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Optimisation of microalgal starch formation for the biochemical production of biobutanol

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Abstract

The current global energy demands have led to an overuse of petroleum resources. In consequence, biofuels have emerged as a suitable and renewable replacement for fossil fuels. A potential replacement for gasoline is biobutanol due to its high heat of combustion, among several other properties (Abdehagh et al., 2014). Biobutanol is produced through ABE fermentation, a renowned microbial process. An efficient feedstock selection, however, is still necessary to overcome current production challenges (Kumar and Gayen, 2011). Although microalgal biomass has largely been examined as a biodiesel feedstock due to its ability to accumulate oil bodies (Markou et al., 2012), only recently has it been considered a promising substrate for ABE fermentation because its structure contains starch, a polymeric carbohydrate. Simple microalgal cultivation strategies such as nitrogen and phosphorus limitation have been shown to further enhance starch contents (Markou et al., 2012), but such strategies must be effectively implemented to reduce the expected trade-off in growth. Kinetic models capable of predicting cell dynamics during cultivation represent a robust tool for establishing optimized strategies. Nevertheless, most modelling approaches have been constructed under a single-nutrient basis and/or are only able to predict lipid formation. Thus, this work aims to develop a novel predictive multi-parameter kinetic model for the optimization of starch formation during microalgae cultivation. The algal growth rate is co-limited by nitrogen, phosphorus, and the carbon source, and follows a compartmentalized structure considering three intracellular pools: active biomass, starch, and lipids. The model was fitted and successfully validated against experimental datasets generated from lab-scale cultures of *Chlamydomonas reinhardtii* CCAP 11/32C grown mixotrophically under various nutrient concentrations. Fitting of the model parameters was carried out through an in-house developed optimization algorithm linking stochastic and deterministic methods avoiding getting trapped in local optima. The predictive performance of the model is exploited by establishing the optimal conditions for maximum starch formation.

Keywords: microalgae; *chlamydomonas*; kinetic modelling; biofuels.

1. Introduction

Global warming and crude oil depletion have shifted research and industry attention to the area of biofuels and their development. The current sugar-based biofuel market has been dominated by bioethanol, but a higher energy content and compatibility with

existing infrastructure make biobutanol a more suitable candidate for the energy sector (Kumar and Gayen, 2011). Biobutanol is biochemically produced by the ABE (Acetone-Butanol-Ethanol) fermentation, a microbial process whereby *Clostridia* species metabolise carbohydrates into fermentation solvents. Although food-based and lignocellulosic feedstocks have been commonly studied for their viability as fermentation substrates, these sugar-rich compounds create challenges due to their competition with food destined for human use and arable land. Recently, microalgae have been considered as a suitable ABE fermentation substrate due to their ability to accumulate starch. Nutrient-limited strategies, such as nitrogen and phosphorus deprivation, have been successfully employed to further increase starch accumulation in microalgal cells, but a simultaneous trade-off in biomass production affects overall productivity (Markou et al., 2012). Optimisation of the microalgal cultivation stage is necessary to establish the optimal initial nutrient concentrations for the formation of starch. Thus, in this work, an integrated experimental and computational study was undertaken to develop a multi-parameter kinetic model for the prediction of starch formation in response to nitrogen, phosphorus, and acetate (carbon) concentrations.

2. Materials and Methods

2.1. Strain and lab-scale cultivation

Lab-scale experiments were carried out with the wild-type strain *Chlamydomonas reinhardtii* CCAP 11/32c, grown mixotrophically in Tris-Acetate-Phosphate (TAP) medium. In order to evaluate nutrient-limitation and calibrate the proposed model, six different cultures were grown under various nitrogen ($0.3543 - 0.3824 \text{ gN L}^{-1}$), phosphorus ($0.01 - 0.1 \text{ gPO}_4 \text{ L}^{-1}$), and acetate ($0.42 - 0.84 \text{ gC L}^{-1}$) concentrations. Culture vessels contained 500 mL of medium and 1 mL of algal inoculum, and were kept at 25 °C and 150 rpm while providing constant illumination of $125 \mu\text{mol m}^{-2} \text{ s}^{-1}$ in a light/dark cycle of 16/8 hours. Samples of the biomass and residual medium were taken at regular intervals throughout the cultivation period until cells attained the stationary phase.

