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Kinetic models for astaxanthin production by high cell density mixotrophic culture of the microalga *Haematococcus pluvialis*

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High cell density cultivation of *Haematococcus pluvialis* for astaxanthin production was carried out in batch and fed-batch modes in 3.7-L bioreactors with stepwise increased light intensity control mode. A high cell density of 2.65 g L^{-1} (batch culture) or 2.74 g L^{-1} (fed-batch culture) was obtained, and total astaxanthin production in the fed-batch culture (64.36 mg L^{-1}) was about 20.5% higher than in the batch culture (53.43 mg L^{-1}). An unstructured kinetic model to describe the microalga culture system including cell growth, astaxanthin formation, as well as sodium acetate consumption was proposed. Good agreement was found between the model predictions and experimental data. The models demonstrated that the optimal light intensity for mixotrophic growth of *H. pluvialis* in batch or fed-batch cultures in a 3.7-L bioreactor was 90–360 μmol m^{-2} s^{-1}, and that the stepwise increased light intensity mode could be replaced by a constant light intensity mode.

Keywords: *Haematococcus pluvialis*; mixotrophic culture; light irradiance; astaxanthin production; kinetic model

Introduction

Astaxanthin is receiving commercial interest due to its use as a preferred pigment in the feeds of farmed salmon and trout, as a potential prophylactic agent against skin cancer [18] and as a possible chemopreventive agent for bladder carcinogenesis [22]. Despite the availability of synthetic astaxanthin which may contain other undesirable compounds during the processing, there is renewed interest in using microorganisms such as *Haematococcus pluvialis* (microalga) and *Phaffia rhodozyma* (yeast) as potential natural sources of the product.

H. pluvialis can grow heterotrophically on acetate in the dark [9]. Dark-grown cultures of *H. pluvialis* accumulate a trace amount of astaxanthin (mainly as mono-esters), and the specific growth rate was significantly lower under heterotrophic conditions than in the same acetate-based medium under mixotrophic conditions [6]. Recently, papers have been published on the effects of nutritional and environmental factors such as light intensity, temperature and acetate concentration on the growth of *Haematococcus* and accumulation of astaxanthin [2–3,10–13].

Large-scale synthesis of astaxanthin by *Haematococcus pluvialis* is hampered by problems associated with process design [13]. Low productivity with typical cell densities of 0.5–1 g L^{-1} is a major obstacle to the successful commercialization of astaxanthin production [8]. The optimization and control of bioprocesses often require the establishment of a mathematical model that describes the kinetics of process variables (microbial growth, substrate uptake and product formation). Despite impressive progress made recently in developing structured models for microbial growth, the unstructured models or semimechanistic models are still the most popular ones used in practice [28].

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However, little information is available concerning kinetic models for mixotrophic microalgal culture systems involved in cell growth, acetate consumption, and product formation simultaneously, although the relationship between specific growth rate of microalgae and substrate or light intensity is sometimes involved in the investigations [5,7,15,19,21,24].

The objectives of the present study were to investigate astaxanthin production by high cell density cultures of *Haematococcus pluvialis* in batch and fed-batch modes, to develop an unstructured kinetic model and to analyze the dynamics of microalgae growth and astaxanthin accumulation under mixotrophic culture conditions.

Materials and methods

Microorganism and culture medium

Haematococcus pluvialis (*H. lacustris*, UTEX No. 16) was obtained from the University of Texas Culture Collection (Austin, TX, USA). The medium consisted of (per litre): 300 mg KNO_3, 30 mg Na_2HPO_4, 35.5 mg NaH_2PO_4, 24.6 mg $MgSO_4 \cdot 7H_2O$, 73 mg $CaCl_2 \cdot 2H_2O$, 6.7 mg EDTANa $2H_2O$, 8.3 mg $FeSO_4 \cdot H_2O$, 0.014 mg $ZnSO_4$, 0.003 mg H_3BO_3, 0.0005 mg $CoCl_3 \cdot 2H_2O$, 0.012 mg $CuSO_4 \cdot 5H_2O$, 0.016 mg $MnSO_4 \cdot 5H_2O$, 0.001 mg $Na_2MoO_4 \cdot 2H_2O$ and 1 g sodium acetate. The initial pH was adjusted to 7.0. The culture medium was sterilized at 121°C for 15 min before use.

