CHAPTER V

THE EFFECTS OF *RHODOBACTER* SP. TM11B ON GROWTH PERFORMANCE, PIGMENTATION, BLOOD PARAMETERS AND DISEASE RESISTANCE IN BLACK TIGER SHRIMP

Introduction

In the aquaculture industries of South East Asia, black tiger shrimp is the most importance economic species which the desirable bright red color after boiling must be reproduced for acceptability in international markets. According from the previous studies by Tanaka *et al*. (1976) and Chein and Jeng (1992), the natural color of shrimps and other crustaceans is usually due to carotenoids, especially to astaxanthin and its complexes with protein. However, shrimp cannot synthesize these carotenoids *de novo* and therefore have to obtain these pigments from dietary sources. Many studies have been demonstrated that the supplementation of astaxanthin in the last 6-8 weeks of culture period will improve the market value of the shrimp products (Yamada *et al*., 1990 ; Lieo *et al*., 1993 ; Hunter *et al*., 2000). With the exception of pigmentation, carotenoid are also involve in many physiological processes in a number of aquatic animals, Storebakken and Goswami (1996) reported on the positive effect of dietary carotenoid on the growth performance of rainbow trout, as well as, improvement of broodstock performance (Verakunpiriya *et al*., 1997) and enhanced of immune response in many fish species (Thorisen *et al*., 1995; Tachibawa *et al*., 1997 ; Amar *et al*., 2000). But no studies have been report on such phenomenon in shrimp.

However, supplementation of shrimp feed with synthetic astaxanthin leads to the higher production costs. Several attempts have been made to find alternative sources of astaxanthin, e. g. the yeast *Xanthophyllomyces* (formerly

Phaffia) (Sanderson and Jolly, 1994) the alga *Haematococcus* (Sommer *et al*., 1991) and *Chlorella* (Gouveia *et al*., 2002). Although, Tanaka *et al.* (1976) have been reported that astaxanhin was the most effective carotenoid for pigmentation in kuruma shrimp (*Penaeus japonicus*), but high efficiencies of pigmentation of black tiger shrimp (*Penaeus monodon*) were achieved with other carotenoids such as zeaxanthin and β-carotene (Liao *et al*., 1993; Boonyaratpalin *et al*., 2001). Microbial sources of these carotenoids, e.g. *Spirulina* (Liao *et al*., 1993) and *Dunaliella* (Boonyaratpalin *et al*., 2001) have been shown to be cost-effective in pigmentation in black tiger shrimp. However, no information on the utilization and metabolism of other carotenoids in the black tiger shrimp have been reported, and this lead to the further observation.

Photosynthetic bacteria also contain high level of carotenoids (Tanaka *et al*., 1994). These microorganisms are cost effective to produce in the bioreactor with varieties of substrate from agricultural industries by- product (Prasertsan *et al*., 1993). Kobayashi and Kurata (1978) ; Varati (1984) and Noparatnaraporn and Nagai (1986) reported on the use of photosynthetic bacterial cells as an animal feed ingredient and source of single cell protein in poultry. Recently, many photosynthetic bacterial cells products (PSB) have been produced as feed supplement and introduced as source of protein, vitamin and carotenoid in Asian aquaculture industries. Normally, anoxygenic photosynthetic bacteria contain high levels of acyclic carotenoids such as spheroidene and spheroidenone (Takaishi, 2001). It is highly unlike that these carotenoids could be converted into astaxanthin by any metabolic process (Latscha, 1991). But no report on the application of photosynthetic bacteria as feed supplement in aquatic animal has been made, especially as carotenoid source in black tiger shrimp. In this work, shrimp feed was supplemented with mechanical cell wall disruption dried material from *Rhodobacter* sp. TM11B or with synthetic astaxanthin

(CAROPHILL pink®, Roche) as positive control, in order to evaluate the effects of these supplements on growth performance, feed utilization, pigmentation, immune response and disease resistance in black tiger shrimp.

Materials and Methods

Test animals

One thousand healthy shrimps with a weight range of 2-3 g were purchased from a good-management private shrimp farm. Shrimp were acclimated and were fed with commercial shrimp feed 5 times daily in a 10 tons concrete tank for one week before the start of the experiment. Twenty shrimp were transferred to each 250 l glass aquaria, this system was equipped with a 200 l filtration tank and the temperature was kept constant at 29 $\pm$ 0.5 ^{0} C with 300 W glass heaters. Shrimp were acclimatized in this test system for 2 weeks before the start of the feed supplement experiment. Uneaten feed and fecae were removed daily and 25 % of the water was exchanged every 2 days.

Photosynthetic bacterial cells

Rhodobacter sp. TM11B which has the highest potential use in the black tiger shrimp (result from Chapter III) was used. The bacteria were grown in optimized-modified G5 medium with yield the highest total carotenoid production (by the addition of 0.1 mM ferric citrate) under anaerobic conditions with illumination of 1,500 lux for 96 hr (result from Chapter IV). Each culture was harvested by centrifugation at 5,000 g for 15 min to obtain concentrated cells. Bacterial cells were disrupted by sonication (Vibra Cell™ Sonicator and material Inc.). Broken cells of this PSB were lyophilized by freeze-drying (LABCONCO® freezone 1).

Experimental diet

Six isonitrogenous and isocaloric experimental diets were fed to the test shrimp for a period of 8 weeks. Diet 1 was a negative control without carotenoid supplementation, diet 2 (positive control) containing 100 ppm synthetic astaxanthin (CAROPHILL pink®), diet 3 supplemented with 3% freezed-dry *Spirulina*, diet 4-6 contained 1-5 % of lyophilized *Rhodobacter* sp. TM11 B (Table 5-1). The test diets were prepared by a method described by Boonyaratpalin *et al*. (2001). All ingredients were mixed and processed by a meat grinder and were stabilized with about 30% moisture. Then the spaghetti-like feed was broken into pellets. This process was followed by four hours of drying in an air flow oven at 60 ^{0}C until the moisture content was lower than 10%. The dry pellets were kept in two layer plastic bags at -14 ^{0}C until use. The proximate analysis of nutrients in each test diet were done by the method described by AOAC (1990).

Effects of the bacterial cell material

For each treatment (5 replicates), the shrimp were fed each experimental diet to satiation 5 times daily for 8 weeks. Data for growth performance were recorded every two weeks *i.e*. average body weight, weight gain, survival, feed intake and FCR. After 8 weeks of feeding, 8-10 shrimps from each treatment were collected and the color was recorded by immersing shrimp from each treatment in boiling water for 3 min and then comparing the color of the boiled shrimp.

Carotenoid analysis

After 8 weeks of feeding trial, 12 shrimp from each treatment were randomly sampled for analysis of carotenoid composition. The shrimp from each treatment were immediately

immersed in liquid nitrogen and stored at -70^{0}C before being lyophilized (LABCONCO® freezone 1). Quantitative spectrophotometry for the analysis of total carotenoids in freeze-dried shrimp samples and in test diets was carried out by the method described by Sommer *et al*. (1991). The pooled samples were homogenized and extracted with acetone and then transferred to petroleum ether, dehydrated over anhydrous sodium sulphate and finally made up to volume (25 ml) with petroleum ether. The extracts were scanned between 400-600 nm in a scanning spectrophotometer (Perkin Elmer, Lambda 25). The total carotenoid concentration was calculated from the peak absorbance in petroleum ether using an absorption coefficients ($A^{1\%}_{1cm}$) of 2500. Thin layer chromatography (TLC) according to the method described by Yamada *et al*. (1990) was used for the analysis of free astaxanthin, astaxanthin mono-ester and astaxanthin di-ester; the crude carotenoids were separate by TLC silica (Merck), with diethyl ether : petroleum ether (1:1) as developing solvent. Astaxanthin esters were identified by comparison with chromatographic behavior of standard astaxanthin mono-ester and astaxanthin di-ester. Quantitative analysis of the astaxanthin was carried out by scraping off the TLC band corresponding to each form of astaxanthin, eluting in diethyl ether and concentrating with an evaporator. The concentrated astaxanthin fractions were made to volume (10 ml) with petroleum ether and then determined the light absorption spectra using a scanning spectrophotometer (Perkin Elmer, Lambda 25). Astaxanthin and astaxanthin ester concentrations were calculated (as astaxanthin equivalents) by use of an absorption coefficient value ($A^{1\%}_{1cm}$= 2500) in petroleum ether.

