Purification and characterization of uricase enzyme produced from*Pseudomonas aeruginos*a 7

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Summary :

uricase enzyme was partially purified from culture filtrate of *P. aeruginosa 7* by two steps :

Precipitation with ammonium sulfate 70 % saturation and ion exchange chromatography on DEAE – Cellulose . uricase was purified by 7.2 fold with yield 43.79 % .

Uricase showed optimum pH (8.5 – 9) and pH stability was in the range of (8 – 9.5) . the temperature optimum of uricase enzyme was 35 C^o .and the thermostable for uricase enzyme to observe that the enzyme was retain reserved by perfect activity at (25 – 40) C^o and for 20 min.

The kinetic of uricase enzyme that representation by K_m and V_{max} was investigation and to be whole estimation by followed by three method , the rang value of K_m was reached to 0.0091 mg / ml . However the V_{max} was reached to 6.68 µM / ml . min. , by using the uric acid as a substrate.

The effective of some metal ion was studied and the result showed that the NiCl , $AgCl_2$ and HgClare inhibiters , inaddition to that using 1 mM from $FeCl_2$ cause to lose 88.93 % from enzyme activity. Whereas NaCl enhance the enzyme activity that reached to 216.448 % at 1 mMconcentration , $Mgcl_2$,$CaCl_2$ were consider enhancer to the enzyme activity at concentration of 5mM and the residue activity was reached to(170.35 and129.62)% respectively .

تنقية و توصيف انزيم اليوريكيز المنتج من بكتريا *Pseudomonas aeruginosa 7*

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كلمة المفتاح: أنزيم اليوريكيز ، حامض اليوريك

الخلاصة :

تم تنقية انزيم اليوريكيز جزئياً من الوسط الزرعي لبكتريا *P. aeruginosa* بأستخدام خطوتين :

الترسيب بوساطة كبريتات الأمونيوم (70% تشبع) و التبادل الايوني بأستخدام المبادل الايوني DEAE –Cellulose إذ تم الحصول على عدد مرات تنقية 7.2 مرة و حصيلة انزيمية 42.8% وبلغ الاس الهيدروجيني الامثل لفعالية الانزيم (, 9

8.5) وان الانزيم ثابت في مدى أس هيدروجيني بين (8 , 9.5) . وان درجة الحرارة المثلى لفعالية الانزيم تكون هي 35 مْ ويبقى الانزيم محتفظاً بفعاليتهِ كاملةً عند درجة حرارة (25 - 40) مْ ولمدة 20 دقيقة .

أظهرت الدراسات الحركية لانزيم اليوريكيز ان العلاقة بين تركيز الركيزة (حامض اليوريك) وسرعة التفاعل تخضعان الى معادلة ميكالس منتن وقدرت الثوابت الحركية المتمثلة بثابت ميكالس K_m والسرعة القصوى V_{max} للأنزيم وتم تقديرها بأتباع طرائق ثلاث , إذ بلغت معدلات قيم ثابت ميكالس K_m هو 0.0091 ملغم \ مل , اما السرعة القصوى V_{max} فقد بلغت 6.68 مايكرومول \ مل . دقيقة , عند استخدام حامض اليوريك كركيزة .

دُرس تاثير بعض الايونات الفلزية حيث أوضحت النتائج ان كلوريدات النيكل الزئبق و الفضة من مثبطات الانزيم , كما ان استخدام كلوريد الحديد وبتركيز 1 ملي مولر يُفقد من الانزيم فعاليتهُ 88.93% .

بينما يُعد كلوريد الصوديوم منشطاً قوياً لفعالية الانزيم اذ بلغت الفعالية المتبقية 216.44% عند استخدامهُ بنسبة 1 ملي مولر , كما ويُعد كلوريد المغنسيوم وكلوريد الكالسيوم من المنشطات ايضاً عند استخدامهما بتركيز 5 ملي مولر لتبلغ الفعالية المتبقية (129.62 , 170.35) % .

Introduction :

Uricase or urate oxidase [E.C. 1.7.3.3] the enzyme that catalyzes the oxidation of uric acid to allantoin, CO_2 and H_2O_2 . Uricase is used in medicine ,occupies a pivotal position in the chain of enzymes responsible for the metabolism of purines [1] . This enzyme is widely present in most vertebrates but is absent in humans . It was first found in bovine kidney and in variant source such as bacteria , fungi and high organism [2 , 3] . Higher primates (apes and human) lake the functional uricase and excreted the uric acid as the end product of purine degradation [4] . In some individuals , uric acid precipitation , leading to gout symptoms . gout treatment generally include Allopurinol , it is a potent competitive inhibitors of xanthine oxidase , the enzyme that catalyzes the conversion hypoxanthine to xanthine and xanthine to uric acid , as a result to prevent formation uric acid in the body . In other cases, the precipitation of uric acid lead to formation the renal calculi [5] , and obstruction in valve of the heart , necrosis in the intestinal wall and skin calcification [5] .The cause to absent of uricase enzyme in human and some higher primates as a result of two mutations in the gene coding sequence , which cause premature termination of the translation process[2]. The an important application of uricase enzyme in the clinical biochemistry as a diagnostic reagent for measurement of uric acid in blood and other biological fluids[6] .The second used for this enzyme in treatment the acute gout that associated with the renal complication , direct injection of uricase allows a much more rapid to uric acid [7] .The most organism (prokaryote and eukaryote) can be useful from uric acid and metabolism as nitrogen source and energy .Uricase was originally isolated from mammalian organisms. Recently interest was concentration on microbial preparations from various fungi , yeast and bacteria [8] . The uricase enzyme is inducible and therefore , the presence of uric acid in the medium is necessary for enzyme production [2] . Uricase enzyme has high specialization toward it's substrate (uric acid) [9]. Several investigators studied the purification and characterization of uricase enzyme by microorganisms .

