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Production of lipid-based fuels and chemicals from microalgae: An integrated experimental and model-based optimization study

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ABSTRACT

Cultivation of microalgae is a promising long-term, sustainable candidate for biomass and oil for the production of fuel, food, nutraceuticals and other added-value products. Attention has been drawn to the use of computational and experimental validation studies aiming at the optimisation and the control of microalgal oil productivity either through the improvement of the growth mechanism or through the application of metabolic engineering methods to microalgae. Optimisation of such a system can be achieved through the evaluation of organic carbon sources, nutrients and water supply, leading to high oil yield. The main objective of this work is to develop a novel integrated experimental and computational approach, utilising a microalgal strain grown at bench-scale, with the aim to systematically identify the conditions that optimise growth and lipid production, in order to ultimately develop a cost-effective process to improve the system economic viability and overall sustainability. To achieve this, a detailed model has been constructed through a multi-parameter quantification methodology taking into account photo-heterotrophic biomass growth. The corresponding growth rate is based on carbon substrate concentration, nitrogen and light availability. The developed model also considers the pH of the medium. Parameter estimation was undertaken using the proposed model in conjunction with an extensive number of experimental data taken at a range of operating conditions. The model was validated and utilised to determine the optimal operating conditions for bench-scale batch lipid oil production.

1. Introduction

Fossil fuels provide a non-renewable form of energy that is also finite [12,31]. The use of non-renewable resources negatively impacts on the environment since it leads to the production of harmful greenhouse gas (GHG) emissions [17]. On the contrary, renewable forms of energy sources such as solar and wind energy as well as biomass, are environmentally sustainable [24]. Various biomass sources such as energy crops, animal fat, agricultural residues and fungal or bacterial microbes have been used for the commercial production of biofuels [2]. Biodiesel production is a well-established platform [20], with soybeans, canola oil, palm oil, corn oil, animal fat and waste cooking oil, the most common commercial sources.

Microalgal oil consists of the neutral lipid Triacylglycerol (TAG), which is stored in cytosolic and/or plastidic lipid bodies [18]. The accumulation of such lipid bodies can be enhanced by abiotic stress, including deprivation of nutrients like nitrogen (N) and phosphorus (P), and factors such as light intensity and temperature stress [5,19]. Depending on the fatty acid characteristics, the oil can be utilised directly or it can be processed into biolubricants, surfactants, nutritional lipids like omega-3 fatty acids, and importantly, into liquid fuels and gas. The use of microalgal oil for biodiesel production has not yet been exploited commercially as the current price of production is still too high compared to fossil fuel diesel. Approximately 60–75% of the total cost of microalgal biodiesel comes from microalgae cultivation, mainly due to the high cost of the carbon source, the fertilizer requirements and the high cultivation facility costs relative to often low oil productivity [22].

However, production of biofuels from microalgal oil bears several advantages both in terms of environmental impact and of sustainability. The main ones are the rapid growth rate of microalgae and high oil productivity per area of land used [26], the reduction of GHG emissions due to the avoidance of fossil fuel combustion and to the use and fixation of available inorganic (CO_2) and/or waste organic carbon (e.g. waste glycerol), the use of less resources (freshwater and nutrient fertilizer), particularly for marine or wastewater cultivated microalgae [43], and no competition for agricultural land and simple growing needs (light, N, P, potassium (K) and CO_2) [11,21]. Although microalgal oil has an immense potential in biotechnological applications, metabolic productivity needs to be enhanced to realise economic viability. Strain

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Nomenclature

TAG	Triacylglycerol
TAP	Tris-acetate-phosphate
DCW	Dry cell weight
N	Nitrogen
K	Phosphorus
S	Substrate
I	Light intensity
L	Lipid
X	Oil-free biomass
AA	Acetic acid
GA	Glycolic acid
FA	Formic acid
μ	Specific growth rate
μ_{max}	Maximum specific growth rate of biomass
K_S	Substrate saturation constant
K_{iS}	Substrate inhibition constant
μ_X	Specific growth rate of oil-free biomass
μ_{Xmax}	Maximum specific growth rate of oil-free biomass
K_{XS}	Acetate saturation constant
K_{iXS}	Acetate inhibition constant
K_{XN}	Nitrogen saturation constant
K_{iXN}	Nitrogen inhibition constant
q_L	Specific growth rate of lipid
q_{Lmax}	Maximum specific growth rate of lipid
K_{LS}	Acetate saturation constant
K_{iLS}	Substrate inhibition constant
K_{iNL}	Nitrogen inhibition constant
$Y_{X/S}$	Yield coefficient for oil-free biomass production with respect to substrate
$Y_{X/N}$	Yield coefficient for oil-free biomass production with respect to N
K_H	pH rate constant
$Y_{L/S}$	Yield coefficient for lipid production with respect to substrate
K_{XI}	Light saturation constant
K_{iXI}	Light inhibition constant
K_{LI}	Light saturation constant
K_{iLI}	Light inhibition constant
σ	Molar extinction coefficient
k_1	Parameter of the mathematical model
K_{GAS}	Acetate saturation constant
K_{GAN}	Nitrogen saturation constant
K_{iGAN}	Nitrogen inhibition constant
k_2	Parameter of the mathematical model
K_{FAS}	Acetate saturation constant
K_{FAN}	Nitrogen saturation constant

development by genetic manipulation, mutagenesis or natural selection is one approach that is being actively evaluated [27]. Alternatively, cultivation conditions and metabolic productivity can be optimized based on an integrated combination of mathematical modelling and growth experiments at different scales.

A critical component of sustainable microalgae-derived biofuel productivity is the balance between biomass growth and lipid accumulation, whereby conditions of extreme nutrient starvation that drive substantial cellular lipid accumulation can also significantly inhibit cell growth, and thus net volumetric lipid productivity is low [28]. For this reason, integrated experimental and theoretical studies to model and experimentally validate changes in microalgal metabolism and metabolite yield are an important tool to predict improvements to oil productivity [6,9,35]. The combination of predictive models and experiments allows the development of a framework that will reveal the relationship between microlgal growth and lipid accumulation which can be used to optimise the balance of biomass and oil productivity from algal strains, in order to ultimately achieve a positive energy balance for a cost-efficient and sustainable scaled-up biodiesel production.

Experimental studies have shown that both microalgae growth and lipid production can be simultaneously and antagonistically affected by two or more nutrients and environmental variables, such as carbon and nutrient concentrations, light intensity, pH and temperature [13,15,19]. However, the majority of the previously developed kinetic models are expressed either as a function of a single nutrient or environmental variable concentration, or as a function of multiple nutrient concentrations. Monod [40] formulated a kinetic model, the so-called Monod model, to analyse the effect of a single nutrient limitation on biomass growth, while the inhibition effects of the nutrient and of other growth parameters were not considered. Andrews [4] constructed an improved version of the Monod model to take into account both the single nutrient limitation and the nutrient inhibition effects, but this study did not take into consideration the inhibition effect of the other growth parameters. Such models have been extensively employed to analyse the effect of a single nutrient. The effect of light was analysed by [29], the effects of one substrate (S) and of pH were investigated by Zhang et al. [50], and the effect of temperature was explored by Bernard and Rémond [10].