Effects of the bacterial cell material on blood parameters

After 8 weeks of culture period, 15 shrimp from each treatment were randomly sampled for analysis of blood parameters as follows :

Total hemocyte count

Blood from each shrimp was collected from the base of a walking leg by means of a 1 ml plastic syringe with 25 G needle. After withdrawal, the blood was diluted with 0.15% trypan blue solution, and hemocytes were counted by means of a hemacytometer and calculated as number of blood cells (total hemocytes) / cm^3 (Boonyaratpalin *et al*., 2000)

Phenoloxidase activity

Phenoloxidase activity (PO) was measured by a method modified from that of Smith and Soderhall (1983), The blood samples were withdrawn by using a 1 ml plastic syringe with 0.1 ml of 3% L-cysteine in K-199 medium as an anticoagulant. The hemocytes were washed with K-199 and preserved in 1 ml of cacodylate buffer. Hemocyte lysate was prepared in a cacodylate buffer pH 7.4 by using a sonicator (Vibra Cell™ Sonicator and material Inc.) at 30 amplitude for 20 seconds and then centrifuged at 10,000 *g* for 10 min. Phenoloxidase activity was analysed by using L-dihydroxyphenylalanine (L-DOPA) as the substrate. Enzyme activity was analysed spectrophotometrically by measuring absorbance at 630 nm. The protein concentration was analysed by the Lowry method (Lowry *et al.*, 1951). Phenoloxidase activity was expressed as units/min /mg-protein.

Total antioxidant status and superoxide dismutase

Total antioxidant status (TAS) was analysed by means of a RANDOX test kit (Randox Laboratories Ltd., UK). Blood samples were collected from the base of a walking leg, using 0.1 ml of 1 % Tris-EDTA buffer (pH 7.2) as anticoagulant. After centrifugation at 6,000 *g* for 2 min, the plasma was transferred to another microcentrifuge tube and TAS analysed by the method described in the Randox laboratory manual. The superoxide dismutase (SOD) activity in hemocytes were analyzed by collecting the blood sample, using 0.1 ml of 1 % Tris-EDTA buffer (pH 7.2) as anticoagulant. After centrifugation at 6,000 *g* for 2 min the hemocytes were washed 3 time with 0.9 % NaCl and then lysed in 0.1 ml of sterile double deionized water. The hemocyte lysate was analysed for superoxide dismutase activity, by use of a RANDOX test kit (Randox Laboratories Ltd., UK) with the method described in the Randox laboratory manual. The protein concentration was analyzed by the Lowry method (Lowry *et al.*, 1951). Superoxide dismutase activity was expressed as unit/min/mg-protein.

Stress tolerance

At the end of experiment, 10 shrimp from each treatment were transferred to duplicates of stress test chambers (100 l glass aquaria). Low dissolved oxygen conditions were maintained by stop the water flow through system and aeration, using plastic sheet overlying on the water surface in each stress test chamber for 10 hr / day. Dissolved oxygen were linearity decreased to below 1 ppm within 10 hr. Mortality of the shrimp in each group was recorded for 7 days.

Disease resistance

At termination of the experiment, the shrimp from each treatment were transferred to duplicates of 150 l glass aquaria. Each shrimp were injected with 0.1 ml of *Vibrio harveyi*

suspended in 1.5 % NaCl at the concentration of LD_{50} (1 x 10^6 cfu/ml). The mortality was recorded every day for the periods of 14 days.

Histological study

At the end of the experiment, 12-15 shrimp from each treatment were sampled and examined of histological changes. Living shrimp were injected with Davidson's fixative by use of a 1 ml syringe. The site of injection and process of fixation were carried out as described by Bell and Lightner (1988). After 72 hr of preservation, each sample was cut into an appropriate size and then further processed as paraffin sections. Haematoxylin and eosin (H&E) were used for staining (Humason, 1979). Stained sections were examined under a light microscope (Olympus, CH30).

Table 5-1 Feed formulation of test diets.

% in diet	T1 control	T2 100 ppm astaxanthin	T3 3% Spirulina	T4 1% TM11 B	T5 3% TM11 B	T6 5% TM11 B
Fish meal	34	34	31	33	31	29
Squid meal	10	10	10	10	10	10
Wheat gluten	6	6	6	6	6	6
Soybean meal	10	10	10	10	10	10
Wheat fluor	20	20	20	20	20	20
Rice flour	9.1	8.975	9.1	9.1	9.1	9.1
Fish oil	2	2	2	2	2	2
Lecithin	2	2	2	2	2	2
Cholesterol	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin mix*	0.33	0.33	0.33	0.33	0.33	0.33
Choline	0.3	0.3	0.3	0.3	0.3	0.3
Vitamin E	0.15	0.15	0.15	0.15	0.15	0.15
Vitamin C	0.1	0.1	0.1	0.1	0.1	0.1
Mineral mix**	4	4	4	4	4	4
Zeolite	1.5	1.5	1.5	1.5	1.5	1.5
CAROPHILL pink®	-	0.125	-	-	-	-
Spirulina	-	-	3	-	-	-
PSB	-	-	-	1	3	5
BHT	0.02	0.02	0.02	0.02	0.02	0.02
Total carotenoid (ppm)	- *	78.2	172.2	27.2	68.8	95.9

* Vitamin mixture (mg/ kg diet): thiamine 22.5; riboflavin 20; nicotinic acid 36.7; Ca pantothenate 24; inositol 98; biotin 0.5; folic acid 1.68; vitamin B_{12} 0.005; menadione

13.28; vitamin A 1150 IU; vitamin D_3 230 IU; BHT 1; PABA 20

** Mineral mixture (g/kg diet) KH_2PO_4 1; $CaHPO_4$ 1; NaH_2PO_4 1.5; KCL 0.5

Results

Growth performances

After 2-4 weeks of the feeding trial, the average body weights of shrimp fed with control diet and 100 ppm synthetic astaxanthin were significantly higher than others ($P<0.05$) (Table 5-2). During week 6-8 of the feeding trial, body weight of the shrimp fed 100 ppm astaxanthin was significantly lower than the control group. Moreover, growth retardation of the shrimp fed 3 % *Spirulina* and 1-5 % *Rhodobacter* sp. TM11B were observed. At the end of the experiment, average body weight, weight gain and FCR were significantly different ($P<0.05$). Shrimp fed with control diet had the highest weight gain. Feed conversion ratio (FCR) of the shrimp fed control and 100 ppm astaxanthin were not significantly different. However, a negative correlation in FCR and level of PSB in test diet (R^2 = - 0.96) was found in the group fed the test diet supplemented with *Rhodobacter* sp. TM11B There were no significant differences in survival among the treatments.

Table 5-2 Average body weight (g), Weight gain (%), Survival (%) and FCR of the shrimp fed each experimental diet for 8 weeks periods.

