CHAPTER II

MATERIALS AND METHODS

Materials

2.1 Instruments

Autoclave: Model HA 30, Hirayama Manufacturing Cooperation, Japan

Autopipette: Pipetman, Gilson, France

Centrifuge, refrigerated centrifuge: Model J-21C, Beckman Instrument Inc, USA

Digital Lux Meter FT710: Taiwan

Electrophoresis unit: model Mini-protein II Cell: Bio-Rad, USA

Glass Microanalysis Filter Holder: Millipore, USA

Illuminated/Refrigerated Orbital: Sanyo, England

Laminar Flow BVT-124: International Scientific Supply, Thailand.

Microcentrifuge: Kubota, Japan

Microscope: Olympus, USA

pH meter: PHM 83 Autocal pH meter, Radiometer, Denmark

Power Supply: Pharmacia, England

Scintillation Counter: Pharmacia LKB Wallac, Reckbeta 1218, England

Spectrofluorophotometer RF-5300PC: Shimadzu, Japan

Spectrophotometer UV-240: Shimadzu, Japan, and Du series 650: Beckman, USA

Ultracentrifuge: Hitachi, Japan

Vacuum Dry: Taitech, Japan

Vortex: Model K-550-GE: Scientific Industries, USA

Water Bath: Charles Hearson, England

Water Bath Shaking: Heto lab Equipment, Denmark

2.2 Chemicals

Acetylcholine: Sigma, USA

Acetic acid: Merck Ag Darmstadt, Germany

Acridine Orange: Katayama Chem, Japan

Acrylamide: Merck Ag Darmstadt, Germany

Boric acid: Merck Ag Darmstadt, Germany

β-mercaptoethanol: Katayama Chem, Japan

Calcium Chloride: Merck Ag Darmstadt, Germany

Citric acid: Fluka, Switzerland

Cobalt chloride: Merck Ag Darmstadt, Germany

Coomassie Brilliant Blue G-250: Sigma, USA

Cupric sulfate: Merck Ag Darmstadt, Germany

Dialysis Tubing: Sigma, USA

Dinitrophenol: Merck Ag Darmstadt, Germany

Dowex 50W(50x4-200, H^+ from): Sigma, USA

DTT(Dithiothreitol): Sigma, USA

EDTA(Ethylenediaminetetraacetic acid): Sigma, USA

Ethanol: Katayama Chem, Japan

Ethanolamine: Sigma, USA

Ethylene Dichloride(1, 2- dichloroethane): Sigma, USA

Ferric Sulfate: Mallinckrodt Chemical, USA

Glycerol: Merck Ag Darmstadt, Germany

Glycine: Sigma, USA

Glycine betaine: Sigma, USA

Gramicidin D: Sigma, USA

Hepes: Sigma, USA

Hydrogen peroxide: Sigma, USA

L-Alanine: BDH, England

L-Arginine: BDH, England

L-Carnitine: Sigma, USA

L-Cysteine : The British Drug Houses Ltd, England

L-Glutamic acid : BDH, England

L-Lysine: BDH, England

L-Phenylalanine: BDH, England

L-Proline: The British Drug Houses Ltd, England

L-Serine: BDH, England

L-Threonine: BDH, England

L-Valine: BDH, England

Lysozyme: Sigma, USA

Magnesium Chloride: Merck Ag Darmstadt, Germany

Magnesium Sulfate: Merck Ag Darmstadt, Germany

Manitol: BDH, England

Methanol: Merck Ag Darmstadt, Germany

[*methyl*-^{14}C]-choline: Amersham, England

N-Ethylmaleimide: Sigma, USA

Nitro Blue Tetrazolium: Sigma, USA

N,N'-methylene-bis-acrylamide: Sigma, USA

Nitrocellulose Membrane: BioRad, USA

Phosphoric acid: Sigma, USA

Phosphorylcholine: Sigma, USA

POPOP: Sigma, USA

PPO: Sigma, USA

Potassium cyanide: Merck Ag Darmstadt, Germany

Potassium chloride: Merck Ag Darmstadt, Germany

Potassium phosphate monobasic: Merck Ag Darmstadt, Germany

Soduim arsenate: BDH, England

Sodium carbonate: La Jota, Spain

Sodium chloride: BDH, England

Sodium fluoride: BDH, England

Sodium Dodecyl Sulfate: Sigma, USA

Sodium molybdate: BDH, England

Sodium nitrate: BDH, England

