

The Optimum use of Salinity, Nitrate and Pond Depth for β -Carotene Production of *Dunaliella salina*

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ABSTRACT

Dunaliella salina, halotolerant green algae was collected from the East Coast of Thailand. It has a massive accumulation of β -carotene when grown under defined growth conditions such as high light intensity, high salt concentration and nitrate deficiency. The present study investigated the optimization of salinity, nitrate and pond depth for cell growth and β -carotene production of the alga in question. Cultivation was done in three stages. These were indoor cell growth cultivation, outdoor cell growth cultivation and outdoor β -carotene production. The optimum salinity for cell growth of indoor and outdoor cultivation (5 l) was 9% NaCl, which has specific growth rate (μ) of 0.579 (d^{-1}) and 0.981 (d^{-1}). The optimum salinity for outdoors β -carotene production (5 l) when use 40% inoculum (2.16×10^6 cell ml^{-1}) was 12% NaCl. This produced β -carotene content of $51.73 \mu g ml^{-1}$. In addition, the concentrations of medium used were 100%, 75% and 50% to decrease nitrate concentration in the starter ponds for increasing β -carotene production in the next step. The results showed that 50% medium gave minimum nitrate concentration of $4.5 mg l^{-1}$. For the effect of pond depth, the light expose of the alga were used 9, 11, 13 and 18 cm the ratios of carotenoid to chlorophyll were 7.48, 6.25, 5.54 and 3.35, respectively. Therefore the suitable pond depth for β -carotene production from *D. salina* was 9 to 11 cm.

Key words: β -carotene production, *Dunaliella salina*, pond depth, salinity, nitrate

INTRODUCTION

Dunaliella is a unicellular green algae capable of growing in a wide range of salt concentrations from 0.2% to saturation (around 35%). It produces and accumulates large amounts of β -carotene when cultivated under high light intensity, high salt concentration and nitrate deficiency conditions. More than 10% of the dry weight of *D. salina* is β -carotene (Ben-Amotz and Avron, 1983). β -carotene has important nutritional characteristics, as it is the most effective precursor of vitamin A. Moreover, β -carotene is also used as

food and cosmetics coloring agent; as pro-vitamin A in animal feed; in medical treatment of disease such as erythropoietic protoporphyria (EPP) (Ben-Amotz and Avron, 1990); and as a potent antioxidant which reduced the incidence of cancer in humans (Ziegler, 1989). The aim of the present study was to optimized the culture conditions for *D. salina* β -carotene production.

MATERIALS AND METHODS

Stain

D. salina DS1197 was collected from the

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Eastcoast of Thailand. It was isolated and cultured in Borowitzka's medium (Borowitzka, 1988).

Optimum salinity for indoor cell growth

The algae were cultured in 150 ml transparent plastic cone tubes placed in a water bath. The inoculum was approximately 0.5×10^6 cell ml $^{-1}$. Cells were grown in a modified Borowitzka's medium having 0.5 g l $^{-1}$ of KNO₃ at 30°C. Light intensity was supplied to the culture tube surface at 8 klx on daylight for 16 hrs. A mixture of CO₂ and air (2% CO₂) was provided at 12 ml min $^{-1}$. Salinity of 6%, 9% and 12% NaCl were used for cell growth.

Optimum salinity for outdoor cell growth

The cells from indoor cell growth (maximum cell density) were centrifuged at 2,000 rpm for 10 min. The living cell pellets were transferred to culture medium to make up a starting cell of about 0.5×10^6 cell ml $^{-1}$. The cells were grown in a 5 l plastic tray with controlled shaking of 12 rpm in the open air outdoor. Pure CO₂ was supplied to the culture at the rate of 72 ml min $^{-1}$ for 8 hr daylight. As for indoor culture, cells were grown in a medium of 9% salinity.

Optimum salinity for outdoor β -carotene production

Cell culture of outdoor cell growth was used to inoculate 40% of the culture volume (2.16×10^6 cell ml $^{-1}$) for further study of β -carotene production. The outdoor culture was carried out in 5 l plastic trays at 100% outdoor light exposure. Pure CO₂ was supplied at the rate of 72 ml min $^{-1}$ for 8 hr daylight. The cultures were cultivated in a Borowitzka's medium without KNO₃ at different salinity of 9%, 12%, 15% and 18% NaCl.

