RESEARCH ARTICLE

Production, purification, and characterization of thermostable α-amylase from thermophilic *Geobacillus stearothermophilus*

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The α-amylase (α-1-4-D-glucan glucanohydrolase; EC 3.2.1.1) secreted by *Geobacillus stearothermophilus* was purified and characterized. Maximum enzyme production was achieved after 24 h cultivation at pH 7.0 and 55°C. The enzyme was active in a broad temperature range, between 50 and 80°C, with an optimum at 70°C; and maximum activity was at pH 7.0. The enzyme was purified using 80% ammonium sulfate precipitation, dialysis, Sephadex G-100 gel filtration, and DEAE-cellulose column chromatography, with a 46-fold and 65% recovery and showed a MW of 63 kDa by SDS-PAGE. It was determined that the purified enzyme was stable at 50 and 60°C, and pH 7.0. It was determined that the purified enzyme was stable at 50 and 60°C at the end of 2 h. The enzyme retained 100% activity pH 7.0 at the end of 3 h. The enzyme was activated by Ca^{2+}, Mn^{2+}, and Triton X-100, but strongly inhibited by Cu^{2+}, Zn^{2+}, Fe^{2+}, and Hg^{2+}. The enzyme follows Michaelis–Menten kinetics with K_m and V_{max} values of 0.051 mM and 1.424 μmol/min, respectively.

Keywords:
α-Amylase / *Geobacillus stearothermophilus* / Production / Purification

1 Introduction

Amylases (1,4-α-D-glucan-4-glucanohydrolase, EC 3.2.1.1) are extracellular endoenzymes that randomly hydrolyze starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units [1]. Amylases also play a significant role in starch, detergent, beverage, and textile industries and its commercial production from microorganisms represent 25–33% of the world enzyme market [2].

Thermostable α-amylases have had extensive commercial applications in brewing, baking, sugar production, paper industry, desizing in textile industries, and in detergent manufacturing processes [3]. Production of thermostable amylases is of special interest as they could be used for saccharification processes occurring at high temperatures [4]. Thermophilic fermentation is also considered quite useful for technical and environmental purposes. The advantages are, for instance, a reduction in cooling costs, a better solubility of substrates, a lower viscosity allowing accelerated mixing and pumping, and reduced risk of microbial contamination. Furthermore, they are resistant to denaturing agents, solvents, and proteolytic enzymes [5]. Thus, these properties make them extremely attractive for industrial processes.

α-Amylases from genus *Bacillus* have been extensively studied in terms of structure, function, secretion, and industrial applications [6, 7]. Some bacterial α-amylases can hydrolyze raw starch granules, notably the enzymes from certain strains of *Geobacillus thermodenitrificans HRO10*, *Geobacillus caldoxylosilyticus TK4*, and *Geobacillus sp. LH8* [8–10].

In the present study, we report the optimum conditions for the production, purification, and characterization of extracellular α-amylase from thermophilic *Geobacillus stearothermophilus*.

2 Materials and methods

2.1 Materials

DEAE-Cellulose DE-32, Sephadex G-100, and DNS (3,5-dinitrosalicylic acid) were purchased from Sigma (Sigma–Aldrich, USA). All culture media were commercially

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purchased from Merck (Merck & Co., Inc.). All other chemicals used were of analytical grade.

2.2 Microorganisms

Geobacillus stearothermophilus (DSM 5934 and ATCC 7953) was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen).

2.3 Growth and culture conditions

The microorganism was grown at 55°C in nutrient broth (NB; peptone 5.0, meat extract 3.0) and the pH was adjusted to 7.0 before autoclaving. Amylase production was carried out by inoculating 25 mL of the medium in a 100 mL flask with 2 mL of a 1-day-old culture. Cultures were incubated at 55°C on a shaker at 120 rpm. The cultures were centrifuged at 10 000 rpm for 10 min at 4°C, and the cell-free supernatants were used for estimation of α-amylase activities. Bacterial growth was determined by measuring OD at 460 nm in a spectrophotometer (UV-6450; Jenway, UK).

2.4 Enzyme assay

α-Amylase activity was assayed by adding 0.1 mL of enzyme to 0.2 mL soluble starch (0.5% w/v) in 0.1 M Tris-HCI buffer, pH 7.0, for 30 min at 70°C. The reaction was stopped and the reducing sugars determined with dinitrosalicylic acid (DNS) according to the method of Bernfeld [11]. One unit of amylase activity was defined as the release of 1 μmol of reducing sugar per min at 70°C and a pH of 7.0.

