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The Comparison of Lutein Production by *Scenesdesmus* sp. in the Autotrophic and the Mixotrophic Cultivation

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Abstract The results of this study indicate that an increase in CO_2 percentage to 30% can enhance *Scenedesmus* sp. growth in autotrophic cultivation to a maximum of 0.85 g/l as compared with 0.6 g/l obtained in the batch with air (after 6 days of cultivation). However, while the CO_2 was higher than 30%, it showed a negative impact on cell growth. A mixotrophic cultivation with 3 g/l of glycerol can achieve 0.38 g $l^{-1}day^{-1}$ of the maximum biomass productivity compared with that of 0.21 g $l^{-1}day^{-1}$ in autotrophic cultivation. Nevertheless, the lutein content of the mixotrophic cultivation was 0.08–0.1% lower than 0.2–0.25% obtained in autotrophic cultivation, which led to a lower lutein productivity of 0.36 mg $l^{-1}day^{-1}$ in the mixotrophic batch compared with 0.44 mg $l^{-1}day^{-1}$ obtained in the autotrophic batch. The limitation of cell growth in the mixotrophic cultivation would be the contributing factor regarding the lower lutein productivity. The mixotrophic cultivation of repeated batch to remove potential inhibitive metabolic products from glycerol catabolism does not show an obvious improvement on biomass. Conclusively, mixotrophic cultivation achieves higher biomass productivity with lower lutein content than that of autotrophic cultivation, which leads to lower lutein productivity. Therefore, the autotrophic cultivation is preferred in the lutein production.

Keywords Mixotrophic · Luedeking–Piret · Lutein · Autotrophic · *Scenedesmus*

Introduction

Lutein is an important carotenoid which has been used for the pigmentation of animal tissues and products in the foods, drugs, and cosmetics industries. The commercial market of lutein in the USA was estimated to be about US $150 million per year [1]. Lutein has been also proposed for the prevention of cancer and diseases related to retinal degeneration [2]. Therefore, the large scale production of lutein in a more economically competitive way has gained much attention recently.

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A marigold flower contains abundant amounts of a valuable antioxidant compound lutein, which is currently the primary commercial lutein production source [1, 3]. Two different parts of marigold (petals and calyces) are often adopted for lutein production through traditional chemical extraction procedures. The petals of the marigold are rich in lutein and lutein fatty acid esters which, on the whole, represent over 90% of the pigments identified in this plant. However, marigold presents drawbacks as a lutein source; the flowers must be periodically harvested and the petals separated prior to extraction. The lutein content in marigold petals is variable and could be as low as 0.03% [3]. As a result, lutein production from marigold petals is a labor-intensive process that demands the use of large tracts of land, making it infeasible in many developing economies. Recently, a new process involving the production of lutein using microalgae instead of marigold has been shown to be an attractive alternative. Several microalgae have been proposed as potentially adequate lutein sources, such as *Muriellopsis* sp. [4], *Chlorella zofingensis* [5], or *Chlorella protothecoides* [6]. *Muriellopsis* sp. is a chlorophycean microalga which can achieve a high lutein production concentration (up to 35 mg/l) under specific culture conditions as well as a high growth rate and high cell density. The maximal biomass concentration and lutein content of *C. protothecoides* CS41 cultivated heterotrophically with 9 g/l glucose in a 3.7-L fermentor were 4–4.6 g dry cells/l and 4.4–4.6 mg lutein/g dry cells on different culturing medium, respectively. The heterotrophic cultivation process was scaled up successfully to a 30-L fermenter. Thus, there has been a development of processes that allows the industrial production of microalgae rich enough in lutein so as to supply a daily recommended uptake of lutein consisting of 6 mg per day.

