J. Microbiol. Biotechnol. (2000), **10**(2), 201–207

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

© The Korean Society for Applied Microbiology

Effects of Nutrient Levels on Cell Growth and Secondary Carotenoids Formation in the Freshwater Green Alga, *Chlorococcum* sp.

LIU, BEI HUI[1], DAO HAI ZHANG[2], AND YUAN KUN LEE[1]*

[1]*Department of Microbiology, Faculty of Medicine, The National University of Singapore, 5 Science Drive 2, Singapore 117597*
[2]*Laboratory of Biosignal and Bioengineering, Institute of Molecular Agrobiology, 1 Research Link, Singapore 117604*

Received: November 1, 1999

Abstract The freshwater green alga *Chlorococcum* sp. grew on NH_4^+, NO_3^-, urea, yeast extract, and peptone as the nitrogen source showing similar patterns of growth and secondary carotenoid (SC) production. However, the most suitable nitrogen source for the induction of SC was urea. The effects of nutrient levels (urea, phosphate, sulfate, ferrous iron, and salt) on growth and SC production were studied by varying the concentration of each nutrient in batch cultures. High biomass production was achieved in cultures containing 20–28 mM urea, 4.8–10 mM phosphate, 1.6 mM sulfate, 70 mM NaCl, and 20–100 μM iron. The highest SC content in biomass was obtained when cultured at 5–10 mM urea, 1–2 mM phosphate, 1.6 mM sulfate, 170 mM NaCl, and 50 μM iron. The optimum concentrations of nutrients for biomass and for the SC accumulation in biomass were evaluated and the two media for achieving high biomass production and SC production were thus developed. The extent to which each parameter to stimulate the formation of SC in the alga were varied and the potentially improved SC production by manipulating the nutrient levels in the modified media were discussed.

Key words: Astaxanthin, secondary carotenoids, *Chlorococcum* sp.

It is well known that a number of microalgae contain the ability to accumulate extra-plastic secondary carotenoids (SC) under stressed growth conditions such as nitrogen starvation, high light intensity, and increased salinity [14, 30, 36]. The best-known examples are the production of β-carotene by the halophilic flagellate alga *Dunaliella salina* [3] and astaxanthin by the freshwater microalga *Haematococcus pluvialis* [10] along with *Chlamydomonas nivalis* [8]. The commercial production of β-carotene from *Dunaliella* is well established in many countries today [2]. The large-scale production of astaxanthin by *Haematococcus* sp. is hampered by its relatively slow growth rate, low growth temperature, and susceptibility to contamination and predation. Therefore, commercial cultivation of *Haematococcus* sp. for the production of astaxanthin is limited. In addition to the biological functions of astaxanthin [17, 24, 27, 34], it has economical value as a feed supplement for farmed salmon, trout, and chicken to provide the coloration of meat and eggs since the animals are not able to perform *de novo* biosynthesis of the pigment [33]. The most expensive ingredient in salmon feed is astaxanthin. It has been estimated that the market for astaxanthin is >US$80 million per year [37]. At this time, the market is mainly satisfied by synthetic astaxanthin. However, the chemical synthesis of astaxanthin is complex and costly due to the presence of chiral centers in its molecular structure. A considerable interest is being generated within the aquacultural industry in terms of natural sources of astaxanthin for commercial purposes.

The influence of nutrient levels on cell growth and carotenogenesis in microalgae has been investigated in recent years [4, 18, 20, 21, 23]. There are, however, still considerable controversy over their roles in carotenogenesis. In the early studies of *Haematococcus* sp., a nitrogen-deficient condition was claimed to induce carotenogenesis [11]. In contrast, Boussiba and Vonshak found that nitrogen-deficiency was ineffective for carotenoid induction when the alga grew autotrophically on CO_2 [5]. Recent studies have shown that nitrogen or phosphate starvation stimulated astaxanthin formation, however, the highest accumulation rate was obtained at high irradiance [23] and under salt stress [16]. In *Chlorella zofingiensis*, maximal growth was achieved at a nitrogen concentration of 15 mM KNO_3 and a low light irradiance, while the maximal accumulation of carotenoids occurred in the cells starved of nitrogen under a high light irradiance [29]. Nevertheless, in *Chlorococcum wimmei*, the concentration of carotenoids was primarily a function of light intensity and independent of cell age and of nitrogen content in the culture medium [7]. It is

*Corresponding author
Phone: 65-874-3284; Fax: 65-776-6872;
E-mail: micleeyk@nus.edu.sg

difficult to draw general conclusions on the effects of nutrients on growth and carotenogenesis in microalgal cultures.