2.5 Protein determination

The protein concentration was determined by the method of Lowry [12] using BSA as standard.

2.6 Effect of pH, temperature, $CaCl_2$, carbon, and nitrogen sources on amylase production

The effect of time, temperature, and pH on enzyme production was investigated by cultivating the organism at different times (4–48 h), different temperatures (25–80°C), and different pHs using appropriate buffers at a concentrations of 0.1 M (4.0–6.0, sodium citrate; 7.0–9.0, Tris–HCl; 10.0 glycine-NaOH).

The effect of various concentrations (10–60 mM) of $CaCl_2$ on enzyme production was tested in the NB medium at 55°C in a shaker and 120 rpm for 24 h.

G. stearothermophilus was grown in medium containing different carbon and nitrogen sources at a concentration of 1% w/v, in order to study their effect on enzyme production. Various carbon sources such as glucose, lactose, maltose, sucrose, galactose, fructose, and soluble starch were evaluated for their effect. Different nitrogen sources examined were peptone, tryptone, sodium nitrate, beef extract, yeast extract, ammonium chloride, ammonium nitrate, ammonium sulfate, corn steep liquor, and urea.

2.7 Effect of pH and temperature on α-amylase activity

Soluble starch (0.5%) was prepared in 50 mM buffers at different pHs. Sodium citrate buffer, Tris-HCl buffer, and glycine-NaOH buffer were used for pH 4.0–6.0, 7.0–9.0, and 10.0–11.0, respectively.

The enzyme–substrate mixtures were then incubated at various temperatures (35–90°C) for 30 min and residual activity was measured under standard assay conditions.

2.8 Purification of α-amylase

Ammonium sulfate was added to the crude culture supernatant to 80% saturation. After centrifugation, the precipitates were dissolved in a minimum volume of 0.1 M potassium phosphate buffer (pH 7.0), and dialyzed overnight against the same buffer. The dialyzed crude enzyme was applied to Sephadex G-100 column on previously equilibrated with 5 mM acetate buffer (pH 7.0), and eluted with the same buffer containing 0.1 M NaCl at a flow rate of 15 mL/h. The active fractions were pooled, concentrated, and dialyzed.

The dialyzed crude enzyme was applied on a DEAE-cellulose (DE 32) column (5 cm × 20 cm glass column, flow rate 15 mL/h) equilibrated with 0.01 M phosphate buffer (pH 7.0). The protein was then eluted with a linear gradient of NaCl (0.1–1 M) in the same buffer. The active fractions were pooled, dialyzed, and lyophilized. This purified enzyme was used for further biochemical characterization.

2.9 Electrophoresis

The MW of the purified α-amylase was estimated by SDS–PAGE according to the method of Laemmli [13]. The purified enzyme was loaded onto 0.75 mm thick 10% polyacrylamide gel together with molecular size markers. After completion of electrophoresis, the gel was stained with CBB R-250.

Discontinuous native-PAGE was done in 10% acrylamide gel with Bio-Rad mini gel electrophoresis. All the buffer system used for native-PAGE was prepared without SDS. The gel was stained with iodine reagent (KI-I_2 solution). One half of the gel was incubated in 3% soluble starch at 37°C for 30 min. Then, the gel was washed with distilled water, and stained with a solution containing 0.1% I_2 in 1% KI.

2.10 Determination of kinetic parameter

The kinetic parameters V_{max} and K_m were determined by incubating the amylase with various concentrations of soluble

starch ranging from 0.1 to 0.7% w/v in buffer at pH 7.0 for 30 min at 70°C. V_{max} and K_m values were obtained from Lineweaver–Burk plot.

2.11 Effect of pH and temperature on amylase stability

Stability of the enzyme at different pH values was determined by measuring the residual activity after incubating the enzyme at pH 6.0–8.0 for 3 h at optimum temperature.

In the testing of thermal stability were incubated at various temperatures ranging from 50 to 70°C for 15–120 min in 0.1 M Tris-HCI buffer, pH 7.0 and residual activity was measured under standard assay conditions.

