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Dynamics and stability analysis of the growth and astaxanthin production system of *Haematococcus pluvialis*

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This paper investigated high cell density cultivation of *Haematococcus pluvialis* for astaxanthin production in 3.7-L bioreactors. A biomass concentration of 2.74 g L^{-1} and an astaxanthin yield of 64.4 mg L^{-1} were obtained. Based on the experimental results, a new and simple dynamic model is proposed, differing from Monod kinetics, to describe cell growth, product formation and substrate consumption. Good agreement was found between the model predictions and experimental data. The model revealed that there was cell growth inhibition on product formation and product feedback compensation for substrate consumption, but no substrate inhibition or product inhibition of cell growth. Stability analysis demonstrated that no multiplicity of steady states was observed; the unique positive steady state was locally asymptotically stable; and the effect of dilution rate on steady states was greater than that of the initial substrate concentration.

Keywords: *Haematococcus pluvialis*; mixotrophic culture; astaxanthin production; dynamic model; stability analysis

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 [15] and a possible chemopreventive agent for bladder carcinogenesis [17]. *Haematococcus pluvialis* was able to grow heterotrophically on acetate in the dark [9]. However, dark-grown cultures accumulated only trace amounts 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, numerous papers have been published on the effects of nutritional and environmental factors such as light intensity, temperature and acetate concentration on growth of *Haematococcus* and its accumulation of astaxanthin [2,3,9,10,12,13].

Large-scale production of astaxanthin by *H. pluvialis* is hampered by problems associated with process design [13], whereas the optimization and control of bioprocesses often requires establishment of mathematical models that describe the kinetics of process variables (microbial growth, substrate uptake and product formation). In particular, due to the development of new biological products and tight regulations on product quality, the stability and consistency in a bioprocess has received sustained interest. Knowledge of culture dynamics and stability is needed, which would help to understand, optimize and control the process. Although the dynamic behaviour of microbial cultures has been the objective of a number of theoretical works [4,5,8,19,21], little information is available about the stability of microalgal culture systems on mixotrophic culture. On the other hand, it is now well accepted that Monod kinetics and some of its modifications can only be used under restrictive conditions and there are some difficulties in understanding the steady state behavior of microorganisms, particularly in understanding sustained oscillation and chaos [19].

The objective of this study was to investigate the growth of *Haematococcus pluvialis* and the production of astaxanthin in mixotrophic culture. Based on the experimental results, a new and simple dynamic model was proposed, differing from Monod kinetics, to describe cell growth, product formation and substrate consumption and the steady state of this system was determined.

Materials and methods

Microorganism and culture medium

Haematococcus pluvialis (*H. lacustris*, UTEX No. 16) was obtained from the University of Texas Culture Collection. The Hong Kong medium was used which 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 $FeSO_4 \cdot 7H_2O$, 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.

Fed-batch culture

The experiment was carried out in a 3.7-L bioreactor (Bioengineering AG, 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 culture temperature was set at 30°C. Two hundred millilitres of an inoculum (approximately 22000 cells ml^{-1}) were used. Irradiance was provided by surrounding cool white fluorescent tubes. Forty milliliters of a solution containing 1 g sodium acetate were added daily to the bioreactor for 4 days from day 4.

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Analytical methods

Cell dry weight was determined by filtering 10 ml of the culture fluid through a predried filter membrane (Millipore 0.45-μm pore size) and subsequently drying the membrane containing the algal cells in a vacuum oven at 85°C overnight to constant weight. Astaxanthin was determined as previously described [20].

Derivation of mathematical models and theory

In a microbial culture system, there exist three primary state variables: biomass, substrate and product. Substrate makes cells grow, cells metabolize product, and product may exert a feed-back impact (inhibition or stimulation) on the growth of cells. They are interactive. The following assumptions were made about the kinetics of the microalgal culture system: (1) The growth rate of cells in the absence of substrate inhibition and product inhibition satisfies the logistic law, a model widely used in population ecology:

$$\frac{\mathrm{d}X}{\mathrm{d}t} = r_{\mathrm{x}}\left(1 - \frac{X}{K}\right) \tag{1}$$

