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Purification and Characterization of Thermostable and Detergent-Stable α-Amylase from *Anoxybacillus* sp. AH1

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Summary

A thermostable and detergent-stable α-amylase from a newly isolated *Anoxybacillus* sp. AH1 was purified and characterized. Maximum enzyme production (1874.8 U/mL) was obtained at 24 h of incubation. The amylase was purified by using Sephadex G-75 gel filtration, after which an 18-fold increase in specific activity and a yield of 9 % were achieved. The molecular mass of the purified enzyme was estimated at 85 kDa by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The optimum pH and temperature values of the enzyme were 7.0 and 60 °C, respectively. The enzyme was highly stable in the presence of 30 % glycerol, retaining 85 % of its original activity at 60 °C within 120 min. K_m and v_{max} values were 0.102 μmol and 0.929 μmol/min, respectively, using Lineweaver-Burk plot. The enzyme activity was increased by various detergents, but it was significantly inhibited in the presence of urea. Mg^{2+} and Ca^{2+} also significantly activated α-amylase, while Zn^{2+}, Cu^{2+} and metal ion chelators ethylenediaminetetraacetic acid (EDTA) and 1,10-phenanthroline (phen) greatly inhibited the enzyme activity. α-Amylase activity was enhanced by β-mercaptoethanol (β-ME) and dithiothreitol (DTT) to a great extent, but inhibited by *p*-chloromercuribenzoic acid (PCMB). Iodoacetamide (IAA) and *N*-ethylmaleimide (NEM) had a slight, whereas phenylmethylsulfonyl fluoride (PMSF) had a strong inhibitory effect on the amylase activity.

Key words: detergent-stable α-amylase, *Anoxybacillus* sp. AH1, enzyme activity inhibition, enzyme purification

Introduction

Alpha-amylases (α-1,4-glucan-4-glucanohydrolase; EC 3.2.1.1) are a family of endoamylases that randomly cleave α-1,4-glucosidic linkages in starch and related carbohydrates to produce oligosaccharides of different lengths and glucose in the α-anomeric form (*1–3*). α-Amylases can be obtained from different sources, including plants, animals and microorganisms (*4,5*). Microbial enzymes are generally preferred in several industrial applications due to advantages such as wide use, efficient production, stability and cost-effectiveness (*3,6*). Amylases are one of the most important families of enzymes used in starch processing, brewing and sugar production (*7,8*), desizing in textile industries, in detergent manufacturing processes, drugs and pharmaceuticals (*2*), in filling of pores of paper, in removing food and starch stains in dry cleaning (*9*), and production of corn and chocolate syrup (*10*).

Amylases have been obtained from thermophilic bacteria for several years. In recent studies, amylases have been studied from thermophilic *Bacillus* species such as *Bacillus subtilis* (*8,11*), *Bacillus licheniformis* (*7,12,13*), *Bacillus amyloliquifaciens* (*14*), *Bacillus cereus* (2), *Bacillus thermooleovorans* (*15*), *Anoxybacillus flavithermus* (*16*), *Anoxybacillus amylolyticus* (*17*), *Anoxybacillus* sp. (*3,9,18*), *Geobacillus*

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stearothermophilus (*19*), *Geobacillus thermooleovorans* (*20*) and *Geobacillus* sp. (*21,22*).

There are a few studies regarding purification and characterization of α-amylases in *Anoxybacillus* species. The purpose of this study is to purify and characterize a biotechnologically important α-amylase, rather stable in several detergent formulations, produced by thermophilic *Anoxybacillus* sp. AH1 isolated from Dargeçit hot spring in Turkey.

Materials and Methods

Materials

Sephadex G-75, 3,5-dinitrosalicylic acid (DNS), bovine serum albumin (BSA), 1,10-phenanthroline (phen), dithiothreitol (DTT), *p*-chlorobenzoic acid (PCMB), *N*-ethylmaleimide (NEM), iodoacetamide (IAA), phenylmethanesulfonyl fluoride (PMSF), sodium dodecyl sulphate (SDS), Tween 40, Triton X-100 and α-amylase (Type II-A≥1500 U/mg from *Bacillus* sp.) were purchased from Sigma (Sigma–Aldrich, St Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA) and β-mercaptoethanol (β-ME) and all culture media (nutrient broth) were provided by Merck (Darmstadt, Germany). All chemicals were of analytical grade.

