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Characterization of Membrane-bound Nitrate Reductase from Denitrifying Bacteria *Ochrobactrum anthropi* SY509

Seung Hwan Kim[1], Seung Hoon Song[2], and Young Je Yoo[1,3]*

[1] Interdisciplinary Program for Biochemical Engineering and Biotechnology, Seoul National University, Seoul 151-742, Korea
[2] Bio-MAX Institute, Seoul National University, Seoul 151-742, Korea
[3] School of Chemical and Biological Engineering, Seoul National University, Seoul 151-742, Korea

Abstract In this study, we have purified and characterized the membrane bound nitrate reductase obtained from the denitrifying bacteria, *Ochrobactrum anthropi* SY509, which was isolated from soil samples. *O. anthropi* SY509 can grow in minimal medium using nitrate as a nitrogen source. We achieved an overall purification rate of 15-fold from the protein extracted from the membrane fraction, with a recovery of approximately 12% of activity. The enzyme exhibited its highest level of activity at pH 5.5, and the activity was increased up to 70℃. Periplasmic and cytochromic proteins, including nitrite and nitrous oxide reductase, were excluded during centrifugation and were verified using enzyme essay. Reduced methyl viologen was determined to be the most efficient electron donor among a variety of anionic and cationic dyestuffs, which could be also used as an electron donor with dimethyl dithionite. The effects of purification and storage conditions on the stability of enzyme were also investigated. The activity of the membrane-bound nitrate reductase was stably maintained for over 2 weeks in solution. To maintain the stability of enzyme, the cell was disrupted using sonication at low temperatures, and enzyme was extracted by hot water without any surfactant. The purified enzyme was stored in solution with no salt to prevent any significant losses in activity levels.

Keywords: nitrate reductase, denitrification, enzyme stability, *Ochrobactrum anthropi*

INTRODUCTION

The excessively accumulated ammonia in an environment is aerobically oxidized to nitrate through a bacterial nitrification process, then the resulting nitrate is converted to nitrogen gas by denitrifying microorganisms and released into the atmosphere. Denitrification is a primary biotic process resulting in the loss of fixed nitrogen from the environment as well as the removal of excess nitrate from surface-water and wastewaters, which is accumulated mainly as a result of agricultural N-fertilizer application [1]. When reduced to ammonia, the nitrogen is not removed from the environment, but transformed into a less mobile form. In either case, the reduction of nitrate plays a central role in preventing leaching and pollution of the ground-water [2,3].

The specific enzymes, dissimilatory nitrate reductases, which mediate the reduction of nitrate to nitrite in both denitrifiers and bacteria that dissimilate nitrate in nitrate reducing bacteria, have been the focus of the physiological and biochemical studies [2,4]. Most attention has been given to the membrane-bound nitrate reductase within the nitrate-reducing bacterium. Recently, however, there has been a huge increase in the quantity of studies conducted regarding enzymes from other organisms, as well as novel classes of respiratory nitrate reductases, which differ with regard to location and/or activity [5,6].

Proteins bounded in membrane can be easily denaturated when extracted from the cell membrane. The easy with which this denaturation can occur renders the purification of membrane-bound nitrate reductase unpopular and prevents its use in the process of enzymatic denitrification. Thus, the maintenance of the stability of membrane-bound nitrate reductase during purification is meaningful in that it extends the application of the enzyme [7,8].

Denitrifying microorganisms are usually distributed in the bacteria, but also in the eukaryotic microorganisms and the archaea. *Pseudomonas* sp. is the most commonly referenced denitrifying organism [9]. Other bacteria, including *Achromobacter*, *Alcaligenes*, *Bacillus*, *Flavobacterium*, and *Thiobacillus*, are also known to denitrify. As the nitrate reductase inherent to the process of denitrification is oxygen-sensitive, denitrification generally occurs in environments with low levels of oxygen, such as in waterlogged soils [1].

The denitrifying bacteria, *Ochrobactrum anthropi* SY509, which was isolated from soil exhibited excellent activity

***Corresponding author**
Tel: +82-2-880-7411 Fax: +82-2-887-1659
e-mail: yjyoo@snu.ac.kr

on dissimilative nitrate reduction in anaerobic condition. It was identified as a novel class of bacteria that can reduce nitrate to nitrogen gas [10].

