# CHAPTER III

# EFFECTS OF SYNTHETIC ASTAXANTHIN AND PHOTOSYNTHETIC BACTERIAL CELLS ON GROWTH PERFORMANCE, PIGMENTATION AND IMMUNE RESPONSES IN BLACK TIGER SHRIMP (*PENAEUS MONODON*)

## Introduction

The natural color of shrimps and other crustaceans is usually due to carotenoids, especially to astaxanthin and its complexes with protein. In aquaculture, the desirable color must be reproduced for acceptability in international markets. This can be achieved by feeding astaxanthin in the last 6-8 weeks of culture. However, supplementation of shrimp feed with synthetic astaxanthin leads to 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) and the alga *Haematococcus* (Sommer *et al*., 1991). However, dietary β-carotene has been reported to be converted into astaxanthin and deposited in the shrimp body (Tanaka *et al.*, 1976) though different results 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). Natural 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. Kobayashi and Kurata (1978) reported on the use of photosynthetic bacterial cells as an animal feed ingredient and source of single cell protein (Varati, 1984; Noparatnaraporn and Nagai, 1986). Photosynthetic bacteria also contain high level of carotenoids (Prasertsan *et al*., 1993; Tanaka *et al*., 1994). However, no report on the application of photosynthetic bacteria as a carotenoid source in black tiger shrimp has been made. In this work, shrimp feed was supplemented with cell material from four strains of photosynthetic bacteria or with synthetic astaxanthin (CAROPHILL pink®, Roche) as a control, in order to evaluate the effects of these supplements on growth performance, pigmentation and

immune response in black tiger shrimp. The photosynthetic bacteria used (*Rhodobacter*) do not contain astaxanthin, but have high concentration of acyclic carotenoids such as spheroidene and spheroidenone. It is highly unlike that these carotenoids could be converted into astaxanthin by any metabolic process, but they could possibly be incorporated to some extent into shrimp body.

**Materials and Methods**

**Test animals**

The experiment was carried out at the Aquatic Science Research Station, Department of Aquatic Science, Faculty of Natural Resources, Prince of Songkla University, Thailand. Twenty - thousand high quality black tiger shrimp larva (PL-15) were grown on commercial shrimp feed with minced fresh fish in a 10 tons concrete tank with 15 ppt seawater. After the shrimp attained a weight of 0.5-1 g, they were graded to obtain approximate size of 1 g and transferred to 250 l glass aquaria at a density of 20 shrimp / aquarium. The system was equipped with a 200 l filtration tank and the temperature was kept constant at 28 $\pm$ 0.5 $^{0}$C. 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

Four photosynthetic bacteria (PSB) isolated from shrimp ponds in Songkhla and Satun province (*Rhodobacter sphaeroides* SV-2, *Rhodobacter sphaeroides* TN5, *Rhodobacter* sp. TM3 and *Rhodobacter* sp. TM11B) were used. The pigment compositions (Spheroidene series) and other features of these strains are characteristic of *Rhodobacter* species or similar member of the Rhodospirillaceae. The bacteria were grown in

G5 medium under anaerobic conditions with illumination of 1,500 lux for 72 hr (Prasertsan *et al.*, 1993). 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 each 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 3-6 contained 5% of lyophilized photosynthetic bacterial strain (Table 3-1). 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 by added distilled water to contain 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.

