# Purification and characterization of extracellular α-amylase from a thermophilic *Anoxybacillus thermarum* A4 strain

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## ABSTRACT

*α-Amylase from Anoxybacillus thermarum A4 was purified using ammonium sulphate precipitation and Sephadex G-100 gel filtration chromatography, with 29.8-fold purification and 74.6% yield. A4 amylase showed best performance for soluble potato starch hydrolysis at 70 °C and pH 5.5-10.5. A4 amylase was extremely stable at +4 °C, and the enzyme retained over 65% of its original α-amylase activity at 70 °C and 43% at 90 °C. The enzyme's $K_m$ values for soluble starch, amylopectin and amylose substrates were obtained as 0.9, 1.3 and 0.5 mg/mL, respectively. EDTA, $Hg^{2+}$, $B_4O_7^{2-}$, $OH^-$, $CN^-$ and urea exhibited different inhibition effects; their $IC_{50}$ values were identified as 8.0, 5.75, 16.5, 15.2, 8.2 and 10.9 mM, respectively. A4 amylase exhibited extreme stability toward some surfactants and perfect match for a wide variety of commercial solid and liquid detergents at 55 °C. So, it may be considered to be potential applications for detergent and other industrial uses.*

**Key words:** *Anoxybacillus thermarum* A4, Extracellular α-Amylase, Highly thermostable, Purification

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## INTRODUCTION

α-Amylases (EC 3.2.1.1, 1,4-α-D-glucan glucanohydrolase, and endoamylase) are glycoside hydrolases and have been classified in family 13[1]. The enzymatic hydrolysis of starch occurs with the interdependent action of endo-amylases (α-amylases), exo-amylases (β-amylase and glucoamylase), debranching enzymes (pullulanase), and other enzymes[2,3]. The most important amylases are glucoamylase and α-amylase for industrial and biotechnological applications. α-amylase carries out hydrolysis of starch, glycogen and related polysaccharides to produce different sizes of oligosaccharides by randomly cleaving internal α-1,4-glycosidic bonds. Additionally, the α-amylase family is one of the most important industrial enzymes and most important families of enzymes in biotechnology[3,4] and can be obtained from several sources[4].

These are broadly dispersed in different bacteria, fungi, plants and animals, and play an important role in the usage of polysaccharides. However, for industrial applications they are generally outcome of bacteria from the genus *Bacillus,* such as *Bacillus licheniformis, B. amyloliquefaciens, B. stearothemophilus* and *B. subtilis* [5]. They have diverse applications in a wide variety of industries like food, fermentation, textiles, paper, detergents, pharmaceuticals and sugar[6]. They are not only used as used as an additive in detergent, but also they can be used for such purposes as the removal of starch sizing from textiles, the liquefaction of starch, and the appropriate structure of dextrin in baking. In the starch industry, the natural pH of starch slurry is usually between 4.5-5.5 [7]. The maximum pH's for most industrial α-amylases are approximately 6.5, but they are less stable at lower pH values. Consequently, the liquefaction level in the starch processing industry is now dealing with a limited range of pH values (5.5–6.5), and the thermal stability of the enzymes at low pH therefore needs to be improved in order to eliminate the pH adjustment step[8,9]. Recently, global business has concentrated on raw starch-digesting amylases capable of acting at low pH and high temperatures, which could be valuable in terms of efficiently simplifying the process of starch conversion. Microbial amylases have successfully replaced the chemical hydrolysis of starch in industries[10].

This study is the first report of α-amylase being purified and characterized from a thermostable micro-organism, *Anoxybacillus thermarum* A4 strain (GenBank Number: KC310455) from a hot mineral spring in the province of Erzurum, Turkey. This study describes the purification of thermostable extracellular α-amylase from *A. thermarum* A4, using very a economical method, characterization of this single protein and potential applications such as new detergent formulations, textiles, starch liquefaction and other areas of industry.

## MATERIALS AND METHODS

### Materials
DNS (3,5-dinitrosalicylic acid), glucose, soluble starch, amylose, amylopectin, glycogen, Amicon ultrafiltration membrane and β-cyclodextrin were purchased from Sigma (Sigma-Aldrich, USA). Sephadex G-100 was purchased from Pharmacia (Pfizer and Pharmacia, Sweden). All culture medias, sodium salts and their additives were commercially obtained from Merck (Merck & Co., Inc.). The molecular mass marker was bought from Fermentas (Fermentas Life Science, USA). All related chemicals applied were of analytical grade.

### Optimization of medium for extracellular amylase production
Four different medias were chosen for selecting the optimum medium for extracellular amylase production; 1- 0.5% tryptone, 1% potato starch, 1% NaCl, 1% $CaCl_2$ and 2.5% $MgSO_4$; 2- 1% potato starch, 0.5% NaCl, 1% $CaCl_2,$ 0.1 M $NH_4Cl$ and 2.5% $MgSO_4$; 3- 1.0% potato starch; 2.5% tryptone; 1.0% NaCl; 0.1% $CaCl_2$ and 0.25% $Mg_2SO_4.7H_2O$ and 4- 0.5% yeast extract, 0.5% NaCl and 1% starch. The effects of different pH values and at different incubation times, i,e., 5, 8, 16, 20, 24, 36, 40, 48, 55, 60, 65 and 72 h, on extracellular amylase production were examined at 55°C.

### Micro-organism, culture, media, and growth conditions
The *A. thermarum* A4 strain (GenBank Number: KC310455) isolated from a hot mineral spring in the province of Erzurum in Turkey was routinely grown in Luira-Bertani (LB) broth composed of (g/L): peptone 10.0; yeast extract 5.0 and NaCl

5.0. The growth medium used for extracellular α-amylase production was composed of (g/L) insoluble potato starch 10.0; triptone 2.5; NaCl 10.0; $CaCl_2$ 0.1 and $Mg_2SO_4.7H_2O$ 2.5. The medium pH was adjusted to 7.0 and autoclaved at 120 °C for 20 min. And then, 50 mL medium was transferred into 500 mL Erlenmeyer flasks. Growth of cell was carried out at 55 °C for 48 h in a rotary shaker at 150 rpm, after which the cells were separated by centrifugation. The culture was centrifuged at 15000 x g for 45 min and the cell-free supernatant was used as a crude enzyme source.