The aim of study describes the purification of uricase and properties study from *P. aeruginosa 7* .

Material and methods

The suitable bacterial isolated that used as source to uricase enzyme and in this study was brought from Babylon university, this bacterial strain was tested for uricase production using uric acid medium : uric acid 0.3 % ; dates juice 0.05 % ; KH_2PO_4 0.75% and $MgCl_2$ 0.1 % , pH was adjusted at 6.5 , 37 C^0 for 24 hour and at 150 rpm[7] .

Uricaseassay :

Uricase was assayed by following the disappearance of uric acid , detected by decrease in absorbance at 293 nm to the substrate suspended comparison with the same suspended free from enzyme activity , the reaction mixture contain : 2ml from substrate solution (10 µm from uric acid per1ml from borate buffer 0.2 M , pH = 9) , 0.3 ml of distill water and 0.5 ml of crude enzyme at 30 C^0 were added , after 15 min. , 0.2 ml of 0.2 M potassium cyanide solution was added to the mixture to stop the enzyme reaction , in the reference sample , the solution of potassium cyanide was added to the mixture before addition of the crude enzyme. The difference between the absorbance of the sample and reference is equivalent to the decrease in uric acid during the enzyme reaction. One unit of uricase enzyme was equal to the amount of enzyme which convert 1µmol of uric acid to allantoin per min. at 30 C^0 .

Determination of protein content :

Total protein concentration of cell free supernatant was assayed by the method of Bradford using a standard curve established with bovine serum albumin (BSA) as a standard and coomasie brilliant blue dye (CBB) G – 250. Appropriate volume (0.1 ml)was taken from the protein solution, then 5 ml of CBB dye was added and the contents were well mixed. The absorbance of the mixture was measured after 5 minutes at 595 nm against reagent blank. The protein content calculated from the standard curve[21].

Purification of uricase enzyme

The following steps were performed for purification of uricase produced by *P. aeruginosa*7 under all optimal condition .

A) Uricase production and preparation of cell free filtrate:

*P.aeruginosa*7 was allowed to grow on uric acid medium under all previous studied optimal condition (at 37 C^0 , pH = 6.5 , rotatory incubation period shaker at 150 rpm for 24 hours) at the end of the incubation period , the obtained extract was filtrated by centrifugation at 8000 rpm for 15 min under cooling conditions to obtain the cell free filtrate. Uricaseactivity and protein content were determined and then the obtained cell free filtrate was preserved in the refrigerator as a crude uricaseenzyme[7] .

B) Precipitation by ammonium sulphate:

Different concentrations of ammonium sulphate (20, 40, 60 and 70 % w/v)were used. Solid ammonium sulphate was added slowly to the culture filtrate with gentle stirring on ice bath until the required saturation of ammonium sulphate was reached after which the mixture was

allowed to stand at 4 ^{0}C overnight. The mixture was then centrifuged at 8000 rpm for 30 minutes at 4^{0}C[7]. both enzyme activity and protein content were determined for each separation fraction .

C) Uricase concentration using :

This purification step was carried out to remove the trace of ammonium sulphate .the resultant precipitate was dissolved in 10 ml 0.01 M Tris – HCl buffer pH 8.5 and dialyzed overnight against 1 liter of the same buffer in a cellophane bag. The concentrated and dialyzed cell free supernatant became ready to applied on further purification step , i.e. , column chromatography technique .

D) purification of uricase by ion exchange chromatography on DEAE-cellulose :

The concentrated and dialyzed cell free supernatant was applied to a column (21x1.8 cm) containing DEAE-cellulose , which previously was equilibrated with 10 mMTris-HCl, pH 8.5. The column was washed with 3 time volumes of 10 mMTris – HCl, pH 8.5 at a flow rate of 60 ml / hr , and the bound proteins were eluted with a linear NaCl gradient (0 – 0.3 M) in the same buffer, and collected the fraction and analyzed by UV spectrophotometer at 280 nm to detect the protein concentration and enzyme activity at 293 nm[7] .

Properties study of the purified uricase :

A) Determination of the suitable concentration of uric acid and determination the kinetic constants:

The substrate (uric acid) was applied into the reaction mixture at different concentration (0.005, 0.01, 0.015, 0.02, 0.025, 0.03, 0.035, 0.040 mg/ml of borate buffer , pH 9) .and determination the kinetic ((K_m and V_{max}) by the following three method (Line weaver and Burk method (1/[S] vs 1/V , Eadie – Hoffstee plot (V/[S] vs V , and Hanes - Woolf plot [S] vs [S]/V).

B) Determination of optimum pH for enzyme activity :

The purified enzyme reaction mixture were incubated at different pH values (sodium phosphate buffer 0.1 M was used from pH 7, 7.5 borate buffer 0.1 M for 8, 8.5, 9, 9.5, and Tris – HCl0.1 M for pH 10 and 10.5) .

C) Determination the optimum pH stability :

Equal volumes from enzyme and pH solution buffer at different pH values were mixed (sodium phosphate buffer 0.1 M was used from pH 7, 7.5 borate buffer 0.1 M for 8, 8.5, 9, 9.5, Tris – HCl 0.1 M for pH 10, 10.5 and Tris – base 0.1 M for 11, 11.5, 12) .and incubated on the water bath at 30 ^{0}C for 20 minutes .

D) Determination of optimum temperature for enzyme activity :

The purified enzyme reaction mixture were incubated at different temperature (25, 30, 35, 40, 45, 50, 55, 60 and 65 ^{0}C) .

E) Determination of thermal stability of uricase enzyme :

The purified enzyme was incubated with the different temperatures (25, 30, 35, 40, 45, 50, 55, 60 and 65 ^{0}C) at 20 minutes , and at the end of incubation period at those temperature degrees, the replicate tubes were cooled and assay for uricase activity .

