CHAPTER VI

EFFECT OF VARIOUS CAROTENOIDS ON GROWTH PERFORMANCE, IMMUNE RESPONSE AND DISEASE RESISTANCE IN THE BLACK TIGER SHRIMP

Introduction

Carotenoid pigments play an important roles in aquaculture. These include using of pigments in the diet of aquarium fish, salmon culture and others aquatic animal cultures (Simpson *et al.*, 1981; Sommer *et al.*, 1991). Shrimp culture is becoming a big industry worldwide and carotenoids such as astaxantine and β-carotene from Dunaliella were proved to enhance the body color and value of shrimp (Chein and Jeng, 1992).

Tanaka *et al.* (1978) and D' Abramo *et al.* (1983) have been demonstrated that many carotenoid pigments were converted to astaxanthin in kuruma shrimp (*Penaeus japonicus*) and several attempt have been made to investigate on the utilization of various carotenoid sources in shrimp. From previous study dietary synthetic astaxanthin, canthaxanthin and β-carotene were deposited as astaxanthin in shrimp body, but astaxanthin was the most effective substance for pigmentation (Yamada *et al.*, 1990). However, the effect of synthetic β-carotene (Lucarotin®) on pigmentation of black tiger shrimp (*Penaeus monodon*) has not been studied. Since synthetic β-carotene is much cheaper than canthaxanthin and astaxanthin, it would have economical benefit to use synthetic β-carotene as a carotenoid source in shrimp feed. Moreover, carotenoids supplemented in feed can enhance immune function and disease resistance in higher animals and fish (Latscha, 1991; Estermann, 1994). Little information is available for aquatic animal, especially shrimp. In this study, the different carotenoids will be used in shrimp feed and effects of these substances on health

condition, immune function and disease resistance in black tiger shrimp will be evaluated.

Materials and methods

Test animal

Trial 1 : Small shrimp

High quality PL-15 were grown on commercial shrimp feed for one and a half month until weight attained 0.5-1 g, then grade to the same size and were kept in 5-ton tanks equipped with seawater follow-through system for further use.

Trial 2 : Juvenile shrimp

Healthy shrimp with a weight range of 10-12 g (approximately 2 mouths old) were purchased from a shrimp farm where no severe disease outbreak has been recorded. Hundred shrimps were stocked in 3-ton tank equipped with aeration and seawater flow-through system. Before start of the experiment the shrimp will be fed with commercial shrimp feed 5 time daily until satiation.

Test diet

Isonitrogenous and isocaloric diets which containing 45% protein and 12% fat (Boonyaratpalin *et al.*, 2000) was formulated to obtain equal amounts of all ingredients except the sources of carotenoid as follows

Test diet for small shrimp : Six isonotrogenous and isocaloric experimental diets were fed to five replicates per treatment of small shrimp for a period of 8 weeks. Diet 1 was negative control without carotenoid while diet 2 to 6 contain 50 ppm astaxanthin (Lucanthin® pink, BASF), 125 ppm β-carotene (Lucarotin®, BASF), 200 ppm β-carotene (Lucarotin®, BASF), 125 ppm Betatene® (β-carotene extract from *Dunaliella* sp., Cognis) and 3 % dried spirulina.

Test diet for juvenile shrimp : The six diets were fed to juvenile shrimp for a period of 5 weeks. Diet 1 was negative control without carotenoid while diet 2 to 6 contain 100 ppm astaxanthin (Lucanthin® pink, BASF), 125 ppm β-carotene (Lucarotin®, BASF), 250 ppm β-carotene (Lucarotin®, BASF), 250 ppm Betatene® (β-carotene extract from *Dunaliella* sp., Cognis) and 6 % dried spirulina.

