CHAPTER III

RESULTS

The strain of Spirulina originally isolated from Makkasan pond by Miss Duangrat Inthorn was used for the present study. A clonal culture was obtained by isolating a single trichome and appeared a blue-green filament composed of cylindrical cells arranged in unbranched, helicoidal trichomes as shown in Figure 7.

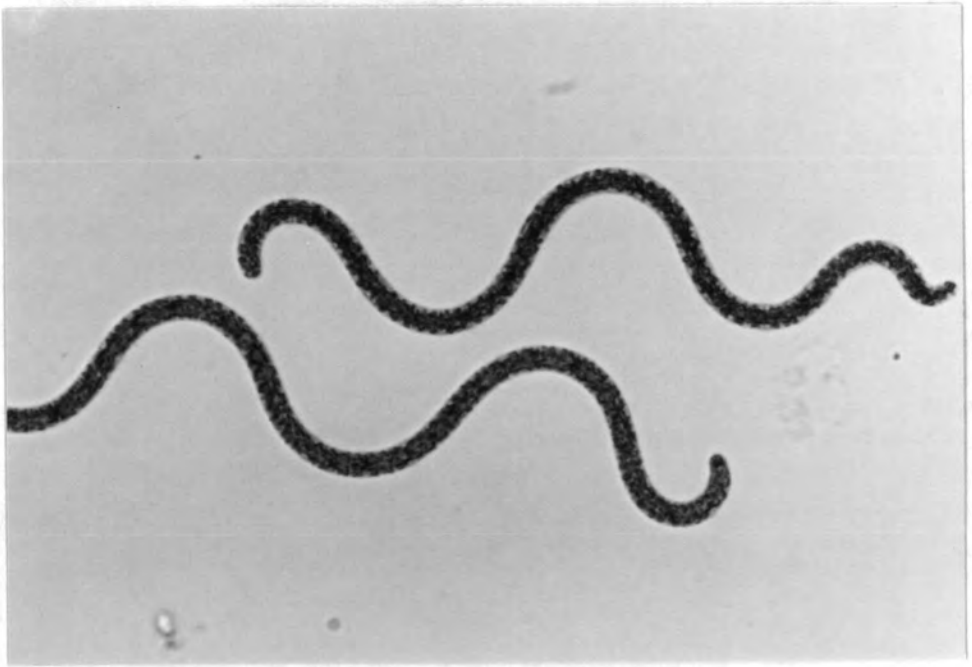

Figure 7 Spirulina from Makkasan pond (x200)

1. Type of Solvents for Beta-carotene Extraction

Ten ml of Spirulina culture was extracted by various types of solvent for example absolute methanol, 90 % acetone, a mixture of acetone and methanol(4:6) and dichloromethane before analysis for pigments by HPLC. Beta-carotene extraction with absolute methanol gave the highest yield at 2.63 mg/g dry weight which was arbitrarily set for 100 % extraction (Figure 8). For acetone:methanol (4:6), 90 % acetone and dichloromethane, beta-carotene extraction percents were 90, 76 and 36, respectively (Figure 8). Therefore, in later experiments methanol was selected to be the best solvent for beta-carotene extraction.

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

2.1 Effect of Cultivation Temperature on Growth and Beta-carotene Content

Spirulina was grown in Zarrouk medium with different cultivation temperatures of 25, 30, 35 and 40^{o}C for 5 days. A 50 ml of culture in a 125 ml flask was shaken on a Psycrotherm with illumination at 4,000 lux and shaking speed at 200 rpm. Figure 9 shows that cultivation temperature could affect growth of Spirulina. The cultivation

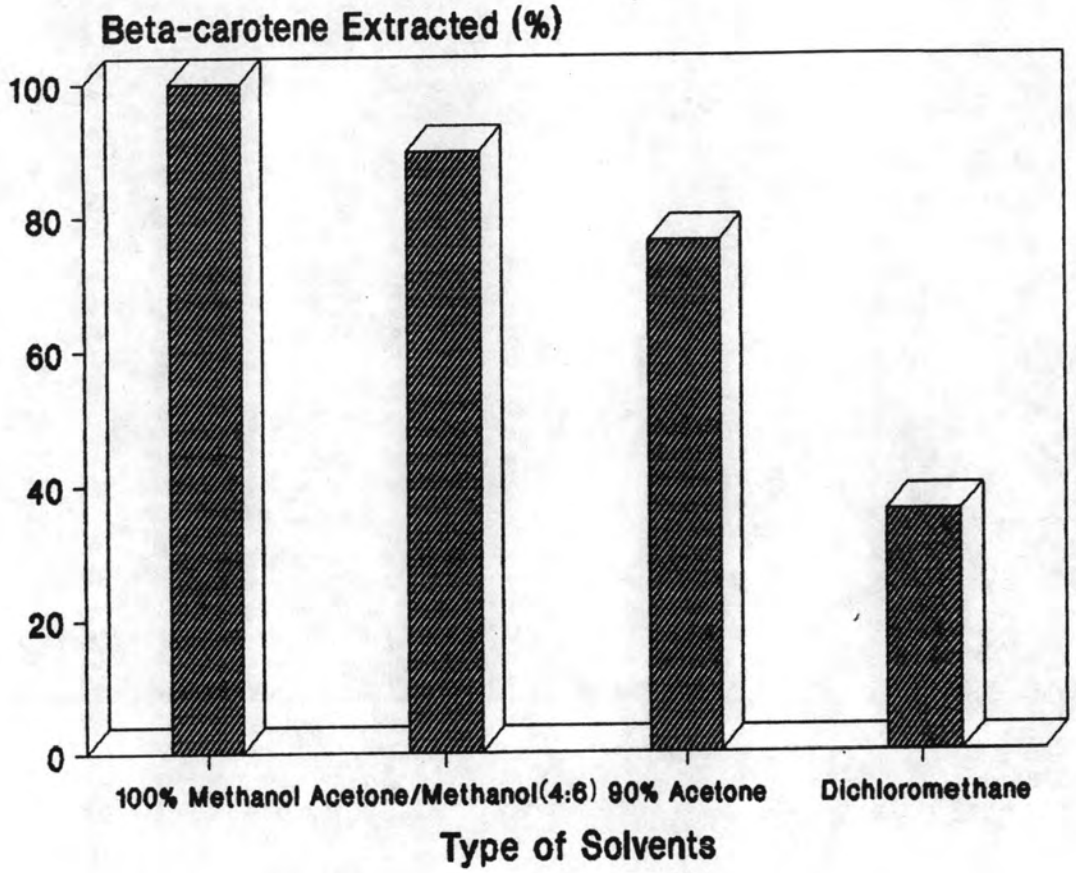

Figure 8 Beta-carotene extraction efficiency by various solvents

temperature giving the highest growth was 35°C and that giving the lowest growth was 40°C at day 4 (Figure 9). As shown in Figure 10, beta-carotene content was not different at temperatures 25, 30 and 35°C. At 40°C, beta-carotene content was the lowest. However, 30°C was chosen for cultivating Spirulina because not only Spirulina was naturally grown at 30°C but also beta-carotene content was high at this temperature.

2.2 Effect of Initial pH on Growth and Beta-carotene Content

Spirulina was grown in Zarrouk medium with different initial pH values of 8, 8.5, 9, 9.5, 10, 10.5 and 11 for 1 week in conditions as described in section 2.1 at 30°C. Figure 11 shows that the lowest growth occurred at initial pH 11. The cells ceased to grow after 3 days of cultivation at initial pH 10.5 and 11.0. At initial pH between 8 to 10, the growth was not different. The pH changes occurring during 7 days cultivation is shown in Figure 12. The pattern of pH changes converged to the pH between 11 to 12 at the end of the cultivation period. Initial pH 10.5 gave the maximum beta-carotene content of 5.00 mg/g dry weight at day 7 (Figure 13). However, initial pH 10.5 was not appropriate for cell growth. So, in later experiments, the initial pH was fixed between 8 and 9.

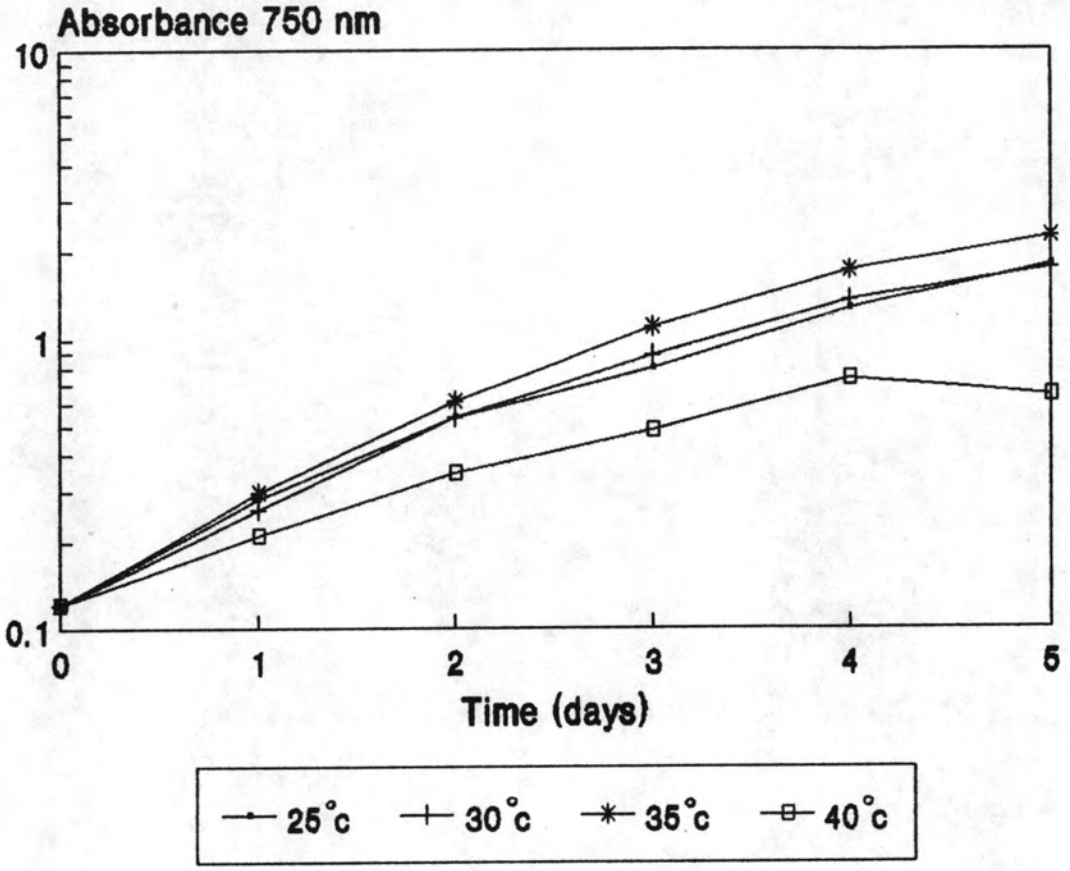

Figure 9 Growth of Spirulina in Zarrouk medium under various cultivation temperatures for 5 days

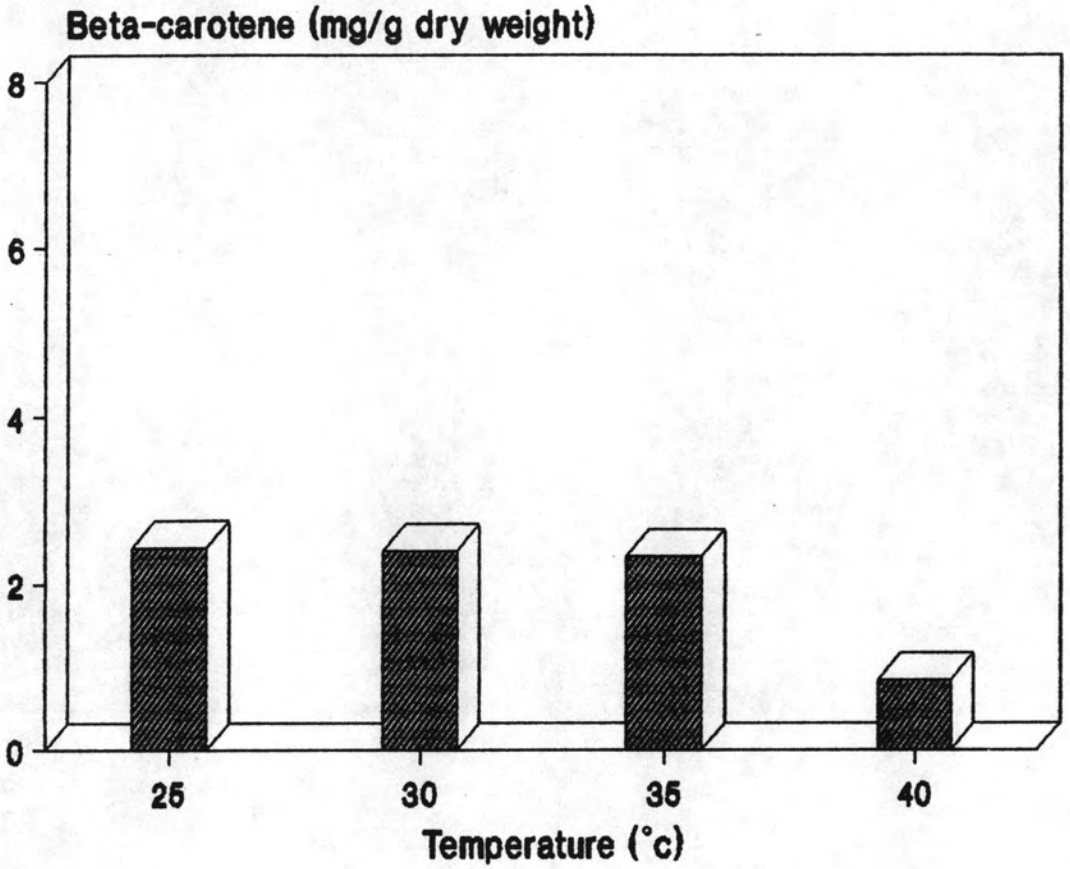

Figure 10 Effect of cultivation temperature on beta-carotene content of Spirulina at day 4

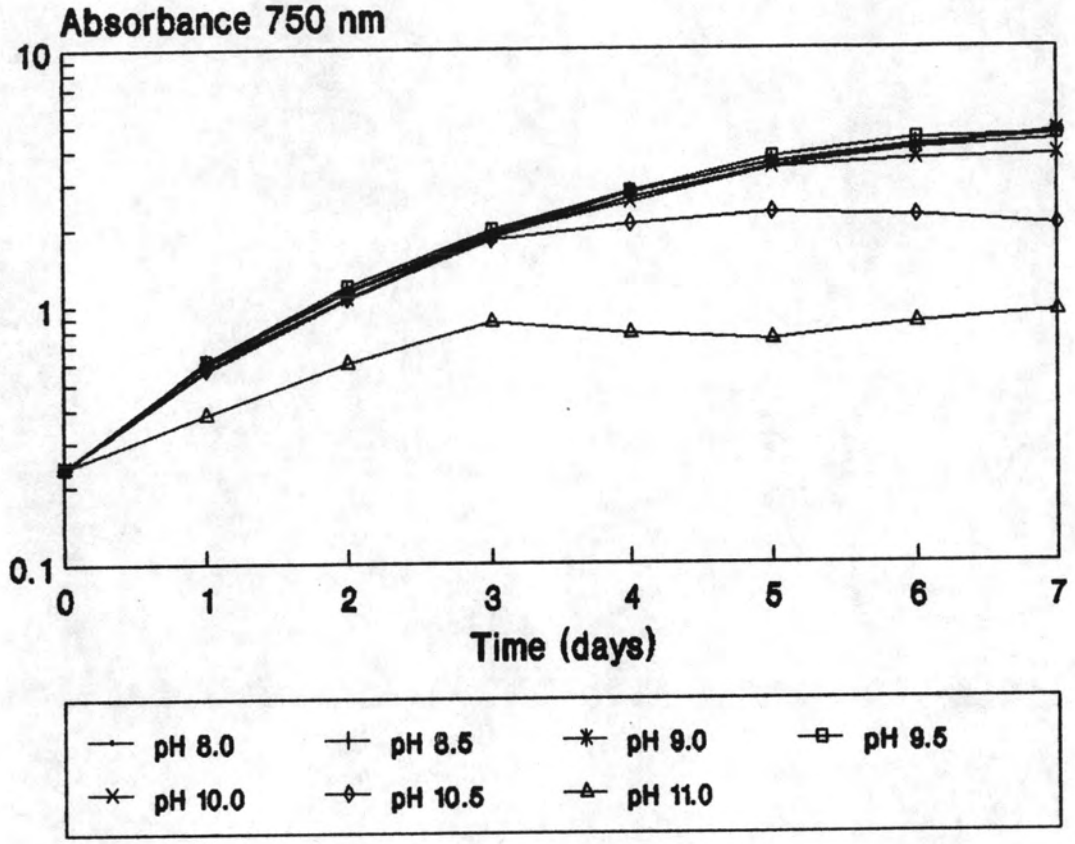

Figure 11 Growth of Spirulina in Zarrouk medium under various initial pH for 1 week