F) **Effect the metal ions and some chemical substance on the enzyme activity** :

Same volume from purified enzyme was mixed with the solution content different metal ions such as ($AgCl_2$, KCl_2, NaCl, $HgCl_2$, $NiCl_2$, $CaCl_2$, $FeCl_2$, $MgCl_2$, and EDTA) at tow concentration (1 , 5 mM) and incubated at 35^{0}C for 10 minutes after incubation period assay for uricase activity and determination the percentage of uricase enzyme compared with uricase activity that with out metal ions .

Results and discussion :

Purification of uricaseenzyme :

These experiments were carried out to obtain the pure uricase from the crude compound which was collected from the fermentation experiments of *P.aeruginosa*7.

Precipitation by ammonium sulphate :

Different concentrations of ammonium sulphate(20, 40, 60 and 70% W/V) were added to the crudeuricase. The Results presented in Table (1) indicatethat ammonium sulphate 70% saturation proved tobe high uricolytic activity (1.18 U/ml) and specificactivity (34.705 U/mg protein) comparing with crudeuricase and other concentrations followed byammonium sulphate 60% saturation.

Table (1): purification of uricase from *P. aeruginosa 7* by ammonium sulphate .

Ammonium sulphate concentration%	Uricase activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg protein)
0.0	0.72	0.025	28.8
20	0.0	0.023	0.0
40	0.5	0.030	16.667
60	0.9	0.038	23.684
70	1.18	0.034	34.705

These results are in agreementwith those of Saeed who found that *P.aeruginosa*uricase enzyme was purified using ammonium sulphate (70% saturation) [(7)] and agreement with those of Mabrouk who found that*G. Guog*uricase enzyme was purified using ammonium sulphate (70% saturation) [(3)]. Also Adamek[(6)] showed that crude enzyme extract from *C. utilis* was

precipitated with 60% ammonium sulphate. These results were in disagreement with those of Azab[10] who reported that the proteins of the culture supernatants (of *Proteus* and *Streptomyces*) were precipitated with 80% ammonium sulphate.

Ion exchange chromatography on DEAE – cellulose :

The obtained uricase after partially purified with 70 % ammonium sulphate was dialyzed and then applied to the top of DEAE – cellulose column chromatography **(1.8 x 21)** which previously equilibrated with 0.01 M buffer at pH 8.5 under cooling conditions. At first , the unbound protein was removed by washing with 3 times volume of 0.01 M Tris – HCl buffer. Uricase was bound to the ion exchange column at pH 8.5 eluted with linear NaCl gradient (0 – 0.3M). The result illustrated in fig. (1) show that tow protein peaks were detected .Also most of the uricolytic activity was detected in the second protein peak , first protein peak showed lesser enzyme activity than the second protein peak. The purification results which summarized in Table (2) record that the enzyme was purified almost 7.012 - fold with a specific activity and recovery of 114.588 and 42.85%, respectively. These result agreement with Mabrouk[3] who used DEAE – cellulose as ion exchangeto purified the enzyme from *G. guog*, there found the purification 33.2- fold, and recovery 17.6%. But these results are in disagreement with those of Saeed[7] Who showed that *P. aeruginosa*uricase enzyme was purified using ion exchange chromatography on Qsepharose fast flow anion exchange and sephadex G- 50 column. They found that upon using Q- Sepharose fast flow column gave the most of uricolytic activity was found in the 0.5M NaCl gradient and the enzyme was purified almost 29.71- fold with a specific activity and recovery of 592 and 31% respectively. However, Adamek[6]found that*C.utilis*uricase enzyme was purified using ionexchange chromatography on DEAE – cellulose andthe active fraction of uricase was released from thecolumn at a low ionic strength (0.1- 0.2M sodiumchloride).However , Kai was obtain the 19.7 – fold and recovery 31% [11] .

Table (2): purification steps of uricase enzyme from *P.aeruginosa 7.*

Purification steps	Volume (ml)	Enzyme activity U/ml	Protein concentration mg /ml	Specific activity (U/mg protein)	Total units	Purification fold	Recovery %
Crud enzyme	95	1.34	0.082	16.34	127.3	1	100
Pellet after 70 % ammonium sulphate and dialysis	25	2.71	0.095	28.52	67.75	1.75	53.22
After DEAE-cellulose column	14	3.896	0.034	114.588	54.54	7.012	42.85

