# DEVELOPMENT OF AN EFFECTIVE SWINE MANURE-BASED ALGAL CULTIVATION SYSTEM FOR BIOFUEL & ANIMAL FEED PRODUCTION AND WASTEWATER TREATMENT

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# Dedication

This work is dedicated to my gentle father Fudong Hu, loved mother Hongna Liu, and dear sister Ling Hu.

# Abstract

Microalgae have great potential to replace current crop feedstocks for biofuel and animal feed production. However, the algal industry is still far from being economically applicable. The dissertation was inspired by the idea of integrating algal biomass production and wastewater treatment. The overall objective of the study was to develop an effective algal cultivation system in which inorganic nutrients and organic substances in swine manure could be utilized by robust microalgae strains for the production of algal biomass with high lipids or protein contents.

The first step in the study was bioprospecting for mixotrophic microalgae strains that adapted well to diluted swine manure. Through the multi-step screening strategy, two locally isolated microalgae strains, UMN271 and UMN266, were found to be facultative heterotrophic, manure-tolerant, and obviously robust in the algae storage including 98 UTEX strains and 50 indigenous strains. The two strains were identified as *Chlorella* sp. and *Hindakia* sp., respectively, through morphological observation and genetic identification, and were utilized for further studies in the dissertation.

Since algae growth on highly diluted swine manure was still relatively low, a second step in the study was to assess the limiting factors of using anaerobically decomposed swine manure as nutrient supplement for algae cultivation. In the study, *Chlorella* sp. (UMN271) and *Hindakia* sp. (UMN266) were used to investigate the effects of two potential factors, which were trace elements and carbon compounds in swine manure, on algal growth and waste nutrient recovery. The results indicated that the algal growth and nutrient removal rates were independent of trace metal content in diluted swine manure,

but were significantly improved when the initial liquid COD content in swine manure was high. Moreover, it was demonstrated that *Chlorella* sp. (UMN271) was able to utilize acetic, propionic and butyric acids, the main water-soluble organic carbon compounds in digested swine manure, for algal growth, lipid production, and waste nutrient removal. Therefore, it was considered that algal growth in highly diluted swine manure was limited by the deficiency of volatile fatty acids (VFAs).

The third step was to modify anaerobic digestion process for liquid swine manure (LSM) rich in VFAs, so that the liquid effluent could be more suitable than the conventionally decomposed LSM effluent as nutrient for the cultivation of *Chlorella* sp. (UMN271). The results showed that the modified acidogenic digestion successfully promoted VFA concentrations in swine manure. The obtained acidogenically digested LSM supported the growth of *Chlorella* sp.(UMN271) in a 5-day batch experiment with higher algal growth rates and fatty acid contents in comparison with those on the conventionally decomposed LSM. High removal efficiencies on water-soluble nutrients, including COD, $PO_4$-P, TN and $NH_3$-N, were also observed for the raw, acidogenically digested manure sample (58.02%, 44.74%, 31.58%, and 44.73%, respectively). Finally, the fatty acid profile analysis showed that harvested algal biomass could be used as feedstock to produce high-quality biodiesel.

In the fourth step, central composite design (CCD) was used to investigate the influences of two key parameters, namely wastewater dilution rate (DR) and hydraulic retention time (HRT), on algal biomass productivity and waste nutrient removal rates. According to the response surface analyses of the CCD results, statistically valid quadric models for

the response variables, including algal biomass productivity and the liquid nutrient removal rates including COD, $PO_4$-P, TN and $NH_3$-N, were obtained. The regression analyses illustrated that both DR and HRT had significant influences on the five response variables. The optimal conditions estimated from the significant second-order quadratic models ($p<0.05$) were 8-fold DR and 2.26-d HRT.

The final step was the development of an effective algal cultivation system for the semi-continuous production of algal biomass and waste nutrient removal. In the study, the predicted optimal conditions were applied in a bench-scale multi-layer photobioreactor (PBR) for algae growth on acidogenically digested swine manure. The 17-day semi-continuous cultivating experiment achieved high and stable algal productivity and nutrient removal rates, which fitted the predictive models well. Moreover, relatively high and stable protein and lipid contents (58.78% and 26.09% of the dry weight, respectively) were observed for the harvested algal sample, indicating the suitability of the algal biomass as ideal feedstock for both biofuel and animal feed production.

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# CHAPTER 1. INTRODUCTION

## 1.1 Background and significance of the research

The use of traditional crops (such as oil seeds, sugar crops and wheat) as sources of renewable energy and animal feed is well developed. Mature commercial markets for crop-based biofuel in transportation and crop-based animal feed in husbandry applications are both in place (Sims et al., 2010; Khush, 2005). However, the industries are faced with challenges, such as a significant increase of crop prices (Vidal, 2010), an intensive competition among people, vehicles and animal for food sources caused by a rapid human population growth (Tenenbaum, 2008), ecological consequences of agricultural intensification (Erb et al., 2012), and a relatively low crop yield in comparison with the excessive demand for biofuel and animal feed (EPA, 2011; Bouwman et al., 2012). Therefore, it is greatly urgent to develop alternative feedstock for biofuel and animal feed production.

Microalgae have the potential to replace current feedstock crops by providing oil for biodiesel, crude oil and carbohydrate compounds for bioethanol and biobutanol, and protein and other nutritional substances for animal feed. The nutritional composition of microalgae is similar to agricultural crops (Burtin, 2003). Compared with crops, microalgae have rapid growth rates but no growing season or altitude limits (Sheehan et al., 1998). Previous research suggested that some microalgae species could produce oil up to 80% of their dry weight and hence, could be good sources for biofuel production (Spolaore et al., 2006; Chisti, 2007a). Some reviews and studies have shown that microalgae have acceptable animal feed and/or non-food feedstock qualities (Joy, 1969;

Sheehan et al., 1998; Banerjee et al., 2002; Chisti 2007). Harvested microalgal biomass is a high-grade protein source, and most microalgae species are rich in the essential amino acids (Wilkie and Mulbry, 2002). Also, microalgal biomass is microbial sources of polyunsaturated fatty acids (PUFAs) containing eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), which are important dietary compounds (Ackman et al., 1964).

However, there are many constraints on the commercialization of microalgae-based biofuel and animal feed production. Most of the microalgal biomass production processes investigated had been based on photoautotrophic growth, which was hard to reach a high algal cell concentration because of light insufficiency for photosynthesis, which was caused by self-shading at high culture density (Chen, 1996). The high costs for the harvesting and drying processes were also big drawback to implementing the technology (Sander and Murthy, 2010).

To increase the biomass productivity, one of the methods is to develop a cultivating process known as mixotrophy. Microalgae species with the mixotrophic mode can simultaneously drive photoautotrophy and heterotrophy to utilize both inorganic ($CO_2$) and organic carbon substances in culture. Compared with photoautotrophic cultivation, mixotrophic processes could bring higher growth rates (Lee et al., 1996; Chen and Zhang, 1997; Cid et al., 1992). It was reported that the specific growth rate of microalgae in the mixotrophic mode represented the fastest way of algal mass production, since it was approximately the sum of autotrophic and heterotrophic growth rates (Lee et al., 1989; Xu et al., 2004). Mixotrophic cultivation is also good for the production of lipids,

especially various omega-3 unsaturated fatty acids. The ratio of algae-available carbon ($CO_2$ and organic carbon in total) over nitrogen in a mixotrophic culture is higher than that in an autophotrophic culture. This means that the amount of carbon available to make carbohydrate and lipids is higher, while the amount of carbon beside the algae-available nitrogen being taken up by the cell for protein synthesis remained the same. It was reported that highly PUFAs (C16:3 and C18:3) were mainly produced under photosynthetic conditions in microalgae cells, with an exception for the diatoms *N. laevis*, *N. alba*, and *Tetraselmis spp*., in which the productivities of EPA and DHA were higher under heterotrophic conditions (Day et al., 1991, Gladue and Maxey, 1994, Chen et al., 2007). So far, the number of mixotrophic species that can be employed is limited, and the cultivation cost is still high because the traditional media is expensive. Common organic substances for this process consist of glucose, glycerol, acetate pricing at around \$0.07 $lb^{-1}$, \$0.80 $lb^{-1}$ and \$0.2 $lb^{-1}$, respectively (Liang et al., 2009; Huang et al., 2002).

The integration of algal biomass production with wastewater treatment is considered to be one of the most viable strategies for the cost reduction in algal feedstock production, as first suggested by Oswald and Golueke (1960). In previous studies, raw and anaerobically digested swine manure effluents, rich in organic carbon substances and other nutrients such as nitrogen and phosphorus were investigated for the ability of supporting algal growth (Ayala and Bravo, 1984; Jimenez-Perez et al., 2004; Olguin, 2003). The major forms of water-soluble organic carbon in swine manure are monosaccharides and volatile fatty acids (VFAs) such as acetic acid, propionic acid and butyric acid, which are available substances for many microalgal species under

heterotrophic or mixotrophic conditions (Paul and Beauchamp, 1988; Perez-Garcia et al., 2010). The major form of soluble nitrogen in swine manure is ammonia-nitrogen ($NH_3$-N), which is the predominant form of nitrogen being assimilated by algae with glutamate dehydrogenase (GDH) and glutamine synthetase (GS) (Yang et al., 2000).

The focus in this study is on bioprospecting for robust mixotrophic microalgal strains with the ability to uptake various nutrients in swine manure, and on optimizing environmental factors and culture strategies for an effective algal mass production and wastewater treatment process. It is hypothesized that selected mixotrophic microalgae strains could significantly recover waste nutrients such as nitrogen, phosphorus, and organic carbon in the swine manure culture for the production of algal protein and lipids.

Significances of the proposed work are summarized as follows: 1) it will enhance the manufacturing practice in applying microalgae-based biofuel and animal feed production systems; 2) it will reduce threats of pig effluents containing high nitrogen and phosphorus loads to the aquatic environment; 3) it will provide key information concerning with pilot-scale and commercial-scale algal biomass and/or dietary nutrients accumulation based on swine manure.

### 1.2 Objectives

One goal of this study is to employ potential microalgae strains adapting well in swine manure wastewater. A second goal is to determine and remove the nutritional limitation in swine manure for an efficient algal mass production and waste nutrient removal. A

third goal of this study is to develop an effective system under the optimum operation strategy that can facilitate biomass production of microalgae with high lipids and/or protein content as well as high nutrient removal rates from swine manure effluents in different-scale culture systems. The specific objectives of the research consist of five major portions:

1) Bioprospect microalgal species that adapt well in anaerobically digested swine manure with relatively high biomass productivities as well as superior efficiency on nutrients removal;

2) Determine the nutritional limitation in swine manure that constraint algal biomass accumulation during the mixotrophic growth;

3) Modify anaerobic digestion of swine manure to obtain manure effluents that could be used for enhanced algal growth, lipids and protein accumulation, and nutrient removal;

3) Optimize the key parameters, including dilution rate and hydraulic retention time, in the semi-continuous culture systems to maximize algal biomass productivity and the removal rates of water-soluble manure nutrients, as well as the valuable chemical contents in dried algae;

5) Investigate a bench-scale cultivation process under the optimal conditions for the development of economic production of animal feed and/or biofuel in the microalgae-manure system in the future.

# CHAPTER 2. LITERATURE REVIEW

## 2.1 Overview of microalgae

Microalgae are typically unicellular eukaryotic organisms, and are mainly distributed in the aquatic habitats with a few species found on the surface of all types of soils (Chen et al., 2009; Richmond, 2004). They have played crucial roles in the evolution of land plants and the global ecosystem for millions of years (Fig. 1; Bowman et al., 2007). The biodiversity of microalgae is enormous, and there are about $2\times10^5 \sim 8\times10^5$ species widewide of which around $3.5\times10^4$ are described. Based on plastid phylogeny, they can be categorized into at least 12 major divisions, among which some frequently mentioned

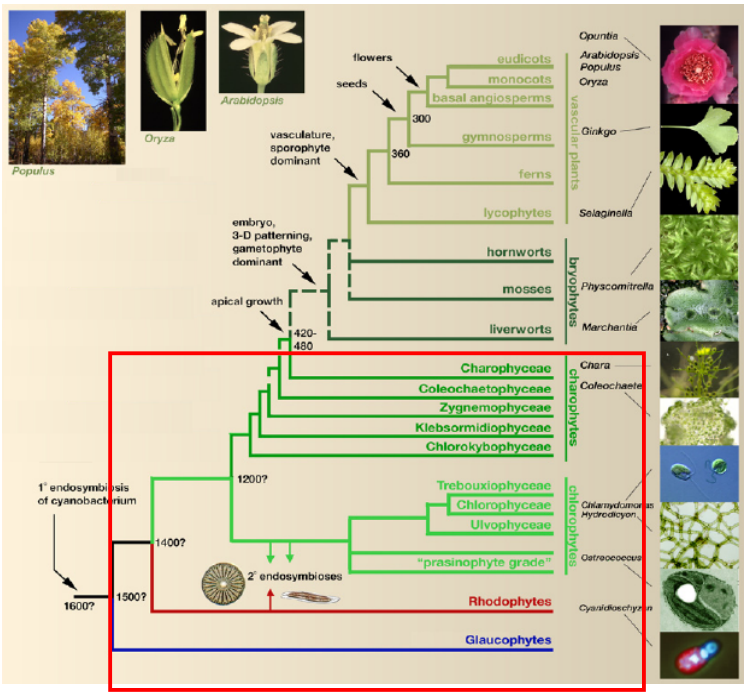


**Figure 1**. Phylogenetic relationship among algae and higher plants. The three lineages of algae, including glaucophytes (freshwater algae; blue), rhodophytes (red algae; red), chlorophytes and charophytes (green algae; green) are marked in the red box. (Bowman et al., 2007)

classes include green algae, red algae, and yellow-green algae (Chen et al., 2009). Nearly all microalgae have photosynthetic machinery, and a wide range of microalgae species in each group grow through photosynthesis within chloroplasts just like higher plants by converting solar energy into chemical energy.

### 2.2 Potential of biofuel production from algal biomass

Microalgae have long been considered as an alternative feedstock to produce biofuels as they possess high growth rate, provide carbohydrate fraction for biomethanol, bioethanol, biobutanol and/or acetone production, offer lipid fraction for biodiesel production, and need fewer acres of farmland than traditional crops for the growth (Harun et al., 2010). It is reported that algae can commonly double their biomass within 24 hours, and grow 10 times faster than sugar cane and 20 ~ 30 times faster than food crops (McDill, 2009). The amount of carbohydrates and lipids from some microalgae species could reach up to 50% and 80% by weight under stressed environment, respectively, leading them to be productive feedstock of biofuel production (Harun et al., 2010; Christi, 2007a). Only 3% of the U.S. cropping area is needed if all petroleum-derived transport fuel required by the United States annually is to be replaced with biodiesel produced from microalgae with 30% oil content, while it would be 61% if the raw material for biodiesel production is oil palm, one of the most productive oil crops (Chisti, 2007b).

The idea of using microalgae as a source of fuel is not new. Algae-based biofuels were first mentioned in the report of MIT feasibility tests for $CO_2$ conversion during early 1950s (Benemann, 2008). Although research on microalgal biofuels has been developed

into various orientations, such as biodiesel synthesis from algal oil through transesterification, methane and/or ethanol production from algal biomass through anaerobic digestion, and photobiological hydrogen production from energy-rich organic compounds through water photolysis, the studies are still some way from being commercially viable (Gavrilescu and Chisti, 2005; Spolaore et al, 2006; Kapdan and Kargi, 2006). Most algal biofuel-based companies are still entangled in the nontrivial economic hurdles with existing technologies. To overcome the issue for the cost-effective production of algal biomass and extraction of biofuels from the feedstock, people need to attain high productivity while reducing capital and operating costs like on biomass harvesting and dewatering.

### 2.3 Potential of animal feed production from algal biomass

Microalgae have certain well-known characteristics that are considered to be a potential alternative source for the production of animal feed. Firstly, some microalgae species contain rich sources of vitamins, minerals, and/or ω-3 PUFAs, which are ideal for the health of farmed animals and for the improvement of the nutritional value of animal meat and products. (Thajuddin and Subramanian, 2005; Mendes et al., 2009; Franklin et al., 1999; Spolaore et al., 2006; Chisti, 2007a). Secondly, dried algae cake has high protein content, sometimes up to 60% of the dry matter, and has been demonstrated as a valuable substitute for conventional protein sources like soybean meal (Kyndt, 2011; Becker, 2007). Thirdly, algae grow extremely fast and commonly double their biomass within 24 hours. During exponential phase, biomass doubling time is as short as few hours and

hence, microalgae strains with high yield and good composition can be produced relatively easily (Chisti, 2007a). Finally, algae culture can be done in space which is normally unused by massive agriculture crops, so that there is no need to compete for land. Once microalgal materials satisfy the Safety and International Standards regulating feed production and get the approval for their incorporation as dietary supplements or additives, microalgae incorporation as feed supplements could be regarded.

Algae-based animal feed production has been investigated and received increasing commercial attention since the early 1970s (Joy, 1969). The early research was focused on photosynthetic microalgae strains as crude protein and/or PUFA resources in aquaculture (Tamiya, 1975; Yongmanitchai and Ward, 1989; Monlina Grima et al., 1994). Unfortunately, it will be hard to commercialize photosynthetically grown algae-based husbandry of animals, especially on land animals. Protein synthesis in phytoplankton is demonstrated to be decreased during photosynthesis since most intracellular carbon flow from the carbon metabolism pool used for protein synthesis into the pool of photosynthetic polymers like chloroactic acid (TCA)-soluble materials (Cuhel et al., 1984). The PUFAs productivity is relatively low due to the low biomass concentrations in phototrophic cultivation (Barclay et al., 1994). People are working on the bioreactor design to enhance the production of biomass (Pirt et al., 2008), species screening and/or genetic modification for trains with high content of crude protein and/or high-quality lipids (Zhou et al., 2011; Rosenberg et al., 2008), technical and/or mechanical design to increase the harvesting and/or dewatering efficiencies (Millamena et al., 1990), or on the integration of nutrition, aquaculture, pharmaceuticals, and/or biofuels.(Harun et al., 2010).

## 2.4 Integration of algal biomass production with swine manure treatment

The integration of algal biomass industry and wastewater treatment is a potential strategy for the cost reduction in algae-based biofuel and/or feed industries. Cultivation of algae in wastewater has been in research for more than 50 years (Oswald and Golueke, 1960). Previous studies have demonstrated the success of using algae to clean wastewater rich in nitrogenous and phosphorus compounds (Oswald et al., 1978). Compared with the conventional wastewater treatment process, which applies activated sludge, a biological floc, to degrade organic carbonaceous matter to $CO_2$, algae can assimilate organic pollutants into cellular constituents such as lipids and carbohydrates, thus achieve pollutant reduction in a more environmental friendly way.

Swine manure is effluent from anaerobic or aerobic lagoons. It is low in solids, allowing easier transportation, and is significant in the amount of mineral components and nutrients, such as nitrogen (mainly in the forms of uric acid and ammonia) and phosphorus (mainly as phytic acid and other complexes resulting from the growth and digestion processes of pigs), allowing wide application in agriculture (Hatfield et al., 1998). Faced with the fact that large-scale swine production is rapidly increasing in the United States, from 2.6 million head in 1990 (Vanotti and Hunt, 1999) to 65.8 million head on June 1, 2012 (USDA, 2012), the cropland for manure application is limited. The increasing odors and gaseous emissions from swine manure storage systems and the exceeded capacity of the local farmland for swine manure lead to the environmental effects on the qualities of air, surface water, and groundwater. It is reported that confined

animal feeding operations (CAFOs) housing of swine has been geographically concentrated in the United States over the last 30 years, and has led to the intensified impacts on soil condition, environment and human health (Starmer, 2007). Alternative disposal methods should be set up to recover the dissolved nutrients in liquid swine manure (LSM) before the wastes are stored or spread on land.

After the comparison of the mineral composition of several classic mass culture media and LSM, it was concluded that LSM appeared to be a suitable medium for the growth of microalgae, in which the algal cells could assimilate nutrients such as nitrogen and phosphorus to get algal lipids in the forms of free fatty acids, triacylglyceride (TAG), phospholipids and glycolipid, as well as proteins (Barlow et al., 1975; Wang et al., 2010; Boersma et al., 1975). In previous studies, swine manure was collected to test the ability of supporting algae growth in different scales as shown in Table 1. From the literature cited bellow we can see that the systems were focused only on nutrient removal by using a small number of commercial photoautotrophic algal strains from the family of *Cyanophyceae* and *Chlorophyceae*, in which *Chlorella* were most commonly used. According to previous research, algae biomass productivities on LSM ranged from 0.68 to 27.7 g $m^{-2}$ $d^{-1}$ with very high variation, which were equal or lower than the regular algal mass productivity of 10~30 g $m^{-2}$ $d^{-1}$ on municipal wastewater (Banat et al., 1990; Su et al., 2011), of 2.52 ~ 61 g $m^{-2}$ $d^{-1}$ on industrial wastewaters, such as carpet mill effluents (Chinnasamy et al., 2011) and rubber factory wastewater (Phang et al., 2001), and of 2.5~ 25.65 g $m^{-2}$ $d^{-1}$ on diluted dairy manure effluents (Mulbry et al., 2008; Johnson and Wen, 2010). The productivities of algal biomass and different nutritional

components in algae depend on many factors, such as algae species (Illman et al., 2000), medium composition (Valenzuela-Espinoza et al, 2002), culturing method and/or systems (Wen and Chen, 2000), algae growth phase (Xu et al., 2008), and so on. It is important to overcome the limitations in the algae-swine manure system for the scale-up production of algae-based biofuels and/or nutritional animal feeds.

**Table 1.** Examples of microalgae cultivated in swine manure

| Algae species | Dilution of manure (fold) | nutrient concentration after dilution(mg $L^{-1}$) | | | Culture type | Scale | Purpose | growth rate (g $m^{-2}$ $d^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|---|---|
| | | N | P | COD or BOD | | | | | |
| *Chlorella vulgaris* | - | 330 | 84 | - | Semi-continuous culture | Mini-pond | Protein feed | 22 | Barlow et al. (1975) |
| *Chlorella vulgaris* | 9 | 137.2 | 18.79 | 1617 (BOD) | Continuous culture | Pilot-plant scale with raceway | Manure management & biomass accumulation | 7.65~32.95 | Fallowfield and Garrett (1985) |
| *Phormidium.bohneri* | 100 | 34 | 27.7 | 77 (COD) | Semi-continuous culture | Lab-scale reactor | Manure management & biomass accumulation | 16.67 | de la Noüe and Bassères (1989) |
| Mixture of *Scenedesmus falcatus*, *S.quadricauda*, and *Chlorella* sp. | 40~50 | 40~57.5 | <50 | 280~350 (COD) | Batch culture | Pilot-plant scale raceways | Manure management & biomass accumulation | 0.68~9 | Sevrin-Reyssac (1998) |
| *Spirulina* | 50 | 28.1~30.38 | 3.28~12.4 | 54.92~83.14 (COD) | Semi-continuous culture | Pilot-plant scale raceways | Biomass accumulation | 11.8 | Olguín et al. (2003) |
| Filamentous green algae including *Microspora willeana Lagerh*, *Ulothrix ozonata Kütz*, *Rhizoclonium hieroglyphicum Kütz*, and *Oedogonium* sp.. | 2.5 | 480±70 | 142±37 | 2536±28 (COD) | Semi-continuous culture | Lab-scale algal turf scrubber(ATS) unit | Manure management & biomass accumulation | 9.4±2.2 | Kebede-Westhead et al. (2006) |
| *Chlamydomonas* spp., *Microspora* spp., *Chlorella* spp. *Ankistrodesmus* spp., *Nitzschia* spp., *Achnanthes* spp., etc. | 10 | 302±126 | - | 2418±481 (COD) | Continuous culture | Pilot-plant scale with high rate algal pond (HRAP) | Manure management & biomass accumulation | 27.7 | de Godos et al. (2009) |
| | 20 | 154±54 | - | 1221±294 (COD) | | | | 21.3 | |

Note: P represents total phosphorus, N represents total nitrogen, COD represents Chemical Oxygen Demand, and TOC represents Total Organic Carbon.

## 2.5 Potential of mixotrophic microalgae strains for biofuel and livestock feed production

Although many microalgae species are typically photoautotrophic, deriving energy from photosynthesis, some algae species also possess the ability of heterotrophy, which means that they can take up some organic substances from the environment and convert them into building blocks and storage compounds in the dark. The metabolic mode in which microorganisms exhibit both photosynthesis and heterotrophy is called mixotrophy. It was reported that higher specific growth rate for certain algal strains was obtained when they were grown mixotrophically than when they were grown either photoautotrophically or heterotrophically alone (Martínez and OrÚs, 1991; Kobayashi et al., 1992; Boëchat et al., 2007). Several studies showed that the specific growth rates of some microalgae strains in mixotrophic mode were approximately the sum of autotrophic and heterotrophic growth rates, representing the fastest way of growing algae biomass (Lee et al., 1989; Xu et al., 2004). Examples of microalgal species reported to be able to grow mixotrophically along with the suitable organic substances are summarized in Table 2 (Richmond, 2004; Andrade and Costa, 2007).

**Table 2** Examples of microalgae species that can be cultivated mixotrophically

| Microalgae species | Organic carbon substance | References |
|---|---|---|
| *Anabaena variabilis* | Fructose, glucose | Pearce et al. (1969)<br>Valiente et al. (1992) |
| *Brachiomonas submarina* | Acetate | Tsavalos and Day (1994) |
| *Chlorella minutisima* | Methanol | Kotzbasis et al. (1999) |
| *C.regularis* | Acetate | Endo et al. (1977) |
| *C. Sorokiniana* | Glucose | Lee et al. (1996) |
| *C.vulgaris* | Glucose | Ogwa and Aiba (1981) |
| *Cyclotella cryptica* | Glycerol, glucose | Wood et al. (1999) |
| *Euglena gracilis* | Glucose, glycerol, galactose, ethanol | Tanim and Tsumura (1989) |
| *Haematococcus pluvialis* | Acetate | Droop (1955) |
| *Nannochloropsis CCAP879/5* | Glycerol, glucose | Wood et al. (1999) |

| | | |
|---|---|---|
| *Nannochloropsis CCAP211/78* | Glycerol | Wood et al. (1999) |
| *Navicula saprophila* | Acetate | Kitano et al. (1997) |
| *Phaeodactylum tricornutum* | Glycerol | Garcia et al. (2000) |
| *Rhodomonas reticulata* | Glycerol, glucose | Wood et al. (1999) |
| *R.salina* | Acetate | Kitano et al. (1997) |
| *Scenedesmus acutus* | Glucose | Ogwa and Aiba (1981) |
| *S.obliquus* | Methanol | Kotzabasis et al. (1999) |
| *Spirulina platensis* | Molasses | Andrade and Costa (2007) |

Mixotrophic growth of microalgae has the potential for not only the enhancement of algal mass production, but also the accumulation of protein and lipids containing different ω-3 PUFAs. The mixotrophic cells of some volvocalean biflagellates (Chlorophyceae) such as *Chlamydomonas* were characterized by higher protein contents than those in autotrophic and heterotrophic cells (Laliberté and de la Noüe, 1993). Although the reason for the phenomenon are not clear, it seems that protein accumulation in mixotrophic cells partly depends on photophosphorylation which is responsible for ATP formation. If ATP is formed by cyclic photophosphorylation through photosystem I, small organic compounds such as acetate and glucose in algal cells are preferred to be converted to amino acids and then proteins via the glyoxylate cycle, the tricarboxylic acid (TCA) cycle, and glyoxylate cycle, leading to the high protein content in mixotrophic cells (Wiessner, 1979). It is reported that highly polyunsaturated fatty acids (C16:3 and C18:3) are mainly produced under photoautotrophic conditions, while the saturated fatty acids are the favored products of microalgae under heterotrophic growth (Somerville et al., 2000). However, it was shown that the heterotrophic production systems of the diatoms *Nitzschia laevis*, *Nitzschia alba*, and *Tetraselmis* spp. can exhibit higher EPA and DHA productivities than those of the autotrophic systems. (Wen and Chen, 2000; Day et al., 1991, Gladue and Maxey, 1994, Chen et al., 2007). Due to the combination of biochemical benefits in autotrophic and heterotrophic metabolic pathways, mixotrophs

can synthesize different PUFAs which are essential for many physiological functions and energy storage. Fernández Sevilla et al. (2004) found that fed-batch mixotrophic growth of *Phaeodactylum tricornutum* UTEX640 in glycerol in pilot-scale devices effectively promoted the algal mass concentration to 25.4 g $L^{-1}$ and EPA productivity to 56 mg $L^{-1}d^{-1}$.

The mixotrophic algae-swine manure system is potential for algae-based biofuel and/or animal feed production and waste nutrient removal. As mentioned in 2.3, swine manure is significant in the amount of mineral components and nutrients, and is considered to be a cheaper organic-carbon source compared with media containing glucose, glycerol, acetate and/or other regular additives. However, the system is not cost-effective because of two main reasons. Firstly, there are few available mixotrophic species with high lipid content and/or high nutritional value for the system. Secondly, since the renewed interest in wastewater-grown algae is not long of history, there are few reports on the effects of the culture strategy, bioreactor styles, and cultivation conditions on algal biomass accumulation, protein content and lipid composition in the system, and thus, an integral, large-scale process of the algae-based swine manure treatment system for biofuel and/or feed production has not been set up yet (Mulbry et al., 2008).