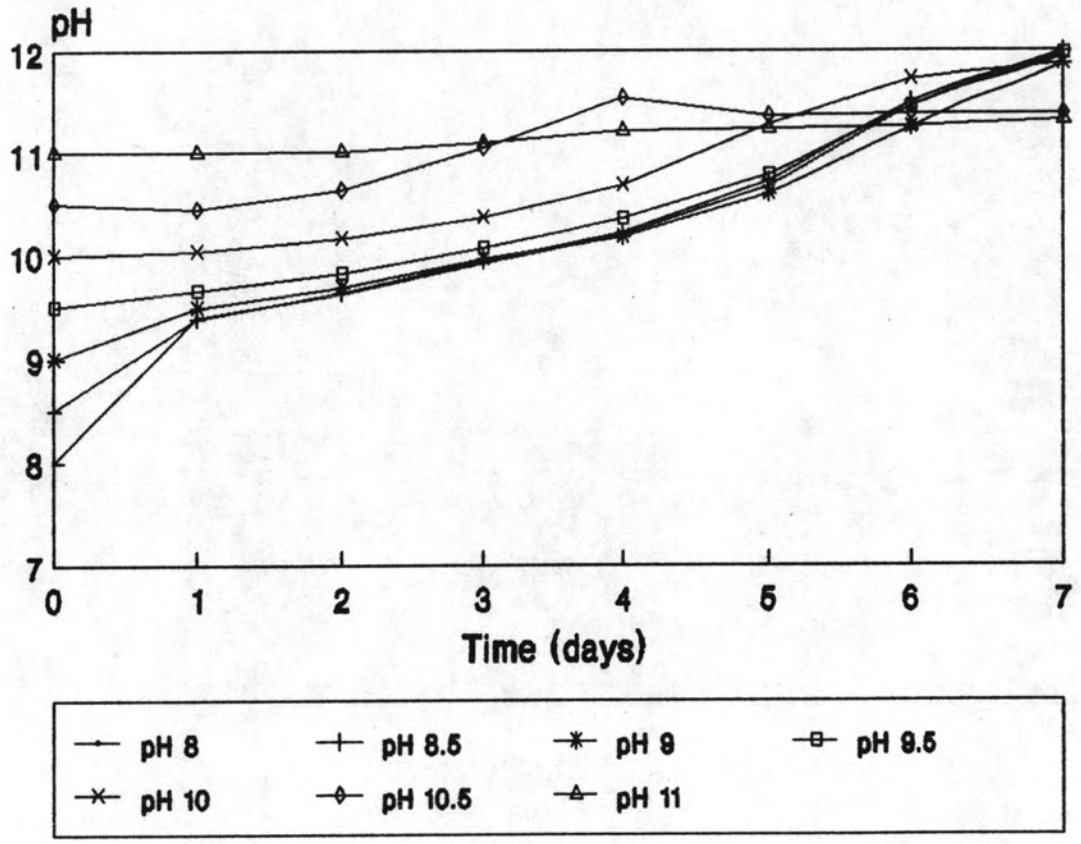

Figure 12 The change of pH during Spirulina cultivation for 1 week

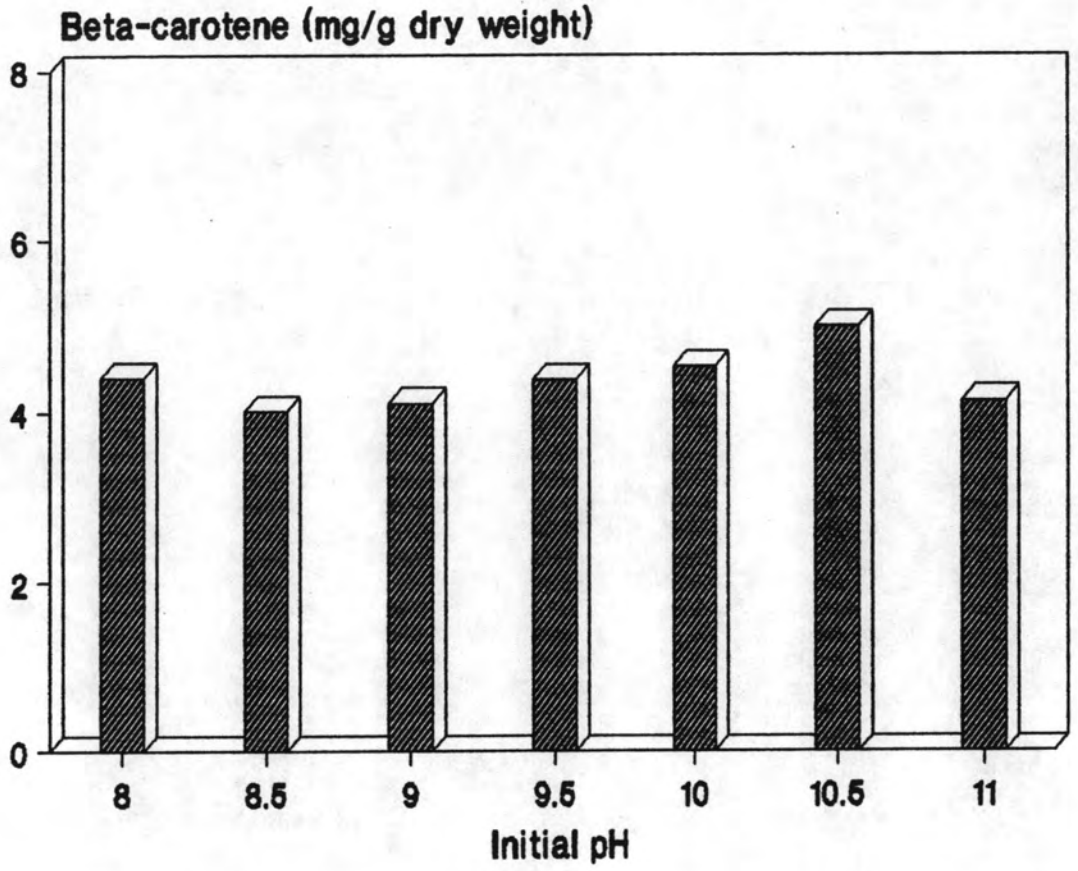

Figure 13 Effect of initial pH on beta-carotene content of Spirulina at day 7

2.3 Effect of $NaNO_3$ on Growth and Beta-carotene Content

Cells grown in Zarrouk medium with the final $NaNO_3$ content of either 0, 0.31, 0.62, 1.25, 2.50, 3.75 or 5.0 g/l for 1 week were also performed in the same manner. The cell growth was obviously decreased when grown under 0 g/l of $NaNO_3$ (Figure 14). In the range of $NaNO_3$ content between 0.16 g/l and 5.0 g/l, no difference of growth was observed (Figure 14). The highest beta-carotene content yielded 4.01 mg/g dry weight at day 7 when cells were grown under 1.25 g/l of $NaNO_3$ and beta-carotene content decreased when grown at above or below this concentration (Figure 15).

2.4 Effect of K_2SO_4 on Growth and Beta-carotene Content

Cells grown in Zarrouk medium with the final K_2SO_4 content of either 0, 0.25, 0.5, 0.75, 1.0, 1.5 or 2.0 g/l for 1 week were also performed in the same manner. Figure 16 shows that K_2SO_4 concentrations did not appear to cause changes in growth of Spirulina. The omission of K_2SO_4 (0 g/l) in the growth medium was the best condition for beta-carotene production which yielded 4.19 mg/g dry weight at day 7 (Figure 17). Beta-carotene content was slightly decreased when K_2SO_4 was included in the growth medium at various concentrations (Figure 17).

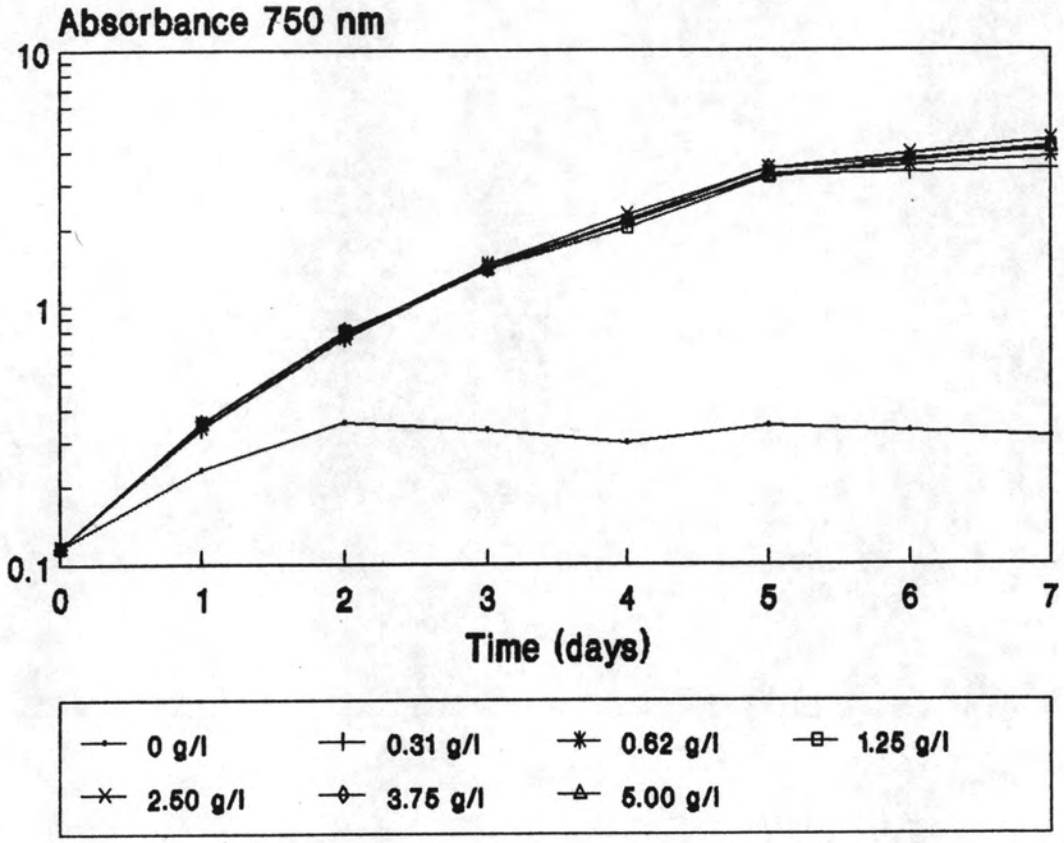

Figure 14 Growth of Spirulina in Zarrouk medium containing various $NaNO_3$ concentrations for 1 week

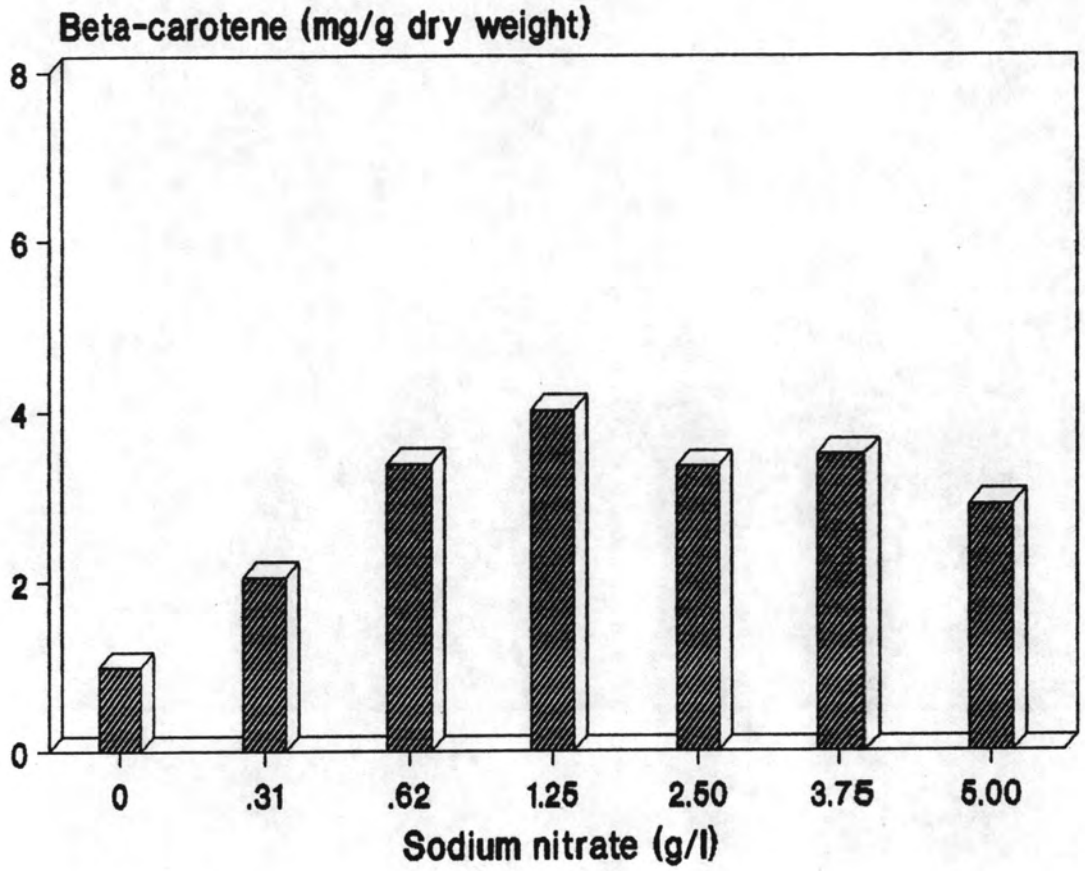

Figure 15 Effect of $NaNO_3$ on beta-carotene content of Spirulina at day 7

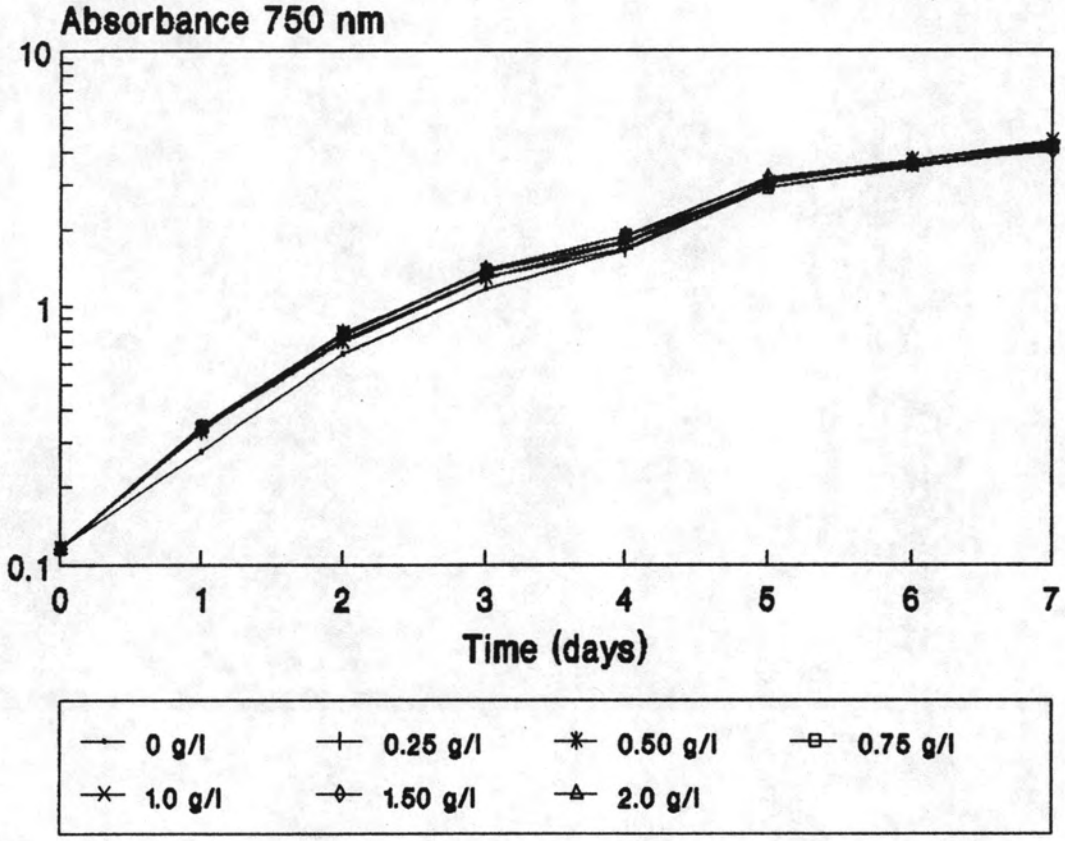

Figure 16 Growth of Spirulina in Zarrouk medium containing various K_2SO_4 concentrations for 1 week

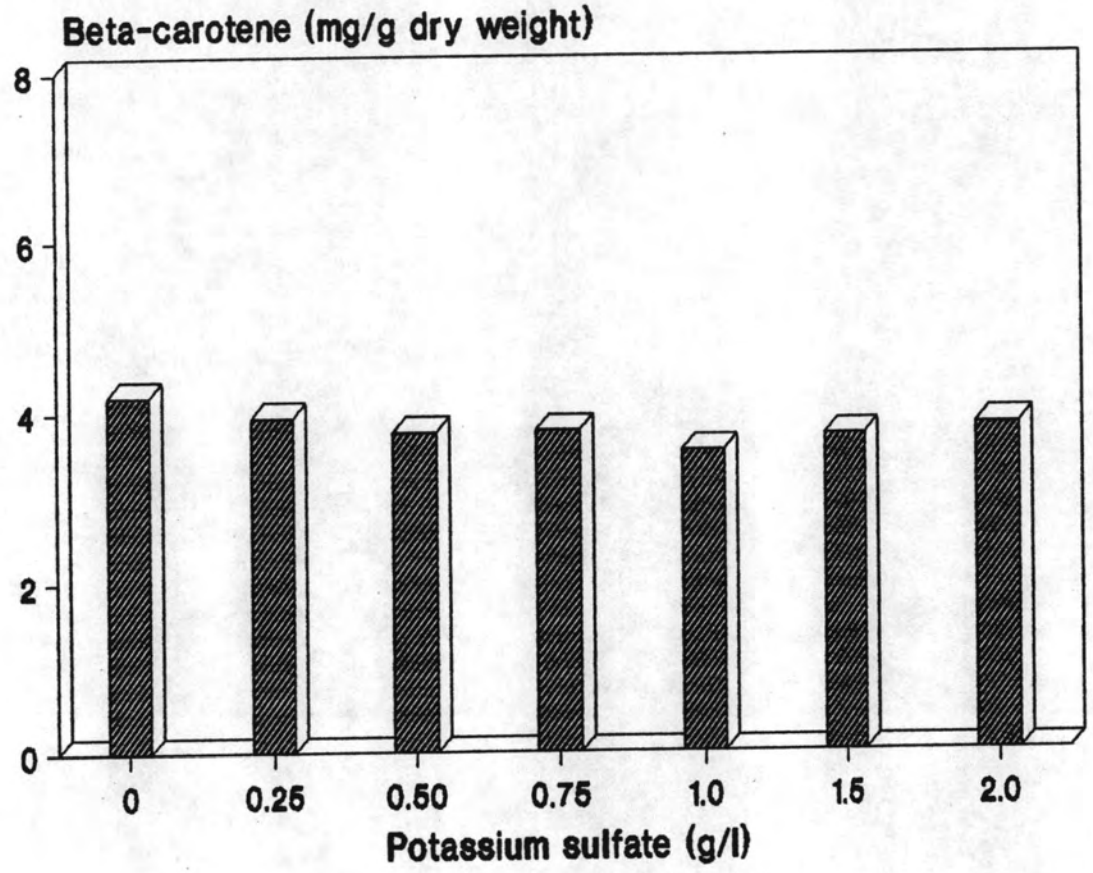

Figure 17 Effect of K_2SO_4 on beta-carotene content of Spirulina at day 7

2.5 Effect of K_2HPO_4 on Growth and Beta-carotene Content

Cells grown in Zarrouk medium with the final K_2HPO_4 content of either 0, 0.25, 0.5, 1.0, 2.5, 5.0 or 10.0 g/l for 1 week were also performed in the same manner. Similar to the result of $NaNO_3$, the omission of K_2HPO_4 affected growth of Spirulina. Figure 18 shows that growth was clearly declined when grown under 0 g/l of K_2HPO_4 and in the range of K_2HPO_4 content between 0.25 and 10.0 g/l, the cell growth were not different. The K_2HPO_4 deprivation resulted in the lowest beta-carotene content yielding only 0.75 mg/g dry weight at day 7 (Figure 19). On the other hand, cells grown under 0.25 g/l of K_2HPO_4 gave the highest yield of beta-carotene content at 4.24 mg/g dry weight at day 7. In addition, beta-carotene content was slightly decreased when K_2HPO_4 in the medium was higher than 0.25 g/l.