	Average body weight (g)	**Weight gain (%)**	**Survival (%)**	**FCR**
T1 control	9.49 ± 0.84 ^{a}	200.43 ± 26.37 ^{a}	86.00 ± 6.52 ^{ab}	1.91 ± 0.31 ^{a}
T2 Asx 100 ppm	8.45 ± 0.73 ^{b}	166.97 ± 23.04 ^{b}	86.00 ± 8.94 ^{ab}	2.06 ± 0.14 ^{a}
T3 *Spirulina* 3%	7.51 ± 0.89 ^{c}	136.75 ± 27.66 ^{c}	84.00 ± 7.42 ^{ab}	2.11 ± 0.19 ^{ab}
T4 *Rhodobacter* 1%	7.83 ± 0.47 ^{bc}	148.34 ± 15.21 ^{bc}	90.00 ± 7.91 ^{a}	2.11 ± 0.26 ^{ab}
T5 *Rhodobacter* 3%	7.24 ± 0.22 ^{c}	129.28 ± 7.67 ^{c}	75.00 ± 7.91 ^{bc}	2.78 ± 0.48 ^{bc}
T6 *Rhodobacter* 5%	7.09 ± 0.52 ^{c}	124.42 ± 15.18 ^{c}	70.00 ± 15.00 ^{c}	3.10 ± 1.04 ^{c}

Means within columns not sharing the same superscript are significantly different ($P<0.05$).
ns = not significant ($P>0.05$)

Blood parameters

Total hemocyte count were not significantly difference among the group fed control, 100 ppm astaxanthin, 3% Spirulina and 1% *Rhodobacter* sp. TM11B, whereas the lower total hemocytes count was found in the group fed the diet supplemented with 3-5% *Rhodobacter* sp. TM11B. Superoxide dismutase activity were not significantly different among the groups (Table 5-3). Phenoloxidase activity in hemocytes from shrimp fed with test diet containing 5% *Rhodobacter* sp. TM11B was significantly higher than the others ($P<0.05$).

Table 5-3 Blood parameter of the shrimp fed each experimental diet for 8 week.

	Total hemocytes (x 10^7 cell / ml)	**Phenoloxidase activity (U/min/mg prot.)**	**Superoxide dismutase activity (U/mg prot.)**	**Total antioxidant status (mmole/l)**
T1 control	1.79 ± 0.41 ^{ab}	808.38 ± 151.06 ^{a}	1.44 ± 1.07 ^{ns}	1.29 ± 0.21 ^{ns}
T2 Asx 100 ppm	1.75 ± 0.47 ^{ab}	1065.20 ± 245.11 ^{a}	0.88 ± 0.99 ^{ns}	1.23 ± 0.22 ^{ns}
T3 *Spirulina* 3%	1.72 ± 0.55 ^{ab}	977.59 ± 375.43 ^{a}	0.76 ± 0.98 ^{ns}	1.32 ± 0.20 ^{ns}
T4 *Rhodobacter* 1%	2.08 ± 0.42 ^{a}	1014.46 ± 335.43 ^{a}	0.83 ± 0.98 ^{ns}	1.26 ± 0.30 ^{ns}

T5 *Rhodobacter* 3%	1.67 ± 0.37 [b]	977.31 ± 238.57 [a]	1.20 ± 2.29 [ns]	1.25 ± 0.25 [ns]
T6 *Rhodobacter* 5%	1.48 ± 0.66 [b]	1311.42 ± 355.20 [b]	0.90 ± 0.83 [ns]	1.25 ± 0.15 [ns]

Means within columns not sharing the same superscript are significantly different (P<0.05).
ns = not significant (P>0.05)

Carotenoid analysis

At termination of the feeding trial, the shrimp fed with diet supplemented with 100 ppm synthetic astaxanthin and 3% *Spirulina* were dark brown, shrimp fed with diet containing 5% *Rhodobacter* sp. TM11B were greenish-brown, but the others remained pale-blue (Figure 5-1). The colors of boiled shrimp from each treatment are shown in figure 5-2. The thin layer chromatogram of the carotenoids extracted from shrimp is similar in all groups fed each test diet (Figure 5-3). The carotenoid contents of the shrimp are summarized in table 5-4. Total carotenoid was highest in the shrimp fed the diet supplemented with 3% *Spirulina*. The total carotenoid content in the groups fed 1-3% *Rhodobacter* sp. TM11B was not significantly different from that of the control group (p>0.05). Total carotenoid content in the shrimp fed 5% *Rhodobacter* sp. TM11B was higher than the control group. From spectrophotometric analysis, and saponified technique to converted all astaxanthin to astacene, it was found that the total astaxanthin (free-, mono ester and di ester) are major carotenoids in the shrimp samples, which found in the amounts of 84.1.-88.6 % in all extractable carotenoid from shrimp materials, but small amount of β-carotene also found in all samples (Figure 5-4 to 5-7). However, there were not significantly difference in the concentration of astaxanthin in all group fed the diet supplemented with *Rhodobacter* sp. TM11B except the amount

of diester-astaxanthin in the group fed 5% *Rhodobacter* sp. TM11B.

Table 5-4 Total carotenoid and astaxanthin content in the shrimp fed each experimental diet for 8 weeks.

	Di-ester astaxanthin (ppm)	**Mono-ester astaxanthin (ppm)**	**Free Astaxanthin (ppm)**	**Total Carotenoids (ppm)**
T1 control	4.5 ± 0.1 ^{c}	1.7 ± 0.3 ^{b}	5.0 ± 0.3 ^{c}	23.0 ± 2.3 ^{a}
T2 Asx 100 ppm	35.3 ± 5.4 ^{a}	43.9± 1.3 ^{a}	14.8 ± 0.6 ^{b}	106.1 ± 10.3 ^{c}
T3 *Spirulina* 3%	19.2 ± 4.8 ^{bc}	52.0 ± 10.9 ^{a}	39.8 ± 2.9^{a}	206.5 ± 24.2 ^{d}
T4 *Rhodobacter* 1%	5.9 ± 0.5 ^{c}	1.6 ± 0.3 ^{b}	7.2 ± 0.3 ^{c}	39.7 ± 1.9 ^{ab}
T5 *Rhodobacter* 3%	8.2 ± 0.5 ^{c}	1.4 ± 1.2 ^{b}	8.5 ± 0.3 ^{c}	49.4 ± 3.4 ^{ab}
T6 *Rhodobacter* 5%	12.2 ± 1.6 ^{bc}	1.8 ± 0.2 ^{b}	7.4 ± 2.67 ^{c}	61.8 ± 6.7 ^{b}

Means within columns not sharing the same superscript are significantly different (P<0.05).
ns = not significant (P>0.05)

Figure 5-1 Body color of shrimp fed each test diet for 8 weeks.

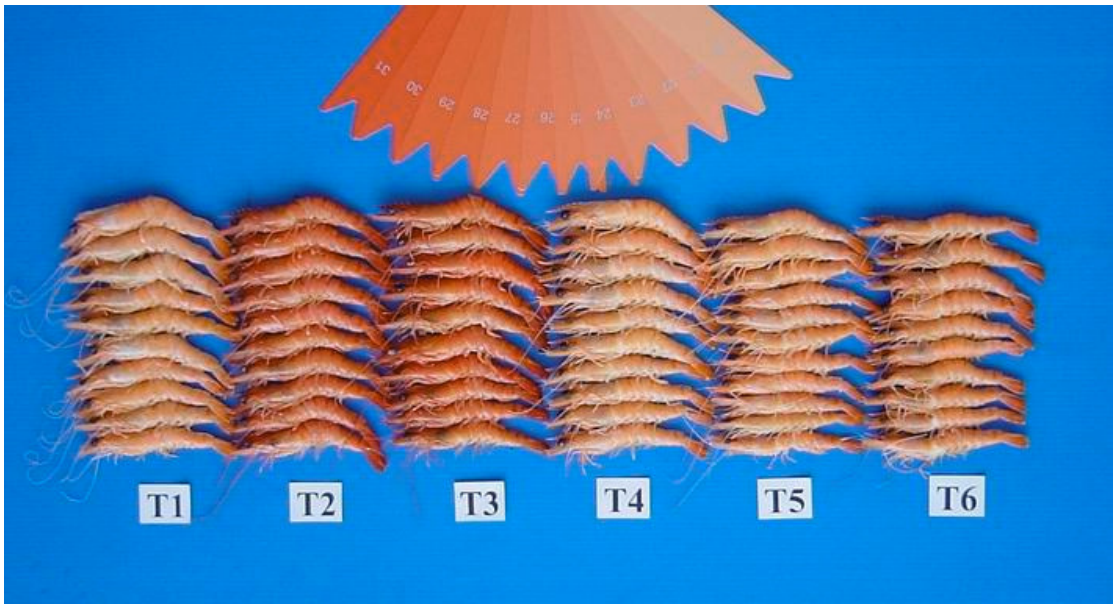

Figure 5-2 Body color of shrimp fed each test diet after boiling for 3 min.