Autotrophic cultivation has often been adopted for the growth of microalgae. However, mixotrophic cultivation can provide a more optimal environment for the growth of microalgae, which often leads to a higher growth rate compared with conventional autotrophic cultivation [7]. The mixotrophic cultivation is an alternative to the photoautotrophic production of biomass. The growth rates and biomass concentrations increase due to a synergistic effect of light and the organic substrate [8, 9]. Among all possible substrates for the mixotrophic cultivation, the use of glycerol as feedstock for the cultivation has gained more attention recently. Notably, glycerol is a by-product of the biodiesel production process, estimated to be as high as 10% (*w*/*w*) of the output. It is estimated that, globally, nearly 11 M tones of biodiesel (merely a third of name-plate capacity) will be manufactured. About 1.1 M tones of the glycerol by-product was produced in 2008 [10]. In the production of eicosapentaenoic acid (EPA) by diatom *Phaeodactylum tricornutum*, glycerol was added to the medium in the range of 0–0.1 M. These results suggest that 0.1 M was an optimal concentration for growth and EPA production. However, a lag phase was observed in the batch with a high concentration of glycerol [9]. In the autotrophic cultivation of *Scenedesmus* for lutein production, the lutein content was generally less than 0.5% (5 mg lutein/g dry cell weight) [1]. Theoretically, such values would not have much of an economic impact. As such, the improvement of lutein production can come from two advancements. The first one involves directly increasing the lutein content while the second method involves enhancing the biomass productivity but maintain the lutein content. Notably, the mixotrophic cultivation was known to have a higher growth rate than the autotrophic cultivation. However, not much information about the mixotrophic cultivation of *Scenedesmus* was released in the literature. In this study, we attempted to use mixotrophic cultivation instead of autotrophic cultivation to increase biomass productivity for lutein production.

This study targeted the investigation of cell growth and lutein production under mixotrophic cultivation of *Scenedesmus* sp. using glycerol. This is compared with lutein production under conventional photoautotrophic cultivation. A repeated batch operation

employing mixotrophic cultivation was performed to investigate the effects of biomass enhancement by the potential inhibitive metabolic products removed.

Materials and Methods

Microorganism and Medium

The microalgae *Scenedesmus* sp. was generously donated by Prof. Jo-Shu Chang (National Cheng Kung University, Taiwan). The modified basal medium supplemented with designed amounts of glycerol (from 1 to 10 g/l) was used for mixotrophic cultivation in flasks. Details of the modified basal medium preparation are described in past research [11]. For autotrophic and mixotrophic cultivation, the initial biomass concentration was controlled at around 100 mg/l, which was transferred from the previous prepared algal stock solution.

Cultivation

The mixotrophic cultivation was grown at 25±1 °C, bubbled with 0.2-vvm air supplemented with 10% (*v*/*v*) CO_2 in a 500-ml flask (containing 400-ml medium). The flask was agitated using a stir bar at around 100 rpm and continuously illuminated with white fluorescent lamps (FL-20D, 12000 LUX measured at the surface of the flask). The conditions for autotrophic cultivation were similar to the mixotrophic process described above. Notably, in the latter, there was no glycerol supplement in the culture medium.

Repeated Batch Operation

A medium with 3 g/l of glycerol was prepared for mixotrophic cultivation grown at 25±1 °C and 0.2-vvm air supplemented with 10% (*v*/*v*) CO_2 in a 500-ml flask (containing 400-ml medium). After 3 days of cultivation, the supernatant was removed through centrifugation at 5,000 rpm for 10 min. The remaining biomass was transferred to another sterilized fresh medium containing 3 g/l of glycerol for mixotrophic cultivation. A total of three repeated batches were performed in this study.

Glycerol and Biomass Analysis

Glycerol was analyzed using a high-performance liquid chromatography (HPLC) system (Agilent 1100 LC) under the following conditions: column, Vercopack inertsil 7 ODS-3 (250×4.68 mm); temperature, ambient (27±1°C); mobile phase, 0.01 N sulfuric acid; flow rate, 0.6 ml/min; refractive index detector; then, 50 μl was injected into the column [12]. The biomass concentration was estimated by absorbance at 560 nm, and the relationship between optical density (OD_{560}) and dry cell weight (DCW) is given as DCW (g/l)= $0.8939 \times OD_{560} - 0.0351$

