TWO-STAGE HETEROTROPHIC AND PHOTOTROPHIC CULTURE TECHNOLOGY FOR MICROALGAL BIOFUEL PRODUCTION

By

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ABSTRACT

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Microalgae are attractive feedstocks for producing renewable biofuels. In this dissertation, I developed a two-stage heterotrophic and phototrophic microalgae culture system for biofuel production. Heterotrophic cultures could not only serve as seed for subsequent phototrophic growth, but also produ.ce microalgal biomass and lipid separately by feeding with organic wastes.

I demonstrated that the heterotrophically cultured microalga *Chlorella sorokiniana* was more efficient to be used as seed for subsequent phototrophic growth, due to the higher productivity and similar performance compared with its phototrophic counterpart. High inoculation of heterotrophically produced seeds was a potential tool for contamination control.

High cell density heterotrophic cultivation of *C. sorokiniana* was achieved through two-stage fed-batch fermentation. With the optimized culture conditions, the algal biomass and lipid reached high concentrations of 103.8 g L^{-1} and 40.2 g L^{-1}. The lipid of *C. sorokiniana* contained a large amount of neutral lipids (92.9% of total lipids), triacylglycerols (82.8% of neutral lipids), and high contents of palmitic, oleic and linoleic acids, which were desirable feedstocks for biofuel production.

I evaluated the feasibility to culture *C. sorokiniana* for lipid production with cellulosic materials through a simultaneous saccharification and fermentation (SSF) process. The culture with substrate loading of 3% (w/v) and enzyme loading of 30 FPU/g cellulose was an appropriate combination for lipid production. Elevated temperature improved the SSF efficiency and the highest lipid concentration (2.98 g L^{-1}), yield (99.2 mg g^{-1}) and productivity (20.7 mg L^{-1} h^{-1}) were achieved at 40°C.

I studied D-xylose uptake and the related metabolism in *C. sorokiniana*. The sugar uptake kinetic analysis suggested that an inducible hexose symporter might be responsible for the transport of D-xylose. The maximum D-xylose transport rate was 3.8 nmol min^{-1} mg^{-1} cell with K_m value of 6.8 mM. The enzymatic activities of NAD(P)H-linked xylose reductase (XR) and $NADP^+$-linked xylitol dehydrogenase (XDH) were detected in *C. sorokiniana*. Culturing *C. sorokiniana* under light improved D-xylose utilization due to additional NADPH from the light-dependent reaction of photosynthesis. The results presented in this study suggested that the two-stage heterotrophic and phototrophic process was a promising technology for microalgal biofuels production.

Dedication

This dissertation is dedicated to my parents and my wife

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Chaper 1

Introduction

1.1. Microalgal biofuels

Current energy supply and use are not sustainable and friendly to the environment. Fossil fuels account more than 80% of the primary energy consumption in the world. Greenhouse gas (GHG) accumulation, mainly due to the combustion of fossil fuels for transportation, manufacturing and domestic heating, greatly affects the global climate and human systems. There is a great demand for compatible mitigation strategies to neutralise the excess carbon dioxide since natural processes can only remove about 40% of the total amount. Increasing the usagae of renewable energy is one of the salient strategies to reduce GHG emissions and alleviate the environmental stress (Brennan & Owende, 2010; Pienkos & Darzins, 2009).

Liquid biofuel is a promising renewable energy and play a significant role in replacing transportation fossil fuels, because minimal changes to distribution infrastructure and vehicle engine are required. First generation biofuels produced from food and oil crops can hardly meet the energy demands due to the competition with food, land and water. Second generation biofuels produced from lignocellulosic and microalgal biomass can significantly reduce the GHG emissions and improve carbon footprint and environmental damage (Luque, 2010). The Energy Independence and Security Act (EISA) passed in the United States in 2007 mandates specific yearly targets of 36 billion gallons renewable transportation fuels by 2022. Of the total, 21 billion gallons must be met with advanced biofuels, defined as cellulosic ethanol and other biofuels derived from feedstock other than corn starch (Sissine, 2007). Microalgae are

considered as ideal feedstock for producing renewable fuels (Fig 1.1), such as (1) ethanol, hydrogen and methane produced through biochemical conversion; (2) bio-oil and syngas produced through thermochemical conversion; (3) biodiesel produced by transesterification of algal lipid; (4) green diesel, green jet fuel, and green gasoline produced by hydroprocessing and catalytic cracking of algal lipid (Brennan & Owende, 2010; Pienkos & Darzins, 2009).

There are many advantages for microalgae based biofuels: (1) microalgae grow very fast and have high lipid contents (Chisti, 2007). Normally the growth rate is in the range of 1-4 doublings per day and during the exponential phase the biomass can double in 3.5 hours. The lipid content is about 10% to 30% of dry biomass in general culture conditions, however, this value can double or triple under nutrient depletion, for instance, nitrogen deficiency led to an increase in the lipid content of *Ankistrodesmus* (from 24.5% to 40.3%), *Boekelovia* (from 27% to 59%), *Isochrysis* (from 7.1% to 26.0%) (Chisti, 2007; Sheehan et al., 1998); (2) microalgae have high photosynthetic efficiency (PE). The PE of natural terrestrial plant is below 1% while most farming crops range from 1-4%. Sugarcane is one of the top efficiency plant that can reach 8% (Moore, 1995). The estimate PE for algae is in the range of 10 to 20% (Huntley & Redalje, 2007; Pirt et al., 1980). It has been reported that the PE of *Chlorella vulgaris* reached 20% in indoor cultures illuminated with artificial light (Tamiya, 2003), *Phaeodactylum tricornutum* obtained 15-20% in outdoor tubular photobioreactors (David et al., 2003; Fernández et al., 1998), and *Tetraselmis suecica* averaged 13-19% in a shallow outdoor flume (Laws et al., 1986). As a result, algae biomass productivity can be about 30 times greater than that of crop plants (Sheehan et al., 1998); (3) microalgae can be used efficiently to capture CO_2 from concentrated source such as the flue gas from a power plant; (4) microalgae can be grown using non-arable land and non-agricultural waters, such as salt water, brackish water and municipal wastewater. Growing

microalgae under these conditions does not compete with food crops for land and water resources; (5) microalgae have the capability to produce a wide range of co-products. After the lipid is extracted for biofuel production, the biomass residues that mainly contain protein and polysaccharides can be used as the feedstock for producing a variety of valuable products for different sized markets (Brennan & Owende, 2010).

1.2. Technologies for microalga cultivation

Although it is generally and correctly perceived that algae growth requires light and CO_2, a growth mode often referred to as photoautotrophy, some algae can grow on organic carbon without needing light, a growth mode called heterotrophy (Chen & Chen, 2006). Furthermore, some algae can use a nutritional strategy that combines autophototrophic and heterotrophic growth. This type of growth mode is referred to as mixotrophy (Norbert & Jörg, 2009; Troost et al., 2005). Research and development of viable algae fuel technologies may need to exploit all of these growth modes.

1.2.1. Phototrophic culture

Phototrophic growth systems are the most studied among the three growth modes. Phototrophic algae culture can be conducted in either open systems, such as ponds, or in closed systems such as photobioreactors (PBRs). The open culture is investigated by many researchers due to its relative low cost. However, the main disadvantages of open ponds are susceptibility of contamination by invading species and insects (Rusch & Malone, 1998; Theegala et al., 1999) and relatively low biomass productivity due to the poor carbon dioxide utilization efficiency (Chisti, 2007). Compared with open ponds, PBRs provide a better mass transfer and light

penetration environment for algae growth, and the volumetric biomass productivity is 13 times greater (Chisti, 2007). However, the cost for construction of the PBR is relative expensive. Generally, phototrophic algae culture has a low productivity due to unsatisfactory light penetration and carbon dioxide fixation which makes phototrophic algal biomass relatively costly (Li et al., 2008). Commercial phototrophic culture systems have been reported for production of high-value products (Borowitzka, 1999; Olaizola, 2003) which helps alleviate culture costs.

1.2.2. Heterotrophic culture

Heterotrophic algae growth is less studied than its phototrophic counterparts. Heterotrophic algae are capable of utilizing organic substrates as energy and carbon sources. Consequently, heterotrophic algae culture has the advantage of fast growth without subjection to light limitation. Thus, heterotrophic culture can be accomplished in large vessels and achieve high biomass concentration. The biomass is easy to harvest and process because of the high concentrations obtained. It has been reported that the biomass concentration of green algae *Chlorella protothecoides* achieved 51 g L^{-1} in 167 h in a 5-l bioreactor by feeding with glucose. Heterotrophic culture systems have been successfully applied to commercial production of nutraceuticals such as docosahexaenoic acid (DHA). More than 100 g L^{-1} of DHA was obtained in a batch-fed fermentation of the algae *Schizochytrium* (Bailey et al., 2003). The main limitation of heterotrophic algae culture is the high cost associated with operation and sourcing organic carbon substrates.

1.2.3. Mixotrophic culture

Mixotrophic growth is also less studied than phototrophy. Mixotrophic algae have the capability to use both carbon dioxide and organic substrates and normally the growth rate is higher than that of phototrophic algae (Samejima & Myers, 1958a). It has been reported that the biomass concentration of mixotrophic culture of *Spirulina platensis* could reach 10.24 g L^{-1} (dry weight) which was 5.1-fold that obtained in the phototrophic culture using the same fermentor (Chen & Zhang, 1997). Another study found that in mixotrophically growing *Phaeodactylumtricornutum*, feed with glycerol, the biomass concentration (16.2 g L^{-1}) and maximum biomass productivity (61.5 mg L^{-1} h^{-1}) increased remarkably, and were 9 and 8 fold higher than in the phototrophically grown control respectively (Garcia et al., 2000). But light is still often required for the mixotrophic algae culture, just as with phtotrophic culture. Moreover, the cultures must be conducted in closed sterilized PBRs, and cannot be applied to low-cost open ponds, due to potential contamination by feeding with organic substrates.

1.2.4. Integrated sequential culture

Taking advantage of both phototrophic and heterotrophic cultures, some researchers developed combined process based on the dual-trophic characteristics of specific algae species. The dual-trophism is defined as the ability of algae to be cultured at both phototrophic and heterotrophic conditions. Example algae species include strains from *Chlorella*, *Scenedesmus*, *Chlamydomonas* and some species of diatoms (Table 1.1). Ogbonna et al. (1999) proposed a sequential heterotrophic-phototrophic algae culture system for α-tocopherol production. In this system, algae were heterotrophically cultured to a high concentration and then illuminated with light to induce α-tocopherol production. A similar culture strategy to produce astaxanthin by *Haematococcus pluvialis* was demonstrated by Hata et al. (2001). These processes significantly

increased the algal productivity than solely phototrophic or solely heterotrophic culture, but it was still limited to high-value pigments production because the high-cost specifically designed PBR was required in these processes. Moreover, Oyler (2008) described a process for production of oil from algae via a reverse sequential phototrophic and heterotrophic growth to overcome single phototrophic or heterotrophic culture disadvantages. However, it still needs a large part of organic carbon and faces the risk for contamination from open pond.

1.3. Scope and objectives

To develop an efficient microalgae culture system, overcoming year-round production and potential contamination issues, we developed a two-stage heterotrophic and phototrophic strategy (Fig. 1.2). This process takes advantages of both heterotrophic culture's high efficiency and phototrophic culture's low cost. On one hand, heterotrophic culture can provide an efficient way for seed cells production and used as inoculums in the subsequent phototrophic open pond cultivation for lipid production, which cannot only save the illuminated area but also have the potential for contamination control. On the other hand, the heterotrophic process can produce algal biomass and lipid separately by feeding with organic wastes, such as cellulosic residues, during the winter time to provide a year-round production capability. The green microalga *Chlorella sorokiniana* will be used in this process since it has been proved with dual-trophic capability (Table 1.1) and had the potential to accumulate more than 30% lipid (Chen & Johns, 1991). To achieve the aim of this project, the following specific objectives will be investigated:

Objective #1 Heterotrophic culture of the microalga C. sorokiniana as seeds for open pond phototrophic system (Chapter 2)

The working hypotheses of this task are: (1) Heterotrophic growth provides high efficiency

algal seed preparation; (2) Heterotrophic seed has comparable performance with phototrophic seed; (3) Contamination control can be achieved by high inoculation of heterotrophic seed.

Objective #2 High density heterotrophic culture of C. sorokiniana for lipid production (Chapter 3)

The goals of this task are: (1) Improvement of the heterotrophic culture conditions; (2) High density fermentation for biomass and lipid production; (3) Evaluation of the characteristics of algal lipid for biofuel production.

Objective #3 Heterotrophic culture of C. sorokiniana with sugars derived from lignocellulosic materials (Chapter 4 and 5)

The working hypotheses of this task are: (1) *C. sorokiniana* can heterotrophically grow with cellulose through a simultaneous saccharificiaton and fermentation (SSF) process; (2) *C. sorokiniana* can utilize D-xylose as the carbon source.

1.4. Dissertation structure

This dissertation consists of an introduction and four main chapters. Chapter 1 introduces the background of microalgal biofuels and the technologies for the cultivation, and provides an overview of the major objectives of the thesis. Chapter 2 describes the two-stage heterotrophic and phototrophic microalgae culture system. This chaper shows that heterotrophic grown microalgae can serve as seeds for phototrophic cultivation. Contamination control can be achieved by high inoculation of the heterotrophic microalgal seeds. The production efficiency of the heterotrophic process is critical for the overall performance of this two-stage system. Chapter 3 improves the heterotrophic cultivation through a fed-batch strategy. The effects of nitrogen source and pH on the microalgae growth and their relationship during the fermentation are

elucidated. This chapter also demonstrates that high density microalgal biomass and lipid can be obtained through heterotrophic fermentation, which is essential to the two-stage process since the system requires the heterotrophic process with the capability to produce algal biomass and lipid in parallel. Utilization of ligocellulosic materials (mainly cellulose and hemicellulose) as the feedstock can help reduce the production cost of heterotrophic cultivation and make the whole process ecomical feasible. Chapter 4 presents a simultaneous saccharification and fermentation (SSF) process for microalgal lipid production by using cellulose as the feedstock. In this chapter, the effects of substrate and enzyme loadings on microalgal lipid accumulation are evaluated. The process effiency is improved by elevated temperature. Xylose utilization is also important since it is the major component of hemicellulose. Chapter 5 investigates xylose uptake and the related metabolism by the microalgae. In this chapter, the uptake kinetics reveals the mechanism for xylose transportation across microalgal membrane. The discovery of two metabolic enzymes demonstrates the capability for xylose utilization by the microalgae. Finally, the major conclusions of this study are presented in Chapter 6. Tables and figures are shown at the end of each chapter and references are listed at the end of the dissertation.

1.5. Tables and figures

Table 1.1. Algae species with dual-trophic capability

Available species	References
Chlorella sorokiniana	Chen and Johns (1991)
Chlorella vulgaris	Liang et al. (2009)
Chlorella protothecoides	Yan et al. (2011)
Chlorella pyrenoidosa	Chen (1996)
Chlorella saccharophila	Chen (1996)
Chlorella zofingiensis	Liu et al. (2010b)
Tetraselmis suecica	Azma et al. (2011)
Scenedesmus obliquus	Abeliovich and Weisman (1978)
Scenedesmus acutus	Shamala et al. (1982)
Chlamydomonas reinhardtii	Chen & Johns (1995)
Euglena gracilis	Ogbonna et al. (1999)
Haematococcus pluvialis	Hata et al. (2001)
Nitzschia closterium	Tan & Johns (1996)
Navicula pelliculosa	Tan & Johns (1996)

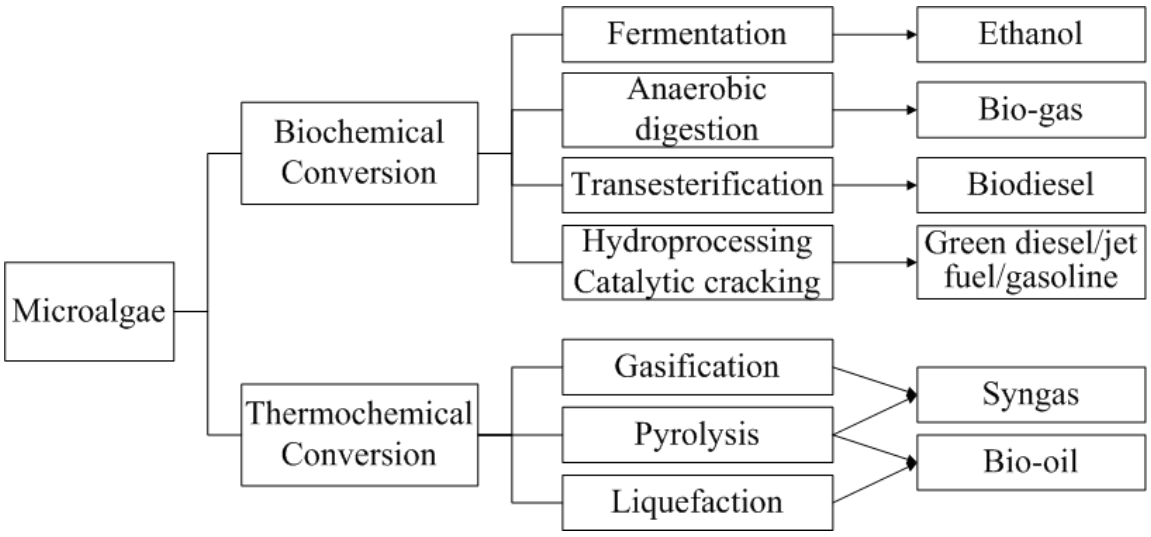


Fig. 1.1. Potential microalgal biomass conversion processes (Brennan & Owende, 2010; Pienkos & Darzins, 2009).

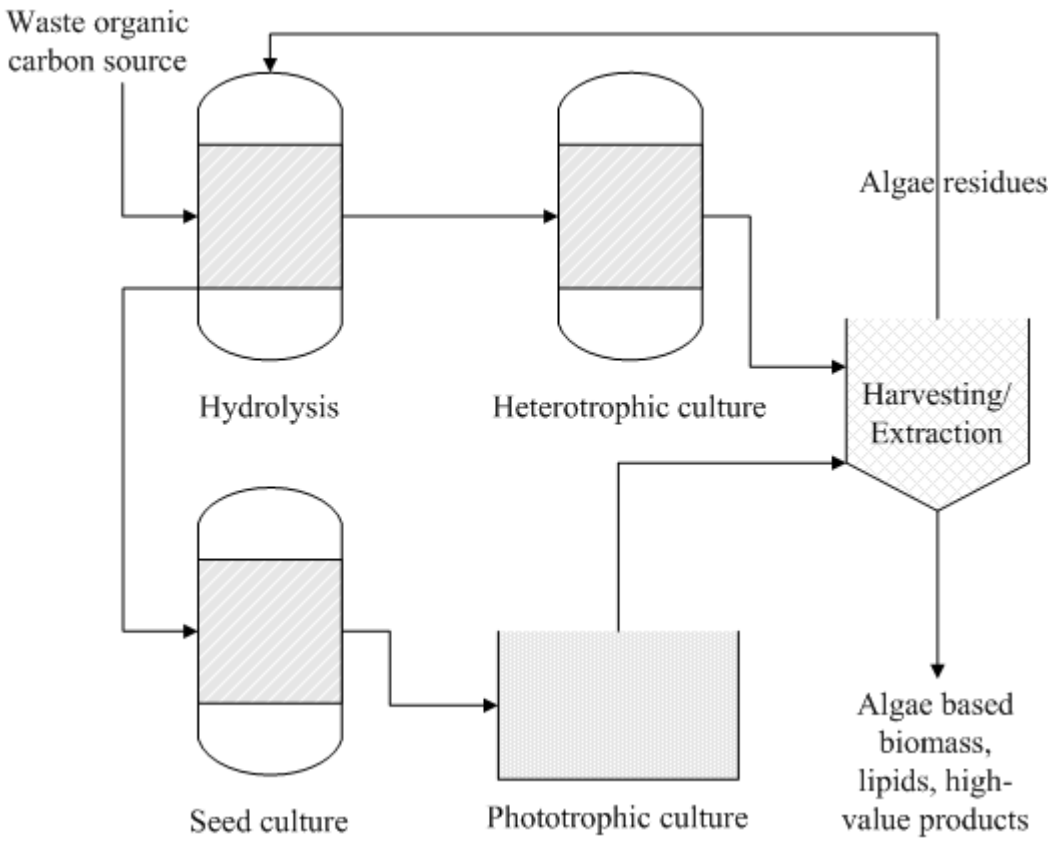


Fig. 1.2. Overview for an integrated system of the two-stage heterotrophic and phototrophic culture of microalgae

Chapter 2

Two-stage heterotrophic and phototrophic culture strategy for algal biomass and lipid production

2.1. Abstract

A two-stage heterotrophic and phototrophic culture strategy for algal biomass and lipid production was studied, wherein high density heterotrophic cultures of *Chlorella sorokiniana* serve as seed for subsequent phototrophic growth. The data showed growth rate, cell density and productivity of heterotrophic *C. sorokiniana* was 3.0, 3.3 and 7.4 times higher than phototrophic counterpart, respectively. Hetero- and phototrophic algal seeds had similar biomass/lipid production and fatty acid profile when inoculated into phototrophic culture system. To expand application, food waste and wastewater were tested as feedstock for heterotrophic growth, and supported cell growth successfully. These results demonstrated the advantages of using heterotrophic algae cells as seeds for open algae culture system. Additionally, high inoculation rate of heterotrophic algal seed can be utilized as an effective method for contamination control. This two-stage heterotrophic phototrophic process is promising to provide a more efficient way for large scale production of algal biomass and biofuels.

Key words: Microalgae; *Chlorella sorokiniana*; heterotrophic; phototrophic; lipid.

2.2. Introduction

Algal lipid is considered as an ideal feedstock for transportation fuels (Pienkos & Darzins, 2009). However, prior to industrial scale application, a series of key challenges have to be resolved. For example, in northern climates, phototrophic biomass production is limited in the winter because of the cold temperature and lack of available sun light. Even in summer months, open algae cultures have relatively low growth rates and biomass productivity (Chisti, 2007). Usually, light limitation is the major limiting factor, since light penetration is inversely proportional to the cell concentration (Chen, 1996). Although a relatively higher biomass productivity can be achieved in photobioreactors (PBRs), its high cost in facility and operation leads to a lower economical viability than open pond (Chisti, 2007). Additionally, open systems are continuously threatened by invading species, such as undesired algae and bacteria. Due to these problems, phototrophic algae are only commercially used to produce high value products (Spolaore et al., 2006). Large scale culture of phototrophic algae for biofuel production still has too high production costs, compared to the produced value (Pienkos & Darzins, 2009).

Compared to phototrophic growth, heterotrophic algae culture takes advantage of fast growth, high production rate, and convenient harvesting. A series of heterotrophic microalgae species were successfully used in industry-scale polyunsaturated fatty acids production (Chi et al., 2009). Recently, heterotrophic microalgae culture to produce biodiesel was reported and showed the promise of this process, however, a high cost of organic carbon is one of limiting factors for this process (Chi et al., 2011a).

To develop an efficient phototrophic process and overcome potential contamination issues, we have investigated a process that takes advantages of both heterotrophic culture's high efficiency and phototrophic culture's low cost. In this two-stage heterotrophic and phototrophic

culture process, heterotrophic culture provided an efficient way for seed cells production, which can be used as inoculums in the subsequent phototrophic open pond cultivation for algal biomass and lipid production.

2.3. Materials and methods

2.3.1. Organism and medium

Chlorella sorokiniana (UTEX 1602) was obtained from the Culture Collection of Alga at the University of Texas (Austin, TX, USA). Kuhl medium was used for phototrophic culture (Kuhl & Lorenzen, 1964). Heterotrophic culture was supplement with different concentrations of glucose, as indicated in individual experiments.

2.3.2. Culture conditions

Flask cultures were conducted in 0.25 L Erlenmeyer flasks. Phototrophic cultures contained 0.2 L Kuhl medium and were bubbled with air supplemented with 0.9% CO_2 at a rate of 0.08 L min^{-1}. Heterotrophic cultures contained 50 mL Kuhl medium with glucose respectively, and incubated at a rotary rate of 200 rpm. For the experiments with bioreactors, phototrophic cultures were carried out with 1-L and 5-L closed PBR containing 1.0 L and 3.0 L Kuhl medium, and bubbled with 0.9% CO_2 in air at the rate of 0.32 and 0.96 L min^{-1}, respectively. Heterotrophic cultures were performed in 5-L NBS Bioflo 110 fermentors (New Brunswick Scientific) with 3.0 L Kuhl medium (20 g L^{-1} glucose). The dissolved oxygen (DO) concentration was maintained at 50% with cascading DO control to agitation speed, and the aeration rate was 3.0 L min^{-1}.

