CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Southeast Asia's contribution to global aquaculture production in 1995 was 8.8% by weight and 15.3% by value. Its share of total fisheries production by weight increased from 12.6% in 1984 to 17.7% in 1995. Shrimp production in Southeast Asia is dominated by penaeid shrimps, especially the giant tiger prawn, *Penaeus monodon*. In 1995, the sub-region produced 0.48 million mt of *P. monodon* which is 96.4% share of the total world production. Other crustacean species cultured include *P. merguiensis*, *P. indicus*, *Metapenaeus* spp. *and Macrobrachium rosenbergii*, Total shrimp production in the sub-region increased (with some fluctuation) within all major producing countries from 1984 to 1994. The highest average production for crustaceans in this region was recorded by Thailand (33.0 %) (FAO, 2003). Thailand became the world's top producer of cultured shrimp in 1991 by adopting appropriate culture systems from Taiwan. This has also been due to its suitable climatic conditions, soil and water temperature, availability of wild broodstock, long experience in aquaculture, seafood processing, established trade links, good infrastructure and transport facilities, and locally produced farm equipment and feeds (Kongkeo, 1994). However, the production of Thai shrimp decreased by 2.1% during 1994-1995, owing to the shrimp viral disease outbreak, such as Monodon baculo virus (MBV), Yellow-head virus disease (YHV), and white spot syndrome virus (WSSV), which have spread through the sub-region at epizootic levels, causing severe economic damage to the entire Asian region (Flegel, 1996). Moreover, unregulated and unplanned expansion, resulting in environmental problems such as self-pollution of water supplies and the introduction of exotic pathogens through unregulated transboundary movement of broodstock and post-larvae may also have contributed to the problem (Subasinghe *et al.*, 1996).

In recent years, the culture of white shrimp (*Penaeus vannamei*) has attracted significantly investment from entrepreneurs and has rapidly replaced the culture of black tiger shrimp. *P. vannamei* as white shrimp is fast-growing with high productivity per unit area. From 2001-2003, the total Thai black tiger shrimp production decreased year by

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shrimp. *P. vannamei* as white shrimp is fast-growing with high productivity per unit area. From 2001-2003, the total Thai black tiger shrimp production decreased year by year as shown in Table 1-1 (FAO, 2003). At present, however, the international market price of the black tiger shrimp is higher than the current price of white shrimp so some shrimp farmers continue to produce the black tiger shrimp using appropriate culture systems to yield high quality black tiger shrimp products. As a result of this, production of black tiger shrimp in the last 2 years has increased slightly.

Table 1-1. Black tiger shrimp production (mt) from aquaculture in Thailand 1995-2003.

Year	Metric tons
1995	257,062
1996	235,875
1997	223,551
1998	247,458
1999	271,019
2000	304,988
2001	276,000
2002	160,000
2003	176,000

From : FAO (2003)

Shrimp produced from good aquaculture practice systems are in high demand on international markets. Recently, the Department of Fisheries have been encouraging shrimp farmers to use applied biotechnology techniques in shrimp culture systems, thus reducing reliance on chemical treatment and using biological processes to manage the overall quality of production more effectively. This strategy, which is in agreement

with international guidelines for best practice in organic agricultural production systems, is of real benefit to the producers as it results in higher export prices for the shrimp market. (Department of Fisheries, 2005).

In intensive aquaculture systems, feed and feed stuffs are the most significant factors in determining production cost. More than 40% of the production costs in modern aquaculture arise from feeds and feed stuffs (Halver, 2003). According to the "Aquaculture Development Beyond 2000: the Bangkok Declaration and Strategy" (NACA / FAO, 2000), development of fish and shellfish nutrition can be improved by incorporating better feed quality enhancements in feedstuffs which will ultimately lead to a reduction in overall feeding costs. There have been several attempts to develop similar strategies in Thailand, for example, in selecting more suitable biological materials for fishmeal or fish oils replacement in the feed ingredients or the application of improved biological processing methods to enhance the digestibility/utility of the feed stuffs. In addition to the above, biological treatment methods have also been applied in the area of disease prevention. Microbial or microbial products have been used as feed ingredients for the enhancement of fish and shellfish immunity, and for the control of pathogens in cultured species which have led to improved production efficiency in the aquaculture industry as a whole.

The production of seafood products which meet consumer requirements is also important and can lead to increased market prices. Colour is a major factor in determining the price of black tiger shrimp on international markets. It has been shown that dietary supplements of carotenoid pigments can lead to higher pigmentation in the astaxanthin) or crude carotenoids (oleoresin, paprika, crayfish waste extract, corn gluten, alfalfa and *Spirulina*) in diets have also been shown to improve pigmentation in crustaceans (Katayama *et al*., 1972 ; Tanaka *et al*., 1976 and D' Abramo *et al*., 1983). Although the highest pigmentation levels have been obtained with astaxanthin

supplementation (Yamada *et al*., 1990 ; Chien and Jeng, 1992), synthetic carotenoids, such as astaxanthin are currently too expensive for use in working aquaculture systems. Several attempt have been made to find alternative sources of astaxanthin and other carotenoids, such as yeast *Phaffia* sp. (Sanderson and Jolly, 1994), or various algal species (Liao, *et al*., 1993 ; Sommer *et al*., 1991 ; Boonyaratpalin *et al*., 2001).

Black tiger shrimp are unable to biosynthesis carotenoid *de novo* but can convert β-carotene, zeaxanthin and canthaxanthin in feed and finally deposit in the body as astaxanthin, in both free or esterified form (Tanaka *et al*., 1976 ; Boonyaratpalin *et al*., 2001). Therefore the supplement price of these pigments and the efficiency of conversion are also important factors in determining the most appropriate pigment to use in shrimp feed. In addition to beneficial pigmentation effects, astaxanthin and others carotenoids have also been shown to have positive effects on the immunological and stress responses in aquatic animals (Lastcha, 1991 ; Estermann, 1994), mostly through their antioxidant activity and also the activity of pro-vitamin A in astaxanthin. However, there is little information available with specific reference to aquatic animals, especially shrimp. These facts have encouraged many researchers to work on the nature of disease resistance and immunostimulants in shrimp (Merchie *et al*., 1998 ; Chien *et al*., 2002). Several attempts have been made to find an alternative pigment source for aquatic animal feed, espectially the microbial origins (Gentles and Haard, 1991 ; Sommer *et al*., 1991 ; Liao *et al*., 1993). In the aspect of dietary carotenoids, microorganisms containing high carotenoids levels have been used in aquatic animal feed before (Latscha, 1991). However, the efficiency of the supplement depends on several factors, such as the initial composition of the diet (Nickell and Bromage, 1998), the type of carotenoids present, (Yamada *et al*., 1990), and their subsequent digestion and absorption by the particular aquatic animal (Genteles and Haard, 1991). Photosynthetic bacteria have significant potential for use as a carotenoid source in animal

feed. They are rich in protein (50%-70% w/w), and contain a relatively high content of vitamin B_{12}, ubiquinone and carotenoids (Vrati, 1984; Noparatnaraporn and Nagai, 1986). Thus, it can be used as either a nutritional carotenoid source and/or an immonulogical enhancer in aquatic feed. This study was therefore undertaken to investigate the appropriate photosynthetic bacterial strain and other carotenoids-rich microorganisms for potential use as sources of carotenoid as well as immunostimulant in black tiger shrimp.

Literature review

Biological properties of carotenoid

Carotenoids are terpenoid compounds synthesized *de novo* by bacteria, fungi, algae and higher plants. These compounds are probably the most widely distributed and are certainly among the most importance. They are found both photosynthetic and non-photosynthetic tissues of all organism throughout the plant kingdom, some genera of monera and fungi. Carotenoids are responsible, wholly or in part, for the colors of many animals, notably birds, fish, and insects and other invertebrates. Almost all carotenoids are, or are derived from tetraterpenes, C_{40} compounds with a carbon skeleton buit up from eight C_5 isoprene units (Figure 1-1). The basic skeleton of carotenoid is symmetrical, and consists of two C_{20}, which attach in head to head pattern. The similar form of carotenoid is lycopene, which is the red pigment from tomatoes (Figure 1-2). However, the fundamental structure of carotenoid may be modified by the presence of six-membered (or five-membered) ring at one or both end of the molecule, for the example, β-carotene (Figure 1-3), This compound is the yellow-orange pigment of carrot root, and is generally considered as the parent of all carotenoid group (Britton, 1983).
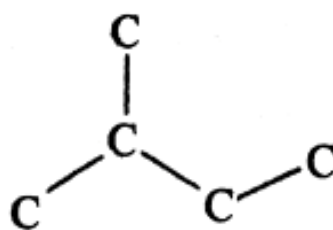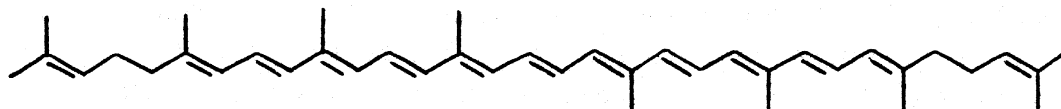

Figure 1-1. Isoprene unit
From :   Britton (1983)

Figure 1-2. Lycopene
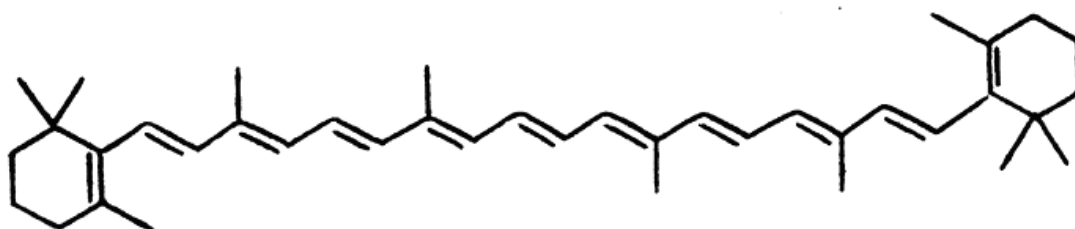**From :**   Britton (2004)

Figure 1-3. β-carotene
From :   Britton (2004)

Molecule of carotenoid in the part of hydrocarbons are known as carotene. All derivatives which contained oxygen functions are xanthophylls. Such oxygen-containing functional groups are hydroxyl-, methoxy-, epoxy-, oxo-,aldehyde-,and carboxylic acid. Appropriate groups may be esterified or glycosylated.

Each organism have some difference carotenoid synthesis pathway. In general carotenoids are synthesized in the terpenoid pathway, which are originated from the acetyl CoA by the several steps for conversion to the first carotenoid 'phytoene'.

Carotenoids are usually tetraterpenoids consisting of 8 isoprene units, which are biosynthesized by the normal isoprenoid pathway which give rise also to other important natural products such as rubber, steroids, terpenes in essential oils and the sidechains of the electron transport quinines (Britton, 1983).

The carotenoid biosynthesis pathway started from the formation of the C_{20} intermediate, geranylgeranyl pyrophosphate, the first isoprenoid precursor is considered to be acetate, as acetyl-CoA. The biosynthesis from acetyl-CoA to geranylgeranyl pyrophosphate (GGPP) is summarized in figure 1-4. Acetyl-CoA is converted to acetoacetyl-CoA into 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The HMG-CoA undergoes a two-step reduction to mevalonic acid (MVA) by means of HMG-CoA reductase. In the next steps, MVA is phosphorylated twice to give mevalonic acid-5-phosphate and -5-pyrophosphate by kinase enzymes and ATP. The MVA-5-pyrophosphate is decarboxylated to give the isoprene unit 'isopentenyl pyrophosphate' (IPP) and dimethylallyl pyrophosphate (DMAPP). By the activity of prenyl transferase enzymes, the DMAPP undergoes condensation with IPP to give the C_{10} intermediate ' geranyl pyrophosphate' (GPP). Sucessive addition of two molecules of IPP give the C_{15} 'fernesyl pyrophosphate' (FPP), which is precursor to sesquiterpenes, steroids and triterpenes and the C_{20} GGPP. However, geranylgeranyl pyrophosphate (GGPP) may be used to form C_{20} diterpenes, including phytol, which is the sidechain of chlorophyll (Britton, 1983).

The first biosynthesis of carotenoids is that in which two molecules of GGPP are use to form C_{40} cyclopropane intermediate 'prephytoene pyrophosphate' (PPPP) and then, the final intermediate (PPPP) is stabilized by proton loss to give phytoene as shown in figure 1-5 (Armstrong, 1994).
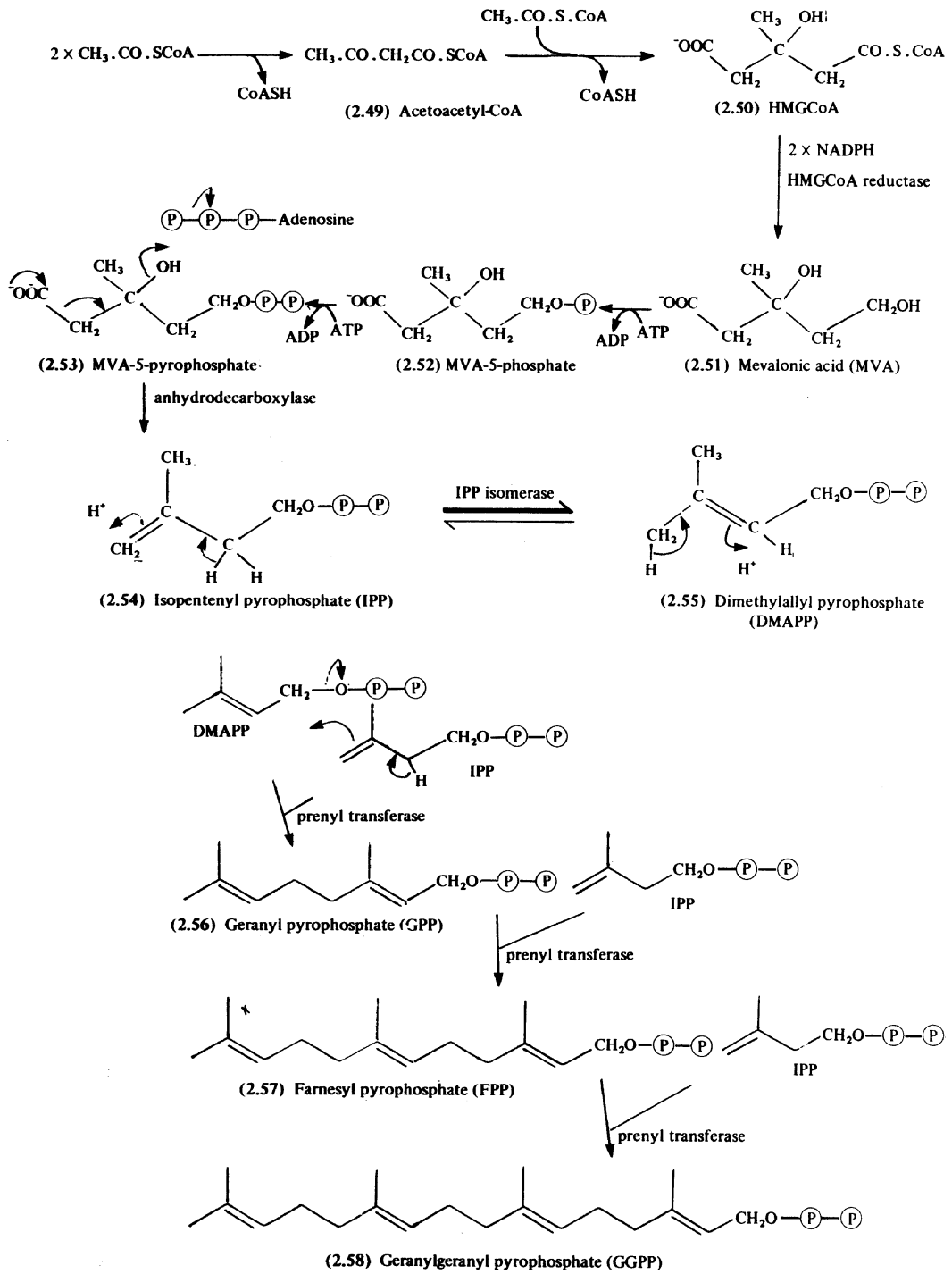
$2 \times CH_3.CO.SCoA \longrightarrow CH_3.CO.CH_2CO.SCoA \longrightarrow$

