

## Purification and Characterization of Nitrate Reductase (NAR) from *Pseudomonas* sp. SH7 Isolate

تنقية وتوصيف انزيم Nitrate Reductase (NAR) المنتج من بكتيريا *Pseudomonas* sp. العزلة SH7

Shaymaa H. Al-Rajhi

Majed H. Al-Gelawi

Ghazi M. Aziz \*

Biotechnology Dept., College of Science, Al-Nahrain University

\*Biotechnology Dept., College of Science, Baghdad University

غازي منعم عزيز\*

ماجد حسين الجلاوي

شيماء حسين الراجحي

قسم التقانة الاحيائية - كلية العلوم - جامعة النهرين

\*قسم التقنيات الاحيائية - كلية العلوم - جامعة بغداد

### Abstract

Sixty five soil samples and fifteen water samples were collected from different places in which previously explosions were occurred in Iraq. Seven isolates showed ability to utilize 0.1mM trinitrotoluene (TNT) and/or 0.2mM glycerol trinitrate (GTN) as a sole carbon and nitrogen source and one of these isolates showed the highest nitrate reduction which was classified and coded as *Pseudomonas* sp. SH7. The highest nitrate reductase activity extracted by sonication while optimum conditions for enzyme production in minimal media pH 7 containing 0.25 mM GTN at 35°C for 3 days under aerobic condition. Nitrate reductase was purified by 40-60% ammonium sulphate, ion exchange and gel filtration. Nitrate reductase molecular weight determined by SDS-PAGE was 115 kD. The characterization of purified enzyme activity and stability was higher at a pH between 6.5-7.5 and. Maximum activity was at 35°C and stable at 30-40°C for 15 min., while for heat sensitivity 100% activity observed at 45°C for 20 min. Treatment with 200 µM azide and 500 µM cyanide inhibited the activity by 76 and 91% respectively.

### المستخلص

جمعت خمسة وستون عينة تربة وخمسة عشر عينة من الماء من مختلف المناطق التي تعرضت للانفجارات في العراق. لقد تميزت سبع عزلات بأعطائها نسبة اختزال عالية 0.1 mM من (TNT) و 0.2 mM من (GTN) كمصدر كاربوني و نيتروجيني وحيد. وبعد عدة خطوات من الغربلة الاولى انتخبت العزلة SH7 كونها العزلة الاعلى اختزالا للنترات مقارنة مع بقية العزلات هي من نوع *Pseudomonas*. ان اعلى فعاليتها لانزيم NAR تم الحصول عليها عند معاملة الخلايا بالامواج فوق الصوتية. بينما الظروف المثلى للانتاج كانت بتسمية الخلايا في الوسط الادنى المحتوي على 0.25 mM من (GTN) عند رقم هيدروجيني 7.0 وحضن بدرجة حرارة 35م لمدة ثلاثة ايام تحت ظروف هوائية. تضمنت خطوات تنقية الـNAR الترسيب بكبريتات الامونيوم بنسبة اشباع 40-60% والتبادل الايوني والترشيح الهلامي. كما ان الوزن الجزيئي للانزيم 115 كيلو دالتون عند تعيينه بأجراء الترحيل الكهربائي في هلام متعدد الاكريل امايد بوجود المادة الماسخة للبروتين SDS. و اظهرت النتائج ان اعلى فعالية وثبات للانزيم كانت من الرقم الهيدروجيني 6.5-7.5. ولوحظ ان اقصى فعالية للانزيم كانت عند درجة حرارة 35م واحتفظ الانزيم بفعاليتها عند حضن لمدة 15 دقيقة بدرجات حرارة (30 - 40)م. كما احتفظ الانزيم بكامل فعاليتها عند حضن لمدة 20 دقيقة بدرجة حرارة 45 م°. وقد وجد ان فعالية الانزيم قد تثبطت عند معاملته بـ 200 µM من الازايد و 500 µM من السيانيد الى انخفاض في الفعالية بلغ (76، 91) %، على التوالي.

Key words: TNT, GTN, *Pseudomonas*, Nitrate reductase, Purification.

## Introduction

Life on this planet is based on the continuous cycling of elements. In the recent years the massive mobilization of natural resources and the industrial synthesis of chemicals have generated a number of environmental problems as a consequence of the limited incorporation of natural and synthesized molecules into ongoing biological cycles. This is particularly true for xenobiotic compounds, which exhibit structural elements or substituents that are rarely found in natural products. Nitroaromatic compounds are xenobiotics that have found multiple applications in the synthesis of foams, pharmaceuticals, pesticides, and explosive. These compounds are toxic, recalcitrant and degraded relatively slowly in the environment by the microorganisms [1].

