8-2016

Mixotrophic Cultivation of Microalgae for Biomass Production Optimization Using Statistical Methods

Ning Zhang
Clemson University, ningz@g.clemson.edu

MIXOTROPHIC CULTIVATION OF MICROALGAE FOR BIOMASS PRODUCTION OPTIMIZATION USING STATISTICAL METHODS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Biosystems Engineering

---

by
Ning Zhang
August 2016

Approved by:
Dr. Terry H. Walker, Committee Chair
Dr. David A. Ladner
Dr. William C. Bridges

ABSTRACT

Microalgae have received considerable attention as a potential source for the third generation biofuel. Various products including biodiesel could be obtained from the algal biomass. In this study, the green algae *Chlorella protothecoides* was cultured in mixotrophic mode – under the light from a LED lamp and supplemented with organic carbon and nitrogen sources. The carbon and nitrogen sources were selected for their capabilities to sustain algae growth, and their low cost as industrial by-products. Crude glycerol was derived from the biodiesel production process and the spent brewer's yeast was the remaining yeast after beer fermentation. Both were subject to certain pretreatment steps before being mixed with the culturing medium. To assess their effects on algae growth, both the glycerol and the yeast were evaluated based on their carbon (g C/L) and nitrogen concentration (g N/L).

To study the effects of light intensity ($\mu mol/m^{-2}\ s^{-1}$), carbon concentration in the medium (g C/L), and nitrogen concentration (g N/L), experiments were performed based on a three-factor, three-level full factorial design, with algal biomass (g/L) as the response. Data was fitted into a response surface model to investigate the impact on biomass of 3 factors. Initially a regular response surface model was obtained, yet the lack of fit was quite significant. Then another modified model was fitted to the data. Compared to the regular response surface model, the new model had better fit with the response from the experiment data.

DEDICATION

This thesis is dedicated to all who have supported me throughout my studies here at Clemson. It is dedicated to my parents: Xianzhou Zhang and Liying Gao. It is dedicated to all my friends here: Rui Xiao, Karthik Gopalakrishnan, David Thornton, Wilson Beita, Meric Selbes, Jasmin Taylor, and Xiaoyu Feng.

ACKNOWLEDGEMENTS

I would like to thank my committee members, especially my supervisor Dr. Terry H. Walker for his patience and support through my whole master studies. Also I would like to thank Dr. William Bridges for his help with the experiment design and data analysis during my research. And I would like to thank Dr. David Ladner for his advices in designing the project.

My thanks also go to Dr. Caye Drapcho and Dr. Yi Zheng for their support and encouragement during my studies at the Biosystems Engineering at Clemson. And I want to thank all other faculty, staff and students in the EEES department for all the assistance I received.

I would like to thank all current and previous lab members, David, Karthik, Shwetha, Xiaoyu, and Arpan for their help with my research. Meanwhile I want to thank Dr. Nishanth Tharayil and his student Amith Maroli for their help with the chromatography analysis. Finally, I would like to thank all my friends and my parents for their continuing support of me.

TABLE OF CONTENTS

Table of Contents (Continued)

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

ABBREVIATIONS

TOC	Total Organic Carbon
TN	Total Nitrogen
YE	Yeast Extract
RSM	Response Surface Methodology

CHAPTER I

INTRODUCTION

The International Energy Agency (IEA) has predicted that the world energy demand will continue growing, which is mainly driven by developing countries and regions (https://www.iea.org/Textbase/npsum/WEO2015SUM.pdf). Meanwhile, growing concerns about global warming has led to increasing efforts for less dependence on fossil fuels. This awareness creates many opportunities to develop biofuels, especially biodiesel.

1.1 Biomass yield improvement

Much expectation has been given to microalgae derived biodiesel, because microalgae can accumulate considerable amount of lipids and the algae cultivation will not compete with food crops for land usage (Chisti, 2007). However, the production cost is still prohibitively high for the microalgae biodiesel. To tackle this issue, the total algal biomass yield needs to be enhanced in order to reduce the production cost per unit of algal biomass. Heterotrophic or mixotrophic cultivation can be employed as either culturing mode has been demonstrated to lead to faster growth if compared to autotrophic cultivation (Mitra, van Leeuwen and Lamsal, 2012a; Liang, Sarkany and Cui, 2009b; Heredia-Arroyo, Wei and Hu, 2010b). And furthermore, culturing conditions have to be optimized for maximal algal biomass yield. Usually, statistical methods such as the response surface methodology (RSM) would assist in the optimization step. The RSM modeling can investigate the interaction between different variables, instead of studying one single factor each time (Chen and Wang, 2013; Mopkar Anand, Sankar and Daniel, 2013).

1.2 Low cost nutrients

To lower the algal biomass production cost, inexpensive nutrients such as industrial or municipal waste effluents can be used, which would simultaneously solve the waste water treatment problem. During the biodiesel production, glycerol is generated as a by-product that also contains various impurities, and the refining process would incur high cost in order to obtain pure glycerin, resulting in a decreasing price (Chen and Walker, 2011). As microalgae can grow in different carbon sources, including glycerol, the aforementioned crude glycerol could be used as a low cost carbon source. Nitrogen source is also a crucial factor for algae cultivation, and the commercial yeast extract is a widely used nitrogen source in many laboratories. As an alternative of the expensive yeast extract, the brewery spent yeast could be a cheap source of nitrogen. It is rich in protein, thus nitrogen, and vitamin B complex, while being inexpensive because many small breweries would discharge it into the drainage systems, which would cost them for waste water treatment (dos Santos Mathias et al., 2014; Mussatto, 2009). Therefore, spent yeast could be collected for algae cultivation, and in combination with crude glycerol, would also decrease the financial cost and energy consumption from the waste effluent treatment of the respective industries.

1.3 Objectives

In this research, the microalgae *Chlorella protothecoides* was grown under LED light, while the culturing medium was supplemented with biodiesel derived crude glycerol and brewery spent yeast. A three-factor by three-level factorial design was used for the experiments, and the response surface model was attempted to fit into the data. The major objectives of this research are:

1. To grow the microalgae *Chlorella protothecoides* under mixotrophic condition;

2. To evaluate the crude glycerol and brewery spent yeast for their potential to sustain the algae growth;

3. To statistically model the algae biomass production on three variables – LED light intensity, carbon concentration and nitrogen concentration in the medium.

CHAPTER II

LITERATURE REVIEW

Human began consuming microalgae as a food source for thousands of years, but algae cultivation only started a few hundred years ago (Spolaore et al., 2006). According to history records, the Aztec population was the first to cultivate *Arthrospira spirulina* as a food source (Pulz and Gross, 2004). Then in 1731, people attempted to cultivate brown algae which would be processed to obtain iodine and soda. When it came to the 20th century, the Nazi Germany started large-scale algae cultivation for protein in the Second World War, probably due to the lack of animal source proteins that had been cut off by the allied blockade. After the war, in the 1950s researchers from Carnegie Institution contributed to this field by demonstrating that the lipid and protein contents of Chlorella cells could be altered by changing environmental conditions. In 1955, Meier proposed that algae be processed to turn the hydrocarbon contents into methane gas, which marked the first time that microalgae were considered an energy source (Hu et al., 2008). Then in 1957 Japanese scientists carried out outdoor mass cultivation of Chlorella, and thus Japan became the first country to produce and sell Chlorella biomass as a healthy food (Vonshak, 1997). Since the 1960s, all kinds of commercial algae culturing facilities have been established in the U.S., Germany, Japan, Israel and China. Besides biomass, other products have been extracted from the microalgae cultures, such as β-carotenoids and astaxanthin. In just a few decades, the microalgae industry has expanded and diversified and now it is generating 1.25 billion US dollars per year (Ben-Amotz, 2004). The development is due to the advancing of the knowledge on all aspects of algae — their chemical compositions, metabolic pathways, biochemical mechanisms

for specific physiological functions, and processing techniques to manufacture desired algal products. Below is a summary of such knowledge and progresses.

2.1 Microalgae

Microalgae have been found in diverse ecological habitats including fresh water, brackish water and marine environments, and thus vary from one species to another in growth conditions such as temperature, pH, and salinity. Till now, there are approximately 100,000 species that have been found, with even more yet to be identified (Sheehan et al., 1998). The currently discovered algae can be classified into the following categories: cyanobacteria (Cyanophyceae), diatoms (Bacillariophyceae), green algae (Chlorophyceae), golden algae (Chrysophyceae), red algae (Rhodophyceae), brown algae (Phaeophyceae), yellow-green algae (Xanthophyceae), dinoflagellates (Dinophyceae) and 'pico-plankton' (Prasinophyceae and Eustigmatophyceae). Among these taxa, the first four are found to be the most abundant (Hu et al., 2008).

2.1.1 Chemical compositions of microalgae

The worldwide algae consumption can be explained by the chemical compositions of the algae biomass. Microalgae are rich in proteins, which contain all amino acids, including the essential amino acids. The protein contents of several algal strains are comparable to animal sources of proteins like meat and milk (Spolaore et al., 2006). That is the reason behind the great nutritional value of algae-containing food. Carbohydrates in microalgae are easy to digest, which made early consumption of dry algae biomass possible. Lipid content varies from 10% to 70% on the basis of dry weight biomass, depending on specific strains and culturing conditions (Metting,

1996). Normally microalgae synthesize lipids for building membranes in various organelles, mainly in the form of polar lipids. Yet under stressed conditions such as nutrient deficiency or high salinity, the lipid contents of microalgae would be greatly increased. For instance, oleaginous green algae show an average total lipid content of 25.5% of dry weight under optimal conditions, while under stress, the total lipids would reach an average of 45.7% of dry weight. And there is no evidence for significant difference in the capacity of lipid synthesis and accumulation (Hu, Zhang and Sommerfeld, 2006; Hu et al., 2008).

2.2.2 Heterotrophic / mixotrophic cultivation

As the most primitive plants, microalgae usually grow in autotrophic mode, in which they absorb the solar energy through the photosynthetic apparatus, and assimilate inorganic carbon source such as CO_2 to produce carbohydrates. Meanwhile, some algal species can also be cultivated in hetertrophic mode, in which organic nutrients are provided in the absence of light. Shi has studied heterotrophic cultivation of different Chlorella species for lutein production (Shi et al., 1997). And Chen and Walker grew *Chlorella protothecoides* heterotrophically in a fed-batch mode for algal lipids (Chen and Walker, 2011). Compared to autotrophic growth, heterotrophic algal cultures can achieve more biomass yield in faster growth, and accumulate higher lipid contents in the cell (Xu, Miao and Wu, 2006). However, the heterotrophic cultivation also has its down side: 1. high energy consumption due to the necessity of mechanic mixing, 2. high capital investment for the building of bioreactors, 3. medium cost due to the organic nutrients, especially if glucose is used as the carbon source, 4. the risk of contamination because of the organic nutrients in the medium.