CoASH

(2.49) Acetoacetyl-CoA

$CH_3.CO.S.CoA$

CoASH

$^-OOC \quad \overset{CH_3 \quad OH}{\underset{CH_2 \qquad CH_2}{C}} \quad CO.S.CoA$

(2.50) HMGCoA

$2 \times NADPH$

HMGCoA reductase

(P)—(P)—(P)—Adenosine

$^-OOC \quad \overset{CH_3 \quad OH}{\underset{CH_2 \qquad CH_2}{C}} \quad CH_2O-(P)(P)$

ADP ATP

(2.53) MVA-5-pyrophosphate·

$^-OOC \quad \overset{CH_3 \quad OH}{\underset{CH_2 \qquad CH_2}{C}} \quad CH_2O-(P)$

ADP ATP

(2.52) MVA-5-phosphate

$^-OOC \quad \overset{CH_3 \quad OH}{\underset{CH_2 \qquad CH_2}{C}} \quad CH_2OH$

(2.51) Mevalonic acid (MVA)

anhydrodecarboxylase

$H^+ \quad \overset{CH_3}{\underset{CH_2}{C}} \quad \overset{C}{\underset{H \quad H}{}} \quad CH_2O-(P)—(P)$

(2.54) Isopentenyl pyrophosphate (IPP)

IPP isomerase

$\overset{CH_3}{\underset{CH_2 \qquad C}{C}} \quad CH_2O-(P)—(P)$

$\underset{H \qquad H^+}{}$

(2.55) Dimethylallyl pyrophosphate
(DMAPP)

$CH_2-O-(P)—(P)$

DMAPP

$CH_2O-(P)—(P)$ IPP

H

prenyl transferase

$CH_2O-(P)—(P)$

(2.56) Geranyl pyrophosphate (GPP)

$CH_2O-(P)—(P)$

IPP

prenyl transferase

$CH_2O-(P)—(P)$

(2.57) Farnesyl pyrophosphate (FPP)

$CH_2O-(P)—(P)$

IPP

prenyl transferase

$CH_2O-(P)—(P)$

(2.58) Geranylgeranyl pyrophosphate (GGPP)

Figure 1-4. Formation of geranylgeranyl pyrophosphate (GGPP) by the basic
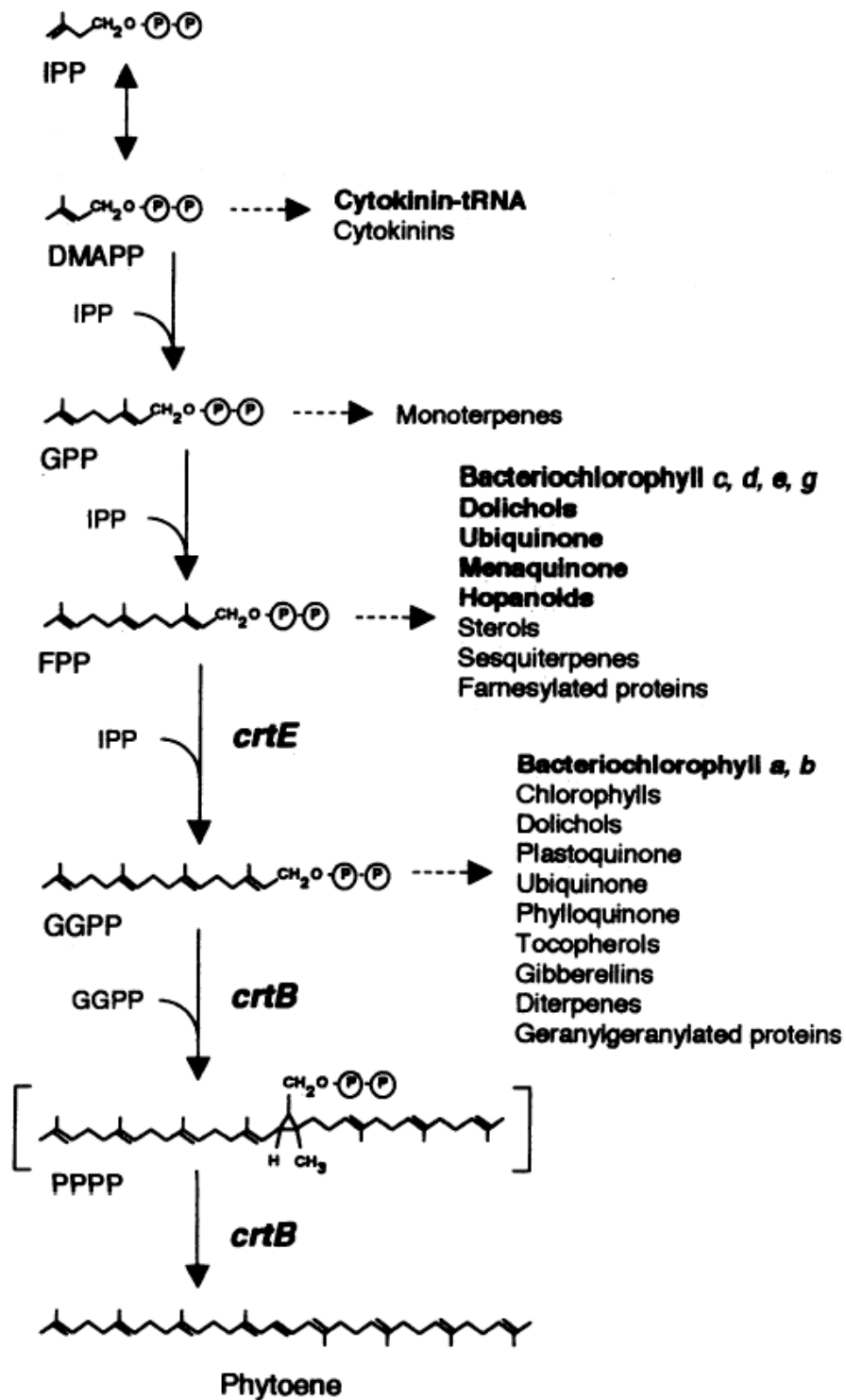isoprenoid biosynthesis pathway from acetyl-CoA.
From : Britton (1983)

Figure 1-5. General isoprenoid biosynthesis pathway and the mechanism for the
formation of phytoene
From :   Armstrong (1994)
The C_{40}, phytoene, is formed by phytoene synthase whose reaction needs ATP (Sandmann, 1997). The carotenogenesis are difference among the group of organisms (Armstrong, 1994).

Carotenoid in photosynthetic bacteria

In photosynthetic bacteria, four main pathways for carotenogenesis were proposed by Schmidt (1978) e.g. : the spirilloxanthin pathway, the spheroidene pathway, the okenone pathway and the isorenieratene pathway. But in recently, five main carotenogenesis pathway in photosynthetic bacteria are now suggested :

1). Spirilloxanthin pathway (normal spirilloxanthin, unusual spirilloxanthin, spheroidene and carotenal pathways)
2). Okenone pathway (okenone, and keto-carotenoid pathways)
3). Isorenieratene pathway (isorenieratene, and chlorobactene pathways)
4). γ and β-carotene pathway
5). Diapocarotene pathway

Most of photosynthetic bacteria so far described have the spirilloxanthin pathway, but some also have unusual carotenoids, which difference among each group of such bacteria (Table 1-2).

Table 1-2. Carotenogenesis pathways and their distribution within anaerobic
photosynthetic bacteria

Carotenogenesis pathways	Family
Spirilloxanthin	
Normal spirilloxanthin	Rhodospirillaceae, Chromatiaceae, Eutothiorhospiraceae
Unusual spirilloxanthin	Rhodospirillaceae, Chromatiaceae, Eutothiorhospiraceae
Spheroidene	Rhodospirillaceae
Carotenal	Rhodospirillaceae, Chromatiaceae
Okenone	
Okenone	Chromatiaceae
R.g.-Keto carotenoid	Rhodospirillaceae
Isorenieratene	
Isorenieratene	Chlorobiaceae
Chlorobactene	Chlorobiaceae
γ and β-carotene	Chloroflexaceae
Diapocarotene	Heliobacteriaceae
Additional pathway	
Glucoside	Rhodospirillaceae, Ectothiorhodospiraceae
Glucoside ester	Ectothiorhodospiraceae, Chlorobiaceae, Chloroflexaceae

From : Takaichi (2001)

Biosynthesis and distribution of carotenoid in photosynthesis bacteria

Phytoene is desaturated to neurosporene or lycopene by the photosynthetic bacteria. These are then converted to carotenes by desaturation, saturation, cyclization and aromatization, and/ or to xanthophylls by introduction of the oxy groups, i.e. hydroxyl, methoxy, keto and aldehyde groups. Phytoene is the first carotenoid in carotenogenesis, and it usually in the 15-cis form. Four desaturation steps are performed in the conversion of phytoene to lycopene by phytoene desaturase in bacteria except for cyanobacteria (Takaichi, 2001). During desaturation, two pathways are involved, i.e. ζ - carotene and asymmetrical ζ- carotene (Figure 1-6). Both of such pathways are found in many difference kind of photosynthetic bacteria, but seem not to be related to the final products of the phytoene desaturase, neurosporene and lycopene, or to the classification of photosynthetic bacteria (Takaichi, 2001).
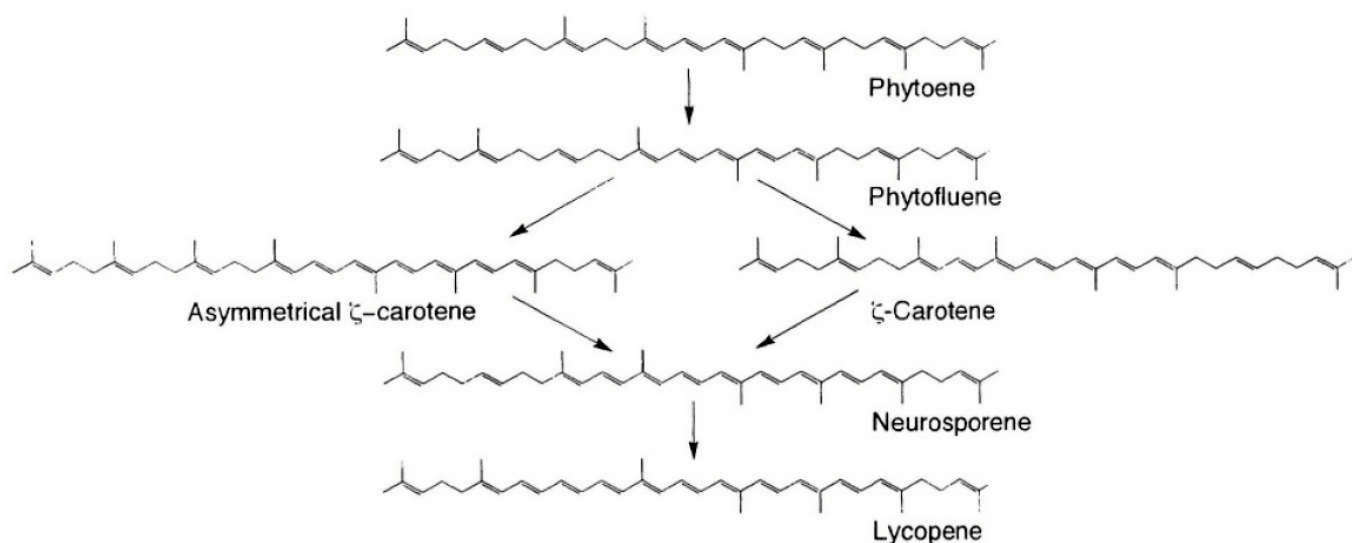
Figure 1-6. Desaturation of phytoene to lycopene by phytoene desaturase.

From :   Takaichi (2001)

Biosynthesis of Spirilloxanthin

Normal Spirilloxanthin

Carotenogenesis of the normal spirilloxanthin pathway is found in a large number of species of purple bacteria : Rhodospirillaceae, Chromatiaceae and Ectothiorhodospiraceae. Spirilloxanthin is a symmetrical carotenoid containing the methoxy groups at C-1 and C-1' and additional double bounds in the C-3,4 and C-3',4' positions. These compound has 13 conjugated double bounds.

A formation of spirilloxanthin leading from lycopene as shown in figure 1-7. The formation includes the successive reaction of (1) hydration at C-1,2 (2) desaturation at C-3,4 and (3) methylation of the tertiary hydroxyl group at C-1. These reactions occur at first on the one half of the molecule and then on another half. So the major component is the final product spirilloxanthin and usually small amounts of all or a few of five intermediates are also found (Takaichi, 2001).