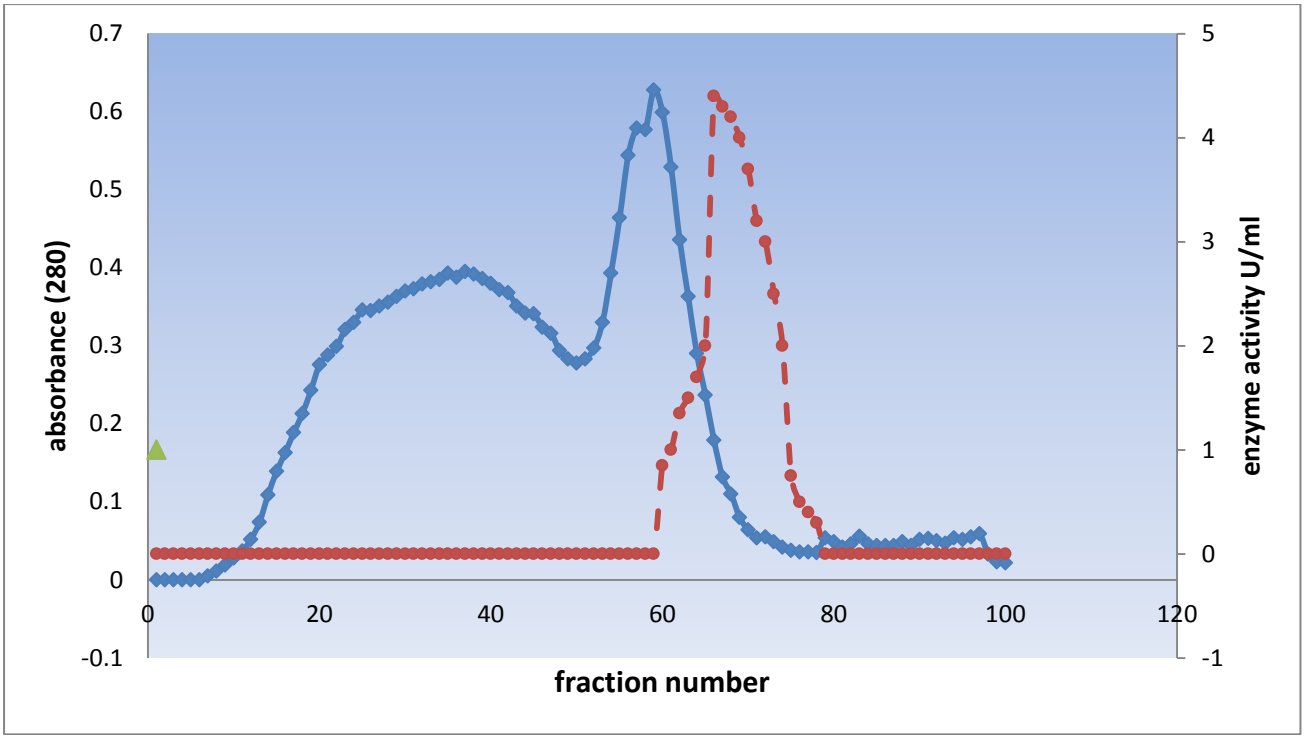


Fig. (1) : fractionation of uricase enzyme on DEAE – cellulose .

Properties study of purified uricase :

A) Determination of the suitable concentration of uric acid and determination the kinetic constants:

This experiment was performed to investigate the effect of different substrate (uric acid) concentrations on the purified enzyme activity. The effect of different substrate concentrations was tested by incubating different substrate concentrations(0.005, 0.01, 0.015, 0.020, 0.025, 0.030, 0.035 and 0.040 mg/ml) with the same amount of the enzyme. The enzyme activity was plotted against substrate concentration. The results in Fig.(2) demonstrate that, 20µg of uric acid substrate proved to be high uricolytic activity (5.409 U/ml) and then uricolytic activity persist with increasing uric acid concentration.
These results are in agreement with those of Saeed[7]who found that 20µg of uric acid (substrate) was enough to be used in the reaction mixture. But Mabrouk[3] find the high uricolytic activity at 10 µg of uric acid as a substrate .

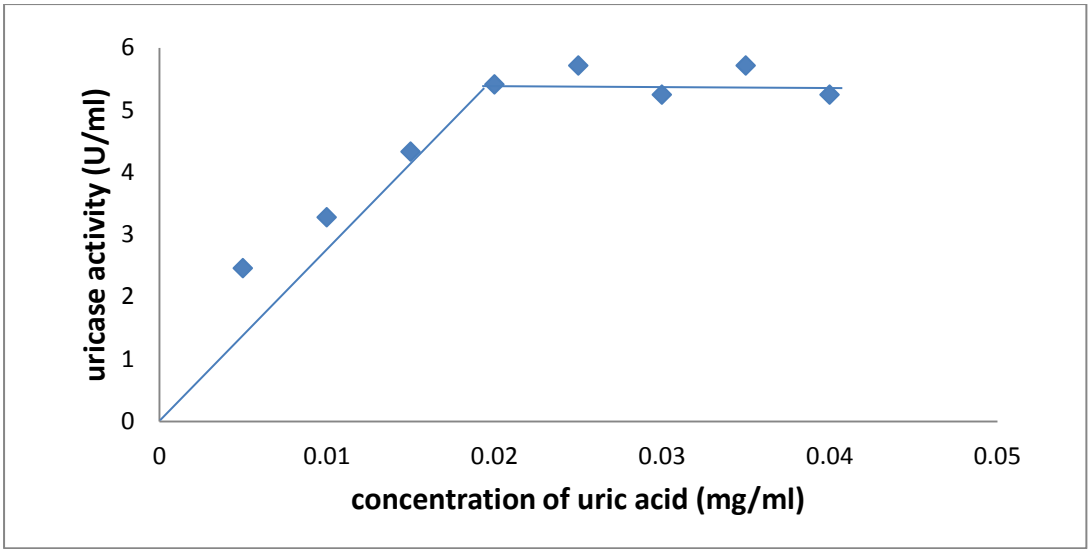
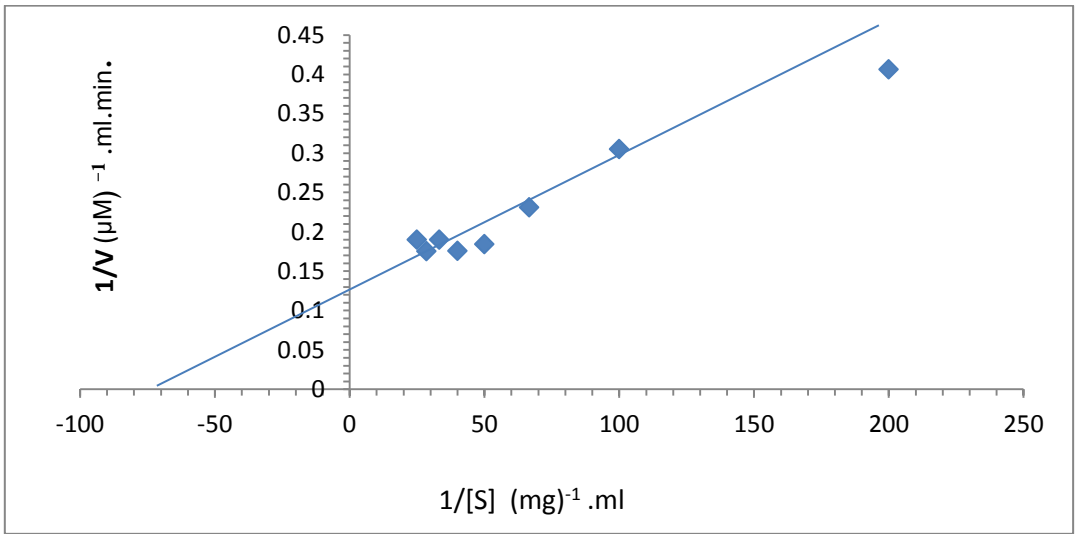


