CHAPTER III

MATERIALS AND METHODS

3.1 Isolation of *Chlorella* spp. and *Scenedesmus* spp.

Water samples from Samut Sakhon province and Lard Prao area in Bangkok were centrifuged at 3,000 rpm for 10 mins at room temperature. Cell pellets were streaked on Bold Basal agar Medium (BBM). Composition of the BBM medium was as described in Appendix A. Isolates purified by streak plate method were kept in BBM agar slants at 4 °C. Cells grown in BBM medium under approximately 3,000 lux continuous light intensity for 3, 6, 9 and 12 days were taken for photography under a microscope.

3.2 PCR fingerprinting

3.2.1 Extraction of DNA

Chlorella spp. and *Scenedesmus* spp. cells kept on agar slants were reactivated by plating onto BBM agar. Contamination check was performed by inoculating reactivated cells into NB medium and inoculated at 37 °C for 1 week. If the NB medium was turbid, the algal culture(s) was found to be contaminated. In this case, the fingerprints obtained were discarded and pure algal cultures be obtained again. Mid-log phase cells grown in BBM liquid medium under continuous illumination of approximately 3,000 lux were used for DNA extraction by phenol: chloroform: isoamylalcohol method. Sterilized spatula was used to harvest cells from agar plate to an eppendorf tube. Cells were broken by vortexing with 2/3 volumes sterilized glass beads (Sigma), 400 μl TE buffer, 10% SDS for 2 mins three times followed by centrifugation at 12,000 rpm, 4 °C for 10 mins. DNA was precipitated from the aqueous layer with ice-cold absolute ethanol, washed with 70% ethanol, air-dried, and dissolved in 20 μl sterilized distilled water. Quantity and quality of extracted DNA were checked by optical density readings at 260 nm and 280 nm and 1.25% agarose gel electrophoresis according to standard methods (Sambrook and Russel, 2001).

3.2.2 PCR fingerprinting

Fingerprints of all *Chlorella* spp. and *Scenedesmus* spp. isolates were obtained by RAPD-PCR method with either CRL-7 or 27f or 1492r as the primers. Nucleotide sequences of the primers as well as PCR mixture and PCR program were as follows:

Nucleotide sequences of primers (Mathis and McMillin, 1996; Dorsch & Stackebrandt, 1992)

CRL-7	5' GCCCGCCGCC 3'
27f (9-27)*	5' GAGTTTGATCCTGGCTCAG3'
1492r (1492-1512)*	5' ACGGCTACCTTGTTACGACTT3'

* Position of nucleotides on 16S rDNA of *E.coli*

Mixture			Program		
10x PCR buffer with 20mM $MgCl_2$	2.0	µl	94 °C	4 minutes	
dNTP mixture (2.5 mM each)	2.0	µl	94 °C	30 seconds	30 cycles
5 -10 µM primer			45°C	60 seconds	
DNA template (60-100 ng)	1.0	µl	72 °C	120 seconds	
i *Taq* ™ DNA polymerase (5U.µl^{-1})	0.2	µl	72 °C	10 minutes	
High quality double distilled water up to	20.0	µl			
Total	20.0	µl			

PCR products were separated by 1.25 % agarose gel electrophoresis, stained in 0.5 µg.ml^{-1} Ethidium bromides by standard method (Sambrook and Russell, 2001) and viewed and photographed on a UV transilluminator (Bio-rad).

3.3 Scanning Electron Microscope

Scenedesmus spp. SS4, SS5 and SS9 were grown on agar plates containing BBM medium, incubated at 25 ^{0}C for 12 days. The plates were sent to the Scientific and Technological Research Equipment Center, Chulalongkorn University, for scanning electron microscope determinations of surface structure. Cells in a colony were suspended in 0.1 M phosphate buffer solution (PBS), pH 7.2 and filltrated on to 0.45 µm cellulose membrane filter. The specimens were immersed in 2.5% Glutaraldehyde in 0.1

M phosphate buffer solution (PBS), pH 7.2 for 1 hour, rinsed with 2 changes of 0.1 M phosphate buffer solution (PBS), pH 7.2 (5 min duration for each rinse). and 1 change of H_2O, dehydrated with 5 min immersion in a series of ethanol 30 %, 50%, 70%, 90%, and 100% (three). The samples were dried on the critical point dryer (Balzers, CPD 020), mounted onto the metal stubs, coated with gold 15 mA, 3 min in an ion sputtering (Balzers, SCD 040). The samples were observed under a scanning electron microscope (JEOL, JSM-5410LV) at 15 kV.