2.6 Effect of $MgSO_4$ on Growth and Beta-carotene Content

Spirulina grown in Zarrouk medium with the final $MgSO_4$ of either 0, 0.1, 0.2, 0.4 or 0.8 g/l for 1 week were also performed in the same manner. From Figure 20, the data indicate that after 4 days cultivation, the growth was decreased when grown in Zarrouk medium without $MgSO_4$ and the maximum growth was at 0.1 g/l of $MgSO_4$. Beta-carotene content was the lowest at 0 g/l of $MgSO_4$ yielding 1.81 mg/g

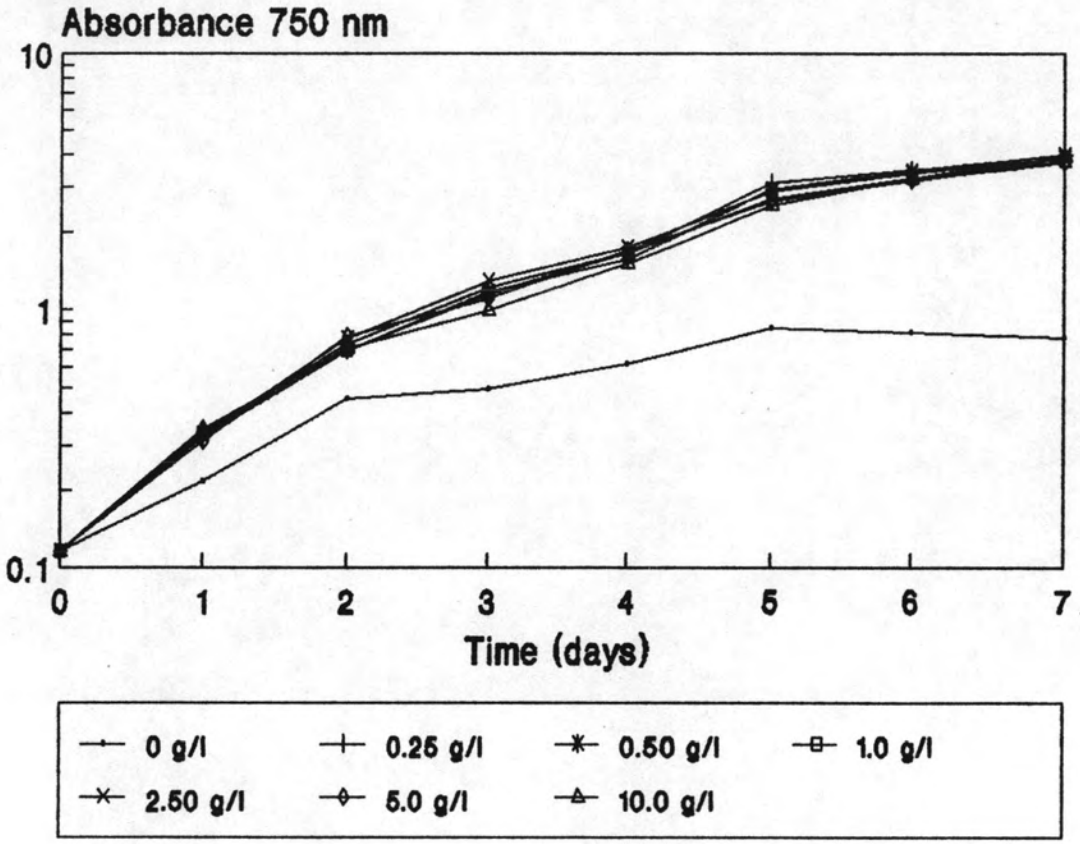

Figure 18 Growth of Spirulina in Zarrouk medium containing various K_2HPO_4 concentrations for 1 week

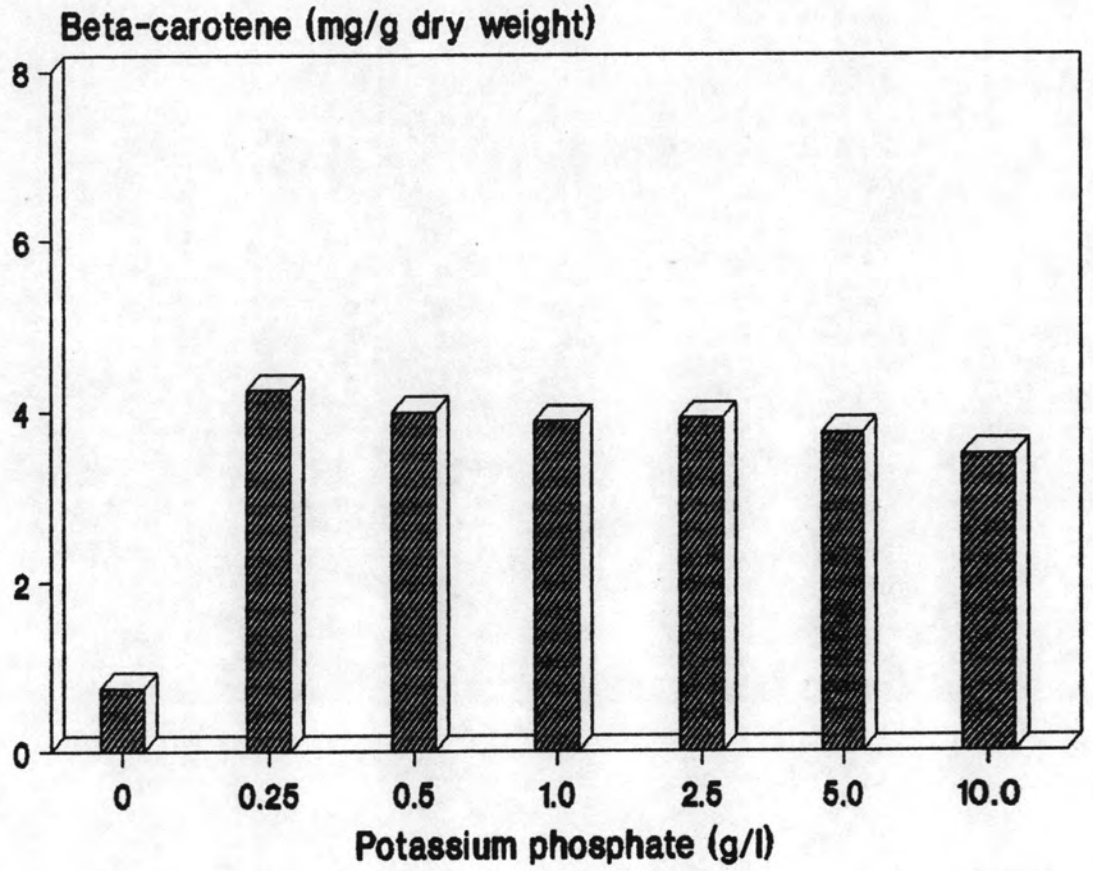

Figure 19 Effect of K_2HPO_4 on beta-carotene content of Spirulina at day 7

dry weight and the highest at 0.1 g/l of $MgSO_4$ yielding 5.03 mg/g dry weight at day 7 (Figure 21).

2.7 Comparison of Growth and Beta-carotene Content between Cells Grown in Optimized and Zarrouk Medium

Spirulina was grown in optimized medium containing 1.25 g/l of $NaNO_3$, 0.25 g/l of K_2HPO_4, 0.1 g/l of $MgSO_4$ and without K_2SO_4 for 9 days. Other components were the same as those of Zarrouk medium. Figure 22 shows that there were no differences on growth of Spirulina when grown in either optimized or Zarrouk medium. Cells grown in optimized and Zarrouk medium gave the same beta-carotene yield after one day cultivation (Figure 23). Afterwards, beta-carotene content of cells grown in optimized medium was higher than that in Zarrouk medium (Figure 23). At day 9, beta-carotene content of cells grown in optimized medium yielded 3.87 mg/g dry weight and that grown in Zarrouk medium yielded 3.35 mg/g dry weight.

2.8 Effect of NaCl on Growth and Beta-carotene Content

Effect of changes of NaCl content in Zarrouk medium from 1 to 20, 40 g/l as well as from 20 to 30 and 40 g/l were investigated on growth and beta-carotene content of

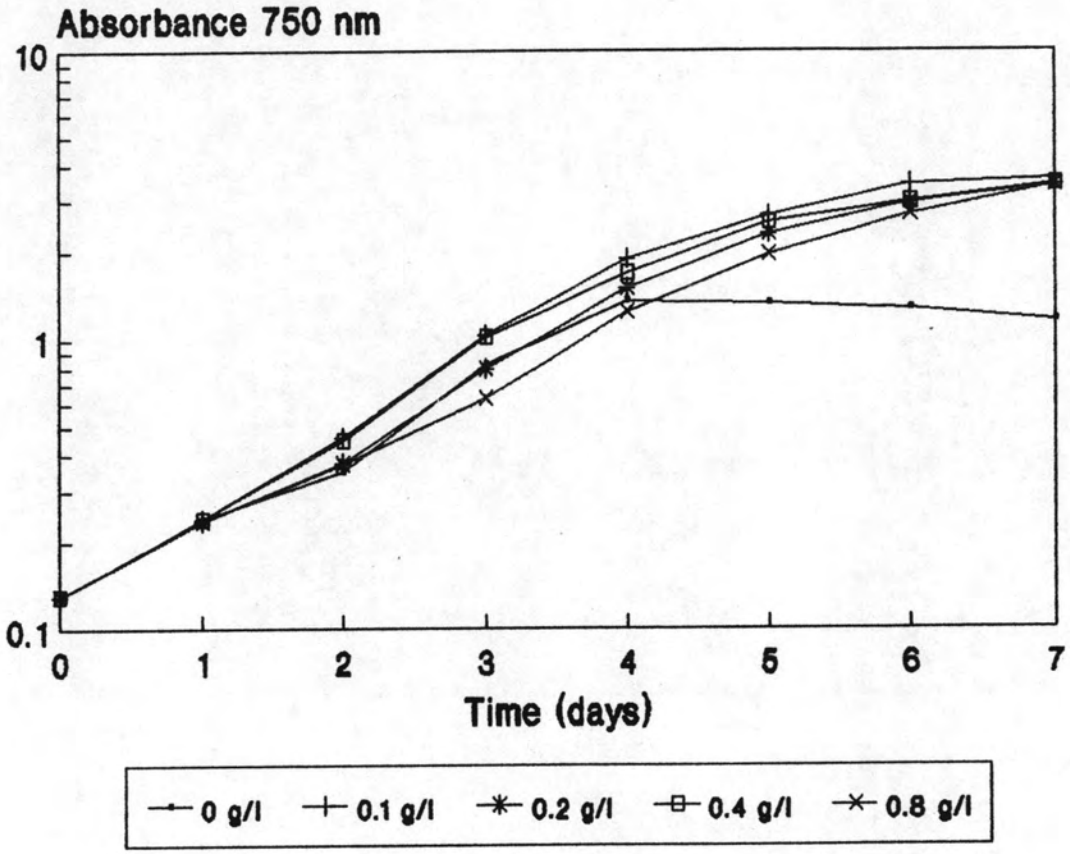

Figure 20 Growth of Spirulina in Zarrouk medium containing various $MgSO_4$ concentrations for 1 week

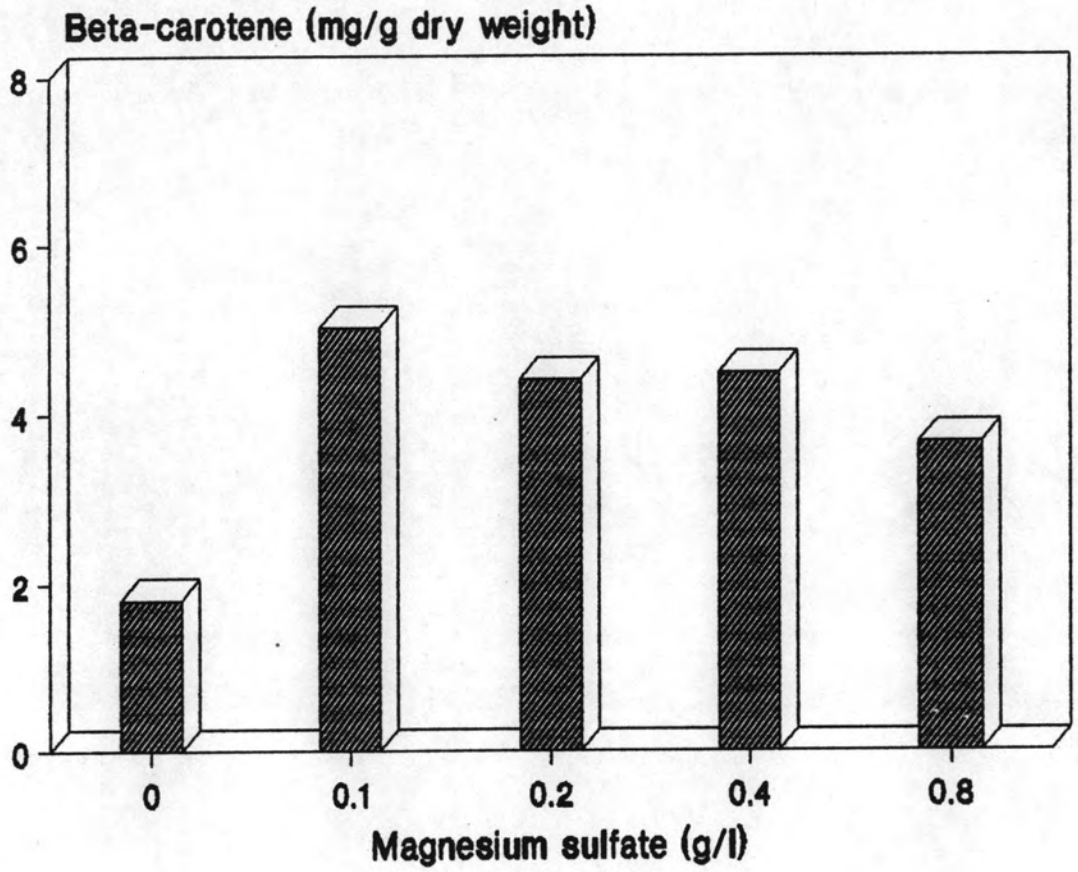

Figure 21 Effect of $MgSO_4$ on beta-carotene content of Spirulina at day 7

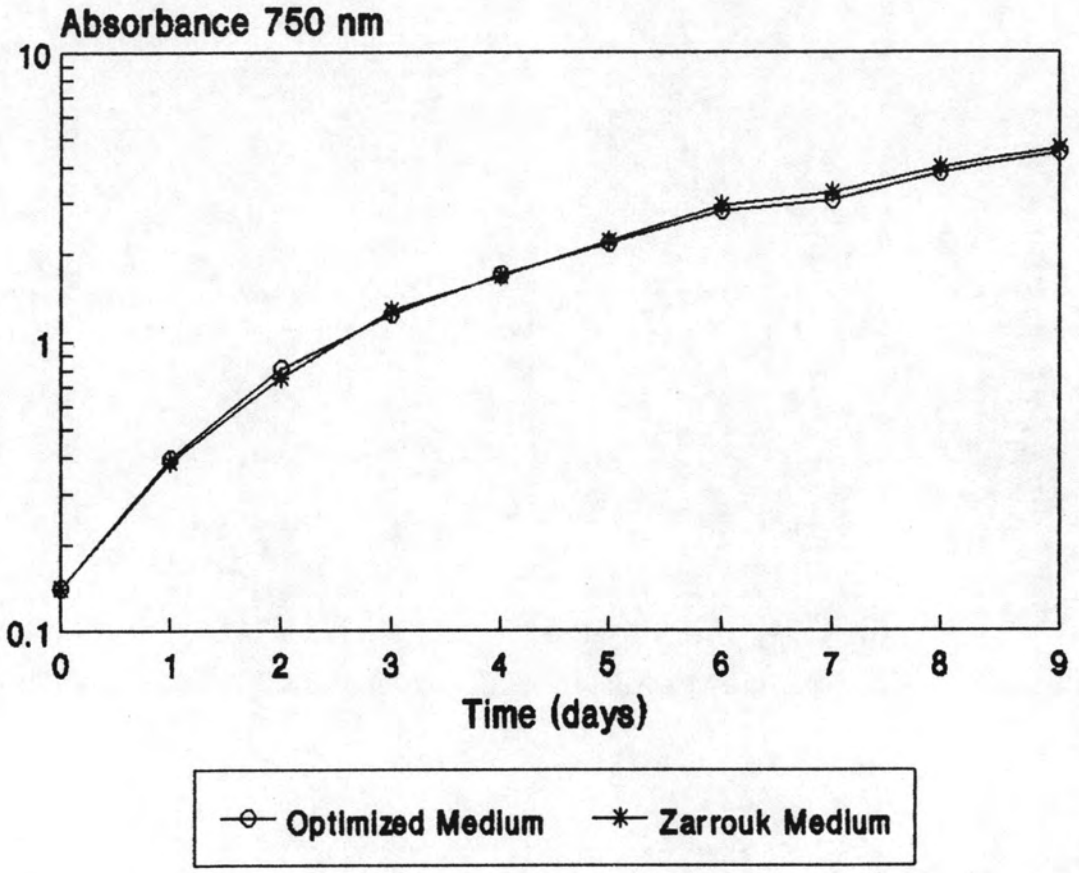

Figure 22 Comparison of growth between Spirulina grown in optimized and Zarrouk medium for 9 days

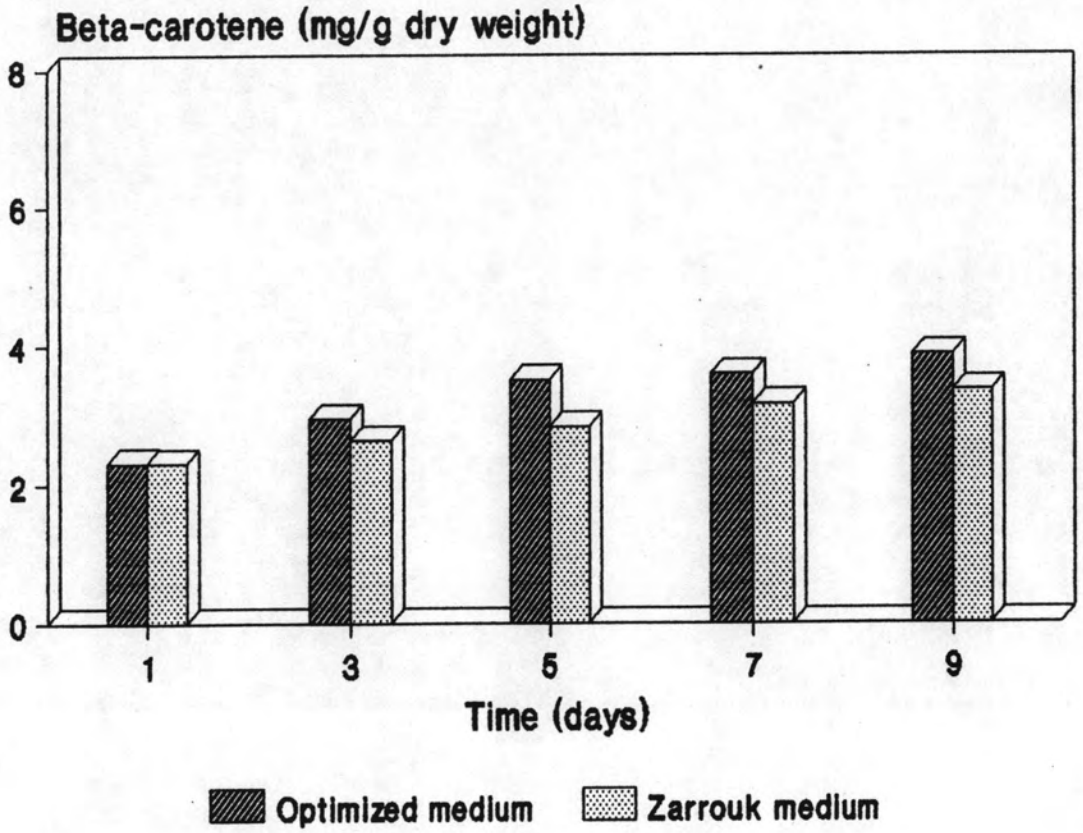

Figure 23 Comparison of beta-carotene content between Spirulina grown in optimized and Zarrouk medium for 9 days

Spirulina. From the data in Figure 24, the maintenance of NaCl at 1 g/l gave the highest growth followed by changing of NaCl content from 1 to 20 g/l and from 20 to 30 g/l, respectively. On the other hand, cells showed a lag period of 2 days adjusting for growth when NaCl content was changed from 1 to 40 g/l. When NaCl content was changed from 20 to 40 g/l, cells could grow in the first day after which they became stationary for 3 days before growth was resumed. The highest beta-carotene content was 7.52 mg/g dry weight when NaCl content was changed from 20 to 30 g/l accounting for about 1.6 times of that when NaCl content was maintained at 1 g/l (Zarrouk medium) (Figure 25).

2.9 Effect of Adding NaCl on Growth and Beta-carotene Content after 4-day Cultivation

Spirulina cultures of day 4 were added with NaCl to final concentrations of 1, 10, 20, 30 and 40 g/l, respectively. During the first 4 days of cultivation, cells manifest usual growth pattern (Figure 26). However, after adding various concentrations of NaCl, it was found that the increment of NaCl content reduced growth of Spirulina. Nevertheless, increased NaCl content gave the highest beta-carotene content yielding 5.22 mg/g dry weight at 40 g/l of NaCl (Figure 27).

