CHAPTER V

DISCUSSIONS

Production of astaxanthin from H. pluvialis NIES144

The culture of H. pluvialis NIES144 which was incubated at 25 °C under 14 hr light intensity (1.5-3.0 klux) and 10 hr dark illumination can promote growth of vegetative cells showed by increasing of chlorophyll and total carotenoids content. It has been reported that low light intensity and low salinity promoted rapid expansion of the culture (Spencer, 1989). But at this condition, there was no physiological morphology change from green to red aplanospore as reported by Goodwin (1954). Under stress condition by increasing light intensity (10 klux continuously), cells of H. pluvialis became yellow then red due to a formation of large amounts of carotenoids. There was a report that light is important for regulation of carotenogenesis in a wide variety of organisms included H. pluvialis (Boussiba and Vonshak, 1991; Fan, Vonshak and Boussiba, 1994; Lee and Soh, 1991). And the major pigments in H. pluvialis are generally β-carotene and its keto-derivatives such as echinenone, canthaxanthin and mainly astaxanthin (Choubert and Heinrich, 1993; Kobayashi et al., 1992A).

H. pluvialis accumulates secondary carotenoid, mainly astaxanthin, in the cytoplasm, vicinity of the nucleus, possibly in endoplasmic reticulum that is different from the primary carotenoids which are intregrated into the lamella of chloroplasts. And also different from β-carotene in Dunaliella bardawil which is located in the interthylakoid space of the chloroplast (Boussiba and Vonshak, 1991).

H. pluvialis was both rapid growth and proficient production of astaxanthin (Bubrick, 1991; Spencer, 1989). Culture of H. pluvialis NIES144 can grow well in autotrophic and heterotrophic condition (doubling was 2.67 days) because the medium was supplemented with completed organic carbon source, nitrogen source and vitamin. Acetate is used as carbon source, urea is the nitrogen source and thiamin strands for vitamin. The satisfaction of this study showed that dry cysts of H. pluvialis NIES144 contained high protein content (about 40%). Kobayashi et al. (1992A) suggested that supplementation of acetate to the vegetative growth phase of H. pluvialis induced cysts cell formation, which was closed associated with a concomitant increase in the DNA content and decrease in the protein content.

Studies of the factors controlling astaxanthin accumulation in Haematococcus sp. have yielded inconclusive results. A different hypothesis has been shown that the accumulation of astaxanthin is favored by agents which prevent cell division without impairing the ability of the alga to assimilate carbon or the carbon-nitrogen balance in the medium (Boussiba

and Vonshak, 1991). It has been assigned a role in photoprotection (Lee and Soh, 1991) which explaines that Haematococcus sp. has adopted a protective mechanism through an accumulation of secondary carotenoids in the cytoplasm which most of the other microalgae develop effective repair mechanism for damage caused by high photon flux density (Lee and Ding, 1992; Yong and Lee, 1991). However, Kobayashi et al. (1992B) demonstrated that Haematococcus sp. can be grown heterotrophically on acetate in the dark as well as mixotrophically on acetate in the light and astaxanthin accumulation is induced in these conditions.

Astaxanthin esters are the primary pigment in H. pluvialis cysts, typically ranging from 60% to 80% by weight of the total pigment content (Spencer, 1989). Kobayashi et al. (1992B) showed that H. pluvialis NIES144 accumulates astaxanthin mainly esterified form; [monoester (69%), diester (20%)] and cannot detect in free form. In synthetic astaxanthin, free form of astaxanthin is the major compound. The predominant configurational isomer in H. pluvialis is shown to be 3S, 3'S (Johnson and An, 1991). This 3S, 3'S is also found in lobster (Homarus gammarus) eggs and yeast (Phaffia rhodozyma).

Effect of sodium chloride on cyst formation

In the stimulation of cyst formation by NaCl alone and NaCl plus high light intensity in culture of H. pluvialis NIES144, it was shown that NaCl plus high light intensity could stimulate accumulation of astaxanthin and alga cells formed full red aplanospore while the group added NaCl alone had only red spot in the centre of the cells. High light intensity may affect cyst forming of H. pluvialis more than NaCl. Furthermore, the more concentration of NaCl added in the media, the less of cells number produced. Boussiba and Vonshak (1991) indicated that NaCl may affect growth of H. pluvialis in order to disturb in cell division. However, our result was in contrast with Spencer (1989) which reported that NaCl concentration about 50 mM (0.3%) found to promote encystment and Boussiba and Vonshak (1991) showed that an addition of 0.8% NaCl to the growth medium caused complete cessation of growth and accompanied to massive accumulation of astaxanthin.

