# CHAPTER I

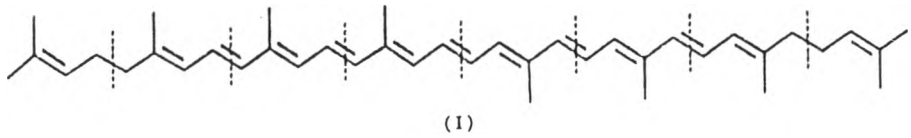
## INTRODUCTION

Carotenoids are unique pigments synthesized in photosynthetic higher plants and photosynthetic microorganisms which serve essential functions in the protection against singlet oxygen-generated damage during photosynthesis process. In the plants, carotenoids participate in light harvesting reactions and may act as a protective covering for certain higher plant species. In animals, carotenoids have attracted considerable interest, beginning over fifty years ago when it was discovered that they are precursors of vitamin A and derivatives. Mammalian species do not synthesize carotenoids or vitamin A. These essential substances are therefore derived solely from plant carotenoids, which are absorbed and stored in tissues and subsequently metabolized.

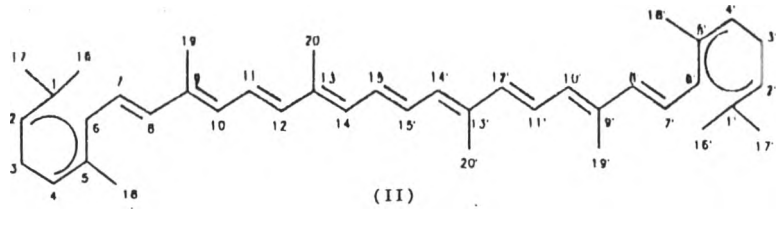
From a biomedical and biotechnological viewpoint, the carotenoids have also received considerable attention recently. In numerous epidemiological studies, the dietary intake of foods rich in carotenoids. In addition to their role as vitamin A precursor, carotenoids are known to act as antioxidant, and to inactivate (quench) highly reactive chemical species such as singlet oxygen, triplet photochemical sensitizers and free radicals which would otherwise induce potentially harmful process (e.g. lipid peroxidation). Through these actions, it has been suggested that carotenoids may play important rules in cancer protection (Parker, 1992 ; Ong and Tee, 1992).

## Nomenclature and Structure

Carotenoids are a class of hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls) They consist of eight isoprenoid units joined in such a manner that the arrangement of isoprenoid units is reversed at the center of the molecule so that two central methyl groups are in a 1, 5 - position relationship. All carotenoids may be formally derived from the acyclic $C_{40}H_{56}$ structure, having a long central chain of conjugated double bonds, by (1) hydrogenation, (2) dehydrogenation, (3) cyclization, or (4) oxidation, or any combination of these processes. Carotenoids also include compounds that arise from certain rearrangements or degradations of the carbon skeleton, provided that the two central methyl groups are retained (Figure 1).



(I)



(II)

Figure 1 Acyclic $C_{40}H_{56}$ structure (I), carotene structure(II) (Frender,1992)

Carotenoids are a well-characterized class of compound present in microorganisms, algae, higher plants, animals and humans. More than 600 carotenoids occur in nature. Ever since the elucidation of structure of β-carotene and other carotenoids much effort has been devoted to the synthesis of carotenoid. Today six synthetic carotenoids have become commercially important : β - apo - 8' - carotenol (XIII) , β - apo - 8' - carotenoic acid ethyl ester (XV) , citranaxanthin (XVI) , β - carotene (III) , canthaxanthin (XI) and racemic astaxanthin (XII) (Figure 2)

