Journal of Industrial Microbiology & Biotechnology (1999) 23, 503–507

http://www.stockton-press.co.uk/jim

A kinetic model for lutein production by the green microalga *Chlorella protothecoides* in heterotrophic culture

X-W Zhang, X-M Shi and F Chen

Department of Botany, the University of Hong Kong, Pokfulam Road, Hong Kong

Production of lutein by the green microalga *Chlorella protothecoides* grown heterotrophically in a fermentor using glucose as the carbon source and urea as the nitrogen source was investigated. An unstructured kinetic model was proposed to describe the microalgal culture system including cell growth, lutein formation, as well as glucose and nitrogen consumption. The inhibition potentials of biomass, product and substrates on growth and lutein formation were examined and incorporated into the kinetic model. Values of the kinetic model parameters were estimated. The resulting model predictions were in good agreement with the experimental results. The model can be helpful in scale-up, optimization and control of the *C. protothecoides* culture process, and can also be used as a guideline for similar microalgal cultivation systems.

Keywords: *Chlorella protothecoides*; heterotrophic culture; lutein production; unstructured kinetic model

Introduction

Carotenoids have been proposed as cancer prevention agents, life extenders, and inhibitors of ulcer, heart attack and coronary artery disease [9]. Lutein is one of the most prominent carotenoids in human serum and in foods and has been used for pigmentation of animal tissues and products, as well as for coloration of foods, drugs and cosmetics [12]. Microalgae are one of the major sources of naturally occurring lutein [8].

At present, almost all industrial microalgal processes are based on open pond technology [3]. The inability to control environmental factors is a major unsolved problem which limits its development [4]. An alternative to overcome or minimize the problem with the photoautotrophic strategies is to develop heterotrophic culture technology using sugars or other organic compounds as the sole energy and carbon source(s). In this study, heterotrophic cultivation of *Chlorella protothecoides* was carried out in a fermentor using glucose as the carbon source and urea as the nitrogen source for lutein production.

Efforts have also been made to develop mathematical models to describe bioprocesses. Recently Bailey [2] made a comprehensive review of this subject. Structured models may be attractive due to their general applicability, but they suffer from the shortcomings of unknown or complicated mechanisms of regulation of cell culture, and often contain a large number of model parameters. Identification and estimation of these parameters are often difficult [23]. Therefore, unstructured kinetic models are the most frequently employed for modeling microbial systems because of their simplicity, and because they are adequate for technical purposes [11]. However, little information is available concerning kinetic models for heterotrophic microalgal culture systems with respect to cell growth, substrate consumption and product formation, although the relationship between specific growth rate and substrate or light intensity was sometimes involved in investigations under photoautotrophic conditions of growth [10,14,19,20].

The aim of this work was to develop an unstructured kinetic model for lutein production using *C. protothecoides*. This model takes into account dependencies of growth and production on nutrients together with product inhibition and autoinhibition to analyze the dynamics of growth and metabolism of microalgae grown under heterotrophic culture conditions.

Theoretical aspects

Cell growth model

In the literature, two classes of models to describe the specific growth rate μ are distinguished:

(1) Monotonic kinetics. The kinetics increase as a function of the substrate concentration C_s, such as the well-known Monod growth model [15]:

$$\mu = \frac{\mu_m C_S}{K_s + C_S} \tag{1}$$

where μ_m is the maximum specific growth rate. K_S is the Monod saturation constant, a kinetic parameter which indicates how fast the maximum specific growth rate is reached. According to Eqn (1), $\mu \rightarrow \mu_m$ when $K_S \rightarrow 0$. Unfortunately, the Monod model often fails to account for substrate inhibition of growth at higher substrate concentrations. To overcome the drawback, another model is employed:

(2) Non-monotonic kinetics. The non-monotonic is used, the Haldane model [1], as a function of the substrate concentration C_S:

$$\mu = \frac{\mu_m C_S}{K_s + C_S + \frac{C_S^2}{K_i}} \tag{2}$$

Correspondence: Dr F Chen, Department of Botany, the University of Hong Kong, Pokfulam Rd, Hong Kong. E-mail: sfchen@hkuusa.hku.hk
Received 28 January 1999; accepted 27 August 1999

where K_i is the inhibition constant. The smaller K_i, the larger the inhibition effect of the substrate.