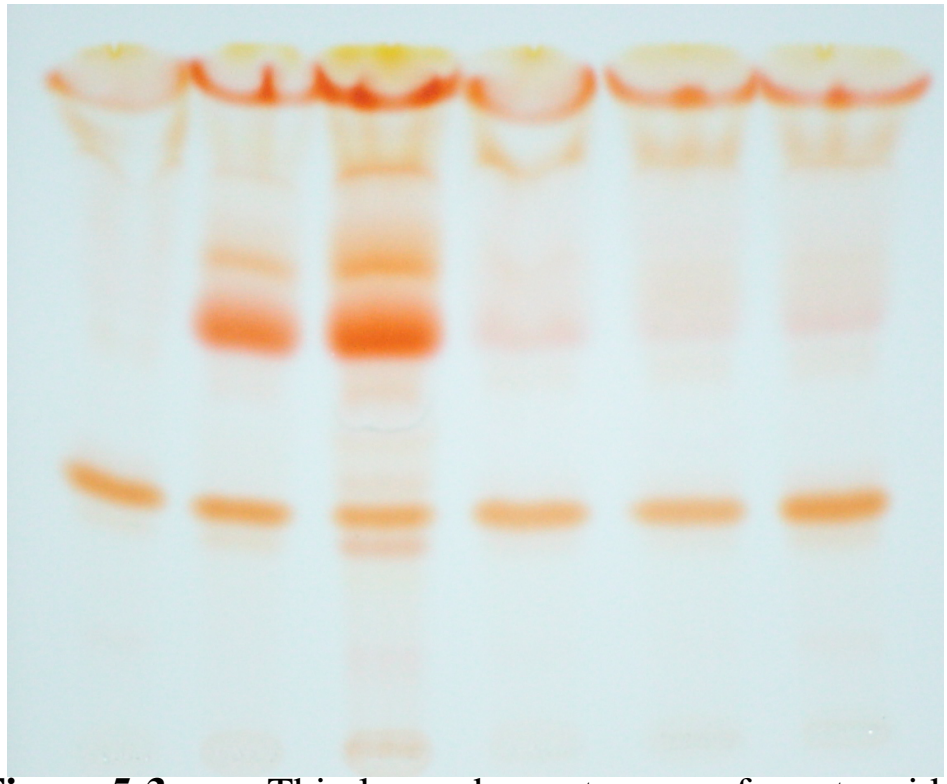

Figure 5-3 Thin layer chromatogram of carotenoids extracted from the shrimp fed test diet for 8 weeks (A = β-carotene, B = diester astaxanthin, C = monoester astaxanthin D = free astaxanthin)

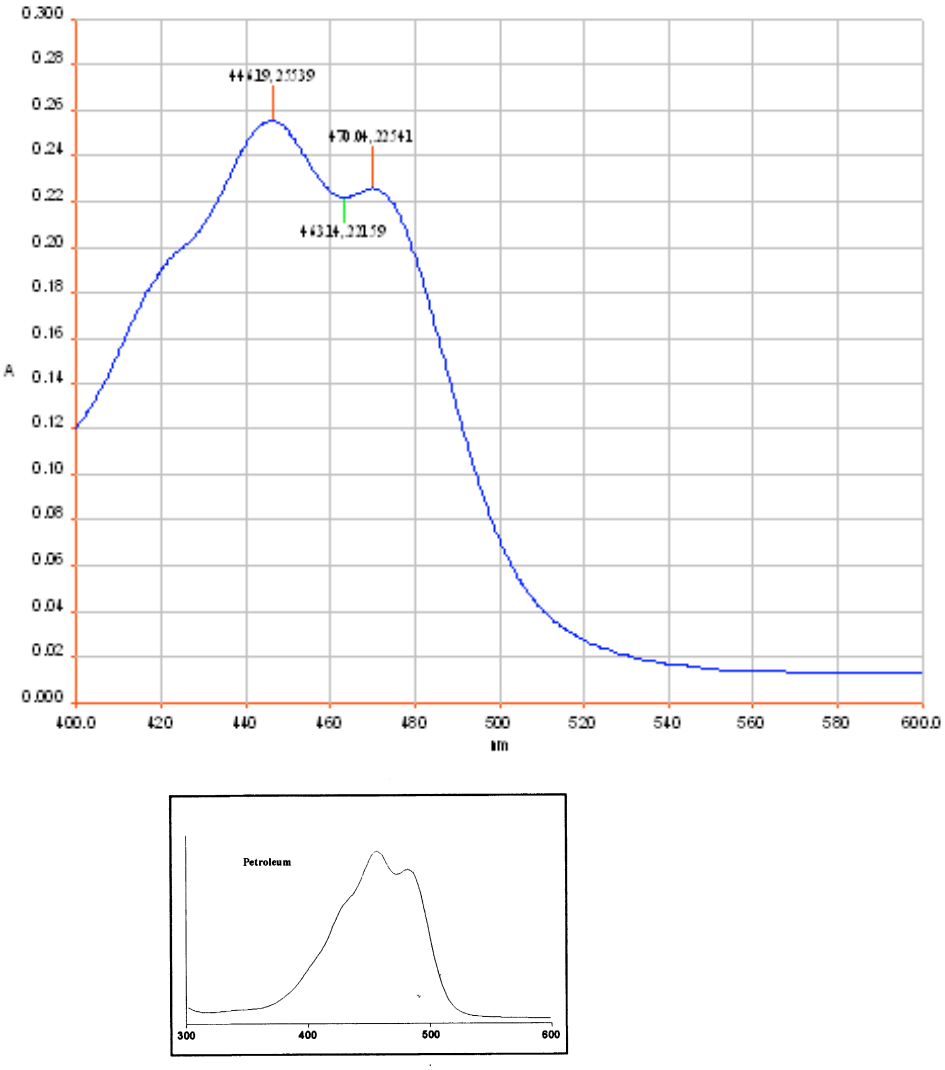

Figure 5-4 VIS spectrum of the pigment A (β-carotene) extracted from the shrimp fed diet 1-6 for 8 weeks compared to the standard UV/VIS spectrum (Britton *et al.*, 2004).

