CHAPTER III

RESULTS

3. Growth rate and nitrate reductase activity determination

3.1 Effect of salinity on growth rate and nitrate reductase activity

3.1.1 Effect of salinity on growth rate of *Aphanothece halophytica*

A.halophytica is a short cylindrical shape cyanobacterium surrounded with mucous membrane which multiplies by binary fission as shown in Figure 3.1. The cells were grown photoautrophically at 30 °C in BG_{11} plus 18 mM $NaNO_3$ and Turk Island Salt Solution plus modified BG_{11} medium at 0.5 M NaCl concentration for normal condition and 2.0 M NaCl for salt-stress condition. The growth rate was monitored spectrophotometrically by the increase of the optical density at 750 nm. Figure 3.2 shows the growth rate of *A.halophytica*, the maximal growth occurred in the medium containing 0.5 M NaCl. At 2.0 M NaCl (salt stress condition), a reduction in growth was observed.

3.1.2 Effect of salinity on nitrate reductase activity

We tested nitrate reductase activity at each growth period including lag phase, log phase and stationary phase. The cells grown under normal condition (the medium plus 0.5 M NaCl) and salt stress condition (the medium plus 2.0 M NaCl) were harvested and determined for nitrate reductase activity. The nitrate reductase activity showed maximal activity in the log phase of growth. Also, the nitrate reductase activity level at 0.5 M NaCl is maximal, with significant lower activity at 2.0 M NaCl as shown in Figure 3.3. The influence of salinity on the nitrate reductase activity was due to the short – term inhibition of nitrate reductase activity and nitrate

uptake. The specific activities of nitrate reductase in cells grown under normal and salt-stress conditions were 0.278 and 0.140 nmol/min/mg protein, respectively (Table 3.1).

3.2 Effect of nitrogen–sources on growth and nitrate reductase activity of *A. halophytica*

We investigated the effect of different nitrogen – sources on the growth rate and nitrate reductase activity level of *A. halophytica.*

3.2.1 Effect of nitrogen – sources on growth rate

In normal culture condition, *A. halophytica* was grown in nitrate as the sole nitrogen–source. Effect of nitrogen – source was investigated by used L-glutamine and ammonium chloride (NH_4Cl) in place of nitrate at the same concentration (18 mM). Figure 3.4 shows that *A. halophytica* was able to grow with nitrate, L–glutamine and ammonium chloride. The replacement of nitrate by ammonium chloride and L–glutamine affected growth rate. We found that ammonium chloride caused a drastic decrease in growth rate. When L–glutamine was used as nitrogen–source, the high growth rate was observed, while in the presence ammonium chloride only a very slow growth rates was observed up to 6 days.

3.2.2 Effect of nitrogen–sources on nitrate reductase activity

We investigated the effect of nitrogen–sources on nitrate reductase activity by measuring the nitrite formation. Figure 3.5 shows nitrate reductase activity was only observed in cells grown with nitrate. In the presence of L–glutamine and ammonium chloride, the nitrate reductase activity was hardly detected. Replacement of nitrate by ammonium chloride and L-glutamine at same concentration affected both growth rate and nitrate reductase activity level. These

results suggested that the nitrate reductase is induced by nitrate and repressed by L–glutamine and ammonium chloride. The inhibition of nitrate reductase activity by L–glutamine was due to metabolism of L–glutamine formed by glutamine synthetase activity. The repression of nitrate reductase activity by ammonium chloride was due to inhibited nitrate uptake. In normal condition, where nitrate was nitrogen source, nitrate functioned as efficient inducer to enhance nitrate reductase activity. The specific activities of nitrate reductase were 0.278, 0.02 and 0.02 nmol/min/mg protein when the medium contains nitrate, ammonium chloride and glutamine as nitrogen-source, respectively (Table 1).

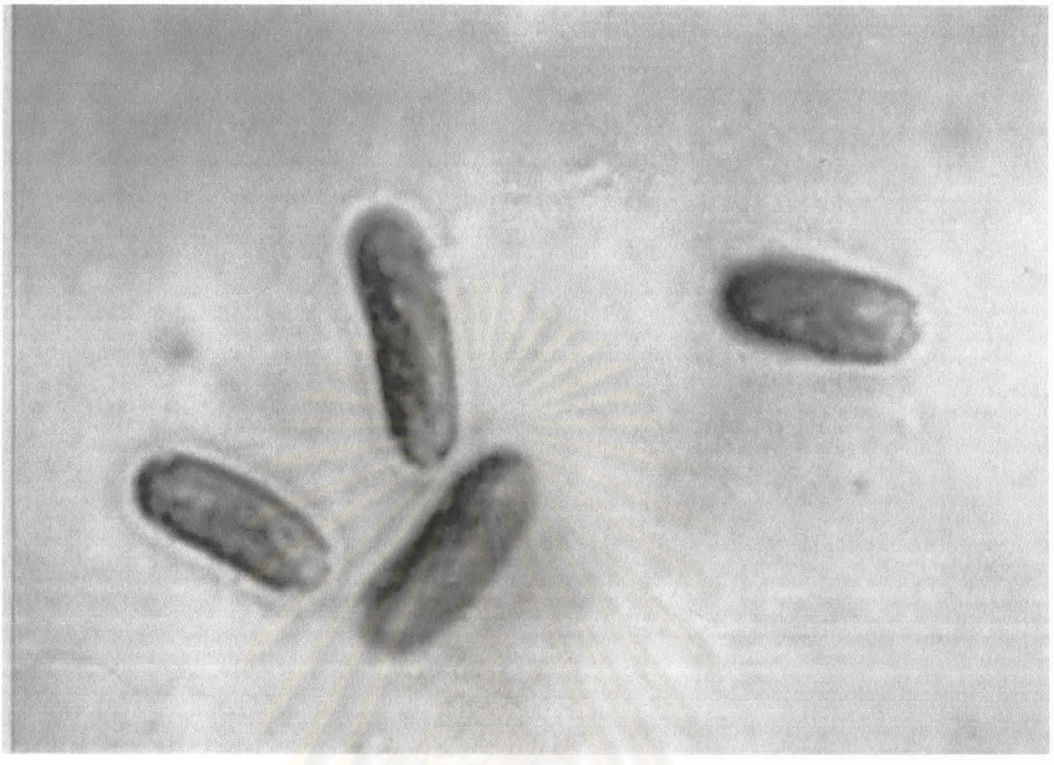


Figure 3.1: Microscopic picture of *Aphanothece halophytica* grown in Turk Island Salt Solution plus BG_{11} after 8 days (x 2250).