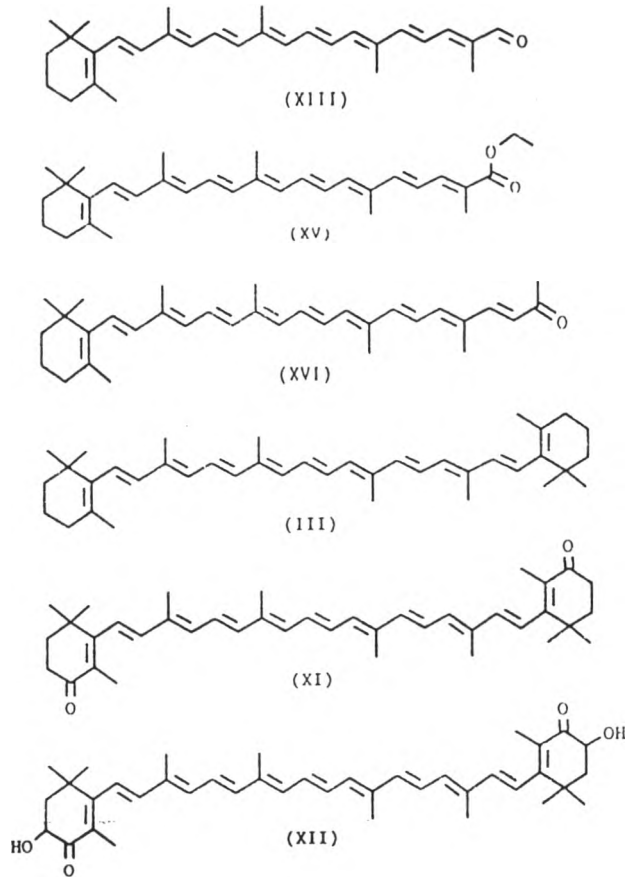



Figure 2 Structure of six synthetic carotenoids (Frander,1992)

## Astaxanthin

Astaxanthin (3,3' - dihydroxy - β - β'- carotene - 4 - 4' - dione) is a red pigment common in many marine animals contributing to the pinkish colour of their flesh. Only few animals can synthesize it *de novo* from other carotenoids and most of them acquire it in their food (Lu and Soh, 1991 ; Yong and Lee,1991). There has been a growing interest in the use of this pigment as a colourant for egg yolk in the poultry (Boussiba, Fan, and Vonshak,1992) and in aquaculture where it is used as a feed supplement in the production of salmon, trout, and shrimp. (Boussiba and Vonshak,1991;Kakizono, Kobayashi and Nakai, 1992 (a, b)) . Astaxanthin has been shown to posses higher antioxidant activity than β -carotene and α - tocopherol (Miki,1991; Tjahjono, Hayama, Kakizono, Terada, Nishio and Nakai,1994)

To date, there have been two major industrial sources of astaxanthin

1. Synthetic astaxanthin

F.Hoffman-La Roche, Basal Switzerland accomplished the synthesis trans-astaxanthin which is marketed as "carophyll pink" It contains 8 % astaxanthin. Synthetic astaxanthin is commonly used in diets of aquaculture farm to produce colouration similar to that obtained by natural which would lead to market acceptance(Sommer, D'Souza and Morrissly, 1992).

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2. Natural astaxanthin

2.1 Crustacean meals

Astaxanthin composition in grass shrimp (*Panaeus monodon*) meals were studied to obtain basic information for any future application of the natural colourants. The main caroteniods in the grass shrimp meals are composed of astaxanthin and its ester. (Wu and Hwang, 1993) But Crustacean meals have relatively low contents of astaxanthin and high levels of moisture, ash, chitin (Johnson and An,1991) and minerals, which cause several practical problems in feed formation that limit their usefulness in animal feed (Bubrick, 1991).

