CHAPTER II

MATERIALS AND METHODS

Materials

2.1 Equipments

Autoclave	:	Hirayama Manufacturing Cooperation, Japan
Autopipette	:	Pipetman, Gilson, France
Centrifuge, Refrigerated	:	Model J-21C, Beckman Instrument Inc, USA
Electrophoresis unit	:	Model MiNi-protein II Cell: Bio-Rad, USA
Fraction collector	:	Model 2211 Pharmacia LKB, Sweden
Incubator	:	Model G76D, Scientific co.inc., USA
Larminar flow	:	BVT-124, International Scientific Supply Co.Ltd., Thailand
Microcentrifuge	:	Kubota, Japan
Peristaltic pump	:	Pharmacia LKB, Sweden
pH meter	:	PHM 83 Autocal pH meter, Radiometer, Denmark
Power supply	:	Pharmacia, England
Rotary shaker	:	New Brunswick Scientific Edison, USA
Spectrophotometer	:	UV-240 :Shimadzu, Japan and du series 650,
Ultracentrifuge	:	Hitachi, Japan
Vortex	:	Model K-550-GE, Scientific Industries, USA
Water bath	:	Charles Hearson, England
Water bath shaking	:	Heto Lab Equipment, Denmark

2.2 Chemicals

Acetic acid	:	Merck Ag Darmstadt, Germany
Acrylamide	:	Merck, USA
Ammonium chloride	:	Sigma, USA
Bio-Gel Hydroxyapatite	:	Bio-red, USA
Calcium chloride	:	Merck Ag Darmstadt, Germany
Coomassie brillant blue G-250	:	Sigma, USA
Coomassie brillant blue R-250	:	Sigma, USA
DEAE-cellulose	:	Sigma, USA
DEAE-Toyopearl	:	Japan
Dialysis Tubing	:	Sigma, USA
DTT (Dithiothreitol)	:	Sigma, USA
EDTA (Ethylenediamine tetraacetic acid)	:	Sigma, USA
Ethanol	:	Katayama Chem, Japan
Ferric sulfate	:	Mallinckrodt Chemical, USA
Glycerol	:	Merck Ag Darmstadt, Germany
Glycine	:	Sigma, USA
Hepes	:	Sigma, USA
L-Glutamic acid	:	BDH, England
Lysozyme	:	Sigma, USA
Magnesiam sulfate	:	Merck Ag Darmstadt, Germany
Magnesium chloride	:	Merck Ag Darmstadt, Germany
β-mercaptoethanol	:	Katayama Chem, Japan
Methanol	:	Merck Ag Darmstadt, Germany
Methyl viologen	:	Sigma, USA
N,N'-methylene-bis-acrylamide	:	Sigma, USA
N-(1-naphthyl)ethylendiamine dihydrochloride	:	Sigma, USA
Phosphoric acid	:	Sigma, USA
Potassium chloride	:	Merck Ag Darmstadt, Germany
Potassium phosphate monobasic	:	Merck Ag Darmstadt, Germany

Sodium chloride	:	BDH, England
Sodium dodecyl sulfate	:	Sigma, USA
Sodium molybdate	:	BDH, England
Sodium nitrate	:	BDH, England
Sodium nitrite	:	Fluka AG, Switzerland
Sodium dithionite	:	M&B, England
Sodium hydrogencarbonate	:	Sigma, USA
Sodium *p*-chloromercuribenzoate	:	Sigma, USA
Sobitol	:	BDH, England
Standard molecular weight marker protein	:	Sigma, USA
Sucrose	:	Katayama Chem, Japan
Sulfanilamide	:	Fluka AG, Switzerland
Toluene	:	BDH, England
Tris – hydrochloride	:	Katayama Chem, Japan
Triton x-100	:	Packard, USA
Zinc sulfate	:	Fisher, USA

2.3 Kits

Standard molecular weight marker protein, USA

Centricon concentrator PM10

2.4 Bacterial strains

Aphanothece halophytica was initially isolated from Solar lake in Israel. The organisms was kindly provided by Dr. T. Takabe of Nagoya University, Japan.

