CHAPTER II

MATERIALS AND METHODS

The strain selection and culture of D. salina for β-carotene production were carried out in 4 steps, i.e. [1] field survey and water sampling for D. salina clone selection, [2] selection the clones yielding high β-carotene, [3] optimizing the culture condition for β-carotene production in laboratory, and [4] outdoor mass culture. The laboratory experiments were conducted at Marine Plankton Culture laboratory, Department of Marine Science, Chulalongkorn University, Bangkok. Outdoor mass cultivation was conducted at Ang-sila Marine Science Station, Chulalongkorn University, located on Ang-sila District, Chon Buri Province.

Field survey and isolation of Dunaliella spp. from hyper-saline water collected from salt ponds.

Salt ponds in four Provinces, Chon Buri, Chachoengsao, Samut Songkhram and Chanthaburi were chosen for sample collection (Figure 4). Hyper-saline water samples were collected for cell isolation and for nutrient, salinity and pH determination. Samples for nutrient analysis were collected in 1 liter plastic bottle and frozen in ice box before taken back to the laboratory. The nutrients, i.e. nitrate and phosphate, were analyzed by the method described by Strickland and Parsons (1977). The salinity

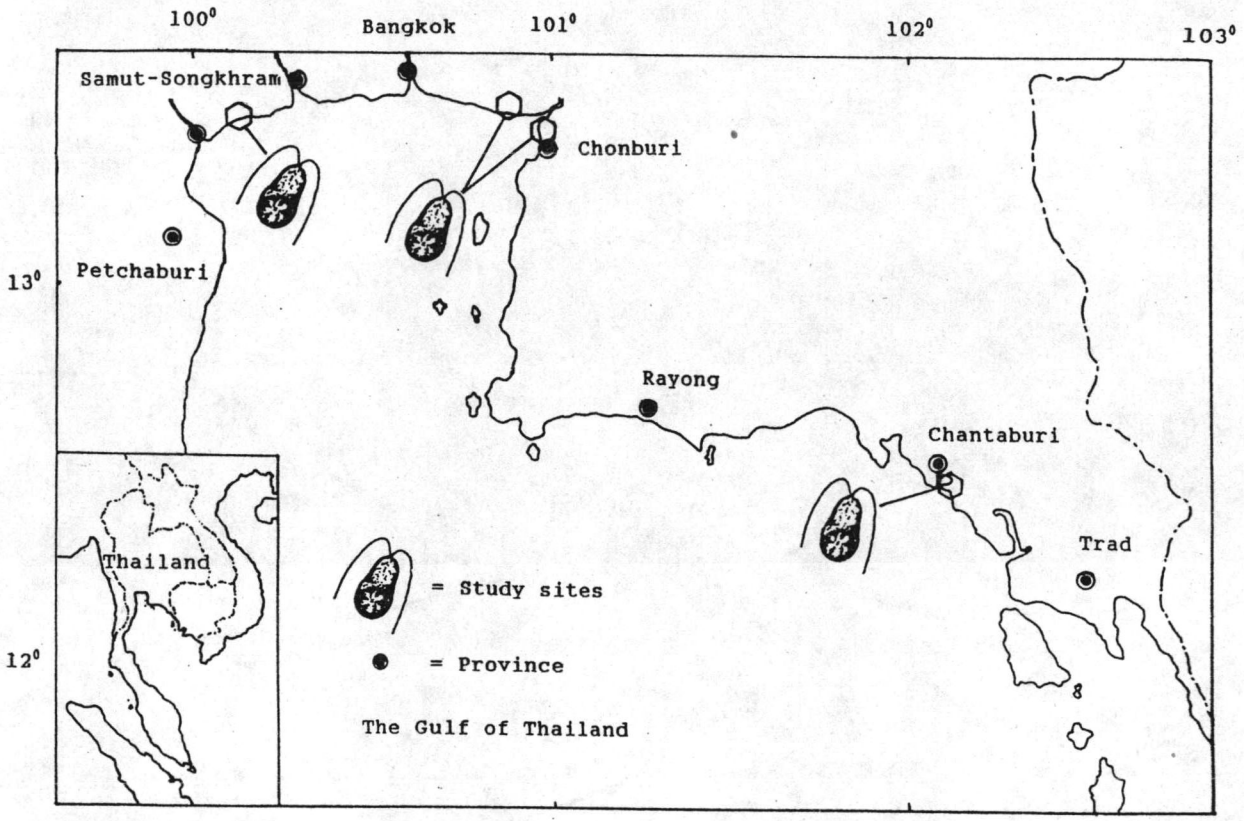


Figure 4. Map of the study sites.

and pH were examined by a hand refractometer and portable pH meter, respectively. Cell density of Dunaliella spp. in each sample was determined by haemacytometer.