The test diets for the small shrimp and the juvenile shrimp trial were processed by a meat grinder and was 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 C until the moisture content is lower than 10%. The dry pellets were kept in two layer plastic bags in a refrigerator until use. The composition and nutrient content of the test diets are shown in Table 6-1 and 6-2. After feed preparation all test diet were sampled for analysis of carotenoids (astaxanthin, β-carotene and canthaxanthin) by BASF laboratory (Table 6-4 and 6-5). The test diets were sample and extracted of the total carotenoids by the method described by Britton *et al.* (1995) follow by the modification processes by the BASF Fine Chemical Division (1998, 1999). For the analysis of β-carotene and canthaxanthin, the grinded samples were added with 1 g EDTA and digested with the mixture of 20 mg pronase, 50 mg trypsin and 50 mg pepsin at 50^0 C for 10 min. Add 2:5:1 of ethanol : cyclohexane : ethyl acetate 140 ml mixed for 10 min. The non-polar phase were separated by washed with 70 ml of saturated NaCl solution, the upper phase were collected and

filtered before injected (20 ul) to the HPLC system (column : Lichrosorb Si 60, Merck mobile phase : 5:1 cyclohexane : ethyl acetate) with the flow rate of 0.65 ml/min. Each carotenoid were detacted by UV-VIS detector (Visio-Duo, Linear Instrument Corp.) at 470 nm the concentration of each carotenoid were calculated by the calibration solution of β-carotene and canthaxanthin, respectively. For the analysis of astaxanthin, 10 g of the grinded samples were extracted with 2 : 5 of ethanol : butyl methyl ether 140 ml and mixed for 10 min. The non-polar phase were separated by washed with 70 ml of saturated NaCl solution, the upper phase were collected and evaporated over sodium sulphate and then added with 86 : 14 of n-heptane : acetone, filtered and injected into the HPLC system (column : Lichrosorb Si 60, Merck mobile phase : 86 : 14 of n-heptane : acetone) with the flow rate of 2 ml/min. The astaxanthin were detected by the photodiode array detector at 470 nm, the concentration of astaxanthin were calculated from the internal standard.

Table 6-1 Composition of the test diet for small shrimp (1-5g) in trial 1.

Ingradient	Percent in diet					
	1	2	3	4	5	6
Fish meal	28	28	28	28	27.8	25
Shrimp head meal	10	10	10	10	10	10
Squid meal	5	5	5	5	5	5
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	10.1	10.05	9.975	9.9	9.675	10.1

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	4	4	4	4	4	4
BHT	0.02	0.02	0.02	0.02	0.02	0.02
Zeolite	1.5	1.5	1.5	1.5	1.5	1.5
Lucantinpink (10%)	0	0.05	-	-	-	-
Lucarotene (10%)	0	0	0.125	0.20	-	-
Betatene (2%)	0	0	0	0	0.625	-
Spirulina (%)	0	0	0	0	0	3
Total	**100**	**100**	**100**	**100**	**100**	**100**

Table 6-2 Composition of the test diet for juvenile shrimp (>10g) in trial 2.

Ingradient	Percent in diet					
	1	2	3	4	5	6
Fish meal	25	25	25	25	24.6	19
Shrimp head meal	10	10	10	10	10	10
Squid meal	5	5	5	5	5	5
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	13.1	13	12.975	12.85	12.25	13.1
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	4	4	4	4	4	4
BHT	0.02	0.02	0.02	0.02	0.02	0.02
Zeolite	1.5	1.5	1.5	1.5	1.5	1.5
Lucantinpink (10%)	0	0.1	0	0	0	0
Lucarotene (10%)	0	0	0.125	0.25	0	0
Betatene (2%)	0	0	0	0	1.25	0
Spirulina	0	0	0	0	0	6
Total	**100**	**100**	**100**	**100**	**100**	**100**

Table 6-3 Analyzed of carotenoid compositions in each test diet for small shrimp in
trial 1.

	Astaxanthin (ppm)	Canthaxanthin (ppm)	β-carotene (ppm)
T1 control	0.4	< 0.2	-
T2 astaxanthin 50 ppm	42.4	< 0.5	-

T3 β-carotene 125 ppm	0.9	0.6	72.7
T4 β-carotene 250 ppm	0.6	0.8	130.0
T5 Betatene 125 ppm	0.5	0.8	51.3
T6 Spirulina 3 %	1.3	3.0	34.2

Table 6-4 Analyzed of carotenoid compositions in each test diet for juvenilel shrimp in trial 2.