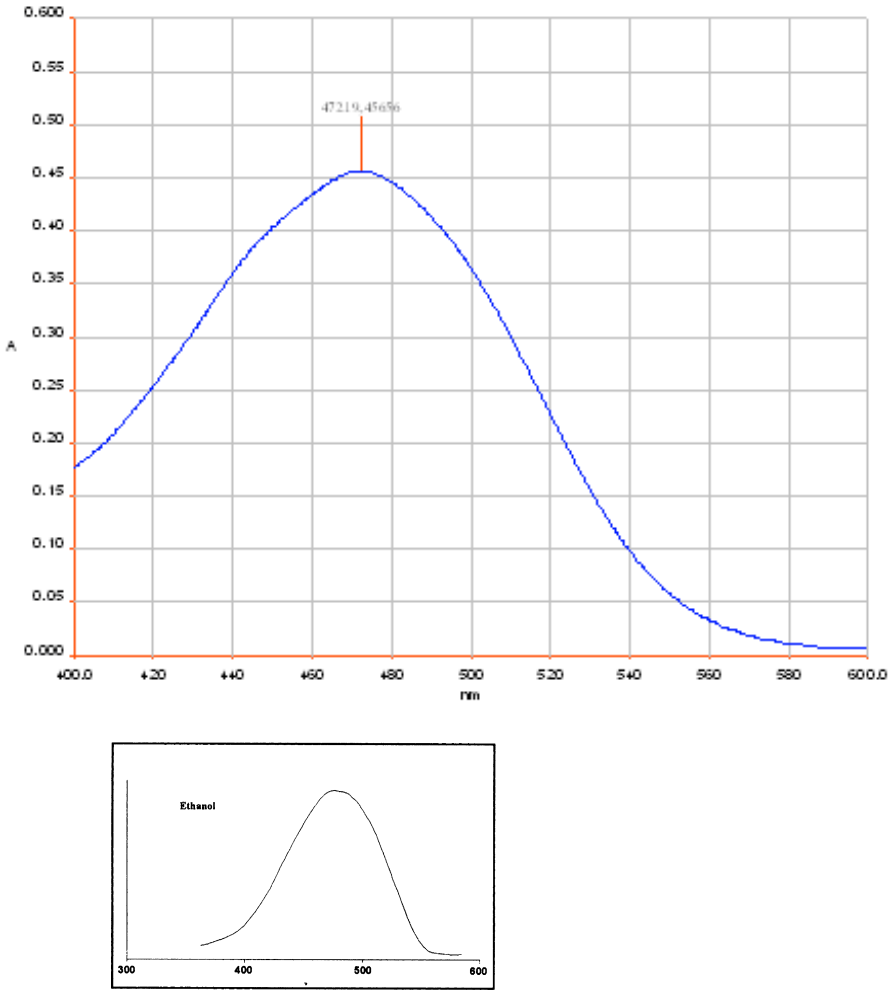

Figure 5-5 VIS spectrum of the pigment B (diester astaxanthin) extracted from the shrimp fed diet 1-6 for 8 weeks compared to the standard UV/VIS spectrum (Britton *et al.*, 2004).

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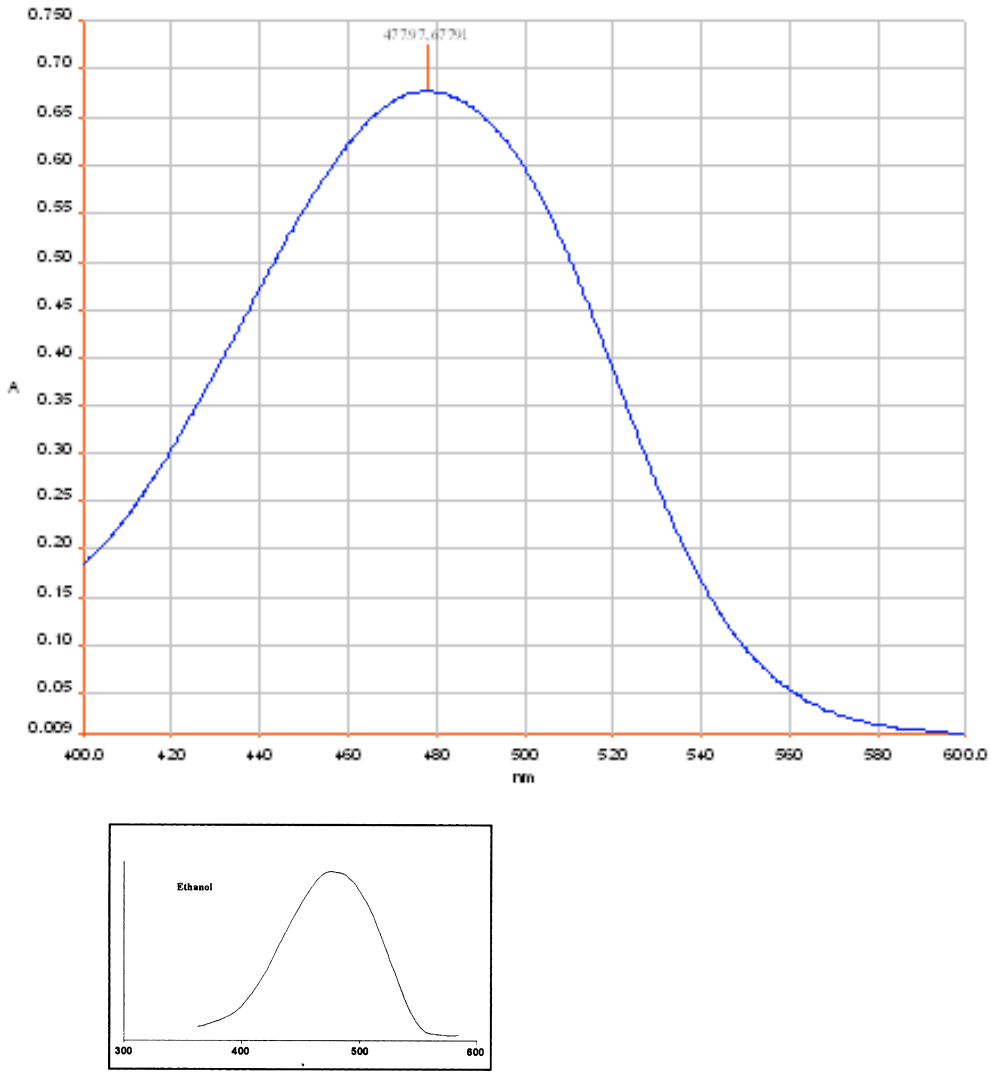


Figure 5-6 VIS spectrum of the pigment C (mono-ester astaxanthin) extracted from the shrimp fed diet 1-6 for 8 weeks.