**Table 3-1** Feed formulation of test diets.

| **% in diet** | **T1 control** | **T2 100 ppm astaxanthin** | **T3 5 % PSB SV2** | **T4 5 % PSB TN5** | **T5 5% PSB TM 3 T** | **T6 5% PSB TM 11 B** |
|---|---|---|---|---|---|---|
| Fish meal | 34 | 34 | 29 | 29 | 29 | 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 flour | 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 |
| CAROPHIL L pink® | - | 0.125 | - | - | - | - |
| PSB | - | - | 5 | 5 | 5 | 5 |
| BHT | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
| **Total** | 100 | 100 | 100 | 100 | 100 | 100 |

**Table 3-1 (continued)**

| **Analysis of chemical composition in test diet** | | | | | | |
|---|---|---|---|---|---|---|
| **% in diet** | **T1 control** | **T2 100 ppm astaxanthin** | **T3 5 % PSB SV2** | **T4 5 % PSB TN5** | **T5 5% PSB TM 3 T** | **T6 5% PSB TM 11 B** |
| Moisture (%) | 6.43 | 6.23 | 6.52 | 6.78 | 6.12 | 6.31 |
| Protein (%) | 39.51 | 39.43 | 39.71 | 39.22 | 39.33 | 39.41 |
| Fat (%) | 7.75 | 7.63 | 7.49 | 7.53 | 7.80 | 7.55 |
| Ash(%) | 7.08 | 7.12 | 6.21 | 6.45 | 6.52 | 6.62 |
| Total carotenoid (ppm) | - * | 78.2 | 133.6 | 144.6 | 143.7 | 122.0 |

* Below of detection limit

**Growth performance and body color**

The shrimp in each treatment (7 replicates), were fed the 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, feed conversion rate (FCR) and color changes. 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 by scoring with Salmo fan$^{TM}$ (Roche) by the method modified from Boonyaratpalin *et al*. (2001).

**Carotenoid analysis in shrimp**

After 8 weeks of feeding, 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 300-900 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 separated 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 photosynthetic bacterial cell on blood parameters**

After 8 weeks of culture, 10 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$^{TM}$ 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 analysed by the Lowry method (Lowry *et al.*, 1951). Superoxide dismutase activity was expressed as unit/min/mg-protein.

## 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).

## Results

## Effects of photosynthetic bacterial cells on growth and pigmentation

## Growth performances

After 2-8 weeks of the feeding trial, the average body weights of shrimp fed with control diet, 100 ppm synthetic astaxanthin or 5% *Rhodobacter* sp. TM3 supplemented diet were significantly higher than others ($P<0.05$) (Table 3-3). At the end of the experiment, average body weight, weight gain and FCR were significantly different ($P<0.05$). Shrimp fed with control diet or with 100 ppm synthetic astaxanthin had the highest growth performance (average body weight, weight gain and FCR). Whereas, it was not different between the groups fed with 5% *Rhodobacter* sp. TM3 or *Rhodobacter* sp. TM11 B. The lowest growth performance was found in the shrimp fed with *Rhodobacter sphaeroides* SV2 and *Rhodobacter sphaeroides*

TN5. There were no significant different in survival and feed intake among the treatments. (Table 3-4)

**Pigmentation**

Shrimp in all treatments were pale-blue to greenish-blue before the start of the experiment. After 1 week of feeding, the shrimp fed with diet containing 100 ppm synthetic astaxanthin exhibited dark blue coloration compared to the others, By week 3, dark-green to brown coloration was observed in shrimp fed with synthetic astaxanthin, while the color of the shrimp in other groups was not different from that of the control. At termination of the feeding trial, the shrimp fed with diet supplemented with 100 ppm synthetic astaxanthin were dark brown, shrimp fed with diet containing 5% PSB strain *Rhodobacter* sp. TM11 B were greenish-brown, but the others remained pale-blue (Figure 3-1). The colors of boiled shrimp from each treatment are shown in figure 3-2, Salmofan scores were highest in the group that were fed with synthetic astaxanthin (Table 3-2).