Bacterial strain and medium

The strain AH1 used in this study was isolated from Dargeçit hot spring in Turkey and then identifed and characterized by morphological, physiological and biochemical tests and 16S rRNA sequence analysis by Acer *et al*. (*23*). A volume of 1 mL of culture was inoculated in a 100-mL Erlenmeyer flask containing 25 mL of nutrient broth composed of (in g/L): beef extract 10, peptone 10 and NaCl 5, and incubated overnight at 60 °C for 24 h in a shaker. Growth was followed by absorbance measurements at 540 nm. The culture was centrifuged at 8200×*g* and 4 °C for 10 min and the cell-free supernatant was used for the estimation of amylolytic enzyme activity.

Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA and purification of the PCR products were performed as described previously (*24,25*). The 1134 nucleotides of the 16S rRNA gene were specified (*23*). A BLAST search (*26*) was used in order to compare these sequences with other *Anoxybacillus* species that produce amylolytic enzymes. The 16S rRNA gene sequences of the species most closely related to our strain were retrieved from the database. The CLC Sequence Viewer v. 6.0 software package (*27*) was used for the construction of phylogenetic tree.

Enzyme activity assay

The enzyme activity was determined according to Bernfeld (*28*): 50 μL of enzyme solution were added into 200 μL of soluble starch (Merck) (0.5 %, by mass per volume) in 0.1 M Tris-HCl buffer, pH=7.0, at 60 °C for 30 min. The reaction was stopped by the addition of 0.4 mL of 3,5-dinitrosalicylic acid (DNS) reagent and the mixture was boiled for 5 min. After cooling to room temperature, the mixture was diluted with 3.0 mL of distilled water and the absorption was then measured at 489 nm. One unit of amylase activity was defined as the amount of enzyme that released 1 μmol of maltose per minute per mL under the assay conditions. The protein content in the extracellular extracts was determined by the method of Lowry *et al.* (*29*) using bovine serum albumin (BSA) as a standard in this procedure.

Effect of incubation time on bacterial growth and α-amylase production

In order to determine the effect of incubation time on bacterial growth and α-amylase production, 1 mL of the isolate was inoculated into 100 mL of nutrient broth and the samples were taken every 3 hours over a 72-hour period. The growth was determined by measuring the increase in absorbance at 540 nm. After centrifugation, the supernatant was used for measuring the enzyme activity.

Purification of α-amylase

The strain AH1 was grown in nutrient broth for 24 h and was removed by centrifugation at 8200×*g* and 4 °C for 10 min. The supernatant was precipitated using ammonium sulphate to 80 % saturation. The precipitate was then dissolved in 0.1 M Tris-HCl buffer (pH=7.0), and dialyzed overnight against the same buffer. Gel filtration of the precipitate was done on a Sephadex G-75 column (1.5 cm ×30 cm), pre-equilibrated with 0.1 M Tris–HCl, pH=7.0. An elution was performed with the same buffer at a flow rate of 3 mL/min. The enzyme containing fractions was collected and concentrated by ultrafiltration. Protein content and enzyme activity were determined after each step. All purification procedures were carried out at 4 °C.

Determination of purified α-amylase molecular mass and activity

SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out for the determination of purity and molecular mass of the amylase as described by Laemmli (*30*). Samples were heated at 100 °C for 5 min before electrophoresis. Gels were stained with Coomassie Brilliant Blue R250. The molecular mass of the enzyme was estimated using molecular mass markers (catalog number SDS7B2, Sigma): α-2-macroglobulin (180 kDa), β-galactosidase (116 kDa), lactoferrin (90 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactic dehydrogenase (36.5 kDa) and triosephosphate isomerase (26.6 kDa). For zymography of amylase activity, the native gel containing 0.2 % soluble starch was used. The gels were stained with iodine solution after electrophoresis. Clear bands indicated the presence of amylase activity. The enzyme activity band was compared with commercial α-amylase band (58 kDa) from *Bacillus* sp.

