CHAPTER IV

DISCUSSIONS

1. Type of Solvents for Beta-carotene Extraction

Solvents for testing beta-carotene extraction efficiency from Spirulina were absolute methanol, 90% acetone, a mixture of acetone and methanol in the ratio of 4:6 and dichloromethane. There are several reasons for selecting each solvent for beta-carotene extraction. Absolute methanol had been reported to be an efficient extractant of chlorophyll a (Holm-Hansen, 1978; Stauffer *et al.*, 1979) and there was no difference of pigment extraction between 90% methanol and 100% methanol. In case of 90% acetone or a mixture of methanol and acetone, they were found to be the preferred solvents for either trichromatic spectrophotometric or chromatographic analyzes of lipophilic pigments in prokaryotic and eukaryotic phytoplankton. Dichloromethane was used for beta-carotene extraction process of Dunaliella in United States Patent number 4,439,629 in 1984. Spirulina require repeated extraction to remove completely pigments from cells. Methanol is structurally composed of methane and water so it

contains a lipophilic, alkane-like group and a hydrophillic, water-like hydroxyl group (Morrison and Boyd, 1989). Because of the presence of the C-OH group, methanol is polar compound and there is extensive hydrogen bonding between methanol molecules in the pure liquid (Brown,1982). Meckel and Kester (1980) had reported that methanol was 100% effective in pigment extraction in 13 organisms, acetone extraction was complete in 6 organisms, however, they failed to demonstrate significant correlation between pigment extraction patterns and taxonomic status at the generic level. In most of previous reports, pigments of Spirulina were extracted with acetone:methanol (4:6), pigments of Dunaliella were extracted with 90% acetone and of Scenedesmus were methanol but variation of both among genera and among species of the same genus was unclear. There are many reasons to explain why methanol is the best solvent for complete pigment extraction. Firstly, the cell wall or some component in the cell wall of Spirulina, act as a physical barrier to extraction. Disruption of cell, enabling total removal of the pigment from cells, may be obtained by heating from boiling methanol procedure. In case of 90% acetone and methanol:acetone, sonication was used for breaking cells, and this may not completely disrupt cells. Secondly, the degree of extractability is influenced by the physical location of the carotenoids. Binding of carotenoid in the cell affects their extractability ; for example, in

photosynthetic organisms, carotenoids were associated with protein (Ke, 1971) which might profoundly affect their solubility. It is possible to postulate that methanol may remove more carotenoids from protein in comparison with acetone and the mixtures and sometimes heating from boiling methanol can completely disrupt the covalent bond of carotenoids associated with protein. Thirdly, methanol has less polarity than acetone, and was able to be an optimal solvent for beta-carotene extraction. Finally, We noticed that extracted pigment with methanol had a green-yellow color and extracted pigment with acetone had a yellow color which was expected to be beta-carotene devoid of chlorophyll a.

2. Effect of Environmental Factors on Spirulina Cultivation for High Beta-carotene Production

Temperature is an important factor for algal growth influencing the composition of cell structures, metabolic regulatory mechanisms and specificity of enzyme reactions. In addition, temperature affects cell reaction rates. Therefore, Spirulina cultivation in optimal temperature is essential for high yield. From the result in Figure 9, the lowest growth occurred at 40°C cultivation due to broken filaments. According to Zarrouk (1966), the optimal growth temperature for Spirulina was between 35 and 40°C. The

study of Richmond (1986) with different strains of Spirulina indicated the optimal temperature for growth was between 35 and 37°C, 40°C being definitely injurious. In a report of Ciferri (1983), above 45°C massive breakage of trichomes followed by cell lysis had been observed and even a brief period (e.g. 10 min) of exposure at temperature around 50°C results in death of the cultures. Figure 9 showed the temperature for the highest growth rate at 35°C and there was no difference in growth between 25 and 35°C. Consistent with growth, beta-carotene yield was the highest at temperature between 25 and 35°C of cultivation and actually lowest at 40°C cultivation. The primary cause was the death of cells after 4 days cultivation in Figure 9. It was anticipated that no cell division and no pigments production for photosynthesis occurred. However, we chose temperature at 30°C for Spirulina cultivation in subsequent experiments because there was no difference on growth and beta-carotene production between 25 and 35°C and cultivation temperature in nature and in our laboratory for stock culture was 30°C.