Unusual spirilloxanthin pathway

When one enzyme of the normal spirilloxanthin pathway is lacking or impaired activity, the composition will be expected to change. Lycopene is accumulated in *Rhodobactor marinum* (Dilling *et al.*, 1995), which caused by the low activity of C-1,2 hydration. Matsuura and Shimada (1993) reported that *Rhodopseudomonas photometricum* and *Rhodopseudomonas molischianum* accumulated rhodopin as major carotenoid, which may be due to low activity of C-3,4 desaturation, and the 1-hydroxy- ψ end group may not to be a suitable substrate for the methylation enzyme. Rhodovibrin has been reported to be the major component in the type strains of *Rhodopseudomonas photometricum* and *Rhodopseudomonas palustris* (Takaichi, 2001).
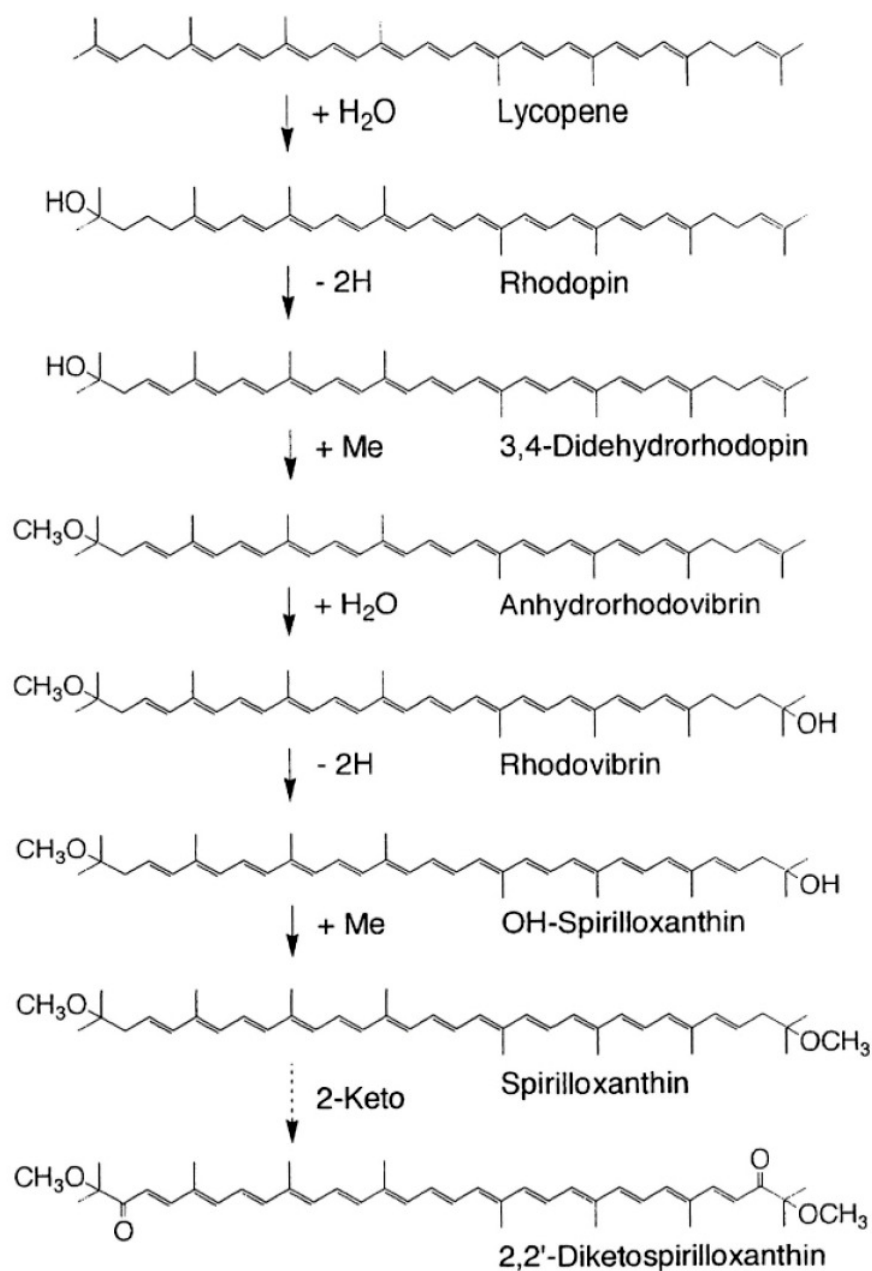
Figure 1-7. The predicted pathway for the biosynthesis of spirilloxanthin.

From : Takaichi (2001)

Spheroidene pathway

The spheroidene pathway is found only from 4 genera, *Rhodobacter*, *Rhodoferax*, *Rubrivivax* and *Rhodovulum*, and some species of aerobic photosynthetic bacteria. Spheroidene is an asymmetrical compound containing the same end group as spirilloxanthin on one side and the 7,8-dihydro- ψ end group on the other side (Figure 1-8). The phytoene desaturase produces neurosporene from phytoene in 3 desaturation steps. The next sequence includes the successive reaction of (1) hydration of C-1,2 by hydroxyneurosporene synthase to give chloroxanthin (hydroxyneurosporene), (2) desaturation at C-3,4 by methoxyneurosporene dehydrogenase to yield demethylspheroidene, and (3) methylation at the C-1 hydroxy group by hydroxyneurosporene-*O*-methyltransferase to give spheroidene. Further, hydroxyneurosporene synthase can also hydrate at the 1',2'– dihydro-π end group to yield OH-spheroidene. In semi-aerobic condition, spheroidene monooxygenase introduces the keto group at C-2 to give spheroidenone.
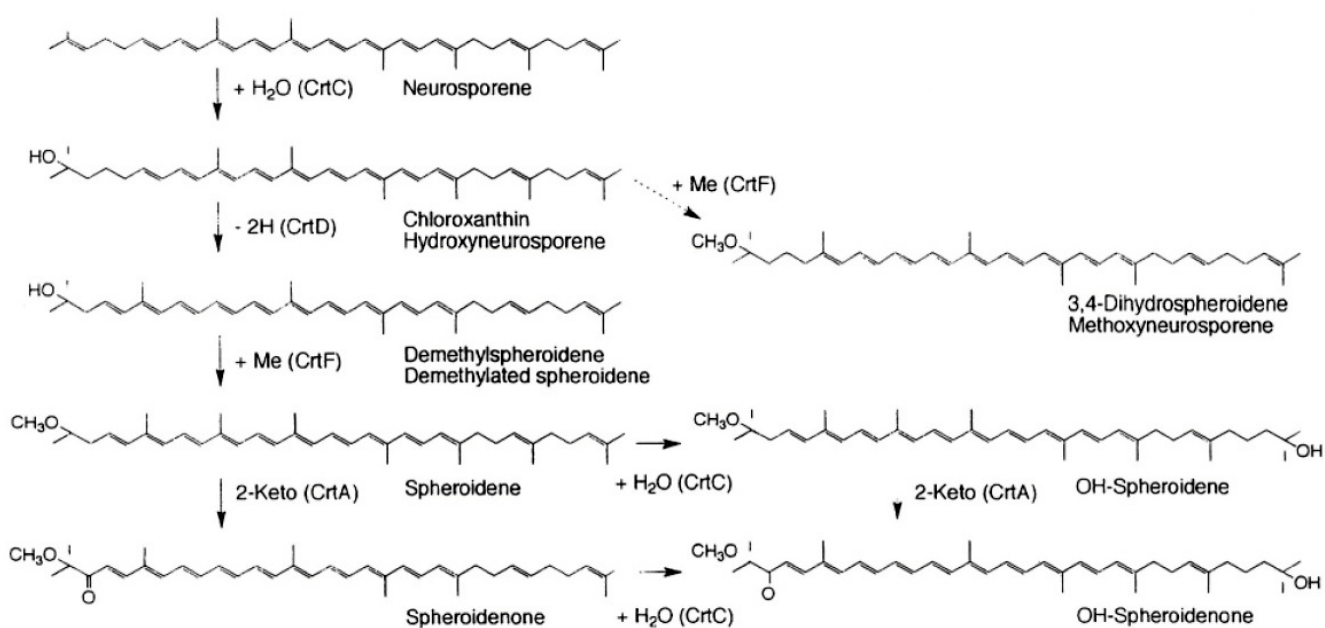
Figure 1-8.   The pathway for the biosynthesis of spheroidene.
From :   Takaichi (2001)

Carotenal pathway

Rhodopinal, which is a major carotenoid in the Rhodospirillaceae and the Chromatiaceae has an aldehyde group at C-20 of rhodopin with the 13-cis form (Figure 1-9). Usually, small amounts of rhodopinal, lycopenol and lycopenal can also found in Rhodospirillaceae and Chromatiaceae. From the structure of these carotenals, it is postulated that a branched path for each of the derivative of cross-conjugated carotenals. Rhodopin and lycopene are hydroxylated at C-20 to give rhodopinol and lycopinol, then the hydoxygroups are oxidized to the aldehyde groups to yield rhodopinal and lycopenal, respectively. Whether lycopenol and lycopenal are precursors of rhodopinol and rhodopinal has not been confirmed. Since all of these carotenals have a C-3,4 single bond, the C-3,4 desaturase may be inactive for these. Further, the activity of methylation to the hydroxyl groups at C-1 is either absent or low. Additional enzymes for hydroxylation at C-20 and for oxidation to the aldehyde group may be involved in this pathway, whereas nothing is known about how the aldehyde group is introduced under the anaerobic condition. However, the methyl group of chlorophyll a is oxidized to the aldehyde group of chlorophyll b using molecular oxygen, catalyzed by chlorophyll a monooxygenase which contains methyl-monooxygenase and alcohol dehydrogenase activity (Tanaka *et al.*, 1998). The position is necessarily at C-20, not C-20'. They take necessarily the 13 cis form, since this form is more stable than the all-trans form due to the hydroxyl or the aldehyde groups at C-20.
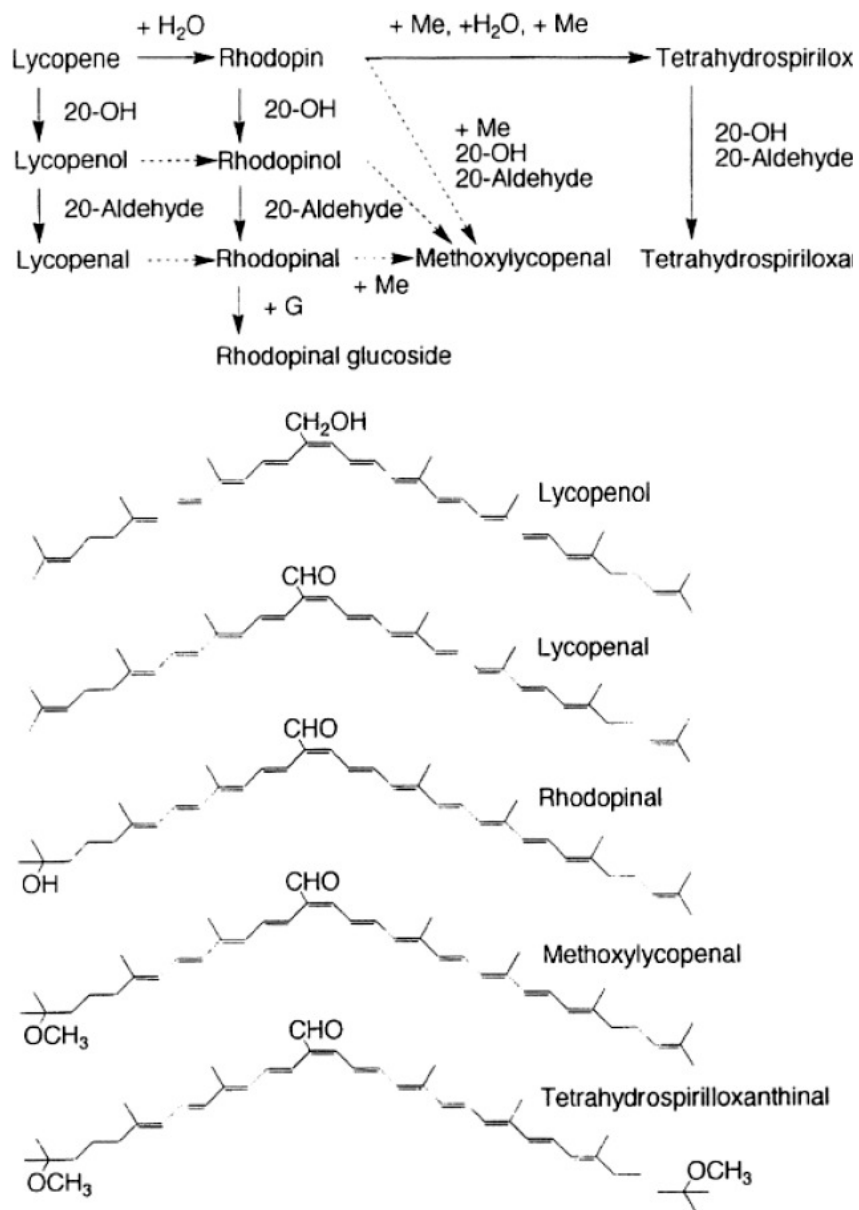
Figure 1-9. The predicted pathway for the biosynthesis of cross-conjugated carotenals
         and their structure.
From :   Takaichi (2001)

Biosynthesis of Okenone

Okenone has one aromatic end group and one aliphatic end group substituted with the methoxy group at C-1 and the keto group at C-4. The keto group of okenone is in the single bond trans-conformation around the conjugated double bond (Fujii *et al*., 1998). *R.g.*-keto III is a symmetrical carotenoid having end groups the same as one end of okenone. The biosynthesis of both of these carotenoids may be closely related, and these pathways may be distinguished at the level of the cyclase, lycopene cyclase or γ-carotene synthase (Takaichi, 2001).