where r and K are positive constants, named the intrinsic growth rate and the carrying capacity, respectively [11] and X is cell concentration. The latter here means a critical concentration of cells above which cells themselves produce autoinhibition. (2) The contribution of one component to the other component's effect on growth rate is represented by a term proportional to their concentrations, such as the contribution of product to the cell's growth rate is cXP, enhancement or inhibition depends on positive c, or negative c, respectively. These terms like XP can be thought of as representing the conversion of energy from one source to another [14]. (3) The product has an intrinsic formation rate within the cell, a term proportional to the concentration itself, $r_{\mathrm{p}}P$, this represents an intrinsic ability of the cell in forming the product, which is dependent on the species and strains of microorganisms studied. (4) Without consideration of other factors, the effects of dilution rate (D) on each component result in exponential decay, that is a term like ($-DX$).

Thus, the following model system and governing equations are derived:

$$\begin{aligned}
\frac{\mathrm{d}X}{\mathrm{d}t} &= -DX + r_{\mathrm{x}}X\left(1 - \frac{X}{K}\right) + aPX + bSX \\
\frac{\mathrm{d}P}{\mathrm{d}t} &= -DP + r_{\mathrm{p}}P + cXP + eSP \\
-\frac{\mathrm{d}S}{\mathrm{d}t} &= -D(S_{\mathrm{o}} - S) + fXS + gPS
\end{aligned} \tag{2}$$

where X (g L^{-1}), P (mg L^{-1}) and S (g L^{-1}) are the concentrations of cell, product and substrate, respectively. S_{o} is the initial substrate concentration (g L^{-1}). r_{x} (day^{-1}), r_{p} (day^{-1}) and r_{s} (day^{-1}) are the intrinsic growth rate of cell, the intrinsic formation rate of product and the intrinsic consumption rate of substrate, respectively. K is a positive constant, a critical concentration of the cell above which the cell itself produces autoinhibition. a, b, c, e, f and g are constants, which represent the interactions among X, P and S.

It is useful to write the system in nondimensional form. Although there is no unique way of doing this, it is often a good idea to relate the variables to some key relevant parameters. Here, let us write

$$\begin{aligned}
&u(\tau) = \frac{X(t)}{K},\ v(\tau) = \frac{P}{K},\ w(\tau) = \frac{S}{S_{\mathrm{o}}},\ \tau = r_{\mathrm{x}}t, \\
&\alpha_1 = \frac{aK}{r_{\mathrm{x}}},\ \alpha_2 = \frac{bS_{\mathrm{o}}}{r_{\mathrm{x}}}\ \beta_1 = \frac{r_{\mathrm{p}}}{r_{\mathrm{x}}},\ \beta_2 = \frac{cK}{r_{\mathrm{x}}}, \\
&\beta_3 = \frac{eS_{\mathrm{o}}}{r_{\mathrm{x}}},\ \theta_1 = \frac{D}{r_{\mathrm{x}}},\ \theta_2 = \frac{fK}{r_{\mathrm{x}}},\ \theta_3 = \frac{gK}{r_{\mathrm{x}}}
\end{aligned}$$

Substituting these notations into Eqn (2), the following dimensionless equations are obtained:

$$\begin{aligned}
\frac{\mathrm{d}u}{\mathrm{d}\tau} &= -\theta_1 u + u(1-u) + \alpha_1 uv + \alpha_2 uw = F(u,v,w) \\
\frac{\mathrm{d}v}{d\tau} &= -\theta_1 v + \beta_1 v + \beta_2 uv + \beta_3 vw = G(u,v,w) \\
\frac{\mathrm{d}w}{\mathrm{d}\tau} &= -\theta_1(1-w) + \theta_2 uw + \theta_3 vw = H(u,v,w)
\end{aligned} \tag{3}$$

