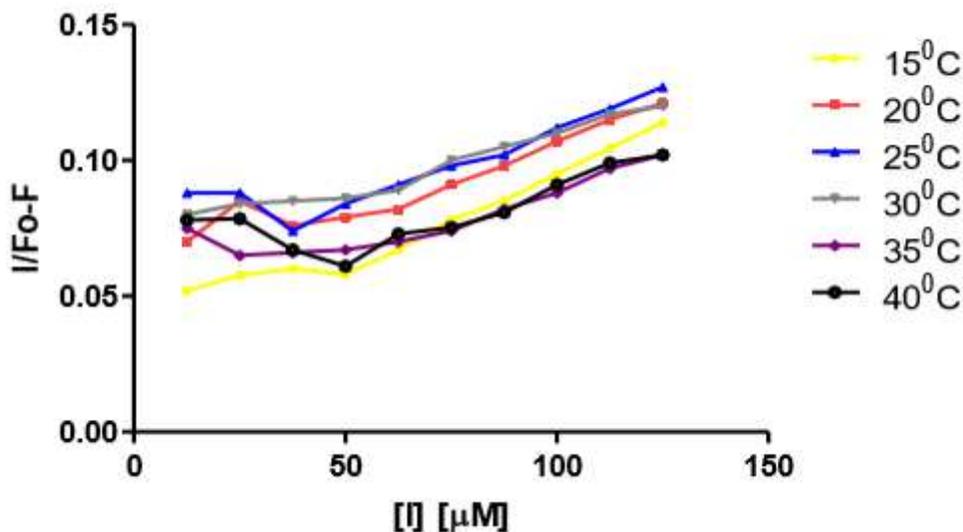


Figure 12

Intensity of fluorophore and its dissociative properties by modified Stern-Volmer plots for the determination of the dissociation constant ( $k_d$ ) of  $\beta$ -amylyase by Epicatechin at different temperatures 288K-313K