Nitrate reductases can be performed with three different types: the utilization of nitrate as a nitrogen source for growth (nitrate assimilation), the generation of metabolic energy by using nitrate as a terminal electron acceptor (nitrate respiration), and the dissipation of excess reducing power for redox balancing (nitrate dissimilation). Fourth type of nitrate reductases catalyze the two-electron reduction of nitrate to nitrite was the eukaryotic assimilatory nitrate reductases while the other three distinct bacterial enzymes, comprising the cytoplasmic assimilatory (Nas), membrane-bound respiratory (Nar), and periplasmic dissimilatory (Nap) nitrate reductases. All eukaryotic and bacterial nitrate reductases contain a molybdenum cofactor at their active sites. The basic structure of the eukaryotic cofactor is molybdopterin, a 6-alkyl pterin derivative with a phosphorylated C4 chain with two thiol groups binding the Mo atom [2].

Nitrate-reducing and true denitrifying bacteria have been shown to harbor either the membrane-bound or the preiplasmic nitrate reductase or both types of reductases. A study focusing on the fluorescent *Pseudomonads* community showed that 56% of the isolated strains had nar gen only, 51% had the nap gene and 15.5% possessed both nitrate reductase genes [3].

Enzyme application in biotechnology and environmental fields requires a highly purified nitrate reductase with maximum specific activity. Thus, isolate specific strain that had efficient nitrate reductase. Studied the optimization, purification and characterization of nitrate reductase produced from *Pseudomonas* sp. SH7 isolate.

## Materials and Methods

**Sampling:** Soil and water from different places in which previously explosions were occurred from (Baghdad, Najaf, Karbala, Basra, Waset, Mousel and Al-Anbar). Two or three tablespoons of soil and 5ml of water were collected from each locations. Transported in sterile bags, then the samples were stored at 4°C until use.

**Isolation and Identification:** Samples were identified as the criteria of Bergey's Manual of Systematic Bacteriology [4].

**Preparation of Cell Extract:** Culture of *Pseudomonas* isolate SH7 was grown on minimal media (composed of the following (g/L):  $\text{KH}_2\text{PO}_4$ ;3,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 0.12,  $\text{K}_2\text{HPO}_4$ ; 1, NaCl; 0.5 and 1ml of trace element solution which contained the following (g/L):  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ;0.06,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ;0.03,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ;0.31,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ; 0.04,

Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O;0.03, H<sub>3</sub>BO<sub>3</sub>; 0.57, FeCl<sub>3</sub>·6H<sub>2</sub>O;0.24) containing 0.2mM GTN at 35°C for 3 days, then harvested by centrifugation at 6000rpm for 20 min., washed, and re-suspended in 0.1M phosphate buffer. Cells were disrupted by three different methods one of them was sonication for 15 min at 4°C using 20KHz. Residual whole cells and cell membrane fragments were removed by centrifugation 6000rpm for 20 min. The other method was done by adding SDS to the cell suspension with a final concentration of 1% and the mixture was incubated in shaker water bath at 37°C for 30 min. Samples were taken from the mixture, centrifuged at 6000rpm at 4°C for 20 min. While the third method was freezing and thawing in which a 20-ml amount of the cell suspension was placed in a 100-ml container and stored at -23°C until completely frozen. The sample was then placed at 37°C until completely thawed. The complete cycle was repeated 5 times with a total elapsed time of 2 to 4hr. Cell suspension from the three methods were centrifuged at 6000rpm for 20min. The resulting supernatant was used as the starting point to establish nitrate reductase activity in crude cell extracts.

**Assay of nitrate reductase:** Nitrate reductase activity was assayed with reduced β-NADH as an electron donor [5].

**Protein determinations:** Protein was performed by Bradford, [6] with bovine serum albumin used as standard

**Optimal conditions for nitrate reductase production:** Several factors were studied to determine the optimal conditions for nitrate reductase production from *Pseudomonas* sp. SH7 isolate and as following:

- 1. Determination of optimal substrate concentrations:** Minimal media broth (pH 7.0) was prepared with different concentrations of glycerol trinitrate 0.05, (0.1, 0.15, 0.2, 0.25, 0.3, 0.4 and 0.5mM).
- 2. Determination of nitrate reductase incubation period:** Minimal media were inoculated and incubated at 35°C for (1, 3, 5, 7, 9, 12) days).
- 3. Determination of optimal aeration conditions for nitrate reductase production:** Minimal media broth (pH 7.0) was inoculated with bacterial isolate and incubated at 35°C for 3 days under aerobic and anaerobic conditions.
- 4. Determination of optimal pH for nitrate reductase production:** Minimal media broth with different pHs (5, 6, 7, 8 , 9) were inoculated and incubated at 35°C for 3 days.
- 5. Determination of optimal temperature for nitrate reductase production:** *Pseudomonas* sp. SH7 isolate was grown in minimal media broth (pH 7.0) and incubated at different temperatures 30, 35, 40, 45 , 50)°C for 3 days. Cells from all above factors were harvested by centrifugation at 6000 rpm for 10 min. Cell extracts were prepared by sonication treatment and nitrate reductase assays were performed as described above.

**Enzyme purification:** All steps for purification of nitrate reductase were carried out at 4°C, and all buffers contained 2% glycerol as an enzyme stabilizer.

**A. Ammonium sulfate fractionation:** The supernatant was fractionated with ammonium sulfate at (40–60) % saturation, and the precipitate obtained after

centrifugation at 6000 rpm for 30 min was suspended in 50 mM phosphate buffer (pH 7.0) and the enzyme activity and protein concentration were measured.