2.3.3. Heterotrophic algae culture with food waste and wastewater

Food waste was collected from a cafeteria at Washington State University, Pullman, WA. The preparation for Food Waste Hydrolyzed Broth (FWHB) was described by Chi et al. (2011a). The primary wastewater was collected from Pullman Wastewater Treatment Plant at Pullman, WA. Heterotrophic cultures were conducted in 5-L fermentors containing 3.0 L mixture of FWHB and primary wastewater (1:1, v/v).

2.3.4. Algal seeds comparison

After heterotrophic culture with the mixture of FWHB and wastewater, the produced algal seeds were harvested as the hetero-seed. The phototrophic cultured algal seeds in the 5-L PBR were used as the photo-seed. For closed system comparison, the hetero- and photo-seeds were inoculated into the 1-L PBR for phototrophic culture. For open system comparison, the hetero- and photo-seeds were inoculated into open tanks containing 40 L Kuhl medium with the depth of 0.15 meters. The open tank was placed in a greenhouse and the temperature was kept around 23°C. The cultures were mixed with air bubbling at the rate of 4.0 L min^{-1}, and pure CO_2 was injected at the rate of 0.2 L min^{-1}. The average photon flux density was 400 μmol m^{-2} s^{-1} during the daytime with sunlight, and 200 μmol m^{-2} s^{-1} in the night with artificial light.

2.3.5. Contamination test

C. sorokiniana was used as the desired alga species, while a native green alga *Chlamydomonas sp.* (with a higher growth rate of 0.75 d^{-1} compared with *C. sorokiniana*) isolated from the northwest pacific area in US and *E. coli* (Top 10, Invitrogen) were used as the contaminants. Cultures were performed in 0.25 L flasks phototrophically. The experimental

groups contained both *C. sorokiniana* and *Chlamydomonas sp.*, or *C. sorokiniana* and *E. coli*, while the control group only contained *C. sorokiniana*.

2.3.6. Analytical procedure

The analytical methods of cell density, dry cell weight (DCW), fatty acids, sugars and chemical oxygen demand (COD) were described by Chi et al. (2011b).

2.4. Results and discussion

2.4.1. Heterotrophic and phototrophic culture of *C. sorokiniana* for seed cells production

Flasks cultures were conducted to investigate the effect of initial glucose on the alga *C. sorokiniana* growth. As shown in Table 2.1, the final algal cell density increased when glucose went up from 5 to 20 g L^{-1}, but the high concentration glucose 40 g L^{-1} showed inhibitory effects. At 20 g L^{-1} glucose, *C. sorokiniana* reached the highest growth rate, cell density and productivity of 1.48 d^{-1}, 397 × 10^6 cells mL^{-1} and 182 × 10^6 cells mL^{-1} d^{-1}, respectively. Compared with heterotrophic growth, phototrophic culture of *C. sorokiniana* showed a shorter lag time of 24 hours. However, the growth rate, final cell density and average productivity was only 0.73 d^{-1}, 261 × 10^6 cells mL^{-1} and 62 × 10^6 cells mL^{-1} d^{-1}, respectively. These results showed that the heterotrophic culture had higher growth rate and cell productivity for the green alga *C. sorokiniana*.

For dual-trophic algae, such as many *Chlorella* species, the uptake of glucose is based on an inducible hexose/H^+ symport system (Tanner, 2000). In the presence of inducer glucose, algae can change the trophic mode in a short time. Additionally, when algae switch from phototrophy to heterotrophy, the transporter activity can increase more than 200 fold. Under heterotrophic

conditions, growth rate, DCW, ATP generated from the supplied energy and the yields of biomass on ATP are higher than those of phototrophic cultures (Liang et al., 2009; Liu et al., 2010b; Yang et al., 2000). Many other algal species outside of the *Chlorella* genus also exhibit higher growth rates under heterotrophy (Azma et al., 2011; Chen & Johns, 1995; Ogbonna et al., 1999), and can be potentially applied for this two-stage heterotrophic and phototrophic system.

To study algal productivity at a larger scale, *C. sorokiniana* was cultured in larger size bioreactors. For heterotrophic culture, a much higher final cell density of 542×10^6 cells mL^{-1} was achieved in the fermentor than that in the 0.25 L flask (Table 2.1), which attributed to the well controlled DO and mixing. However, for phototrophic culture, the scaling up had negative effects on algae growth. The growth rate, final cell density and average productivity in 5-L PBR was only 0.48 d^{-1}, 165×10^6 cells mL^{-1} and 24×10^6 cells mL^{-1} d^{-1}, respectively, which were much lower than those in 0.25-L flask (Table 2.1). In small-size flasks (0.25-L), the growth rate, final cell density and average productivity of heterotrophic culture (20 g L^{-1} glucose) was 2.0, 1.5 and 2.9 times higher than those of phototrophic culture, respectively. In larger scale bioreactors (5-L), the advantage of heterotrophic growth was more obvious, which gave 3.0 fold growth rate, 3.3 fold final cell density and 7.4 fold average productivity compared with phototrophic culture.

Traditionally, seed cells for algae culture are generated in PBRs and/or in open ponds. For these phototrophic culture processes, light is a significant limiting factor. It is suggested that the light is hardly disperse efficiently and evenly inside the PBR at operational volumes of 50-100 L or higher, which leads to a low productivity in large scale production (Perez-Garcia et al., 2011). Heterotrophic culture is more easily to be scaled up and does not compete with illuminated surface area of the dense open cultures, e.g. the heterotrophic cultivation of microalgae has been successfully scaled-up to 50000 L (Chen, 1996). In addition, the heterotrophic culture can use

the existing infrastructures, which results in a significant reduction in costs (Perez-Garcia et al., 2011). It is clearly demonstrated that heterotrophic culture has significant advantages over phototrophic culture in large scale application.

2.4.2. Heterotrophic algae seed culture with food waste and wastewater

The mixture of FWHB and wastewater was used for the heterotrophic culture of *C. sorokiniana* in 5-L fermentor. The results showed that *C. sorokiniana* was able to reach a high cell density of 463×10^6 cells mL^{-1}, which was 2.8 fold higher than that of phototrophic culture in 5-L PBR. Generally the cost of substrates is considered as one of major limitations of heterotrophic fermentation (Chen et al., 2010; Perez-Garcia et al., 2011), but it can be reduced if the low value waste materials is utilized. Our results suggested that FWHB and wastewater were good feedstock for the heterotrophic culture of *C. sorokiniana*, which potentially expanded the application of our approach.

2.4.3. Comparison of phototrophic and heterotrophic algae seeds

To test the two-stage heterotrophic and phototrophic algae culture system, the performances of hetero- and photo-seeds were studied with closed and open systems respectively. In closed PBR, the hetero-seed had a comparable growth curve with the photo-seed (data not shown). The DCW after 336 hours' culture was 2.84 and 2.78 g L^{-1} for hetero- and photo-seeds, respectively. No additional lag time was observed for the hetero-seed, indicating heterotrophically cultured *C. sorokiniana* cells could adapt to phototrophic conditions rapidly. This kind of transition investigated by Vernotte et al. (1992) on cyanobacterium showed the heterotrophic-grown cells could recover a full photosynthetic activity within only 24–48 hours under illumination.

Open ponds represent a more natural algal growth environment and are a more favorable option for industrial cultivation rather than closed PBRs (Perez-Garcia et al., 2011). Therefore, the growths of hetero- and photo-seeds were investigated in the open system. Unlike the performance in closed PBR, the cell density of the hetero-seed was lower than that of photo-seed within first 96 hours. The reason might be the lower recovery rate of the photosynthetic activity in a larger scale open pond. After 96 hours' culture, the growth of the hetero-seed was almost the same as the photo-seed, which might result from the complete recovery of photosynthetic activity. The final DCW was 0.82 and 0.80 g L^{-1} for hetero- and photo-seeds, respectively. The residue sugar from the hetero-seed was completely consumed, and the COD also reduced to a very low level of 0.2 g L^{-1} compared with the initial COD 54.6 g L^{-1} in the FWHB (Table 2.2). These results indicated this two-stage algae culture system could also benefit waste treatment. Moreover, no significant difference of the lipid content and fatty acid profile was detected for these two types of algal seeds after 312 hours of open pond culture (Table 2.3). Clearly, these results demonstrated the feasibility of our two-stage heterotrophic and phototrophic algae culture strategy in both closed PBR and open culture systems.

2.4.4. Contamination control with the hetero-seed

As aforementioned, open ponds algae cultures must compete with foreign species. Here, we investigated varying inoculum magnitude of *C. sorokiniana* under the contamination of invading alga *Chlamydomonas sp.* and bacterial *E.coli* as a way to maintain monocultures. As shown in Fig. 2.1A, when the inoculum size for *C. sorokiniana* was 0.1×10^6 cells mL^{-1}, the competition for nutrients and light was very drastic between these two algae. After 168 hours' culture, *Chlamydomonas sp.* reached a cell density of 38×10^6 cells mL^{-1}, which was about three times

higher than that of *C. sorokiniana*. In Fig. 2.1B, the cell density of *Chlamydomonas sp.* was 26 × 10^6 cells mL^{-1}, whereas, the tenfold inoculum size (1 × 10^6 cells mL^{-1}) brought *C. sorokiniana* to the dominant, and its cell density reached 111 × 10^6 cells mL^{-1}. However, the cell density of *C. sorokiniana* was still about 2.7 fold lower than that of the control, which indicated that the competition from *Chlamydomonas sp.* still had significant negative effects. When the inoculum size reached 10 × 10^6 cells mL^{-1}, *C. sorokiniana* exhibited a comparable growth curve with the control (Fig. 2.1C). The final cell density of *C. sorokiniana* reached 289 × 10^6 cells mL^{-1} (control 300 × 10^6 cells mL^{-1}), while *Chlamydomonas sp.* was only 2 × 10^6 cells mL^{-1}. These results clearly showed that increasing the inoculum size of the desired algal species could overcome the contamination from the invading algae.

To test the effect of inoculum size in competation with bacteria, *E.coli* was inoculated along with the hetero-seed of *C. sorokiniana*. As shown in Fig. 2.2, the cell density of *E. coli* reached the maximum point at the 24^{th} hour, but decreased to a very low density after 48 hours, because there was no additional organic carbon to support its growth. The cell density of *E.coli* increased from 1.3 to 4.2 × 10^6 cells mL^{-1} with inoculums of *C. sorokiniana* from 0.1 to 10 × 10^6 cells mL^{-1}, because more sugars were introduced with higher inoculation of the hetero-seed. Clearly, the growth of *E. coli* adversely affected *C. sorokiniana*. In Fig. 2.2A and Fig. 2.2B, the negative effects of *E. coli* resulted in a lower cell density of *C. sorokiniana*. However, with the highest inoculum size of 10 × 10^6 cells mL^{-1}, the final cell density of *C. sorokiniana* was almost at the same level as the control (Fig. 2.2C). Although the higher inoculum brought more organic carbon to support the growth of bacteria, which would generate negative factors to the growth of algae, it made the algae be dominant and successfully adapted rapidly to the unfavorable environment.

One disadvantage of open pond culture systems is maintenance of monocultures for desired alga (Lee, 2001). Some environmental factors, such as flow rates and retention time, have proven to control the foreign species for outdoor aquaculture production (Theegala et al., 1999). Beyond the algae culture, the concept of inoculum effect has been ascribed for many microorganisms and antimicrobial agents (Steels et al., 2000). Actually it can be used as a tool to control contamination, because increasing the inoculum size can strengthen the ability of microbes to resist unfavorable conditions, whereas, it is not feasible for the traditional phototrophic processes, especially for the open cultures, due to their low cell density and productivity. This contamination control strategy is in accordance with our heterotrophic and phototrophic algae culture system, because the heterotrophic process can produce high density seeds more efficiently than phototrophic cultivation. On the other hand, the low inoculation rate usually has a long lag phase in the culture process, which significantly affects the final productivity. Although semi-continuous culture is used as the strategy for traditional phototrophic process to avoid this problem, the invading species will replace the desired strain gradually if it has a higher specific growth rate. Inoculating open pond system with high rates of heterotrophic seeds can lead to a shorter lag phase, improve the productivity and allow for a more controlled and contaminant-free down-stream phototrophic growth.

Another drawback of the open pond system is highly dependent on local weather conditions, which is hardly controlled and makes the production seasonal (Perez-Garcia et al., 2011). This two-stage algae culture system can operate the heterotrophic and phototrophic culture processes in parallel, allowing the use of both organic and inorganic carbon source to support year-round production, against inclement seasonal weather and/or phototrophic contamination. In that case, the phototrophic algae culture process will be shut down, but the heterotrophic process using

organic waste as feedstock will be run alone to produce oil-enriched algae biomass, which then can be processed into biofuel. Data in Table 2.2 showed that the heterotrophic cultivated algal biomass could accumulate about 23.3% lipid by feeding with FWHB and wastewater without any optimization. These organic waste streams are often readily available and the tipping fee makes it have a higher economic viability. Therefore, this algae culture system has the potential to overcome seasonal weather variations in cold climates, recycle waste nutrients and enhance contamination control, while lowering illuminated surface area.

2.5. Tables and figures

Table 2.1. Heterotrophic and phototrophic culture of *C. sorokiniana* under different conditions. Phototrophic cultures were illuminated with continuously white light at a photon flux density of 150 μmol m^{-2} s^{-1}; heterotrophic cultures were in darkness. The temperature was 27°C. The initial cell density was 2 × 10^6 cells mL^{-1}. Data are represented as the average ± standard deviation of two independent replicate cultures.

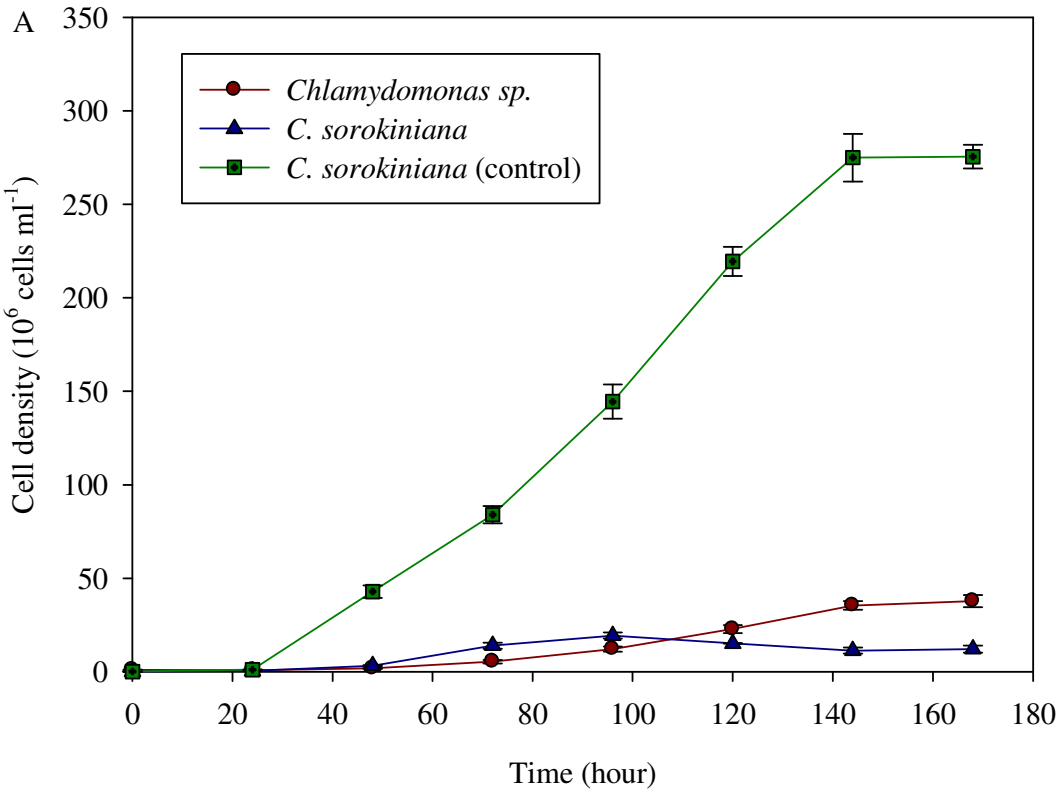
Growth mode	Glucose (g L^{-1})	Reactor	Growth rate (d^{-1})	Cell density (10^6 cells mL^{-1})	Productivity (10^6 cells mL^{-1} d^{-1})	Lag time (h)
Hetero	5	0.25 L flask	1.20 ± 0.02	318 ± 25	131 ± 8	48
Hetero	10	0.25 L flask	1.35 ± 0.04	376 ± 12	169 ± 5	48
Hetero	20	0.25 L flask	1.48 ± 0.04	397 ± 17	182 ± 3	48
Hetero	40	0.25 L flask	1.08 ± 0.07	303 ± 6	112 ± 6	72
Hetero	20	5 L fermentor	1.42 ± 0.03	542 ± 23	178 ± 8	24
Photo	0	0.25 L flask	0.73 ± 0.05	261 ± 29	62 ± 7	24
Photo	0	1 L PBR	0.59 ± 0.01	258 ± 12	48 ± 3	24
Photo	0	5 L PBR	0.48 ± 0.01	165 ± 16	24 ± 2	24

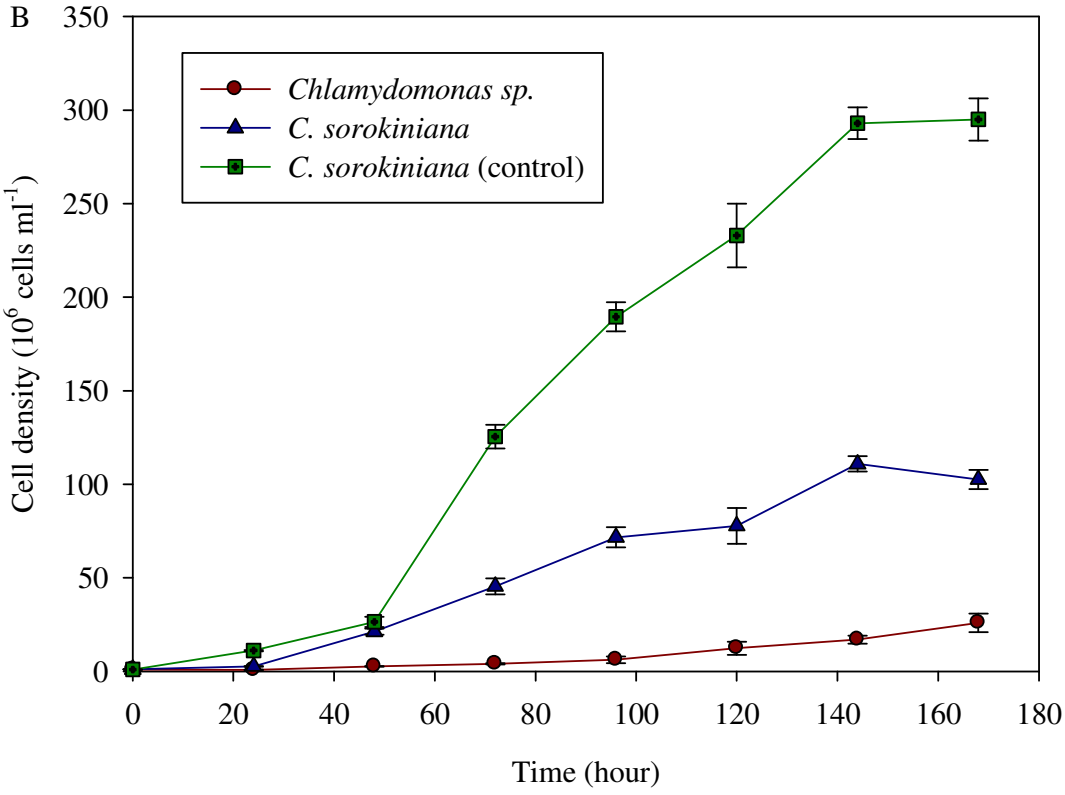
Table 2.2. Culture of *C. sorokiniana* with FWHB and wastewater. Heterotrophic cultures were in 5-L fermentors with the mixture of FWHB and wastewater in the dark. Then the algal seeds were inoculated into 40 L open ponds for phototrophic growth. Data are represented as the average ± standard deviation of two independent replicate cultures.

	FWHB	Wastewater	Heterotrophic culture	Open pond culture
DCW (g L^{-1})	-	-	4.72 ± 0.19	0.82 ± 0.09
Lipid (% dry wt.)	-	-	23.3 ± 2.0	12.8 ± 0.9
Sugar (g L^{-1})	29.7	0.0	4.3 ± 0.3	0.0 ± 0.0
COD (g L^{-1})	54.6	0.4	9.4 ± 0.5	0.2 ± 0.0

Table 2.3. Fatty acid profiles of *C. sorokiniana* in open ponds culture. Hetero- and photo-seeds were inoculated into 40 L open ponds, respectively, with the same initial cell density of 2×10^6 cells mL^{-1}. After 312 hours growth, the algae were harvested. Data are represented as the average ± standard deviation of two independent replicate cultures. There is not a statistically significant difference between these two groups ($p = 0.957$).

	Hetero-seed	Photo-seed
Total Lipid (% dry wt.)	12.8 ± 0.9	12.2 ± 0.6
Fatty acid (% total fatty acid)		
C14:1	2.2 ± 0.1	2.5 ± 0.1
C14:2	7.4 ± 0.7	8.8 ± 0.4
C16:0	13.7 ± 0.4	12.8 ± 0.5
C16:1	4.6 ± 0.3	3.6 ± 0.5
C16:2	5.0 ± 0.6	5.7 ± 0.3
C18:1	14.4 ± 0.5	16.1 ± 0.3
C18:2	14.1 ± 0.6	14.5 ± 0.2
C18:3	26.4 ± 0.4	26.1 ± 0.4


A
Chlamydomonas sp.
C. sorokiniana
C. sorokiniana (control)
Cell density (10^6 cells ml^-1)
Time (hour)
0
50
100
150
200
250
300
350
0
20
40
60
80
100
120
140
160
180


B
Chlamydomonas sp.
C. sorokiniana
C. sorokiniana (control)
Cell density (10^6 cells ml^-1)
Time (hour)
0
50
100
150
200
250
300
350
0
20
40
60
80
100
120
140
160
180