The effect of multiple nutrient concentrations can be examined through the use of two other frameworks; the threshold and the multiplicative models [37]. The threshold model considers that the growth is only affected by the growth parameter with the lowest concentration, and therefore, the model takes the form of a single substrate growth model. On the contrary, the multiplicative model takes into account two or more growth parameters that contribute to microalgae growth equally. The threshold model was employed by Spijkerman et al. [46] for the investigation of the effects of substrate and of P concentration, while the multiplicative model was used by Bernard [8] for the analysis of the effects of light intensity and of N concentration. Although the aforementioned models are deemed to be accurate enough to predict the effects of the nutrients, they are not able to predict the simultaneous effects of other factors such as nutrient factors and environmental factors with the same accuracy. Moreover, although the control of microalgal growth and lipid accumulation by multiple factors (such as multiple limiting nutrients) has been investigated on a theoretical basis, the published data are limited and they do not allow conclusions on the kinetic relationship between microalgal growth and lipid accumulation with respect to the concentrations of the limiting nutrients [36].

Here, we present a comprehensive multiplicative kinetic model to describe microalgal growth and the relevant lipid oil production under photo-heterotrophic conditions. The formulated model takes into account the effects of four different growth-promoting resources: acetate (organic carbon substrate for the heterotrophic component of growth), nitrogen, light intensity and pH. The model simulates all of the effects simultaneously and it is capable of predicting the microalgal biomass growth and the lipid accumulation with high accuracy. To efficiently estimate the kinetic parameters that are crucial for accurate system simulations and to validate the developed model, experiments were performed using the well-studied chlorophyte microalgal species *Chlamydomonas reinhardtii* [5,39,45]. We demonstrate that such an integrated experimental-computational framework can be used to provide insights on biomass growth and lipid metabolism, and eventually to enable robust system design and scale-up.

2. Materials and methods

2.1. Strain and culture conditions

C. reinhardtii (CCAP 11/32C) was used here as the experimental microalgal strain, obtained from the Culture Collection of Algae and

Protozoa, UK. The strain was cultivated under photo-heterotrophic conditions in batch cultures [5]. Preculture of the strain was carried out in an environmentally-controlled incubation room at 25 °C, using 250 mL conical flasks containing 150 mL of Tris-acetate-phosphate (TAP) medium [30] (TAP constituents are given in Table S1) on an orbital shaker at 120 rpm for 7–10— days. A 4 ft. long 20 W high power led T8 tube light was used for illumination at a constant 125 $\mu Em^{-2} s^{-1}$ light intensity. Once sufficient cell density was reached, an algal inoculum of 1 mL was added to the experimental culture vessels, Small Anaerobic Reactors (SARs, 500 mL), containing 500 mL of modified TAP culture medium (described below) at the same temperature and light conditions as preculturing. The initial cell density of 0.024×10^6 cells per mL was identical for all the treatments. The number of cells was determined through the measurement of living cells using a Nexcelom Cellometer T4 (Nexcelom Biosciences). 20 μL of the sample was injected into the cellometer counting chamber and the chamber was then inserted into the apparatus. Once the sample was placed, the following specifications were defined: cell diameter min 1.0 μm and max 1000 μm, roundness 0.30 and contrast enhancement 0.30. Subsequently, the lens was focused in order to count all the cells. The acetate (referred to as substrate, S) and N (as NH_4Cl) concentration in standard TAP medium was 1.05 g L^{-1} and 0.098 g L^{-1}, respectively. The TAP culture media was also modified to contain different concentrations of N and acetate in order to induce N or acetate starvation and excess, respectively. Overall, we used six different acetate concentrations: 0 g L^{-1}, 0.42 g L^{-1}.1.05 g L^{-1}, 2.1 g L^{-1}, 3.15 g L^{-1} and 4.2 g L^{-1}; and seven different N concentrations: 0.0049 g L^{-1}, 0.0098 g L^{-1}.0.049 g L^{-1}, 0.098 g L^{-1}, 0.196 g L^{-1}, 0.98 g L^{-1} and 1.96 g L^{-1}. When the concentrations of N were manipulated, the concentration of acetate was kept constant at, 1.05 g L^{-1}, and when the concentration of acetate were manipulated, the concentration of N was kept constant at 0.098 g L^{-1}. The initial pH value of all media was set at pH = 7.

C. reinhardtii growth was determined at set time points by biomass measurement. The biomass concentration was measured in terms of dry cell weight (DCW) concentration. DCW was measured by centrifuging 500 mL cultures for 3 min at 3000 g in an Eppendorf Centrifuge 5424. The obtained pellet was then washed with cold distilled water. The washed pellet was centrifuged again for 3 min at 3000 g and weighed on a fine balance (Sartorius - M-Pact AX224, Germany) to determine the wet biomass. Subsequently, the wet biomass was dried overnight at 70 °C to determine the dry biomass weight. The pH of the samples was analysed through the use of a bench type pH meter (Denver UltraBasic Benchtop Meters, USA). The supernatant and the biomass of the samples were kept stored at −20 °C for quantification of specific metabolites. All data was statistically analysed by one-way ANOVA using Tukey post-hoc test performed using Prism v.6.04 (GraphPad).

2.2. Metabolite analysis

2.2.1. HPLC analysis of organic acids

The concentrations of organic acids produced and/or consumed were quantified using a High Performance/Pressure Liquid Chromatographer (HPLC) equipped with a Hi- Plex 8 μm 300 × 7.7 mm column. Glacial acetic acid (AA) as well as glycolic acid (GA) and formic acid (FA), were included as standards, as these were either growth media substrate (AA) or secreted microalgal by-products of the cultivation as also corroborated by Allen [51]. Sulphuric acid solution (0.05% *v*/*v*) was used as a mobile phase. The flow rate of the system was set at 0.6 mL min^{-1}, with a pressure value around 45 bars and a temperature of 50 °C, while the detection wavelength was fixed at 210 nm. Filtration through 0.45 μm filter membranes was undertaken for the sample preparation.

2.2.2. TOC/TN analyser

The total dissolved N concentration in the growth media was quantified by the use of a Total Organic Carbon/Total Nitrogen analyser (TOC/TN) (TOC-VCSH/TNM-1 Shimadzu). Ammonium chloride (NH_4Cl), added to the growth media as a nutrient, was used to prepare standard solutions. Three different ammonia (NH_3) sources can be found in TAP media; Ethylenediaminetetraacetic acid (EDTA), Tris-hydroxymethyl-aminomethane (TRIS) and NH_4Cl, which is the form assimilated by the microalgae for biomass growth. In order to quantify the NH_4Cl-originated N, the samples were initially analysed to determine the total N concentration in the media. Then, 100 μL of supernatant first diluted to 1 mL and then mixed with 200 μL of NaOH, and placed into hot water to enable the evaporation of the formed NH_3 (produced from NH_4Cl through NH_4^+). Finally, the samples were analysed again to determine the total N left in the media. The difference between the two aforementioned measurements equals to the amount of N originated by NH_4Cl.

