หอสมุดกลาง สถาบันวิทยบริการ จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER II

MATERIALS AND METHODS

Materials

Mercuric chloride and ferrous sulfate were purchased from Mallinckrodt Chemical Works. Cobalt chloride, cadmium chloride, potassium chloride, magnesium sulphate, sodium hydrogen carbonate, sodium carbonate, zinc sulfate, potassium cyanide and ethylenediaminetetra-acetic acid were from BDH chemicals Ltd. Lead nitrate, magnesium chloride, manganese chloride, sodium nitrate and boric acid were from May & Baker Ltd. Sodium chloride, calcium chloride, potassium di-hydrogen phosphate, potassium sulphate and D-sorbitol were obtained from Sigma Chemical Company. Zinc chloride, glucose, di-potassium hydrogen phosphate were from Fluka Chemie AG. Water soluble siliconizing fluid was from PIERCE.

Lead and zinc atomic absorption standard solutions were procured from Aldrich Chemical Company.

Hollow cathode lamps of lead and zinc were obtained from Varian Techtron Pty. Ltd.

Methods

1. Cultivation of Aphanothece halophytica

The blue green alga Aphanothece halophytica, provided by Dr. T. Takabe (Nagoya University), was maintained in Turk Island Salt Solution combined with BG11 medium + NO_3 solution.

1.1 Preparation of Turk Island Salt Solution

Stock Solution A: KCl 33.3 g

$MgCl_2.6H_2O$ 275.0 g

$CaCl_2.2H_2O$ 73.3 g

and made up to 5 liters with deionized water

Stock Solution B: $MgSO_4.7H_2O$ 347.0 g and then made up to 5 liters with deionized water

To make Turk Island Salt Solution, 500 ml of Stock Solution A was added to 500 ml of Stock Solution B. To this mixture 140.8 g of NaCl was added and the final volume was adjusted to 5 liters with deionized water.

1.2 Composition of BG11+NO_3 Solution

$NaNO_3$	(75 g/500 ml)	50 ml
KH_2PO_4	(8 g/200 ml)	5 ml
$MgSO_4.7H_2O$	(15 g/200 ml)	5 ml
$CaCl_2.2H_2O$	(7.2 g/200 ml)	5 ml
Na_2CO_3	(4 g/200 ml)	5 ml
Citric acid	(1.2 g/200 ml)	5 ml

$EDTA.Na_2$	(0.2 g/200 ml)	5 ml
$FeSO_4.7H_2O$	(1.2 g/200 ml)	5 ml
*Trace element A_5 Solution + Co		5 ml

*Trace element A_5 Solution + Co contained the following components in gram per liter; H_3BO_4: 2.86; $ZnSO_4.7H_2O$: 0.22; $CuSO_4.5H_2O$: 0.08; $MnCl_2.4H_2O$: 1.81; $Na_2MoO_4.2H_2O$: 0.39; and $Co(NO_3)_2.6H_2O$: 0.049

Culture medium of A. halophytica was prepared by adding all solutions of item 1.2 at indicated volume to five liters of Turk Island Salt Solution and the pH was adjusted to 7.6 by slowly adding 2 M NaOH. This medium can be stored in the cold room until the culturing of A. halophytica is required. Culturing was done by autoclaving the culture medium at 15 lb/in^2 for 15-20 min and inoculating this autoclaved medium (after it was cooled down to room temperature) with stock culture of A. halophytica (ratio of medium to stock culture is approximately 10:1). The culture was then placed on a shaker with illumination (840-1,720 lux) at room temperature (25-30 °C). The organism was harvested at the sixth day of growth and used for experiments.

2. Cultivation of Spirulina platensis

The blue green alga, Spirulina platensis isolated from Bueng Makkasan by Duangrat Inthorn, was

maintained in Zarrouk's medium (Zarrouk 1966) containing the following components in gram per liter.

$NaHCO_3$	16.80
K_2HPO_4	0.50
$NaNO_3$	2.50
NaCl	1.00
$MgSO_4.7H_2O$	0.20
$FeSO_4.7H_2O$	0.01
K_2SO_4	1.00
$CaCl_2.2H_2O$	0.04
EDTA	0.08
A_5 Solution	1 ml/l
B_6 Solution	1 ml/l
pH	8-10

The A_5 Solution contained the following in mg/l; H_3BO_4: 2.86; $MnCl_2.4H_2O$: 1.81; $ZnSO_4.7H_2O$: 0.22; MoO_3: 0.01;and $CuSO_4.5H_2O$: 0.08.

The B_6 Solution contained the following in g/l; NH_4VO_3: 22.9; $NiSO_3.7H_2O$: 47.8; Na_2WO_4: 17.9; $Ti(SO_4)$:40.0; and $Co(NO_3)_2.6H_2O$: 4.4.

