

CHAPTER III

RESULTS

Field survey and isolation of Dunaliella spp. from salt ponds.

Water samples were collected from salt pond in different areas. Locations and characteristics of water samples are listed in Table 1. Dunaliella spp. were found abundantly in high saline water samples from Samut Songkhram Province. D. salina was always a dominant species in water samples that salinity was higher than 200 ppt.

D. viridis was found coexistence with D. salina in high saline water samples. The isolation of D. viridis could be done simply by ESM medium provided from water sample which contained algal cells. However, D. salina was not successfully isolated because it could not grow within the enriched medium which contained salinity equal to its origin and therefore discarded. ESM medium with different salinity (255, 180, 105 and 30 ppt) were prepared for the cell isolation by adding of low salinity ESM medium (30 ppt.) into high salinity ESM medium at different proportions. The results showed that D. salina could survived and grew better in the lower salinity isolated medium than the original high salinity medium (Table 2.).

Table 1. Location and characteristics of water samples.

Location	Date	Salinity (ppt.)	pH	Present of <u>Dunaliella</u> spp. or dominant algal species	Remarks:
Shrimp farm, Angsila Chon Buri	8 Nov 90	30	-	-	small evaporation pond
Salt pond, Chon Buri	8 Nov 90	45	-	-	Artemia culture pond
Salt pond, Bang Pakong Chachoengsao	8 Nov 90	74	-	<u>Chroomonas</u> sp. (Cryptophyta)	
Salt pond, Samut Songkhram	26 Nov 90	35	-	-	low salinity
Salt pond, Chon Buri	26 Nov 90	40	-	-	Evaporation pond of Artemia culture pond
Salt pond, Samut Songkhram	22 Dec 90	42	-	-	
	22 Dec 90	96	-	<u>Dunaliella viridis</u>	
	26 Jan 91	90	-	Protozoa, flagellate algae	
	26 Jan 91	150	-	<u>D. salina</u> , <u>D. viridis</u>	
	26 Jan 91	255	-	<u>D. salina</u>	
Salt pond, Samut Songkhram	26 Jan 91	>300	-	<u>D. salina</u>	
	5 Feb 91	88	8.1	-	
	5 Feb 91	184	7.6	<u>D. salina</u> , <u>D. viridis</u>	
	5 Feb 91	244	7.49	<u>D. salina</u>	
	5 Feb 91	260	7.46	<u>D. salina</u>	
Salt pond, Samut Songkhram	5 Feb 91	270	7.45	<u>D. salina</u>	
	12 Jan 92	70	-	-	
	12 Jan 92	120	-	Flagellate algae (Cryptophyta)	
	12 Jan 92	230	-	<u>D. salina</u> (4×10^5 cell/ml)	with lower amount of <u>D. viridis</u>
	12 Jan 92	245	-	<u>D. salina</u> (1.1×10^4 cell/ml)	
12 Jan 92	250	-	<u>D. salina</u> (1×10^3 cell/ml)		
Salt pond, Chanthaburi	16 Jan 92	82	-	Flagellate algae, Dinoflagellate	
	16 Jan 92	290	-	-	
Salt pond, Samut Songkhram	18 Feb 92	90	8.66	<u>Chroomonas</u> sp. (4.7×10^4 cell/ml)	
	18 Feb 92	120	8.17	<u>D. salina</u> (3.8×10^3 cell/ml)	
	18 Feb 92	212	7.66	<u>D. salina</u> (8.8×10^3 cell/ml)	
	18 Feb 92	300	7.16	<u>D. salina</u> (1.5×10^4 cell/ml)	
	18 Feb 92	320	6.81	<u>D. salina</u> (3.8×10^3 cell/ml)	

Table 2. Percent survival of the isolated D. salina in different salinity of ESM medium.

Salinity (ppt) (Initial=255)	Number of isolation (cell)	Number of survival (cell)	Survival (%)
255	12	0	0
180	12	0	0
105	12	10	83.3
30	12	2	16.6

Clones of Dunaliella spp. that isolated from high salinity water samples were identified by the key as described by Butcher (1959). Two species of Dunaliella that found in water samples were D. salina and D. viridis. The D. salina could be easily distinguished from D. viridis by its size, color and swimming behavior. The former was larger in size (more than 15 μ), orange to orange-red color and slowly migrated throughout water column while the later was smaller, greenish color even in high salinity water and active swimming. Pictures of orange and green stage of D. salina and D. viridis are showed in Figure. 7,8 and 9 respectively. For this research, 10 clones of D. salina, 24 clones of D. viridis were isolated and then cultured as a unialgal culture (Table 2). Thereafter, six clones of D. salina were selected for Experiment 2.

In addition, some environmental factors in the salt pond at Samut Songkhram were investigated. It was found that nitrate and phosphate concentration tend to rise with increasing salinity. On the other hand, pH decreased significantly with decreasing salinity. The correlation between pH and salinity was $\text{pH} = 9.17 \times 0.007 \text{ Salinity}$.



Figure 7. Photomicrograph of orange *D. salina* cultured in 20%NaCl J/1 medium, 20,000 lux light intensity.



Figure 8. Greenish stage of *D. salina* cultured in 20% NaCl J/1 medium, 4,000 lux light intensity.



Figure 9. D. viridis cultured in 20% NaCl J/1 medium at 15,000 lux light intensity.

Table 3. Some environmental parameters in salt ponds at Samut Songkhram Province.

Pond No	Salinity (ppt.)	pH	NO ₃ (μg-at N/l) (±SE)	PO ₄ (μg-at P/l) (±SE)	<u>D. salina</u> x10 ⁴ cell/ml
1	320	6.81	1.67±0.02	0.028±0.002	0.39
2	300	7.16	0.89±0.04	0.011±0.007	1.50
3	212	7.66	0.41±0.02	0.012±0.002	0.89
4	120	8.17	0.18±0.07	0.012±0.000	0.39
5	90	8.66	0.25±0.07	0.012±0.005	-

(n=2)

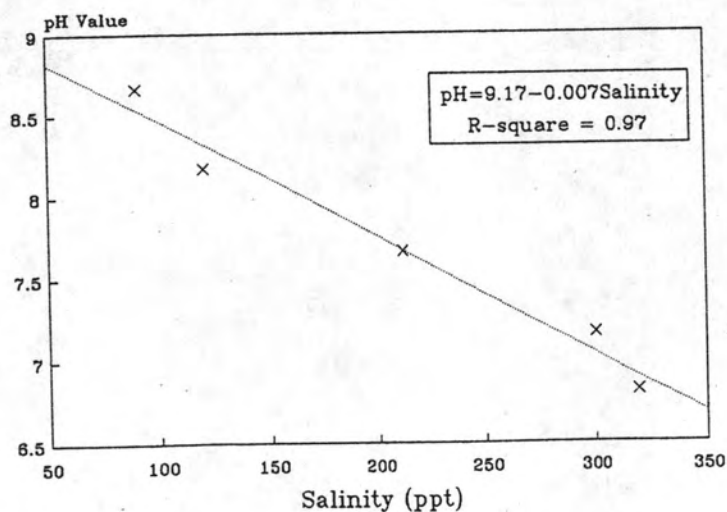


Figure 10. Correlation between pH and salinity in salt ponds at Samut Songkhram Province

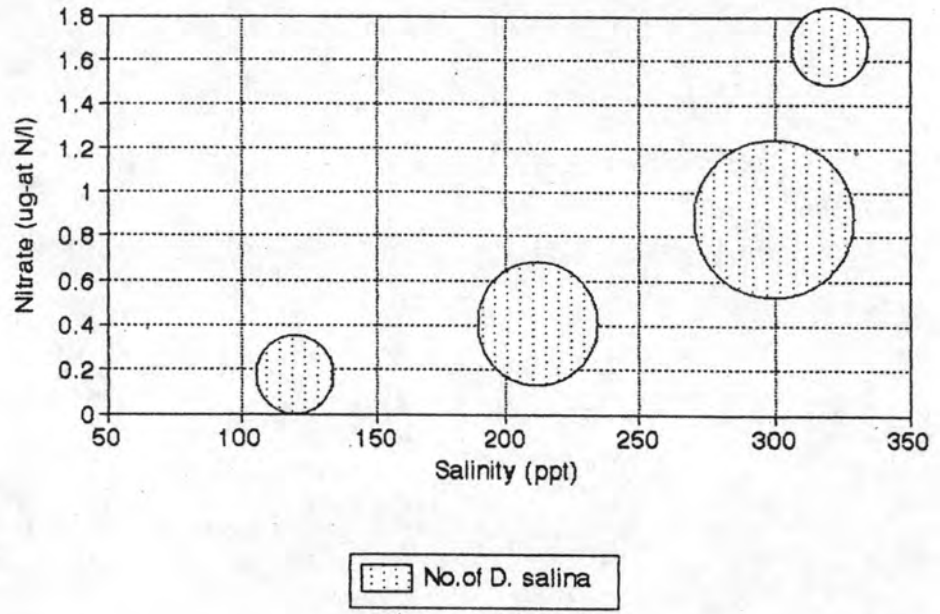


Figure 11. Bubble graph represent correlation between salinity, nitrate concentration and the number of D. salina in salt evaporation ponds.

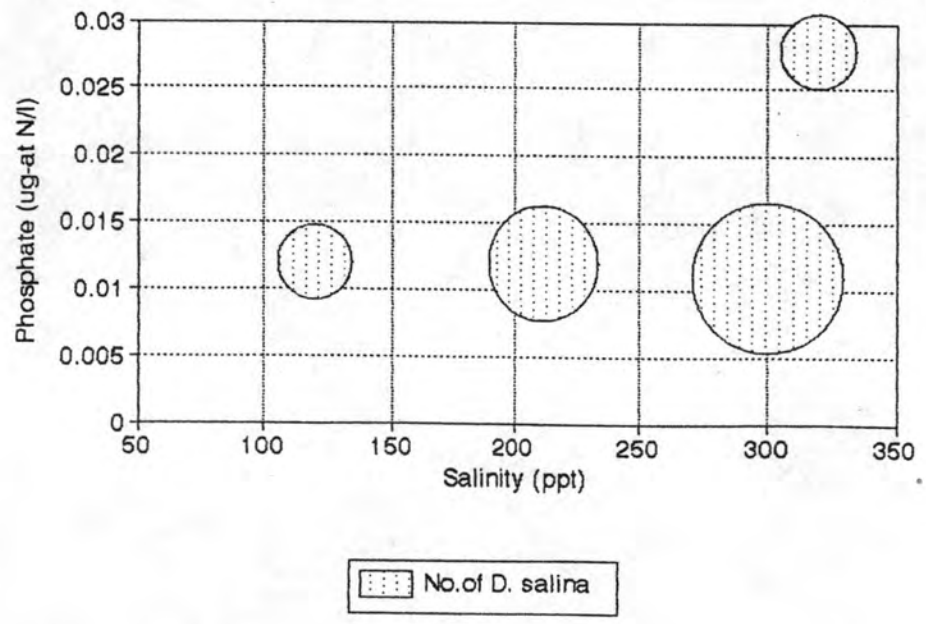


Figure 12. Correlation between salinity, phosphate concentration and number of D. salina in salt evaporation ponds.

selection of D. salina clone yielded the highest carotenoid content.

