Purification and characterization of amylase from local isolate *Pseudomonas sp.*SPH4

تنقية وتوصيف انزيم الاميليز من العزلة المحلية لبكتيريا الـ *Pseudomonas sp.* SPH4

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Abstract

The amylase produced from local isolate *Pseudomonas sp.* SPH4 was purified by precipitation with 30% saturation ammonium sulphate, followed by ion-exchange chromotography using DEAE-cellulose column, and Gel filtration using Sephacryl S-300 column.The two iso-enzymes (a, b) were purified to (2.83, 3.47) times in the last step with an enzymes yields of (32.36, 76.34)% respectively. Enzyme characterization of the two iso-enzymes indicated that the optimum pH for the two iso-enzymes a and b were (7, 7.5) respectively, while the optimum pH for the iso-enzymes stability were (6.5, 7) respectively. The maximum activity for iso-enzymes (a, b) appeared at 45ºC and stable for 15 min at 30-50ºC and lost approximately 50% of it's activity at rang above 75ºC. Enzyme characterization results showed that the chlorides of silver and mercury had inhibitory effect on enzyme activity, the remaining enzyme activity for the iso-enzymes (a, b) were (46.66, 36.36)% for silver ions and (41.33, 33.63)% for mercury ions at 5 mM respectively, and (28, 28.18)% for silver ions and (25.33, 19.09)% for mercury ions at 10 mM respectively. The iso-enzymes a and b were affected by chelating agent ethylene diamine tetra acetic acid (EDTA) at concentration 2mM the remaining activity (45.33, 43.63)% respectively, and 5mM the remaining activity (28, 28.18)% respectivily, and these iso-enzymes (a, b) refered to metalloenzymes. The iso-enzymes (a, b) were kept their activity when treated by reducing agent (2-mercaptoethanol) at 2 mM the remaining activity (92, 92.72)% respectively, and 5 mM the remaining activity (85.3, 89.09)% respectivily. The iso-enzymes (a, b) were kept their activity when treated by phenyl methyl sulphonyl fluoride (PMSF) at concentration 1mM the remaining activity (93.33, 90.90)% respectivily,and 5 mM the remaining activity (90.66, 87.27)% respectivily, and these indicated that these iso-enzymes didnot referred to serine amylases group.

المستخلص

نقي انزيم الاميليز من العزلة المحلية لبكتيريا الـ *Pseudomonas sp.*SPH4 بخطوات عدة تضمنت الترسيب بكبريتات الامونيوم بنسبة اشباع 30% وتقنية كروموتوغرافيا التبادل الايوني و الترشيح الهلامي . لوحظ ظهور متناظرين للانزيم (a،b) عند خطوة التبادل الايوني باستعمال المبادل الايوني DEAE-cellulose والترشيح الهلامي بواسطة هلام Sephacryl S-300 و قد بلغ عدد مرات التنقية للمتناظرين 2.83(2.83، 3.47) بحصيلة انزيمية مقدارها(32.36 ، 76.34)% على التوالي . بينت نتائج التوصيف للمتناظرين (a ، b) ان الارقام الهيدروجينية المثلى لفعالية المتناظرين (a ، b) ضمن 7 ،7.5 على التوالي ، وتراوح الرقم الهيدروجيني الامثل لثبات المتناظرين 6.5 ، 7 على التوالي . وظهرت اعلى فعالية للمتناظرين عند درجة 45 م . واحتفظ المتناظران بكامل فعاليتهما عند حضنهما مدة 15 دقيقة في درجة حرارة (30-50)م بينما فقدا 50% من فعاليتهما عند حضنهما بدرجة حرارة اعلى من 75م . اوضحت نتائج تاثير بعض