pH of the medium affects directly and indirectly on the metabolism of alga. Ciferri (1983) reported that laboratory cultures of Spirulina platensis exhibited a wide pH optimum (8 to 11), but growth is evident also at pH values close to 7 and as high as 11.3. Our results indicated that initial pH between 8 and 10 provided the maximum growth

while initial pH 11 was unsuitable for growth of Spirulina and initial pH 10.5 was unsuitable after 3 days cultivation. This was attributed to the fact that pH played important roles in the solubility of NaCl in the medium. In addition, when pH of the medium increased, essential metal compounds precipitated and growth was decreased because of the depletion of some essential metals (Richmond, 1986). In general, the alga exploits CO_2 or the other forms of carbon to produce organic substances. In Zarrouk medium, carbon source is $NaHCO_3$ and the buffer system is on $CO_2 \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- \rightleftharpoons CO_3^{2-}$ system essential for adjusting pH of medium to be an alkaline. As a result of photosynthesis, the changing in carbon source, whether by CO_2 or HCO_3^- utilization and also OH^- production, would raise the pH. Typical for HCO_3^- utilization is the plateau found around pH 8 where the HCO_3^- concentration reaches a maximum (Prins and Elzenga, 1989). The efficiency of HCO_3^- utilization at high pH is low and there is no indication for the use of CO_3^{2-} so cell growth and division are decreased. Under visual observation, the color of extracted pigment in Spirulina cultivated in pH 10-11 was mostly yellow whereas that cultivated in pH below 10 was green. We expected that at higher pH, Spirulina would lose chlorophyll a during cultivation. The maximum beta-carotene content was at pH 10.5 (Figure 13). However, growth of Spirulina at this pH was actually suppressed. Spirulina cultivated at initial pH between 8 and 10 gave

similar growth and similar beta-carotene content. Fox (1983) reported that initial pH of the medium suitable for Spirulina cultivation was between 7.8 and 8.5 depending on nutrients. However, Tangjaitrong (1990) used initial pH between 8.5 and 9.5 for satisfactory cultivation of Spirulina. As above-mentioned, we selected initial pH between 8 and 9 for subsequent growth of Spirulina.

Most recognized role of nitrogen in the plant is its presence in the structure of the protein molecule. In addition, nitrogen is found in such important molecules as purines, pyrimidines, porphyrins, and coenzymes. Purines and pyrimidines are found in the nucleic acids, RNA and DNA, essential for protein synthesis. The porphyrin structure is found in such metabolically important compounds as the chlorophyll pigments and the cytochromes. Coenzymes are essential to many enzymes (Devlin & Witham, 1983). Inorganic nitrogen source, $NaNO_3$, was utilized for complete growth of Spirulina in Zarrouk medium. It was indicated that $NaNO_3$ was essential chemical for growth. Growth of Spirulina cultured in Zarrouk medium without $NaNO_3$ was slightly increased in the first 2 days and then leveled off until day 7. We assumed, from the fact that nitrogen is an essential constituent of protein, that nitrogen deficiency must cause a decrease in protein synthesis, which subsequently cause a decrease in cell size and especially