The medium was sterilized by autoclaving at 15 lb/in^2. for 15-20 min. The cultivation of this organism was 10% inoculum, maintained in 500 ml flask on a shaker with illumination (840-1720 lux) at room temperature (25-30°C). The organism was harvested at the fifth day of growth and used for experiments.

3. Determination of Biomass

Biomass of A. halophytica was determined by centrifugation at 7,700 g for 10 min (BECKMAN J-21C Centrifuge). The cells were washed with distilled water and dried to constant weight at a temperature of 110°C, prior to final weighing. Optical density determinations at 650 nm using a Spectronic 20 D (Milton Roy Company) correlated well with dry weight (Figure 1).

Biomass of S. platensis was determined by filtering through a filter paper (Whatman No. 42). The cells were washed by distilled water and dried to constant weight in a 50 watt NEC microwave oven, prior to final weighing. Optical density determinations at 650 nm using a Spectronic 20 D also correlated well with dry weight (Figure 2).

4. Metal Accumulation by A. halophytica and S. platensis

4.1 Metal Accumulation by A. halophytica

The cells were harvested by centrifugation at 7,700 g for 10 min at 20°C, washed once with 10 mM PIPES buffer pH 6.5 in 0.6 M sorbitol and then suspended in the buffer to make a density of cells in the range 0.15 - 0.22 mg dry wt/ml for the study of zinc accumulation and 0.41 - 0.46 mg dry wt/ml for the study of lead accumulation. The reaction was started by adding metal

solution to make the final concentration of 500 ppm for lead and 100 ppm for zinc into 1 ml cell suspension and immediately mixing. The reaction mixture was shaken and incubated for 40 min and 1 hour for lead and zinc respectively at room temperature. The reaction was stopped by centrifugation at 10,500 g for 1 min (TOMY SEIKO MC-15A) and the cells were washed once with PIPES-sorbitol buffer. The cell pellets were transferred to another test tube by rinsing with double distilled water and dried overnight in an oven at 110°C. The sample without cells was used as a blank.

4.2 Metal Accumulation by S. platensis

The cells were harvested by filtration through a 20 micron plankton net, washed and resuspended in 10 mM acetate buffer pH 5.5 to make a density of cells in the range of 0.37-0.41 mg dry wt./ml for the study of lead and 10 mM PIPES buffer pH 6.5 to make a density of cells in the range of 0.14-0.18 The reaction was started by adding metal solution to make a final concentration of 500 ppm into 1 ml cell suspension and immediately mixing. The reaction mixture was shaken and incubated for 1 hour and 30 min for lead and zinc respectively at room temperature. The reaction was stopped by filtration through a Whatman No. 42 filter paper. The cells were washed with the buffer and dried overnight in an oven at 110°C. The sample without cells was used as a blank.

017747

4.3 EDTA Washing

The reactions of cells and metal ion were performed as described in sections 4.1 and 4.2. After stopping the reactions, the cells of A. halophytica were washed once with 1 ml of PIPES-Sorbitol buffer containing 10 mM EDTA and washed again with PIPES-Sorbitol buffer whereas the cells of S. platensis were washed once with 1 ml of PIPES buffer containing 10 mM EDTA and washed again with PIPES buffer.

4.4 Effect of Metabolic Inhibitors

The harvested cells were preincubated with 0.1, 1.0 mM NaN_3; 0.01, 0.1 mM DNP and 40 mM DCCD in the buffer as described in sections 4.1 and 4.2 for 30 min. After the preincubation, the reaction was started by adding metal solution and proceeded as described in sections 4.1 and 4.2. The DCCD treated sample was illuminated at 850 lux during preincubation and incubation.

4.5 Effect of pH

The reactions of cells and metal ion were performed as described in sections 4.1 and 4.2. The pH's of the buffer were varied from 4.0 to 5.5 using 10 mM acetate buffer and 6.0 to 7.0 using 10 mM PIPES buffer.

4.6 Effect of Cation

The reactions of cells and metal ion were performed as described in sections 4.1 and 4.2. The cells in the presence of lead or zinc were contacted with the cations at equimolar concentration.

4.7 Effect of Metal Concentration

The reactions of cells and metal ion were performed as described in sections 4.1 and 4.2 The final lead concentrations were varied from 50 to 800 ppm. The final zinc concentrations were varied from 20 to 150 ppm for A. halophytica and 50 to 500 ppm for S. platensis.

4.8 Effect of Cells Density

The reactions of cells and metal ion were performed as described in sections 4.1 and 4.2. The densities of cells were varied in the range 0.5 to 10 times of that used in other experiments.

4.9 Aging Effect

The cells were harvested at various stages of growth and used in the experiment as described in section 4.1 and 4.2.