## Enzyme assay and protein concentration
## Reducing sugar estimation
α-Amylase process was evaluated using the dinitrosalicylate (DNS) method described by Miller[11]. α-Amylase activity was found out by measuring amount of the reducing sugars released during starch hydrolysis. Two sets of reaction mixtures (blank and sample) were taken for the test of amylase activity, and the contents in the tubes were mixed uniformly. The α-amylase activity assay was performed at 70 °C for 20 min in a 600 µL sample reaction mixture containing 300 µL of 2% (w/v) soluble potato starch in 50 mM of MOPS buffer, pH 7.0, and 300 µL of suitably diluted enzyme solution. The blank contained 300 µL 50 mM of MOPS buffer instead of the enzyme solution. After 20 min incubation, to each tube 600 µL DNS reagent was added and then mixed. Then the tubes were heated in boiling water bath for 5 min; and when it was cooled to room temperature the amount of liberated reducing sugar released from starch was measured at 540 nm. Under test conditions, one unit of amylase activity was described as the amount of enzyme that released 1 µM of reducing end groups per minute. D-glucose was used to construct a standard curve.

## Protein estimation
Protein concentration was determined using the Lowry method[12] with BSA as standard.

## Purification of the amylase
Every purification step was done at 4 °C, and with 50 mM of MOPS buffer (pH 7.0). The culture broth was centrifuged (13,000 x g for 45 min at 4 °C) and the cell-free supernatant was subjected to ammonium sulphate fractionation. Solid ammonium sulphate was then added slowly to the supernatant fluid with constant stirring at 4 °C. The precipitate in the saturation range of 40–80% (w/v) ammonium sulphate solution was achieved by centrifugation for 15 min at 10,000 x g. The precipitate was suspended in MOPS buffer. The enzyme solution was washed and then concentrated with an Amicon ultrafiltration membrane (MWCO: 30 kDa).

## Sephadex G-100 chromatography
The enzyme solution (14 mL) obtained from the step described above was loaded onto a Sephadex G-100 column (3 x 60 cm) pre-equilibrated with MOPS buffer. The column was eluted using 400 mL of the same buffer, at a flow rate of 0.5 mL/min. Fractions of 4.0 mL were collected, checked for enzyme activity and monitored by measuring the absorbance at 280 nm protein content.

## Polyacrylamide gel electrophoresis
SDS-PAGE was carried out according to the method of Laemmli[13] using 5% (w/v) stacking gel and 12% (w/v) separating gel. Samples were heated at 100 °C for 5 min before electrophoresis. The protein bands were stained with Coomassie brilliant blue R-250 (Sigma). Relative molecular weight of the enzyme was estimated by comparison with molecular mass standard markers in the range 250.0–10.0 kDa (Fermentas, Germany).

## Zymography of the enzyme
Nondenaturing PAGE was done as identified by Laemmli et al.[13]. The protein sample was mixed with β-mercaptoethanol free loading buffer and was electrophoresed without heating at room temperature until the surveillance dye migrated to the bottom of the gel. The gel was incubated at 55 °C 30 min in 50 mM MOPS buffer (pH 7.0). After incubation, the gel was stained with iodine solution, which left a white halo zone pattern where the starch had been hydrolyzed by amylase.

## Characterization of the purified amylase
## The effects of pH and temperature on enzyme activity
The effect of pH on amylase activity was determined using soluble potato starch as a substrate in the presence of 100 mM acetate buffer, pH 4.5-5.5; 100 mM MOPS buffer, pH 6.5-7.5 and 100 mM Tris-HCl buffer, pH 8.5-9.5 at 70 °C. Samples were assayed at various temperatures

in the range of 20–90 °C in order to determine the effect of temperature on the enzyme activity over an initial 20 min period. The reaction mixtures (at pH 7.0) containing all the reagents and enzyme were incubated for 20 min, at related temperatures, as described above. Results were shown as the percentage of residual activity obtained at either the optimum pH or the optimum temperature by measuring the activity after incubating the reaction mixture at various temperatures and pHs.

**pH and thermal stability of α-amylase**
Thermal stability of the enzyme was determined by incubating the enzyme at 4, 55, 70 and 90 °C for up to 10 days. After heat treatment, the enzyme solution was cooled, and the residual activity was determined under the optimum enzyme assay procedure. The non-heated enzyme was used as a control sample at 100%. The pH stability of α-amylase was examined by incubating the enzyme solution for 1 to 4 days at +4 °C for storage temperature), 55 °C (microorganism growth and detergent industry temperature), 70 °C (optimum temperature) and 90 °C (for the starch industry) for up to 10 days with different buffers, and then determined the residual activity using the standard activity assay procedure. The activity of the enzyme at the start of the test was considered as 100% activity. The following buffer systems were used: 100 mM acetate buffer, pH 4.5-5.5; 100 mM MOPS buffer, pH 6.5-7.5 and 100 mM Tris-HCl buffer, pH 8.5-9.5.

**Effect of metal ions, anions and enzyme inhibitors**
The effect of various metal ions (at final concentrations of 1, 5, 10 and 20 mM) on enzyme activity was investigated using $BaCl_2$, $MgCl_2$, $MnCl_2$, $HgCl_2$, $NiCl_2$, $ZnCl_2$, $CaCl_2$, $CoCl_2$, $FeCl_2$ and $CuCl_2$. The activity of the α-amylase was determined using 1% potato starch as a substrate by incubating the enzyme in the presence of various metal ions for 20 min at 70 °C (optimum temperature) in 50 mM pH 7.0 MOPS buffer. The effect of various anions (at the final concentrations of 1, 5, 10 and 20 mM) on the enzyme activity was investigated using NaOH, $NaClO_4$, $NaNO_2$, $Na_3PO_4$, $NaN_3$, NaCN, NaCl, $Na_2HPO_4$, $Na_2CO_3$, $NaCH_3COO$, $Na_2CO_4$, $NaNO_3$, $NaHCO_3$, $Na_2SO_4$, $NaSO_3$, $NaC_8H_{11}N_2O_3$, $Na_2WO_4$ and $NaHSO_3$. The effect of EDTA and urea on enzyme activity was also investigated at the same concentrations.

The effect of various enzyme inhibitors at two concentrations (1 and 10 mM) on α-amylase activity was investigated by using phenylmethylsulphonyl fluoride (PMSF) and 2-mercaptoethanol (2-ME). For these purposes, the enzyme, without any additive, was taken as 100%. The purified enzyme was pre-incubated with inhibitors at 20 °C for 20 min, after which the remaining activity was calculated using 1% potato starch as a substrate under standard assay conditions. Relative activity was calculated by comparing with control (enzyme incubated without inhibitors).