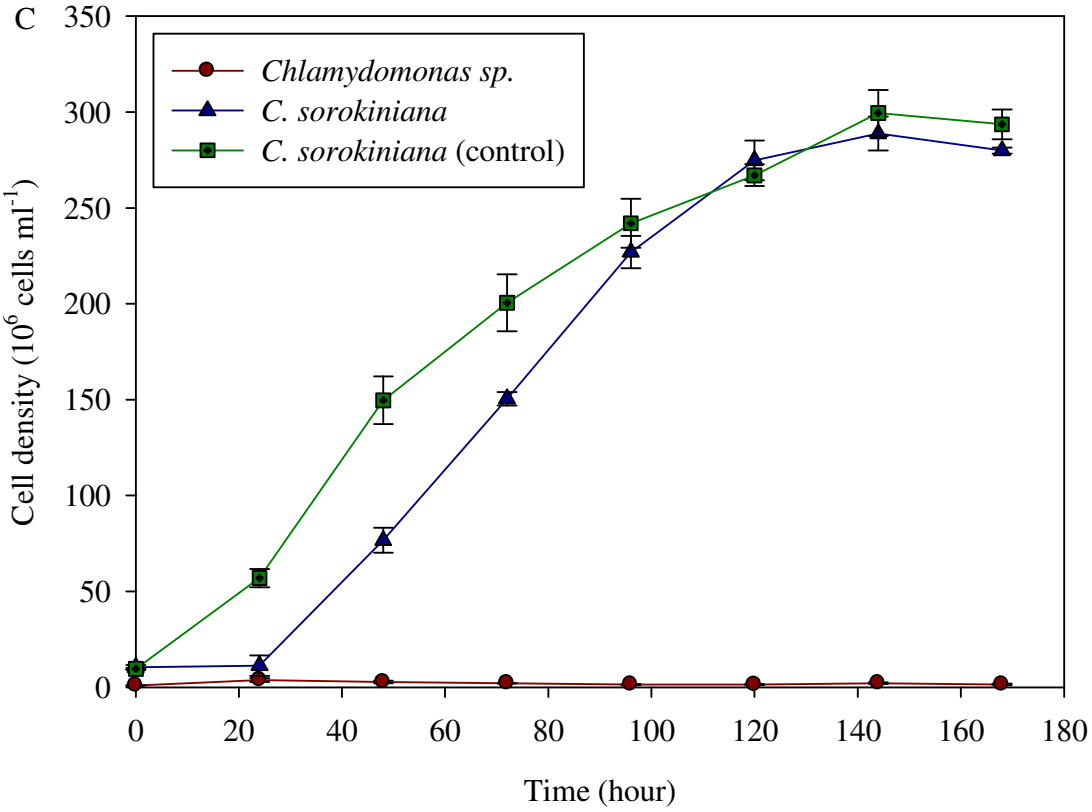
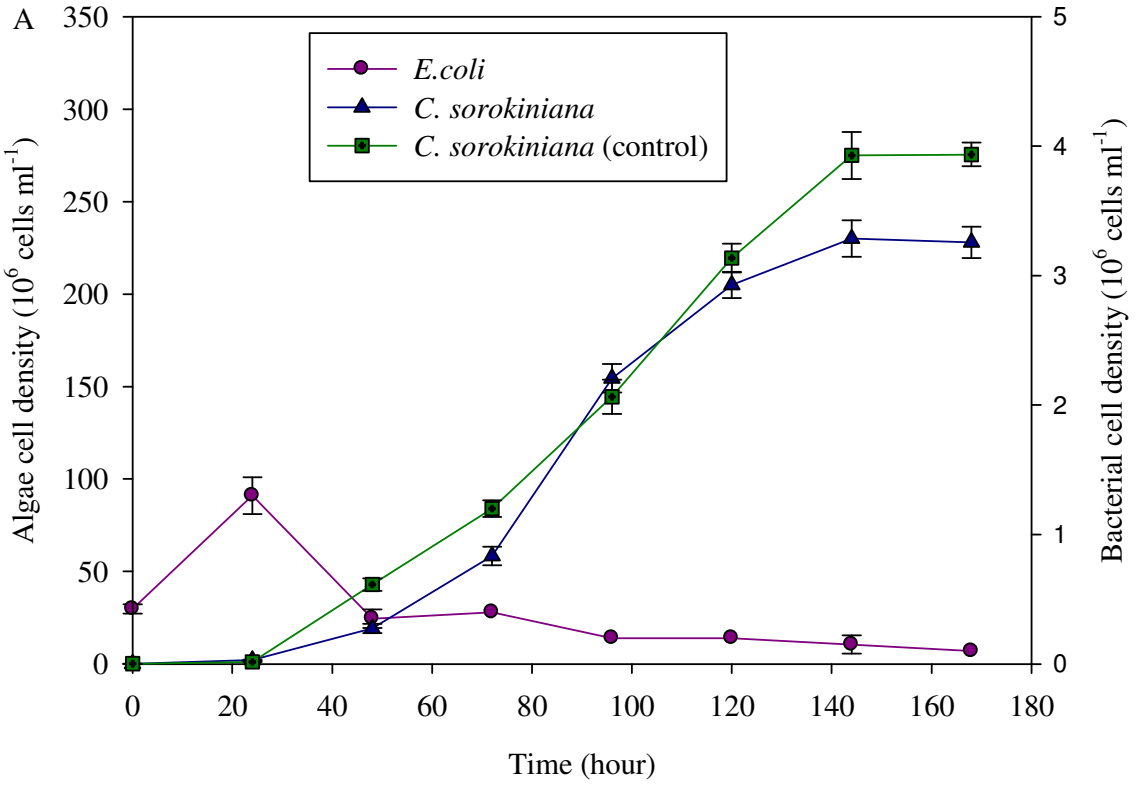
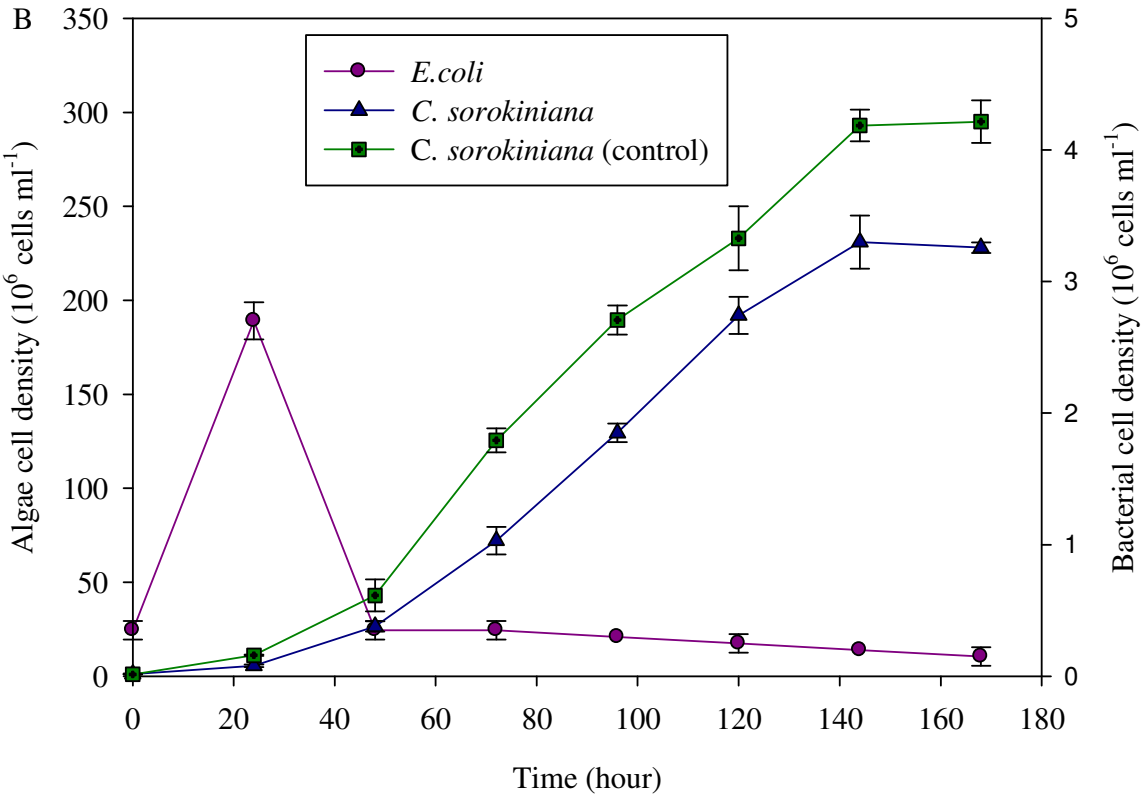


Fig. 2.1. Growth curves of *C. sorokiniana* versus *Chlamydomonas sp.*. Initial cell density of *C. sorokiniana* (10^6 cells mL^{-1}): (A) 0.1; (B) 1.0; (C) 10. The inoculum size of *Chlamydomonas sp.* was 1.0×10^6 cells mL^{-1}. Cultures were bubbled with air supplemented with 0.9% CO_2 at a rate of 0.08 L min^{-1} and illuminated with continuously white light at a photon flux density of 150 µmol m^{-2} s^{-1}. The temperature was 27°C. Data are represented as the average ± standard deviation of two independent replicate cultures.


A
E.coli
C. sorokiniana
C. sorokiniana (control)
Algae cell density (10^6 cells ml^-1)
Bacterial cell density (10^6 cells ml^-1)
Time (hour)
0
20
40
60
80
100
120
140
160
180
0
50
100
150
200
250
300
350
0
1
2
3
4
5


B
E.coli
C. sorokiniana
C. sorokiniana (control)
Algae cell density (10^6 cells ml^-1)
Bacterial cell density (10^6 cells ml^-1)
Time (hour)
0
20
40
60
80
100
120
140
160
180
0
50
100
150
200
250
300
350
0
1
2
3
4
5

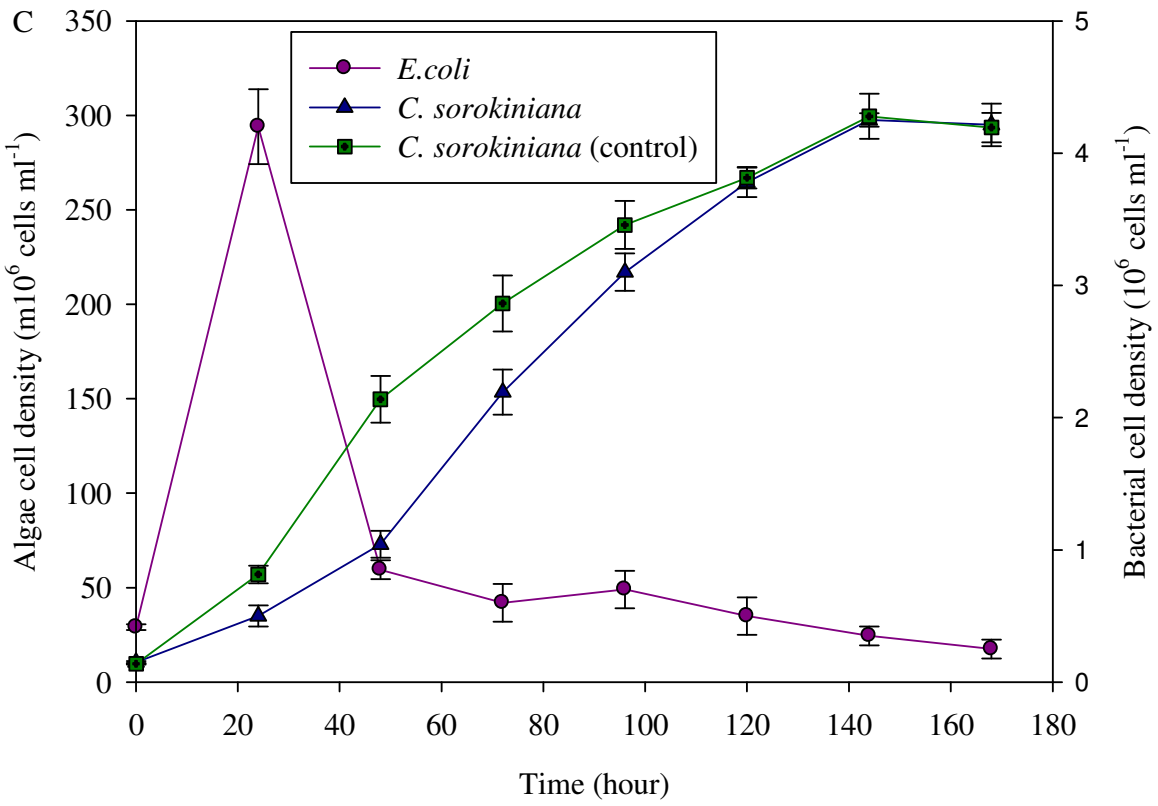


Fig. 2.2. Growth curves of *C. sorokiniana* versus *E. coli*. Initial cell density of *C. sorokiniana* (10^6 cells mL^{-1}): (A) 0.1; (B) 1.0; (C) 10. The inoculum size of *E. coli* was 0.4×10^6 cells mL^{-1}. Cultures were bubbled with air supplemented with 0.9% CO_2 at a rate of 0.08 L min^{-1} and illuminated with continuously white light at a photon flux density of 150 µmol m^{-2} s^{-1}. The temperature was 27°C. Data are represented as the average ± standard deviation of two independent replicate cultures.

Chapter 3

High-density fed-batch culture of a thermotolerant microalga *Chlorella sorokiniana* for biofuel production

3.1. Abstract

Culturing microalgae heterotrophically for producing lipid-based biofuels such as biodiesel and renewable hydrocarbons has attracted increasing attention due to the advantages of fast growth and high lipid yield under this growth mode without being subjected to light limitation. High cell density in the culture broth is desirable for reducing downstream processing costs. Oleaginous microalga *Chlorella sorokiniana* was investigated for high cell density culture with glucose as the carbon source. Best growth performance was obtained first with batch culture at pH 7.0 when ammonium was the nitrogen source. Then, two-stage fed-batch fermentation was conducted under the optimal conditions. The algal biomass grew linearly in the first stage with a productivity of 24.2 g L^{-1} d^{-1}, and the lipid content increased from 14.5% to 38.7% in the second stage. This fermentation strategy resulted in algal biomass and lipid concentrations of 103.8 g L^{-1} and 40.2 g L^{-1} respectively. Analysis of lipid and fatty acid profiles showed *C. sorokiniana* accumulated a large amount of neutral lipids (92.9% of total lipids), triacylglycerols (82.8% of neutral lipids), and high contents of palmitic, oleic and linoleic acids, which are ideal form of lipid for making biodiesel. These results suggest that heterotrophic culture of *C. sorokiniana* holds great potential for lipid-based biofuel production.

Keywords: *Chlorella sorokiniana*; algae; heterotrophic; fed-batch culture; lipid; biofuel.

3.2. Introduction

Microalgal biomass as a feedstock for producing renewable fuels has attracted great attention in the recent years due to primarily its high potential productivity (Pienkos & Darzins, 2009). Phototrophy and heterotrophy are two growth modes of microalgae that both have commercial potential. Phototrophic culture of algae in open ponds is a more favorable option than closed photobioreactor systems due to the low production costs (Davis et al., 2011; Perez-Garcia et al., 2011). However, prior to large scale application of phototrophic microalgal biomass cultivation for biofuel production, a series of key challenges have to be resolved, i.e., susceptible to contamination, light limitation, low productivity and difficulties in harvesting (Zhang & Hu, 2012; Zheng et al., 2012a). Heterotrophic cultivation eliminates the requirement for light and takes advantage of fast growth, high production rate, high degree of process control and low cost harvesting due to the high cell density (Chen & Chen, 2006). Heterotrophic cultivation can be accomplished with mature fermentation technologies and facilities, such as those used for industrial beverages, medicines and food additives production, which results in a significant reduction in costs compared with closed photobioreactor systems (Eriksen, 2008; Perez-Garcia et al., 2011). A major limitation of heterotrophic algal cultivation is the sustainability and cost of organic carbon source, but using of lignocellulose-derived sugars offers a solution (Zheng et al., 2012b).

Among algal species with industrial potential, the green microalga *Chlorella* is attractive because it can grow both phototrophically and heterotrophically with a high biomass concentration (Liu et al., 2010a). *Chlorella* is one of the commercially important microalgae with world annual sales of more than US$ 38 billion (Spolaore et al., 2006). Many high-value chemicals can be produced from *Chlorella*, such as β-1,3-glucan (an active immunostimulator, a

free radical scavenger and a reducer of blood lipids), and carotenoids (protection against UV-caused skin damage, macular degeneration, cancers and age-related degenerative diseases) (Spolaore et al., 2006; Wang & Chen, 2008). Recently, heterotrophic culture of *Chlorella* has shown promise for biodiesel production, which is attributed to its high growth rate and oil content (Liu et al., 2010a). Biodiesel made from heterotrophic *Chlorella* meets the ASTM standard in terms of density, viscosity, flash point, cold filter plugging point, solidifying point, and heating value (Xu et al., 2006). Liu et al. (2010b) also demonstrated that the oil extracted from heterotrophically grown cells was more feasible for biodiesel production than the photoautotrophic cells due to the higher yields of total lipids, neutral lipids, triacylglycerol (TAG) and oleic acid. Cell density is one of the most important factors affecting the economics of algae based biofuels. Studies on high density culture were performed in order to reduce the process cost. Liu et al. (2010a) cultured *Chlorella zofingiensis* with fed-batch fermentation, and the lipid concentration reached 20.7 g L^{-1}. Higher cell density of 51.2 g L^{-1} and lipid concentration of 25.8 g L^{-1} were achieved by *Chlorella protothecoides* (Xiong et al., 2008). However, the biomass concentration is still low compared to some commercial oleaginous microalgae like *Crypthecodinium cohnii* and *Schizochytrium* sp. that were reported to reach biomass concentration higher than 100 g L^{-1} (Chen & Chen, 2006; Chi et al., 2009).

It was demonstrated that the green alga *Chlorella sorokiniana* could grow at a higher rate on glucose in the dark compared with the phototrophic cultivation (Zheng et al., 2012a). In our previous work, we found that the thermotolerant characteristics of *C. sorokiniana* (tolerate up to 42°C with optimal growth at 37°C) greatly enhanced its growth performance. Cell density of 37.6 g L^{-1} with the lipid content of 31.5% was obtained in only 72 hours under optimal batch fermentation conditions (Li et al., 2013). The high growth rate makes *C. sorokiniana* a potential

species for high density cultivation. However, further optimization is necessary since our data showed that the consumption of different nitrogen sources by *C. sorokiniana* might result in pH change of the culture broth, which subsequently affected the algal growth. Moreover, screening a less expensive nitrogen source (i.e. ammonium) can benefit the cost reduction. Actually, not all lipids produced from microalgae are equally suitable for biofuel production. Only neutral lipids especially triacylglycerols (TAGs) are ideal starting material to make high energy density transportation fuels, i.e., biodiesel and renewable hydrocarbons (Pienkos & Darzins, 2009). Therefore, detailed lipid and fatty acid profiles of *C. sorokiniana* will be useful for assessing their appropriateness as biofuel feedstocks.

The objective of this study was to further improve the biomass productivity and lipid concentrations of *C. sorokiniana*, and investigate the potential of this algal species as a feedstock for lipid based biofuel production. Firstly, the relationship between pH change and nitrogen consumption was studied in order to improve the growth rate and select a low cost nitrogen source for *C. sorokiniana*. Then, fed-batch cultivation was tested for achieving high cell density and oil accumulation. Finally, evaluation of the lipid and fatty acid profiles extracted from *C. sorokiniana* were performed for the purpose of making biodiesel.

3.3. Materials and methods

3.3.1. Organism and medium

The green microalga *Chlorella sorokiniana* (UTEX 1602) was obtained from the Culture Collection of Algae at the University of Texas (Austin, TX, USA). This strain was maintained at 4^{o}C on an agar slant of Kuhl medium (Kuhl & Lorenzen, 1964) supplemented with 10 g L^{-1} glucose. The minimal medium was used in all batch and fed-batch cultivations and consisted of

(per L) 621 mg $NaH_2PO_4 \cdot H_2O$, 89 mg $Na_2HPO_4 \cdot 2H_2O$, 246.5 mg $MgSO_4 \cdot 7H_2O$, 9.3 mg EDTA, 0.061 mg H_3BO_3, 14.7 mg $CaCl_2 \cdot 2H_2O$, 6.95 mg $FeSO_4 \cdot 7H_2O$, 0.287 mg $ZnSO_4 \cdot 7H_2O$, 0.01235 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.169 mg $MnSO_4 \cdot H_2O$, and 0.00249 mg $CuSO_4 \cdot 5H_2O$.

3.3.2. Batch culture

To investigate the effects of pH on the growth of *C. sorokiniana*, batch cultures were carried out in a 1-L fermentor (New Brunswick Scientific, CT, USA) containing 0.5 L minimal medium supplemented with 20 g L^{-1} glucose and 2.0 g L^{-1} KNO_3. The pH values were maintained at 5.0, 6.0, 7.0, 8.0 and 9.0 by feeding with 2 mol L^{-1} NaOH or H_2SO_4 solutions. To study the nitrogen sources on cell growth, NH_4Cl, KNO_3, NH_4NO_3 and yeast extract at the same nitrogen concentration of 20 mmol L^{-1} were added into the culture medium respectively with or without pH control.

3.3.3. Fed-batch culture

In order to achieve high biomass and lipid concentration, *C. sorokiniana* was cultured in fed-batch mode. Primary fed-batch culture was conducted in a 5-L fermentor (New Brunswick Scientific, CT, USA) containing 2.0 L minimal medium supplemented with 20 g L^{-1} glucose and 2.0 g L^{-1} KNO_3 (C/N ratio 29/1) (Li et al., 2013). The glucose concentration was maintained between 10 g L^{-1} to 60 g L^{-1} by feeding with concentrated culture medium containing 500 g L^{-1} glucose, 50 g L^{-1} KNO_3 and 25 × minimal medium. The pH value was maintained at 6.0 by feeding with 2 mol L^{-1} H_2SO_4 solution.

To improve the biomass and lipid concentration, a two-stage fed-batch fermentation strategy was conducted. The initial culture contained 2.0 L minimal medium supplemented with 20 g L^{-1}

glucose and 1.1 g L^{-1} NH_4Cl. Nitrogen stock solution containing 185 g L^{-1} NH_4Cl and 50 × minimal medium was fed with glucose stock solution (700 g L^{-1} pure glucose) at C/N ratio of 29/1 in the first 48 h of cultivation, and later on only glucose stock solution was added. The pH value was maintained at 7.0 by feeding with 10 mol L^{-1} NaOH solution.

The fermentors for batch and fed-batch cultures were covered with aluminum foil to keep the cultures in darkness. The dissolved oxygen concentration was maintained at 50% by changing the agitation speed and the aeration rate. The temperature was controlled at 37°C. All the media including the stock solution were autoclaved at 121°C for 20 min before cultivation.

3.3.4. Analytical procedure

For algal biomass determination, 5 mL cell suspension samples were transferred to a centrifuge tube and centrifuged at the speed of 1000 × g for 5 min. The cell pellet was washed three times with distilled water to remove the residual sugars and chemicals, and then dried in a pre-weighed aluminum dish at 105°C for 3 hours. Glucose and nitrate concentrations were analyzed according to Li et al. (2013). The high range (2–47 mg L^{-1}) ammonia TNTplus testing kit (Hach Company, CO, USA) was used in ammonium nitrogen analysis, and Hach Method 10205 was followed.

The lipids were extracted with a solvent mixture of chloroform-methanol-water (2:1:0.75, v/v) based on the modified Folch procedure (Christie, 2003). The analysis of lipid and fatty acid profiles was performed according to the description by Bates and Browse (2011). Individual lipid class was separated with one-dimensional thin-layer chromatography (TLC), silica gel 60 20 x 20 cm glass plates (EMD Millipore, MA, USA), using a solvent mixture of hexane-diethyl ether-acetic acid (70:30:2, v/v). Visualization was carried out by staining with primuline (Sigma,

MO, USA) and lipid bands were identified under UV light. After identification, individual lipid bands were scraped out, and 1 mL 2.5% H_2SO_4 (v/v) in methanol was added. Fatty acid methyl esters (FAMEs) were prepared by heating the mixture at 80°C for 90 min, and then extracted with 200 μL hexane and 1.5 mL 0.5% NaCl (w/v). FAME composition and quantity were analyzed on an Agilent 6890 gas chromatograph (GC) (Palo Alto, CA, USA) with flame ionization detection on an EC Wax column, 30 m × 0.53 mm i.d. × 1.20 μm. The auto injector injected 5 μl of the sample in hexane into the inlet at 250°C with a split ratio of 10:1. Initial oven temperature was 210°C held for 1 min, and increased to 240°C at 10°C/min held for 2 min. The carrier gas was helium. The detector temperature was kept at 250°C. Pentadecanoic acid (C15:0) was used as the internal standard for quantification.

3.4. Results and discussion

3.4.1. Effects of pH on *C. sorokiniana* growth

The growth parameters of *C. sorokiniana* at different culture pH were tabulated in Table 3.1. Among the tested pH ranged from 5.0 to 7.0, the highest specific growth rate of 0.132 h^{-1} and biomass productivity of 10.2 g L^{-1} d^{-1} were obtained at pH 7.0. Comparable specific growth rate and biomass productivity were exhibited when *C. sorokiniana* was cultured at pH 6.0 and pH 8.0. Although the alga grew much faster at pH 7.0, the maximum biomass was at the same level after 48 hours' cultivation from pH 6.0 to 8.0. Very poor growth was observed when the environmental pH reached 9.0. The specific growth rate was only 0.031 h^{-1} and biomass productivity was approximately ten times lower than that at pH 7.0. However, pH did not play a significant role in the biomass yield (the amount of produced biomass per gram of sugar consumed), which indicated that the capability to convert glucose to algal biomass for *C.*

sorokiniana might not be sensitive to pH (in the range of 6.0 to 9.0). Moreover, acidic environment was deleterious to *C. sorokiniana* because it could not survive at pH 5.0. In summary, the environmental pH from 6.0 to 8.0 was a suitable range for the alga *C. sorokiniana* growth and the optimal pH was 7.0.

Microalgae have the capability to grow over a wide pH range, but the suitable pH for growth is largely species-dependent. For instance, *Chlamydomonas* (UTCC 121) is an acid-tolerant green alga that can survive at pH 2.5 (Balkos & Colman, 2007). Many blue-green algal strains are alkali-tolerant and can grow at pH up to 11.0 (Summerfield & Sherman, 2008). The culture pH plays a significant role in the growth of microalgae. Gehl and Colman (1985) reported that the external pH could affect the internal pH for the green alga *Chlorella*. They found that relative constant internal pH of *Chlorella* was kept at pH 7.3 over the external pH range from 5.0 to 7.5, however, a gradual decrease in the internal pH to 6.3 was observed when external pH was below 5.0. Tanner (2000) demonstrated that hexose/H^+-symporter proteins of *Chlorella* were fully active at an outside pH of 6.0, whereas they were almost complete inactive and the sugar uptake rate was reduced by 90% when the internal pH was around 6.0. These phenomenon were in agreement with our results that the glucose consumption rate was maintained at a high level between pH 6.0 to 7.0 but the uptake was almost ceased at pH 5.0 (Table 3.1).