	Astaxanthin (ppm)	Canthaxanthin (ppm)	β-carotene (ppm)
T1 control	0.7	< 0.3	-
T2 astaxanthin 50 ppm	93.1	1.1	-
T3 β-carotene 125 ppm	1.2	0.7	74.0
T4 β-carotene 250 ppm	0.3	0.8	101.0
T5 Betatene 125 ppm	0.4	1.1	71.2
T6 Spirulina 3 %	4.2	6.4	69.2

Growth performance analysis

The feeding trial for small shrimp experiment, the stocking rate was 20 shrimp per 200 L aquarium equipped with seawater flow-through system. Shrimp was fed to satiation 5 times daily for 8 weeks. Data for growth performance was recorded in the small shrimp experiment, *i.e.* growth, weight gain, survival, FCR and color changes. Data recorded in small shrimp was determined every two weeks for the 8 weeks periods. Disease resistance against white spot virus (by immersion) was performed by the end of 8 weeks in small shrimp. At termination of the experiment, 15 small shrimp were samples for preliminary evaluation on the effect of pigment source on pigmentation, the shrimp in each treatment were immersed in boiling water for 5 min and then comparison the color of boiled shrimp by scoring with Salmo fan™.

Carotenoid analysis

At termination of the experiment 15-20 shrimp in each treatment were frozen with liquid nitrogen and storage in -70 ^{0}C for analysis of carotenoid by BASF laboratory. The sample were freeze dried, grinded and extracted of the carotenoids by the conventional method (Britton *et al.*, 1995) with the enzymatic digestion which modified by BASF Fine Chemical Division (1998, 1999) as described above.

Immune function and disease resistance

The body color of juvenile shrimp after immersed in boiling water for 5 min was compared after 5 week of feeding period using salmo fan. Immune function and disease resistant were performed in the test group of juvenile shrimp as follows :

Total hemocyte count : Blood from each shrimp was collected by using a 1 ml syringe with 25 G needle from the base of a walking leg. After withdrawal, the hemocytes was diluted with 0.15% trypan blue solution and the number of hemocyte

from each shrimp was counted using hemacytometer and calculated as number of blood cell (total hemocytes) / mm^3.

Phenoloxidase activity : Fifteen shrimps from each experimental group were collected on week 5 after feeding with test diet. PO activity from hemocytes was measured by the following methods. Blood was collected from each shrimp using a 1 ml plastic syringe. L-cycteine was used as an anticoagulant (50 mg/ml). Hemocyte was washed with K-199 and hemocyte lysate was prepared in a cacodylate buffer pH 7.4 by using sonicator (Vibra Cell™) at 30 amplitude for 20 second. PO activity from hemocyte lysate was measured by modified method of Smith and Soderhall (1983). L-3,4-dihydroxyphenylalanine (L-DOPA) was used as a substrate and enzyme activity was measured at 630 nm. Protein content in hemocyte lysate was measured by using Lowry's method (Lowry *et al.*, 1951). PO activity was defined by the increase of OD /min/mg protein.

Ability to remove bacterial cell from the circulation system

At the end of the five week feeding period, 15 shrimp from each experimental group was determined for their ability to remove bacterial cell from the blood system. These parameter was determined by the modified method of Martin *et al.* (1993).

Bacterial suspension was prepared from 24 h cultured of *Vibrio harveyi*. Bacterial cell was adjusted to 2.7 $x10^7$ cell/ml in a sterile 1.5% NaCl solution. A bacterial cell suspension of 0.1 ml was injected into the tail muscle of each shrimp. Injected shrimp was kept in aquarium for 3 h. Blood was collected from each shrimp without an anticoagulant. A two-fold dilution of whole blood was made using sterile 2.5% NaCl solution and 20 ul of each dilution was dropped on PCA agar plus 1.5% NaCl. The number of bacterial cells in the hemolymph was reported as cfu/ml after 18-24 h of incubation periods at 35 ^{0} C.