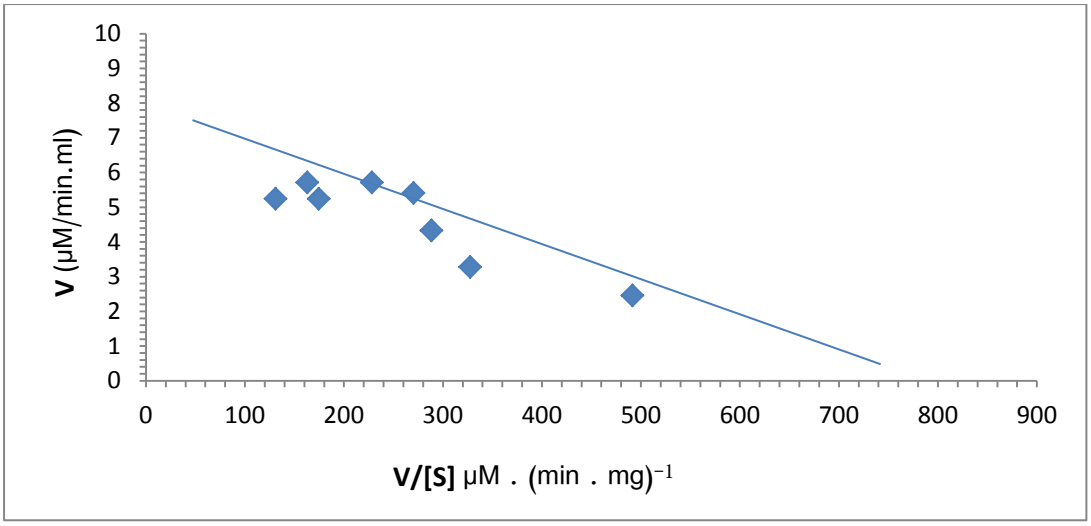
Fig (2):Was showed theeffect of different concentration of substrate (uric acid) on the activity of purified uricase.

B) Determination the kinetic constant of uricase :

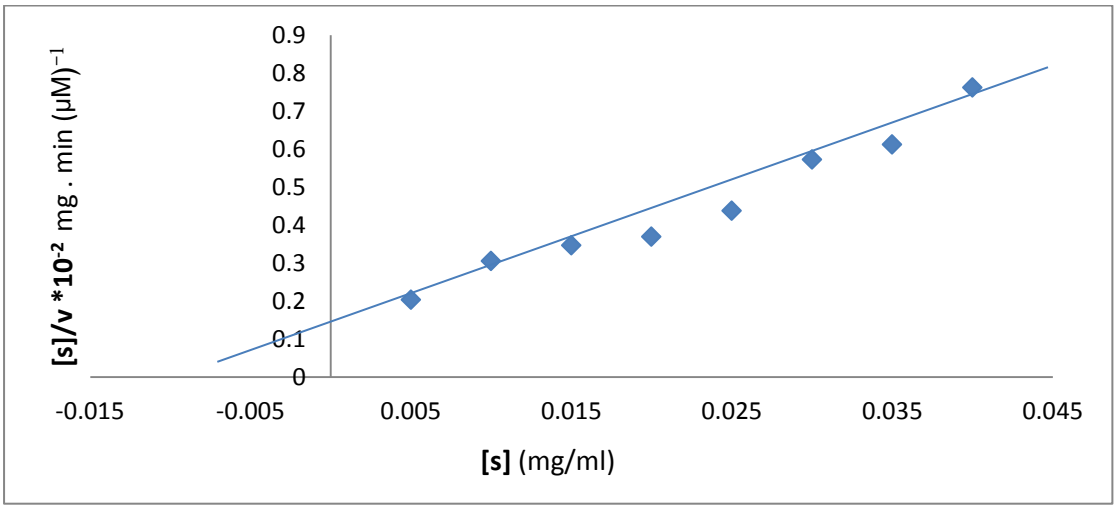
This experiment was carried out to investigate the K_m and V_{max} of purified uricase enzyme that produced from *P. aeruginosa 7*. Using the three mode for draw the relation between the velocity (v) against substrate concentration [s] and the result show in the fig (3) and the value of this constant explain in the table (3). Since the rang value of $K_m$and V_{max}reach to 0.0091 mg/ml and 6.68 µmol/ml.min respectively. This result was disagreementwith Wang and Marzluf[12] who said the value of K_m was 4.2x 10^{-5}mol for uricase enzyme that produced from*Neurosporacrassa*, and from plant leafs was equal to (9 – 24) µmol[13]. With respect to V_{max} the value was reached to 0.033922 mmol/min [14]. And from fungi *A. flavus* was reached to 8 µmol/min.[15] .



(A) Lineweaver-Burk plot.



(B): Eadie-Hoffsteeplot .



(C) : Hanes plot .

Fig (3): Determination of kinetic constant for uricase enzyme that produced from *P. aeruginosa7* .