2.2.3. Soxhlet solvent extraction using Soxtec

The lipid concentration was quantified by extracting the lipid using the Soxtec 1043 automated solvent extraction system. The freeze-dried algal biomass was homogenised through a double cycle of liquid N_2 immersion and pulverisation in a mortar with pestle. The pulverized biomass were then placed into cellulose extraction thimbles and located in the Soxtec unit. The procedure followed to quantify the lipid concentration was boiling for 2 h, rinsing for 40 min and solvent recovery for 20 min. The extraction temperature for the selected solvent, Hexane (ACS spectrophotometric grade, ≥98.5%, Sigma Aldrich, Dorset, UK), was 155 °C [52]. Following the oil extraction performed through the use of Soxtec 1043, the extracted lipids were dried at 100 °C for 1 h, were placed in a vacuum applied desiccator for 1 h, and were weighed to define the lipid concentration gravimetrically.

3. Mathematical modelling

3.1. Growth kinetics

A number of experiments we conducted in our laboratory, demonstrated that high substrate concentrations act as system inhibitors, and they can significantly reduce the biomass growth and the lipid accumulation rates [7]. To account for substrate inhibition on the transient cell behaviour, a modified Monod equation, the Haldane equation, is extensively applied [4,23,42]:

$$\mu = \mu_{max} \cdot \frac{S}{S + K_s + \frac{S^2}{K_{iS}}} \quad \text{Eq. 1}$$

Here μ is the specific growth rate, μ_{max} the maximum specific growth rate, S the substrate concentration, K_s the substrate saturation constant, and K_{iS} the substrate inhibition constant.

The depletion of N is known to increase the oil accumulation while it inhibits biomass growth [32,47]. Additionally, light intensity plays a crucial role on microalgae growth and lipid accumulation ([29], [33]). Therefore, the Haldane equation (expressed by Eq. 1) needs to be enhanced to account for the additional effects of N concentration and of light intensity.

Due to the contrasting effect of N on biomass concentration and on lipid accumulation, two different expressions for the N effect as a substrate, similar to the ones presented by Economou et al. [23], were employed here to describe the specific (oil-free) biomass growth and the lipid accumulation rate. Furthermore, the Aiba model [3,49] was taken into consideration for the simulation of the effect of light intensity as a pseudo-substrate.

Table 1
Estimated kinetic parameters along with bounds available in the literature.

Parameter	Value (units)	Standard deviation (σ)	Variance to mean ratio $\frac{\sigma^2}{\mu}$	Reference value	Species	Sources
μ_{Xmax}	0.227 h^{-1}	0.005	0.021	0.2274	*C.·reinhardtii*	[25]
K_{XS}	0.050 g S L^{-1}	0.000	0.010	0.028 − 2.295	*C.·reinhardtii*	([15], [50])
K_{iXS}	9.923 g S L^{-1}	0.130	0.013	0.1557 − 1.76	*C.·reinhardtii*	([50], [16])
K_{XN}	0.065 g N L^{-1}	0.000	0.007	This study		
K_{iXN}	0.500 g N L^{-1}	0.001	0.002	This study		
q_{Lmax}	0.121 g L g $X^{-1}h^{-1}$	0.002	0.013	This study		
K_{LS}	6.554 g S L^{-1}	0.063	0.010	This study		
K_{iLS}	0.110 g S L^{-1}	0.002	0.014	This study		
K_{iNL}	380.023 g N L^{-1}	3.154	0.008	This study		
$Y_{X/S}$	1.470 g X g S^{-1}	0.010	0.007	0.7104 − 15.6	*C.·reinhardtii*	([50], [16])
$Y_{X/N}$	6.883 g X g N^{-1}	0.183	0.027	18.9		[23]
K_H	0.879 L g S^{-1}	0.018	0.020	0.8759	*C.·reinhardtii*	[50]
$Y_{L/S}$	0.064 g X g S^{-1}	0.005	0.074	0.24	*C.·protothecoide*	[41]
K_{XI}	19.519 μ E $m^{-2}s^{-1}$	0.731	0.037	81.38	*C.·reinhardtii*	[25]
K_{iXI}	2053.924μE $m^{-2}s^{-1}$	33.755	0.016	2500	*C.·reinhardtii*	[25]
K_{LI}	15.023 μ E $m^{-2}s^{-1}$	0.461	0.031	This study		
K_{iLI}	2152.918μE $m^{-2}s^{-1}$	43.688	0.020	This study		
σ	34.104 g X^{-1}L m^{-1}	1.221	0.0036	This study		
k_1	0.329	0.013	0.040	This study		
K_{GAS}	1.456 g $S^{-1}L^{-1}$	0.031	0.021	This study		
K_{GAN}	12.976 g $N^{-1}L^{-1}$	0.189	0.015	This study		
K_{iGAN}	2.533 g $N^{-1}L^{-1}$	0.040	0.016	This study		
k_2	1.4055	0.008	0.006	This study		
K_{FAS}	12.976 g $S^{-1}L^{-1}$	0.450	0.035	This study		
K_{FAN}	2.533 g $N^{-1}L^{-1}$	0.059	0.023	This study		

Thus, the specific oil-free biomass growth rate, μ_X, is described by a pseudo-triple substrate expression as:

$$\mu_X = \mu_{Xmax} \cdot \frac{S}{S + K_{XS} + \frac{S^2}{K_{iXS}}} \cdot \frac{N}{N + K_{XN} + \frac{N^2}{K_{iXN}}} \cdot \frac{I(l)}{I(l) + K_{XI} + \frac{I(l)^2}{K_{iXI}}} \quad \text{Eq. 2}$$

where μ_{Xmax} is the maximum specific growth rate of oil-free biomass on acetate substrate (denoted as substrate onwards), depending on the concentration of nitrogen, N, and on the local light intensity, $I(l)$. Here, K_{XS}, K_{XN} and K_{XI} are the saturation constants and K_{iXS}, K_{iXN} and K_{iXI} the inhibition constants for oil-free biomass growth based on substrate, nitrogen concentration and light intensity, respectively. The local light intensity $I(l)$ is expressed by the Beer-Lambert Equation [6]:

$$I(l) = I_0 \cdot \exp(-\sigma X l) \quad \text{Eq. 3}$$

where l is the distance between the local position and the external surface of the system, I_0 the incident light intensity, σ the molar extinction coefficient and X the oil-free biomass concentration [6].