Optimum nitrate concentration for outdoor cell growth

The cells from indoor cell growth

cultivation (maximum cell density) were transferred to culture medium to make up a starting cell of about 2×10^5 cell ml $^{-1}$. The cultures were grown in 300 l of outdoor raceway pond under 100% outdoors light exposure. Culture agitation was provided by a paddle wheel. Three different Borowitzka's media concentration of 100%, 75% and 50% were supplied to brine (9% NaCl) to reduce nitrate concentration in order to increase β -carotene production in the next step.

Optimum pond depth for outdoor β -carotene production

The cells from outdoor cell growth (optimized nitrate concentration) were transferred to culture medium (without KNO₃) to make up starting cell of about 2×10^5 cell ml $^{-1}$. The cells were grown in an outdoor raceway pond under 100% outdoors light exposure. CO₂ was bubbled into the pond to maintain the culture pH of 8.0. There were 2 separated sets of experiment.

Experiment 1: The pond depths of 9 cm (250 l working volume) and 18 cm (500 l working volume).

Experiment 2: The pond depths of 11 cm (300 l working volume) and 13 cm (350 l working volume).

Pigment analysis

Chlorophyll and carotenoid were extracted from the alga pellet using 90% acetone and assayed as described by Borowitzka (1991).

β -carotene analysis

The filtrate of 5-10 ml of culture through whatman GF/C filter was wrapped in an aluminum foil and frozen at - 20°C until analysis. The extraction was done in the dim light 10-15 ml cold 90% acetone with gentle grinding. The supernatant was collected by centrifuging at 3,000 rpm for 15 min. β -carotene in supernatant was detected by HPLC as described by Borowitzka (1991).

RESULTS AND DISCUSSION

1. Effect of salinity for indoor cell growth

The results showed that higher salinity medium affected the intracellular mechanism by inhibit cell division of *D. salina* more than lower salinity medium (Figure 1). The specific growth rates (μ) of 6%, 9% and 12% NaCl were 0.463, 0.597 and 0.511 d^{-1} , respectively. Thus, the optimal salinity for indoor cell growth of *D. salina* were 9% and 12% NaCl same in the previous report found by Borowitzka (1988).

2. Effect of salinity on outdoor cell growth (5 liter)

The problem of outdoor culture was protozoan contamination when growth under low salinity that effected cell growth and yield. Although the 9% NaCl were optimal for cell growth of the indoor culture, it also good for outdoor cell growth. The cultures at 9% and 12% NaCl were investigated for cell growth and protozoan contamination. The results were similar to those of the indoor cultures at the same salinity. The specific growth rate of culture at 9% NaCl was higher than that of 12% NaCl. Specific growth rates of the cultures at 9% and 12% NaCl were 0.98 and 0.51 d^{-1} , respectively (Figure 2). Protozoan

contamination was not found in either culture. The salinity of 9% NaCl was therefore considered optimal for outdoor cell growth culture of *D. salina* DS1197.

3. Effect of salinity on outdoor β -carotene production (5 liter)

The culture was cultivated by using inoculum size of 40% of the culture. The inoculum culture was $2.16 \times 10^6 \text{ cell ml}^{-1}$. The culture was investigated for β -carotene production at different salinity of 9%, 12%, 15% and 18% NaCl for 15 days. The highest β -carotene of the culture at 12% NaCl was $51.73 \mu\text{g ml}^{-1}$ (Table 1).

4. Effect of nitrate concentration on outdoor cell growth (300 liter)

The source and concentration of nitrogen can provoke important change in the growth and biochemical composition of microalgal. Manipulation of nitrogen concentration of the culture medium was found to be a simple technique to effect significant differences in the protein, carbohydrate, lipid and pigment content of *Dunaliella* (Uriarte *et. al.*, 1993). *D. salina* can accumulate highest β -carotene when growth under nitrate deficiency. Thus, the medium concentration of 100%, 75% and 50% were used to reduce nitrate

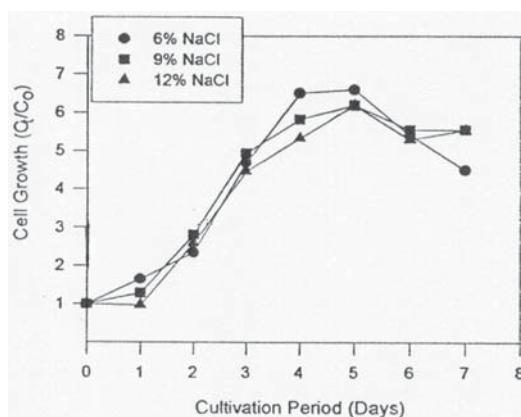


Figure 1 Effect of salinity on cell growth in indoor cultivation of *D. salina*.