Methods

2.5 Growth rate determination

2.5.1 Growth of *A. halophytica* in various NaCl concentrations

Ten percents inoculum of *A. halophytica* was inoculated into a 250 ml flask containing 100 ml of Turk Island Salt Solution plus modified BG_{11} medium. The medium contained 18 mM $NaNO_3$ as nitrogen-source, the pH of the medium was adjusted to 7.6 (see Appendix 1). Cells were grown on a rotary shaker with 160 rpm at 30°C without CO_2 supplement, 2,000 lux of continuous illlumination. The concentration of NaCl in the culture medium was 0.5 M for normal condition and 2.0 M for salt-stress condition. At various time intervals, an aliquot of the culture was withdrawn and its turbidity was measured by a spectrophotometer at 750 nm.

2.5.2 Growth of *A.halophytica* in different nitrogen-sources

A.halophytica was grown photoautotrophically in BG_{11} medium plus 18 mM $NaNO_3$ and Turk Island Salt Solution with 0.5 M NaCl, the pH of the medium was adjusted to 7.6 for growth under normal condition. L-glumine or ammonium chloride (NH_4Cl) was used instead of $NaNO_3$ at same concentration (18 mM).

For growth under different nitrogen-source conditions, the culture was grown in 250 ml flasks containing 100 ml of medium on a rotary shaker with 2,000 lux of continuous illumination at 30°C without CO_2 supplementation. At various time intervals an aliquot of the culture was withdrawn and its turbidity was measured by a spectrophotometer at 750 nm.

2.6 Determination of nitrate reductase activity

2.6.1 Effect of NaCl concentration on nitrate reductase activity

After eight days, *A.habphytica* culture in BG_{11} medium plus 0.5 M or 2.0 M NaCl attained an exponential growth phase. The cells were harvested by centrifugation at 5000 g for 20 min, washed twice in 50 mM Tris-HCl buffer, pH 7.5, containing 1.0 M sorbitol to protect the cell from lysis. For the estimation of cellular nitrate reductase activity levels, an in situ assay was used. To 1 ml of this cyanobacterial suspension, 20 µl toluene was added and the mixture was shaken rigorously for 2 min. The reaction mixture (in a final volume of 1 ml) contained $NaHCO_3$-Na_2CO_3 buffer (100 µmol, pH 10.5), $NaNO_3$ (20 µmol), methyl viologen (4 µmol), $Na_2S_2O_4$ (10 µmol in 0.1 ml of 0.3 µmol $NaHCO_3$) and toluenized cyanobacterial cells. After 5 min incubation at 30°C under light, the nitrite (NO^-_2) formed was determined. Nitrite formed was determined by adding 200 µl of 10% sulfanilamide in 3.0 M HCl and 200 µl of 0.2% (w/v) N-(1-naphthyl)ethylendiamine dihydrochloride according to the method by Snell and Snell (1949). Then this mixture was centrifuged at 12,000 rpm for 10 min, the clear supernatant was measured by a spectrophotometer at 540 nm. Correction for the amount of nitrite present at zero time was also done. The values are the average of three replicates of independent cultures. One unit of enzyme activity is defined as the amount of nitrite formed (nmol) per min under the assay condition.

2.6.2 Effect of different nitrogen sources on nitrate reductase activity

An experiment was done to compare the effect of nitrogen-sources among L-glutamine , ammonium chloride (NH_4Cl) and nitrate at the same concentration. *A. halophytica* was grown in medium containing different nitrogen sources, harvested and the assay of nitrate reductase activity was performed

as described in section 2.5.1. The culture medium containing nitrate as nitrogen-source is the control condition.