It is obvious that both models examine only the effect of a single substrate and ignore the inhibition potential of other environmental factors such as the nitrogen source, the cell itself and the product.

Nitrogen is another essential substrate for cell growth, and here a Monod-type expression $C_N/(K_{XN} + C_N)$ was employed to describe the effect of nitrogen. On the other hand, the expression $(1 - C_p/C_{p_m})$ was used to describe product inhibition, as in glycerol fermentation [22]. Thus the Haldane model may be extended as follows:

$$\mu = \mu_m \frac{C_S}{K_S + C_S + \frac{C_S^2}{K_{xi}}} \frac{C_N}{K_{XN} + C_N} \left(1 - \frac{C_P}{C_{P_m}}\right) \tag{3}$$

where μ is the specific growth rate (h^{-1}), μ_m is the maximum specific growth rate (h^{-1}), C_P, C_S, C_N are the product concentration (mg L^{-1}), the glucose concentration (g L^{-1}) and the urea (nitrogen source) concentration (g L^{-1}), respectively. C_{P_m} is the maximum product concentration (mg L^{-1}). K_S, K_{XN}, K_{xi} are respectively the glucose saturation constant, the nitrogen saturation constant and the glucose inhibition constant of cell growth (g L^{-1}).

Product formation model

The Luedeking–Piret kinetic model for product formation is Equation [13]:

$$\frac{dC_P}{dt} = Y_{PX} \frac{dC_X}{dt} + \mu_{PX} C_X \tag{4}$$

The model states that the product formation rate of cells can be attributed to a growth-associated part and a non-growth-associated part. Apparently, the model does not take into account the inhibition effects of glucose and nitrogen. In order to account for glucose inhibition of product formation, a Haldane-type expression $\frac{C_S}{K_{PS} + C_S + \frac{C_S^2}{K_{pi}}}$ was incorporated. Similarly, a Monod-type expression $C_N/(K_{PN} + C_N)$ was formulated accordingly to describe nitrogen inhibition as in modeling the growth of hybridoma cells [22]. Therefore, the Luedeking–Piret equation for product formation is extended as follows:

$$\frac{dC_P}{dt} = \left(Y_{PX} \frac{dC_X}{dt} + \mu_{PX} X\right) \frac{C_S}{K_{PS} + C_S + \frac{C_S^2}{K_{pi}}} \frac{C_N}{K_{PN} + C_N} \tag{5}$$

where C_X, C_P, C_S, C_N are the same as above. K_{PS}, K_{PN} and K_{pi} are the glucose saturation constant, the nitrogen saturation constant and the glucose inhibition constant of product formation (mg L^{-1}), respectively. Y_{PX} is the yield coefficient of product formation due to cell growth in the early phase of culture (mg g^{-1}). When in early cultivation, the cell concentration is lower, according to Eqn (4), $dC_P/dt \approx Y_{PX}dC_X/dt$, ie $Y_{PX} = dC_P/dC_X$. μ_{PX} is the specific formation rate of product in the later phase of culture (h^{-1}). In fact, according to Eqn (4), $\mu_{PX} = dC_P/Xdt$ when $dC_X/dt = 0$, ie the concentration of cells is at the maximum value, the cells cease to grow.

