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# Production, Purification and Characterization of Uricase Produced by *Pseudomonas aeruginosa*

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**Abstract**

In this study, detection of uricase production from *Pseudomonas aeruginosa* isolates was done by applying colorimetric method, Uricase was purified from the most potent isolate by precipitation using ammonium sulphate (80% saturation) then purification was achieved using DEAE –Cellulose ion exchange and Sepharose 6B gel filtration chromatography column, 16.4% of total enzyme was recovered with specific activity 2337.5U/mg and 22.21folds of purification. Characterization of uricase involved detection of optimal conditions for uricase activity, the maximal activity was obtained at temperature 45ºC,while uricase appeared to be stable at 40ºC. Uricase showed optimal activity at pH 9 while pH stability was in the range of 7.5-9. Molecular weight of uricase obtained in this study determined by sodium dedosyl sulfate electrophoresis (SDS-PAGE) technique was approximately 35kDa.

**Keywords:** Uricase, *P.aeruginosa*, Quantitative screening, Purification, Characterization.

# انتاج ، تنقية وتوصيف انزيم اليوريكيز المنقى من بكتريا *Pseudomonas aeruginosa*

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**الخلاصة**

في هذا البحث تم انتاج انزيم اليوريكيز من بكتريا *Pseudomonas aeruginosa* كمياً باعتماد الطريقة االلونية كما تم تنقيته باستخدام الترسيب بملح كبريتات الامونيوم عند نسبة اشباع 80% ثم كروماتوغرافيا التبادل الايوني باستخدام المبادل DEAE-Cellulose والثانية باستخدام كروماتوغرافيا الترشيح الهلامي باستخدام هلام Sepharose 6B حيث تم الحصول على نسبة ارجاع بلغت16.4 % من الانزيم وفعالية نوعية 2337.5وحدة/ملغم وبعدد مرات تنقية بلغت22.21 . اظهر الانزيم فعالية مثلى عند درجة حرارة 45 درجة مئوية ثباتية عند 40 درجة مئوية وفعالية مثلى عند الاس الهيدروجيني 9 والثباتية عند اس هيدروجيني تراوح بين(9–7.5) و قدر الوزن الجزيئي للانزيم بتقنية الترحيل الكهربائي باستخدام الاكريل امايد المتعدد SDS-PAGE حيث بلغ الوزن الجزيئي للانزيم تقريبا 35 كيلودالتون.

## Introduction

Urate oxidase or uricase (oxidoreductase :EC 1.7.3.3), is a peroxisomal enzyme that catalyzes uric acid oxidation in most mammals [1]. Uricase enzyme is usually present in most vertebrates but it is absent in higher primates (apes and humans) who excrete uric acid as the end product of degradation of purine. During evolution of primate, the inactivation of the uricase gene in hominoid was caused by

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independent frameshift or nonsense mutations [2]. It has been purified from various sources, and its properties investigated [3].Uricase can be also used as a protein drug to reduce toxic urate accumulation [4].

The enzyme was induced in the producing organisms by different concentrations of different inducers, and uric acid was the most potent inducer[5]. Uricase has substantial importance; it is used in the treatment of nephrolithiasis and pharmaceutical preparation. This enzyme also used in the uric acid determination in biological fluids after isolation and purification from different animal or microbial sources [6]. During evolution of primate, a major factor in lengthening life-span and decreasing rates of age-specific cancer may have been improved protective mechanisms against radicals of oxygen. One of these protective systems is plasma uric acid, the level of which increased markedly during evolution of primate as a consequence of a series of mutations [7].There are different microbial sources for enzyme production such as *Pseudomonas aeruginosa* [8,9]; *Mucor hiemalis*[10]; *Candida utilis* [11]and *Bacillus fastidious*[12].

The aim of the present study was to detect the potent *Pseudomonas aeruginosa* uricase producer then purify uricase from this isolate and study some properties of this enzyme.

## Materials and Methods

### Bacterial Isolation

*Pseudomonas aeruginosa* (43)isolates were collected from different clinical specimens and identified by bacteriological biochemical assays for uricase production by qualitative and semi quantitative methods [13] and were used in present study.

### Detection of uricase production by quantitative method

### Enzyme Production

Bacterial culture were allowed to grow at 30 °C in shaker incubator (Gallenkamp/England) at 200 rpm, in 250 ml conical flasks volume containing 50 ml of pre-culture basal medium consist of(g/l): Glucose 30g(Fluka/Switzerland); Peptone, 20g (Himedia/India); $KH_2PO_4$ 1g(BDH/England); $MgSO_4$-$7H_2O$,0.5g (BDH/England) and uric acid, 3g (BDH/England) which dissolved in 1 liter D.W., adjust pH to7, distributed each 50ml in a flask and sterilized by autoclaving for 15min at 121°C.Then 0.5 ml of overnight bacterial culture was washed with sterile saline , centrifuged, and used as an inoculum for the basal production medium consists of the following composition (g/l): Glucose, 3.5g (Fluka/Switzerland); $K_2HPO_4$, 10g (BDH/England) ,used instead of $K_3PO_4$; $MgCl_2$, 1g(BDH/England); and uric acid, 3g(BDH/England), then distributed each 50ml in flask and sterilized by autoclaving at 121°C for 15min.