Lutein Concentration and Pigment Measurement

Five milliliters of thoroughly mixed culture fluid from the fermentor was transferred to a 15-ml conical tube. The culture was then centrifuged at 8,000 rpm for 15 min, and the supernatant was discarded. Five milliliters of methanol was subsequently added to the mixture for the extraction of lutein ultrasonically. The mixture was then centrifuged at

8,000 rpm for 15 min at 4 °C [11]. Pigments were analyzed using an HPLC system under the following conditions: column, N 5ODS (250×4.68 mm); temperature, ambient (27±1 °C); mobile phase, initially consisted of 60% solvent A (acetonitrile/methanol, 20/80 *v*/*v*) and 40% solvent B (methanol/acetone, 80/20 *v*/*v*), which was finally brought to 30% solvent A and 70% solvent B over a period of 15 min. The column was subsequently returned to its original solvent composition of 60% solvent A and 40% solvent B over the next 6 min prior to the injection of a new sample; flow rate, 1.0 ml/min; and UV detector (Hitachi) at 450 nm [13].

Results and Discussion

Lutein Production Under Autotrophic Cultivation

Under autotrophic conditions, CO_2 was used as the carbon source for cell growth through the Calvin cycle. Therefore, the increase in CO_2 percentage in the inlet gas might enhance the cell growth. The results of adjusting CO_2 percentages in the inlet gas are shown in Fig. 1. The results indicate that an increase of CO_2 in the inlet gas could efficiently enhance the algal biomass as compared an environment with using only air. While the CO_2 percentage increased from 10% to about 30% of the inlet gas, not much difference in the growth was observed. The maximum biomass concentration obtained 0.85 g/l after 140 h of cultivation as compared with 0.6 g/l obtained in air. However, the biomass level fell rapidly while the CO_2 concentration was over 40%. It became obvious that excessive CO_2 concentrations will impede cell growth. Lee and Tay had reported the similar results such that the steady state biomass production rate and bioenergetic growth yield were related inversely to CO_2 in the range of 2–90 kPa (2–89%) of CO_2 in a light-limited context in the cultivation of *Chlorella pyrenoidosa* [14]. The time course of algal growth and lutein production under autotrophic conditions (supplemental with 10% CO_2) is shown in Fig. 2. As seen in the figure, the algal biomass and lutein concentration increase up to 2.1 g/l and 5.6 mg/l after 11 days of culturing. The maximum lutein production rate is about 0.51 mg $l^{-1}day^{-1}$. However, the cell growth rate gradually decreases with the increase of biomass. The decrease of cell growth rate is possibly due to an insufficient light intensity supplied under the condition of high biomass density. The lutein content of the algal biomass is mostly in the range of 0.2–0.25% (grams of lutein per gram of algal biomass)

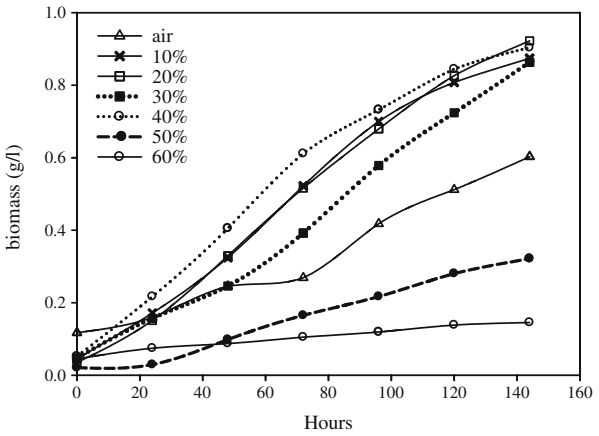

Fig. 1 Effects of CO_2 percentage in the inlet gas on the growth of *Scenedesmus* in the autotrophic cultivation

