Investigation of microalgae cultivation and anaerobic codigestion of algae and sewage sludge for wastewater treatment facilities

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INVESTIGATION OF MICROALGAE CULTIVATION AND ANAEROBIC CO-
DIGESTION OF ALGAE AND SEWAGE SLUDGE FOR WASTEWATER
TREATMENT FACILITIES

A Dissertation Presented
by
MENG WANG

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2013

Department of Civil and Environmental Engineering
INVESTIGATION OF MICROALGAE CULTIVATION AND ANAEROBIC CO-DIGESTION OF ALGAE AND SEWAGE SLUDGE FOR WASTEWATER TREATMENT FACILITIES

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by

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To my loving parents and my husband
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Thank you all!
ABSTRACT

INVESTIGATION OF MICROALGAE CULTIVATION AND ANAEROBIC CO-
DIGESTION OF ALGAE AND SEWAGE SLUDGE FOR WASTEWATER
TREATMENT FACILITIES

MAY 2013

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The main goals of this research are to investigate the anaerobic digestibility of
algae and to investigate the effects of growth media on the growth rates, nutrient removal
kinetics, and extracellular polymeric substances (EPS) characteristics of wild type green
algae. Anaerobic co-digestion of algae with sewage sludge is proposed to improve the
digestibility of algae. It is hypothesized that the addition of sewage sludge improves the
hydrolysis rate of algae, which is often the rate-limiting step for anaerobic digestion. It is
also hypothesized that the composition and concentration of nutrients in growth media
will affect the kinetics of nutrient removal and the content of EPS, which will influence
algae flocculation and subsequent anaerobic digestion.

In this research, algae collected from a local wastewater treatment plant were
cultivated in synthetic medium, primary wastewater effluent and pure or diluted
anaerobic sludge centrate. Light cycles and the level of CO₂ addition were varied at
different stages of cultivation for nutrient removal and physiochemical properties of algae.
Harvested algae were then anaerobically co-digested with varying proportions of sewage sludge under mesophilic condition.

Results showed that when algae were digested alone (i.e. no sludge addition) with a small amount of seed sludge, algae were poorly digested. When algae were co-digested with sewage sludge, the gas yield was improved and the gas phase (CH₄ generation) was reached faster. The biogas yield of algae increased to a comparable level to that of digestion of waste sludge when 44\% (by VS) of seed sludge was inoculated for digestion. The addition of sewage sludge improved the hydrolysis rate and the overall digestibility of algae. Algae grown in primary effluent, which had a balanced N/P ratio showed a higher nutrient removal efficiency. The P-limitation in sludge centrate led to lower nutrient removal efficiency and higher EPS production compared to algae grown in primary effluent, indicating that sludge centrate was a harsher medium for algae growth.

In conclusion, microalgae can grow in primary effluent and anaerobic sludge centrate for nutrient removal. Anaerobic co-digestion of algae with waste sludge was strongly recommended to enhance the biogas generation.
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CHAPTER 1

INTRODUCTION

1.1 Problem statement and hypotheses

High-energy requirement and greenhouse gas production have led to challenges in current methods of wastewater treatment (WWT). Microalgae-based WWT has gained interest these days because of their high growth rate, absorption of CO₂ during photosynthesis and ability to remove nutrients from different sources of wastewater (Park et al., 2010; Wang et al., 2010a; Yuan et al., 2012). Algal biomass harvested from cultivation can then be used as a bioenergy feedstock. Thus, algae cultivation at a wastewater treatment plant (WWTP) could be an economic method to simultaneously achieve nutrient removal, bioenergy generation and the reduction of greenhouse gas production.

Bioenergy can be achieved by biodiesel production from microalgae. The oil yield from algae can reach 1000-6500 gallons/acre/yr, which is much higher than that gained from soil plants (such as soybean, oil palm or sunflowers), in the range of 48-635 gallons/acre/yr (Chisti, 2007; Wigmosta et al., 2011). However, the high cost of lipid extraction and the high amount of unsaturated fatty acid in biodiesels from algae hinder large scale application of microalgae for biodiesel generation (Chisti, 2007; Sialve et al., 2009; Singh et al., 2011).

Anaerobic digestion of algae could be another straightforward and feasible method for energy recovery. Sialve et al. (2009) recommended that anaerobic digestion was a suitable method for bioenergy generation when the lipid content of algae was less than 40%. Extensive research has been conducted to increase the biomass productivity
and further energy production by anaerobic digestion (Packer, 2009; Park et al., 2011b; Yuan et al., 2011). However, the low biogas yield from anaerobic digestion of algae has reduced the benefit of gaining energy by digesting algae and, thus, methods to improve the efficiency of gas generation from anaerobic digestion of algae has become an important issue in the field.

Anaerobic digestion of algae showed a lower gas yield compared to sewage sludge due to the thick cell wall and high protein content in algal biomass (Golueke et al., 1957; Yen & Brune, 2007). Co-digestion of algae with sewage sludge in an anaerobic digester at a WWTP could be a possible method to increase biogas yield from algae. It is hypothesized that high concentrations of bacteria in sludge will enhance the hydrolysis rate of algae, presumed to be the rate limiting step for algal anaerobic digestion, thus, improving the overall digestibility of microalgae. In order to verify this hypothesis, batch anaerobic digestion of algae with different proportions of sewage sludge was performed in this research. Digestion of sewage sludge and algae as sole feedstocks was also performed to compare with algae-sludge co-digestion.

Various strategies have been proposed to incorporate bioenergy generation and greenhouse gas mitigation into wastewater treatment, and one of the strategies that includes cultivation and anaerobic digestion of algae is described in Figure 1.1 (Rusten and Sahu, 2011). In this system, centrate from dewatered anaerobic sludge and CO₂ from a combined heat and power unit (CHP) are recycled for algae cultivation; harvested algae will be digested in an anaerobic digester for bioenergy recovery. Instead of returning sludge centrate back to the headwork of the activated sludge process, it is used for algae cultivation in a photobioreactor (PBR). The large amount of N and P released from
anaerobic digestion can be fixed in algal biomass and could be reused in the proposed process (Figure 1.1).

![Diagram of algae digestion process](image)

**Figure 1.1** Integration of algae cultivation for nutrient removal and bioenergy generation into existing wastewater treatment system

In spite of the ability to mitigate nutrient and greenhouse gases by algae, there are some concerns about successful application of algae-based wastewater treatment. The existence of bacteria in wastewater along with other environmental factors, such as light supply, inorganic carbon sources, and nutrients, will affect the metabolism of algae which will in turn lead to different physiological and morphological characteristics. These will further affect downstream algae treatment process such as harvesting and digestion. In this regard, it is hypothesized that extracellular polymeric substance (EPS) could be an indicator of the metabolism and physiology changes of algae. EPS of sludge have been...
shown to be closely related to bioflocculation, dewatering, and digestibility (Park et al., 2006; Subramanian et al., 2010; Yang & Li, 2009; Yu et al., 2008). Thus, it is reasonable to assume that the EPS in algal biomass are also related to algae growth and bioflocculation, which will affect algae harvesting and their anaerobic digestion. However, little is currently known about algal EPS and its association with anaerobic digestion of algae. Therefore, research on the characteristics of algal EPS will be important to improve understanding of the bioflocculation of algae and the effects of EPS on anaerobic digestion.

1.2 Research objectives

The overall goals of this research are to investigate the characteristics of algae grown in different types of wastewater, including the growth rate, nutrient removal efficiency, and characteristics of EPS, and to investigate the anaerobic digestion of algae and algae co-digestion with sewage sludge. This research investigated the cultivation of several species of microalgae, including *Spirulina, Chlorella* and *Micratinium*, along with varying light conditions and CO₂ addition to maximize nutrient removal from wastewater. The characteristics of algal EPS under different growth conditions have been investigated in this study. Co-digestion of waste activated sludge (WAS) and harvested algae grown in different media was conducted to study whether co-digestion with WAS improves the digestibility of algae and how the addition of algae affects the digestion of WAS.

1.3 Organization of chapters

In this dissertation, Chapter 1 introduces the background, research hypotheses and objectives. Chapter 2 presents a literature review of the application of algae-based wastewater treatment, the metabolism of algae, progress in anaerobic digestion of algae
and research on EPS. The research approaches and main results of the research are presented from Chapter 3 to Chapter 7. The main research objectives of Chapter 3 to Chapter 7 are:

Chapter 3: Anaerobic co-digestion of microalgae *Chlorella sp.* and waste activated sludge

The main objective of this study was to evaluate the effect of co-digestion with WAS on the digestibility of *Chlorella* and on other characteristics of digestion product including dewatering rates and nutrient release. *Chlorella sp.*, cultivated in the modified Zarrouk medium, was used in this study.

Chapter 4: Cultivation and anaerobic co-digestion of microalgae for wastewater treatment systems

The objective of this study was to evaluate nutrient removal by microalgae and anaerobic co-digestion of microalgae and sewage sludge for wastewater treatment systems. In this study, two wildtype microalgae, *Chlorella* and *Micractinium*, were cultivated under different types of wastewater and light conditions to study their growth and nutrient removal. No external CO₂ was provided for cultures. Anaerobic digestion of cultivated microalgae and waste activated sludge (WAS) was also performed to investigate the effect of a small addition of algal biomass on the digestibility of sewage sludge.

Chapter 5: Investigation of nutrient removal, cation uptake, and expression of extracellular polymeric substances of microalgae, *Chlorella sp.* and *Micractinium nov.*, during wastewater treatment

The objectives of this study were to evaluate the kinetics of nutrient removal by the growth of two species of green algae in different wastewater with different N/P ratios
and the effect of N/P ratio on the cation uptake and the characteristics of EPS. Wild type algae, *Chlorella* and *Micractinium*, were cultivated under periodical light conditions with external CO$_2$ addition in this study.

Chapter 6: Investigation of cultivation of green algae in sludge centrate and their anaerobic digestion under mesophilic condition

The objective of this study was to evaluate anaerobic digestibility of two green algae, *Chlorella* and *Micractinium*, cultivated in effluent from anaerobic digestion of sewage sludge and their co-digestion with waste activated sludge. The effluent quality from the algal digestion and co-digestion was also monitored to evaluate the possibility for recycling for further algae cultivation.

Chapter 7: Pilot study of algae cultivation

The objective of this study was to demonstrate nutrient removal by algae in a pilot scale PBR under natural light condition. Algae harvesting method was also evaluated in this pilot study.
CHAPTER 2

LITERATURE REVIEW

2.1 Algae classification and metabolism

2.1.1 Properties of algae and algae classification

Microalgae contain pigments that can use solar energy as the energy source for metabolism. The classifications of algae divisions are based on their pigment, photosynthetic storage product, and other morphological features. Microalgae are a diverse group of eukaryotic and prokaryotic organisms, composed mainly of cyanobacteria (or blue-green algae). About 40,000 algal species have been found in fresh water and marine environments, of which about 8,000 species are green algae (Packer, 2009). All green algae contain Chlorophyll a. The main species of microalgae commercially used include Chlorella, Spirulina, Dunaliella, Isochrysis and Chaetoceros (Carlsson et al., 2007; Lee, 1997), among which Dunaliella, Isochrysis and Chaetoceros are commonly seen in marine environments (Sun et al., 2008). Species which grow in fresh water environments will be meaningful for algae application in wastewater treatment.

The Chlorella species generally exist in soil and freshwater environments and belong to the green algae, which show the color of chlorophylls because of the presence of accessory pigments (carotenoids) does not shade the color of chlorophylls. Chlorella are small spherical or ellipsoidal cells (2-12μm) (Grobbelaar, 2007). The Chlorella species are easy to grow in culture and therefore are frequently used for commercial and research purposes. Chlorella have been shown to grow well in various types of wastewater, including municipal, industrial and agricultural wastewaters and sludge
centrate (Li et al., 2011; Lim et al., 2010; Tam & Wong, 1989; Wang et al., 2009). 

*Chlorella* can also grow heterotrophically in the presence of organic carbon sources (Sansawa & Endo, 2004).

*Spirulina platensis* are a species of cyanobacteria that have been studied for wastewater treatment by a number of researchers (Converti et al., 2006; Radmann et al., 2007; Rodrigues et al., 2010). Known as blue-green algae, cyanobacteria constitute a phylum of obligate photoautotrophic bacteria; however, the presence of simple organic substrates such as glucose can stimulate mixotrophic growth in these organisms (Andrade & Costa, 2007). *Spirulina platensis* has been shown to tolerate free ammonia even at high pH (Boussiba, 1989). Due to its multicellular structure and ability to self-aggregate, simple gravity settling works well for harvesting *Spirulina platensis* (Olguin, 2003).

### 2.1.2 Autotrophic, heterotrophic and mixotrophic metabolisms of microalgae

Microalgae contain pigments that can use solar energy for their metabolism. When algal biomass undergoes autotrophic metabolism, inorganic carbon is used as the carbon source and the energy comes from solar energy. Nowadays, most microalgae cultivations undergo autotrophic growth because all microalgae are photosynthetic and most algal species are obligate autotrophs (Perez-Garcia et al., 2011). Sufficient light, either naturally or artificially applied to algae cultivation, is critical for massive algae production. Large open ponds and numerous closed photo-bioreactors (PBR) have been built to cultivate algae by natural or artificial light for food supplements, lipids, enzymes, wastewater treatment and biofuels (Chisti, 2008; Lee, 2001; Park et al., 2011a; Yuan et al., 2011). However, several factors limit the growth rate of algae. A large reactor volume will reduce the light dispersion efficiency inside the PBR and the biofilm formed
on the surface of a PBR will lead to the limitation of light penetration. A feasible way to overcome the light limitation is to use algae species that have heterotrophic growth capacity.

Heterotrophic organisms utilize organic compounds as carbon and energy sources for their metabolism. Some algae can undergo heterotrophic growth in dark conditions. The heterotrophic growth of algae can eliminate the limitation of illumination capacity in a PBR and reduce the energy cost for most algae cultivation processes (Prathima Devi et al., 2012). Commonly used carbon sources for heterotrophic algae are glucose, glycerol, acetate and organics from wastewater (Perez-Garcia et al., 2011). In order to apply algae to wastewater treatment, this feature becomes extremely important. The metabolism of algae use the same respiration pathway as other plants under dark conditions, where oxygen is consumed and carbon dioxide is produced. In general, the respiration rate is intimately related to the growth rate and cell division of algae (Perez-Garcia et al., 2011).

Glucose is a favorable carbon source that supports higher rates of growth and respiration because it produces a higher energy output than other substrates (Perez-Garcia et al., 2011). The metabolism pathways for glucose (glycolysis) include the Embden-Meyerhof Pathway (EMP) and Pentose Phosphate Pathway (PPP) under aerobic conditions (Neilson & Lewin, 1974; Perez-Garcia et al., 2011). Anaerobic metabolism of glucose by algae is not feasible due to the insufficient energy and low level of enzyme lactate dehydrogenase (Neilson & Lewin, 1974). The ability to metabolize carbon sources is species related. For example, *Chlorella* can uptake glucose from the medium, however, *Prymnesium parvum* and *Dunaliella tertiolecta* are unable to assimilate glucose.
even though they possess the enzymes necessary for their metabolism (Neilson & Lewin, 1974).

With different carbon sources and light conditions, the metabolism pathway will change and affect the physiology, cell sizes, storage materials, such as lipid and protein, and chlorophyll of algae (Andrade & Costa, 2007; Perez-Garcia et al., 2011; Prathima Devi et al., 2012; Sansawa & Endo, 2004). Environmental factors also play an important role in the metabolism of carbon sources. For example, blue light of the visible spectrum inhibits the uptake of glucose, glycine, proline, and arginine, but enhances the uptake of oxygen and nitrate (Kamiya, 1997; Kamiya & Saitoh, 2002).

Considering the natural conditions of light and the ability to removal nutrient and organic carbon, mixotrophic growth of algae will be a better choice for algae application in a WWTP. In mixotrophic growth mode, organic carbon and CO$_2$ will simultaneously be utilized as carbon sources (Perez-Garcia et al., 2011). The rate of mixotrophic growth is the sum of autotrophic and heterotrophic growth (Martinez & Orús, 1991; Perez-Garcia et al., 2011).

Large scale cultivation of an algae species requires that that species possess the following characteristics: ability to survive and adapt to environmental changes; have an overall low cost of cultivation, i.e., can use common carbon sources; and that the metabolites have significant economic worth. To meet these features, a limited number of species can be used for wastewater treatment.

2.2 Effect of nitrogen sources, pH and CO$_2$ on algae growth

Algae growth refers to the increase of cell numbers or total mass of algal cells. Microalgae growth is affected by several chemical and physical conditions. When
substrate concentrations or other factors become limiting, or toxic compounds accumulate, the growth rate will decrease and the production of secondary metabolites will take place (Grobbeelaar, 2007). In some cases, the decline of cells will also be accompanied by the formation of spores or similar structures, which may survive and overcome the negative conditions and produce new individuals when favorable conditions return.

2.2.1 Nitrogen sources

Nitrogen accounts for 1-10% of algae cell dry weight and the metabolism of nitrogen is very important for algae growth (Perez-Garcia et al., 2011). Microalgae can assimilate various sources of nitrogen and can be used for nutrient removal in a WWTP. Ammonium is the preferred nitrogen source for algae because less energy is required for its metabolism compared to nitrate or urea. The main pathway for ammonium incorporation into algae is catalyzed by glutamine synthase (GS; EC6.3.1.2) and glutamate synthase (GOGAT; EC 1.4.1.14). Another way to incorporate ammonium into algae is catalyzed by glutamate dehydrogenase (GDH, EC1.4.1.2), which is activated under stress conditions (Lu et al., 2005).

Nitrate is another major nitrogen source for algae growth. Nitrate is metabolized through the reduction of nitrate to ammonium through nitrate reductase and nitrite reductase. Different metabolite pathways are expressed under different conditions. The nitrate reductase is expressed when nitrate is the only nitrogen source while activity is repressed when there is sufficient ammonium in the environment (Cannons and Pendleton, 1994). Nitrogen deficiency is regarded as the main factor to stimulate the accumulation of lipid or carbohydrates in algae cells (Li et al., 2010; Rhee, 1978). The
accumulation of lipids is a response to the depletion of nutrients, especially nitrogen. Limitation of nitrogen will decrease the content of thylakoid membrane whose main content is glycolipid, activate acyl hydrolase and stimulate the hydrolysis of phospholipid, which lead to the accumulation of fatty acid acyl-CoA. At the same time, nitrogen limitation will activate the diacylglycerol acyltransferase, which will convert fatty acid acyl-CoA to triglyceride (Takagi et al., 2000). Therefore, limitation of nitrogen not only increases the intercellular lipid content but also the triglyceride of algae (Li et al., 2010; Takagi et al., 2000). The commercial use of algae will be highly affected by their intercellular composition. Meanwhile, the intercellular content of algae will also affect algal digestibility because of different biogas yield from lipids, proteins and polysaccharides. Anaerobic digestion of lipids yield the most methane and proteins are the second macromolecule type that has high methane production (Cirne et al., 2007). Therefore, the control of algae growth conditions is important to deplete the nitrogen in the medium and to get the desired end product from algae cultivation.

