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Influence of Daily Collection and Culture Medium Recycling on the Growth and β-Carotene Yield of *Dunaliella salina*

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The halophilic green alga *Dunaliella salina* has the potential to be cultivated for β-carotene-rich biomass, however, open-air systems need to be further improved in order to become more competitive and more economical, rather than leave the major β-carotene consuming market derived from artificially synthesis. A set of daily collection ratios was designed and scaled up with the aim to harvest cell biomass and β-carotene from *D. salina* at logarithmic phase; the yields were comparable to the normal culture without daily removal of culture. Daily collection of 1/7.5 volume of algal culture was found to be appropriate to keep the balance between the cell biomass and β-carotene accumulation. Light intensity as one of the important factors would affect both cell growth and β-carotene content synchronously. Further, the method of recycling 1/7.5 volume of culture after removal of algae cells was developed in order to decrease input cost for the effective production of β-carotene, and both the resulting yields of the cell biomass and β-carotene gained an advantage over those from the normal *D. salina* culture.

KEYWORDS: *Dunaliella salina*; β-carotene accumulation; daily collection volume; culture medium recycling

1. INTRODUCTION

Carotenoids as high-value molecules are receiving growing attention due to their beneficial effects on human health and potential pharmaceutical applications (*1, 2*). Several microalgae like *Chlorella*, *Dunaliella*, *Haematococcus*, and *Spirulina* are currently commercially cultivated for the production of astaxanthin, β-carotene and other fine biochemicals of high value (*3*). Although industrial scales have not yet been reached, some other microorganisms, such as freshwater green alga *Botryococcus braunii* (*4*) and food yeast *Candida utilis* (*5*) and even noncarotenogenic bacteria like *Escherichia coli* (*6*), have shown the potential for industrial uses for carotenoid production. The microalgal species successfully employed share a common feature that they could grow in highly selective or even extreme environmental conditions, meaning that they may grow in open system cultures and still remain relatively free of contamination by other organisms (*7*). Taken as a good example, *Dunaliella salina* could grow at very high salinity and it may not need to be cultivated in closed systems (*8*), especially considering the cost of biochemical-rich algal biomass production.

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It is an expensive work to produce microalgae, so in order to achieve cost-efficient modes for mass cultivation of these organisms, various systems have been designed for the production of microalgae on a large scale (*9–11*). Currently, open-air systems are much cheaper to construct and operate for outdoor cultivation of microalgae (*12*). Culture systems all around the world mostly consist of large open ponds, tanks, or raceways. In this paper, the green flagellate *D. salina* was selected for open-air culture experiments; it is the most suitable organism for the mass production of β-carotene, even up to 14% of cell dry weight (*13*). Commercial plants in large outdoor ponds for β-carotene-rich *Dunaliella* exist today in Israel, the United States, Australia, Spain, and China (*14, 15*). There are three categories of products derived from *D. salina*, including β-carotene extracts, β-carotene-rich algal powder for human consumption as an ingredient of dietary supplements and functional foods, and dried *Dunaliella* for feed use (*16*). Moreover, *D. salina* could produce natural stereoisometric compositions of β-carotene by properly regulating growth conditions (*14, 17*), which is superior to the artificially synthesized *all-trans*-β-carotene with a high ratio of 9-cis form to all-trans form. This fact might suggest the basis for an

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alternative way to produce high-quality carotenoids or carotene-rich algae for the health food market.

Algal harvesting in an open culture system is likely to remain an active area of study, including not only techniques like flocculation but also methods related to the relationship between the harvest time point and the yield of both cell biomass and expected biocompound. The aims of this study were to design a set of programs with different daily collection ratios of culture to extend the cultivation time of harvesting objective product β-carotene from *D. salina*, and to assess the possible effect of recycling culture medium on cell growth and β-carotene accumulation, with an attempt to decrease input cost for the effective production of β-carotene-rich biomass.

2. MATERIALS AND METHODS

2.1. Strain and Growth Conditions. *Dunaliella salina* (1/2 SW), from the Institute of Hydrobiology, Chinese Academy of Sciences, was grown in a culture medium according to the method of Hejazi and Wijffels (*18*). The pH of the medium after addition of 100 mM Tris-HCl buffer was adjusted to 7.5 with 2 M HCl. The medium was sterilized before inoculation.