After incubation, 2 ml of the growing cultures from basal production medium were centrifuged at 8000 rpm for 2 min. The cell free supernatant was used as crude enzyme preparation for further determinations [14].

### Meassurment of Enzyme Activity

Crude enzyme solution(0.1ml ) was mixed with 0.6ml sodium borate buffer (pH 8.5, 0.1M) containing 2mM uric acid(BDH/England), 0.15ml 4-aminoantipyrine (30mM) (BDH/England), 0.1ml phenol (1.5%)(Himedia/India), 0.05ml peroxidase (15U/ml) (Promega/USA)and incubated at 37 °C for 20 min .The reaction then stopped by addition of 1.0ml ethanol (GCC/England), and the absorbance at 540 nm was read against the blank by a spectrophotometer. One unit of enzyme can be defined as the amount of enzyme that can produce 1.0 mmol of $H_2O_2$ per minute under the standard assay conditions [15].

### Production, Purification and Characterization of uricase

### Production of uricase:

The cells were aerobically cultured in 1 liter flask at 37°C with rotator shaking 200 rpm for 18 hr in the production medium mentioned above, then the culture was centrifuged at 8000 rpm for 20 min. at 4 °C and supernatant was collected while the pellet was discarded [15].

### Precipitation by Ammonium sulphate and dialysis:

Different saturation percentages(50%, 60%, 70%, 80%, 85%) of solid ammonium sulphate (Himedia/India) were added to the crude extract. Solid ammonium sulfate was slowly added at 4 C° with gentle stirring on ice bath, after dissolving of ammonium sulphate,the mixture was allowed to stand at 4°C for overnight, and centrifuged at12000 rpm for 30 minutes at 4 °C. Pellet were dissolved in 5 ml of 0.02M Tris-HCl pH8.5 ,then dialyzed against 2 liters of 0.02M Tris-HCl pH8.5 overnight

under cooled conditions (4ºC) [9]. Uricase activity was measured as mebtioned above and protein concentration were determinate by Bradford method [16].

**Purification of uricase by ion exchange chromatography on DEAE-cellulose**

A column (2.5x7cm) was packed with DEAE-cellulose (Pharmacia/Sweeden). The concentrated and dialyzed cell free supernatant was applied to a column, which previously was equilibrated with 20mM Tris-HCl pH8.5. The column was washed with 3 beds volumes of 20mM Tris-HCl pH8.5at a flow rate of 60ml/hour and the bound proteins were eluted with linear gradient of NaCl (0- 0.75M) in the same buffer. The volume of collected fractions was 5ml, optical density was measured for each fraction at 280nm and uricase activity was measured for active fractions . Fractions containing uricase enzyme activity were pooled and concentrated by using polyethylene glycol.

**Purification of uricase by Gel filteration Column (Sepharose 6B)**

The concentrated and active fractions collected by DEAE-Cellulose were applied to sepharose 6B column(1.5x45cm)which had been equilibrated with 0.02M Tris-HCl pH8.5.Slow flow rate (0.6ml/min)were used to allow optimal separation of the enzyme form other proteins. Fractions containing uricase activity were pooled and concentrated by using polyethylene glycol.

**Enzyme characterization**

**Effect of temperature on enzyme activity and stability**

To study the effect of temperature on uricase activity, the purified enzyme reaction mixture was incubated at different temperatures (30, 35, 40, 45, 50, 55, and 60 °C) for 30min, and uricase activity was detected ,while to study the effect of temperature on uricase stability, the enzyme solution was pre-incubated at different temperatures 40, 50, 60 ,70 and 80°C for 30 min. then the enzyme activity was measured at the optimum temperature [15].

**Effect of pH value on enzyme activity and stability**

To investigate the effect of pH on the enzymatic activity of the uricase, the enzyme was assayed at different pH; 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0. For 6.0–8.0 pH, phosphate buffer was used , while borate buffer was used for 8.0–10.0 pH .Then enzymatic activity was measured at optimum temperature ,while to investigate the effect of pH on the stability of uricase, 0.1ml of the enzyme was incubated with same amount of different buffer solutions at different pH value; 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0 for 30 min, then enzyme activity was measured at optimum temperature and optimum pH value as described previously [15].

**SDS-PAGE protein Electrophoresis**

The molecular weight of purified uricase was detected by SDS-PAGE electrophoresis and the size of polypeptide chains was determined by comparing its electrophoretic mobility in SDS-PAGE gel with mobility marker proteins (Promega/USA) of known molecular weight.