2.2.2 Carbon Dioxide (CO₂) and pH

Carbon dioxide emission to the atmosphere is regarded as one of the main sources of global warming. Another benefit of microalgae use for wastewater treatment is their ability to decrease CO₂ emission. Sung et al. (1999) indicated that Chlorella has a tremendous tolerance to a high range of CO₂ and pH conditions. Chlorella can maintain good growth rate using air containing up to 10-30% CO₂ (Chinnasamy et al., 2009; Sung et al., 1999). Mayo and Noike (1994) found no significant effect on the survival of Chlorella vulgaris for pH up 10.5 when glucose was the substrate. However, the optimal pH for the highest specific growth rate Chlorella vulgaris was 6.3-6.8 (Mayo, 1997). The
flue gas from combustion of natural gas contained about 6-8% of CO₂ and can be used for algae cultivation (Doucha et al., 2005). The high tolerance of pH enables that algae can be used to treat acidic or basic wastewater.

2.3 Reduction of CO₂, N and P by microalgae cultivation

With the presence of light and CO₂, algae can utilize CO₂ as a carbon source and light as an energy source for autotrophic growth. During algae cultivation, large amounts of nitrogen (N) and phosphorus (P) can be fixed, reducing concerns about eutrophication due to the waste discharge. Harvested algae can be used as potential biofuels, foods, pharmaceuticals and food supplements (Carlsson et al., 2007; Chisti, 2007; Kumar et al., 2010). When ammonium is used as a nitrogen source, the photosynthesis of algae in the presence of sunlight is expressed as (Grobbeelaar, 2007):

$$106CO_2 + 236H_2O + 16NH_4^+ + HPO_4^{2-} \rightarrow C_{106}H_{181}O_{45}N_{16}P + 118O_2 + 171H_2O + 14H^+$$

Equation 2.1

The main mechanism of nitrogen removal includes uptake of nutrients by algae and stripping of ammonia due to the increase in pH from photosynthesis (Aslan & Kapdan, 2006). The chemical equilibria of inorganic carbon is:

$$H_2O + CO_2 \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-}$$

Equation 2.2

When algae perform photosynthesis, CO₂ is consumed and the equilibration shifts towards the left and leads to an increase of pH in the surrounding medium. Ammonia may be volatilized at high pH. Nutrient removal by microalgae, if successfully implemented, may have several advantages over conventional biological nutrient removal processes used in WWTPs: low capital and operational costs, no need of additional
organic carbon for nutrient removal, simultaneous mitigation of CO$_2$ output, and production of oxygen during treatment process (Aslan & Kapdan, 2006; Li et al., 2010).

Yun et al. (1997) indicated that about 1.8 kg of CO$_2$ could be fixed by the production of 1 kg of *Chlorella vulgaris* cultivated in wastewater from a steel-making facility, whose main nitrogen content was ammonium. This value is quite similar to the theoretical value predicted in Equation 2.1. The uptake of CO$_2$ will rely mainly on the growth rate of algae under autotrophic and mixotrophic metabolisms.

The mechanisms of phosphorus removal are metabolic uptake by algae and adsorption and precipitation of inorganic phosphorus at pH over 8 (Li et al., 2011; Song et al., 2002). Li et al. (2011) suggested that over 76% of phosphorus was removed by inorganic P sedimentation and less than 23% of phosphorus was assimilated by algae.

2.4 Progress in anaerobic digestion of algae

Microalgae are cultivated as sources for biofuels, such as biodiesel and bioethanol. Anaerobic digestion is another way to stabilize biomass and generate bioenergy by combustion of methane. Sialve et al. (2009) recommend anaerobic digestion as a suitable way for bioenergy generation when the lipid content in algal biomass is less than 40%. In spite of the potential for energy recovery by anaerobic digestion of algae, digestibility and gas yield are still poorly understood.

A few studies indicated that anaerobic digestion of algae showed a low yield due to the characteristics of strong and thick cell walls and to the high protein content in algal biomass, which can lead to a significant release of ammonia, inhibiting anaerobic digestion (Chen et al., 2008; Takáčová et al., 2012). One possible way to increase digestibility was physical-chemical pretreatment, including mechanical shearing.
sonication, thermal pretreatment, alkaline pretreatment or a combination of processes. These pretreatments can help to make the substrate more accessible to anaerobic microorganism, thus improving the degradability of substrates (Chu et al., 2002; Elliott & Mahmood, 2007; Liu et al., 2008; Muller et al., 2007). However, whether the profit from increased methane production can balance the cost of chemical addition or energy required for pretreatment is still being debated.

Co-digestion could be another way to improve the anaerobic digestibility of algae by improving the substrate composition for anaerobic digestion. Carbon rich sources such as primary and secondary sludge, oil-greases, waste papers and various food wastes may be introduced into algae anaerobic digesters to increase the C/N ratio and reduce the possibility of toxication of ammonia. Yen and Brune (2007) reported the co-digestion of waste paper with algae increased the gas yield by 50%, but the availability of waste paper reduced its feasibility for large scale application.

Waste activated sludge, which has high carbon content and microorganisms, can be used as a co-substrate for algae digestion. Scarce research has been done on the anaerobic co-digestion of algae and sewage sludge. Samson and Leduy (1983) found that addition of half sewage sludge (by volatile solids) increased the biogas yield from digestion of *Spirulina maxima* 2.1 fold. Cecchi et al. (1996) studied the co-digestion of sewage sludge and macroalgae from lagoons under mesophilic and thermophilic conditions. Their experiments showed that around 30% (total solids based) addition of macroalgae had similar levels of methane yield as sewage sludge, while thermophilic digestion showed inhibition of methanogens. Large amounts of waste activated sludge need to be properly treated which can make this waste stream a more convenient algal co-
substrate compared with food waste or waste paper. However, there is still no available information on the anaerobic co-digestion of sewage sludge with ubiquitous green algae, such as Chlorella which are commonly found in WWTPs and widely used for wastewater treatment (Gonzalez et al., 1997; Wang et al., 2010a). Whether the addition of WAS will improve the digestibility of microalgae and how the addition of algae will affect the anaerobic digestion of WAS are still unknown. Because digestion will be the key step for recycling nutrients as well as for energy recovery, research on anaerobic digestion of microalgae and sewage sludge will be critical to evaluate the feasibility of integrating algae cultivation into existing WWTPs.

2.5 Extracellular substances of algae

2.5.1 EPS of sludge flocs

Extracellular polymeric substances (EPS), which are located outside of cells, have been shown to be a major organic component of sludge flocs (Wagner et al., 2009). The EPS of sludge are composed of metabolites of microbial activity, intracellular material released by cell lysis, and organic matter adsorbed from wastewater (Dignac et al., 1998; Jorand et al., 1995; Park & Novak, 2007; Yu et al., 2008). It has been seen that short term variations in environmental conditions affected the secretion of EPS by microbial communities (Yang & Li, 2009). EPS in turn has been shown to govern many sludge properties including floc formation, settleability, and dewaterability, as well as effluent quality (Dignac et al., 1998; Li & Yang, 2007; Liu & Fang, 2003; Neyens et al., 2004; Park & Novak, 2009; Subramanian et al., 2010; Yu et al., 2009).
2.5.2 Extracellular polymeric substances of algae

The EPS of activated sludge play a significant role in successful wastewater treatment and have been widely studied. The EPS of microorganisms are composed of metabolism substances, products of cell lysis, and some organic compounds from the growth medium (Frølund et al., 1996; Park & Novak, 2007; Yu et al., 2009). As discussed above, the algal metabolism pathway will shift under different growth conditions including nutrients, light and other environmental stresses. Different metabolites from algae will result in changes in quality and quantity of the EPS.

It is hypothesized that extracellular substances of microalgae are also related to algae growth, flocculation, and anaerobic digestion. Proper utilization of EPS from microalgae will also benefit biomass harvesting, which is the bottleneck for bulk diluted microalgae culturing. Currently, microalgae harvesting relies mainly on centrifugation, chemical flocculation, and dissolved air flotation (DAF), which all increase the cost of commercial algae application. Salim et al. (2011) reported that harvesting of algae from open ponds by centrifugation accounted for about 30% of the total cost. Salim et al. (2011) successfully harvested non-flocculating Chlorella by adding another autoflocculating algae for bioflocculation. Similar positive effects of bioflocculation on harvesting microalgae with bacteria by sedimentation were also observed (Lee et al., 2009). Salim et al. (2011) indicated that cationic polymers outside the cell surface may explain the mechanism of cell aggregation. The bioflocculation characteristics of bacteria will be important for algae harvesting if they worked with nonflocculated algae. On the other hand, microorganisms grown in harsh conditions were likely to produce more EPS which negatively affected anaerobic digestion (Wang et al., 2010b). It is hypothesized
that algae grown in different growth media will have different expression of EPS, which may in turn affect anaerobic digestion. However, there is little information on the extraction methods of algal EPS and their characteristics. Therefore, fundamental knowledge of the characteristics of algal EPS, and their production conditions may be critical to find possible economic ways to harvest algae and to predict their digestibility.
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CHAPTER 3

ANAEROBIC CO-DIGESTION OF MICROALGAE CHLORELLA SP. AND 
WASTE ACTIVATED SLUDGE*

*Submitted to Bioresource Technology (under review)

3.1 Abstract

The study investigated the growth characteristics of environmental algal strain, Chlorella, in the modified Zarrouk medium and its anaerobic co-digestion with waste activated sludge (WAS). Analysis of extracellular polymeric substances (EPS) in algal culture and WAS indicated that Chlorella secreted more EPS into the surrounding liquid than formed floc-associated EPS as in activated sludge. Mesophilic anaerobic digestion of algae alone required an extended digestion period to produce methane, with biogas yield at 262 mL/gVSfed after 45 days of digestion. When algae was co-digested with varying amounts of WAS, 59-96% in mass, not only biogas yield of microalgae improved but the gas phase was reached more quickly. The dewaterability of co-digestion products were also better than two controls digesting WAS or algae only. These results suggest that anaerobic co-digestion of algae and sludge improves the digestibility of microalgae and could also bring synergistic effects for the dewaterability of digested products for existing anaerobic digesters.

Keywords

Chlorella, waste activated sludge, anaerobic co-digestion, biogas yield, extracellular polymeric substances (EPS)
3.2 Introduction

Due to the steady depletion of fossil fuels, efforts have been made toward finding alternative renewable energy sources. Microalgae have gained high attention in the field of bioenergy because of their higher productivity and higher biofuel yield compared to terrestrial plants. The oil yield from algae can achieve 1000-6500 gallons/acre\textperthousand yr, which is about 20 times higher than that gained from soil plants, such as soybean, oil palm or sunflowers (Chisti, 2007; Wigmosta et al., 2011). However, high costs for lipid extraction and high amount of unsaturated fatty acid in algal biofuels have hindered large-scale application of microalgae for biofuel generation (Chisti, 2008; Sialve et al., 2009; Singh et al., 2011).

Anaerobic digestion could be another method for energy recovery from grown algal biomass. Sialve et al. (2009) recommended that anaerobic digestion is actually a suitable method for bioenergy generation when the lipid content of algae is less than 40%. A few studies, however, indicated that anaerobic digestion of algae led to a low biogas yield due to the characteristics of strong and thick cell walls, limiting effective anaerobic digestion of algae (Chen et al., 2008; Takáčová et al., 2012). One possible way to increase the digestibility of algae then could be physical-chemical pretreatment, including mechanical shearing, sonication, thermal process, alkaline treatment, or a combination of these processes. These pretreatments can help to make the substrate more accessible to anaerobic microorganisms, thus improving the degradability of substrates (Chu et al., 2002; Elliott & Mahmood, 2007; Liu et al., 2008; Muller et al., 2007). However, whether the benefit from increased methane production can justify the cost of chemical addition or energy required for pretreatment is still unsure.
Studies have shown that co-digestion could increase the anaerobic digestibility of algae by improving the substrate composition for anaerobic digestion. Carbon-rich substrates, such as primary and secondary sludge, oil-greases, waste papers and various food wastes, can be introduced into algae anaerobic digesters to increase the C/N ratio and to reduce the chance of ammonia toxicity. Yen and Brune (2007) reported that the co-digestion of algae with waste paper increased the gas yield of algae by 50%.

Sludge, which has high carbon content and active microorganisms, can also be used as a co-substrate for algae digestion, and it is an attractive co-substrate because a large amount of sludge is produced daily at wastewater treatment plants (WWTPs) where anaerobic digesters are also often located. A few studies of anaerobic co-digestion of algae and sewage sludge has been done. Samson and Leduy (1983) found that addition of primary sludge (50% by volatile solids) increased the biogas yield of cyanobacteria Spirulina maxima 2.1 fold. Cecchi et al. (1996) studied the co-digestion of sewage sludge and macroalgae from lagoons under mesophilic conditions. The study showed that addition of macroalgae at around 30% (total solids based) led to a similar level of methane yield as digestion of sludge alone.

Although earlier studies indicated co-digestion of algae with sludge is promising, whether the addition of waste activated sludge (WAS) improves the digestibility of microalgae and how this co-digestion affects the anaerobic digestion of WAS are still not clearly known. Furthermore, there is little information on the anaerobic co-digestion of sludge with ubiquitous freshwater green algae, such as Chlorella, which are commonly found in WWTPs, and that have recently been used for wastewater treatment (Gonzalez et al., 1997; Wang et al., 2010a). Because anaerobic digestion could be a key step for
recycling nutrients for algal cultivation as well as for energy recovery at WWTPs, research on anaerobic digestion of microalgae and sewage sludge will be important for the future of WWTPs.

The main objective of this study was to evaluate the effect of co-digestion with WAS on the digestibility of Chlorella and on other characteristics of digestion products including dewatering rates and nutrient release. Chlorella sp. originally collected from a local WWTP (Amherst, MA) and inoculated in a laboratory culture was used for anaerobic co-digestion in this study. During the study, the characteristics of algal EPS and sludge EPS were also investigated to understand the physiological characteristics of both algae and WAS before anaerobic digestion.

3.3 Material and Methods

3.3.1 Cultivation and harvesting of algae

Green algae were collected from a primary settling tank at the Amherst WWTP (Amherst, MA). Microscopic analysis of algae showed a sphere shape green algae identified as Chlorella sp. Algae were cultivated in the modified Zarrouk medium containing the following chemicals: NaHCO₃ (13.61 g/L), Na₂CO₃ (4.03 g/L), K₂HPO₄ (0.5 g/L), NaNO₃ (1.25 g/L), NH₄Cl (0.82 g/L), K₂SO₄ (1.0 g/L), NaCl (1.0 g/L), MgSO₄•7H₂O (0.2 g/L), and CaCl₂•2H₂O (0.04 g/L). Trace elements (Co, Mn, Fe, Mo and B12) were added to the medium based on instructions from UT Austin’s Algae Culture Collection (web.biosci.utexas.edu/utex/). Algae were inoculated in 20°C at a temperature-controlled room in 1 L Erlenmeyer flasks with a working volume of 700 mL. The flasks were covered with parafilm and had a side arm for gas exchange. Cultures were kept suspended by shaking at 145 rpm using a G10 gyratory shaker (New Brunswick
Scientific, Inc., Edison, NJ). A 24 hour continuous light condition (~ 5,000 Lux) was provided by six 40-W fluorescent lamps for algal cultivation. Algae were cultivated in batches with a cultivation time of 6 days. After each batch algae were divided into two new flasks which were then filled to the same working volume (700 mL) with fresh medium. Microalgae were continuously inoculated to obtain and maintain pure culture. The algal biomass was measured by total suspended solids. Algal productivity per unit illuminated surface (g/m²/d; hereafter referred to algal productivity P) was calculated following the method described in Yuan et al. (2012). The specific growth rate ($\mu$, d⁻¹) was calculated based on biomass density as:

$$\mu = \frac{C_n - C_m}{(n-m)C_m}$$

where, $\mu$ is specific growth rate (d⁻¹); $C_m$ and $C_n$ are suspended solids concentrations of algae on day m and n; m and n are days of algae cultivation.

Algae were harvested at the end of batch cycle when a sufficient quantity of biomass was generated for digestion. The harvested algae were washed three times to remove excessive salts used in medium by centrifuging the algae at 3000 rpm for 5 minutes, and resuspending in tap water.

### 3.3.2 Mesophilic anaerobic co-digestion

All digestion was performed in 150 mL serum bottles with a working volume of 100 mL. Digestion was conducted in duplicate. All digestion bottles contained about 4% of anaerobic digestion seed sludge by mass of VS, or 10% by volume. This anaerobic seed sludge was collected from a lab-scale mesophilic anaerobic digester (5L), with an SRT of 60 days fed with WAS from the Amherst WWTP. WAS for anaerobic co-digestion was prepared by thickening activated sludge collected from the aeration basin.
of the Amherst WWTP, first by decanting of liquid after 1hr settling and by subsequent centrifuging (3,000 rpm, 5 min) to target approximately 1% total solids. The harvested algae were also concentrated to 1% total solids. Different volumes of algae and WAS were then added to the co-digestion bottles to achieve the following mass (VS) composition of algae in the digester: 0%, 4%, 11%, 41%, and 100%. Note that these numbers only represent the mass of algae in the substrate (i.e., algae + sludge), not including the mass of seed sludge. Anaerobic digestion was conducted in a 37 °C (mesophilic conditions) constant temperature room with a multiple magnetic stirrer to provide identical mixing for each digestion bottle.

3.3.3 Biogas measurement

The biogas volume was measured every 2 or 3 days by injecting a needle tightly attached to a 50 mL glass syringe through the digester’s rubber septa and reading the volume of the ejected plunger. This procedure allowed for the release of generated gas from the closed batch digester. As digestion progressed, the biogas measurement became less frequent due to declination in gas generation in the later digestion period. Digestion was performed for 45 days. The composition of methane and carbon dioxide in biogas (500 μL of gas sample) were measured on the 13th, 29th, and 42nd day of digestion using a gas chromatograph with thermal conductivity detector (Gow-Mac instrument Co. GC 550 series).

