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Characteristics of a Novel Highly Thermostable and Extremely Thermophilic Alkalitolerant Amylase from Hyperthermophilic *Bacillus* Strain HUTBS71

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Abstract: Problem statement: This study reported the purification and characterization of a novel highly thermostable alkaline amylase from a newly isolated *Bacillus* strain HUTBS71. **Approach:** The enzyme was purified using ammonium sulfate precipitation, ion exchange and gel filtration chromatography. **Results:** Maximum amylase activity (72 U mL^{-1}) was obtained at 100°C after 10 min of incubation. The enzyme was purified 24 fold with 12.5% yield and showed a monomer band with a molecular weight of 58.8 kDa by SDS-PAGE. This enzyme exhibited maximum activity at pH and temperature, 7.8 and 100°C, respectively. It performed stability over a broad range of pH and temperature, 5.2-10.0 and 80-115°C, respectively. The half-life of the enzyme at 90 and 100°C was estimated to be 3 h. The activation energy of denaturation of purified enzyme was 2.53 kJ moL^{-1}. The enzyme was activated by 5 mM of $CoCl_2$, $MgSO_4$, $MnCl_2$, $ZnSO_4$ and $MnSO_4$ (relative activity was 133, 126, 133, 106.6 and 103%, respectively). It was strongly inhibited by $CuSO_4$ and $CdCl_2$ but less affected by NaCl, $CaCl_2$, $FeCl_3$, $ZnCl_2$ and EDTA. **Conclusion:** The present purified amylase therefore could be defined as a highly thermostable, extremely hyperthermophilic and alkalitolerant with new properties make the present enzyme applicable for many starch processing and food industries.

Key words: *Bacillus*, alkalitolerant, amylase, hyperthermophilic, high thermostability

INTRODUCTION

The discovery of hyperthermophilic bacteria has provided a valuable tool for the analysis of protein stability. The intrinsic thermal stability of the enzymes isolated from these sources makes it possible to study the molecular mechanisms governing structure and function in a system adapted for elevated temperatures. The thermostability exhibited by these enzymes is maintained without any components unique to thermophiles, suggesting that the increase in molecular stability is accomplished through the same stereochemical interactions found in their mesophilic counterparts. Through analysis of these enzymes it should be possible to determine the stabilizing interactions by which the enzymes maintain activity at extreme temperatures[1].

Alkaline amylases are a favorable choice for thermophilic enzymes due to the wealth of data currently available about this enzyme family. The industrial importance of this enzyme also makes it a popular subject for study. Horikoshi[2] first reported an alkaline amylase of an alkalophilic *Bacillus* sp. strain A-40-2. Alkaline amylases have been identified from a variety of species such as *Bacillus subtilis* JS-2004[3], *Bacillus* sp. A3-15[4], *Bacillus* sp. GM8901[5] and *Bacillus* sp. NRRL B-3881[6].

A thermophilic *Bacillus* strain HUTBS71 is a facultative anaerobes with an optimal growth temperature of 70°C, isolated by Hazem and Manar[7] from hot-spring located near to the Dead Sea, Jordan. Amylase activity has been reported in the cell homogenate and growth medium of *Bacillus* strain HUTBS71[8] but purification was not completed. In this report we present data on the purification of an amylase from *Bacillus* strain HUTBS71 and effect of different environmental factors on its properties.

MATERIALS AND METHODS

Bacterial strain and enzyme production: *Bacillus* strain HUTBS71, used in the present study, was isolated from water samples obtained from hot spring near to the Dead Sea, Jordan. The strain was left to

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grow in a medium containing (w/v): 0.5% yeast extract, 1.0% soluble starch, 4% casein, 2.5% NaCl, 0.02 g L^{-1} $CaCl_2$ and 0.01 g L^{-1} $MgSO_4$. The culture was incubated in bioreactor (Electrolab 351 EMC, Switzerland) at 50°C for 50 h under a continuous stir (at 100 rpm) and then centrifuged at 14000 × g for 30 min at 25°C and the clear supernatant was recovered. The crude enzyme supernatant was assayed for proteolytic activity and used for further purification.