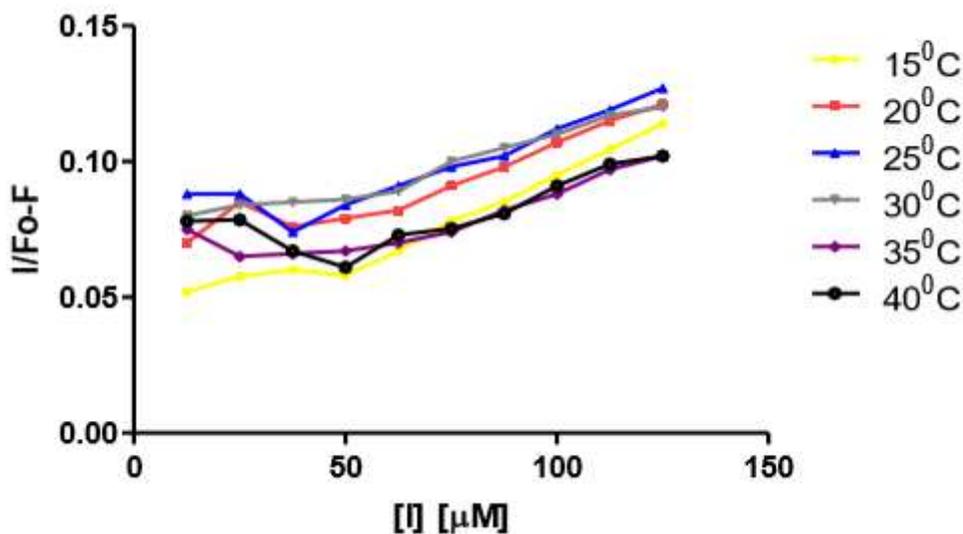


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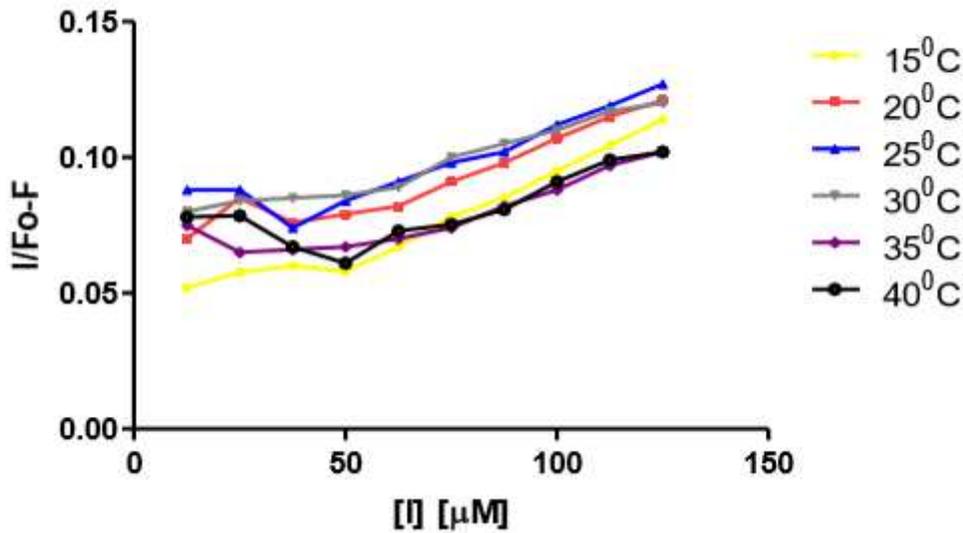


Figure 12

Intensity of fluorophore and its dissociative properties by modified Stern-Volmer plots for the determination of the dissociation constant ( $k_d$ ) of  $\beta$ -amylase by Epicatechin at different temperatures 288K-313K

