Integrated Computational and Experimental Studies of Microalgal Lipids Production

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Abbreviations

GHGs	Greenhouse gases
TAG	Triacylglycerol
TAP	Tris-acetate-phosphate
OPs	Open ponds
PBRs	Photobioreactors
DCW	Dry cell weight
N	Nitrogen
P	Phosphorus
S	Substrate
I	Light intensity
T	Temperature
L	Lipid
X	Oil-free biomass
AA	Acetic acid
GA	Glycolic acid
FA	Formic acid
OA	Oxalic acid
TRIS	Tris-hydroxymethyl-aminomethane
EDTA	Ethylenediaminetetraacetic acid
HPLC	High Pressure (Performance) Liquid Chromatography
TOC/TN	Total organic carbon / Total nitrogen analyzer

Nomenclature

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
I_0	Local light intensity
T_0	Reference temperature
l	The distance between the local position and the external surface of the system
A_{0_X}	Frequency factors
B_{0_X}	Frequency factors
E_{a_X}	Activation energy of oil-free biomass growth
E_{b_X}	Activation energy of oil-free biomass degradation
A_{0_L}	Frequency factors
B_{0_L}	Frequency factors
E_{a_L}	Activation energy of oil production
E_{b_L}	Activation energy of oil degradation
m_1	Maintenance term

Abstract

Microalgal biomass and its lipids are long-term promising candidates for the production of fuels, food, nutraceuticals and other added-value products. Due to irreversible depletion of fossil fuel reserves for very large demands of transportation and escalating greenhouse gas emissions (GHGs) into the atmosphere, serious consideration has been given to microalgae-derived biodiesel production due to several outstanding characteristics inherent to microalgae. However, the current production cost of microalgal biodiesel is still too expensive to compete with conventional fuels. Although microalgal lipids have an immense potential in biotechnological applications, in order to improve the sustainability of microagal biodiesel and also to enable its economic viability, microalgal biomass and lipid productivities need to be enhanced.

Metabolic modifications by genetic manipulation, mutagenesis or natural selection are approaches that have been actively evaluated to develop high productivity strains. On the other hand, a combination of kinetic modelling with growth experiments at different scales is widely utilised to optimise cultivation conditions and metabolic productivities. Optimisation of the microalgae growth media composition and environmental factors such as carbon source, nutrient, light intensity and temperature can lead to high metabolic productivities.

The aim of this Thesis is the development of a novel integrated experimental and computational framework to systematically identify optimal growth conditions for biomass growth and lipid accumulation and to ultimately result to a cost-effective scaled-up process. To achieve this, experiments were initially conducted with heterotrophic growth of a well-studied chlorophyte microalgae species *Chlamydomonas reinhardtii* at bench scale under different acetate and nitrogen concentrations, light intensity and temperature. Based on high-fidelity experimental observations and on existing literature, a detailed kinetic model was constructed through a multi-parameter quantification methodology. The developed model was based on a multiplicative modelling approach, which assumes equal contribution of growth limiting factors: substrate (acetate), nitrogen, light intensity and temperature. The model was validated and utilized in optimisation studies to predict the optimal acetate and nitrogen concentrations, light intensity and temperature in order to achieve the highest lipid productivity possible. It was found that the lipid productivity can be increased by 50.9 % compared to a base case.

Scale-up of the process offers a potential pathway to produce substantial amount of lipids for biodiesel production. Therefore, the quadruple substrate kinetic model was adapted to be applied in large-scale raceway open ponds to assess the applicability of the developed kinetic model in scaled-up applications. Experiments with photoautotrophic growth of *C. reinhardtii* in a 2 m^3 raceway open pond were screened. The open pond model proposed in this study was a function of light intensity, temperature and nitrogen. The kinetic parameters of the model were estimated using in-house obtained experimental data performed in 2m^3 raceway open pond. The model was validated and is able to predict both biomass growth and lipid accumulation with high accuracy.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Mesut Bekirogullari

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Sevgili Annem, Babam ve Kardeşlerim’e armağan ediyorum. Onlarsız bu dünya tüm güzelliğini ve eğlencisini kaybederdi.

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Hayatım boyunca bana şükretmeyi ve insanlar ile güzel dostluklar kurmayı öğrettikleri için Anneme, Babama ve tüm aile bireylerine (İbrahim, Asiye, Engin, Meral, Eda, Gülce, Gökçe, Gülper, Onur, Ela ve Begüm) teşekkür ve şükranlarımı sunuyorum. Tüm bunlarla beraber bana her zaman kişisel ve mesleki alanlarda kendimi geliştirmem için teşvikte bulundular. Onların teşviki ve desteği olmadan, şu an olduğum yerde olamazdım. Onlara hayatımı borçluyum ve onları her zaman mutlu etmek için elimden gelenin en iyisini yapacağım. Umarım bu çalışma onları gururlandırır.

Son olarak Manchester'da ki arkadaşlarıma (Bahar, Halis, Burak, Emre, Tuğrul, Halil, Yusuf, Harun, Lütfi, Enbiya, Kemal, Elif, Merve, Vildan, Melisa ve Tuba) arkadaşlıkları, dostlukları ve sevgileri için şükranlarımı sunuyorum. Ayrıca, Türkiye'de bulunan can dostlarım Onur, Suna, Emre, Tuğba, Esra, Hüseyin ve Kadir'e dostlukları ve paylaştığımız mutlu ve güzel günler için çok teşekkür ediyorum.

Associated Publications

Conference proceedings

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Journal publications

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Bekirogullari, M., Fragkopoulos, I. S., Pittman, J. K. & Theodoropoulos, C. 2017. “Kinetic Modelling of Microalgal Cultivation for Optimised Biofuel Production under Multiple Environmental Factors”. (Submitted to Biotechnology for Biofuels).

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Chapter 1

Introduction and scope of the Thesis

Unsustainable energy resources satisfies 88% of the global energy demand, 35% of which is oil consumption, 29% coal and 24% natural gas. (Brennan and Owende, 2013). The continuous rise in urbanization and industrialization in the developing countries will increase the energy demand by 40% in the period from 2007 to 2030 (Brennan and Owende, 2010). Thus, fossil fuel usage will likewise continue to increase (Pittman et al., 2011, Brennan and Owende, 2010). Fossil fuels provide a non-renewable form of energy that is limited due to depleting resources and will ultimately run out due to the fast growing worldwide energy demand (Brennan and Owende, 2010, Hoel and Kverndokk, 1996, Thomas, 2017). A recent study by Thomas (2017) showed that with the 2014 consumption rate without any imports, the Europe & Eurasia would consume all of its available oil reserves in 24 years, and all of its natural gas reserves in 72 years, while their coal reserves would last for more than 600 years. On average, the world would consume all of its oil reserves in 64 years, natural gas in 60 years and coal over 380 years based on 2014 consumption rates. Additionally, the use of non-renewable resources leads to the production of harmful greenhouse gases (GHG), carbon dioxide (CO_2), nitrogen dioxide(NO_2), sulphur dioxide (SO_2), carbon monoxide (CO), and subsequently it impacts the environment on a negative manner generating acid rains due to sulphur dioxide (SO_2) and global warming (Chiari and Zecca, 2011, Coady et al., 2017). For instance, as the GHG build-up in the atmosphere, they act like a blanket covering the planet and heat is trapped inside the atmosphere. This is known as the greenhouse effect and leads to a rise in the global temperature, the so-called global warming (Montzka et al., 2011). On the contrary, renewable forms of energy such as solar and wind energy as well as biofuels, are environmentally sustainable sources (Efroymson and Dale, 2015). Although the generation of energy by geothermal, hydropower, solar and wind means is successfully substituting the fossil fuel demand in several sectors, the increasing energy demand in the transportation sector could only be tackled by just a limited number of alternative renewable energy resources among which biofuels is the most promising (Demirbas, 2010). Bioethanol, biodiesel, biobutanol, biohydrogen and biomethane are the primary types of biofuels which have been commercially produced by various biomass sources such as forestry, energy crops, animal fat, agricultural

residues and fungal or bacterial microbes (Ahmad et al., 2011, Shankar and Shikha, 2017). According to the agreement with the Renewable Energy Directive for 2020, the EU needs to replace 10% of the fossil fuels demand with biofuels. In 2012, the annual EU transportation energy demand was about 300Mtoe and biofuels supplied around 10Mtoe - mainly biodiesel (about 80%) produced from rape seed and bioethanol (about 20%) from wheat, maize, beet and sugar cane (EASAC, 2012).

Biofuels can be classified into four different categories based on the feedstock selection, first, second, third and fourth generation biofuels.First-generation biofuels - derived from starch, sugar, animal fats and vegetable oil by conventional extraction techniques - represent the majority of the current commercially available biofuels, such as bioethanol or biodiesel (Nigam and Singh, 2011). Second generation biofuels are produced from lignocellulosic biomass such as wood, bagasse, forest residues and agricultural residues such as short rotation forest residue and grasses (Nigam and Singh, 2011). Although first and second generation biofuels are renewable and environmentally friendly, the use of food crops for the production of biofuels has been debated by public and non-governmental organizations due to its conflict with the food supply (Driver et al., 2014). Additionally, they are too expensive due to high percentage of free fatty acid content which requires extra pre-treatment and raw material processing cost. Third generation biofuels produced from natural (isolated and screened from natural habitats) or engineered (the lipid metabolism of the strains is genetically modified to achieve higher productitivities) microalgae has been the proposed alternative energy source as it does not affect the agricultural production. Fourth generation biofuels produced are through photobiological solar fuels and electrofuels based on use of raw materials that are inexhaustible, cheap and widely available (Aro, 2016). Fourth generation biofuels are likely to bring fundamental breakthroughs to the biofuels field. Despite the advantages that fouth generation biofuels bear, the technology is still under development. Production of biofuels from microalgae was investigated in this thesis due its unique traits.

1.1. Biofuels

Biofuels production is a well-established workflow (Demirbas and Fatih Demirbas, 2011), with soybeans, canola oil, palm oil, corn oil, animal fat and waste cooking oil, as the most common commercial sources. Traditional forestry plants have also been used in commercial scale to produce biofuels. However, the use of such existing biomass sources without any appropriate compensation may result in serious environmental problems such as deforestation (Schoneveld et al., 2010, Field et al., 2008). On the other hand, a wide selection of agricultural plants such as oil crops, rape seed, sunflower and cellulosic crops, such as switch grass and sugar canes, has been used for the production of biofuels raising a debate between food and fuel. Due to modern practices such as industrialisation, urbanisation, land degradation and desertification, dedicating agricultural land specifically for the production of biofuels is unwise (Jean Vasile et al., 2016). Oldeman (1994) studied the impact of degradation of agricultural land and found that since 1945, around 2 billion hectares out of the world's 8.7 billion hectares agricultural land, pastures, forests and woodlands have been degraded.

The production of biofuels from microalgae exhibits a great potential in the bio-energy field, overcoming the competition for agricultural land which is the major limitation of plant derived fuels (Chisti, 2007a, Mercer and Armenta, 2011). Microalgae species can be used for the production of several types of renewable biofuels: methane production by anaerobic digestion of microalgal biomass (Amin, 2009), biodiesel production from microalgal oil (Demirbas and Fatih Demirbas, 2011, Lai, 2014, Dragone et al., 2010), alcoholic fuels (bioethanol, biobutanol) from microbial fermentation of carbohydrate and hydrogen production by photobiology (Chisti, 2007a). Additional fuel types can also be generated from thermochemical treatment of algal biomass using methods such as pyrolysis and hydrothermal liquefaction. Furthermore, microalgae species are useful for bioremediation processes and they can be used as nitrogen fixing fertilizers (Kong et al., 2010, Chisti, 2007a, Zhou et al., 2015).

The idea of using microalgae as a source for renewable fuel production is not new (Chisti, 2007a), but it is now being seriously considered due to the irreversible depletion of fossil fuels, and more importantly, the increasing concern about global warming which is mainly associated with fossil fuel usage. However, the use of microalgal oil for biodiesel production has not yet been exploited commercially as the current price for its production is still too high compared to

the fossil fuel diesel one (Kotasthane, 2017). Approximately 60-75% of the total cost of microalgal biodiesel comes from the microalgae cultivation, mainly due to the high cost of the carbon source, the fertilizer requirements and the high cultivation facility costs relative to the often low oil productivity (Driver et al., 2014). In order to bring the technology a step closer to industrialisation, sustainability and economic viability of the microalgal biofuel market, its production needs to be improved.

1.2. Optimization of Microalgal Oil Production

There two main methodologies that have been widely evaluated to optimise the lipid production are strain development and integration of mathematical modelling with growth experiments at different scales. The strain development is carried out mainly by genetic manipulations, mutagenesis or natural selection. Several studies have attempted to overexpress or knock out the genes that are involved in lipid biosynthesis to understand their roles in lipid accumulation and most of these transgenic overexpression studies, resulted in the enhanced accumulation of TAGs in microalgal cells (Radakovits et al., 2010, Trentacoste et al., 2013, Carrier et al., 2014).

Furthermore, an integration of detailed mathematical kinetic models and in-house produced growth experimental data at different scales can be exploited for the evaluation of the growth-limiting factors in order to achieve optimal cultivation conditions and metabolic productivities (Goncalves et al., 2015, Thornton et al., 2010, Pfaffinger et al., 2016, Han et al., 2015).

Experimental studies have revealed that both the microalgal biomass growth and the lipid accumulation can be simultaneously and antagonistically affected by the growth media composition and environmental factors such as carbon source and nitrogen concentration, and light intensity and temperature, respectively (Breuer et al., 2015, Chen and Johns, 1994, Converti et al., 2009, Blair et al., 2014). Microalgal storage lipids comprise of the neutral lipid Triacylglycerol (TAG), stored in cytosolic and/or plastidic lipid bodies (Chisti, 2007b). The production rate of such lipid bodies can be increased by abiotic stress, supply nutrient deprivation such as of nitrogen (N) and phosphorus (P), and environmental factors such as light intensity and temperature stresses (Bajhaiya et al., 2016, Converti et al., 2009). The balance between biomass growth and lipid accumulation under extreme nutrient starvation drives substantial cellular lipid accumulation while significantly inhibiting cell growth, and thus net

volumetric lipid productivity, is a critical component (Griffiths and Harrison, 2009). Coupling detailed kinetic models and experiments at different scales could be a very useful framework to be employed to understand the relationship between growth limiting factors, lipid accumulation and biomass growth. Such a framework can then be utilised for the optimisation of the biomass growth and lipid accumulation, in order to ultimately achieve realize a positive energy balance for a cost effective and sustainable scaled-up biodiesel production (Béchet et al., 2013, Bernard et al., 2016).

Co-limitation of both growth media composition (nutrients and carbon substrate availability) and environmental factors (light intensity and temperature) is commonly observed in natural environments (Lee et al., 2015, Béchet et al., 2013, Chisti, 2007b). Autotrophic and heterotrophic microalgal biomass growth are mainly limited by the carbon, nitrogen and phosphorus concentrations, and light intensity and temperature. In order to reveal the interactions between biomass growth, lipid accumulation and limiting factors, and to get a better understanding of microalgae growth process, the co-limitation concept has been widely applied in the kinetic modelling field (Solimeno et al., 2015, Mairet et al., 2011a, Franz et al., 2012, Shi et al., 2000, Beran and Kargi, 2005). The main assumption behind the co-limitation approach is that both growth media composition and environmental factors as well as their interactions control the microalgal biomass growth and the lipid accumulation.

This phenomenon has been studied through two other kinetic modelling frameworks: the threshold and multiplicative models. The threshold model considers that the biomass growth is only influenced by the growth limiting factor with the lowest concentration, and consequently, the model becomes a single substrate growth model. On the contrary, the multiplicative modelling approach considers the co-limitation of two or more growth limiting parameters that contribute to microalgal biomass growth and lipid accumulation equally.

Although both the threshold and the multiplicative kinetic modelling approaches are able to precisely predict the effects of the growth limiting factors, they are not capable of predicting the simultaneous effects of other co-limiting factors such as carbon and nitrogen concentrations, and light intensity and temperature with the same accuracy. Mathematical modelling approaches has widely been utilized on a theoretical basis to investigate both microalgal biomass growth and lipid accumulation under the simultaneous and antagonistic effect of

multiple growth limiting factors (Zhang et al., 1998, Yang et al., 2011, Xin et al., 2010, Yoo et al., 2014, He et al., 2012). However, the published data are limited and they do not allow conclusions to be made on the kinetic interactions between microalgal biomass growth and lipid production with respect to the growth-limiting factors.

1.3. Thesis objective and research contribution

The main objective of this work is to understand the simultaneous and antagonistic effect of nutrient and substrate concentrations, light intensity and temperature on microalgae growth with a particular focus on lipid accumulation. To achieve this, a kinetic model was developed to provide an understanding of microalgal growth and lipid accumulation so that in conjunction with high fidelity experimental data, cultivation conditions could be optimised in order to improve the sustainability and competitiveness of algal derived biofuels industry. Additionally, the effect of nutrient stress, carbon substrate stress and environmental variables (light intensity and temperature) stress, which result in maximum lipid productivity with the least negative effect on microalgae growth rate, was explored. Screening of macrospic/semi-empirical microalgae growth kinetic models, and consequently understanding the phenomena behind them, was also undertaken, and a new kinetic model overcoming the problems associated with these kinetic models was developed. The adaptation of the developed kinetic model to be used at a raceway open pond scale is the last part of the research contribution presented in this Thesis.

1.4. Thesis Structure

This thesis is presented in “journal format” as a series of papers one published and two submitted for publication in scientific journals. Previous studies related to the experimentation and kinetic modelling of microalgal biomass growth and lipid accumulation are extensively reviewed in Chapter 2. The research contributions of this work are summarised as follows:

1. A kinetic modelling of microalgal biomass production and lipid accumulation is constructed here in conjunction with experiments of various growth media compositions to define the optimal growth conditions resulting in maximum biomass growth and lipid accumulation. In order to investigate the interactions between the biomass growth, the lipid accumulation and

the nutrient and substrate stresses, bench scale experiments were carried out for different nutrient and acetate concentrations using the well-studied chlorophyte microalgal species *Chlamydomonas reinhardtii*.

To develop a novel bench scale kinetic model, all existing single and multiple (threshold models and multiplicative) substrate models were identified and the phenomena behind them along with the weaknesses and strengths of each model were studied. Based on the experimental observations and the existing literature, a novel multi-parameter, predictive kinetic model was constructed to describe algal growth and lipid accumulation in laboratory-scale batch systems. The constructed model takes into consideration the effects of three different growth limiting parameters: acetate (organic carbon substrate for the heterotrophic component of growth), nitrogen, light intensity as well as pH.

An in-house developed stochastic algorithm, based on Simulated Annealing (SA), was coupled with a deterministic method, Sequential Quadratic Programming (SQP) applied through the "fmincon" function in MATLAB, to identify key parameters of the process leading to optimal microalgae growth and productivity for the bench-scale batch system. The developed kinetic model is then validated against a different set of data. Ultimately, the validated model was used in an optimization study to predict the optimal growth condition to achieve the highest biomass and the lipid productivities under the simultaneous effect of N, S, I. The optimal growth condition was found to be 2.1906 g L^{-1} acetate and 0.0742 g L^{-1} nitrogen. The lipid productivity was increased by 32.85%, which corresponds to 20.5 mg L^{-1} increase compare to the base case. The resulting optimization results were finally validated experimentally. This study is presented in Chapter 3.

2. Environmental factors, light intensity and temperature variations are also responsible for the biomass growth and they have an inhibitory effect on microalgal biomass growth and lipid accumulation. In order to investigate the effect of light intensity and temperature, lab scale experiments were performed at different light intensities and temperatures and also using the well-studied chlorophyte microalgal species *Chlamydomonas reinhardtii*.

   The kinetic model developed (see Chapter 3) was extended to take into account the co-limitation of environmental factors, light intensity and temperature. Consequently, the biomass growth and lipid accumulation rates of the improved model were expresses as

functions of the simultaneous effect of carbon substrate concentration, nitrogen concentration, light intensity and temperature.

The expanded model was then validated and utilized in an optimization study to determine the optimal light intensity and temperature to achieve the maximum lipid productivity through the use of the predefined optimal acetate and nitrogen concentrations (2.1906 g L^{-1} acetate and 0.0742 g L^{-1} nitrogen). It was found that the computed optimal lipid productivity increased by 50.9 % compared to a base case, and by 13.6% compared to the previously computed optimal case (chapter 3). The resulting optimization results were ultimatelly validated experimentally. This work is described in detail in Chapter 4.

3. The scale-up of the microalgal biomass growth process is necessary in order to investigate its economic viability and to bring the technology one step closer to commercialisation and industrialisation. Here, experiments were carried out in 2 m^3 raceway open ponds to assess the applicability of the detailed kinetic model constructed earlier (see Chapter 4). The kinetic model was constructed and validated for the heterotrophic growth of *C.* reinhardtii. Due to preliminary experiments demonstrating problems of microbial contamination in open ponds through the use of acetate, different strategies were employed such as reduced initial acetate, nitrogen and biomass concentrations. However, all these strategies still resulted in contamination. Hence, experiments and modelling were conducted for photoautotrophic growth of C. reinhardtii in the absence of acetate. This work is described in Chapter 5.

   As the microalgae strain uses available atmospheric CO_2 to perform photosynthesis, the effect of carbon source on biomass growth and lipid production has been removed from the corresponding kinetic model. The final open pond model considers the simultaneous effect of three growth limiting factors: light intensity, temperature and nitrogen. The developed model was used in conjunction with the in-house produced experimental data performed in 2m^3 raceway open ponds to define key parameters of the process which lead to the optimal microalgae growth and productivity.Subsequently, the model was validated and used for the optimization and the control of microalgal growth process to define the optimal operating conditions which lead to the highest lipid productivity at the open pond scale.

Chapters 3 to 5 each contain a preface and the paper submitted to the scientific journals with the discussion of the corresponding study. Finally, conclusions of this work are summarised in Chapter 6.

2. Literature Review

2.1. Introduction

From the previous chapters, it becomes clear that it is essential to identify alternative renewable sources of fuel that need to be carbon neutral so as to mitigate with CO_2 emission and also to reduce dependence on fossil fuels towards sustainable development of biodiesel industry. Use of microalgae for the production of biodiesel due to is unique advantages. The economic viability of biodiesel production from microalgae is strictly dependent on the metabolic productivities of the strains. In this chapter, the state of the art in production of biodiesel from microalgae is presented. Initially, in section 2.2., the effects of cultivation conditions on the production of microalgal oils and biofuels are explained. Subsequently, in section 2.3., the effect of nutrient starvation and environmental factors on microalgal oil accumulation was discussed. Then, microalgae culture systems were studied with particular focus on the potential of each of the technology. Finally, optimization of microalgal lipid productivity was studied.

2.1.1 Microalgal oil and biofuels

Microalgae are able to produce significant amounts of lipids, proteins and carbohydrates that can be converted into biofuels, foods, feeds and high-value bio-products (Chisti, 2007b). Microalgal biomass has been considered as an alternative feedstock for biodiesel production for more than 50 years (Driver et al., 2014, Chisti, 2007b). This is due to the intrinsic advantages that microalgae bear, such as rapid growth rate of microalgae and high oil productivity per area of land used (Georgianna and Mayfield, 2012). Additionally, a marine strain can be cultivated in saline water, or a strain may be cultivated in wastewater leading to the reduction of the use of resources (fresh water, nutrient fertilizer) and also mitigating air pollution. Furthermore, microalgae can grow on non-arable land that would not be used for traditional agricultural activities, considerably increasing agricultural land availability and bearing simple growing requirements (light, nitrogen, potassium and CO_2) (Borowitzka, 1999, Dragone et al., 2010). Ultimately, the use of microalgae as a feedstock for the production of biomass for markets such as food, feed, energy, and CO_2 mitigation, is considered to be a most sustainable, renewable, effective and environmentally friendly response to climate change and food–feed security

concern, and it has the potential to solve some of the future limitations of traditional biomass sources (Ziolkowska and Simon, 2014, Mata et al., 2010). Most importantly, it has been claimed that microalgal-based fuels are the only renewable energy source that has the potential to replace the traditional fuels and meet the global energy demand in the log-term (Vassilev and Vassileva, 2016).

Microalgal oil comprises of a neutral lipid triacylglycerol (TAG) which is stored in cytosolic and/or plastidic oil bodies (Chisti, 2007b). The microalgal production of such oil bodies can be improved by abiotic stress, including starvation of nutrients like nitrogen (N) and phosphorus (P), and environmental factors such as light intensity and temperature stress (Bajhaiya et al., 2016, Converti et al., 2009). Depending on the fatty acid characteristics, the lipid can be processed directly or it can be utilised into biolubricants, surfactants, nutritional lipids like omega-3 fatty acids, and most importantly, into liquid fuels and gas. The use of microalgal oil as feedstock for biodiesel production has not yet been commercially exploited as the current price of algal oil production is still too high to compete with fossil fuel diesel. Cultivation of microalgae accounts for 60-75% of the total cost for microalgal biodiesel production, mainly due to the high cost of carbon and nutrients input and the high cultivation facility costs relative to usually low oil productivity (Driver et al., 2014). Collet et al. (2011) recently performed a life cycle analysis (LCA) to assess biogas production from microalgae *Chlorella vulgaris*. The study shows that production of methane from microalgae is strongly related with the electricity demand and it suggests that in order to commercialise this technology, the most energy consuming steps, mixing and circulation costs needs to be decreased or the efficiency of the anaerobic process needs to be enhanced.

Despite the disadvantages of this technology, the use of microalgal oil for biodiesel production offers several advantages in terms of environmental impact and sustainability. The advantages are: rapid growth rate and high oil content per area of land required (Georgianna and Mayfield, 2012), reduction of GHG emissions by avoiding fossil fuel combustion and the fixation and use of atmospheric CO_2 and/or waste organic carbon (e.g. waste glycerol), mainly for marine and wastewater cultivated microalgae (Pittman et al., 2011) where there is no competition for agricultural land, nutrients (N,P and CO_2) and light (Dragone et al., 2010, Borowitzka, 1999). Despite the immense potential of microalgae oil and carbohydrate in biotechnological applications, metabolic productivity of microalgae needs to be improved in order to enhance its

economic viability. Strain selection and development by genetic manipulation, mutagenesis or natural selection are approaches that have been actively considered (Goncalves et al., 2015).

On the other hand, kinetic modelling can be used to optimise the cultivation conditions and the metabolic productivity. Furthermore, production of biofuels from microalgae can be improved through understanding the balance between biomass growth and lipid accumulation, whereby extreme nutrient starvation conditions that lead to substantial cellular oil production can also significantly inhibit biomass growth (Béchet et al., 2013). For this reason, integrated mathematical and experimental studies can be an important tool to explore the enhancement of the lipid production process by modelling and experimentally validating microalgal metabolism and metabolite yields (Béchet et al., 2013, Jørgensen, 1976, Bernard et al., 2016). Therefore, attention has been drawn to these combined experimental/modelling methodologies to develop a framework that could utilise algal strains in an integrated and cost effective way in order to improve the sustainability and competitiveness of the algal-derived biofuels industry.

2.2. Effects of Cultivation Conditions

Growth characteristics and composition of microalgae strongly depend on cultivation conditions. There are three major cultivation conditions: autotrophic, heterotrophic and mixotrophic.

2.2.1. Autotrophic cultivation

The most common microalgae cultivation method is the autotrophic cultivation (Perez-Garcia et al., 2011), in which the strain uses natural or artificial light as an energy source and carbon dioxide as a carbon source to form biomass through photosynthesis. The autotrophic cultivation cycle is represented in more detail in Figure 2.1 (Chen et al., 2011). Autotrophic cultivation provides several advantages: (a) the strain can convert sunlight into valuable chemicals by using inexpensive natural resources (CO_2 and H_2O), (b) also leading to CO_2 reduction, and (c) microalgae grows at areas where other crops cannot grow due to salty water, excessive sun light and lack of essential nutrients (Chisti, 2007b, Liang et al., 2009).

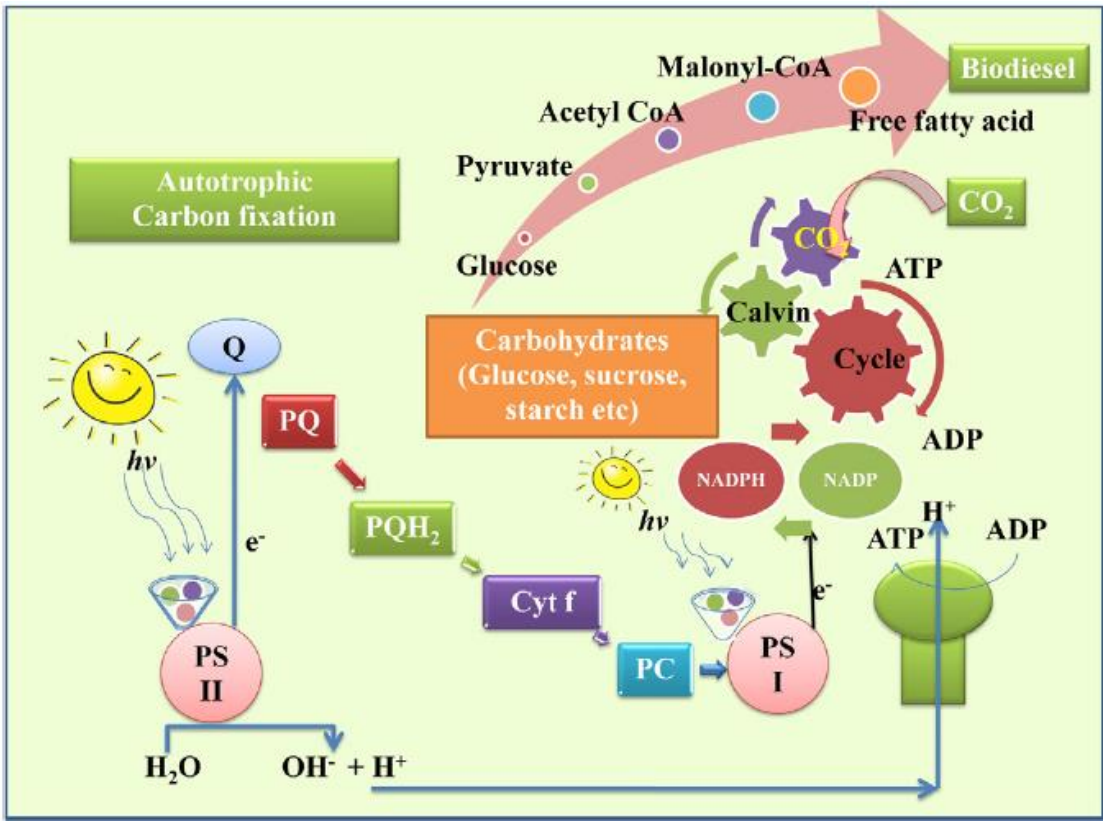

Figure 2.1 Autotrophic cultivation (Venkata Mohan et al., 2015). In this type of cultivation, in order to incorporate CO_2 into glucose, three ATP and two NAPDH are required, which are produced through charge separation, light absorption, proton gradient and water-splitting. The dark phase (Calvin cycle) of autotroptic cultivation consists of three main steps: CO_2 fixation, reduction and regeneration.

The provision of sufficient natural or artificial light triggers massive growth and high densities of microalgae cells. However, light supply in an autotrophic cultivation system should be limited to certain values - below which the more the light the higher the growth - depending on the microalgae species of interest (Perez-Garcia et al., 2011). Exceeding these light supply limits causes photo oxidation, which in turn damages the light receptors of algae and reduces the photosynthetic rate and productivity (Hu et al., 2008b, Van Wagenen et al., 2012). Moreover, it is difficult to achieve high-density microalgae culture due to both light penetration being inversely proportional to cell density and mutual shading of cells which result in very low biomass and lipid productivity (Liang et al., 2009).

2.2.2. Heterotrophic cultivation

Microalgae can also use organic carbons such as sugars and organic acids as a carbon source in order to be able to survive (see

Figure 2.2). External carbon sources provide prefabricated chemical energy, which is usually stored by algae as lipid, starch, or protein depending on the route of metabolism of the external carbon and the metabolic needs of the cell at that time. This mode of cultivation is called heterotrophic (Chen et al., 2011). Heterotrophic cultivation takes place both in the presence and absence of light. In photo-heterotrophic cultivation both light and carbon serve as energy sources while in dark conditions, organic carbon is the sole energy source. Heterotrophic cultivation eliminates the light dependency and thus, it provides the possibility of a significant increase in culture density and productivity (Chen and Johns, 1996) without any light source supply.

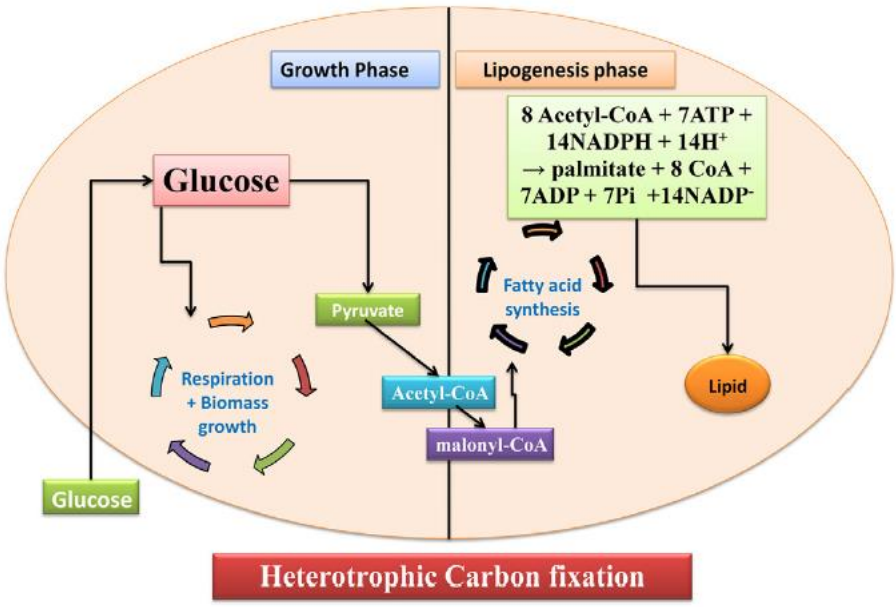

Figure 2.2 Heterotrophic cultivation (Venkata Mohan et al., 2015). In heterotrophic cultivation, an organic carbon source is utilised through the respiration metabolism to generate biomass. The Acetyl-CoA is produced from an extra cellular organic carbon source available in Pyruvate and is then transformed into lipids in the lipogenesis phase.

Heterotrophic cultivation processes can potentially provide a cost effective massive biomass growth and high-density microalgae culture method of cultivation for some algae species

(Perez-Garcia et al., 2011, Chen and Johns, 1996). To be able to grow heterotrophically, microalgae species must bear the following characteristics: (a) the ability of cell division and of having active metabolism without light, (b) the ability to grow in cheap and easily sterilized media, (c) the ability to adapt quickly to environmental changes, and (d) the ability to resist hydrodynamic stresses in the fermenters (Chen and Chen, 2006). *Chlamydomonas*, *Chlorella, Tetraselmis*, and *Nitzschia* are good examples of heterotrophically grown microalgae species (Gao et al., 2010, Chen and Johns, 1996, Nan and Dong, 2004). Glucose, organic acids, sugars, sugar phosphates, monohydric alcohols and sugar alcohols are the most commonly used carbon sources for heterotrophic cultivation (Perez-Garcia et al., 2011). Although the achieved cell densities are significantly higher compared to autotrophic cultivation, the organic carbon cost is very high when compared to all other supplementary nutrients (Meireles dos Santos et al., 2017). To make such systems economic viable, a cheap carbon source (e.g. crude glycerol derived from biodiesel production) should be used.

2.2.3. Mixotrophic Cultivation

Mixotrophic cultivation occurs when photosynthetic and respiratory metabolisms operate simultaneously (see Figure 2.3), where microalgae uses both organic compounds and CO_2 as a carbon source to form biomass (Lee, 2007). This means that the strain is able to survive under either heterotrophic or phototrophic conditions, or under both. CO_2 is assimilated through photosynthesis which is affected by light intensity, and organic carbons are fixed through the respiratory metabolism which is influenced by organic carbon concentration (Wang et al., 2014a). In this mode of cultivation, lowlight intensity and low organic carbon concentration may limit the algae growth. On the other hand, highlight intensity and high carbon concentration may inhibit algae growth.

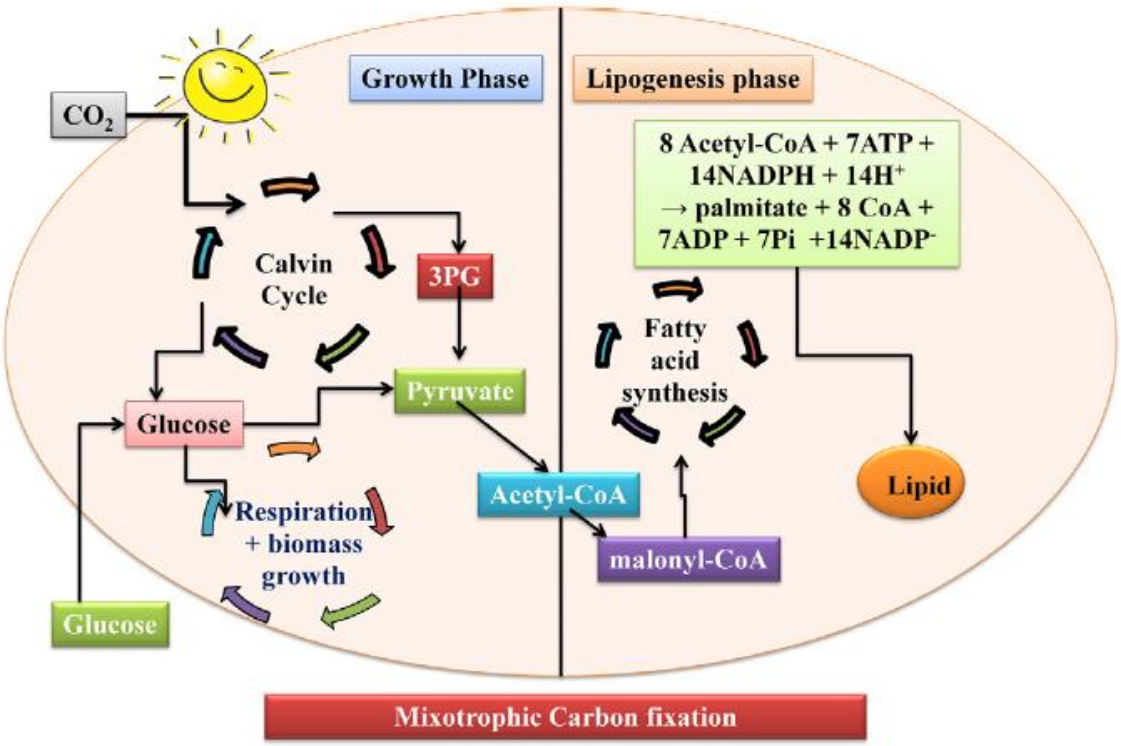

Figure 2.3 Mixotrophic cultivation (Venkata Mohan et al., 2015). In mixotrophic cultivation, both inorganic and organic carbon sources are utilised through the photosynthetic and respiration metabolism, respectively, to generate biomass. Acetyl-CoA is generated from both the carbon sources through CO_2 fixation (Calvin cycle) and the extra cellular organic carbon, and is then transformed into lipids in the lipogenesis phase.

The main difference with this mode of cultivation is that the organic carbon can also be used as energy source, while photoheterotrophy requires only light as energy source. Consequently, photoheterotrophy is rarely used for biodiesel production due to the need for both organic carbon and light at the same time (Chen et al., 2011).

As organic carbon can be assimilated under mixotrophic cultivation, the growth of microalgae does not completely depend on photosynthesis and light is not an absolute constraint for microalgal biomass growth. Consequently, photolimitation and photoinhibition can be decreased in mixotrophic cultivation when light intensity levels are too high or too low (Wang et al., 2014a).

Compared to the heterotrophic cultivation mode that solely relies on organic carbons, mixotrophic cultivation offers higher productivities with an identical organic carbon source. Furthermore, as the organic carbon source contributes to about 45% of microalgae cultivation

cost (Ogbonna and Moheimani, 2015), microalgal lipids production cost may also be reduced when cultivated under mixotrophic cultivation as it achieves higher productivities compare to heterotrophic cultivation (Wang et al., 2014a).

Hetrotrophic cultivation of *Chlorella* on organic carbon substrate (acetate and glucose) has been used in Japan with approximately 550 t produced in 1996 (Borowitzka, 1999). *Tetraselmis* species were also grown heterotrophically in UK for a short period, however the production cost was too high to compete with the conventional methods and therefore the focus was given on autotrohically grown algae (Borowitzka, 1999). Heterotrophic culture has also being used in the USA for the production of long-chain polyunsaturated fatty acids (Borowitzka, 1999). Despite the disadvantages of heterotrophic growth, not being possible for all microalgae species and composition of the strain often changes under heterotrophic conditions, the culture offers several advantages, namely: (i) fermentation process is well studied and there is wide knowledge in their design and operation, (ii) high cell densities can be achieved. Consequently, production of microalgal lipids under heterotrophic conditions has more potential to bring the microalgal biodiesel production technology one step closer to industrialisation.

2.3. Effect of nutrient starvation and environmental factors on microalgal oil accumulation

The development of microalgae-derived biofuels faces great challenges due to the high production cost of microalgae compared to fossil-derived fuels. Several studies have been undertaken to improve the economic feasibility of microalgae-derived biofuels production by enhancing strain performances, algae culture systems, harvesting and extraction techniques (Zhu et al., 2014).

Under desirable conditions, microalgae commonly produce cell wall carbohydrates, proteins and membrane lipids for cell structure. Moreover, under abiotic stress, including nutrients starvation like nitrogen (N) and phosphorus (P), and growth conditions stress like light intensity and temperature (Bajhaiya et al., 2016, Converti et al., 2009, Zhu et al., 2014), many microalgae species cease cell division and switch from photosynthetic carbon partitioning to forming energy rich storage compounds such as lipid and/or starch which can be processed into biofuels (Hu et al., 2008a, Li et al., 2012, Zhu et al., 2014). Consequently, to advance microalgal biofuel

production it is important to reveal the interactions between growth-limiting factors and metabolic productivities that results in high biomass and storage compounds production.

2.3.1. Nutrients starvation and substrate variation

Experiments with numerous microalgae strains have shown that biomass and storage compounds accumulation can be manipulated by varying the growth media composition. Growth media manipulation is mainly achieved through nutrient starvation. N-limitation and P-limitation can significantly influence the microalgae biomass composition, hence they are considered to be the most critical nutrient limitations. Controlling these nutrients and mainly the concentration of N can significantly enhance lipid accumulation. For this reason, nutrients' concentration manipulation is deemed to be the most affordable and convenient approach (Hu et al., 2008a).

Several studies have been carried out to demonstrate the effect of N-limitation on microalgal lipid accumulation and are summarised in Table 2-1. A general trend of nutrient starvation reveals that the TAG synthesis in microalgae increases upon N-starvation. N-limitation studies with *Botryococcus* species, *Chlorella vulgaris*,*Chlamydomonas reinhardtii* and *Haematococcus pluvialis* show substantial increases in lipid concentration upon N-starvation. As a result of the decline in cell protein as well as chlorophyll content and the reduced cell division upon N-limitation, the total culture volume biomass concentration decreases (Courchesne et al., 2009).

Biomass growth and lipid accumulation are strictly dependent on substrate (organic and inorganic carbon depends on the cultivation condition) availability (Fan et al., 2012). High substrate concentration stimulates the N-limitation induced lipid production. On the other hand, low substrate concentration favours algae growing. The substrate uptake is induced by light in growing microalgae cells and it leads to an increase in storage compounds such as lipid and starch. This effect suggests that in dark-grown algae, increasing the substrate concentration does not increase the lipid accumulation during N-limitation (Fan et al., 2012). Some studies with *Chlamydomonas Reinhardtii* reveal the inhibitory effect of different substrate types and concentrations (see Table 2-1). As can be seen in Table 2-1, the lipid content increased 10-15% upon nitrogen starvation and different growth rates have been observed when grown in different nitrogen sources (urea, nitrate and ammonia) with the highest growth rate recorded for the

microalgae growth in urea and lowest for the growth in ammonia. The effect of carbon source has also been studied in Table 2-1. As can be seen, increasing the acetate concentration increases the biomass growth until a point, and then the inhibition takes place. The table also shows that the carbon source availability increases in the number and size of oil bodies given acetate boost and also the amount of oil increases steadily as the acetate concentration increases to the levels several-fold higher than that of the standard growth medium.

Table 2-1 General impact of nitrogen and acetate on the biochemical composition of oil producing algae.

Factors	Organisms	Conditions	Observations	References
Nitrogen	*Botryococcus sp-TRG*	Nitrogen starvation	Lipid content increased from 25.8% to 35.9%	(Yeesang and Cheirsilp, 2011)
	Botryococcus sp-KB	Nitrogen starvation	Lipid content increased from 17.8% to 30.2%	
	Botryococcus sp-SK	Nitrogen starvation	Lipid content increased from 15.8% to 28.4%	
	Botryococcus sp-PSU	Nitrogen starvation	Lipid content increased from 5.7% to 14.7%	
	M. aeruginosa	Nitrogen starvation	Higher polysaccharide content under nitrogen starvation condition	(Yang et al., 2012)
	C. vulgaris	Nitrogen starvation (75% decrease)	Increase in lipid synthesis from 5.90% to 16.41%	(Converti et al., 2009)
	H. pluvialis	Nitrogen starvation	Increase in carotenoid formation (13% w/w)	(Borowitzka et al., 1991)
	C. reinhardtii	Nitrogen Source	Growth in urea 0.071 h^{-1} in nitrate 0.062 h^{-1} and in Ammonia 0.058 h^{-1}	(Zhang et al., 1999a)
Acetate	*C. reinhardtii*	C availability	Increase in the number and size of oil bodies given acetate boost	(Goodson et al., 2011)
	C. reinhardtii	C availability	The amount of oil increased steadily as the acetate concentration increased to the levels several-fold higher than that of the standard growth medium	(Fan et al., 2012)
	C. reinhardtii	C availability	Growth yield increased from 0.4 g/L acetate to 3.4 g/L and decreased for 5.1 g/L acetate	(Chen and Johns, 1996)

2.3.2. Environmental factors

Apart from the essential carbon availability and macronutrients as well as micronutrients, having favourable environmental conditions (light intensity and temperature) are also necessary in algae cultivation processes to manipulate algal growth, reproduction, lipid accumulation, algae chemical composition and photosynthetic activity, as well as economic viability of the processes (Rosso et al., 1995, Singh et al., 2015, Hu et al., 2008a). Therefore, the supply and efficient utilisation of these two environmental factors need to be controlled in order to improve algal biomass growth and lipid accumulation for biofuel production.

Many photosynthetic microalgae species are capable of heterotrophic growth, utilizing organic carbon under dark conditions to produce biomass and valuable products. However, for some algae strains, light is also required for the efficient biomass and storage compounds production, since the biomass growth rate of such mixotrophic cultures is dependent on a combination of both heterotrophic and photoautotrophic conditions (Li et al., 2012). In such cases, the light requirement is less than the one in photoautotrophic growth. Therefore, in order to achieve a desired biomass concentration with high concentration in desired products, the light intensity and quality needs to be controlled.