Batch culture

The experiment was carried out in a 3.7-L bioreactor (Bioengineering, Wald, Switzerland) containing 2.5 L medium. The culture was agitated at 350 rpm and sterile air was supplied to the culture at a flow rate of 100 L h^{-1}. The temperature was set at 30°C and 200 ml of an inoculum (approximately 22000 cells ml^{-1}) were used. Light irradiance was provided by cool white fluorescent tubes which surrounded the vessel. Three experiments were run. In the first, which was used to build up the model, light

intensity was gradually increased from 45 μmol m^{-2} s^{-1} (the first 4 days) to 90 (days 5–7), 120 (days 8–11), 150 (days 12–15) and 180 (after day 15). In the second and third runs, which were used to test the model, the light intensity was kept at 180 μmol m^{-2} s^{-1} till the end of the experiment in the second run, and the light intensity was changed according to the relationship $I = 40 + 10t$ where $t = 1,2,\ldots,20$ (days) in the third run. Each run was repeated three times.

Fed-batch culture

The experiment was also carried out in a 3.7-L bioreactor containing 2.5 L medium. The temperature, agitation rate, light intensity, and aeration rate were the same as in the batch culture. A solution 40 ml containing 1 g sodium acetate was added daily to the bioreactor for 4 days from day 4. The initial pH was adjusted to 7.0.

Analytical methods

Cell dry weight was determined by filtering 10 ml of the culture fluid through a predried membrane filter (Millipore 0.45-μm pore size, Bedford, MA, USA) and subsequently drying the membrane in a vacuum oven at 85°C overnight to constant weight. Astaxanthin was determined as described by Yuan *et al* [26], ie HPLC was conducted on a Waters liquid chromatograph equipped with two 510 pumps and a 996 photodiode array (PDA) detector. The pigment extract filtered through 0.45-μm filters was separated using a 250 × 4.6 mm Ultrasphere C_{18} (5 μm) column (Beckman, Fullerton, CA, USA) at 25°C. A Resolve C_{18} column (Waters, Milford, MA, USA, 300 × 3.9 mm, 5 μm) was also tested. The mobile phase consisted of methanol (69.0%), dichloromethane (17.0%), acetonitrile (11.5%), and water (2.5%). The flow rate was set at 1.0 ml min^{-1}. Samples were injected with a Rheodyne 7725 valve with a 20-μl loop. A tridimensional chromatogram was recorded from 300 to 700 nm. Peaks were measured at a wavelength of 450 nm. Astaxanthin was identified using a PDA detector by comparing its spectrum with published data [24,25] and the concentration was measured by area comparison with β-carotene [4].

Model development

Cell growth model: The most widely used unstructured model for the specific growth rate, μ, is the Monod equation, which increases monotonically as a function of the substrate concentration C_s [16]:

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

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. In fact, according to Eqn (1), $\mu \rightarrow \mu_m$ when $K_S \rightarrow 0$, ie the smaller K_S, the more rapidly the maximum specific growth rate is reached. Unfortunately, the Monod model often fails to account for substrate inhibition of growth at high substrate concentrations. To overcome the drawback, another well-known model, the non-monotonic Haldane model as a function of the substrate concentration C_S [1]:

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

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 light intensity, the cell itself and the product.