**Table 3-2** Salmofan scores of the shrimp fed the experimental diets for 8 weeks.

| | **Salmofan scores** |
|---|---|
| T1 (control) | 19-20 |
| T2 (100 ppm astaxanthin) | 29-30 |
| T3 (5% SV2) | 19-20 |
| T4 (5% TN5) | 19-20 |
| T5 (5% TM3) | 19-20 |
| T6 (5% TM11 B) | 21-22 |


T 1
T 2
T 3
T 4
T 5
T 6

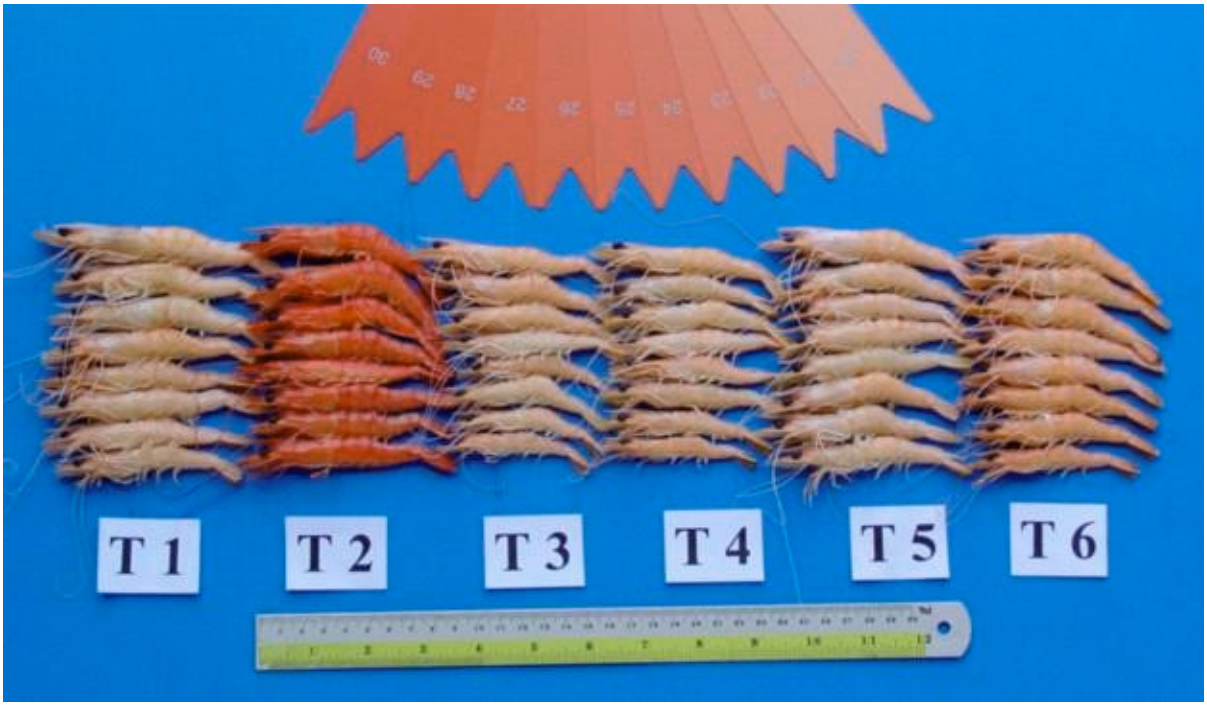



**Figure 3-1** Body color of shrimp fed with each experimental diet for 8 weeks (Upper : before boiling Lower : after boiling for 3 min.)

**Table 3-3** Average body weight (g) of shrimp fed with each experimental diet during 8 weeks of feeding period.

| | **0 weeks** | **2 weeks** | **4 weeks** | **6 weeks** | **8 weeks** |
|---|---|---|---|---|---|
| T1 (control) | 0.78 ± 0.01 $^{ns}$ | 1.51 ± 0.20 $^{a}$ | 2.70 ± 0.41 $^{ab}$ | 4.21 ± 0.49 $^{a}$ | 6.21 ± 0.65 $^{a}$ |
| T2 (100 ppm astaxanthin) | 0.78 ± 0.01 $^{ns}$ | 1.40 ± 0.09 $^{ab}$ | 2.85 ± 0.42 $^{a}$ | 4.29 ± 0.66 $^{a}$ | 5.64 ± 0.67 $^{a}$ |
| T3 (5% PSB SV2) | 0.77 ± 0.00 $^{ns}$ | 1.24 ± 0.18 $^{bc}$ | 1.94 ± 0.25 $^{c}$ | 2.95 ± 0.33 $^{bc}$ | 3.56 ± 0.19 $^{c}$ |
| T4 (5% PSB | 0.78 ± 0.01 $^{ns}$ | 1.15 ± 0.06 $^{c}$ | 1.95 ± 0.12 $^{c}$ | 2.75 ± 0.33 $^{c}$ | 3.49 ± 0.56 $^{c}$ |

