ISSN 1330-9862
(FTB-2153)

scientific note

Biochemical System Analysis of Lutein Production by Heterotrophic *Chlorella pyrenoidosa* in a Fermentor

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Received: September 10, 2008
Accepted: March 25, 2009

Summary

Chlorella is a promising alternative source of lutein, as it can be cultivated heterotrophically with high efficiency. In this study, the carotenoids in *Chlorella pyrenoidosa* heterotrophically cultivated in a 19-litre fermentor have been analyzed and determined by using HPLC and HPLC-MS. A biochemical system theory (BST) model was developed for understanding the regulatory features of carotenoid metabolism during the batch cultivation. Factors that influence lutein production by *C. pyrenoidosa* were discussed based on the model. It shows that low flux for lycopene formation is the major bottleneck for lutein production, while by-product syntheses and inhibitions affect the cellular lutein content much less. However, with further increase of the cellular lutein content, the inhibition on lycopene formation by lutein may become a limiting factor. Although speculative, these results may provide useful information for further elucidation of the regulatory mechanisms of carotenoid biosynthesis in *Chlorella* and modifying its metabolic network to enhance lutein production.

Key words: Chlorella pyrenoidosa, lutein, heterotrophic cultivation, biochemical system analysis, modelling

Introduction

Lutein is a natural colorant applied widely in food or food materials. It can also play an important role in delaying some chronic diseases (*1*,*2*) and preventing the loss of sight caused by age-related macular degeneration (*3*). French marigold is currently the most widely applied source of lutein production. However, mass plantation of marigold occupies large land area and it is easily influenced by season and climate. Recently, production of lutein by microalgae such as *Chlorella* has drawn increasing attention of researchers (*4*,*5*). Although these algae were found to accumulate more lutein in autotrophic cultivations, the restrictions associated with illumination and other cultivation parameters limit their development (*6*,*7*). Previous studies in our laboratory showed that heterotrophically cultivated *Chlorella* contained considerable amount of lutein, which is comparable to that in marigold (*8*). The ability of heterotrophic growth in fermentors with high efficiency makes *Chlorella* a potential alternative resource for commercial production of lutein (*9*,*10*).

However, compared to the high cellular content of β-carotene in other carotenoid recourses such as *Dunaliella salina* and *Blakeslea trispora* (*11*,*12*), the cellular lutein content of *Chlorella* obtained presently, usually between 2–4 mg/g (*8*), is much lower. This shows both the necessity and the possibility of further enhancing lutein production by *Chlorella*. As optimizing culture parameters (such as pH, temperature and medium components) does not seem very efficient to attain this goal (*13*,*14*), modifying metabolic network may be required in the future. Understanding the regulatory features of carote-

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noid metabolism is undoubtedly the prerequisite for this purpose. Unfortunately, the information on the regulation of carotenoid metabolism, either qualitative or quantitative, is very limited, although the biochemical pathways of carotenoid biosynthesis have been elucidated for many organisms (*15,16*). The complexity of the metabolic network, as well as the difficulties in separating and assessing the enzymes involved, make investigating its regulatory mechanisms by experimenting an extremely tough task (*17,18*).

As complementary to experimental approaches, biochemical system theory (BST) provides a framework for system analysis by using power-law expansions in the variables of the system to represent the biochemical processes (*19–21*). With the simplicity in model construction and analysis, BST model is effective in analyzing complicated metabolic paths and testing the hypotheses of their regulatory features even when detailed information is unavailable (*22,23*).

The aim of this study is to develop a BST model to understand the regulatory features of lutein production by heterotrophic *Chlorella pyrenoidosa*, and provide information for future optimization of the metabolic network to enhance lutein production by this alga.

Materials and Methods

Alga, medium and cultivation conditions

Chlorella pyrenoidosa 15-2070 obtained from Carolina Biological Supply Co., Burlington, VT, USA was used in this study. The modified basal medium contained (in g/L): KH_2PO_4 1.25, $MgSO_4$ 1.0, EDTA 0.5, H_3BO_3 0.1142, $CaCl_2$ 0.111, $FeSO_4$ 0.0498, $ZnSO_4$ 0.0882, $MnCl_2$ 0.0142, MoO_3 0.0071, $CuSO_4$ 0.0157 and $Co(NO_3)_2$ 0.0049 supplemented with 40 g/L of glucose and 7 g/L of KNO_3.

