

UTILIZATION OF WASTE RESOURCES FOR
LOW-COST ALGAE-BASED BIOFUEL
PRODUCTION AND WASTEWATER
BIOREMEDIATION

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Xiaochen Ma

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Dedication

This dissertation is dedicated to my parents Yanhe Ma and Jing Liu.

Abstract

Microalgae have great potential to replace current crop feedstocks for biofuel production. However, the algal biofuel industry is still far from being economically available. This dissertation was inspired by the idea of coupling algae-based biofuel generation and municipal wastewater treatment, and developing a biofuel production system that can recycle its own wastes, and thus significantly improve its environmental friendliness and economic viability. The objectives of this study are to fully utilize wastewater and by-products from biodiesel production process to grow mixotrophic algae strains for simultaneous biomass accumulation, biodiesel production and waste stream nutrient removal.

The previous study has shown that *Chlorella vulgaris* grew well on centrate wastewater, which is the best among all municipal wastewater streams for both algae yield and wastewater nutrient removal. For the large scale algal biomass production, wastewater-borne bacteria are expected to influence the algal biomass accumulation. Therefore, different levels of initial algal inoculums were tested to determine the appropriate algae inoculation levels that would allow algae to compete favorably with the wastewater-borne bacteria and dominate the culture. The effect of algae and wastewater-borne bacteria interaction on the algal biomass accumulation and wastewater nutrients removal were studied. Raw and autoclaved centrate was used as the media for algae growth. The results showed that algae can promote bacterial growth, and the presence of bacteria had a significant influence on algal growth

pattern, suggesting symbiotic relationship between algae and bacteria at the initial stage of algae cultivation. Moreover, bacteria could increase the algal growth rate and nutrients removal rate at the initial stage of the cultivation. The maximum algal biomass of 2.01 g/L with 0.1 g/L initial algal inoculums concentration can be obtained during algae cultivation in raw centrate medium.

The bacteria community profiles tested during cultivation period showed the abundant and diverse microbial community in the samples. Ten phyla and 37 genera were identified. Phylum Bacteroidetes dominate the culture in the centrate wastewater while genus *Prevotella* was most abundant accounting for 87.1% of the total sequences. At the end of cultivation, microbial community was dominated by phylum Proteobacteria, Bacteroidetes, and Firmicutes. Four bacteria genus, *Acinetobacter*, *Bacteroidales_norank*, *Megasphaera*, and *S24-7_norank*, may have a strong influence on algal growth.

Lipid-extracted microalgal biomass residues (LMBRs) are the leftover biomass from algae-based biodiesel production. The enzymatic hydrolysates of this waste resource, which contain mainly proteins and carbohydrates, were recycled and used as nutritional sources for microalgal cultivation and lipid production. Effect of temperature and substrate concentration on algae growth and lipid production using hydrolyzed LMBRs were studied. The results showed that *C. vulgaris* could grow mixotrophically in a wide range of temperatures (20~35 °C). The optimal temperature for cell growth and lipid accumulation of the mixotrophic cultivation of *C. vulgaris* was between 25 and 30 °C. The neutral lipids of the algal culture at 25 °C

accounted for as much as 82 % of the total lipid content in the microalga at culture Day 8. Fatty acid composition analysis showed that the increase of saturated fatty acids was proportional to the increase in cultivation temperature. The maximum biomass concentration of 4.83 g/L and the maximum lipid productivity of 164 mg/L/day were obtained at an initial total LMBRs hydrolyzed sugar concentration of 10 g/L and an initial total concentration of LMBRs hydrolyzed amino acids of 1.0 g/L but decreased at lower and higher substrate concentrations.

Waste glycerol generated from biodiesel production using wastewater scum as oil feedstock was recycled and added into wastewater to provide carbon source, support microalgal lipid production, and nutrient removal of wastewater, and further increased the economic viability of scum-based biodiesel production. Effect of crude glycerol and pretreated glycerol concentration and initial pH on algae growth and lipid production of *C. vulgaris* were tested. The results showed that nutrient removal was improved and lipid production of *C. vulgaris* was enhanced with the addition of waste glycerol into the wastewater to improve its C/N ratio. The optimal concentration of pretreated glycerol for *C. vulgaris* cultivation was 10 g L⁻¹ to achieve the biomass concentration of 2.92 g L⁻¹ and lipid productivity of 163 mg L⁻¹ d⁻¹, and the removal of 100% ammonia and 95% of total nitrogen. Alkaline conditions promoted cell growth and lipid accumulation of *C. vulgaris* and simultaneously stimulated nutrient removal.

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CHAPTER 1. INTRODUCTION

1.1 Background and significance of the research

Because of the depletion of fossil fuel supplies and global warming caused by carbon dioxide emission, sustainable use of conventional petroleum sourced fuels is widely recognized as untenable. Bio-fuels may offer a promising alternative because of their characteristics similar to those of the petroleum fuel (Antolin, Tinaut et al., 2002; Chisti, 2008). Biodiesel is a potential renewable fuel that has attracted tremendous attentions. Most biodiesel is produced from conventional agricultural oil crops that require high-quality agricultural land for growth. In April 2008, United Nations officially claimed that the US and the EU took a criminal path by contributing to the global food crisis by using food crops for bio-fuel production (Xu and Mi, 2011). It is urgent to develop alternative feedstock for biodiesel production.

Microalgae have long been considered an alternative feedstock for biofuels production because they possess high growth rate, provide lipids fraction for biodiesel production while they do not need large acres of farmland for the growth (Harun et al., 2010). In addition, microalgae are a perfect candidate for CO₂ fixation and reduction through CO₂ photoautotrophic or mixotrophic growth (Wang, Min et al. 2010). Numerous researchers have put much effort into producing biofuels from autotrophic microalgae (Rawat et al., 2013; Wang et al., 2013). Unfortunately, it is hard to obtain a high biomass concentration at present due to associated issues such

as light penetration and limited inorganic carbon supply. Some microalgae could reach a high biomass concentration under heterotrophic conditions. However, the heterotrophic growth of microalgae has high production costs and does not lead to a reduction in CO₂ emissions because of the use of organic compounds. The mixotrophic growth of microalgae could have a relatively high biomass concentration, mitigate CO₂ emissions, and reduce production costs.

Microalgae require no cropland and have high oil productivity per unit land area. The Aquatic Species Program sponsored by the US Department of Energy estimated that algal oil yield of over 5,000 to 10,000 gallons per acre per year is possible compared with 50 to 100 gallons per acre per year for traditional oil crops such as soybean (Kong, Li et al. 2010). It was reported that microalgae can produce up to 250 times the amount of oil per acre of soybeans, and 7 to 31 time greater oil than palm oil (A. B.M. Sharif Hossain 2008).

To minimize the costs associated with algal biomass production, free or low-cost nutrients, water and light must be supplied. Use of waste resources containing enormous amounts of water and nutrients (e.g. carbon, nitrogen and other minerals) is a viable solution to the cost issue concerning the sustainable renewable algae-based biodiesel production. It was reported that algae can grow in three type of wastewater: municipal wastewater, animal wastewater, and industrial wastewater (Li et al., 2012; Chinnasamy et al., 2010; Hu et al., 2012). However, many challenges have impeded the wastewater based algae cultivation technology, which includes culture crash risks due to the contamination by bacteria and other microorganisms,

low algal biomass and lipid accumulation, lack of algae strain that could tolerate wastewater media (Zhou et al., 2014).

During algae-based biodiesel production through transesterification of lipid, crude glycerol is produced as the major byproduct, which, after refined, can be sold as a commercial product. However, the cost of refining the crude glycerol is about \$0.20/lb, which is higher than the price of glycerol (\$0.05/lb) (Chi et al., 2007). Lipid-extracted microalgal biomass residues (LMBRs) are the leftover biomass from microalgal biodiesel production, which contains mainly proteins and carbohydrates and is a potential nutrients source for the mixotrophic growth of microalgae after appropriate pretreatment such as enzymatic hydrolysis (Zheng et al., 2012). If we can use these waste resources to produce algal biomass mixotrophically, we will be able to develop a biodiesel production system that can recycle its own wastes, and thus significantly improve its environmental friendliness and economic viability.

1.2 Objectives

The overall goal of this study was to fully utilize wastewater nutrients and by-products from biodiesel production process to grow mixotrophic algal strains for simultaneous biomass accumulation, biodiesel production, and waste stream nutrient removal. The specific objectives of the research were to:

- 1) Determine the appropriate algae inoculation levels that would allow algae to compete favorably with the wastewater-borne bacteria and dominate the culture;
- 2) Study the effect of algae and wastewater-borne bacteria interaction on the algal

biomass accumulation and wastewater nutrients removal;

3) Test the bacteria profiles in the centrate wastewater, monitor and analyze the variation of the microbial community during algae cultivation by using the second generation sequencing method to obtain the information on their interactions with algae;

4) Develop schemes to recycle nutrients in byproducts and residues for algae production with high biomass and lipid productivity;

5) Understand key operation parameter interactions to maximize algal growth rate, biomass and lipid production using waste resources.

CHAPTER 2. LITERATURE REVIEW

2.1 Algal biomass production using wastewater

Growing algae in wastewater has been under study for more than half a century (Oswald and Golueke, 1960). Algae growth on municipal wastewater and animal wastewater has been widely studied because these wastewaters are extensively available while industrial wastewater is less variable (Li et al., 2012; Chinnasamy et al., 2010; Hu et al., 2012). However, animal wastewater contains high ammonia concentration, which could inhibit algal growth; and a large amount of fresh water is needed to dilute the high nutrient concentration in animal waste (Martin et al., 1985; Wang et al., 2010).

In municipal wastewater treatment plants, four different types of wastewater streams are generated in different stages (Figure 2.1). Previous studies evaluated the growth characteristics of algae grown on different wastewater streams including effluent from secondary treatment tank (Oswald et al., 1978; Shelef et al., 1978), extract from activated and digested sludge (Cheung and Wong, 1981), and other streams. Wang et al. (2009) investigated the feasibility of cultivating *Chlorella* sp. In wastewater before primary settling, wastewater after primary settling, wastewater after activated sludge tank, and centrate. Centrate is a highly concentrated municipal wastewater stream generated from dewatering of sludge after primary and secondary settling, which is rich in phosphorus, ammonia, organic nitrogen, and a variety of minerals such as Ca, Mg, K, Fe, Cu and Mn (Zhou et al., 2011). The average specific growth

rates in the exponential period were 0.412, 0.429, 0.343, and 0.948 day⁻¹ for 4 wastewater streams, respectively (Wang et al., 2009). Li et al. (2011) further demonstrated that centrate wastewater was proved to be able to support the growth of algal strain *Chlorella sp.* which removed ammonia, total nitrogen, total phosphorus, and COD as high as 93.9%, 89.1%, 80.9%, and 90.8%, respectively. The fatty acid methyl ester (FAME) content in the algae cultivated in the raw centrate was 11.04% (dry biomass), and the biodiesel productivity was 0.12 g-biodiesel/L-algae culture solution (Li et al., 2011). Other previous studies also showed that this nutrient-rich wastewater stream is the best among all municipal wastewater streams for algal cultivation with high biomass accumulation and high-efficiency wastewater nutrient removal (Zhou et al., 2012). These results suggested that cultivating algae on municipal wastewater was a good option for high yield biomass production.

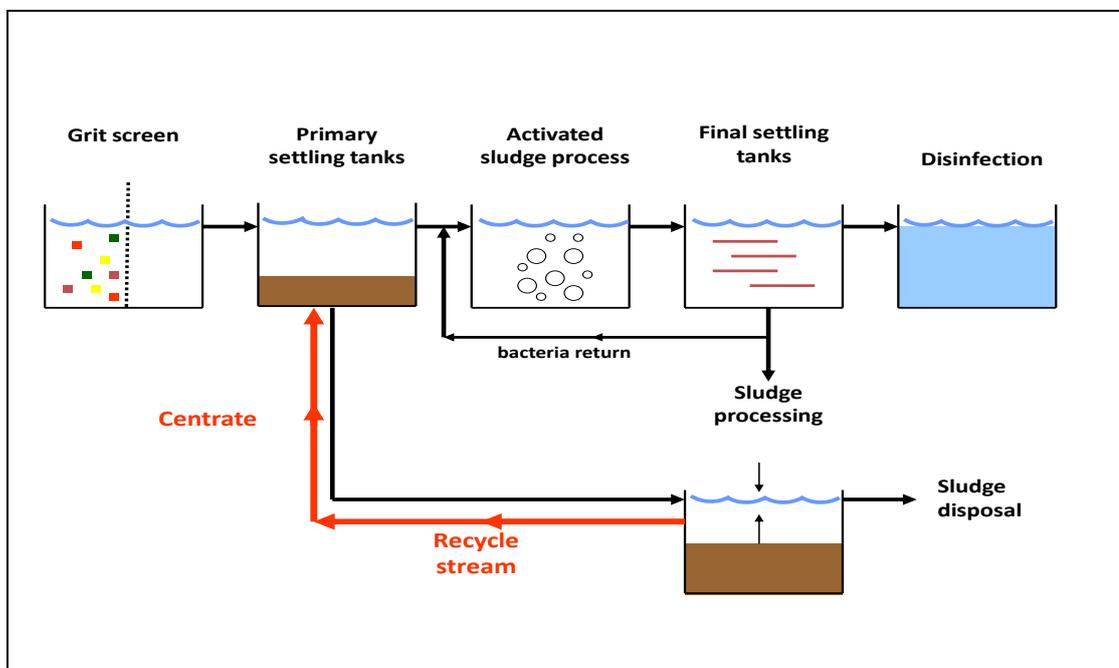


Figure 2.1: Typical wastewater treatment schematic in municipal wastewater treatment plant

When cultivated in centrate medium, algae exhibited growth curves with all characteristic growth phases except the lag phase of algae in batch culture. The exponential growth in the autoclaved centrate media lasted for 4–5 days, followed by a stationary phase until the end of cultivation. In the raw centrate medium, the algal biomass reached its maximum level on Day 2 and then declined followed by a stationary phase. The initial rapid algal growth led to the rapid decline in carbon supply. During the period of algae growth, the concentration of COD decreased dramatically in the first two days of cultivation. 90.4% and 90.9% of COD in the autoclaved centrate and raw centrate were removed, which were almost the final COD removal rate. Besides, centrate has a highly unbalanced N: P ratio compared with TAP media. The concentration of total nitrogen and total phosphorus in the raw centrate wastewater was 116.1 mg/L and 212.0 mg/L, respectively. The phosphorus concentration in centrate was ten times higher than that in TAP media while the COD, nitrogen and $\text{NH}_4\text{-N}$ concentration was only about 60%, 34% and 38% of that in TAP media, respectively. Compared to the high COD removal amount, relatively large amount of N and P remain in the culturing medium, only 59% and 81% of total N and total P were removed (Zhou et al., 2012). The low carbon source and unfavorable nutrients ratio might be the reason for the sooner onset of declining algae growth phase in centrate media (Lau et al., 1995). In order to completely utilize and remove N and P from the wastewater and obtain maximal algal biomass feedstock for biodiesel application, the carbon source may be replenished by low-cost carbon source.

2.2 The effect of bacteria on algae growth

In large scale algae production using wastewaters, autoclaving even a small portion of the wastewater in any size municipal wastewater treatment plants is impractical. Therefore, attention should be paid to cultivating algae using raw centrate. However, centrate wastewater contains numerous species of bacteria. Bacterial activities are expected to influence algae growth and hence the algal biomass accumulation, lipid content, and nutrients removal. Algae and bacteria have a complex relationship. Both inhibition and stimulation of algal growth by bacteria have been reported. Some bacteria could kill the algae by releasing enzymes to break down the algal cell wall (Cole, 1982). Extracellular substances produced by bacteria could cause algae lysis (Fergola et al., 2007; Morris, 1962). When nutrients, such as phosphate, are limited, the competition for nutrients between bacteria and algae suppresses algae growth (Rhee, 1972). On the other hand, some bacteria and algae in the co-existing system have a mutually beneficial relationship. Bacteria can help degrade intractable compounds to ammonium, nitrogen, phosphate and carbon dioxide, which can be easily used by algae (Zhang et al., 2012). Algae can also supply nutrients to bacteria for synthesizing required products, like vitamin B₁₂ (Croft et al., 2005). Other research reported that certain bacteria can increase the population of microalgae *Chlorella vulgaris* during autotrophic growth (Gonzalez and Bashan, 2000). However, the objective of most paper that studied the relationship of algae and bacteria was to improve the natural environment, like contaminated lake. Some researchers also added specific bacteria to help algae growth. Little research was

focused on the interaction between algae and bacteria in the wastewater for wastewater treatment and algae cultivation purposes. Therefore, the effect of wastewater-borne bacteria on algal growth and nutrients removal in wastewater deserves a closer look.

2.3 Feasibility of fully utilizing waste resources from biodiesel production

2.3.1 Overview of algae-based biodiesel production

Because of diminishing petroleum reserves and the deleterious environmental consequences caused by burning of fossil fuels, biodiesel has attracted significant attention during the past few decades as an environmentally friendly fuel. Compared with petro-diesel, biodiesel is renewable, has higher combustion efficiency, and a more favorable combustion emission profile, such as low emissions of carbon monoxide, particulate matter and unburned hydrocarbons (Canakci and Sanli 2008). Microalgae as an alternative bio-fuel source have gained much attention recently because of its high biomass productivity and high oil content, without encroaching on arable land suitable for food production, short production life cycle, etc.

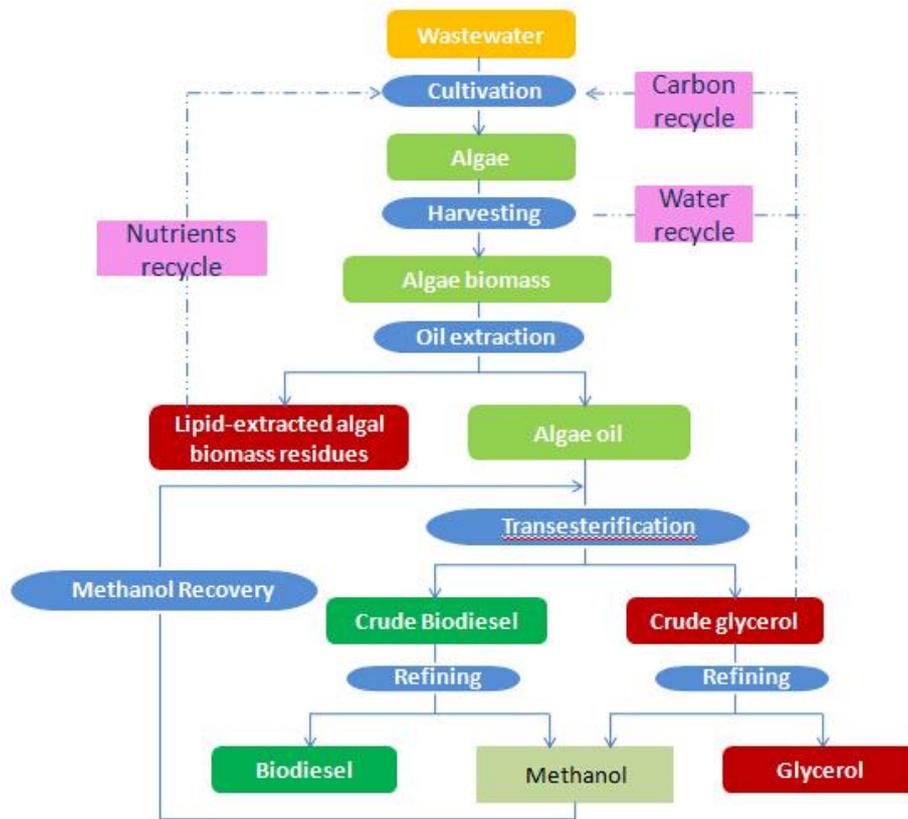


Figure 2.2 Basic scheme for algae-based biodiesel production

From the Figure 2.2, there are two major by-products from algae-based biodiesel production which contain notable amounts of nutrients for algae growth, namely lipid-extracted algal biomass residues and crude glycerol. Lipid-extracted microalgal biomass residues (LMBRs) are the leftover biomass from microalgal biodiesel production. LMBRs contain mainly proteins and carbohydrates, which could be converted to products such as amino acids and sugars by using appropriate technologies (Mooibroek et al., 2007). During biodiesel production via lipid transesterification, crude glycerol is produced as the major byproduct. In 2013, global biodiesel production has reached 236.3 billion liters, and about 10% of the glycerol by weight was produced (Renewables global status report, 2014). It is worthwhile to investigate the effectiveness of using LMBRs and crude glycerol as

substrates for mass cultivation of algae.

2.3.2 Utilization of lipid-extracted microalgae biomass residues

LMBRs are the leftover biomass after the extraction of lipid from microalgal biomass, but little attention is given to this high nutrient waste resource. From previous research, LMBRs were used to produce other forms of carbon resource or added to livestock feeds as a nutrient additive. Most research was focused on recovering carbohydrate compound remaining in the biomass after lipid extraction, producing biogas through anaerobic digestion. Chisti (2008) pointed out the potential of LMBRs to meet most of the energy demands of the preceding processes and estimated that an average heating value of 9360 MJ/metric t of microalgae residues can be recovered as methane. The anaerobic digestion of LMBRs was further examined by several researchers. Ehimen et al. (2009) investigate the practical CH₄ yields from LMBRs after biodiesel production using both the conventional and *in situ* transesterification methods. A recoverable energy of 8.7–10.5MJkg⁻¹ of dry algae biomass residue was obtained using the lipid extracted and transesterified microalgae samples. The result indicated that the lipid extraction solvent utilized in the conventional transesterification process could inhibit following CH₄ production. LMBRs were also used to produce maltodextrin through the carbohydrate hydrolysis (Lam et al., 2014).

Zheng et al. (2012) treated the LMBRs by using cellulase, neutrase and alcalase, and a two-step enzymatic hydrolysis of LMBRs was studied. The dry LMBRs contained

high contents of proteins and carbohydrates (34% and 26%, respectively), suggesting they are a good potential nitrogen and carbon sources for algal cultivation and lipid production. LMBRs were hydrolyzed into amino acids and sugars and were used as a nutrients resource for algae cultivation under non-aerated and aerated conditions. Aeration was favorable for cell growth and lipid accumulation and a biomass of approximately 3.28 g L^{-1} , lipid content of 35% and lipid productivity of $116 \text{ mg L}^{-1}\text{d}^{-1}$ were obtained. Furthermore, the effect of CO_2 concentration and aeration rate on microalgal growth and lipid accumulation were investigated. The most favorable concentration and an aeration rate of CO_2 were 5% and 0.5 vvm, respectively. Although the use of LMBRs could contribute to the economy of microbial biodiesel production, further research is needed to better understand lipid metabolism and to solve several technical limitations, such as low lipid productivity, unclear lipid metabolism regulation, and insufficient usage of LMBRs.

2.3.3 Utilization of crude glycerol

Glycerol, a trihydric alcohol, is a colorless, odorless and viscous liquid, and miscible with water and ethanol (Ayoub and Abdullah, 2012). In making biodiesel, transesterification uses 3 mol of alcohol for each mole of triglyceride to produce 3 mol of methyl esters and 1 mol of glycerol. Crude glycerol is produced as the major byproduct (10% of the product by weight) in this process (Johnson, 2007). However, the cost of refining the crude glycerol is about \$0.20/lb, which is higher than the price of glycerol (\$0.05/lb) (Chi et al., 2007). It is clear that

alternative uses for biodiesel derived crude glycerol are needed.

Numerous articles have been published on the utilization of crude glycerol as animal feed and chemical feedstock. Glycerol is a good energy resource which could be converted to glucose in the liver of animals, and could be used as a feed ingredient for chicken, pig, lambs, and cows (Yang et al., 2012; Lammers et al. 2008; Gunn et al., 2010). However, the impurities in the crude glycerol will influence the animal growth performance and nutrient digestibility. For instance, the residual methanol is toxic and excess potassium may result in wet litter or imbalances in dietary electrolyte balance in broilers (Cerrate et al., 2006).

Glycerol could also be used as feedstock for synthesis of numerous chemicals, such as 1,3-propanediol, 1,2-propanediol, dihydroxyacetones, hydrogen, polyglycerols, succinic acid, and polyesters (Mu et al., 2006; Sabourin-Provost and Hallenbeck, 2009). The two main methods to convert crude glycerol to chemicals were biotransformation and conventional chemical catalysis. Fermentative production is the most common method for biotransformation. Crude glycerol could be used directly for the production of 1, 3-propanediol, citric acid, lipids, PHA, and other chemicals in fed-batch cultures of bacteria, yeast, fungi, and algae, and resulting products were very similar to those obtained from glucose (Ashby et al., 2004; Mu et al., 2006). For conventional chemical catalysis, crude glycerol was reported to produce hydrogen or syngas through gasification, acrolein by vaporizing glycerol into a fluidized bed reactor, and monoglycerides via glycerolysis of triglyceride with crude glycerol (Yoon et al., 2010; Sereshki et al., 2009; Chetpattananondh and

Tongurai, 2008). On the other hand, the challenges of utilizing crude glycerol as feedstock for chemicals were same as an animal feedstock. The impurities in crude glycerol will inhibit the growth of microorganism or poison the catalyst, which will affect the conversion of glycerol into the desirable products. Additionally, high concentrations of glycerol had negative effects on cell growth during fermentation (Liang et al., 2010).

As mentioned in Chapter 2.1, depleting carbon source is the reason for the sooner onset of declining algae growth phase in centrate wastewater. Crude glycerol is potentially an energy resource because it is of low cost and contains high level carbon. The combination of crude glycerol treatment and algae cultivation could serve the dual role of waste reduction and biomass production.

CHAPTER 3. Effect of wastewater-borne bacteria on algal growth and nutrients removal in wastewater based algae cultivation system

Summary

Centrate, a type of nutrient-rich municipal wastewater was used to determine the effect of wastewater-borne bacteria on algal growth and nutrients removal efficiency in this study. The characteristics of algal and bacterial growth profiles, wastewater nutrient removal, and effect of initial algal inoculums were systematically examined. The results showed that initial algal concentration had an apparent effect on bacterial growth, and the presence of bacteria had a significant influence on algal growth pattern, suggesting a symbiotic relationship between algae and bacteria at the initial stage of algae cultivation. The maximum algal biomass of 2.01 g/L with 0.1 g/L initial algal inoculums concentration can be obtained during algae cultivation in raw centrate medium. The synergistic effect of centrate-borne bacteria and microalgae on algae growth and nutrient removal performance at initial fast growth stage has great potential to be applied to pilot-scale wastewater-based algae system cultivated in continuous or semi-continuous mode.

3.1 Introduction

Microalgae have attracted considerable attention in recent years because of their unique advantages of fast growth, high oil content, not competing with arable land, synergy with CO₂ biofixation and wastewater bioremediation. Furthermore, algal

biomass can be used in a wide range of applications, including biofuels, fertilizers, nutraceuticals, fish and animal feeds, cosmetics and wastewater treatment, etc. (Brennan and Owende, 2010; Chen et al., 2009; Milledge, 2010). However, many of these applications remain uneconomical due to the high cost of algae cultivation and harvesting (Van Beilen, 2010). Use of waste streams containing enormous amounts of water and nutrients (e.g. carbon, nitrogen and other minerals) is considered to be a viable solution to the cost issue in algal cultivation (Zhou et al., 2012a).

Centrate, a highly concentrated municipal wastewater (CMW) stream generated from dewatering of sludge after primary and secondary settling, is rich in phosphorus, ammonia, organic nitrogen, and a variety of minerals such as Ca, Mg, K, Fe, Cu and Mn (Zhou et al., 2011). Right now, centrate are recycled back for repeat treatment which adds very significant load for the energy intensive wastewater treatment process (Li et al., 2011). Therefore, using centrate as a resource for algae cultivation could serve dual purposes: improving wastewater treatment efficiency and algal biomass production. Our previous studies have shown that this nutrient-rich wastewater stream is the best among all municipal wastewater streams for algal cultivation with high biomass accumulation and high-efficiency wastewater nutrient removal (Wang et al., 2010).

However, centrate contains numerous species of bacteria. Bacterial infection is expected to influence the algal biomass accumulation, lipid accumulation, and nutrients removal. Both inhibition and stimulation of algal growth by bacteria have been reported. Some bacteria could kill the algae by releasing enzyme to break down

the algal cell wall (Cole, 1982). Extracellular substances produced by bacteria could cause algae lysis (Fergola et al., 2007; Morris, 1962). When nutrients, such as phosphate, are limited, the competition for nutrients between bacteria and algae suppresses algae growth (Rhee, 1972). On the other hand, some bacteria and algae in the co-existing system have a mutually beneficial relationship. Bacteria can help degrade intractable compounds to ammonium, nitrogen, phosphate and carbon dioxide, which can easily be used by algae (Zhang et al., 2012). Algae can also supply nutrients to bacteria for synthesizing required products, like vitamin B₁₂ (Croft et al., 2005). Other research reported that certain bacteria can increase the population of microalgae *Chlorella vulgaris* during autotrophic growth (Gonzalez and Bashan, 2000). Therefore, algae and bacteria have a complex relationship, which may be symbiotic, competitive and antagonistic, and deserves a closer look.