Substrate consumption model

The most widely used substrate consumption model can be expressed as:

$$-\frac{dC_S}{dt} = \frac{1}{Y_{XS}} \frac{dC_X}{dt} + \frac{1}{Y_{PS}} \frac{dC_P}{dt} + \mu_{SX} C_X \tag{6}$$

which is used as a glucose consumption model. C_X, C_P, C_S, C_N are the same as above. Y_{XS} is the yield coefficient of cells on glucose (g g^{-1}), Y_{PS} is the yield coefficient of product on glucose (mg g^{-1}). μ_{SX} is specific consumption rate of glucose (h^{-1}) in the later phase of culture. In fact, $\mu_{SX} = -dC_S/C_Xdt$, when $dC_X/dt = dC_P/dt = 0$, ie the concentrations of cell and product arrive at maximum values, the cell and product cease to accumulate, so the parameter is also considered to be related to maintenance functions of cells.

Similarly, the following equation was used to describe nitrogen consumption:

$$-\frac{dC_N}{dt} = \frac{1}{Y_{XN}} \frac{dC_X}{dt} + \frac{1}{Y_{PN}} \frac{dC_P}{dt} + \mu_{NX} C_X \tag{7}$$

where C_X, C_P, C_S, C_N are the same as above. Y_{XN} is the yield coefficient of cells on nitrogen (g g^{-1}), Y_{PN} is the yield coefficient of product on nitrogen (mg g^{-1}). μ_{NX} is the specific consumption rate of nitrogen (h^{-1}) in the later phase of culture, and can be considered to be a parameter related to maintenance requirements of cell metabolism like μ_{SX}; the analysis method is the same as for Eqn (6).

Consequently, an unstructured kinetic model (Eqns (3), (5), (6), (7)) for lutein production by heterotrophic cultivation of the microalga *Chlorella protothecoides* is proposed in this work. This model contains a total of 16 parameters, which have important physiological meanings; each one exhibits a process-state during the microalga cultivation.

Parameter estimations were performed using a Simplex Search Method; the principal idea of this method is to search the best value of the objective function in a space of points, P_i which represent the feasible solutions. The first point P_1 is arbitrarily selected as the starting point (base point). The second point P_2 is chosen and compared with P_1. If P_2 is found to be a better solution than P_1, then P_2 is selected as the new base point; if not, P_1 stays as the base point. This process is continued until the best operating point is found. The details were described previously [23]. The starting values of model parameters were determined based on physiologically meaningful ranges. The fitting result was evaluated by the root-square residuals:

$$E = \left(\frac{\sum_{i=1}^{Q} (Y_{exp} - Y_{cal})^2}{Q} \right)^{1/2}$$

where E is the fitting error, Y_{exp} and Y_{cal} are experimental and calculated values, respectively and Q is data number.

Materials and methods

Microalgal strain and medium

C. protothecoides CS-41 was obtained from the CSIRO Marine Laboratory, Hobart, Australia. The modified Basal medium supplemented with 40 g L^{-1} glucose and 3.6 g L^{-1} urea was used [16].

Cultivation

Sterilized medium (121°C, 15 min) was inoculated with 5% exponentially growing inocula. Heterotrophic cultivation of *C. protothecoides* CS-41 was initially carried out in a 250-ml Erlenmeyer flask containing 150 ml medium at 28 ± 1°C under continuous shaking (180 rpm) in the dark. Further heterotrophic cultivation was performed in a 3.7-L fermentor (Bioengineering AG, Wald, Switzerland) containing 2.5 L medium. The cultivation conditions in the fermentor were controlled as follows: pH 6.6 ± 0.1; temperature 28°C; agitation 480 rpm; dissolved oxygen concentration 50% saturation.

Analytical method

The dry cell weight concentration was determined according to Chen *et al* [6]. Glucose and nitrate concentrations were determined as previously described [18]. Lutein concentration was determined according to Shi and Chen [16,17]. Specific growth rate, μ, was determined according to Chen and Johns [5].

