CHAPTER II

MATERIALS AND METHODS

Materials

Equipments

1. Controlled Environment Incubator Shaker, Psycrotherm. New Brunswick Scientific Edison, N.J., U.S.A
2. Spectrophotometer Shimadzu UV-20 Shimadzu, Japan.
3. Oven Temperature 60°C Memmert, Germany.
4. pH Meter PHM83 Radiometer Copenhagen, Denmark.
5. Autoclave Model HA-3D Hirayama Manufacturing Corporation, Japan.
6. Laminar Flow Model BVT-124 International Scientific Supply Co.Ltd, Thailand.
7. Lux Meter Digicon LX-50.
8. Freeze Dryer Labconco Corporation, Kansas City, U.S.A.
9. Filter Whatman GF/C 4.7cm Whatman International Ltd. Maidstone, England.

High Performance Liquid Chromatography Equipments

at Marine Biotechnology Unit, Chulalongkorn University.

1. Waters Model 501 HPLC Pump Solvent Delivery System, Operator's Manual, Millipore Corporation.

2. Waters Model 486 Tunable Absorbance Detector,Operator's Manual, Millipore Corporation.
3. Baseline 810 Chromatography Workstation Start-up Guide.
4. Waters Temperature Control Module,Millipore Corporation.
5. Nova-Pak C18 HPLC Column, 4 micron Spherical, size 3.9x150 mm., Part No. 86344, Lot.No. T10532, Millipore Corporation.
6. μBondapak C18 Guard Column, Part No. 88070, Lot.No. T10462, Millipore Corporation.

Chemicals

Acetonitrile, Methanol, Dichloromethane and Milli Q H_2O were HPLC grade from J.T.Baker Inc., U.S.A.

Standard beta-carotene was Type IV from carrots No.C-0126 Sigma Chemical Company, U.S.A.

Silica gel G-60 for Thin-layer Chromatography Art.7731 Merck, Germany.

Specimen

The strain of Spirulina originally isolated from Makkasan pond by Miss Duangrat Inthorn was used for the present study. A clonal culture was obtained by isolating a single trichome using a modified method of single cell isolation technique (Hoshow and Rosowski, 1973). This clone

was used for all subsequent cultures. Trichomes were grown in batch cultures in Zarrouk medium (Zarrouk, 1966) (see Appendix 1) on rotary shaker with 160 rpm at 27-30 ^{o}C. Four 20-W fluorescent lamps placed overhead provided the flask with 1,500 lux of continuous, illumination.

Methods

1. Type of Solvents for Beta-carotene Extraction

Spirulina was grown in Zarrouk medium on rotary shaker with 160 rpm at 27-30^{o}C with continuous illumination of 1,500 lux for 7 days. Cultures were extracted by various types of solvent.

1.1 Extraction with Methanol

Ten millilitre of *Spirulina* culture was filtered through Whatman GF/C. Pigments were extracted from the alga with 10 ml of boiling methanol at 70^{o}C for 10 minutes. This procedure was repeated until no more pigments could be detected in methanol. The combined extracts were stored immediately in the dark at -20^{o}C before analysis for pigments by High Performance Liquid Chromatography (HPLC).

1.2 Extraction with 90% Acetone

Ten millilitre of Spirulina culture was filtered through Whatman GF/C. Ten millilitre of 90% acetone was added to the filter and sonicated for 10 minutes. Repeated extraction, storage and pigment analysis were performed as described in section 1.1.

1.3 Extraction with Acetone:Methanol (4:6)

Ten millilitre of Spirulina culture was filtered through Whatman GF/C. Ten ml of acetone:methanol (4:6) was added to the filter and sonicated for 10 minutes. Repeated extraction, storage and pigment analysis were performed as described in section 1.1.

1.4 Extraction with Dichloromethane

Ten millilitre of Spirulina culture was heated at about 100°C for 2 hours with 1.7 g of calcium hydroxide. After cooling to about 50°C,the mixture was suction filtered and washed with water. The filter was then completely dried and extracted with dichloromethane (or methylene chloride) in a soxhlett apparatus until the extract is almost colorless. The pigment extracts were analyzed by HPLC to determine beta-carotene content.

Quantitative Determination of Beta-carotene by HPLC

The analysis of pigments was run on a Water 486 HPLC system which consisted of two pumps and a UV/VIS detector set at 452 nm. Separation was carried out using a 3.9x150 mm. Novapak C18 column with a precolumn. The sample volume was always 20 μl. To separate chlorophylls, carotenes and xanthophylls the elution solvent contained 79.9% acetonitrile, 10% dichloromethane, 10% methanol and 0.1% milli Q water. The flow rate was 1.0 ml/min. Beta-carotene was identified by using an authentic beta-carotene.