This study mainly focused on how the centrate-borne bacteria influenced algal growth and nutrient removal efficiency in the wastewater-based algae cultivation system. Different levels of initial algal inoculums were tested to determine the effect of algae and bacteria interaction on the algal cultivation and if the bacteria will cause algal cultivation to crash. Raw centrate and autoclaved centrate were used as the media for algal growth. The effects of different ratios of bacteria and algal inoculums on both algae and bacteria growth and wastewater treatment were studied.

3.2 Materials and Methods

3.2.1 Pretreatment and characteristics of wastewater

The centrate was collected from the Metropolitan Wastewater Treatment Plant located in Saint Paul, Minnesota. The large solid particles in the centrate were removed by sedimentation and filtration with filter cloth (Wypall X70, Kimberly-Clark Professional). After filtration the centrate was divided into two equal portions. One portion, labeled as autoclaved centrate, was autoclaved at 121 °C for 30 min and the other untreated portion, labeled as raw centrate, was directly used for experiments. The composition of raw and autoclaved centrate samples was shown in Table 3.1.

Table 3.1. Main nutrients of raw centrate and autoclaved centrate.

| Characteristics | Raw centrate | Autoclaved centrate |
|--------------------------|---------------|---------------------|
| COD (mg/L) | 2613.3 ± 28.9 | 2786.7 ± 20.8 |
| Total nitrogen (mg/L) | 141 ± 0.6 | 157 ± 5.0 |
| Total phosphorous (mg/L) | 178 ± 3.8 | 130 ± 7.1 |
| Ammonia | 91.7 ± 0.6 | 92.4 ± 1.2 |

3.2.2 Algae strain and culture conditions

Chlorella vulgaris UTEX 2714 was purchased from the Culture Collection of Algae at the University of Texas. Prior to transferring onto the centrate, the algae cells were conserved in Tris-Acetate-Phosphorus (TAP) media (Harris, 1989) containing following ingredients (mg/L): NH₄Cl 400, MgSO₄ · 7H₂O 100, CaCl₂ · 2H₂O 50,

K₂HPO₄ 108, KH₂PO₄ 56, Tris (hydroxymethyl) aminomethane 2420, 1 mL/L glacial acetic acid, 1 mL/L trace elements solution consisted of 50 g/L Na₂EDTA, 22 g/L ZnSO₄ ·7H₂O, 0.05 g/L CaCl₂ ·2H₂O, 11.4 g/L H₃BO₃, 5.06 g/L MnCl₂ ·4H₂O, 4.99 g/L FeSO₄ ·7H₂O, 1.61 g/L CoCl₂ ·6H₂O, 1.57 g/L CuSO₄ ·5H₂O, 1.10 g/L (NH₄)₆Mo₇O₂₄ ·4H₂O, and 16 g/L KOH.

Algae cultivation was done using 250mL Erlenmeyer flasks containing 100mL TAP on two different centrate media. The flasks were kept on a shaker at 100 rpm rotation speed. In all cases, the algae were grown at 25 ±2 °C with illumination at light intensity of 50 μmol m⁻²s⁻¹. All of the inoculations and cultivations were performed in triplicates. Samples were taken at the designated times for evaluation of growth rate, nutrients consumption, pH value, and bacterial counts as described below.

3.2.3 Analytical procedures

3.2.3.1 Nutrients analysis

Algae samples were collected daily from each flask starting from inoculation. A volume of 2 mL algae suspension was collected and centrifuged at 2044g for 15 min. The supernatant was collected and properly diluted for analyses of chemical oxygen demand (COD), total nitrogen (TN), and total phosphorus (TP). The measurements were performed following the Hach DR 5000 Spectrophotometer Manual. The value of pH was determined by using a pH meter.

3.2.3.2 Algal growth determination

Algal biomass was determined as the total biomass minus the biomass in control

experiments, where raw centrate or autoclaved centrate was not inoculated with algae. Algal biomass was expressed as total volatile suspended solids (TVSS), which represents biomass concentration and was determined according to the standard method (APHA, 1995) using 5 mL of algal suspension from the flasks.

The growth rate (k) is usually determined from the exponential phase by following this equation:

$$\ln TVSS = \ln TVSS_0 + kt$$

where $TVSS_0$ and $TVSS$ represent the biomass concentration at the beginning and any given time t , respectively.

3.2.3.3 Bacterial counts determination

Algae samples were collected from each flask starting from inoculation. A volume of 1 mL algae suspension was collected and properly diluted for inoculation on the plate. The inoculated plates were incubated at 37 °C for 12 hours. The bacterial number were determined using the method of Reed and Muench (Reed, 1938).

3.3 Results and Discussion

3.3.1 Effect of the centrate-borne bacteria on algal growth

Figure 3.1 shows the growth curves of *C. vulgaris* inoculated at different levels in raw centrate (RC) and autoclaved centrate (AC), respectively. The growth patterns for AC and RC are totally different. In the AC, the lag phase lasted one to two days, and the algal growth peaked on Day 3 followed by a stationary phase until the end of the experiment. After seven days, the algal biomass in the AC were 0.71, 0.68, 0.77,

and 0.81 g/L for different initial algal inoculums of 0.02, 0.05, 0.1 and 0.2 g/L, respectively. In the RC, when the initial algae inoculums were above 0.02 g/L, the algal growth did not show a lag phase, and the algal biomass reached its maximum level on Day 2 and then declined in the next two days followed by a stationary phase. At 0.02 g/L algae inoculation level, the growth curve appears different with the growth peaked on Day 5. Within two days, the algal culture in the RC had a maximum biomass of 1.17, 2.01, and 1.3 g/L for initial algal inoculums of 0.05, 0.1 and 0.2 g/L, respectively. At 0.02 g/L algal inoculum level, the maximum algal biomass of 1.21 g/L was reached on Day 5. After seven days, the algal biomass in the RC was 0.69, 0.53, 0.57, and 0.70 g/L for initial algal inoculums of 0.02, 0.05, 0.1 and 0.2 g/L, respectively.

The final biomass in AC is a little higher than that in RC at the end of the experiment, but the coexisting bacteria did not significantly diminish the final algal biomass. The statistical significance of the effect of bacteria to the final algal biomass was evaluated by using ANOVA technique. The p-values of the four inoculum levels were 0.712, 0.234, 0.051, and 0.356 with initial algal inoculums of 0.02 g/L, 0.05 g/L, 0.1 g/L, and 0.2 g/L, respectively. The p-values were all more than 0.05, indicating that the effects of bacteria on the final algal biomass were not significant.

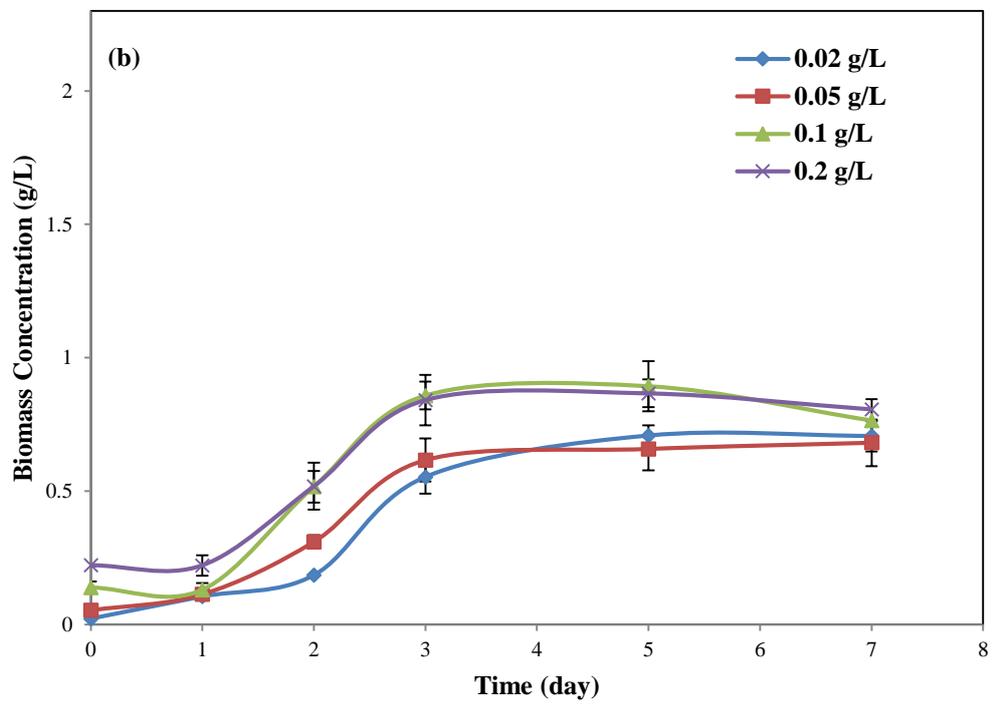
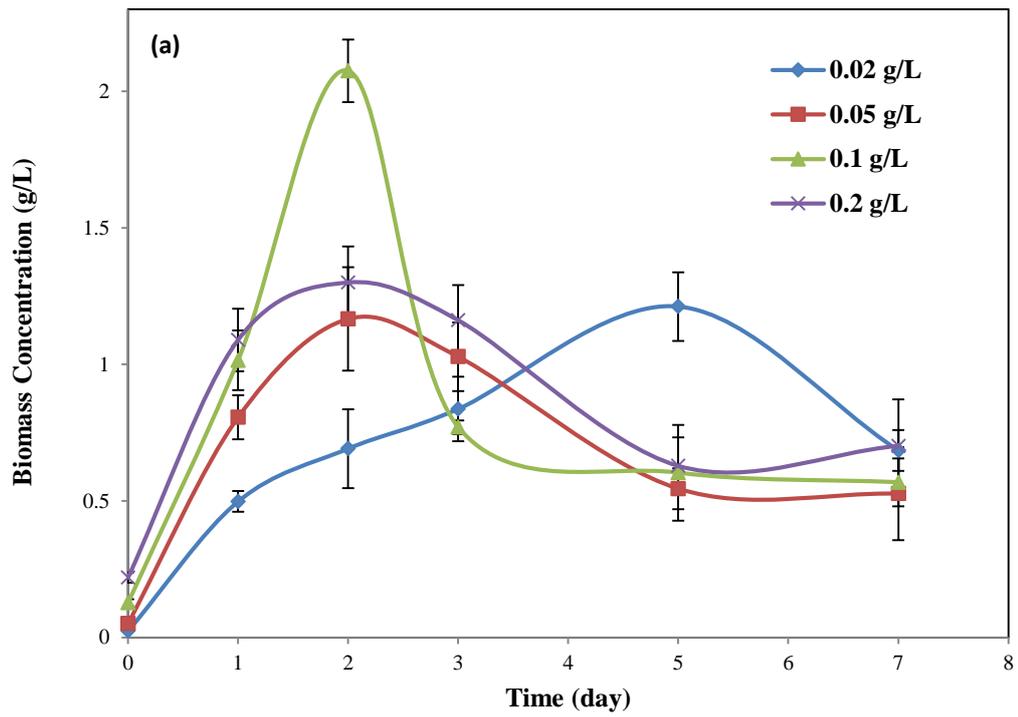


Figure 3.1: Growth profile of algae grown in the (a) raw centrate medium and (b) autoclaved centrate medium with different initial algal inoculums

However, the growth rate at the initial stage of the cultivation in RC is much higher

than that in AC. During the first two days, the growth rate of algae in AC were 1.07, 0.88, 0.66, and 0.42 d⁻¹ for different initial algal inoculums of 0.02, 0.05, 0.1 and 0.2 g/L, respectively. In RC, the growth rate were 1.67, 1.56, 1.39, and 0.89 d⁻¹ for different initial algal inoculums. This suggests that centrate-borne bacteria has the positive effects on algal growth and biomass accumulation during the exponential phase. The later declining and stationary periods could be due to the media nutrient limitation in the batch culture. To further examine the effects of bacteria on algal biomass, the number of bacteria in the RC medium during the cultivation was monitored.

3.3.2 Bacterial growth

The bacterial counts in the RC as a function of growth time are shown in Figure 3.2. It was clear that the growth profiles of the bacteria at different initial algal inoculums including the control where no algae were inoculated followed the same trend. The initial number of bacteria was about 3.9×10^5 /mL. The bacteria grew rapidly at the beginning of cultivation and reached its maximum level on Day 2. After that, the number of bacteria in the media decreased dramatically and maintained at a very low level on Day 5, about 1.6×10^4 /mL. The maximum bacterial numbers were 1.1×10^6 , 9.5×10^5 , 8.0×10^5 , 7.5×10^5 and 7.1×10^5 /mL with initial algal inoculums of 0.02 g/L, 0.05 g/L, 0.1 g/L, 0.2 g/L and control, respectively. These data indicate that (1) all cultures with algae inoculum showed higher bacterial counts than the control, suggesting that algae facilitated bacterial growth; and (2) the higher the initial

inoculation, the lower the number of bacteria at maximum, suggesting that algae also competed with bacteria for certain nutrients or produced certain substance that limited bacteria growth. These conflicting observations point to a complex relationship between algae and bacteria. The fact that the maximum bacterial numbers for 0.02 g/L algal inoculums concentration was about 1.5 times higher than for the control suggests that low initial algal concentration may be more favorable for bacterial growth, and too low of initial algal concentration may cause bacteria to bloom and algae to crash.

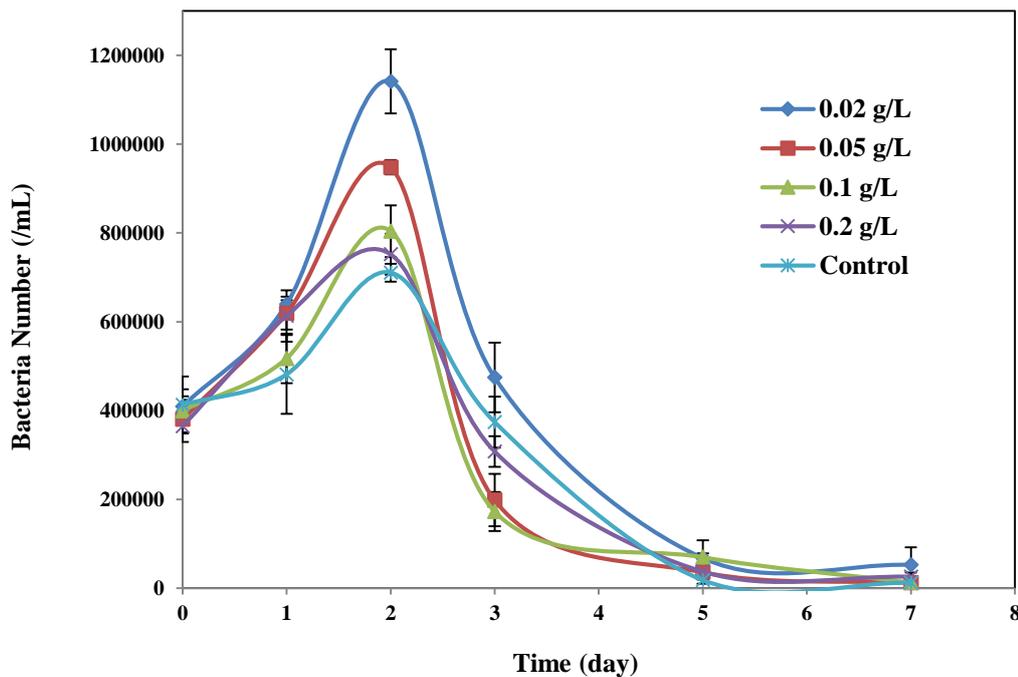


Figure 3.2: The number of bacteria in raw centrate (RC) with different initial algal inoculums

The bacterial number at high initial algal inoculation was similar to that of the control and the algal biomass was determined as the total biomass minus the biomass in control experiments, so the algal biomass in this study truly reflected the real algal

growth.

From the above results, it is obvious that centrate-borne bacteria have strong influences on algal growth pattern and the biomass accumulation. The growth of bacteria matched with algal biomass changes in RC, except for algal inoculum concentration of 0.02 g/L. Both of them reached the highest yields on Day 2, which indicated that algae can facilitate the bacteria growth with all inoculum concentration level within first two days, suggesting a mutually beneficial relationship between algae and centrate-borne bacteria for the algae inoculum concentration levels used. The statistical significance of the effect of bacteria to the maximum algal biomass was evaluated by using ANOVA technique. The p-values of the four inoculum levels were 0.012, 0.016, 0, and 0.006 with initial algal inoculum of 0.02 g/L, 0.05 g/L, 0.1 g/L, and 0.2 g/L, respectively. The p-values were all far less than 0.05, indicating that the effects of bacteria on maximum algal biomass were significant at high confidence levels.

This result is partially different from other reports which claimed that high algal cell concentration would inhibit the bacterial growth completely probably due to different microbial community (Xavier, 2002), while the synergistic relationship was observed in this study (Figure 3.2). There are several reasons that wastewater-borne bacteria and algae may have a symbiotic relationship. First, bacteria could supply sufficient carbon dioxide which is required for algal bloom during the fast growth stage. Under favorable conditions, bacteria could deliver a large amount of CO₂ in a supersaturated state (Kuentzel, 1969). From our previous study, the microalgae strain

used in this study could grow well in organic-rich wastewater with the mixotrophic mode to achieve high biomass concentration and high lipid content simultaneously (Zhou et al., 2012b). The CO₂ generated by bacteria would favor the algal growth and bloom which could explain why algal growth did not have a visible lag phase at the initial stage during the cultivation. Second, bacteria participated in the nutrients and pollutants degradation process and transformed complex substrates to small organic acid molecules and ammonium which could be easily used by algae (Zhang et al., 2012). In addition, the generation of large amount of CO₂ led to the maximum concentration of CO₂ presented at the surfaces of algal and bacterial cells. When these cells are in close proximity, this CO₂ rich environment is a favorable condition for algal growth. Algae could also utilize incomplete metabolic products released by the bacteria, and similarly the bacteria may benefit from vitamins and growth factors leached from algal cells (Humenik and Hanna, 1971).

For the low algal inoculum concentration (0.02 g/L), the algal biomass increased slowly and reached the highest biomass on Day 5. In this case, it is clear that the centrate-borne bacteria competed well against algae in growth and nutrient uptake due to the limited algal cell number. Therefore, algae growth was significantly retarded initially, and it took 5 days to reach just over 1 g/L, while with appropriate algae inoculation level, such as 0.1 g/L, there was an obvious symbiotic effect between algae and bacteria (Figure 3.1a and Figure 3.2). There are significant since the control test with autoclaved centrate medium without bacteria did not seem to have any complication as expected (Figure 3.1b). And the bacteria number count data

also reached over 2 g/L in just two days. It is worth noting that when algae inoculation level is too high (0.2 g/L in this case), the mutual symbiotic relationship between algae and bacteria was not observed. Therefore, in order to obtain the highest algal biomass, the optimized initial algae inoculum size of 0.1 g/L should be chosen. From the above data, appropriate hydraulic retention time, in other words, the period that centrate was retained in the system, should be two days. In order to further understand the interaction between algae and centrate-borne bacteria, additional research should focus on monitoring the microbial community compositions with different initial algal inoculums. And scaling-up and grown in semi-continuous and continuous mode are recommended for future research.

3.3.3 Effects of centrate-borne bacteria on the centrate nutrient removal

The changes in COD with time in raw centrate and autoclaved centrate are shown in Figure 3.3. The profiles of COD reduction were similar in both media with different initial algal inoculums. In raw centrate medium, the concentration of COD decreased dramatically from 2613 mg/L to 545, 496, 456 and 452 mg/L within the first two days with initial algal inoculums of 0.02, 0.05, 0.1 and 0.2 g/L, respectively. The corresponding COD removal efficiencies by the end of cultivation were 87.1%, 87.2%, 87.0% and 87.2%, respectively. In autoclaved centrate, the concentration of COD also decreased significantly from 2787 mg/L to 737, 576, 617 and 467 mg/L with initial algal inoculums of 0.02, 0.05, 0.1 and 0.2 g/L, respectively. The corresponding COD removal efficiencies by the end of cultivation were 86.8%,

88.0%, 88.6% and 88.2%.

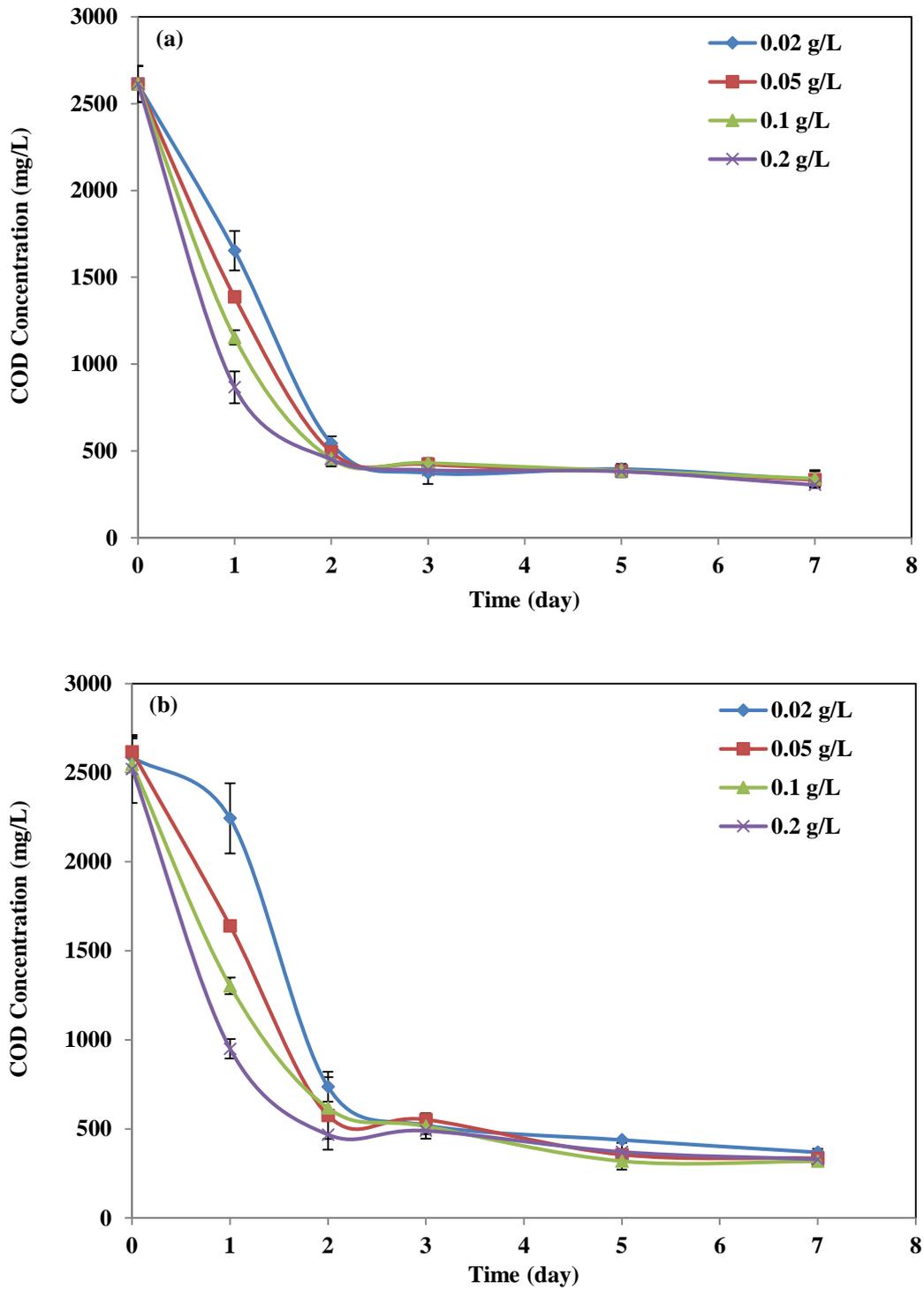


Figure 3.3: The COD concentration profile in (a) RC medium and (b) AC medium with different initial algal inoculums

In general, higher algae inoculation level removed more COD in both RC and AC

cases, and in RC the symbiotic effect between algae and bacteria did improve the COD removal as expected (Figure 3.3a, day 1). After Day 2, the COD levels already dropped significantly, leaving only difficult to use organic carbons in the batch media, and therefore causing nutrient deficiency for further algae growth. Previous studies reported that bacteria can break down complex organic compounds into smaller molecules as nutrients usable for algae (Zhang et al., 2012). That may explain the higher COD removal rate in the first two days and the symbiotic relationship between centrate-borne bacteria and algae at the initial stage during the cultivation.

In the autoclaved media, higher algal inoculum concentration gave higher nutrients removal rate during the cultivation, which suggested that nutrients removal was largely attributed to the absorption and degradation by algae.

The RC and AC did not show significant differences in final COD removal rate. This result was similar to some previous studies that bacteria do not have a significant effect on COD removal (Su et al., 2012). However, in the first two days, the COD removal efficiencies in RC were higher than the AC indicating that centrate-borne bacteria participated in the degradation process and utilized the organic compounds as a carbon source.

Figure 3.4 shows the change in total phosphorus during the experiment. In RC, similar to the removal of COD, most of the phosphorus degradations occurred during the first two days, total phosphorous was drastically reduced from 178 mg/L to 57.7, 58.2, 55.3 and 47.3 mg/L with initial algal inoculums of 0.02, 0.05, 0.1 and 0.2 g/L, respectively. The concentration increased slightly and stayed at a similar level until

the end of the cultivation. The corresponding final total phosphorus removal efficiencies were 65.4%, 69.6%, 65.3% and 63.5%, respectively. In AC, the total phosphorus concentration decreased much slower and the removal curve was relatively smooth. However, during the seven days cultivation, total phosphorus was also reduced from 130 mg/L to 44.3, 41.5, 47.9 and 43.4 mg/L with initial algal inoculums of 0.02, 0.05, 0.1 and 0.2 g/L, respectively. The corresponding final total phosphorus removal efficiencies were 65.9%, 68.1%, 63.2% and 66.6%, respectively, which is similar to the efficiencies in RC. Previous studies reported that both algal biomass uptake and phosphate precipitation can account for the removal of phosphorus and abiotic phosphorus removal normally occurred at pH 9 to 11 (Godos et al., 2009). The pH value was tested and did not exceed 8.5 at the end of cultivation (Figure 3.5); therefore the phosphate precipitation might have only minor influence here. The main mechanism of such phosphorus removal was accumulation into the biomass. This result matched the algal biomass changes in RC and AC in the earlier discussion.