2.7 The localization of nitrate reductase in *A. halophytica* cells

2.7.1 Isolation of periplasmic proteins by cold osmotic shock.

A. halophytica was grown in medium containing 0.5 M NaCl to exponential growth phase. At 8 days, the cells from 100 ml of culture were harvested by centrifugation at 5,000 g for 20 min and washed twice with 20 ml cold wash buffer [10 mM Tris-HCl (pH 7.6) and 0.5 M NaCl]. The cells were resuspended in 20 ml of plasmolysis buffer [1.0 M sorbitol, 10 mM Tris-HCl (pH 7.6) and 0.5 M NaCl] , supplemented with EDTA to a final concentration of 1 mM, shaken gently for 30 min at room temperature, and centrifuged as above. The cells were resuspended in 10 ml cold deionised water. The suspension was frozen at –80 °C, 30 min and thawed at 37°C, 30 min. After centrifugation, the supernatant was centrifuged once more to remove remaining cells. This clear supernatant represented the fraction of periplasmic proteins, which was concentrated by centricon. Protein content was estimated by the Bradford's method (Bradford, 1976).

2.7.2 Preparation of membrane and cytoplasmic fraction

After eight days, *A. halophytica* culture in BG_{11} medium plus 0.5 M or 2.0 M NaCl attained an exponential growth phase. The cells were harvested by centrifugation at 5,000 g for 20 min, washed twice in 50 mM Tris-HCl buffer, pH 7.5, containing 1.0 M sorbitol to protect the cell from lysis. The cells were resuspended in 10 ml of the resuspened buffer [50 mM Tris-HCl buffer, pH 7.5, 0.5 M NaCl , 1 mM EDTA, 20% glycerol (v/v), 10 mM DTT and 4 mM benzamidine]. This suspension was treated by sonic disintegration for 7 min below 15°C. After sonication, the mixture was centrifuged by ultracentrifugation in Ti 70 rotor at 40,000 rpm, 120 min (Gangeswaran *et.al*, 1993). The high speed supernatant

was collected and the pellet (membrane fraction) was washed once by suspending it in the 50 mM Hepes-NaOH pH 7.5, 10% glycerol (v/v), 10 mM EDTA and 5 mM DTT that also contained 0.3 % Triton X-100 and centrifuging again at 40,000 rpm for 60 min. The final pellet was resuspended in the same buffer.

2.7.3 Spectrophotometric assay for nitrate reductase activity

The fractions of periplasmic, membrane and cytoplasmic were assayed to determine nitrite formed. Methyl viologen-linked nitrate reductase was used for routine assay at pH 7.5 with the use of excess dithionite. The assay mixture contained 100 mM Tris-HCl buffer, pH 7.5, 40 mM $NaNO_3$, 20 mM methylviologen and 10 mM of $Na_2S_2O_4$ in 1 ml of 0.2 M $NaHCO_3$. The mixture was incubated at 30°C for 3-5 min. Nitrite formation was determined by the diazo color reaction. The suspension was centrifuged at 12,000 rpm for 10 min to give a clear supernatant. The spectrophotometer reading for nitrate reductase activity was set at 540 nm.

2.8 Purification of nitrate reductase

Nitrate reductase was purified from *A.halophytica* by four steps of purification including ultracentrifuge, ammonium sulfate precipltation ($(NH_4)_2SO_4$), DEAE-Toyopearl chromatography column and Bio-Gel hydroxyapatite chromatography column. All purification processes were performed at 4°C.

2.8.1 Ultracentrifugation

The frozen cells of *A .halophytica* were thawed in 20 ml of the resuspension buffer [50 ml of 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1 mM EDTA, 20% glycerol (v/v) and 4 mM benzamidine]. The cell suspension was treated by sonic disintegration for 7 min below 15°C. The pooled crude extract from 10 g (wet wt.) cells was centrifuged by ultracentrifugation in Ti 70 rotor at 40,000 rpm, for 120

min. The blue-green supernatant contained nitrate reductase activity from *A.halophytica.*

2.8.2 Ammonium sulfate $(NH_4)_2SO_4$ precipitation

The solid ammonium sulfate was added to the blue-green supernatant in four ranges from 0-20 % saturation, 20-40 % saturation, 40-60 % saturation and 60-80 % saturation. After the precipitate from each range was collected by centrifugation at 15,000 g for 20 min, it was dissolved in 10 ml of buffer A [50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% (v/v) glycerol] and assayed for nitrate reductase activity. The enzyme was desalted by dialysis against buffer A at 4 ^{o}C with 3 changes of buffer A.