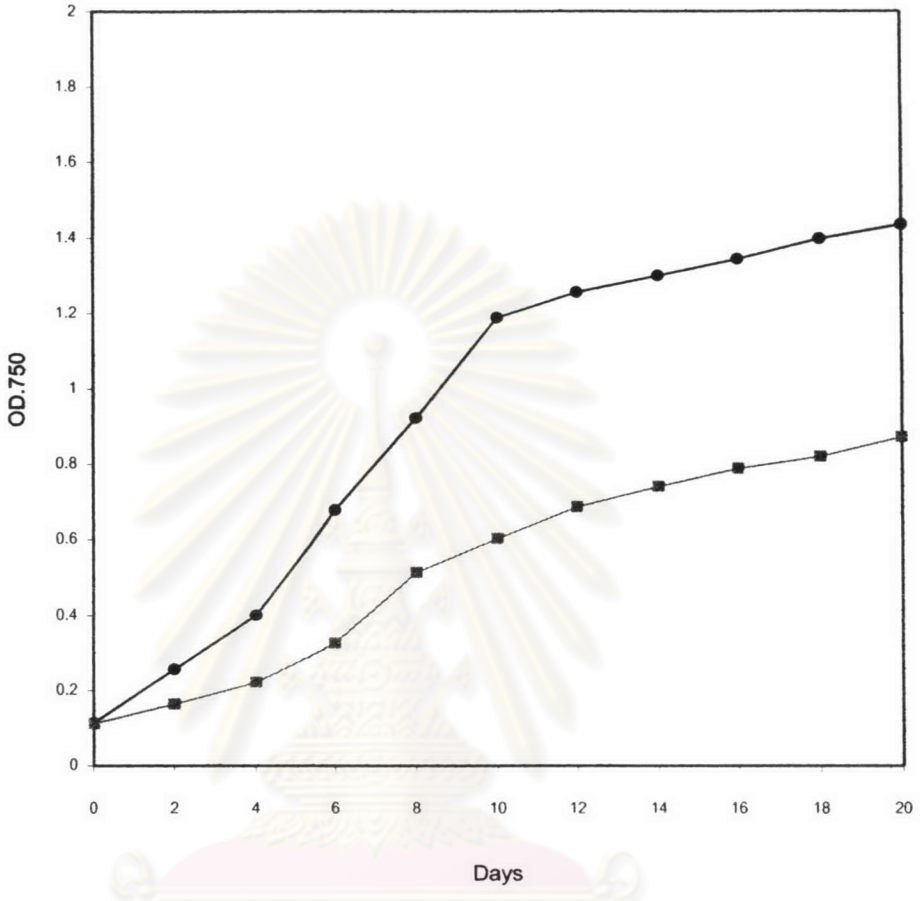


Figure 3.2: Growth of *A.halophytica* in Turk Island Salt Solution plus modified BG_{11} medium containing 0.5 M NaCl for normal condition and 2.0 M NaCl for salt - stress condition.

● 0.5 M NaCl ■ 2.0 M NaCl

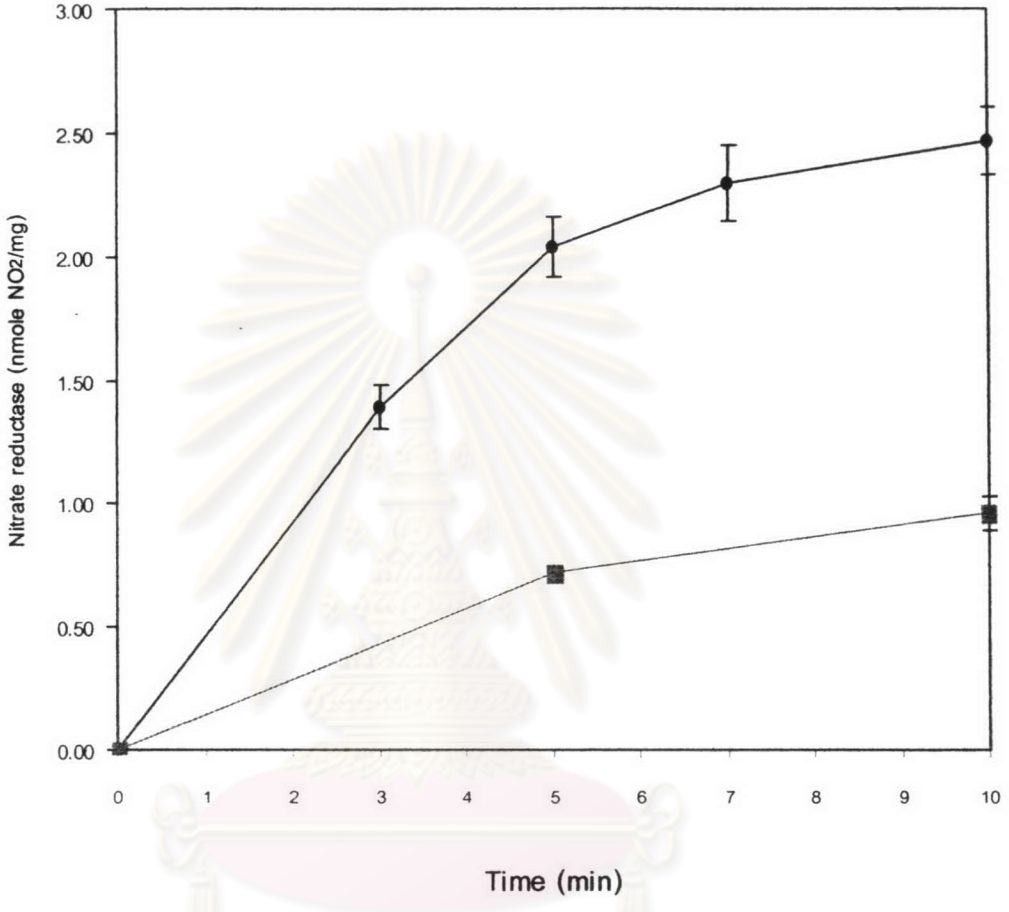


Figure 3.3: The time course of nitrate reductase activity of *A. halophytica* grown under normal (0.5 M NaCl) and salt - stress (2.0 M NaCl) conditions for 8 days.