Six clones of D. salina, DS91001, DS91002, DS91007, DS91008, DS91009 and DS91010 isolated from Samut Songkhram salt pond were cultured in J/1 medium at 20,000 lux light intensity. As shown in Figures 13, 15 and 17, the growth rates of the six clones grown within same salinity were different. The growth rate decreased with increasing salinity. At the salinity of 10% NaCl, clone number DS91009 had highest growth rate (0.361 d^{-1}) while at salinity 20% NaCl the DS91008 had the highest growth rate (0.254 d^{-1}). At 30% NaCl, clone number DS91010 had the highest growth rate (0.123 d^{-1}).

Nevertheless, carotenoid content analysis showed that the clone number DS91008 had highest carotenoid content in every salt concentration (Figure 14, 16 and 18 respectively). The maximum carotenoid content of DS91008 clone at 30% NaCl was 80.4 pg/cell. With this respect, Dunaliella salina clone number DS91008 was selected for the further experiments.

Effect of light intensity, nutrient concentration and initial pH on growth and carotenoid content of D. salina.

This experiment was performed to determine whether various culture conditions could effect growth and carotenoid concentration of D. salina.

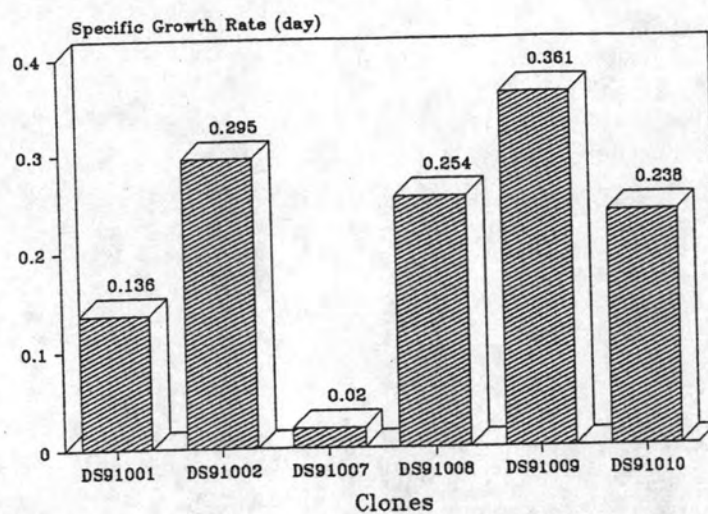


Figure 13. Specific growth rate of *D. salina* clones cultured in J/1 medium at salinity 10% NaCl.

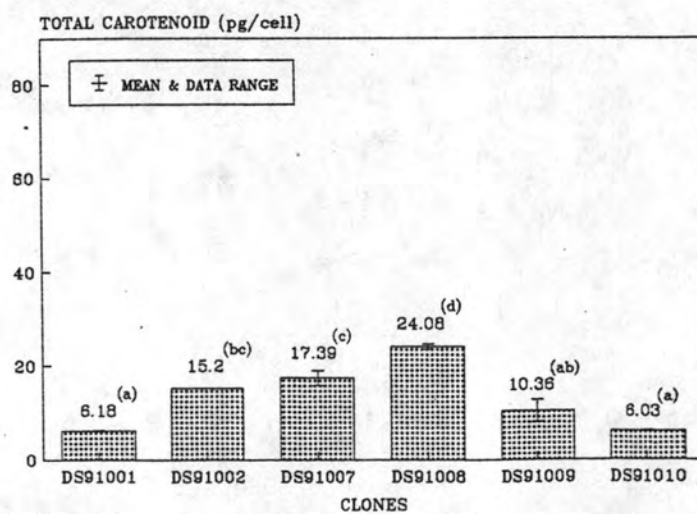


Figure 14. Carotenoid content of *D. salina* clones cultured in J/1 medium at salinity 10% NaCl. a, b, c and d denoted significant difference in mean ($P < 0.05$).

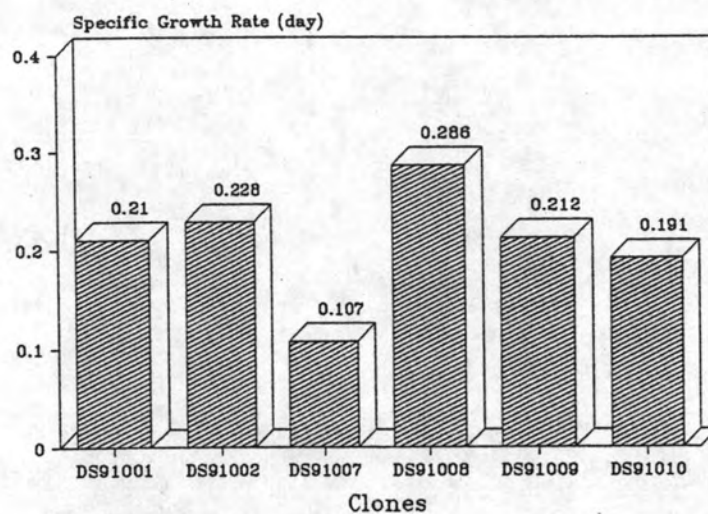


Figure 15. Specific growth rate of *D. salina* clones cultured in J/1 medium at salinity 20% NaCl.

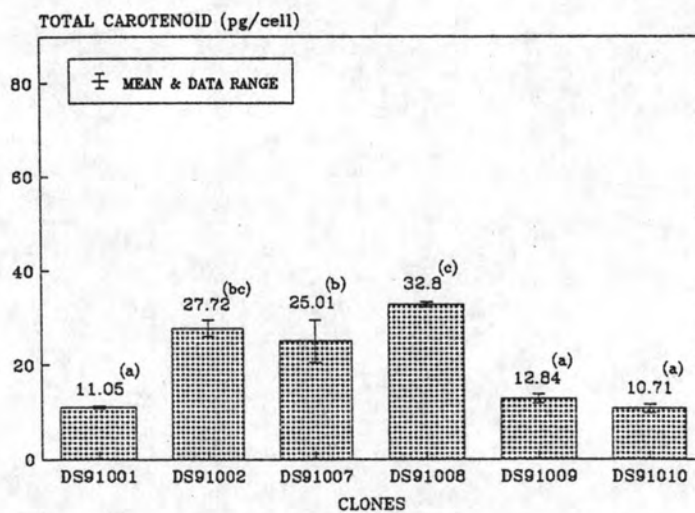


Figure 16. Carotenoid content of *D. salina* clones cultured in J/1 medium at salinity 20% NaCl. a, b, and c denoted significant difference in mean ($P < 0.05$).

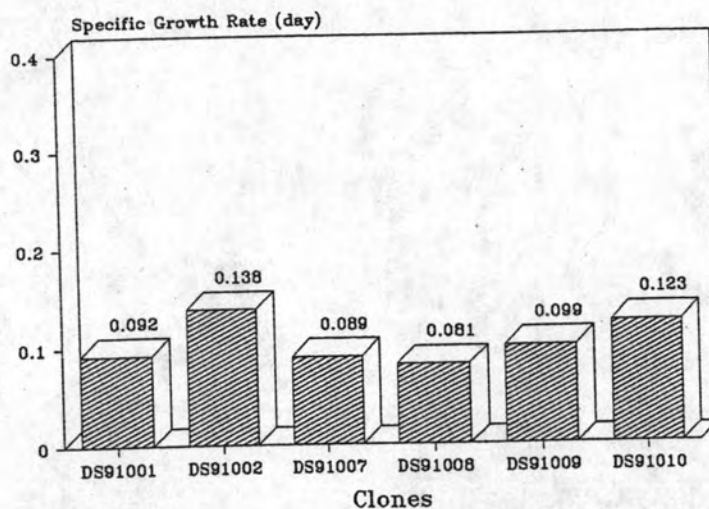


Figure 17. Specific growth rate of *D. salina* clones cultured in J/1 medium at salinity 30% NaCl.

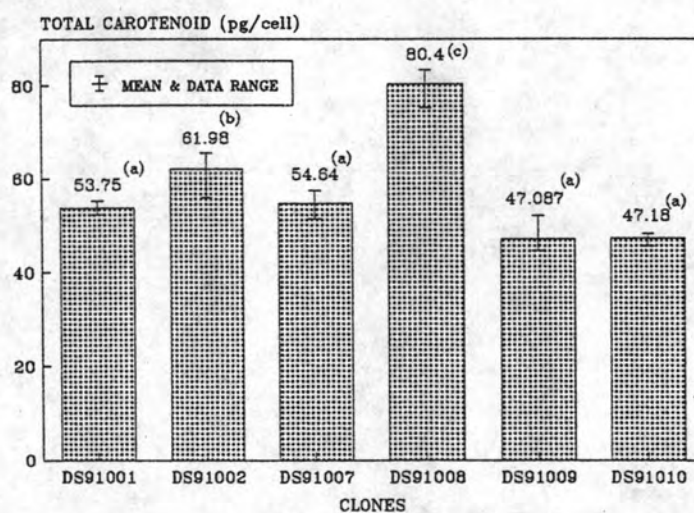


Figure 18. Carotenoid content of *D. salina* clones cultured in J/1 medium at salinity 30% NaCl.

a, b, and c denoted significant difference in mean ($P < 0.05$).

a) Effect of light intensity.

D. salina was cultured in three light intensities: 5,000, 10,000 and 15,000 lux (70, 136, 203 $\mu\text{E}/\text{m}^2/\text{s}$ respectively) in J/1 medium at 20% NaCl. The result showed that specific growth rate of each light intensity was not significantly different (Figure 19). However, as shown in Figure 20, total carotenoid content was significantly higher in high light intensity than the lower one. The colour of algal cell changed from green to orange at light intensity over than 10,000 lux.

Absorption spectra of D. salina which grew in different light intensities are show in Figure 21. The orange cells of D. salina cultured at 15,000 lux had higher peak of carotenoid (400-500 nm) while chlorophyll peak (>600 nm) was lower than D. salina which grew at 10,000 lux. On the other hand, green D. salina had different spectra pattern of β -carotene (452 nm) from the other two higher light intensities.

The HPLC analysis showed that β -carotene was major pigment of algal carotenoid (Figure 20). The overlay HPLC chromatogram is shown in Figure 22 and HPLC printout chromatograms are shown in Appendix 8. It was found that β -carotene peak area at the retention time about 13 minute increased with the increasing light intensity. At 5,000 lux (green algal cell) about 72% of carotenoid was β -carotene while β -carotene proportion increased up to 98% in 15,000 lux (orange stage). The β -carotene isomer separation analysis showed that β -carotene of D. salina was consisted of cis and trans isomer with approximately equal amount (Figure 23, 24).

