CHAPTER III

MATERIALS AND METHODS

The study consists of two parts. The first part deals with the production of astaxanthin from H. pluvialis NIES144. The second part deals with effect of astaxanthin from H. pluvialis NIES144 and the synthetic astaxanthin on growth, survival and salinity tolerance of P. monodon larvae.

Part 1 : Production of astaxanthin by H. pluvialis NIES144.

Cultivation of H. pluvialis NIES144

Algal strain : H. pluvialis NIES144 purchased from the National Institute for Environmental Studies (NIES), Japan, was used in this study.

Culture conditions : An axenic starter culture of H. pluvialis was prepared in Modified Bold's basal medium (Appendix1) pH 7.3. The culture was incubated at 25 ^{o}C under 14 hr light (1.5-3.0 klux) and 10 hr dark illumination with manually shaking twice a day (Fig1). In this condition the algae remained green and motile. When they reached the stationary phase, the light intensity was increased to be 10 klux continuously then algal cells began massive accumulation of astaxanthin. When the entire cells turned red, the algae was harvested.

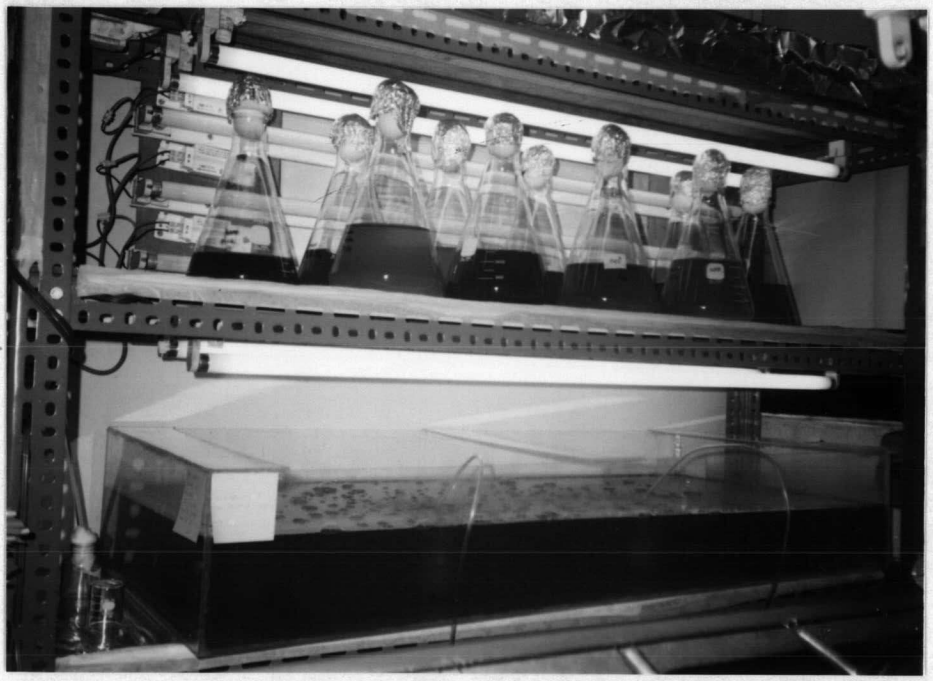

Figure 5 Culture of H. pluvialis NIES144 at 25 °C under 1,500 lux light intensity

Harvest and storage : To collect red cells of H. pluvialis, the culture was centrifuged at 3,500 rpm and the separated cells were then dried by freezed dryer. The dried cells were kept under nitrogen gas in a dark pack at 4 ^{o}C until use. Red cells were ground in liquid nitrogen and then used for feed preparation.

Growth and pigment analysis : The algal growth was determined by cell counting with a haemacytometer. Growth rate (μ) and doubling time (t_d ; the time for cell division in days) were calculated by the following equations :

$$\mu = (\ln x_2 - \ln x_1)/t_2 - t_1$$

$$t_d = 0.6931/\mu$$

where x_2 and x_1 are the number of cells at time t_2 and t_1 , respectively.

For carotenoids and chlorophyll determination, the culture at known volume was filtered through Millipore filter. The filtrates were extracted with 90% acetone. The carotenoid concentrations were determined at 480 nm (Extinction coefficient$_{1\%,\ 1cm}$ = 2,500). The total chlorophyll concentrations were determined at 665, 645 and 630 nm (Strickland & Parsons, 1977). Detail of the method was shown in Appendix 2. Dry weight and ash free dry weight of the algae were determined according to Powtongsook (1993) (Appendix 3). Astaxanthin was determined according to Davies (1976) and confirmed by HPLC method (Weber, 1990). Proximate analysis of dried algae was done according to AOAC (1980).