| | | | | | |
|---|---|---|---|---|---|
| TN5) | | | | | |
| T5 (5% PSB TM3) | 0.78 ± 0.01 $^{ns}$ | 1.38 ± 0.13 $^{ab}$ | 2.35 ± 0.38 $^{bc}$ | 3.58 ± 0.52 $^{b}$ | 4.65 ± 0.45 $^{b}$ |
| T6 (5% PSB TM11 B) | 0.78 ± 0.01 $^{ns}$ | 1.25 ± 0.12 $^{bc}$ | 2.05 ± 0.21 $^{c}$ | 3.33 ± 0.40 $^{bc}$ | 4.39 ± 0.50 $^{b}$ |

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

**Table 3-4** Growth performance of the shrimp fed with each experimental diet for 8 weeks.

| | **Average weight (g)** | **Weight gain (%)** | **Survival (%)** | **FCR** | **Feed intake (g/shrimp)** |
|---|---|---|---|---|---|
| T1 (control) | 6.21 ± 0.65 $^{a}$ | 801.52 ± 79.81 $^{a}$ | 50.00 ± 13.69 $^{ns}$ | 1.16 ± 0.09 $^{c}$ | 6.24 ± 0.48 $^{ns}$ |
| T2 (100 | 5.64 ± | 725.27 ± | 46.00 ± | 1.35 ± | 6.44 ± |

| | | | | | |
|---|---|---|---|---|---|
| ppm astaxanthin) | 0.67 [a] | 87.11 [a] | 10.84 [ns] | 0.28 [bc] | 0.79 [ns] |
| T3 (5% SV2) | 3.56 ± 0.19 [c] | 459.01 ± 24.31 [c] | 47.00 ± 8.37 [ns] | 2.10 ± 0.27 [a] | 5.82 ± 0.36 [ns] |
| T4 (5% TN5) | 3.49 ± 0.56 [c] | 449.19 ± 71.95 [c] | 47.00 ± 12.04 [ns] | 2.11 ± 0.65 [a] | 5.49 ± 0.83 [ns] |
| T5 (5% TM3) | 4.65 ± 0.45 [b] | 597.57 ± 59.83 [b] | 49.00 ± 10.84 [ns] | 1.59 ± 0.21 [bc] | 6.08 ± 0.52 [ns] |
| T6 (5% TM11 B) | 4.39 ± 0.50 [b] | 564.08 ± 64.23 [b] | 40.00 ± 7.07 [ns] | 1.72 ± 0.31 [ab] | 6.11 ± 0.49 [ns] |

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

**Carotenoid analysis**

The carotenoid contents of the shrimp are summarized in Table 3-5. Total carotenoid and concentration of astaxanthin were highest in the shrimp fed the diet supplemented

with synthetic astaxanthin. Except for *Rhodobacter* sp. TM11 B, the total carotenoid content in the groups fed with all strains of photosynthetic bacteria was not significantly different from that of the control group ($p>0.05$). These was more carotenoid present in the shrimp fed with *Rhodobacter* sp. TM11 B when compared to the control group. However, the increase was due to the presence of some unidentified carotenoids that appear to be derived from the bacteria, and not due to an increase in free or esterified astaxanthin.