Stress tolerance

Salinity stress was performed in small shrimp. The shrimp were immediately transferred from normal seawater (30 ppt) to 5 ppt for 5 h and return to normal condition. These processes were carried on for 5 consecutive days. Mortality was daily recorded in each group for 13 days.

Disease resistant

Disease resistant was performed in small and juvenile shrimp fed with experimental diet. After 5 weeks of feeding period, shrimp was injected with white spot virus suspension at 10^{-7} dilution (LD-50 of stock virus was 1×10^{-7}). Mortality was recorded for 10 days post injection.

Results

Trail 1 Experiment in small shrimp

There were not significantly difference in the average weight, weight gain, FCR and survival rates between the shrimp fed carotenoids supplemented diet and control shrimp after 8 weeks of feeding trial (Table 6-5). The survivals of shrimp injected with WSSV are shown in figure 6-1. There was not significantly different in survival of the shrimp after challenged with WSSV at a concentration of either $1 : 10^{7}$ and $1 : 10^{10}$. In stress test, survival rate of control shrimp was lower than carotenoids-fed shrimp during salinity stress for 10 days. However, it was not statistically difference among each treatment (Fig. 6-2).

Table 6-5 Growth performance of small shrimp (avg. weigh 1-5 g) after feeding with each carotenoid pigments for 8 weeks.

	Avg. weight (g)	Weight gain (%)	Survival rate (%)	FCR
T1 control	4.53 ± 0.82 ns	455.76 ± 62.29 ns	64.00 ± 15.17 ns	1.42 ± 0.20 ns
T2 astaxanthin 50 ppm	4.53 ± 0.63 ns	485.08 ± 77.71 ns	83.00 ± 9.75 ns	1.47 ± 0.21 ns
T3 β-carotene 125 ppm	4.69 ± 0.81 ns	468.27 ± 59.59 ns	70.00 ± 11.73 ns	1.40 ± 0.18 ns
T4 β-carotene 250 ppm	4.21 ± 0.63 ns	446.53 ± 66.63 ns	68.00 ± 16.81 ns	1.38 ± 0.22 ns
T5 Betatene 125 ppm	4.62 ± 0.42 ns	478.51 ± 23.16 ns	73.00 ± 13.04 ns	1.28 ± 0.08 ns
T6 Spirulina 3 %	4.63 ± 0.46 ns	479.17 ± 47.54 ns	73.00 ± 6.71 ns	1.37 ± 0.09 ns