Outdoor cultures were performed at the experimental site in the central district of Guangzhou City, China (E 113°15′, N 23°08′), which lies in the subtropical belt. Temperature and light were almost not controlled and reflected those available in this area. *D. salina* cells in Erlenmeyer flasks on a shaker table (60 rpm was applied to facilitate gas exchange) were grown with a 14/10 h dark/light cycle and were collected at the log phase or late log phase.

2.2. Experimental Setup. A set of daily collection ratios was designed in program A as 1/6, 1/7.5, 1/10, 1/15, and 1/30 volume of the *D. salina* culture at the early or middle log phase. In the preliminary phase of this work, algal cultures of 25, 20, 15, 10, and 5 mL would be removed, respectively, and then equal volumes of fresh culture medium were added to maintain the original culture volumes. The negative control was set as the normal cultivation. Program B was set up with 10 times the volume of program A and the collection ratios were broadened as 1/3, 1/3.75, 1/5, 1/7.5, and 1/15 in five tested groups.

On the basis of the results of daily collection experiments, reuse of the "aged" culture medium to increase both the algal biomass and β-carotene yield or to maintain the production balance between them was attempted. *D. salina* culture (1/7.5 volume) at late log phase was collected daily, and after thorough removal of cells, the aged culture medium was recycled into the original algal culture. A positive control was set by adding an equal volume of fresh culture medium after collection; a negative control was set as normal culture without daily collection. All experiments were conducted in parallel or repeated twice.

2.3. Determination of Growth. Cell growth was estimated by determining cell dry weight, cell number, and/or cell growth rate (*19*). The OD_{630} values for algal culture solutions with different concentrations were measured. Then the cells were harvested by centrifugation at 4000 rpm for 20 min and transferred to constant-weight filter paper before being rinsed 2∼3 times rapidly with distilled water to remove salts. The cell pellet was subjected to vacuum drying at 50∼60 °C before being weighed. A relationship curve of the dry weight of cells (grams per liter) versus OD_{630} value was plotted, and the biomass of the *D. salina* culture was calculated according to the regression equation $y = 0.7449x + 0.0146$ ($R^2 = 0.9986$), where y is the biomass dry weight and x is the OD_{630} value.

Cell numbers were determined by the optical density method. Light absorbance values (OD_{700}) of the samples at 700 nm were measured first, and then they were plotted against the counted cell numbers in 1 mL of algal culture. The sample cell number was calculated according to the regression equation $N = 157.34OD_{700} - 1.72$, where N is the cell numbers per milliliter of algal culture, with the unit as thousands. Cell growth rate was calculated on the basis of the unicellular algal growth rate equation: $R = (\ln N_t - \ln N_0)/t$, where N_0 and N_t are the cell numbers at the beginning and termination of cultivation, respectively.

2.4. Extraction and Quantification of β-Carotene. The standard curve of β-carotene was drawn with different OD_{453} values versus the corresponding concentrations of the β-carotene/acetone standard solutions. The regression equation was $y = -0.0037x^2 + 0.1082x$ ($R^2 = 0.9901$), where y is the β-carotene concentration (grams per liter) and x is the OD_{453} value.

Extraction of β-carotene from *D. salina* culture in darkness followed previously described protocols (*19, 20*). Two milliliters of the algal culture with daily collection and/or recycled old culture medium was centrifuged at 4 °C (4000*g*, 10 min); the pellet was dissolved in acetone. The sample was agitated until all pigments were extracted. After incubation at ice-cold temperature, the pigment layer was transferred to a new tube. The extraction was repeated till the algal pellet turned white. Saponification of the pigment mixtures was performed in 60% (w/v) ethanol/KOH (9:1) for 3 h at 45 °C in order to remove chlorophylls and neutral fat. The β-carotene was extracted with ether after the addition of NaCl to a final concentration of 1.2%. The sample was dried and dissolved in acetone to measure its OD_{453} value.