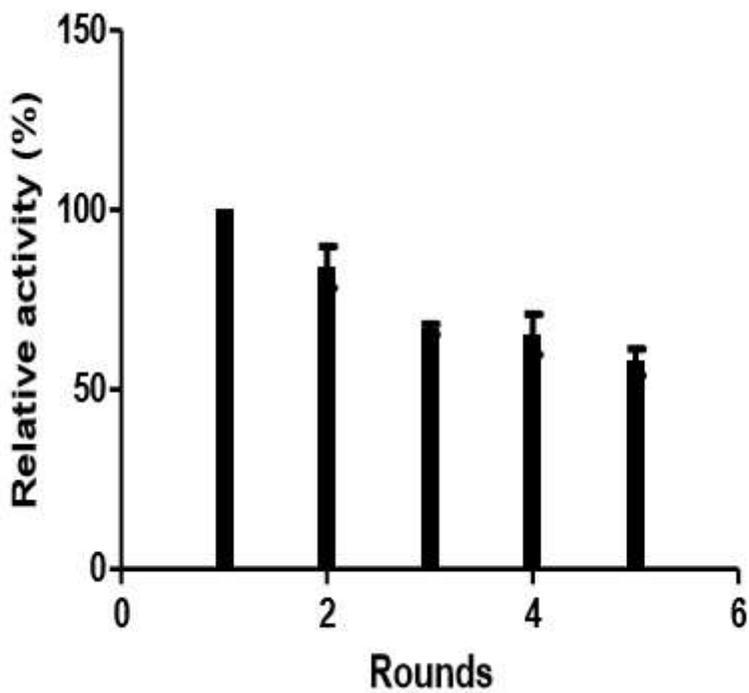


Figure 13

Reusability of  $\beta$ -amylase

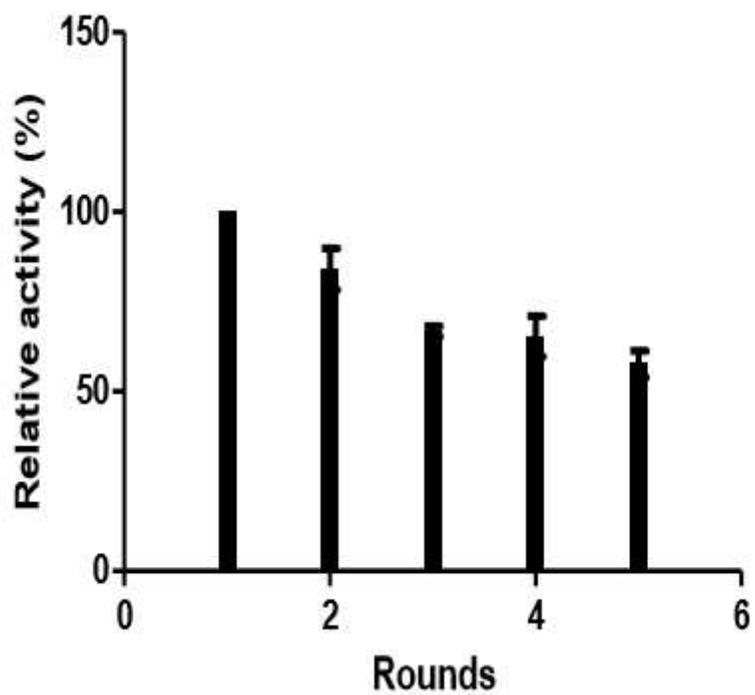


Figure 13

Reusability of  $\beta$ -amylase

