CHAPTER I

INTRODUCTION

The Carotenoids comprise a class of bright yellow to violet pigments widely distributed throughout both the plant and animal kingdoms. Carotenoids are found largely in yellow plants and in green plants along with chlorophyll. Many of the yellow, red, and purple colors seen in living organisms are due to the presence of these compounds. The isolation of carotene was first accomplished by Wackenroder in 1831, who obtained the crystalline pigment from the root of the carrot (Goodwin, 1980). The next important developments happened from exploiting newly discovered technique of chromatography, spectroscopy, nuclear magnetic resonance and mass spectrometry. The number of known carotenoids has now grown to over 400.

Carotenoids are carbon skeleton compounds basically consisting of eight isoprenoid units. The arrangement of these isoprenoid units is reversed at the center of the molecule such that the two central methyl groups are in the 1,6-position and the remaining nonterminal methyl groups are in the 1,5-position, as shown in Figure 1 (Spurgeon and Porter, 1980).

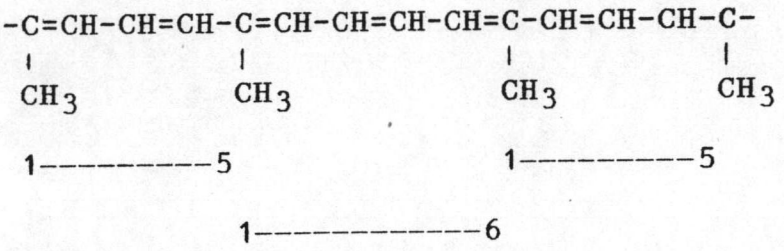

Figure 1 The arrangement of isoprenoid unit

The structures of all carotenoids can be derived from the acyclic polyene lycopene (Figure 2) which has a central chain of 11 conjugated double bonds giving a completely conjugated system of alternate doubles, which is the chromophore giving its color and the chemical formula $C_{40}H_{56}$.

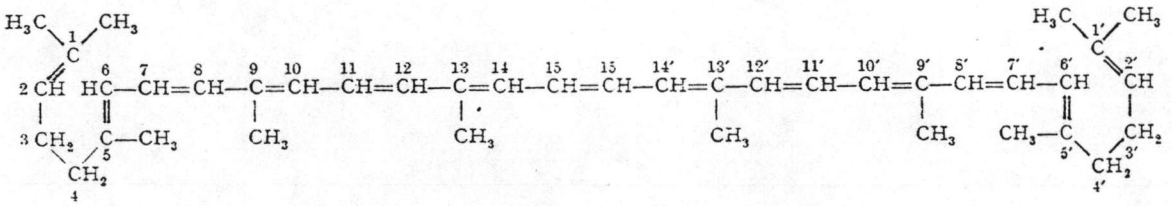

Figure 2 Structure of lycopene

In general, natural carotenoids may be divided into 4 groups.

1. The carotenes, carotenoid hydrocarbons, $C_{40}H_{56}$, which include alpha-, beta-, gamma-carotenes and lycopene.
2. The xanthophylls, oxy and hydroxy derivatives of the carotenes, which include among others, cryptoxanthin, $C_{40}H_{55}OH$ and lutein, $C_{40}H_{56}(OH)_2$.

3. The xanthophyll esters, esters of the xanthophylls with fatty acids.
4. The carotenoid acids, carboxyl derivatives of the carotenes.

Carotenes are named by the specific end groups which they contain. The end groups and their prefixes are indicated in Table 1 (Goodwin, 1980).

Table 1 : End group designation of carotenes

Type	Prefix
Acyclic	Psi
Cyclohexane	Beta
Methylenecyclohexane	Gamma
Cyclopentane	Kappa
Aryl	Phi,Chi

The structure of the carotenoid pigments is characterized by an aliphatic chain with attached methyl groups and a system of conjugated double bonds which is responsible for the deep red to yellow color of these compounds. The conjugated double bond system of the carotenoid pigments is subject to cis-trans isomerization,

which the stereoisomers differ from each other in biological potency and in certain physical properties such as adsorption affinity and absorption spectra.

