Chapter II

Materials and Methods

Materials

Chemicals

Magnesium sulfate, sodium hydrogen carbonate were from BDH chemicals Ltd. Magnesium chloride, sodium nitrate were from May & Baker Ltd. Sodium chloride, calcium chloride, potassium sulfate , potassium di-hydrogen phosphate were from Sigma Chemical Company, U.S.A.

DEAE cellulose (fine mesh) was from Sigma Chemical Company, USA., Sephadex G-150 (fine particle) was from Pharmacia Fine Chemicals, Sweden.

Glycerol,acrylamide,N,N'-methylene bis acrylamide (BIS) were from Sigma Chemical Company ,U.S.A. N,N,N,N ' tetramethylenediamine (TEMED) , coomassie brilliant blue were from BDH Laboratory Chemicals Ltd., England. 2-Mercaptoethanol was from Fluka, Switzerland.

Standard protein: bovine serum albumin (BSA,grade VI), ovalbumin(grade II), chymotrypsinogen A (bovine pancreas Type II), myoglobin, cytochrome c were from Sigma Chemical Company USA.

All chemicals mentioned above were analytical grade except methanol. Other chemicals were from Sigma Chemicals, USA.; BDH, England; and Fluka Company, Switzerland.

Specimens

Three strains of Spirulina of which the first two were isolated from turtle pond in Wat Benjamaborpit, designated as BP and from Makkasan pond, designated as MP. The third strain TS-H-02 was kindly donated from the National Inland Fisheries Institute, Department of Fisheries, Thailand and designated as NIFI.

Methods

1. Collection of samples from natural water sources

Samples were collected at the surface of water from various sources. The pH of the water was measured and the morphology of Spirulina was observed under a microscope (100 X). The cells were counted by a Haemacytometer under a microscope with a magnification of 100 X.

2. Collection and cultivation of Spirulina from natural water sources

2.1 Cultivation in a 50 ml flask

2.1.1 One hundred and fifty ml of water sample was filtered through a 40 μ nylon sieve. The cells retained on the nylon sieve were suspended in 20 ml of Zarrouk medium (see Appendix) in a 50 ml flask and grown on a rotary shaker (160 rpm) at 27-30°C with continuous illumination of 1500 lux provided by fluorescent lamps for 2 weeks.

2.1.2 The experiment was done as described in 2.1.1 but the pH of Zarrouk medium was adjusted to 7.0,7.5,8.0 and 8.5.

2.2 Cultivation in a 500 ml glass chamber

2.2.1 Four liters of water sample from Makkasan Pond was filtered through a 40 μ nylon sieve. The cells retained on the nylon sieve were suspended in a glass chamber containing 500 ml of Zarrouk medium. The culture was aerated by an air pump and grown for 2 weeks.

2.2.2 The experiment was done as described in 2.2.1 but the pH of Zarrouk medium was adjusted to 7.0,7.5,8.0 and 8.5.

The collection and isolation of Spirulina from Benjamaborpit pond were done by the same method as described for Spirulina from Makkasan pond.

3. Isolation of Spirulina to obtain unialgal culture

3.1 Purification of Spirulina from natural water sources.

Purification was done by a modification of Single Cell Isolation Technique (Hoshaw and Rosowski, 1973) with three Spirulina samples. Spirulina was initially grown in Zarrouk medium for 10-14 days.

Two or three drops of the growing culture were added to 6-8 drops of Zarrouk medium previously placed on a watch glass. The set up is shown in Figure 3 . The same dilution process was repeated on another watch glass. The next step was to pick out a single cell