Practically, the most important environmental factor for the algal culture is light, which primarily concerns photosynthesis. In a manner to that of the Tamiya model [20], a Monod-type expression $I/(K_{XI} + I)$ was employed to describe the effect of light. On the other hand, Weiss and Ollis [23] proposed a model depending on biomass concentration only by means of a logistic equation:

$$\mu = \mu_m \left(1 - \frac{C_X}{C_{X_m}}\right) \tag{3}$$

hence the expression $(1 - C_X/C_{X_m})$ was employed to describe autoinhibition of the cell itself. Also, the expression $(1 - C_p/C_{p_m})$ was used to describe product inhibition, as in glycerol fermentation by Zeng *et al* [27]. Thus the Haldane model may be extended as follows:

$$\mu = \mu_m \frac{C_S}{K_S + C_S + \frac{C_S^2}{K_{xi}}} \frac{I}{K_{XI} + I} \left(1 - \frac{C_X}{C_{X_m}}\right) \left(1 - \frac{C_P}{C_{P_m}}\right) \tag{4}$$

where μ is the specific growth rate (day^{-1}), μ_m is the maximum specific growth rate (day^{-1}), C_X, C_P, C_S are the cell concentration (g L^{-1}), the product concentration (mg L^{-1}), the sodium acetate concentration (g L^{-1}), respectively. C_{X_m} and C_{P_m} are the achievable maximum cell concentration (g L^{-1}) and maximum product concentration (mg L^{-1}), respectively. I is light intensity (μmol m^{-2} s^{-1}). K_S, K_{XI} and K_{xi} are, respectively, the sodium acetate saturation constant, the light saturation constant and the sodium acetate inhibition constant of cell growth (g L^{-1}).

Product formation model: The much-discussed kinetic model for product formation is the following Equation [14]:

$$\frac{dC_P}{dt} = Y_{PX} \frac{dC_X}{dt} + \mu_{PX} C_X \tag{5}$$

The model states that the product formation rate of cells can be attributed to a growth-associated part and a non-growth-asociated part. Apparently, the model does not take into account the inhibition effects of sodium acetate, of the product itself and light intensity. To account for sodium acetate inhibition of product formation, a Haldane-type expression $\frac{C_S}{K_{PS} + C_S + \frac{C_S^2}{K_{pi}}}$ was incorporated. In fact,

Moraine and Rogovin [17] used the following equation to describe xanthan production:

$$\frac{dC_P}{dt} = \frac{\mu_{PX} C_X C_S}{K_{PS} + C_S} \tag{6}$$

Similarly, Monod-type expressions ($I/(K_{PI} + I)$) and ($C_P/K_P + C_P$) were formulated accordingly to describe light influence and product inhibition, respectively, as used in modeling growth of hybridoma cells by Zeng *et al* [29]. Therefore, the Luedeking–Piret equation for product formation is extended as follows:

$$\frac{dC_P}{dt} = \left(Y_{PX}\frac{dC_X}{dt} + \mu_{PX}C_X\right) \frac{C_S}{K_{PS} + C_S + \frac{C_S^2}{K_{pi}}} \frac{C_P}{K_P + C_P} \frac{I}{K_{PI} + I} \tag{7}$$

where C_X, C_P, C_S, I are the same as above. K_{PS}, K_{pi}, K_P and K_{PI} are the sodium acetate saturation constant, the sodium acetate inhibition constant, the autoinhibition constant and light inhibition constant of product formation (mg L^{-1}), respectively. Y_{PX} is the instantaneous yield coefficient of product formation due to cell growth (mg g^{-1}), which reflects the ability of product formation with the increased cell yield. μ_{PX} is the specific formation rate of product (day^{-1}). In fact, according to Eqn (5), $dC_P/dt \approx Y_{PX}\, dC_X/dt$ (when $C_X \rightarrow 0$), ie $Y_{PX} = dC_P/dC_X$; $\mu_{PX} = 1/X\,(dC_P/dt)$ when $dC_X/dt = 0$, ie the concentration of cells arrives at the maximum value, the cells cease to grow, so the non-growth-associated part is often considered to be related to maintenance functions of cells.