Mixotrophic cultivation combines the autotrophic and heterotrophic growth modes. Algae could obtain energy from both the light source and the organic nutrients, while both assimilating CO_2 through photosynthesis and evolving it by aerobic respiration. This symbiotic effect of different metabolic pathways might explain why biomass productivities in mixotrophic cultivations are equal to those of heterotrophic growth, or even higher (Deschênes, Boudreau and Tremblay, 2015).

2.2 Valuable products from microalgae

The algal biomass is the major product in the microalgae industry. It is harvested from various kinds of culturing systems, then processed and sold in the form of a green or orange colored powder. The destination is usually the human health food market (Pulz and Gross, 2004). The nutrition value of the algal biomass can be comparable to some common food sources, as shown in table 2.1. Records show that each year worldwide production of the top three algae biomass exceed 6000 tons in total, and from 1975 to 1999, the annual production of Spirulina was increased from less than 100 tons to about 3400 tons.

Today, the consumption of algal biomass is confined to a limited number of species, such as *Arthrospira spirulina*, Chlorella, Dunaliella and the lesser used Nostoc and Aphanizomenon, with Chlorella and Spirulina dominating the market. Algal products come in various forms, with 75% being converted into powder, tablets, capsules and pastilles. Besides, microalgae could be added to pastas, edible oils and beverages (Brooks et al., 2010; Liang et al., 2004; Yamaguchi, 1996).

Feed quality is a very important factor influencing animal health, especially for animals raised under intensive breeding conditions. Researchers have found evidence that very small amounts of algal biomass, mainly from the genera Chlorella,

Scenedesmus and Spirulina, can significantly improve the physiology of animals (Belay et al., 1993). These results have led to a significant increase in the use of algal biomass as feed additives, especially in the poultry industry. In addition, pet food can also be supplemented with microalgae biomass or extracts, not just to improve the health of them, but also to enhance their appearance, such as shiny hair and beautiful feathers (Pulz and Gross, 2004).

Microalgal products, such as the biomass or certain extracts have been widely used in aquaculture, either as feed or food additives, which can either improve the immune systems of the fish, or enhance the color of the fish muscles. For instance, microalga-derived astaxanthin has been added to salmon feed, which would provide salmon with the red color (Pulz and Gross, 2004). Other than health food and feed additives, microalgae biomass can also be converted to fertilizer (Metting, 1996).

Currently algal products have gained a firm foothold in the market. More sophisticated products have been gathering popularity, especially in the nutrient supplement industry. For instance, algae have been found to excrete compounds which exhibit anti-oxidative effects, polyunsaturated fatty acids (PUFA), heat-induced proteins, or immunologically effective, virostatic compounds (Pulz and Gross, 2004).

Microalgae have evolved to adapt themselves to extreme habitats over billions of years. As autotrophic species, they are exposed to high oxygen and thus high radical stresses, which has led to the development of numerous efficient protective mechanisms against oxidative and radical stressors. Those mechanisms are able to prevent the accumulation of free radicals and reactive oxygen species and counteract cell-damaging activities. This is done by algae cells through accumulating anti-oxidative scavenger molecules, which protect cells from free radicals. And as a

natural source of anti-oxidative components, their application in the cosmetics industry is developing fast (Spolaore et al., 2006).

Algae cells contain several pigment substances. Besides chlorophyll, they can synthesize β-carotenoids and astaxanthins. And the latter two can also be applied to cosmetics as anti-oxidative components, in addition to their use as food colorants (Pulz and Gross, 2004).

Microalgae Lipids are discussed about either as triacylglycerols (TAGs) or the basic building blocks — fatty acids (FA). Among fatty acids of various lengths, poly unsaturated fatty acids (PUFA) are of special interest. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are two well-known PUFA for being added into baby formula as supplements, because they are important for brain and eye development in infants and cardiovascular health in adults (Kroes et al., 2003). Although people used to obtain these fatty acids from fish oil, the unpleasant fish smell is not desirable for them. Now algae derived DHA and EPA have been commercially available, yet other healthy fatty acids of algal source are still daunted by high cost in production (Apt and Behrens, 2002). Several microalgae derived products in the health market are listed in Table 2.2.

2.3 Biodiesel production from microalgae

Although algae are mainly marketed for their nutritional values now, increasing efforts are being diverted to the exploration of their role in the renewable energy industry. The content of TAGs, the major neutral lipids in algal cells is significantly increased under stress conditions, so the extracting these lipids and converting them into fatty acid methyl esters (FAME), or biodiesel, has become another major focus in the microalgae technology field. There have been numerous algal species that are

being considered for biodiesel production, mainly due to their capabilities to accumulate high lipid contents. Table 2.3 listed some of these algae species with their oil/lipid contents.

Biodiesel, produced by the transesterifying TAGs with methanol, is an alternative to the current fossil fuel (Durrett, Benning and Ohlrogge, 2008). As shown in Fig 2.1, Properties of biodiesel are largely determined by the structure of its component fatty acid esters, and usually consideration needs to be taken on properties including ignition quality or cetane number (CN), cold-flow properties and oxidative stability (Knothe, 2005). And the saturation and fatty acid profile can affect the properties of the fuel product. For example, saturated fatty acids produce a biodiesel with superior oxidative stability and a higher cetane number, but rather poor low temperature properties, thus fuels are more likely to gel at ambient temperatures. Biodiesel produced from feedstocks that are high in PUFAs, on the other hand, has good cold-flow properties. However, these fatty acids are particularly susceptible to oxidation. Therefore, biodiesel produced from feedstock enriched with these fatty acid species tends to have instability problems after long period of storage.

2.4 Large-scale microalgae cultivation

As many different products as can be generated from microalgae, the first step is always to grow the relevant algae strain to reach a high biomass concentration. Growing microalgae generally costs more than growing crops. Autotrophic cultivation of algae requires light, carbon dioxide, water and inorganic salts. The facility should keep the temperature generally within 20-30°C. Growth medium must provide the inorganic elements that constitute the algal cell. Essential elements include nitrogen (N), phosphorus (P), iron (Fe) and in the case of diatom, silicon (Si). Minimal

nutritional requirements can be estimated using the approximate molecular formula of the microalgae biomass — $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$. Nutrients such as phosphorus must be supplied in significant excess because the phosphates form complex with metal ions, therefore, not all the added phosphorus is available to cells (Chisti, 2007). Then the culturing method needs to be decided. This involves the choice between two main methods of large scale cultivation — open raceway ponds and enclosed photo-bioreactors (Fukuda et al., 2001).

In open pond culturing, the mostly used is what is called raceway ponds, which is a closed loop shaped recirculation channel system, the scheme of which is shown in Figure 2.2. Raceway ponds are built in concretes or compacted earth, may be lined with white plastic, and is typically about 0.3 m deep (Chisti, 2007). A paddlewheel creates the driving force for mixing of the culture. Flow is guided around bends by baffles placed in the flow channel. During daylight, the culture is fed continuously in front of the paddlewheel where the flow begins. The biomass is harvested behind the paddlewheel, on completion of the circulation loop. The paddlewheel operates all the time to prevent sedimentation. The largest raceway-based biomass production facility occupies an area of 440,000 m^2, which is owned by Earthrise Nutritionals and used to produce cyanobacteria biomass as a food source. In raceway ponds, cooling can only be done by evaporation. Temperature fluctuates within a diurnal cycle and seasonally. Water loss due to evaporation can be significant. And productivity is further lowered by contamination with undesired algae species and algae grazers. The biomass concentration remains low because raceways are poorly mixed and cannot sustain an optically dark zone. Production of microalgal biomass for making biodiesel has been extensively evaluated in raceway ponds in studies sponsored by the United States

Department of Energy (Sheehan et al., 1998). Despite the low productivities, raceway ponds are cheap to build and operate, giving them economical edge over other cultivation methods.

Photo-bioreactors could sustain a rather pure culture for a longer period compared to the open raceway ponds, which is one of its advantages over the latter. They have been successfully used for producing large quantities of algal biomass. Among various photo-bioreactor designs, the most common type is a tubular photo-bioreactor. As shown in Figure 2.3, it consists of an array of parallel straight transparent tubes that are usually made of plastic or glass. This tubular array is where the microalgae are cultivated and the sunlight is captured. The tubes are generally 0.1 m or less in diameter. This restriction in tube diameter is because light does not penetrate too deep into the dense culture broth that is necessary for ensuring a high biomass productivity of the photo-bioreactor (Chisti, 2007). The algae broth is circulated from a reservoir to the solar collector and back to the reservoir. Continuous culture operation is used. Biomass sedimentation in tubes is prevented by either a mechanical pump or a gentler airlift pump to maintain a highly turbulent flow. Periodically, photo-bioreactors must be cleaned and sanitized. This is easily achieved using automated clean-in-place operations. During the cultivation, large quantities of oxygen would be generated. Dissolved oxygen levels much greater than the air saturation values inhibit photosynthesis. Furthermore, a high concentration of dissolved oxygen in combination with intense sunlight produces photo-oxidative damage to algal cells (Carlozzi, 2002). To avoid inhibition and damage, the highest concentration of dissolved oxygen should be set at 400% of air saturation value. Oxygen cannot be removed within a photo-bioreactor tube. This limits the maximum length of a

continuous run tube before oxygen removal becomes necessary. The culture must periodically be flowed to a degassing column which is coupled to the photo-bioreactor to remove the extra oxygen and cooled down (Pulz, 2001).

Comparing the raceway pond with the photo-bioreactor reveals that the former has a lower building and operating cost, yet low productivity as well as the high contamination risk; while photo-bioreactors could keep contamination and evaporation losses under control, though with higher operation cost. Thus a new system incorporating the individual advantages of two systems while circumventing their weaknesses might be the final answer to a large scale microalgae cultivation that is economically viable (Greenwell et al., 2010; Sato et al., 2006).

After the set biomass concentration is reached, the algae biomass would be harvested, dewatered, and extracted for oil contents. Many new techniques have been developed for these purposes (Kale, 2011; Acién Fernández et al., 2001; Janssen et al., 2002).

2.5 Low cost nutrient sources

One issue has to be solved before mass production of microalgae in heterotrophic / mixotrophic mode becomes reality, which is finding the right organic nutrient sources. Ideal carbon / nitrogen source needs to be able to sustain fast growth of microalgae while not being expensive. For this purpose, much research effort has been directed towards finding an agricultural or industrial by-product or effluent that can support the growth of algae strain under investigation. For *Chlorella protothecoides*, a range of carbon sources were studied such as waste molasses, sweet sorghum juice, hydrolysate of Jerusalem artichoke, and biodiesel-derived crude

glycerol (Cheng et al., 2009; Gao et al., 2010; Yan et al., 2011; Chen and Walker, 2011).