المركبات في فعالية الانزيم ان لكل من كلوريد الزئبق والفضة تاثيرا تثبيطيا واضحا في فعالية المتناظرين و لم تتجاوز الفعالية الانزيمية المتبقية لهما عن (46.66 ، 36.36)% بالنسبة لايونات الفضة و (41.33 ، 33.63)% بالنسبة لايونات الزئبق عند التركيز 5 ملي مولر، على التوالي ولم تتجاوزالفعالية الانزيمية المتبقية لهما عن (28 ، 28.18)% بالنسبة لايونات الفضة و (25.33 ، 19.09)% بالنسبة لايونات الزئبق عند التركيز 10 ملي مولر، على التوالي . وتاثيرا مثبطا للمادة الكلابية الـ (EDTA) Ethylene Diamine Tetra Acetic acid عند معاملةالمتناظرين بـ 2 ملي مولر من المادة الكلابية اذ لم تتجاوز الفعالية الانزيمية المتبقية لهما عن (45.33، 34.63)% على التوالي ، و5 ملي مولر من المادة الكلا بية اذ لم تتجاوز الفعالية الانزيمية المتبقية لهما عن (28 ، 28.18)% على التوالي مما يدل ان الانزيم من الانزيمات المعدنية (Metalloenzymes) . فضلا عن ذلك فقد احتفظ المتناظرين (a،b) بفعاليتهما تقريبا عند معاملتهما بـ 2 ملي مولرمن المركبتوايثانول 2-mercaptoethanol اذ لم تتجاوز الفعالية الانزيمية المتبقية لهما عن (92 ، 92.72)% على التوالي ، و 5 ملي مولرمن المركبتوايثانول mercaptoethanol -2 اذ لم تتجاوز الفعالية الانزيمية المتبقية لهما عن (85.3 ، 89.09)% على التوالي . واحتفظ المتناظرين (a ، b) بفعاليتها تقر يبا عند معاملتهما بـ 1 ملي مولر من المركب phenyl methyl (PMSF) sulphonyl flouride اذ لم تتجاوز الفعالية الانزيمية المتبقية لهما عن (93.33 ، 90.90)% على التوالي ، و5 ملي مولر من المركب phenyl methyl sulphonyl flouride (PMSF) اذ لم تتجاوز الفعالية الانزيمية المتبقية لهما عن (90.66 ، 87.27)% على التوالي مما يؤكد عدم انتماء المتناظرين الى مجموعة الاميليزات السيرينية .

Introduction

Amylases are enzymes which hydrolyze the starch molecules into simple polymers composed of glucose units. Amylases are ubiquitous in distribution, with plants, bacteria and fungi being the predominat sources [1]. Amylases hydrolysis the α-1, 4 glycosidic linkage of starch in a random endo-amylolytic fashion [2]. Amylases find potential application in a number of industrial processes such as in the food, fermentation, textiles and papers industries. Amylases were isolated from numerious bacteria such as *Pseudomonas amylodermosa*, which has the highest producing ability, *Bacillus amyloliquefaciens, B.stearothermophilus, B.licheniformis, B.subtilis, B.flavothermus, Escherichia coli, Lactobacillus amylovorus* and *Flavobacterium sp*. [3]. Alpha-amylase was purified from *Thermus sp*. till homogenity,the fold of purification was 360 [4].While in *Bacillus sp*. alpha-amylase was purified by concentration of crude extract first by using ultrafiltration, then passed on ion-exchange (Hi trap column), then pass on gel filtration column (Sephacryl S-300) folds of purification were 209 fold and the enzyme recovery was 13.2% [5]. Amylase enzyme was purified from *Pseudomonas stutzeri* by MO-precipitation (20-40)%, ion-exchange chromatography with (DEAE-Toyopeal) 650S, the purity of each fraction containing enzyme activity was checked by sodium dodocyl sulphate-polyacrylamide gel electophoresis and the fraction giving a single protein band were pooled and used for further studies [6]. The effect of some compounds in enzyme activity was monitered that different metal ions had different effect on the activity of amylase. Many studies showed that calcium ions have the large effect on amylase activity and stability and made the amylase more stable in high temperature [7]. All studies showed that the metal ions such as silver, mercury, aluminum, and copper have inhibitory effect on amylase activity [3]. The effect of different chemical materials in amylase activity such as PMSF (Phenyl Methyl Sulphonyl Fluoride). The activity of alpha-amylase produced from *Bacillus sp*. didn't affected by 10 mM of this

compound [5]. Studies the effect of chelating EDTA (Ethylene Diamine Tetra Acetic Acid) on amylase produced from *Bacillus sp.*K-12 it has inhibitor effect on amylase activity [8]. The activity of amylase produced from *Bacillus stearothermophilus* didn´t affect by using the reducing agent (ß-mercaptoethanol) [9]. This study aimed to purification and characterization of amylase from local isolate *Pseudomonas sp*.SPH4.