The net algae growth rate increases with an increase in light intensity and temperature, until a certain point where the biomass growth rate is at its maximum. Increasing the light intensity and temperature beyond this point does not increase algal growth rate. On the contrary, increasing the light intensity causes photo oxidation, damages the light receptors of algae and reduces the photosynthetic rate and thus the productivity. Various studies have utilised microalgae species like *Chlorella minutissima*, Chlorella vulgaris, Enteromorpha species, Chlamydomonas reinhardtii, Botryococcus species, Botryococcus braunii, Nannochloropsis species and Dunaliella virdis to show the effect of light intensity and temperature variations, and are summarised in Table 2-2. A more detailed review on light intensity and temperature variations can be found in Singh et al. (2015) and Ras et al. (2013). The effect of light intensity studies with respect to different microalgae species listed in Table 2-2 shows that increasing the light intensity increases biomass and lipid growth rates several fold higher than the low light

intensities. The table also shows the inhibitory effect of light intensity where high light intensities reduce the biomass and lipid growth rates. The effect of different light colours has also been shown in the table and the maximum specific growth rates monitored are as follows: blue>white>green>red. Table 2-2 also shows the influence of temperature on biomass and lipid growth rates where similar observations as of light intensity are observed. The table shows that increasing the temperature until optimal point reduces the doubling time and increases the biomass growth rate and exceeding the optimal temperature, microalgae growth rate sharply decreases and at high temperatures where the strain cannot survive the growth rate becomes zero.

Table 2-2 General impact of temperature and light on the biochemical composition of oil producing algae.

Factors	Organisms	Conditions	Observations	References
Temperature	*C. minutissima*	increased from 10 to 30	growth increased from 0.12 d^{-1} to 0.66 d^{-1}	(Aleya et al., 2011)
	C. vulgaris	reduced from 27 to 5	doubling time increased from 8.6 h to 48.5 h	(Maxwell et al., 1994)
	B. braunii	increased from 5,15,20,25,30,35,38 and 45 ^{0}C	maximum specific growth rate increases up to a point where the maximum specific growth rate becomes zero (0.095, 0.207, 0.392, 0.431, 0.496, 0 0 and 0 d^{-1} respectively)	(Yoshimura et al., 2013)
		increased from 25 to 32	lipid content decreased from 22% to 5%	(Kalacheva et al., 2002)
Light	*Enteromorpha sp*	increased to 90 µE m^{-2} s^{-1} from 20 and 40 µE m^{-2} s^{-1}	Biomass increased compared to other two treatments	(Sousa et al., 2007)
	C. reinhardtii	Light Availability	Chl content was 10.1 mg L-1 for dark growth, 15.3 g.L-1 for 12-12h dark/light cycle and 18.5 mg.L-1 for continues illumination	(Tamburic et al., 2011)
	Botryococcus sp	increased to 82.5 µE m^{-2} s^{-1} from 33 and 49.5 µE m^{-2} s^{-1}	lipid content increased in all four strains with increasing light intensity from 33 to 49.5 but decreased with increasing light intensity from 49.5 to 82.5 µE m^{-2} s^{-1}	(Yeesang and Cheirsilp, 2011)
	B. braunii	increased to 538 µE m^{-2} s^{-1} from 87.5 and 200 µE m^{-2} s^{-1}	highest Lipid content was observed in 538 µE m^{-2} s^{-1} while highest biomass concentration was observed in 87.5 µE m^{-2} s^{-1}	(Ruangsomboon, 2012)
	Nannochloropsis	Light Colour	maximum specific growth rate: blue>white>green>red	(Das et al., 2011)
	C. vulgaris	Light Colour	growth rate: red>white>yellow>purple>blue>green	(Yan et al., 2013)

2.4. Microalgae Culture Systems

Several microalgae production systems are currently under development, ranging from open ponds, photobioreactors (PBRs) and fermenter vessels to hybrid systems, where all the above technologies can be combined into a single process.

In many cases, maximizing the growth of algae for the production of biofuels, chemicals or other value adding industrial products, is the main approach followed towards the design of a sustainable microalgae culture system. The viability of each system is often a function of its structural properties, properties of the microalgae under use and climatic as well as operating conditions. This section reviews the microalgae growth technologies that are currently being deployed around the world.

2.4.1. Open Ponds

Algae cultivation in open pond systems has been operated since the 1950s and is the most common algae cultivation system, already used commercially all over the world for the production of nutritional products and wastewater treatment. Open pond systems are generally one-foot deep ponds in which algae uses sunlight to perform photosynthesis and convert natural solar radiation into biomass. Open-culture systems have lower energy input, operating cost and less installation cost. Also, open pond systems are easy to clean and suitable for large scale applications (Mata et al., 2010, Brennan and Owende, 2010).

Open pond systems can be categorised into natural waters (lakes, lagoons and ponds) and artificial ponds or containers. The most commonly used artificial type is the raceway open pond (see Figure 2.4) (Jiménez et al., 2003, Mata et al., 2010). Raceway open ponds are commonly employed for algae cultivation as they are easy to clean and operate and their operating cost is low compared to PBRs and fermenters (Davis et al., 2011). Although the open race-way ponds are widely employed, it is difficult to achieve dense microalgae cultures. They are also limited by environmental factors (light and temperature) and are very sensitive to contamination and pollution (Pulz, 2001, Jorquera et al., 2010, Carvalho et al., 2006).

In order to avoid contamination and pollution from other microalgae and bacteria, selective environments need to be maintained by manipulating growth factors such as pH, salt level, and nutrients (Brennan and Owende, 2010). For instance, species of *Chlorella* (adaptable to nutrient-rich media), *Dunaliella salina* (adaptable very high salinity) and Spirulina (adaptable to high alkalinity) can thrive under these extreme conditions (Brennan and Owende, 2010).

Figure 2.4 Graphical representation of a raceway open pond.

Additionally, with respect to biomass productivity, open ponds usage leads to lower productivity if compared to PBRs. This is attributed to several factors, such as evaporation losses, temperature fluctuation, light limitation, inefficient mixing and inefficient CO_2 fixation, which are difficult to control in such systems. However, as the capital and operating cost of OPs are lower compare PBR, which will be explained in the following section, the use of open pond systems for microalgal-derived biofuels gets more attention. For instance, Sapphire Energy is constructing a commercial scale open pond production facility, aiming to produce millions of gallons of biofuel annually by 2017 in New Mexico (Sapphire Energy, 2012).

2.4.2. Photobioreactors (PBRs)

Photobioreactors are closed systems in which specific conditions can be maintained for respective species. The PBRs allow monoculture cultivation of microalgae and much higher growth rates which in turn makes them appropriate for use in the cosmetic and the pharmaceutical industries to produce high value products (Brennan and Owende, 2010). As closed systems, PBRs can also prevent the contamination by weed algae and bacteria. On the other hand, depending on the economic value of the final product, the high capital cost of PBRs remains the major drawback of the system (Mata et al., 2010).

PBRs differ from open ponds in that the algae is enclosed in a transparent vessel oriented horizontally or vertically. In some cases, additional artificial light is used to enhance production and some PBRs rely solely on artificial lights (generally laboratory-scale PBRs). The most popular closed systems are the tubular, the flat plate and the column photobioreactors (Carvalho et al., 2006). The tubular PBR consists of two main parts, an airlift system and a solar receiver. The airlift system is responsible for the transfer of CO_2 into the system and transfer of O_2 out of the system. The solar receiver offers a platform for algae to grow. The tubular PBRs are the most commonly employed PBR designs. The tubular photobioreactors consist of transparent solar tubes and tubular array that can be aligned helix, inclined horizontally or vertically (Zhou et al., 2015). The graphical representation of four main types of tubular PBRs is given in Figure 2.5. The flat-plate PBR is the earliest form of PBRs, which consists of narrow panels that provides large surface area for the uniform distribution of light and high density cultures. Common types of flat-plate PBRs are represented in Figure 2.6. The production of dissolved O_2 in flat-plate PBRs is lower and the photosynthetic efficiency is higher compared to tubular PBRs. Hence, the flat-plate PBRs are considered to be more suitable for mass production of microalgal biomass than the tubular ones (Zhou et al., 2015).

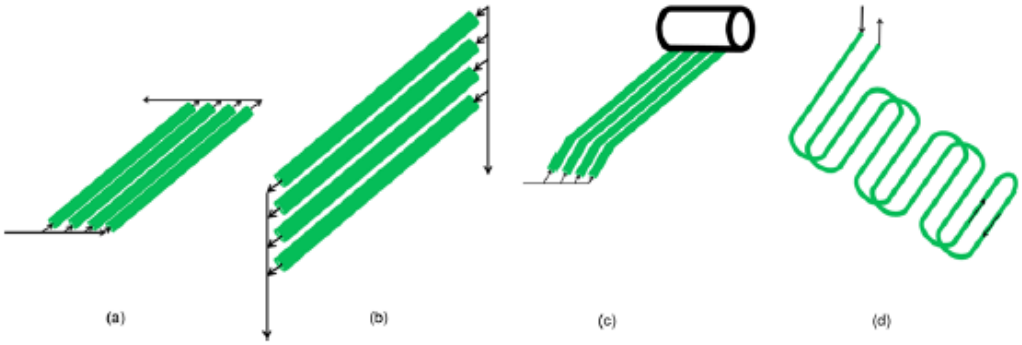

Figure 2.5 Graphical representation of tubular PBRs: (a) parallel run horizontal tubes, (b) fence-like layout, (c) near horizontal tube and (d) three-typed helical tubes (Zhou et al., 2015).

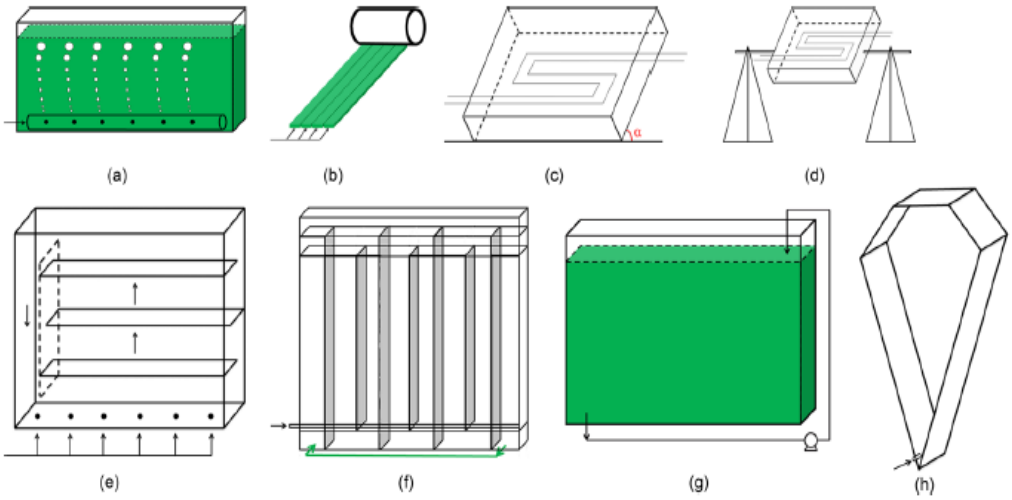

Figure 2.6 Graphical representation of flat panel PBRs: (a) vertical flat plate with bubbling at the bottom, (b) near horizontal flat plate, (c) inclined flat plate, (d) flat plate rocking reactor, (e) flat plate with baffles, (f) vertical alveolar plates, (g) vertical flat plate with recirculation and (h) V-shaped flat plate (Zhou et al., 2015).

A graphical representation of three different configuration of column PBRs is shown in Figure 2.7. The column photobioreactors are compact and easy to operate; their production cost is relatively low, and most importantly, their performance is better than that of the tubular photobioreactors. Column PBRs exhibit better performance compared to flat-plate and tubular PBRs due to their best-mixing effect, higher volumetric mass transfer rate, and better controllable mono-culturing (Zhou et al., 2015).

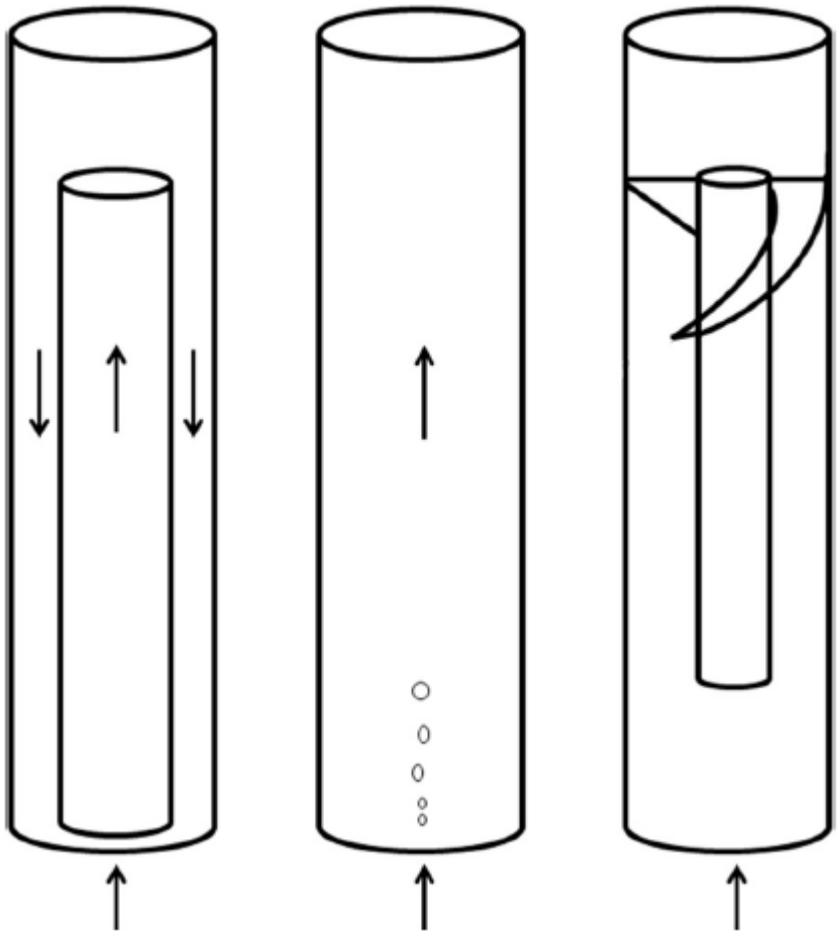

Figure 2.7 Graphical representation of column PBRs: (a) air-lift column, (b) bubble column and (c) air-lift column with helical flow promoter (Zhou et al., 2015).

2.4.3. Heterotrophic Cultivation in Fermenter Vessels

Another method of cultivation is the heterotrophic growth in fermenter vessels, which is becoming increasingly used in industry. This so-called heterotrophic fermentation technology is an alternative approach to growing algae using natural light radiation, which allows growing algae on organic carbon in dark. In heterotrophic fermentation, algae converts organic carbons into valuable oils, chemicals, nutritional and pharmaceutical products (Borowitzka, 1999).

Microalgae cultivation in fermenter vessels (as in Figure 2.8) is well developed and bears several advantages over PBRs and open ponds such as: (a) low operating cost, (b) removal of light requirements, (c) large and existing fermentation technology database and (d) non-dependency on weather and climatic conditions (Alabi et al., 2009). This type of cultivation allows the production of algae with high enrichment of desired components which in some

cases is twenty-fold higher than in phototrophic production. However, heterotrophic cultivation of microalgae in fermenters requires sufficient oxygen supply, necessary for the catabolism of organic carbons and high organic carbon input (Alabi et al., 2009), which increases the operating cost.

Figure 2.8 Representation of fermenter vessels.

The contamination, the space required, the water losses and the CO_2 losses for microalgal oil production in open ponds, PBRs and fermenters are listed in Table 2-3.

Table 2-3 Advantages and disadvantages of open ponds, photobioreactors and fermenters (Pulz, 2001, Chisti, 2007b, Richardson et al., 2012, Alabi et al., 2009).

Parameter	**Open ponds**	**PBR systems**	**Fermenters**
Contamination	Extremely high	Low	Very low
Water losses	Extremely high	Almost none	Almost none
CO_2 losses	High	Almost none	Almost none
Space required	High	Low	Very low

Although PBR systems and fermenters are deemed to be better than the open pond systems in terms of contamination, space required, water losses and CO_2 losses, in order to make an appropriate selection of the cultivation method, an economic comparison between different systems needs to be carried out. A techno-economic analysis of the open pond and PBR production systems has been undertaken by Davis et al. (2011) where baseline economics for two microalgae pathways were studied using a set of assumptions were considered and the corresponding results are given in Figure 2.9. The production scales for both pathways were set at 10 million gallons per year of raw algal oil. In this techno-economic analysis, rigorous mass balances were undertaken using Aspen Plus software and the resulting costs were assessed on a unit level basis. The analysis considered both operating costs (labour, maintenance, insurance, taxes and plant life), and capita cost (land, ponds and PBRs systems, CO2 delivery, harvesting, extraction, digestion, inoculum system, osbl equipment and hydrotreating costs).

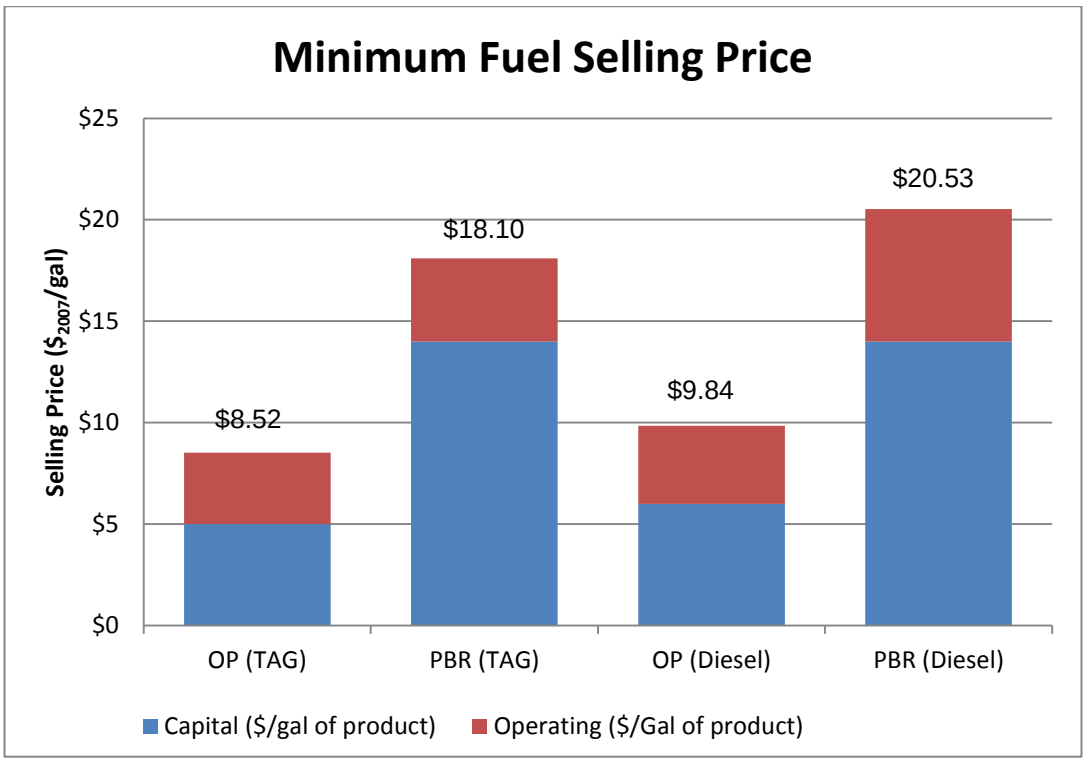

Figure 2.9 Minimum selling price required to achieve 10% rate of return for algal TAG and diesel production (OP = open ponds, PBR = photobioreactors) (Davis et al., 2011).

As per Figure 2.9, although the contribution of the operating cost to the selling price of the microalgal biodiesel is similar for both open ponds and PBRs, their economics are mainly driven by the capital cost. Figure 2.9 shows that the capital cost of PBRs systems is several times higher than the one of open pond systems. Several studies have been published where the influence of the capital cost on the algal biodiesel potential is examined; while this is beyond the scope of this Thesis, a thorough techno-economic analysis for the comparison of open ponds and PBRs is provided by Davis et al. (2011), Brentner et al. (2011) and Richardson et al. (2012). Therefore, due to less operating and capital cost and lower energy input, open ponds are the most promising long term sustainable candidates for the microalgal biodiesel production and hence, it has been selected in this study to assess the predictviness of the developed kinetic model for large scale applications.

2.5. Optimization of Microalgal Lipid Productivity

2.5.1. Genetic Engineering of Algae for Enhanced Lipid Production

Although many studies showed that the nutrient starvation can enhance the lipid accumulation, the drawbacks associated with this approach such as decrease in cell division and hence cell number and subsequently a decrease in overall biomass concentration are yet to be overcome. Therefore, understanding the metabolic processes at the genetic and transcriptomic level is necessary to overcome these limitations.

Radakovits et al. (2010) have suggested that the fatty acid supply helps to understand the regulation of lipids and therefore, some attempts have been made knock out or overexpress enzymes and proteins that are involved in the fatty acid synthesis. Several studies have attempted overexpress the enzymes that plays crucial role in lipid biosynthesis and they resulted in increased accumulation of TAGs in algal cells.

A recent study showed that introducing genes for enzymes related to lipid synthesis, such as acetyl-CoA carboxylase (ACC), 3-ketoacyl-acyl carrier protein synthase III (KAS) III, and ATP:citrate lyase (ACL) into higher plants like Arabidopsis, Brassica napus, and tobacco resulted in higher production of lipids. However, no increase of lipid synthesis was observed when the ACC gene introduced into two different algae species, C. cryptica and Navicula saprophila (Gan et al., 2016). On the other hand, several successful stories have been reported such as Trentacoste et al. (2013) have reported that knockdown of a multifunctional lipase/phospholipase/acyltransferase improved lipid yields by 2.4-3.3 fold without negatively affecting growth in the diatom *Thalassiosira pseudonana* and Niu et al. (2013) have reported that overexpression of diacylglycerol acyltransferase (DGAT) stimulated more oil bodies, and the neutral lipid content increased by 35 %. Although there is a lack of successful stories up to date, the genetic and transcription factor engineering approaches have a great potential in lipid overproduction. (Courchesne et al., 2009).

2.5.2. Kinetic Modelling microalgae growth

Production of biofuels from microalgae has received increasing attention as an alternative energy source workflow (Lee et al., 2015, Borowitzka, 1992, Dragone et al., 2010). Microalgae cultivation is an important step for successful biomass production in the biofuel industry.

Recently, attention was drawn to the cultivation of microalgae in order to increase microalgal biomass productivity and lipid productivity, achieve positive energy balance and environmental sustainability as well as improve competitiveness of algal derived biofuels industry, through kinetic modelling of the process (Lee et al., 2015, Flynn, 2003). In order to further develop novel harvesting techniques and oil extraction methods at the industrial scale, undertaken studies simulate closed batch systems to identify the key parameters of the process which lead to microalgae growth and lipid accumulation.

Depending on type of the knowledge that the models are based on, mathematical modelling of biological systems can be classified into three different perspectives, white-box, black-box and grey-box. White-box modelling, so-called first-principles, are obtained from a prior process knowledge and they are based on mass balance and phenomenological equations (Surisetty et al., 2010). Black-box modelling approach, so-called data-driven model, obtained by directly mining the process data using statistical tools, and do not need any prior knowledge of the process mechanism and consider that the output of the model at any time is a function of previous system states of interest (Gormley et al., 2007). On the other hand, grey-box modelling frameworks are the combination of all existing white-box and black-box modelling approaches. This modelling technique is utilized where knowledge of internal working of a process is limited and the knowledge of fundamental aspects of the process is known. Grey-box modelling technique provides combined benefits of both white-box and black-box models. There are some successful applications of grey-box modelling has been reported (Flassig et al., 2016, Baroukh et al., 2016, Chapman et al., 2015).

Although the white-box and grey-box models can provide better deep understanding of the physicochemical relationships of the process, black-box models offer an alternative pathway for capturing the essential behaviour and dynamics of the biological processes utilizing a simplified model structure. Therefore, black-box modelling approach has been considered in this study for mathematical modelling of microalgal biomass growth and lipid accumulation. The black-box

kinetic models developed can be grouped into two categories: (i) single substrate or environmental factor models and (ii) multiple factor models which take into account both substrate and environmental variables.

2.5.2.1. Single substrate growth kinetic models

Microalgae growth rate depends on the availability of nutrients such as N and C sources and the availability of light in aquatic media. Most of the existing kinetic models such as the Monod model, the Haldane model, the Droop model, the Martinez-Sancho model and the Caperon Meyer model, are expressed as a function of a single nutrient or environmental variable (see Table 2-4) (Droop, 1968, Andrews, 1968, Haldane, 1930, Monod, 1949, Martínez Sancho et al., 1997, Caperon and Meyer, 1972), and they can be categorised into two groups, the external and the internal (compartmental model) nutrient concentration models.

External nutrient concentration models like the Monod, Haldane and Martinez-Sancho models, assume that the growth rate is dependent on an external nutrient (extracellular) concentration (Lee et al., 2015, Monod, 1949, Flynn, 2003). In other words, the growth rate is controlled by a nutrient concentration in the growth media. This group of models is utilized to predict biomass growth rate as measuring external nutrient concentration is easy.

The Monod model is the first model developed and it considers only nutrient limitation conditions. Due to its simplicity, the Monod model has been widely used to reveal the relationship between microalgae growth and nutrient (N,P,C) limitation (Eriksen et al., 2006, Wang et al., 2014a, Chen and Johns, 1994, D'Elia and DeBoer, 1978, Klasson et al., 1993). However, as the model does not account for nutrient inhibition, it fails to reveal the relationship between biomass growth and nutrient inhibition under excessive nutrient concentrations. In order to overcome this limitation, Haldane modified the Monod model by adding a new term (S^2/K_i) in the denominator of the growth rate expression (see Table 2-4) to describe the nutrient inhibition effect on microalgae growth rate at high nutrient concentrations. The Haldane inhibition model is mainly used in the field of enzymes and it is also known as the Andrew model in microbial growth context. The Andrew model has been widely applied to address high nutrient concentrations on biomass growth rate (Chen and Johns, 1994, Zhang et al., 1999a, Mayo, 1997).

Another limitation of the Monod model is that it is not capable of predicting the microalgae growth under nutrient absence conditions. In reality, although there is no nutrient in the growth media, algae can still grow due to nutrient concentrations stored internally in their cells (Droop, 1968, Flynn, 2005). To consider this characteristic, Martinez-Sancho proposed a modification to the Monod model by introducing an additional maximum specific growth rate (μ_{m2}) parameter. Consequently, in the absence of nutrient (S), the growth rate (μ) is equal to the maximum specific growth rate (μ_{m2}) (Martínez et al., 1999).

Internal nutrient concentration models like the Droop and Caperon Meyer models, assume that the growth rate depends on an internal nutrient concentration in the cells, which is the amount of the intracellular nutrient concentration per cell, given by cell quota. These type of models are known as quota models. These models may predict the growth rate more realistically as they can address the biomass growth in the absence of external nutrients by accounting for internal nutrient concentration. Although the applicability of intracellular nutrient concentration models is limited compared to that of the extracellular ones due to the difficulty in measuring cell quota, they have been widely utilised to describe microalgal biomass growth rate (Kwon et al., 2013, Mairet et al., 2011b, Grover, 1991)..

The first model developed that falls into this group of models is the Droop model. Due to the nature of the mathematical equation (see Table 2-4), when the growth rate (μ) reaches the maximum specific growth rate (μ_{max}), quota (q_s) should approach infinity which is infeasible in reality. In order to overcome this issue, Caperon-Meyer proposed an improved formula - a combination of the Droop model and the Monod model - which introduces a constant (K_c) to the denominator of the Droop model (Caperon and Meyer, 1972). Although the Caperon-Meyer model is better than the Droop model in terms of determining the biomass growth rate, it requires the estimation of an extra parameter (K_c) that increases the complexity of the model (Lee et al., 2015).

Table 2-4 Single substrate microalgal growth kinetic models.

Models	Equations	Considered Variables	Strains	References
Monod	$\mu = \mu_{max}\frac{S}{S+K_S}$	N	*C. reinhardtii UTEX 2337*	(Eriksen et al., 2006)
			G. folifera	(D'Elia and DeBoer, 1978)
			N. baileyi	(D'Elia and DeBoer, 1978)
			Tetraselmis sp.	(Molina et al., 1991)
		P	*Chlorella sp.*	(Wang et al., 2014b)
			Micractinium sp.	(Wang et al., 2014b)
		C	*C. reinhardtii*	(Chen and Johns, 1994)
		I	*P. cruentum*	(Sada et al., 1989)
			R. rubrum	(Klasson et al., 1993)
			P. tricornutum	(Bitaubé Pérez et al., 2008)
			C. vulgaris	(Sasi et al., 2011)
			C. pyrenoidosa	(Martínez Sancho et al., 1997)
Andrew	$\mu = \mu_{max}\frac{S}{S+K_S+\frac{S^2}{K_i}}$	C	*C. reinhardtii CS-51*	(Chen and Johns, 1994)
			S. platensis UTEX 1926	(Zhang et al., 1999b)
		I	*S. platensis UTEX 1926*	(Zhang et al., 1999b)
		pH	*C. vulgaris*	(Mayo, 1997)
Martinez-Sancho	$\mu = \frac{\mu_{m1}S+\mu_{m2}K_s}{K_s+S}$	P	*S. obliquus*	(Martínez Sancho et al., 1997)
Droop	$\mu = \mu_{max}(1-\frac{q_{S_0}}{q_s})$	N	*I.aff. galbana*	(Mairet et al., 2011b)
			Achnanthes sp.	(Kwon et al., 2013)
			Amphora sp.	
			Navicula sp.	
			Nitzschia sp.	
		P	*Achnanthes sp.*	
			Amphora sp.	
			Navicula sp.	
			Nitzschia sp.	
			Tetraselmis subcordiformis	(Nan and Dong, 2004)
			Nitzschia sp.	(Yamamoto et al., 2012)
			Scenedesmus sp.	(Grover, 1991)
			Chlorella sp.	
			U. pertusa	(Nan and Dong, 2004)
Caperon-Meyer	$\mu = \mu_{max}(\frac{q-q_{S_0}}{K_{q,s}+q-$	N	*T. pseudonana CCMP 1335*	(Davidson and Gurney, 1999)
			H. carterae (Chatonella sp.) HA1V	
			A. minutum AL2V	
		P	*S. quadricauda*	(Yao et al., 2011)
			S. quadricauda	(John and Flynn, 2000)

2.5.2.2. Multiple substrate growth kinetic models

Microalgae growth and lipid accumulation can be simultaneously and antagonistically influenced by one or more nutrients (N, P), the substrate (C) and environmental factors (light intensity, temperature) (Chen and Johns, 1994, Converti et al., 2009, Breuer et al., 2015, Lee et al., 2015). In order to provide more accurate estimations of microalgae growth along with a better understating of the growth dynamics, the co-limitation concept has been applied in the development of mathematical models. The main assumption behind this concept is that the biomass growth is controlled by the multiple nutrients and the light, as well as the interactions between them. The co-limitation models can be grouped into two categories, the threshold and the multiplicative models.

The threshold models assume that the biomass growth is only controlled by the growth parameter with the concentration of the limited nutrient, and therefore, the final kinetic model takes the form of a single substrate growth model similar to the growth kinetic models considering a single parameter. However, the threshold models are based on the co-limitation concept, as all possible resources were taken into account while constructing the kinetic model. The most commonly used models are tabulated in Table 2-5. They describe the effect of two parameters on biomass growth in combination with the Droop equation.

Table 2-5 Microalgal growth kinetic threshold models.

Equations	Organisms	Parameters	References
$\mu = \mu_{max,i} min\left(1 - \frac{Q_{min.i}^{N}}{Q_i^{N}}\right), \left(1 - \frac{Q_{min.i}^{P}}{Q_i^{P}}\right), \left(1 - \frac{Q_{min.i}^{Fe}}{Q_i^{Fe}}\right), \frac{Si}{k_i^{Si}+Si}; \frac{1}{\sum_{i=1}^{n} \alpha_i \beta_i + \alpha_{bg}} \frac{1}{Z_{mix}} Log\left[\frac{I_{in}+k_i^{I}}{I+k_i^{I}}\right]$	*Coccolithophores*	N,P,Si, Fe,I	(Litchman et al., 2006)
$\mu = \mu_{\infty min}\left(1 - \frac{Q_{min,1}}{Q_1}, 1 - \frac{Q_{min,2}}{Q_2}\right) B - mB$	*Scenedesmus sp*	Double Substrate	(Klausmeier et al., 2004)
$\mu = \mu_{max} min\left(\frac{1 - \frac{q_{No}}{q_N}}{1 - \frac{q_{No}}{q_{Nexp}}}, \frac{1 - \frac{q_{Po}}{q_P}}{1 - \frac{q_{Po}}{q_{Pexp}}}\right)$	*S. minutum*	N, P	(Bougaran et al., 2010)
$\mu = \mu_{max} \frac{\frac{S_1}{K_1}}{1 + \frac{S_1}{K_1}} \quad \frac{S_1}{K_1} < \frac{S_2}{K_2}$ $\mu = \mu_{max} \frac{\frac{S_2}{K_2}}{1 + \frac{S_2}{K_2}} \quad \frac{S_2}{K_2} < \frac{S_1}{K_1}$	*Various species*	N, C	(Bader, 1978)
$\mu = \mu_{max} min\left(\frac{P}{K_P + P}, \frac{C}{K_C + C}\right)$	*C. acidophila*	CO_2,P	(Spijkerman et al., 2011)

The multiplicative models assume that all the growth parameters contribute to microalgae growth equally. Such models were commonly utilised to describe simultaneous co-limitation of N, P, I and C on the growth of biomass (Solimeno et al., 2015, Franz et al., 2012). As it can be seen in Table 2-6, the Monod is the most common model that has been employed to express the contribution of each parameter to the growth rate (Zhang et al., 1998, Shi et al., 2000). These types of models take into account multiple co-limitations and they are based on internal nutrient concentrations, external nutrient concentrations or the combination of both. Although this type of models provide more accurate estimations of biomass growth and also a better understanding of the co-limitation of nutrients, their complexity and the high number of parameters need to be estimated require high computational effort.

Although the presented single substrate modelling approaches are deemed good enough to predict biomass growth and lipid accumulation, they fail to account for the co-limitation of both growth media composition and environmental factors such as carbon source, nutrient availability, light intensity and temperature. Consequently, multiplicative kinetic modelling approach has been considered in this study to account for the equal contribution of multiple growth-limiting factors.

Table 2-6 Microalgal growth kinetic multiplicative models.

Equations	Organisms	Parameters	References
$\mu = Ae^{-E/RT}\left(\frac{S}{K_s(T)+S}\right)$	*Various species*	T + S	(Goldman and Carpenter, 1974)
$\mu = \mu_{max}\frac{q_P - q_{P_0}}{K_P+(q_P-q_{P_0})}\frac{q_N - q_{N_0}}{K_P+(q_N-q_{N_0})}$	*Scenedesmus sp*	N+P	(Rhee, 1978)
$\mu = \mu_{max} f_{T,FS}(T)\eta_{PS}(I,SO_2)\frac{S_{CO2}+S_{HCO3}}{K_{C,Alg}+S_{CO2}+S_{HCO3}+\frac{{S_{CO2}}^2}{I_{CO2,Alg}}}\frac{S_{NH3}+S_{NH4}}{K_{N,Alg}+S_{NH3}+S_{NH4}}$	*Scenedemus sp.*	S+N+I+T	(Solimeno et al., 2015)
$\mu = \frac{\mu_{max}}{1+\frac{H^+}{K_H}+\frac{K_{OH}}{H^+}}\frac{C_{Gly}}{C_{Gly}+K_S}\left(1-\frac{C_{HAc}}{C^*_{HAc}}\right)\left(1-\frac{C_{HBu}}{C^*_{HBu}}\right)\left(1-\frac{C_{EtOH}}{C^*_{EtOH}}\right)\left(1-\frac{C_{PD}}{C^*_{PD}}\right)\left(1-\frac{C_{Gly}}{C^*_{Gly}}\right)$	*C. butyricum*	Multiple product inhibition and pH	(Zeng et al., 1994)
$\mu = \mu_{max}\frac{S}{S+K_S}\frac{N}{N+K_N}$	*C. prototheocoides*	S+N	(Shi et al., 2000)
$\mu = \mu_{max}\frac{C_S}{K_S+C_S+\frac{{C_S}^2}{K_S}}\frac{I}{K_{XI}+I}\left(1-\frac{C_X}{C_{X_m}}\right)\left(1-\frac{C_P}{C_{P_m}}\right)$	*S. platensis*	S+I+P+Cell Concentration	(Zhang et al., 1998)
$\mu = \mu_{max}\left(1-\frac{q_o}{q}\right)\left(\frac{S}{K_S+I}\right)\left(\frac{I}{K_I+I+\frac{I^2}{K_{Ii}}}\right)$	*C. prototheocoides*	N+S+I	(Yoo et al., 2014)
$\mu = \mu_{max} Min[f_1(N), f_2(I)]f_3(pH)f_4(T)$	*Chlamydomonas sp. and Euglena sp*	(N, I)+pH+T	(Beran and Kargi, 2005)
$\mu = \mu_{max}\frac{1}{K_{h1}+\frac{K_{h2}}{H^+}+\frac{H^+}{K_{h3}}}\frac{S}{K_S+S+\frac{S^2}{K_i}}$	*C. reinhardtii*	S+N	(Zhang et al., 1999a)
$\mu = \mu_{max}\frac{PPFD}{K_{PPFD}+PPFD}\frac{C_{CO_2}}{C_{CO_2}+K_{CO_2}}\frac{C_{nu_i}}{C_{nu}+K_{nu}}$	*C. reinhardtii*	I+C+N	(Franz et al., 2012)

2.5.2.3. Modelling effect of temperature and light distribution

As it was discussed in section 2.3.2, the light source and its magnitude and also temperature play a crucial role in both enhancing microalgal biomass growth rate and lipid production rate. Therefore, it is essential to understand the time-dependent dynamics of both light distribution and temperature fluctuations in order for the optimization microalgal biomass and lipid production. Arrhenius equation is widely employed to describe the effect of temperature on the specific rate constant of chemical reactions:

$$\frac{dlnK}{dT} = \frac{E}{RT^2} \qquad \text{Eq. 1}$$

However, the expression does not describe the effect of temperature in field of microbial growth. Ratkowsky et al. (1982) proposed a modified version of Arrhenius equation in which the reaction rate constant, K, is replaced with the specific growth rate constant, μ,:

$$\mu = A_0 \cdot exp\left(-\frac{E}{RT}\right) \qquad \text{Eq. 2}$$

Alternatively, in order to account for effect of temperature on the deactivation of microbial growth, Roels (1983) also proposed a improved version of the Arrhenius equation:

$$\mu(I) = \mu_{m,0}(I)\frac{exp\left(-\frac{E_a}{kT}\right)}{1 + K \cdot exp\left(-\frac{E_a{}'}{kT}\right)} \qquad \text{Eq. 3}$$

The effect of temperature has also been considered through use of an empirical temperature-limited model, called Cardinal temperature model with inflexion (CTMI) in which the growth rate is function of minimum, maximum and optimal temperatures:

$$\emptyset(T) = \left[\frac{(T - T_{max})(T - T_{min})^2}{(T_{opt} - T_{min})\left[(T_{opt} - T_{min})(T - T_{opt}) - (T_{opt} - T_{max})(T_{opt} + T_{min} - 2T)\right]}\right] \qquad \text{Eq. 4}$$

Although both the Arrhenius equation and the CTMI has four parameters that need to be estimated, the Arrhenius expression is commonly employed in the field of microbial growth and it is easy find the relevant data in the literature. Consequently, in this Thesis, the Arrhenius formula was used to describe both the activation and deactivation effect of temperature.

The effect of light distribution on microbial growth is commonly expressed with the Beer-lambert law:

$$I(l, X) = I_0 \exp(-\sigma X l) \qquad \text{Eq. 5}$$

As it can be seen from the expression, the law assumes that the light irradiance at the external surface of an algal system declines as the light travels through the culture broth due to growing biomass. The Beer-lambert law can be applied when culture is well-mixed to ensure the cells do not scatter the light and system is isotropic.

Béchet et al. (2013) proposed alternative expressions which account for light scattering:

$$I(l) = I_0 \exp\left(-\frac{k_1 X l}{k_2 + X}\right) \qquad \text{Eq. 6}$$

$$I(l) = I_0 \exp\left(-\frac{k_1 X}{k_2 + X} l\right) \qquad \text{Eq. 7}$$

$$I(l) = I_0 \exp(-(k_1 + k_2 X_P) X l) \qquad \text{Eq. 8}$$

We assume that all our cultures are well-mixed and since this expression includes two parameters that need to be estimated while Beer-lambert law only include one, we utilized Beer-lambert formula to account for the effect of light intensity on both microalgae growth and lipid accumulation. A more detailed and comprehensive explanation for modelling effect of temperature and light irradiance can be found in Béchet et al. (2013).

2.5.3. Parameter estimation and optimization of the process

In order to ensure the best fit of the model to experimental results, estimation of kinetic parameters of dynamic models, which cannot be determined directly, is essential. Parameter estimation can be done through two different methodologies: (i) by globally minimizing an objective function which measures the quality of the fit and (ii) by optimizing a cost function such as maximum likelihood. The first parameter estimation methodology, minimizing an objective function, is widely employed in the field of microbial growth (Bitaubé Pérez et al., 2008, Vlysidis et al., 2011, Adesanya et al., 2014, Figueroa-Torres et al., 2017), and therefore it was selected and implemented throughout this Thesis.

Genetic algorithm (GA) and simulated annealing (SA) are global optimization methods that have been commonly employed to solve optimization problems. GA is an adaptive search method that is designed to find near-optimal solutions of large-scale optimization problems with multiple local maxima while SA algorithm is a general purpose optimization technique.

Simulated annealing and sequential quadratic programming (SQP) (Fmincon in MATLAB) optimization methodologies have been extensively employed throughout this Thesis (Chapters 3-5). Simulated annealing, a stochastic optimization algorithm, originally develop by (Kirkpatrick et al., 1983) to define global optimum of a given problem in large parameter subspace. The methodology is based physical annealing of metals which is in molten state at high temperatures and it reaches different states of minimum energy when cooled down. Depending on the cooling temperature, the metal can take two forms: (i) crystalline structure when cooled down slowly and (ii) metastable state with higher energy compare to crystalline structure when cooled down quickly. In an optimization problem, the crystalline structure represents the global optimum while metastable states represent local optima (Kirkpatrick et al., 1983). The controlling parameters for the simulated annealing algorithm that need to carefully selected are starting temperature, acceptance and convergence criterion, cooling rate and the number of iterations applied for every temperature step.

Initially, a feasible set of initial parameters, starting temperature and cooling rate needs to be selected. Subsequently, the simulation sets new set of parameters value with random moves which modify the initial guess and the value of the objective function is then calculated and

comparison is made compare to previous parameter values. If the new set of parameters meets the acceptance criterion, the old set is replaced the old set and a new iteration starts until reaching convergence criterion.

In this work, a feasible set of initial parameters are selected and the objective function is calculated based on this initial guesses. Annealing temperature was set 100 ºC and cooling rate was set 1%. Bounds of the parameters were set according experimental behaviour of the process and existing literature. The entire simulated annealing procedure was repeated several times starting from different initial conditions increase chance of defining a set of solution at or very close to the global optimum. The SA pseudo-code (Şendrescu and Selişteanu, 2015) is as follows:

1. generate initial solution θ_i

2. initialize I_{max} and T

3. for k=1 to I_{max} do

4. while stopping criteria not met do

5. compute $\theta_K \in N(\theta_i)$ (neighbour to current solution)

6. compute $\Delta j = J(\theta_K) - J(\theta_i)$ and generate r (random number)

7. if $\Delta j<0$ or $r<\exp(-\Delta j/T)$ then $\theta_i = \theta_K$

8. end while

9. Reduce T

10. end for

The results from the simulated annealing was then fed to a deterministic optimization algorithm (Fmincon in MATLAB) to check if the solutions from the simulated annealing could be improved. The coupled simulated annealing and SQP algorithm was then utilized to define optimal growth conditions for maximum biomass and lipid productivities. Similar to the parameter estimation methodology, a set of initial concentration was set and bounds were set according to

experimental behaviour of the process. The coupled algorithm was run several times to improve chance of obtaining global optimum.

Chapter 3

Production of Lipid-Based Fuels and Chemicals from Microalgae: An Integrated Experimental and Model-based Optimization Study

3.1. Preface

Microalgal oil bodies contain cytosolic and/or plastidic lipid bodies in the form of neutral lipid Triacylglycerol (TAG). These lipid bodies can be used directly or can be processed into biolubricants, surfactants, nutritional lipids like omega-3 fatty acids, and more importantly for this work, liquid fuels and gas. Abiotic stress such as the deprivation of nutrients nitrogen (N) and phosphorus (P), and stress caused by light intensity and temperature have been employed to trigger accumulation of algal lipid bodies (Yeesang and Cheirsilp, 2011, Spijkerman et al., 2011, Ruangsomboon, 2012, Yoshimura et al., 2013). Although abiotic stress has been exploited experimentally, computational exploration of stresses is yet to be employed.

As reviewed in chapter 2, the aim of kinetic models is to precisely represent the interactions between growth-limiting factors as well as cellular components. Kinetic models allow a deeper understanding of the cell behaviour, biomass growth, lipid accumulation, and provide useful knowledge for the robust design, control and scale-up of microalgal oil production, which can help to bring this important technology closer to commercialisation and industrial applicability.

In this regard, integrated computational and experimental approaches are valuable tools to get insights on biomass growth, lipid accumulation and interactions between growth limiting factors. Additionally, a carefully constructed and experimentally validated kinetic model, which is predictive over a wide range of experimental conditions, can then be used in an optimization study to maximise cellular productivities such as biomass and lipid productivity. For this reason, integrated experimental and theoretical studies to model and experimentally validate changes in microalgal cell compositions are an important tool to predict improvements to oil productivity.

Single substrate and multiple substrate models are widely employed to express the effects of one or multiple growth limiting factors, respectively, on biomass growth and lipid accumulation (Kwon et al., 2013, Bitaubé Pérez et al., 2008, Bougaran et al., 2010, Yoo et al., 2014). Although the aforementioned models are good enough to predict simultaneous and antagonistic effects of growth media composition and environmental factors they are not able to precisely predict both biomass growth and lipid accumulation individually due to the assumption made in these modelling studies, the biomass growth and lipid accumulation are considered as a single variable.

In order to ultimately achieve a positive energy balance for a cost-efficient and sustainable scaled-up biodiesel production, the objective of the work described in this chapter is to: 1) experimentally quantify the impact of varying starting substrate (acetate) and nutrient (N) composition of the growth medium on the system behaviour and 2) based on the experimental observations to develop a kinetic model which considers biomass growth and lipid accumulation as two individual variables to take advantage of abiotic stress towards maximizing lipid accumulation..

Experiments have shown that both microalgae growth and lipid accumulation production can be simultaneously and antagonistically affected by two or more nutrients and environmental variables, such as carbon and nutrient concentrations, light intensity and pH. Based on experimental observations and existing literature (Lee et al., 2015, Flynn, 2003), a detailed kinetic model has been constructed considering the effects of four different growth-promoting resources: acetate (carbon substrate), nitrogen, light intensity and pH to describe both microalgal biomass growth and lipid accumulation.