● 0.5 M NaCl ■ 2.0 M NaCl.

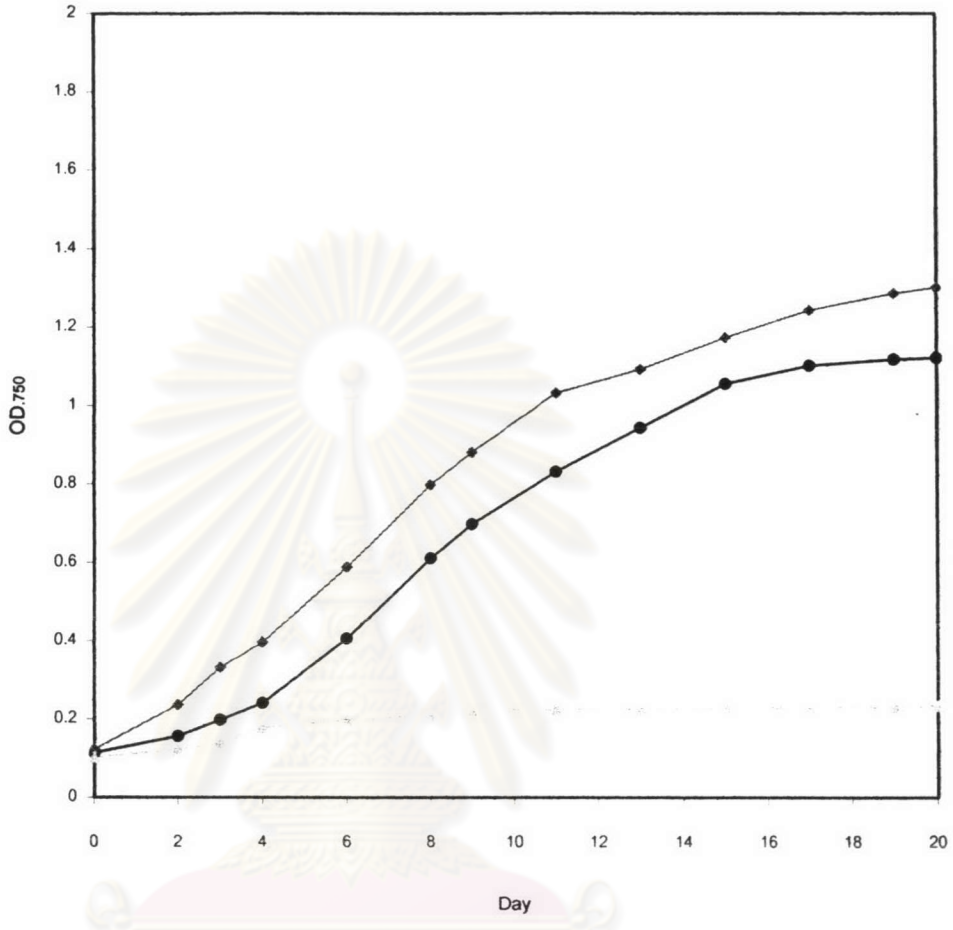


Figure3.4: Growth of *A. halophytica* under normal condition containing sodium nitrate, L-glutamine and ammonium chloride at 18 mM as nitrogen-sources.

