Kinetic modeling of lutein production by heterotrophic *Chlorella* at various pH and temperatures

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Kinetics of lutein production by heterotrophic *Chlorella protothecoides* was investigated with respect to pH and temperature. Flask cultures with initial pH 5.0 – 8.0 were carried out, and it was found that pH 6.0 was optimal for the algal growth. Further tests in fermentors showed that the highest biomass concentration, maximum cellular lutein content and lutein yield were achieved at pH 6. 6. In addition, it was shown that optimal biomass concentration and lutein yield were obtained at 28°C, while application of 35°C resulted in the highest cellular lutein content. A mathematical model was developed for the description of the processes under these cultivation conditions and the kinetic model fitted well to the experimental data. The obtained results may contribute to the commercial production of lutein by *C. protothecoides*.

Keywords: *Chlorella protothecoides* / Heterotrophic culture / Lutein / pH / Temperature

Received: March 14, 2006; revised: April 24, 2006; accepted: April 25, 2006

1 Introduction

Chlorella is a unicellular microalga that was first used for mass heterotrophic cultivation research [1, 2]. It is known that *Chlorella* is abundant in nutritious substances, such as proteins, polysaccharides, carotenoids, vitamins, and unsaturated fatty acids, some of which have been shown to have biological functions for human health [3, 4]. Lutein, the main carotenoid in *Chlorella*, is not only an important natural colorant and additive in food or feed but has also been shown to be effective in the treatment of age-related macular degeneration [5, 6]. The higher cellular content of lutein in *Chlorella* compared to most of the other microorganisms makes it a promising candidate for commercial lutein production [7, 8].

Currently the cultivation of microalgae is mostly carried out with photoautotrophic systems. However, there are some limitations associated with illumination and other cultivation parameters, which severely limit its development [9–11]. Heterotrophic culture technology has been regarded as a promising alternative for microalgal mass cultivation [9, 12]. Recently, a highly efficient lutein production system using heterotrophic *Chlorella* has been developed in our laboratory [7, 13–15].

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Further optimization of process parameters, including pH and temperature, is important and has been noted for their effect on both cell growth and carotenoids accumulation [16, 17]. However, very limited information is available for the heterotrophic cultivation of *Chlorella*. The purpose of this study was to develop a kinetic model for lutein production by heterotrophic cultivation of *Chlorella* at various pH and temperatures, and to provide valuable information for the commercial production of lutein by *C. protothecoides*.

2 Materials and methods

2.1 Microalgal strain

The microalgal strain used in this study was *Chlorella protothecoides* CS-41 obtained from the CSIRO Marine Laboratory, Hobart, Australia.

2.2 Medium

A modified basal medium [13] supplemented with 40 g/L glucose and 7.6 g/L $NaNO_3$ was used in all studies.

2.3 Cultivation

For the pH study, a sterilized medium (121°C, 15 min) was inoculated with 5% v/v exponentially growing inocula. Het-

erotrophic cultivations were carried out in both flasks and 16-L fermentors (Bioengineering AG, Wald, Switzerland). The cultivation conditions in 250 mL Erlenmeyer flasks containing 150 mL medium were: temperature, 26°C; shaking speed, 180 rpm; initial pH: 5.0, 6.0, 7.0, and 8.0; all experiments were carried out in triplicates in the dark. The cultivation conditions in fermentors were as follows: pH 5.8, 6.2, 6.6, and 7.0; temperature, 26°C; agitation, 480 rpm; and dissolved oxygen concentration 50% air saturation.

For the temperature study, cultivations were carried out in 3.7-L fermentors (Bioengineering AG). Cultivation was controlled as stated above, except that pH was set at 6.6, and the temperature was varied (24, 26, 28, 30, 35, and 40°C).

2.4 Analytical methods

The dry cell weight was determined according to literature [18]. Glucose, nitrate, and lutein concentrations were measured according to the methods previously reported [13]. Standard lutein was obtained from Sigma Chemical (St. Louis, MO). All solvents were of HPLC-grade obtained from BDH Laboratory Supplies (Pool, UK).