Table (3) The kinetic const\ant of uricase.

plot	K_m (mg/ml)	V_{max}(µM/ml . min)
Linwiever-Burk reciprocal plot	0.01	6.66
Hanes-Woolf plot	0.009	6.6
Eadie –Hoffstee plot	0.0087	6.8

C) Determination of optimum pH for enzyme activity :

This experiment was performed to investigate the effect of different pH-values on the purified uricase activity. The enzyme activity was determined as mentioned before. The results in Fig (4) show that the maximum purified uricase activity was detected at pH 8.5 and 9.0 reaching 6.557 U/ml. These results are in agreement with those of [16, 6]who found that purified enzyme exhibited maximum uricolytic activity at pH 8.5 and 9 respectively.And agreement with those of [17] who found that the high activity of uricase at pH 8.5.

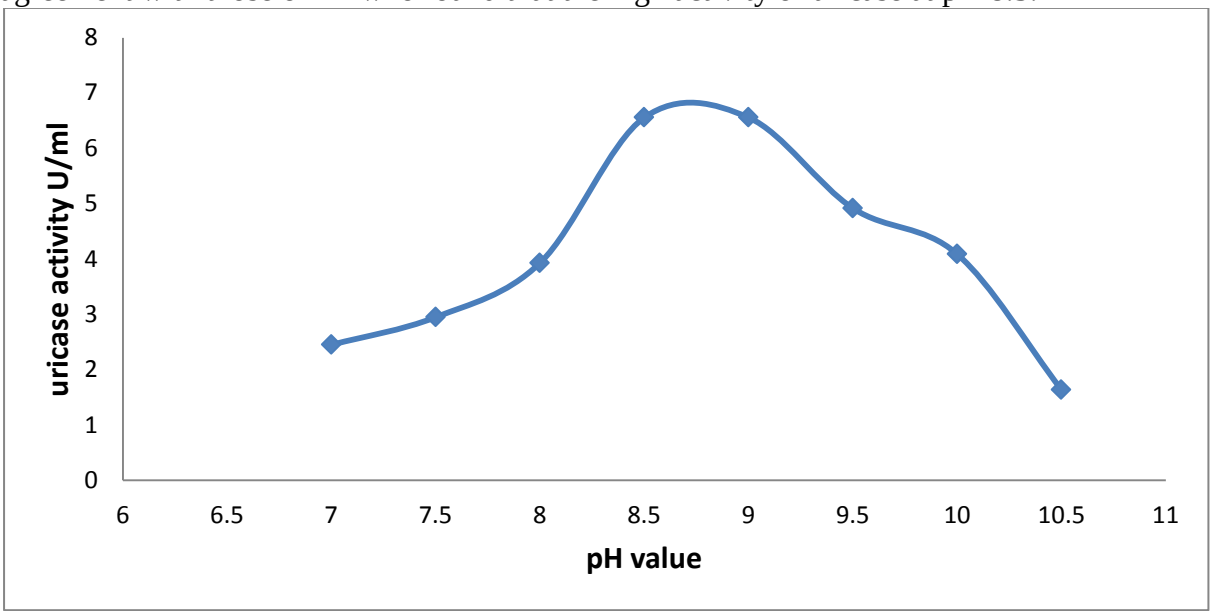


Fig (4) : The optimum pH for activity of purified uricase .

D) Determination the optimum pH stability:

This experiment was carried out to determine the optimum pH stability of purified uricase, the result show at fig (5) explain the enzyme maintain to all activity at about pH (8 to 9.5) and decrease the activity at Ph (7.5 and 10) it preserve to 90.35 and 96.85 % from activity respectively. This result was agreement with [18]who was show the pH stability for uricase that purified from *Candida* SP. at (8.5 to 9.5). But Huang [18] refer to the pH stability of purifieduricase at pH (6 to 9).

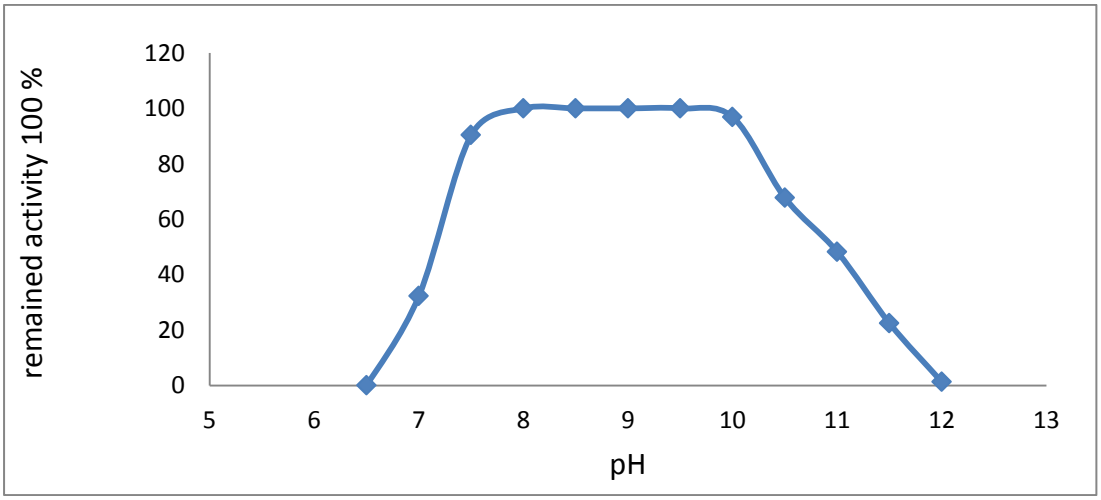
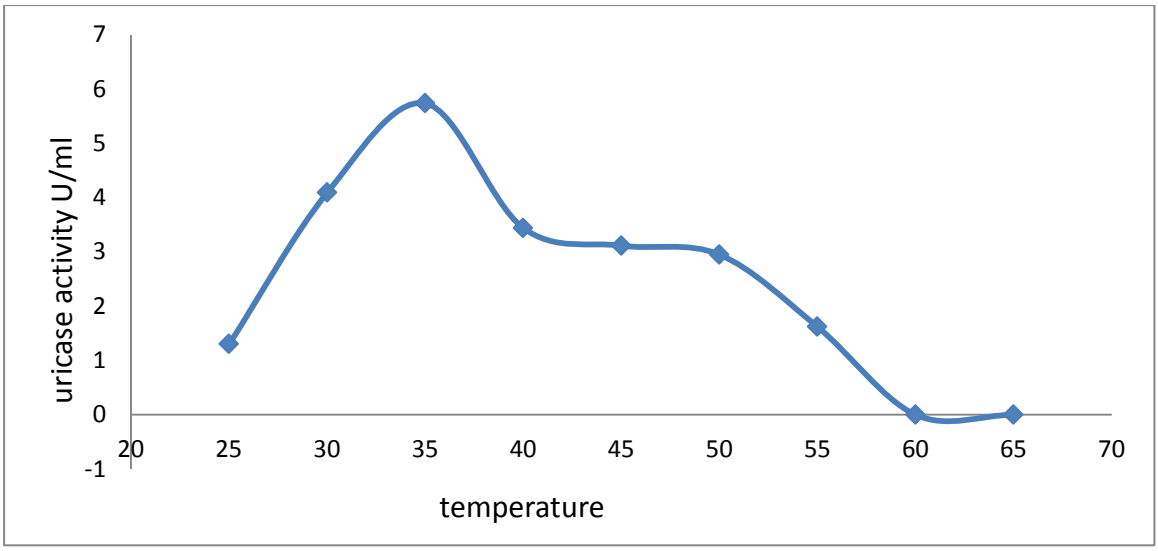