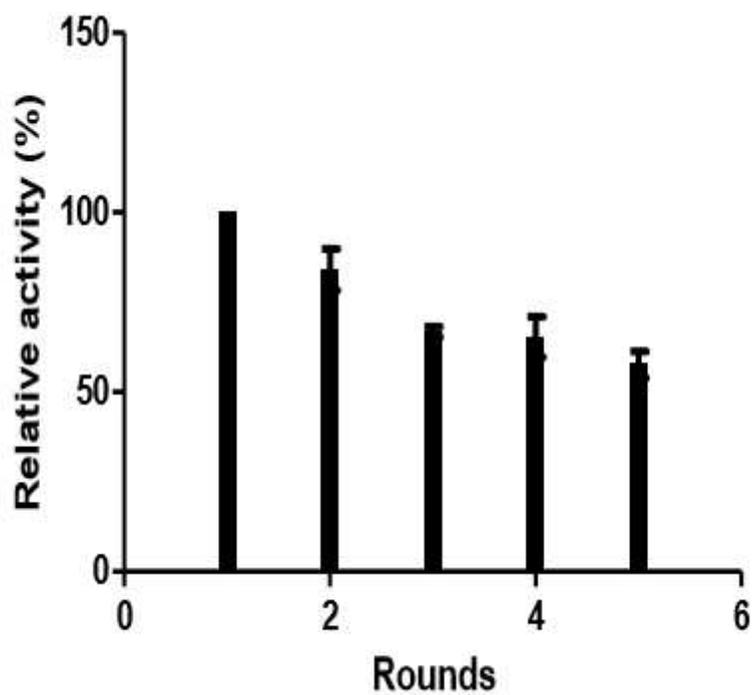


Figure 13

Reusability of  $\beta$ -amylase

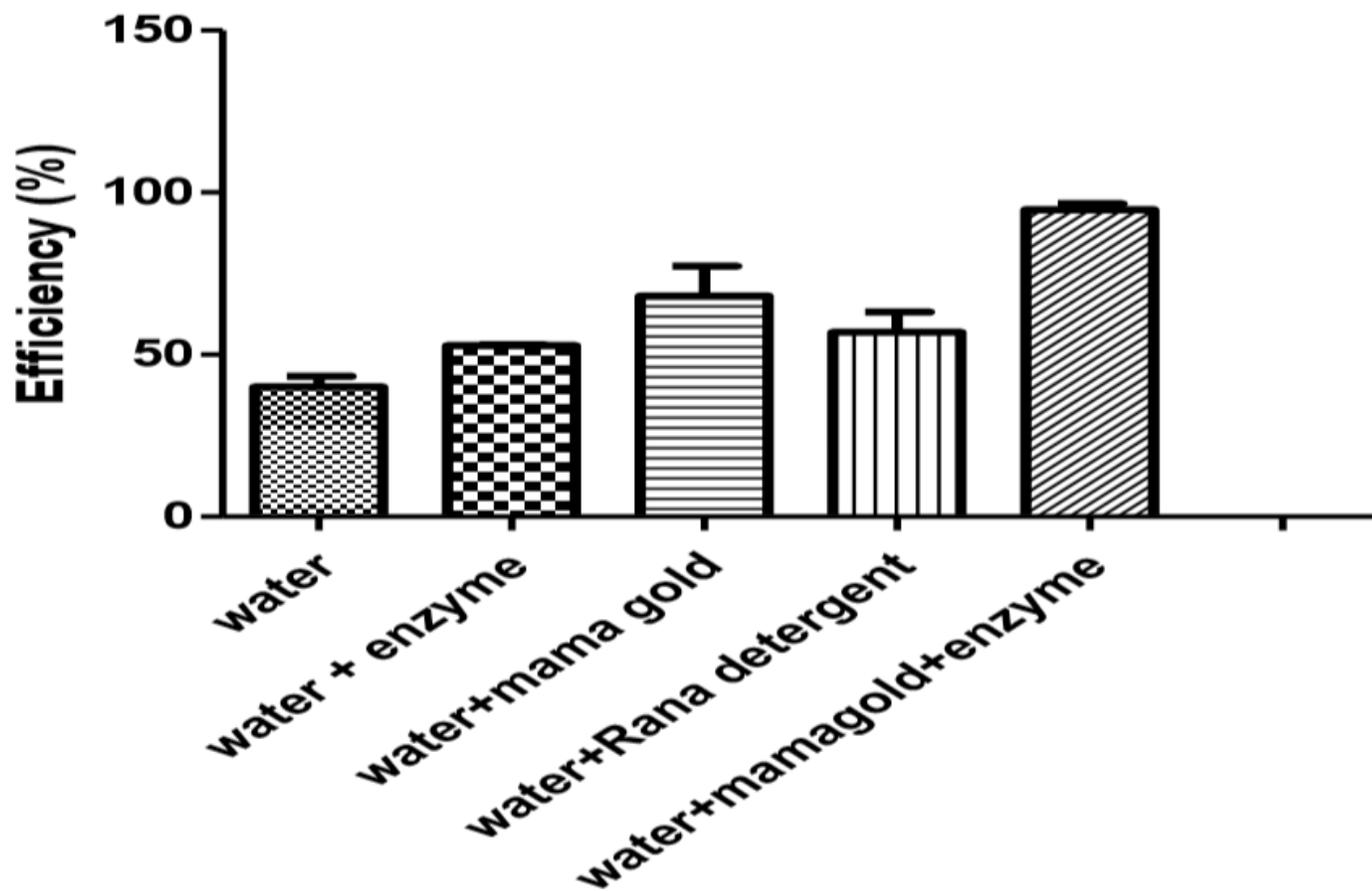


Figure 14