●sodium nitrate , ◆ L-glutamine , ▲ ammonium chloride

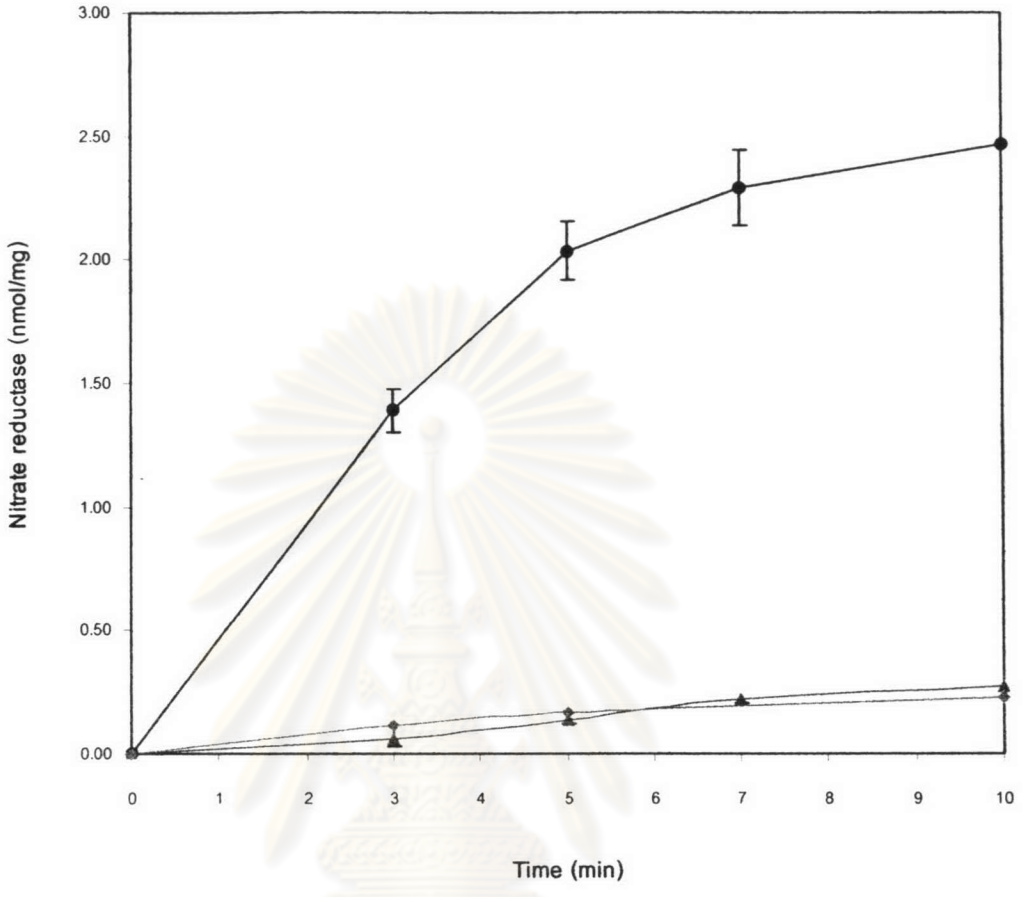


Figure 3.5: The time course of nitrate reductase activity of *A. halophytica* grown in medium containing different nitrogen-sources as $NaNO_3$, glutamine and NH_4Cl at 18 mM for 8 days.

●sodium nitrate , ◆ L-glutamine , ▲ ammonium chloride.

Table3.1: The effect of salt and nitrogen-source in the growth medium on specific activity of nitrate reductase.

Growth Conditions	Specific Activity (nmol/min/mg protein)
Effect of salt	
0.5 M NaCl	0.278
2.0 M NaCl	0.140
Effect of nitrogen-sources	
18 mM Nitrate*	0.278
18 mM Ammonium*	0.02
18 mMGlutamine*	0.02

* in medium containing 0.5 M NaCl

3.3 Localization of the nitrate reductase from *A. halophytica* cells

A. halophytica was grown in medium plus 0.5 M NaCl to exponential growth phase. Periplasmic proteins, membrane fraction and cytoplasmic proteins were isolated by cold osmotic shock and sonicator method. We found that almost all nitrate reductase activity was localized in cytoplasmic fraction (Figure 3.6). These results were observed in organisms that posses assimilatory nitrate reductase involved in protein metabolism for growth of cell. Ammonia, the end product of nitrate reduction, is incorporated into carbon skeletons by the sequential reaction of two enzymes: glutamine synthetase (GS)–glutamate synthetase (GOGAT) pathway.

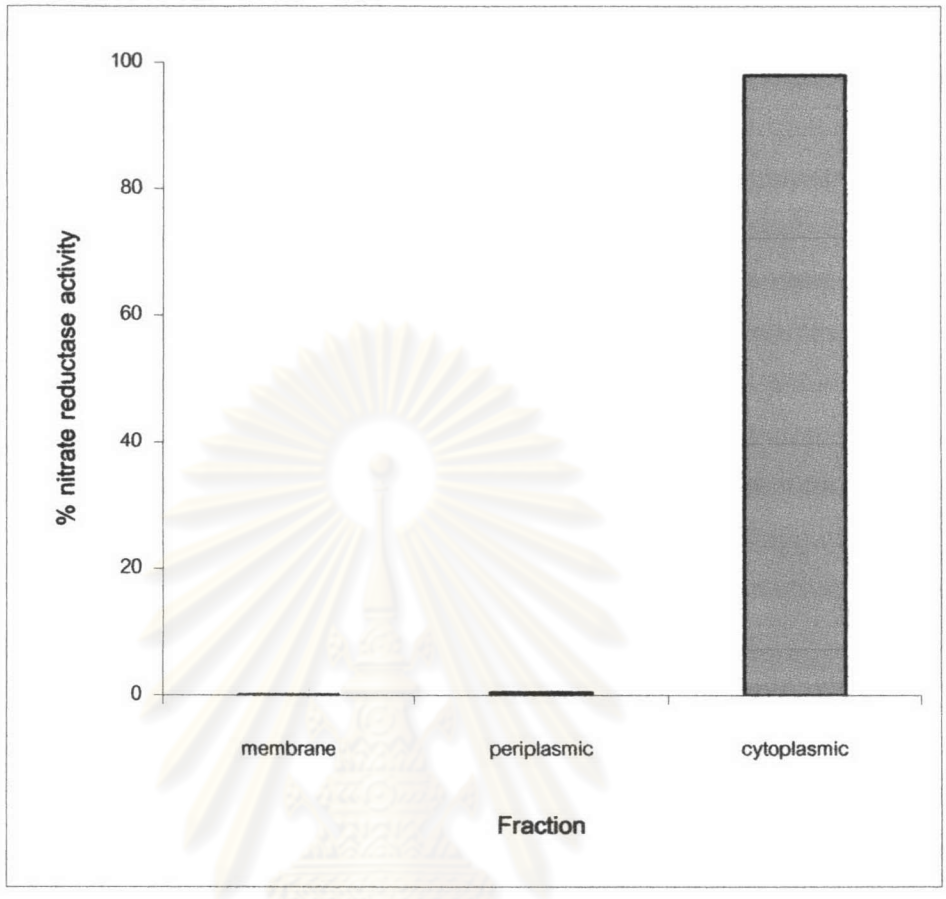


Figure3.6: The localization of nitrate reductase activity in *A. halophytica* cells.

3.4 Purification of nitrate reductase from *A. halophytica*

The nitrate reductase (NaR) linked to reduced methyl viologen from *A. halophytica* was purified by ultracentrifugation, ammonium sulfate [$(NH_4)_2SO_4$] precipitation, DEAE–Toyopearl chromatography and Bio–Gel hydroxyapatite chromatography.

3.4.1 Preparation of crude enzyme solution

Crude nitrate reductase was prepared from 10 g of *A. halophytica* as described in section 2.7.2. Crude enzyme solution contained 5225 mg proteins with 548 units of nitrate reductase activity (Table 3.2).

3.4.2 Ultracentrifugation

The crude enzyme was further purified by ultracentrifugation at 40,000 rpm using Ti70 rotor as described in section 2.8.1, which can separate cytoplasmic proteins from other parts. The nitrate reductase activity was found approximately more than 90% in cytoplasmic fraction from *A. halophytica.* The amount of protein obtained was 2920 mg with 476 units of nitrate reductase activity.