There has been no report to investigate the biofuel and feed potential of swine manure-grown algae. With the above discussion comes the research hypothesis for my doctoral proposal, which is that the swine manure may be a candidate cultivation medium that contains sufficient nutrients for algae cell growth to supply feedstock for biofuel and animal feed production. Since the aim of this project is to treat wastewater and to produce algal biomass from animal waste for biofuels and animal feed, I focused the study on the screening for robust mixotrophic microalgae strains of superior tolerance to swine

manure cultures, and the modification of the waste nutrient composition for efficient algae assimilation conducting to high algal growth rates beside with high lipids content and/or high nutritional values. A second objective of the project is the design of a cultivation model with proper culture conditions and culture strategies which is suitable for the algal mass growth and waste nutrient removal.

# CHAPTER 3. BIOPROSPECTING FOR MIXOTROPHIC MICROALGAE STRAINS TO BE GROWN ON DILUTED SWINE MANURE


## Abstract

Successful and economically viable algae based biofuel and/or animal feed industries mainly depend on the selection of appropriate algal strains. The main focus in the chapter was bioprospecting for mixotrophic microalgae strains that were adapted well to diluted swine manure. By using multi-step screening strategy, 19 candidate strains were found to be facultative heterotrophic, manure-tolerant, and relatively robust in storage containing 98 UTEX strains and 50 indigenous strains. Among the 19 candidate strains, UMN271, which was locally isolated from Loon Lake, Waseca, MN, was chosen as the top-performing strain because of its obviously high growth rate (1.051 $d^{-1}$) during the 7-day batch cultivation on sterilized 20-fold diluted fresh swine manure, and UMN266, which was locally isolated from 3M Innovation Plant lake, St. Paul, MN, was selected as a second top-performing strain with the growth rate of 0.803 $d^{-1}$. The two strains were identified as *Chlorella* sp. and *Hindakia* sp. through morphological observation and genetic identification, and would be utilized for further studies in the thesis.


## 3.1 Introduction

Microalgae have the potential to replace current feedstock crops such as corn and soybean for biofuel and animal feed production due to their ability to grow rapidly (Chisti,

2007), to synthesize and accumulate large amounts of lipids (approx 20 ~ 50% of dry weight; Mutanda et al., 2011), protein (approx 6~71% of dry weight; Becker, 2007), and carbohydrates (approx 8 ~ 57% of dry weight; Edwards, 2008), and to expand at less land area and on lower-quality land than agricultural crops (Singh et al., 2011). However, depleting water resources and high costs in microalgae production have put the feasibility of algae-based biofuel and animal feed production in question (Chinnasamy et al., 2010; Hu et al., 2012a).

The integration of algal biomass production and wastewater treatment is considered to be one of the most viable strategies to overcome the hurdles of insufficient water resource and high costs in algae-based biofuel and/or animal feed industries. Algae are able to assimilate inorganic pollutants in wastewater streams, such as nitrogen and phosphorus, and convert them into cellular constituents during the photoassimilation of $CO_2$, resulting in pollutant reduction and algal feedstock production in an environmentally friendly and productive way. Cultivation of microalgae has been tested on different wastewater streams including municipal wastewater (Lau et al., 1996; Hernandez et al., 2005; Li et al., 2011), industrial wastewater (Chinnasamy et al., 2011; Phang et al., 2011), fresh and digested dairy manure (Mulbry & Wilkie, 2001; Wang et al., 2010), and agricultural runoff (Spatharis et al., 2007).

Microalgae-swine manure system is considered to be feasible for waste nutrient recovery and algal mass accumulation. LSM, rich in organic carbon substances and other nutrients such as nitrogen (N) and phosphorus (P), is an excellent source of fertilizer in agriculture. However, confined animal feeding operations (CAFOs) housing of swine has been geographically concentrated in the United States over the last thirty years, which led to

the intensified impacts on soil condition, environment and human health (Starmer, 2007). An algal treatment system could provide a holistic solution to the problems. Based on its mineral composition, LSM is a suitable medium for algae growth. The major form of soluble nitrogen in swine manure is ammonia nitrogen ($NH_3$-N), which is the predominant form of nitrogen assimilated by algae with glutamate dehydrogenase and glutamine synthetase (Yang et al., 2000). Phosphorus exists in both mineral and organic forms in swine manure, of which the soluble orthophosphate form is biologically available to algae (Sonzoghi et al., 1982). The other macro-nutrients, including sulfur, magnesium and potassium, and micro-nutrients, such as zinc, manganese, copper and boron, could be found in swine manure and appear adequate to support algal growth (Barlow et al, 1975). The ability of raw and anaerobically digested LSM to support algae growth at different scales was investigated in previous studies (Ayala and Bravo, 1984; Jimenez-Perez et al., 2004; Olguin, 2003).

However, significant obstacles, such as unsuitable algae species, ammonia inhibition, turbidity, and bacteria contamination, must be overcomed before large-scale algae production systems on swine manure can be commercialized (Hu et al, 2012b). de Godos et al. (2010) reported that *Chlorella sorokiniana* and *Euglena viridis* were capable of growing on 4-fold diluted swine slurries with activated sludge bacteria, while *Spirulina platensis* was not, suggesting that only specific algae strains may perform the tolerance in swine manure. de Pauw (1980) reported that the yield of *Chlorella saccharophila* on diluted aerated LSM was about 20% ~ 30% lower than that on inorganic fertilizers due to the high turbidity resulting from various particles and pigments in swine manure.

Some microalgae species are facultative heterotrophic strains, in which both the photoassimilation of $CO_2$ and oxidative assimilation proceed concomitantly. The metabolic mode in which microorganisms exhibit the capability of utilizing both organic and inorganic carbon sources is called mixotrophy (Andrade and Costa. 2007). For the same microalgae strains, mixotrophic cultivation is more economically feasible than the autotrophic growth. Marquez et al. (1995) found that the biomass and photopigment concentrations of *Spirulina platensis* produced mixotrophically were around 1.5~2.0 times higher than those produced in autotrophic growth at the same light intensity of $5.07\times10^{-3}$ kJ $cm^{-2}$ $h^{-1}$. Perez-Garcia et al. (2010) found that *Chlorella vulgaris* immobilized in alginate beads under the mixotrophic regime had higher efficiencies of algal growth and nutrient uptake from synthetic wastewater than those in autotrophic regime.

In the light of the above discussion, it is hypothesized there are facultative heterotrophic microalgal strains which have the ability to survive in diluted LSM, the capability of growing to high cell density robustly, and the capability of waste nutrient recovery. The specific objective of this analysis was (1) to collect microalgae from existing microalgal culture collections of UTEX and from freshwater and wastewater habitats in Minnesota; (2) to isolate and identify facultative heterotrophic microalgal strains adapted well in the swine manure; (3) to characterize selected strains for their biomass productivities when grown in diluted swine manure so that the most promising strains for effective wastewater treatment as well as biofuel production and/or nutritional animal feed production could be determined; (4) to identify species of the top-performing microalgal strain with the techniques of morphological analysis and genetic identification.

## 3.2 Materials and Methods

### 3.2.1 Preparation of algal sample storage

The microalgae strains from the sample storage used in this study were obtained through two ways. Ninety-eight samples (Table 3) were purchased from the University of Texas Culture Collection (Austin, USA), while the remaining 50 strains (Table 4) were obtained from five different types of water bodies in Minnesota, including lakes, rivers, creeks, ponds and wastewaters. For the local strains, samples from different locations were collected, labeled, and stored in sterilized transparent plastic bottles and sent to the laboratory within one day for algal cell purification and amplification. In the study, one water body had one water sample in most cases, but several large lakes, such as Lake Johanna, Arden Hills, MN and Loon Lake, Waseca, MN, had more than one samples collected from different locations in the lakes so that multiple algal species might be obtained. The isolation of the unialgal strains was done by following the protocol of Zhou et al. (2011).

### 3.2.2 Characterization of the swine manure

The fresh and anaerobically digested swine manure effluents were collected from a farm 5 miles northeast of the University of Minnesota Outreach, Research and Education (UMore) Park. The characteristics of the two types of swine manure, including pH, water-soluble nutrients such as ammonia-nitrogen ($NH_3$-N), total nitrogen (TN), orthophosphate-phosphorus ($PO_4$-P), and chemical oxygen demand (COD), as well as total suspended solid (TSS), total volatile suspended solid (TVSS), and micronutrient concentrations, were determined. All samples were collected and measured in triplicates, and the averages were presented in Table 5. It is shown that the similar nutrient

characteristics were observed for both types, except on COD and $NH_3$-N concentrations and the solid content. The obvious differences were reasonable, because some of the volatile solids and nitrogen in manure were converted to methane and ammonia, respectively, in these lagoons during the anaerobic digestion (Hatfield et al., 1998). Considering that fresh swine manure had higher carbon content, it was chosen for the bioprospecting for microalgae strains that could survive in swine manure in this study. The undigested swine manure effluent was divided into two equal portions. One portion was autoclaved at 121ºC for 15 minutes (Hu et al., 2012a), while the untreated portion was stored at 4ºC until in use in experiments. As shown in Table 5, the nutrient composition and contents in the sterilized fresh swine manure were similar with those in the raw fresh manure. The pH of the fresh swine manure samples were adjusted to around 7.0 with sulfuric acid ($H_2SO_4$) solution or sodium hydroxide (NaOH) solution before being used for algae incubation in the study. Since the swine manure had a high concentration of TSS which led to poor transmission of light, and the substance concentrations in swine manure were almost 20 times higher than those in BG11 medium, 20-fold diluted sterilized swine wastewater was used during the bioprospecting process.

**Table 3** List of UTEX strains for the selection in this study

| UTEX ID | Species | UTEX ID | Species | UTEX ID | Species |
|---|---|---|---|---|---|
| 16 | *Haematococcus lacustris* | 302 | *Cosmarium botrytis* | 1779 | *Chlorococcum paludosum* |
| 20 | *Chlorella ellipsoidea* | 305 | *Cosmarium subtumidum* | 1782 | *Chlorococcum oviforme* |
| 25 | *Chlorella protothecoides* | 325 | *Selenastrum gracile* | 1786 | *chlorococcum salsugineum* |
| 26 | *Chlorella vulgaris* | 326 | *Selenastrum minutum* | 1787 | *Chlorococcum sphacosum* |
| 32 | *Chlorella zofingiensis* | 343 | *Chlorella fusca var.fusca* | 1788 | *Chlorococcum texanum* |
| 46 | *Protosiphon botryoides f.pariet* | 398 | *Chlorella kessleri* | 1789 | *Chlorococcum typicum* |
| 55 | *Haematococcus droebakensis* | 414 | *Scenedesmus dispar* | 1904 | *Chlamydomonas zebra* |
| 63 | *Crucigenia tetrapedia* | 415 | *Selenastrum Acuminatus* | 2096 | *Characium bulgariense* |
| 78 | *Scenedesmus obliquus* | 416 | *Scenedesmus acutiformis* | 2097 | *Characium californicum* |
| 79 | *Scenedesmus basiliensis* | 417 | *Scenedesmus dimorphus* | 2108 | *Characium typicum* |
| 101 | *Ankistrodesmus falcatus var.* | 572 | *Botryococcus braunii* | 2168 | *Chlorella sp.* |
| 117 | *Chlorococcum minutum* | 580 | *Chlorella sp.* | 2219 | *chlorella minutissima* |
| 120 | *Tetraedron bitridens* | 674 | *Navicula pelliculosa* | 2222 | *Chlorococcum aquaticum* |
| 127 | *Dictyochloris pulchra* | 748 | *Ankistrodesmus falcatus var.* | 2240 | *chlorella minutissima* |
| 151 | *Monodus subterraneus* | 750 | *Ankistrodesmus braunii* | 2248 | *Chlorella sp.* |
| 187 | *Ankistrodesmus braunii* | 773 | *Tetracystis aplanosporum* | 2252 | *Dictyochloris schumacherensis* |
| 189 | *Ankistrodesmus angustus* | 972 | *Chlorococcum ellipsoideum* | 2341 | *chlorella minutissima* |
| 190 | *Ankistrodesmus densus* | 1054 | *Chlamydomonas moewusii var.* | 2438 | *Chlorococcum sp.* |
| 208 | *Chlamydomonas sphaeroides* | 1230 | *chlorella sorokiniana* | 2442 | *Coelastrum astroideum* |
| 228 | *Chlamydomonas dorsoventralis* | 1233 | *Chlorococcum scabellum* | 2445 | *Tetrastrum heteracantum* |
| 230 | *Chlamydomonas applanata* | 1236 | *Scenedesmus longus* | 2459 | *Scenedesmus minutum* |
| 241 | *Ankistrodesmus angustus* | 1237 | *Scenedesmus dimorphus* | 2498 | *Chlorococcum pamirum* |
| 242 | *Ankistrodesmus falcatus var.* | 1338 | *Chlamydomonas noctigama* | 2502 | *Nannochloris eucaryotum* |
| 244 | *Ankistrodesmus braunii* | 1344 | *Chlamydomonas debaryana var* | 2505 | *Haematococcus pluvialis* |
| 245 | *Ankistrodesmus braunii* | 1450 | *Scenedesmus obliquus* | 2527 | *Dictyochloris pulchra* |
| 246 | *chlorella sorokiniana* | 1591 | *Scenedesmus sp.* | 2532 | *Scenedesmus subspicatus* |
| 251 | *Chlorella fusca var.vacuolata* | 1648 | *Selenastrum capricornutum* | 2551 | *Scenedesmus armatus* |
| 252 | *Chlorella fusca var.vacuolata* | 1767 | *Chlorococcum arenosum* | 2629 | *Botryococcus sudeticus* |

| | | | | | |
|---|---|---|---|---|---|
| 256 | *Chlorella protothecoides* | 1768 | *Chlorococcum aureum* | 2630 | *Scenedesmus obliquus* |
| 261 | *chlorella sorokiniana* | 1769 | *Chlorococcum citriforme* | 2714 | *chlorella vulgaris* |
| 280 | *Coelastrum microporum* | 1774 | *Chlorococcum macrostigmatum* | 2805 | *chlorella sorokiniana* |
| 287 | *Oocystis marssonii* | 1776 | *Chlorococcum loculatum* | 2911 | *Chlorella saccharophila* |
| 299 | *Cosmarium impressulum* | 1777 | *Chlorococcum microstigmatum* | | |

**Table 4** Collection dates and sites of local microalgal strains established in the study

| Local strain ID | Collection date | Collection site | Local strain ID | Collection date | Collection site |
|---|---|---|---|---|---|
| UMN220 | Oct, 2006 | a Twin Cities lake | UMN253 | Jun, 2006 | Theodore Wirth Lake 2 on the beach |
| UMN221 | Apr, 2006 | RoseLawn Pond | UMN254 | Jun, 2006 | Lake Calhoun |
| UMN223 | Apr, 2006 | Como Park Golf Course Pond #2 | UMN255 | Jun, 2006 | Moore Lake |
| UMN224 | Apr,2006 | Como Lake | UMN258 | Jun, 2006 | Rice Creek after the bridge inside the park |
| UMN225 | Apr, 2006 | McCarrons Lake | UMN259 | Jun, 2006 | MayFlower drainage pond |
| UMN226 | Apr, 2006 | Rosville Park, Lexington south of County Rd C | UMN260 | Jun, 2006 | Drainage pond behind Bachmans |
| UMN227 | Apr, 2006 | Oxford and County Rd C | UMN261 | Jun, 2006 | Pond of Assisting Living banfill acrss apartments |
| UMN228 | Apr, 2006 | County Rd C and Victoria: north Pond | UMN263 | Jun, 2006 | Marlenes's drainage at the park |
| UMN229 | Apr, 2006 | County Rd C and Victoria: north Pond | UMN264 | Jun, 2006 | Metro Wastewater Treatment Plant dreft side |
| UMN230 | Apr, 2006 | Falls to the lake on County Rd C and Victoria | UMN265 | Jun, 2006 | Kaller Lake |
| UMN231 | Apr, 2006 | Lake Johanna, west side | UMN266 | Jun, 2006 | 3M Innovation Plant lake |
| UMN232 | May, 2006 | swamp in west side of Lake Johanna across the road | UMN267 | Jun, 2006 | Pond on Keller Lake |
| UMN233 | May, 2006 | next swamp across Lake Johanna | UMN268 | Jun, 2006 | Maplewood: Lakewood and Maryland |
| UMN238 | May, 2006 | Lake Josephine east side | UMN269 | Jun, 2006 | Mcarron's lake |
| UMN240 | May, 2006 | Drainage to Lake Josephine #2 | UMN270 | Jun, 2006 | Margolis pond on Lapenteur |
| UMN241 | May, 2006 | Pond #1 across Rosville High School | UMN271 | Jul, 2006 | Loon Lake, Waseca |
| UMN242 | May, 2006 | Pond #1 across Rosville High School | UMN272 | Jul, 2006 | Loon Lake, Waseca |
| UMN243 | Jun, 2006 | Como Park lake | UMN273 | Jul, 2006 | White Bear Lake |
| UMN244 | Jun, 2006 | Channel on Ripley road, Litchfield (at the golf course) | UMN274 | Jul, 2006 | Bold Lake, east site |

| | | | | | |
|---|---|---|---|---|---|
| UMN245 | Jun, 2006 | Lake Ripley picnic area, Lithfield | UMN275 | Jul, 2006 | Amelia Lake |
| UMN246 | Jun, 2006 | Pond between County Rd 1 and County Rd 23, Litchfield | UMN276 | Jul, 2006 | Coon Rapids Dam #1 |
| UMN247 | Jun, 2006 | Lake Hope, Litchfield | UMN277 | Jul, 2007 | Pond at Marine City |
| UMN250 | Jun, 2006 | Theodore Wirth Parkway, Pond #3 on the right coming from 394 | UMN278 | Jul, 2007 | Pond at Marine City |
| UMN251 | Jun, 2006 | Theodore Wirth Parkway, left, right Pond #2b around the bridge | UMN279 | Jul, 2007 | Spring brook 1, Fridley |
| UMN252 | Jun, 2006 | Theodore Wirth Lake 1, farther than the beach | UMN281 | May, 2006 | Itasca main lake |

**Table 5** Characteristics of swine manure

| Characteristics | Digested manure | Raw fresh manure | Autoclaved fresh manure |
|---|---|---|---|
| pH | 8.48±0.29 | 7.45±0.31 | 9.63±0.27 |
| Ammonia-nitrogen (mg $NH_3$-N $L^{-1}$) | 3630.1±1250.0 | 2820.3±225.7 | 2594.3±534.3 |
| Total nitrogen (mg N $L^{-1}$ ) | 4317.0±1263.2 | 3272.1±323.6 | 3196.4±456.4 |
| Orthophosphate-phosphorus (mg $PO_4$-P $L^{-1}$) | 100.70±9.91 | 131.51±6.74 | 150.74±14.26 |
| COD[a] (mg $L^{-1}$) | 8933±666.7 | 14707±3668.9 | 14748.9±3891.1 |
| Total suspended solid (TSS, g $L^{-1}$) | 3.21±0.36 | 3.23±0.29 | 2.92±0.17 |
| Total volatile suspended solid (TVSS, g $L^{-1}$) | 2.38±0.25 | 2.50±0.31 | 2.15±0.18 |
| Al (mg $L^{-1}$) | 1.9 | 2.32 | 2.45 |
| B (mg $L^{-1}$) | 2.5 | 2.5 | 3.21 |
| Ca (mg $L^{-1}$) | 99.46 | 64.02 | 57.67 |
| Cu (mg $L^{-1}$) | 1.4 | 1.06 | 1.18 |
| Fe (mg $L^{-1}$) | 11.66 | 11.14 | 8.67 |
| K (mg $L^{-1}$) | 3389.2 | 3494.8 | 3772.1 |
| Mg (mg $L^{-1}$) | 133.66 | 81.92 | 101.38 |
| Mn (mg $L^{-1}$) | 0.38 | 0.2 | 0.31 |
| Na (mg $L^{-1}$) | 973.5 | 970.76 | 972.43 |
| Ni (mg $L^{-1}$) | 0.64 | 0.64 | 0.68 |
| Zn (mg $L^{-1}$) | 4.94 | 4.14 | 4.41 |

COD[a] is short for Chemical Oxygen Demand.

### 3.2.3 Multi-step screening strategy for top-performing candidate strains

In order to identify strains that showed superior growth on diluted swine manure, the following five-step process was conducted using solid media with 2% agar in petri dishes followed by liquid media in flasks on orbital shakers:

Step 1, liquid BG11 medium was used to enrich the algae strains listed in Table 3 and Table 4. BG11 medium is widely used for green algae cultivation with the following ingredient profile: 0.04 g $K_2HPO_4 \cdot 3H_2O$ $L^{-1}$, 0.075 g $MgSO_4 \cdot 7H_2O$ $L^{-1}$, 0.036 g $CaCl_2 \cdot 2H_2O$ $L^{-1}$, 0.006 g citric acid $L^{-1}$, 0.006 g Ferric ammonium citrate $L^{-1}$, 0.001 g EDTA $L^{-1}$, 1.5 g $NaNO_3$ $L^{-1}$, 0.02 g $Na_2CO_3$ $L^{-1}$, and 1.0 mL of trace metal mix A5. Trace metal mix A5 solution consisted of 2.86 g $H_3BO_3$ $L^{-1}$, 1.81 g $MnCl_2 \cdot 4H_2O$ $L^{-1}$, 0.222 g $ZnSO_4 \cdot 7H_2O$ $L^{-1}$, 0.39 g $NaMoO_4 \cdot 2H_2O$ $L^{-1}$, 0.079 g $CuSO_4 \cdot 5H_2O$ $L^{-1}$, and 0.05 g $CoCl_2 \cdot 6H_2O$ $L^{-1}$ (Rippka et al., 1979).

Step 2, unialgal strains adapted well in BG11 media were placed in two types of solid media, which were BG11 medium for autotrophic growth and BG11 medium enriched with glucose other than $NaHCO_3$ for heterotrophic growth. For the autotrophic medium, strains were cultivated at light intensity of 100 μmol $m^{-2}$ $s^{-1}$. For the heterotrophic medium, the strains were cultivated under light (100 μmol $m^{-2}$ $s^{-1}$) or completely dark.

Step 3, strains that survived in autotrophic media and heterotrophic media under both light and dark conditions were screened in solid medium of 20-fold diluted, fresh swine manure under light intensity of 100 μmol $m^{-2}$ $s^{-1}$.

Step 4, strains adapted well in the petri dishes in step3 were cultivated in 15 mL glass tubes containing 5~7 mL sterilized, 20-fold diluted fresh swine manure liquid under light intensity of 100 μmol $m^{-2}$ $s^{-1}$ for 2 ~ 3 weeks.

Step 5, algal cultures that could turn green in step4 were transferred into 150 mL Erlenmeyer flasks containing 100 mL sterilized 20-fold diluted fresh swine manure liquid, and then was incubated on orbital shakers under light intensity of 100 μmol $m^{-2}$ $s^{-1}$ for 7 days. The growth data for each strain was determined during the 7-day batch culture to get top-performing strains which had obviously high growth rates.

### 3.2.4 Growth and chemical analyses

3.2.4.1 Sampling and algal growth determination

Algal growth was monitored through testing the total volatile suspended solids (TVSS), which represented biomass concentration and was determined according to the standard method (APHA, 1995). A volume of 4 mL samples was taken from the well mixed culture broth in the flask, and then a vacuum was used to pass the samples through the Whatman™ 934-AH glass fiber filter paper sheet (GE Healthcare Bioresciences Corp, Pittsburgh, PA, USA). Next, the filter with the non-filterable residues was dried at 103 ~ 105°C for 24 hours, and then was cooled in a desiccator and weighed ($m_1$). Then, the filter with the dry residue was left in a muffle furnace at 550°C for at least 30 minutes to ignite volatile residue, and was transferred to the desiccator for final cooling and weight measurement ($m_2$). The algal biomass density based on TVSS could be calculated using Eq.1 as follows:

$$\text{TVSS (g L}^{-1}) = \frac{m_1 - m_2}{4} \times 1000 \quad \text{Eq.1}$$

The basic microalgal growth equation is expressed in term of mass based on TVSS as follows:

$$R_{TVSS}\ (d^{-1}) = \frac{\ln\left(\frac{TVSS_t}{TVSS_0}\right)}{t} \quad \text{Eq.2}$$

Where $R_{TVSS}$ is the growth rate based on TVSS, which is one important way of expressing the relative ecological success of a species or strain in adapting to its environment imposed upon it. $TVSS_0$ and $TVSS_t$ are the TVSS at the beginning and in the end of the exponential growth phase, respectively. t is the time interval in the unit of day between $TVSS_t$ and $TVSS_0$.

3.2.4.2 Nutrient analysis

A volume of 3 mL samples was taken from the well mixed culture broth at the beginning and ending of the culture period. The samples were first centrifuged at 7000 rpm for 10 minutes, and then the supernatants were properly diluted for the determination of the concentrations of COD, $NH_3$-N, TN, and $PO_4$-P in the supernatants by following the instructions for nutrient analysis in the Hach DR5000 spectrophotometer manual (Hach, 2008). The calculation of nutrient removal efficiencies is as the following equation.

$$R_i\ (\%) = \frac{C_{i0} - C_{it}}{C_{i0}} \times 100\% \quad \text{Eq.3}$$

Where $R_i$ is the removal efficiency of substance i, $C_{i0}$ is the initial concentration of i in the batch culture system, $C_{it}$ is the final concentration of i in the culture system after t-day batch cultivation.

Statistical analysis was conducted using one-way analysis of variance (ANOVA) with the JMP8.0 software package (SAS Institute Inc., NC). Any treatment with a *p*-value less than 0.05 was considered significant.

### 3.2.5 Species identification of the top-performing microalgae

#### 3.2.5.1 Algae strains and culture conditions

The top-performing microalgae strains selected in section 3.3.3 were cultivated in 250 mL Erlenmeyer flasks each containing sterilized 100 mL BG11 medium for 2 weeks. During the 2-week batch incubation, the flasks were kept at 25±2°C on a shaker at 100 rpm under a continuous cool-white fluorescent light intensity of 100 μmol $m^{-2}s^{-1}$.

#### 3.2.5.2 Morphological analysis

Morphological characters of the top-performing microalgae strains, including cell dimensions, reproductive features, and the presence of pyrenoids/contractile vacuoles/starch/flagella, were evaluated from the samples collected from the 2-week-old cultures using a microscope (Olympus IX70, USA). Features were compared with the published descriptions (Ettl and Gärtner 1995) and incorporated with molecular analysis to achieve the cell identification.

#### 3.2.5.3 DNA profiling analysis

The genetic identification of the strain was based on DNA extraction, polymerase chain reaction (PCR) amplification and sequencing.

At the beginning, the algal cells were separated from the 2-week-old culture broth through centrifuge at 2000 rpm for 10 min, and were washed with deionized water. The

centrifugation-washing-centrifugation process was repeated two times. The genomic DNA of the algal cells was extracted using Qiagen DNeasy® Plant Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instruction with minor modification. The process of the extraction was similar with that mentioned by Zhou et al. (2011). The details of the extraction were listed as follows: firstly, around 0.25 g clean cells were suspended with 200 μL of buffer AP1 and 4 μL of RNase-A stock, and then the mixture was kept at 65 °C for 15 minutes to lyse the cells (mixed several times during the incubation); secondly, the lysate was ground, mixed with 130 μL of buffer AP2, and then kept on ice for 5 minutes for the precipitation of detergents, proteins and polysaccharides; thirdly, the mixture was centrifuged at 14000 rpm for 5 minutes to separate the solid from the lysate; fourthly, the lysate was transferred to a QIA shredder spin column for a 2-min centrifugation at 14000 rpm to obtain the supernatant which was then mixed with 1.5 mL of buffer AP3/E in the DNeasy mini spin column; fifthly, the column was centrifuged at 6000 rpm for 1 minutes, and then placed in a clean microcentrifuge tube with 500 μL buffer AW, which was centrifuged at 6000 rpm for 1 minute to discard the filtrate; sixthly, 500 μL of buffer AW was added to the column and was centrifuged at 14000 rpm for 2 minutes to wash the membrane; seventhly, the washed column was placed in a clean 1.5 mL tube, and was covered with 100 μL of buffer AE at 65°C; eighthly, the sample was incubated at 25°C for 2 minutes, followed by a 1-min centrifugation at 6000 rpm to elute the DNA; finally, step 8 was repeated once to get a final volume of 200 μL DNA solution.

For polymerase chain reaction (PCR) amplification, a diluted portion of extracted DNA (5, 10, 20, 50, or 100 times) was used to amplify the 18S rRNA gene with two primers16S1N and 16S2N whose nucleotide sequences were given in Table 6 (Grzebyk et al., 1998). Around 50 ~ 100 ng of genomic DNA template was mixed with the PCR mixture containing 50 μL of 1× PCR buffer (TaKaRa, Otsu, Japan), 0.2 mM of dNTP, 0.2 μM of 16S1N and 16S2N primers, respectively, and 2.5 U of Taq polymerase (TaKaRa, Otsu, Japan), and then was denatured at 94°C for 5 minutes. The PCR program ran for 30 seconds at 94°C, 30 seconds at 50°C, 2 minutes at 72°C for 40 cycles, and a 7-min final extension at 72°C. The PCR products were purified using QiAquick gel extraction kit (Qiagen, Germantown, MD, USA), and then were sent to Biomedical Genomics Center at University of Minnesota (Saint Paul, MN, USA) for DNA sequencing. The resulting 18S rRNA gene sequences were aligned and compared to the nucleotide sequences of some known microorganisms in GenBank database of the National Center for Biotechnology Information by using Basic Local Alignment Search Tool (BLAST).