2.12 Effect of metal ions and inhibitors on amylase activity

Enzyme assays were performed in presence of different metal ions, all at 1.5 mM final concentration. All metals were used as chloride. The activity of the enzyme alone in 0.1 M Tris-HCI buffer was taken to be 100%. The effect of metal ions and inhibitors (EDTA, PMSF, β-mercaptoethanol, and DTT) on amylolytic activity was determined by pre-incubating the enzyme in the presence of inhibitor for 30 min at 37°C, and then performing the assay in the presence of the same inhibitor concentration at optimum temperature for 30 min.

2.13 Effects of detergents on amylase activity

The effect of some surfactants (SDS, Tween-40, Tween-80, and TritonX-100) on enzyme stability was studied by preincubating the enzyme for 30 min at 37°C. The residual activity was measured at pH 7.0 and 70°C. The activity of the enzyme without any additive was taken as 100%.

3 Results and discussion

3.1 Effect of time, pH, temperature, calcium, carbon, and nitrogen sources on amylase production

The extracellular α-amylase production of the bacterium was studied through SmF (Submerged fermentation) method. In our study *G. stearothermophilus* showed maximum α-amylase yield at pH 7.0 (4125 U/mg) (Fig. 1). Asgher reported the optimum pH 7.0 for α-amylase production from *Bacillus subtilis* JS-2004 [1]. Similar results were also reported by Hamilton et al. and Konsoula and Liakopoulou-Kyriakides [14, 15].

Maximum enzyme production was achieved at 55°C (5444 U/mg) (Fig. 2). Temperature optima for activity of *B. stearothermophilus* α-amylases are generally in the range of 50–70°C [16]. Lin et al. (1998) similarly observed that

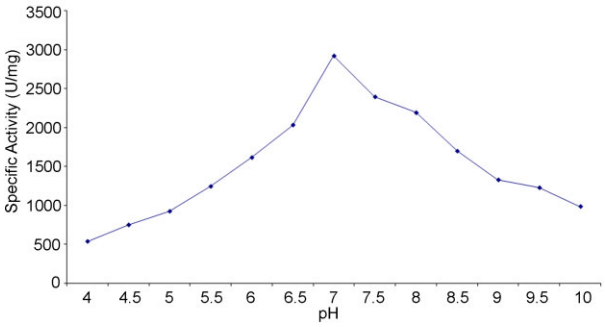

Figure 1. Effect of pH on enzyme production.

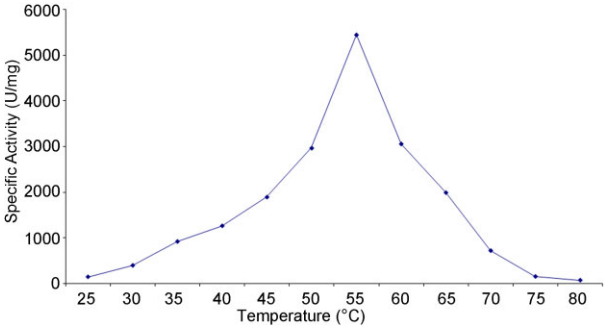

Figure 2. Effect of temperature on enzyme production.

Bacillus sp. TS-23 had production optimum at 55°C [17]. A wide range of temperature (35–80°C) has been reported for α-amylase production in bacteria [15, 18].

The highest α-amylase production was obtained in the 24th hour at optimum pH 7.0 and optimum temperature (2429 U/mg) (Fig. 3). Since it is important to produce enzyme in a short time in the field of biotechnology, therefore a 24-hour incubation time is as well important in terms of biotechnology. Burhan et al. reported maximum amylase production from *Bacillus* sp. ANT-6 after 24 hrs [19].

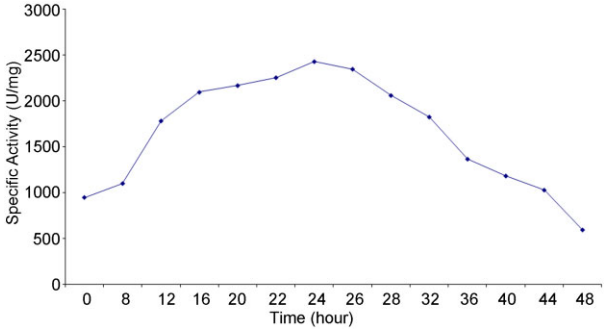

Figure 3. Effect of time on enzyme production.

Similar findings have been reported on *Bacillus amyloliquefaciens* and *Bacillus flavothermus* [18, 20]. Reaching highest amylase production from *G. stearothermophilus* after 24 hours provides economical advantages.