The results and discussion of this study are presented in the paper that follows, where the effect of varying nitrogen and acetate (carbon source) concentrations on biomass growth and lipid accumulation is evaluated in detail along with the development of the kinetic modelling framework. In order to take advantage of abiotic stress biomass growth and lipid accumulation, are considered as two separate state variables. The parameter estimation problem was solved using an in-house developed stochastic optimisation algorithm (Simulated Annealing), coupled with a non-linear programming (NLP)–based deterministic optimization algorithm. The procedure of estimation of kinetic parameter values is also explained along with the optimisation

study to obtain maximum lipid productivity. K.T. and J.K.P. contemplated and supervised the research, I.S.F. helped with the fitting and optimization coding in MATLAB and M.B. designed the research plans, performed the research, analysed data and wrote the manuscripts.

3.3. Publication 1

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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.

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Keywords: *Chlamydomonas reinhardtii*, Biofuels, Kinetic modelling, Microalgal oil, Nitrogen starvation, Acetate utilization.

Nomenclature

TAG	Triacylglycerol
TAP	Tris-acetate-phosphate
DCW	Dry cell weight
N	Nitrogen
P	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

1. Introduction

Fossil fuels provide a non-renewable form of energy that is also finite (Brennan and Owende, 2010, Hoel and Kverndokk, 1996). The use of non-renewable resources negatively impacts on the environment since it leads to the production of harmful greenhouse gas (GHG) emissions (Chiari and Zecca, 2011). On the contrary, renewable forms of energy sources such as solar and wind energy as well as biomass, are environmentally sustainable (Efroymson and Dale, 2015). 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 (Ahmad et al., 2011). Biodiesel production is a well-established platform (Demirbas and Fatih Demirbas, 2011), 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 (Chisti, 2007). 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 (Bajhaiya et al., 2016, Converti et al., 2009). 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 (Driver et al., 2014).

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 (Georgianna and Mayfield, 2012), 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 fertiliser), particularly for marine or wastewater cultivated microalgae (Pittman et al., 2011), and no competition for agricultural land and simple

growing needs (light, N, P, potassium (K) and CO_2) (Dragone et al., 2010, Borowitzka, 1999). Although microalgal oil has an immense potential in biotechnological applications, metabolic productivity needs to be enhanced to realise economic viability. Strain development by genetic manipulation, mutagenesis or natural selection is one approach that is being actively evaluated (Goncalves et al., 2015). Alternatively, cultivation conditions and metabolic productivity can be optimised 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 (Griffiths and Harrison, 2009). 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 (Béchet et al., 2013, Jørgensen, 1976, Bernard et al., 2016). 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 (Chen and Johns, 1994, Converti et al., 2009, Breuer et al., 2015). 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 (1949) 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 (1968) 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 Grima et al. (1994), the effects of one substrate (S) and of pH were investigated by Zhang et al. (1999), and the effect of temperature was explored by Bernard and Rémond (2012).

The effect of multiple nutrient concentrations can be examined through the use of two other frameworks; the threshold and the multiplicative models (Lee et al., 2015). 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. (2011) for the investigation of the effects of substrate and of P concentration, while the multiplicative model was used by Bernard (2011) 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 (Kovárová-Kovar and Egli, 1998).

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* (Bajhaiya et al., 2016, Miller et al., 2010, Siaut et al., 2011). 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

Chlamydomonas 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 (Bajhaiya et al., 2016). Preculture of the strain was carried out in an environmentally-controlled incubation room at 25ºC, using 250 mL conical flasks containing 150 mL of Tris-acetate-phosphate (TAP) medium (Harris, 1989) (TAP constituents are given in Table S1) on an orbital shaker at 120 rpm for 7-10 days. A 4ft long 20W high power led T8 tube light was used for illumination at a constant $125\ \mu\mathrm{E}\mathrm{m}^{-2}\mathrm{s}^{-1}$ 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 aparatus. Once the sample was placed, the following specifications were defined: cell diameter min 1.0 micron and max 1000micron, 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 70ºC 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 -20ºC for quantification of specific metabolites. All data was statistically analyzed by one-way ANOVA using Tukey post-hoc test performed using Prism v.6.04 (GraphPad).

2.2. Metabolite Analysis

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 300x7.7 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, 1956). 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 50ºC, while the detection wavelength was fixed at 210 nm. Filtration through 0.45 µm filter membranes was undertaken for the sample preparation.

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.

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 ºC (McNichol et al., 2012). Following the oil extraction performed through the use of Soxtec 1043, the extracted lipids were dried at 100 ºC 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 (Bekirogullari et al., 2015). To account for substrate inhibition on the transient cell behaviour, a modified Monod equation, the Haldane equation, is extensively applied (Andrews, 1968, Economou et al., 2011, Ogbonna et al., 1995):

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

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 (James et al., 2011, Tevatia et al., 2012). Additionally, light intensity plays a crucial role on microalgae growth and lipid accumulation (Grima et al., 1994, Jeon et al., 2005). 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. (2011), were employed here to describe the specific (oil-free) biomass growth and the lipid accumulation rate. Furthermore, the Aiba model (Aiba, 1982, Zhang et al., 2015) was taken into consideration for the simulation of the effect of light intensity as a pseudo-substrate.

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 (Béchet et al., 2013):

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

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 (Béchet et al., 2013).

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:

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

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

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

The substrate consumption rate can be calculated through a mass conservation equation (Vlysidis et al., 2011):

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

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 (Zhang et al., 1999):

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

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:

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

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

Here k_1 and k_2 are kinetic constants, K_{GAS}, K_{FAS} are substrate and K_{GAN}, K_{FAN} nitrogen saturation constants; $\mathrm{K_{iGAN}}$ 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 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 (Zhang et al., 1999):

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

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 (Eq. 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 (Vlysidis et al., 2011):

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

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, $\varepsilon=\left(C_{k,l,m}^{pred}(kk) - C_{k,l,m}^{exp}\right)$. Here the weights were set to $W_{k,l,m}$ = 1/ $C_{k,l,m}^{exp}$, where $C_{k,l,m}^{pred}$ are the predicted state variables (computed by Eq. 5 to 11) and $C_{k,l,m}^{exp}$ the experimentally obtained ones.

The estimation problem was solved using an in-house developed stochastic algorithm, based on Simulated Annealing (SA) (Vlysidis et al, 2011), 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 Figure 1.

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	(Fouchard et al., 2009)
K_{XS}	0.050 g S L^{-1}	0.000	0.010	0.028 – 2.295	C.Reinhardtii	(Chen and Johns, 1994, Zhang et al., 1999)
K_{iXS}	9.923 g S L^{-1}	0.130	0.013	0.1557 – 1.76	C.Reinhardtii	(Zhang et al., 1999, Chen and Johns, 1996)
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	(Zhang et al., 1999, Chen and Johns, 1996)
$Y_{X/N}$	6.883 g X g N^{-1}	0.183	0.027	18.9		(Economou et al., 2011)
K_H	0.879 L $g\ S^{-1}$	0.018	0.020	0.8759	C.Reinhardtii	(Zhang et al., 1999)
$Y_{L/S}$	0.064 g X g S^{-1}	0.005	0.074	0.24	C.Prototheocoide	(O'Grady and Morgan, 2010)
K_{XI}	19.519 µ E $m^{-2}s^{-1}$	0.731	0.037	81.38	C.Reinhardtii	(Fouchard et al., 2009)
K_{iXI}	2053.924 µ E $m^{-2}s^{-1}$	33.755	0.016	2500	C.Reinhardtii	(Fouchard et al., 2009)
K_{LI}	15.023 µ E $m^{-2}s^{-1}$	0.461	0.031	this study		
K_{iLI}	2152.918 µ E $m^{-2}s^{-1}$	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

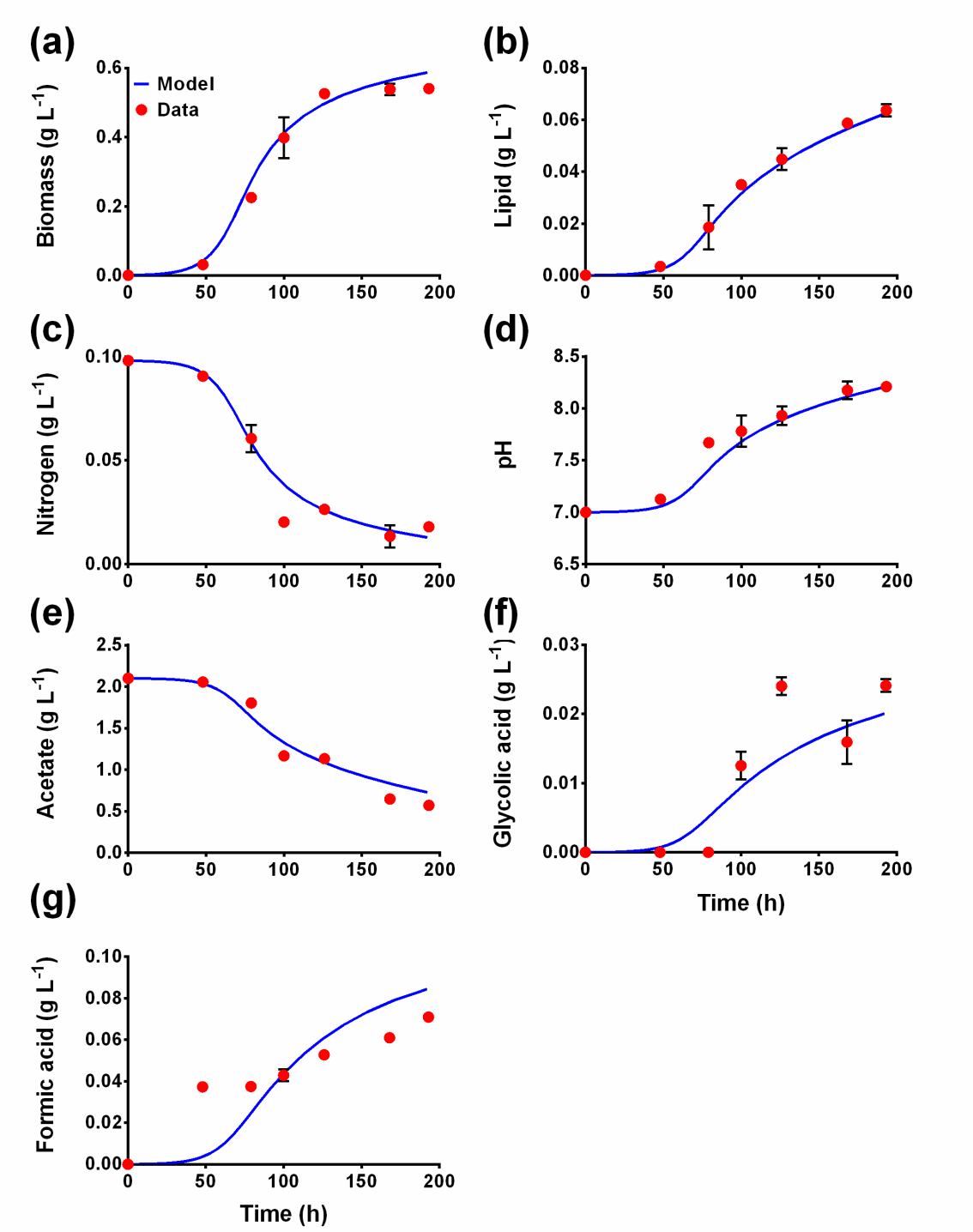

Fig. 1. Fitting of model predictions (lines) to experimental data (symbols with error bars) for: (a) biomass, (b) lipid concentration, (c) substrate (acetate) consumption, (d) N consumption, (e) pH change, (f) oxalic acid production, (g) glycolic acid production and (h) formic acid production, using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

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.

4.1. Experimental Results

Measurements of microalgal growth, as determined by biomass concentration, and lipid accumulation (Fig. 2 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. 2a). 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 (Lowrey et al., 2016). 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 (Johnson and Alric, 2013, Chapman et al., 2015). 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 (Johnson and Alric, 2013). 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 (Ramanan et al., 2013). 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. 2b) 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. (2013) 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 (Chapman et al., 2015).

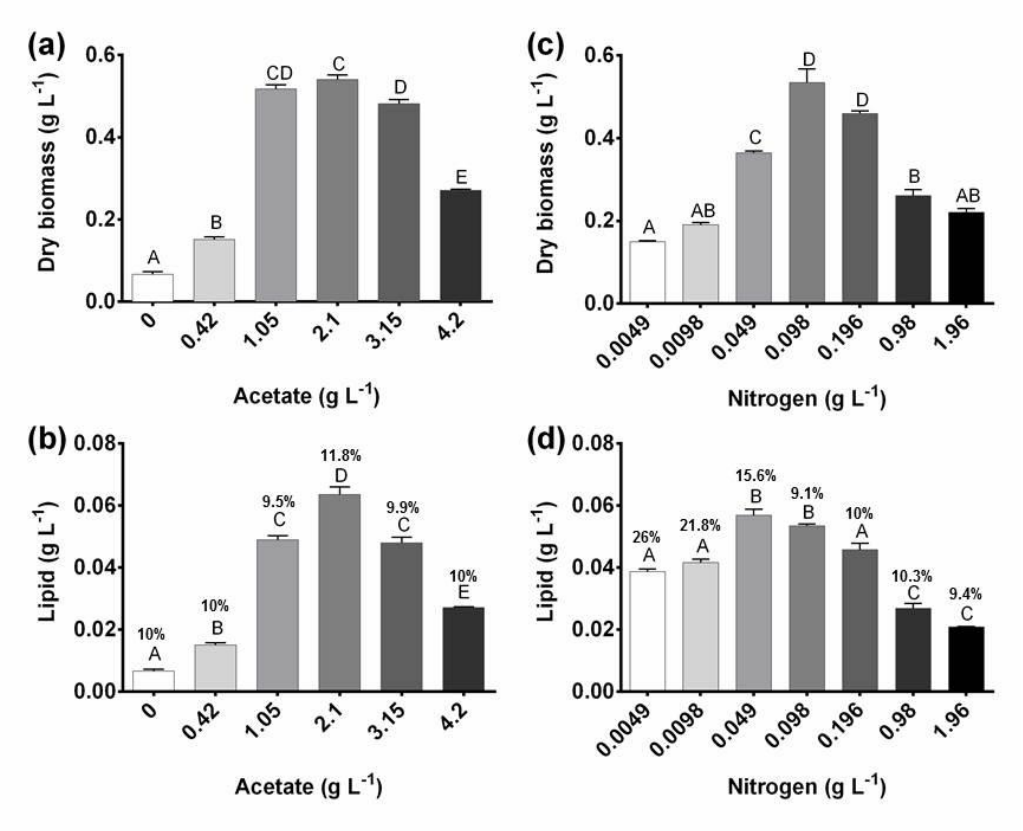

Fig. 2. The effect of carbon substrate (acetate) (a, b) and nutrient (nitrogen, N) (c, d) concentrations on dry weight biomass concentration (a, c) and total lipid concentration (b, d) after photo-heterotrophic growth for 8 d. The starting N concentration for the acetate range treatment experiments was 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 lowercase 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.

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. 2c). 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. 2c). 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%, 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 (Bajhaiya et al., 2016). 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 Figure 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 = 25ºC and the light illumination (I_0) is considered constant and equal to $125\ \mu\mathrm{Em}^{-2}\mathrm{s}^{-1}$. 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 behavior of bench-scale batch experiments.

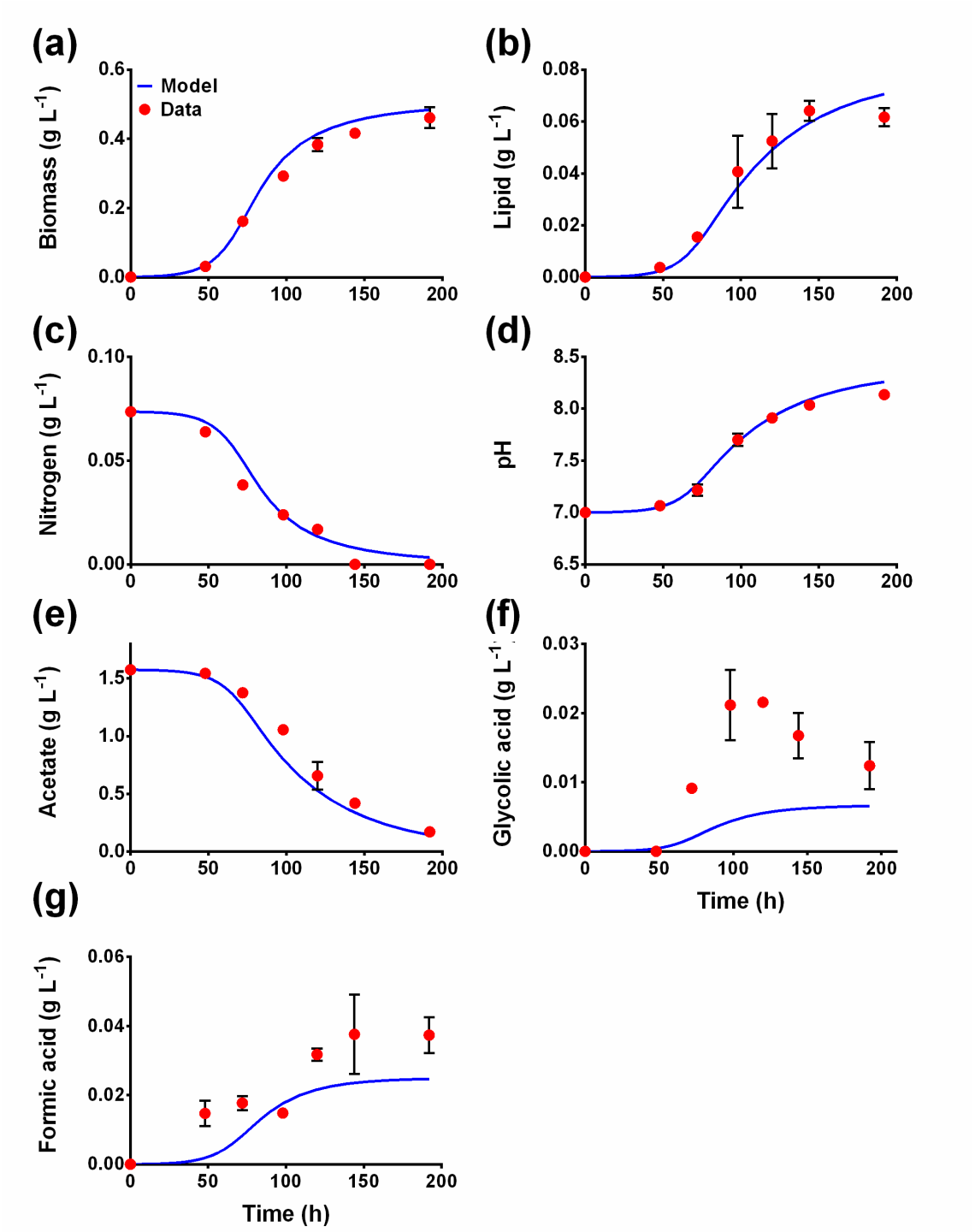

Fig. 3. Validation of model predictions (lines) by experimental data (symbols with error bars) for: (a) biomass, (b) lipid concentration, (c) substrate (acetate) consumption, (d) N consumption, (e) pH change, (f) oxalic acid production, (g) glycolic acid production and (h) formic acid production, using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

4.3. 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:

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

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

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

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

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

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 Figure 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 (Adesanya et al., 2014, Fouchard et al., 2009, Lee et al., 2015).

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

Initial Conditions	Base Case Runs	Optimisation Runs		
Biomass Concentration	$0.001\ \text{g}\ L^{-1}$	$0.005\ \text{g}\ L^{-1}$		
Acetate Concentration	$2.1\ \text{g}\ L^{-1}$	$2.1906\ \text{g}\ L^{-1}$		
Nitrogen Concentration	$0.098\ \text{g}\ L^{-1}$	$0.0742\text{g}\ L^{-1}$		
Resulted Measures	**Base Case Results**	**Optimized Results**	**Change**	**Experimental Results**
Lipid Concentration	$62.4\ \text{mg}\ L^{-1}$	$82.9\ \text{mg}\ L^{-1}$	$+20.5\ \text{mg}\ L^{-1}$	$84.7\ \text{mg}\ L^{-1}$
Lipid Productivity	$7.8\ \text{mg}\ L^{-1}\ d^{-1}$	$10.3625\text{mg}\ L^{-1}\ d^{-1}$	$+32.85\%$	$10.5875\ \text{mg}\ L^{-1}\ d^{-1}$
Biomass Concentration	$586.8\ \text{mg}\ L^{-1}$	$498.4\ \text{mg}\ L^{-1}$	$-88.4\ \text{mg}\ L^{-1}$	$458.6\ \text{mg}\ L^{-1}$
Biomass Productivity	$73.85\ \text{mg}\ L^{-1}\ d^{-1}$	$62.3\ \text{mg}\ L^{-1}\ d^{-1}$	-15.65%	$57.325\ \text{mg}\ L^{-1}\ d^{-1}$

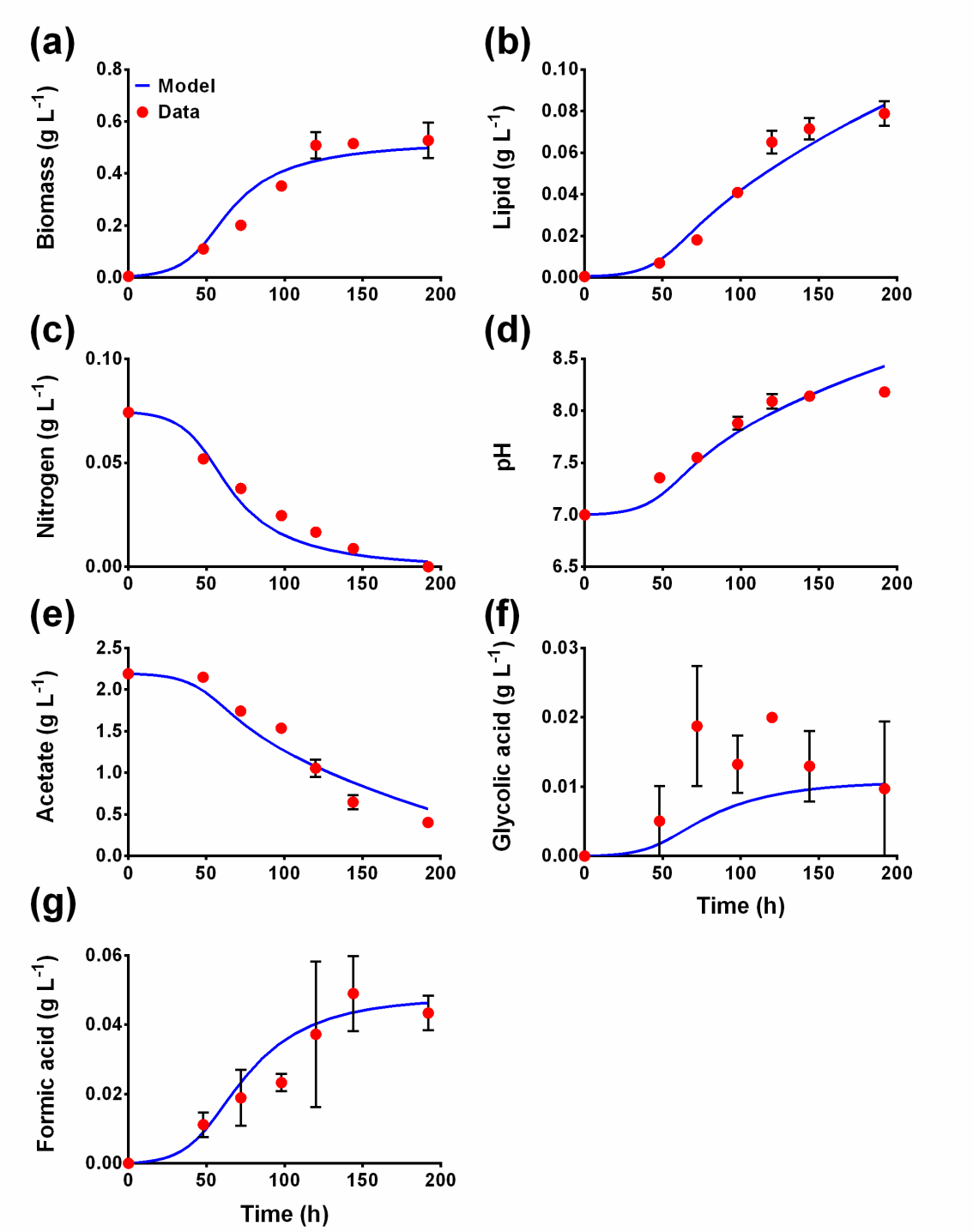

Fig. 4. Optimization of model predictions (lines) by experimental data (symbols with error bars) for: (a) biomass, (b) lipid concentration, (c) substrate (acetate) consumption, (d) N consumption, (e) pH change, (f) oxalic acid production, (g) glycolic acid production and (h) formic acid production, using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

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 behavior 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 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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References

Adesanya, V. O., Davey, M. P., Scott, S. A. & Smith, A. G. 2014. Kinetic modelling of growth and storage molecule production in microalgae under mixotrophic and autotrophic conditions. *Bioresource Technology,* 157**,** 293-304.

Ahmad, A. L., Yasin, N. H. M., Derek, C. J. C. & Lim, J. K. 2011. Microalgae as a sustainable energy source for biodiesel production: A review. *Renewable and Sustainable Energy Reviews,* 15**,** 584-593.

Aiba, S. 1982. Growth kinetics of photosynthetic microorganisms. *Microbial Reactions.* Berlin, Heidelberg: Springer Berlin Heidelberg.

Andrews, J. F. 1968. A mathematical model for the continuous culture of microorganisms utilizing inhibitory substrates. *Biotechnology and Bioengineering,* 10**,** 707-723.

Bajhaiya, A. K., Dean, A. P., Driver, T., Trivedi, D. K., Rattray, N. J. W., Allwood, J. W., Goodacre, R. & Pittman, J. K. 2016. High-throughput metabolic screening of microalgae genetic variation in response to nutrient limitation. *Metabolomics,* 12**,** 1-14.

Béchet, Q., Shilton, A. & Guieysse, B. 2013. Modeling the effects of light and temperature on algae growth: State of the art and critical assessment for productivity prediction during outdoor cultivation. *Biotechnology Advances,* 31**,** 1648-1663.

Bekirogullari, M., Pittman, J. & Theodoropoulos, C. 2015. Integrated Computational and Experimental Studies of Microalgal Production of Fuels and Chemicals. *In:* Krist V. Gernaey, J. K. H. & Rafiqul, G. (eds.) *Computer Aided Chemical Engineering.* Elsevier.

Bernard, O. 2011. Hurdles and challenges for modelling and control of microalgae for CO2 mitigation and biofuel production. *Journal of Process Control,* 21**,** 1378-1389.

Bernard, O., Mairet, F. & Chachuat, B. 2016. Modelling of Microalgae Culture Systems with Applications to Control and Optimization. *In:* Posten, C. & Feng Chen, S. (eds.) *Microalgae Biotechnology.* Cham: Springer International Publishing.

Bernard, O. & Rémond, B. 2012. Validation of a simple model accounting for light and temperature effect on microalgal growth. *Bioresource Technology,* 123**,** 520-527.

Borowitzka, M. A. 1999. Commercial production of microalgae: ponds, tanks, tubes and fermenters. *Journal of Biotechnology,* 70**,** 313-321.

Brennan, L. & Owende, P. 2010. Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews,* 14**,** 557-577.

Breuer, G., Lamers, P. P., Janssen, M., Wijffels, R. H. & Martens, D. E. 2015. Opportunities to improve the areal oil productivity of microalgae. *Bioresource Technology,* 186**,** 294-302.

Chapman, S. P., Paget, C. M., Johnson, G. N. & Schwartz, J.-M. 2015. Flux balance analysis reveals acetate metabolism modulates cyclic electron flow and alternative glycolytic pathways in Chlamydomonas reinhardtii. *Frontiers in Plant Science,* 6**,** 474.

Chen, F. & Johns, M. R. 1994. Substrate inhibition of Chlamydomonas reinhardtii by acetate in heterotrophic culture. *Process Biochemistry,* 29**,** 245-252.

Chen, F. & Johns, M. R. 1996. Heterotrophic growth of Chlamydomonas reinhardtii on acetate in chemostat culture. *Process Biochemistry,* 31**,** 601-604.

Chiari, L. & Zecca, A. 2011. Constraints of fossil fuels depletion on global warming projections. *Energy Policy,* 39**,** 5026-5034.

Chisti, Y. 2007. Biodiesel from microalgae. *Biotechnology Advances,* 25**,** 294-306.

Converti, A., Casazza, A. A., Ortiz, E. Y., Perego, P. & Del Borghi, M. 2009. Effect of temperature and nitrogen concentration on the growth and lipid content of Nannochloropsis oculata and Chlorella vulgaris for biodiesel production. *Chemical Engineering and Processing: Process Intensification,* 48**,** 1146-1151.

Demirbas, A. & Fatih Demirbas, M. 2011. Importance of algae oil as a source of biodiesel. *Energy Conversion and Management,* 52**,** 163-170.

Dragone, G., Fernandes, B. D., Vicente, A. A. & Teixeira, J. A. 2010. Third generation biofuels from microalgae.

Driver, T., Bajhaiya, A. & Pittman, J. K. 2014. Potential of Bioenergy Production from Microalgae. *Current Sustainable/Renewable Energy Reports,* 1**,** 94-103.

Economou, C. N., Aggelis, G., Pavlou, S. & Vayenas, D. V. 2011. Modeling of single-cell oil production under nitrogen-limited and substrate inhibition conditions. *Biotechnology and Bioengineering,* 108**,** 1049-1055.

Efroymson, R. A. & Dale, V. H. 2015. Environmental indicators for sustainable production of algal biofuels. *Ecological Indicators,* 49**,** 1-13.

Fouchard, S., Pruvost, J., Degrenne, B., Titica, M. & Legrand, J. 2009. Kinetic modeling of light limitation and sulfur deprivation effects in the induction of hydrogen production with Chlamydomonas reinhardtii: Part I. Model development and parameter identification. *Biotechnology and Bioengineering,* 102**,** 232-245.

Georgianna, D. R. & Mayfield, S. P. 2012. Exploiting diversity and synthetic biology for the production of algal biofuels. *Nature,* 488**,** 329-335.

Goncalves, E. C., Wilkie, A. C., Kirst, M. & Rathinasabapathi, B. 2015. Metabolic regulation of triacylglycerol accumulation in the green algae: identification of potential targets for engineering to improve oil yield. *Plant Biotechnology Journal***,** n/a-n/a.

Griffiths, M. J. & Harrison, S. T. L. 2009. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. *Journal of Applied Phycology,* 21**,** 493-507.

Grima, E. M., Camacho, F. G., Pérez, J. a. S., Sevilla, J. M. F., Fernández, F. G. A. & Gómez, A. C. 1994. A mathematical model of microalgal growth in light-limited chemostat culture. *Journal of Chemical Technology & Biotechnology,* 61**,** 167-173.

Harris, E. H. 1989. *The Chlamydomonas Sourcebook: A Comprehensive Guide to Biology and Laboratory Use,* San Diego, Academic Press, Inc.

Hoel, M. & Kverndokk, S. 1996. Depletion of fossil fuels and the impacts of global warming. *Resource and Energy Economics,* 18**,** 115-136.

James, G. O., Hocart, C. H., Hillier, W., Chen, H., Kordbacheh, F., Price, G. D. & Djordjevic, M. A. 2011. Fatty acid profiling of Chlamydomonas reinhardtii under nitrogen deprivation. *Bioresource Technology,* 102**,** 3343-3351.

Jeon, Y.-C., Cho, C.-W. & Yun, Y.-S. 2005. Measurement of microalgal photosynthetic activity depending on light intensity and quality. *Biochemical Engineering Journal,* 27**,** 127-131.

Johnson, X. & Alric, J. 2013. Central Carbon Metabolism and Electron Transport in Chlamydomonas reinhardtii: Metabolic Constraints for Carbon Partitioning between Oil and Starch. *Eukaryotic cell,* 12**,** 776-793.

Jørgensen, S. E. 1976. A eutrophication model for a lake. *Ecological Modelling,* 2**,** 147-165.

Kovárová-Kovar, K. & Egli, T. 1998. Growth Kinetics of Suspended Microbial Cells: From Single-Substrate-Controlled Growth to Mixed-Substrate Kinetics. *Microbiology and Molecular Biology Reviews,* 62**,** 646-666.

Lee, E., Jalalizadeh, M. & Zhang, Q. 2015. Growth kinetic models for microalgae cultivation: A review. *Algal Research,* 12**,** 497-512.

Lowrey, J., Armenta, R. E. & Brooks, M. S. 2016. Nutrient and media recycling in heterotrophic microalgae cultures. *Applied Microbiology and Biotechnology,* 100**,** 1061-1075.

Miller, R., Wu, G., Deshpande, R. R., Vieler, A., Gärtner, K., Li, X., Moellering, E. R., Zäuner, S., Cornish, A. J. & Liu, B. 2010. Changes in transcript abundance in Chlamydomonas reinhardtii following nitrogen deprivation predict diversion of metabolism. *Plant physiology,* 154**,** 1737-1752.

Monod, J. 1949. The Growth of Bacterial Cultures. *Annual Review of Microbiology,* 3**,** 371-394.

O'grady, J. & Morgan, J. A. 2010. Heterotrophic growth and lipid production of Chlorella protothecoides on glycerol. *Bioprocess and Biosystems Engineering,* 34**,** 121-125.

Ogbonna, J. C., Yada, H. & Tanaka, H. 1995. Kinetic study on light-limited batch cultivation of photosynthetic cells. *Journal of Fermentation and Bioengineering,* 80**,** 259-264.

Pittman, J. K., Dean, A. P. & Osundeko, O. 2011. The potential of sustainable algal biofuel production using wastewater resources. *Bioresource Technology,* 102**,** 17-25.

Ramanan, R., Kim, B.-H., Cho, D.-H., Ko, S.-R., Oh, H.-M. & Kim, H.-S. 2013. Lipid droplet synthesis is limited by acetate availability in starchless mutant of Chlamydomonas reinhardtii. *FEBS Letters,* 587**,** 370-377.

Siaut, M., Cuiné, S., Cagnon, C., Fessler, B., Nguyen, M., Carrier, P., Beyly, A., Beisson, F., Triantaphylidès, C., Li-Beisson, Y. & Peltier, G. 2011. Oil accumulation in the model green alga Chlamydomonas reinhardtii: characterization, variability between common laboratory strains and relationship with starch reserves. *BMC Biotechnology,* 11**,** 1-15.

Spijkerman, E., De Castro, F. & Gaedke, U. 2011. Independent Colimitation for Carbon Dioxide and Inorganic Phosphorus. *PLoS ONE,* 6**,** e28219.

Tevatia, R., Demirel, Y. & Blum, P. 2012. Kinetic modeling of photoautotropic growth and neutral lipid accumulation in terms of ammonium concentration in Chlamydomonas reinhardtii. *Bioresource Technology,* 119**,** 419-424.

Vlysidis, A., Binns, M., Webb, C. & Theodoropoulos, C. 2011. Glycerol utilisation for the production of chemicals: Conversion to succinic acid, a combined experimental and computational study. *Biochemical Engineering Journal,* 58–59**,** 1-11.

Zhang, D., Dechatiwongse, P., Del Rio-Chanona, E. A., Maitland, G. C., Hellgardt, K. & Vassiliadis, V. S. 2015. Modelling of light and temperature influences on cyanobacterial growth and biohydrogen production. *Algal Research,* 9**,** 263-274.

Zhang, X.-W., Chen, F. & Johns, M. R. 1999. Kinetic models for heterotrophic growth of Chlamydomonas reinhardtii in batch and fed-batch cultures. *Process Biochemistry,* 35**,** 385-389.

Supplementary Table:

Table S1: Concentration of each constituent in the TAP medium.

Constituent	mg L^{-1}	Constituent	mg L^{-1}
NH_4Cl	187.5	$MnCl_2 \cdot 4H_2O$	5.06
$MgS0_4 \cdot 7H_2O$	50	$CoCl_2 \cdot 6H_2O$	1.61
$CaCl_2 \cdot 2H_2O$	2.5	$CuSO_4 \cdot 5H_2O$	1.57
K_2HPO_4	0.108	$(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$	1.10
KH_2PO_4	0.054	$FeSO_4 \cdot 4H_2O$	4.99
$EDTA, disodium\ salt$	50	$Glacial\ acetic\ acid$	1050
$ZnSO_4 \cdot 7H_2O$	22	$Tris$	2420
H_3BO_3	11.4		

Supplementary Figure:

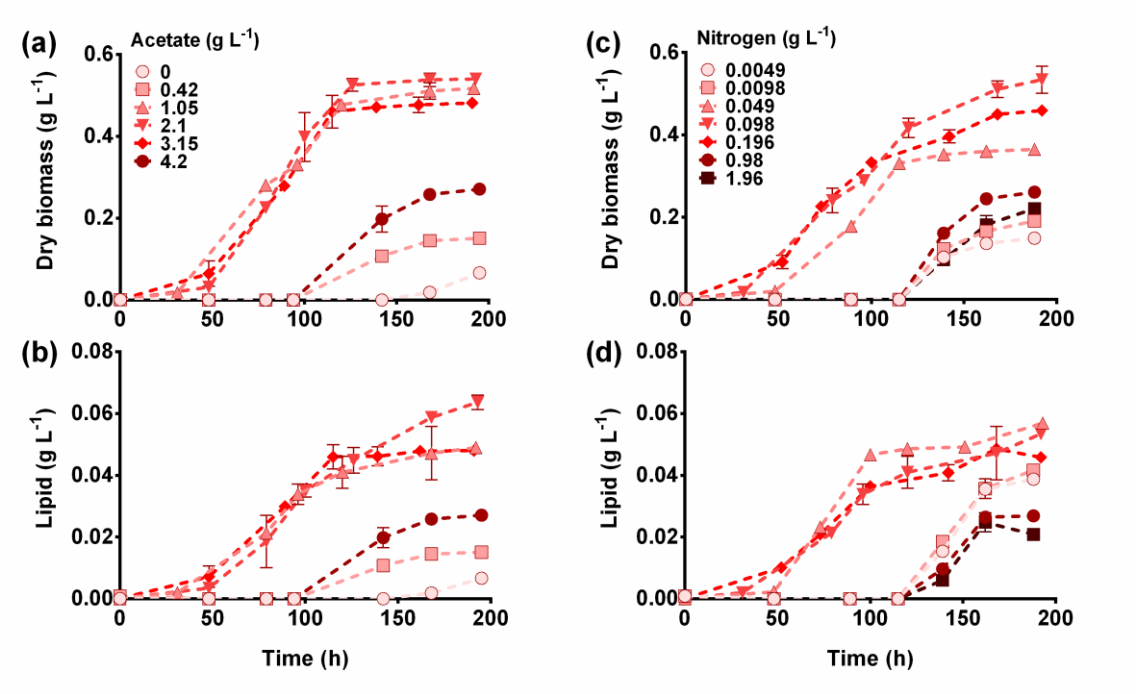

Fig. S1. The effect of carbon substrate (acetate) (a, b) and nutrient (nitrogen, N) (c, d) concentrations on dry weight biomass concentration (a, c) and total lipid concentration (b, d) over time during photo-heterotrophic growth for 8 d. The starting N concentration for the acetate range treatment experiments 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.

Supplementary Information

Appendix A: Fitting of model predictions to experimental data given as experiment 2 in section 3.3. parameter estimation

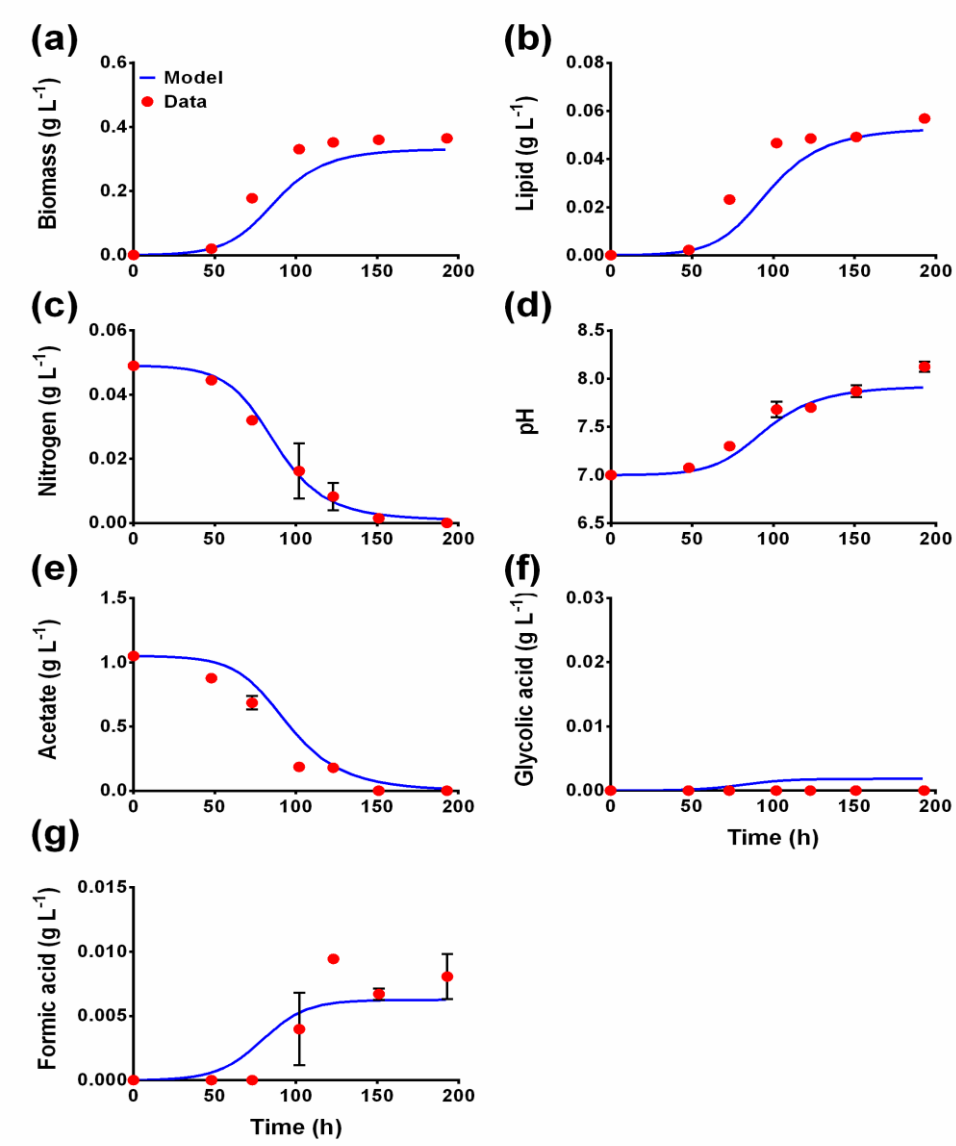

Fig. A.1 Fitting of model predictions (lines) to experimental data (symbols with error bars) for: (a) biomass, (b) lipid concentration, (c) substrate (acetate) consumption, (d) N consumption, (e) pH change, (f) oxalic acid production, (g) glycolic acid production and (h) formic acid production, using 1.05 g L^{-1} acetate and 0.049 g L^{-1} N.

Appendix B: Upper and lower bounds of the estimated parameters used in section 3.3 parameters estimation problem

Table B.1 Upper and lower bounds of the estimated parameters

Parameters	Lower bounds	Upper bounds	Parameters	Lower bounds	Upper bounds
μ_{Xmax}	0.2	0.25	K_{XI}	10	100
K_{XS}	0.02	2	K_{iXI}	2000	2500
K_{iXS}	5	15	K_{LI}	10	50
K_{XN}	0.05	0.075	K_{iLI}	2000	2500
K_{iXN}	0.03	0.06	σ	10	50
q_{Lmax}	0.1	0.2	k_1	0.2	0.5
K_{LS}	5	10	K_{GAS}	1	2
K_{iLS}	0.075	0.15	K_{GAN}	5	25
K_{iNL}	350	600	K_{iGAN}	0.5	5
$Y_{X/S}$	0.7	15	k_2	1	2
$Y_{X/N}$	5	10	K_{FAS}	10	20
K_H	0.075	1	K_{FAN}	1	5
$Y_{L/S}$	0.05	0.1			

Appendix C: Upper and lower bounds of the estimated optimal growth conditions used in section 4.3 process optimization problem

The decision variables in the optimization problem were initial biomass concentration (X_0), substrate (acetate) (S_0) and nitrogen (N_0) concentrations. The upper and lower bounds of the optimization problem were set according to experimental behaviour of system.

Table C.1 Upper and lower bounds of the decision variables used in optimization problem

Parameters	Lower bounds	Upper bounds
$\boldsymbol{X_0}$	0.001	0.01
$\boldsymbol{S_0}$	0.1	3.15
$\boldsymbol{N_0}$	0.01	1

Appendix D: The basic assumptions for the general case of the model are:

- Microalgal growth takes place in a homogeneous reactor. Biomass is cultivated in a well-mixed environment containing acetate as substrate.
- The microalgal biomass growth depends on acetate and nitrogen concentrations and light intensity.
- Lipid production starts when nitrogen limitation occurs and carbon is in excess.
- Acetate is utilized for both biomass and lipid synthesis.
- Nitrogen is only consumed for fat-free biomass synthesis.

Appendix E. Sensitivity analysis

A sensitivity analysis was carried out for the kinetic model proposed in this work which consists of 25 parameters. The analysis was performed by calculating the sensitivity (Eq. (A.1)), for all 7 dynamic variables with respect to each parameter at eight different cultivation times (t=25h, 50h, 75h, 85h, 125h, 150h, 175h and 190h).

$$Sensitivity = abs\left(\frac{Prediction(t, P + \Delta P) - Prediction(t, P - \Delta P)}{2 * \Delta P}\right) \quad \text{Eq. A.1}$$

Sensitivity of the parameters was computed with a %10 change (ΔP) in parameters values. The sensitivity analysis results of 25 kinetic parameters are presented in Table E.1. The threshold for sensitivity was set to 0.01, meaning parameters with sensitivities lower than 0.01 were considered not-sensitive and sensitivities higher than 0.01 deemed to be sensitive and they are highlighted.

Table E.1. Sensitivity analysis results of the proposed model kinetic parameters.