cell division. So the reduction of growth brought about primarily the lack of sufficient proteins and nucleic acids for continued production of new cells. Not surprisingly, nitrogen deficiency limited growth more severely than deficiency of other elements. Algae completely deprived of nitrogen are frequently unable to grow larger and regenerate (Greulach, 1973). From Figure 15, beta-carotene content was the lowest at 0 g/l of $NaNO_3$ and higher along with increasing $NaNO_3$ content up to 1.25 g/l. The highest beta-carotene was 4.01 mg/g dry weight at 1.25 g/l of nitrate content and it then decreased while $NaNO_3$ was increasing. There had been numerous reports to indicate that nitrogen deficiency led to an increase of carotenoids in cyanobacteria and beta-carotene in Dunaliella (Aasen *et al.*, 1969; Loeblich, 1982; Ben-Amotz and Avron, 1983; Al-Hasan *et al.*, 1987; Borowitzka and Borowitzka, 1988; Junmin, 1990). As nitrogen deficiency develops, the amount of chlorophyll in the cells decreases faster than the total nitrogen content. Nothing is known of the changes in enzymatic balance which may occur (Lewn, 1962). The most easily observed characteristic of nitrogen deficiency was the yellowing or chlorosis which probably resulted not only because of inadequate nitrogen for chlorophyll synthesis but also because of reduced chloroplast protein production. Cell was stimulated to produce higher beta-carotene content so that they could be exploited in photosynthesis and to be

an energy for growth. From the results of the effect of $NaNO_3$ content on beta-carotene synthesis, we summarized that $NaNO_3$ at 1.25 g/l was the optimal content to produce the highest beta-carotene content in Spirulina. However, beta-carotene content decreased when nitrate content dropped to 0, 0.31, and 0.62 g/l. Similar results were reported in Dunaliella by Ben-Amotz and Avron (1983) and Powtongsook (1993) and in Chlorella by Thongprasong (1989). They found that beta-carotene content was dropped when nitrate content in the medium reached near zero. In general, Zarrouk medium contains 2.5 g/l of nitrate, therefore, there was no effect on beta-carotene content when grown in 2.5, 3.75 and 5 g/l of nitrate because of sufficient nitrogen level.

Sulphur is a constituent of a variety of organic compounds which are essential to the structure and metabolism of alga. Sulphur is found in some other coenzymes e.g. biotin, thiamine and coenzyme A and in the amino acids, cystine, cysteine and methionine which occur in proteins. Sulphur bridges (-S-S) have an important role in determining protein structure and sulphydryl groups (-SH) are often part of the active center of enzymes. Also, sulphur is important in Fe-S proteins in photosynthesis, nitrogen metabolism, and ferredoxin synthesis (Devlin and Witham, 1983). Sulphur is exclusively present as sulphate. From the result in Figure 16, no difference in growth of Spirulina cultivation at K_2SO_4

concentration between 0 and 2.0 g/l was observed indicating the non-requirement of K_2SO_4 for growth because of the presence of other sulphate forms such as $MgSO_4$ and $FeSO_4$ in the medium. However, K_2SO_4 deprivation (0 g/l) gave the highest yield of beta-carotene content at 4.19 mg/g dry weight (Figure 17). The results was consistent with the previous result of Dunaliella (Ben-Amotz and Avron, 1983). Beta-carotene per cell of Dunaliella was the highest in the absence of K_2SO_4 and when increasing K_2SO_4 concentration, beta-carotene per cell declined. Thus, we selected the optimum K_2SO_4 concentration for growth and beta-carotene content at 0 g/l (or without K_2SO_4) because it gave the highest beta-carotene content and did not affect growth.

Phosphate is one of the major important nutrient elements required for normal growth of cells. As in all living organisms, compounds containing phosphorus play important roles in nearly all phases of metabolism, particularly in energy transformation reactions. In nutrient solution, as generally in nature, phosphorus is exclusively present as phosphate. It seems quite natural that the concentration of this compound in the medium would influence the rate of its uptake by alga cells (Lewn, 1962). The growth of a wide variety of algae in their natural environment as well as in the laboratory has been shown to depend on the amount of available phosphorus. Phosphate