The effect of various concentration of $CaCl_2$ on the enzyme production was examined. Maximum enzyme production was achieved in the presence of 10 mM $CaCl_2$. A decline in amylase production in parallel with an incline in $CaCl_2$ concentration was found. According to this result, it cannot be concluded that $CaCl_2$ increases enzyme production because it is considerable that Ca^{+2} ion creates a stable situation on α-amylase and an increase is possible only by this or there is an increased amount of enzymes in the media because enzyme is released within the cell and then emitted outside the cell. In the presence of 5 mM Ca^{+2} an increase of enzyme activity was reported by Lin [17]. Additionally, Prakash et al. determined that 50 mM $CaCl_2$ included in the media results in an increase of amylase production by 29% [21].

In our study, the effects of different carbon and nitrogen sources on the production of α-amylase were examined. When control was compared with carbon sources, it was determined that maltose valued closely with control, that enzyme production significantly decreased in other carbon sources and completely disappeared in glucose, sucrose, and lactose. It was reported that in many *Bacillus* types, glucose and sucrose repressed amylase synthesis [1, 5, 17]. It has been reported by Saxena et al. that synthesis of carbohydrate degrading enzyme in most species of *Bacillus* sp. PN5 leads to catabolic repression by readily metabolizable substrates such as glucose and fructose [5]. Haseltine et al. observed that glucose represses the production of amylase in the hyperthermophilic archeon *Sulfolobus solfataricus* [22]. Maximum α-amylase production was obtained from medium containing 0.6% starch for *Bacillus* sp. PN5 by Saxena et al., 4% lactose for *Bacillus* sp. IMD 435 by Hamilton et al., 1% fructose for *Bacillus* sp. AB 04 by Behal et al. and soluble starch for thermophilic *Bacillus* sp. SMIA-2 by Carvalho et al. [5, 14, 23, 24].

It was observed that nitrogen sources did not influence the enzyme production. Likewise, when compared with the control, a lower level of amylase activity was obtained in all nitrogen resources. The highest α-amylase production as a nitrogen source was achieved in NBs; by Saxena et al. with 0.5 pepton and 0.3 yeast extract, by Hamilton et al. with 2% yeast extract, by Behal et al. with 1% meat extract, by Carvalho et al. with pepton [5, 14, 23, 24].

3.2 Effect of pH and temperature on α-amylase activity

When examined at different pH values, activity showed that the enzyme was active in the pH range of 5–11.0 with maximum activity at pH 7.0 (Fig. 4). The optimum pH of the enzyme was found to be 7.0. It is of significance in terms of liquefaction of starch and the use of it in the food industry that enzyme pH was neutral. The optimum temperature of extracellular α-amylase enzyme obtained from *G. stearothermophilus* was found to be 70°C. The temperature profile of this α-amylase showed that it has to be optimum temperature of 70°C, with 63% activity retained at 80°C (Fig. 5). High temperature of enzyme is important in terms of industrial point of view because processes used by industrial enzymes are generally carried out in high temperatures.

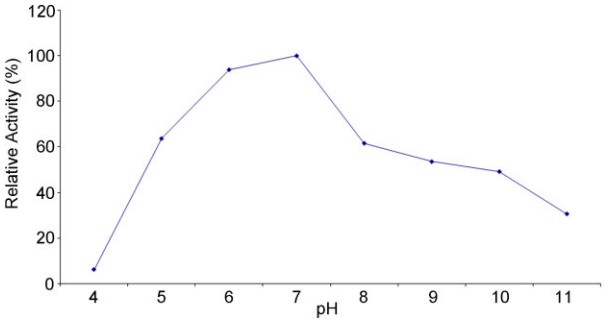

Figure 4. Effect of pH on α-amylase activity.

The optimal pH and temperatures reported for α-amylase from *Geobacillus thermodenitrificans* HRO10 (5.5 and 80°C), *Bacillus* sp. I-3 (7.0 and 70°C), *Bacillus* sp. SMIA-2 (7.5 and 70°C), *Bacillus subtilis* JS-2004 (8.0 and 70°C), and *Geobacillus* sp. LH8 (5–7.0 and 80°C) [1, 8, 10, 25, 26].

3.3 Purification of the α-amylase

α-Amylase produced by *G. stearothermophilus* was purified by Sephadex G-100 gel filtration, followed by DEAE-cellulose ion exchange chromatography. An overall purification of about 65-folds with an activity yield of 46% was achieved. Acquiring α-amylase in high purity and efficiency reveals its availability to be used in industrial applications. The results of the purification are summarized in Table 1. These results are in accordance with other researchers findings [27, 28].