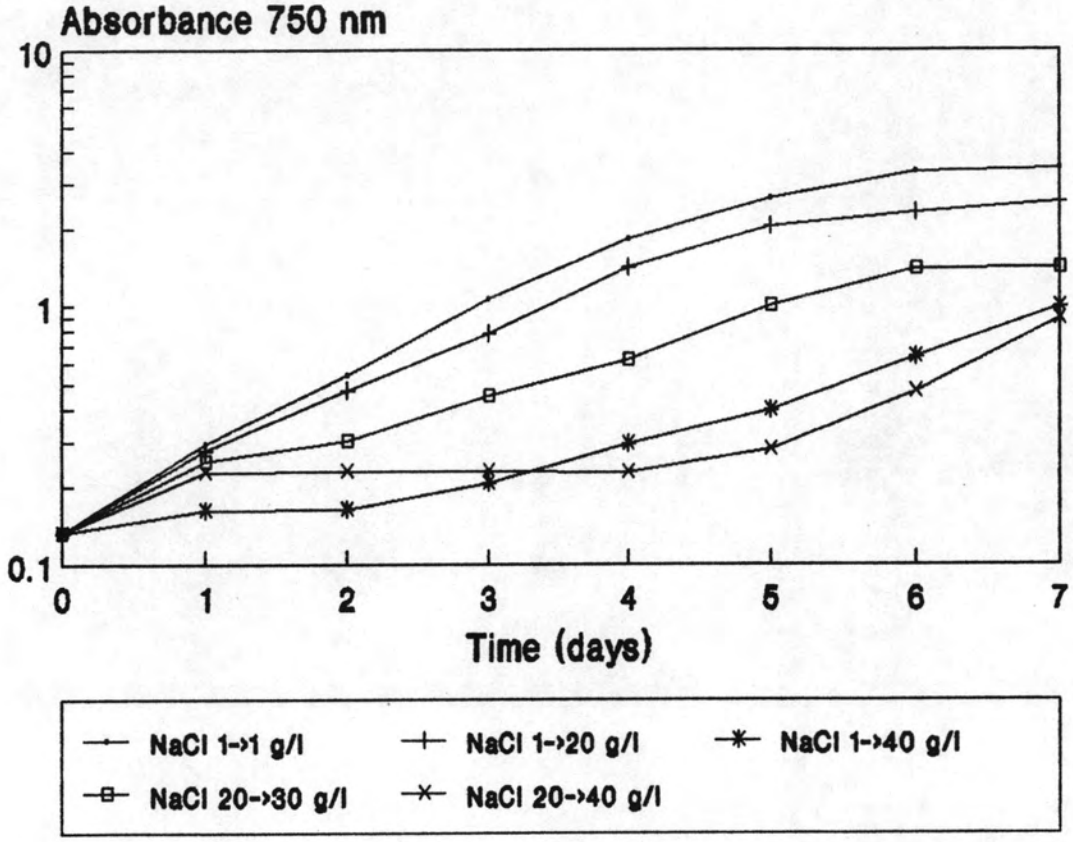

Figure 24 Growth of Spirulina in Zarrouk medium containing various level of NaCl for 1 week

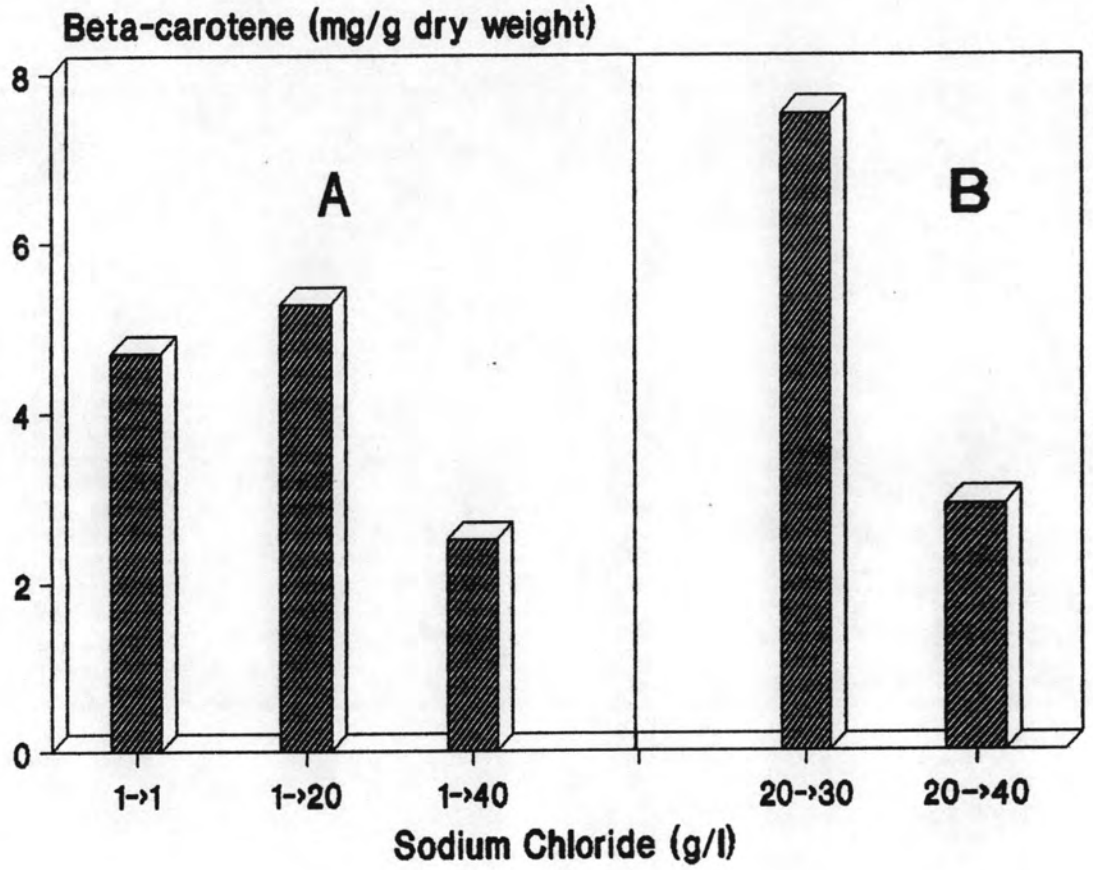

Figure 25 Effect of changing level of NaCl on beta-carotene content of Spirulina at day 7

A) Changing of NaCl content from 1 to 20 and 40 g/l

B) Changing of NaCl content from 20 to 30 and 40 g/l

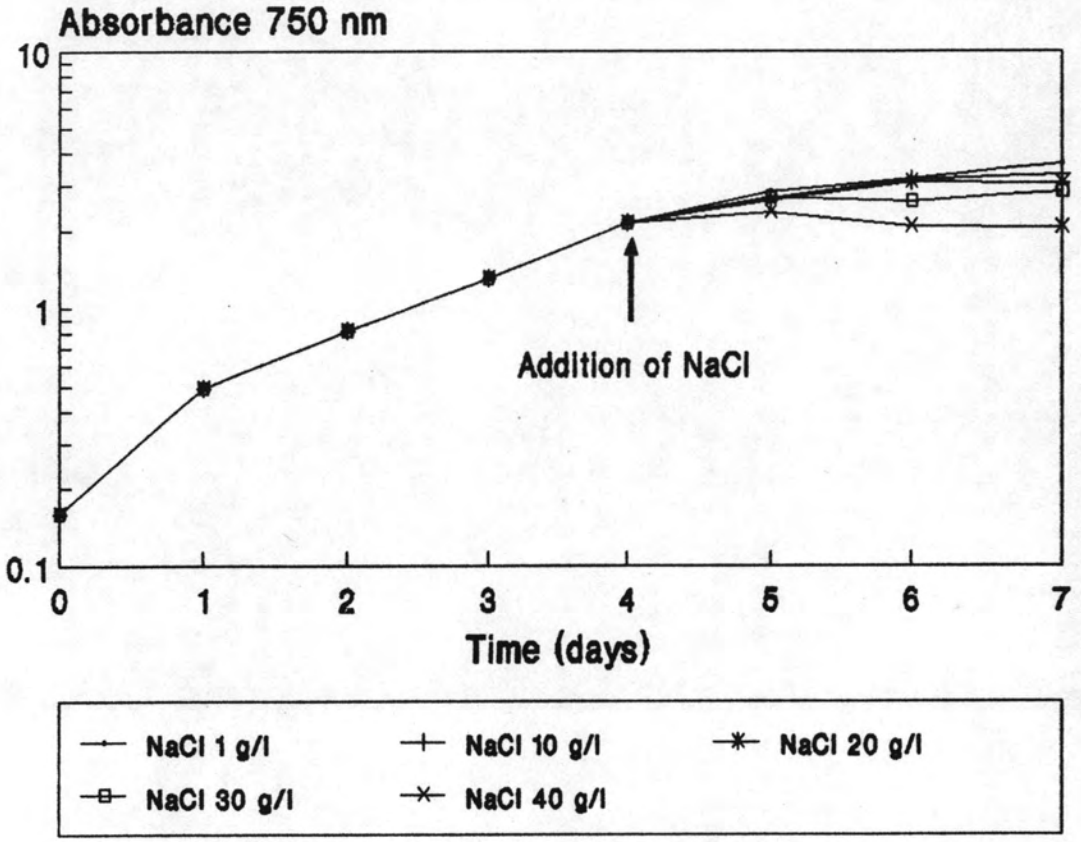

Figure 26 Growth of Spirulina in Zarrouk medium containing various NaCl contents after 4-day cultivation

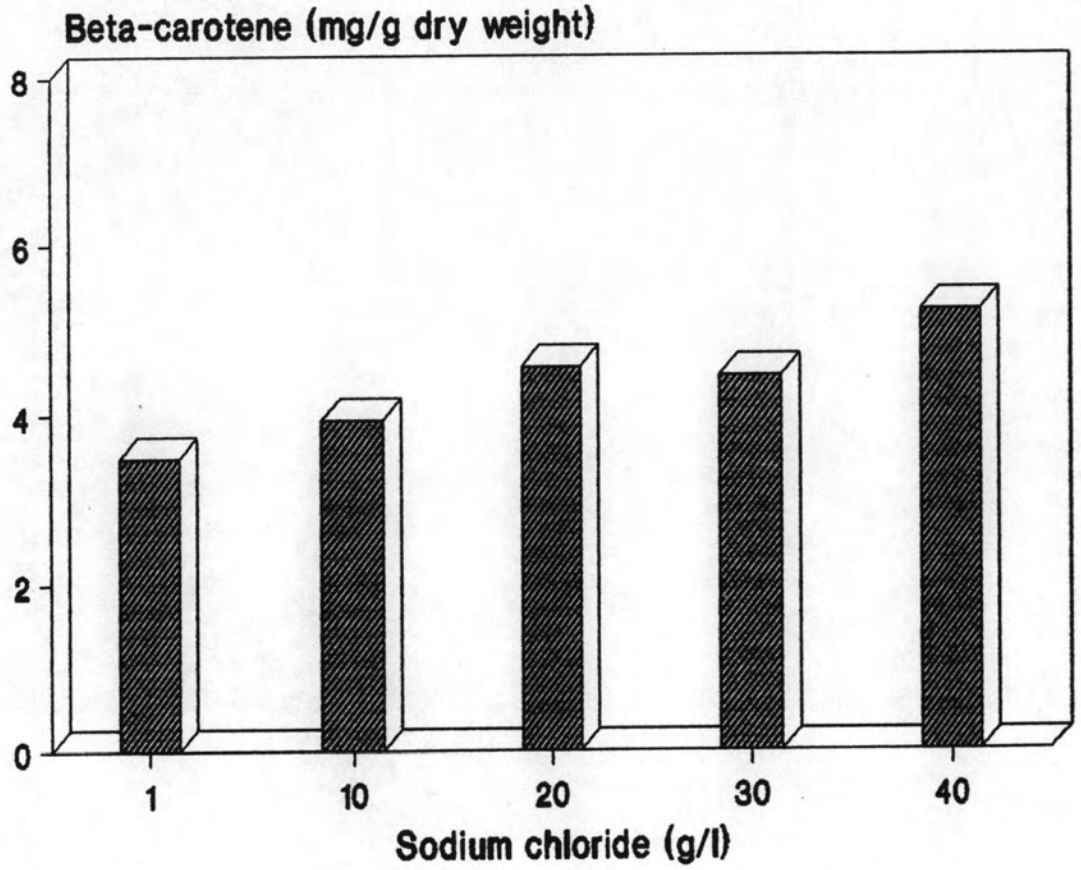

Figure 27 Effect of adding various NaCl contents after 4-day cultivation on beta-carotene content of Spirulina at day 7

2.10 Effect of NaCl on Growth and Beta-carotene Content after Acclimation to NaCl at Various Concentrations

After acclimation to various concentrations of NaCl at 1, 5, 10, 15, 20, 30 and 40 g/l for 1 week, the cultures were used as an initial stock on Zarrouk medium containing equal NaCl concentration. Figure 28 shows that growth was decreased when NaCl concentration increased. The highest growth was at 1 g/l of NaCl whereas the lowest growth was at 40 g/l of NaCl. In contrast, beta-carotene content increased when grown under increased NaCl concentration (Figure 29). Zarrouk medium containing 40 g/l of NaCl gave the highest beta-carotene yielding 7.55 mg/g dry weight, approximately 2.3 times that of Zarrouk medium (composed of 1 g/l NaCl).

2.11 Effect of Light Intensity on Growth and Beta-carotene Content

Spirulina was grown in Zarrouk medium with different light intensities at 1,500, 3,000, 6,000, 10,000 and 14,000 lux for 15 days. Spirulina had the lowest growth when grown under illumination at 1,500 lux (Figure 30). Cells grown under illumination at 3,000 and 6,000 lux, had similar growth but lower than those at 10,000 and 14,000 lux (Figure 30). During 9-day cultivation, growth of cells under light

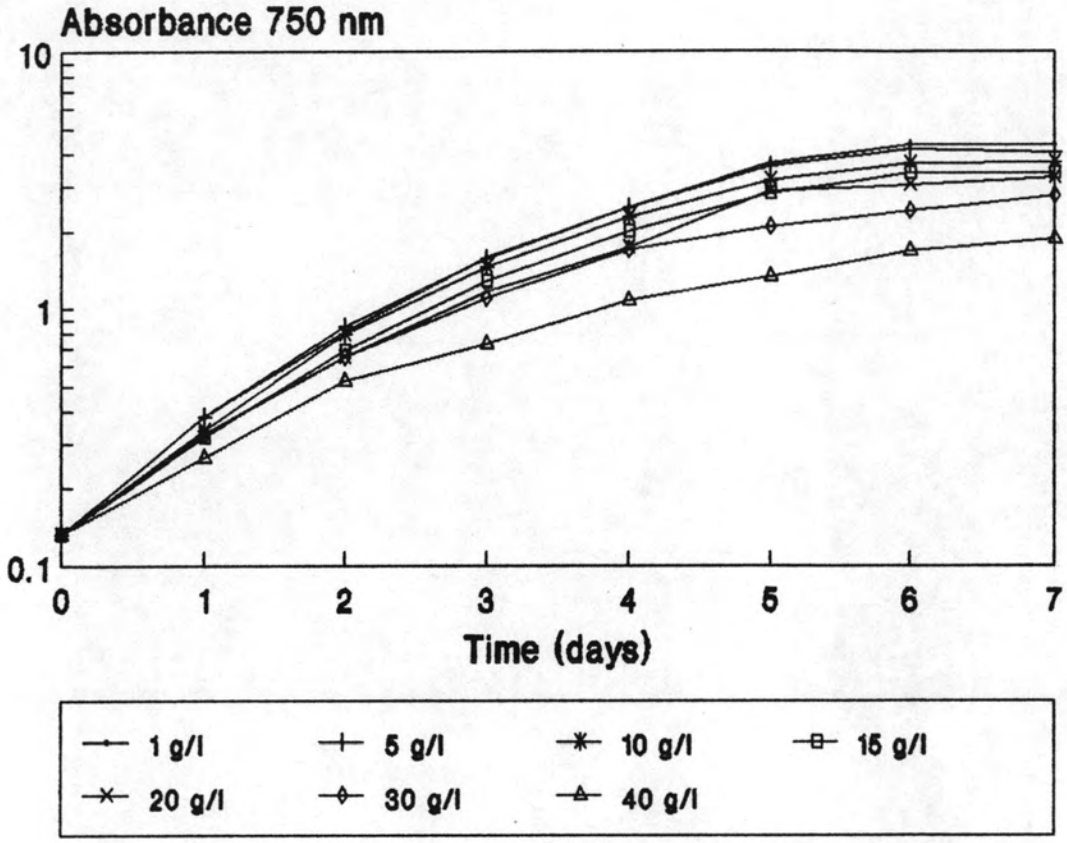

Figure 28 Growth of Spirulina in Zarrouk medium after acclimation to NaCl at various concentrations for 1 week

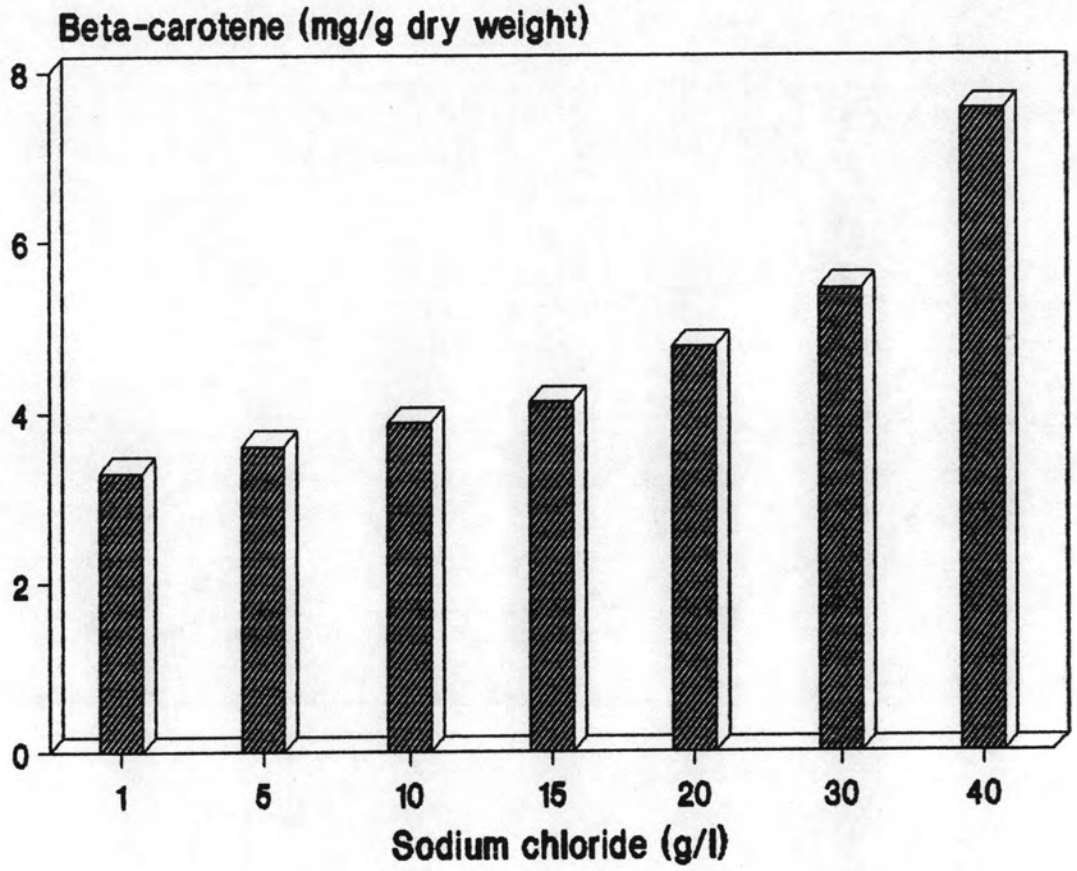

Figure 29 Effect of NaCl on beta-carotene content after acclimation to NaCl at various concentrations at day 7

intensity at 10,000 lux and 14,000 lux resembled each other but after day 9, growth of cells under 10,000 lux was higher than that under 14,000 lux. Beta-carotene content was the highest at light intensity 10,000 and 14,000 lux yielding 4.43 and 4.39 mg/g dry weight, respectively at day 12 (Figure 31). The results showed that under higher intensity, Spirulina produced higher beta-carotene content.

2.12 Effect of Light Quality on Growth and Beta-carotene

Spirulina was grown in Zarrouk medium under white, red and blue light at photosynthetic photon flux density of 70 μmol photon $m^{-2}s^{-1}$ for 1 week. Measurement of the photon flux was performed with a Li-cor quantum meter Li-189. Optical characteristics of the red and blue light is shown in Figure 32. Growth was the highest when grown under white light followed by those under red and blue light, respectively (Figure 33). In contrast, beta-carotene content was the lowest when grown under white light and the highest when grown under red light. Beta-carotene content yielded 4.50, 3.68 and 2.87 mg/g dry weight at day 7 when grown under red, blue and white light, respectively (Figure 34).