The specific lipid accumulation rate, μ_L, is expressed as:

$$\mu_L = q_{Lmax} \cdot \frac{S}{S + K_{LS} + \frac{S^2}{K_{iLS}}} \cdot \frac{K_{iNL}}{N + K_{iNL}} \cdot \frac{I(l)}{I(l) + K_{LI} + \frac{I(l)^2}{K_{iLI}}} \quad \text{Eq. 4}$$

where q_{Lmax} is the maximum lipid specific growth rate, K_{LS} and K_{LI} the saturation constants and, K_{iLS} and K_{iLI} the inhibition constants for lipid accumulation based on substrate concentration and light intensity, respectively; K_{iNL} is an inhibition constant used here to describe the lipid production dependent on nitrogen concentration.

3.2. Rate equations

The dynamic model developed in this work consists of a set of ordinary differential equations (ODEs) employed for the simultaneous simulation of microalgal growth, lipid accumulation, substrate and nitrogen consumption, by-product formation and pH change rates.

The microalgal (oil-free biomass) growth rate is expressed as:

$$\frac{dX}{dt} = \mu_X . X \quad \text{Eq. 5}$$

The lipid accumulation (lipid production) rate is described by:

$$\frac{dL}{dt} = \mu_L . X \quad \text{Eq. 6}$$

The substrate consumption rate can be calculated through a mass conservation equation [48]:

$$\frac{dS}{dt} = -\frac{1}{Y_{\frac{X}{S}}} \cdot \frac{dX}{dt} - \frac{1}{Y_{\frac{L}{S}}} \cdot \frac{dL}{dt} \quad \text{Eq. 7}$$

where $Y_{\frac{X}{S}}$ is the yield coefficient for oil-free biomass production with respect to substrate and $Y_{\frac{L}{S}}$ is the yield coefficient for lipid production with respect to substrate.

The N consumption rate is given by [50]:

$$\frac{dN}{dt} = -\frac{1}{Y_{\frac{X}{N}}} \cdot \frac{dX}{dt} \quad \text{Eq. 8}$$

where $Y_{\frac{X}{N}}$ is the yield coefficient for oil-free biomass production with respect to N.

For byproduct formation, only two acids are taken into account in our model: glycolic acid (GA) and formic acid (FA). The formation rates of GA and FA can be described by a multiplicative model, including the effects of acetate and N as follows:

$$\frac{dP_{GA}}{dt} = k_1 \cdot \frac{S}{S + K_{GAS}} \cdot \frac{N}{N + K_{GAN} + \frac{N^2}{K_{iGAN}}} \quad \text{Eq. 9}$$

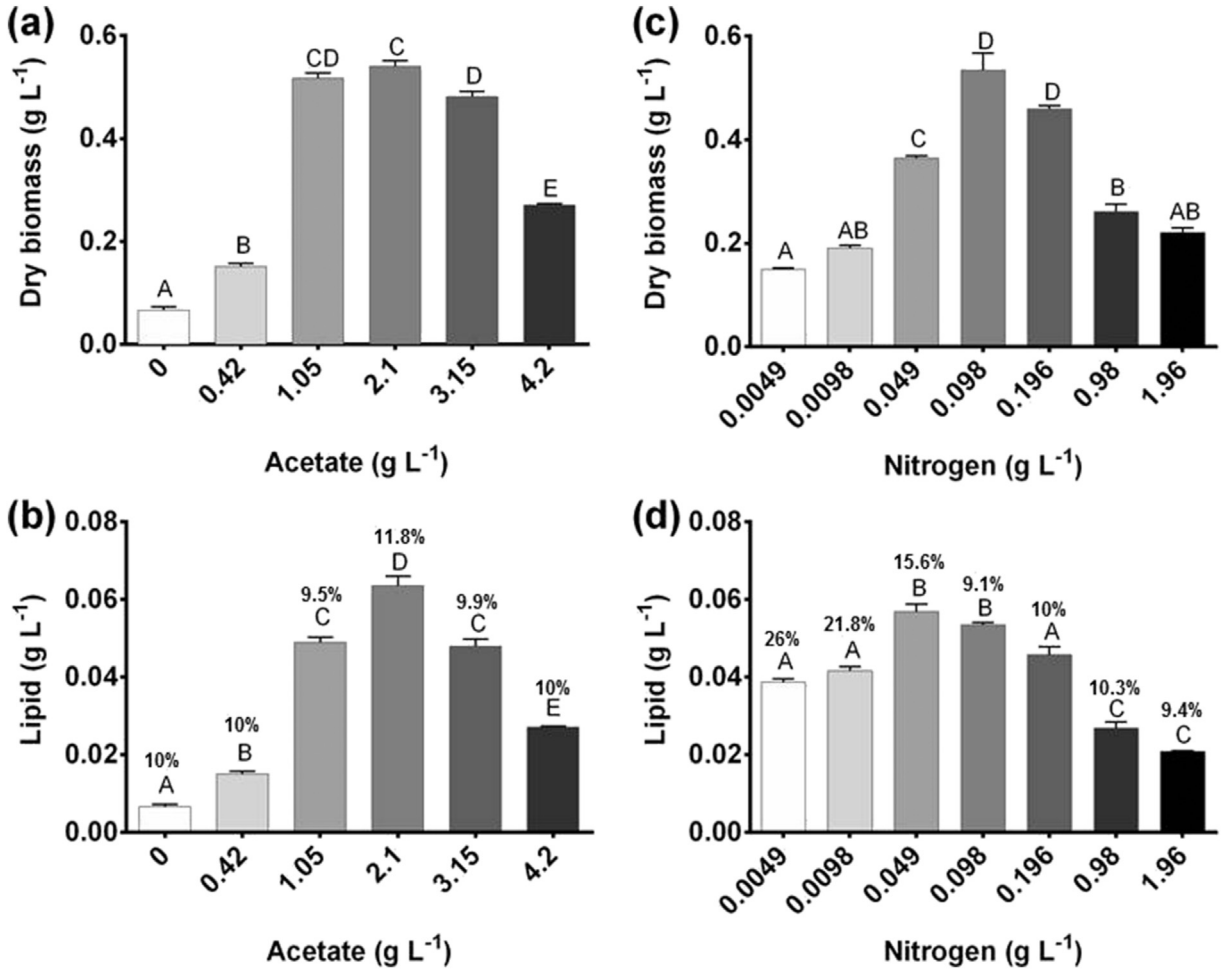

Fig. 1. The effect of carbon substrate (acetate) (a, b) and nutrient (nitrogen, N) (c, d) concentrations on dry weight biomass concentration (a, c) and total lipid concentration (b, d) after photo-heterotrophic growth for 8 d. The starting N concentration for the acetate range treatment experiments was 0.098 g L^{-1} and the starting acetate concentration for the N range treatment experiments was 1.05 g L^{-1}. All data are mean ± SE values of 2–3 biological replicates. Treatments that do not share uppercase letters are significantly different ($p < 0.05$), as determined by one-way ANOVA. The percentage lipid value as a proportion of dry weight biomass is indicated above each bar in panels b and d.

$$\frac{dP_{FA}}{dt} = k_2 \cdot \frac{S}{S + K_{FAS}} \cdot \frac{N}{N + K_{FAN}} \quad \text{Eq. 10}$$

here k_1 and k_2 are kinetic constants, K_{GAS}, K_{FAS} are substrate and K_{GAN}, K_{FAN} nitrogen saturation constants; K_{iGAN} is the nitrogen inhibition constant.