**B. DEAE - Cellulose column chromatography:** The sample was applied to a DEAE-cellulose (Whatman, DE52) column (3×25 cm) previously equilibrated with 50 mM phosphate buffer (pH 7.0). The protein was washed with the same buffer and eluted with a linear salt gradient containing 0–0.3M NaCl. The enzymatic activity for each fraction was assayed as described above; the curve of enzymatic activity (unit/ml) was plotted against O.D.<sub>280</sub> nm. The fractions that revealed significant peak of activity were mixed together.

**C. Gel filtration chromatography:** The gel (Sephadex G-200) was prepared according to the instruction of the manufacturer. The fractions collected from the DEAE-cellulose column chromatography were applied to a Sephadex G-200 column (2×90 cm) previously equilibrated with 0.1 M phosphate buffer (pH 7.5). Elution was performed with the same buffer, the fractions that revealed the protein and enzymatic activity in the same peak were mixed and transferred to a new sterile tube for further study.

**SDS-Polyacrylamide electrophoresis:** Molecular weight and proteins were determined by denaturing gel electrophoresis in 7.5% polyacrylamide resolving gels, using a Tris-glycine-SDS buffer system with  $\beta$ -mercaptoethanol as a reducing agent. Proteins were visualized by staining with Coomassie brilliant blue R-250, there molecular weight were determined by comparison with standard proteins.

**Enzyme characterization assays:** Some of the characteristics of partially purified nitrate reductase were determined and as the following:

**Determination of optimal temperature activity and thermal stability for nitrate reductase:** 0.1 ml of partially purified nitrate reductase was added to 0.9 ml of 0.1M potassium phosphate buffer, containing 0.2mM glycerin trinitrate as a substrate, was incubated for 15 min. at different temperatures (25, 30, 35, 40, 50, 60)°C then immediately transferred into an ice bath. Enzymatic activity was measured against the temperature.

While for thermal stability, equal volumes of partially purified nitrate reductase and 0.1M potassium phosphate buffer solution were incubated in water bath at (25, 30, 35, 40, 50, 60)°C for 15min., and immediately transferred into an ice bath. Enzymatic activity was measured and the remaining activity (%) was plotted against the temperature.

**Determination of thermal sensitivity for nitrate reductase at 45°C:** The partially purified nitrate reductase was incubated in a water bath 45°C for different times (10, 20, 30, 40, 50, 60) min. and immediately transferred into an ice bath. Enzymatic activity was measured against the temperature.

**Determination of pH effects on nitrate reductase activity and stability:** 0.1 ml of Partially purified nitrate reductase was added to 0.9 ml of 0.1M potassium phosphate buffer, each one of different pH (5.5 - 8.5) containing 0.2mM glycerin trinitrate as a substrate and nitrate reductase assays were performed.

While for stability equal volumes of partially purified enzyme and 0.1M potassium phosphate buffer solution with pH range (5.5 - 8.5) were incubated in a water bath at

35°C for 30 min. then transferred immediately into an ice bath. The enzymatic activity for each one was measured. The remaining activity (%) for nitrate reductase, optimal pH for enzyme stability.

**Determination of inhibitors effects on nitrate reductase activity:** Equal volume of partially purified nitrate reductase were incubated in a water bath with (0, 25, 50, 100, 150, 200, 300)  $\mu\text{M}$  of sodium azide and (0, 50, 100, 200, 300, 400, 500, 600)  $\mu\text{M}$  of potassium cyanide at 35°C for 10 min., then immediately transferred into an ice bath. The enzymatic activity was measured.

### Results and Discussion

**Isolation and identification of microorganisms:** Sixty five soil samples and fifteen water samples were collected from different places in which previously explosions were occurred.

Only one isolate was chosen which was SH7, the highest nitrate and nitrite analysis among them and was identified as *Pseudomonas* isolate by the criteria of Bergey's Manual of Systematic Bacteriology [4].

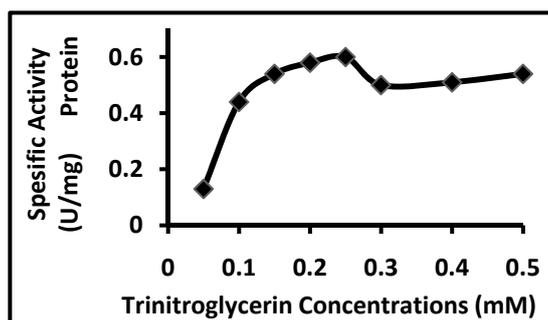
**Extraction of nitrate reductase:** Three of the most known extraction methods were used to determine nitrate reductase activity. Results showed that, *Pseudomonas* sp. SH7 cells washed with 0.1M phosphate buffered, treated with sonication have maximal nitrate reductase activity (1.5 U/ml) compared with SDS treated cells (0.85 U/ml) while the freezing and thawing show very little nitrate reductase activity (0.2 U/ml).