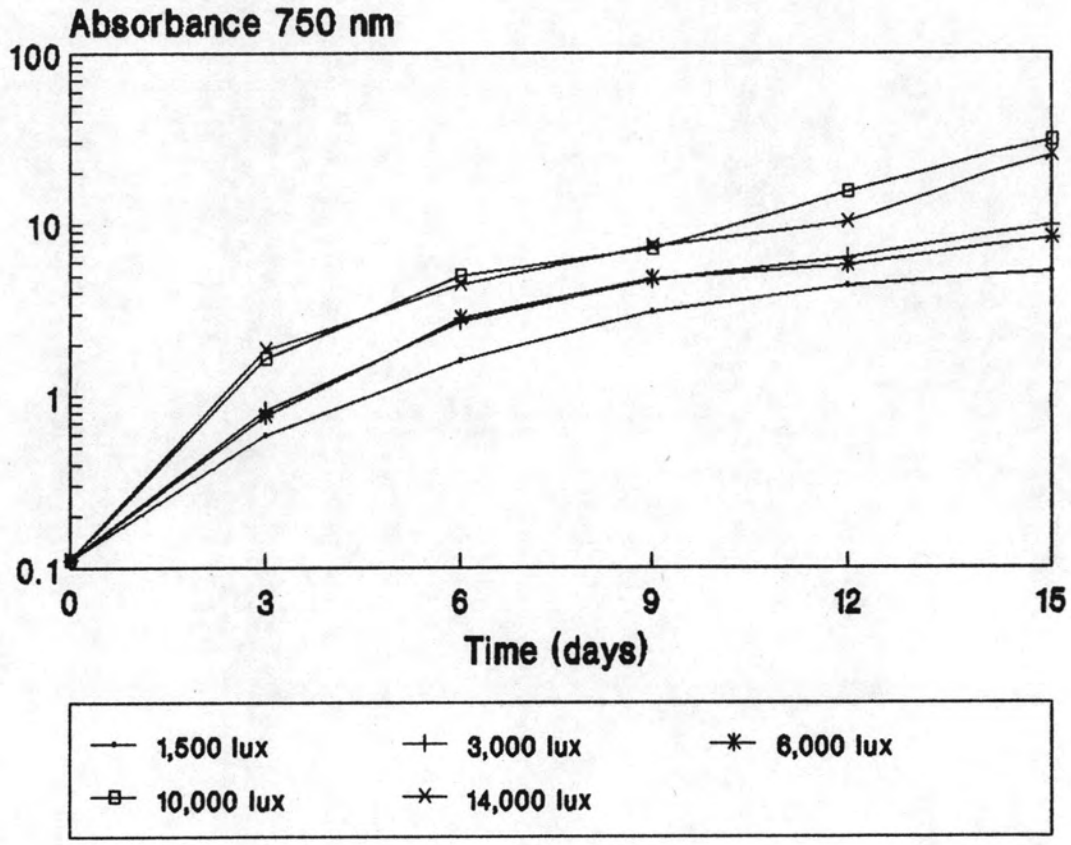

Figure 30 Growth of Spirulina in Zarrouk medium under various light intensities for 15 days

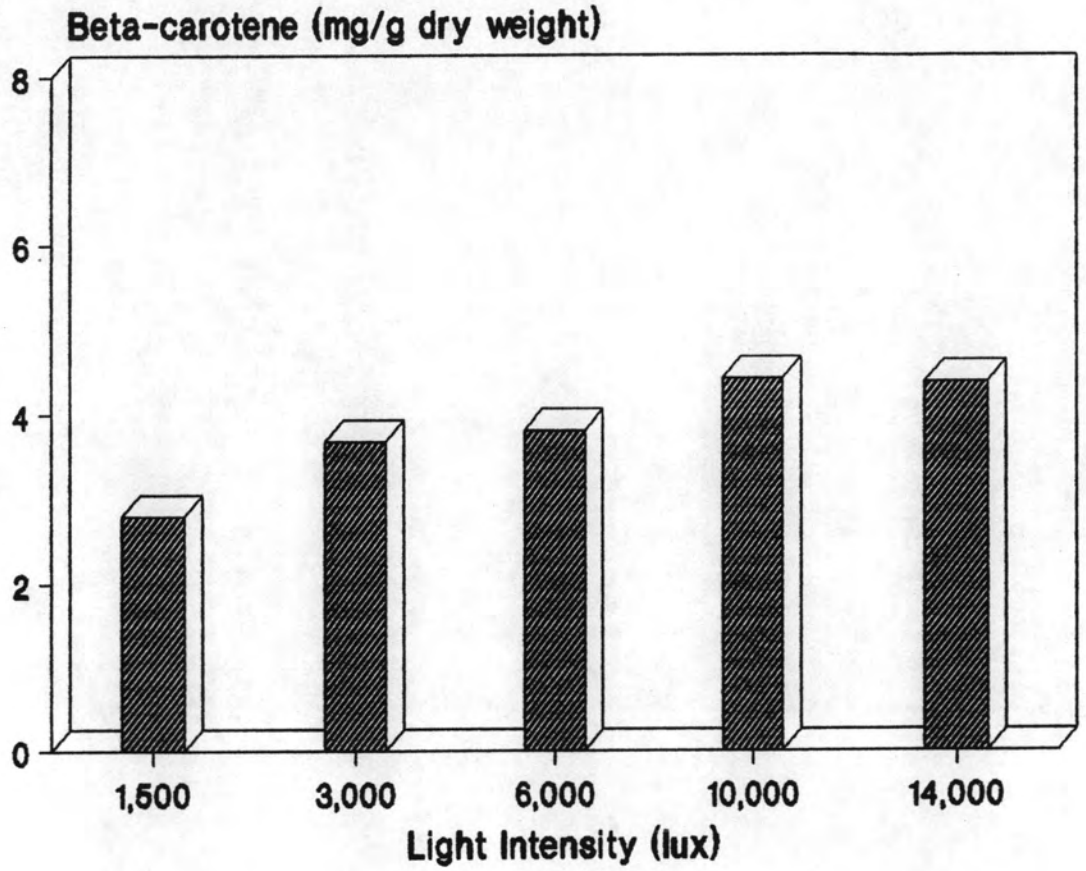

Figure 31 Effect of light intensities on beta-carotene content of Spirulina at day 12

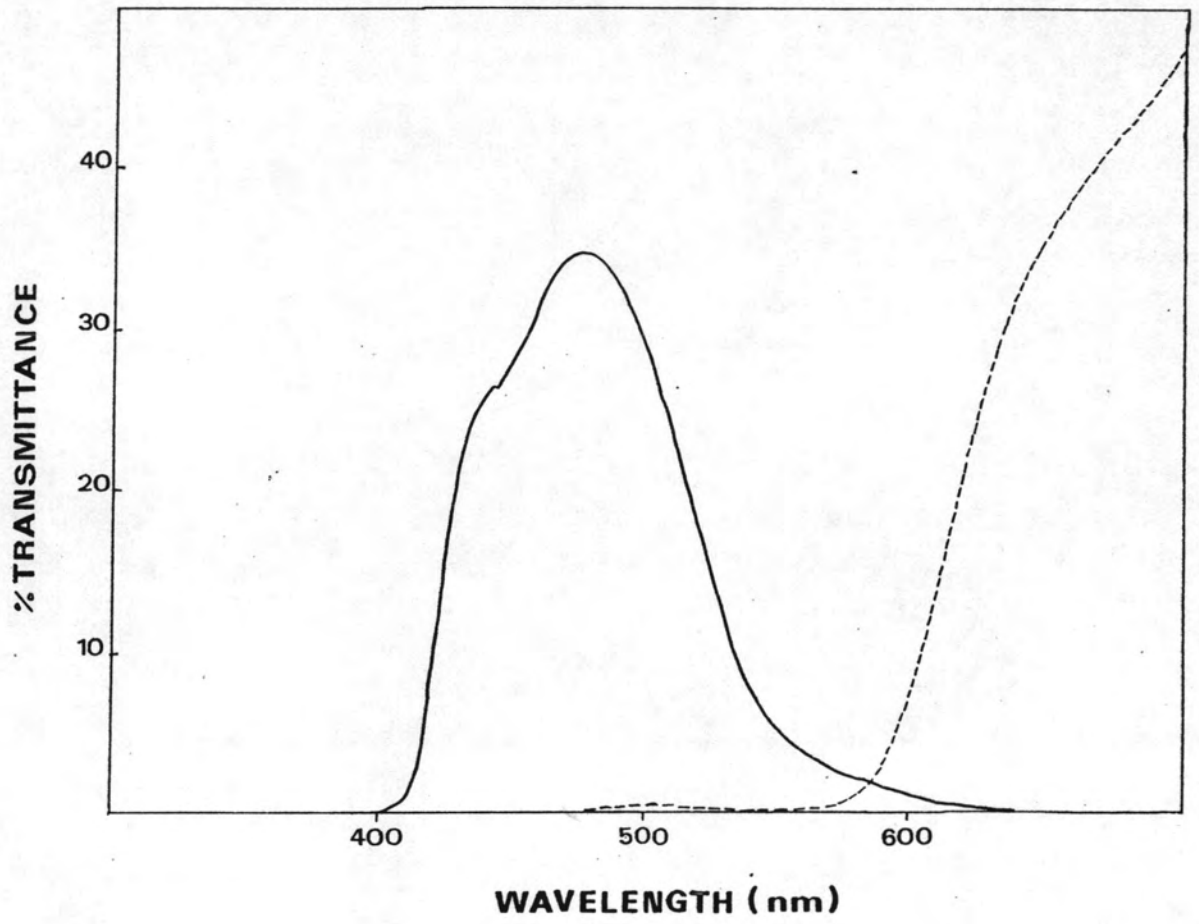

Figure 32 Optical characteristics of the red and blue lights

---------- red light

_____ blue light

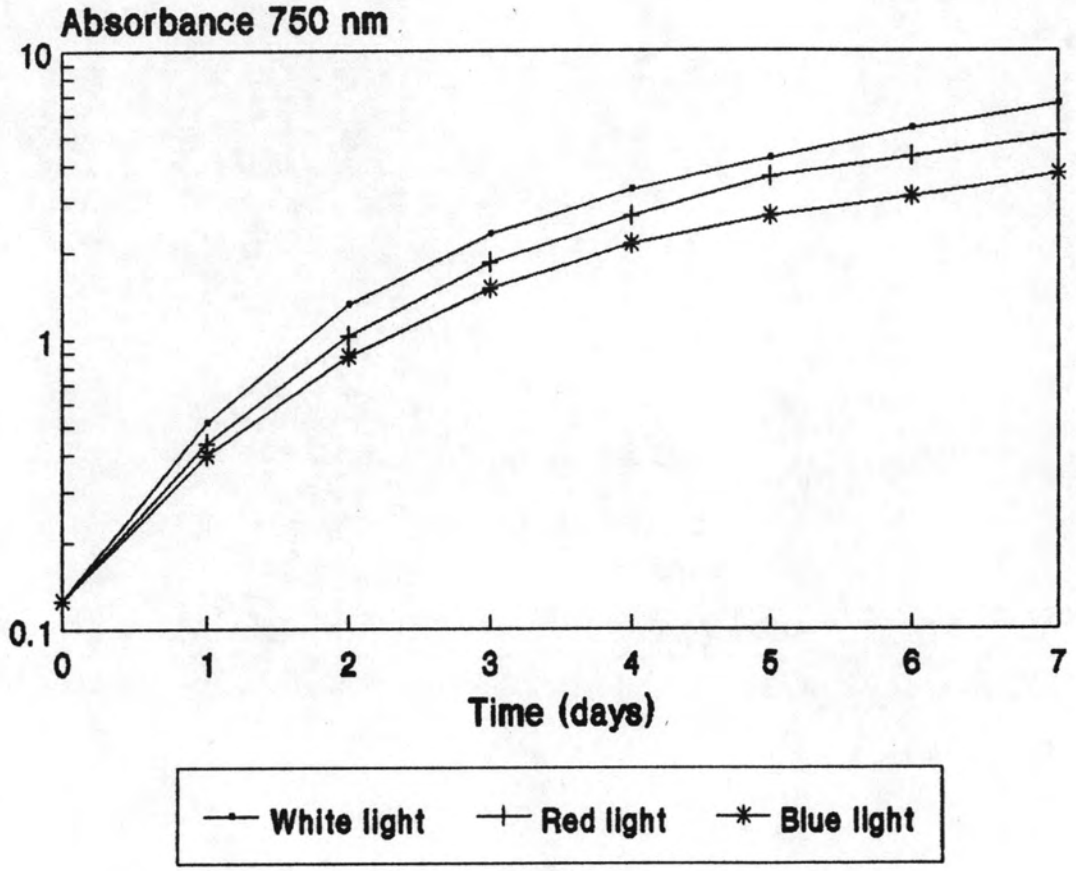

Figure 33 Growth of Spirulina in Zarrouk medium under white, red and blue light at photosynthetic photon flux density of 70 μmol photon m^{-2} s^{-1} for 7 days

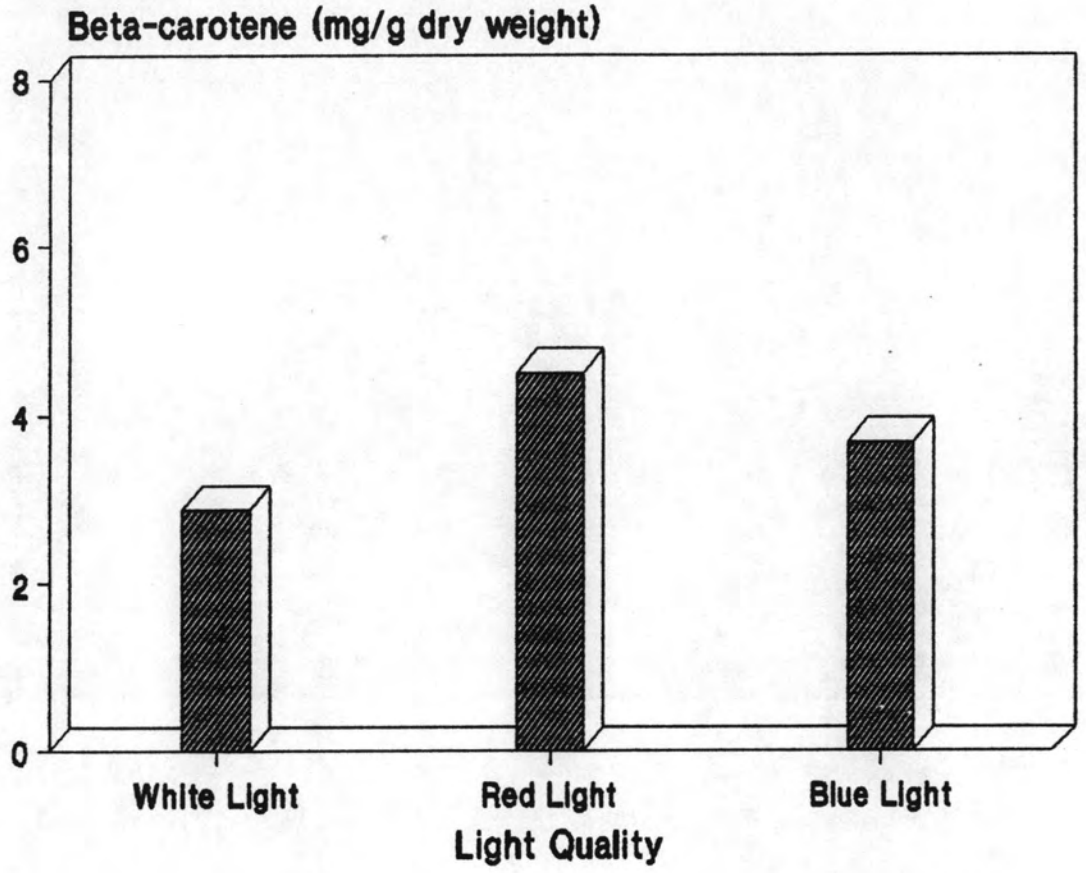

Figure 34 Effect of light quality on beta-carotene content of Spirulina at photosynthetic photon flux density of 70 μmol photon m^{-2} s^{-1} at day 7

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

3.1 Effect of Norflurazon on Growth and Beta-carotene Content

Spirulina was cultivated in Zarrouk medium containing 0, 0.1, 0.2, 0.5, 1.0 and 2.0 μM of norflurazon for 4 days. Figure 35 shows that no difference in growth among 5 concentrations of norflurazon was observed. Beta-carotene content in Spirulina grown in Zarrouk medium containing 2.0 μM of norflurazon at day 4 was the lowest (Figure 36).

3.2 Effect of Diphenylamine on Growth and Beta-carotene Content

Spirulina was cultivated in Zarrouk medium containing 0, 0.1, 1.0, 10 and 100 μM of diphenylamine for 4 days. The results showed that 100 μM of diphenylamine could affect both growth and beta-carotene content in Spirulina. Growth was similar under 0, 0.1, 1.0 and 10 μM. However a slight decrease was observed after 2 days in the medium containing 10 μM of diphenylamine (Figure 37). Increased diphenylamine concentration up to 10 μM did not cause an apparent change in beta-carotene content (Figure 38). At very high concentration of diphenylamine (100 μM), cell

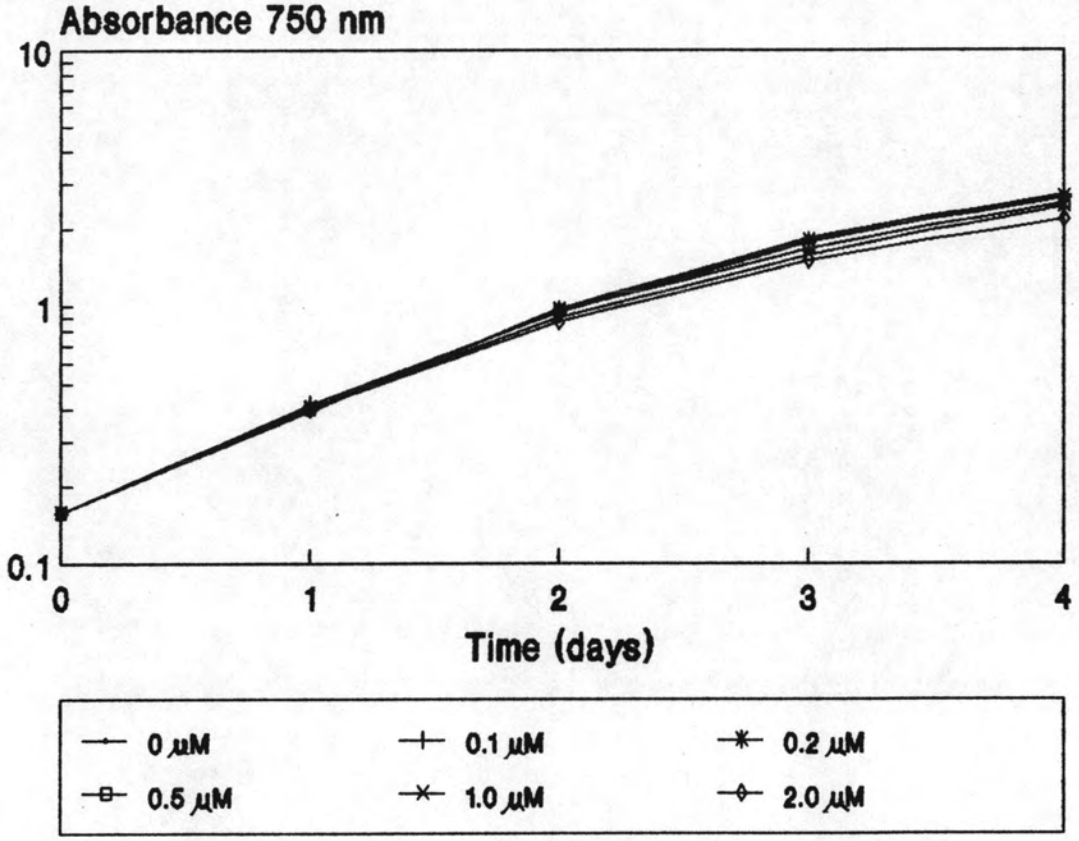

Figure 35 Growth of Spirulina in Zarrouk medium containing various concentrations of norflurazon for 4 days

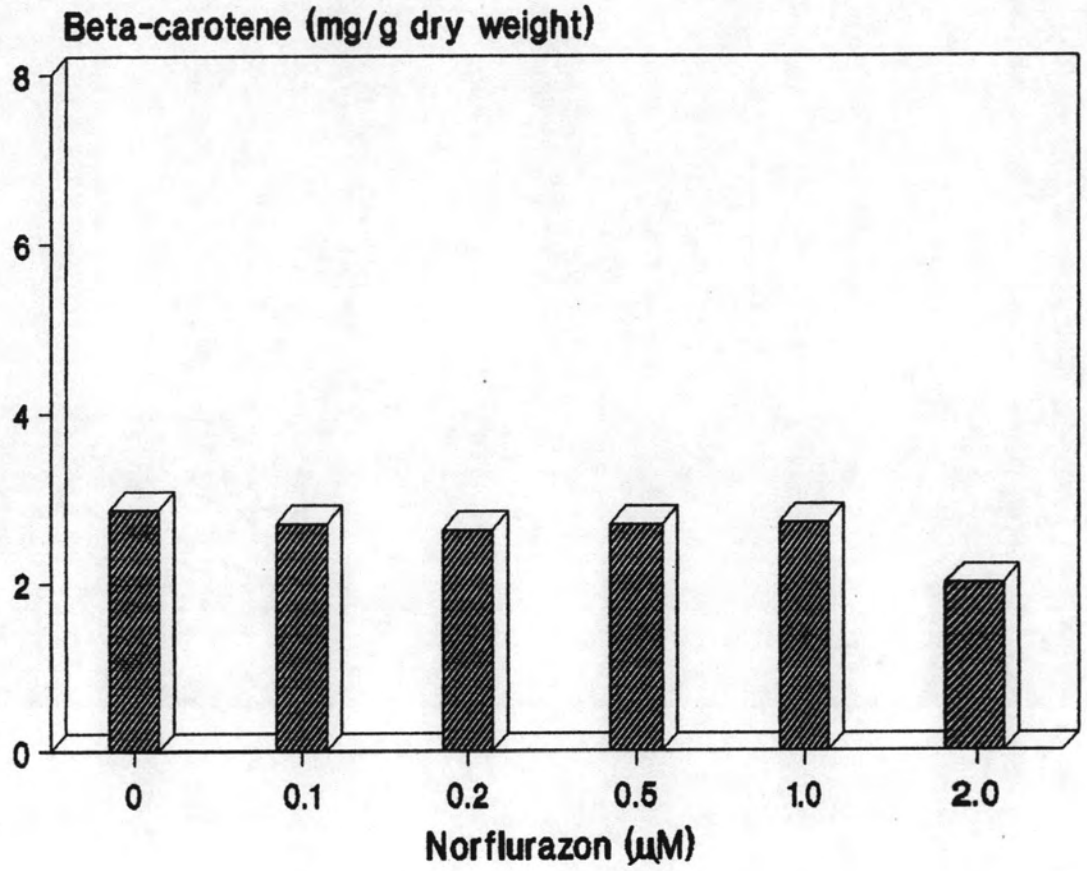

Figure 36 Effect of norflurazon on beta-carotene content of Spirulina at day 4

death occurred after 2 days (Figure 37) and the near complete loss of beta-carotene content was observed at 100 μM of diphenylamine after 4 days (Figure 38).