3.3.4 Extraction and analysis of EPS of Chlorella and activated sludge

In order to study the EPS of Chlorella and activated sludge, unthickened fresh algae and activated sludge were subjected to sonication and base extraction. Sonication extraction was performed on 30 mL of activated sludge (VS of 1,700 mg/L) and 20 mL
of fresh algal biomass (VS of 3,700 mg/L). These were centrifuged at 9,000 rpm for 15
minutes to separate supernatant and solids. The EPS in supernatant was considered
soluble EPS. The pellet of WAS and algae was resuspended to a volume of 20 mL in a
phosphate buffer solution (10 mM NaCl, 1.2 mM KH$_2$PO$_4$ and 6 mM Na$_2$HPO$_4$). A 400
watt Sonic Dismembrator was used for sonication extraction. The sonication was
conducted at 10% strength for 40 sec. Sonication extraction took place in a beaker
surrounded by crushed ice to avoid heating the extraction sample. The sample was again
centrifuged at 9,000 rpm for 15 minutes and the supernatant was collected as sonication-
extracted EPS. Base extraction followed the procedure of Park and Novak (2007). Pellets
from initial centrifugation were quickly resuspended with 10 mM NaCl. The pH was
adjusted to ~10.5 with 1 M NaOH. The resuspended samples were then stirred on a
shaking table for 2 hours at 425 rpm at 4°C in a temperature-controlled room. After
extraction, the sample was again centrifuged at 9,000 rpm for 15 minutes and the
supernatant was collected as base-extracted EPS. The protein, polysaccharide and
chemical oxygen demand (COD) content in EPS were analyzed. Protein concentration
was quantified by the modified Lowry Method (Frølund et al., 1996) using bovine serum
albumin as standard. Polysaccharide was measured by the Dubois et al. (1956) method
with glucose used as the standard. COD was measured following Standard Methods
(APHA et al., 2005).

3.3.5 Other analytical methods

Measurements of biomass dry weight (DW), total solids (TS), volatile solids (VS),
total suspended solids (TSS), and total volatile suspended solids (VSS) were performed
according to Standard Methods (APHA et al., 2005). Ammonium concentration was
measured using Hach Reagent kits (Hach Inc., Loveland, CO). Nitrate and phosphate concentrations were measured using a Metrohm Peak 850 Professional AnCat ion chromatography system (Metrohm Inc., Switzerland). A calibrated Orion GS9156 pH meter (Thermo Fisher Scientific Inc., Waltham, MA) was used to measure pH. The dewaterability of sludge was indicated by capillary suction time (CST) following a designated method in *Standard Methods* (APHA et al., 2005). Triton Type 319 multi-CST equipment was used to measure CST.

### 3.4 Results and discussion

#### 3.4.1 Growth rate of algae

The highest specific growth rate of *Chlorella* 0.45 d⁻¹, was achieved when the algae dry weight increased from 605 mg/L to 875 mg/L within one day. The maximum productivity of algae was 6.5 g/m²/d, with the specific growth rate of 0.26 d⁻¹, when algal biomass increased from 1,380 mg/L to 1,735 mg/L in one day. The highest specific growth rate or the maximum productivity were achieved when algae was provided with fresh medium after adapting to the same environment for about 20 days. The highest specific growth rate was achieved when initial algae concentration was 605 mg/L, while a higher initial algae concentration, such as 1,380 mg/L, led to a lower specific growth rate. A similar trend was also observed from the growth of *Scenedesmus sp.* in livestock wastewater (Park et al., 2010). This study showed that the specific growth rate decreased from 0.091 d⁻¹ to 0.038 d⁻¹ when the dry weight of seeding cells increased from 500 mg/L to 1,500 mg/L. Adversely, the maximum productivity of algae was achieved with higher initial algae concentration.
The specific growth rate seen in our study is not low compared to other studies. Lim et al. (2010) showed a growth rate of *C. vulgaris* at 0.05-0.39 day⁻¹ when cultivated in diluted textile wastewater. Chinnasamy et al. (2009) showed a specific growth rate of *Chlorella* at 0.22 d⁻¹ when the culture was bubbled with 6% CO₂. Yuan et al. (2012) showed that the specific growth rate of a wildtype green algae ranged from 0.19-0.32 d⁻¹ with 2% CO₂ bubbling. It is known that the growth rate and productivity of algae vary considerably depending on species and cultivation conditions. In general, the nutrient in growth medium, carbon dioxide, pH, light condition and temperature will affect algae growth. For *chlorella*, the growth rate will increase proportionally with the increase of temperature up to 35°C (Chinnasamy et al., 2009; Sung et al., 1999). Since our cultivation temperature was only 20°C, the growth rate of *Chlorella* in our cultivation could have been higher with higher temperature.

*Chlorella* has a tolerance of CO₂ and has shown good growth in air containing 10-30% CO₂ (Chinnasamy et al., 2009; Sung et al., 1999). In our study, external CO₂ was not provided and gas transfer was achieved only through shaking the culture bottles, where gas transfer efficiency was much lower than gas bubbling. However, additional carbonate and bicarbonate included in the culture medium also served as inorganic carbon sources for algae growth. Our cultivation experiment without bicarbonate and carbonate addition showed an immediate attenuated growth rate of *Chlorella*. Park et al. (2010) also showed a decreased algal (*Scenedesmus sp.*) growth rate when bicarbonate or carbonate was consumed from the livestock wastewater and CO₂ was not provided during growth. Therefore, CO₂ requirement could be reduced when algae are cultivated in wastewater containing high alkalinity, such as anaerobic digested sludge centrate.
Chlorella has also been reported to grow in a wide range of pH. Sung et al. (1999) showed that Chlorella can grow well when pH is above 4. During algae cultivation in our study, pH was maintained between 9.3-10.6, and no significant inhibition was observed.

3.4.2 Characteristics of algal and sludge EPS

EPS are composed of metabolites of microbial activity, intracellular materials released by cell lysis, and organic matter adsorbed from influent media, such as wastewater (Dignac et al., 1998; Jorand et al., 1995; Park & Novak, 2007; Yu et al., 2008). The characteristics of sonication- and base-extracted EPS from Chlorella and activated sludge are shown in Table 3.1. The data show that algal culture contained more organic substances (COD) in the supernatant, presumably soluble microbial products (SMPs) or soluble EPS, than activated sludge. The activated sludge contained more organic matter that was attached onto the sludge biomass (i.e., extracted EPS). This fraction has been known to play an important role in bioflocculation of activated sludge and biofilms (Park et al., 2006; Yu et al., 2009).

Compared to activated sludge, Chlorella were less susceptible to sonication and base extraction. Algae were particularly resistant to base extraction, possibly because Chlorella were cultivated in high pH (9.3-10.6) medium and had a tolerance to a high pH. Though sonication was more effective for extracting EPS from algae, this method still extracted much less EPS compared to the extraction from sludge. These results suggest that Chlorella in our study did not significantly produce bound or attached EPS during their growth while they released relatively high soluble EPS into the growth medium. The soluble protein and polysaccharide in algal supernatant should have been from the
biological activity of *Chlorella*, because there was no organic matter in the original cultivation medium.

The quantity and quality of EPS are closely related to microbial activity. It has been reported that a short-term variation in environmental conditions affect the secretion of EPS by microbial communities (Yang & Li, 2009). The algal EPS should also be related to their growth characteristics and will in turn affect cell aggregation and subsequent biomass harvesting. In related context, Henderson et al. (2010) studied the relationship between coagulation by alum and algal organic matter in four different algal species including *Chlorella vulgaris*, and reported that the composition of EPS would be important in determining the effectiveness of algal harvesting and subsequent anaerobic digestion. Therefore, further study is necessary to gain fundamental knowledge of the characteristics of algal EPS and factors that affect production of EPS and algal aggregation.

### 3.4.3 Anaerobic mesophilic co-digestion of algae and WAS

Mesophilic anaerobic digestion was performed for 45 days and the average volatile solids reductions (VSR) of anaerobic co-digestion are shown in Figure 3.1. A 100% algae digestion set showed the highest VSR while the digesters with 100% WAS led to the lowest VSR, which is consistent with the previous finding from the study of (Yuan et al., 2012).

The cumulative biogas data for each digestion set are presented in Figure 3.2. The digester with WAS-alone (i.e., 0% algae) produced 483 mL/g VS<sub>fed</sub>. On the other hand, digestion of 100% algae (no WAS addition) produced 262 mL/gVS<sub>fed</sub>. Importantly, the algae and sludge co-digestion sets nearly doubled this gas yield. The gas yields of co-
digestion with 4%, 11%, and 41% of algae addition, by mass of VS, were 463, 453, and 468 mL/g VS, respectively and these gas yield values were 77%, 73%, and 79% larger than the gas yield from pure algae digestion sets.

Although pure algae digestion sets showed the highest VSR (Figure 3.1), it produced the lowest biogas, which indicates that high VSR in this digestion was not mediated via CH₄ generation but likely via volatilization of volatile organics produced in the digester during solids measurement. The pH of 100% algae digestion was only 6.5 (after 45 days of digestion), while the co-digestions and WAS digestion had pH ranging between 6.8 and 7.0. As the gas generation data were similar for digesters with WAS-only and all co-digestions, higher VSR values seen from various co-digestion sets are meaningful and indicate that some addition of algae into existing anaerobic digesters at a WWTP could provide a benefit of higher solids reduction.

The data in Figure 3.2 further show that *Chlorella* (in 100% algae set) were poorly digested during the early period of digestion while gas yield slowly increased from approximately after 20 days of digestion, suggesting that a long SRT is needed for anaerobic digestion of algae solely. On the other hand, none of co-digestion sets that contained both *Chlorella* and WAS showed such a pronounced lag period in gas generation, indicating that algae in these digestion sets were relatively easily bioavailable and degraded. Northcote et al. (1958) showed that the cell wall of *Chlorella* accounted for 13.6% of dry weight of the whole cell and the cell wall contained about 31% of hemicellulose, 15.4% of α-cellulose and 5.2% of ash. The cellulose is composed of over hundreds to thousands of glucose units and binds strongly to each other; thus, it is relatively difficult to break down compared to other polysaccharides. When *Chlorella*
were co-digested with WAS, the high amount of and diverse microorganisms in WAS are thought to help the hydrolysis of algal cells thus, leading to the improved digestibility of algae.

The digestion time for pure algae digestion was extended to 69 days. The gas yield increased to 360 mL/g VS, which is about a 37% increase in biogas compared to 45 days of digestion. However, the VSR increased only by 2%. This strongly indicated that most of acid hydrolysis occurred early for this set and more hydrolyzed products converted to biogas during later digestion period, which is also much longer than typical digestion of WAS at a WWTP.

Concentrations of methane in the biogas from different digesters, at different phase of digestion, are listed in Table 3.2. As the data clearly show, the concentration of methane from 100% algae digestion at day 13 was substantially less than others but significantly increased from day 13 to day 29. In contrast, all co-digestion sets showed similar CH₄ content compared to the digestion with WAS only. These data support the data in Figure 3.2 that the gas phase was reached quickly in co-digestion although some digestion sets, some as the 41%-algae addition, contained a large amount of algae as digestion feed. It is therefore concluded that co-digestion of algae with WAS should be a better approach than extended SRT in order to obtain a higher gas yield of microalgae.

The methane yield of pure algae digestion in our study was 123 mL CH₄/g VS_{fed} on day 45 and 54 mL CH₄/g VS_{fed} on Day 30. Compared with gas yield of algae digestion from other studies (160 -240 mL CH₄/g VS or VSS) (Ras et al., 2011; Samson & Leduy, 1983), our study showed a relatively lower biogas yield. The different gas yields might have resulted from different species of algae and different growth condition
used in this study, which is expected to lead to the different characteristics of algae and thus digestibility.

During this study, we also “calculated” a gas yield value for each co-digestion set with an assumption that gas generation by WAS alone and algae alone can be applied to co-digestion of different mass compositions. To do so, we used the following equation:

\[
\text{Calculated gas yield} = Y_s \times C_s + Y_a \times C_a
\]

Where \(Y_s\) is the gas yield of WAS, \(C_s\) is the fraction of WAS by mass in each digestion set, \(Y_a\) is the gas yield of algae, and \(C_a\) is the fraction of algae by mass in each digestion set. The expected gas yield from co-digestion sets, based on the equation above, are 461, 446, 380 mL/g VS fed for 4%, 11% and 41% addition of algae, respectively. Those values were lower than experimental values of 463, 453 and 468 mL/g VSfed. The 41% algal addition set had a 23% higher gas yield than the calculated value. This is another evidence that co-digestion of \textit{Chlorella} and WAS increased the gas yield of algae while maintaining similar gas yield from WAS. These results mean that more efficient bioenergy harvesting from microalgae could be achieved when microalgae is co-digested with sludge.

3.4.4 Dewaterability and characteristics of algae and WAS digested product

Dewatering is an important step taking place immediately after anaerobic digestion. Hence, the effect of co-digestion on dewatering was also investigated. Capillary suction times (CST), used as a proxy parameter for dewaterability, of the digested products were measured and normalized by TS. In the mesophilic digestion, 4% and 11% \textit{Chlorella} sets not only improved the biogas yield of algae but also improved the dewatering rate compared to two control digestion sets (WAS or algae only). Especially
co-digestion with 11% algae led to a CST value even less than a half of CSTs from control digestions, indicating significant improvement in dewatering. As shown in Table 3.3, the co-digestion with algae at 11% also showed a lower soluble COD. The 41% *Chlorella* set showed a relatively higher CST which might have been caused by the high amount of soluble materials present in this digested product. The well performed digestion sets (WAS, 4%, 11% and 41% of algae co-digestion sets), which showed decent methane generation, also had lower polysaccharide and COD after digestion. The lower COD in solution indicated that a large amount of organic matter was changed to methane through mesophilic digestion.

One of the potential applications of anaerobic digestion of algae and sludge is to recycle the released nutrients for additional algal growth at WWTP. As shown in Table 3.3, 591 mg/L of ammonium-N and 202 mg/L of PO$_4^{3-}$-P were released from mesophilic digestion of WAS, while those released from 100% algae digestion set were only 392 mg/L and 36 mg/L. The co-digestion set showed ammonium-N concentrations in the range of 477 mg/L to 593 mg/L and PO$_4^{3-}$-P concentrations ranged between 107 mg/L to 189 mg/L. Nutrients released from 4% and 11% algae were very similar to WAS control digestion. Consequently, some of the ammonium and phosphate released from algae and sludge co-digestion can be reutilized for cultivating algae at a WWTP.

### 3.5 Conclusions

The study showed that co-digestion with WAS is an effective way to improve the biogas yields of microalgae *Chlorella*. The specific conclusions drawn from this study are listed as follows:
• Chlorella grown in the synthetic media produced more soluble EPS than cell or floc-associated EPS as activated sludge.

• Anaerobic co-digestion of Chlorella with varying amounts of WAS increased biogas yields of Chlorella by 73-79%.

• Co-digestion sets with 4% and 11% of algae significantly improved the dewatering rate of digestion product compared to the controls that digested only WAS or algae.

• Mesophilic anaerobic co-digestion of algae and sludge was a suitable approach to increase the biogas yield of algae and also improved the solids reduction of WAS.

Acknowledgements

The authors would like to thank the Research Council of Norway and Biowater Technology AS, Norway for their financial support on this research.
References


### Table 3.1 Soluble and biomass-associated (extracted) EPS in *Chlorella sp.* and waste activated sludge

<table>
<thead>
<tr>
<th></th>
<th>Soluble EPS (mg/g VS)</th>
<th>Sonication-extracted EPS (mg/g VS)</th>
<th>Base-extracted EPS (mg/g VS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pn</td>
<td>ps</td>
<td>COD</td>
</tr>
<tr>
<td>Activated sludge</td>
<td>2.8</td>
<td>0.2</td>
<td>15.8</td>
</tr>
<tr>
<td><em>Chlorella</em></td>
<td>N.A</td>
<td>12</td>
<td>39.1</td>
</tr>
</tbody>
</table>

N.A: not available because of high salt in solution interfering protein measurement  
 pn*: protein  
 ps**: polysaccharide

### Table 3.2 Concentration of methane in mesophilic (37°C) anaerobic digestion at different digestion time

<table>
<thead>
<tr>
<th>Feed composition of algae by mass (VS) in digester (%)</th>
<th>CH₄ day 13 (%)</th>
<th>CH₄ day 29 (%)</th>
<th>CH₄ day 42 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>49.5</td>
<td>54.5</td>
<td>62.6</td>
</tr>
<tr>
<td>4</td>
<td>46.8</td>
<td>55.3</td>
<td>64.5</td>
</tr>
<tr>
<td>11</td>
<td>45.9</td>
<td>62.3</td>
<td>60.1</td>
</tr>
<tr>
<td>41</td>
<td>40.0</td>
<td>63.2</td>
<td>63.2</td>
</tr>
<tr>
<td>100</td>
<td>4.9</td>
<td>34.7</td>
<td>47.4</td>
</tr>
</tbody>
</table>

*This composition is only based on the feed (algae + WAS). Each digestion bottle also included approximately 4% mass (VS) of anaerobic seed sludge

### Table 3.3 Dewatering rates and chemical properties of digested product

<table>
<thead>
<tr>
<th>Feed Composition</th>
<th>Normalized CST (sec L/g TS)</th>
<th>Protein (mg/L)</th>
<th>Polysaccharide (mg/L)</th>
<th>COD (mg/L)</th>
<th>NH₄⁺-N (mg/L)</th>
<th>PO₄³⁻-P (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% WAS</td>
<td>494</td>
<td>27.3</td>
<td>36.0</td>
<td>1023</td>
<td>591</td>
<td>202</td>
</tr>
<tr>
<td>4% algae</td>
<td>349</td>
<td>60.1</td>
<td>36.9</td>
<td>1051</td>
<td>593</td>
<td>189</td>
</tr>
<tr>
<td>11% algae</td>
<td>244</td>
<td>119.5</td>
<td>36.9</td>
<td>854</td>
<td>583</td>
<td>167</td>
</tr>
<tr>
<td>41% algae</td>
<td>724</td>
<td>54.9</td>
<td>39.3</td>
<td>1118</td>
<td>477</td>
<td>107</td>
</tr>
<tr>
<td>100% algae</td>
<td>694</td>
<td>5.5</td>
<td>75.0</td>
<td>2556</td>
<td>392</td>
<td>36</td>
</tr>
</tbody>
</table>

44
Figure 3.1 Efficiency of volatile solids reduction (VSR) in mesophilic digestion with varying amounts of *Chlorella* and waste activated sludge.