Efficiency of starch stain removal by  $\beta$ -amylase

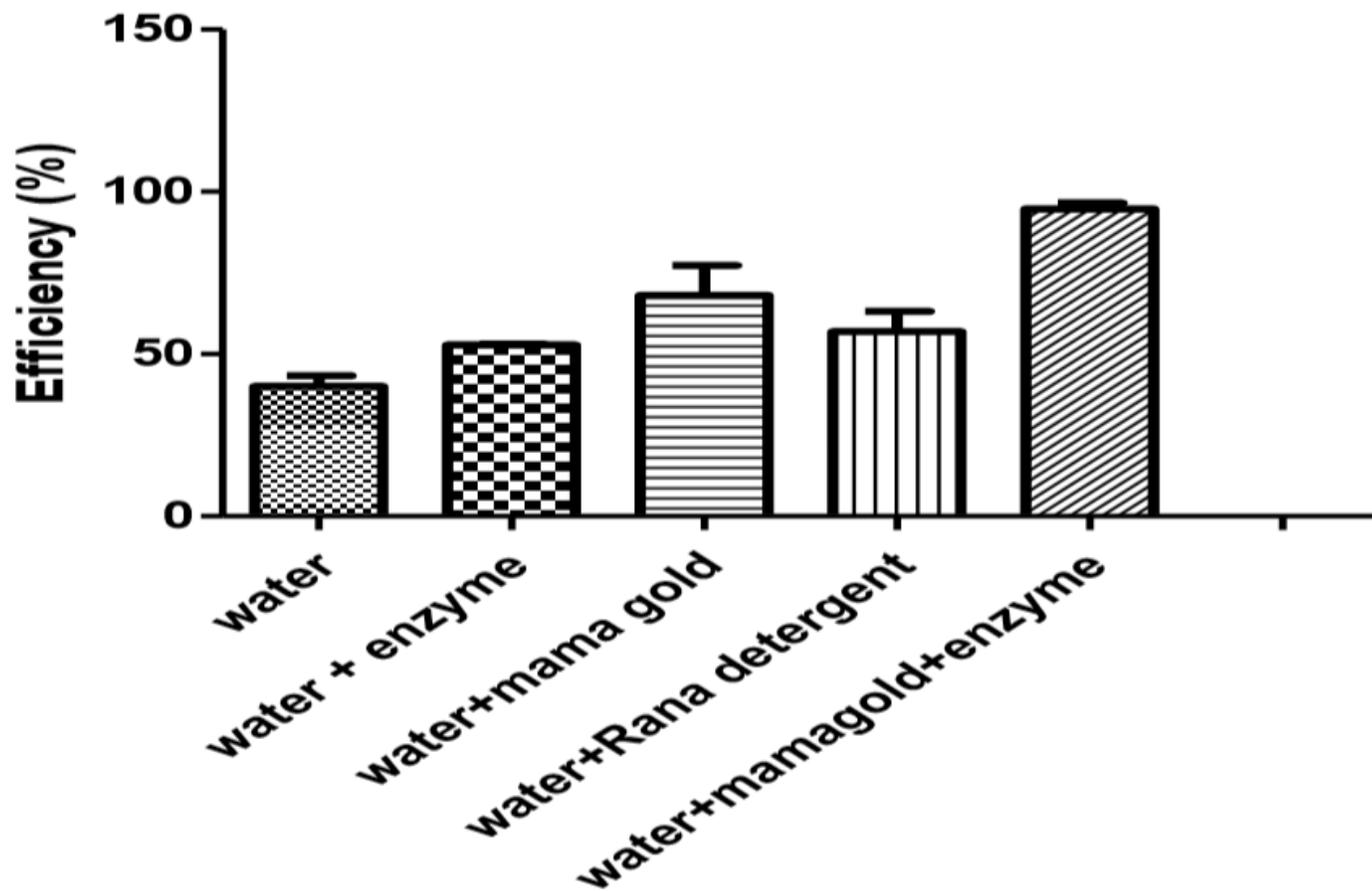


Figure 14

Efficiency of starch stain removal by  $\beta$ -amylase