Fig (5): The pH stability of purified uricase .

E) Determination of optimum temperature for enzyme activity :

The velocity of enzyme reaction increase at limit rang from temperature due to increase the kinetic energy to the molecule , in this experimental investigation to the optimum temperature of purified uricase, the result showed in fig(6), the optimum temperature at 35^0C and the activity was decrease at 40^0C and above due to the Denaturation of enzyme. And this result was agreement with Ohe and Watanabe [19]who refer tothe optimum temperature of uricase that purified from *Streptomyces cyanogenus*at 35^0C, but Alamillo[20] , who refer to optimum temperature for purified uricase from *Chlamydomonasreinhardtii* at 40^0C .



Fig(6) : the optimum temperature of purified uricase from *P. aeruginosa 7.*

F) Determination of thermal stability of uricase enzyme :

This experiment was designed to determine the temperature range within the partially purified uricase enzyme maintains its activity and also to illustrate the enzyme thermal stability under study. This experiment was carried out by incubating the purified enzyme at temperatures (25, 30, 35, 40, 45, 50, 55, 60 and ºc) and for(20min.), at the end of incubation period at those temperature degrees, the enzyme activity was determined as described before. The results presented in fig.(7) illustrate that purified uricase enzyme retained all its activity after exposure to temperature from 25 to 40 ºc for 20 minutes. Also retained 90% of its activity after exposure for 20 min. at the 45^0C, Thus the purified *p. aeruginosa 7*uricase enzyme was lack all it's activity at 60ºC. These results are in approximate with those of Ohe and Watanabe[19] who found that *Streptomyces cyanogenus*uricase enzyme maintain to all its activity at 50^oc after exposure for 10 minutes. Also,Mabrouk[3] reported that purified uricase enzyme from *G. guog*was retain with 66.06% from original it's activity if incubated at 40^oc for 10 min.

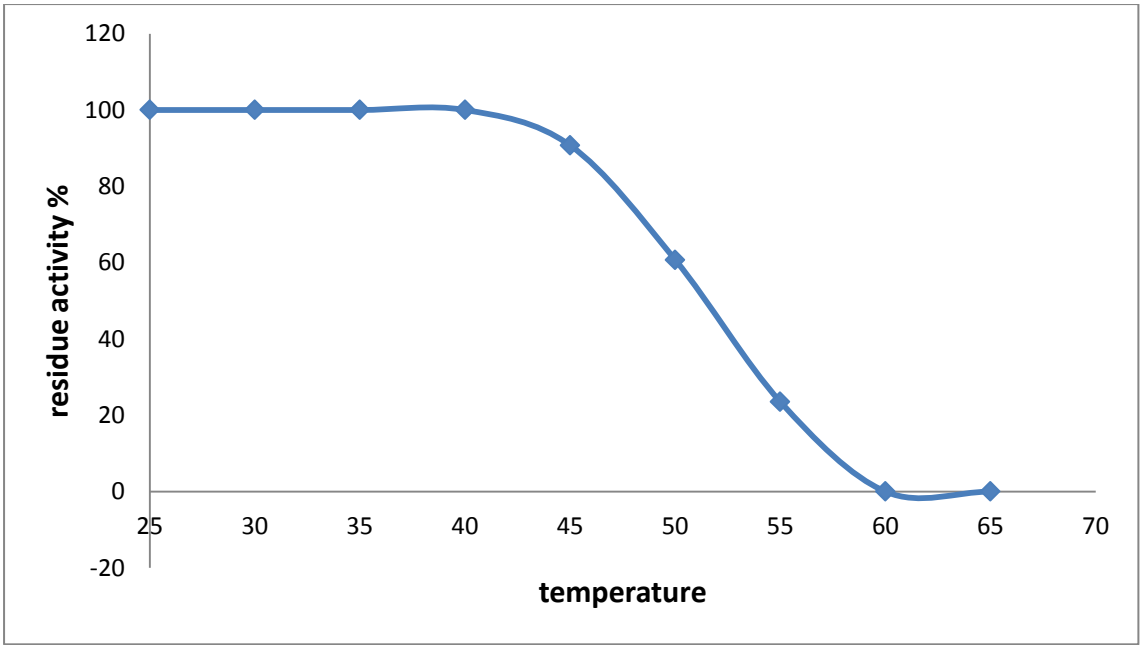


Fig.(7): Thermal stability of the purified uricase from*P. aeruginosa 7.*

G) **Effect the metal ions and some chemical substance on the enzyme activity** :