Crude glycerol is produced in the transesterification reaction during biodiesel production, and about 10 gallons of it would be generated for every 100 gallons of biodiesel being produced (Liang et al., 2010). As increasing amount of biodiesel is produced, excessive crude glycerol comes along in low value effluent. Various impurities exist in the crude glycerol, such as methanol, glycerides, fatty acids and catalyst used in transesterification. These impurities would require more resources to refine the crude glycerol than the value of the resulting pure glycerin. Thus, researchers have been developing new processes to utilize the crude glycerol, mostly bio-conversion of it into high value products. Liang et al. studied the effect of crude glycerol in fermentation of *Schizochytrium limacinum* SR21 for lipid production (Liang et al., 2010). Pyle et al. used the same alga cultured in medium supplemented with the crude glycerol for docosahexaenoic acid (DHA) production (Pyle, Garcia and Wen, 2008). Wijesekara et al. used pretreated crude glycerol in cultivation of *Clostridium butyricum* to produce 1,3-propanediol (Wijesekara et al., 2008).

Brewer's yeast is recycled in the beer industry 4-6 times before being discarded. Despite of this recycling effort, spent brewer's yeast is the second major by-product generated by the brewery industry, only after spent grain in volume (Ferreira et al., 2010). Brewery yeast is mainly composed of proteins, between 35% - 60% in dry weight, and also carbohydrates, lipids, minerals and vitamin B complex (dos Santos Mathias et al., 2014). Until now, the main application for the spent yeast is either being offered as animal feed, or combined with other brewery waste for anaerobic digestion. Yet more potential applications are being investigated, especially as a nutrient

supplement for microbial fermentation. For example, Jiang et al. used spent yeast as a nitrogen source for succinic acid production in the fermentation of *Actinobacillus succinogenes* (Jiang et al., 2010). Ryu et al. cultivated *Aurantiochytrium sp.* with spent yeast for docosahexaenoic acid (DHA) (Ryu et al., 2013).

2.6 Response surface methodology

Statistical modelling such as response surface methodology (RSM) has been widely used in researches to study effects of several independent factors on response. Usually, when it comes to investigating different variables, one single factor would be studied in several levels while all others remain constant. Then the rest variables are studied in the same way, one at a time. Such approach is simple and straightforward, yet it would neglect the possible interactions between different variables. Response surface methodology (RSM) can include the interaction terms in its model, therefore eliminating such potential errors (Mopkar Anand, Sankar and Daniel, 2013). Another advantage of RSM is that it requires less experiment trials to be performed, thus saving both time and resources. However, this would also lead to loss of certain information, compared to a full factorial design. Therefore, a combination of the two might generate more accurate models, in which a full factorial design is used for the experiment, while the response surface method could be used later to model the data. Such combination could guarantee the completeness of the information in the data, while being able to investigate the possible interactions between different factors.

Table 2.1 Compositions of major nutrients in common food sources and algae (% dry weight).

Food source	Protein	Carbohydrate	Lipids
Baker's yeast	39	38	1
Meat	43	1	34
Milk	26	38	28
Rice	8	77	2
Soybean	37	30	20
Anabaena cylindrica	43-56	25-30	4-7
Chlamydomonas rheinhardii	48	17	21
Chlorella vulgaris	51-58	12-17	14-22
Dunaliella salina	57	32	6
Porphyridium cruentem	28-39	40-57	9-14
Scenedesmus obliquus	50-56	10-17	12-14
Spirulina maxima	60-71	13-16	6-7
Synechococcus sp.	63	15	11

Adapted from Spolaore et al., 2006

Table 2.2 Microalgae derived health products.

Company	Algae	Product	Activity
Martek (DSM)	Crypthecodinium	DHA	Brain development
Cyanotec	Haematococcus	Astaxanthin	Anti-oxidant
MERA	Haematococcus	Astaxanthin	Anti-oxidant
OceanNutrition	Chlorella	Carbohydrate	Immunity
InnovalG	Odontella	EPA	Anti-inflammatory
Panmol/Madaus	Spirulina	Vitamin B_{12}	Immunity
Nutrinova/Celanese	Ulkenia	DHA	Brain development
BSV	Rhodophyta (mix)	Biomass	Bowel candidiasis

Source: Pulz and Gross, 2004.

Table 2.3 Lipid contents of several algae species.

Microalgae	Lipid content (% dry weight)
Botryococcus braunii	25-75
Chlorella sp.	28-32
Crypthecodinium cohnii	20
Cylindrotheca sp.	16-37
Dunaliella primolecta	23
Isochrysis sp.	25-33
Monallanthus salina	>20
Nannochloris sp	20-35
Nannochloropsis sp	31-68
Neochloris oleoabundans	35-54
Nitzschia sp.	45-47
Phaeodactylum tricornutum	20-30
Schizochytrium sp.	50-77
Tetraselmis sueica	15-23

Source: Chisti, 2007.

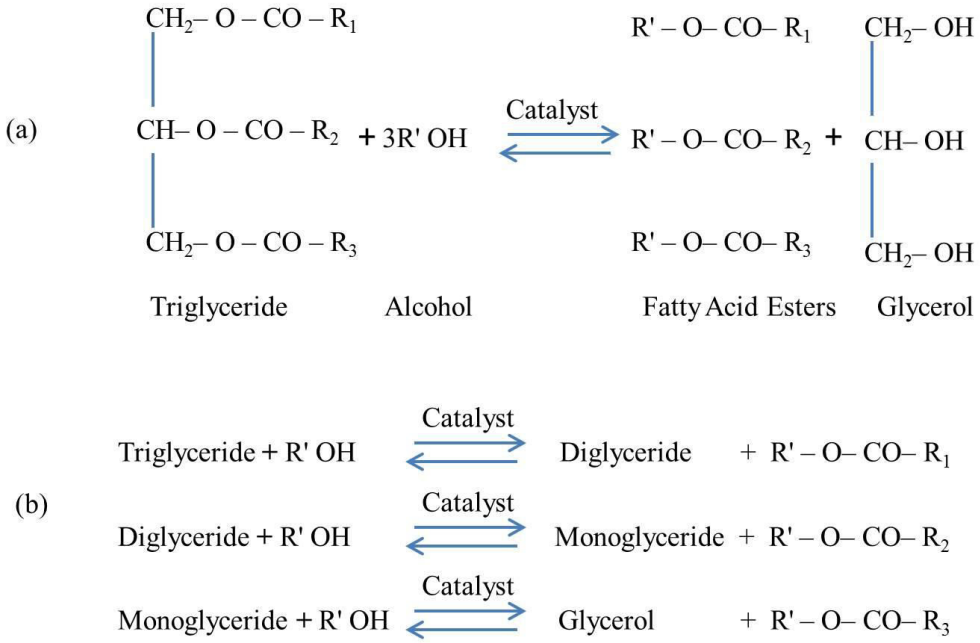

Fig 2.1 Transesterification of triglyceride with alcohol.
(a) General equation; (b) Three consecutive and reversible reactions. R_1, R_2, R_3 and R' represent alkyl groups.
Source: Adapted from Fukuda et al., 2001.

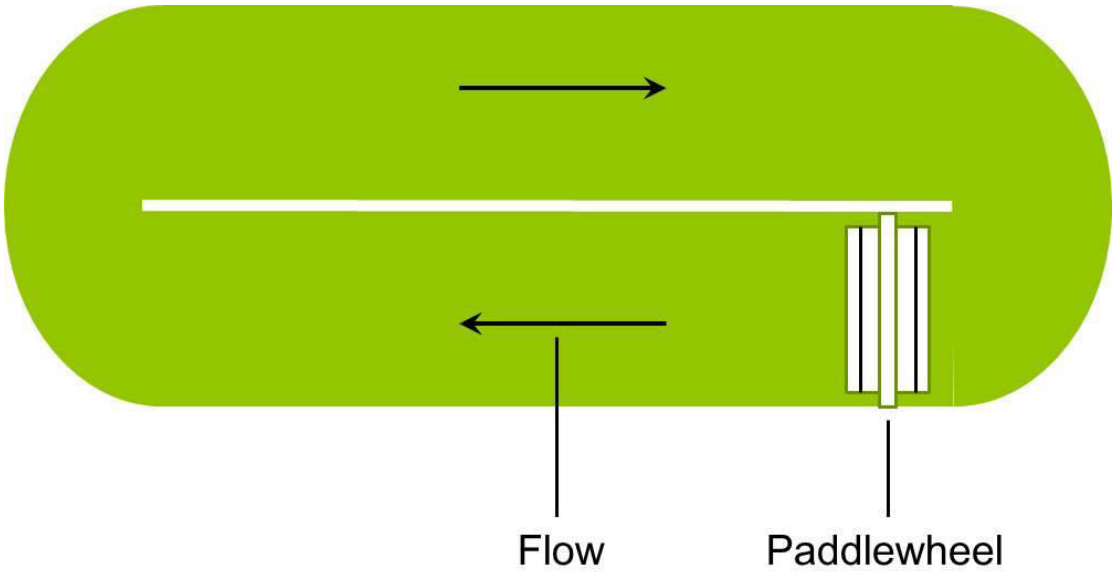

Figure 2.2 Scheme of a raceway pond.

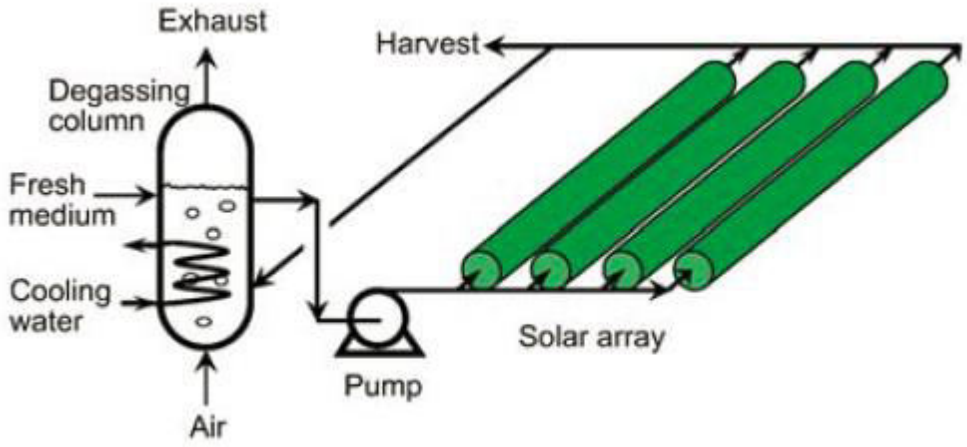

Figure 2.3 The scheme of a tubular photo-bioreactor system.
Source: Chisti, 2007.