**Stability in the presence of solid and liquid detergents and denaturing agents**
The effect on enzyme stability of various commercial solid and liquid laundry detergents at three concentrations (0.5%, 1% and 2%) was investigated by pre-incubating the enzyme for 20 min at 55 °C. The remaining activity was measured at pH 7.0 and 70 °C. Remaining activity was calculated and enzyme solution without detergents was used to compare as reference. Similarly, the effect on enzyme stability of various surfactants (Triton X-114, Triton X-100, Tween-80, Tween-20, SDS) at final concentrations of 0.5%, 1% and 2% and oxidizing agent (sodium perborate) at four concentrations of 1, 5, 10 and 20 mM was examined by pre-incubating the enzyme for 20 min at 55 °C and then the remaining activity was calculated by comparing with control (enzyme incubated without surfactants).

**Chromatographic analysis of the starch hydrolysis products**
The reaction products produced by the enzyme for soluble potato starch were identified using high performance liquid chromatography (HPLC). The enzyme was incubated with a 1% soluble starch final concentration at pH 7.0 and 70 °C. Sugars produced by the enzyme after hydrolysis of starch were identified by HPLC (Prominence, SHIMADZU), eluted with 80% acetonitrile at 25 °C as the mobile phase.

**Substrate specificity**
0.3 mL containing 2.0% (w/v) different substrates solutions (potato starch, corn starch, wheat starch, glycogen, amylopectin, amylose, cellulose or β-cyclodextrin) in 50 mM MOPS buffer (pH 7.0) were mixed with 0.3 mL of suitably diluted

enzyme solution and then the enzyme assay was carried out at 70 °C for 20 min.

**Kinetic constants**

Initial rate measurements with purified amylase at pH 7.0 at 70 °C with increasing substrate concentration for potato starch, amylopectin and amylose as a substrate were performed to determine the kinetic parameters such as maximum reaction rate ($V_{max}$) and Michaelis–Menten constant ($K_m$). The kinetic parameters were estimated from the Lineweaver–Burk equation plot.

## RESULTS AND DISCUSSION

### Effect of various media on bacterial growth and enzyme production

The growth of *Anoxybacillus thermarum* A4 microorganism and the production of α-amylase depended on the type of growth medium and incubation time has been described by numerous authors for microbial α-amylase synthesis. The present research demonstrated that medium 3 supported maximum extracellular amylase production at pH 7.0 for 48 h of incubation, demonstrating that this medium influenced the α-amylase synthesis machinery in the bacteria, as shown in Figure 1A and B. Potato starch was determined to be a very good substrate for the induction of extracellular α-amylase secretion in medium by *A. thermarum* A4.
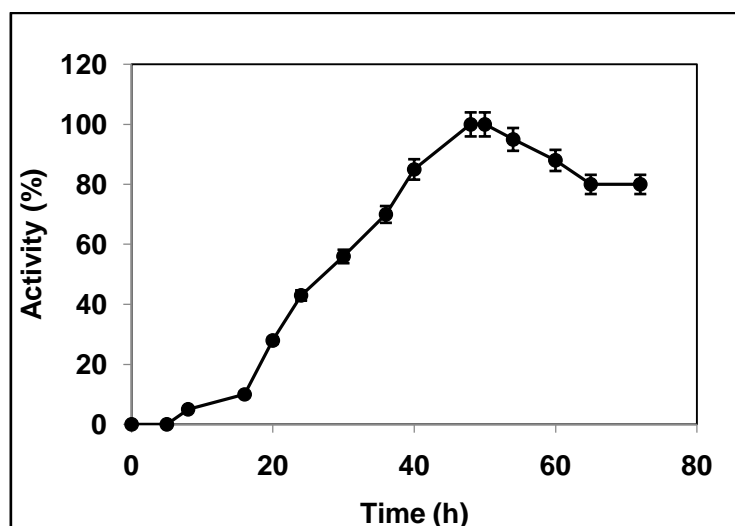


(A)
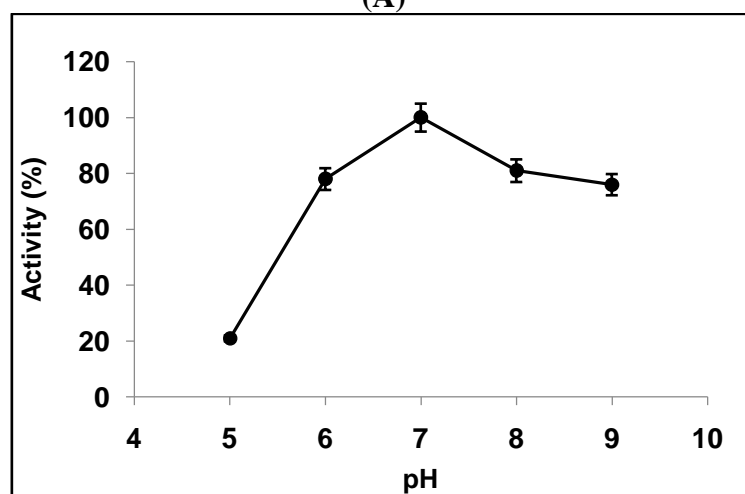


(B)

**Figure 1-** Effect of time (a) and pH (b) on α–amylase production in medium 3 (1.0% potato starch; 0.5% triptone; 1.0% NaCl; 0.1% CaCl$_2$ and 0.25% Mg$_2$SO$_4$.7H$_2$O)

**Purification of α-amylase**

Extracellular α-amylase from *Anoxybacillus thermarum* A4 strain was purified to homogeneity by 40-80% ammonium sulfate precipitation and Sephadex-G 100 gel filtration chromatography as described in Section 2. In the first step, the culture supernatant was precipitated with ammonium sulphate. No activity was detected in the 0-40% saturated supernatant and the final supernatant. The fraction (40-80%) which had maximum α-amylase activity was subjected to gel filtration on a Sephadex G-100 column. The gel filtration chromatography Sephadex G-100 is shown in Figure 2. This procedure yielded a single peak of α-amylase activity. Fractions (fraction number of 30-43) containing α-amylase activity were pooled and used as a pure enzyme source. The results of the purification steps are summarized in Table 1.