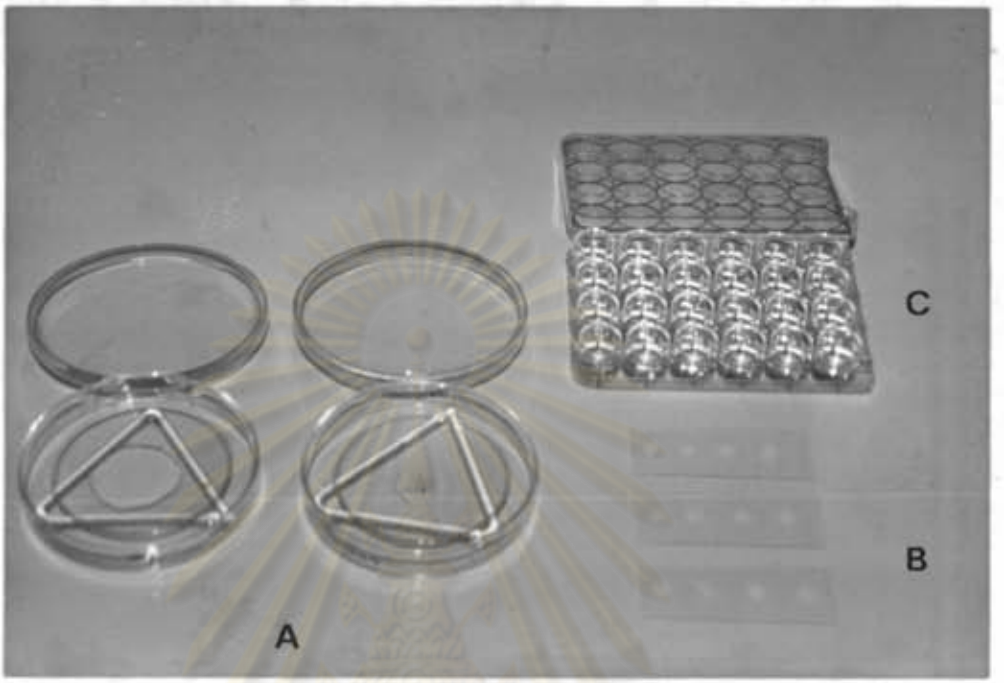

Figure 3 Isolation dishes

A. Isolation dishes arranged with a watch glass supported by a triangle glass rod.

B. Slides with four drops of sterile Zarrouk medium.

C. Culture plates for growing Spirulina

of Spirulina using a specially made fine capillary Pasteur pipette (Figure 4). This was done under a stereomicroscope (Figure 5). The single cell that was picked out was then subjected to a series of successive washing on a glass slide containing 4-6 drops of Zarrouk medium arranged as shown in Figure 3 B.

When the washing was finished, the single cell was transferred to a culture plate containing 1.5 ml of Zarrouk medium (Figure 3 C) and incubated at 30°C with light intensity of 3,000 lux (intermittent illumination at 14 hr light and 10 hr dark) for 2 weeks or until the culture turned green.

This single cell transfer was repeated until no contamination was detected. To scale up the culture, cells from each well of the culture plate were inoculated into a 50 ml flask containing 20 ml of Zarrouk medium and grown on a rotary shaker (160 rpm) at 27-30°C with continuous illumination of 1500 lux provided by white fluorescent lamps. Later the growing cells in a 50 ml flask were inoculated into a 500 ml flask containing 250 ml of Zarrouk medium.

4. Growth of Spirulina in Zarrouk medium

5 % inoculum of Spirulina was inoculated into a 500 ml flask containing 250 ml of Zarrouk medium and grown on a rotary shaker (160 rpm) at 27-30°C with