Materials and methods

Isolation of amylase enzyme

Amylase enzyme was extracted from local isolate *Pseudomonas sp*. SPH4 identified in previous study [10]. After inoculating the isolate in the production media composed of the starch as carbon source 1%, ammonium sulphate as nitrogen source 1%, calcium chloride 0.05%, sodium chloride 0.05%, magnesium phosphate 0.05%, and di-hydrogen sodium phosphate 0.16% and the pH was adjusted at 8. The production media was inoculated with 1×10^8 cell/ml, after 24 hours of incubation in 37 ºC shaker incubator at 150 rpm/min, cultures was centrifuged at 6000 rpm for 20 min. Enzyme activity and protein concentration were then estimated in the crude filtrates. Activity of amylase was estimated according to reducing sugar liberated after hydrolysis of starch by the enzyme [11, 12]. Protein concentration was estimated according to the procedure of [13] .The unit of enzyme activity was defined as the amount of enzyme that liberates 1mM of reducing sugars (maltose) in one minute at reaction conditions[14].

Purification of amylase

Precipitation by ammonium sulphate

The supernatant (crude extract) was fractionated with ammonium sulphate at (20, 30, 40, 50, 60, 70, 80)% saturation then the precipitate obtained by centrifugation at 10000 rpm for 30 min was suspended in 5 ml of 0.05 M phosphate buffer pH7 and the enzyme activity and protein concentration were measured.

Ion exchange chromatography

DEAE-cellulose ionic exchange was prepared according to [15]. The sample obtained after ammonium sulphate precipitation with saturation ratio 30% after dialysis the crude enzyme in distilled water for 24 hr. then the concentrated enzyme solution was applied to a DEAE-cellulose column (2×18 cm) previously equilibrated with 50 mM phosphate buffer pH7. The column was washed with the same buffer and eluted with a linear salt gradient with the same buffer containing (0.1-1)M NaCl. The fractions were collected in test tubes at flow rate 30ml/hour. Protein concentration in each fraction was monitored spectrophotometrically at 280 nm. Fractions of the protein peaks were assayed for amylase activity. Fractions containing enzymatic activity were collected and concentrated for further experiment.

Gel filtration chromatography

The gel Sephacryl S-300 was prepared according to the instruction of the manufacturer (Pharmacia Fine Chemical). The fractions collected from DEAE-cellulose column chromatography were applied to a Sephacryl S-300column (2.5×37) cm previously equilibrated with 0.2 M phosphate buffer pH7. Elution was performed with the same buffer. The fractions were collected in test tubes at flow rate 30

ml/hour. Protein concentration in each fraction was monitored spectrophotometrically at 280nm. Fractions containing enzymatic activity were collected and stored in refrigerator for further characterization.

Enzyme characterization

Effect of pH on amylase activity

Buffer solutions of different pHs (4-10) were prepared 0.1 ml. of partially purified amylase was added to 0.9ml. of a buffer solution containing 1% soluble starch as a substrate, enzyme activity was assayed for each pH.

Effect of pH on amylase stability

Equal volumes of partially purified enzyme and buffer solution with pH ranging from (4,10) were incubated in a water bath at 35ºC for 30 min. Then transferred immediately into an ice bath. The enzymatic activity for each pH was measured.

Effect of temperature on amylase activity

Amylase activity was assayed at various temperature (25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80)ºC. Activity of amylase was plotted against the temperatures to determine the optimal temperature for amylase activity.

Effect of temperature on amylase stability

Equal volumes of partially purified enzyme were incubated for 15min at different temperature (25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80)ºC then immediately transferred into an ice bath. Enzymatic activity was measured and the remaining activity (%) was plotted against the temperature.

Effect of PMSF on enzyme activity

Equal volumes of partially purified enzyme were mixed with (1, 5) mM of PMSF (dissolved in methanol) and incubated in water bath at 35ºC for 15 min then immediately transferred into an ice bath. The remaining activities (%) were estimated.

Effect of metal ions, reducing and chelating agents on enzyme activity

Equal volumes of partially purified enzyme were mixed with (5, 10) mM of different metal salts ($CuSO_4$, AgCl , $HgCl_2$, $CaCl_2$) and incubated in water bath at 35ºC for 15 min.These metal ions were prepared by dissolving these metal salts in phosphate buffer pH7.

Equal volumes of partially purified enzyme were mixed with (2, 5) mM of chelating agent Ethylene Diamine Tetra Acetic acid (EDTA) and reducing agent (ß-mercaptoethanol) and incubated in water bath at 35Cº for 15 min. These compounds were prepared by dissolving these compounds in phosphate buffer pH7. The remaining activity (%) was estimated.