2.2. Analytical methods

The cell dry weight was determined gravimetrically by drying the wet biomass for 24 h at 70 °C. Starch concentration was measured by means of a colorimetric Total Starch Kit as per the method supplied by *Megazyme International*, Ireland. Lipid concentration was quantified by solvent extraction in a SOXTEC Unit 1043, where oil extraction was carried out for 2h using hexane at 160 °C. Extraction was then followed by rinsing and evaporating stages of 20 min and 30 min, respectively. Extracted oil was then measured gravimetrically. The residual nutrient concentrations were determined as follows: nitrogen was measured in a TOC-V_{CSH} /TNM-1, Shimadzu (Total Organic Carbon/ Total Nitrogen); phosphorus was quantified in a Varian Vista MPX ICP-OES at $\lambda=213\text{nm}$ (Inductively Coupled Plasma - Optical Emission Spectroscopy); acetic acid was quantified by HPLC (High Pressure Liquid Chromatography) in a Hi-Plex column (8 m, 300x7.7mm) coupled with a UV detector at $\lambda=210 \text{ nm}$. H_2SO_4 5mM was used as mobile phase at 50 °C and a flow rate of 0.6 mL min^{-1} .

3. Kinetic Modelling

The multi-parameter model presented here is based on our previous modelling work (Figueroa-Torres et al., 2016), which was expanded to account for phosphorus-limited

growth. The model considers the following 9 state variables: Total biomass (X , gC L⁻¹), starch (S , gC L⁻¹), lipids (L , gC L⁻¹), active biomass (x^* , gC L⁻¹), nitrogen (N , gN L⁻¹), nitrogen quota (q_N , gN gC⁻¹), phosphorus (P , gPO₄ L⁻¹), phosphorus quota (q_P , gPO₄ gC⁻¹), and acetate (A , gC L⁻¹). All kinetic parameters are defined and presented in Table 1. Following the approach of Mairet et al. (2011), total biomass is made up of three carbon-based elements: starch, lipids, and active biomass (i.e. $X = S + L + x^*$). Starch and lipid dynamics incorporate formation and degradation rates and depend mainly on the internal nitrogen ($N_{int} = q_N \cdot X$) and carbon ($A_{int} = A - A_o$) concentrations. Accumulation rates for these elements are described by Eq.(1) – Eq.(4).

$$\frac{dX}{dt} = \mu \cdot X \quad (1)$$

$$\frac{dS}{dt} = r_1 \cdot \frac{N_{int}^{n_S}}{N_{int}^{n_S} + K_{s,S}^{n_S} + (N_{int}^2/k_{i,S})^{n_S}} \cdot \frac{k_1}{k_1 + N/N_o} \cdot \left(1 + \frac{1}{\mu} \cdot e^{\phi_S \cdot A_{int}}\right) \cdot \mu \cdot x^* - \frac{r_2}{q_N} \cdot X \quad (2)$$

$$\frac{dL}{dt} = r_3 \cdot \frac{N_{int}^{n_L}}{N_{int}^{n_L} + K_{s,L}^{n_L} + (N_{int}^2/k_{i,L})^{n_L}} \cdot \frac{k_2}{k_2 + N/N_o} \cdot \left(1 + \frac{1}{\mu} \cdot e^{\phi_S \cdot A_{int}}\right) \cdot \mu \cdot x^* - \frac{r_4}{q_N} \cdot X \quad (3)$$

$$\frac{dx^*}{dt} = \frac{dX}{dt} - \left(\frac{dS}{dt} + \frac{dL}{dt}\right) \quad (4)$$

Here, a new mathematical expression for the algal specific growth rate, μ , was constructed so as to portray mixotrophic microalgal growth under either nitrogen or phosphorus limitation. Mixotrophic conditions are expressed by the sum of the heterotrophic and phototrophic growth rates, μ_H and μ_I , both modelled as Haldane functions to account for inhibition. The growth rates limited by nitrogen and phosphorus, μ_N and μ_P , are modelled as Droop functions subject to a minimum law as shown in Eq.(5).