Biosynthesis of isorenieratene

Isorenieratene and Chlorobactene pathways

Aromatic carotenoids with the β-end group are found only in the Chlorobiaceae. With respect to their carotenogenesis, these bacteria can be divided into two types: the isorenieratene and the chlorobactene pathways. In the isorenieratene pathway, both end group are cyclized by lycopene cyclase to give β-carotene and further aromatized to yield β-isorenieratene and isorenieratene. In the chlorobactene pathway, monocyclic chlorobactene is always a predominant product, and small amount of a hydroxylated compound, OH-chlorobactene, are also found (Figure 1-10). Therefore, two pathway may be distinguished at the level of cyclization of lycopene, although it is unknown whether γ-carotene is synthesized by lycopene cyclase or γ-carotene synthase (Takaichi, 2001)

Biosynthesis of γ- and β- carotene

The γ- and β- carotene, found in the Chloroflexaceae, are not the typical end products of carotenogenesis in photosynthetic bacteria. These pigments are more characteristic of cyanobacteria, algae, higher plant and non-photosynthesis bacteria. Moreover, OH-γ- carotene and its esters are also found. In green algae, molecular oxygen is used for the insertion of the keto group. A small amount of β- carotene has also found in two species of the Rhodospirillaceae, *Rhodomicrobium vannielii* and *Rhodopseudomonas acidophilus* strain 7050 (Britton *et al*., 1975)

Biosynthesis of Diapocarotene

The Heliobacteriaceae only have C_{30} acyclic carotene, 4,4'-diapocarotene, instead of the usual C_{40} carotenoids. 4,4'-diaponeurosporene is the major carotene, and diapophytoene, diapophytofluene, diapo-ζ-carotene and diapolycopene are also found as minor components. Dehydrosqualene synthase converts two molecules of farnesyl pyrophosphate into 4,4'-diapophytoene in similar way to the one in which phytoene synthase combines two C_{20} units in the production of phytoene. Diapophytoene is then successively desaturated by diapophytoene desaturase to give 4,4'-diaponeurosporene, analogous to the activity of phytoene desaturase.
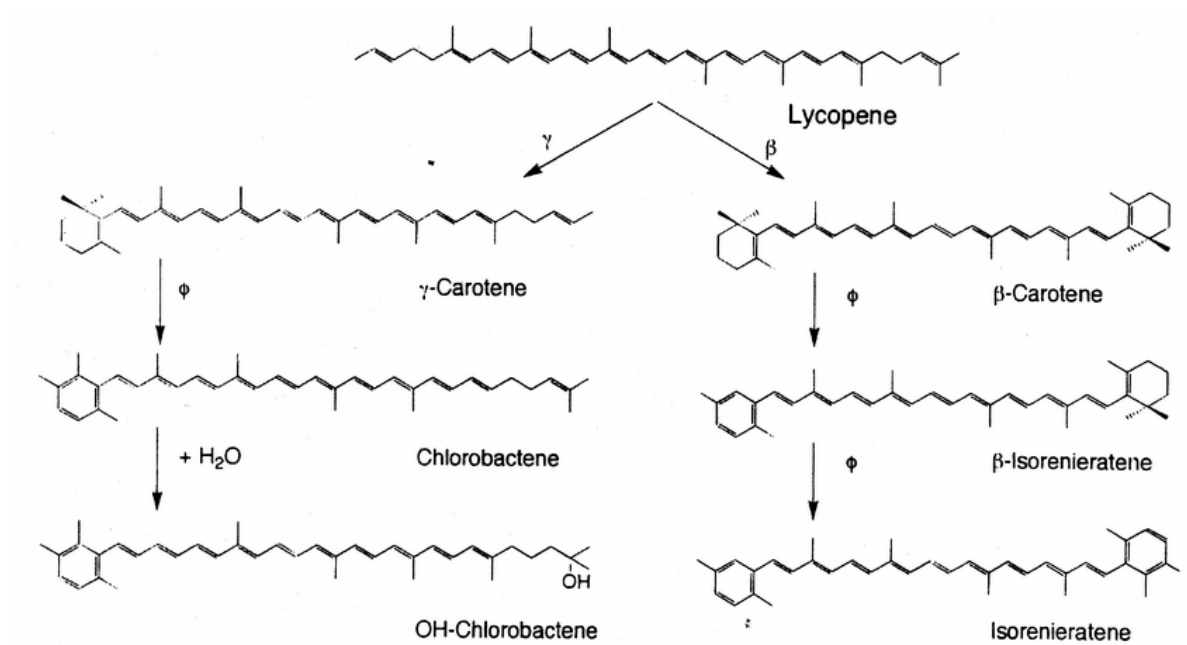
Figure 1-10. The predicted pathway for the biosynthesis of isorenieratene and
    Chlorobactene.
From : Takauchi (2001)

Distribution of carotenoids in Photosynthetic bacteria

Anaerobic photosynthetic bacteria

Rhodospirillaceae : The Rhodospirillaceae are purple nonsulfur bacteria and belong to the α- and the β-subclass of the proteobacteria (Imhoff, 1995). The spirilloxanthin pathway is found in all the Rhodospirillaceae except for one genus, Rhodophila, which has the okenone pathway. Most genera have the normal spirilloxanthin or the unusual spirilloxanthin pathways. The spheroidene pathway is found only in genera from the α3-subclass of the Rhodospirillaceae, *Rhodobacter* and *Rhodovulum. Rhodoferax* and *Rubrivivax*, which belong to the β-subclass of the Rhodospirillaceae, have both the normal spirilloxanthin and the spheroidene pathways. The carotenal pathway is found in *Rhodocyclus* and *Rhodopseudomonas acidophila*. The R.g.-keto carotenoid pathway is found only in *Rhodopila globiformis*. β-carotene is found only in *Rhodomicrobium vanielii*.

Chromatiaceae : The Chromatiaceae are purple sulfur bacteria and belong to the γ-subclass of the Proteobacteria (Imhoff, 1995). The Chromatiaceae have either the spirilloxanthin pathway (normal spiriloxanthin, unusual spirilloxanthin and carotenal pathways) or okenone of the okenone pathway except for one species, *Thiocapsa halophila*, which has both pathways. Even in the same genus, some species have the spirilloxanthin and others have the okenone pathway. Therefore, the carotenogenesis pathways are not well related to the bacteria's classification in the Chromatiaceae.

Ectothiorhodospiraceae : The Ectothiorhodospiraceae are a group of haloalkaliphilic purple sulfur bacteria and belong to the γ-subclass of the Proteobacteria. Only the spirilloxanthin pathway is found. Some species have the unusual spirilloxanthin pathway. *Halorhodospira halochloris* and *Halorhodospira abdelmalekii* contain in addition carotenoid glycosides and their ester as major components.

Chlorobiaceae : The Chlorobiaceae are green sulfur bacteria. They forms a tight phytogenetic group, and grow only under strictly anaerobic conditions. The Chlorobiaceae have the isorenieratene pathway only can be divided the isorenieratene and the chlorobactene pathways. The species which mainly contain isorenieratene always have bacteriochlorophyll c or d as major component, and those which chlorobactene always have bacteriochlorophyll e (Imhoff, 1995). Mostly the carotenes are located in chlorosome, accompanied by bacteriochlorophyll c.

Chloroflexaceae : The Chloroflexaceae are green filamentous bacteria. They form a deep division in the eubacterial line and have an interesting combination of the characteristics found in very different and diverse groups of photosynthetic bacteria. The Chloroflexaceae only have the γ- and β- carotene pathway. In Chloroflexus, most of carotene and bacteriochlorophyll c are located in the chlorosomes, which are very similar to those of the Chlorobiaceae.

Heliobacteriaceae : The Heliobacteriaceae are heliobacteria. They are strictly anaerobic photosynthetic bacteria that contain bacteriochlorophyll g as a major pigment. All the Heliobacteriaceae only have C_{30} carotenes, 4,4' –diapocarotenes. 4,4' –diaponeurosporene is the dominant pigment and trace amounts of C_{30} diapocarotenes are also present. Furthermore, the esterifying alcohol of bacteriochlorophyll g is farnesol (C_{15}) instead of the usual phytol (C_{20}) of bacteriochlorophyll a and b. Both phytoene and phytol are produced from geranylgeraniol (C_{20}). It is thus likely that the Heliobacteriaceae are unable to produce geranylgeraniol from farnesol (Takaichi, 2001).

Aerobic photosynthetic bacteria

More than 10 genera (about 30 species) of aerobic photosynthetic bacteria have been found. These baceria are distinguished from typical anaerobic photosynthetic bacteria in that they synthesized bacteriochlorophyll only under aerobic condition and can not grow without O_2 even in the light. In some species, photosynthetic activities have been demonstrated. The low content of bacteriochlorophyll, unique composition of carotenoids, and presence of 'non-photosynthetic' carotenoids, which have no photosynthetic activities, are also marked characteristics.

Species of aerobic photosynthetic bacteria are distributed rather widely within the α-subclass of the Proteobacteria (Shimada, 1995), furthermore one specie belong to the β-subclass has been found (Suyama *et al.*, 1998). In some species, identification of the pigments is very secure, while in others the carotenoids were not even analyzed. The only bacteriochlorophyll found was phytol bacteriochlorophyll a. Exceptionally, the acidiophilic genus *Acidiphilium* has Zn-bacteriochlorophyll a, where central metal is zinc instead of the usual magnesium, but small amount of Mg- bacteriochlorophyll a is also present (Hiraishi *et al.*, 1998)

The carotenoids compositions of most of these bacteria are different from those of the more typical purple bacteria. Most species so far investigated contain spirilloxanthin, but the amount are varies from low to high depending on the species. The aerobic photosynthetic bacteria can be classified into 5 groups based upon their carotenoid composition.

(1). The group which has spirilloxanthin and its precursors, and spirilloxanthin is dominant. *Acidophilium* (α 1), *Rhizobium* (α 2) and *Roseateles* (β) belong to this group.

(2). The group which has spheroidene and its derivatives,and spheroidene in dominant. *Roseobacter* (α 3) and *Erythromonas* (α 4) belong to this group.

(3). The group which has a small amount of spirilloxanthin and large amounts of other carotenoids. *Craurococcus* (α 1), *Paracraurococcus* (α 1) and *Methylobacterium* (α 2) contains unidentified carotenoic acids, and *Brandyrhizobium* (α 2) contain canthaxanthin.

(4).The group which has the diapocarotene derivative, di (acyl-glucosyl)-diapo-carotene dioate (includes species such as *Roseococcus thiosulfatophilus* (α 1) and *Methylobacterium rhodinum* (α 2).

(5). The group which has unique Erb-type carotenoids including spirilloxanthin and its precursors, γ-carotene and its cross-conjugated aldehyde derivative, β-carotene and its poly-hydroxyl derivatives, and carotenoids sulfates. This group is found only in the α4-subclass of the Proteobacteria: *Erythrobacter*, *Porphyrobacter* and *Erythromicrobium.*

In *Methylobacterium radiotolerans*, spirilloxanthin is the dominant component in the RC-LH I complex (Saitoh *et al.*, 1995), while carotenoic acids are found in the outer membranes accompanied by no bacteriochlorophylls and have no photosynthetic functions. Two species of *Methylobacterium* have also similar carotenoic acids with *Methylobacterium tolerans*, and two species of the forth group described above have polar carotenoids, diapocarotenoic acid derivatives. These hightly polar carotenoids in the third, the forth and the fifth groups may be 'nonphotosynthetic' carotenoids, which are not bound to the photosynthetic pigment-protein complexes. Although their function are not known, there is the possibility that they protect the photosynthetic apparatus from the outside aerobic conditions (Takaichi, 2001). In conclusion, most of the aerobic photosynthetic bacteria have the purple bacteria-like photosynthetic apparatus including bacteriochlorophyll a, and many species have spirilloxanthin, as well as additional polar 'non-photosynthetic' carotenoids.

Biosynthesis and distribution of carotenoid in algae and plant

In higher plants, carotenoids are biosynthesized by normal isoprenoids pathway which gave rise also to other important natural products such as rubber, steroids, the mono-, sesqui-, and diterpenes present in many essential oils, and the sidechains of the electron transport quinines. The carotenoid biosynthesis pathway in higher plants may be considered in several stages :

Formation of geranylgeranyl pyrophosphate : The first general isoprenoid precursor is usually considered to be acetate, as acetyl-coenzyme A. Acetyl CoA is converted, via acetoacetyl-CoA into 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) and undergoes a two-step reduction to mevalonic acid (MVA). This HMG-CoA reductase reaction is major control point in cholesterol biosynthesis. MVA is the first compound that serves solely as an isoprenoid intermediate (Britton, 1983). In the next steps, MVA is phosphorylated twice to give mevalonic acid-5-phosphate and -5-pyrophosphate by kinase enzymes and ATP. The MVA-5-pyrophosphate is then decarboxylated to gave the 'isoprene unit' calls intermediate isopentenyl pyrophosphate, IPP. An isomerase enzyme catalyses the reversible isomerisation of IPP and dimethylallyl pyrophosphate (DMAPP).
These two molecules are the first substrates of the prenyl tranferase enzymes by which isoprenoid chain are built up. DMAPP acts as a 'primer' molecules and undergoes condensation with IPP to gave the C_{10} intermediate geranyl pyrophosphate, GPP, precursor of monoterpenes. Successive addition of two further molecules of IPP gives the C_{15} farnesyl pyrophosphate, FPP, precursor to sesquiteterpenes, steroids and triterpenes, and the C_{20} GGPP. The chain-lengthening process may continue, to give long-chain polyprenols, or the GGPP may be used to form C_{20} diterpenes, including phytol, the sidechain of chlorophyll, or to give the C_{40} cerotenoids.

Formation of phytoene : Two molecules of GGPP are used to form the C_{40} carotenoid intermediate called phytoene. The formation of phytoene involves a C_{40} cyclopropane intermediate, prephytoene pyrophosphate (PPPP), which was stabilized the final 'carbonium ion' intermediate by proton loss to give phytoene (Armstrong, 1994).

Desaturation : The formation of colored carotenoids from phytoene first involved a series of four desaturation, each step resulting in the introduction of a double bond and consequent extention of the polyene chromophore by two conjugated double bonds. The intermediate in this sequence are phytofluene, ζ-carotene and neurosporene and the final product of the desaturations is lycopene.

Later reactions-general : The sequence of desaturation reactions does not go to completion to produce the fully conjugated pentadecaene, but stops at the stage of lycopene, in which the C-3,4 bonds remain saturated. The C-1,2 double bonds are therefore isolated and not part of the main polyene chromophore. In most carotenogenic systems, however, lycopene is not an end product but is nerely an intermediate in the biosynthesis of the normal main carotenoids present.

Later reaction-acyclic carotenoid biosynthesis : Several reaction can occur at the C-1,2 double bond, the simplest are seen in the acyclic carotenoid series. The most obvious example is the addition of water to give the 1-hydroxy- and 1-methoxycarotenoids and the another is the hydration of the C-1,2 double bond of lycopene (Britton, 1983).