Figure 3.2 Cumulative biogas yields obtained from mesophilic anaerobic digestion sets with varying amounts of *Chlorella* and waste activated sludge. The % indicates the mass proportion of *Chlorella* in each digester in terms of VS.
CHAPTER 4

CULTIVATION AND ANAEROBIC CO-DIGESTION OF MICROALGAE FOR WASTEWATER TREATMENT SYSTEMS

4.1 Abstract

In this study, two wildtype microalgal species harvested from a local WWTP, *Micractinium* and *Chlorella*, were cultivated in primary effluent, sludge centrate, and a mixture of centrate and primary effluent. An algal co-digestion study was also performed by digesting *Chlorella* grown in the synthetic medium and waste activated sludge (WAS) under mesophilic conditions. The cultivation study results showed that primary effluent supported better growth of both algal species, which also led to higher soluble nitrogen (84-92%) and phosphorous (59-74%) removal. The phosphorous-limited condition led to overall relatively low growth rate for both species in all media investigated. Microscopic analysis indicated that different species of microalgae pose different growth characteristics in real wastewater, which could be important information for harvesting of algae. The results from the co-digestion study showed that addition of microalgae did not bring negative effects on anaerobic digestion of sewage sludge. These results suggest that a microalgae-based process has the potential to decrease nutrient loads into wastewater treatment systems and to produce bioenergy by co-digestion of harvested algae with sewage sludge in existing anaerobic digesters.

**Keywords:** *Chlorella, Micractinium, microalgae, nutrient removal, anaerobic co-digestion*
4.2 Introduction

Microalgae have gained high attention as renewable bioenergy resources due to their fast photosynthetic growth rate (Chisti, 2007) and their use for production of biodiesel. Despite the convenience to obtain direct biofuel from algal biomass, the high cost of lipid extraction from algae and the high content of unsaturated fat within their biomass make the direct production of algal biodiesel a challenge in real application (Chisti, 2007; Sialve et al., 2009; Singh et al., 2011).

Sialve et al. (2009) suggested that anaerobic digestion should be the optimal method for the energy recovery from algal biomass if the lipid content in the algae is not greater than 40%. Nevertheless, anaerobic digestion of pure algal biomass showed relatively low gas yield compared to sewage sludge. The first algal digestion study by Goluke et al. (1957) showed that anaerobic digestion of microalgae is feasible but the digestion efficiency was 60-70% less than that of the digestion of raw sludge. This is likely due to the characteristics of strong and thick cell walls of the photosynthetic organisms and to the high protein content in algal biomass which can lead to a significant release of ammonia potentially inhibiting the anaerobic digestion process (McCarty, 1964; Yen and Brune, 2005; Chen et al., 2008). Later, Samson and LeDuy (1983) and our recent study (Yuan et al., 2012) showed that co-digestion with sewage sludge could improve the digestibility of microalgae, possibly due to the enhanced hydrolysis of algal biomass by sludge bacteria.

Microalgae have also shown potential to remove nitrogen and phosphorus from different types of wastewater (Wang et al., 2010; Sahu and Rusten, 2011; Yuan et al., 2012). The main mechanism of algal nutrient removal includes uptake of nutrients by
algae and stripping of ammonia due to the increase in pH from photosynthesis (Aslan and Kapdan, 2006). Nutrient removal by microalgae, if successfully implemented, may have several advantages over the conventional biological nutrient removal processes used in WWTP and they include low capital and operational costs, no need of addition of organic carbon for nutrient removal, simultaneous mitigation of CO$_2$ footprint, and production of oxygen during treatment process (Aslan and Kapdan, 2006; Li et al., 2010). In addition, harvested algal biomass can be used for anaerobic digestion at a WWTP for energy generation and the nitrogen and phosphorus released from anaerobic digestion could be recycled for algae growth.

In spite of these increasing interests in algal cultivation and anaerobic digestion of algae, little study has been performed to combine these two topics together, especially for cultures grown in the real wastewater. Hence, much still needs to be learned about cultivation of algae in different types of wastewater and direct anaerobic digestion of cultivated algae. The objective of this study was therefore to evaluate nutrient removal by microalgae and anaerobic co-digestion of microalgae and sewage sludge for wastewater treatment systems. In this study, two wildtype microalgae collected and isolated from the primary clarifiers from a local WWTP were cultivated under different types of wastewater and light conditions to study their growth and nutrient removal. Anaerobic digestion of cultivated microalgae and waste activated sludge (WAS) was also performed to investigate the effect of a small addition (less than 10% by VS) of algal biomass on the digestibility of sewage sludge.
4.3 Methods

4.3.1 Microalgae strains and cultivation in synthetic media

Microalgae were first collected from the wall of the primary clarifier at a local WWTP at different time. The collected algae underwent continuous inoculation steps with modified Basal Medium (Stein, 1973; Andersen, 2005) to obtain and maintain pure cultures of these microalgae. Algae were inoculated at 20°C constant culture room in 1 L Erlenmeyer flasks with a working volume of 700 mL. The flasks were covered with cotton and had side arm for gas exchange. Cultures were kept suspended by shaking at 145 rpm using a G10 gyratory shaker (New Brunswick Scientific, Inc., Edison, NJ). A 24 hour continuous light condition (~ 5,000 Lux) was provided by six 40-W fluorescent lamps for algal cultivation. Algae cultures were continuously inoculated with fresh medium to maintain pure culture.

4.3.2 Batch cultivation of algae in different types of wastewater

Primary effluent and sludge centrate which is centrate from dewatering of anaerobic digested sludge collected from a local WWTP were pretreated by filtration through filter paper with pore size of 20-25 µm to remove large particles (Whatman Filter Papers, Grade 4). The pretreated primary effluent and sludge centrate were stored in 4°C constant temperature room and used within 24 hours for the cultivation experiment. *Micractinium* and *Chlorella* cultivated in the modified Basal Medium were added to the targeted wastewater medium. The size of the containers and shaking methods were the same as that used for cultivation in the modified Basal Medium. Both 24hr continuous light (~5,000 Lux) and 16hr light/8hr dark conditions were used to cultivate these microalgae. The matrix of algal cultivation is summarized in Table 4.1. Algal biomass
concentrations were monitored by dry weight (total suspended solids) and concentrations of N and P were also measured. During cultivation, each sample was monitored with the Olympus microscope and images were taken with the phase contrast microscope and Normaski Differentiate Interference microscope at the time of chemical analysis.

4.3.3 Anaerobic digestion study

Batch anaerobic co-digestion of WAS and microalgae was conducted under mesophilic conditions (37°C) for 30 days. Activated sludge was collected from the aeration basin of the local WWTP and thickened first by 1hr settling and subsequent centrifuging (3,000 rpm, 5 min) to target approximately 1% total solids. All digestions were performed in 2 L bottles with working volumes of 1.8 L. Biogas was collected in 2.5 L gas bags from the top of each digester. Magnetic stirrers were used to provide continuous mixing for each digester. Digestion was conducted in triplicate. Anaerobic digestion seed sludge was added and resultant mass portion in the digester was 8%. This seed sludge was collected from a lab-scale mesophilic anaerobic digester maintained at an SRT of 60 days fed with WAS. Chlorella grown in the synthetic medium was harvested by centrifuging at 3,000 rpm for 5 minutes. Harvested algae was then added to the digesters to achieve 5% by mass, while WAS accounted for the remaining 95% of the feed to digesters. Anaerobic digestion of WAS was also performed as a control. The study also had time-phased digestion to gain deeper insight of anaerobic algal co-digestion. Ten serum bottles (20 mL) of batch digesters were also setup for each composition (i.e., feed with 5% algae + 95% WAS or 100% WAS). Duplicate serum bottles from each digestion set were collected for measurement on day 0, 3, 5, 13 and 37.
Ammonium ion, total solids, volatile solids, and pH were measured during each sampling event.

4.3.4 Analytical methods

Measurements of total suspended solids (TSS), volatile suspended solids (VSS), total solids (TS), and total volatile solids (VS) were performed according to Standard Methods (APHA et al., 2005). Anions and cations were measured using a Metrohm Peak 850 Professional AnCat ion chromatography system (Metrohm Inc., Switzerland). A calibrated Orion GS9156 pH meter (Thermo Fisher Scientific Inc., Waltham, MA) was used to measure pH. The concentration of methane and carbon dioxide in the anaerobic biogas (500 μL of gas sample) was measured using a gas chromatograph with a thermal conductivity detector (Gow-Mac instrument Co. GC 550 series).

4.4 Results and discussion

4.4.1 Cultivation of microalgae in different types of wastewater

Microscopic and genetic analysis of the wildtype microalgae obtained from the local WWTP indicated that one was a novel species of Micractinium and the other was Chlorella sp.. These algae were cultivated in primary effluent, sludge centrate, and their mixture (50/50, v/v) under 24hr light and 16hr light/8hr dark conditions. Figure 4.1 shows the biomass dry weight obtained during the lab cultivation of Micractinium nov. sp. (hereafter Micractinium) and Chlorella sp. (hereafter Chlorella) In general, both species grew well in the primary effluent regardless of light conditions. The highest biomass dry weight was achieved with the cultivation of Chlorella in the primary effluent under 16hr light/8hr dark condition. Micractinium grown under the same condition also achieved high biomass dry weight. In contrast to the primary effluent medium, sludge centrate did
not support the growth of both types of algae under 16hr light/8hr dark condition and only *Chlorella* in 24hr light showed some growth after 14 days of lag period. This indicates that *Chlorella* were more tolerant than *Micractinium* to the harsh medium condition present in the sludge centrate. The cultivation in the mixture of centrate and primary effluent supported similar growth for both species under 24hr light while the periodic light condition led to better growth for *Micractinium*. These data indicate that primary effluent is better media and growth was generally promoted when a longer light condition was provided.

Among twelve cultivation sets, *Micractinium* grown under 24hr light in the primary effluent resulted in the highest specific growth rate of 0.22 d\(^{-1}\) in the first five days. The average specific growth rate of *Chlorella* and *Micractinium* grown in primary effluent ranged between 0.1 and 0.21 d\(^{-1}\). Algae grown under 24hr light conditions had relatively higher specific growth rate for both species. The highest specific growth rate of 0.21 d\(^{-1}\) for *Chlorella* was achieved for the sludge centrate + 24hr light condition after two weeks of cultivation. The highest specific growth rate of *Micractinium* at 0.17 d\(^{-1}\) under the same condition was achieved after 3 weeks of cultivation. The specific growth rate obtained in this study was generally lower than those reported in other studies that also used primary effluent as growth media. Lau et al. (1995) found a specific growth rate of 0.28 d\(^{-1}\) for *Chlorella vulgaris* cultivated in the primary effluent. The specific growth rates of *Chlorella* *sp.* cultivated in sludge centrate ranged from 0.19 and 0.68 d\(^{-1}\) (Li et al., 2011; Yuan et al., 2012). In general, availability of carbon dioxide, light, and nutrients are considered to be the limiting factors for algae growth. The absence of CO\(_2\) purging and low phosphorous (see below) in wastewater used in this study might have
caused lower algal growth rate. Low P concentration in the wastewater media could be one particular reason for the low growth rate. *Chlorella vulgaris* showed optimal nutrient removal at a N/P ratio of 8 (Kapdan and Aslan, 2008). The empirical formula of microalgae is C_{106}H_{263}O_{110}N_{16}P (N/P ratio = 7.2:1) (Grobelaar, 2007). Li et al., (2010) reported a similar optimal N/P ratio between 5:1 and 8:1 for freshwater microalgae Scenedesmus sp. In this study, the N/P ratios in primary effluent and sludge centrate were 48:1 and 86:1, respectively, indicating severe phosphorous-limited conditions for algae growth.

### 4.4.2 Nitrogen and phosphorous removal during algae cultivation

The N removal efficiency for microalgae grown in the primary effluent reached 84 to 92% (Figure 4.2). The dark/light condition showed the highest N removal efficiency at 92% for both species. Meanwhile, the continuous light condition led to 90% N removal from the cultivation of *Chlorella* and 84% from the cultivation of *Micractinium* (84%). As for the combination of primary effluent and sludge centrate, *Chlorella* showed only 38% removal of soluble N under dark/light condition, while the other cultures showed the N removal efficiency between 61% and 69%.

The P removal efficiency ranged between 60% and 74% for the cultivation in the primary effluent (Figure 4.3). Furthermore, a total of 75-90% of soluble P was removed from the mixed media. *Chlorella* under 24hr light condition led to the highest P removal (90%), while dark/light condition showed only 75% of P removal. *Micractinium* had the same P removal efficiency under two light conditions when they were grown in the combined wastewater.
Sludge centrate used in this study was not a good medium for algal growth. Only *Chlorella* under continuous light condition showed some growth with the maximum soluble N and P removal efficiencies of 51% and 85%, respectively. The other three cultures showed 39-42% N removal and 25-46% removal of soluble P.

As noted above, the empirical chemical formula of microalgae is $C_{106}H_{263}O_{110}N_{16}P$. Although this composition could vary based on different species and growth characteristics of microalgae, nutrient removal by algae can be expected to occur in a N/P ratio of around 7.2:1. The N/P removal ratio obtained in the current study, however, did not show such a value. For example, the initial N/P uptake (removal) ratios for *Chlorella* and *Micractinium* grown in the primary effluent with 24hr light were 38:1 and 43:1, respectively, and later reduced to 13:1 and 19:1. These results indicated an unbalanced uptake of N and P and there was an over-uptake of nitrogen in both microalgal cells. A similar result (over uptake of nitrogen) was also found when *Scenedesmus sp.* was cultivated under high N/P ratios (Li et al., 2010).

### 4.4.3 Microscopic analysis for algae cultivation

Microscopic images of *Chlorella* and *Micractinium* were taken during cultivation. Although algal cells grew and divided in the primary effluent (Figure 4.4) and the mixture medium, they hardly divided in the medium of 100% sludge centrate, consistent with the biomass dry weight data shown in Figure 4.1. *Chlorella* grew and divided well under both light and dark/light conditions. Their growth pattern as picoplanktonic growth in the primary effluent was the same as that in the synthetic regular medium (Figure 4.4 a, b). On the other hand, *Micractinium* grew more slowly under 24hr light but well under the 16hr light/8hr dark condition. They originally formed clusters in the modified Basal
Medium (data not shown). However, *Micractinium* in the primary effluent did not form clusters but were agglutinated with bacteria grown in the cultivation. Although *Micractinium* in both continuous and periodic light conditions became agglutinated with bacteria, since bacteria grew more under the dark condition, they formed more agglutination with bacteria under 16hr light/8hr dark condition (Figure 4.4 c,d).

Karakashina (1970) studied the changes of cell structure of *Chlorella* including chloroplast, lamella, pyrenoids, and starches using a transmission electron microscope. The study showed that *Chlorella* grown heterotrophically in continuous darkness had abundant starch and lipid droplets. A more recent study also showed that *Chlorella* was able to maintain and grow heterotrophically on glucose during the dark condition (Perez-Garcia et al, 2011). Heterotrophic and mixotrophic growth of *Micractinium pusillum* was also reported and growth in the dark + glucose was found to be more important than growth in the light + acetate (Bouarab et al., 2004). In the current study both species of algae formed thick cell walls in the harsh wastewater environment. Cell sizes were smaller than regular algal cells in the Basal Medium. However, the cell sizes of *Chlorella* increased over 31 days of cultivation. In 16hr light/8hr dark, mother cells were rarely four-celled with dividing autospores, but were mostly two- or three-celled. These observations indicate that different species of microalgae pose different growth characteristics in real wastewater, which could be important information for harvesting of algae. The microscopic analysis also showed that more bacteria were present in the dark/light condition for both species.
4.4.4 Anaerobic co-digestion of algae and WAS

Our previous algal co-digestion study showed an improvement of biogas generation of Chlorella when co-digested with WAS (Wang et al., under review). The study showed that the concentration of methane in the biogas from the digestion of pure algae was only 37%. Poor digestibility of pure algae is very likely due to the thick cell walls of microalgae, providing resistance to anaerobic hydrolysis and further degradation. To better understand the effect of addition of algae on existing sewage sludge digestion, both one-time batch and time-phased anaerobic co-digestion were performed for Chlorella cultivated in the synthetic medium in the current study. Figure 4.5 shows similar biogas yield and methane composition between the co-digestion set and control digestion (WAS only) set.

The results from the time-phased digestion also showed very similar degradation trends between the algal co-digestion set and the control (Figure 4.6). The ammonia released from the hydrolysis of proteins mainly happened in the first five days of digestion from both sets along with a pH drop to 6.1 (Figure 4.6a, 4.6d). This data also correlated with the trends of VS reduction data (Figure 4.6c) and, therefore, the released ammonia was linearly related to the amount of VS destroyed (Figure 4.6b). All these data indicated there were no detrimental effects of the addition of microalgae on the anaerobic digestion compared to the control digestion set digesting only WAS. These are significant results because anaerobic co-digestion of algae and sewage sludge will increase the digestibility of algae without causing negative effects on the existing anaerobic digesters, thus permitting more biogas generation from the added algal feedstock.
4.5 Conclusions

This study investigated the cultivation of *Micractinium* and *Chlorella* and their nutrient removal in different types of wastewater. *Chlorella* from the synthetic medium were also co-digested with WAS to investigate the biogas yield and the effect of co-digestion on the digestibility of WAS. Major findings of this study are listed as follows:

- *Micractinium* and *Chlorella* in the primary effluent showed better growth and higher soluble nitrogen (84-92%) and phosphorous (59-74%) removal than from other two media.
- Sludge centrate did not support the growth of algae. Only *Chlorella* under 24hr light conditions showed some growth after 13 days of cultivation.
- *Chlorella* showed better ability to adapt to a harsh medium condition compared to *Micractinium*.
- The specific growth rates of *Micractinium* and *Chlorella* in all cultivation conditions were relatively low compared to previously published studies, possibly due to phosphorous and CO₂ limitations.
- Microscopic analysis indicated that different species of microalgae pose different growth characteristics in real wastewater, which could have influence on the downstream harvesting strategy.
- Co-digestion of algae and WAS did not cause detrimental effects on anaerobic digestion of WAS.
References


Table 4.1 Matrix of microalgae cultivation performed in this study

<table>
<thead>
<tr>
<th>Species of microalga</th>
<th>Light conditions</th>
<th>24h Light</th>
<th>16h Light / 8h Dark</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ratio of sludge centrate to primary effluent</td>
<td>Ratio of sludge centrate to primary effluent</td>
<td></td>
</tr>
<tr>
<td><em>Chlorella sp.</em></td>
<td>1:0</td>
<td>1:1</td>
<td>0:1</td>
</tr>
<tr>
<td><em>Micractinium nov. sp.</em></td>
<td>1:0</td>
<td>1:1</td>
<td>0:1</td>
</tr>
</tbody>
</table>

Figure 4.1 (a) Biomass dry weight under 24hr light conditions and (b) biomass dry weight under 16hr light and 8hr dark conditions

In the legend, S: anaerobic sludge centrate, 1E: primary effluent, S1E: one portion of sludge centrate and one portion of primary effluent
Figure 4.2 (a) Soluble N of *Chlorella* and *Micractinium* grown in primary effluent; (b) soluble N of *Chlorella* and *Micractinium* grown in mixture of sludge centrate and primary effluent; (c) soluble N of *Chlorella* and *Micractinium* grown in sludge centrate.