Shrimp diets

Microparticulated diets: algal astaxanthin-added diet (AAD), synthetic astaxanthin-added diet (SAD) and pigment-free diet (CD), prepared for P. monodon larvae were dried by freezed drying method which was the best method in order to keep good the quality of nutrients. Thamrujikul (1990) reported that freezed drying method could maintain vitamin C in feed higher

than cabinet dry and vacuum dry. For present diets, we approved moisture content of diets after drying (about 5%) which was lower than 7% moisture content regulation for commercial feeds. The protein and fat content; 47% and 8%, respectively, were in a suitable range for shrimp larvae.

In preparation of diet, astaxanthin concentration was controlled at 200 ppm, but the AAD contained astaxanthin 188.90 ± 4.84 ppm and the SAD was 208.0 ± 4.26 ppm. Errors of astaxanthin content probably comes from measurement and analysis. In H. pluvialis, the pigment composition included astaxanthin esters, α-carotene, β-carotene, lutein, violaxanthin, neoxanthin, chlorophyll a and b, free astaxanthin as well as trace amounts of lutein epoxide, zeaxanthin, antheraxanthin, echinenone, canthaxanthin and various keto-carotenoids (Spencer, 1989), and wavelength at maximum absorption is very closed to one another. For synthetic astaxanthin, all pigments occured in free astaxanthin.

Effect of dietary astaxanthin on shrimp larvae

1. Effect on survival

Determine to zoea stage, survival rate of zoea fed CD, AAD and natural food (NF) were significantly higher than the zoea fed SAD ($P<0.05$). The result showed that zoea can accept natural astaxanthin better than synthetic astaxanthin. Commonly, zoea was fed with phytoplankton, such as Chaetoceros sp., Tetraselmis sp. and Skeletonema sp. but Chaetoceros sp.

is better (Brown, Jeffrey and Garland, 1991). They gain natural carotenoids (and convert to astaxanthin) from these algae. In the case of AAD, H. pluvialis accumulates natural astaxanthin and various nutrients (from the rich medium) which are familiar to zoea. For SAD, synthetic astaxanthin probably may contaminate with other chemical substances which was easy to affect to zoea (that was too sensitive) then survival rate of zoea fed synthetic diet was lower than other groups. The finding indicated that AAD can be used instead of NF for zoea.

In mysis stage, the results showed that survival rate of mysis fed AAD and NF was similar and significantly higher than mysis fed CD and SAD ($P<0.05$). At this stage, zoea still accept natural astaxanthin (from AAD and NF) better than synthetic astaxanthin (from SAD). Normally, mysis can gain astaxanthin from small zooplankton such as Artemia sp. and rotifer. The results showed the similar survival rate between AAD and NF, this implied that AAD can be replaced NF for mysis stage.

In postlarval stage, the best survival rate for postlarva 15 was the larvae fed AAD which was significantly higher than the larvae fed NF. Survival rate of larvae fed CD and SAD was similar. However, there was no significant different survival rate of the larvae fed AAD, SAD and CD, so we may conclude that natural astaxanthin probably improves on survival of the postlarvae, but the synthesis one had less effect to postlarvae survival.

Negre-Sadargues et al. (1993) and Yamada et al. (1990) commented unclear result of carotenoid pigments on survival rate. However, we found that astaxanthin may effect on survival rate of shrimp larvae. Survival rate of larvae fed SAD is higher in larger larval stage. Synthetic astaxanthin may be useful to postlarvae more than zoea and mysis when natural astaxanthin still more effective in zoea to postlarvae.

Efficiency of astaxanthin in postlarvae fed AAD is higher than that fed NF (Artrmia sp.). Tanaka et al. (1975) explained that Artemia sp. accumulated mainly canthaxanthin and in the synthesis of astaxanthin in Artemia sp., it takes two steps to convert canthaxanthin to astaxanthin (according to Fig 3) which indicates the low efficacy of using astaxanthin in Artemia sp. But in case of cyst of H. pluvialis, shrimp larvae can use astaxanthin directly.

Our results agree with the experiment of Chindamaikul and Phimonchinda (1990) which showed that the survival rate on nursing zoea, mysis until postlarva 15 of P. monodon with natural feed (Chaetoceros sp. and Artemia sp.) plus artificial feed was higher than the nursing with natural feed or artificial feed only.