Purification of thermostable amylase: All works were carried out at 25°C. The strain was grown at 50°C for 50 h, centrifuged at 14000 × g for 30 min and the supernatant retained as the source of extracellular enzyme. The supernatant was precipitated with ammonium sulfate. After 20 min, the precipitate was collected using centrifugation at 14000 × g, the pellet obtained resuspended in minimum volume of 66 mM Tris-HCl buffer at pH 7.8 and dialyzed in the same buffer. The dialyzed sample was passed through a Sephadex G 100 column (Fluke, Switzerland; 1.5×27 cm) equilibrated with 66 mM Tris-HCl buffer pH 7.8. The flow rate was 0.3 mL min^{-1} and 3 mL fractions were collected and analyzed for protein content and amylase activity. Fractions with high amylase activities were pooled, concentrated by filter tube (Sartoruis, Germany) and passed through DEAE-Sepharose fast flow column (Pharmacia, Biotechnology, Uppsala, Sweden) equilibrated with 66 mM Tris-HCl buffer pH 7.8 and eluted with 10 mM sodium chloride. Again, the flow rate was 0.3 mL min^{-1} and 3 mL fractions were collected and analyzed for protein content and amylase activity. Fractions with high amylase activities were again pooled and concentrated by filter tube. The sample was examined by sodium-dodecyl sulfate (7.5%) polyacrylamide gel electrophoresis (SDS-PAGE). The total protein was determined by the method of Lowry *et al.*[9].

Polyacrylamide gel electrophoresis: Polyacrylamide gel electrophoresis was carried out at 7.5% (w/v) isolation gel and 10% (w/v) concentration gel according to the method of Laemmli[10] with 2 mercaptoethanol was used as reducing agent. The molecular weight was determined by interpolation from a linear semi-logarithmic plot of relative molecular mass versus the R_f value (relative mobility) on Bio-Rad Mini Protein Cell with Promega molecular weight standards 25, 35, 50, 75, 100, 150 and 225 kDa (Promega, Wisconsin, USA).

Amylase assay: The activity of a-amylase was assayed by incubating 0.5 mL of the diluted enzyme (0.55 mg mL^{-1}) with 0.5 mL soluble starch (0.5%, w/v) prepared in 0.05 M Tris-HCl buffer, pH 7.8. After incubation at 90°C for 10 min the reaction was stopped and the reducing sugars released were assayed calorimetrically by the addition of 1 mL of 3-5-dinitrosalicylic acid reagent[11]. One enzyme activity Unit (U) was defined as the amount of enzyme releasing 1 µmol of glucose from the substrate in 1 min at 90°C.

Effect of pH-values on amylase activity: The effect of pH on the activity of amylase was measured by incubating 0.5 mL of the diluted enzyme (0.55 mg mL^{-1}) and 0.5 mL of buffers presenting pH from 4.6-9.0, containing 0.5% soluble starch for 30 min at 100°C. The buffers used were: Sodium acetate buffer, pH 4.6-5.6; sodium phosphate buffer, pH 6.8-7.5; Tris-HCl buffer, pH 7.8-9.0 and sodium carbonate, pH 9.6-10.8. The stability of the enzyme at different pH values was also studied by incubating the enzyme at various pH values ranging from 4.6-9.0 for 2 h and then estimating the residual activity.

Effect of temperature on amylase activity and stability: The effect of temperature on the enzyme activity was determined by performing the previously described standard assay procedure for 10 min at pH 7.8 within a temperature range of 25-120°C. Thermostability was determined by incubation of the lyophilized enzyme at temperatures ranging from 50-100°C for 8 h in a constant-temperature water bath. After treatment the residual enzyme activity was assayed.