CHAPTER III

OPTIMIZATION OF *CHLORELLA PROTOTHECOIDES* BIOMASS PRODUCTION UNDER MIXOTROPHIC CONDITION USING STATISTICAL MODELLING

3.1 Introduction

The human society has been utilizing microalgae since ancient times (Spolaore et al., 2006). Yet mass cultivation of microalgae in the industrial scale only began after World War II (Spolaore et al., 2006). Several algae species are currently mass produced for various purposes, such as food, nutritional supplements, valuable chemicals, etc. (Pulz and Gross, 2004; Spolaore et al., 2006). With the idea of sustainability gaining popularity among modern societies, substantial efforts have been devoted to the development of biofuels, especially microalgae based biofuels (Chisti, 2007; Chen and Walker, 2011; Huang et al., 2010; Xu, Miao and Wu, 2006). While great challenges are encountered to lower the cost of microalgae-based products, biodiesel in particular, diversifying the product line to overcome this issue has received greater focus. For instance, Campenni et al. (2013) cultivated *Chlorella protothecoides* to produce both carotenoids and lipids. Others are incorporating waste-water treatment with microalgae cultivation, as was accomplished by the Offshore Membrane Enclosures for Growing Algae (OMEGA) project (Wiley, 2013).

Chlorella protothecoides has attracted considerable attention in the research field for its capability of accumulating lipids under certain metabolic conditions, which could reach a lipid content as high as 55.2 wt% of the algal biomass (Miao and Wu,

2004). While *Chlorella protothecoides* can grow in autotrophic, heterotrophic mode, or mixotrophic modes, researchers tend to prefer heterotrophic or mixotrophic cultivation because organic carbon or nitrogen sources that are added to the medium significantly enhance the algae biomass production when compared to the autotrophic growth that relies solely on carbon dioxide as the carbon source (Miao and Wu, 2004; Xu, Miao and Wu, 2006; Chen and Walker, 2011).

Recently mixotrophic growth, where both light and organic carbon source are provided to the microalgal cells, has been extensively investigated for various algae strains; some algae strains achieved higher biomass accumulation in mixotrophic mode than purely heterotrophic growth (Heredia-Arroyo et al., 2010a; Liang et al., 2009a; Mitra et al., 2012b).

For mass cultivation of any microalgae to provide feedstock for biofuels, the high cost of algal biomass has been a problem, and a constant target among scientists and engineers. Therefore, development of a process that can maximize the algal biomass yield while keeping the cost at a minimum is necessary. One solution is to use a carbon and/or nitrogen source that is of low or no cost to the algae industry. Crude glycerol has been generated by large quantities with the current biodiesel production. Since it is not cost efficient to refine much of this biodiesel by-product to produce pure glycerol, crude glycerol could serve as a substitute carbon source for more expensive glucose, thus converting a waste into a potentially valuable resource, which has been demonstrated by previous researches (Chen and Walker, 2011; Feng et al., 2014). Similarly, finding a cheaper alternative for the yeast extract typically used as the nitrogen source in heterotrophic cultivation is desired. Spent yeast from brewery

waste could serve this purpose. During the beer production process, spent yeast is generated as a by-product after the fermentation is complete, which may make up 1.5-3% of the total volume of the beer produced (Fillaudeau, Blanpain-Avet and Daufin, 2006). Common recycling practices, if any, include selling the spent yeast as animal feed or generating methane through anaerobic digestion (dos Santos Mathias et al., 2014; Mussatto, 2009; Neira and Jeison, 2010). Yet since spent yeast is rich in protein and vitamins, it can also become a suitable nitrogen source in heterotrophic or mixotrophic algae cultivation after appropriate pretreatments (dos Santos Mathias et al., 2014) .

To maximize the biomass yield, culturing conditions should be optimized. Traditional optimization methods, no matter for the best growing conditions of algae or for the optimal trans-esterification parameters, tend to tackle one single factor at a time, while keeping all other factors constant. However, this one-factor approach does not take into account the interactions among different factors by splitting individual factors (Mopkar Anand, Sankar and Daniel, 2013). In this study, response surface methodology (RSM) was used to obtain the best combination of the following 3 factors: light intensity, carbon concentration and nitrogen concentration in the medium. Also the feasibility of incorporating both crude glycerol and spent yeast in the culturing medium was explored.

3.2 Materials & Methods

3.2.1 Materials and chemicals

All chemicals in this project were obtained from commercial sources and of analytical grade. Crude glycerol was obtained from Clemson University Sustainable

Biodiesel Lab (Clemson, SC). The spent yeast was kindly provided by Thomas Creek Brewery (Greenville, SC).

3.2.2 Microorganism and culture maintenance

Chlorella protothecoides UTEX 256 was originally purchased from the algae collection at the University of Texas at Austin (Austin, TX), and maintained on 1.5% agar plates of proteose medium under ambient light. Single algal colonies are streaked onto fresh agar plates on a regular basis. The components of proteose medium (per liter) are as follows: 1g proteose peptone, 0.25g $NaNO_3$, 0.025g $CaCl_2 \cdot 2H_2O$, 0.075g $MgSO_4 \cdot 7H_2O$, 0.075g K_2HPO_4, 0.175g KH_2PO_4, 0.025g NaCl. Sterile operations were practiced in culture maintenance, inoculum preparation and batch cultivation.

3.2.3 Inoculum preparation

Seed culture or inoculum was prepared by picking single colonies from agar plates, which were then used to inoculate 200 ml of basal medium contained in 500 ml shake flasks. The basal medium was supplemented with 30 g/L of pure glycerol (Fisher Scientific) and 4 g/L of yeast extract (Alfa Aesar). The components of basal medium (per liter) are as follows: 0.7g KH_2PO_4, 0.3g K_2HPO_4, 0.3g $MgSO_4 \cdot 7H_2O$, 25mg $CaCl_2 \cdot 2H_2O$, 25mg NaCl, 3mg $FeSO_4 \cdot 7H_2O$, 0.01 mg vitamin B_1, and 1ml of A5 solution. The seed culture was grown mixotrophically for 8 days on a shaking incubator under the same light intensities as the culture inoculated by it. The incubator was set at 200 rpm and kept in a dark room with constant temperature of 28ºC, and the only light source was a LED light with adjustable light intensities. A light regime of 12-hour light, 12-hour darkness was used.

3.2.4 Mixotrophic batch growth of Chlorella protothecoides

The batch culture was carried out in 500 ml shake flasks. 10 ml of seed culture was inoculated into 190 ml of basal medium supplemented with partially refined crude glycerol and treated spent yeast to achieve an inoculation ratio of 1/20. Then the culture was grown under the same light intensity and light regime as the seed culture. The temperature was maintained at 28ºC and the shaking incubator was set at 200 rpm. A 5 ml sample was drawn on a daily basis. Samples were centrifuged at 3000 rpm for 15 min, washed with distilled water, and freeze-dried overnight for dry weight biomass (g/L) measurement.

3.2.5 Pretreatment of crude glycerol and spent yeast

The crude glycerol was obtained from Clemson University Sustainable Biodiesel Lab as a by-product of biodiesel production. Biodiesel was produced through the alkali-catalyzed trans-esterification process, in which waste-cooking oil was catalyzed by KOH to react with excessive amount of methanol. Therefore the by-product glycerol would usually have a pH range of about 9-10, and contain methanol as impurity.

To pretreat the crude glycerol, 12N of hydrochloric acid was added to adjust the pH to around 7.0. Then the glycerol was centrifuged at 3000 rpm for 15 min and 3 layers were formed that respectively corresponded to biodiesel, glycerol and soap from top to bottom. The top two layers that formed the supernatant were transferred to a separatory funnel, and glycerol was then collected from the bottom once the two layers were formed again. Finally, the collected glycerol was heated to 65ºC with stirring in the fume hood so remaining methanol was evaporated. The pretreated

glycerol was autoclaved and refrigerated. A Shimadzu HPLC system and a Shimadzu TOC-V / TMN-1 system were used to analyze the glycerol concentration and total organic carbon / total nitrogen (TOC / TN) in the stock.

Spent yeast was collected at the Thomas Creek Brewery (Greenville, SC). Sodium chloride was added to the slurry of spent yeast to achieve a final concentration of 2% (W/V) (Sugimoto, Takeuchi and Yokotsuka, 1976). The slurry was heated with stirring for 72 hours while the temperature was maintained at 40ºC. The mixture was then centrifuged at 3000 rpm for 30 min and the supernatant was collected, autoclaved and refrigerated as the pretreated spent yeast stock. A Shimadzu TOC-V / TMN-1 system was used to analyze the total organic carbon/total nitrogen (TOC/TN) of the stock.

3.2.6 Experiment design and data analysis

Using a full factorial design as shown in Tables 1 and Table 2, we would study the effects of light intensity (L), carbon concentration (C) and nitrogen concentration (N) on biomass production. The biomass data is collected as the response, then fitted to a second-order polynomial model as follows:

$$Y = \varepsilon + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2.$$

In this equation, Y is the predicted response, i.e. the algal biomass (g/L), and X_1, X_2 and X_3 are the coded values of 3 factors, light intensity, carbon and nitrogen concentrations, respectively. A response surface was then generated with its contour

plots to find out the factor combination that yields the optimal response. All data were subjected to the least squares technique using the software package SAS JMP11.

3.3 Results & Discussions

The pretreatment released considerable amount of nitrogen, presumably proteins in the spent yeast, as shown in Table 3.1. In the untreated spent yeast, the total organic carbon concentration was 30.78 g/L, and total nitrogen was 0.68 g/L. While in the treated yeast, the total organic carbon became 52.47 g/L, and total nitrogen was 11.86 g/L. This indicates the autolysis of the yeast cells so intracellular carbohydrates and proteins were released into the supernatant medium, which facilitated the consumption by microalgae.

Response surface methodology has been widely used in microalgae researches. Chen and Wang (2013) used RSM design to optimize the concentrations of glucose, $NaNO_3$, and $MgSO_4 \cdot 7H_2O$ in the culturing medium of *Chlorella zofingiensis* (Chen and Wang, 2013). Muge I et al. (2012) applied RSM design to optimize glucose, glycerol and peptone in the *Chlorella saccharophila* cultures for biomass and lipid production (Isleten-Hosoglu et al., 2012). Medium ingredients could be conveniently manipulated through the RSM design. Meanwhile, in this research, a full factorial design was used instead of the response surface method design that would have fewer experiment trials. This was due to the fact that only one LED lamp is available to the researchers, thus only one light intensity level could be applied during one single batch. Therefore the light intensity would become a blocking factor. And more information could be obtained by using a full factorial design than a RSM design that only involved a fraction of the former one.