ns = non significant

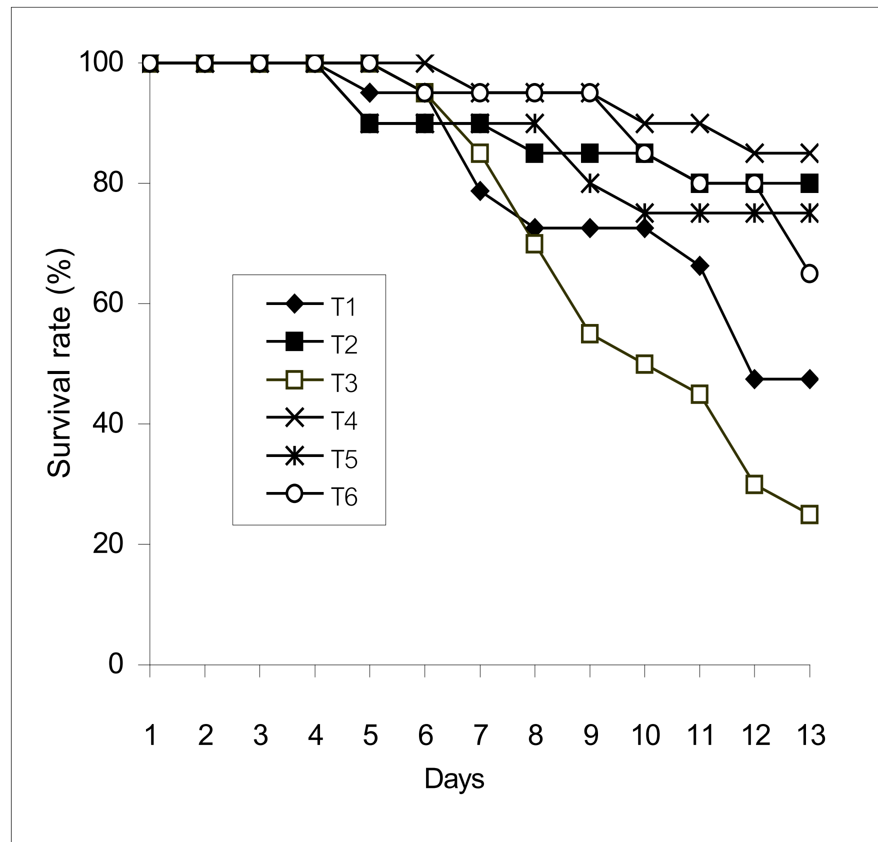

Figure 6-1 Survival rates of small shrimp fed diet containing with each carotenoid pigments after challenged with WSSV at a concentration of 1 : 10^7 for 13 days. (T 1=control, T2 to T6 = 50 ppm astaxanthin, 125 ppm β-carotene, 200 ppm β-carotene, 125 ppm Betatene® and 3 % dried spirulina)

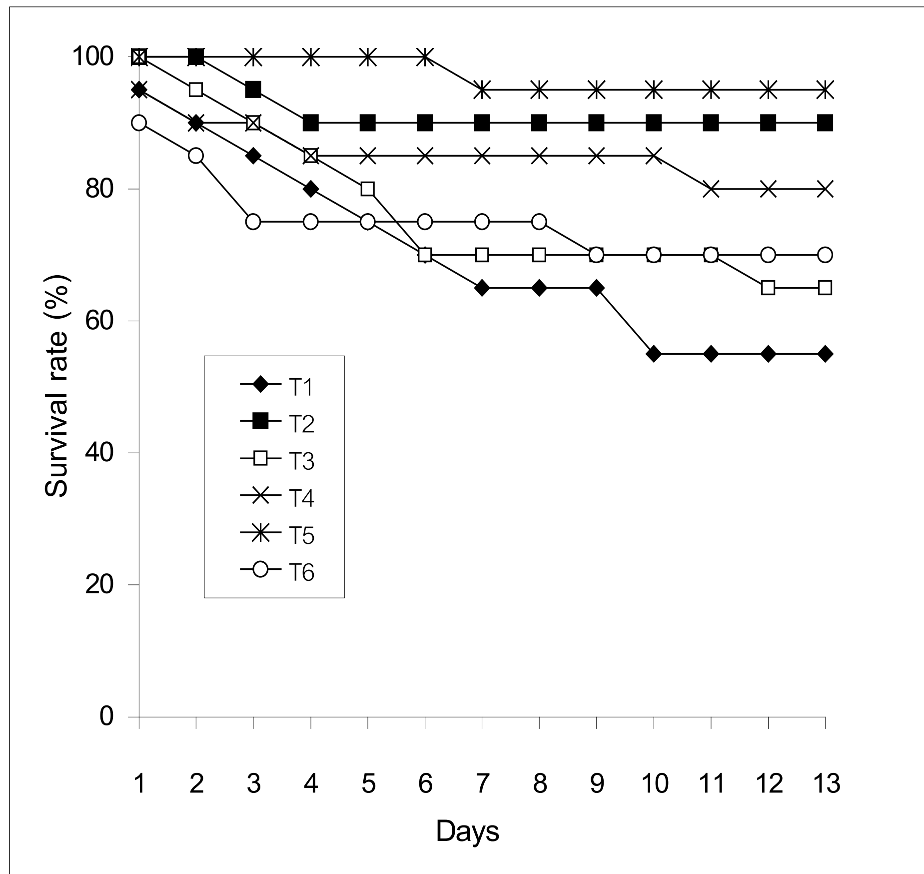

Figure 6-2 Survival rates of small shrimp fed diet containing with each carotenoid pigments during salinity stress for 13 days. (T 1=control, T2 to T6 = 50 ppm astaxanthin, 125 ppm β-carotene, 200 ppm β-carotene, 125 ppm Betatene® and 3 % dried spirulina)