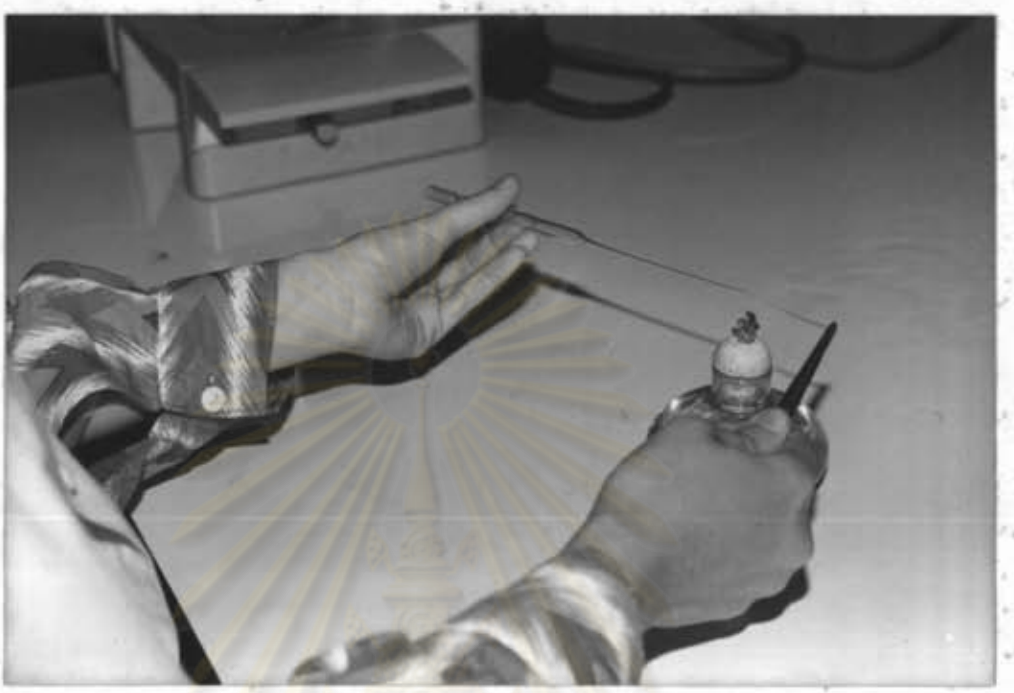

Figure 4 Preparation of a fine capillary pipette using a flame of an alcohol burner.

Figure 5 Isolation of a single cell by a capillary pipette under a stereomicroscope.

continuous illumination of 1500 lux. The organisms were harvested at the 12 th day of growth and used for subsequent experiments.

5. Effect of $NaHCO_3$ content

Spirulina was grown in Zarrouk medium containing 0 ,2.1 ,4.2 ,8.4 ,16.8 and 25.2 g/l $NaHCO_3$, the pH of the medium was adjusted to 8.2 . A 50 ml culture in 125 ml flask was shaken on a rotary shaker (160 rpm) at 27-30°C with 1,500 lux illumination arranged as 14 hr light and 10 hr dark cycle (figure 6). At 3 days intervals, the culture was collected for growth analysis and for phycocyanin determination, 2 ml of the day 12 culture was used.

6. Effect of $NaNO_3$

The experiment was performed as described in section 5 in which $NaHCO_3$ content in Zarrouk medium was kept at 8.4 g/l and the final contents of $NaNO_3$ were 0, 1.25, 2.5, 3.75 and 5 g/l.

7. Effect of K_2HPO_4

The experiment was performed as described in section 5 in which $NaHCO_3$ content in Zarrouk medium was kept at 8.4 g/l , $NaNO_3$ contents was kept at 2.5 g/l and the final contents of K_2HPO_4 were 0.093, 0.185, 0.370,

Figure 6 Cultivation of Spirulina in a 125 ml flask on a rotary shaker (160 rpm) at 27°C with continuous illumination of 1500 lux provided by fluorescent lamps.

0.56 and 0.74 g/l.

8. Effect of NaCl

The experiment was performed as described in section 5 in which $NaHCO_3$ content in the Zarrouk medium was 8.4 g/l, $NaNO_3$ content was 2.5 g/l and K_2HPO_4 content was 0.185 g/l. The final contents of NaCl were 1, 5, 10, 15 and 20 g/l.

9. Effect of light intensity

Spirulina was grown in 200 ml Zarrouk medium contained in a 500 ml flask. The intensities of light were 1,500 , 5,000 , 6,500 and 8,000 lux and the aeration was provided by an air pump.

10. Effect of light quality

Cells were grown in 750 ml of Zarrouk medium in a 1 l flask under normal white light at the photosynthetic photon flux density (PPFD) of 270 $\mu Es^{-1}m^{-2}$, under red light with the PPFD of 35 $\mu Es^{-1}m^{-2}$ and also under green light with the PPFD of 35 $\mu Es^{-1}m^{-2}$. The red or green light was provided by interposing the red or green plastic filter between the white light and the culture flask.

11. Effect of changing level of nitrate

Two regimes of changing level of nitrate experiments were performed as follows :

11.1 Adjusting level of $NaNO_3$ from low to that of Zarrouk medium

Cells were initially grown in Zarrouk medium containing low $NaNO_3$ content of 0.33 g/l in the same manner as described in section 4. After 7 days the cells were harvested and inoculated into Zarrouk medium containing 2.5 g/l $NaNO_3$. At various time intervals, aliquots were removed for the analysis of phycocyanin content ,OD 750 and dry weight. The control experiments were also done in which the cells were inoculated into Zarrouk medium containing 0.33 g/l $NaNO_3$.

11.2 Adjusting level of $NaNO_3$ from that of Zarrouk medium to high content

The experiment was as described in 11.1 except that the cells were initially grown in Zarrouk medium and after 7 days were inoculated into Zarrouk medium containing 10 g/l $NaNO_3$.

12. Partial purification of phycocyanin

12.1 Growth of Spirulina

Spirulina cells were grown in 3 l of Zarrouk medium in a 4 l flask as described in section 4 but the aeration was done by using an air pump and the illumination was provided by white light at 4,000 lux and with a cycle of 14 hr light and 10 hr dark (Figure 7). After 14 days, the cells were harvested by filtering through a 40 μ nylon sieve and frozen at -70°C until required for the purification of phycocyanin.