5. Determination of Cell Mortality

A. halophytica and S. platensis were treated with lead and zinc ion as described in sections 4.1 and 4.2. The cells were collected and suspended in 0.5 M Tris-HCl pH 7.5 containing 0.5 M NaCl for A. halophytica and in 0.5 M Tris-HCl pH 7.5 for S. platensis. The cells were then stained with Erythrosine B staining to determine cell mortality.

Dye solution consisting of Erythrosine B 0.4 g, sodium chloride 0.81 g, potassium dihydrogen

phosphate 0.06 g and methyl-p-hydroxybenzoate 0.05 g in 100 ml distilled water was prepared. The pH of dye solution was adjusted to 7.2.

Cell suspension was mixed with the dye solution at a ratio of 4.5 : 0.5 (by volume). The mixture was incubated at room temperature for 5 min before observation under light microscope. Only dead cells were stained by the dye pigment.

6. Removal of Lead and Zinc from Waste Water

Glass or polyethylene containers used in the experiments were previously coated with a silicone compound.

6.1 Removal of Lead and Zinc by A. halophytica

The cells were harvested by centrifugation at 7,700 g for 10 min at 20°C. The cells were suspended in 20 ml metal containing waste water previously filtered through a Whatman No.1 filter paper to remove particles and oils contamination. The mixture was incubated at room temperature, continuously shaken for 1 hour for lead and 3 or 5 hours for zinc removal experiments. After incubation, the cells were removed by centrifugation at 7,700 g for 10 min at 20°C. The supernatant was directly measured by atomic absorption spectrophotometer to determine the remaining metal concentration after removing the cells by centrifugation. The results were expressed as

% efficiency which is calculated as follows:

$$\% \text{ efficiency} = \frac{(\text{initial metal concentration} - \text{remaining metal concentration}) \times 100}{\text{initial metal concentration}}$$

The % efficiency shown in the result was the value that was subtracted by % metal removal in the absence of cells which might occur due to the pH effect.

6.2 Removal of Lead and Zinc by S. platensis

The cells were harvested by filtration through a 20 micron plankton net and suspended in 20 ml metal containing waste water previously filtered through a Whatman No.1 filter paper to remove particles and oils contamination. The mixture was incubated at room temperature and continuously shaken for 3 hours for lead and 2 hours for zinc removal experiments. After incubation, the cells were removed by vacuum filtration through a Whatman No.42. The filtrate was processed for measurement by atomic absorption spectrophotometer as described in section 8.2 to determine the remaining metal concentration. The efficiency of metal removal was calculated as described in section 6.1.

7. Reutilization of Cells

7.1 Reutilization of A. halophytica

The cells which had been incubated with the heavy metals in the waste water as described in 6.1

were washed with 5 ml of 10 mM EDTA in 0.6 M sorbitol and washed again with 10 ml of 0.6 M sorbitol. The washed cells were resuspended in 20 ml waste water and processed as described in 6.1.

7.2 Reutilization of S. platensis

The cells which had been incubated with the heavy metals in the waste water as described in 6.2 were washed with 5 ml of 10 mM EDTA and washed again with double distilled water. The washed cells were resuspended in 20 ml waste water and processed as described in 6.2.

8. Preparation of sample for atomic absorption analysis

8.1 Algae Cell Sample

The cells were digested with 1 ml concentrated nitric acid at 70°C until the sample completely dissolved. The digests were diluted with double distilled water, taken to appropriate volumes, and cooled at 20°C. To remove fat, the digests were filtered through Whatman No. 1 filter papers.

The contents of metals were analyzed by an Atomic Absorption Spectrophotometer (Shimadzu). The metal concentration was expressed as ug metal accumulation per mg dry weight after subtracting the control (Cells incubated in the same manner but without metal).

8.2 Waste Water Sample

The method was modified from total metals

analyses in Standard Methods for the Examination of Water and Waste Water (American Public Health Association, American Water Works Association and Water Pollution Control Federation 1975). Ten ml of well-mixed sample was transferred to a beaker and then added with 1 ml concentrated nitric acid. The mixture was evaporated to near dryness, making certain that the sample did not boil. After the beaker cooled, another 2 ml of acid was added and the beaker was covered with a watch glass. The mixture was heated to cause a reflux action. During heating acid was added as necessary until digestion was complete. This was indicated by a light colored residue. The residue was dissolved by 1 to 2 ml concentrated nitric acid and slightly warmed. Double distilled water was used to wash down the sample from the beaker walls and watch glass. The sample volume was adjusted to 10 ml with double distilled water and filtered through a Whatman No.1 filter paper to remove silicate and other insoluble materials that could clog the atomizer. The sample was then ready for analysis.

9. Sedimentation of A. halophytica and S. platensis

The cells were harvested and incubated in waste water by the processes as described in 6.1 and 6.2. The mixtures were left standing in either light or dark condition.