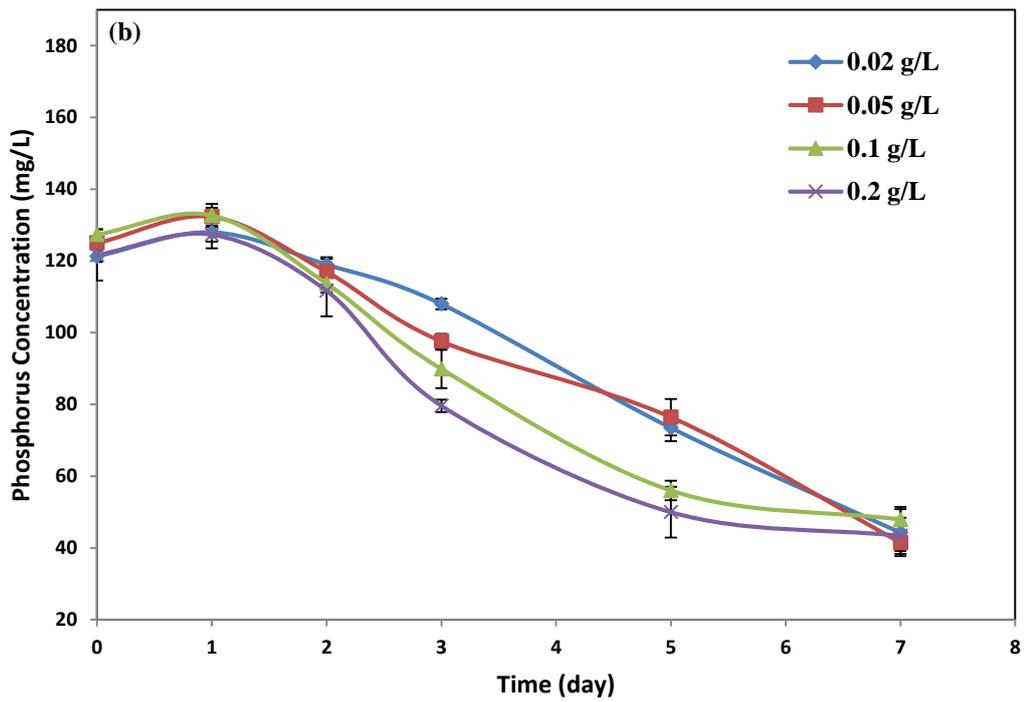
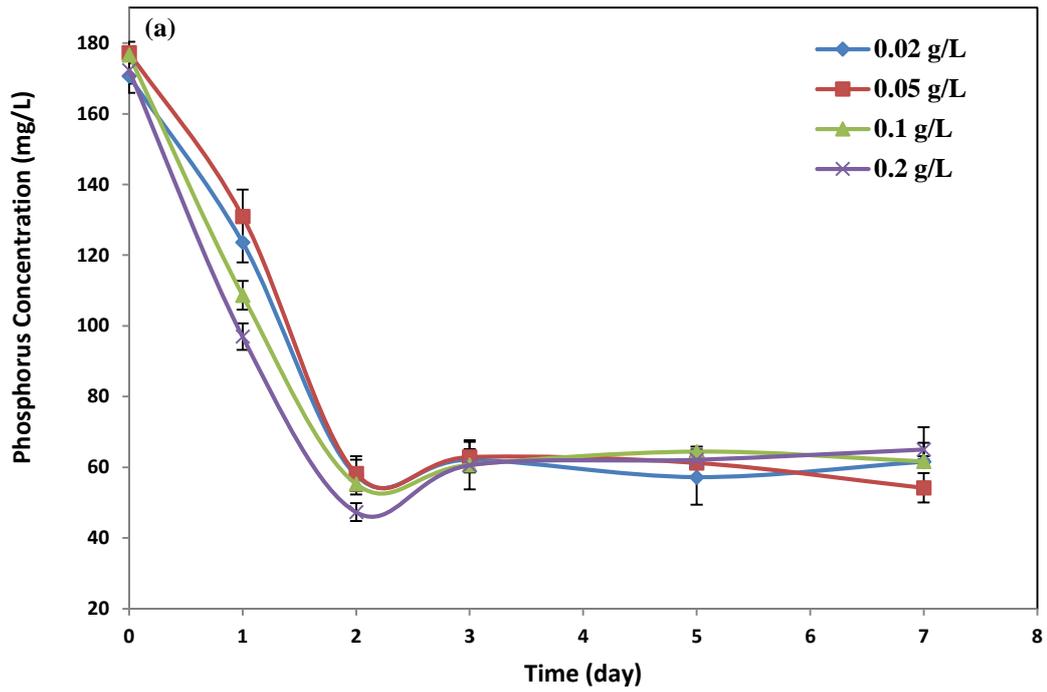


Figure 3.4: The total phosphorus concentration profile in (a) RC medium and (b) AC medium with different initial algal inoculums

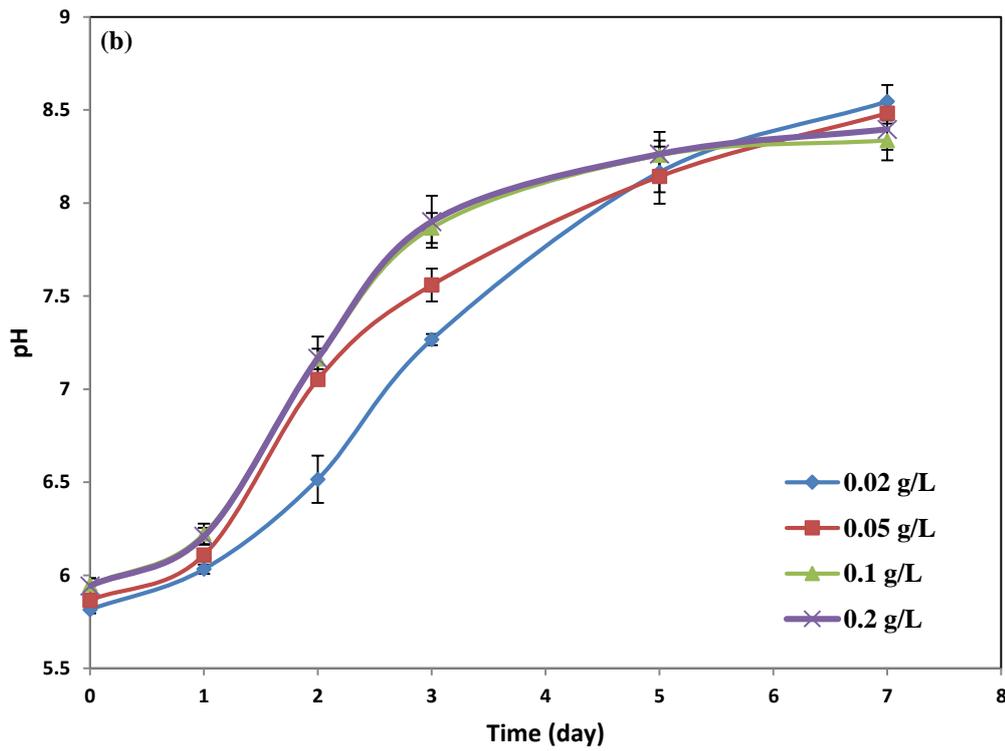
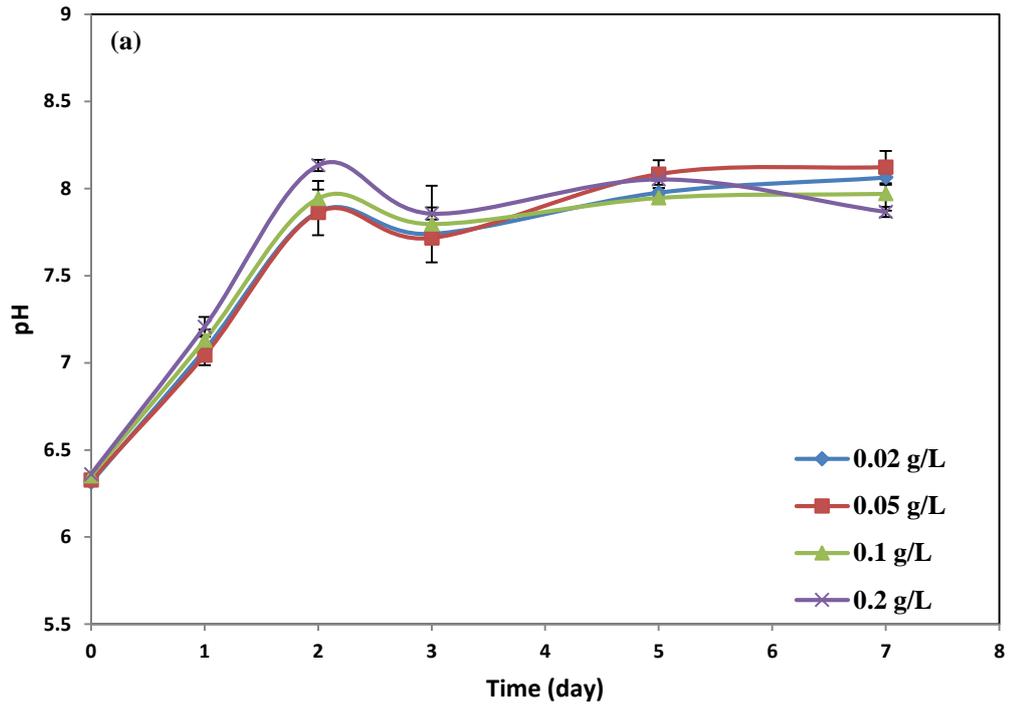


Figure 3.5: The pH value change in (a) RC medium and (b) AC medium with different initial algal inoculums

The changes in total nitrogen concentration were also investigated in this study

(Figure 3.6). In raw centrate medium, the concentration of total nitrogen decreased rapidly from 141 mg/L to 67.4, 65.5, 62.0 and 57.5 mg/L within the first two days with initial algal inoculums of 0.02, 0.05, 0.1 and 0.2 g/L, respectively. Then the concentration increased in the next day followed by a gradual increase in the remaining days of the experiments. The final concentration is 109.2, 107.6, 106.0 and 109.9 mg/L, respectively. In autoclaved centrate, similar to the removal of other nutrients, total nitrogen concentration decreased slowly and the removal curve was smooth. During the seven days cultivation, total nitrogen was reduced from 157 mg/L to 84.3, 88.5, 85.9 and 87.2 mg/L with initial algal inoculums of 0.02, 0.05, 0.1 and 0.2 g/L, respectively. At the initial stage of the cultivation, the total nitrogen removal rate in RC was much higher than that in AC, which indicated that centrate-borne bacteria participated in nitrogen degradation and assisted the removal of nitrogen. After two days, the number of bacteria in RC decreased dramatically; at the same time, nitrogen concentration began to increase. Therefore, the death and degradation of bacteria (Figure 3.2) and algae (Figure 3.1a) might be the main reason for following increase in total nitrogen concentration in the raw centrate medium (Li et al., 2012). That again indicated that algae and bacteria had a symbiotic relationship.

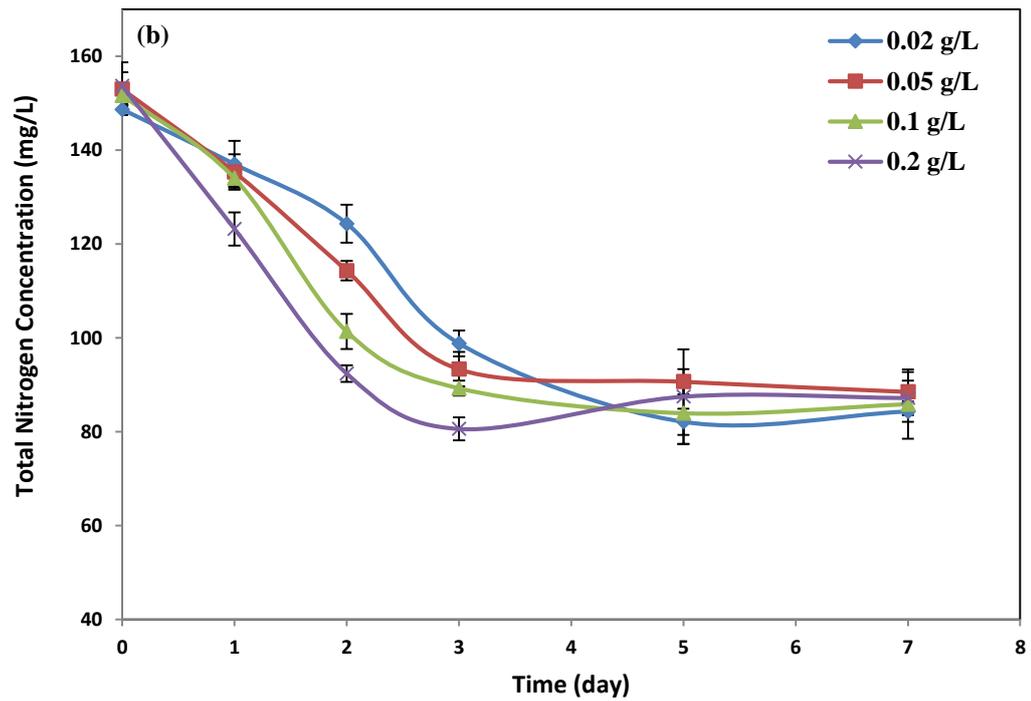
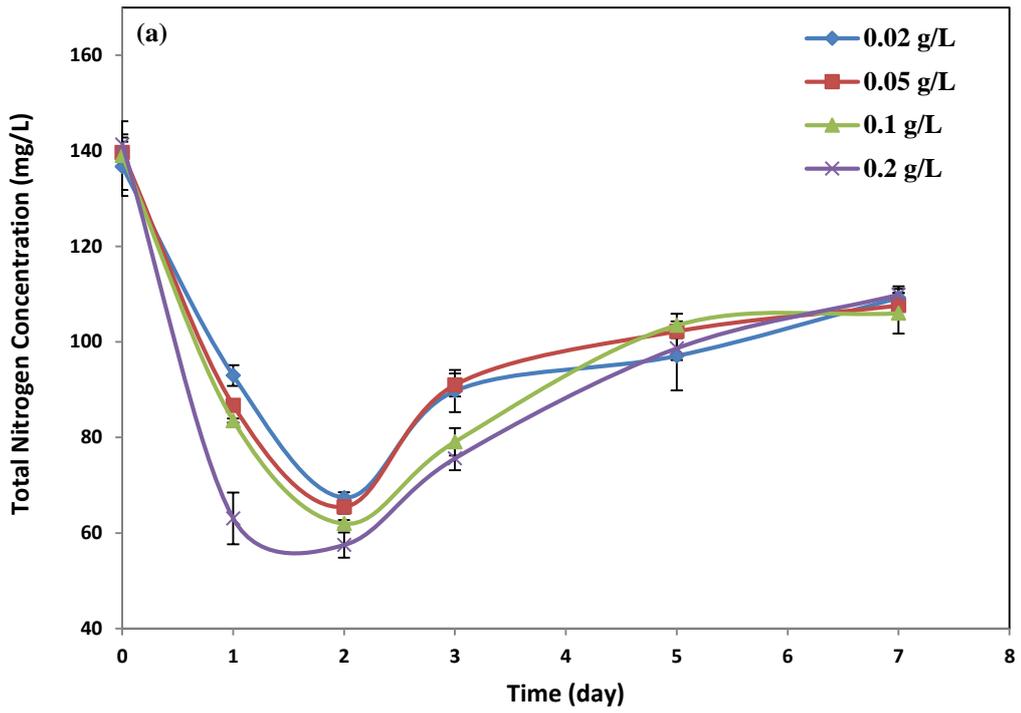


Figure 3.6: The total nitrogen concentration profile in (a) RC medium and (b) AC medium with different initial algal inoculum

3.4 Conclusions

A mutual compound relationship between algae and centrate-borne bacteria has been demonstrated in this study. Algae can promote bacterial growth and optimal initial algal concentration may be more favorable for bacterial growth. Moreover, bacteria could increase the algal growth rate and nutrients removal rate at the initial stage of the cultivation, which is very important in the continuous or semi-continuous cultivation process. To obtain the maximum algal biomass, semi-continuous and/or continuous cultivation and the appropriate hydraulic retention time of two days are recommended. This result could provide a guideline for efficient microalgae-based organic-rich wastewater treatment and wastewater energy crop production.

CHAPTER 4. Microbial community profile analysis during algae cultivation

Summary

The microbial community profile in the raw centrate wastewater and dynamics during 7 days of cultivation with algae were analyzed using Illumina Miseq sequencing. The results showed that 10 phyla occurred in the centrate wastewater samples, phylum Bacteroidetes occupied the top position in the communities with the relative abundance of 88.76%. The other dominant phyla were Firmicutes and Proteobacteria. At the end of cultivation, microbial community was dominated by phylum Proteobacteria. Thirty-seven genera were identified and 4 bacteria genus, Acinetobacter, Bacteroidales_norank, Megasphaera, and S24-7_norank, were found have strong impact on the synergetic relationship with algae.

4.1 Introduction

In the previous experiment, the initial algal concentration and the ratio of algae versus bacteria in raw centrate wastewater had an apparent effect on bacterial growth, and the presence of bacteria had a significant influence on the algal growth pattern, suggesting potential synergetic relationship between algae and bacteria at the initial stage of algae cultivation. The maximum algal biomass of 2.01 g/L with 0.1 g/L initial algal inoculums concentration can be obtained during algae cultivation in raw centrate medium. In order to further understand the interaction between algae and centrate-borne bacteria, additional research should focus on monitoring the microbial community variation duration cultivation. It is hypothesized that one or more

bacteria species could stimulate algae growth and elucidate the potential mechanism of synergistic relationship.

The specific objectives of this study were (1) to analyze the bacteria profile in the centrate wastewater; (2) to monitor the changes in the microbial community during algae cultivation by using the second generation sequencing to obtain the information on their interactions with algae.

Microbial community profiles can be investigated using several techniques, including denaturing gradient gel electrophoresis (DGGE), cloning library of 16S rRNA genes, terminal restriction fragment length polymorphism (T-RFLP), and Single-Strand Conformation Polymorphism (SSCP) (Boon et al., 2002; Schütte et al., 2008; Xiao et al., 2015). However, the sequences these methods generated cannot be directly translated to taxonomic information because only a few sequences can be examined (Osborn et al., 2000). High-throughput sequencing technology such as Illumina MiSeq sequencing was conducted to obtain sample's detail microbial community information by using specific primers to provide an insight into the diversity of bacterial groups at greatest coverage (Tago et al., 2014). At present, Illumina MiSeq sequencing has been developed as an effective tool for better access to microbial diversity because of the low cost per sequence as well as high read quantity and quality (You et al., 2015).

4.2 Materials and Methods

4.2.1 Pretreatment and characteristics of wastewater

The centrate was collected from the Metropolitan Wastewater Treatment Plant located in Saint Paul, Minnesota. The large solid particles in the centrate were removed by sedimentation and filtration with filter cloth (Wypall X70, Kimberly-Clark Professional).

4.2.2 Algae strain and culture conditions

Chlorella vulgaris UTEX 2714 was purchased from the Culture Collection of Algae at the University of Texas. Prior to transferring onto the centrate, the algae cells were conserved in Tris-Acetate-Phosphorus (TAP) media (Harris, 1989) containing following ingredients (mg/L): NH₄Cl 400, MgSO₄ ·7H₂O 100, CaCl₂ ·2H₂O 50, K₂HPO₄ 108, KH₂PO₄ 56, Tris (hydroxymethyl) aminomethane 2420, 1 mL/L glacial acetic acid, 1 mL/L trace elements solution consisted of 50 g/L Na₂EDTA, 22 g/L ZnSO₄ ·7H₂O, 0.05 g/L CaCl₂ ·2H₂O, 11.4 g/L H₃BO₃, 5.06 g/L MnCl₂ ·4H₂O, 4.99 g/L FeSO₄ ·7H₂O, 1.61 g/L CoCl₂ ·6H₂O, 1.57 g/L CuSO₄ ·5H₂O, 1.10 g/L (NH₄)₆Mo₇O₂₄ ·4H₂O, and 16 g/L KOH.

Algae cultivation was done using 250mL Erlenmeyer flasks containing 100mL centrate media. The flasks were kept on a shaker at 100 rpm rotation speed. In all cases, the algae were grown at 25 ±2 °C with illumination at a light intensity of 50 μmol m⁻²s⁻¹. All of the inoculations and cultivations were performed in triplicates. Samples were taken at the designated times for evaluation of growth rate, bacterial

counts, and microbial diversity analysis as described below.

4.2.3 Algal growth determination

Algal biomass was determined as the total biomass minus the biomass in control experiments, where raw centrate or autoclaved centrate was not inoculated with algae. Algal biomass expressed as total volatile suspended solids (TVSS), which represents biomass concentration and was determined according to the standard method (APHA, 1995) using 5 mL of algal suspension from the flasks.

4.2.4 Bacterial counts determination

Algae samples were collected from each flask starting from inoculation. A volume of 1 mL algae suspension was collected and centrifuged at 2044g for 10 min. The supernatant was collected and properly diluted for inoculation on the plate. The inoculated plates were incubated at 37 °C for 12 hours. The bacterial number were determined using the method of Reed and Muench (Reed, 1938).

4.2.5 DNA extraction and PCR amplification

Microbial DNA was extracted from the samples using the E.Z.N.A Soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to manufacturer's protocols. The V4-V5 region of the 16S rRNA gene were amplified by PCR (95 °C for 2 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 5 min) using primers 515F 5'-barcode-GTGCCAGCMGCCGCGG)-3' and 907R 5'-CCGTCAATTCMTTTRAGTTT-3',

where barcode is an eight-base sequence unique to each sample. PCR reactions were performed in triplicate 20 μ L mixture containing 4 μ L of 5 \times FastPfu Buffer, 2 μ L of 2.5 mM dNTPs, 0.8 μ L of each primer (5 μ M), 0.4 μ L of FastPfu Polymerase, and 10 ng of template DNA.

4.2.6 Illumina MiSeq sequencing

Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using QuantiFluor™ -ST (Promega, U.S.). Purified amplicons were pooled in equimolar and paired-end sequenced (2 \times 250) on an Illumina MiSeq platform according to the standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database.

4.2.7 Processing of sequencing data

Raw fastq files were demultiplexed, quality-filtered using QIIME (version 1.17) with the following criteria: (i) The 300 bp reads were truncated at any site receiving an average quality score <20 over a 50 bp sliding window, discarding the truncated reads that were shorter than 50bp. (ii) exact barcode matching, 2 nucleotide mismatch in primer matching, reads containing ambiguous characters were removed. (iii) only sequences that overlap longer than 10 bp were assembled according to their overlap sequence. Reads which could not be assembled were discarded.

Operational Units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1 <http://drive5.com/uparse/>) and chimeric sequences were identified and

removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier (<http://rdp.cme.msu.edu/>) against the silva (SSU115)16S rRNA database using confidence threshold of 70%

4.3 Results and Discussion

The microbial community profile and dynamics during 7 days of cultivation were analyzed using the Illumina Miseq sequencing technique. Sequence numbers per sample ranged from 21208 (day 1) to 33272 (day 2) with a mean of 27369 and the average length is approximately 445 bp. A 97% of similarity cut-off was used to group OTUs (Operational Taxonomic Units) for downstream analysis. Table 4.1 summarizes the corresponding number of OTUs, Shannon index, Chao, and other estimators.

Table 4.1 Characteristic of bacterial phylotype coverage and diversity estimators

| Sample | Reads | OTUs | ACE | Chao | Coverage (%) | Shannon | Simpson |
|--------|-------|------|-----|------|--------------|---------|---------|
| Day0 | 17033 | 130 | 150 | 155 | 99.84 | 0.95 | 0.7356 |
| Day1 | 17033 | 192 | 211 | 209 | 99.82 | 2.19 | 0.3256 |
| Day2 | 17033 | 195 | 212 | 212 | 99.85 | 3.33 | 0.0835 |
| Day3 | 17033 | 181 | 202 | 202 | 99.83 | 3.06 | 0.1287 |
| Day5 | 17033 | 183 | 199 | 201 | 99.85 | 3.32 | 0.0832 |
| Day7 | 17033 | 176 | 181 | 183 | 99.92 | 3.5 | 0.0631 |

The coverage was used to assess whether the library was sufficiently large to obtain meaningful and stable richness estimates. The calculated coverage of each sample

was all above 99%, indicating the clone library yield sufficiently stable richness estimates. Ace and Chao are richness estimators, which are commonly used in ecology to estimate the total number of species. A greater value of Ace and Chao indicates larger variety of species exist in a sample. Shannon and Simpson are diversity indexes for an OTU definition, a larger value of Shannon and a smaller value of Simpson means higher community diversity of a sample. Results showed that OTU numbers of these samples ranged from 130 to 195 based on the effective reads, suggesting the diversity of microbial community was abundant in the samples. Microbial community and relative abundance by phylum is shown in Figure 4.1. It was obvious that phylum Bacteroidetes occupied the top position in all communities with the relative abundance of 88.76%. The other dominant phyla were Firmicutes (6.07%) and Proteobacteria (4.14%), and the rest of divisions were all present as minor components less than 1%. During the algae cultivation period, the relative abundances of phylum Proteobacteria and Firmicutes increased extensively, while the relative abundances of phylum Bacteroidetes decreased to a relatively low level. The rest 7 phylum were present in less than 0.5% until the end of cultivation. On day 7, microbial community was dominated by phylum Proteobacteria (50.31%), followed by Bacteroidetes (37.65%), and Firmicutes (11.69%).

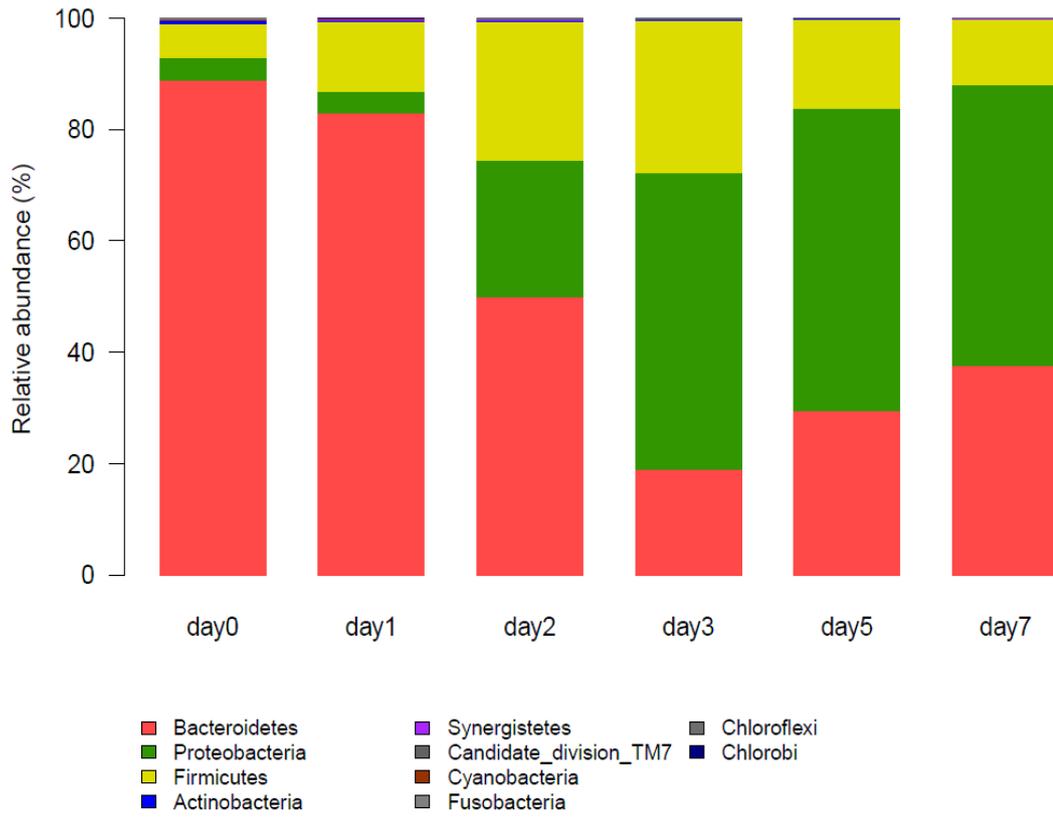


Figure 4.1: Bacterial community and relative abundance by phylum

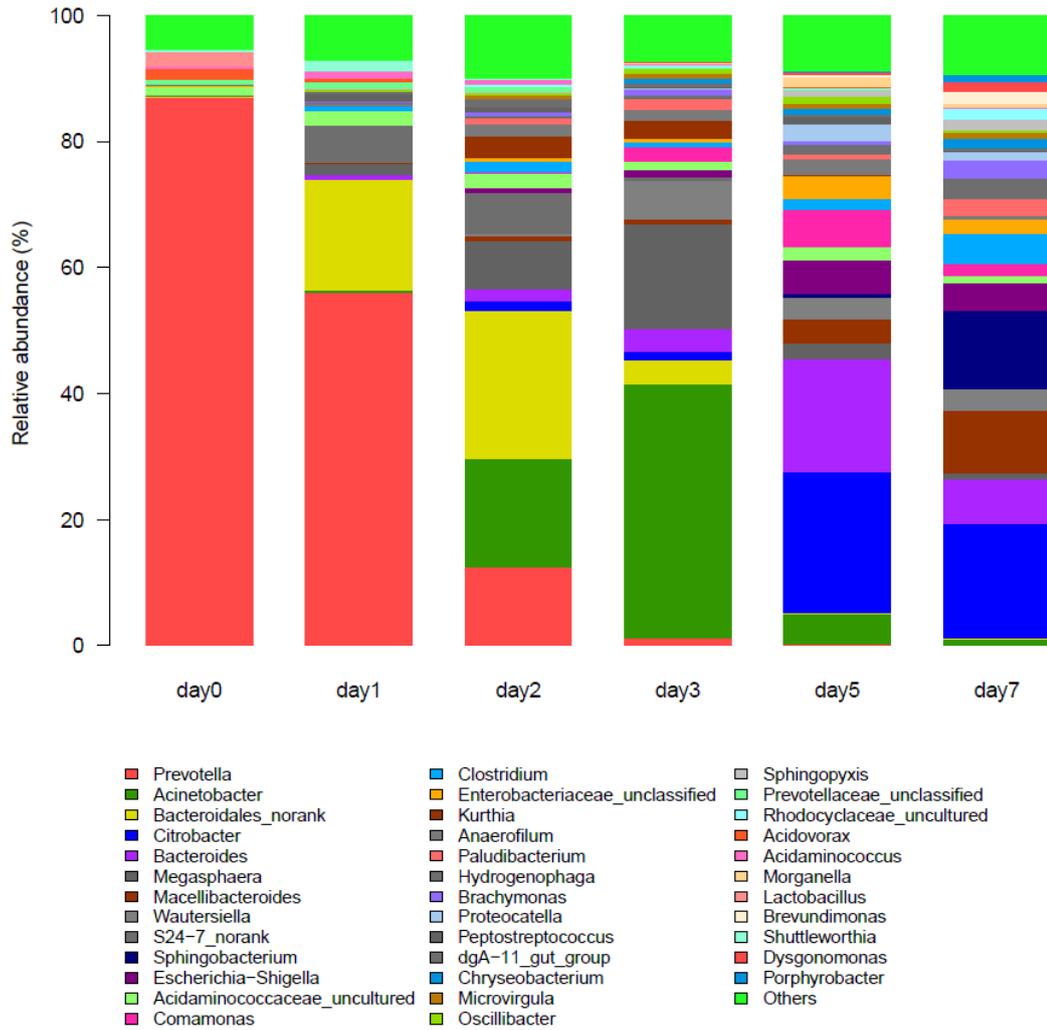


Figure 4.2: Bacterial community and relative abundance by genus

Figure 4.2 shows the genera detected in the total microbial population and their percentage within the experimental process. The sequences that could not be classified into any known group were designated as no_rank, sequences were named as unclassified, and sequences that had relative abundance of less than 1% were grouped into others.