Results and discussion

Extraction and purification of enzyme

The crude amylase produced by the locally isolate *Pseudomonas sp.*SPH4 under the optimum conditions had specific activity 31.7 U/mg protein.

Precipitation of enzyme by ammonium sulphate

In order to concentrate the crude extract of amylase and remove a much of water and some protein molecules as possible, ammonium sulphate were used at (20, 30, 40, 50, 60, 70, 80)% saturation, the saturation ratio 30% was used. It achieved specific activity 58.33 U/mg protein, 1.84 purification fold with 88.33% yield. Protein

precipitation by ammonium sulphate depends on the salting out phenomenon. Since ammonium sulphate has the ability to neutralize charges at the surface of the protein and to disrupt the water layer surrounding the protein, it will eventually cause a decrease in the solubility of the protein which, in turn will lead to the precipitation of the protein by the effect of salt [16,17]. Ammonium sulphate are widely used because of its availability, high solubility, low cost and it stabilizes the proteins [18].

Ion-Exchange chromatography

Purification of amylase was done by ion-exchange chromatography by (DEAE-cellulose). Results in Table (1) showed amylase purified by using anion-exchange column chromatography using DEAE-cellulose. Figure (1) showed the wash and elution of DEAE-column. The wash step of DEAE-column contained two protein peaks without amylase activity, while the eluted fractions of DEAE-cellulose revealed three protein peaks one peak for protein and two peaks had amylase activity. This results indicates that *Pseudomonas sp.* SPH4 amylase have negative charge. Appearance of two peaks for amylase activity they reffered to the appearance of iso-enzymes of amylase. The fractions which had amylase activity were collected together giving specific activity of 66.66 U/mg protein with 2.1 fold of purification and 37.85% yield for the first peak (iso-enzyme a) (the fractions 91-100) and 78.94 U/mg protein with 2.94 fold of purification and 80.44% yield for the second peak (iso-enzyme b) (the fractions 101-112). These result are similar to those found by [19] who purified alpha-amylase from *Bacillus stearothermophilus* by DEAE-cellulose chromatography and which revealed two peaks of amylase activity in the elution step each peak have alpha-amylase activity.

Gel filtration chromatography

The fractions of two peaks containing amylase activity from DEAE-cellulose column chromatography were applied to a Sephacryl S-300 column. Figure (2, 3) showed that there are two peaks, with specific activity 90 U/mg proteins, fold of purification 2.83 and yield 32.36 %, for first peak and with specific activity 110 U/mg proteins, fold of purification 3.47 and yield 76.34% for second peak. Amylase produced from *Bacillus sp*. was purified by Hi prep Sephacryl S- 200, a fold of purification was 709 with a yield1 3.2% (5).

Table (1): Purification steps of amylase (iso-enzyme a and b) produced by *Pseudomonas sp.*SPH4

Steps of purification	Volume (ml)	Activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Total activity (U)	Fold of purification	Yield (%)
Crude extract	25	6.34	0.2	31.7	158.5	1	100
Precipitation by saturation ammonium sulphate 30%	8	17.5	0.3	58.33	140	1.84	88.33
Ion exchange chromatography by DEAE-cellulose (Iso-enzyme a)	15	4	0.06	66.67	60	2.1	37.85
(Iso-enzyme b)	17	7.5	0.095	78.95	127.5	2.49	80.44
Gel filtration chromatography by Sephacryl S-300 (Iso-enzyme a)	19	2.7	0.03	90	51.3	2.84	32.37
(Iso-enzyme b)	22	5.5	0.05	110	121	3.47	76.34

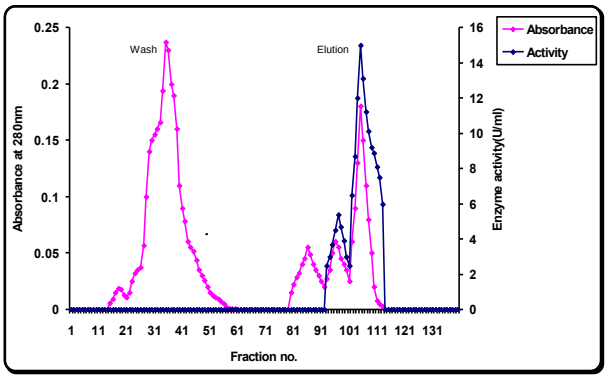
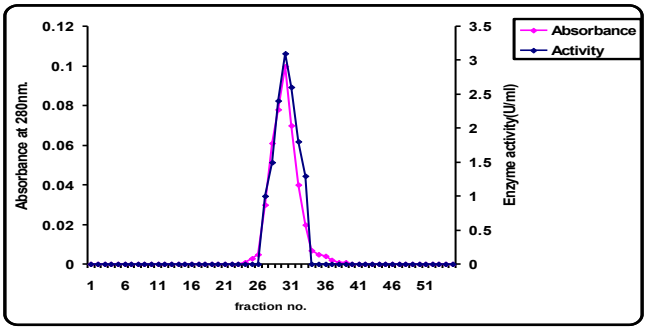


