An Integrated Biorefinery for Anaerobic Digestion of Thin Stillage and Microalgae Cultivation for Nutrient Recycling, Bioenergy and Bioproduct Production

by

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To my father who passed away during the preparation of this thesis, to my mother who always believed in me and to my wife who always stood by me through thick and thin

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ABSTRACT

Corn grain is one of the main sources for bioethanol production. However, corn bioethanol plants consume a significant amount of energy and water, compete with the food supply chain and may lead to eutrophication as the result of high nitrogen and phosphorous content of waste streams. All these issues challenge the sustainability of bioethanol production from corn grain in terms of energy, water and lands used. In this study, integration of anaerobic digestion-microalgae cultivation with an existing corn-bioethanol-plant is proposed, which can improve the energy balance and reduce the capital and operating cost of the plant. To evaluate the feasibility of the proposed integrated process, thin-stillage was digested in a conventional anaerobic baffled reactor (ABR). The limitations of the conventional ABR were addressed by introducing a novel ABR, in which the chemical oxygen demand (COD) and sulfate removal efficiency and CH_4 yield were improved as a result of reduced biomass washout and enhanced phase separation. Furthermore, the effect of operating parameters such as OLR and recycle ratio (RR) on the performance of the novel ABR were studied. According to the results, when the OLR increased from 3.5 to 6 kg COD m^{-3} d^{-1}, the COD and sulfate removal efficiency and methane yield changed from 92.5±3.2%, 97±1.6% and 305±6 mL CH_4 g^{-1} $COD_{removed}$ to 78.9±3.4%, 92.9±1.4% and 275±5 mL CH_4 g^{-1} $COD_{removed}$ at RR of 15. But, reducing the RR from 20 to 10 did not change those parameters significantly. The concentration of nutrients in resulting digestate was reduced by struvite recovery. Various microalgae species in different dilutions of struvite-removed-digestate were grown and *C. sorokiniana* in two times dilution was selected for cultivation in the photobioreactor. The microalgae biomass concentration reached 1.62±0.11 g/L and the removal efficiencies of nitrogen and phosphorus were 95.3±1% and 78.3±1.1% at the end experiment. The protein, starch and lipid contents of biomass were 37.8±3.4%, 17.8±0.8% and 8.9±0.3%, respectively. The findings for nutrient recovery from anaerobic digestate of thin-stillage in the form of struvite and bioproducts accumulated in microalgal biomass show the potential of integrated biorefinery for improving the sustainability and energy balance of existing corn-bioethanol-plants.

LIST OF ABBREVIATIONS USED

AEBR	Anaerobic expanded bed reactor
ABR	Anaerobic baffled reactor
ACP	Anaerobic contact process
AD	Anaerobic digestion
AF	Anaerobic filter
AFBR	Anaerobic fluidized bed reactor
AMBR	Anaerobic migrating blanket reactor
AnMBR	Anaerobic membrane bioreactor
ASBR	Anaerobic sequential batch reactor
CABR	Carrier anaerobic baffled reactor
Chl.	Chlorophyll
CHP	Combined heat and power generation
COD	Chemical oxygen demand
Comp.	Compartment
CSTR	Continuously stirred tank reactor
DAF	Downflow anaerobic filter
DDG	Dried distillers' grains
DDGS	Distiller's dried grains with solubles
DW	Dry weight
EGSB	Expanded granular sludge bed
F/M	Food to microorganism ratio
GC	Gas chromatography
HRT	Hydraulic retention time
IC	Internal circulation reactor
ICP-OES	Inductively coupled plasma optical emission spectrometry
K_a	Dissociation constant
NPQ	Non-photochemical quenching
OLR	Organic loading rate
PBR	Photobioreactor
PVA	Polyvinyl alcohol
Q_{in}	Influent flow rate
Q_{out}	Effluent flow rate
RR	Recycle ratio
SEM	Scanning electron microscope
SLR	Sulfate loading rate
SMA	Specific methanogenic activities
SRB	Sulfate reducing bacteria
SRT	Solid retention time
SCOD	Soluble chemical oxygen demand
TA	Total alkalinity
TAG	Triacylglyceride

TBOD	Total biochemical oxygen demand
TCD	Thermal conductivity detectors
TCOD	Total chemical oxygen demand
TN	Total nitrogen
TP	Total phosphorus
TS	Total solid
TSS	Total suspended solid
UAF	Upflow anaerobic filter
UASB	Upflow anaerobic sludge bed
USSB	Upflow staged sludge bed reactor
VFA	Volatile fatty acids
VS	Volatile solid
VSS	Volatile suspended solid
V_R	Volume of reactor
WDG	Wet distillers' grains
Y	Biomass yield

ACKNOWLEDGEMENTS

CHAPTER 1   INTRODUCTION

1.1   Background

Energy is one of the most important elements for social and economic development. Currently, fossil fuels are the dominant source for energy production (Lim et al., 2012). The growing demand for energy has increased the consumption of fossil fuels significantly. The fossil fuels are finite and are exhausting. Therefore, the increasing demand for energy cannot be met solely by the conventional fuels in the near future. The conversion of the fossil fuels to energy also releases CO_2, which has caused a significant increase in the greenhouse effect and global warming (Goldemberg, 2007). Hence, alternative renewable energy sources that are economically competitive and environmentally friendly have gained increasing attention in recent years (Mussgnug et al., 2010). One of the renewable energy sources is bioenergy. Biomass can be converted to heat or energy carriers using thermochemical or biological methods. Thermochemical conversion processes are pyrolysis, liquefaction, combustion and gasification in which combustion produces heat, gasification produces heat and syn-gas, pyrolysis generates heat, syn-gas, bio-oil and biochar and liquefaction produces bio-crude (McKendry, 2002). The biological methods include anaerobic digestion and fermentation of organics to produce energy carriers such as biogas, biohydrogen, biobutanol and bioethanol (Skjånes et al., 2007).

Bioethanol is the most widely used biofuel, which is mainly produced from sugar based crops such as corn and sugarcane (Harun et al., 2010). Taking into the consideration the increasing demand for food and freshwater requirements for plant cultivation,

lignocellulosic sources such as agricultural wastes (e.g. bagasse, corn stover and rice straw) can be considered as an alternative source for bioethanol production (Sims et al., 2010). Furthermore, these feedstock are rich in carbohydrate and available at a stable and low price (Zabed et al., 2016). Currently, 12 cellulosic commercial scale bioethanol plants are in operation in the world (Halder et al., 2019). However, the main drawback of lignocellulosic materials is the high content of lignin leading to generating wastewater that is difficult to treat (Mood et al., 2013). Moreover, several conversion steps are required to make lignocellulosic materials available for natural microorganisms (Zabed et al., 2016). Therefore, the cost of converting the lignocellulosic biomass to bioethanol is relatively high (Lynd, 1996).

On the other hand, microalgae-based carbohydrate does not contain lignin. Moreover, microalgae are considered as an alternative third generation feedstock, which do not compete for arable land or portable water. Microalgae use the process of photosynthesis to convert CO_2, water and light into biomass (Domozych et al., 2007). Using microalgae for bioethanol production has several advantages over crops-based feedstocks including high growth rate, high volumetric productivity and short harvesting cycle (1–10 days), minor pretreatment due to no lignin and low content of hemicellulose, being able to grow on salty or wastewater streams or to grow in areas unsuitable for agricultural purposes such as desert and seashore lands (Harun et al., 2010). However, providing chemical fertilizers and the application of energy intensive biomass harvesting and biofuel conversion technologies raises several issues such as operational and capital cost which are needed to be addressed before microalgae to biofuel production becomes cost effective (Chen et al., 2015; Singh

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and Gu, 2010). For producing ethanol from microalgae, several processes are required; namely, starch liquefaction, pre-saccharification, fermentation and distillation. The starch-based-crops ethanol plants such as corn-ethanol plants already have the equipment for microalgae to ethanol conversion. Thus, microalgal biomass can be used as a supplement to substitute part of the primary feedstocks such as corn, taking advantage of existing technologies and facilities (Chen et al., 2013b). The residues from the distillation process in corn-ethanol plants is usually centrifuged and the liquid part from the centrifugation is called thin stillage. Thin stillage is rich in nutrients and can be used as a water and nutrient source for microalgae cultivation. But, thin stillage also contains high concentration of organics which are conventionally processed by evaporation and drying and sold as animal feed (Wilkie et al., 2000).

Generally, corn-based ethanol can be produced via dry milling or wet milling plants (Bothast and Schlicher, 2005). In a wet milling process, the corn oil and gluten feed are extracted first from the milled corn and the rest is sent to a fermenter. However, in a dry milling process, a fermenter receives the milled corn and converts it into ethanol. The remaining part is distillers' grains with solubles (DGS), which can be sold as a commercial animal feed (Wang et al., 2011). The dry milling plants account for almost 90% of the total U.S. capacity by 2010, which is the result of better energy balance and less costly equipment in dry milling compared to wet milling (Agency, 2010; Halder et al., 2019; Wang et al., 2011). A dry milling corn ethanol plant is divided into two sections; namely, the bioethanol production part and the downstream stillage processing (Figure 1-1). In the first section, hammer milled corn kernels are cooked and then hydrolyzed in a liquefaction

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tank by enzymes. The hydrolyzed substrate is fermented to produce CO_2 and ethanol. Then, the ethanol is separated by distillation and purified by molecular sieves. Whole stillage, which is the residue of enzymatic hydrolysate of corn fermentation mixture, is usually centrifuged to produce a liquid fraction (thin stillage) and a solids fraction (wet distillers' grains (WDG)). The thin stillage is concentrated in evaporators and mixed with dried WDG (DDG) to form distiller's dried grains with solubles (DDGS), which is used as feed for livestock (Chatzifragkou and Charalampopoulos, 2018). Each liter of ethanol produced can produce up to 20 L of stillage with chemical oxygen demand (COD) of 100 g L^{-1}, total nitrogen of 2000 mg L^{-1} and phosphorous of 1500 mg L^{-1} (Wilkie et al., 2000). This large amount of wastewater needs to be treated. Processing the stillage, including drying and evaporating, is the major challenge for corn-ethanol plants since it accounts for 46.8% of the total energy consumption of a bioethanol plant (Khalid et al., 2011). Also, not all bioethanol plants have a livestock farms nearby to sell the animal feed or the plants produce too much animal feed to be handled by nearby farms (Eskicioglu et al., 2011). Moreover, phosphorus and nitrogen of DDGS can end up in the animal feed and be subsequently be discharged, which can cause environmental issue such as eutrophication (Arora et al., 2011). Phosphorus is an essential parameter in food security but it is finite and non-renewable; therefore, recycling and closing phosphorus loop is critical (Scholz et al., 2013; Yang et al., 2019).
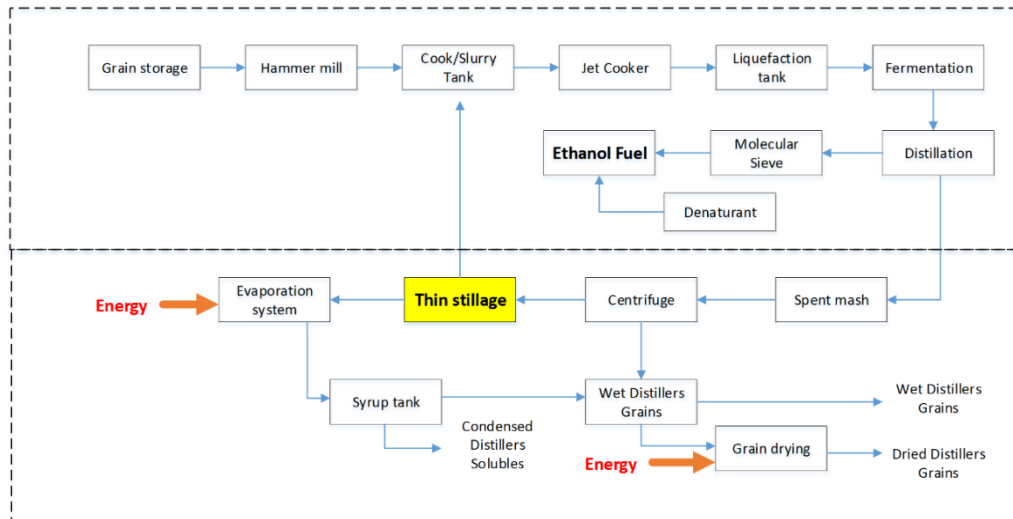
Figure 1-1: A schematic view of a conventional bioethanol plant

Alternative technologies for thin stillage treatment such as anaerobic digestion can be applied to remove the organic materials and improve the energy balance of the process since the biogas produced from the digesters presents an alternate energy source for the plant (Wilkie et al., 2000). There are different types of anaerobic digester such as continually stirred tank reactors (CSTR), upflow anaerobic sludge blanket (USAB), anaerobic filter (AF) and anaerobic baffled reactor (ABR) (Khanal, 2008). Various anaerobic digester configurations have been invented for the treatment of waste streams with a diverse range of characteristics. Treating waste streams with a high organic loading rate (OLR) such as thin stillage is not possible for every type of anaerobic digesters. Also, anaerobic digesters such as USAB are not able to work well with wastewaters containing high suspended solid levels (e.g. thin stillage) due to sludge washout from the digester and low sludge formation rate (Schmidt and Ahring, 1996). Moreover, thin stillage has a relatively high sulfur content (approximately 500 mg L^{-1} (Alkan-Ozkaynak and Karthikeyan, 2011)), which is undesirable since sulfides can inhibit the activity of methane

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producing bacteria and the presence of hydrogen sulfide in the produced biogas is inevitable (Alkan-Ozkaynak and Karthikeyan, 2011).

ABRs offer significant advantages over other digesters. ABR is a compartmentalized reactor, which can foster a two-phase system, allowing optimal conditions for methanogenic and acidogenic bacteria, which is ideal for high OLR (Fang, 2010a). Also, sulfate in the influent stream will lead to sulfidogenesis and sulfur removal primarily in the first compartment of the ABR and as a result mainly biogas from the first compartment contains the hydrogen sulfide (Saritpongteeraka and Chaiprapat, 2008b). The other advantages of ABR include higher stability to organic and hydraulic shock loadings and longer solid retention time (SRT). Among the benefits, the phase separation is the most important one since it provides increased protection against toxic materials. Besides, it enhances the stability of the system to fluctuation in environmental conditions such as temperature and pH (Zhu et al., 2015). Moreover, the anaerobic digestion of thin stillage does not need heating to the optimum temperature (35 °C) since the temperature of thin stillage in bioethanol plants is approximately 70 °C (Tomczak-Wandzel et al., 2012). Therefore, the conventional treatment systems for thin stillage such as evaporator in bioethanol plants can be replaced by ABR. Using the ABR not only reduces the energy consumption significantly but also it produces methane that can be used as an energy source in power plants for combined heat and power generation (CHP).

The drawback of anaerobic digestion is that it is not able to reduce the nutrient in thin stillage such as nitrogen and phosphorus efficiently (Wilkie et al., 2000). However, this disadvantage can be an opportunity to improve the performance of bioethanol plant. The

effluent of the ABR, which is rich in N and P, can be applied as a resource for microalgae cultivation. Many studies have proven that microalgae are capable of using the nutrients from different wastewaters and digestates (Razzak et al., 2013). However, the high concentration of ammonia in the digestate is inhibitory to the growth of microalgae. On the other hand, the optimal molar N/P ratio is 16 for microalgae growth (Choi and Lee, 2015; Kafle and Kim, 2013; Li et al., 2011) while the N/P ratio in thin stillage digestate is close to 2.1. Therefore, a process such as struvite recovery can reduce the concentration ammonia and its toxic effect and also increase the N/P ratio of digestate (Sayedin et al., 2019). Furthermore, phosphorus limitation provides a more suitable environment for microalgae to overcompete bacteria in a medium (Marcilhac et al., 2014).

Struvite is a value-added product, which can be used for producing fertilizer (Muhmood et al., 2019). Then, the struvite removed digestate is ready to be utilized for microalgae growth. The microalgae grown on struvite removed digestate can be mixed with corn for bioethanol production within the plant. This will increase the yield of ethanol production per same input amount of corn as feedstock. Also, protein content of microalgae will end up in DDG and increase its protein content and improve the quality of animal feed. Collectively, the integrated system of anaerobic digestion and microalgae cultivation can potentially enhance the sustainability of corn-ethanol plants. The schematic view of the proposed plant is shown in Figure 1-2. As shown in the figure, the thin stillage goes to the ABR and it produces digestate and methane. The methane is used in the power plant and the digestate after struvite recovery is used for microalgae cultivation. Then, the algal biomass is sent to the process for bioethanol production. The integration of microalgae

cultivation into an existing corn ethanol plant eliminates the need for costly and energy intensive microalgal harvesting processes.
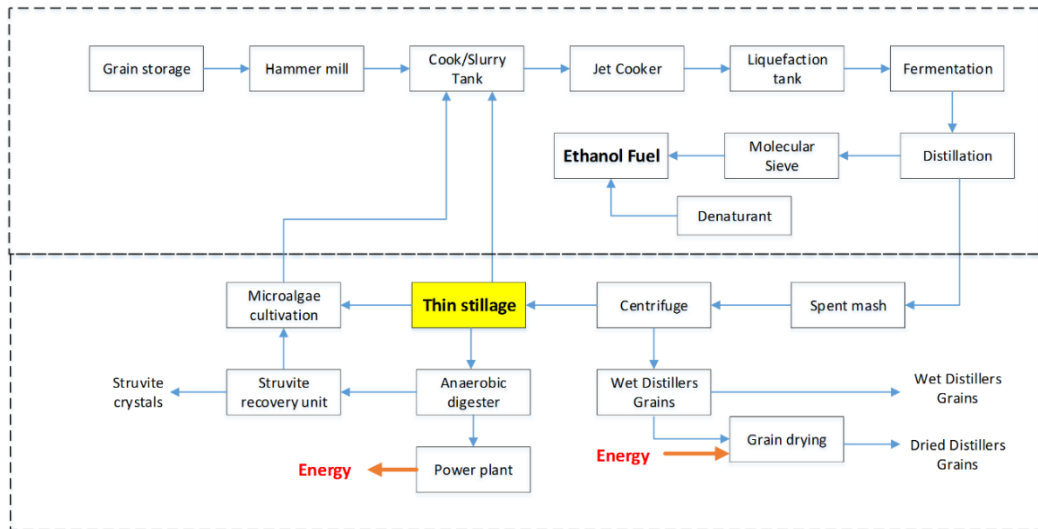
Figure 1-2: A schematic view of a bioethanol plant with the proposed treatment system

The simplified diagram of the proposed configuration is illustrated in Figure 1-3. In the figure, in addition to mentioned advantages, the potential of CO_2 capturing from CHP and bioethanol plants by microalgae is shown.
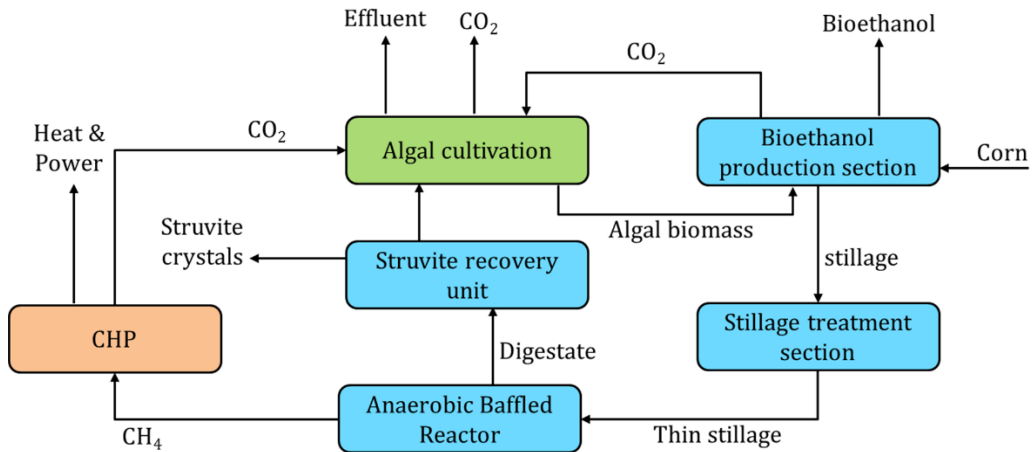
Figure 1-3: Simplified diagram of an integrated anaerobic treatment, algal cultivation and corn-microalgae bioethanol production

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Based on energy and environmental considerations, this study proposes a new configuration of corn-based bioethanol plants including an anaerobic baffled reactor (ABR) and a microalgae cultivation system. The proposed system has the following advantages:

- Bioethanol production from microalgae as the third generation of feedstock

- Recycling nutrients in the bioethanol production process

- Capturing a part of the produced CO_2 from bioethanol plant

- Efficient treatment of organic material in thin stillage by an ABR

- Producing methane by an ABR as a new energy source for heat and power generation and reducing the total energy consumption of plant

- Producing struvite as a value-added product

- Treating thin stillage by anaerobic digestion and microalgae cultivation on digestate for biofuel and bioproduct production

- An opportunity for a closed-loop system by recycling starch-rich microalgae into the front-end of the corn-ethanol plant to partially replace corn

1.2 Research Objectives

This PhD research is aimed at understanding the requirements and characteristics of anaerobic digestion of thin stillage by an anaerobic baffled reactor and microalgae cultivation in anaerobically digested thin stillage for carbohydrate production. The

anaerobic digestion and microalgae cultivation system can be integrated into the existing corn-ethanol plants. The specific goals of this study are as follows:

- Enhance the sustainability of corn-ethanol by innovation at multiple scales

- Develop an understanding of the strengths and shortcomings of two-phase systems for anaerobic digestion of thin stillage and innovate system design to overcome the challenges

- Reduce/eliminate dilution of digestate used as growth medium for microalgal growth by process design

- Identify robust algae species capable of growing on thin stillage digestate and develop an understanding of the biomass composition

- Develop an understanding of the synergy of bacterial population and microalgae for nutrient and carbon conversion

1.3   Research Significance

The anaerobic treatability of thin stillage in different digesters, including CSTR (Lee et al., 2011; Moestedt et al., 2013; Schaefer and Sung, 2008), anaerobic sequencing batch reactors (ASBR) (Agler et al., 2008), anaerobic fluidized bed reactor (AFBR) (Andalib et al., 2012) and anaerobic membrane bioreactor (AnMBR) (Dereli et al., 2012; Dereli et al., 2014), has already been investigated (Agler et al., 2008; Andalib et al., 2012; Dereli et al., 2012; Dereli et al., 2014; Lee et al., 2011; Moestedt et al., 2013; Schaefer and Sung, 2008). Thin stillage has a high COD and total suspended solid. The phase-separated anaerobic

digesters such as ABR are designed for wastewater with high COD (Fang, 2010a). ABRs have been applied for treatment of wastewaters such as soybean protein processing (Zhu et al., 2008), whisky distillery (Akunna and Clark, 2000), pulp and paper mill black liquor (Grover et al., 1999) and high sulfur wastewater (Saritpongteeraka and Chaiprapat, 2008a). This study shows the challenges and limitations regarding the phase separation and operation of ABR for treating thin stillage. Also, the strategies to avoid digester failure and to improve the efficiency of system with respect to COD removal efficiency and methane productivity is investigated, which provides insight about the application of this technology on a pilot or full scale. Moreover, the evaluation of biogas production from ABR can reveal the potential of energy savings in the bioethanol plant. In the present study a novel configuration of ABR is introduced to address the challenges regarding the conventional ABR.

Although anaerobic digestion technology can efficiently remove the organic material, it is not able to reduce the nutrients such as phosphorus and nitrogen in the waste stream significantly. Therefore, the wastewater cannot be discharged into the environment. Microalgae were successfully cultivated on anaerobically digested wastewaters such as digested dairy manure (Wang et al., 2010b), digested poultry litter (Singh et al., 2011), digested cheese whey (Riaño et al., 2016), digested starch (Yang et al., 2015), digested piggery effluent (Kumar et al., 2010) and digested cheese factory effluents (Blier et al., 1995) and removed the nutrients from them. However, the anaerobically digested thin stillage from an ABR has not been used for microalgae cultivation despite its high potential (rich in nutrient). This research shows the ability of different microalgae species to grow

on the anaerobic effluent and remove nutrients as well as organics. Furthermore, the effect of different digestate dosages and pretreatment methods is investigated. Using the results from the experiments, the maximum biomass concentration and nutrient removal efficiency were determined.

Also, in most studies the microalgae cultivation on anaerobic digested materials is used for lipid production (Bjornsson et al., 2013; Cai et al., 2013b; Cho et al., 2013; Olguín et al., 2015; Riaño et al., 2016; Wang et al., 2010b; Woertz et al., 2009; Yang et al., 2015) and its application for carbohydrate production has rarely been studied (Singh et al., 2011). Microalgae cultivation under different environmental stresses for the purpose of carbohydrate production has been mainly conducted in synthetic medium (Ho et al., 2012; Ho et al., 2013a; Ho et al., 2013c; Ho et al., 2013d; Hosono et al., 1994; Khalil et al., 2010; Siaut et al., 2011; Sukenik and Wahnon, 1991; Yao et al., 2012). In this study, the potential of carbohydrate production using microalgae cultivation on thin stillage digestate has been investigated. To the best of our knowledge, the use of anaerobic digestate of thin stillage for microalgae cultivation in general and for carbohydrate production has not been previously studied. Use of thin stillage digestate as microalgal growth media provides an opportunity for integrating the first and third generations of biofuel, resulting in enhancing the sustainability of the bioethanol plant. Besides carbohydrate, determining the protein and lipid content of the produced biomass gives insight about the potential of converting an existing corn ethanol plant to a more economically sustainable and environmentally friendly biorefinery plant.

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CHAPTER 2   LITERATURE REVIEW

2.1 Basic Principles of Anaerobic Digestion

Industrial wastewaters are one of the main sources of the water pollution because of the high concentration of organic matters. The characteristics of wastewaters are different based on the type of industry (Rajeshwari et al., 2000). High strength wastewater, (wastewater with chemical oxygen demand (COD) concentration higher than 4000 mg L^{-1} (Chan et al., 2009)) needs to be treated to remove the organic matters. For this purpose, typically, a biological treatment approach is used. The biological process is preferably anaerobic (break down of organic material by microorganism in the absence of oxygen) due to its potential for providing methane as an energy source and producing low amount of sludge compared to aerobic (microbial activity in the presence of oxygen) systems. Moreover, aerobic systems are suitable for treating low strength wastewater (COD lower than 1000 mg L^{-1}) and treatment of wastewater with COD higher than 4000 mg L^{-1} with them is not feasible (Hamza et al., 2016). In fact, the advantages of anaerobic system for treating high strength wastewater such as low sludge production, low energy requirement and methane production outweigh the advantages of aerobic digestion (Chan et al., 2009).

Anaerobic processes are biological processes in which organic materials are metabolized in an environment where oxygen is not present. This multi-step process includes bioconversion of organics to methane and CO_2. A specific group of bacteria is responsible for each mentioned step (Parkin and Owen, 1986). The basic metabolic routes of anaerobic digestion are shown in Figure 2-1.
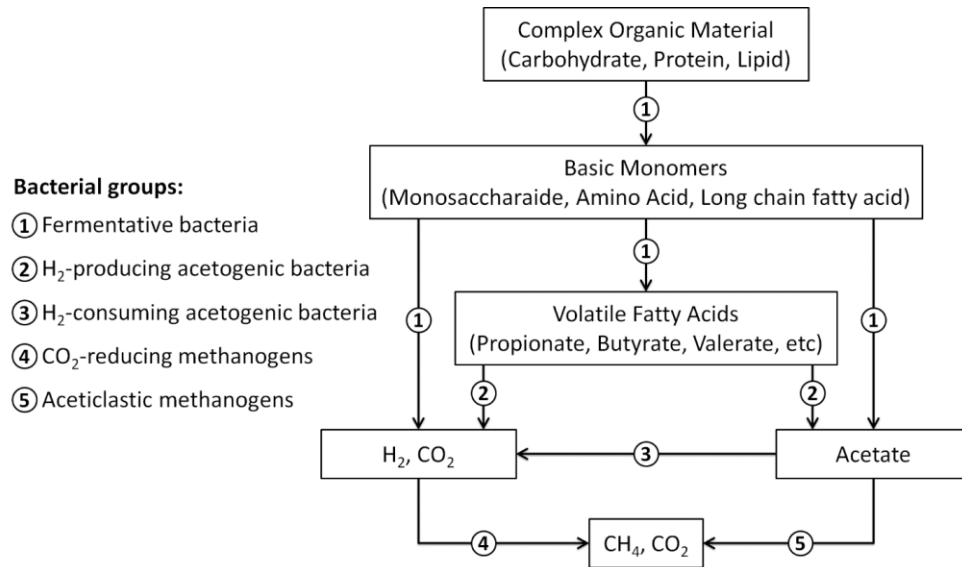
Figure 2-1: Basic metabolic pathway of anaerobic digestion

The first step in anaerobic digestion is the hydrolysis of complex organic material like proteins, polysaccharides and fats to its basic monomers by the exo-enzymes excreted by fermentative bacteria. For instance in the case of lactose which is a polysaccharides, it breaks down to glucose (a monosaccharide) and galactose by following reaction (Pawlowski, 1982).

$$Lactose + H_2O \rightarrow Galactose + Glucose \tag{2.1}$$

During the hydrolysis, the lipids, mainly triglycerides are converted to three fatty acids and a glycerol as shown in the following reaction (Henze, 2008).

$$Triglyceride + 3H_2O \rightarrow Glycerol + 3\ fatty\ acids + 3H^+ \tag{2.2}$$

The next stage of the anaerobic digestion process is acidogenesis or acidification in which hydrolyzed products convert into smaller molecules with a lower molecular weight such as volatile fatty acids (VFA), aldehydes, alcohols and gases like CO_2, H_2 and NH_3. A very diverse group of bacteria affect acidification, in which the majority of them are strictly

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anaerobic (the presence of oxidants like oxygen or nitrate is toxic). However, for these strict anaerobes, there are always bacteria that use oxygen whenever it is available. For removing all oxygen that might be introduced into the system, the presence of these bacteria is very important. The acidogenic bacteria can metabolize organic material down to a very low pH (around 4) (van Haandel and van der Lubbe, 2007). As an example, the acidogenic reactions of sucrose as a substrate is given as follows (Henze, 2008):

$$C_{12}H_{22}O_{11} + 9H_2O \rightarrow 4CH_3COO^- + 4HCO_3^- + 8H^+ + 8H_2 \tag{2.3}$$

$$C_{12}H_{22}O_{11} + 5H_2O \rightarrow 2CH_3CH_2CH_2COO^- + 4HCO_3^- + 6H^+ + 4H_2$$

$$C_{12}H_{22}O_{11} + 3H_2O \rightarrow 2CH_3COO^- + 2CH_3CH_2COO^- + 2HCO_3^- + 6H^+ + 2H_2$$
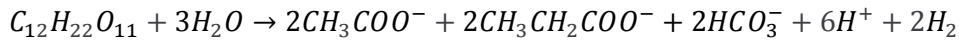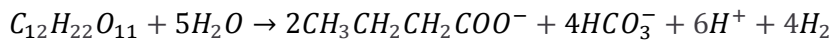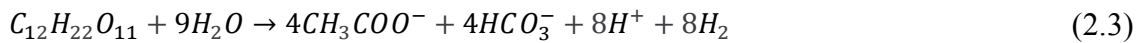
In the third step (acetogenesis), acetogenic bacteria convert the products of the acidogenesis into acetic acid, hydrogen, and carbon dioxide. The first three steps of anaerobic digestion are usually grouped together and called acid fermentation. It should be noted that the acid fermentation process does not remove organic material from the liquid phase. In fact, it is converted into a suitable form for the subsequent process of methanogenesis (van Haandel and van der Lubbe, 2007). Selected acetogenic reactions for conversion of propionate and butyrate to acetate are illustrated below (Khanal, 2008):

$$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + H^+ + HCO_3^- + 3H_2 \tag{2.4}$$

$$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2$$
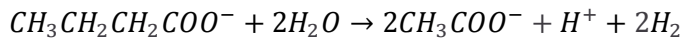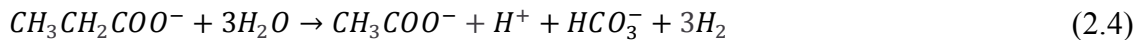
In the last step of anaerobic digestion, archaeal methanogens use hydrogen and acetic acid generated by obligate hydrogen producing acetogens to produce methane. Methane production from acetic acid and from H_2 and CO_2 is conducted by acetoclastic methanogens and hydrogenotrophic methanogens, respectively. Only then, the organic

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material will be removed, as the produced methane gas will largely desorb from the liquid phase (McCarty, 2012). Selected reactions related to methanogens are as follows:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \tag{2.5}$$

$$4HCOO^- + 2H^+ \rightarrow CH_4 + CO_2 + 2HCO_3^-$$

$$CH_3COO^- + H_2O \rightarrow CH_4 + 2HCO_3^-$$
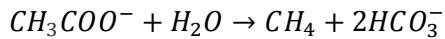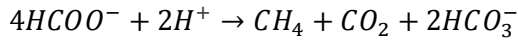
COD is an indirect measurement for the amount of organic material in the waste stream. It equals the amount of oxygen needed to fully oxidize all the organic compounds. The methane production in the anaerobic system can be calculated by COD balance so that the COD lost is accounted for methane production. The COD of methane is the amount of O_2 for oxidation of methane as follows.

$$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O \tag{2.6}$$

Based on the above reaction, each mole of methane (16 g) needs 2 moles (64 g) of O_2 (COD) to be oxidized. The volume of 1 mole CH_4 at standard condition is 22.414 L. Thus, the CH_4 production per COD conversion will be 22.414 L / 64 g = 0.350 L CH_4 g^{-1} COD at standard conditions (1 atm and 0 °C) (Riffat, 2012).