			Sensitivity							
Parameter	**Value**	**Variable**	**25h**	**50h**	**75h**	**85h**	**125h**	**150h**	**175h**	**190h**
μ_{xmax}	0.2272	X	6.16E-02	8.56E-01	3.05E+00	2.80E+00	1.31E+00	9.58E-01	7.61E-01	6.76E-01
		L	1.92E-03	3.64E-02	1.67E-01	1.57E-01	6.36E-03	4.89E-02	8.52E-02	1.01E-01
		AA	7.19E-02	1.15E+00	4.69E+00	4.37E+00	9.90E-01	1.14E-01	8.16E-01	1.12E+00
		N	8.96E-03	1.24E-01	4.43E-01	4.06E-01	1.90E-01	1.39E-01	1.11E-01	9.82E-02
		pH	6.32E-02	1.01E+00	4.12E+00	3.84E+00	8.70E-01	1.00E-01	7.17E-01	9.89E-01
		GA	6.24E-04	1.10E-02	4.81E-02	5.25E-02	2.71E-02	1.14E-02	4.44E-04	6.12E-03
		FA	3.12E-03	5.42E-02	2.21E-01	2.32E-01	1.07E-01	4.51E-02	3.68E-03	1.49E-02
K_{XS}	0.0503	X	5.35E-03	7.47E-02	2.89E-01	2.65E-01	1.45E-01	1.21E-01	1.06E-01	1.02E-01
		L	1.66E-04	3.18E-03	1.54E-02	1.39E-02	2.22E-03	8.60E-03	1.35E-02	1.57E-02
		AA	6.24E-03	1.01E-01	4.38E-01	3.97E-01	6.37E-02	5.22E-02	1.38E-01	1.76E-01
		N	7.78E-04	1.08E-02	4.19E-02	3.85E-02	2.10E-02	1.76E-02	1.55E-02	1.48E-02
		pH	5.49E-03	8.83E-02	3.85E-01	3.49E-01	5.60E-02	4.59E-02	1.21E-01	1.55E-01
		GA	5.42E-05	9.60E-04	4.40E-03	4.73E-03	2.05E-03	2.62E-04	1.31E-03	2.09E-03
		FA	2.71E-04	4.74E-03	2.02E-02	2.08E-02	7.98E-03	1.25E-03	4.20E-03	6.69E-03
K_{iXS}	9.923	X	2.40E-04	3.29E-03	1.20E-02	1.05E-02	4.27E-03	2.99E-03	2.19E-03	1.89E-03
		L	7.47E-06	1.41E-04	6.75E-04	6.16E-04	6.69E-05	1.03E-04	2.13E-04	2.57E-04
		AA	2.80E-04	4.45E-03	1.87E-02	1.68E-02	3.95E-03	4.20E-04	1.85E-03	2.73E-03
		N	3.49E-05	4.78E-04	1.74E-03	1.52E-03	6.21E-04	4.34E-04	3.18E-04	2.75E-04
		pH	2.46E-04	3.91E-03	1.65E-02	1.47E-02	3.48E-03	3.69E-04	1.62E-03	2.40E-03
		GA	2.43E-06	4.27E-05	1.91E-04	2.05E-04	1.09E-04	5.77E-05	1.98E-05	4.03E-06
		FA	1.22E-05	2.11E-04	8.80E-04	9.04E-04	4.28E-04	2.26E-04	8.96E-05	3.80E-05
K_{XN}	0.0648	X	7.68E-02	1.08E+00	4.17E+00	3.85E+00	2.10E+00	1.70E+00	1.45E+00	1.34E+00
		L	2.39E-03	4.56E-02	2.21E-01	1.98E-01	3.57E-02	1.28E-01	1.95E-01	2.25E-01
		AA	8.96E-02	1.45E+00	6.29E+00	5.72E+00	8.73E-01	8.44E-01	2.06E+00	2.60E+00
		N	1.12E-02	1.56E-01	6.06E-01	5.59E-01	3.06E-01	2.47E-01	2.11E-01	1.95E-01
		pH	7.88E-02	1.27E+00	5.53E+00	5.02E+00	7.67E-01	7.42E-01	1.81E+00	2.29E+00
		GA	7.78E-04	1.38E-02	6.31E-02	6.80E-02	2.89E-02	3.30E-03	1.80E-02	2.85E-02
		FA	3.89E-03	6.81E-02	2.90E-01	2.99E-01	1.13E-01	1.63E-02	5.67E-02	9.05E-02
K_{iXN}	0.5004	X	2.93E-03	3.98E-02	1.37E-01	1.16E-01	4.04E-02	2.61E-02	1.78E-02	1.48E-02
		L	9.11E-05	1.71E-03	8.02E-03	7.38E-03	1.80E-03	2.75E-04	6.63E-04	1.01E-03
		AA	3.42E-03	5.38E-02	2.19E-01	1.94E-01	5.56E-02	2.21E-02	1.72E-03	5.71E-03
		N	4.25E-04	5.78E-03	2.00E-02	1.68E-02	5.88E-03	3.79E-03	2.58E-03	2.16E-03
		pH	3.00E-03	4.73E-02	1.92E-01	1.71E-01	4.89E-02	1.94E-02	1.51E-03	5.02E-03
		GA	2.97E-05	5.18E-04	2.27E-03	2.42E-03	1.40E-03	8.98E-04	5.46E-04	4.08E-04
		FA	1.49E-04	2.56E-03	1.04E-02	1.07E-02	5.49E-03	3.46E-03	2.13E-03	1.65E-03
q_{Lmax}	0.1205	X	1.44E-05	1.14E-03	2.01E-02	2.87E-02	3.41E-02	3.25E-02	2.66E-02	2.35E-02
		L	2.69E-03	2.29E-02	1.31E-01	1.97E-01	3.93E-01	4.69E-01	5.25E-01	5.50E-01
		AA	4.21E-02	3.59E-01	2.06E+00	3.11E+00	6.16E+00	7.37E+00	8.23E+00	8.62E+00
		N	2.09E-06	1.66E-04	2.92E-03	4.18E-03	4.95E-03	4.72E-03	3.86E-03	3.41E-03
		pH	3.70E-02	3.16E-01	1.81E+00	2.73E+00	5.42E+00	6.47E+00	7.23E+00	7.57E+00

		GA	3.40E-07	2.20E-05	8.94E-04	2.47E-03	1.71E-02	2.96E-02	4.29E-02	5.08E-02
		FA	4.11E-06	2.75E-04	9.07E-03	2.26E-02	1.24E-01	1.98E-01	2.70E-01	3.09E-01
K_{LS}	6.5544	X	3.57E-08	2.86E-06	5.48E-05	8.30E-05	1.18E-04	1.24E-04	1.06E-04	9.63E-05
		L	6.68E-06	5.84E-05	3.85E-04	6.33E-04	1.62E-03	2.16E-03	2.64E-03	2.90E-03
		AA	1.05E-04	9.16E-04	6.06E-03	9.96E-03	2.54E-02	3.38E-02	4.14E-02	4.55E-02
		N	5.19E-09	4.16E-07	7.96E-06	1.21E-05	1.72E-05	1.81E-05	1.54E-05	1.40E-05
		pH	9.19E-05	8.05E-04	5.32E-03	8.75E-03	2.23E-02	2.97E-02	3.64E-02	4.00E-02
		GA	8.43E-10	5.58E-08	2.51E-06	7.40E-06	6.20E-05	1.16E-04	1.80E-04	2.20E-04
		FA	1.02E-08	6.96E-07	2.53E-05	6.72E-05	4.45E-04	7.67E-04	1.11E-03	1.31E-03
K_{iLS}	0.1099	X	1.30E-05	1.02E-03	1.78E-02	2.51E-02	2.86E-02	2.66E-02	2.16E-02	1.90E-02
		L	2.43E-03	2.05E-02	1.14E-01	1.68E-01	3.12E-01	3.59E-01	3.89E-01	3.99E-01
		AA	3.80E-02	3.22E-01	1.79E+00	2.65E+00	4.90E+00	5.64E+00	6.09E+00	6.26E+00
		N	1.89E-06	1.49E-04	2.59E-03	3.65E-03	4.15E-03	3.86E-03	3.14E-03	2.76E-03
		pH	3.34E-02	2.83E-01	1.58E+00	2.33E+00	4.30E+00	4.96E+00	5.36E+00	5.50E+00
		GA	3.07E-07	1.97E-05	7.85E-04	2.14E-03	1.41E-02	2.39E-02	3.38E-02	3.96E-02
		FA	3.70E-06	2.47E-04	7.98E-03	1.97E-02	1.03E-01	1.61E-01	2.14E-01	2.44E-01
K_{iNL}	380.023	X	1.19E-12	9.23E-11	1.49E-09	2.00E-09	2.01E-09	1.72E-09	1.40E-09	1.20E-09
		L	2.21E-10	1.82E-09	8.82E-09	1.21E-08	1.88E-08	2.04E-08	2.12E-08	2.14E-08
		AA	3.46E-09	2.86E-08	1.39E-07	1.91E-07	2.95E-07	3.21E-07	3.33E-07	3.35E-07
		N	1.73E-13	1.34E-11	2.16E-10	2.91E-10	2.92E-10	2.50E-10	2.03E-10	1.74E-10
		pH	3.04E-09	2.51E-08	1.22E-07	1.68E-07	2.59E-07	2.82E-07	2.92E-07	2.95E-07
		GA	2.79E-14	1.76E-12	6.35E-11	1.64E-10	9.39E-10	1.51E-09	2.07E-09	2.39E-09
		FA	3.38E-13	2.21E-11	6.50E-10	1.52E-09	6.91E-09	1.03E-08	1.33E-08	1.49E-08
$Y_{X/S}$	1.4700	X	9.57E-07	7.48E-05	1.23E-03	1.67E-03	1.68E-03	1.50E-03	1.19E-03	1.03E-03
		L	1.67E-07	1.25E-05	3.35E-04	6.61E-04	1.74E-03	2.02E-03	1.97E-03	1.80E-03
		AA	2.79E-03	2.33E-02	1.17E-01	1.63E-01	2.60E-01	2.85E-01	2.99E-01	3.02E-01
		N	1.39E-07	1.09E-05	1.79E-04	2.43E-04	2.44E-04	2.17E-04	1.72E-04	1.50E-04
		pH	2.46E-03	2.05E-02	1.03E-01	1.43E-01	2.28E-01	2.51E-01	2.62E-01	2.66E-01
		GA	2.26E-08	1.43E-06	5.31E-05	1.39E-04	8.14E-04	1.32E-03	1.82E-03	2.11E-03
		FA	2.73E-07	1.80E-05	5.42E-04	1.28E-03	5.97E-03	8.97E-03	1.17E-02	1.31E-02
$Y_{X/N}$	6.8829	X	1.56E-06	1.34E-04	3.48E-03	6.40E-03	1.76E-02	2.40E-02	3.00E-02	3.34E-02
		L	3.17E-08	3.42E-06	6.34E-05	2.30E-05	1.07E-03	2.11E-03	3.26E-03	3.97E-03
		AA	1.56E-06	1.45E-04	3.36E-03	4.71E-03	4.75E-03	1.67E-02	3.06E-02	3.94E-02
		N	1.27E-04	1.03E-03	4.55E-03	5.96E-03	7.93E-03	7.95E-03	7.70E-03	7.48E-03
		pH	1.37E-06	1.27E-04	2.95E-03	4.14E-03	4.18E-03	1.46E-02	2.69E-02	3.47E-02
		GA	7.89E-08	5.84E-06	1.73E-04	4.05E-04	2.05E-03	3.25E-03	4.42E-03	5.07E-03
		FA	3.85E-07	2.83E-05	8.00E-04	1.82E-03	8.56E-03	1.32E-02	1.75E-02	1.98E-02
K_H	0.8789	X	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
		L	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
		AA	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
		N	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
		pH	9.13E-03	7.64E-02	3.98E-01	5.71E-01	1.01E+00	1.17E+00	1.30E+00	1.37E+00
		GA	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
		FA	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
$Y_{L/S}$	0.0639	X	2.74E-05	2.17E-03	3.83E-02	5.48E-02	6.48E-02	6.14E-02	4.99E-02	4.36E-02
		L	4.80E-06	3.63E-04	1.06E-02	2.20E-02	6.67E-02	7.94E-02	7.64E-02	6.67E-02
		AA	8.02E-02	6.84E-01	3.93E+00	5.93E+00	1.18E+01	1.40E+01	1.57E+01	1.64E+01
		N	3.99E-06	3.15E-04	5.57E-03	7.96E-03	9.42E-03	8.93E-03	7.24E-03	6.34E-03
		pH	7.05E-02	6.01E-01	3.45E+00	5.21E+00	1.03E+01	1.23E+01	1.38E+01	1.44E+01
		GA	6.48E-07	4.19E-05	1.71E-03	4.72E-03	3.28E-02	5.68E-02	8.22E-02	9.73E-02
		AA	7.82E-06	5.24E-04	1.73E-02	4.31E-02	2.37E-01	3.78E-01	5.14E-01	5.89E-01
K_{XI}	19.519	X	9.33E-05	1.40E-03	7.45E-03	8.26E-03	6.61E-03	5.78E-03	5.11E-03	4.79E-03
		L	2.88E-06	5.75E-05	3.15E-04	2.62E-04	3.90E-04	6.98E-04	9.29E-04	1.03E-03
		AA	1.09E-04	1.85E-03	9.99E-03	9.72E-03	1.61E-03	6.98E-03	1.10E-02	1.29E-02
		N	1.36E-05	2.03E-04	1.08E-03	1.20E-03	9.60E-04	8.40E-04	7.42E-04	6.95E-04
		pH	9.55E-05	1.63E-03	8.78E-03	8.54E-03	1.42E-03	6.14E-03	9.71E-03	1.14E-02
		GA	9.40E-07	1.74E-05	9.12E-05	1.01E-04	3.68E-06	7.28E-05	1.40E-04	1.74E-04
		FA	4.71E-06	8.58E-05	4.18E-04	4.43E-04	1.18E-05	2.60E-04	4.78E-04	5.80E-04
K_{iXI}	2053.92	X	3.31E-07	4.22E-06	1.23E-05	9.74E-06	3.04E-06	1.90E-06	1.27E-06	1.05E-06
		L	1.04E-08	1.87E-07	8.10E-07	7.58E-07	3.32E-07	2.24E-07	1.57E-07	1.31E-07
		AA	3.87E-07	5.80E-06	2.10E-05	1.85E-05	7.27E-06	4.80E-06	3.32E-06	2.76E-06
		N	4.81E-08	6.13E-07	1.79E-06	1.42E-06	4.41E-07	2.76E-07	1.85E-07	1.53E-07
		pH	3.41E-07	5.10E-06	1.85E-05	1.63E-05	6.39E-06	4.22E-06	2.92E-06	2.43E-06
		GA	3.38E-09	5.67E-08	2.28E-07	2.40E-07	1.51E-07	1.09E-07	7.81E-08	6.54E-08
		FA	1.69E-08	2.80E-07	1.05E-06	1.06E-06	5.94E-07	4.10E-07	2.85E-07	2.37E-07
K_{LI}	15.0228	X	1.20E-08	1.05E-06	3.03E-05	5.65E-05	1.08E-04	1.30E-04	1.05E-04	9.82E-05
		L	2.27E-06	2.31E-05	2.83E-04	5.77E-04	1.78E-03	2.33E-03	2.75E-03	2.96E-03
		AA	3.56E-05	3.62E-04	4.45E-03	9.07E-03	2.79E-02	3.66E-02	4.32E-02	4.63E-02
		N	1.75E-09	1.52E-07	4.41E-06	8.21E-06	1.57E-05	1.88E-05	1.52E-05	1.43E-05
		pH	3.13E-05	3.18E-04	3.91E-03	7.97E-03	2.46E-02	3.21E-02	3.79E-02	4.07E-02
		GA	2.85E-10	2.13E-08	1.58E-06	5.73E-06	6.37E-05	1.23E-04	1.91E-04	2.32E-04
		FA	3.44E-09	2.62E-07	1.55E-05	5.08E-05	4.51E-04	8.02E-04	1.17E-03	1.37E-03
K_{iLI}	2152.92	X	3.92E-11	2.81E-09	3.21E-08	3.73E-08	2.87E-08	1.79E-08	1.68E-08	1.25E-08

		L	7.24E-09	5.09E-08	1.34E-07	1.47E-07	1.63E-07	1.65E-07	1.65E-07	1.64E-07
		AA	1.13E-07	7.98E-07	2.12E-06	2.33E-06	2.57E-06	2.60E-06	2.60E-06	2.57E-06
		N	5.69E-12	4.08E-10	4.66E-09	5.42E-09	4.16E-09	2.60E-09	2.45E-09	1.82E-09
		pH	9.96E-08	7.01E-07	1.86E-06	2.05E-06	2.26E-06	2.28E-06	2.28E-06	2.26E-06
		GA	9.21E-13	5.11E-11	1.15E-09	2.45E-09	1.01E-08	1.51E-08	1.95E-08	2.20E-08
		FA	1.11E-11	6.50E-10	1.23E-08	2.37E-08	7.70E-08	1.06E-07	1.30E-07	1.42E-07
σ	34.1036	X	8.56E-07	7.01E-05	2.97E-03	5.73E-03	1.04E-02	1.07E-02	1.05E-02	1.02E-02
		L	3.83E-08	3.43E-06	2.00E-04	4.73E-04	1.56E-03	2.09E-03	2.53E-03	2.75E-03
		AA	1.18E-06	1.01E-04	5.16E-03	1.13E-02	3.15E-02	4.00E-02	4.67E-02	5.00E-02
		N	1.24E-07	1.02E-05	4.32E-04	8.33E-04	1.51E-03	1.56E-03	1.53E-03	1.49E-03
		pH	1.04E-06	8.90E-05	4.53E-03	9.92E-03	2.77E-02	3.51E-02	4.10E-02	4.39E-02
		GA	6.49E-09	5.12E-07	1.38E-05	1.71E-05	1.61E-04	3.69E-04	5.96E-04	7.32E-04
		FA	3.25E-08	2.52E-06	5.89E-05	5.57E-05	8.50E-04	1.77E-03	2.72E-03	3.26E-03
k_1	0.3285	X	0.00E+00	0.00E+00	0.00E+00	0.00E+00	9.78E-11	6.65E-10	5.98E-11	6.48E-11
		L	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.69E-11	1.06E-11	1.43E-11	4.54E-12
		AA	0.00E+00	0.00E+00	0.00E+00	0.00E+00	5.82E-10	1.94E-10	2.24E-10	4.55E-13
		N	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.29E-11	8.82E-11	9.65E-12	5.98E-12
		pH	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.38E-10	4.32E-10	6.69E-11	8.95E-11
		GA	3.24E-04	2.64E-03	1.33E-02	1.97E-02	4.15E-02	5.07E-02	5.75E-02	6.08E-02
		FA	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.01E-11	1.13E-11	1.01E-11	2.07E-12
K_{GAS}	1.4555	X	0.00E+00	0.00E+00	0.00E+00	0.00E+00	3.73E-06	6.24E-07	1.77E-06	1.65E-07
		L	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.99E-07	2.00E-08	1.87E-07	2.25E-08
		AA	0.00E+00	0.00E+00	0.00E+00	0.00E+00	7.21E-06	1.12E-07	4.13E-06	4.64E-07
		N	0.00E+00	0.00E+00	0.00E+00	0.00E+00	5.42E-07	9.07E-08	2.57E-07	2.40E-08
		pH	0.00E+00	0.00E+00	0.00E+00	0.00E+00	6.34E-06	9.83E-08	3.63E-06	4.07E-07
		GA	3.00E-05	2.47E-04	1.30E-03	2.00E-03	4.61E-03	5.84E-03	6.81E-03	7.29E-03
		FA	0.00E+00	0.00E+00	0.00E+00	0.00E+00	3.73E-07	1.69E-08	3.49E-07	2.70E-08
K_{GAN}	12.976	X	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.27E-08	2.03E-09	5.59E-09	5.04E-10
		L	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.01E-09	6.04E-11	5.89E-10	6.79E-11
		AA	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.45E-08	4.21E-10	1.30E-08	1.40E-09
		N	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.85E-09	2.94E-10	8.12E-10	7.32E-11
		pH	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.16E-08	3.72E-10	1.14E-08	1.23E-09
		GA	8.23E-06	6.70E-05	3.37E-04	5.02E-04	1.06E-03	1.29E-03	1.47E-03	1.55E-03
		FA	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.26E-09	4.46E-11	1.10E-09	7.82E-11
K_{iGAN}	2.5328	X	0.00E+00	0.00E+00	0.00E+00	0.00E+00	4.77E-09	7.52E-10	2.11E-09	1.94E-10
		L	0.00E+00	0.00E+00	0.00E+00	0.00E+00	3.80E-10	8.62E-12	2.23E-10	2.65E-11
		AA	0.00E+00	0.00E+00	0.00E+00	0.00E+00	9.21E-09	5.68E-10	4.95E-09	5.55E-10
		N	0.00E+00	0.00E+00	0.00E+00	0.00E+00	6.93E-10	1.16E-10	3.07E-10	2.89E-11
		pH	0.00E+00	0.00E+00	0.00E+00	0.00E+00	8.08E-09	5.14E-10	4.34E-09	4.84E-10
		GA	1.22E-08	9.33E-08	3.44E-07	4.27E-07	5.50E-07	5.69E-07	5.77E-07	5.79E-07
		FA	0.00E+00	0.00E+00	0.00E+00	0.00E+00	4.61E-10	3.10E-11	4.16E-10	3.10E-11
k_2	1.4055	X	0.00E+00	2.22E-06	1.21E-05	6.36E-06	1.78E-05	8.62E-06	9.49E-06	1.35E-06
		L	0.00E+00	1.19E-07	9.49E-07	8.98E-07	1.18E-06	4.19E-07	8.84E-07	2.19E-07
		AA	0.00E+00	3.38E-06	2.31E-05	1.84E-05	3.06E-05	1.24E-05	2.03E-05	4.34E-06
		N	0.00E+00	3.23E-07	1.76E-06	9.24E-07	2.59E-06	1.25E-06	1.38E-06	1.97E-07
		pH	0.00E+00	2.97E-06	2.03E-05	1.62E-05	2.69E-05	1.09E-05	1.78E-05	3.82E-06
		GA	0.00E+00	3.91E-08	2.28E-07	2.72E-07	1.89E-07	8.94E-08	4.02E-07	1.13E-07
		FA	3.80E-04	3.07E-03	1.50E-02	2.18E-02	4.31E-02	5.13E-02	5.71E-02	5.98E-02
K_{FAS}	12.976	X	0.00E+00	2.07E-07	1.11E-06	5.83E-07	1.65E-06	7.93E-07	8.71E-07	1.23E-07
		L	0.00E+00	1.11E-08	8.71E-08	8.24E-08	1.09E-07	3.86E-08	8.13E-08	1.98E-08
		AA	0.00E+00	3.15E-07	2.12E-06	1.69E-06	2.83E-06	1.14E-06	1.86E-06	3.94E-07
		N	0.00E+00	3.00E-08	1.61E-07	8.46E-08	2.39E-07	1.15E-07	1.27E-07	1.79E-08
		pH	0.00E+00	2.77E-07	1.86E-06	1.48E-06	2.48E-06	1.00E-06	1.64E-06	3.46E-07
		GA	0.00E+00	3.64E-09	2.10E-08	2.49E-08	1.75E-08	8.30E-09	3.70E-08	1.03E-08
		FA	3.57E-05	2.90E-04	1.42E-03	2.09E-03	4.20E-03	5.04E-03	5.63E-03	5.91E-03
K_{FAN}	2.5328	X	0.00E+00	1.35E-06	7.33E-06	3.86E-06	1.07E-05	5.22E-06	5.74E-06	8.28E-07
		L	0.00E+00	7.28E-08	5.75E-07	5.44E-07	7.11E-07	2.53E-07	5.35E-07	1.34E-07
		AA	0.00E+00	2.06E-06	1.40E-05	1.11E-05	1.84E-05	7.51E-06	1.23E-05	2.66E-06
		N	0.00E+00	1.97E-07	1.06E-06	5.60E-07	1.56E-06	7.58E-07	8.35E-07	1.20E-07
		pH	0.00E+00	1.81E-06	1.23E-05	9.79E-06	1.62E-05	6.60E-06	1.08E-05	2.34E-06
		GA	0.00E+00	2.38E-08	1.38E-07	1.64E-07	1.13E-07	5.37E-08	2.43E-07	6.93E-08
		FA	2.05E-04	1.66E-03	8.12E-03	1.19E-02	2.36E-02	2.82E-02	3.14E-02	3.29E-02

Chapter 4

Kinetic Modelling of Microalgal Cultivation for Optimised Biofuel Production under Multiple Environmental Factors

4.1. Preface

Integrated experimental and computational studies of microalgal biomass growth and lipid accumulation bring numerous advantages and applications such as understanding the interactions between the cellular components such as lipid, starch and protein and improving process productivities through the optimisation of the growth media composition and the environmental factors.

The kinetic modelling of the microalgal growth process allows us to compute optimal cultivation strategies through the manipulation of the growth media composition and environmental factors to achieve higher lipid and biomass productivities. In order to develop a detailed kinetic model for microalgae growth process, all the growth-limiting parameters need to be considered and the effect of their variations needs to be studied through a combination of experiments and computations.

Microalgal growth is affected by both the growth media composition (carbon substrate and nutrients) and the environmental factors (light and temperature). As shown in Chapter 3, the addition of carbon substrate can increase biomass concentration while excess carbon addition can inhibit growth. On the other hand, N limitation led to an increase in lipid accumulation while excess of N did not inhibit cellular lipid accumulation but biomass concentration. Variations of these two growth media were tested experimentally and a kinetic model was then developed considering the effect of both carbon substrate and nitrogen under constant light illumination. Furthermore, experiments with light intensity and temperature variations showed that these environmental factors also have big influence on biomass growth and lipid accumulation.

The model developed in Chapter 3 was used to establish an optimal strategy for lipid accumulation in terms of the substrate and nitrogen concentration variations. Here, this model is expanded in order to develop an improved strategy taking into account light and temperature

variations as well. To accomplish this, experiments with seven different light intensities and temperatures were carried out and resulting biomass growth and lipid accumulation were analysed along with the substrate and nitrogen consumption. Based on the experimental observations and existing literature, a comprehensive quadruple substrate kinetic model was developed considering the simultaneous and antagonistic effects of carbon, nitrogen, temperature and light intensity.

The results and discussion of this study are presented in the paper that follows where the effect of temperature and light intensity variations on biomass growth and lipid accumulation are assessed and discussed in detail. Additionally, the development of the kinetic modelling framework is discussed. The details of the kinetic parameter values estimation and the optimisation study towards maximization of lipid accumulation are also explained.

K.T. and J.K.P. contemplated and supervised the research and M.B. designed the research plans, performed the research, analysed data and wrote the manuscripts.

4.2. Publication 2

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Kinetic Modelling of Microalgal Cultivation for Optimised Biofuel Production under Multiple Environmental Factors

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Abstract

Background: Microalgae-derived biofuels have the potential to make a sustainable contribution to global fuel supply, due to reduced greenhouse gas emissions and lack of conflict with food production. However, the near-term economic viability of algal-derived biofuels remains a major concern. Enhancing cultivation methods in order to increase microalgae productivity has been given serious consideration in order to reduce the cost of algal biofuel production. It has been shown that it is more economically beneficial to target enhancements in lipid content by manipulating cultivation media conditions and environmental factors, rather than biomass growth rate.

Results: The present study aims to develop a novel kinetic model that will consider four major growth parameters: carbon substrate, nitrogen concentration, light intensity and temperature, in order to develop a cost-effective process to improve the economic viability of the system and its overall sustainability. To accomplish this, a detailed kinetic model has been constructed through a multi-parameter quantification methodology in conjunction with experiments taking into account photo-heterotrophic biomass growth. The growth rate of the proposed model is based on carbon and nitrogen concentration, light intensity and temperature. Parameters of the proposed model were estimated through an extensive number of experimental data from microalgae laboratory and bench-scale cultures. Consequently, predictive capabilities of the model were assessed and the validated model was utilised to determine the optimal operating

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conditions for bench-scale batch lipid production. The computed optimal conditions were experimentally tested demonstrating excellent agreement with the optimisation results.

Conclusions: Such comprehensive predictive kinetic modelling approaches can be exploited for the robust design, control and optimization of microalgal oil production as well as for process scale-up, which can help to reduce overall operating cost and bring this important technology one step closer to commercialization and industrialization.

Keywords

Chlamydomonas reinhardtii, Biofuels, Dynamic kinetic modelling, Microalgal lipid, Cultivation optimization, Variable light intensity and temperature.

Nomenclature

TAG	Triacylglycerol
TAP	Tris-acetate-phosphate
DCW	Dry cell weight
N	Nitrogen
S	Substrate
I	Light intensity
I_0	Local light intensity
L	Lipid
X	Oil-free biomass
T	Incubation temperature
T_0	Reference temperature
AA	Acetic 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
l	The distance between the local position and the external surface of the system
A_{0_X}	Frequency factors
B_{0_X}	Frequency factors
E_{a_X}	Activation energy of oil-free biomass growth
E_{b_X}	Activation energy of oil-free biomass degradation
A_{0_L}	Frequency factors
B_{0_L}	Frequency factors
E_{a_L}	Activation energy of oil production
E_{b_L}	Activation energy of oil degradation

1. Introduction

The growing energy demand and concerns about climate change have led to an increased utilization of renewable resources, particularly biomass, for the sustainable production of fuels

and chemicals. In this regard, microalgae have been proposed as an alternative renewable source for the production of fuel, chemicals and added-value products due to their outstanding characteristics: mainly producing high volume of oils and rapid growth rate compare to other terrestrial plants [1, 2]. Microalgal oil consists of glycerolipid triacylglycerol (TAG) that can be converted into biodiesel, or used for the synthesis of other bioproducts or for other added-value products such as nutraceuticals. With high oil and biomass productivities, algae can produce a considerably higher concentration of oil and biomass per acre than other terrestrial biomass [3].

Microalgal metabolic activities are highly versatile and flexible, which make many of them adaptable to different cultivation conditions [4]. This potential of microalgae can be utilized to control and maximise the production of a targeted compound within microalgae cells. The chemical composition of photoheterotrophic microalgae is affected by a number of factors such as organic and/or inorganic carbon substrate, essential nutrient availability, temperature and light intensity [5, 6]. If an organic carbon substrate is used, its cost accounts for up to 50% of the total media cost used for the photoheterotrophic algae cultivation [7, 8]. As biodiesel is a low-value product, the production cost of its feedstock needs to be reduced to make it cost-competitive and sustainable [9].

In both natural and modified systems, the microalgae culture can be exposed to various environmental factors such as organic carbon and nutrient concentrations, light intensity, pH and temperature that can simultaneously and antagonistically affect both the biomass production and the lipid accumulation [6, 10, 11]. Thus, in order to produce oil-rich microalgal bodies, abiotic stress including nitrogen and phosphorus deprivation as well as high light intensity and temperature stress can be applied [12-16].

Integrated experimental-computational frameworks that have the ability to predict biomass growth and lipid accumulation under different growing conditions will help to optimize the process performance, operating conditions and scale-up of cultivation systems for commercialization and industrial applicability [17-19]. Droop, Monod and Andrew models have been extensively applied to predict biomass growth rate as a function of a single substrate or nutrient concentration such as phosphorus [20], nitrogen [21], carbon [10] or light [22]. Some researchers have also considered the simultaneous effect of multiple substrate and environmental factors, such as temperature and carbon [23], nitrogen and phosphorus [24], CO_2, nitrogen, light and temperature [25], or carbon and nitrogen [26]. However, kinetic modelling and experimental validation of simultaneous co-limitation of growth media elements

(nitrogen and acetate) and environmental factors (light and temperature) has not been considered yet. Additionally, while lipid accumulation has been considered to be proportional to biomass growth, we have recently shown the effects of abiotic stress towards enhancement of lipid productivity through a new kinetic model considering biomass growth and lipid accumulation as two different dynamic variables [18]. The model took into account the effect of acetate (as an organic carbon substrate) and nitrogen (as a replete or limiting nutrient) variation under constant light illumination on microalgal biomass growth and lipid production, and allowed the optimization of the process for maximum lipid productivity [18]. Nevertheless, it has been observed that other environmental factors, such as light and temperature, will have a positive or negative effect on microalgal growth and lipid accumulation [27-29]. However, there is currently no kinetic model available to describe both biomass growth and lipid accumulation dynamics simultaneously as a function of carbon substrate (C), nutrient (N), light (I) and temperature (T) under photo-heterotrophic growth conditions.

Here we present a kinetic model describing in detail biomass growth and lipid accumulation in the model green microalga *Chlamydomonas reinhardtii*, and including the simultaneous effect of four limiting factors, C, N, I and T, under photo-heterotrophic growth conditions. The predictive ability of the developed photoheterotrophic model for biomass growth rate and lipid accumulation was tested over a range of growth conditions and environmental conditions. The validated model was then used to compute optimal initial conditions for maximum lipid accumulation, producing excellent results. The integrated experimental-computational framework that we present here can be used to confidently predict biomass growth and lipid accumulation, and ultimately to enable robust system design and scale-up.

2. Materials and Methods

2.1. Microorganism and Culture Conditions

Chlamydomonas reinhardtii (CCAP 11/32C), obtained from the Culture Collection of Algae and Protozoa, UK, was used here as the experimental microalgal strain. The strain was maintained under photo-heterotrophic conditions in batch cultures as described previously [5]. Prior to bench-scale batch experiments, preculture of the strain was carried out in an environmentally-controlled incubation room at 25ºC, using 250 mL Erlenmeyer flasks containing 150 mL of Tris-

acetate-phosphate (TAP) medium [30]. The inoculum was then placed on an orbital shaker at 120 rpm for 7-10 days and constant light illumination was provided by a 4ft long 20W high power led T8 tube light, 125 $\mu Em^{-2} s^{-1}$ light intensity. After achieving the sufficient cell density (7-10 days), experiments were carried out in experimental culture vessels, Small Anaerobic Reactors (SARs, 500 mL), containing 500 mL of sterile TAP culture medium and 1 mL of algal inoculum. The initial concentration of inoculum, 0.001 g/mL, was identical for all the treatments. Initial concentration was determined through the measurement of dry cell weight (DCW) by centrifuging 1 mL inoculum culture for 3 min at 3000 g in an Eppendorf Centrifuge 5424. The obtained wet 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 ºC to determine the DCW.

The initial environmental factor values were 125 $\mu Em^{-2} s^{-1}$ and 25 ºC for the light intensity and temperature, respectively. The light intensity and temperature were varied in order to analyse the effect of light intensity and temperature variations on biomass growth and lipid accumulation. Overall, seven light intensities were analysed: 0 $\mu Em^{-2} s^{-1}$, 5 $\mu Em^{-2} s^{-1}$, 45 $\mu Em^{-2} s^{-1}$, 105 $\mu Em^{-2} s^{-1}$, 125 $\mu Em^{-2} s^{-1}$, 135 $\mu Em^{-2} s^{-1}$ and 155 $\mu Em^{-2} s^{-1}$; and seven temperatures; 5 ºC, 10 ºC, 15 ºC, 20 ºC, 25 ºC, 30 ºC and 35 ºC. All culture vessels were placed into the growth chamber (Fitotron® SGC 120 Plant Growth Chamber) one day before inoculation to allow the culture vessels to reach the set temperature. Constant light illumination in the growth chamber was provided by 4ft long standard white fluorescent tubes. When light intensity was manipulated, the temperature was kept constant at 25 ºC, and when temperature was manipulated the light intensity was kept constant at 125 $\mu Em^{-2} s^{-1}$. At the start of treatments the pH value was set at pH=7. 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 ºC 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. Analytical Methods

Biomass concentration, lipid accumulation in dried biomass, acetate and total nitrogen concentration (linked to NH_4Cl concentration), as well as pH of the growth media were measured and quantified over time during the experiments. The supernatant samples were filtered through 0.45 µm nitrocellulose membranes (Millipore Ltd.) and then diluted appropriately with HPLC grade water. The concentration of acetate consumed was quantified using a High Performance/Pressure Liquid Chromatographer (HPLC) equipped with a Hi- Plex 8 µm 300x7.7 mm column. Here the mobile phase was sulphuric acid solution (0.05% v/v) and the flow rate of the system was 0.6 mL min^{-1}. The total nitrogen concentration was quantified by the use of a Total Organic Carbon / Total Nitrogen analyser (TOC/TN) (TOC-V_{CSH}/TNM-1 Shimadzu). The quantification of lipid concentration was performed by extracting the lipid from dried biomass using the SOXTEC 1043 automated solvent extraction system. Hexane was used as organic solvent. After extraction, the hexane was removed by evaporation and the accumulated lipid was determined gravimetrically. The detailed procedure for the use of HPLC, TOC-TN and SOXTEC 1043 has been described previously [18].

3. Mathematical Model Construction

3.1. Growth kinetics

We have previously demonstrated [18] that excess carbon supply in the form of acetate can be inhibitory as seen by dramatically reduced biomass growth and lipid production. We have also shown that reduced N supply can enhance lipid accumulation while inhibiting biomass growth. Therefore, two different kinetic equations were used to describe specific (oil-free) biomass growth rate (Eq. 1) and lipid production rate (Eq. 2) involving the simultaneous effect of acetate substrate (denoted as substrate, S, onwards) nitrogen, N, and local light intensity illumination, $I(l)$:

$$\mu_X = \mu_{Xmax} \cdot \frac{S}{S + K_{XS} + \frac{S^2}{K_{iXS}}} \cdot \frac{N}{N + K_{XN} + \frac{N^2}{K_{iXN}}} \cdot \frac{I(l)}{I(l) + K_{XI} + \frac{I(l)^2}{K_{iXI}}} \quad \text{Eq. 1}$$

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

Here μ_{Xmax} is the maximum specific growth rate of oil-free biomass and q_{Lmax} the maximum lipid specific growth rate. K_{XS}, K_{XN}, K_{XI}, K_{LS} and K_{LI} are the saturation constants and K_{iXS}, K_{iXN}, K_{iXI}, K_{iLS}, K_{iLI} the inhibition constants for oil-free biomass growth based on substrate, nitrogen concentration and light intensity, respectively. K_{iNL} is an inhibition constant used here to describe the lipid production dependent on nitrogen concentration.

The local light intensity $I(l)$ is expressed by the Beer-Lambert equation [27]:

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

where l is the distance between the local position and the external surface of the system (measured as 0.25 m), I_0 the incident light intensity, σ the molar extinction coefficient and X the oil-free biomass concentration [27].

The final biomass concentration of the acetate-absent treatment that was performed in [18] was almost zero which points out that the influence of CO_2 is negligible and therefore it has been discounted in the final biomass growth and lipid accumulation expressions. The model in Bekirogullari et al. [18] was a function of substrate and nitrogen under constant incident light intensity, I_0 as expressed by Eq. 1 and Eq. 2.

In this work, the effect of temperature and of incident light intensity variations on biomass growth and lipid accumulation has been taken into consideration, hence accounting for environmental changes on microalgal cultivation processes. Hence, the triple substrate expressions (Eq. 1 and 2) have been modified into quadruple substrate expressions:

$$\mu_X = \mu_{Xmax} \cdot f(S)_X \cdot f(N)_X \cdot f(I)_X \cdot f(T)_X \quad \text{Eq. 4}$$

$$\mu_L = q_{Lmax} \cdot f(S)_L \cdot f(N)_L \cdot f(I)_L \cdot f(T)_L \quad \text{Eq. 5}$$

The effect of temperature has been considered through two different expressions: (i) cardinal temperature model with inflexion (CTMI) and (ii) Arrhenius equation. Although both the Arrhenius equation and the CTMI has same number of parameters (four) that need to be

estimated, the Arrhenius expression is widely utilized in the field of microbial growth and it is easy find the relevant data in the literature. Therefore, the effect of temperature on (oil-free) biomass growth and lipid accumulation $f(T)$ is expressed by a modified version of the Arrhenius equation which accounts for both saturation and inhibition effect of temperature [31]:

$$f(T)_X = A_{0_X} \exp\left[\frac{-E_{a_X}}{R}\left(\frac{1}{T}-\frac{1}{T_0}\right)\right] - B_{0_X}\exp\left[\frac{-E_{b_X}}{R}\left(\frac{1}{T}-\frac{1}{T_0}\right)\right] \quad (6)$$

$$f(T)_L = A_{0_L} \exp\left[\frac{-E_{a_L}}{R}\left(\frac{1}{T}-\frac{1}{T_0}\right)\right] - B_{0_L}\exp\left[\frac{-E_{b_L}}{R}\left(\frac{1}{T}-\frac{1}{T_0}\right)\right] \quad (7)$$

The first and the second parts of Eq. 6 and 7 represent the promotion and inhibition effects of temperature, respectively. E_{a_X}, E_{a_L} and E_{b_X}, E_{b_L} are the activation energies of growth and cellular degradation, respectively (kcal mol^{-1}), R is the gas constant (kcal mol^{-1}), T the incubation temperature (K), T_0 the reference temperature (K), and A_{0_X}, A_{0_L} and B_{0_X}, B_{0_L} the corresponding frequency factors (h^{-1}).

3.2. Rate equations

The kinetic model developed in this work consists of a set of ordinary differential equations (ODEs) employed for the simultaneous simulation of microalgal biomass growth, lipid production, substrate and nitrogen consumption and pH change rates.

The microalgal (oil-free biomass) growth rate is described by:

$$\frac{dX}{dt} = \mu_X . X \quad \text{Eq. 7}$$

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

$$\frac{dL}{dt} = \mu_L . X \quad \text{Eq. 8}$$

The substrate consumption rate can be calculated through a mass conservation equation [18]:

$$\frac{dS}{dt} = -\frac{1}{Y_{\frac{X}{S}}} \cdot \frac{dX}{dt} - \frac{1}{Y_{\frac{L}{S}}} \cdot \frac{dL}{dt} \quad \text{Eq. 9}$$

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

The nitrogen consumption rate is given by [18]:

$$\frac{dN}{dt} = -\frac{1}{Y_{\frac{X}{N}}} \cdot \frac{dX}{dt} \qquad \text{Eq. 10}$$

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

The pH change throughout the cultivation period of the microalgae cultivation system was observed to be proportional to the substrate consumption rate [32] and is described by:

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

where H is the pH of the medium, and K_h is a constant. Consequently, the developed model consists of 5 ODEs (Eq.7 to 11), with 5 state variables describing the dynamics of biomass growth, lipids accumulation, substrate and nitrogen consumption as well as pH change. The model contains 26 parameters (listed in Table 2, below), which have been estimated through the procedure discussed in section 3.3 below.

3.3. Parameter Estimation

As far as we know, this work is the first to develop a kinetic model for photoheterotrophic microalgae growth and lipid accumulation by considering the simultaneous effect of an organic carbon substrate (acetate), nitrogen, light intensity and temperature, and therefore, the kinetic parameter values for such a system are not available in the literature. Consequently, we carried out a parameter estimation study using the constructed model (Eq.7 to 11) in conjunction with in-house derived experimental data. Data fitting was performed using a non-linear weighted least squares method [33]:

$$Z(kk) = \min \sum_{k=1}^{n_k} \sum_{l=1}^{n_l} \sum_{m=1}^{n_m} W_{k,l,m} \left(C_{k,l,m}^{pred}(kk) - C_{k,l,m}^{exp} \right)^2 \qquad \text{Eq. 14}$$

Here *kk* is the vector of the 26 model parameters, n_k is the number of experiments (n_k=2), n_l is the number of state variables (n_l =5), n_m is the number of experimental measurements in time (n_m=7), and $W_{k,l,m}$ are the weights for each variable used to effectively normalise the computed errors, $\varepsilon = \left(C_{k,l,m}^{pred}(kk) - C_{k,l,m}^{exp} \right)$, where $C_{k,l,m}^{pred}$ are the predicted state variables and $C_{k,l,m}^{exp}$ the experimentally obtained ones. Here the weights were set to $W_{k,l,m} = 1/ C_{k,l,m}^{exp}$,.

The two set of experiments that have been used for the fitting problem are tabulated as Experiment 1 and Experiment 2 in Table 1. The initial concentrations of each experiment were used as the initial values of the state variables in the ODEs (Eq.7 to 11). The kinetic parameter values were restricted with an upper and lower bound, which were set according to the experimental behaviour of the system and values found in the literature.

Table 1: Experiments used for the fitting and validation process

Experiment	Acetate (g L^{-1})	Nitrogen (g L^{-1})	Light (µEm^{-2} s^{-1})	Temperature (^{0}C)
1	1.05	0.098	125	30
2	1.05	0.098	105	25
3	1.365	0.074	110	28

The parameter estimation problem was solved using an in-house developed stochastic optimisation algorithm (Simulated Annealing) [33], coupled with a non-linear programming (NLP) –based deterministic optimization algorithm. In order to improve the chance of obtaining solutions in the neighbourhood of the global optimum, the simulated annealing algorithm was used with multiple restarts. The solution from the simulated annealing algorithm was then used as an initial guess to compute the final optimum using the MATLAB function fmincon. The optimisation procedure followed here has been described more in detail previously [18].

Here 10 stochastic optimization runs (restarts) have been used to ensure that the local minima were avoided. By using the procedure explained above, the values of the 26 parameters as well as their standard deviations were found and tabulated in Table 2. The values of the two constants, T_0 and l used in the simulations are also given in Table 2. The resulting time profiles of the 5 state variables, computed by the kinetic model, and comparisons against experimental datasets including biomass growth, lipid accumulation, acetate and nitrogen consumption, pH change of the system are discussed in section 4.2 below.

Table 2: Estimated kinetic parameters along with bounds available in the literature.

Parameter	Value (Units)	Standard Deviation (σ)	Reference value	Species	Reference
μ_{Xmax}	0.2226 h^{-1}	0.008	0.227	*C. reinhardtii*	[18]
K_{XS}	0.060 g S L^{-1}	0.003	0.05	*C. reinhardtii*	[18]
K_{iXS}	11.9076 g S L^{-1}	0.063	9.923	*C. reinhardtii*	[18]
K_{XN}	0.078 g N L^{-1}	0.001	0.065	*C. reinhardtii*	[18]
K_{iXN}	0.700 g N L^{-1}	0.003	0.5	*C. reinhardtii*	[18]
K_{XI}	6.010 μEm^{-2} s^{-1}	0.012	this study		
K_{iXI}	234 μEm^{-2} s^{-1}	2.835	this study		
A_{0_X}	0.6175 h^{-1}	0.022	0.26	*P. tricornutum*	[31]
E_{a_X}	25.9243 kcal mol^{-1}	0.283	28	*P. tricornutum*	[31]
B_{0_X}	0.1101 h^{-1}	0.004	0.18	*P. tricornutum*	[31]
E_{b_X}	48.0151 kcal mol^{-1}	0.349	39	*P. tricornutum*	[31]
q_{Lmax}	0.1452 g L g X^{-1} h^{-1}	0.0002	0.121	*C. reinhardtii*	[18]
K_{LS}	5.2432 g S L^{-1}	0.0033	6.554	*C. reinhardtii*	[18]
K_{iLS}	0.1320 g S L^{-1}	0.0011	0.110	*C. reinhardtii*	[18]
K_{iNL}	442.14 g N L^{-1}	8.468	380.02	*C. reinhardtii*	[18]
K_{LI}	5.221 μEm^{-2} s^{-1}	0.196	this study		
K_{iLI}	224 μEm^{-2} s^{-1}	0.449	this study		
A_{0_L}	0.7 h^{-1}	0.022	this study		
E_{a_L}	20 kcal mol^{-1}	0.013	this study		
B_{0_L}	0.08 h^{-1}	0.0017	this study		
E_{b_L}	32 kcal mol^{-1}	0.146	this study		
$Y_{X/S}$	1.176 g X g S^{-1}	0.002	1.470	*C. reinhardtii*	[18]
$Y_{L/S}$	0.0512 g X g S^{-1}	0.002	0.064	*C. reinhardtii*	[18]

$Y_{X/N}$	5.5064 g X g N^{-1}	0.017	6.883	*C. reinhardtii*	[18]
K_H	0.7032 L g S^{-1}	0.018	0.879	*C. reinhardtii*	[18]
σ	10 g X^{-1} L m^{-1}	0.641	this study		
l	0.25 m				
T_0	293 K				

4. Results and Discussion

A set of experiments were conducted to analyse the effect of varying light intensity and temperature on biomass growth and lipid accumulation as well as acetate and nitrogen consumption and pH change. The constructed model was then used to compute kinetic parameter values, which are of crucial significance for precise system simulations, using the procedure described above. Validation of the model with the computed kinetic parameters was subsequently performed using sets of experimental data produced at different operating conditions. The validated model was then used to compute optimal process operating conditions, for maximum lipid and biomass production.

