CHAPTER IV

RESULTS

Cultivation of H. pluvialis NIES144

Under 14 hr light (1.5-3.0 klux) and 10 hr dark cycle at 25 °C, H. pluvialis NIES144 showed as green, biflagellate swimmers with a characteristic cell wall. The wall appeared as a halo, separated from the plasma membrane connecting by a series of cytoplasmic strands (Fig 11). Size of vegetative cell was approximate 10 micrometers. The vegetative cells took about 10 days in exponential phase and 5 days in stationary phase before decreasing of cells numbers (Fig 7). The specific growth rate (μ) and doubling time were 0.26 and 2.67 days, respectively. Chlorophyll content, total carotenoids, dry weight and ash free dry weight of vegetative cells were increased by cells number as shown in Fig 8 and Fig 9. After leaving the culture up to 25 days in this condition, there was no morphological change from green cell to red aplanospore.

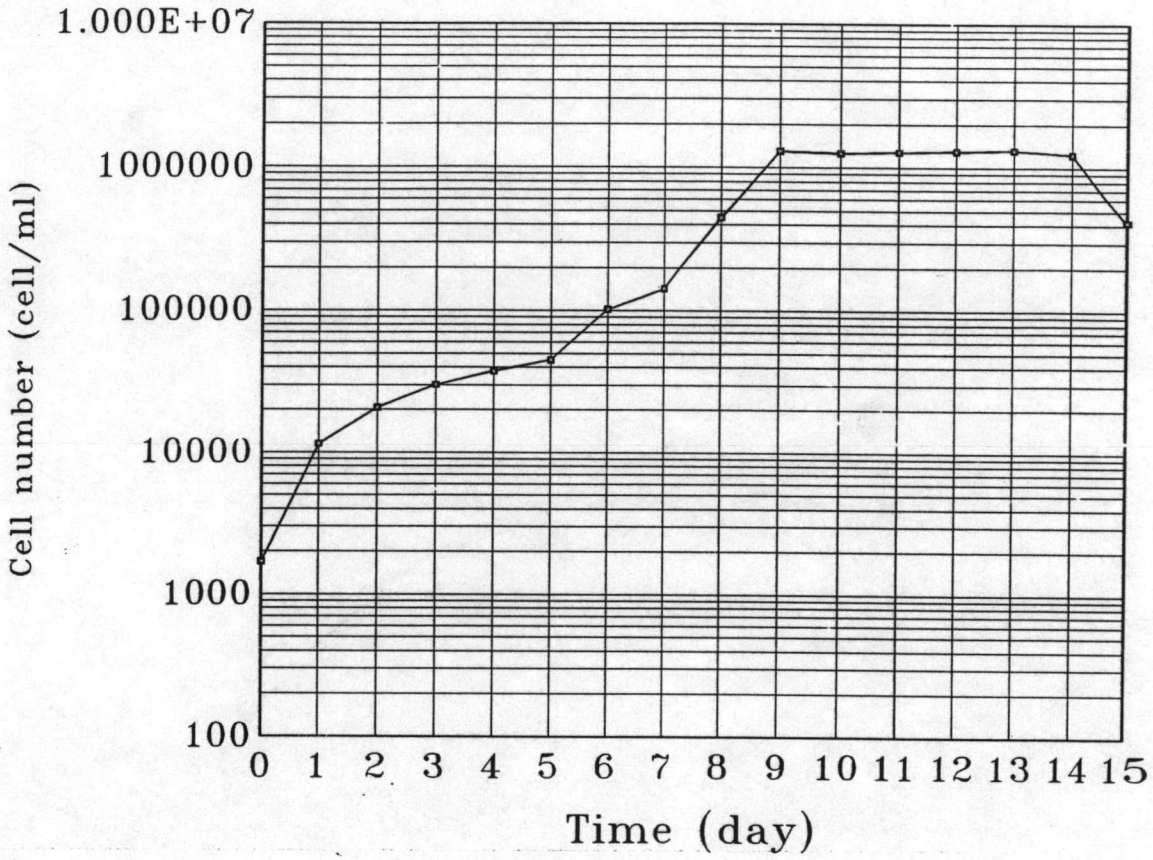

Figure 7 Growth curve of H. pluvialis NIES144 cultured under 14 hr light (1.5-3.0 klux) and 10 hr dark cycle at 25 °C.

Correlation between chlorophylls & total carotenoids content and culture period was observed (Fig 8). The regression analysis showed linear correlation. The regression equations for chlorophylls & total carotenoids calculation were as follow:

Chlorophyll a = 45.322 day - 22.373

Chlorophyll b = 31.672 day - 33.288

Chlorophyll c = 18.451 day - 17.573

Total carotenoids = 27.631 day - 26.579

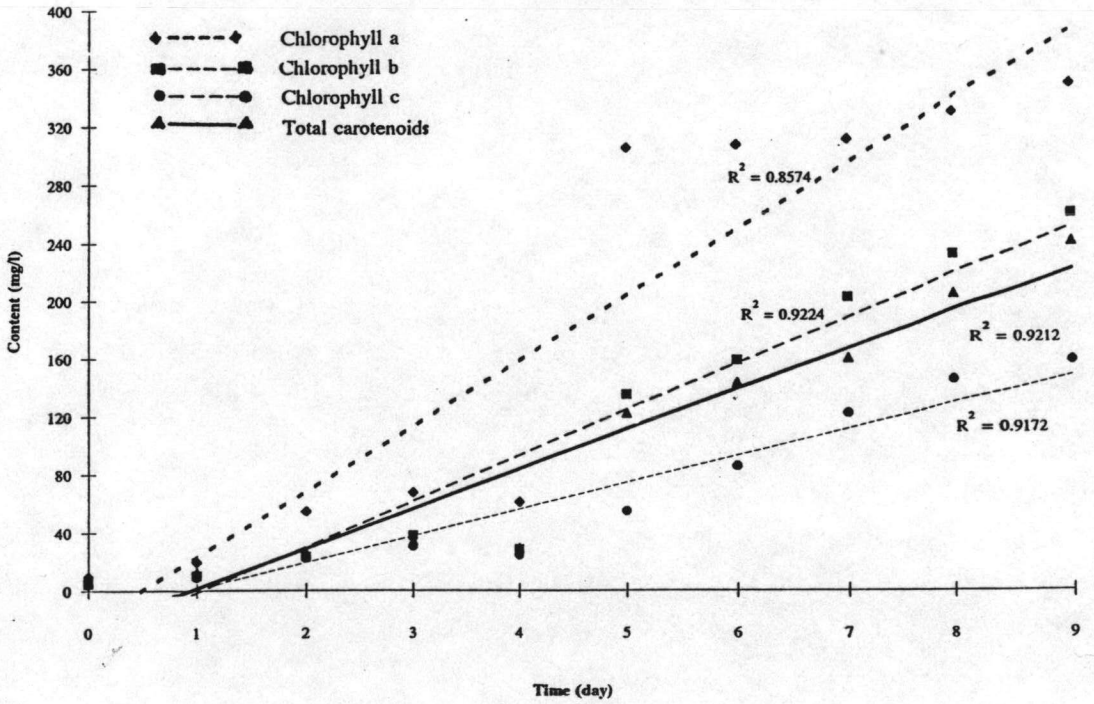

Figure 8 Chlorophyll and total carotenoids contents of H. pluvialis NIES144 at different cell concentrations.

Correlation between cell number and dry weight & AFDW was also determined (Fig 9). The regression analysis showed linear correlation. The regression equations for dry weight and AFDW were as follow:

Dry weight = 0.0005 cell number + 0.3127

AFDW = 0.0005 cell number + 0.2425

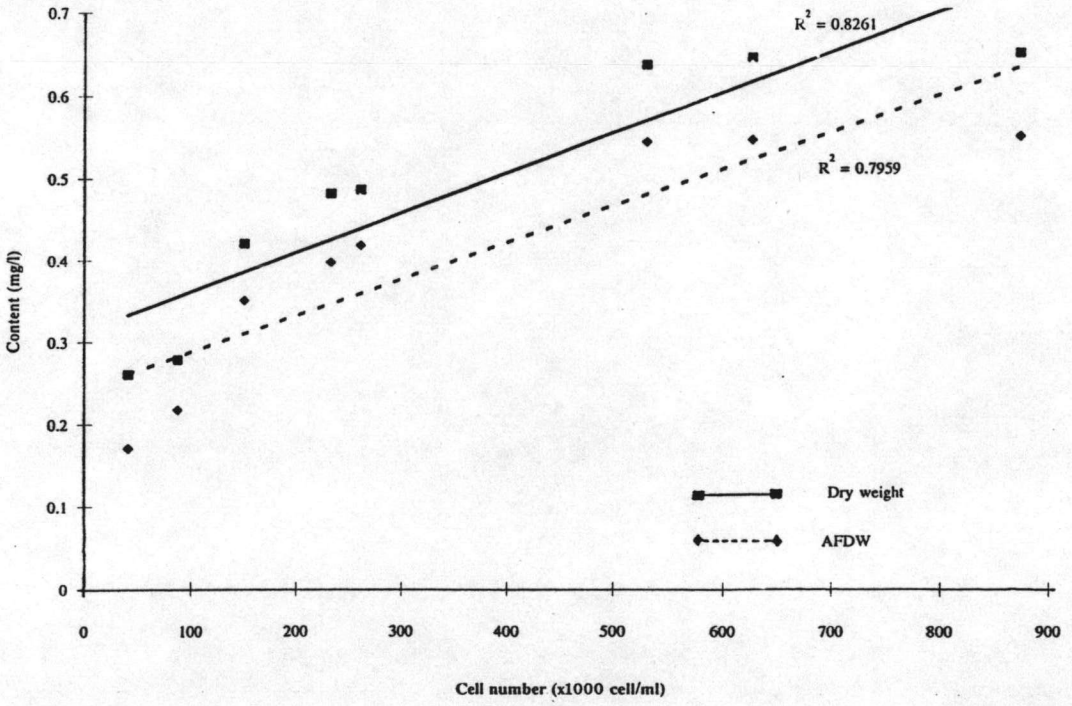

Figure 9 Dry weight and ash free dry weight of H. pluvialis NIES144 cultured at normal condition.

When a cell density has been reached in the range from about 10^5-10^6 cells/ml, encystment of the algal cells could be promoted by increasing of light intensity. The cells lost their flagella and formed a thick, persistent cyst wall. Simultaneously, cell began the massive accumulation of astaxanthin but decreased cell numbers as in Fig10. Astaxanthin deposition first occured around the nucleus and proceeded radially until the entire protoplast was red. Size of cyst (Fig11) was approximate 50 micrometers (about 4 times larger than vegetative cell). Accumulation of astaxanthin in H. pluvialis NIES144 is shown in Fig 11. Comparison of culture between vegetative stage and cyst stage was shown in Fig 12 .

When the entire cell of H. pluvialis NIES144 was red, its cyst was harvested. Because each cell did not accumulate astaxanthin at the same time, fully mature cysts were solely harvested by centrifugation during 10-12 days after stressed cells occurred. After freezed drying, a yield of the dried cyst was approx. 0.2 g/l. Proximate analysis of cyst is shown in Table 4.

