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Production and purification of α-amylase from halobacterium sodomense

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ABSTRACT

The halophilic bacterial strain *Halobacterium sodomense* was shown to produce extracellular, halotolerant, alkali-stable and thermophilic α-amylases activity. The culture conditions for higher amylases production were optimized with respect NaCl, temperature and substrates. Maximum amylase production was achieved in a medium containing 25% NaCl at 55ºC in the presence of 1% soluble starch and Yeast extract. The enzyme was purified to homogeneity with an overall recovery of 24.2 % and specific activity of 4133 U/mg. The native protein showed a molecular mass of 149 kDa composed of a homodimmer of 78 kDa polypeptide by SDS-PAGE. The optimum pH and temperature of the amylase were 6.0 and 55ºC, respectively. The purified enzyme was stable from pH 7.5 to 9.0 and able to prolong its thermal stability up to 70ºC. The amylase was activated by Na^+, K^+, Mn^{2+}, Mg^{2+}, and Fe^{2+}. However, it was inhibited by Ca^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}, and EDTA. The purified amylase shows interesting properties useful for industrial applications.

KEYWORDS

Production;
Purification;
Amylase;
Characterization;
Halobacterium sodomense.

INTRODUCTION

Halophilic microorganisms are found as normal inhabitants of highly saline environments and thus are considered extremophiles. Several extreme and moderate halophiles have been isolated and exploited for various biotechnological applications, including the production of polymers, enzymes, and biocompatible solutes; enhanced oil recovery, cancer detection; drug screening and the biodegradation of industrial toxic compounds[37]. Halophilic enzymes function in up to 4 M NaCl and in conditions of low water activity[23]. The proteins themselves appear halophilic in that they are only stable in solvents of very high salt concentration in which proteins from more familiar physiological environments are likely to aggregate, precipitate or even unfold, depending upon the type of salt[39]. In certain industrial organic syntheses, enzymes are required to function in the presence of a high concentration of substrate, i.e. at low water activities. Enzymes from normal microorganisms are often inactive in these situations but clearly those from extremely halophilic archaebacteria may by of use[14].

Halophilic amylases have been characterized from halophilic microorganisms such as *Haloarcula hispanica*[15]; *Micrococcus halobius*[31] *Micrococcus varians* subspecies *halobius*[21], *Micrococcus* sp., *Halobacterium salinarum*[10,33], *Halomonas*

meridiana[6] and *Bacillus dipsosauri*[7]. In addition to these halophilies, the search for new microorganisms that can be used for α-amylase production is a continuous process. Bacterial amylase find used in the preparation of starch-derived adhesives, in brewing, in the preparation of grain alcohol and in the production of moist cakes and fruit cakes. They are also used in pharmaceutical animal feeds, sewage treatment, detergents and confectionary[22]. No much detailed study has been done on the amylase of *Halobacterium sodomense*. Therefore, the present investigation was undertaken to study the production and purification of extracelular, alkali-stable and extremely halotolerant α-amylase from *Halobacterium sodomense*.

MATERIALS AND METHODS

Screening, isolation and identification of bacterial strains

The sediment samples for screening were obtained from Wady El-Natroon lakes. The organism was enriched on a screening agar plate containing (g/l): NaCl, 250; $MgSO_4.7H_2O$, 20; KCl, 3; $Na_3C_6H_5O_7.2H_2O$, 2; Peptone, 10; corn starch, 10 and agar, 20 in distilled water and final pH 7.2[32]. The plates were incubation at 50-65°C for 24-48 h, then the plates were stained with Gram's iodine solution (0.1 % I_2 and 1 % KI) and the colonies with the largest halo-forming zone were isolated for further investigation. Microbiological properties of the isolated strain were determined according to the methods described in Bergey's Manual of Determinative Bacteriology[13]. Biolog GP2 MicroPlate™ gives a characteristics reaction pattern called a 'metabolic fingerprint', wherein the metabolic fingerprint patterns were compared and identified using the MicroLog™ database software[9].