Cyclisation : Cyclisation in carotenoids is limited to the formation of a single six-membered ring at one end or both ends of the acyclic precursor molecule. Cyclisation of carotenoid intermediates may be considered as an addition process initiated by proton attack at C-2 of the terminal C-1,2 double bond. Cyclisation then occurs to give a 'carbonium ion' which can be stabilized by proton lose from either C-6, C-4 or C-18 to give the β ring, ε ring or some rare case γ ring. There are two major points at which cyclisation may occur. If normal desaturation goes to completion before cyclisation then lycopene is the immediate precursor of the monocyclic γ carotene and δ carotene and then of the bicyclic β- carotene, α- carotene and ε carotene. Alternatively, if cyclisation occurs before desaturation is complete, neurosporene and β- and α-zeacarotene are key intermediates. In all case, cyclisation are occur in a carotenoid-half molecule that has reached the lycopene level of desaturation; end-groups with a C-7,8 single bond cannot cyclise.

Final modifications : The most common additional structural feature found in cyclic carotenoids is the hydroxyl-group. Hydroxylation occurs most frequently at C-3, but 2-hydroxy- and 4-hydroxycarotenoids are also quite common. The latter are often oxidized further to 4-oxocarotenoids such as canthaxanthin. Hydroxy-groups are also sometimes found in other positions in the molecule, e.g. C-19. Of these processes, only the introduction of the C-3hydroxy-group has been studied. In plants and algae zeaxanthin is formed by hydroxylation of β-carotene. The most abundant leaf xanthophylls, lutein is probably formed similarly from α- carotene. The chloroplast xanthophylls violaxanthin and neoxanthin contain 5,6-epoxy-groups.

Distribution of carotenoids in higher plants

Two types of tissue shown the difference of distribution of carotenoids in higher plants. Photosynthetic tissues, in all green tissue of higher plants contain the same

major carotenoids, which are located in the chloroplasts. These pigments are β- carotene, lutein, violaxanthin and neoxanthin often accompanied by lesser amounts of α- carotene, zeaxanthin, β- cryptoxanthin and β- antheraxanthin. The carotenoids are located in the chloroplast grana, as chromoproteins. Whereas, in the non-photosynthetic tissues such as yellow flowers and orange fruits have their carotenoid, which are normally located in chromoplast structures. Yellow flowers often contain large amounts of carotenoids epoxides, such as violaxanthin, whereas orange and red fruits, respectively, are often colored by β-carotene and lycopene or their simple hydroxyl-derivatives.

Distribution of carotenoids in algae

Carotenoids are normally found in the chloroplasts of algae, including the seaweed. The chloroplasts carotenoids of the various classes of algae show considerable qualitative differences. Carotenoid composition in the algae are similar to that of higher plants, and includes α-carotene, β-carotene, neoxanthin, lutein, violaxanthin, antheraxanthin and zeaxanthin, integrated in chloroplast lamellae and generally designated as primary carotenoids (Young, 1993). These are in contrast to secondary carotenoids, which may include equinenone, hydroxyequinenone, canthaxanthin and astaxanthin, as such or esterified to higher or lesser degrees. Biosynthesis of carotenoids is species dependent, the primary carotenoids β-carotene and lutein, zeaxanthin, as well as the secondary carotenoids astaxanthin and canthaxanthin, are included in microalgal carotenogenesis pathway (Gouveia *et al.*, 1996). In some species, such as *Dunaliella salina*, β-carotene is the predominant carotenoid (Borowitzka and Borowitzka, 1990), in other such as *Haematococcus pluvialis*, carotenogenesis changes go all the way to the secondary astaxanthin and its esters (Harker *et al.*, 1996)

Carotenoids are sometimes present also outside of the chloroplast e.g. in the 'eye spot' of *Euglena* and in reproductive areas of colonial species, *Ulva*. Ketocarotenoids, including astaxanthin, may accumulate outside the chloroplast in some green algae under adverse cultural conditions, notably mineral or nitrogen deficiency. The chloroplast carotenoid compositions of green algae are generally similar to those of higher plant chloroplasts, suggesting a strong evolutionary link. Most other algal classes produce acetylenic or allenic carotenoids; the annual natural production of fucoxanthin. The primitive prokaryotic blue green or cyanobacteria produce β- carotene and several of its simple hydroxyl- and keto- derivatives but many species also accumulate myxoxanthophyll and other glycosidic carotenoids which, structurally, are more typical of non-photosynthetic bacteria.

Distribution of carotenoids in fungi

Carotenoids are found widly, but no means universally, in fungi. Some species, notably *Blakeslea trispora*, synthesise β- carotene in the mycelium in such quantities that commercial production is possible. Most carotenogenic fungi accumulate only carotenes, especially β- carotene and γ-carotene; xanthophylls are rare. Very few of the macrofungi, i.e. mushroom and toadstools, are colored by carotenoids, the chanterelle mushroom *Cantharellus cibarius* is colored yellow by canthaxanthin. The red yeast (*Rhodotorula* sp.) characteristically produce the carotenoid acid torularhodin (Britton, 1983).

Regulation and control of carotenoids biosynthesis

Extensive complementation studies have revealed that only three genes, termed *car R*, *car B* and *car A* , are involved in normal carotenogenesis. Cyclisation of lycopene is carried out by the product of gene *car R*; two copies of its

product i.e. two cyclase enzymes, in an enzyme complex, are considered to be concerned in β- carotene formation (Britton, 1983), however Armstrong (1994) call these gene as *Crt Y* and *Crt L.* Similarly four copies of the product of gene *car B* are considered to act in a dehydrogenase complex which carries out the four successive desaturations required to convert phytoene into lycopene. Two whole of the biosynthesis is suggested to take place on multi-enzyme aggregate containing the desaturases and the cyclase. Light stimulates additional carotenoid synthesis in many fungi and bacteria, however carotenoid synthesis occurs to a very limited or not at all in the dark, but can be initiated in response to a short simultaneous exposure to light and oxygen. After photoinduction, there is usually a time lag for enzyme synthesis before carotenogenesis begins. This photoinduction mechanism ensure that carotenoid is available only when it is needed to protect the organism against the harmful effects of excessive light and oxygen. However, carotenoid which synthesis in the chloroplasts, in the photosynthetic tissue are synthesized as functional chloroplasts are formed. The light effect is thought to be mediated by phytochrome. The carotenoids are an integral part of the chloroplast structure and the regulation of their synthesis is closely interrelated with the synthesis of chlorophyll and other chloroplast constituents (Britton, 1983). The carotenoid compositions of many fungi and bacteria are altered quantitatively and qualitatively by variation in culture conditions. The nature of the carbon and nitrogen sources used, the carbon : nitrogen ratio, the availability of minerals, vitamins and growth factors, the degree of aeration, the pH of the medium, and the growth temperature may all greatly affect carotenoid production and composition. These phenomenons were also found in many unicellular algae, When the *Haematococcus pluvialis* (Chlorophyte) exposed to extreme environmental conditions they can accumulates large quantities of the ketocarotenoid, astaxanthin. Under optimal growth conditions vegetative cells of the alga persist and the alga possesses carotenoids normally found in the Chlorophyta and in

the chloroplasts of higher plants, namely β- carotene, lutein, violaxanthin, neoxanthin and zeaxanthin. Harker *et al.* (1996) reported that when *Haematococcus pluvialis* was cultivated in media deficient in nitrogen, algal growth was limited severely and astaxanthin synthesis greatly stimulated. Similar stimulatory effects on carotenoid syntheis were observed in the presence of elevated levels of ferrous iron and, especially, when the alga was transferred into saline media. In both case algal growth was severely limited. In contrast to these effects, transfer of the alga to phosphate-limiting conditions increased the rate of astaxanthin synthesis but, algal growth was not inhibited greatly. Beihui and Kun (2001) studies on the ability of a cell-free carotenogenesis in the green alga *Chlorococcum* sp. to convert β- carotene into echinenone, canthaxanthin and other endogenous xanthophylls. Optimal carotenogenesis activities were obtained when β-carotene was provided in chloroform and cholis acid as well as L-α-phophatidylcholine was present in the incubation mixture. The investigation also indicated that oxygen was essential for the conversions. The effects of dioxygenase cofactors and monooxygenase cofactor were presentes. It was found that a dioxygenase cofactor mixture of Fe^{++}, ascorbic acid and 2-oxoglutarate was required to the carotenogenesis activities.

Some chemical substances are also stimulate or inhibit carotenoid synthesis or to cause qualitative modifications in the carotenoid composition of microorganisms. One example of chemical control of carotenogenesis in a natural biological system is found in certain heterothallic fungi, *Blakeslea trispora*. This synthesis is induced by trisporic acid, a hormone which is a metabolite of β- carotene and its main function is to stimulate sporulation and reproduction. The stimulation of carotenogenesis is probably part of the mechanism for increased trisporic acid production. In higher plants many substances are known that stimulate or inhibit carotenoid synthesis. Included among these are several herbicides which may blocking desaturation of phytoene and hence preventing proper chloroplast development (Britton, 1983).

Metabolism and distribution of carotenoids in aquatic animal

Although the aquatic animal cannot synthesize carotenoids but can introduce structural modifications into the carotenoids they obtain from the diet. Many oxidative and reductive metabolic pathways have been proposed for the transformations of carotenoids in these organisms, the most important processes perhaps being those that introduce oxygen functions into the β- ring to produce astaxanthin. In almost all case, the proposed pathways are based only on the structures of the compounds present and direct conversions have not been demonstrated. None of the enzymes involved has been isolated (Britton, 1996).

Pigmentation by carotenoids is especially common and important in invertebrate marine animal of almost every class. The typical carotenoids present are keto compounds, such as canthaxanthin and astaxanthin. Other, unusual carotenoids are sometimes found. In many marine invertebrates the main carotenoid is present not in the free form but as a stocheiometric carotenoid-protein complex. Carotenoids and carotenoproteins are commonly found in the epidermis or the shell of invertebrate animals, but sometimes in high concentrations, in reproductive organs and eggs (Britton, 1983).

One of the main roles of carotenoids in organisms seems to relate to light absorption functions such as photosynthesis, photoprotection, phototropism, photoreception, and camouflage effects for concealment from enemies. Carotenoids deactivate reactive chemical species such as singlet oxygen, triplet photochemical sensitizers and free radicals, which would otherwise induce potentially harmful processes in biological systems. They also have an immune function in mammals (Bendich, 1994). Animals in general, however, do not synthesize carotenoids *de novo* and those found in the bodies of animals are either a result of the direct accumulation of carotenoids from food or are partly modified through metabolic

reactions (Britton *et al.*, 1995). Thus, the carotenoid patterns in animals provide a key to tracing the food chain as well as metabolic pathways (Matsuno, 2001).

Shrimps, lobsters, crabs, crayfish, krills and barnacles are typical crustaceans. Which occur in either fresh or salt water, are among the most important members of this class because they serve as food for many species of fishes. In general, keto carotenoids such as echinenone, canthaxanthin, two stereoisomers of phoenicoxanthin, 4-ketozeaxanthin, fritschiellaxanthin, papilioerythrinone and astaxanthins are dominant in crustaceans, and astaxanthin is widely distributed as free, esterified and protein-complexed forms. In many Branchiopoda, however, canthaxanthin is found to be dominant, and there is little or no astaxanthin (Goodwin, 1984 sited by Matsuno, 2001).

Papilioerythrinone, which was first isolated from the swallowtail *Papilio xuthus*, was also obtained from the crab *Paralithodes brevipes* (Harashima *et al.*, 1972 sited by Matsuno, 2001). Fritschiellaxanthin was isolated as the major carotenoid along with astaxanthin, lutein A, zeaxanthin, β-carotene and papilioerythrinone from the crab *Sesarma hematocheir* (Matsuno *et al.*, 1982). 2-Hydroxy-echinenone and 2- hydroxy-canthaxanthin have been isolated from the water fleas *Daphnia magna* (order Branchiopoda), and hydroxy-echinenone has been isolated from *Arthrospira* sp. (Foss *et al.*, 1986 sited by Matsuno, 2001). 2-Hydroxy-β-carotene was found to be the major carotenoid in three species of *Idotea resecata*, *I. granulosa* and *I. montereyensis* (order Isopoda) (Lee and Gilchrist, 1975 sited by Matsuno, 2001).

Almost all crustaceans convert β-carotene and zeaxanthin to the major carotenoid astaxanthin. The positions 4,4',3,3' of β-ionone rings are oxidized in these oxidative metabolic reactions (Goodwin, 1984 sited by Matsuno, 2001). But some crustaceans convert lutein A to papilioerythrinone via fritschiellaxanthin (Matsuno, 2001).

Carotenoids common to many fishes are β-carotene, β-cryptoxanthin, tunaxanthins, luteins, zeaxanthins, diatoxanthin, alloxanthin, β-echinenone, canthaxanthin, 4-ketolutein B (β-doradexanthin), 4- ketozeaxanthin (β-doradexanthin) and astaxanthin Among these, the dominant ones are: tunaxanthins in yellow and blue-green fish; astaxanthins in red marine fish; zeaxanthins in anchovies, some flatfishes, sharks and rays; tunaxanthins, luteins and zeaxanthins in brackish water fish; and luteins and alloxanthin in freshwater fish (Matsuno, 2001). Recently, from the integuments of the red tile- fish Branchiostegus japonicus, two new keto carotenoids, 4-ketolutein D and 4-ketolutein F, have been isolated along with another 19 known carotenoids (Tsushima and Matsuno, 1998). The unusual retro carotenoid rhodoxanthin have been isolated from Tilapia (Matsuno, 2001). Moreover, The unique carotenoids parasiloxanthin and dihydroparasiloxanthin having a 7,8- dihydro-β end group have been isolated from the integuments and eggs of the Japanese common catfish *Parasilurus asotus* (order Siluriformes). From feeding experiments of the Japanese common catfish, it has been concluded that (3R, 3'R)-zeaxanthin is reductively metabolized to (3R, 3'R)-7,8-dihydro- parasiloxanthin via (3R, 3'R)-parasiloxanthin, whereas lutein A is reductively metabolized to 7,8-dihydrolutein A (Matsuno and Nakata, 1980). In Cypriniformes fish, possible oxidative metabolic pathways from (3R, 3'R)-zeaxanthin to (3S, 3'S)-astaxanthin via β-carotene triols and tetrols have been proposed (Tsushima and Matsuno, 1999).