Results and discussion

The experimental results for heterotrophic production of biomass and lutein by *C. protothecoides* in a 3.7-L fermentor are shown in Figures 1–4. By fitting all the experimental data, the following kinetic models proposed in this work were given:

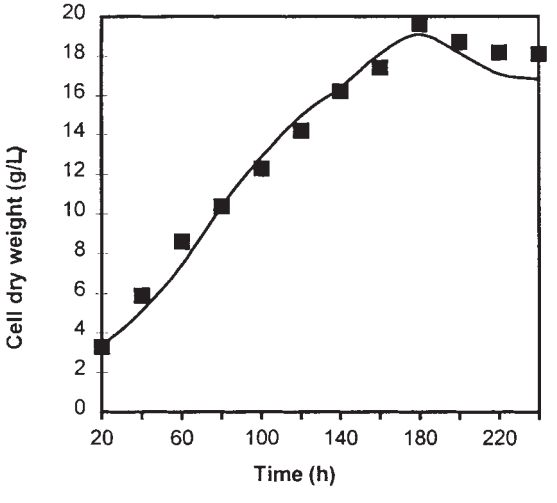

Figure 1 Prediction (——) of growth of *C. protothecoides* under heterotrophic conditions using a kinetic model (Equation 8) compared to the experimental data (■).

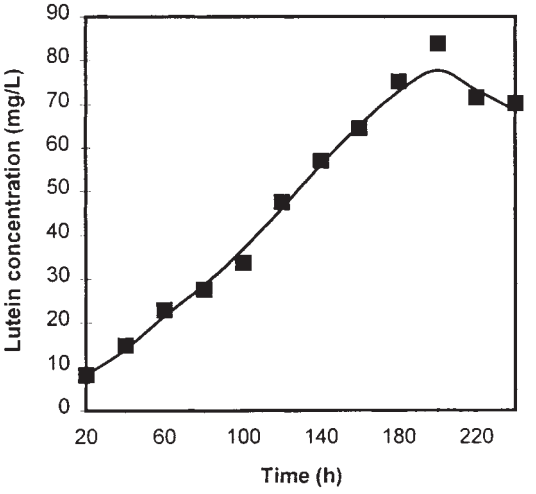

Figure 2 Prediction (——) of lutein concentration in the culture of *C. protothecoides* under heterotrophic conditions using a kinetic model (Equation 9) compared to the experimental data (■).

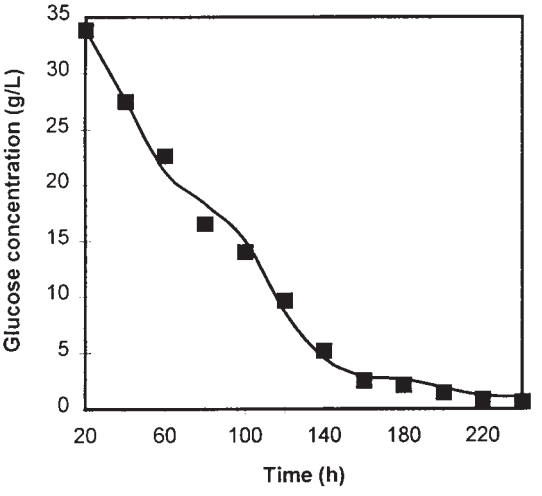

Figure 3 Prediction (——) of glucose consumption in the culture of *C. protothecoides* under heterotrophic conditions using a kinetic model (Equation 10) compared to the experimental data (■).

$$\frac{dC_X}{dt} = \frac{0.0527 C_S C_X}{0.1218 + C_S + \frac{C_S^2}{110.0605}} \frac{C_N}{0.3070 + C_N} \left(1 - \frac{C_P}{76.2734}\right) \quad (8)$$

$$\frac{dC_P}{dt} = \left(2.3584 \frac{dC_X}{dt} + 0.0265 C_X\right) \frac{C_S}{0.7536 + C_S + \frac{C_S^2}{120.1358}} \frac{C_N}{0.0546 + C_N} \quad (9)$$

$$-\frac{dC_S}{dt} = \frac{1}{0.5886} \frac{dC_X}{dt} + \frac{1}{2.2878} \frac{dC_P}{dt} + 0.0128 C_X \quad (10)$$