2.5 Kinetic model

The hypotheses of the model are: (i) cell growth follows the Logistic expression; (ii) lutein accumulation is correlated with cell growth and is inhibited by glucose concentration; (iii) consumption of glucose for lutein formation and energy maintenance can be neglected since both cellular lutein content and the substrate consumption for energy maintenance are relatively low [19–21]; and (iv) the consumption rate of nitrogen is in proportion to that of glucose.

$$\text{Biomass: } \mu = \mu_m(\text{pH}, T)\left(1 - \frac{X}{X_m(\text{pH}, T)}\right) \quad (1)$$

$$\text{Lutein: } q_P = \frac{k_m(\text{pH}, T)}{1 + S/k_i}\mu \quad (2)$$

$$\text{Glucose: } q_S = -\frac{1}{Y_{XS}(\text{pH}, T)}\mu \quad (3)$$

$$\text{Nitrogen: } q_N = -K_{N/S}\, q_S \quad (4)$$

where μ is the specific growth rate (1/h), μ_m is the maximum specific growth rate (1/h), X is biomass concentration (g/L), X_m is the maximum biomass concentration (g/L), q_P is the specific lutein formation rate (mg/g/h), k_m is the maximum ratio of q_P/μ (mg/g), k_i is the inhibition constant of glucose on lutein formation (g/L), q_S is the specific glucose consumption rate (1/h), Y_{XS} is the yield coefficient of biomass on glucose (g/g), μ_N is the specific nitrogen consumption rate (1/h), and T is the temperature (°C).

When temperature was maintained during the cultivation, the expression of $q_m(\text{pH}, T)$ was assumed to be $\mu_m(\text{pH}) = \frac{\mu_m^*}{1 + 10^{-\text{pH}}/k_1 + k_2/10^{-\text{pH}}}$, where μ_m^*, k_1, and k_2 are constants; expression $\mu_m(T) = Ae^{\frac{-E_a}{R(T+273)}}$ was used when pH was maintained constant, where E_a is the active energy (J/mol), R the universal gas constant (= 8.31 J/mol.K), and A is a constant; empirical equations were used for expressions of $X_m(\text{pH}, T)$, $k_m(\text{pH}, T)$, and $Y_{XS}(\text{pH}, T)$. Parameters were estimated by fitting experimental data with Matlab software package.

3 Results and discussion

3.1 Algal growth and lutein production at different pH

Heterotrophic cultivation of *C. protothecoides* was first investigated in flasks with initial pH 5.0–8.0 to find out an appropriate pH range for cell growth. As shown in Table 1, the highest values of specific growth rate and maximum biomass concentration in flasks were found in the culture at an initial pH of 6.0.

Table 1. Effects of different initial pH on cell growth of *C. protothecoides*

Initial pH	Maximum biomass (g/L)	Maximum specific growth rate (1/h)
5.0	16.2	0.034
6.0	16.9	0.045
7.0	16.4	0.041
8.0	15.8	0.035

Based on results from experiments in flasks, the influence of culture pH from 5.8 to 7.0 on the heterotrophic cultivation of *C. protothecoides* was investigated in fermentors, where the pH was continuously controlled. As shown in Fig. 1, the maximum biomass concentration of the culture at pH 6.6 was 18.2 g/L dry cells, which was the highest among the cultures tested. The consumption of carbon and nitrogen sources during the algal growth was monitored during the cultivation; glucose was consumed completely by the alga after the maximum biomass was achieved in the cultures while the nitrate remained at the end of cultivation (Fig. 1).

The culture pH influenced the heterotrophic production of lutein by *C. protothecoides* as well. Both the maximum cel-

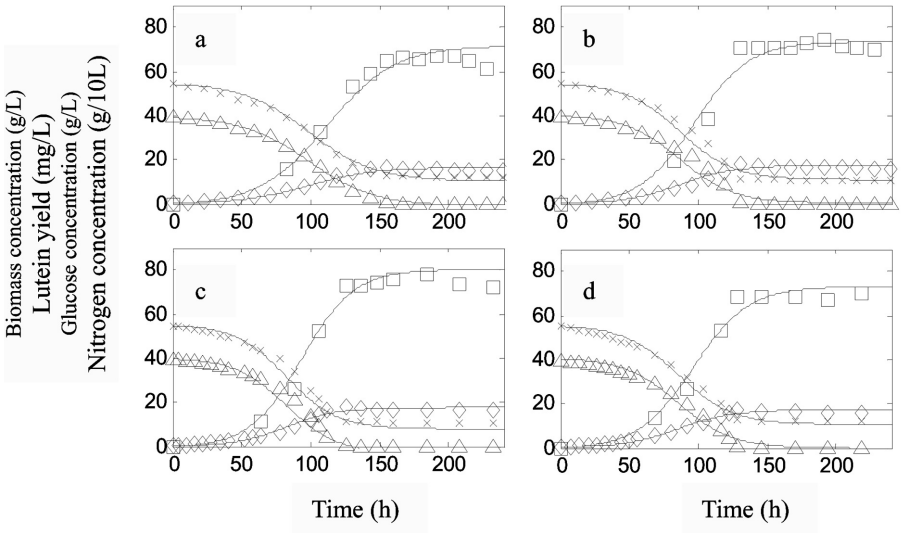

Figure 1. Time course of cell growth (◇), lutein production (□), glucose consumption (△), and nitrogen consumption (×) of the cultures in fermentors at various pH: (a) pH 5.8; (b) pH 6.2; (c) pH 6.6; (d) pH 7.0 (curves were results calculated by models).