Figure (1): Purification of amylase from local isolate *Pseudomonas sp.*SPH4 by DEAE-cellulose ion-exchange chromatography column (2×18 cm) equilibrated with 0.05 M phosphate buffer pH 7, enzyme was eluted with linear salt gradient 0.1-1 M Nacl, flow rate 30 ml/hour



Figure(2): Gel filtration chromatography of amylase (iso-enzyme a) from local isolate *Pseudomonas sp.*SPH4 by Sephadex S-300 column (2.5×37cm) equilibrated with 0.2 M phosphate buffer pH 7,flow rate 30 ml /hour

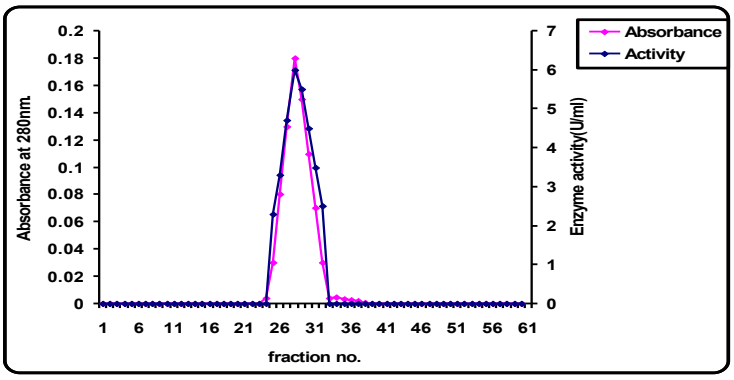


Figure (3): Gel filtration chromatography of amylase (iso-enzyme b) from local isolate *Pseudomonas sp*.SPH4 by Sephadex S-300 column (2.5×37cm) equilibrated with 0.2 M phosphate buffer pH 7, flow rate 30 ml/hour

Enzyme characterization

Optimum pH of amylase activity

Figure (4) showed the effect of pH on the activity of partially purified amylase from *Pseudomonas sp*.SPH4 was studied in pH rang from 4-10. Results showed that the maximum activity of amylase (iso-enzyme a,b) was at pH (7, 7.5), respectivily the activity was 6.5 U/ml for iso-enzyme (a) and 9.5 U/ml for iso-enzyme (b). pH has effect on the ionic state of enzyme by effecting on the amino acid chains necessary for tertiary structure of enzyme and hence its activity may varies. Higher or lower pH from the optimum will lead to denature the enzyme and losing it's activity [15, 16, 20]. The optimum pH for isoamylase activity from the yeast *Lipomyces kononenkoae* was 5.6 [21].

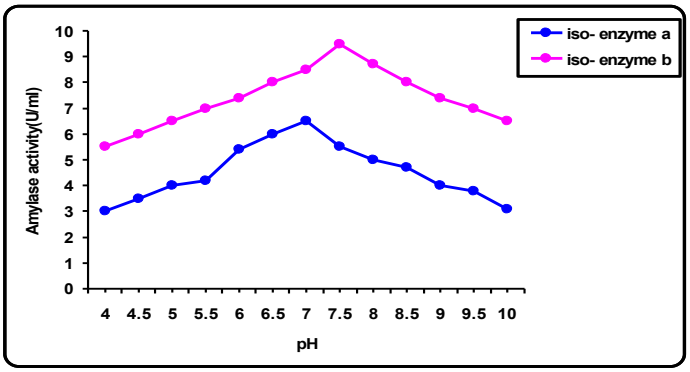


Figure (4): Effect of pH on purified of *Pseudomonas sp*.SPH4 amylase (iso-enzymes)