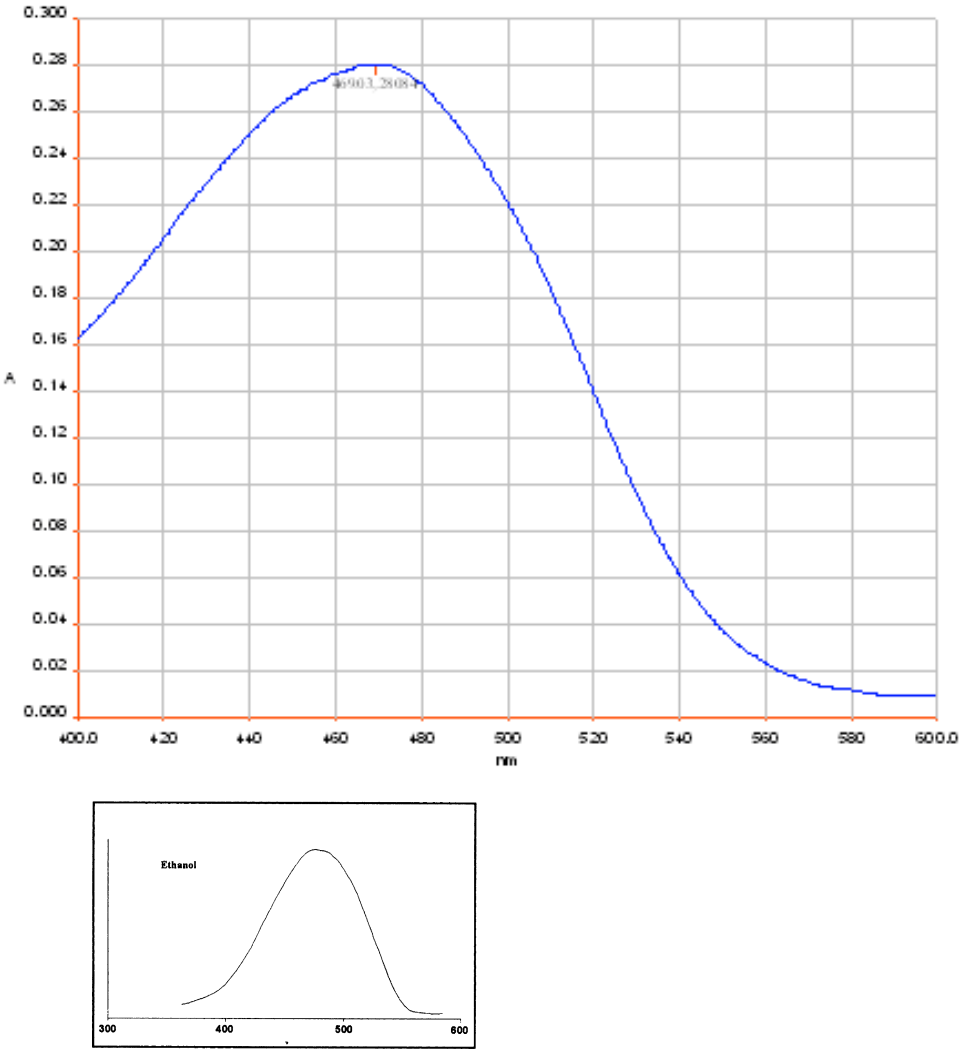


Figure 5-7 VIS spectrum of the pigment D (free-astaxanthin) extracted from the shrimp fed diet 1-6 for 8 weeks.

Stress tolerance

During first 3-4 days of stress period, the survival of shrimp fed diet containing 100 ppm astaxanthin (T2) tend to higher than other treatment, but there was not statistically difference among treatments ($P<0.05$) as shown in figure 5-8. The survival rate of all groups were not significantly difference during 5-7 days of stress periods.

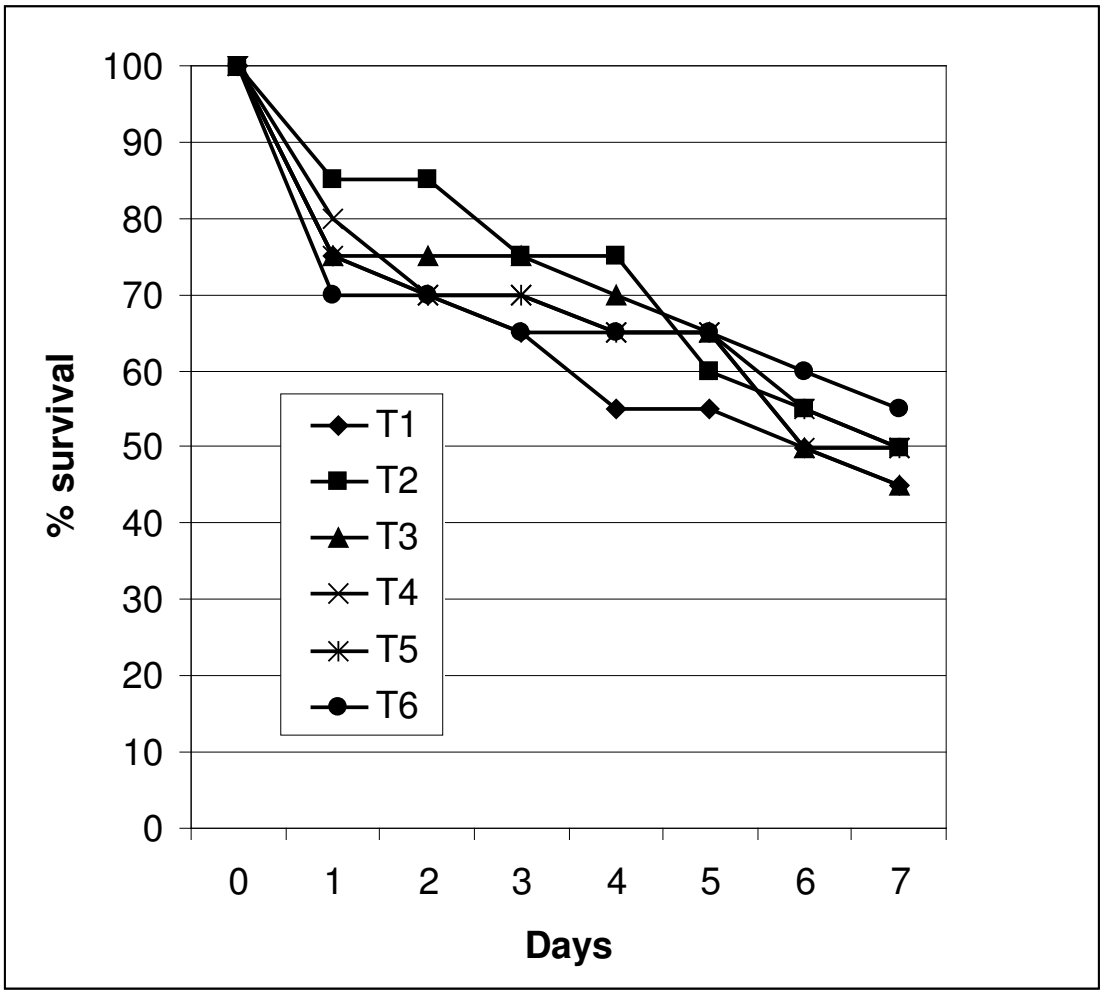


Figure 5-8 Survival rates (%) of the shrimp in each treatment during 7-days of stress period. (T1 = control, T2= 100 ppm astaxanthin, T3= 3 % *Spirulina*, T4-T6= 1-5% *Rhodobacter* sp. TM11B)

Disease resistance

The survival rate after challenged with *Vibrio harveyi* were shown in figure 5-9. There was not significantly different in survival of the shrimp after challenged with *Vibrio harveyi* at a concentration of 1 x 10^6 cfu/ml.