4.1. Experimental Results

In order to analyse the impact of the light intensity and temperature on biomass growth and lipid accumulation and also to guide the model construction and validation process, we conducted a series of experiments using Small Anaerobic Reactors (SARs, 500ml). Biomass concentration, lipid accumulation, pH change of the growth media, and acetate and nitrogen concentrations in the growth media were measured over time for seven different light intensities and seven different temperature treatments mentioned in section 2.1. As can be seen in Fig. 1, most of the cultures from different light and temperature treatments reached stationary phase at about 140h, but to make sure that stationary phase was achieved, the cultures were grown up to 192h. The produced dynamic profiles were also used to aid the understanding of the effects of modifying light intensity and temperature on biomass growth and lipid production rates.

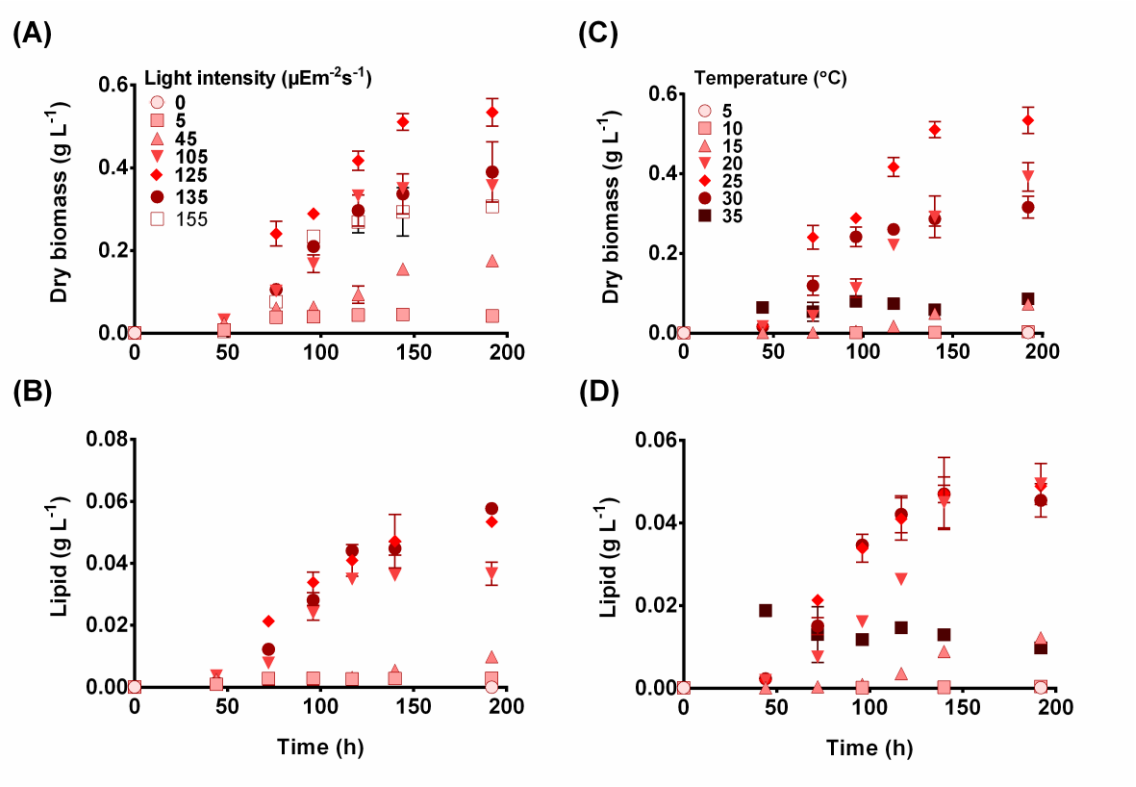

Fig. 1. Effect of light intensity (A, B) and temperature (C, D) on dry cell weight (A, C) and total lipid concentration (B, D) dynamics during photo-heterotrophic growth. Starting temperature for the light intensity variation treatment experiments was 25ºC and starting light intensity for the temperature variation treatment experiments was 125 $\mu Em^{-2} s^{-1}$. All data are mean ± SE values of 2-3 biological replicates.

The experiments revealed that high light intensities act as system inhibitors, as they can dramatically reduce biomass growth and lipid accumulation rates (Fig. 1 and 2), which is in agreement with previous studies [27, 34]. In addition, there was no growth observed for the light-absent (0 μEm^{-2} s^{-1}) media and hence, there was no lipid accumulated (Fig. 2B). For the two low light intensity treatments (5 μEm^{-2} s^{-1} and 45 μEm^{-2} s^{-1}), growth rate was slow, while for the other four treatments under increasingly higher light intensities (105 μEm^{-2} s^{-1}, 125 μEm^{-2} s^{-1}, 135 μEm^{-2} s^{-1} and 155 μEm^{-2} s^{-1}), cells grew increasingly faster with equivalent growth profiles. The growth profiles (Fig. 2) revealed that the biomass concentration increased significantly up to a maximum of 0.517 g L^{-1} as light intensity increased (to 125 μEm^{-2} s^{-1}). Beyond this light intensity value, biomass concentration decreased. Compared to the 125 μEm^{-2} s^{-1} light intensity treatment, biomass concentration decreased significantly ($p < 0.0001$, one-way ANOVA) both for the light-absent treatment (0 μEm^{-2} s^{-1}) and light-deficient (5 μEm^{-2} s^{-1}) treatment by approximately 100% and 92%, respectively (Fig. 1A and 2A). For the other light intensity treatments (45 μEm^{-2} s^{-1},105 μEm^{-2} s^{-1} and 155 μEm^{-2} s^{-1}) biomass decreased by approximately 38%, 25% and 40%, respectively as can be seen in Fig. 1B and 2B. The light intensity treatment with 135 μEm^{-2} s^{-1} did not show any significant difference compared to the 125 μEm^{-2} s^{-1} treatment. The proportion of lipid production within the cell on a total dry cell weight basis for all six light intensity treatments was essentially identical (approximately 10-13%), and the difference in volumetric lipid concentration between the light intensity treatments (Fig. 2) was almost entirely due to the difference in biomass concentration. This finding suggests that under sufficient acetate (1.05 gL^{-1}) and nitrogen (0.098 gL^{-1}) conditions, light is being assimilated mainly for cell growth. While increasing light intensity can indeed increase the biomass concentration and lipid accumulation, as we show here, the inhibition at high light intensities may be either due to photo-oxidation or to reduction of the photosynthetic rate and thus of productivity. It should be noted that while all treatments included acetate, the increased light-dependent growth at increasing light intensities and the inability to grow in the absence of light suggests that the cells do still require light and therefore the acetate can only limit, but cannot fully replace photosynthesis. The cells are therefore growing mixotrophically rather than heterotrophically. The effect of light intensity treatments on biomass concentration and lipid accumulation is in agreement with previously reported data for *C. reinhardtii* where a range of light intensity treatments ($0 - 1200$ μEm^{-2} s^{-1}) were examined [35]. They found that the biomass

concentration increases as the light intensity increases until a point where photoinhibition starts and biomass concentration decreases. Similar observations were made for *C. reinhardtii* by Pottier et al. [36] and Janssen et al. [37]. The impact of light intensity variation treatments on biomass growth rate are also in good agreement with other previously published findings for other microalgae strains, such as *Enteromorpha* sp. [38], *Botryococcus* sp. [39] and *B. braunii* [40].

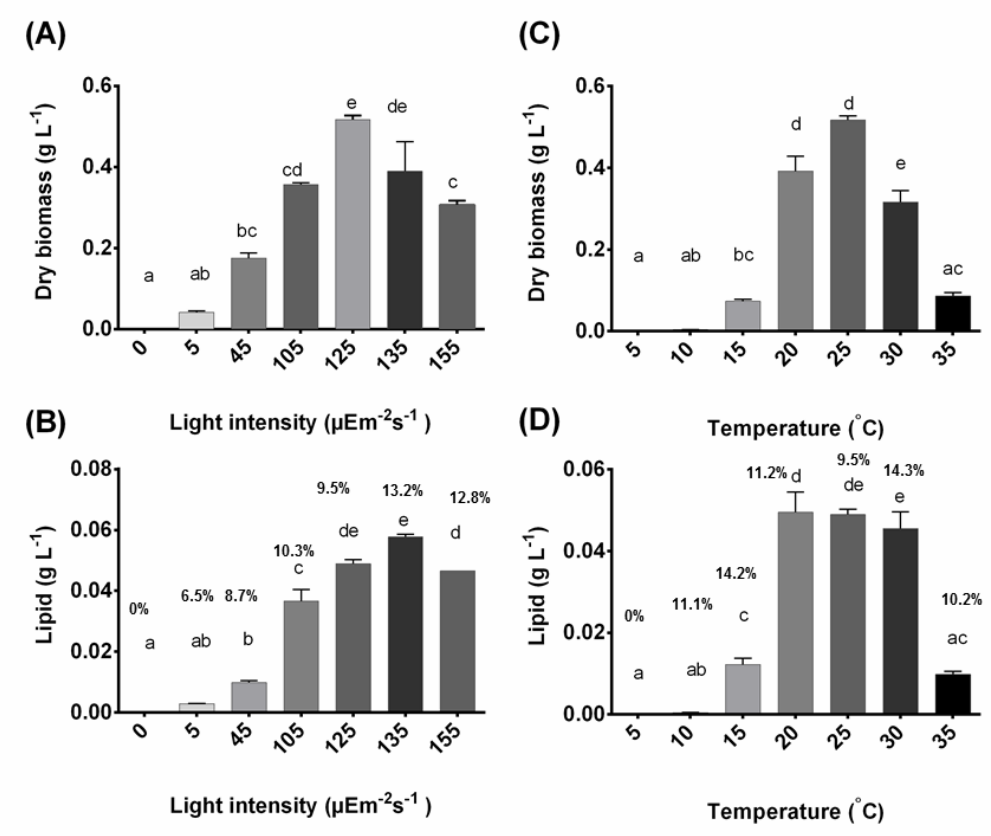

Fig. 2. Effect of light intensity (A, B) and temperature (C, D) on dry cell weight (A, C) and total lipid concentration (B, D) dynamics during photo-heterotrophic growth. The starting temperature for the light intensity variation treatment experiments was 25ºC and the starting light intensity for the temperature variation treatment experiments was 125 μEm^{-2} s^{-1}. All data are mean ± SE values of 2-3 biological replicates. Treatments that do not share lowercase 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.

C. reinhardtii responded to increases in temperature with increased exponential growth rates until reaching the optimum temperature for growth (approx. 25 ºC). Increasing temperature beyond this point led to sharp declines in biomass growth and lipid accumulation, as can be seen in Fig. 1C,D and 2C,D. This temperature-dependent profiles are also in agreement with previous studies (Van Wagenen et al., 2012, James et al., 2011, Ota et al., 2015). For low temperature treatments (5ºC and 10ºC), biomass and lipid concentration were below detectable levels due to slow growth rate (Fig. 1C). For the moderate temperature treatments (15ºC and 35ºC), biomass concentration and lipid accumulation decreased compared to the 25ºC treatment by approximately 86% and 83% (for biomass) and 75% and 80% (for lipid), respectively. For the other three temperature treatments (20ºC, 25ºC, and 30ºC) lipid accumulation growth profiles were essentially the same (Fig. 1C,D) and biomass concentration was also same for (20ºC, 25ºC) and significantly different compare to (30ºC) . Temperature affects the chemical composition of microalgae by impacting the rate of chemical reactions and the stability of cellular components. The net algae growth rates increase exponentially as the temperature increases until a certain point where strains reach their optimum temperature, after which loss of structural integrity leads to sharp declines in biomass growth rate. Increasing temperature beyond these points does not increase algal growth rates and causes damage to a wide range of proteins, molecules and the light receptors of the algae [41]. The impact of temperature variation treatments are in good agreement with previously published data by James et al. [42] for *C. reinhardtii* where 4 different temperatures were examined: 17 ºC, 25 ºC, 32 ºC and 35 ºC. James et al. [42] found that biomass growth increases as temperature increases until the maximum tolerable temperature which was suggested to be between 35 ºC - 38 ºC. The optimal growth temperature in nitrogen sufficient media was reported to be 32 ºC. The effect of temperature variation treatments on biomass growth rate are also in good agreement with other previously reported findings for other microalgae strains, such as *C. minutissima* [43], *C. vulgaris* [44] and *B. braunii* [45].

4.2. Model validation

The constructed ODE-based kinetic model consists of 5 ordinary differential equations (Eq.7 to 11) and 26 kinetic parameters. Values of the kinetic parameters were computed by following the methodology defined in section 3.3. As can be seen in Fig. 3 and Fig. 4, the resulting model is in excellent agreement with experimental data for all 5 state variables for experiment 1 and experiment 2 (Table 1).

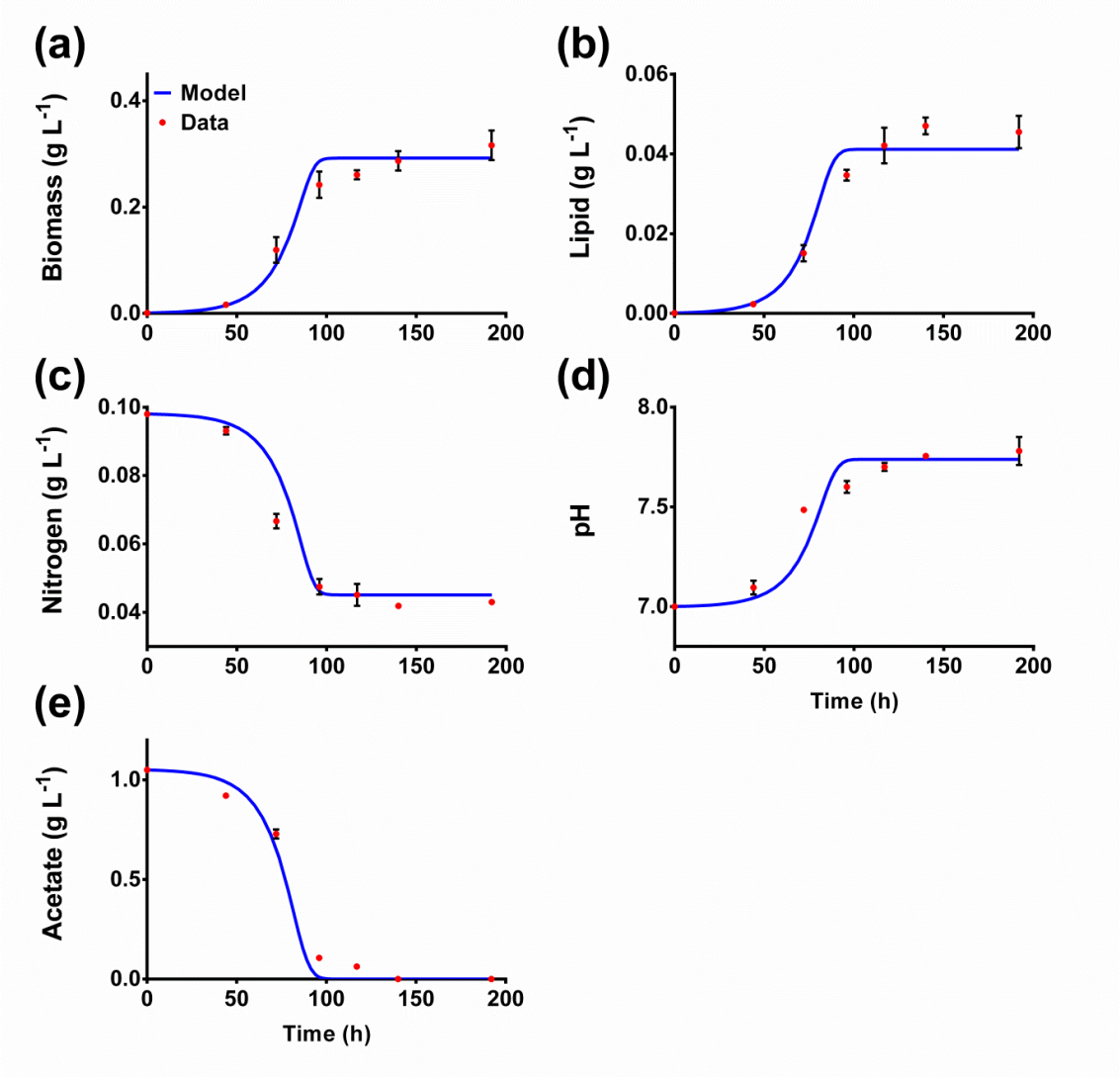

Fig. 3. Comparison of model predictions (lines) with experimental data from Experiment 1 (symbols with error bars) for: (A) biomass, (B) lipid concentration, (C) substrate (acetate) consumption, (D) N consumption, (E) pH change. All data are mean ± SE values of 2-3 biological replicates.

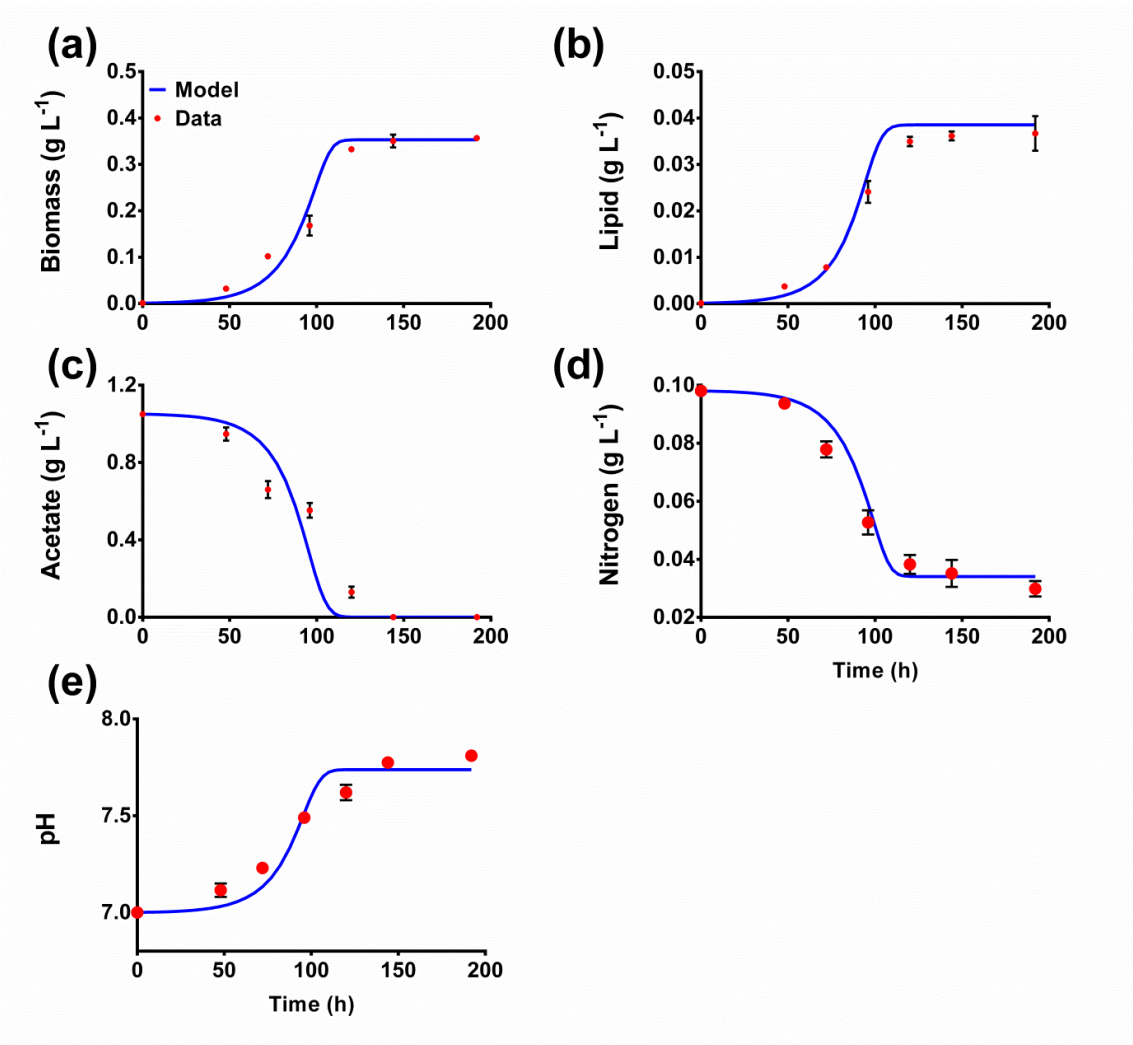

Fig. 4. Comparison of model predictions (lines) with experimental data from Experiment 2 (symbols with error bars) for: (A) biomass, (B) lipid concentration, (C) substrate (acetate) consumption, (D) N consumption, (E) pH change. All data are mean ± SE values of 2-3 biological replicates.

In order to assess the predictive capability of the developed model, we carried out a validation study by using the conditions (initial acetate and nitrogen concentrations, temperature and light intensity) of Experiment 3 (also given in Table 1). It should be noted that all Experiment 3 conditions are different than the ones of Experiments 1 and 2 used for parameter fitting. Fig. 5 depicts the resulting model predictions against the corresponding experimental results. As it can be seen, the kinetic model is able to predict the dynamics of the 5 experimentally obtained state variables (biomass growth, lipid accumulation, acetate and N consumption and pH change) with high accuracy. We can therefore conclude that the detailed interactive (multiplicative) model constructed here can be utilized for accurate prediction of the dynamic behavior of microalgal

batch experiments. Hence, it can be utilized as an optimization tool to compute optimal operating conditions for maximum biomass and lipid accumulation for microalgal cultivation systems operating in batch.

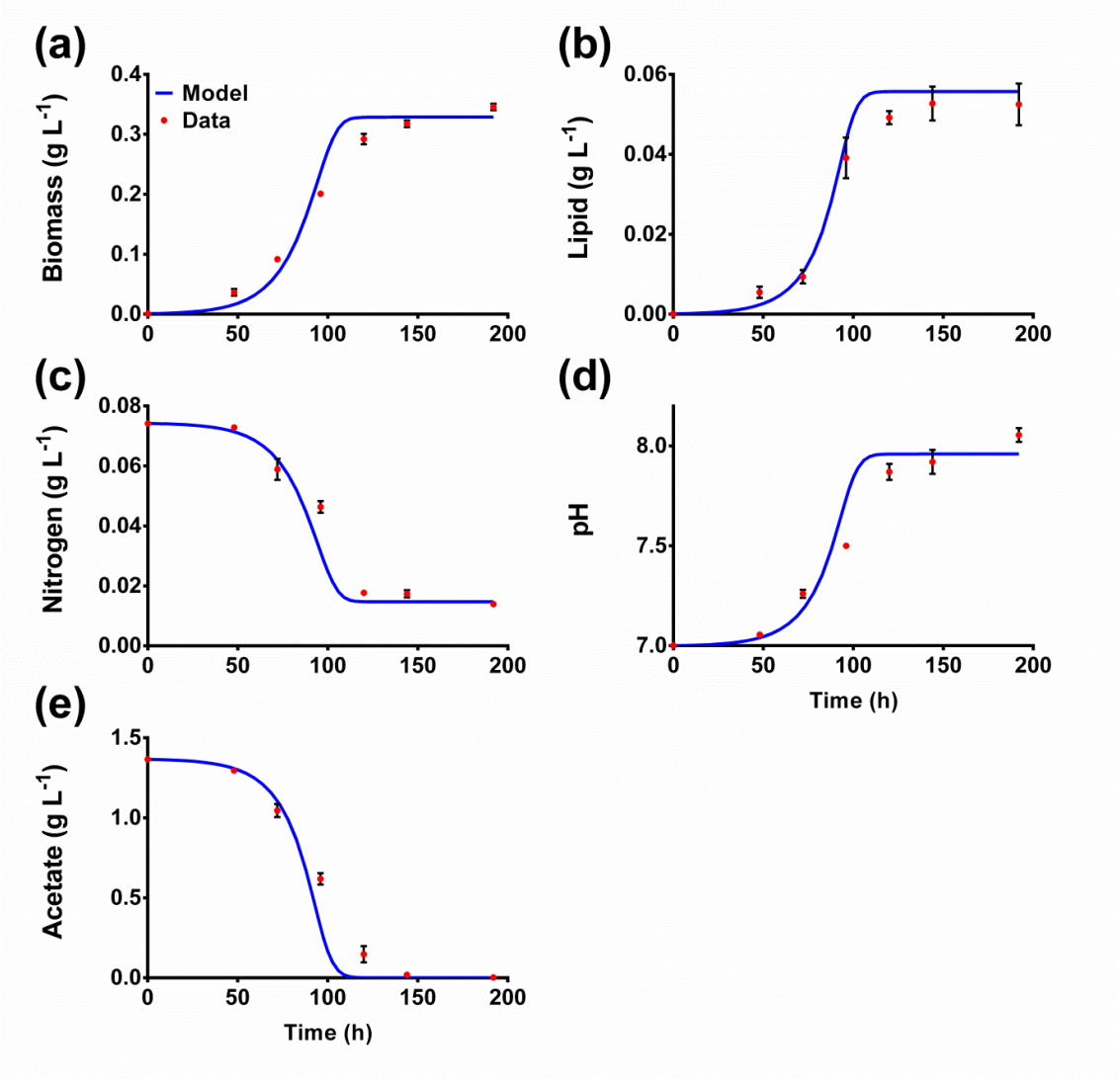

Fig. 5. Comparison of model predictions (lines) with experimental data from Experiment 3 (symbols with error bars) for: (A) biomass, (B) lipid concentration, (C) substrate (acetate) consumption, (D) N consumption, (E) pH change. All data are mean ± SE values of 2-3 biological replicates.

4.3. Process optimization

We employed our validated kinetic model in an optimization study to compute optimal operating light intensity and temperature using the optimal acetate and nitrogen concentrations (2.19 g L^{-1} acetate and 0.074 g L^{-1} nitrogen) computed previously [18] for maximum biomass and lipid productivity. The objective was to maximise the sum of lipid, J_L , and biomass, J_X, productivities subject to the model equations (Eq. 7 to 11):

$$\text{Objective} = \max(J_L + J_X) \qquad \text{Eq. 15}$$

The productivities are defined as:

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

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

Here J_L is the lipid productivity (mg L^{-1} s^{-1}), J_X is the biomass productivity (mg L^{-1} s^{-1}),, L is the final lipid concentration (mg Lipid L^{-1}) calculated by Eq.8, 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.7 and X_0 is the initial biomass concentration (mg Biomass L^{-1}).

The operating light intensity and temperature were degrees of freedom in the optimization process. The resulting optimal operating conditions for light intensity and temperature are $130\ \mu Em^{-2}\ s^{-1}$ and 24ºC, respectively (also tabulated in Table 3). The corresponding optimum lipid productivity is 0.0942 g L^{-1}, which represents an increase of 50.9 % from the Base Case (see Table 3) and an increase of 13.6% compared to the optimal case computed in [18]. We subsequently validated experimentally the computed optimal operating conditions. The computed optimal time profiles along with the corresponding experimental results obtained at the same conditions are depicted in Fig. 6. The model predictions are in excellent agreement with the experimental results, which illustrates the usefulness of our kinetic model for the optimal design of experiments.

Table 3: Computed optimal conditions and maximum productivities.

Initial Conditions	**Base case runs** [18]	**Optimization runs** [18]	**Optimization runs** (This study)		
Biomass concentration	0.001 g L^{-1}	0.005 g L^{-1}	0.005 g L^{-1}		
Acetate concentration	2.1 g L^{-1}	2.19 $g\ L^{-1}$	2.19 g L^{-1}		
Nitrogen concentration	0.098 g L^{-1}	0.074 g L^{-1}	0.074 g L^{-1}		
Light Intensity	125 µEm^{-2} s^{-1}	125 µEm^{-2} s^{-1}	130 µEm^{-2} s^{-1}		
Temperature	25 ºC	25 ºC	24 ºC		
Resulted measurements	**Base case results** [18]	**Optimization results** [18]	**Experimental Results** [18]	**Optimization results (This study)**	**Experimental results (This study)**
Lipid concentration	62.4 mg L^{-1}	82.9 mg L^{-1}	84.7 mg L^{-1}	94.2 mg L^{-1}	93.3 mg L^{-1}
Lipid productivity	7.8 mg L^{-1} d^{-1}	10.362 mg L^{-1} d^{-1}	10.5875 mg L^{-1} d^{-1}	11.775 mg L^{-1} d^{-1}	11.662 mg L^{-1} d^{-1}
Biomass concentration	586.8 mg L^{-1}	498.4 mg L^{-1}	458.6 mg L^{-1}	399.8 mg L^{-1}	415 mg L^{-1}
Biomass productivity	73.85 mg L^{-1} d^{-1}	62.3 mg L^{-1} d^{-1}	57.325 mg L^{-1} d^{-1}	49.975 mg L^{-1} d^{-1}	51.875 mg L^{-1} d^{-1}

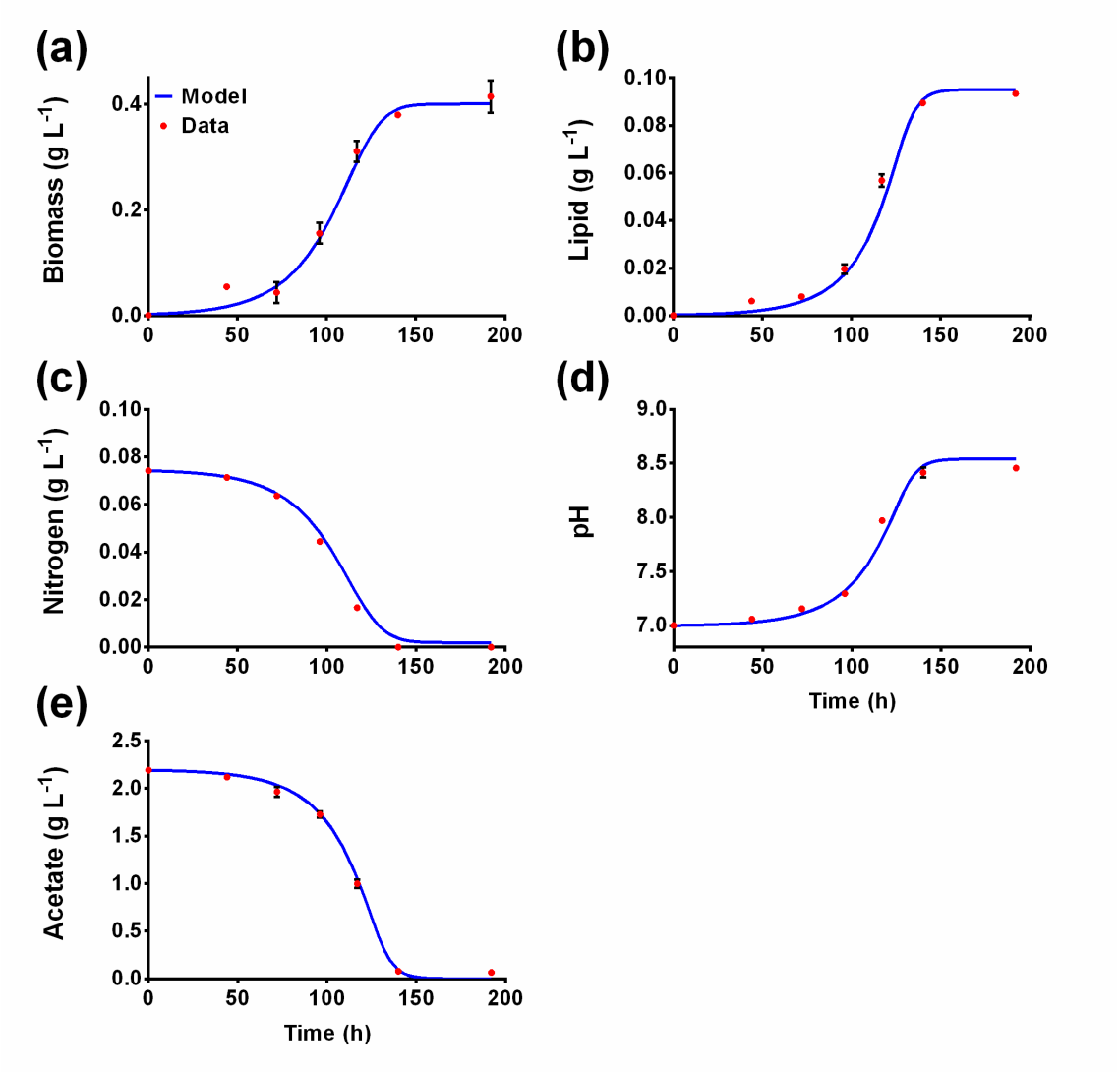

Fig. 6. Comparison of computed optimal system dynamics (lines) with experimental data (symbols with error bars) at the same conditions for: (A) biomass, (B) lipid concentration, (C) substrate (acetate) consumption, (D) N consumption, (E) pH change, using 2.19 g L^{-1} acetate and 0.074 g L^{-1} N. All data are mean ± SE values of 2-3 biological replicates.

Coupled kinetic modelling and experimental design of microalgal cultivation is a powerful tool to predict the interactions between biomass growth, lipid accumulation and environmental growth-limiting factors. A detailed, experimentally validated kinetic model can be used in optimization studies to compute optimal growth conditions that yield maximum biomass and lipid productivities. A few studies have attempted to develop a kinetic model for microalgal biomass growth [25, 46, 47] but not all of them consider the simultaneous and antagonistic effects of substrate concentration, nitrogen starvation, light intensity and temperature. Additionally, these studies did not consider lipid production as a different state variable to take advantage of

nutrient stress, which results in higher lipid accumulation. Solimeno et al. [25] developed a mechanistic model which considers inorganic carbon limitation, nitrogen availability, temperature and light intensity effects but the study only simulated biomass growth, considering that lipid accumulation is proportional to it, which does not allow optimization of biomass and lipid productivities individually. Consequently, previously developed kinetic models do not allow the accurate prediction of microalgal cultivation system dynamics under realistic operating conditions. The detailed model developed and validated in this study is a fully quadruple expression considering co-limitation of acetate (carbon substrate), nitrogen, light intensity and temperature. It also contains separate expressions for biomass growth and lipid accumulation, which allows the optimization of individual productivities to be carried out.

Conclusions

In order to understand the synergistic interactions between substrate, nutrients and environmental factors, we developed a comprehensive kinetic model considering the effect of four different culture variables (C, N, I, T) to accurately predict the dynamic behavior of 5 system state variables, with the aim to develop sustainable high productivity algae systems for the commercial biofuel production. We have conducted a range of experiments for different light intensities and temperatures to investigate the effect of environmental variables on both biomass growth and lipid accumulation. The kinetic parameters of the model were computed by fitting the model outputs to the produced experimental data. Model predictions were subsequently validated through comparisons to different sets of experiments. An optimization study was carried out to identify optimal light intensity and temperature conditions that result in maximum biomass and lipid productivities. It was found that the computed optimal lipid productivity increased by 50.9 % compared to a non-optimal base case, and by 13.6% and compared to a previously computed optimal case. This illustrates not only the usefulness of computer-based optimization studies for the improvement of microalgal-based lipid production, but also the effectiveness of carefully constructed kinetic models for the efficient operation and control of microalgae culture systems. Such predictive modelling approaches can be exploited for the robust design, control and optimization of microalgal oil accumulation as well as for process scale-up, which can help to reduce overall production costs and bring this important technology one step closer to commercialization and industrialization.

Availability of Data and Materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

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Highlights:

We develop a new quadruple substrate kinetic model of microalgal cultivation

The model accounts for substrate, nutrient, temperature & light intensity variations.

We have experimentally validated the model through a series of lab-scale experiments

We have used the model to compute optimal conditions to maximise lipid productivity

We have validated experimentally the computed optimal growth parameters

Bibliography

1. Gangl D, Zedler JAZ, Rajakumar PD, Martinez EMR, Riseley A, Włodarczyk A, et al. Biotechnological exploitation of microalgae. Journal of Experimental Botany. 2015;66(22):6975-90.

2. Liu B, Benning C. Lipid metabolism in microalgae distinguishes itself. Current Opinion in Biotechnology. 2013;24(2):300-9.

3. Wijffels RH, Barbosa MJ. An Outlook on Microalgal Biofuels. Science. 2010;329(5993):796-9.

4. Adesanya VO, Davey MP, Scott SA, Smith AG. Kinetic modelling of growth and storage molecule production in microalgae under mixotrophic and autotrophic conditions. Bioresource Technology. 2014;157(0):293-304.

5. Bajhaiya AK, Dean AP, Driver T, Trivedi DK, Rattray NJW, Allwood JW, et al. High-throughput metabolic screening of microalgae genetic variation in response to nutrient limitation. Metabolomics. 2016;12(1):1-14.

6. Converti A, Casazza AA, Ortiz EY, Perego P, Del Borghi M. Effect of temperature and nitrogen concentration on the growth and lipid content of Nannochloropsis oculata and Chlorella vulgaris for biodiesel production. Chemical Engineering and Processing: Process Intensification. 2009;48(6):1146-51.

7. Cheng Y, Lu Y, Gao C, Wu Q. Alga-Based Biodiesel Production and Optimization Using Sugar Cane as the Feedstock. Energy & Fuels. 2009;23(8):4166-73.

8. Driver T, Bajhaiya A, Pittman JK. Potential of Bioenergy Production from Microalgae. Current Sustainable/Renewable Energy Reports. 2014;1(3):94-103.

9. Acién FG, Fernández JM, Magán JJ, Molina E. Production cost of a real microalgae production plant and strategies to reduce it. Biotechnology Advances. 2012;30(6):1344-53.

10. Chen F, Johns MR. Substrate inhibition of Chlamydomonas reinhardtii by acetate in heterotrophic culture. Process Biochemistry. 1994;29(4):245-52.

11. Breuer G, Lamers PP, Janssen M, Wijffels RH, Martens DE. Opportunities to improve the areal oil productivity of microalgae. Bioresource Technology. 2015;186(0):294-302.

12. Zhu S, Huang W, Xu J, Wang Z, Xu J, Yuan Z. Metabolic changes of starch and lipid triggered by nitrogen starvation in the microalga Chlorella zofingiensis. Bioresource Technology. 2014;152(0):292-8.

13. Wang ZT, Ullrich N, Joo S, Waffenschmidt S, Goodenough U. Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starchless Chlamydomonas reinhardtii. Eukaryotic cell. 2009;8(12):1856-68.

14. Fan J, Yan C, Andre C, Shanklin J, Schwender J, Xu C. Oil accumulation is controlled by carbon precursor supply for fatty acid synthesis in Chlamydomonas reinhardtii. Plant and Cell Physiology. 2012;53(8):1380-90.

15. Singh B, Guldhe A, Singh P, Singh A, Rawat I, Bux F. Sustainable Production of Biofuels from Microalgae Using a Biorefinary Approach. In: Kaushik G, editor. Applied Environmental Biotechnology: Present Scenario and Future Trends: Springer India; 2015. p. 115-28.

16. Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, et al. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J. 2008;54.

17. Koskimaki JE, Blazier AS, Clarens AF, Papin JA. Computational models of algae metabolism for industrial applications. Industrial Biotechnology. 2013;9(4):185-95.

18. Bekirogullari M, Fragkopoulos IS, Pittman JK, Theodoropoulos C. Production of lipid-based fuels and chemicals from microalgae: An integrated experimental and model-based optimization study. Algal Research. 2017;23:78-87.

19. Figueroa-Torres GM, Pittman J, Theodoropoulos C. Kinetic Modelling of Starch and Lipid Formation during Mixotrophic, Nutrient-limited Microalgal Growth Bioresource Technology. 2017;Submitted.

20. Yao B, Xi B, Hu C, Huo S, Su J, Liu H. A model and experimental study of phosphate uptake kinetics in algae: Considering surface adsorption and P-stress. Journal of Environmental Sciences. 2011;23(2):189-98.

21. Eriksen NT, Riisgård FK, Gunther WS, Lønsmann Iversen JJ. On-line estimation of O2 production, CO2 uptake, and growth kinetics of microalgal cultures in a gas-tight photobioreactor. J Appl Phycol. 2006;19(2):161-74.

22. Zhang XW, Zhang YM, Chen F. Application of mathematical models to the determination optimal glucose concentration and light intensity for mixotrophic culture of Spirulina platensis. Process Biochemistry. 1999;34(5):477-81.

23. Goldman JC, Carpenter EJ. A kinetic approach to the effect of temperature on algal growth1. Limnology and Oceanography. 1974;19(5):756-66.

24. Rhee GY. Effects of N:P atomic ratios and nitrate limitation on algal growth, cell composition, and nitrate uptake 1. Limnology and Oceanography. 1978;23(1):10-25.

25. Solimeno A, Samsó R, Uggetti E, Sialve B, Steyer J-P, Gabarró A, et al. New mechanistic model to simulate microalgae growth. Algal Research. 2015;12:350-8.

26. Shi X-M, Zhang X-W, Chen F. Heterotrophic production of biomass and lutein by Chlorella protothecoides on various nitrogen sources. Enzyme and Microbial Technology. 2000;27(3–5):312-8.

27. Béchet Q, Shilton A, Guieysse B. Modeling the effects of light and temperature on algae growth: State of the art and critical assessment for productivity prediction during outdoor cultivation. Biotechnology Advances. 2013;31(8):1648-63.

28. Ota M, Takenaka M, Sato Y, Jr RLS, Inomata H. Effects of light intensity and temperature on photoautotrophic growth of a green microalga, Chlorococcum littorale. Biotechnology Reports. 2015;7:24-9.

29. Lee E, Jalalizadeh M, Zhang Q. Growth kinetic models for microalgae cultivation: A review. Algal Research. 2015;12:497-512.

30. Harris EH. The Chlamydomonas Sourcebook: A Comprehensive Guide to Biology and Laboratory Use. San Diego: Academic Press, Inc; 1989.

31. Bitaubé Pérez E, Caro Pina I, Pérez Rodríguez L. Kinetic model for growth of Phaeodactylum tricornutum in intensive culture photobioreactor. Biochemical Engineering Journal. 2008;40(3):520-5.

32. Zhang X-W, Chen F, Johns MR. Kinetic models for heterotrophic growth of Chlamydomonas reinhardtii in batch and fed-batch cultures. Process Biochemistry. 1999;35(3–4):385-9.

33. Vlysidis A, Binns M, Webb C, Theodoropoulos C. Glycerol utilisation for the production of chemicals: Conversion to succinic acid, a combined experimental and computational study. Biochemical Engineering Journal. 2011;58–59(0):1-11.

34. Gordillo FJL, Goutx M, Figueroa FL, Niell FX. Effects of light intensity, CO2 and nitrogen supply on lipid class composition of Dunaliella viridis. J Appl Phycol. 1998;10(2):135-44.

35. Fouchard S, Pruvost J, Degrenne B, Titica M, Legrand J. Kinetic modeling of light limitation and sulfur deprivation effects in the induction of hydrogen production with Chlamydomonas reinhardtii: Part I. Model development and parameter identification. Biotechnology and Bioengineering. 2009;102(1):232-45.

36. Pottier L, Pruvost J, Deremetz J, Cornet JF, Legrand J, Dussap CG. A fully predictive model for one-dimensional light attenuation by Chlamydomonas reinhardtii in a torus photobioreactor. Biotechnology and Bioengineering. 2005;91(5):569-82.

37. Janssen M, de Bresser L, Baijens T, Tramper J, Mur LR, Snel JFH, et al. Scale-up aspects of photobioreactors: effects of mixing-induced light/dark cycles. J Appl Phycol. 2000;12(3):225-37.

38. Sousa AI, Martins I, Lillebø AI, Flindt MR, Pardal MA. Influence of salinity, nutrients and light on the germination and growth of Enteromorpha sp. spores. Journal of Experimental Marine Biology and Ecology. 2007;341(1):142-50.

39. Yeesang C, Cheirsilp B. Effect of nitrogen, salt, and iron content in the growth medium and light intensity on lipid production by microalgae isolated from freshwater sources in Thailand. Bioresource Technology. 2011;102(3):3034-40.

40. Ruangsomboon S. Effect of light, nutrient, cultivation time and salinity on lipid production of newly isolated strain of the green microalga, Botryococcus braunii KMITL 2. Bioresource Technology. 2012;109:261-5.

41. Bernard O, Rémond B. Validation of a simple model accounting for light and temperature effect on microalgal growth. Bioresource Technology. 2012;123(0):520-7.

42. James GO, Hocart CH, Hillier W, Price GD, Djordjevic MA. Temperature modulation of fatty acid profiles for biofuel production in nitrogen deprived Chlamydomonas reinhardtii. Bioresource Technology. 2013;127:441-7.

43. Aleya L, Dauta A, Reynolds CS. Endogenous regulation of the growth-rate responses of a spring-dwelling strain of the freshwater alga, Chlorella minutissima, to light and temperature. European Journal of Protistology. 2011;47(4):239-44.

44. Maxwell DP, Falk S, Trick CG, Huner N. Growth at Low Temperature Mimics High-Light Acclimation in Chlorella vulgaris. Plant physiology. 1994;105(2):535-43.

45. Yoshimura T, Okada S, Honda M. Culture of the hydrocarbon producing microalga Botryococcus braunii strain Showa: Optimal CO2, salinity, temperature, and irradiance conditions. Bioresource Technology. 2013;133:232-9.

46. Yoo SJ, Kim JH, Lee JM. Dynamic modelling of mixotrophic microalgal photobioreactor systems with time-varying yield coefficient for the lipid consumption. Bioresource Technology. 2014;162:228-35.

47. He L, Subramanian VR, Tang YJ. Experimental analysis and model-based optimization of microalgae growth in photo-bioreactors using flue gas. Biomass and Bioenergy. 2012;41:131-8.

SUPPLEMENTARY INFORMATION

Appendix A. Detailed explanation of light distribution in the reactor from the experimental data

In the case of a planar geometry with a perpendicular light source to the reactor surface, Beer-lambert approximation is commonly employed for the irradiance distribution in the reactor with an exponential decrease:

$$I(l) = I_0.\exp(-\xi l) \quad \text{Eq. A.1}$$

Where light attenuation coefficient is related to biomass, chlorophyll and background turbidity;

$$\xi = (a + b0)X + c \quad \text{Eq. A.2}$$

The optical depth of the Beer-lambert law can be define as: $\lambda = \xi l$, and it reflects how efficiently light energy is absorbed by the growth medium.

$$\lambda = ln\frac{I_0}{I(L)} \quad \text{Eq. A.3}$$

The average irradiance absorbed by culture medium can be expressed as:

$$\bar{I} = \frac{I_0}{L}\int_0^L \exp(-\xi l)dz = \frac{I_0}{\lambda}[1 - \exp(-\lambda)] \quad \text{Eq. A.4}$$

Combination of two previous equations (Eq. A.3 and Eq. A.4) gives:

$$\frac{\bar{I}}{I_0} = \frac{\frac{I(L)}{I_0} - 1}{ln\left(\frac{I(L)}{I_0}\right)} \quad \text{Eq. A.5}$$

By using the latter expression and in-house produced experimental, the following graph has been generated to compare the average irradiance versus irradiance from Beer-Lambert approximation.