A 19-litre fermentor (Bioengineering AG, Wald, Switzerland) was used for batch cultivations of heterotrophic *C. pyrenoidosa*. The cultivation conditions were: working volume 12 L, inocula 10 %, temperature 28 °C, aeration rate 1 vvm; agitation speed was cascaded to the dissolved oxygen and the dissolved oxygen was set at 50 %; pH was maintained at 6.5 during the cultivation.

Analytical methods

The dry cell mass of *C. pyrenoidosa* was measured according to Chen *et al*. (*24*). The specific cell growth rate during the cultivation was calculated as follows:

$$\mu = (1/x_1)\cdot(x_2 - x_1)/(t_2 - t_1) \qquad /1/$$

where μ is the specific cell growth rate (h^{-1}), x_1 is the biomass concentration (g/L) at time t_1 (h), and x_2 is the biomass concentration (g/L) at the next time point t_2 (h).

Lutein and other components in the cell extracts were assayed and determined by using an HPLC system (Waters-Millipore, Bedford, MA, USA) and an HPLC-MS system (HP-1100MSD, Agilent Technologies, Santa Clara, CA, USA), the details of which had been described in literature (*25*). Standard lutein and chlorophyll *a* and *b* were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Software

The PLAS (Power Law Analysis and Simulation) software freely available at *http://www.dqb.fc.ul.pt/docentes/aferreira/plas.html* was used for model construction, the integration of differential equations in the BST model and analyses.

Results and Discussion

Determination of carotenoids in heterotrophic C. pyrenoidosa

Three batch cultivations of *C. pyrenoidosa* were carried out in a 19-litre fermentor, and the typical results are shown in Fig. 1. It can be seen that the specific cell growth rate increased first, attained its maximum at about 50 h, then decreased until the cell growth stopped (Fig. 1a); while an almost opposite trend is observed for the cellular lutein content (Fig. 1b).

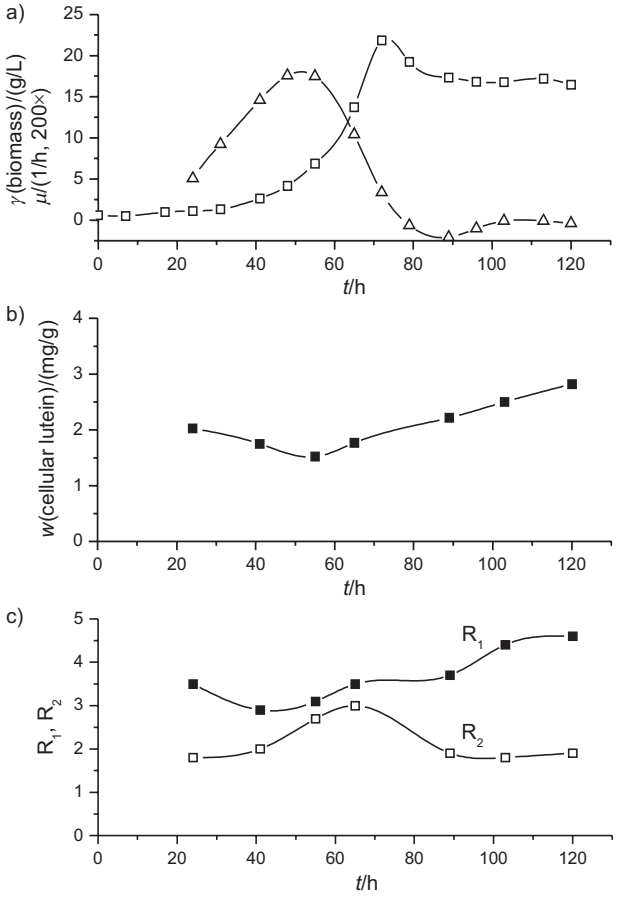

Fig. 1. Time courses of cell growth: (a) □ biomass concentration, △ specific cell growth rate; (b) cellular lutein content, and (c) ratios R_1 and R_2 in a typical batch cultivation of *C. pyrenoidosa* (R_1=(lutein+loroxanthin)/(violaxanthin+neoxanthin); R_2=lutein/loroxanthin)