The significant increase in nitrate reductase activity after sonication treatment could be explained depending on the fact that complete lysis of cells would be sufficient to increase nitrate reductase activity since best results were obtained when the cell wall is completely removed and this increase in activity proves that *Pseudomonas* sp. SH7 isolate having periplasmic nitrate reductase is not membrane-bound nitrate reductase [7].

Recent reports on the isolation of periplasmic nitrate reductase from *D. desulfuricans* described the preparation of cell extracts by sonicated cells suspension for a total of 10 min. using Kubota Isonator Model 20S [8].

**Optimum substrate concentrations for nitrate reductase production:** Different concentrations of trinitroglycerin were studied (0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5) mM in which the optimum concentration was 0.25mM and the specific activity was 0.60 U/mg protein of *Pseudomonas* sp. SH7 isolate was shown in Figure (2). Neubauer and Gotz, [9] studied the effect of nitrate on the nitrate reductase activity of *S. carnosus*. They found that, increasing nitrate from 10mM to 25 mM lead to increase the specific activity from 1.4 to 2.98 U/mg proteins.

A recent study found that the optimum substrate concentration for cotton nitrate reductase was 0.1mM of potassium nitrate and the specific activity at that concentration was 3.2U/mg protein and it decreased to 2.5U/mg protein when the potassium nitrate was 1.5M [10].



Fig(1): Effect of Trinitrolycerin concentrations on nitrate reductase production from *Pseudomonas* SH7

**Optimum incubation period for nitrate reductase production:** The optimum incubation periods were characterized for the nitrate reductase produced from *Pseudomonas* SH7 isolate for (1, 3, 5, 7, 9, 12) days. The maximum production of nitrite occurred when the enzyme was incubated for 3 days in which the specific activity was 0.64 U/mg protein as shown in Figure (2).

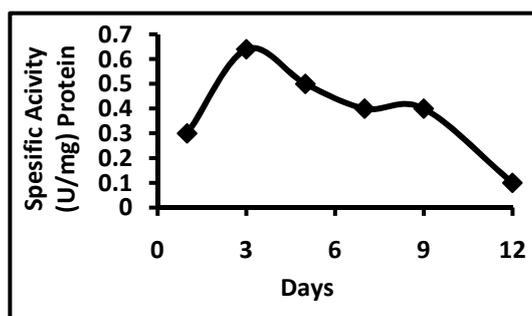


Fig (2): Optimum incubation period for nitrate reductase production from *Pseudomonas* sp. SH7

**Optimum aeration conditions for nitrate reductase production:** *Pseudomonas* sp. SH7 isolate was grown in minimal media containing 0.25mM trinitrolycerin under aerobic and anaerobic conditions. Results showed that the aerobic condition gave a higher specific activity (0.65 U/mg protein) than anaerobic condition (0.5 U/mg protein), this result was much similar to the results obtained by [19], in which the periplasmic nitrate reductase of *T. pantotropha* was expressed under both aerobic and anaerobic conditions and the specific activity was very low under anoxic condition 0.2 and 0.05 U/mg protein respectively. In contrast with the other study, a fourfold increase in enzyme activity was observed with anaerobically grown cells of *S. carnosus*. Specific activity under aerobic was 0.15 while under anaerobic was 0.62 U/mg protein [9].

**Optimum pH for nitrate reductase production:** To investigate the effect of the medium pH on nitrate reductase, *Pseudomonas* sp. SH7 isolate was grown in minimal media containing 0.25mM trinitrolycerin with different pH values (5, 6, 7, 8, and 9). The results in Figure (3), showed that the higher production of nitrate reductase was close to optimal pH of bacterial growth which was 7.0 and the specific activity was

0.68 U/mg protein, while there was low growth at pH values less than 5.0 and more than 9.0, in which we could suggest the higher production linked to the higher growth of the bacteria, that result was similar to [11] reported that the optimal pH for nitrate reductase production was 7.1.

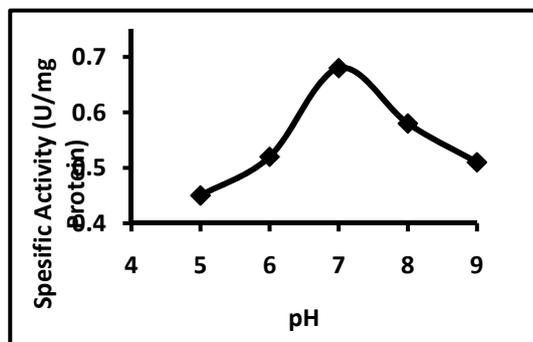


Fig (3): Optimal pH for nitrate reductase production from *Pseudomonas* sp. SH7

**Optimal temperature for nitrate reductase production:** The enzyme activity was assayed at various temperatures (25, 30, 35, 40, 45, 50)°C. The optimum temperature for nitrate reductase production was 35°C and the specific activity was 0.72 U/mg protein Figure (4), this result is similar to [12] in which they found that the optimum temperature for growth and production of nitrate reductase was 35°C.