In order to obtain monoclonal culture, single cell isolation were conducted under an inverted light microscope. Each Dunaliella cell was isolated with a fine sterile Pasture-type pipette (Hoshaw and Rosowski, 1975) and washed 5 times in 5 drops of sterile ESM culture medium (Appendix 1). This medium was prepared from the water sample. Thereafter, the isolated cell was transferred into a sterile tissue culture plate (Nunc, 6x4 wells type) with 1.5 ml ESM medium and incubated at 30°C with light intensity of 3,000 lux. In addition, the effect of salinity on survival rate of the isolated cell was studied. Different salinities, 255, 180, 105 and 30 ppt of ESM medium (prepared by adding lower salinity ESM medium) were used as the culture medium for the single cell isolation process.

The isolated strains were transferred from tissue culture plate into 50 ml culture tube that contained 30 ml of the J/1 culture medium (Appendix 2). The taxonomic study was examined as described by Lerche (1937), Butcher (1959) and Loeblich (1982).

Selection of D. salina clones yielding high carotenoid content.

Clonal cultures of D. salina isolated from salt pond were transferred to J/1 medium at three different salinities, 10%, 20% and 30% NaCl. Each clone was inoculated in 125 ml flat bottom flasks containing 90 ml of culture medium and were slowly shaken with a mechanical shaker at 80 rpm (Figure 5). The light intensity was 20,000 lux.

Cells number of each strain were counted by a haemacytometer every two days for growth determination. Growth rate and division time were calculated by the following equations (Schoen, 1988):

$$\mu = \frac{\ln X_2 - \ln X_1}{t_2 - t_1}$$

Where μ is the growth rate, X_2 and X_1 are the number of cells at time 2 (t_2) and time 1 (t_1) in days, respectively.

Division time, t_g, is the time required for cell division (in days) and can be calcutated from the growth rate;

$$t_g = \frac{0.6931}{\mu}$$

Cells were collected at the end of logarithmic growth phase for the total carotenoid determination. The culture at known volume was separated and then filtered through GF/C glass fibre filter. The filters were freezed in refrigerator at -20°C until the analysis of total carotenoid and chlorophyll.

Figure 5. Rotary shaker illuminated with 8 white fluorescent lamps, at 80 rpm shaking speed.

Total carotenoid was determined by 90% acetone extraction and detected by spectrophotometer at 452 nm (Borowitzka, 1991). For chlorophyll determination, 90% acetone was also used for extraction but detected at wavelength 664 and 647 nm. The detail of method was shown in Appendix 3 and 4.

The clone that yielded the highest carotenoid content was selected for further study.

Effects of light intensities, nutrients and pH on growth and carotenoid content of the selected clone.

a) Effect of light intensity

The selected D. salina clone was cultured in three different light intensities at 5,000, 10,000 and 15,000 lux (70, 136 and 203 $\mu E/m^2/s$ respectively). Cultures were carried out in 125 ml flat bottom flask with 90 ml of J/1 medium. All cultures were maintained in a rotary shaker with 80 rpm shaking speed. The salinity of J/1 medium in this experiment was 20% NaCl. Growth rate of the culture was determined by cell counting. At mid-expenential growth phase and stationary growth phase, algal cells were collected for the carotenoid analysis.

In order to determine the β-carotene content, the samples were extracted with 90% acetone and were later analyzed by High Performance Liquid Chromatography (HPLC). The HPLC system is consisted of Waters Associates model 501 HPLC pump with

BASELINE computerized programmable controller/data system and Waters model 486 UV-VIS tunable absorbance detector. The Nova-pak C-18 3.9x150 mm HPLC column with Guard-pak μ-Bondapak C-18 guard column were used for a reverse-phase chromatography. HPLC analysis method is shown in Appendix 5. The separation of cis and trans isomer of β-carotene was analyzed by HPLC with different method described by Ben-Amotz et al. (1988). The method is shown in Appendix 6.

b) Effect of nitrate

Five KNO_3 concentrations, i.e. 1%, 10%, 50%, 100%, and 200% of J/1 medium (0.01, 0.1, 0.5, 1.0 and 2.0 g/l respectively) were used as culture medium for D. salina. The culture were conducted in a shaker with white fluorescent lamps which provided 20,000 lux light intensity. Salinity of the culture medium was 20% NaCl. Algal cells were counted at every two day for the growth rate determination. Samples for total carotenoid and chlorophyll analysis were collected at the middle of exponential growth phase and at the end of exponential growth phase.

c) Effect of phosphate

Five concentration of KH_2PO_4: 1%, 10%, 50%, 100% and 200% of J/1 medium (0.00035, 0.0035, 0.0175, 0.035, 0.07 g/l respectively) were used as the culture medium. The culture condition was performed in the same maner as described in 3.2.