The purified enzyme exhibited 74.6% yield of the total initial activity with 29.8-fold purification and a specific activity of about 1203.7 U/mg. The molecular mass of the protein showing purified amylase was estimated about 50 kDa, a single band based on SDS-PAGE (Fig. 3C).

The fold purification and yields were comparable to those reported previously; for example, extracellular α-amylase of *Lactobacillus plantarum* A6 (50-70%) was purified to 35.6% yield with 19.5-fold purification by 40-80% ammonium sulfate precipitation and diethyl aminoethyl anion exchange chromatography[14]. Hmidet et al. have reported 15.3-fold purification and 11% yield in case of extracellular *Bacillus mojavensis* A21 α-amylase (50-70%) by steps of gel filtration and ion exchange chromatography[15].
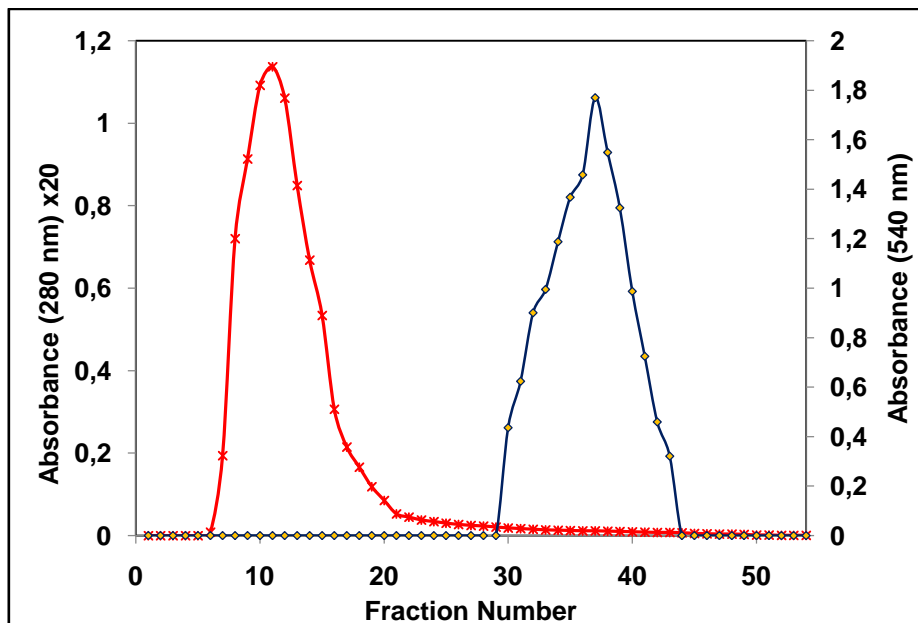


**Figure 2-** Elution profile of sephadeks G-100 column chromatography. Symbols: (♦) Amylase activity at 540 nm; (∗) OD at 280 nm. Column 3x60 cm; flow rate: 0.5 ml/min.
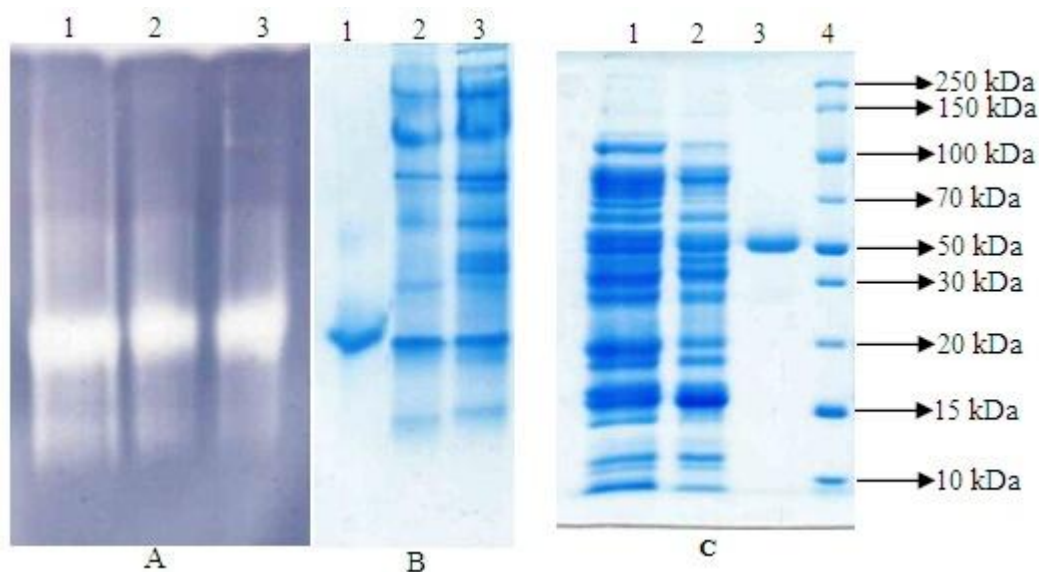
**Figure 3-** Polyacrilamide gel electrophoresis of the purified α−amylase from *Anoxybacillus thermarum A4*. (A) Zymography of purified enzyme. Lane 1: purified enzyme; Lane 2: Precipitate (40-80)% As and concentration with Amicon filter; Lane 3: crude enzyme extract. (B) Native SDS-PAGE. Lane 1: purified enzyme; Lane 2: Precipitate (40-80)% As and concentration with Amicon fitler; Lane 3: crude enzyme extract, (C) SDS-PAGE. Lane 1: crude enzyme extract; Lane 2: Precipitate (40-80)% As and concentration with Amicon fitler; Lane 3: purified enzyme; Lane 4: marker proteins for the determination of molecular weight of the enzyme

**Table 1 -** Summary of the purification of extracellular α−amylase from *Anoxybacillus thermarum* A4

|  | Total Volume (mL) | Total Protein (mg) | Total Activity (U) | Spesific activity (U/mg protein) | Purification fold | Yield (%) |
|---|---|---|---|---|---|---|
| **Crude enzyme extract** | 9900 | 538.6 | 21780 | 40.4 | 1 | 100 |
| **Concentraion with Amicon filter after precipitate (40-80)% As[a]** | 15 | 92.25 | 16375 | 177.5 | 4.4 | 75.2 |
| **Sephadex G-100** | 15 | 13.5 | 16250 | 1203.7 | 29.8 | 74.6 |