Effect of pH and temperature, and kinetic properties of purified enzyme

The effect of pH on amylase activity was determined at 60 °C for 30 min in different buffers (0.1 M citric acid

buffer, pH=4.0–5.5; sodium phosphate buffer, pH=6.0–6.5; Tris-HCl buffer, pH=7.0–9.0; and glycine-NaOH buffer, pH=9.5–11.0). The effect of temperature on amylase activity was determined by assaying the enzyme activity in the range from 30 to 90 °C for 30 min.

In order to test the thermostability of the purified enzyme, the residual enzyme activity was measured after incubating an aliquot of the enzyme at 60 °C for 20, 40, 60, 80 and 120 min. The enzyme was also incubated with 30 % glycerol. Aliquots were withdrawn at desired time intervals and the remaining activity was measured under enzyme assay conditions. The non-heated enzyme was considered as control (100 %).

Soluble starch was used for determination of Michaelis constant (K_m) and the rate of reaction (v_{max}). The enzyme was assayed at various soluble starch mass per volume ratios ranging from 0.5 to 3 % in Tris-HCl buffer. All reactions were carried out at 60 °C and pH=7.0 for 30 min. K_m and v_{max} values were estimated from the Lineweaver-Burk plot.

Effect of different metal ions and chemical reagents

To study the effect of different metal ions (Cu^{2+}, Zn^{2+}, Ca^{2+} and Mg^{2+}), chelating agents (EDTA and phen), some chemicals (DTT, β-ME, PCMB, PMSF, NEM and IAA), various surfactants (SDS, Tween 40, Triton X-100 and commercial detergent) and urea on amylase activity, the purified enzyme was preincubated with all agents for 15 min. The remaining activity was calculated using the enzyme assay under standard assay conditions. Activity in the absence of any additives (control) was taken as 100 %. All of the used metals were in the chloride form. Divalent metals, chelating agents, chemicals, surfactants and urea were dissolved in 0.1 M Tris-HCl buffer (pH=7.0), whereas PMSF and NEM were dissolved in ethanol, and phen in methanol.

Results and Discussion

The comparison of 16S rRNA sequence of the strain AH1 with those of the amylase-producing *Anoxybacillus* species showed high similarity with *A. flavithermus* (Fig. 1). The strain AH1 was identified and named *Anoxybacillus* sp. AH1 [DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) Deposit Number: 23210, GenBank Accession Number: KP172526] (*23*).

α-Amylase production and purification

The time-dependent amylase production was maximum (1874 U/mL) at 24 h (Fig. 2). There are several studies on thermophilic *Anoxybacillus* species that possess the ability to produce amylases (*3,9,16,18,31*). The steps used for the α-amylase extraction from *Anoxybacillus* sp. AH1 and purification are shown in Table 1. It can be seen clearly that α-amylase was purified up to 18-fold with a yield of 9 % of the pure enzyme.

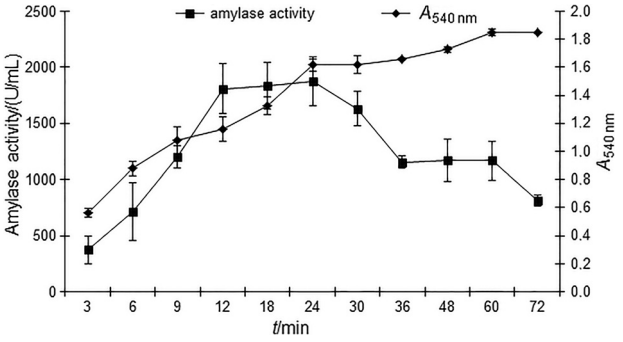

Fig. 2. Time course of bacterial growth and *Anoxybacillus* sp. AH1 amylase production. The cells were incubated at pH=7.0 and 60 °C for 72 h. The results represent the mean values of three experiments, and bars indicate standard deviation. Absence of bars indicates that errors were smaller than symbols