Effect of various metal ions on amylase activity: The effect of metal ions on a-amylase activity was measured incubating the enzyme at 100°C for 2 min with various metal ions at a concentration of 5 mM. The enzyme assay was carried out in the presence of Ca^{2+}, Zn^{2+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Na^{2+} and Cd^{2+} chlorides and Mg^{2+}, Zn^{2+}, Mn^{2+} and Cu^{2+} sulfates.

Effect of different concentrations of EDTA on amylase activity: Purified enzyme preparation was pre-incubated in 3.71 mM Tris-HCl buffer, pH 7.8 containing various EDTA concentrations ranged from 0-15 mM for 30 min at 100°C in the assay mixture and activity of the pure enzyme was measured.

RESULTS AND DISCUSSION

The purification of amylases is important from the perspective of developing a better understanding of the functioning of the enzyme[12]. Precipitation is the most commonly used method for the isolation and recover of

proteins from crude biological mixtures[13]. It also performs both purification and concentration steps. Precipitation by ammonium sulfate is used in acidic and neutral pH solutions[14]. The obtained data showed that the fractions from 60% ammonium sulfate saturation correlated with high proteolytic and specific activities compared with the crude amylase and other concentrations. Similar concentration of 60% saturation ammonium sulfate was used by Carvalho *et al*.[15] to precipitate amylase from thermophilic *Bacillus* sp. strain SMIA2. Whereas, amylase from *Bacillus* strain GM8901 precipitated by 80% saturation of ammonium sulfate[5].

The purification procedures of the amylase secreted by tested bacteria are shown in Table 1. The results showed that the enzyme was purified 6.9 fold with a specific activity of 2487 U mg^{-1} proteins after ammonium sulfate fractionation. The enzyme was then purified with Sephadex G-100 and showed 18 fold enzyme purification with a specific activity of 6535 U mg^{-1} protein. The final purification step presented 24-fold enzyme purification with a specific activity of 8716 U mg^{-1} proteins. These results indicated that the effectiveness of purification method applied in this research. However, the yield of the enzyme after purification was found to be low (12.5%) (Fig. 1 and 2). This might be due to the result of autolysis of the enzyme in each purification step.

Analysis of the purified enzyme by SDS-PAGE revealed a monomer band with a molecular mass of 58.8 kDa (Fig. 3). Vieille and Zeikus[16] reported that the molecular mass for amylase range from 50-60 kDa. Different molecular masses for different amylases have been reported by gel filtration: 76-53 kDa for *Bacillus* WN11[17]; 56 kDa for *Bacillus* sp. YX-1[18]; 86-60 kDa for *Bacillus* A3-15[4] and 97 kDa for *Bacillus* GM8901[5] (Table 2).

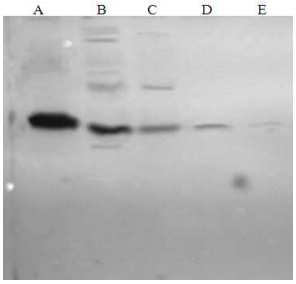

Fig. 1: PEG (7.5%) patterns of proteins isolated at different purification steps from *Bacillus* HUTBS71 strain stained with Coommassie brilliant blue R-250. Where; Lane (A): Standard amylase (Marmar Bio. Inc, USA); Lane (B): Media supernatant; Lane (C): Ammonium sulfate fraction; Lane (D): Gel filtration fraction; Lane (E): Ion exchange chromatography fraction

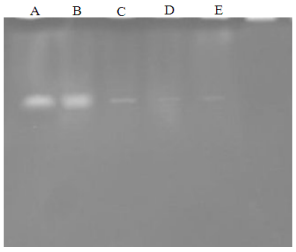

Fig. 2: PEG (7.5%) of amylase purified from Bacillus strains HUTBS71 at different purification steps. The bands of protein that were associated with amylase activity were seen as clear and colorless zones with a blue background. Where, Lane (A): Media supernatant; Lane (B): Ammonium sulfate fraction; lane C: Gel filtration fraction; Lane (D and E): Ion exchange chromatography fraction