In the recent studies conducted by our research group, it was found that the microalga *Chlorococcum* sp. could accumulate a significant quantity of secondary carotenoids, including astaxanthin and its esters [25, 38, 39]. Due to its tolerance to temperatures of up to 40°C and easy cultivation in outdoor photobioreactors using sunlight, *Chlorococcum* sp. is a promising source for industrial production of astaxanthin [38, 39]. A clear understanding of influences of growth conditions may provide crucial information for optimization of astaxanthin production. In the present study, the effects of nutrient levels including nitrogen, phosphorous, ferrous iron, sulfate, salt, and nitrogen sources on cell growth and formation of secondary carotenoids in *Chlorococcum* sp. were investigated, with each parameter tested independently of each other, enabling the contribution of each to cell growth and secondary carotenoid accumulation to be determined.

Materials and Methods

Organism and Growth Medium

The microalga *Chlorococcum* sp. was isolated in Malaysia, obtained from S. M. Phang (University of Malaya, Kuala Lumpur) and maintained in our laboratory. The alga was cultivated in A9 medium [22]. Six nitrogen sources, namely, urea, peptone, yeast extract, $NaNO_3$, NH_4Cl, and NH_4NO_3 at a concentration of 28 mM were examined. The urea concentrations investigated were 0, 5, 10, 20, and 28 mM. The phosphate concentrations investigated were 0, 1, 2, 4.8, 10, and 20 mM of KH_2PO_4. Tested iron concentrations were 0, 20, 50, 100, and 200 µM, added as EDTA-chelated $FeSO_4 \cdot 7H_2O$. Sulfur concentrations were varied by adjusting the level of $MgSO_4 \cdot 7H_2O$ to 0, 0.5, 1.6, 5, and 10 mM. Various salt concentrations were achieved by altering the level of NaCl and KCl in the culture medium. Sets of three 250-ml flasks containing 50 ml of various media were prepared and inoculated with 2 ml of algal suspension in the logarithmic stage. The flasks were incubated on an orbital shaker rotating at 200 rpm and the cultures were maintained at 28°C. CO_2 was provided to the cultures by purging the flasks with air containing 5% (v/v) CO_2 once every day and capped. Continuous light was supplied by cool white fluorescent tubes at an irradiance of 250 µmol photons $m^{-2}s^{-1}$.

Analytical Methods

Biomass was determined by filtering 5 ml of samples through preweighed filter paper (Whatman No. 1) and washed three times with distilled water. The filtered papers were then weighed after drying at 80°C for 24 h. Total secondary carotenoids were determined by the method described by Boussiba and Vonshak *et al.* [6].

Results and Discussion

Effects of Nitrogen Sources

Figure 1 shows the effects of different nitrogen sources on cell growth and SC synthesis. *Chlorococcum* sp. was able to grow on all nitrogen sources tested with almost the same growth pattern. Cultures reached to a stationary stage at about day 8–10 (Fig. 1a). Peptone was the best nitrogen source for cell growth with a final biomass concentration of 4.5 g/l, followed by yeast extract and urea. $NaNO_3$, NH_4Cl, and NH_4NO_3 were relatively poor nitrogen sources for biomass production in this microalga. As for the cellular production of SC, it seems to be very different. Figure 1b shows that urea and NH_4Cl were preferred compared to other nitrogen sources including peptone, yeast extract, $NaNO_3$, and NH_4NO_3 for SC production. SC concentrations in urea and NH_4Cl cultures were the highest (about 3.8 mg/g dry weight), followed by peptone and yeast extract (about 2.6 mg/g dry weight). On the other

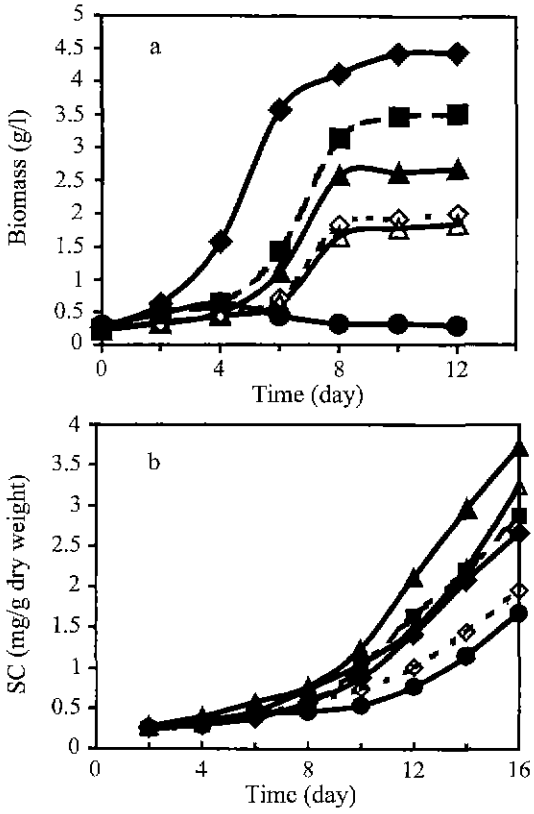

Fig. 1. Effects of nitrogen sources on biomass growth (a) and SC accumulation in biomass (b) in *Chlorococcum* sp.
Symbols. – ● – NH_2NO_3; – △ – NH_4Cl; --◇-- $NaNO_3$; – ▲ – urea; – ■ – yeast extract; – ◆ – peptone. Each data point represents the average of three cultures.