For maintaining an anaerobic sludge with a high metabolic activity, it is essential to provide desirable environmental conditions. In this regard, the most important factors are temperature, pH, nutrients and the absence of toxic materials. The methanogens are very susceptible to adverse environmental conditions and that is why the environmental conditions are always maintained close to optimal conditions for these bacteria (Akindele and Sartaj, 2018; van Haandel and van der Lubbe, 2007). These optimal conditions will be explained in detail in the next section.

2.2 Parameters Influencing Anaerobic Digestion

1.1.1 pH, alkalinity and volatile fatty acids

Each group of bacteria has a different optimal pH range. Methanogenic bacteria are extremely susceptible to pH and their optimum range is 6.5–7.2 (Boe and Angelidaki, 2006). The fermentative microorganisms are less sensitive and can be active in a wider range of pH (4.0–8.5) (Hwang et al., 2004). The optimum pH is 5.5–6.5 for acidogens. At a low pH (around 4) the main products of acidogenesis are acetic and butyric acid, whereas mainly acetic and propionic acid are produced at a pH of 8.0 (Boe and Angelidaki, 2006). This occurs because of the change in the dominant microbial populations, from butyric-acid-producing-bacteria to propionic-acid-producing-bacteria when the pH of reactor increases from 4 to 8 (Horiuchi et al., 2002).

Since methanogenesis is the rate-limiting stage in anaerobic digestion, where both groups of bacteria are present, it is very important to control the reactor pH close to neutral. The pH of an anaerobic system can be controlled by self-produced alkalinity or natural alkalinity. The destruction of organic matter, primarily the proteins, produces NH_3. Each mole of organic nitrogen (e.g. amino acid ($RCH(NH_2)COOH$)) theoretically produces one mole equivalent of alkalinity (Moosbrugger et al., 1990). Ammonia–N reacts with CO_2 generated during the biochemical reaction to produce ammonium bicarbonate, which contributes to alkalinity (equation 2.7). Only wastes with high organic nitrogen (e.g., protein) such as thin stillage and bean curd manufacturing waste can adequately contribute to alkalinity (Khanal, 2008).

$$RCH(NH_2)COOH + 2H_2O \rightarrow RCOOH + NH_3 + CO_2 + 2H_2 \qquad (2.7)$$

$$NH_3 + H_2O + CO_2 \rightarrow NH_4^+ + HCO_3^-$$
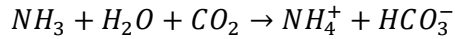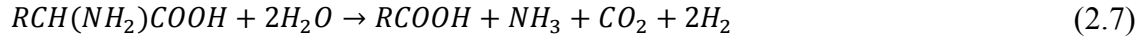
Likewise, the treatment of high-sulfate/sulfite wastewater such as molasses fermentation, seafood processing, pharmaceutical plant and edible oil refinery also produces alkalinity due to sulfate/sulfite reduction. In fact, reduction of 1 g SO_4 provides 1.04 g of alkalinity as $CaCO_3$ (equation 2.8)(Greben et al., 2000). In the neutral pH range, which is optimal for anaerobic treatment, alkalinity mainly exists in the bicarbonate form.

$$H_2 + SO_4^{2-} + CO_2 \rightarrow HS^- + HCO_3^- + 3H_2O \qquad (2.8)$$

$$CH_3COO^- + SO_4^{2-} \rightarrow HS^- + 2HCO_3^-$$
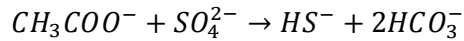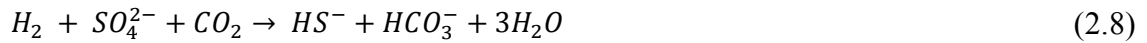
The alkalinity in anaerobic treatment processes is usually between 1,000 and 5,000 mg L^{-1} as $CaCO_3$ (Metcalf, 2003). The stability of an anaerobic system can be determined by VFA/TA (total alkalinity) ratio. A VFA/TA ratio of 0.1–0.25 is usually desirable without the risk of acidification while a ratio beyond 0.3–0.4 indicates digester upset, and corrective measures are necessary. When the ratio is 0.8 or higher, inhibition of methanogenesis occurs and leads to digester failure (Khanal, 2008; Li et al., 2014; Nigam and Pandey, 2009). In cases where the natural alkalinity is not enough to provide the desirable range of VFA/TA, chemicals such as sodium bicarbonate, sodium carbonate, ammonium hydroxide, gaseous ammonia, lime, sodium, and potassium hydroxide are used to maintain an optimum pH in the bioreactor. Among them, sodium bicarbonate is the preferred source due to its high solubility, low toxicity and long-lasting impact. In addition, direct addition of bicarbonate ions leads to a direct pH increase contributing gas-phase carbon dioxide (Rajeshwari et al., 2000).

2.2.1 Temperature

Temperature can greatly affect the physicochemical features of substrates in the anaerobic digestion system. It also has an effect of metabolism of bacteria and as a result their population in the digester. Among the bacteria, methanogens are the most susceptible population to increasing temperature (Rehm et al., 2000). The optimum temperature for the anaerobic digestion can be 37 °C (in mesophilic condition) or 55 °C (in thermophilic condition). Thermophilic conditions have some advantages including increased solubility of the organic compounds, enhanced reaction rates and higher death rate of pathogens (Qi et al., 2018). On the other hand, in thermophilic conditions, because of the higher temperature compared to mesophilic conditions, there would be an increase in the fraction of free NH_3 which is inhibitory to the bacteria. The reason is that the dissociation constant of NH_3 is temperature sensitive and the dissociation constant increases with increasing temperature (Boyd and Tucker, 2012). Moreover, an increase in temperature increases pKa for VFA production and consequently increases the undissociated fraction of VFA which makes the system more suitable to inhibition and more difficult to control (Boe and Angelidaki, 2006). In both thermophilic and mesophilic conditions, a stable operating temperature should be maintained since the frequent and intense fluctuation in the temperature has an adverse effect on methanogens and may cause failure of anaerobic systems. The other important property of the anaerobic bacteria is their low decay rate under 15 °C which makes it possible to preserve the bacteria for a long period of time (Rajeshwari et al., 2000).

2.2.2 Solids and hydraulic retention time

The solids retention time (SRT) is the average time the solids spend in the digester. A decrease in SRT means the sludge leaves the reactor faster and a fraction of the anaerobic bacteria is lost. Thus, the SRT should be at a level so that the biomass growth can compensate the sludge washout from the reactor (Turovskiy and Mathai, 2006). In this regard, studies show that a SRT lower than 5 days is not enough for a stable anaerobic digestion and consequently will lead to accumulation of VFA in the system due to washout of methanogens from the reactor (Appels et al., 2008). The SRT can be calculated using the following equation (Burke and Dennis, 2001).

$$SRT = \frac{V_R . VSS_R}{Q_{out} . VSS_{out}} \tag{2.9}$$

where V_R is the volume of the reactor, VSS_R is the concentration of volatile suspended solids inside the reactor (amount of sludge), Q_{out} is the flowrate of effluent and VSS_{out} is the volatile suspended solids in the effluent.

Based on the equation (2.9), higher VSS in the effluent decreases the SRT; therefore, many different reactor configurations have been developed to decrease the biomass washout from the reactor (Khanal, 2008).

The hydraulic retention time (HRT) is the average time the liquid spends in the digester. HRT is function of digester's volume and flowrate of influent (Q_{in}) as follows:

$$HRT = \frac{V_R}{Q_{in}} \tag{2.10}$$

In anaerobic systems, the minimum HRT is determined with respect to the growth rate of methanogens which are the slowest one in the anaerobic system. The reason is the system may fail in shorter HRT due to washout of methanogens from the system since lower HRT (higher influent flowrate) decreases the SRT (Khanal, 2008). However, the benefit of shorter HRT is the reduction in size of the digester and consequently the capital cost. Moreover, a decrease in HRT will increase the biogas production rate, however, it will decrease the methane fraction of the biogas since there is not enough time for efficient breakdown of VFA to methane (de Lemos Chernicharo, 2007).

2.2.3   Organic loading rate

The organic loading rate (OLR) is the main parameter used for planning the size of digesters and it greatly affects the performance of anaerobic digestion processes. It is defined as the amount of organic matters, which is introduced daily per unit volume of the digester. The maximum OLR that a digestion system can handle depends on the type of digesters. The high rate digesters are capable of treating wastewater with an OLR of 10-40 kg m^{-3} d^{-1} (Khanal, 2008).

$$OLR = \frac{COD_{in} . Q_{in}}{V_R} \qquad (2.11)$$

where COD_{in} is the COD in the influent.

OLR has a great effect on the performance of anaerobic digesters. Increasing the OLR will reduce the COD removal efficiency and methane fraction of biogas while it increases the biogas production rate (Zhu et al., 2015).

2.2.4 Biomass yield

Biomass yield is a measure of cell growth in a system (in the form of volatile suspended solids (VSS)) for a given substrate (COD). The biomass yield (Y) is given as:

$$Y = \frac{increase\ in\ biomass\ concentration\ (mg\ VSS/L)}{decrease\ in\ substrate\ concentration\ (mg\ COD/L)} = \frac{\Delta X}{\Delta S} \qquad (2.12)$$

The yield coefficient of acidogens (0.15 kg VSS kg^{-1} COD) is significantly higher than for methane-producing bacteria (0.03 kg VSS kg^{-1} COD). The overall yield coefficient of an anaerobic system is considered as 0.18 kg VSS kg^{-1} COD (Khanal, 2008). The aerobic treatment process has an approximately constant yield coefficient (around 0.4 VSS kg^{-1} COD (Metcalf, 2003)) for biodegradable COD regardless of the type of waste. Carbohydrate and protein will increase both acetogenic and methanogenic bacteria, whereas acetate and hydrogen only increase the biomass of methanogens (Khanal, 2008).

2.2.5 Food to microorganism ratio

Food to microorganism ratio (F/M) is the ratio of available substrate to the quantity of bacteria in the anaerobic system which can consume the substrate (Strezov and Evans, 2014). The ratio can be calculated by following equation:

$$\frac{F}{M} = \frac{Q_{in}.COD_{in}}{V_R.VSS_R} \qquad (2.13)$$

where Q_{in} is the flowrate of influent, COD_{in} is the COD of influent, VSS_R is the concentration of bacteria in the reactor and V_R is the volume of reactor. The ratio can greatly affects COD removal efficiency, microbial composition and sludge properties (Ghangrekar et al., 2005). In general, lower F/M ratio increases COD removal efficiency and enhances

sludge flocculation and settleability. But too low F/M ratio will limit bacterial growth rate and sludge flocculation. Also, excessive F/M ratio will result in accumulation of VFA in the system due to higher activity of hydrolysis and acidification compared to methanogenesis (Liu et al., 2012b). The suitable ratio of F/M can promote the growth rate of sludge inside the reactor (Liu et al., 2012b). In the literature different F/M ratios have been reported depending on the type of reactor and stage of the process. For the startup period of the reactor, a F/M ratio of 0.3-0.6 g COD g^{-1} VSS d^{-1} is used while in the full operation mode a F/M ratio of 1-3 g COD g^{-1} VSS d^{-1} is applied (Chen et al., 2010; Fang and Chui, 1993; Najafpour et al., 2006).

2.2.6 Nutritional condition and trace metals

For biochemical operations, both macronutrients (nitrogen and phosphorus) and micronutrients (trace minerals) are necessary for the anaerobic digestion to support the formation of new biomass. The calculation of macronutrient requirements is based on wastewater strength (COD). The theoretical minimum COD:N:P ratios of 1,000:7:1 for lightly loaded (<0.5 kg COD kg^{-1} VSS d^{-1}) and 350:7:1 for highly loaded (0.8–1.2 kg COD kg^{-1} VSS d^{-1}) anaerobic systems can be used to calculate the nitrogen and phosphorus needs (Khanal, 2008).

As mentioned before, the methanogens are the most sensitive group of bacteria in an anaerobic system to environmental conditions. Also, the biomass yield of them is five times lower than acidogens. Therefore, providing good conditions for growth of methanogens is essential for success of an anaerobic system. These nutrients (in decreasing order of importance) are essential for the growth of methanogens: nitrogen, sulfur, phosphorus,

iron, cobalt, nickel, molybdenum, selenium, riboflavin and vitamin B_{12} (de Lemos Chernicharo, 2007).

2.2.7 Granulation

A granule is an aggregation of packed diverse microbial groups and the process of formation of the granule is called granulation (Khanal, 2008). The granulation process allows higher loading rates compared to conventional activated sludge processes. Two main factors make these high loading rates possible:

1) The superior settling features of granules. Settling velocity of 60 m h^{-1} for granular sludge is common, whereas the superficial upflow velocity in the case of upflow anaerobic sludge blanket (UASB) reactors are usually below 2 m h^{-1}. This allows an extreme uncoupling of the HRT from the SRT so that SRT of 200 days can be achieved at HRT of only 6 h (Pol et al., 2004).

2) The high specific methanogenic activities (SMA) of granules. It could be shown that high OLR of over 50 kg COD m^{-3} d^{-1} could be well handled under mesophilic conditions, with SMA of more than 2 kg COD kg^{-1} VSS d^{-1} (Pol et al., 2004). Micromorphology of the granules showed that colonies of acetogenic bacteria and micro-colonies of hydrogenotrophic methanogenic archaea are closely linked which provides an efficient interspecies hydrogen transfer and as a result, high degradation rates (Pol et al., 2004).

It is important to examine the stage of granulation. In this regards, Ahn (2000) proposed a granulation model in which the maturity of granules can be determined by their

appearance using a scanning electron microscope (SEM). In the first stage, the filamentous methanogens start to grow, and, in the last stage, it will be the growth of large granules with multilayer structure and diameter of 2-5mm.

Several studies have concluded that the process of granulation in single-stage reactors can be affected by conditioning the sludge with polymers (Uyanik et al., 2002a; Uyanik et al., 2002b; Wirtz and Dague, 1996). Moreover, it has been shown that some metal ions, such as Ca^{2+}, Fe^{2+}, Al^{3+} and Mg^{2+} enhance the granulation and play an important role in microbial aggregation (Schmidt and Ahring, 1993; Yu et al., 2001). Besides, the abiotic environment, like temperature, mixing, ionic strength and hydrogen-ion concentration can influence the granulation process (Pol et al., 2004; Schmidt and Ahring, 1996; Wang et al., 2018a). Yu et al. (2001) studied the effect of aluminum chloride ($AlCl_3$) and calcium chloride ($CaCl_2$) on the sludge granulation process. The 4,000 mg COD L^{-1} of soluble synthetic wastewater was used to feed an UASB reactor at OLR of 2.0 kg COD m^{-3} d^{-1}. The results illustrated that the addition of $AlCl_3$ at a concentration of 300 mg L^{-1} reduced the sludge granulation time by approximately one month. In addition, increasing the $CaCl_2$ concentration from 150 to 300 mg L^{-1} enhanced the biomass accumulation and granulation process (Yu et al., 2001).

2.2.8 Upflow velocity

The upflow velocity is an important factor in the performance of anaerobic digesters such as ABR, UASB and anaerobic fluidized/expanded bed reactor (AFBR/AEBR), expanded granular sludge bed (EGSB) and upflow anaerobic filter (UAF) in which the liquid upflow velocity is the main mechanism for providing contact between sludge and

organic materials. It can affect the performance of anaerobic digesters in several ways. The upflow velocity of liquid creates a continuous selective pressure on the microorganisms, which start to adhere together. Therefore, it causes formation of dense high quality granules which improve the efficiency of system (O'flaherty et al., 1997; Wang et al., 2018a). Moreover, the sludge bed can be expanded by controlling the upflow velocity in order to provide effective contact between wastewater and active sludge. However, the upflow velocity should be maintained below a limit since a too high upflow velocity will lead to washout of biomass from the system. The limit depends on density of suspended solids and granules so that higher upflow velocity is possible with denser granules (de Lemos Chernicharo, 2007). Also, upflow velocity depends on the type of reactor. For instance the upflow velocity for USAB reactor is between 1-3 m h^{-1} while the upflow velocity for EGBR is around 5-10 m h^{-1} (Rajeshwari et al., 2000; Wang et al., 2007). In general, the upflow velocity is maintained at low level (0.3-0.4 m h^{-1}) in the startup period since the sludge are fine and can be easily washout from the reactor (Barber and Stuckey, 1999; Rollon, 2005).

2.2.9 Mixing

Mixing can provide efficient transfer of organic matters to microbial communities. Depending on the configuration of digester, mixing can have different functions such as preventing sedimentation of denser particulates or releasing gas bubbles trapped in the medium (Burton and Turner, 2003; Liu et al., 2018). Furthermore, it is shown that the low speed mixing can absorb the disturbance of organic shocks in the system. On the other hand, high speed mixing can disrupt the granule structure. The granules have a positive effect on the performance of digesters, therefore low mixing speed is recommended to

maintain the granule structure (Ong et al., 2002). Diverse methods of mixing can be used. In some digesters, propellers are used which are appropriate for low viscosity liquids. Also, in order to avoid using moving parts inside the digester, the recirculation of biogas into the bottom of the reactor or recycling of digestate to the influent of the reactor can be applied (Karim et al., 2005). Recycling the effluent stream can reduce the COD removal efficiency since the reactor approaches to a completely mixed system and as a result the mass transfer driving force for substrate removal decreases in spite of a small increase in the OLR (Barber and Stuckey, 1999).

2.2.10 Start-Up

The start-up time is an important factor in anaerobic digestion systems. Lower start-up times can increase competitiveness of the high-rate anaerobic systems. The startup time for mesophilic condition (37 °C) is usually 2-4 months while under thermophilic condition (55 °C) it may take up to 1 year (Khanal, 2008). Also, the biomass initial inventory can affect the startup time so that more initial inventory will result in less startup time. The recommended value for initial sludge is around 30-60% of reactor volume (approximate volatile solid of 30 g L^{-1}) (Cervantes et al., 2006; Hutňan et al., 1999). However, depending on the type of reactor, different inoculum amount is suggested. For fixed film reactors such as UAF, DAF and AnMBR, the quantity of seed should be at least 10% of reactor volume. On the other hand, for suspended growth biomass reactors such as UASB, EGSB and ABR, a seed quantity of 30% reactor volume is required (Stronach et al., 1986). Also, different amounts for initial biomass concentration in the reactor are reported ranging from 4.1-30

g VSS L^{-1} (Barber and Stuckey, 1999; Stronach et al., 1986). The start-up can be basically obtained in the following three ways (de Lemos Chernicharo, 2007):

1) With seed sludge adapted to the target wastewater: the start-up of the system occurs fast, in a satisfactory way, as the sludge do not need to be acclimated.

2) With seed sludge not adapted to the target wastewater: in this case, the start-up of the system goes under acclimatization.

3) With no use of seed sludge: this is considered the most undesirable form to start up the system. As the concentration of microorganisms in the wastewater is very small, the time required for the retention and selection of a large microbial mass can be very long (4 to 6 months).

Furthermore, different initial OLR is considered for the startup period of an anaerobic digester (between 0.4 to 4.3 kg COD m^{-3} d^{-1}) (Barber and Stuckey, 1999). In general, OLR of 20% of the design volatile solids loading capacity is recommended for the first 20 days of operation (Khanal, 2008).

2.2.11 Inhibitory factors

2.2.11.1 Volatile fatty acids

Short chain fatty acids at a high concentration can have an inhibitory effect on methanogens. The study of Siegert and Banks (2005) showed that the fermentation of glucose can be inhibited at total VFA concentrations above 4 g L^{-1}. Also, individual fatty acids have different inhibitory effect. For example, propionic and butyric acids are more inhibitory to the methanogens than acetic acid. Hill et al. (1987) examined the organic acid

levels in the digester and found that, in the digestion of cow or swine manures, acetic acid levels greater than 0.8 g L⁻¹ and propionic to acetic acid ratios greater than 1:1.4 indicate impending failure. Based on the literature, the tolerance concentration of acetic, propionic and butyric acid for sludge is 6, 4 and 8 g L⁻¹, respectively (Liu et al., 2018; Yuan and Zhu, 2016).

2.2.11.2 Ammonia

The degradation of nitrogen compounds, especially protein and urea produces ammonia. The free ammonia in the anaerobic system is more toxic than ammonium. The free ammonia concentration is mainly a function of total ammonia concentration, pH and temperature (Hansen et al., 1998). The ammonia-ammonium equilibrium can be described by following reaction and equation of dissociation constant (K_a):

$$NH_4^+ \leftrightarrow NH_3 + H^+ \tag{2.14}$$

$$K_a = \frac{[NH_3][H^+]}{[NH_4^+]} \tag{2.15}$$

The dependence of ammonia-ammonium equilibrium on temperature (T [°C]) is given as (Emerson et al., 1975):

$$pK_a = \frac{0.09108 + 2729.92}{273.2 + T} \tag{2.16}$$

Based on the above equation, an increase in the temperature decreases the pK_a and consequently increases the K_a. As a result, the fraction of free NH_3 will increase at equilibrium. The fraction of free NH_3 is also function of pH as presented in following equation.

$$Unionised\ NH_3\,(\%) = \frac{100}{1 + 10^{(pK_a - pH)}}$$
(2.17)

The free ammonia fraction is 0.3% and 4.7% at a pH of 6.8 and 8, respectively. Therefore, an increase in temperature or pH will increase the ratio of free ammonia to ammonium (Hansen et al., 1998; Sung and Liu, 2003). An extensive range of inhibition concentration (1.7 to 14 g L^{-1}) for ammonia is reported in the literature which causes 50% reduction in methane production (Chen et al., 2008; Yuan and Zhu, 2016). In order to decrease the concentration of ammonia, dilution of the feed can be used. Moreover, for removing the ammonia, air striping and precipitation are two practical techniques at high concentration of ammonia (Chen et al., 2008).

2.2.11.3 Sulfide

Many wastewater streams contain sulfate such as wastewaters from molasses fermentation, seafood processing, pharmaceutical plant and edible oil refinery. Under anaerobic conditions, sulfate reducing bacteria (SRB) use sulfate as an electron acceptor and therefore convert it to sulfide (Chen et al., 2008). There are two groups of SBR. The first group converts components such as lactate to acetate and CO_2 while the second group consumes the acetate to produce CO_2 and HCO_3 (bicarbonate alkalinity). Therefore, two different inhibitions can happen. The first one is toxicity of sulfide for the microorganism and the second one is the competition of SRB with other bacteria for substrate. The second one is important since the SRB compete with methanogens for substrate like acetate and hydrogen (Boe and Angelidaki, 2006; Chen et al., 2008; Rehm et al., 2000). Moreover, non-dissociated H_2S is toxic for both methanogens and SRB. Regarding the inhibitory level

of concentration, controversial amounts are reported. It is reported that at the sulfide concentration of 150 mg L^{-1} a stable methanogenesis can still happen (Rehm et al., 2000), while another study reports that a total sulfur concentration of 96-192 mg L^{-1} is considered inhibitory to microorganisms (Boe and Angelidaki, 2006). According to the literature, 100–800 mg L^{-1} of dissolved sulfide or 50–400 mg L^{-1} of undissociated H$_2$S can be inhibitory to anaerobic digestion process (Yuan and Zhu, 2016). The dissociation reaction of H$_2$S is:

$$H_2S \leftrightarrow HS^- + H^+ \tag{2.18}$$

The equilibrium and the fraction of free H$_2$S depends on pH and temperature so that an increase in pH or temperature decreases the fraction of free H$_2$S in the solution (Hughes et al., 2009).

2.2.11.4 Sodium

The presence of sodium is necessary for methanogenic bacteria. However, high concentration of Na can be inhibitory to methanogens so that the optimum concentration of Na is 350 mg Na$^+$ L^{-1}, a moderate inhibition can occur at concentration of 3500-5500 mg Na$^+$ L^{-1} and strong inhibition may happen with the concentration of 8800 mg Na$^+$ L^{-1} under mesophilic condition (Chen et al., 2008; Gagliano et al., 2017). Also, the presence of other cations such as potassium and calcium at optimum concentration (400 mg L^{-1} and 200 mg L^{-1}, respectively) can reduce the toxicity of sodium (Chen et al., 2008).

2.2.11.5 Potassium

A potassium concentration lower than 400 mg L^{-1} has a positive effect on the performance of both mesophilic and thermophilic systems. But, a higher concentration is

inhibitory to digestion systems especially under thermophilic condition so that the concentration of 28.86 g L^{-1} may reduce the activity of methanogens by 50% (Kugelman and McCarty, 1965). Similar to sodium, the other cations such as magnesium and calcium at optimum concentration (720 mg L^{-1} and 200 mg L^{-1} respectively) can reduce the inhibitory effect of potassium (Chen et al., 2008).

2.2.11.6 Heavy metals

Industrial activities may introduce heavy metals such as zinc, copper, chromium, nickel, cadmium and lead (Juliastuti et al., 2003). The presence of these metals in high concentration can inhibit the activity of microorganism in an anaerobic system. The main cause of this toxic effect is the disruption of enzyme structure due to bonding of heavy metals to the enzymes (Massé and Droste, 2000). The inhibitory level of concentration for some heavy metals is presented in Table 2-1.

Table 2-1: Inhibitory concentration of some heavy metals in anaerobic system (Appels et al., 2008; Turovskiy and Mathai, 2006)

Substance	inhibitory concentration (mg L^{-1})
Cu^{2+}	0.5 (soluble), 50–70 (total)
Cr^{6+}	3.0 (soluble), 200–250 (total)
Cr^{3+}	2.0 (soluble), 180–240 (total)
Ni^{2+}	30 (total)
Zn^{2+}	1.0 (soluble)
Arseniate and arsenite	> 0.7
Lead-containing compounds	5
Iron-containing compounds	>35
Copper-containing compounds	1

2.2.12 Reactor design

The first anaerobic digesters were low rate reactors without a mixing mechanism such as septic tank and the lmhoff tank. The low rate term refers to the long time (4 to 6 weeks) that the organic needs to spend in the reactor to be significantly biodegraded. The reason for the long time was that there was no continuous contact between non-settleable organic and the underlying active sludge in the tanks (Tauseef et al., 2013). To overcome this problem, high rate anaerobic digesters were introduced in the early 20th century in which a continuous intimate contact between active sludge and wastewater was provided by mixing (Khanal, 2008). Moreover, a part of sludge was recycled to the reactor in order to increase the concentration of microorganism in the reactor and, as a result, decrease the food to microorganism ratio since washout of sludge from the reactor increases the ratio and may overload the system (Nemerow, 2010).

2.2.12.1 Continuously stirred tank reactor (CSTR)

The first developed high rate reactor was a continuously stirred tank reactor (CSTR), in which intense mechanical mixing was introduced into the reactor (Figure 2-2). The COD removal efficiency of the reactor was improved 2-3 times compared to the unstirred one. However, its HRT (10 to 20 days) was still high. Moreover, it has the drawback of biomass washout from the reactor (Harrison et al., 1974). In these reactors, there is a high possibility of failure at low HRT since they have no recycling or solid separation system to prevent the biomass washout from the reactor. Also, higher HRTs need a bigger reactor size which is costly (de Lemos Chernicharo, 2007). Therefore, in this type of reactor, the HRT must be optimized to prevent failure due to biomass washout (Dareioti and Kornaros, 2015).