2.2 Yeast

*Phaffia rhodozyma* has desirable properties of astaxanthin and other nutrients in salmon diets, including rapid heterotrophic metabolism and production of high cell density in fermentors. However, its content of astaxanthin in wild strains is only 200 to 300 μg/g yeast (0.02 - 0.03%). The content of astaxanthin varies substantially depending on strain and method of culture. (Johnson, Villa, and Lewis, 1980)

2.3 Algae

The biflagellate green alga, *H. pluvialis* has recently received much attention due to its capacity to accumulate large amount of astaxanthin. Under unfavorable growth condition, or following different types of environment stress, cells of this alga form cyst and accumulate massive amount of astaxanthin in their cytoplasm to the extent that their colour changes to red. *Haematococcus* contains a high concentration of astaxanthin (0.2 to 2 %) (Vonshak, 1990). Moreover, astaxanthin can be produced from other strains of algae such as *Chlamydomonas nivalis*

(Czygan,1986) , *Euglena rubida* (Czeczuga,1974) and *Acetabularia mediterranea* (Czeczuga,1974)

**Chemical Properties of Astaxanthin**

Astaxanthin is an ketocarotenoid with a molecular formula $C_{40}H_{52}O_4$ and molecular weight 596.86. Isolated crystalline astaxanthin has the appearance of a fine, dark violet-brown powder. Its melting point is approximately 224 °C. It is insoluble in aquous solution and most organic solvents but can be dissolved at room temperature in non-polar solvents such as acetone, dimethyl-sulfoxide. Because carotenoids contain a long conjugated double bond, they are less stable than other isoprenoids and precaution must be taken to avoid artifacts and destruction of the pigments. Light, heat acid and oxygen are particularly detrimental to carotenoids and enzymatic destruction also can occur during extraction from biological samples (Stanier, Kunizawa, and Cohen-Baxire, 1971).

Astaxanthin has two asymetric carbon atoms at the 3 and 3' position and can exist in four configurations, including the identical enantiomers (3S , 3'S ; 3R, 3'R) and meso forms (3R , 3'S ; 3'R 3S) (Renstrom et al,1981). Chemical synthesis from racemic precursors gives equal mixture of the configurational isomer. The isomers can be separated by reacting (-) - camphanic diester by HPLC and by TLC on silica gel. (Foss et al,1987)

Configurations of astaxanthin are shown in figure 3 :