2. Effect of Environmental Factors on Spirulina Cultivation for High Beta-carotene Production

2.1 Effect of Cultivation Temperature on Growth and Beta-carotene Content

Spirulina was grown in Zarrouk medium on a photosynthetic growth incubator (New Brunswick Psycrotherm). The temperature of the incubator was set at either 25, 30, 35 or 40^{o}C with a shaking speed at 200 rpm. The illumination at about 4,000 lux was provided by cool white fluorescent lamps. The culture was daily analyzed for growth up to 7 days by measuring the turbidity at 750 nm which was the the representative of algal dry weight. Correlation between

absorbance 750 nm and dry weight was shown in Appendix 3. The culture (10 ml) of day 7 was used for beta-carotene determination with the best solvent selected from the results in section 1.

2.2 Effect of Initial pH on Growth and Beta-carotene Content

Spirulina was grown in Zarrouk medium with different initial pH values of 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5, and 12. A 50 ml of culture in a 125 ml flask was shaken on a psycrotherm at 30°C with illumination at 4,000 lux and shaking speed at 200 rpm. The culture was daily analyzed for growth up to 7 days and at day 7, 10 ml of culture was collected for beta-carotene determination.

2.3 Effect of $NaNO_3$ on Growth and Beta-carotene Content

The experiment was performed as described in section 2.2 except the pH in Zarrouk medium was in the range between 8 and 9 and the final $NaNO_3$ content was either 0, 0.31, 0.62, 1.25, 2.50, 3.75 or 5.0 g/l.

2.4 Effect of K_2SO_4 on Growth and Beta-carotene Content

The experiment was done as described in section

2.2 except the pH in Zarrouk medium was in the range between 8 and 9 and the final K_2SO_4 content was either 0, 0.25, 0.5, 0.75, 1.0, 1.5 or 2.0 g/l.

2.5 Effect of K_2HPO_4 on Growth and Beta-carotene Content

The experiment was done as described in section 2.2 except the pH in Zarrouk medium was in the range between 8 and 9 and the final K_2HPO_4 content was either 0, 0.25, 0.5, 1.0, 2.5, 5.0 or 10.0 g/l.

2.6 Effect of $MgSO_4$ on Growth and Beta-carotene Content

The experiment was done as described in section 2.2 except the pH in Zarrouk medium was in the range between 8 and 9 and the final $MgSO_4$ content was either 0, 0.1, 0.2, 0.4 or 0.8 g/l.

2.7 Comparison of Growth and Beta-carotene Content between Cells Grown in Optimized and Zarrouk Medium

Spirulina was grown in optimized medium containing 1.25 g/l $NaNO_3$, 0.25 g/l K_2HPO_4, 0.1 g/l $MgSO_4$ and without K_2SO_4. Other components were the same as those of Zarrouk medium. A 200 ml of culture in a 500 ml flask was shaken

on conditions as described in section 2.2. Cells grown in Zarrouk medium were also performed in the same manner. At 24 hours intervals, the culture was collected for growth determination and beta-carotene determined at day 1, 3, 5, 7 and 9.

2.8 Effect of NaCl on Growth and Beta-carotene Content

2.8.1 Changing of NaCl Content from 1 to 20 and 40 g/l

Spirulina was initially grown in Zarrouk medium containing NaCl content of 1 g/l in the same conditions as described in section 2.2. After 7 days, cultures were inoculated into Zarrouk medium containing 1 (as a control), 20 and 40 g/l of NaCl and continuously cultivated and collected for growth and beta-carotene determination after 7 days.

2.8.2 Changing of NaCl Content from 20 to 30 and 40 g/l

The experiment was done as described in section 2.8.1 but the initial content of NaCl was 20 g/l and was transferred up to 30 and 40 g/l of NaCl

2.9 Effect of Adding NaCl on Growth and Beta-carotene Content after 4-day Cultivation

Spirulina was grown in Zarrouk medium in conditions as described in section 2.2. Cultures of day 4 were added up with NaCl to final concentrations of 1, 10, 20, 30 and 40 g/l, respectively. Consequently, growth of cultures was daily monitored spectrophotometrically at 750 nm and beta-carotene content determined at day 7.

2.10 Effect of NaCl on Growth and Beta-carotene Content after Acclimation to NaCl at Various Concentrations

Spirulina was grown in Zarrouk medium containing various NaCl concentrations at 1, 10, 20, 30 and 40 g/l for 1 week. After acclimation to NaCl for 1 week, the cultures were used as an initial stock on Zarrouk medium containing equal NaCl concentration and cultivation was done as described in section 2.2.

2.11 Effect of Light Intensity on Growth and Beta-carotene Content

Spirulina was grown in a 1 l flask containing 500 ml of Zarrouk medium. The culture was aerated by an air pump

with a flow rate of 1.5 l/min at 30-35°C for 15 days and the light intensities were 1,500, 3,000, 6,000, 10,000 and 14,000 lux. At 3 days intervals, culture was collected for growth analysis and 10 ml culture of the day 12 was used for beta-carotene determination.