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

$$-\frac{dC_S}{dt} = \frac{1}{Y_{XS}}\frac{dC_X}{dt} + \frac{1}{Y_{PS}}\frac{dC_P}{dt} + \mu_{SX}C_X \tag{8}$$

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

Consequently, an unstructured kinetic model (Eqns 4, 7 and 8) for astaxanthin production by mixotrophic cultivation of the microalga *Haematococcus pluvialis* is proposed in this work. This model contains 15 parameters, which have important physiological meanings; each one exhibits a process-state during cultivation of the microalga.

Parameter estimations were performed using a Simplex Search Method [30]; 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 feasible solutions. The first point, P_i 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 starting values of model parameters were determined by physiologically meaningful ranges.

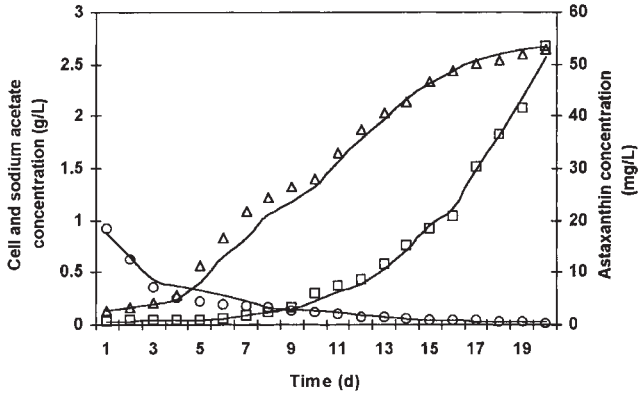

Figure 1 Time courses of cell growth, sodium acetate consumption and astaxanthin formation for *Haematococcus pluvialis* in batch mixotrophic culture. The triangle (△), the box (□) and the circle (○) represent the experimental data for cell, astaxanthin and sodium acetate concentration, respectively; the line (——) represents the calculated values.

Results and discussion

The results (the average values of three experiments) of batch and fed-batch culture experiments in the first run are shown in Figures 1 and 2. There is no significant difference between the cell dry weight concentrations in the batch culture (2.65 g L^{-1}) and the fed-batch culture (2.74 g L^{-1}), but, by feeding sodium acetate during cultivation, the total astaxanthin production of the fed-batch culture (64.36

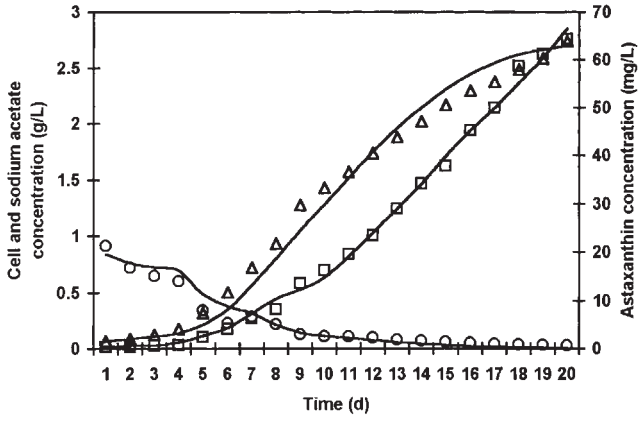

Figure 2 Time courses of cell growth, sodium acetate consumption and astaxanthin formation for *Haematococcus pluvialis* in fed-batch mixotrophic culture. The triangle (△), the box (□) and the circle (○) represent the experimental data for cell, astaxanthin and sodium acetate concentration, respectively; the line (——) represents the calculated values.

mg L^{-1}) was about 20.5% higher than that of the batch culture (53.43 mg L^{-1}).

By fitting the experimental data in the first run, the parameter values of the kinetic models proposed are given in Table 1. The average relative errors, *E* for cell and product concentrations in batch culture are 7.6% and 8.2%, respectively. While in fed-batch culture the corresponding errors, *E*, are 10.6% and 9.3%, respectively. In general, the fit was good except for one or two points (Figures 1 and 2).