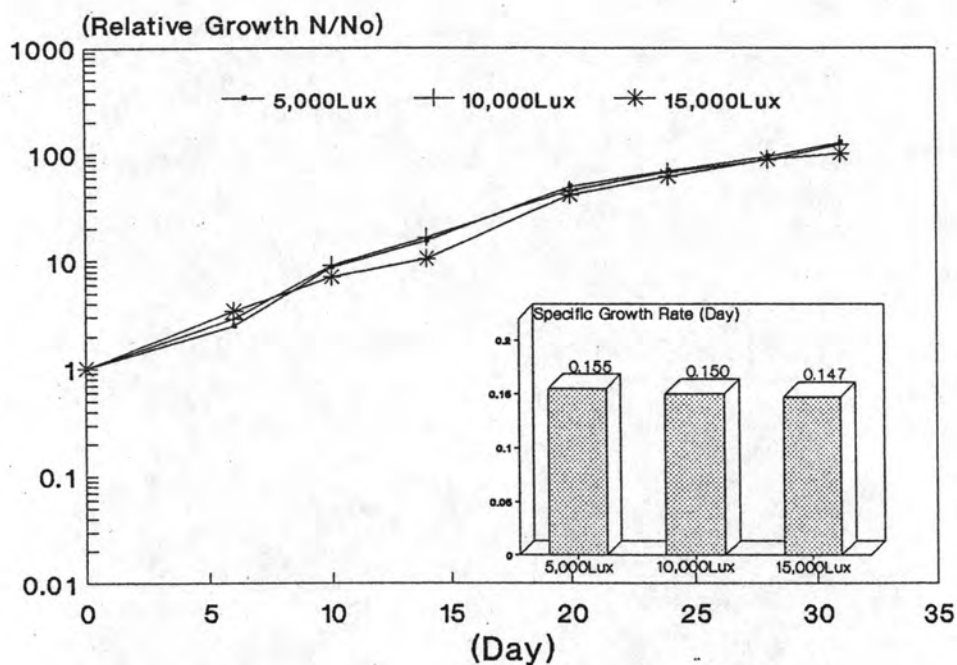


Figure 19. Specific growth rate of *D. salina* cultured in three light intensities.

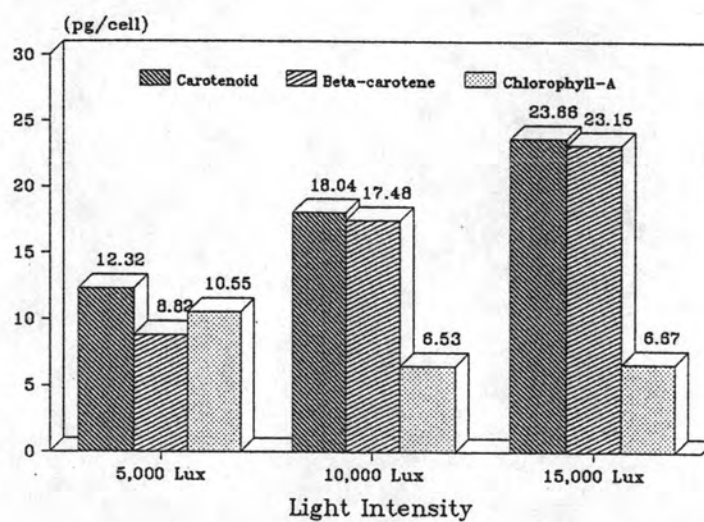


Figure 20. Carotenoid, β -carotene and Chlorophyll-A content of *D. salina* cultured in three light intensities.

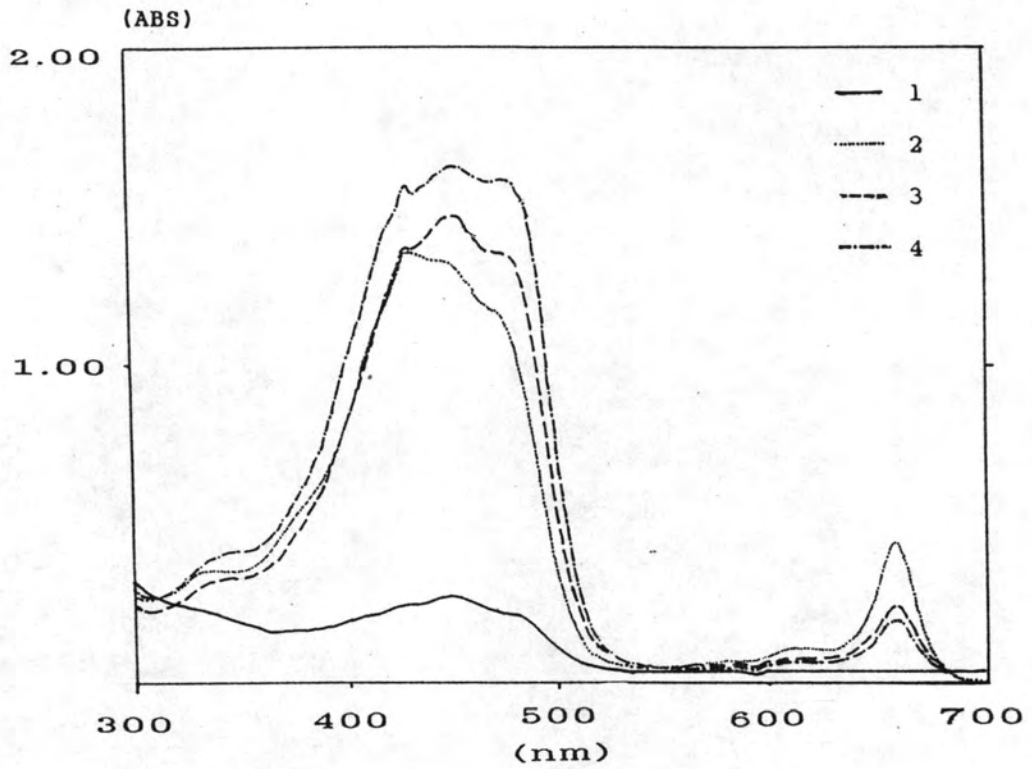


Figure 21. Absorption spectra of *D. salina* cultured in different light intensities.

- (1) Standard β -carotene
- (2) *D. salina* cultured in 5,000 lux
- (3) *D. salina* cultured in 10,000 lux
- (4) *D. salina* cultured in 15,000 lux

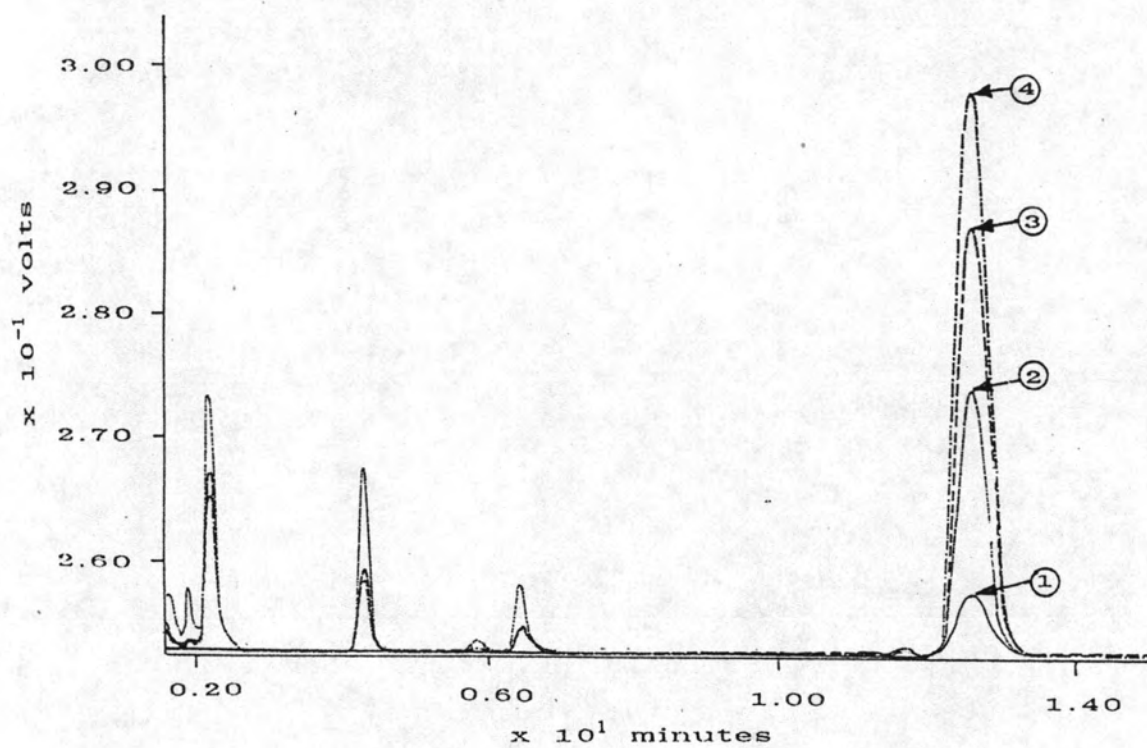


Figure 22. HPLC overlay chromatogram of *D. salina*

- (1) Standard β -carotene
- (2) *D. salina* cultured in 5,000 lux
- (3) *D. salina* cultured in 10,000 lux
- (4) *D. salina* cultured in 15,000 lux