The HPLC chromatograms of *C. pyrenoidosa* extracts are presented in Fig. 2. Six peaks were observed with the UV/VIS detector. Peaks 4, 5 and 6 were identified as lutein, chlorophyll *b* and chlorophyll *a*, respectively, by comparing their retention times and spectra with those

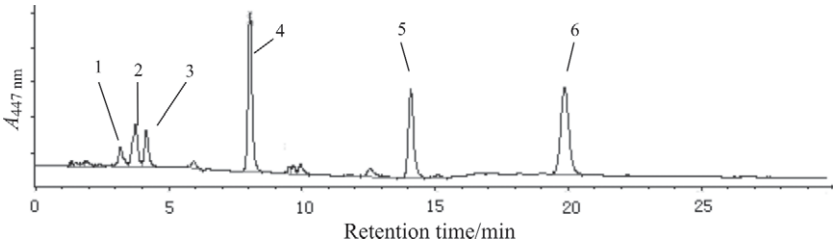

Fig. 2. HPLC separation of *C. pyrenoidosa* extracts. Peak identification: 1 neoxanthin, 2 loroxanthin, 3 violaxanthin, 4 lutein, 5 chlorophyll *b*, 6 chlorophyll *a*

of the corresponding standards. Peaks 1, 2 and 3 were tentatively identified as neoxanthin, loroxanthin and violaxanthin, respectively, by comparing their retention times and spectra with the published data (*26–28*). All these carotenoids are closely related with the metabolism of lutein (*14*).

Two ratios, R_1=(lutein+loroxanthin)/(violaxanthin+neoxanthin) and R_2=lutein/loroxanthin, which determine the flux distribution between lutein and by-products, were calculated according to their peak areas in the HPLC chromatograms, with the consideration of their similar absorption coefficients (*29*). It was observed that these two ratios varied during the cultivation (Fig. 1c), implying the regulation of carotenoid metabolism.

BST model for lutein production by C. pyrenoidosa

The pathway of carotenoid metabolism in the microalga has been reported in literature (*14,30*). As only four carotenoids (neoxanthin, loroxanthin, violaxanthin and lutein) were detected in our experiments, the pathway of carotenoid metabolism in *C. pyrenoidosa* can be simply schemed (Fig. 3). Here some intermediate reactions were lumped and the carotenoids that were not detected in our experiments were excluded. Though lycopene was not detected either, it should be considered as a key branch point for carotenoid metabolism. On the basis of the simplified metabolic pathway, a BST model was developed to understand and analyze the regulation features of carotenoid metabolism in *C. pyrenoidosa*.

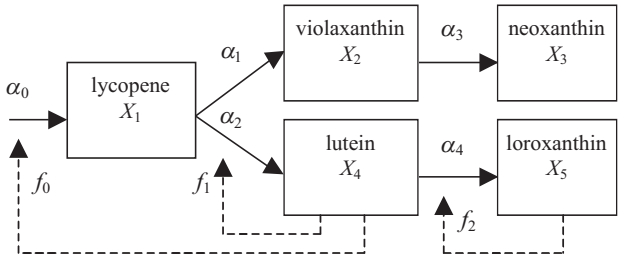

Fig. 3. Simplified metabolic pathway of carotenoid formation by *C. pyrenoidosa*

The hypotheses of the model are as follows: (*i*) most of the transforming rates are in proportion to corresponding substrate contents. Although there is no experimental evidence for this assumption, it seems rational as the carotenoid content in *C. pyrenoidosa* is relatively low, and thus is not likely to saturate the corresponding enzymes (*20*); (*ii*) the syntheses of lycopene and lutein are influenced by cellular lutein content, while the formation of loroxanthin is influenced by its content in the cell. This assumption was made to ensure the variations of ratios R_1 and R_2 and cellular lutein content observed in the experiments. Obviously, these variations are determined by flux distributions, for which feedback control is the most possible reason.