This experiment was carried out in order to study the effect of the tested metal ions to the reaction mixture on the enzyme activity. Metal ions (1 – 5 Mm) such as Ca^{2+}, Mn^{2+}, Na^{2+}, Fe^{2+}, Hg^{+2}, Ni^{+2}, K^{+1} andEDTA at 5 Mmwere incubated with same volume fromenzyme at 30^0C , pH 9 for 10 min. The enzyme activity was determined as mentioned before, metal ions free reaction mixture was carried out as control. The results illustrated in table (4)

Table (4) effect of some compound on uricaseactivity .

Compound or metal ions	Concentration Mm	Uricase residual activity %
None or control	-	100
AgCl	1	51.85
	5	18.504
NaCl	1	216.448
	5	100
$HgCl_2$	1	37.03
	5	11.07
$MnCl_2$	1	37.03
	5	170.35
$NiCl_2$	1	55.558
	5	66.65
$CaCl_2$	1	48.124
	5	129.62
$FeCl_2$	1	11.07
	5	73.88
KCl	1	59.195
	5	25.915
EDTA	5	85.178

Showed that the enzyme was retain to all it's activity when incubated with sodium chloride at 5Mm , but the residual enzyme activity was increased to reach to 216.448 % when incubated with this salt at 1 Mm. However, metal ions such as Ca^{2+} ,Mn^{+2} and Ni^{+2} strongly enhance the uricase activity when increase the concentration from 1 to 5 Mm from this ions. One possible explanation for the results that, some metal ions bind to the uricase enzyme and alter the enzymatic activity by stabilization or destabilization of the enzyme's conformation. On the other hand the ions for Hg^{+2} , Ag^{+2} and K^{+1} was have inhibited effect on enzyme activity when the increase the concentration from 1 to 5. The effect of EDTA was investigated, and show the activity was reduced little because the EDTA was taking back some ions that necessary for enzyme stability . Some investigator were studies effect the metal ions on the uricase activity , Mabrouk(3) who showed the magnesium chloride and manganese sulphate were considered as activated for enzyme, but ferric chloride , magnesium sulphate and cobalt chloride was inhibitor for uricase activity.

Reference :

1) **Bertrand , K.E**., N. Mathieu, G. Inocet and F.K. HonorePak. J. Biol. Sci., 11:1646-1649 , , (2008) .
2) **Farley P. C.** and Santosa S. , J. of Microbiology , vol. 48, NO. 12, p. 1104- 1108 (2002) .
3) **Mabrouk ,** A. M. ; Hamed E.R. ; M.M.Farag and Ahmed N.E. , Gate 2 biotech , 2 (11) : p. 1-13(2010) .
4) **Friedman, T. B.,** Polanco, G. E. Appold, J. C. and Mayle, J. E. Comparative Biochemistry and Physiology 81B, 653-659(1985).
5) **Cammalleri , L. and** Malaguarnera ,M. , J. Med . sci. 2007, 4 (2) : 83-93(2007) .
6) **Adamek, V.,** Kralova, B., Suchova, M., Valentova, O.andDemnerova, K. *Journal of Chromatography* 497, 268-275 (1989) .
7) **Saeed H.M. ;**yousry Y.A. ; Gohar M. ; and Elbaz M.A. , journal of microbiology , vol. 53 , No.1, 45-52(2004) .
8) **Liu J.** ; Li G. ; Lio H. and Zhou X. , J. of biochemistry and biotechnology, p. 57- 63 (1993).
9) **Vogels , G. D.** and Drift , C. V. D. , American society for microbiology, Vol. 40 , No. 2 , P.403 – 468(1976) .
10) **Azab E.A. ,**Ali M. Magda and Fareed M. F. , J. of biology , 2005 , Vol. 7, p. 44 – 54(2005).
11) **Kai, L.** ; Xiao-Hang, M. ; Zhou, X. ; Jia , X. ; Li, X. and Guo, K. , J. microbiology and biotechnology, vol. 24, No. 3, p. 401-406(2007) .
12) **Wang, L. C.** and Marzlf G. A. , Biochemistry and Biophysics , vol. 201, No. 1, p. 185-193(1979) .
13) **Montalbini , P. ;** Redondo , J. ; Caballero , J. L. ; Cárdenas J. and Pineda , M. , J.ofplanta 202, p.277-283(1997) .
14) **Sarkar, P.** Biosensor for uric acid estimation(2010) .
15) **Bomalaski**, J. S. ; Holtsberg, F. W. ; Ensor, C. M. and Clark, M. A. , vol. 29, No. 9, p. 1924-1949(2002) .
16) **Bongaerts GPA**, Sin LI, Peters JLJ, Vogels DG. Biochem. Biophys. Acta; 499: 111-118 (1977).
17) **Rajoka,** M. I. ;Rehman, K. ; Tabish, T. and Zia, M. A. , J. of microbiology & biotechnology 22,p . 288-291(2006) .
18) **) Huang, S.** and Wu ,T. , Eur. J. Biochem. 271 , p. 517-523 (2004) .
19) **)Ohe , T.** and Watanabe , Y. , biochem , 1981,vol. 89, No.6 p. 1769-1779(1981) .
20) **Alamillo,** J. M. ; Cárdenas, J. and Pineda, M. Biochimica et biophusicaActa (BBA) – protein structure and Molecular Enzymology , vol. 1076, p. 203-208,(2003) .
21) **Bradford, M. M.** dye binding Ana. Biochem .72 : 248 -254(1976).