Table 1: Purification steps of amylase from *Bacillus* strain HUTB71

Purification steps	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U $mg^{-1)}$	Yield (%)	Purification (fold)
Culture medium	100	3286	9.200	357	100.0	1.0
60% ammonium sulfate precipitation	12	789	0.317	2487	24.0	6.9
Sephadex-G100 gel filtration	8	592	0.090	6535	18.0	18.0
DEAE-chromatography	5	411	0.047	8716	12.5	24.0

Table 2: Comparison of various characteristics for amylase from *Bacillus* sp. strain HUTBS71 with other *Bacillus* species

Source	Optimum pH	pH stability	Optimum temperature (°C)	MW (kDa)	References
Bacillus HUTBS71	7.8	5.2-10.0	100	58.8	This study
Bacillus YX-1	5.0	4.5-11.0	40-50	56	Liu and Yan[18]
Bacillus GM8901	10.5	6.0-13.0	50	97	Kim *et al*.[5]
Bacillus subtilis JS-2004	8.0	5.5-10.0	70	-	Asgher *et al*.[3]
Bacillus ANT6	10.5	9.0-13.0	100	-	Burhan *et al*.[21]
Bacillus WN11	5.5	5.6-9.0	75-80	76; 53	Mamo and Gessesse[17]
Bacillus A3-15	8.5	6.0-12.0	60	86; 60	Burhan[4]
Bacillus licheniformis 07	7.5	6.0-7.0	70	-	Iraj *et al*.[19]

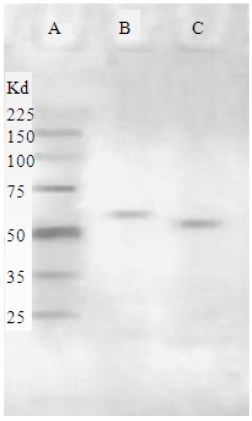

Fig. 3: SDS-PAGE of amylase from HUTBS71. Where, Lane (A): Protein marker: 25 kDa, trypsinogen; 35 kDa, carbonic anhydrase; 50 kDa, ovalbumin; 75 kDa, bovine serum albumin; 100 kDa, phosphorylase b; 150 kDa, albumin; Lane (B): Purified amylase from HUTBS71 and Lane (C): Purified amylase from HUTBS62

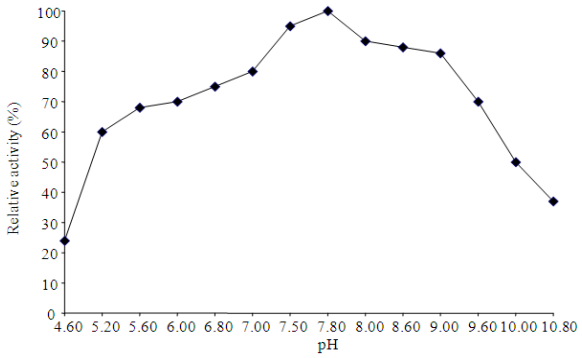

Fig. 4: Effect of pH-values on the amylase activity from *Bacillus* strain HUTBS71 at temperature 100°C

Most of the *Bacillus* amylase have optimum pH values of from 5.0-8.5[3,4,18,19] and an acidic amylase from *Bacillus acidocaldarius* has an optimal pH of 3.5[20]. Alkaline amylases reported previously had optimum pH values of 9.0 to 11.0[5,6,21,22]. However, our amylase has an optimal pH value of 7.8 and is stable in an acidic and an alkaline pH range of 5.2-10.0; more than 50% enzyme activity remained after 30 min of incubation in this pH values, indicating that the enzyme is an alkalitolerant (Fig. 4). The comparison of various characteristics of alkaline amylase from *Bacillus* strain HUTBS71 with other *Bacillus* species are shown in Table 2.