## Zymography of the enzyme and SDS-PAGE

In order to determine the amylolytic activity, the purified protein was run on native polyacrylamide gel (10%) including 1% (w/v) soluble starch. For activity staining, the gel containing nondenaturing protein was placed into a petri dish containing an iodine solution for 10 min. The appearance of a hoary zone, where the starch had been degraded on the native gel, verified the existence and the location of the purified enzyme having amylolytic activity (Fig. 3A). SDS-PAGE profile of the purified α-amylase revealed a single protein band of the same mobility, suggesting that the enzyme consisted of a single polypeptide chain. When the relative molecular weight of the α-amylase was estimated by comparison with molecular mass standard marker proteins, the molecular mass of

the protein showing α-amylase activity was determined about 50 kDa, based on SDS-PAGE (Fig. 3B). The masses of α-amylases from various microbial sources showed variety from 10 to 210 kDa although the majority were within the range 50-70 kDa[16]. However, molecular masses of 28, 22.5, 23.5, 60 and 27 kDa, have also been reported from *B. licheniformis* CUMC 305[17], *B. licheniformis* 584[18], *B. licheniformis* BLM1777[19], *Anoxybacillus flavithermus*[20] and *B. cereus* Ms6[21], respectively.
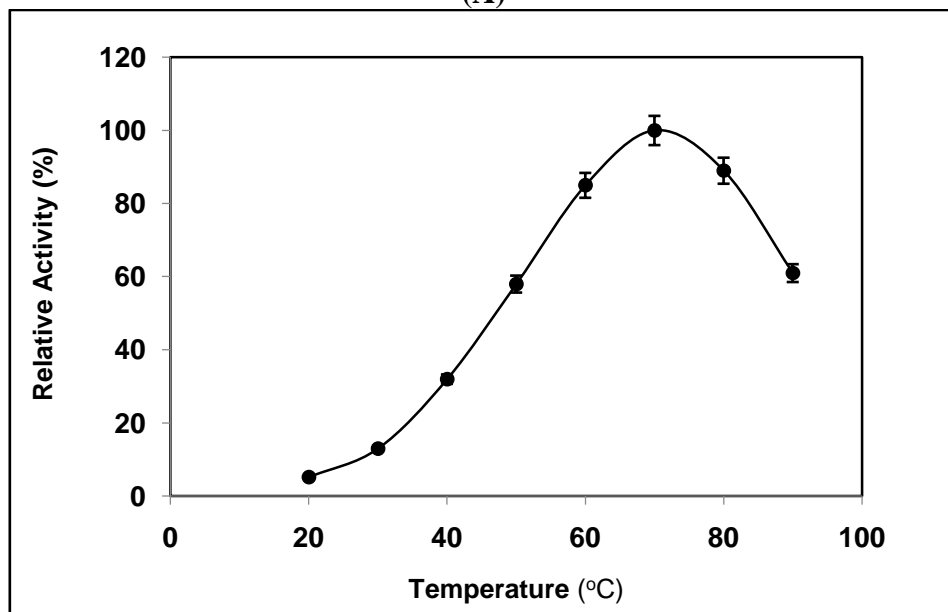
## Effect of pH and temperature on the enzyme activity

The optimal pH for the α-amylase activity was determined a broad range of pH activity from 5.5 to 10.5 in the presence of soluble potato starch as a

substrate (Fig. 4A). This finding confirmed the previously reported results for *Bacillus* strain HUTBS71 (pH 5.2-10) [22] and *B. licheniformis* NH 1 (pH 5-10)[23]. To determine effect of the temperature on *A. thermarum* A4 α-amylase activity was assayed over a range from 20 °C to 90 °C (Fig. 4B). The results showed that the purified enzyme exhibited optimum activity at 70 °C which is similar or very close to other α-amylase reported from *Bacillus* sp. *Ferdowsicous* (70 °C)[24], *B.*

*mojavensis* A21[15], *Geobacillus* sp. LH8[25], *B. stearothermophilus* ATCC 12980[26] and *Bacillus* sp. KR-8104 (80 °C)[8]. The enzyme retained 60% of its maximum activity at 90 °C (Fig. 4B). In addition, optimum temperature of 90 °C has also been reported for α-amylase *B. licheniformis* NH 1[23]. The broad range of pH activity profile and activity profile at 90 °C therefore make this enzyme highly attractive for both basic research and industrial processes.
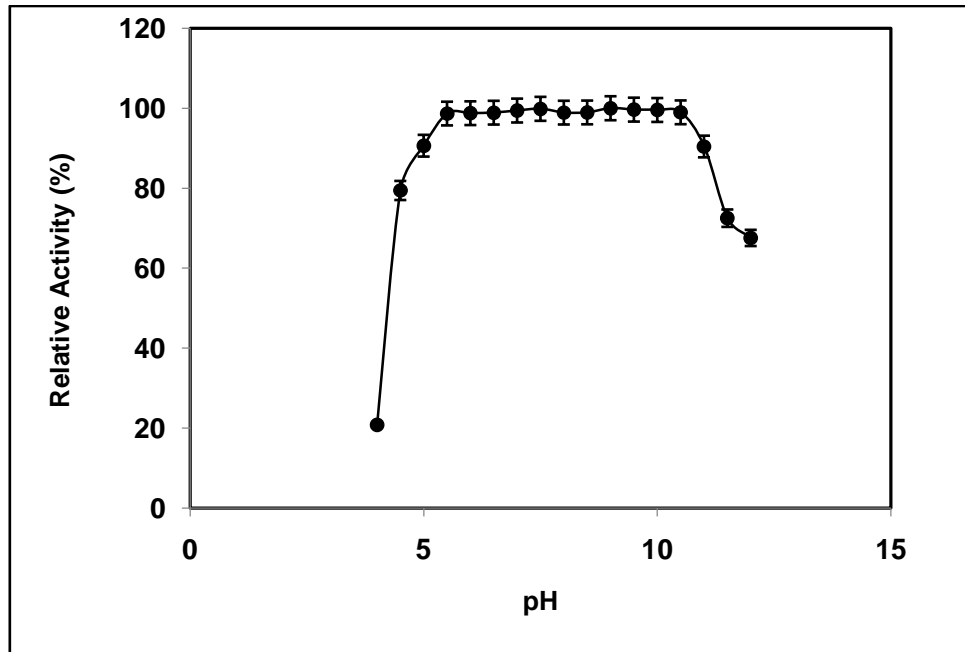
**(A)**