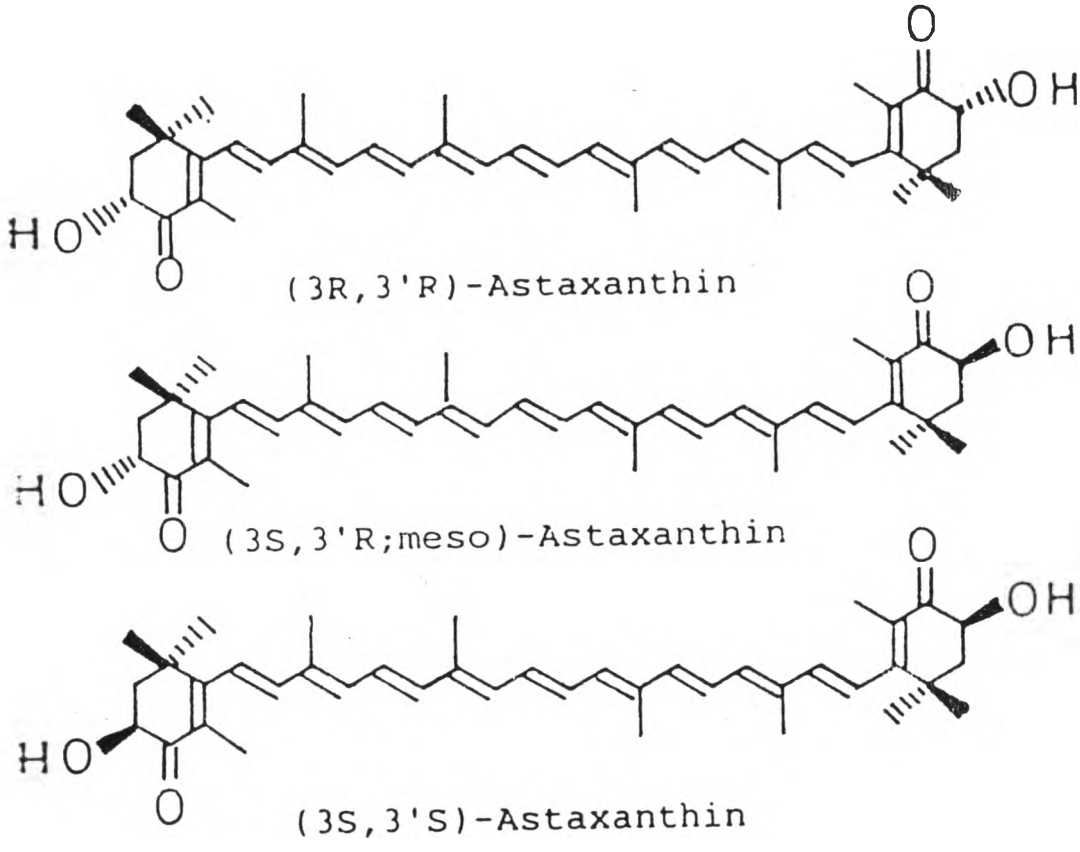


Figure 3 Three configurations of astaxanthin (Johnson and An, 1991)

**Taxonomy** (Prescott, 1964)

| | |
|---|---|
| Phylum | Chlorophyta |
| Class | Chlorophyceae |
| Order | Volvocales |
| Family | Chlamydomonadaceae |
| Genus | Haematococcus |

**Characteristics and reproduction of *H. pluvialis***

Motile cells of *Haematococcus* are solitary, biflagellate and enclosed by a wall that is broadly ellipsoid to ovoid. The protoplasm lies some distance inward from the wall and is connected with it by numerous delicate strands of cytoplasm. The intervening space between the wall and the protoplasm is filled with a watery gelatinous substance. There are flagella at the interior end of a cell and the portion of each flagellum between the wall and protoplasm lies within a gelatinous canal. There is an eyespot in the equatorial region, contractile vacuole here and there beneath the plasma membrane and several pyrenoids within chloroplast. (Smith, 1950).

Asexual reproduction of *Haematococcus* may take place by division of free-swimming cells into two or four daughter cells (macrospores). Sexual reproduction, gamatic union is isogamous, and the colonies are homotallic, but apparently with a fusion of gametes from different cells. Four or eight gametes are formed within a cell. A pair of fusion gametes become apposed at the anterior ends, and the flagella persist after fusion is completed (Donkin, 1976 ; Gudin and Chaumont, 1991 ; Zlonik, Sukenik and Dubinsky , 1993)

Under optimal environment condition, *Haematococcus* produces chlorophyll a and b and primary carotenoids, especially β-carotene and lutein (Rockette, 1970). The cells are green and ellipsoid, two flagella provide motility and growth rate is high. But under condition of growth limitation such as nitrogen limitation, the alga produces secondary carotenoids such as echinenone, canthaxanthin and astaxanthin following a decrease in chlorophyll and primary carotenoids (Droop, 1954 ; Goodwin et al, 1954 ; Lee et al, 1991 ; Zlonik et al, 1993). *Haematococcus* decreases its growth rate significantly, acquires spherical shape, loses its flagella and motility and builds a new thick cell wall, thereby becoming transformed into aplanospore (Santos et al,1984). Concomitantly, cells begin the massive accumulation of astaxanthin. Astaxanthin deposition is first noted around the nucleus and proceeds radically until the entire protoplast is red. The two processes, encystment and astaxanthin deposition, while usually coupled, are in fact distinct processes which can be experimentally separated in time. Fully mature cysts contain up to 5% by weight astaxanthin, predominantly in the form of monoesters of fatty acids (Renstrom et al, 1981 ; Bubrick, 1991 ; Bridigare et al, 1993 ; Lee and Ding, 1992).