Effect of sodium chloride on cyst formation

Stimulation of cyst formation of H. pluvialis NIES144 was studied by adding NaCl at 6 concentrations: 0, 0.125, 0.5, 1, 3 and 5 g/l into 30 ml culture (cell concentration approximate 1.09 x 10^5 cell/ml). Algae was cultured in test tubes and separated into 2 groups. The first group was incubated at 25 ^{o}C under 14 hr light (1.5-3 klux) and 10 hr dark illumination. The other group was incubated under 10 klux continuously. The culture was shaken twice a day and number of cysts were observed.

Part 2 : Effect of astaxanthin on growth, survival and stress resistance of P. monodon larvae.

Experimental Design

The study was carried out at the Department of Marine Science and the Marine Biotechnology Research Unit, Chulalongkorn University. A complete randomized design was used for four diets with five replications. The diets were natural food (NF), control diet (astaxanthin-free diet: CD), algal astaxanthin-added diet (diet containing astaxanthin from H. pluvialis: AAD) and synthetic astaxanthin-added diet (diet containing synthetic astaxanthin: SAD). The amount of astaxanthin in the diet was controlled at 200 mg/kg diet (ppm). The feeding experiments were divided into three larval stages; zoea I-III, mysis I-III and postlarva 1-15.

Shrimp nauplii obtained from a commercial hatchery in Chonburi province were used in this experiment. The feeding tests were separated exactly into three stages of larvae. Test 1: from zoea I to zoea III, test 2: from mysis I to mysis III and test 3: from postlarva 1 to postlarva 15. Numbers of larvae were counted from the begining of each stage. The larvae were assigned to each aquarium at a density of 100 larvae/l (or 300 larvae/aquarium) for zoea, 80 larvae/l (or 240 larvae/aquarium) for mysis and 30 larvae/l (or 100 larvae/aquarium) for postlarva. The aquarium contained water (3.5 l) made from plastic bottle, sealed the top, holed at the bottom and put upside down when the tests were done (Fig. 6).

The larvae were fed *ad libitum* with five feeding periods; 8.00, 11.00, 14.00, 17.00 and 20.00 hr for all experimental units. The first feeding regime for all treatment, Chaetoceros sp., was fed to the larvae until the larvae reached zoea II, then the designed diets were delivered to fed the shrimp. For natural diet, Chaetoceros sp. was introduced for zoea I to mysis II and live Artemia sp. was obtained for mysis II to postlarva 15. For purified diet, the diet size $\leq$ 106 microns was for zoea and mysis and size 106-250 microns for postlarvae.

The rearing condition was in a static system with partial water change (about 2/3 of total volume) everyday. Filtered and chlorinated seawater was used. Salinity was controlled at 30 ppt continuously with gentle airation.

Water temperature during experiment was 27-30 ^{o}C. The rearing period was carried out about one month.