deficiency could affect growth in Spirulina because phosphorus, like nitrogen, is extremely important as a structural part of many compounds, notably nucleic acids and phospholipids. In addition, phosphorus plays an indispensable role in energy metabolism, the high energy of hydrolysis of pyrophosphate and various organic bonds used to drive chemical reactions (Greulach, 1973). Spirulina cultured in Zarrouk medium without phosphate was expected to contain reduced quantities of ATP, NAD, NADP and the various other important compounds, resulting in disruption of various metabolic pathways. Therefore, beta-carotene content was the lowest yield at 0.75 mg/g dry weight in Spirulina cultured in Zarrouk medium without phosphate. It was clear that phophorus compounds were involved in many essential reaction of phosphorylation and photosynthesis. In Dunaliella, the carotenoid content was slightly increased when phosphate concentration declined (Junmin, 1990; Powtongsook, 1993) and the optimal phosphate concentration was about 0.02-0.025 g/l (Borowitzka and Borowitzka, 1988). In contrast, excess phosphate concentration inhibited carotenoid synthesis in Chlorella (Thongprasong, 1989). Our results were consistent with the above investigations. Suitable phosphate concentration was 0.2 g/l and produced the highest beta-carotene yield at 4.24 mg/g dry weight (Figure 19). Phosphate concentrations higher than 0.25 g/l inhibited beta-carotene synthesis.

Magnesium is an essential constituent of chlorophyll molecule, without which photosynthesis would not occur (Devlin and Witham, 1983). Magnesium ions are the natural activitors of a number of enzymes including nearly all of those acting on phosphorylated substrates. Magnesium was used in magnesium sulphate form. For the present experiment, Spirulina received sufficient sulphate for cell growth exploiting K_2SO_4 and $FeSO_4$ in the medium. Therefore the results expressed were due to the effect of magnesium on growth and beta-carotene content. Growth of Spirulina declined after 4 day cultivation under 0 g/l of magnesium sulphate because lack of magnesium limited chlorophyll synthesis and also retarded photosynthesis and cell division. However, only 0.1 g/l of magnesium sulphate was enough for the highest growth as shown in Figure 20. For beta-carotene content, Spirulina cultivation under 0.1 g/l of magnesium sulphate gave the highest beta-carotene content because of limitation of chlorophyll synthesis (Figure 21). Thus, cell was somehow stimulated to produce higher beta-carotene and xanthophyll content so that they could be exploited in photosynthesis and to be an energy for growth. On the other hand, magnesium sulphate concentration of 0.2, 0.4 and 0.8 g/l did not affect beta-carotene content. It was possible that magnesium at 0.1 g/l was sufficient for cell to produce chlorophyll, therefore, cell was not in a need to produce more beta-carotene content.

The role of NaCl in plant metabolism is still uncertain. The observation that chloride is essential for production of oxygen by isolated chloroplasts has led to the view that chloride acts as an electron-transporting agent in photophosphorylation. Zarrouk medium is composed of 1 g/l of NaCl and at this concentration, growth of Spirulina was the highest. Growth is impaired if the concentration in the medium of either essential or non-essential elements exceeds a certain level. In general, macronutrients are much less toxic than micronutrients and their concentration can be raised appreciably above the optimum without significantly affecting growth. Spirulina can grow under a wide range of environments of highly diversified osmotica. Hence, the photosynthetic apparatus must adapt to different degrees of salinity in the habitat by means of either osmoregulation, or modification of enzyme structure. Plant and algal cell regulate the intracellular osmoticum against high external salt by accumulation of glycerol, mannitol, proline and other low molecular weight substances. Tel-Or and Melamed-Harel (1981) studied adaptation to salt of the photosynthetic apparatus in Cyanobacteria. They found that S. platensis was much more sensitive to NaCl. However, the salt sensitive enzyme in S. platensis could acquire tolerance to NaCl during growth in salt containing medium. Saltstress injured the chloroplast and elevated the carotene to chlorophyll ratio, apparently to combat this injury. This