2. Effect on growth

The effect of dietary carotenoids on growth of shrimp larvae was observed. The larvae fed the pigmented diets had higher length than those

fed the pigment-free diet. Length of the larvae fed CD and SAD was significantly lower than the larvae fed AAD and NF. The highest growth found in the larvae fed AAD and NF. Indicating that astaxanthin from H. pluvialis NIES144 (mostly in esterified form) performed significantly better growth than free astaxanthin from the synthetic one.

Reviewed by Brown et al. (1991) showed that shrimps fed on diet plus alga grow faster and have a better survival than shrimps fed only one diet. This is because while one diet may lack a particular nutrients, but alga may contain those nutrients, so it can supply adequate amount of nutrients to the larvae. H. pluvialis NIES144 was cultured in rich medium (contained thiamin as a growth factor) and the dry cysts contained 40% protein content and 1% fat content. Furthermore, Bidigare et al. (1993) reported that Haematococcus sp. contained fatty acid (especially n-3 polyunsaturated fatty acid) which was useful for shrimp. It appeared that various nutrients in cyst of H. pluvialis was responsible for the higher growth. The use of H. pluvialis cyst in the diet is adventage more than synthetic astaxanthin or control diet.

Cohen (1986) reported that dietary xanthophylls from algae are incorporated into the exoskeleton of prawns and lobster (as astaxanthin) play a major role in pigmentation but other functions are poorly defined. Boonyaratpalin et al. (1994) found that experimental feeding with synthetic

astaxanthin and canthaxanthin diet pigments did not effect growth or feed efficiency of juvenile P. monodon.

3. Effect on pigmentation

Carotenoids content in the larvae fed different diet was significant difference ($P<0.05$). The results showed that the pigment concentration in shrimp fed pigment-free diets was less than the groups fed the natural pigment diets. It indicated that shrimp larvae can accumulate carotenoids (mainly astaxanthin) from the diets.

Astaxanthin content in shrimp fed NF (Artemia sp.) was higher than other groups it may be because Artemia sp. accumulated various carotenoids, such as astaxanthin, violaxanthin, zeaxanthin, echinenone, β-carotene, lutein and mainly canthaxanthin with two oxygen atom. Many carotenoid can be converted to astaxanthin. Moreover, all carotenoids have similar wavelength at maximum absorption that may overlap or interfere during an analysis by spectrophotometer.

Comparison between shrimp fed SAD and AAD, our results showed that the larvae fed AAD contained higher amount of carotenoid. This may be due to; first, shrimp larvae can gain esterified astaxanthin and accumulate in body tissue better than free astaxanthin. Secondly, the larvae are accustomed to natural astaxanthin more than synthetic astaxanthin so they can accept AAD more than SAD (notice from survival and growth).

However, the factors controlling pigment absorption, transfer and excretion among various kind of tissues are not known. Herring (1969) speculated that when a pigment is absorbed in excess the animal's requirement, it is later excreted.

4. Effect on stress resistance

During stressed period, many of shrimps died at first hour then still decline later, showing less resistance (or weaken of the non-healthy shrimp). Determining the 50% cumulative mortality, we found that the larvae fed AAD could endure the change of salinity from 30 ppt to 2 ppt better than the larvae fed NF, SAD and CD. Tolerance of shrimp fed pigmented diet (AAD and SAD) was higher than the larvae fed pigment-free diet (CD).

In the case of AAD and SAD, esterified astaxanthin (in cyst) appear to be storaged form and accumulate dissolved in lipids in the hepatopancreas from which they are transferred to other sites in the body by haemolymph (Ghidalia, 1985) as the same of free astaxanthin (in synthetic astaxanthin) which accumulate in the hepatopancreas. However, P. monodon accumulated esterified astaxanthin (85.7%) more than free astaxanthin (14.3%) of all astaxanthin in the whole body (Latscha, 1989).

Animals especially crustacea which located in euryhaline sites need more energy (O_2) when moved from high to low salinity in order to maintain the mineral level in whole body. Because astaxanthin contain a long

conjugated double bond system, they are less stable and usually offer oxygen atom to cell (Stanier et al., 1971). In the low salinity stress test, astaxanthin accumulated in cell will provide oxygen source. The larvae contained high astaxanthin would also provide high energy but did not the larvae fed NF (which had the most carotenoids accumulation but may be not only astaxanthin). Now, the adequate astaxanthin content, the excess astaxanthin in cell and the oxygen consumption in the larvae is not reported clearly. Although the mechanism of astaxanthin on stress test did not explain, the better results of the efficacy of natural astaxanthin (from H. pluvialis NIES144) more than the synthetic one on growth, survival and stress resistance of shrimp larvae should be benefit information for further developing shrimp culture.