2.12 Effect of Light Quality on Growth and Beta-carotene Content

Spirulina was grown in 500 ml of Zarrouk medium in a 1 litre flask under white, red and blue light at the photosynthetic photon flux density of 70 μmol photon $m^{-2}s^{-1}$ with aeration rate of 1.5 l/min at 30-35°C for 7 days. The red and blue light were provided by red and blue fluorescent lamps, respectively.

3. Effect of Inhibitors on Growth and Beta-carotene Content

3.1 Effect of Norflurazon on Growth and Beta-carotene Content

Spirulina was cultivated in Zarrouk medium containing 0, 0.1, 0.2, 0.5, 1.0 and 2.0 μM of norflurazon in conditions as described in section 2.2 for 4 days before determining growth and beta-carotene content.

3.2 Effect of Diphenylamine on Growth and Beta-carotene Content

Spirulina cultivation was done as described in section 3.1 and the Zarrouk medium was added with 0, 0.1, 1, 10 and 100 μM of diphenylamine.

3.3 Effect of 2,4-Dinitrophenol on Growth and Beta-carotene Content

Spirulina cultivation was done as described in section 3.1 and the Zarrouk medium was added with 0, 0.1, 1, 10 and 100 μM of 2,4-dinitrophenol.

4. The Pilot Scale Production of Spirulina

Spirulina cultivating ponds which were of cylindricral shape with approximately 130 cm diameter and 59 cm deep made from polyvinylchloride were located outdoor at Marine Biotechnology Research Unit, Department of Marine Science, Chulalongkorn University.

Chemical substances such as sodium bicarbonate, sodium nitrate and sodium chloride used in the culture medium were commercial grades. Spirulina was cultivated in a 500 l Zarrouk medium with different NaCl contents of 1 and 30 g/l. Stock culture used in this experiment was

obtained by growing cells in a 4 l flask containing 2 l of medium with different NaCl contents at 1 and 20 g/l for 1 week as shown in Figure 4 and then transferring into 10 l of medium with NaCl contents at 1 and 30 g/l, respectively for 1 week (Figure 5). Spirulina was then transferred to culture in a 500 l Zarrouk medium with different NaCl contents of 1 and 30 g/l for 2 weeks (Figure 6). Air was circulated by pumping external air throughout the medium. Environmental parameters such as temperature,light intensity and pH of the outdoor culture were monitored. Growth rate and beta-carotene content were daily determined for 14 days.

5. Effect of Method of Drying on Beta-carotene loss

Spirulina acquired from the outdoor cultivation was dried by various kinds of drying i.e. sun drying, oven drying and freeze drying. For sun drying, Spirulina was filtered through 200 μm of nylon sieve and washed 2 times with tap water. Afterwards, cells were spread on aluminum tray and placed in outdoor for 24 hours and powdery ground in a mortar. For oven drying, Spirulina was spread on aluminum tray and placed in an oven at 60^{o}C for 24 hours instead of drying under the sun. In case of freeze drying, pressure and temperature of the system was 5 microns Hg and -50^{o}C, respectively.

Figure 4 Cultivation of Spirulina in 4 l flask containing 2 l of medium. The aeration was provided by an air pump whereas illumination at 4,000 lux was provided by four white fluorescent lamps

Figure 5 Cultivation of Spirulina in 15 l jar containing 10 l of medium. The aeration was provided by an air pump whereas illumination at 4,000 lux was provided by four white fluorescent lamps

Figure 6 The outdoor cultivation of Spirulina on pilot scale with 500 l of medium

6. Partial Purification of Beta-carotene

Ten mg of Spirulina acquired from freeze drying was added with 50 ml absolute ethanol, 10 ml 50% KOH and 10 ml petroleum ether and incubated in water bath at 40°C for 10 minutes. The solvent was then filtered and extracted with petroleum ether in separating funnel until the extract was almost colorless. Carotenoid was evaporated under gas N_2 and then dissolved in 2 ml hexane. Carotenoid solution was then loaded on a column containing Silica G-60. Beta-carotene was eluted with 5% ether in hexane. Other xanthophylls were eluted with acetone:chloroform (1:1). Beta-carotene and xanthophylls were evaporated under N_2 and then dissolved in hexane and quantitatively determined by HPLC.

7. Effect of Storage Temperature on Beta-carotene Loss

Freeze-dried Spirulina was stored in the dark with different temperature conditions such as 30°C, 4°C, -20°C and -70°C. In addition, they were added with sodium metabisulphite at levels of 0.1 and 1%. Beta-carotene in Spirulina was analyzed every 2 weeks over a period of 8 weeks.