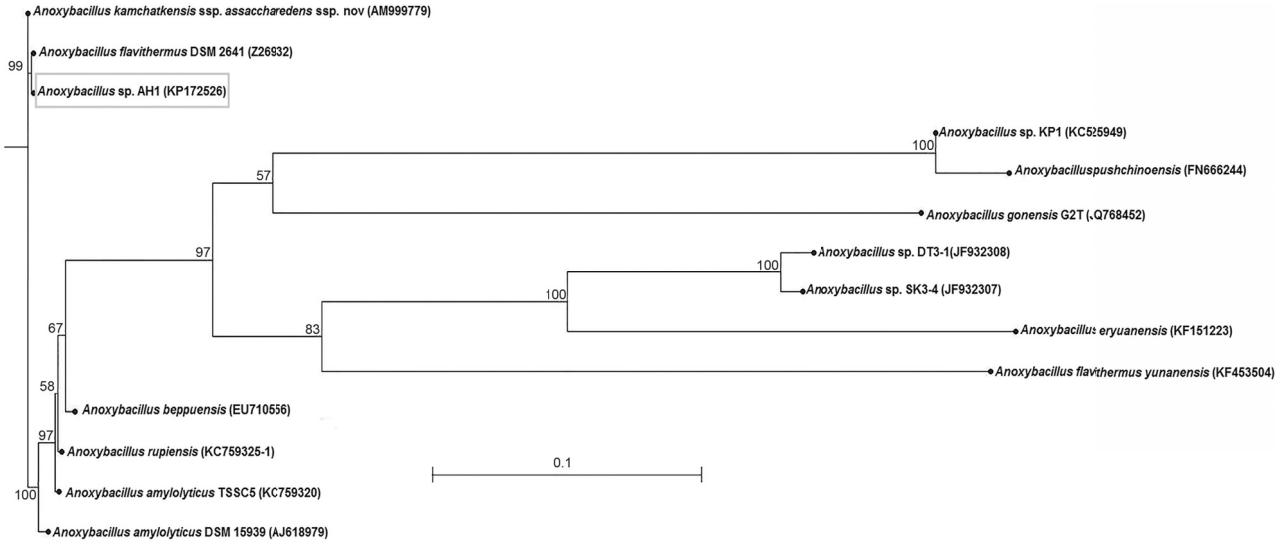

Fig. 1. 16S rDNA sequence-based phylogenetic neighbour-joining tree showing the phylogenetic relationship of AH1 strain relative to other amylase-producing strains of the genus *Anoxybacillus*. The tree topology was obtained by calculation using the CLC Sequence Viewer v. 6 program (*27*). Bootstrap values (%) from 1000 replicates are shown. Bar indicates 0.1 nucleotide substitutions per position

Table 1. Purification steps of α-amylase

Purification step	*m*(total protein)	Total activity	Specific activity	Purification (fold)	Yield
	mg	U	U/mg		%
Crude extract	11.2	17024	1520	1	100
Ammonium sulphate and precipitation/dialysis	0.268	4559.21	17012	11	27
Sephadex G-75	0.055	1488.57	27065	18	9

SDS-PAGE showed that the molecular mass of the α-amylase from *Anoxybacillus* sp. AH1 determined by Commassie staining was around 85 kDa (Fig. 3). Non-denaturing PAGE and zymogram analyses also show the presence of α-amylase activity. The molecular masses of the α-amylases from various bacilli were reported to be 43, 50, 60.5–86, 91 and 97 kDa from *Anoxybacillus beppuensis* TSSC-1 (*18*), *Anoxybacillus* sp. SK3-4 (*32*), *Bacillus* sp. A3-15 (*4*), *Bacillus* sp. AAH-31 (*33*) and *Geobacillus* sp. IIPTN (*21*), respectively.