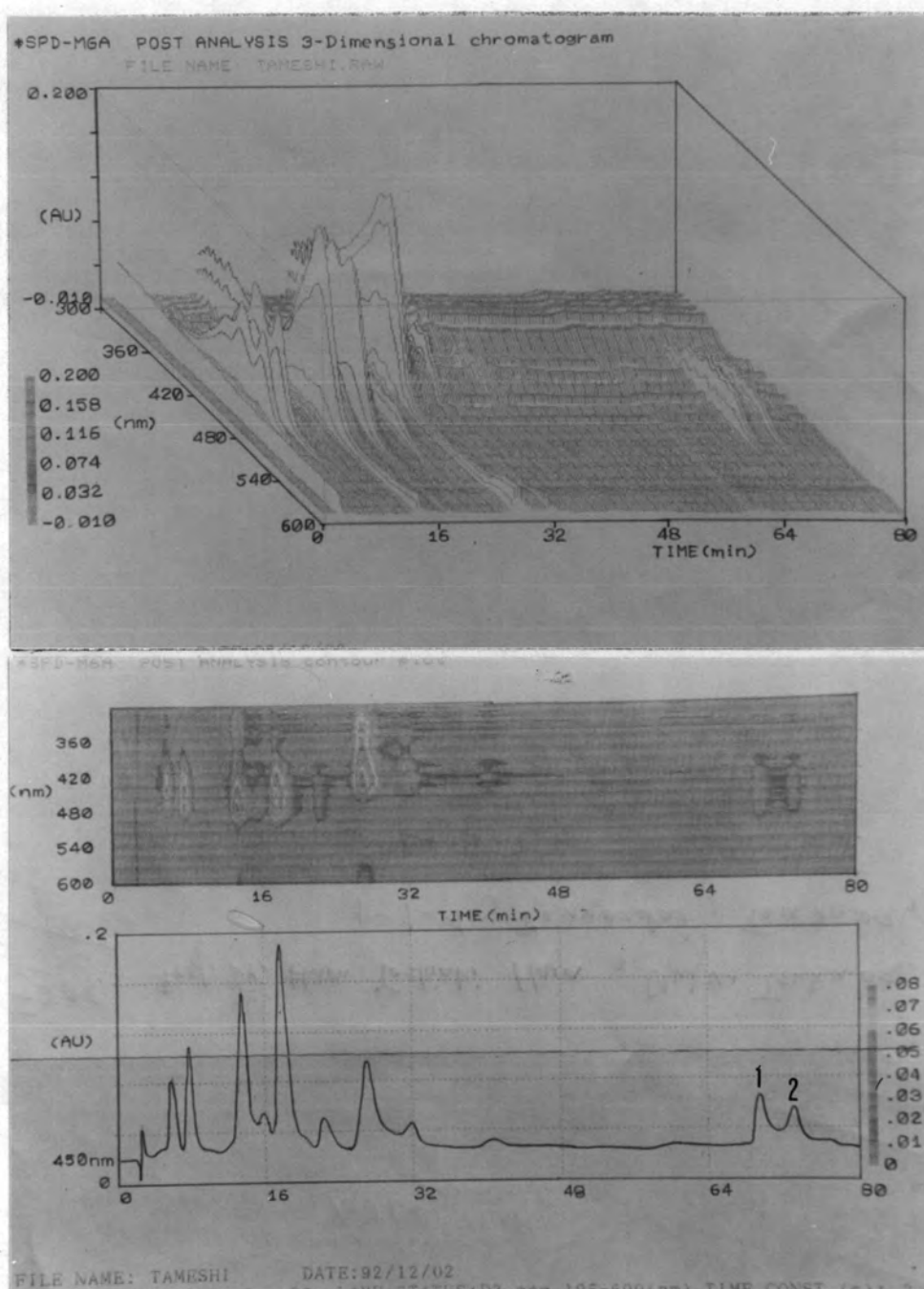


Figure 23. (a) 3-dimensional HPLC chromatogram of β -carotene isomer separation analysis of greenish stage D. salina
 (b) HPLC chromatogram and wavelength scanning chromatogram of greenish stage D. salina.
 [1] all-trans β -carotene
 [2] 9-cis β -carotene

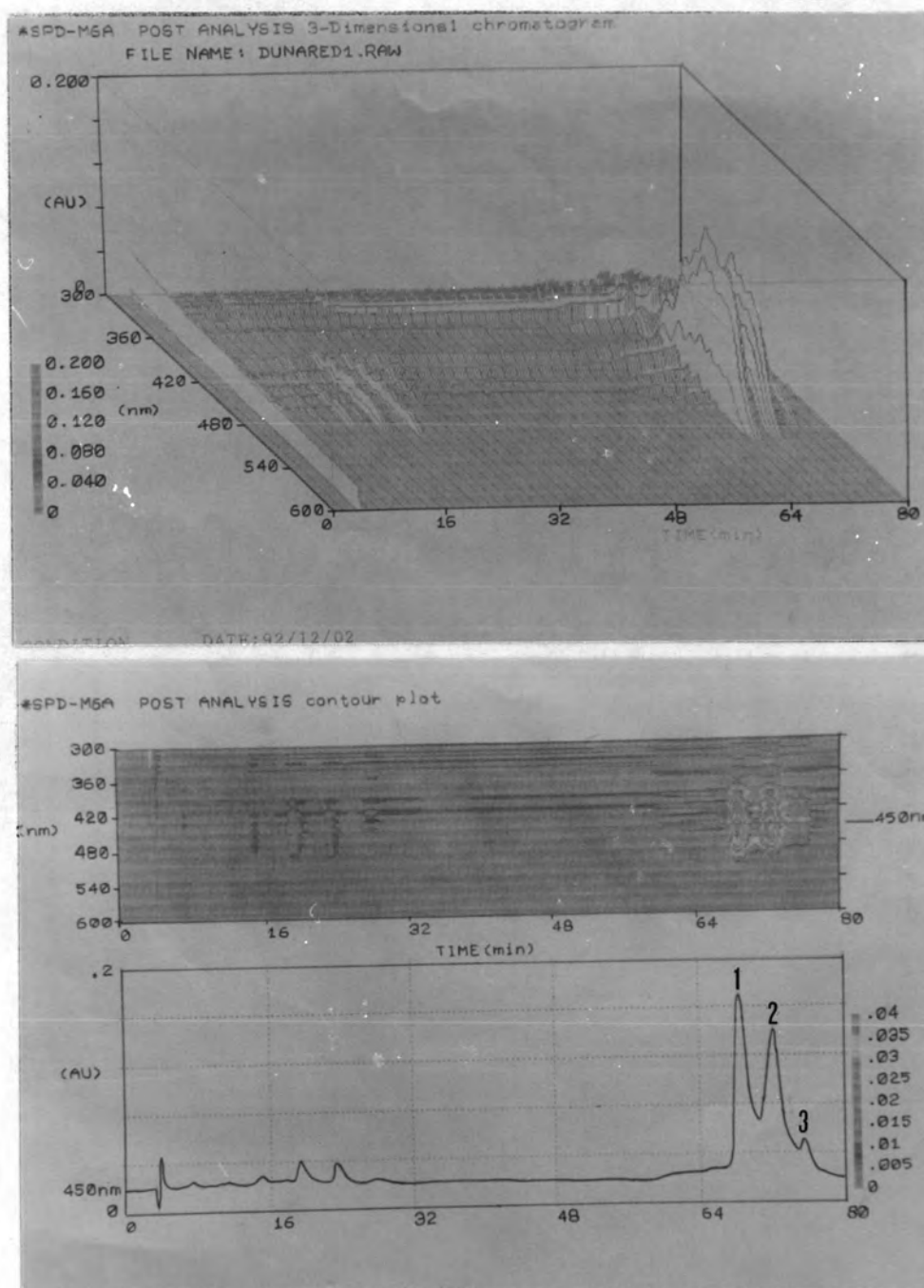


Figure 24. (a) 3-dimensional HPLC chromatogram of β -carotene isomer separation analysis of orange stage D. salina

(b) HPLC chromatogram and wavelength scanning chromatogram of orange stage D. salina.

[1] All-trans β -carotene

[2] 9-cis β -carotene [3] 13-cis β -carotene

D. salina which cultured in the high light intensity (high carotenoid content) was significant higher in both cell length and width. The discriminant statistical analysis showed that D. salina which cultured at 3,000 lux (green stage) had significant smaller size and could be separated from D. salina which cultured at 5,000, 10,000 and 15,000 lux by cell dimensions (Figure 25).

b) Effect of nitrate concentrations.

The specific growth rate of D. salina at various KNO_3 concentration in J/1 medium are shown in Figure 26. The data indicate that the specific growth rate rapidly decreased with decreasing nitrate concentration from 200% KNO_3 to 1% KNO_3 .

Nevertheless, as shown in Figure 27, total carotenoid content in logarithmic growth phase increased at 1% and 10% KNO_3 concentration. The maximum carotenoid content appeared at 10% KNO_3 concentration (137.2 pg/cell or 12% β -carotene/AFDW). Whereas at the stationary growth phase there were slight difference in total carotenoid contents among five nitrate concentrations. Similarly, the chlorophyll-a content increased with the decreasing growth rate. As shown in Figure 28, chlorophyll-a increased with the same pattern of carotenoid. The maximum chlorophyll-a content was at 10% KNO_3 in logarithmic growth phase. However, the carotenoid to chlorophyll-a ratio in this experiment was not significantly different (Figure 29).

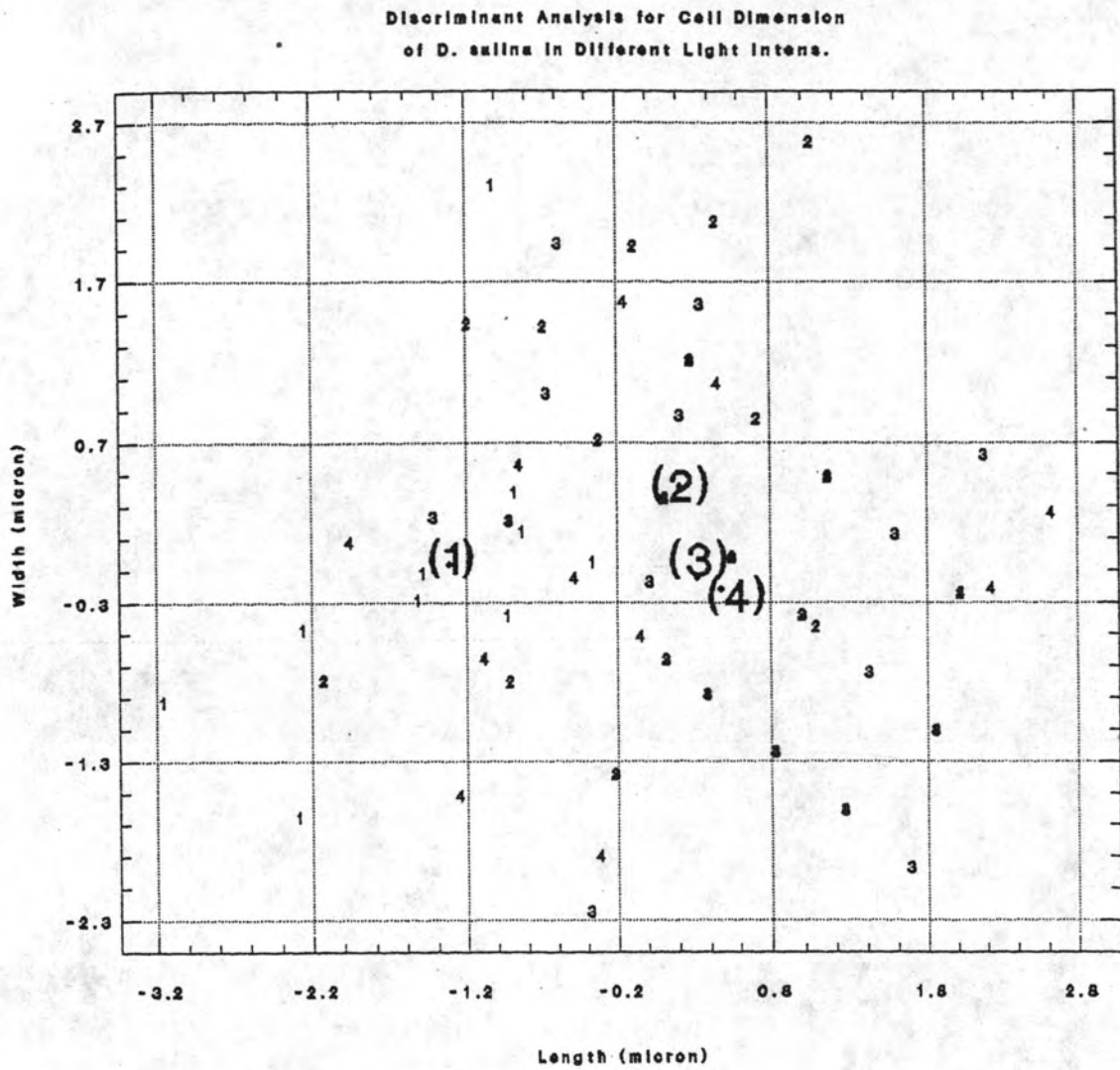


Figure 25. Discriminant Analysis for cell length and width of *D. salina* cultured in 4 light intensities.