**Results and Discussion**

**Screening of Uricase Production by Quantitative Method**

The results showed that the isolates produce the enzyme uricase at different degrees of activity the isolate PA51showed the potential activity, which has reached to 13.1U/ml and the specific activity reached to 262U/mg, table -1 .Anderson and Vijayakumar [15] reported that the activity of uricase enzyme which extracted from *P.aeruginosa* as a crude extract was measured by colorimetric method was reached to 40U/ml after precipitation by 70% ammonium sulphate.

**Table 1**- Activity produced by *P.aeruginosa* uricase producers using quantitative method

| Isolates | Activity (U/ml) | Protien (mg/ml) | Specific activity(U/mg) |
|---|---|---|---|
| PA7 | 3.1 | 0.08 | 38.75 |
| PA9 | 2 | 0.03 | 66.66 |
| PA10 | 2 | 0.07 | 28.57 |
| PA15 | 1.3 | 0.07 | 18.57 |
| PA17 | 4.1 | 0.04 | 102.5 |
| PA18 | 3.1 | 0.04 | 77.5 |
| PA19 | 1.5 | 0.06 | 25 |
| PA29 | 1.1 | 0.06 | 18.3 |
| PA35 | 8 | 0.06 | 133.3 |

| | | | |
|---|---|---|---|
| PA36 | 1 | 0.09 | 11.1 |
| PA37 | 1.5 | 0.05 | 30 |
| PA38 | 5.1 | 0.05 | 102 |
| PA40 | 5.2 | 0.05 | 104 |
| PA42 | 3.4 | 0.06 | 56.6 |
| PA49 | 12.1 | 0.07 | 172.85 |
| PA51 | 13.1 | 0.05 | 262 |
| PA52 | 11 | 0.05 | 220 |
| PA53 | 10 | 0.07 | 142.85 |
| PA61 | 5 | 0.05 | 100 |
| PA62 | 1.4 | 0.07 | 20 |
| PA66 | 5.4 | 0.07 | 77.41 |
| PA67 | 1.4 | 0.06 | 23.33 |
| PA68 | 0.5 | 0.05 | 10 |
| PA69 | 5 | 0.04 | 125 |
| PA70 | 9.4 | 0.06 | 156.6 |
| PA71 | 1.6 | 0.04 | 40 |
| PA72 | 3.1 | 0.05 | 62 |
| PA74 | 5.4 | 0.05 | 108 |
| PA75 | 0.5 | 0.05 | 10 |
| PA76 | 6.4 | 0.03 | 213.33 |
| PA77 | 6.1 | 0.06 | 101.6 |
| PA80 | 12 | 0.07 | 171.42 |
| PA81 | 1.2 | 0.07 | 17.14 |
| PA82 | 8.2 | 0.06 | 136.6 |
| PA83 | 2 | 0.07 | 28.57 |
| PA24 | 0.5 | 0.07 | 7.14 |
| PA84 | 7.2 | 0.04 | 180 |
| PA85 | 11.2 | 0.06 | 186.6 |

**Purification of uricase**

**Precipitation by Ammonium sulphate**

The results indicated that 80%of saturated precipitates of crude uricase showed 35.2 U/ml while the specific activity was 234.66U/mg, table -2 .In previous study [5] reported that the proteins of the culture supernatants of *Proteus* and *Streptomyces* were precipitated with 80% ammonium sulphate.

**DEAE-Cellulose Column Chromatograpgy**

*P.aeruginosa* uricase enzyme was purified by using DEAE-Cellulose as a second step of purification. The results showed that the wash fractions had no enzyme activity of uricase, fractionation pattern of uricase enzyme upon using DEAE-Cellulose ion exchange chromatography showed that *P.aeruginosa* uricase was bound to the column at pH 8.5 and eluted with Tris-HCl containing NaCl gradients (0-0.75M) . The enzyme was detected in the third protein peak at fraction numbers (21-27), eluted in the 0.5 M of NaCl, purified almost 12.35 fold with a specific activity and recovery of 1300U/mg and 30.10%, respectively figure-1. The results of present study were in agreement with those of Saeed [9] who showed that after purification of uricase of *P. aeruginosa* uricase using an ion exchange chromatography on Q-sepharose fast flow anion exchange and sephadex G- 50 column, most of uricase activity was found in the third protein peak eluted by the 0.5M NaCl gradient with 29.71- purification fold and recovery of 31% respectively.