Beta-carotene (Figure 3) is lycopene containing 11 ethylenic linkages which is shifted to be six-membered rings (cyclohexane) at the 2 end groups.

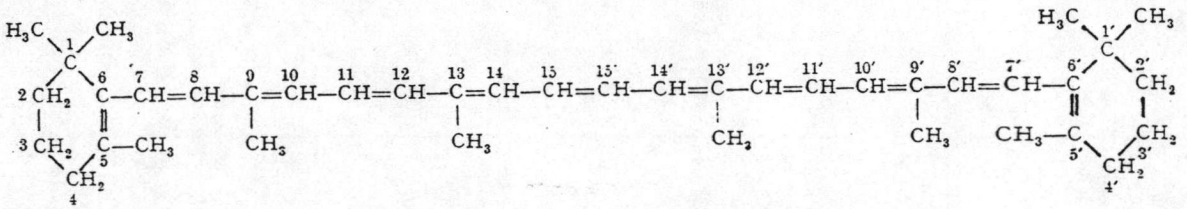

Figure 3 Structure of beta-carotene

In general, beta-carotene has the following physical and chemical properties.

1. It is fat-soluble.
2. It is readily soluble in chloroform, benzene, carbon disulfide, petroleum ether.
3. It is sensitive to oxidation, autooxidation, and light.
4. It is stable to heat in an oxygen-free atmosphere, except for some stereoisomeric changes.
5. It has characteristic absorption spectra, closely related to one another, the positions of the maxima differing with the solvents used.

Beta-carotene is a lipid-soluble photopigment which absorbs light energy in the 300-500 nm range. It serves several functions. The functions of these beta-carotene pigment have been reviewed extensively. Under high light conditions, they protect chlorophyll a from photo-oxidation as protective pigment. Under low light conditions, they serve as accessory pigment, in absorbing low wavelength (400-550 nm) as well as near UV irradiation (360-400 nm) and having the ability to transfer captured radiant energy to chlorophyll a thereby increasing photosynthetic production potentials (Goedheer, 1969; Fork, 1977; Goodwin, 1980; Siefermann-Harms, 1980; Paerl, 1984). It may also have a structural function (Szalontai and Czatorday, 1979; Hladik *et al.*, 1983). Beta-carotene is usually associated with the chlorophyll in the thylakoids where it acts as a light harvesting pigment. Beta-carotene is localized mostly in the chloroplast periphery forming lipoidal globules or "carotene droplets" playing a dual role as light-harvesting pigment and as a photo-protector of the photosynthetic apparatus from high irradiance (Ben-Amotz, 1989).

Now beta-carotene is exploited as natural food coloring in food technology. Although natural food coloring has a fairly long history, many synthetic pigments are now preferred by the industry. However, since certain synthetic pigments are not only harmful to man but may also be

carcinogenic, many countries have limited their uses in the food industry. Beta-carotene has a high coloring index ($E^{1\%}_{1cm}$ =2500) and can also provide vitamin A, it can therefore provide both color and nutrition and is now widely used for coloring soft-drinks, margarine, fruit juices, bread, fish products, creamcakes, ice-cream, etc., as well as being used as a nutritional supplement (Xihai, 1990). Beta-carotene is a pro-vitamin A,i.e., since one molecule of beta-carotene can be broken to two molecules of vitamin A, it can be used as a safe source of vitamin A to alleviate vitamin A deficiency without exceeding tolerance limits of vitamin A in the human body (Vitamin A is known to be toxic when taken in large amounts). Beta-carotene is more valuable than vitamin A in preventing tumors, protecting the human body from radiation damage by UV-rays and retarding cell and tissue degeneration on aging. Many international studies have indicated that beta-carotene can afford some protection against skin cancer, lung cancer, and bladder cancer, etc. (Dexin *et al.*, 1990).