2.8.3 DEAE-Toyopearl chromatography

DEAE-Toyopearl was washed with excess volume of 0.5 N HCl for 3 h followed by distilled water until pH was about 7.0 and then washed with 0.5 N NaOH for 3 h followed by distilled water to obtain pH about 7.5. The DEAE-Toyopearl was equilibrated with buffer A. The desalted enzyme solution was applied to a DEAE-Toyopearl column (2.5 x 18 cm). The unbounded proteins were eluted from the column with the same buffer. The bounded proteins were eluted from the column with linear gradient of NaCl concentration formed with 250 ml of buffer A and 250 ml of the same buffer containing 0.5 M NaCl with a flow rate of 1 ml min^{-1}. The fractions of 5.0 ml were continuously collected. The elution profile was monitored for protein by measuring the absorbance at 280 nm. The enzyme activity was determined as described in section 2.6.3. The active fractions were pooled for further purification step. The enzyme fractions were pooled and concentrated by aqua sorb.

2.8.4 Bio-Gel hydroxyapatite chromatography

Bio – Gel hydroxyapatite was washed with excess volume of water and buffer B [50 mM potassium phosphate, pH 7.5, 0.1 mM EDTA]. The washed Bio-Gel hydroxyapatite was packed into a column (2.2x20 cm) using peristaltic pump at flow rate of 15 $ml.h^{-1}$. The Bio-Gel hydroxyapatite column was equilibrated with buffer B for 5 column volumes. The pooled enzyme fraction from section 2.7.3 was desalted by dialysis against buffer B at 4 °C with 3 changes of buffer B. The desalted enzyme was applied to a Bio-Gel hydroxyapatite column. The column was washed with 500 ml of buffer B to remove the unbounded proteins. Then, the column was further eluted with a linear gradient of phosphate concentration formed with 200 ml of 50 mM phosphate, buffer pH 7.5 and 200 ml of 500 mM phosphate buffer, pH 7.5. The enzyme fractions were pooled and concentrated with the Centricon PM-10 concentrator.

2.9 Characterization of nitrate reductase

2.9.1 Kinetics of nitrate reductase

The purified nitrate reductase was characterized for the kinetic constants of initial rates of the nitrate reductase activity. The Michaelis constant (Km) and maximum velocities were calculated from x-axis and y-axis intercepts respectively of the double-reciprocal plot, i.e., 1/[s] vs 1/[v].

2.9.2 Effect of various inhibitors on nitrate reductase

The nitrate reductase was quite sensitive to a variety of agents that affect protein structure, as well as inhibitors of biochemical protein structure (p-chloromercuribenzoate, iodoacetamide) and electron transport (KCN). The inhibition of the enzyme nitrate reductase was studied by observing the initial rates of nitrate reductase activity. The nitrate reductase activity was measured as described in

section 2.7.3. The nitrate reductase activity was expressed as the percentage of remaining nitrate reductase activity compared to the control.