- (1) 3,000 lux
- (2) 5,000 lux
- (3) 10,000 lux
- (4) 15,000 lux

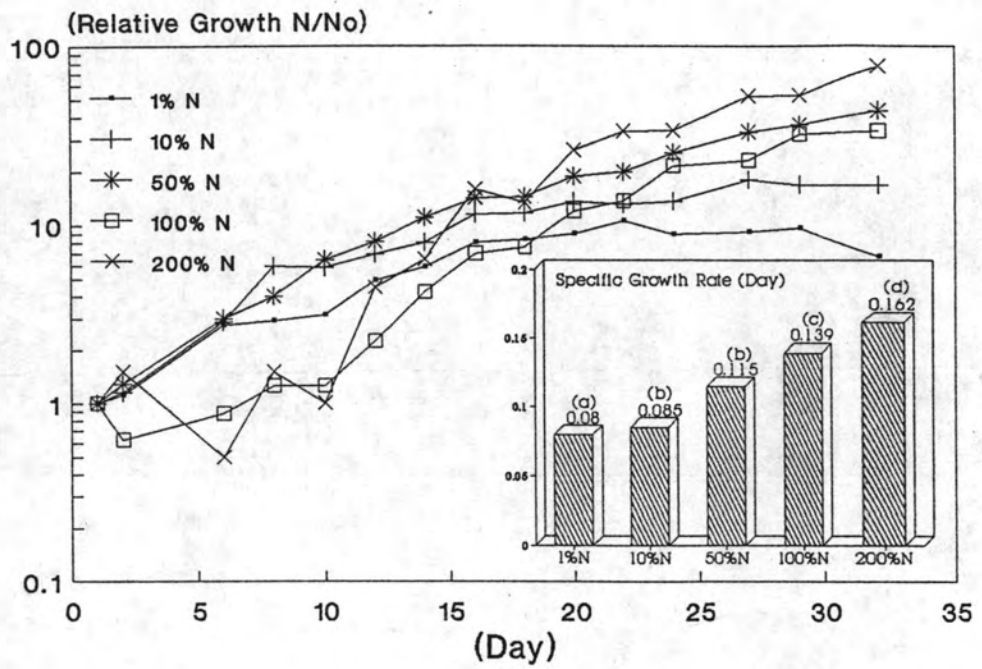


Figure 26. Effect of KNO₃ concentration in J/1 medium on specific growth rate of *D. salina*.

a, b, c, and d denoted significant difference in growth rate ($P < 0.05$).

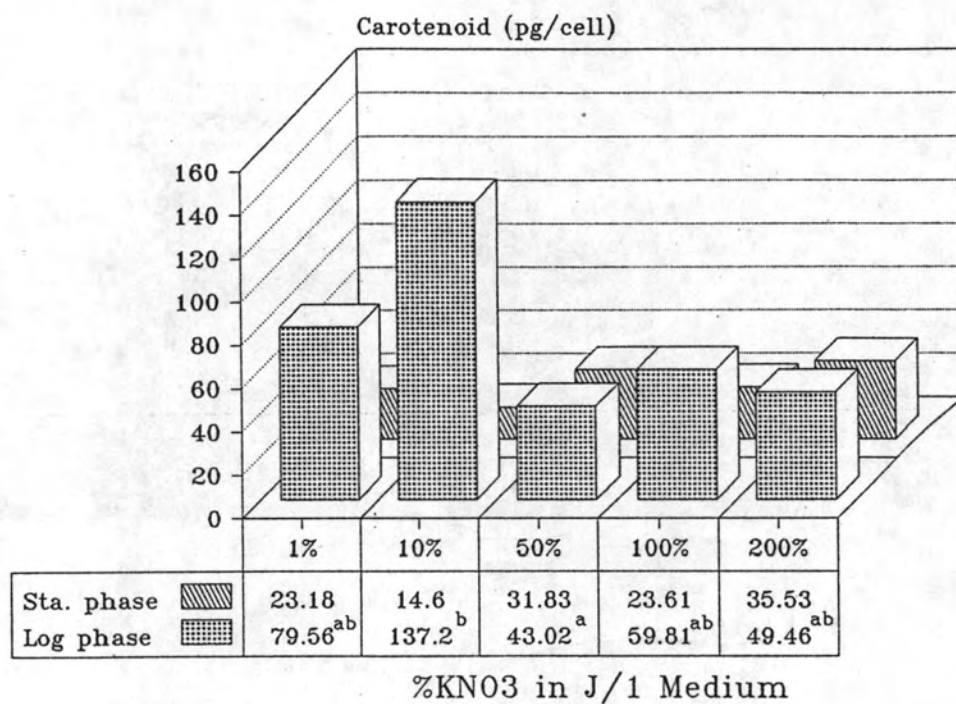


Figure 27. Effect of KNO₃ concentration on carotenoid content of D. salina.

a and b denoted significant difference in mean (P<0.05).

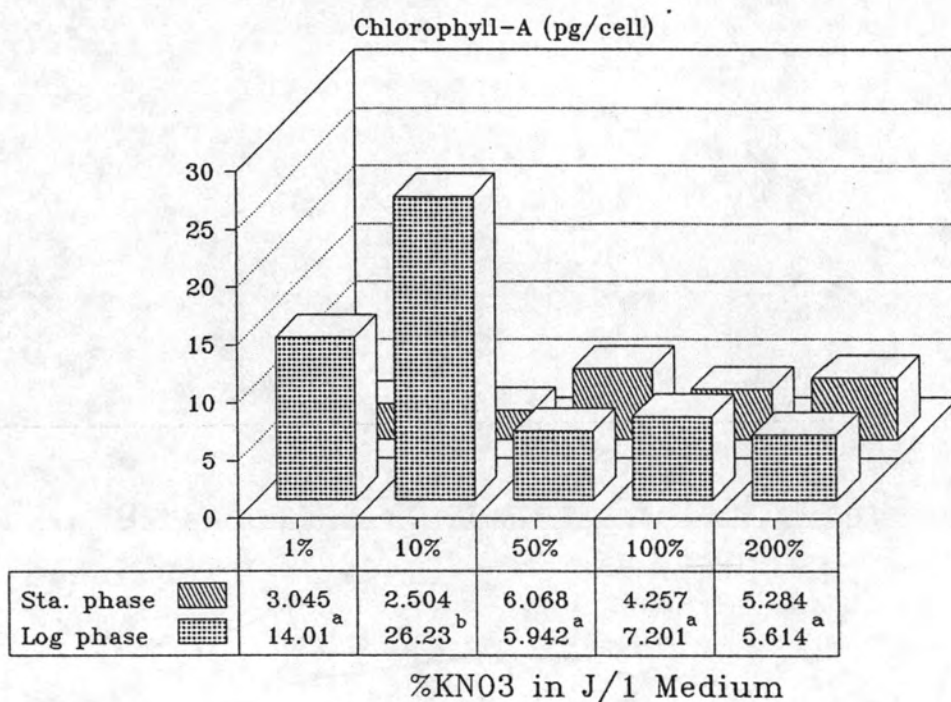


Figure 28. Effect of KNO₃ concentration on chlorophyll-a content of D. salina.

a and b denoted significant difference in mean (P<0.05).

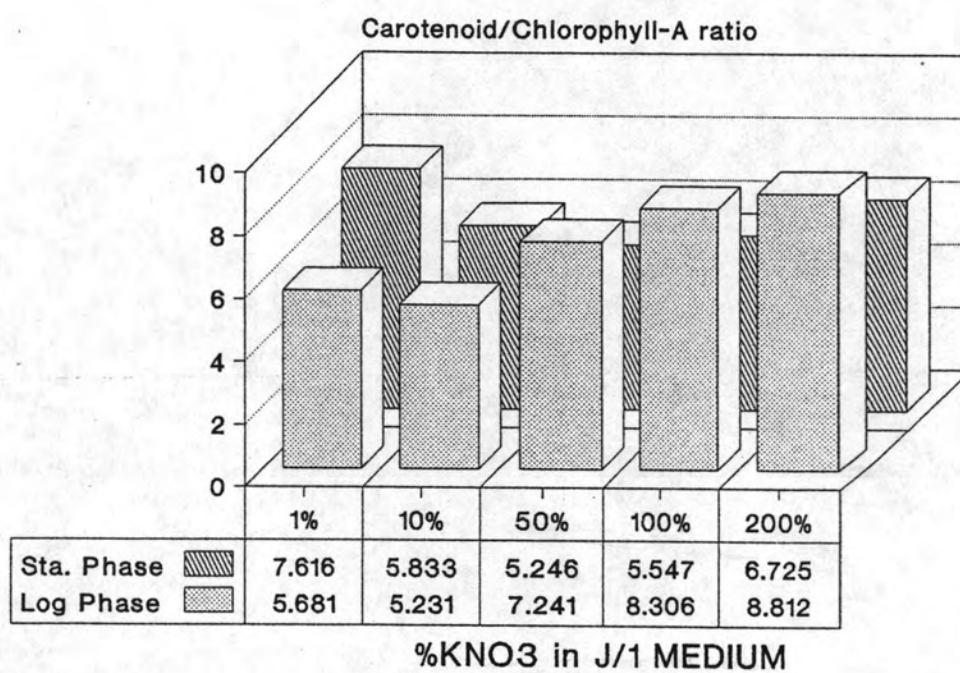


Figure 29. Effect of KNO₃ concentration on carotenoid to chlorophyll-a ratio.

c) Effect of phosphate concentrations.

Figure 30 shows specific growth rate of D. salina at different KH_2PO_4 concentration in J/1 medium. Similar to the effect of nitrate, specific growth rate significantly decreased with the decreasing phosphate concentrations from 100% to 1%. However, the specific growth rates at 100% and 200% KH_2PO_4 were not significantly different. The total carotenoid contents rose insignificantly with decreasing phosphate in both logarithmic and stationary phases (Figure 31). The chlorophyll-a content in various phosphate concentrations is shown in Figure 32. The carotenoid to chlorophyll-a ratio was not significant different in various phosphate concentration (Figure 33).

d) Effect of initial pH.

The growth response of D. salina to various initial pH increased from 6.36 to 8.70 is shown in Figure 34. In this experiment, tris buffer was added into culture medium to insure that pH would not changed rapidly by buffering capacity of the medium itself. The pH variation throughout the experiment is shown in Figure 35. The result indicated that, the initial pH at 7.4 (J/1 medium recommended at 7.5) provided the maximum growth rate. Specific growth rate would decreased if initial pH were changed above or below this value. Carotenoid and chlorophyll-a content in various initial pH differed at stationary phase (Figure 36, 37). The maximum carotenoid content was at pH 7.36 in logarithmic growth phase and pH 8.31 in stationary phase. At stationary phase, carotenoid increased with decreasing of growth rate.