Sodium *p*-chloromercuribenzoate: Sigma, USA

Sorbitol: BDH, England

Standard Molecular Weight Marker Protein: Sigma, USA

Sucrose: Katayama Chem, Japan

Toluene: BDH, England

Triethylamine : Sigma, USA

Tris-hydrochloride: Katayama Chem, Japan

Triton X-100: Packard, USA

Zinc sulfate: Fisher, USA

2.3 Supplies

Nitrocellulose membrane: Millipore Coorperation, USA

Whatmann 3 MM paper: Whatmann International, England

2.4 Kit

Standard molecular weight: Sigma, USA

2.5 Bacterial strains

Aphanothece halophytica was initially isolated from Solar Lake in Israel. The organism was kindly provided by Dr. T. Takabe of Research Institute of Meijo University, Japan

Methods

2.6 Growth rate determination.

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

A. halophytica was grown photoautotrophically in a BG_{11} medium plus 18 mM $NaNO_3$ and Turk Island Salt Solution, pH of the medium was adjusted to 7.6 (see Appendix 1). The culture were grown in 250 ml flasks containing 100 ml of medium on a rotary shaker with three 20 W fluorescent lamps placed overhead

provided the flasks with 2,000 lux of continuous illumination at 30 °C without CO_2 supplementation. The concentration of NaCl in the culture medium was adjusted by adding NaCl as required (0.5 M, 1.0 M, 1.5 M and 2.0 M). At various time intervals an aliquot of the culture was withdrawn and its turbidity was measured by a spectrophotometer at 750 nm.

2.6.2 Choline functions as an osmoprotectant in *A. halophytica.*

A. halophytica was grown photoautotrophically in BG_{11} medium plus 18 mM $NaNO_3$ and Turk Island Salt Solution with 0.5 M and 2.0 M NaCl, the pH medium was adusted to 7.6. Choline and glycine betaine were added to high osmilality growth medium in a final concentration of 1 mM. The culture were grown in 250 ml flasks containing 100 ml of medium on a rotary shaker with three 20 W fluorescent lamps placed overhead provided the flasks 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.

2.6.3 Effect of choline concentration on growth at diferrent salinity of *A. halophytica.*

A. halophytica was grown in BG_{11} medium plus 18 mM $NaNO_3$ and Turk Island Salt Solution in a wide range of choline concentrations with (0-50 mM) 0.5 M and 2.0 M NaCl, the pH medium was adusted to 7.6. The culture were grown in 250 ml flasks containing 100 ml of medium on a rotary shaker with three 20 W

fluorescent lamps placed overhead provided the flasks 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.

2.6.4 Effect of choline on the salinity growth range of *A. halophytica.*

A. halophytica was grown in BG_{11} medium plus 18 mM $NaNO_3$ and Turk Island Salt Solution in a wide range of salt concentrations in the absence of any added osmoprotectant, the pH medium was adjusted to 7.6. To test the effect of choline on the growth of *A. halophytica* at different salinities, 10% of cells was inoculated into medium plus 0.25 M to 4.0 M NaCl in the presence or absence of 1 mM choline. The culture were grown in 250 ml flasks containing 100 ml of medium on a rotary shaker with three 20 W fluorescent lamps placed overhead provided the flasks 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.