3.4 Extraction and Determination of β-carotene contents (Vonshak, 1996).

Mid-log phase cells (20 days) grown in 1,500 ml BBM medium in a 2 l flask at 25 $^{\circ}C$, 150 rpm, and 3000 lux continuous light intensity were harvested by centrifugation at 8,000 rpm, 10 mins, and 4 $^{\circ}C$. Cells were lyophilized and stored in a desiccator until use. β-carotene was extracted from 50 mg lyophilized cells which were broken in a polyallomer centrifuge tube containing 10 ml 95% Ethanol and 1 ml 60% KOH with shaking in a 50 $^{\circ}C$ water bath at 230 rpm for 10 mins. Cell debris was removed by centrifugation at 8,000 rpm at 4 $^{\circ}C$. Extraction of pigments was carried out as above for one more time. Combined pigment solutions were extracted with 20 ml diethyl ether and 20 ml 9% NaCl in a separating funnel which was shaken on a funnel reciprocal shaker (HSIANGTA) at setting speed 6.5 for 3 min. Solution was left standing for phase separation for 10 mins. The lower chlorophyll layer was discarded. Twenty ml 9% NaCl was added to the separating funnel to extract carotenoids. Chlorophyll layer was discarded as previously described. Water in the carotenoid layer was removed by adding 2 spatula-full of Na_2SO_4 which was removed by filtration with Whatman No. 1 filter paper. Diethyl ether was added to adjust the carotenoid layer to 25 ml in a 25 ml volumetric flask. Three ml of the carotenoid layer were used to measure optical density at 450 nm. The solvent in the remaining 22 ml was evaporated at 34 $^{\circ}C$. 500 μl mobile phase (Acetonitrile: Dichloromethane: Methanol = 70: 20: 10 by volumn) was added to the residues, filtered through a syringe 13 mm filter with 0.45 μm nylon membrane (TITAN Filtration system) before injection into Waters HPLC equipped with Nova-Pak ® C_{18} column (Milford, MA, USA) id: 4 μm. ; 3.9 mm x 150 mm, flow rate of mobile phase

1.5 ml.min^{-1}. Detection with uv/vis detector at 450 nm. Data were analyzed with Breeze software. β-carotene standard (Merck) in the range of 200, 400, 600 and 800 μg/ml in mobile phase was extracted in a separating funnel as previously described to construct a standard curve of standard β-carotene after extraction in order to determine the extent of loss due to the extraction process. External standard curve of β-carotene was also constructed with direct injection of filtered β-carotene standard (Merck). Each β-carotene concentration determination is repeated at least three times.

3.5 Determination of Quercetin contents (Hertog et al., 1992)

3.5.1 Hydrolysis of dried algal cells

Mid-log phase cells (20 days) grown in 1,500 ml BBM medium in a 2 l flask at 25 °C, 150 rpm, and 3000 lux continuous light intensity were harvested by centrifugation at 8,000 rpm, 10 mins, and 4 °C. Cells were lyophilized and stored in a desiccator until use. Hydrolysis of dried algal cells was carried out by reflux under N_2 and cooling at 15 °C. 200 mg lyophilized algae cells in 2.5 ml 0.2 % Tert butyl hydroquinone (Merck: 8.41424) in Methanol, 1.9 ml distilled water and 0.6 ml 10 M HCl at 90 °C for 1 hour with occasional mixing. The hydrolyzate was cooled down before adding 0.1 ml 0.1% Ascorbic acid (Sigma: A5960) in Milli Q-water. The volumn was adjusted to 5 ml with absolute methanol and sonicated for 2 minutes before injection into reversed-phase HPLC.

3.5.2 Determination of Quercetin

Mid-log phase cells (20 days) grown in 1,500 ml BBM medium in a 2 l flask at 25 °C, 150 rpm, and 3000 lux continuous light intensity were harvested by centrifugation at 8,000 rpm, 10 mins, and 4 °C. Cells were lyophilized and stored in a desiccator until use. Quercetin was extracted from dried algal cells by sonicator 200 mg lyophilized algae cells in 5.0 ml 0.2 % Tert butyl hydroquinone (Merck; 8.41424) in methanol at room temperature for 30 minutes and adding 0.1 ml 0.1% Ascorbic acid (Sigma; A5960)

in Milli Q-water. The volumn was adjusted to 5 ml with absolute methanol and sonicated for 2 minutes.

Mobile phase (0.025 M KH_2PO_4 pH 2.4: Milli Q-water: Acetonitrile: = 1: 9: 3.6). Samples were filtered through a syringe 13 mm filter with 0.45 μm nylon membrane (TITAN Filtration system) before injection into Waters HPLC equipped with Nova- Pak ® C_{18} column (Milford, MA, USA) id: 4 μm. ; 3.9 mm x 150 mm, Flow rate of mobile phase was 1.0 $ml.min^{-1}$. Detection with uv/vis detector at 370 nm. Quercetin-3- β-D-glycoside (Fluka: 17793) in the range of 0.5, 1.0, 1.5, 4.0 and 20.0 μg/ml was prepared in sample matrix solution (Appendix B). External standard curve of Quercetin was also constructed with direct injection of filtered Quercetin standard (Sigma: Q-0125).