The stereoisomers of beta-carotene have two major isomers:all-trans and 9-cis. The 9-cis to all-trans ratio is proportional to the integral light intensity in a similar manner as the total content of beta-carotene (Ben-Amotz *et al.*, 1987, 1988). The 9-cis beta-carotene, present in higher proportions in natural beta-carotene than in

synthetic beta-carotene, is easily assimilated by the human body. Like natural alpha-carotene, it can inhibit the growth of cancer cells but has no apparent effect on normal cells. Natural beta-carotene may therefore be more effective than synthetic beta-carotene for prevention of cancer.

Beta-carotene pigment is widely distributed in nature. Plants as well as many fungi and bacteria synthesize beta-carotene de novo, but animals do not. In animals, beta-carotene is obtained in the diet and then enzymatically altered (Singh and Cama, 1975). Carotenoids, especially beta-carotene, are found in many common foods, e.g. tomatoes, carrots, oranges, spinach, and other fruits and vegetables (Xihai,1990). Since in these foods the contents of beta-carotene are usually quite low, it is not practicable to extract the pigment from these sources and they take a long time to grow. So there are a lot of attentions in beta-carotene production from alga. Algal sources, Spirulina or Dunaliella, offer a wide scope for production of large quantities of natural beta-carotene directly in an edible state in a shorter duration compared with any other natural sources (Seshadri *et al.*, 1991). This has given a considerable impetus to the algal industry. Numerous strains of Spirulina are easily cultured and harvested, and are rich in carotenoids and other valuable

products. For this reason, Spirulina culture has received increased attention, including an interest in optimizing the production of high-value compounds such as beta-carotene.

Spirulina was observed and reported for the first time in 1890 by Dangeard and for the second time in 1959 by Brandily that a type of naturally grown alga is traditionally consumed as food by some tribes living in the Kanem area, the northern part of the Republic of Chad (Faucher *et al.*, 1979). Spirulina or Saa Rai Kliew Thong is a member of the prokaryote and belongs to the genus Spirulina, the family Oscillatoriaceae, the order Nostocales, and the division Cyanophyta. It is an ubiquitous, multicellular, filamentous cyanobacterium. Under the microscope, Spirulina appears a blue-green filament composed of 1 to 12 micron diameter cylindrical cells arranged in unbranched, helicoidal trichome. Most members of the Oscillatoriaceae have unbranched, cylindrical filaments of indefinite length, and, since trichome color and sheath characteristics vary considerably according to environmental conditions, a classical taxonomy of this group is largely confined to considerations of cell size and shape. The helical shape of the trichome is characteristic of the genus Spirulina, but helix pitch, length and helix dimensions vary with the species and even within the same species, differences have been observed in these parameters (Rich, 1931) or may be

induced by changing the environmental conditions such as growth temperature (Eykelenburg, 1979). The helical shape is maintained only in liquid media, the filaments becoming true spirals on solid media (Eykelenburg, 1980). The filaments are motile, gliding along their axis and have no heterocysts. Spirulina can grow rapidly and can be found in widely differing environments such as soils, marshes, fresh, brackish and seawaters and thermal spring, appearing to be capable of adaptation to very different habitats and colonization certain environments in which life for other microorganisms is, if not impossible, very difficult (Ciferri, 1983). The life cycle of Spirulina in laboratory is rather simple and short. Sexual reproduction is absent in Spirulina and it reproduces by binary fission (Rippka *et al.*, 1979).

The chemical composition of Spirulina reflects its potential as human food, animal feed and as a source of natural products. Spirulina has a high nutritional value, especially high protein content which ranges from 55 to 77 % (Zafaralla *et al.*, 1985). The protein content of Spirulina appears to be high also when compared with that of unicellular algae and other cyanobacteria. It contains all of the essential amino acids (Clement *et al.*, 1967; Ciferri, 1983). The amino acid spectrum of Spirulina protein is similar to that of other microorganisms and, in comparison