Other studies on the effect of temperature showed that *P. denitrificans* cannot grow at temperatures above 40°C while the optimal temperature for nitrate reduction was found to be about 38°C [11], while optimum temperature of thermostable nitrate reductase from the hyperthermal archaeon *Pyrobaculum aerophilum* was 95°C and optimal growth temperature was 100°C [13].

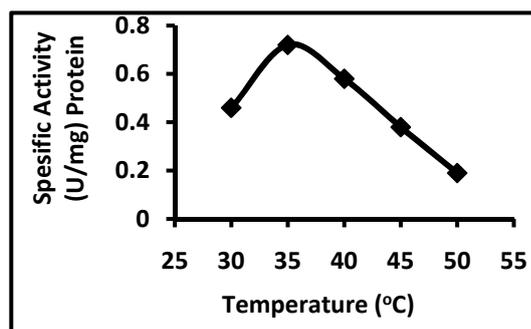


Fig (4): Optimal temperature for nitrate reductase production from *Pseudomonas* sp. SH7

**Enzyme purification:** The purification steps for nitrate reductase from *Pseudomonas* sp. SH7 are summarized in Table (1).

#### A. Ammonium Sulfate Precipitation

In order to concentrate the crude extract of nitrate reductase and remove as much water and some protein molecules as possible, the saturation ratio (40-60) % was used. It achieved specific activity 4.95 U/mg, with 7.5 purification folds with 54.4%

yield. From these results approximately there was duplication in activity within (40-60) % comparing with the crude extract, in addition to an increase in the specific activity. So the ammonium sulfate precipitation is recommended to this particularly stage of purification. Concerning the other studies, those results are similar to those of [14] they found that the specific activity with 60% ammonium sulfate of dissimilarly nitrate reductase purified from denitrifier *P. aeruginosa* was 24.6 U/mg, while the crude specific activity was 64.8 U/mg while [15], obtained a specific activity of 0.9 U/mg from *E. coli* by using 30% saturation ratio. Others found that purification of nitrate reductase from *P. nautica*, strain 617 used 40% sodium sulfate instead of ammonium sulfate. In general, ammonium sulfate is favored in the precipitation step due to its high solubility, availability, being cheap and that it does not damage most enzymes [16].

**Table (1): Purification Steps of Nitrate Reductase Produced by *Pseudomonas* sp. SH7**

Steps of Purification	Volume (ml)	Activity (U/ml)	Protein Conc. (mg/ml)	Specific Activity (U/mg)	Total Activity (U)	Folds of Purification	Yields (%)
Crude Extract	100	1.5	2.27	0.66	150	1	100
40 – 60% Ammonium Sulfate Saturation	30	2.72	0.55	4.95	81.6	7.5	54.4
Ion Exchange Chromatography by DEAE–Cellulose	20	2.49	0.4	6.23	49.8	9.43	33.2
Gel Filtration Chromatography by Sephadex G-200	10	2.24	0.19	11.79	22.4	17.86	14.9

**B. Ion – exchange and gel filtration chromatography:** DEAE-cellulose is a weak anion exchanger with excellent flow properties and high capacity for protein of most pI values with a high resolution. The ion exchange functional group is diethylaminoethyl that remains charged and maintains consistently high capacities over the entire working range.

The results in Table (1) showed nitrate reductase purified by anion ion-exchange column chromatography using DEAE-cellulose (3 by 25 cm). Figure (5) showed the wash of DEAE-cellulose column had two protein peaks without nitrate reductase activity, while Figure (6), showed that there was one peak for protein and one peak for nitrate reductase activity coincidence with each other in the elution fraction. Therefore, it could be concluded that nitrate reductase has been bounded onto the matrix of the exchanger, those protein fractions were washed out. Then the elution was run using the same buffer with linear salt gradient 0 M to 0.3 M NaCl (pH 7.0) which could detect as in. Then the fractions were collected together giving specific activity of 6.23 U/mg with 9.43 folds of purification and 33.2% yield.

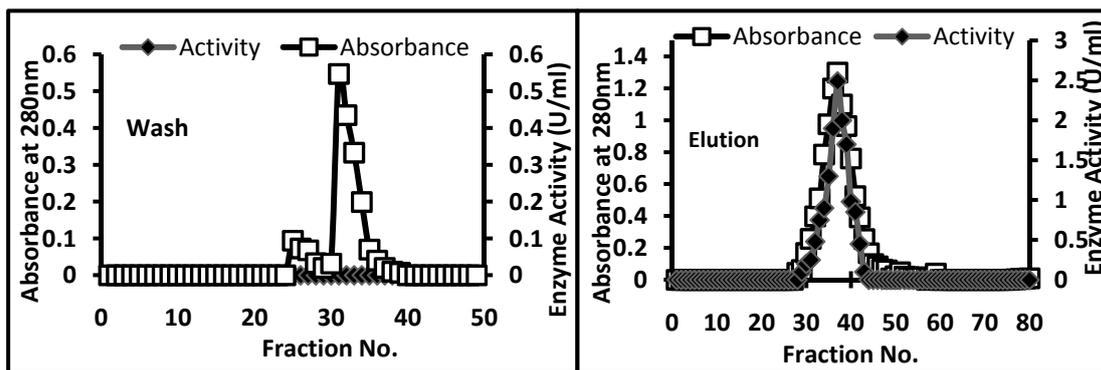


Fig (5): DEAE-Cellulose ion-exchange chromatography column (3×25cm) equilibrated with 0.1M phosphate buffer pH 7.2, Fraction volume: 3ml/tube