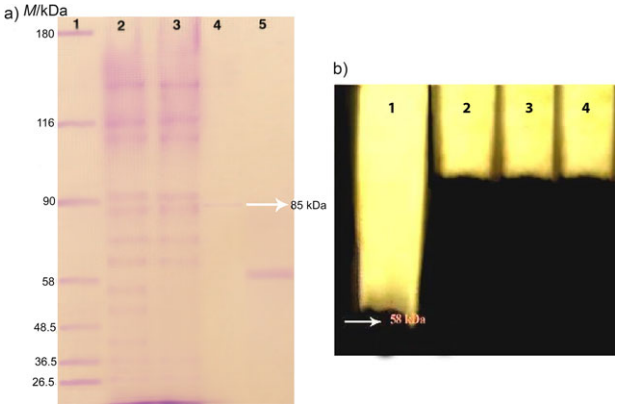

Fig. 3. Amylase activity determined by: a) SDS-PAGE staining with Coomassie Brilliant Blue R250: lane 1=molecular mass markers [catalog number SDS7B2, Sigma: α-2-macroglobulin (180 kDa), β-galactosidase (116 kDa), lactoferrin (90 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactic dehydrogenase (36.5 kDa), triosephosphate isomerase (26.5 kDa)], lanes 2, 3, 4 and 5=staining of crude extract and purified amylase (ammonium sulphate precipitation/dialysis and Sephadex G-75), respectively; and b) zymogram: lane 1=commercial α-amylase (58 kDa); lanes 2, 3 and 4=iodine solution staining of crude extract and purified amylase (ammonium sulphate precipitation/dialysis and Sephadex G-75 column), respectively

Influence of pH, thermal and kinetic properties of purified enzyme

As it can be seen in Fig. 4, the purified enzyme exhibited maximum activity at pH=7.0, and retained amylase activity of 80, 88 and 83 % at pH=6.5, 7.5 and 8.0, respectively. Similar optimum pH value has been reported for α-amylase from *Anoxybacillus* sp. KP1 (*9*), *A. beppuensis* TSSC-1 (*18*), *Bacillus stearothermophilus* (*34*), *Bacillus* sp. 1–3 (*35*) and *Anoxybacillus gonensis* A4 (*36*).

The thermostable starch-digesting amylases are rather important in the process of starch hydrolysis because the industrial aplications involving α-amylases operate at high temperatures exceeding 50 °C, due to higher reaction rates at these temperatures (*35*). The purified amylolytic activity was assayed at different temperatures exhibiting maximum activity at 60 °C, and displayed 92 and 99 % of its peak activity at 50 and 55 °C, respectively (Fig. 5a). In recent studies, the optimum temperature of 60 °C has been reported for a few α-amylases from *Anoxybacillus* species (*3,9,32*). As shown in Fig. 5b, the enzyme was highly stable up to 1 h and retained 93 % of the original activity at 60 °C. However, the enzyme activity decreased

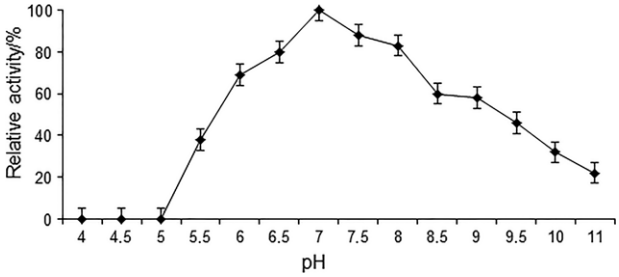

Fig. 4. Effect of pH on the activity of *Anoxybacillus* sp. AH1 amylase. The values are shown as percentages of the maximum enzyme activity observed at pH=7.0 and 60 °C, which is taken as 100 %

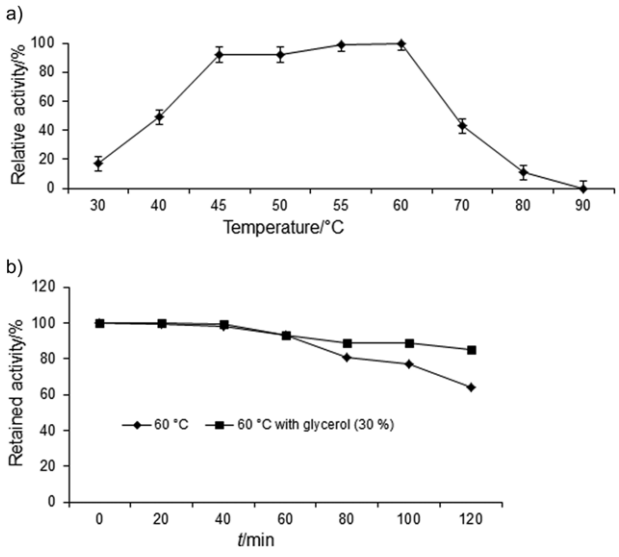

Fig. 5. Effect of: a) temperature on the activity of *Anoxybacillus* sp. AH1 amylase. The values are shown as percentages of the maximum enzyme activity observed at 60 °C, which is taken as 100 %, and b) effect of temperature on the stability of *Anoxybacillus* sp. AH1 amylase. The α-amylase was incubated at 60 °C for different time periods (20–120 min). The stability of unheated crude enzyme was taken as 100 %. The remaining amylolytic activity was measured under standard assay conditions