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These drawbacks reduce the competitiveness of CSTR compared to other high rate anaerobic reactors.
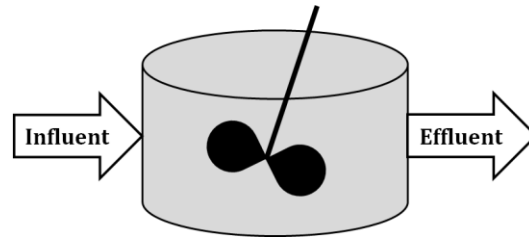
Figure 2-2: Schematic view of a completely mixed reactor

2.2.12.2 Anaerobic contact process (ACP)

A solution for the problem of biomass washout can be that the biomass lost can be compensated by recycling the part of microbial population in the effluent to the reactor. For this purpose, the anaerobic contact process (ACP) was introduced where there is a settling tank after the reactor as shown in Figure 2-3. Therefore, the settled biomass will be recycled to the reactor which can increase the SRT significantly. The ACP is useful for treating wastewater with high suspended solids such as food industry wastewater (Şentürk et al., 2013) or pulp and paper mills (Capela et al., 2009). The biomass concentration in the reactor is typically 4–6 g L^{-1}, (maximum concentrations is 25–30 g L^{-1}). They usually can handle an OLR up to 8-10 kg COD m^{-3} d^{-1} with COD removal of 78–95% (Capela et al., 2009; Khanal, 2008; Şentürk et al., 2010; Şentürk et al., 2013). The main drawback of ACP is the need for degasifier which limits the rate of treatment (Tauseef et al., 2013).
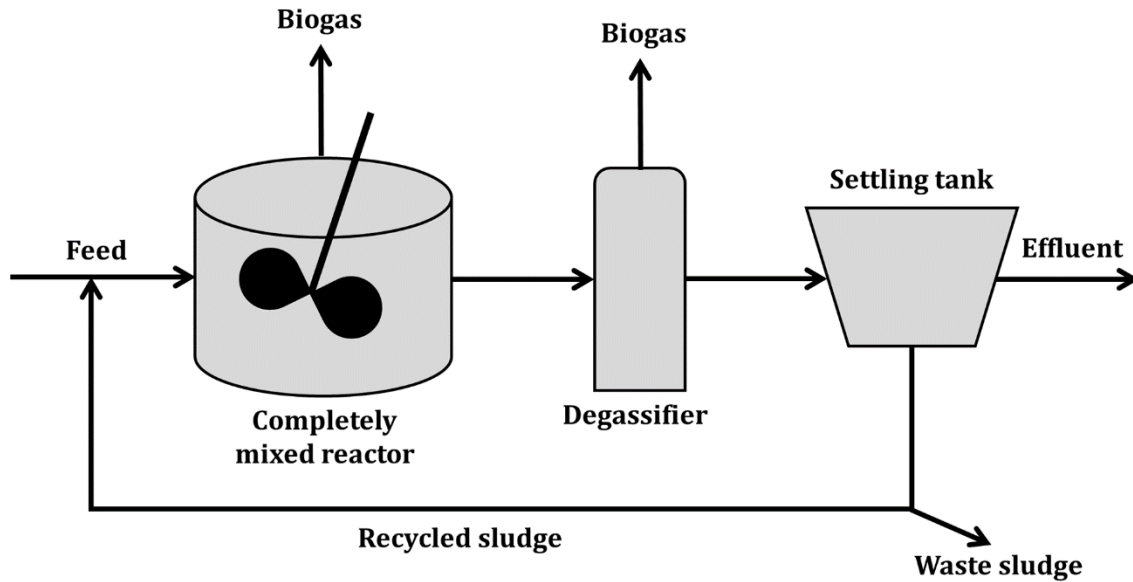
Figure 2-3: Configuration of anaerobic contact digester

2.2.12.3 Anaerobic filter (AF)

The first demonstration of anaerobic filter was an UAF, treating distillery wastewater. Figure 2-4(A) shows an UAF reactor. In the reactor, a support media for bacterial film is provided to attach themselves to it (Chan et al., 2009). Therefore, the microorganisms are able to grow on the media or in the space inside the media particles. In the reactor, the wastewater is introduced from the bottom of the reactor, which provides an intimate contact between waste stream and bacteria. The UAF is employed for treating high strength wastewater such as cheese whey with a COD as high as 80 g L^{-1} (Patel and Madamwar, 1997). The benefit of UAF is that they are able to treat industrial wastewater since the media retains the active sludge for a long time (High SRT), therefore, the reactor can be applied to wastewater with fluctuation in flowrate. The reason is that the high concentration of microorganisms makes the digester less susceptible to fluctuation in pH, temperature

and OLR (Carl et al., 1997). The main drawback of UAF is the accumulation of sludge at the bottom of reactor, which will lead to blockage or may cause formation of hydraulic short circuits (de Lemos Chernicharo, 2007). This issue can be solved by periodically backwashing the filter; however, a more efficient method is needed (Carl et al., 1997). In order to overcome this problem, downflow anaerobic filter (DAF) is used where the wastewater enters the top of the reactor and passes downward through the filter (Figure 2-4(B)). Thus, an upflowing produced biogas in the reactor disperses the downflowing wastewater and facilitates biodegradation (Duff and Kennedy, 1983). These reactors are successfully used for coking wastewater (Huang et al., 2016), greywater (do Couto et al., 2015) and rice winery wastewater (Jo et al., 2015).
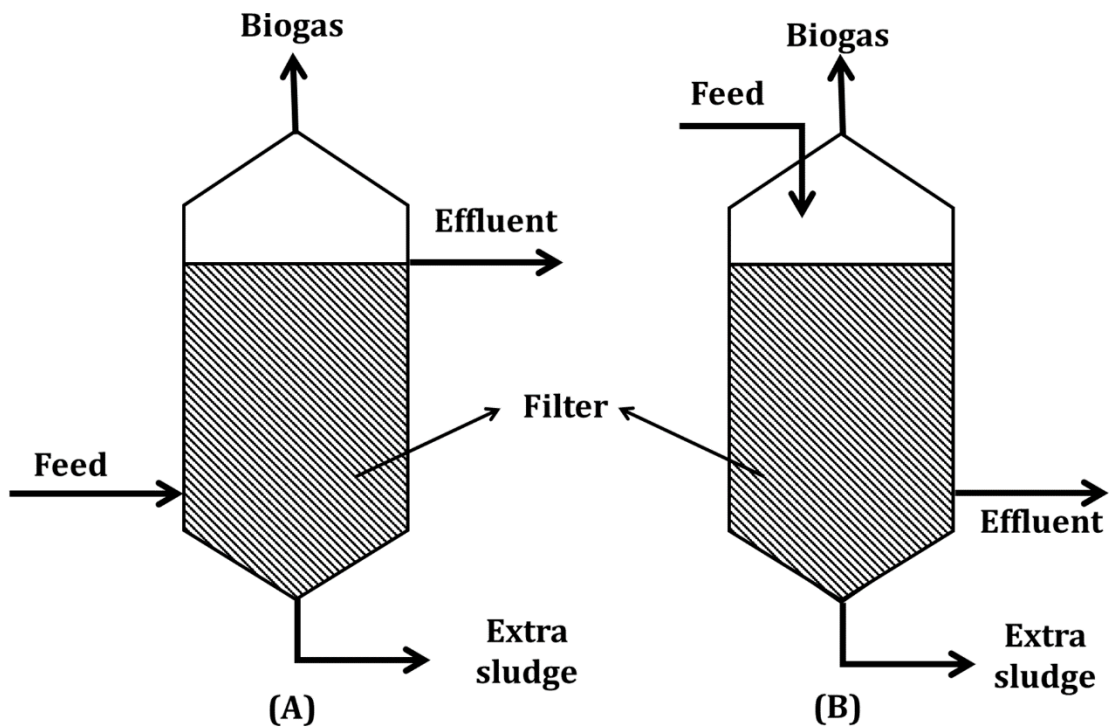
Figure 2-4: Schematic view of (A) upflow and (B) downflow anaerobic filter

2.2.12.4 Upflow anaerobic sludge blanket (UASB)

Lettinga and coworkers (Lettinga et al., 1980) introduced upflow anaerobic sludge blanket (UASB) in Netherlands for treatment of sugar-rich soluble wastewater. In their experiment, they noticed that a support media is not necessary for retaining a high amount of active sludge in the reactor. In fact, this objective can be obtained more efficiently by formation of dense granular sludge in the reactor (Lettinga et al., 1980). As illustrated in Figure 2-5, the process includes an upflow wastewater stream passing through a sludge bed with high microbial activity. In steady full scale reactors, the upflow velocity is usually 2 m h^{-1}, while in the startup period, velocity of 0.3-0.5m h^{-1} is considered as optimal for formation of granules (Panesar and Marwaha, 2013). The mixing in the reactor is performed by upflow stream and gas bubbles. Then, the effluent passes through a gas–liquid–solid separation equipment where the granules as solids and biogas are separated from liquid effluent (de Lemos Chernicharo, 2007). The problem of plugging the filter in anaerobic filter does not occur in UASB since the granules are dense and consequently a higher concentration of bacteria is achievable per unit volume of reactor. Therefore, USAB can handle wastewater with higher COD compared to AF (Abbasi and Abbasi, 2012). The major parameter in success of UASB is the formation of high quality dense granules. The formation of granules depends on the composition of the wastewater so that there is suitable granulation with wastewater containing high amount of VFA and sugar while the granulation may not even happen with some types of wastewater (Aiyuk et al., 2006; Durai and Rajasimman, 2011). Due to its benefits, UASB is the most used high rate reactor type throughout the world. In fact, around 90% of high-rate anaerobic reactors were

conventional UASB reactors, or its modified ones (50–50,000 m^3 in volume) until 2008 (Van Lier, 2008). However, the performance of the reactor largely depends on the quality of granules and usually the formation of large granules needs a long startup time (Mao et al., 2015).
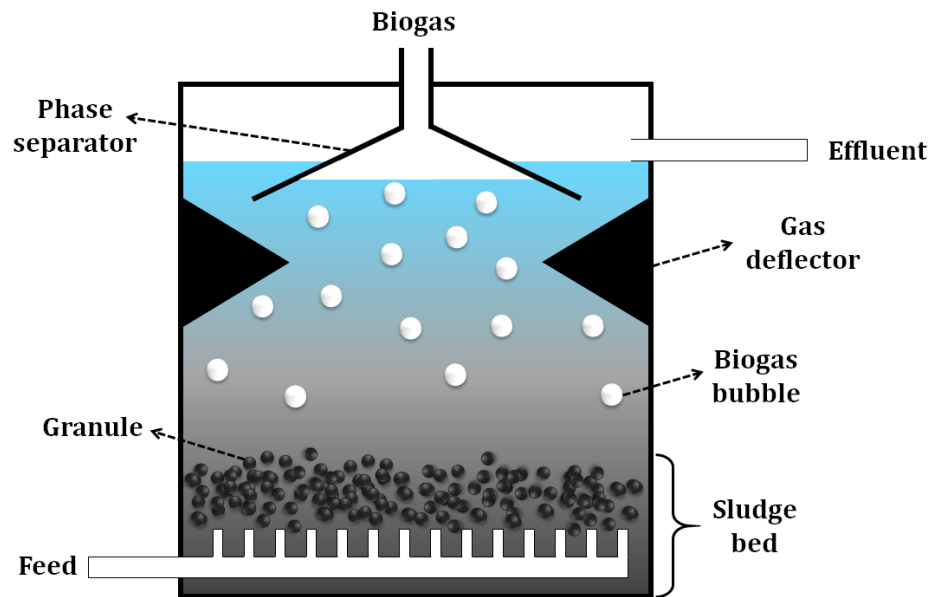
Figure 2-5: Configuration of an Upflow Anaerobic Sludge Blanket

2.2.12.5 Anaerobic fluidized bed reactor (AFBR), anaerobic expanded bed reactor (AEBR)

In these reactors the microbial population is grown on biocarriers such as sand, granular activated carbon or synthetic plastic media. These biocarriers are maintained suspended by high upflow velocity of wastewater. A greater upflow velocity leads to higher expansion of the sludge bed and, based on the extent of expansion, the reactor can be fluidized bed (>25–300% expansion) or expanded bed (15–25% expansion) (Khanal, 2008). These reactors are more effective for treating wastewater containing soluble or easily biodegradable suspended organics such as thin stillage (Andalib et al., 2012),

municipal wastewater sludges (Mustafa et al., 2014) or cheese whey (Ottaviano et al., 2017) and sugarcane vinasse (Ramos and Silva, 2018). The reactors are able to treat high strength wastewater such as brewery effluent with COD of 40 g L^{-1} (Borja et al., 1993). The advantages of AFBR and AEBR include small reactor size due to high sludge concentration and activity in unit volume, compact and thin biofilms due to shear stress of fluidization, minimal effect of toxic material in the reactor because of mixing regime and no significant blockage problem (Cervantes et al., 2006). However, the system has some disadvantages including difficulty in control of biolayer thickness, high energy consumption due to high recirculation ratio (Hamza et al., 2016). Figure 2-6 demonstrates an Anaerobic Fluidized/Expanded Bed Reactor.
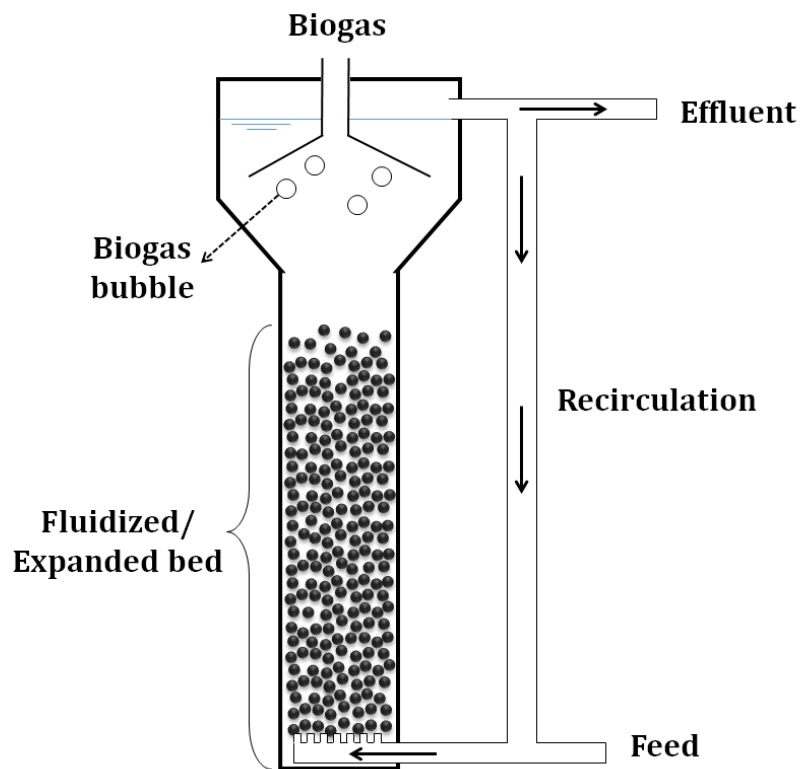
Figure 2-6: Schematic diagram of an Anaerobic Fluidized/Expanded Bed Reactor

2.2.12.6 Anaerobic sequential batch reactor (ASBR)

Dague et al. (1992) introduced the anaerobic sequential batch reactor (ASBR) in the early 1990s. The ASBR is suitable with high strength wastewater with medium solid content (1-4%). In this type of digester, both reaction and settling occur in one tank because of the sequential operation of this system. Thus, a high concentration of biomass in the reactor is achievable regardless of HRT. The ASBR is able to retain sludge because of granulation (Khanal, 2008). Therefore, the system performance depends on sludge settling, self-immobilization which happens naturally within the reactor. However, the problem is that self-immobilization does not provide good settleability. Besides, the reactor needs mixing to provide sufficient contact between sludge and wastewater which means more energy consumption. The benefits of using ASBR are operational simplicity, flexibility of use and high biogas yield (Ratusznei et al., 2000; Singh and Srivastava, 2011). These reactors have been used for treatment of palm oil mill effluent (Nasir et al., 2019), tequila vinasses (Arreola-Vargas et al., 2016), poultry slaughterhouse wastewater (Rajab et al., 2017) and tuna cooking wastewater (Militon et al., 2015). The ASBR operation has four stages: feed, react, settle and decant. These stages are illustrated in Figure 2-7. In the feed stage, the wastewater is introduced to a completely mixed reactor. Then, the react step starts with the conversion of organics to biogas and the reactor is kept in a mixed condition. Afterwards, in the settle stage, the mixing will be turned off which allows the sludge to settle. The final step is decant in which the decanted volume is equal to fed volume in order to maintain HRT (Zaiat et al., 2001).
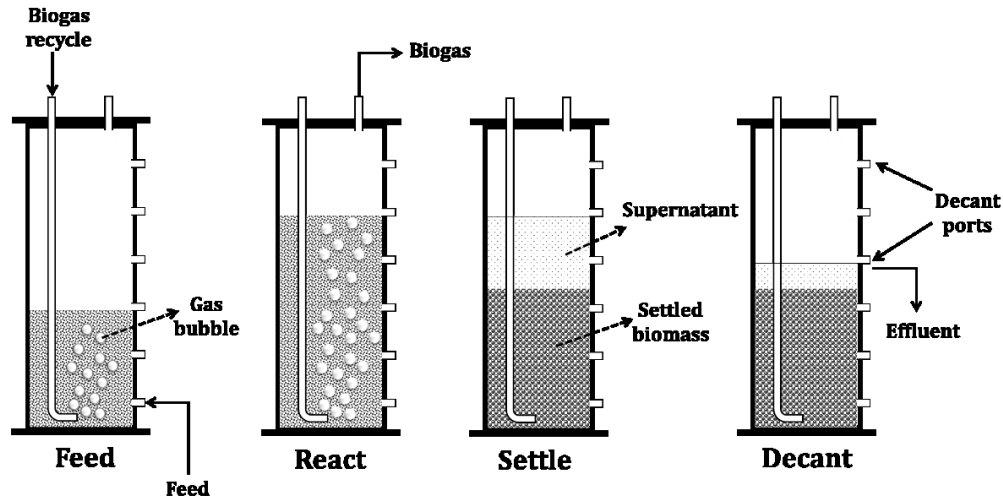
Figure 2-7: Different stages for Anaerobic Sequential Batch Reactor

2.2.12.7 Expanded granular sludge blanket (EGSB) reactor

Expanded granular sludge blanket was introduced by Lettinga and co-workers (Lettinga et al., 1980). The design of the reactor is based on modification in UASB. EGSB is the second most widely used high rate anaerobic digester after UASB (Van Lier, 2008). The process of EGSB is similar to UASB except in EGSB, the sludge granular bed is expanded because of higher liquid upflow velocity (5-10 m h^{-1}) compared to UASB (0.5-1.5 m h^{-1}) (Khanal, 2008; Van de Last and Lettinga, 1992).The difference between a EGSB and a AEBR is the presence of biocarriers in AEBRs. EGSB is a tall reactor with a small footprint (Figure 2-8). In the reactor, the high hydraulic and gas loads enhance the contact between wastewater and sludges (Kato et al., 1994). This reactor is suitable for complex and toxic wastewater such as chemical and petrochemical wastewaters since the high recycling rate can reduce the effect of toxicity. However, the reactor is not able to remove particulate organic matters efficiently (de Lemos Chernicharo, 2007). The EGSB reactors

can be applied for different wastewater such as for domestic sewage (Xu et al., 2018), ethylene glycol wastewater (Jin et al., 2016), cephalosporin wastewater (Li et al., 2019) and so on. Because of its advantages, more than 200 full scale EGSB reactors are constructed around the world ranging from 30–5000 m^3 in volume until 2008 (Tauseef et al., 2013).
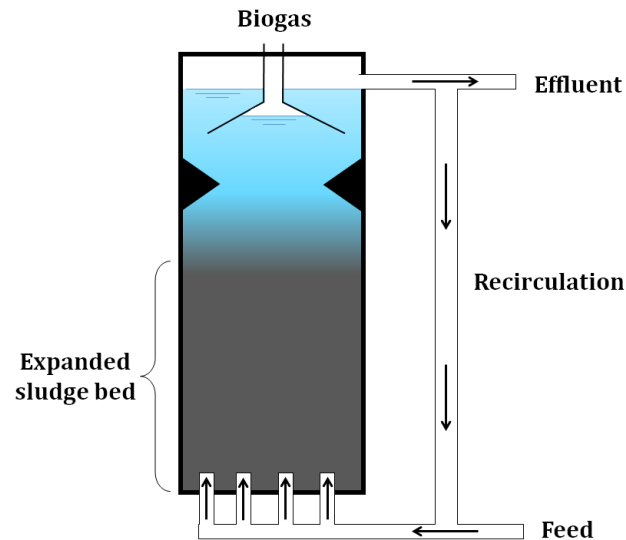
Figure 2-8: Schematic diagram of an EGSB reactor

2.2.12.8 Internal circulation (IC) reactor

Internal circulation (IC) reactor includes two UASB reactors as two compartments connected to each other. Figure 2-9 shows the configuration of an IC reactor. In fact, IC is a modified UASB reactor, which is designed for high organic loads (up to 40 kg m^{-3} d^{-1}). Treatment of high OLR requires a suitable strategy for separation of gas, solid and liquid since high biogas production in the reactor reduces the SRT (de Lemos Chernicharo, 2007). Therefore, two stages are considered for the separation of solid, liquid and gas. In the first

step, the produced biogas carries the sludge and liquid goes upward to the gas-liquid separator. Then, the gas leaves the reactor from the top and the mixture of sludge and liquid flow downward to the bottom of reactor which provides internal circulation (Kassam et al., 2003). IC reactors have the advantages of both fluidized bed and UASB reactors. It has a very high upflow velocity compared to UASB (8-20 times higher) (Pereboom, 1994). Thus, it can operate at higher OLR than EGSB can. It is able treat high strength wastewater (COD up to 23 g L^{-1}) with high OLR (up to 35 kg m^{-3} d^{-1}) (Driessen and Yspeert, 1999; Lettinga et al., 1997). It has been used for various applications worldwide. Until 2003, 161 IC reactors were used in different industries such as brewery and soft drink, pulp and paper, food, distillery (Tauseef et al., 2013), dyeing (Yang et al., 2018b) and pharmaceutical (Chen et al., 2019).
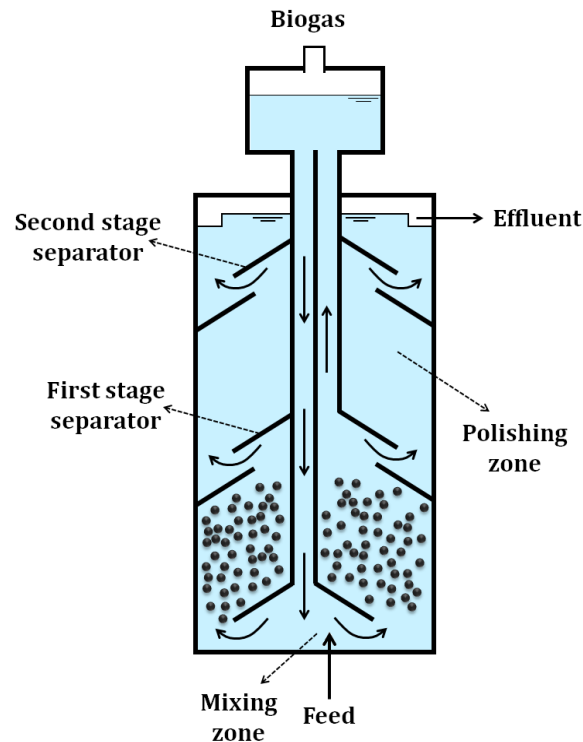
Figure 2-9: Schematic view of an Internal Circulation (IC) reactor

2.2.12.9 Anaerobic migrating blanket reactor (AMBR)

Anaerobic migrating blanket reactor (AMBR) was developed by Angenent and Sung (Angenent and Sung, 2001). AMBR is a continuously fed, baffled reactor which does not need gas, solid and liquid separation device, feed distribution system and recycling. In this reactor, the contact between active biomass and wastewater is provided by a mechanical mixer in each compartment (Fang, 2010b). In AMBR, the wastewater enters from one side, passes horizontally through the compartments and then exits from the other side. The last compartment receives less organic matter, therefore less biogas will be produced in the last compartment. As a result, better settling occurs in the last compartment, which leads to less biomass washout (Tauseef et al., 2013). Angenent and Sung (2001) showed that higher OLR is achievable with this reactor compared to USAB and ASBR. The shortcoming of this technology is the loss of active sludge because of excessive bed expansion. Also, because of the mechanical mixing, the AMBR consumes more energy than reactors such as UASB (Angenent and Sung, 2001; Ebrahimi et al., 2018). Figure 2-10 illustrates an AMBR.
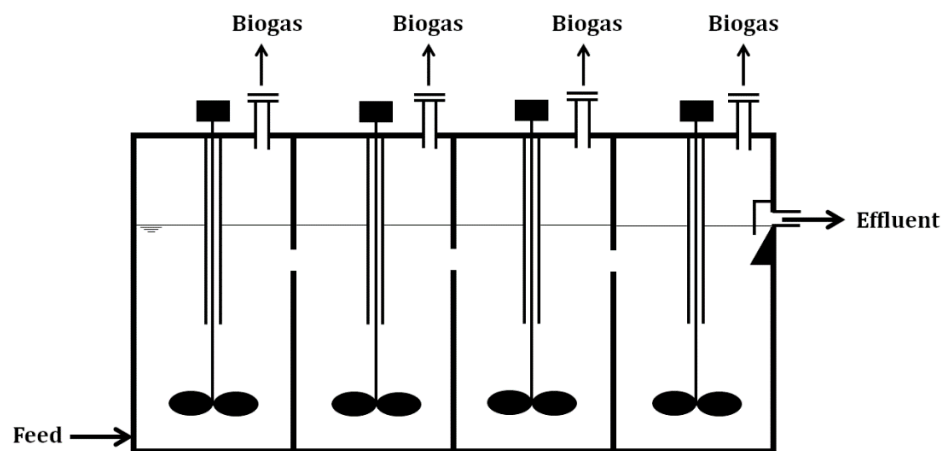
Figure 2-10: Configuration of an Anaerobic Migrating Blanket Reactor

2.2.12.10   Anaerobic baffled reactor (ABR)

In phase separated reactors the purpose is to separate different processes of anaerobic digestion such as hydrolysis, acidogenesis, acetogenesis and methanogenesis. The phase separation improves the efficiency of the system since each group of bacteria has different optimal conditions and in each phase a better environmental condition can be provided for each group (Syngellakis, 2014). Anaerobic baffled reactor is one of the phase separated reactors.

Anaerobic baffled reactor was developed by McCarty (1982). It can be described as a UASB reactor including vertical baffles (Figure 2-11). Its configuration forces the wastewater to pass through the sludge in the reactor with downflow and upflow movement in order to provide intimate contact between the wastewater and the active microbial population (de Lemos Chernicharo, 2007). The advantages of ABR include higher stability to organic and hydraulic shock loadings, longer SRT and phase separation between acidogenic and methanogenic bacteria along the reactor. Among the benefits, the phase separation is the most important one since it provides increased protection against toxic materials. Besides, it enhances the stability of the system to fluctuation in environmental conditions such as temperature and pH (Zhu et al., 2015). The disadvantages of these reactors include inadequate mixing and settleability of the microbial granules (Mao et al., 2015). The drawback of pilot/full scale ABR is that the reactor should be shallow to maintain suitable gas and liquid upflow velocities. Moreover, controlling an even distribution of the influent is difficult (Barber and Stuckey, 1999). ABRs have been applied for various types of wastes such as vegetable/food waste (Ahamed et al., 2015; Gulhane et

al., 2017), raw municipal wastewater (Hahn and Figueroa, 2015), baker's yeast manufacturing wastewater (Pirsaheb et al., 2015), thin stillage (Sayedin et al., 2018) and blackwater (Moges et al., 2018).
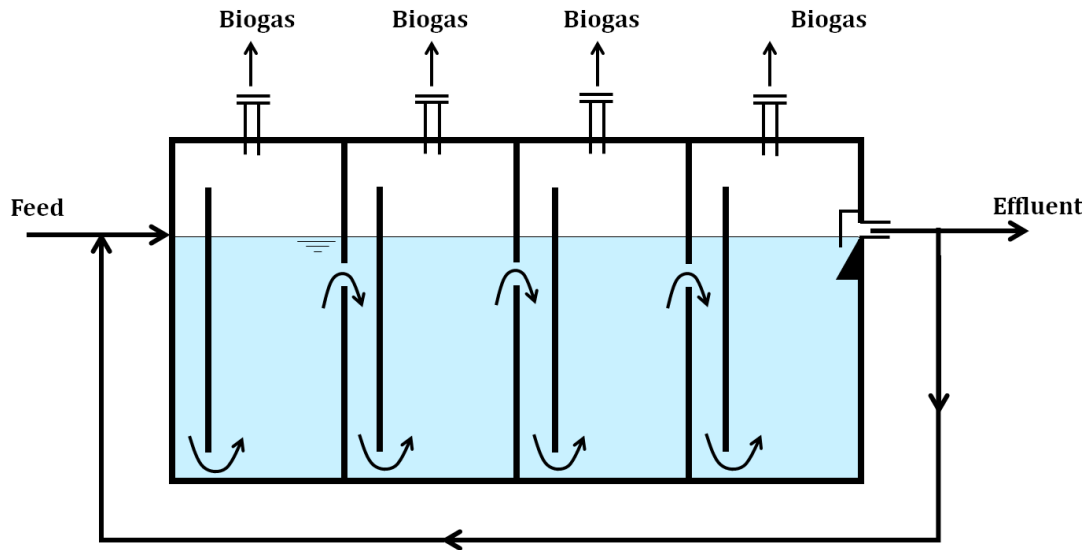
Figure 2-11: Schematic diagram of an anaerobic baffled reactor

2.2.12.11   *Upflow staged sludge bed (USSB) reactor*

Upflow staged sludge bed (USSB) reactor was introduced by Lettinga and co-workers (Van Lier et al., 1994). The main motivation for design of the reactor was to solve the problem of VFA accumulation in conventional UASB reactors as a result of inadequate mixing. For this purpose, several tilted baffles were added to the reactor to increase turbulence (Figure 2-12). Therefore, partial compartmentalization occurs along the reactor which leads to separation of reactions. As a result, optimal condition for hydrolysis, acidogenesis and methanogenesis is achievable by suitable positioning of baffles (Sevilla-

Espinosa et al., 2010). USSB can handle higher OLR (30 kg m^{-3} d^{-1}) than UASB can (20

kg m^{-3} d^{-1}) (Lens et al., 1998).
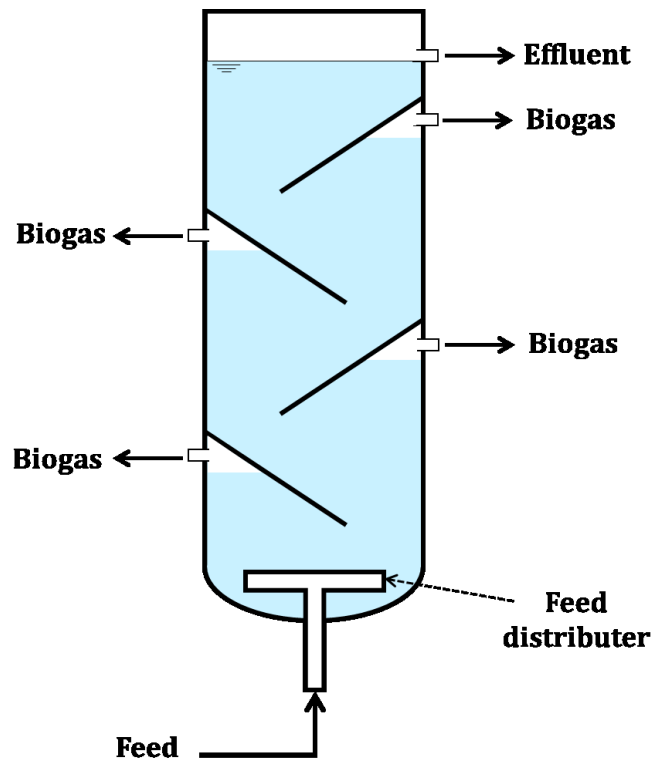
Figure 2-12: Configuration of an upflow staged sludge bed reactor

2.2.12.12   Hydrolysis upflow sludge bed (HUSB) reactor

The HUSB reactor is designed for wastewater with high solid content since

introducing this kind of waste into the conventional UASB reactor may cause reduction in

methanogenesis activity and deformation of granules. To avoid this, first, the wastewater

is introduced to the HUSB reactor in which the hydrolytic acidogenic reaction occurs.