BST is a framework for steady state analysis. However, only batch cultivation data were available in our study. In order to make the system run near its steady state, the cellular content of the carotenoids, instead of carotenoid concentration in the culture, was chosen to be the model variable. In this case, dilution items determined by the specific growth rate, μ, should be considered. To simplify the calculations, model variables were expressed in mg/g, and transformation coefficients were neglected as the molecular mass of these carotenoids is similar.

Based on the assumptions above, a BST model for carotenoid metabolism in *C. pyrenoidosa* was constructed as follows:

$$X_1 = \alpha_0 X_4^{f_0} - (\alpha_1 + \alpha_2 X_4^{f_1} + \mu) X_1 \qquad /2/$$

$$X_2 = \alpha_1 X_1 - (\alpha_3 + \mu) X_2 \qquad /3/$$

$$X_3 = \alpha_3 X_2 - \mu X_3 \qquad /4/$$

$$X_4 = \alpha_2 X_1 X_4^{f_1} - (\alpha_4 X_5^{f_2} + \mu) X_4 \qquad /5/$$

$$X_5 = \alpha_4 X_4 X_5^{f_2} - \mu X_5 \qquad /6/$$

where X_1–X_5 are the cellular content of lycopene, violaxanthin, neoxanthin, lutein and loroxanthin, respectively (in mg/g), μ is the specific cell growth rate of *C. pyrenoidosa* (h^{-1}), α_0–α_4 are rate constants for corresponding reactions, and f_0–f_2 are kinetic orders of feedback control (Fig. 3).

The model parameters were estimated based on the experimental results. Briefly, the specific cell growth rate μ was determined according to the experimental data and set at a moderate value of 0.045 h^{-1} (the specific cell growth rate in Fig. 1 ranged from 0 to 0.09 h^{-1}). The steady values of X_2–X_5 were set at moderate values (0.62, 0.25, 2.5 and 1 mg/g, respectively) referring to the corresponding cellular carotenoid content observed in our experiments (including cultivations both in fermentor and in flasks, details not shown). The steady value of X_1 was arbitrarily set at a low value of 0.01 mg/g, as lycopene was not detected in our experiments and simulations showed that different assumptions of this steady value did not influence model predictions significantly

when it was low. Rate constants α_0–α_4 were calculated based on the steady values of X_1–X_5 after the kinetic orders f_0–f_2 had been determined. Kinetic orders f_0–f_2 were estimated by manual adjustment of their values in the range between –1 and 1 (*19,20*) until the values of X_4, R_1 and R_2 under varied specific cell growth rates predicted by the model were close to those observed in the experiments. Cellular lutein content (X_4) was observed to vary between 1.5–3.5 mg/g in our experiments (including cultivations both in fermentor and in flasks, details not shown). R_1 and R_2, ranging from 3 to 5 and from 1.5 to 3.5 respectively, in the experiments were obtained using the following equations:

$$R_1 = \frac{\text{lutein} + \text{loroxanthin}}{\text{violaxanthin} + \text{neoxanthin}} = \frac{X_4 + X_5}{X_2 + X_3} \qquad /7/$$

$$R_2 = \frac{\text{lutein}}{\text{loroxanthin}} = \frac{X_4}{X_5} \qquad /8/$$

Parameters estimated for the model and sensitivity analysis are shown in Table 1. No extraordinarily high sensitivities of the model parameters were observed (the variation ranges of lutein content corresponding to all model parameters are much less than 10 %, which is the variation range of specified model parameters), suggesting the robustness of the system. Parameters μ and α_0 have relatively high sensitivities, implying that the specific cell growth rate and the lycopene formation rate are two major factors affecting the cellular lutein content in *C. pyrenoidosa*.

Table 1. Parameters estimated for the BST model and sensitivity analysis

Parameter	Value	Predicted steady cellular lutein content of +/–10 % changes in specified model parameters	
		10 %	–10 %
μ	0.0450	–4.8	4.8
α_0	0.4496	5.6	–6.0
α_1	3.9150	–1.2	1.2
α_2	13.1127	1.2	–1.2
α_3	0.01814	0	0
α_4	0.01800	–1.2	1.2
f_0	–0.9	0	0
f_1	0.4	0	0
f_2	–0.5	0	0

Sensitivities of kinetic orders f_0–f_2 were zero. This means that these parameters are unimportant for lutein production at present steady state (μ=0.045 h^{-1}). However, simulations with varied specific cell growth rate showed that f_0–f_2 were important to ensure appropriate trends and ranges of the variables. For example, the negative value of f_0 reflects the inhibition of lycopene formation by lutein. Without considering this inhibition, cellular lutein content is much higher than that observed in the experiments when the specific cell growth rate decreases. The positive value of f_1 suggests the stimulation of lutein production by itself, which is to ensure the decrease of R_1 when the specific growth rate increases. Although the latter may also be attributed to the inhibition of violaxanthin formation by itself (or by lutein), simulations showed that these different assumptions had similar results.