Effect of pH on amylase stability

In order to determine the optimum pH for amylase stability, the enzyme was incubated in buffer solution with pH range (4-10) at 35ºC for 15 min. Results indicated in Figure (5) showed that amylase (iso-enzyme a and b) high stability at pH range between (6.5-7.5) the activity of amylase (iso-enzyme a) was 7.5 U/ml and the activity of (iso-enzyme b) was 10.1 U/ml, in which it kept more than 90% of it's total activity. The effect of pH on the enzyme stability could be explained in the formation of ionic form of enzyme or the active sites, irreversible inactivation. The stability of the enzyme depends on many factors such as temperature, ionic strength, chemical nature of buffer, concentration of various preservatives, concentration of metal ions, substrate and enzyme concentration [16].Whitaker indicated that most amylase from

Pseudomonas sp. have optimum pH (3.5-5.5) [15]. The amylase produced from *Bacillus sp*. have a wide range of pH between (6-11) [5].

Effect of temperature on amylase activity

The results in Figure (6) indicated that the activity of amylase (iso-enzyme a and b) reaches the maximum (7.4, 10.2) U/ml respectively at 45ºC, and decreased to (1.9, 3.1) U/ml respectively at 80ºC, which may be attributed to the denaturation of the enzyme after incubation at high temperature due to structural and conformational changing of the protein molecule, this will influence the binding of enzyme and substrate [22]. The optimum temperature for isoamylase activity from *Pseudomonas amylodermosa* was 50ºC [23].

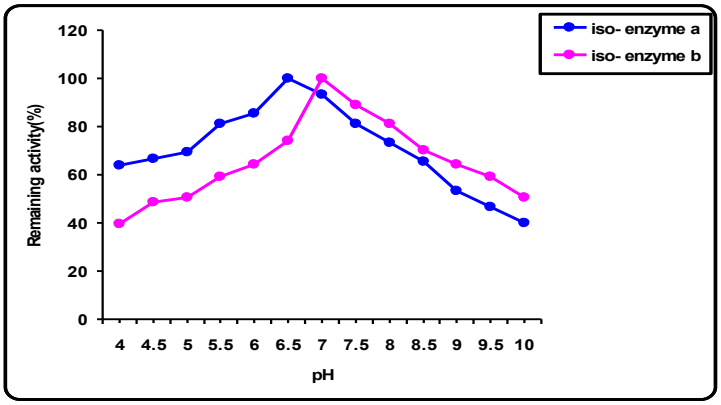


Figure (5): Effect of pH on amylase (iso-enzymes) stability purified from *Pseudomonas sp*.SPH4

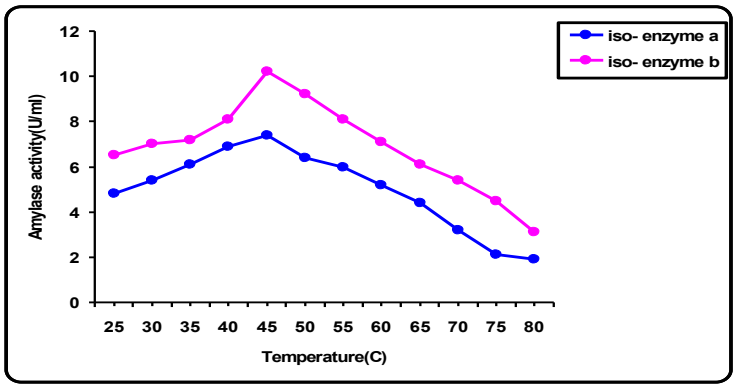


Figure (6): Effect of temperature on *Pseudomonas sp*.SPH4 amylase (iso-enzymes) activity

Effect of temperature on amylase stability

The stability of amylase iso-enzyme a and b from *Pseudomonas sp*. SPH4.was examined by enzyme incubation at various temperature (30- 80)ºC for 15min.The results in Figure (7) revealed that the iso-enzyme are stable at 30-50ºC, the activity declined at higher temperature, although at 60 ºC about 60% of the activity remained. The enzyme was suppressed at 80ºC, about 20% of the activity remained. The activity of amylase (iso-enzyme a) was 7.5 U/ml and the activity of amylase (iso-enzyme b) was 11.1 U/ml. The amylase produced by *Bacillus sp*. is stable at temperature over 60 ºC, and lost its activity at 95 ºC after 10 min [5].