3.3 Effect of 2,4-Dinitrophenol on Growth and Beta-carotene Content

Spirulina was cultivated in Zarrouk medium containing 0, 0.1, 1.0, 10 and 100 μM of 2,4-dinitrophenol for 4 days. 2,4-dinitrophenol did not appear to affect growth of cells during 4 days (Figure 39). Beta-carotene was slightly decreased when concentrations of 2,4-dinitrophenol was increased (Figure 40).

4. The Pilot Scale Production of Spirulina

The results of the pilot scale production of Spirulina showed that growth of Spirulina under 1 g/l of NaCl (Zarrouk medium) was higher than that under 30 g/l of NaCl (Figure 41). In addition, growth rate of Spirulina on pilot scale in Zarrouk medium was lower than that on laboratory scale in the same Zarrouk medium. In contrast to growth, beta-carotene content of Spirulina grown under 30 g/l was obviously higher than cells grown under 1 g/l (Figure 42). The highest beta-carotene content was 4.00

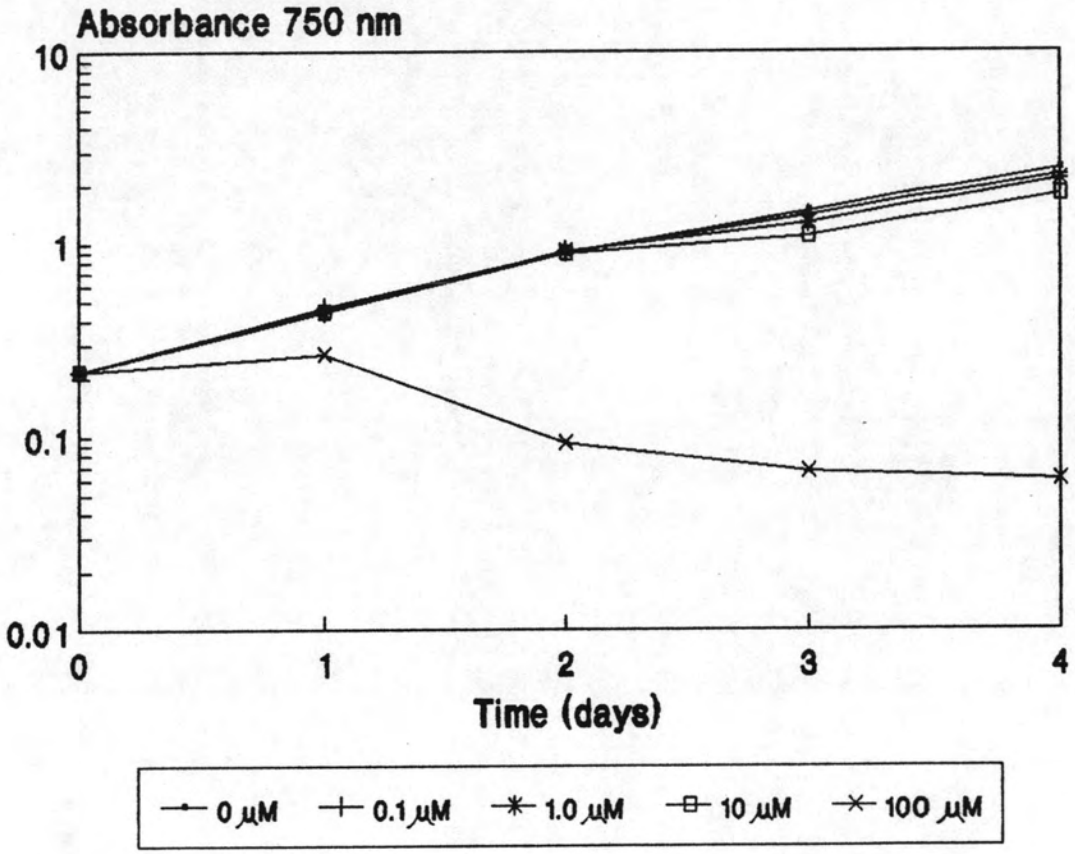

Figure 37 Growth of Spirulina in Zarrouk medium containing various concentrations of diphenylamine for 4 days

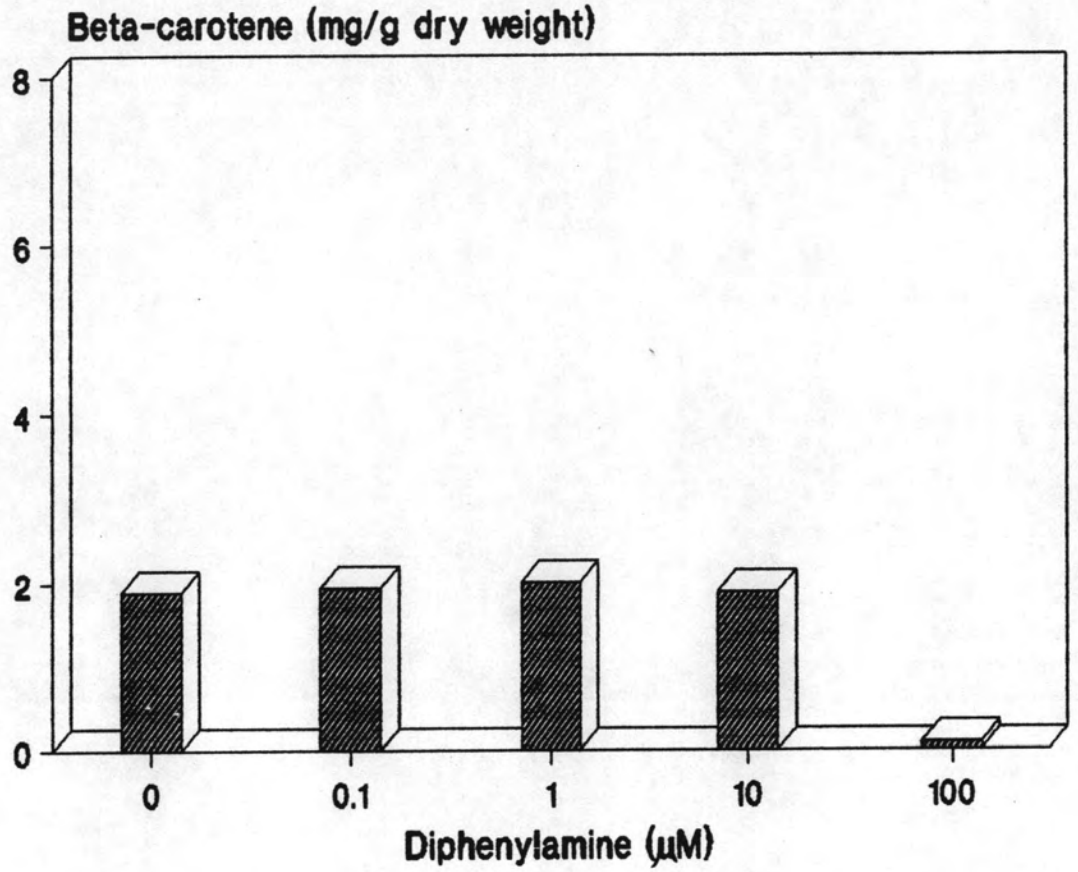

Figure 38 Effect of diphenylamine on beta-carotene content of Spirulina at day 4

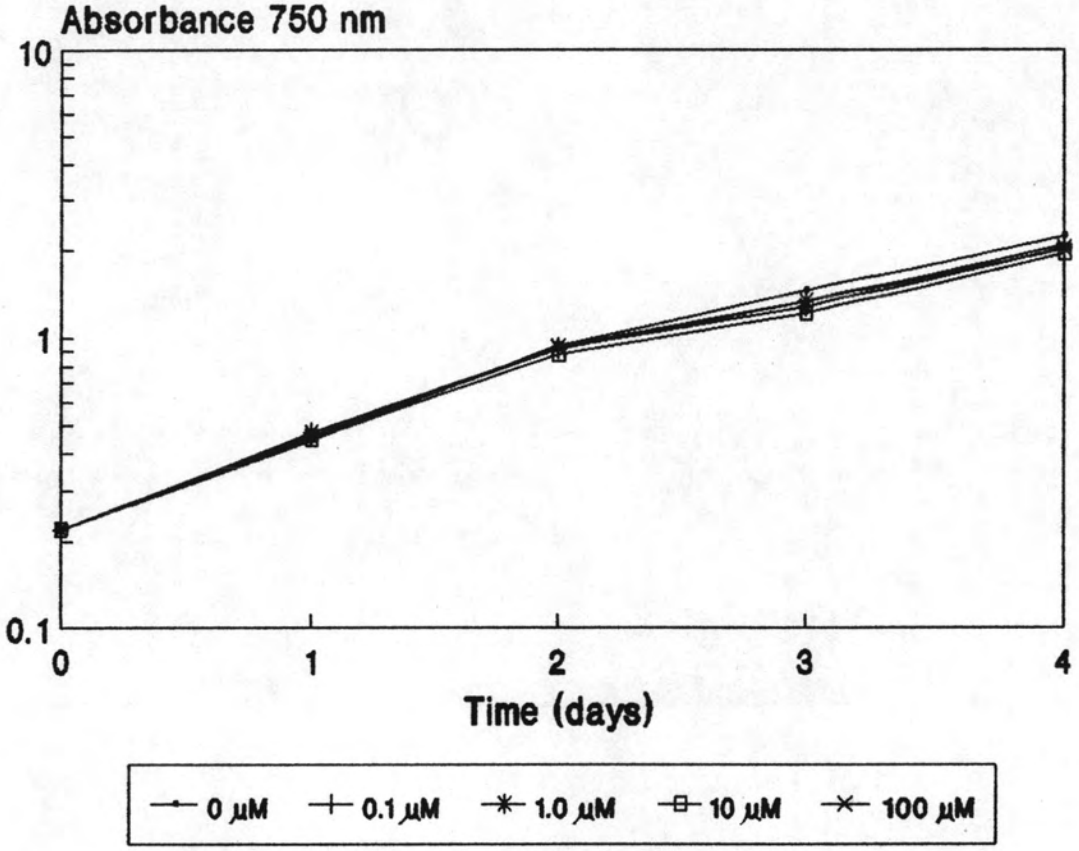

Figure 39 Growth of Spirulina in Zarrouk medium containing various concentrations of 2,4-dinitrophenol for 4 days

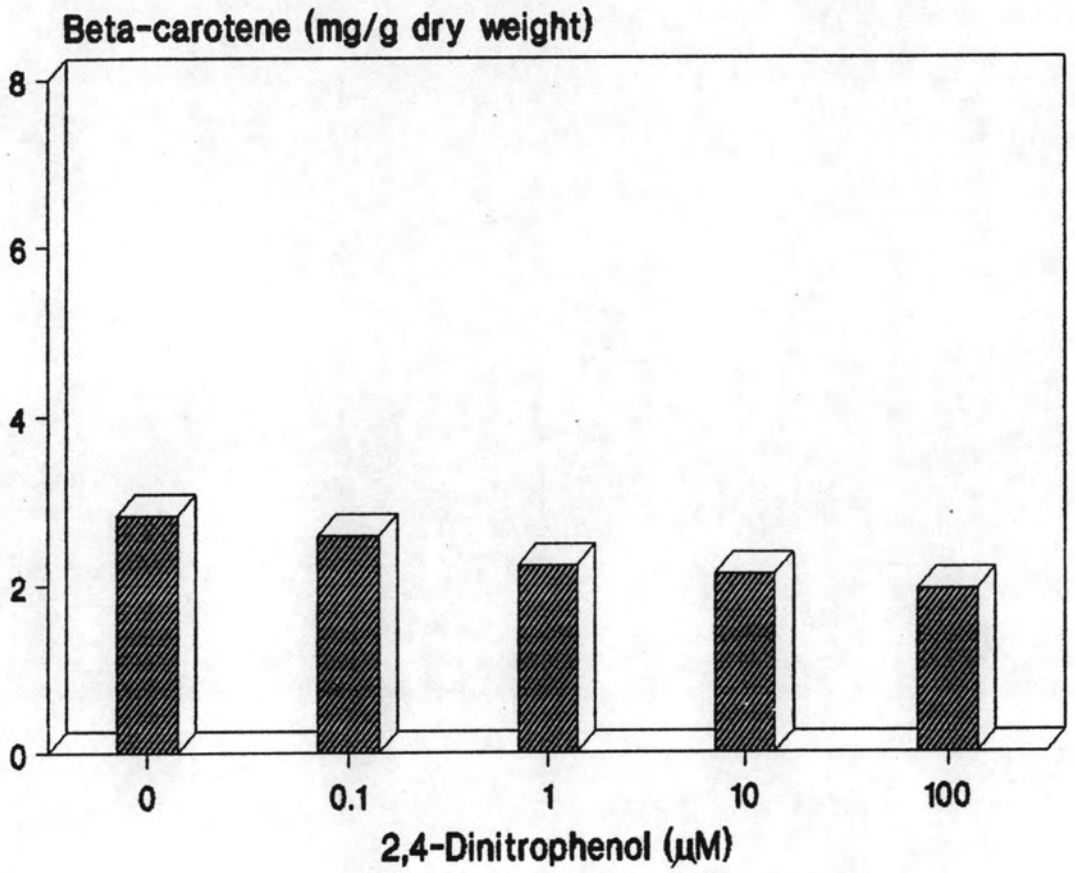

Figure 40 Effect of 2,4-dinitrophenol on beta-carotene content of Spirulina at day 4

mg/g dry weight at day 14 of cultivation under 30 g/l of NaCl and was 3.44 mg/g dry weight under 1 g/l of NaCl. Productivity of beta-carotene during 14 days of Spirulina cultivation under 30 g/l of NaCl was 95.4 mg/l/day and that under 1 g/l of NaCl was 133.7 mg/l/day. During 14 days of cultivation, pH in 500 l culture was increased slowly and was in the range between 8.6 and 9.7 (Figure 43). Light intensity was measured by lux meter between 8 a.m. and 6 p.m. At the period of 8 to 11 a.m., light intensity was at 5,000 to 13,400 lux and from 11 a.m. to 2 p.m., it was the highest at about 38,000-44,000 lux. Light intensity was sharply decreased after 4 p.m. and could not be detected after 6 p.m. (Figure 44). Figure 45 shows that air temperature had higher variation than medium temperature during the day time.

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

Spirulina acquired from the outdoor cultivation was dried by various methods of drying i.e. sun drying, oven drying and freeze drying. It was found that freeze drying was the best way of drying because of the lowest percent beta-carotene loss of 0.9 (Figure 46). Percent beta-carotene losses by sun drying and oven drying were 19.6 and 4.5, respectively. Furthermore, moisture content of Spirulina by freeze drying was the lowest at 1.72% and by sun and oven drying were 6.33 and 5.72%, respectively (Figure 47).

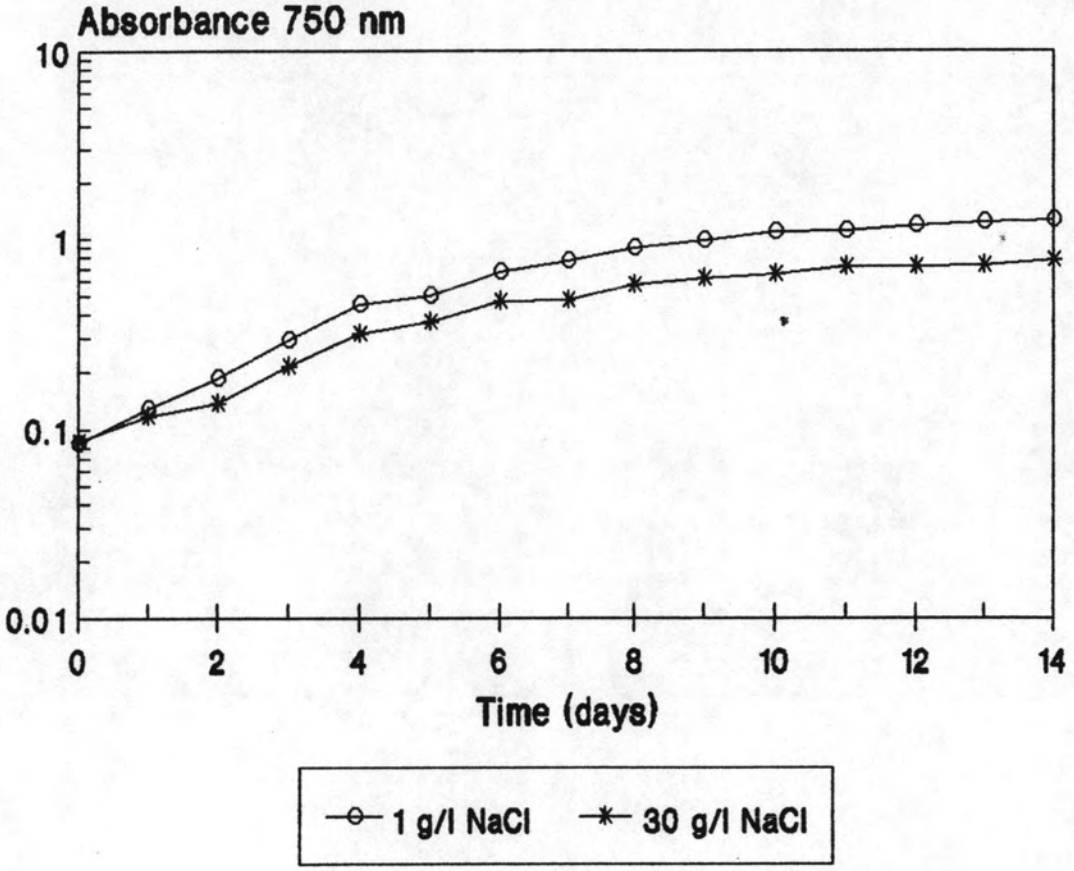

Figure 41 Growth of the outdoor cultivation Spirulina in Zarrouk medium containing 1 and 30 g/l of NaCl for 2 weeks