In this study, all experiments were based on a 3×3 full factorial design to investigate the individual impact and interaction of light intensity, carbon concentration and nitrogen concentration on the biomass yield of *Chlorella protothecoides*. The coded levels and corresponding actual values are explained in Table 3.2. Each factor was designed with 3 levels, coded as -1, 0, 1, for the low, medium and high respectively. In total 27 experiment runs were performed, with each run being represented by the average of 3 replicates. The response of the predicted model was the algal biomass (g/L). After fitting the data using a response surface method, the following model was obtained:

$$Y = 13.01 - 3.15X_1 - 4.31X_2 - 1.06X_3 - 1.84X_1X_2 + 1.44X_1X_3 - 5.02X_2^2 + 1.40X_3^2$$

Where Y was the predicted response, i.e. the algal biomass (g/L) and X_1, X_2, X_3 were light intensity (μmole m^{-2} s^{-1}), carbon concentration (g C/L), nitrogen concentration (g N/L) respectively. The fitness of the model was examined by the analysis of variance (ANOVA) as shown in Table 3. The model had an F ratio of 36.97, which was quite significant ($p<0.0001$) and indicated a good fitness of the model. Among all terms in the model equation, only 2 were insignificant with a p-value larger than 0.05. They were the quadratic term of light intensity (X_1^2), and the interaction between carbon and nitrogen concentrations (X_2X_3).

However, the lack of fit of this model is quite significant ($p<0.0001$), with an F ratio of 47.23. Therefore more interaction terms were added to the model, as shown in Table 4. After 10 of these interaction terms were added, the lack of fit was rendered insignificant, as shown in Table 5, the ANOVA table of the modified model.

Compared to the regular RSM model, the modified model also had a higher R-square at 0.99, while it was only 0.83 in the first RSM model with fewer interaction terms. This could also be demonstrated by the plot of the actual response against the predicted response. As shown in figure 3.1, the predicted responses generated by the modified model were more closely correlated with the actual responses, in comparison with responses generated by the regular RSM model.

After modelling the data set, the JMP software also provided the treatment combination that would generate the largest response within the range of the factors used in this study. According to data analysis report for the modified RSM model, the highest response could be at 22.5g/L, when X_1=-1, X_2=-0.014, X_3=-1. This could also be indicated in the contour plots generated by the new model. As shown in figure 3.2, when the nitrogen factor, X_3 was fixed at -1, the response would increase to over 20g/L at a low level of light intensity ($-1 < X_1 < -0.5$), while the carbon factor should be around the medium level ($-0.5 < X_2 < 0.5$). To reach 22g/L in response, the carbon factor should be set more closely to 0, the medium level, while a low level of light could be maintained for reducing the production cost. In figure 3.3, similar trend could be observed for the carbon factor X_2, where the response increased to 22g/L when X_2 was between -0.25 and 0.25. Meanwhile, the nitrogen factor X_3 can be kept at a low level and high biomass concentration can still be achieved, according to the contour plot. In figure 3.4, the contour plot of the X1, X3 showed that to obtain the highest biomass predicted by this model, 22g/L, both the light and nitrogen factors are to be kept at a low level. This would be desirable in the actual production process, since high biomass could be produced with even low energy and nutrient input. However, due to the restraint of the factor levels, the data could show little

information about the response if the 3 factors were set beyond their respective range in this study. For example, in figure 3.4, it would be difficult to predict the trend of the response, if the light and nitrogen factors were set lower than the "-1" level in this research. And this should be investigated in future researches.

Also, since cultivation duration in this work lasts between 7 to 14 days, water loss through evaporation became a significant issue. The loss was noted to reach 15ml in day 11, and 30ml if the culture was maintained to day 19. This would lead to confusion in biomass data collection, since the vapor loss would increase the recorded biomass concentration. And in order to record and compensate for the water vapor loss, the batch would usually be terminated. Therefore, a degree of inaccuracy was introduced as to where exactly the biomass would reach its highest possible value. To circumvent this issue, future research should focus on fed-batch, or continuous mode cultivation, in which water loss could be countered by introducing fresh nutrients into the reactor.

When all data were collected after the experiment, a regular RSM model was fitted to the data at first, resulting in a significant lack of fit. To eliminate the lack of fit, all 26 possible terms of $X_1^a * X_2^b * X_3^c$ (a, b, c are integers that can only be 0, 1, 2) were added to the model. Then insignificant terms were removed to generate a less complicated model while the lack of fit was kept insignificant. Finally a model with 19 terms was obtained, including those 9 terms as in a regular RSM model. The X_1 linear term, though insignificant, was still kept in the model because the current range of light intensity might not be high enough to trigger the photo-inhibition.

Table 3.1 TOC and TN concentrations in nutrient sources.

	TOC (g/L)	TN (g/L)
Untreated spent yeast	30.78	0.68
Treated spent yeast	52.47	11.86
Treated crude glycerol	373.68	____

Table 3.2 Coded and uncoded levels of variables used in the RSM design.

Variables	Coded-variables levels (x_j)			Δj
	-1	0	1	
Light(μmol m^{-2} s^{-1})	100	200	300	100
Carbon (g/L)	14	28	42	14
Nitrogen (g/L)	0.7	1.4	2.1	0.7

Table 3.3 Factorial design of experiment runs. (n=3)

	Coded levels of factors		
Treatment	light(X_1)	carbon(X_2)	nitrogen(X_3)
1	0	1	1
2	-1	1	-1
3	1	0	0
4	-1	0	1
5	1	-1	-1
6	1	-1	1
7	1	-1	0
8	-1	1	1
9	1	0	-1
10	0	1	0
11	-1	-1	-1
12	0	-1	0
13	-1	0	-1
14	0	1	-1
15	0	0	1
16	-1	1	0
17	1	1	0
18	-1	0	0
19	1	1	-1
20	0	0	-1
21	1	0	1
22	-1	-1	0
23	0	-1	1
24	0	-1	-1
25	0	0	0
26	1	1	1
27	-1	-1	1

Table 3.4 Analysis of variance for the RSM model.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	9	2294.0212	254.891	36.9677	<0.0001
X_1	1	557.2866	557.287	80.8250	<0.0001
X_2	1	1107.9630	1107.963	160.6913	<0.0001
X_3	1	60.2281	60.228	8.7351	0.0043
X_1*X_2	1	122.5639	122.564	17.7758	<0.0001
X_1*X_3	1	74.5920	74.592	10.8183	0.0016
X_2*X_3	1	3.2882	3.288	0.4769	0.4922
X_1*X_1	1	3.9960	3.996	0.5796	0.4491
X_2*X_2	1	443.1800	443.180	64.2758	<0.0001
X_3*X_3	1	31.9961	31.996	4.6405	0.0348
Error	68	468.8586	6.895		
Total	77	2762.8798			

Table 3.5 Estimates of parameters in the modified model.

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	13.006353	0.219352	59.29	<0.0001*
X_1	0.4866667	0.30462	1.60	0.1153
X_2	-4.069917	0.13623	-29.88	<0.0001*
X_3	-1.57	0.175873	-8.93	<0.0001*
X_1*X_1	0.4937111	0.175873	2.81	0.0067*
X_1*X_2	-0.768762	0.215399	-3.57	0.0007*
X_2*X_2	-5.023379	0.175873	-28.56	<0.0001*
X_1*X_3	3.3916667	0.215399	15.75	<0.0001*
X_2*X_3	1.52	0.215399	7.06	<0.0001*
X_3*X_3	1.3978698	0.175873	7.95	<0.0001*
$X_1*X_1*X_3$	2.915	0.278079	10.48	<0.0001*
$X_2*X_2*X_1$	-3.577905	0.373082	-9.59	<0.0001*
$X_1*X_3*X_3$	-6.038333	0.373082	-16.19	<0.0001*
$X_1*X_2*X_3$	1.7433333	0.15231	11.45	<0.0001*
X_1* $X_1*X_2*X_3$	-1.826667	0.263809	-6.92	<0.0001*
X_1*X_2* X_2*X_3	-2.928333	0.263809	-11.10	<0.0001*
$X_1*X_2*X_3*X_3$	-1.614571	0.263809	-6.12	<0.0001*
X_1* $X_1*X_2*X_2*X_3$	-3.245	0.263809	-12.30	<0.0001*
X_1*X_2* $X_2*X_3*X_3$	6.2529048	0.45693	13.68	<0.0001*
X_1* $X_1*X_2*X_3*X_3$	-0.573416	0.204345	-2.81	0.0067*

Table 3.6 Analysis of variance of the modified model.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F
Model	19	2734.956533	143.945081	258.5402	<0.0001
Lack Of Fit	7	4.416872	0.630982	1.1532	0.3448
Pure Error	54	29.545543	0.54714		
Total Error	61	33.962416			
C. Total	80	2768.918949			

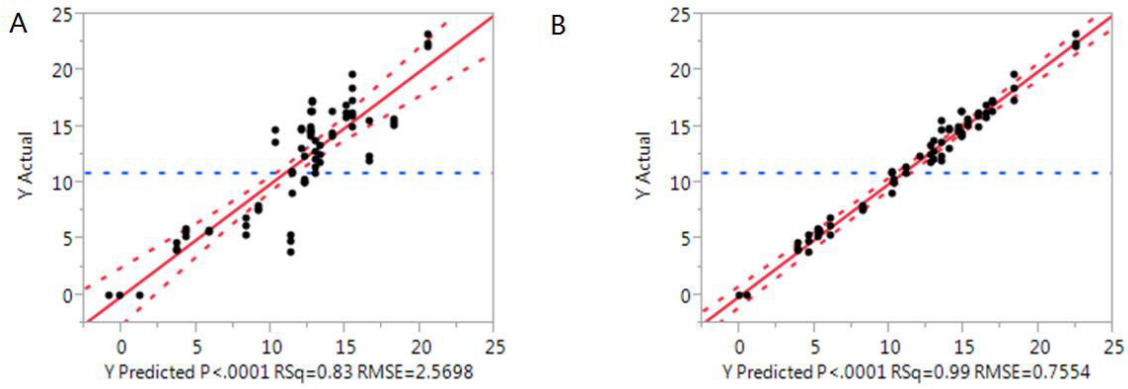

Fig 3.1 Plot of actual responses vs. predicted values by the two models. (A: regular RSM model; B: modified model)

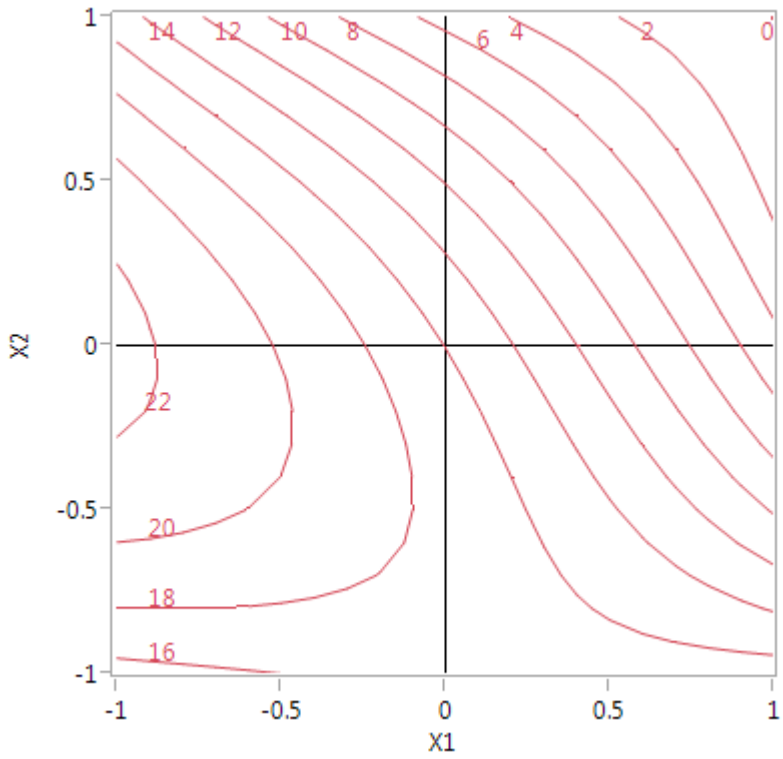

Fig 3.2 Contour plot of the response for factor X_1 and X_2, while X_3 was fixed (X_3= -1).