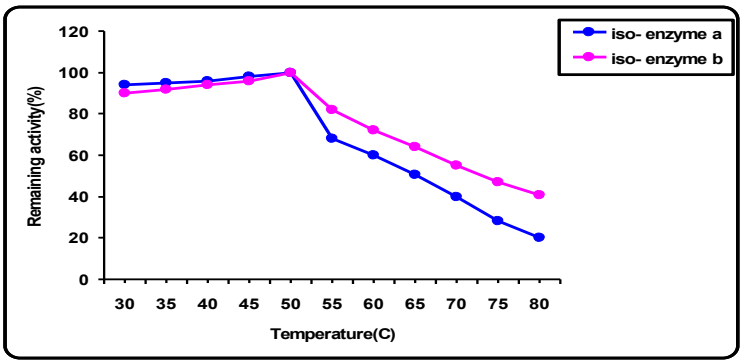


Figure (7): Effect of temperature on the purified *Pseudomonas sp*.SPH4 Amylase (iso-enzymes) stability

Effect of some chemical compounds on enzyme activity

Effect of metal ions on enzyme acyivity

The results of amylase (iso-enzymes) treated with metal ions Table(2) showed that the amylase activity iso-enzymes (a, b) was decreased when treated with (5, 10) mM of $HgCl_2$, AgCl and $CuSO_4$.The inhibition of amylase activity iso-enzymes (a, b) by $HgCl_2$, AgCl and $CuSO_4$ may indicated the presence of SH group in the active site of the enzyme leading to oxidize them by $HgCl_2$, further more the presence of $HgCl_2$, AgCl and $CuSO_4$ in the substrate working solution may form a complexes with the enzyme which prevent it from binding to the substrate and form the product. The activity of amylase enzyme produced by *Pseudomonas sp*. enhanced by Mg^{+2} , Ca^{+2} ,Rb^{+}, at 1mM, while the activity was inhibited by Ag^{+}, Hg^{+2},and Cu^{+2} at 1 Mm [19].

Table (2): Effect of metal ions on purified *Pseudomonas sp*.SPH4 amylase iso-enzymes activity

Metal salt	Concentration (mM)	Remaining activity%	
		iso-enzyme a	iso-enzyme b
Control	-	100	100
CuSO4	5	66.6	72.72
	10	53.3	64.54
AgCl	5	46.66	36.36
	10	28	28.18
HgCl2	5	41.33	33.63
	10	25.33	19.09
CaCl2	5	96	97.2
	10	101.3	101.8

Effect of reducing and chelating agents on enzyme activity

The results in the Table (3) revealed the effect of reducing and chelating agents on amylase iso-enzymes a and b activity, the amylase (iso-enzymes a and b) activity was not inhibited when it was incubated with (2, 5) Mm of 2- mercaptoethanol, these results indicated the presence of SH group in or near the active site. The reducing compounds aid the SH group to be stable. The amylase produced by *Bacillus sp*. remained active when treated with reducing agent 2-mercaptoethanol at 1 mM. [5]. *Pseudomonas sp*.SPH4 amylase (iso-enzyme a and b) was inhibited by chelating agent (EDTA) at (2, 5) mM, these results indicated that these iso-enzymes refered to

metalloamylases on which the activity of enzyme is dependent on the some kinds of ions. Additional chelating agents to the reaction medium it forms complexes with the ions in the active site which cause inhibition of enzyme activity. The amylase produced by *Bacillus stearothermophilus* was inhibited by chelating agent (EDTA) at 10mM [19]. The amylase iso-enzyme a and b did not affected by the present of PMSF in the reaction mixture at (1, 5) mM indicated that these iso-enzymes are not belong to serine amylases. The activity of amylase produced by *Bacillus sp*. did not affected in the presence of 10 mM of PMSF [5].

Table (3): Effect of reducing and chelating agents on *Pseudomonas sp.*SPH4 amylase (iso-enzymes) activity.

Compound	Concentration (mM)	Remaining activity%	
		iso-enzyme a	iso-enzyme b
Control	-	100	100
EDTA	2	45.33	43.63
	5	28	28.18
2-mercaptoethanol	2	92	92.72
	5	85.3	89.09
PMSF	1	93.33	90.90
	5	90.66	87.27

References

1. Reddy, N.S.; Nimmagadda, A. and Rao, K. R. S. S. (2003). An overview of the microbial α - amylase family. Afri. J. Biotechnol. 2(12): 645-648.