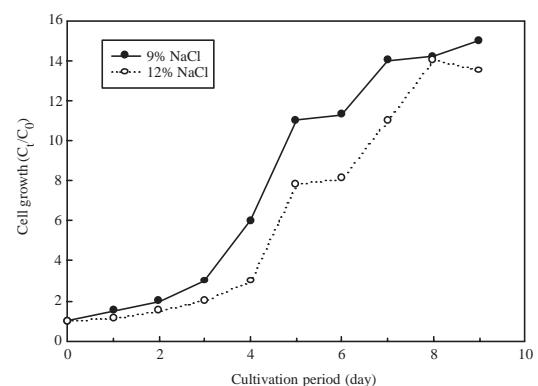


Figure 2 Effect of salinity on outdoor cell growth of *D. salina*.

concentration were 36, 12 and 4.5 mg.l⁻¹ respectively (Figure 3) and increase β -carotene production. There were non-significant differences in the cell concentration is shown in Figure 4. The medium concentration selected for further culture in the present study was 50% of Borowitzka's medium due to gave minimum nitrate concentration.

5. Effect of pond depth on β -carotene production

Pond depth is an one factor for β -carotene production which there has relationship with light quality and intensity absorbed by this alga. Thus, different pond depths were use to search for optimum β -carotene production. The results were as follows:

Experiments 1: The cultivation was carried out during August 1999. Pond depths of 9 and 18

cm were used gave carotenoid to chlorophyll ratios to be 7.48 and 3.35, respectively (Figure 5).

Experiment 2: The cultures were carried out during September 1999. Pond depths of 11 and 13 cm were used gave carotenoid to chlorophyll ratios of 6.25 and 5.54, respectively (Figure 6).

According to the result of these experiments, the pond depth suitable for further culture of β -carotene production was 9 to 11 cm due to gave highest carotenoid to chlorophyll ratios.

Large scale outdoor mass culture where the system is more complex and other factors interact to affect growth and carogenesis. These factors included perdition by protozoa, variable mixing of the culture and the interactions of these factors with nutrient supply, salinity, temperature and light. In addition, cell density and pond depth as an important factor for β -carotene production due to

Table 1 β -carotene production of outdoor cultivation in different salinity.

	Salinity			
	9 % NaCl	12 % NaCl	15 % NaCl	18 % NaCl
Cultivation period (d)	15	15	15	15
Initial cell $\times 10^6$ cell ml ⁻¹ (C ₀)	2.16	2.16	2.16	2.16
Final cell $\times 10^6$ cell ml ⁻¹ (C _f)	1.79	1.66	1.38	1.12
Initial β -carotene (μ g ml ⁻¹)	9.48	9.01	7.58	6.32
Final β -carotene (μ g ml ⁻¹)	48.71	51.73	43.33	38.04

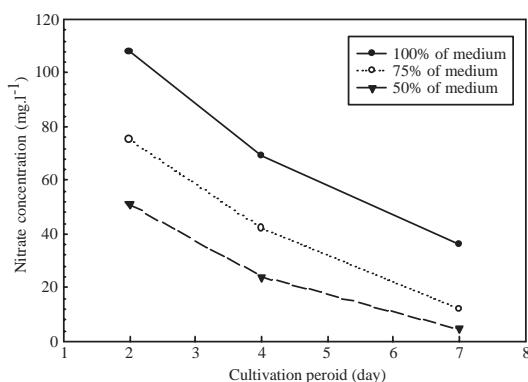


Figure 3 Effect of medium concentration on nitrate concentration.