Afterwards, the produced VFAs exit the HUSB reactor and enter into the methanogenic

UASB reactor. Thus, the solid content of the wastewater is retained in the HUSB reactor

(Tauseef et al., 2013). The configuration of a HUSB reactor is shown in Figure 2-13.
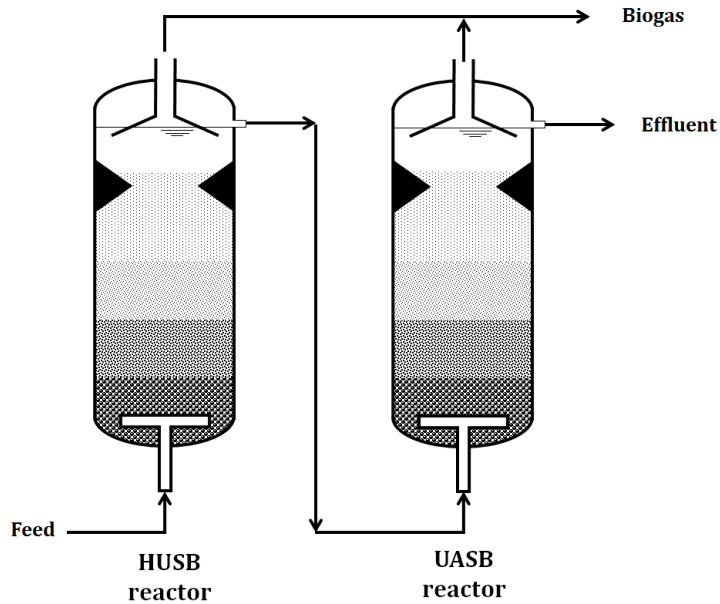
Figure 2-13: Schematic view of a hydrolysis upflow sludge bed reactor

In AnMBRs, the membrane enhances the solid-liquid separation inside the reactor which causes long SRT regardless of HRT. This feature makes AnMBR suitable for wastewater with a high solid content. Moreover, the membrane retains the sludge and reduces biomass washout from the reactor (Khanal, 2008). Using a membrane in the anaerobic system enhances sludge activity, reduces plant size and makes higher OLR achievable (Cicek et al., 1998). Polymeric and ceramic membranes are the most widely used membranes in AnMBRs (Mutamim et al., 2013; Yue et al., 2015). In AnMBR, a membrane can be placed in an external loop (Figure 2-14a) or immersed (submerged) within the reactor (Figure 2-14b). The first commercial AnMBR was developed in the early 1980s for treating high-strength wastewater from whey processing. The reactor was only applied at the pilot scale (not full scale) because of high membrane cost (Liao et al., 2006).

In fact, membrane fouling (precipitation of particulates on the membrane) and related operating costs and maintenance are the main barriers for widespread application of AnMBRs for wastewater treatment (Lin et al., 2013). Wastewaters such as pharmaceutical wastewater (Svojitka et al., 2017), municipal wastewater (Seib et al., 2016), thin stillage (Dereli et al., 2014) and so on are treated by AnMBRs.
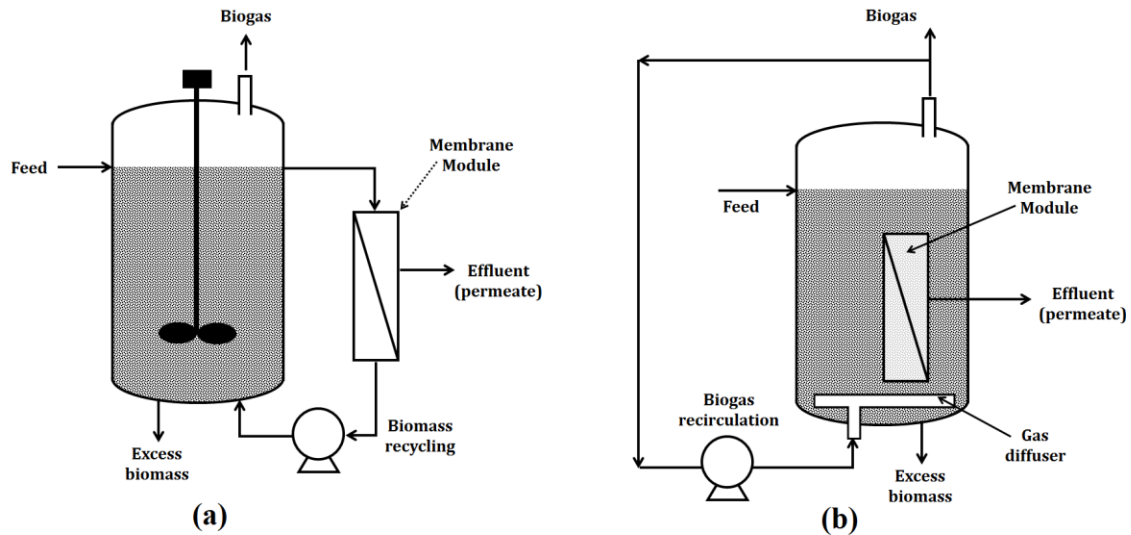
Figure 2-14: Anaerobic membrane bioreactors (A) membrane in the external loop (B) membrane immerse on the reactor

2.3 Treatment of Thin Stillage

Corn ethanol plants process thin stillage (a liquid, nutrient rich by-product of corn ethanol production) by energy intensive methods such as evaporation and drying. Anaerobic digestion can be an effective approach to replace those methods to remove organic materials (COD) from thin stillage and produce methane that can be easily used in bioethanol plants to produce energy. This can improve the energy balance of the plant and

reduce the operating costs (Andalib et al., 2012). Many parameters such as OLR, HRT and COD of influent and reactor type can affect the performance of the anaerobic digestion process for COD removal and methane production. The effect of these parameters on anaerobic treatment of corn-thin stillage is summarized in Table 2-2. Alkan-Ozkaynak and Karthikeyan (2011) investigated the anaerobic digestion of thin stillage in a batch system (250 mL serum bottle) to produce biogas and remove COD under mesophilic condition. They determined that inoculum-to-substrate ratio and alkalinity have a great effect on digestibility of thin stillage so that the ratio of 2 g volatile solid (VS) inoculum g^{-1} VS substrate is optimal according to high biogas production level of 763 mL biogas g^{-1} volatile solids added and 80.6% COD removal. The anaerobic digestion of thin stillage is also studied in different continuous reactors. Lee et al. (2011) studied the mesophilic anaerobic digestion of corn-thin stillage in a CSTR. In their experiment, HRT ranging from 25 days (OLR=4.5 kg COD $m^{-3}d^{-1}$) to 40 days (OLR=2.6 kg COD $m^{-3}d^{-1}$) did not have an effect on the COD removal efficiency. However, the maximum methane yield (0.77 L CH_4 g^{-1} $VS_{removed}$) was achieved with HRTs between 30 and 40 days. Schaefer and Sung (2008) tested the thermophilic anaerobic digestion of corn thin stillage in a CSTR. The highest COD removal (88%) was obtained at the HRT of 20 days (OLR=6.1 kg COD $m^{-3}d^{-1}$). They also faced accumulation of VFA and digester failure at 12-day HRT (OLR=7.6 kg COD $m^{-3}d^{-1}$). The treatability of thin stillage in a thermophilic anaerobic sequencing batch reactor was examined by Agler et al. (2008). Their reactor operated at OLR of 9.5 kg COD $m^{-3}d^{-1}$ and HRT of 10 days. The reactor was able to reach the COD removal efficiency of 90%. Dereli et al. (2014) used an AnMBR for treating thin stillage under mesophilic

condition. They achieved very high COD removal efficiency (99%) at OLR of 8 kg COD m^{-3}d^{-1} and SRT of 20 days. In another study, Dereli et al. (2012) tested a pilot scale AnMBR with working volume of 12m^3. The reactor operated in mesophilic condition and removed TSS and COD up to 99% and 98%, respectively. Also, the methane yield for the system was 0.31 L CH$_4$ g^{-1} COD$_{removed}$. Moestedt et al. (2013) discussed the operation of a full scale anaerobic digestion plant for treating thin stillage under mesophilic condition. The plant had two CSTR (2000 m^3 and 1800 m^3) and its annual biogas production in 2011 was 3.5 million m^3 with an average methane content of 55%. Andalib et al. (2012) examined the treatability of thin stillage with total COD of 130 g L^{-1} and Total Suspended Solids (TSS) of 47 g L^{-1} using an anaerobic fluidized bed reactor. The reactor demonstrated 88% total COD and 78% TSS removal at OLR of 29 kg COD m^{-3} d^{-1}. According to the literature, the ABR has not been applied for treating thin stillage. Therefore, its ability and challenges for treatment of thin stillage is not clear.

Table 2-2: Characteristic of various anaerobic reactors for treating thin stillage

Reactor type	Volume (L)	Influent COD (g L^{-1})	Influent TSS (g L^{-1})	OLR (kg COD m^{-3} d^{-1})	COD removal (%)	HRT (d)	Methane yield	Ref
CSTR	18	105-131	66-79	2.9-4.5	84-86	25-40	0.68-0.77[a]	(Lee et al., 2011)
CSTR	10	97-121	69-90	3.2-6.1	82-88	20-30	0.748-0.631[a]	(Schaefer and Sung, 2008)
ASBR	5	74-100	42	9.5	90	10	0.284[b]	(Agler et al., 2008)
AFBR	17.6	130	47	29	88	3.5	0.31[b]	(Andalib et al., 2012)
AnMBR	10	72	16.5	8.3	99	10-12	0.26[b]	(Dereli et al., 2014)
AnMBR	12000	16.2	5.4	4.5–7	98	17 ± 4	0.31[b]	(Dereli et al., 2012)
CSTR	3.8x10^6	-	-	2.4–3.2[c]	-	45-60	-	(Moestedt et al., 2013)

[a] L CH$_4$ g^{-1} VS $_{removed}$
[b] L CH$_4$ g^{-1} COD $_{removed}$
[c] kg VS m^{-3} d^{-1}

2.4 Application of Anaerobic Baffled Reactor

Although ABRs have not been applied for treatment of thin stillage, they have been used successfully for treating different wastewaters such as soybean protein processing (Zhu et al., 2008), sugarcane vinasse (Vuitik et al., 2019), whisky distillery (Akunna and Clark, 2000), pulp and paper mill black liquor (Grover et al., 1999) and high sulfur wastewater (Saritpongteeraka and Chaiprapat, 2008a), food waste (Ahamed et al., 2015), vegetable waste (Gulhane et al., 2017). Zhu et al. (2008) used a laboratory scale ABR with total volume of 20 L for treating soybean protein processing wastewater under mesophilic condition. In the experiment, the COD removal efficiency of 97 and 92% was obtained at the OLR of 1.2 (HRT=40 h and influent COD=2 g L^{-1}) and 6 kg COD m^{-3} d^{-1} (HRT=40 h and influent COD=10 g L^{-1}) respectively. Also, it was found that the dominant compounds in the 1st compartment were propionate and butyrate and in the 2nd compartment it was acetate. Moreover, 93% of VFA was removed in 3rd and 4th compartments. According to the results, the biogas composition from first compartment was mainly H_2 (30%) and CO_2 (63%), while in the last compartment it was up to 80% methane. ABR was also applied by Akunna and Clark (2000) in the treatment of a whisky distillery wastewater. The reactor included 10 compartments with an effective volume of 35 L. The reactor was able to remove up to 80% of the COD at OLR of 4.75 kg COD m^{-3} d^{-1}, HRT of 2 days and influent COD of 9.5 g L^{-1}. It was observed that acidogens were mostly non-granular while methanogens were granular. Grover et al. (1999) studied the effect of different pH (6.5-9.5), temperature (25-40 °C), HRT (2-5 d) and OLR (2-6 kg COD m^{-3} d^{-1}) on the COD removal efficiency of ABR. A maximum COD reduction of 60% was obtained at OLR of

5 kg COD m^{-3} d^{-1}, HRT of 2 d, pH of 8 and temperature of 35 °C. At the OLR of 5 kg COD m^{-3} d^{-1}, the methane content was 65% and the influent COD was 10 g L^{-1}. The results showed that the OLR above 6 kg m^{-3} d^{-1} were toxic and destabilized the reactor system. ABR is also applied for high sulfur wastewater. Saritpongteeraka and Chaiprapat (2008a) used a 23 L ABR for treating concentrated rubber latex wastewater pretreated with NaOH (approximate COD of 6 g L^{-1} and sulfate of 1.8 g L^{-1}) under different pH and recycling ratios. The HRT changed from 10 d to 1.25 d and the highest COD (82.7%) and sulfate (96.2%) removal efficiencies were obtained at HRT of 10d. Also, increasing recycling ratio from 0 to 0.5 at HRT of 1.25d raised the hydraulic loading on the system and decreased the COD removal efficiency (66.8 to 63.3%) and methane content (65.1 to 54.7%) but it did not change the sulfate reduction significantly.

2.5   Nutrient Recovery from Anaerobic Digestate

The resulting digestate from anaerobic digestion of thin stillage is rich in nutrient such as nitrogen and phosphorus. Phosphorus is a non-renewable resource and it is essential for food security (Cordell et al., 2009). The nutrient can be recovered through biological (e.g. uptake by microalgae) and chemical mechanism (precipitation of orthophosphate with magnesium, iron, calcium or aluminum) (Sengupta et al., 2015). Among chemical methods, the reaction of orthophosphate with magnesium and ammonium forms struvite crystals which can be used as a source of fertilizer. The focus of this study is the nutrient removal by struvite recovery and further removal of nitrogen and phosphorus by microalgae cultivation on struvite-removed-digestate.

2.5.1 Struvite recovery from digestate

The drawback of anaerobic digestion is that it is not able to reduce the nutrients in thin stillage such as nitrogen and phosphorus efficiently (Wilkie et al., 2000). The recovery of the nutrients is essential for food production and agriculture, especially phosphorus due to its non-renewable nature (Nghiem et al., 2017). Therefore, many technologies have been developed to recover phosphorus from wastewater (Kataki et al., 2016; Oliveira et al., 2019). Phosphorus can precipitate in the form of struvite, however, it causes blockage in wastewater treatment systems (Kataki et al., 2016). Different parameters such molar ratio of Mg:P:N and pH can affect the rate of precipitation. For example, within the pH range 7.5 to 9.5, the higher rate of precipitation happens at pH range of 8.9-9.25 (Nelson et al., 2003). Also, the effect of different range of Mg:P ratio such as 1:1-1.6:1 (Rahaman et al., 2008) and 1.5:1-3.6:1 (Quintana et al., 2005) is studied so that the higher ratio of Mg:P increases the precipitation rate.

The common chemical methods for precipitation of phosphorus are the addition of external Mg or creating alkaline conditions (Kataki et al., 2016). $MgCl_2$, $MgSO_4$ or MgO is used as a source Mg and pH is adjusted by adding NaOH (Bouropoulos and Koutsoukos, 2000). In small scale laboratory systems, usually stirred batch reactors are used, while at a larger scale, fluidized bed reactors are frequently applied for crystallization and phosphorus removal (Ueno and Fujii, 2001; Xu et al., 2012). The problem with the stirred batch reactors is the production of fine struvite particles due to high mixing energy, which is non-recoverable. The problem can be solved by recycling of the fine crystals to the precipitating reactor in order to act as seeding agents (Le Corre et al., 2009; Ueno and Fujii, 2001). As

mentioned, commercially available large scale struvite recovery technologies such as NuReSys, Phosnix, Pearl Ostara and AirPrex use CO_2 stripping and NaOH addition for increasing pH. Moreover, in all technologies Mg is added for production of struvite crystals (Desmidt et al., 2015).

NuReSys technology (Figure 2-15) for nutrient recovery was developed by Belgian company Akwadok. It includes two reactors for CO_2 striping and struvite crystallization (CSTR tank). The purpose aeration and CO_2 striping is to increase the pH of digestate. The system works in a continuous mode and controls the pH also by mixing intensity and addition of 29% NaOH solution between 8-8.5 (Moerman et al., 2009). The technology is applied for phosphorus removal from digester supernatant with removal efficiency of 90% (Egle et al., 2015).
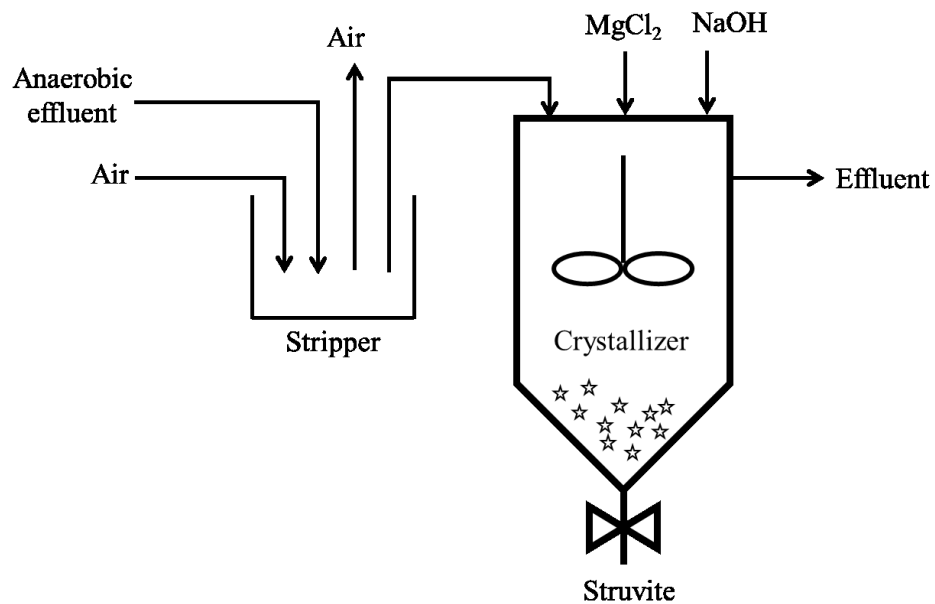
Figure 2-15: Overview of NuReSys technology

Phosnix technology was developed by Unitika Ltd Environmental and Engineering Div in Japan. In the process, the wastewater is pumped into the bottom of a fluidized bed reactor (Figure 2-16). The granulated struvite in the column acts as a seed for crystal growth. In order to achieve the Mg:P ratio of 1:1, $Mg(OH)_2$ is added to the reactor. Moreover, the pH is maintained 8.2-8.8 by addition of NaOH (Le Corre et al., 2009). A retention time of 10 days in the reactor is used for the growth of crystals to 0.5 and 1 mm in size. Also, the fine crystals are returned to the reactor to provide new seeds for formation of struvite crystals. The treated water in the Phosnix process can also be sent to primary clarifier in a wastewater treatment plant (Ueno and Fujii, 2001).
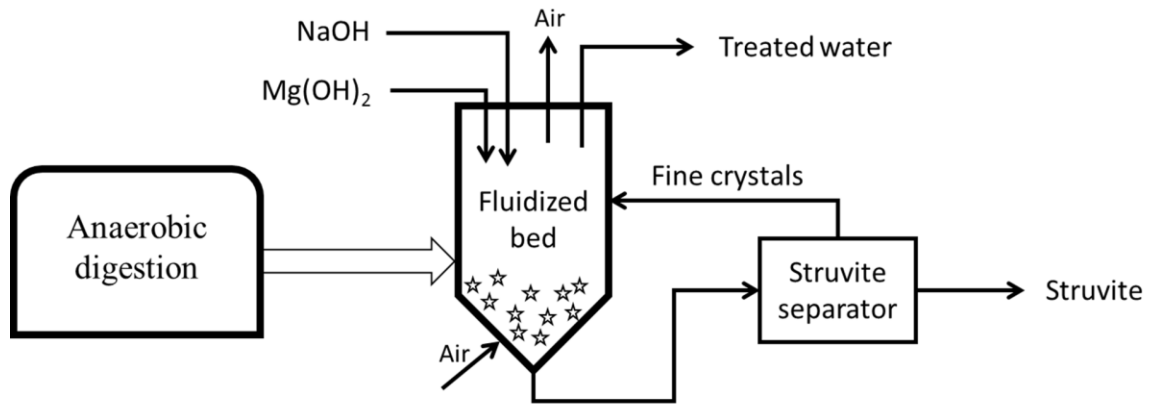
Figure 2-16: Schematic view of Phosnix technology

In the AirPrex technology, the digested sludge is mixed by air upflow in a cylindrical reactor (Figure 2-17). The aeration provides internal recycle flow allowing struvite crystals grow to a certain size that enables them to escape from the flow and settle. The smaller crystals can settle in the second tank (Desmidt et al., 2015). A Mg:P ratio of 1.5:1 is used

in this process and a phosphorus removal efficiency of 80 to 90% is reported. The technology is used for phosphorus removal from digested sludge (Egle et al., 2015).
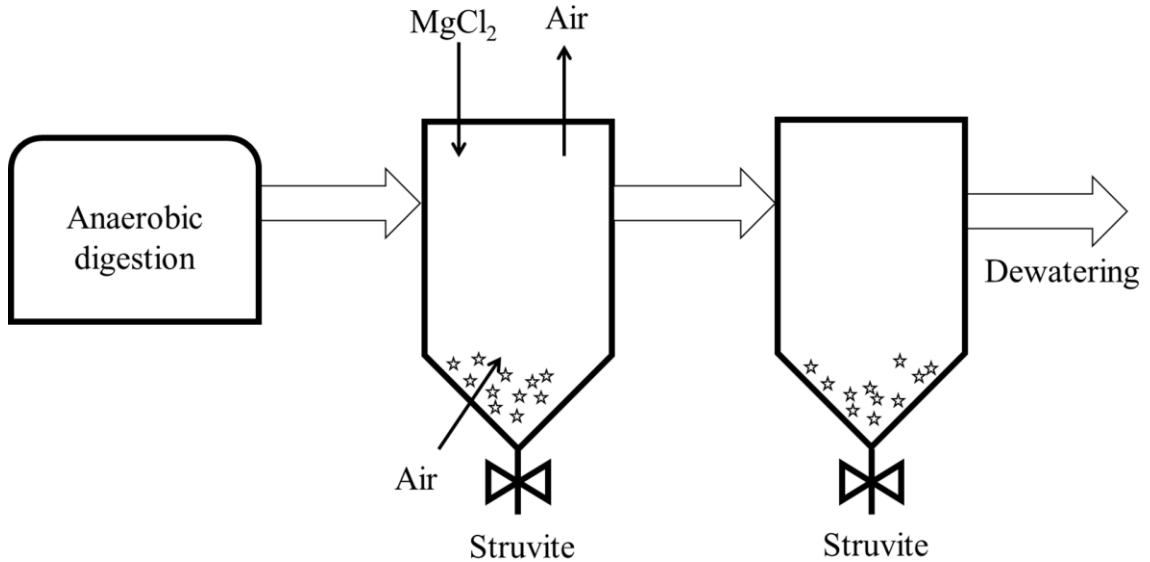
Figure 2-17: Schematic diagram of AirPrex process

The Ostara Pearl technology was developed in the University of British Columbia, Canada. In this process (Figure 2-18), different reaction zones are designed in an up flow fluidized bed to increase the diameter of crystal (Prasad et al., 2007). The configuration of the reactor allows large struvite pellets (1.5 to 4.5 mm) to stay suspended in the bottom of reactor without washout the fine particle from top of the reactor. Moreover, the washout of residual sludge from the lower section of reactor due to high upflow velocity results in more pure struvite crystals. The technology has been applied to remove phosphorus from sludge liquor of anaerobic digesters with removal efficiency of 90% (Desmidt et al., 2015).
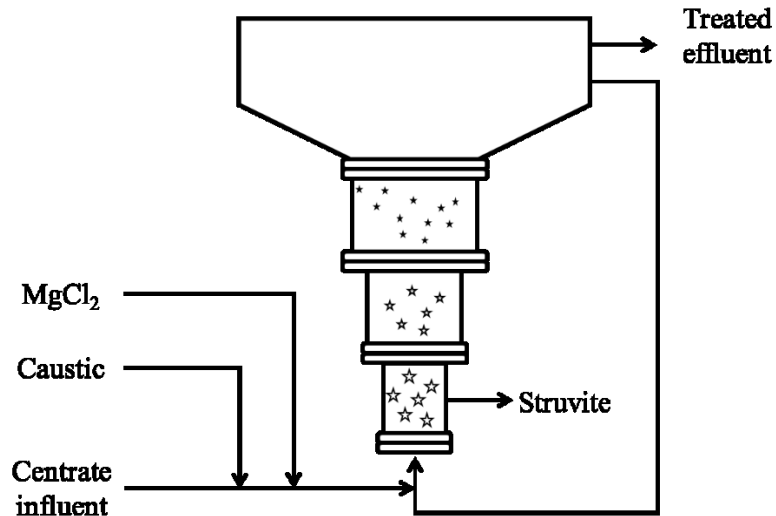
Figure 2-18: Overview of Ostara Pearl technology

The chemical methods have been used extensively for anaerobic treatment systems (Le Corre et al., 2009). Struvite can also be recovered by electrochemical, ion exchange and biomineralisation approaches, however, these methods have only been applied in laboratory scales (Kataki et al., 2016). In an electrochemical method, a voltage is applied to an electrochemical cell. Then, struvite precipitates on the cathode and hydrogen is released on the anode. This increases the pH around the cathode and causes a higher rate of struvite deposition, therefore, there is no need for addition of a chemical for pH adjustment (Wang et al., 2010a). The downside of the technology is the use of costly material such as platinum. In an ion exchange approach, NaCl is usually used as a regenerating solution. The exchange of ammonium in a cationic exchanger (zeolite based) and phosphate in an anionic exchanger (sulphonic/carboxylic based) allows for their reaction with Mg and formation of struvite (Ortueta et al., 2015). The disadvantages of this method are the limited availability of anion exchanger for phosphate and production of

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high suspended solid effluent (Gönder et al., 2006; Petruzzelli et al., 2004). The basic principle of biomineralisation methods is that certain bacteria are able to precipitate struvite to harden their structural tissue. The drawback of biomineralisation is the slow rate of precipitation (Da Silva et al., 2000).

2.5.2 Microalgae cultivation on anaerobic digestate

Struvite recovery from thin stillage digestate can efficiently remove phosphorus from digestate, however, a significant amount of nitrogen-ammonia will remain in digestate. Microalgae can effectively remove nitrogen and phosphorus from wastewaters and convert it to value added products such as biofuels (e.g. carbohydrate) under a controlled environment. In this section, the effect of environmental parameters on microalgal growth, application of microalgae for nutrient removal and the carbohydrate production from microalgae will be discussed.

2.5.2.1 *Microalgal growth parameters*

Irradiance

Microalgae use light as an energy source for photosynthesis. Light intensity is one of the major parameters affecting the growth rate and cell composition of microalgae. When the light intensity is too low (e.g. below the compensation point), there is no growth. Above this point, the growth rate increases with rising light intensity until the light saturation point where the photosynthesis rate is maximum. After the light saturation point, an increase in light intensity may cause photoinhibition and decrease in growth rate (Ho et al., 2014). The extent of light intensity effects on microalgae is species dependent. The light intensity also

can affect the cell composition. When the light intensity remains beyond the saturation point, the microalgae acclimate to the new condition by making some changes in their cellular components. For instance, increasing the irradiance can cause photoadaptation, which is the down regulation of pigments synthesis at high irradiance (Geider et al., 1998). Also, it should be noted that the up and down-regulation of pigments is relatively slow. Therefore, when the irradiance increases very fast, it activates the photoprotective mechanisms such as xanthophyll cycles and state transitions to reduce photodamage caused by high light intensity. Xanthophyll cycles dissipate energy by pigment interconversion (it reduces the energy transfer to reaction center). In state transition, the energy is directed to a futile cycle (it reduces the light absorption in photosystem II (PSII)) (MacIntyre et al., 2000). Non-photochemical quenching (NPQ) is a measure of photoprotective energy dissipation and indicates the photoprotective capacity of a phytoplankton. If these photoprotective mechanisms cannot dissipate the excess energy, it may cause photoinhibition which is the loss of photosynthetic competence, due to over-excitation of PSII reaction centers and loss of D1 protein (MacIntyre et al., 2000). In this respect, one should mention that there are two types of response to light intensity. The first one is genotypic responses which are constrained by genetic constitution (e.g. difference between the ratio of Chl.b:Chl.a (Antenna size:Core) in two ecotypes). Second is phenotypic response, which is constrained by genetic expression/physiology (e.g. differences within an ecotype at different light levels) (Rocap et al., 2003).

Moreover, the cell composition changes during dark and light period. During the dark period when there is no photosynthesis and carbon accumulation, the energy storage

reserves are used and nitrogen is assimilated. There are two reasons for this phenomenon. First, for synthesis of amino acids, the required carbon for amino acid structure is obtained from the reserves. Second, the processes of amino acid synthesis and nitrogen assimilation need energy which is provided by the respiration of particulate carbon (the N:C ratio increases) (Geider et al., 1998). However, when the growth rate is considered over a 24 hours interval, there is no significant change in rate of different indices so it is called balanced growth in dynamic equilibrium (MacIntyre and Cullen, 2005). Under a low light, the nitrogen uptake rate is limited by the rate of photosynthesis due to the requirement for carbon skeletons for amino acid synthesis (Geider et al., 1998).

An appropriate light supply, which is different according to the microalgae species, can increase the content of neutral lipids significantly (primarily TAG) as energy-storage compounds (Iasimone et al., 2018; Khotimchenko and Yakovleva, 2005; Sukenik et al., 1989). The relation between light intensity and carbohydrate synthesis is not clear yet (Ho et al., 2014). However, the changes in protein, lipid and carbohydrate content of microalgae in response to increased light intensity is species dependent (Kumar et al., 2019).

Temperature

Microalgae growth rate is maximum in an optimum growth temperature. The dependence of growth rate on temperature is different between microalgae species. Temperature variations can affect enzymatic activity, cell compositions and nutritional requirements (Razzak et al., 2013). An increase in temperature increases the phytoplankton carbon-specific nitrate uptake rate and the carbon specific rate of photosynthesis due to increase in turnover rate of enzymes. However, it continues until the temperature reaches

the optimum. Beyond the optimum temperature, the number of functional enzyme molecules and as a result net catalytic rate and growth rate decrease (Gao et al., 2000). Temperature can have different effects on growth rate and cell composition. Wu et al. (2013) increased the temperature of *Monoraphidium sp. SB2* culture from 25 to 35 °C and as a result the lipid content decreased from 33 to 29%. However, the biomass concentration increased (577 to 650 mg L^{-1}) first by increasing temperature from 25 to 30 °C, then the concentration decreased (650 to 499 mg L^{-1}) when the temperature increased from 30 to 35 °C. Regarding carbohydrate accumulation, Hosono et al. (1994) showed that when the temperature is reduced from 25 to 5 °C, the carbohydrate content of *Chlorella vulgaris SO-26* increased from 20 to 30%.

Nutrients

Nitrogen and phosphorus (Si in case of diatoms) are considered as a macronutrients for microalgae growth. In addition, microalgae need vitamins and trace metals (Andersen, 2005). Several studies have shown that N or P limitation during microalgae cultivation can enhance the accumulation of energy storage compounds (Chen et al., 2017; Iasimone et al., 2018). Among the nutrients in the culture medium, nitrogen is the most critical one influencing the lipid and carbohydrate accumulation in microalgae (Chen et al., 2013a; Chisti, 2007). Many studies have shown that microalgae allocate their carbon molecules to energy-rich lipids or carbohydrates when they are under conditions of nitrogen limitation (Flynn et al., 1994; Hu et al., 2008; John et al., 2011). The magnitude of increase in energy storage compounds depends on species. For example, under the nitrogen starvation, *Tetraselmis subcordiformis* and *Chlorella vulgaris* accumulated starch up to 54.3 % and

38% of their dry weight (DW), respectively (Brányiková et al., 2011; Yao et al., 2013). Also, the lipid content in *Chlorella emersonii* and *eustigmatophyte Nannochloropsis sp.* increased to 63% and 60% of their DW, respectively, under nitrogen starvation (Illman et al., 2000; Rodolfi et al., 2009).

pH

Another influencing factor is pH of the culture medium which affects many biological processes associated with microalgal growth, metabolism, and uptake of ions (Khalil et al., 2010). Indeed, the optimum pH for growth is species dependent. For instance, the optimal pH for *Dunaliella bardawil* and *Chlorella ellipsoidea* are about 7.5 and 10, respectively (Khalil et al., 2010). Most algae are tolerant to a fairly wide range of pH. However, suboptimal pH can inhibit the growth. Rachlin and Grosso (1991) considered 100% growth rate for *Chlorella vulgaris* at pH of 6.9 and they showed that when the pH increased from 6.9 to 9, the growth rate reached to 34% and when the pH decreased from 6.9 to 3, the growth rate decreased to 27%. In most cases, freshwater eukaryotic algae prefer acidic environments (pH 5–7), while cyanobacteria prefer alkaline environments (pH 7–9) (Myint, 2014; Qiu et al., 2017; Rachlin and Grosso, 1991).