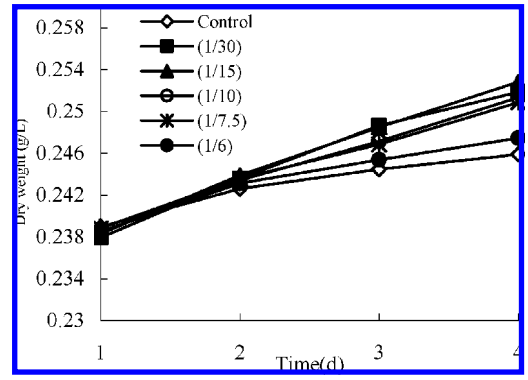

Figure 1. Time courses of cell growth with various daily collection ratios. Culture medium was collected at ratios of 1/30, 1/15, 1/10, 1/7.5, and 1/6, and then fresh medium was added to maintain the original volume. Normal culture was set as the control group. Values in the curves are the means of triplicate measurements.

3. RESULTS

3.1. Effects of Daily Gathering Volume on *D. salina* Growth and β-Carotene Accumulation. At the fixed date of the late log phase, *D. salina* culture solutions were collected according to program A. As shown in **Figure 1**, cell biomass in the remaining cultures increased after four days as the daily collection ratio decreased from 1/6 to 1/15, but not for the 1/30 group: a difference of 0.0121 g/L for the 1/7.5 group was close to that of the 1/15 group (0.0145 g/L) and higher than that of the control (0.0069 g/L). Cell density mean values of each group were higher than those at the beginning of the experiment, because the range of collection volumes selected therein was relatively narrow. Even after collection of 1/6 volume of culture per day, the *D. salina* cell numbers increased stably, but the cell growth rate was lower than that for the collection ratio of 1/7.5 group, which together with the collection ratio 1/10 group kept a stable growth rate. Therefore, the collection ratio 1/7.5 was preliminarily selected as a compromise between the collection volume and cell biomass growth.

In order to optimize the gathering volume from the mature microalgal culture at the late log phase, a 10 times scaled-up collection program (program B) was set up, based on the results of program A. **Figure 2** shows the cell growth course of the remaining cultures after gathering different volumes of culture per day. In the whole range of collection ratios the *D. salina* biomass showed an obvious deviation. The biomass curves in the three groups of 1/3, 1/3.75, and 1/5 collection ratios were descending in comparison with the control. Daily collecting 1/7.5 volume of the algal culture had an effect on increasing

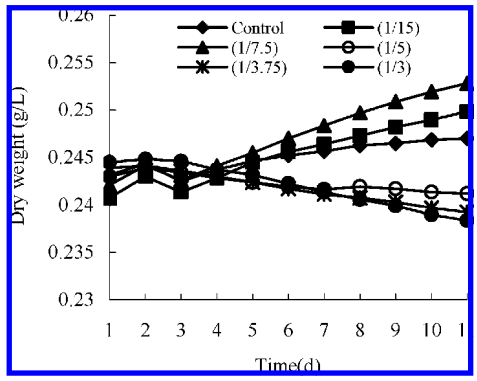

Figure 2. Time courses of cell growth in the remaining cultures with various daily collection ratios in program B. Culture was collected at ratios of 1/15, 1/7.5, 1/5, 1/3.75, and 1/3, and then fresh medium was added to maintain the original volume. Normal culture was set as the control. Values in the curves are the means of triplicate measurements.

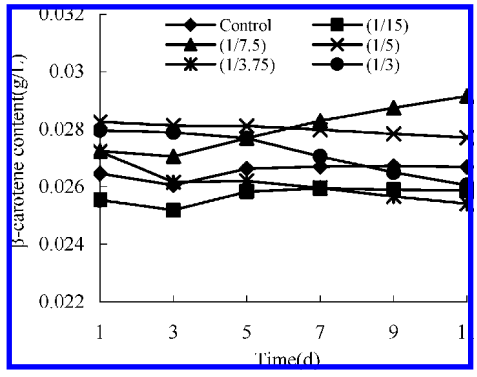

Figure 3. Time courses of β-carotene production after daily collection in program B. Culture medium was collected at ratios of 1/15, 1/7.5, 1/5, 1/3.75, and 1/3, and then fresh medium was added to maintain the original volume. Values in the curves are the means of triplicate measurements.