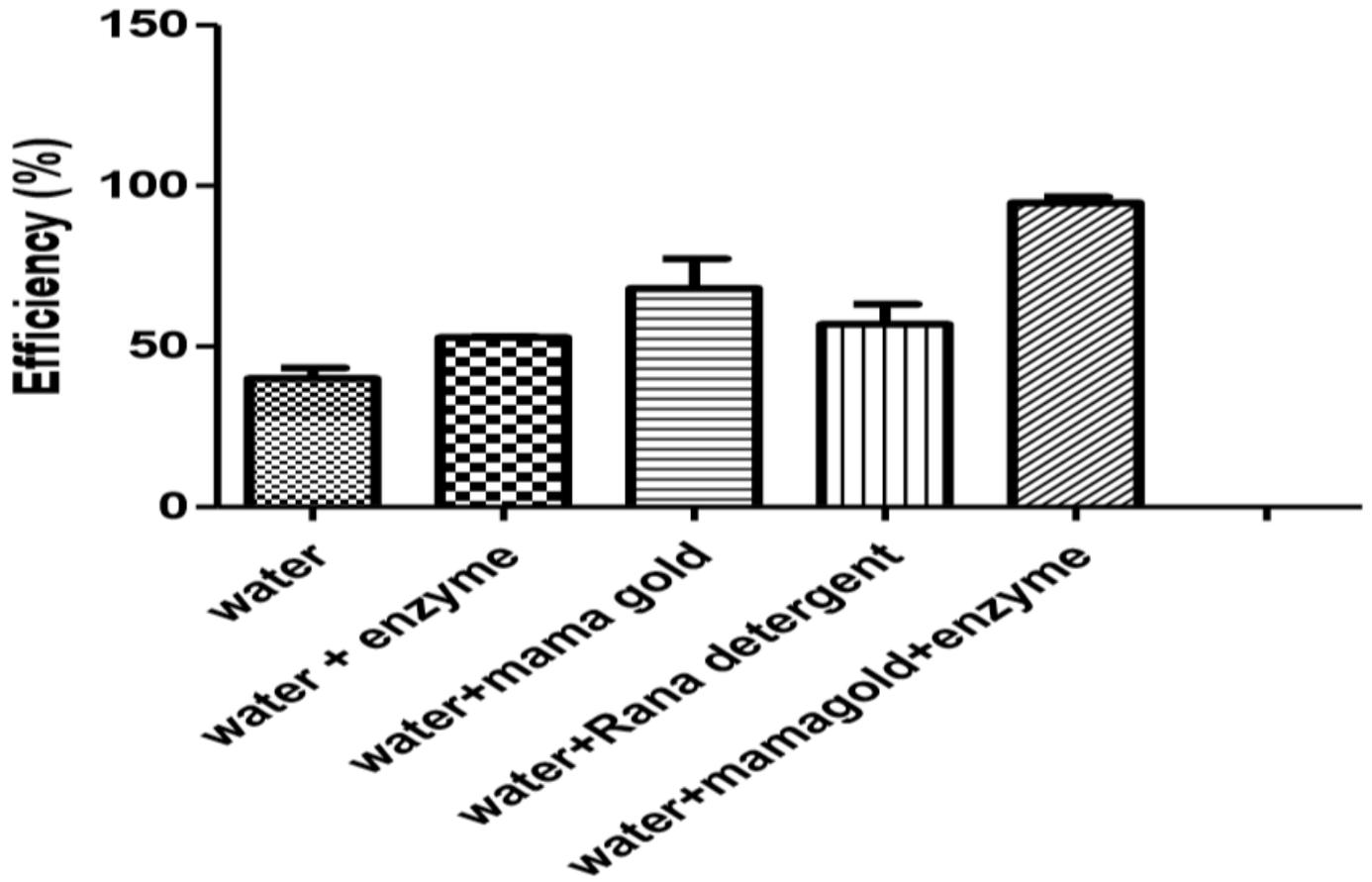


Figure 14

Efficiency of starch stain removal by  $\beta$ -amylase

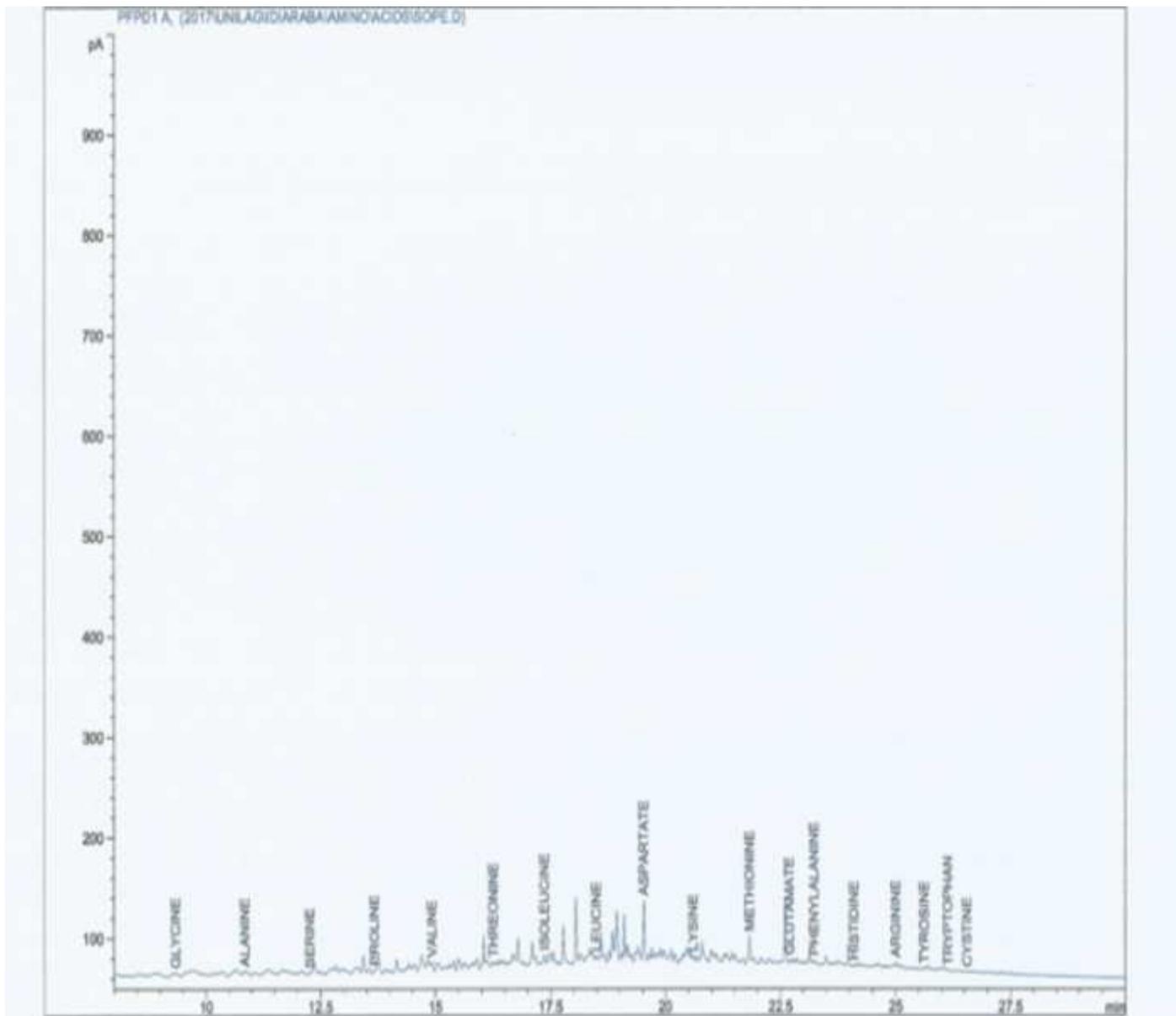


Figure 15