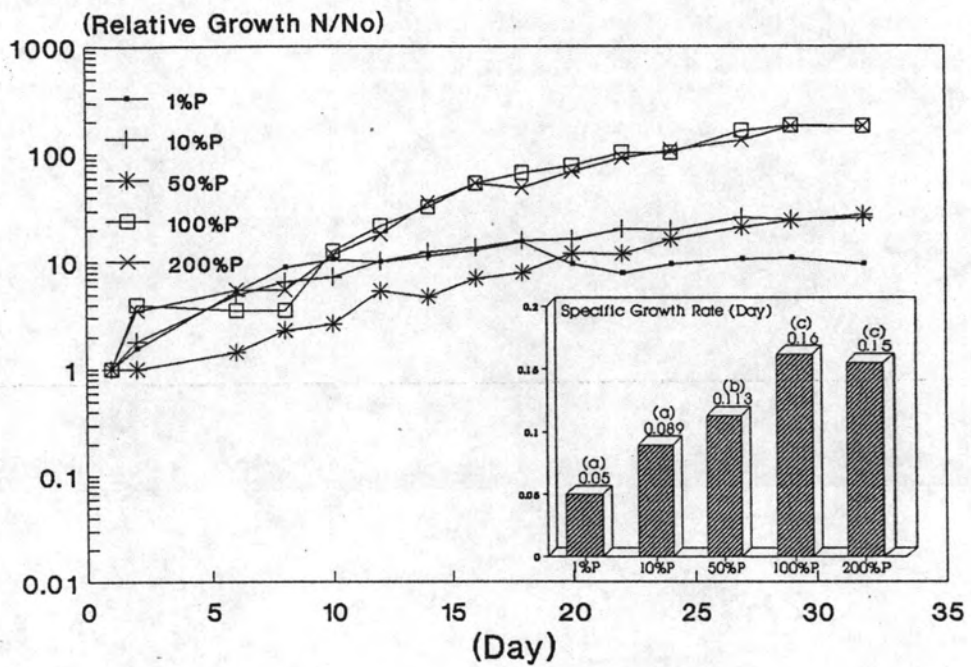


Figure 30. Effect of KH_2PO_4 concentration on specific growth rate of D. salina

a, b, and c denoted significant difference in growth rate ($P < 0.05$).

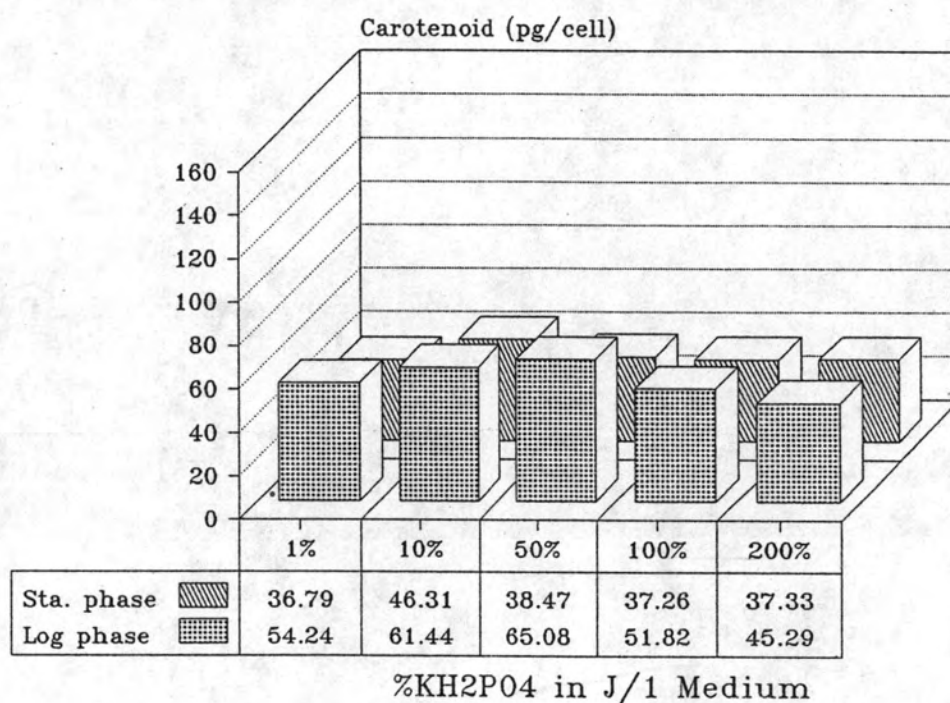


Figure 31. Effect of KH_2PO_4 concentration on carotenoid content of D. salina.

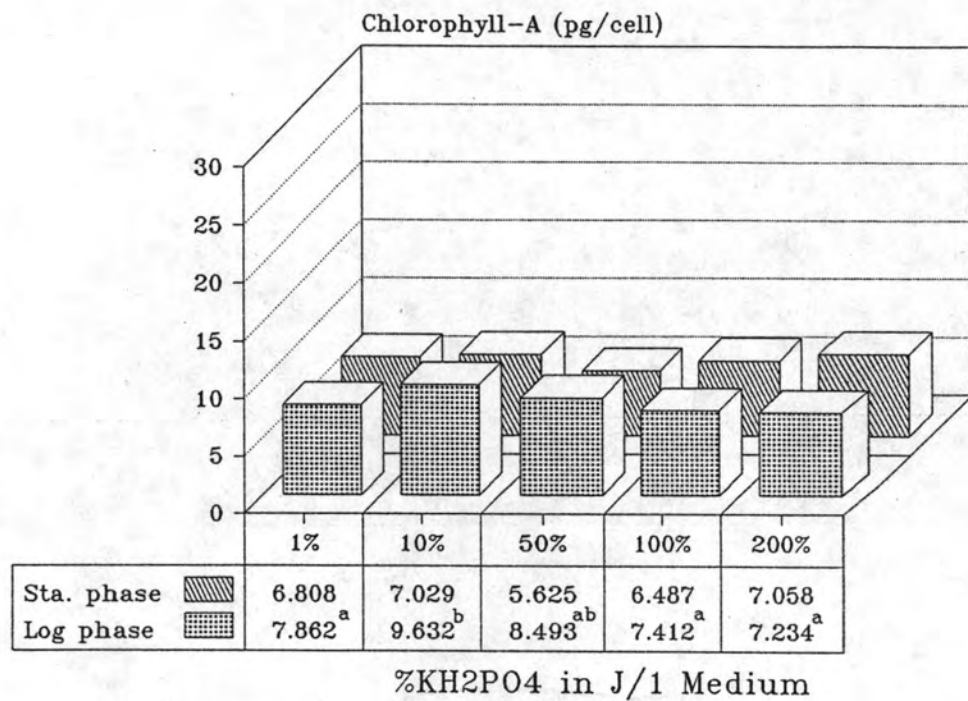
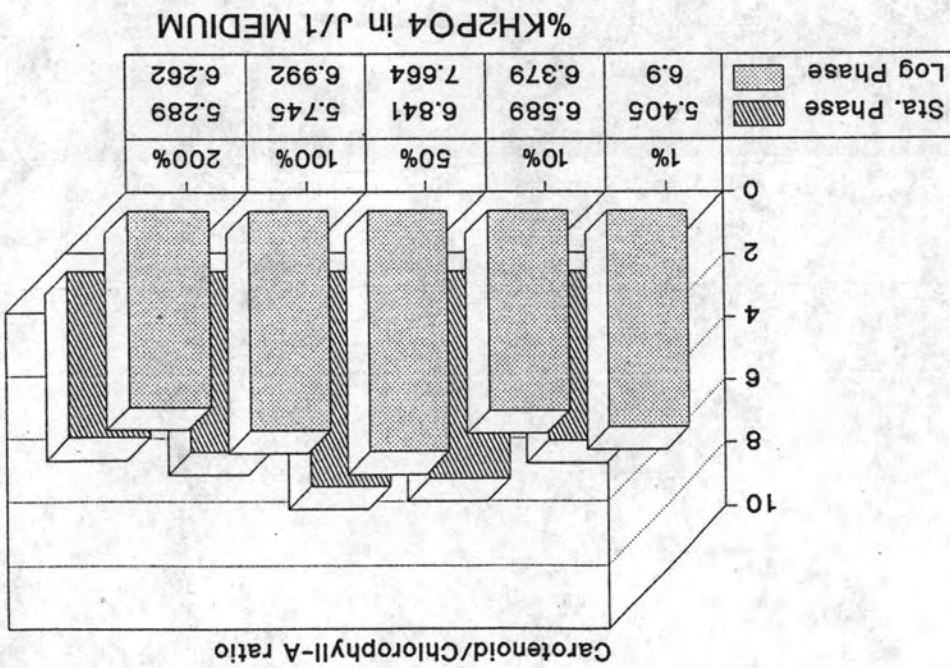


Figure 32. Effect of KH_2PO_4 concentration on chlorophyll-a content of D. salina.

a and b denoted significant difference in mean ($P < 0.05$).

Figure 33. Effect of KH_2PO_4 concentration on carotenoid to chlorophyll-a ratio of *D. salina*.



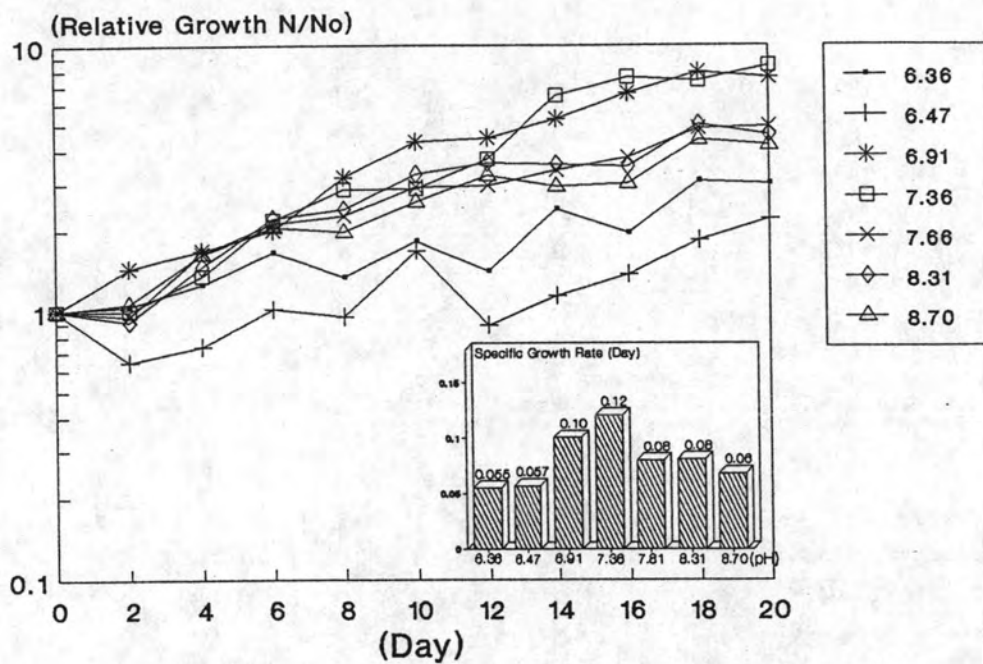


Figure 34. Effect of pH on specific growth rate of *D. salina*. a, b, and c denoted significant different in growth rate ($p < 0.05$).