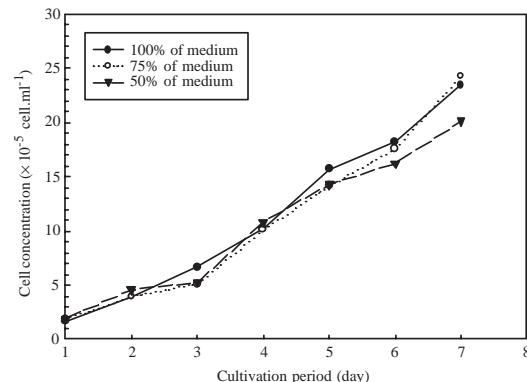


Figure 4 Effect of medium concentration on cell concentration.

relationship with light quality and intensity absorbed by this alga. The optimum cell density is not fix, but is influenced by the type of algae culture, culture depth (pond depth), turbulence and environmental conditions (temperature a available irradiant). At cell density below the optimum, productivity decreases, because the biomass present cannot absorb all the available light energy. Above optimum cell density productivity decrease, because a portion of the culture is in the dark and biomass is lost due to respiration (Grobbelaar, 1995). When β -carotene production in plastic tray (lab scale) and in raceway pond (pilot scale) were compared. The results (Table 2) show that β -carotene production of lab

scale was higher than that of pilot scale because of the difference in cell density and pond depth. The lab scale had higher cell density and less pond depth than pilot scale. Thus, light energy absorbed by this alga would increase to give maximum β -carotene accumulation and production.

CONCLUSION

The optimal conditions for cell growth and β -carotene production of *D. salina* DS1197 as follows: The optimum salinity of indoor and outdoor cell growth was 9% NaCl while the optimum salinity for outdoor β -carotene production was 12% NaCl. The minimum nitrate concentration

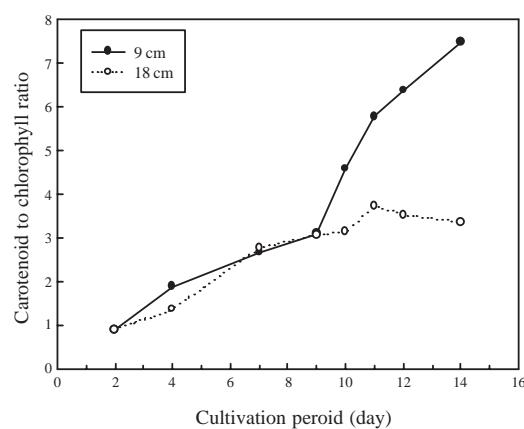


Figure 5 Effect of pond depth, 9 and 18 cm. on carotenoid to chlorophyll ratios.

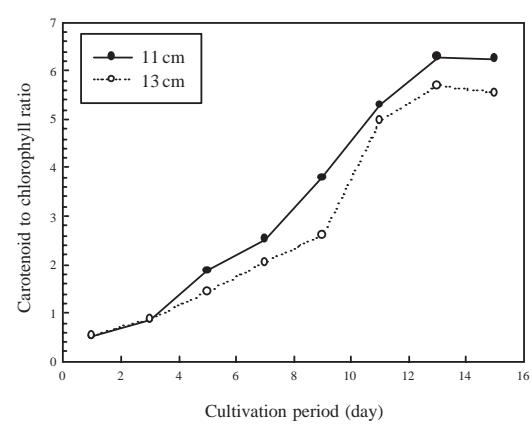


Figure 6 Effect of pond depth, 11 and 13 cm. on carotenoid to chlorophyll ratios.

Table 2 Comparison of β -carotene production lab scale (plastic tray, 5 l) and pilot scale (raceway pond, 250-300 l).

	Laboratory scale (5 L)	Pilot scale	
		9 cm (250 l)	11 cm (300 l)
Cultivation period (d)	15	14	15
Initial cell (cell ml^{-1})	2.16×10^6	2.03×10^5	1.95×10^5
Final cell (cell ml^{-1})	1.66×10^6	3.70×10^5	5.12×10^5
Initial beta-carotene ($\mu\text{g ml}^{-1}$)	9.01	1.36	0.95
Final beta-carotene ($\mu\text{g ml}^{-1}$)	51.73	3.65	3.84
beta-carotene (pg cell^{-1})	31.10	9.80	7.29

was 4.5 mg l⁻¹ when growth under 50% Borowitzka's medium and optimum of raceway pond depth for β -carotene production was 9 to 11 cm.

ACKNOWLEDGEMENTS

The research was supported by KURDI, Kasetsart University under National Research Council, Bangkok, Thailand.

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