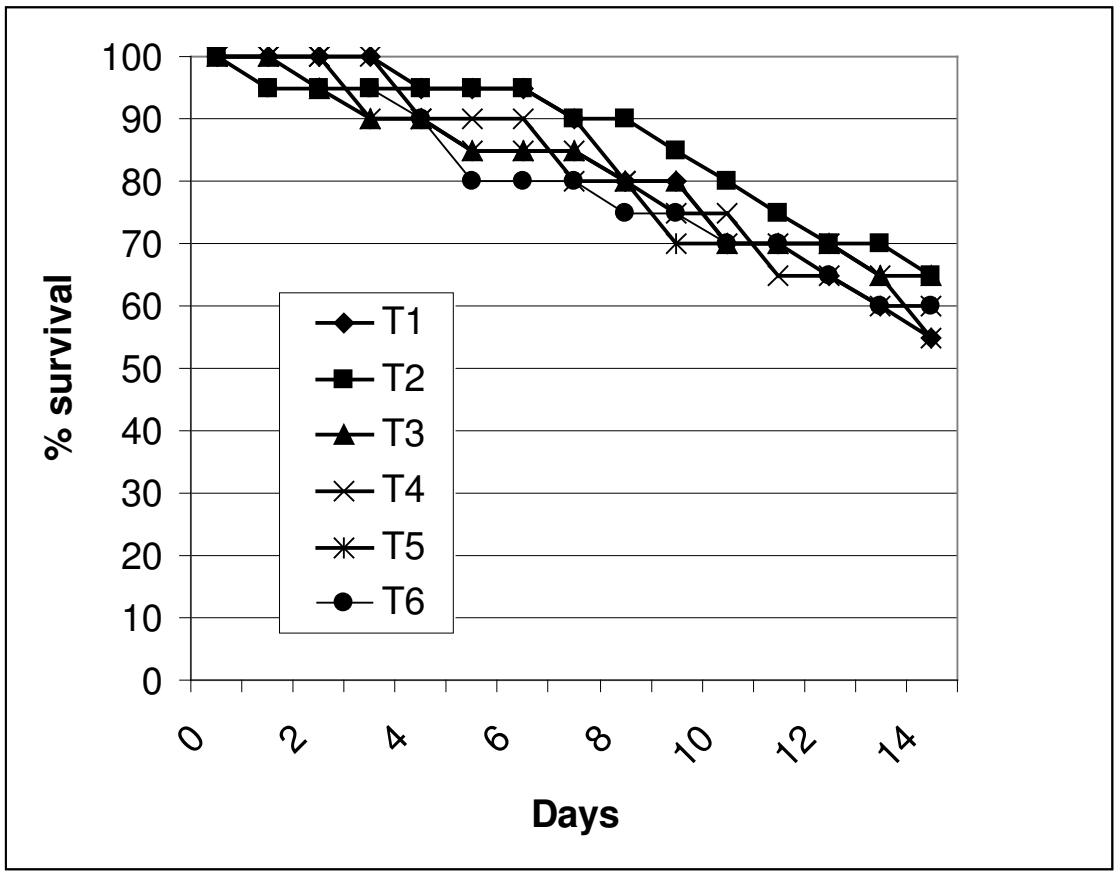
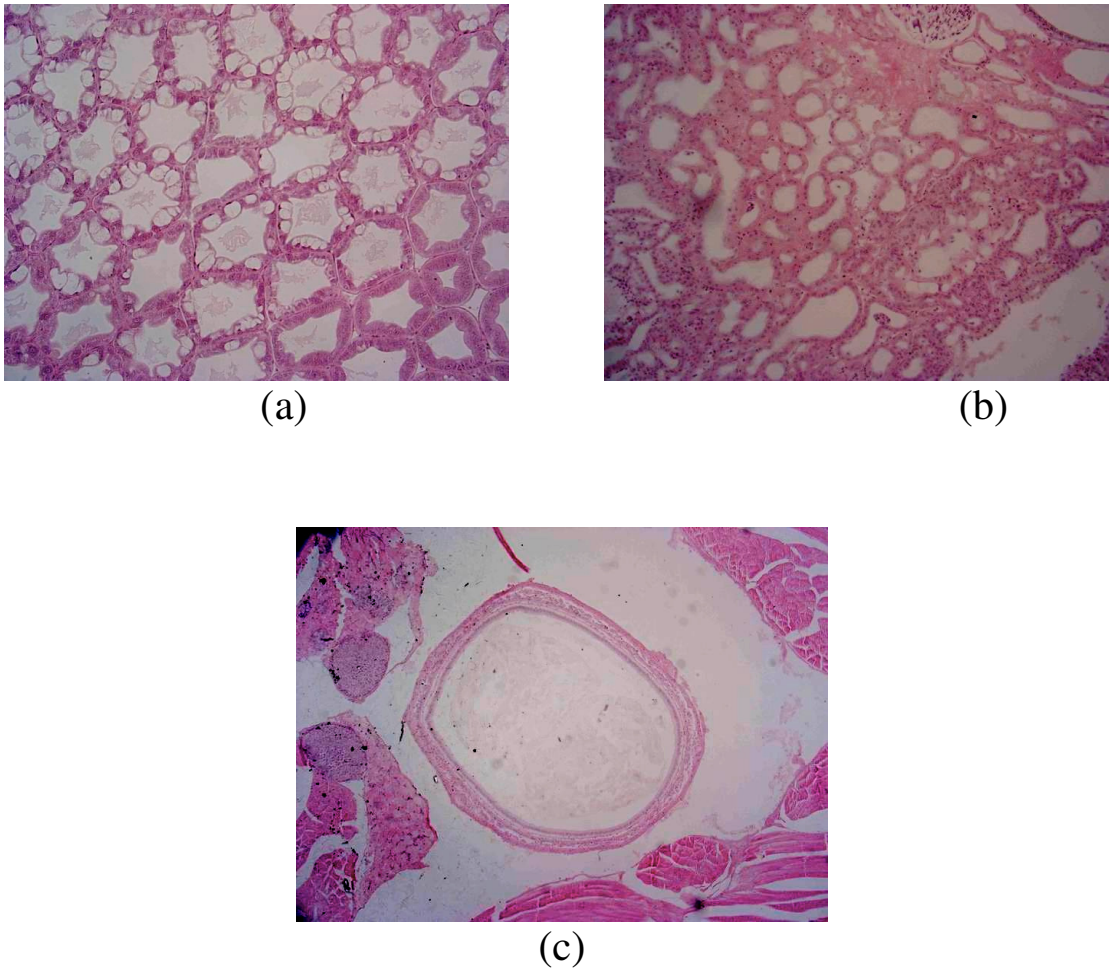


Figure 5-9 Survival rates (%) of the shrimp in each treatment after challenged with *Vibrio harveyi* for 14 days. (T1 = control, T2= 100 ppm astaxanthin, T3= 3 % *Spirulina*, T4-T6= 1-5% *Rhodobacter* sp. TM11B)

Histological studies

After 8 weeks of feeding trial, all shrimp fed diet 1-6 did not showed sign of histological changes in the hepatopancreas, antennal gland and intestine. The structure of hepatopancreatic tubles are similar in all shrimp fed all diet, the antennal gland which functions as excretion and osmotic regulation are normal in all treatments, No sign of degeneration of the intestinal epithelium in all shrimp examined in this study (Fig. 5-10).



(a) (b)

(c)

Figure 5-10 (a) Hepatopancreas of the shrimp fed diet supplemented with 5% *Rhodobacter* sp. TM11B (b) Antennal gland of the shrimp fed diet supplemented with 5% *Rhodobacter* sp. TM11B (c) Intestinal epithelium of the shrimp fed diet supplemented with 5% *Rhodobacter* sp. TM11B (Hematoxylene & Eosin, H & E).

Discussion

Although the photosynthetic bacterial products are wildly available in the aquaculture industries, they was no scientific data to support on the beneficial effect of these products, especially, the effects on the growth, pigmentation and immune enhancements in penaeid shrimp. From our results, supplementation of photosynthetic bacteria into shrimp diet did not show beneficial effect on growth performance in black tiger shrimp. Moreover, high levels of photosynthetic bacterial cell material cause retardation of growth and feed conversion rate. Normally, the limiting factors for the use of microbial cells in aquatic feed are poor digestibility due to the lack of enzymes for cell wall digestion (Halver, 2002). From the study on application of alkane yeast as a source of protein in coho salmon, only 25% yeast substitution was judged to be acceptable in the Oregon Moist Pellet diet for marine-cultured coho salmon, causing less than 4% reduction in growth. Growth was depressed further at fish meal substitution levels greater than 25% (Conrad *et al.*, 1980). In our study, cell wall disruption using sonication is not suitable to the practical work, other methods is seem to be possible such as the alkaline or enzyme disruptions, Gentle and Haard (1991) reported that the mechanical cell wall disruption is the most effective treatment for enhance of carotenoids utilization from *Phaffia rhodozyma* in rainbow trout, but the pigment of fish fed dried yeast with enzymatic treatment (funcelase) was higher than the control without cell wall distruption treatment. Some single cell proteins are imbalanced in amino acid composition (Conrad *et al.*, 1980).