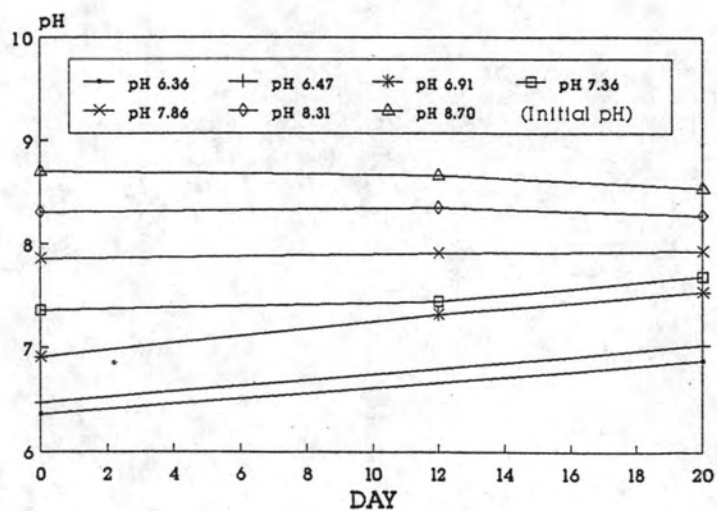


Figure 35. The pH variation throughout experiment.

Carotenoid to chlorophyll-a ratio was effected by high pH value (Figure 38). It was found that the ratio tended to be increased with increasing pH value, and reached to the highest amount at pH 8.7 in stationary phase which significant differed from others.

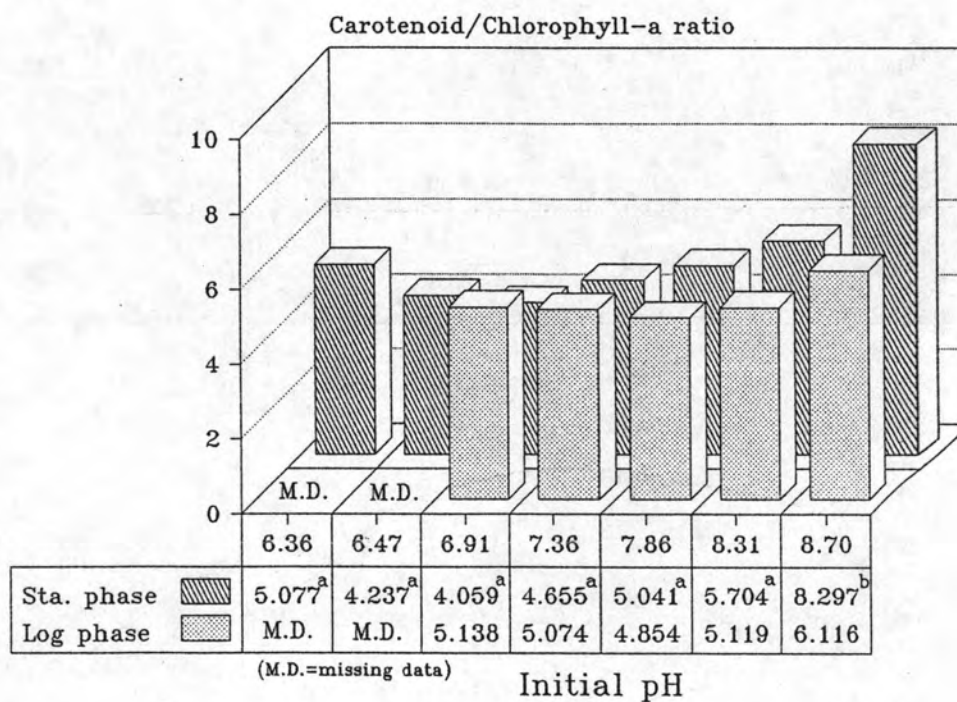


Figure 38. Effect of pH on carotenoid to chlorophyll-a ratio of *D. salina*.

a and b denoted significant different in mean ($P < 0.05$).

Correlation between cell number and ash free dry weight (AFDW) was also determined (Figure 39). The regression analysis showed linear correlation (R-square = 0.79). The regression equation for AFDW calculation was $AFDW=8.19 \times 10^{-10} \times \text{cell number}$.

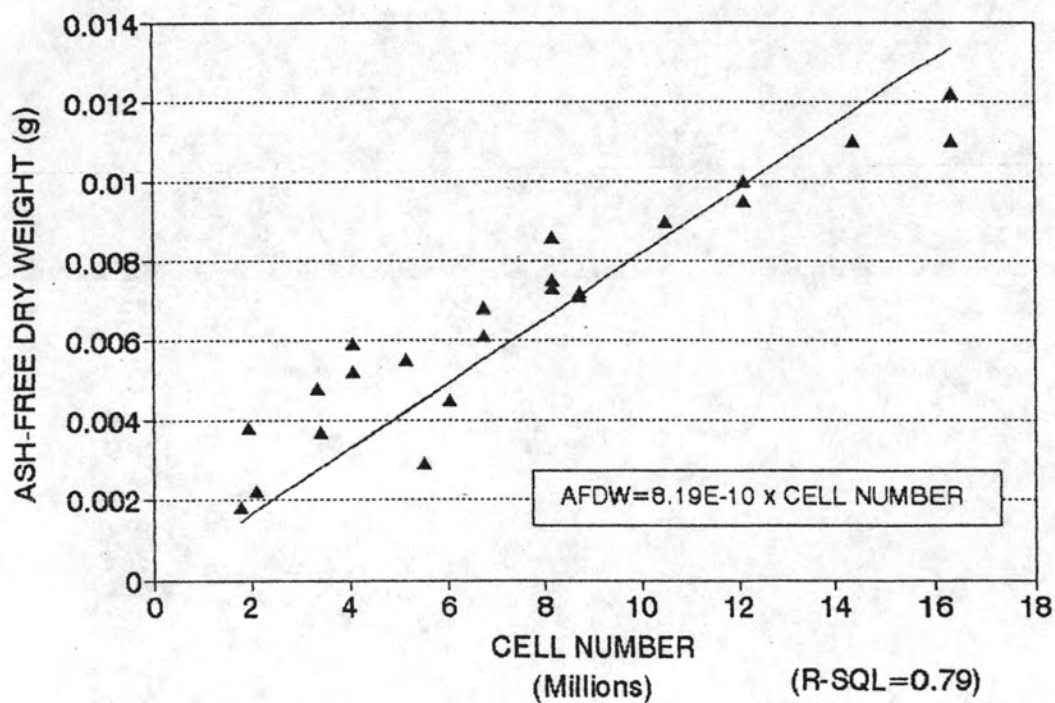


Figure 39. Correlation between cell number and AFDW of D. salina.

Mass cultivation of D. salina in outdoor raceway pond.

Mass culture of D. salina was operated in a pilot-scale 9.1 m² raceway pond with paddle wheel (Figure 40, 41). Throughout the entire period of outdoor cultivation (1 month), the culture salinity was maintained at 200 ppt by the addition of freshwater. Because the experiment was conducted in the rainy season (September - October, 1992), there were frequent rains in the evening and night. Transparency plastic sheet (6x4 m²) was used as a roof to protect the rain. Fortunately, the mid day was usually clear.

The D. salina clone number DS91008 was cultivated under the laboratory condition and then transferred to the pond. Since the starter algal stock from laboratory was in greenish stage, lag phase occurred for 3 days until algal could adapted for new environmental conditions and cells colour would eventually changed to orange. The growth curve of D. salina in outdoor pond is shown in Figure 42. During the logarithmic growth phase, an average specific growth rate (μ) of 0.15 or doubling time of 4.62 days was recorded.

During the outdoor experiment, the climate changed rapidly. This caused fluctuating temperature (Figure 43, Table 5) and light intensity. The plotting between light intensity and dissolved oxygen in the pond showed that algal photosynthesis depended on light intensity (Figure 44). The photosynthesis was satisfactory in a sunny day while it was lower in the cloudy day. Dissolved oxygen increased with increasing light intensity which indicated that no photoinhibition was detected.



Figure 40. D. salina outdoor raceway pond.



Figure 41. Four blade paddle wheel driven by motor and reducing gear for the water circulation.

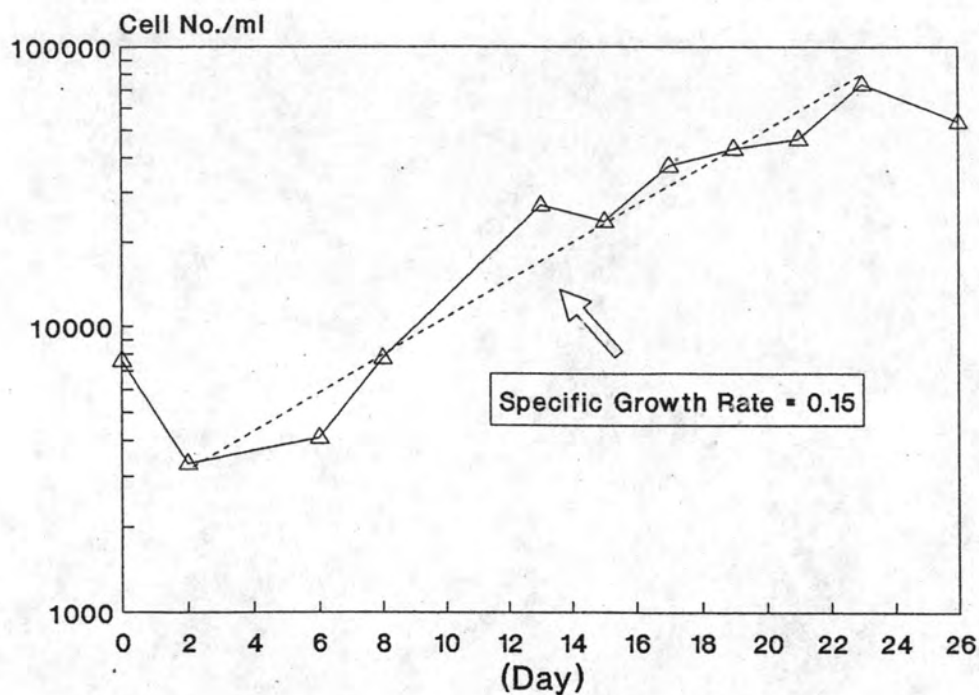


Figure 42. Growth curve of *D. salina* outdoor cultivation.

Table 4. Environmental condition during the outdoor cultivation (September - October 1992).