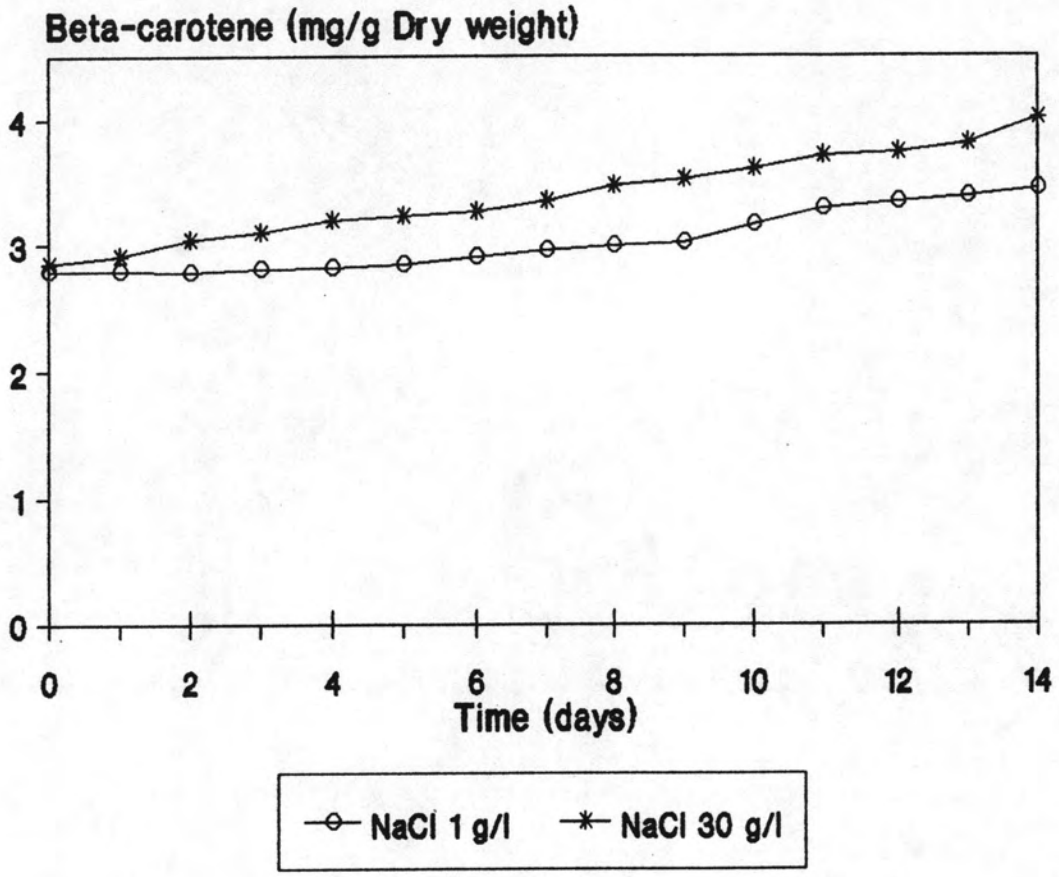

Figure 42 Beta-carotene content of Spirulina in Zarrouk medium containing 1 and 30 g/l of NaCl for 2 weeks

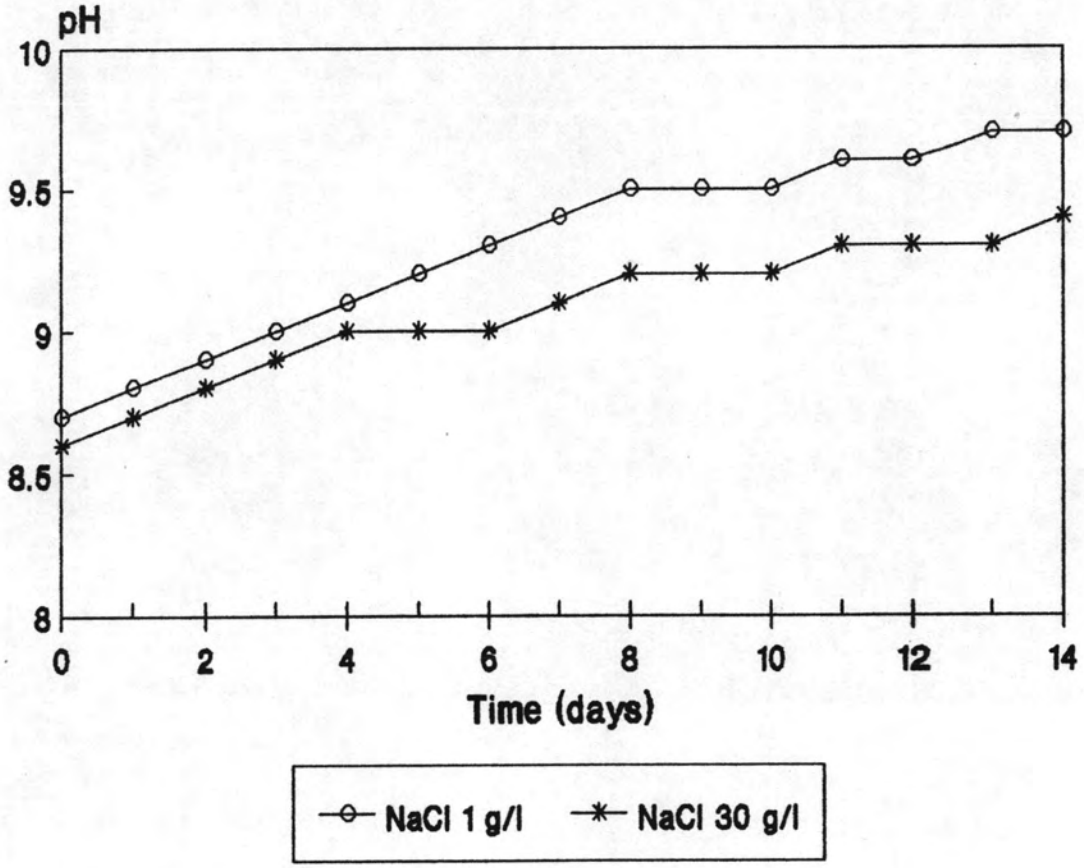

Figure 43 pH profile of medium during 14 days of the outdoor cultivation

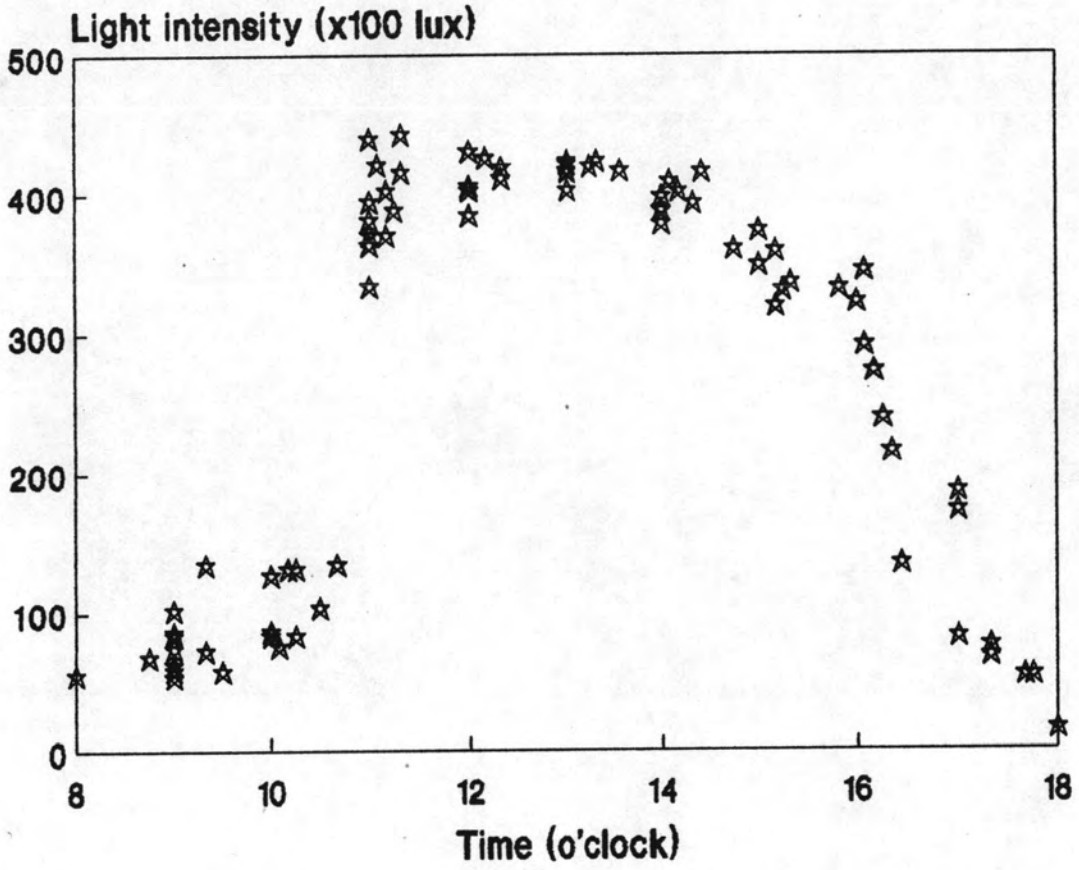

Figure 44 Light intensity between 8 a.m. and 6 p.m. during 14 days of the outdoor cultivation from April 24 to May 8, 1993

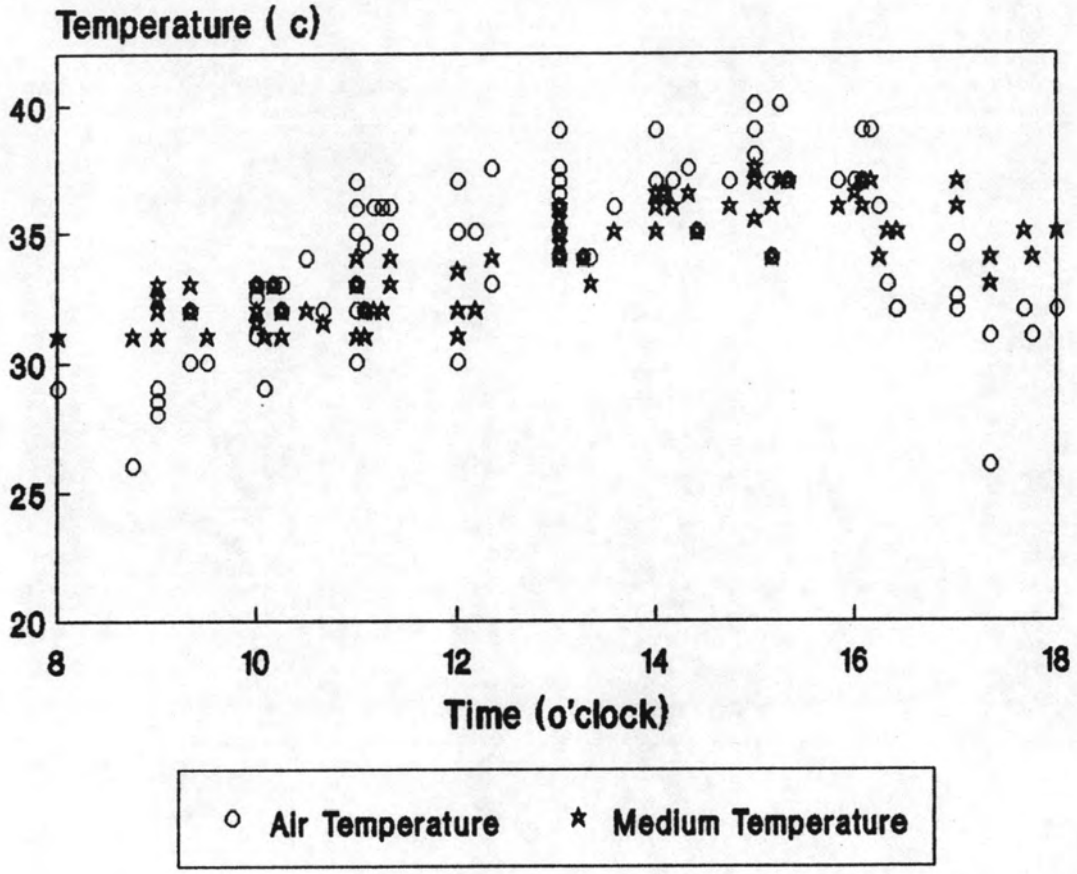

Figure 45 Comparison of air temperature and medium temperature between 8 a.m. and 6 p.m. during 14 days of the outdoor cultivation from April 24 to May 8, 1993

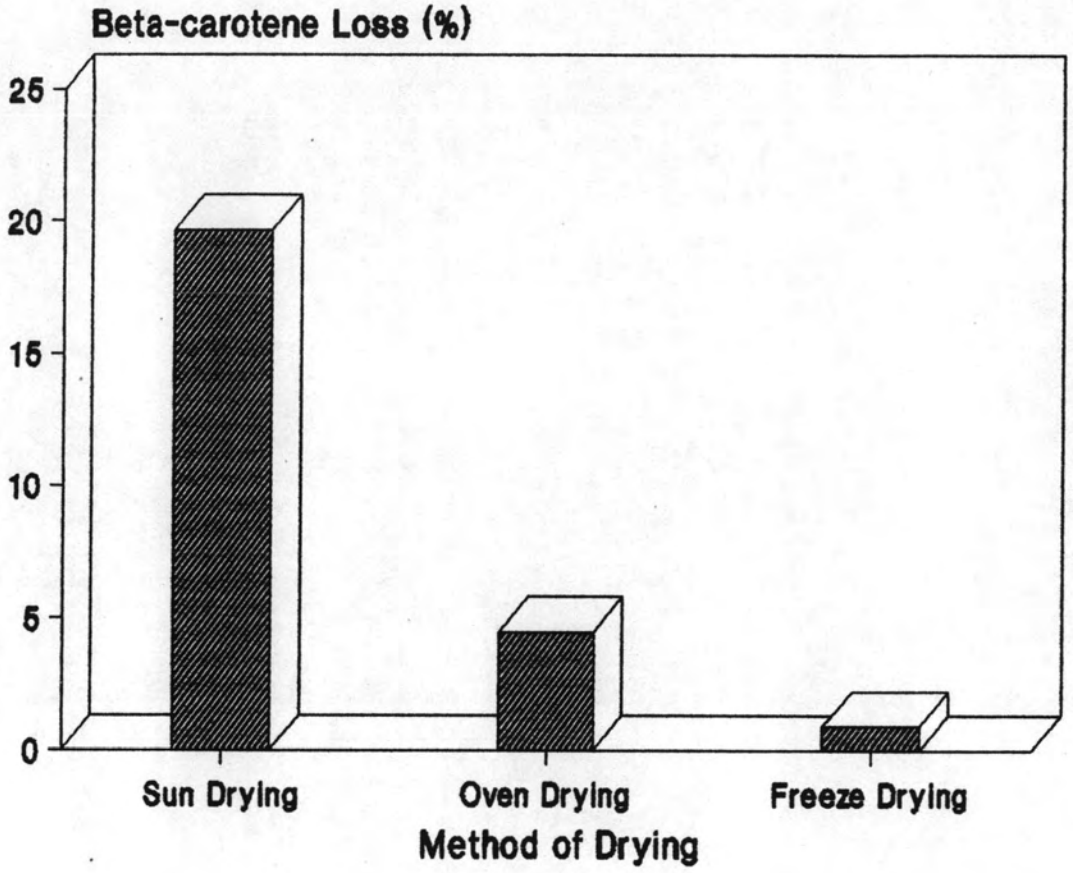

Figure 46 Effect of method of drying on beta-carotene loss

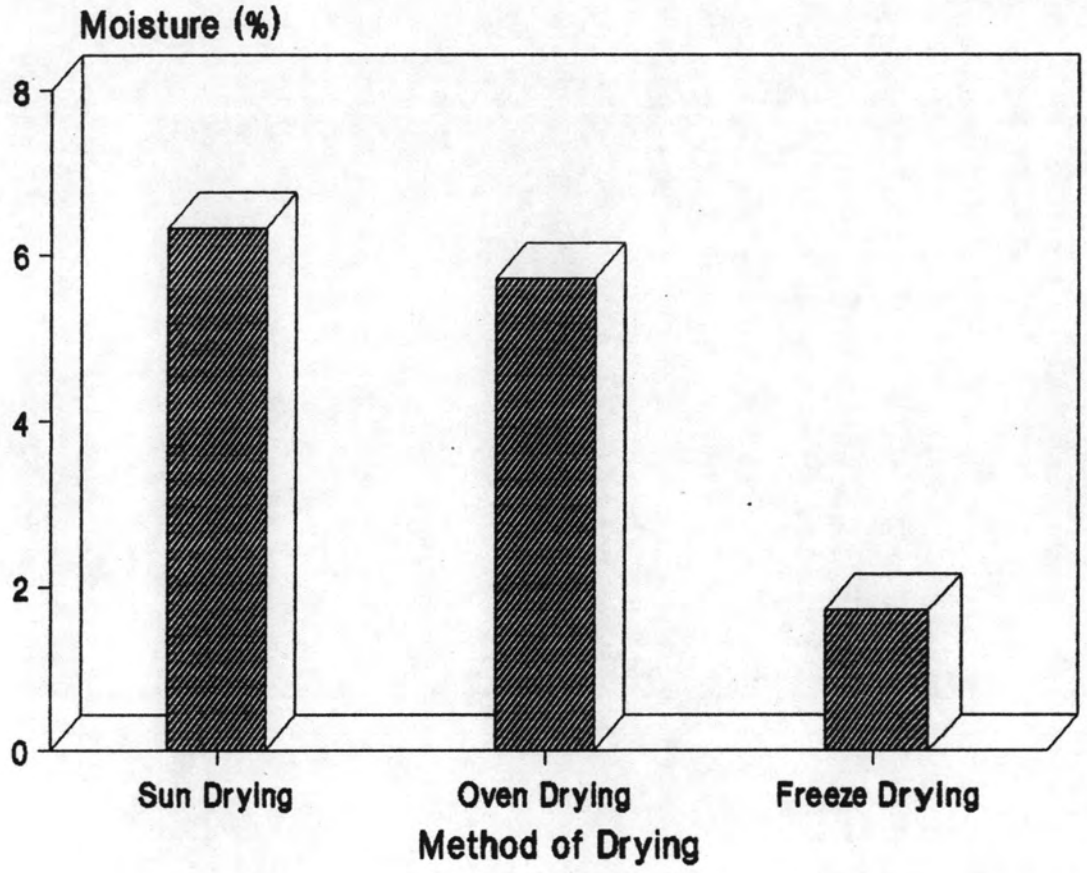

Figure 47 Moisture content of Spirulina after various methods of drying

6. Partial Purification of Beta-carotene

Carotenoids from Spirulina extracted with absolute ethanol, 50% KOH and petroleum ether were evaporated under N_2 gas and dissolved in 2 ml hexane before analysis by HPLC. HPLC chromatogram of carotenoids is shown in Figure 48. Beta-carotene peak was at retention time 11.8 minutes. Carotenoids solution was then loaded on a column containing Silica G-60. Beta-carotene eluted with 5% ether in hexane was evaporated under N_2 gas and dissolved in hexane. HPLC chromatogram of beta-carotene is shown in Figure 49 where beta-carotene was eluted at retention time 11.8 minutes. Other xanthophylls were eluted from Silica G-60 column with acetone : chloroform (1:1), evaporated under N_2 gas and dissolved in hexane. HPLC chromatogram of xanthophylls is shown in Figure 50. Absorption spectra of carotenoids, beta-carotene and xanthophylls solution are shown in Figure 51. Table 2 shows beta-carotene quantities during purification procedures.