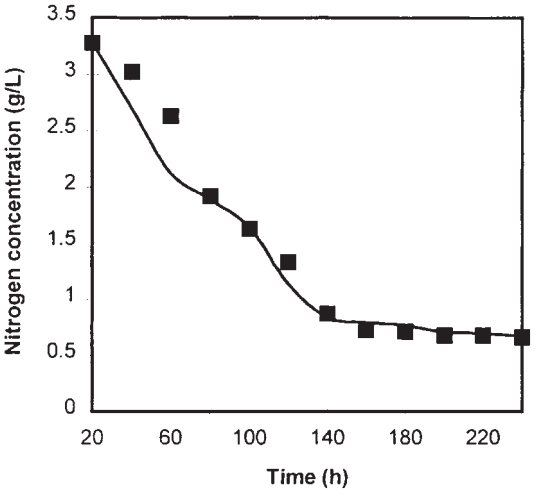

Figure 4 Prediction (——) of nitrogen consumption in the culture of *C. protothecoides* under heterotrophic conditions using a kinetic model (Equation 11) compared to the experimental data (■).

$$-\frac{dC_N}{dt} = \frac{1}{6.3871}\frac{dC_X}{dt} + \frac{1}{25.8963}\frac{dC_P}{dt} + 0.0013C_X \qquad (11)$$

The fitting errors (E) were 0.5877, 1.4825, 0.9994, 0.2258 for cell growth, lutein formation, glucose consumption and nitrogen consumption, respectively. The calculated results for Eqns (8)–(11) are also shown in Figures 1–4.

Equation (8) revealed that the maximum specific growth rate was reached more slowly for consumption of glucose than for consumption of nitrogen, because the velocity indication parameter K_S = 0.1218 g L^{-1} was smaller than K_{NX} = 0.3070 g L^{-1}. This suggested that glucose might be a limiting substrate. In fact, in the cultivation, glucose was used up by the microalga ahead of the nitrogen. On the other hand, based on the inhibition parameter K_{xi} = 110.0605 g L^{-1}, it demonstrated that glucose exhibited an inhibitory effect on cell growth to some extent. Moreover, according to Eqn (8) the maximum lutein yield was C_{pm} = 76.2734 mg L^{-1}, and the maximum specific growth rate was μ_m = 0.0527 (h^{-1}), while the experimental values were 83.81 mg L^{-1} and 0.0425 (h^{-1}), respectively. This showed a satisfactory fit between the model predictions and experimental data.

From Eqn (9) the yield coefficient of product formation due to cell growth (Y_{PX}) and specific formation rate of product in the early phase of cell growth (μ_{PX}) can be calculated to be 2.3584 (mg g^{-1}) and 0.0265 (h^{-1}), respectively. While the corresponding values determined by experimental data were 2.6289 mg g^{-1} in the early phase of culture (t = 20 h) and 0.0232 (h^{-1}) in the later phase of culture (t = 140 h) (cell growth approaches the maximum value 19.6 g L^{-1} at 142 h). These were again in good agreement. Also, based on the inhibition constant K_{pi} = 120.1358 mg L^{-1}, glucose had an inhibitory effect on product formation.

Furthermore, with Eqns (10) and (11) the following information may be obtained: the yield coefficient of cells on glucose Y_{PS} is 0.5886 g g^{-1}, the yield coefficient of produt on glucose Y_{PS} is 2.2878 mg g^{-1}, the specific consumption rate of glucose μ_{SX} in the later phase of culture is 0.0128 (h^{-1}), while the experimental values of corresponding parameters were 0.48 g g^{-1}, 2.08 mg g^{-1}, 0.0159 (h^{-1}) in the later phase of culture (t = 120 h), respectively. The yield coefficient of cells on nitrogen, Y_{XN}, is 6.3871 g g^{-1}, the yield coefficient of product on nitrogen Y_{PN}, is 25.8963 mg g^{-1} and the specific consumption rate of nitrogen, μ_{NX}, in the later phase of culture is 0.0013 (h^{-1}) while the experimental values of corresponding parameters were 5.44 g g^{-1}, 23.2806 mg g^{-1} and 0.0016 (h^{-1}) in the later phase of culture (t = 120 h), respectively. Consistency was achieved between the predicted data and experimental results.