3.4.3 Ammonium sulfate precipitation

The cytoplasmic fraction was further purified by ammonium sulfate precipitation as mentioned in section 2.8.2. To determine the suitable ammonium sulfate concentration for precipitation of nitrate reductase, preliminary experiment was performed by step–wise increase of ammonium sulfate at 0 % to 80 %. Most of enzyme activity was found in the 0.2–0.4 saturation and 0.4–0.6 saturation. Therefore, to harvest most of the enzyme, protein fractionation was carried out in the range of 20 – 60 % saturated ammonium sulfate precipitation. The protein remaining was 972 mg with enzyme activity recovery of 330 units.

3.4.4 DEAE – Toyopearl chromatography

The enzyme precipitate from 20–60 % saturated ammonium sulfate was dissolved and dialysed. The enzyme solution was applied onto DEAE–Toyopearl column as described in section 2.8.4. The unbound proteins were eluted from DEAE–Toyopearl column with buffer A. The bound proteins were then eluted with linear salt gradient from 0 to 0.5 M sodium chloride concentration. The enzyme was eluted at approximately 0.2–0.25 M sodium chloride concentration as indicated in the profile (Figure 3.7). The fractions with active nitrate reductase were pooled, concentrated by Aqua sorb to reduced enzyme volume. The protein remaining from this step with 225 units of enzyme activity. The enzyme activity was purified for about 11 folds and recovery was about 45 % compared with crude enzyme.

3.4.5 Bio – Gel Hydroxyapatite chromatography

The pooled active fraction from DEAE–Toyopearl column was further purified by the Bio–Gel Hydroxyapatite column as described in section 2.8.6. The chromatographic profile is shown in Figure 3.8. The unbound proteins were eluted from the column with buffer B. The enzyme bound to Bio–Gel hydroxyapatite column was then eluted with linear phosphate gradient of 0.05 to 0.5 M potassium phosphate solution. The enzyme was eluted at about 0.1–0.15 M potassium phosphate solution as indicated in the profile. The active fractions with nitrate reductase activity were pooled, concentrated by Aqua sorb. The protein remaining from this step was 2.12 mg with 86 units of enzyme activity. The purify from this step led to a 406 folds with about 15.7 % recovery with respect to crude enzyme. This last step in the purification can efficiently separated the enzyme from the bulk of proteins.

3.4.6 Summary of nitrate reductase purification

The nitrate reductase from *A. halophytica* was purified by ultracentrifugation, ammonium sulfate precipitation, DEAE–Toyopearl chromatography and Bio–Gel hydroxyapatite chromatography as described

previously. The summary of purification of this enzyme is shown in Table 3.2. At the final step, nitrate reductase was purified to homogeneity with 406 purification folds and 15.7 % yield.

3.4.7 Determination of enzyme purity and protein pattern on non – denaturing polyacrylamide electrophoresis

The enzyme from each step of purification was analysed for purity and protein by non-denaturing polyacrylamide gel electrophoresis as described 2.10.1. The result is shown in Figure 3.9. The enzyme in lane 5 shows a single protein band on native-PAGE and molecular weight of monomer subunit is 58 kDa from SDS–PAGE.

3.5 Characterization of nitrate reductase enzyme

3.5.1 Molecular weight determination of nitrate reductase

The purified nitrate reductase from Bio–Gel hydroxyapatite chromatography was used for the molecular weight determination of nitrate reductase by SDS–polyacrylamide gel electrophoresis (Figure 3.10) which included a series of standard proteins in the run. From the mobility in SDS – PAGE, the molecular weight of the enzyme protein monomer was calculated to be 58 kDa.

3.5.2 Initial velocity studies for nitrate reduction

The purified enzyme was characterised for Km and Vmax for nitrate of nitrate reductase. The Km value of nitrate is 465 μM and Vmax value of nitrate is 32 nmol/min/mg protein for nitrate reductase from *A. halophytica* in methyl viologen assay as described in section 2.6.1.