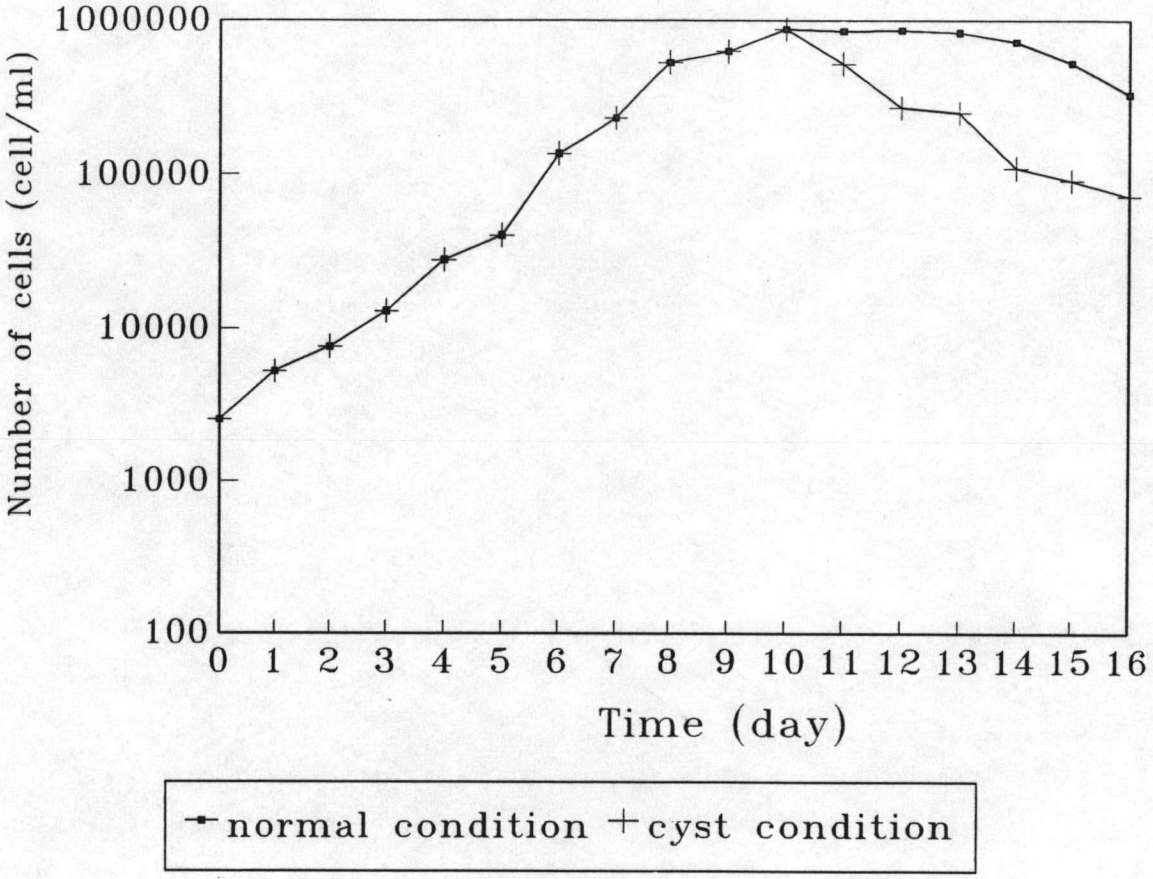

Figure 10 Comparison of growth curve of H. pluvialis NIES144 in normal condition and in stress condition (10 klux light intensity continuously).