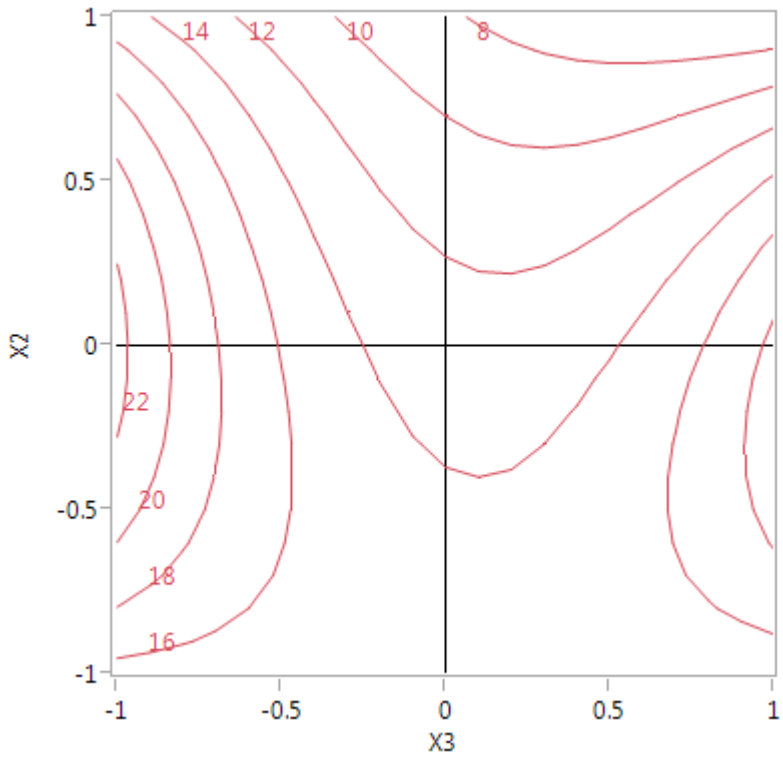

Fig 3.3 Contour plot of the response for factor X_2 and X_3, while X_1 was fixed (X_1= -1).

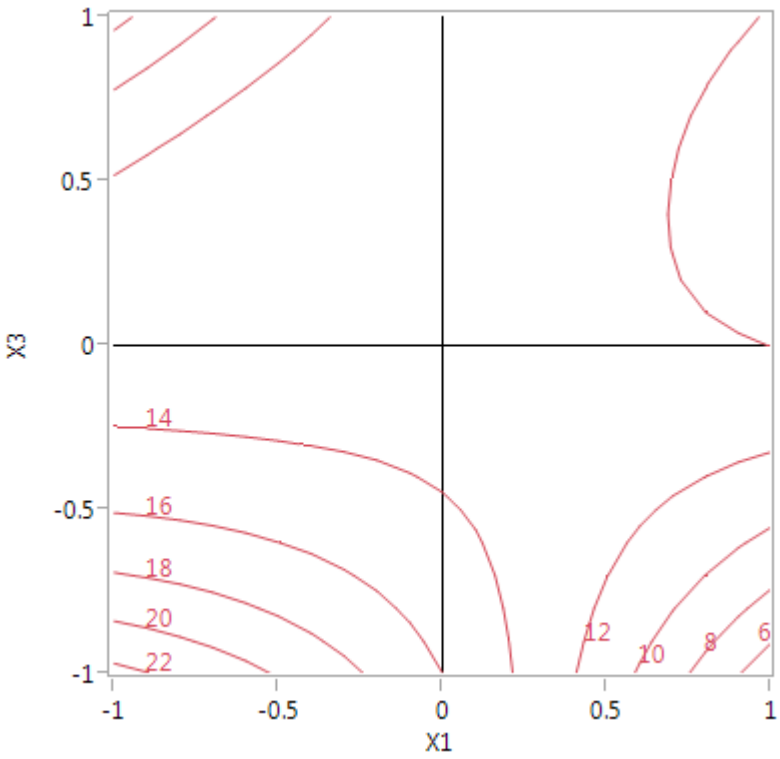

Fig 3.4 Contour plot of the response for factor X_1 and X_3, while X_2 was fixed (X_2= -0.01).

CHAPTER IV

CONCLUSION AND FUTURE PLAN

In this study, biodiesel derived crude glycerol and spent brewer's yeast were pretreated in order for the microalgae to better consume the carbon and nitrogen. The culturing medium supplemented with the two nutrient sources was able to sustain the growth of *Chlorella protothecoides*.

The alga was cultivated mixotrophically in this study. To investigate the impact of light intensity, carbon and nitrogen on algal biomass yield, a full factorial design was used and data was analyzed through response surface methodology. The obtained model, which was modified from a regular RSM model, had better fit with the actual data and therefore could better predict the response.

For future investigation, the pretreatment of crude glycerol and spent yeast would need to be improved, as considerable amount of acid was consumed to adjust the pH of the glycerol. And the pH adjustment caused a drastic increase in salinity of the nutrients, thus the salinity in the medium was quite high. A combination of the pretreatments of the two nutrients could be desirable, since crude glycerol was highly basic, while the spent yeast was acidic. Directly mixing the two at an appropriate ratio could eliminate the necessity of acid addition, thus further reducing the cost, and better sustain the algae growth due to lowering of salinity.

Also, the design of the experiment could be improved as a broader range of light intensity could be used to better investigate the effect of LED light intensity on algae growth. Besides, the nutrient level might also be adjusted to have a larger coverage of the variable levels. Therefore, more data could be generated to improve the modelling.

In addition, different cultivation strategies could be applied other than batch studies as used in this research. Algae could be grown in fed-batch or continuous mode for a higher biomass accumulation.

Finally, future research might be more focused on products, such as lipids, or lutein, since *Chlorella protothecoides* has been shown to have high lutein content. Preliminary result on lipids in this research has demonstrated that the mixotrophic culture of *Chlorella* has displayed a quite different profile in fatty acid composition compared to that of the heterotrophic culture, as shown in the appendix table. And more data on lipids is needed to support this observation. Meanwhile it would be more economically reasonable to combine the production of lipids with lutein, which has a higher market value in the present time.

APPENDIX A

Table A. Fatty acid profile comparison of mixotrophic culture with heterotrophic culture.

Fatty Acid	Weight %	
	Hetertrophic	Mixotrophic (this study)
C6:0	—	13.34
C14:0	2.10	—
C16:0	11.06	13.53
C18:0	3.14	8.70
C18:1	58.76	20.17
C18:2	21.38	44.25
C18:3	1.20	—

APPENDIX B

Figure B.1 Reactor setup of mixotrophic *Chlorella protothecoides* cultures in the dark room. Left, LED light was turned on; Right, the light source was toggled to white light.

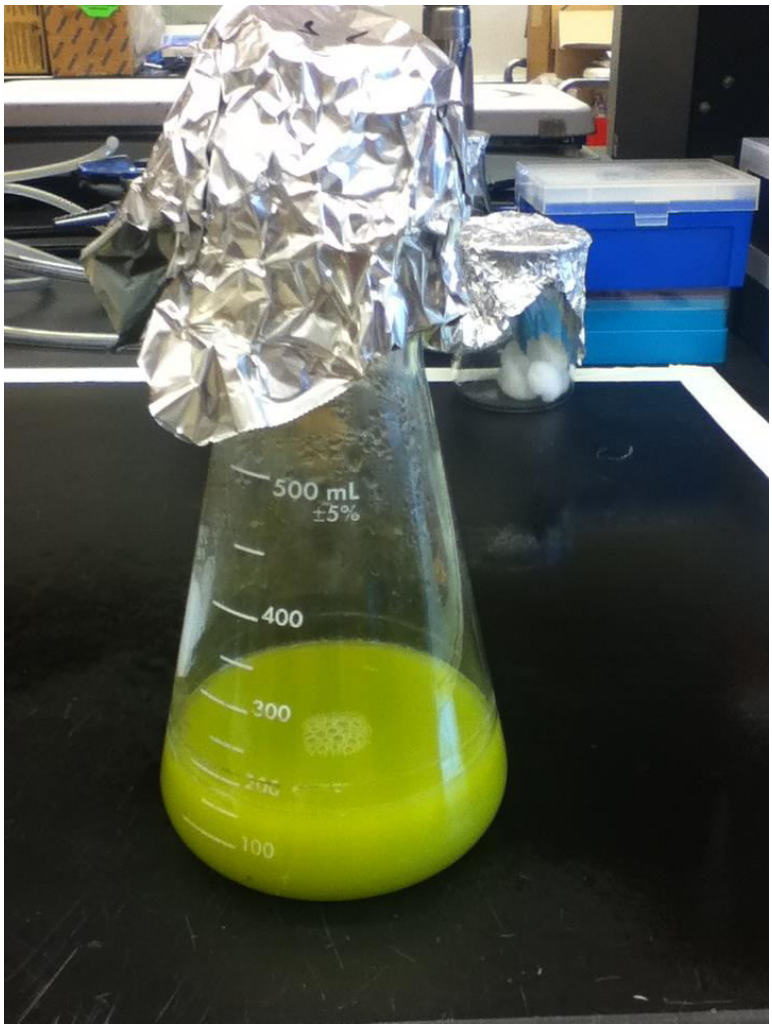

Figure B.2 Mixotrophic *Chlorella protothecoides* culture that was used as the inoculum.