It should be noted here that oxalic acid production was also observed experimentally. The concentration of the oxalic acid (OA) for all the N and acetate treatments remains essentially constant at 0.015 g L^{-1} throughout the growth process, which signifies that OA is not a product of the metabolism. Hence its formation was not included in the kinetic model.

The pH change rate of the microalgae cultivation system is proportional to the substrate consumption rate and is expressed by [50]:

$$\frac{dH}{dt} = -K_h \cdot \frac{dS}{dt} \quad \text{Eq. 11}$$

where H describes the process pH, and K_h is a constant. Hence our model consists of 7 ODEs, corresponding to 7 state variables describing the dynamic evolution of biomass and lipids as well as that of the substrate, nutrients, pH and byproducts. The model includes 25 parameters, outlined in Table 1 and estimated through the procedure discussed in Section 4.2 below.

3.3. Parameter estimation

To the best of our knowledge, this study is the first attempt to model microalgae growth and lipid accumulation by taking into account the simultaneous effect of three growth-promoting resources (N, S, I), and thus, the reaction kinetics for such a system are not available in the literature. For this reason, we undertook a parameter estimation study using the constructed ODE-based system (Eqs. 5 to 11) in conjunction with high fidelity in-house produced experimental data. Two of the experiments discussed above were used (2.1 g L^{-1} acetate, 0.098 g L^{-1} N –experiment 1-, and 1.05 g L^{-1} acetate, 0.049 g L^{-1} N –experiment 2-with 1 mg L^{-1} biomass, and pH 7, and with starting by-product concentrations all at 0 g L^{-1}) The parameter estimation is set up as a non-linear weighted least squares method [48]:

$$Z(kk) = \min \sum_{k=1}^{n_k} \sum_{l=1}^{n_l} \sum_{m=1}^{n_m} W_{k,l,m} \left(C^{pred}_{k,l,m}(kk) - C^{exp}_{k,l,m} \right)^2 \quad \text{Eq. 12}$$

Here kk is the vector of the 25 model parameters, n_k is the number of experiments ($n_k = 2$), n_l is the number of state variables ($n_l = 7$), n_m is the number of experimental measurements in time ($n_m = 7$), and $W_{k,l,m}$ are the weights used to effectively normalise the computed errors, $\varepsilon = (C^{pred}_{k,l,m}(kk) - C^{exp}_{k,l,m})$. Here the weights were set to $W_{k,l,m} = 1/C^{exp}_{k,l,m}$, where $C^{pred}_{k,l,m}$ are the predicted state variables (computed by Eqs. 5 to 11) and $C^{exp}_{k,l,m}$ the experimentally obtained ones.

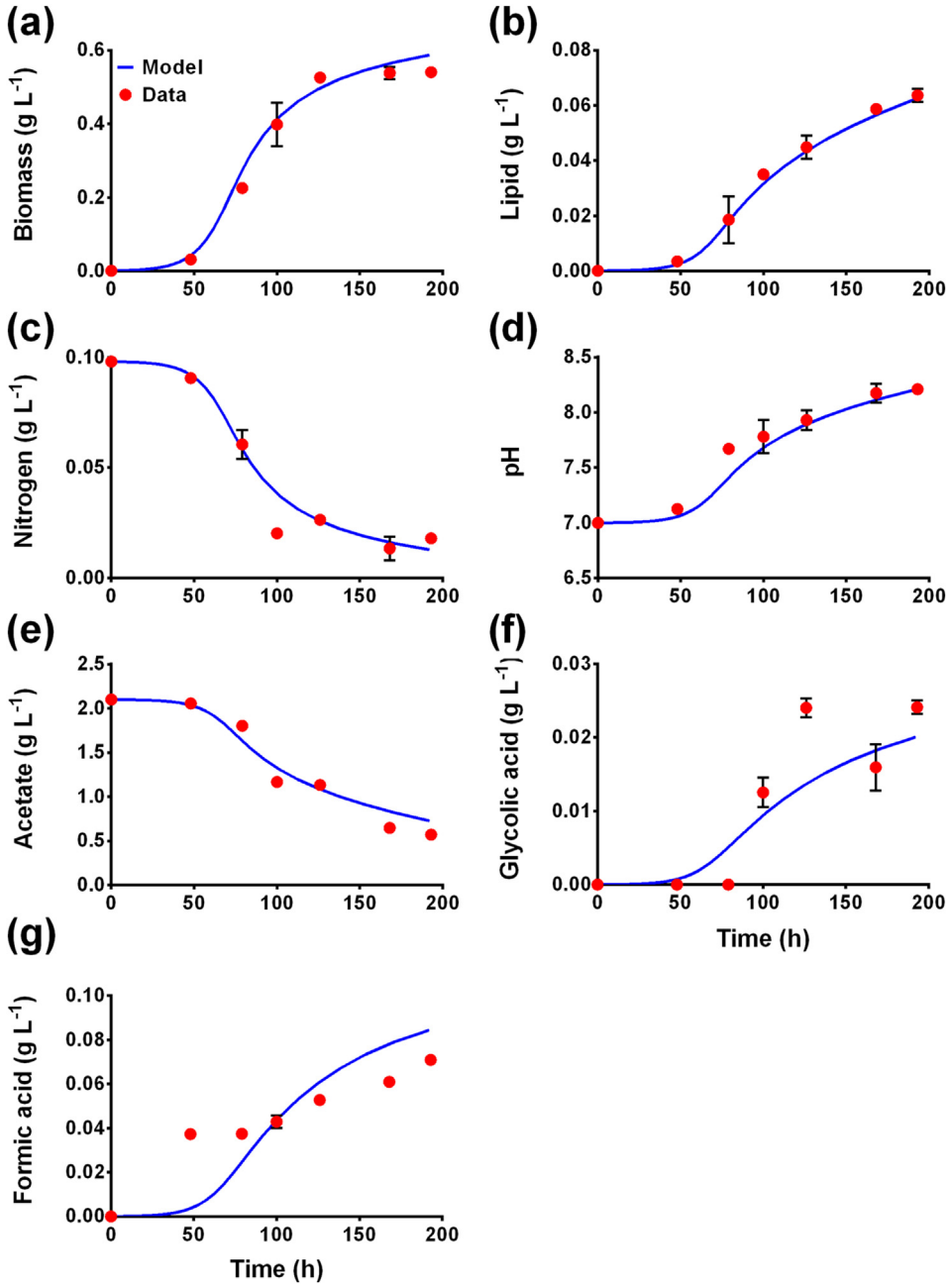

Fig. 2. Fitting of model predictions (lines) to experimental data (symbols with error bars) for: (a) biomass, (b) lipid concentration, (c) substrate (acetate) consumption, (d) N consumption, (e) pH change, (f) oxalic acid production, (g) glycolic acid production and (h) formic acid production, using 2.1 g L^{-1} acetate and 0.098 g L^{-1} N.