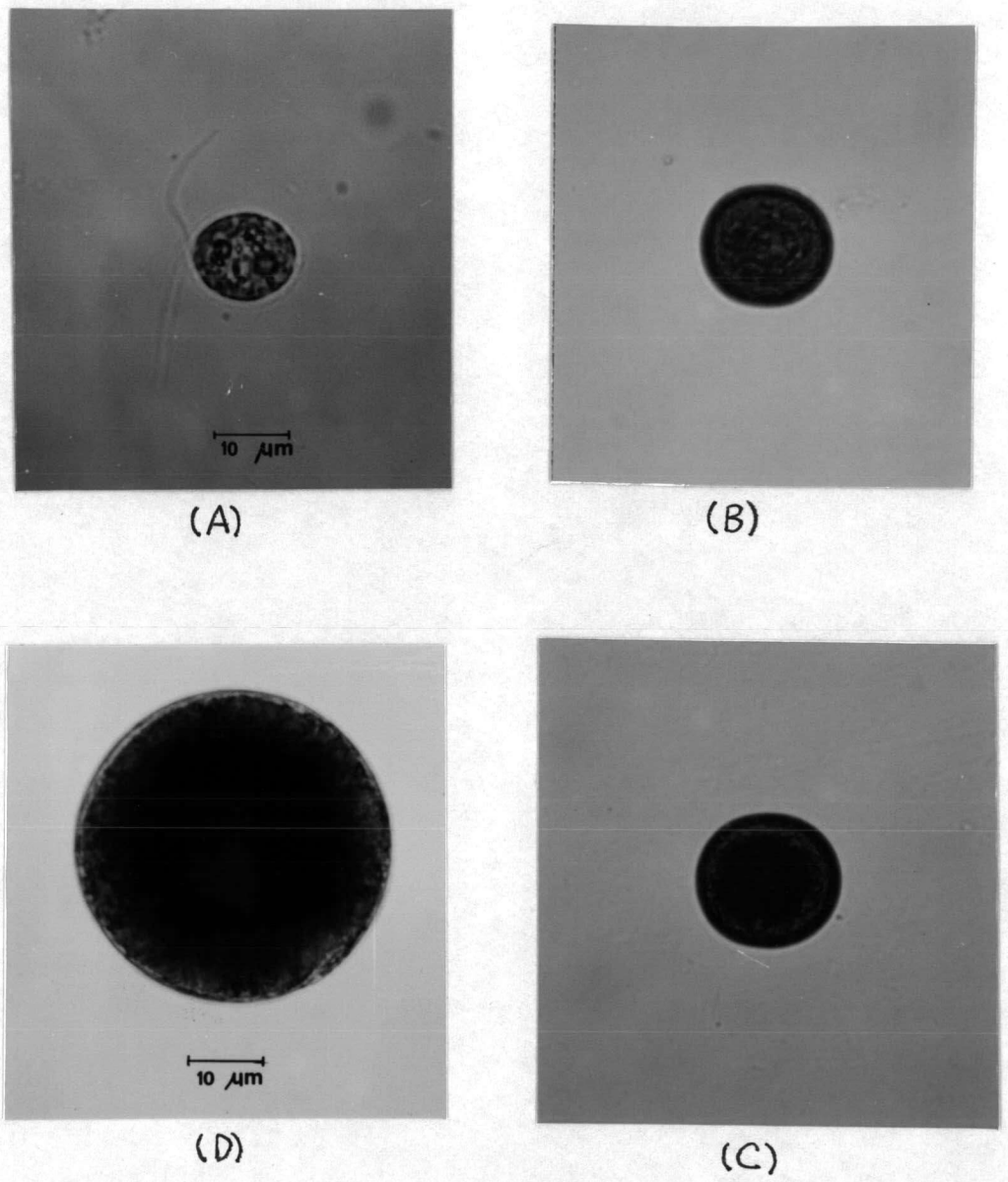

Figure 11 Change of H. pluvialis NIES144 from vegetative cell into cyst which showed the accumulation of astaxanthin (A) vegetative cell (B),(C) a change from vegetative cell into cyst (D) mature cyst.

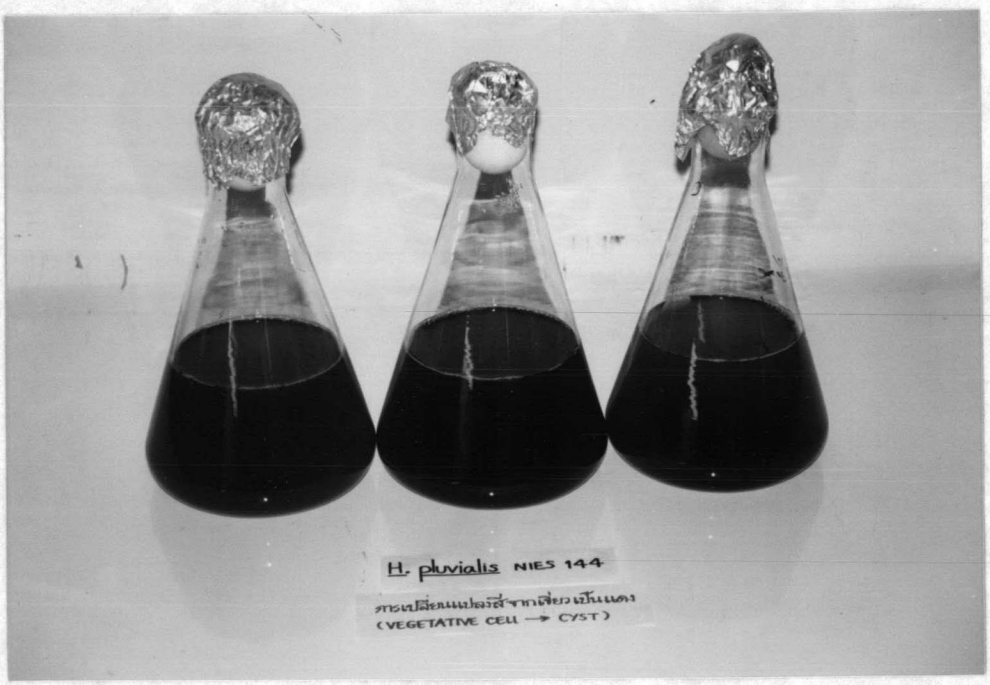

Figure 12 Comparison of culture in vegetative stage (left) and cyst stage (right) of H. pluvialis NIES144.

Table 4 Proximate analysis of cyst of H. pluvialis NIES144.

Content	MEAN ± SD (%)
Crude protein	39.14 ± 0.82
Crude fat	0.99 ± 0.42
Fibre	7.01 ± 0.25
Ash	3.25 ± 0.13
Moisture	7.45 ± 0.42

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 and compared cysts number in culture which incubated under normal and high light intensity. The results showed that after 2 days of experiment, the group incubated at high light intensity started accumulation of astaxanthin and formed red aplanospore while another group did not and had only red spot in the center and number of cells decreased after 15 days. Number of cysts incubated at high light intensity in all concentrations of 0, 0.125, 0.5, 1 and 3 g/l was similar (Table 5) except at 5 g/l the cyst number was much lower than others. On the other hand, number of cells in culture containing various concentrations of NaCl incubated at normal light intensity was

different. Cell number in culture added NaCl at concentration of 0 and 0.125 g/l was higher than that in concentrations of 0.5, 1, 3 and 5 g/l. It seemed to show that NaCl affected growth of H. pluvialis NIES144. It indicated that high light intensity affected cyst formation more than NaCl.

Table 5 Effect of NaCl concentration on cyst formation after 15 days of cultivation under 1.5-3 klux and 10 klux light intensity.