Fig (6): DEAE-Cellulose ion-exchange chromatography column (3×25cm), enzyme recovered with linear salt gradient 0-0.3M NaCl

The fraction from DEAE-cellulose column chromatography was applied to a Sephadex G200 column (2 mm× 90 cm) previously equilibrated with 0.1 M phosphate buffer. Gel filtration step for partially purified nitrate reductase was done, Figure (7), showed that there are two peaks, one peak for protein without any enzyme activity, while other peak for the enzyme in the eluted fraction from 27<sup>th</sup> to 40<sup>th</sup> with specific activity 11.79 U/mg, fold of purification 17.86 and yield 14.9%, which are considered as a good result when compared with other studies.

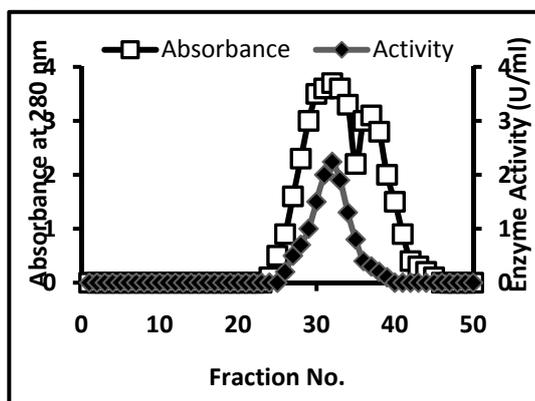
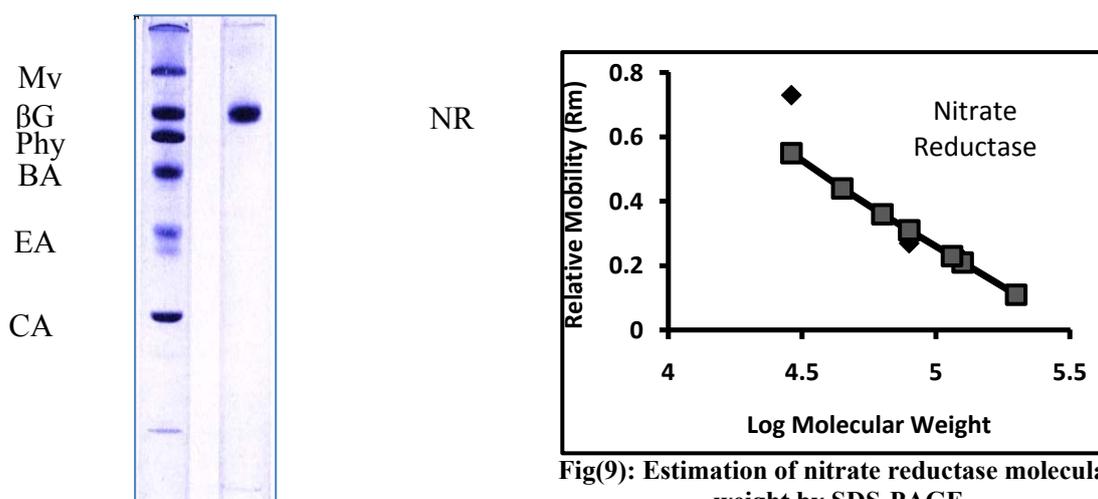


Fig (7): Gel filtration chromatography by sephadex G200 column (2×90 cm) equilibrated with 0.1 M phosphate buffer pH 7.0, fraction volume: 3ml/tube.

[17] purified nitrate reductase from *P. fluorescence* and *P. putida* by DEAE-cellulose chromatography followed by Q-Sepharose, showed specific activity of 0.35 and 0.33 U/mg with a yield of 71 and 33% respectively. [15] purified nitrate reductase from *E. coil* by alkali-acetone treatment followed by Sephadex G200 filtration and Bio-gel A15 filtration to get enzyme with specific activity of 15.8 U/mg and purification folds 52.5 with yield 1.6%, while Burke and Lascelles, [18]; could purify nitrate reductase enzyme by Bio-gel A1.5m with specific activity 7.7 U/mg and yield 71% followed by ECTEOLA-cellulose, with specific activity of 12.4 U/mg and yield 33%.

Furthermore, nitrate reductase purified from *A. eutrophus* H16 by Alkyl Superose and Mono Superose with specific activity 2.4 and 32.9 U/ml and yield 26 and 9% respectively [19].