The estimation problem was solved using an in-house developed stochastic algorithm, based on Simulated Annealing (SA) [48], with multiple restarts in order to increase the chances of obtaining solutions in the neighbourhood of the global optimum. A refining step using a deterministic method, Sequential Quadratic Programming (SQP) implemented through the "fmincon" function in MATLAB, was subsequently carried out using as initial guess the result from SA.

The initial values of the state variables used in the ODEs were set to the initial concentration values of each experiment. Multiple optimization runs have been used to ensure that the local minima were avoided. The values of the parameters as well as their standard deviation estimated using the above procedure are shown in Table 1. The system dynamics obtained using our model were compared to the experimental results described above, including biomass and lipid growth, pH changes and formation of organic acids, GA and FA. The resulting model shows very good agreement with the experimental data for all state variables, as can be seen in Fig. 2.

4. Results and discussion

An experimental study was carried out to quantify the effect of varying starting substrate (acetate) and nutrient (N) composition of the growth medium on the system behaviour. A parameter estimation study was then performed using the constructed mathematical model, to compute parameter values that are of crucial importance for accurate system simulations. The model was subsequently validated against experimental data at different operating conditions, and was then used in optimisation studies to determine optimal operating conditions.

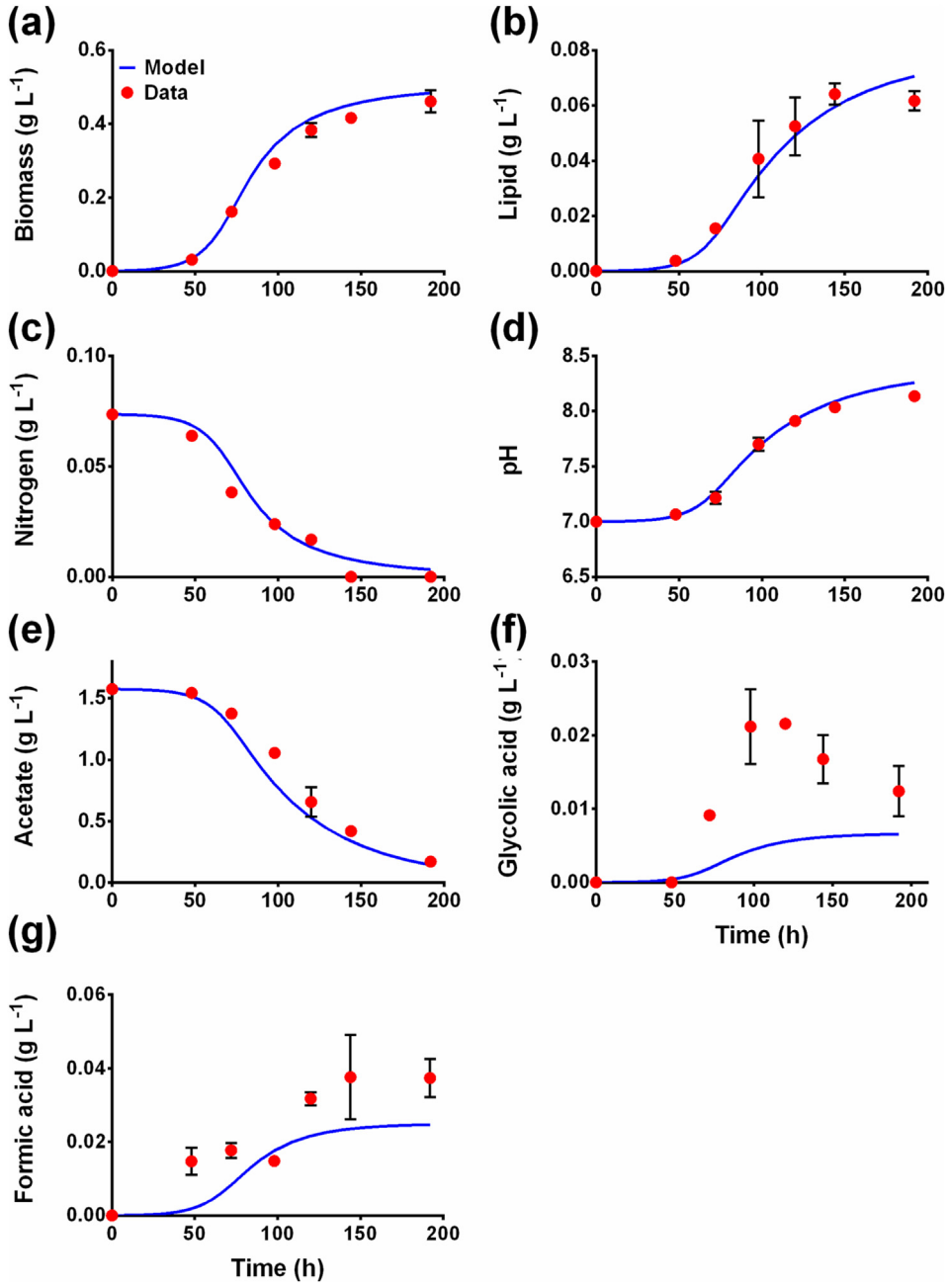

Fig. 3. Validation of model predictions (lines) by experimental data (symbols with error bars) for: (a) biomass, (b) lipid concentration, (c) substrate (acetate) consumption, (d) N consumption, (e) pH change, (f) oxalic acid production, (g) glycolic acid production and (h) formic acid production, using 1.575 g L^{-1} acetate and 0.0735 g L^{-1} N.