Concentration of NaCl (g/l)	No. of cysts under 10 klux light intensity (cell/ml)	No. of cysts under 1.5-3 klux light intensity (cell/ml)
0	4.71×10^4	6.2×10^5
0.125	3.77×10^4	5.8×10^5
0.50	3.33×10^4	8.8×10^4
1.0	3.24×10^4	5.8×10^4
3.0	3.28×10^4	3.6×10^4
5.0	3.81×10^3	1.0×10^4

Astaxanthin content in cyst

Astaxanthin concentration in cyst was analysed by HPLC and spectrophotometry method. The results are shown in Table 6. Chromatograms of astaxanthin analysis in cyst and in synthetic astaxanthin showed in Appendix 11. In Table 6, astaxanthin concentration determined by 2 methods was different. The spectrophotometric analysis, the data showed all forms of astaxanthin while the HPLC method the results showed only astaxanthin free-form. Pigment composition in H. pluvialis NIES144 composed of all form of astaxanthin; monoester, diester and free form and other xanthophyll such as echinenone, zeaxanthin, lutein, neoxanthin etc. (Spencer, 1989), while synthetic astaxanthin composed of only free astaxanthin in beadlets.

Table 6 Astaxanthin concentration in cyst and in synthetic one.

	Astaxanthin concentration (%)	
Analysis method	Cyst of H. pluvialis	Synthetic astaxanthin
Spectrophotometry	1.44 ± 0.35	6.95 ± 0.42
HPLC	0.07 ± 0.05	7.06 ± 1.46

Astaxanthin in diets

Four diets; algal astaxanthin-added diet (AAD), synthetic astaxanthin-added diet (SAD), control diet (CD), and natural food (NF) were prepared for shrimp larva. The color of particulated diets varied from yellowish to red orange depending on the source of astaxanthin (Fig 13). The appearence of diet dissolved in water are shown in Fig 14-16. Nutritional values of the diets and astaxanthin content in feeds are shown in Tables 7 and 8 .

Figure 13 Microparticulated diets for shrimp larva; control diet (CD), algal astaxanthin-added diet (AAD) and synthetic astaxanthin-added diet (SAD)

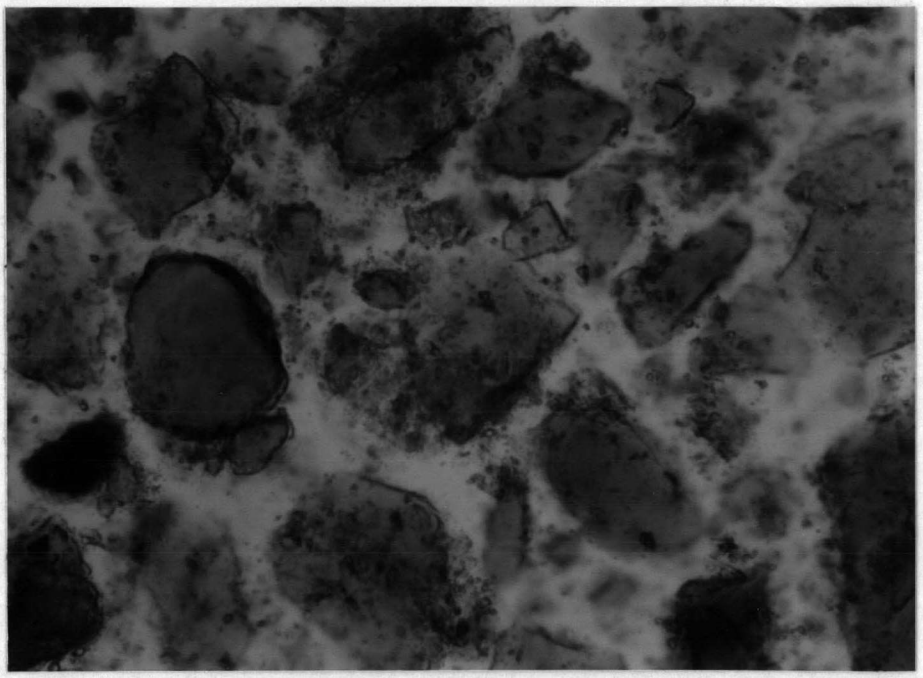

Figure 14 Characteristics of control diet dissolved in water (x 400x)

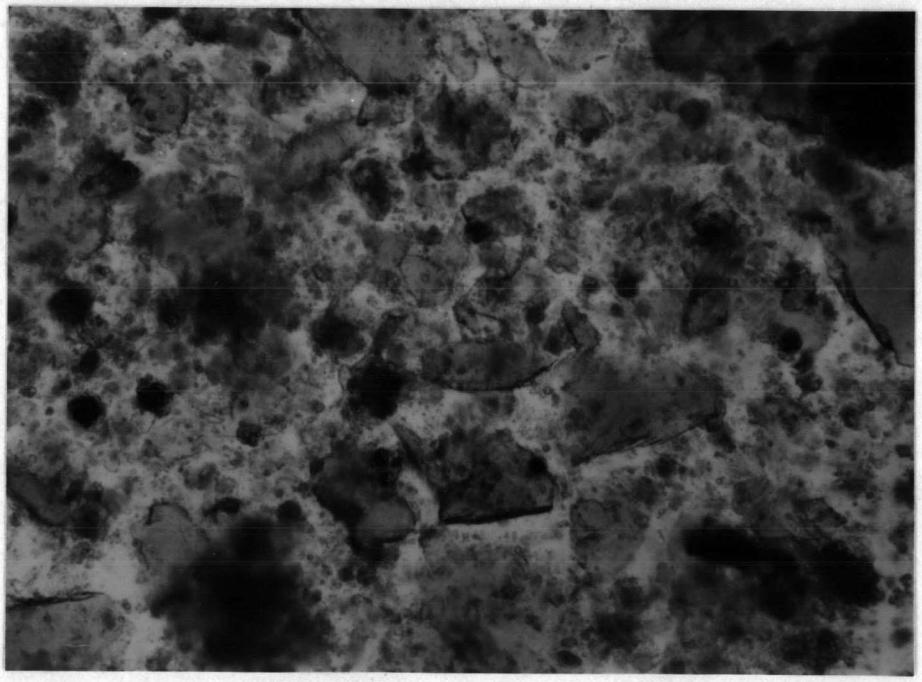

Figure 15 Characteristics of algal astaxanthin-added diet dissolved in water (x 400x)