2. Cornelis, P. (1987).Microbial amylase. Microbiol. Sci. 4(11): 342 – 343.

3. Pandey, A.; Nigma, P.; Soccal, C.R.; Soccal, V.T.; Singh, D. and Mohan, R. (2000). Advances in microbial amylase. Biotechnol. Appl. Biochem. 31: 135- 152.

4. Shaw, J. F.; Lin, F. P. S. C. and Chen, H. C. (1995). Purification and properties of a cellular α-amylase from *Thermus sp*. Bot. Bull. Acad. Sin. 36: 195 – 200.

5. Lin, L. L.; Hsu, W. H. and Chu, W. S. (1998). Production and properties of a raw starch- degrading amylase from the thermophilic and alkaliphilic *Bacillus* sp. Ts- 23. Biotechnol. Appl.Biochem. 28: 61-68.

6. Fujita, M.; Torigoe, K.; Nakada, T.; Tsusaki, K.; Kubota, M.; Sakai, S.and Tsujisaka, Y. (1989). Cloning and nucleotide sequence of the gene (*amy P*) for maltotetraose-forming amylase from *Pseudomonas stutzeri* MO-19.J. Bacteriol. 171(3): 1333-1339.

7. Nielsen, J. E.; Beier, L.; Otzen, D.; Borehert, T. V.; Frantzen, H. B; Andersen , K. M. and Svendsen, A. (1999). Electrostatics in the active site of α-amylase. Eur. J. Biochem. 264: 816 – 824.

8. Kiran, Ö.; Com lekcioglu, U. and Akrian. B. (2005). Effect of carbon sources and various chemicals on the production of a novel amylase from a thermophilic *Bacillus* sp. K-12. Turk. J. Biol. 29: 99 – 103.

9. Srivastava, R. A. K. (1987). Purification and chemical characterization of thermostable amylase produced by *Bacillus streaothermophilus*. Enzyme. Microbiol. Technol. 9: 749 – 754.

10. Ali, M. Hala. Determination of the optimum conditions for the production of

Amylase from local isolate *Pseudomonas sp.* SPH4 proceeding of 3rd scientific conference Of the College of Science, University of Baghdad 24 to 26 March 2009.(In Arabic).

11. Toye Ekunsaumi (2001). Uw–Washington country. Laboratory production and assay of amylase by Fungi and Bacteria. (Internet).

12. Lin, L. L.; Hsu, W. H. and Chu, W. S. (1997). A gene encoding for α - amylase from thermophilhc *Bacillus sp*.strainTs-23and it's expression in *Escherichia coli*. J. Appl. Microbiol. 82: 325- 334.

13. Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72: 248 – 254.

14. Aiyer, P. V. D. (2004). Effect of C:N ratio on alpha–amylase production by *Bacillus licheniforms sp*T 27. African. J. Biotechnol. 3(10): 519 – 522.

15. Whitaker, J. R. and Bernard, R. A. (1972). Experiment for Introduction to Enzymology. The Wiber Press Davis. Inc.New York, USA. Segel, I. H. (1976). Biochemical Calculations, 2nd edition, John and Sons. Inc. New York.

16. Englard, S., and Seifter, S. (1990).Precipitation techniques In: Methods in Enzymology (ed.Murray, E. D. and Dentscher, P.).182: 425-441.

17. Volesky, B., and Loung, L. (1985). Micrbial enzymes production, purification and isolate (CRC) (Critical Review in Biotechnology. 2:119.

18. Srivastava, R. A. K. (1987). Purification and chemical characterization of thermostable amylase produced by *Bacillus streaothermophilus*. Enzyme. Microbiol. Technol. 9: 749 – 754.

19. Neilsen, J.E.; Borchert,T.V.; and Vriend, G.(2001) . The determinants of α-amylase pH activity profiles. Protein.Engineer.14 (7): 505-512.

20. Spencer-Martin, (1982) .Extracellular isoamylase produced by the yeast *Lipomyces kononenkae* . Eur.J.Appl.Microbiol.44 (60):1253-1257.

21. Wang, H. (1999). Biochemical characteristics of cholesroloxidase immobilized in a polyaniline film. Biochem. Biophys. Res. Commun. 56:22-30.

22. Katsuya, Y., Mezaki,Y., Kubota, M. and Matsuura, Y.(1998).Three-dimensional structure of *Pseudomonas* isoamylase at 2.2Aºresolution.J. Mol. Biol. 281:885-897.