**(B)**

**Figure 4-** pH-activity and optimum temperature profile on α-amylase activity. Effect of pH on enzyme activity in the presence of soluble patato starch as a substrate was assayed at different pH values range from 3.0 to 9.0 using 50

mM (Acetate (pH 4.5–5.5), (MOPS (pH 6.5–7.5) and Tris–HCl (pH 8.5–9.5)) buffers (A). The activity, as a function of temperature, was examined under standard assay conditions, in the range of 20–90 °C (B)

**pH and thermal stability**

In order to evaluate the pH-stability of α-amylase, purified enzyme mixed buffers having different pH values (4.5–9.5) were incubated at 4 °C, 50 °C, 70 °C and 90 °C for up to 10 days, and the enzyme activity was assayed as described above (Fig. 5A, B, and C). The enzyme was significantly stable at all pH values except for pH 4.5 at 4°C, and maintained almost all of its original activity after a 10-day incubation period. However, α-amylase retained 80% of its original activity when incubated at 4 °C for 10 days at pH 4.5. After 10 days' incubation at 90 °C, the incubation temperature used in the starch industry, the

enzyme retained 40% of its original activity at all pH values.

In terms of thermal stability for evaluation of α-amylase, the experiments were carried out at 4 °C, 55 °C, 70 °C and 90 °C for up to 10 days. The thermal stability profile for the purified enzyme, presented in the form of residual activity, is shown in Fig. 5d. After one-day incubation, no loss of original activity was observed at nearly all investigated temperatures. The α-amylase lost 10% of its original activity when incubated at 55 °C at the end of 10-days' incubation. A4 amylase maintained 43% of its original activity at 90 °C, the incubation temperature of the enzyme in the starch industry, at the end of 10 days.



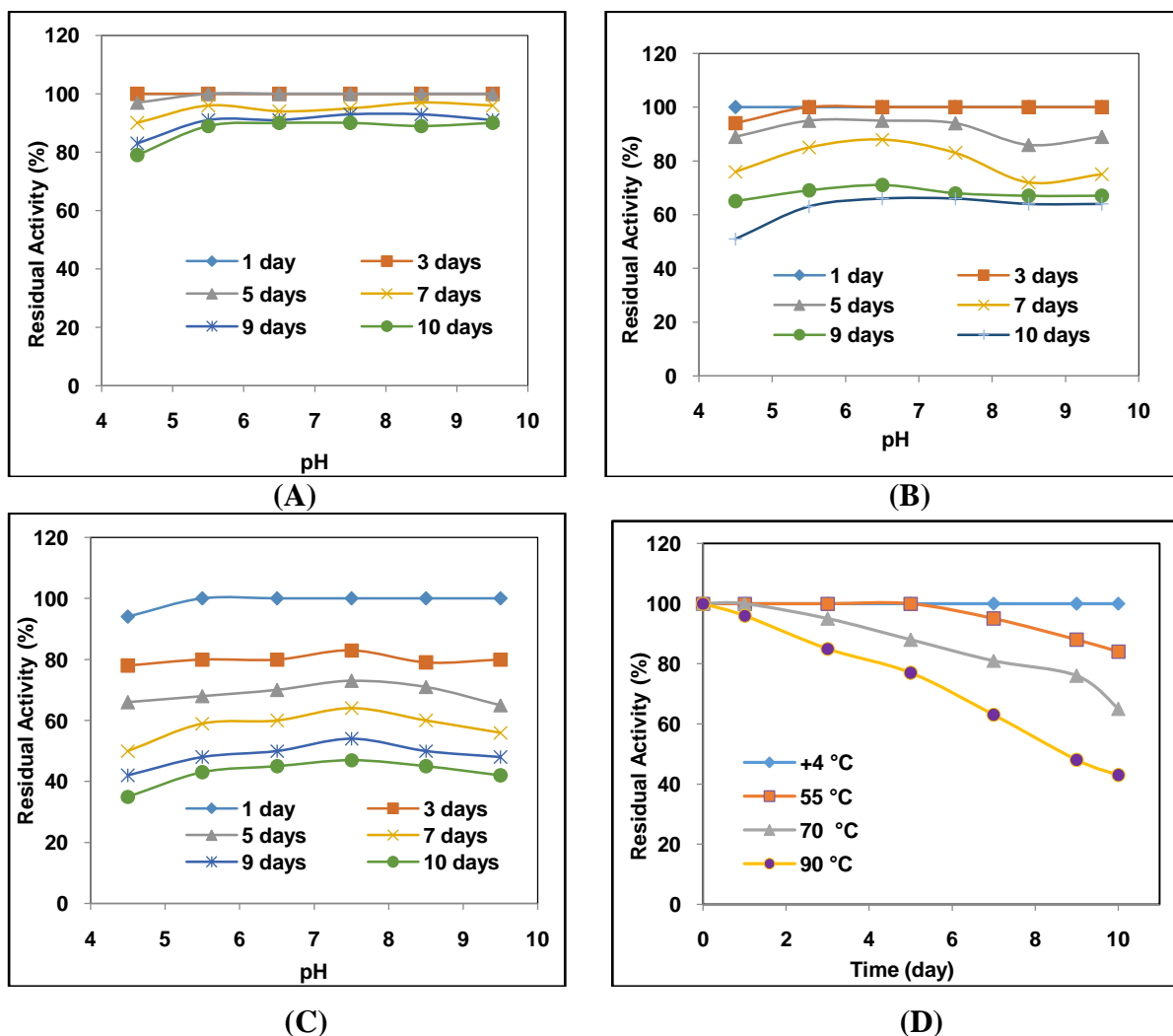**(A)**



**(B)**



**(C)**



**(D)**

**Figure 5-** Effect of pH stability at different temperatures (A, B, C) of the purified amylase. The enzyme activities were assayed as described in Section 2. (A) pH stability at 55 °C; (B) pH stability at 70 °C; (C) pH stability at 90

°C. (D); The remaining enzyme activity was measured after incubation of the enzyme solution in 50 mM MOPS buffer (pH 7.0) at 4 °C, 55 °C, 70 °C and 90 °C for up to 10 days

**Effects of metal ions, anions, enzyme inhibitors and chemical reagents**

The effects of various cations on enzyme activity were assessed at final concentrations of 1 mM, 5 mM, 10 mM and 20 mM (see Table 2). While the activity of purified α-amylase was slightly inhibited by $Ni^{2+}$, $Mg^{2+}$ and $Hg^{2+}$ at levels of 2.3%, 26.2% and 49%, respectively, $Mn^{2+}$, $Ca^{2+}$, $Cu^{2+}$, $Ba^{2+}$, $Co^{2+}$ and $Zn^{2+}$ increased the activity (by 48%, 29%, 24%, 6%, 17% and 3%, respectively) at a final concentration 5 mM. $IC_{50}$ value was 5.75 mM for $Hg^{2+}$. Changes in enzyme activity at other concentrations are shown in Table 2.