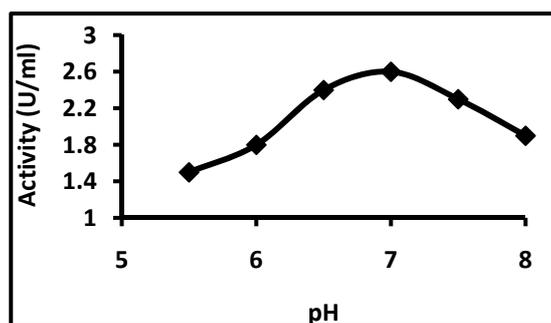
**Determination of enzyme purity:** The purified enzyme was electrophoresed under denaturing conditions using 7.5% SDS-PAGE in the presence of  $\beta$ -mercaptoethanol. Single protein band appear in the gel, when stained by Coomassie blue R250 Figure (8), indicating that nitrate reductase purified till homogeneity. The molecular weight of nitrate reductase was about 115 kD when compared with the molecular weight of standard proteins as shown in Figure (9). This value is similar to those reported for other nitrate reductase purified from *S. aureus*, *P. aeruginosa* and *P. isachenkovii* which had a molecular weight 112, 115 and 118 kD respectively [7, 14, 18].



**Fig(8): SDS gel electrophoresis of nitrate reductase** The gel on the right is Nitrate Reductase (NR) after gel filtration. The gel on the left is standard protein (from top): Myosin (My) (205KD),  $\beta$ -Galactosidase (116KD), Phosphorylase (Phy) (97KD), Bovine Albumin (BA) (66KD), Egg Albumin (EA) (45KD), and Carbonic Anhydrase (CA) (29KD)

**Optimum pH on nitrate reductase activity:** The effect of pH on purified nitrate reductase from *Pseudomonas* sp. SH7 was studied in pH range from 5.5–8, as shown in figure 10. The optimum pH was observed at values between (6.5–7.5), but the enzyme was active at pH 7.0, the activity was 2.9 U/ml. The active sites on enzyme are frequently composed of ionizable groups that must be in the proper ionic form in order to maintain the conformation of the active sites or change the configuration of the enzyme itself [20], which could be explain the decrease in activity at extreme values of acidity (5.5 , 6.5) and alkalinity (8).

Concerning the results of the other studies, the optimal pH for the nitrate reductase activity produced by *Haloferax mediterranei* was 7.2. While others found that the optimum pH for nitrate reductase purified from *Haloarcula marismortui* was 9.2. [21].

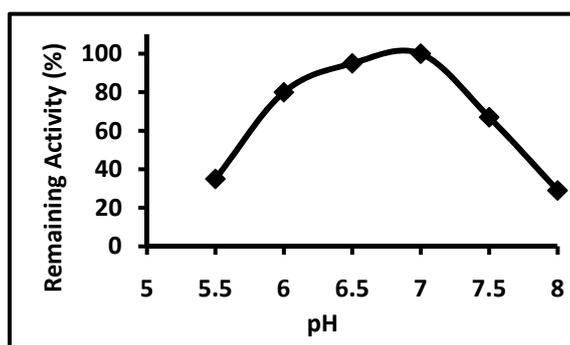


Fig(10): Optimal pH for nitrate reductase activity purified from *Pseudomonas* sp. SH7

**Effect of pH on nitrate reductase stability:** In order to determine the optimal pH for nitrate reductase stability, the enzyme was incubated in a buffer solution with a pH range from 5.5 to 8 at 35°C for 30 min.

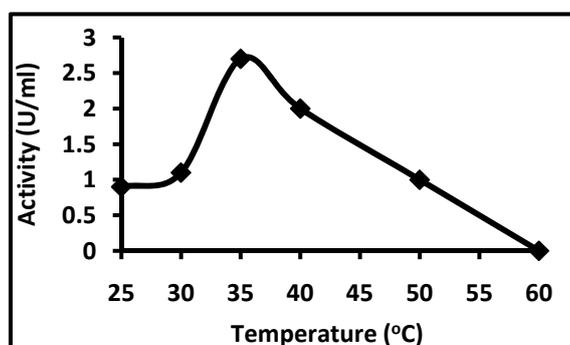
Best stability was observed at pH 7.0 where more than 95% of the activity remained as shown in Figure (11).

Studies on *P. denitrificans* nitrate reductase showed that the enzyme was stable in the pH from 6 to 9 and more than 80% of the activity remained at pH 7.0 while enzymatic activity eliminated at pH 3.0 [11]. [8], stated that *D. desulfuricans* nitrate reductase had high stability at pH 6.5.



Fig(11): Nitrate reductase stability at different pH values

**Effect of temperature on nitrate reductase activity:** The maximum activity of nitrate reductase was shown at 35°C, as in Figure (12), and decreasing in enzyme activity was observed whenever temperature increased more than 35°C.