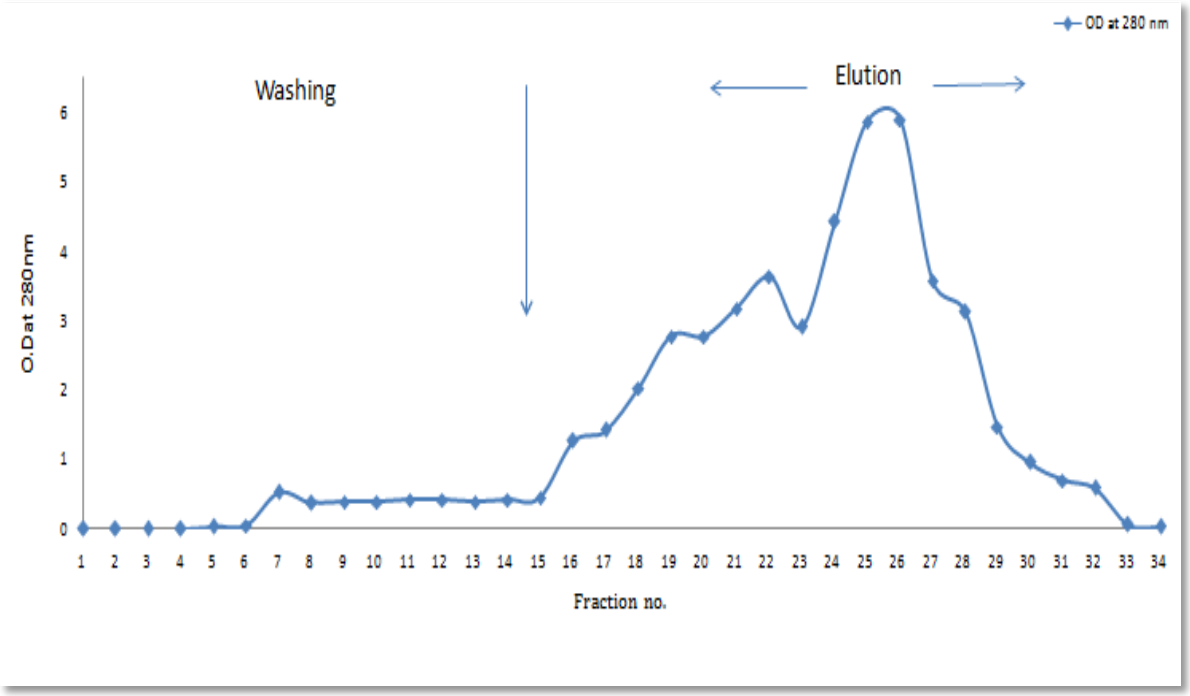


**Figure 1**- Fractionation pattern of *P.aeruginosa* uricase enzyme by DEAE-Cellulose chromatography column (2.5x7cm). washed with 20mM Tris-HCl pH8.5. eluted with a NaCl (0- 0.75M) in the same buffer and the fraction volume (5ml).

**Sepharose 6B Column Chromatograpgy**

Figure -2 showed only one peak of protein were obtained, the highest protein concentration with the highest uricase activity was determined in the fractions from (21 to 25). 16.4% of uricase was recovered with specific activity 2337.5 U/mg and 22.21 fold of purification table -2 . The results of this step were somewhat similar with those of Saeed [9] who found that only second protien peak has uricase activity. Uricase was purified to 31.93 fold with specific activity of 636.360U/mg and recovery of 11.5%.

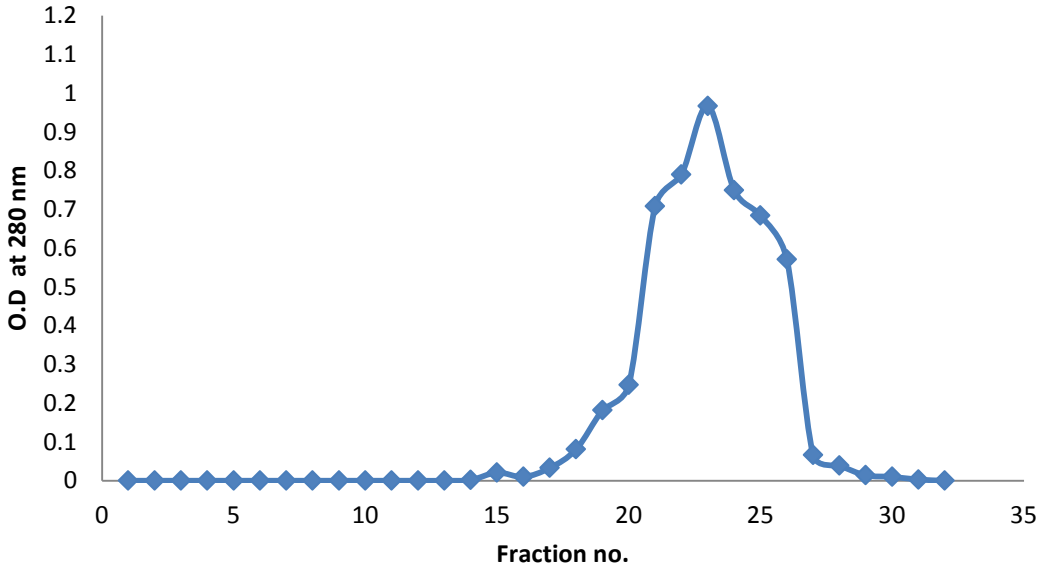


**Figure 2-** Fractionation pattern of *P.aeruginosa* uricase enzyme by gel filteration( sepharose 6B) chromatography column (1.5x45cm). washed with 0.02M Tris-HCl pH8.5.the fraction volume (5ml).