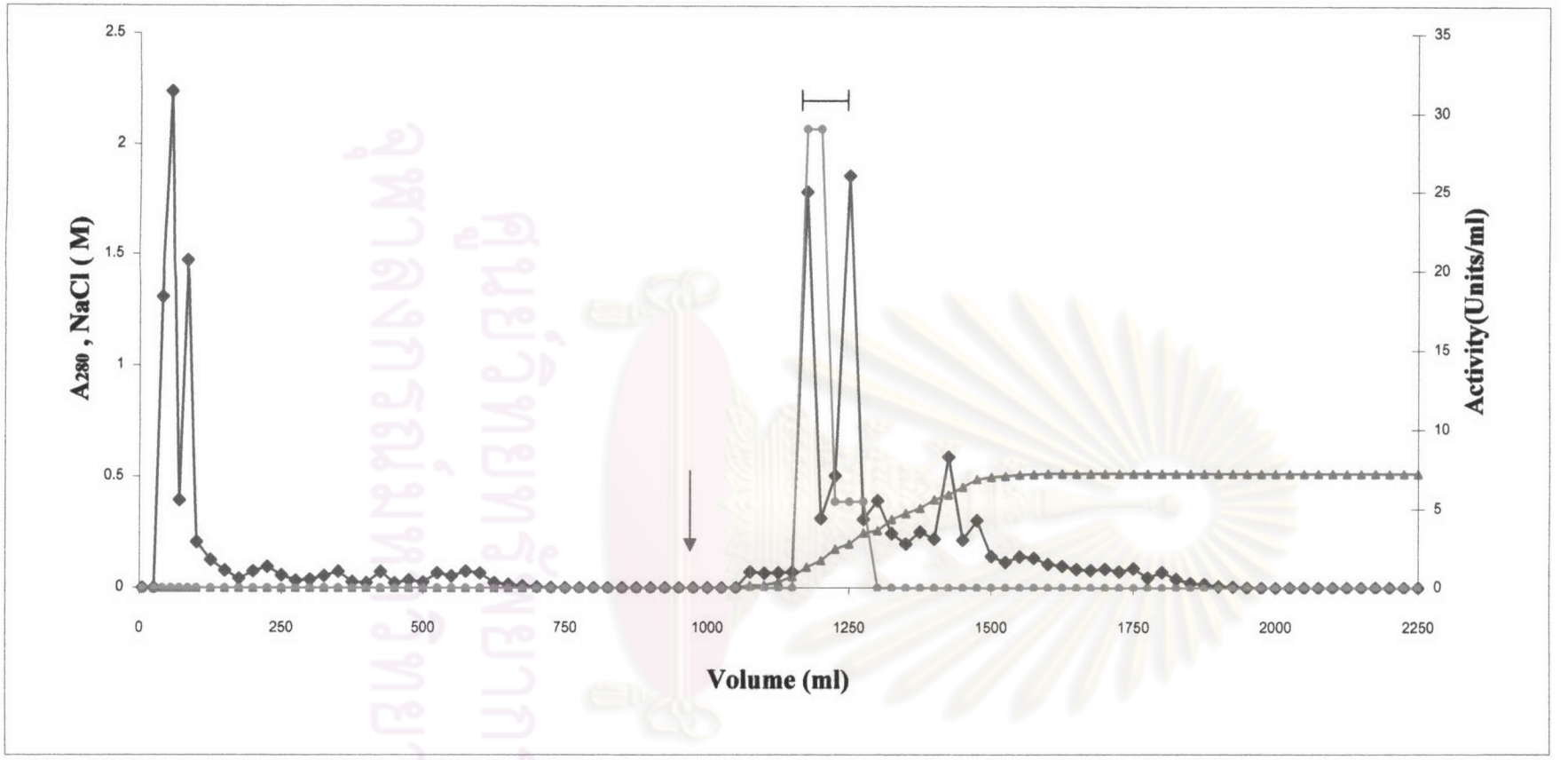


Figure 3.7 Purification on DEAE-Toyopearl chromatography eluted with linear gradient range 0 – 500 mM NaCl concentration.

◆ A_{280} ▲ conductivity[NaCl] ● nitrate reuctase activity ⊢⊣ the pool fractions

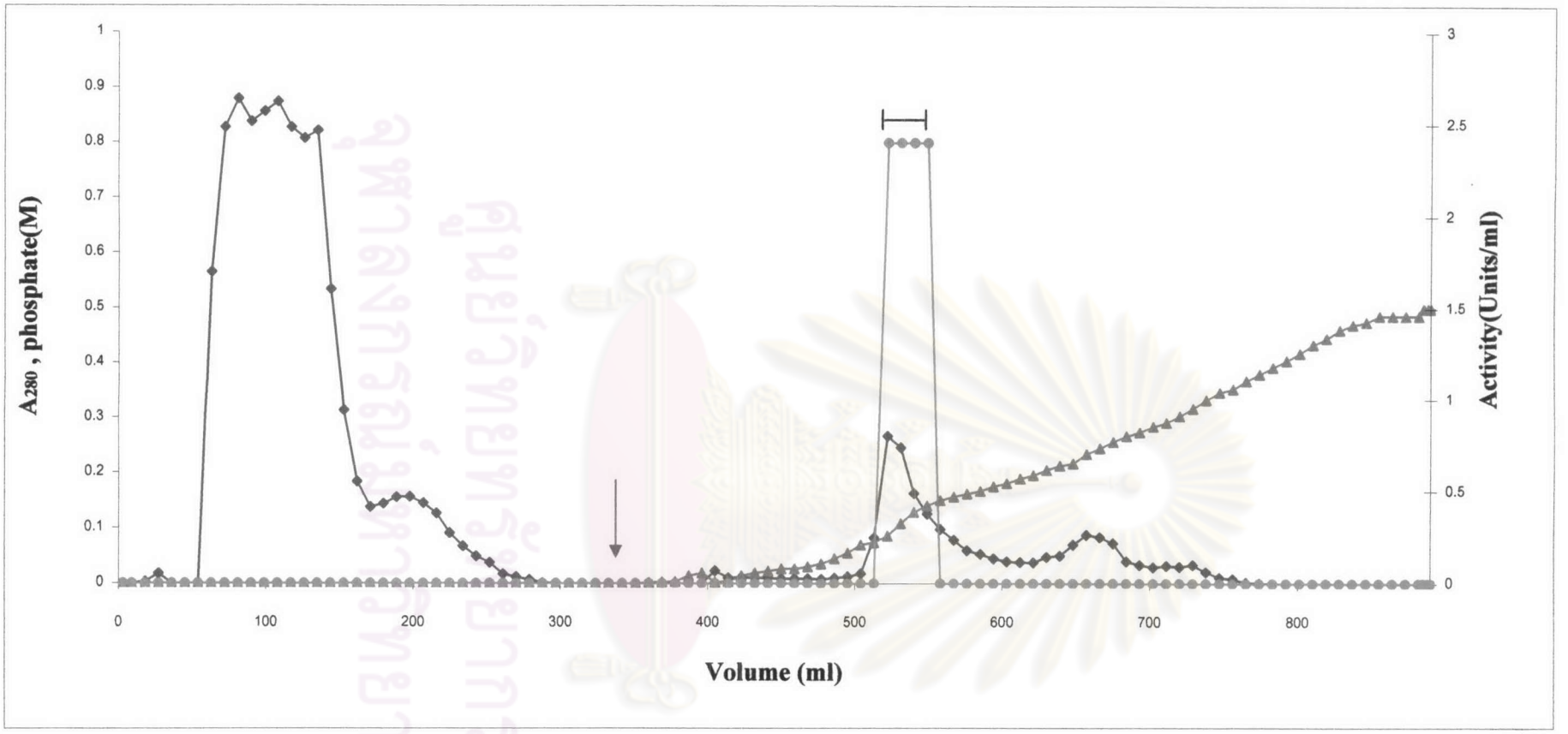


Figure 3.8 Purification on Bio-Gel Hydroxyapatite column eluted with linear gradient range 50 - 500 mM of phosphate buffer .

◆ A_{280} ▲ conductivity [phosphate] ● nitrate reductase activity ⊢⊣ the pooled fractions

Table 3.2: Purification of the nitrate reductase from *A. halophytica*.