hand, $NaNO_3$ and NH_4NO_3 were the poorest nitrogen sources for SC accumulation. Contrary to this result, Borowitzka *et al.* [4] reported that KNO_3 was the best nitrogen source for SC formation in *Haematococcus pluvialis*, while NH_4Cl and urea inhibited carotenogenesis. Similarly, it was found that carotenoid formation in *Dunaliella salina* was inhibited in a medium containing ammonium [3]. In *Phaffia rhodozyma*, urea and NH_4Cl had similar effects on astaxanthin formation and some of the amino acids, such as valine and leucine, could stimulate astaxanthin synthesis [26]. In the case of *Chlorococcum* sp. growing in high nitrogen concentration (28 mM), carotenogenesis was induced after the growth ceased on the 10th day, and the presence of amino acids as the carbon and nitrogen substrates in the complex media (peptone and yeast extract) suppressed the rate of carotenogenesis. These may imply that the synthesis of SC in *Chlorococcum* sp. acquired carbon from photosynthetic reaction. These results demonstrate that the form of nitrogen source influences the accumulation of SC and the effect was probably a result of different physiological responses and metabolic pathways in the different organisms [35]. Since urea was suitable for both growth and carotenogenesis in *Chlorococcum* sp., it was chosen as the nitrogen source in subsequent experiments.

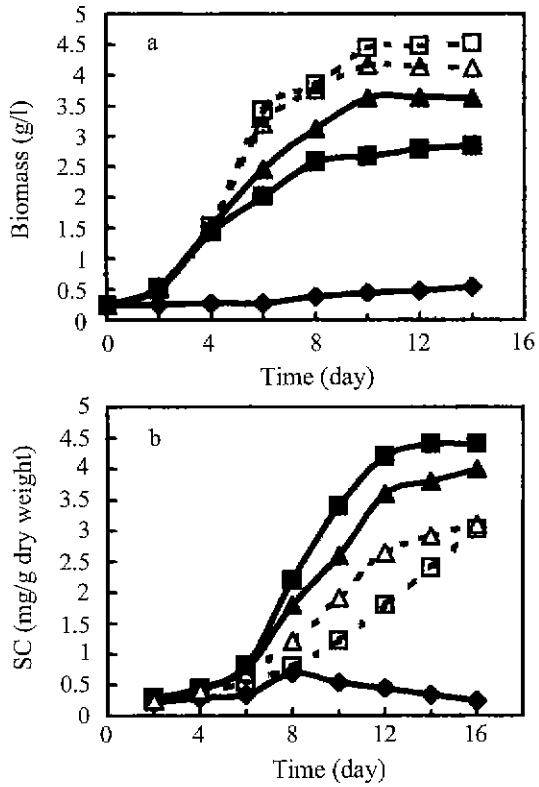

Fig. 2. Effects of urea concentration on biomass growth (a) and SC accumulation in biomass (b) in *Chlorococcum* sp.
Symbols: -◆- 0 mM, -■- 5 mM, -▲- 10 mM, --△-- 20 mM, --□-- 28 mM. Each data point represents the average of three cultures.

Effects of Urea Concentration

It was previously reported that nitrogen limitation was an important factor triggering SC synthesis in *Haematococcus* sp. [11, 12, 13, 23, 32]. Figure 2 shows the effects of urea concentration on growth and SC accumulation in *Chlorococcum* sp. Urea-limited growth was observed at urea concentrations of 0–20 mM, whereas urea was saturated at the concentration of 28 mM, for biomass did not further increase correspondingly with increased urea concentrations from 20 mM to 28 mM (Fig. 2a). It should be mentioned here that the alga is not a nitrogen-fixer and could not grow without any nitrogen source. Urea concentration significantly affected the rate of SC accumulation. As shown in Fig. 2b, the optimum nitrogen concentration for SC accumulation was 5 mM, at which 4.5 mg/g dry weight SC was synthesized in 14 days. Both higher and lower nitrogen concentrations resulted in lower value of SC accumulation. In agreement with Boussiba and Vonshak [6], cultures in nitrogen starvation (0 mM urea) did not accumulate significant quantity of SC, which may be explained by the fact that *de novo* synthesis of some enzymes was necessary during the induction of carotenogenesis. On the contrary, Goodwin and Jamikorn [11], Harker *et al.* [16], and Droop [12] reported that astaxanthin accumulation in *Haematococcus* sp. could be completely independent of nitrogen supply. In the later studies, it was not clear whether the *Haematococcus* cells conserved nitrogen substrate, which would be the case if the nitrogen substrate was not growth-limiting in the inoculum culture. The conserved nitrogen substrate could be used for the synthesis of enzymes involved in carotenogenesis. It is clear that carotenogenesis could be induced by nitrogen-limitation and was independent of growth stage (Fig. 2). The accumulation of SC in the nitrogen-limited culture (5–20 mM urea) was observed in the growth phase and the rate of accumulation was inversely proportion to the growth rate of the culture. In the nitrogen saturated culture (28 mM urea), carotenogenesis occurred only after growth stopped on the 10th day. The same result was observed as shown in Fig. 1. As for the volumetric production of SC, it increased as the nitrogen concentration increased and reached the similar level in 14 days of cultivation by the three concentrations (5, 10, 20 mM) (data not shown). The result may imply that nitrogen content determines the induction and rate of carotenogenesis, while the volumetric content of SC in *Chlorococcum* sp. culture is determined by other growth parameters, possibly light intensity.