Trial 2. Experiment in juvenile shrimp

Shrimp fed with astaxanthin, Betatene and Spirulina supplemented diet exhibited dark blue or dark brown color while pale blue in control group. After boiling for 5 min, the body color turn bright red in the group fed with astaxanthin, Betatene and spirulina whereas pale orange in control group. Salmo fan score, astaxanthin, canthaxanthin and β-carotene in shrimp body were shown in Table 6-6. Blood parameters and immune response of shrimp at the end of feeding trial were shown in Table 6-7. Total hemocytes count after feeding with each experimental diet for 6 weeks were higher ($P>0.05$) in the group fed with control, astaxanthin, β-carotene and 6 % Spirulina-supplemented diet. The remaining bacteria in hemolymph (clearance ability) and PO activity were not significantly difference among each treatment. The survival rates of shrimp challenged with WSSV are shown in figure 6-3. On day 10 all shrimp died in the control group. However, there was not significantly difference in survival among treatment throughout day 10 of challenging period.

Table 6-6 Salmo fan score, astaxanthin and total carotenoid in juvenile shrimp after fed each experimental diet for 6 weeks.

	Salmo fan score	Astaxanthin content (ppm)	Canthaxanthin content (ppm)
T1 control	21-22	21.7	1.8
T2 astaxanthin 100 ppm	29-30	30.4	2.5
T3 β-carotene 125 ppm	30-31	31.8	3.6
T4 β-carotene 250 ppm	27-28	32.0	4.2
T5 Betatene 250 ppm	28-29	31.7	3.4
T6 Spirulina 6 %	30-31	30.1	3.2

Table 6-7 Blood parameters and immune response of juvenile shrimp fed diet containing with carotenoid pigments for 6 weeks.

	Total hemocytes count ($x10^4$ cell / ml)	Clearance ability (cfu / ml)	PO activity (unit/min/mg protein)
T1 control	5.91 ± 2.52[a]	2083.33 ± 1599.30[ns]	382.57 ± 226.37[ns]
T2 astaxanthin 100 ppm	5.32 ± 2.03[a]	1361.54 ± 1296.86 [ns]	329.05 ± 186.49 [ns]
T3 β-carotene 125 ppm	4.52 ± 1.34[ab]	638.33 ± 842.87 [ns]	435.11 ± 177.23 [ns]
T4 β-carotene 250 ppm	3.26 ± 1.54[b]	1191.67 ± 1552.72 [ns]	471.58 ± 153.27 [ns]
T5 Betatene 250 ppm	3.32 ± 2.82[b]	1057.14 ± 1691.11 [ns]	407.63 ± 207.03 [ns]
T6 Spirulina 6 %	4.77 ± 2.04[ab]	846.67 ± 1820.61 [ns]	528.44 ± 218.11 [ns]

ns = non significant

Means not sharing a common superscript are significantly different ($P<0.05$)