3.4.2. pH dependent nitrogen source screening

The relationship between nitrogen consumption and pH change was shown in Fig. 3.1. The pH dropped dramatically from 7.0 to 4.0 with the consumption of ammonium from 19.7 mmol L^{-1} to 15.8 mmol L^{-1} in the first 6 hours. From 6 to 24 hours, ammonium was slowly consumed from 15.8 mmol L^{-1} to 14.6 mmol L^{-1}, resulting in continuous pH decrease to 3.1. After that, the

consumption of ammonium stopped and pH did not further decrease. The biomass also increased from 0.8 g L^{-1} to 1.0 g L^{-1} in the first 6 hours and then began to decrease, which was identical with our previous result that *C. sorokiniana* could not grow at pH 5.0 or lower. On the other hand, the pH increased gradually until the nitrogen was exhausted when *C. sorokiniana* was grown on nitrate. In batch culture without pH control, the uptake of ammonium led to pH decrease, while nitrate consumption resulted in increase in pH. This might be the reason why nitrate was more suitable nitrogen source for *C. sorokiniana* in flask culture (without pH control). The environmental pH does not only affect the sugar uptake for the microalgae but also the nitrogen metabolism. It has been reported that the glutamate dehydrogenase (for ammonium metabolism) and nitrate reductase (for nitrate metabolism) isolated from *Chlorella* preferred neutral pH, so the acidic or alkali condition would inactivate the enzymes and inhibit nitrogen metabolism (Gronostajski et al., 1978; Kay & Barber, 1986). Thus the change of pH due to the consumption of ammonium or nitrate may be the major factor affecting the growth of *C. sorokiniana*. Besides the environmental pH, other factors such as temperature, carbon/nitrogen sources, and C/N ratio also play a significant role in the cultivation of the microalgae (Chen & Chen, 2006; Li et al., 2013).

To eliminate the effect of pH change on the algal growth, *C. sorokiniana* was cultured with different nitrogen sources with controlled pH at 7.0. As shown in Table 3.2, the algal biomass reached 12.5 g L^{-1} on ammonium with controlled pH, while no growth was observed without pH control. When nitrate was utilized as nitrogen source, the controlled pH led to a slightly higher biomass than that without pH control. Comparable specific growth rate and biomass productivity were obtained for using ammonium and nitrate, but the nitrogen consumption rate and biomass yield per gram nitrogen consumed on ammonium were higher than those on nitrate. Perez-Garcia

et al. (2011) indicated that the most preferred nitrogen source for algae was ammonium due to the less energy requirement for its uptake, while the nitrate assimilation consumed more energy, carbon, and protons. Moreover, yeast extract did not result in significant pH change (data not shown) and comparable biomass was obtained without or with pH control. Our results showed that yeast extract was a less preferred nitrogen source for *C. sorokiniana* compared to ammonium and nitrate, although the organic nitrogen source has typically been more suitable for *Chlorella* growth without causing drastic pH fluctuations (Shi et al., 2000; Xiong et al., 2008).

In the presence of both ammonium and nitrate, *C. sorokiniana* assimilated ammonium in priority of nitrate under controlled pH (Fig. 3.2). In the first 9 hours, ammonium was consumed quickly from 10.0 mmol L^{-1} to 1.1 mmol L^{-1}, while nitrate concentration remained at a constant level. After ammonium was depleted, the alga began to uptake nitrate. Therefore, without pH control, the consumption of ammonium prior to nitrate would cause a drop in the pH and inhibit the algal growth. Generally, it is considered that the reduction of nitrate uptake rate in the presence of ammonium is not only due to the inherent preference of ammonium, but also ammonium inhibits the nitrate transporter and nitrate reductase activities (Kobayashi et al., 1997). The preference for ammonium is important because ammonium is much cheaper than nitrate and organic nitrogen sources (the amount of consumed acid and base for pH control was almost at the same level during the fed-batch fermentation), which may make the large scale application more economical (Erisman et al., 2007; Lofgren et al., 2008).

3.4.3. Fed-batch fermentation

Inhibitory effects on *C. sorokiniana* were observed when initial glucose concentration was above 80 g L^{-1} in our previous study (Li et al., 2013). To achieve much higher cell density, fed-

batch cultivation was conducted to maintain the glucose concentration at a proper range for the alga growth. The time course of biomass, lipid and glucose for primary fed-batch fermentation is shown in Fig. 3.3A. Over a period of 132 h, 79.1 g L^{-1} biomass and 11.4 g L^{-1} lipid were produced with the productivity of 14.3 g L^{-1} d^{-1} and 2.1 g L^{-1} d^{-1} respectively. Although higher algal biomass concentration was obtained compared with our previous batch fermentation (37.6 g L^{-1}), the lipid content was only 14.4% in this study by feeding with concentrated medium at the fixed C/N ratio (Li et al., 2013).

Thus, two-stage fed-batch strategy was adopted to improve the lipid production: carbon and nitrogen substrates were fed to the culture together at the ratio of 29:1 in the first stage, and during the second stage the culture was only supplemented with pure carbon source. Ammonium was used as the nitrogen source and the optimal pH 7.0 was maintained by feeding with 10 mol L^{-1} NaOH solution. As shown in Fig. 3.3B, a two-stage growth curve, the first stage (0–72 h) and the second stage (72–228 h), was presented for *C. sorokiniana*. During the period of 6–24 h, the algae grew exponentially with an estimated specific growth rate of 0.137 h^{-1}, which was much higher than those of the commercial oleaginous microalgae *C. cohnii* and *Schizochytrium* sp. (de Swaaf et al., 2003a; Ganuza et al., 2008; Qu et al., 2011). The algal biomass increased linearly from 1.3 g L^{-1} to 73.9 g L^{-1} in the first stage with the productivity of 24.2 g L^{-1} d^{-1}, and the biomass yield was 0.47 g g^{-1} glucose. However, only 10.7 g L^{-1} lipid (14.5% of the dry biomass) was obtained in this stage. As the second stage set in, the lipid content increased until 228 h and a maximum lipid content of 38.7% was achieved. The lipid yield was 0.19 g g^{-1} glucose and 29.5 g L^{-1} lipid was produced during this period, which was almost equal to the increased amount of algal biomass (29.9 g L^{-1}). This indicated that biosynthesis of lipid was the major activity for *C. sorokiniana* in the second stage of cultivation. After 228 h, the biomass and lipid concentration

began to decrease although there was still more than 30 g L^{-1} glucose in the culture medium. The purpose to maintain a high glucose concentration at the end of fermentation was to prove that the cell density already reached the highest point and carbon source was not the limiting factor for the further biomass increase. In practice, the feeding strategy can be improved to avoid excessive glucose residue. If there are still lots of glucose remained, they can be recycled during the algae biomass separation process.

Fed-batch cultivation is considered as an effective fermentation technology to enhance the cell density and lipid content of oleaginous microbes by avoiding substrates inhibition. However, suitable algal strains with the capability to efficiently uptake organic substrates like glucose are required in order to achieve high cell density and volumetric productivity. Therefore, only a few heterotrophic algal species have been evaluated with the fed-batch technology especially for lipid production. Table 3.3 summarizes the performances of some oleaginous microalgae in fed-batch fermentation when glucose was used as the substrate. The final biomass 103.8 g L^{-1}, lipid 40.2 g L^{-1} and lipid productivity 4.2 g L^{-1} d^{-1} were achieved for *C. sorokiniana* in this study, which were the highest values compared with other reported oleaginous *Chlorella* species (Liu et al., 2010a; Xiong et al., 2008). In addition, comparable biomass and lipid concentrations with *Schizochytrium* sp. were observed for *C. sorokiniana*. *Schizochytrium* sp. is a commercial heterotrophic microalga for the production of polyunsaturated fatty acids (PUFAs), like docosahexaenoic acid (DHA; 22:6 n-3), which has clinical benefits in prevention of cardiovascular and inflammatory diseases, and development of brain (Chen & Chen, 2006; Chi et al., 2009; Qu et al., 2011). These results indicate that the lipid producing thermotolerant microalga *C. sorokiniana* has great promise for industrial application. However, the lipid content of *C. sorokiniana* was only 38.7% that was lower than *Schizochytrium* sp. and other *Chlorella*

species. As described by Li et al. (2013), although the thermotolerant characteristics of *C. sorokiniana* could greatly enhance the growth rate and biomass productivity, the high temperature played a negative impact on the lipid accumulation. Therefore, low temperature may be adopted during the second stage to increase the lipid content for this two-stage fed-batch fermentation process in the future.

3.4.4. Lipid and fatty acid profiles

Heterotrophic culture of microalgae with fed-batch technology greatly enhances the lipid production, but not all lipids extracted from microalgae are suitable for biodiesel production. To evaluate the potential to produce biodiesel from *C. sorokiniana*, the lipid and fatty acid profiles were analyzed. The fractionation of lipid extracted from heterotrophically grown *C. sorokiniana* is shown in Fig. 3.4. *C. sorokiniana* accumulated a large amount of neutral lipids (92.9% of total lipids), which were comprised of mainly 82.8% TAG and 12.0% free fatty acid (FFA). Although TAG is a suitable feedstock for biodiesel production, the high content of FFA is very difficult to be converted to biodiesel with an alkaline catalyst, attributed to the feedstock losses from neutralization and negative effects of soap on glycerin separation. However, this problem can be resolved by adding an acid-catalyzed pretreatment to esterify FFA prior to the alkali-catalyzed transesterification (Canakci & Gerpen, 2001). In addition, 7.1% polar lipids was observed in the total lipids of *C. sorokiniana*, which was higher than the amount derived from *Schizochytrium* (4.1%) but much lower than that of *C. zofingiensis* (19.1%) (Fan et al., 2007; Liu et al., 2010b). Polar lipids, especially phospholipids, are not desirable for biodiesel production due to the phosphate moiety. High content of phosphorus plays a negative role in the transesterification process, and also affects the performance of the catalytic converters (Lu et al., 2009). However,

similar with FFA, a pretreatment process, removal of the phosphate moiety by hydrolysis or phospholipase enzymes treatment, can be adopted for biodiesel production from phospholipids (Foley et al., 2011).

The amounts of each fatty acid present in the feedstocks are critical to the physical properties of biodiesel because transesterification does not change the fatty acid composition (Ramos et al., 2009). Thus, it is necessary to investigate the fatty acid composition of *C. sorokiniana* in terms of biodiesel production. Table 3.4 shows the fatty acid distribution in individual lipid class of *C. sorokiniana*. Palmitic (C16:0), oleic (C18:1) and linoleic (C18:2) acids were the major fatty acids in both polar and neutral lipids, which was similar with the fatty acid composition of soybean oil (Canakci & Gerpen, 2001). It was observed that steroid ester (SE) was rich in saturated fatty acids mainly from palmitic (C16:0) and stearic (C18:0) acids. As the major lipid class, TAG contained the highest PUFA content of 40.2%, but it was still much lower than soybean oil (60.5%). Indeed, the high content of PUFA will lead to satisfactory fuel properties at low temperatures (Zheng et al., 2012b). However, excess unsaturated fatty acids especially long-chain PUFA play an adverse effect on the oxidative stability of biodiesel, and too many double bonds will result in low cetane numbers that tend to increase gaseous and particulate exhaust emissions attributed to incomplete combustion (Luque, 2010; Ramírez-Verduzco et al., 2012). For example, the iodine value of 137 g I_2/100 g biodiesel and cetane number 46.6 were obtained for soybean oil, while the European biodiesel standard (EN14214) specifies the iodine value should be lower than 120 and cetane number higher than 51.0 respectively. EN14214 standard also requires that the polyunsaturated methylester (>= 4 double bonds) should be lower than 1%. In this case, the microalga *Schizochytrium* rich in DHA is undesirable feedstock for biodiesel production due to its high content of PUFA (38.3% >= 4 double bonds), as well as high iodine

value (172 g I_2/100 g) and low cetane number (38.1), although it shows the highest lipid productivity in fed-batch fermentation compared with other heterotrophic oleaginous microalgae (Table 3.3). This is in accordance with the description by Luque (2010) that not all the microalgal species are suitable for biodiesel production although they can produce more than 50% lipid of their biomass. Compared with *Schizochytrium* and soybean, the estimated biodiesel properties from the lipid of *C. sorokiniana* matched the EN14214 criteria very well, in terms of iodine value (< 120), cetane number (> 51.0), viscosity (3.5–5.0 $mm^2 s^{-1}$, 40°C), density (860–900 kg m^{-3}), PUFA (>= 4 double bonds, < 1%) and linolenic acid (C18:3, < 12%).

Growing microalgae heterotrophically can lead to high cell density and oil content which will benefit the downstream process and reduce production cost. It has been reported that the lipid produced from heterotrophically grown microalgae was more suitable for biodiesel production than phototrophic cells because of the better lipid and fatty acid profiles (Liu et al., 2010b). However, the hurdle is the availability and cost of organic carbon source. As an alternative resource, lignocellulosic materials can be used as the feedstock to culture *C. sorokiniana* due to their abundance and relatively low cost. It was estimated that over 1.3 billion dry tons of lignocellulosic biomass could be produced annually in the US on a sustainable basis for biofuel production (Perlack et al., 2005). Taking wheat straw as an example, 4.4 million tons glucose can be obtained based on the annual yields 11 million dry tons in US (Zheng et al., 2012b). From our fed-batch fermentation, the highest lipid yield was 0.19 g g^{-1} glucose, which could lead to annual biodiesel yield of 214 million gallon only using wheat straw as feedstock (if 90% neutral lipid was converted to biodiesel). In previous research, we have successfully demonstrated the feasibility to culture yeasts and fungi for lipid production with wheat straw (Yu et al., 2011; Zheng et al., 2012b). We also found *C. sorokiniana* could utilize avicel cellulose as the substrate

efficiently if the strategy of simultaneous saccharification and fermentation (SSF) was applied, because its thermotolerant characteristics (37–42 °C) was favorable for the enzymatic hydrolysis of cellulose (unpublished data). It was suggested that 70% cost reduction could be achieved by using lignocellulosic biomass derived sugars compared with the commercial raw sugars (Zheng et al., 2012b). Thus, further study on cultivation of *C. sorokiniana* with lignocellulosic materials, such as wheat straw, corn stover and switch grass, will be conducted to reduce the production cost.

3.5. Tables and figures

Table 3.1. Cell growth parameters of *C. sorokiniana* at various pH

Parameters	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 9.0
μ (h^{-1})	-[a]	0.098	0.132	0.100	0.031
$X_{biomass}$ (g L^{-1})	-	11.1	11.2	11.1	2.9
$P_{biomass}$ (g L^{-1} d^{-1})	-	8.2	10.2	8.2	1.0
R_{Glu} (g L^{-1} d^{-1})	-	17.5	20.5	16.8	2.1
$Y_{biomass/glu}$ (g g^{-1})	-	0.47	0.50	0.49	0.48

μ, specific growth rate; $X_{biomass}$, biomass concentration; $P_{biomass}$, biomass productivity; R_{Glu}, glucose consumption rate; $Y_{biomass/glu}$, biomass yield per gram glucose consumed.

[a] The algae did not grow at pH 5.0.

Table 3.2. Cell growth parameters of *C. sorokiniana* on ammonium and nitrate

Nitrogen	pH	μ (h^{-1})	$X_{biomass}$ (g L^{-1})	$P_{biomass}$ (g L^{-1} d^{-1})	R_N (mmol L^{-1} d^{-1})	$Y_{biomass/N}$ (g g^{-1})
Ammonium	w/o	-[a]	-	-	-	-
	w/	0.133	13.0	10.7	25.2	49.1
Nitrate	w/o	0.114	10.7	9.1	17.5	40.1
	w/	0.132	11.2	10.2	18.5	42.0
Yeast extract	w/o	0.098	3.8	2.7	/	/
	w/	0.100	3.9	2.7	/	/

R_N, nitrogen consumption rate; $Y_{biomass/N}$, biomass yield per gram nitrogen consumed.

[a] The algae did not grow on ammonium without pH control.

Table 3.3. High-density fed-batch cultivation of microalgae for lipid production with glucose

Strain	Nitrogen source	pH	T °C	$X_{biomass}$ g L^{-1}	C_{lipid} %	X_{lipid} g L^{-1}	P_{lipid} g L^{-1} d^{-1}	Reference
Crypthecodinium cohnii	YE	6.5	27	26.0	15.0	3.8	0.8	(de Swaaf et al., 2003b)
Schizochytrium sp.	GL	-	25	92.7	50.3	46.6	7.0	(Qu et al., 2011)
Chlorella zofingiensis	NI	6.5	25	42.5	48.7	20.7	1.3	(Liu et al., 2010a)
Chlorella protothecoides	YE	6.5	28	51.2	50.3	25.8	3.3	(Xiong et al., 2008)
Chlorella Sorokiniana	AM	7.0	37	103.8	38.7	40.2	4.2	This study

YE, yeast extract; GL: glutamate; AM, ammonium; NI, nitrate; T, temperature; C_{lipid}, lipid content (w/w); X_{lipid}, lipid concentration; P_{lipid}, lipid productivity.

Table 3.4. Fatty acid compositions of the individual lipid classes of *C. sorokiniana*

	C. sorokiniana[a]						*Schizochytrium*[b]	Soybean[c]
	PL	FFA	DAG	TAG	SE	TL	TL	TL
C14:0	0.0	0.3	0.0	0.4	5.5	0.4	3.3	-
C14:1	0.0	0.0	0.0	0.1	2.0	0.1	-	-
C15:0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	-
C16:0	29.2	35.9	25.9	16.7	21.6	20.1	50.4	10.6
C16:1	7.7	4.8	0.0	3.2	6.6	3.7	-	-
C16:2	4.2	3.4	0.0	2.3	0.0	2.4	-	-
C16:3	1.0	0.6	0.0	0.9	0.0	0.9	-	-
C18:0	3.0	9.4	8.4	4.4	28.0	5.6	1.1	4.8
C18:1	29.1	20.0	29.0	34.1	13.5	31.5	0.1	22.5
C18:2	24.1	22.9	36.7	34.5	22.7	32.2	-	52.3
C18:3	1.6	2.7	0.0	2.0	0.0	2.0	0.1	8.2
C22:5	0.0	0.0	0.0	0.0	0.0	0.0	8.1	-
C22:6	0.0	0.0	0.0	0.0	0.0	0.0	29.7	-
SFA	32.3	45.6	34.3	22.4	55.1	26.9	61.3	15.3
PUFA	30.9	29.6	36.7	40.2	22.7	37.8	38.6	60.5
PUFA4[d]	0.0	0.0	0.0	0.0	0.0	0.0	38.3	-
IV[e]	93	80	93	110	62	104	172	137
CN[f]	56.0	59.1	56.9	53.3	63.3	54.5	38.1	46.6
KV	4.1	4.2	4.3	4.2	4.5	4.2	3.8	3.9
DE	874	873	876	877	872	876	863	868
HHV	39.6	39.6	39.7	39.7	39.7	39.7	39.6	39.1

[a] PL, polar lipids; FFA, free fatty acids; DAG, diacylglycerols; TAG, triacylglyceride; SE, steroid ester; TL, total lipids; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; IV, iodine value; CN, cetane number; KV, kinematic viscosity; DE, density; HHV, higher heating value

[b] The fatty acid compositions of *Schizochytrium* (Fan et al., 2007).

[c] The fatty acid compositions of soyben (Canakci & Gerpen, 2001).

[d] PUFA4 means PUFA with more than four double bonds.

[e] The iodine value (g of I_2/100 g) was calculated according to Knothe (2002).

[f] Cetane number, kinematic viscosity ($mm^2\ s^{-1}$, 40°C), density ($kg\ m^{-3}$) and higher heating value ($MJ\ kg^{-1}$) were calculated according to Ramírez-Verduzco et al. (2012).

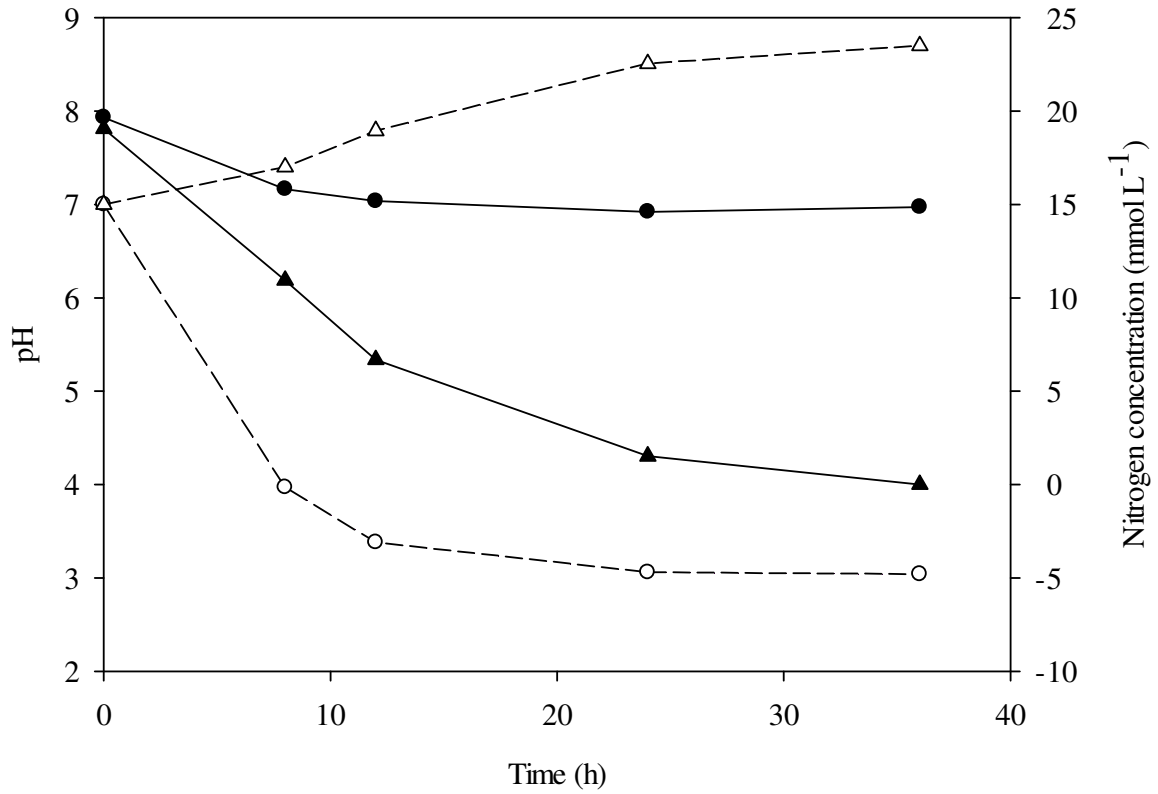


Fig. 3.1. The change of pH with the consumption of ammonium and nitrate. Dash and solid lines represent pH and nitrogen concentration respectively (ammonium, circle; nitrate, triangle).

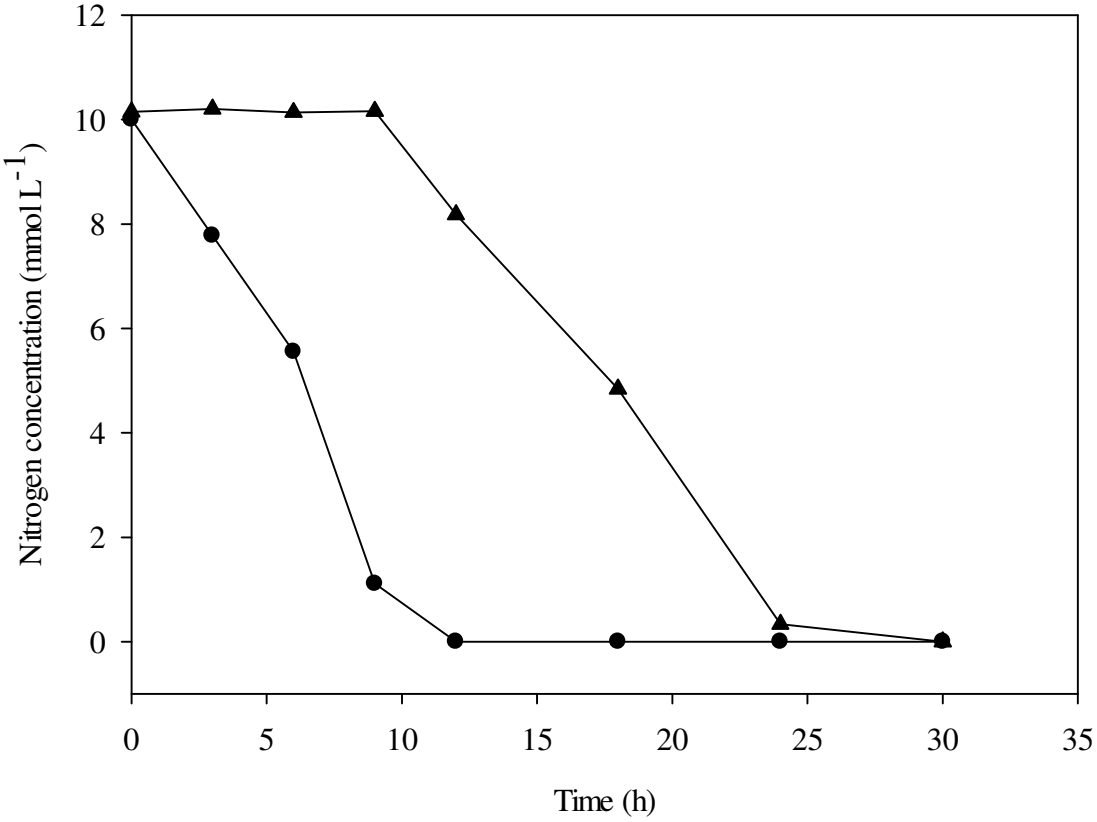


Fig. 3.2. The nitrogen consumption curve when *C. sorokiniana* was cultured with ammonium nitrate under controlled pH at 7.0 (ammonium, circle; nitrate, triangle).