Enzyme production

The medium composition used for α-amylase production was the same as above contained in addition 1% (w/v) soluble starch and 0.1mM $ZnSO_4$, which increased the production of α-amylase[33]. Medium (100 ml) was inoculated with 0.2 ml (OD_{600}, 1.0) of a one week old culture of *Halobacterium sodomense.* Cultures were incubating at 50ºC on a rotary shaker at 180 rpm. Flasks were removed at regular intervals and the contents were centrifuged at 5000 rpm for 20 min at 4ºC to remove the bacteria. The supernatant was used for amylase activity. Bacterial growth was monitored by measuring absorbance at 600 nm in a spectrophotometer (UV-visible 2401PC Shimadzu, Kyoto, Japan).

Amylolytic activity

This was done according to Bergmann et al. (1988) by estimating the released reducing sugars from 1% starch in 0.05 M citrate-phosphate buffer (pH 6.0) at 50ºC. Amylase activity was determined by measuring released reducing sugars using the DNS method[27]. One unit of enzyme activity (U) was defied as the amount of protein that produced 1 mg of reducing sugar per ml of enzyme solution at 50ºC in 30 min.

Protein determination

Protein was determined either by measuring the absorbance at 280 nm[38] or by the method of Bradford[4] using bovine serum albumin as a standard.

Effect of incubation time, temperature and NaCl concentration on bacterial growth and enzyme production

The effect of incubation time, temperature and NaCl concentration on enzyme production was investigated by cultivating the organism at (a) different incubation time (3-11 days), (b) different temperature (25-60ºC) and (c) different concentration of NaCl (10-30%). The amylolytic activity and biomass were measured.

Effect of carbon and nitrogen source on bacterial growth and enzyme production Halobacterium sodomense

It was grown in the mineral salt medium containing different carbon and nitrogen sources, in order to study their effect on bacterial growth and enzyme production. The various kinds of starch, soluble starch, corn, rice and potato were used at concentration (0.5-2.5%, w/v). The nitrogen sources tested were casein, Soya meal, meat extract, peptone, yeast extract and beef extract.

Purification of α-amylase production from Halobacterium sodomense

The purification of α-amylase was carried out in

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three steps.

Grounded ammonium sulfate was gradually added to the chilled enzyme solution with stirring until 80 % saturation was obtained. The solution was the stirred at 0ºC for 2 h. The precipitate was collected by centrifugation at 5000 rpm at 4ºC for 30 min[12]. The protein pellet was dissolved in a minimal volume of 0.05 M citrate-phosphate buffer pH 6.0. The enzyme solution was dialyzed overnight against the same buffer at 4ºC. The dialyzed fraction (70 % saturation ammonium sulfate) was then applied directly to ion exchanger DEAE-cellulose column (2.4×70 cm, i.d.). The adsorbed amylase was eluted by a linear gradient of NaCl from 0 to 0.3 M in the same buffer. Fractions in 5 ml volume were collected at a flow rate of 0.7 ml/min. The eluted fractions were monitored at 280 nm for protein and assayed for enzyme activity[38]. In final step, the active fractions were pooled, concentrated and dialyzed against the same buffer and loaded onto a gel filtration column (2.4×80 cm, i.d.) Sephadex G-150 and flow rate was maintained at 0.3 ml/min. The eluted fractions were monitored at 280 nm for protein and assayed for enzyme activity.

Polyacrylamide gel electrophoresis

Electrophoresis under non-denaturing conditions was performed in 10 % (w/v) acrylamide stab gel according to the method of Davis[8] using a Tris-glycine buffer, pH 8.3. Protein bands were located by stained with Coomassie Brilliant Blue R-250.

Molecular weight determination

Molecular weight was determined by gel filtration technique using a Sephadex G-150[1,2]. The column (2.4×80 cm, i.d.) was calibrated with pepsin (35,000), egg albumin (45,000) and phosphorylase b (97,000). Dextran blue (2,000,000) was used to determine the void volume (V_o). Subunit molecular weight was determined by SDS-polyacrylamide gel electrophoresis[24]. SDS-denatured phosphorylase b (97,000), bovine serum albumin (67,000), ovalbumin (45,000) carbonic anhydrase (30,000), Trysin inhibitor (20,000) and α-Lactalbumin (14,000) were used as standard protein molecular weight markers.

Enzyme characterization

The purified enzyme was characterized with respect to its optimum pH, temperature, stability at different temperature and pH values, effect of metal ions on activity and stability.