In this paper, in order to elucidate the effects of differing purification conditions on the stability and yield of active membrane-bound enzyme purified from bacteria, we conducted a series of purifications under a variety of disruption and extraction conditions, and also characterized the purified membrane-bound nitrate reductase extracted from the bacteria, *O. anthropi* SY509.

MATERIALS AND METHODS

Medium Composition and Cell Culture

The medium used in the present study contained the following components; 10.0 g/L Glucose, 10.0 g/L Yeast extracts, 7.0 g/L KNO_3, 4.47 g/L $Na_2HPO_4 \cdot 12H_2O$, 1.7 g/L KH_2PO_4, 0.3 g/L $MgSO_4 \cdot 7H_2O$, 2.0 g/L NH_4Cl, 0.03 g/L $CaCl_2 \cdot 2H_2O$, 0.05 g/L $FeSO_4 \cdot 7H_2O$, 0.28 g/L $Na_2 \cdot EDTA \cdot 2H_2O$, 0.012 g/L $Na_2MoO_4 \cdot 4H_2O$, and 0.002 g/L $CuSO_4 \cdot 7H_2O$. *O. anthropi* SY509 was pre-cultured at 30°C in a 250 mL flask containing 100 mL of autoclaved medium at 30°C with 200 rpm in a shaking incubator for 24 h. After the cells were harvested, they (OD_{660} 0.1) were inoculated in a 100 mL bottle containing 90 mL of autoclaved medium. The cells were then cultured about for 24 h at 30°C with 200 rpm in a shaking incubator. Next, the cells were harvested by the centrifuge. The soluble fraction was discarded and the cells were washed several times with distilled water and twice in a potassium phosphate buffer. Potassium phosphate was dissolved to make 80 mM solution and pH was set to 7.0. The downed cells were stored at −70°C until experimental uses.

Enzyme Activity Assay

The activities of nitrate and nitrite reductase were assayed based on the reported methods [2,6]. The assay mixture (total volume, 1.5 mL) was composed of a potassium phosphate buffer (80 mM, pH 7.0), containing methyl viologen (1 mM) as an artificial electron donor, and the reducing agent dithionite (10 mM). The addition of excess dithionite facilitated the handling of the solution without an anaerobic chamber. The enzyme was added, and the mixture was stirred at 30°C. Subsequently the reaction was started by the addition of potassium nitrate (10 mM). Nitrate reduction was stopped by vigorous vortexing the mixture to oxidize all dithionite and methyl viologen. After the removal of the cell extracts by centrifugation, the nitrite concentrations in the samples were measured.

One unit of nitrate reductase will produce 1.0 μmol of nitrite-N per (min·mg cell extracts) in the presence of methyl viologen at pH 7.0 and 30°C. The nitrite reductase assay was performed as described for nitrate reductase. The 1 unit of nitrite reductase will reduce 1.0 μmol of nitrite-N per (min·mg cell extracts) in the presence of benzyl viologen at pH 7.0 and 30°C.

Purification of Nitrate Reductase

The purification method was modified from Yoshimatsu's method [3]. Cultured bacterial cells (30 g wet weight) were resuspended in 180 mL of 80 mM potassium phosphate buffer (pH 7.0). The cells were disrupted by ultrasonication in an ice/ethanol bath (maintained at −10°C). After ultrasonication, the suspension was centrifuged at 6,000 rpm for 30 min at 4°C in order to precipitate the intact cells. And then the cell-free extract obtained was centrifuged at 15,000 × g for 60 min at 4°C. The precipitant was resuspended in an 80 mM potassium phosphate buffer (pH 7.0) and centrifuged again at 15,000 × g for 60 min at 4°C. The precipitant thus obtained was employed as a membrane fraction for the purification of nitrate reductase.