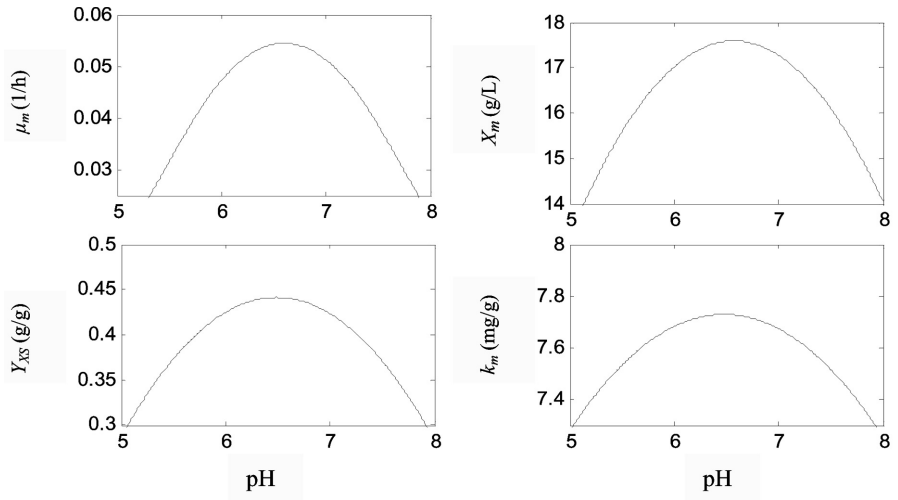

Figure 2. Influence of pH on kinetic parameters.

lular lutein content (4.75 mg/g dry cells) and the total lutein yield (77.92 mg/L) in the culture at pH 6.6 were the highest among the four cultures under study (Fig. 1).

By fitting experimental data of batch cultivations at different pH, the kinetic models for cell growth, lutein production, consumption of glucose and nitrogen were given:

$$\mu = \frac{0.063}{1 + 10^{-\mathrm{pH}}/(3.20 \times 10^{-6}) + (1.97 \times 10^{-8})/10^{-\mathrm{pH}}} \cdot \left(1 - \frac{X}{(-1.745\,\mathrm{pH}^2 + 22.94\,\mathrm{pH} - 57.81)}\right) \quad (5)$$

$$q_\mathrm{P} = \frac{(-0.2047\,\mathrm{pH}^2 + 2.6528\,\mathrm{pH} - 0.8659)}{1 + S/20.83}\mu \quad (6)$$

$$q_\mathrm{S} = -\frac{1}{(-0.0694\,\mathrm{pH}^2 + 0.9612\,\mathrm{pH} - 2.484)}\mu \quad (7)$$

$$q_\mathrm{N} = -0.1101 q_\mathrm{S} \quad (8)$$

Effect of pH on several kinetic parameters was calculated by the kinetic model. Comparison of μ_m, X_m, Y_XS, and k_m showed that the optimal pH for both cell growth and lutein formation was between 6.0 and 7.0 (Fig. 2). Similar results on cell growth of *Chlorella* were reported by others [22, 23]. However, no report was found about the influence of pH on lutein formation by *Chlorella* although Del Campo *et al.* [16] indicated that pH 6.5 was optimal for lutein accumulation in cultivation of *Muriellopsis* sp. (Chlorophyta). Moreover, those reported experiments were carried out photoautotrophically in flasks or open-pond systems.