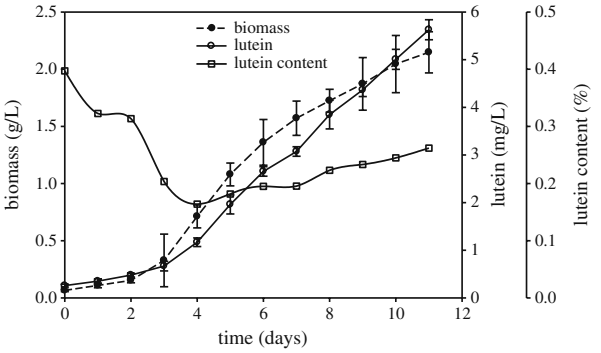

Fig. 2 Biomass, lutein concentration, and lutein content in the autotrophic cultivation of *Scenedesmus* with 10% of CO_2 supplemental in the inlet gas

during the whole process. As shown in the literature, an isolated new strain of *Scenedesmus* could potentially achieve 0.54% lutein content under autotrophic conditions after the culturing optimization using the response surface methodology [15]. Notably, the lutein content yields a maximum value of 0.39% at the onset of culturing. However, the value decreases rapidly with the increase of culturing time.

Logistic [16] and Luedeking–Piret [17] equation were adopted for the simulations of cell growth and lutein production in the autotrophic growth at 10% CO_2.

$$\frac{1}{X}\frac{\mathrm{d}X}{\mathrm{d}t} = \mu_{\mathrm{m}}\left(1 - \frac{X}{X_{\mathrm{m}}}\right) \text{Logistic equation}$$

Where μ_{m} is the maximum specific growth rate (1/day) and X_{m} is the maximum attainable biomass concentration.

$$\frac{\mathrm{d}P}{\mathrm{d}t} = \alpha\frac{\mathrm{d}X}{\mathrm{d}t} + \beta X \text{ Luedeking} - \text{Piret equation}$$

Where P is the lutein concentration and X is the biomass concentration measured. The genetic algorithm was adopted for the estimation of equation parameters. The kinetic parameters of μ_{m}, X_{m}, α, and β obtained in Logistic and Luedeking–Piret were 0.656 (1/day), 2.113 (g/l), 1.160 (mg/g), and 0.250 (mg/g day). The comparison of experimental data and simulation results was shown in Fig. 3. Both simulation curves of biomass and lutein production had R^2 values of 0.993 and 0.997, which indicated both equations of Logistic and Luedeking–Piret could be quite well fitting to the experimental data. Under the autotrophic conditions with 10% CO_2, the maximum attainable biomass was 2.113 g/l according to the

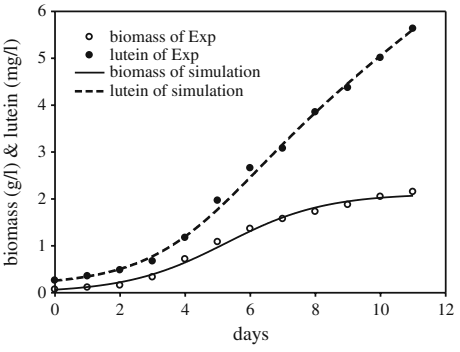

Fig. 3 Comparison of cell growth and lutein production between experimental data and simulation results in the autotrophic cultivation of *Scenedesmus* with 10% of CO_2 supplemental in the inlet gas

simulation results of Logistic. The high value of α (1.16) compared with 0.25 of β shown in Luedeking–Piret equation suggested that lutein production might intend to be growth associated. Therefore, an extended growth phase could possible lead to more lutein produced.