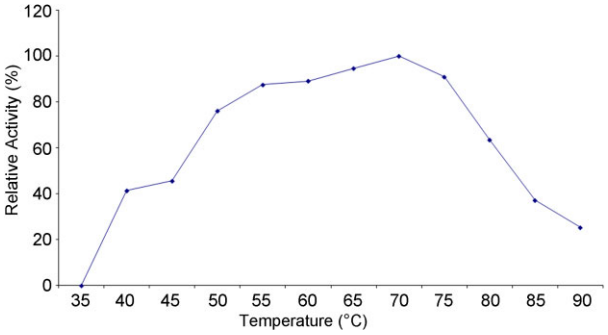

Figure 5. Effect of temperature on α-amylase activity.

Table 1. Summary of the purification of α-amylase from *Geobacillus stearothermophilus*

Purification steps	Specific activity (U/mg)	Total activity	Total protein (mg/mL)	Yield (%)	Purification fold
Crude extract	2396	209 991	87.651	100	1
Precipitation of 80% ammonium sulfate and dialysis	6914	110 238	15.944	52	3
Sephadex G-100	45 199	106 944	2.366	51	19
DEAE cellulose	154 947	96 510	0.623	46	65

3.4 Molecular mass determination

The MW of purified α-amylase from *G. stearothermophilus* was 63 kDa, as estimated by SDS-PAGE (Fig. 6). MW of most of the α-amylase enzymes were between 45 and 70 kDa. Ezeji and Bahl, determined the MW of α-amylase enzyme purified from *G. thermodenitrificans* HRO10 as 58 kDa [8].

Other molecular masses for different amylases have been reported: 63 kDa for *Bacillus* sp. IMD 435; 52 kDa for *Geobacillus* sp. LH8; 58 kDa for *B. licheniformis* NH1 and 58 kDa for *B. licheniformis* [14, 24, 29, 30].

Table 2. Effect of metal ions on α-amylase activity

Metal ions (1.5 mM)	Relative enzyme activity (%)
Control	100
Mn^{+2}	122
Fe^{+2}	55
Zn^{+2}	29
Mg^{+2}	97
Hg^{+2}	17
Co^{+2}	96
Cu^{+2}	19
Ca^{+2}	123

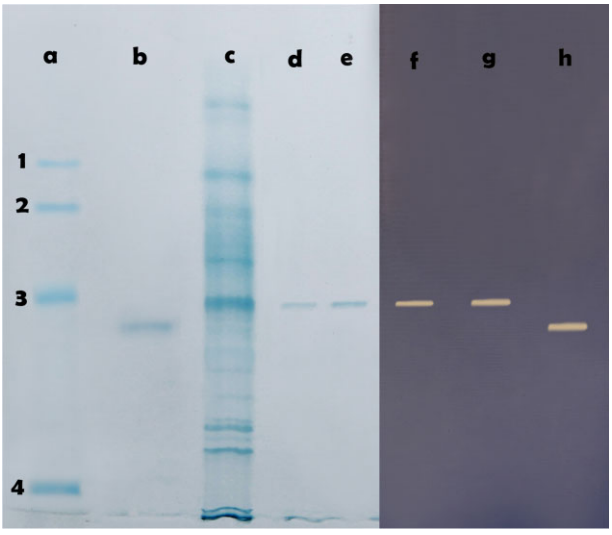

Figure 6. The α-amylase showed a molecular mass. SDS-PAGE Lane a: standard proteins; β-galactosidase (116 kDa), phosphorylase (97 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa); Lane b: standard α-amylase from *Bacillus subtilis* (58 kDa); Lane c: dialyze; Lane d: purified enzyme (5 μL) from sephadex G-100; Lane e: purified enzyme (2 μL) from DEAE cellulose; Native-PAGE (using iodine stain) Lane f: purified enzyme from sephadex G-100; Lane g: purified enzyme from DEAE cellulose; Lane h: standard α-amylase.

3.5 Kinetic parameter determination

Kinetic studies of α-amylase were performed under standard assay conditions using soluble starch as the substrate. The K_m and V_{max} values were calculated from a Lineweaver–Burk plot (Fig. 7). The Michaelis–Menten constant (K_m), for α-amylase was found to be 0.051 mM and V_{max} value to be 1.424 μmol/min, respectively. Aguilar et al., determined the K_m value of purified α-amylase enzyme as 3.44 mg/mL [31].