2.7 Determination of [*methyl*-^{14}C]choline uptake system.

2.7.1 Uptake of [*methyl*- ^{14}C]choline upon hyperosmotic stress.

Choline uptake was measured with [*methyl*-^{14}C]choline by a method similar to that of Perroud and Le Rudulier. Ten percents inoculum of *A. halophytica* was incubated into flask containing 100 ml of Turk Island Salt Solution plus modified

BG_{11} medium with 0.5 M NaCl, the pH medium was adjusted to 7.6 and grown on a rotary shaker with 160 rpm at 30°C, 2,000 lux of continuous illumination. After ten days, cell culture 100 ml was harvested at the exponential growth phase. The cells were harvested by centrifugation at 10,000 rpm for 20 min, washed twice in the 50 mM Hepes-NaOH buffer pH 7.5. Cell pellet was rersuspended in 10 ml of the same buffer and incubation with osmotic stress experiment (control; 0.5 M NaCl and stress; 2.0 M NaCl). Uptake was carried out by adding [*methyl*-^{14}C]choline at 0.1 μCi/μmol final concentration 50 μM. Cell culture was incubated at 37 °C with continuous shaking at 200 rpm. At various time intervals at 5-90 min, 1,000 μl reaction mixture was withdrawn and terminated by rapid filtration through a membrane filter (cellulose-acetate memebrane; 0.45 μm pore size, 25 mm diameter). The cells were then quickly washed twice at room temperature with the same buffer (Figure 2.1). The membrane filter was transferred to a scintillation vial, added with 15 % H_2O_2 200 ml and incubated at 40 °C for 2 hours to allow for H_2O_2 decoloration of the cells, and finally it was heated at 80 °C overnight to oxidize H_2O_2. The fillter was solubilized in 2 ml scintillation fluid. The radioactive choline accumulated by the cells was determinded by scintillation counting. The protein content of cell suspensions measured routeinly was determined by Bradford's method (Bradford, 1976), according to the specifications of the supplier.

2.7.2 Inhibition of choline uptake by chloramphenicol.

An experiment was carried out with the protein synthesis inhibitor chloramphenicol to determine whether choline acts as a genetic inducer of its own

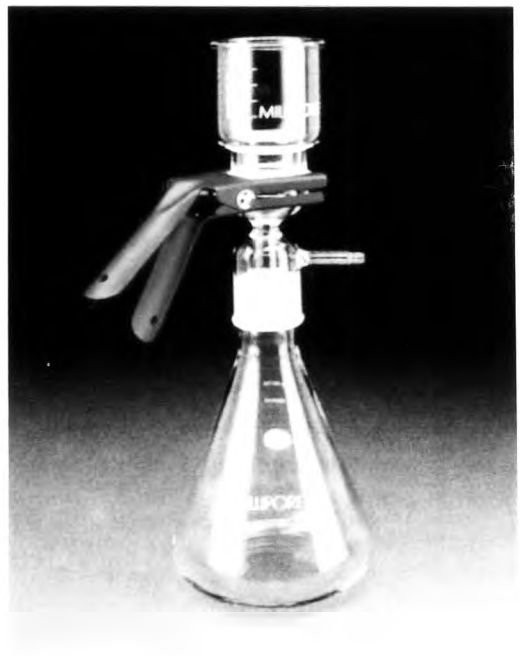

Figure 2.1 Glass Microanalysis Filter Holder (for 25 mm disc filter).

uptake. *A. halophytica* was grown in the medium, harvested and osmotic stress experiment was performed as described in 2.7.1. The cells were preincubated for 37 °C with continuous shaking at 200 rpm with 100 and 500 μg/ml of chloramphenicol in stress conditions (2.0 M NaCl), for 30 min. The uptake experiments were as described in 2.7.1.

2.7.3 Effect of various osmoticum on choline uptake.

An experiment was done to compare the effect of osmotic stress between NaCl and various osmoticums such as mannitol, sorbitol and sucrose at the same osmolarity. *A. halophytica* was grown in medium, harvested and osmotic stress experiment was performed as described in 2.7.1. All solutes were added to the control conditions (0.5 M NaCl) at 3 M and incubated for 37 °C with continuous shaking at 200 rpm, for 30 mins. The uptake experiments were as described in 2.7.1.

2.8 Characterization of choline uptake system.

2.8.1 Kinetics of choline uptake.

A. halophytica was grown in the medium, harvested and osmotic stress experiment was done as described in 2.7.1. To determine the kinetic constants of the choline uptake system, initial rates of uptake were determined over a wide range of [*methyl*-^{14}C]choline concentrations (100 to 700 μM) in the presence of osmotic stress conditions (0.5 M and 2.0 M NaCl). The Michaelis constant (K_m) and maximum

velocity (V_{max}) were calculated from x-axis and y-axis intercepts respectively of the double-reciprocal plot, i.e., 1/[s] vs 1/[v].