Parameters	value
Light Intensity	max 135,000 lux
Light:Dark period	about 12:12 hours
pH	7.8 - 9.2
Temperature (air)	24.0 - 35.8°C
(medium)	29.2 - 40.7°C
Salinity	200 - 216 ppt.
Dissolved Oxygen	0.2 - >20 mg/l

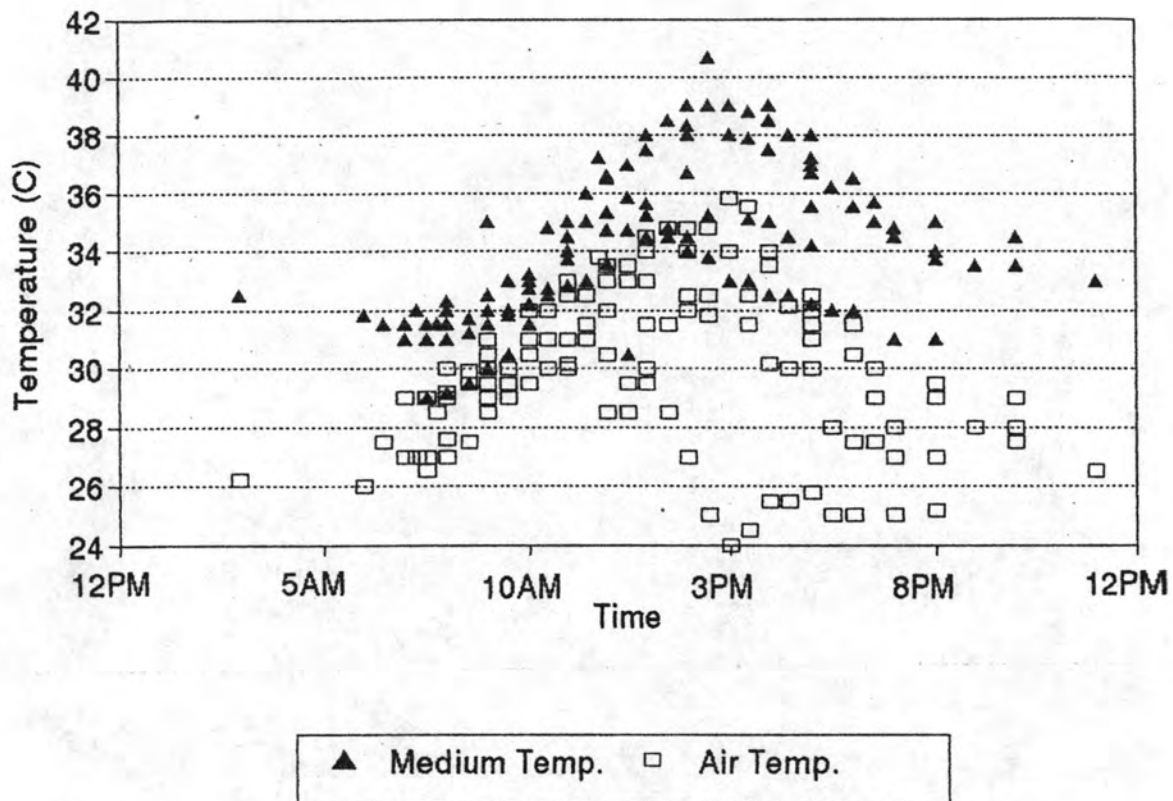


Figure 43. Scatter plot of culture medium temperature and air temperature during the outdoor experiment.



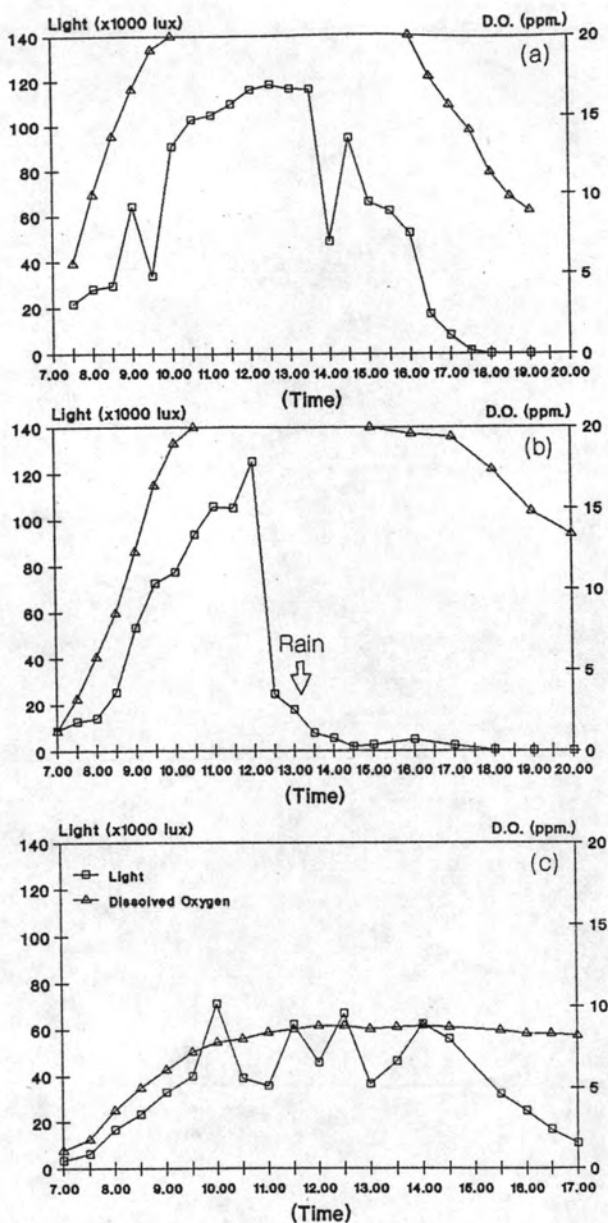


Figure 44. Plotting between light intensity and dissolved oxygen in algal pond.

(a) sunny day

(b) half day cloud

(c) cloudy day

Note: maximum detection limit of the D.O. meter (YSI model 57) was 20 ppm.

The maximum algal biomass of the outdoor experiment was 12.04 g-AFDW/m² at day 22.

Throughout the entire period of outdoor cultivation, the culture contaminants were examined by microscopic observation. It was found that D. salina was contaminated with a little amount of green D. viridis and flagellate protozoa identified as Heteramoeba sp. (Figure 45 and 46).

However, after 25 days of cultivation, pond salinity suddenly dropped because the plastic roof was leaked. The protozoa Heteramoeba sp. then started to bloom in the algal pond. Because of this, D. salina culture was harvested by the flocculation with aluminum sulphate. The flocced algae floated at the surface and were later collected by the 20 micron plankton filter net.

After harvesting, algal soup was centrifuged and washed 4 times with freshwater. Thereafter, algae was dried by freeze drying and oven drying (70°C). Algal flake was homogenated to algal powder. The humidity analysis showed that average percent humidity of the Dunaliella powder was 6.5%, unfortunately, % Ash of algal powder was high (>50%) because of remaining salt. The HPLC analysis showed that freeze dried algae had more β -carotene content than oven dried algae (Table 5).

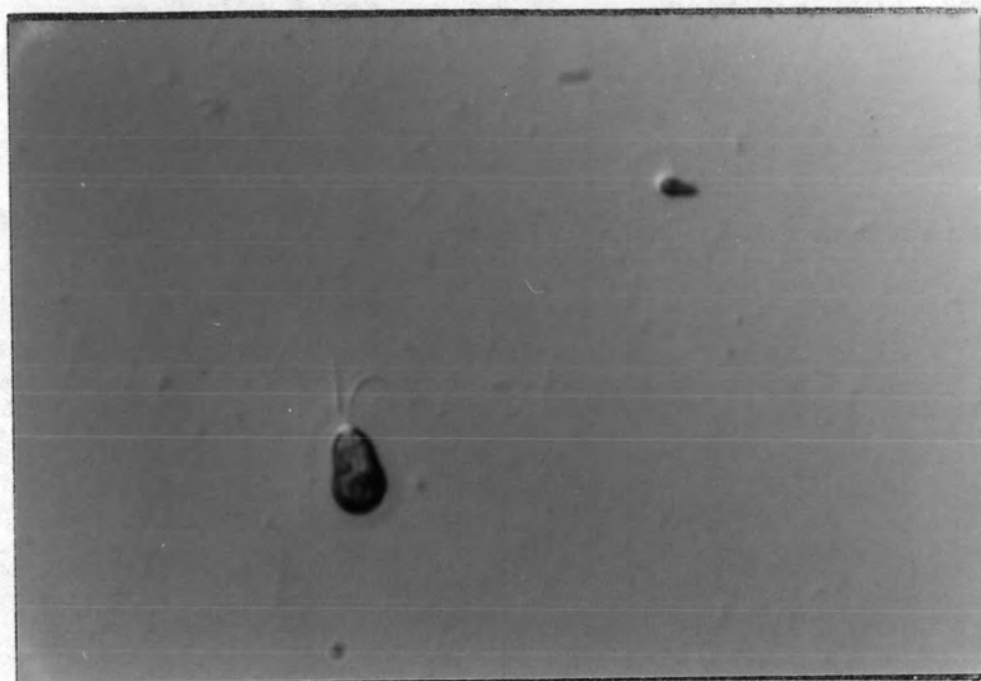


Figure 45. Green D. viridis (small cell) contaminated in D. salina mass culture pond.

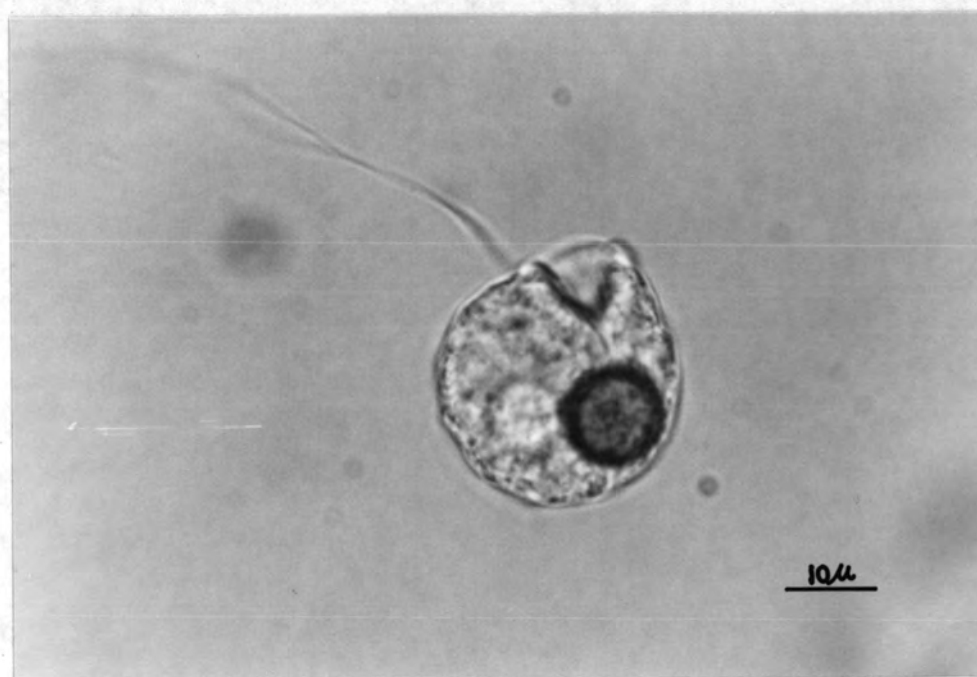


Figure 46. D. salina was eaten by the protozoa. The algal cell could be seen as an orange spot inside the cell.

Table 5. Effect of drying methods on β -carotene content in D. salina.

Drying Method	% Humidity	% Ash (\pm S.E.)	% β -carotene/AFDW
Freeze drying	6.39 \pm 0.14	53.72 \pm 1.02	5.90 \pm 1.43
Oven drying	6.50 \pm 0.19	56.15 \pm 0.39	1.94 \pm 0.68