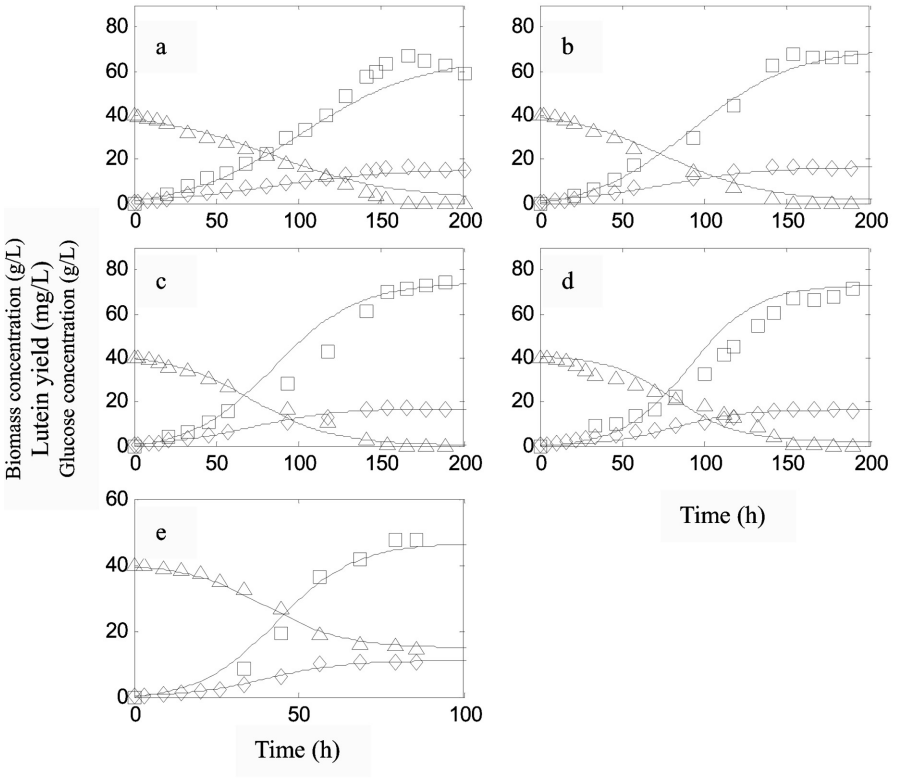

Figure 3. Time course of cell growth (◇), lutein production (◇), and glucose consumption (△) in heterotrophic cultivation at different temperatures: (a) 24°C; (b), 26°C; (c), 28°C; (d) 30°C; (e) 35°C (curves were results calculated by models).

Equation (8) showed that the consumption ratio of nitrogen was stable, which implies that the cellular protein content of *Chlorella* has relative stability at varied pH. Other studies in our laboratory supported this assumption (details not shown).

3.2 Algal growth and lutein production at different temperatures

The influence of temperature was investigated in fermentors using temperatures set at 24, 26, 28, 30, 35, and 40°C, respectively. *C. protothecoides* grew well at temperatures 24–35°C (Fig. 3), while at 40°C the algal growth lasted only 1 day after inoculation, the cell size of the alga grown at 35 or 40°C appeared larger than that at lower temperatures (data not shown).

The maximum biomass concentrations of the cultures grown between 24 and 30°C were 16.2–17.4 g/L dry cells, and the highest value was obtained at 28°C; while much lower value was observed at 35°C, which was 10.7 g/L (Fig. 3).

The maximum cellular lutein content increased from 4.25 to 4.59 mg/g with the increase of temperature from 24 to 35°C. The maximum lutein yield in the culture grown at 35°C was much lower than that at lower temperatures though the highest cellular lutein content was found at this temperature. The highest lutein yield (74.29 mg/L) was observed in the culture at 28°C.

By fitting experimental data of batch cultivations at different temperatures, the following kinetic models were obtained:

$$\mu = 0.5001 \times 10^{13} \mathrm{e}^{\frac{-81\,167}{R(T+273)}} \cdot \left(1 - \frac{X}{(-0.1096T^2 + 6.0718T - 67.2974)}\right) \quad (9)$$

$$q_{\mathrm{P}} = \frac{(0.0242T^2 - 1.3076T + 25.9514)}{1 + S/20.83}\mu \quad (10)$$

$$q_{\mathrm{S}} = -\frac{1}{(0.0034T + 0.3057)}\mu \quad (11)$$

The effect of temperature on kinetic parameters is depicted in Fig. 4. It can be found that the values of μ_{m} and Y_{XS} increased when temperature was elevated. Temperatures between 25 and 30°C were optimal for X_{m}. The value of X_{m} was zero at 40°C, and this implies that cells could not grow. This calculated results are consistent with the observations of our experiments.

According to the literature [24–27], *Chlorella* strains can be divided into two groups in terms of optimum temperature for growth: low-temperature strains with an optimum range of 25–30°C and high-temperature strains with an optimum range of 35–40°C. The *Chlorella* strain used in this study belongs to the former. A culture temperature close to room