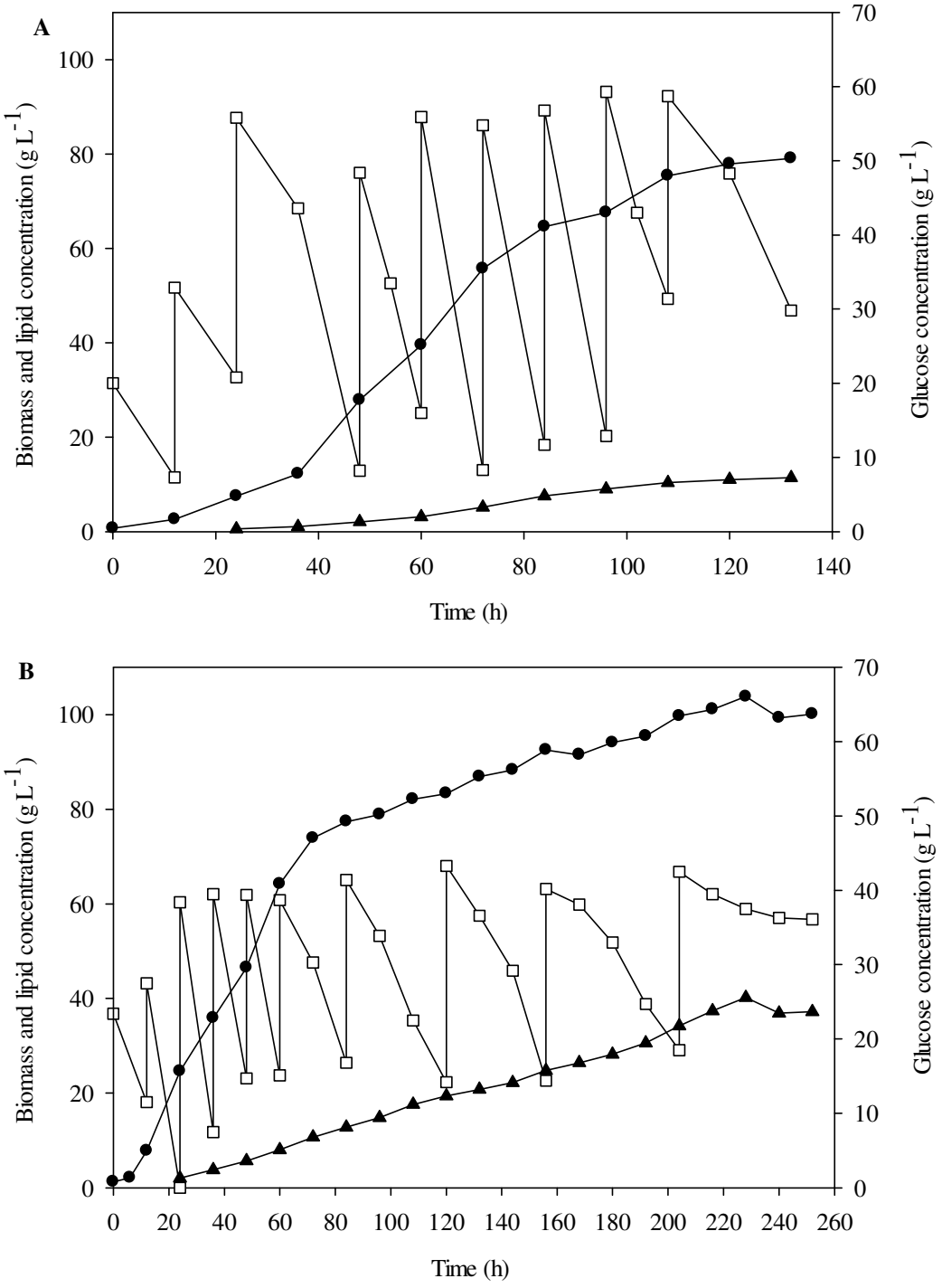


Fig. 3.3. Glucose consumption, biomass and lipid production of *C. sorokiniana* in (A) primary fed-batch fermentation; (B) two-stage fed-batch fermentation (closed circle, biomass; closed triangle, lipid; open square, glucose).

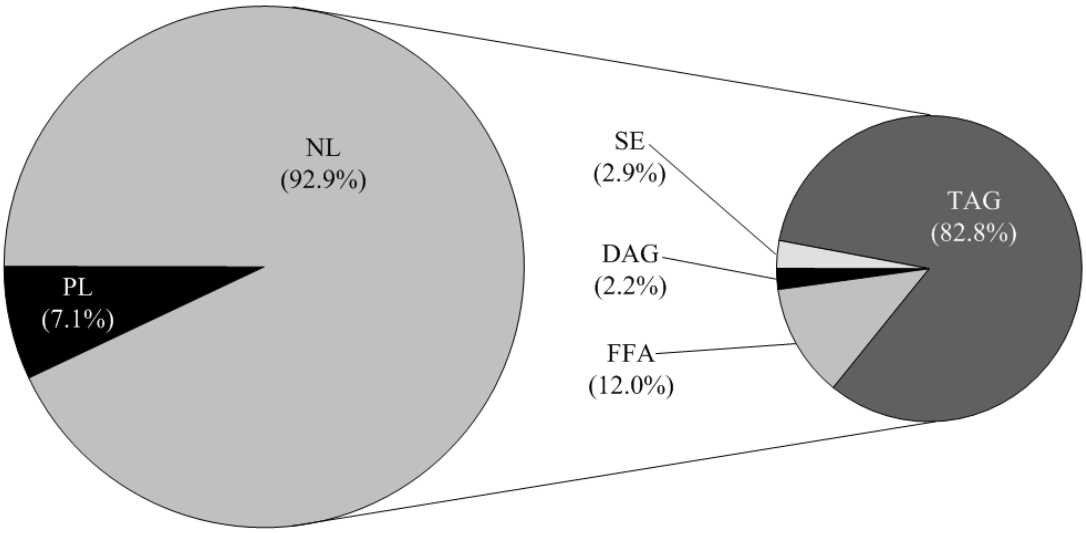


Fig. 3.4. Fractionation of lipids extracted from *C. sorokiniana*. Lipid proportions are based on relative weight of fatty acids in each fraction. (A) Distribution of PL and NL in lipids; (B) Distribution of lipid classes in NL. PL, polar lipids; Neutral lipids, NL; FFA, free fatty acids; DAG, diacylglycerols; TAG, triacylglyceride; SE, steroid ester.

Chapter 4

Simultaneous saccharification and fermentation of cellulosic materials by a microalga *Chlorella sorokiniana* for lipid production

4.1. Abstract

Although heterotrophic culture of microalgae is attractive for lipid-based biofuel production, the cost and sustainable supply of organic carbon are major limiting factors. Simultaneous saccharification and fermentation (SSF) of lignocellulosic biomass is considered as one of the few alternatives. A thermotolerant microalga *Chlorella sorokiniana* was tested for lipid production in an SSF process using cellulosic materials as feedstock. The results showed the cellulase enzyme mixture was not inhibitory for *C. sorokiniana* growth. The culture with substrate loading of 3% (w/v) and enzyme loading of 30 FPU/g cellulose exhibited the best performance for lipid production within the evaluated range. Elevating temperature improved the SSF efficiency and the highest lipid concentration (2.98 g L^{-1}), yield (99.2 mg g^{-1}) and productivity (20.7 mg L^{-1} h^{-1}) were achieved at 40°C. This study suggested SSF with cellulosic materials as the substrate could be a potential process for lipid-based biofuel production via heterotrophic microalgae cultivation.

Keywords: Microalgae; *Chlorella sorokiniana*; simultaneous saccharification and fermentation (SSF); cellulosic materials; biofuel.

4.2. Introduction

Microalgal lipid is considered as a promising feedstock for making high energy density transportation fuels like biodiesel, green diesel, green jet fuel and green gasoline (Pienkos & Darzins, 2009). Heterotrophic culture of microalgae using organic substances as carbon and energy sources has attracted increasing attention in recent years. This growth mode offers a strategy to overcome one of the major limitations during mass algal cultivation–the difficulty to deliver light which leads to high production cost and low production efficiency (Perez-Garcia et al., 2011). Compared with phototrophic growth, heterotrophic cultivation can achieve much higher growth rate, cell density and lipid productivity in common stirred-tank fermentors. Other major advantages include low costs for harvesting biomass and being easy to be scaled up (Chen & Chen, 2006; Miao & Wu, 2006; Perez-Garcia et al., 2011). However, the major limitation factor is the high cost of organic carbon source for the heterotrophic microalgal cultivation in terms of biofuel production.

Some attempts have been made to seek cheaper feedstocks or waste materials for heterotrophic culture. Chi et al. (2007) cultured the microalga *Schizochytrium limacinum* with biodiesel-derived crude glycerol, and the lipid content reached 50.6% of the dry cell weight, which was comparable with the controls on pure glycerol and glucose. As a by-product from the fermentative hydrogen production, acetate has also proven to be a suitable carbon source to support heterotrophic growth of microalgae for lipid accumulation (de Swaaf et al., 2003b; Heredia-Arroyo et al., 2010). Additionally, it has been reported that some oleaginous *Chlorella* species could grow on hydrolysates of various low value materials, such as Jerusalem artichoke tuber, cassava starch, food waste (Cheng et al., 2009; Lu et al., 2010; Zheng et al., 2012a). However, the availability of these types of materials is limited and may not meet the increasing

demand for biofuel sustainably.

Lignocellulosic biomass has been considered as a sustainable feedstock for biofuel production due to its abundance and relatively low cost. Cellulose and hemicellulose are the major carbohydrates of lignocellulosics. For cellulosic biofuel production, one option is separate hydrolysis and fermentation (SHF) in which the cellulose is first hydrolyzed by cellulases into glucose followed by fermentation of the sugar into target products. SHF is an easy and practical process but the disadvantages of SHF is (1) end-product inhibition of cellulose hydrolysis by glucose and cellobiose and (2) additional investment in separate hydrolysis and fermentation vessels (Olofsson et al., 2008). These drawbacks can be overcome by another strategy called simultaneous saccharification and fermentation (SSF), in which the enzymatic hydrolysis of cellulose is coupled with the fermentation. The SSF process takes advantages of reducing capital costs, contamination risk and enzyme requirement, as well as increasing productivity (Suryawati et al., 2008).

Although intensive studies have been investigated on the SSF process for ethanol production, culturing microalgae heterotrophically with cellulosic materials through SSF for lipid production has not been examined (Olofsson et al., 2008). A major challenge in such an application is the difference in optimum temperature for saccharification and fermentation. The optimum temperature for enzymatic hydrolysis is between 40 and 50 °C, while most oleaginous microalgae prefer the temperature ranging from 15 to 30 °C (Chi et al., 2007; Jiang & Chen, 2000; Miao & Wu, 2006; Suryawati et al., 2008; Wen & Chen, 2001). Some studies even showed that low temperature would induce lipid accumulation resulting in a high lipid content, whereas, it was reported that the use of low temperature had negative effects on enzyme activity and increased fermentation time and/or enzyme usage (Chi et al., 2007; Jiang & Chen, 2000;

Suryawati et al., 2008). Therefore, the SSF process can be greatly improved if thermotolerant oleaginous microalgae are utilized. We found in our previous study that the green microalga *Chlorella sorokiniana* could tolerate the temperature around 40°C and the elevated temperature greatly increased the growth rate (Li et al., 2013). In fed-batch fermentation with glucose as the carbon source, the cell density and lipid concentration could reach 103.8 g L^{-1} and 40.2 g L^{-1} respectively (Zheng et al., 2013).

The aim of this work was to investigate the feasibility to culture *C. sorokiniana* for lipid production through the SSF process with cellulosic materials as the feedstock. Firstly, the effect of enzyme on the growth of *C. sorokiniana* was studied. Then, a suitable buffer system was screened in order to control the pH in flask cultivations. Evaluations on lipid production were performed during the SSF process with different substrate and enzyme loadings. Finally, the lipid yield and productivity by *C. sorokiniana* at different temperatures in SSF were compared.

4.3. Materials and methods

4.3.1. Organism and inoculum preparation

The green microalga *Chlorella sorokiniana* (UTEX 1602) was obtained from the Culture Collection of Algae at the University of Texas (Austin, TX). The minimal medium used in all the cultivations contained (per L) 621 mg $KH_2PO_4 \cdot H_2O$, 89 mg $Na_2HPO_4 \cdot 2H_2O$, 246.5 mg $MgSO_4 \cdot 7H_2O$, 9.3 mg EDTA, 0.061 mg H_3BO_3, 14.7 mg $CaCl_2 \cdot 2H_2O$, 6.95 mg $FeSO_4 \cdot 7H_2O$, 0.287 mg $ZnSO_4 \cdot 7H_2O$, 0.01235 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.169 mg $MnSO_4 \cdot H_2O$, and 0.00249 mg $CuSO_4 \cdot 5H_2O$. For inoculum preparation, *C. sorokiniana* was cultured in 250 mL Erlenmeyer flasks containing 50 ml of minimal medium supplemented with 20 g L^{-1} glucose and 2.0 g L^{-1} KNO_3. The culture was incubated at 37°C in an orbital shaker at a rotary rate of 200 rpm. After

24 hours growth, the algal cells were harvested and washed three times with sterile distilled water before inoculation. All the media were sterilized by passing through a 0.22 μm membrane (Millipore, MA) before utilization.

4.3.2. Effects of enzyme on algae growth

Commercial enzyme mixture used in this study was Cellic® CTec2 (kindly provided by Novozymes, USA) with a cellulase activity of 202 FPU/mL (Adney & Baker, 1996) and a β-glucosidase activity of 4483 CBU/mL (Ghose, 1987). To investigate whether the cellulase had an inhibitory effect on the growth of *C. sorokiniana*, different amounts of enzyme were added to the culture medium (with 20 g L^{-1} glucose as the sole carbon source). The enzyme loadings were 1.5, 4.5 and 7.5 mL per liter culture of medium, which were equivalent to the amounts of required enzymes at 30 FPU/g cellulose with 1%, 3% and 5% (w/v) substrate loading respectively.

4.3.3. Buffers for pH control

C. sorokiniana was grown in the minimal medium with 20 g L^{-1} glucose for 72 hours. Different concentrations (50 mM, 100 mM, 500 mM) of sodium phosphate and sodium citrate buffers (pH 6.0) were tested for pH control when 2.0 g L^{-1} KNO_3 was the nitrogen source. The cultures with 1.1 g L^{-1} NH_4Cl as the nitrogen source were supplemented $CaCO_3$ at different concentrations (10 mM, 20 mM, 40 mM, 60 mM) with initial pH 6.0.

4.3.4. SSF

To investigate the effects of substrate and enzyme loadings, SSF was carried out in 250 mL Erlenmeyer flasks containing 50 mL of minimal medium supplemented with 1.1 g L^{-1} NH_4Cl.

Avicel PH101 (Sigma-Aldrich, MO), a microcrystalline cellulose was used as the substrate with different loadings (1%, 3%, 5%, w/v). Enzyme loadings of 5, 15 and 30 FPU/g cellulose were applied. The initial pH was 6.0 and 40 mM $CaCO_3$ was added for pH maintenance. The SSF experiments were run for 144 h at a constant temperature of 37°C. To study the effects of temperatures, SSF was performed with substrate loading of 3% (w/v) and enzyme loading of 30 FPU/g cellulose at 30, 37, 40 and 45°C respectively.

4.3.5. Analytical procedure

To determine the algal dry cell weight (DCW), 5 mL cell suspension was centrifuged at the speed of 1000 × g for 5 min. The cell pellet was washed three times with distilled water, and then dried in a pre-weighed aluminum dish at 105 °C for 3 hours.

The concentrations of sugar residues were analyzed using a Dionex ICS-3000 ion chromatography (Dionex Corp., CA), which was equipped with a CarboPac TM PA 20 (4 × 50 mm) analytical column, and CarboPac TM PA 20 (3 × 30 mm) guard column(Yu et al., 2011). The filtered samples were injected and eluted isocratically with 0.01 M NaOH. Analytes were detected and quantified based on standard curves.

For lipid analysis, cells were harvested and prepared according to the procedure reported by O'Fallon et al. (2007). The total fat was quantified by an Agilent 7890A gas chromatography equipped with an auto-sampler (Agilent 7683B) (Agilent Technologies, CA), a flame ionization detector (FID) and a FAMEWAX column (30 m × 320 µm × 0.25 µm) (Restek Corp., PA). Injector temperature was kept at 280°C and 1 µl sample was injected with a split ratio of 30:1. Initial oven temperature was 120°C, and increased to 240°C at 3°C/min held for 20 min. The FID temperature was set at 250°C. Helium was used as the carrier gas. Tridecanoic acid (C13:0)

was used as the internal standard.

All the flask cultures were carried out in triplicate. The experimental data were statistically analyzed with ANOVA using SAS 9.2 (SAS Institute, NC).). All values were presented as the average of independent measurements with significance declared at $P < 0.05$.

4.4. Results and discussion

4.4.1. Effects of enzyme on the growth of *C. sorokiniana*

Fig. 4.1 shows the growth rate, DCW and lipid of *C. sorokiniana* grown with different enzyme loadings. The control group (without addition of enzyme) produced 10.40 g L^{-1} DCW and 2.93 g L^{-1} lipid with the specific growth rate of 1.26 d^{-1}. With the increase of enzyme loadings, enhanced growth performance was observed for *C. sorokiniana*. The highest growth rate (1.37 d^{-1}), DCW (12.11 g L^{-1}) and lipid (3.29 g L^{-1}) were achieved when 7.5 mL L^{-1} cellulase enzyme mixture was added to the culture medium. The improvement of the growth for *C. sorokiniana* with addition of enzyme might be attributed to a high concentration of sugar residues remained in the enzyme cocktail. These results indicated that the enzyme mixture Cellic® CTec2 did not have an inhibitory effect on the microalga *C. sorokiniana* growth.

To investigate whether cellulase enzyme had inhibitory effects on the microalgae growth is critical for the SSF process, since the cell wall of *Chlorella* is composed of up to 80% polysaccharides including cellulose (Rodrigues & Bon, 2011). Unlike some terrestrial plants, lignin is not present in the cell wall structure, but a polysaccharide and glycoprotein matrix consisted in the microalgal cell walls provides a highly recalcitrant defense against its environment (Gerken et al., 2013). Some *Chlorella* species have a two-layer cell wall structure. The outer layer contains algaenan, a nonhydrolyzable aliphatic biopolymer, which is postulated

to be responsible for the resistance of algal cell wall (Rodrigues & Bon, 2011). It has been suggested that *C. sorokiniana* belonged to another group with only a single homogeneous layer, but it had a high resistance to the degradation by polysaccharide-degrading enzymes including cellulase, hemicellulase, pectinase (either alone or in combination) (Russell, 1995). Although the algal cell wall is resistant to enzyme degradation, the enzyme may still play an inhibitory role on algal growth by interfering the cell wall construction during growth or cell division. Gerken et al. (2013) demonstrated that cellulase or enzyme mixes containing cellulase such as driselase and macerozyme was inhibitory to *Chlorella emersonii* CCAP211/11N and *Oocystis pusilla* OOCYS1 growth. However, no inhibition was observed for *C. sorokiniana* in this study in the presence of the cellulase enzyme mixture Cellic® CTec2. This result was in accordance with the data reported by Russell (1995), which suggested that cellulose was not a major cell wall polysaccharide of *C. sorokiniana*.

4.4.2. Buffer system selection

Effects of *different* concentrations of phosphate and citrate buffers on pH maintenance and DCW production of *C. sorokiniana* grown with nitrate are shown in Table 4.1. For the control group (no extra buffer was added), the pH of the fermentation broth increased from 6.1 to 7.7, and the DCW of *C. sorokiniana* reached 10.40 g L^{-1} after 72 hours cultivation. With addition of 50 mM phosphate buffer, the final pH was 6.8 and DCW decreased to 9.53 g L^{-1}. There was no significant pH change when 500 mM phosphate buffer was present, but the DCW of *C. sorokiniana* was only 1.04 g L^{-1}. Addition of phosphate buffer could help to maintain the pH and smaller fluctuation was observed with the increase of buffer concentrations. The higher phosphate concentration provided a higher buffer capacity, but it inhibited the growth of *C.*

sorokiniana. Similar results reported by Jeong et al. (2003) showed critical inhibitory effect on the microalga *C. vulgaris* growth when phosphate concentration reached 100 mM. In addition, the pH did not changed significantly (6.0 to 6.3) even 50 mM citrate was applied, but the DCW of *C. sorokiniana* was only 2.83 g L^{-1}. When the concentration of citrate increased from 50 to 500 mM, the DCW of the microalgae decreased to 0.74 g L^{-1}. The citrate buffer was more deleterious than phosphate buffer on the algal growth, although it showed a better pH control capability.

As shown in Table 4.2, the pH decreased significantly from 6.0 to 3.6 when ammonium was used as the nitrogen source for *C. sorokiniana* without pH control. The DCW was only 0.61 g L^{-1} (almost equal to the inoculum concentration). Similar with the cultures grown on nitrate, inhibition was observed when phosphate and citrate buffers were utilized (data not shown). However, the final pH and DCW increased gradually with addition of $CaCO_3$. When 40 mM $CaCO_3$ was added into the culture medium, the pH was kept constantly and the DCW reached 10.14 g L^{-1}, which was comparable with our previous result under pH control (Zheng et al., 2013). Further increasing the concentration of $CaCO_3$ to 60 mM did not have negative effect on the pH maintenance and the algae growth.

The environmental pH is an important factor not only for the enzyme activity but also for the microbial growth. Our previous studied showed that *C. sorokiniana* preferred the pH ranging from 6.0–8.0 and could not grow at pH 5.0 or lower. The pH increased with the consumption of nitrate but decreased with the uptake of ammonium in batch culture without pH control (Zheng et al., 2013). Meanwhile, the suitable pH range for the enzyme Cellic® CTec2 is 5.0–6.0. Therefore, it is advantageous to maintain the pH around 6.0 for the SSF process.

Phosphate and citrate buffers are widely used in SSF for ethanol fermentation by yeasts or

fungi, but our data show that they have inhibitory effects on *C. sorokiniana* growth. Actually, phosphate is not only used as buffer for pH control, but also serves as nutrition for the algae growth. The uptake of phosphorus compound is repressed if high external phosphate concentrations are present, which results in inhibition on cell growth (Krichnavaruk et al., 2005). The inhibitory effect of citrate is more serious. Midgley and Dawes (1973) observed preferential utilization of citrate in the presence of both glucose and citrate. Mischak et al. (1984) reported that citrate inhibited glucose uptake in *Aspergillus niger* by affecting a high affinity glucose transport system. It was also suggested that high concentrations of cytosolic citrate could inhibit glycolysis by suppressing the key glycolytic enzyme phosphofructokinase (Christophe et al., 2012). Moreover, citrate could inhibit the growth of microorganisms by chelating metal ions. For instance, as a chelator of ferric, citrate exhibited strongly inhibitory effect (>95% inhibition at 20 mM citrate) on Fe-uptake complex, a unique system in fungi and algae (Ziegler et al., 2011). The consumption of ammonium led to pH decrease, calcium carbonate acted as a buffer to maintain pH by neutralization of generated protons and did not show inhibitory effect on *C. sorokiniana* growth. Xia et al. (2011) also described that calcium carbonate could be utilized for pH adjustment, the final pH of the fermentation broth was 5.3 with addition of 40 mM $CaCO_3$ while the pH of the control (without $CaCO_3$) was 3.0. According to the data shown in Table 2, ammonium was used as the nitrogen source and 40 mM $CaCO_3$ would be adopted for pH control in the SSF process.