For batch culture, the maximum biomass concentration was C_{Xm} = 2.92 g L^{-1} (dry weight), the maximum astaxanthin yield was C_{pm} = 55.6 mg L^{-1}, and the maximum specific growth rate was μ_m = 0.5258 (day^{-1}) (Table 1); the experimental values were 2.65 g L^{-1}, 53.43 mg L^{-1}, and 0.4928 (day^{-1}), respectively. For fed-batch culture, the maximum biomass concentration was C_{Xm} = 3.31 g L^{-1} (dry weight), the maximum astaxanthin yield was C_{pm} = 72.7 mg L^{-1}, and the maximum specific growth rate was μ_m = 0.6727 (day^{-1}) (Table 1); the experimental values were 2.74 g L^{-1}, 64.4 mg L^{-1} and 0.5820 (day^{-1}), respectively. Furthermore, for batch and fed-batch culture, the following information may be obtained from Table 1: the yield coefficient of cells on sodium acetate Y_{XS} is 2.6872 g g^{-1} and 2.7629 g g^{-1}, respectively, and the yield coefficient of product on sodium acetate Y_{PS} is 60.4 mg g^{-1} and 66.7 mg g^{-1}, respectively. While the experimental values of corresponding parameters were 2.65 g g^{-1} and 2.74 g g^{-1}, 53.4 mg g^{-1} and 64.4 mg g^{-1}, respectively. These showed a good fit between the model predictions and experimental data.

The maximum specific growth rate was reached more slowly for batch culture than fed-batch culture (Table 1), because the velocity indication parameter in batch culture (K_S = 0.0211 g L^{-1}) was remarkably larger than that in fed-batch culture (K_S = 0.0109 g L^{-1}). Since the instantaneous yield coefficient of product formation due to cell growth (Y_{PX} = 30.2 mg g^{-1}) in fed-batch culture was larger than that in batch culture (Y_{PX} = 20.8 mg g^{-1}), astaxanthin formation with the increased number of cells in fed-batch culture was stronger than that in batch culture.

Figures 3 and 4 show the predicted results of effects of light intensity on cell growth and astaxanthin formation.

Table 1 Parameter values for different models

Model	Parameters	Batch culture	Fed-batch culture
(4)	μ_m (day^{-1})	0.5258	0.6727
	K_S (g L^{-1})	0.0211	0.0109
	K_{xi} (g L^{-1})	56.6813	60.7680
	K_{XI} (g L^{-1}	53.26	71.8983
	C_{Xm} (g L^{-1})	2.9161	3.3081
	C_{Pm} (g L^{-1})	55.6192	72.6912
(7)	μ_{PX} (day^{-1})	6.0679	2.7266
	Y_{PX} (mg g^{-1})	20.7502	30.1732
	K_{PS} (mg L^{-1})	0.0017	0.0138
	K_{pi} (mg L^{-1})	63.9593	71.1257
	K_{PI} (mg L^{-1})	72.5525	50.7622
	K_P (mg L^{-1})	19.1078	3.0782
(8)	μ_{SX} (day^{-1})	1.8216	1.0362
	Y_{XS} (g g^{-1})	2.6872	2.7629
	Y_{PS} (mg g^{-1})	60.3616	66.6776