Salinity

Salinity is one of the important factors in microalgae growth. Every microalgae has a different optimal range of salinity in which higher salinity can inhibit the growth of microalgae because it can change the water pressure between media and cells and consequently change the shape and structure of cells (Mata et al., 2010). For instance, Harwati et al. (2012) increased the NaCl concentration from 0 to 2% in the culture medium

of *Chlorococcum sp* (culture condition: 54 µE m^{-2} s^{-1} and 28 °C for 10 days), which caused a lipid content increase from 10.3 to 29.8%. Also, biomass productivity of algae decreased from 60 to 14 mg L^{-1} d^{-1}.

2.5.2.2 *Application of microalgae cultivation on wastewaters*

The utilization of microalgae as robust cellular species for biological nutrient removal from industrial and domestic wastewater streams has gained a great interest. The reason is the significant capacity of microalgae for photosynthetic uptake of high concentrations of minerals and organics while simultaneously capturing carbon dioxide (Molinuevo-Salces et al., 2019; Zeng et al., 2015).

Anaerobic digestates contain nitrogen and phosphorus so microalgae can grow on them and remove nutrients. It makes microalgae cultivation more cost-effective and provides an opportunity for capturing CO_2, nutrients removal from wastewater and producing feedstock for biofuel production, without using freshwater (Ho et al., 2014).

In order to use wastewaters with higher concentration of growth inhibitory elements (e.g. ammonia) for microalgal cultivation, the adaptive process for microalgal cells or the dilutions of wastewater are needed. Due to the complexity of different wastewaters, the screening and isolation of high tolerance microalgae species and strains is crucial to achieve high growth efficiency (Chiu et al., 2015). Bohutskyi et al. (2016) showed that when the dosage of anaerobic digestion centrate (from the Back River Wastewater Treatment Plant, Baltimore, MD) in the culture of *Chlorella vulgaris* was increased to 5-10%, the growth rate increased from 0.4 d^{-1} to 0.8 d^{-1}. However when the dosage increased to 20%, the growth rate reduced to 0.6 d^{-1}. The nutrient characteristics of several anaerobic

digestates and the ability of microalgae strains to remove the nutrients are summarized in Table 2-3.

The anaerobic digestion effluent has lower carbon levels compared to typical agricultural, municipal, and industrial wastewater due to COD removal by bacterial activity (Wang et al., 2010b). Also, the nitrogen in the effluent of anaerobic digestion (AD) is mainly in the form of ammonia. Cultivation of microalgae on AD effluent includes some challenges which are turbidity, high concentration of ammonia and contamination by bacteria. Therefore, AD effluent is usually diluted before feeding to algae since dilution reduces the inhibitory effect of turbidity and ammonia concentration on microalgae growth (Wang et al., 2010c). Furthermore, as AD contains a considerable amount of bacteria, an appropriate pretreatment such as filtration or autoclave may be needed to prevent the contamination of microalgae by bacteria (Wang et al., 2010b).

Cho et al. (2013) used the mixture effluent from an anaerobic digestion tank and the conflux of wastewaters rejected from sludge-concentrate tanks of a municipal wastewater treatment plant to cultivate *Chlorella sp. 227* and remove the nutrients from the wastewater. They reached a very high biomass production (3.01 g-dry cell weight per liter) after 5 days of cultivation. Also, the microalgae was able to remove TP and TN up to 95%. Wang et al. (2010b) cultivated *Chlorella sp.* on diluted digested dairy manure. The microalgae not only removed nutrient as mentioned in following table but also removed COD from the waste stream by 27.4–38.4% under mixotrophic conditions. A poultry litter anaerobic digester (AD) effluent was used by Singh et al. (2011) to grow a consortium of mixotrophic microalgae. The consortium removed TN and TP by 16% and 60% respectively. Moreover

the cell composition of the consortium after 4 days was 9.6 % lipid, 11.3% carbohydrate and 40% protein. In the study of Riaño et al. (2016), *Chlorella sorokiniana* was grown on anaerobic digested cheese whey (10% AD) to remove ammonium and TP. They used a semi-continuously fed microalgal-based system to examine biomass productivity and lipid accumulation during a period of 77 days. Maximum biomass productivity (12.0 g m^{-2} d^{-1}) and lipid content (12.3%) were obtained at ammonium loading rate of 12.9 mg L^{-1} d^{-1}, OLR of 0.43 kg COD m^{-3} d^{-1} and HRT of 5 days. Also, the microalgae culture had a soluble COD removal efficiency of 94%. Bjornsson et al. (2013) investigated the ability of *Scenedesmus sp. AMDD* to remove nutrients from a mixture of anaerobic digested of swine manure and algal biomass. In the experiment, the algae were able to remove ammonium and TP by 99.6 and 92.2%, respectively. They also conducted another experiment with the same microalgae strain to remove nutrients from a swine manure digestate supplemented with MgSO$_4$ (3.04×10^{-4} mol L^{-1}). In this case, the microalgae consumed almost all ammonium in the culture and 65.2% of TP. The study showed that the microalgae strain has different nutrient removal efficiency and biomass productivity in the two wastewaters. Olguín et al. (2015) studied a dual purpose system for the treatment of the anaerobic effluents from pig waste using *Neochloris oleoabundans* and investigating its growth, lipid content and nutrient removal ability. The culture was subjected to an N deficiency since day 5 of cultivation. With this method they achieved a biomass productivity of 45 mg L^{-1} d^{-1} and lipid content of 25.4%. In the study of Woertz et al. (2009), anaerobic digested dairy wastewater was treated outdoors in bench-scale open batch cultures using a mixture of *Actinastrum, Scenedesmus*, *Chlorella* and *Micractinium*. In the photobioreactor, the

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ammonium and TP were removed by 96% and 99%, respectively. Also, the highest lipid

content during the 13 days of cultivation was 29.

Table 2-3: The characteristics of microalgae grown on anaerobically digested wastewaters

Wastewater types	Microalgae type	$NH_4^+ - N$	TN	TP	TN/ TP	Biomass productivity (mg L⁻¹ d⁻¹)	Lipid/Carb. content (%)	Operational conditions	Ref.
		Content (mg L⁻¹) Removal (%)							
Digested dairy manure (20x dilution)	*Chlorella sp.*	112 100%	173 78.3%	13 71.6%	13.3	81	13.6/-	Batch culture, Flask (0.25L), 25 °C, 200 µmol m⁻² s⁻¹, 24 h d⁻¹ photoperiod, removal efficiency period: 21 days	(Wang et al., 2010b)
Diluted anaerobically digested poultry litter effluent	Consortium of *Chlorella minutissima*, *Chlorella sorokiniana*, *Scenedesmus bijuga*	-	84 16%	7 50-60%	12	71	9.6/11.3	Batch culture, Flask (0.25L), 25 °C, 75–80 µmol m⁻² s⁻¹ light intensity, 12 h d⁻¹ photoperiod, removal efficiency period: 4 days	(Singh et al., 2011)
Anaerobic digested cheese whey (10% AD)	*Chlorella sorokiniana*	74 84%	-	8.7 20%	-	12	12.3/-	semi-continuous, PBR (3L), 27 °C, 24 h d⁻¹ photoperiod, 54 µE m⁻² s⁻¹, removal efficiency period: 1 day	(Riaño et al., 2016)
Anaerobic digested starch wastewater	*Chlorella pyrenoidosa*	243	283 91.6%	29 90.7%	9.8	58	25.4/-	Batch culture, PBR (2L), 25 °C, 127 µmol m⁻² s⁻¹, 12 h d⁻¹ photoperiod, removal efficiency period: 9 days	(Yang et al., 2015)
Swine manure and algal biomass co-digestion	*Scenedesmus sp. AMDD*	22 99.6%	-	6 92.2%	-	70	-	Batch culture, Flask (0.25L), 25 °C, 85-90 µmol m⁻² s⁻¹, 24 h d⁻¹ photoperiod, removal efficiency period: 6 days	(Bjornsson et al., 2013)
10% cattle anaerobic digester effluent	*Chlorella sorokiniana UTEX 2714*	89 72.2%	231 87.4%	112 64.1%	2.1	9	12.8/22.2	80 L polyethylene Hanging bag, 25 °C, 160 µmol m⁻² s⁻¹, removal efficiency period: 21 days	(Bjornsson et al., 2013)
Digested pig waste	*Neochloris oleoabundans*	41 98%	42.3	5 99%	8.5	45	22.4/-	Batch culture, Flat PBR (4L), 35 °C; 134 µmol m⁻² s⁻¹; 16 h d⁻¹ photoperiod, removal efficiency period: 9 days	(Olguín et al., 2015)
Anaerobic digested municipal wastewater (4x)	*Nannochloropsis salina*	546 100%	640 87%	91 99%	7	68	~21/-	Batch culture, PBR (2L), 35 °C, 200 µmol m⁻² s⁻¹, 24 h d⁻¹ photoperiod, removal efficiency period: 10 days	(Cai et al., 2013b)
Digested dairy manure (25% dilution)	Mix of *Actinastrum*, *Scenedesmus*, *Chlorella and Micractinium*	30 96%	81	2 99%	40	69	10-29/-	Batch culture, 40-L rectangular PBR, average temperature, 30.6 °C, average daily solar radiation was 203Wm⁻², removal efficiency period: 13 days	(Woertz et al., 2009)
Anaerobically treated cheese factory effluents (20x dilution)	*Phormidium bohneri,*	25 24%	-	16 18%	-	82	-	Batch cultures, Plastic bottle (1.8L), 21 °C, 80 µmol m⁻² s⁻¹; 15 h d⁻¹ photoperiod, removal efficiency period: 1 day	(Blier et al., 1995)
	Micractinium pusillum	25 37%	-	16 16%	-	34	-		
Anaerobically digested piggery effluent	*Chlorella vulgaris*	21 54%	-	0.43 88%	-	-	-	Batch (11L plastic bag), 25 °C, 15 h d⁻¹ photoperiod, removal efficiency period: 6 days	(Kumar et al., 2010)

2.5.2.3   Carbohydrate production from microalgae

Microalgae can accumulate a significant amount of energy-rich compounds such as carbohydrate or starch. Moreover, they have the ability to up-regulate the cell quota of the compounds under stress condition such as high irradiance, high salinity, extreme temperature and nutrient limitation. The ability of microalgae to accumulate carbohydrate is different between species so that species from genera such as *Scenedesmus*, *Chlorella*, and *Chlamydomonas* can accumulate carbohydrate above 50% of their dry weight. The carbohydrate content of a microalgae cell and the growth rate depend significantly on the cultivation strategy and type of stress. The cultivation and stress conditions and their effects on different microalgae are summarized in Table 2-4.

Ho et al. (2012) studied the effect of light intensity on cell growth and carbohydrate and lipid productivity of *Scenedesmus obliquus CNW-N*. They applied the light intensity ranging from 60-540 μmol m^{-2} s^{-1}. The highest biomass productivity (840.56 mg L^{-1} d^{-1}), lipid productivity (96.50 mg L^{-1} d^{-1}) and carbohydrate productivity (321.52 mg L^{-1} d^{-1}) were obtained at the light intensity of 420 μmol m^{-2} s^{-1}. The increase in carbohydrate content with increasing light intensity has been shown by the study of Sukenik and Wahnon (1991) on *I. Isochrysis galbana* at different light intensities. The study showed that both growth rate and carbohydrate content increased 3 fold when the light intensity increased from 30 to 400 μmol m^{-2} s^{-1}. They also tested the effect of nitrogen limitation on carbohydrate content by decreasing the nitrogen load from 140 to 28 μmol NO_3^{-1} L^{-1} d^{-1}. This caused a 3-fold increase in carbohydrate content.

Table 2-4: The operation strategy and its effects on different microalgae species

Microalgae	Operation strategy	Stress condition	Ref.
Scenedesmus obliquus CNW-N	PBR (1L), batch culture, HRT: 82h, 28 °C, pH: 6.2, CO_2 concentration, 2.5%; CO_2 flow rate, 0.4 vvm, medium: prepared synthetic feed	**Stress**: Increase in light intensity from 60 to 420 µmol m^{-2} s^{-1} **Effect**: Carbohydrate content increased from 15 to 38%. Biomass productivity increased around 3-fold.	(Ho et al., 2012)
I. Isochrysis galbana	PBR (2.6L), turbidostat culture, pH: 7.8, 25 °C, medium: prepared synthetic feed	**Stress:** Increase in light intensity from 30 to 400 µmol m^{-2} s^{-1} **Effect:** Carbohydrate content increased almost 3-fold, from 2.7 to 7.0 pg $cell^{-1}$ and also the growth rate increased almost 3-fold	(Sukenik and Wahnon, 1991)
I. Isochrysis galbana	PBR (2.6L), Chemostat culture, 25 °C, 175 µmol m^{-2} s^{-1}, medium: prepared synthetic feed	**Stress:** Nitrogen load was decreased from 140 to 28 µmol $NO_3^{-1}L^{-1}$ d^{-1} **Effect:** Carbohydrate content increased almost 3-fold, from 5.3 to 16.5 pg $cell^{-1}$	(Sukenik and Wahnon, 1991)
Chlorella vulgaris SO-26	Flask (0.5L), Batch culture, pH:5.5, 20kLx light intensity, 24h d^{-1} photoperiod, medium: prepared synthetic feed	**Stress:** Increase in temperature from 5 to 20°C **Effect:** Carbohydrate content decreased from 70 to 50%. Around 3 fold increase in the Specific growth rate increased.	(Hosono et al., 1994)
Chlorella ellipsoidea	Flask (0.25L), Batch culture, 28 °C, 78 µE m^{-2} s^{-1}, 24h d^{-1} photoperiod, medium: prepared synthetic feed	**Stress:** pH values increased from 4 to 9 **Effect:** Carbohydrate content increased from ~19% to ~34%. Also the dry weigh increased from ~340 mg L^{-1} to ~720 mg L^{-1}	(Khalil et al., 2010)
Scenedesmus obliquus CNW-N	PBR (1L), Batch culture, 220-240 µmol m^{-2} s^{-1}, pH: 6.2, 28 °C, pH: 6.2, CO_2 concentration, 2.5%; CO_2 flow rate, 0.4 vvm, medium: prepared synthetic feed	**Stress:** 2 days of nitrogen starvation **Effect:** Carbohydrate content increased from 21 to 49% and the growth rate slightly decreased.	(Ho et al., 2013c)
Chlorella vulgaris FSP-E	PBR (1L), Batch culture, 450 µmol m^{-2} s^{-1}, pH: 6.2, 28 °C, pH: 6.2, CO_2 concentration, 2%; CO_2 flow rate, 0.2 vvm, medium: prepared synthetic feed	**Stress:** 2 days of nitrogen starvation **Effect:** Carbohydrate content increased from 15 to 51% and growth rate slightly decreased	(Ho et al., 2013a)
Chlamydomonas reinhardtii CC-124	Batch culture, 150 µmol m^{-2} s^{-1}, 24 °C, medium: prepared synthetic feed	**Stress:** salinity stress by increasing NaCl concentration from 0 to 1.0 M **Effect:** Starch and TAG content increased around 4-fold and 5-fold respectively	(Siaut et al., 2011)
Tetraselmis subcordiformis	PBR (0.6L), 200 µmol m^{-2} s^{-1}, 25 °C CO_2 concentration, 3%; CO_2 flow rate, 0.4 vvm, medium: prepared synthetic feed	**Stress:** Sulfur-deprived condition **Effect:** Increase in starch content from 17.6% to 62.1% after 2 days	(Yao et al., 2012)
Tetraselmis subcordiformis	PBR (0.6L), 50 µmol m^{-2} s^{-1}, 25 °C CO_2 concentration, 3%; CO_2 flow rate, 0.4 vvm, medium: prepared synthetic feed	**Stress:** Nitrogen-deprived condition **Effect:** Increase in starch content from ~25% to 54.3% after 1 day	(Yao et al., 2012)
Scenedesmus obliquus CNW-N	PBR (1L), two stage Batch culture, 210-230 µmol m^{-2} s^{-1}, pH: 6.2, 28 °C, pH: 6.2, CO_2 concentration: 2.5%; CO_2 flow rate, 0.4 vvm, medium: prepared synthetic feed	**Stress:** Nutrient replete condition until day 4 and transfer to DI water after day for to provide nutrient starvation **Effect:** Increase in carbohydrate content from 16.9% to 51.8 after 1 day	(Ho et al., 2013d)

Hosono et al. (1994) examined the effect of temperature on the carbohydrate content of *Chlorella vulgaris SO-26*. They realized that when the temperature increased from 5 to 20 °C, the carbohydrate content decreased from 70% to 50% but the maximum specific growth rate increased approximately from 0.015 to 0.05 h^{-1}. The effect of pH on the carbohydrate content of *Chlorella ellipsoidea* was studied by Khalil et al. (2010). They increased the pH of culture from 4 to 9 using NaOH solutions. As a result, the carbohydrate content increased approximately from 19% to 34%. Also, the dry weigh increased approximately from 340 mg L^{-1} to 720 mg L^{-1}. In another research, Ho et al. (2013a) evaluated the effect of nutrient starvation on *Chlorella vulgaris FSP-E*. In their experiment, after 2 days of nitrogen starvation, the carbohydrate content increased from 15 to 51%. One of the environmental stresses that can be applied to microalgae is salinity. Siaut et al. (2011) showed that starch and triacylglycerol (TAG) content of *Chlamydomonas reinhardtii CC-124* increased around 4-fold and 5-fold, respectively, when the NaCl concentration is increased from 0 to 1.0 M. Yao et al. (2012) studied the effect of sulfur and nitrogen starvation on the starch content of *Tetraselmis subcordiformis*. Under sulfur-deprived condition, the carbohydrate content increased from 17.6% to 62.1% after 2 days, and under nitrogen-deprived condition, starch content increased from ~25% to 54.3% after 1 day since the nitrogen-deprived cells ceased starch accumulation on the first day.

As mentioned, nutrient starvation is the main stress applied for increasing carbohydrate content of microalgae cell but it reduces the growth rate. Therefore, high biomass productivity conflicts with high carbohydrate content. The problem can be solved by employing a two-stage cultivation method. In the first stage, high biomass productivity

is achieved by maintaining the optimal growth conditions. In the second stage, the produced biomass is transferred to a stressful environment such as a nutrient deficient medium where the microalgae up-regulate the carbohydrate content of cell. This cultivation strategy has already been used in both small lab scale and large outdoor scale for production of lipids (Huntley and Redalje, 2007; Mujtaba et al., 2012; San Pedro et al., 2013; Xia et al., 2013) and carbohydrates (Ho et al., 2013d). Ho et al. (2013d) used a 1 L PBR to cultivate *Scenedesmus obliquus CNW-N* under nutrient rich conditions to maximize the biomass productivity for 4 days. Then, they transferred the produced biomass to a nutrient deficient media. Using the method, the carbohydrate content increased 3.5 fold and they achieved the biomass productivity and carbohydrate productivity of 681.4 and 352.9 mg L^{-1} d^{-1} respectively. Based on the study of San Pedro et al. (2013), a reasonable cultivation method for the first stage is continuous operation process in order to maximize the biomass productivity by optimizing the dilution rate. Then, different kinds of stress can be applied to the biomass. For an industrial-scale, Huntley and Redalje (2007) suggested using a photobioreactor for first stage since optimal growth conditions are best maintained in a photobioreactor (PBR). Thereafter, the biomass can be transferred to an open pond to expose the cells to nutrient deprivation or any other stress. They applied this method for industrial-scale lipid production from *Haematococcus Pluvialis* (Huntley and Redalje, 2007).

CHAPTER 3 ANAEROBIC DIGESTION OF THIN STILLAGE OF CORN ETHANOL PLANT IN A NOVEL ANAEROBIC BAFFLED REACTOR

3.1 Abstract

In this study, the performance of a conventional anaerobic baffled reactor (ABR) and a novel configuration of hybrid ABR for the treatment of thin stillage was evaluated. The hybrid ABR achieved the chemical oxygen demand (COD) removal, sulfate removal and methane yield of 97-94%, 97-94% and 294-310 mL CH_4 g^{-1} $COD_{removed}$, respectively at organic loading rate (OLR) of 1 to 3.5 kg COD m^{-3} d^{-1}. On the other hand, the value of COD and sulfate removal and methane yield for the conventional ABR were 75-94%, 67-76% and 140-240 mL CH_4 g^{-1} $COD_{removed}$, respectively at OLR range of 1.1 kg COD m^{-3} d^{-1} to 1.8 kg COD m^{-3} d^{-1}. The enhanced performance and robustness of the novel ABR was demonstrated to be the result of incorporation of solid/liquid/gas separators into the configuration of the conventional ABR, leading to reduced biomass washout, higher solid retention time and significantly improved phase separation.

3.2 Introduction

Bioethanol is the most widely used biofuel, which is mainly produced from sugar based crops such as corn and sugarcane (Arapoglou et al., 2010; Harun et al., 2010) with

increasing annual production volume of 3.4 million gallons to 14.3 million gallons per year from 2004 to 2014 (Koza et al., 2017). Each liter of ethanol produced can generate up to 20 L of thin stillage, an aqueous by-product from the distillation of ethanol with chemical oxygen demand (COD) of approximately 100 g L^{-1} (Wilkie et al., 2000). The current treatment of thin stillage relies on evaporation and drying, accounting for 46.8% of total energy consumption of the bioethanol plant (Khalid et al., 2011).

Alternative technologies for thin stillage treatment such as anaerobic digestion have been proposed for the removal of organic materials and improving the energy balance of the process, given that the biogas produced, presents an alternate energy source for the plant (Wilkie et al., 2000). Different types of anaerobic digesters have been applied for the treatment of thin stillage with organic loading rate (OLR) range of 2.9-29 kg COD $m^{-3}d^{-1}$ and COD removal of 82-99% (Agler et al., 2008; Andalib et al., 2012; Dereli et al., 2014; Lee et al., 2011; Schaefer and Sung, 2008).

Anaerobic baffled reactor (ABR), which is a compartmentalized reactor and thus can foster optimal environmental conditions for methanogenic and acidogenic bacteria in a two-phase system (Fang, 2010a) has not previously been employed for the digestion of thin stillage. Sulfate in the influent stream will lead to sulfidogenesis and sulfur removal primarily in the first compartment of the ABR due to lower Gibbs free energy of the reaction compared to methanogenesis and as a result, mainly biogas from the first compartment contains the hydrogen sulfide (Saritpongteeraka and Chaiprapat, 2008a). Given that sulfides can inhibit the activity of methane producing bacteria (Alkan-Ozkaynak and Karthikeyan, 2011) and thin stillage has a relatively high sulfur content of

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approximately 500 mg L^{-1} (Alkan-Ozkaynak and Karthikeyan, 2011), the two-phase configuration of ABR is advantageous. The other advantage of ABR is a long solid retention time (SRT) (612 to 42d (Grobicki and Stuckey, 1991)). Two phase systems enhance the stability of the system to fluctuation in environmental conditions such as temperature and pH (Zhu et al., 2015). ABR has been successfully used for treating different wastewater such as soybean protein processing (Zhu et al., 2008), whisky distillery (Akunna and Clark, 2000), pulp and paper mill black liquor (Grover et al., 1999) and high sulfur rubber latex wastewater (Saritpongteeraka and Chaiprapat, 2008a).

Conventional ABR has not been applied and evaluated for anaerobic digestion of thin stillage to the best of our knowledge, and its performance and operation has yet to be explored. The low biomass growth rate and high biomass washout are the main problems of conventional ABR (Barber and Stuckey, 1999). Since the introduction of conventional ABR, different modifications to its configuration have been suggested in order to improve the stability and treatment efficiency of the reactor including the use of carrier to support the growth of microorganisms (Faisal and Unno, 2001) and using compartments of different sizes (Elreedy et al., 2015; Malakahmad et al., 2011) or using more number of compartments (Boopathy, 1998). Therefore, the carrier anaerobic baffled reactor (CABR) was introduced to support the growth of biomass to decrease the washout and increase the biomass concentration inside the reactor. Modifications of ABR configuration are well documented in the literature (Barber and Stuckey, 1999; Zhu et al., 2015). The drawback of using carriers is the cost of carriers as well as the blockage caused by accumulated sludge (Zhu et al., 2015). Moreover, building an ABR with a large first compartment as a settler

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or an ABR with more number of compartments results in a significantly higher construction cost compared to a conventional ABR. In the present study, a novel hybrid ABR in which a solid/liquid/gas separator is incorporated into the configuration of conventional ABR, is evaluated for anaerobic digestion of thin stillage. The suggested modifications in this study are easy and practical to perform on an existing reactor without imposing any considerable cost. It has been hypothesized that this novel configuration enables handling a higher OLR at a higher removal efficiency due to reduced sludge wash out and enhanced phase separation and robustness compared to the conventional ABR. To verify this hypothesis, the performance of the novel hybrid ABR was evaluated and compared with the conventional ABR with respect to robustness, sludge washout, sulfate and COD removal efficiency and biogas production.

3.3 Materials and Methods

3.3.1 Thin stillage characterization

The corn thin stillage was obtained from IGPC Ethanol Inc. (Aylmer, ON, Canada). After collection, the thin stillage sample was stored in a refrigerator at 4 °C to avoid degradation. Physical and chemical characteristics of the thin stillage used in this study were characterized by a number of different analysis methods. The elemental analysis (K, Ca, Mg, S, Zn, Mn, Fe, Cu, Al and Na) of the thin stillage was conducted at the Minerals Engineering Center at Dalhousie University (Halifax, Nova Scotia, Canada) using inductively coupled plasma optical emission spectrometry (ICP-OES) in which the samples were diluted into 5% nitric acid prior to measurement. COD, biological oxygen demand

(BOD), total solids (TS), volatile solids (VS), total suspended solids (TSS), and volatile suspended solids (VSS) analyses were based on standard methods (Eugene et al., 2012). The total nitrogen (TN) was determined by HACH analysis kit, and UV-vis spectrophotometer (DR6000, HACH). The thin stillage was filtered before introducing to the reactor due to high solid content. The characteristics of filtered thin stillage such as TS, VS, TSS and VSS were determined according to standard methods (Apha, 1985) and other features (TN, total phosphorus, sulfate and ammonia) were measured by HACH analysis kit.

3.3.2   ABR start-up

The COD of feed was adjusted by diluting thin stillage with tap water. Souring due to the accumulation of volatile fatty acids (VFAs) often leads to process failure (Chua et al., 1997; Yu et al., 2002). In order to control pH and prevent souring, pH adjustment is done by the addition of $NaHCO_3$ to the feed, leading to an increase in alkalinity and buffering capacity of the system. The stability of an anaerobic system can be determined by VFA/TA (total alkalinity) ratio. The VFA/TA ratio of 0.1–0.25 is usually desirable without the risk of acidification while the ratio beyond 0.3–0.4 indicates digester upset, and corrective measures are necessary (Li et al., 2014; Liu et al., 2012a; Nigam and Pandey, 2009). The downside of $NaHCO_3$ addition is an increase in the operating cost of anaerobic digestion especially in a large scale but the alkalinity supplementation is usually added to the anaerobic digestion plants (Khanal, 2008; Metcalf and Eddy, 2003). On the other hand, the provided phase separation in the hybrid ABR results in enhanced activity of methanogenic

bacteria and consequently higher consumption rate of VFAs. Thus, it reduces the risk of acidification/reactor failure and its associated costs.
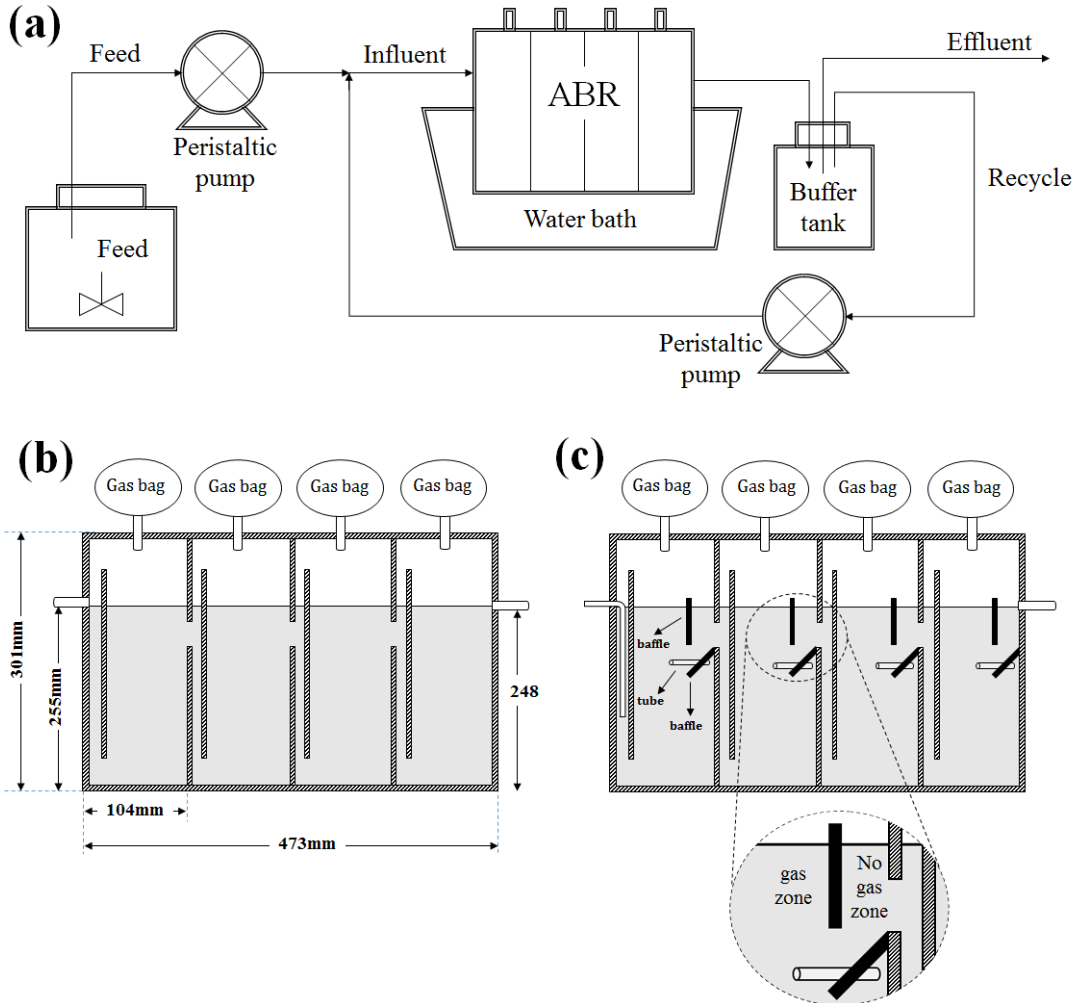
Figure 3-1: Schematic view of ABR system (a) overview of ABR system (b) Conventional ABR (c) Hybrid ABR

A lab scale ABR was operated with a total and working volume of 40 L and 27.5 L, respectively (Figure 3-1a). The reactor includes four compartments with a working volume of 6.9 L in each compartment. The prepared feed was fed continuously to the ABR using a peristaltic pump (feeding pump) (Cole Parmer, Master flex L/s). A water bath was used

to maintain the temperature of reactor constant at 35 °C. The reactor was sealed and the top of each compartment was connected to a 25 L Tedlar® gas sampling bag to collect the produced biogas. The effluent from the ABR was collected in the buffer tank (Figure 3-1a) and then recycled to the inlet by a peristaltic pump (recycle pump) to be mixed with the fresh feed. The OLR of the reactor was increased step by step. The system was monitored on daily basis with respect to VFA and alkalinity and once it reached to stable condition, different parameters such as biogas production rate, COD, sulfate, biomass washout were measured.