Table 1 Parameter values and fitting errors for different models of cell growth

Model	Parameter	Error	Reference
Monod model (Equation 1)	μ_m = 0.0147 (h^{-1}) K_s = 3.1618 (g L^{-1})	1.7896	[15]
Haldane model (Equation 2)	μ_m = 0.0150 (h^{-1}) K_S = 3.1618 (g L^{-1}) K_i = 594.1771 (g L^{-1})	1.8308	[1]
Equation 3	μ_m = 0.0527 (h^{-1}) K_S = 0.1218 (g L^{-1}) K_{XI} = 110.0605 (g L^{-1}) K_{XN} = 110.0605 (g L^{-1}) C_{pm} = 76.2734 (mg L^{-1})	0.5877	This work

High cell-density culture of *Chlorella* in a batch system requires comparatively high concentrations of nitrogen in the medium. It was reported that urea at 2.0 g L^{-1} (0.14 M) nitrogen in the medium had ill effects on the growth of *C. pyrenoidosa* [7]. A similar effect was observed by [10] when *C. vulgaris* was cultivated in the presence of nitrate at a concentration as high as 0.097 M. In contrast, a high concentration (10 g L^{-1}) of nitrate and urea was also used in the cultivation of *C. pyrenoidosa*, with no ill effect found [21]. In this study, there was no inhibitory effect observed on growth of *C. protothecoides* when the concentration of urea was maintained at 3.6 g L^{-1} (0.12 M) nitrogen in the medium. These observations show that the tolerance of various *Chlorella* strains towards the concentraton of nitrogen sources might be different.

Finally, to compare the simulations, the classical models such as the Monod (Eqn (1)), the Haldane (Eqn (2)) and the Luedeking–Piret (Eqn (4)) models were employed to fit the experimental data. The parameters and fitting errors for different models estimated with the data of cell growth or product formation are given in Tables 1 and 2.

Table 2 Parameter values and fitting errors for different models of product formation

Model	Parameter	Error	Reference
Luedeking–Piret model (Equation 4)	μ_{PX} = 0.0229 (h^{-1}) Y_{PX} = 1.7441 (mg g^{-1})	1.6294	[13]
Equation 5	μ_{PX} = 0.0265 (h^{-1}) Y_{PX} = 2.3584 (mg g^{-1}) K_{PS} = 0.7536 (mg L^{-1}) K_{Pi} = 120.1358 (mg L^{-1}) K_{PN} = 0.0546 (mg L^{-1})	1.4825	This work

Equations (1) and (2) describe the cell growth data with the average errors (E) 1.7896 and 1.8308, respectively, larger than that of Eqn (3) proposed in this work (E = 0.5877). The fitting error for Eqn (4) to describe lutein production was 1.6294, larger than that of Eqn (5) proposed in this work (E = 1.4825). Furthermore, all these models do not take into account the effects of nitrogen- and product-inhibition. Therefore, the kinetic model for lutein production by microalga culture (Eqns (3), (5)–(7)) gave a superior fit over the other classical models. What is most important is that a number of kinetic parameters of physiological significance can be determined, and can be used as guidelines for other microalgal culture systems.

Acknowledgements

This research was supported by the Research Grants Council of Hong Kong and the University of Hong Kong Committee on Research and Conference Grants.