**Effects of the photosynthetic bacterial cell on blood parameters**

Total hemocyte count and superoxide dismutase activity were not significant different among the groups (Table 3-6). Phenoloxidase activity in hemocytes from shrimp fed with test diet containing 5% *Rhodobacter* sp. TM3 or *Rhodobacter* sp. TM11 B was significantly higher than in the others ($P<0.05$). There was a marked reduction in total antioxidant status in hemolymph in shrimps fed with the control diet or 5% *Rhodobacter sphaeroides* SV2 ($P<0.05$) (Table 3-6).

**Table 3-5** Total carotenoid, free astaxanthin, astaxanthin mono-ester and di-ester in shrimp fed with each experimental diet for 8 weeks.

| | **Total carotenoid (ppm)** | **Free astaxanthin (ppm)** | **Mono-ester astaxanthin (ppm)** | **Di-ester astaxanthin (ppm)** |
|---|---|---|---|---|
| T1 (control) | 9.59 ± 3.46 $^{b}$ | 5.23 ± 0.85 $^{c}$ | 1.32 ± 0.16 $^{b}$ | 1.51± 0.21 $^{b}$ |
| T2 (100 ppm astaxanthin) | 121.96 ± 42.91 $^{a}$ | 36.27 ± 0.04 $^{a}$ | 34.70 ± 0.93 $^{a}$ | 31.57 ± 1.48 $^{a}$ |
| T3 (5% SV2) | 10.22 ± 0.72 $^{b}$ | 4.80 ± 0.34 $^{c}$ | 1.12 ± 0.13 $^{b}$ | 0.87 ± 0.12 $^{b}$ |
| T4 (5% TN5) | 11.39 ± 1.96 $^{b}$ | 4.91 ± 0.17 $^{c}$ | 0.69 ± 0.09 $^{b}$ | 1.10 ± 0.01 $^{b}$ |
| T5 (5% TM3) | 9.49 ± 0.32 $^{b}$ | 3.15 ± 0.12 $^{d}$ | 0.65 ± 0.14 $^{b}$ | 1.05 ± 0.02 $^{b}$ |
| T6 (5% TM11 B) | 25.61 ± 0.86 $^{b}$ | 7.77 ± 0.76 $^{b}$ | 1.44 ± 0.12 $^{b}$ | 1.88 ± 0.19 $^{b}$ |

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

**Table 3-6** Total hemocytes, phenoloxidase activity, superoxide dismutase activity and total antioxidant status of the shrimp fed with each experimental diet for 8 weeks.

| | **THC ($x10^4$ cell / $mm^3$)** | **PO (U/min/mg prot.)** | **SOD (U / mg prot.)** | **TAS (mmole/l)** |
|---|---|---|---|---|
| T1 (control) | 5.90 ± 2.20 $^{ns}$ | 782.28 ± 103.24 $^{b}$ | 6.40 ± 6.62 $^{ns}$ | 0.38 ± 0.26 $^{bc}$ |
| T2 (100 ppm astaxanthin) | 6.11 ± 2.75 $^{ns}$ | 876.29 ± 329.14 $^{b}$ | 12.76 ± 6.87 $^{ns}$ | 0.69 ± 0.41 $^{ab}$ |
| T3 (5% SV2) | 6.18 ± 3.66 $^{ns}$ | 1032.01 ± 364.04 $^{ab}$ | 4.88 ± 7.60 $^{ns}$ | 0.27 ± 0.15 $^{c}$ |
| T4 (5% TN5) | 6.21 ± 1.76 $^{ns}$ | 951.43 ± 300.42 $^{b}$ | 5.06 ± 7.90 $^{ns}$ | 0.60 ± 0.41 $^{ab}$ |
| T5 (5% TM3) | 6.68 ± 2.05 $^{ns}$ | 861.41 ± 192.86 $^{b}$ | 13.64 ± 17.58 $^{ns}$ | 0.67 ± 0.32 $^{ab}$ |
| T6 (5% TM11 B) | 7.13 ± 1.98 $^{ns}$ | 1211.68 ± 298.89 $^{a}$ | 5.55 ± 3.30 $^{ns}$ | 0.75 ± 0.18 $^{a}$ |