Chromatogram of amino composition of  $\beta$ -amylase

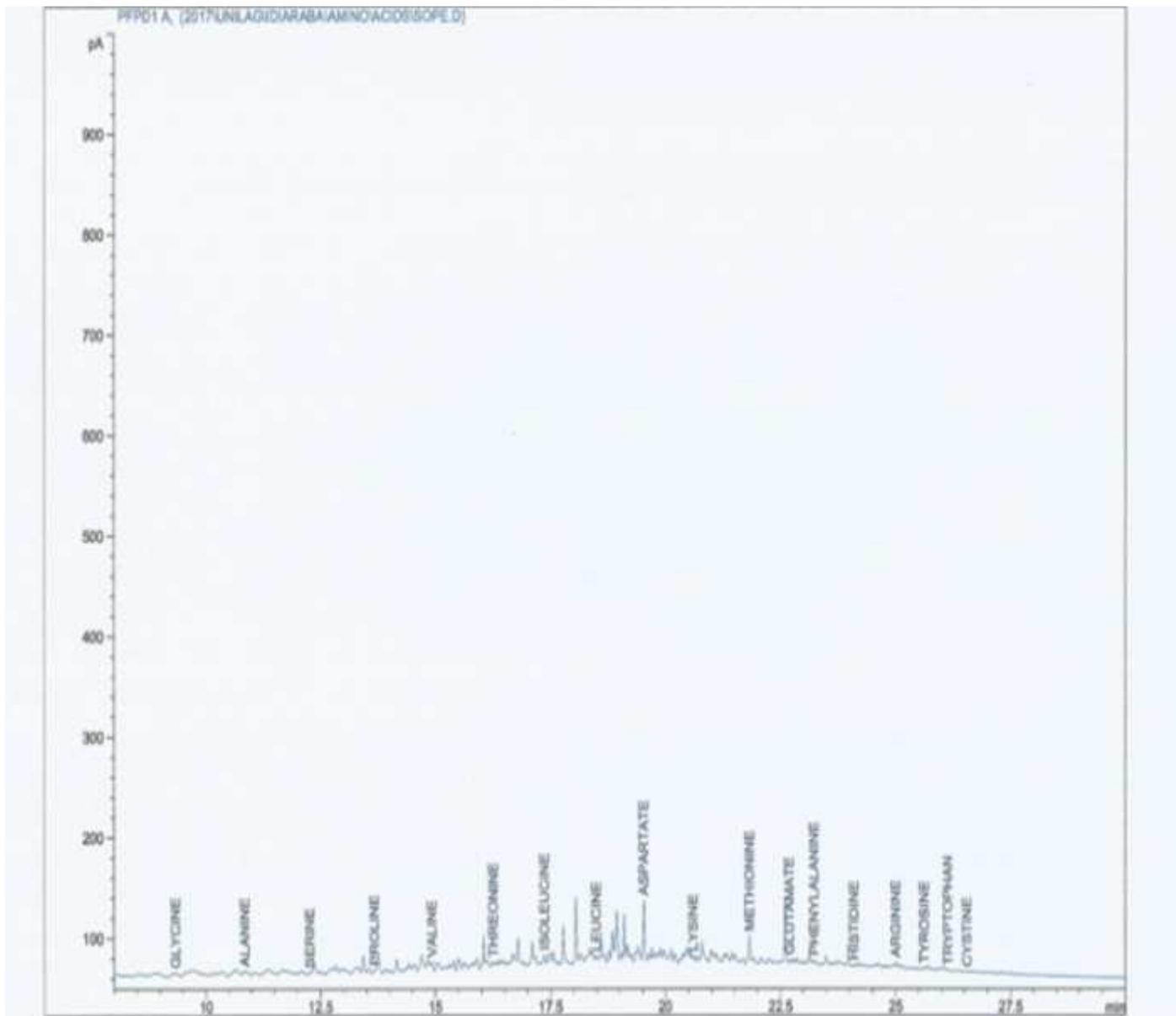


Figure 15

Chromatogram of amino composition of  $\beta$ -amylase