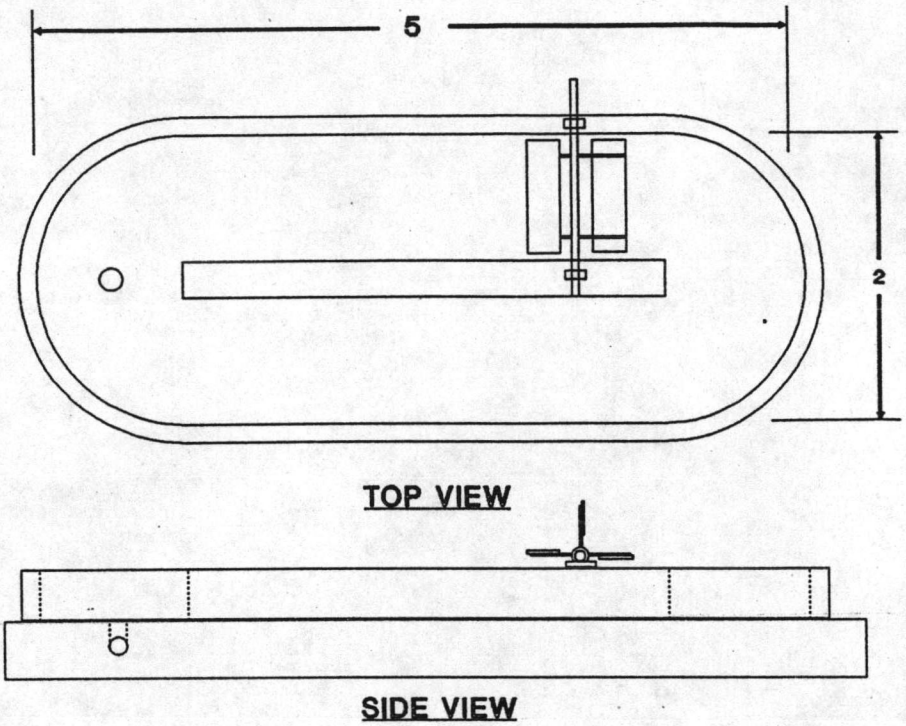
d) Effect of initial pH

The experiment was performed in the same maner as described in experiment 3.2. Tris-buffer were added into J/1 medium to controlled a constant pH value. The pH in J/1 medium was adjusted to seven pH values, i.e. 6.36, 6.47, 6.91, 7.36, 7.86, 8.31 and 8.70 (measured after autoclaving). Growth rate were determined by cell counting every two days. Total carotenoid and chlorophyll were determined at mid-exponential and at the late exponential growth phase.

In addition, correlation between cell number and ash free dry weight (AFDW) was determined. D. salina was cultured in the modified J/1 medium containing KNO_3 10% of full formulation medium at salinity 20% NaCl. Cells were collected for counting and AFDW determination (Appendix 7). The correlation result was examined by the the regression analysis.

Outdoor mass culture of D. salina.

The 9.1 m^2 outdoor raceway pond was constructed at Ang-Sila Marine Biological Station, Ang Sila District, Chon Buri Province. The pond design is shown in Figure 6. Four blade paddle wheel were driven by 2 horses power motor at 1:60 gear reducer. The final paddle wheel speed was 13 rpm and water circulation speed was 0.15 meter per second. White Epoxy rasin was painted inside the pond for the maximum light reflection. A transparent plastic sheet was used to protected pond from rain during the night.



Pond specification:

Pond area	9.1 m^2
Length	5 m
Width	2 m
Depth	30 cm
Culture volume	1,500 l
Culture depth	20 cm

Figure 6. Construction design of the outdoor raceway pond.

The culture medium, 1,500 liters in volume, was enriched with KNO_3 and KH_2PO_4, 0.1 and 0.035 g/l respectively. Fe-solution and trace element was added according to J/1 medium. Depth of the culture was maintained at 20 cm by adding the fresh water in order to control salinity. The pond was inoculated at a cell concentration of $1x10^4$ cell/ml. CO_2 was supplied to maintain the pH in the range of 7-8.5.

Environmental parameters such as temperature, light intensity, dissolved oxygen and pH of the outdoor culture were monitored. Cells number were counted by haemacytometer for the growth rate determination.

Biomass of D. salina was harvested by flocculation with aluminium sulphate. The flocced algae was centrifuged and dried. The β-carotene analysis of algal powder was determined by the HPLC (appendix 5).

The statistical analyses were carried out by using the statistic computer software. The analysis of covariance was computed by SPSS PC$^+$ studentware (SPSS Inc.) and other hypothesis test were computed by STATGRAPHICS V 5.0 (Statistical Graphics Corporation).