The membrane fraction was suspended in an 80 mM potassium phosphate buffer (pH 7.0) at a protein concentration of 5 mg/mL. The resulting suspension was gently stirred for 20 min at 60°C, and centrifuged at 15,000 × g for 60 min. The obtained supernatant was subjected to anion-exchange chromatography on a DEAE-Sepacryl column (1.0 × 30 cm), which had been equilibrated with an 80 mM potassium phosphate buffer (pH 7.0) [11]. After the column had been washed with 200 mL of an 80 mM potassium phosphate buffer (pH 7.0) containing 100 mM NaCl, the enzyme that was adsorbed on the column was eluted with a linear gradient solution generated from 100 mL each of 80 mM potassium phosphate buffers (pH 7.0) containing 100 mM NaCl and the buffer containing 500 mM NaCl. The elute that showed nitrate reducing activity was collected, and then dialyzed against an 80 mM potassium phosphate buffer (pH 7.0). The enzyme preparation was concentrated with a small size DEAE-Sepacryl column (0.5 × 2.0 cm), then subjected to a Sephacryl S-200 (Pharmacia, Uppsala, Sweden) gel-filtration column (2.0 × 30 cm) equilibrated with an 80 mM potassium phosphate buffer (pH 7.0) containing 300 mM NaCl. The elute manifesting enzymatic activity was harvested and employed as the purified enzyme preparation. All of the purification procedures were conducted at 4°C.

RESULTS AND DISCUSSION

Purification Conditions and Stability of Membrane-bound Nitrate Reductase

Dissimilatory nitrate reductase was purified from bacteria, *O. anthropi* SY509, which had been cultivated anaerobically in the presence of nitrate. After disrupting the cultivated cells with an ultrasonic oscillating device, the membrane-bound nitrate reductase was released into the solution by heat solubilization. The centrifugation of the disrupted cells was conducted in two steps to separate the intact cell and the metabolites.

It is well known that membrane bound proteins are less stable than soluble proteins and easily deactivated during purification and storage, and also the abrupt decrease in

Table 1. Yield of cell debris under various disruption conditions

Disruption condition	Cell debris yield (%)
Sonication at 0°C without pulse[a]	20.4
Soniaction at −10°C with pulse[b]	23.2
French press at 10,000 psi at 4°C [c]	8.4
French press at 14,000 psi at 4°C [c]	18.0

[a]5 times of 2 min. ultrasonication with 1 min. cooling.
[b]10 times of 2 min. ultrasonication with 1 min. cooling.
[c]twice of French press.

the activity of nitrate reductase was observed during purification. Therefore, in order to avoid the activity decrease, the deactivation of nitrate reductase during cell disruption and extraction was observed and the condition for the minimizing of nitrate reductase deactivation was optimized.

Table 1 and Fig. 1 depict the effects of two typical cell disruption methods, ultrasonication and the french press, on the stability of enzyme and the yield of membrane fraction. In Table 1, it can be seen that ultrasonication is a more efficient disruption method than the french press. A membrane fraction yield in excess of 20% was readily obtained when the cells were disrupted by ultrasonic wave without significant decrease of enzyme activity under some conditions.

Reduction in enzyme activity was observed in an extracted membrane fraction during storage, in a sample which had been disruptured by the french press. A significant decrease in activity was also observed in samples disruptured by ultrasonication at 0°C. When the cells were disrupted by ultrasonication without pulsing at 0°C, the temperature of the cell suspension was increased to 7°C even though the suspension was cooled for 1 min after every 2 min of sonication. It suggests that the aforementioned instability might be attributable to the effects of ultrasonic energy at relatively high temperatures. After 110 h, the residual activity of the membrane fraction showed no significant changes when the cells were disrupted by ultrasonication with pulsing at −10°C and maintained 90% of its initial activity. However, the enzymes separated by sonication at 0°C did not exhibit activity almost after 70 h.

The extracted fraction with the french press showed a relatively high residual activity. The difference in residual activity between the two conditions might be due to the stress induced during cell disruption. The yield of the membrane fraction acquired through distruption at 14,000 psi was over two times than at 10,000 psi, but

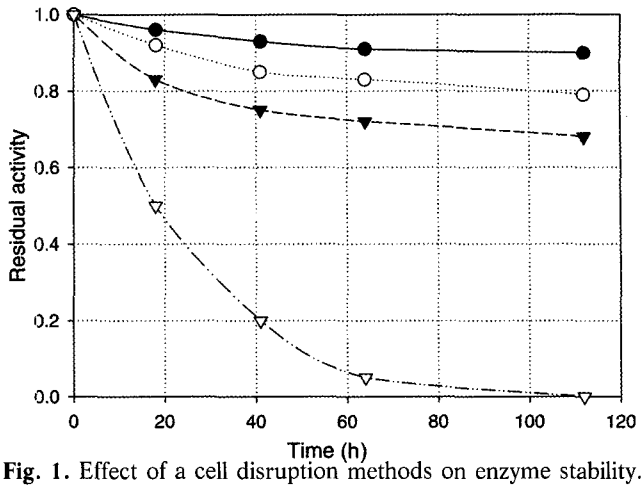

Fig. 1. Effect of a cell disruption methods on enzyme stability. Each fraction in Table 1 was stored at 4°C and residual activities were compared to the initial activity: ●, Disrupted by ultrasonication with pulse at −10°C; ▼, by french press at 10,000 psi, 4°C; ○, by french press at 14,000 psi, 4°C; ▽, by ultrasonication at 0°C.

showed relatively low stability. Fig. 1 shows the activity changes of the membrane fraction over 110 h.