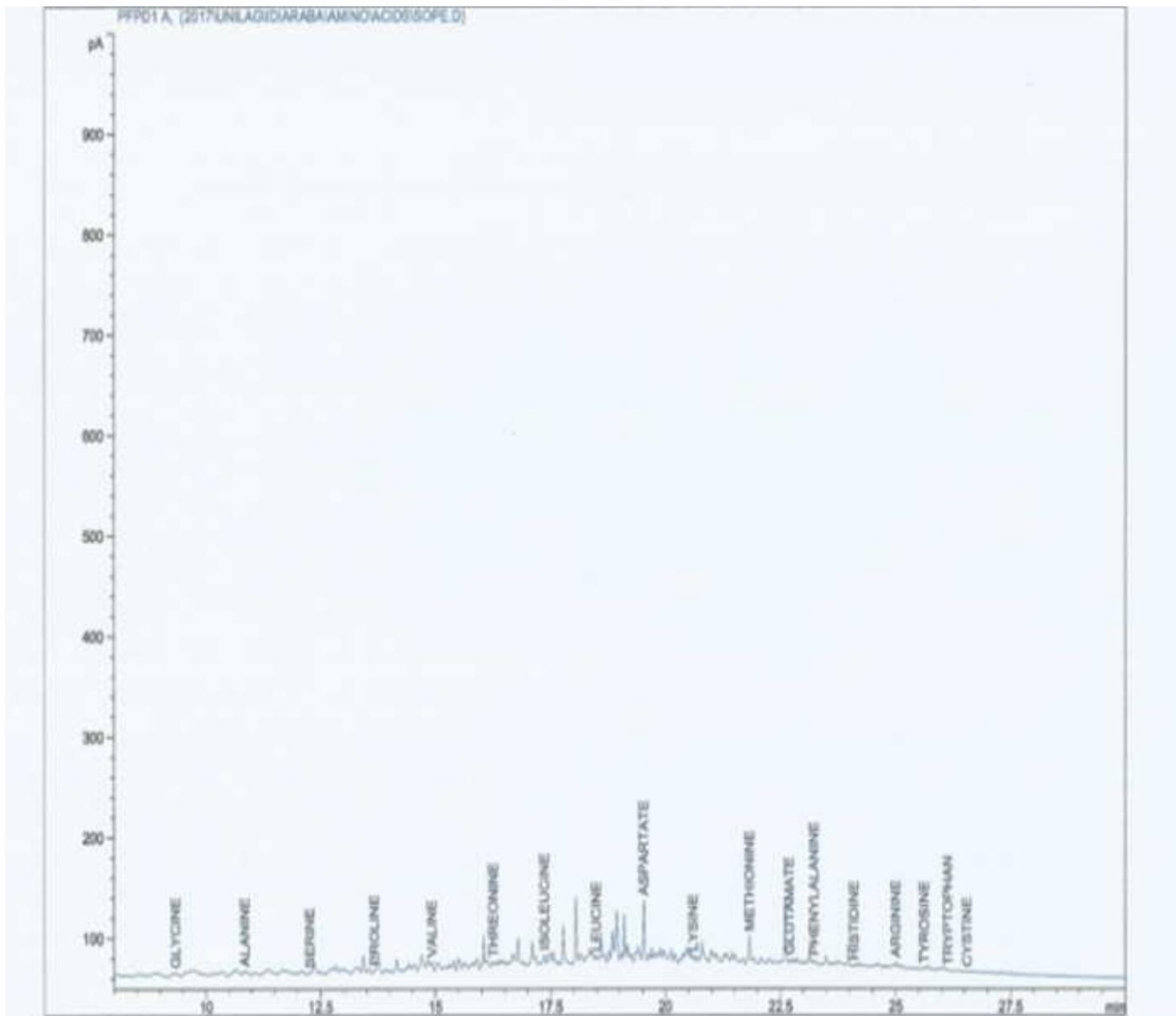


Figure 15

Chromatogram of amino composition of  $\beta$ -amylase

## Supplementary Files

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