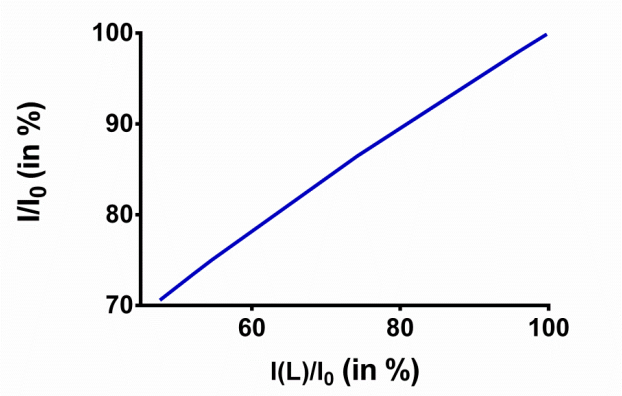

Fig. A.1 Comparison of average irradiance versus irradiance from Beer-Lambert approximation

Appendix B. Sensitivity analysis

A sensitivity analysis was carried out for the kinetic model proposed in this work which consists of 26 parameters. The analysis was performed by calculating the sensitivity (Eq. (A.1)), for all 5 dynamic variables with respect to each parameter at eight different cultivation times (t=25h, 50h, 75h, 85h, 125h, 150h, 175h and 190h).

$$Sensitivity = abs\left(\frac{Prediction(t, P + \Delta P) - Prediction(t, P - \Delta P)}{2 * \Delta P}\right) \qquad \text{Eq. A.1}$$

Sensitivity of the parameters was computed with a %10 change (ΔP) in parameters values. The sensitivity analysis results of 26 kinetic parameters are presented in Table A.1. The threshold for sensitivity was set to 0.01, meaning parameters with sensitivities lower than 0.01 were considered not-sensitive and sensitivities higher than 0.01 deemed to be sensitive and they are highlighted.

Table B.1. Sensitivity analysis results of the proposed model kinetic parameters.

			Sensitivity							
Parameter	**Value**	**Variable**	**25h**	**50h**	**75h**	**85h**	**125h**	**150h**	**175h**	**190h**
K_{XI}	6.01	X	0.0001	0.0009	0.0046	0.0061	0.0010	0.0010	0.0010	0.0010
		L	0.0000	0.0001	0.0007	0.0014	0.0000	0.0000	0.0000	0.0000
		AA	0.0002	0.0021	0.0167	0.0330	0.0000	0.0000	0.0000	0.0000
		N	0.0000	0.0002	0.0008	0.0011	0.0002	0.0002	0.0002	0.0002
		pH	0.0001	0.0015	0.0118	0.0232	0.0000	0.0000	0.0000	0.0000
K_{iXI}	234	X	0.0040	0.0041	0.0043	0.0045	0.0049	0.0045	0.0040	0.0040
		L	0.0040	0.0040	0.0040	0.0040	0.0043	0.0042	0.0040	0.0040
		AA	0.0040	0.0041	0.0047	0.0052	0.0110	0.0083	0.0040	0.0040
		N	0.0040	0.0040	0.0041	0.0041	0.0042	0.0041	0.0040	0.0040
		pH	0.0040	0.0041	0.0045	0.0049	0.0089	0.0070	0.0040	0.0040
K_{LI}	5.221	X	0.0000	0.0000	0.0000	0.0001	0.0010	0.0010	0.0010	0.0010
		L	0.0000	0.0000	0.0002	0.0006	0.0000	0.0000	0.0000	0.0000
		AA	0.0001	0.0007	0.0047	0.0115	0.0000	0.0000	0.0000	0.0000
		N	0.0000	0.0000	0.0000	0.0000	0.0002	0.0002	0.0002	0.0002
		pH	0.0001	0.0005	0.0033	0.0081	0.0000	0.0000	0.0000	0.0000
K_{iLI}	224	X	0.0090	0.0090	0.0090	0.0090	0.0090	0.0090	0.0090	0.0090
		L	0.0090	0.0090	0.0090	0.0090	0.0091	0.0090	0.0090	0.0090
		AA	0.0090	0.0091	0.0092	0.0093	0.0106	0.0091	0.0090	0.0090
		N	0.0090	0.0090	0.0090	0.0090	0.0090	0.0090	0.0090	0.0090
		pH	0.0090	0.0090	0.0091	0.0092	0.0101	0.0091	0.0090	0.0090
σ	10	X	0.0000	0.0001	0.0016	0.0024	0.0001	0.0001	0.0001	0.0001
		L	0.0000	0.0000	0.0003	0.0008	0.0000	0.0000	0.0000	0.0000
		AA	0.0000	0.0005	0.0075	0.0179	0.0000	0.0000	0.0000	0.0000
		N	0.0000	0.0000	0.0003	0.0004	0.0000	0.0000	0.0000	0.0000
		pH	0.0000	0.0003	0.0053	0.0126	0.0000	0.0000	0.0000	0.0000
A_{0X}	0.618	X	0.0531	0.4530	1.9295	2.0576	0.2701	0.2434	0.2434	0.2434
		L	0.0029	0.0387	0.3324	0.5233	0.0062	0.0106	0.0106	0.0106
		AA	0.1021	1.1406	8.1328	11.9709	0.1087	0.0001	0.0000	0.0000
		N	0.0096	0.0823	0.3504	0.3737	0.0491	0.0442	0.0442	0.0442
		pH	0.0718	0.8021	5.7190	8.4180	0.0764	0.0001	0.0000	0.0000
A_{0L}	0.7	X	0.0000	0.0004	0.0074	0.0126	0.1254	0.1260	0.1260	0.1260
		L	0.0018	0.0099	0.0526	0.1059	0.0057	0.0055	0.0055	0.0055
		AA	0.0354	0.1943	1.0345	2.0797	0.0052	0.0000	0.0000	0.0000
		N	0.0000	0.0001	0.0013	0.0023	0.0228	0.0229	0.0229	0.0229
		pH	0.0249	0.1366	0.7275	1.4625	0.0037	0.0000	0.0000	0.0000
μ_{Xmax}	0.223	X	0.0745	0.6221	2.9382	3.4163	0.3326	0.3278	0.3278	0.3278
		L	0.0041	0.0532	0.4553	0.8836	0.0133	0.0143	0.0143	0.0143
		AA	0.1435	1.5682	11.3911	20.1636	0.0221	0.0000	0.0000	0.0000
		N	0.0135	0.1130	0.5336	0.6204	0.0604	0.0595	0.0595	0.0595
		pH	0.1009	1.1027	8.0102	14.1791	0.0156	0.0000	0.0000	0.0000
K_{XS}	0.06	X	0.0063	0.0528	0.2745	0.3611	0.1901	0.1898	0.1898	0.1898
		L	0.0003	0.0045	0.0394	0.0847	0.0082	0.0083	0.0083	0.0083
		AA	0.0121	0.1325	1.0027	1.9611	0.0022	0.0000	0.0000	0.0000
		N	0.0011	0.0096	0.0499	0.0656	0.0345	0.0345	0.0345	0.0345
		pH	0.0085	0.0932	0.7051	1.3790	0.0015	0.0000	0.0000	0.0000
K_{iXS}	11.91	X	0.0002	0.0017	0.0081	0.0095	0.0004	0.0004	0.0004	0.0004
		L	0.0000	0.0001	0.0012	0.0025	0.0000	0.0000	0.0000	0.0000
		AA	0.0004	0.0044	0.0308	0.0570	0.0000	0.0000	0.0000	0.0000
		N	0.0000	0.0003	0.0015	0.0017	0.0001	0.0001	0.0001	0.0001

		pH	0.0003	0.0031	0.0217	0.0401	0.0000	0.0000	0.0000	0.0000
K_{XN}	0.078	X	0.1046	0.8825	4.4855	5.5402	0.6991	0.6952	0.6952	0.6952
		L	0.0058	0.0749	0.6603	1.3742	0.0295	0.0303	0.0303	0.0303
		AA	0.2017	2.2139	16.7097	31.5506	0.0183	0.0000	0.0000	0.0000
		N	0.0190	0.1603	0.8146	1.0061	0.1270	0.1263	0.1263	0.1263
		pH	0.1418	1.5568	11.7503	22.1864	0.0129	0.0000	0.0000	0.0000
K_{iXN}	0.7	X	0.0012	0.0093	0.0405	0.0454	0.0013	0.0013	0.0013	0.0013
		L	0.0001	0.0008	0.0064	0.0127	0.0001	0.0001	0.0001	0.0001
		AA	0.0022	0.0236	0.1593	0.2871	0.0001	0.0000	0.0000	0.0000
		N	0.0002	0.0017	0.0074	0.0082	0.0002	0.0002	0.0002	0.0002
		pH	0.0016	0.0166	0.1120	0.2019	0.0001	0.0000	0.0000	0.0000
q_{Lmax}	0.145	X	0.0001	0.0017	0.0284	0.0503	0.4872	0.4894	0.4894	0.4894
		L	0.0070	0.0384	0.2036	0.4104	0.0221	0.0213	0.0213	0.0213
		AA	0.1368	0.7515	4.0011	8.0589	0.0172	0.0000	0.0000	0.0000
		N	0.0000	0.0003	0.0052	0.0091	0.0885	0.0889	0.0889	0.0889
		pH	0.0962	0.5284	2.8136	5.6671	0.0121	0.0000	0.0000	0.0000
K_{LS}	5.243	X	0.0000	0.0000	0.0001	0.0002	0.0071	0.0072	0.0072	0.0072
		L	0.0000	0.0001	0.0009	0.0025	0.0003	0.0003	0.0003	0.0003
		AA	0.0005	0.0026	0.0178	0.0485	0.0003	0.0000	0.0000	0.0000
		N	0.0000	0.0000	0.0000	0.0000	0.0013	0.0013	0.0013	0.0013
		pH	0.0003	0.0019	0.0125	0.0341	0.0002	0.0000	0.0000	0.0000
K_{iLS}	0.132	X	0.0000	0.0015	0.0253	0.0437	0.2302	0.2307	0.2307	0.2307
		L	0.0064	0.0347	0.1754	0.3260	0.0102	0.0100	0.0100	0.0100
		AA	0.1247	0.6796	3.4468	6.4044	0.0042	0.0000	0.0000	0.0000
		N	0.0000	0.0003	0.0046	0.0079	0.0418	0.0419	0.0419	0.0419
		pH	0.0877	0.4779	2.4238	4.5036	0.0029	0.0000	0.0000	0.0000
K_{iNL}	442.1	X	2.80E-12	9.05E-11	1.38E-09	2.25E-09	9.94E-09	9.96E-09	9.96E-09	9.96E-09
		L	3.85E-10	2.02E-09	8.67E-09	1.42E-08	4.42E-10	4.34E-10	4.34E-10	4.34E-10
		AA	7.53E-09	3.95E-08	1.71E-07	2.80E-07	1.81E-10	2.73E-13	1.01E-14	6.48E-16
		N	5.09E-13	1.64E-11	2.51E-10	4.09E-10	1.80E-09	1.81E-09	1.81E-09	1.81E-09
		pH	5.30E-09	2.78E-08	1.20E-07	1.97E-07	1.27E-10	1.59E-13	2.58E-14	3.33E-14
$Y_{X/S}$	1.176	X	0.0000	0.0001	0.0012	0.0020	0.0108	0.0108	0.0108	0.0108
		L	0.0000	0.0000	0.0008	0.0023	0.0141	0.0141	0.0141	0.0141
		AA	0.0064	0.0340	0.1512	0.2517	0.0003	0.0000	0.0000	0.0000
		N	0.0000	0.0000	0.0002	0.0004	0.0020	0.0020	0.0020	0.0020
		pH	0.0045	0.0239	0.1063	0.1770	0.0002	0.0000	0.0000	0.0000
$Y_{X/N}$	5.506	X	0.0000	0.0004	0.0093	0.0267	0.0554	0.0554	0.0554	0.0554
		L	0.0000	0.0000	0.0007	0.0029	0.0024	0.0024	0.0024	0.0024
		AA	0.0000	0.0008	0.0223	0.0797	0.0004	0.0000	0.0000	0.0000
		N	0.0003	0.0014	0.0044	0.0044	0.0028	0.0028	0.0028	0.0028
		pH	0.0000	0.0005	0.0157	0.0560	0.0003	0.0000	0.0000	0.0000
K_H	0.703	X	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
		L	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
		AA	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
		N	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
		pH	0.0272	0.1454	0.6780	1.2282	2.1905	2.1906	2.1906	2.1906
$Y_{L/S}$	0.051	X	0.0001	0.0048	0.0816	0.1392	1.4194	1.4251	1.4251	1.4251
		L	0.0001	0.0022	0.0565	0.1751	1.7956	1.7978	1.7978	1.7978
		AA	0.3918	2.1536	11.4873	23.1130	0.0429	0.0001	0.0000	0.0000
		N	0.0000	0.0009	0.0148	0.0253	0.2578	0.2588	0.2588	0.2588
		pH	0.2755	1.5144	8.0779	16.2531	0.0301	0.0001	0.0000	0.0000
E_{aX}	25.92	X	0.0015	0.0135	0.0528	0.0532	0.0080	0.0067	0.0067	0.0067
		L	0.0001	0.0012	0.0098	0.0135	0.0001	0.0003	0.0003	0.0003
		AA	0.0029	0.0340	0.2373	0.3092	0.0052	0.0000	0.0000	0.0000
		N	0.0003	0.0025	0.0096	0.0097	0.0015	0.0012	0.0012	0.0012
		pH	0.0021	0.0239	0.1668	0.2174	0.0036	0.0000	0.0000	0.0000
E_{aL}	20	X	0.0000	0.0000	0.0002	0.0004	0.0041	0.0041	0.0041	0.0041
		L	0.0001	0.0003	0.0017	0.0034	0.0002	0.0002	0.0002	0.0002
		AA	0.0011	0.0062	0.0332	0.0667	0.0001	0.0000	0.0000	0.0000
		N	0.0000	0.0000	0.0000	0.0001	0.0007	0.0007	0.0007	0.0007
		pH	0.0008	0.0044	0.0233	0.0469	0.0001	0.0000	0.0000	0.0000
B_{oX}	0.11	X	0.1440	1.2025	5.6964	6.6471	0.6417	0.6331	0.6331	0.6331
		L	0.0079	0.1029	0.8797	1.7184	0.0259	0.0276	0.0276	0.0276
		AA	0.2776	3.0315	22.0260	39.2147	0.0401	0.0000	0.0000	0.0000
		N	0.0262	0.2184	1.0345	1.2072	0.1165	0.1150	0.1150	0.1150
		pH	0.1952	2.1317	15.4887	27.5758	0.0282	0.0000	0.0000	0.0000
E_{bX}	48.02	X	0.0007	0.0060	0.0257	0.0285	0.0048	0.0037	0.0037	0.0037
		L	0.0000	0.0005	0.0043	0.0070	0.0000	0.0002	0.0002	0.0002
		AA	0.0014	0.0151	0.1061	0.1610	0.0046	0.0000	0.0000	0.0000
		N	0.0001	0.0011	0.0047	0.0052	0.0009	0.0007	0.0007	0.0007
		pH	0.0010	0.0106	0.0746	0.1132	0.0032	0.0000	0.0000	0.0000

B_{oL}	0.08	X	0.0000	0.0008	0.0127	0.0226	0.2197	0.2205	0.2205	0.2205
		L	0.0031	0.0172	0.0911	0.1839	0.0099	0.0096	0.0096	0.0096
		AA	0.0612	0.3361	1.7896	3.6102	0.0057	0.0000	0.0000	0.0000
		N	0.0000	0.0001	0.0023	0.0041	0.0399	0.0401	0.0401	0.0401
		pH	0.0430	0.2364	1.2584	2.5387	0.0040	0.0000	0.0000	0.0000
E_{bL}	32	X	0.0000	0.0000	0.0000	0.0001	0.0008	0.0008	0.0008	0.0008
		L	0.0000	0.0001	0.0003	0.0007	0.0000	0.0000	0.0000	0.0000
		AA	0.0002	0.0012	0.0066	0.0132	0.0000	0.0000	0.0000	0.0000
		N	0.0000	0.0000	0.0000	0.0000	0.0001	0.0001	0.0001	0.0001
		pH	0.0002	0.0009	0.0046	0.0093	0.0000	0.0000	0.0000	0.0000

Appendix C. Plots of the evolution of the various f functions used in the proposed model

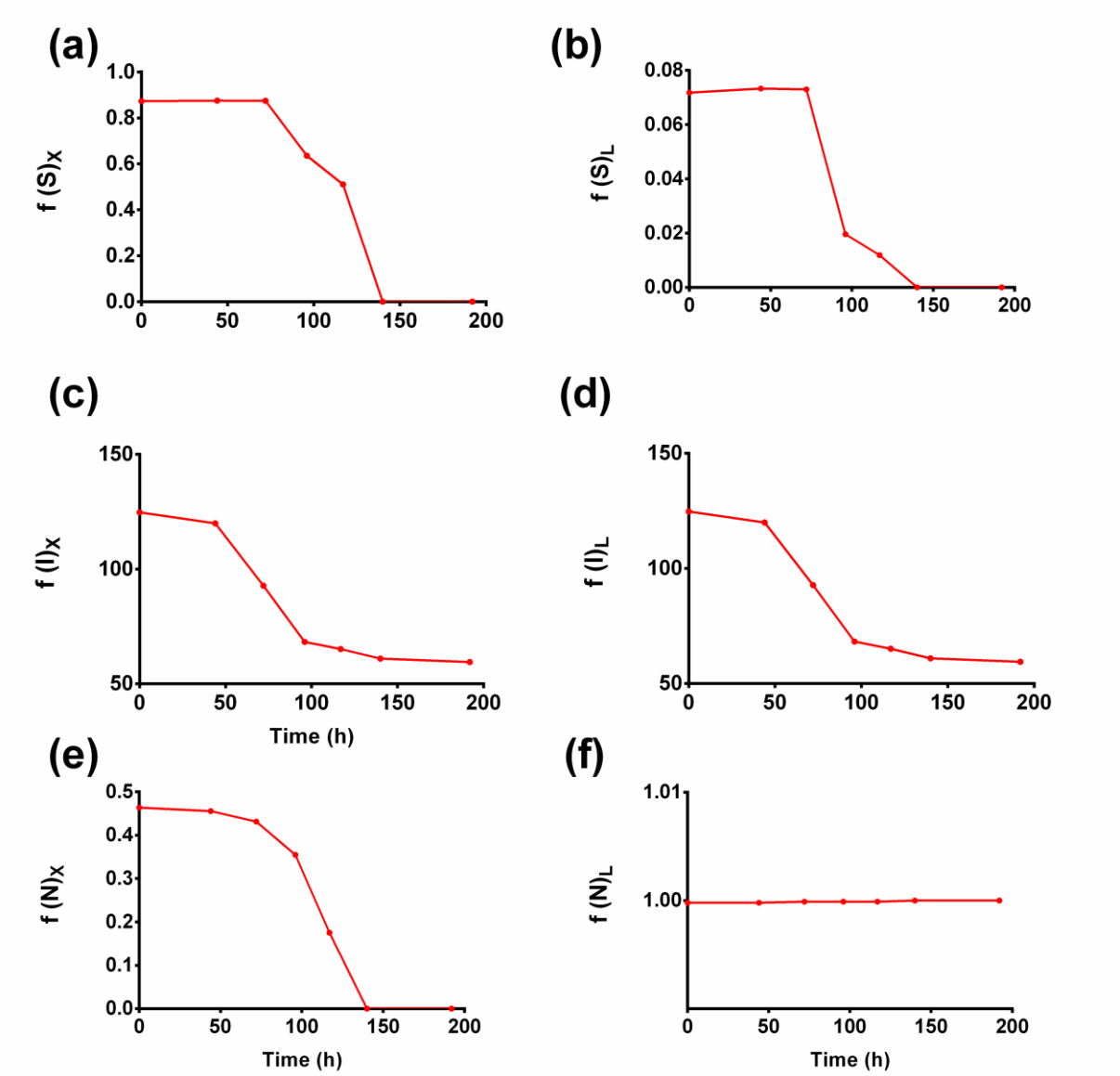

Fig. C.1 Evolution of the various f functions used in the proposed model: (a) $f(s)_X$, (b) $f(s)_L$, (c) $f(I)_X$, (d) $f(I)_L$, (e) $f(N)_X$ and (f) $f(N)_L$.

Appendix D. Comparison profiles of model results to experimental data

Two additional set of data presented here to show predectiveness of the proposed model.

Table D.1 Additional experiments used for the predectiveness of the proposed model.

Experiment	Acetate (g L^{-1})	Nitrogen (g L^{-1})	Light (µEm^{-2} s^{-1})	Temperature (^{0}C)
1	1.05	0.098	125	25
2	1.05	0.098	155	25

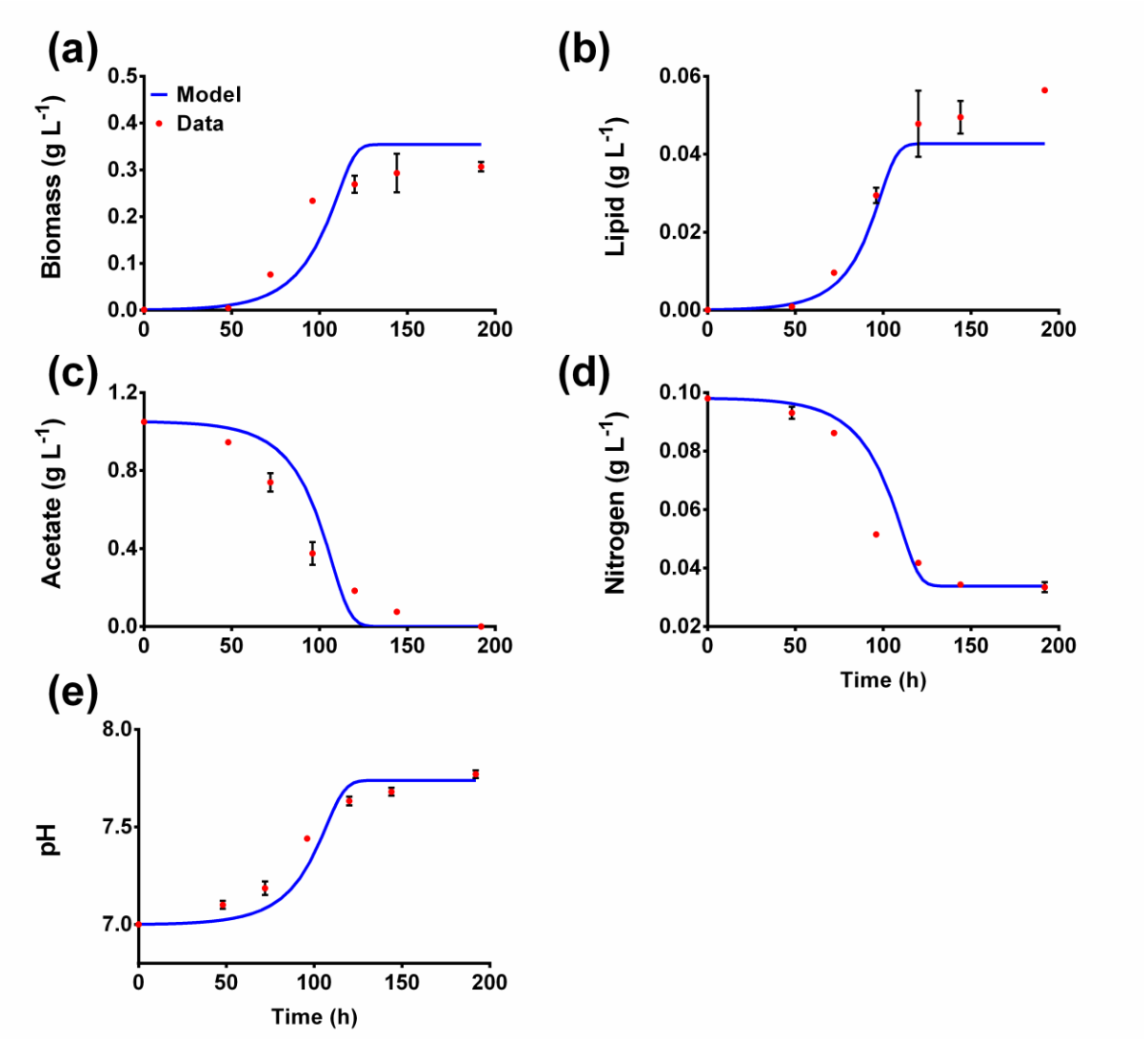

Fig. D.1 Comparison of model predictions (lines) with experimental data from Experiment 1 (symbols with error bars) for: (A) biomass, (B) lipid concentration, (C) substrate (acetate) consumption, (D) N consumption and (E) pH change. All data are mean ± SE values of 2-3 biological replicates.

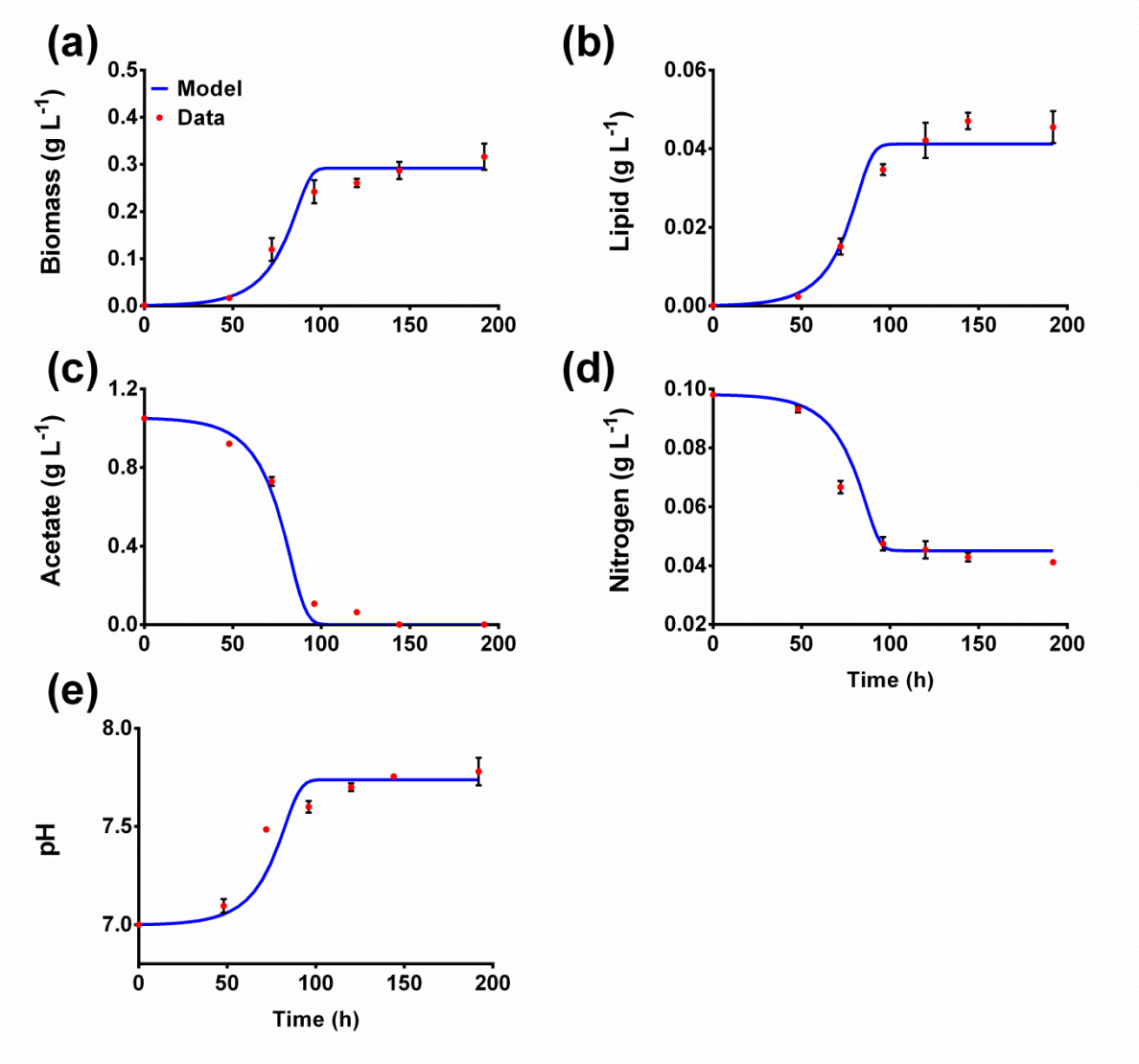

Fig. D.2 Comparison of model predictions (lines) with experimental data from Experiment 2 (symbols with error bars) for: (A) biomass, (B) lipid concentration, (C) substrate (acetate) consumption, (D) N consumption and (E) pH change. All data are mean ± SE values of 2-3 biological replicates.

Appendix E: Upper and lower bounds of the estimated parameters used in section 3.3 parameters estimation problem

The parameters that are related to substrate and nitrogen have been taken from the first paper and in order to obtain a better fitting of the model to experimental data, the parameters were allowed to change within a range of ±10%. Hence, some of the parameters are slightly different (most within 1-2%) than the ones computed in the original paper.

Table E.1 Upper and lower bounds of the estimated parameters

Parameters	Lower bounds	Upper bounds	Parameters	Lower bounds	Upper bounds
K_{XI}	6	6.05	K_{LS}	5.2432	7.8648
K_{iXI}	220	240	K_{iLS}	0.088	0.132
K_{LI}	5	6	K_{iNL}	304	456
K_{iLI}	220	240	$Y_{X/S}$	1.176	1.764
σ	8	11	$Y_{X/N}$	5.5064	8.2596
A_{0X}	0.6	0.7	K_H	0.7032	1.0548
A_{0L}	0.6	0.8	$Y_{L/S}$	0.0512	0.0768
μ_{Xmax}	0.1816	0.2724	E_{aX}	25	26
K_{XS}	0.03	0.06	E_{aL}	20	25
K_{iXS}	7.9384	11.9076	B_{oX}	0.1	0.13
K_{XN}	0.052	0.078	E_{bX}	45	50
K_{iXN}	0.4	0.7	B_{oL}	0.08	0.1
q_{Lmax}	0.0968	0.1452	E_{bL}	30	35

Appendix F: Upper and lower bounds of the estimated optimal growth conditions used in section 4.3 process optimization problem

The decision variables in the optimization problem were light intensity (I_0) and inoculum temperature (T). The upper and lower bounds of the optimization problem were set according to experimental behaviour of system.

Table F.1 Upper and lower bounds of the decision variables used in optimization problem

Parameters	Lower bounds	Upper bounds
I_0	75	250
T	293	303

Chapter 5

Kinetic Modelling and Experimental Studies of Microalgal Lipids Production in Raceway Open Ponds

5.1. Preface

Microalgae can artificially grow in photobioreactors (PBRs) and open ponds (OP) where light is provided to a growth medium, which contains essential nutrients and organic and/or inorganic carbon source enabling/driving the photosynthesis process. In comparison with open ponds, PBRs can achieve higher biomass and lipid productivities, while contamination of monocultures is easily avoided. A number of PBR and open pond designs were reviewed in Chapter 2. As described in Chapter 2, although the PBRs provide a number of technical advantages, they suffer from significantly higher operating and capital costs than open ponds. Hence, the use of open ponds for large scale microalgal cultivation is still considered to be a more practical option. Despite the practicability of open ponds, their biodiesel production cost is still too high to compete with the conventional fossil diesel one. Consequently, it is essential to improve the biomass and the lipid productivity to make the use of this technology feasible and economically viable.

As shown in the preceding chapters, kinetic modelling of microalgal cultivation has become a significant tool that can help towards the understanding of the mechanism of microalgal growth, the lipid accumulation and the utilization of essential nutrients, which can then be used to predict the process performance and optimise the operating conditions to achieve maximum biomass and lipid productivities.

The aim of this chapter is to adapt the quadruple substrate kinetic model developed in chapter 4 to be applied in large-scale raceway open ponds. The model developed in chapter 4 considered the photoheterotrophic growth of *Chlamydomonas reinhardtii*. In order to use the developed model, initially, experiments were conducted in 2 m^3 raceway open ponds which contained 500

L of Tris-acetate-phosphate (TAP) medium (32cm height, 80cm width and 2m length). The media got contaminated immediately after the inoculation and this was thought to be due to the organic carbon source which is attractive to heterotrophic microorganisms.

After establishing that we could not perform photoheterotrophic growth experiments with *C. reinhardtii* in open ponds, we decided to grow the strain photoautotrophically using a fertilizer medium consisting of diluted Wilko liquid tomato feed (100mgl^{-1} NH4-N, 55mgL^{-1} PO_4^{-3}-P). In order to achieve that, a lab-scale adaptation process was carried out prior to experimentation. The strain was adapted to photoautotrophic growth conditions by simultaneously increasing the fertilizer concentration in the growth medium and decreasing the TAP medium concentration.

Photoautotrophic microalgal growth in open ponds is mainly affected by environmental factors (light intensity and temperature) and growth media composition (nitrogen and CO_2). The detailed kinetic model that was developed in chapter 4, for photoheterotrophic growth of *C. reinhardtii* in bench-scale, was a function of carbon substrate and nitrogen concentration, light intensity and temperature. The growth of photoautotrophic algal cells is a result of photosynthesis which is affected by CO_2 and nitrogen concentration, light intensity and temperature. During the cultivation period, no organic carbon was added or CO_2 was pumped into the ponds and the algal broth utilised available atmospheric CO_2. Therefore, the influence of carbon source was reduced in the final biomass growth and lipid accumulation kinetic expressions. The resulting model is a function of nitrogen concentration, light intensity and temperature. In Chapter 3, the effect of nitrogen on lipid accumulation was expressed by Monod type equation. Under continues light irradiance and available atmospheric CO_2, the cells perform photosynthesis and produce biomass and lipids. However, there is a limit to lipid concentration within cells. In this chapter, the Monod function was replaced by Andrew equation to include nitrogen inhibition as there was not any other limiting variable for lipid accumulation.

The results and discussion of this work are presented in the following paper, where the adaptation process for *C. reinhardtii* to grow photoautotrophically and the development of the kinetic modelling framework are explained in detail.

Experimental observations such biomass growth, lipid accumulation as well as nutrient uptake and the differences between cultivation growth conditions (photoautotrophic and

photoheterotrophic) are assessed. Details about the estimation of kinetic parameter values and the optimisation study which results in the maximisation of lipid production are also explained.

K.T. and J.K.P. contemplated and supervised the research and M.B. designed the research plans, performed the research, analysed data and wrote the manuscripts.

5.2. Publication 3

Bekirogullari, M., Pittman, J. K. & Theodoropoulos, C. 2017. Kinetic Modelling and Experimental Studies of Microalgal Lipids Production in Raceway Open Ponds. Submitted to Biotechnology and Bioengineering.

Kinetic Modelling and Experimental Studies of Microalgal Lipid Production in Raceway Open Ponds

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Abstract

The use of microalgal lipids and carbohydrates as feedstock for biodiesel production has the potential to make a significant contribution to the world energy market, due to several outstanding characteristics inherent to microalgae. However, due to uncertainties and difficulties associated with the scale-up of the technology, the algal biofuel production cost is too high. In order to improve the economic viability of microalgal-based biofuels, attention has been drawn to investigate coupled kinetic models and experimental studies with the aim of optimization and control of microalgal biomass growth and lipid accumulation through the improvement of the growth mechanism. In this work, experiments with photoautotrophic growth of *Chlamydomonas reinhardtii* in a raceway open pond were performed with the aim to develop a kinetic model to predict and optimize biomass growth and lipid accumulation in the scaled-up microalgae cultivation process. We have previously developed and validated a quadruple substrate kinetic model (C, N, I, T) for bench-scale cultivation of photoheterotrophic growth of *C. reinhardtii.* Photoautotrophic microalgal growth in open ponds is mainly affected by environmental factors (light intensity and temperature) and growth media composition (nitrogen and CO_2). During the cultivation period, no organic carbon was added or CO_2 was pumped into the ponds and the algal broth utilised available atmospheric CO_2. Therefore, the influence of carbon source was reduced in the final biomass growth and lipid accumulation kinetic expressions. The resulting kinetic model is a function of light intensity, temperature and nitrogen. The model was used in conjunction with the in-house produced experimental data performed in 2 m^3 raceway open

ponds which contained 500 L fertilizer medium (32cm height, 80cm width and 2m length) to define kinetic parameters of the scaled-up level process and subsequently enable the optimisation of the microalgal growth process. Moreover, the model was validated against a different set of in-house obtained data and it was able to predict the microalgal biomass growth and lipid production, as well as the N consumption of the process in a satisfactory way. Simulations of the scaled-up level with the aid of a comprehensive modelling framework can enhance the competitiveness and sustainability of the microalgal biofuel industry and correspondingly enable the system industrial applicability and commercialization. Moreover, the numerical simulations of the microalgal biomass cultivation can help to evaluate the impacts of various changes to system conditions (nitrogen, light intensity and temperature) without excessive experimental trial and error.

1. Introduction

An alternative and sustainable fuel source is needed due to the worldwide increase in energy consumption and increase in atmospheric CO_2 coupled with global warming (Jorquera et al., 2010, Raupach et al., 2007). Microalgal biomass is one of the long-term promising sources of energy as it can be either directly utilized as biomass feedstock or it can be processed for a comparatively high oil content (Davis et al., 2011). With respect the latter, depending on the algal oil characteristics, the oil can be utilised directly into fuels or it can be upgraded into a wide selection of products of interest for different industrial sectors such as biolubricants, surfactants, and nutritional lipids like omega-3 fatty acids (Spolaore et al., 2006, Metting, 1996, Mata et al., 2010).

Microalgal growth is divided into three major classes: autotrophic, heterotrophic and mixotrophic. Autotrophic algae use natural or artificial light as the energy source and carbon dioxide as the carbon source to form biomass through photosynthesis, while heterotrophic algae utilize organic carbons such as sugars and organic acids as a carbon source derived from other biomass sources (Chen et al., 2011, Chen and Johns, 1996). On the other hand, mixotrophic algae use both organic compounds and CO_2 as a carbon source to form biomass, where photosynthetic and respiratory metabolism operate simultaneously (Lee, 2007). In this work, we focused on the autotrophic algal growth as it is easy to maintain a monoculture and it directly utilizes available atmospheric CO_2.

Depending on the selected algal strain and its growth requirements such as ideal environmental conditions and availability of carbon source and nutrients, the selection of the culture system is also crucial. Frequently, optimizing microalgal cultivation conditions and reducing the operating cost are the main reason taken to choose a microalgae cultivation system. There are two main cultivation systems: open ponds (OPs) and highly-controlled closed culture systems called photobioreactors (PBRs) (Mata et al., 2010, Borowitzka, 1999). Open pond systems are easy to build and operate and therefore much less expensive. However, this type of cultivation is very inefficient due to low biomass growth and large land requirement compared to PBRs. PBRs are flexible systems in which the growth conditions can be optimized with respect to the biological and physiological characteristics of the species (Mata et al., 2010). Although PBRs are deemed to be better than the OPs in terms of contamination, space required, water losses and CO_2 losses (Pulz, 2001, Chisti, 2007, Richardson et al., 2012), in order to make an appropriate selection of the cultivation method, an economic comparison between different systems needs to be taken into consideration. A life cycle analysis carried out by Pulz (2001) showed that the maximum achievable biomass concentration for photoautotrophic growth of microalgae in PBRs is 2-8 g L^{-1} while it is only 0.1-0.2 g L^{-1} for OPs. A recent study on economic comparison study of OPs to PBRs showed that the capital and the annual operating expenses for PBRs are 2.6 and 1.5 times higher than capital expenses and operating expenses for OPs, respectively (Richardson et al., 2012). Hence, despite the advantages that PBRs have over OPs, it is not likely that the PBRs will have substantial contribution in the near term on any product or process that can be attained in raceway open ponds.

Although microalgal biomass is a superior long-term feedstock for biodiesel production and it has immense potential in biotechnological applications, the production cost is still too expensive to compete with the conventional fossil diesel production (Doshi et al., 2016) . In order to improve the competitiveness and sustainability of microalgal biodiesel industry, metabolic productivity needs to be enhanced by defining optimal growth conditions through the use of an integrated computational and experimental framework (Bekirogullari et al., 2017). Kinetic modelling is one of the commonly used computational tools to reveal the relationship between microalgal biomass growth and lipid production which can then be used for the optimization of biomass growth, lipid accumulation and scale-up of the microalgal cultivation process (Adesanya et al., 2014, Lee et al., 2015).

The black-box kinetic modelling frameworks can be categorized and organized into two main groups: non-interactive and interactive models. Non-interactive, so-called single substrate, models are widely utilized to predict biomass growth rate and they are expressed as a function of a single substrate or environmental variable such as the Monod, Haldane and Martinez-Sancho models which depend on external nutrient concentrations and the Droop model which depends on internal nutrient concentration (Lee et al., 2015, Monod, 1949, Flynn, 2003, Droop, 1968). Non-interactive models are generally applied for bench-scale heterotrophic microalgal biomass growth. However, this is usually not the case as microalgal biomass growth is co-limited by multiple factors such as substrate and nutrient concentration, light intensity and temperature. In order to take into consideration the co-limitation of multiple factors, interactive, so-called multiple substrate, modelling frameworks have emerged. Interactive modelling approaches, despite their complexity, are often preferred to predict biomass growth rate as they take into account interactions between substrate, nutrients and environmental factors (Yoo et al., 2014, Bekirogullari et al., 2017, He et al., 2012, Franz et al., 2012). This modelling approach is mainly applied to predict heterotrophic, autotrophic and mixotrophic algal biomass growth in order to reveal the interactions between the co-limiting factors in OPs and PBRs. Despite their accuracy to predict biomass growth, the aforementioned models are not able to predict simultaneous effect of nutrients and environmental factors on both lipid accumulation and biomass growth as these models describe both the biomass growth and the lipid accumulation as one state variable. However, it is known that the lipid accumulation can be boosted by abiotic stress, including nutrient deprivation like starvation of nitrogen (N) and phosphorus (P), and factors such as light intensity (I) and temperature stress (T) (Bajhaiya et al., 2016, Converti et al., 2009, Bekirogullari et al., 2017).

Several attempts have been made to model microalgal biomass growth in outdoor raceway open ponds. Huesemann et al. (2013) modelled the microalgal growth as a function of light in which the light distribution was considered through the Beer–Lambert law. The proposed model was capable of predicting the biomass growth successfully. However, in real outdoor cultivation of microalgae, light is not the only growth-limiting factor. Temperature and nutrient availability contribute significantly to biomass growth and lipid production. James and Boriah (2010)and Malek et al. (2015) proposed two different expressions for microalgal biomass growth as a function of light intensity, temperature and nutrient availability and both models successfully

predicted biomass growth. However, these two models considered biomass growth and lipid accumulation as one state variable which does not allow optimizing productivities individually. On the other hand, several studies have attempted to use computational fluid dynamics (CFD) for characterising the hydrodynamics in microalgae culture systems, such as ANSYS Fluent based on existing CFD codes. This technique has been applied to simulate both closed PBRs, and raceway open ponds (Bernard et al., 2016). However, the application of CFD codes still needs to be established and validated experimentally. As described above, only a few studies have attempted to develop a kinetic model for microalgal biomass growth and lipid accumulation in open ponds but none of these studies have considered the biomass growth and lipid accumulation as two different state variables to take advantage of the promotion effect of N, light intensity and temperature towards lipid accumulation.

This study is the first, to the best of our knowledge, to attempt to develop a kinetic model for photoautotrophic growth of microalgal (oil-free) biomass and of lipid accumulation by considering the simultaneous effects of three limiting growth parameters, N, light intensity and temperature. In this work, we present a detailed multiplicative kinetic model to investigate biomass growth and relevant lipid accumulation of a well-studied chlorophyte microalgal species, *Chlamydomonas reinhardtii*, under photo-autotrophic growth conditions in raceway open ponds. The model considers the simultaneous influence of three different growth-promoting resources: N, light intensity and temperature. Experiments were carried out in 2 m^3 raceway open ponds containing 500 L of fertilizer medium (32cm height, 80cm width and 2m length) to accurately determine kinetic parameters of the model that are important for precise process simulations and validation of the model for the scaled-up level. The model was then used in conjunction with in-house produced experimental data to predict biomass growth and lipid accumulation and also to reveal the interactions between the limiting factors. A stochastic algorithm, based on Simulated Annealing (SA) and a deterministic MATLAB optimization function (fmincon) was employed to estimate the key parameters used in the large-scale raceway open pond. Moreover, the predictive capability of the constructed model was assessed with a different set of experiments, which was designed with reduced initial nitrogen concentration and different daily light intensities and temperature.

2. Materials and Methods

2.1. Strain and culture conditions

Experiments were conducted with a wild-type strain of *C. reinhardtii* (CCAP 11/32C), obtained from the Culture Collection of Algae and Protozoa, UK. The strain was initially maintained under batch mixotrophic conditions in Tris-acetate-phosphate (TAP) medium at a temperature of 25 °C and a light intensity of 125 $\mu Em^{-2} s^{-1}$. Prior to experimentation, in order to grow the strain photo-autotrophically in raceway open ponds, the strain was adapted for growth in a lower nutrient and organic carbon-free fertilizer medium consisting of diluted liquid tomato feed (Wilko) containing N and P to a final concentration of 100 mg L^{-1} NH_4-N and 55 mg L^{-1} PO_4-P, respectively. The adaptation process was performed by simultaneously increasing the fertilizer medium concentration in the growth medium while decreasing the TAP medium concentration. Prior to raceway open pond experimentation, an initial fertilizer-adapted algal inoculum was grown in an environmentally-controlled growth chamber at 25 °C and constant light illumination of 125 $\mu Em^{-2} s^{-1}$ using 250 mL conical flasks containing 150 mL of fertilizer medium on an orbital shaker at 120 rpm until the late exponential phase (40-45 days). An algal inoculum of 1L was then added to two raceway open ponds (2 m^3), located in a greenhouse, each containing 500 L of fertilizer medium (32cm height, 80cm width and 2m length). No artificial light was supplied and there was no temperature control in the greenhouse. The growth process was sustained for 45 days operating in batch mode and daily samples were collected to evaluate the biomass growth, lipid production, nitrogen consumption and pH change. The set of data was used in fitting was obtained in early autumn staring form 12th of September and the validation data was obtained in late winter starting from 27th of February. The daily temperature and local light intensity of the parameter fitting data was measured once a day (noon time) for 45 days and the mean values were found to be 289 K and 122 $\mu Em^{-2} s^{-1}$, respectively. The daily temperature and local light intensity of the kinetic model validation data was also measured once a day (noon time) for 45 days and the mean values were found to be 291 K and 158 $\mu Em^{-2} s^{-1}$, respectively.

2.2. Metabolite analysis

2.2.1. Biomass growth

C.reinhardtii biomass growth was measured in terms of dry cell weight (DCW). The DCW of the samples was quantified by centrifuging 500 mL cultures for 3 min at 3000 g in an Eppendorf Centrifuge 5424. After separating the supernatant, the obtained wet biomass pellet was dried for 24h at 70 °C to determine the DCW. The DCW was determined gravimetrically by weighing the dry pellet on a fine balance (Sartorius - M-Pact AX224, Germany). The supernatant and the dry biomass of the samples were stored in 50 mL falcon tubes and they were kept stored at −20 °C for quantification of specific metabolites nutrient consumption and lipid production, respectively. A bench type pH meter (Denver UltraBasic Benchtop Meters, USA) was used to analyse the pH of the samples. A light meter (Fisher Scientific Light Meter, Leicestershire, UK) was used to measure daily light illumination.