2.8.2 Na^+ requirement of the uptake system.

Cells were grown in the medium, harvested and osmotic stress experiment was done as described in 2.7.1. Initial uptake rates were determined in the presence of various Na^+ concentrations by using 50 μM [*methyl*-^{14}C]choline.

2.8.3 Substrate specificity of choline uptake.

A series of organic compounds were tested for their effects on the short term uptake of [*methyl*-^{14}C]choline. These included potential competitive inhibitors (acetylcholine, phosphorylcholine, ethanolamine, trimethylamine, glycine betaine aldehyde, glycine betaine, glycine, L-carnitine and amino acid). The specificity of the system was studied by observing the initial rates of [*methyl*-^{14}C]choline uptake in the presence of 100 fold excesses of unlabled compounds. The uptake experiments were as described in 2.7.1. The choline uptake system was expressed as the percentage of remaining choline compared to the control.

2.8.4 Effect of various inhibitors on choline uptake.

Choline uptake was quite susceptible to a variety of agents that affect protein structure, as well as inhibitor of biochemical energy generation (*N*-ethylmaleimide, sodium *p*-chloromercuribenzoate), ATP formation (sodium arsenate), electron

transport (KCN), the proton motive force (dinitrophenol) and Na^+ gradients (gramicidin D). The inhibition of the system was studied by observing the initial rates of [*methyl*-^{14}C]choline uptake in the presence of 100 fold excesses of unlabelled compounds. The uptake experiments were as described in 2.7.1. The choline uptake system was expressed as the percentage of remaining choline compared to the control.

2.9 Choline oxidation by choline dehydrogenase.

2.9.1 Preparation of cells and enzyme fraction.

2.9.1.1 Oxidation *in vivo*.

A. halophytica was grown in medium plus 0.5 or 2.0 M NaCl to exponential growth phase. After ten days, the cells were harvested by centrifugation at 10,000 rpm for 20 min and washed twice in the 50 mM Hepes-NaOH buffer pH 7.5. Cell pellet was resuspended with the same buffer with ratio 1 g cell pellet per 1 ml buffer. The solution was assayed for the content of protein and choline dehydrogenase activity.

2.9.1.2 Oxidation by crude enzyme fraction.

A. halophytica was grown in medium plus 0.5 or 2.0 M NaCl to exponential growth phase. After ten days, the cells were harvested by centrifugation at 10,000 rpm for 20 min and washed twice in the 50 mM Hepes-NaOH buffer pH 7.5. Cell pellet was suspended with grinding buffer (50 mM Hepes-NaOH, pH 7.5, 10% glycerol, 10 mM EDTA and 5 mM DTT) containing 2 mg/ml lysozyme and stirred at 4 °C for

90 min. After lysozyme treatment, the mixture was centrifuged at 3,500 rpm, 10 min and the supernatant was collected. The solution was assayed for the content of protein and choline dehydrogenase activity.

2.9.1.3 Oxidation by membrane and cytoplasmic fraction.

A. halophytica was grown in medium plus 0.5 or 2.0 M NaCl to exponential growth phase. After ten days, the cells were harvested by centrifugation at 10,000 rpm for 20 min and washed twice in the 50 mM Hepes-NaOH buffer pH 7.5. Cell pellet were suspended with grinding buffer (50 mM Hepes-NaOH, pH 7.5, 10% glycerol, 10 mM EDTA and 5 mM DTT) containing 2 mg/ml lysozyme and stirred at 4 °C for 90 min. After lysozyme treatment, the mixture was centrifuged in Ti 50 rotor at 19,000 rpm, 20 min to remove whole cells and large fragments. The supernatant was then centrifuged at 44,000 rpm in the same rotor for 60 min. The high speed supernatant was collected and the pellet (membrane fraction) was washed once by suspending it in the grinding buffer and centrifuging again at 44,000 rpm for 60min. The final pellet was resuspended in the same buffer.