**Table 2-** Purification of uricase enzyme using DEAE-cellulose and Sepharose 6B column chromatography

| Step | Volume (ml) | Activity (U/ml) | Total Activity (U) | Protien mg/ml | Total Protien mg | Specific activity U/mg | Fold of purification | Recovery % |
|---|---|---|---|---|---|---|---|---|
| Crude | 285 | 10 | 2850 | 0.095 | 27.07 | 105.26 | 1 | 100 |
| Ammonium sulphate precipitation | 26 | 35.2 | 915.2 | 0.15 | 3.9 | 234.66 | 2.22 | 32.11 |
| DEAE-Cellulose | 11 | 78 | 858 | 0.06 | 0.66 | 1300 | 12.35 | 30.10 |
| Sepharose 6B | 5 | 93.5 | 467.5 | 0.04 | 0.2 | 2337.5 | 22.21 | 16.4 |

**Effect of Temperature on Enzyme Activity:**

Figure -3 , showed that uricase activity increased with increasing of temperature until reached 45ºC while, sharp decreasing in uricase activity were appeared above 45ºC. The result of this study showen that uricase gave high activity at 45 °C was agreed with those of other study [17], who found that maximum activity of uricase from the unicellular green alga *Chlamydomonas reinhardtii* was obtained at 40 ºC. Aly and co-workers [18] also reported that the optimum temperature for maximum activity of uricase from *Streptomyces exfoliatus* was 45 °C.While this result was higher than those obtained by Saeed [9] and Mabrouk [19], who study the effect of temperature on purified uricase produced by *P.aeruginosa* and *Gliomastix gueg*, respectively and found that the purified uricase gave highest uricase activity at 35 ºC. The highest uricase activity of *P.aeruginosa* was obtained at 35°C [15].

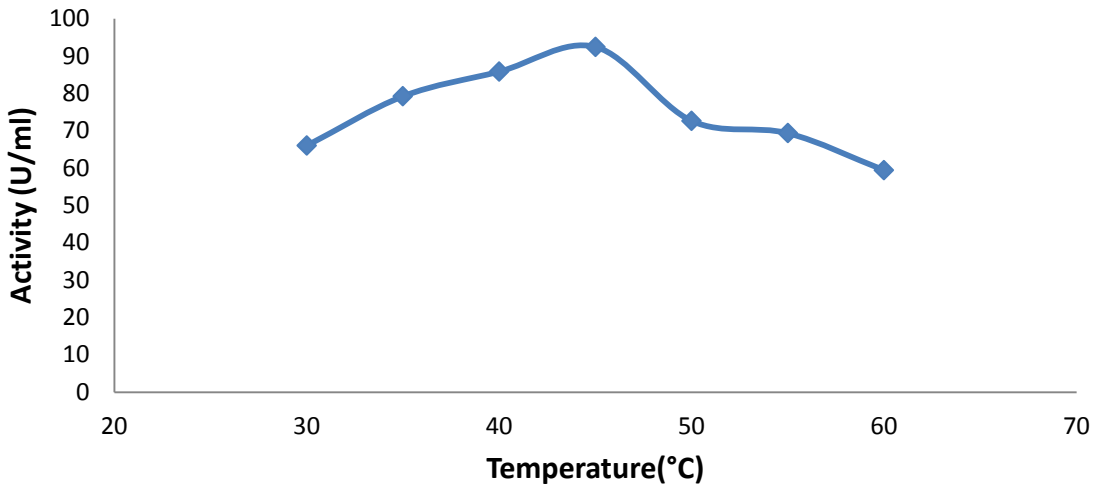


**Figure 3-** Effect of temperature on uricase activity

**Effect of Temperature on Enzyme Stability**

Uricase enzyme appear to be stable at 40ºC, with slightly decrease in 50ºC and 60 ºC (remaining activity 88.88% ,8333%) , respectively while at 70ºC and 80ºC showed sharp decreasing in the stability with remaining activity 16.16% and 11.11% , respectively figure -4. Studying effect of temperature on enzyme stability showed that uricase was stable at 40°C ,many researchers study the thermostbility of uricase, these results agreed with those of Ohe and Watanabe [20], who showed that uricase of *Streptomyces cyanogenus* was stable below 50 ºC (pH 7.8, 10 min). While in another study Zhou and co-workers [21] reported that uricase of new isolate belongs to the genus *Microbacterium* , retained about 100% of their initial activity after thermal treatment at 70 °C for 30 min. The results of

this study also agreed with those of Aly and co-workers [18] who found that the purified uricase produced by *Streptomyces exfoliatus* was stable at 45 °C for one hour.

Nishimura and co-workers [22] reported that *C. utilis* uricase contains three cysteines, of which one is located near the uricase active site but is not necessary for its activity. It is known that *Candida* uricase subunits can associate to form an enzyme with lower specific activity under aerobic conditions, *C. utilis* uricase usually contains four cysteines residues, and the substitution or oxidation of Cys168 can result in uricase activity reduction.