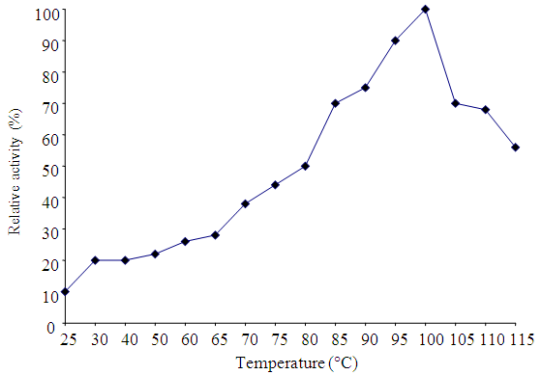

Fig. 5: Effect of temperature on amylase activity for *Bacillus* strain HUTBS71. The activity of the purified enzyme was measured in 50 mM Tris-HCl buffer pH 7.8 for 10 min at different temperatures

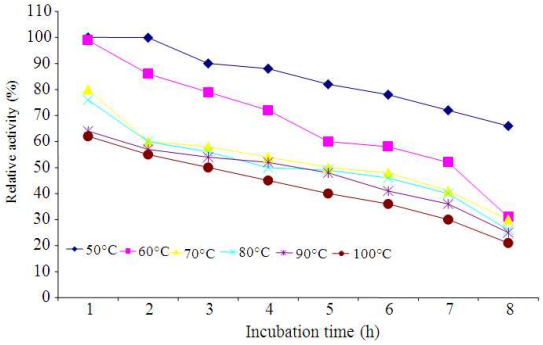

Fig. 6: Effect of temperature on the thermostability of the amylase purified from *Bacillus* strain HUTBS71. Activity of the purified enzyme was measured after incubation for designated time and perform enzyme assay as usual in 50 mM Tris-HCl buffer pH 7.8 at 100°C

The supernatant amylolytic activities were assayed at different temperatures ranging from 25-120°C at optimum pH (Fig. 5). Enzyme activity increased with temperature within the range of 80-115°C. A reduction in enzyme activity was observed at temperatures less than 75°C and above 115°C. The optimum temperature of amylase activity was 100°C, which is comparable to that described for other *Bacillus* amylase[23,24]. Our enzyme is extremely thermophilic when compared with other *Bacillus* species: 40[18]; 50[5]; 60[4] and 70°C[3,19] (Table 2).

The heat stability of amylase in the range of 50-100°C was studied for a period 1-8 h and results are seen in Fig. 6. Thermostability profile indicated that the enzyme retained 100% of its activity at 50 and 60°C

when incubated 1 h, respectively. After incubation for 3 h at 90 and 100°C, the enzyme retained 52 and 50% of the original activity, respectively; on the other hand 4 h incubation at 70 and 80°C, the enzyme retained 54 and 52% of the original activity, respectively. Thermostability for 1 h at 80°C has been reported for amylase from *Bacillus subtilis* JS-2004[3]. *Bacillus licheniformis* CUMC305 amylase was stable after 4 h incubation at 100°C[25]. *Bacillus* species ANT6 amylase was stable after overnight (85%) and 24 h (55%) incubation at 100°C and pH 10.5[21]. Carvalho *et al.*[15] reported that the enzyme was stable for 1 h at temperatures ranging from 40-50°C while at 90°C, 66% of its maximum activity was lost. A novel strain *Bacillus licheniformis* Shahed-07 had a highly thermostable amylase. The optimum temperature and pH for the activity of this enzyme was 70°C and 7.5[19]. Amylases from *Bacillus* genus are heat stable and this is a desirable property for industrial starch liquefication[3,19]. The activation energy (Ea) for denaturation of purified enzyme was calculated from the slope of Arrenhenius plots to be 2.53 kJ mol^{-1} (Fig. 7). Alkalitolerant, high stability, extreme thermophilic and a longer shelf-life of the enzyme produced by *Bacillus* HUTBS71 could be encouraging factors to consider further studies on its industrial application.