3.3.2.1 Conventional ABR

An initial run was performed in the conventional ABR (Figure 3-1b) with a feeding flowrate of 6.55 L d^{-1} and a recycle flowrate of 66 L d^{-1} (Stage I) (overall hydraulic retention time (HRT) of 4.2 d and internal HRT of 0.4 d). In this study, the overall HRT is considered as the length of time the liquid remain in the reactor (Henze, 2008) while the internal HRT is calculated considering the recycle stream (Serna-Maza et al., 2014).

The OLR of the system was increased stepwise from 0.75 to 1.8 kg COD m^{-3} d^{-1} by increasing the COD of feed from 3450±79 mg L^{-1} to 8150±228 mg L^{-1}. The OLR is calculated based on the COD concentration of wastewater, feeding flowrate and working volume of the reactor (Metcalf and Eddy, 2003). To control the OLR precisely, the feeding flowrate were measured and checked every day. Moreover, for each round of feed preparation, the COD of feed was measured. Due to the accumulation of high concentration of VFA (917±28 mg L^{-1} in the 4th compartment), the operating parameters of system such as feeding and recycle flowrate were changed as well as biomass concentration inside the

reactor. Therefore, in order to have a better control on the system, the feeding flowrate was decreased from 6.55 to 2.52 L d^{-1} and the recycle flowrate was increased from 66 to 144 L d^{-1} (recycle ratio (RR) of 57, overall HRT of 11.0 d and internal HRT of 0.2 d) while the OLR was maintained at 1.8 kg COD m^{-3} d^{-1} by increasing the COD of feed from 8150±228 to 19500±429 mg L^{-1}. The higher recycle flowrate of the effluent provides higher capacity to toxic substrate and high concentration wastewater by diluting the influent and maintaining the buffer capacity (Zhu et al., 2015). Increasing the overall HRT by decreasing the feeding rate increases the COD removal efficiency and consequently decreases the VFA concentration in the reactor (Castillo et al., 2007; Kuşçu and Sponza, 2005; Nachaiyasit and Stuckey, 1997b). A high biomass concentration in the reactor indicates a low food to microorganism ratio (F/M) resulting in an increase in COD removal efficiency (Ghangrekar et al., 2005). Thus, at that step of operation, 75 g VSS (3.9L) from IGPC methanator's sludge was added to the ABR (18.8 g VSS, 980 mL was added to each compartment to increase the sludge amount in each compartment from 69.3 g VSS to 88.3 g VSS), resulting in the increase in the ratio of inoculation volume:compartment volume from 4.6:6.9 to 5.0:6.9. The OLR was increased to 2.9 kg COD m^{-3} d^{-1} stepwise by increasing the COD to 31200±593 mg L^{-1}. However, at the OLR of 2.9 kg COD m^{-3} d^{-1}, overall HRT of 11.0 d and recycle ratio of 57, the experiment was stopped because of the biomass washout from the reactor and accumulation of VFA in the 4th compartment to 613±20 mg L^{-1} despite the high COD removal efficiency (91.3%). At that step, the high VFA to total alkalinity ratio reached around 0.4 in the last compartment which is an early indicator of process failure (Li et al., 2014) and it could inhibit methanogenesis and disrupt

the performance of the anaerobic digestion (Li et al., 2013). Therefore, corrective measures were needed to prevent the system failure before losing the metanogenic bacteria.

3.3.2.2 Hybrid ABR

To overcome the challenges, the conventional ABR configuration (Figure 3-1b) was modified by installing solid/liquid/gas separators and baffles to retain the biomass in each compartment as shown in Figure 3-1c. The black baffles (vertical and 45°) effectively reduced the sludge that was brought up by produced biogas. The tubes installed horizontally were designed to release the trapped biogas under 45° plates, and the vertical plates would prevent the sludge from moving with the flow of liquid. As can be seen in Figure 3-1c, two different zones are created by the baffles. The tube installed on the 45° baffle results in discharging the gas further from the connection of the two compartments, resulting in the formation of "gas zone" and "no-gas zone". This configuration will lead to higher biomass concentration and SRT of each compartment compared to the conventional configuration. The operation in modified (hybrid) ABR started with OLR of 1 kg COD m^{-3} d^{-1} and then increased by steps of 0.5 kg COD m^{-3} d^{-1} to OLR of 3.5 kg COD m^{-3} d^{-1}. The feeding and recycle flowrate were 2.52 and 25.2 L d^{-1}, respectively (the overall HRT of 11 d and internal HRT of 1 d).

3.3.3 Liquid digestate characterization

During the operation of ABR, the characteristics of liquid samples from each compartment and effluent of reactor such as COD, sulfate, total nitrogen, total phosphorus and ammonia were measured using HACH analysis kit, and UV-vis spectrophotometer

(DR6000, HACH). Moreover, the pH of samples was measured by VWR symphony pH meter. The values of VFA and alkalinity of samples were determined using titration (Anderson and Yang, 1992). For measuring the individual VFA including acetic, propionic and butyric acid, the samples from each compartment were acidified to pH 2 using concentrated sulfuric acid (EMD, ACS grade) and filtered with 0.2 μm syringe filters. 1 mL of the acidified filtered sample was shaken with 1 mL of diethyl ether (Anachemia) for 1 min for extraction of VFA into the organic phase (Manni and Caron, 1995). 2 μL of the supernatant ether phase containing the extracted VFA was injected into a Gas Chromatograph (Agilent 7890) equipped with a flame ionization detector and a HP-PLOT/U column (dimensions 30 m × 0.32 mm and 10 μm film thickness). The column operating temperature was 125 °C for 1 min, then 10 °C/min to 190 °C, hold for 15 min. The injector and detector temperature were 180 °C and 200 °C, respectively. The carrier gas was helium with split ratio of 10. Two series of standard mixture of VFA containing acetic acid (Fisher, ACS reagent grade), propionic acid (Sigma, 99.5%) and butyric acid (Alfa Aesar, 99%) in concentration ranging from 10 to 1000 mg L^{-1} of each VFA, were prepared in the same manner as samples for the calibration curves. The VFA test was performed in duplicate.

3.3.4   Characterization of struvite

The struvite crystals were collected from ABR effluent and elemental analysis for Mg and phosphorus was performed using ICP-OES at the Minerals Engineering Center at Dalhousie University (Halifax, Nova Scotia, Canada). Then, the struvite crystals were

dissolved in deionized water and the total nitrogen content was measured with the HACH test kit.

3.3.5 Biomass characteristics and washout measurement

For determining the biomass washout, the samples were collected from the effluent of reactor. Also, the sludge samples for each compartment were collected from the top, middle and bottom of sludge blanket to measure the biomass concentration. The VSS test for both biomass washout and biomass concentration was performed according to standard method (Apha, 1985). The size distribution of sludge granules was analyzed by ImageJ software (http://rsb.info.nih.gov/ij/).

For determining the sludge microbial population, samples were collected from the sludge in each compartment of ABR during the operation of hybrid ABR at the OLR of 3.5 kg COD m^{-3} d^{-1}. In the same day (for preventing any temporal variability), the samples were submitted to the Integrated Microbiome Resource (IMR) at Dalhousie University (Halifax, Canada) for DNA extraction, sequencing and analysis following the procedure of IMR (www.cgeb-imr.ca). The fresh sludge samples from each compartment were stored at -80˚C. The samples thawed and DNA was extracted using the PowerSoil DNA isolation kit (MoBio, Carslbad, CA). Then, the extracted DNA were stored at -80˚C. Quantification and quality-checks are performed using Qubit PicoGreen reagents after thawing the extracted DNA. Amplicons were generated according to Earth Microbiome Project protocols (http://www.earthmicrobiome.org/emp-standard-protocols/).

Amplicon fragments are PCR-amplified from the DNA in duplicate using different dilutions (1:1 and 1:10) using the high-fidelity Phusion polymerase. A single round of PCR

is performed applying "fusion primers" (Illumina adaptors + indices + specific regions) targeting the 16S V6-V8 (Bacteria/Archaea; ~440-450 bp) regions. PCR products are verified using Invitrogen 96-well E-gel and then purified and normalized by the SequalPrep 96-well Plate Kit (Invitrogen). For making one library 380 samples are pooled and then quantified fluorometrically. Pooled samples were run on an Illumina MiSeq using 300+300 bp paired-end V3 chemistry. For bioinformatics Analyses, the Microbiome Helper standard operating procedure (Comeau et al., 2017) was used to process the 16S rRNA gene data.

3.3.6 Biogas collection and measurement

The produced biogas from different compartments of the ABR was collected in 25L Tedlar® gas bags for a time period of 18 hours and was measured using water displacement method (Kafle and Kim, 2013; Liang and McDonald, 2015). The composition of biogas including CH_4 and CO_2 was determined by a gas chromatography (GC, 490 Micro GC, Agilent Technologies) equipped with a 10-metre MS5A and a 10-metre PPU column and thermal conductivity detectors (TCDs). Helium was used as a carrier gas and the temperature of both columns was 80 °C. Also, the injector temperature was 110 °C.

3.4 Results and Discussion

3.4.1 Characteristics of thin stillage

Table 3-1 shows the composition of raw thin stillage. For each test, three samples were analyzed (n = 3). During the operation of ABR, different batches of thin stillage were received from IGPC Ethanol Inc. The characteristics of thin stillage were slightly different

from batch to batch. For example the value of total COD (TCOD) varied from 108.8 to 117.3 g L^{-1}. The source of metals in thin stillage is the mineral nutrition of yeasts employed in fermentation processes (Walker, 2004). Sulfur in the thin stillage originated from the addition of sulfuric acid for pH regulation in bioethanol production process (Bajpai, 2013). The composition of raw thin stillage is comparable with the reported values in the literature as shown in Table 3-1 (Alkan-Ozkaynak and Karthikeyan, 2011; Andalib et al., 2012).

Table 3-1: The characterization of thin stillage

Parameters	Test results of this study	Alkan-Ozkaynak and Karthikeyan (2011)	Andalib et al. (2012)
TS (g L^{-1})	59.8±1.5	72.6	71.5 ± 0.72
VS (g L^{-1})	45.8±2.9	62.7	64.8 ± 0.6
TSS (g L^{-1})	23.7±0.5	26.21	46.4 ± 3.9
VSS (g L^{-1})	23.5±0.4	25.63	46.2 ± 3.7
TCOD (g L^{-1})	111.9±1.2	85.04	129.3 ± 6.3
SCOD (g L^{-1})	48.5±1.2	57.04	62 ± 4.5
TBOD (g L^{-1})	54.9±0.5	-	68.3 ± 0.8
TN (mg L^{-1})	1960±92	2000	-
TP (ppm)	1495.8±17.4	1508.47	-
K (ppm)	2539.6±30.6	2386.26	-
Ca (ppm)	29.1±0.3	27.31	-
Mg (ppm)	629±9.1	586.42	-
S (ppm)	942.7±19.7	527.62	-
Zn (ppm)	4.7±0.1	6.75	-
Mn (ppm)	1.6±0.05	3.9	-
Fe (ppm)	7.6±0.5	8.12	-
Cu (ppm)	0.4±0.03	0.17	-
Al (ppm)	0.4±0.1	<1	-
Na (ppm)	270.5±6.0	402.6	-

As indicated in Table 3-1, the solid content of thin stillage is very high. Therefore, direct feeding of ABR with thin stillage may cause clogging the system. For this reason, the thin stillage was filtered to reduce the amount of solid. The characteristics of the filtered

thin stillage are measured and shown in Table 3-2 (the tests were performed in triplicate). As shown in Table 3-2, the solid contents (TS, TSS, VS and VSS) and nutrients (TCOD, TP and TN) of thin stillage were reduced considerably as a result of filtration.

Table 3-2: The characterization of filtered thin stillage

Parameters	Test results
TS (g L^{-1})	32.2±0.4
VS (g L^{-1})	31.1±0.5
TSS (mg L^{-1})	940±53
VSS (mg L^{-1})	870±46
TCOD (g L^{-1})	69.8±2.9
TN (mg L^{-1})	1220±53
TP (mg L^{-1})	1191±49
SO$_4^{2-}$ (mg L^{-1})	2936±76
Ammonia (mg L^{-1})	104±4

3.4.2 Biomass concentration, sludge size distribution and washout

The biomass concentration in each compartment at different depths of the bioreactor was measured at various OLRs during the operation of the bioreactor. The values presented in Table 3-3 for biomass concentrations represent the average biomass concentration at various depths of the bioreactor. Biomass concentration increased in response to increase in OLR and the highest biomass concentration of 15.2±0.2 gVSS L^{-1} was observed at the highest OLR (3.5 kg COD m^{-3} d^{-1}) that the hybrid ABR was operated (Table 3-3). Biomass washout was lower in hybrid ABR than the conventional ABR at the same range of OLR (Table 3-3), which is attributed to the incorporation of solid/liquid/gas separator (Figure 3-1c). Table 3-3 also indicates that the biomass washout increased when the OLR in hybrid ABR increased from 1 to 3.5 kg COD m^{-3} d^{-1}, which is attributed to higher biogas production at higher OLRs, resulting in higher amount of sludge removal from the

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bioreactor (statistical analysis is provided in Appendix E). Moreover, the values of SRT show that the retention time of sludge in hybrid ABR is higher than the conventional ABR. The range of SRT for OLR of 1.1 to 1.8 kg COD m^{-3} d^{-1} was 312 to 101 days in the conventional ABR while the range was 646 to 150 days for OLR of 1 to 2.5 kg COD m^{-3} d^{-1} in the hybrid ABR. The values of SRT are comparable with the obtained results in the study of Grobicki and Stuckey (1991) on treatment of a synthetic feed using a 8.2L ABR containing 4 compartments. They applied the OLR of 1.2 to 4.8 kg COD m^{-3} d^{-1} (HRT of 80 to 20h) and the SRT of 612 to 42 days was achieved. In another study, the range of SRT in an ABR (with 4 compartments) for treating nitrobenzene at HRT of 10.4 days and OLR of 0.29-0.43 kg COD m^{-3} d^{-1} was 521-670 days (Kuscu and Sponza, 2009). The formation of granular sludge makes SRTs over 200 days in an upflow anaerobic sludge bed reactor achievable at HRTs as low as 6h (Pol et al., 2004).

Table 3-3: The biomass washout and concentration for conventional and hybrid ABR

Configuration/Stage	OLR (kg COD m^{-3} d^{-1})	Biomass concentration (g VSS L^{-1})	Biomass washout (g VSS d^{-1})	SRT (d)
Conventional ABR/ Stage I	1.1	10.2±1.2	0.9±0.03	312±46
	1.8	10.1±0.5	-	-
Conventional ABR/ Stage II	1.8	11.3±0.8	3.09±0.02	101±8
Hybrid ABR	1	13.9±0.8	0.59±0.02	646±57
	1.5	-	1.04±0.08	-
	2	-	2.14±0.02	-
	2.5	14.8±0.6	2.71±0.12	150±12
	3	-	3.09±0.18	-
	3.5	15.2±0.2	3.15±0.09	132±5

A long SRT is not desirable for aerobic treatment where the presence of slow growing bacteria (e.g. nitrifying bacteria) is not beneficial since it causes sludge bulking/foaming,

excessive oxygen demand and acid production. However, a long SRT is advantageous for anaerobic treatment where the treatment ability relies on the slow growing bacteria such as methanogens (Rittmann and McCarty, 2012). The higher SRT and less biomass washout in the hybrid ABR than the conventional ABR also reduce the transfer of microbial population from one compartment to the other.

In this study, the formation of granules was promoted by gradual increase of OLR, biogas production and liquid upflow velocity which is recommended in the literature (Barber and Stuckey, 1999). The granulation is also observed in the operation of ABR in other studies when they applied low OLR in the startup period (Boopathy and Tilche, 1991; Xing and Tilche, 1992). In addition to the above mentioned parameters that promote granular sludge formation, the unique configuration of the novel ABR introduced in this study contributed to the formation of granular sludge by separating the solid via a gas/liquid/solid separator and consequently retaining the sludge in the reactor for a longer period. Since the granules have superior settling capability, the reactor can operate at higher upflow velocity (low HRT) and as a result it can handle higher OLR.

Figure 3-2 shows the distribution of sludge size in each compartment. As can be seen in the graph, in the first compartment, the possibility of the presence of bigger granules is higher than other compartments. The probability of presence of bigger granules decreases along the reactor so that the last compartment has the highest number of small granules.
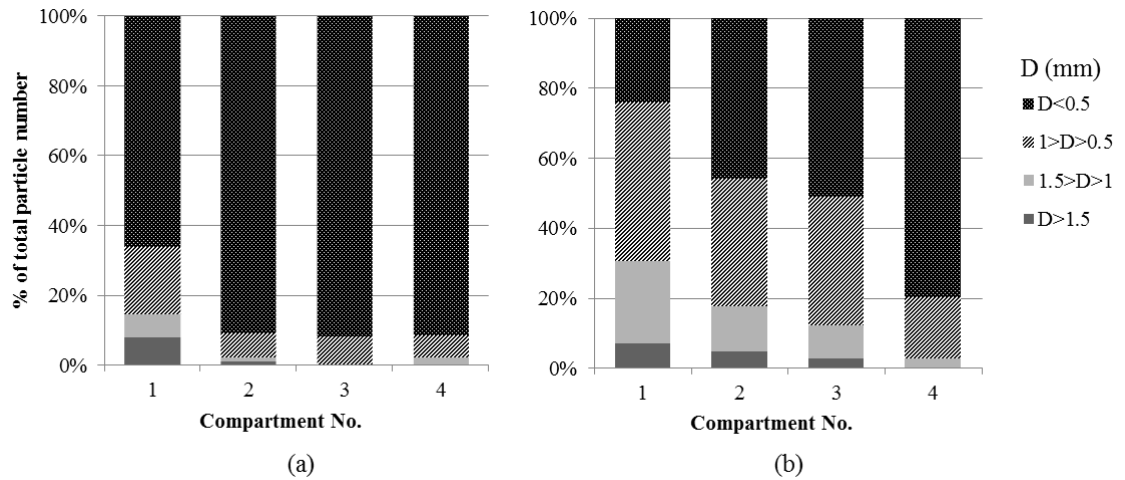
Figure 3-2: The size distribution of granules in each compartment in hybrid ABR (a) OLR of 1 kg COD m^{-3} d^{-1} (b) OLR of 3.5 kg COD m^{-3} d^{-1}

The size of sludge granules in all compartments increased in response to increasing the OLR from 1 to 3.5 kg COD m^{-3} d^{-1}. At the OLR of 1 kg COD m^{-3} d^{-1}, the size distribution of sludge in 2nd, 3rd and 4th compartment was almost similar. However, at the OLR of 3.5 kg COD m^{-3} d^{-1}, the sludge size in 2nd, 3rd and 4th compartment increased significantly which was related to the high rate of biogas production and mixing at higher OLRs. The change in the size of sludge in the 4th compartment was least significant among all compartments, which was expected due to the lower biogas production in 4th compartment compared to the other compartments. Lower biogas production rate results in poor mixing condition and lower shear stress, which are the influencing factors on granulation (Bhunia and Ghangrekar, 2008) (images and size distribution of sludge granules can be found in Appendix C).

3.4.3 Microbial community analysis

Sludge washout from one compartment to the other is a significant challenge in ABR, which was the main motivation for the new design. The microbial population depends on the environmental condition in each compartment as well as feedstock (Barber and Stuckey, 1999). However, a high mixing condition due to generation of biogas can reduce the phase separation and mix the bacterial community in each compartment. The new design results in reduced washout due to the unique configuration, leading to solids/gas/liquid separation. Additionally, upward and downward movement of sludge, results in the formation of granular sludge, which is heavier and consequently sludge wash out from one compartment to the other is reduced. The significance of the incorporation of the solid/liquid/gas separator will be more realized in industrial scale through avoiding contamination of subsequent steps. The microbial population analysis also shows that the novel configuration enables an enhanced phase separation. Diverse microbial population observed in each compartment was due to the different environmental condition (pH and VFA concentration) achieved in each compartment. The unique configuration, allowed for an enhanced separation of microbial population.

The microbial communities of bacteria and archaea at phylum and class level in the four compartments of hybrid ABR are shown in Figure 3-3a and Figure 3-3b, respectively. The dominant phylums of bacteria in the microbial community in the 1st compartment were *Bacteroidetes* (45.2%), *Synergistetes* (13.1%), *Firmicutes* (12.8%), *Proteobacteria* (10%) and *Spirochaetes* (5.8%). The majority of bacteria under the phylum of *Bacteroidetes* and *Firmicutes* has cellulolytic and hemi-cellulolytic properties and are responsible for the

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initial degradation of organic substrates (Gulhane et al., 2017). The dominance of those bacteria phylum showed the role of 1st compartment in hydrolysis and acidogenesis which is expected in a phase separated system such as ABR. However, the composition changed along the reactor so that the relative abundance of *Bacteroidetes* changed from 45.2% in the 1st compartment to 20.8% in the 4th compartment. Moreover, the relative abundance of *Firmicutes* reduced from 12.8% in the 1st compartment to 6.9% in the 4th compartment. On the other hand, the relative abundance of *Synergistetes*, *Thermotogae* and *Proteobacteria* increased from 1st compartment to the later compartments. This observation is in agreement with study of Gulhane et al. (2017) on microbial community plasticity for anaerobic digestion of vegetable waste in an ABR. In the study, the relative abundance of *Bacteroidetes* and *Firmicutes* reduced from 52% and 39% in 1st compartment of ABR to 18-21% and 6-10%, respectively in the last three compartments.

The dominant classes of archaea in the microbial population in the 1st compartment were *Methanomicrobia* (4.4%) and *Methanobacteria* (0.61%). The relative abundance *Methanomicrobia* and *Methanobacteria* changed downside of the reactor and reached 6.3% and 4.4% respectively in the 4th compartment which shows the increase in population of methanogens along the reactor. The dominance of *Methanomicrobia* and *Methanobacteria* in archaea is common in anaerobic waste and wastewater sludges (Narihiro and Sekiguchi, 2007).

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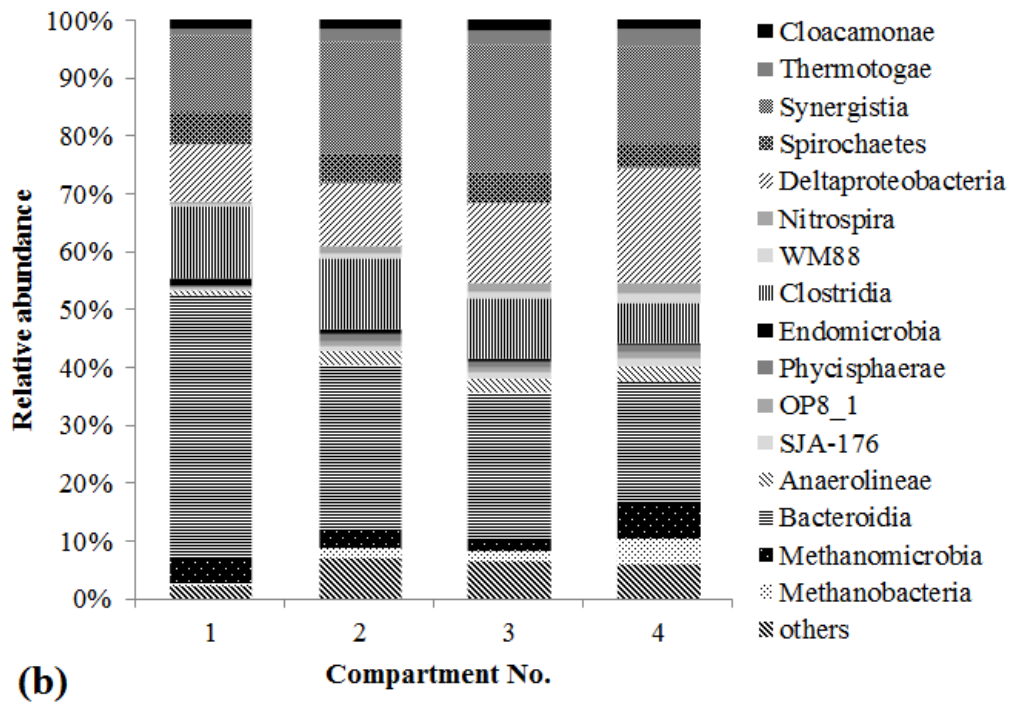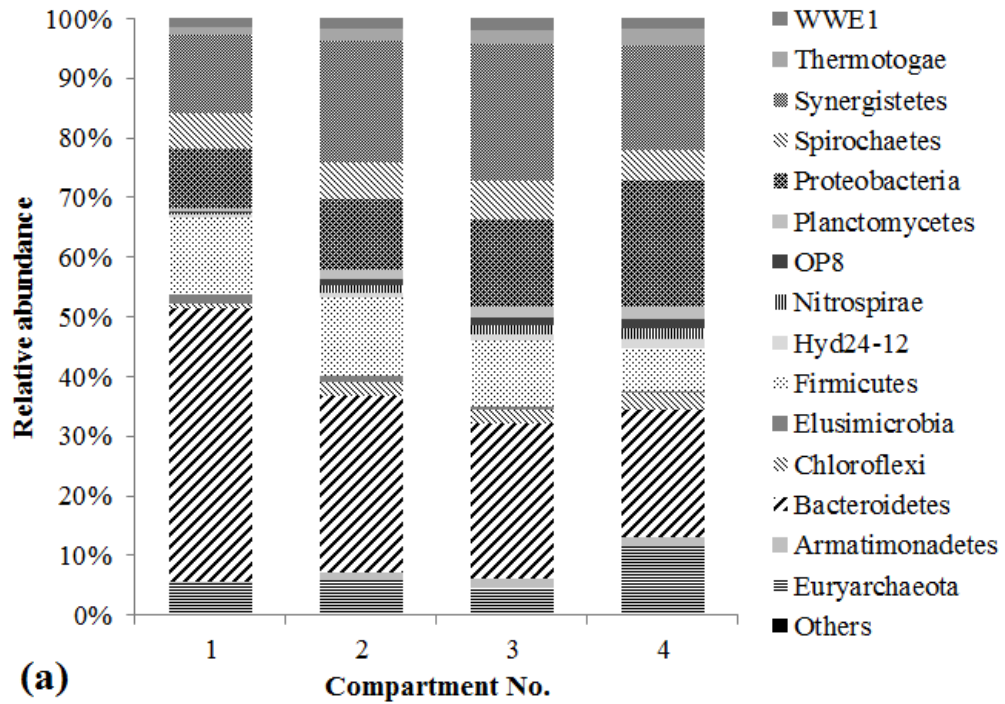
Figure 3-3: Taxonomic distribution of bacterial and archaeal diversity at (a) phylum and (b) class level of four compartments at OLR of 3.5 COD m^{-3} d^{-1}. Others refers to the taxa with a maximum abundance of <1%.

3.4.4 Phase separation in conventional and hybrid ABR

Maintaining pH at the desired level for the optimum activity of microorganisms is essential for an optimum anaerobic digestion process (Ganesh et al., 2014). The specific configuration of ABR enables separation of acidogenesis and methanogenesis longitudinally down the reactor. In general, the first compartment is dominated by fermentative bacteria and acetogens with an optimum activity under acidic condition with pH value ranging from 5 to 6.5, while the fourth compartment majoring in methanogens with the optimum pH ranging from 6.6 to 7.5 (Zhu et al., 2015).

The VFA concentration in the 1st and 4th compartment at different OLRs and configurations/stages is shown in Figure 3-4. In the conventional ABR/stage I (Figure 3-4a), the VFA concentration in both compartments increased sharply between OLR of 1.5 and 1.8 kg COD m^{-3} d^{-1}, and VFA/TA ratio in the 4th compartment increased to 0.38 (the VFA concentration increased from 483±17 to 917±28 mg L^{-1}). Therefore, the operation was modified by increasing the overall HRT from 4.2d to 11d and recycle ratio from 10 to 57. Moreover, the biomass concentration was increased in the conventional ABR from 10.1±0.5 to 12.5±0.4 g VSS L^{-1} by adding 75 g VSS (980 mL, 18.8 g VSS to each compartment) of methanator's sludge from IGPC plant and the operation was started at the OLR of 1.8 kg COD m^{-3} d^{-1} (stage II, Figure 3-4a). Likewise, at this stage, there was a sharp rise in VFA concentration (468±18 to 613±20 mg L^{-1} in the 4th compartment) in response to increasing the OLR of 2.6 kg COD m^{-3} d^{-1} to 2.9 kg COD m^{-3} d^{-1}. The reason for the increase in VFA concentration was the frequent blockage of reactor output with sludge. In the novel hybrid ABR configuration, the VFA concentration was lower

compared to the conventional ABR (Figure 3-4b) and it was more stable, which showed the positive effect of the physical modification of the reactor configuration on the performance of reactor. The difference between the VFA of 1st and 4th compartment (which is an indicator of phase separation) in the stage I (feeding flowrate of 6.55 L d^{-1} and recycle flow of 66 L d^{-1}) varied between 108 to 176 mg L^{-1} and the range for stage II (feeding flowrate of 2.52 L d^{-1} and recycle flow of 144 L d^{-1}) was 123 to 212 mg L^{-1}. This shows there was not a significant difference between the stage I and II of the conventional ABR regarding the phase separation. However, VFA concentration gradient was higher in the hybrid ABR, indicating an enhanced phase separation in comparison with the conventional ABR. The VFA concentration difference between the 1st and 4th compartment in hybrid ABR increased from 111 to 822 mg L^{-1} in response to increase in OLR from 1 to 3.5 kg COD m^{-3} d^{-1}. The improved phase separation in hybrid ABR is due to its higher SRT (646 day at OLR of 1 kg COD m^{-3} d^{-1}) compared to conventional ABR (312 days at OLR of 1.1 kg COD m^{-3} d^{-1}).