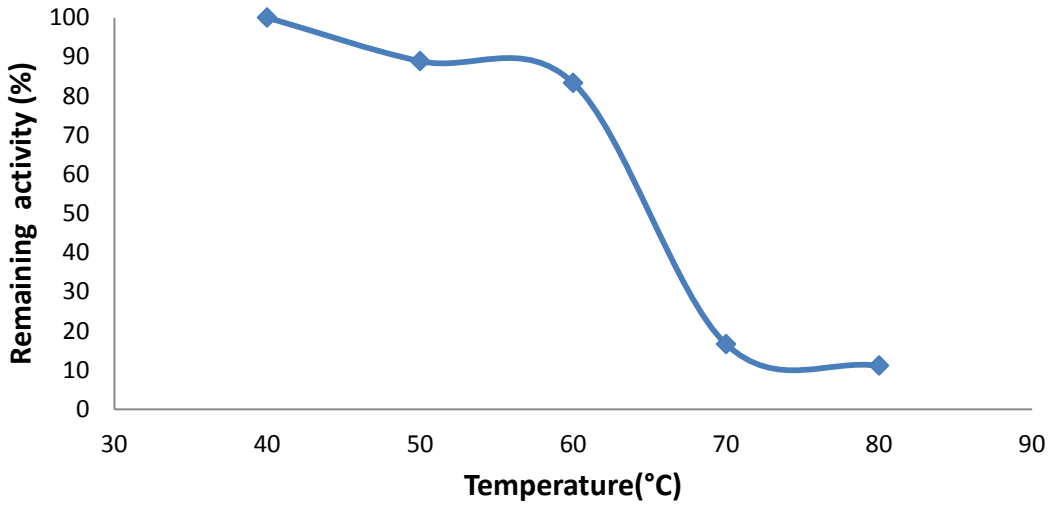


**Figure 4-** Effect of temperature on uricase stability

**Effect of pH on Enzyme Activity**

The results of pH effect on activity indicated that optimum pH for maximum uricase activity was (pH=9) while higher and lower pH values showed decreased activity of the enzyme. Uricase activity increased gradually with increasing of pH values from 6 towards alkaline pH and reached to maximum activity at pH 9, then activity decreased above pH 9, figure -5. The maximum activity of purified uricase from *Gliomastix gueg* was detected at pH 9.0 [19]. Saeed and co-workers [9] found that the purified uricase of *P.aeruginosa* exhibited maximum uricase activity at pH 9.