The enzyme activity was inhibited by 17%, 35%, 67% and 73% respectively at final concentrations of 1, 5, 10 and 20 mM EDTA. $IC_{50}$ value was 8 mM for EDTA. Among all the inhibitors tested, the chelating agent EDTA inactivated the enzyme, indicating that A4 amylase is a metalloenzyme[23] (see Table 2). These findings are in line with earlier reports showing that calcium cation is essential for enzyme folding[27,] *Aspergillus oryzae* strain S2[28,] *Bacillus stearothermophilus*[29]. The effect of EDTA alkaliphilic *Bacillus* species varies considerably, some being unaffected at EDTA concentrations as high as 100 mM[30], while others were completely inhibited in the presence of low EDTA concentration, e.g. the α-amylase of *Bacillus* sp. IMD370 was completely inhibited by 1 mM EDTA[31].

**Table 2 -** Effects of some metal ions on the α–amylase activity

| Metal ions | Remaining activity (%) | | | |
|---|---|---|---|---|
| | 1 mM | 5 mM | 10 mM | 20 mM |
| None | 100 | 100 | 100 | 100 |
| $Mn^{2+}$ | 132 | 148 | 156 | 175 |
| $Ca^{2+}$ | 120 | 129 | 134 | 141 |
| $Cu^{2+}$ | 121 | 124 | 125 | 132 |
| $Ba^{2+}$ | 102 | 106 | 112 | 130 |
| $Co^{2+}$ | 100 | 117 | 138 | 148 |
| $Zn^{2+}$ | 100 | 103 | 107 | 114 |
| $Fe^{2+}$ | 95.94 | 102 | 119 | 123 |
| $Ni^{2+}$ | 95.61 | 98.67 | 114 | 117 |
| $Mg^{2+}$ | 95.03 | 74.83 | 70.33 | 65.98 |
| $Hg^{2+}$ | 66.31 | 51.3 | 42.4 | 37.3 |
| EDTA | 82.9 | 64.8 | 43.1 | 27 |

The effects of various anions on enzyme activity were assessed in final concentrations of 1, 5, 10 and 20 mM (see Table 3). The activity of purified α-amylase was slightly inhibited by $HCO_3^-$, $HPO_4^{-2}$, $HSO_3^-$, $WO_4^{2-}$ and $C_8H_{11}N_2O_3^-$ at a final concentration 10 mM. $IC_{50}$ values of *A. thermarum* A4 amylase for $B_4O_7^{-2}$, $OH^-$, $CN^-$ and urea inhibitors were determined as 16.5, 15.2, 8.2 and 10.9 mM respectively. Changes in enzyme activity at other concentrations are shown in Table 3.

**Table 3 -** Effects of some anions on the α-amylase activity

| Anions | Remaining activity (%) | | | |
|---|---|---|---|---|
| | 1 mM | 5 mM | 10 mM | 20 mM |
| None | 100 | 100 | 100 | 100 |
| $ClO_4^-$ | 100 | 100 | 100 | 100 |
| $Cl^-$ | 100 | 100 | 100 | 100 |
| $Ac^-$ | 100 | 100 | 100 | 100 |
| $NO_3^-$ | 100 | 100 | 100 | 100 |
| $SO_4^{-2}$ | 102 | 104 | 105 | 106 |
| $N_3^-$ | 98.98 | 89.94 | 100 | 100 |
| $C_2O_4^{-2}$ | 110 | 106 | 102 | 101 |
| $HCO_3^-$ | 103 | 101 | 95.16 | 88.71 |
| $HPO_4^{-2}$ | 100 | 97.66 | 94.27 | 90.97 |
| $HSO_3^-$ | 99.58 | 97.90 | 95.96 | 89.11 |

| | | | |
|---|---|---|---|
| $WO_4^{2-}$ | 98.77 | 95.24 | 93.46 | 83.22 |
| $C_8H_{11}N_2O_3^-$ | 99.1 | 94.75 | 92.77 | 82.5 |
| $SO_3^-$ | 99.19 | 97.18 | 85.40 | 71.53 |
| $CO_3^{-2}$ | 98.56 | 92.82 | 81.37 | 60.48 |
| $PO_4^{-3}$ | 96.4 | 80.56 | 72.58 | 58.62 |
| $B_4O_7^{-2}$ | 97.1 | 81.93 | 61.69 | 43.54 |
| $OH^-$ | 98 | 91.20 | 65.73 | 37.1 |
| $CN^-$ | 97.75 | 73.79 | 40.16 | 5.65 |
| $N_2H_4CO$ | 93.9 | 67.2 | 52.1 | 23.41 |

**Stability in the presence of solid-liquid detergents and denaturing agents**

Stability was also investigated by incubating the enzyme in the presence of SDS, Tween 20, Triton X-114, Tween-80 and Triton X-100 as surfactants for 20 min at 40 °C. As shown in Table 4, α-amylase appears to be quite stable in the presence of the non-ionic surfactants such as Tween 20, Triton X-114, Tween-80 and Triton X- 100. In addition, the enzyme was highly stable in the presence of the strong anionic surfactant SDS, retaining approximately 96% of its initial activity when incubated in the presence of 2% SDS. α-Amylase was also relatively stable towards oxidizing agents, retaining 62% of its activity after 20-min incubation in the presence of 10 mM sodium perborate.

**Table 4 -** Stability of the α–amylase from *Anoxybacillus thermarum* A4 in the presence of denaturing agents, various commercial solid and liquid detergents

| Denaturing agents, various commercial solid and liquid detergents | Remaining activity (%) | | |
|---|---|---|---|
| | 0.5 % | 1 % | 2 % |
| None | 100 | 100 | 100 |
| Triton X-114 | 113 | 114 | 115 |
| Triton X-100 | 109 | 107 | 104 |
| Tween-80 | 100 | 100 | 100 |
| Tween-20 | 100 | 100 | 100 |
| SDS | 100 | 98 | 95.4 |
| Liquid dishwashing machine detergent | 100 | 100 | 100 |
| Liquid hand dishwashing detergent | 100 | 98 | 95.6 |
| Liquid laundry detergent | 100 | 99 | 97 |
| Solid laundry detergent | 98.3 | 95.8 | 92.7 |
| Spot remover | 100 | 98.2 | 96 |

Solid detergents and diluted tap water were used at final concentrations of 0.5, 1 and 2% (w/v) (to stimulate washing conditions). The data presented in Table 4 demonstrate that the enzyme is extremely stable in the presence of commercial solid and liquid washing machine detergents. Additionally, the enzyme was highly stable in the presence of commercial liquid washing machine detergents.