4.4.3. SSF with different substrate and enzyme loadings for lipid production

Fig. 4.2 shows the lipid accumulation by *C. sorokiniana* through SSF process with different substrate and enzyme loadings. At the lowest substrate loading (1%, w/v), an increased rate of

cellulose hydrolysis with increased enzyme loading was observed at the first 4 h (Fig. 4.2A). From 24 to 144 h, the residual glucose concentration remained below 0.10 g L^{-1}, independent of the cellulase activity. Lipid concentrations increased continuously from the beginning of the fermentation and reached the maximum concentration after 96 h for the runs with 5 and 15 FPU/g cellulose. The cultures with enzyme loading of 30 FPU/g substrate had still not reached the maximum concentration after 144 h. When the substrate loading increased to 3% (w/v), the time required to reach the maximum lipid concentration was the same as that of the runs with 1% (w/v) solid loading (Fig. 4.2B). However, the curves for the residual glucose concentration were different. For the cultures with the lowest enzyme loading of 5 FPU/g cellulose, the residual glucose increased to the highest concentration after 4 h and then decreased quickly. When the enzyme loading increased to 15 FPU/g cellulose, the glucose concentration achieved the highest (2.70 g L^{-1}) at 8 h and dropped below 0.10 g L^{-1} after 48 h. Further increasing the enzyme loading to 30 FPU/g substrate resulted in much better hydrolysis of cellulose. The residual glucose concentration increased to the highest point of 9.09 g L^{-1} after 24 h. There was sufficient glucose remained in the fermentation broth during the first 96 h and then the concentration kept below 0.10 g L^{-1} until 144 h. In the cultivation with 5% (w/v) substrate loading, both lipid and residual glucose kept increasing during the SSF (Fig. 4.2C). Higher enzyme loading led to higher concentration of lipid as well as higher residual glucose. Glucose accumulation ranged from 1.83 to 2.98 g L^{-1} at the end of the fermentation in the cultures with enzyme activity of 5–30 FPU/g cellulose.

Cellulase enzyme played a significant role in lipid production for all the cultures (Table 4.3). Higher lipid concentration and yield were obtained during the fermentation period at higher enzyme loading. There was no saturation of the cellulase loading in the investigated scope from

5 to 30 FPU/g substrate, but the positive effect from cellulase was more significant at lower substrate concentrations. For instance, the lipid yield increased more than 50% when the enzyme loading increased from 15 to 30 FPU/g cellulose for the runs with the substrate loading of 1–3% (w/v), while only 12% increase in the lipid yield was observed at 5% (w/v) cellulose. The availability of the carbon source might be the reason for this phenomenon. In Fig. 2, glucose was limited during the fermentation for the runs with 1–3% (w/v) substrate as evidenced by the low residual glucose concentration (< 0.10 g L^{-1}) after a period of fermentation, so the higher cellulase loading led to more available sugar for lipid accumulation. However, at substrate loading of 5% (w/v), there was sufficient glucose present till the end of SSF even at 5 FPU/g cellulose, so the further increase in enzyme loading did not significantly improve the lipid production.

Higher substrate loading resulted in an increase in lipid concentration when the same cellulase enzyme loading was applied (Table 4.3). The lipid concentration increased about 3 times for the cultures on pure glucose with substrate loading from 1% to 3% (w/v) (data not shown), meanwhile, 7 times increase in the lipid concentration was observed for the SSF of cellulose (30 FPU/g substrate). The relative lipid yield with 3% (w/v) substrate was approximately twice higher than that with 1% (w/v) substrate. This result was different with the SSF of cellulose for ethanol production, wherein the increase in substrate loading from 2% to 5% led to only 11% increase in the relative ethanol yield with cellulase loading of 32 FPU/g substrate (Stenberg et al., 2000). Unlike ethanol, the function of lipid is energy storage. Oleaginous microbes tend to accumulate lipid when extra carbon source is present in the environment. At the lowest cellulose loading (1%, w/v), available carbon source was limited and the residual glucose concentration maintained at very low level after 12 h even at the highest

enzyme loading (Fig. 4.2A), which resulted in poor lipid yields. Thus, higher substrate loading is more beneficial for lipid production during the SSF process. However, the results showed that the lipid concentration only increased by 15% from 3% to 5% (w/v) when the cellulase loading was 30 FPU/g substrate. Although the cultures with 5% (w/v) cellulose led to the highest lipid concentration of 2.98 g L^{-1}, the highest lipid yield of 86.2 mg g^{-1} was achieved at 3% (w/v) substrate loading. Similarly, Liu et al. (2012) reported that the lipid production performance was poorer with 15% solid than that with 10% solid for the yeast *Trichosporon cutaneum* although a higher lipid concentration was achieved at higher substrate loading. It was suggested that high solid loading might limit the oxygen transfer, which negatively affected the lipid accumulation since the lipid fermentation was an aerobic process. Therefore, 3% (w/v) was a suitable substrate loading for lipid production by the microalga *C. sorokiniana* in the SSF process.

4.4.4. Effects of temperature

Fig. 4.3 shows the SSF of cellulose (3%, w/v) by *C. sorokiniana* with the enzyme loading of 30 FPU/g substrate at different temperatures of 30, 37, 40 and 45°C. An increase in glucose concentration was recorded with increased temperature at 4 h, which indicated that the elevated temperature improved the hydrolysis of cellulose. Glucose assimilation by *C. sorokiniana* also increased when temperature increased from 30 to 37°C as shown by residual glucose measured during SSF (Fig. 4.3A). At 30°C, the residual glucose concentration increased continuously until 96 h and 8.81 g L^{-1} glucose was accumulated at the end of the fermentation. Meanwhile, the residual glucose reached the highest concentration of 9.09 g L^{-1} at 24 h and then decreased to the concentration below 0.10 g L^{-1} after 96 h for the cultures at 37°C. Further increasing the temperature from 37 to 40°C resulted in higher residual glucose concentration, but glucose

fermentation by *C. sorokiniana* did not occur as fast as the hydrolysis as evidenced by the similar consumption rate after 48 h. There was no significant difference in lipid production in the first 24 h when temperature was at 40°C or below (Fig. 4.3B). The final lipid concentration increased from 1.50 to 2.98 g L^{-1} with increasing temperature from 30 to 40°C. The highest lipid yield of 99.2 mg g^{-1} and lipid productivity of 20.7 mg L^{-1} h^{-1} were achieved at 40°C, approximately twice higher than those at 30°C (Table 4.4). When the SSF temperature increased to 45°C, lipid could not be formed, instead, only hydrolysis of cellulose occurred and glucose was accumulated in the fermentation broth. These results suggested that the elevated temperature (in the range of 30–40°C) could not only improve the saccharification rate but also the lipid production performance in the SSF process.

Heterotrophic cultivation of microalgae takes advantages of high growth rate, high cell density, low cost harvesting and desirable lipid profiles for biofuel production (Zheng et al., 2013). The cost from organic carbon sources that limits the application for making biofuel can be potentially reduced if lignocellulosic materials are utilized as the feedstock (Zheng et al., 2012b). SSF, especially at elevated temperature, is considered as a more cost-effective and practical process for biofuel production from cellulosic materials. A high temperature fermentation process could significantly help the costs reduction associated with cooling. Abdel-Banat et al. (2010) reported that a 5°C increase only in the fermentation temperature could reduce the biofuel production costs greatly. The fermenting strain with the capability to resist higher temperature could minimize the yield loss due to cell death from temperature fluctuations during large scale fermentation. The elevated temperature of SSF could reduce the enzyme addition into the reactor and the time required for SSF to complete. However, as mentioned before, 15 to 30°C is the optimal temperature for many lipid producing microalgae. High temperature displays inhibitory

effects on lipid accumulation for some algal species, which limits the application of SSF for lipid production by heterotrophic algal cultivation using cellulosic materials (Chi et al., 2007; Hur et al., 2002; Xin et al., 2011). Thus, there is demand to explore thermotolerant microalgae capable accumulation of lipid at high temperature. Our results showed that the microalga *C. sorokiniana* could tolerate much higher temperatures than many other oleaginous microalgal species without the loss of lipid yield. The highest lipid yield and productivity were achieved at 40°C in the SSF process, which were twofold higher than those obtained at 30°C. It would lead to enzyme cost reduction up to 50%, since the cellulase enzyme mixture Cellic® CTec2 also showed a twofold higher activity at 40°C compared with its activity at 30°C (data not shown).

4.5. Tables and figures

Table 4.1. Culture of *C. sorokiniana* on nitrate with phosphate and nitrate buffers

Buffer	Concentration (mM)	Initial pH	Final pH	DCW ($g\ L^{-1}$)
Control	-	6.1	7.7	10.40
Phosphate	50	5.9	6.8	9.53
	100	5.9	6.4	7.31
	500	6.1	6.1	1.04
Citrate	50	6.0	6.3	2.83
	100	6.0	6.2	1.92
	500	6.2	6.2	0.74

Table 4.2. Culture of *C. sorokiniana* on ammonium with addition of $CaCO_3$.

$CaCO_3$ (mM)	Initial pH	Final pH	DCW ($g\ L^{-1}$)
0	6.0	3.6	0.61
10	6.0	4.9	5.70
20	6.1	5.2	8.73
40	6.1	6.1	10.14
60	6.1	6.2	10.21

Table 4.3. Lipid yield during SSF process with different substrate and enzyme loadings

Substrate (%, w/v)	Enzyme (FPU/g)	Lipid ($g\ L^{-1}$)	Yield[a] ($mg\ g^{-1}$, w/w)	Relative yield[b] (%, w/w)
1	5	0.16	15.9	13.1
1	15	0.25	24.8	20.3
1	30	0.38	38.4	31.5
3	5	0.73	24.4	20.9
3	15	1.51	50.3	43.0
3	30	2.59	86.2	73.7
5	5	1.83	36.6	48.4
5	15	2.67	53.4	70.6
5	30	2.98	59.6	78.8

[a] Lipid yield: milligrams lipid produced by *C. sorokiniana* per gram original cellulose.

[b] Relative lipid yield: the percentage of lipid yield on cellulose of the lipid yield on glucose.

Table 4.4. Lipid yield during SSF process with different temperatures

Temperature (°C)	Lipid ($g\ L^{-1}$)	Yield[a] ($mg\ g^{-1}$, w/w)	Productivity ($mg\ L^{-1}\ h^{-1}$)
30	1.50	50.1	10.4
37	2.59	86.2	18.0
40	2.98	99.2	20.7
45	-	-	-

[a] Lipid yield: milligrams lipid produced by *C. sorokiniana* per gram original cellulose.

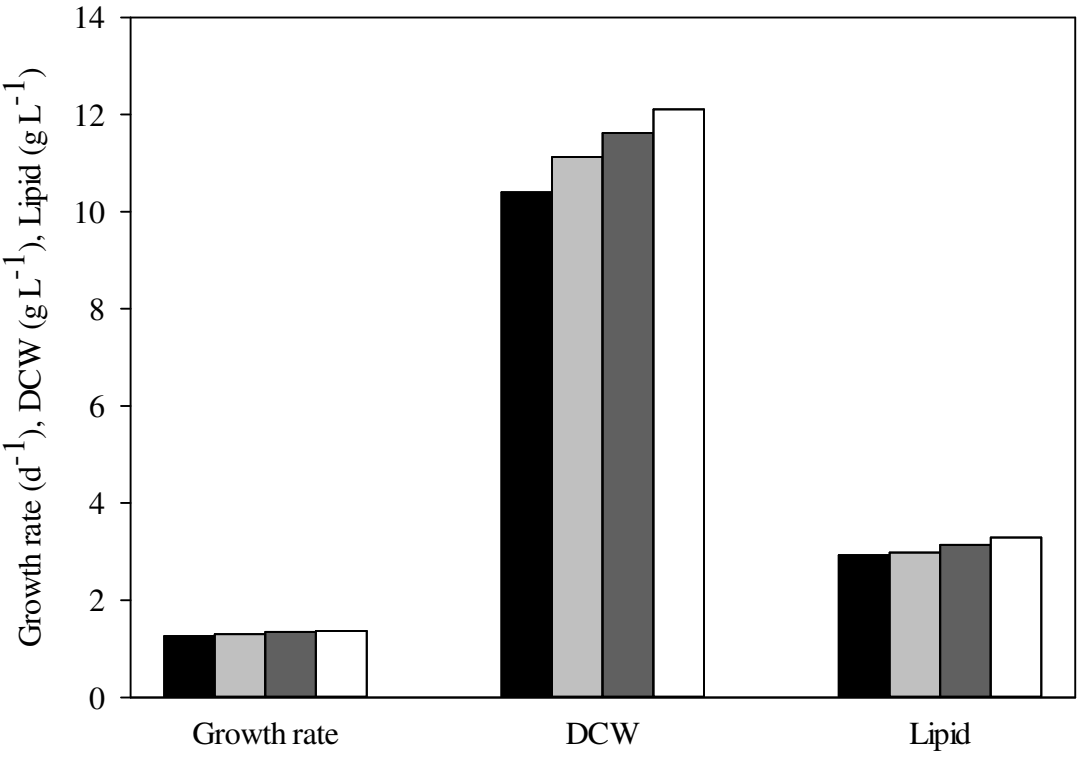
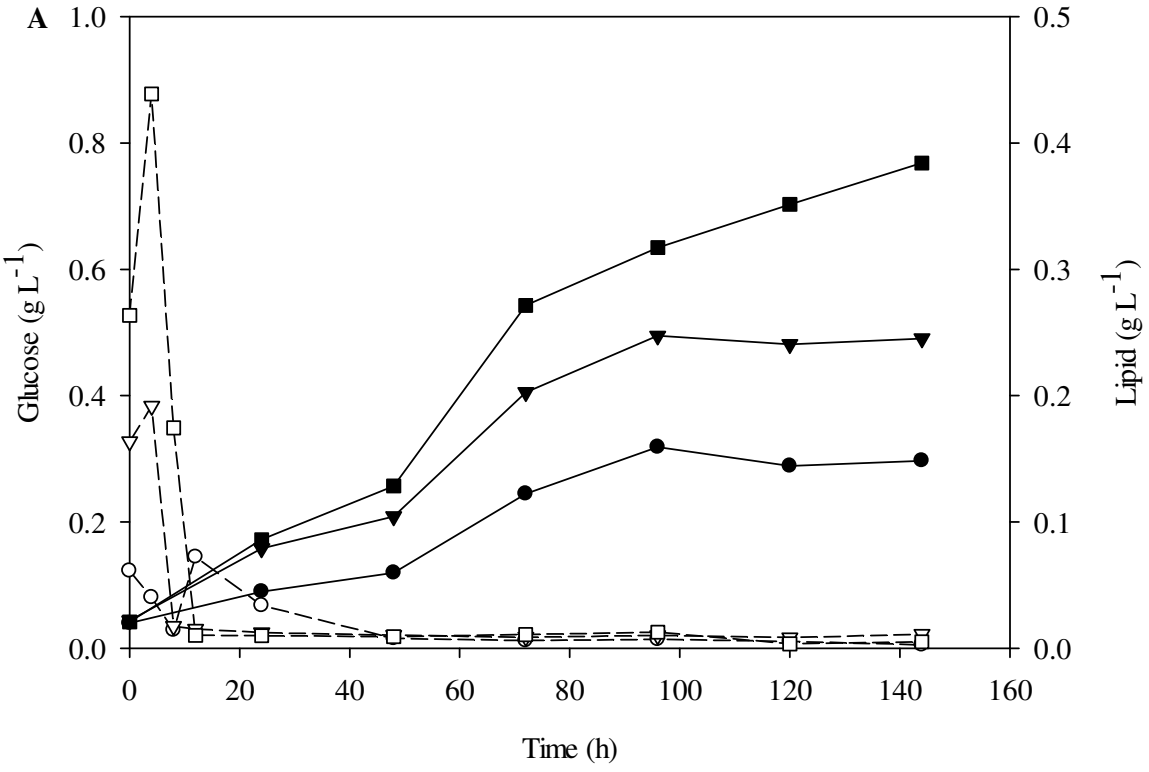
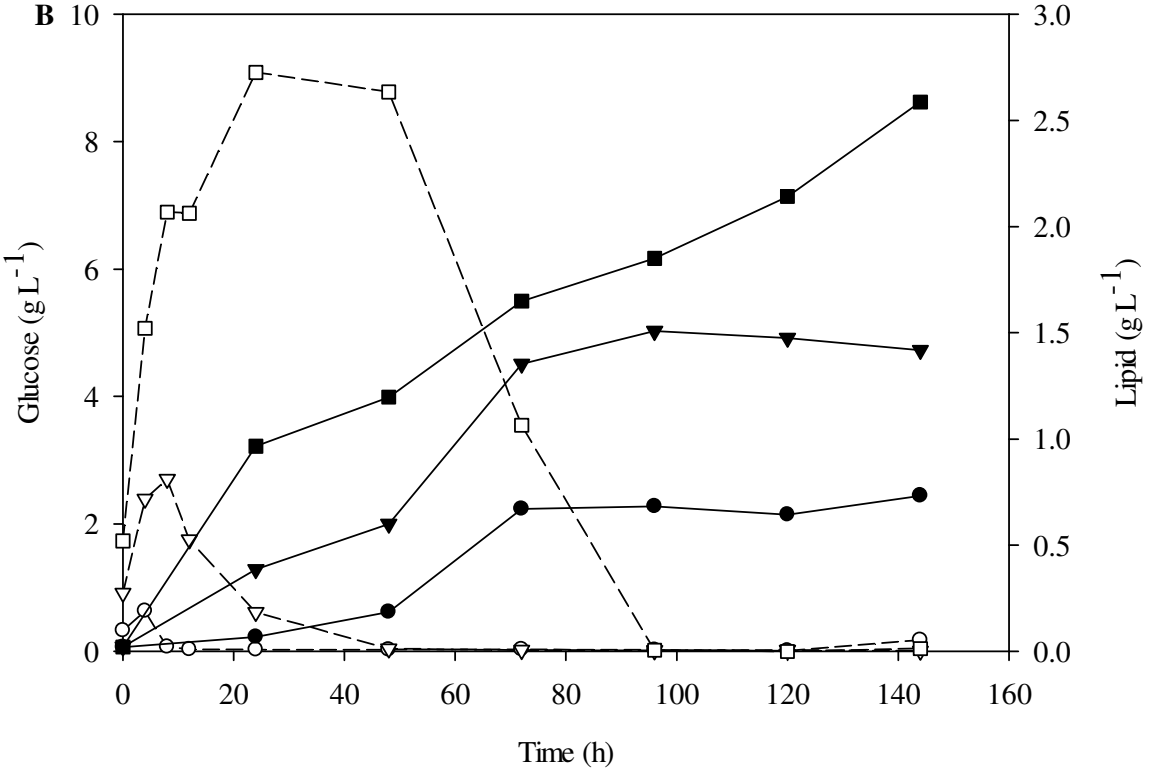


Fig. 4.1. Culture of *C. sorokiniana* with different enzyme loadings, per liter medium containing enzyme: black, 0 mL; grey, 1.5 mL; dark grey, 4.5 mL; white, 7.5 mL.


A
Glucose (g L-1)
Lipid (g L-1)
Time (h)
0.0
0.2
0.4
0.6
0.8
1.0
0.1
0.3
0.5
0
20
40
60
80
100
120
140
160


B
Glucose (g L-1)
Lipid (g L-1)
Time (h)
0
2
4
6
8
10
0.5
1.0
1.5
2.0
2.5
3.0
0
20
40
60
80
100
120
140
160

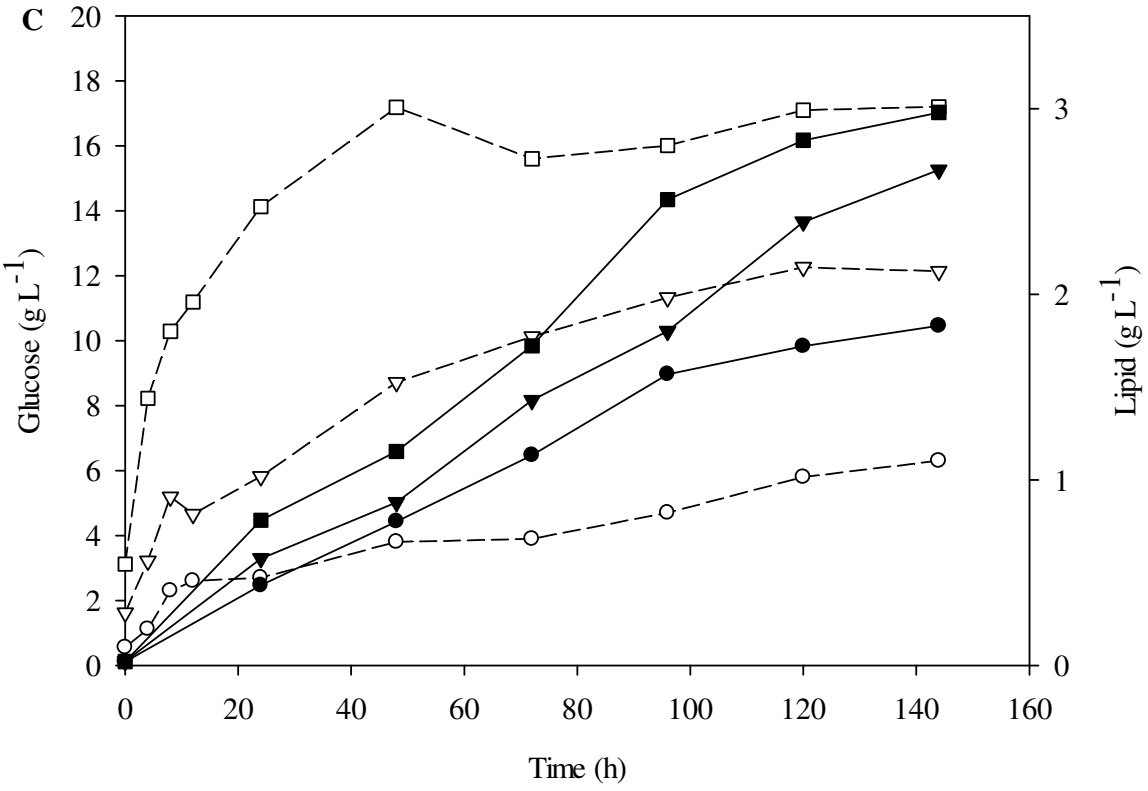


Fig. 4.2. Time course for glucose (dash line) and lipid (solid line) during SSF process with different enzyme loadings (circle, 5 FPU/g cellulose; triangle, 15 FPU/g cellulose; square, 30 FPU/g cellulose). (A) 1% (w/v) substrate loading; (B) 3% (w/v) substrate loading; (C) 5% (w/v) substrate loading.

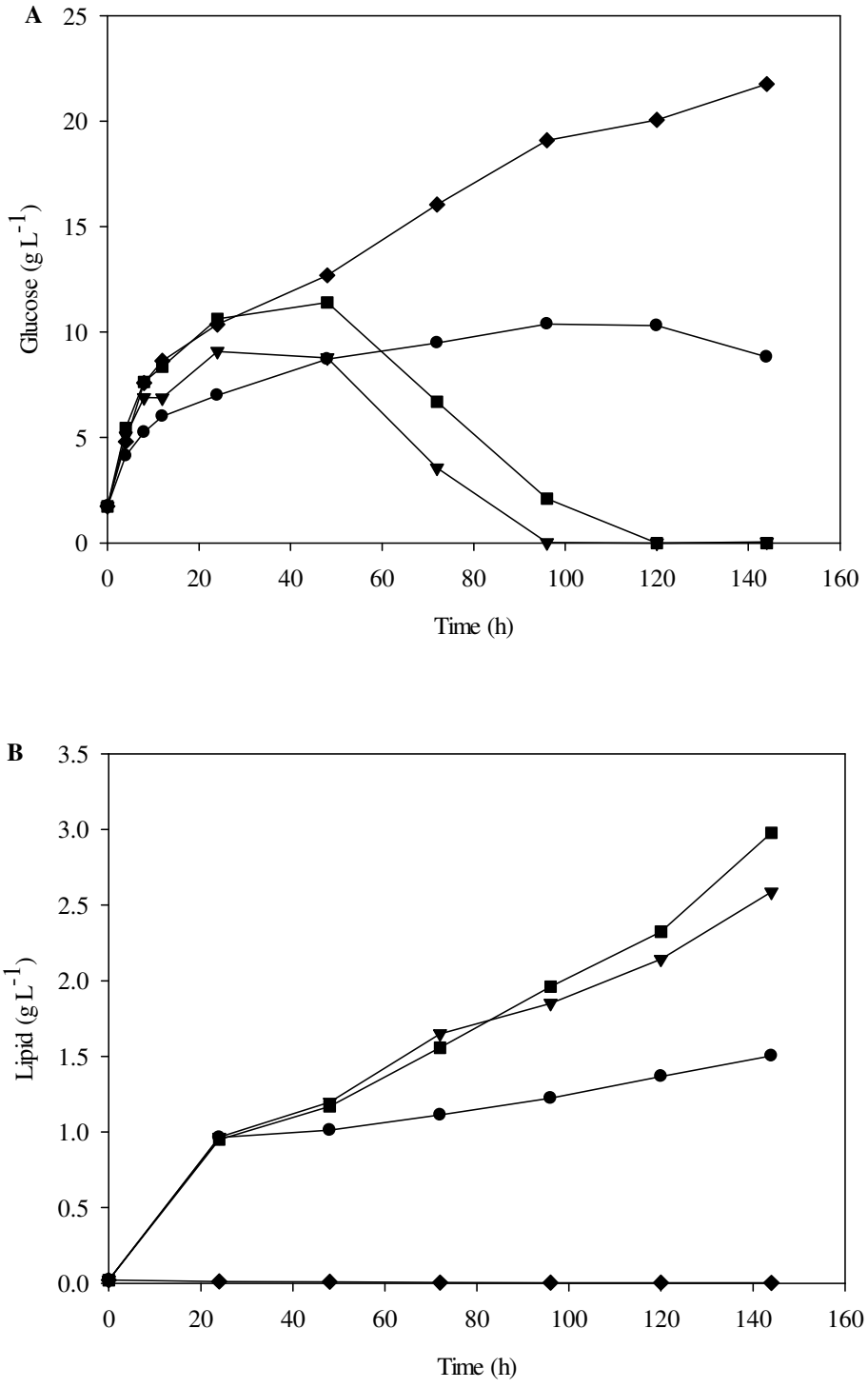


Fig. 4.3. Time course for glucose (A) and lipid (B) during SSF process (3% substrate loading, 30 FPU/g cellulose) with different temperatures (circle, 30°C; triangle, 37°C; square, 40°C; diamond, 45°C).