**Table 6** Oligonucleotide primer sequences used to amplify and sequence the 18s ribosomal RNA of eukaryotic algae

| Name of primer | Primer sequence (5' ~ 3') |
|---|---|
| 16S1N (forward) | TCCTGCCAGTAGTCATATGC |
| 16S2N (reverse) | TGATCCTCT/CGCAGGTTCAC |

## 3.3 Results and Discussion

### 3.3.1 Bioprospecting for facultative heterotrophic microalgae strains on swine manure

The multi-step screening strategy was used to identify candidate strains which showed facultative heterotrophic capability and were capable to robustly grow on swine manure wastewater. By using the strategy, all the strains in the algae storage in the study, including 98 UTEX strains and 50 local strains, could grow on classic BG11 media under light condition, proving the general capability of the media for the growth of the strains in the study, which coincided with other reports (Chinnasamy et al., 2010). One hundred strains which could grow photoautotrophically and heterotrophically under light and in dark conditions were obtained out of the 148 strains, among which 80 strains grew colonies on the 20-fold diluted swine manure agar plates. These 80 strains were further investigated for their growth capability in liquid medium of swine manure by cultivating strains in glass tubes with autoclaved 20-fold diluted undigested swine manure under continuous illumination for weeks, among which 42 trains adapted well in cultures without further acclimation (Table 7). These candidate strains were further screened based on their growth rates in terms of $R_{TVSS}$ in 7-day batch cultivation in sterilized, 20-fold diluted fresh swine manure on an orbital shaker under light intensity of 100 μmol $m^{-2}$ $s^{-1}$. $R_{TVSS}$ for the 42 strains were summarized in Table 6, in which 19 strains with high growth rates were in bold types. Among the 19 candidate strains, 14 strains were from the local sites. Thus, it seems reasonable to prospect that species that naturally develop in wastewater or real water bodies could perform better than most others in commercial-

scale cultivation on wastewaters (Jiménez-Pérez et al., 2004). Since the locally isolated microalgal strains, namely UMN271 and UMN266, had obviously higher growth rates (1.051 $d^{-1}$and 0.803 $d^{-1}$, respectively) than the other strains in Table 7, they were selected as the top-performing strains to be used in the following studies.

**Table 7** List of strains adapted well in swine manure with the growth rates in batch cultures

| Strain ID | $R_{TVSS}$ [a] ($d^{-1}$) | Strain ID | $R_{TVSS}$ [a] ($d^{-1}$) | Strain ID | $R_{TVSS}$ [a] ($d^{-1}$) |
|---|---|---|---|---|---|
| UTEX26 | 0.252 | UTEX2714 | 0.280 | UMN259 | 0.499 |
| UTEX78 | 0.328 | **UMN220** | **0.569** | UMN260 | 0.431 |
| UTEX230 | 0.323 | **UMN224** | **0.608** | UMN263 | 0.370 |
| **UTEX326** | **0.502** | UMN228 | 0.361 | **UMN264** | **0.505** |
| **UTEX343** | **0.511** | UMN231 | 0.436 | **UMN265** | **0.636** |
| UTEX251 | 0.215 | UMN232 | 0.432 | **UMN266** | **0.803** |
| UTEX252 | 0.223 | UMN238 | 0.461 | UMN267 | 0.322 |
| **UTEX1230** | **0.533** | UMN240 | 0.152 | **UMN269** | **0.604** |
| UTEX1236 | 0.253 | UMN242 | 0.039 | **UMN270** | **0.579** |
| **UTEX1591** | **0.517** | UMN243 | 0.498 | **UMN271** | **1.051** |
| UTEX1787 | 0.410 | **UMN244** | **0.750** | **UMN274** | **0.599** |
| **UTEX2240** | **0.530** | **UMN245** | **0.501** | **UMN276** | **0.533** |
| UTEX2498 | 0.307 | **UMN247** | **0.696** | UMN277 | 0.258 |
| UTEX2551 | 0.168 | UMN251 | 0.393 | **UMN279** | **0.532** |

$R_{TVSS}$ [a] represents the growth rate of microalgae. The bold types are tag for strains with relatively high growth rates.

### 3.3.2 Phylogenetic analysis of top-performing microalgae strains

Experiments on the microalgal species identification have been performed on UMN266 and UMN271.

The cell morphology of each microalgae train was demonstrated in Fig.2. It was observed that both of the strains were green, planktonic microalgae, but the strains slightly differed

morphologically in cell size and form. UMN266 were oval cells (5 ~ 7 µm in long axis) connected with mucilaginous stalks, whereas UMN271 was solitary algae with spherical morphology (3 ~ 5 µm in diameter).

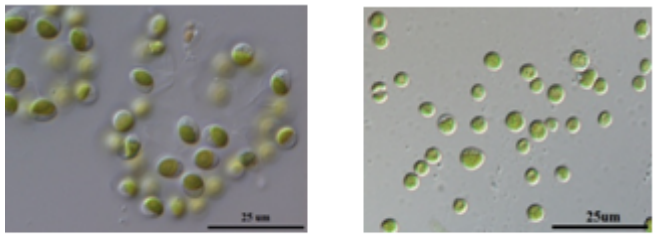


**Figure 2** Microscope images of UMN266 and UMN271 with the scale bar as 25 µm. The cells were cultured in classic BG11 medium at 25°C under continuous illumination (100 µmol $m^{-2}$ $s^{-1}$).

Extraction, PCR amplification, and sequencing of the 18s rRNA gene of the microalgae isolates were applied to aid phylogenetic studies of the microalgae strains. The agarose gel electrophoresis of amplified 18s rRNA genes of UMN266 and UMN271 were shown in Fig.3. For the PCR product detection, a freshwater strain *chlorella vulgaris* was used as a positive control (Fig.3, Lane 1) and $dH_2O$ was used as a negative control (Fig.3, Lane 4). The 18S rDNA genes of the two strains were successfully amplified from the whole genomic DNA using the primer pairs, namely 16S1N and 16S2N (Fig.1, Lane 2 and Lane 3). According to the blast result from algae gene library (Table 8), combined with morphological observation, UMN266 and UMN271 were identified as *Hindakia* sp. and *Chlorella* sp., respectively.

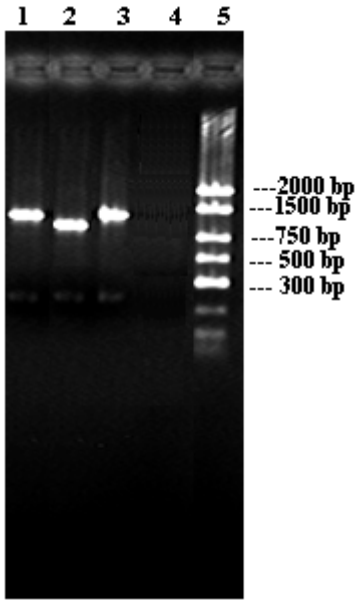


**Figure 3**. Detection of the PCR products of 18s rDNA with agarose gel electrophoresis. Lane 1*: Chlorella vulgaris* (positive control); Lane 2: UMN266; Lane 3: UMN271: Lane 4: deionized water (negative control); Lane 5: DNA marker.

**Table 8** Homology among 18s rRNA gene sequences of microalgal isolates and GenBank strains

| Strain number | rDNA length (bp) | Database strain | GenBank accession no. | Homology (%) |
|---|---|---|---|---|
| UMN266 | 796 | *Hindakia tetrachoroma* CCAP 222/69 | GQ867590.1 | 97 |
| | | *Hindakia tetrachoroma* CCAP 222/60 | GQ867589.1 | 97 |
| | | *Chlorella* sp. ZJU0204 | JX097056.1 | 97 |
| | | *Chlorella sorokiniana* MIC-G5 | JF834706.1 | 97 |
| | | *Chlorella vulgaris* KMMCC FC-42 | HQ702285.1 | 97 |
| | | *Chlorella* sp. UTEX938 | FM205862.1 | 97 |
| UMN271 | 1036 | *Chlorella* sp. ZJU0204 | JX09056.1 | 99 |
| | | *Chlorella* sp. KMMCC | HQ702284.1 | 99 |

| | | | |
|---|---|---|---|
| | FC-26 | | |
| | *Chlorella vulgaris* strain KMMCC FC-42 | HQ702285.1 | 99 |
| | *Chlorella sorokiniana* isolate BE1 | GQ122327.1 | 99 |
| | *Cholrella sorokiniana* | AB488573.1 | 99 |
| | *Chlorella* sp. IFRPD 1018 | AB260898.1 | 99 |

According to previous phylogenetic analyses of 18s rRNA gene and rDNA internal transcribed spacer (ITS) sequences, it is found that *Hindakia* sp. and *Chlorella* sp. should be sister taxa within the Chlorella clade of the Trebouxiophyceae/Chlorellaceae (Fig.4). It is not surprising that the top-performing strain UMN271 is a Chlorella species. Previous researches reported that Chlorella strains could be used for the treatment of different wastewater streams, such as animal manure, primary settled wastewater, settled and activated sewage, and industrial wastewater (Tam and Wong, 1990; Chinnasamy et al., 2010; Wang et al., 2010). There are few studies reported using *Hindakia* sp. for wastewater treatment. However, Hindakia species have the potential for wastewater treatment as alternative to Chlorella, since Bock et al. (2010) demonstrated that *Hindakia sp.* have the polyphyletic origin of morphotype within the Chlorellaceae, suggesting the species normally exist in freshwater bodies and wastewaters.

The selected strains have potential for feed or biofuel production. *Chlorella* species are considered to be important sources of PUFAs production (Tokuşoglu and Ünal, 2006). For *Hindakia sp*, the lipid productivity was reported to be 77.8mg $L^{-1}$ $d^{-1}$, which was the

second highest among the 17 strains in the batch cultures of concentrated municipal wastewater mentioned in the literature of Zhou et al. (2011).

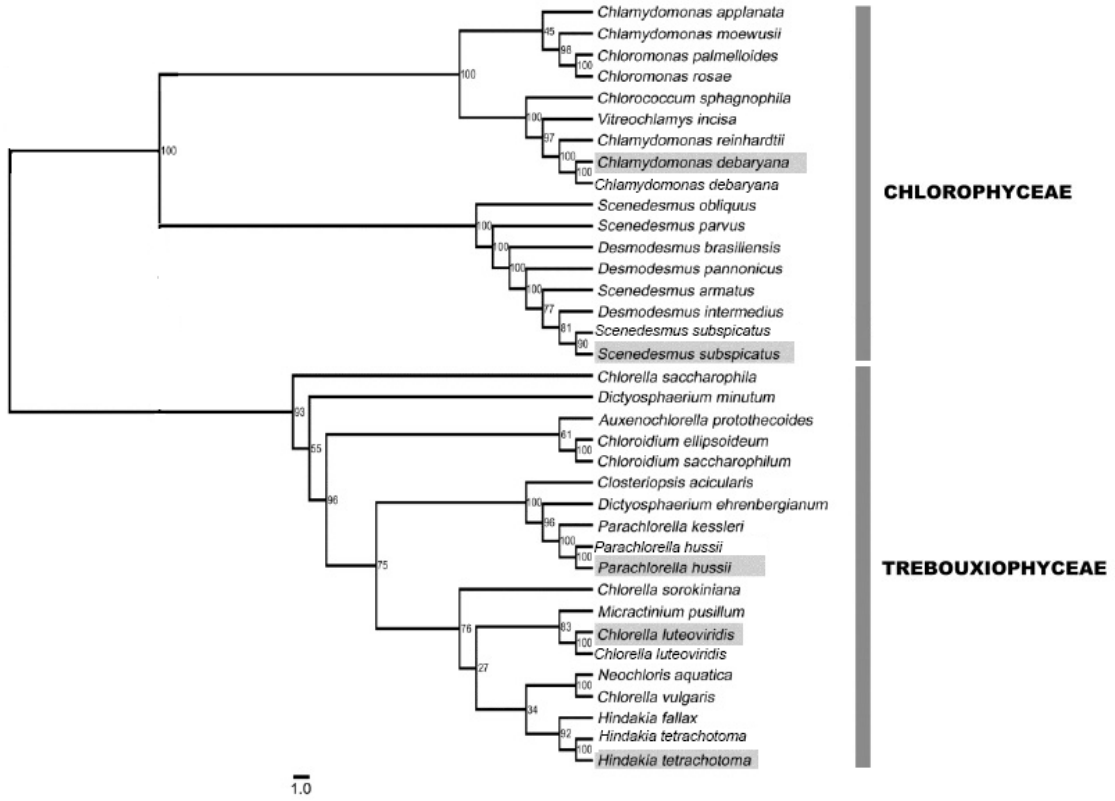


**Figure 4**. Phylogenetic tree of Chlorophyta taxa based on combined 18s and ITS RNA sequences. Branch lengths correspond to evolutionary distances; numbers around the nodes represent the bootstraps. (Modified from Osundeko, 2012)

### 3.4 Conclusions

Mass cultivation of microalgae on animal wastewater heavily depends on the performance of the microalgae strains used. By using multi-step strategy in the study, I screened 19 candidate strains which could potentially be used in cultivation on swine manure wastewater, of which 2 strains, namely UMN271 and UMN266, were selected as the top-performing strains to be used in the following study because of their relative high

growth rates on swine manure. Based on the morphological and genetic analyses, UMN271 and UMN266 were identified to be *Chlorella* sp. and *Hindakia* sp, respectively.

# CHAPTER 4. DETERMINATION OF FACTORS IN SWINE MANURE LIMITING ALGAE GROWTH


## Abstract

Although swine manure is rich in nutrients, such as nitrogen and phosphorus, and is considered to be an excellent source of fertilizer in agriculture, it was not a very suitable medium for algae growth after the high-rate dilution. The objective in the chapter was to assess the compositions in anaerobically decomposed swine manure that limited the growth of algae. In the study, two locally isolated facultative heterotrophic strains, *Chlorella* sp. (UMN271) and *Hindakia* sp. (UMN266), were separately inoculated on 20-fold diluted LSM with 0.1% (v/v) BG11 trace element A5 stock for a 7-day batch cultivation, but the algal growths were not obviously different from those on cultures without exogenous trace elements. However, the algal growth and nutrient removal rates by UMN271 were significantly improved when the initial COD content in diluted LSM increased from 460 mg $L^{-1}$ to 1968 mg $L^{-1}$. UMN271 was found to be able to utilize acetic, propionic and butyric acids, the main water-soluble organic carbon compounds in digested swine manure, for algal growth, lipid production, and $PO_4$-P, $NH_3$-N and TN removal from the swine wastewater. Therefore, it was considered that algal growth in highly diluted swine manure was limited by the deficiency of volatile fatty acids (VFAs).

## 4.1 Introduction

People have increased interest in integrating algal cultivation into swine manure disposal. Production of pork is a major agricultural enterprise in the States, but one of the primary issues associated with pig husbandry is the disposal of swine wastewater and the odor associated with pig and manure storage facilities (Hatfield et al., 1998). Traditionally, swine manure has been expanded to the farmland in the manner of the organic fertilizer because of some obvious advantages including low costs, a good source of inorganic nutrients (such as N, P, K, and Zn), local availability, high organic substance contents, and the ability to enhance soil characteristics (Adeli et al., 2008). However, land of manure application is limited faced with the expansion of pork production, and the overloading of swine manure on land could lead to the environmental effects particularly with respect to surface water, groundwater, and air quality. It is reported that some microalgae strains can grow in swine manure effluents and recover the waste nutrient for algal mass production, which could be a potential alternative method for swine manure application (Table 1).

According to our previous study in Chapter 3, several facultative heterotrophic microalgae strains show adaptation in diluted swine manure, but the biomass productivities of the trains in pig manure barely exceeded 0.8 g $L^{-1}$, while the maximum biomass density of some algae could be over 1.6 g $L^{-1}$ in standard growth medium BG11 with glucose (Liang et al., 2009).The current concentration of algae cells grown on swine manure are not satisfactory for algal biomass accumulation to produce animal feed and biofuel (Deng et al., 2009; Sialve et al., 2009; Um and Kim, 2009). Also, it might not be

satisfactory for wastewater treatment considering the fact that the overall pattern of algae growth rate presents a positive relationship between nutrient consumption and algal growth (Chen, et al. 2012).

One critical parameter that might limit algal cultivation on swine manure is trace element deficiency. It is known that swine manure effluent from anaerobic lagoons is significant in the amount of N mainly in the forms of uric acid and ammonia, and P mainly in the forms of phytic acid and other complex, while the amount of other mineral components being available for algae are on various levels (Hatfield et al., 1998). The comparison between elemental composition between classic BG11 medium and 20-fold diluted anaerobically digested swine manure shows that the concentrations of several trace metals in diluted swine manure, including B, Ca, Fe, and Mn, are more or less lower than those in BG11 medium (Table 9). It is reported that a deficiency of bioactive trace elements, particularly Mn, Fe, Co, Ni, Cu, and Zn, can inhibit various metabolic functions in phytoplankton and thus, inhibit plankton growth (Bruland et al., 1991).

**Table 9.** Elemental characteristics of BG11 medium and 20x swine manure

| Characteristics | BG11 | 20x digested manure |
|---|---|---|
| PH | 7.2±0.1 | 8.48±0.29 |
| Ammonia-nitrogen (mg $NH_3$-N/L) | 0.445 | 181.5±62.5 |
| Total nitrogen (mg N/L ) | 247.5 | 215.9±63.2 |
| Total phosphorous (mg $PO_4^{3-}$P/L) | 5.43 | 5.04±0.50 |
| Al (mg/L) | - | 0.095 |
| B (mg/L) | 0.5 | 0.125 |
| Ca (mg/L) | 9.8 | 4.973 |
| Cu (mg/L) | 0.02 | 0.07 |
| Fe (mg/L) | 1.05 | 0.583 |
| K (mg/L) | 13.66 | 169.46 |
| Mg (mg/L) | 7.3 | 6.68 |
| Mn (mg/L) | 0.503 | 0.019 |
| Na (mg/L) | 414.84 | 48.68 |
| Ni (mg/L) | - | 0.032 |
| Zn (mg/L) | 0.05 | 0.247 |

Another potential limiting factor is the surfeit of algae-available carbon resource in highly diluted swine manure. Gonzålez (2008) reported that the major soluble organic carbon substances in LSM were volatile fatty acids (VFAs) (approximately 80%), of which acetic and propionic acids were the dominant forms (around 64% of the VFAs carbon). Butyric acid was the other major VFA, with isobutyric, pentanoic and isopentanoic acids as the minor components (Paul and Beauchamp, 1989). Barlow et al. (1975) found that the growth rate of *Chlorella vulgaris* had positive relationship with the LSM concentration, suggesting that the growth response may be due to the higher organic matter content of the more concentrated waste. Baumgarten et al. (1999) achieved a good growth of an isolated alga, *Chlorella* sp. and enhanced reduction rates of $NH_4^+$ and total organic carbon (TOC) from the diluted swine manure when bacteria naturally living in LSM were added into the microalga-swine waste system to supply $CO_2$.

It is hypothesized that the nutrients in the manure medium is not complete or balanced for the algae growth. The objective of the present analysis is to determine if the capability of microalgae growing on swine manure could be improved by the compositional changes of the wastewater.

## 4.2 Materials and Methods

### 4.2.1 Microalgae strains and seed growth conditions

*Hindakia* sp. (UMN266) and *Chlorella* sp. (UMN271) obtained from the candidate strain panel in Chapter 3 were used in this section. The alga seeds were enriched in 250 mL

Erlenmeyer flasks each containing 100 mL BG11 medium with 2 g $L^{-1}$ glucose. Before the alga seeds were inoculated, the flasks with the organic BG11 medium were autoclaved at 121 °C for 15 min (Hu et al., 2012a). After the inoculation, the flasks were kept at 25±2°C on a shaker at 100 rpm under a continuous cool-white fluorescent light intensity of 100 μmol $m^{-2}s^{-1}$. After 5-day batch cultivation, the seeds were separated from the culture broth through centrifuge at 2000 rpm for 10 min, and were washed with deionized water. The centrifugation-washing process was repeated two times before the inoculation in Experiment I, II, and III.

### 4.2.2 Manure medium preparation

Anaerobically digested LSM effluents collected from UMore Park, Waseca, MN, were used in the Chapter. Due to the high turbidity, the LSM samples were centrifuged at 1500 rpm with a Damon/IEC Division IEC EXD centrifuge (IEC, Inc. USA). In the study, the digested LSM samples were diluted with deionized water to obtain 1/5 and 1/20 (v/v) manure concentrations. Because 5-fold diluted LSM had a high ammonia content which inhibited algal growth, the manure sample was pretreated with air aeration at 0.5 vvm for ammonia volatilization to make the initial ammonia concentration be at the same level with 20-fold diluted swine manure (78~180 mg $NH_3$-N $L^{-1}$) before being used for algae cultivation.

### 4.2.3 Algae growth on swine waste with trace elements (Experiment I)

This experiment was conducted to observe the growth of microalgae in LSM with or without the exogenous trace elements. The seeds of *Hindakina* sp. (UMN266) and *Chlorella* sp. (UMN271) were inoculated in 250 mL Erlenmeyer flasks containing 150

mL 20-fold diluted LSM with or without 0.1% (v/v) BG11 trace element A5 stock, which contained 2.86 g $H_3BO_3$ $L^{-1}$, 1.81 g $MnCl_2.4H_2O$ $L^{-1}$, 0.222 g $ZnSO_4.7H_2O$ $L^{-1}$, 0.079 g $CuSO_4.5H_2O$ $L^{-1}$, 0.391 g $NaMoO_4.2H_2O$ $L^{-1}$, and 0.05 g $CoCl_2.6H_2O$ $L^{-1}$, to get the initial biomass concentrations of approx 0.3 g $L^{-1}$, respectively. The algal biomass concentration in the form of TVSS was quantified daily during 11-day incubation under growth conditions as mentioned in section 3.2.4.1.

### 4.2.4 Algae growth on swine manure with low dilution rate (Experiment II)

The objective of this experiment was to determine if the growth of microalgae on LSM had relationship with the initial nutrient concentrations. The seeds of *Chlorella* sp. (UMN271) were inoculated into 250 mL Erlenmeyer flasks containing 150mL pretreated 5-fold diluted swine manure with an initial biomass concentration of 0.3g $L^{-1}$, and then the flask was kept at 25±2°C on a shaker at 100 rpm under a continuous cool-white fluorescent light illumination at 100 μmol $m^{-2}s^{-1}$ for 8 days. *Chlorella* sp. (UMN271) grown on 20-fold diluted LSM was taken as the control in the experiment. Each treatment was done in four replicates (three for sampling and the left one for refilling). Culture samples were taken at the beginning and in the end of the incubating period for the measurement of TVSS, concentrations of COD, $NH_3$-N, TN, and $PO_4$-P, and water-soluble volatile organic carbon composition.

### 4.2.5 Algae growth on swine manure with the exogenous VFAs (Experiment III)

This experiment was used to observe the growth of microalgae in LSM with or without the exogenous VFAs to study the connection between algal growth and VFAs such as acetic, propionic and butyric acid concentrations, and to understand the implication of the

dearth of VFAs in anaerobically digested LSM for microalgae growth. Seven-day batch cultivation of *Chlorella* sp. (UMN271) was carried out on four different culture media containing sterilized 20-fold diluted digested LSM with 0.1% (v/v) of exogenous distilled water (control), acetic acid, propionic acid and butyric acid, respectively. The microalgae inoculation level was approx 0.3 g $L^{-1}$. The pH of all the cultures were adjusted to around 7.0 with sulfuric acid ($H_2SO_4$) solution or sodium hydroxide (NaOH) solution at the beginning of the incubation. Every treatment was done in four replicates (three for sampling and the left one for refilling). All the cultures were incubated at 25±2°C under a continuous cool-white fluorescent light intensity of 60 μmol $m^{-2}s^{-1}$. TVSS, pH, COD, $NH_3$-N, TN and $PO_4$-P concentrations in the cultures were monitored daily, the VFA concentrations were detected at the beginning and the end of the culture period, and total lipid content analysis was performed on the harvested algal samples after the 7-day cultivation.

### 4.2.6 Growth and chemical analyses

4.2.6.1 Sampling and algal growth determination

Same as section 3.2.4.1

4.2.6.2 Nutrient analysis

Same as section 3.2.4.2

4.2.6.3 VFA content analysis

The algal culture samples were centrifuged at 7000 rpm for 10 minutes, and then the liquid phase was passed through a Fisherbrand 0.45 μm syringe filter (Thermo Fisher

Scientific Inc., Pittsburgh, PA, USA) to get clear filtrates. The quantitative characterizations of water soluble volatile organic fractions in the filtrate, including acetic, propionic and butyric acids were measured with an Agilent 7820A gas chromatography with flame ionization detector (GC-FID) according to the method of Zhou et al. (2012a). The operating conditions were summarized in Table 10.

**Table10.** GC-FID operating conditions for VFA analysis

| | | | | |
|---|---|---|---|---|
| Instrumentation | Agilent 7820A gas chromatography with an FID | | | |
| GC column | Agilant DB-WAX column with 30 m (length) × 0.25 mm (I.D.) × 0.25 um (thickness) | | | |
| Flow rate | Helium, 2 mL $min^{-1}$ | | | |
| **Oven program** | | | | |
| | Rate (°C $min^{-1}$) | Temperature (°C) | Hold time (min) | Run time (min) |
| Initial | | 80 | 1 | 1 |
| Ramp | 15 | 250 | 2.5 | 14.833 |

| **Inlet** | |
|---|---|
| Heater | 250 °C |
| Pressure | 10.744 psi |

| **FID-back detector** | |
|---|---|
| Heater | 300 °C |
| $H_2$ flow rate | 40 mL $min^{-1}$ |
| Air flow rate | 350 mL $min^{-1}$ |
| Makeup flow rate | 30 mL $min^{-1}$ |

4.2.6.4 Total lipid content and lipid composition analyses

After the batch cultivation, algal cells were harvested by centrifugation and then dried with a freeze-drier (Savant Instruments Inc., USA) for algal lipid analyses.

Total lipid contents in the harvested algae cells were analyzed using the one-step extraction method adapted from Folch method (Folch et al., 1956). The detailed steps are listed as follows: firstly, around 0.1 g of dry algal powder was suspended with 10 mL of chloroform-methanol mixture (2:1 in volume) in a 25 mL fresh screw-top glass tube, and then the tube was kept at 30 °C for 30 minutes in a water bath (Cole-Parmer Inc., USA) for lipid extraction; secondly, the suspension was passed through a Fisherbrand 0.45 μm syringe filter to discard the solid residues; thirdly, the filtrate was washed with 2 mL of 0.9% NaCl solution in another 25 mL fresh glass tube to wash out water-soluble components, and then the liquid volume was measured, of which 3 mL was transferred into an empty 5 mL fresh glass tube (with the weight of $m_2$ in unit of g) to be dried under the N-EVAP Analytical Nitrogen Evaporator (Organomation Associate Inc., USA); finally, the tube with dried lipid extracts were weighed. The lipid content (% of the dry weight) was calculated according to Eq.4.

$$C_L\ (\%) = \frac{(m_3 - m_2) \times V}{3m_1} \times 100\% \qquad \text{Eq.4}$$

where $C_L$ (%) is the total lipid content in dry algae cells; $m_1$ (g) is the initial weight of the dried algae powder; $m_2$ (g) and $m_3$ (g) are the weights of the 5mL glass tube with and without dried lipid extracts, respectively; V (mL) is the liquid volume in step 2.

The total lipid production, another index for the measurement of total lipids which means the lipid yield per liter algal culture at the end of the incubation, was calculated according to Eq.5.

$$P_L\ (g\ L^{-1}) = C_L \times TVSS \qquad \text{Eq.5}$$

where $P_L$ (g $L^{-1}$) is the total lipid production, TVSS (g $L^{-1}$) is the algal cell concentration in form of TVSS at the end of the batch cultivation.

The fatty acid composition of the harvested cells were determined by following two consecutive steps including the preparation of fatty acid methyl ester (FAME) from a one-step extraction-transesterification method and GC-MS analysis using an Agilent 7890-5975C gas chromatography – mass spectrometry with an HP-5 MS capillary column (Li et al., 2011). The detailed steps of the one-step method are shown as follows: firstly, around 0.1 g dry algae powder was well suspended with the chloroform-methanol-sulfuric acid mixture with the proportion of 5:4.75:0.25 (v/v/v) in a 25 mL fresh screw-top glass tube; secondly, the tube was tightly covered and was kept at 90°C for 90 minutes in the water bath for transesterification; thirdly, the suspension after the reaction was passed through a Fisherbrand 0.45 μm syringe filter to obtain the clear filtrate containing FAME being used for GC-MS analysis. For the GC-MS analysis, the oven temperature of the GC was set at 80°C and was kept for 5 minutes, and then raised to 290°C at the rate of 4°C $min^{-1}$ and was kept at 290°C for 5 minutes, while the temperatures in the injector and the detector were set to be 250°C and 230°C, respectively. The carrier gas (He) flow rate was controlled at 1.2 mL $min^{-1}$. The separated compounds in GC column were identified in the NIST Mass Spectral Database, and the FAME data were reported using Agilent data analysis software.

Statistical analysis was conducted using *t*-test and one-way analysis of variance (ANOVA) with the JMP8.0 software package (SAS Institute Inc., NC). Any treatment with a *p*-value less than 0.05 was considered to be significant.

## 4.3 Results and Discussion

### 4.3.1 Effect of exogenous trace elemental substances on algae growth (Exp I)

Trace elements including B, Mn, Zn, Cu, Co and Mo were added in 20-fold diluted swine manure medium at the same level as in standard BG11 medium. As shown in Fig.5, in the entire mixotrophic cultivation period, the biomass growth of microalgae strains *Hindakia* sp. (UMN266) and *Chlorella* sp. (UMN271) in cultures containing the BG11 trace elements had no significant difference with those in cultures without the additives ($p$>0.05), indicating that the lack of trace elements was not the main reason of the weak algal growth.