The solubilized fraction was treated to DEAE-Sephacryl anion exchange chromatography and Sephacry S-200 size exclusion chromatography (SEC) and finally the purified enzyme was obtained. The activity of the purified enzyme preparation was determined to be almost 18.7 unit/mg protein at pH 7.0 and 30°C, and was obtained with 54% from solubilized fraction through two chromatographic steps as summarized in Table 2.

Enzymes in the membrane fraction could be extracted using a surfactant and hot water. The effects of two typical extraction methods on enzyme stability and yield were observed in Fig. 2. The extraction yield observed using a surfactant, Triton X 100, was almost twice of that obtained using heat solubilization. However, in this case, the residual activity after 24 h was only 25% of its initial activity, whereas the heat solubilization exerted only nominal effects on enzyme activity. Therefore, surfactant solution, although showed efficacious with regard to the extraction of enzymes, exerted deleterious effects on the activity of these stored enzymes.

The extraction time using heat solubilization at 60°C was optimized as shown in Fig. 3. The significant de crease in enzyme activity was observed at 10 min, but there was almost no change in the activity of the extracted enzyme after 10 min. This result indicates that

Table 2. Specific activity of nitrate reductase during purification

Fraction	Protein (mg)	Total activity (unit)	Specific activity (unit/mg protein)
Membrane fraction	223.00	276.0	1.24
Solubilized fraction	21.20	63.2	2.98
After DEAE-Sephacryl ion exchange	4.73	56.1	11.80
After Sephacryl S-200	1.81	33.8	18.70

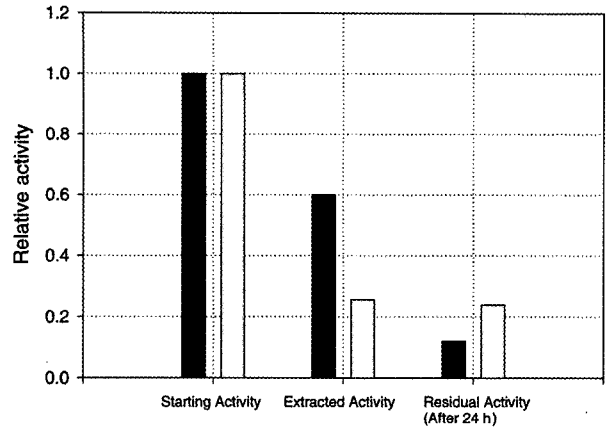

Fig. 2. Effect of an extraction method on enzyme stability. Extracted enzyme was stored at 4°C and compared to initial activity: ■, extracted by 1.5% non-ionic surfactant Triton X-100 at 4°C for 90 min; □, extracted by heat at 60°C for 30 min.

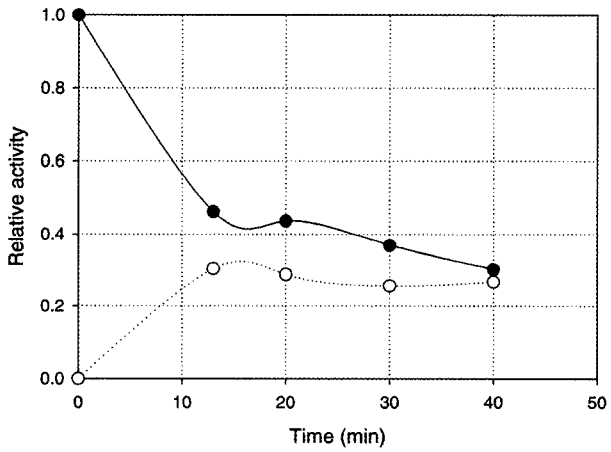

Fig. 3. Effect of extaction time on yield of enzyme. Enzyme was extracted at 60°C according to time: ●, Residual activity of nitrate reductase in the solution after heat treatment; ○, Extracted nitrate reductase activity in the solution.

the loss of activity associated with extraction might occur during the extraction procedure itself and the optimal condition for solubilization should be required to achieve higher yield.