Chapter 5

D-xylose utilization by a green microalga *Chlorella sorokiniana*

5.1. Abstract

Heterotrophic and mixotrophic culture of microalgae for biofuel production is generating considerable interest, and lignocellulosic materials are attractive feedstocks to make the process cost-effective. As one of the major fermentable sugars in the lignocellulosic hydrolysates, D-xylose has not been well examined as carbon source for microalgae. To fill this knowledge gap, a green microalga *Chlorella sorokiniana* UTEX 1602 was investigated for its capability to utilize D-xylose. The sugar uptake kinetics analysis showed that the algal cells exhibited a remarkably increased D-xylose uptake rate after incubating with D-glucose. The maximum D-xylose transport rate was 3.8 nmol min^{-1} mg^{-1} cell with K_m value of 6.8 mM. D-xylose uptake was suppressed in the presence of D-glucose, D-galactose and D-fructose but not L-arabinose and D-ribose. The enzymatic activities of NAD(P)H-linked xylose reductase (XR) and $NADP^+$-linked xylitol dehydrogenase (XDH) were detected in *C. sorokiniana* grown on D-xylose. Compared with the culture in the dark, the consumption of D-xylose increased 2 fold under light but decreased to the same level with addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). In summary, this study first revealed the D-xylose uptake mechanism and the related metabolism for the microalga *C. sorokiniana*.

Keywords: *Chlorella sorokiniana*; microalgae; D-xylose; xylose reductase; xylitol dehydrogenase.

5.2. Introduction

Microalgal biomass is considered as potential feedstock for making biofuels and high-value chemicals (Amin, 2009; Chen & Chen, 2006). Phototrophic culture of microalgae is difficult to reach a high density due to poor light penetration under high cell concentration conditions, resulting in high production cost associated with the downstream harvesting (Liang et al., 2009). Growing microalgae heterotrophically or mixotrophically by feeding with organic carbon shows great potential to overcome this limitation. It has been reported that much higher growth rate, cell density and biomass productivity could be achieved under heterotrophic and mixotrophic conditions for some microalgal species (Garcia et al., 2000; Zheng et al., 2012a). Based on the analysis of energetics and carbon metabolism during growth of microalgae, Yang et al. (2000) demonstrated that the heterotrophic and mixotrophic cultures produced more ATP and biomass from the supplied energy than the phototrophic culture. However, addition of organic carbon increases the cost and makes the process economically unfeasible. Although some low value materials have been explored in order to reduce the cost, such as crude glycerol, acetate, food waste and cassava starch (Chi et al., 2007; Lu et al., 2010; Zheng et al., 2012a), the availability and sustainability of the organic carbon supplies are limiting factors for large scale applications.

Lignocellulosic materials are promising feedstocks due to its abundance and relatively low cost (Zheng et al., 2012b). Mono-sugars can be obtained from hydrolysis of lignocellulosics and serve as carbon sources for heterotrophic or mixotrophic culture of microalgae. D-glucose (mainly from cellulose) and D-xylose (from hemicellulose) are two major carbohydrates in the lignocellulosic hydrolysates. D-glucose has been proven as a suitable carbon source for the growth of many microalgae, such as *Crypthecodinium cohnii*, *Schizochytrium* sp. *Nitzschia laevis*, *Euglena gracilis*, *Galdieria sulphuraria* and *Chlorella* species (Bumbak et al., 2011). Li

et al. (2011) successfully demonstrated that the microalga *Chlorella pyrenoidosa* could be cultured on the rice straw hydrolysates primarily containing D-glucose for lipid production. The mechanism of D-glucose uptake in microalgae has also been determined by using *Chlorella* as the model strain. Tanner (2000) revealed that the uptake of D-glucose was based on an inducible hexose/H^+ symport system. In the presence of the inducer D-glucose, *Chlorella* could synthesize the transporter only in 15 minutes and the uptake rate could increase more than 400 fold (Haass & Tanner, 1974). After the transportation, the assimilated D-glucose is metabolized via the Embden–Meyerhof Pathway (EMP) and the Pentose Phosphate Pathway (PPP) (Perez-Garcia et al., 2011).

Although culturing microalgae by feeding with D-glucose has been intensively studied, the report on D-xylose utilization is very limited. Hawkins (1999) screened a *Chlorella* strain with the capability to grow on D-xylose mixotrophically after UV irradiation. However, to the best of our knowledge, wild-type microalgae with the capability to utilize D-xylose as the sole carbon source have not been reported. A lack of efficient uptake system and/or the related metabolic pathway may be the major reasons. Neish (1951) evaluated various carbohydrates on the growth of *Chlorella vulgaris*. The results showed that D-glucose, D-fructose, D-galactose, and β-glucosides were good sources of carbon and energy for algal growth, but pentose sugars including D-xylose caused little or no simulation of growth. Similarly, Samejima and Myers (1958b) found that *C. pyrenoidosa* and *Chlorella ellipsoidea* could hardly grow on xylose although glucose and galactose supported continued growth. Moreover, Hassall (1958) demonstrated that D-xylose could not be used as a substrate for chemosynthesis by the microalga *C. pyrenoidosa*, even more, it acted as a specific inhibitor for the photosynthesis. In the presence of 0.5 per cent D-xylose (33 mM), cell division was arrested and the color of microalgae was lost

within a few days.

In our previous study, we reported a green microalga *Chlorella sorokiniana* (UTEX 1602) with great potential for biofuel production due to its high growth rate, cell density and lipid content by feeding with D-glucose (Li et al., 2013; Zheng et al., 2013). However, the utilization of D-xylose, the second most abundant sugar in lignocellulosic biomass, was also critically required for cost-effective microalgal biofuel production. The aim of this study was to investigate D-xylose utilization by *C. sorokiniana* and the related metabolic pathway. Firstly, D-xylose uptake kinetics was analyzed for heterotrophically and phototrophically grown algal cells. The effects of different mono-sugars were examined on D-xylose uptake. Then, enzyme assay was performed to detect the related metabolic enzymes. The enzyme characteristics including pH, temperature and cofactors were evaluated. Finally, the effect of light on D-xylose utilization was studied.

5.3. Materials and methods

5.3.1. Organism, media and cultivation

The green microalga *Chlorella sorokiniana* (UTEX 1602) was obtained from the Culture Collection of Algae at the University of Texas (Austin, TX). The minimal medium used in all the cultivations was according to our previous description (Zheng et al., 2013).

Flask cultures were conducted in 250 mL Erlenmeyer flasks. Phototrophic cultures contained 200 mL minimal medium supplemented with 10 mM KNO_3 and were bubbled with air (1.0% CO_2) at a rate of 100 mL min^{-1} under light. Heterotrophic cultures containing 50 mL minimal medium were supplemented with 20 mM KNO_3 and 100 mM D-glucose, and incubated at a rotary rate of 200 rpm in dark. The temperature for all the cultures was maintained at 37°C.

5.3.2. ^{14}C-labeled D-xylose uptake assay

The method for ^{14}C-labeled D-xylose uptake assay was carried out according to the description by (Du et al., 2010). ^{14}C-labeled D-xylose was obtained from American Radiolabeled Chemicals (St. Louis, MO). Algal cells were harvested at the exponential phase and washed with ice-cold water three times. The cells were then resuspended to about 60 mg dry cell weight (DCW) per mL in potassium phosphate buffer (50 mM, pH 7.0). One milliliter cell suspension was dried in a pre-weighed aluminum dish at 105°C for 3 h to determine the DCW. The prepared cell suspension was kept on ice before use. D-xylose uptake kinetics was initiated by mixing 160 μL cell suspension (5 min preincubation at 37°C) with 40 μL labeled D-xylose of various concentrations. Sugar inhibition studies were performed with 20 μL labeled D-xylose (5 mM) and 20 μL unlabeled sugar (5 mM). The reaction was stopped by adding 10 mL ice-cold water after 30 s. The zero time point was handled by adding cell suspension and ice-cold water simultaneously. The mixture was filtered through a Whatman GF/C filter (Whatman, NJ) presoaked in 40% D-xylose and washed twice with 10 mL ice-cold water. Samples were counted with a Beckman LS6500 scintillation counter (Beckman Coulter, CA) in the scintillation cocktail (National Diagnostics, GA). Three independent assays were used to determine kinetic values. The uptake rate was recorded as nanomoles (nmol) of labeled D-xylose min^{-1} mg^{-1} of cell.

5.3.3. Kinetic analysis

The sugar uptake data were analyzed with non-linear regression using SigmaPlot 12.0 (Systat Software, CA). The analysis was performed with the following two models (Alcorn & Griffin, 1978):

Model I, Michaelis-Menten function for a single carrier.

$$V = V_{max1} \bullet [S]/(K_{m1} + [S]) \quad (1)$$

Model II, Michaelis-Menten function for two carriers.

$$V = V_{max1} \bullet [S]/(K_{m1} + [S]) + V_{max2} \bullet [S]/(K_{m2} + [S]) \quad (2)$$

where K_{m1} and V_{max1} indicate K_m value and maximum xylose transport rate for one transporter, while K_{m2} and V_{max2} are the corresponding parameters for another transporter.

5.3.4. D-xylose utilization under heterotrophic and mixotrophic conditions

Heterotrophically and phototrophically grown algal cells were washed and resuspended to 8 mg dry cell weight (DCW) per mL in 50 mL minimal medium supplemented with 5 mM KNO_3 and 40 mM D-xylose. The cultures were performed in 250 mL Erlenmeyer flasks and shaken with or without light for 24 h at 37°C.

To evaluate the effect of photosynthesis on D-xylose utilization, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was added to the cultures at the concentration of 10 μM.

5.3.5. Enzyme assay

The algal cells were harvested and washed with ice-cold water three times. The cell extracts for enzyme assay were prepared by disrupting the cells with glass bead in 50 mM potassium

phosphate buffer (pH 7.0) containing 0.5 mM DTT and 1 mM phenylmethylsulfonyl fluoride.

The method for enzyme assay was according to the description by Freer et al. (1997). Xylose reductase (XR) activity was assayed in reaction mixtures (1.0 mL) containing 50 mM potassium phosphate buffer (pH 5.5–7.0) or Tris buffer (pH 7.5–9.0), 50 mM D-xylose, 0.34 mM NAD(P)H and 0.1 mL enzyme preparation. Xylitol dehydrogenase (XDH) was assayed in a similar manner, except 50 mM xylitol and 2 mM $NAD(P)^+$ was used in the reaction mixture. Temperature ranged from 30°C to 70°C. The oxidation or formation of NAD(P)H was recorded at 340 nm using a UV-Vis spectrophotometer (UV-2550, Shimadzu, Kyoto, Japan). Background enzyme activities without addition of cofactor were also measured. One unit of enzyme activity was defined as one micromole (μmol) of cofactor converted per minute. Specific enzyme activity was expressed as U mg^{-1} protein, based on the determination of protein concentration according to the procedure described by Bradford (1976) using bovine serum albumin as the standard.

5.3.6. Other analysis

D-glucose, D-xylose and xylitol were analyzed using a Dionex ICS-3000 ion chromatography (Dionex Corp., CA), which was equipped with a CarboPac TM PA 20 (4 × 50 mm) analytical column, and CarboPac TM PA 20 (3 × 30 mm) guard column (Yu et al., 2011). The filtered samples were injected and eluted isocratically with 0.01 M NaOH. Analytes were detected and quantified based on standard curves.

All the flasks cultures were repeated in triplicate. The experimental data were statistically analyzed with ANOVA using SAS 9.2 (SAS Institute, NC).). All values were presented as the average of independent measurements with significance declared at $P < 0.05$.

5.4. Results

5.4.1. D-xylose uptake in *C. sorokiniana*

The kinetic parameters of two different models for D-xylose transport in C. sorokiniana are listed in Table 5.1. For heterotrophically grown algal cells, there was no significant difference of variances between these two models. From Fig. 5.1, the two-carrier model fitted the experimental data better than the single-carrier model. However, the V_{max2} and K_{m2} of the two-carrier model were extremely high, which was unreasonable to represent the transportation kinetics. Thus, the single-carrier model was selected as the representation of D-xylose transport in heterotrophically grown *C. sorokiniana*. Non-linear regression analysis showed that the maximum D-xylose transport rate was 3.8 nmol min^{-1} mg^{-1} cell with K_m value of 6.8 mM.

Phototrophically grown *C. sorokiniana* also exhibited the capability to take up D-xylose, but with a comparatively low uptake rate. For instance, the uptake rate of 2.0 nmol min^{-1} mg^{-1} cell was observed at 5 mM D-xylose for heterotrophically grown algae, while phototrophically cultured cells only showed a rate of 0.1 nmol min^{-1} mg^{-1} cell. The two-carrier model gave a lower variance than the single-carrier model for phototrophically grown algal cells, but the V_{max2} and K_{m2} values were too high to make the model reasonable. According to the kinetic parameters of the single-carrier model, the maximum D-xylose uptake rate was only 0.3 nmol min^{-1} mg^{-1} cell, ten times lower than that of heterotrophically cultured algae, but the K_m value (5.5 mM) was almost at the same level. Thus, these results indicated that the transporters had comparable affinities to D-xylose for the algae grown under heterotrophic and phototrophic modes, however, heterotrophically grown cells exhibited much higher capacity.

5.4.2. Effect of mono-sugars on D-xylose uptake

The effects of different mono-sugars on D-xylose uptake in the microalga *C. sorokiniana* are tabulated in Table 5.2. D-xylose uptake rate was significantly inhibited in the presence of D-glucose, independent of the cultivation modes, which indicated that D-glucose competed with D-xylose for the transporter and had much higher affinity. Similarly, D-galactose also performed as a competitor to the transporter, but it showed much stronger inhibition in phototrophically grown cells. A different inhibitory manner was observed on D-fructose, which did not inhibit D-xylose uptake in phototrophically cultured cells but slightly suppressed the transport in heterotrophically cultured cells. The pentose sugars (L-arabinose and D-ribose) did not play a significant negative role in D-xylose uptake for both heterotrophically and phototrophically grown *C. sorokiniana*, which might be attributed to the low affinity or even the transporter lacked the capability to take up these two sugars.

5.4.3. D-xylose metabolic enzymes

The activities of XR and XDH present in crude cell-free extract of *C. sorokiniana* cultivated under different conditions are shown in Table 5.3. When the algal cells grew on D-glucose, the enzyme activity was not detectable, independent of culture modes. After incubated with D-xylose for 24 h, the heterotrophically grown *C. sorokiniana* exhibited XR and XDH activities of 0.021 and 0.026 U mg^{-1} protein respectively, indicating that the expression of XR and XDH was induced by D-xylose. However, the enzyme activity was not detected for phototrophically cultured algae although the cells were capable of taking up D-xylose. The reason might be the enzyme activity was too low to be detected or the intracellular D-xylose was not sufficient to induce the enzyme expression due to the low sugar uptake rate.

Fig. 5.2 shows the effects of pH and temperature on the activity of XR and XDH, present in crude cell-free extract obtained from heterotrophically grown *C. sorokiniana* after incubation with D-xylose. The activity of XR increased from 0.018 to 0.021 U mg^{-1} protein with an increase of pH from 5.5 to 6.0, and then decrease until 0.012 U mg^{-1} protein at pH 9.0. In the same tested pH range, XDH achieved the highest activity of 0.026 U mg^{-1} protein at pH 8.0. At pH 6.0 (the optimal pH for XR), the activity of XDH decreased 92% from its highest value. Meanwhile, only a loss of 30% was observed on XR activity at pH 8.0 (the optimal pH for XDH). Therefore, acidic environmental was more detrimental to XDH than the negative effect of alkalinity to XR. The optimal temperature for XDH was 50°C and further increasing the temperature to 70°C resulted in a 93% activity loss. XR was more stable at higher temperature than XDH. The elevated temperature improved the performance of XR and the highest activity of 0.027 U mg^{-1} protein was obtained at 70°C.

5.4.4. Cofactors for XR and XDH

Table 5.4 shows the activities of XR and XDH present in crude cell-free extract of *C. sorokiniana* with different cofactors. Both NADPH and NADH could be used as the cofactor for the reaction from D-xylose to xylitol catalyzed by XR, but the enzyme had a preference for NADPH. The activity of XR with NADPH was about 5 fold higher than that with NADH. Different with XR, XDH could catalyze the oxidation of xylitol to D-xylulose only in the presence of the cofactor $NADP^+$.

5.4.5. Effect of light on D-xylose utilization

Fig. 5.3 shows D-xylose consumption and xylitol production by *C. sorokiniana* after 24 h

incubation under different conditions. The highest D-xylose consumption of 19.1 mM was observed for the cultures with light. Heterotrophically grown *C. sorokiniana* also had the capability to utilize D-xylose in the dark but the consumption was more than 2 fold lower compared with the cultures under light. The stimulation of D-xylose utilization by light was vanished in the presence of DCMU, evidenced by a comparable D-xylose consumption with the cultures in the dark. However, DCMU did not play a significant inhibitory role in D-xylose utilization in the dark conditions. Xylitol was formed during the fermentation for all the cultures. *C. sorokiniana* converted 50–60% of the consumed D-xylose to xylitol.

5.5. Discussion

The first step for D-xylose metabolism is the uptake of the sugar through the plasma membrane. A lack of transport system may be one of the major reasons for most microalgae without the capability to utilize D-xylose as the sole carbon source. There are two kinetically distinct D-xylose transport systems–the high-affinity system (specific for D-xylose) and the low-affinity system (shared with D-glucose) for native D-xylose-metabolizing microbes. Meanwhile, D-xylose also can be taken up by the D-glucose transporters for non-native D-xylose utilizing species, i.e., the ethanol producing yeast *Saccharomyces cerevisiae*, but D-glucose strongly inhibits D-xylose transportation due to the different affinities for these two sugars (Jeffries & Jin, 2004).

Our results showed that *C. sorokiniana* could take up D-xylose through a single-carrier system. The maximum D-xylose transport rate of 3.8 nmol min^{-1} mg^{-1} cell with K_m value of 6.8 mM was obtained for *C. sorokiniana* after incubated with D-glucose heterotrophically. It has been reported that *Chlorella* cells possessed an inducible active hexose/H^+ symport system

responsible for uptake of D-glucose from the environment (Tanner, 2000). Thus, the green microalga *C. sorokiniana* might take up D-xylose through the inducible hexose symporter (Fig. 5.4). Tanner (2000) stated that the symporter could take up various hexoses and two pentoses (D-arabinose and D-xylose, but not D-ribose) with different affinities. This was in agreement with our results that D-xylose uptake was suppressed in the presence of D-glucose, D-galactose and D-fructose, but the degrees of inhibition were different due to the different affinities to these hexoses. *C. sorokiniana* might not be able to transport the pentose sugars (L-arabinose and D-ribose) by using the hexose symporter, because no significant inhibitory effect on D-xylose assimilation was observed for these sugars.

Interestingly, the phototrophically grown *C. sorokiniana* were also able to take up D-xylose and had similar affinity (K_m) to D-xylose compared with heterotrophically incubated cells. Thus, the phototrophically cultured cells might still utilize the hexose symporter for D-xylose assimilation, which was also evidenced by the severe inhibition of D-xylose uptake in the presence of D-glucose and D-galactose. However, the capacity (V_{max}) was much lower than that in heterotrophically cultured algae, indicating a limited quantity of transporter presented in phototrophically grown cells. Tanner (2000) suggested that a small amount of transport protein constitutively presented in the algal membrane. Haass and Tanner (1974) reported that noninduced *Chlorella* strains had the sugar uptake capability and the uptake rate increased 2–412 fold after induction by D-glucose. This was in accordance with our results of D-xylose uptake for phototrophically grown cells, and the data also demonstrated that *C. sorokiniana* exhibited a remarkably increased D-xylose uptake rate after incubated with D-glucose heterotrophically.

After D-xylose is transported into the cells, it will be reduced to xylitol by XR and then converted to D-xylulose by XDH in most eukaryotic microorganisms. Subsequently, D-xylulose

is converted to D-xylulose 5-phosphate (D-xylulose 5-P) by xylulokinase before entering the PPP pathway (Hahn-Hägerdal et al., 2001). In this study, NAD(P)H-linked XR and $NADP^+$-linked XDH activities were detected in crude cell-free extract obtained from heterotrophically grown *C. sorokiniana*. In fact, XDH from most reported microorganisms exclusively uses NAD^+ as the cofactor, while XR prefers NADPH (Hahn-Hägerdal et al., 2001; Verho et al., 2003). The imbalance of redox cofactors will occur due to the different coenzyme usage, resulting in high amounts of xylitol accumulation. A notable quantity of xylitol was also produced during the fermentation of D-xylose by *C. sorokiniana*, but inappropriate cofactors balance might not be the reason because the XDH of *C. sorokiniana* showed a unique characteristic by using $NADP^+$ as the cofactor. The formation of xylitol is not solely a consequence of coenzyme imbalance and some metabolic factors may also cause its production, i.e., the activity ratios of XR and XDH. It has been reported that a high ratio of 10:1 of XDH and XR activities were essential to improve the conversion of D-xylose to D-xylulose (Hahn-Hägerdal et al., 2007). However, our data showed that the activity ratio of XDH and XR was only 1.8:1 at pH 8.0 (optimal pH of XDH). Actually, *Chlorella* tends to maintain intracellular pH relative constant around 7.0 (Gehl & Colman, 1985; Lane & Burris, 1981). In this condition, the activity of XR was even higher than XDH (Fig. 5.2A) and the equilibrium of the reaction favored xylitol formation.

Unlike yeast and fungi, microalgae are photosynthetic organisms and have the capability to capture light energy through photochemical reactions. During the first stage of photosynthesis, light energy is converted into chemical energy such as NADPH, which can potentially serve as the coenzyme for D-xylose metabolism (Fig. 5.4). Our results showed that the D-xylose consumption was 2 fold higher under light compared with the cultures in the dark. However, in the presence of DCMU, an herbicide specifically blocking electron flow from photosystem II and

inhibiting NADPH production, the consumption of D-xylose was almost identical with that of the cultures in the absence of light. This result also reflected that the improvement of D-xylose utilization by light was attributed to the extra chemical energy from the light-dependent reaction.

Although *C. sorokiniana* could take up D-xylose through the inducible hexose symporter and express XR and XDH for D-xylose catabolism, the growth efficiency was very low since the cell number and DCW did not increase during the fermentation (data not shown). There are several possible reasons for this phenomenon. The synthesis of the hexose symporter could be induced by D-glucose, D-galactose and D-fructose, but the induction was not achievable by pentoses (Tanner, 2000). Although D-glucose induced algae can take up D-xylose efficiently, the reproduced algal cells may not possess such a transport system (or only a very few amount of constitutive transporter), which subsequently hinders the expression of XR and XDH and eventually results in the death of the reproduced cells. Hahn-Hägerdal (2001) declared that the inability of *S. cerevisiae* to utilize D-xylose was due to the low expression of XR and XDH although the genes encoding these enzymes were present in its genome. Additionally, the low activity ratio of XDH and XR limits D-xylose toward central metabolism. Our data showed that more than half of the D-xylose carbon was secreted out in the form of xylitol, which would lead to inefficient ATP generation (Kötter & Ciriacy, 1993). Actually, microalgae require a significant amount of ATP just for maintenance (Yang et al., 2000). As a result, the energy and the carbon are inadequate to support the cell reproduction and biomass accumulation.