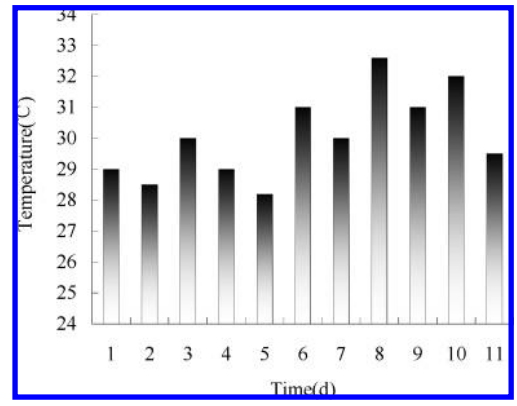

Figure 4. Temperature variation at the fixed time point per day of algal culture collection. Data were measured from *D. salina* cultures inoculated in triplicate.

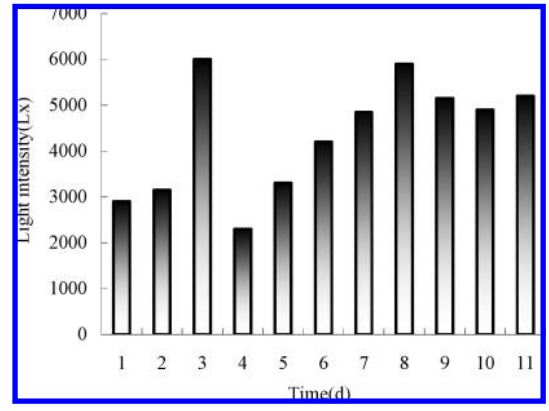

Figure 5. Light intensity variation at the fixed time point per day of collection. Data were measured at the surface of algal cultures inoculated in triplicate.

the algal biomass, and the cell mean densities of each day were higher than the control (data not shown); the incremental quantity (0.0107 g/L) was higher than that of the 1/15 collection ratio group (0.0091 g/L). This was even true when the cultivation time was prolonged for another 7 days, indicating that application of daily collecting 1/7.5 volume of the algal culture could maintain or extend the log phase of *D. salina* cultivation.

The relationship of the β-carotene contents accumulated in the cultures of program B to the cultivation date was plotted (**Figure 3**). After cultures were gathered according to program B, the β-carotene contents of the five collection ratios showed similar accumulating trends with their corresponding biomass curves, the β-carotene content differences among the five groups were enlarged when the cultivation time was lengthened to 11 days. In both 1/7.5 and 1/15 collection ratio groups, the β-carotene contents increased along with cultivation time: 0.0019 and 0.0003 g/L within the 11-day period, respectively, displaying an advantage of β-carotene accumulation over the neatly constant β-carotene content in the control. Although the ratio 1/5 and 1/7.5 groups had relatively stable accumulation, β-carotene production of the former appeared to decrease from the ninth day. The other three collection ratios (1/5, 1/3.75, and 1/3) led to decreased β-carotene synthesis.

From the above results, it was found that collecting a certain volume from the *D. salina* culture would be helpful to keep the green algae cell growing at a log phase, as well as to produce more β-carotene. The more algal culture medium was withdrawn per day, the less *D. salina* cell biomass was produced as well as less β-carotene accumulated. A balance between the daily yields of β-carotene and the stable growth of *D. salina* cells was established: herein, the daily collection ratio of 1/7.5 was considered to be more feasible even when the cultivation volume was scaled up, and it was used for subsequent experiments.

3.2. Effects of Temperature and Light Intensity on Cell Growth and β-Carotene Content. At the collection time point of each day, the temperature data at fixed locations of three parallel culture samples of *D. salina* were measured. As shown in **Figure 4**, the temperatures of the algal cultures fluctuated within a narrow range from 28.6 to 31.2 °C during a cultivation period of 11 days. Together with both curves of the cell growth and β-carotene accumulation in **Figures 2** and **3**, it could be found that the culture temperature fluctuation did not pose observable influences on the biomass and β-carotene production, both of which in the algal cultures, for instance, in the control and 1/7.5 collection ratio groups, kept a stable growing state.