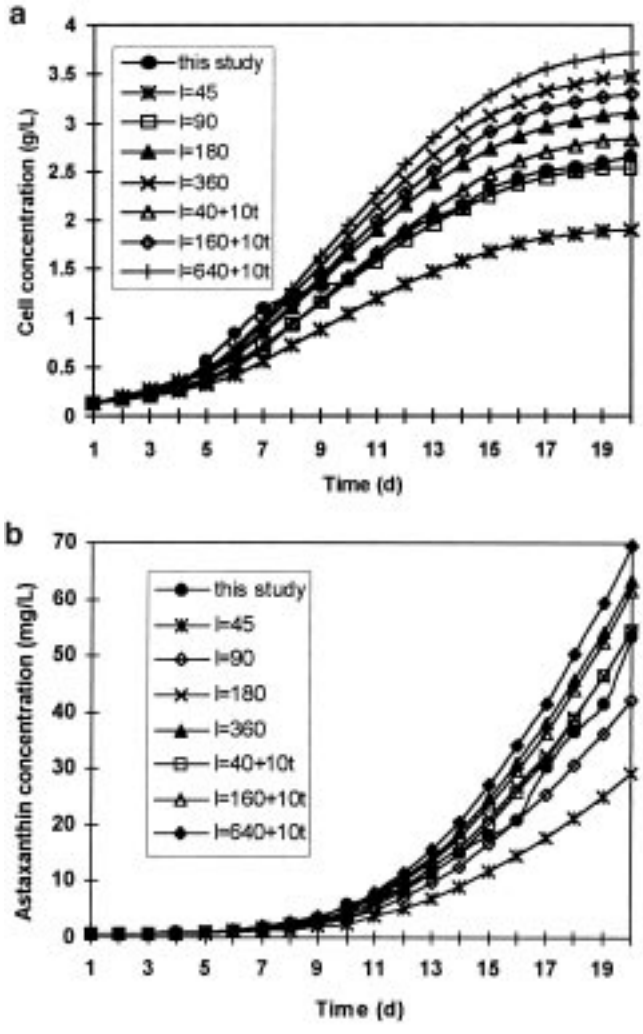

Figure 3 Simulation curves for the effect of different light intensity on cell growth (a) and astaxanthin formation (b) in batch mixotrophic culture.

The results demonstrate that for batch culture the maximum cell concentration in this study was 39.7% higher than that in the constant light intensity mode, I = 45, and approached the level at the constant light control mode, I = 90, or the variant light control mode, I = 40+10t (ie the light intensity change complied with the law I = 40+10t where t = 1,2,…,20 (days), and the maximum astaxanthin concentration in this study was 26.4%–81.6% higher than that in the constant light intensity modes I = 45 and I = 90, and approached the level at the constant light control programme, I = 180, or the variant light control programme, I = 40 + 0t. While for fed-batch culture the maximum cell concentration in this study was 45.8% higher than that in the constant light intensity mode, I = 45, and approached the level at I = 90 or the variant light control mode, I = 40 + 10t; the maximum astaxanthin concentration in this study was 43.5% higher than that in the constant light intensity, I = 45, and approached the level at I = 90 or the variant light control mode, I = 40 + 10t. These indicated that the stepwise increased light intensity mode could be replaced by a constant light intensity mode; lower light intensity (<90 μmol m^{-2} s^{-1}) produces the effect of light limitation,