2.2.2. Lipid Quantification

Total cellular lipid concentration was determined by extracting the lipids using Soxtec 1043 automated solvent unit as per the Soxhlet solvent extraction method (Bekirogullari et al., 2017). Prior to solvent extraction, disruption of the freeze-dried algal cells was performed through a double cycle of liquid nitrogen immersion and pulverization with mortar and pestle. The dried pulverized algal cells were then placed into cellulose extraction thimbles and loaded in the Soxtec equipment. Extraction of the lipids was carried out by boiling the pulverized algal cells for 2 hours at 160 °C using a suitable solvent (Hexane, ACS spectrophotometric grade, ≥98.5%, Sigma Aldrich, Dorset, UK) followed by rinsing for 40 min. After the extraction process, solvent recovery with evaporation was carried out for 20 min. Following the lipid extraction carried out through the use of Soxtec 1043, the extracted lipids were isolated and dried at 100 °C for 1 h, were placed in a vacuum applied desiccator for 1 h, and finally the algal lipids concentration was quantified gravimetrically.

2.2.3. Total Nitrogen Quantification

The total dissolved N concentration in the culture broth was determined using a Total Organic Carbon/Total N measuring unit (TOC/TN) (TOC-VCSH/TNM-1 Shimadzu) as per the total N

quantification method (Karamerou et al., 2017). Prior to dissolved nitrogen determination, a calibration curve was prepared using NH_4Cl. All supernatant samples collected by centrifugation were diluted properly and injected into the analyser. Zero grade air was used as carrier gas to transport the samples to a combustion unit where nitrogen was catalytically transformed into NO and the flow rate of the carrier gas was set to 150 cm^3 min^{-1}. Subsequently, the formed NO concentration was measured through a chemiluminescence detector and was then converted into total nitrogen (g L^{-1}) with the use of a previously created calibration curve.

3. Kinetic Modelling

3.1. Biomass Growth and Lipid Accumulation Kinetic

Various kinetic models have been proposed to describe the algal biomass growth and to account for the interactions between the limiting factors (Aiba, 1982, Fouchard et al., 2009, Goldman and Carpenter, 1974, Franz et al., 2012, Adesanya et al., 2014). A detailed kinetic modelling framework was constructed by Bekirogullari et al. (2017) to describe heterotrophic biomass growth rate as a function of carbon substrate and N concentration and light intensity for bench scale microalgal growth:

$$\mu_X = \mu_{Xmax} \cdot f(S) \cdot f(N) \cdot f(I) \quad (1)$$

where μ_{Xmax} is the maximum specific growth rate of biomass and $f(S)$, $f(N)$ and $f(I)$ represent the effect of substrate, N and light, respectively.

The kinetic model of Bekirogullari et al. (2017) is adapted here to be utilized for the prediction of the dynamic behaviour of photoautotrophic algal biomass growth in large-scale raceway open pond systems. For photoautotrophic algal cells, the biomass growth is the result of photosynthesis. Consequently, the biomass growth rate is affected by CO_2 and N concentration, light intensity and temperature. The algal broth in our experiments uses available atmospheric CO_2 as we did not pump or add any additional carbon source. Therefore, the effect of CO_2 variation was discounted in the final biomass and lipid growth rate expressions. In this study, we have modified the ordinary differential equation- (ODE) based model to predict the microalgal biomass growth and lipid accumulation processes (Bekirogullari et al., 2017).

N depletion is known to boost lipid accumulation while it inhibits biomass growth (James et al., 2011, Tevatia et al., 2012, Bekirogullari et al., 2017). In order to take advantage of N starvation over lipid accumulation, two different expressions were used for both oil-free biomass growth and lipid production. The specific growth rate of biomass and of lipids, μ_X and μ_L respectively, are described by a multiplicative form (pseudo-triple) of substrate expression as follows:

$$\mu_X(N,I,T) = \mu_{Xmax} \cdot f(N)_X \cdot f(I)_X \cdot f(T)_X \quad (2)$$

$$\mu_L(N,I,T) = q_{Lmax} \cdot f(N)_L \cdot f(I)_L \cdot f(T)_L \quad (3)$$

where μ_{Xmax} is the maximum specific growth rate of biomass, q_{Lmax} is the maximum specific growth rate of lipid, $f(N)$, $f(I)$ and $f(T)$ represent the effect of N, light and temperature, respectively.

The Haldane equation was employed to express the influence of N on both biomass growth and lipid production as follows:

$$f(N)_X = \frac{N}{N + K_{XN} + \frac{N^2}{K_{iXN}}} \quad (4)$$

$$f(N)_L = \frac{N}{N + K_{LN} + \frac{N^2}{K_{iLN}}} \quad (5)$$

where N is the N concentration ($g\ L^{-1}$), K_{XN} and K_{LN} are the saturation constants (g L^{-1}), and K_{iXN} and K_{iLN} are the inhibition constants of N (g L^{-1}).

The Haldane equation was also used to describe the effect of light intensity on both biomass growth and lipid accumulation:

$$f(I)_X = \frac{I}{I + K_{XI} + \frac{I^2}{K_{iXI}}} \quad (6)$$

$$f(I)_L = \frac{I}{I + K_{LI} + \frac{I^2}{K_{iLI}}} \quad (7)$$

where I is the local light intensity (μEm^{-2} s^{-1}), K_{LI} and K_{XI} are the saturation constants (μEm^{-2} s^{-1}), and K_{iXI} and K_{iLI} are the inhibition constants (μEm^{-2} s^{-1}) of light intensity.

The light intensity distribution, I, is expressed by a widely used expression, the Beer-Lambert law, which assumes that the light illumination decreases from the external surface of the cultivation system as the light travels through culture system (Béchet et al., 2013);

$$f(I) = I_0 exp(-\sigma \mathrm{X} l) \tag{8}$$

where l is the distance between the local light position and the external surface of the cultivation system (the value of l is given in Table 2), I_0 is the local light intensity (μEm^{-2} s^{-1}) and σ is the molar extinction coefficient.

To account for variations in the photoperiod of natural light (the modelled photoperiod of natural light is assumed to be 16 hours of light and 8 hours of dark cycles), we have attained the following quadratic expression for the photoperiodic light change (Fig. 1A):

$$\begin{cases} I_0 = -1101 \cdot c^2 + 733.5 \cdot c & when \quad 0 \le c \le 0.66 \\ I_0 = 0 & when \quad 0.66 \le c \le 1 \end{cases} \tag{9}$$

where C is the proportion of light/dark cycle ($0 \le c \le 1$).

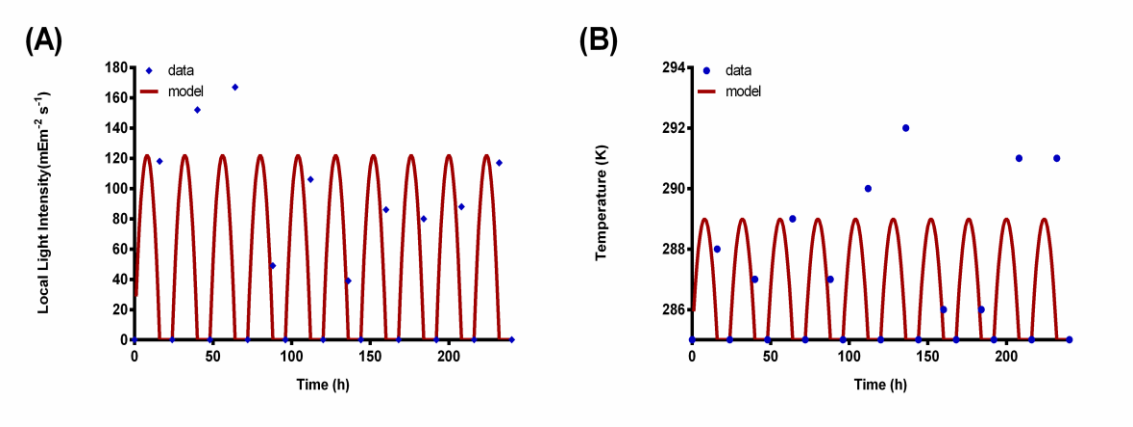

Figure 1. Modelled photoperiodic (day/night cycle) local light intensity and periodic (day/night cycle) temperature change (lines) with measured experimental data (symbols) used for fitting of the model.

The constant coefficient values that have been used in the quadratic Eq.9 were obtained based on in-house produced data. The local light illumination was measured for 45 days and the mean value was found to be 122 μEm^{-2} s^{-1}.

General form a quadratic formula is: $ac^2 + b \cdot c + d = 0$.

$$\begin{cases} when\ c = 0, \quad d = 0; \\ when\ c = 0.33; \\ a \cdot 0.33^2 + b \cdot 0.33 = 122\ ; \\ 2 \cdot a \cdot 0.33 + b = 0; \end{cases} \tag{10}$$

A modified version of the Arrhenius equation was used here to describe the influence of temperature on both biomass growth and lipid accumulation, which accounts for the saturation and inhibition effect of temperature (Bitaubé Pérez et al., 2008):

$$f(T)_X = A_{0_X} \exp\left[\frac{-E_{a_X}}{R}\left(\frac{1}{T} - \frac{1}{T_0}\right)\right] - B_{0_X} \exp\left[\frac{-E_{b_X}}{R}\left(\frac{1}{T} - \frac{1}{T_0}\right)\right] \tag{11}$$

$$f(T)_L = A_{0_L} \exp\left[\frac{-E_{a_L}}{R}\left(\frac{1}{T} - \frac{1}{T_0}\right)\right] - B_{0_L} \exp\left[\frac{-E_{b_L}}{R}\left(\frac{1}{T} - \frac{1}{T_0}\right)\right] \tag{12}$$

Here the first and the second part of the expression represent the promotion and inhibition effect of temperature, respectively. E_{a_X}, E_{a_L} and E_{b_X}, E_{b_L} are the activation energies of growth and cellular degradation, respectively (kcal mol^{-1}), R is the gas constant (kcal mol^{-1}), T the incubation temperature (K), T_0 the reference temperature (K), and A_{0_X}, A_{0_L} and B_{0_X}, B_{0_L} are the corresponding frequency factors (h^{-1}).

In order to account for the day/night periodic change of temperature (the modelled periodic change of temperature assumed to be ±4 K), we obtained the following quadratic formula (Fig. 1B):

$$\begin{cases} T = -36.036 \cdot c^2 + 24 \cdot c + 285 & when \quad 0 \le c \le 0.66 \\ T = 285 & when \quad 0.66 \le c \le 1 \end{cases} \tag{13}$$

where again C is the proportion of light/dark cycle ($0 \le c \le 1$).

The method used in Eq.9 to determine values of the constants was also used here to define the values of the Eq.13 as shown below in Eq. 14. The daily temperature was measured for 45 days and the mean value was found to be 289 K.

$$\begin{cases} when\ c = 0, \quad d = 285; \\ when\ c = 0.33; \\ a \cdot 0.33^2 + b \cdot 0.33 + 285 = 289\ ; \\ 2 \cdot a \cdot 0.33 + b = 0; \end{cases} \tag{14}$$

3.2. Rate Expressions

The dynamic kinetic model constructed in this work consists of a set of ODEs employed to simulate algal biomass growth, lipid production and N consumption rates simultaneously.

The oil-free biomass growth rate is described as:

$$\frac{dX}{dt} = \mu_X \cdot X \tag{15}$$

The oil accumulation rate is expressed as:

$$\frac{dL}{dt} = \mu_L \cdot X + m_1 \cdot X \tag{16}$$

Here: m_1 is the maintenance coefficient (g L g X^{-1}) introduced to the lipid accumulation rate in order to account for lipid accumulation when nitrogen depletion takes place.

The nitrogen consumption rate is represented as:

$$\frac{dN}{dt} = -\frac{1}{Y_{X/N}} \cdot \frac{dX}{dt} - m_2 \cdot X \tag{17}$$

where $Y_{X/N}$ is the yield coefficient for oil-free biomass production with respect to N, and m_2 is the maintenance coefficient (g N g X^{-1}) introduced to the nitrogen consumption rate for the biomass synthesis.

Hence, the constructed model contains 3 ODEs (Eq.15 to 17), corresponding to 3 state variables describing the oil-free biomass growth and lipid production and N consumption of the process. The model contains 19 parameters (listed in Table 2, below), which have been estimated through the procedure discussed in section 3.3 below.

3.3. Parameter Estimation

In this work, a multi-parameter kinetic model was developed for the photoautotrophic growth of microalgal (oil-free) biomass growth and lipid accumulation considering three growth-limiting factors nitrogen concentration, light intensity and temperature. However, due to lack of kinetic modelling of photoautotrophic growth of microalgal processes with respect to aforementioned growth-limiting factors, the values of the kinetic parameters for such a system are not available in the literature. Hence, we conducted a parameter estimation study subject to developed model (Eq.15 to 17) in conjunction with in-house produced experimental data. Data fitting was carried out by using a non-linear weighted least squares method (Bekirogullari et al., 2017):

$$Z(kk) = \min \sum_{k=1}^{n_k} \sum_{l=1}^{n_l} \sum_{m=1}^{n_m} W_{k,l,m} \left(C_{k,l,m}^{pred}(kk) - C_{k,l,m}^{exp} \right)^2 \qquad \text{Eq. 18}$$

where kk is the vector of the 19 kinetic parameters, n_k the number of experiments ($n_k = 1$), n_l the number of state variables ($n_l = 3$), n_m the number of experimental measurements in time ($n_m = 45$), and $W_{k,l,m}$ the weights the weights for each variable used to effectively normalise the computed errors. Here, $C_{k,l,m}^{pred}$ are the predicted state variables (computed by Eq. 15 to 17) and $C_{k,l,m}^{exp}$ the experimental measured ones. The initial concentrations of each experiment were used as the initial conditions in the ODEs. The experiment that has been used for the fitting problem is tabulated as Experiment 1 in Table 1.

Table 1: Experiments used for the fitting and validation process.

Experiment	Nitrogen (g L^{-1})	Light intensity (day) (µ$Em^{-2}s^{-1}$)	Light intensity (Night) (µEm^{-2} s^{-1})	Temperature (day) (K)	Temperature (Night) (K)
1	0.108	122	0	289	285
2	0.08	158	0	291	287

An in-house developed stochastic optimization algorithm (SA) was linked to a deterministic MATLAB optimization function (fmincon) for the estimation of the key parameter values. The procedure that was followed here was described previously in detail (Bekirogullari et al., 2017).

Here 10 stochastic optimization restarts have been used to increase the probability of avoiding local minima. The values of the 19 parameters as well as their standard deviations were found by using the method explained above and tabulated in Table 2. The values of the two constants, T_0 and l used in the simulations are also given in Table 2. The resulting time profiles of the 3 state variables, computed by the constructed kinetic model, and comparisons against experimental datasets including biomass growth, lipid production and N consumption of the process are discussed in section 4.2 below.

Table 2: Estimated kinetic parameters along with bounds available in the literature.

Parameter	Description	Value	Unit	SD
$\boldsymbol{\mu_{Xmax}}$	Maximum specific growth rate of oil-free biomass	0.1312	h^{-1}	0.0008
$\boldsymbol{K_{XN}}$	Nitrogen saturation constant	0.4243	g N L^{-1}	0.0018
$\boldsymbol{K_{iXN}}$	Nitrogen inhibition constant	0.4758	g N L^{-1}	0.0018
$\boldsymbol{K_{XI}}$	Light saturation constant	3.7017	μEm^{-2} s^{-1}	0.0844
$\boldsymbol{K_{iXI}}$	Light inhibition constant	153.5258	μEm^{-2} s^{-1}	1.62788
$\boldsymbol{A_{0X}}$	Frequency factors	0.9889	h^{-1}	0.0086
$\boldsymbol{E_{aX}}$	Activation energy of oil-free biomass growth	7.6018	kcal mol^{-1}	0.0378
$\boldsymbol{B_{0X}}$	Frequency factors	0.1659	h^{-1}	0.0025
$\boldsymbol{E_{bX}}$	Activation energy of oil-free biomass degradation	51.4027	kcal mol^{-1}	1.0593
$\boldsymbol{q_{Lmax}}$	Maximum specific growth rate of lipid	0.1202	g L g X^{-1} h^{-1}	0.0015
$\boldsymbol{K_{LN}}$	Nitrogen saturation constant	0.1025	g N L^{-1}	0.0012
$\boldsymbol{K_{iLN}}$	Nitrogen inhibition constant	0.5996	g N L^{-1}	0.0027
$\boldsymbol{K_{LI}}$	Light saturation constant	2.5538	μEm^{-2} s^{-1}	0.0205
$\boldsymbol{K_{iLI}}$	Light inhibition constant	92.0299	μEm^{-2} s^{-1}	1.2863
$\boldsymbol{A_{0L}}$	Frequency factors	0.1107	h^{-1}	0.0029
$\boldsymbol{E_{aL}}$	Activation energy of oil production	13.8844	kcal mol^{-1}	0.2307
$\boldsymbol{B_{0L}}$	Frequency factors	0.0219	h^{-1}	0.0016
$\boldsymbol{E_{bL}}$	Activation energy of oil degradation	2.2478	kcal mol^{-1}	0.01369
$\boldsymbol{\sigma}$	Molar extinction coefficient	0.2095	g X^{-1} L m^{-1}	0.0028
$\boldsymbol{Y_{X/N}}$	Yield coefficient for oil-free biomass production with respect to N	1.8288	g X g N^{-1}	0.1623
$\boldsymbol{m_1}$	Maintenance term	0.0001	g L g X^{-1}	0
$\boldsymbol{m_2}$	Maintenance term	0.0006	g N g X^{-1}	0
$\boldsymbol{l}$	The distance between the local position and the external surface of the system	0.25	m	
$\boldsymbol{R}$	Gas constant	0.001986	kcal mol^{-1}	
$\boldsymbol{T_0}$	Reference Temperature	293	K	

4. Results and Discussion

An experimental study was performed in 2 m^3 raceway open pond to quantify the effect growth limiting factors, light intensity, temperature and N concentration on the microalgal growth and lipid productivity. A parameter estimation study was then undertaken subject to constructed

model to compute parameter values that are essential for accurate process simulations. The developed model was subsequently validated and used in an optimization study to determine optimal operating conditions for maximum lipid productivity.

4.1. Experimental Results

C. reinhardtii biomass production, volumetric lipid yield and N consumption, as well as daily light intensity and temperature, were measured during a 45-day cultivation in 2 m^3 raceway ponds. As seen in Figure 3 and 4, the cells were in lag phase for an average for 17 days and then the cells grew rapidly with equivalent growth profiles in the exponential phase until reaching the stationary phase on average for 40 days. To make sure that stationary phase concentrations were reached, the cultures were grown further up to 45 days.

At the end of the cultivation period, the maximum achievable biomass concentration in the open ponds was 0.15 g L^{-1}. The proportion of lipid accumulation within the cell on a total dry cell weight basis was between 10-15 % during the cultivation period and at the end of the cultivation period, the final lipid concentration was 0.023 g L^{-1} corresponding to 13.6 % of dry cell weight. The proportion of lipid production was essentially similar to the reported value for photoheterotrophic growth of *C. reinhardtii* in TAP media, 13,2%, and the difference in volumetric lipid concentration between fertilizer and TAP media growth was almost entirely due to the difference in biomass concentration (Bekirogullari et al., 2017).

The pH of the growth medium increased from 6.68 to 7.07, which was due to carbon uptake-assimilation and fixation from atmospheric CO_2. In the open pond conditions the algae are exposed to available atmospheric concentrations of CO_2. As the cells divide and grow, the rate of inorganic carbon uptake and fixation exceeds the rate of CO_2 supply from the atmosphere and therefore, pCO_2 of the medium decreases and the pH increases.

4.2. Model Validation

Values of the kinetic parameters were estimated using the procedure explained in section 3.3. As can be seen in Figure 3, the resulting model shows excellent agreement with the experimental data for all state variables.

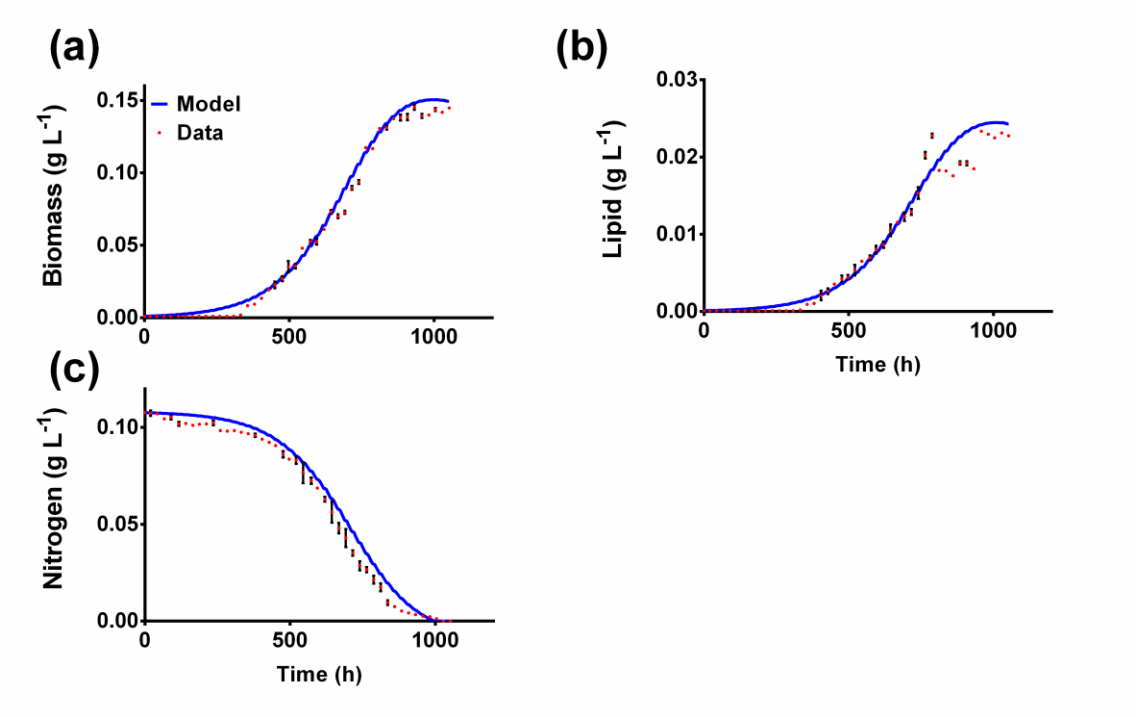

Figure 3. Comparison of model predictions (lines) with experimental data from Experiment 1 (symbols with error bars) for: (a) biomass concentration, (b) lipid concentration, (c) N consumption. All data are mean ± SE values of 2-3 biological replicates.

A validation study was subsequently performed in order to assess the predictive capability of the developed model by using the conditions (initial N concentrations, temperature and light intensity) of Experiment 2 (also given in Table 1). It should be noted that all Experiment 2 conditions are different than the ones used for parameter fitting. The local light intensity was measured for 45 days and the mean value was found to be $158\ \mu Em^{-2}\ s^{-1}$. To account for day/night photoperiodic light cycle the following quadratic expression is used for validation data (Fig. 2A);

$$\begin{cases} I_0 = -1423 \cdot c^2 + 948 \cdot C & when \quad 0 \le c \le 0.66 \\ I_0 = 0 & when \quad 0.66 \le c \le 1 \end{cases} \tag{19}$$

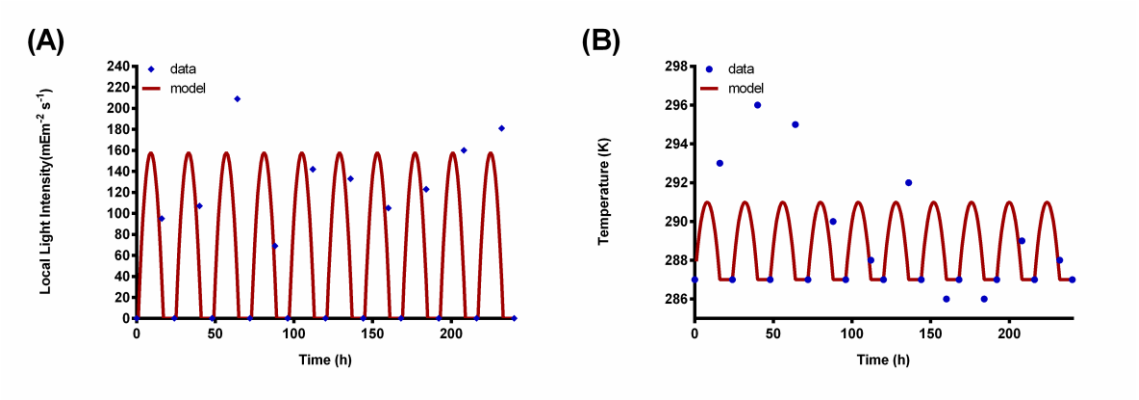

Figure 2. Modelled photoperiodic (day/night cycle) local light intensity and periodic (day/night cycle) temperature change (lines) with measured experimental data (symbols) used for validation of the model.

The temperature was measured for 45 days and the mean value was found to be $291\,\mathrm{K}$. To account for day/night periodic change of temperature, the following quadratic expression is used for validation data (Fig. 2B);

$$\begin{cases} T = -36.036 \cdot c^2 + 24 \cdot c + 287 & when \quad 0 \le c \le 0.66 \\ T = 287 & when \quad 0.66 \le c \le 1 \end{cases} \tag{20}$$

The kinetic model was capable of predicting the dynamics of the 3 experimentally obtained state variables (oil-free biomass growth, lipid accumulation and N consumption) with high precision as can be seen in Figure 4. We therefore can conclude that the detailed kinetic model developed in this study can be utilized for precise prediction of the dynamic behaviour of microalgal growth in large-scale raceway open ponds. Consequently, it can be used as an optimization tool to compute optimal operating conditions for maximum biomass growth and lipid production for microalgal cultivation systems.

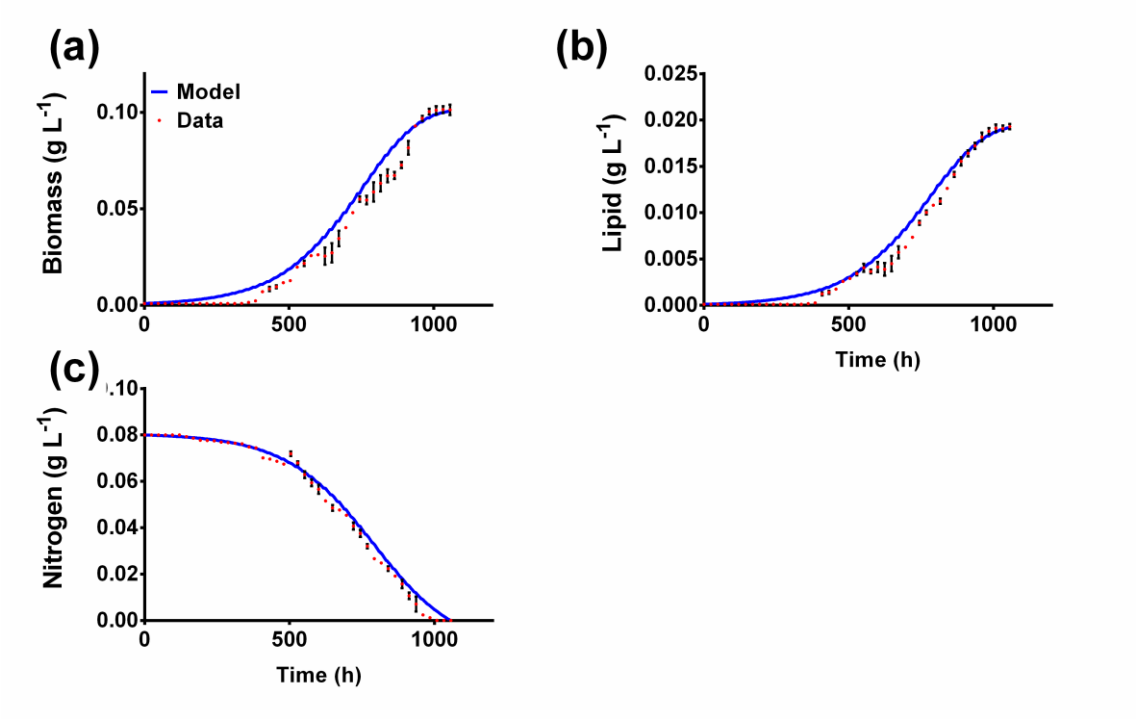

Figure 4. Comparison of model predictions (lines) with experimental data from Experiment 2 (symbols with error bars) for: (a) biomass concentration, (b) lipid concentration, (c) N consumption. All data are mean ± SE values of 2-3 biological replicates.

5. Conclusions

Several different kinetic modelling approaches have been suggested for photoautotrophic cultivation of microalgae in both open ponds and photobioreactors. Not all of these studies consider all growth-limiting factors. On the other hand, lipid accumulation either has not been modelled or has been considered to be proportional to the biomass growth, which does not allow accurate prediction and optimization of lipid accumulation. In this work, a detailed kinetic model in response to N, light intensity and temperature was developed to understand the synergistic interactions between nutrients and environmental factors and also to predict the microalgal biomass growth and lipid accumulation in raceway open ponds. Two different expressions were used for both biomass growth and lipid accumulation to take advantage of nutrient starvation towards maximal lipid accumulation. We have carried out a range of experiments for two different N concentrations where the environmental factors also (I, T) varied based on daily light and temperature conditions. The kinetic parameters of the constructed model were computed by fitting the model outputs to in-house produced experimental data. Model validation was subsequently carried out through comparisons to different sets of

experimental data. The developed kinetic model was able predict satisfactorily microalgal biomass growth and lipid production, as well as N consumption of the process. This illustrates the usefulness of the integrated computational and experimental frameworks for the optimization of microalgal-based lipid accumulation. Such carefully constructed comprehensive kinetic models can be exploited for the robust design, control and optimization of microalgal biomass and lipid accumulation processes, which can improve the viability and sustainability of this important technology.

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References

Adesanya, V. O., Davey, M. P., Scott, S. A. & Smith, A. G. 2014. Kinetic modelling of growth and storage molecule production in microalgae under mixotrophic and autotrophic conditions. *Bioresource Technology,* 157**,** 293-304.

Aiba, S. 1982. Growth kinetics of photosynthetic microorganisms. *Microbial Reactions.* Berlin, Heidelberg: Springer Berlin Heidelberg.

Bajhaiya, A. K., Dean, A. P., Driver, T., Trivedi, D. K., Rattray, N. J. W., Allwood, J. W., Goodacre, R. & Pittman, J. K. 2016. High-throughput metabolic screening of microalgae genetic variation in response to nutrient limitation. *Metabolomics,* 12**,** 1-14.

Béchet, Q., Shilton, A. & Guieysse, B. 2013. Modeling the effects of light and temperature on algae growth: State of the art and critical assessment for productivity prediction during outdoor cultivation. *Biotechnology Advances,* 31**,** 1648-1663.

Bekirogullari, M., Fragkopoulos, I. S., Pittman, J. K. & Theodoropoulos, C. 2017. Production of lipid-based fuels and chemicals from microalgae: An integrated experimental and model-based optimization study. *Algal Research,* 23**,** 78-87.

Bernard, O., Mairet, F. & Chachuat, B. 2016. Modelling of Microalgae Culture Systems with Applications to Control and Optimization. *In:* Posten, C. & Feng Chen, S. (eds.) *Microalgae Biotechnology.* Cham: Springer International Publishing.

Bitaubé Pérez, E., Caro Pina, I. & Pérez Rodríguez, L. 2008. Kinetic model for growth of Phaeodactylum tricornutum in intensive culture photobioreactor. *Biochemical Engineering Journal,* 40**,** 520-525.

Borowitzka, M. A. 1999. Commercial production of microalgae: ponds, tanks, tubes and fermenters. *Journal of Biotechnology,* 70**,** 313-321.

Chen, C.-Y., Yeh, K.-L., Aisyah, R., Lee, D.-J. & Chang, J.-S. 2011. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. *Bioresource Technology,* 102**,** 71-81.

Chen, F. & Johns, M. R. 1996. Heterotrophic growth of Chlamydomonas reinhardtii on acetate in chemostat culture. *Process Biochemistry,* 31**,** 601-604.

Chisti, Y. 2007. Biodiesel from microalgae. *Biotechnol Adv,* 25.

Converti, A., Casazza, A. A., Ortiz, E. Y., Perego, P. & Del Borghi, M. 2009. Effect of temperature and nitrogen concentration on the growth and lipid content of Nannochloropsis oculata and Chlorella vulgaris for biodiesel production. *Chemical Engineering and Processing: Process Intensification,* 48**,** 1146-1151.

Davis, R., Aden, A. & Pienkos, P. T. 2011. Techno-economic analysis of autotrophic microalgae for fuel production. *Applied Energy,* 88**,** 3524-3531.

Doshi, A., Pascoe, S., Coglan, L. & Rainey, T. J. 2016. Economic and policy issues in the production of algae-based biofuels: A review. *Renewable and Sustainable Energy Reviews,* 64**,** 329-337.

Droop, M. R. 1968. Vitamin B12 and Marine Ecology. IV. The Kinetics of Uptake, Growth and Inhibition in Monochrysis Lutheri. *Journal of the Marine Biological Association of the United Kingdom,* 48**,** 689-733.

Flynn, K. J. 2003. Modelling multi-nutrient interactions in phytoplankton; balancing simplicity and realism. *Progress in Oceanography,* 56**,** 249-279.

Fouchard, S., Pruvost, J., Degrenne, B., Titica, M. & Legrand, J. 2009. Kinetic modeling of light limitation and sulfur deprivation effects in the induction of hydrogen production with Chlamydomonas reinhardtii: Part I. Model development and parameter identification. *Biotechnology and Bioengineering,* 102**,** 232-245.

Franz, A., Lehr, F., Posten, C. & Schaub, G. 2012. Modeling microalgae cultivation productivities in different geographic locations – estimation method for idealized photobioreactors. *Biotechnology Journal,* 7**,** 546-557.

Goldman, J. C. & Carpenter, E. J. 1974. A kinetic approach to the effect of temperature on algal growth1. *Limnology and Oceanography,* 19**,** 756-766.

He, L., Subramanian, V. R. & Tang, Y. J. 2012. Experimental analysis and model-based optimization of microalgae growth in photo-bioreactors using flue gas. *Biomass and Bioenergy,* 41**,** 131-138.

Huesemann, M. H., Van Wagenen, J., Miller, T., Chavis, A., Hobbs, S. & Crowe, B. 2013. A screening model to predict microalgae biomass growth in photobioreactors and raceway ponds. *Biotechnology and Bioengineering,* 110**,** 1583-1594.

James, G. O., Hocart, C. H., Hillier, W., Chen, H., Kordbacheh, F., Price, G. D. & Djordjevic, M. A. 2011. Fatty acid profiling of Chlamydomonas reinhardtii under nitrogen deprivation. *Bioresource Technology,* 102**,** 3343-3351.

James, S. C. & Boriah, V. 2010. Modeling algae growth in an open-channel raceway. *Journal of Computational Biology,* 17**,** 895-906.

Jorquera, O., Kiperstok, A., Sales, E. A., Embiruçu, M. & Ghirardi, M. L. 2010. Comparative energy life-cycle analyses of microalgal biomass production in open ponds and photobioreactors. *Bioresource Technology,* 101**,** 1406-1413.

Karamerou, E. E., Theodoropoulos, C. & Webb, C. 2017. Evaluating feeding strategies for microbial oil production from glycerol by Rhodotorula glutinis. *Engineering in Life Sciences,* 17**,** 314-324.

Lee, E., Jalalizadeh, M. & Zhang, Q. 2015. Growth kinetic models for microalgae cultivation: A review. *Algal Research,* 12**,** 497-512.

Lee, Y.-K. 2007. Algal Nutrition – Heterotrophic Carbon Nutrition. *Handbook of Microalgal Culture.* Blackwell Publishing Ltd.

Malek, A., Zullo, L. C. & Daoutidis, P. 2015. Modeling and dynamic optimization of microalgae cultivation in outdoor open ponds. *Industrial & Engineering Chemistry Research,* 55**,** 3327-3337.

Mata, T. M., Martins, A. A. & Caetano, N. S. 2010. Microalgae for biodiesel production and other applications: A review. *Renewable and Sustainable Energy Reviews,* 14**,** 217-232.

Metting, F. B. 1996. Biodiversity and application of microalgae. *Journal of Industrial Microbiology,* 17**,** 477-489.

Monod, J. 1949. The Growth of Bacterial Cultures. *Annual Review of Microbiology,* 3**,** 371-394.

Pulz, O. 2001. Photobioreactors: production systems for phototrophic microorganisms. *Applied Microbiology and Biotechnology,* 57**,** 287-293.

Raupach, M. R., Marland, G., Ciais, P., Le Quéré, C., Canadell, J. G., Klepper, G. & Field, C. B. 2007. Global and regional drivers of accelerating CO2 emissions. *Proceedings of the National Academy of Sciences,* 104**,** 10288-10293.

Richardson, J. W., Johnson, M. D. & Outlaw, J. L. 2012. Economic comparison of open pond raceways to photo bio-reactors for profitable production of algae for transportation fuels in the Southwest. *Algal Research,* 1**,** 93-100.

Spolaore, P., Joannis-Cassan, C., Duran, E. & Isambert, A. 2006. Commercial applications of microalgae. *Journal of Bioscience and Bioengineering,* 101**,** 87-96.

Tevatia, R., Demirel, Y. & Blum, P. 2012. Kinetic modeling of photoautotropic growth and neutral lipid accumulation in terms of ammonium concentration in Chlamydomonas reinhardtii. *Bioresource Technology,* 119**,** 419-424.

Yoo, S. J., Kim, J. H. & Lee, J. M. 2014. Dynamic modelling of mixotrophic microalgal photobioreactor systems with time-varying yield coefficient for the lipid consumption. *Bioresource Technology,* 162**,** 228-235.

Appendix A. Sensitivity analysis

A sensitivity analysis was carried out for the kinetic model proposed in this work which consists of 22 parameters. The analysis was performed by calculating the sensitivity (Eq. (A.1)), for all 3 dynamic variables with respect to each parameter at eight different cultivation times (t=25h, 50h, 75h, 85h, 125h, 150h, 175h and 190h).

$$Sensitivity = abs\left(\frac{Prediction(t, P + \Delta P) - Prediction(t, P - \Delta P)}{2 * \Delta P}\right) \quad \text{Eq. A.1}$$

Sensitivity of the parameters was computed with a %10 change (ΔP) in parameters values. The sensitivity analysis results of 22 kinetic parameters are presented in Table A.1. The threshold for sensitivity was set to 0.01, meaning parameters with sensitivities lower than 0.01 were considered not-sensitive and sensitivities higher than 0.01 deemed to be sensitive and they are highlighted.

Table A.1. Sensitivity analysis results of the proposed model kinetic parameters.

			Sensitivity							
Parameter	**Value**	**Variable**	**25h**	**50h**	**75h**	**85h**	**125h**	**150h**	**175h**	**190h**
μ_{xmax}	0.1312	X	1.12E-01	4.28E-01	1.24E+00	3.23E-01	1.14E-01	3.21E-01	3.92E-01	4.03E-01
		L	4.13E-03	2.70E-02	9.56E-02	1.03E-01	1.96E-01	2.29E-01	2.25E-01	2.06E-01
		N	6.41E-02	2.52E-01	8.04E-01	4.09E-01	1.75E-01	4.72E-02	1.38E-02	4.43E-02
K_{XN}	0.4243	X	2.67E-02	1.01E-01	3.09E-01	8.09E-02	3.13E-02	8.59E-02	1.06E-01	1.08E-01
		L	9.45E-04	6.03E-03	2.20E-02	2.95E-02	5.23E-02	5.96E-02	5.74E-02	5.07E-02
		N	1.53E-02	5.93E-02	2.00E-01	1.02E-01	4.15E-02	7.86E-03	9.14E-03	1.65E-02
K_{iXN}	0.4758	X	2.21E-03	4.49E-03	1.03E-02	1.68E-03	1.69E-03	2.88E-03	3.11E-03	2.15E-03
		L	1.68E-04	3.07E-04	1.08E-03	3.01E-04	1.12E-03	1.46E-03	1.57E-03	1.39E-03
		N	1.27E-03	2.72E-03	6.94E-03	2.96E-03	1.11E-03	3.12E-04	3.43E-05	3.56E-04
K_{XI}	3.7017	X	3.07E-04	6.75E-04	2.16E-03	4.58E-04	1.88E-04	6.04E-04	4.95E-04	5.32E-04
		L	1.94E-05	3.09E-05	1.27E-04	2.31E-04	3.55E-04	4.22E-04	3.53E-04	3.10E-04
		N	1.74E-04	4.03E-04	1.42E-03	6.46E-04	2.96E-04	4.04E-05	6.48E-05	1.09E-05
K_{iXI}	153.526	X	3.35E-05	1.19E-04	3.40E-04	8.69E-05	3.50E-05	9.22E-05	1.15E-04	1.19E-04
		L	1.58E-06	7.61E-06	2.54E-05	2.99E-05	5.54E-05	6.43E-05	6.43E-05	5.89E-05
		N	1.92E-05	7.02E-05	2.21E-04	1.12E-04	4.62E-05	1.08E-05	8.28E-06	1.73E-05
q_{Lmax}	0.1202	X	4.41E-04	7.05E-03	1.08E-03	2.47E-03	4.05E-04	5.70E-04	1.93E-03	9.16E-04
		L	1.13E-02	3.52E-02	1.30E-01	1.88E-01	1.79E-01	1.58E-01	1.33E-01	1.09E-01
		N	2.62E-04	4.03E-03	2.22E-04	2.83E-03	1.76E-03	1.19E-03	4.35E-04	2.00E-03
K_{LN}	0.1025	X	2.26E-03	5.96E-03	1.78E-04	8.51E-03	4.12E-03	2.60E-03	2.76E-03	1.46E-04
		L	6.54E-03	1.99E-02	8.76E-02	1.47E-01	1.35E-01	1.08E-01	7.66E-02	4.42E-02
		N	1.27E-03	3.49E-03	1.37E-03	4.85E-03	2.80E-03	2.13E-03	2.45E-03	8.32E-04
K_{LI}	2.5538	X	4.31E-05	2.85E-04	1.03E-04	1.82E-04	8.44E-05	2.50E-04	7.98E-05	1.71E-04
		L	1.40E-05	1.08E-05	2.37E-04	3.96E-04	3.55E-04	3.56E-04	2.16E-04	2.29E-04
		N	2.36E-05	1.63E-04	3.53E-05	1.00E-04	5.48E-05	1.55E-04	2.43E-05	1.23E-04
K_{iLI}	92.0299	X	8.96E-07	1.79E-06	2.36E-05	1.04E-06	9.49E-07	7.91E-06	5.20E-06	7.78E-06
		L	6.41E-06	2.01E-05	7.87E-05	1.07E-04	1.03E-04	8.89E-05	7.57E-05	6.15E-05
		N	5.24E-07	1.06E-06	1.51E-05	2.31E-06	2.25E-06	1.80E-06	7.87E-07	2.69E-06
$Y_{X/N}$	1.8288	X	2.11E-05	1.61E-03	2.68E-02	6.00E-02	5.92E-02	5.11E-02	4.08E-02	3.13E-02
		L	1.31E-05	1.51E-04	3.37E-03	9.46E-03	9.46E-03	7.72E-03	5.27E-03	2.99E-03
		N	3.23E-03	8.56E-03	1.59E-02	3.15E-03	1.46E-03	3.66E-03	4.55E-03	5.22E-03
σ	0.2095	X	7.16E-05	1.69E-03	7.45E-03	2.38E-03	1.55E-04	1.16E-03	1.71E-03	2.16E-03
		L	2.65E-05	2.32E-04	1.33E-03	2.20E-05	5.05E-05	2.64E-04	3.14E-04	3.81E-04
		N	2.18E-05	9.77E-04	4.73E-03	2.61E-03	1.44E-03	7.02E-04	3.05E-04	5.55E-05
A_{0X}	0.9888	X	1.60E-02	5.91E-02	1.69E-01	4.39E-02	1.56E-02	4.45E-02	5.47E-02	5.57E-02
		L	6.40E-04	3.68E-03	1.28E-02	1.45E-02	2.70E-02	3.16E-02	3.12E-02	2.83E-02
		N	9.18E-03	3.49E-02	1.10E-01	5.61E-02	2.41E-02	6.35E-03	2.36E-03	6.26E-03
E_{aX}	7.6018	X	4.66E-04	1.77E-03	5.50E-03	1.36E-03	5.52E-04	1.45E-03	1.71E-03	1.73E-03
		L	1.42E-05	1.02E-04	4.22E-04	4.78E-04	8.78E-04	1.01E-03	9.88E-04	8.95E-04
		N	2.66E-04	1.05E-03	3.57E-03	1.77E-03	7.36E-04	1.79E-04	6.27E-05	1.80E-04
B_{0X}	0.1659	X	4.31E-03	1.62E-02	3.77E-02	6.25E-03	5.76E-03	8.84E-03	1.09E-02	1.33E-02
		L	2.13E-04	1.23E-03	2.78E-03	4.30E-03	6.65E-03	6.59E-03	5.86E-03	5.67E-03
		N	2.47E-03	9.52E-03	2.49E-02	1.07E-02	4.08E-03	1.92E-03	1.72E-04	1.91E-03
E_{bX}	51.4027	X	1.93E-05	6.41E-05	2.03E-04	5.00E-05	1.52E-05	5.23E-05	7.19E-05	6.94E-05
		L	7.04E-07	2.73E-06	1.33E-05	2.23E-05	3.53E-05	4.00E-05	4.17E-05	3.69E-05
		N	1.11E-05	3.76E-05	1.30E-04	6.22E-05	2.72E-05	4.66E-06	1.01E-05	1.33E-05

A_{0L}	0.1106	X	6.37E-04	2.13E-03	1.42E-02	2.61E-03	2.76E-03	4.67E-03	9.77E-04	1.42E-03
		L	1.72E-02	5.26E-02	1.94E-01	2.84E-01	2.72E-01	2.41E-01	2.02E-01	1.64E-01
		N	3.64E-04	1.13E-03	8.70E-03	3.41E-03	4.95E-04	7.69E-04	2.24E-03	2.37E-03
E_{aL}	13.8844	X	7.59E-06	3.89E-05	2.15E-04	3.40E-05	2.58E-05	5.75E-05	6.47E-05	9.33E-07
		L	6.07E-05	1.80E-04	6.59E-04	1.02E-03	9.73E-04	8.69E-04	7.34E-04	5.84E-04
		N	4.21E-06	2.17E-05	1.35E-04	5.82E-06	8.17E-06	1.18E-05	1.99E-05	1.40E-05
B_{0L}	0.02194	X	1.02E-02	2.01E-03	7.70E-02	4.94E-03	1.86E-03	2.35E-02	7.53E-03	4.59E-03
		L	2.42E-02	7.62E-02	2.73E-01	4.21E-01	4.00E-01	3.60E-01	3.01E-01	2.47E-01
		N	5.75E-03	1.49E-03	4.46E-02	6.73E-04	2.14E-03	1.06E-02	2.23E-03	8.27E-04
E_{bL}	2.2478	X	1.53E-06	3.51E-04	6.27E-04	4.05E-06	3.10E-05	1.23E-04	2.12E-05	1.01E-04
		L	2.03E-05	9.56E-06	8.52E-05	2.99E-04	3.00E-04	2.24E-04	1.98E-04	1.90E-04
		N	2.35E-06	2.00E-04	3.80E-04	4.44E-05	2.27E-05	1.06E-04	5.76E-05	1.36E-05
K_{iLN}	0.5996	X	6.23E-04	3.10E-04	3.13E-03	1.65E-03	2.51E-04	5.66E-04	1.42E-03	1.30E-03
		L	9.68E-05	4.46E-04	6.60E-04	9.30E-04	1.47E-03	1.52E-03	1.74E-03	1.76E-03
		N	3.52E-04	2.28E-04	1.94E-03	1.59E-03	5.81E-04	3.69E-04	1.56E-04	1.74E-04
m_1	0.0001	X	3.69E-01	8.03E+00	3.10E+01	9.37E+00	2.33E+00	1.16E+01	1.05E+01	7.74E+00
		L	1.80E-02	1.11E+00	3.87E+00	4.37E+00	9.20E+00	1.38E+01	1.54E+01	1.62E+01
		N	1.97E-01	4.56E+00	2.06E+01	1.11E+01	4.83E+00	6.62E-01	8.13E-01	2.46E-01
m_2	0.00061	X	1.93E-01	3.72E-01	1.89E+01	5.20E+01	7.09E+01	8.71E+01	9.87E+01	1.09E+02
		L	3.12E-02	2.68E-02	2.64E+00	8.93E+00	1.34E+01	1.77E+01	2.11E+01	2.42E+01
		N	1.29E+00	4.07E+00	8.16E+00	1.38E+01	1.48E+01	1.53E+01	1.64E+01	1.61E+01

Appendix B: Upper and lower bounds of the estimated parameters used in section 3.3 parameters estimation problem

Table B.1 Upper and lower bounds of the estimated parameters

Parameters	Lower bounds	Upper bounds	Parameters	Lower bounds	Upper bounds
μ_{xmax}	15	0.15	A_{0X}	0.4	0.6
K_{XN}	0.1	0.3	E_{aX}	5	9
K_{iXN}	0.1	0.8	B_{0X}	0.1	0.2
K_{XI}	1	8	E_{bX}	5	51
K_{iXI}	9	154	A_{0L}	0.1	0.2
q_{Lmax}	1	0.5	E_{aL}	8	14
K_{LN}	0.2	0.5	B_{0L}	0.002	0.005
K_{LI}	15	6	E_{bL}	2	5
K_{iLI}	0.1	90	K_{iLN}	0.4	0.5
$Y_{X/N}$	0.1	8	m_1	0.0001	0.0002
σ	1	0.8	m_2	0.0001	0.0002

Chapter 6

Conclusions and Future Work

6.1. Conclusions

Microalgae-based biofuel production has gained increasing attention as an alternative energy source due to its unique traits. However, the use of microalgal oil as an alternative feedstock for the production of biofuel has not yet been exploited commercially as the current price for its production is still too high compared to the fossil fuel diesel. The high cost of the growth media (carbon source and fertilizer requirements) and the high cultivation facility costs are responsible for approximately 60-75% of the total cost of microalgal biodiesel production. Despite the immense potential of microalgae in biotechnological applications, the metabolic productivities of microalgae need to be enhanced in order to enable economic viability of this technology. Nowadays, many tools are available for the improvement and control of the microalgae cultivation process such as genetic manipulations and mathematical modelling, with the aim to develop sustainable high productivity processes for commercial scale biofuel production.