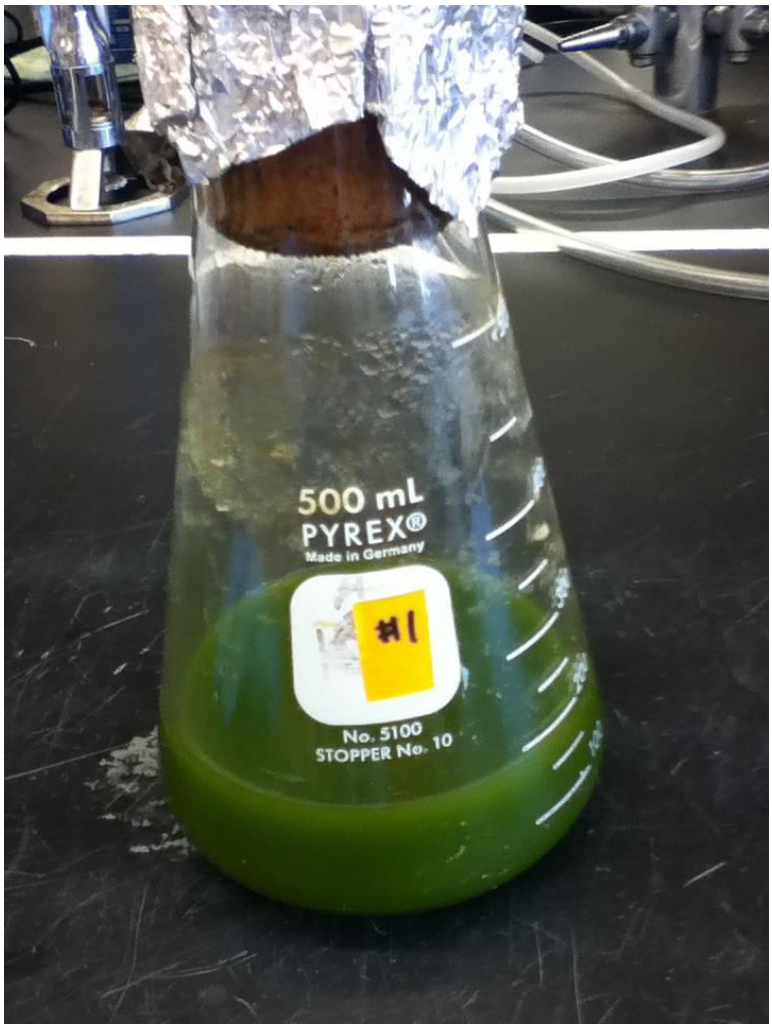

Figure B.3 Mixotrophic *Chlorella protothecoides* culture that was used for data collection.

APPENDIX C

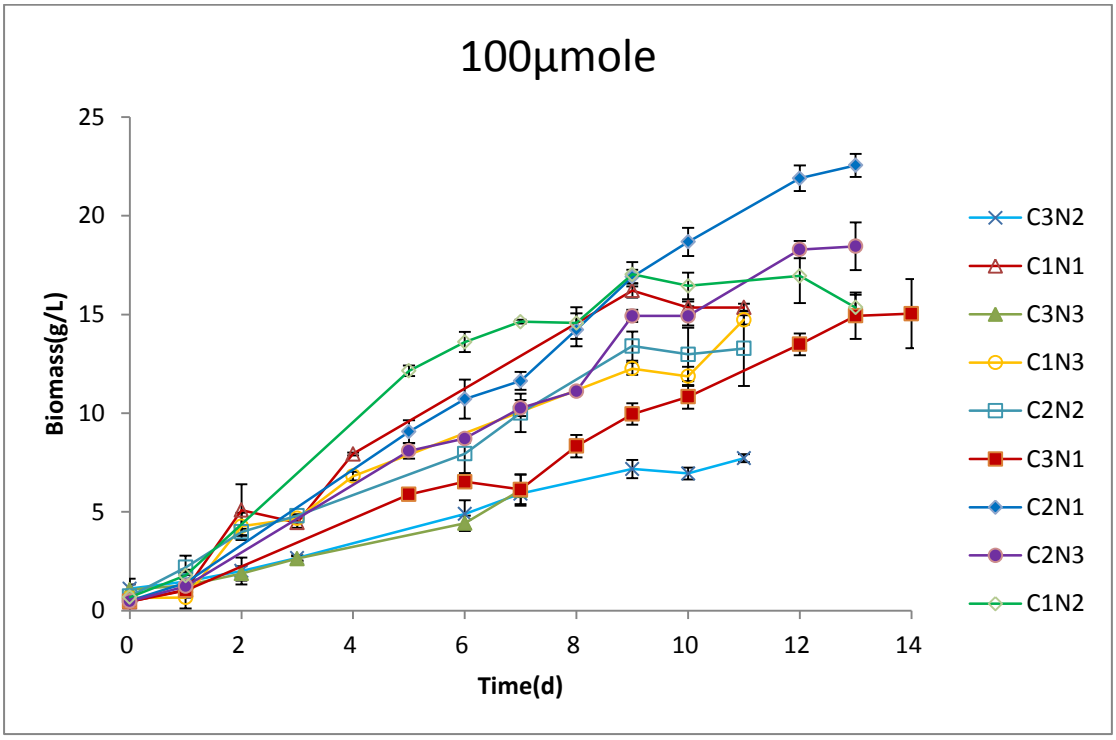

Figure C.1 Growth curve of mixotrophic cultures under 100µmol/m^{-2} s^{-1} of LED light. C, carbon concentration; N, nitrogen concentration; 1,2,3 were codes for factor levels. For example, C1N3 refers to low carbon concentration (X_1=-1) and high nitrogen concentration (X_3=1), and C2N2 refers to medium carbon and medium nitrogen (X_2=0, X_3=0).

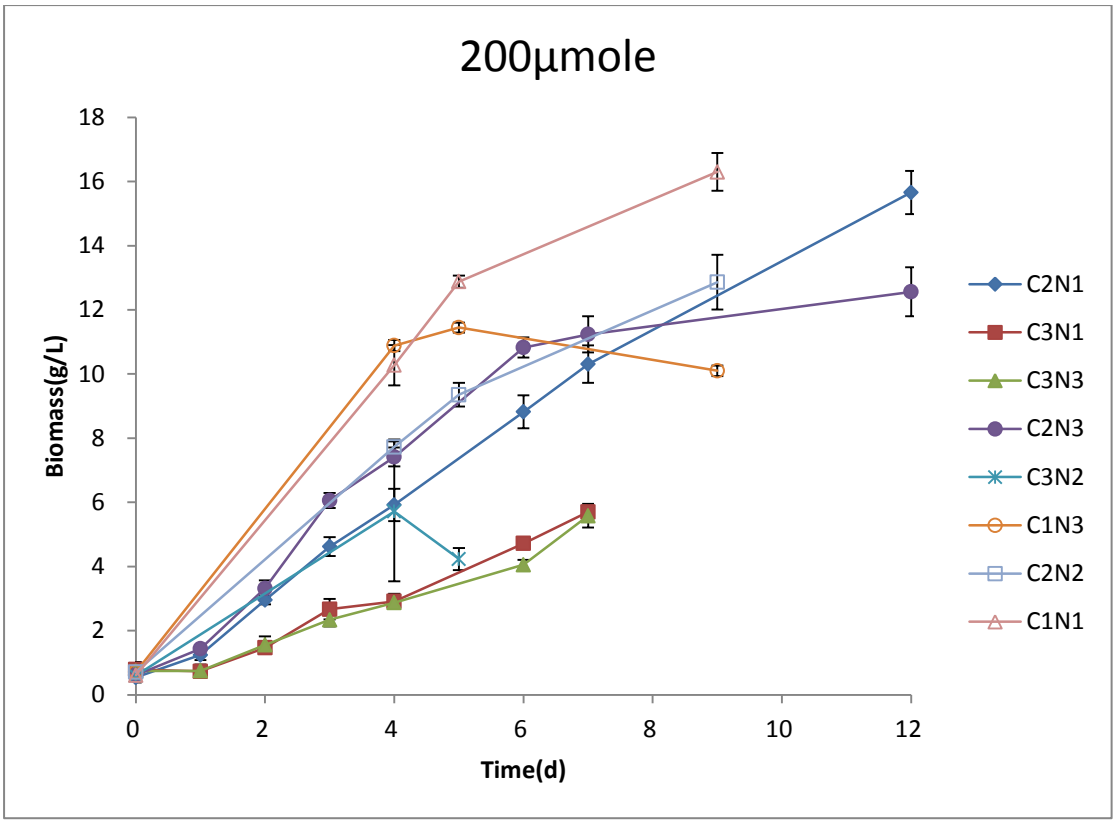

Figure C.2 Growth curve of mixotrophic cultures under 200µmol/m^{-2} s^{-1} of LED light.

C, carbon concentration; N, nitrogen concentration; 1,2,3 were codes for factor levels.

APPENDIX D

Table D.1 Maximum biomass concentration of all treatments.

X_1	X_2	X_3	Max Biomass (g/L)
−1	−1	−1	15.34
−1	−1	−1	15.66
−1	−1	−1	15.06
−1	−1	0	16.28
−1	−1	0	14.1
−1	−1	0	14.42
−1	−1	1	14.7
−1	−1	1	14.98
−1	−1	1	14.5
−1	0	−1	22.08
−1	0	−1	23.2
−1	0	−1	22.36
−1	0	0	15.46
−1	0	0	12.38
−1	0	0	12
−1	0	1	19.68
−1	0	1	17.28
−1	0	1	18.4
−1	1	−1	16.28
−1	1	−1	14.1
−1	1	−1	14.42
−1	1	0	7.64
−1	1	0	7.57
−1	1	0	7.95
−1	1	1	6.9
−1	1	1	6.1
−1	1	1	5.32

Table D.2 Maximum biomass concentration of all treatments.

X_1	X_2	X_3	Max Biomass (g/L)
0	−1	−1	15.74
0	−1	−1	16.24
0	−1	−1	16.92
0	−1	1	10.28
0	−1	1	10.06
0	−1	1	9.96
0	0	−1	16.18
0	0	−1	15.9
0	0	−1	14.9
0	0	0	12.1
0	0	0	12.7
0	0	0	13.78
0	0	1	13.36
0	0	1	12.48
0	0	1	11.84
0	1	−1	5.7
0	1	−1	5.76
0	1	−1	5.64
0	1	0	4.62
0	1	0	3.96
0	1	0	4.12
0	1	1	5.94
0	1	1	5.2
0	1	1	5.6

Table D.3 Maximum biomass concentration of all treatments.