3.6 Effects of pH and temperature on amylase stability

In order to determine pH stability of the enzyme, α-amylase activity determination has to be carried out subsequent to pre-incubation of different pH values at optimum temperature for 3 h. It was observed that enzyme activity was preserved by 100% for pH 6–8.0 at the end of 1 h of pre-incubation (Fig. 8). After incubation for 3 h at pH 6.0, 7.0, and 8.0 the enzyme retained 67, 52, and 50%

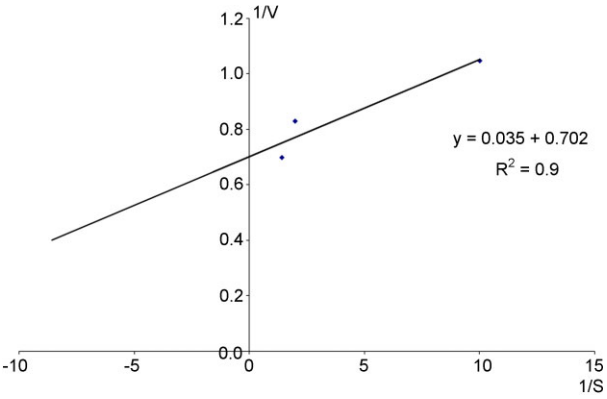

Figure 7. Lineweaver–Burk plot of purified α-amylase from *G. stearothermophilus*.

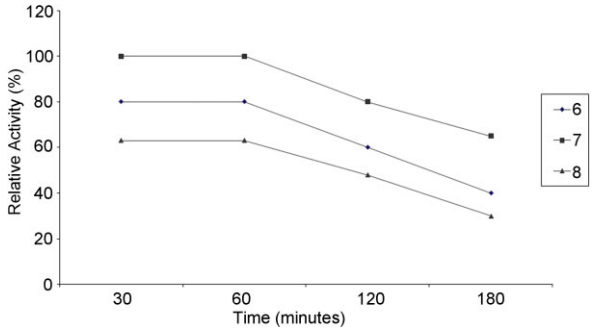

Figure 8. Effects of pH on amylase stability.

of the original activities, respectively. α-Amylases are generally stable in a wide pH range from 4 to 11; however, α-amylases with stability in a narrow range have also been reported [28, 32]. Saxena et al. determined that *Bacillus* sp. PN5 α-amylase activity was retained by 80% at pH 10.0 following a 1 h pre-incubation while Behal et al. determined that *Bacillus* sp. AB 04 α-amylase was stable between pH 7.0 and 10.0, Liu and Xu determined that *Bacillus* sp. YX-1 α-amylase was stable between pH 4.5 and 11.0 [5, 23, 32].

To determine the thermal stability of pure enzyme, pre-incubation process was carried out with the sole use of enzyme for 15, 30, 45, 60, and 120 min in 50, 60, and 70°C heat values (Fig. 9). Enzyme activity was determined following pre-incubation. It was observed that the enzyme was stable between 50 and 60°C at the end of 2 h and that it lost 50% of its activity at 70°C. Corderio et al. have determined that α-amylase activity was stable at 50°C over 2 h, and that it lost 4, 13, and 38% of its activity at 60, 70, and 90°C, respectively [26]. Asoodeh et al. reported that purified α-amylase preserved 75% of its activity at 75°C′ at the end of 45 min [33]. Behal et al. determined that α-amylase enzyme they obtained from *Bacillus* sp. AB 04 was stable between 50 and 80°C. [23].

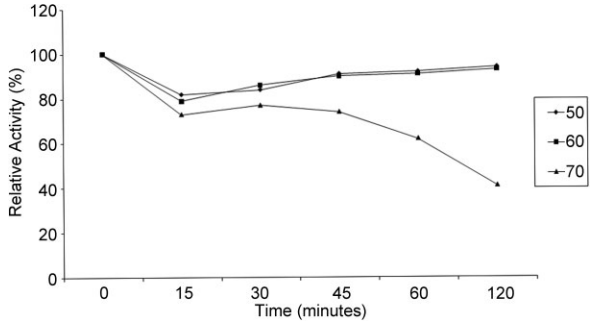

Figure 9. Effects of temperature on amylase stability.