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

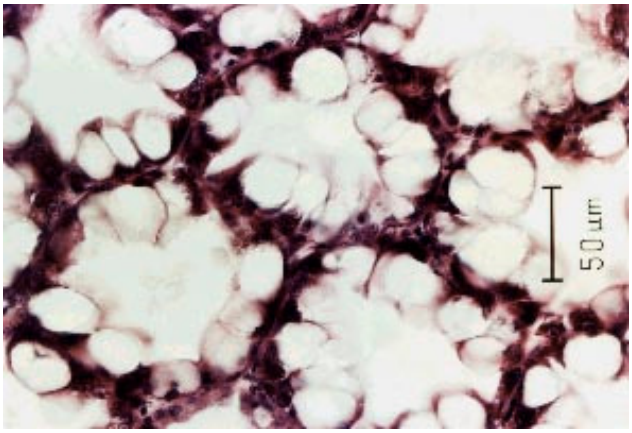
## Histological changes

At the end of the feeding trial, histological changes in the hepatopancreas were found in the shrimp fed with test diet containing 5% *Rhodobacter sphaeroides* SV2 or *Rhodobacter sphaeroides* TN5. The levels and frequency of histological changes are shown in Table 3-7. Twenty five percent of histological samples from the *Rhodobacter sphaeroides* SV2-supplemented and 10 % from *Rhodobacter sphaeroides* TN5 supplemented exhibited signs of atrophy in the tubular epithelium of the hepatopancreas (Fig 3). About 25 % and 20 % of the *Rhodobacter sphaeroides* SV2 and *Rhodobacter sphaeroides* TN5 groups showed cell necrosis and degeneration of tubules with the infiltration of haemocytes (Fig. 3-4). The shrimp fed with the control, or supplemented with 100 ppm astaxanthin, *Rhodobacter* sp. TM3 or *Rhodobacter* sp. TM11 B showed no sign of histological changes.

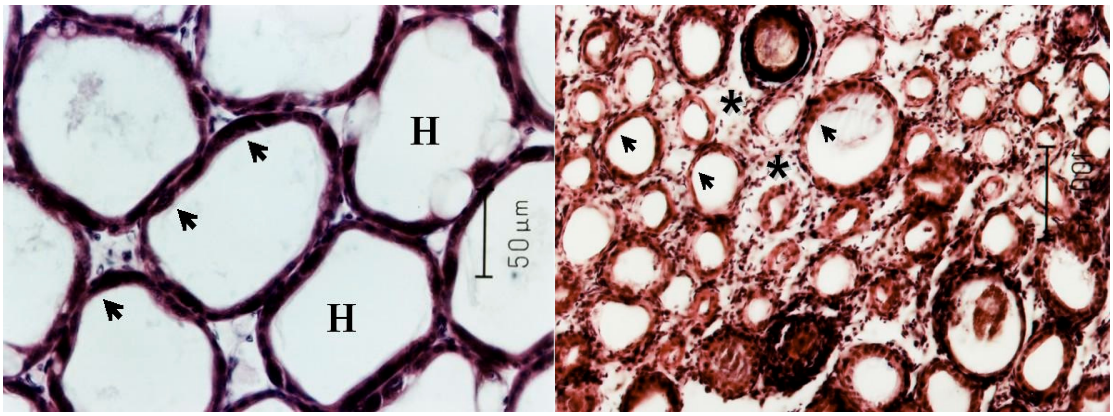
**Table 3-7** Levels and frequency of histological changes (%) in black tiger shrimp fed with each experimental diet for 8 weeks.