is reflected in the reduction of growth rates (Ben-amotz *et al.*, 1982). However, the maintenance of activity functioning at photosynthetic sites within cells of Spirulina appeared to be an essential prerequisite for survival of this alga at extreme salinity. The higher photosynthetic activity is essential under saltstress in order to produce high levels of the osmoregulator or perhaps beta-carotene, in addition to the cell material needed for the relatively slow growth. Al-Hasan *et al* (1987) concluded that saltstress lowered the growth rate of D. salina and injured the chloroplast, but the thylakoids that remained intact became better qualified for photosynthesis. The study of the effect of changing level of NaCl indicated that when the content of NaCl drastically changed, the growth of Spirulina was obviously decreased. Changing NaCl content from 1 to 40 g/l and from 20 to 40 g/l mostly affected on growth (Figure 24). In contrast, beta-carotene content was the highest at 7.52 mg/g dry weight when NaCl content was changed from 20 to 30 g/l (Figure 25). The result indicated that saltstress reduced the growth and promoted beta-carotene production of Spirulina. However, we attempted to increase Spirulina cell by means of adding different concentrations of NaCl after cell was in log phase (4 day cultivation) (Figure 26). Spirulina grown under 40 g/l of NaCl gave the highest beta-carotene content at 5.22 mg/g dry weight but beta-carotene content was much less than the previous

experiment (Figure 27). Therefore, Spirulina cell acclimated to NaCl for 1 week was used to be an initial stock culture. Spirulina was able to adapt to high NaCl and produced the highest beta-carotene content at 7.55 mg/g dry weight under 40 g/l of NaCl (Figure 29). The growth of Spirulina acclimated to NaCl for 1 week was similar to its acclimation to NaCl for 5 weeks therefore a period of 1 week was sufficient for growth adaptation. Nevertheless, the highest beta-carotene content possible was 9.35 mg/g dry weight when stock culture was acclimated to 40 g/l of NaCl for 5 weeks.

Light initiates the process of photosynthesis through photosynthetic apparatus, equipped with an intricate array of membranes and pigments. Algal and higher plant photosynthesis requires the presence of light as an energy source. In recent years scientists have directed considerable attention to the possible role of carotenoids in plants. At least 2 probable roles of carotenoids were evident.

1) They protect against the photooxidation of chlorophyll
2) They absorb and transfer light energy to chlorophyll a

The light intensities in our experiment were 1,500, 3,000, 6,000, 10,000 and 14,000 lux. When the light intensity was increased, rate of photosynthesis was increased resulting in increased growth and beta-carotene

content. We found that Spirulina could not grow in Zarrouk medium in the dark (0 lux) because of lack of light energy for photosynthesis. However, Spirulina tolerated to a certain extent of high light intensity because it could not grow under light intensity about 18,000 lux. This was because high light intensity inhibited the cellular respiration or induced photooxidation. In addition, higher light intensity was usually accompanied by the higher temperature. The increase in beta-carotene content was thought to be an adaptation necessary to photoprotect, absorb and transfer light energy to chlorophyll molecules at higher intensities. There had been reported that beta-carotene could serve either as a light harvesting or structural role in Photosystem I (Olaizola and Duerr, 1990). The increase in the cellular level of beta-carotene might be due to an accelerated rate of biosynthesis resulting from enhanced activity or de novo synthesis of carotenogenic enzymes or a reduced rate of its degradation or secretion. In laboratory condition where temperature is lower than in outdoor condition the optimum light intensity of Spirulina cultivation was 8,000-10,000 lux (Ciferri, 1983), 7,000 lux (Venkataraman, 1983), and, 5,000-10,000 lux (Tangjaitrong, 1990). In conclusion, light intensity at 10,000 lux was appropriate to produce higher growth and beta-carotene content in Spirulina. The quantitative data on the cellular pigment content of beta-carotene of Spirulina showed that in

both red light and blue light beta-carotene content was higher than that in white light. Furthermore, it was noted that in red light beta-carotene content was higher than that in blue light. Our results indicated that Spirulina can respond to the changes in the spectral distribution of growth irradiance by changing beta-carotene level. The reasons for a preferential accumulation of carotenoids under red and blue light have yet to be discovered. We speculated that the blue light transmitted wavelength in the range of beta-carotene absorption. In case of the red light, it transmitted wavelength in the range of chlorophyll absorption, therefore, resulting in higher chlorophyll and also beta-carotene content.