Amylase activity was assayed at different pH values (pH 4.5-10.0) using different buffers 0.05 M such as citrate-phosphate buffer (pH, 4.5-7.0), sodium phosphate buffer (pH, 6.5-8.0), tri-HCl buffer (pH, 7.0-8.5) and glycin NaOH buffer (pH, 8.6-10.0). To determine pH stability, amylase preparations in buffer at different pH ranging from 4.5-10.0 were activity assayed under standard conditions.

Amylase activity was assayed at different temperatures ranging from 35-80ºC at pH 6.0 in citrate-phosphate buffer (0.05 M). To determined thermo stability, amylase preparation was incubated at temperature ranging from 50-90ºC.

The effects of metal ions and EDTA on amylase activity were determined by the residual activity after 30 min incubation at 55ºC in 0.05 M Tris-HCl (pH 6.0) buffers containing various metal ions at a concentration of 5 mM.

RESULTS AND DISCUSSION

Screening of microorganisms

The bacterial strain exhibited large clear zone around the colony on starch agar plate was tested of the characters according the methods described in Bargey's Manual, and Biolog GP2 MicroPlate™ database, the organism selected was identified and designated as *Halobacterium sodomens*.

Effect of incubation period and NaCl concentration on bacterial growth and amylase production

The time course of incubation period form 3-9 days was followed in liquid shaken culture media at 50°C, and pH 7.2. Data presented in TABLE 1, show that there gradual increased in halophilic α-amylase unit up to 9 day of incubation at which maximum activity (1.6 U/mg protein). The salinity was found to be a significant factor in the production of halophilic α-amylase. The α-amylase production was optimum in the medium containing 25 % NaCl (TABLE 2). Similar behavior has been described for other moderate halophiles producing amylase[33].

TABLE 1 : Effect of incubation period on α-amylase production by *Halobacterium sodomense*

Incubation time (day)	Cell growth ($O.D_{600nm}$)	Protein (mg)	Enzyme activity (U)	Specific activity (U/mg protein)
3	0.23	0.39	0.16	0.41
5	0.31	0.51	0.24	0.47
7	0.63	0.85	0.79	0.93
9	1.27	0.96	1.12	1.16
11	1.36	1.07	1.09	1.02

TABLE 2 : Effect of NaCl concentration on α-amylase production by *Halobacterium sodomense*

NaCl (% w/v)	Cell growth ($O.D_{600nm}$)	Protein (mg)	Enzyme activity (U)	Specific activity (U/mg protein)
10	0.43	0.65	0.57	0.87
15	0.75	0.69	0.72	1.04
20	1.11	0.81	1.03	1.27
25	1.27	0.79	1.16	1.47
30	0.92	0.74	0.88	1.19

TABLE 3 : Effect of temperature on α-amylase production by *Halobacterium sodomense*

Incubation Temperature	Cell growth ($O.D_{600nm}$)	Protein (mg)	Enzyme activity (U)	Specific activity (U/mg protein)
25	0.83	0.82	0.73	0.89
30	0.89	0.71	0.76	1.07
35	1.06	0.77	0.89	1.15
40	1.22	0.76	0.93	1.22
45	1.29	0.82	1.11	1.35
50	1.37	0.78	1.06	1.36
55	1.41	0.85	1.25	1.47
60	1.17	0.75	1.04	1.38

Effect of temperature concentration on bacterial growth and amylase production

The production of enzyme and bacterial growth were determined at different temperature rang in from 25-60°C. The optimum enzyme production waned at 55°C (TABLE 3). At 60°C the enzyme production decreased 17 % than at 55°C. the results agree with data previously published by Onishi (1972).

Effect of carbon and nitrogen source on bacterial growth and amylase production

The organism produced amylase without added starch (TABLE 4); production was increased significantly in the presence of starch, with soluble starch being the best inducer than the other types of starch tests. The maximum production (1.38 U/mg protein) was obtained with 1 % soluble starch[33]. Among the different nitrogen sources tested (TABLE 5), Soya meal was found to be a good nitrogen source for both bacterial growth and amylase production (1.46 U/mg protein). This data investigated that the organism required equal amount of carbon and nitrogen source for bacterial growth and amylase production. *Halobacterium sodomense* can use various carbohydrates as carbon sources, but the enzyme was secreted only in the presence of soluble starch, and starch-containing flours. Inductive production of amylase was reported in *Bacillus sp.* strain TSCVKK and *Bacillus sp.* 64[18,19], whereas it was constitutive in *Halobacillus sp.* strain MA-2[1]. TVSP 101 showed repression of amylase production when glucose was included in the medium