At the beginning of the cultivation, genus *Prevotella* was most abundant, accounting for 87.1% of the total sequences. Other dominant genera were *Lactobacillus* (2.25%), *Acidovorax* (1.73%), and *Acidaminococcaceae* (1.34%). The rest of divisions were

the minor groups (less than 0.5%). During the algae cultivation period, the relative abundances of genus *Prevotella* decreased to low level, while genus *Bacteroidales_norank*, *Acinetobacter*, *Citrobacter*, *Bacteroides*, *Megasphaera* showed increasing at different levels. On day 7, microbial community was dominated by genus *Citrobacter* (18.09%), followed by *Sphingobacterium* (12.27%), and *Macellibacteroides* (10.04%).

However, the result cannot confirm the real changes in each bacteria genus, because the bacterial amount varied during the 7-day algae cultivation process. To further understand the interaction between algae and centrate-borne bacteria, the amount of each genus per mill liter culture was estimated by the percentage of each genus multiplied by the total amount of bacteria in the samples. From the result, 4 bacteria genus showed a similar growth pattern with algae: *Acinetobacter*, *Bacteroidales*, *Megasphaera*, and S24-7. The bacterial counts at genera level as a function of cultivation time are shown in Figure 4.3.

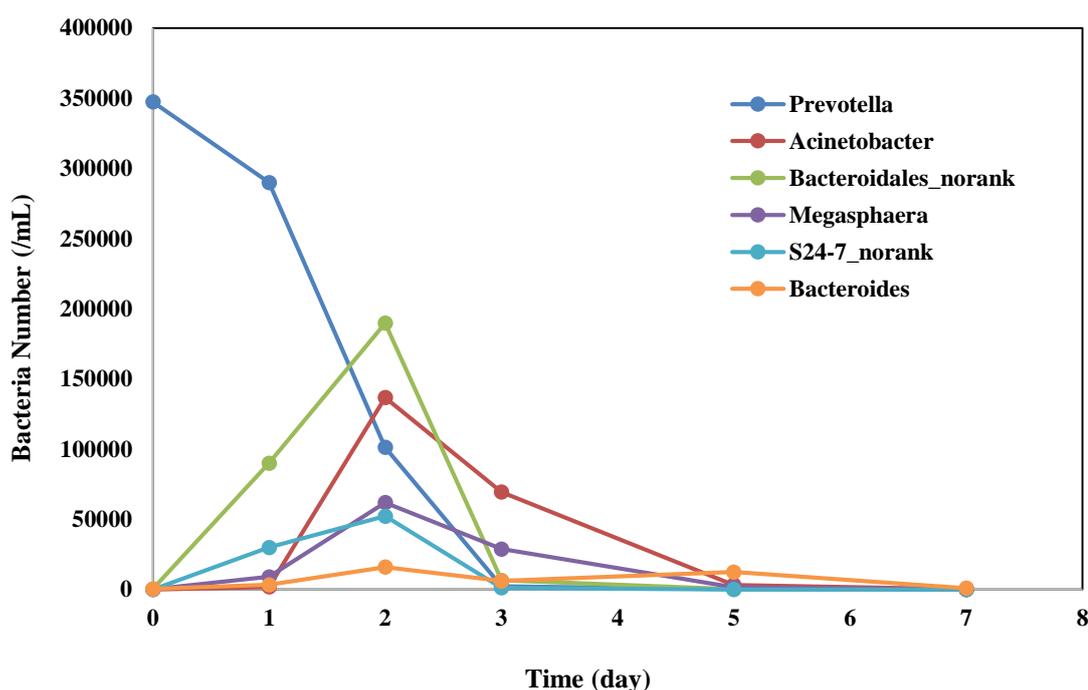


Figure 4.3: The number of bacteria at the genera level during cultivation process

Genus *Prevotella* (phylum Bacteroidetes) is the largest component of the raw centrate wastewater, the initial amount was about 3.5×10^5 /mL. Then the number of bacteria decreased dramatically and maintained at a very low level until the end of cultivation. The amount of genus *Prevotella* on Day 7 was about 22.4 /mL. Among the total bacterial population, the growth profiles of genus *Acinetobacter* (phylum Proteobacteria), *Bacteroidales_norank* (phylum Bacteroidetes), genus *Megasphaera* (phylum Firmicutes), and *S24-7_norank* (phylum Bacteroidetes) showed the similar growth pattern and followed the same trend with algae growth (Figure 3.1). Other genus, like *Bacteroides*, only appeared in low abundance and remained relatively constant during the 7-day cultivation. These four kinds of bacteria grew rapidly at the beginning of cultivation and reached its maximum level on Day 2. The maximum bacterial numbers of Genus *Acinetobacter*, Order *Bacteroidales*, Genus *Megasphaera*,

and Order S24-7 was 1.4×10^5 , 1.9×10^5 , 6.2×10^4 , and 5.2×10^4 /mL. After Day 2, COD levels dropped significantly, leaving only difficult to use organic carbons in the batch media, and, therefore, causing nutrient deficiency for further bacterial growth. Therefore, the number of bacteria in the media decreased and maintained at a low level until the end of cultivation. This result suggested that algae and some specific centrate-borne bacteria genus may have a symbiotic relationship.

Genus *Prevotella* are strictly anaerobes and Gram-negative bacteria. They could be isolated from municipal wastewater and food wastewater (Jang et al., 2015; Lee et al., 2015). The sequences of *Bacteroidales_norank* and *S24-7_norank* belong to known order (*Bacteroidales* and *S24-7*) but were simply deposited without classifications. Species of the genus *Acinetobacter* are strictly aerobic, non-fermentative, Gram-negative bacilli and found in many environments, including water, soil, sewage and food (Zhang et al., 2009). Several *Acinetobacter* species can cause life-threatening infections. Genus *Acinetobacter* has been reported to be found in municipal wastewater and industrial wastewater (Zakaria et al., 2007; Bark et al., 1992). It was reported that genus *Acinetobacter* has been used for removal of phosphate and heavy metal from the wastewater and other chemical substances like phenol (Cloete and Steyn, 1988; Deinema et al., 1980; Cordova-Rosa et al., 2009). This result also explains the previous result that the phosphate removal efficiencies in RC were much higher than the AC at the early stage of algae cultivation. Genus *Megasphaera* is strictly anaerobic, Gram-negative bacteria. They have been reported to be isolated from the human feces and intestinal, and spoilage of bottled beer (Cai

et al., 2014; Haikara and Helander, 2001). Genus *Megasphaera* is also found in the rumen of cattle and sheep which metabolizes lactate to acetate and propionate (Prabhu et al., 2012).

4.4 Conclusion

The bacteria community profiles during cultivation period were tested, the diversity of microbial community was abundant in the samples. Ten phyla and 37 genera were identified. Phylum Bacteroidetes dominate the culture in the centrate wastewater, genus *Prevotella* was most abundant accounting for 87.1% of the total sequences. At the end of cultivation, microbial community was dominated by phylum Proteobacteria, Bacteroidetes, and Firmicutes. Four bacteria genus, *Acinetobacter*, *Bacteroidales_norank*, *Megasphaera*, and *S24-7_norank*, might have strong influence on algal growth.

CHAPTER 5. Effect of temperature and substrate concentration on lipid production by *Chlorella vulgaris* using hydrolyzed LMBRs as substrate

Summary

The enzymatic hydrolysates of the lipid-extracted microalgal biomass residues (LMBRs) from biodiesel production were evaluated as nutritional sources for the mixotrophic growth of *Chlorella vulgaris* and lipid production at different temperature levels and substrate concentrations. Both parameters had a significant effect on cell growth and lipid production. It was observed that *C. vulgaris* could grow mixotrophically in a wide range of temperatures (20~35 °C). The optimal temperature for cell and lipid accumulation through the mixotrophic growth of *C. vulgaris* was between 25 and 30 °C. The neutral lipids of the culture at 25 °C accounted for as much as 82 % of the total lipid content in the microalgae at culture Day 8. Fatty acid composition analysis showed that the increase in saturated fatty acids was proportional to the increase in temperature. The maximum biomass concentration of 4.83 g/L and the maximum lipid productivity of 164 mg/L/day were obtained at an initial total sugar concentration of 10 g/L and an initial total concentration of amino acids of 1.0 g/L but decreased at lower and higher substrate concentrations. The present results show that LMBRS could be utilized by the mixotrophic growth of *C. vulgaris* for microalgal lipid production under the optimum temperature and substrate concentration.

5.1 Introduction

Along with the exhaustion of fossil fuels and the worsening of global warming by CO₂ emissions grows increasing interest in sustainable microalgal biofuel. Microalgae use sunlight and CO₂ to synthesize organic matter through photosynthesis, and some species can accumulate lipids simultaneously, thus having great potential to alleviate the problems of CO₂ emissions and energy shortage (Rawat et al., 2013). Microalgae are considered to be suitable for fixing CO₂ to produce biodiesel based on their merits such as high surface area to volume ratio and a comparatively high photosynthetic efficiency (Wang et al., 2013). Many species of microalgae including *Chlorella vulgaris* can also use the organic carbon in the culture medium and show rapid growth mixotrophically (Zhou et al., 2011). However, current efforts on microalgal biofuel production have been economically undermined (Nobre et al., 2013). The main constraints include the limited amount of biomass which can be obtained with currently available photobioreactors and low lipid productivity.

Lipid-extracted microalgal biomass residues (LMBRs) is the leftover biomass from microalgal biodiesel production, which mainly contains proteins and carbohydrates, are thus ideal nutritional supplements for the mixotrophic growth of microalgae after appropriate pretreatment such as enzymatic hydrolysis (Zheng et al., 2012; Zheng et al., 2012). Nutrient supplies highly influence the cost and sustainability of biodiesel production from microalgae (Stephens et al., 2010). Full utilization of these residues can reduce the production waste and improve environmental viability (Ehimen et al.,

2011). Moreover, LMBRs have been enzymatically hydrolyzed into amino acids and sugars and successfully utilized as nutrient sources for a new crop of microalgae (Zheng et al., 2012; Zheng et al., 2012).

Temperature drastically influences cell growth and lipid production of microalgae under both autotrophic and heterotrophic conditions (Sheng et al., 2011; Jiang and Chen, 2000). The lower temperature (22 °C) and higher temperature (44 °C) severely inhibit *Synechocystis sp.* PCC6803 growth under autotrophic conditions, and the lower temperature (18 °C) promotes the accumulation of unsaturated fatty acids (Sheng et al., 2011). When the temperature is increased from 20 to 25 °C, the lipid content of autotrophic *Nannochloropsis oculata* is virtually doubled; while that of autotrophic *C. vulgaris* is reduced by about 60% when the temperature is increased from 25 to 30 °C (Converti et al., 2009). The polyunsaturated fatty acids (PUFAs) content of the heterotrophic growth of *Cryptocodinium cohnii* is enhanced at low temperature to increase membrane flexibility (Jiang and Chen, 2000). Compared with autotrophic and heterotrophic microalgae, the mixotrophic growth of microalgae could have a relatively high biomass concentration, mitigate CO₂ emissions, and reduce production costs, especially by utilizing LMBRs. So far, the effect of temperature on the growth and neutral lipid accumulation of the mixotrophic growth of microalgae using LMBRs remains unexplored.

Previous research found that autotrophic *Neochloris oleoabundans* with lower sodium nitrate concentration (3 mM) or higher sodium nitrate concentration (20 mM) resulted in lower yields of biomass and lipid production than that of 5 mM sodium

nitrate (Li et al., 2008). The maximum biomass and lipid production of the heterotrophic growth of *Chlorella saccharophila* are attained at 20 g/L glucose, decreasing with increasing or decreasing glucose concentration (Muge et al., 2012). Since both lower and higher substrate concentrations are detrimental to cell growth and lipid production, determining a suitable substrate concentration is critical in microalgal cultivation and lipid production mixotrophically using LMBRs.

Because of several technical limitations, such as low lipid productivity, unclear lipid metabolism regulation, and insufficient usage of LMBRs, associated with existing technologies in the production of economically-viable algal oil, further research is needed to better understand lipid metabolism and accumulation in algal cells. Therefore, the aim of this study is to understand the effects of temperature and substrate concentration on the mixotrophic growth of *C. vulgaris* using low-cost culture media from LMBRs to enhance lipid production. In addition, this study also provides some information on the regulation of neutral lipid biosynthesis through temperature regulation and variation in substrate concentration.

5.2 Materials and methods

5.2.1 Materials

Cellulase derived from *Trichoderma* with an enzymatic activity of 80 U/mg, neurase derived from *Bacillus subtilis* and alcalase derived from *Bacillus licheniformis* with enzymatic activities of 100 U/mg were provided by Nanjing Genetime Biotechnology Co. Ltd., Jiangsu Province, China.

5.2.2 Microalgal strain and cultivation conditions

The microalga *C. vulgaris* was obtained from the China Center for Type Culture Collection, Wuhan, China. The strain was preserved in 20% (v/v) glycerol at $-80\text{ }^{\circ}\text{C}$. For temperature experiments, the culture medium was composed of instant ocean synthetic sea salt (Aquarium Systems, Inc., USA), 34 g/L; initial total sugar concentration, 5 g/L; and initial total concentration of amino acids, 0.5 g/L in the hydrolysates of LMBRs. The hydrolysates were sterilized using a sterile 0.45- μm membrane filter (Millipore Corporation, USA). The culture temperatures were regulated by water recycled in the outer layer of the photobioreactor (Figure 5.1) using a thermostatic water bath (501, Jintan Guowang Experimental Instrument Factory, Jiangsu Province, China). For substrate concentration experiments, the culture medium was composed of instant ocean synthetic sea salt, 34 g/L; and the cultures were cultured at $25\text{ }^{\circ}\text{C}$ at different substrate (sugar and amino acid) concentrations. A 10-L bubble column photobioreactor (25.0 cm in height, 8.0 cm in diameter, a closed system) was used with a working volume of 8 L. In all cases, *C. vulgaris* was inoculated at 1:10 (v/v) ratio into the photobioreactor. The initial biomass concentration of 0.10 g/L was used in all runs. The culture temperatures were regulated by water recycled in the outer layer of the photobioreactor. Ten fluorescent lamps were arranged around the photobioreactor to supply illumination of $300\text{ }\mu\text{mol photons/m}^2/\text{s}$ with a 12/12 h light/dark cycle. A gas sparger was located at the bottom of the reactor. The cultures were aerated with 0.6 vvm (volume gas per volume medium per minute) aeration of 3.0% sterile CO_2 prepared with the

combination of room air and pure CO₂ from a compressor. The initial pH of the medium for each run was adjusted to 6.5 with 0.5 M HCl or 0.5 M NaOH. The cultivation cycle of all runs was 10 days. Sample pH was directly determined using a Mettler Toledo Delta 320 pH meter (Mettler-Toledo, Greifensee, Switzerland). The cells were centrifuged at 1,600×g for 5 min at room temperature, washed with distilled water three times, and prepared for further analysis. After biomass harvesting, the culture media were used for the analysis of amino acids and sugars.

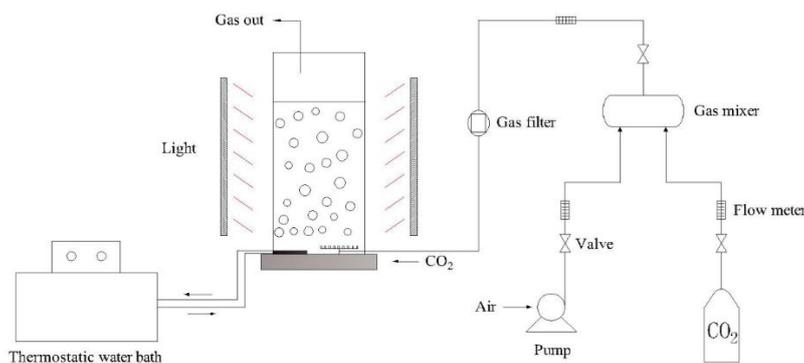


Figure 5.1: The photobioreactor.

5.2.3 Enzymatic hydrolysis of LMBRs

C. vulgaris biomass was obtained from a previous study (Zheng et al., 2011). The methods for preparing and hydrolyzing LMBRs from *C. vulgaris* biomass were the same as outlined by Zheng et al. (Zheng et al., 2012). A sample of suspension from the photobioreactor was concentrated using a centrifuge (1600g for 5 min). The pellets obtained were dissolved in the original suspension to attain the desired

biomass concentration of 100 g/L for cell disruption through cellulose hydrolysis (microalgal suspension pH adjusted to 4.8 with acetic acid before disruption). The cellulase concentration was 500.00 mg/L. The cells were broken at 55 °C in a water bath for 10 h and the lipids were extracted from the cell slurries according to the method section of gravimetric determination of total lipids and fatty acid profile analysis from the cell slurries. The aqueous phase was centrifuged at 4800g for 5 min, the pellet was washed with distilled water three times and LMBRs were obtained. Subsequently, LMBRs were subjected to a two-step enzymatic hydrolysis process. The purpose of the first step was to convert carbohydrates to sugars by dissolving LMBRs in distilled water to a volume of 1200 mL and enzymatically hydrolyzing them with the same cellulase under exactly the same conditions as the cell disruption process for a further 10 h. The second step involved the hydrolysis of proteins (pH adjusted to 6.0 with acetic acid before hydrolysis) with neutrase and alcalase both at concentration 500.00 mg/L. The hydrolysis was carried out at 60 °C in a water bath for 24 h. The enzymes were inactivated by placing the hydrolysates in a water bath at 100 °C for 5 min. The hydrolysates were centrifuged at 4800g for 5 min, and the supernatants were diluted 1:10 with ethanol prior to high performance liquid chromatography (HPLC) analysis of amino acids and sugars. The samples and mobile phase were filtered separately with a 0.45- μ m membrane filter.

5.2.4 Analytical methods

5.2.4.1 Analysis of sugars and amino acids

The xylose, glucose and arabinose analyzed by HPLC were presented by Yan et al. (Yan et al., 2009). Amino acids were also analyzed using the HPLC method as described by Zheng et al. (Zheng et al., 2012). Amino acids in the hydrolysates and culture medium were analyzed using a Dionex Ultimate 3,000 HPLC system equipped with a Sepax AA column (300×4.6 mm, 5 μm, Sepax Technologies, Inc., Delaware, USA). Amino acid standard solutions and the supernatants of the hydrolysates and culture medium were derivatized with phenylisothiocyanate using a slightly modified version of the method described by Henrikson and Meredith (Henrikson and Meredith, 1984). After 1 h of derivatization, 200 μL of hexane was added and mixed, and the mixture was left unagitated for 10 min, then the derivatives in the lower layer were filtered with a 0.45-μm membrane filter before injection (20 μL). HPLC analysis was carried out using a 4:1 (cyanomethane: water) mobile phase, a flow rate of 0.4 mL/min and a 36 °C column temperature. The amino acids were identified by comparison of the retention times with those of authentic standards. The quantification was based on the external standard method.

5.2.4.2 Measurement of microalgal cells and growth rates

The biomass concentration (Y, g/L) was measured by the spectrophotometer as described by Zheng et al. (Zheng et al., 2012) using the following equation:

$$Y=0.573X+0.062 \quad (R^2=0.995)$$

(1)

where X is the optical density measurements at 680 nm.

Specific growth rate μ (/d) was expressed as:

$$\mu = \frac{\ln(X_2 / X_1)}{t_2 - t_1}$$

(2)

where X1 (g/L) is the microalgal cell concentration at culture time t1 (d) and X2 (g/L) is that at culture time t2 (d).

5.2.4.3 Chlorophyll-a content measurement

The method for chlorophyll-a content measurement was the same as presented previously (Zheng et al., 2012). A sample of 10 mL *C. vulgaris* was centrifuged at 1,600×g for 5 min, washed with distilled water three times and the pellets were resuspended in 10 mL of acetone. The mixture was disrupted using a 600 W ultrasonic cell disintegrator (GA92-IID, Wuxi Shangjia Biotechnology Co., Ltd, Jiangsu Province, China) for 30 s with 5 s intervals for a total working time of 10 min followed by centrifugation at 4,800×g for 5 min. The relationship between chlorophyll-a concentration (Ca, mg/L) and the optical density (OD) was presented by Wellburn (Wellburn, 1994) as:

$$Ca = 15.65 \times OD_{666} - 7.34 \times OD_{653}$$

(3)

Chlorophyll-a content (mg/g) was calculated as:

$$\text{Chlorophyll-a content} = \frac{C_a}{\text{Biomass concentration}}$$

(4)

5.2.4.4 Nile red fluorescence determination of neutral lipids

The measurement of neutral lipids by the Nile red was a slight revision from that presented by Chen et al. (Chen et al., 2009). Dimethyl sulfoxide (DMSO) was added

to 5 mL of *C. vulgaris* suspension to attain a final concentration of 20% (v/v) prior to staining. Standard triolein (Sigma, USA) dissolved in acetone was used to create a calibration curve according to the above method. The fluorescence intensity measurements (F) were used to determine the concentrations of the neutral lipids (N, g/L) in the microalga quantitatively as below:

$$F=9245N+108 \quad (R^2=0.998) \quad (5)$$

Each sample of 10 mL *C. vulgaris* suspension was used for its fluorescence intensity measurement. Samples were properly diluted to give a desired biomass concentration (0.1-0.5 g/L). The neutral lipid content was expressed as:

$$\text{Neutral lipid content} = \frac{\text{Dry weight of the neutral lipids}}{\text{Biomass dry weight}} \times 100\%$$

(6) **5.2.4.5 Gravimetric determination of total lipids and fatty acid profile analysis**

Each 200-mL microalgal suspension sample with the pretreated LMBRs as the culture medium was disrupted by cellulase. The cell slurries were extracted with a mixture of hexane-methanol (1:1 v/v) with the samples in a proportion of 1:2 using a modified Bligh-Dyer method (Bligh and Dyer, 1959). The mixtures were shaken for 5 min in a separatory funnel, the organic phases were allowed to separate and evaporate at 30 °C using a rotary evaporator to obtain lipids. The weight of lipids from the organic phases was measured using an electronic scale. The total lipid content was calculated as:

$$\text{Total lipid content} = \frac{\text{Dry weight of the extracted lipids}}{\text{Biomass dry weight}} \times 100\% \quad (7)$$

The composition analysis of fatty acids was established using gas chromatography–mass spectrometry (GC–MS) (Thermo Finnigan, USA). The methylation of fatty acids was based on the method described by Metchalfe & Schmitz (Metchalfe and Schmitz, 1961). The GC–MS method was adopted from Ren et al. (Ren et al., 2010).

5.2.5 Experimental design and data analysis

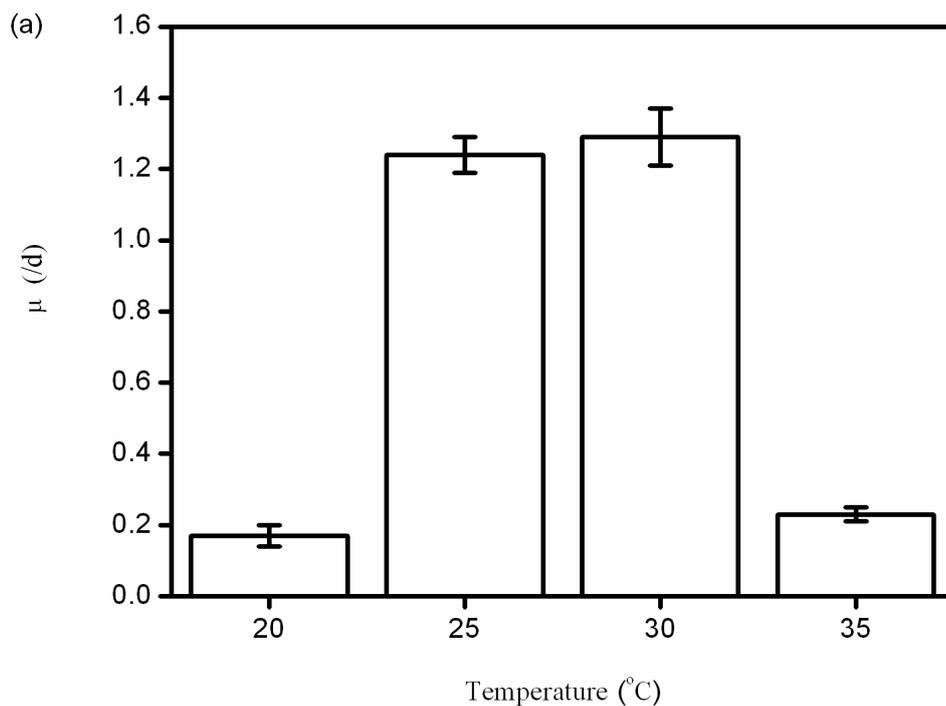
The experiment was designed and carried out at random. For temperature experiments, different temperature levels, including 20, 25, 30, and 35 °C were tested. The substrate concentration of the media was adjusted by addition of the enzymatic hydrolysates of LMBRs or distilled water for the substrate concentration experiment. The initial total sugar and amino acids concentrations at four different levels were 1 and 0.1, 5 and 0.5, 10 and 1.0, and 15 and 1.5 g/L, respectively. Analysis of variance (ANOVA) was applied to the generated data. When ANOVA indicated no less than one significantly different result, Duncan's multiple range tests (Duncan, 1955) were carried out. Statistical software SPSS 13.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the data from the temperature and substrate concentration experiments to identify differences.

5.3 Results and discussion

5.3.1 Effect of temperature and substrate concentration on cell growth

The mixotrophic growth of *C. vulgaris* with 5 g/L sugars and 0.5 g/L amino acids from the hydrolysates of LMBRs was tested at four temperatures (20, 25, 30, and 35 °C). Specific growth rates (μ) of the cells from the cultures at different temperatures

were calculated (Figure 5.2a). The mixotrophic growth of *C. vulgaris* was observed from 20 to 35 °C. The relatively higher μ up to 1.24 and 1.29 /d were obtained at the culture temperatures of 25 and 30 °C, respectively. However, the lower or higher culture temperature led to, a decrease of μ (0.17 /d at 20 °C, 0.23 /d at 35 °C), indicating lower temperatures (20 °C) or higher temperatures (35 °C) were not suitable for cell growth. The highest μ obtained in this study is 3 times that of the mixotrophic growth of *C. vulgaris* using CO₂ and hydrolyzed cheese whey solution (5 g/L glucose and 5 g/L galactose) as the carbon source (Abreu et al., 2012).



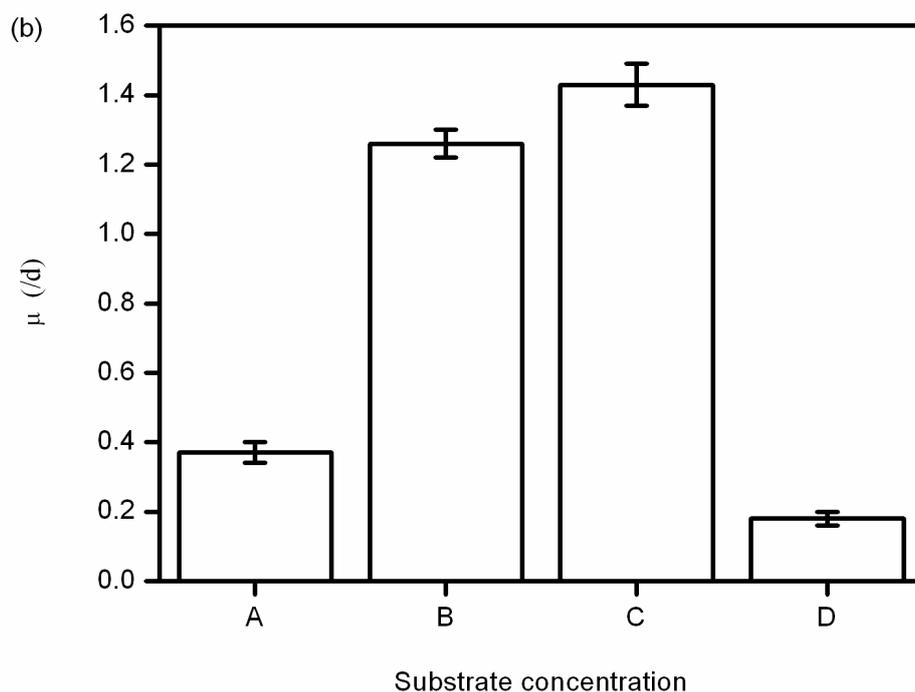


Figure 5.2: Specific growth rate (μ) of the mixotrophic growth of *C. vulgaris* (A. Initial total sugar concentration 1 g/L, initial total concentrations of amino acids 0.1 g/L; B. Initial total sugar concentration 5 g/L, initial total concentrations of amino acids 0.5 g/L; C. Initial total sugar concentration 10 g/L, initial total concentrations of amino acids 1.0 g/L; D. Initial total sugar concentration 15 g/L, initial total concentrations of amino acids 1.5 g/L; same for Figures 5.3, 5.4, & 5.6 and Tables 5.2, & 5.3 where applicable.)