3. Effect of Inhibitors on Growth and Beta-carotene Content

The biosynthetic pathway of carotenoids is the target for various bleaching herbicides. Bleaching herbicides inhibit carotenogenic pathway of many plants, resulting in photooxidative destruction of pigments. One of the bleaching herbicides, norflurazon, has been reported on target of inhibition by direct interaction with the enzyme phytoene desaturase. It has been shown recently that norflurazon acts as a reversible noncompetitive inhibitor of phytoene desaturase (Linden *et al.*, 1990). Masamoto (1992) reported that at low concentrations of norflurazon (0.2 μM)

the beta-carotene hydroxylation was inhibited resulting in beta-carotene accumulation in Synechococcus PCC7942. On the other hand, at high concentration of norflurazon (1 μM) it had been shown to block carotenogenic pathways by inhibiting phytoene desaturase resulting in the reduction of beta-carotene synthesis. From our results, there had been no difference in growth and beta-carotene content of Spirulina among 6 concentrations of norflurazon (Figure 35). Beta-carotene content at 2.0 μM of norflurazon was slightly lower than other concentrations (Figure 36). It was shown that norflurazon did not affect growth and originally inhibited beta-carotene accumulation at 2.0 μM. This contradicted Masamoto's results (Masamoto, 1992). However, we could only say that treatment of Spirulina with norflurazon may block the biosynthesis of colored carotenes. In case of diphenylamine, concentration of 100 μM inhibited completely both growth and beta-carotene synthesis (Figure 37 and 38). Goodwin (1980) has suggested that diphenylamine may have a dual role, first as an inhibitor of dehydrogenation reactions leading from colorless carotenoids to highly unsaturated aliphatic and alicyclic carotenoids, and second as a stimulator of the formation of phytoene, phytofluene and similar aliphatic compounds (Olson and Knizley, 1962). 2,4-dinitrophenol affected beta-carotene content of Spirulina much less than that by diphenylamine because concentration of 2,4-dinitrophenol at 100 μM did not sharply cause the

decrease of beta-carotene (Figure 40). There had been reported that 2,4-dinitrophenol stimulated carotenoid content in Dunaliella salina (Goodwin, 1980). Nevertheless, our results showed that the increase in 2,4-dinitrophenol concentration inhibited slightly beta-carotene content and did not affect growth of Spirulina (Figure 39 and 40).

4. The Pilot Scale Production of Spirulina for High Beta-carotene Production

Mass production of Spirulina was reviewed in order to achieve high productivity and low cost of production (Vonshak and Richmond, 1988; Santillan, 1982; Vonshak, 1990). Our experiment for pilot scale production was aimed to produce high beta-carotene content from Spirulina. NaCl concentration of 1 g/l and 30 g/l in Zarrouk medium were used for the comparison of growth and beta-carotene content. The results indicated that growth and beta-carotene content of Spirulina cultivated in outdoor were much less than those in laboratory. The main points of problems and limitations for production of Spirulina in mass culture were discussed below: Firstly, strain of Spirulina was not suitable for growth and beta-carotene content. Spirulina isolated from Makkason pond was sensitive to increased NaCl, so growth was lower when NaCl concentration increased. In contrast, marine Spirulina can adapt to increased NaCl (Tel-Or and

Melamed-Harel, 1981). In our experiment, if we used marine Spirulina strain, the result would be higher growth and beta-carotene content. Secondly, design of the open cultivating container was one of the important factors in growth limitation. Almost all commercial reactors for production of Spirulina were based on shallow raceways in which algal cultures were mixed in a turbulent flow sustained by a paddle wheel. At production sites for algal mass cultivation in the USA, Israel, India, Thailand, and Taiwan, 2 types of open raceway ponds were used: the first, the most capital intensive, lined by concrete (Thailand, USA); the second was a shallow earthen tunnel lined with PVC or some other durable plastic (Vonshak and Richmond, 1988). In general, open raceway pond should be shallow allowing penetration of sunlight throughout the cultures. Spirulina cultivating container in our experiments had a 59 cm depth of the medium. This was too deep for light penetration to the bottom of the container. Thus, cultures in such depth had a lower growth and beta-carotene content. The third was controlling the photoinhibition. The upper layer of an outdoor algal culture was exposed, even in densely populated cultures, to very high solar irradiance. In most cases some photoinhibition may be observed with light intensity at 60 to 70 % of full sunlight. In many algal species prolonged exposure to high light intensity may cause photooxidative death (Vonshak and Richmond, 1988). The suggestion for