Effects of Glycerol Concentration on Mixotrophic Cultivation

Mixotrophic cultivation allows for a higher growth rate than autotrophic cultivation. Therefore, an investigation using glycerol to improve cell growth under mixotrophic conditions was performed. The light intensity in the mixotrophic conditions was as the same as that in autotrophic condition and the CO_2 was supplemented at 10% in the inlet gas. As shown in Fig. 4 regarding the mixotrophic cultivation of *Scenedesmus* with different concentrations of glycerol adding (1, 3, 5, 7, and 10 g/l), the increase of initial glycerol concentration slightly increased the biomass concentration to a maximum of 1.9 g/l after 72 h of cultivation in the batch using 3 g/l glycerol. While the glycerol concentration was higher than 3 g/l, the cells could not completely digest it and large amounts of residual glycerol were observed in the medium. The yield of biomass produced per gram of glycerol consumed is in the range of 0.7–1.3 g/g under the mixotrophic cultivation. However, the lutein content decreased from 0.12% to about 0.06% with the increase of glycerol concentration from 1 to 10 g/l. The results suggest that, with a limited glycerol supply, the algal cell would also utilize CO_2 as the carbon source for cell growth. Therefore, the biomass yield would be as high as 1.3 g biomass formed per gram of glycerol consumed in the batch with 1 g/l of glycerol. Additionally, the increase of glycerol concentration would have negative impact to lutein production. The lutein production rate would decrease from 0.53 to 0.32 mg $l^{-1}day^{-1}$ with the increase of glycerol from 1 to 10 g/l. Therefore, in mixotrophic cultivation for lutein production, the adding glycerol concentration would have an optimal value. In the production of EPA, the optimal glycerol concentration of 0.1 M (0.92 g/l) was obtained by *P. tricornutum* under mixotrophic conditions [9]. To further investigate the effects of glycerol on mixotrophic cultivation, a batch with mixotrophic cultivation at 3 g/l of glycerol was performed to compare the performance of lutein and biomass production using autotrophic cultivation.

Comparison of Lutein Production Between Autotrophic and Mixotrophic

The comparison of biomass and lutein production between autotrophic and mixotrophic conditions is shown in Fig. 5. The results indicate that the biomass production in mixotrophic cultivation with 3 g/l of glycerol was faster than autotrophic cultivation. The maximum

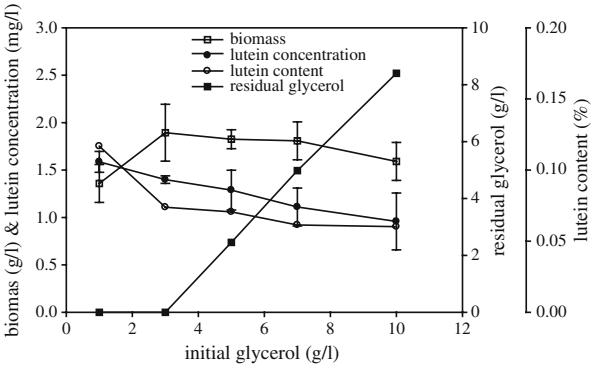

Fig. 4 Effects of initial glycerol concentration (1, 3, 4, 7, and 10 g/l) on the mixotrophic growth of *Scenedesmus* sp. after 72-h cultivation

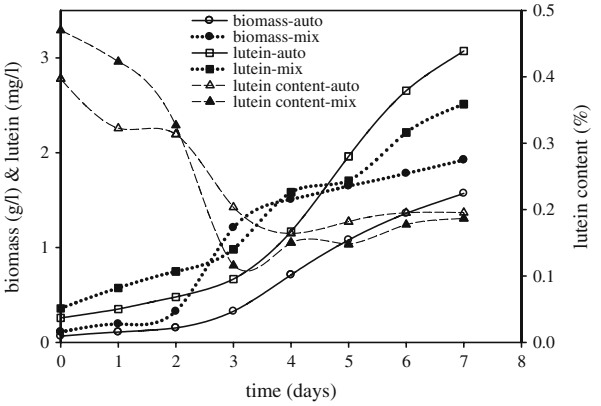

Fig. 5 Comparison of biomass, lutein concentration, and lutein content under the conditions of autotrophic and mixotrophic cultivation with 3 g/l of glycerol