2.9.3 Electron donor availability

The ability to use electron donor as the physiological electron donor was investigated for nitrate reductase from *A . halophytica.* Nitrate reductase was assayed under different electron donor including NADH, NADPH, ferredoxin and methyl viologen (used for routine assay). Nitrate reductase was assayed in a system of 1 ml, containing 100 μmol Tris-HCl buffer, pH 7.5, 30 μmol $NaNO_3$, 20 μmol dithionite in 0.2 M of $NaHCO_3$ and enzyme. For instance varying NAD(P)H concentrations were used as electron donor. After incubation at 30°C for 10 minutes, the reaction was stopped by the addition of 200 μl of 10% sulfanilamide in 3.0 M HCl after which 200 μl of 0.2% (w/v) N-(1-naphthyl)ethylendiamine dihydrochloride was added. The absorption of the resultant color was determined at 540 nm with spectrophotometer of nitrite formed. For the determination of the activity of NADH oxidation, the decrease in absorbance at 340 nm was monitored. To test whether ferredoxin can replace methyl viologen as electron donor, ferredoxin – nitrate reductase was assayed by the reaction system of 1.0 ml containing 100 μmol Tris – HCl, pH 7.5 , 30 μmol $NaNO_3$, 2 μmol NADPH, 0.06 mg ferredoxin and 0.025 mg ferredoxin – NADP reductase. The assay mixture was incubated at 30° C for 15 min. The reaction was terminated by adding 0.1 ml of 0.5 M zinc acetate and heating in boiling water for 1 min. The assay mixture was centrifuged to give a color supernatant to which 5 μl of 0.3 mM phenazine methosulfate was added and was left to stand for 30 min . Nitrite formation was determined as described in the methyl viologen assay.

2.10 Determination of enzyme purity by non–denaturing polyacrylamide gel electrophoresis (native PAGE)

The enzyme from each step of purification was analyzed by native PAGE to determine the native protein pattern and its purity according to the method of Bollag *et al* .(1996). Electrophoresis conditions and protein detection were described below.

2.10.1 Non–denaturing gel electrophoresis

Discontinuous PAGE was performed on the slab gel (10 x 10 x 0.75) of a 7 % (w/v) separating gel and a 5% (w/v) stacking gel. Tris – glycine buffer, pH 8.3, was used as electrode buffer. Preparation of solutions and polyacrylamide gels was described in Appendix B. The enzyme from each step was mixed with 5x sample buffer by ratio 5:1 and loaded onto the gel. The electrophoresis was run from cathode towards anode at constant voltage of 100 volts at room temperature.

2.10.2 Detection of protein bands

After electrophoresis, proteins in the gel were visualized by Coomassie blue staining. The gel was stained with staining solution [0.1% (w/v) Coomassie brilliant blue R-250 in 10% (v/v) acetic acid and 45% (v/v) methanol] for at least 20 minutes on slow shaker. Destaining was performed by immersing the gel in destaining solution (10% (v/v) acetic acid and 10% (v/v) methanol) followed by several changes of destaining solution until gel background was clear.

2.11 Molecular weight determination of nitrate reductase

2.11.1 SDS–Polyacrylamide gel electrophoresis (SDS–PAGE)

The denaturing gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). The slab gel (10 x 10 x 0.75 cm) system consisted of 0.1% (w/v) SDS in 12% (w/v) separating gel and 5.0% (w/v) stacking gel, 25 mM Tris, 192 mM glycine (pH 8.8) containing 0.1% SDS was used as an electrode buffer (see Appendix C). Sample to be analyzed was treated with sample buffer (see Appendix C) and boiled for 5 min prior to application to the gel. The electrophoresis was performed at constant current of 20 mA per slab in Midget LKB 2001 electrophoresis unit from cathode toward the anode, the gel was stained with Coomassie blue. The protein molecular weight markers were phosphorylase B (97,400 dalton), bovine serum albumin (66,200 dalton), ovalbumin (45,000 dalton), carbonic anhydrase (31,000 dalton), soybean trypsin inhibitor (21,000 dalton) and lysozyme (14,400 dalton). After electrophoresis, proteins in the gel were visualized by coomassie blue staining as described above. Relative molecular weight of the protein sample was estimated from a standard curve plotted on semilog scale between the molecular weight of protein marker and the relative electrophoretic mobility (R_f).

The R_f was calculated by:

$$R_f = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$