Simulations with varied specific cell growth rates are illustrated in Fig. 4. With the decrease of the specific cell growth rate, R_1 and cellular lutein content X_4 increased, while R_2 decreased, and new steady states were attained after about 20–40 h. As the specific cell growth rate varies continuously in batch cultivation, model predictions here cannot be compared directly and strictly to experimental data. Nevertheless, they are similar both in trend and in range (Fig. 1).

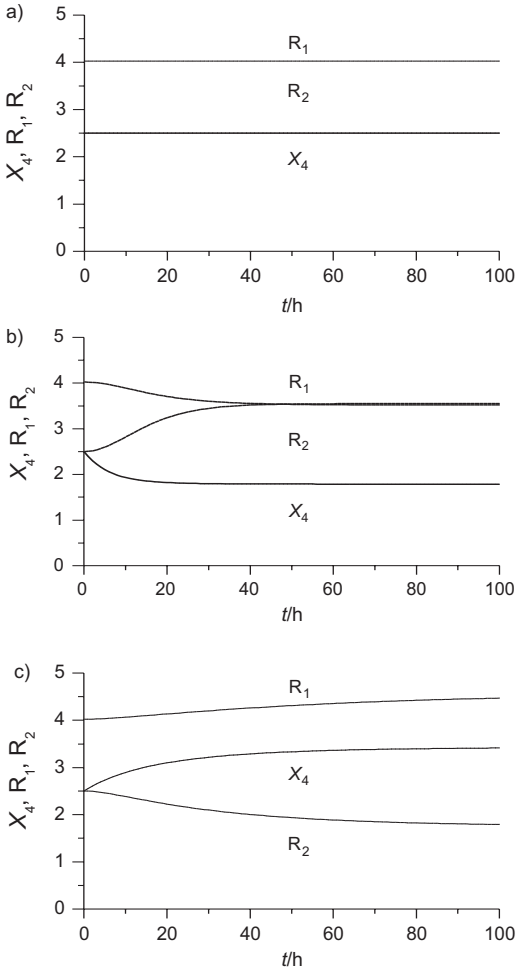

Fig. 4. Kinetics of R_1, R_2 and cellular lutein content, X_4, predicted by BST model under various specific cell growth rates (a: μ=45; b: μ×2; c: μ×0.5)

Factors influencing lutein production and possible strategies for evaluating the cellular lutein content in C. pyrenoidosa

The rationality of the model encouraged us to further analyze the factors influencing lutein accumulation and compare several possible strategies for evaluating cellular lutein content in *C. pyrenoidosa*. This may provide useful information for future metabolic network