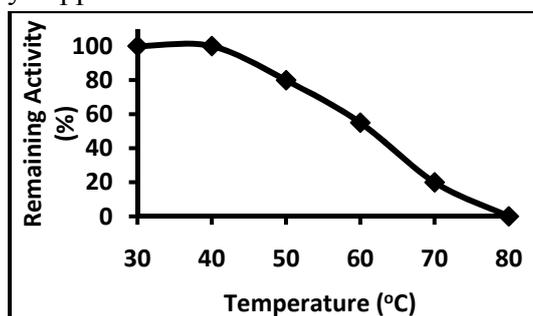


Fig(12): Optimal temperature for nitrate reductase activity purified from *Pseudomonas* sp. SH7

The maximal activity of thermostable nitrate reductase from the hyperthermophilic archaeon *Pyrobaculum aerophilum* was above 95°C [15], while the optimal activity temperatures for *P. ambigue*, *E. coli* DH5 $\alpha$  and *V. harveni* KCTC 2720 were 55, 30 and 30°C respectively [22]. Other study found that the optimum activity of nitrate reductase isolated from *H. mediterranei* was 70°C [21].

**Effect of temperature on nitrate reductase stability:** The stability of nitrate reductase from *Pseudomonas* sp. SH7 was examined by incubation enzyme at various temperature ranges from 30°C to 80°C for 15 min.

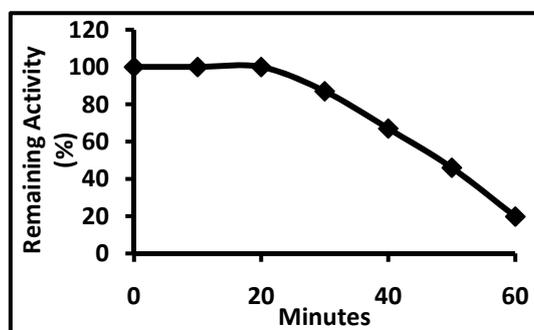
The results in Figure (13), revealed that the enzyme was active up to 50°C, maximal enzyme activity was observed at (30-40)°C, the activity declined when incubated at higher temperature, although 80% of the activity remained at 50°C. The enzyme activity was completely suppressed at 80°C for 15 min.



Fig(13): The effect of temperature on nitrate reductase stability purified from *Pseudomonas* sp. SH7

To determine the stability of the enzyme, purified nitrate reductase was incubated at 45°C. Decrease in enzyme activity was observed when the enzyme was incubated more than 20 min. and less than 20% of the activity was remained after incubation for 60 min., result in Figure (14).

[23] found that the optimal temperature for activity of nitrate reductase from *B. fragilis* was 35°C and this enzyme was heat sensitive since it lost more than 60% of the activity after incubation at 42°C for 20 min., while nitrate reductase from denitrifier *P. aeruginosa* was heat stable since it could be heated for 10 min. at 65 to 70°C, or for more than 90 min. at 50°C, without loss in activity [14].



Fig(14): Stability of nitrate reductase at 45°C for different time values purified from *Pseudomonas* sp. SH7

**Effect of inhibitors on nitrate reductase activity:** Sodium azide and potassium cyanide are known as inhibitors of nitrate reductase. The effects of different concentrations of each inhibitor were investigated.

Nitrate reductase from *Pseudomonas* sp. SH7 treated with (50, 100, 150, 200)  $\mu\text{M}$  sodium azide showed inhibition in activity by (11, 24, 46, 76) % respectively, whereas (100, 200, 300, 400, 500)  $\mu\text{M}$  potassium cyanide treatment inhibited the activity by (35, 58, 75, 86, 91) % respectively, as in Figure (15,16).

Cyanide and Azide exhibited a pattern of inhibition of the activities of nitrate reductase; this data suggests that the metal components may be functioning in nitrate reductase. [7], found that nitrate reductase purified from *P. isachenkovii* was very sensitive to low concentrations of cyanide (40 $\mu\text{M}$ ) and azide (20 $\mu\text{M}$ ), this inhibition because of the presence of a metal atom in the active site.

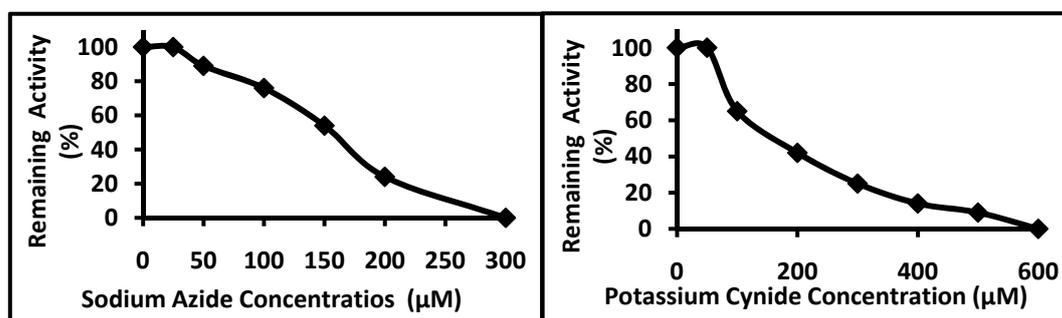


Fig (15): The effect of sodium azide concentrations (0-300 $\mu\text{M}$ ) on nitrate reductase activity

Fig(16): The effect of potassium cyanide concentrations (0-600 $\mu\text{M}$ ) on nitrate reductase activity

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