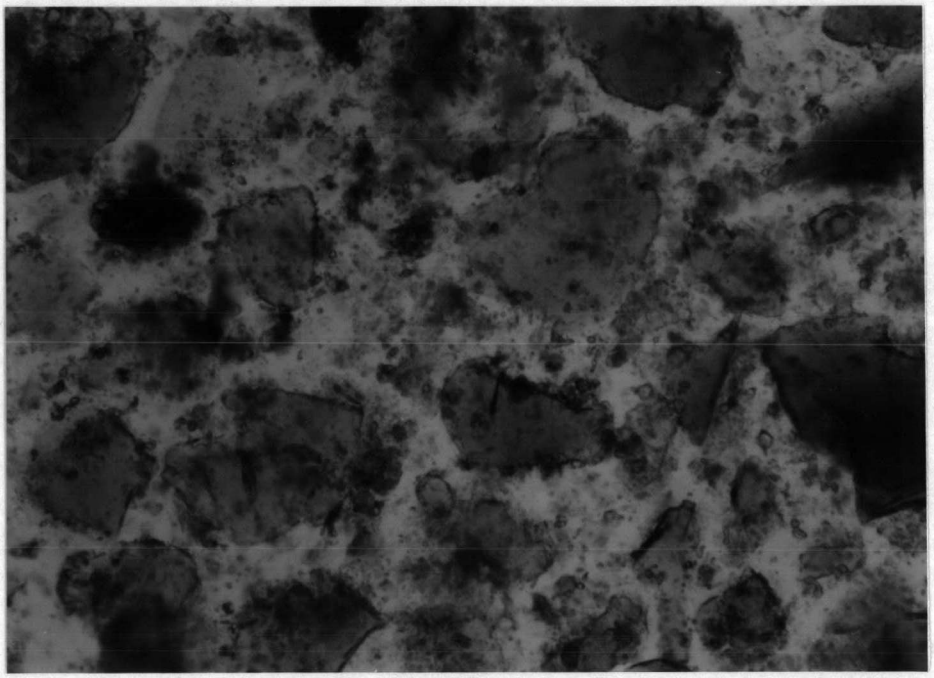

Figure 16 Characteristics of synthetic astaxanthin-added diet dissolved in water (x 400x)

Table 7 Proximate analysis of the microparticulated diets.

Content (%)	CD	AAD	SAD
Crude protein	46.76 ± 0.90	46.75 ± 0.54	47.49 ± 0.35
Crude fat	8.21 ± 0.12	8.36 ± 0.02	8.38 ± 0.31
Fibre	1.24 ± 0.06	1.61 ± 0.88	1.34 ± 0.01
Ash	7.25 ± 0.20	7.84 ± 0.10	7.42 ± 0.03
Moisture	5.93 ± 0.14	1.27 ± 0.06	5.83 ± 0.11

Table 8 Astaxanthin concentration in the diets.

	Astaxanthin concentration (ppm) in diets		
Analysis method	CD	AAD	SAD
Spectrophotometry	0	188.90 ± 4.84	208.60 ± 4.26
HPLC	0	7.02 ± 2.52*	189.05 ± 5.40

* HPLC method showed only 7.02 ± 2.52 ppm of astaxanthin, because this technique detected only a free form of astaxanthin. Naturally, the alag H. pluvialis contains very low content of a free form of astaxanthin.

Effect of astaxanthin on survival and growth of Penaeus monodon larvae

Survival rate of larvae

The results of survival rate of shrimp larva (3 stages): zoea, mysis and post larva 1-15 fed different diets are shown in Table 9 and Fig 17. Survival rate of zoea fed NF, CD, and AAD was significantly higher than the zoea fed SAD ($P<0.05$). In mysis stage, survival rate of larva fed AAD and NF was similar and significantly higher than the larvae fed CD and SAD. The best survival rate for postlarva was the larvae fed AAD, which was significantly higher than the postlarvae fed NF. However, there was no significant different survival rate of the larvae fed AAD, SAD and CD. The analysis of variance data is shown in Appendix 9.

Table 9 Percentage survival of larval stages fed different diets.

	Survival of larval stage (%)		
Diets	Protozoea	Mysis	Post larva
NF*	82.0 ± 3.30 [a]	76.7 ± 8.61 [a]	55.2 ± 6.14 [b]
CD	74.0 ± 5.19 [a]	57.3 ± 5.01 [b]	68.6 ± 5.73 [ab]
AAD	82.5 ± 3.53 [a]	69.7 ± 12.05 [a]	76.0 ± 9.46 [a]
SAD	27.8 ± 4.01 [b]	58.1 ± 0.29 [b]	64.4 ± 11.86 [ab]

Means in the same column with different superscripts are significantly different at $P<0.05$.

* NF (Natural food) for zoea1-mysis2 was Chaetoceros sp. and for mysisII-postlarva15 was Artemia sp.