Chlorophyll-a contents and pH value from different temperatures as a function of culture time were investigated. Table 5.1 shows that both chlorophyll-a content and pH increased during the initial 5 days for all temperatures, after which time a decrease was observed. The culture temperature of 30 °C had the maximum chlorophyll-a content (28 mg/g) on Day 5. The maximum pH of 9.9 was obtained at

30 °C. The culture with high chlorophyll-a content and pH had a high biomass concentration. Biomass concentrations of the cultures at different temperatures increased with increasing culture time except at 35 °C (Figure 5.3a). The culture at 35 °C reached a plateau stage after 5 days of cultivation and the cells decomposed afterwards. The results show that higher temperature (35 °C) prompts the microalga to reach a plateau stage and the microalga could not tolerate long-term high temperature. The relatively higher biomass concentrations up to 4.19 and 4.24 g/L were obtained at the temperatures of 25 and 30 °C, respectively. Compared with a previous study, biomass concentration of the mixotrophic growth of *C. vulgaris* using sugars and CO₂ in this work was twice that of autotrophic *C. vulgaris* using CO₂ (Zheng et al., 2011) and the optimal temperature for the mixotrophic growth of *C. vulgaris* was 25~30 °C. Microalgal growth was inhibited at 20 °C compared with the optimal growth temperature, possibly due to less photosynthetic light utilization efficiency at a lower temperature (Borowitzka, 1998). The optimal growth temperature of microalgae is possibly species-dependent, as that of the mixotrophic growth of *Chlorella sorokiniana* was 37 °C (Li et al., 2014).

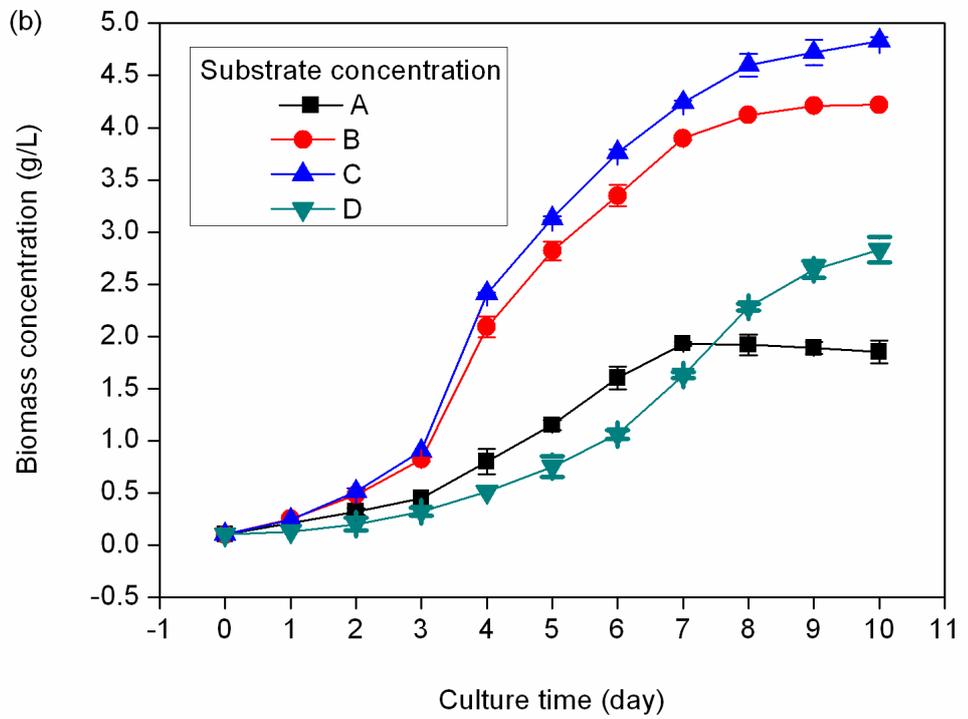
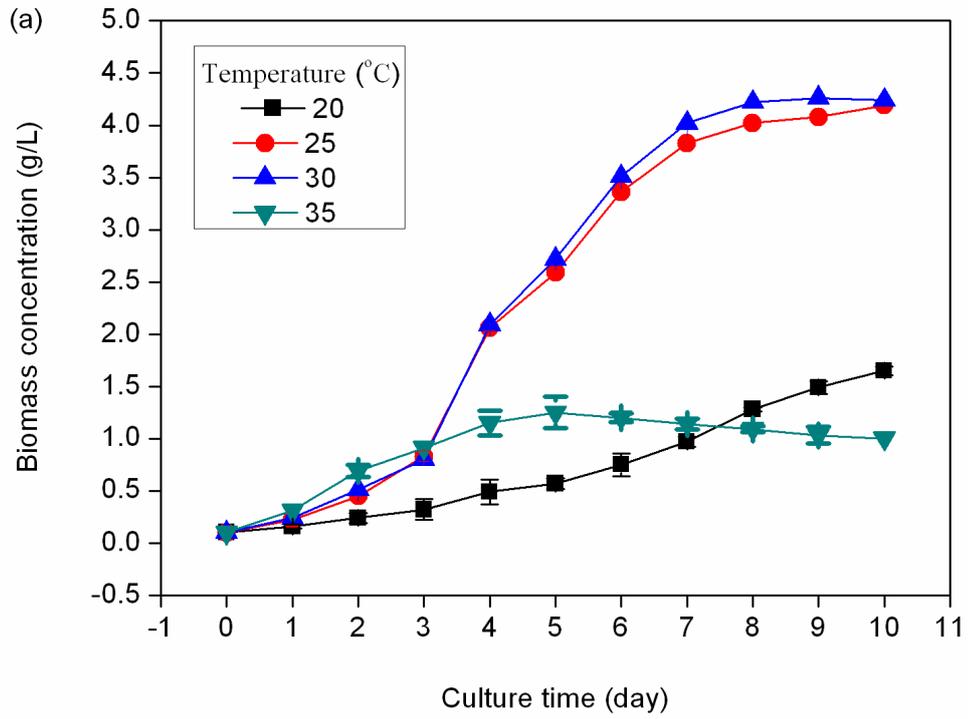


Figure 5.3: Biomass concentration of the mixotrophic growth of *C. vulgaris*

Table 5.1 Biomass concentration, chlorophyll-a content, total lipid content, and pH variation of the mixtrophic growth of *C. vulgaris* in response to different temperatures

| Parameters | Results | | | | | | | | | | | |
|------------------------------|----------|-----------|-----------|----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| | 20 | | | 25 | | | 30 | | | 35 | | |
| Culture time (day) | 0 | 5 | 10 | 0 | 5 | 10 | 0 | 5 | 10 | 0 | 5 | 10 |
| Biomass concentration (g/ L) | 0.10±0.0 | 0.57±0.07 | 1.65±0.00 | 0.10±0.0 | 2.59±0.09 | 4.19±0.00 | 0.10±0.00 | 2.72±0.10 | 4.24±0.05 | 0.10±0.00 | 1.25±0.10 | 1.00±0.00 |
| Chlorophyll-a content (mg/g) | 13.4±0.0 | 19.6±0.0 | 22.7±0.9 | 13.2±0.0 | 25.9±0.3 | 18.6±0.0 | 13.3±0.0 | 28.0±0.5 | 18.1±0.4 | 13.5±0.0 | 15.0±0.7 | 7.8±0.8 |
| pH | 6.5±0.0 | 8.2±0.5 | 7.8±0.4 | 6.5±0.0 | 9.7±0.8 | 9.0±0.7 | 6.5±0.0 | 9.9±0.3 | 9.2±0.5 | 6.5±0.0 | 7.7±0.9 | 6.1±0.6 |
| Total lipid content (%) | 15±0.0 | 18±0.0 | 28±1.0 | 16±0.0 | 19±0.4 | 35±0.0 | 15±0.0 | 20±0.5 | 37±1.0 | 16±0.0 | 21±0.7 | 15±0.8 |

Table 5.2 Biomass concentration, chlorophyll-a content, total lipid content, and pH variation of the mixotrophic growth of *C. vulgaris* in response to different substrate concentrations.

| Parameters | Results | | | | | | | | | | | |
|------------------------------|----------|-----------|-----------|----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| | A | | | B | | | C | | | D | | |
| Substrate concentration | | | | | | | | | | | | |
| Culture time (day) | 0 | 5 | 10 | 0 | 5 | 10 | 0 | 5 | 10 | 0 | 5 | 10 |
| Biomass concentration (g/ L) | 0.10±0.0 | 1.15±0.08 | 1.85±0.06 | 0.10±0.0 | 2.82±0.12 | 4.22±0.00 | 0.10±0.00 | 3.13±0.15 | 4.83±0.02 | 0.10±0.00 | 0.75±0.12 | 2.83±0.16 |
| | 0 | | | 0 | | | | | | | | |
| Chlorophyll-a content (mg/g) | 13.0±0.0 | 23.1±0.8 | 12.2±0.7 | 13.3±0.0 | 26.8±0.3 | 18.6±0.2 | 13.1±0.0 | 28.9±0.8 | 19.6±0.5 | 13.2±0.0 | 22.9±0.5 | 29.3±0.2 |
| pH | 6.5±0.0 | 8.5±0.6 | 7.1±0.3 | 6.5±0.0 | 10.2±0.9 | 9.5±0.5 | 6.5±0.0 | 9.8±0.6 | 9.1±0.3 | 6.5±0.0 | 7.5±0.6 | 8.9±0.5 |
| Total lipid content (%) | 15±0.0 | 21±0.2 | 38±0.7 | 16±0.0 | 18±0.2 | 36±0.0 | 15±0.0 | 18±0.4 | 34±0.5 | 15±0.0 | 16±0.3 | 22±0.6 |

The mixotrophic growth of *C. vulgaris* was cultivated at 25 °C using sugars and amino acids from the hydrolysates of LMBRs in different concentrations. Eighteen varieties of amino acids and three sugars were used in this study (Zheng et al., 2012). The effect of substrate concentration on the microalgal growth is shown in Figure 5.2b & 3b and Table 5.2. The optimal substrate concentration was 10 g/L sugar and 1.0 g/L amino acids from the hydrolysates of LMBRs with a maximum μ of 1.43 /d, a maximum chlorophyll-a content of 28.9 mg/g and a maximum biomass concentration of 4.83 g/L. The μ , maximum chlorophyll-a content and maximum biomass concentration of mixotrophic growth of *C. vulgaris* all increased before they decreased along with increasing substrate concentration. Microalgal growth cultured with 1 g/L sugars and 0.1 g/L amino acids from the hydrolysates of LMBRs showed a decrease compared with microalgal growth cultured with 10 g/L sugar and 1.0 g/L amino acids. This may be due to the lower concentration of substrate in the medium, and thus inefficiency in its supply of carbon and nitrogen sources for microalgal growth. The present results showed that initial total sugar concentration above 10 g/L and initial total concentration of amino acids above 1.0 g/L caused substrate inhibition of the mixotrophic growth of *C. vulgaris*. Substrate inhibition was also observed in the mixotrophic growth of *C. vulgaris* cultivated with diluted monosodium glutamate wastewater (Ji et al., 2014).

5.3.2 Effect of temperature and substrate concentration on substrate consumption

The substrate consumption of the mixotrophic growth of *C. vulgaris* at different temperatures is shown in Figure 5.4a. Temperature had a significant effect ($P<0.05$) on sugar and amino acid consumption. Consumption of sugars increased significantly

($P < 0.05$) with increasing temperature up to 30 °C and decreased significantly ($P < 0.05$) above 30 °C. These observations are in line with assimilation of sugars starting at hexose phosphorylation catalyzed by hexokinase, the activity of which was decreased at a lower temperature (20 °C) or higher temperature (35 °C) (Guan et al., 2011). Amino acids of the test cultures at 25 and 30 °C were exhausted but residual amino acids at 20 and 35 °C were 0.15 and 0.21 g/L, respectively, showing that amino acids assimilation of the mixotrophic growth of *C. vulgaris* was inhibited at temperature stress (20 or 35 °C). The cultures at 25 and 30 °C possibly have more suitable transport systems for amino acids than those at lower temperatures (20 °C) or higher temperatures (35 °C) (Liu and Hellebust, 1974). Amino acids are considered a less preferable nitrogen sources for microalgae (Perez-Garcia et al., 2011). However, amino acids combined with CO₂ and sugars as a substrate in this work showed good performance. Glucose induces microalgae to express two transport systems for the transfer of amino acids through membranes (Perez-Garcia et al., 2011). Moreover, the eighteen amino acids contained in the enzymatic hydrolysates from LMBRs may serve as buffer agents, nitrogen and carbon sources simultaneously, in the culture of microalgae, and this may work better than a single nitrogen source. Carbon and nitrate assimilation of microalgae inhibited by temperature stress is also found in a report by Converti et al. (Converti et al., 2009).

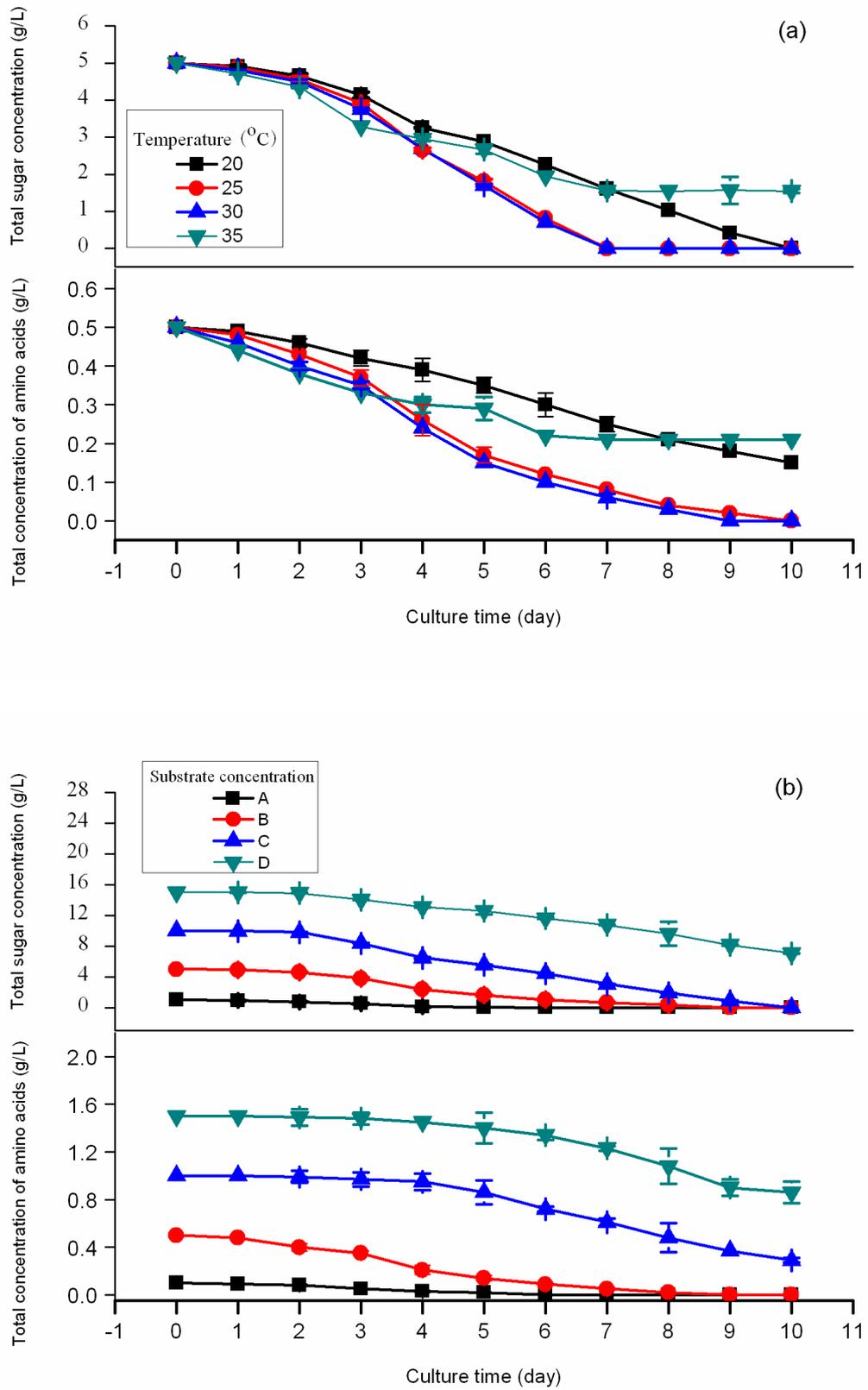


Figure 5.4: Substrate consumption of the mixotrophic growth of *C. vulgaris*

Sugars and amino acids from the enzymatic hydrolysates of LMBRs were used as substrates for the mixotrophic growth of *C. vulgaris* at 25 °C. Substrate concentration had a significant effect ($P<0.05$) on substrate consumption (Figure 5.4b). After 10 days of cultivation, sugar (15 g/L) and amino acid (1.5 g/L) concentrations of the test culture maintained concentrations of 7.05 and 0.86 g/L, respectively. This indicated that substrate inhibition of the mixotrophic growth of *C. vulgaris* occurred at concentrations of 15 g/L sugar and 1.5 g/L amino acid. Similar findings were obtained in autotrophic *Neochloris oleoabundans* using 20 mM sodium nitrate as a substrate (Li et al., 2008). Compared with those cultured with 10 g/L sugars and 1.0 g/L amino acids by Day 3 (Figure 5.3b), substrate inhibition of the culture with 15 g/L sugars and 1.5 g/L amino acid concentrations was obvious. However, the culture with 15 g/L sugars and 1.5 g/L amino acid showed better growth at the later stage. Both sugars and amino acids of the test culture with 1 g/L sugars and 0.1 g/L amino acids were consumed almost fully by Day 6. The results showed that both sugars and amino acids were exhausted earlier at lower concentrations of sugars and amino acids. Glucose, xylose, and arabinose accounted for 62%, 6%, and 32% of the total sugars, correspondingly, in the enzymatic hydrolysates. The corresponding concentrations of the three kinds of sugars had similar variation tendency with those of the total sugars during culture time for all runs (data not shown).

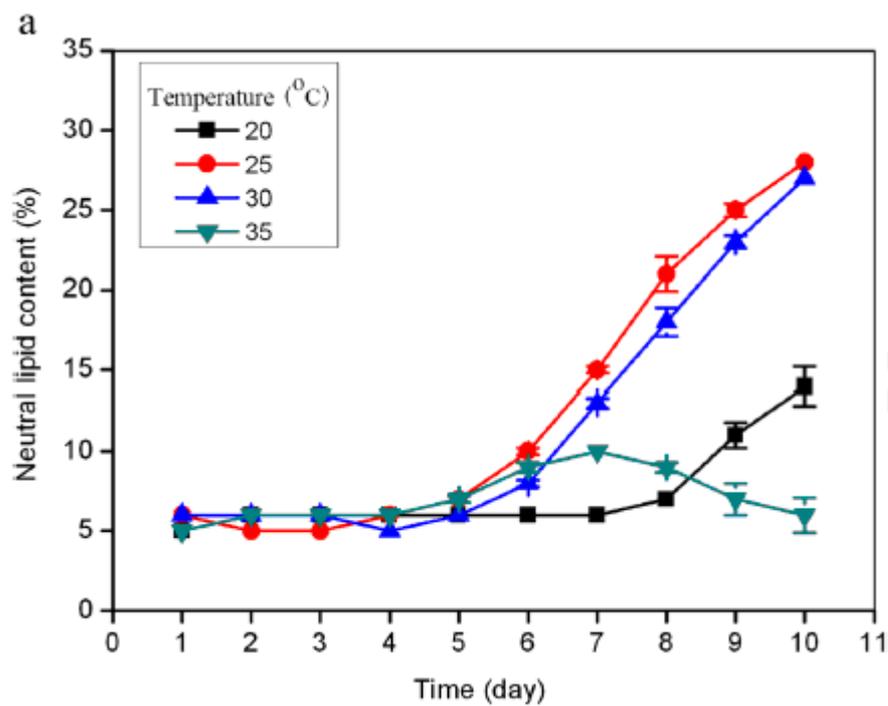
5.3.3 Effect of temperature and substrate concentration on lipid accumulation

Previous studies have reported that microalgae generally accumulate neutral lipids (triglycerides), which can easily be converted into biodiesel (Rawat et al., 2013; Wang et al., 2013; Hu et al., 2008). Thus, neutral lipid content can be applied to assess the potential of the microalgal lipids as an excellent biodiesel feedstock. In

this study, neutral lipid content of the cultures at different temperatures was measured by Nile red. Nile red measurement of neutral lipids is quick and has a high-throughput (Chen et al., 2009). The applications of Nile red staining in microalgal production include: (1) screening microalgal strains with a high lipid content (Thi et al., 2011), (2) creating a calibration curve between crude lipid content and Nile red fluorescence for autotrophic *Nannochloropsis oculata* to quantify lipid content (Chiu et al., 2009), (3) creating a calibration curve between standard triolein and Nile red fluorescence for autotrophic *C. vulgaris*, autotrophic *Nannochloris* sp., etc. to quantify neutral lipid content (Chen et al., 2009). However, using Nile red to determine neutral lipids in the mixotrophic growth of microalga cultured using LMBRs, known to be more complicated than the commonly used synthetic medium, has not yet been reported.

Lipid accumulation by the mixotrophic growth of *C. vulgaris* using 5 g/L sugars and 0.5 g/L amino acids in the enzymatic hydrolysates from LMBRs in the cultures at different temperatures is shown in Figs. 5a & b. It has been confirmed that amino acids promote lipid accumulation of microalgae due to their ability in offering a suitable environment to maintain high metabolic efficiency (Biel et al., 2007). In all the experiments, temperature had no significant effect ($P>0.05$) on neutral and total lipid content at the early growth stage. However, temperature affected neutral lipid and total lipid contents significantly ($P<0.05$) at the late growth stage (Figure 5.5a & b). Amino acids were utilized as a nitrogen source and at the end of the culture, their total concentrations were relatively low due to the fast uptake by the microalga. Results in Figure 5.4a may indicate that the mixotrophic growth of *C. vulgaris* was nitrogen-limiting at this stage. Neutral and total lipid contents at the end of the culture may be increased by nitrogen limitation. Similar findings were also reported

by Daroch et al. & Molina Grima et al. (Wang et al., 2013; Molina Grima et al., 1996). After 10 days of culture in temperature experiments, the culture at 35 °C had the lowest neutral and total lipid contents of 6 and 15%, respectively. And the culture at 25 °C had the highest neutral lipid content of 28% while the culture at 30 °C had the highest total lipid content of 37%. Calculated by biomass concentration (Figure 5.3a) and total lipid content (Figure 5.5b), the lipid productivity at 25 and 30 °C were 147 and 156 mg/L/d, respectively.



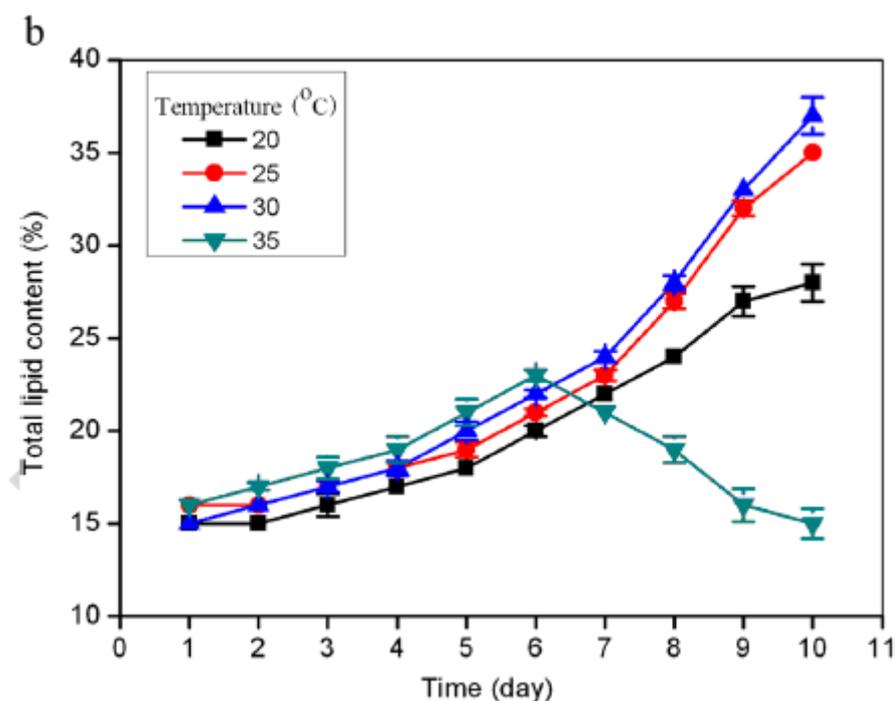


Figure 5.5: Lipid accumulation during the mixotrophic growth of *C. vulgaris* culture at different temperatures

The neutral lipids of the culture at 25 °C accounted for as much as 82% of the total lipids in the microalga by culture Day 8. It was the maximum ratio of neutral lipids to total lipids in the temperature experiments. The minimum ratio of neutral lipids to total lipids of 27% was obtained at 20 °C on culture Day 7. Lower temperature (20 °C) was not beneficial to accumulate neutral lipids (Figure 5.5a). Lower or higher temperature reduces carbon metabolism (Masojć et al., 2004) and nitrogen assimilation (Figure 5.4a). The cultures were under nitrogen sufficient conditions; therefore neutral lipid accumulation was inhibited. Both neutral and total lipid contents of the culture at 35 °C decreased significantly ($P < 0.05$) during the late growth stage.

The composition of dominant fatty acids of the mixotrophic growth of *C. vulgaris* at different temperatures was measured by GC-MS (Table 5.3). The common eight kinds of fatty acids were confirmed, but their percentages changed. C16 and C18

were the dominant fatty acids in the mixotrophic growth of microalga at different temperatures. When the cultivation temperature increased, saturated fatty acids increased, which confirmed the results of autotrophic *Scenedesmus* sp. obtained by Li et al. (Li et al., 2011). Unsaturated fatty acids increased with decreasing temperature. Greater degree of unsaturation could increase membrane fluidity to adapt to low temperature (Hu et al., 2008). In particular, more than 25% of the fatty acids in the mixotrophic growth of *C. vulgaris* at different temperatures were saturated fatty acids. The lipids with ~17% of saturated fatty acids content obtained from soybean oil meet the US Standard Specification for Biodiesel Fuel (ASTM D6751) (Fallen et al., 2011). A previous report showed that higher proportions of saturated fatty acids can result in larger cetane number (indicating shorter ignition delay) in biodiesel, reduced NO_x exhaust emissions, and higher oxidative stability (Bello et al., 2012). Therefore, the fatty acids from the mixotrophic growth of *C. vulgaris* are a potentially good source of high quality biodiesel, but more detailed investigations are needed to further validate it.