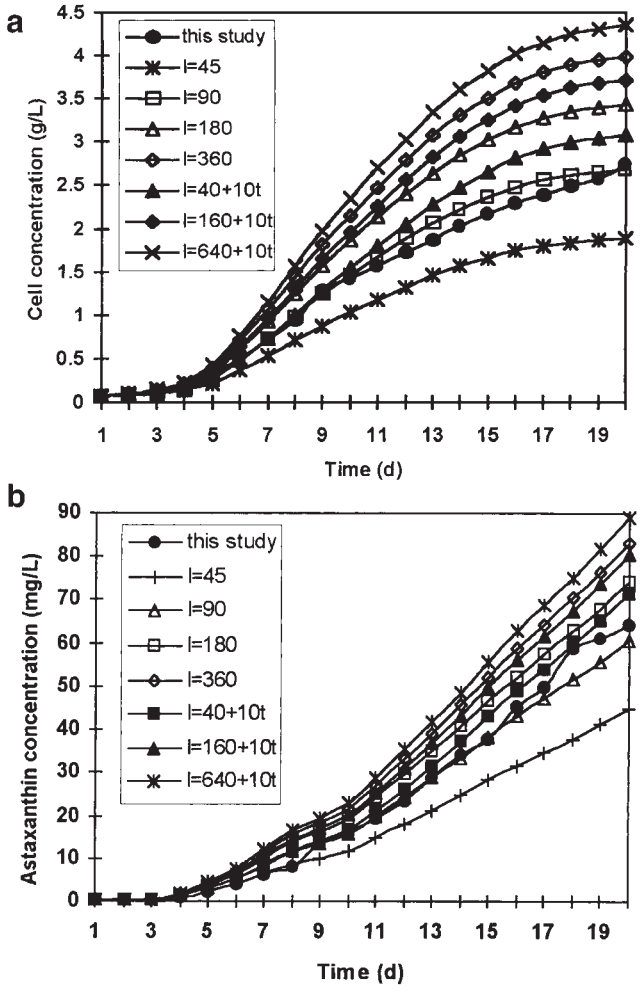

Figure 4 Simulation curves for the effect of different light intensity on cell growth (a) and astaxanthin formation (b) in fed-batch mixotrophic culture.

and in batch culture the effect of light limitation on cell growth was not as serious as the effect of light limitation on astaxanthin formation.

On the other hand, it can also be seen that for the constant light intensity mode, when light intensity increases from $I = 45$ μmol m^{-2} s^{-1} to $I = 180$ μmol m^{-2} s^{-1}, the cell concentration increased by 63.3% (batch culture) or 82.5% (fed-batch culture), and the astaxanthin concentration increased 85.7% (batch culture) or 65.4% (fed-batch culture); while with the further increase in light intensity, up to $I = 360$ μmol m^{-2} s^{-1}, the cell concentration only increased by 12.3% (batch culture) or 16.3% (fed-batch culture), the astaxanthin concentration only increased by 15.7% (batch culture) or 12.3% (fed-batch culture). For the variant light intensity mode, when light intensity changed from $I = 40 + 10t$ (μmol m^{-2} s^{-1}, where $t = 1,2,\ldots,20$) to $I = 160 + 10t$ (μmol m^{-2} s^{-1}, where $t = 1,2,\ldots,20$), the cell concentration increased by 16.3% (batch culture) or 21.2% (fed-batch culture), and the astaxanthin concentration increased by 12.5% (batch culture) or 12.3% (fed-batch culture); while with further increase in light intensity, up to $I = 640 + 10t$ (μmol m^{-2} s^{-1}, where $t = 1,2,\ldots,20$), the cell concentration increased by only 12.4% (batch culture) or 16.9% (fed-batch culture), the astaxanthin concentation increased by only 11.8% (batch culture) or 10.7% (fed-batch culture). These suggested that higher light intensities (>360 μmol m^{-2} s^{-1}) result in the effect of photoinhibition.

Thus according to the model predictions made above, the optimal light intensity for mixotrophic growth of *H. pluvialis* in batch or fed-batch cultures using a 3.7-L bioreactor was found to be 90–360 μmol m^{-2} s^{-1} and the stepwise increased light intensity mode could be replaced by a constant light intensity mode.

Furthermore, the comparison between the experimental data (the average values of three experiments) from the second and third runs is shown in Figures 5 and 6. For cell concentration and product concentration in batch culture, the errors, *E*, between the two groups of data from the second run and the third run are 7.4% and 8.9%, respectively; and in fed-batch culture the corresponding errors, *E*, for cell concentration and product concentration are 5.9% and 8.2%, respectively. It means that the result of the second run with a constant light control mode ($I = 180$) approched the result of the third run with a variant light control mode ($I = 40 + 10t$), ie, the variant light control mode can be replaced by a constant light intensity mode. This showed that the model prediction and the experimental result were consistent, hence the proposed model is applicable.