improving mass culture in such condition was to isolate Spirulina strains which were more resistant to high light intensities. Fourthly, Spirulina cultures naturally received illumination of sunlight for only 8 hours/day. This caused the limitation of growth, and photosynthesis of alga. Fifthly, air flow rate in outdoor mass culture was much less than in laboratory. Thus, increase of air flow rate is able to increase growth. Sixthly, diurnal fluctuation in temperature was of considerable interest. During the morning, when irradiance was high enough to support intense photosynthesis and high growth rates, water temperature was still below air temperature and could be much below the optimal for growth. At midday, culture temperature was increased but lower than air temperature. Spirulina was essentially adapted to diurnal fluctuation in temperature and strains with a wide optimum temperature range for growth would be advantageous in outdoor cultivation. Finally, sensitivity to high osmoticum affected growth and beta-carotene content of Spirulina both in laboratory scale and in outdoor cultivation scale. Water evaporation from medium led to a continuous increase in the salt concentration of the medium. Thus an algal strain well adapted to increasing osmoticum without a decreased growth and photosynthetic apparatus will be advantageous for mass cultivation.

5. Effect of Method of Drying on Beta-carotene loss

Three methods of drying were performed: sun drying, oven drying and freeze drying. Freeze drying was the best type of drying because the beta-carotene loss and moisture content of Spirulina were the lowest. In addition, physical characteristics of Spirulina were softer and coarser than other types of drying. The advantages of freeze drying on Spirulina was no loss of alga or the highest recovery because low temperature was used for dehydration of Spirulina. But the disadvantage of freeze drying was the high cost of equipment. The disadvantages of sun drying were to bring algal cake griding again and had the losses of beta-carotene and much more moisture content. Oven drying is economized and has their disadvantages similar to sun drying. The selection of type of drying depended on many factors such as available equipment, the size of cultivation and the budgets.

6. Partial purification of Beta-carotene

Partial purification was based on the use of petroleum ether as a solvent on carotenoid extraction from alga. Chlorophyll had not been extracted with petroleum ether because it was saponified with KOH and dissolved in ethanol. The separation of beta-carotene and other

xanthophylls depended on the polarity of solvents. 5% petroleum ether in hexane was much less polar than acetone:chloroform (1:1), therefore, it was able to elute beta-carotene from Silica G-60 column. After that, other xanthophylls were much more polar and eluted with acetone: chloroform (1:1). Beta-carotene and xanthophylls profiles as determined by HPLC indicated that beta-carotene was pure after column separation. Alpha-carotene and beta-carotene could not be separated each other by this method. Carotene of Spirulina is composed of only beta-carotene (Goodwin, 1980) so we could use this method for partial purification of beta-carotene with high efficiency.

7. Effect of Storage Temperature on Beta-carotene Loss

The algal product brought into the retailer's shop must be capable of retaining most of the original beta-carotene over a period of time, therefore, the protection during storage must be specially careful. Because of sensitivity of beta-carotene to high temperature, we found that high temperature during storage affected beta-carotene in freeze-dried Spirulina. The best storage temperature was -70°C followed by -20°C, 4°C and 30°C. But in practice, the algal storage at-70°c was difficult and expensive. To solve this problem, the delivery from the manufacturer to the retailer's shop should be performed very rapidly or

include some kinds of antioxidant in algal product. Antioxidants are widely employed to protect the oxygen sensitive components from the atmosphere oxygen. Sodium metabisulphite, an antioxidant was recommended at levels between 0.1 and 1.0 % in the products to be preserved (Blacow, 1972). From our result, there had been a litte difference of beta-carotene in Spirulina added with 0, 0.1, 1.0% of sodium metabisulphite. This was a result of limitation of pigment extraction which used a small quantity of Spirulina and used much less sodium metabisulphite. Thus, we expected that sodium metabisulphite or other antioxidants were efficient to preserve the Spirulina in an industrial scale.