to standard alimentary proteins such as those of eggs or milk, it is somewhat deficient in methionine, cysteine and lysine (Clement *et al.*, 1967; Aaronson and Dubinsky, 1982). Thus, it appears that the high concentration of protein together with its amino acid composition make Spirulina a source of nonconventional protein of considerble interest. There had been reports that Spirulina had a high lipid content of 11 to 16.6 % of dry weight (Hudson and Karis, 1974; Tornabene *et al.*, 1985). The presence of high concentrations of gamma-linoleic acid seems to be a characteristic of Spirulina. Carbohydrates, accounting for 15 to 20 % of dry weight, are represented in Spirulina platensis essentially by a branched polysaccharide, composed of only glucose and structurally similar to glycogen. Furthermore, it is to serve as a vitamin food because of relatively high vitamin B1, B2 and particularly B12 contents and all vitamins have been found in Spirulina and their concentrations have been evaluated (Santillan, 1982).

Among the pigments, chlorophyll a is the most abundant pigment in Spirulina accounting for 0.8 to 1.5 % of dry weight (Tel-or *et al.*, 1980; Santillan, 1982) and chlorophyll b as accessory pigment is absent. Several papers have already been published for carotenoid composition of Spirulina which varied widely in quantities (Choubert, 1979; Miki *et al.*, 1986). Beta-carotene and myxoxanthophyll

are the major carotenoids, their content representing approximately 0.23 and 0.12-0.15 % of dry weight, respectively. Phycobillins amount up to 12-15 % of dry weight (Santillan, 1982). The pigments of Spirulina also make this alga useful in aquaculture, particularly as feed for rainbow trout (Choubert, 1979) and sweet smelt Plecoglossus altivelis (Mori *et al.*, 1987).

Carotenoid extractions were accomplished by using several organic solvents such as acetone (Meckel and Kester, 1980; Paerl, 1984; Bowles *et al.*, 1985; Ben-Amotz *et al.*, 1988; Fawley, 1988; Millie *et al.*, 1990), methanol (Meckel and Kester, 1980; Bowles, 1985; Coufal *et al.*, 1989; Humbeck, 1990) and the mixture of both (Miki *et al.*, 1986). Dichloromethane extraction process for beta-carotene was discovered and recognized by Riiegg in United States Patent (Riiegg, 1984). In addition, extraction efficiency for lypophilic pigments is reliant on such factors as phytoplankton type, intracellular pigment location and orientation, pigment affinities for membranes and extraction solvents, solvent-membrane interactions, reactions of solvent and pigment and most importantly, selectivity for the pigment of interest (Davies,1965; Goodwin, 1974). As test solvents we chose 90% (v/v) acetone (routinely used for chlorophyll a extraction by physiologists and ecologists), absolute methanol (often recommended for blue-green algae) (Holm-

Hansen, 1978; Stauffer *et al.*, 1979)

The optimal medium composition and quality for the highest growth of Spirulina were studied by Zarrouk (Zarrouk, 1966). The major nutrients of Zarrouk medium contain carbon, nitrogen, sulfur, phosphorus, magnesium, chloride, iron, and other micronutrients.

The environmental stress such as high light intensity, high NaCl concentration, extreme temperature, extreme pH value or nutrient deficiency were important factors for beta-carotene content in the cell of Dunaliella (Aasen *et al.*, 1969; Loeblich, 1982; Ben-Amotz and Avron, 1983; Al-Hasan *et al.*, 1987; Borowitzka and Borowitzka, 1988; Junmin, 1990). Sodium nitrate is the best nitrogen source for Spirulina cultivation. Beta-carotene was increased by depletion of nitrogen content in the growth medium. In Dunaliella, under nitrate starvation at 1 mM the cell had maximum beta-carotene per cell and when increasing nitrate, beta-carotene became clearly decreased (Ben-Amotz and Avron, 1983). De loura *et al* (1987) reported the effects of nitrogen deficiency on pigments and lipids of cyanobacteria Pseudoanabaena sp. and Oscillatoria splendida. They found that nitrogen deficiency does not affect the cellular content in chlorophyll a, but it causes a selective loss in phycobilliproteins, carotenoid content increases with