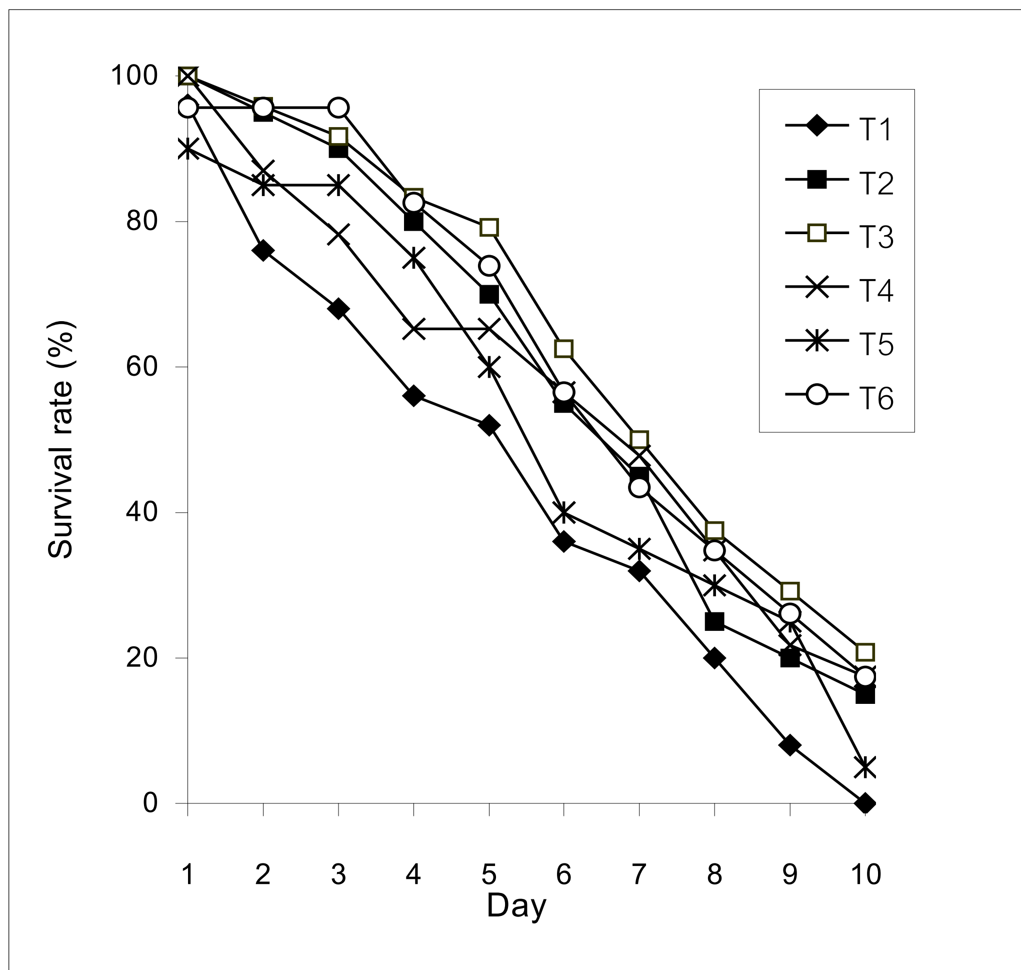

Figure 6-3 Survival rates of juvenile shrimp fed diet containing with each carotenoid pigments after challenged with WSSV at a concentration of 1 : 10^7 for 10 days. (T 1=control, T2 to T6 = 100 ppm astaxanthin, 125 ppm β-carotene, 250 ppm β-carotene, 250 ppm Betatene® and 6 % dried spirulina)

Discussion

All carotenoid pigments in our study *i.e.* synthetic astaxanthin, synthetic β-carotene or both natural β-carotene from *Dunaliella* and *Spirulina* can improve the body color in black tiger shrimp. The results can be supported by the studies in kuruma shrimp which converted β-carotene to astaxanthin through the biosynthetic scheme described by Tanaka *et al*. (1976). Although, astaxanthin was the most effective substance for pigmentation in shrimp, the alternative source for carotenoid pigments like β-carotene or spirulina can be used. Chein and Jeng (1992) reported that shrimp fed either synthetic β-carotene or algal meal (*Dunaliella salina*) have higher pigment concentrations than group fed control diet. Moreover, they found that the pigment concentration ratio of head to shell (HSR) in the shrimp fed diet contained 500 mg β-carotene/kg was higher than the group fed 500 mg astaxanthin/kg, so they concluded that low level of β-carotene (500 mg/kg diet) resulted in greater pigment distribution in shrimp body than astaxanthin. β-carotene has been known to have an antioxidation activity for reduced of oxidative stress in many biological processes. From the study by Liao *et al.* (1993) the incorporation of 3 % *Spirulina* meal in diet gave a higher growth rate of black tiger shrimp than the group fed diet supplemented with *Phaffia* yeast, which are highly astaxanthin content (Sanderson and Jolly, 1994). However, the efficiency of *Phaffia* yeast in shrimp may limited by the lack of enzyme for cell wall disruption in shrimp intestine. As well as, Tangeras and Slinde (1994) who suggested that the thick cell wall in *Phaffia rhodozyma* restricted pigment availability, because no enzyme