Effects of Phosphate Concentration

Phosphate starvation has been reported previously to act as a trigger for the accumulation of astaxanthin [5]. The effects of phosphate concentration on cell growth and

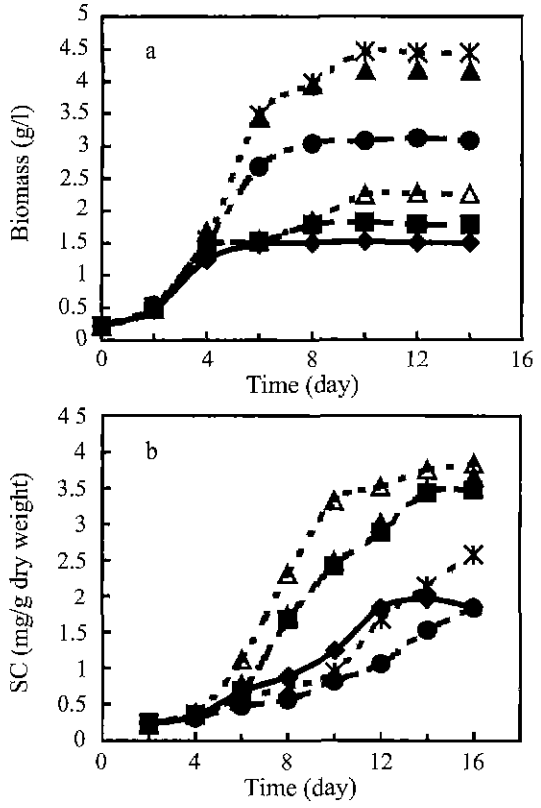

Fig. 3. Effects of phosphate concentration on biomass growth (a) and SC accumulation in biomass (b) in *Chlorococcum* sp. Symbols: -◆- 0 mM; -■- 1 mM; --△-- 2 mM; -▲- 4.8 mM; --×-- 10 mM; -●- 20 mM. Each data point represents the average of three cultures.

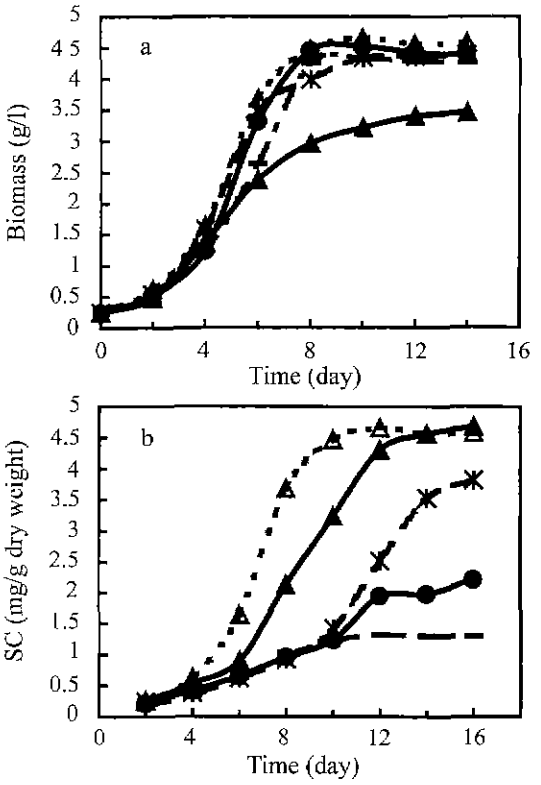

Fig. 4. Effects of $FeSO_4$ concentration on biomass growth (a) and SC accumulation in biomass (b) in *Chlorococcum* sp. Symbols. -◆- 0 mM; -●- 20 mM, --△-- 50 mM; --×-- 100 mM; -▲- 200 mM. Each data point represents the average of three cultures