phycocyanin depletion. Thongprasong (1989) studied a green alga Chlorella spp. and found that 12.36 mM of nitrate was the optimal concentration for maximum carotene production and either higher or lower than 12.36 mM of nitrate caused a reduction in carotene production. Apart from nitrate, phosphate is another important nutrient because it is a constituent of nucleic acids, phospholipids, the coenzymes NAD and NADP, and, most important, as a constituent of ATP and other high energy compounds (Devlin and Witham, 1983). Blum and Begin-Heick (1967) indicated that during phosphate deprivation the carotenoid content of Euglena increased more than three folds. On the other hand, Chlorella sp. produced higher carotene and xanthophyll in response to increased phosphate content in the medium (Thongprasong, 1989). There are few reports on the effect of sulphate (Ben-Amotz and Avron, 1983) on growth and beta-carotene of Dunaliella. High salt concentration caused a decrease in the content of chlorophyll per cell and an increase in the amount of beta-carotene per cell (Ben-Amotz and Avron, 1983; Al-Hasan *et al.*, 1987; Powtongsook, 1993).

A number of nonphotosynthetic microorganisms produce carotenoids only in response to an exposure to light. This photoinduced carotenoid synthesis can be divided into two parts,the light dependent step and a series of metabolic reactions which do not require light. The result of these

reactions is the synthesis of substantial quantities of carotenoid de novo (Rilling, 1964). The photoinduction reaction requires oxygen and is temperature independent (Rilling, 1962, 1964; Batra and Rilling, 1964). In case of alga, pigment mutant C-6D of the green alga Scenedesmus obliquus exhibits a light-dependent chloroplast differentiation. During the first 30 minutes of illumination the amount of beta-carotene increases very fast and then levels off (Humbeck, 1990). In the halotolerant alga Dunaliella, Ben-Amotz and Avron (1983) tested the effect of light intensity on growth and pigment content. They found that beta-carotene content per cell increased sharply with light intensity in Dunaliella bardawil but decreased slightly in D. salina and a maximal beta-carotene to chlorophyll ratio of 13 was observed in high light-grown D. bardawil, whereas D. salina grown under the same conditions had a ratio of only 0.8 (Ben-Amotz and Avron, 1983; Lers *et al.*, 1990).

The quantitative and qualitative effects of light on carotenoid production by Spirulina were studied by Olaizola and Duerr (1990). The growth rate of Spirulina increased rapidly to a maximum of approximately 2.7 doublings/day at a light irradiance level of 465 μmol photon $m^{-2}s^{-1}$. Beta-carotene decreased as percent dry weight when light irradiance increased from 66 to about 750 μmol photon

$m^{-2}s^{-1}$. At light irradiance levels above this point, beta-carotene increased. Under red and blue light, they found decreased values of myxoxanthophyll, while beta-carotene increased and lutein, zeaxanthin and echinenone showed little changes.

There has been a report that the losses of beta-carotene while drying the alga can be minimized by lowering the drying temperature and it is possible to retard the degradation rate of beta-carotene in Spirulina by using antioxidants (Seshadri *et al.*, 1991).

The studies of beta-carotene production were mostly done in the halotolerant Dunaliella spp. and recently, Powtongsook (1993) studied strain selection and culture of Dunaliella salina (Chlorophyceae) for beta-carotene production. However, there are many disadvantages of Dunaliella cultivation for example low growth rate, difficult to harvest and cultivation is only possible in habitats which must have sea water or high NaCl contents. For this reason, the interest of cultivating blue green alga, Spirulina, for beta-carotene production, was started. Spirulina spp. used in this experiment was isolated from Makkason pond, Bangkok. It has a high growth rate and can be cultivated either in freshwater or seawater. Furthermore it has high nutrition value and can be conveniently

harvested. At present industrial scale cultivation of Spirulina is in operation at Siam Algae Co.Ltd., situated at the outskirt of Bangkok, Thailand.

Objectives

The objectives of this research are:

1. To select the solvent for beta-carotene extraction from Spirulina.
2. To study the effect of environmental factors on Spirulina cultivation for high beta-carotene production.
3. To study the pilot scale production of Spirulina for high beta-carotene.
4. To study the strategy for drying and storing Spirulina to minimize beta-carotene loss.