after 1 h, after which 30 % glycerol was found to help the enzyme stability at 60 °C up to 2 h by retaining 85 % of the original activity. The stabilizing effect of glycerol on thermostability of the enzyme has also been reported in previous studies (*37,38*). The results show that this thermostable enzyme could be a good candidate for the efficient and quick hydrolysis of starches.

As shown in Fig. 6, kinetic studies of the enzyme were carried out using various concentrations of soluble starch as substrate under standard assay conditions. Using the Lineweaver–Burk plot, the K_m and v_{max} values of 0.102 μmol and 0.929 μmol/min were obtained, respectively.

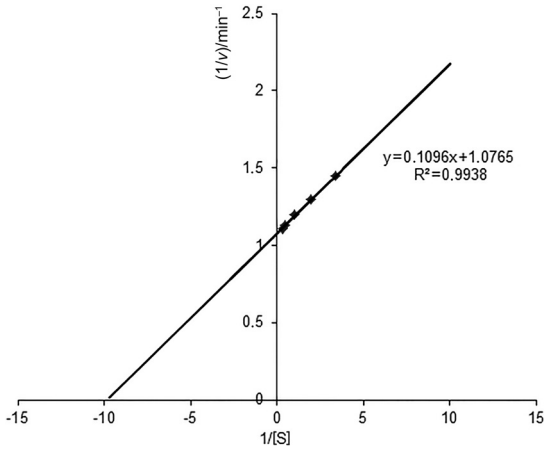

Fig. 6. Lineweaver–Burk plot for K_m and v_{max} values of the amylase in the presence of different concentrations of soluble starch [S]

Influence of different metal ions and chemical reagents

As shown in Table 2, α-amylase was significantly activated by Mg^{2+} (41 % at 8 mM) and Ca^{2+} (70 % at 8 mM). Calcium ion is well known to activate most amylases to a great extent. Arikan (*4*) and Srivastava (*39*) reported that α-amylase activity was enhanced by Ca^{2+}. In addition, Rao and Satyanaryana (*40*) also found that the amylase activity was increased in the presence of Mg^{2+}. Stability of an enzyme in the presence of metal salts plays a crucial role in their industrial application. It is already known that most enzymes require the presence of metal ion activators to express their full catalytic activity (*41,42*).

In the present study, enzyme activity was greatly inhibited by Zn^{2+} (93 % at 1 mM) and Cu^{2+} (76 % at 0.5 mM and 100 % at 1 mM) as well as by the metal ion chelators EDTA (63 % at 10 mM) and phen (22 % at 10 mM). It is known that Cu^{2+} (*43,44*), Zn^{2+} and phen (*45*) have inhibitory effects on α-amylase. EDTA generally shows non-competitive inhibition of amylase activity and a slight inhibition in the present study may indicate that it is a metalloenzyme (*4,46,47*).

β-ME and DTT containing SH groups were found to enhance α-amylase activity for 64 and 106 %, respectively, at 10 mM. However, the enzymatic activity was inhibited by PCMB (52 % at 4 mM). This result shows the presence of at least one essential cysteine residue in the active site of the enzyme, modified by the chemicals. Previous studies showed that the amylase activity increased in the presence of β-ME (*41,43*) and DTT (*43*). Srivastava (*39*) found a similar inhibition effect by PCMB. IAA and NEM had little effect on the enzyme activity. This phenomenon further supported the existence of cysteine on the active sites of purified amylase. Hsieh *et al*. (*48*) also determined that IAA and NEM partially inhibited amylase activity. PMSF inhibited the enzyme strongly (60 % at 4 mM) (Table 2). The inhibition of α-amylase from *Anoxybacillus* sp. AH1 by PMSF suggested the importance of the seryl hydroxyl group in enzyme catalysis. Arikan (*4*), Rao and Satyanaryana (*40*), Shafiei *et al*. (*44*) and Tatar (*45*) also reported that the activity of α-amylase was inhibited by PMSF.