SC synthesis in *Chlorococcum* sp. were studied over the concentration range of 0 to 20 mM, as shown in Fig. 3. Cell growth was dependent on phosphate below the concentration of 4.8 mM, and at or above this concentration, the culture was phosphate saturated, and thus it did not further increase biomass growth. Biomass concentration was at the highest at the concentration of 10 mM phosphate and further increase of phosphate concentration inhibited cell growth (Fig. 3a). The overall effect of phosphate-limiting condition in *Chlorococcum* sp. was similar to the effect observed in nitrogen-limited cultures, i.e, phosphate limitation induced SC synthesis in this alga. This is in agreement with previous results obtained in *Haematococcus* sp. [5, 6]. These results generally supported that low phosphate levels induced SC formation in microalgal cells. However, it is important to note that in those phosphate-limited (0–2 mM) cultures of *Chlorococcum* sp., the cellular SC content increased with increasing concentration of phosphate in the medium (Fig. 3b). The volumetric production of SC achieved the highest level in the cultures containing 4.8 mM phosphate (data not shown). Cultures with high phosphate concentration (e.g. 10 mM) could be suitable for extended cultivation as the SC production continued to increase at the end of the cultivation process.

Effects of Iron Concentration

Five concentrations of iron (0 to 200 μM), added as EDTA chelated $FeSO_4 \cdot 7H_2O$, were tested. There were no significant differences between the five treatments on cell growth, except that growth inhibition was observed with 200 μM, as indicated in Fig. 4a. Iron is a trace element for microbial cells. Therefore, the trace amount of contaminant in the other substrates may be enough to support cell growth. At high concentration levels (50 to 200 μM), ferrous iron exerted stimulating effects on SC accumulation, as indicated in Fig. 4b. In terms of volumetric SC production, the best iron concentration was 50 μM since the highest SC concentration (20 mg/l) was obtained on the 14th day (data not shown). It is known that ferrous iron gives rise to free radical formation via the Fenton reaction [15]. It has been suggested that free radicals may play a role in the accumulation of β-carotene in *Dunaliella* [1]. It was reported that enhancement of astaxanthin formation in *Haematococcus pluvialis* caused by ferrous iron could be inhibited by the inclusion of potassium iodide that scavenges HO, thus suggesting that HO, formed in an iron-catalyzed Fenton reaction, is

required for the enhancement of astaxanthin biosynthesis in *Haematococcus pluvialis* [19]. The same authors also reported that four active oxygen species (namely $^1O_2$, H_2O_2, peroxyl radical, and the superoxide anion radical) also stimulated the formation of astaxanthin in this alga. It was also reported that in the astaxanthin-accumulating yeast *Phaffia rhodozyma*, exposure to reactive oxygen species, especially $^1O_2$, stimulated carotenoid synthesis [37]. These findings support the hypothesis that oxidative stress may be one of the mechanisms leading to astaxanthin formation. Further studies are needed to clarify the mechanism of the effects of the Fenton reaction and oxygen radicals on carotenoid synthesis.

Effects of Sulfur Concentration

The effects of sulfur concentration were studied over the concentration range of 0 to 10 mM. Maximum biomass production was obtained at about 1.6 mM (Fig. 5a), thus, culture was sulfur-limited at concentrations below 1.6 mM. High concentration of sulfur (>1.6 mM) inhibited cell growth and produced a relatively low yield of biomass. SC formation was less sensitive to sulfur concentrations ranging between 0.5 and 5 mM (Fig. 5b). Under sulfur starvation, cell growth and SC formation were not obvious, whereas, at the growth inhibitory sulfur level, SC formation was also suppressed. The result shows that sulfur is an important element for biomass production but less crucial for SC formation, which was only observed after growth ceased on the 8-10th day in this microalga. Contrary to our results, Shaish *et al.* [31] and Ben-Amotz and Avron [1] reported that sulfur starvation caused massive accumulation of both β-carotene and chlorophyll in *Dunaliella bardawil*. In *Haematococcus pluvialis*, sulfur starvation induced an accumulation of astaxanthin under high light illumination, but the accumulation rate was much lower than that in nitrogen and phosphate starvation in addition to salt stress [6]. Our data shows that sulfur limitation induced the SC accumulation at a slightly lower level than those obtained by manipulating nitrogen and phosphate levels in the growth medium. With respect to the volumetric SC production, it reached the highest level with 1.6 mM of sulfur in the cultures.

Effects of Salt Concentration

Figure 6a illustrates the growth of *Chlorococcum* sp. at several NaCl concentrations. The optimal growth occurred at 70 mM NaCl and cell growth was severely inhibited at the 170 mM level. The low salt tolerance of this microalga was similar to that of *Haematococcus pluvialis*, in which