Light intensity at the culture surface was determined with an illumination photometer. During the 11-day experiments of collecting culture medium, the light intensity at the collection time showed a relatively strong fluctuation, ranging from 2300 to 5900 Lx (**Figure 5**). A steep rise in the light intensity mean value of three parallel cultures was observed on the third day; this caused a systematic decrease of both OD_{453} and OD_{630} values (**Figures 2** and **3**). We supposed that the combination effects of withdrawing a certain volume of *D. salina* culture solutions and high irradiance could limit the cell proliferation and further reduce the β-carotene accumulation. Similarly to the strong drop of light intensity that may occur in the currently

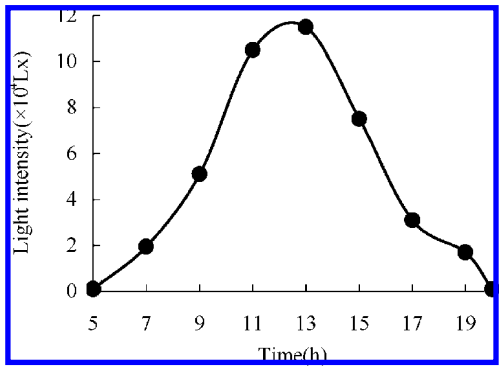

Figure 6. Light intensity variation during a cultivation daytime. Data were measured at the surface of algal cultures.

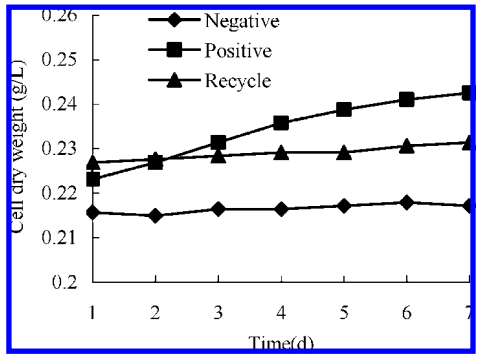

Figure 7. Cell growth curve of *D. salina* cultivated with recycled culture medium. The recycle volume was 1/7.5 of the original culture. Values in the curves are the means of triplicate measurements.

frequently adopted open-pond cultivation, the experiments with natural sunlight in this work had the same problem (**Figure 6**). The curve of light intensity, measured at the experimental site, may change its peak height and peak width along with the four seasons, but the basic peak shape did not change. In order to keep the relative balance of light intensity during the 14 h semicycle cultivation during several different seasons, a sunshade may be used at noon or additional light illumination with a fluorescence lamp may be applied in the morning and afternoon to the culture. The light intensity ranging from 4000 to 6300 Lx was proved to be suitable for the current work (detailed data not shown).

3.3. Effects of Aged and Recycle of Culture Medium on Cell Growth and *β*-Carotene Accumulation. Adding the recycled culture medium to the original culture daily showed a limited advantage over the normal culture (**Figure 7**): the cell biomass had an incremental quantity 0.0045 g/L (from 0.2269 to 0.2314 g/L) within a 7-day period, and the amplitude variation was about 4 times less than that of the positive culture and 3 times more than that of the negative control. In the negative culture group, the original cell density kept at a high point and the culture medium had aged to a certain extent, while recycling 1/7.5 volume of the culture medium without the *D. salina* cells, that is to say, daily harvest of a quantity of algal biomass, would cause a dilution effect on the cell density. On the other hand, the recycled culture medium could not supply fresh nutrition for the cell growth as done in the positive culture group, it is supposed that the aged recycled culture medium would cause a sort of inhibition on *D. salina* cell proliferation. Therefore, this method of cyclically reusing the culture medium was feasible to a certain extent for *D. salina* biomass production.

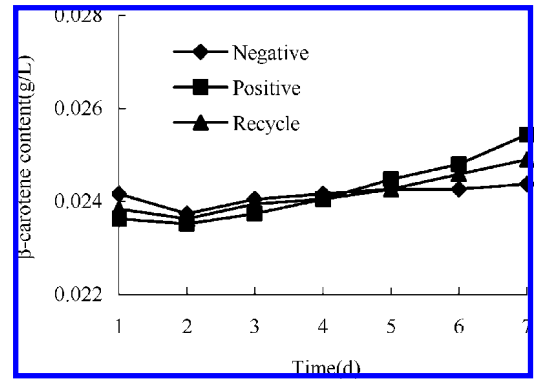

Figure 8. *β*-Carotene accumulation curve of *D. salina* cultivated with recycled culture medium. The recycle volume was 1/7.5 of the original culture. Values in the curves are the means of triplicate measurements.