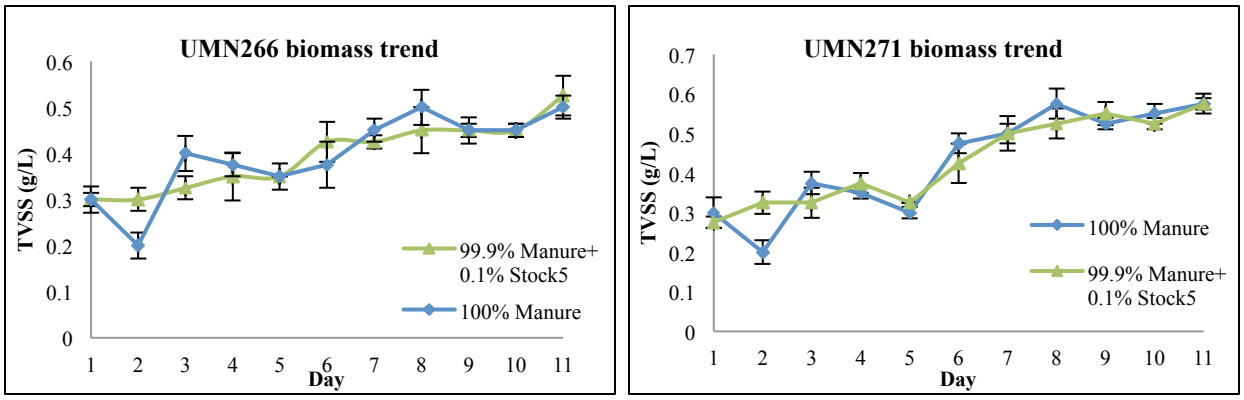


**Figure 5.** Biomass production by UMN266 and UMN271 cultivated in 20-fold diluted swine manure with or without 0.1% (v/v) BG11 trace element stock

### 4.3.2 Effect of waste nutrient concentrations on algae growth (Exp II)

Alga strain *Chlorella* sp. (UMN271) was incubated in either 20-fold diluted LSM or pretreated 5-fold diluted LSM for 7 days, and the changes in algal cell density and waste nutrient concentrations are shown in Fig.6. The algal biomass concentration in 5-fold

diluted LSM increased from 0.3 g $L^{-1}$ to 0.81 g $L^{-1}$ after 7-day cultivation, which was obviously higher than that in 20-fold diluted LSM (0.53 g $L^{-1}$), indicating that algae growth was enhanced in LSM with higher waste nutrient concentrations (Fig.6a). As shown in Fig.6b, 6c, and 6d, the algal growth was accompanied by decreases in water-soluble COD, $PO_4$-P, $NH_3$-N and TN contents in the media, suggesting that UMN271 was able to treat swine manure. Since it was observed that nutrient consumption was positively correlated with algal growth, it was important to determine the substances in pretreated 5-fold diluted LSM that enhance algae growth, so that the system could be feasible for both algal feedstock production and swine manure treatment.

Among the monitored water-soluble nutrients including COD, $PO_4$-P, $NH_3$-N, and TN, it was found that only COD content was significantly higher in the pretreated 5-fold diluted LSM (1968 mg COD $L^{-1}$) than that in the control culture (460 mg COD $L^{-1}$; Fig.6b). COD test is commonly used to indirectly measure the amount of organic pollutants in wastewater (Zielińska and Wojnowska-Baryla, 2004). $NH_3$-N and TN contents had comparatively small increases (2-time raise from 78.80 to 159.40 mg $NH_3$-N $L^{-1}$, 2.24-time raise from 107 to 240 mg N $L^{-1}$ ) when the dilution rate of LSM dropped from 20 to 5 times, mainly due to ammonia volatilization during air aeration operated on 5-fold diluted LSM before algae inoculation (Fig.6d). Tam and Wong (1996) reported that the growth rates of *Chlorella vulgaris* had no significant difference at $NH_3$-N concentrations between 20 and 250 mg N $L^{-1}$, and maximal cell densities attained. For phosphorus, although the $PO_4$-P content in 5-fold diluted LSM (8.88 mg $L^{-1}$) was slightly higher than that in 20-fold diluted LSM (7.20 mg $L^{-1}$), it was not considered to be a key factor

affecting algal growth. Nayak et al. (2009) reported that the $PO_4$-P at levels as low as 0.05 mg $L^{-1}$ could support algae growth. Others (Fong et al., 1993) found that the growth rates of many microalgae species were independent of phosphate concentrations when the phosphate level was above 0.03 mg $L^{-1}$. Therefore, it is considered that the algae growth was limited by algae-available carbon deficiency in highly diluted LSM in the study.

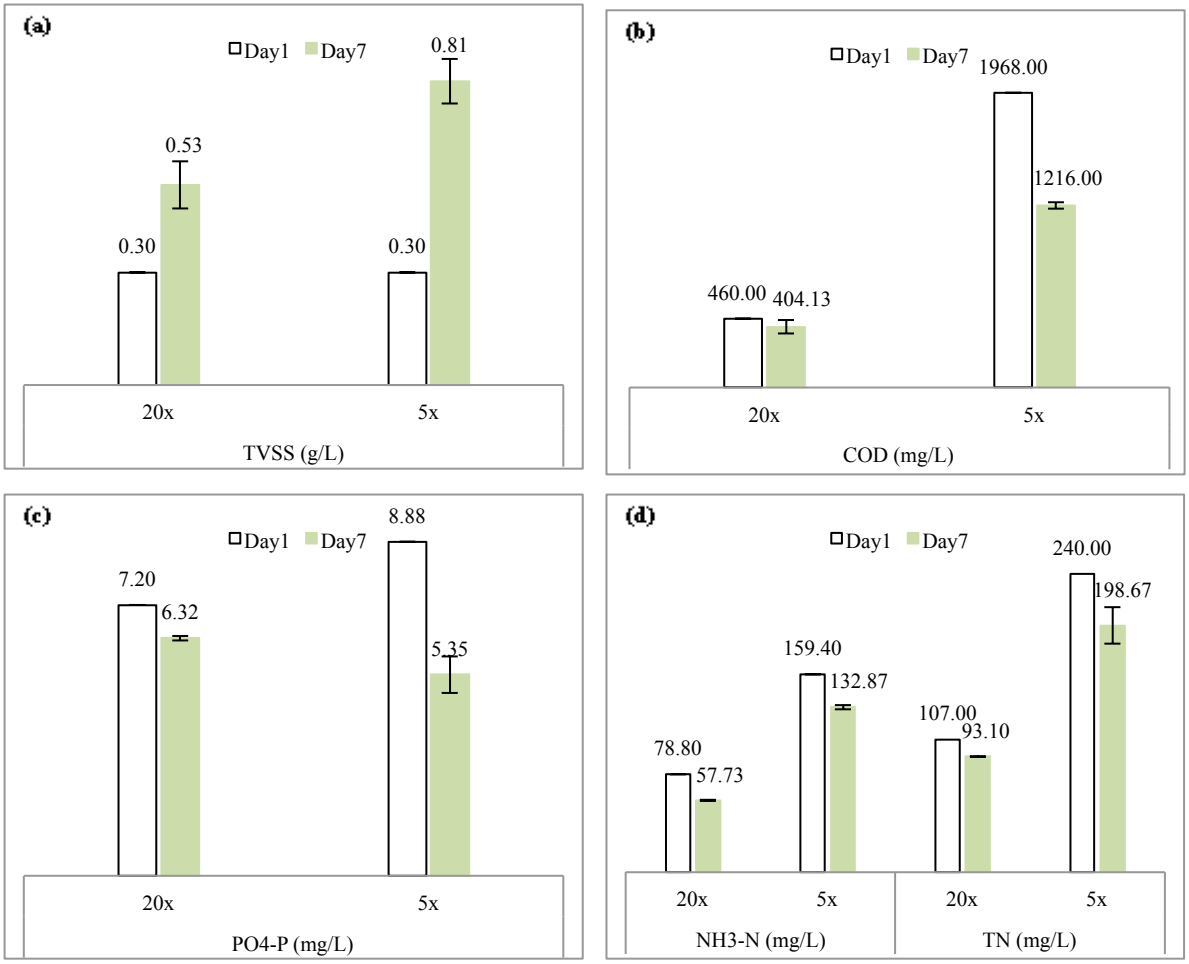


**Figure 6**. Changes on algal TVSS (a), concentrations of COD (b), $PO_4$-P (c), $NH_3$-N, and TN (d) before and after the 7-day batch cultivation of UMN271 on 20-fold diluted LSM and pretreated, 5-fold diluted LSM

Water-soluble carbon compositions in algae cultures before and after the 7-day batch cultivation were analyzed using GC-FID, and the data are shown in Fig.7. The major water-soluble volatile organic compounds in the 20-fold diluted LSM were found to be VFAs including acetic, propionic, and butyric acids (Fig.7a). The result was in agreement with those reported by Paul and Beauchamp (1988) and González (2008) who concluded that the significant components of water-soluble carbon in digested animal manure were VFAs, of which acetic, propionic, and butyric acids were dominant with lower concentrations of isobutyric, valeric, and isovaleric acids. According to the CG-FID analysis, the initial concentrations of acetic, propionic, and butyric acids were calculated to be 89.2, 30.7, and 14.6 mg $L^{-1}$, respectively. For the carbon composition in the culture after 7-day algae growth, as shown in Fig.7b, the peaks representing acetic and propionic acids were not observed, and the butyric acid concentration decreased to be 1.96 mg $L^{-1}$. It is very possible that the removal of acetic, propionic, and butyric acids in the diluted LSM was mainly attributed to the carbon uptake by UMN271. Bollman and Robinson (1977) found that some microalgae could live on dissolved carboxylic acids, such as VFAs, fumaric acid, pyruvic acid and succinic acid. However, there has been no report on any Chlorella species which has the ability to utilize not only acetic acid, but also propionic and butyric acids. The deduction was investigated in Exp III.

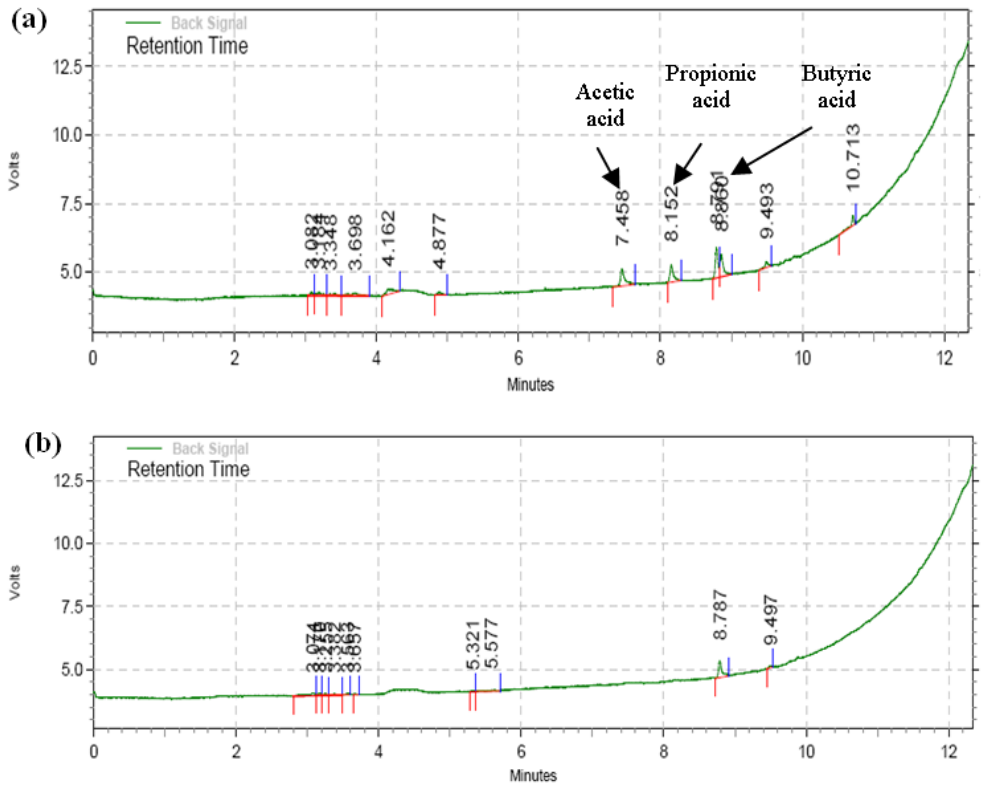


**Figure 7.** GC-FID curves for the detection of water-soluble volatile organic carbon compounds in media before (a) and after (b) the 7-day batch cultivation of UMN271

### 4.3.3 Effect of exogenous VFAs on algae growth (Exp III)

Algal growth in terms of TVSS on 20-fold diluted, digested LSM with or without VFA additives is shown in Fig.8. The growth varied dramatically with different types of culture media. The biomass accumulation of *Chlorella* sp. (UMN271) was greatly enhanced by added VFA. The addition of exogenous acetic, propionic and butyric acids increased the algal growth rates from 0.398 $d^{-1}$ to 0.640, 0.821 and 0.825 $d^{-1}$, respectively (Table 11). As shown in Fig.8, UMN271 survived in all culture media containing swine manure with 1~2-day lag phase periods. It was noticed that algae on 20-fold diluted LSM

with acetic acid experienced the shortest lag phase (1 day) while algae on the other media started exponential growth on day 3. After the 3rd day, the algae in all treatments steadily accumulated algal biomass, except for algae on the medium with butyric acid which was in a step raise. For the control (without VFAs), the growth was at much lower level compared with others and leveled off from day 4. The final biomass concentrations in terms of TVSS after 7-day batch growth were 0.53 g $L^{-1}$ for the control treatment, 1.15, 1.58 and 1.25 g $L^{-1}$ for cultures with acetic, propionic, and butyric acids, respectively.

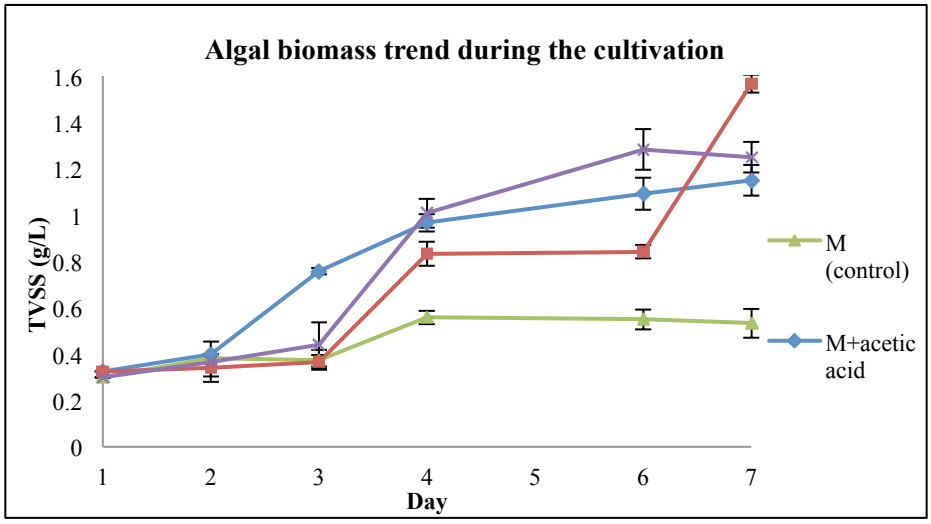


**Figure 8.** Biomass production of UMN271 grown in batch modes on 20-fold diluted digested LSM samples with distilled water (control; green), acetic acid (blue), propionic acid (red) and butyric acid (purple)

The water-soluble COD, $PO_4$-P, $NH_3$-N, and TN in manure samples were measured daily during the 7-day culture, and the data are shown in Fig.9 and Table 11. In our experiment, the initial CODs varied with the 4 treatments (387.6, 1296, 1676 and 1940 mg $L^{-1}$, respectively), because the exogenous acetic, propionic and butyric acids had different densities (1.049, 0.99 and 0.9595 kg $L^{-1}$) and theoretical COD values (1.07, 1.51 and 1.82

g COD $g^{-1}$, respectively) (Pitter and Chudoba, 1990). As shown in Fig.9a, the decrease in COD for cultures with VFA additives showed a lag phase similar to the algae growth, and the exponential decrease last only for one day. No apparent changes in COD for culture with butyric acid were observed during the entire culture period. The net increase of COD in the control treatment (-4.27%) was probably due to secretion of soluble materials such as polysaccharides and other organic compounds from the algal cells (de al Noüe and Bassères, 1989). Because the cultivation was carried out in airtight environment, evaporation of the added VFAs can be ruled out, and hence the observed decrease in the COD could be due to algae consumption. It is therefore concluded that acetic, propionic and butyric acids are assimilable to the locally isolated strain UMN271 as carbon sources, and the concentrations of the three VFAs in which it would had an inhibitory effect on the growth of UMN271 strain was higher than 0.1% (v/v), respectively. The positive correlation between initial CODs and the final TVSS among the 4 treatments further implicated that the dearth of carbon sources in LSM was a key limiting factor affecting algal growth.

In contrast to the algae growth and decrease in COD, the decrease in $PO_4$-P followed an exponential trend without an apparent lag phase (Fig.9b), suggesting that P removal did not have a linear relationship with algae growth. Nayak et al. (2009) reported that $PO_4$-P at levels as low as 0.05 mg $L^{-1}$ could support algae growth. Others (Fong et al., 1993) found that the growth rates of many microalgae species were independent of phosphate concentrations when the phosphate level was above 0.03 mg $L^{-1}$. Similar to the results on other nutrient removal, algae in the culture with acetic acid and the culture without

exogenous VFA performed the best and worst in terms of P removal (Table 11). However, a *t*-test indicates that the P removal efficiencies were not significantly different among the three VFA-added manure samples ($p$>0.05).

The $NH_3$-N removal (Fig.9c) followed quite well the reversed patterns of the growth curves (Fig.8), so did the total nitrogen content reduction (Fig.9d). Ammonium was almost completely removed within 5 days from the two cultures with propionic and butyric acids, respectively, and was significantly removed from the culture with acetic acid within the 7-day growth period, confirming the feasibility of algae-based LSM treatment approach. However, only 26.73% of $NH_3$-N was removed for the control treatment, once again suggesting the lack of carbon source limited the growth of algae consumed ammonia. At the end of the 7-day culture, 12.99%, 51.78%, 55.82% and 55.85% of total nitrogen were removed from the control culture and culture broths with acetic, propionic, and butyric acids, respectively. Since $NH_3$-N in cultures with VFA additives was mostly removed as described above, the remaining approx 44~49% nitrogen compounds that were not converted to $NH_3$-N or directly assimilated by the algae may be mainly in the forms of organic nitrogen (Chadwick et al., 2000).

**Table 11**. Growth rates and nutrient removal efficiencies of UMN271 in Experiment III

| Treatment | $R_{TVSS}$ [a] ($d^{-1}$) | Average nutrient removal efficiency (%) | | | |
|---|---|---|---|---|---|
| | | $R_{COD}$ [b] | $R_{PO4\text{-}P}$ [c] | $R_{NH3\text{-}N}$ [d] | $R_{TN}$ [e] |
| M (control) | 0.398 | -4.27 | 79.08 | 26.73 | 12.99 |
| M + acetic acid | 0.640 | 62.45 | 87.07 | 88.99 | 51.78 |
| M + propionic acid | 0.821 | 68.66 | 87.55 | 99.98 | 55.82 |
| M + butyric acid | 0.825 | 72.58 | 88.56 | 99.99 | 55.85 |

$R_{TVSS}$[a] is the algal growth rate based on TVSS; $R_{COD}$[b], $R_{PO4\text{-}P}$[c], $R_{NH3\text{-}N}$[d], and $R_{TN}$[e] are removal efficiencies on water-soluble COD, $PO_4$-P, $NH_3$-N, and TN, respectively.

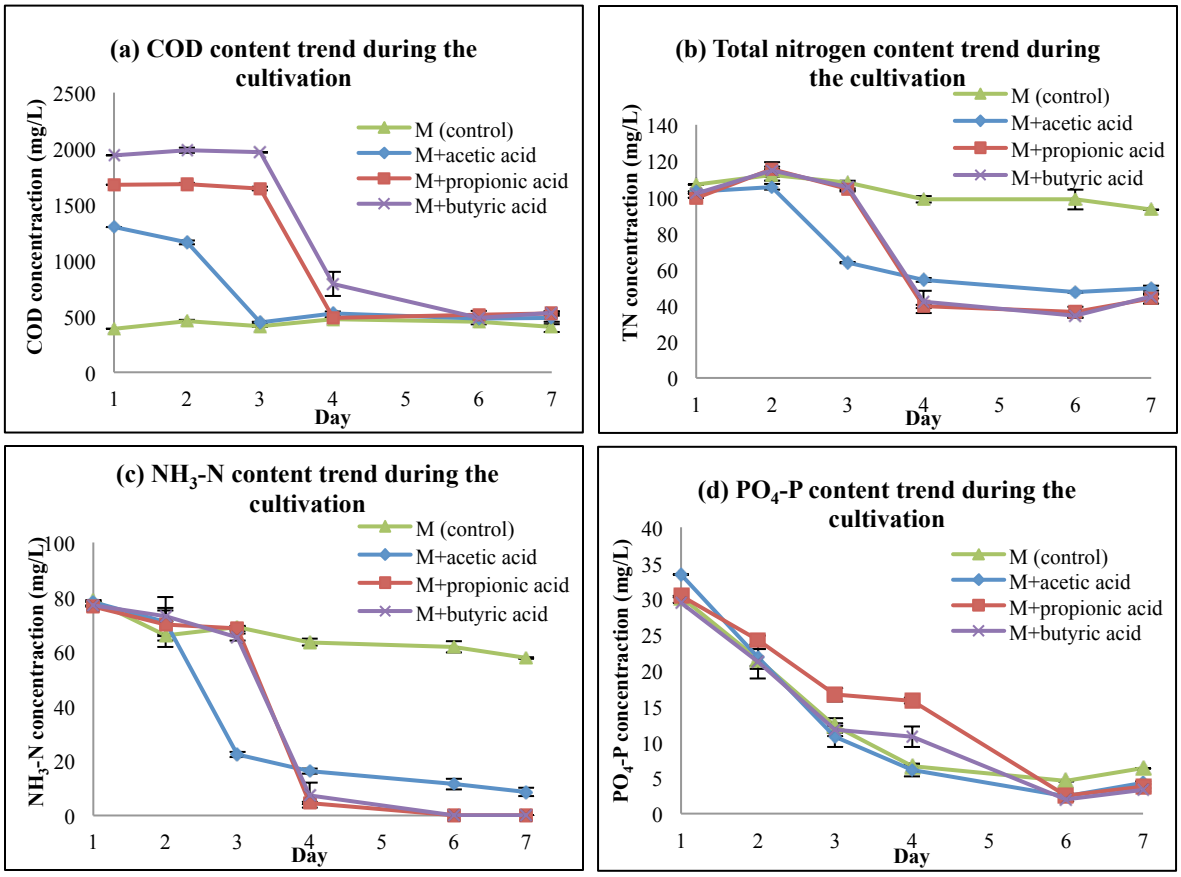


**Figure 9.** Comparison of removal of COD (a), $PO_4$-P (b), $NH_3$-N (c), and total nitrogen (d) by UMN271 on LSM media with distilled water (control; green), acetic acid (blue), propionic acid (red), and butyric acid (purple) during the 7-day batch cultivation

Algal cells were harvested and dried after the 7-day batch experiment for total lipid content measurement. As shown in Fig.10, the percentages of lipids in the dried algae samples collected from the control culture and from the cultures with 0.1% (v/v) acetic, propionic, and butyric acids were 29.97%, 26.74%, 26.25% and 27.43%, respectively. There was no significant difference in the total lipid contents among the algae samples cultivated on swine manure with and without VFAs ($p$>0.5). Generally, total lipid

contents of microalgae range from 1% to 85%, with values higher than 40% being typically reached under environmental stresses, such as nutrient limitation, low temperature, high pressure and irradiance (Rodolfi, 2008). The ~ 30% lipid contents of our locally isolated strain *Chlorella* sp. was relatively high under normal growth conditions. The relatively high lipid content could be attributed to the heterotrophic mode during algae grown on manure, since it was found that the lipid contents in some species of Chlorella under heterotrophic conditions were significantly higher than those under autotrophic conditions (Liu et al, 2011; Isleten-Hosoglu et al., 2012). In the experiment, the total lipid production (mg $L^{-1}$) was significantly higher for algae on VFA-added LSM than that for algae on the control LSM ($p<0.05$). Therefore, the wild-isolated *Chlorella* sp. grown on VFA-enriched LSM had the potential as raw materials for biofuel production in terms of the quantity. Quality of algal lipids is also important for biofuel production. Murakami et al. (1997) found that lipids in Chlorella grown on both organic and inorganic media consisted of neutral lipids, phosopholipids, glycolipids, and free fatty acids.

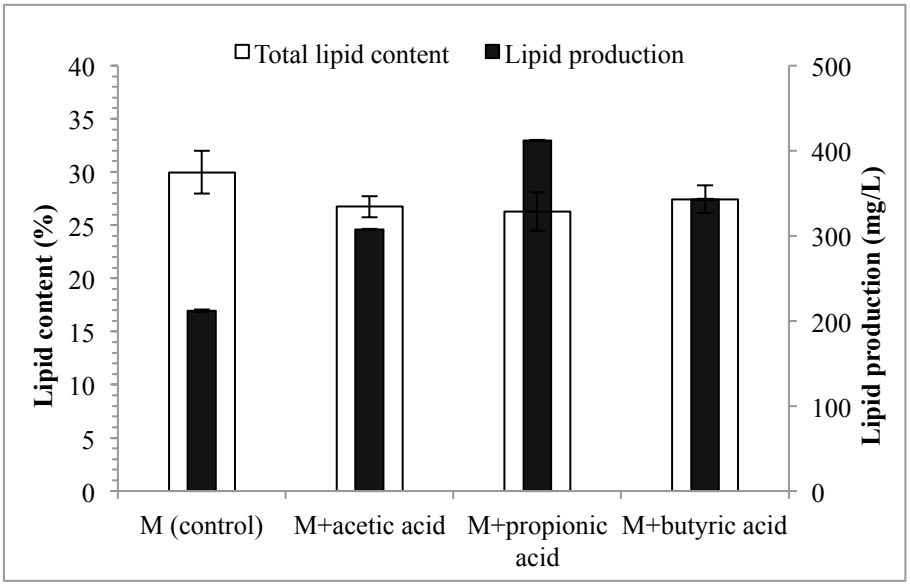


**Figure 10**. Lipid content of algae harvested from cultures with or without VFA additives. White columns indicate the total lipid content (%) of dry weight; Black columns indicate the total lipid production (mg $L^{-1}$) of TVSS on day7

The ability of our microalgae strain *Chlorella* sp. (UMN271) to use not only acetic acid but also propionic and butyric acids for growth is reported for the first time. It has been known for decades that several microalgae species could live on dissolved carboxylic acids, such as VFAs, fumaric acid, pyruvic acid and succinic acid (Bollman and Robinson, 1977). Acetic acid (or acetate) is carried by coenzyme A in microalgal cytosol, and then the formed acetyl coenzyme A (acetyl-CoA) is assimilated into algal carbohydrates via glyoxylate cycle in cooperation with tricarboxylic acid cycle (TCA) (Kovács et al., 2000; Sijtsma et al., 2005). However, acetic acid could be toxic for microalgae at high concentrations as reported in the literature. Chen and Johns (1994) found that the growth of *Chlamydomonas reinhardtill* was progressively inhibited by acetate at the concentration above 0.4 g $L^{-1}$. It is believed that propionic acid (or

propionate) could alleviate acetate inhibition on algae growth and improve cellular growth, since propionic acid (or propionate) can be converted to oxaloacetate to keep a proper balance between oxaloacetate and acetyl-CoA for an efficient TCA cycle process (Perez-Garcia et al., 2011). The efficiency of propionate metabolism varied in algal species. Many microalgae strains cannot degrade propionic acid, such as *Chlorella sorokiniana* (Imase et al., 2008). Several species, such as *Arthrospira platensis* and *Rhodobacter sphaeroides*, could remove propionate from the culture media, but revealed poor growth and nutrient removal (Lodi et al., 2005; Ogbonna et al., 2000). Until now, there is no report on microalgae species that live robustly on propionate acid. For butyric acid (or butyrate), mechanisms on algae metabolism of butyric acid remain to be investigated, since few studies have provided the availability of butyric acid on algae growth.

### 4.4 Conclusion

In the chapter, it was demonstrated that (1) the growth of the candidate microalgae strains were independent of trace element concentrations in highly diluted LSM; (2) carbon deficiency in highly diluted LSM limited the algae growth; (3) acetic, propionic, and butyric acids, the main organic carbon components in digested swine manure, can be used as organic carbon and energy sources for UMN271 cell growth, algal lipids production, and nutrients removal in anaerobically decomposed LSM. Therefore, VFA enrichment in swine manure is considered to be an important LSM compositional-change scenario for the enhanced biomass production and waste nutrient removal in the future.