Table 5.3 Composition of fatty acids (percentage of total fatty acids) of the mixotrophic growth of *C. vulgaris* at different temperatures and substrate concentrations

| Fatty acids | Factors | | | | | | | |
|-----------------|------------------|------------|------------|------------|-------------------------|------------|------------|------------|
| | Temperature (°C) | | | | Substrate concentration | | | |
| | 20 | 25 | 30 | 35 | A | B | C | D |
| C14:0 | 2.53±0.11 | 3.27±0.25 | 2.85±0.17 | 2.92±0.19 | 3.15±0.20 | 3.01±0.12 | 4.25±0.17 | 5.17±0.25 |
| C16:0 | 16.66±0.40 | 18.23±0.52 | 19.95±0.86 | 22.74±0.37 | 17.11±0.32 | 20.55±0.49 | 21.38±0.68 | 20.51±0.33 |
| C16:1 | 27.60±0.72 | 25.38±0.85 | 27.02±0.90 | 24.92±0.81 | 26.41±0.53 | 23.58±0.36 | 24.49±0.59 | 24.16±0.62 |
| C16:2 | 4.73±0.56 | 4.03±0.22 | 2.18±0.16 | 4.15±0.55 | 4.19±0.28 | 3.26±0.07 | 4.35±0.10 | 4.04±0.18 |
| C18:0 | 2.04±0.11 | 1.35±0.00 | 2.32±0.21 | 1.59±0.13 | 1.41±0.05 | 1.87±0.04 | 2.33±0.09 | 2.53±0.08 |
| C18:1 | 37.61±0.62 | 39.56±0.91 | 37.62±0.75 | 35.78±0.57 | 39.33±0.86 | 38.68±0.75 | 34.24±0.44 | 34.62±0.70 |
| C18:2 | 4.25±0.26 | 3.88±0.24 | 4.20±0.18 | 4.42±0.13 | 4.89±0.33 | 5.71±0.22 | 4.66±0.13 | 5.11±0.27 |
| C20:0 | 4.58±0.15 | 4.30±0.29 | 3.86±0.38 | 3.48±0.16 | 3.51±0.20 | 3.34±0.11 | 4.30±0.08 | 3.86±0.06 |
| C16 fatty acids | 48.99±0.56 | 47.64±0.75 | 49.15±0.61 | 51.81±0.52 | 47.71±0.40 | 47.39±0.57 | 50.22±0.91 | 48.71±0.82 |
| C18 fatty acids | 43.90±0.83 | 44.79±0.64 | 44.14±0.86 | 41.79±0.44 | 45.63±0.41 | 46.26±0.38 | 41.23±0.32 | 42.26±0.25 |
| Σ sat | 25.81±0.40 | 27.15±0.52 | 28.98±0.68 | 30.73±0.57 | 25.18±0.63 | 28.77±0.64 | 32.26±0.53 | 32.07±0.44 |
| Σ monounsaturat | 65.21±0.73 | 64.94±0.92 | 64.64±0.86 | 60.70±0.69 | 65.74±0.85 | 62.26±0.24 | 58.73±0.84 | 58.78±0.35 |
| Σ polyunsaturat | 8.98±0.38 | 7.91±0.53 | 6.38±0.64 | 8.57±0.72 | 9.08±0.55 | 8.97±0.16 | 9.01±0.27 | 9.15±0.23 |

Lipid accumulation of the mixotrophic growth of *C. vulgaris* at 25 °C using sugars and amino acids in the hydrolysates of LMBRs at different concentrations is shown in Figure 5.6a and b. The culture with 1 g/L sugars and 0.1 g/L amino acids had higher neutral and total lipid contents than others. Of all the tests, the culture with 15 g/L sugars and 1.5 g/L amino acids had the lowest neutral and total lipid contents. As shown in Figure 5.4b, the test culture with 15 g/L sugars and 1.5 g/L amino acid had 7.05 g/L sugars and 0.86 g/L amino acids residual at the end of cultivation. However, sugars and amino acids of the three other test cultures were almost exhausted. The results indicated that the three test cultures were nitrogen limitation. Neutral lipid accumulation is often triggered by nitrogen limitation (Wang et al., 2013; Hu et al., 2008). Though there were almost no sugars for the three cultures at the end of culture, microalga could still use the aerated CO₂ as a carbon source to synthesize lipids. The maximum ratio of neutral lipids to total lipids of 87% in the substrate concentration experiments was obtained at 1 g/L sugars and 0.1 g/L amino acids on culture Day 9. The results indicated that lower concentration of substrate (sugar and amino acid) stimulated neutral lipids accumulation. In the substrate concentration experiments, neutral lipid content increased slightly in the initial 5 days and increased significantly ($P<0.05$) in the last 5 days. Total lipid content increased with increasing culture time. The results indicated that total lipid content increased at the late growth stage was mainly induced by neutral lipid accumulation. After 10 days of culture in substrate concentration experiments, the culture with 1 g/L sugars and 0.1 g/L amino acids had the highest neutral and total lipid contents of 32 and 38%, respectively. The total lipid content of different substrate concentrations.

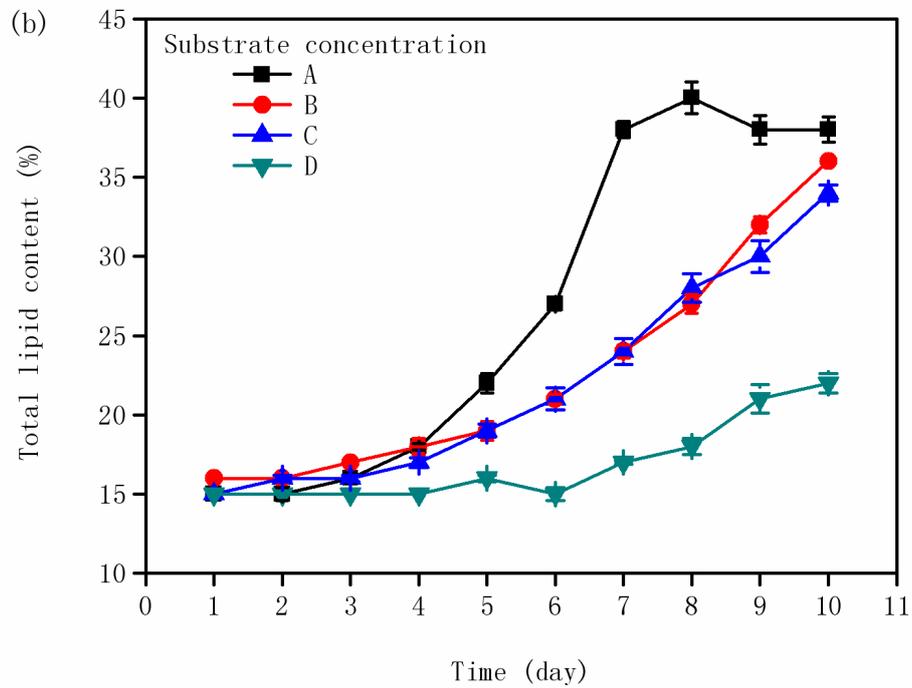
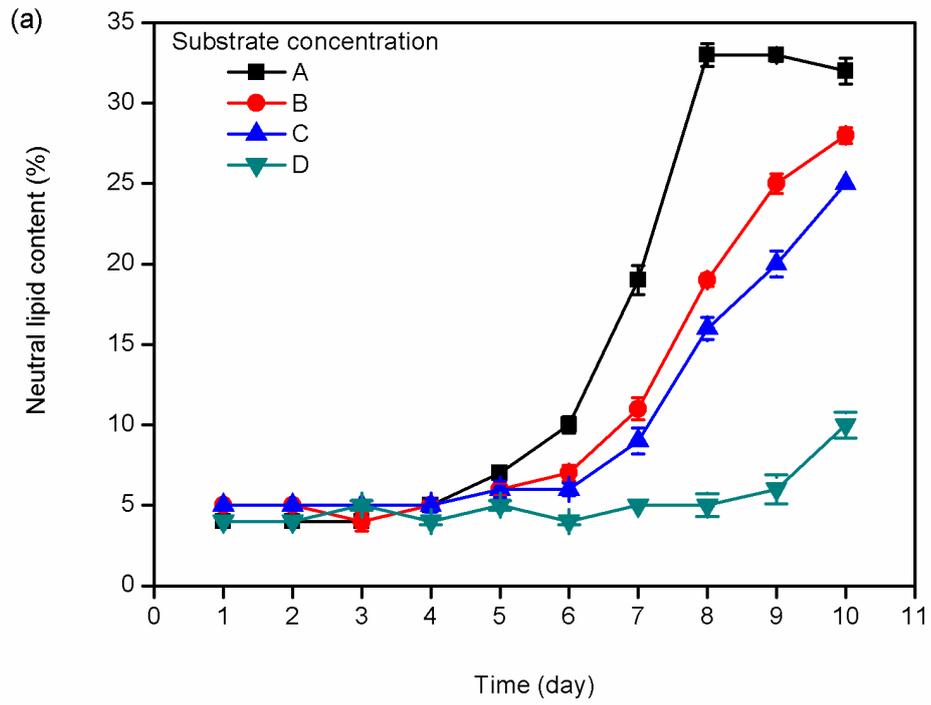


Figure 5.6: Lipid accumulation during the mixotrophic growth of *C. vulgaris* culture at different substrate concentrations

The mixotrophic growth of *C. vulgaris* using sugars and CO₂ in this work was higher than that of autotrophic *C. vulgaris* using CO₂ (Zheng et al., 2011). Similar results reported by Miao and Wu indicated that the lipid content of the heterotrophic growth of *Chlorella protothecoides* using glucose is much higher than that of autotrophic microalga (Miao and Wu, 2006). The culture with 15 g/L sugars and 1.5 g/L amino acids had the lowest neutral and total lipid contents of 10 and 22%, respectively. The optimal substrate concentration was 10 g/L sugars and 1.0 g/L amino acids leading to the maximum lipid productivity of 164 mg/L/d.

The common eight types of fatty acids were identified using the GC-MS system, and little difference in the relative content of the fatty acids from the cultures with different substrate concentration was observed (Table 5.3). The contents of unsaturated fatty acids of the cultures ranged from 67.74 to 74.82%. And the contents of saturated fatty acids of the cultures ranged from 25.18 to 32.26%. Lower sugar and amino acid concentration stimulated the accumulation of unsaturated fatty acids. In particular, lipids of the cultures at different temperatures had similarly high saturated fatty acid content. Therefore, fatty acids from the mixotrophic growth of *C. vulgaris* under different substrate concentrations are also likely suitable for the production of good-quality biodiesel.

5.4 Conclusion

Sugars and amino acids in the enzymatic hydrolysates from LMBRs were successfully used as nutrient for microalgal cultivation and lipid production. Lipid production by the mixotrophic growth of *C. vulgaris* using the obtained sugars and amino acids were shown to be largely dependent on temperature and substrate concentration. The mixotrophic growth of *C. vulgaris* achieved a high biomass

concentration and high lipid productivity, which would facilitate downstream processing and reduce production waste. The extracted lipids were probably suitable to be used as feedstock for the production of good-quality biodiesel. As an alternative resource, LMBRs was confirmed to be a recyclable feedstock for the mixotrophic growth of *C. vulgaris* in this study, nevertheless, further research is needed to disclose the more clear composition of the enzymatic hydrolysates of LMBRs and reduce the cost of enzymes used, and to further prepare biodiesel using the extracted lipids to evaluate its actual properties.

CHAPTER 6. Cultivation of *Chlorella vulgaris* in wastewater with waste glycerol: Strategies for improving nutrient removal and enhancing lipid production

Summary

To improve nutrient removal from wastewater and enhance microalgal lipid production, cultivation of *Chlorella vulgaris* in wastewater with waste glycerol generated from biodiesel production using scum derived oil as feedstock was studied. The results showed that nutrient removal was improved and lipid production of *C. vulgaris* was enhanced with the addition of waste glycerol into the synthetic wastewater to regulate its C/N ratio. The optimal concentration of the pretreated glycerol for *C. vulgaris* was 10 g L⁻¹ to achieve the biomass concentration of 2.92 g L⁻¹, lipid productivity of 163 mg L⁻¹ d⁻¹, and the removal of 100% ammonia and 95% of total nitrogen. Alkaline conditions prompted cell growth and lipid accumulation of *C. vulgaris* while stimulating nutrient removal. The application of the integration process can lower both wastewater treatment and biofuel feedstock costs.

6.1 Introduction

In the United State, an estimated 6.2 million wet tons of scum are generated in the wastewater treatment plant, which is a floatable material skimmed from the surface of primary and secondary settling tanks (Kargbo, 2010). Scum contained a variety of wastes, such as animal fat, waste cooking oil, food wastes, plastic material, soaps, and many other impurities discharged from restaurants, households, and other facilities. Previous studies show that 70% of dried and filtered scum could be

converted to biodiesel (Bi et al., 2015), and biodiesel production generates about 18% (w/w) glycerol as the main byproduct (Yang et al., 2012). Thus, about 0.5 million tons of glycerol will be generated in the United States each year by using scums as feedstock for biodiesel production. A technology needs to be developed to make full use of glycerol generating from biodiesel production using scum as oil feedstock to reduce the biodiesel production cost and environmental impact.

The previous research (Chapter 3) shows that algae consume organic carbon source in the centrate wastewater quickly while relatively large amount of N and P remain in the culture broth. In order to completely utilize and remove N and P from the wastewater, carbon source may be replenished through addition of glycerol from the scum to biodiesel conversion process. Additionally, photo deficiency is problematic for autotrophic dominated growth of microalgae at high cell concentration (Ma et al., 2014). Compared with the photoautotrophic cultivation, a hetero-photoautotrophic two-stage cultivation process could improve wastewater nutrient removal and enhance algal lipid accumulation (Zhou et al., 2012). To further improve nutrient removal to satisfy wastewater discharge standards and decrease biofuel production cost, heterotrophic dominated growth of microalgae is explored instead of autotrophic dominated growth of microalgae. The integration of biofuel production and microalgae-based wastewater treatment with the addition of organic carbon sources into wastewater is a viable means. However, using crude glycerol from waste oil-based biodiesel production as supplemental organic carbon sources to develop a heterotrophic dominated microalgae culturing mode for efficiently coupling wastewater treatment and low-cost biofuel production, to the best of our knowledge, has not yet been reported.

To prevent the effect of the differences among batches of concentrated municipal

wastewater for lipid production and nutrient removal, synthetic wastewater simulating concentrated municipal wastewater (Zhou et al., 2011), was used as surrogate in this study followed by real wastewater as verification. The aim of the present study was to develop the integration of microalgal cultivation with wastewater treatment by supplying glycerol generating from biodiesel production using scum as oil feedstock to enhance nutrient removal and lipid production and improve the economic viability of scum-based biodiesel production. Furthermore, the obtained lipids were analyzed to evaluate its potential as biodiesel feedstock.

6.2 Materials and methods

6.2.1. Materials

Crude glycerol is the byproduct of biodiesel production from scum using potassium methoxide-catalyzed transesterification (Bi et al., 2015). It is a dark brown moderately thick liquid after the removal of methanol with a viscosity comparable to molasses. The liquid separated into two layers, a small top layer referred to as the “rag layer” composed of polar impurities and soap, and the bottom glycerol layer.

6.2.2. Microalgal strain and culture conditions

Chlorella vulgaris UTEX 2714 was purchased from the Culture Collection of Algae at the University of Texas. The strain was preserved in Tris–Acetate–Phosphorus (TAP) media (Harris, 1989), which was composed of (mg L⁻¹): NH₄Cl 400, MgSO₄ · 7H₂O 100, CaCl₂ · 2H₂O 50, K₂HPO₄ 108, KH₂PO₄ 56, Tris (hydroxymethyl) aminomethane 2420, glacial acetic acid 1, 1 mL L⁻¹ trace elements solution consisted of 50 g L⁻¹ Na₂EDTA, 22 g L⁻¹ ZnSO₄ · 7H₂O, 0.05 g L⁻¹ CaCl₂ · 2H₂O, 11.4 g L⁻¹ H₃BO₃, 5.06 g L⁻¹ MnCl₂ · 4H₂O, 4.99 g L⁻¹ FeSO₄ · 7H₂O, 1.61 g L⁻¹ CoCl₂ · 6H₂O,

1.57 g L⁻¹ CuSO₄ · 5H₂O, 1.10 g L⁻¹ (NH₄)₆Mo₇O₂₄ · 4H₂O, and 16 g L⁻¹ KOH.

The experiments was carried out in synthetic wastewater, which was composed of (mg L⁻¹): NH₄Cl 230, MgSO₄ · 7H₂O 400, CaCl₂ · 2H₂O 100, K₂HPO₄ 200, KH₂PO₄ 50, Na₂HPO₄ 100, NaNO₃ 100, NaCOOCH₃ 100, 1 mL L⁻¹ trace elements solution consisted of 50 g L⁻¹ Na₂EDTA, 22 g L⁻¹ ZnSO₄ · 7H₂O, 11.4 g L⁻¹ H₃BO₃, 5.06 g L⁻¹ MnCl₂ · 4H₂O, 4.99 g L⁻¹ FeSO₄ · 7H₂O, 1.61 g L⁻¹ CoCl₂ · 6H₂O, 1.57 g L⁻¹ CuSO₄ · 5H₂O, 1.10 g L⁻¹ (NH₄)₆Mo₇O₂₄ · 4H₂O and 16 g L⁻¹ KOH.

An experiment to determine the effects of waste glycerol on nutrients removal and lipid production from synthetic wastewater by *C. vulgaris* was carried out. Synthetic wastewater with different concentrations of crude glycerol (0, 1, 5 and 10 g L⁻¹), pretreated glycerol (1, 5, 10 and 15 g L⁻¹), and pure glycerol (25 g L⁻¹) was tested at initial pH 7.0. The initial pH values of the media for all runs except initial pH experiment were adjusted to 7.0 with 0.5 M HCl or 0.5 M NaOH. For the pH value experiment, the initial pH values of the media were adjusted to 5.0, 7.0, and 9.0 with 10 g L⁻¹ pretreated glycerol. The media were autoclaved at 121 °C for 30 min. Microalga was cultured in 250 mL Erlenmeyer flasks containing 150 mL media. In all cases, *C. vulgaris* was inoculated at 1:10 (v/v) ratio into the flask. The cultivation of *C. vulgaris* was carried out with continual illumination of 50 μmol photons m⁻² s⁻¹. The initial biomass concentration was about 0.10 g L⁻¹ in all runs. All treatments were cultivated for 7 days at 25 ± 2 °C.

6.2.3. Pretreatment of crude glycerol

Crude glycerol was pretreated to remove soaps with the hydrochloric acid method as described in a previous study (Pyle et al., 2008). Crude glycerol was mixed with

distilled water at a ratio of 1/4 (v/v) to decrease the viscosity of the fluid. Sample pH of crude glycerol was adjusted to 3.0 using 2 M HCl. The whole content was then centrifuged at $4,800 \times g$ for 20 min. The top layer which was dark was mainly free fatty acids. Glycerol was in the obtained bottom layer.

6.2.4. Analytical methods

6.2.4.1. Crude glycerol analysis

The sample of 1 mL was dried to constant weight in an oven at 105 °C for 24 h to evaluate the moisture content. A 1 mL sample was dried to constant weight in an oven at 550 °C for 4 h to evaluate the ash content. The crude glycerol was measured using an electronic scale and a graduated cylinder. The crude glycerol density (g/mL) is the glycerol weight per unit volume.

The amount of catalyst in the crude glycerol was determined as follows: the solvent solution was prepared by mixing 55 mL of acetone with 5 mL of distilled water and 5 drops of phenolphthalein. Then the acids in the solvent solution were neutralized according to a previous study (Frankel et al., 1965) by addition of a small amount of 0.1 N KOH until the solution turned light pink. Each sample of 0.7 g crude glycerol was put into the neutralized solution. The solution would turn pink if there was catalyst present. It was titrated with 0.1 N HCl until the solution turned clear. The catalyst content was calculated by multiplying the volume of the titrant required by its normality and its molecular weight; then dividing by 1000 mg g⁻¹ and the mass of the crude glycerol sample used. The soap content experiment was performed using 0.1 N HCl in conjunction with the catalyst content. Once the catalyst had been completely titrated, 4 drops of Bromophenol blue indicator were added to the same sample and the solution was titrated to the Bromophenol blue end point (yellow

color). The difference between the catalyst end point and the Bromophenol blue yellow end point was the amount of soap present. The soap content was calculated by multiplying the volume of the titrant required by its normality and its molecular weight; then dividing by 1000 mg g^{-1} and the mass of the crude glycerol sample used. The micro-nutrient (metal ion) profiles of the crude glycerol were analyzed at the Earth Science Geology and Geophysics Lab at the University of Minnesota using an inductively coupled plasma atomic emission spectrometer (ICP-AES, Perkin Elmer Optima 3000, the United States).

6.2.4.2. Measurement of glycerol concentration

To determine the glycerol concentration in the crude glycerol and the medium, the samples were analyzed by a spectrophotometric method as described in Bondioli & Bella's study (2005). Each sample of the crude glycerol was diluted 500 times with distilled water. Ethanol (47.5%, v/v) of 5 mL was added to 5 mL of the above diluted sample. The resulting solution was mixed with 1.2 mL of 0.2 M acetylacetone solution and 1.2 mL of 10 mM sodium periodate solution. Test tube with the above mixture was at $70 \pm 2 \text{ }^\circ\text{C}$ in a water bath for 1 min; then immediately cooled to $20 \pm 2 \text{ }^\circ\text{C}$ using a water bath. Finally the absorbance of the solution was determined at 410 nm by using the spectrophotometer.

6.2.4.3. Measurement of biomass concentration and composition

A volume of 5 mL microalgal suspension was taken from the well-mixed culture broth in the flasks and filtered through a glass microfiber filter (Whatman, GE Healthcare Bio-Sciences, the United States) followed by drying the microalgal pellets in the dry oven at $105 \text{ }^\circ\text{C}$ over-night. Cell dry weight was measured with an electronic scale after drying. Specific growth rate $\mu \text{ (d}^{-1}\text{)}$ was expressed as:

$$\mu = \frac{\ln(X_2 / X_1)}{t_2 - t_1} \quad (1)$$

where X_1 (g L^{-1}) is the microalgal cell concentration at culture time t_1 (day) and X_2 (g L^{-1}) is that at culture time t_2 (day).

6.2.4.4. Lipid extraction and fatty acid analysis

After 7-day cultivation, total carbohydrates in *C. vulgaris* cells were analyzed according to the phenol-sulphuric acid method (Ben-Amotz et al., 1985). Protein contents in *C. vulgaris* cells (dry biomass) were determined from the nitrogen content data evaluated with a CE-440 elemental analyzer (Exeter Analytical Inc., North Chelmsford, the United States), using the nitrogen-to-protein conversion factor of 6.35 (Safi et al., 2013). Each sample of 0.2 g microalga was disrupted by cellulase. Microalgal suspension pH was adjusted to 4.8 by acetic acid before cell disruption. The cellulase concentration was 500.00 mg L^{-1} . The cells were broken at $55 \text{ }^\circ\text{C}$ in a water bath for 10 h. Then the lipids were extracted using a chloroform-methanol method as described in a previous study (Zheng et al., 2011). The weights of the obtained lipids were determined with an electronic scale.

The extracted lipids of 0.1 mL were analyzed by gas chromatography–mass spectrometry (GC-MS) (Thermo Finnigan, the United States). Prior to GC-MS analysis, the methylation of fatty acids with boron trifluoride and methanol was carried out at $100 \text{ }^\circ\text{C}$ for 120 min (Doan & Obbard, 2012). Methylated fatty acids were dissolved in 2 mL hexane and dehydrated by 0.1 g anhydrous sodium sulfate for 30 min, followed by 1.0 μL injection for GC-MS analysis. The GC-MS method was according to Doan & Obbard's study (2012).

6.2.4.5. Nutrient analysis

The samples for nutrient analysis were first centrifuged at 2044g for 10 min and then

the supernatants were collected and properly diluted and analyzed for total phosphorus (TP), ammonium ($\text{NH}_4^+\text{-N}$), total nitrogen (TN), and chemical oxygen demand (COD) following the Hach DR 5000 Spectrophotometer Manual (Hach, 2008). Removal rates ($\text{mg L}^{-1} \text{d}^{-1}$) for the above nutrients were calculated by dividing the difference between the first time (t_3, d) and second time (t_4, d) concentrations by the difference of the two times. Removal efficiencies (%) for the above nutrients were calculated by dividing the difference between the first day and final day concentrations by the first day concentration and then multiplied by 100.

6.2.5. Data Analysis

The experiments were carried out in triplicate. Samples were taken at the designated times for evaluation of glycerol characterization and consumption, cell growth and lipid accumulation, and nutrients consumption. Results were reported as mean \pm standard deviation values. Analysis of variance (ANOVA) of the data was carried out. When ANOVA gave at least one significantly different result, Duncan's multiple range test was performed (1955).

6.3 Results and discussion

6.3.1. Crude glycerol characterization

The crude glycerol density is 0.95 g mL^{-1} and its pH value is 13.8. On weight basis, crude glycerol sample contained glycerol, soap, catalyst, water, total nitrogen, and others as 38.5%, 2.6%, 1.2%, 28.2%, 0.1%, and 27.3%, respectively (Table 6.1).

Table 6.1 Composition of crude glycerol sample

| Composition | % (w/w) |
|-------------|---------|
|-------------|---------|

| | |
|----------------|----------|
| Glycerol | 38.5±0.8 |
| Moisture | 28.2±0.5 |
| Catalyst | 1.2±0.1 |
| Soap | 2.6±0.2 |
| Ash | 2.1±0.1 |
| Total nitrogen | 0.1±0.0 |
| Others | 27.3±0.6 |

Table 6.2 Elemental composition of crude glycerol sample by ICP-AES analysis

| Elements | Concentration (mg L ⁻¹) |
|-----------|-------------------------------------|
| Calcium | 3.1±0.2 |
| Potassium | 1446.3±22.6 |
| Magnesium | 0.3±0.0 |
| Iron | 0.2±0.0 |
| Sodium | 5.0±0.1 |
| Silicon | 7.7±0.8 |
| Zinc | 0.3±0.0 |
| Tin | 0.2±0.0 |
| Selenium | 0.2±0.0 |

Of all the analyzed elements in crude glycerol, potassium had the highest concentration of 1446.3 mg L⁻¹ (Table 6.2). Potassium is an essential element for microalgae. The compositions of crude glycerol in present study were different from previous studies (Chen & Walker, 2011; Liang et al., 2010). This is mainly due to different oil feedstocks and biodiesel production processes. In particular, methanol in

crude glycerol was found to negatively influence the microalgal growth and docosahexaenoic acid production of *Schizochytrium limacinum* (Plye et al., 2008). Therefore, crude glycerol without methanol in this work has the potential to be better carbon source for microalgal growth.

6.3.2. Effect of glycerol concentration and initial pH on cell growth of *C. vulgaris* and nutrient removal

6.3.2.1. Effect of glycerol concentration on cell growth of *C. vulgaris* and nutrient removal

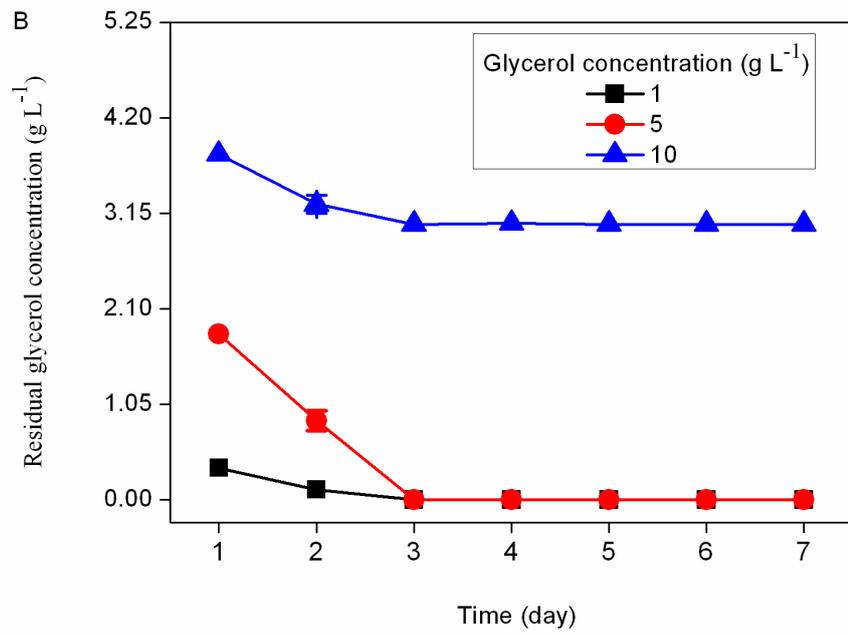
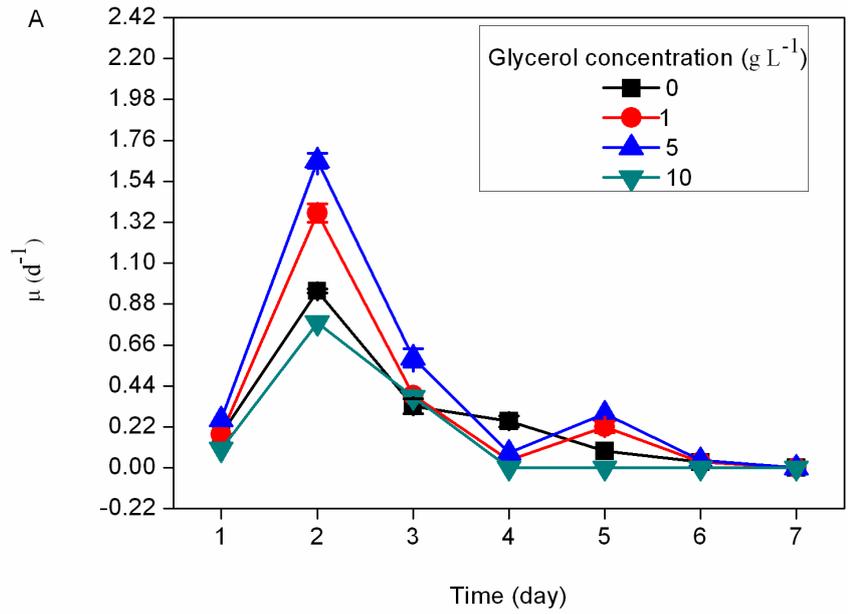
To investigate crude glycerol derived from biodiesel production using scum as oil feedstock on *C. vulgaris* growth and nutrient removal, three different concentrations of crude glycerol (1, 5, and 10 g L⁻¹) were carried out in synthetic wastewater at initial pH 7.0. The culture of *C. vulgaris* grown in the synthetic wastewater without crude glycerol (0 g L⁻¹) was used as control. The effect of the concentration of crude glycerol on the microalgal growth and nutrient removal is shown in Table 6.3 & Figure 6.1.