The major carotenoids in the gold fish *Carassius auratus* (order Cypriniformes) and the fancy red carp *Cyprinus carpio* (order Cypriniformes) are (3*S*, 3'*S*)-astaxanthin, fritschiellaxanthin and 4-ketolutein B (α-doradexanthin). These fish convert (3*R*, 3'*R*)-zeaxanthin and lutein A to (3*S*, 3'*S*)-astaxanthin, and [4- ketolutein B, fritschiellaxanthin, respectively (Ookubo *et al.*, 1999). They can oxidize the 4,4',3,3' positions

of the β- end group and epimerize the 3' position. The most familiar reactions of carotenoid metabolism in animals are essentially oxidative (Matsuno *et al.*, 2001). However, Schiedt *et al.* (1985) have been reported on the reductive metabolic pathways of keto carotenoids in the rainbow trout *Salmo gairdneri* (order Salmoniformes) (i) Canthaxanthin to echinenone to β-carotene (ii) Adonirubin (3-hydroxycanthaxanthin) to asteroidenone (3'- hydroxy-echinenone) to cryptoxanthin and (iii) Astaxanthin to adonixanthin (4-ketozeaxanthin) to zeaxanthin to antheraxanthin to deepoxyneoxanthin. These are reductive metabolic reactions involving the stepwise removal of the keto groups at C-4 and C-4'.

The type of carotenoid that can be utilized varies with species. Astaxanthin is by far the major pigment found in aquatic animals including salmon and shrimp (Britton, 1996). Shrimp and other crustaceans can convert cantaxanthin or zeaxanthin into astaxanthin. In contrast, salmon has very limited capability for converting other carotenoids into its primary pigment astaxanthin. Red sea bream apparently has the capability to metabolize lutein or zeaxanthin but not β-carotene or cantaxanthin into tunaxanthin or astaxanthin. Similarly fancy carp are reported to convert zeaxanthin or lutein into astaxanthin (Boonyaratpalin, 2000).

Dietary carotenoids are absorbed in the gut along with other lipids. A most important process, following absorption, is the conversion of β- carotene and other suitable molecules, that is those having one unsubstituted β- ring, into vitamin A (retinol) (Olson, 1986). This conversion occurs mainly in the small intestine, although there are reported of its occurrence also in other tissues, such as kidney and liver. The accepted mechanism of conversion is central cleavage, which requires a 15,15'-dioxygenase enzyme and can provide two molecules of retinal from one molecule of β - carotene. In addition to central cleavage, it is likely that excentric cleavage by oxidation of double bonds also occurs to give apocarotenals of different chain

lengths (C_{22} - C_{30}), which undergo further oxidation to give retinal. The retinal that is produced by either of those processes is then reduced enzymatically to retinol, which is transported by retinol-binding protein to its required sites of action or to the liver for storage as acyl esters. Carotenoids that are absorbed intact are also transported on blood lipoprotein, and can be deposited in tissues. β- carotene and other carotenes are associated mainly with the low-density lipoprotein (Britton, 1996). When excessive amounts of carotenoids are supplied they may be deposited in many tissues and can accumulate in concentrations sufficient to cause yellow-orange coloration of the skin. Absorptoin of carotenoids is facilitated by the presence of fats and agents such as lecithin, which aid emulsification. Chan *et al.* (2002) reported that the coho salmon (*Oncorhynchus kisutch*) fed diets containing the higher lipid levels (23 and 30% lipid) had higher astaxanthin content in raw flesh. The muscle lipid content was affected by dietary lipid levels and astaxanthin concentration in the flesh was positively correlated (r^2 = 0.68) with the muscle lipid content. These effect of dietary lipid on pigment deposition could be a result of increased astaxanthin digestibility. Furthermore, in fish muscle astaxanthin was bound to the acto-myosis of the muscle after the astaxanthin was transported to the muscle tissue. Increasing the turnover rate of muscle protein would likewise increase the turnover rate of deposited astaxanthin. When salmon were fed diets containing higher dietary energy, possibly less muscle protein would have been used for energy production purposes, and the turnover rates for both muscle protein and the deposited astaxanthin may have been decreased. Carotenoids and the antioxidant vitamins are labile compounds that may protect each other during oxidative degradation. A diet rich in antioxidants may preserve the individual compounds during the absorptive and the post-absorptive phase. Christiansen *et al.* (1995) reported that the adding 60 ppm astaxanthin to semi-purified diets for juvenile Atlantic salmon led to an improved antioxidant status. Bjerkeng *et al.* (1999) investigated the effects of vitamin E (α-tocopheryl

acetate) on the flesh deposition of astaxanthin in Atlantic salmon, improved flesh deposition of astaxanthin a 8-14% was achived for fish fed diets with 30 and 50 ppm astaxanthin respectively, by the dietary addition of 800 ppm compared with 200 ppm of α-tocopheryl acetate. These results concluded that the dietary addition of vitamin E appears to increase astaxanthin fillet deposition in salmonids and may reduce the demand for astaxanthin supplementation in the diets, which give the beneficial result for the large scale production.

Even astaxanthin is the most effective pigment in rainbow trout, the esterified form of these carotenoids (mono- and di ester astaxanthin) seem to less effective in the term of digestion and deposition in fish body. After ingestion of astaxanthin esters, intestinal hydrolysis is required before absorbtion can occur. Synthetic astaxanthin dipalmitate is poorly utilized in comparison to astaxanthin (Foss *et al.*, 1987). And Sommer *et al.* (1991) and Sommer *et al.* (1992) have reported poorer pigmentation when using feeds supplemented with *Haematococcus pluvialis* compared with those supplemented with free astaxanthin. The rate of hydrolysis of astaxanhtin esters to free astaxanthin appears to be the limiting factor, and this may explain observed differences in deposition. Several factors may affect the rate of astaxanthin ester hydrolysis. Firstly, the action of digestive enzymes on nutrients and contact time to the absorptive sites in the intestinal tract are affected by the transit rate of the feed bolus (Choubert and Storebakken, 1996). Secondly, there may be differences in esterase activity along the length of the salmonid intestine. Thirdly, there may be differences in carotenoid absorption along the length of the salmonid intestine (Torrisen 1986). White *et al.* (2002) studies on the effect of esterification on the absorption of astaxanthin in rainbow trout. Rainbow trout fed diets supplemented with either esterified astaxanthin or free astaxanthin at similar levels (50 ppm). After 56 days of feeding trial, the steady-state serum astaxanthin concentrations were higher in the group fed diet supplemented with free astaxanthin (2.0 ug/ml) when compared

to those fed test diet contained esterified astaxanthin (1.3 ug/ml). However, there was not significantly difference in the absorption of astaxanthin from the single meal supplemented with free or esterified astaxanthin. From the analysis of astaxanthinin the intestinal, higher absorption of astaxanthin by the ileal compared with the posterior intestine was recorded. This result comfirmed the role of the anterior intestine in carotenoid absorption. White *et al.* (2003) also reported that the degree of esterification of astaxanthin affect to the intestinal absorption of astaxanthin in rainbow trout. The absorption of astaxanthin from diets (30 ppm) supplemented with either unesterified astaxanthin; isolated astaxanthin monoesters, diesters or a cell-free carotenoid extract from *Haematococcus pluvialis* were difference in rainbow trout. After consumption of a single meal, peak serum astaxanthin levels at 32 h were significantly higher in fish fed unesterified astaxanthin and astaxanthin monoester, compared to fish fed astaxanthin diester and the cell free extract. However, no significant differences were recorded in serum astaxanthin uptake rates between sources of astaxanthin. Results suggest that the extent of carotenoid esterification negatively influences the peak serum levels of astaxanthin in rainbow trout. There are some contradictions about the efficiency of free- and esterified astaxanthin in salmonid, Bowen *et al.* (2002) studied on the utilization of astaxanthin acyl esters in rainbow trout. Mono-esterified and di-esterified astaxanthin were purified from the green microalga *Haematococcus pluvialis* and incorporated into extruded diets and compared with diets containing synthetic astaxanthin and a total carotenoid extract from the alga. The result of these studies revealed that both the isolated *Haematococcus* mono- and diesters of astaxanthin were as equally well utilized as the synthetic unesterified astaxanthin. Similarly the `cell-free' total carotenoid extract from *Haematococcus* was effective in pigmenting rainbow trout. The present study also suggests that cleavage of the astaxanthin esters may not be a limiting step for the deposition of astaxanthin. But the algal cell wall is the main limiting factor.

The different in geometrical isomers of astaxanthin are also affecting to the absorption and utilization of astaxanthin in salmonids. Bjerkeng *et al.* (1997) reported that the apparent digestibility coefficients (ADC) of total astaxanthin were significantly higher in trout fed test diet supplemented with all-E-astaxanthin (79%) compared to the trout fed the streoisomer mixture (64%). Apparent digestibility coefficients of all-E-astaxanthin was higher than that of 9Z- and 13Z- astaxanthin, and apparent digestibility coefficients of 13Z- astaxanthin was higher than that for 9Z- astaxanthin. This indicated that Z-astaxanthin are less utilized to the same extent as all-E-astaxanthin for flesh pigmentation. But, the retention of all-E-astaxanthin was higher in trout fed the stereoisomer mixture of astaxanthin than in the group fed all-E-astaxanthin. This indicates that a considerable isomerization is taking place after resorption of the carotenoids, presumably in the liver. The tendency for higher retention of digestible astaxanthin in trout fed a stereoisomer mixture of astaxanthin suggests that the stereoisomer composition of the dietary astaxanthin may influence the absorption capacity, probably by increasing micellar load of total astaxanthin.

Biological function of carotenoid in aquatic animal

Carotenoids are structurally related to retinol and β-carotene, the main source of vitamin A for animals. Generally aquatic animals are not capable of synthesizing vitamin A which is essential for vision, growth, normal development of epidermal tissue and mucosa, resistance to various bacteria and fungal diseases (Latscha, 1991). Katsuyama and Matsuno (1988) reported that dihydroxy-carotenoids, such as astaxanthin, zeaxanthin, lutein and tunaxanthin were bioconverted into vitamin A alcohol in Nile tilapia (*Oreochromis niloticus*). Carotenoids also affect the endocrine system with respect to gonadal development and maturation, fertilization, hatching viability and growth, particularly in fish and crustaceans. Craik

(1985) concluded that 1-3 mg of carotenoids per gram of salmonid eggs was associated with hatching percentages above 60 %, whereas lower carotenoid levels resulted in reduced percentage of hatching to below 50 %. The active redistribution on astaxanthin from the muscle tissue to the skin in male and to the overy in female of the salmonid has led to speculation that the carotenoids play an important role in oogenesis or embryogenesis. The transfer of astaxanthin to skin results in coloring of male fish and the active movement of astaxanthin to the overy has speculation that to the same phenomenon in mammalian, which increase the concentration of β- carotene in the corpus luteum (Bird and Savage, 1990). It is noted that the decreasing of maternal blood-carotenoid associated with embryonic development and increasing of carotenoids in the embryo (β- carotene in mammalian, zeaxanthin in avian and astaxanthin in salmonids). Some of the chemical characteristics of carotenoids may play an important role in the protection of the developing embryo (Bird and Savage, 1990). Tacon (1981) suggests a carotenoid role similar to that of α-tocopherol citing data that β- carotene is 50 time better than α-tocopherol in quenching singlet oxygen, which is a reactive and pathologically damaging oxygen species.

Schiedt *et al.* (1985) reported on the convertion of astaxanthin to vitamin A1 and A2 in rainbow trout fed diet deficient in vitamin A. Astaxanthin may play a similar role in the development of the embryo if a hypovitaminosis arises (Bird and Savage, 1990). Vitamin A plays a central role in many essential biological process, including growth promotion, reproduction and bone development as well as vision. Vitamin A deficiency changed the differentiation of epithelial cells. Moreover, vitamin A are also known to be involved in fetal development and in regulating the proliferation and differentiation of many cells (Zile, 1998). Choubert *et al.* (1998) studies on the relationship between dietary keto-carotenoids (canthaxanthin and astaxanthin) and reproductive performance in female rainbow trout. There was no significant difference on the frequency of

maturing females or the date of maturation, egg and larval survival among fish fed carotenoids-supplemented and control diet were not different. But from the pigment analysis revealed that canthaxanthin fed to the female parent was transferred into the eggs and therefore to the larvae. From the studies in Japanese flounder, *Paralichthys olivaceus*, the fish fed diet supplemented with vitamin A have longer egg production period (2.5 month) when compared to the control group (1.5 month), and the percentage of normal larvae in the control group was significantly lower than in the vitamin A supplemented group (Furuita *et al.*, 2003).

Linan-Cabello *et al*. (2002) suggested that dietary carotenoids are the sole biological precursors of retinoids in crustaceans. The importance of carotenoids as bioactive molecules reside to a large degree on their conversion to retinoids (Figure 1-11) are involved in the activation of hormonal nuclear receptors. The presence of receptors of retinoic acid in crustaceans and the finding of retinoids in the neuroendocrine complex and in reproductive tissue, as well as the enchancement of the ovarian development in shrimp suggests an important role of retinoids in shrimp physiology.
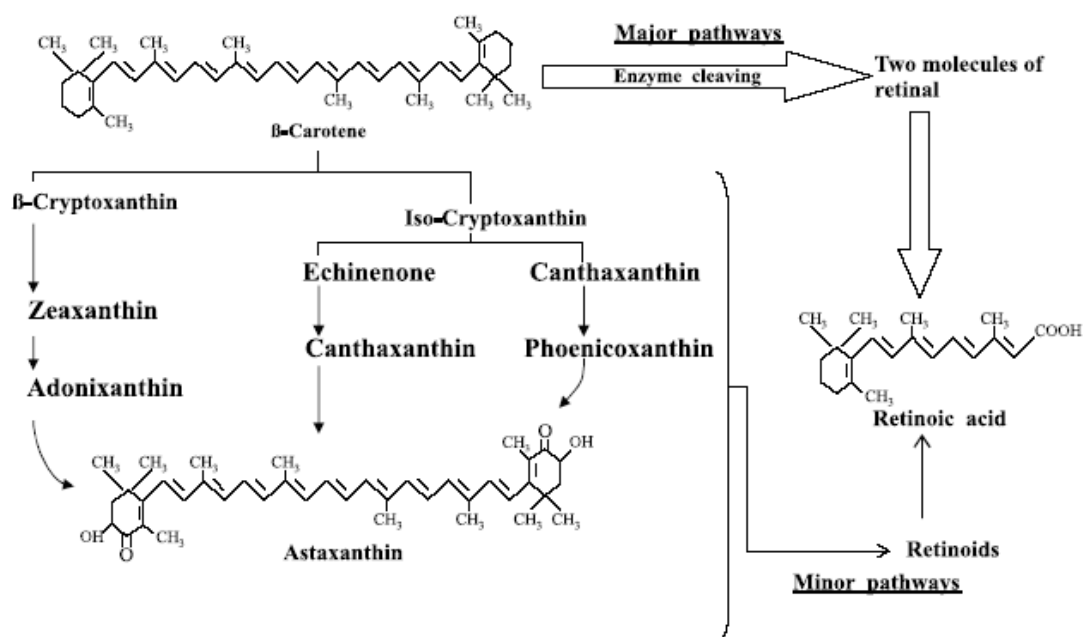
Figure 1-11. Bioconversion pathways of carotenoids in the penaeid shrimp and proposed
conversion pathway to retinoids.
From : Linan-Cabello *et al*. (2002)

These noval hypotheses on the role of carotenoids and retinoids in shrimp production have been proved in many shrimp species. The higher levels of vitamin A in broodstock diet shown positive effects on fecundity and larval quality in *Penaeus chinensis*, (Mengqing *et al*., 2004). The broodstock shrimp fed the diet with highest level of vitamin A acetate (60 ppm) exhibited higher fecundity, hatching rate and survival of the larvae. As well as in the crayfish, Linen-Cabello *et al*. (2004) reported that the crayfiah, *Cherax quadricarinatus*, which were injected with retinol palmitate show the greatest inductive effect on the primary vitellogenic phase and on indicators of ontogenic oocyte development and followed by the groups which were injected with β- carotene and astaxanthin.