In the legend, L: 24hr light; DL: 16hr light+8hr dark; 1E: primary effluent; S1E: mixture of sludge centrate and primary effluent; S: sludge centrate.
Figure 4. 3(a) Soluble P of *Chlorella* and *Micractinium* grown in primary effluent; (b) soluble P of *Chlorella* and *Micractinium* grown in mixture of sludge centrate and primary effluent; (c) soluble P of *Chlorella* and *Micractinium* grown in sludge centrate.

In the legend, L: 24hr light; DL: 16hr light+8hr dark; 1E: primary effluent; S1E: mixture of sludge centrate and primary effluent; S: sludge centrate.
Figure 4. 4 Microscopic images of *Chlorella* and *Micractinium* grown in primary effluent for 31 days

(a) *Chlorella* under 24hr light condition; autospores (most of them are four cells in a mother cell) are ready to divide. (b) *Chlorella* 16hr light /8hr dark condition; majority is two–celled dividing autospores. (c) *Micractinium* under 24hr light condition; (d) *Micractinium* under 16hr light/ 8hr dark condition, two- or three-celled dividing autospores are abundant. Magnifications of a, b, c and d are X400.

Figure 4. 5 Anaerobic co-digestion of algae and WAS: (a) biogas yield of anaerobic co-digestion; (b) methane concentration in biogas
Figure 4.6 Time phase anaerobic digestion of 5% of algae (co-digestion with 95% of WAS) and 100% of WAS. (a) Ammonia concentration over time; (b) relationship of ammonia and volatile solids reduction; (c) volatile solids over digestion time; (d) pH changes over digestion time.
CHAPTER 5

INVESTIGATION OF NUTRIENT REMOVAL, CATION UPTAKE, AND
EXPRESSION OF EXTRACELLULAR POLYMERIC SUBSTANCES OF
MICROALGAE, CHLORELLA SP. AND MICRACHTINUM NOV., DURING
WASTEWATER TREATMENT

5.1 Abstract

Two species of green algae, Chlorella sp. and Micractinium nov., obtained from the
primary clarifier’s wall at a local wastewater treatment plant were cultivated in primary
effluent and in a mixture of sludge centrate and primary effluent (SC1) to study nutrient
removal and expression of extracellular polymeric substances (EPS) during growth. The
nitrogen to phosphorous (N/P) ratio of the primary effluent was around 9 and the N/P of
SC1 was larger than 50 indicating P-limited conditions. Both Chlorella sp. and
Micractinium nov. grew well in these two media, with average specific growth rates from
0.19 to 0.30 d⁻¹ for both species. The higher initial N concentration in SC1 led to higher
specific N removal rate. The high N/P in growth media resulted in more uptake of N than
for algae grown in a balanced N/P growth media. The first order reaction rate constant for
N removal in the primary effluent was 0.16 d⁻¹ which is about 3 times higher than that
from SC1. The first order reaction rate constant for P removal was similar for both
cultivation media. The algae grown in SC1 also showed a higher specific removal rate of
K⁺ (above 0.5 mg mg⁻¹ chl a d⁻¹) but a much lower Ca²⁺ reduction rate (below 0.1
mg mg⁻¹ chl a d⁻¹) for both Chlorella and Micractinium. Algae grown in SC1 produced
higher EPS for both species which was due to the unbalanced N/P and high nutrient load
in SC1. For the algae grown in the same medium, Micractinium produced more protein-
EPS than Chlorella. The differences of EPS may affect the subsequent harvesting and anaerobic digestion.

**Key words:**

Microalgae, Chlorella, Micractinium, nutrient removal, N/P ratio, cations and EPS.

**5.2 Introduction**

Microalgae-based wastewater treatment has gained much attention these days because algae have high photosynthetic growth rate, absorption of CO$_2$ during photosynthesis, and ability to remove nutrients from different sources of wastewater (Park et al., 2009; Wang et al., 2009; Yuan et al., 2012). Algal biomass harvested from wastewater treatment can then be used as feedstock for biofuels. Nutrient removal by microalgae has several advantages over conventional biological nutrient removal processes used in a wastewater treatment plant (WWTP) including: no need of additional organic carbon for nutrient removal, simultaneous mitigation of CO$_2$ output, production of oxygen during treatment process, and low capital and operational costs (Aslan & Kapdan, 2006; Li et al., 2010). With these merits, algae based wastewater treatment would contribute to the better management of wastes.

The mechanisms of nitrogen removal from wastewater could be N uptake by algae biomass and the stripping of ammonia due to the increase of pH during algae growth (Bich et al., 1999). Phosphorus is also removed by algae cultivation through bioassimilation, adsorption, and chemical precipitation above pH 8 (Li et al., 2011; Song et al., 2002). Many factors could affect the growth characteristics of algae and the kinetics of nutrient removal, which include light conditions, CO$_2$ purging, and nutrient concentrations (Hu et al., 2012; Li et al., 2010; Li et al., 2012; Ruiz et al., 2011). Since
the main mechanism of nutrient removal is algal assimilation, the nutrient concentration in the growth medium is a key factor to regulate growth characteristics of algae and the nutrient removal kinetics.

The extracellular polymeric substances (EPS) of microorganisms in wastewater are composed of metabolites of microbial activity, intracellular material released by cell lysis, and organic matter adsorbed from wastewater (Dignac et al., 1998; Jorand et al., 1995; Park & Novak, 2007; Yu et al., 2008). It has been seen that short term variations in environmental conditions affected the secretion of EPS by microbial communities (Yang & Li, 2009). EPS in turn has been shown to govern floc formation, settleability, and dewaterability of microorganisms, as well as effluent quality (Dignac et al., 1998; Li & Yang, 2007; Liu & Fang, 2003; Neyens et al., 2004; Park & Novak, 2009; Subramanian et al., 2010; Yu et al., 2009). The algal metabolism pathway will be shifted under different growth conditions including nutrients, light and other environmental stresses. Different metabolites from algae will result in changes in quality and quantity of the EPS, which may affect algae harvesting and digestibility. The nutrient level and composition for different streams of wastewater will be significantly different, which may affect the expression of algal EPS. However, there is little research on the characteristics of algal EPS. Fundamental research on the characteristics of algal EPS will be helpful to better understand their effects on subsequent anaerobic digestion.

The microalgae species commonly used for nutrient removal are *Scenedesmus, Chlorella*, and *Spirulina* (Bich et al., 1999; Li et al., 2010; Yuan et al., 2012). However, little research on another microalgal species, *Micractinium*, has been done regarding their ability for nutrient removal and their growth characteristics. In this
study, two wildtype green algal species, *Chlorella* sp. and a novel species of *Micractinium nov.*, which were originally collected from a local WWTP, were cultivated in different streams of wastewater. The objectives of this study were to evaluate the kinetics of nutrient removal by the growth of two species of green algae in different wastewater with different N/P ratios and the effect of N/P ratio on cation uptake and characteristics of EPS, which could be important for physiological characteristics of algae and their subsequent effect on bioenergy harvesting.

**5.3 Methods**

**5.3.1 Microalgae strains**

The environmental samples of microalgae were collected, in different seasons, from the wall of the primary settling tank at a local WWTP. The collected algae underwent continuous inoculation with Basal Media (Andersen, 2005; Stein, 1973) to obtain and maintain pure cultures. One algae species was identified as a novel species of *Micractinium nov.*, based on genetic analysis (S. Dolan and Germany Prof, 2012), which is the same family as *Chlorella* but different genus and species. The other species was identified as *Chlorella* sp., which is commonly found in freshwater and wastewater environments. Algae were inoculated at a 20°C in a temperature-controlled room in 1 L Erlenmeyer flasks with a working volume of 700 mL. 24 hour continuous external illuminations were provided by six 40-W fluorescent lamps. The light intensity in the middle of the culture bottles was around 60 \( \mu \text{mol m}^{-2} \text{ s}^{-1} \). The flasks were covered with cotton and further had a side-arm for gas exchange. Cultures were kept suspended by shaking at 145 rpm using a G10 gyratory shaker (New Brunswick Scientific, Inc., Edison,
NJ). Both algae cultures were continuously inoculated with fresh medium to maintain pure culture.

5.3.2 Microalgae cultivation in different wastewater

The primary effluent and sludge centrate from dewatering process of anaerobic digested sludge were collected from a local WWTP. The two types of wastewater were filtered through filter paper with a pore size of 20-25 μm to remove large particles (Whatman Filter Papers, Grade 4) and no additional autoclave was applied. Preliminary experiment indicated that undiluted sludge centrate was too strong as algae were unable to survive in pure sludge centrate. The filtered sludge centrate was then diluted by 3 times with primary effluent, and this mixture of wastewater is called SC1 hereafter. The soluble N concentration in SC1 was around 200 mg/L for algae cultivation. N and P removal by algae were performed in a batch mode in a 2 L Pyrex bottle. The volume of thickened algae inoculum accounted for 10 % of the total volume of all batch cultivation, and targeted an initial biomass concentration of around 200 mg/L as dry weight. The light intensity was the same as above for pure cultures. Periodic light of 16-hr light/8-hr dark was applied for algae cultivation. The batch reactors were also bubbled with house air mixed with 1.5% (v/v) CO₂ addition. Magnetic stirrers were used to mix and keep the culture suspended. The growth matrix of algae is shown in Table 5.1.

5.3.3 Extraction of algae EPS

EPS of algae were evaluated by sonication and base extraction at the end of cultivation. Algae were centrifuged at 9000 rpm for 15 minutes and the supernatant was removed. The pellet after centrifuge was resuspended in 10 mM NaCl solution for based extraction. The procedure for base extraction followed the method described in Park and
Novak (2007). The method for sonication was performed following the method in Wang et al. (in preparation). The quantity of protein of EPS was measured by modified Lowry Methods (Frølund et al., 1996; Lowry et al., 1951). The concentration of polysaccharide was measured by the Dubois et al. (1956) method.

5.3.4 Analytical methods

The biomass dry weight, Chlorophyll a, soluble and total COD, nitrogen and phosphorus were measured during cultivation. Soluble samples referred to a 0.45 μm filtered sample. The dry weight, Chlorophyll a and COD were measured by standard method (APHA, 1998). The N and P were measured by Hach TNTplus chemical kits (Hach, US). The size distribution and zeta potential for algae were also measured at the end of cultivation. The size distribution was measured by Malvern Mastersizer 2000 (Malvern Instruments Ltd, UK) and the Zeta potential was measured by Malvern ZetaMaster (Malvern Instruments Ltd, UK). The main groups of anions and cations were monitored by a Metrohm Peak 850 Professional AnCat ion chromatography system (Metrohm Inc., Switzerland) during algae cultivation.

5.4 Results and discussion

5.4.1 Characteristics of algae grown in different wastewater

The chemical composition of primary effluent and anaerobic sludge centrate was substantially different. The effluent from anaerobic digester (sludge centrate) contained high amount of ammonium which is usually returned to the headwork of the treatment process and increases the N load to the wastewater treatment system. Different compositions of wastewater will support different growth properties of algae. Both Chlorella and Micractinium grew well in primary effluent and SC1 (Figure 5.1). High
nitrigen concentration in sludge centrate promoted algae growth and resulted in higher average specific growth rate of both Chlorella and Micractinium (Table 5.2). The average specific growth rates of Chlorella and Micractinium grown in SC1 were 0.27 d⁻¹ and 0.30 d⁻¹, respectively, which were higher than those from primary effluent (0.19 and 0.21 d⁻¹, respectively). These data indicated that growth medium rather than the species led to different algal productivity for these two green algae.

The average size of these two algal species were approximate 4.2 to 4.8 μm and much smaller than the average size of the same algae grown in synthetic Basal media (8.05 μm and 5.43 μm for Chlorella and Micractinium, respectively), implying that the bacteria and nutrient level in real wastewater posed stress for their growth, resulting in smaller cell sizes. Meanwhile, Chlorella and Micractinium grown in the same wastewater also showed similar values of zeta potential (Table 5.2). The algae was cultivated with CO₂ addition to avoid the pH increase during algae growth and the pH of the cultures was controlled between 6.3 and 7.4.

5.4.2 Nitrogen and phosphorus removal

Nitrogen and phosphorus removal from different wastewater by Chlorella and Micractinium were evaluated under batch cultivation mode. Both Chlorella and Micractinium demonstrated good ability for nitrogen and phosphorus removal from different types of wastewater. The changes of N and P concentrations with cultivation time are shown in Figure 5.2. More than 95% of the soluble P in primary effluent and SC1 was removed from the media when the initial P concentrations were 4 and 3.5 mg/L for primary effluent and SC1, respectively. The final P concentrations in all growth media were below 0.17 mg/L. The soluble N in the primary effluent sets were reduced by
95.7% and 93.9 % for Chlorella and Micractinium, respectively. The N concentrations in primary effluent were 1.5 and 2.2 mg/L for Chlorella and Micractinium, respectively. The initial N concentration in SC1 was around 200 mg/L. Soluble nitrogen in SC1 mixture was decreased by 50.6% and 45.7 % for Chlorella and Micractinium, respectively. The nitrogen removal efficiency was lower when the nutrient from the sludge centrate was at a high level. Nitrogen and phosphorus removals by microalgae were highly dependent on the composition of the growth medium. The initial N/P ratio in the primary effluent and SC1 were 9 and 56, respectively. The SC1 medium was phosphorus limited for algae growth. The P-limited condition in SC1 should have hindered further assimilation of N by microalgae. Nevertheless, a significant amount of N was removed by the algae. The P in SC1 was below 0.17 mg/L, while the N in the media was approximate 100 mg/L for both algae species at the end of cultivation. This highly unbalanced N and P ratio in the media reduced the further removal of nitrogen from the growth media. The algae in the SC1 increased significantly, and the Chlorophyll a for Chlorella increased from 2.2 to 13 mg/L and that of Micratinium increased from 1.7 to 16 mg/L within 14 days of cultivation.

5.4.2 Kinetics of nitrogen and phosphorus removal

The nitrogen and phosphorus removal rates were determined using Equation 5.1.

\[
R = \frac{s_0 - s_i}{t_i - t_o}
\]  

Equation 5.1

Where \( R \) represents the removal rate of nutrients (nitrogen or phosphorus), \( s_0 \) represents the initial concentration of nutrients, \( s_i \) is the nutrient concentration at time \( t_i \), and \( t_i \) is the time when there was no significant change in the nutrient concentration. The
specific nutrient removal rate was obtained by normalizing R by the initial chlorophyll a (chl a) concentration.

The specific N removal rates (R_N) for *Micractinium* grown in SC1 and primary effluent and *Chlorella* grown in SC1 and primary effluent were 4.4, 1.9, 3.8 and 1.8 mg mg⁻¹ chl a d⁻¹, respectively. The maximum R_N (4.4 mg mg⁻¹ chl a d⁻¹) was achieved by *Micractinium* grown in SC1, which is higher than the maximum rate of 3.0 mg mg⁻¹ chl a d⁻¹ reported by Aslan and Kapdan (2006). The initial nitrogen in the growth medium of SC1 was 197 mg/L, while the initial nitrogen concentration in primary effluent was 36.1 mg/L. These results indicated that the higher initial N led to higher R_N. A similar trend was reported in Aslan and Kapdan (2006). The specific nitrogen removal rates increased from 0.5 to 3 mg mg⁻¹ chl a d⁻¹ with the increase of initial NH₄-N, from 13.2 mg/ L to 410 mg/L (Aslan & Kapdan, 2006). The real wastewater lead to a higher specific N removal rate compared to the synthetic medium used by Aslan and Kapdan (2006).

Although the N in sludge centrate was very high, the P in the sludge centrate was very low due to chemical P removal performed at WWTP for the anaerobically digested sludge. The initial P in the SC1 (3.5 mg/L) was similar to that in the primary effluent (4.0 mg/L). The specific P removal rates (R_P) for *Chlorella* and *Micractinium* grown in SC1 were 0.15 and 0.19 mg mg⁻¹ chl a d⁻¹ respectively. R_P for *Chlorella* and *Micractinium* grown in primary effluent were 0.25 and 0.38 mg mg⁻¹ chl a d⁻¹, respectively. In general, the higher initial P concentration led to slightly higher R_P in primary effluent compared to that achieved in SC1. This data is also similar to that of
0.2 mg mg\(^{-1}\) chl \(a\) d\(^{-1}\) reported by Aslan and Kapdan (2006) when the initial concentration of P was 7.7 mg/L.

N and P removal can be regarded as first order reaction. Therefore, the reaction of N and P removal can be expressed by Equation 5.2:

\[ S_t = S_0 \times e^{-kt} \]  \hspace{1cm} \text{Equation 5.2}

Where, \( S_t \) represents the nutrient concentration, \( S_0 \) represents the initial concentration of nutrient, and \( k \) is the first order reaction rate constant.

The yield of biomass/nutrient was calculated using Equation 5.3:

\[ Y = \frac{C-c_0}{s_0-s} \]  \hspace{1cm} \text{Equation 5.3}

Where \( Y \) is the yield of biomass linked with consumption of nutrients (N or P); \( S_0 \) is initial nutrient concentration, \( S \) is the concentration of nutrients when there was no significant decrease of substrate, \( C \) represents for the concentration of Chlorophyll a with respect to the nutrient \( S \), and \( c_0 \) is the initial concentration of Chlorophyll \( a \) (chl \( a \)). The initial N/P ratio, the yield of biomass and the specific nutrient removal rates are shown in Table 5.3.

As shown in Table 5.3, the \( Y_N \) for \textit{Chlorella} and \textit{Micratinium} grown in primary effluent were 0.18 and 0.23 mg chl \( a \) mg\(^{-1}\) N, respectively. While the \( Y_N \) for \textit{Chlorella} and \textit{Micratinium} grown in SC1 were 0.10 and 0.12 mg chl \( a \) mg\(^{-1}\) N, respectively, which are lower than those cultivated in primary effluent. The \( Y_P \) for \textit{Chlorella} and \textit{Micratinium} grown in primary effluent were 1.6 and 2.0, and \( Y_P \) were 2.4 and 2.9 for the algae grown in SC1. The algae grown in primary effluent had a higher yield of biomass based on N consumption, while the SC1 had a higher yield based on P consumption. As shown in Table 5.3, the ratio of \( Y_P / Y_N \) for primary effluent was much lower than that in SC1,
which indicated that more nitrogen was consumed to produce the same amount of algae biomass when algae was grown in SC1. The ratio of $Y_p / Y_N$ indicated the $N_{\text{uptake}}/P_{\text{uptake}}$ ratio required to produce per unit biomass. The theoretical formula of microalgae is $C_{106}H_{181}O_{45}N_{16}P$ (Groppelaar, 2007), where the $N_{\text{uptake}}/P_{\text{uptake}}$ is 7.2. The $Y_p / Y_N$ for Chlorella and Micractinium grown in primary effluent were 8.9 and 8.7, respectively, which are very close to the theoretical value. But the $Y_p / Y_N$ for algae grown in SC1 was larger than 20 (Table 5.3), which is much higher than the theoretical value, indicating an over uptake of N. The initial N/P ratio in the SC1 (> 50) was much higher than that in primary effluent (9). The SC1 was P-limited for algae growth and led to the over uptake of N for algae growth, which is the same phenomenon as seen in our previous study (Wang et al., 2012).