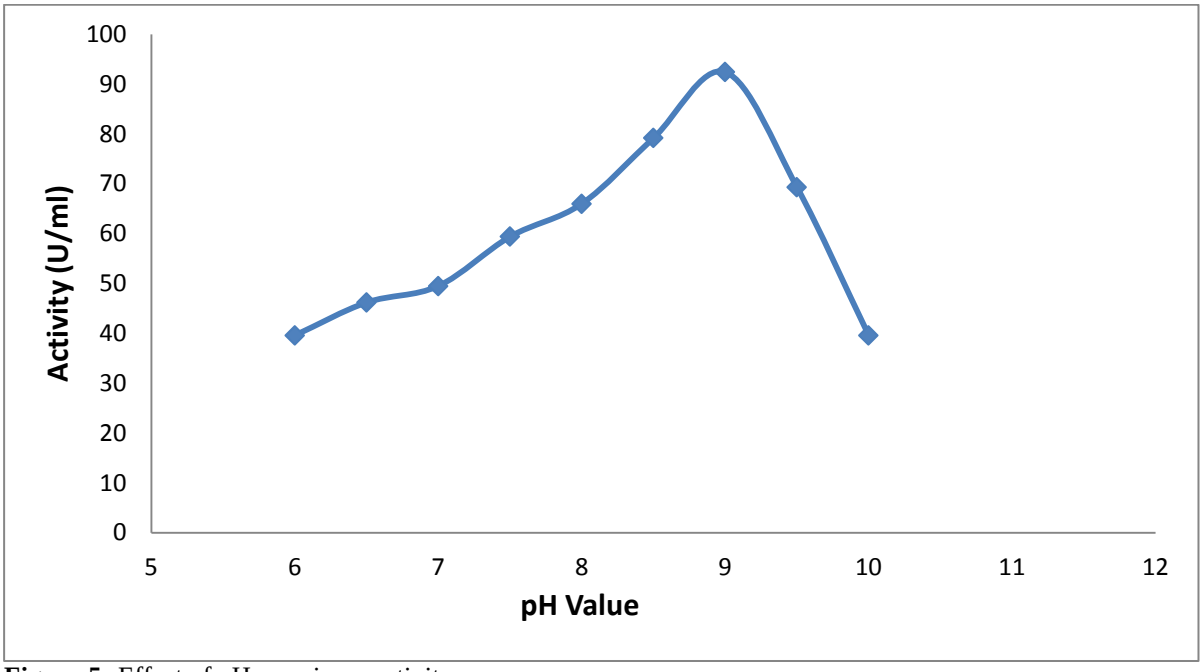


**Figure 5-** Effect of pH on uricase activity

**Effect of pH on Enzyme Stability**

Uricase was subjected to different pH (6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5,10)for 30 min , the results indicated that it is stable at a wide range of pH values, its more stable at the neutral and slightly alkaline pH and there is no changes in enzymatic activity when the enzyme was incubated in pH (7.5-9), figure -6. The results of present study were agreed with those obtained by Ohe and Watanabe [20]who reported that the uricase enzyme of a strain of *Streptomyces cyanogenus* has highest activity at pH 8 and was stable between pH 6 and 11 (35 ºC, 1 h) and below 50 ºC (pH 7.8, 10 min) , Studying the stability of uricase from *Candida* sp. in different pH values showed that uricase was stable at a pH ranged from 8.5-9.5. [24] .The stability at different pH values (pH 6–11) showed that uricase from both wild-type and mutant *of Bacillus subtilis* are at highest activity at pH values from 6-10 [25]. The uricase produced by *Microbacterium* sp. was stable at pH between 7 – 10. [26].

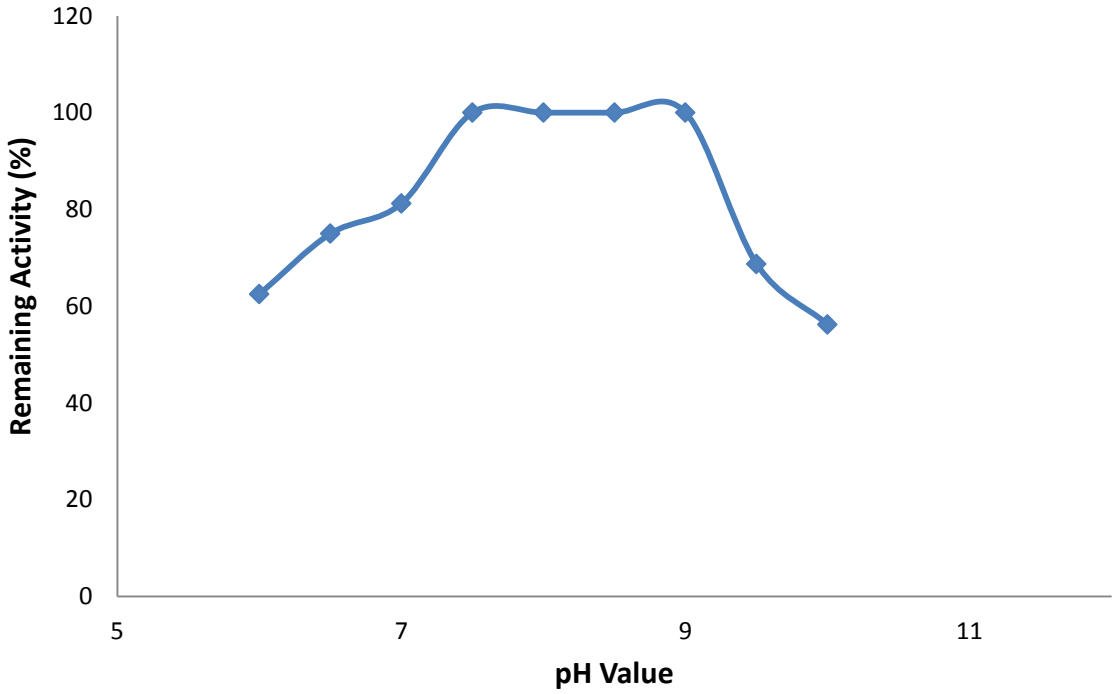


**Figure 6-** Effect of pH on uricase stability.

**Sulphate polyacrylamid gel electrophoresis (SDS-PAGE)**

Sodium dodecyl Sulphate polyacrylamid gel electrophoresis was used for determination of molecular weight ,uricase was migrate as one band by using Sodium dodecyl Sulphate polyacrylamid gel electrophoresis (SDS-PAGE) and the molecular weight obtained in this study was approximately 35kDa, figures -7,-8. The result for determination of uricase molecular weight that showed a molecular weight of approximately 35KDa, agreed with those of Anderson and Vijayakumar [15] who found that The molecular weight of purified uricase enzyme produced by *P.aeruginosa* was detected to be 33 kDa by SDS-PAGE profile and with results of Geweely and Nawar [27] who proved that the molecular weight of *A. niger* extracellular uricase isoenzymes UI, UII, UIII and UIV were 39.70, 30.50, 55.30 and 18 KDa, respectively.