Purification step	Total Protein (mg)	Total Activity (unit)	Specific Activity (unit/mg protein)	Yield (%)	Purification (fold)
Crude extract	5225	548	0.1	100	1
Supernatant of crude extract	2920	476	0.16	86.8	1.6
Ammonium sulfate (20 – 60%)	972	330	0.34	60.2	3.4
DEAE–Toyopearl	225	246	1.1	44.9	11
Bio–Gel hydroxyapatite	2.12	86	40.6	15.7	406

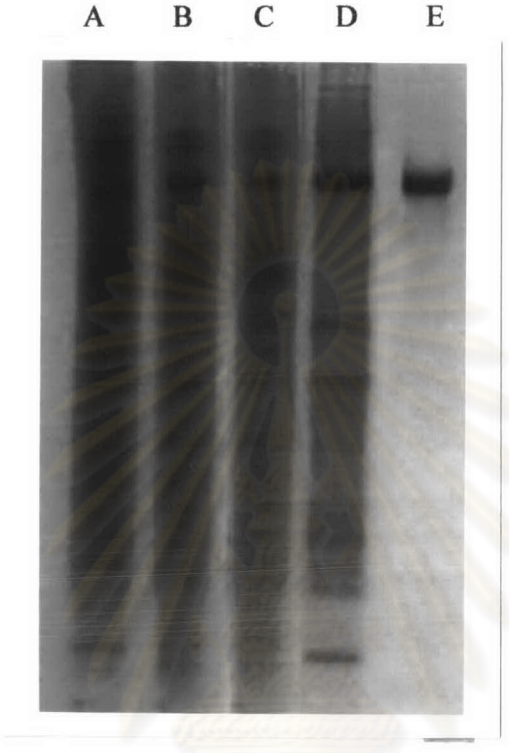


Figure 3.9: Non–denaturing PAGE of the *A.halophytica* nitrate reductase from each step of purification.

Protein staining

Lane A	crude enzyme	75	μg protein
Lane B	ultracentrifugation	65	μg protein
Lane C	20-60 % ammonium sulfate	55	μg protein
Lane D	DEAE–Toyopearl column	30	μg protein
Lane E	Bio-Gel hydroxyapatite	10	μg protein

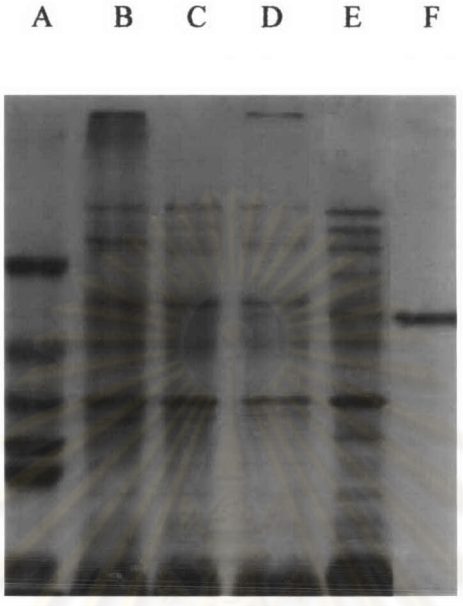


Figure 3.10: SDS-PAGE of the purified nitrate reductase fractions in 10% acrylamide gel.

Lane A: Mr markers ; BSA (66200) ; egg albumin (45000) ; glyceraldehyde- 3-phosphate dehydrogenase (36000) ; carbonic anhydrase (29000) ; trypsinogen (PMSF) (24000) ; trypsin inhibitor (20100) ; α - lactalbumin (14200)

Lane B: crude extract

Lane C: supernatant after centrifugation by ultracentrifugation.

Lane D: 20 – 60% (w/v) ammonium sulfate precipitation.

Lane E: The fraction obtained from DEAE-Toyopearl column.

Lane F: The fraction obtained from Bio-Gel hydroxyapatite column (7 μg).

Protein content of each Lane A to E is 50μg.

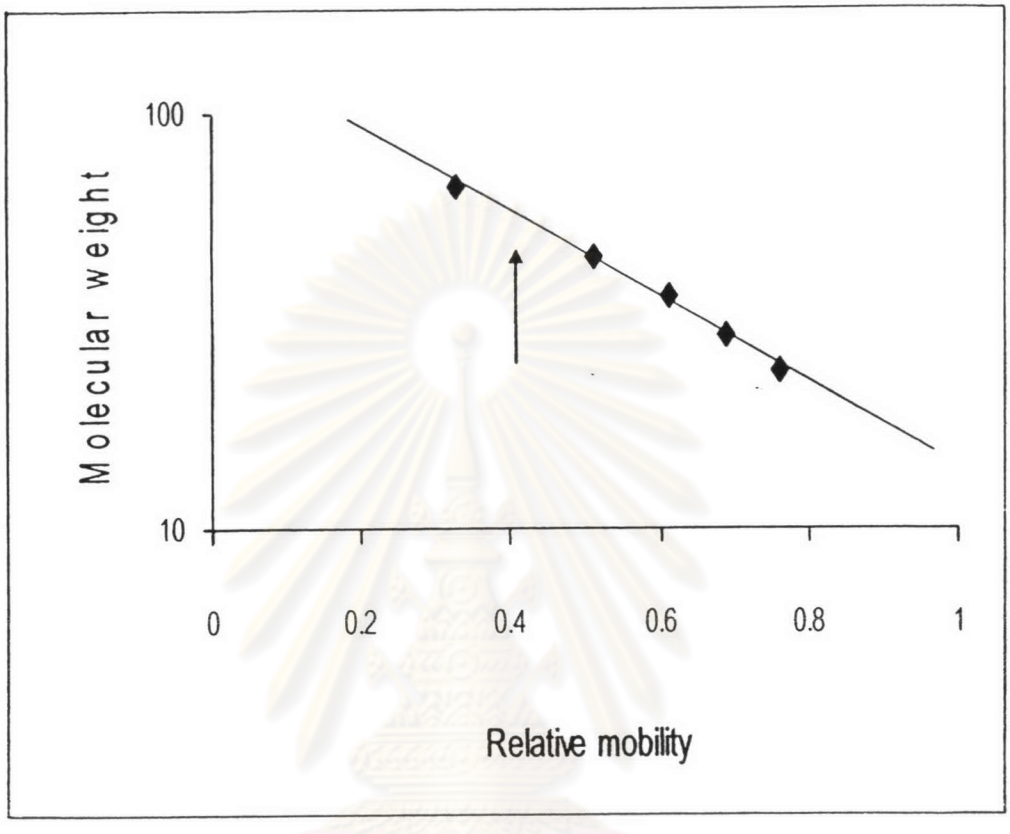


Figure 3.11: Calibration curve for molecular weight of purified nitrate reductase from *A.halophytica* on SDS–PAGE.