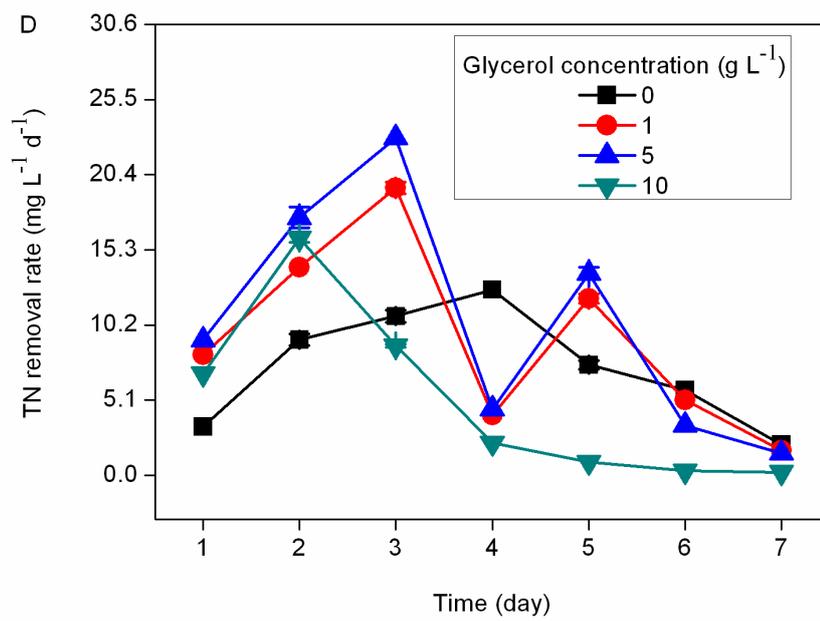
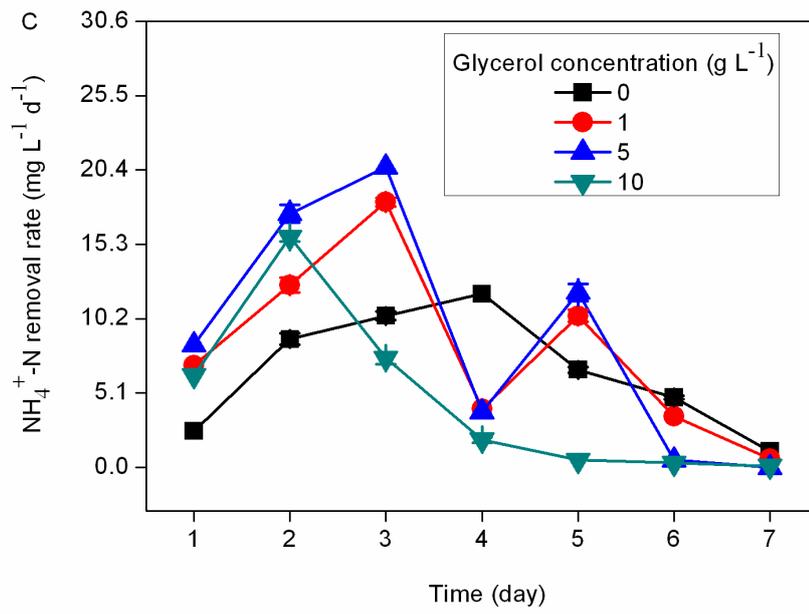
The optimal concentration of crude glycerol was 5 g L⁻¹ with the maximum biomass concentration of 1.82 g L⁻¹, the maximum biomass productivity of 260 mg L⁻¹ d⁻¹, the maximum specific growth rate (μ) of 1.65 d⁻¹, the maximum NH₄⁺-N removal efficiency of 100%, the maximum TN removal efficiency of 92%, the maximum TP removal efficiency of 88%, and the COD removal efficiency of 94%. This result is different from other reports which claimed that the optimal concentrations of crude glycerol for a marine microalga, *Schizochytrium limacinum* SR21, and a freshwater microalga, *Chlorella protothecoides* in artificial media were 25 and 30 g L⁻¹, respectively (Liang et al., 2010; Chen & Walker, 2011). The optimal concentration of

crude glycerol varies for different microalgal species. And compared with other media, wastewater had negative influence on microalgal growth, thus inhibiting glycerol consumption.

Table 6.3 Cell growth and lipid production of *C. vulgaris* and nutrient removal using different glycerol concentrations.

| Parameters | Crude glycerol (g L ⁻¹) | | | | Pretreated glycerol (g L ⁻¹) | | | | Pure glycerol (g L ⁻¹) |
|--|-------------------------------------|-----------|-----------|-----------|--|-----------|-----------|-----------|------------------------------------|
| | 0 | 1 | 5 | 10 | 1 | 5 | 10 | 15 | 25 |
| Biomass concentration (g L ⁻¹) | 0.62±0.01 | 0.93±0.06 | 1.82±0.11 | 0.35±0.03 | 1.14±0.08 | 1.95±0.10 | 2.92±0.00 | 0.64±0.07 | 5.73±0.09 |
| Biomass productivity (mg L ⁻¹ d ⁻¹) | 89±2 | 133±9 | 260±11 | 50±4 | 163±8 | 279±15 | 417±13 | 91±5 | 819±11 |
| Carbohydrate content (%) | 15±1 | 19±1 | 25±2 | 18±2 | 20±2 | 27±1 | 30±0 | 16±0 | 32±1 |
| Protein content (%) | 61±2 | 50±3 | 31±1 | 55±0 | 48±1 | 28±2 | 20±3 | 57±4 | 17±0 |
| Lipid content (%) | 10±0 | 18±1 | 32±3 | 18±3 | 20±1 | 34±0 | 39±2 | 17±1 | 42±1 |
| Lipid productivity (mg L ⁻¹ d ⁻¹) | 9±0 | 24±2 | 78±4 | 9±1 | 33±4 | 95±5 | 163±7 | 15±2 | 344±9 |
| TN removal efficiency (%) | 65±1 | 82±2 | 92±1 | 45±2 | 88±2 | 93±2 | 95±1 | 68±3 | 98±0 |
| TP removal efficiency (%) | 58±0 | 75±2 | 88±2 | 32±2 | 83±2 | 90±0 | 95±3 | 51±1 | 96±0 |
| NH ₄ -N removal efficiency (%) | 74±3 | 90±0 | 100±1 | 52±2 | 94±1 | 100±0 | 100±1 | 75±3 | 100±0 |
| COD removal efficiency (%) | 100±0 | 97±1 | 94±0 | 42±4 | 98±2 | 98±0 | 98±0 | 55±5 | 100±0 |





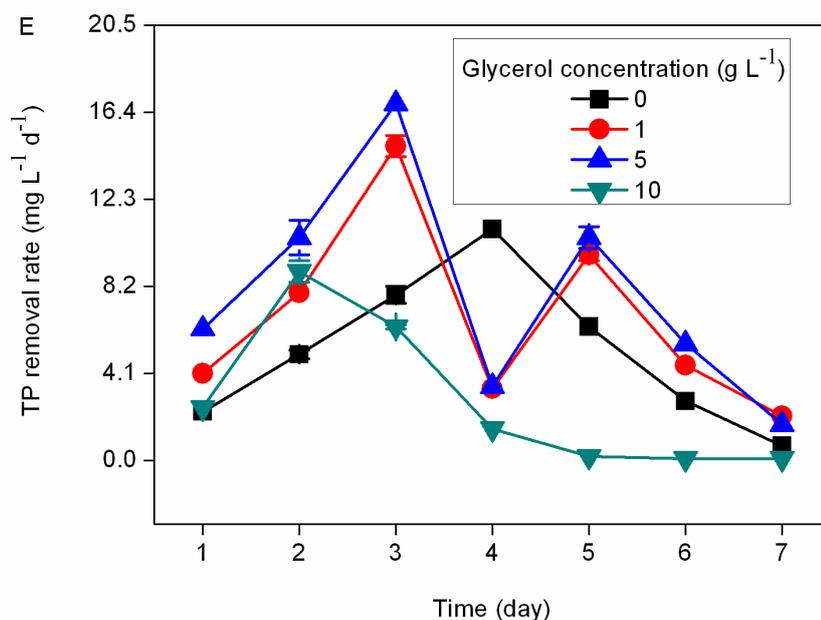


Figure 6.1: Effect of concentration of crude glycerol on cell growth of *C. vulgaris* and nutrient removal (A: Specific growth rate; B: Residual glycerol concentration; C: $\text{NH}_4^+\text{-N}$ removal rate; D: TN removal rate; and E: TP removal rate)

During the 7-day cultivation with the crude glycerol of different concentrations, biomass concentration, biomass productivity, μ of *C. vulgaris* and the removal rates of $\text{NH}_4^+\text{-N}$, TN, and TP all increased before they decreased. The results showed that the microalgal growth was improved and nutrients removal was enhanced with the addition of crude glycerol of 1 and 5 g L^{-1} compared with control (0 g L^{-1} crude glycerol). The possible reason was that crude glycerol addition balanced the C/N ratio of the synthetic wastewater, stimulating microalgal growth. In addition, heterotrophic growth was possible for *C. vulgaris* with more organic carbon source. Finally, glycerol as an osmoticum enhanced the osmotic strength of the wastewater, kept the osmotic equilibrium in cells and was a very compatible solute for enzymes and membranes (Perez-Garcia et al., 2011). However, the concentration of crude

glycerol above 5 g L⁻¹ could be harmful to microalgal cells and thus decreased nutrients removal. High concentration of the crude glycerol might result in substrate inhibition, high dose of impurities, catalyst and soap in the synthetic wastewater, leading to cell growth reduction or even cell death. COD removal efficiency decreased with the increasing concentration of the crude glycerol when the concentration of crude glycerol increased from 0 to 5 g L⁻¹. This was because some organic compounds, such as soap and impurities in the crude glycerol, cannot be used by *C. vulgaris*.

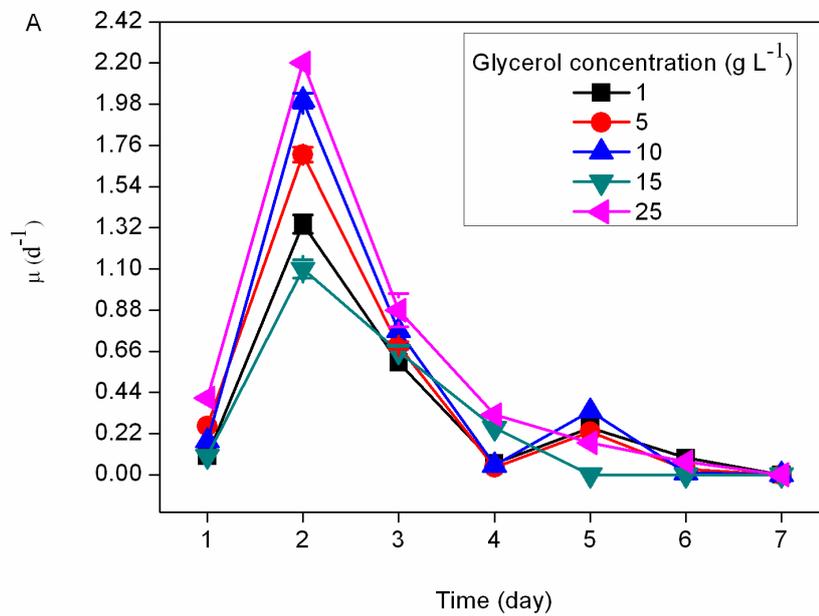
As shown in Figure 6.1, the μ , NH₄⁺-N, TN, and TP removal rates of the cultures with 1 and 5 g L⁻¹ crude glycerol had two peaks, respectively. The residual glycerol concentrations of the cultures with 1 and 5 g L⁻¹ crude glycerol both decreased with increasing culture time during the initial 3 days, and approached 0 g L⁻¹ on day 3. The results indicated that the cultures of *C. vulgaris* with 1 and 5 g L⁻¹ crude glycerol mainly used glycerol as organic carbon source to grow during the first 3 days, thus it was under a heterotrophic dominated culturing mode. After that time period, they mainly used CO₂ from the air as inorganic carbon source to grow and it was under an autotrophic dominated culturing mode. A similar two-stage growth pattern was observed for *Auxenochlorella protothecoides* cultivated in concentrated municipal wastewater with the exogenous CO₂ as inorganic carbon sources (Hu et al., 2012). When glycerol in the cultures with 1 and 5 g L⁻¹ crude glycerol was exhausted, *C. vulgaris* cells switched from heterotrophic to autotrophic nutrition on Day 4, but more detailed investigations are needed to further validate this hypothesis. Therefore, the μ and nutrients removal rates were relatively low on Day 4. During the heterotrophic dominated stage, the μ of the cultures with 1 and 5 g L⁻¹ crude glycerol had one peak on Day 2 and the NH₄⁺-N, TN, and TP removal rates showed

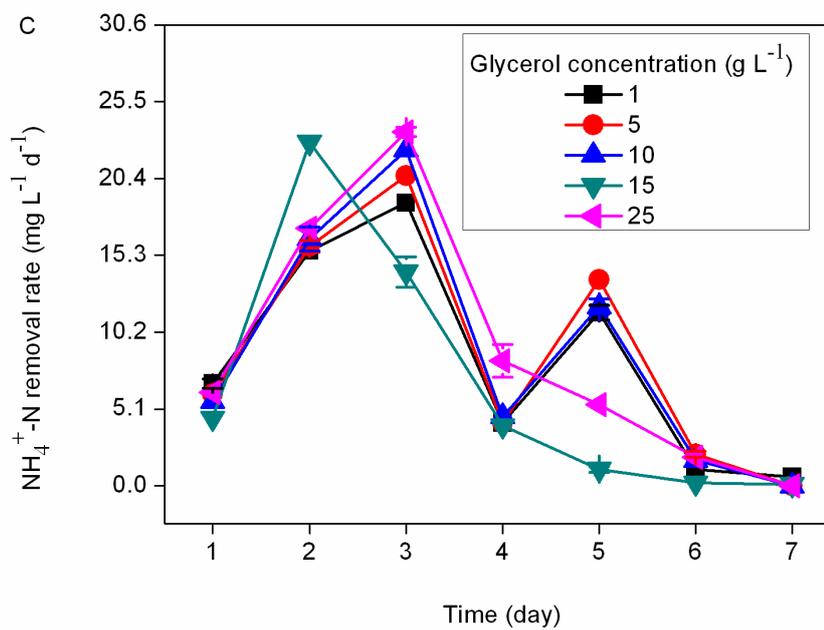
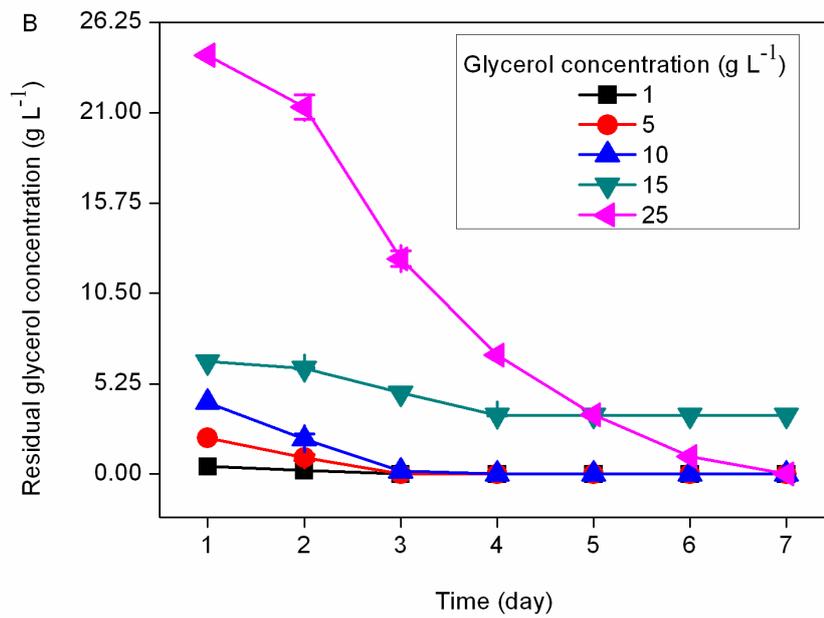
corresponding peaks on Day 3. This is probably due to biomass productivity of the cultures on Day 3 was higher than that on Day 2. During the autotrophic dominated stage, the μ of the cultures with 1 and 5 g L⁻¹ crude glycerol had one peak on Day 5 and the NH₄⁺-N, TN, and TP removal rates showed corresponding peaks at the same day.

The peaks of the μ and NH₄⁺-N, TN, and TP removal rates of the cultures with 1 and 5 g L⁻¹ crude glycerol at the heterotrophic dominated stage were higher than those at the autotrophic dominated stage, respectively. The results showed that microalga under the heterotrophic dominated culturing mode had higher biomass production and nutrient removal ability than those under an autotrophic dominated culturing mode. This may be due to the fact that *C. vulgaris* shows serious photo deficiency at the late growth stage since light penetration was inversely proportional to the cell concentration. Cells growing faster required more nitrogen and phosphorus to synthesize biomass. Similar results were obtained in a previous study using a hetero-photoautotrophic two-stage cultivation process to treat concentrated municipal wastewater (Zhou et al., 2012) with longer cultivation cycle. The culture with 10 g L⁻¹ crude glycerol and control had only one peak of the μ and nutrients removal rates of NH₄⁺-N, TN, and TP, respectively. This is because that COD of control was low and *C. vulgaris* was under an autotrophic dominated culturing mode during the whole cultivation cycle. And cell growth of the culture with 10 g L⁻¹ crude glycerol was highly inhibited. A fed-batch process will be necessary with regard to glycerol utilization if maximum rates of microalgal lipid production and nutrient removal are desired.

Previous studies have shown that soap in the glycerol restricts microalgal growth (Plye et al., 2008; Liang et al., 2010). Consequently, the crude glycerol was

pretreated to remove the free fatty acids from the soap in this study. To investigate the effect of pretreated glycerol on *C. vulgaris* growth and nutrients removal, four different concentrations of the pretreated glycerol (1, 5, 10, and 15 g L⁻¹) were applied in the synthetic wastewater at pH 7.0. The culture with the optimal concentration of pure glycerol (25 g L⁻¹) was carried out as control. Figure 6.2 showed the effect of concentration of pretreated glycerol on cell growth of *C. vulgaris* and nutrient removal.





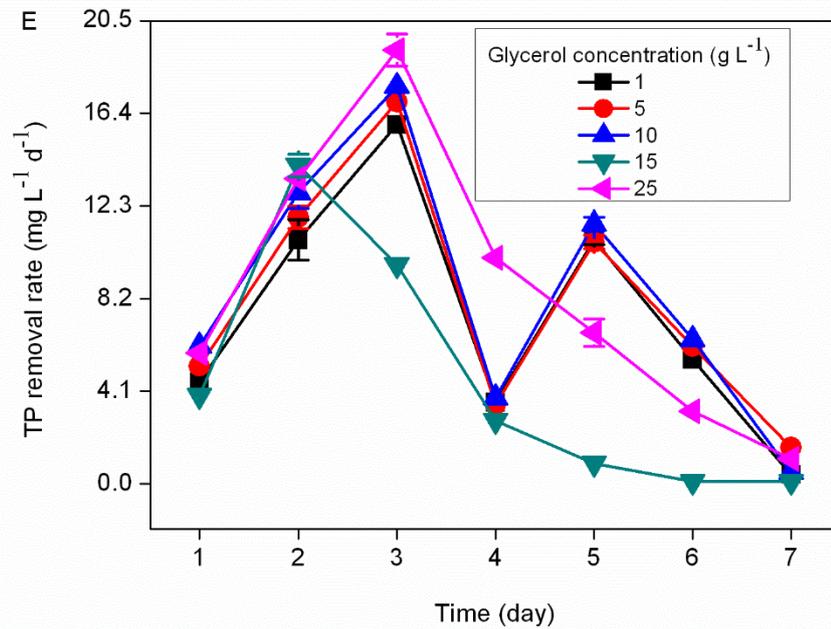
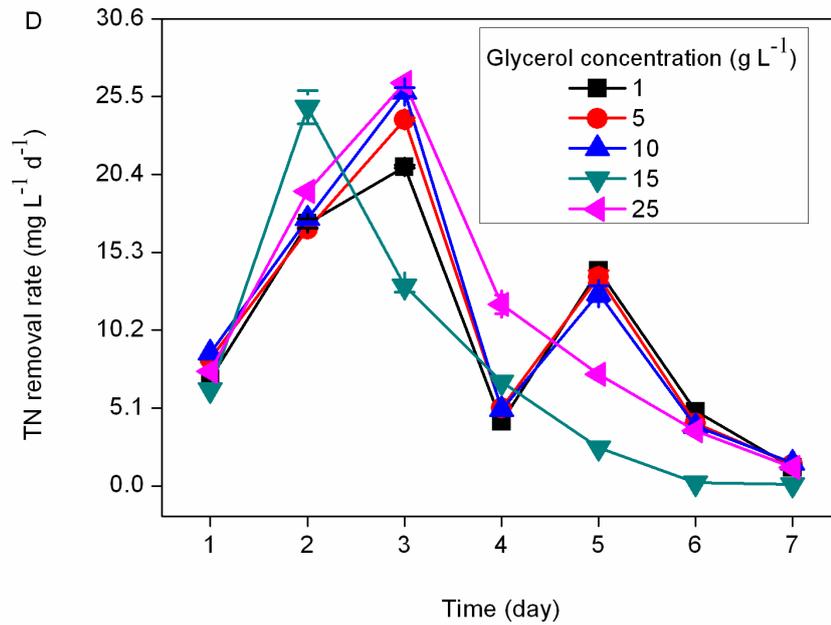


Figure 6.2: Effect of concentration of pretreated glycerol on cell growth of *C. vulgaris* and nutrient removal (A: Specific growth rate; B: Residual glycerol concentration; C: NH₄⁺-N removal rate; D: TN removal rate; and E: TP removal rate)

For the same glycerol concentration (1, 5, or 10 g L⁻¹) of the pretreated and the crude

glycerol, the pretreated glycerol generally yielded higher biomass (Table 6.3). This was because glycerol content increased after pretreatment, meaning more organic carbon source for *C. vulgaris* in the heterotrophic growth. Furthermore, the inhibition of cell growth could be partially mitigated by removing free fatty acids and some impurities from the crude glycerol. The optimal concentration of the pretreated glycerol was 10 g L⁻¹ with the maximum biomass concentration of 2.92 g L⁻¹, the maximum biomass productivity of 417 mg L⁻¹ d⁻¹, the maximum μ of 2.00 d⁻¹, the maximum NH₄⁺-N removal efficiency of 100%, the maximum TN removal efficiency of 95%, TP removal efficiency of 95%, and COD removal efficiency of 98% (Table 6.3), which were much higher than those observed in previous studies without the addition of crude glycerol into concentrated municipal wastewater (Ma et al., 2014; Li et al., 2011). The results also showed that *C. vulgaris* performed better at higher dose of pretreated glycerol (10 g L⁻¹) than that of crude glycerol (5 g L⁻¹). The culture of *C. vulgaris* had a good performance at 25 g L⁻¹ pure glycerol. These results further confirmed that the soap and some impurities in the crude glycerol inhibited cell growth. As shown in Figure 6.2, the μ , NH₄⁺-N, TN, and TP removal rates of the cultures with 1, 5, and 10 g L⁻¹ pretreated glycerol had two peaks, respectively. And the residual glycerol concentrations of the cultures with 1, 5, and 10 g L⁻¹ pretreated glycerol decreased with increasing culture time during the initial 3 days, with no glycerol left after Day 3. The culture with 15 g L⁻¹ pretreated glycerol and 25 g L⁻¹ pure glycerol had only one peak of the μ and nutrients removal rates, respectively. This is due to the presence of glycerol at the late growth stage (Figure 6.2). COD removal efficiency of the culture with 25 g L⁻¹ pure glycerol can achieve 100%. However, the highest COD removal efficiency of the culture with the pretreated glycerol was 98%. The possible reason is that some organic compounds in the crude

glycerol, which were not removed by the pretreatment, were not able to be absorbed by *C. vulgaris*.

6.3.2.2. Effect of initial pH on cell growth of *C. vulgaris* and nutrient removal

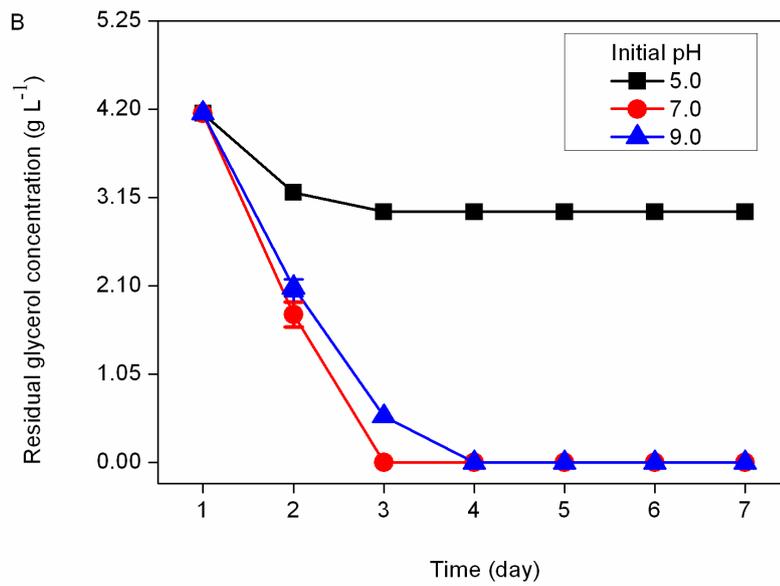
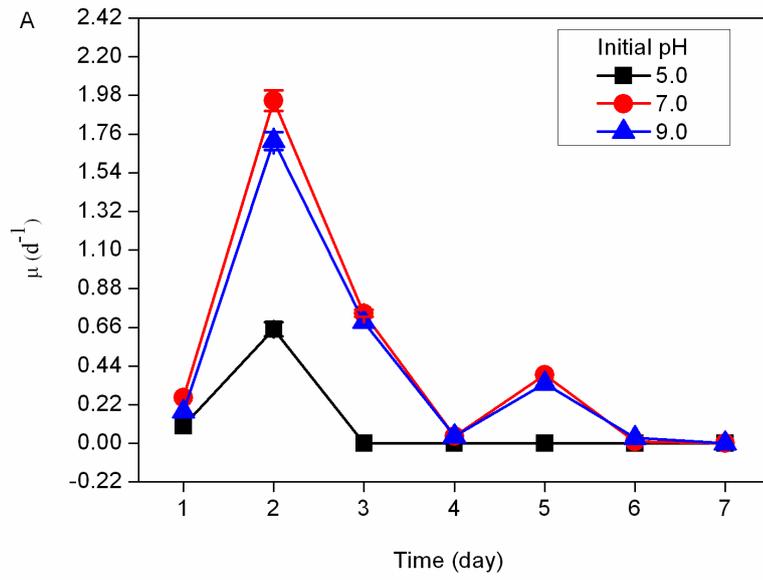
The value of pH is an important factor of microalgal growth (Hu, 2013). The crude glycerol was alkaline because of the base catalyzed transesterification process while pretreated glycerol was acidic by adding acid to remove the free fatty acid. Therefore, the synthetic wastewater with crude or pretreated glycerol became alkaline or acid. The effect of initial pH (5.0, 7.0, and 9.0) on cell growth of *C. vulgaris* and nutrient removal with 10 g L⁻¹ pretreated glycerol was tested and the results were shown in Table 6.4 and Figure 6.3.