References

1 Andrews JF. 1968. A mathematical model for the continuous culture of microorganisms utilizing inhibitory substrates. Biotechnol Bioeng 10: 707–723.
2 Bailey JE. 1998. Mathematical modeling and analysis in biochemical engineering: past accomplishments and future opportunities. Biotechnol Prog 14: 8–20.
3 Borowitzka MA. 1994. Large-scale algal culture sysems: the next generation. Australasian Biotechnol 4: 212–215.
4 Chaumont D. 1993. Biotechnology of algal biomass production: a review of systems for outdoor mass culture. J Appl Phycol 5: 593–604.
5 Chen F and MR Johns. 1996. Relationship between substrate inhibition and maintenance energy of *Chlamydomonas reinhardtii* in heterotrophic culture. J Appl Phycol 8: 15–19.
6 Chen F, YM Zhang and SY Guo. 1996. Growth and phycocyanin formation of *Spirulina platensis* in photoheterotrophic culture. Biotechnol Lett 18: 603–608.
7 Davis EA, J Dedrick, CS French, HW Milner, J Myers, JHC Smith and HA Spoehr. 1953. Laboratory experiments in *Chlorella* culture at the Carnegie Institution of Washington Department of Plant Biology. In: Algal Culture—From Laboratory to Pilot Plant (Burlew JS, ed), pp 85–102, The Kirby Lithographic Company, Washington, DC.
8 Goodwin TW. 1980. The Biochemistry of the Carotenoids, 2nd edn. Vol I, Plants. Chapman and Hall, London.
9 Jacobs MM. 1993. Diet, nutrition, and cancer research: an overview. Nutr Today May/June:19.
10 Jeanfils J, MF Canisius and N Burlion. 1993. Effect of high nitrate concentrations on growth and nitrate uptake by free-living and immobilized *Chlorella vulgaris* cells. J Appl Phycol 5: 369–374.
11 Kennedy JF and IJ Bradshaw. 1984. Production, properties and applications of xanthan. Prog Ind Microbiol 19: 319–371.
12 Klaui H. 1982. Industrial and commercial uses of carotenoids. In: Carotenoid Chemistry and Biochemistry (Britton G and TW Goodwin, eds), pp 309–328, Pergamon Press, Oxford.
13 Luedeking R and EL Piret. 1959. A kinetic study of the lactic acid fermentation. J Biochem Microbiol Technol Eng 1: 393–412.
14 Marsot P, A Cembella and L Houle. 1991. Growth kinetics and nitrogen—nutrition of the marine diatom *Phaeodactylum tricornutum* in continuous dialysis culture. J Appl Phycol 3: 1–10.
15 Monod J. 1949. The growth of bacterial cultures. Ann Rev Microbiol 3: 371–394.
16 Shi XM and F Chen. 1997. Stability of lutein under various storage conditions. Nahrung/Food 41: 38–41.
17 Shi XM and F Chen. 1999. Production and rapid extraction of lutein and the other lipid-soluble pigments from *Chlorella protothecoides* grown under heterotrophic and mixotrophic conditions. Nahrung/Food 43: 109–113.
18 Shi XM, F Chen, JP Yuan and H Chen. 1997. Heterotrophic production of lutein by selected *Chlorella* strains. J Appl Phycol 9: 445–450.
19 Tam NFY and YS Wong. 1996. Effect of ammonia concentrations on growth of *Chlorella vulgaris* and nitrogen removal from media. Biores Technol 57: 45–50.
20 Tan CK, YK Lee and KK Ho. 1993. Effect of light intensity and ammonium-N on carotenogenesis of *Trentepohlia odorata* and *Dunaliella bardawil*. J Appl Phycol 5: 547–549.
21 Theriault RJ. 1965. Heterotrophic growth and production of xanthophylls by *Chlorella pyrenoidosa*. Appl Microbiol 13: 402–416.
22 Zeng A-P, WD Deckwer and W-S Hu. 1998. Determinants and rate laws of growth and death of hybridoma cells in continuous culture. Biotechnol Bioeng 57: 642–654.
23 Zhang XW, DX Gu, X Liu and YZ Guan. 1997. A three-dimensional model for a fermentation process: fermentation plane. Enzyme Microb Technol 21: 165–169.