3.7 Effect of metal ions and inhibitors on amylase activity

The effect of different metal ions and inhibitors were investigated on purified enzyme pre-incubated at 37°C for 30 min. The residual activity was measured at optimum pH and temperature. The enzyme was activated by Ca^{2+} and Mn^{2+}, but strongly inhibited by Fe^{2+}, Zn^{2+}, Cu^{2+}, and Hg^{2+} (Table 2). Noman reported α-amylase to be strongly inhibited by Fe^{2+}, Zn^{2+}, Cu^{2+} [34]. Lin suggested that enzyme was activated with Ca^{2+}, while it was inhibited in the presence of Hg^{2+}, Pb^{2+}, Zn^{2+}, and Cu^{2+} [17]. Mamo and Gessesse observed enzyme inhibition in the presence of Hg^{2+}, Cu^{2+}, and Fe^{3+} metals [35]. Shafiei et al. found that α-amylase activity increased with Ca^{2+}, and that it was inhibited with Fe^{2+}, Zn^{2+}, Cu^{2+}, and Al^{+3} ions [36]. Our results are consistent with findings of other studies.

Effects of some inhibitors such as EDTA, PMSF, DTT, and β-mercaptoethanol on purified enzyme were studied. The highest inhibitory effects were obtained with 1 mM EDTA and 10 mM EDTA. An increasing inhibitory effect on enzyme activity was determined with increasing of concentration (Table 3).

3.8 Effect of detergents on amylase activity

To examine the effects of some detergents on purified α-amylase enzyme activity, 0.5% SDS, Tween-40, Tween-80, Triton X-100 were used. Relative activity was calculated by comparing their remaining enzyme amounts with control. Remaining amounts of enzymes, in comparison to control, were determined as SDS 84%, Tween-40 93%, Tween-80 95%, and TritonX-100 115% (Fig. 10). It was observed that enzyme resisted against detergents and activity increased

Table 3. Activity remaining after incubation for 30 min at 37°C

Agent	Concentration (mM)	Relative activity (%) of α-amylase
PMSF	1	93
	2	85
	4	67
	10	23
DTT	1	96
	2	83
	4	73
	10	41
β-Mercaptoethanol	1	86
	2	81
	4	67
	10	61
EDTA	1	35
	2	28
	4	21
	10	11

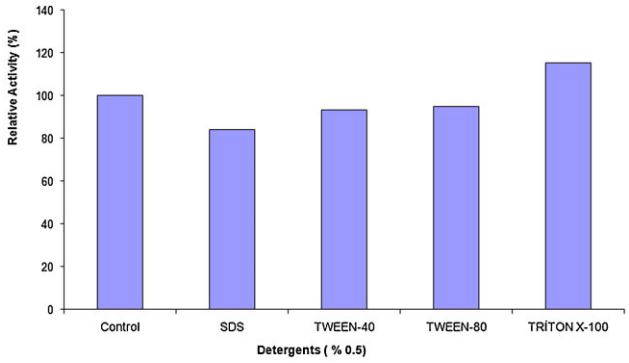

Figure 10. Effect of detergents on amylase activity.

with TritonX-100. It was declared that due to the presence of disulfide bonds in the enzyme and oxidation of amino acids, thermo stability in the enzyme increased and thereby the enzyme is not affected by oxidizing agents. This result achieved may indicate that the enzyme is not affected by oxidizing agents and that amino acids change with temperature and cause resistance. Asoodeh et al. determined that α-amylase activity increased with Triton X-100. Shafiei et al. determined that α-amylase enzyme was quite stable against 0.5%SDS, 0.2%Triton X-100, Tween 80, and Tween 20 detergents [33, 36]. Results achieved by these researchers are in conformity with our study.

4 Conclusions

In our study, due to obtaining maximum α-amylase activity economically, in short time period, in high temperature and with neutral pH, α-amylase enzyme purification was carried out from *G. stearothermophilus*. With low loss and high purity, α-amylase compatible for industrialized use was obtained. More recently, many authors have presented good results in developing α-amylase purification techniques, which enable applications in pharmaceutical and clinical sectors which require high purity amylases. That the purified enzyme was thermo-stable and showed high tolerance against different denatured conditions in the media reveals that it is functional in terms of enzyme technology and bio-technology. Thermo-stabilization of purified enzyme shows that it is usable biotechnologically because it is highly tolerant against diverse denaturing agents within the medium.

The authors have declared no conflict of interest.

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