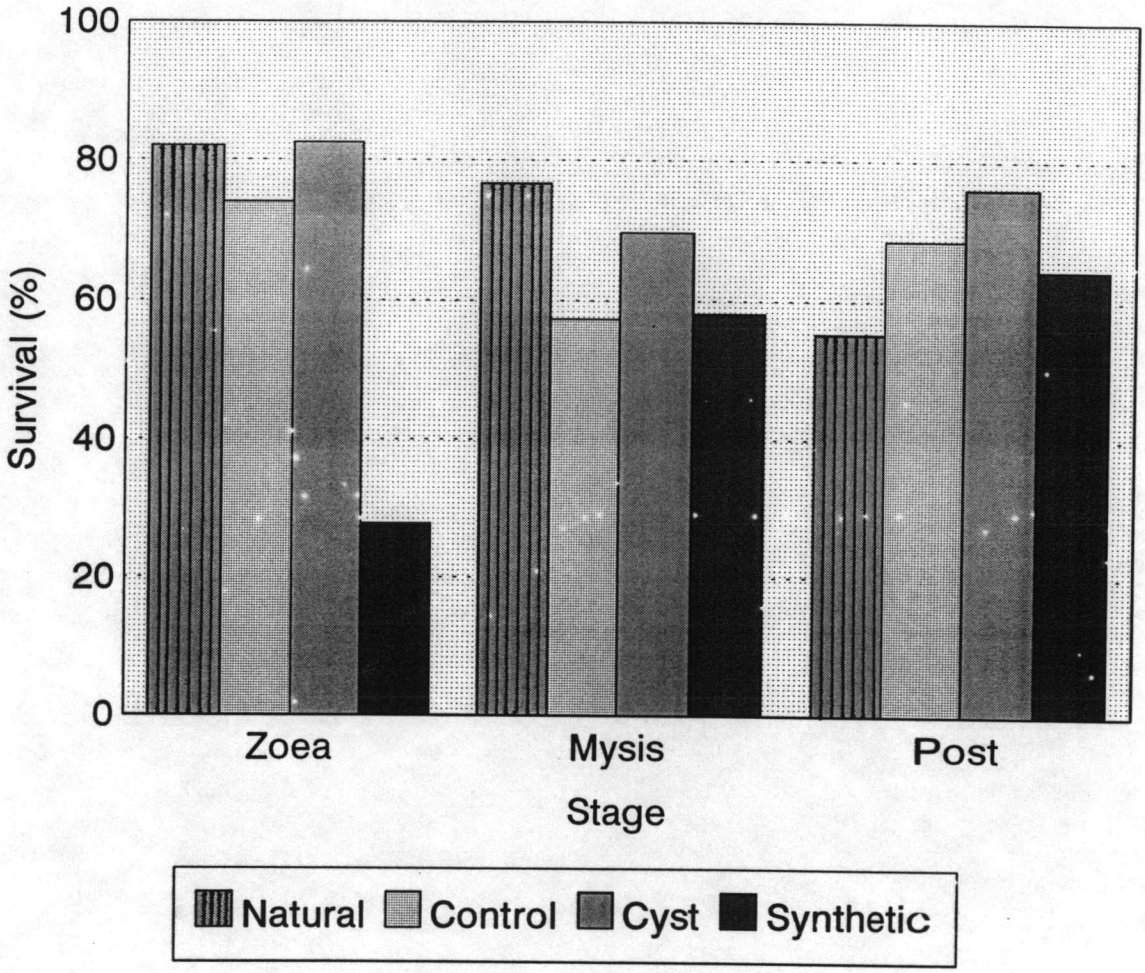

Figure 17 Survival rate of zoea, mysis and post larvae of P. monodon fed different diets.

Growth of postlarvae fed different diets

The growth in term of total length of postlarva 15 fed different sources of astaxanthin diet were shown in Table 10. The length of the larvae fed CD and the group fed SAD was significantly lower than the larvae fed AAD and NF ($P<0.05$). Nevertheless, there was no significant difference within the larva fed NF and AAD. The analysis of variance data is shown in Appendix 9.

Table 10 Length of post larva 15 fed different diets

Diets	Length of PL 15 (mm) MEAN + SD
NF	9.843 ± 0.73^{ab}
CD	9.674 ± 0.74^{b}
AAD	10.019 ± 0.67^{a}
SAD	9.652 ± 0.75^{b}

Means with different superscripts are significantly different at $P<0.05$

Tolerance of postlarva 15 fed different diets on stress test.

Tolerance of postlarva 15 was done by sudden stress from salinity 30 ppt to 2 ppt for 2 hr. The percent cumulative mortality of larvae on the stress test is shown in Table 11 and Fig 18.

The larvae fed all groups of diets started to die at 10 minutes of salinity stress. At first hour, the highest number of cumulative mortality (CM) of dead shrimp was found in the larvae fed NF (76%), followed by the larvae fed CD (68%), SAD (66%) and AAD (62%). The mortality rate declined after the first hour. At time 90 minutes, CM of the shrimp fed AAD and SAD was (76%), and that fed NF and CD were 80% and 86%, respectively. After 120 minutes, some shrimp in all treatment fed diets could resist to 2 ppm salinity and showed no sight of mortality up to 140 minutes of the exposed period. To examine 50% CM of post larva15, probit analysis (Appendix 10) was used to interpret the data. The results showed that 50% CM of shrimp fed NF was 27.99 min, CD group was 32.23 min, SAD group was 36.63 min and AAD was 44.86 min (Table12).

Table 11 Cumulative mortality of postlarvae 15 fed different astaxanthin diets at 2 ppt salinity

Stress time (min)	Cumulative mortality(%)			
	NF	CD	AAD	SAD
0	0	0	0	0
10	16	14	8	12
20	40	34	20	28
30	54	48	34	44
40	66	58	44	60
50	68	66	58	62
60	76	68	62	66
70	78	74	62	74
80	80	78	72	74
90	80	86	76	76
100	80	86	76	76
110	84	88	76	76
120	90	92	80	80
130	90	92	80	80
140	90	92	80	80