Figure 3-4c shows the profile of VFA concentration in the four compartments in hybrid ABR at various OLRs. As can be seen, the increase in the OLR has the highest and lowest impact on the VFA concentrations in the 1st compartment and 4th compartment, respectively. The increase in the OLR from 1 to 3.5 kg COD m^{-3} d^{-1} resulted in four fold increase in the VFA concentration in the 1st compartment, while only two fold increase in the VFA concentration in the 4th compartment was observed. Figure 3-4c also shows the phase separation in the system indicating that the 1st compartment has highest VFA concentration and lowest pH (6.6-6.8) among the compartments whereas the last

compartment has the lowest VFA concentration and highest pH (7-7.2). Therefore, each

compartment has a different environmental condition (phase), which makes it suitable for

a specific community of bacteria (e.g. acidogens and methanogens).
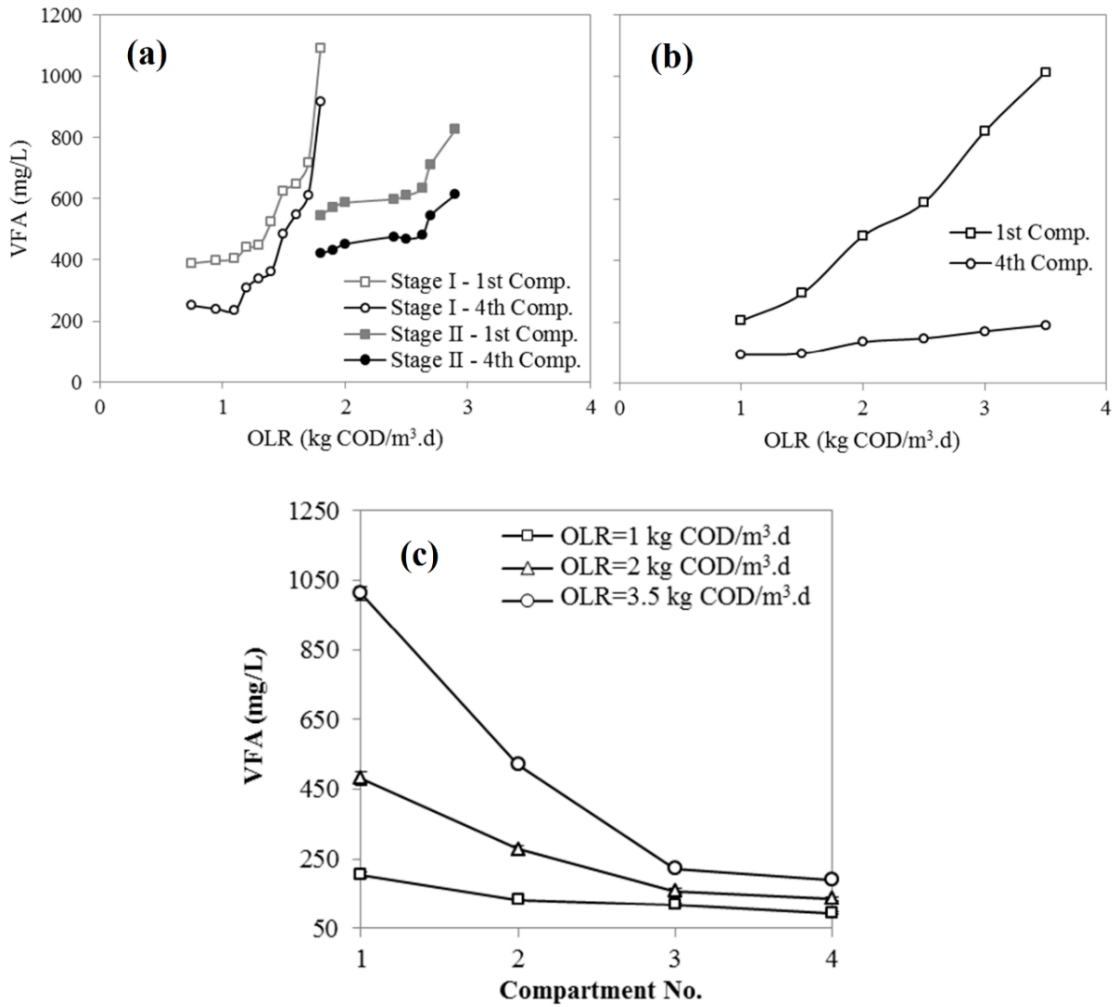
Figure 3-4: Variation of VFA in 1ˢᵗ and 4ᵗʰ compartment with OLR (a) conventional ABR (b) Hybrid ABR (c) all compartments of hybrid ABR

The difference of the VFA concentration in 1ˢᵗ and 2ⁿᵈ compartment was 71 and 492

mg L^{-1} at the OLR of 1 and 3.5 kg COD m^{-3} d^{-1}, respectively. While the difference of VFA

concentration in compartment 3 and 4 increased from 25 to 32 mg L^{-1} with an increase in OLR from 1 to 3.5 kg COD m^{-3} d^{-1}. According to Figure 3-4c, the hybrid ABR without considering the last compartment is able to provide the phase separation of 790 mg L^{-1} VFA (difference between the VFA concentration in 1st and 3rd compartment) at OLR of 3.5 kg COD m^{-3} d^{-1} along the reactor which is approximately close to the phase separation with four compartments (822 mg L^{-1} VFA difference between the concentration in 1st and 4th compartments at OLR of 3.5 kg COD m^{-3} d^{-1}). Therefore, the last compartment did not play a significant role in the term of phase separation at the OLR range of 1 to 3.5 kg COD m^{-3} d^{-1} and as a result only three compartments can be considered adequate for implementation of the hybrid ABR in full scale up to OLR of 3.5 kg COD m^{-3} d^{-1}. However, a more comprehensive study is required to identify the limitations of the hybrid ABR scale up.

The profiles of VFAs in different compartments of hybrid ABR at various OLRs are shown in Figure 3-5. The concentration of individual VFAs in anaerobic reactors are also important since the ratio of propionic to acetic acid has been suggested as an indicator of process instability. It is proposed that propionic to acetic acid ratio higher than 0.71 indicates impending failure (Ahring et al., 1995; Marchaim and Krause, 1993). In this study, the ratio in different compartments of hybrid ABR varied from 0.32 to 0.58 at various OLRs. As can be seen from Figure 3-5, among the three VFAs, acetic acid is considered as a key intermediate for methane production. The accumulation of acetic acid was observed in the 1st compartment and decreased along the length of the reactor (from 1st compartment to 4th compartment) which is due to utilization of acetic acid by Methanogenic bacteria. Propionic and butyric acids also followed a similar trend to acetic

acid along the reactor. On the other hand, no significant change in the composition of VFA was observed along the reactor or at different OLRs. The reduction in concentration of acetic, propionic and butyric acids along ABRs is also mentioned in the study of Wang et al. (2004) on performance of an 5-compartment anaerobic baffled reactor treating synthetic wastewater containing glucose. Furthermore, in the study, in each compartment of ABR, acetic acid and butyric acid had the highest and lowest concentration among VFAs, respectively.
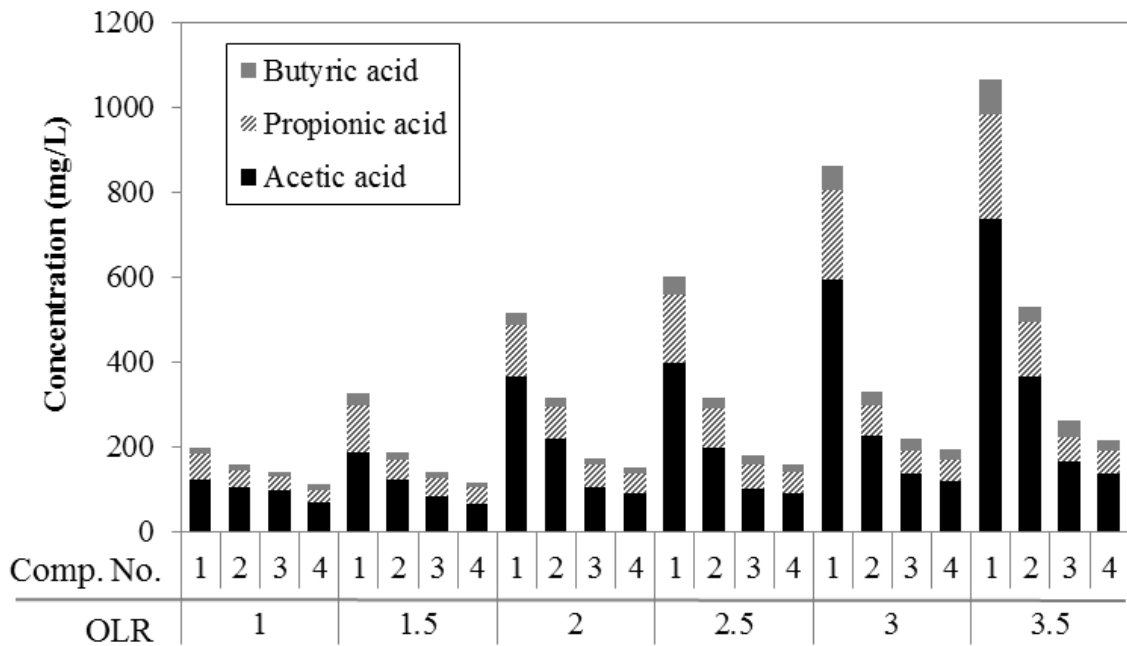
Figure 3-5: Individual VFA concentration in each compartment at various OLR (kg COD m^{-3} d^{-1})

3.4.5 COD removal and biogas production

The COD removal efficiency in different configurations/stages is depicted in Figure 3-6a. The figure indicates that the COD removal in the conventional ABR/stage I is lower and less stable than stage II. In the stage I, the COD removal efficiency reduced from 83% to 75% in conventional ABR in response to an increase in OLR from 1.7 to 1.8 kg COD m^{-3} d^{-1} (Figure 3-6a). The COD removal efficiency increased again in stage II due to the changes in the operating conditions (increasing the overall HRT from 4.3 to 11d, recycle ratio from 10 to 57 and biomass concentration from 10.1 to 12.5 g VSS L^{-1}). Operation of the conventional ABR was stopped at stage II because of a high rate of biomass washout and reactor blockage and high concentration of VFA, and the conventional ABR was physically modified to a novel hybrid ABR. The COD removal efficiency in the hybrid ABR was higher than that in the conventional ABR due to high SRT (646 days at OLR of 1 kg COD m^{-3} d^{-1} in hybrid ABR versus 312 days at OLR of 1.1 kg COD m^{-3} d^{-1} in the conventional ABR). The higher SRT in the hybrid ABR compared to the conventional ABR is because of novel configuration of hybrid ABR and solid/liquid/gas separator inside each compartment.

The COD removal and biogas production rate in each compartment for hybrid ABR is shown in Figure 3-6b and Figure 3-6c. The majority of COD removal and consequently biogas production occurred in the 1st compartment (Figure 3-6b). At a low OLR (1 kg COD m^{-3} d^{-1}), the 1st compartment contributed to 66% of COD removal, while the contribution from the 2nd, 3rd and 4th compartment was 26%, 4% and 3%, respectively. For the higher OLRs (1.5 to 3 kg COD m^{-3} d^{-1}), the contribution of the 1st compartment to COD removal

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decreased, while the contribution of the 2nd compartment increased. No significant change was observed in the contribution of 3rd and 4th compartment to COD removal at the OLRs of 1.5 and 2 kg COD m^{-3} d^{-1}. However, at OLR of 2.5 kg COD m^{-3} d^{-1}, the contribution for the 3rd compartment increased to 10%, while it did not change for the 4th compartment. On the other hand, from the OLR of 3 to 3.5 kg COD m^{-3} d^{-1}, the contribution of 4th compartment increased from 4% to 7%. The data shows that in ABR, the contribution of later compartments to COD removal increases with increasing OLR. Based on the Monod equation, the rate of COD consumption by bacteria is proportional to COD concentration. The 1st compartment received the highest concentration of COD and as a result had the highest rate of COD conversion and biogas production. At higher OLRs, higher concentration of COD was received at later compartments and therefore, the contribution of 2nd and 3rd compartment to total COD removal increased. However, the 4th compartment did not receive high concentration of COD to increase its contribution to COD removal until the OLR of 3.5 kg COD m^{-3} d^{-1}. The rate of biogas production from each compartment (Figure 3-6c) followed the same pattern as the COD removal. For example, from the OLR of 1 to 3 kg COD m^{-3} d^{-1}, the contribution of 4th compartment to total biogas production rate was in the range of 3-4% while the contribution increased to 6% at the OLR of 3.5 kg COD m^{-3} d^{-1}. Considering the hybrid ABR at the OLR of 3.5 kg COD m^{-3} d^{-1} without the 4th compartment, the OLR applied to these three compartments will be 4.5 kg COD m^{-3} d^{-1}. This shows the hybrid ABR is able to handle higher OLRs without a significant change in its treatment ability (mass balances for COD are provided in Appendix B).
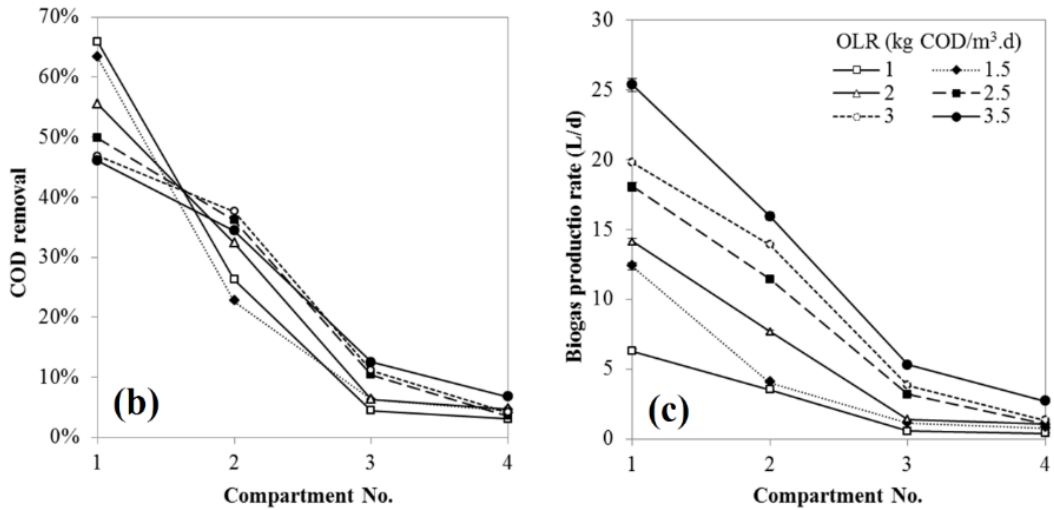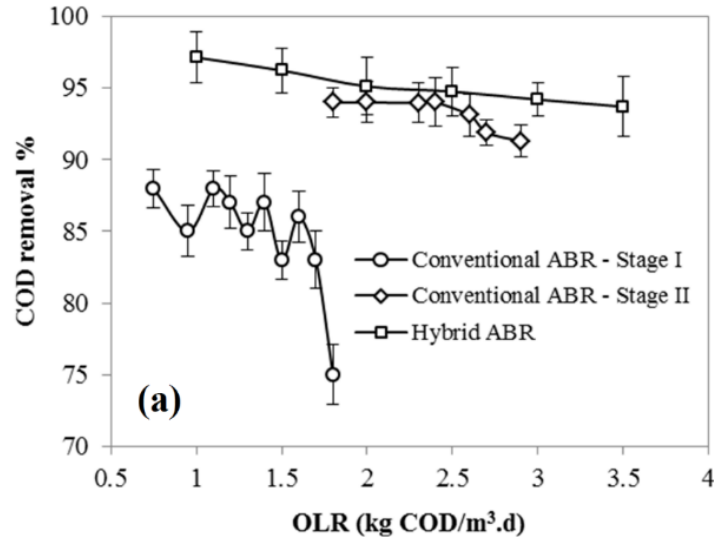
Figure 3-6: (a) The variation of COD removal efficiency with OLR at various configurations/stages (b) The COD removal and (c) biogas production rate in each compartment in hybrid ABR at different OLRs

The biogas production rate and methane yield for different OLRs at various configuration/stages is given in Table 3-4. As can be seen, for the same range of OLR, the biogas production rate in the hybrid ABR is considerably higher than the conventional ABR.

Table 3-4: Biogas production rate and methane yield

Configuration/Stage	OLR (kg COD m^{-3} d^{-1})	Total biogas (L d^{-1})	Biogas yield (L g^{-1} COD$_{removed}$)	Methane yield (L CH$_4$ g^{-1} COD$_{removed}$)
Conventional ABR/ Stage I	1.1	5.8	0.20	0.14
	1.2	9.8	0.34	0.24
	1.4	11.4	0.34	0.23
Conventional ABR/ Stage II	1.8	12.8	0.30	0.19
	2.2	16.9	0.32	0.20
Hybrid ABR	1	10.7	0.39	0.28
	1.5	18.3	0.46	0.31
	2	24.2	0.48	0.30
	2.5	33.7	0.52	0.30
	3	38.9	0.50	0.30
	3.5	49.4	0.55	0.29

Generally, the methane concentration in the biogas decreases when the OLR increases regardless of feedstock or reactor configuration (Kuşçu and Sponza, 2009; Lee et al., 2011; Pandian et al., 2011). The reason lies in the fact that the acidogenesis exceeds the methanogenesis when the OLR increases resulting in lower concentration of methane in the biogas (Kuşçu and Sponza, 2009). The same phenomenon was observed in this study in which the total methane concentration decreased from 71% to 54% when the OLR increased from 1 to 3.5 kg COD m^{-3} d^{-1} in the hybrid ABR. However, the response to the increase in OLR can be different. Faisal and Unno (2001) increased the OLR from 1.6 to 5.3 kg COD m^{-3} d^{-1} resulting in the decrease in methane concentration from 71.2 to 69.1% for treating palm oil mill wastewater in an ABR. On the other hand, Kuşçu and Sponza (2009) increased OLR from 0.31 to 3.25 kg COD m^{-3} d^{-1} and the methane concentration decreased from 47% to 31% in an anaerobic migrating blanket reactor treating a synthetic wastewater containing para-nitrophenol.

Configuration of a digester greatly influences the highest achievable organic loading rate, the extent of removal efficiency and biogas production yield as well as energy consumption rate. The anaerobic fluidized bed reactor (AFBR) was able to handle the highest OLR of thin stillage (29 kg COD m^{-3}d^{-1}) among other digesters (Andalib et al., 2012). In the AFBR, small inert particles are used as a medium for bacterial attachment, which are kept suspended by upward liquid flow of wastewater (recycle ratio of 105 and recycle flowrate of 5.4m^3 d^{-1} which is approximately 320 L$_{Recycle}$ L$_{Reactor}$$^{-1}$ d^{-1}). However, the systems have some drawbacks namely difficulty in control of biolayer thickness and high energy consumption due to high recirculation ratio (Hamza et al., 2016). The recycling accounts for 10-13% energy of produced biogas (Heijnen et al., 1989). On the other hand, the highest COD removal from thin stillage was obtained in an anaerobic membrane bioreactor (AnMBR). Dereli et al. (2014) used an AnMBR for treating thin stillage under mesophilic condition. They obtained the COD removal efficiency of 99%. In AnMBR, the membrane enhances the solid-liquid separation inside the reactor which causes long SRT regardless of HRT. This feature makes AnMBR suitable for wastewater with a high solid content such as thin stillage. Using membrane in anaerobic system enhances sludge activity, reduces plant size and makes higher OLR achievable (Cicek et al., 1998). However, membrane fouling (precipitation of particulates on the membrane) and related operating costs and maintenance are the main barriers for widespread application of AnMBRs for wastewater treatment (Lin et al., 2013). In the mesophilic anaerobic digestion of corn-thin stillage in a continuous stirred tank reactor (CSTR), the OLR of range of 2.6 to 4.5 kg COD m^{-3}d^{-1} was applied and the COD removal efficiency of 84-86% was obtained

(Lee et al., 2011). In a CSTR, microorganisms are maintained suspended in the reactor with intermittent or continuous mixing which consumes a significant amount of energy. Moreover, it has the drawback of biomass washout from the reactor (Harrison et al., 1974). On the other hand, the comparison between the thermophilic (Schaefer and Sung, 2008) and mesophilic (Lee et al., 2011) anaerobic digestion of thin stillage in CSTRs indicates that there is not a considerable difference between their performance in the term of COD removal and methane yield.

In the case of anaerobic sequencing batch reactor (ASBR), the reactor achieved the COD removal efficiency and methane yield of 90% and 0.254 L CH_4 g^{-1} COD_{fed}, respectively, at OLR of 9.5 kg COD $m^{-3}d^{-1}$ under thermophilic condition (Agler et al., 2008). In ASBRs, both reaction and settling occur in one tank because of the sequential operation of these systems. Thus, high concentration of biomass in the reactor is achievable regardless of HRT. The ASBR is able to retain sludge because of granulation (Khanal, 2008). The problem is that self-immobilization does not provide good settleability. Furthermore, the reactor needs mixing to provide sufficient contact between sludge and wastewater which means more energy consumption. The benefits of using ASBR is operational simplicity, flexibility of use and high biogas yield (Ratusznei et al., 2000; Singh and Srivastava, 2011).

The COD removal efficiency and methane yield in the present study (hybrid ABR) are within the range of reported values in the literature for anaerobic digestion of thin stillage, however, the influent COD is lower than other studies due to filtration and dilution of thin stillage with tap water in this study. In the other studies, addition of trace element and

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dilution is applied as pretreatment of thin stillage. The OLRs applied in this study is not the maximum achievable OLR since the objective of this study was to investigate the treatability of thin stillage in a conventional and hybrid ABR and compare their performance with respect to phase separation, SRT, COD and sulfate removal and biogas production. Those parameters reveal the robustness and stability of the hybrid ABR since OLR is not a comprehensive indicator of reactor performance. Unlike other digesters mentioned above, ABR configuration can provide the phase separation which causes increased protection against toxic materials and fluctuations in pH and temperature (Zhu et al., 2015). Due to compartmentalized configuration of ABR, sulfate is mainly removed in the first compartment (Barber and Stuckey, 1999; Saritpongteeraka and Chaiprapat, 2008a) and as a result the hydrogen sulfate mainly exists in the biogas from the first compartment. The biogas needs to be desulfurizated before using to prevent damage to gas utilization units (Weiland, 2010). Therefore, considering the desulfurization cost per unit volume of biogas, less costly biogas treatment is needed for the produced biogas from ABR. Moreover, ABR is capable of underground installation (Deng et al., 2016) with low energy consumption but the drawback of pilot/full scale ABR is that the reactor should be shallow to maintain suitable gas and liquid upflow velocities. Also, controlling an even distribution of the influent is difficult (Barber and Stuckey, 1999).

Regarding the application of ABR for various feedstocks, the range of OLR and COD removal efficiency was 6-20 kg COD $m^{-3}d^{-1}$ and 54-97%, respectively (Akunna and Clark, 2000; Boopathy and Tilche, 1991; Grover et al., 1999; Hutňan et al., 1999; Zhu et

al., 2008). For the present study, the removal efficiency can be considered high in the range reported in the literature for digestion of different feedstock with ABR.

Furthermore, the thin stillage was filtered in this study to reduce solids content and prevent clogging the system. However, various solid concentration in wastewaters for treatment in ABRs are reported in the literature (8-24 g L^{-1} total solid) (Akunna and Clark, 2000; Zhu et al., 2008). Using an ABR for high solid wastewater (around 15%) results in the accumulation of solids in the 1st compartment which reduces hydrolysis rate due to the declined contact between microorganism and substrate. It also physically displaces the active biomass out of the reactor (Barber and Stuckey, 1999). Therefore, some modification such as increasing the volume of 1st compartment or agitation should be performed on ABR in order to handle high solid content wastewater (Boopathy and Sievers, 1991). In general, solid removal as pretreatment is a common practice in full scale wastewater treatment. In the case of thin stillage, solid recovered from thin stillage can be further dried for application as animal feed. For solid separation from thin stillage in industrial scale, various methods such as clarifying agents, size-exclusion medium, and fermentative coagulation can be used (Ratanapariyanuch et al., 2017).

3.4.6 Sulfate removal

The sulfate removal efficiency in different configurations/stages is presented in Table 3-5. Sulfate removal efficiency in stage II was higher than stage I in the conventional ABR. It can be mainly due to the higher overall HRT in the stage II since a higher HRT results in an increase of the sulfate removal efficiency (Saritpongteeraka and Chaiprapat, 2008a). In the hybrid ABR, the sulfate removal efficiency was higher than that in the conventional

ABR. The modified configuration in the hybrid ABR allows for higher concentration of sulfate reducing bacteria resulting in higher sulfate removal compared to conventional ABR. In the hybrid ABR, the effluent concentration of sulfate did not change considerably in response to increasing OLR from 1.5 to 3.5 kg COD m^{-3} d^{-1}. This shows that the system can handle higher input concentration of sulfate.

Table 3-5: Sulfate removal in various Configuration/Stage of ABR

Configuration/Stage	OLR (kg COD m^{-3} d^{-1})	Feed concentration (mg L^{-1} SO_4)	Effluent concentration (mg L^{-1} SO_4)	Removal efficiency %
Conventional ABR/ Stage I	1.1	145±23	48±8	67±11
Conventional ABR/ Stage II	1.8	249±10	59±2.4	76±3.1
Hybrid ABR	1	462±2	29±1.4	93.7±4
	1.5	747±3	41±0.2	94.5±0.4
	2	954±7	49±0.3	94.9±0.7
	2.5	1190±7	49±0.3	95.9±0.6
	3	1396±20	55±0.8	96.1±1.3
	3.5	1832±33	59±1.1	96.8±1.8

Saritpongteeraka and Chaiprapat (2008a) also used an ABR for treatment of high sulfate rubber latex wastewater. They applied the OLR of 0.66 to 4.43 kg COD m^{-3} d^{-1} and they obtained the sulfate removal efficiency of 97 to 87%. In their study, the influent concentration of sulfate was 1800 mg L^{-1} approximately and the effluent concentration varied between 78 to 206 mg L^{-1} depending on the OLR. The sulfate removal efficiency from thin stillage and its response to the change in OLR has not been addressed to the best of our knowledge in the literature.

3.4.7 Nitrogen and phosphorus removal

The anaerobic digestion of thin stillage is not able to reduce the nutrient in thin stillage efficiently (Wilkie et al., 2000). However, nitrogen and phosphorus can be removed through struvite precipitation in anaerobic digestion of thin stillage (Agler et al., 2008; Dereli et al., 2014). The amount of total phosphorus (TP), total nitrogen (TN) and ammonia were measured in the hybrid ABR. The change in the concentration of TP, TN and ammonia in the effluent was 145-383, 253-550 and 231-415 mg L^{-1}, respectively when the OLR changed from 1 to 3.5 kg COD m^{-3} d^{-1}. The ammonia concentration can have an inhibitory effect on the anaerobic digestion process. However, the inhibitory concentration range (3000-5000 mg L^{-1}) of ammonia (Duan et al., 2012; Van Velsen, 1979; Yenigün and Demirel, 2013) is considerably higher than the concentration in this study. During the operation of system in this study, the presence of a significant amount of white solids in buffer tank, effluent tank and recycle tube was observed. The test results of elemental analysis and total nitrogen were consistent with the structure of struvite. The result of phosphorus removal in the present study (37-59%) is comparable with phosphorus removal of 68% in the study of Andalib et al. (2012) on treatment of thin stillage in an AFBR. Usually, in a full scale wastewater treatment plant, a struvite recovery unit including a crystallizer reactor is installed to recover phosphorus from the effluent of anaerobic digestion (Desmidt et al., 2015).

3.5 Conclusions

A novel ABR was developed and its performance was compared with the conventional ABR for bioenergy production from thin stillage. The significantly improved robustness

and performance of the novel ABR was attributed to the enhanced phase separation, reduced washout and increased SRT. The hybrid ABR was very efficient in the treatment of thin stillage and achieved high COD and sulfate removal efficiency. Future research must focus on the optimization of reactor with respect to highest achievable OLR and investigating the effect of hydraulic retention time and recycle ratio. Due to the biogas production and the geometry of the 45 degree baffle, there would be mixing beneath the baffle. The extent of mixing, however should be investigated through trace study and CFD simulation. As a future plan, the hydrodynamics and mixing condition will be investigated through CFD simulation.

3.6 Acknowledgements

CHAPTER 4   EVALUATING THE POTENTIAL OF A NOVEL ANAEROBIC BAFFLED REACTOR FOR ANAEROBIC DIGESTION OF THIN STILLAGE: EFFECT OF ORGANIC LOADING RATE, HYDRAULIC RETENTION TIME AND RECYCLE RATIO

This chapter has been published in journal of *Renewable Energy*:

4.1   Abstract

Anaerobic digestion of thin stillage in a novel anaerobic baffled reactor (ABR) was evaluated with respect to the selected operating conditions including organic loading rate (OLR), hydraulic retention time (HRT) and recycle ratio (RR). The hybrid ABR achieved the chemical oxygen demand (COD) removal, sulfate removal and methane yield of 92.5-78.9%, 97-93% and 305-275 mL CH_4 g^{-1} $COD_{removed}$, respectively at OLR of 3.5-6 kg COD m^{-3} d^{-1}, HRT of 20-11.7d and RR of 15. However, the COD and sulfate removal and methane yield did not change significantly at the RR range of 10-20 and OLR of 3.5 kg COD m^{-3} d^{-1} (HRT of 20d). Results showed that, increasing RR from 10 to 20, increased the contribution of later compartments to COD removal from 9% to 16%. On the other hand, the composition of VFA changed in response to the change in OLR. The removal of

nitrogen and phosphorus from thin stillage digestate was around 37% and 49% in the novel ABR, respectively due to struvite precipitation. Struvite precipitation from the effluent of novel ABR with the addition of magnesium led to further nitrogen and phosphorus removal of 44% and 81%, respectively, indicating the potential of digestate for nutrient recycling.

4.2 Introduction

Anaerobic digestion has gained a lot of attention due to its advantages over aerobic treatment. It is more cost effective compared to aerobic treatment since it does not need aeration and produces a small amount of excess sludge. More importantly, anaerobic digestion generates methane as an energy source (Lavagnolo et al., 2018; Wang et al., 2018b).

Thin stillage is an aqueous by-product from the distillation of ethanol in corn bioethanol plants. A common method for treatment of thin stillage is based on evaporation and drying, which is energy intensive (Khalid et al., 2011). The presence of high organic materials as well as micro and macro nutrient makes thin stillage a suitable feedstock for anaerobic digestion and biogas production (Alkan-Ozkaynak and Karthikeyan, 2011). The traditional treatment method of thin stillage treatment can be replaced with anaerobic digestion to remove the organic material from thin stillage and to enhance the energy balance of the plant since the produced biogas from anaerobic digestion can be used for power generation (Wilkie et al., 2000).

Thin stillage has been treated in various types of anaerobic digesters including anaerobic fluidized bed reactor (AFBR), continuous stirred tank reactor (CSTR), anaerobic membrane bioreactor (AnMBR), anaerobic sequencing batch reactor (ASBR) and

anaerobic baffled reactor (ABR) with a COD removal range of 82 to 99% and organic loading rate (OLR) range of 2.9-29 kg COD m^{-3} d^{-1} (Agler et al., 2008; Andalib et al., 2012; Dereli et al., 2014; Lee et al., 2011; Sayedin et al., 2018; Schaefer and Sung, 2008). Among these reactors, the conventional anaerobic baffled reactor (ABR) can provide a two phase system due to its compartmentalized configuration (Fang, 2010a) and has been evaluated for treating various wastewater such as raw municipal wastewater (Hahn and Figueroa, 2015), baker's yeast manufacturing wastewater (Pirsaheb et al., 2015), sweet potato starch wastewater (Xu et al., 2017), alkali-decrement wastewater of polyester fabrics (Yang et al., 2018a), high sulfur rubber latex wastewater (Saritpongteeraka and Chaiprapat, 2008a), whisky distillery (Akunna and Clark, 2000), soybean protein processing (Zhu et al., 2008) and pulp and paper mill black liquor (Grover et al., 1999). The most important features of ABR configuration are the long solid retention time (SRT) (612 to 42 days according to Grobicki and Stuckey (1991)) and improved stability in response to fluctuation in OLR due to compartmentalized configuration (Jürgensen et al., 2018).