The equilibrium or steady state concentrations (u^*,v^*,w^*) are solutions of $\mathrm{d}u/\mathrm{d}\tau = 0$, $\mathrm{d}v/\mathrm{d}\tau = 0$, $\mathrm{d}w/\mathrm{d}\tau = 0$, namely:

$$F(u^*,v^*,w^*) = 0, \qquad G(u^*,v^*,w^*) = 0, \qquad H(u^*,v^*,w^*) = 0$$

We are only concerned here with positive solutions u^*, v^* and w^* (except the trivial steady state $u = 0$, $v = 0$, $w = 0$). We can attempt to determine behavior near (u^*,v^*,w^*) by expanding the right-hand side of Eqn (3) in a Taylor's series about (u^*,v^*,w^*) and neglecting all terms of second and higher in the deviations $u(\tau) - u^*$, $v(\tau) - v^*$ and $w(\tau) - w^*$, since they are presumed small. Then we obtain the following linear approximation system:

$$\begin{pmatrix} \frac{\mathrm{d}u}{\mathrm{d}\tau} \\ \frac{\mathrm{d}v}{\mathrm{d}\tau} \\ \frac{\mathrm{d}w}{\mathrm{d}\tau} \end{pmatrix} = \mathbf{J}\begin{pmatrix} u \\ v \\ w \end{pmatrix}, \tag{4}$$

$$\mathbf{J} = \begin{pmatrix} \frac{\partial F}{\partial u}, & \frac{\partial F}{\partial v}, & \frac{\partial F}{\partial w} \\ \frac{\partial G}{\partial u}, & \frac{\partial G}{\partial v}, & \frac{\partial G}{\partial w} \\ \frac{\partial H}{\partial u}, & \frac{\partial H}{\partial v}, & \frac{\partial H}{\partial w} \end{pmatrix} (u^*,v^*,w^*)$$

where $\mathbf{J}$ is called Jacobian matrix or stability matrix. The

local stability of a particular steady state will be of greatest concern. A steady state is said to be locally asymptotically stable if the system will return to the pertinent state after a sufficiently small and arbitrary perturbation from the steady state. Local stability is determined in most cases by the eigenvalues λ of the matrix **J**, namely, λ satisfies the characteristic equation:

$$\mathbf{Det}(\mathbf{J} - \lambda\mathbf{I}) = 0, \qquad \mathbf{I} \text{ is the identity matrix}$$

Det means the value of determinant. If the real parts of all the eigenvalues are negative, $\mathbf{Re}(\lambda)<0$, the steady state is said to be locally asymptotically stable.

The steady state solutions of differential equations were obtained by evaluating the derived analytical expressions using standard and readily available software packages: Microsoft EXCEL Ver. 5.0 for spreadsheet computations and MATHEMATICA Ver. 3.0 for symbolic calculations [18].

Results and discussion

The results of a fed-batch culture experiment are shown in Figure 1. A cell dry weight concentration of 2.74 g L^{-1} and an astaxanthin yield of 64.4 mg L^{-1} were obtained. The experimental data were to fit Equation (2) and yields (D = 0.016 day^{-1}, S_o = 0.917 g L^{-1}):

$$\frac{dX}{dt} = -DX + 0.1697X\left(1 - \frac{X}{1.1720}\right) + 0.0038PX + 0.8986SX \tag{5}$$

$$\frac{dP}{dt} = -DP + 0.3364P - 0.1245XP + 0.6824SP$$

$$-\frac{dS}{dt} = -D(S_o - S) + 0.9840XS - 0.0511PS$$

It can be seen from Eqn (5) that no substrate inhibition exists for cell growth (0.8986>0) and product formation (0.6824>0). But cell growth exerts an inhibitory impact on product formation (–0.1245<0), although no product inhibits cell growth (0.0038>0). Moreover, cell growth accelerates the consumption of substrate (0.9840>0), but product formation has a feedback compensation for substrate consumption (–0.0511<0). This can be understood in such a way: the formation of product, due to occupation of some spaces, reduces the quantity of cell growth (although it does not inhibit it), hence reduces the demand of substrate and slows down the rate of substrate consumption, this is equivalent to a compensation.

A good agreement was found between the calculated values and the experimental data (Figure 1); 60 sets of data produced 8.73% of the average relative error. After mathematical calculations, no multiplicity of steady states is found, and the unique positive nondimensional steady state is obtained:

$$(u^*, v^*, w^*) = (2.5714, 46.7561, 0.0876)$$

transforming back to the dimensional variables:

$$(X^*, P^*, S^*) = (3.0137, 54.7982, 0.0803)$$

and all eigenvalues satisfy: $\mathbf{Re}(\lambda)<0$. Thus the above steady state is locally asymptotically stable. The trajectory of the three-variable system (Eqn 5) and the surrounding vector field are shown in Figure 2 (transformed back from dimensionless variables), directed towards the local stable point.