Properties of Membrane-bound Nitrate Reductase

Purified enzyme of *O. anthropi* SY509 was tested with various mediators for optimizing electron transfer. Tested mediators were involved a set of well-known mediators [12] and dye-stuffs. The molecular weights are ranged from 257 (methyl viologen) to 997 (Chicago sky blue). Five mediators, including methyl viologen, feature their own reducible sites (called electrophore) which are able to receive electrons from an electron donor and four mediators negatively charged to give channels for electron transfer. All of the charges of the tested mediators were

Table 3. Relative activities of various mediators to methyl viologen when tested in nitrate reductase assay condition

Mediator	Relative activity
Cationic Mediators	
Methyl viologen (Control)	1.00
Benzyl viologen	0.88
Azure A	0.31
Crystal violet	0.02
Safranin O	0.72
Anionic Mediators	
Bromophenol blue	0.79
Cresol red	0.45
Cibacron blue	0.13
Chicago sky blue	0.01

stabilized by conjugated double bonds such as phenyl and azo groups. The mediators were soluble to water by charged functional groups, however some of the mediators such as benzyl viologen became more insoluble than in their salt form, due to the reduction of their charged sites.

The relative activities of the tested mediators to methyl viologen were shown in Table 3. Nitrate ion was reduced to nitrite ion by reduced nitrate reductase which was reduced by the mediated electron. The reaction media were maintained under reduction condition for mediator reduction using 10 mM disodium dithionite and neutral pH was done using 80 mM potassium phosphate buffer.

Methyl viologen was found to be the most effective mediator among the tested mediators, when reduced by disodium dithionite. Benzyl viologen and safranin O also exhibited good activity for the mediated enzyme reaction in the cationic mediators, Azure A manifested a relatively low level of activity and crystal violet showed almost no activity of electron transfer.

Bromophenol blue had the best activity of electron transfer in anionic mediators. Bromophenol blue exhibited 80% activity of methyl viologen, whereas cresol red, Cibacron blue and Chicago sky blue showed 45, 15, and ~0% of activity observed with methyl viologen. Five cationic mediators were electrically reducible, as they had their own reducible sites that are usually negativity charged nitrogen or sulfur.

Nitrate reductase activity exhibited maximum activity at pH 5.5 at 30°C and showed higher levels of activity at higher temperature to 70°C. As shown in Fig. 4, the activity of the purified enzyme was decreased drastically by the application of low or high pH values. These results due to not only with regard to the optimality of nitrate reductase in terms of pH, but also to the reducibility of methyl viologen at such pH value. Especially, at extreme pH levels, such as 2 and 3, no reduction of methyl viologen by dithionite was observed in the enzyme solution, indicating that no enzyme reaction had occurred.

Enzyme activity was 3.5 times more pronounced at 70°C than at 30°C, whereas the activity at 40°C showed 1.7 times than at 30°C. This result was consistent with

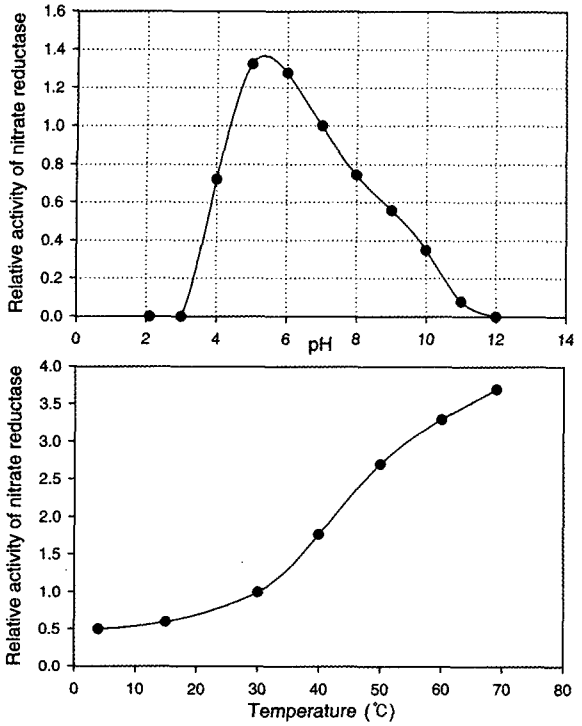

Fig. 4. Effect of temperature and pH on the activity of purified nitrate reductase. Methyl viologen was used as a mediator and disodium dithionate was used as an electron donor. Relative activities indicate relative values to activity tested at 30°C, pH = 7.0.

those reported by Antipov *et al.* [13]. The observed reduction in activity was attenuation of enzyme activity.