TABLE 4 : α-amylase production in the presence of different starches (under shaking condition at 55°C in medium containing 25% (w/v) NaCl) from *Halobacterium sodomense*

Starch	Concentration (w/v)	Cell growth ($O.D_{600nm}$)	Protein (mg)	Enzyme activity (U)	Specific activity (U/mg protein)
Soluble starch	0.5	0.96	0.81	0.85	1.05
	1.0	1.27	0.83	1.15	1.38
	1.5	1.18	0.93	1.02	1.09
	2.0	1.03	0.76	0.76	1.00
	2.5	0.89	0.72	0.62	0.86
Corn starch	0.5	0.56	0.67	0.32	0.48
	1.0	0.67	0.74	0.38	0.51
	1.5	0.59	0.73	0.27	0.37
	2.0	0.48	0.69	0.25	0.36
	2.5	0.49	0.66	0.6	0.39
Rice starch	0.5	0.62	0.59	0.45	0.76
	1.0	0.78	0.81	0.57	0.70
	1.5	0.73	0.76	0.48	0.63
	2.0	0.61	0.72	0.39	0.54
	2.5	0.54	0.69	0.33	0.49
Potato starch	0.5	0.65	0.56	0.51	0.91
	1.0	0.89	0.86	0.59	0.68
	1.5	0.82	0.77	0.48	0.62
	2.0	0.78	0.67	0.44	0.65
	2.5	0.68	0.71	0.42	0.59

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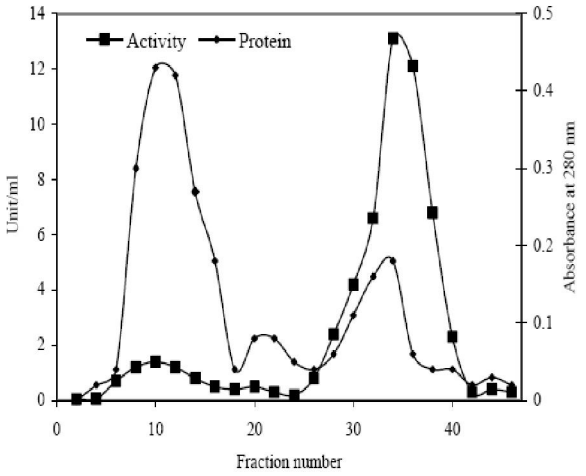

Figure 1 : Typical elution profile for the chromatography of amylase on DEAE-cellulose column (50 × 2.0 cm, i.d), previously equilibrated with 10 mM phosphate buffer, pH 7.0 at a flow rat 0.7 ml/min and 5 ml fractions

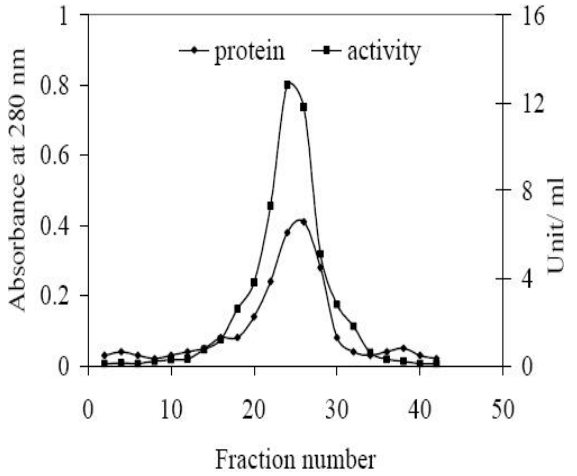

Figure 2 : Gel filtration for the chromatography of Amylase DEAE-cellulose faction (A1) on Sephadex G-100 column (90 × 2.6 cm, i.d) the column was equilibrated with 10 mM phosphate buffer, pH 7.0 at a flow rate of 0.3 ml/min and 5 ml fractions

TABLE 5 : Effect of different nitrogen sources (each 1.0 % (w/v)) on α-amylase production (under shaking condition at 55ºC in the presence of 1.0 % (w/v) starch and 25% (w/v) NaCl) from *Halobacterium sodomense*