12.2 Ammonium sulfate precipitation of crude extract

About 10 g wet weight of Spirulina was frozen at -70°C and thawed at 37°C for 3 cycles to rupture the cell wall. The suspension obtained was centrifuged at 2,000 x g for 10 min and the blue supernatant was collected. The precipitate was washed with 0.02 M sodium phosphate buffer pH 7.5 until nearly all of phycocyanin was removed from the precipitate which could be observed by the appearance of an olive green color of the residue. The washings were combined with the previously collected blue supernatant. The crude phycocyanin was then subjected to 20-65 % ammonium

Figure 7 Cultivation of Spirulina in 4 l flask.The aeration was provided by an air pump whereas illumination at 4,000 lux was provided by white fluorescent lamps.

sulfate precipitation. The final pellet was suspended in 20 ml of 0.02 M sodium phosphate buffer pH 7.5 and dialyzed overnight in the same buffer.

12.3 DEAE-cellulose column chromatography

About 10 g of DEAE-cellulose was swelled in 1 liter of distilled water and then washed several times at room temperature to remove the fine particles. The resin was activated by washing sequentially with excess volume of 0.1 M HCl for 30 min followed by distilled water until the pH was 7. The resin was then treated with 0.1 M NaOH for 30 min followed by distilled water until the pH was 7. The activated resin was pre-equilibrated with 0.20 M sodium phosphate buffer pH 7.5 for 30 min and then equilibrated with 0.02 M sodium phosphate buffer overnight. The prepared DEAE-cellulose was packed into a glass column (2.5x 40 cm) at the height of 30 cm. It was equilibrated with 0.02 M sodium phosphate buffer pH 7.5 overnight at least for 300 ml at the flow rate of 25-30 ml/hr. Twenty ml of the protein solution containing about 150 mg was loaded and allowed to be absorbed. Elution was carried out by a continuous linear gradient of 0 to 0.5 M NaCl prepared in the buffer. A flow rate of 25-30 ml/hr was maintained and 4 ml fractions were collected. The concentration of NaCl in the fraction was estimated by using a conductivity

meter. The absorbances of the fractions were read at 280 nm and 620 nm.

12.4 Sephadex G-150 column chromatography

About 5 g hydrated Sephadex G-150 was swelled in 1 liter of 0.02 M sodium phosphate buffer pH 7.5 by boiling for 5 hr. The Sephadex G-150 suspension was poured into a 1.5x60 cm column at the height of 50 cm. It was equilibrated with 0.02 M sodium phosphate buffer pH 7.5 at least for 180 ml. Three ml of dialyzed phycocyanin extract (about 20-30 mg) was layered on the column. Elution was carried out by the same buffer and the flow rate was maintained at 12-15 ml/hr. Two ml fractions were collected and the absorbances of the fraction were read at 280 nm and 620 nm.

12.5 Hydroxylapatite column chromatography

10 g of Hydroxylapatite was swelled in 50 ml of distilled water. The resin was activated by washing sequentially with 500 ml of 0.1 M HCl for 30 min followed by distilled water until the pH was 7. The resin was then treated with 0.1 M NaOH for 30 min followed by distilled water until the pH was 7. The activated resin was equilibrated with 2.5 mM sodium phosphate buffer pH 7 overnight. The prepared Hydroxylapatite was packed into a plastic column, 1.4 x

11 cm at the height of 9 cm. It was equilibrated with 2.5 mM sodium phosphate buffer pH 7.5 at least for 30 ml at the flow rate of 30 ml/hr. About 2-4 ml of the protein solution containing about 7-18 mg was loaded and allowed to be absorbed. Elution was carried out by a stepwise gradient of 2.5, 10, 30, 50 and 80 mM of phosphate. A flow rate of 30 ml/hr was maintained and 0.5 ml fractions were collected. The absorbances of the fractions were read at 280 nm, 620 nm and 650 nm.

13. Determination of Protein

Protein content was determined by Lowry's method (Lowry and Rosenbrough, 1951). The sample was diluted to contain suitable concentration of protein. This diluted sample (0.5 ml) was mixed with 3 ml of Reagent A and 0.3 ml of Reagent B and left in the dark for at least 30 min. The absorbance was then read at 750 nm. The standard curve was constructed using bovine serum albumin as a protein sample.