5.4.3 Removal of cations during algae growth

Cations concentrations were monitored during cultivation in primary effluent and SC1 (Figure 5.3). The sodium remained constant during cultivation, and no significant decrease in sodium was observed. Cations are removed by algae through transport through the cell membrane, bioadsorption and precipitation (Malik, 2004). The yield of biomass based on cation reduction was calculated by Equation 5.3. The biomass yield based on Mg $^{2+}$ was in the range of 2.24 to 3.35 mg chl a mg$^{-1}$ Mg $^{2+}$ for all cultures. The yields of Chlorophyll a in the primary effluent based on K$^+$ consumption were 1.36 and 2.36 mg Chl a mg$^{-1}$K$^+$, for Chlorella and Micractinium, respectively. The microalgae yields based on K$^+$ for Chlorella and Micractinium grown in SC1 were only 0.51 and 0.71 mg Chl a mg$^{-1}$K$^+$, respectively, which are much lower than those grown in primary effluent.
The sludge centrate contained higher amounts of Mg\(^{2+}\) and K\(^+\) compared to primary effluent, due to the release of cations during anaerobic digestion process. When using sludge centrate and primary effluent for algae growth, the levels of intracellular cations of different algae species in different growth medium will also differ.

The specific cation removal rate is shown in Table 5.4. The specific K\(^+\) remove rate is high, which is the same trend as for the growth rate of algae. The specific K\(^+\) reduction rate is much higher for algae grown in SC1; rates for *Chlorella* and *Micractinium* were 0.57 and 0.59 mg mg\(^{-1}\) *chl a* d\(^{-1}\), respectively. These values are higher than that of algae grown in primary effluent. The requirement of K\(^+\) for algae growth was higher compared to other trace elements. K\(^+\) was one of the major cations support the growth of cells. The main function of K\(^+\) is to maintain electroneutrality and osmotic equilibrium (Rodriguez-Navarro & Rubio, 2006). K\(^+\) can also regulate some biochemical reactions where protein activation are affected by irreplaceable K\(^+\)-protein interaction, which makes K\(^+\) a necessary element for algae growth (Rodriguez-Navarro & Rubio, 2006). Compared to the requirement for other cations, K\(^+\) had the highest algal uptake rates. The specific removal rates for Na\(^+\) and Mg\(^{2+}\) did not vary a lot for all the cultures (Table 5.4). However, the specific removal rate of Ca\(^{2+}\) from algae grown in primary effluent was much higher than that for algae growing in SC1. The removal of Ca\(^{2+}\) in an algae treatment system may result from precipitation and from uptake by algae. The unchanged Na\(^+\) along with algae growth indicated a balanced osmotic system between intracellular and outside environments.
5.4.4 EPS of algae grown in different wastewater

Sonication and base extraction data indicated that the former extraction was more effective in harvesting EPS from algae than the latter (Figure 5.4). Algae are usually tolerant to high pH conditions (Wang et al., under review), therefore, base extraction did not extract much EPS from algal cultures. Both protein and polysaccharide in algal EPS were reduced during cultivation. The protein content in *Micractinium*-EPS was higher compared to *Chlorella*-EPS at the end of cultivation for both media. For microalgae grown in primary effluent, the quantities of extracted protein and polysaccharide were similar to each other while for the same algae cultivated in SC1, protein was more abundant than polysaccharide.

The quantity and quality of EPS could be related to the physiology of microorganisms. Previous studies (Wang et al. 2010) showed that microorganism grown in harsh condition produce a larger amount of EPS. It could be clearly seen that overall EPS was much greater for microalgae grown in SC1 than that from primary effluent cultivation, indicating that growth of microalgae in the presence of sludge centrate led to much higher EPS expression. The high nutrient load and the extremely unbalanced N/P ratio in SC1 could have been a harsh condition for algae growth, which is thought to lead to the expression of higher EPS. EPS will affect the bioflocculation, settling and the digestibility of biomass (Liu & Fang, 2003; Park & Novak, 2009). The digestibility and settling ability of biomass or sludge usually deteriorate with increased amounts of EPS (Liu & Fang, 2003; Wang et al., 2010). The higher amount of protein content in sonication extracted EPS for algae grown in SC1, especially *Micractinium*, probably poorly digested under anaerobic condition. Further research on the relationship of algal
EPS and their digestibility would be helpful to predict the performance of anaerobic digestion for bioenergy generation.

5.5 Conclusions

Wild type *Chlorella* and *Micratinium* were cultivated in different wastewaters to study the kinetics of nutrient removal, the cation removal and the expression of EPS. The main conclusions from this study are:

- *Chlorella* and *Micratinium* can grow in primary effluent and SC1 along with good nutrient removal from growth media.
- Over 93% of N and 95% of P in primary effluent were removed by microalgae. The N removal efficiency from SC1 was relatively low due to P-limited conditions.
- Higher initial N concentration resulted in higher specific N removal rate ($R_N$).
- The P-limited condition in SC1 led to the over-uptake of N by both algae species.
- The specific removal rates of $K^+$ were higher compared to the rates for other cations. A high N/P ratio induced a higher specific $K^+$ removal rate but a lower $Ca^{2+}$ removal rates.
- Algae grown in balanced N/P media produced less EPS compared to algae grown in harsh media SC1, which had high nutrient load and extremely limited P for algae growth.
References


### Table 5.1 Basic conditions of microalgae cultivation

<table>
<thead>
<tr>
<th>Species of microalgae</th>
<th>Ratio of sludge centrate to primary effluent</th>
<th>Light intensity (μmol m⁻² s⁻¹)</th>
<th>Light period</th>
<th>Aeration</th>
<th>Temperature (°C)</th>
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<tbody>
<tr>
<td>Chlorella sp.</td>
<td>0:1</td>
<td>1:2</td>
<td>60</td>
<td>~1.5% CO₂ combined with house air</td>
<td>23±2</td>
</tr>
<tr>
<td>Micractinium nov.</td>
<td>0:1</td>
<td>1:2</td>
<td>60</td>
<td>~1.5% CO₂ combined with house air</td>
<td>23±2</td>
</tr>
</tbody>
</table>

### Table 5.2 Various characteristics of microalgae grown in different wastewater

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average specific growth rate (d⁻¹)</th>
<th>pH at light period</th>
<th>Zeta potential (mV)</th>
<th>Mean size (μm)</th>
<th>Soluble N removal efficiency (%)</th>
<th>Soluble P removal efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chl-1’</td>
<td>0.19</td>
<td>6.35</td>
<td>-22.0±0.8</td>
<td>4.19</td>
<td>95.7%</td>
<td>96.4%</td>
</tr>
<tr>
<td>Mic-1’</td>
<td>0.21</td>
<td>6.30</td>
<td>-22.2±1.9</td>
<td>4.73</td>
<td>93.9%</td>
<td>96.1%</td>
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<td>Chl-SC1</td>
<td>0.27</td>
<td>7.41</td>
<td>-20.0±0.8</td>
<td>4.17</td>
<td>50.6%</td>
<td>95.3%</td>
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<tr>
<td>Mic-SC1</td>
<td>0.30</td>
<td>7.25</td>
<td>-18.7±1.0</td>
<td>4.85</td>
<td>45.7%</td>
<td>95.2%</td>
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</table>

### Table 5.3 Kinetics parameters of nutrient removal in different wastewater

<table>
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<tr>
<th>Sample</th>
<th>Initial N/P</th>
<th>Yₚ (mg Chl a mg⁻³ N)</th>
<th>Yₚ (mg Chl a mg⁻¹P)</th>
<th>Yₚ/Yₚ/N</th>
<th>Kₚ (d⁻¹)</th>
<th>Kₚ’ (d⁻¹)</th>
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<td>0.19</td>
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<td>0.32</td>
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<td>2.9</td>
<td>24.2</td>
<td>0.049</td>
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Table 5. 4 The specific cations reduction rate for algae grown in different media

<table>
<thead>
<tr>
<th>Sample</th>
<th>Na(^+) (mg mg(^{-1}) chl a d(^{-1}))</th>
<th>K(^+) (mg mg(^{-1}) chl a d(^{-1}))</th>
<th>Ca(^{2+}) (mg mg(^{-1}) chl a d(^{-1}))</th>
<th>Mg(^{2+}) (mg mg(^{-1}) chl a d(^{-1}))</th>
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<tr>
<td>Mic-1'</td>
<td>0.02</td>
<td>0.19</td>
<td>0.25</td>
<td>0.13</td>
</tr>
<tr>
<td>Chl-SC1</td>
<td>0.03</td>
<td>0.57</td>
<td>0.03</td>
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<td>Mic-SC1</td>
<td>0.04</td>
<td>0.59</td>
<td>0.08</td>
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</tr>
</tbody>
</table>

Dry weight along with cultivation

![Dry weight graph](image)

**Figure 5. 1** Dry weight of *Chlorella* and *Micractinium* grown in different wastewater

In the legend, Chl: *Chlorella*, Mic: *Micractinium*. 1': primary effluent, SC1: one portion of sludge centrate and two portion of primary effluent by volume.
Figure 5.2 (a) Soluble N and (b) soluble P of *Chlorella* and *Micractinium* grown in primary effluent (1') and SC1

Figure 5.3 Cation along cultivation (a): Na⁺ (b) K⁺; (c): Ca²⁺; (d) Mg²⁺
Figure 5.4 Algal EPS extracted by sonication and base extraction at the end of cultivation
CHAPTER 6

INVESTIGATION OF CULTIVATION OF GREEN ALGAE IN A MIXTURE OF SLUDGE CENTRATE AND PRIMARY WASTEWATER EFFLUENT AND THEIR ANAEROBIC DIGESTION UNDER MESOPHILIC CONDITION

6.1 Abstract

Two species of green algae, *Micractinium nov.* and *Chlorella sp.*, were cultivated in a mixture of anaerobic sludge centrate and primary wastewater effluent to evaluate nutrient removal and to study their anaerobic digestion under mesophilic condition. Results showed that sludge centrate supported the growth of algae when diluted with 2 parts of wastewater primary effluent by volume. Cultivation of both species of algae in centrate and primary effluent mixture led to a reduction of phosphorous from 3.5 to 0.17 mg/L and the nitrogen from about 200 to 100 mg/L. Analysis of extracellular polymeric substances (EPS) of both algal species showed that *Micractinium* generated larger quantity of EPS-proteins than *Chlorella*. Anaerobic digestion of harvested algae showed that the CH₄ yield of *Chlorella* and *Micractinium* were 296 and 248 mL g⁻¹ volatile solids fed, respectively. It was presumed that different growth characteristics of two types of algae (different quantity of EPS) affected their anaerobic digestibility differently. Co-digestion of algae with waste activated sludge (WAS) improved the volatile solids reduction efficiency as well as the biogas yield of both algae species.

Key words

*Chlorella, Micractinium, Microalgae, Nutrient removal, Anaerobic digestion, WAS, Methane production, N/P ratio*
6.2 Introduction

Microalgae, due to its ability to reduce nutrients and abate CO₂ footprint, have been used in treating different types of wastewater including effluent from anaerobic digestion, animal wastewater, and municipal wastewater (González-Fernández et al., 2010; Guzzon et al., 2008; Lau et al., 1995; Park et al., 2009; Park et al., 2011; Singh et al., 2011; Wang et al., 2009; Yun et al., 1997). The algal biomass produced from wastewater treatment can also be used as an energy feedstock. Algae draw attention in the bioenergy field because of a high photosynthesis rate and the ability for lipid accumulation (Lardon et al., 2009). Harvesting of energy from microalgae can be achieved by producing biodiesel and generating methane by anaerobic digestion. Production of biodiesel from microalgae requires accumulation of high lipid content, effective biomass harvesting, and oil extraction to maintain the economical and energy balance of this system (Lardon et al., 2009; Sialve et al., 2009). Accumulation of intracellular lipid usually resulted from the limitation of nitrogen (Illman et al., 2000). However, municipal wastewater is usually rich in nitrogen. Consequently, algae grown in municipal wastewater typically showed a lipid content in the range of 4.9% and 11.3% of the volatile suspended solids (Woertz et al., 2009), which is a much lower fraction of lipid content than recommended for economical biodiesel production.

Anaerobic digestion to produce methane is another feasible way to harvest bioenergy from grown algae, regardless of lipid content. The protein and polysaccharide as well as lipid in biomass can all be utilized for biogas generation by anaerobic digestion. Biogas production by anaerobic digestion of algae ranged from 0.14 to 0.36 CH₄/g VS fed (Lakaniemi et al., 2011; Ras et al., 2011; Zamalloa et al., 2012; Zeng et al., 2010),
which are comparable to digestion of municipal sludge of 0.19 to 0.43 CH4/g VS fed (Nallathambi Gunaseelan, 1997).

By using anaerobic digestion for grown algal biomass, it is possible to integrate algae cultivation and energy production in the existing wastewater treatment plants (WWTP), which will also contribute to recycling of nitrogen and phosphorus, absorbing CO2, and producing bioenergy from wastewater. One diagram of the proposed processes is shown in Figure 6.1 (Rusten and Sahu, 2011). In this process, the high ammonium remaining in anaerobically digested sludge centrate, which is usually returned to the upstream of the wastewater treatment process, can be used for algae cultivation, decreasing the nitrogen load to existing wastewater treatment process. The CO2 generated from combustion of methane gas can also be fed and recycled for algae growth in order to reduce CO2 emission and to enhance algal growth. Algal biomass harvested from wastewater treatment can then be digested alone or co-digested with sewage sludge for biogas production.

The hypothesis of this study is that species of microalgae and their cultivation conditions (e.g., different types of wastewater) will affect the digestibility of microalgae and biogas yields. It is also worth stating that there has been little research conducted on studying anaerobic digestion of microalgae that are directly cultivated in real wastewater. The aim of this study was to evaluate the growth of two different species of microalgae (Chlorella sp. and a novel species of Micractinium nov.) in real wastewater, their ability to remove nutrient, and finally their anaerobic digestibility under mesophilic condition. The effluent quality from the algal digestion and co-digestion was also monitored to
evaluate the possibility for recycling the dewatered liquid post digestion for further algae cultivation.

6.3 Methods

6.3.1 Microalgae cultivation

Wildtype *Micractinium nov.* and *Chlorella sp.* were initially isolated from the wall of a primary clarifier at a local WWTP and were inoculated in Basal Media (Andersen, 2005; Stein, 1973) to maintain pure cultures. The anaerobic sludge centrate, collected after dewatering of anaerobically digested sewage sludge, was also collected from the same local WWTP and used for algae cultivation. The sludge centrate was filtered through Grade 4 Whatman Filter Papers, which had a pore size around 20-25 μm, to remove large particles. Preliminary research indicated that pure sludge centrate was too harsh for algae growth. Therefore, sludge centrate combined with primary effluent at a ratio of 1 to 2 (V/V), which had total N at around 200 mg/L, was used for algae cultivation and this culture media is hereafter called SC1. Algae were cultivated in 2 L Pyrex bottle with the working volume of 1.8 L in batch mode. The volume of algae inoculum accounted for 10% of the total working volume. The light intensity was 60 μmol m⁻² s⁻¹ provided by cool-white fluorescent light at 16-hr light/8-hr dark cycle. External CO₂ combined with 98.5% house air was added continuously to provide additional carbon source for algae. The temperature of the culture room was 23±2°C and algae were kept in suspension by magnetic stirrers. The size distribution of *Chlorella* and *Micractinium* harvested at the end of cultivation were measured by Mastersizer 2000 (Malvern Instruments Ltd, UK) and the Zeta potential was measured by a laser electrophoresis meter (Zeta Master, Malvern Instruments Ltd, UK).
6.3.2 Extraction of algae EPS

Sonication and base extraction were performed on algal biomass separated from the wastewater growth media at the beginning and the end of cultivation to evaluate the extracellular polymeric substances (EPS) of algae. The base extraction was performed following the method described in Park and Novak (2007). The sample after base extraction was centrifuged at 9000 rpm for 15 minutes and the supernatant was collected as base-extracted EPS. For sonication extraction, the pellets of algae after removing liquid by centrifuge at 9000 rpm for 15 minutes were resuspended in a phosphate buffer solution (10 mM NaCl, 1.2 mM KH$_2$PO$_4$ and 6 mM Na$_2$HPO$_4$) and subjected to sonication by a Fisher Scientific Sonic Dismembrator (400-watt max) at 40% strength for 120 sec. The strength of sonication was determined by preliminary experiments that had shown the maximum EPS release without disruption of algal cells. The sample after sonication extraction was again centrifuged at 9000 rpm for 15 minutes and the supernatant was collected as sonication-EPS. Protein concentration of EPS was quantified by the modified Lowry Methods (Lowry et al., 1951; Frølund et al., 1996) using bovine serum albumin as standard. The polysaccharide was measured by the Dubois et al. (1956) method and dextrose was used as standard.

6.3.3 Anaerobic digestion

Micractinium nov. and Chlorella sp grown in SC1 were harvested by lab centrifuge at 3000 rpm for 5 minutes for the anaerobic digestion study. The mixed liquor from an aeration basin from a local WWTP was settled for one hour and then centrifuged to get thickened sludge, $\sim$7000mg/L, for anaerobic digestion. The feed for co-digestion was 21% algae+79% thickened sludge by VS. Anaerobic digestions of 100% sludge, 100%
*Micractinium nov.*, and 100% *Chlorella sp.* were also performed. The digestion was performed in a serum bottle with a working volume of 100 mL. Muti-magnetic stirrers were used to provide continuous mixing for digestion. All digestion sets were performed under mesophlic conditions (37°C) for 20 days. The digestion was performed in duplicate with 44% by VS of field anaerobic digestion sludge as a seed sludge. Gas was released every 2-3 days by a 50 mL glass syringe attached to a gas-tight needle and the gas volume was recorded.

Methane generated in the gas phase of the digestion was analyzed by a gas chromatograph equipped with TCD detector and HP-PLOT/Q column. Helium was used as the carrier gas at a flow rate of 8.6 mL/min. 500μL of gas sample was injected for methane measurement in triplicate.

In order to assess the hydrolysis of different feedstock during anaerobic digestion, the following equation was used to calculate hydrolysis efficiency:

\[
S = \frac{sCOD + V_{CH_4} \times COD_{CH_4}}{tCOD}
\]

Where \(sCOD\) is the soluble COD from the digested sludge, \(V_{CH_4}\) is the cumulative methane production at standard temperature and pressure, \(COD_{CH_4}\) is theoretical conversion coefficient from methane to COD (350 L CH\(_4\) kg\(^{-1}\) COD)(Costa et al., 2012).