Nitrogen source	Cell growth ($O.D_{600nm}$)	Protein (mg)	Enzyme Activity (U)	Specific activity (U/mg protein)
Casein	0.51	0.49	0.35	0.71
Soya meal	1.06	0.76	1.11	1.46
Meat extract	0.96	0.72	0.75	1.04
Peptone	1.33	0.93	1.23	1.32
Yeast extract	0.76	0.69	0.98	1.42
Beef extract	0.49	0.52	0.39	0.75

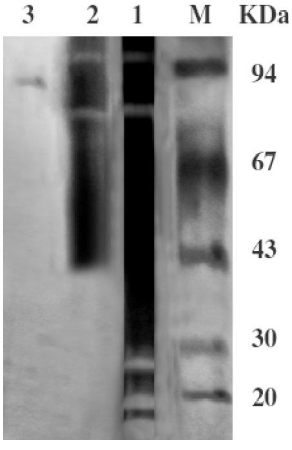

Figure 3 :Polyacrylamide gel electrophoresis for *H. sodomense* during purification steps.1- Crude enzyme; 2-Ammonium sulphate (70%) fraction; 3-Sephadex G-150 A2; M-Standard protein (phosphorylase b, 94,000), bovine serum albumin, 67,000), ovalbumin, 43,000 and carbonic anhydrase, 30,000), (Soya trypsin inhibitor, 20,000)

TABLE 6 : Purification of α-amylase from *Halobacterium sodomense*

Purification steps	Activity (U)	Protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
Supernatant	201	75.11	2.67	1..00	100
$(NH_4)_2SO_4$					
(70 %)	171	34.1	4.93	1.84	85
DEAE-cellulose					
A1	27.3	23.2	1.18	0.44	13.6
A2	153.6	3.83	6.63	2.48	76.4
Sephadex G-150					
A2	48.6	0.85	57.0	21.3	24.2

TABLE 7 : Effect of metal ions and EDTA on *H. sodomense* amylase

Metal ions	% Relative activity
Control	100
K^+	110
Na^+	135
Ca^{2+}	95
Cd^{2+}	80
Mn^{2+}	122
Mg^{2+}	115
Fe^{2+}	109
Ag^+	69
Co^{2+}	92
Zn^{2+}	85
EDTA	87

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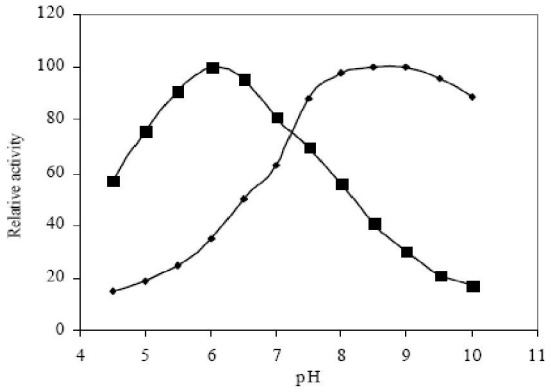

Figure 4 : pH optimum (-■-), and pH stability (-●-) of *Halobacterium sodomense* amylase

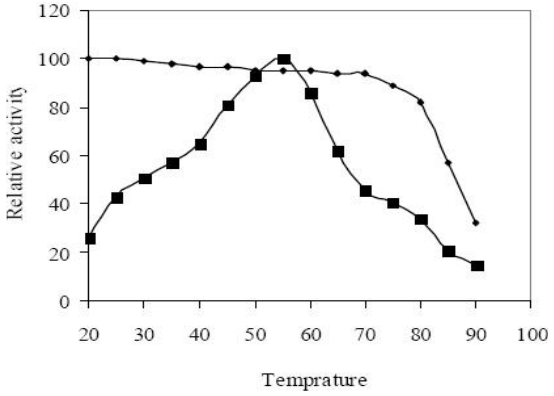

Figure 5 : Temperature optimum (-■-), and temperature stability (--) of *Halobacterium sodomense* amylase

together with soluble starch. Similar results have been reported in other halophilic organisms[6,18,19]. Media containing starch rich flours such as rice, wheat, sorghum, and maize were suitable for the production of α-amylase. Starch derived from wheat and cassava served as the best source of α-amylase production by *Clostridium thermosulfurogenes* SV9, *Bacillus stearothermophilus*, and *Thermoactinomyces thalpophilus*[25,36]. Natural sources could serve as economical and readily available raw material for production of valuable enzymes. Hence, these starch-rich flours may prove useful as cheaper alternative sources of carbon and energy for the bacterial production of amylases. Among the nitrogen sources tested, Soya meal was found to enhance amylase secretion. Tryptone and peptone have been reported as a good nitrogen sources for amylase secretion in *Halobacterium salinarum* and *Bacillus thermooleovorans*[26,33].