Carotenoids in conjugation with protein moieties (carotenoproteins) appears to improve the stabilization of proteins and their tertiary structure. Such interaction are belived

to be involved in olfactory reception and chemoreception in various animals as well as in human. Carotenoids are also involved in cross-membrane calcium transfer in the neuronal respiratory chain as oxygen reservoirs and in photoprotection and biological antioxidant in protecting sensitive tissue and reactive compounds from damage due to oxidation (Latscha, 1991). Astaxanthin has antioxidant activity as a oxygen quencher and free radical scavenger (Miki, 1991). Astaxanthin has been shown to be more efficient than β-carotene and zeaxanthin in retarding hydroperoxidation of methyl lonoleate (Terao, 1989). Astaxanthin has been reported to be 200 times (molar basis) more effective than vitamin E and 10 times more effective than BHA in preventing hydroperoxidation of lipids (Sanderson and Jolly, 1994).

The antioxidant activity of carotenoids leads many scientists to study the effect of dietary carotenoids on the immunological response in aquatic animals. Thompson *et al.* (1995) experimented on rainbow trout fed diets supplemented with vitamin A and astaxanthin, astaxanthin alone, vitamin A alone and neither vitamin A nor astaxanthin, to investigate the immunomodulatory effects of these compounds in fish. It was found that total serum antiprotease activity was significantly lower in the group fed the diet without astaxanthin and vitamin A. Further studies on higher doses and in other cultured animals such as shrimp remain to be done.

Since vitamin A has a number of significant effects on innate and specific responses in homeotherms (West *et al.*, 1991), and it has been shown that dietary vitamin A intake affects both humoral and cell-mediated immunity in Atlantic salmon (Thompson *et al.*, 1994). The whole requirement of vitamin A in aquatic animal must be supplied in the diet. This may occur directly by adding vitamin A to the diet, or indirectly through metabolism of carotenoid precursors (Schiedt *et al.*, 1985). Apart from the vitamin A precursor, the other importance effects of carotenoids in immunity is a very high scavenging affinity for toxic oxygen redicals which result, mainly from lipid

peroxidation but they are also generated during the respiratory burst of phagocytes (Thompson *et al.*, 1995). From the studies on the effect of dietary vitamin A and astaxanthin on the immunocompetence of rainbow trout, The fish fed test diet supplemented with 18 ppm vitamin A and 100 ppm astaxanthin increasred the total serum antiprotease activity and classical serum complement activity when compared to the fish fed diet without vitamin A and astaxanthin, or diet with vitamin A or astaxanthin alone (Thompson *et al.*, 1995).

Amar *et al.* (2001) examined the influence of different carotenoids on some immune indices in rainbow trout. The fish in each group fed test diets contain astaxanthin, canthaxanthin and β-carotene, at 100 ppm, each of them with vitamins A, C and E either added or omitted. The two control diets contained no carotenoids and were either with or without the vitamins. Serum complement activity and serum lysozyme activity in both β-carotene groups and the vitamin containing astaxanthin group were significantly higher than both the control fish. Phagocytic activity was also high in the vitamin- containing β-carotene and astaxanthin groups compared with the controls. But, for phagocytic index, the vitamin-containing canthaxanthin group gave better results compared with the controls. The vitamin-containing astaxanthin and β-carotene groups also exhibited better nonspecific cytotoxicity for the peripheral blood lymphocytes, so β-carotene and astaxanthin elevated humoral factors as well as cellular factors, and they concluded that in the presence of the vitamins the carotenoids exerted a greater influence on the bio-defense mechanisms of rainbow trout. Because the cost of synthetic carotenoid, which is not suitable for the application in farm-system, Amar *et al.* (2004) studied on the application of carotenoids from natural products, such as *Dunaliella salina* and *Phaffia rhodozyma* as source of β-carotene and astaxanthin. Fish were fed test diets containing either β-carotene and astaxanthin at 100 and 200 ppm from *Dunaliella salina* and *Phaffia rhodozyma* respectively, among the humoral

factors, serum alternative complement activity increased in all carotenoid supplemented groups. However, the serum lysozyme activity increased in the *Dunaliella* group but not in the *Phaffia* group, whereas plasma total immunoglobulin levels were not altered by the feeding treatments. For the cellular responses, the superoxide anion production from the head kidney were not difference while the phagocytic activity and phagocytic index in all supplemented group were higher than the control. These finding demonstrate that dietary carotenoids from *Dunaliella salina* and *Phaffia rhodozyma* can modulate some of the innate defense mechanisms in rainbow trout.

Little in known about the mechanisms by which astaxanthin and other carotenoids may involve in performance improvement and stress response in marine animals. Several researchers have hypothesized that these benefits may derive from astaxanthin unique oxygen quenching capacity (Hunter, 2000; Chein *et al.*, 2002). Studies in trout was found that serum glutamic-oxoloacetic transaminase activity were higher in the group fed diet supplemented with astaxanthin than non-supplemented fish. In addition astaxanthin were found to have reduced lipid peroxide levels and reduced hepatosomatic index in trout. Moreover, the studies in trout and shrimp have shown reduced levels of oxidative products in phagocytes and hemocytes in astaxanthin supplemented animals (Hunter, 2000). Chien *et al.* (1999) determine the effect of oxygen depletion stress on mortality and lethal course of black tiger shrimp fed high level of dietary astaxanthin. The results indicated that those shrimp exposed to oxygen depletion stress trial, the shrimp fed diet contained 360 ppm astaxanthin had better survival than control shrimp. Lower mortality in the group fed test diet supplemented with 360 ppm astaxanthin than in control shrimp could be attributed to higher astaxanthin in the shrimp body. Lethal dissolved oxygen levels of the shrimp fed diet contained 360 ppm astaxanthin was significantly lower than control group. When measuring of the oxygen consumption of both groups after exposed to low dissolved oxygen condition for 2 hr, the oxygen

consumption rate of the shrimp fed 360 ppm astaxanthin was lower than control shrimp. It appeared that astaxanthin may play a buffer role on oxygen intake according to the environment dissolved oxygen conditions.

Merchie *et al.* (1998) studied on the effect of astaxanthin on the resistance of salinity stress in juvenile black tiger shrimp. Shrimp were subjected to salinity stress consisting of a transfer from 27 ppt to 0 ppt salinity water for 1 hr. Shrimp supplemented with 810 ppm astaxanthin had a cumulative stress index which was significantly lower than that of postlarvae fed a diet containing 230 ppm astaxanthin diet. While an organism is subjected to stresses such as chemical, physical, biological upon sudden shortage of oxygen, abnormal oxidative reactions in the aerobic metabolic pathway result in the formation of excess amounts of singlet oxygen and the subsequently generated radicals. These radicals can impair lipids, proteins, carbohydrates and nucleotides which are important parts of cellular constituents. Radical damage can be significant because it can proceed as a chain reaction. Consequently, mortality can occur due to severe destruction by massive radicals generated from acute stresses or long-term chronic stresses. Because astaxanthin contains a long conjugated double bond system with relatively unstable electron orbitals, it may scavenge oxygen radicals in cells and therefore reduce cellular damage and enhance resistance. The same speculation could be applied to thermal stress because thermal stress also involves oxidation for energy. Chein *et al.* (2003) examined the resistance to thermal and osmotic stress by black tiger shrimp which were fed diets supplemented with astaxanthin. The shrimp fed 80 ppm astaxanthin for 8 weeks had significantly higher average recovery (56% recovery) than the control shrimp (48% recovery) suggested that astaxanthin supplement had improved the resistance against thermal (change in water temperature from 27 ^{0}C to 5 ^{0}C for 5 min) and osmotic stress (change in salinity from 32 ppt to 0 ppt for 5 min). Total antioxidant status was improved and superoxide dismutase activity was reduced by the presence

of dietary astaxanthin. In these studies shrimp hepatopancreatic function may have been improved by dietary astaxanthin since hemolymph aspartate aminotransferase (AST) of the control shrimp was significantly higher than that of the treated shrimp.

Dietary astaxanthin also enhanced the resistance to ammonia stress, Pan *et al*. (2003) evaluated the resistance to chemical stress in black tiger shrimp by means of the survival rate, hemolymph aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Black tiger shrimp (PL 5) were fed diets supplemented with 0 and 71.5 ppm astaxanthin for 8 weeks. Shrimps were then subjected to 72 h exposure of ammonia at 0.02, 0.2, 2 and 20 ppm. The survival rates of the shrimp fed astaxanthin were higher than the control shrimp under all levels of ammonia except 20 ppm indicating that the resistance of black tiger shrimp to ammonia stress had been improved by dietary astaxanthin. Moreover, shrimp fed diet contained astaxanthin had higher total antioxidant status than control shrimp at ammonia levels which higher than 0.02 ppm and lower SOD at all ammonia levels suggested that antioxidation capability had been enhanced by dietary astaxanthin. AST in shrimp fed astaxanthin was lower than control shrimp and ALT was either lower than or equal to that in control shrimp under various levels of ammonia. These results indicated that shrimp hepatopancreatic function had been improved by dietary astaxanthin.

Applications of carotenoids for pigmentation in aquatic animal

Upon uptake by marine animals, carotenoids can be deposited unchanged or in modified or complex forms in various organs such as skin, flesh and exoskeleton, thereby imparting spectacular pigmentation. Since pigmentation is generally perceived as a quality parameter, dietary supplementation of carotenoids is widely practiced in aquaculture.

Bjerkeng *et al*. (1992) studied the efficiency of astaxanthin and canthaxanthin for pigmentation of rainbow trout.

The fish were fed with diets supplemented with either 100 ppm astaxanthin, 100 ppm canthaxanthin and without carotenoids added to the diet for 140 week. They found that the carotenoid concentrations in skin and flesh of trout fed astaxanthin tended to be higher than that of the fish fed canthaxanthin, Moreover, the optical isomer of astaxanthin in the flesh was not significantly different from that in feed. Skin of fish fed astaxanthin mainly contained astaxanthin esters and skin of fish fed canthaxanthin contained canthaxanthin and its reductive metabolites, mainly zeaxanthin.

However, deposition of carotenoids in salmonid flesh occurs as a result of several processes: absorption of pigments in the digestive tract, transport of pigment in the blood, retention in the muscle and metabolism of carotenoids. Values for deposition effciencies of carotenoids (the amount of astaxanthin or canthaxanthin measured in the flesh (ppm) compared with the amount of astaxanthin or canthaxanthin in the feed) given to the fish reported vary. Buttle *et al.* (2001) reported that the Atlantic salmon, *Salmo salar* L. (220 g initial weight) were given feeds where the pigment source was astaxanthin only, canthaxanthin only or a astaxanthin/ canthaxanthin mix. As the proportion of dietary canthaxanthin increased, flesh pigment levels also showed an increase; the pigment content in the muscle of canthaxanthin-only fed fish was 14% higher than that of the astaxanthin-only fed fish, with the mixed pigment fed fish being intermediate between the two extremes. Results of crosssection assessment for redness (a*) values and Salmofan scores also showed an increase in colour with increasing proportions of canthaxanthin in the feed. The data reported clearly indicates that *Salmo salar* deposit canthaxanthin more efficiently than they do astaxanthin. These results contrast with those obtained by other authors with rainbow trout, *Oncorynchus mykiss* (Walbaum), and imply that the absorption or utilization of the pigments differs between species. Moreover, Hatlen *et al.* (1997) reported that the reproductive status, genetically origin and rearing condition also effect to the efficiency of each carotenoid in Arctic charr

Salvelinus alpinus, when compered fillet color (L*, a* ,b* and C*) and carotenoids concentration of the fish samples, it was found that increasing carotenoid concentrations led to increased redness (a*) yellowness (b*) and chroma (C*) and decreased lightness (L*). But yellowness was higher in sexually mature than in immature fish.   This was possibly a result of the higher propotions of idoxanthin in mature fish, which cause by the different in proximate composition of fillets of mature and immature fish. Because, the idoxanthin differs from astaxanthin in having keto group in the 4- position reduced to a hydroxyl group, so these carotenoid shown slightly changes in absorption characteristics and resulting in a more yellowness appearance of the fillets (Britton *et al.*, 1995). Muscles of mature Arctic charr had higher percentage water and lower protein contents than the muscles of immature fish, and the introduction of a hydroxyl group into carotenoid molecules leads to increased polarity and this may influence solubility and interaction with other components in the muscle cells. Moreover, Henmi *et al* (1987) reported that the binding capacity of carotenoids in fish muscle seems to increase with introduction of 3-hydroxy and 4-keto groups into the β-endgroup of carotenoid molecules. Although, the astaxanthin shown its highest efficiency for pigmentation in salmonid, but the efficiency of astaxanthin in rainbow trout are also effected by the degree of esterification of these pigment. White *et al.* (2002) reported the peak serum astaxanthin in the fish fed a single meal of astaxanthin were significantly higher in fish fed unesterified astaxanthin and astaxanthin monoester compared to fish fed astaxanthin diester.