4.1. Experimental results

Measurements of microalgal growth, as determined by biomass concentration, and lipid accumulation (Fig. 1 and Fig. S1) were taken alongside measurements of growth media pH change and organic acid concentrations, for the six different acetate concentrations and the seven different N concentrations mentioned in Section 2.1, in order to examine the effect of the change in nutrient and substrate concentration on the overall biomass and lipid concentrations. For the acetate-absent and acetate-deficient (0 g L^{-1} and 0.42 g L^{-1}) as well as the acetate-excess (4.2 g L^{-1}) media, dry biomass was below detectable levels for the first 120 h due to slow growth rate (Fig. S1a). Thus lipid concentration was also undetectable (Fig. S1b). Cells grown in the other acetate concentrations (1.05 g L^{-1}, 2.1 g L^{-1} and 3.15 g L^{-1}) grew rapidly with equivalent growth profiles. Compared to the 1.05 g L^{-1} acetate treatment, biomass concentration decreased significantly ($p < 0.0001$, one-way ANOVA) both for the acetate excess (4.2 g L^{-1}) treatment, by approximately 50%, and for the acetate-deficient (0.42 g L^{-1}) and absent (0 g L^{-1}) treatments, by approximately 80% (Fig. 1a). In contrast, biomass concentration was essentially the same for the 1.05 g L^{-1}, 2.1 g L^{-1} and 3.15 g L^{-1} acetate treatments. Many chlorophyte microalgae species such as *C. reinhardtii* are able to efficiently grow heterotrophically and this is increasingly being considered as a more commercially viable method of high-productive cultivation [38]. While organic carbon addition such as acetate can indeed increase biomass concentration, as we show here, the inhibition of growth by excessive concentrations of acetate may either be due to acetate toxicity or a saturation of acetate assimilation and metabolism, coupled to the acetate-induced inhibition of photosynthesis [14,34]. Acetate is metabolised via the glyoxylate cycle, but can also be converted into acetyl-CoA in an

ATP-dependent mechanism and then used as a substrate for fatty acid synthesis and then TAG metabolism [34]. Increase in lipid concentration as acetate concentration increases might therefore be predicted and indeed this has been previously observed in *C. reinhardtii* under both N sufficient and N limited conditions [44]. However, we found that the proportion of lipid accumulation within the cell on a total dry weight basis was essentially identical for all acetate treatments (approximately 10% lipid), and therefore the difference in volumetric lipid concentration between the treatments (Fig. 1b) was almost entirely due to the difference in biomass. This therefore suggests that under these N sufficient (0.098 g L^{-1} N) conditions, assimilated acetate is being used predominantly for cell growth. It is also worth noting that the study of Ramanan et al. [44] evaluated acetate addition in a mutant strain of *C. reinhardtii* that was unable to produce starch, whereas in wild type strains acetate addition has been suggested to drive carbon allocation preferentially towards starch accumulation rather than lipid [14].

For the N deficient (0.0049 g L^{-1} and 0.0098 g L^{-1}) and N excess (0.98 g L^{-1} and 1.96 g L^{-1}) media, dry biomass concentration (and therefore lipid concentration) was again below level of detection for the first 120 h due to slow growth rate (Fig. S1c and d). As expected for an essential nutrient, and in agreement with previous studies, N limitation significantly inhibited growth compared to the 0.098 g L^{-1} N replete treatment ($p < 0.0001$ for 0.0049 g L^{-1} and 0.0098 g L^{-1} N; $p = 0.0009$ for 0.049 g L^{-1} N, one-way ANOVA), with the lowest biomass concentration (0.149 g L^{-1}) seen for the 0.0049 g L^{-1} N concentration (Fig. 1c). However, the highest N concentrations (0.98 g L^{-1} and 1.96 g L^{-1}) also significantly inhibited growth ($p < 0.0001$, one-way ANOVA), possibly due to partial toxicity when ammonium concentration is too high (Fig. 1c). As anticipated, N limitation led to an increase in lipid accumulation compared to the higher N concentrations, with the 0.049, 0.0098 and 0.0049 g L^{-1} N treatments inducing cellular (per dry weight) lipid content values of 15.6%, 21.8% and 26%,

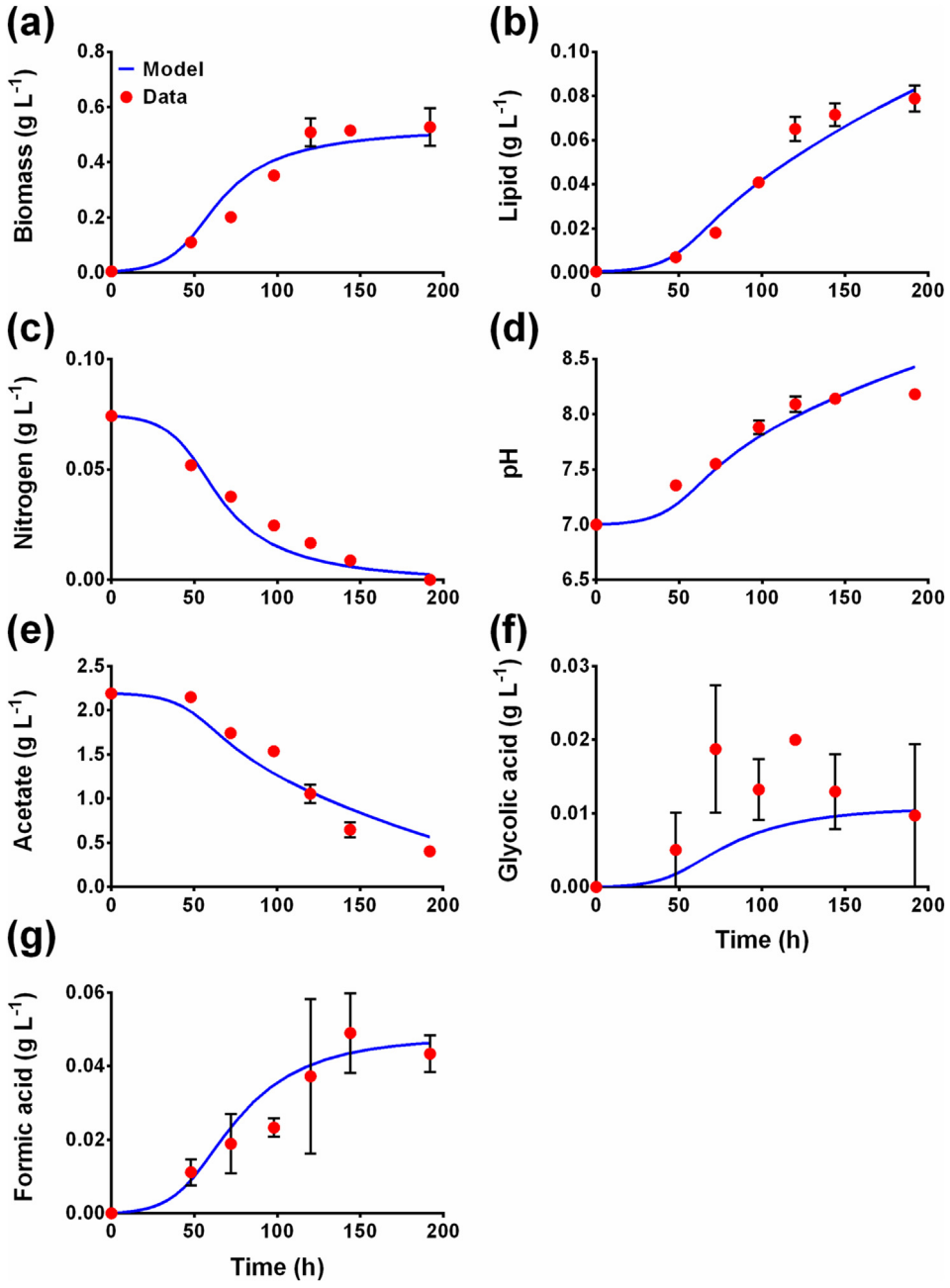

Fig. 4. Optimization of model predictions (lines) by experimental data (symbols with error bars) for: (a) biomass, (b) lipid concentration, (c) substrate (acetate) consumption, (d) N consumption, (e) pH change, (f) oxalic acid production, (g) glycolic acid production and (h) formic acid production, using 2.1906 g L^{-1} acetate and 0.0742 g L^{-1} N.