Lack of palatability in microbial meal is another reason to decrease feed intake of the test animal. Another disadvantage of microbial cell is that some antinutritional factor

or toxins were produced during the growth phase in a fermentor. Kiessling and Askbrandt (1993) reported that the supplementation of commercially available bacterial protein products derived from *Brevibacterium lactofermentum* and *Bacterium glutamaticum* into rainbow trout feed resulted in a reduction in growth with the increasing levels of *Bacterium glutamaticum*. Moreover, the low hepatosomatic index (HIS) and gonadosomatic index (GSI) found in the groups fed with high level of *Bacterium glutamaticum* indicated a metabolic disorder caused by an unknown toxic compound in this single cell protein. Shrimp fed test diet supplemented with 100 ppm synthetic astaxanthin in our study have a slightly reduction in growth rate during 6-8 week of the study. A concentration of synthetic astaxanthin in our study (100 ppm) was adopted from the previous study in Kuruma shrimp (Yamada *et al*., 1990) and this level is the recommended level for use in the practical farm feed for penaeid shrimp (Boonyaratpalin *et al.*, 2000). Normally, 100 ppm of synthetic astaxanthin dose not affected the growth performance in most aquatic animal including Red cray fish, *Cherax quadrinacantus* (Harpez *et al*., 1998); Black tiger shrimp (Hunter *et al*., 2000) Kuruma shrimp (Yamada *et al.*, 1990 ; Chein and Jeng, 1992). However, many factors involved in the absorption and deposition of astaxanthin in aquatic animal, such as dietary lipid content and form of astaxanthin, Torrisen *et al*. (1995) has reported that the digestibility of astaxanthin and carotenoid deposition in rainbow trout and Atlantic salmon (Einen and Roem, 1997) increased with increasing of dietary lipid levels. Recently, Chan *et al*. (2002) studied the effect of dietary lipid on the deposition of astaxanthin in Coho salmon *Onchorhynchus kisutch*, and reported that the concentration of flesh astaxanthin was positively correlated to the lipid level in the test diet. Moreover, the absorption of astaxanthin in the rainbow trout in terms of the peak serum levels of astaxanthin was negatively influenced by the extention of carotenoid esterification in test diet (White *et al*., 2003).

Although, from our result, the high level of astaxanthin reduced growth performance in black tiger shrimp within 8 weeks. But, in the shrimp pigmentation strategy in Asian shrimp culture, to minimized the production cost, 100 ppm astaxanthin are recommended to be used in farm feed for 4 weeks before harvesting, This standard strategy is applicable to our results and also advantage to prevent the negative effect of long-term fed high level of astaxanthin on growth performance in the black tiger shrimp. Reduction of body weigh in the shrimp fed 3% *Spirulina* in our study is contrary to the results which were reported by Liao *et al*. (1993) who found that the body weight of black tiger shrimp fed test diet supplemented with 1-3% *Spirulina* was not differene from the control, but a marked decrease in shrimp body weight was found in the group fed 5% *Spirulina* supplemented diet. These contrasts may be caused by the difference species of Spirulina used in the studies, The nutritive composition of *Spirulina maxima* which was used by Liao *et al*. (1993) especially protein and lipid content are different to the nutritional composition of *Spirulina platansis* (Borowitzka and Borowitzka, 1988).

No evidence supported a positive effect of 100 ppm astaxanthin on the blood parameters of black tiger shrimp, in term of total hemocytes, phenoloxidase activity, superoxide dismutase and total antioxidant status. However, dietary carotenoids show a positive effect on the immune response in many fish species, Thompson *et al*. (1995) found that astaxanthin with vitamin A increased the serum antiprotease activity in rainbow trout, but did not affect growth or other humoral and cellular immune responses. However, Tachibana *et al*. (1997) found increased spleen lymphocyte proliferation in parrot fish larvae fed β-carotene-enhanced rotifers and attributed the high survival of parrot fish larvae to β-carotene supplementation.

From the study in wild white shrimp (*Penaeus vanamei*), carotenoid concentration and range of SOD activity,

which is an enzymatic indicator of oxidative stress in shrimp body were significantly higher in captive than in wild shrimp. It is possible that the wild shrimp with access to greater quantity and diversity of dietary carotenoids had lower enzymatic antioxidant activity (SOD) (Linan-Cabello *et al*., 2003). Recently, immunological activity of carotenoids are believed to result from the provitamin A activity and/or retiniods activity. Linan-Cabello *et al*. (2002) have proposed that the bioactive roles of carotenoids is resided to a large degree to their conversion to retinoids that are involved in the activation of hormonal nuclear receptors. Retinoids play a prominent role in many developmental processes, including embryonic development and differentiation of various cell types. Amar *et al*. (2001) reported that the production of reactive oxygen species by head kidney leukocytes and plasma total immunoglobulin levels did not vary with the groups of rainbow trout fed test diet supplemented with 100 ppm astaxanthin, canthaxanthin and β-carotene. But serum complement activity in both β-carotene groups and the astaxanthin group were significantly higher than both the control fish. Serum lysozyme activity in the vitamin-containing β-carotene and astaxanthin groups were higher than control groups. Phagocytic activity was also high in the fish fed vitamin- containing β-carotene and astaxanthin compared with the controls. The vitamin-containing canthaxanthin gave better phagocytic index compared with the controls. The vitamin-containing astaxanthin and β-carotene groups exhibited better nonspecific cytotoxicity for the peripheral blood lymphocytes.

From the analysis of carotenoid in shrimp body in terms of total carotenoid, free astaxanthin and astaxanthin esters, the results shown that 3% freeze-dried *Spirulina* was the most effective material for increasing free astaxanthin in shrimp body compared to the group fed 100 ppm astaxanthin. The major carotenoids in *Spirulina platensis* are β-carotene and zeaxanthin (Borowitzka and Borowitzka, 1988), and both carotenoids were

readily converted into astaxanthin and deposited in black tiger shrimp (Liao *et al*., 1993; Boonyaratpalin *et al*., 2001). Normally, dietary astaxanthin and some carotenoids were absorbed and transformed into both, astaxanthin ester and free astaxanthin which was combined with protein into shrimp body (Britton, 1996). However, all carotenoids analyzed data in the group fed 1-3% *Rhodobacter* sp. TM11B were not different to those in the shrimp fed control diet, the major carotenoids in photosynthetic bacteria mainly composed of the non-cyclic carotenoids, such as spheroidene and spheroidenone which can not be converted to astaxanthin in any reported pathway (Britton, 1996). However, some carotenoid presented in the photosynthetic cell materials are absorbed and transferred to shrimp tissue. These may be act as the antioxidant, which allow other cyclic carotenoids remained and accumulated in the shrimp body as found in the group fed 5% of *Rhodobacter* sp. TM11B which is the highest level of photosynthetic bacterial cell supplementation in this study. This phenomenon was also reported in the application of *Rhodobacter capsulatus* as bioencapsulation feed for juvenile Japanese flounder (Okimasa *et al.*, 1992). They found that the amount of lipid peroxide in flounder fed rotifers reared with photosynthetic bacteria was lower than that in the control group. These findings suggested that the photosynthetic bacterial material inhibited the initiation and progression of lipid peroxidation of egg phosphatidylcholine liposomes. However, the chromatographic procedures they used gave only fractions and not pure, characterized pigments. Moreover, Pradal (1993) reported that the rainbow trout fed test diet incorporated of 0.01 % *Rhodobacter capsulatus* have similar body weight as those fed the unsupplemented diet or the diet supplemented with astaxanthin. Moreover, the coloration of the rainbow trout flesh was almost at the same level as that of fish fed with diet supplemented with astaxanthin. However, the pigment responsible for this observed coloration was not identified. The results of these study was found a slightly increment of total carotenoids and di-ester astaxanthin were

observed in the shrimp fed 5% *Rhodobacter* sp. TM11 B when compared to the control group. However, the efficiency of this photosynthetic baterial isolate is much lower than *Spirulina* and synthetic astaxanthin-supplemented diet. However, the application of dried *Spirulina* in shrimp feed will be increase the production cost. By the economically reason, it is nescessary to study on other natural source of carotenoids compared to the *Spirulina*-supplemented diet, in order to improve body color and reduced the feeding cost in black tiger shrimp.

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