growth rates were 0.21 and 0.38 g $l^{-1}day^{-1}$ in the batches of autotrophic and mixotrophic, respectively. However, the batch with autotrophic cultivation would have greater lutein content than that of the mixotrophic cultivation. Obviously, while photosynthesis was the major route for the cell growth, it could accumulate more lutein. Conclusively, the mixotrophic growth using glycerol as the carbon source could enhance the biomass growth rate, but decrease the lutein content as compared with the values obtained in autotrophic cultivation. Therefore, the overall lutein production rate in the mixotrophic batch would be 0.36 mg $l^{-1}day^{-1}$, which was lower than 0.44 mg $l^{-1}day^{-1}$ obtained in the autotrophic batch after 7 days of cultivation. Notably, the metabolic products of glycerol used in mixotrophic cultivation seemed to show a feedback inhibition to cell growth, which led to the limitation of final biomass concentration in mixotrophic cultivation. As seen in Fig. 4, the maximum biomass was less than 2 g/l in mixotrophic cultivation. Even with a higher initial glycerol concentration in the medium, the biomass concentration was still restricted to less than 2 g/l. Since the lutein content in mixotrophic cultivation was lower than the value obtained in autotrophic cultivation, the total biomass in mixotrophic cultivation would be detrimental to the total lutein production. More accumulated biomass would efficiently increase the total lutein production. Therefore, a repeated batch operation using mixotrophic cultivation was performed to remove the possible growth-inhibited products derived from glycerol in order to enhance the cell growth.

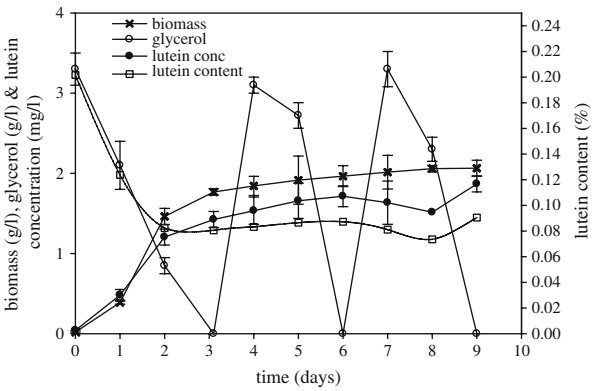

Fig. 6 The results of repeated fed-batch (3 days for each batch) with cell retained under the mixotrophic conditions with 3 g/l of glycerol

Lutein Production in a Repeated Batch Under Mixotrophic Conditions

A repeated batch with cell recycling was performed to investigate the growth effects of potential inhibition derived from the metabolic products of glycerol catabolism. In the repeated batch operation, the total biomass was retained after the broth was removed using centrifugation. If the metabolic products of glycerol remain to be possible reasons leading to the inhibition of cell growth, a repeated batch operation could efficiently enhance the cell growth. However, the operation of a repeated batch to remove potential inhibitive products would not have positive effects to the cell growth after three repeated batches, as the results show in Fig. 6. As seen in the figure, a total of three cycles of mixotrophic cultivation (3 days for each batch) were performed. The glycerol was consumed completely at the end of each batch. The biomass ceased to increase after the first batch, which led to a 2 g/l of maximum biomass observed. The lutein content had an almost constant value of 0.08–0.1% obtained during the overall repeated batches. The results of this repeated batch operation indicates that potential inhibition coming from the metabolic production of glycerol is not the leading reason for the slowing and stopping of cell growth in mixotrophic cultivation. The reasons for the limitation of cell growth in mixotrophic cultivation are still not clear.

Conclusions

Lutein production from algal biomass was an attractive idea and has gained much attention recently. The growth of *Scenedesmus* under autotrophic conditions can obtain an average lutein content of 0.2–0.25%. However, the maximum biomass growth rate was shown to be 0.21 g $l^{-1}day^{-1}$. The simulation results by Luedeking–Piret equation indicated that the production of lutein under autotrophic conditions might be regarded as a growth-associated production. In mixotrophic cultivation with 3 g/l of glycerol, the biomass growth rate showed a notable increase to 0.38 g $l^{-1}day^{-1}$. Nevertheless, the lutein content decreased to about 0.08–0.1%. Besides the low lutein content, the biomass concentration was limited to not more than 2 g/l in mixotrophic cultivation. No obvious improvement on biomass growth was observed; even a repeated batch operation was adopted. Conclusively, glycerol, as the carbon source in mixotrophic cultivation for lutein production, had some advantages to autotrophic cultivation. However, the mixotrophic cultivation led to lower lutein productivity as presented in this study, which resulted in a conclusion that the autotrophic cultivation would be preferred in lutein production.

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