Figures 3 and 4 show the effects of dilution rate and initial substrate concentration on the steady state concentration (all these steady states are locally asymptotically stable). These data indicate that the steady state cell density decreased by 13% and the steady state product concentration decreased by 25%, when dilution rate varied from 0.008 (day^{-1}) to 0.2 (day^{-1}). Furthermore only 7% of the increase and 2% of the reduction in the steady state concentrations of cell and product respectively were observed when the initial substrate concentration increased from 0.4585 (g L^{-1}) to 2.751 (g L^{-1}). On the other hand, no great changes in the resulting cell and product concentrations were observed (Figures 5 and 6) transformed back from

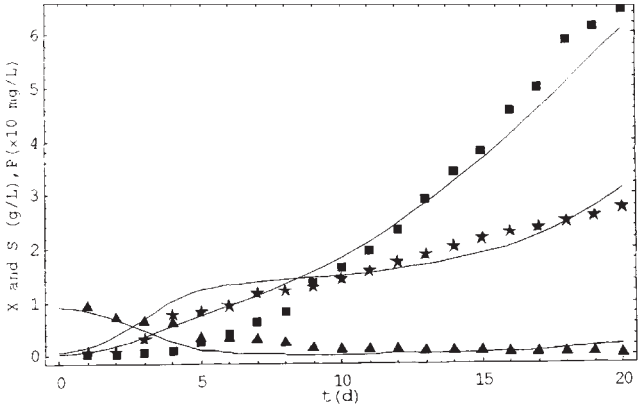

Figure 1 Time courses of cell growth, sodium acetate consumption and astaxanthin formation for *Haematococcus pluvialis* in fed-batch mixotrophic culture. Star, filled box and filled triangle refer to the experimental data for cell, astaxanthin and sodium acetate concentrations, respectively; lines refer to the calculated values.

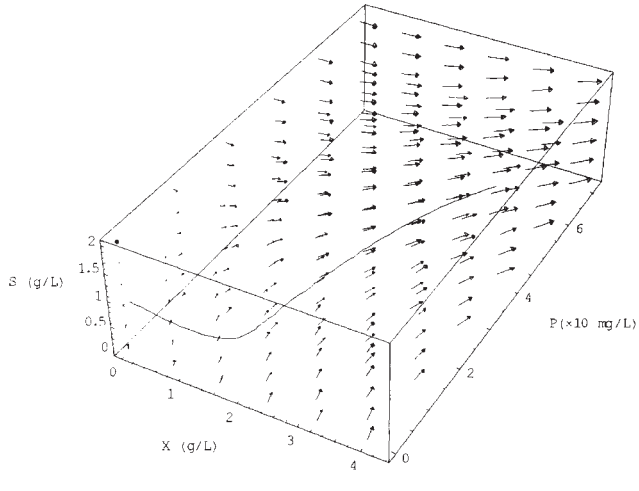

Figure 2 The trajectory and the surrounding vector field of the three-variable system (Eqn 2). The arrows are directed towards the steady state.

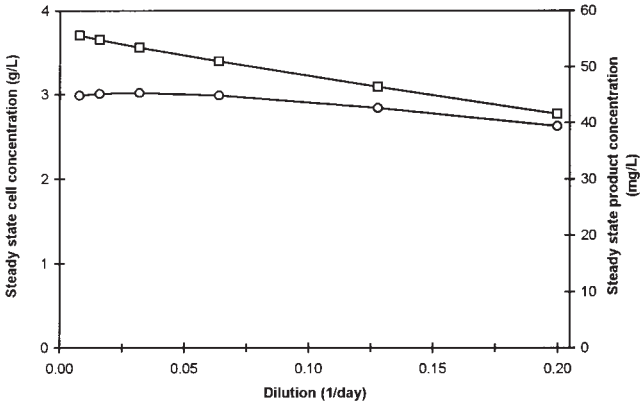

Figure 3 The effect of dilution rate on steady state concentrations. The open circle and open box refer to the steady state concentrations of cell and product, respectively.