# CHAPTER 5. MODIFICATION OF ANAEROBIC DIGESTION PROCESS FOR VOLATILE FATTY ACID-ENRICHED SWINE MANURE


## Abstract

The objective was to modify anaerobic digestion process for liquid swine manure (LSM) rich in volatile fatty acids (VFAs), so that the liquid effluent could be more suitable than the conventionally decomposed LSM effluent as a nutritional medium for the cultivation of *Chlorella* sp. (UMN271), a locally isolated facultative heterotrophic microalga strain. The results showed that the acidic pretreatment on fresh LSM followed by an anaerobic digestion process at pH5.3 successfully promoted VFA concentrations in swine manure. The obtained acidogenically digested LSM supported the growth of *Chlorella* sp. in a 5-day batch experiment, with higher algal growth rates (0.85 and 0.90 $d^{-1}$ on sterilized and raw manure samples, respectively) and fatty acid contents (9.14% and 10.93% of the dry weight on sterilized and raw manure samples, respectively) in comparison with those on the conventionally decomposed LSM. High removal efficiencies on chemical oxygen demand (COD), orthophosphate-phosphorus ($PO_4$-P), ammonia-nitrogen ($NH_3$-N), and total nitrogen (TN) were observed for the raw acidogenically digested manure sample (58.02%, 44.74%, 31.58%, and 44.73%, respectively). Finally, the fatty acid profile analysis showed that *Chlorella* sp. grown on acidogenically digested manure could be used as feedstock to produce high-quality biodiesel.

## 5.1 Introduction

Cultivation of algae with piggery wastewater is considered to be a potentially practical and economical strategy for the algal feedstock production and wastewater treatment. However, algae growth on swine wastewater has not been industrialized yet, although algae-based swine wastewater treatment has been investigated since 1980s (De Pauw, et al., 1980). According to previous studies, the algal biomass productivity and nutrient removal rates on swine wastewater were always lower than those on other types of wastewaters such as municipal and industrial wastewaters (Hu et al., 2012a; Chinnasamy et al., 2010). It is considered that the limitation in algae production and incomplete assimilation of nutrients in swine wastewater by algae are mainly attributed to the lack of suitable algae species and the dearth of algae-available carbon compounds in the wastewater (de Godos et al., 2010; Woertz et al., 2009; Chapter 3&4 in the dissertation).

A few researchers have studied the production of algae on anaerobically digested LSM effluents with the addition of exogenous carbon compounds, such as $CO_2$ (Sevrin-Reyssac, 1998), and with the addition of bacteria naturally living in liquid manure to supply $CO_2$ (Baumgarten et al., 1999). However, little information is available regarding the modification of the initial carbon profile of swine slurry for algae growth.

Increasing the availability of carbons in the form of small organics is considered to be a feasible approach to enhance nutrient assimilation and algae growth on swine manure. Gonzålez (2008) reported that the major soluble organic carbon substrates in LSM were volatile fatty acids (VFAs) (approximately 80%), of which acetic and propionic acids were the dominant forms (around 64% of the VFAs carbon). Butyric acid was the other

major VFA, with isobutyric, pentanoic and isopentanoic acids as the minor components (Paul and Beauchamp, 1989). Acetic acid (or acetate) was one of the most commonly used organic carbon substrates for mixotrophic or heterotrophic culture of microalgae (Takechi, 1971; Perez-Garcia et al., 2011). O'Brien and Taylor (1977) suggested that propionic acid (or propionate) could be a precursor of oxaloacetate through the tricarboxylic acid cycle in cells and improve cellular growth. Until now, however, research on microalgae species that could tolerate and consume propionic acid is limited. Smith and Lucas (1971) found that suspensions of the wild-type *Anacystis nidulans* and *Gloeocapsa alpicola* assimilated [2-$^{14}$C]propionate on the medium containing low concentrations of propionate (0.5 mM) under the light with 5% $CO_2$ (v/v). Gonzålez et al. (2008) found that *Chlorella sorokiniana* consumed acetic and propionic acids in pretreated LSM completely in the first 30 and 60 hours, respectively. There are few reports on the availability of butyric acid to algae. Cook (1968) demonstrated that *E. gracills* var. *bacillaris* was able to use butyric acid in heterotrophic cultures, among a few other organic substrates. However, there is little evidence to suggest that specific microalgae strains were capable to utilize all the three VFAs, including acetic, propionic and butyric acids.

Our previous results showed that locally isolated facultative heterotrophic microalga strain *Chlorella* sp. (UMN271) was capable to adapted well in anaerobically digested LSM without further acclimation, and could utilize not only acetic acid, but also propionic and butyric acids, which were the major water-soluble organic carbon substances in swine wastewater (Zhou et al., 2012b; Hu et al., 2012b).

Anaerobic digestion converts organic matters in fresh swine slurry into biogas, which is mostly methane ($CH_4$) and $CO_2$, by a consortium of microorganisms through a complex biochemical process including four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. In addition to $H_2$, VFAs are produced during the first three steps and are rapidly consumed by methanogens in the last step (Fig.11). Therefore, it is important to suppress methanogenesis in order to accmulate VFAs in LSM, with biohydrogen as byproduct. To suppress methanogenesis, two techniques, namely heat treatment and low-pH treatments of the inoculums, have been commonly used to kill methanogenic bacteria from LSM (Oh et al., 2003). Studies on modification of anaerobic digestion have focused on only biohydrogen production (Wu et al., 2009; Kotsopoulos et al., 2009). Investigation of the utilization of organic acids in manure effluents from modified anaerobic digestion process by algae is still lacking.

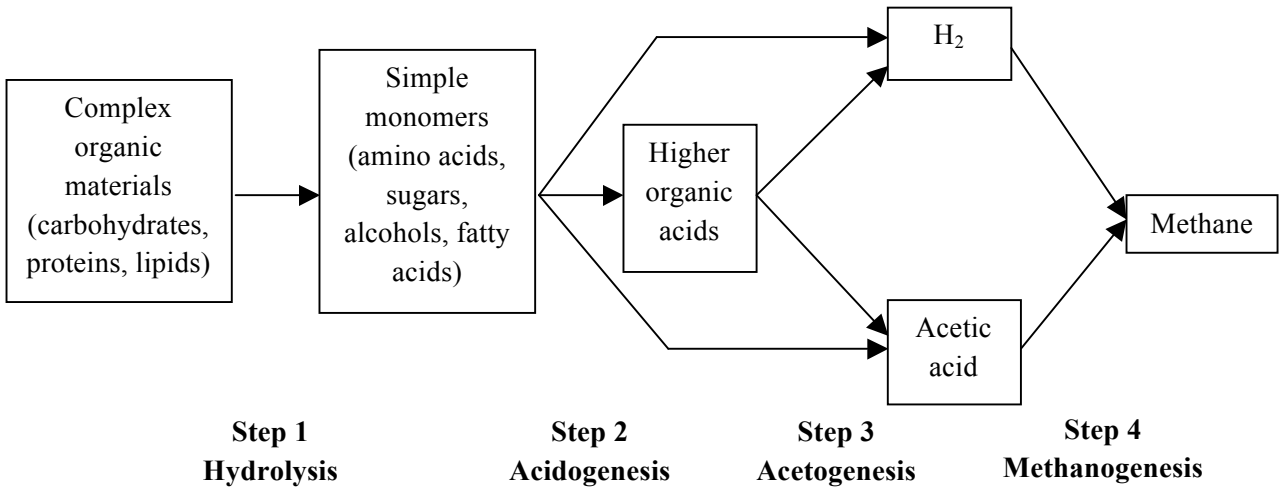


**Figure 11.** Scheme for anaerobic conversion of biomass to methane

In the light of the above discussion, this work investigated the modification of anaerobic digestion process for the production of VFA-enriched swine manure to support the

growth of *Chlorella* sp. (UMN271) as well as swine wastewater treatment. The detailed objectives of the study were to determine how VFA composition changes during our acidogenic fermentation of fresh LSM, and to determine how algae growth and oil yield could be affected by the LSM after the modified anaerobic digestion.

## 5.2 Materials and Methods

### 5.2.1 Algae strain and seed culture conditions

Alga strain *Chlorella* sp. (UMN271), collected from Loon Lake, Waseca, MN, and isolated and identified in our laboratory, was used in the study. The alga seeds were enriched in 250 mL Erlenmeyer flasks each containing 100 mL sterilized BG11 medium with 2g $L^{-1}$ glucose (Hu et al., 2012). The culture conditions were the same with those in section 4.2.1. After 5-day batch cultivation, the seeds were separated from the culture broth through centrifuge at 2000 rpm for 10 min, and were washed with deionized water. The centrifugation-washing process was repeated two times before the inoculation in Experiment II as described in section 5.2.4.

### 5.2.2 Manure resource

Fresh swine manure samples from UMore Park were collected. Due to the high turbidity, the LSM samples were centrifuged as described in section 4.2.2.The characteristics of the two LSM supernatants, including pH, chemical oxygen demand (COD), phosphate-phosphorus ($PO_4$-P), ammonia-nitrogen ($NH_3$-N), and total nitrogen (TN), were determined. All samples were collected and measured in triplicates, and the averages

were presented in Table 12. In this study, the raw LSM sample was divided into two equal portions for experiment I as described in section 5.2.3. Because LSM had a high concentration of total suspended solids which led to poor transmission of light, and a high ammonia content which inhibited algal growth, the manure samples were diluted with deionized water so that the initial ammonia concentrations was 0.5% (78~180mg $NH_3$-N $L^{-1}$) before being used for algae cultivation.

**Table 12** Characteristics of liquid swine manure used in the study

| Parameter | Value |
|---|---|
| pH | 7.45±0.31 |
| COD (mg $L^{-1}$) | 20820±45.05 |
| $PO_4$-P (mg $L^{-1}$) | 367±1.46 |
| $NH_3$-N (mg $L^{-1}$) | 5190±9.21 |
| TN (mg $L^{-1}$ ) | 6880±6.14 |

### 5.2.3 Acidogenic fermentation of raw LSM (Experiment I)

The objective of this experiment was to determine if VFAs-enriched manure effluents could be produced from swine manure using a modified anaerobically digestion process. Raw LSM was divided into two aliquots, one for conventional anaerobic digestion as control and the other for modified anaerobic digestion. The two types of treatments were labeled as A1 (control) and A2, respectively. The traditional anaerobically digestion process on fresh swine manure was done at 38 ± 1°C in a 4 L fermenter for 15 days as a control. Before the digestion period, the fermenter was covered airtight and purged with nitrogen gas for about 5 minutes to remove oxygen left in the headspace. The other

portion had the pH adjusted to approx 5.3 before being placed in a 4 L Erlenmeyer flask to fill up the working volume of 4 L. The flask was covered airtight and excluded oxygen in the headspace in the same way with treatment A1, and maintained at 38 ± 1°C. The pH was controlled at 5.3 for acidogenic fermentation for 72 hours, which was determined by preliminary trials showing that a peak of VFAs accumulation could normally be achieved within that period (data not shown). Samples were taken for VFA concentration test at the beginning and the end of the fermentation period in treatment A1 and every 24 hours in treatment A2. All samples were collected and analyzed in triplicates for statistical analysis.

### 5.2.4 Lab-scale cultivation of algae on acidogenically fermented LSM (Experiment II)

The objective of this experiment was to determine whether algal biomass productivity, lipid accumulation and wastewater treatment efficiencies were improved on acidogenically fermented manure obtained in Exp I. In this experiment, UMN271 seeds were inoculated of approx 0.3 g $L^{-1}$ on 150 mL culture medium in a 250 mL Erlenmeyer flask. Four types of culture media were tested. They were raw 20-fold diluted A1 manure effluent (treatment B1), sterilized 20-fold diluted A1 manure effluent (treatment B2), raw 20-fold diluted A2 manure effluent (treatment B3), and sterilized 20-fold diluted A2 manure effluent (treatment B4). Five-day batch cultivation was operated at 25±2°C under a continuous cool-white fluorescent light intensity of 60 μmol $m^{-2}s^{-1}$. All the treatments were done in four replicates (three for sampling and the left one for recharging). TVSS, pH, COD, $PO_4$-P, $NH_3$-N, and TN were analyzed daily, VFAs contents at the beginning

and the end of the culture period were determined, and fatty acid profiles were analyzed on the harvested algal samples after the 5-day cultivation.

### 5.2.5 Analytical methods

#### 5.2.5.1 VFA composition analysis

The manure effluent samples or algal culture samples were centrifuged at 7000 rpm for 10 minutes, and then the liquid phase was passed through a Fisherbrand 0.45 μm syringe filter (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA) to get clear filtrates. The quantitative characterizations of VFAs in the filtrate, including acetic, propionic and butyric acids were measured using an Agilent 7820A gas chromatography with flame ionization detector (GC-FID) according to the method in section 4.2.6.3.

#### 5.2.5.2 Algal biomass growth analysis

The daily algal biomass growth was determined in form of total volatile suspended solid (TVSS). The details of the measurement were described in section 3.2.4.1

#### 5.2.5.3 Nutrient analysis

Water-soluble COD, $NH_3$-N, TN and $PO_4$-P were analyzed in accordance with the standard methods (APHA, 1998) and instructions in the Hach DR5000 spectrophotometer manual (Hach, 2008), which were similar with those in section 3.2.4.2

#### 5.2.5.4 Algal lipid content and composition analysis

Total lipid contents and the fatty acid composition of the harvested algal cells were determined using the methods in section 4.2.6.4

Statistical analysis was conducted using *t*-test and one-way analysis of variance (ANOVA) with the JMP8.0 software package (SAS Institute Inc., NC). Any treatment with a *p*-value less than 0.05 was considered significant.

## 5.3 Results and Discussion

### 5.3.1 Acidogenic fermentation of fresh swine manure for VFA accumulation (Exp I)

In this experiment, fresh LSM with acidification pretreatment, which was used to inactivate methanogens, was anaerobically digested for 72 h at pH 5.3 to promote VFA accumulation in treatment A2, by taking 15-day anaerobic digestion of untreated LSM as a control (treatment A1). As shown in Table 13, VFAs content in effluent of A2 (11748.32 mg $L^{-1}$) was significantly higher than that from treatment A1 (8586.20 mg $L^{-1}$), indicating that the modified anaerobic digestion was effective in promoting VFA production in LSM. The VFA concentration of 8586.20 mg $L^{-1}$ in the conventionally digested swine manure was similar to those (2230 ~ 17330 mg VFA $L^{-1}$ swine slurry) reported in the literature (Cooper and Cornforth, 1978). For treatment A2, the concentration of acetic acid increased from 2899.12 to 3553.84 mg $L^{-1}$ immediately after the acidification pretreatment (0 h), and then increased to 5744.43 mg $L^{-1}$ after the 72 h acidified digestion (72 h); the propionic acid content rose from 3540.26 mg $L^{-1}$ in fresh manure to 3923.73 mg $L^{-1}$ at 0 h and 5678.02 mg $L^{-1}$ at 72 h, while the butyric acid content was slightly reduced to 325.87 mg $L^{-1}$ from 333.52 mg $L^{-1}$ (fresh manure). Based on the data in Table 13, it is concluded that the best hydraulic retention time (HRT) in

this particular study was at 48 h when the highest total VFA concentration (13811.91 mg $L^{-1}$) could be obtained.

**Table 13.** VFA concentrations (mg $L^{-1}$) in swine manure

| | Fresh | Treatment | Treatment A2 | | | |
|---|---|---|---|---|---|---|
| | Manure | A1 | 0 h | 24 h | 48 h | 72 h |
| Acetic acid | 2899.12 | 4535.2 | 3553.84 | 3797.15 | 6487.45 | 5744.43 |
| Propionic acid | 3540.26 | 3517.8 | 3923.73 | 4181.7 | 7066.33 | 5678.02 |
| Butyric acid | 333.52 | 533.2 | 264.96 | 293.41 | 258.13 | 325.87 |
| Total | 6772.9 | 8586.2 | 7742.53 | 8272.26 | 13811.91 | 11748.32 |

Although VFA–enriched LSM can be considered as potential resources for algae production, there is few study available to evaluate its significance in manure management, which may be due to the fact that the developers and operators of well-established traditional digestion process for methane production from animal manure have not been presented any convincing incentives to deviate from their stable process. However, investigation on the modification of anaerobic digestion on swine manure to get effective production of VFAs for algae growth is of profound significance, since suppressing methanogenesis in anaerobic digestion process will also increase the production of $H_2$, a potential clean fuel with a high energy density between 120 and 142 kJ $g^{-1}$ to replace hydrocarbon fuels (Aceves-Lara et al., 2008). The idea of biohydrogen production from organic wastes through anaerobic fermentation is not new. The process has been investigated on different wastewaters, such as municipal wastewater, brewery wastewater, rice winery wastewater and dairy wastewater (Wang et al, 2003; Vijayaraghavan et al., 2007; Yu et al., 2003; Mohan et al., 2007). Up to now, research on

biohydrogen production from animal wastes has mainly focused on chemical, heat and/or pH pretreatment (Mohan et al, 2008), pH control and additional feeding (Wu, 2009) under mesophilic (Cheong et al., 2007) and/or thermophilic conditions (Kotsopoulos et al., 2009). Knowledge from the previous works could be used to develop a high-efficient swine manure digestion system for both VFA-enriched LSM and $H_2$ production.

### 5.3.2 Lab-scale cultivation of algae on acidogenically digested LSM (Exp II)

In the experiment, the diluted LSM effluents from treatments A1 and A2 were used for the growth of *Chlorella* sp. The initial contents of acetic, propionic and butyric acids of the diluted manure samples used in the experiment are shown in Table 14. The acidogenically fermented manure media used in treatments B3 and B4 were obtained from the treatment A2 effluent as described in section 5.3.1 with a 20-fold dilution rate, while the traditionally digested manure media in treatments B1 and B2 were from the diluted treatment A1 effluent. Therefore, it was reasonable that the total VFA contents in treatments B3 and B4 (442.26 and 568.27 mg $L^{-1}$) were higher than those in treatments B1 and B2 (375.75 and 429.31 mg $L^{-1}$). Also, it was observed that the concentrations of each acid were reduced after the sterilization of the manure medium, leading to similar VFA concentrations between B1 and B4 media. After the 5-day batch growth, all the three acids completely disappeared from the cultures (data not shown).

**Table 14**. The initial VFA concentrations in manure samples for experiment II

| Treatment | medium | Acetic acid (mg $L^{-1}$) | Propionic acid (mg $L^{-1}$) | Butyric acid (mg $L^{-1}$) | Total (mg $L^{-1}$) |
|---|---|---|---|---|---|
| B1 | 20x raw, conventionally digested manure | 226.76 | 175.89 | 26.66 | 429.31 |

| | | | | | |
|---|---|---|---|---|---|
| B2 | 20x sterilized, conventionally digested manure | 204.38 | 147.21 | 24.16 | 375.75 |
| B3 | 20x raw, acidogenically fermented manure | 304.04 | 237.26 | 26.97 | 568.27 |
| B4 | 20x sterilized, acidogenically fermented manure | 241.55 | 177.95 | 22.76 | 442.26 |

The trends in the pH of the cultures during the 5-day batch cultivation of *Chlorella* sp. for treatments B1 ~ B4 are shown in Fig.12. As shown in the figure, the pH values of the treatments were all adjusted to the neutral level at the beginning of the experiment. During the 5-day growth of algae, the pH curves of treatments B1 and B2 essentially agreed with each other, which were characterized by a rapid increase in the first 4 days followed by a steady level at pH8.5 for the rest of the culture period, while the pH values of treatments B3 and B4 rose to around 8.0 in the first 3 days with the similar trends. This indicated that sterilization of manure media had no significant effect on pH changes during algal growth. Also, it was found that pH trends varied dramatically between the cultures with traditionally digested LSM and acidogenically fermented LSM, and the pH level of cultures in treatments B1 and B2 were always higher than those in treatments B3 and B4. Water alkalinity usually occurs during algae cultivation because dissolved $CO_2$ in the water is consumed during algal photosynthesis, resulting in a rise in water pH. The data in Table 14 and Fig.17 suggest that a higher concentration of assimilable carbon compounds in B3 and B4 samples led to a less requirement for water dissolved $CO_2$ which prevented a further alkalinity and resulted in a lower pH compared with the B1 and B2 cultures.

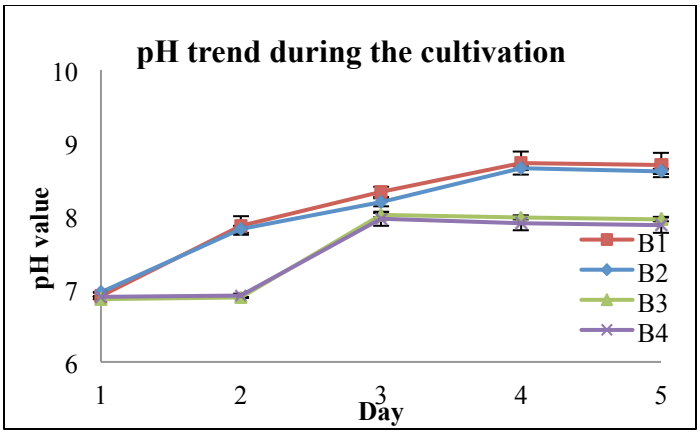


**Figure 12**. pH changes during the 5-day batch experiment on 20-fold diluted raw and sterilized traditionally digested LSM (B1 and B2, respectively), and raw and sterilized acidogenically fermented LSM (B3 and B4, respectively)

After the 5-day batch growth, the biomass accumulation of *Chlorella* sp. was highest from B3 culture (0.65 g TVSS $L^{-1}$) and lowest from B2 culture (0.50 g TVSS $L^{-1}$) (Fig 13a), indicating that the acidogenically digested LSM could promote the algal growth in comparison to the conventionally digested LSM. An exponential phase followed by a subsequent rise to the final stationary state was observed in all four growth curves, but the algal growth rates during the exponential phase according to Eq.2 for treatments B3 and B4 (0.898 and 0.845 $d^{-1}$, respectively) were significantly higher than those for treatments B1 and B2 (0.490 and 0.375 $d^{-1}$, respectively). Different from the algal growth in Fig.8 (in section 4.3.3), the growth of *Chlorella* sp. on the four manure media did not show any obvious lag phase (Fig.13a). We do not know the exact mechanisms responsible for the lack of lag phenomena for the strain grown on digested LSM. However, it was previously reported that the lag phase duration varied with environmental conditions (Duarte, 1990). A close correlation between algal growth rate and the initial ratio of VFA to total nitrogen (TN) was found ($R^2 = 0.7455$, $p < 0.05$),

indicating that VFA/TN ratio was an important factor affecting the suitability of LSM for algae growth (Fig.13b). Chen and Johns (1991), working on *Chlorella sorokiniana*, found that carbon was the growth-limiting nutrient when the C/N value of the medium was below 20~25. In our study, initial VFA/TN ratios of LSM with the exogenous acetic, propionic and butyric acids were 10.18, 9.94 and 9.41, respectively, while initial VFA/TN ratios of raw and sterilized acidogenically fermented LSM samples were 2.58 and 2.03, respectively, indicating that algae-available carbon content in the study significantly affected the growth of algae. Since the initial VFA contents in LSM with the exogenous acetic, propionic and butyric acids (1049, 990, 959.5 mg $L^{-1}$, respectively) were significantly higher than those in raw and sterilized acidogenically fermented LSM samples (568.27 and 442.26 mg $L^{-1}$, respectively), it is not surprising that the algae biomass productivities in exogenous VFA-added LSM (section 4.3.3) were significantly higher than those in the acidogenically fermented LSM.

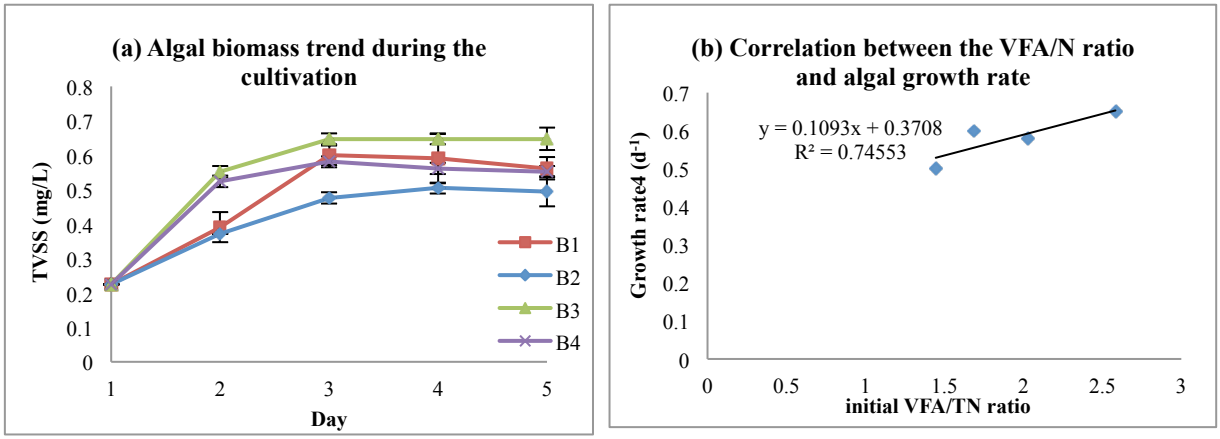


**Figure 13**. Biomass production of *Chlorella* sp. in treatments B1, B2, B3 and B4 (a), and the correlation between the initial VFA concentrations on manure media and algal growth rate (b) during the 5-day batch experiment

The changes in nutrients in the 4 cultures during the 5-day batch cultivation of *Chlorella* sp. and the nutrient removal efficiencies are shown in Fig.14 and Table 15, respectively. The initial concentrations of COD, $PO_4$-P $NH_3$-N, and TN in the sterilized manure cultures were similar with those in the raw manure cultures, indicating that sterilization had no significant effect on the quantitative change of manure nutrients. It was observed that the COD removal efficiencies for all treatments were in a narrow range of 58.02 ~ 61.67%. It was interesting to note that the initial amounts of COD in treatments B1 and B2 were higher than those in treatments B3 and B4 (Fig.14a). Ye et al. (2008) found that 46% of COD concentration in aquatic products processing wastewater was lost in the stage of hydrolytic acidification treatment. Therefore, it is possible that organic compounds in fresh manure were partially oxidized during the acidification process in Exp I so that the amounts of oxygen consumed per liter of B3 and B4 solutions were measured to be as low as ~786 mg COD $L^{-1}$.

As shown in Table 15, $PO_4$-P removal efficiencies by *Chlorella* sp. on the two acidogenic fermented manure samples in treatments B3 and B4 (34.16% and 23.37%, respectively) were significantly lower than those on the traditionally digested manure samples in treatments B1 and B2 (70.86% and 68.48%, respectively). However, the quantities of $PO_4$-P recovered by algae in treatments B3 and B4 (12.03 and 17.87 mg $PO_4$-P $L^{-1}$, respectively) were higher than those in treatments B1 and B2 (7.72 and 7.53 mg $PO_4$-P $L^{-1}$, respectively) which may be attributed to the enrichment of orthophosphate on acidogenically fermented manure effluents. As shown in Fig.14b, compared with the initial $PO_4$-P concentrations of 10.9 and 11.0 mg $PO_4$-P $L^{-1}$ in treatments B1 and B2,

respectively, the $PO_4$-P concentrations in treatments B3 and B4 were as high as 51.5 and 52.3 mg $L^{-1}$, which were higher than those reported in literatures (Mallarino and Wittry., 2010; Wu et al., 2009). The differences should be attributed to hydrolysis of phytic acid during the acidification pretreatment in Exp I. It was reported that phytic acid, the major form of the organic phosphorus in manures, mainly existed as insoluble compounds in the solid fraction of LSM, but could become water soluble under acidic conditions (Turner and Leytem, 2004).

$NH_3$-N was not completely removed within the 5-day growth period for any of the 4 diluted samples with the initial concentrations in the range of 163~190 mg $L^{-1}$ (Fig.14c). Although the $NH_3$-N removal efficiencies in this experiment were much lower (42.57%, 31.58%, 44.73% and 36.89% for treatments B1 ~ B4, respectively) than those in Exp III in Chapter 4 (Table 11), the amounts of $NH_3$-N removed from the acidogenically fermented LSM by *Chlorella* sp. (73.80 and 60.13 mg $NH_3$-N $L^{-1}$) were similar to those removed from the exogenous VFA-added LSM by the same strain (69.59 ~ 76.89 mg $NH_3$-N $L^{-1}$), which were equal to or higher than those reported in literatures. Park et al. (2010) reported that the amount of ammonium-nitrogen removed from an anaerobically digested swine effluent by *Scenedesmus* sp. with different initial seeding cell concentrations ranged from 52.0 to 64.6 mg $L^{-1}$. We believe that the removal of $NH_3$-N in the experiment was mainly attributed to the N uptake by algae other than ammonia volatilization, since the pH of the cultures (Fig.12) were always below pH9.3 which was reported as pKa of $NH_4^+/NH_3$ at room temperature (Hu et al., 2012a).

The time courses of TN decline from the manure media generally followed those of $NH_3$-N, as shown in Fig.14d and Table 15. Though the TN reduction efficiencies in the experiment (22.32% ~ 33.62% in the 4 treatments) were only half of those in Exp III in Chapter 4 (section 4.3.3), the amounts of TN removed from the acidogenically fermented manure samples in treatment B3 (69.47 mg TN $L^{-1}$) was higher than those from exogenous VFA-added manure samples (53.33 ~ 56.97 mg TN $L^{-1}$), indicating the stronger TN removal ability of *Chlorella* sp. on our manure samples without additives. For treatments B1 and B2, the amounts of TN removed during the 5-day growth of *Chlorella* sp. were 85.40 and 74.53 mg $L^{-1}$, respectively, and were slightly higher than the quantities of $NH_3$-N removed (78.33 and 60.00 mg $NH_3$-N $L^{-1}$, respectively), indicating that ammonia was the dominant form of recovered nitrogen compounds by *Chlorella* sp. in the traditionally digested manure samples. For treatment B4, it was interesting that the amounts of removed TN (48.67 mg $L^{-1}$) were lower than the amount of removed $NH_3$-N (60.13 mg $L^{-1}$). This situation was also observed in treatment B3. Regarding algal metabolism, it was not really surprising. We speculate that a slight secretion of soluble, organic nitrogen substances from the algal cells beside with the uptake of $NH_3$-N by algal cells occurred during the 5-day growth on acidogenically fermented manure media.