### 6.3.4 Analytical methods

The solids and sludge properties before and after anaerobic digestion were examined for different digestion scenarios. The soluble sample was prepared by filtration through 0.45 membrane filter. The biomass dry weight (TSS), TS, VS, total and soluble COD were measured according to standard method (APHA et al., 2005). Total and soluble N and P were measured using Hach TNTplus chemistry kits (Hach, US). The
concentrations of NH₄-N in liquids after digestion were monitored by a Metrohm Peak 850 Professional AnCat ion chromatography system (Metrohm Inc., Switzerland).

6.4 Results and discussion

6.4.1 Cultivation of microalgae

The average specific growth rates of *Chlorella* and *Micractinium* grown in SC1 were 0.27 d⁻¹ and 0.30 d⁻¹, respectively. Both *Chlorella* and *Micractinium* showed a good ability for nutrient removal. Phosphorous in wastewater decreased from 3.5 to 0.17 mg/L at the end of cultivation for both species. More than 95% of soluble P was removed from SC1 by both cultures (Table 6.1). The soluble N for *Chlorella* decreased from 204 mg/L to 97.3 mg/L, and the soluble N for *Micractinium* declined from 189 mg/L to 107 mg/L. The soluble N in SC1 was reduced by 52.5% and 43.4 % for *Chlorella* and *Micractinium*, respectively (Table 6.1). No further reduction of N was observed for both species possibly due to nearly complete removal of P during their growth. The soluble P in both cultures at the end of cultivation was lower than 0.2 mg/L when the soluble N in the effluent was still around 100 mg/L (500 times the soluble P). The P-limitation most likely induced an lower N removal efficiency, but still a significant amount of N was consumed during algal cultivation.

The zeta potential and the average size of the two species are shown in Table 6.1. The average sizes of *Chlorella* and *Micractinium* were 4.17 to 4.85 μm, respectively. No significant differences in size between these two species were observed. These sizes of *Chlorella* and *Micractinium* in our study are larger than algae cultivated in sludge centrate reported by Rusten and Sahu (2011), but are smaller than the size of algae cultivated in Basal medium. The relatively small size of algae in this study resulted in
ineffective settling ability and additional flocculation or centrifuge was required for algae harvesting. The Zeta potential of microalgae was slightly less negative than typical activated sludge (around -30 mV) (Chitikela & Dentel, 1998; Chang et al., 2001).

Sonication and base extraction of algae EPS data on Day 0 and Day 14 (the end of cultivation) are shown in Figure 6.2. Sonication extraction was more effective than base extraction, which was consistent with our previous research (Wang et al., under review). EPS harvested from base extraction was less than half of that from sonication extraction for both species. The protein concentrations of sonication-extracted EPS on day 0 were 82.3 and 112.9 mg/g VS for Chlorella and Micratinium, respectively. The protein decreased to 50.8 and 61.9 mg/g VS after 14 days of cultivation in SC1 media for Chlorella and Micratinium, respectively. The EPS extracted on day 0 showed higher EPS for both algae species than that cultivated on day 14. Nevertheless, Micratinium always showed higher content of proteins in EPS compared to that in Chlorella. This may explain that higher EPS-protein expression in Micratinium likely support their typical growth in clusters, compared to more picoplanktonic growth of Chlorella. The trend of EPS-polysaccharide was similar to that of protein at the beginning of cultivation for both species. However, the difference in extracellular polysaccharide concentrations between Chlorella and Micratinium became smaller after 14 days of cultivation.

6.4.2 Chemical characteristics of different algal feedstocks for anaerobic digestion

For algae digestion, algae grown in SC1 were harvested on D14 when the growth medium was almost depleted of P. In this study, Chlorella, Micratinium, and a mixture of each species with thickened sludge (21% algae and 79% thickened sludge by VS) were used as the feed for anaerobic digestion.
The characteristics of different digestion feedstock (substrates) for anaerobic digestion are shown in Table 6.2. The COD/TN ratio was in the range of 10.3 and 13.4, indicating that there was not such a high difference for algae and the mixture of algae and thickened sludge. A low COD/N ratio can potentially indicate high protein content in the biomass (Sialve et al., 2009; Yen & Brune, 2007). Assuming the conversion coefficient from nitrogen to protein is 6.25, the protein contents in both algae species were 47 and 52% for *Chlorella* and *Micractinium*, which is similar to values reported in other studies (Zamalloa et al., 2012). The initial concentrations of total P (TP) in *Chlorella* and *Micractinium* were low compared to that in other digestion sets. The initial pH before digestion was around 7.6 for all the digesters, which was in the optimum range of 6.6 to 7.8 for anaerobic digestion (Mata-Alvarez et al., 2000).

### 6.4.3 Digestibility of algae in anaerobic digestion

The cumulative biogas production is shown in Figure 6.3. The co-digestion of thickened sludge and algae showed higher biogas yield than other digestion sets during the early stage of digestion. The biogas yield from the digestion of sludge alone was lower than the co-digestion and algae digestion sets in this study. After 20 days of digestion, the cumulative biogas yield from *Chlorella* showed the highest biogas production as 534 mL/g VS fed, while *Micratinium* digestion alone showed the lowest biogas production of 450 mL/g VS fed. The digestion of *Micratinium* and sludge alone showed the similar cumulative biogas yield, but the higher CH₄ concentration for WAS digestion resulted in a higher CH₄ yield from WAS digestion (Table 6.3). The final CH₄ yield of *Chlorella* and Chl+WAS showed the same, and highest, biogas yield of 296 mL/g VS fed. The final CH₄ yield from digestion of *Micratinium* was the lowest (248
mL/g VS fed). The hydrolysis efficiency of anaerobic digestion was calculated based on equation 1 and showed that the digestion of Micractinium had the smallest hydrolysis of 25%, which also had the lowest CH₄ yield.

Volatile solids reduction (VSR) data for different feeds are shown in Table 6.3. The VSR from digestion of Chlorella and Micractinium were around 42% and 40%, respectively, which are much lower than the digestion of WAS (60%). The higher gas yield from Chlorella and relatively lower VSR indicated that the gas generated per unit of VS reduced was higher for Chlorella. The co-digestion of algae and WAS increased the VSR of Chlorella and Micractinium by 33% and 47.5%, respectively. In this digestion study the ratio of inoculum (anaerobic digested seed sludge) to feed was 0.8 by VS. The seed inoculum was collected from stably operated egg-shaped digesters treating sewage sludge with an average VSR over 60%, and the VSR from seed sludge itself during further anaerobic digestion was negligible. Assuming that the VSR of algae and WAS alone can be applied to the co-digestion sets, the VSR can be calculated through the following equation:

\[
\text{Calculated VSR} = VSR_a \times C_a + VSR_w \times C_w \tag{Equation 6.2}
\]

Where \(VSR_a\) is the VSR of algae (Chlorella or Micractinium), \(C_a\) is the fraction of algae by VS, \(VSR_w\) is the VSR of WAS and \(C_w\) is the fraction of WAS by VS. The calculated VSR for digestion of Chlorella, Micractinium, WAS and Chl+WAS were the same as experimental value (Table 6.3). The calculated VSR for Mic+WAS was 56%, which is lower than the experimental value of 59%. The experimental VSR of co-digestion of WAS and Micractinium showed a higher value than the calculated VSR, indicating that co-digestion significantly improved the VSR for Micractinium.
The cumulative CH₄ yield from this digestion study is high compared to other studies. As shown in Table 6.4, the CH₄ yield from anaerobic digestion of microalgae ranged from 140 to 360 mL/g VS in the reported studies. The different biogas yield may be attributed to different species and growth conditions. In this study, even though the cultivation condition for *Chlorella* and *Micractinium* was the same, and both species showed similar growth characteristics and high nutrient removal efficiency, the two species still digested differently. The different digestibility of algae in this study may also be attributed to the different quality and quantity of EPS. Some protein in EPS generated from the metabolism of microorganisms may be hard to degrade, which will lead to ineffective digestibility of biomass. Previous research indicated that the protein concentration from sonication extraction was negatively related to the digestion of sludge (Wang et al., 2010). The higher the EPS-protein, the lower the digestibility of the biomass. In this study, *Micractinium* showed higher EPS-protein than that from *Chlorella*, but a lower CH₄ yield. The different expression of EPS of these two species during cultivation could be one reason why they showed different digestibility. Different growth conditions for the same algae species will also result in different digestibility of algae. N limited conditions will lead to higher intracellular lipid accumulation in algae (Li et al., 2010; Perez-Garcia et al., 2011). The protein, carbonhydrates and lipids have different value for methane production and the different molecular composition of algae could result in different biogas yield.

Compared to the digestion of secondary activated sludge (WAS) which is relatively hard to digest, digestion of microalgae showed similar or even higher methane yield. This becomes even more significant when considering the substantially different
VSR between digestion of algae and WAS. The gas yield from unit VS of WAS reduced was much lower compared to algae. The co-digestion of *Chlorella* and WAS showed higher gas yield than WAS digestion alone. These results suggest that algae cultivated in sludge centrate had the potential for biogas generation and can be used as a bioenergy source. The co-digestion of algae and sewage sludge could be also beneficial to get higher biogas yield for existing anaerobic digestion at a WWTP.

**6.4.4 Characteristics of centrate after anaerobic digestion**

In order to evaluate the possibility to recycle nutrients in sludge centrate from anaerobic co-digestion for additional algae growth, the characteristics of centrate after algae digestion or co-digestion was also evaluated (Table 6.5). The pH of all digesters after 20 days digestion was 6.9. The soluble *N* after anaerobic digestion was in the range of 385 to 484 mg/L, and the soluble *P* was in the range between 29.1 and 42.9 mg/L. In all digestion solutions, NH₄-N accounted for about 80% of total soluble *N*. The *N* released from anaerobic digestion of different feedstock in this study was in the same order of magnitude of sludge centrate from domestic WWTPs (Rusten & Sahu, 2011), which has been confirmed to support algae growth. The cultivation of other feedstock also had sufficient nutrient released for algae growth. The *N/P* ratios in the digestion of algae sets were relatively higher than those of co-digestion and digestion of WAS, possibly due to their growth in the P-limited condition. However, the *N/P* ratio after digestion is still in the range for proper nutrient removal and algae growth of 5:1 to 20:1 as suggested by Li et al. (2010). The optimal *N/P* ratio found for *Chlorella vulgaris* for nutrient removal was 8:1 and the optimal ratio for mixture of microalgae from lagoon was 14:1 (Karapinar Kapdan & Aslan, 2008; Lee et al., 2013). The balanced nutrient load, as well as the
nearly neutral pH made the effluent from anaerobic digestion of algae and their co-digestion with WAS good sources for algae cultivation. The nutrients released from anaerobic digestion can be recycled and utilized by algae as shown in Figure 6.1. The goals of nutrient recycling, CO₂ emission reduction and energy generation can be achieved by integrated algae cultivation and anaerobic digestion at current WWTPs. Further energy balance analysis and life cycle assessment would be beneficial to evaluate the sustainability of this process.

6.5 Conclusion

This study integrated the algae cultivation and anaerobic digestion. Two species, *Chlorella* and *Micratinium*, originated from a WWTP, were cultivated in a mixture of sludge centrate and primary effluent (SC1) and the harvested algae were directly digested under mesophilic condition with or without addition of thickened WAS. The major findings from this study are:

- The sludge centrate diluted with primary effluent supported the growth of *Chlorella* and *Micratinium* collected from the WWTP.
- The protein content in EPS produced from *Micratinium* was higher than that from *Chlorella*. The high protein content in EPS was negatively correlated with the digestibility of biomass.
- The cumulative CH₄ yield from digestion of *Chlorella* showed the highest value of 296 mL / VS fed. The digestion of *Micratinium* showed the lowest CH₄ yield of 248 mL / VS fed.
- Co-digestion of algae with WAS improved the VSR of algae and improved the biogas yield of poorly digested biomass.
• The centrate after lab batch digestion had N/P ratios between 10.3 and 15.7, which are proper for algae cultivation.
References


Table 6. 1 Various characteristics of microalgae grown in different wastewater

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average specific growth rate (d⁻¹)</th>
<th>Zeta potential (mV)</th>
<th>Mean size (μm)</th>
<th>Soluble N removal efficiency (%)</th>
<th>Soluble P removal efficiency (%)</th>
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<tr>
<td>Chl-SC1</td>
<td>0.27</td>
<td>-20.0±0.8</td>
<td>4.17</td>
<td>52.5%</td>
<td>95.3%</td>
</tr>
<tr>
<td>Mic-SC1</td>
<td>0.30</td>
<td>-18.7±1.0</td>
<td>4.85</td>
<td>43.4%</td>
<td>95.2%</td>
</tr>
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</table>

Table 6. 2 Chemical characteristics of different substrates before anaerobic digestion

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<tr>
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<th>Chl+WAS</th>
<th>Mic+WAS</th>
<th>WAS</th>
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<td>pH</td>
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</tr>
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<td>sCOD (mg/L)</td>
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<td>251</td>
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<td>758</td>
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<td>84.2</td>
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</tr>
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<tr>
<td>N/P ratio</td>
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<td>5.3</td>
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<tr>
<td>COD/TS ratio</td>
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<td>1.1</td>
<td>1.1</td>
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</table>

Table 6. 3 Biogas production and VSR at the end of digestion

<table>
<thead>
<tr>
<th>Digestion Samples</th>
<th>Cumulative biogas yield (mL/g VS fed)</th>
<th>CH₄ composition (%)</th>
<th>CH₄ yield (mL/g VS fed)</th>
<th>Experimental VSR (%)</th>
<th>Calculated VSR (%)</th>
<th>Hydrolysis efficiency</th>
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<td>296</td>
<td>42%</td>
<td>42%</td>
<td>28%</td>
</tr>
<tr>
<td>Micratinum um</td>
<td>450</td>
<td>55.1</td>
<td>248</td>
<td>40%</td>
<td>40%</td>
<td>25%</td>
</tr>
<tr>
<td>Chl + WAS</td>
<td>505</td>
<td>58.7</td>
<td>296</td>
<td>56%</td>
<td>56%</td>
<td>30%</td>
</tr>
<tr>
<td>Mic + WAS</td>
<td>491</td>
<td>56.5</td>
<td>278</td>
<td>59%</td>
<td>56%</td>
<td>28%</td>
</tr>
<tr>
<td>WAS</td>
<td>458</td>
<td>62.2</td>
<td>285</td>
<td>60%</td>
<td>60%</td>
<td>30%</td>
</tr>
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</table>

Table 6. 4 Methane yield of microalgae from other anaerobic digestion studies
<table>
<thead>
<tr>
<th>Species</th>
<th>Algae classification</th>
<th>Algae growth medium</th>
<th>Digestion temperature (°C)</th>
<th>Digestion time (d)</th>
<th>CMY (a) (mL/g VSS fed)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green algae</td>
<td>Marine algae</td>
<td>Collected from sea shore</td>
<td>35</td>
<td>60</td>
<td>256±28</td>
<td>Gurung et al. (2012)</td>
</tr>
<tr>
<td><em>Microcystis</em> <em>sp.</em></td>
<td>Freshwater microalgae</td>
<td>Collected from lake</td>
<td>35</td>
<td>30</td>
<td>140.48</td>
<td>Zeng et al. (2010)</td>
</tr>
<tr>
<td><em>Scenedes mus obliquus</em></td>
<td>Fresh water microalgae</td>
<td>Artificial nutrient solution</td>
<td>33±2</td>
<td>30</td>
<td>240</td>
<td>Zamalloa et al. (2012)</td>
</tr>
<tr>
<td><em>Phaeodactylum tricornutum</em></td>
<td>Marine microalgae</td>
<td>Artificial sea water</td>
<td>33±2</td>
<td>30</td>
<td>360</td>
<td>Zamalloa et al. (2012)</td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em></td>
<td>Freshwater microalgae</td>
<td>Jaworski's medium</td>
<td>37</td>
<td>50</td>
<td>286</td>
<td>Lakaniemi et al. (2011)</td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em></td>
<td>Freshwater microalgae</td>
<td>Modified Z-8 nutrient broth</td>
<td>35</td>
<td>28</td>
<td>240 (b)</td>
<td>Ras et al. (2011)</td>
</tr>
<tr>
<td><em>Chlorella sp.</em></td>
<td>Freshwater microalgae</td>
<td>Sludge centrate</td>
<td>35</td>
<td>20</td>
<td>296</td>
<td>This study</td>
</tr>
<tr>
<td><em>Micractinium</em></td>
<td>Freshwater microalgae</td>
<td>Sludge centrate</td>
<td>35</td>
<td>20</td>
<td>248</td>
<td>This study</td>
</tr>
</tbody>
</table>

\(a\) CMY: cumulative methane yield  
\(b\) unit of CMY is mL/ g VSS

**Table 6.5 Parameters of sludge after anaerobic digestion**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Protein (mg/L)</th>
<th>Polysaccharide (mg/L)</th>
<th>sCOD (mg/L)</th>
<th>sTN (mg/L)</th>
<th>sTP (mg/L)</th>
<th>NH(_4)-N (mg/L)</th>
<th>N/P ratio</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlorella</em></td>
<td>24.0</td>
<td>31.1</td>
<td>281</td>
<td>379.6</td>
<td>24.2</td>
<td>294.7</td>
<td>15.7</td>
<td>6.90</td>
</tr>
<tr>
<td>Micractinium</td>
<td>21.6</td>
<td>27.5</td>
<td>247</td>
<td>385.2</td>
<td>29.1</td>
<td>299.3</td>
<td>13.3</td>
<td>6.85</td>
</tr>
<tr>
<td>Chl + WAS</td>
<td>21.3</td>
<td>29.7</td>
<td>247</td>
<td>428</td>
<td>41.4</td>
<td>346.2</td>
<td>10.3</td>
<td>6.86</td>
</tr>
<tr>
<td>Mic+ WAS</td>
<td>16.6</td>
<td>32.4</td>
<td>281</td>
<td>444</td>
<td>41.5</td>
<td>341.0</td>
<td>10.7</td>
<td>6.86</td>
</tr>
<tr>
<td>WAS</td>
<td>22.0</td>
<td>31.6</td>
<td>266</td>
<td>484</td>
<td>42.9</td>
<td>362.4</td>
<td>11.3</td>
<td>6.87</td>
</tr>
</tbody>
</table>

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Figure 6.1 Integration of algae cultivation for nutrient removal and bioenergy generation
Figure 6.2 Protein and polysaccharide in sonication and base extracted algal EPS on day 0 and day 14 (the end of algae cultivation)
Figure 6.3 Cumulative biogas yield of anaerobic digestion of *Chlorella* (left) and *Micractinium* (right) with or without WAS
CHAPTER 7
PILOT STUDY OF ALGAE CULTIVATION

7.1 Overview of the project

Previous research indicated that algae can utilize nutrients in sludge centrate for their growth and the harvested algae can undergo anaerobic digestion for energy production. The pilot study of algae cultivation was carried out at FREVAR, Norway to demonstrate the ability for nutrient removal by algae from centrate of anaerobically digested sludge under natural light condition. A green house was built at FREVAR for the pilot photobioreactor operation and lab sample analysis. The project was conducted from June to September, 2010 with the normal daylight around 15 hours. The light intensity at noon for a sunny day was around 74500 LUX, and the light intensity for a cloudy day is around 9000 LUX. *Chlorella* *sp.* from University of Life Sciences (Norway) was used for this pilot study. Sludge centrate from an anaerobic digester was pretreated by coagulation and anthracite filtration. Continuous operated multi-level open pond photobioreactor (MLOP) and batch mode Vertical Photobioreactor were tested for algae cultivation. The algae harvesting strategy was also studied.