**Environment Effect on Growth and Astaxanthin Accumulation in *H. pluvialis***

1. Nutritional Types

According to Kobayashi, Kakizono, Yamaguchi, Nishio, and Nagai (1992), *H. pluvialis* is able to grow heterotrophically on acetate in the dark as well as mixotrophically on acetate in the light. The photosynthetic pigments ; chlorophyll and astaxanthin are formed even in the dark heterotrophic growth condition. The specific growth rate of the mixotrophic condition (acetate + light) corresponded well to the sum of the specific growth rate of heterophic (acetate + dark) and autotrophic (no acetate + light) conditions.

2. Light

Light is important for growth and accumulation of astaxanthin in a wide variety of organisms. According to Boussiba and Vonshak (1991), Optimal light intensity for growth and astaxanthin accumulation of *H. pluvialis* Flotow were 85 and 170 μ mol $m^{-2}s^{-1}$ Moreover, Kobayashi et al (1992, a) showed that under higher light intensity over 50 μmol $m^{-2}s^{-1}$, morphological changed from vegetative cell to cyst and tended to process together with carotenoids formation, whereas morphological changes did not take place below 50 μ mol $m^{-2}s^{-1}$ and that carotenoid formation was more efficiently enhanced under blue light than under red light.

3. Temperature

Temperature is a factor that affects growth and the accumulation of astaxanthin in a wide strain of algae. Under mixotrophic

growth condition, *H. pluvialis* NIES144 showed no morphological change from green vegetative cells to enlarged red cyst cells at 20 °C although lower cell numbers were observed at these higher temperatures.

4. Macronutrient

4.1 Carbon and Nitrogen

According to Kobayashi et al, 1991, acetate is a main carbon source in the basal medium. Therefore it is of interest to know why the addition of acetate can cause the cyst formation. It seems likely that acetate addition in excess may generate a relative shortage of nitrogen. This is because under the high C/N balance, the astaxanthin level stayed at nearly the same level as that with no addition(Kakizono et al, 1992 b) In addition, *H. pluvialis* grows rapidly at high nitrogen concentration and induce the formation of reddish aplanospore at low nitrogen concentration. The rate of astaxanthin accumulation was dependent on high light intensity (Borowitza, Huisman, Osborn, 1991 ; Boussiba et al, 1992 ; Kobayashi et al, 1991)

4.2 Phosphate

Boussiba et al (1992) showed that logarithmic cells of *H. pluvialis* grown in a phosphate - free medium contained low concentration of nitrogen under high light intensity. The astaxanthin content was estimated to be 58.7 pg /cell

4.3 Sodium Chloride

When NaCl was added to a final concentration of 0.8% (W/V) to the logarithmic cells under optimal light intensity, complete cessation of growth was observed (Boussiba et al,1991 ; Vonshak, 1990). Growth arrest was accompanied by a massive accumulation of astaxanthin amounting to 47.2 pg /cell after 4 days

5. Micronutrient

5.1 Iron

According to Kobayashi, Kakizono and Nagai (1992), the astaxanthin formation of *H. pluvialis* was enhanced in $Fe^{2+}$ rich modified medium. It was shown that $Fe^{2+}$ would possibly function as an $OH^-$ generator via an iron-catalyzed Fenton reaction that plays an essential role in the enhanced carotenoid formation in the algae cyst cells (Kobayashi et al, 1993).

5.2 Vitamin

In addition to inorganic nutrient, some *H. pluvialis* strains require exogenously supplied vitamins such as cyanocobalamin (B12), thyamine (B1) (Pringsheim,1966). Furthermore, cell division inhibitor such as vinblastine or dichlorophenyl dimethyl urea can cause cessation of growth followed by an increase in the accumulation of astaxanthin in the resting cells (Boussiba et al, 1992).