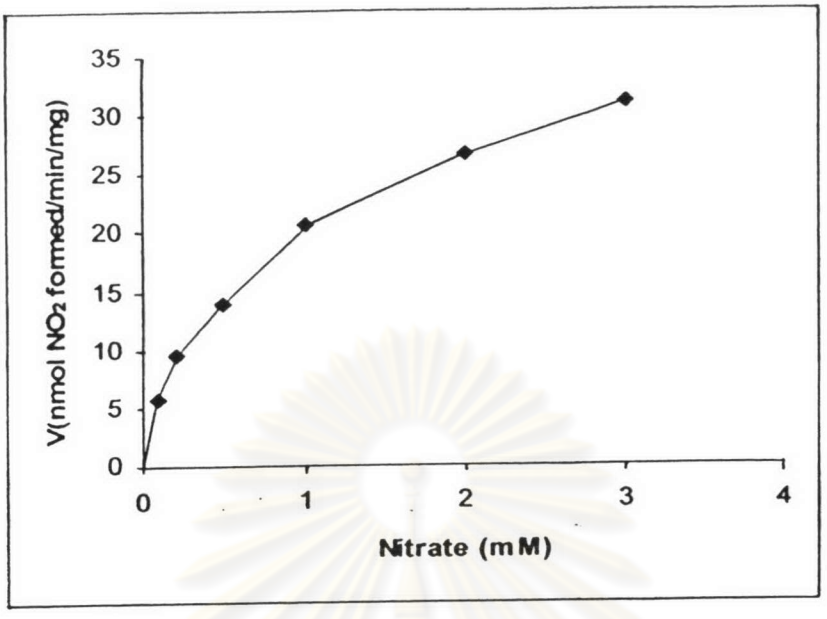
3.5.3 Electron donor availability

In the present study we had tested NADH , NADPH and ferredoxin as described in section 2.9.3 for nitrate reductase of *A. halophytica*. The purified nitrate reductase from *A. halophytica* could use ferredoxin as physiological electron donor. Table 3.3 shows electron donor specificity for the purified nitrate reductase, when used different physiological electron donor. The concentration of NADH and NADPH are vary from 5-10 mM.

3.5.3 Inhibitory effect of various agents on nitrate reductase activity

Inhibitory effect of various reagents, which were not the substrates of nitrate reductase was tested. The result of percent inhibition is shown in Table 3.4. *p*-Chloromercuribenzoate, iodoacetamide and N-Ethylmaleimide caused more than 80 % inhibition of nitrate reductase of *A. halophytica*. Also, potassium cyanide (KCN) caused more than 80 % inhibition at 0.1 mM. Sodium azide (NaN_3) did not inhibit nitrate reductase at 1 mM. Substrate analog, chlorate (ClO_3), can bind to nitrate reductase with competitive inhibition to nitrate. The end product of the reaction, nitrite (NO_2), did not affect nitrate reductase activity.

A.



B.

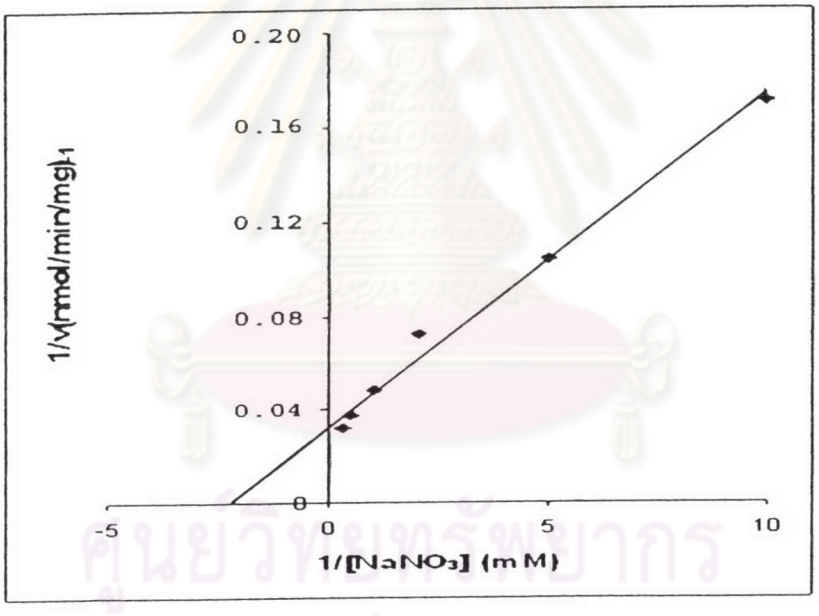


Figure 3.12 Kinetic studies of nitrate reductase activity with nitrate as substrate.

A) Saturation curve

B) Lineweaver – Burk plot of nitrate for nitrate reductase.

Table 3.3: Electron donor specificity.

Conditions	**Specific activity** nmol NO_2/min/mg
Methyl viologen	32.4
Ferredoxin	14.6
NADH	0
NADPH	0.1

Table 3.4: Effect of inhibitors on nitrate reductase activity.

Addition	Concentration (mM)	Inhibition (%)
None		0
p-Chloromercuribenzoate	0.1	90
Iodoacetamide	1	84
	0.2	79
N-Ethylmaleimide	2.5	81
KCN	1	100
	0.1	88
	0.01	65
NaN_3	1	2
ClO_3	100	83
	40	51
$NaNO_2$	100	20
	40	4

100% activity represents 40.6 nmol/min/mg