7.2 Material and methods

7.2.1 Pretreatment of sludge centrate

Sludge centrate obtained from the de-watering unit for anaerobically digested sludge at FREVAR was proposed for algae cultivation. The sludge centrate contained a high amount of total suspended solids (TSS) and nutrients. Pretreatment by coagulation and upflow anthracite filter was required to remove the color and TSS before feeding to the photobioreactors for algae cultivation.
In the coagulation step, one liter of 25 g/L Zetag 8125 (CIBA) polymer solution was added to 100 L of sludge centrate to reach a polymer concentration of 0.25 g/L. The coagulation was conducted in a 120 L tank equipped with driller and stander for mixing (Figure 7.1). The mixture of polymer and sludge centrate was briefly mixed rapidly using a drill for 30 seconds (rapid mixing step). The speed of the drill was reduced and the mixture of sludge liquor and polymer was slowly mixed for 10 minutes (floculation step). Then the sludge centrate was settled for 30 mins and the supernatant were pumped to the upflow anthracite filter.

The supernatant from coagulation was pumped to the filter at a flow rate of 0.520 L/minute, resulting in an empty bed contact time of 120 min. Backwash by combined air and water was conducted to clean the anthracite when needed. The diagram of anthracite filter (7.2 a) and picture of back wash (7.2 b) are shown in Figure 7.2. The effluent from the anthracite filter was collected in a 100 L black plastic container as the feed for algae cultivation. The appropriate volume of phosphoric acid was added to the pre-treated diluted sludge centrate to achieve an N: P ratio of 6.3. The light transmittance (T) at 670 nm of sludge centrate after pretreatment increased from 0.1% to 77%.

7.2.2. Vertical Photobioreactor

Pretreated sludge centrate with varying dilutions with non-chlorinated effluent from Frevar WWTP (Fredrikstad, Norway) were fed to the photobioreactors. Four of the thin-walled polyethylene tubes were filled with 5 L of algae grown on sludge centrate and 10 L of a mixture of pre-treated sludge centrate and secondary effluent from WWTP. The ratios of sludge centrate to secondary effluent by volume in these four vertical
reactors were: 1:4 (V1), 3:2(V2), 1:0(V3) and 1:0 (V4). The diameter of the reactor was 156mm. The setup of the Vertical Photobioreactor is shown in Figure 7.3. The reactors were sparged with CO₂ from the bottom for 20 minutes twice per day. Algae sample was taken from outlets at the bottom of reactor for analysis.

7.2.3 Multi-Level open pond photobioreactor (MLOP)

The setup and dimensions of MLOP is shown in Figure 7.4. The total volume of the reactor was 210 L and operated with a desired retention time of 7 days. The water depth in each tray is 9 cm with the capacity of 11 cm. The system was sparged with CO₂ for 10 minutes every three hours from 8:00 am to 11:00 pm. No sparging was carried out overnight since there was little light for growth of algae. The CO₂ inlet was maintained at 1.5 psi.

7.2.4 Analytical methods

Total suspended solids (TSS) were measured three times a week according to standard method (APHA, 1998). Chemical parameters measured included nitrate, phosphate, ammonium, total nitrogen, chemical oxygen demand (COD) and total phosphorous concentration. Nitrate (NO$_3^-$-N), phosphate (PO$_4^{3-}$-P), and ammonium (NH$_4^+$-N) concentrations were measured 3 times per week using Dr. Lange kits (Hach Lange, German). The COD, total nitrogen and total phosphorous concentrations were measured once a week using Dr. Lange kits (Hach Lange, German).

The algae growth rate and the removal efficiency of nutrient were investigated in the pilot scale Vertical Photobioreactor and MLOP. The volumetric growth rate of algae was calculated by the following equation:
\[ R_v = \frac{\sum TSS}{\text{mg} \times \text{Total days}} \]  

Equation 7.1

Where, \( R_v \) is volumetric growth rate of algae (g/L/d), and TSS is the dry weight of biomass (mg/L).

The aerial productivity of algae was calculated by Equation 7.2:

\[ R_A = \frac{\sum v \times TSS}{A \times t} \]  

Equation 7.2

Where, \( R_A \) is the aerial productivity of algae; \( V \) is the volume of reactor, \( A \) is the illuminated surface area of the reactor; TSS is the concentration of biomass.

7.3 Results

7.3.1 Algae cultivation

7.3.1.1 Vertical Photobioreactor

The algae productivity and the nutrient removal rate are shown in Table 7.1.

The V1, where sludge centrate was diluted with 4 parts of secondary effluent (by volume), showed the highest volumetric growth rate, growth rate based on illuminated area and highest \( \text{NH}_4^+ \)-N and \( \text{PO}_4^{3-} \)-P removal rate. V2 and V3 showed slight COD removal, but COD removal was not seen for the other reactors. The pH during day time was 8.5 ± 0.17 when there was no CO\(_2\) sparging. The \( \text{NH}_4\)-N removal was caused by bioassimilation and volatilization of NH\(_3\) at high pH. The \( \text{PO}_4^{3-} \)-P was removed by bioassimilation of algae and precipitation at pH above 8 (Li et al., 2010; Song et al., 2002). In V2, 110 g NH\(_4\)-N and 15 g \( \text{PO}_4^{3-} \)-P were removal when producing 1kg of biomass. Algae grown in pure sludge centrate also demonstrated a strong ability of nutrient removal and high growth rate under natural light conditions as show in Table 7.1.
7.3.1.2 Performance of MLOP

The influent of MLOP was 1 part of sludge centrate with 4 parts of secondary effluent, which was the same as the composition of V1. The MLOP was started up by step feeding and internal recycle in the first 10 days. The reactor started continuous feeding when the NH$_4^+$-N and PO$_4^{3-}$-P removal in the reactor was stabilized. The NH$_4^+$-N and PO$_4^{3-}$-P of influent and effluent are shown in Figure 7.5. The average NH$_4^+$-N and PO$_4^{3-}$-P reduction rates were 70.7 % and 34.5 %, respectively. The NH$_4^+$-N and PO$_4^{3-}$-P removal efficiency in MLOP was lower than the batch mode operated V1. The average $R_A$ was 3.4 g/m$^2$-d, which was also much lower than $R_A$ in V1. The lower light transmission efficiency in MLOP may attribute to the relatively lower removal efficiency and lower algae growth rate in MLOP.

7.3.2 Algae harvesting

7.3.2.1 Cationic polymers

Algae grown in diluted sludge centrate were used for harvesting experiments. The cationic polymers used for algae harvesting included C-496, C-492, C-491, Zetag 8125, Zetag 7550 (BASF, Norway). The T% of algae raw sample at 670 nm ranged from 2% to 3.5% and TSS varied from 455 mg/L to 485 mg/L. The improvement of T% of supernatant at 670 nm after coagulation was used to indicate the effect of polymers in pre-screening experiments. Higher T% of supernatant at 670 nm after coagulation indicated better performance of coagulant. The T% of supernatant at 670 nm after coagulation is shown in Figure 7.6. The T% of supernatant after coagulation increased with increased dosage of cationic polymers. Compared to other cationic polymers,
25mg/L of Zetag 7550 increased the T% of supernatant to 70.0% after coagulation, indicating that Zetag 7550 was the most effective coagulant in algae harvesting.

7.3.2.1 Anion polymers

Anionic polymers investigated for algae harvesting included: Magna Floc 919, Magna Floc 342, Magna Floc 155 and A-120 (CIBA, Norway). The algae for harvesting with original T% at 670nm varied from 2.3% to 7.2%. The TSS of algae ranged between 377 and 585 mg/L. T% of supernatant at 670nm after coagulation indicated the effect of polymers (Figure 7.7).

Little increase of T% was seen after coagulation with anionic polymers. Higher dosage of polymer also did not work on algae coagulation. There was no floc formation during fast mixing. The results indicated that anionic polymers did not help coagulation.

In general, Chlorella. sp grown in sludge liquor was quite small and the majority cells were below 4 µm, which were difficult to settle down. Coagulation was necessary to neutralize the negative charges on the surface of algae and help to form larger particles. Cationic polymers were better choice for coagulation because of the negative charges on algae surface. Zetag 7550 showed to be the most effective coagulant among all the other polymers tested.

7.4 Conclusion

Chlorella. Sp grown in pilot scale Vertical Photobioreactor under natural light condition demonstrated high nutrient removal efficiency when fed with 5 time diluted sludge centrate. The algae can also survive in undiluted sludge centrate in the Vertical Photobioreactor. The algae grown in continuous fed MLOP, showed a lower nutrient removal rate and growth rate compared to algae grown in the Vertical Photobioreactor
with the same feed. The low light penetrate efficiency in MLOP and the lower retention
time hindered the growth of algae. Cationic polymer Zetag 7550 (BASF, Norway)
showed effective coagulation for algae.

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Table 7.1 Biomass productivity and nutrient removal by Vertical Photobioreactor

| Reactor No. | $R_v$  
| (g/L-d) | $R_A$  
| (g/m²-d) | NH₄-N removal (%) | PO₄-P removal (%) | COD Removal (%) | g NH₄-N removal/kg TSS produced | g PO₄³⁻-P/kg TSS produced |
|---|---|---|---|---|---|---|
| V1 | 0.81 | 13.5 | 96.8 | 80.3 | No | 110 | 15 |
| V2 | 0.65 | 11.25 | 67.2 | 50.5 | 17.4 | 158 | 20 |
| V3 | 0.73 | 8.75 | 66.6 | 69.3 | 17.2 | 133 | 14 |
| V4 | 0.96 | 6.36 | 74.8 | 22.7 | No | 223 | 17 |

Figure 7.1 Mixing system for the pre-treatment of sludge liquor
Figure 7.2 (a) Diagram of the anthracite filter; (b) Anthracite filter during backwashing procedure

(1) anthracite column (2) outlet for treated sludge liquor exiting filter (3) outlet for backwash effluent (4) inlet valve for sludge liquor, tap water, and air (5) tube to distribute sludge liquor along the bottom of the anthracite column.

Figure 7.3 Assembled vertical photobioreactor
Figure 7.4 Setup and dimensions of the MLOP

Figure 7.5 Nutrient removal by MLOP
Figure 7.6 T% of algae after different amount of cationic polymer addition

Figure 7.7 T% of algae after different amount of anionic polymer addition
APPENDIX A

PRELIMINARY RESEARCH ON ANAEROBIC DIGESTION OF SPIRULINA AND CHLORELLA WITH WASTE ACTIVATED SLUDGE (WAS)*

* This research was published in Water Environment Research (2012), 84(5), 396-404.

Batch anaerobic co-digestion experiments were performed to study the effect of algae addition on the digestibility of waste activated sludge (WAS). WAS was obtained from the Amherst WWTP. Harvested algae were washed in tap water to remove excess salts. Note that the algae used in these experiments were grown on either the modified Zarrouk or Bristol media. Experiments were set up in 500 mL glass batch anaerobic digesters, which were seeded with 100 mL of anaerobically digested sludge (~ 1,500 mg L⁻¹) obtained from the laboratory digester described previously. The reactors were purged with N₂ gas and digested under mesophilic conditions (37 °C) for 28 days with mixing.

In the first set of digestion experiments, S. platensis and WAS were added to the digesters to achieve the following volumetric algae/WAS ratios: 100%, 70%, 50%, 30%, and 0%. As the algal biomass concentration was lower than that of the WAS, the resulting algae/WAS mass ratios were: 100%, 52%, 32%, 17% and 0%. In the second set of anaerobic digestion experiments both S. platensis and Chlorella were studied. As the amount of harvested algae available in a WWTP is expected to be smaller than the amount of WAS available, the algae fraction was decreased for these experiments. Harvested algae and WAS were added to the digesters to achieve the following algae/WAS ratios: 100%, 15%, 5% and 0%. During this stage, algae were harvested by
centrifugation at 3,000 rpm for 10 min and both WAS and algae biomass densities were similar, therefore the mass and volumetric ratios were similar.

Initial experiments investigated co-digestion of *S. platensis* and WAS. The volatile solids reduction (VSR) data from five different batch digestion sets at varying algae/WAS mass ratios is shown in Figure A.1. The digestion of pure algal biomass resulted in approximately 57% VSR and this was even greater than 47% VSR observed for WAS alone. As the data further shows, overall VSR generally increased with increasing algal composition in the digester. The VSR value did not, however, change once the algal mass fraction became greater than 32%. The digestion sets with 52% and 32% algae performed much better than the set with WAS only and slightly better than 100% algae.

![Figure A.1 Volatile solids reduction for anaerobic co-digestion sets with harvested S. platensis/WAS at varying ratios](image)

$R^2 = 0.9238$
Data from the second set of algal co-digestion experiments are shown in Table A.1. The digestion of 100% *Chlorella* and 100% *S. platensis* resulted in 68.1% and 51.3% VSR, respectively. These values were much higher than the VSR (41.1%) obtained from digestion with WAS alone, which is consistent with the data shown in the earlier phase. Addition of 15% of *Chlorella* or *S. platensis* into the anaerobic digesters led to an improvement in VSR, as seen by the increase in VSR from 41.1% to 45.3% and 46.6%, respectively. These results again show the benefit of anaerobic co-digestion of algae and WAS and indicate that addition of microalgae will improve sludge digestibility and generate more useful biogas.

The effect of anaerobic co-digestion on sludge dewaterability was also investigated during the second stage of anaerobic co-digestion experiments. The dewaterability of all samples deteriorated after anaerobic digestion, as shown by increases in CST before and after digestion. When 5% or 15% of *S. platensis* was co-digested with WAS, the dewaterability of digested product improved compared to WAS alone. This is worth noting, as addition of *S. platensis* improved not only VSR but generated a digested product with better dewaterability than digestion of WAS alone. In contrast, addition of 5% or 15% of *Chlorella* led to a decrease in dewaterability in spite of better VSR, indicating that addition of different algal species can lead to different conditioning requirements and dewatering performance following anaerobic co-digestion. From the current investigation, *S. platensis* appears to be a better algal species for anaerobic digestion due to better dewaterability and improved VSR when 15% biomass was added.
Table A. 1 Volatile solids (VS) reduction and initial and final capillary suction time (CST) for anaerobic co-digestion of WAS with harvested *Chlorella* and *S. platensis*

<table>
<thead>
<tr>
<th>Mass fraction algae/WAS (%)</th>
<th>Algal species</th>
<th>VS reduction (%)</th>
<th>CST (s)</th>
<th>Before digestion</th>
<th>After digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100% WAS</td>
<td>41.1</td>
<td>40</td>
<td>471</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td><em>Chlorella</em></td>
<td>44.8</td>
<td>29</td>
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<td><em>Chlorella</em></td>
<td>45.3</td>
<td>26</td>
<td>862</td>
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</tr>
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<td><em>Chlorella</em></td>
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<tr>
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<td><em>S. platensis</em></td>
<td>42.8</td>
<td>44</td>
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<td></td>
</tr>
<tr>
<td>15</td>
<td><em>S. platensis</em></td>
<td>46.6</td>
<td>49</td>
<td>346</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td><em>S. platensis</em></td>
<td>51.3</td>
<td>19</td>
<td>893</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX B

EFFECT OF 2-BROMOETHANESULFONIC ACID (BESA) ON MESOPHILIC ANAEROBIC DIGESTION OF CO-DIGESTION OF ALGAE AND WAS

2-bromoethanesulfonic acid (BESA) was added into some digesters to investigate the effect of methanogen inhibition on the performance of conventional digestion (WAS only) and co-digestion in the presence of algae. The gas yield data from the digesters with and without BESA addition are shown in Figure B.1. As the data show, gas generation was significantly inhibited from day 3 when 1.4 mM of BESA was present in the digesters. It was found that about 90% less gas was generated from both sets of digestion with BESA regardless of the presence of algae. These data may suggest that the small amount of algae did not help reduce the negative effect of BESA on anaerobic digestion. However, it should also be noted that the addition of BESA with initiation of digestion might have overwhelmed the systems and different results could be seen if BESA or other inhibitory effects are applied to the digesters that are established with typical anaerobic digestion environment and designated community.
**Figure B. 1** Gas generation with and without BESA addition under mesophilic condition, % shows the mass fraction of algae in digestion research
APPENDIX C

THERMOPHILIC ANAEROBIC DIGESTION OF ALGAE AND WAS

Thermophilic digestion was also performed during this phase of research. The gas generation data for the thermophilic digesters are compared with those from the mesophilic sets in Figure C.1. It can be clearly seen that thermophilic digestion did not lead to good gas generation. The thermophilic and mesophilic digesters were started up with the same anaerobic seed sludge, which was collected from our lab-scale mesophilic anaerobic digester with 3 to 4 % of seed sludge by mass in each digester. The low gas evolution from both WAS and codigestion sets suggest that thermophilic bacteria and methanogens were not properly established in these batch digesters, which hindered the performance of digestion and investigation of algae co-digestion under thermophilic conditions. Further research on thermophilic digestion will be necessary. For the future research, thermophilic digestion can be seeded directly with thermophilic seed sludge and relatively a large amount of seed sludge could be used. This means one needs to have access to ongoing thermophilic digesters or should cultivate or maintain a thermophilic digester for the purpose of seed sludge for co-digestion study.
Figure C. 1 Gas generation from mesophilic and thermophilic digestion of algae and WAS
APPENDIX D

SONICATION EXTRACTION OF ALGAE

The strength of sonication was tested using a Sonic Dismembrator Model 500 (Fisher Scientific, USA) with varying intensity. The release of COD after sonication extraction is shown in Figure D. 1.

![Sonication extraction method of algae](image)

**Figure D. 1** COD release by sonication extraction of algae
APPENDIX E

SET UP OF FLOCCULATION AND COAGULATION JAR TEST

Figure E. 1 Effect of cationic polymers for algae harvesting

Figure E. 2 Effect of anionic polymers for algae harvesting
BIBLIOGRAPHY