**Table 15**. Nutrient removal efficiencies (%) of *Chlorella* sp. (UMN271) in Experiment II

| Treatment | COD | $PO_4$-P | $NH_3$-N | TN |
|---|---|---|---|---|
| B1 | 61.67 | 70.86 | 42.57 | 33.62 |
| B2 | 58.82 | 68.48 | 31.58 | 28.67 |
| B3 | 58.02 | 34.16 | 44.73 | 31.58 |

| | | | | |
|---|---|---|---|---|
| B4 | 58.16 | 23.37 | 36.89 | 22.32 |

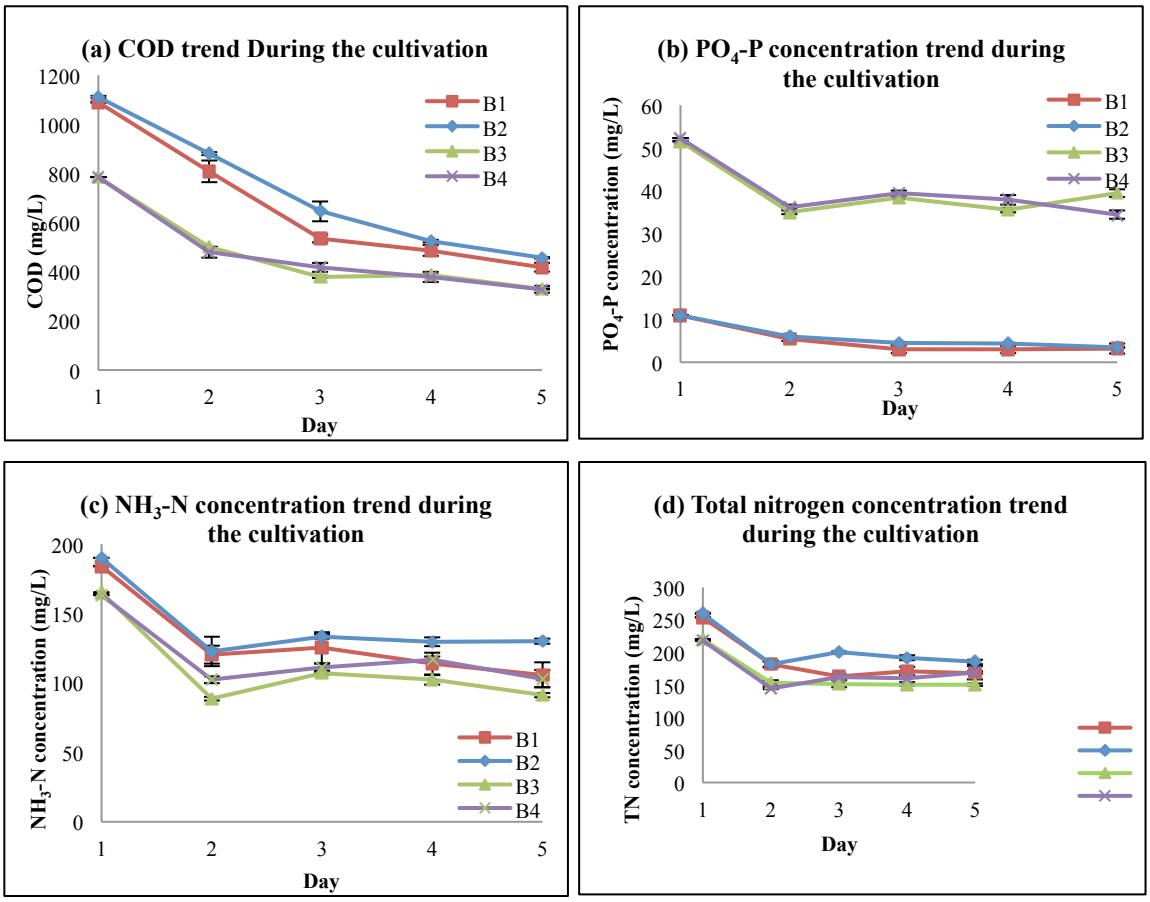


**Figure 14**. Content trends of COD (a), $PO_4$-P (b), $NH_3$-N (c), total nitrogen (d) in cultures containing 20-fold raw (B1, B3) and sterilized (B2, B4), conventionally digested and acidogenically fermented LSM during the 5-day batch growth of *Chlorella* sp.

The fatty acid profiles of the harvested algae samples (dry weight base) after the 5-day cultivation are shown in Table 16. Algae cultivated on raw and sterilized, acidogenically fermented manure samples showed higher fatty acid content (10.93% and 9.14%, respectively) than the rest two. It was found that the initial VFA/TN ratios in the diluted LSM samples correlated well with the fatty acid content in the harvested algae ($R^2$ =

0.9967), indicating that the ration of assimilable C and N in media was an important factor affecting the algal oil yield (Fig.15a). This result coincided with the previous reports that an increase in C:N ratio in nutrient broths could improve lipid production and change lipid composition in algal cells (Meng et al., 2009). GC-MS analysis showed that microalgal lipids for all the four algae samples were mainly composed of saturated and polyunsaturated fatty acids (PUFAs) with C16 and C18 fatty acids as the major compounds, which was good for animal feed production, since PUFAs in animal diets could affect the fatty acid composition in animal products, such as egg, meat, and milk, and thus influence consumer demands for the health benefits of PUFAs (Kouba and Mourot, 2011). For biodiesel production, it is reported that linolenic acid (C18:3) should be specially noted among the fatty acid compounds, since a limit of 12% linolenic acid was specified for a quality biodiesel according to EN14214 standard (2004). As shown in Table 16, only the fatty acid profile of algae in treatment C3 had linolenic acid content (10.51%) within the specification, which meant that oil from *Chlorella* sp. grown on acidogenically digested LSM may be used for good quality biodiesel. Also, as shown in Fig.15b, the fatty acid production from algae cultivated on raw, acidogenically fermented LSM was as high as 70.78 mg $L^{-1}$, which was 1.51 and 1.91 times of those from algae grown on the raw and sterilized, conventionally digested LSM samples, respectively. Therefore, algae grown on acidogenically fermented LSM could be used as feedstock for biodiesel and other biofuel production.

**Table 16.** Fatty acid profiles derived from triacylglycerol, phospholipid and free fatty acids in *Chlorella* sp. after 5-day batch cultivation on different manure samples

| | Treatment B1 | Treatment B2 | Treatment B3 | Treatment B4 |
|---|---|---|---|---|
| Total fatty acid/dry weight (%) | 8.33 | 7.48 | 10.93 | 9.14 |
| Saturated fatty acids (% of total fatty acids) | 48.37 | 43.16 | 57.1 | 50.68 |
| C14:0 | 0.28 | NA[a] | 0.26 | 0.38 |
| C16:0 | 17.37 | 22.02 | 16.84 | 20.72 |
| C18:0 | 28.15 | 19.14 | 37.83 | 27.61 |
| C20:0 | 1.06 | 0.88 | 1.01 | 1.02 |
| C24:0 | 1.51 | 1.12 | 1.16 | 0.95 |
| Monounsaturated fatty acids (% total fatty acids) | 9.95 | 8.6 | 7.91 | 8.17 |
| C16:1 | 2.57 | 2.71 | 1.21 | 1.84 |
| C18:1 | 7.38 | 5.89 | 6.7 | 6.33 |
| Polyunsaturated fatty acids (% total fatty acids) | 41.68 | 48.24 | 35 | 41.15 |
| C16:2 | 5.87 | 6.26 | 5.05 | 3.97 |
| C16:3 | 5.46 | 5.98 | 4.25 | 5.62 |
| C18:2 | 16.87 | 20.09 | 15.19 | 15.88 |
| C18:3 | 13.48 | 15.91 | 10.51 | 15.68 |

NA [a] means 'not available'.

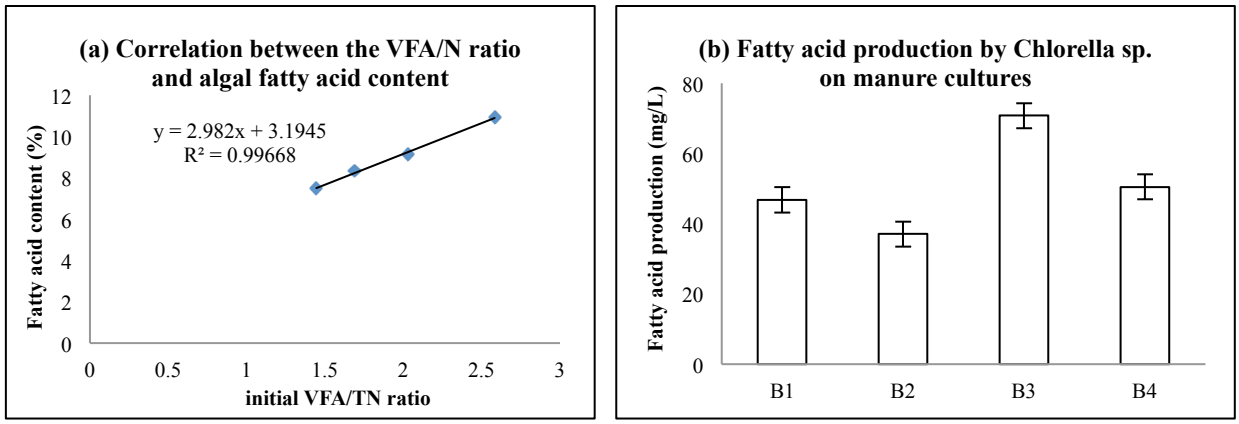


**Figure 15**. The correlation between the initial VFA/TN ratios on manure media and algal fatty acid content (a) and the algal fatty acid accumulation (b) from 5-day batch cultures in treatments B1 ~ B4

## 5.4 Conclusions

Algae growth on wastewater nutrients could provide a holistic solution to nutrient management problems on pig farm and associated production cost problems on algal biofuel industry. One of the challenges for LSM-based algal biomass production is the dearth of carbon resources in diluted LSM. In the study, the experimental results demonstrated that acidic pretreatment of fresh LSM and anaerobic digestion of the pretreated LSM at pH5.3 could successfully promote the VFA accumulation, and the microalgae-acidogenically fermented LSM system was proved to be effective on wastewater treatment and algal biomass accumulation for biofuel and animal feed production using a locally isolated, robust microalga strain *Chlorella sp.* UMN271. Also, GC-MS analysis showed that the harvested algae samples after the 5-day growth could be used as raw material for good-quality biodiesel and animal feed production without restrictions. Based on the results from this study, a process combining acidogenic

fermentation and algae cultivation can be proposed as an effective method to convert nutrients in LSM into biofuel and animal feed as well as to reduce environmental pollution and farmland requirement for manure spreading.

# CHAPTER 6. OPTIMIZATION OF THE GROWTH CONDITIONS FOR ENHANCED MICROALGAE GROWTH AND NUTRIENT REMOVAL FROM ACIDOGENICALLY DIGESTED SWINE MANURE


## Abstract

In the study, central composite design (CCD) was used to investigate the influences of two key parameters, namely wastewater dilution rate (DR) and hydraulic retention time (HRT), on algal biomass productivity and nutrient removal rates, including COD, $PO_4$-P, TN, and $NH_3$-N, and to optimize the semi-continuous process for enhanced growth rates of *Chlorella* sp. on acidogenically digested swine manure and waste nutrient removal rates. According to the response surface analyses of the CCD results, statistically valid quadric models for the response variables of algal biomass productivity and nutrient removal rates including COD, $PO_4$-P, TN and $NH_3$-N were obtained. The regression analyses illustrated that both DR and HRT had significant influences on the five response variables, and the interaction between DR and HRT played an important role in algal biomass productivity and COD removal rates only. The optimal conditions for algal biomass productivity and waste nutrient removal rates were estimated from the significant second-order quadratic models ($p<0.05$) to be 8-fold DR and 2.26-d HRT.

## 6.1 Introduction

In previous chapters, it was showed that the locally isolated facultative heterotrophic microalga strain *Chlorella* sp. (UMN271) was capable to utilize volatile fatty acids (VFAs) including acetic, propionic and butyric acids, which were the major soluble organic carbon substrates in swine wastewater. This *Chlorella* sp. grew well on the diluted VFA-enriched swine wastewater effluent from the acidogenic fermentation, with the algal growth rate as high as 0.90 $d^{-1}$, the lipid content of approx 30%, and the nutrient removal of 458.4 mg COD $L^{-1}$, 12.03 mg $PO_4$-P $L^{-1}$ and 69.5 mg N $L^{-1}$ after the 5-day lab-scale batch cultivation (Hu et al. 2012b).

The next step is to build a system to continuously produce algal feedstock, in which the real suitable cultural condition for high biomass production and nutrient assimilation should be established. In semi-continuous mode, a proportion of the culture is replaced with fresh media when the microalgae reach late logarithmic phase, and then the culture is maintained for days to increase cell density before a second replacement. The repeated harvest-regrowing process can be maintained for a week or for several months without apparent growth decline in the system (Brown et al., 1993). Among the limited amount of factors that could be artificially adjusted in a pilot-scale semi-continuous culture, hydraulic retention time (HRT) and medium dilution rate (DR) are important to influence algal growth and wastewater treatment.

The ratio of the recovery period to the replaced proportion is the HRT, which is a key factor to influence algae growth and nutrient uptake. Olguín (2003) reported that a high-rate algal pond could be operated at short HRTs in the range of 4 ~ 10 days. Uwimana et

al. (2012) found that HRT was an influential factor to enhance the removal efficiency of pathogens from the algal ponds fed with municipal wastewater.

Wastewater DR is responsible for turbidity and nutrient concentrations in the algae cultures, which is also an important factor for algae growth. Cheunbarn and Peerapornpisal (2010) reported that *Spirulina platensis* grown on high diluted anaerobically treated swine wastewater (5-fold and 10-fold dilutions) could obtained higher cell densities than those on low diluted wastewater (1-fold ~ 3.33-fold dilutions) during the 2-week batch study due to the fact that the high color and turbidity in low diluted swine wastewater significantly affected algal photosynthesis efficiency. Wang et al. (2010) reported that the growth rates and nutrient removal rates by *Chlorella* sp. on 10-fold and 15-fold diluted manure were initially slower than those on 20-fold and 25-fold diluted manure, but caught up in the latter part of the 21-day batch cultivation probably due to the continued algal growth supported by the higher nutrient concentrations.

The Box-Wilson Central Composite Design (CCD) is a useful mathematical approach widely applied in the optimization of cultivation processes, in which treatment time and process variability could be reduced, the predictive responses could be closer to the target achievement, and interactions of two or more variables could be studied simultaneously (Box and Hunter, 1957). Kim et al. (2012) used CCD to optimize the culture conditions (initial pH, nitrogen and phosphate concentrations) for the mass production of three green algae *Chlorella* sp., *Dunaliella salina* DCCBC2 and *Dunaliella* sp.. Khataee et al. (2010) used CCD to optimize the biological decolorization of textile wastewater by macroalgae

*Chara* sp. However, there is still little research using CCD for the optimization of culture conditions for both algal mass production and wastewater treatment.

In the light of the above discussion, CCD was used in the study to develop a quadratic mathematical model to determine the influences of HRT and DR on algal biomass productivity and nutrient removal rates in the microalga-acidogenically digested LSM. Another objective of this study was to predict the optimum HRT and DR from the quadric models on the *Chlorella* sp. mass production and the removal of swine wastewater nutrients.

## 6.2 Materials and Methods

### 6.2.1 Algae strain and seeds preparation

Alga strain *Chlorella* sp. (UMN271), which was isolated from Loon Lake, Waseca MN, was used in the study. The preparation and maintenance of the inoculums was accomplished using 250 mL Erlenmeyer flasks containing 100 mL BG11 medium with 2 g $L^{-1}$ glucose. The culture details were the same as those in section 4.2.1. After 1 ~ 2-week cultivation at 25 ± 2°C under a continuous cool-white fluorescent light illumination of 100 μmol $m^{-2}$ $s^{-1}$, the algal cells were separated from the culture broth using centrifuge at 2000 rpm for 5 minutes, followed by washing with deionized water and another centrifugation-suspension process.

### 6.2.2 Characteristics of swine wastewater

The fresh swine manure and the inoculum sludge were collected from the University of Minnesota Southern Research and Outreach Center, Waseca MN. The fresh swine manure was used as the substrate during the acidogenic digestion in the study. The sludge was anaerobically cultivated for 5 days with 5 g $L^{-1}$ glucose at temperature 38 ±1 °C to get the activated and concentrated inoculum, and then was heat-treated at 80 °C with a water bath (Thermo Fisher Scientific Inc., Waltham, MA) for 30 minutes to kill methanogenic bacteria from the community according to Wang et al. (2009). An Erlenmeyer flask with the working volume of 4 L was used as the acidogenically anaerobic digester. 1.0 L concentrated inoculum was added into the reactor containing 3.0 L fresh manure substrate. The mixture was adjusted to approx pH 5.3 with sulfuric acid ($H_2SO_4$) solution, and was maintained at pH 5.3 ~ 5.6 and 38 ± 1 °C for the acidogenic fermentation for 48 h as described by Hu et al. (2012b). The drained effluent was used in the following experiments for algae growth. The characteristics of the fresh and acidogenically digested LSM effluents are shown in Table 17.

**Table 17**. Characteristics of fresh swine manure

| Parameter | Fresh LSM | Acidogenically digested LSM |
|---|---|---|
| pH | 7.58±0.31 | 6.09 |
| TVSS (mg $L^{-1}$) | 2580.01±300.01 | 2462.50 |
| Total nitrogen (mg TN $L^{-1}$) | 2031.43±66.19 | 1673.50 |
| Ammonia-nitrogen (mg $NH_3$-N $L^{-1}$) | 1602.86±84.72 | 1423.75 |
| Orthophosphate-phosphorus (mg $PO_4$-P $L^{-1}$) | 407.43±99.58 | 613.50 |
| COD (mg $L^{-1}$) | 15240±816.66 | 16242.5 |

| | | |
|---|---|---|
| Total VFAs (mg $L^{-1}$) | 7676.26±576.37 | 10965.85 |
| Acetic acid (mg $L^{-1}$) | 4957.08±357.48 | 6793.12 |
| Propionic acid (mg $L^{-1}$) | 1612.03±116.13 | 2576.86 |
| Butyric acid (mg $L^{-1}$) | 1107.16±105.48 | 1595.87 |

### 6.2.3 Optimization of conditions for the semi-continuous mode

6.2.3.1 Factorial design

The experiment was performed to build a mathematical model for the response variables, including biomass productivities and nutrient removal rates, and to predict the optimum DR and HRT for biomass production and wastewater treatment. The experiments were designed according to a $2^2$ circumscribed central composite response surface methodology (RSM) to build a second order model for the response variables without employing a completely full factorial experiment design. Each factor was designed with 5 coded levels ($-\alpha$, -1, 0, +1, $+\alpha$) which could constitute 25 combinations of DR and HRT at different levels. The typical value of $\alpha$ is a function of the number of variables $\alpha = (2^k)^{1/4}$, where k is the number of independent variables. According to the two-variable CCD approach, only 9 variable level setting combinations with the $\alpha$ value of 1.41 were needed for experiment runs in the study, which were presented in the forms of 1 center point, 4 factorial points and 4 star points in Fig.16. The design matrix of the DR and HRT in both coded levels and actual values, as shown in Table 18, was created using SAS 8.1 software (SAS Institute Inc, Cary, NC).

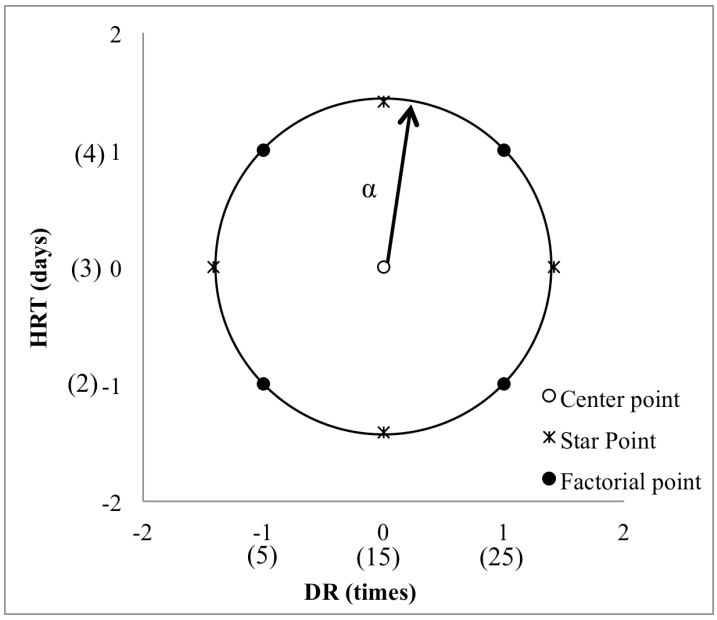


**Figure 16.** Two-variable central composite design

**Table 18** Coded levels and true values of potential significant variables

| Independent variable | Variable | ($-\alpha^a$) level | (-1) level | 0 level | (+1) level | ($+\alpha^a$) level |
|---|---|---|---|---|---|---|
| Dilution rate (DR, time) | $X_1$ | 1 | 5 | 15 | 25 | 30. |
| Hydraulic retention time (HRT, day) | $X_2$ | 1.6 | 2 | 3 | 4 | 4.4 |

$\alpha$ [a] is the distance from the center of the design space to a star point.

6.2.3.2 Algae growth experiments

The designed 13 runs listed in Table 19 were carried out in 250 mL Erlenmeyer flasks containing 150 mL of unsterilized acidogenically digested swine manure with a variety of DRs and an initial *Chlorella* sp. biomass concentration of 0.3 g $L^{-1}$. All the cultures were adjusted to around pH7.0 at the beginning of the incubation, and were maintained at 25 ± 2 °C under 100 μmol $m^{-2}$ $s^{-1}$ white fluorescent light on a 16:8 h light/dark cycle for one week to get the stationary-phase algae. The one-week batch cultivation was followed by

10-day semi-continuous modes with various HRTs (Table 19) at the same temperature and light conditions as described above. Samples were taken from the daily harvested cultures and feed solutions for the TVSS and nutrient concentration test. The data collected after the cultivation system reached quasi steady states were used for the statistical analysis in section 6.2.3.3. To avoid bias, 13 runs were performed in a totally random order.

**Table 19**. Full factorial central composite design matrix

| Experimental runs | Coded value | | Real value | |
|---|---|---|---|---|
| | $X_1$ | $X_2$ | DR (time) | HRT (day) |
| 1 | -1 | -1 | 5 | 2 |
| 2 | -1 | +1 | 5 | 4 |
| 3 | +1 | -1 | 25 | 2 |
| 4 | +1 | +1 | 25 | 4 |
| 5 | -α | 0 | 1 | 3 |
| 6 | +α | 0 | 30 | 3 |
| 7 | 0 | -α | 15 | 1.6 |
| 8 | 0 | +α | 15 | 4.4 |
| 9 | 0 | 0 | 15 | 3 |
| 10 | 0 | 0 | 15 | 3 |
| 11 | 0 | 0 | 15 | 3 |
| 12 | 0 | 0 | 15 | 3 |
| 13 | 0 | 0 | 15 | 3 |

6.2.3.3 Quadratic model analysis

Data of algal biomass and nutrient contents collected from samples on the 9$^{th}$ and 10$^{th}$ days of the semi-continuous cultivation were used to calculate the algae biomass

productivity ($\mu_1$, mg $L^{-1}$ $d^{-1}$) and removal rates of chemical oxygen demand (COD), orthophosphate-phosphorus ($PO_4$-P), total nitrogen (TN), and ammonia-nitrogen ($NH_3$-N) ($\mu_2 \sim \mu_5$, respectively, mg $L^{-1}$ $d^{-1}$) based on Eq.6

$$\mu_i = | i_{10} - (i_9 \frac{X_2 - 1}{X_2} + i_{M9} \frac{1}{X_1 X_2}) | \quad \text{Eq.6}$$

where $\mu_i$ is the daily change of i's concentration; $i_9$ and $i_{10}$ are concentrations of i in the harvested cultures on day 9 and 10, respectively; $i_{M9}$ is i's concentration in the feeding swine manure on day 9; $X_1$ and $X_2$ are the values of DR and HRT for the experiment run, respectively.

The results of $\mu_1 \sim \mu_5$ were analyzed statistically through analysis of variance (ANOVA) at 95% confidence interval and RSM with SAS8.1 ADX Interface software. Second-order quadratic models were established to evaluate the effects of DR and HRT on the responses, including algal biomass productivity ($Y_1$, mg $L^{-1}$ $d^{-1}$), COD removal rate ($Y_2$, mg $L^{-1}$ $d^{-1}$), $PO_4$-P removal rate ($Y_3$, mg $L^{-1}$ $d^{-1}$), TN removal rate ($Y_4$, mg $L^{-1}$ $d^{-1}$), and $NH_3$-N removal rate ($Y_5$, mg $L^{-1}$ $d^{-1}$), as in Eq.7 by using the method of least squares:

$$Y_i = a_{i0} + a_{i1}X_1 + a_{i2}X_2 + a_{i12}X_1X_2 + a_{i11}X_1^2 + a_{i22}X_2^2 \quad \text{Eq.7}$$

where $Y_i$ is the predicted response; $X_1$ and $X_2$ are the real values of DR and HRT, respectively; $a_{i0}$, $a_{i1}$, $a_{i2}$, $a_{i12}$, $a_{i11}$ and $a_{i22}$ are the coefficients in the mathematical model of $Y_i$.

### 6.2.4 Analytical methods

6.2.4.1 Algal biomass growth analysis

The daily algal biomass growth was determined in form of total volatile suspended solid (TVSS). The details of the measurement were described in section 3.2.4.1

6.2.4.2 Nutrient analysis

COD, $NH_3$-N, TN and $PO_4$-P were analyzed in accordance with the standard methods (APHA, 1998) and instructions in the Hach DR5000 spectrophotometer manual (Hach, 2008), which was similar with those in section 3.2.4.2

## 6.3 Results and Discussion

### 6.3.1 Algal biomass accumulation and nutrient removal rates in the acidogenically digested swine manure cultures

The $2^2$ CCD with 5 repeated center points was used to find the optimal values of DR and HRT for algal biomass production and nutrient removal from the acidogenically digested swine manure. Thirteen runs were established by using the SAS 8.1 software (Table 19). The experimental results, including algal biomass productivity ($\mu_1$, mg $L^{-1}$ $d^{-1}$), COD removal rate ($\mu_2$, mg $L^{-1}$ $d^{-1}$), $PO_4$-P ($\mu_3$, mg $L^{-1}$ $d^{-1}$), TN ($\mu_4$, mg $L^{-1}$ $d^{-1}$), and $NH_3$-N ($\mu_5$, mg $L^{-1}$ $d^{-1}$), for each CCD run are listed in Table 20. Among the 13 treatments, run 1 (DR = 5 times, HRT = 2 d) had the largest biomass productivity and nutrient removal rates, while run 6 (DR = 30 times, HRT = 3 d) had the smallest $\mu_1$ and $\mu_2$ and the second smallest $\mu_3 \sim \mu_5$. The responses in run 4 (DR = 25 times, HRT = 4 d) were lower than those in run 2 (DR = 5 times, HRT = 4 d), and the differences were more significant between run 3 and run 1, whose DRs were 25 and 5 times, respectively, and HRTs were

both 2 days. It can be supposed that the low manure concentration limited algae growth; the shorter the HRTs, the more obvious the limitation is on algae growth and nutrients uptake. However, the behavior was contradictory to run 5 (DR = 1 times, HRT = 3 d) and run 9~13 (DR = 15 times, HRT = 3 d), in which the smaller $\mu_1 \sim \mu_5$ were obtained in the treatment with higher manure concentration. Similar results were observed by Mezzomo et al. (2010) who investigated the influences of the swine wastewater concentration and sodium biocarbonate concentration on the growth of microalgae *Spirulina platensis*. They suggested that the swine manure was toxic to the microalgae when used without dilution because of sunlight penetration problem caused by the coloration of swine manure, the toxic effect of ammonia, and the competition from other microorganisms in the wastewater.