Table 2
Optimal system initial conditions and resulted productivity and yield measures.

Initial conditions		Base case runs		Optimisation runs
Biomass concentration		0.001 g L^{-1}		0.005 g L^{-1}
Acetate concentration		2.1 g L^{-1}		2.1906 g L^{-1}
Nitrogen concentration		0.098 g L^{-1}		0.0742g L^{-1}
Resulted measures	Base case Results	Optimized results	Change	Experimental results
Lipid concentration	62.4 mg L^{-1}	82.9 mg L^{-1}	+20.5 mg L^{-1}	84.7 mg L^{-1}
Lipid productivity	7.8 mg L^{-1} d^{-1}	10.3625mgL^{-1} d^{-1}	+32.85%	10.5875 mg L^{-1} d^{-1}
Biomass concentration	586.8 mg L^{-1}	498.4 mg L^{-1}	−88.4 mg L^{-1}	458.6 mg L^{-1}
Biomass productivity	73.85 mg L^{-1} d^{-1}	62.3 mg L^{-1} d^{-1}	−15.65%	57.325 mg L^{-1} d^{-1}

respectively, compared to 9 to 10% lipid content in the N replete (0.098 g L^{-1}) cells. This is in agreement with many previous N limitation studies where substantial lipid induction can be observed as N availability becomes starved [5]. N excess did not inhibit cellular lipid accumulation but on a volumetric basis, lipid concentration was lowest with 0.98 g L^{-1} and 1.96 g L^{-1} N (0.261 g L^{-1}, 0.221 g L^{-1} respectively) and highest with 0.049 g L^{-1} and 0.098 g L^{-1} N (0.3645 g L^{-1}, 0.5335 g L^{-1} respectively) (Fig. 1d), with the low lipid yield at the highest N concentrations explained by the reduced biomass at these concentrations (Fig. 1c).

4.2. Model validation

We have subsequently carried out a validation study for our constructed model to assess its predictive capabilities. In Fig. 3, the model predictions for the experimental results, obtained at base line conditions (1.5735 g L^{-1} acetate, 0.0735 g L^{-1} N, 1 mg L^{-1} biomass, and pH 7, and with starting organic acid (GA and FA) by-product concentrations all at 0 g L^{-1}) are presented. The system was operated at room temperature $T = 25$ °C and the light illumination (I_0) is considered constant and equal to 125 $\mu Em^{-2}s^{-1}$. The model was capable of predicting the experimentally obtained concentrations of biomass, lipid, acetate, N, and the pH change with high precision as well as the concentrations of organic acid by-products with reasonable accuracy (Error = 2.9819). Thus, the detailed multiplicative model proposed in this study can be used for precise prediction of the dynamic behaviour of bench-scale batch experiments.

4.3. Process optimization

The validated model was further exploited in an optimization study to determine the optimal operating conditions for such bench-scale systems. Here, the optimization problem was set up to calculate the maximum lipid and biomass productivities:

$$\text{Objective} = \max(J_L + J_X) \quad \text{Eq. 13}$$

subject to the governing system equations (Eqs. 5 to 11). The productivities are defined as:

$$J_L = \frac{L - L_0}{t_p - t_{p_0}} \quad \text{Eq. 14}$$

$$J_X = \frac{X - X_0}{t_p - t_{p_0}} \quad \text{Eq. 15}$$

where J_L is the productivity of lipid ($mg\, L^{-1}s^{-1}$), J_X is the productivity of biomass ($mg\, L^{-1}s^{-1}$), L is the final lipid concentration (mg Lipid L^{-1}) calculated by Eq. 6, L_0 is the initial lipid concentration (mg Lipid L^{-1}), t_p is the process time (h), X is the final biomass concentration (mg Biomass L^{-1}) calculated by Eq. 5 and X_0 is the initial biomass concentration (mg Biomass L^{-1}).

The substrate, nitrogen and inoculum initial concentrations were the degrees of freedom in the optimization process. The computed optimum is tabulated in Table 2. Optimum lipid productivity is achieved using initial concentrations of acetate, N and inoculum equal to 2.1906 g L^{-1}, 0.0742 g L^{-1} and 0.005 g L^{-1}, respectively. This represents a 32.85% increase in the lipid oil productivity compared to the base case, which illustrates the effectiveness of computer-based optimisation for such systems. The optimization results were experimentally validated. The computed optimal dynamics along with the corresponding experimental results obtained at the optimal operating conditions are presented in Fig. 4. The agreement between the computed and experimental results is very good (error = 2.6249), which illustrates the usefulness of our model for optimal design of experiments, minimizing the need of time-consuming and potentially expensive trial-and-error runs [1,25,37].

5. Conclusions

Few studies have attempted to model microalgal biomass growth and lipid accumulation but none of these previously developed models have considered the simultaneous and antagonistic effect of nutrient starvation, substrate concentration and light intensity on the rate of lipid production and rate of biomass growth. Consequently, these models do not allow the accurate analysis of the culture system behaviour under different operating conditions. A multi-parameter model was developed in this study to predict the dynamic behaviour of all 7 system state variables accurately, by considering the effect of three different culture variables (S, N, I). Experimental studies were conducted for the investigation of the effect of varying substrate (acetate) and nutrient (N) on biomass growth and on lipid accumulation rates, and used in conjunction with the constructed model for the estimation of kinetic parameters that are essential for accurate system simulations. The model was validated for a different set of initial concentrations. Optimization of the process was carried out to determine the optimal system operating conditions and it was found that a 32.85% increase in the lipid oil productivity was achieved using 2.1906 g L^{-1} acetate, 0.0742 g L^{-1} N and 0.005 g L^{-1} starting biomass inoculum. This illustrates the usefulness not only of computer-based optimisation studies for the improvement of microalgal-based production, but also of carefully constructed predictive models for the accurate simulation of these systems. Such predictive models can be exploited for the robust design, control and scale-up of microalgal oil production, which can help to bring this important technology closer to commercialization and industrial applicability.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2016.12.015.

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