5.6. Tables and figures

Table 5.1. Kinetic parameters for D-xylose transport in *C. sorokiniana*.

	Heterotrophic		Phototrophic	
Parameter	Model I	Model II	Model I	Model II
V_{max1} (nmol min^{-1} mg^{-1} cell)	3.8	3.0	0.3	0.1
V_{max2} (nmol min^{-1} mg^{-1} cell)	-	1.9×10^{6}	-	9.0×10^{4}
K_{m1} (mM)	6.8	4.7	5.5	0.6
K_{m2} (mM)	-	1.2×10^{8}	-	2.2×10^{7}
Variance	3.6×10^{-2}	3.7×10^{-2}	1.7×10^{-3}	4.0×10^{-4}

Table 5.2. Effect of mono-sugars on the rate of D-xylose transport by *C. sorokiniana*.

	Heterotrophic		Phototrophic	
	V (nmol min^{-1} mg^{-1})	Inhibition[a] (%)	*V* (nmol min^{-1} mg^{-1})	Inhibition (%)
D-glucose	0.044	92.4	0.011	89.4
D-galactose	0.134	76.7	0.003	97.0
D-fructose	0.504	12.5	0.120	0.0
L-arabinose	0.583	0.0	0.140	0.0
D-ribose	0.567	1.5	0.128	0.0

[a] Percentage of inhibition was calculated based on the control without addition of other mono-sugars.

Table 5.3. The activity of XR and XDH present in crude cell-free extract obtained from disrupted cells of *C. sorokiniana*[a].

	Heterotrophic		Phototrophic	
	D-xylose	D-glucose	D-xylose	D-glucose
XR[b] (U mg^{-1} protein)	0.021	ND[d]	ND	ND
XDH[c] (U mg^{-1} protein)	0.026	ND	ND	ND

[a] The washed heterotrophically and phototrophically grown algal cells were resuspended in the minimal medium containing 40 mM D-xylose or D-glucose and shaken under light for 24 h.

[b] The activity of XR was measured at pH 6.0 and 40°C with NADPH as cofactor.

[c] The activity of XDH was measured at pH 8.0 and 40°C with $NADP^+$ as cofactor.

[d] Not detected.

Table 5.4. The activity of XR and XDH of *C. sorokiniana* with different cofactors[a].

	NADPH	NADH	$NADP^+$	NAD^+
XR[b] (U mg^{-1} protein)	0.021	0.004	-	-
XDH[c] (U mg^{-1} protein)	-	-	0.026	ND[d]

[a] The washed heterotrophically grown algal cells were resuspended in the minimal medium containing 40 mM D-xylose and shaken under light for 24 h.

[b] The activity of XR was measured at pH 6.0 and 40°C.

[c] The activity of XDH was measured at pH 8.0 and 40°C.

[d] Not detected.

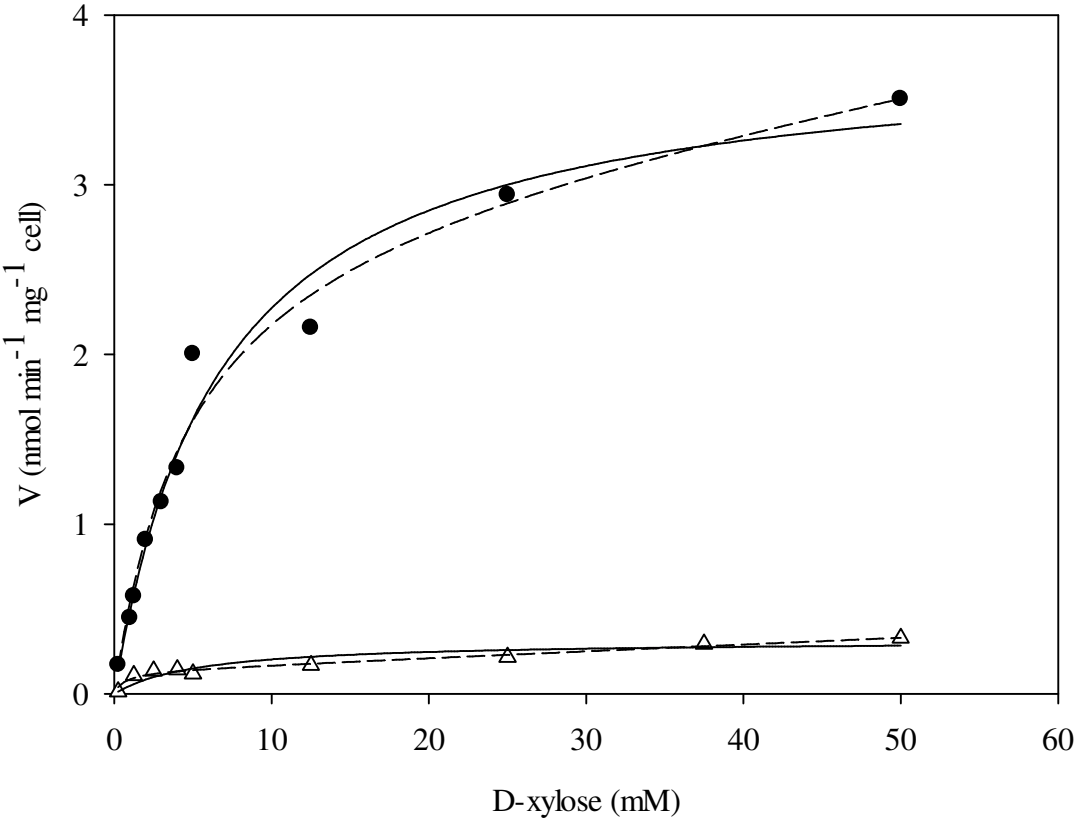


Fig. 5.1. D-xylose uptake kinetics of heterotrophically (black circle) and phototrophically (white triangle) grown *C. sorokiniana* using two different models: solid line, Model I (single-carrier); dash line, Model II (two-carrier).

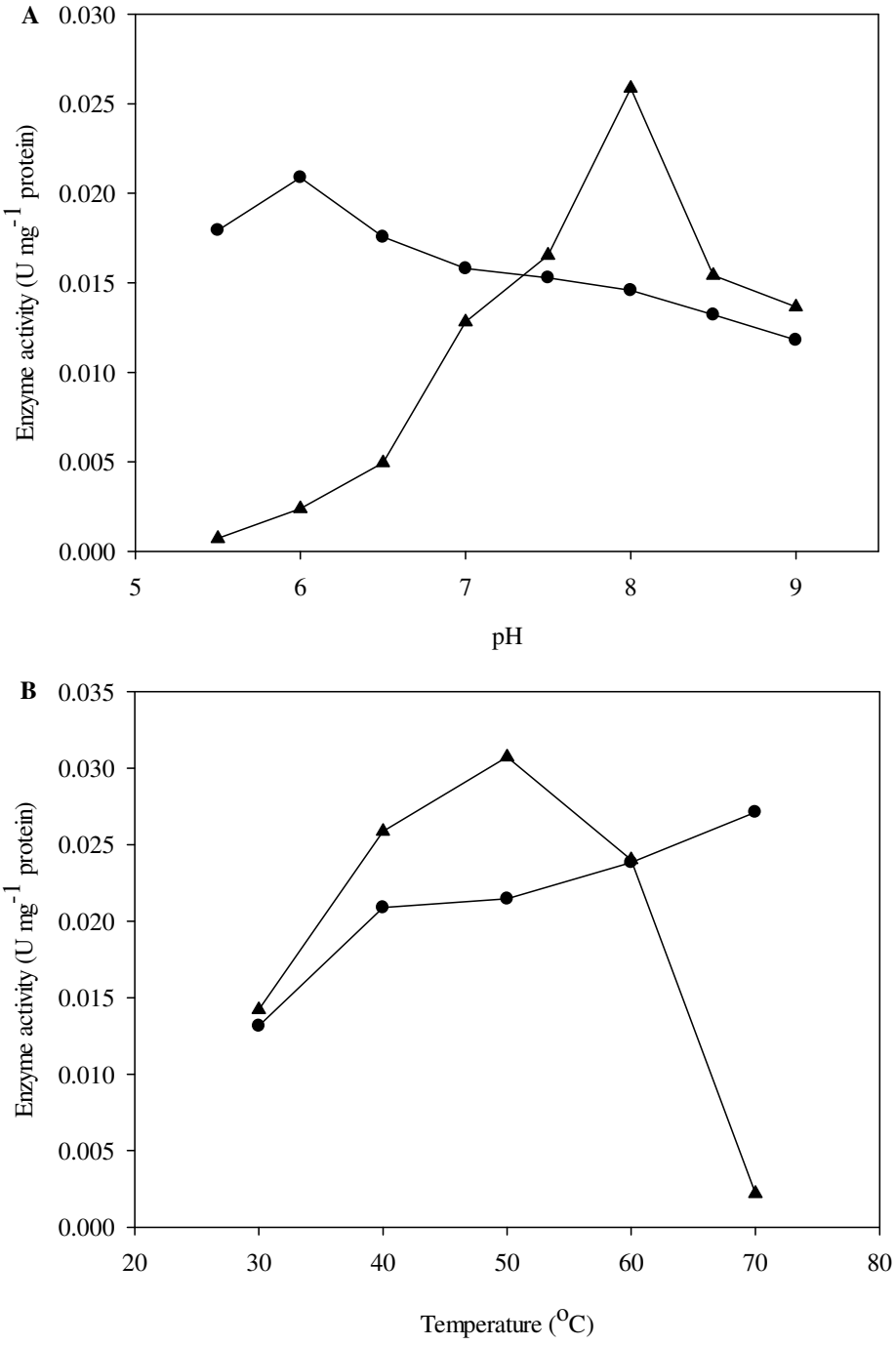


Fig. 5.2. Effect of pH (A) and temperature (B) on the activity of XR (circle) and XDH (triangle) present in crude cell-free extract obtained from *C. sorokiniana*. The washed heterotrophically grown algal cells were resuspended in the minimal medium containing 40 mM D-xylose and shaken under light for 24 h.

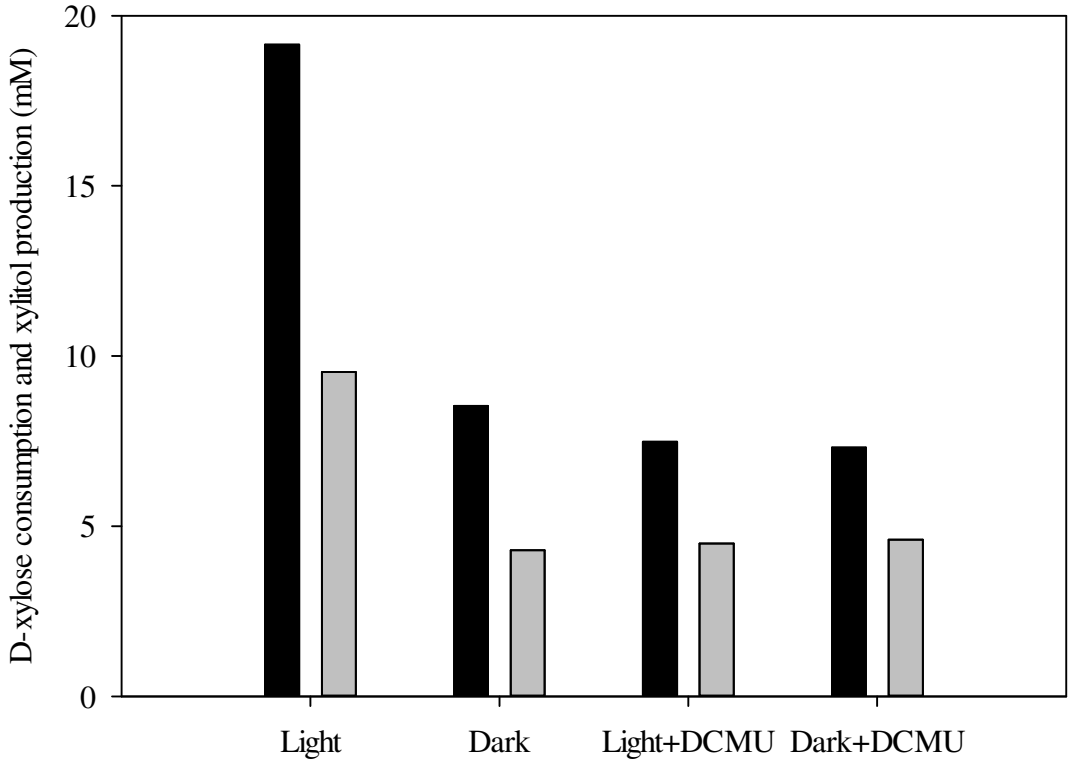


Fig. 5.3. D-xylose consumption (dark column) and xylitol production (grey column) by *C. sorokiniana*. The washed heterotrophically grown algal cells were resuspended in the minimal medium containing 40 mM D-xylose under different conditions for 24 h.

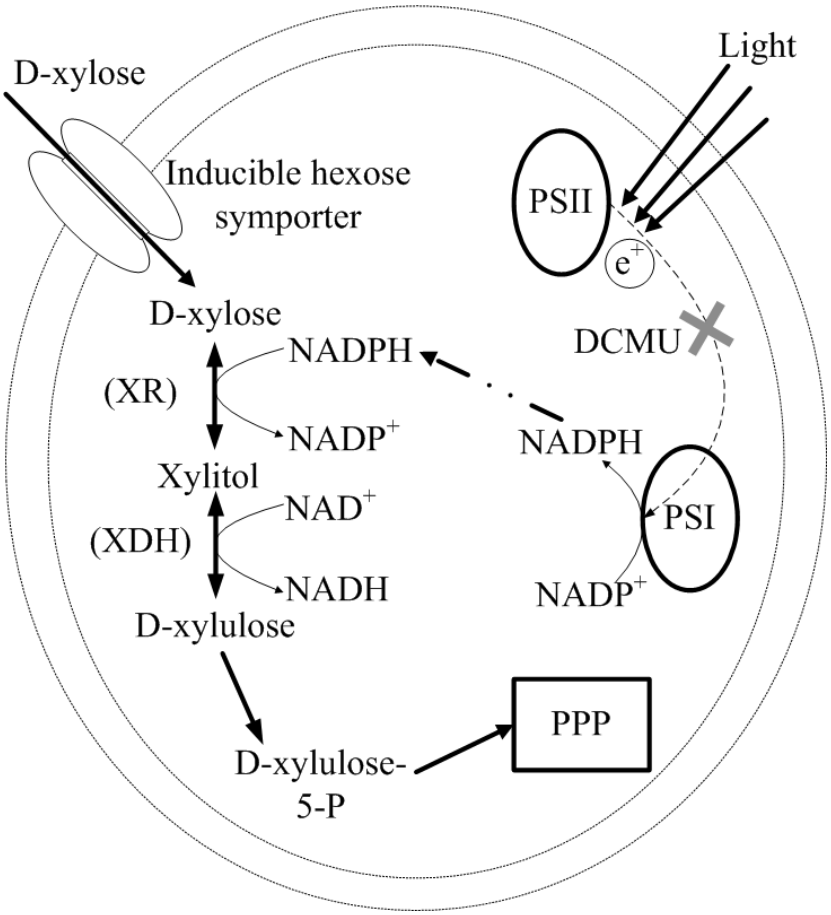


Fig. 5.4. D-xylose metabolic pathway in the green microalga *C. sorokiniana*.

Chapter 6

Summary and conclusions

In this dissertation, I developed a two-stage heterotrophic and phototrophic system for microalgal biomass and lipid production. The major conclusions of this dissertation are:

- Heterotrophic culture can be used as a better process to produce seed cells for the alga *C. sorokiniana* in large scale open systems, since it has much higher productivity but similar performance compared with its phototrophic counterpart. Organic waste and municipal wastewater can be served as good feedstock for this heterotrophic process. In addition, high inoculation rate of heterotrophically produced seed cells can be served as an effective method for contamination control.
- The feasibility for high cell density heterotrophic cultivation of *C. sorokiniana* was demonsrated through two-stage fed-batch fermentation. With the optimized culture conditions, the algal biomass and lipid reached high concentrations of 103.8 g L^{-1} and 40.2 g L^{-1} respectively. The lipid produced by *C. sorokiniana* was a desirable feedstock for biodiesel production in terms of the high content of neutral lipids and appropriate fatty acid composition.
- Culture of *C. sorokiniana* for lipid production with cellulosic materials through SSF was evaluated. High enzyme loading led to high hydrolysis rate and more available sugar for lipid accumulation. Increasing cellulose concentration also played a positive role on *C. sorokiniana* growth, but a moderate substrate loading (3%, w/v) was more suitable for lipid production. Elevated temperature greatly improved SSF efficiency and the highest

lipid concentration (2.98 g L^{-1}), yield (99.2 mg g^{-1}) and productivity (20.7 mg L^{-1} h^{-1}) were achieved at 40°C with 3% (w/v) cellulose and 30 FPU/g substrate.

- The green microalga *C. sorokiniana* had the capability to utilize D-xylose. The analysis on sugar uptake kinetics suggested that the inducible hexose symporter might be responsible for the transport of D-xylose across the cell membrane. Two key enzymes, NAD(P)H-linked XR and $NADP^+$-linked XDH, for D-xylose metabolism were discovered from *C. sorokiniana*. The unique characteristic of XDH using $NADP^+$ as the cofactor would not lead to redox imbalance, but its relative low activity compared with XR might be the reason to cause xylitol accumulation. Culturing *C. sorokiniana* mixotrophically could improve D-xylose utilization due to additional NADPH from the light-dependent reaction of photosynthesis.

The heterotrophic process can operated in sequence with phototrophic culture to enhance the productivity and stability of the system. In the first stage, algae grow heterotrophically by feeding with waste organic carbon source. The purpose of this stage is to produce seed algae cells with high density. This process takes the advantage of the high growth kinetics of heterotrophic growth and algae can reach a very high density that is desirable for the subsequent phototrophic culture. The requirement for organic substrate during the initial cell growth stage is much less than that of single heterotrophic culture. The high density algae seed is used as an inoculum for the second open pond phototrophic culture. During this stage, algae can accumulate biomass by assimilation of carbon dioxide produced from the first stage, or other resources, e.g., power plants.

The heterotrophic process can be operated in parallel with phototrophic culture to make a year-round production capability. The open pond system is highly dependent on local weather

conditions, which is hardly controlled and makes the production seasonal, especially in the cold climate areas (Perez-Garcia et al., 2011). In the winter time, the phototrophic algae culture process will be shut down, but the heterotrophic process using organic waste as feedstock will be run alone to produce oil-enriched algae biomass, which then can be processed into biofuel. The organic waste streams like agricultural residues are often readily available and the tipping fee makes it have a higher economic viability. So, using heterotrophic process in algal biomass production improves the practical operability, and makes it not only relying on CO_2 and sunlight.

The essence of this technology is to integrate phototrophic microalgae fuel production with heterotrophic production to increase the overall productivity. The novelty of this approach is to utilize different growth modes in sequence or parallel to improve algal biomass production in a manner that is difficult to accomplish under natural environments. This two-stage heterotrophic and phototrophic microalgae culture system has some potential advantages for industrial application:

(1) High efficiency microalgal seed preparation

The traditional microalgae seed preparation was achieved by using raceway pond and/or PBR. The microalgal biomass productivity is about 25 and 48 g m^{-2} d^{-1} for large scale raceway pond and PBR systems, with volumetric productivity of 0.083 and 1.535 kg m^{-3} d^{-1} respectively (Table 6.1). Actually, it is very difficult to maintain this kind of productivity for phototrophic culture because the microalgae growth is greatly affected by the weather conditions. Meanwhile, heterotrophic cultivation is operated in well controlled fermentor and exhibits notable high growth rate. Our data showed that the biomass productivity could reach 24.2 kg m^{-3} d^{-1}, which was 292 and 16 fold higher than that of the raceway pond and PBR systems.

(2) Low land and water requirement

The illuminated surface area is required for microalgal seed preparation through phototrophic process, which can occupy about 30% of the total cultivation land (Huntley & Redalje, 2007). Heterotrophic culture does not need light, so the land for seed production can be saved for the second stage phototrophic growth. In addition, large amount of water will be used for phototrophic culture due to the low cell density. Evaporation brings more serious problem for the open pond systems. However, heterotrophic process was operated in closed fermentor and the high density leads to a much lower water usage compared with phototrophic culture (Table 6.1).

(3) Contamination control

Open ponds are easily contaminated by invading species and mono-culutures can be maintained only for a short period of time. Although the PBR system can reduce the risk, contamination is still possible. Heterotrophic culture is performed under sterilized conditions, which can avoid the contamination occurring during seed preparation stage and guarantee the purity of the seed cells. Moreover, high inoculation is a feasible tool to reduce the contamination in the second phototrophic culture stage, since heterotrophic process can provide a high density seed stream very efficiently.

(4) Low production cost

The cost of feedstock is the major limiting factor for heterotrophic culture, but utilizing lignocellulosic materials offers a solution. According to the latest estimations released from the National Renewable Energy Laboratory, the estimated microalgal biomass production cost is about \$0.44 kg^{-1} by using corn stover as the feedstock, even a lower cost compared with the raceway open pond system (table 6.2 and 6.3). For PBR systems, the high production cost is from the facility since specifically designed reactor is required. Heterotrophic cultivation can be accomplished with mature fermentation technologies and facilities, such as those used for

industrial beverages, medicines and food additives production, which results in a significant reduction in costs compared with PBR systems. Additionally, the low requirement of land and water makes contributions to reduce the cost. Credits also come from the recycle of carbon dioxide generated during the heterotrophic cultivation.

(5) High biomass productivity

It has been reported that much higher algal biomass productivity could be achieved by using higher cell density as inoculums (Aflalo et al., 2007). However, it is not feasible for traditional phototrophic seed preparation due to the low cell density. This strategy can be realized by heterotrophic seed production system, which can provide very high dense of algal seed in a short time.

Tables and figures

Table 6.1. Comparison of phototrophic and heterotrophic microalgal biomass production methods.

Variable	Raceway pond	PBR	Heterotrophy
Microalgal biomass (ton)	100	100	100
Area productivity (kg m^{-2} d^{-1})	0.025[a]	0.048[b]	-
Volumetric productivity (kg m^{-3} d^{-1})	0.083[a]	1.535[b]	24.2[c]
Area (m^2)	12,121	6,313	200
CO_2 (ton)	183[b]	183[b]	−129[d]
Sugar (ton)	-	-	213[c]
Water (MM gal)	7.3[a]	2.2[a]	0.2[c]

[a] Based on Davis et al. (2011).

[b] Based on Chisti (2007).

[c] Based on fermentation data in this study.

[d] Based on the sugar conversion rate in this study and chemical composition of *Chlorella* described by Spoehr and Milner (1949).

Table 6.2. Microalgae production costs of raceway pond and PBR systems.

Variable	Raceway pond	PBR
Capital cost ($)		
Equipment and installation	303,025[a]	3,105,918[b]
Land	14,976[c]	7,800[c]
Infrastructure	24,242[a]	2,563,078[b]
Total capital cost	342,243	5,676,796
Depreciation (10 years, $ y^{-1})	34,224	567,680
Operating cost ($ y^{-1})		
CO_2	9,150[c]	9,150[c]
Fertilizers	5,140[a]	5,140[a]
Water	584[c]	176[c]
Labor	14,545[a]	14,545[a]
Electricity	3,636[a]	176,764[b]
Total operating cost ($ y^{-1})	33,056	205,775
Total production cost ($ y^{-1})	67,280	773,455
Biomass production cost ($ kg^{-1})	0.67	7.73

[a] Calculated according to Ben-Amotz (2008).

[b] Calculated according to Grima (2009).

[c] Calculated according to Sun et al. (2011), CO_2 is derived from power plant.

Table 6.3. Microalgae production costs of heterotrophic systems with lignocellulosic materials as the feedstock.

Variable	Heterotrophic system
Feedstock + Handling	22114[a]
Sulfuric acid	734[a]
Fertilizer	5140[b]
Glucose (enzyme production)	5773[a]
Other raw materials	3865[a]
Waste disposal	734[a]
Net electricity	0[a,c]
CO_2 credit	-6450[d]
Fixed costs	5235[a]
Capital depreciation	6556[a]
Total production cost	43700
Biomass production cost ($\$ kg^{-1}$)	0.44

[a] Calculated according to Humbird et al. (2011).

[b] Calculated according to Ben-Amotz (2008).

[c] Various organic by-product streams, such as the lignin and the unconverted cellulose and hemicellulose from the feedstock, are burned to provide steam and electricity for the ethanol plant. The generated electricity is higher than the demand and additional revenue can be made. Unlike ethanol fermentation, heterotrophic culture of microalgae is an aerobic process, so we assume that the credit of the extra electricity is used to compensate the additional energy required for aeration and agitation.

[d] Calculated according to Sun et al. (2011). Heterotrophoic culture is a CO_2 generatio process, which can be used for phototrophic microalgae growth.

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