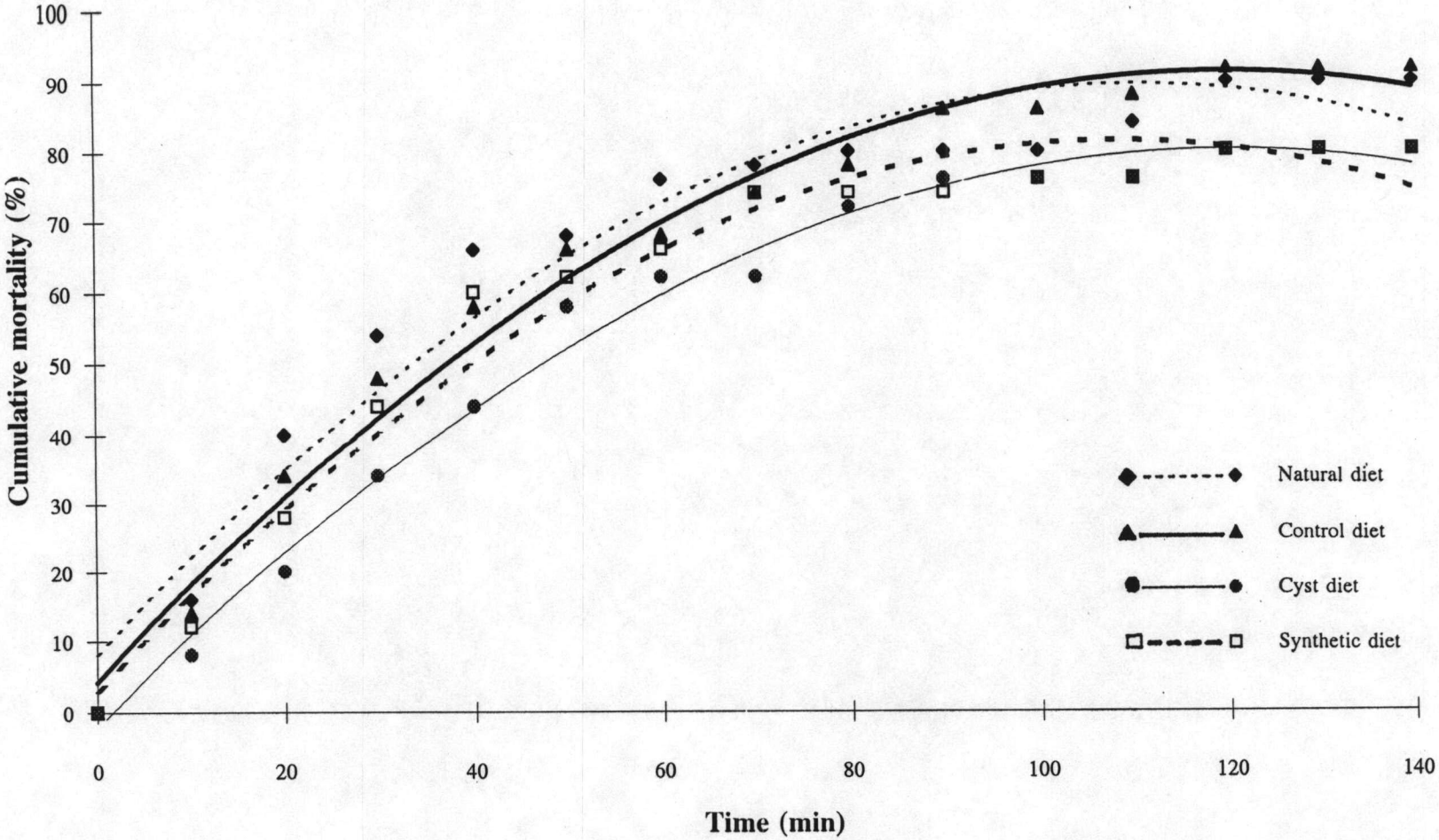

Figure 18 Cumulative mortality of postlarva 15 fed different astaxanthin diets at 2 ppt salinity

Table 12 Time of 50% cumulative mortality of larvae fed different diets in stress resistance (from probit analysis)

Diets	Time of 50% CM (min)
NF	27.99
CD	32.23
AAD	44.86
SAD	36.63

Water quality

Water quality of the rearing unit in this study is shown in Table 13. Water quality parameters were similar among four diets and were in normal ranges throughout the whole period of the experiment.

Table 13 Water quality of the rearing units at different stages of larvae rearing.

Diets	Larval stage	Ammonium (mg/l)	Nitrate (mg/l)	pH	Temp. (°C)	Salinity (%)
NF	Z_1-Z_3	0.5-1	25	7.7	28-30	30
	M_1-M3	0.5-1	25	7.7	28-29	30
	PL_1-PL_{15}	0.5-1	10	7.7	27-28	30
CD	Z_1-Z_3	1-2	10	7.4-7.7	28-30	30
	M_1-M_3	2-3	25	7.4-7.7	28-29	30
	PL_1-PL_{15}	1-2	10	7.4-7.7	27-28	30
AAD	Z_1-Z_3	2-3	10	7.4-7.7	28-30	30
	M_1-M_3	2-3	25	7.4-7.7	28-29	30
	PL_1-PL_{15}	1-2	10	7.4-7.7	27-28	30
SAD	Z_1-Z_3	1-2	10	7.4-7.7	28-30	30
	M_1-M_3	2-3	25	7.4-7.7	28-29	30
	PL_1-PL_1	1-2	10	7.4-7.7	27-28	30

Carotenoids in shrimp larvae

Because of the small size of postlarva15, the method for analysis carotenoids content was determined by wet weight basis using spectrophotometric method. The results of carotenoids content are shown in Table 14. Carotenoids content in shrimps fed all groups of diet was significantly different at $P<0.05$ (Appendix 9). The highest carotenoids accumulation occurred in the shrimp fed NF, followed by the shrimp fed AAD, SAD and CD.

Table 14 Carotenoids concentration (μg/g body wet weight) in the prawn (PL15)

Source of fed shrimp	Carotenoids concentration (ppm)
NF	179.54 ± 0.65^{a}
CD	97.33 ± 3.42^{b}
AAD	122.57 ± 5.62^{c}
SAD	109.07 ± 0.47^{d}

Means with different superscripts are significantly different at $P<0.05$