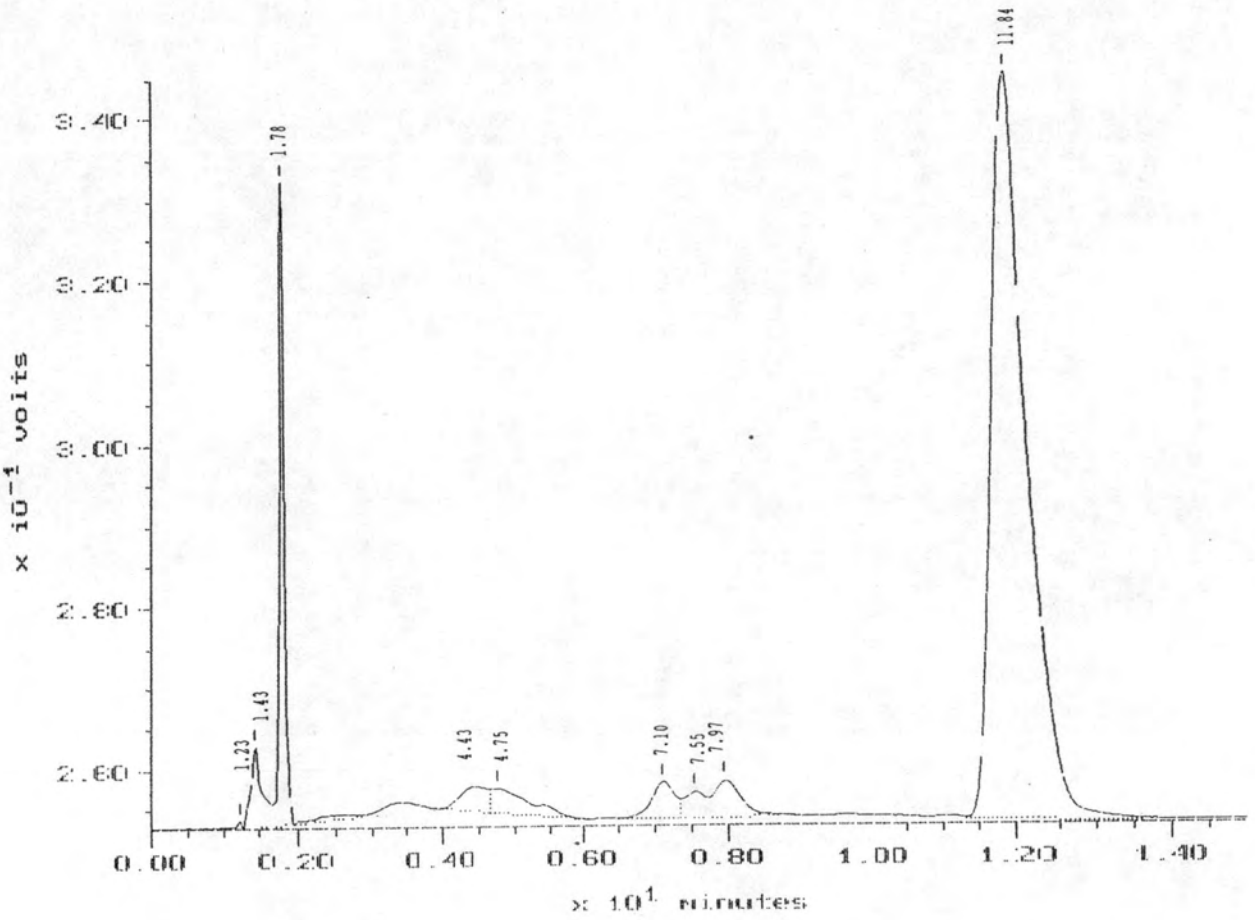

Figure 48 HPLC chromatogram of carotenoids in hexane using Novapak C_{18} column and the elution solvent contained 79.9% acetonitrile, 10% dichloromethane, 10% methanol and 0.1% milliQ water with a flow rate of 1.0 ml/min

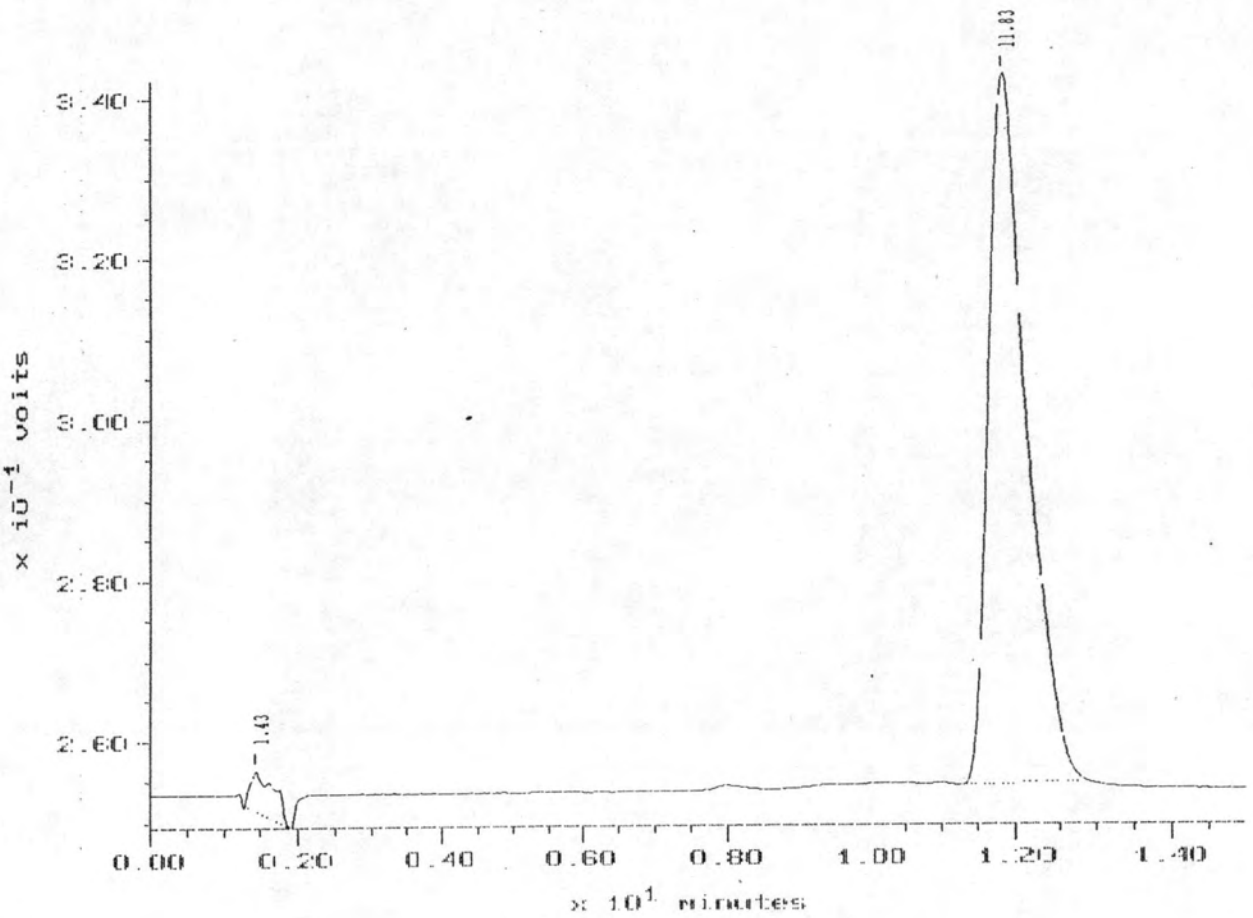

Figure 49 HPLC chromatogram of beta-carotene in hexane using Novapak C_{18} column and the elution solvent contained 79.9% acetonitrile, 10% dichloromethane, 10% methanol and 0.1% milliQ water with a flow rate of 1.0 ml/min

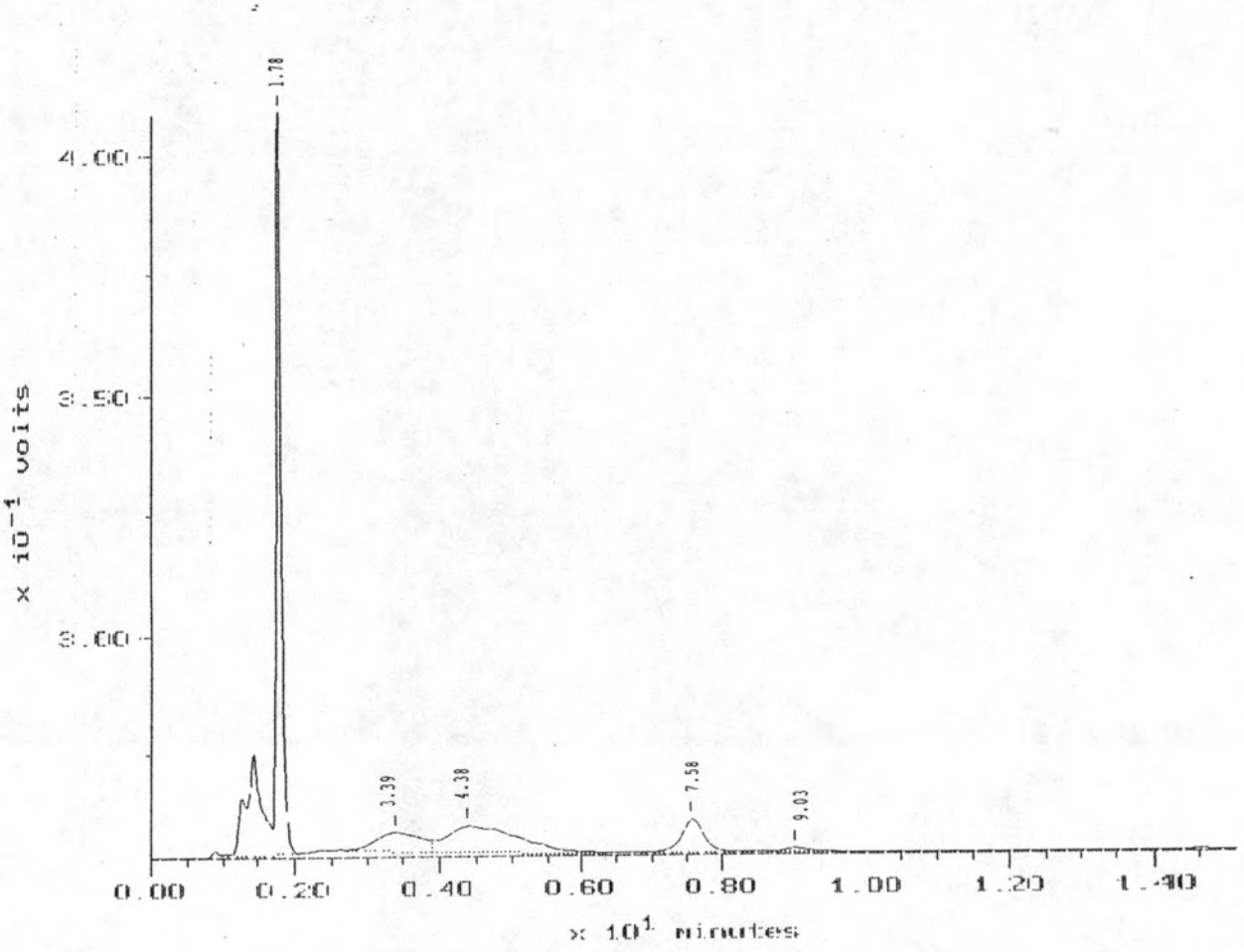

Figure 50 HPLC chromatogram of xanthophylls in hexane using Novapak C_{18} column and the elution solvent contained 79.9% acetonitrile, 10% dichloromethane, 10% methanol and 0.1% milliQ water with a flow rate of 1.0 ml/min

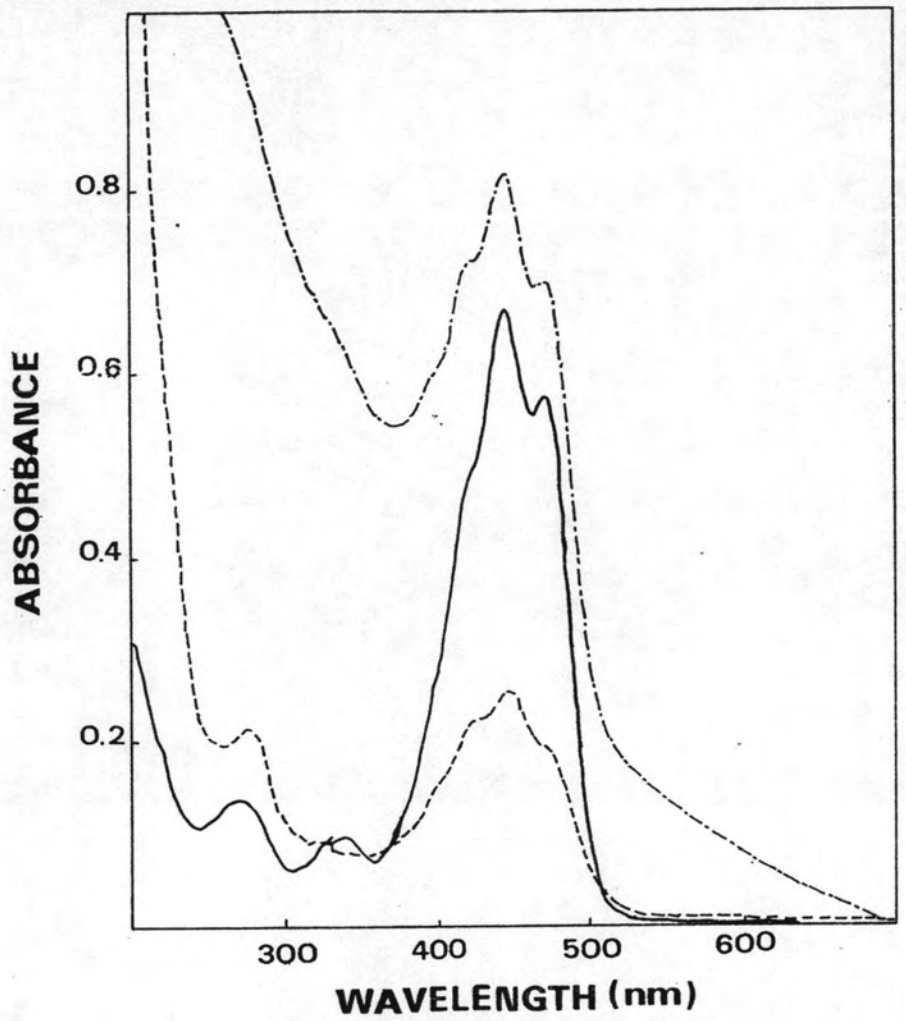

Figure 51 Absorption spectra of carotenoids, beta-carotene and xanthophylls in hexane

———— carotenoids

———— beta-carotene

———— xanthophylls

Table 2 : Efficiency of beta-carotene purification

Procedure of Purification	Beta-carotene (mg/g dry weight)	Beta-carotene Retained (%)
Before loading on Silica G-60 column	4.80	100
After elution with 5% ether in hexane	4.65	97
After elution with acetone:chloroform (1:1)	-	-

7. Effect of Storage Temperature on Beta-carotene Loss

Freeze-dried Spirulina was stored in the dark with different temperature conditions such as 30°C, 4°C, -20°C and -70°C. Storage temperature at -70°C gave the highest percent beta-carotene retained when compared to other storage temperatures over a period of 8 weeks (Figure 52). Fifty percent of beta-carotene was retained at storage temperature 30°C after 6 weeks and at storage temperature 4°C after 8 weeks (Figure 52).

Cells stored in the presence of 10% sodium metabisulphite gave higher percent beta-carotene retained than that in the presence of 1 % sodium metabisulphite and that without sodium metabisulphite (Figure 53).

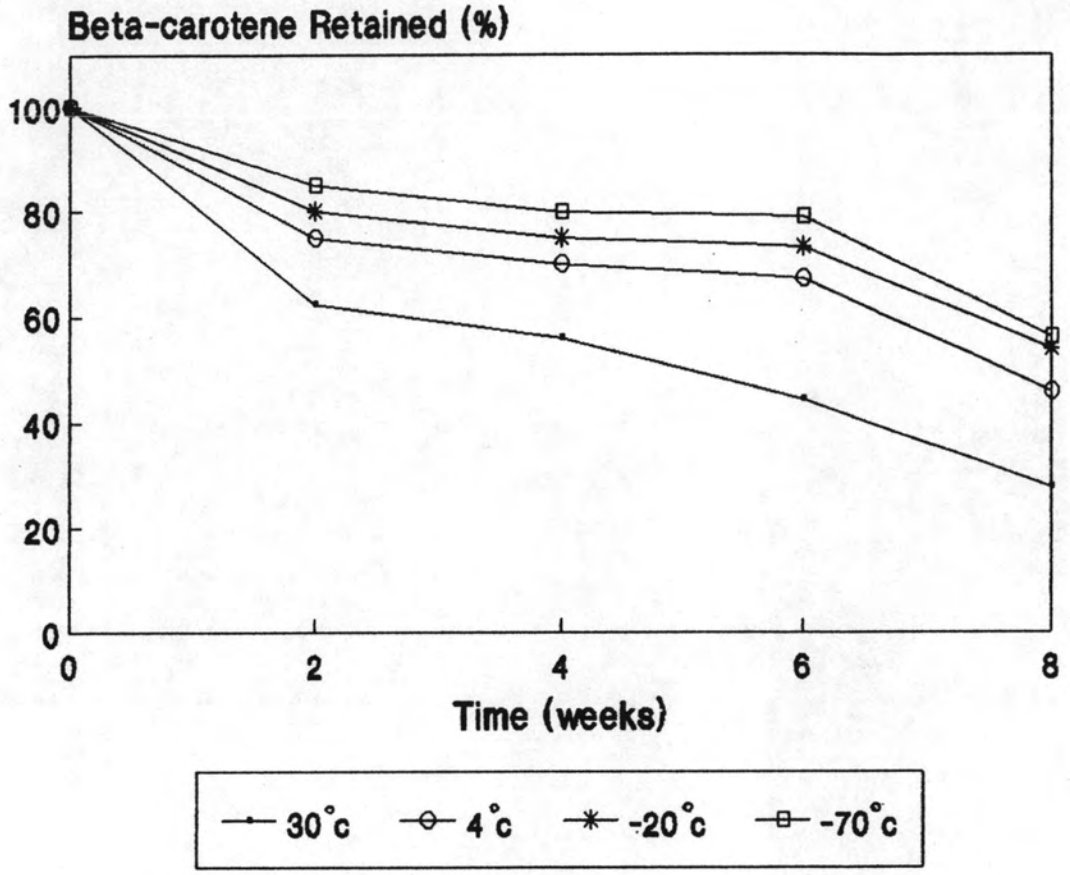

Figure 52 Effect of storage temperature on beta-carotene loss

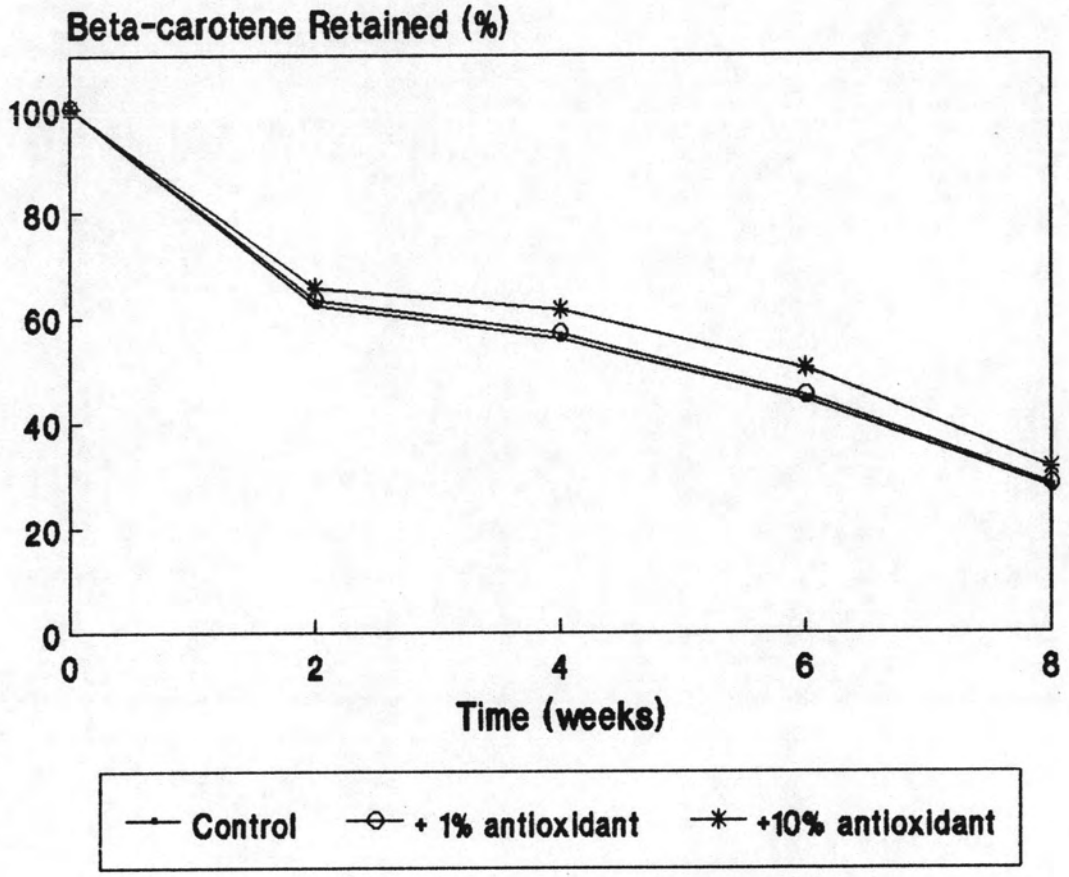

Figure 53 Effect of antioxidant on beta-carotene loss