| **Levels of histological changes** | **Test diets** | | | | | |
|---|---|---|---|---|---|---|
| | **T1 control** | **T2 100 ppm astaxanthin** | **T3 5 % PSB-SV2** | **T4 5 % PSB-TN5** | **T5 5 % PSB-TM3** | **T6 5 % PSB-TM11B** |
| 0 | 100 % | 100 % | 33.33 % | 70 % | 100 % | 100 % |
| 1 | - | - | 25 % | 10 % | - | - |
| 2 | - | - | 25 % | 20 % | - | - |
| 3 | - | - | 16.67 % | | - | - |

0 = normal
1 = atrophic changes of the epithelium in hepatopancreatic tubules
2 = cell necrosis and hemocytic infiltration in hepatopancreas
3 = degeneration of hepatopancreas
- = no sign of histological change




**(a)**




**(b)** **(c)**

**Figure 3-2** (a) Normal hepatopancreatic epithelium in shrimp fed control diet (b) Atrophic changes of tubular epithelium of hepatopancreas (Arrow heads) (c) Cell necrosis and degeneration of tubules with the infiltration of haemocytes into intertubular space (Asterisks) in shrimp fed with *Rhodobacter sphaeroides* SV2 and *Rhodobacters sphaeroides* TN5 (H = Hepatopancreatic tubules). (Hematoxylene & Eosin staining, H & E)

## Discussion

Of the materials studied, synthetic astaxanthin is the most effective substance for pigmentation in penaeid shrimp, as reported by Yamada *et al.* (1990) and Chein and Jeng (1992). Tanaka *et al.* (1976) reported a metabolic pathway, which converted β-carotene into astaxanthin *via* canthaxanthin or zeaxanthin in kuruma shrimp, but a later study found that kuruma shrimp have only a low capability to use β-carotene and canthaxanthin as carotenoid source (Yamada *et al*., 1990). This is markedly different from the black tiger shrimp, which has a high efficiency for using β-carotene from *Dunaliella* extract as carotenoid source and converting it into astaxanthin (Boonyaratpalin *et al.*, 2001). Moreover, Liao *et al.* (1993) found a similar phenomenon in shrimp fed with diets containing β-carotene, *Spirulina*, *Phaffia* yeast or krill oil. The highest increase of carotenoid content in the carapace was observed in the group fed with *Spirulina* supplemented diets. Our study indicates a marked reduction in growth performance in shrimp fed a diet supplemented with photosynthetic bacteria, especially with strains *Rhodobacter sphaeroides* SV2 and *Rhodobacter sphaeroides* TN5. One explanation of this finding may be the high substitution of PSB to fishmeal in test diet, which affects palatability or the amino acid composition of the test diet. Liao *et al*. (1993) reported a marked retardation of growth in shrimp receiving a diet containing 5 % *Spirulina*. Normally, the imbalance of amino acid in fish feed can be improve by the supplementation of crystalline amino acid into the test diets (Yamamoto *et al*., 2005). Moreover, some strains of PSB may produce or transfer gene of an anti-nutritional factor or intracellular microbial toxin, which may cause pathological

changes in the excretory and digestive system (Allgarier *et al*., 2003). High supplementation of single cell protein also showed a negative effect on growth in rainbow trout (Kiessling and Askbrandt, 1993), when commercially available bacterial protein products derived from *Brevibacterium lactofermentum* and *Bacterium glutamaticum* were substituted for fish meal in test diets. The results indicated that growth of the fish was depressed with increasing levels of *Bacterium glutamaticum*. Moreover, the low hepatosomatic index (HIS) and gonadosomatic index (GSI) found in the groups fed with diet with added *Bacterium glutamaticum* could be argued to indicate a metabolic disorder caused by an unknown toxic compound in this single cells protein. In another study, Pradal (1993) examined the coloration and growth of rainbow trout, using diet supplemented with synthetic astaxanthin or the photosynthetic bacterium *Rhodobacter capsulatus* compared to unsupplemented diet. It was found that 0.01 % incorporation of *Rhodobacter capsulatus* was able to increase fish growth compared with 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. The pigment responsible for this observed coloration was not identified.