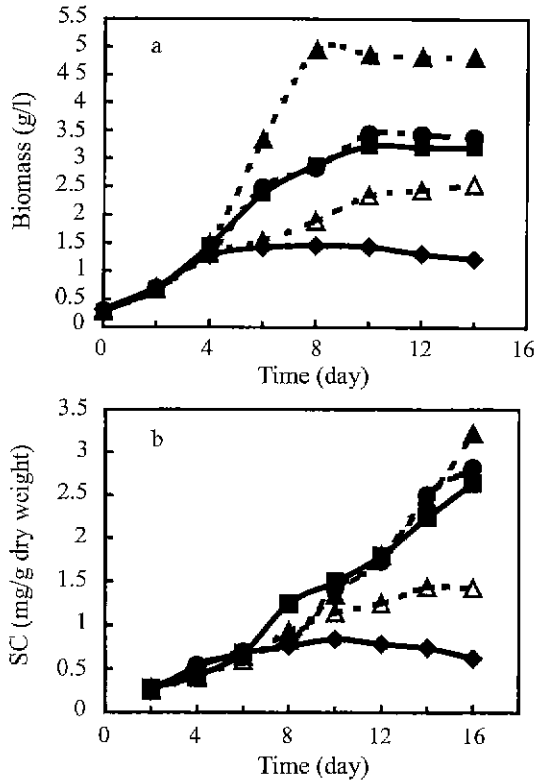

Fig. 5. Effects of $MgSO_4$ concentration on biomass growth (a) and SC accumulation in biomass (b) in *Chlorococcum* sp.
Symbols: -◆- 0 mM; -■- 0.5 mM; -▲- 1 6 mM, -●- 5 mM; -△- 10 mM Each data point represents the average of three cultures.

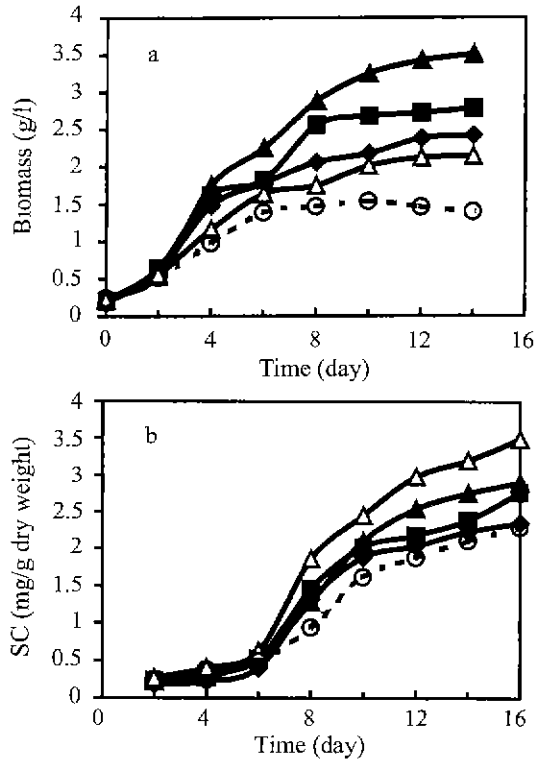

Fig. 6. Effects of NaCl concentration on biomass growth (a) and SC accumulation in biomass (b) in *Chlorococcum* sp.
Symbols -◆- 0 mM; -■- 35 mM -▲- 70 mM; -△- 170 mM; -○- 350 mM Each data point represents the average of three cultures.

140-170 mM NaCl was known to be lethal to cell growth [5, 6]. However, salt stress did not significantly enhance the cellular accumulation of SC in the *Chlorococum* sp. as reported in *Haematococcus pluvialis*, in which more than 3% of astaxanthin in biomass was obtained by adding 35 mM of NaCl [9], while 550 pg of carotenoid per cell was obtained by adding 100 mM of NaCl [16]. The SC content was relatively higher at 170 mM than at other salt concentrations (Fig. 6b). Cells cultured in media containing 0-70 mM of NaCl accumulated a similar amount of SC (2.5-3.1 mg/g dry weight). However, the volumetric SC production was higher in the cultures containing 70 mM of NaCl (data not shown). The effect of adding KCl to the algal cultures was also investigated. Even at low concentration levels (i.e. 40 mM), cell growth was severely inhibited (data not shown). The results show that the addition of K^+ to the cultures even at low concentrations produced a phytotoxic effect and this is in agreement with other observations made by Harker *et al.* [16] on *Haematococcus pluvialis*. These results support the hypothesis that algal cells do not possess an efficient extrusion mechanism for K^+, as they do for Na^+ and Cl^- [28].

Conclusion

The nutrient parameters investigated in the present study affected cell growth and carotenogenesis, with each parameter exerting a different effect on biomass and/or SC production in *Chlorococcum* cultures. Under the growth conditions used, higher nutrient concentration in the medium supported higher biomass growth and volumetric SC production, while the SC production was simultaneously a growth- and nongrowth-associated process depending on the growth-limiting nutrient in this microalga. The SC content in biomass was minimal in the exponential growth period and reached the highest after the cell growth ceased. As the levels of nutrient resulted in differential biomass and/or SC production, it is important to identify the conditions for obtaining high biomass and/or SC production. As shown in Table 1, two types of media (A and B) were designed in the present study. Medium A could be used for the production of biomass to produce a dense algal culture, while medium B could be used for the induction of SC accumulation in biomass in the second step. Present study further indicated that nutrimental factors could invoke differential responses within the cells with respect to the carotenogenesis. Among the parameters tested, all the nutrients play important factors in affecting carotenogenesis. Therefore, by manipulating mainly for nutrient levels in the medium, high SC productivity could be achieved by a two-step process.