**Table 20.** Central composite design matrix and the results of the response variables including biomass productivity ($\mu_1$, mg $L^{-1}$ $d^{-1}$), COD removal rate ($\mu_2$, mg $L^{-1}$ $d^{-1}$), $PO_4$-P ($\mu_3$, mg $L^{-1}$ $d^{-1}$), TN ($\mu_4$, mg $L^{-1}$ $d^{-1}$), and $NH_3$-N ($\mu_5$, mg $L^{-1}$ $d^{-1}$) in the CCD runs

| Run | DR (times) | HRT (days) | $\mu_1$ (mg $L^{-1}$ $d^{-1}$) | $\mu_2$ (mg $L^{-1}$ $d^{-1}$) | $\mu_3$ (mg $L^{-1}$ $d^{-1}$) | $\mu_4$ (mg $L^{-1}$ $d^{-1}$) | $\mu_5$ (mg $L^{-1}$ $d^{-1}$) |
|---|---|---|---|---|---|---|---|
| 1 | 5(-1) | 2(-1) | 332.67 | 1027.33 | 6.77 | 36.20 | 29.33 |
| 2 | 5(-1) | 4(+1) | 121.89 | 349.18 | 0.42 | 14.06 | 10.41 |
| 3 | 25(+1) | 2(-1) | 98.82 | 299.33 | 0.19 | 9.57 | 8.95 |
| 4 | 25(+1) | 4(+1) | 95.35 | 285.58 | 0.08 | 5.69 | 5.12 |
| 5 | 1(-α) | 3(0) | 89.26 | 275.33 | 0.39 | 6.56 | 6.48 |
| 6 | 30(+α) | 3(0) | 76.10 | 254.88 | 0.14 | 6.39 | 6.28 |
| 7 | 15(0) | 1.586(-α) | 164.27 | 445.18 | 0.72 | 15.70 | 14.83 |
| 8 | 15(0) | 4.414(+α) | 102.20 | 243.10 | 0.12 | 6.12 | 5.89 |
| 9 | 15(0) | 3(0) | 220.50 | 678.90 | 2.75 | 22.75 | 16.35 |
| 10 | 15(0) | 3(0) | 221.50 | 688.90 | 2.81 | 24.35 | 15.80 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 11 | 15(0) | 3(0) | 225.50 | 692.90 | 2.65 | 24.55 | 14.98 |
| 12 | 15(0) | 3(0) | 219.50 | 704.10 | 2.92 | 21.05 | 18.21 |
| 13 | 15(0) | 3(0) | 215.50 | 691.00 | 2.55 | 20.98 | 17.56 |

### 6.3.2 Model analyses to response to algal biomass productivity and nutrient removal rates

The SAS ADX interface program was used to analyze the CCD data sets in Table 20 to build up the quadratic mathematical models of DR and HRT responding to the algal biomass productivity and removal rates of COD, $PO_4$-P, TN and $NH_3$-N. According to the statistical analysis of the variable estimates (data not shown), the independent variables of DR ($X_1$, $X_1^2$) and HRT ($X_2$, $X_2^2$) had significant effects on the biomass production and the removal of COD, $PO_4$-P, TN and $NH_3$-N, while the interactions between DR and HRT ($X_1X_2$) had low significances on *Chlorella* sp to remove $PO_4$-P, TN and $NH_3$-N ($p$>0.05). The quadric models predicted for the response variables of $Y_1$ ~ $Y_5$ using significant coefficients are given as Eq.8 ~ 12 in Table 21.

**Table 21** Regression equations for the response variables, including the algal biomass productivity and removal rates of COD, $PO_4$-P, TN and $NH_3$-N ($Y_1$ ~ $Y_5$, respectively)

| Model | Equation label |
|---|---|
| $Y_1 = 2.143\times10^2 + 1.397X_1 + 25.234X_2 - 0.186X_1^2 + 1.082X_1X_2 - 8.505X_2^2$ | Eq.8 |
| $Y_2 = 4.132\times10^2 - 9.489X_1 + 3.658\times10^2X_2 - 1.559X_1^2 + 15.554X_1X_2 - 1.189\times10^2X_2^2$ | Eq.9 |
| $Y_3 = -1.104\times10^2 + 4.062X_1 + 84.115X_2 - 0.182X_1^2 - 16.145X_2^2$ | Eq.10 |
| $Y_4 = 8.169 + 1.315X_1 + 15.375X_2 - 0.054X_1^2 - 3.183X_2^2$ | Eq.11 |
| $Y_5 = 31.660 + 1.863X_1 + 16.752X_2 - 0.078X_1^2 - 4.003X_2^2$ | Eq.12 |

In the study, the quadratic models predicted for the response variables of $Y_1 \sim Y_5$ are all statistically valid. The statistical significance of each quadratic model was evaluated by using ANOVA technique as shown in Table 22. It was observed that the *F*-values of the 5 models were more or less higher than the critical *F*-value (critical *F*=3.972), and the *p*-values of the 5 models were all relatively low ($p<0.05$), indicating that the regression models were significant at high confidence levels.

**Table 22.** ANOVA for the quadratic models predicted for the response variables of the algal biomass productivity and removal rates of COD, $PO_4$-P, TN and $NH_3$-N ($Y_1 \sim Y_5$, respectively)

| | Source | SS [a] | DF [b] | MS [c] | *F*-value ($p < 0.05$) |
|---|---|---|---|---|---|
| $Y_1$ | Model error | 2.292 | 5 | 0.458 | 8.205 ($p = 0.008$) |
| | Residual error | 0.391 | 7 | 0.056 | |
| | Total | 2.683 | 12 | | |
| | $R^2$ [d] | 85.42% | | | |
| | Adjusted $R^2$ [e] | 75.01% | | | |
| | RMSE [f] | 0.236 | | | |
| | CV [g] | 4.706 | | | |
| $Y_2$ | Model error | $5.853 \times 10^5$ | 5 | $1.171 \times 10^5$ | 5.048 ($p = 0.028$) |
| | Residual error | $1.623 \times 10^5$ | 7 | $2.319 \times 10^4$ | |
| | Total | $7.476 \times 10^5$ | 12 | | |
| | $R^2$ [d] | 78.29% | | | |
| | Adjusted $R^2$ [e] | 62.78% | | | |
| | RMSE [f] | 152.279 | | | |
| | CV [g] | 29.836 | | | |
| $Y_3$ | Model error | 24.612 | 5 | 4.922 | 13.482 ($p = 0.002$) |
| | Residual error | 2.556 | 7 | 0.365 | |

| | | | | | |
|---|---|---|---|---|---|
| | Total | 27.168 | 12 | | |
| | $R^{2\,d}$ | 90.59% | | | |
| | Adjusted $R^{2\,e}$ | 83.87% | | | |
| | RMSE [f] | 0.604 | | | |
| | CV [g] | -224.715 | | | |
| | Model error | 3.839 | 5 | 0.768 | 4.914 ($p = 0.030$) |
| | Residual error | 1.094 | 7 | 0.156 | |
| | Total | 4.932 | 12 | | |
| $Y_4$ | $R^{2\,d}$ | 77.83% | | | |
| | Adjusted $R^{2\,e}$ | 61.99% | | | |
| | RMSE [f] | 0.395 | | | |
| | CV [g] | 15.052 | | | |
| | Model error | 2.799 | 5 | 0.560 | 5.601 ($p = 0.022$) |
| | Residual error | 0.700 | 7 | 0.100 | |
| | Total | 3.498 | 12 | | |
| $Y_5$ | $R^{2\,d}$ | 80% | | | |
| | Adjusted $R^{2\,e}$ | 65.72% | | | |
| | RMSE [f] | 0.316 | | | |
| | CV [g] | 12.943 | | | |

SS [a] represents 'Sum of Square';

DF [b] represents 'Degree of Freedom';

MS [c] represents "Mean Squares";

$R^{2\,d}$ means "Coefficient of Correlation";

Adjusted $R^{2\,e}$ is "Coefficient of determination";

RMSE [f] is short for "Root-mean-square Error";

CV [g] is short for "Coefficient of Variation".

The three-dimensional (3D) response surface plots for $Y_1 \sim Y_5$ against the two experimental factors of DR and HRT are depicted in Fig.17. The plots for $Y_1$, $Y_2$, $Y_4$ and $Y_5$ showed that higher biomass productivities and nutrient removal rates were generally obtained with short HRT, but there was a strong effect of DR (Fig.17a, 17b, 17d and 17e). Considerably lower biomass production and nutrient removal rates could be attained at high DR values and short HRTs. From the $Y_3$ surface plot (Fig.17c) and the counter plot (not shown), it can be seen that $PO_4$-P removal rate was sensitive to higher values of both DR and HRT.

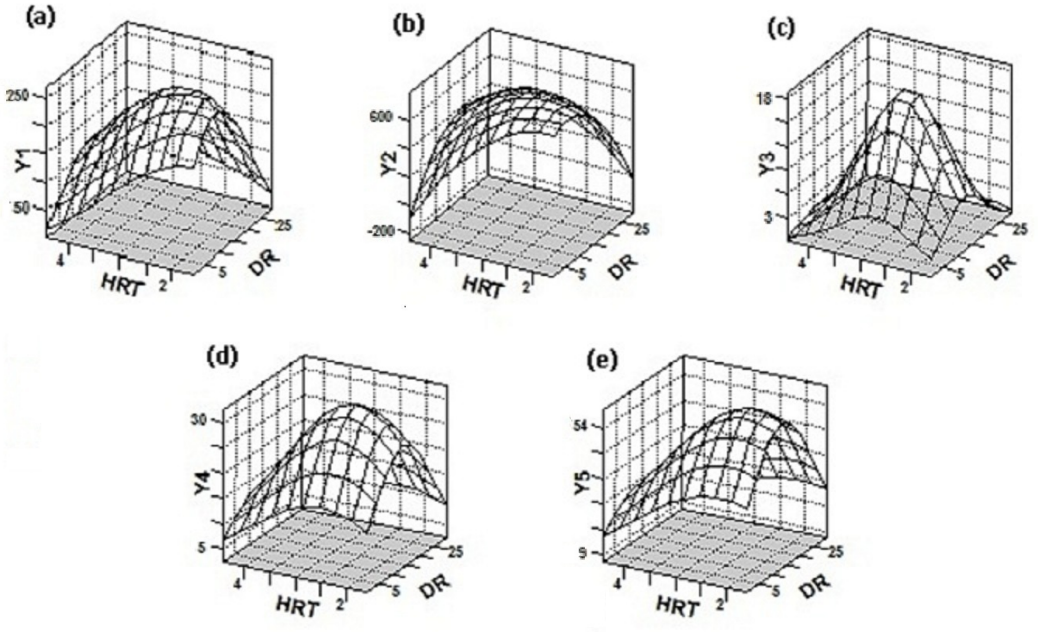


**Figure 17.** Response surface plots for biomass productivity, $Y_1$ (a), COD removal rate, $Y_2$ (b), $PO_4$-P removal rate, $Y_3$ (c), TN removal rate, $Y_4$ (d), and $NH_3$-N removal rate, $Y_5$ (e) as functions of DR and HRT

### 6.3.3 Optimization of DR and HRT in algal mass production and waste nutrient removal

Generally, it is identified that the highest biomass productivity, COD, TN and $NH_3$-N removal rates were obtained at the DR of 8 times and the HRT of 2.26 days. According to the $Y_3$ surface plot (Fig.17c) and the counter plot (not shown), the maximum $PO_4$-P removal rate was observed at the point of 11.4-time DR and 2.66-d HRT. Nevertheless, since the drop of $PO_4$-P removal rate from the apex of the $Y_3$ surface plot (21.80 mg $PO_4$-P $L^{-1}$ $d^{-1}$) to the point with DR of 8 times and HRT of 2.26 d (18.11 mg $PO_4$-P $L^{-1}$ $d^{-1}$) was not very much, the optimal values of DR and HRT in the microalgae – acidogenically digested swine manure system is still determined to be 8 times and 2.26 d, respectively. According to the models, the theoretically predicted response values ($Y_1$ ~ $Y_5$) at the optimal point are algal productivity of 246.76 mg TVSS $L^{-1}$ $d^{-1}$, 738.04 mg COD $L^{-1}$ $d^{-1}$, 18.11 mg $PO_4$-P $L^{-1}$ $d^{-1}$, 33.73 mg TN $L^{-1}$ $d^{-1}$, and 58.96 mg $NH_3$-N $L^{-1}$ $d^{-1}$, respectively.The research presented herein was the first to use CCD for the optimization of culture conditions for both algal mass production and wastewater treatment.

## 6.4 Conclusions

Response surface methodology with CCD was effective in system optimization for algal biomass production using acidogenically digested swine manure in semi-continuous culture. The regression analysis showed that DR, HRT, and the interaction between DR and HRT were significant in the quadric models for algal biomass productivity and

nutrient removal rates. The optimal parameters estimated from the significant second-order quadratic models ($p<0.05$) were 8-fold DR and 2.26-d HRT.

# CHAPTER 7. MODEL APPLICATION AND DEVELOPMENT: SEMI-CONTINUOUS CULTIVATION OF MICROALGAE ON ACIDOGENICALLY DIGESTED SWINE MANURE IN A BENCH-SCALE PHOTOBIOREACTOR


## Abstract

An effective semi-continuous process was developed to grow a locally isolated green microalga *Chlorella* sp. on acidogenically digested swine wastewater under the optimal dilution rate (DR) of swine manure and the optimal hydraulic retention time (HRT), which were investigated in Chapter 6, in bench scale for improved algal biomass production and waste nutrient removal. The cultivating experiment in a bench-scale multi-layer photobioreactor (PBR) with the optimized conditions (($DR_{opt}$ = 8.00 times, $HRT_{opt}$ = 2.26 d) achieved stable algal productivity of 276.18 mg $L^{-1}$ $d^{-1}$ and nutrient removal rates of 751.33 mg COD $L^{-1}$ $d^{-1}$, 20.21 mg $PO_4$-P $L^{-1}$ $d^{-1}$, 38.35 mg TN $L^{-1}$ $d^{-1}$, and 60.39 mg $NH_3$-N $L^{-1}$ $d^{-1}$, which fitted the predictive models well. Moreover, relatively high and stable protein and lipid contents (58.78% and 26.09% of the dry weight, respectively) were observed for the collected algae sample, indicating the suitability of the algal biomass as ideal feedstock for both biofuel and animal feed production.

## 7.1 Introduction

It is potentially practical to cultivate algae with swine manure for algal feedstock production and wastewater treatment (Hu et al., 2012b). According to my previous studies, *Chlorella* sp. (UMN271), locally isolated facultative heterotrophic microalga strain, was capable of growing well on swine manure (Chapter 3); acidogenically digested swine manure was high in volatile fatty acids (VFAs), and enhanced *Chlorella* sp. biomass production and waste nutrient removal (Chapter 5); and algal biomass productivity and nutrient removal rates were considered to be further increased under the predicted optimal culture conditions based on central composite design (CCD) and response surface analysis (Chapter 6).

For the production of microalgae-based biofuel and animal feed in industry, cultivation of specific microalgal species on swine manure should be in large-scale production systems for best productivity and quality. Pilot-scale cultivation of algae for wastewater treatment has been studied in pond systems such as open ponds and high rate algal ponds (HRAPs) since late 1950s (Oswald and Golueke, 1960).Outdoor open ponds are the most common production systems (Chen et al., 2011). They are the most widely used system for large-scale outdoor microalgae cultivation in Southeast Asia, Australia, Middle East for food and medicine supplements during the last few decades, since they are commercially economical, easy to build and operate (Borowitzka, 1993). However, there are many limitations that were widely discussed on outdoor pilot-scale systems, such as large land requirement, low productivity, high evaporation, and contamination by predators (Chen et al., 2011; Craggs et al., 2012).

Min et al. (2011) investigated the growth characteristics of a *Chlorella* strain in concentrated municipal wastewater stream using an innovative pilot-scale semi-open photobioreactor (PBR), and found that the unique PBR system was effective for biomass production coupled with wastewater treatment and could be highly scalable. Although these results were encouraging, it has been unknown whether the system could be extrapolated to the cultivation of microalgae with other types of wastewater with different hydraulic retention times (HRTs).

In light of the above discussion, the next step in the study was to investigate the feasibility of the predicted culture conditions, as reported in previous chapters, for good performance of *Chlorella* sp. in the microalgae-acidogenically digested swine manure system by using a novel bench-scale PBR, which could be utilized for the development of an effective outdoor pilot-scale algae production process using acidongeically digested swine manure.

## 7.2 Materials and Methods

### 7.2.1 Algae strain and seed culture preparation

Alga strain Chlorella sp. (UMN271) was used in the study. The preparation and maintenance of the algal seeds were the same with section 6.2.1.

### 7.2.2 Characteristics of swine manure

The fresh swine manure and the inoculum sludge were collected from the University of Minnesota Southern Research and Outreach Center, Waseca MN. The fresh swine

manure was used as the substrate during the acidogenic digestion in the study, and its characteristics are shown in Table 23. The sludge was anaerobically cultivated for 5 days with 5 g $L^{-1}$ glucose at temperature 38 ±1 °C to get the activated and concentrated inoculum, and then was heat-treated at 80 °C with a water bath (Thermo Fisher Scientific Inc., Waltham, MA) for 30 minutes to kill methanogenic bacteria from the community.

**Table 23**. Characteristics of fresh swine manure

| Parameter | Value |
|---|---|
| pH | 7.58±0.31 |
| TVSS (mg $L^{-1}$) | 2580.01±300.01 |
| Total nitrogen (mg TN $L^{-1}$) | 2031.43±66.19 |
| Ammonia-nitrogen (mg $NH_3$-N $L^{-1}$) | 1602.86±84.72 |
| Orthophosphate-phosphorus (mg $PO_4$-P $L^{-1}$) | 407.43±99.58 |
| COD (mg $L^{-1}$) | 17240±816.66 |
| Total VFAs (mg $L^{-1}$) | 7676.26±576.37 |
| Acetic acid (mg $L^{-1}$) | 4957.08±357.48 |
| Propionic acid (mg $L^{-1}$) | 1612.03±116.13 |
| Butyric acid (mg $L^{-1}$) | 1107.16±105.48 |

### 7.2.3 Acidogenic digester setup and operation

An Erlenmeyer flask with the working volume of 4 L was used as the anaerobic digester. The reactor was operated in semi-continuous mode. At the beginning, 1.0 L concentrated inoculum was added into the reactor containing 3.0 L fresh manure substrate. The mixture was adjusted to approx pH 5.3 with sulfuric acid (4 mol $H_2SO_4$ $L^{-1}$) solution, and was maintained at pH 5.3 ~ 5.6 and 38 ± 1 °C for the acidogenic fermentation for 48 h as described by Hu et al. (2012b). The 2-day batch fermentation was followed by a 10-day

semi-continuous operation with the HRT of 3 days determined in the previous study (Hu et al., 2012b). During the operation, 1.33 L of the mixed liquor was drained from the reactor and was replaced with an equal volume of fresh manure substrate at the beginning of each day. The pH and temperature were kept at 5.3 ~ 5.6 and 38 ± 1 °C, respectively, during the 10-day operation. Samples were taken from the daily effluent and feeding substrate for VFA concentration test. In the study, the daily drained effluent was used in the experiment as described in section 7.2.4.

### 7.2.4 Cultivation of microalgae on acidogenically digested swine manure in a bench-scale multi-layer photobioreactor

The objective of this experiment was to confirm the feasibility of the algae–acidogenically digested swine manure system using the bench-scale photobioreactor with the predicted optimal DR and HRT according to the regression models in Chapter 6. The novel bench-scale photobioreactor system consisted of a proprietary 2-layer reactor and a recycling peristaltic pump (Cole-Parmer Co, Vernon Hills, IL). Due to the pending patent process, a detailed structure and description of the system are omitted here. In the experiment, *Chlorella* sp. seeds were inoculated at approx 0.2 g $L^{-1}$ in the 2-layer photobioreactor containing 4 cm-high unsterilized manure effluents from the 8-fold diluted acidogenic digester. Seven-day batch cultivation followed by 10-day semi-continuous cultivation with the predicted optimal HRT of 2.26 days was operated at 25 ± 2 °C under 100 μmol $m^{-2}s^{-1}$ white fluorescent light on a 16:8 h light/dark cycle. During the incubation period, the culture unit was daily refilled with deionized water to compensate for evaporation. TVSS, pH, COD, $PO_4$-P, TN and $NH_3$-N were assayed daily,

while lipid content, protein content, and the fatty acid profile were analyzed on the harvested algae samples on the last three days of the 10-day semi-continuous cultivation.

### 7.2.5 Analytical methods

#### 7.2.5.1 VFA composition analysis

The manure effluent samples or algal culture samples were centrifuged at 7000 rpm for 10 minutes, and then the liquid phase was passed through a Fisherbrand 0.45 μm syringe filter (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA) to get clear filtrates. The quantitative characterizations of VFAs in the filtrate, including acetic, propionic and butyric acids were measured using an Agilent 7820A gas chromatography with flame ionization detector (GC-FID) according to the method as described in section 4.2.6.3.

#### 7.2.5.2 Algal biomass growth analysis

The daily algal biomass growth was determined in form of total volatile suspended solid (TVSS). The details of the measurement were described in section 3.2.4.1

#### 7.2.5.3 Nutrient analysis

COD, $NH_3$-N, TN and $PO_4$-P were analyzed in accordance with the standard methods (APHA, 1998) and instructions in the Hach DR5000 spectrophotometer manual (Hach, 2008), which was similar with those in section 3.2.4.2

#### 7.2.5.4 Algal lipid content and composition analyses

Total lipid contents and the fatty acid composition of the harvested algal cells were determined using the methods in section 4.2.6.4.

7.2.5.5 Algal protein content analysis

The protein content of the freeze-dried algal biomass was determined from the nitrogen content data evaluated with a CE-440 elemental analyzer (Exeter Analytical Inc., Chelmsford, MA), using the nitrogen-to-protein conversion factor (NTP) of 6.35 (Safi et al., 2012).

## 7.3 Results and discussion

### 7.3.1 Semi-continuous acidogenic fermentation of fresh swine manure

Fresh swine manure was inoculated with concentrated manure sludge in which the methanogenic bacteria were reduced, and were anaerobically incubated for 48 h and then acidogenically fermented in 10-day semi-continuous mode with the HRT of 3 days for the production of VFA-enriched swine manure. As shown in Fig.18, the VFAs production reached the steady state on the $10^{th}$ day of the aciodgenic fermentation, with the VFAs productivity of 2002.25 mg $L^{-1}$ $d^{-1}$ on average. The total VFAs concentration in the acidogenically digested manure effluents from the semi-continuous process was in the range of 12500 ~ 14000 mg $L^{-1}$, which was close to the highest VFAs concentration in the acidogenic, batch fermentation of liquid swine manure (LSM) in our previous report (Hu et al., 2012b), indicating that the semi-continuous, acidogenic fermentation was effective in promoting VFAs production in swine manure.

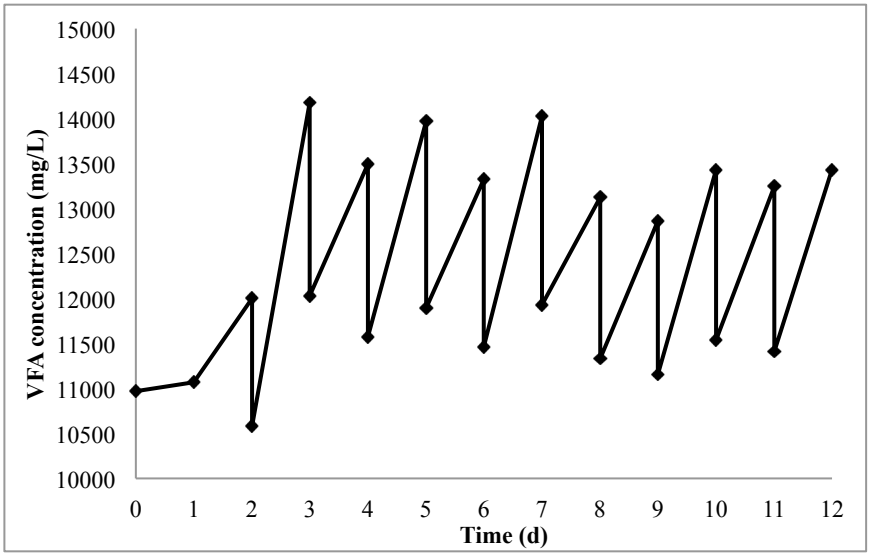


**Figure 18**. Change in total VFAs during acidogenic fermentation of fresh swine manure at 38 ±1 °C during the 2-day batch fermentation followed by a 10-day semi-continuous mode with an HRT of 3 days. The results are average of triplicate determinations

It is observed from Fig.18 that the semi-continuous process had a noticeably higher VFAs yield in the swine manure than the batch mode (12000 mg $L^{-1}$ on day 2 before manure exchange), which was likely due to a slight rise in the reactor pH when fresh manure was fed into the reactor during the semi-continuous process. Rogers (1986) found that the accumulation of organic acids could lead to a drop in the digester pH, and the switch from acidogenesis (a VFAs-production metabolic pathway) to solventogenesis (a ketone-production metabolic pathway) would happen at low pH values so that the acidogenic fermentation process would be inhibited. The moderate-to-high yields obtained with the batch-to-semi-continuous process used in this work demonstrates the advantage of acidogenic, semi-continuous fermentation in the production of VFA-enriched swine manure. The daily drained effluent from the semi-continuous digestion system was used for the cultivation of *Chlorella* sp. in a bench-scale photobioreactor.

### 7.3.2 Applicability of the optimal conditions for fast algae growth and nutrient removal using a bench-scale 2-layer PBR

The predictive optima ($DR_{opt}$ = 8.00 times, $HRT_{opt}$ = 2.26 d), determined in Chapter 6, were utilized for *Chlorella* sp. cultivation on swine manure daily drained effluent obtained from the acidogenic digester as mentioned in above section in a bench-scale 2-layer PBR containing 17 L (4-cm water depth for each layer).

7.3.2.1 Algal growth and nutrient removal

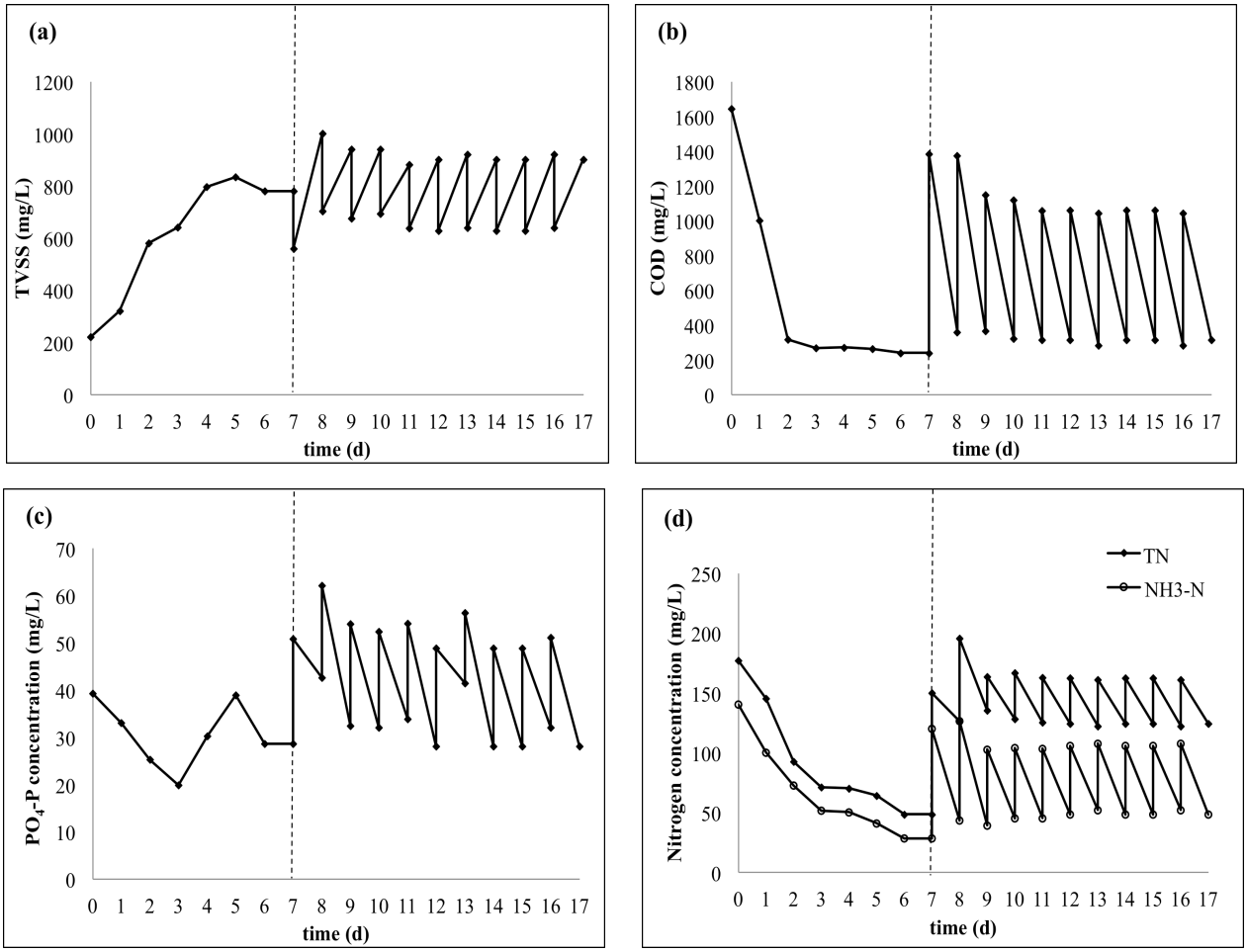

**Figure 19.** Time courses of algal cell density as TVSS (a), COD concentration (b), $PO_4$-P concentration (c), TN and $NH_3$-N concentrations (d) during the 17-day cultivation of *Chlorella* sp. on 8-fold diluted, acidogenically digested swine manure. The vertical dotted line represents the interface between the batch and semi-continuous processes.