The activity of *Bacillus* HUTBS71 amylase buffered with 3.71 mM Tris-HCl (pH 7.8) was assayed in the presence of 5 mM of various metal ions at 100°C for 20 min. As shown in Table 3, the enzyme was activated by $CoCl_2$, $MnCl_2$, $MgSO_4$, $ZnSO_4$ and $MnSO_4$ with the residual activity 133, 113, 126, 106.6 and 103%, respectively. On the other hand, the enzyme activity was strongly inhibited by $CuSO_4$ and $CdCl_2$ (with relative activity 46.6 and 6.6%, respectively) but was slightly inhibited by NaCl, $CaCl_2$, $ZnCl_2$ and $FeCl_3$ resulting in relative activity of 88, 71, 70 and 60%, respectively. Most of amylases are known to be metal ion-dependent enzymes. Calcium was reported to increase amylase activity of *B. stearothermophilus*[26], *Bacillus* strain TS-23[27] and *Bacillus* strain GM8901[5]. The stabilizing effect of Ca^{+2} on thermostability of the enzyme can be explained due to the salting out of hyrophobic residues by Ca^{+2} in the protein, thus, causing the adoption of a compact structure[28]. Calcium-independent enzymes have also been reported. Malhotra *et al.*[29] reported that the amylase from *Bacillus Thermoleovorans* NP54 did not require calcium ions for its activity or production. Similar result was reported by Najafi *et al.*[30] for amylase from *Bacillus subtilis* AX20. The effect of Zn^{+2} also varied between amylases. For instance, it had a potent inhibitory effect on the amylases from *Bacillus* SMIA2 and *Bacillus* A3-15[4,15]; on the other hand, slight inhibition in the activity of the enzyme observed in *Bacillus cohnii* US147 and *Bacillus subtilis* JS-2004[3,31]. Whereas it could have no effect at all on the enzyme of anaerobic archaeon strain HJ21 and *Aspergillus kowashii*[32,33]. The effect of other metal ions on the activity of amylase in *Bacillus* was also investigated by investigators. Cobalt, cadmium, copper and zinc ions strongly inhibited the enzymatic activity from *Bacillus subtilis* JS-2004, *Bacillus* sp. strain SMIA2 and *Bacillus* sp. strain KSM-1378[3,15,34], suggesting that could be due to competition between the exergnous cations and the protein-associated cations.

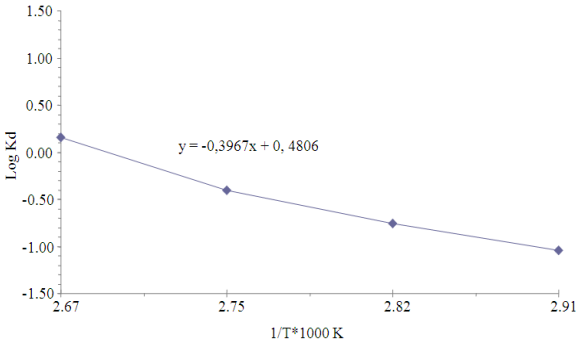

Fig. 7: Arrhenius plot for amylase purified from *Bacillus* strain HUTBS71

Table 3: Effect of metal ions and EDTA on amylase activity of *Bacillus* strain HUTBS71. Activity of amylase from HUTBS71 was determined in the presence of 5 mM of metal ions in 3.71 mM Tris-HCl (pH 7.8) and assayed at temperature 100°C for 30 min. Control for each was the activity in the absence of comparable ion

Metals and chelating agents (5 mM)	Percentage of activity	Enzyme activity (U mL^{-1})
Control (no addition)	100.0	72.0
Ions with chloride:		
$CoCl_2$	133.0	93.6
$MnCl_2$	113.0	81.6
NaCl	88.0	63.4
$CaCl_2$	71.0	51.1
$ZnCl_2$	70.0	50.4
$FeCl_3$	60.0	45.6
$CdCl_2$	6.6	13.6
Ions with sulfur:		
$MgSO_4$	126.0	91.2
$ZnSO_4$	106.6	76.8
$MnSO_4$	103.0	74.4
$CuSO_4$	46.6	38.4