Kiessling *et al.* (2003) studies on the absorption of astaxanthin and canthaxanthin in Atlantic salmon (*Salmo salar*), the results was found that the blood astaxanthin concentration of the fish approached saturation when the fish fed diet supplemented with 30 ppm astaxanthin (1.2 ± 0.04 ug/ml), but in the group fed canthaxanthin blood levels of canthaxanthin continued to increase linearly throughout the inclusion range of 30-60 ppm. However when astaxanthin and canthaxanthin were

presented in the same diet, a reduction in the absorption efficiency of both pigments was observed. These study was in contrast with the several studies with rainbow trout, which shown that astaxanthin is more efficiently absorbed and deposited than canthaxanthin (Torrissen, 1989; Bjerking, *et al.* 1990; Storebakken and Choubert, 1991; Storebakken and No, 1992).

Absorption of carotenoid is also affected with the composition of diet. Lipophilic compounds may interact during absorption. A high dietary fat content is among the factors positively affecting the absorption of astaxanthin in salmonids. Bjerkeng *et al.* (1999) reported that the astaxanthin deposition in fillets was increased by 8-14% by the dietary addition of 800 ppm α- tocopheryl acetate compared with 200 ppm α- tocopheryl acetate.

Tanaka *et al.* (1976) investegated the metabolism of dietary carotenoids in the kuruma shrimp (*Penaeus japonicus*) and suggested the following two oxidative pathways :

1. β-carotene → isocryptoxanthin → echinenone → canthaxanthin →
   phonicoxanthin → astaxanthin

2. Zeaxanthin → 4-ketozeaxanthin → astaxanthin

Moreover, they reported that the shrimp could directly deposit dietary astaxanthin in their tissue. Yamada *et al.* (1990) evaluated the effect of carotenoids on the pigmentation in kuruma shrimp and found that the carotenoids stored in the body were higher in shrimp fed carotenoid supplemented diets than those in the control group. They also found that astaxanthin was the most effective in promoting shrimp pigmentation. However, all forms of carotenoids in this experiment could be converted into astaxanthin esters which were deposited in shrimp body tissue. For the study on the effect of the concentration of

astaxanthin in shrimp, the diets were supplemented with astaxanthin at levels of 0, 50, 100, 200 and 400 ppm. After eight weeks of feeding the concentration of total carotenoids and astaxanthin esters in the shrimp body had increased with increasing dietary astaxanthin level up to 200 ppm. This study concluded that 200 ppm of astaxanthin in a diet is sufficient to improve the pigmentation in kuruma shrimp.

Use of microoganism as a carotenoid source in aquatic animal feed

Carotenoids are found to constituent in many microorganism such as microalgae, yeast and bacteria. Two groups of microorganisms have been studied with the aim of developing an industrial production of astaxanthin. These are microalgae, mainly genus *Haematococcus sp.,* and the yeast *Phaffia rhodozyma*. The green algae has the advantage of containing large amounts of astaxanthin when grown under the right conditions, although such conditions are difficult to obtain in a large-scale process. Another problem associated with large-scale production is foreign algae contamination and predation by protozoan that may consume up to 90% of the algae biomass (Tangeras and Slinde, 1994). While, the yeast can be grown in large fermenters in a process similar to that used for commercial production of baker's yeast. *Phaffia rhodozyma* contains a high level of astaxanthin and it may be applied to use as an astaxanthin source in aquatic feed (Sanderson and Jolly, 1994). Tangeras and Slinde (1994) reported on the application of *Phaffia rhodozyma* as an astaxanthin source in rainbow trout. When three diets containing either *Phaffia rhodozyma*, synthetic astaxanthin or no astaxanthin were given to rainbow trout, the diet supplemented with synthetic astaxanthin gave the highest levels of astaxanthin in the flesh. But, when adjusted for the different levels in the feeds by calculating the amount of astaxanthin accumulated in the flesh per amount of astaxanthin supplemented in the feed, *Phaffia rhodozyma* seems to be a

better astaxanthin source than the synthetic compound. In order to make the astaxanthin avilable to salmonids, at least for Atlantic salmon reared under commercial conditions, cell wall of the yeast must be modified. This can be achieved by chemical, mechanical or enzymatic treatment. Cell wall degradation can be increased by adding hydrolytic enzymes such as β, 1-3 glucanase to the yeast (Sorum and Robertsen, 1991 cited by Tangeras and Slinde, 1994). For mechanical treatment, high-pressure homoginization can be applied. With this technique the cell suspension is forced through a homoginization valve at high pressure.

Gentles and Haard (1991) studied the pigmentation of rainbow trout fed with enzyme-treated and spray-dried *Phaffia rhodozyma* cell supplemented diets. Intact yeast cells were treated by mechanical milling (MY), enzyme-treatment (EY), spray-drying (SY) or extraction of the carotenoids (C) prior to inclusion in the diet of rainbow trout. All treatments resulted in excellent coloration and pigmentation of skin and muscle tissues and significantly differed from each other after 8 weeks of feeding. The flash color of trout fed different diets differed in the following order : MY > EY > SY > C > pigment-free diet. Although mechanically broken cells or enzyme-treated cells of *Phaffia rhodozyma* at 15 % of the diet gave excellent flesh coloration and pigmentation of rainbow trout after 4 weeks of feeding, both treatments may not be practical because of expensive processing costs.

Sommer *et al*. (1991) studied the efficiency of whole and broken algae cell supplements in comparison with synthetic astaxanthin at a concentration of 40 ppm of diet for rainbow trout. The algae *Haematocuccus pluvialis* was used in the study. After a 100 days feeding trial, the flesh of the fish in each treatment was removed and homogenized for pigment analysis. The results confirmed that the control group which fed unsupplemented diet contained only trace levels of flesh carotenoid, whereas the fish fed diets supplemented with levels in the synthetic astaxanthin, broken spore and whole spore

showed steadily increased levels of the pigment with time of experiment. Statistical analysis of the data revealed that these changes were linear. The synthetic astaxanthin treatment produced the highest levels of total carotenoids in the flesh, followed by the broken spore and whole spore.

Choubert and Heinrich (1993) studied the effects of dietary algal (*Haematococcus pluvialis*) incorporation on muscle pigmentation of rainbow trout in comparison with synthetic astaxanthin, canthaxanthin and combination of both pigments. After 4 weeks of feeding, color measurements showed that increased pigmentation of the trout muscle caused an increase in chroma and a reduction in hue and lightness. Muscle of trout fed algae contained carotenoids of 6.2 ppm of muscle whereas the trout fed the combination of the two pigment, synthetic astaxanthin and canthaxanthin contained higly amount of the pigment of 12.7, 11.8 and 10.1 ppm of muscle.

Chien and Jeng (1992) determined the effects of sources and levels of pigments on the pigmentation of kuruma shrimp. Seven pigmented diets containing astaxanthin at 50, 100 and 200 mg / 100 g of diet, β-carotene at 50, 100 and 200 mg / 100 g of diet and algal *Dunaliella salina* meal at 100 mg pigment / 100 g of diet. Astaxanthin was found to be the most effective pigment source. No difference in pigment concentration was found between shrimp fed the β-carotene and algal meal. Shrimp fed the astaxanthin supplemented diet had a higher rate of survival than those fed the β-carotene or algal supplemented diet. A positive correlation between survival rates and pigment concentration in shrimp tissue indicated that pigments may play a role in improving shrimp survival.

Liao *et al*. (1993) studied on the pigmentation of cultured black tiger shrimp by feeding with various pigments sources. In this trial, black tiger shrimp (*Penaeus monodon*) were fed with diets containing different carotenoid sources *eg*. β-carotene, spirulina, *Phaffia* yeast and krill oil. A marked increase of carotenoid content in the carapace was observed when the

shrimp were fed with spirulina diets. With the highest pigment content when supplemented at 3 % of diets. Moreover, the analysis of carapace carotenoids in the shrimp fed spirulina suggested that zeaxanthin, one of major carotenoids in spirulina, was rapidly converted to astaxanthin. Spirulina are also improved protein digestibility of the diets in common carp, *Cyprinus carpio*, Nandeeesha *et al.* (1998) studies on the effect of feeding *Spirulina platensis* (25 -100 % replacing fish meal protein) on growth, carcass composition and digestibility of common carp for 120 days. The final weight gain, specific growth rate, food conversion ratio, and protein efficiency ratio of common carp were not affected by spirulina supplementation. However, the diet with Spirulina as the sole source of protein resulted in better net protein retention. Several attempts have been reported on the efficiency of many micro algae as the alternative carotenoid sources in aquatic animal feeds, Sommer *et al.* (1991) reported that the Western Australian freshwater crayfish *Cherax tenuimanus* (called marron) lose dark exoskeletal coloring when fed artificial diet in indoor tanks, suggesting a carotenoid deficiency in these foods. So the test diets was added either a powder or fresh slurry of the carotenoid-rich microalga, *Dunaliella salina*. Juvenile marron were fed to the diets for 100 days. Thin layer chromatography of pigment extracts revealed that all groups contained astaxanthin, mono- and di- esterified astaxanthin and β- carotene. However, canthaxanthin, adonirubin and esterified adonirubin were observed additionally in the group fed test diet supplemented with algal only. Pigment analyses of marron showed elevated levels of total carotenoid and β- carotene in the algal treatment groups as compared to the control group. Marron on the algal slurry treatment also showed a significantly higher growth rate.

Harpaz *et al.* (1998) examined the effect of dried algal cells prepared from *Dunaliella salina* (main source of β-carotene) compare to synthetic astaxanthin and alfalfa meal in crayfish (*Cherax quadricarinatus*). Crayfish receiving feeds supplemented with all sources of carotenoid exhibited better

body coloration than those in the control group, which were fed a diet without carotenoids added. Growth and survival of the crayfish were not affected by the addition of carotenoids to their diet. *Chlorella* is one of micro algae which contained high level of carotenoids, and seem to shown high potential of carotenoids source in fish feeds. Gouveia *et al.* (2002) studies on the effects of test diets supplemented with dried *Chlorella vulgaris* (0.4 % total carotenoids, lutein 0.3%, β- carotenev1.2 %, canthaxanthin 36.2 %, astaxanthin 55 % and other pigments 7.3 %) compared to the diet supplemented with synthetic astaxanthin and 1:1 dried algae and synthetic astaxanthin in gilthead seabream, *Sparus aurata*. After 9 weeks of feeding trial, carotenoid pigments were significantly deposited in the skin in all treatment. In the muscle, regardless of the astaxanthin source, the concentration of carotenoid measured was very low and not significantly difference. Moreover, no muscle pigmentation was noted in all treatment, and there was no variation in the amount of carotenoid analysed in skin for each treatment. It was concluded that the supplementation of dried *Chlorella vulgaris* in fish feed would be an acceptable practice in aquaculture to improve the market appeal of the gilthead seabream. In the ornamental fish culture, intensity of skin coloris an important quality criterion. Gouveia *et al.* (2003) investigated skin color enchancement in carp and goldfish (*Carassius auratus*) by feeding a dietary carotenoid supplement of freshwater microalgal biomass (*Chlorella vulgaris*, *Haematococcus pluvialis*, and *Spirulina maxima*) in the amount to obtained 80 ppm total carotenoids and using a diet containing 80 ppm synthetic astaxanthin and a control diet with no coloring added for comparison. Growth and feed efficiency were not significantly different between groups administered by the various dietary treatments. Dietary carotenoid supplementation increased total skin carotenoid content. The more efficient coloring for carps was found to be *Chlorella vulgaris* biomass, providing both maximum total carotenoid deposition and red hue. For goldfish the best coloring obtained, as certained by total carotenoid content, was also achieved using

Chlorella vulgaris biomass, and red hue was maximum when using *Haematococcus pluvialis* biomass.

Photosynthetic bacteria and its applications in aquaculture

Photosynthetic bacteria have been studied for their roles in the bioconversion of organic waste or environmental pollutants into useful materials such as single cell protein, biological pesticide and vitamins (Tanaka *et al.*, 1994; Yamada *et al.*, 1997; Holtman and Kobayashi, 1997 cited by Prasertsan *et al.*, 1993). Photosynthetic bacteria can produce many carotenoid pigments e.g. lycopane, rhodopin, spirilloxanthin, chloroxanthin, spheroidene, spheroidenone, okenone, β-carotene and γ-carotene (Staley *et al*., 1989). Carotenoids are derived from the general isoprenoid biosynthetic pathway, along with a varity of other important natural substances. The conversion of two molecules of geranylgeranyl pyrophosphate (GGPP) to phytone, anoxygenic photosynthetic bacteria, nonphotosynthetic bacteria, and fungi desaturate phytone to yeild neurosporene or lycopene respectively. At the level of neurosporene or lycopene, the carotenoid biosynthesis pathway of different organisms branches to generate the tremendous diversities of carotenoids found in nature (Armstrong, 1994).

Prasertsan *et al.* (1993) examined four strains of photosynthetic bacteria isolated from wastewater of a seafood processing plant in Songkhla,and found only one strain which had the ability to utilize high content organic matter. In diluted tuna condensate, it gave a high biomass of 4 g / l, with a cell yield of 0.32 g / g COD and COD removal of 78 %. The cell mass gave the highest carotenoid content and bacteriochlorophyll value at an optimum pH of 7.0 with a low light intensity at 1000 lux.

Pradal (1993) studies the coloration and growth of rainbow trout, using diet supplemented with synthetic astaxanthin, photosynthetic bacteria *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 unsupplemented diet and diet supplemented with astaxanthin. Moreover, the coloration of the rainbow trout flesh was almost at the same level as that of fish fed diet supplemented with astaxanthin. The coloration yields were different between sex in that female had better coloration than male rainbow trout, particularly during sexual maturation. Furthermore, the effect of *Rhodobacter capsulatus* pigments in live feed on the lipid peroxide in the juvenile Japanese flounder was investigated (Okimasa *et al*., 1992). In the administration group of rotifers reared with photosynthetic bacteria, the amount of lipid peroxide in the fish on day 30 after feeding trial was lower than that in the control group. The mortalities on day 30 were also lower than those of the control group. The photosynthetic bacteria pigments inhibited the lipid peroxidation of egg phosphatidylcholine liposomes. These finding suggest that the pigments, especially X^1 and X^3 fractionated by reversed-phase HPLC inhibited the initiation and progression of lipid peroxidation. Although, photosynthetic bacteria have been reported for potential use as carotenoids source in fish, no data have been reported on the bioavailability and utilization of such carotenoid in shrimp. Thus it is necessary to further study the efficiency of photosynthetic bacteria for use as a carotenoid source in shrimp feed.

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Objectives

1. To isolate and identify certain photosynthetic bacteria from seawater and shrimp farms and assess for their potential as carotenoid source in shrimp feed.

2. To optimize the selected isolate for carotenoids production.

3. To assess the potential use of photosynthetic bacteria as a pigment source for body color enhancement, immune response, stress tolerance and in disease resistance in the shrimp.