Table 1. Optimum concentration of tested nutrients for biomass production (medium A) and SC accumulation in biomass (medium B) in *Chlorococcum* sp. under autotrophic condition.

Components	Medium A	Medium B
Urea	20-28 mM	5-10 mM
KH_2PO_4	4.8-10 mM	1-2 mM
NaCl	70 mM	170 mM
$MgSO_4$	1.6 mM	1.6 mM
$FeSO_4$	20-100 μM	50 μM

References

1. Ben-Amotz, A. and M. Avron. 1983. On the factors which determine massive β-carotene accumulation in the halotolerant alga *Dunaliella bardawil*. *Plant Physiol.* **72:** 593-597.
2. Borowitzka, M. A. and L. T. Borowitzka. 1988. Dunaliella, pp. 27-28. *In* M. A. Borowitzka and L. T. Borowitzka (eds.), *Microalgal Biotechnology*, Cambridge University Press, Cambridge, U.K.
3. Borowitzka, M. A. and L. T. Borowitzka. 1988. Limits to growth and carotenogenesis in laboratory and large-scale outdoor cultures of *Dunaliella salina*, pp. 371-381. *In* T. Stadler, H. Mollion, M. C. Verdus, Y. Karamanos, H. Morvan, and D. Chrostiaen (eds.), *Algal Biotechnology*, Elsevier Applied Science, London.
4. Borowitzka, M. A., J. M. Huisman, and A. Osborn. 1991. Cultures of the astaxanthin-producing green alga *Haematococcus pluvialis*. 1. Effect of nutrient on growth and cell type. *J. Appl. Phycol.* **3:** 295-304.
5. Boussiba, S. and A. Vonshak. 1991. Astaxanthin accumulation in the green alga *Haematococcus pluvialis*. *Plant Cell Physiol.* **32:** 1077-1082.
6. Boussiba, S., F. Lu, and A. Vonshak. 1992. Enhancement and determination of astaxanthin accumulation in green alga *Haematococcus pluvialis*. *Meth. Enzymol.* **213:** 386-391.
7. Brown, T. E., F. L. Richardson, and M. L. Vaughn. 1967. Development of red pigmentation in *Chlorococcum wimmeri* (Chlorophyta: Chloroccales). *Phycologia* **6:** 167-184.
8. Bubrick, P. 1991. Production of astaxanthin from Haematococcus. *Bioresource Technol.* **38:** 237-239.
9. Cordero, B., A. Otero, M. Patino, B. O. Arredondo, and J. Fabregas. 1996. Astaxanthin production from the green alga *Haematococcus pluvialis* with different stress conditions. *Biotechnol. Lett.* **18:** 213-218.
10. Donkin, P. 1976. Ketocarotenoid biosynthesis by *Haematococcus lacutris*. *Phytochemistry* **15:** 711-715.
11. Goodwin, T. W. and M. Jamikorn. 1954. Studies in carotenogenesis. II. Carotenoid synthesis in the alga *Haematococcus pluvialis*. *Nature* **57:** 376-381.
12. Droop, M. R. 1954. Conditions governing haematochrome formation in *Haematococcus pluvialis*. *Flotow Arch. Mikrobiol.* **20:** 391-397.
13. Droop, M. R. 1955. Carotenogenesis in *Haemotococcus pluvialis*. *Nature* **175:** 42-48.