The purified enzyme was more stable in the absence of NaCl as also reported by Yoshimatsu *et al.* [3]. To observe the effect of temperature and NaCl concentration on storage stability of the purified nitrate reductase, the enzyme was incubated at 4 and −20°C in the presence of 0 and 0.2 M NaCl (in an 80 mM phosphate buffer at pH 7.0), and the residual activity was observed according to the incubation time. It was suggested that the stability of the nitrate reductase was dependent on the salt concentration, and was also influenced by temperature.

According to these results, the activity of enzyme decreased by more than 10% when enzyme was stored at −20°C after 20 h, and no activity was observed after 40 h. When enzymes were stored at −20°C in the presence of 0.2 M NaCl, however, it was observed a 35% decrease in their activity. A significant reduction in activity was also observed after freezing, in this case, freezing condition might be influenced on the stability of the purified enzyme.

Fig. 5 shows the stability of the purified enzyme. According to this figure, it could be deduced that the enzyme could be stored for 120 h without any significant loss of activity at 4°C, in an 80 mM phosphate buffer at pH 7.0.

As shown in Fig. 6, the heat solubilized nitrate reductase of *O. anthropi* SY509 has an apparent K_m value for

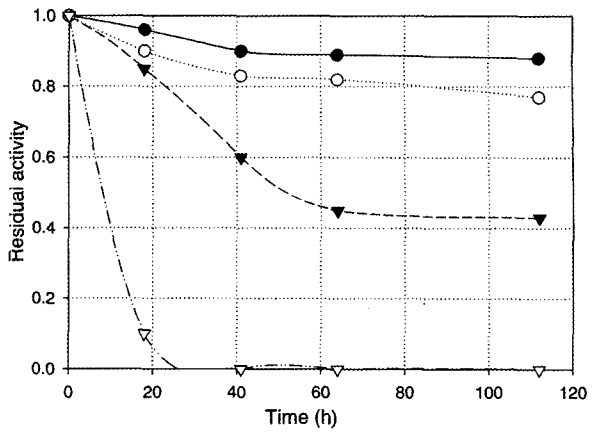

Fig. 5. Storage stability of purified nitrate reductase under various conditions: ●, enzyme was stored at 4°C in an 80 mM potassium phosphate buffer (pH = 7.0); ○, at 4°C in an 80 mM potassium phosphate buffer (pH = 7.0) with 0.2 M NaCl; ▼, at −20°C with freezing in an 80 mM potassium phosphate buffer (pH = 7.0); ▽, at −20°C with freezing in an 80 mM potassium phosphate buffer (pH = 7.0) with 0.2 M NaCl.

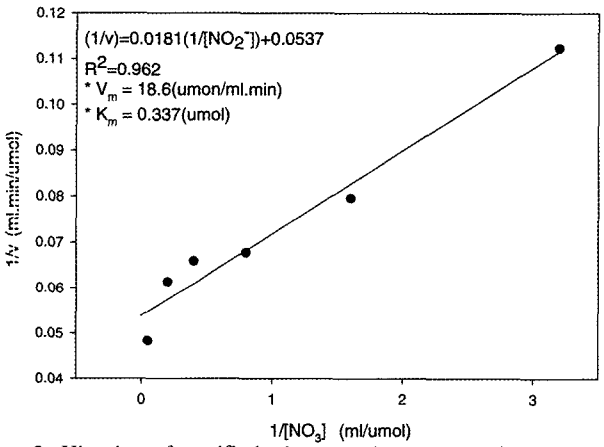

Fig. 6. Kinetics of purified nitrate reductase. Methyl viologen was used as a mediator and disodium dithionite was used as an electron donor at 30°C in an 80 mM phosphate buffer (pH = 7.0).

nitrate of 0.34 mM with reduced methyl viologen as an electron donor and apparent mV values for nitrate of 18.6 unit/min. The enzyme was active also with benzyl viologen and other mediators, but these were less effective than methyl viologen.

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