able to degrade the cell wall components is present in digestive tract of salmonid fish. β-Carotene were effective in inhibiting neoplastic transformation and have a anticarcinogenic effect, which caused by increased transcription of connexin 43 in cultured cells (Pung *et al.*, 1988; Peto *et al.*, 1981; Zang *et al.*, 1991). Each isomer of β-carotene are difference in the biological activity, Hieber *et al.* (2000) reported that 9-cis β-carotene from betatene (commercial Dunaliella extract) was less active than all trans β-carotene in reducing proliferation and upregulating expression of connexin 43 in 10T/2 cells. Moreover, many factors affect on the utilization of carotenoid pigments in aquatic animal, such as dietary lipid content and form of carotenoid in diet (Nickle and Bromage, 1998). Barbosa *et al.* (1999) reported that astaxanthin concentration in serum of rainbow trout fed diet contained synthetic astaxanthin and natural astaxanthin from *Haematococcus* sp. was not difference when dietary lipid level was high, they also found that natural astaxanthin in esterified forms were better absorbed in digestive tract than synthetic astaxanthin in fish fed diet contained low lipid level. As well as, Liao *et al.* (1993) reported that 3 % of dry spirulina has the best effect for pigmentation and growth performance in black tiger shrimp. Moreover, carotenoid in the carapace of shrimp fed diet containing synthetic β-carotene were lower than group fed spirulina supplemented diet. However, *Spirulina* has β-carotene and zeaxanthin as major carotenoids. Thus, the efficiency of spirulina for pigmantation in black tiger shrimp may derived from zeaxanthin, which have a shorter step for convertion to astaxanthin via 4-ketozeaxanthin (Tanaka *et al.*, 1976). In our study, a bioavialability of synthetic β-carotene was not difference from natural β-carotene either from *Dunaliella* nor *Spirulina* for use as pigments sources in black tiger shrimp.

Different concentration of synthetic β-carotene are not effect on pigmentation, growth performance and disease resistance. Our results are in agreement with Chein and Jeng (1992) who concluded that β-carotene from Betatene requires

several conversions before it converted to astaxanthin in shrimp body, this long conversion process may have been resulted in no differences in the pigmentation of shrimp fed all levels of dietary β-carotene.

No evidences were observed on the effect of dietary carotenoids for immune response and diseases resistance in black tiger shrimp. In contrast, Thompson (1995) reported that rainbow trout fed diets supplemented with vitamin A and astaxanthin, astaxanthin alone, vitamin A alone and neither vitamin A nor astaxanthin have higher total serum antiprotease activity than the group fed the diet without astaxanthin and vitamin A. The concentration of carotenoids in our study may lower than an effective dose for improving of the disease resistance and stress tolerance. Merchie *et al.* (1998) reported that an increase in dietary astaxanthin (810 ppm) resulted in a significantly improve survival in black tiger shrimp postlarvae during salinity shock condition. Carotenoid may perform some physiological function as an intracellular oxygen reserve in the low dissolved oxygen condition (Balon, 1979; Chein and Jeng, 1992), dietary carotenoid requirement in aquatic animal which survive in low dissolved oxygen condition may higher than the species in normal condition. According to the previous study, astaxanthin requirement in black tiger shrimp for growth and pigmentation seem to much lower than kuruma shrimp, which hide or bury in substratum where dissolved oxygen was low (Yamada *et al.*, 1990; Boonyaratpalin *et al.*, 2000). However, effects of higher dietary carotenoid for stress tolerance have not been reported. A further study will be required to investigate the effect of high concentration of dietary β-carotene in the immune response and stress tolerance.

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