$$\mu = \mu_{\max} \cdot [w_H \cdot \mu_H(A) + w_I \cdot \mu_I(I)] \cdot \min[\mu_N(q_N), \mu_P(q_P)] \quad (5)$$

$$\mu = \mu_{\max} \cdot \left(w_H \cdot \frac{A}{A + K_{s,A} + A^2/k_{i,A}} + w_I \cdot \frac{I}{I + K_{s,I} + I^2/k_{i,I}} \right) \cdot \min\left(1 - \frac{q_{N,0}}{q_N}, 1 - \frac{q_{P,0}}{q_P}\right) \quad (6)$$

In Eq. (5), w_H and w_P , are weighing functions accounting for the contribution of the heterotrophic or phototrophic rates to overall growth. Light distribution, I , along the culture vessel (with a depth equivalent to z , m) is described by the Beer-Lambert law:

$$I = I_o \exp(-\alpha X z) \quad (7)$$

Consumption of acetate, nitrogen, and phosphorus over the cultivation period is described by Eq.(8) - Eq.(10), respectively.

$$\frac{dA}{dt} = -\frac{1}{Y_{X/A}} \cdot \frac{\mu_H(A)}{\mu_H(A) + \mu_I(I)} \cdot \frac{dX}{dt} \quad (8)$$

$$\frac{dN}{dt} = -\rho_N \cdot X = -\bar{\rho}_{N,\max}(X) \cdot \frac{N}{N + K_{s,N} + N^2/k_{i,N}} \cdot \frac{A}{A + K_{s,A:N} + A^2/k_{i,A:N}} \cdot X \quad (9)$$

$$\frac{dP}{dt} = -\rho_P \cdot X = -\bar{\rho}_{P,\max}(X) \cdot \frac{P}{P + K_{s,P} + P^2/k_{i,P}} \cdot \frac{A}{A + K_{s,A:P} + A^2/k_{i,A:P}} \cdot X \quad (10)$$

The specific uptakes rates of nitrogen and phosphorus, ρ_N and ρ_P , are expressed by inhibited-type kinetics so as to account for the inhibition of growth observed at high nutrient concentrations. Furthermore, since nutrient consumption was found to stop at high cell densities, the maximum uptake rates of nitrogen and phosphorus are both regulated by a decreasing function of total biomass, as shown in Eq.(11) and Eq.(12). In line with experimental observations, the maximum nitrogen uptake is additionally a function of both the initial nitrogen concentration and the phosphorus quota.

$$\bar{\rho}_{N,\max}(X) = \rho_{N,\max} \cdot e^{-\phi_N X} \cdot \frac{N_o^n}{N_o^n + K_{N_o}^n} \cdot \left(1 - \frac{K_{q_P}}{q_P}\right) \quad (11)$$

$$\bar{\rho}_{P,\max}(X) = \rho_{P,\max} \cdot e^{-\phi_P X} \quad (12)$$

Finally, the dynamics of the intracellular nitrogen and phosphorus quotas are obtained by differentiation with respect to time, which yields:

$$\frac{dq_N}{dt} = \rho_N - q_N \cdot \mu \quad (13)$$

$$\frac{dq_P}{dt} = \rho_P - q_P \cdot \mu \quad (14)$$

4. Results and discussion

From the six lab-scale batch cultures grown under various N-P-A regimes, four experimental datasets were used to fit model parameters and two were used for validation. The values of each parameter (Table 1) were estimated by minimizing an objective function defined as the sum of squared relative error (Error) between experimental data and model predictions. Minimization was carried out through a stochastic optimization algorithm coupled with a deterministic method as per the methodology of Vlysidis et al., (2011). The results from the preceding method are presented in Figure 1, showing predicted time-profiles of biomass, starch and lipids against their corresponding experimental values only for two different datasets.

Table 1. Kinetic parameters: definitions and corresponding values.