modification to enhance lutein production by this alga: (*i*) to decrease the specific cell growth rate. Both model predictions and experimental results showed the possibility of elevating the cellular lutein content with this strategy (Figs. 1 and 4). However, as lutein is a cellular component of *C. pyrenoidosa*, lowering the specific growth rate severely decreases lutein productivity, which makes it unacceptable for commercial productions; (*ii*) to block the syntheses of by-products. Simulations showed that the cellular lutein content rose slightly by blocking the synthesis of loroxanthin or violaxanthin, and blocking their syntheses simultaneously had a better effect (Fig. 5). The reason why cellular lutein content cannot be increased significantly with this strategy may be attributed to the relative low flux for by-product formations. Therefore, blocking by-product syntheses does not seem to be an effective strategy to increase the cellular lutein content of *C. pyrenoidosa*; (*iii*) to increase the flux for lycopene formation. Simulations showed that the cellular lutein content increased remarkably with this strategy (Fig. 6), suggesting that the low flux for lycopene formation is a bottleneck for lutein production. Evidence of this deduction can also be found in literature (*31*,*32*), where supplementing carotenoid precursors (isopentenyl pyrophosphate or geranylgeranyl diphosphate) promoted markedly carotenoid accumulation in metabolically engineered *Escherichia coli*. Additionally, it has already been established that phytoene synthesis and desaturation are two control steps in the carotenoid biosynthesis pathway in many organisms (*33–35*). Thus, there is reason to believe that increasing the flux for lycopene formation can be a promising strategy to increase the cellular lutein content of *C. pyrenoidosa;* (*iv*) to remove the inhibition of lycopene formation. Simulations showed that kinetic orders f_1 and f_2 contributed little to elevating cellular lutein content under different conditions (details not shown). As for f_0, its low sensitivity (Table 1) also means that the inhibition of lycopene formation will not affect the cellular lutein content at present. However, simulations indicated that removing this inhibition would increase the cellular lutein content remarkably after by-product syntheses had been blocked, and especially after the flux for lycopene formation increased (Figs. 5 and 6). Therefore, it can be inferred that the inhibition of lycopene formation may become a limiting factor with further increase of the cellular lutein content. To elucidate the cause of this inhibition requires further investigation. There are two possible reasons for this inhibition according to the literature: interference with the balanced regulation pathway, and/or the limitation in carotenoid storage of the cell (*14*). As *Chlorella* is a natural carotenoid producer, the latter reason is especially noteworthy.

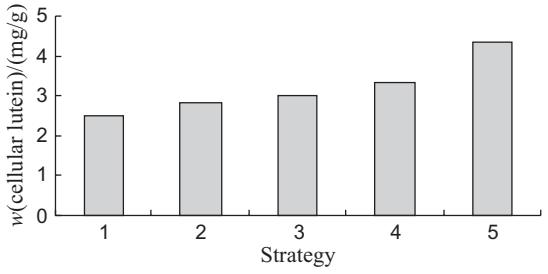

Fig. 5. Model predictions of cellular lutein content after blocking the by-product synthesis. Strategies: 1 – control; 2 – blocking violaxanthin synthesis (α_1=0); 3 – blocking loroxanthin synthesis (α_4=0); 4 – blocking the syntheses of both violaxanthin and loroxanthin (α_1=α_4=0); 5 – blocking the syntheses of both violaxanthin and loroxanthin, and removing the inhibition of lycopene formation simultaneously (α_1=α_4=f_0=0)

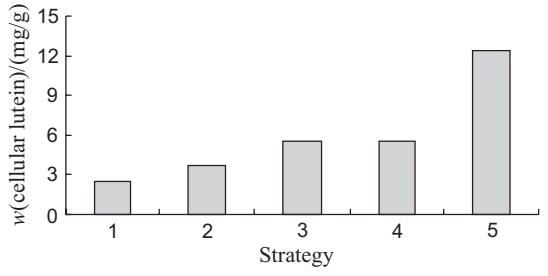

Fig. 6. Model predictions of cellular lutein content with the increase of flux for lycopene formation. Strategies: 1- control; 2 – twofold increase of flux for lycopene formation (α_0×2); 3 – fourfold increase of flux for lycopene formation (α_0×4); 4 – twofold increase of flux for lycopene formation and removal of the inhibitions of lycopene formation at the same time (α_0×2, f_0=0); 5 – fourfold increase of the flux for lycopene formation, and removal of the inhibition of lycopene formation simultaneously (α_0×4, f_0=0)

Conclusion

The BST model developed in this paper suggests the possible regulatory features of carotenoid biosynthesis in *C. pyrenoidosa* and agrees well with the experimental data. Model simulations showed that the bottleneck for lutein production by *C. pyrenoidosa* was the low flux for lycopene formation, while by-product synthesis and inhibitions of lycopene formation did not influence the cellular lutein content much; however, the inhibition of lycopene formation by lutein might also become a limiting factor with further increase of the cellular lutein content. Therefore, future efforts to enhance lutein production by metabolic network modification should be put on both expanding the flux for lycopene formation and removing the inhibition from it.

Acknowledgements

This work was supported by the Ministry of Science and Technology of the People's Republic of China (863 Project No. 2006AA02Z226).

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