The compositions of Reagent A and B were

Reagent A : 2 % Na_2CO_3 in 0.1 M NaOH

1 % Na_2-Tartrate

1 % CuSO4

These 3 solutions were mixed at 100:1:1 ratio

Reagent B : 1 M phenol reagent

14. Determination of phycocyanin

The cells were collected from aliquots of culture by centrifugation at 2,000 x g for 15 min. Cell pellets were then suspended in a small volume of 0.1 M sodium phosphate buffer pH 7. The suspension was frozen at -70°C and thawed at 37°C 3 times before centrifuging at 2,000 x g for 10 min. The supernatant was collected and the pellet was re-extracted with 0.1 M sodium phosphate buffer pH 7 before centrifuging at 2,000 x g for 10 min. The blue supernatant was combined with the previously collected supernatant and kept in the dark before measuring the absorbance at 620 nm. The content of phycocyanin was calculated using $E^{1\%}_{1cm} = 73$ (Boussiba and Richmond, 1979)

15. Determination of dry weight

For dry weight determination, a 10 ml culture (except for the experiment on the effect of light quality whereby 20 ml culture was used) was filtered through a Whatman No.42 paper by means of a suction pump. Prior to filtration the paper was dried in a microwave oven until the constant weight was obtained. The Spirulina residue on the paper was dried in a microwave oven until no further change in the weight occurred. The dry weight of the Spirulina was then determined by the difference between the weight of the paper before and after

filtration.

16. Polyacrylamide gel electrophoresis

Preparation of the slab gel

The slab gel electrophoresis was done according to Laemmli (Laemmli, 1970) either in the presence or absence of SDS.The gel was formed between 18 x 16 cm. glasses assembled with 1 mm thick spacer. In SDS-PAGE all slab gels were prepared using a linear gradient of 10-18 % (w/v) of acrylamide for separating gel and 3 % acrylamide for stacking gel. The concentration of acrylamide was prepared from a stock solution as shown below. In non-denaturing polyacrylamide gel electrophoresis (PAGE) 10 % acrylamide of separating gel were formed.

A. Stock solution

a) 29.2 % (w/v) of acrylamide and 0.8 % (w/v) of N',N'-methylene bisacrylamide (BIS)

b) 1.5 M Tris-HCl pH 8.8 (Separating buffer)

c) 0.5 M Tris-HCl pH 6.8 (Stacking buffer)

d) 10 % Ammonium persulphate

e) 0.025 M Tris, 0.192 M glycine (electrode buffer)

f) 20 % SDS

B. Working solution prepared from stock solution:

Running solution:

for 10 % gel : 6 ml of stock a, 4.5 ml of stock b , 0.07 ml of d, 7.5 ml of distilled water, 0.36 ml of stock f and 0.01 ml of TEMED

for 18 % gel : 10.5 ml of stock a, 4.5 ml of stock b,0.07 ml of d, 3.0 ml of distilled water,0.36 ml of stock f and 0.01 ml of TEMED

Stacking gel solution:

0.9 ml of stock a,1.5 ml of stock c,0.018 ml of d, 3.6 ml of distilled water, 0.03 ml of stock f and 0.006 ml of TEMED

C.Electrode buffer for normal gel : 0.025 M Tris, 0.192 M glycine and distilled water to 1 liter.

D.Electrode buffer for denaturing gel : 0.025 M Tris , 0.192 M glycine ,0.1 % SDS and distilled water to 1 liter.

E.Staining solution : Coomassie brilliant blue R 0.2 % , methanol 50 % and acetic acid 10 %

F.Destaining solution : Acetic acid 7 % and methanol 5%

Preparation of sample for electrophoresis

The phycocyanin was characterized by SDS-PAGE.The sample was dissolved in SDS-sample buffer containing adding the SDS sample buffer with final concentration was 0.0625 M Tris-HCl pH 6.8, 2 % SDS, 10 % glycerol, 5 % mercaptoethanol and 0.02 % bromphenol blue as the dye. Before loading the sample, the protein in the sample buffer was dissociated by boiling for 5 mins.

Running condition

The SDS-PAGE was carried out at 20°C and run from cathode to anode. A constant current of 30 mA was applied until the dye marker was 1 cm from the bottom of the gel (about 4-5 hours). In non-SDS PAGE the gel was run at 4°C.

Staining and destaining

After electrophoresis, the proteins in the slabs were fixed for 2 hr with freshly prepared staining solution at room temperature and destaining was performed by repeated changes of destaining solution until the clear band was observed.