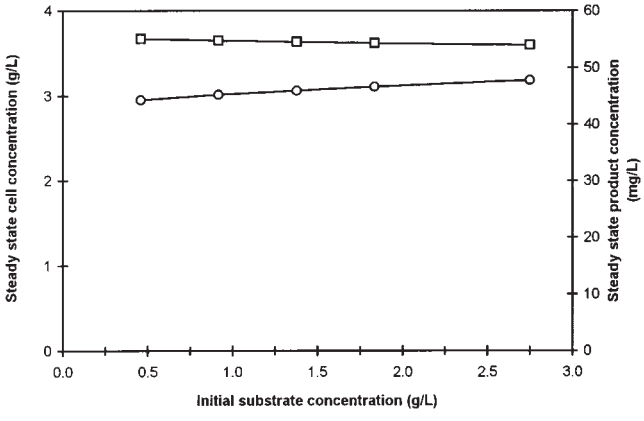

Figure 4 The effect of initial substrate concentration on steady state concentrations. The open circle and open box refer to the steady state concentrations of cell and product, respectively.

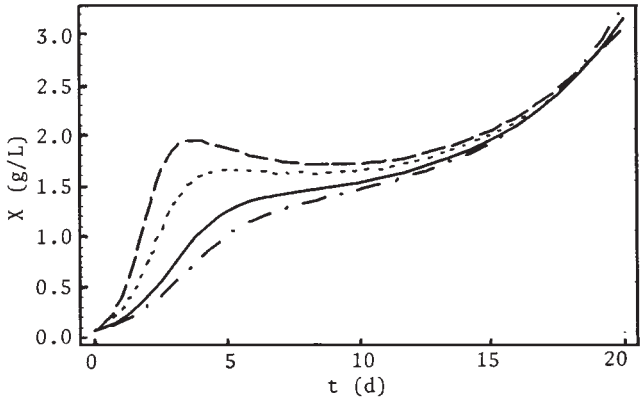

Figure 5 The effect of changing initial substrate concentration on the resulting cell concentration. Solid line, this work with initial substrate concentration S_o; dotted line, with 50% increase in S_o; dashed line, with 100% increase in S_o; dot-dashed line, with 50% reduction in S_o.

dimensionless variables) when increasing or decreasing the initial substrate concentration. So the priority of changing operation conditions should consider dilution rate rather than initial substrate concentration.

So far there have been some culture examples in which

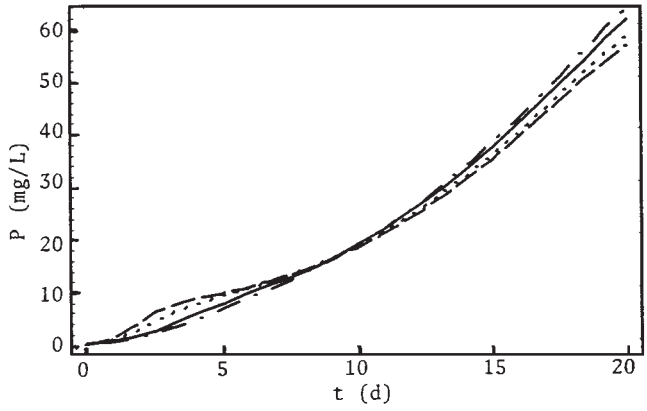

Figure 6 The effect of changing initial substrate concentration on the resulting product concentration. Solid line, this work with initial substrate concentration S_o; dotted line, with 50% increase in S_o; dashed line, with 100% increase in S_o; dot-dashed line, with 50% reduction in S_o.

no multiplicity is found. The yeast culture *Trichosporon cutaneum* has been extensively used for chiometric and energetic studies and for reactor characterization [7,16]. Multiplicity has not been found due to substrate overflow and no extracellular metabolites were found in the culture. Negative examples for the occurrence of multiplicity include a large number of continuous cultures limited by substrate [19]. Though product inhibition may be involved in these cultures, there is no metabolic overflow due to substrate limitation. In *Klebsiella pneumonia* grown on glycerol [19] and ethanol fermentation by *S. cerevisiae* [1], multiplicity was always observed. The authors claimed that product inhibition or the increased formation of a toxic product under conditions of substrate excess was the main reason for the occurrence of stable multiple steady states in a certain operation range of continuous culture. This could be used to explain that no multiplicity was found in this study due to the absence of substrate inhibition, product inhibition and the increased formation of a toxic product.

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