14. Grung, M., F. M. L. D'Souza, M. Borowitzka, and S. Liaaen-Jensen. 1992. Algal carotenoids 51. Secondary carotenoids 2. *Haematococcus pluvialis* aplanospores as a source of (3S, 3'S)-astaxanthin esters. *J. Appl. Phycol.* **4:** 165–168.
15. Halliwell, B. and J. M. C. Gutteridge (eds.). 1989. *Free Radicals in Biology and Medicine*. Oxford University Press, Oxford, U.K.
16. Harker, M., A. J. Tsacalos, and A. J. Young. 1996b. Factors responsible for astaxanthin formation in the Chlorophyte *Haematococcus pluvialis*. *Bioresource Technol.* **55:** 207–214.
17. Jyonouchi, H., H. L. Zhang, and Y. Tomita. 1993. Studies of immunomodulating actions of carotenoids. II. Astaxanthin enhances *in vitro* antibody production to T-dependent antigens without facilitating poly-clonal B-cell activation. *Nutr. Cancer* **19:** 269–278.
18. Kobayashi, M., T. Kakizono, N. Nishio, and S. Niagai. 1992. Effect of light intensity, light quality and illumination cycle on astaxanthin formation in a green alga, *Haematococcus pluvialis*. *J. Ferment. Bioengng.* **74:** 61–63.
19. Kobayashi, M., K. Toshihide, and S. Nagai. 1993. Enhanced carotenoid biosynthesis by oxidative stress in acetate-induced cyst cells of a green unicellular alga *Haematococcus pluvialis*. *Appl. Environ. Microbiol.* **59:** 867–873.
20. Lee, Y. K. and S. Y. Ding. 1994. Cell cycle and accumulation of astaxanthin in *Haematococcus lacustris* (Chlorophyta). *J. Phycol.* **30:** 445–449.
21. Lee, Y. K. and S. Y. Ding. 1995. Effect of dissolved oxygen partial pressure on the accumulation of astaxanthin in chemostat cultures of *Haematococcus lacutris* (Chlorophyta). *J. Phycol.* **31:** 922–924.
22. Lee, Y. K. and S. J. Pirt. 1981. Energetics of photosynthetic algal growth: Influence of intermittent illumination in short (40s) cycle. *J. Gen. Microbiol.* **124:** 433–452.
23. Lee, Y. K. and C. W. Soh. 1991. Accumulation of astaxanthin in the *Haematococcus lacutris*. *J. Phycol.* **27:** 575–577.
24. Lee, Y. K. and D. H. Zhang. 1999. Production of astaxanthin by *Haematococcus*, pp. 173–195. *In* Z. Cohen (ed.), *Chemicals from Microalgae*, Tayer and Francis, London, U.K.
25. Liu, B. H. and Y. K. Lee. 1999. Composition and biosynthetic pathways of carotenoids in the astaxanthin-producing green alga *Chlorococcum* sp. *Biotechnol. Lett.* **21:** 1007–1010.
26. Meyer, P. S., J. C. du Preez, and S. G. Kilian. 1993. Selection and evaluation of astaxanthin-overproducing mutants of *Phaffia rhodozyma*. *World J. Microbiol. Biotechnol.* **9:** 514–520.
27. Miki, W. 1991. Biological functions and activities of animal carotenoids. *Pure Appl. Chem.* **63:** 141–146.
28. Pick, U., A. Ben-Amotz, L. Karni, C. J. Seebergs, and M. Avron. 1986. Partial characterisation of K^+ and Ca^+ uptake systems in the halotolerant alga *Dunaliella salina*. *Plant Physiol.* **81:** 875–881.
29. Rise, M., E. Cohen, M. Vishkautsan, M. Cojocaru, H. E. Gottlieb, and S. Arad. 1994. Accumulation of secondary carotenoids in *Chlorella zofingiensis*. *J. Plant Physiol.* **144:** 287–292.
30. Santos, M. F. and J. F. Mesquita. 1984. Ultrastructural study of *Haematococcus lacustris* (Girad.) Rostafinski (Volvocales). I. Some aspects of carotenogenesis. *Cytologia* **49:** 215–228.
31. Shaish, A., A. Ben-Amotz, and M. Avron. 1991. Production and selection of high b-carotene mutant of *Dunaliella bardewil* (Chlorophyta). *J. Phycol.* **27:** 652–656.
32. Spencer, K. G. 1989. Pigmentation supplements for animal feed compositions. U.S. Patent No. 4871551.
33. Storebakken, T., P. Foss, K. Schiedt, E. Austreng, S. Liaaen-Jensen, and U. Manzz. 1987. Carotenoids in diets for salmonids. IV. Pigmentation of atlantic salmon with astaxanthin, astaxanthin dipalmitate and canthaxanthin. *Aquaculture* **65:** 279–292.
34. Tanaka, T., Y. Morishita, M. Suzui, T. Kojima, A. Okumura, and H. Mori. 1994. Chemoprevention of mouse urinary bladder carcinogenesis by the naturally occurring carotenoid astaxanthin. *Carcinogenesis* **15:** 15–19.
35. Turpin, D. H. 1991. Effects of inorganic nitrogen availability on algal photosynthesis and carbon metabolism. *J. Phycol.* **27:** 14–20.
36. Vechtel, B., W. Eichenberger, and H. G. Ruppel. 1992. Lipid bodies in Eremosphaera viridis De Bary (Chlorophyceae). *Plant Cell Physiol* **31:** 41–48.
37. William, A. S., C. Pilar, L. D. Monica, and A. J. Eric. 1996. Selection for carotenogenesis in the yeast *Phaffia rhodozyma* by dark-generated singlet oxygen. *Microbiology* **142:** 2923–2929.
38. Zhang, D. H. and Y. K. Lee. 1999. Ketocarotenoid production by a mutant of *Chlorococcum* sp. in an outdoor tubular photobioreactor. *Biotechnol. Lett.* **21:** 7–10.
39. Zhang, D. H., Y. K. Lee, M. L. Ng, and S. M. Phang. 1997. Composition and accumulation of secondary carotenoids in *Chlorococcum* sp. *J. Appl. Phycol.* **9:** 459–463.