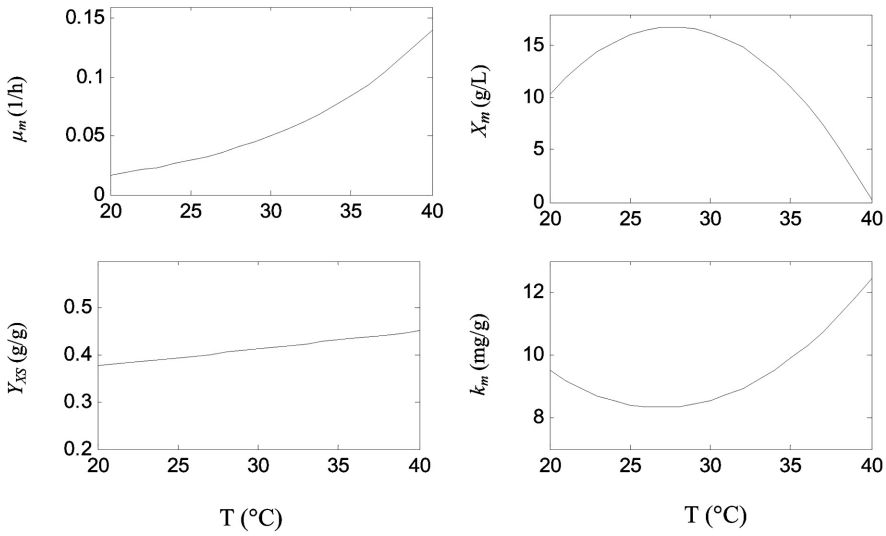

Figure 4. Influence of temperature on kinetic parameters.

temperature is usually favorable for commercial production since the energy supply can be economized. The results obtained from our study demonstrated that the strain *C. protothecoides* CS-41 satisfies this demand.

As shown in Fig. 4, X_m was lower, but Y_{XS} was higher at 35–40°C compared with the temperatures below 35°C. In our experiments, about 15 g/L of glucose remained at the end of cultivation at 35°C. The lower biomass concentration found at the end of the cultivation was then attributed only to the higher residual glucose.

As also shown in Fig. 4, the lowest k_m was obtained between 25 and 30°C; whereas at higher temperature the value of k_m increased significantly, which means that lutein formation can be favored by elevated temperature. This is in agreement with the many reports describing the enhancement of carotenoid production by high temperature for numerous microorganisms, including *C. emersonii* [28], *Haematococcus pluvialis* [29], *Chlorococcum* sp. [30], and *Muriellopsis* [16]. Recently, Wilson *et al.* [31] investigated the effects of temperature on *C. vulgaris* and found that elevation of temperature led to the increase in chlorophyll and light-harvesting complex polypeptide levels. This is a possible reason for enhancement of lutein accumulation, since generally lutein is regarded as an accessorial pigment for light-harvesting.

Varied temperature has opposite effects on k_m and X_m (Fig. 4), which is supported by a former research in our laboratory, where elevated temperature enhanced cellular lutein content at the loss of biomass concentration in culture, and the final lutein yield was almost unimproved [7].

Time courses of lutein formation at various temperatures from 24 to 32°C were calculated by the kinetic model (Fig. 5). It could be found that higher lutein yield at the end of cultivation would be obtained at 26–30°C although the lutein yield in culture increases more quickly when temperature was elevated (Fig. 5).

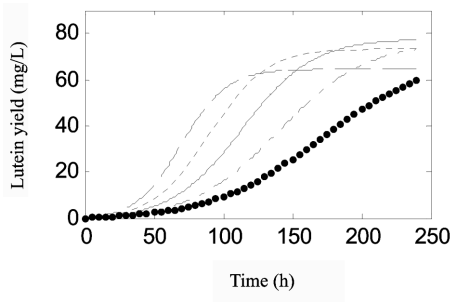

Figure 5. Time courses of lutein production at various temperatures: 32°C (dashed line); 30°C (dotted line); 28°C (solid line); 26°C (dot-dashed line); 24°C (bold dotted line).

In general, environmental stresses induce carotenoids formation while reducing cell growth rate [32]. In this study, however, no opposite trends between k_m and μ_m were found in heterotrophic cultivation of *C. protothecoides* with various pH and temperatures (Figs. 3, 4). The reason for this may be that lutein is a primary carotenoid, whose production is more growth-related compared with secondary carotenoids such as astaxanthin [30, 33].

4 Concluding remarks

It was concluded that pH 6.6 and 28°C were optimal for cell growth and lutein production by *C. protothecoides* based on investigations in controlled stirred fermentors. A kinetic model was developed that satisfactorily describes the heterotrophic cultivations of *C. protothecoides* at various pH and temperatures. Optimal pH and temperature for lutein

production and cell growth calculated by the model agree well with the experimental results. This study could contribute to commercial production of lutein by *C. protothecoides*.

This work was supported by the Research Grant Council of Hong Kong and Ministry of Science and Technology of the People's Republic of China.

5 References

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