Table 2. Effect of divalent metal ions, metal ion chelators and chemicals on the activity of purified *Anoxybacillus* sp. AH1 α-amylase

Divalent metals, ion chelators and chemicals	*c*/mM							
	0.05	0.1	0.5	1	2	4	8	10
	Retained amylase activity /%							
Ca^{2+}	–	–	–	146	152	159	170	–
Mg^{2+}	–	–	–	109	121	133	141	–
Cu^{2+}	65	62	24	0	–	–	–	–
Zn^{2+}	74	32	15	7	0	–	–	–
EDTA	–	–	–	88	58	57	53	37
phen	–	–		99	97	87	–	78
DTT	–	–	–	102	118	124	–	206
β-ME	–	–	–	120	121	144	–	164
PMSF	–	–	–	51	50	40	–	37
PCMB	–	–	86	78	77	48	–	–
NEM	–	–	–	89	ND	ND	ND	ND
IAA	–	–	–	81	ND	ND	ND	ND
urea	–	84	56	37	9	–	–	–

–=not tested, ND=not determined. Phen=1,10-phenanthroline, DTT=dithiothreitol, β-ME=β-mercaptoethanol, PMSF=phenyilmethylsulfonyl fluoride, PCMB=*p*-chloromercuribenzoic acid, NEM=*N*-methylmaleimide, IAA=iodoacetamide

As it can be seen in Table 2, the enzyme activity was significantly inhibited by urea (91 % at 2 mM), which was also confirmed by other authors (*4,41,46*). The reason for this inhibition is that urea denatures the enzyme (*41*).

The use of α-amylases in detergent formulations is known to present problems because it depends on their activity and stability. The enzymes thus need to be stable against detergents (*49*). As it can be seen in Fig. 7, α-amylase activity was increased with the addition of 0.5 % of

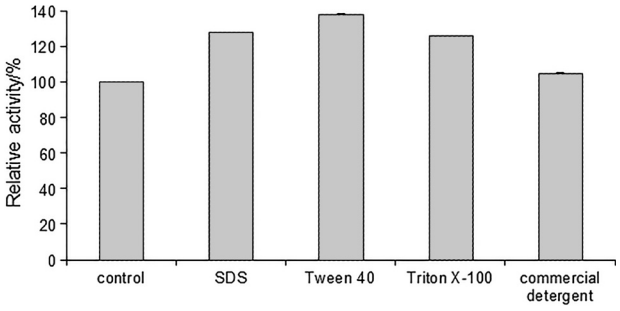

Fig. 7. Effect of surfactants and some commercial detergents on the amylolytic activity. The results represent the mean value of three experiments. Absence of bars indicates that errors were smaller than symbols

several detergents, SDS (28 %), Tween 40 (38 %), Triton X-100 (26 %) and commercial detergent (5 %). The increase of enzyme activity is possibly due to the effect of the surfactant on the folding of substrate moiety as well as an increase in the hydrophobic interactions, which play a crucial role in the stability of the protein tertiary structure and direct interaction with the protein molecule (*41*). Shafiei *et al.* (*44*), Tatar (*45*) and Negi and Banerjee (*41*) reported that α-amylase showed remarkable stability towards 0.5 % SDS and 2 % Triton X-100, Tween 80 and Tween 20. In this study, the thermostable α-amylase was found to be stable against detergents and the α-amylase activity was increased by several detergents compared with other studies, so it may be used as an ingredient in detergent formulations for automatic dishwashers and laundries.

Conclusion

The present study shows that α-amylase from a newly isolated *Anoxybacillus* sp. AH1 is thermostable and detergent stable. The activity of the enzyme was also increased by metal ions such as calcium and magnesium. It is well known that stability of an enzyme in the presence of metal salts and in detergents plays a crucial role in their industrial application. The effect of various inhibitors and chemicals on the amylase activity was also evaluated in the study, which may further clarify the nature of the purified enzyme.

Acknowledgements

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