As for the influence on *β*-carotene accumulation (**Figure 8**), recycling old medium showed a comparable advantage over the negative control culture. The *β*-carotene content of the recycle group had a mean increment value of 0.0011 g/L (from 0.0238 to 0.0249 g/L) during 7 days, which was less than the value of 0.0018 g/L in the positive control group but obviously higher than that of the negative control (0.0002 g/L). Although the recycled culture medium inevitably prohibited *D. salina* cell growth, it made compensation to a certain extent for the *β*-carotene accumulation in comparison with the normal culture mode; this fact implied that reusing the old culture medium without cells would have practicability.

4. DISCUSSION

In this paper we first conducted daily collection experiments of the *D. salina* mature culture and ensured the feasibility of recycling old culture. In order to decrease the operation cost, the algal culture at late log phase was daily recycled into the culture with 1/7.5 volume of original culture to obtain an advantage over the normal *D. salina* culture, in terms of both cell biomass and *β*-carotene yield. The yield of *β*-carotene by *D. salina* in open pond is generally lower than 20 mg/L; the output of *β*-carotene in biophotoreactor is 50% higher than open-pond cultivation (*14*). Both the biomass and *β*-carotene yields in the cultures with daily recycling of 1/7.5 volume of algal culture, which reached 0.2314 g/L biomass and 0.0249 g/L *β*-carotene, were comparable with those in open ponds and the results may suggest that daily recycling of a certain amount of culture medium into the original algal culture was more economical than the normal *D. salina* cultivation. The normal culture solution at the beginning of cultivation showed a color of slight yellow; it turned to a deeper color during the cultivation, and the increased cell density gradually converted the transparent culture into a state of turbidity. *D. salina* cultures that were subject to daily collection and culture medium recycling showed a life-active appearance and the cells were kept at a longer log phase; microscopic observation also showed the algal cells could keep their normal shape during the extended cultivation time (*21*).

The existence of photoinhibition under intense illumination is well-documented for algal cultures (*22*). In our experiments, a steep rise in light intensity occurred that led to a systematic decrease of both the *D. salina* cell number and total *β*-carotene content. This case was not agreeable with the general viewpoint, as high irradiance enhances accumulation of *β*-carotene in *Dunaliella* cells while limiting cell growth (*23*). We employed

relative low light intensity (4000−6300 Lx) by sunshade when needed to make a compromise between the algal cell growth and β-carotene accumulation. Actually, an exception to the general view was once observed that *D. salina* accumulated high levels of the 9-cis isomer when exposed to low irradiances (*24*).

The considerations associated with diminishing the operational inputs (water, salt, energy, land, labors, etc.) and the costs of production are important (*25*). The majority of microalgal production nowadays occurs in outdoor cultivation. As for β-carotene production by *D. salina*, technologically advanced and highly controlled large-scale closed photobioreactors do not seem to operate at present due to much higher production cost than those in open ponds, which require smaller capital investment for construction and operation and are easily scaled up (*9, 12, 26*). However, more than 90% of β-carotene is produced synthetically. Microalgal production systems, especially traditional open-air systems in the majority, need to be further improved in order to become more competitive and more economically feasible. The results in this work suggested that the method of recycling the aged culture medium (originally discarded in the normal case) with a proper daily collection ratio may be helpful to decrease the costs of production.

Furthermore, new thought should be given to exploring the commercial promise of *D. salina*. Genetic modification of carotenoid-producing microalgae is a strategy that has received great attention in the past few years (*27*). Several green algal species including *Chlamydomonas reinhardtii* and *Haematococcus pluvialis* have been transformed (*28, 29*); *D. salina* is also expected to have the feasibility to be genetically modified, based on previous reports, to enhance productivity of specific carotenoids (*11, 30*). This technology together with the improvement of algal culture system would provide broader view of producing desired products and high-value biochemical-rich algal biomass.

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