Solubilized membranes were obtained by dissolving the final membrane pellet in grinding buffer that also contained 0.3 % Triton x-100 (Lanfald and Strom, 1986). The solution was stirred for 60 min at 4 °C and the undissolved material removed by centrifugation (19,000 rpm for 20 min). The solution containing either cytoplasmic or membrane fraction was assayed for the content of protein and choline dehydrogenase activity.

2.10 Radioisotopic assay for choline dehydrogenase.

The incubation mixture for routine assays consists of 5 µl of the cells suspension or solubilized enzyme. The assay was done by addition of 1 µCi [*methyl*-^{14}C] choline 5 mM (55 mCi/mmol), phenazine methosulfate 1 mM and 50 mM Hepes-NaOH buffer pH 7.5 in a final volume of 25 µl. The reactions were performed for 15 min at 37 °C in the dark to prevent nonenzymatic oxidation of phenazine methosulfate. At the end of the incubation period, NaOH (5 µl of 1 N) and H_2O_2 (10 µl of 30 %) were added, and the samples were mixed and then incubated for 1 hr at room temperature to quantitatively convert the product [*methyl*-^{14}C]betaine aldehyde to [*methyl*-^{14}C]glycine betaine. To isolate [*methyl*-^{14}C]glycine betaine, the samples were subjected to ion exchange chromatography. The reaction mixture was applied to a column (0.9 by 1.3 cm) packed with Dowex 50W (50x 4-200, H^+ form, Sigma.).The radioactive glycine betaine was eluted from the resin with 2 ml of H_2O and 5 ml of 2 M NH_3 and collected into a counting vial for measurement of radioactivity by liquid scintillation.

2.10.1 Effect of choline analogues on the choline dehydrogenase activity.

The crude enzyme fraction of stress culture condition or cells grown in medium with 2.0 M NaCl was assayed forcholine dehydrogenase activity. The reaction mixture contained 1 µCi [*methyl*-^{14}C]choline 5 mM (55 mCi/mmol), phenazine methosulfate 1 mM and 50 mM Hepes-NaOH buffer pH 7.5. The substrate analogues (acetylcholine, phosphorylcholine, ethanolamine, glycine betaine, betaine aldehyde or glycine) was present in the reaction mixture with 1 mM. The choline

uptake system was expressed as the percentage of remaining choline compared to the control.

2.10.2 Effect of monovalent cations on choline dehydrogenase activity.

The crude enzyme fraction of stress culture condition cells grown in meduim with 2.0 M NaCl was assayed for choline dehydrogenase activity. The reaction mixture contained 1 μCi [*methyl*-^{14}C]choline 5 mM (55 mCi/mmol), phenazine methosulfate 1 mM and 50 mM Hepes-NaOH buffer pH 7.5, various salts (NaCl and KCl) at the varying concentrations of 0.05, 0.1, 0.25, 0.5 and 1.0 M were added to the reaction mixture, in a final volume of 25 μl.The radioisotopic assay for choline dehydrogenase activity experiments for as described in 2.10. The velocity of enzyme was expressed μmol choline dehydrogenase per min per mg protein.

2.10.3 Effect of metal ions and various reagents on choline dehydrogenase activity.

The crude enzyme fraction of stress culture condition or cells grown in medium with 2.0 M NaCl, was assayed for choline dehydrogenase activity. The reaction mixture contained 1 μCi [methyl-^{14}C]choline 5 mM (55 mCi/mmol), phenazine methosulfate 1 mM and 50 mM Hepes-NaOH buffer pH 7.5. Each inhibitor was added at the concentration as indicated, such as $MnCl_2$, $CuSO_4$, $AgNO_3$, EDTA, sodium *p*-chloromercuribenzoate, sodium arsenate, dinitrophenol and *N*-ethylmaleimide in a final volume of 25 μl. The radioisotopic assay for choline dehydrogenase activity experiments were as described in 2.10. The enzyme activity

was expressed as the percentage of remaining activity compared to the control. The choline uptake system was expressed as the percentage of remaining choline compared to the control.