X_1	X_2	X_3	Max Biomass (g/L)
1	−1	−1	17.14
1	−1	−1	17.28
1	−1	−1	16.36
1	−1	0	9.02
1	−1	0	10.88
1	−1	0	10.96514
1	−1	1	11.32
1	−1	1	10.88
1	−1	1	11.34
1	0	−1	4.76
1	0	−1	3.88
1	0	−1	5.34
1	0	0	14.56
1	0	0	14.62
1	0	0	13.58
1	0	1	14.8
1	0	1	12.98
1	0	1	14.62
1	1	−1	0
1	1	−1	0
1	1	−1	0
1	1	0	0
1	1	0	0
1	1	0	0
1	1	1	0
1	1	1	0
1	1	1	0

References

Acién Fernández, F., Fernández Sevilla, J., Sánchez Pérez, J., Molina Grima, E., Chisti, Y., 2001. Airlift-driven external-loop tubular photobioreactors for outdoor production of microalgae: assessment of design and performance, Chemical Engineering Science. 56, 2721-2732.

Apt, K.E., Behrens, P.W., 2002. Commercial developments in microalgal biotechnology, J. Phycol. 35, 215-226.

Belay, A., Ota, Y., Miyakawa, K., Shimamatsu, H., 1993. Current knowledge on potential health benefits of Spirulina, J. Appl. Phycol. 5, 235-241.

Ben-Amotz, A., 2004. 13 Industrial Production of Microalgal Cell-mass and Secondary Products-Major Industrial Species, Handbook of microalgal culture: Biotechnology and applied phycology. 273.

Brooks, G., Franklin, S., Avila, J., Decker, S.M., Baliu, E., Rakitsky, W., Piechocki, J., Zdanis, D., Norris, L.M., 2010. Edible Oil and Processes for Its Production from Microalgae.

Carlozzi, P., 2002. Dilution of solar radiation through “culture” lamination in photobioreactor rows facing south–north: a way to improve the efficiency of light utilization by cyanobacteria (Arthrospira platensis), Biotechnol. Bioeng. 81, 305-315.

Chen, T., Wang, Y., 2013. Optimized astaxanthin production in Chlorella zofingiensis under dark condition by response surface methodology, Food Science and Biotechnology. 22, 1-8.

Chen, Y., Walker, T.H., 2011. Biomass and lipid production of heterotrophic microalgae Chlorella protothecoides by using biodiesel-derived crude glycerol, Biotechnol. Lett. 33, 1973-1983.

Cheng, Y., Zhou, W., Gao, C., Lan, K., Gao, Y., Wu, Q., 2009. Biodiesel production from Jerusalem artichoke (Helianthus Tuberosus L.) tuber by heterotrophic microalgae Chlorella protothecoides, Journal of Chemical Technology and Biotechnology. 84, 777-781.

Chisti, Y., 2007. Biodiesel from microalgae, Biotechnol. Adv. 25, 294-306.

Deschênes, J., Boudreau, A., Tremblay, R., 2015. Mixotrophic production of microalgae in pilot-scale photobioreactors: Practicability and process considerations, Algal Research. 10, 80-86.

dos Santos Mathias, Thiago Rocha, de Mello, Pedro Paulo Moretzsohn, S&ervulo, E.F.C., 2014. Solid wastes in brewing process: A review, Journal of Brewing and Distilling. 5, 1-9.

Durrett, T.P., Benning, C., Ohlrogge, J., 2008. Plant triacylglycerols as feedstocks for the production of biofuels, The Plant Journal. 54, 593-607.

Feng, X., Walker, T.H., Bridges, W.C., Thornton, C., Gopalakrishnan, K., 2014. Biomass and lipid production of *Chlorella protothecoides* under heterotrophic cultivation on a mixed waste substrate of brewer fermentation and crude glycerol, Bioresour. Technol.

Ferreira, I., Pinho, O., Vieira, E., Tavarela, J., 2010. Brewer's Saccharomyces yeast biomass: characteristics and potential applications, Trends Food Sci. Technol. 21, 77-84.

Fillaudeau, L., Blanpain-Avet, P., Daufin, G., 2006. Water, wastewater and waste management in brewing industries, J. Clean. Prod. 14, 463-471.

Fukuda, H., Kondo, A., Noda, H., 2001. Biodiesel fuel production by transesterification of oils, Journal of bioscience and bioengineering. 92, 405-416.

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

Greenwell, H., Laurens, L., Shields, R., Lovitt, R., Flynn, K., 2010. Placing microalgae on the biofuels priority list: a review of the technological challenges, Journal of the Royal Society Interface. 7, 703-726.

Heredia-Arroyo, T., Wei, W., Hu, B., 2010a. Oil accumulation via heterotrophic/mixotrophic *Chlorella protothecoides*, Appl. Biochem. Biotechnol. 162, 1978-1995.

Heredia-Arroyo, T., Wei, W., Hu, B., 2010b. Oil accumulation via heterotrophic/mixotrophic *Chlorella protothecoides,* Appl. Biochem. Biotechnol. 162, 1978-1995.

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

Hu, Q., Zhang, C., Sommerfeld, M., 2006. Biodiesel from algae: Lessons learned over the past 60 years and future perspectives, 7-12.

Huang, G., Chen, F., Wei, D., Zhang, X., Chen, G., 2010. Biodiesel production by microalgal biotechnology, Appl. Energy. 87, 38-46.

Isleten-Hosoglu, M., Gultepe, I., Elibol, M., 2012. Optimization of carbon and nitrogen sources for biomass and lipid production by *Chlorella saccharophila* under heterotrophic conditions and development of Nile red fluorescence based method for quantification of its neutral lipid content, Biochem. Eng. J. 61, 11-19.

Janssen, M., Tramper, J., Mur, L.R., Wijffels, R.H., 2002. Enclosed outdoor photobioreactors: Light regime, photosynthetic efficiency, scale‐up, and future prospects, Biotechnol. Bioeng. 81, 193-210.

Jiang, M., Chen, K., Liu, Z., Wei, P., Ying, H., Chang, H., 2010. Succinic acid production by *Actinobacillus succinogenes* using spent brewer's yeast hydrolysate as a nitrogen source, Appl. Biochem. Biotechnol. 160, 244-254.

Kale, A., 2011. Methods Of And Systems For Dewatering Algae And Recycling Water Therefrom.

Knothe, G., 2005. Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters, Fuel Process Technol. 86, 1059-1070.

Kroes, R., Schaefer, E.J., Squire, R.A., Williams, G.M., 2003. A review of the safety of DHA45-oil, Food and chemical toxicology. 41, 1433-1446.

Liang, S., Liu, X., Chen, F., Chen, Z., 2004. Current microalgal health food R & D activities in China, Asian Pacific Phycology in the 21st Century: Prospects and Challenges. 45-48.

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

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

Liang, Y., Sarkany, N., Cui, Y., Blackburn, J.W., 2010. Batch stage study of lipid production from crude glycerol derived from yellow grease or animal fats through microalgal fermentation, Bioresour. Technol. 101, 6745-6750.

Metting, F., 1996. Biodiversity and application of microalgae, J. Ind. Microbiol. Biotechnol. 17, 477-489.

Miao, X., Wu, Q., 2004. High yield bio-oil production from fast pyrolysis by metabolic controlling of *Chlorella protothecoides*, J. Biotechnol. 110, 85-93.

Mitra, D., van Leeuwen, J.H., Lamsal, B., 2012a. Heterotrophic/mixotrophic cultivation of oleaginous *Chlorella vulgaris* on industrial co-products, Algal Research. 1, 40-48.

Mitra, D., van Leeuwen, J.H., Lamsal, B., 2012b. Heterotrophic/mixotrophic cultivation of oleaginous *Chlorella vulgaris* on industrial co-products, Algal Research. 1, 40-48.

Mopkar Anand, A., Sankar, V., Daniel, D.K., 2013. Optimization of Light intensity, Nitrate concentration and Cultivation time for Biomass production by *Chlorella minutissima* using Response Surface Methodology, Journal of Applied Sciences Research. 9, 94-99.

Mussatto, S.I., 2009. Biotechnological potential of brewing industry by-products, Anonymous Biotechnology for agro-industrial residues utilisation. Springer, pp. 313-326.

Neira, K., Jeison, D., 2010. Anaerobic co-digestion of surplus yeast and wastewater to increase energy recovery in breweries,.

Pulz, O., 2001. Photobioreactors: production systems for phototrophic microorganisms, Appl. Microbiol. Biotechnol. 57, 287-293.

Pulz, O., Gross, W., 2004. Valuable products from biotechnology of microalgae, Appl. Microbiol. Biotechnol. 65, 635-648.

Pyle, D.J., Garcia, R.A., Wen, Z., 2008. Producing docosahexaenoic acid (DHA)-rich algae from biodiesel-derived crude glycerol: effects of impurities on DHA production and algal biomass composition, J. Agric. Food Chem. 56, 3933-3939.

Ryu, B., Kim, K., Kim, J., Han, J., Yang, J., 2013. Use of organic waste from the brewery industry for high-density cultivation of the docosahexaenoic acid-rich microalga, *Aurantiochytrium sp.* KRS101, Bioresour. Technol. 129, 351-359.

Sato, T., Usui, S., Tsuchiya, Y., Kondo, Y., 2006. Invention of outdoor closed type photobioreactor for microalgae, Energy conversion and management. 47, 791-799.

Sheehan, J., Dunahay, T., Benemann, J., Roessler, P., 1998. A look back at the US department of energy's aquatic species program: biodiesel from algae. National Renewable Energy Laboratory Golden, CO.

Shi, X., Chen, F., Yuan, J., Chen, H., 1997. Heterotrophic production of lutein by selected Chlorella strains, J. Appl. Phycol. 9, 445-450.

Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A., 2006. Commercial applications of microalgae, Journal of bioscience and bioengineering. 101, 87-96.

Sugimoto, H., Takeuchi, H., Yokotsuka, T., 1976. Process for autolysis of yeast.

Vonshak, A., 1997. Spirulina Platensis Arthrospira: Physiology, Cell-Biology And Biotechnology. CRC.

Wijesekara, R., Nomura, N., Sato, S., Matsumura, M., 2008. Pre‐treatment and utilization of raw glycerol from sunflower oil biodiesel for growth and 1, 3‐propanediol production by *Clostridium butyricum*, Journal of chemical technology and biotechnology. 83, 1072-1080.

Wiley, P.E., 2013. Microalgae cultivation using offshore membrane enclosures for growing algae (OMEGA),.

Xu, H., Miao, X., Wu, Q., 2006. High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters, J. Biotechnol. 126, 499-507.

Yamaguchi, K., 1996. Recent advances in microalgal bioscience in Japan, with special reference to utilization of biomass and metabolites: a review, J. Appl. Phycol. 8, 487-502.

Yan, D., Lu, Y., Chen, Y., Wu, Q., 2011. Waste molasses alone displaces glucose-based medium for microalgal fermentation towards cost-saving biodiesel production, Bioresour. Technol. 102, 6487-6493.