The process parameters used in this experiment demonstrated a good performance in growing algae and removing nutrients from the acidogenically digested swine manure. The algal cell density as TVSS, nutrient concentrations including COD, $PO_4$-P, TN and $NH_3$-N in the manure culture, and the culture pH were measured daily during the 17-day cultivation, and the data are shown in Fig.19 and Fig.20. At the end of the period I (7-day batch mode), *Chlorella* sp. reached stationary phase with the cell density of 780 mg $L^{-1}$ (Fig.19a), and the nutrient levels of 238 mg COD $L^{-1}$ $d^{-1}$, 28.6 mg $PO_4$-P $L^{-1}$ $d^{-1}$, 48.3 mg TN $L^{-1}$ $d^{-1}$ and 28.2 mg $NH_3$-N $L^{-1}$ $d^{-1}$(Fig.19b ~ 19d). As shown in Table 24, the cultivation performance achieved stable algal productivity of 276.18 mg $L^{-1}$ $d^{-1}$ and nutrient removal rates of 751.33 mg COD $L^{-1}$ $d^{-1}$, 20.21 mg $PO_4$-P $L^{-1}$ $d^{-1}$, 38.35 mg TN $L^{-1}$ $d^{-1}$, and 60.39 mg $NH_3$-N $L^{-1}$ $d^{-1}$ during period II (10-day semi-continuous mode). It is noticed that TN removal rate was lower than $NH_3$-N removal rate, which was probably due to the fluxes of dissolved organic nitrogen (DON) from the suspended manure particles by the algae strain. Tyler et al. (2001) found that the opportunistic green macroalgae *Ulva lactuca* leaked DON from sediment into water column during its active growth in shallow lagoon in Hog Island Bay, Northampton County, VA. As shown in Table 24, the experimental values were close to the predicted response variables of the quadric models in section 6.3, which was very good for the goodness of fit. The result that the experimental values were slightly higher than the theoretically predicted values

can be associated to the ammonia volatilization from the 2-layer photobioreactor and, therefore for the reduced ammonia content in the culture medium during algae growth. Although ammonia is a good nitrogen source for algae growth, Azov and Goldman (1982) found that the growths of fresh and marine algae *Scenedesmus obliquus*, *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* were all suppressed in intensive cultures with high loadings of ammonia because of the ammonia inhibition on the short-term algal photosynthesis. Chen et al. (2012), studying the optimal pretreated anaerobically digested liquid dairy manure effluent for the cultivation of a selected *Chlorella* strain in a pilot-scale semi-continuous fed raceway pond, observed that the algal growth rate (based on TVSS) significantly decreased from 0.0538 $d^{-1}$ in nutrient loading of 200 mg TN $L^{-1}$ media to approx 0.045 $d^{-1}$ in higher nutrient loading of 300 and 400 mg TN $L^{-1}$ media. Nevertheless, we believe that the removal of $NH_3$-N in the system was mainly attributed to the nitrogen uptake by *Chlorella* sp. other than ammonia volatilization, since the pH of the culture during the course of the growth experiment (Fig.20) was always below pH 9.3 which was reported as $pK_a$ of $NH_4^+/NH_3$ at room temperature (Ferrara and Avci, 1982). Moreover, the time course of pH values in Fig.20 demonstrated that the fed VFA-enriched manure medium could not only be used as a nutrient source, but also had a neutralizing effect against the culture alkalization which was mainly caused by the consumption of dissolved $CO_2$ in the culture during algal photosynthesis (Shiraiwa et al., 1993).

**Table 24**. Theoretically predicted and experimental values for algal biomass productivity, removal rates of COD, $PO_4$-P, TN and $NH_3$-N during the semi-continuous process

| | Biomass productivity ($mg\ L^{-1}\ d^{-1}$) | Nutrient removal rate ($mg\ L^{-1}\ d^{-1}$) | | | |
|---|---|---|---|---|---|
| | | COD | $PO_4$-P | TN | $NH_3$-N |
| Predictive | 246.76 | 738.04 | 18.11 | 33.73 | 58.96 |
| Experimental | 276.18 | 751.33 | 20.21 | 38.35 | 60.39 |

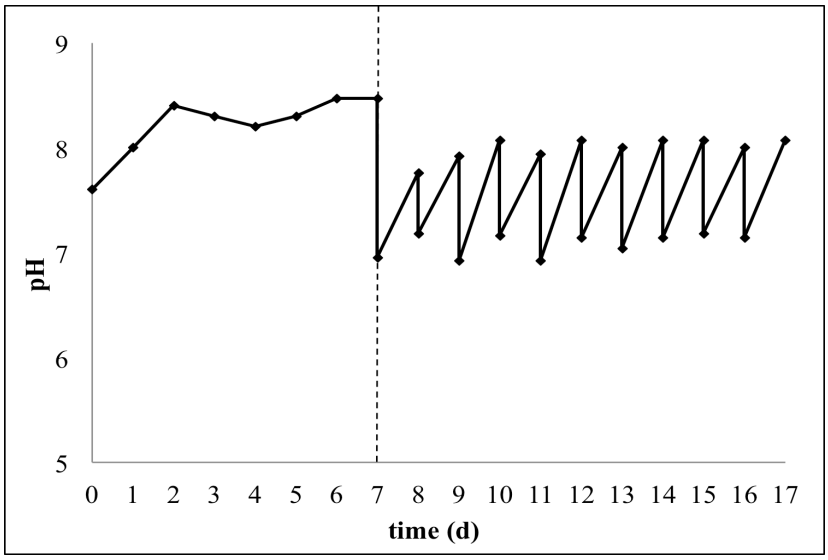


**Figure 20.** pH changes during the 17-day cultivation of *Chlorella* sp. The vertical dotted line represents the interface between the batch and semi-continuous periods

7.3.2.2 Nutritional composition of harvested algae

The chemical compositions of algal biomass collected from the culture effluents on the last three days of the semi-continuous process were measured using Folch extraction, elemental analysis, and GC-MS analysis to elucidate the potential of *Chlorella* sp. as algal feedstock for biofuel and feed production.

As shown in Table 25a, the lipid content (on a dry matter basis) of *Chlorella* sp grown in the semi-continuous system was around 26.09% of dry biomass. According to our previous reports, the alga strain *Chlorella* sp. had similar lipid contents to the one in the study when it was grown on concentrated municipal wastewater (CMW) and 20-fold diluted, conventionally digested swine manure (DSM), which were 27.50% and 23±4.32%, respectively (Zhou et al., 2011; Zhou et al., 2012b). These experimental findings are in agreement with those reported by Woert et al. (2009) who used the green algae community to treat municipal wastewater, 10% and 25% diluted dairy manure, and obtained the algal lipid content as 9.0 ~ 11.3%, 8~14%, and 10~15%, respectively. Therefore, it is considered that the lipid contents of the locally isolated *Chlorella* sp. grown on wastewaters are relatively stable and high, in the range of 25 ~ 30%. According to the data in the study, the lipid productivity of the microalgae-acidogenically digested swine manure system (3.63 g $m^{-2}$ $d^{-1}$) corresponds to about $1.00\times10^4$ L $ha^{-1}$ $y^{-1}$. Though our algal lipid productivity is much lower than the $5.87\times10^4$ ~ $1.37\times10^5$ L $ha^{-1}$ $y^{-1}$ reported for algal oil yield from the artificial media, it is 16.74 times greater than that for soybean oil production (598.6 L $ha^{-1}$ $y^{-1}$; Pradhan et al., 2011), and thus, presents an exciting possibility as a low-cost feedstock for biofuel production.

Around 37.03% (on a dry matter basis) of total lipids in the frozen-dried algal biomass were free fatty acids and fatty acids derived from triacylglycerol and phospholipids (Table 25b). GC-MS analysis showed that the fatty acids in the algal cells were mainly composed of saturated C14 ~ C18 fatty acids and monounsaturated C16 ~ C18 fatty acids (62.83±8.96% and 31.80±8.32% of total fatty acid weight, respectively) with C18:0 and

C18:1 as the major compounds (22.46±2.81% and 19.00±1.62%, respectively). It is considered that transesterification of the fatty acid composition in our study could produce high-quality biodiesel. Xu et al. (2006) found that the biodiesel from algal oil of *Chlorella protothecoides* was abundantly composed of 18 carbon acid methyl esters, and had comparable physical and fuel properties to diesel fuel. Moreover, linolenic acid (C18:3), which should be lower than 12% for a quality biodiesel according to EN14214 standard (2004), was not detected in the fatty acid composition in our study.

The *Chlorella* sp. cells or the remaining biomass fraction after oil extraction from algae can be used as a high protein feed for livestock to further help offset costs of algal mass production. As reported in Table 25a, the crude protein content of the algal strain was 58.78±1.05%, which was comparably high among various microalgal species (6~71% of dry biomass; Becker, 2007). The use of microalgae as animal feed, such as in poultry farms and aquaculture, has been investigated for many years, and the research on waste-grown algae as food is more recent (Marimura and Tamiya, 1954). According to Cook et al. (1963), the protein quality of sewage and organic wastes-grown green algae *Scenedesmus quadricauda* and *Chlorella* spp. incorporated with varying amounts of powdered skim milk, wheat, oat cereals was not inferior to that of milk protein, and the algae-milk-cereal mixture was demonstrated to have no negative effect on the 21-day-old weanling rats. Therefore, it is predicted that the acidogenically digested swine manure-grown algal biomass could be a valuable feed substitute for conventional protein sources.

**Table 25** Chemical composition of *Chlorella* sp.

| (a) Chemical composition of *Chlorella* sp. on a dry matter basis (%) |
|---|

| Composition | Content |
|---|---|
| Protein | 58.78±1.05 |
| Lipids | 26.09±1.13 |
| Others (e.g. carbohydrates and nucleic acids) | 15.13±0.69 |

(b) Fatty acid profile derived from triacylglycerol, phospholipid and free fatty acids in *Chlorella* sp. collected from the semi-continuous microalgae-acidogenically digested swine manure system

| Name of the compounds | Content |
|---|---|
| Total fatty acid/dry weight (%) | 9.66±1.83 |
| Saturated fatty acids (% of total fatty acids) | 62.83±8.96 |
| C14:0 | 12.34±1.69 |
| C15:0 | 5.51±0.45 |
| C16:0 | 17.41±1.57 |
| C18:0 | 19.00±1.62 |
| C20:0 | 8.57±1.53 |
| Monounsaturated fatty acids (% total fatty acids) | 31.80±8.32 |
| C16:1 | 9.34±1.86 |
| C18:1 | 22.46±2.81 |
| Polyunsaturated fatty acids (% total fatty acids) | 1.53±0.08 |
| C20:2 | 1.53±0.08 |

Although the bench-scale culture system developed in our study is considered to be low-cost and effective for algal biomass production and nutrient removal from acidogenically digested swine manure simultaneously, and has the potential for commercialization, we cannot predict the feasibility of the system in an out-door, large-scale operation. Problems such as contamination by zooplankton or other adventitious organisms, evaporation, and temperature volatility could potentially prevent the system from being a

practical strategy for the production of algae-based biofuel or feed (Scott et al., 2010). Therefore, the study needs to be confirmed in a pilot scale before being commercially viable.

### 7.4 Conclusions

It is concluded that by semi-continuously cultivating *Chlorella* sp. on acidogenically digested swine wastewater under the optimal conditions ($DR_{opt}$ = 8.00 times, $HRT_{opt}$ = 2.26 d) using a bench-scale multi-layer photobioreactor, stable mass productivity and nutrient removal rates slightly higher than the predicted values were obtained, and the algal biomass produced in the system can be a good feedstock for biofuel and feed production.

# CHAPTER 8. CONCLUSIONS AND FUTURE WORK

## 8.1 Introduction

Microalgae are photosynthetic microorganisms that convert sunlight, water and carbon dioxide ($CO_2$) to algal lipids, proteins and carbohydrates, which can be used to produce algae-based biofuels or animal feed using existing technology. The potential of microalgae as feedstock for renewable fuels and animal feed production has already been well known (Hu et al., 2012a). As early as 1950s, people suggested that mass culture of algae would help to overcome global energy and protein shortages (Spoehr and Milner, 1949; Burlew, 1953). However, microalgae production with synthetic media is expensive due to the high cost of land, the large requirement of water, and the high price of nutrients (Maira Freire, 2012). Considering the fact that the rapid growth of pork production in the States has produced more swine manure than crops or forage on the croplands could assimilate (Hunt and Vanotti, 2001), cultivating algae on nutrients in swine manure effluents presents an alternative to the current practices of wastewater management and land application, as well as algae-based biofuel and animal feed production (Kebede-Westhead et al., 2006). There are considerable literatures on algae cultivation with raw and/or anaerobically digested swine manure effluent in different types of systems (Ayala and Bravo, 1984; Goh, 1986; Costa et al., 2000; Olguín et al., 2001; Olguín, 2003; Jimenez-Perez et al., 2004).

However, the integration of algal biomass production and swine manure treatment is still far from being economical due to the lack of algal trains which have high growth rates and nutrient removal rates on swine manure. According to previous research, algae

biomass productivities on diluted swine manure ranged from 0.68 to 27.7 g $m^{-2}$ $d^{-1}$ with very high variation, which were equal or lower than the regular biomass productivity of 10~30 g $m^{-2}$ $d^{-1}$ on municipal wastewater (Banat et al., 1990; Su et al., 2011), and of 2.5~ 25.65 g $m^{-2}$ $d^{-1}$ on diluted dairy manure effluents (Mulbry et al., 2008; Johnson and Wen, 2010).

The hypothesis was that some microalgae strains could effectively assimilate inorganic nutrients and organic substances in swine manure through mixotrophic growth for the production of algal biomass with high lipid or protein contents. In order to verified the hypothesis, five step-up researches, including algae strain screening, determination of swine manure compositional limitation, modification of anaerobic digestion, culture condition optimization in lab-scale flasks, and cultivating system development in bench-scale photobioreactor, were conducted in the dissertation.

## 8.2 Summary of the dissertation

Chapter 1 of the dissertation generally described the background and significance of the study, followed by a statement of the research objectives. The overall objective of this study was to develop efficient algal mass production strategy by employing potential microalgae strains on swine manure with suitable nutrient composition and culture conditions.

In Chapter 2, previous reports on algae-based biofuel and animal feed production were reviewed. It has been found that microalgal biomass does have the potential to replace

current feedstock crops but is faced with high-cost production problem. The integration of algal biomass production with swine manure treatment has been considered to be potentially practical and economical. However, according to previous reports, only a small number of commercial photoautotrophic algal strains were most commonly used, and the algae biomass productivities on swine manure were relatively low. Literature review on the related metabolism principals and fundamentals were made in the chapter. Finally, it was concluded in the chapter that bioprospecting for mixotrophic microalgae strains adapt well on swine manure and modification of nutrient composition and culture conditions were needed to develop an efficient microalgae-swine manure system.

In Chapter 3, research was focused on screening for facultative heterotrophic microalgae strains which had the ability of fast growth and waste nutrient recovery in diluted swine manure. By using multi-step screening strategy, 19 candidate strains were found to be facultative heterotrophic, manure-tolerant, and relatively robust in the algae storage including 98 UTEX strains and 50 indigenous strains. Among the 19 candidate strains, 2 locally isolated microalgae strains, namely UMN271 and UMN266, were chosen as the top-two strains because of their obviously high growth rates (1.051 and 0.803 $d^{-1}$, respectively) during the 7-day batch cultivation on sterilized 20-fold diluted undigested swine manure. By using morphological observation and genetic identification, the two strains were identified as *Chlorella* sp. and *Hindakia* sp., respectively.

In Chapter 4, the limitation of using anaerobically decomposed swine manure as a nutritional medium for algae cultivation was assessed. By 7-day batch cultivation of *Chlorella* sp. (UMN271) and *Hindakia* sp. (UMN266) on 20-fold diluted anaerobically

digested swine manure with and without exogenous trace elemental stock, including B, Mn, Zn, Cu, Co and Mo, it was found that the growth of the candidate microalgae strains were independent of trace element concentrations in highly diluted swine manure. However, the algal growth and nutrient removal rates were significantly improved when the initial COD content in diluted manure media increased from 460 mg $L^{-1}$ to 1968 mg $L^{-1}$, indicating that carbon dearth in diluted swine manure limited algal growth. Another experiment was operated to determine the limiting carbon substances in swine manure, and demonstrated that *Chlorella* sp. (UMN271) was able to utilize the exogenous acetic, propionic and butyric acids, the main water-soluble organic carbon compounds in digested swine manure, for algal growth, lipid production, and nutrient removal from the swine wastewater. Therefore, it was considered that algal growth on highly diluted swine manure was limited by the deficiency of volatile fatty acids (VFAs).

In Chapter 5, swine manure anaerobic digestion process was modified to obtain VFA-enriched swine manure for the enhanced algal biomass production and wastewater treatment. In the study, I used acidic and heat pretreatments to suppress methanogenesis in manure sludge. By using the modified anaerobic digestion protocol, VFA concentrations in the swine manure effluent were successfully promoted. The obtained acidogenically digested manure effluents supported the growth of *Chlorella* sp. (UMN271) in a 5-day batch experiment, with higher algal growth rates and fatty acid contents in comparison with those on the conventionally decomposed swine manure. Also, GC-MS analysis showed that the harvested algae samples could be used as raw material for good-quality biodiesel and animal feed production without restrictions.

In Chapter 6, two key operational parameters in the microalgae-swine manure system, namely wastewater dilution rate (DR) and hydraulic retention time (HRT), were optimized using central composite design (CCD) for the improvement of algal biomass production and nutrient removal from acidogenically digested swine manure. The CCD experiments were effective to get the statistically valid quadric models for the response variables of algal biomass productivity and nutrient removal rates including COD, $PO_4$-P, TN and $NH_3$-N. The models showed that both DR and HRT had significant influences on each response variables in the system, while the interaction between DR and HRT had significance on algal biomass productivity and COD removal rate only. Generally, it was predicted that the highest biomass productivity and nutrient removal rates should be obtained at the point of 8-time DR and 2.26-d HRT.

In Chapter 7, the optimal DR and HRT values were applied to a batch-to-semi continuous microalgae-acidogenically digested swine manure system in a bench-scale multi-layer photobioreactor (PBR) for the effective algal mass production and waste nutrient removal. During the 17-day cultivation, *Chlorella* sp. achieved stable algal productivity of 276.18 mg $L^{-1}$ $d^{-1}$ and nutrient removal rates of 751.33 mg COD $L^{-1}$ $d^{-1}$, 20.21 mg $PO_4$-P $L^{-1}$ $d^{-1}$, 38.35 mg TN $L^{-1}$ $d^{-1}$, and 60.39 mg $NH_3$-N $L^{-1}$ $d^{-1}$. The results fitted the predictive models well. Moreover, the harvested algae samples had relatively high and stable protein and lipid contents (58.78% and 26.09% of the dry weight, respectively), indicating the good quality of the algal biomass as alternative feedstock for both biofuel and animal feed production.

## 8.3 Future work

In light of above findings, the process of algae cultivation on acidogenically digested swine manure for simultaneous algal biomass production and wastewater treatment has great potential to become cost effective and thus, play a significant role in meeting the alternative fuel and feed objectives, as well as swine manure disposal objective, in the future. For the commercialization of the process, it is necessary to investigate the feasibility of the process in pilot-scale and then commercial-scale. Also, the developments of low-cost harvesting method and high-efficient refinery process are very important for the substantial economic improvement of the integrated process.

### 8.3.1 Larger-scale operation

Currently, large-scale cultivation of microalgae has been investigated at the outdoor pilot scale either using open ponds or enclosed photobioreactors (PBRs; Johnson and Wen, 2010). The former (see Fig.21a) is made of a closed loop recirculation channel, in which the mixing and circulation are made by paddlewheels (Chisti, 2007a). A closed PBR (see Fig.21b) is made of a reservoir containing microalgal broth and an array of narrow transparent tubes which are oriented for efficient solar collection (Carvalho et al., 2006). Open ponds are simpler and less expensive on the capital and operating costs than closed PBRs, and the operation and engineering of open ponds has been of wide development. The world's largest open-pond system is a 108-acre Spirulina farm built in pure California desert by Earthrise® (www.earthrise.com). However, closed PBRs generally have higher biomass productivities compared with open ponds because of the maximal illumination collection as well as the reduced water evaporation and biomass

sedimentation (Pulz, 2001; Camacho et al., 2007; Tredici, 2002). It is estimated that the volumetric biomass productivity of photosynthetic microalgae in PBRs could be more than 13 times higher than that in open-pond systems (Chisti, 2007a). The worldwide largest closed algae production plant is an 85000 L PBR facility which was built up in 2011 by IGV GmbH Postdam in Spain (IGV-GmbH, 2011).

Researchers do not dwell on the achievements but on the way to develop more efficient system, because, up to now, the operation of extensive experiments on algae cultivation is supported by private and/or public funds but is far from being economical. It is reported that construction of the world's first commercial demonstration algae-to-energy facility consisting of 300-acre algae cultivation ponds has been started by Sapphire Energy, Inc., since Jun 1, 2011 (Sapphire, 2012). The goal of the project is to practically produce 1.5 million gallons of algae-based crude oil per year.

Mixotrophic cultivation of *Chlorella* sp. (UMN271) biomass can be performed on unexploited lands using freely available sunlight with variable light intensity, thus avoiding competition for limited farmlands and extra expense on light energy. It is considered that the system has great potential to produce algae-based biofuel and animal feed in pilot scale and then demonstration scale, so that the national needs for 1$^{st}$-generation fuel and animal feeds could be significantly reduced.

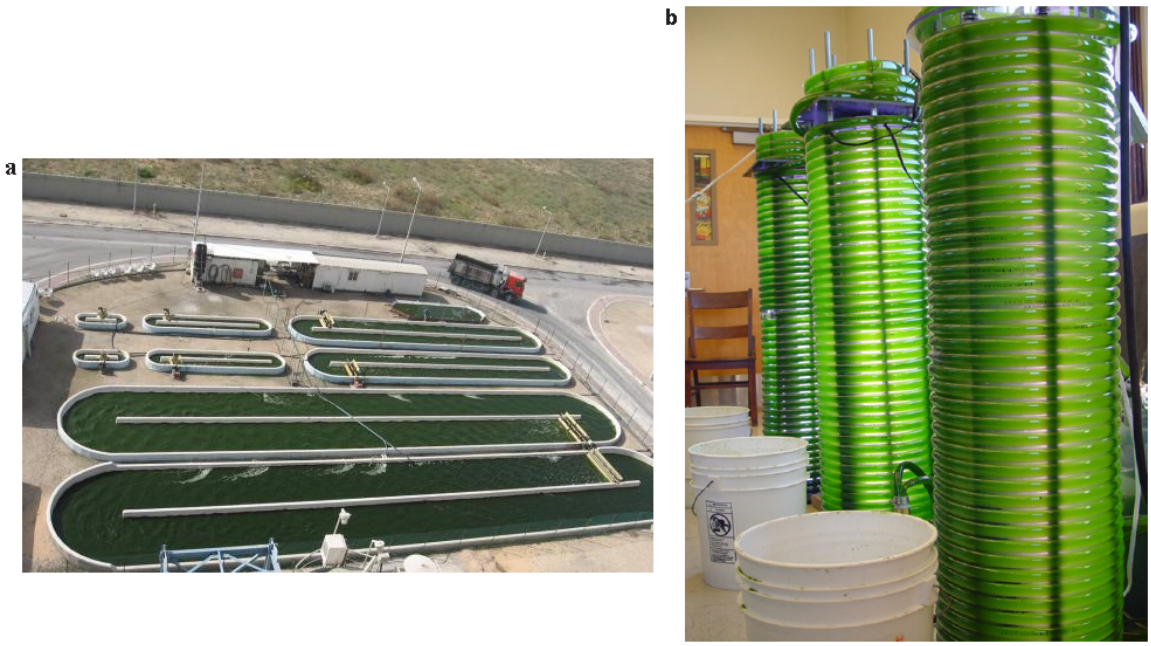


**Figure** 21.Images of an open pond system (a; Seambiotic Co, 2010) and a lab-scale closed PBR (b; Zhou et al., 2012c)

### 8.3.2 Microalgal harvesting technologies

Cost-effective biomass harvesting is important for the development of algal industry. US Department of Energy (DOE) concluded that high harvest cost was a key limitation for algal biofuel production in its final report for the aquatic species program conducted through 1980s ~1990s (Sheehan et al., 1998). Thus, the development of efficient harvesting techniques is of great interest.

The major techniques presently applied in algae harvesting process are centrifugation, flocculation, flotation, sedimentation, filtration, and electrophoresis methods (Chen et al., 2011). Centrifugation is the most commonly used method because of the simple operation, but it is time consuming and costly when processing a large volume of algal cultures (Grima et al., 2003). Flocculating algal cells with inorganic coagulants (i.e.

aluminum sulfate) and/or organic flocculants (i.e. chitosan, cationic starch) for the solid-liquid separation is applicable to treat large quantities of microalgae cultures, but many flocculants are highly sensitive to the environmental factors, such as culture pH, microalgal species, and algal biomass concentrations, and are hard to be removed from the end products after the settling which can affect the application of the harvested algae slurry (Uduman et al., 2010; Edzwald, 1993; Pushparaj et al., 1993). In flotation process, microalgae-liquid separation happens on the cultures surface due to gas bubbles which can attach algal cells and carry them to the liquid surface; in sedimentation process, cell-culture separation depend on gravity settling of the microalgal particles (Yoon and Luttrell, 1989; Brennan and Owende, 2010). Both of them are gravity separation processes, but the former one is considered to be more beneficial and effective (Chen et al., 1998). Filtration involves introducing the algal suspensions through a screen filter, such as microstrainer and vibrating filter. The method has several advantages, such as easy operation, high filtration ratios, physical property maintenance of the collected microalgae, etc (Petrusevski et al., 1995). However, large-scale harvesting using filtration is limited, because of the high cost on screen filter replacement and pumping (Chen et al., 2011). The electrophoresis method is a novel approach to move algae cells out of the solution without the addition of chemicals. In the method, microalgal flocs is adhered by $H^+$ during water electrolysis to form charged algae, and then is forced to move in an electric field (Mollah et al., 2004). The method has several benefits, including energy and cost effectiveness, environmental compatibility, and safety, and several shortages, such as intensive increase of temperature and fouling of the cathodes.

More efficient and economical harvesting technology should be developed to enhance the commercial viability of microalgae industry. Understanding the principles of each existing harvesting techniques and characteristics of algal cells essentially help the development of efficient harvesting methods. Modification and the combination of present technologies are of great potential, such as the investigation of novel filter membrane (Rossignol 1999) and combination of flocculation and air flotation (Uduman et al., 2010).

### 8.3.3 Biorefinery

Algal biorefinery is a conceptual model for the co-production of a spectrum of algae-based biofuel, chemicals, heat, and power from algal biomass feedstock, and thus could make microalgae industry be more economically viable than a single product-based scheme (Fig.22).

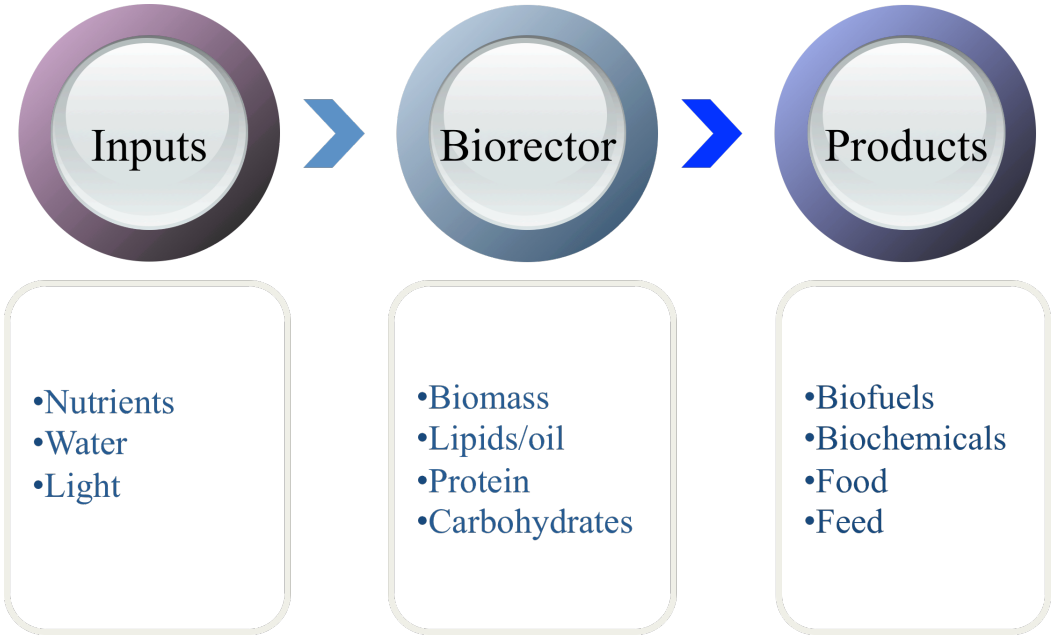


**Figure 22** An algal biorefinery process

There are many techniques in a biorefinery process. They include mechanical pretreatment, heating treatment, chemical and/or enzymatic degradation of cell wall, fermentation, isolation/purification, conversion, etc. (Smith and Consultancy, 2007). The total chain of processing events is always tailor-made and optimized for a given biomass source and the applications aimed at. Biorefineries have been operated on crops (i.e corn and soybean) in several countries, such as Germany, Canada, and the States, for the production and biofuels and other products (Chisti, 2007). For microalgal biomass, the first steps in the total biorefinery process differ strongly from those for higher plant-based materials. Since no lignin or hemicellulose presents, mechanical pretreatment of the crude materials is not necessary. But many algae have cell walls that need to be broken down to allow efficient isolation of compounds of interest. In the later stages where individual compounds are isolated and converted, processes are similar with those for higher plants. For example, the isolated triglycerides (TAGs) and free fatty acids are converted to biodiesel through acid-, alkali-, or lipase enzyme-catalyzed transesterification (Meher et al., 2006); the nutritional compounds in algal cells are converted to marine silage (MS) by lactic acid fermentation (Uchida and Miyoshi, 2010).

In my study, the algal biomass of *Chlorella* sp. (UMN271) harvested from the microalgae-acidogenically digested swine manure system contains around 59% protein, 26% lipids, and 15% other compounds. It is feasible to extract protein and other high-value products such as, pigments, DHA, and/or EPA. Some of the residual biomass after lipid extraction for biofuel production may be used to produce methane by anaerobic digestion, for generating the electrical power necessary for running the microalgal

biomass production facility (Chen et al., 2009; Chisti, 2007), or be collected and processed to form high protein livestock feed. Development of new processes, design of the system, and life cycle analysis are necessary for the development and implementation of biorefinery effectively useful for *Chlorella* sp..

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