The results clearly indicate that enzyme performance is dependent on the nature of the laundry detergent. Considering the high stability of α-amylase in the presence of anionic surfactant, such as SDS, and its stability in the presence of oxidizing agents, such as sodium perborate, and various commercial laundry detergents[23]. *A.*

*thermarum* A4 amylase appears to represent a promising candidate for the detergent industry

**Chromatographic analysis of the starch hydrolysis products**

HPLC analysis indicated that *A. thermarum* A4 amylase hydrolyzed the substrate mainly to glucose, and as do most of the α-amylases.

**Substrate specificity**

Among the tested substrates, purified α-amylase exhibited significant activity towards amylose (113% relative to that for soluble potato starch). Relative activities of corn starch, wheat starch, amylopectin and glycogen to that of soluble potato starch were observed to be 87%, 89%, 93% and 50%, respectively. No hydrolytic activity for

cellulose or β-cyclodextrin was observed (Table 5). Therefore, *A. thermarum* A4 strain amylase has greater affinity for hydrolysis of amylose than for the other α-glycosides.

**Table 5 -** Substrate specificity of the purified enzyme toward various substrates.

| Substrate | Activity (U) | Relative Activity (%) |
|---|---|---|
| Amylopectin | 115.8 | 93 |
| Amylose | 140.8 | 113 |
| Potato starch | 124.9 | 100 |
| Corn starch | 108.6 | 86.9 |
| Wheat starch | 110.8 | 88.7 |
| β-Cyclodextrin | - | - |
| Glycogen | 62.6 | 50 |
| Cellulose | - | - |

**Kinetic constants**

Kinetic investigations were carried out under standard conditions. $K_m$ values of A4 amylase for soluble starch, amylopectin and amylose substrates were determined as 0.9, 1.3 and 0.5 mg/mL, respectively, using the Lineweaver-Burk curve. $V_{max}$ values of A4 amylase for the same substrates

were determined to be 833.3, 1000 and 769 U/mg protein, respectively. $K_m$ values of *Bacillus flavothermus* α-amylase [32], *B. stearothermophilus* ATCC12980 α-amylase [25] and *Anoxybacillus flavithermus*[20] for soluble potato starch as substrate were found as 2.2, 14 mg/mL and 0.005 mM respectively.

$K_m$ values of *B. licheniformis* CUMC 305 α-amylase[17], *Lactobacillus plantarum* A6 extracellular α-amylase[14], *B. subtilis* KIBGE HAS extracellular α-amylase[33] and *B. subtilis* ITBCCB148 extracellular α-amylase[34] in the presence of soluble potato starch substrate were defined as 1.274 mg/mL, 2.38 mg/mL, 2.68 mg/mL, and 2.5 mg/mL, respectively. *A. thermarum* A4 amylase exhibited a lower $K_m$ value than other extracellular α-amylases. The enzyme exhibited lower $K_m$ (higher affinity) for starch compared to some other reported amylases. This feature is a great advantage for the starch industry in terms of starch hydrolysis time. The $K_{cat}/K_m$ values obtained (Table 6) demonstrated that the preferred substrates were, in order, amylose, potato starch (sigma) and amylopectin.

**Table 6 -** Kinetic parameters of the α-amylase produced by *Anoxybacillus thermarum* A4.( The enzyme was incubated with 0–20 mg mL$^{-1}$ soluble starch, amylopectin or amylose in 50 mM MOPS buffer (pH 7.0 at 70 °C)

| | $V_{max}$ (U/mg protein) | $K_M$ (mg/mL) | $k_{cat}$ (s$^{-1}$) | $K_{cat}/K_M$ (mLs$^{-1}$mg$^{-1}$) |
|---|---|---|---|---|
| Potato starch | 833.3±3.7 | 0.9±0.08 | 694.4 | 771.6 |
| Amylose | 769±2.4 | 0.5±0.04 | 640.8 | 1281.7 |
| Amylopectin | 1000±4.5 | 1.3±0.12 | 833.3 | 641.0 |

**CONCLUSION**

This study describes the purification and characterization of a novel thermo- and pH-stable α-amylase from the *A. thermarum* A4 strain. The enzyme was purified 29.8-folds with specific activity of 1203.7 U/mg by using 40-80% ammonium sulfate precipitation and Sephadex G-100 gel filtration chromatography. The purified enzyme showed a single band on SDS-PAGE and molecular weight of it was estimated about 50 kDa. The optimum temperature was determined as 70 °C for amylolytic activity. In addition to being highly active and stable at a low pH, the enzyme demonstrates a considerable stability at a broad range of pH values. These properties are very important in the starch industry and are lacking in the most of α-amylases published to date[35].
IC$_{50}$ values of EDTA, Hg$^{2+}$, B$_4$O$_7$$^{-2}$, OH$^-$, CN$^-$ and urea as inhibitors for α-amylase from the *A.*

*thermarum* A4 strain were determined to be 8.0, 5.75, 16.5, 15.2, 8.2 and 10.9 mM, respectively. While the activity of purified α-amylase was slightly inhibited by Ni$^{2+}$, Mg$^{2+}$, Hg$^{2+}$, Mn$^{2+}$, HCO$_3$$^-$, HPO$_4$$^{2-}$, HSO$_3$$^-$, WO$_4$$^{2-}$ and C$_8$H$_{11}$N$_2$O$_3$$^-$, it was activated by Ca$^{2+}$, Cu$^{2+}$, Ba$^{2+}$, Co$^{2+}$ and Zn$^{2+}$. α- amylase was highly stable in the presence of non-ionic surfactants (such as Tween 20, Triton X-114, Tween-80 and Triton X- 100), strong anionic surfactant SDS, the commercial liquid washing machine detergents and various commercial laundry detergents.

**DECLARATION OF INTEREST**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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