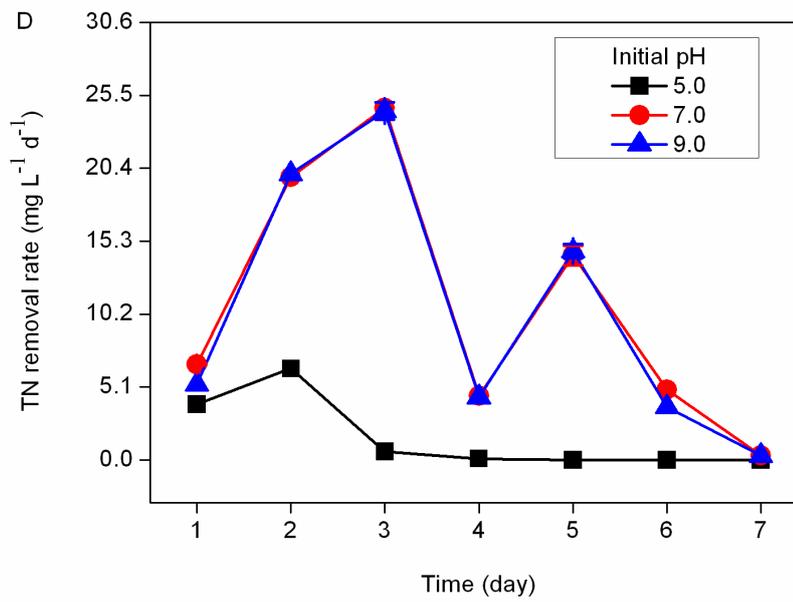
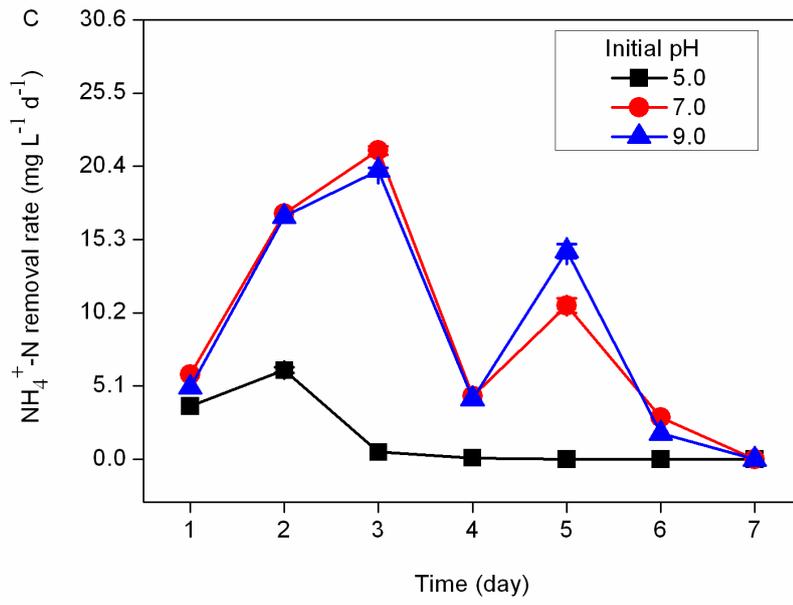
Microalgal cells at an initial pH 5.0 were dead at the late growth stage (Figure 6.3) and most glycerol was left. This result indicated that *C. vulgaris* could not grow well under acidic condition. There was only one peak for every one of μ , nutrients removal rates of NH₄⁺-N, TN, and TP, respectively, showing that *C. vulgaris* at an initial pH 5.0 was under only lone heterotrophic dominated culturing mode. Of all the initial pH values, the culture of *C. vulgaris* at an initial pH 7.0 had the maximum biomass concentration of 2.95 g L⁻¹, the maximum biomass productivity of 421 mg L⁻¹ d⁻¹, the maximum μ of 1.95 d⁻¹, the maximum NH₄⁺-N removal efficiency of 100%, the maximum TN removal efficiency of 95%, the maximum TP removal efficiency of 95%, and COD removal efficiency of 98% (Table 6.4). The culture of *C. vulgaris* at an initial pH 9.0 exhibited only a relatively slight change in the above numbers together with the nutrients removal rates of TN and TP compared with the culture at an initial pH 7.0. The results showed that alkaline condition favored *C. vulgaris* growth instead of acidic ones. Glycerol was exhausted after Day 4 using initial pH values of 7.0 and 9.0. There were two peaks for every pH value of μ ,

nutrients removal rates of $\text{NH}_4^+\text{-N}$, TN, and TP for them, respectively. It indicated that *C. vulgaris* at initial pH values 7.0 and 9.0 was under mixotrophic culturing modes: a heterotrophic dominated culturing mode at the initial growth stage and an autotrophic dominated culturing mode at the late growth stage.

Table 6.4 Effect of initial pH on cell growth and lipid production of *C. vulgaris* and nutrient removal

| Parameters | Initial pH | | |
|---|-----------------|-----------------|-----------------|
| | 5.0 | 7.0 | 9.0 |
| Biomass concentration (g L^{-1}) | 0.21 \pm 0.00 | 2.95 \pm 0.08 | 2.01 \pm 0.12 |
| Biomass productivity ($\text{mg L}^{-1} \text{d}^{-1}$) | 30 \pm 1 | 421 \pm 15 | 287 \pm 10 |
| Carbohydrate content (%) | 18 \pm 2 | 30 \pm 1 | 28 \pm 0 |
| Protein content (%) | 54 \pm 3 | 19 \pm 0 | 26 \pm 1 |
| Lipid content (%) | 17 \pm 2 | 39 \pm 3 | 33 \pm 1 |
| Lipid productivity ($\text{mg L}^{-1} \text{d}^{-1}$) | 5 \pm 0 | 164 \pm 7 | 95 \pm 4 |
| TN removal efficiency (%) | 14 \pm 2 | 95 \pm 0 | 92 \pm 0 |
| TP removal efficiency (%) | 11 \pm 1 | 95 \pm 2 | 91 \pm 3 |
| $\text{NH}_4\text{-N}$ removal efficiency (%) | 17 \pm 1 | 100 \pm 0 | 100 \pm 0 |
| COD removal efficiency (%) | 22 \pm 3 | 98 \pm 0 | 98 \pm 0 |





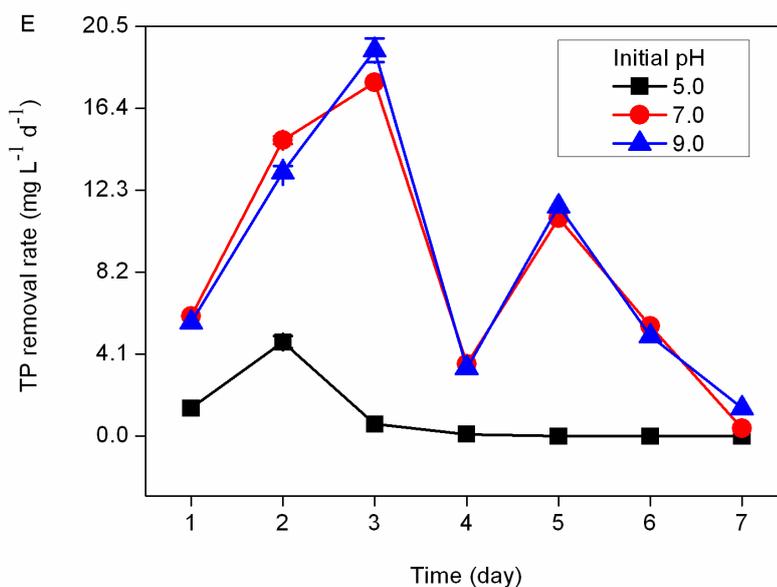


Figure 6.3: Effect of initial pH on cell growth of *C. vulgaris* and nutrient removal (A: Specific growth rate; B: Residual glycerol concentration; C: NH_4^+ -N removal rate; D: TN removal rate; and E: TP removal rate)

6.3.3. Effect of glycerol concentration and initial pH on lipid production of *C. vulgaris*

6.3.3.1. Effect of glycerol concentration on lipid production of *C. vulgaris*

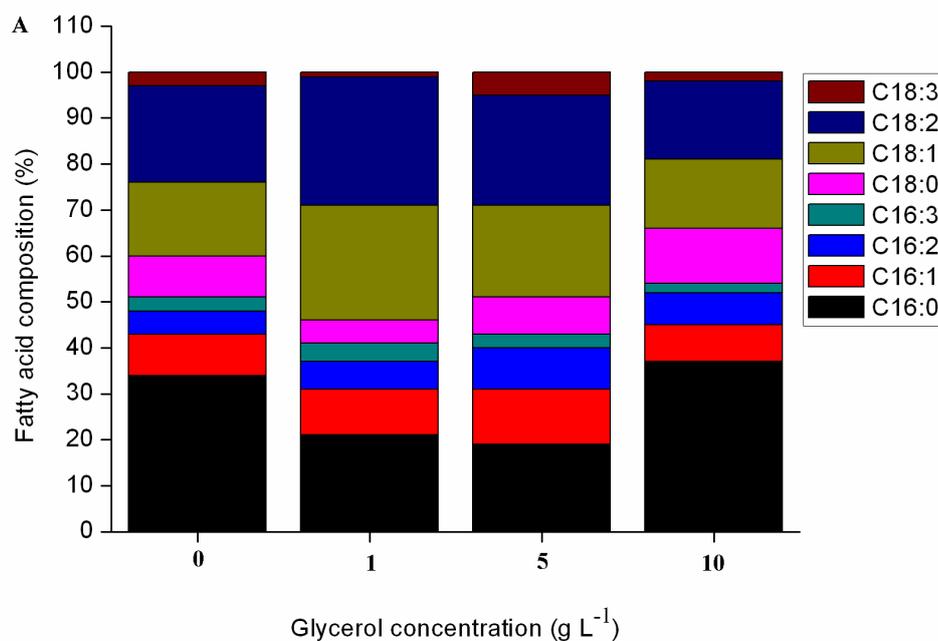
C. vulgaris accumulated lipids in the cultures in the synthetic wastewater with different concentrations of crude glycerol at initial pH 7.0 (Table 6.3). There was a significant difference ($P < 0.05$) in the lipid contents of the cultures. The lipid contents of the cultures increased with the addition of glycerol. However, a high concentration of crude glycerol (10 g L^{-1}) did not provide a reasonable lipid accumulation, which could be explained by nitrogen limitation. The culture of 5 g L^{-1} crude glycerol with TN removal efficiency of 92% was nitrogen-limited at the late growth stage (Table 6.3). Lipid accumulation was often triggered under

nitrogen-limiting conditions (Yeesang & Cheirsilp, 2011; Yeh & Chang, 2012). The maximum lipid content and productivity of 32% and 78 mg L⁻¹ d⁻¹, respectively, were obtained for the culture with 5 g L⁻¹ crude glycerol. Compared with control (with 0 g L⁻¹ crude glycerol), the culture with 1 and 5 g L⁻¹ crude glycerol stimulated lipid accumulation. This was because tri-acyl-glycerols, the storage form of lipids of microalgae, are derived from glycerol and fatty acids. When lipid content of the cultures under different concentrations of crude glycerol was high, protein content was relatively low. Carbohydrate content showed similar tendency as lipid content.

There was a significant difference ($P < 0.05$) in the lipid contents of the cultures with the pretreated glycerol. The optimal glycerol concentration of the pretreated glycerol was 10 g L⁻¹ with the maximum lipid content and productivity of 39% and 163 mg L⁻¹ d⁻¹, respectively. The lipid accumulation increased when the glycerol concentration was increased from 1 to 10 g L⁻¹ (Table 6.3). However, a further increase in glycerol concentration discouraged the lipid accumulation of the strain. As for the pretreated glycerol at 5 g L⁻¹, higher lipid content and productivity have been observed compared with the crude glycerol at the same concentration.

The composition of the major fatty acids of the extracted lipids from the cultures with different glycerol concentrations was determined using a GC-MS system (Figure 6.4). The same eight varieties of fatty acids were identified, but the relative content of the fatty acids varied. C16:0, C18:1, and C18:2 were the three most abundant fatty acids and their total percentage was approximately 60%. The percentage of unsaturated fatty acids increased with increasing concentrations of crude glycerol. The results suggested that high concentration of crude glycerol could promote the degree of unsaturation in fatty acids of *C. vulgaris*. This might be that the increase in concentrations of crude glycerol could lead to high C/N ratio, thus

increasing the content of polyunsaturated fatty acids (Morales-Sánchez et al., 2013). Similar trend was observed in the cultures of *C. vulgaris* with different concentrations of the pretreated and pure glycerol. Compared with soybean oil, the total percentage of C18 fatty acids in *C. vulgaris* was lower (Serio et al., 2006). The content of unsaturated fatty acids in the lipids of *C. vulgaris* was lower than that of soybean oil. C16:1, C16:2, and C16:3 were not present in soybean oil. It is worth pointing out that the fatty acids (primarily C16 and C18) in soybean oil used in biodiesel production were also in those of *C. vulgaris*. Therefore, biodiesel from lipids of *C. vulgaris* and soybean oil, the main current biodiesel feedstock in the United States (Avinash et al., 2014), would have similar physical and chemical properties. Similar fatty acids profile was obtained in the cultures of *C. vulgaris* with different concentrations of the pretreated and pure glycerol. Therefore, fatty acids from *C. vulgaris* under different glycerol concentrations are likely suitable for the production of good-quality biodiesel.



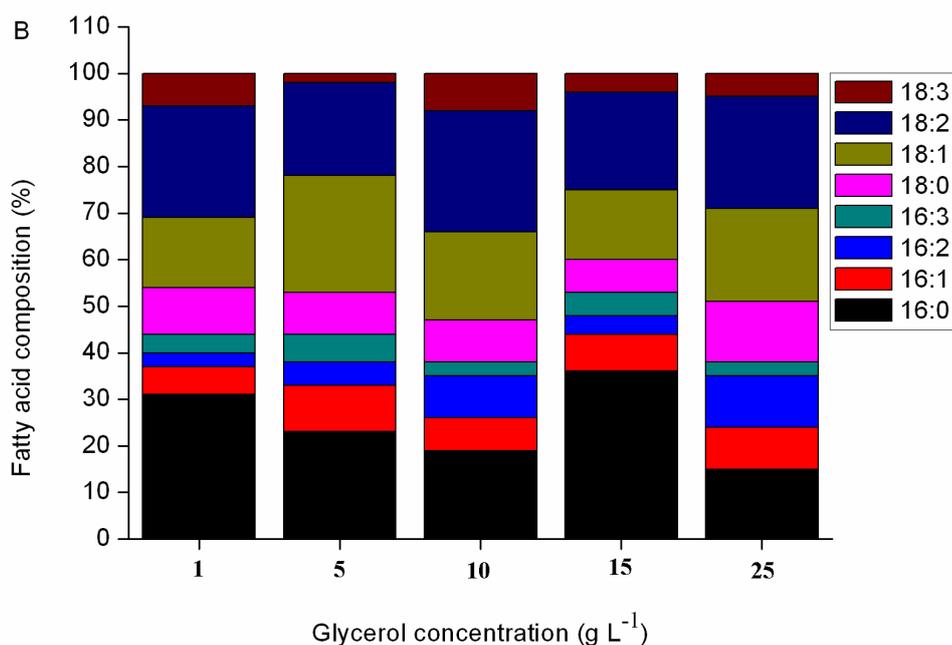


Figure 6.4: Fatty acid profiles of *C. vulgaris* with different glycerol concentrations (A Crude glycerol; B Pretreated glycerol)

6.3.3.2. Effect of initial pH on lipid production of *C. vulgaris*

There was a significant difference ($P < 0.05$) in the lipid contents of the cultures at different initial pH values. The most suitable pH for *C. vulgaris* to accumulate lipids was 7.0 with the maximum lipid content and productivity of 39% and 164 mg L⁻¹ d⁻¹, respectively. The lipid accumulation decreased from 39% to 33% and lipid productivity decreased from 164 to 95 mg L⁻¹ d⁻¹ when the initial pH was increased from 7.0 to 9.0 (Table 6.4). Microalgal cells of the culture at initial pH 5.0 were dead at the late growth stage with their lipid accumulation inhibited greatly. These results indicated that alkaline conditions prompted lipid accumulation of *C. vulgaris*. Low protein content was observed for the culture with high lipid content.

Eight varieties of fatty acids were identified using the GC-MS system, and the relative content of the fatty acids from the cultures with different initial pH values was different (Figure 6.5). The contents of unsaturated fatty acids were higher than

that of saturated fatty acids. There were mainly C16 and C18 fatty acids in the lipids of *C. vulgaris*. This observation is consistent with previous findings that C16 and C18 fatty acids are the main fatty acids of *C. vulgaris* cultivated in the effluent of a low-cost waste fermentation system producing volatile fatty acids (Cho et al., 2015). In addition, lipids of the cultures had similar fatty acid profile to those under different glycerol concentrations. Therefore, fatty acids from *C. vulgaris* under different initial pH values are also likely suitable for the production of good-quality biodiesel.

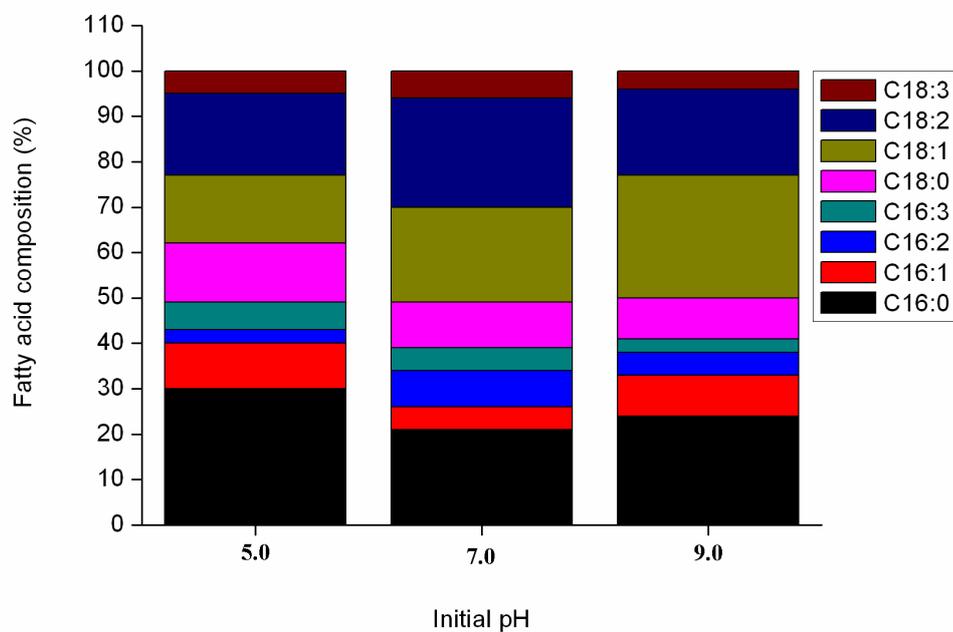


Figure 6.5: Fatty acid profile of *C. vulgaris* with different initial pHs

6.3.4 Cultivation of *C. vulgaris* in concentrated municipal wastewater with waste glycerol

To verify that the integration process of microalgae-based biofuel production and wastewater treatment with the addition of waste glycerol into synthetic wastewater

was applicable to real wastewater, the culture of *C. vulgaris* with 10 g L⁻¹ pretreated glycerol was applied in concentrated municipal wastewater at initial pH 7.0. Prior to inoculation, the medium was autoclaved at 121 °C for 30 min. After 7-day cultivation, the maximum biomass concentration of *C. vulgaris* was 3.38 g L⁻¹ with the maximum NH₄⁺-N removal efficiency of 100%, the maximum TN removal efficiency of 91%, the maximum TP removal efficiency of 90%, the maximum COD removal efficiency of 96%, and the highest lipid content of 37%. Our previous research showed that algae could remove NH₄⁺-N, TN, TP, and COD by 93.9%, 89.1%, 80.9%, and 90.8%, respectively from raw centrate without adding carbon sources, and the FAME content was 11.04% of dry biomass. The results showed that *C. vulgaris* exhibited a good performance on cell growth, lipid production, and nutrients removal in concentrated municipal wastewater with the addition of waste glycerol.

6.4 Conclusions

The results showed that waste glycerol could be metabolized by *C. vulgaris* cultivated in wastewater. Glycerol concentration and initial pH had a significant effect on microalgal growth and lipid accumulation of *C. vulgaris*, and nutrient removal. The respective most favorable concentrations of crude and pretreated glycerol were 5 and 10 g L⁻¹ with the optimal initial pH 7.0 for microalgal lipid production. Eight varieties of fatty acids were identified. The main fatty acids components of the microalga were C16–C18, which are suitable for the production of good-quality biodiesel.

CHAPTER 7. SUMMARY, CONCLUSIONS AND FUTURE WORK

7.1 Summary of the dissertation

Microalgae have long been considered an alternative feedstock for biofuels production because they possess high growth rate, provide lipids fraction for biodiesel production while they do not need large acres of farmland for the production (Harun et al., 2010). However, microalgae production with synthetic media is expensive due to the large requirement of water, high cost of land, and high price of nutrients (Maira Freire, 2012). Cultivating algae on waste resources presents an alternative to the current practices of wastewater management, land application, as well as algae-based biofuel production.

The objectives of this study were to fully utilize wastewater and by-products from biodiesel production process to grow mixotrophic algae strains for simultaneous biomass accumulation, biodiesel production, waste stream treatment, and nutrient removal.

Chapter 1 of this dissertation describes the background, significance of the study, and the specific goals. In Chapter 2, literature was reviewed for the current situation of algal biomass production using wastewater, the effect of bacteria on algae growth, and the feasibility of fully utilizing waste resources from biodiesel production. The utilization of two waste resources generated from algae-based biofuel production process, namely lipid-extracted microalgae biomass residues (LMBRs) and crude glycerol, was reviewed. LMBRs are the leftover biomass after the extraction of lipid

from microalga biomass. Crude glycerol is produced as the major byproduct in transesterification process.

Previous studies have shown that *Chlorella vulgaris* grew well on centrate wastewater, which is the best among all municipal wastewater streams for algal cultivation with high biomass accumulation and high efficiency wastewater nutrient removal. For the large scale algae biomass production, wastewater can be used directly as algae cultivation medium to minimize the costs associated with water, nutrients, and carbon source. However, centrate wastewater contains numerous species of bacteria, which are expected to influence the algal biomass accumulation. Therefore, in Chapter 3 and 4, the appropriate algae inoculation levels that would allow algae to compete favorably with the wastewater-borne bacteria and dominate the culture, and the effect of algae and wastewater-borne bacteria interaction on the algal biomass accumulation and wastewater nutrients removal were studied. A mutual compound relationship between algae and centrate-borne bacteria has been demonstrated in this part of the study. Algae were found to promote bacterial growth and optimal initial algal concentration was favorable for bacterial growth. Moreover, bacteria increased the algal growth rate and nutrients removal rate at the initial stage of the cultivation. The maximum algal biomass of 2.01 g/L with 0.1 g/L initial algal inoculum concentration was obtained during algae cultivation in raw centrate medium. The bacteria community structure during cultivation period were tested. It was found that the diversity of microbial community was abundant in the samples. Ten phyla and 37 genera were identified. Phylum Bacteroidetes dominate the culture

in the centrate wastewater, genus *Prevotella* was most abundant accounting for 87.1% of the total sequences. At the end of cultivation, microbial community was dominated by phylum Proteobacteria, Bacteroidetes, and Firmicutes. Four bacteria genus, *Acinetobacter*, *Bacteroidales_norank*, *Megasphaera*, and *S24-7_norank*, may have influence on algal growth.

In Chapter 5, the enzymatic hydrolysates of lipid-extracted microalgal biomass residues (LMBRs) from biodiesel production were successfully used as nutritional sources for microalgal cultivation and lipid production. Temperature and substrate concentrations had a significant effect on cell growth and lipid production. The optimal temperature for cell growth and lipid accumulation of the mixotrophic growth of *C. vulgaris* was between 25 and 30 °C. The neutral lipids of the culture at 25 °C accounted for as much as 82 % of the total lipid content in the microalga at culture Day 8. The maximum biomass concentration of 4.83 g/L and the maximum lipid productivity of 164 mg/L/day were obtained at an initial total sugar concentration of 10 g/L and an initial total concentration of amino acids of 1.0 g/L. The mixotrophic growth of *C. vulgaris* achieved a high biomass concentration and high lipid productivity, which would facilitate downstream processing and reduce production waste. The extracted lipids were probably suitable to be used as feedstock for the production of good-quality biodiesel.

In Chapter 6, cultivation of *Chlorella vulgaris* in wastewater with waste glycerol generated from biodiesel production using scum derived oil as feedstock was studied. The results showed that nutrient removal and lipid production of *C. vulgaris* was

increased with the addition of waste glycerol into wastewater to balance its C/N ratio. The optimal concentration of pretreated glycerol for *C. vulgaris* was 10 g L⁻¹ with biomass concentration of 2.92 g L⁻¹, lipid productivity of 163 mg L⁻¹ d⁻¹, and the removal of 100% ammonia and 95% of total nitrogen. Alkaline conditions prompted cell growth and lipid accumulation of *C. vulgaris* and simultaneously stimulated nutrient removal. The application of the integration process of microalgae-based biofuel production with wastewater treatment can achieve the dual purpose of lowering both wastewater treatment and biofuel feedstock costs.

7.2 Future work

7.2.1 Specific mechanisms of algae growth and wastewater nutrients removal

Many algae species could use sunlight and CO₂ to synthesize organic matter through photosynthesis, which is called photoautotrophy. Some microalgae could take up some organic substrates from the environment and convert them into building blocks and storage compounds, which is heterotrophy characterized by a higher biomass concentration than photoautotrophy. The metabolic mode in which microorganisms exhibit both photosynthesis and heterotrophy is called mixotrophy. Several studies showed that mixotrophic growth of microalgae have higher biomass concentration and growth rate for certain algal strains than when they were grown either photoautotrophically or heterotrophically alone (Martínez and Orús, 1991; Kobayashi et al., 1992; Boechat et al., 2007). The mechanism involved in uptake of the organic carbon substrate and other nutrients is complicated. It is reported that the

nutrients transported across cell membranes involving diffusing across plasma membrane and inducible active transport system monitored by protein (Tanner, 1969; Haass and Tanner, 1974, Richmond, 2004).

Little research was focused on mechanisms of wastewater nutrients removal by algae and wastewater-borne bacteria for wastewater treatment and algae cultivation purposes. It is reported that both inhibition and stimulation of algal growth by bacteria were observed. Bacteria may harm algae by competing nutrients with algae or release extracellular substances which damage algal cells (Cole, 1982; Fergola et al., 2007); or help degrade intractable compounds to small nutrients particles which can be easily used by algae (Zhang et al., 2012). However, the releasing substrate is hard to test because the complexity of centrate wastewater. Some basic research such as metabolic pathway, genome sequence, molecular map, and phylogenetic analysis of specific bacteria strains which might help algae growth are expected to produce more information about the interaction between algae and wastewater-borne bacteria.

7.2.2 Algae based bio-refinery

Although utilization of waste resources for algal biofuel production is considered as the most viable solution to improve environmental friendliness and economic viability, this production process still faces many challenges before the industry becomes technologically and economically viable in the near future. The solution to these challenges is to develop a bio-refinery system that integrated wastewater-based algal biomass conversion processes and facilities to produce fuels, power,

value-added byproducts from microalgal biomass and at the same time for wastewater treatment (Chisti, 2007). Biorefineries have been operated on crops in several countries, such as Germany, Canada, and the States, for the production of biofuels and other products. For Wastewater-based algal production, A 2000 L and 40,000 L multi-layer pond-like bioreactor was successfully developed and was used to microalgae on centrate wastewaters for effective algal biomass production and efficient nutrient removal, which considered as most feasible and cost-effective culture systems (Min et al., 2011; Min et al., 2014). In harvesting stage, the techniques presently applied in algae harvesting process include centrifugation, flocculation, flotation, sedimentation, filtration, and electrophoresis methods (Chen et al., 2011). Because of the tiny cell size of algae (most 2-50 μ m in diameter) and strong negative charge on the cell surface, the cost of harvesting accounts for at least 20–30% of the total costs of algal biomass production (Zhou et al., 2014). Lack of an efficient and cost-effective algal biomass harvesting technology is another key limiting factor for the commercial algal biofuel industry. Residual biomass after lipid extraction for biofuel production could be used as algae cultivation substrate after treatment, and used to produce methane by anaerobic digestion, for generating the electrical power necessary for running the microalgal biomass production facility (Chen et al., 2010; Chisti, 2007). It is also feasible to extract other high-value products such as DHA or EPA, depending on the specific microalgae used. In summary, development of new processes, design of the system, and life cycle analysis are necessary for the development and implementation of algae based

bio-refinery.

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