Purification of amylase

The culture supernatant was used as a starting material for the purification of amylase from *Halobacterium sodomense*. The enzyme was purified by a three-step strategy including ammonium sulphate precipitation and dialysis, ion exchange chromatography, and gel filtration. The recovery of dialysed enzyme was 85% followed by 76.4% in ion-exchange chromatography and 24.2% in gel filtration (TABLE 6). The overall purification strategy attained 21.3-fold purification of amylase with specific activity of 57.0 U/mg. The molecular weight of the enzyme was about 149 kDa protein using gel filtration techniques. This value was confirmed by SDS-PAGE (Figure 3), where subunit molecular weight of α-amylase was estimated to be 78 kDa protein as dimer subunit. The result obtained from the column was similar to the molecular weight estimated using to SDS-PAGE. Therefore, it can be concluded that α-amylase is a dimeric protein. Other molecular masses for different halophilic amylases have been reported: 55 and 65 kDa protein for *Acinetobacter sp*. amylase I and amylase II, respectively[30]; 56 kDa protein for *Halother- mothrix orenii*[28]; 58 kDa protein for *Haloferax mediterranei*[34]; 74 kDa protein for *Natronococcus sp*. strain Ah-36[20]; 80 kDa protein for *Bacillus dipsosauri*[7]; and 89 kDa protein for *Micrococcus halobius*[31].

Characterization of the purified α-amylase

(1) Effect of pH on enzyme activity and stability

Enzyme activity was measured using the standard assay method by varying the pH values ranging from pH 4.5 to 10.0. The optimum pH of the purified α-amylase was determined as pH 6.0 in 10 mM sodium phosphate buffer (Figure 4), where the enzyme retained 23 % of its activity below pH 7. Meanwhile, the purified α-amylase was stable from pH 8.0 to 9.5 (Figure 4). The results showed that the enzyme was very stable at the pH 9.5 and retained 37 % and 11 % of its activity at pH 7.0 and 10.0, respectively. Therefore the present amylase can have potential applications for hydrolyzing starch under high pH conditions, so it is useful for textile industries and as ingredients in alkaline detergents products[11,29,32].

(2) Effect of temperature on enzyme activity and stability

The activity of the purified α-amylase was measured at different temperatures at pH 6.0 by the standard assay

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method. The optimum temperature for α-amylase from *Halobacterium sodomense* was 55°C (Figure 5). The thermostability was tested by preincubating the enzyme for 1 h and the residual activity was measured. The activity was still retained up to 70°C at pH 6.0. However, it began to lose 54 % of its total activity at around 85°C and was only able to retain 23 % of its remaining activity at 90°C (Figure 5). The optimal temperature for the α-amylases from *Halobacterium sodomense* was in the same range as the optimal temperature 55°C reported for the enzyme from *Natronococcus sp*. strain Ah-36[20]. The enzymes also showed thermostability, but their stability decreased sharply at temperatures above 80°C. The temperatures reported in these work are higher than the ones reported for haloalkaliphilic α-amylase from *H. hispanica* (50°C)[15].

The effects of metal ions on the activity of H. *sodomense* amylase showed the enzyme as a Na^+ dependent enzyme. The amylase was activated by K^+, Mn^{2+}, Mg^{2+}, and Fe^{2+}. However, it was inhibited by Ca^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}, and EDTA (TABLE 7). In accordance with the present study, most amylases activity were inhibited in the presence of Ni^{2+}, Cd^{2+}, Cu^{2+}, Ag^+, Pb^{2+}, Fe^{2+}, and Zn^{2+}. For example, the α-amylases from *Bacillus firmus*[16] and *Bacillus sp.* strain KSM-1378[5] were inhibited by Ni^{2+}, Cd^{2+}, Zn^{2+}, and Hg^{2+} and the α-amylases from *B. subtilis*, *B. amyloliquefaciens* I, and *B. amyloliquefaciens* II were inhibited by Zn^{2+}, Ag^+, Cu^{2+}, and Fe^{2+}[35].

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