Parameter	Value	Units	Parameter	Value	Units
μ_{max} Maximum specific growth rate	0.1106	h^{-1}	Φ_p Uptake regulation coefficient	29.067	L gC^{-1}
$q_{N,0}$ Minimum nitrogen quota	0.8767	gN gC^{-1}	$K_{s,p}$ Half-saturation constant (P)	0.448	$\text{gPO}_4 \text{L}^{-1}$
$q_{P,0}$ Minimum phosphorus quota	0.0092	$\text{gPO}_4 \text{gC}^{-1}$	$K_{i,N}$ Inhibition constant (P)	2.428	$\text{gPO}_4 \text{L}^{-1}$
$K_{s,A}$ Half-saturation constant (A)	1.7892	gC L^{-1}	$K_{s,A,p}$ Half-saturation constant (A)	1.269	gC L^{-1}
$k_{i,A}$ Inhibition constant (A)	0.1097	gC L^{-1}	$k_{i,A,p}$ Inhibition constant (A)	0.206	gC L^{-1}
$K_{s,I}$ Half-saturation constant (I)	1.0782	$\mu\text{mol m}^{-2}\text{s}^{-1}$	r_1 Rate of starch formation	0.3841	gC gC^{-1}
$k_{i,I}$ Inhibition constant (I)	186.5275	$\mu\text{mol m}^{-2}\text{s}^{-1}$	r_2 Rate of starch degradation	0.0025	$\text{gN gC}^{-1}\text{h}^{-1}$
$Y_{X,A}$ Yield coefficient (A to X)	0.0399	gC gC^{-1}	r_3 Rate of lipid formation	0.3908	gC gC^{-1}
σ Light attenuation coefficient	0.6499	$\text{L gC}^{-1} \text{m}^{-1}$	r_4 Rate of lipid degradation	0.0054	$\text{gN gC}^{-1}\text{h}^{-1}$
$\rho_{N,max}$ Maximum nitrogen uptake rate	40.445	$\text{gN gC}^{-1}\text{h}^{-1}$	$K_{s,S}$ Half-saturation constant	0.0477	gN L^{-1}
$K_{N,0}$ Half-saturation constant (No)	0.3125	gN L^{-1}	$k_{i,S}$ Inhibition constant	0.1409	gN L^{-1}
n Shape-controlling parameter	126.767	-	n_S Shape-controlling parameter	3.6418	-
Φ_N Uptake regulation coefficient	137.455	L gC^{-1}	k_j Regulation constant for R_1	0.0354	-
K_{qP} Minimum P quota for N uptake	0.165	$\text{gPO}_4 \text{gC}^{-1}$	Φ_S Regulation constant for R_1	0.0000	L gC^{-1}
$K_{s,N}$ Half-saturation constant (N)	0.163	gN L^{-1}	$K_{s,L}$ Half-saturation constant	0.0481	gN L^{-1}
$k_{i,N}$ Inhibition constant (N)	0.113	gN L^{-1}	$k_{i,L}$ Inhibition constant	0.0275	gN L^{-1}
$K_{s,A,N}$ Half-saturation constant (A)	1.004	gC L^{-1}	n_L Shape-controlling parameter	1.3321	-
$K_{i,A,N}$ Inhibition constant (A)	1.098	gC L^{-1}	k_2 Regulation constant for R_2	0.2434	-
$\rho_{P,max}$ Maximum PO_4 uptake rate	13.997	$\text{gPO}_4 \text{gC}^{-1}\text{h}^{-1}$	Φ_r Regulation constant for R_2	1.0E-05	L gC^{-1}

As seen in Figure 1, a good level of agreement between predictions and experimental data was achieved (Error = 1.956), indicating the model's potential as a robust tool in the establishment of optimized cultivation strategies. Given that microalgal starch is a potential substrate for biobutanol production, a cultivation strategy for optimal starch formation was established in terms of the initial set of nitrogen, phosphorus, and acetate (N-P-A) concentrations.

Figure 1. Predicted time-profile for biomass, starch, and lipids, against experimental data. *Fitting*: culture grown in TAP (0.3824 gN L^{-1} , $0.1 \text{ gPO}_4 \text{ L}^{-1}$, and 1.05 gC L^{-1}); *Validation*: culture grown in A-enrichment and P-limitation (0.3824 gN L^{-1} , $0.01 \text{ gPO}_4 \text{ L}^{-1}$, and 1.26 gC L^{-1}). Error bars are the standard deviation from two replicates.

The optimal N-P-A set was identified in a ternary diagram for starch formation, which was constructed from the predictions generated by the validated model at a cultivation time of 190 h (period of stationary phase). The resulting diagram is presented in Figure 2, showing that a starch concentration of up to 0.06 gC L^{-1} could be obtained by *C. reinhardtii* cultures if grown under the encircled N-P-A concentration sets. When compared with the starch concentration attained by cultures grown in standard TAP medium (0.016 gC L^{-1}), this predicted increase in starch formation translates into a larger availability of the carbohydrate pool that will be ultimately required by Clostridial species to produce biobutanol.