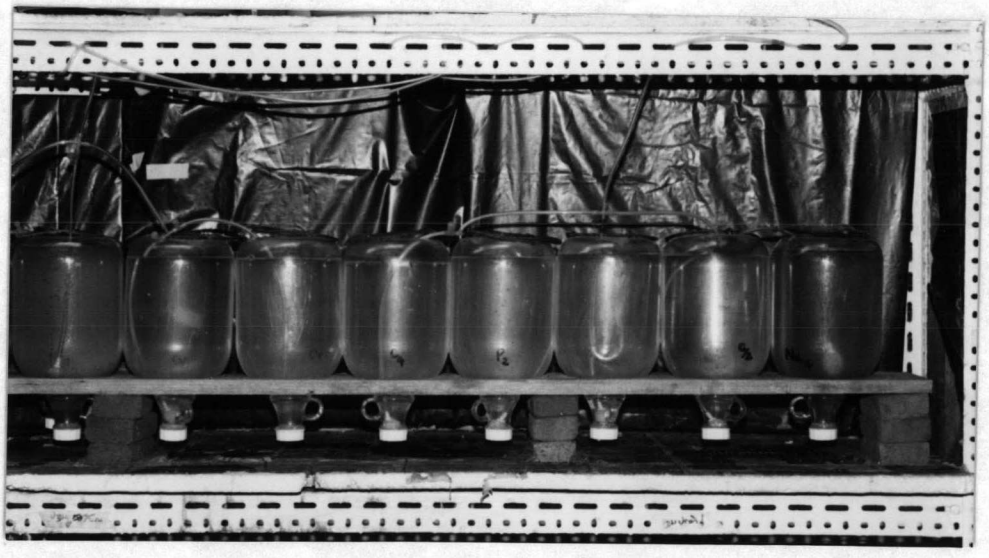

Figure 6 Aquaria for rearing P. monodon larvae

Experimental diet

The diet was a purified microparticulated diet which was modified from Teshima & Kanazawa (1984). Composition of diet is shown in Table 3. Astaxanthin concentration is at 200 mg/kg. Astaxanthin sources were Carophyll Pink (Synthetic, 8% astaxanthin coated with gelatine, carbohydrates and antioxidants, Roche Ltd., Switzerland) and algal astaxanthin (astaxanthin produced by H. pluvialis NIES144, calculated at 1.2% astaxanthin with 38% protein). The control diet had no supplemental pigment. The amount of wheat flour was altered to compensate for astaxanthin elimination and excess protein from algae. The natural diet was Chaetoceros sp. and live Artemia sp. depending on stages of larvae.

Table 3 Composition of the experimental diets.

Ingredients	% Use	Content (g/ feed 100g)		
		Control diet	Cyst diet	Synthetic diet
Casein	56.0	56.0	54.7	56.0
Wheat flour	15.0	15.0	15.0	15.0
Fish oil (refined tuna)	6.0	6.0	6.0	6.0
Na- citrate	0.3	0.3	0.3	0.3
Na-succinate	0.3	0.3	0.3	0.3
Glucosamine-HCl	0.8	0.8	0.8	0.8
Mineral mix	9.4	9.4	9.4	9.4
Vitamin mix *	3.9	3.9	3.9	3.9
Cholesterol 90 % *	1.0	1.0	1.0	1.0
Lecithin	2.0	2.0	2.0	2.0
Rice bran	6.1	6.1	6.1	6.1
Carrageenan	5.0	5.0	5.0	5.0
Synthetic astaxanthin *	200ppm	-	-	0.52
Cyst of H. pluvialis	200ppm	-	3.50	-
Wheat flour (compensated)		0.89	-	0.37

* Provided by Rovithai Ltd., Thailand.

Vitamin mix was shrimp Rovimix (Rovithai, Ltd.) composed of vitamin A,D, E, K, B_2, B_6, B_{12}, niacin, biotin, calcium, folic acid, choline, $FeSO_4$, $CuSO_4$, $MnSO_4$, KI, ZnO, $CoSO_4$, Selenium, $MgCo_3$, SiO_2, antioxidant, rice bran and color.

Mineral mix was composed of K_2HPO_4 2.00 g, $CaSO_4$ 3.58 g, $MgSO_4 .7H_2O$ 3.04 g and $Na_2HPO_4 .2H_20$ 0.79 g in feed 100 g.

Processing of larval diets

The diets were prepared by blending all dry ingredients as a proportion in Table 3 into particle size $\geq$ 20 micron in a beaker, adding fish oil and mixing for 15 min. The mixture was added water 50 ml and heated at 70°C for 20 min. The mixture was cooled to 40°C, then added the mineral mix and vitamin mix which were previously dissolved in 20 ml distilled water. Poured the mixture into the tray, left at room temperature until cool. The diet was cut into small pieces (about 2 x 2 cm) and dried by freezed dryer. When the diets were dried completely, they were blended and sieved for separation into two parts; size $\leq$ 106 micron and 106-250 micron. The microparticulated diets were kept in a dark sealed container, flushed with nitrogen gas and stored at 4 °C until used. Proximate analysis of diets was done according to AOAC (1980).

Salinity stress testing

Stress resistance was observed for postlarva 15. Ten larvae from each replication of diets were transfered from normal salinity 30 ppt to salinity 2 ppt. Number of dead shrimp was recorded every 10 min. The total period for low salt tolerance was done for two hours.

Data collection

Survival of the larvae was recorded at the end of the stage. Survival rate was calculated as the final number in proportion to the initial number of the larvae. Growth was checked in term of length of postlarva 15. Ten larvae from each replication were randomly sampled for growth measurement. For salinity stress test, number of dead shrimp was recorded every 10 min within 2 hr.

About two-thirds of the total water volume was changed everyday. Filtered and steriled seawater was replaced. Salinity was determined by a reflectometer. Levels of ammonium, nitrate and pH of water were monitored by Merck's Test kits once at zoea stage and mysis stages and twice for post larval stage.

Pigment analysis

Astaxanthin content in cyst of H. pluvialis, in synthetic astaxanthin, in diets and in shrimps tissues was determined. The analysis was done by spectrophotometric technique and by HPLC technique as the following method.