Table 4: Comparison of various effects of metal ions on amylase activity from *Bacillus* sp. strain HUTBS71 with other *Bacillus* species (each assay was under different conditions)

	Bacillus strain HUTBS71 100°C for 30 min (5 mM)	*Bacillus Subtilis* JS-2004 60°C for 60 min (1 mM)	*Bacillus* strain GM8901 60°C for 2 h (1 mM)	*Bacillus* strain SMIA2 90°C for 2 min (5 mM)	*Bacillus* strain A3-15 60°C for 60 min (5 mM)
$CoCl_2$	133.0	46	129	-	-
$MnCl_2$	113.0	-	-	28	-
NaCl	88.0	-	-	90	80
$CaCl_2$	71.0	117	154	100	130
ZnCl2	70.0	-	105	70	82
$FeCl_3$	60.0	-	95	-	-
$CdCl_2$	6.6	-	-	-	-
$MgCl_2$	-	-	132	90	-
$CuCl_2$	-	-	120	-	-
$MgSO_4$	126.0	88	-	-	-
$ZnSO_4$	106.6	85	-	-	-
$MnSO_4$	103.0	91	-	-	-
$CuSO_4$	46.6	32	-	10	-
$FeSO_4$	-	70	-	-	-
$CoSO_4$	-	-	-	19	-
References	This study	Asghar *et al*.[3]	Kim *et al*.[5]	Carvalho *et al*.[15]	Burhan[4]

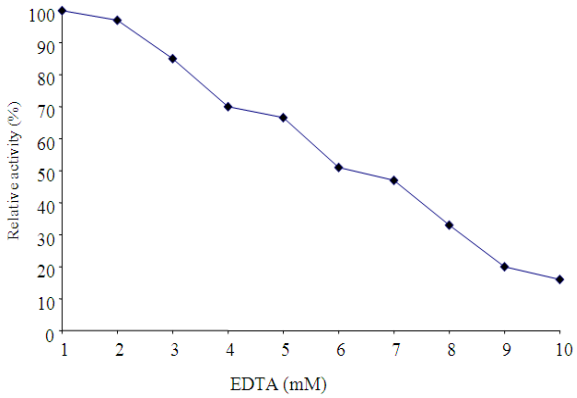

Fig. 8: Effect of different concentrations of EDTA on amylase activity from *Bacillus* strain HUTBS71. Assay was performed in 50 mM Tris-HCl buffer, pH 7.8 containing 0-15 mM EDTA and 100°C. The absence of EDTA from the reaction mixture was used as a control

On the other hand, The enzymatic activity was slightly inhibited in *Bacillus cohnii* US147 and *Bacillus subtillis* JS-2004 in the presence of zinc[3,31]; *Bacillus* KSM-1378 and *Bacillus* TS-23 in the presence of cadmium[27,34]. In addition, a comparison of various effects of metal ions on amylase activity from *Bacillus* strain HUTBS71 with other *Bacillus* species (Each assay was under different conditions) are shown in Table 4.

Our results indicated that the presence of EDTA had strongly inhibitory effect on amylase activity at concentration over 8 mM (Fig. 8). But the enzyme activity retained 100, 97, 85 and 70% activity in the presence of 1, 2, 3 and 4 mM EDTA, respectively. The relative activity of the enzyme was slightly inhibited with 66.6% in the presence of 5 mM EDTA.

Hagihara *et al*.[35] reported that the amylase from *Bacillus* K38 retained full activity in the presence of 1 mM EDTA; while Egas *et al*.[36] reported 88% with 10 mM EDTA. While, Burhan[4] reported 5% activity with 5 mM EDTA.

CONCLUSION

The results of pH and temperature stability in the presence of such high salt and ionic metals concentration of the investigated alkalitolerant amylase from thermophilic *Bacillus* strain HUTBS71 suggesting that it may have potential application in industrial food biotechnology. Further work on this amylase towards cloning and expression of gene responsible for stability is currently underway.

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