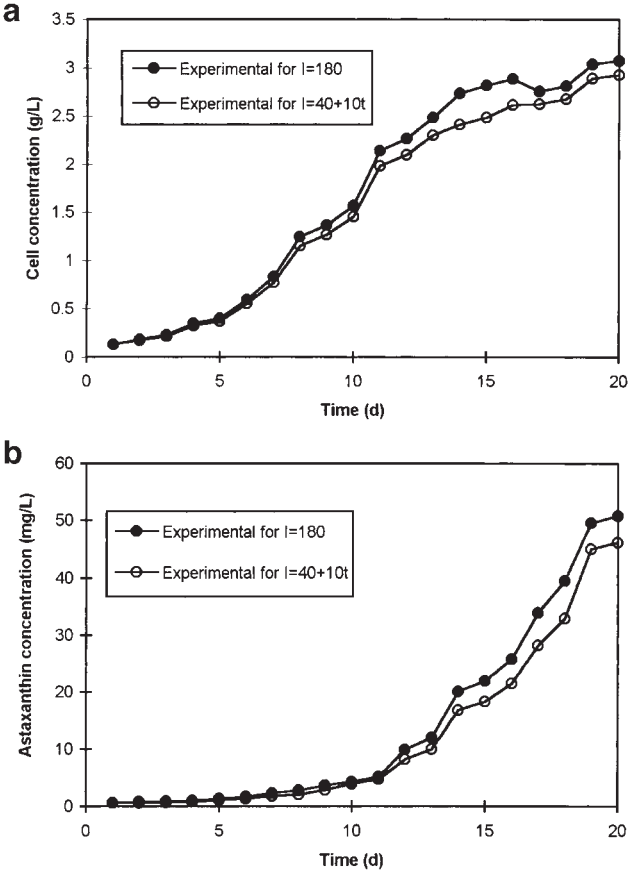

Figure 5 Comparison of experimental results, (a) for cells and (b) for astaxanthin, of the second run with a constant light intensity, $I = 180$, and the third run with a stepwise increased light intensity $I = 40 + 10t$, where $t = 1,2,\ldots,20$ (days) in batch culture.

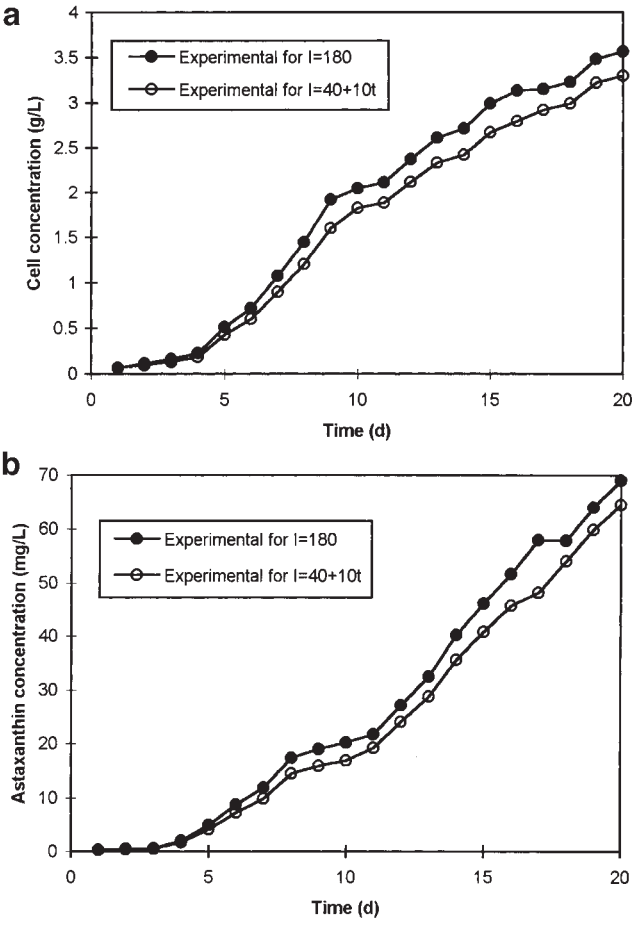

Figure 6 Comparison of experimental results, (a) for cells and (b) for astaxanthin, of the second run with a constant light intensity, $I = 180$, and the third run with a stepwise increased light intensity $I = 40 + 10t$, where $t = 1,2,\ldots,20$ (days) in fed-batch culture.

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