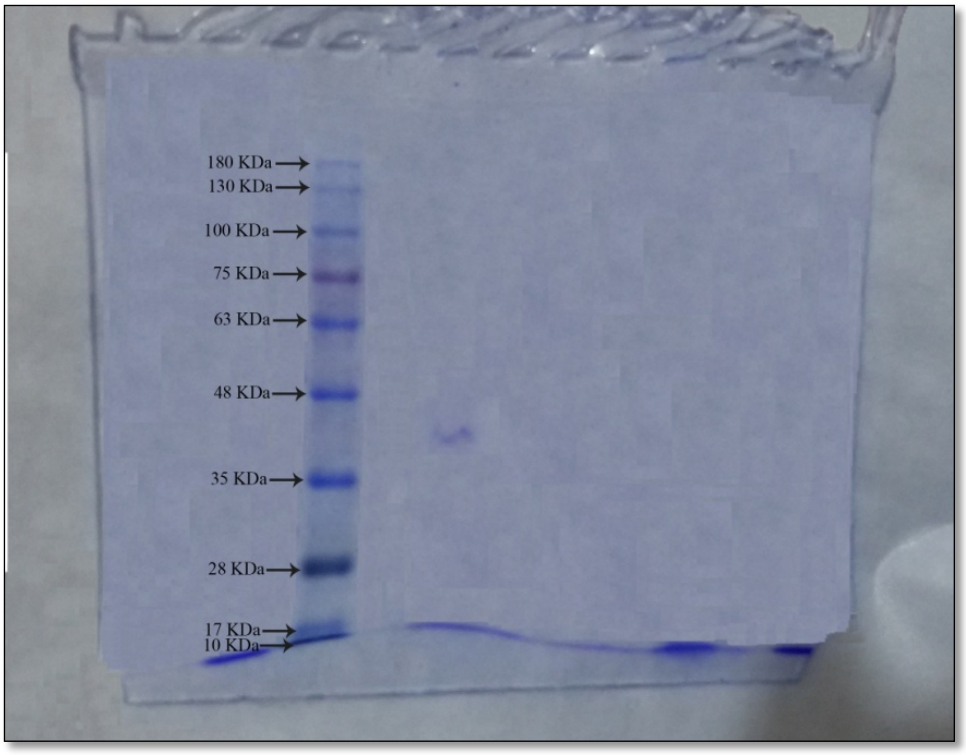


**Figure 7-** Purified uricase from *P.aeruginosa* on Sodium Dedosyl Sulfate Electrophoresis(SDS PAGE).Standard protein markers ranged between (10-180 KDa).

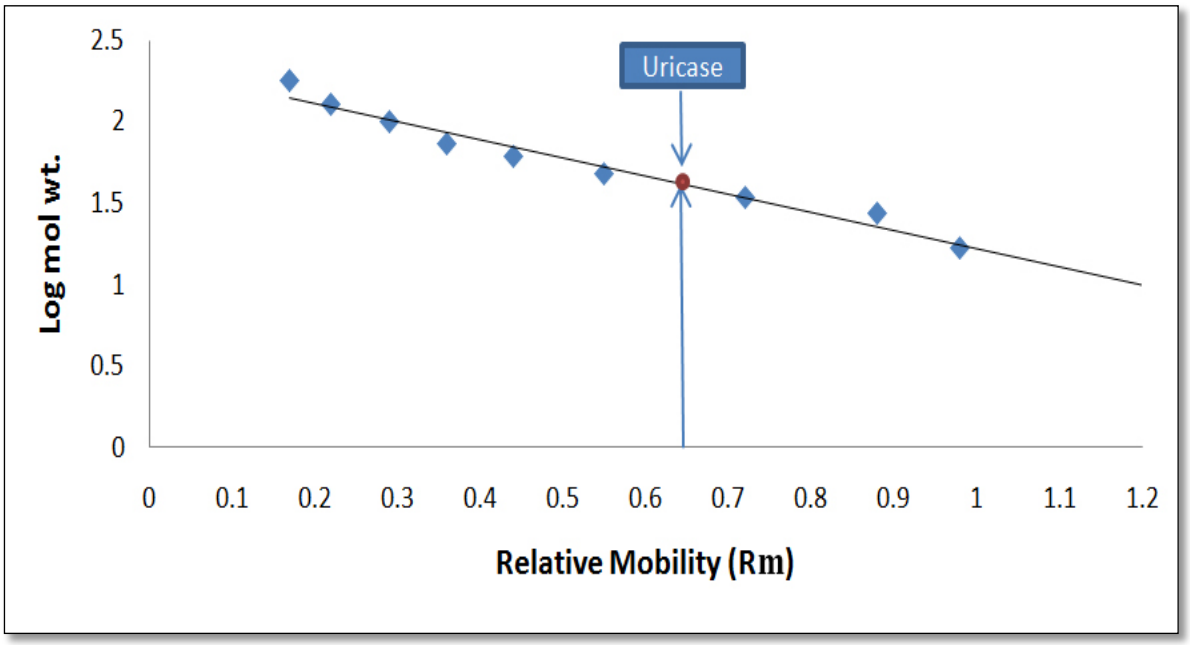


**Figure 8-** Molecular weight standard curve of purified uricase from *P.aeruginosa* . protein markers of molecular weight ranging from 10 to 180 KDa were used.

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