The chemical composition of microalgae is affected by a number of factors such as organic and/or inorganic carbon substrate, essential nutrient availability, temperature and light intensity. The interactions between microalgae and growth-limiting factors determine the cell composition. Abiotic stress such as carbon concentration, nutrients starvation like nitrogen (N) and factors such as light intensity and temperature stress have been employed to trigger accumulation of such lipid bodies. Although the effect of growth-limiting factors has been exploited experimentally, it needs to be integrated with computational studies in order to obtain high productivity algae systems with reasonable experimental effort, i.e. avoiding the costly excessive experimental trial and error. The kinetic modelling and simulation of microalgae growth considering the factors affecting positively or negatively the production of a product of interest provide the background for the successful optimization of the processes.

As discussed in Chapter 1, the aim of this PhD study was to analyse the effect of growth media composition (nitrogen and acetate variations) and environmental factors on microalgae growth

with particular focus on lipid production. Ultimately, to develop a detailed kinetic model considering effect of growth-limiting factors in order to provide an understanding of microalgal growth and lipid production so that cultivation conditions can be optimized with a view of improving the sustainability and competitiveness of microalgal-based biofuels industry.

In this regard, in Chapter 3, the effect of growth media composition with the variations of initial acetate and nitrogen concentrations was first analysed. It was found that the addition of organic carbon such as acetate increases biomass concentration until a certain concentration, while above this limit, excessive acetate concentrations began to inhibit the biomass growth. Additionally, the proportion of lipid accumulation within cell was found to be identical for all acetate treatments, and hence the difference in volumetric lipid concentration between the acetate treatments was almost entirely due to difference in biomass concentration. The experiments with the nitrogen concentration variations showed that under nitrogen starvation conditions substantial lipid induction can be observed.

In Chapter 3, based on the experimental observations of growth media composition variations and existing literature, a semi-empirical model was developed taking into consideration the effect of three growth-limiting culture variables (C, N, I) so to precisely predict the dynamic behaviour of microalgal growth process operating in batch. The developed model was used in conjunction with in-house produced experimental data for the estimation of kinetic parameters. The model was then validated against a different set of data and used in an optimization study to compute optimal system operating conditions. It was found that a 30% increase in the lipid oil productivity can be achieved using 2.19 g L^{-1} acetate and 0.074 g L^{-1} N. The computed optimal results were validated experimentally. The lipid concentrations were measured 62.4 mg L^{-1} for the base case and 84.7 mg L^{-1} for the computed optimal conditions.

The effect of the environmental factors (I and T variations) on biomass growth and lipid accumulation was also evaluated with seven different light intensities and temperatures. The light intensity variation treatment results suggest that the biomass concentration increases as the light intensity rises until a certain point where photoinhibition starts and consequently biomass concentration decreases. Similar observations were made for the temperature variation treatments where the cells responded to increases in temperature with increased exponential growth rates until reaching the optimum temperature for growth. Increasing

temperature beyond this point led to sharp declines in biomass growth and lipid accumulation. The proportion of lipid production within the cells was found to be almost identical for all light intensity and temperature variation treatments, and thus the difference in volumetric lipid concentration between the light intensity and temperature variation treatments was almost entirely due to difference in biomass concentration.

In order to take into account the positive or negative effect of environmental factors, the model developed in Chapter 3 was then expanded to cover the effect of light intensity and temperature variations. The biomass growth and lipid accumulation rates of the improved model were a function of four growth-limiting factors (carbon and nitrogen concentrations, light intensity and temperature). The model was validated and utilised in an optimisation study to determine the optimal light intensity and temperature for such a system. It was found that the lipid productivity can be increased by 50 % compared to a base case, and by 13 % compared to the previously optimal case. The computed optimal results were also validated experimentally. The lipid concentrations were measured 62.4 mg L^{-1} for the base case, 84.7 mg L^{-1} for the previously optimized conditions and 93.3 mg L^{-1} for the new computed optimal conditions.

The applicability of the developed kinetic model to large-scale raceway open pond systems was then assessed. Experiments were carried out in 2 m^3 with photoautotrophic growth of *C. reinhardtii*. As the microalgae strain uses available atmospheric CO_2 to perform photosynthesis, the effect of carbon source on biomass growth and lipid production was removed from the model. The updated open pond model was a function of three growth-limiting factors: light intensity, temperature and nitrogen. The kinetic parameters of the model were re-estimated and the model was validated against a different set of data. The resulting model was able to predict the biomass growth and lipid accumulation with high precision.

Due to the complexity of microalgal growth processes and the uncertainty in biological knowledge, e.g. intracellular interactions of metabolites, and kinetic parameters, only few studies have attempted to develop kinetic models for microalgal growth. However, not all of these studies considered the antagonistic and simultaneous effect of both growth media composition and environmental factors. For instance, Xin et al. (2010) considered the effect of phosphorus and nitrogen, He et al. (2012) inorganic carbon source and light intensity, Yoo et al. (2014) carbon substrate, nitrogen and light intensity and Solimeno et al. (2015) inorganic

carbon, nitrogen, light intensity and temperature. All these kinetic models were capable of predicting the dynamics of microalgal biomass growth. However, kinetic models should not only be able to reflect the dynamics of carbon assimilation towards biomass growth, but also the carbon partitioning between the internal carbohydrate and lipid pools.

Most of the available kinetic models have been constructed with the exclusive goal of precisely predicting microalgal biomass growth in response to different nutrient and carbon substrate concentrations and/or environmental factors, which allows establishing suitable cultivation conditions for optimised biomass production. Enhanced optimisation strategies for microalgae-driven fuel plants could additionally be established by considering algal growth dynamics along with the simultaneous formation of lipids, as well as their response to extracellular or intracellular growth-limiting factors. Until now, we have found four different kinetic modelling frameworks that has considered the lipid accumulation and biomass as different state variables in order to take advantage of nutrient starvation and consequently resulting in higher lipid productivities (Packer et al., 2011, Mairet et al., 2011, Adesanya et al., 2014, Baroukh et al., 2013).

The kinetic model that was developed here considers four growth-limiting factors and also simulates biomass growth and lipid accumulation as two different state variables to take advantage of abiotic stresses. In contrast to the kinetic models available in literature, extensively described in Chapter 2, our models allow for the prediction of biomass and lipid productivities separately (as in Chapters 3 to 5).

This does not only demonstrate the usefulness of computer-based optimisation studies for the enhancement of microalgal-based lipid production, but also the effectiveness of carefully constructed kinetic models for the efficient operation and control of microalgae culture processes. Such predictive kinetic modelling frameworks can be exploited for the robust design, control and optimization of microalgal oil accumulation as well as for process scale-up, which can help to reduce the overall production costs and bring this important technology closer to commercialisation and industrialisation.

6.2. Future work

The comprehensive kinetic modelling and simulation of the complex interactions between cellular components of microalgal cultivation processes are far from being completed at present. This thesis focuses on four growth-limiting factors (C, N, T, I) that could affect the cell composition. However, further studies are needed to reveal the relationship between cellular components with improved precision.

As it has been explained briefly in Chapter 2, phosphorus starvation can also affect the production rate of cellular components. Experiments can be performed with different phosphorus concentrations to understand the effect of phosphorus on cell composition and depending on the change of production rate of product of interest, the effect of phosphorus can also be included in the model.

The triple-substrate kinetic model used in Chapter 5 was a function nitrogen, light intensity and temperature. However, in outdoor microalgal cultivation processes, microalgae are exposed to available atmospheric CO_2. Therefore, the dissolved CO_2 concentration over the cultivation period can be monitored and the effect of carbon source can be included into the model as the one represented in Chapter 4.

The kinetic model proposed in chapter 5 was designed for growth of microalgae in open ponds operating in batch cultures. The model can be utilized to optimize the process for maximal lipid accumulation with respect to nitrogen, light intensity and temperature. However, in outdoor cultivations the light intensity and temperature cannot be controlled. Therefore, the cultivation process can be transformed into fed-batch cultures and depending on the daily light intensity and temperature, the nitrogen concentration can optimized for optimized lipid and biomass productivity.

Due to low available atmospheric CO_2 concentration, the growth rate of microalgae in open ponds is low compared to lab-scale cultivation. Therefore, CO_2 pumping can be provided into cultivation of microalgae in large-scale raceway open ponds to improve the growth rate of microalgae and the effect of CO_2 pumping can be included in the kinetic model for better estimation of dynamics of the system.

Experiments with different microalgae strains under varying growth media compositions and environmental factors can be performed to check the validity of the developed models for other microalgae strains. Based on the experimental observations and process dynamics the model can be modified and utilized for other strains.

C. reinhardtii was per-cultured under constant light intensity at 125 µmol $m^{-2}s^{-1}$ for the experiments. However, microalgae strains can acclimate due to variation in light spectra and their photosynthetic apparatus will be affected which will significantly change quantity and proportion of chlorophyll a and other major light absorbing accessory pigments. Therefore, the effect of different initial acclimation state can be further studied and included in the model for better predictions of the dynamics of microalgal biomass growth processes.

Finally, in this Thesis, model-based optimization technique was utilized to optimize the process. In order to present the effectiveness of such optimization technique, different optimization techniques such as extremum seeking can be used and a comparison can be carried out between different techniques.

6.3. Bibliography

Adesanya, V. O., Davey, M. P., Scott, S. A. & Smith, A. G. 2014. Kinetic modelling of growth and storage molecule production in microalgae under mixotrophic and autotrophic conditions. *Bioresource Technology,* 157**,** 293-304.

Ahmad, A. L., Yasin, N. H. M., Derek, C. J. C. & Lim, J. K. 2011. Microalgae as a sustainable energy source for biodiesel production: A review. *Renewable and Sustainable Energy Reviews,* 15**,** 584-593.

Alabi, A. O., Bibeau, E., Tampier, M. & Council, B. C. I. 2009. *Microalgae Technologies & Processes for Biofuels-bioenergy Production in British Columbia: Current Technology, Suitability & Barriers to Implementation: Final Report*, British Columbia Innovation Council.

Aleya, L., Dauta, A. & Reynolds, C. S. 2011. Endogenous regulation of the growth-rate responses of a spring-dwelling strain of the freshwater alga, Chlorella minutissima, to light and temperature. *European Journal of Protistology,* 47**,** 239-244.

Amin, S. 2009. Review on biofuel oil and gas production processes from microalgae. *Energy Conversion and Management,* 50**,** 1834-1840.

Andrews, J. F. 1968. A mathematical model for the continuous culture of microorganisms utilizing inhibitory substrates. *Biotechnology and Bioengineering,* 10**,** 707-723.

Aro, E.-M. 2016. From first generation biofuels to advanced solar biofuels. *Ambio,* 45**,** 24-31.

Bader, F. G. 1978. Analysis of double-substrate limited growth. *Biotechnology and Bioengineering,* 20**,** 183-202.

Bajhaiya, A. K., Dean, A. P., Driver, T., Trivedi, D. K., Rattray, N. J. W., Allwood, J. W., Goodacre, R. & Pittman, J. K. 2016. High-throughput metabolic screening of microalgae genetic variation in response to nutrient limitation. *Metabolomics,* 12**,** 1-14.

Baroukh, C., Muñoz-Tamayo, R., Steyer, J.-P. & Bernard, O. A new framework for metabolic modeling under non-balanced growth. Application to carbon metabolism of unicellular microalgae. Computer Applied to Biotechnology, 2013.

Baroukh, C., Steyer, J. P., Bernard, O. & Chachuat, B. 2016. dynamic Flux Balance Analysis of the Metabolism of Microalgae under a Diurnal Light Cycle. *IFAC-PapersOnLine,* 49**,** 791-796.

Béchet, Q., Shilton, A. & Guieysse, B. 2013. Modeling the effects of light and temperature on algae growth: State of the art and critical assessment for productivity prediction during outdoor cultivation. *Biotechnology Advances,* 31**,** 1648-1663.

Beran, B. & Kargi, F. 2005. A dynamic mathematical model for wastewater stabilization ponds. *Ecological Modelling,* 181**,** 39-57.

Bernard, O., Mairet, F. & Chachuat, B. 2016. Modelling of Microalgae Culture Systems with Applications to Control and Optimization. *In:* Posten, C. & Feng Chen, S. (eds.) *Microalgae Biotechnology.* Cham: Springer International Publishing.

Bitaubé Pérez, E., Caro Pina, I. & Pérez Rodríguez, L. 2008. Kinetic model for growth of Phaeodactylum tricornutum in intensive culture photobioreactor. *Biochemical Engineering Journal,* 40**,** 520-525.

Blair, M. F., Kokabian, B. & Gude, V. G. 2014. Light and growth medium effect on Chlorella vulgaris biomass production. *Journal of Environmental Chemical Engineering,* 2**,** 665-674.

Borowitzka, M. 1992. Algal biotechnology products and processes — matching science and economics. *Journal of Applied Phycology,* 4**,** 267-279.

Borowitzka, M. A. 1999. Commercial production of microalgae: ponds, tanks, tubes and fermenters. *Journal of Biotechnology,* 70**,** 313-321.

Borowitzka, M. A., Huisman, J. M. & Osborn, A. 1991. Culture of the astaxanthin-producing green algaHaematococcus pluvialis 1. Effects of nutrients on growth and cell type. *Journal of Applied Phycology,* 3**,** 295-304.

Bougaran, G., Bernard, O. & Sciandra, A. 2010. Modeling continuous cultures of microalgae colimited by nitrogen and phosphorus. *Journal of Theoretical Biology,* 265**,** 443-454.

Brennan, L. & Owende, P. 2010. Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews,* 14**,** 557-577.

Brennan, L. & Owende, P. 2013. Biofuels from Microalgae: Towards Meeting Advanced Fuel Standards. *In:* Lee, J. W. (ed.) *Advanced Biofuels and Bioproducts.* Springer New York.

Brentner, L. B., Eckelman, M. J. & Zimmerman, J. B. 2011. Combinatorial Life Cycle Assessment to Inform Process Design of Industrial Production of Algal Biodiesel. *Environmental Science & Technology,* 45**,** 7060-7067.

Breuer, G., Lamers, P. P., Janssen, M., Wijffels, R. H. & Martens, D. E. 2015. Opportunities to improve the areal oil productivity of microalgae. *Bioresource Technology,* 186**,** 294-302.

Caperon, J. & Meyer, J. 1972. Nitrogen-limited growth of marine phytoplankton—II. Uptake kinetics and their role in nutrient limited growth of phytoplankton. *Deep Sea Research and Oceanographic Abstracts,* 19**,** 619-632.

Carrier, G., Garnier, M., Le Cunff, L., Bougaran, G., Probert, I., De Vargas, C., Corre, E., Cadoret, J.-P. & Saint-Jean, B. 2014. Comparative Transcriptome of Wild Type and Selected Strains of the Microalgae Tisochrysis lutea Provides Insights into the Genetic Basis, Lipid Metabolism and the Life Cycle. *PLoS ONE,* 9**,** e86889.

Carvalho, A. P., Meireles, L. A. & Malcata, F. X. 2006. Microalgal Reactors: A Review of Enclosed System Designs and Performances. *Biotechnology Progress,* 22**,** 1490-1506.

Chapman, S. P., Paget, C. M., Johnson, G. N. & Schwartz, J.-M. 2015. Flux balance analysis reveals acetate metabolism modulates cyclic electron flow and alternative glycolytic pathways in Chlamydomonas reinhardtii. *Frontiers in Plant Science,* 6**,** 474.

Chen, C.-Y., Yeh, K.-L., Aisyah, R., Lee, D.-J. & Chang, J.-S. 2011. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. *Bioresource Technology,* 102**,** 71-81.

Chen, F. & Johns, M. R. 1994. Substrate inhibition of Chlamydomonas reinhardtii by acetate in heterotrophic culture. *Process Biochemistry,* 29**,** 245-252.

Chen, F. & Johns, M. R. 1996. Heterotrophic growth of Chlamydomonas reinhardtii on acetate in chemostat culture. *Process Biochemistry,* 31**,** 601-604.

Chen, G.-Q. & Chen, F. 2006. Growing Phototrophic Cells without Light. *Biotechnology Letters,* 28**,** 607-616.

Chiari, L. & Zecca, A. 2011. Constraints of fossil fuels depletion on global warming projections. *Energy Policy,* 39**,** 5026-5034.

Chisti, Y. 2007a. Biodiesel from microalgae. *Biotechnology Advances,* 25**,** 294-306.

Chisti, Y. 2007b. Biodiesel from microalgae. *Biotechnol Adv,* 25.

Coady, D., Parry, I., Sears, L. & Shang, B. 2017. How Large Are Global Fossil Fuel Subsidies? *World Development,* 91**,** 11-27.

Collet, P., Hélias, A., Lardon, L., Ras, M., Goy, R.-A. & Steyer, J.-P. 2011. Life-cycle assessment of microalgae culture coupled to biogas production. *Bioresource Technology,* 102**,** 207-214.

Converti, A., Casazza, A. A., Ortiz, E. Y., Perego, P. & Del Borghi, M. 2009. Effect of temperature and nitrogen concentration on the growth and lipid content of

Nannochloropsis oculata and Chlorella vulgaris for biodiesel production. *Chemical Engineering and Processing: Process Intensification,* 48**,** 1146-1151.

Courchesne, N. M. D., Parisien, A., Wang, B. & Lan, C. Q. 2009. Enhancement of lipid production using biochemical, genetic and transcription factor engineering approaches. *Journal of Biotechnology,* 141**,** 31-41.

D'elia, C. F. & Deboer, J. A. 1978. NUTRITIONAL STUDIES OF TWO RED ALGAE. II. KINETICS OF AMMONIUM AND NITRATE UPTAKE1, 2. *Journal of Phycology,* 14**,** 266-272.

Das, P., Lei, W., Aziz, S. S. & Obbard, J. P. 2011. Enhanced algae growth in both phototrophic and mixotrophic culture under blue light. *Bioresource Technology,* 102**,** 3883-3887.

Davidson, K. & Gurney, W. S. C. 1999. An investigation of non-steady-state algal growth. II. Mathematical modelling of co-nutrient-limited algal growth. *Journal of Plankton Research,* 21**,** 839-858.

Davis, R., Aden, A. & Pienkos, P. T. 2011. Techno-economic analysis of autotrophic microalgae for fuel production. *Applied Energy,* 88**,** 3524-3531.

Demirbas, A. 2010. Biodiesel for Future Transportation Energy Needs. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects,* 32**,** 1490-1508.

Demirbas, A. & Fatih Demirbas, M. 2011. Importance of algae oil as a source of biodiesel. *Energy Conversion and Management,* 52**,** 163-170.

Dragone, G., Fernandes, B. D., Vicente, A. A. & Teixeira, J. A. 2010. Third generation biofuels from microalgae.

Driver, T., Bajhaiya, A. & Pittman, J. K. 2014. Potential of Bioenergy Production from Microalgae. *Current Sustainable/Renewable Energy Reports,* 1**,** 94-103.

Droop, M. R. 1968. Vitamin B12 and Marine Ecology. IV. The Kinetics of Uptake, Growth and Inhibition in Monochrysis Lutheri. *Journal of the Marine Biological Association of the United Kingdom,* 48**,** 689-733.

Easac. 2012. *The current status of biofuels in the European Union, their environmental impacts and future prospects* [Online]. Germany: EASAC. [Accessed 02/05/2017 2017].

Efroymson, R. A. & Dale, V. H. 2015. Environmental indicators for sustainable production of algal biofuels. *Ecological Indicators,* 49**,** 1-13.

Eriksen, N. T., Riisgård, F. K., Gunther, W. S. & Lønsmann Iversen, J. J. 2006. On-line estimation of O2 production, CO2 uptake, and growth kinetics of microalgal cultures in a gas-tight photobioreactor. *Journal of Applied Phycology,* 19**,** 161-174.

Fan, J., Yan, C., Andre, C., Shanklin, J., Schwender, J. & Xu, C. 2012. Oil accumulation is controlled by carbon precursor supply for fatty acid synthesis in Chlamydomonas reinhardtii. *Plant and Cell Physiology,* 53**,** 1380-1390.

Field, C. B., Campbell, J. E. & Lobell, D. B. 2008. Biomass energy: the scale of the potential resource. *Trends in Ecology & Evolution,* 23**,** 65-72.

Figueroa-Torres, G. M., Pittman, J. & Theodoropoulos, C. 2017. Kinetic Modelling of Starch and Lipid Formation during Mixotrophic, Nutrient-limited Microalgal Growth *Bioresource Technology,* Submitted.

Flassig, R. J., Fachet, M., Höffner, K., Barton, P. I. & Sundmacher, K. 2016. Dynamic flux balance modeling to increase the production of high-value compounds in green microalgae. *Biotechnology for Biofuels,* 9**,** 165.

Flynn, K. J. 2003. Modelling multi-nutrient interactions in phytoplankton; balancing simplicity and realism. *Progress in Oceanography,* 56**,** 249-279.

Flynn, K. J. 2005. Modelling marine phytoplankton growth under eutrophic conditions. *Journal of Sea Research,* 54**,** 92-103.

Franz, A., Lehr, F., Posten, C. & Schaub, G. 2012. Modeling microalgae cultivation productivities in different geographic locations – estimation method for idealized photobioreactors. *Biotechnology Journal,* 7**,** 546-557.

Gan, S.-Y., Lim, P.-E. & Phang, S.-M. 2016. Genetic and Metabolic Engineering of Microalgae. *In:* Bux, F. & Chisti, Y. (eds.) *Algae Biotechnology: Products and Processes.* Cham: Springer International Publishing.

Gao, C., Zhai, Y., Ding, Y. & Wu, Q. 2010. Application of sweet sorghum for biodiesel production by heterotrophic microalga Chlorella protothecoides. *Applied Energy,* 87**,** 756-761.

Georgianna, D. R. & Mayfield, S. P. 2012. Exploiting diversity and synthetic biology for the production of algal biofuels. *Nature,* 488**,** 329-335.

Goldman, J. C. & Carpenter, E. J. 1974. A kinetic approach to the effect of temperature on algal growth1. *Limnology and Oceanography,* 19**,** 756-766.

Goncalves, E. C., Wilkie, A. C., Kirst, M. & Rathinasabapathi, B. 2015. Metabolic regulation of triacylglycerol accumulation in the green algae: identification of potential targets for engineering to improve oil yield. *Plant Biotechnology Journal***,** n/a-n/a.

Goodson, C., Roth, R., Wang, Z. T. & Goodenough, U. 2011. Structural Correlates of Cytoplasmic and Chloroplast Lipid Body Synthesis in Chlamydomonas reinhardtii and Stimulation of Lipid Body Production with Acetate Boost. *Eukaryotic cell,* 10**,** 1592-1606.

Gormley, P., Li, K. & Irwin, G. W. 2007. Modelling molecular interaction pathways using a two-stage identification algorithm. *Systems and Synthetic Biology,* 1**,** 145-160.

Griffiths, M. J. & Harrison, S. T. L. 2009. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. *Journal of Applied Phycology,* 21**,** 493-507.

Grover, J. P. 1991. Dynamics of Competition among Microalgae in Variable Environments: Experimental Tests of Alternative Models. *Oikos,* 62**,** 231-243.

Haldane, J. B. S. A mathematical theory of natural and artificial selection.(Part VI, Isolation.). Mathematical Proceedings of the Cambridge Philosophical Society, 1930. Cambridge Univ Press, 220-230.

Han, F., Pei, H., Hu, W., Song, M., Ma, G. & Pei, R. 2015. Optimization and lipid production enhancement of microalgae culture by efficiently changing the conditions along with the growth-state. *Energy Conversion and Management,* 90**,** 315-322.

He, L., Subramanian, V. R. & Tang, Y. J. 2012. Experimental analysis and model-based optimization of microalgae growth in photo-bioreactors using flue gas. *Biomass and Bioenergy,* 41**,** 131-138.

Hoel, M. & Kverndokk, S. 1996. Depletion of fossil fuels and the impacts of global warming. *Resource and Energy Economics,* 18**,** 115-136.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M. & Darzins, A. 2008a. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant J,* 54.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M. & Darzins, A. 2008b. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *The Plant Journal,* 54**,** 621-639.

Jean Vasile, A., Raluca Andreea, I., Popescu, G. H., Elvira, N. & Marian, Z. 2016. Implications of agricultural bioenergy crop production and prices in changing the land use paradigm—The case of Romania. *Land Use Policy,* 50**,** 399-407.

Jiménez, C., CossíO, B. R., Labella, D. & Xavier Niell, F. 2003. The Feasibility of industrial production of Spirulina (Arthrospira) in Southern Spain. *Aquaculture,* 217**,** 179-190.

John, E. H. & Flynn, K. J. 2000. Modelling phosphate transport and assimilation in microalgae; how much complexity is warranted? *Ecological Modelling,* 125**,** 145-157.

Jørgensen, S. E. 1976. A eutrophication model for a lake. *Ecological Modelling,* 2**,** 147-165.

Jorquera, O., Kiperstok, A., Sales, E. A., Embiruçu, M. & Ghirardi, M. L. 2010. Comparative energy life-cycle analyses of microalgal biomass production in open ponds and photobioreactors. *Bioresource Technology,* 101**,** 1406-1413.

Kalacheva, G. S., Zhila, N. O., Volova, T. G. & Gladyshev, M. I. 2002. The Effect of Temperature on the Lipid Composition of the Green Alga Botryococcus. *Microbiology,* 71**,** 286-293.

Kirkpatrick, S., Gelatt, C. D. & Vecchi, M. P. 1983. Optimization by simulated annealing. *Science,* 220**,** 671-680.

Klasson, K. T., Lundbäck, K. M. O., Clausen, E. C. & Gaddy, J. L. 1993. Kinetics of light limited growth and biological hydrogen production from carbon monoxide and water by Rhodospirillum rubrum. *Journal of Biotechnology,* 29**,** 177-188.

Klausmeier, C. A., Litchman, E. & Levin, S. A. 2004. Phytoplankton growth and stoichiometry under multiple nutrient limitation. *Limnology and Oceanography,* 49**,** 1463-1470.

Kong, Q.-X., Li, L., Martinez, B., Chen, P. & Ruan, R. 2010. Culture of Microalgae Chlamydomonas reinhardtii in Wastewater for Biomass Feedstock Production. *Applied Biochemistry and Biotechnology,* 160**,** 9-18.

Kotasthane, T. 2017. Potential of Microalgae for Sustainable Biofuel Production. *Journal of Marine Science: Research & Development,* 7:223.

Kwon, H. K., Oh, S. J. & Yang, H.-S. 2013. Growth and uptake kinetics of nitrate and phosphate by benthic microalgae for phytoremediation of eutrophic coastal sediments. *Bioresource Technology,* 129**,** 387-395.

Lai, E. 2014. Biodiesel: Environmental Friendly Alternative to Petrodiesel. *J Pet Environ Biotechnol,* 5**,** e122.

Lee, E., Jalalizadeh, M. & Zhang, Q. 2015. Growth kinetic models for microalgae cultivation: A review. *Algal Research,* 12**,** 497-512.

Lee, Y.-K. 2007. Algal Nutrition – Heterotrophic Carbon Nutrition. *Handbook of Microalgal Culture.* Blackwell Publishing Ltd.

Li, Y., Zhou, W., Hu, B., Min, M., Chen, P. & Ruan, R. R. 2012. Effect of light intensity on algal biomass accumulation and biodiesel production for mixotrophic strains Chlorella kessleri and Chlorella protothecoide cultivated in highly concentrated municipal wastewater. *Biotechnology and Bioengineering,* 109**,** 2222-2229.

Liang, Y., Sarkany, N. & Cui, Y. 2009. Biomass and lipid productivities of Chlorella vulgaris under autotrophic, heterotrophic and mixotrophic growth conditions. *Biotechnology Letters,* 31**,** 1043-1049.

Litchman, E., Klausmeier, C. A., Miller, J. R., Schofield, O. M. & Falkowski, P. G. 2006. Multi-nutrient, multi-group model of present and future oceanic phytoplankton communities. *Biogeosciences Discussions,* 3**,** 607-663.

Mairet, F., Bernard, O., Lacour, T. & Sciandra, A. 2011a. Modelling microalgae growth in nitrogen limited photobiorector for estimating biomass, carbohydrate and neutral lipid productivities. *IFAC Proceedings Volumes,* 44**,** 10591-10596.

Mairet, F., Bernard, O., Masci, P., Lacour, T. & Sciandra, A. 2011b. Modelling neutral lipid production by the microalga Isochrysis aff. galbana under nitrogen limitation. *Bioresource Technology,* 102**,** 142-149.

Martínez, M. E., Jiménez, J. M. & El Yousfi, F. 1999. Influence of phosphorus concentration and temperature on growth and phosphorus uptake by the microalga Scenedesmus obliquus. *Bioresource Technology,* 67**,** 233-240.

Martínez Sancho, M. E., Jiménez Castillo, J. M. & El Yousfi, F. 1997. Influence of phosphorus concentration on the growth kinetics and stoichiometry of the microalga Scenedesmus obliquus. *Process Biochemistry,* 32**,** 657-664.

Mata, T. M., Martins, A. A. & Caetano, N. S. 2010. Microalgae for biodiesel production and other applications: A review. *Renewable and Sustainable Energy Reviews,* 14**,** 217-232.

Maxwell, D. P., Falk, S., Trick, C. G. & Huner, N. 1994. Growth at Low Temperature Mimics High-Light Acclimation in Chlorella vulgaris. *Plant physiology,* 105**,** 535-543.

Mayo, A. W. 1997. Effects of Temperature and pH on the Kinetic Growth of Unialga Chlorella vulgaris Cultures Containing Bacteria. *Water Environment Research,* 69**,** 64-72.

Meireles Dos Santos, A., Vieira, K. R., Basso Sartori, R., Meireles Dos Santos, A., Queiroz, M. I., Queiroz Zepka, L. & Jacob-Lopes, E. 2017. Heterotrophic Cultivation of Cyanobacteria: Study of Effect of Exogenous Sources of Organic Carbon, Absolute Amount of Nutrients, and Stirring Speed on Biomass and Lipid Productivity. *Frontiers in Bioengineering and Biotechnology,* 5**,** 12.

Mercer, P. & Armenta, R. E. 2011. Developments in oil extraction from microalgae. *European Journal of Lipid Science and Technology,* 113**,** 539-547.

Molina, E., Martínez, M. E., Sánchez, S., García, F. & Contreras, A. 1991. Growth and biochemical composition with emphasis on the fatty acids of Tetraselmis sp. *Applied Microbiology and Biotechnology,* 36**,** 21-25.

Monod, J. 1949. The Growth of Bacterial Cultures. *Annual Review of Microbiology,* 3**,** 371-394.

Montzka, S. A., Dlugokencky, E. J. & Butler, J. H. 2011. Non-CO2 greenhouse gases and climate change. *Nature,* 476**,** 43-50.

Nan, C. & Dong, S. 2004. Comparative studies on phosphorus uptake and growth kinetics of the microalga Tetraselmis subcordiformis and the macroalga Ulva pertusa. *Journal of Ocean University of China,* 3**,** 56-59.

Nigam, P. S. & Singh, A. 2011. Production of liquid biofuels from renewable resources. *Progress in Energy and Combustion Science,* 37**,** 52-68.

Niu, Y.-F., Zhang, M.-H., Li, D.-W., Yang, W.-D., Liu, J.-S., Bai, W.-B. & Li, H.-Y. 2013. Improvement of Neutral Lipid and Polyunsaturated Fatty Acid Biosynthesis by Overexpressing a Type 2 Diacylglycerol Acyltransferase in Marine Diatom Phaeodactylum tricornutum. *Marine Drugs,* 11**,** 4558.

Ogbonna, J. C. & Moheimani, N. R. 2015. Potentials of Exploiting Heterotrophic Metabolism for Biodiesel Oil Production by Microalgae. *In:* Moheimani, R. N., Mchenry, P. M., De Boer, K. & Bahri, A. P. (eds.) *Biomass and Biofuels from Microalgae: Advances in Engineering and Biology.* Cham: Springer International Publishing.

Oldeman, L. 1994. The global extent of soil degradation. *Soil resilience and sustainable land use,* 9.

Packer, A., Li, Y., Andersen, T., Hu, Q., Kuang, Y. & Sommerfeld, M. 2011. Growth and neutral lipid synthesis in green microalgae: A mathematical model. *Bioresource Technology,* 102**,** 111-117.

Perez-Garcia, O., Escalante, F. M. E., De-Bashan, L. E. & Bashan, Y. 2011. Heterotrophic cultures of microalgae: Metabolism and potential products. *Water Research,* 45**,** 11-36.

Pfaffinger, C. E., Schöne, D., Trunz, S., Löwe, H. & Weuster-Botz, D. 2016. Model-based optimization of microalgae areal productivity in flat-plate gas-lift photobioreactors. *Algal Research,* 20**,** 153-163.

Pittman, J. K., Dean, A. P. & Osundeko, O. 2011. The potential of sustainable algal biofuel production using wastewater resources. *Bioresource Technology,* 102**,** 17-25.

Pulz, O. 2001. Photobioreactors: production systems for phototrophic microorganisms. *Applied Microbiology and Biotechnology,* 57**,** 287-293.

Radakovits, R., Jinkerson, R. E., Darzins, A. & Posewitz, M. C. 2010. Genetic engineering of algae for enhanced biofuel production. *Eukaryotic cell,* 9**,** 486-501.

Ras, M., Steyer, J.-P. & Bernard, O. 2013. Temperature effect on microalgae: a crucial factor for outdoor production. *Reviews in Environmental Science and Bio/Technology,* 12**,** 153-164.

Ratkowsky, D. A., Olley, J., Mcmeekin, T. A. & Ball, A. 1982. Relationship between temperature and growth rate of bacterial cultures. *Journal of Bacteriology,* 149**,** 1-5.

Rhee, G. Y. 1978. Effects of N:P atomic ratios and nitrate limitation on algal growth, cell composition, and nitrate uptake 1. *Limnology and Oceanography,* 23**,** 10-25.

Richardson, J. W., Johnson, M. D. & Outlaw, J. L. 2012. Economic comparison of open pond raceways to photo bio-reactors for profitable production of algae for transportation fuels in the Southwest. *Algal Research,* 1**,** 93-100.

Roels, J. 1983. *Energetics and kinetics in biotechnology*, Elsevier Biomedical Press.

Rosso, L., Lobry, J. R., Bajard, S. & Flandrois, J. P. 1995. Convenient Model To Describe the Combined Effects of Temperature and pH on Microbial Growth. *Applied and Environmental Microbiology,* 61**,** 610-6.

Ruangsomboon, S. 2012. Effect of light, nutrient, cultivation time and salinity on lipid production of newly isolated strain of the green microalga, Botryococcus braunii KMITL 2. *Bioresource Technology,* 109**,** 261-265.

Sada, E., Katoh, S., Kheirolomoon, A. & Yokoi, H. 1989. Effects of light intensity on the growth rate of the red alga Porphyridium cruentum. *Journal of Fermentation and Bioengineering,* 67**,** 135-137.

Sapphire Energy, I. 2012. *Algae Farm* [Online]. Available: http://www.sapphireenergy.com/locations/green-crude-farm.html [Accessed 2016.

Sasi, D., Mitra, P., Vigueras, A. & Hill, G. A. 2011. Growth kinetics and lipid production using Chlorella vulgaris in a circulating loop photobioreactor. *Journal of Chemical Technology & Biotechnology,* 86**,** 875-880.

Schoneveld, G., German, L. & Nutakor, E. 2010. *Towards sustainable biofuel development: Assessing the local impacts of large-scale foreign land acquisitions in Ghana*.

Şendrescu, D. & Selişteanu, D. Modeling of bacterial growth bioprocesses based on heuristic optimization techniques. Proceedings of the 2015 16th International Carpathian Control Conference (ICCC), 27-30 May 2015 2015. 470-474.

Shankar, S. & Shikha 2017. Renewable and Nonrenewable Energy Resources: Bioenergy and Biofuels. *In:* Singh, R. L. (ed.) *Principles and Applications of Environmental Biotechnology for a Sustainable Future.* Singapore: Springer Singapore.

Shi, X.-M., Zhang, X.-W. & Chen, F. 2000. Heterotrophic production of biomass and lutein by Chlorella protothecoides on various nitrogen sources. *Enzyme and Microbial Technology,* 27**,** 312-318.

Singh, B., Guldhe, A., Singh, P., Singh, A., Rawat, I. & Bux, F. 2015. Sustainable Production of Biofuels from Microalgae Using a Biorefinary Approach. *In:* Kaushik, G. (ed.) *Applied Environmental Biotechnology: Present Scenario and Future Trends.* Springer India.

Solimeno, A., Samsó, R., Uggetti, E., Sialve, B., Steyer, J.-P., Gabarró, A. & García, J. 2015. New mechanistic model to simulate microalgae growth. *Algal Research,* 12**,** 350-358.

Sousa, A. I., Martins, I., Lillebø, A. I., Flindt, M. R. & Pardal, M. A. 2007. Influence of salinity, nutrients and light on the germination and growth of Enteromorpha sp. spores. *Journal of Experimental Marine Biology and Ecology,* 341**,** 142-150.

Spijkerman, E., De Castro, F. & Gaedke, U. 2011. Independent Colimitation for Carbon Dioxide and Inorganic Phosphorus. *PLoS ONE,* 6**,** e28219.

Surisetty, K., Hoz Siegler, H. D. L., Mccaffrey, W. C. & Ben-Zvi, A. 2010. Robust modeling of a microalgal heterotrophic fed-batch bioreactor. *Chemical Engineering Science,* 65**,** 5402-5410.

Tamburic, B., Zemichael, F. W., Maitland, G. C. & Hellgardt, K. 2011. Parameters affecting the growth and hydrogen production of the green alga Chlamydomonas reinhardtii. *International Journal of Hydrogen Energy,* 36**,** 7872-7876.

Thomas, C. E. 2017. Petroleum and Coal Proven Reserves: The Case for Coal and the Demise of OPEC. *Stopping Climate Change: the Case for Hydrogen and Coal.* Cham: Springer International Publishing.

Thornton, A., Weinhart, T., Bokhove, O., Zhang, B., Sar, D. M. V. D., Kumar, K., Pisarenco, M., Rudnaya, M., Savceno, V., Rademacher, J., Zijlstra, J., Szabelska, A., Zyprych, J., Schans, M. V. D., Timperio, V. & Veerman, F. 2010. Modeling and optimization of algae growth. *In:* Frank, J., Van Der Mei, R., Den Boer, A., Bosman, J., Bouman, N., Van Dam, S. & Verhoef, C. (eds.) *72nd European Study Group Mathematics with Industry.* Amsterdam, the Netherlands: CWI.

Trentacoste, E. M., Shrestha, R. P., Smith, S. R., Glé, C., Hartmann, A. C., Hildebrand, M. & Gerwick, W. H. 2013. Metabolic engineering of lipid catabolism increases microalgal lipid accumulation without compromising growth. *Proceedings of the National Academy of Sciences,* 110**,** 19748-19753.

Van Wagenen, J., Miller, T. W., Hobbs, S., Hook, P., Crowe, B. & Huesemann, M. 2012. Effects of Light and Temperature on Fatty Acid Production in Nannochloropsis Salina. *Energies,* 5**,** 731.

Vassilev, S. V. & Vassileva, C. G. 2016. Composition, properties and challenges of algae biomass for biofuel application: An overview. *Fuel,* 181**,** 1-33.

Venkata Mohan, S., Rohit, M. V., Chiranjeevi, P., Chandra, R. & Navaneeth, B. 2015. Heterotrophic microalgae cultivation to synergize biodiesel production with waste remediation: Progress and perspectives. *Bioresource Technology,* 184**,** 169-178.

Vlysidis, A., Binns, M., Webb, C. & Theodoropoulos, C. 2011. Glycerol utilisation for the production of chemicals: Conversion to succinic acid, a combined experimental and computational study. *Biochemical Engineering Journal,* 58–59**,** 1-11.

Wang, J., Yang, H. & Wang, F. 2014a. Mixotrophic Cultivation of Microalgae for Biodiesel Production: Status and Prospects. *Applied Biochemistry and Biotechnology,* 172**,** 3307-3329.

Wang, M., Kuo-Dahab, W. C., Dolan, S. & Park, C. 2014b. Kinetics of nutrient removal and expression of extracellular polymeric substances of the microalgae, Chlorella sp. and Micractinium sp., in wastewater treatment. *Bioresource Technology,* 154**,** 131-137.

Xin, L., Hong-Ying, H., Ke, G. & Ying-Xue, S. 2010. Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga Scenedesmus sp. *Bioresource Technology,* 101**,** 5494-5500.

Yamamoto, T., Suzuki, M., Kim, K. & Asaoka, S. 2012. Growth and uptake kinetics of phosphate by benthic microalga Nitzschia sp. isolated from Hiroshima Bay, Japan. *Phycological Research,* 60**,** 223-228.

Yan, C., Zhang, L., Luo, X. & Zheng, Z. 2013. Effects of various LED light wavelengths and intensities on the performance of purifying synthetic domestic sewage by microalgae at different influent C/N ratios. *Ecological Engineering,* 51**,** 24-32.

Yang, J., Rasa, E., Tantayotai, P., Scow, K. M., Yuan, H. & Hristova, K. R. 2011. Mathematical model of Chlorella minutissima UTEX2341 growth and lipid production under photoheterotrophic fermentation conditions. *Bioresource Technology,* 102**,** 3077-3082.

Yang, Z., Geng, L., Wang, W. & Zhang, J. 2012. Combined effects of temperature, light intensity, and nitrogen concentration on the growth and polysaccharide content of Microcystis aeruginosa in batch culture. *Biochemical Systematics and Ecology,* 41**,** 130-135.

Yao, B., Xi, B., Hu, C., Huo, S., Su, J. & Liu, H. 2011. A model and experimental study of phosphate uptake kinetics in algae: Considering surface adsorption and P-stress. *Journal of Environmental Sciences,* 23**,** 189-198.

Yeesang, C. & Cheirsilp, B. 2011. Effect of nitrogen, salt, and iron content in the growth medium and light intensity on lipid production by microalgae isolated from freshwater sources in Thailand. *Bioresource Technology,* 102**,** 3034-3040.

Yoo, S. J., Kim, J. H. & Lee, J. M. 2014. Dynamic modelling of mixotrophic microalgal photobioreactor systems with time-varying yield coefficient for the lipid consumption. *Bioresource Technology,* 162**,** 228-235.

Yoshimura, T., Okada, S. & Honda, M. 2013. Culture of the hydrocarbon producing microalga Botryococcus braunii strain Showa: Optimal CO2, salinity, temperature, and irradiance conditions. *Bioresource Technology,* 133**,** 232-239.

Zeng, A. P., Ross, A., Biebl, H., Tag, C., Günzel, B. & Deckwer, W. D. 1994. Multiple product inhibition and growth modeling of clostridium butyricum and klebsiella pneumoniae in glycerol fermentation. *Biotechnology and Bioengineering,* 44**,** 902-911.

Zhang, X.-W., Chen, F. & Johns, M. R. 1999a. Kinetic models for heterotrophic growth of Chlamydomonas reinhardtii in batch and fed-batch cultures. *Process Biochemistry,* 35**,** 385-389.

Zhang, X.-W., Zhang, Y.-M. & Chen, F. 1998. Kinetic models for phycocyanin production by high cell density mixotrophic culture of the microalga Spirulina platensis. *Journal of Industrial Microbiology and Biotechnology,* 21**,** 283-288.

Zhang, X. W., Zhang, Y. M. & Chen, F. 1999b. Application of mathematical models to the determination optimal glucose concentration and light intensity for mixotrophic culture of Spirulina platensis. *Process Biochemistry,* 34**,** 477-481.

Zhou, X., Yuan, S., Chen, R. & Ochieng, R. M. 2015. Sustainable production of energy from microalgae: Review of culturing systems, economics, and modelling. *Journal of Renewable and Sustainable Energy,* 7**,** 012701.

Zhu, S., Huang, W., Xu, J., Wang, Z., Xu, J. & Yuan, Z. 2014. Metabolic changes of starch and lipid triggered by nitrogen starvation in the microalga Chlorella zofingiensis. *Bioresource Technology,* 152**,** 292-298.

Ziolkowska, J. R. & Simon, L. 2014. Recent developments and prospects for algae-based fuels in the US. *Renewable and Sustainable Energy Reviews,* 29**,** 847-853.