Astaxanthin determination by spectrophotometer (Boussiba &Vonshak, 1991; Davies, 1976)

For H. pluvialis, algal sample (5 ml) was centrifuged at 3,500 rpm for 5 min. The collected cells were first treated with a solution of 5% KOH in 30% methanol (5 ml) to destroy chlorophyll. Algal cells were extracted at 70 ^{o}C in a water bath for 10-15 min. The mixture was centrifuged at 3,500 rpm for 5 min, and the supernatant was discarded and pellets were collected. The pellets were added glacial acetic acid (3-5 drops) and dimethylsulfoxide (DMSO) 5 ml, mixed well and leave for 10-15 min. The cells were ground with a manual glass homogeniser and the extract was left at 70 ^{o}C in a water bath for 5 min. The mixture was centrifuged at 3,500 rpm for 5 min. The last step was repeated, if it is necessary until the cell debris was totally white. The absorbance of the combined extracts was determined at 492 nm, and the amount of pigment was calculated using the Extinction coefficient ($E_{1\%}$) at 2,220. Calculation was done by the following equation;

Astaxanthin concentration (μg/ml or mg/l)

= (Optical density at 492 nm) x Factor*

* Factor for Haematococcus sp. = 4.5 (according to $E_{1\%}$= 2,200; see Appendix 12)

Astaxanthin determination by HPLC method (Weber, 1990)

Reagents : (all reagents are AR grade)

Chloroform

n- hexane, free of fluorescence

Diethyl ether, peroxide free

MAXATASE, P440000 encapsulated (International Biosynthetics, Rijswijk, Netherlands)

Siliga gel 60, particle size 0.2-0.5 mm for column chromatography

Ethanol, absolute

Acetone

Ortho-phosphoric acid 85%

Methanol

HPLC Condition :

Modification of the stationary phase : Solution of phosphoric acid in methanol (1g/100ml) is pumped through the packed pre-and main-column (LiChrosorb Si 60, 5 μm, Merck) for 1 hr at a flow rate of 1 ml/min. The

colume is then washed by the mobile phase with a flow rate of 1.2 ml/min for 1 hr at least.

Mobile phase	:	n-hexane:acetone (HPLC grade), 86:14
Flow rate	:	1.2 ml/min
Pressure	:	approx. 80 bar
Temperature	:	28 ^{o}C
Injection volume	:	20 µl
Detection	:	VIS-detection at 470 nm
Run time	:	15 min

Standard astaxanthin preparation (Weber, 1990)

In a 100 ml volumetric flask approximate 3 mg of standard (all-E) astaxanthin crystal was dissolved in 10 ml chloroform and made up to the volume with n-hexane. Five ml of this solution was transferred into a 100 ml volumetric flask, mixed with 4 ml chloroform and made up to the volume with n-hexane. After mixing well, the solution was measured by a spectrophotometer at the maximum of absorption (approx. 470 nm) and calculated according to the following formula ;

$$\text{Astaxanthin (mg/l)} = (\text{Absorption} \times 10{,}000)/\ 2{,}100$$

Method for astaxanthin determination (in algae and synthetic astaxanthin)

Approximate 100 mg of sample were prepared in 250 ml volumetric flask and added 100 mg MAXATASE and 10 ml distilled water. The flask was incubated for 30 min in a water bath at 50 °C, cooled to ambient temperature. The mixture was added 100 ml ethanol, shaked and made almost up to the volume with dichloromethane. The flask was left in the dark until temperature reached ambient. The volume was made up to with dichloromethane and mixed well. The extract (5 ml) was transferred into a 250 ml round-bottom flask, added 30 ml ethanol and evaporated at 50 °C with a rotary evaporator. The residue was dissolved with 10 ml n-hexane/acetone (86:14) and centrifuged at 4,000 rpm for 5 min. The sample can directly be used for HPLC determination.

Method for astaxanthin determination (in diets)

Approximate 10 g of diet, 100 mg of MAXATASE and 80 ml of distilled water were prepared in a weighed 100 ml volumetric flask. The flask was placed for 30 min in a water bath at 50° C, cooled to ambient temperature and then made up the volume with distilled water. The mixture was weighed again. Ten gram of the mixture and 100 ml of ethanol were added into a 250 ml volumetric flask. The mixture was shaked and made

almost up to the volume with dichloromethane. The flask was left in the dark until temperature reached ambient. The solution was made up to the volume with dichloromethane and mixed well. The extract (25 ml) was transferred onto the siliga gel and eluted with 120 ml n-hexane/ether (1:1). The elute was collected and removed the solvent with a rotary evaporator. The residue was dissolved in 5 or 10 ml n- hexane/acetone (86:14). The sample then can be used for HPLC determination.

Statistical analysis

All data were analysed by the Statistic Analysis System (SAS) Program (SAS,1985) and the SPSS-PC program for probit analysis. Means were compared with Duncan's new multiple-range test ($P<0.05$).