**The Biosynthetic Pathway of Astaxanthin in a Green Alga, *H. pluvialis***

Some of the physiological aspects of astaxanthin accumulation in *H. pluvialis* were investigated by several researchers, but the exact pathway by which astaxanthin is synthesized has not yet been elucidated. It is accepted that astaxanthin is formed by hydroxylation reaction of β-carotene.Fan et al,1995 reported that diphenylamine inhibited astaxanthin synthesis in *H. pluvialis*. They demonstrated that (3S, 3'S) astaxanthin was synthesized from β-carotene via echinenone and canthaxanthin (Figure 4).

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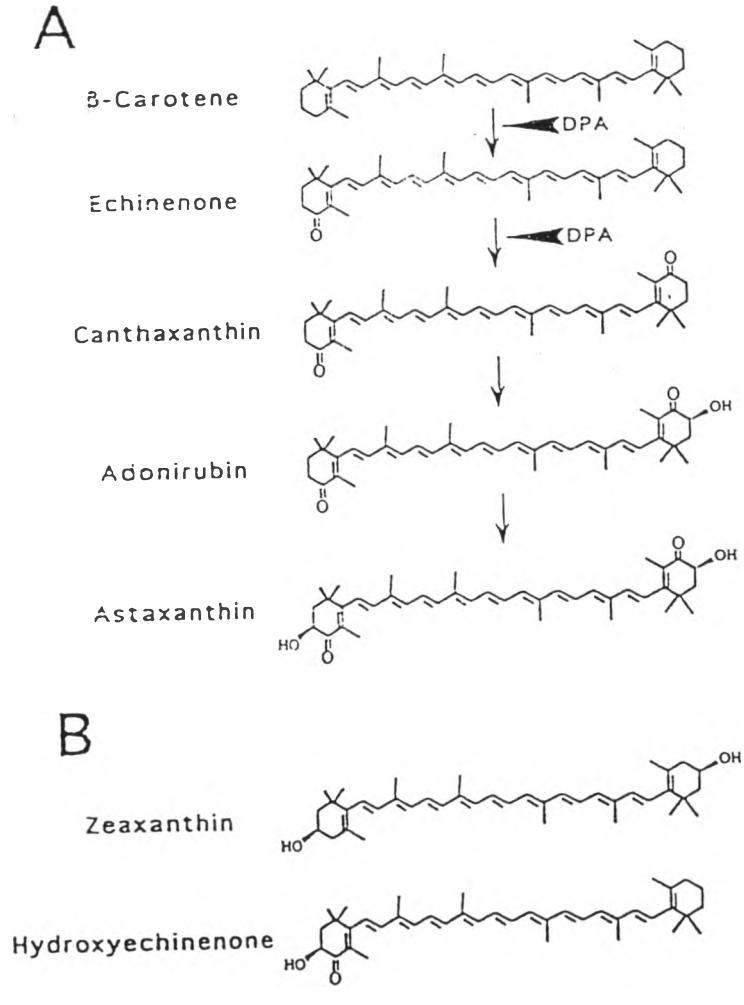


Figure 4 Proposed pathway for astaxanthin biosynthesis in *H. pluvialis* ; (A) related carotenoids, which do not take part in this pathway, are given in (B) (Fan et al,1995)

In this study, the unicellular green alga, *H. pluvialis* was chosen as a source of astaxanthin that could accumulate significant amounts under certain stress conditions which provided a source of pigmentation in aquaculture. Recommended usage is from 25 to 100 ppm pure astaxanthin in the appropriate feed. It is interesting to note that the cost of production of astaxanthin is less than 20 US dollar / kg (based on 1 % W/W astaxanthin / biomass product) and sells for approximately 2000 US dollar / kg astaxanthin, comparable to the price of synthetic astaxanthin.

**Objectives**

The objectives of this thesis are :

1. To select the best culture medium for high growth of *H. pluvialis*
2. To study the effect of environmental factors on *H. pluvialis* for high astaxanthin accumulation
3. To study the extraction of astaxanthin and its analysis