Carotenoid pigments have been reported as immunostimulants in higher animal (Bendich and Shapiro, 1986; Jyonouchi *et al*., 1993; Thompson *et al*., 1995). The antioxidant property of astaxanthin and other carotenoids has also been commented on (Latscha, 1991; Hunter, 2000). In our study, dietary astaxanthin showed no effect on the hemocyte number and phenoloxidase activity in black tiger shrimp. Supplementation with *Rhodobacter sphaeroides* SV2 or *Rhodobacter* sp. TM11 B, however, increased the activity of phenoloxidase. Such enzymes may be activated by various microbial cell wall components such as lipopolysacharide, peptidoglycan and β-glucan (Johansson and Soderhall, 1985;

Itami *et al*., 1998). Moreover, several substances such as lipids, detergents and toxins, can also stimulate phenoloxidase activity (Soderhall and Cerenius, 1992). Yano *et al*. (1989) reported that the structural composition of the glucan from different fungi affects on the potency in enchancing disease resistance in common carp. Schizophyllan derived from *Schizophyllum commune* showed the highest efficiency for improving phagocytic activity of pronephros cells when compared with scleroglucan and lentinan. The alternative complement pathway (ACP) activity, was also highest in carp injected with the suspension of schizophyllan. Moreover, Robertsen *et al*. (1990) compared the efficiency of M-glucan and DL-glucan for enhancement of disease resistance in Atlantic salmon and demonstrated that the protective effect of DL-glucan was much lower than that of M-glucan. Therefore, it is possible that a high phenoloxidase activity in shrimp fed with only photosynthetic bacterial cell strain *Rhodobacter sphaeroides* SV2 or *Rhodobacter* sp. TM11 B may be due to a response of some specific structural cell wall components or specific foreign substances produced by the bacterial strains.

Chien *et al.* (2002) found that total antioxidant status was improved and superoxide dismutase was reduced in black tiger shrimp fed with diet containing with 80 ppm astaxanthin for 8 weeks. These findings correlated with our study, which showed that all carotenoid supplemented diets (except *Rhodobacter sphaeroides* SV2 and *Rhodobacter sphaeroides* TN5, which caused histological changes) increased the total antioxidant status in plasma but had no effect on superoxide dismutase. Our result was also in agreement with Okimasa *et al.* (1992) who studied the effect of fractions of *Rhodobacter capsulatus* extract on the lipid peroxide in the juvenile Japanese flounder. They found that in the group administered rotifers reared with photosynthetic bacteria, the amount of lipid peroxide in the fish on day 30 after starting the feed was lower than that in the control group. They suggested that the photosynthetic bacterial pigments inhibited the initiation and progression of lipid

peroxidation of egg phosphatidylcholine liposomes. However, the chromatographic produced they used gave only fractions and not pure, characterized pigments.

From our study, *Rhodobacter* sp. TM11 B did show a positive effect on coloration, total antioxidant status and phenoloxidase activity. The slight increase in color and total carotenoid content was not due to an increased amount or concentration of astaxanthin but to the presene of some other carotenoids that may be derived from the pigments in the bacteria. However, high-supplementation with dietary bacterial cells may cause retardation of growth in aquatic animals, due to the lack of palatability, imbalance of amino acid profile, or the production of anti-matabolites or toxin during fermentation (Watanabe *et al.*, 1990 ; Kiessling and Askbrandt, 1993). Hence, a need for optimization of growth and nutrition value of photosynthetic bacteria and for further studies on the optimal level of photosynthetic bacterial cells in shrimp diet, for most beneficial effect or immunostimulation and perhaps coloration without retardation of growth. However, the preparation of mechanically broken cells (by sonication), which was used in our study causes a high production cost. It would also be necessary in further studies to find a suitable pretreatment method for application in the industrial scale.

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