2.11 Isolation of periplasmic proteins by cold osmotic shock.

A. halophytica was grown in medium plus 0.5 or 2.0 M NaCl to exponential growth phase. After ten days, the cells from 100 ml of culture were harvested by centrifugation at 8,000 rpm for 20 min and washed twice with 20 ml cold wash buffer [10 mM Tris-HCl (pH 7.6) and 0.5 or 2.0 M NaCl for control and salt adapted cells, respectively]. The cells were resuspended in 20 ml of plasmolysis buffer [1.0 M sorbitol, 10 mM Tris-HCl (pH 7.6) and 0.5 or 2.0 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 aquasorb. Protein content was estimated by the Bradford's method.

2.12 Polyacrylamide gel electrophoresis (PAGE)

2.12.1 Non-denaturing PAGE

Discontinuous PAGE was performed on slab gels (10x8x0.75 cm), consisting of 12.0% (w/v) separating gel, and 5% (w/v) stacking gel. Tris-glycine buffer pH 8.8

was uesd as electrode buffer (see Appendix 3). The sample was treated with sample buffer (see Appendix 3) at a ratio of 4:1 (sample:sample buffer). The electrophoresis was run from cathode toward the anode at constant current of 20 mA per slab in Midget LKB 2001 electrophoresis. The protein bands on the gel were stained with staining solution and destained with destaining solution.

2.12.2 SDS-PAGE

The denaturing gel electrophoresis was performed according to Laemmli (1970). The gel 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 3). Sample to be analyzed was treated with sample buffer (see Appendix 3) 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). Relative molecular weight of the protein sample was estimated from 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 = distance of protein migration/distance of tracking dye migration

2.12.3 Detection of proteins in the slab gel by coomassie blue staining

After electrophoresis, proteins in the gel were stained by coomassie blue. The gel was immersed in 0.2% (w/v) of coomassie brilliant blue R-250 containing 45% (v/v) methanol and 10% (v/v) acetic acid for at least 20 min on slow shaker. It was then destained with a solution of 10 % methanol and 10% (v/v) acetic acid for 1-2 hr, followed by changes of destaining solution until background of the gel was clear.

2.12.4 Choline dehydrogenase activity staining

After non-denaturing polyacrylamide gel electrophoresis, the gel was stained for activivity by incubating the gel in a solution containing 50 mM choline chloride/ 1 mM phenazine methosulfate/ 1 mg/ml nitroblue tetrazolium/ 50 mM Hepes-NaOH, pH 7.5 at 37 °C for 30 min in the dark. The presence of choline dehydrogenase activity was confirmed by the appearance of brown color band.

2.12.5 Radiolabeling of periplasmic protein

Binding activities were detected by running the reaction mixture in non-denaturing polyacrylamide gel electrophoresis using 50 μg periplasmic proteins mixed with 10 μM [*methyl*-^{14}C]choline, and incubating at 20°C for 30 min (Le Rudulier et al., 1991). The gels were quickly dried on Whatman 3 MM paper and autoradiographed with X-OMAT S Kodak films 14 days. The control was run by the mixture without periplasmic proteins.

2.13 Determination of protein.

Protein content was determined by Bradford' s method (Bradford, 1976). The protein sample (maximum 100 µl) was aliquoted into a tube and distilled H_2O was added to make a total volume of 100 µl. This was followed by 1 ml of Bradford working solution. The mixture was mixed well by vortex. Absorbance at 595 nm was read after 2 min but before 1 hr. The standard curve was constructed using 1 mg/ml bovine serum albumin as protein sample.

The compositions of Bradford stock solution and Bradford working solution are as follows:

Bradford stock solution	:	100 ml 95 % ethanol
		200 ml 85 % phosphoric acid
		350 mg serva Blue G
		Stable indefinitely at room temperature.
Bradford working solution	:	425 ml distilled water
		15 ml 95 % ethanol
		30 ml 85 % phosphoric acid
		30 ml Bradford stock solution

Filter through Whatman No. 1 paper, store at room temperature in brown glass bottle. Usable for several weeks but may need to be refiltered.