In addition to the configuration of ABR, the operating parameters such as hydraulic retention time (HRT) and recycle ratio (RR) can considerably affect the performance of ABR. Faisal and Unno (2001) studied the effect of HRT on the performance of a five-compartment ABR and showed a decrease in COD removal efficiency from 95.3% to 77.3% and an increase in effluent volatile fatty acids (VFA) from 608 to 1430 mg L^{-1} when HRT decreased from 10 to 3 days (OLR of 1.60–5.33 kg COD m^{-3} d^{-1}) in treatment of palm oil mill wastewater. In their study, the methane yield remained almost constant at 0.38 L CH$_4$ g^{-1} COD $_{removed}$ in response to the change in HRT. Rongrong et al. (2011) optimized

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operating parameters such as alkalinity of influent (500-2000 mg L^{-1} $CaCO_3$), RR (94-219) and HRT (3-7 d associated with the OLR of 1.92 to 4.48 kg COD m^{-3} d^{-1}) to maximize the removal efficiency of polyvinyl alcohol (PVA) and COD from textile wastewater in an ABR. Optimal COD and PVA removal of 42.0% and 18.0% were achieved, respectively, at HRT of 5d and RR of 94. Saritpongteeraka and Chaiprapat (2008a) studied the effect of HRT and RR on the COD and sulfate removal from rubber latex wastewater in an ABR. Changing HRT from 10 to 1.25 days (OLR of 0.66 to 4.43 kg COD m^{-3} d^{-1}) resulted in a decrease in COD removal from 82 to 67% and a decrease in the sulfate removal from 96.2 to 88% and the methane yield changed from approximately 0.32 to 0.08 L CH_4 g^{-1} COD $_{removed}$. Moreover, in their study, the RR was changed from 0 to 0.5 at the HRT of 1.25d and in response to the change, the COD removal efficiency declined slightly from 67% to 63.3%. However, the sulfate removal efficiency did not change significantly with the RR. Sayedin et al. (2018) investigated the treatability of thin stillage in a conventional and hybrid ABR and compared their performance with respect to phase separation, SRT, COD and sulfate removal and biogas production. However, the effect of operating condition on the performance of the hybrid ABR for treatment of thin stillage and biogas production have not been investigated to date. Additionally, the potential for the recovery of phosphorus and nitrogen in the form of struvite from thin stillage digestate has not been fully explored.

The objective of this study was to develop an understanding on the effect of HRT, OLR and RR on the performance of the novel hybrid ABR for anaerobic digestion of thin stillage with respect to sludge washout, sulfate and COD removal efficiency and biogas

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production rate. The effect of operating condition on phosphorus and nitrogen removal was also investigated in this study. Moreover, the potential of further nutrient recovery in the form struvite recovery was explored.

4.3 Materials and Methods

4.3.1 Start-up and operation of hybrid ABR

Diluted thin stillage was fed to the conventional ABR at the stage of startup. Then, in an attempt to enhance the performance and robustness of the reactor, the conventional ABR was modified to a novel hybrid ABR. The modifications include installing solid/liquid/gas separators and baffles to reduce the biomass washout from the reactor which improved the performance of the reactor significantly (Sayedin et al., 2018). Full detail of the reactor configuration, characteristics of the feedstock as well as the operating parameters during the start-up are described in chapter 3. In the previous chapter, the OLR adjusted in the range of 1 to 3.5 kg COD m^{-3} d^{-1} by diluting the thin stillage with water while in the current study, non-diluted thin stillage was used. In this work, the operation in hybrid ABR started with OLR of 3.5 kg COD m^{-3} d^{-1} and recycle ratio (RR) of 20. Then, at the same OLR and HRT, the RR changed to 15 and 10 in order to study the effect of RR on the performance of the hybrid ABR. For investigating the effect of HRT and OLR, the OLR increased by steps of 0.5 kg COD m^{-3} d^{-1} to OLR of 6 kg COD m^{-3} d^{-1} by increasing the feeding flowrate from 1.38 L d^{-1} to 2.36 L d^{-1}.

Acidification may occur in anaerobic digestion process because of the accumulation of VFA, which results in a pH drop and process failure (Chua et al., 1997). To prevent the

acidification, the pH was routinely controlled by addition of $NaHCO_3$. The ratio of VFA/TA (Total alkalinity) is used as a criterion of system stability so that the ratio of 0.1–0.25 is desirable without the risk of souring, whereas the ratio beyond 0.3–0.4 indicates instability (Khanal, 2008; Li et al., 2014; Nigam and Pandey, 2009). Therefore, the OLR increased incrementally to avoid acidification and to promote the granular sludge formation. After each incremental increase in OLR, the VFA and alkalinity were monitored routinely and when the VFA/TA ratio and COD removal efficiency did not change over time, the system was considered stable. Then, the operating parameters such as COD and sulfate removal, biogas production and composition, individual VFAs, nitrogen and phosphorus were measured.

4.3.2 Liquid digestate characterization

4.3.2.1 pH, volatile fatty acids and alkalinity

Samples were collected from each compartment of the hybrid ABR and pH, VFA and alkalinity were measured. A titration method (Anderson and Yang, 1992) was used for measuring the alkalinity and total VFA of samples applying 0.05M sulfuric acid (EMD, ACS grade) as titrant. The pH of liquid digestate inside the reactor was measured using a VWR Symphony benchtop pH meter. It is well known that the individual VFAs are the key intermediates in the metabolic pathway of methane formation which makes it necessary to examine them during the process of anaerobic digestion (Wang et al., 1999). To determine the individual VFAs (i.e. acetic, propionic and butyric acid), the pH of 1 mL filtered samples (with 0.2 μm syringe filters) were adjusted to 2 by addition of concentrated

sulfuric acid (EMD, ACS grade) and then was mixed with 1 mL of diethyl ether (Anachemia) for 1 min (Manni and Caron, 1995) which resulted in a two-phase solution. The VFA content of supernatant (organic phase) was analyzed using a gas chromatograph (Agilent 7890) using a HP-PLOT/U column (dimensions 30 m × 0.32 mm and 10 μm film thickness). For the analysis, a flame ionization detector with the temperature of 200 °C was used. The injection temperature was set at 180 °C. The injection volume was 2 μL. The column temperature was constant at 125 °C for 1 min, then it increased 10 °C min^{-1} to 190 °C and remained at 190 °C for 15 min. Helium was used as the carrier gas with split ratio of 10.

4.3.2.2   *Elemental and chemical analysis*

The liquid digestate from each compartment and effluent was collected at various HRT and RR and its characteristics such as COD, sulfate, total nitrogen (TN), total phosphorus (TP) and ammonia were determined by HACH analysis kit, and UV-vis spectrophotometer (DR6000, HACH). Inductively coupled plasma optical emission spectrometry (ICP-OES) was used at the Minerals Engineering Center at Dalhousie University (Halifax, Nova Scotia, Canada) to determine the concentration of metals in the digestate. For sample preparation, 6 mL HCl and 2 mL HNO$_3$ were added to 1 mL of sample and digested in situ in hotblocks at 80 °C for 15 minutes. Then the digested solution was made up to 40 mL with deionized water.

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4.3.3 Analysis of precipitants

Elemental analysis of precipitants was conducted at the Minerals Engineering Center at Dalhousie University (Halifax, Nova Scotia, Canada) using ICP-OES. For the sample preparation, 6 mL of HCl and 2 mL of HNO_3 was added to the precipitants, and digested at 80 °C for 30-60 minutes. Deionized water was added to the digested material and made up to 50 mL. Moreover, the nitrogen content of precipitants was determined by dissolving 15 mg of precipitants in 100 mL deionized water (which is lower than the solubility of struvite at 25 °C (169 mg L^{-1})) and using the ammonia HACH test kit to measure ammonia in the resulting solution (Bhuiyan et al., 2007). Struvite has NH_4^+ in it, therefore, the molar concentration of ammonia should be roughly equal to the molar concentration in the precipitates (Wang et al., 2005). X-ray diffraction (XRD) was performed with Rigaku miniflex 600 using Cu Kα radiation at 15mA and 40 kV with step-scanning in the range of 10–50 °2θ at a rate of 5 °min^{-1} (step width 0.05 2θ).

4.3.4 Struvite recovery experiment

The struvite crystals were formed in the digestate of the reactor. For additional removal of the nutrient in the form of struvite, the digestate was collected at OLR of 5.5 kg COD m^{-3} d^{-1}. 160 mL was filtered with 0.45μm filter paper. The amount of ammonia, total phosphorus and Mg in filtered digestate was measured. 50 mL of filtered digestate was added to three 50 mL vials. Based on the amount of Mg and TP, an appropriate amount of $MgSO_4.7H_2O$ was added to each vial to provide the approximate P:Mg molar ratio of 1:1. After 10 days, the digestate in the three vials was filtered with 0.45 μm filter paper and the amount of produced struvite was determined with drying at 40 °C for 24 h (Ali, 2007).

(Ali, 2007)The concentration of ammonia and total phosphorus and Mg was measured in the resulting filtrate as well as precipitants.

4.3.5 Biomass sampling and analysis

The biomass washout was measured by taking samples from the effluent of hybrid ABR. Also, for determining the biomass concentration inside the reactor, the samples were taken from the top, middle and bottom of the sludge blanket. The biomass concentration of samples were measured by VSS test based on standard method (Eugene et al., 2012).

Visual features of the anaerobic granules were studied using scanning electron microscopy (SEM), which was conducted in the Department of Process Engineering and Applied Science at Dalhousie University (Halifax, Nova Scotia, Canada). For sample preparation, a sample of the sludge granules from the middle of sludge blanket in each compartment was taken and washed three times with 0.1 M phosphate buffer (pH 7.4). The samples were fixed with 2.5% glutaraldehyde solution (pH 7.2–7.4) for 4 h at 4 °C and then rinsed again six times with 0.1 M phosphate buffer. Sample dehydration was performed using 50%, 60%, 70%, 80%, 90%, 95% and 100% ethanol (15 min at each ethanol concentration) and air drying. The samples were sputter coated with gold and the morphology of granules was observed by a scanning electron microscope (Hitachi, S-4700 equipped with an Oxford Inca Energy Dispersive X-ray analysis system and a HKL Electron Backscatter Diffraction System). Moreover, ImageJ software (http://rsb.info.nih.gov/ij/) was used to analyze the size distribution of sludge granules.

4.3.6 Biogas collection and measurement

25L Tedlar® gas bags were used for collection of produced biogas from each compartment. The period of gas collection was 18 hours and the water displacement method (Kafle and Kim, 2013) was applied to measure the stored biogas in the bags. The concentration of CH_4 and CO_2 in the gas was measured with a gas chromatograph (GC, 490 Micro GC, Agilent Technologies) including thermal conductivity detectors (TCDs) and a 10-metre PPU column. In the analysis, the carrier gas was Helium, the columns temperature was 80 °C and the temperature of injector was 110 °C.

4.4 Results and Discussion

4.4.1 Biomass concentration, granular size distribution and washout

Sludge washout is one of the main drawbacks of conventional ABRs especially at low HRTs and high upflow velocities, which can lead to lower biomass concentration and consequently lower stability, COD removal and biogas production (Barber and Stuckey, 1999; Jürgensen et al., 2018; Zinatizadeh et al., 2017). The design of hybrid ABR in this study allows for higher sludge retention time and biomass concentration compared to conventional ABRs resulting in better performance. In the present work, the effect of decreasing HRT (increasing OLR) and increasing the RR (i.e. increasing upflow velocity) was investigated for the novel hybrid ABR.

Table 4-1 shows the average biomass concentration at various operating conditions. Biomass concentration did not change considerably during the operation of hybrid ABR and remained at the narrow range of 15.33-15.78 g VSS L^{-1} (Table 4-1). As Table 4-1

shows, the biomass washout decreased from 3.08±0.10 to 2.38±0.05 g VSS d^{-1} when the RR reduced from 20 to 10 (the statistical analysis is provided in Appendix E). Therefore, based on the biomass washout, the solid retention time (SRT) was 137, 146 and 181 days at RRs of 20, 15 and 10, respectively. This was expected since higher RR results in higher hydrodynamic shear (upflow velocity) and mixing which causes more biomass washout from the reactor. Table 4-1 also indicates that the biomass washout increased when the HRT in hybrid ABR decreased from 20d to 11.7d (OLR increased from 3.5 to 6 kg COD m^{-3} d^{-1}), which is associated to higher upflow velocity and biogas production at higher OLRs, resulting in higher amount of sludge washout from the hybrid ABR (statistical analysis is shown in Appendix E). The SRT for the hybrid ABR decreased from 146d to 60d when the OLR increased from 3.5 to 6 kg COD m^{-3} d^{-1} at RR of 15. The calculated SRT values are comparable with the study of Grobicki and Stuckey (1991), which was concerned with the treatment of a synthetic feed using a 4 compartments-ABR. In their study, the OLR of 1.2 to 4.8 kg COD m^{-3} d^{-1} (HRT of 80 to 20h) was applied (without recycle) and the SRT of 612 to 42 days was achieved (sample calculation of SRT can be found in Appendix D).

Table 4-1: The biomass washout and concentration at various operating conditions

OLR (kg COD m^{-3} d^{-1})	HRT (d)	RR	Upflow velocity (cm h^{-1})	Biomass concentration (g VSS L^{-1})	Biomass washout (g VSS d^{-1})	SRT (d)
3.5	20	20	5.8	15.33±0.38	3.08±0.1	137±8
3.5	20	15	4.4	15.56±0.28	2.94±0.1	146±7
3.5	20	10	3.0	-	2.38±0.05	181±7
4	17.5	15	5.0	15.78±0.40	3.34±0.08	130±6
4.5	15.6	15	5.6	15.61±0.56	3.94±0.03	109±5
5	14	15	6.3	-	4.85±0.14	89±6
5.5	12.7	15	6.9	-	5.78±0.31	74±6
6	11.7	15	7.5	15.32±0.44	7.05±0.21	60±3

Another important parameter which allows for higher SRT and biomass concentration in the hybrid ABR is granulation (Pol et al., 2004). The following graph shows the size distribution of granules in different compartments of the hybrid ABR, which was collected at the OLR of 6 kg COD m^{-3} d^{-1}. As can be seen, the first compartment contains more number of bigger granules (Diameter>1mm) compared to other compartment due to a higher rate of biogas production and mixing.
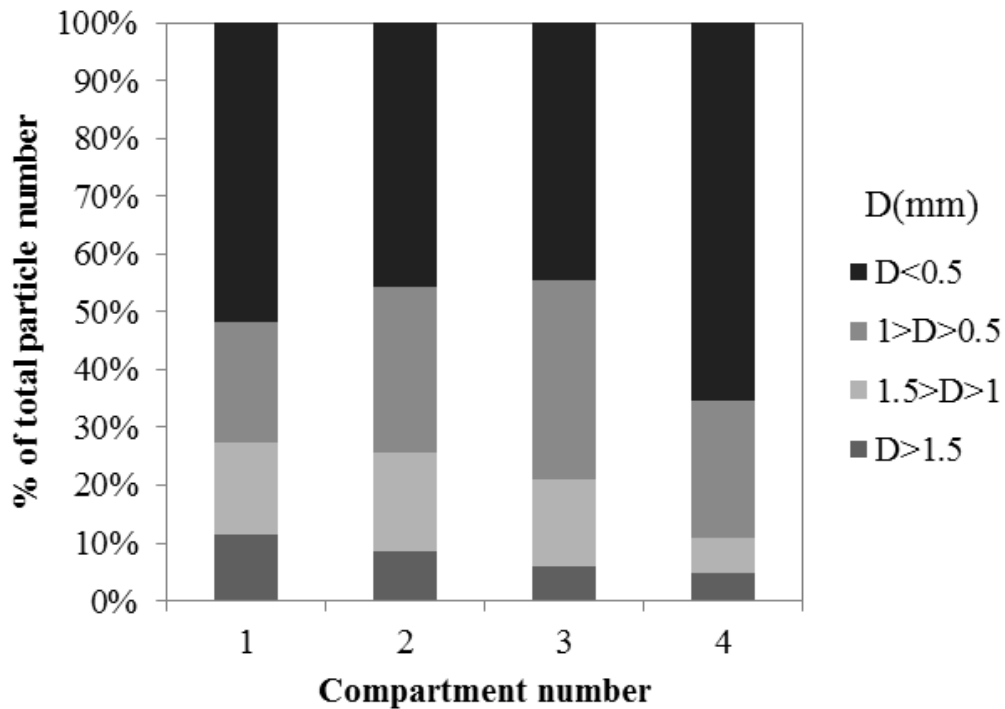
Figure 4-1: The size distribution of sludge granules in the hybrid ABR at OLR of 6 kg COD m^{-3} d^{-1}

The morphology of granules from the hybrid ABR is shown in Figure 4-2. The granules have become dense with spherical shape due to the hydrodynamic shear force caused by the upflow liquid and biogas (Najafpour et al., 2006). It is suggested that the cavities on the granule surfaces were used as the transporting channel of gases, substrate,

or metabolites (Lim and Kim, 2014). Based on the granule composition as proposed by MacLeod et al. (1990), the small rod shape bacteria on the surface of granules are acidogens, which conduct the initial degradation of biopolymers (MacLeod et al., 1990).
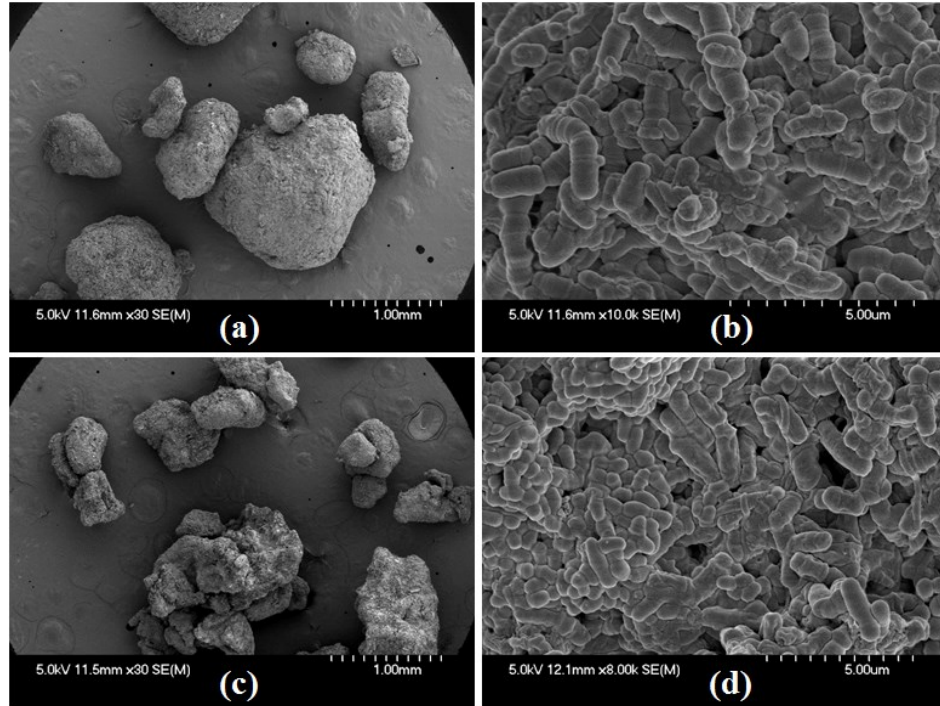
Figure 4-2: Scanning electron microscope (SEM) images of granules from the hybrid ABR (a) and (b) 1st compartment, (c) and (d) 2nd compartment

4.4.2 Phase separation

The single phase reactors such as upflow anaerobic sludge blanket (UASB) reactor, AFBR or CSTR have been widely used in treatment of different wastewaters. However, two-phase reactors such as ABR have gained a lot of attention due to high robustness and enhanced bacterial activity (Ahamed et al., 2015). The activity of microorganism is a strong function of pH. The configuration of ABR provides a gradient of pH and VFA concentration along the reactor resulting in separation of acidogenesis and methanogenesis

longitudinally down the ABR. In fact, the first compartment of ABR is usually dominated by acidogens with the optimal pH of 5 to 6.5, while the microbial population of methanogens increased in the later compartments where the pH ranging from 6.6 to 7.5 (Zhu et al., 2015). The configuration of hybrid ABR allows for higher biomass retention time and less biomass washout (less transfer of microbial population from one phase to another) compared to the conventional ABR resulting in better phase separation.

The profile of VFA in the hybrid ABR is shown in Figure 4-3a and Figure 4-3b. The figures illustrate the phase separation in the system showing that the 1st compartment has highest VFA concentration and lowest pH among the compartments whereas the 4th compartment has the lowest VFA concentration and consequently highest pH. The difference between the compartments creates different environmental conditions (phases), which makes it favorable for a specific type of bacteria (e.g. acidogens or methanogens).
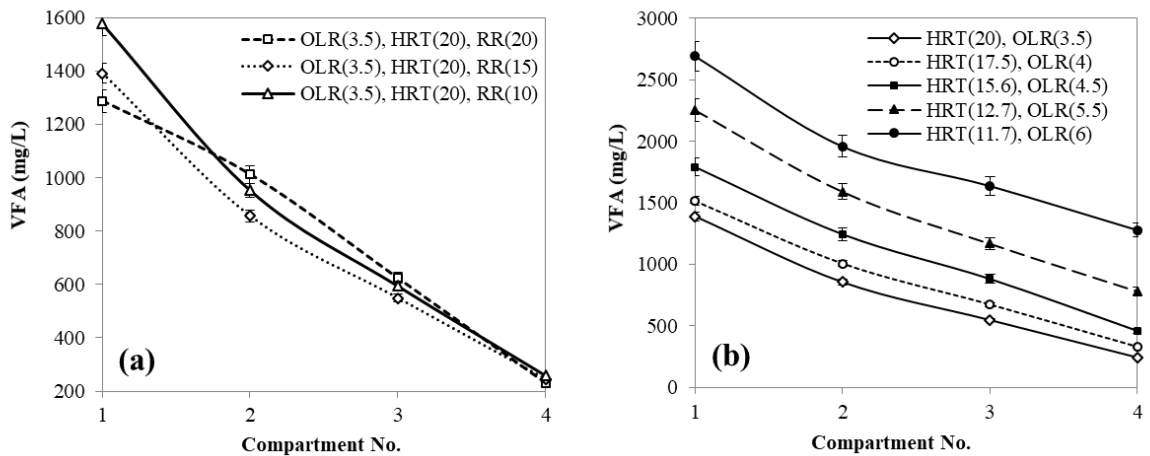
Figure 4-3: Variation of VFA in all compartments of hybrid ABR (a) various RRs with HRT of 20d and OLR of 3.5 kg COD m^{-3} d^{-1} (b) various HRTs (days) and OLR (kg COD m^{-3} d^{-1}) with RR of 15

As the Figure 4-3a shows, the change in the RR ratio has highest influence on the VFA concentration of 1st compartment compared to other compartments. No significant change is observed in the VFA concentration of 4th compartment. The difference between the VFA concentration in the 1st and last (4th) compartment decreased from 1320 to 1057 mg L^{-1} when the RR increased from 10 to 20. Therefore, the average concentration of VFA in the reactor decreased in response to the increase in the RR. It is beneficial to the process since the stability of anaerobic digestion depends on the VFA/TA ratio and lower VFA leads to lower VFA/TA ratio and more robust process (Li et al., 2014). With lower VFA, higher OLR for the reactor is achievable due to reduced risk of acidification. The change in the VFA gradient followed the same pattern in the study of Saritpongteeraka and Chaiprapat (2008a) so that the VFA gradient (difference between the concentration of VFA in the first and fourth compartments) changed from 180 to 142 mg L^{-1} in response to an increase in RR from 0 to 0.5 in a four-compartment ABR. Also, in their study, no considerable change was observed in the VFA concentration of 4th compartment at different RRs. Therefore, the lower RR augments the VFA gradient in the hybrid ABR as shown in Figure 4-3a.

As Figure 4-3b depicts, the VFA concentration in all compartments increased in response to the decrease in HRT. The VFA gradient increased from 1147 to 1413 mg L^{-1} when the HRT decreased from 20 to 11.7d. The comparison between Figure 4-3a and Figure 4-3b indicates that the RR has less effect on the VFA concentration in the last compartment compared to the effect of HRT. The rise in the VFA concentration in response to the decrease in HRT is mentioned in several studies (Akunna and Clark, 2000; Pirsaheb et al., 2015). The reason lies in that at low HRT (high OLR), production rate of VFAs is

generally higher than their biodegradation rate, which is more pronounced at lower HRT (Kuşçu and Sponza, 2009).

Besides total VFA, the concentration of individual VFAs such as acetic, propionic and butyric acids can also be considered as an early indicator of process failure (Buyukkamaci and Filibeli, 2004). It is suggested that the propionic to acetic acid ratio greater than 0.7 shows the instability of process (Marchaim and Krause, 1993). Figure 4-4 shows the composition of VFA in each compartment at different operating conditions. During the whole experiment, the ratio of propionic to acetic varied from 0.33 to 0.6. As can be seen in Figure 4-4b, the composition of VFA did not change significantly in response to the change in RR from 10 to 20. However, the ratio of butyric acid to propionic acid in the first compartment increased from 0.9 to 1.5 when the OLR increased from 4 to 6 kg COD m^{-3} d^{-1} at the recycle ratio of 15. Also, acetic acid concentration in the first compartment did not change considerably from OLR of 5 to 6 kg COD m^{-3} d^{-1}. This phenomenon can be explained by the theory of McCarty and Mosey (1991) in which the microbial population combat the high acidity with production of butyrate instead of acetic or propionic acid. This phenomenon observed in the study of Siles et al. (2007) on anaerobic digestion of wastewater derived from the pressing of orange peel generated in orange juice production. Nachaiyasit and Stuckey (1997a) had the same observation in anaerobic treatment of a synthetic wastewater (carbohydrate-protein) in an ABR.
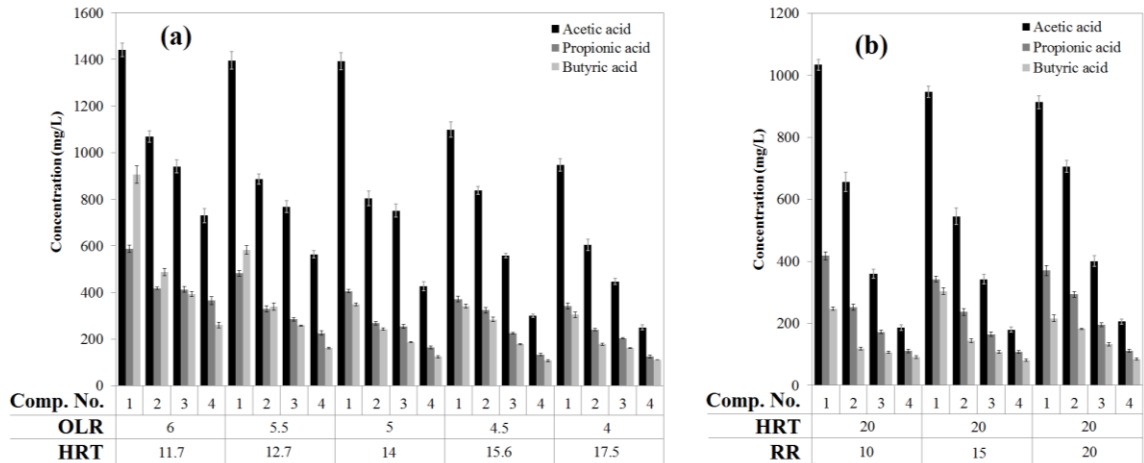
Figure 4-4: Individual VFA concentrations in each compartment of ABR at (a) various OLR (kg COD m^{-3} d^{-1}) and HRT and constant RR of 15 (b) various RR and constant HRT of 20d and OLR of 3.5 kg COD m^{-3} d^{-1}

4.4.3 COD removal and biogas production

In this study, the COD removal efficiency increased slightly from 91.2 to 93.5% when the RR decreased from 20 to 10 (Figure 4-5a). This trend was also observed in the study of Nachaiyasit and Stuckey (1995) in the case of anaerobic digestion of a synthetic carbohydrate (sucrose)-protein substrate using an ABR. In the study, the COD removal efficiency decreased from 98% to 96% when the RR increased from 0.1 to 2. The reason lies in the fact that when the RR increases, the reactor approaches the completely mixed system, which leads to lower mass transfer driving force for COD removal (Barber and Stuckey, 1999). The effect of HRT on COD removal efficiency is also shown in Figure 4-5a. As can be seen, the removal efficiency decreased from 92.5% to 78.9% when the HRT decreased from 20d to 11.7d. The reduction in COD removal as a result of a decrease in HRT is also reported in the literature (Marin et al., 2010; Pirsaheb et al., 2015; Polprasert et al., 1992). Pirsaheb et al. (2015) showed that the COD removal efficiency from a high

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strength baker's yeast manufacturing wastewater in an ABR decreased from 94.3% to 78% when the HRT decreased from 6 to 2 days. The RR did not have any significant effect on the biogas production rate or methane yield. However, the biogas production rate increased and the methane yield decreased when the HRT reduced from 20 to 11.7d and OLR increased from 3.5 to 6 kg COD m^{-3} d^{-1} due to the increase in the feeding flowrate (Figure 4-5b).
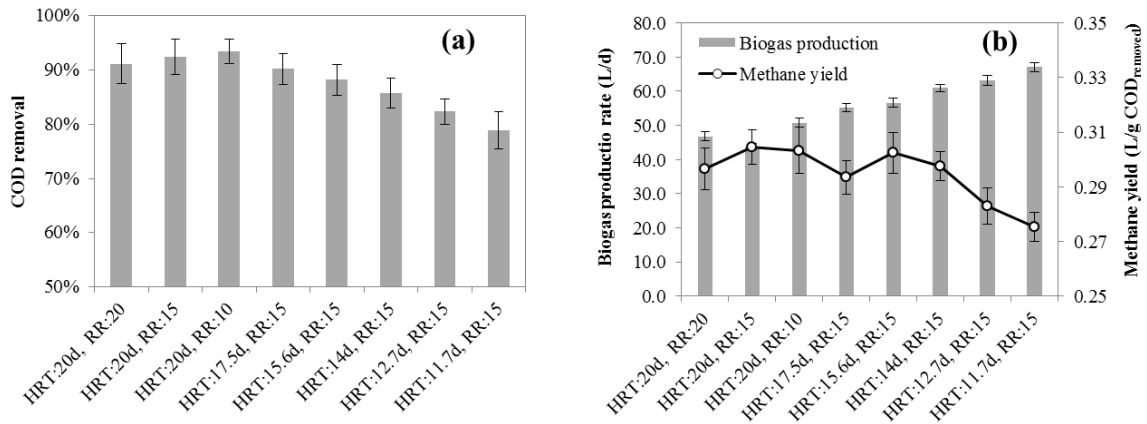
Figure 4-5: The variation of (a) COD removal efficiency and (b) biogas production and methane yield with operating conditions

Unlike total COD removal efficiency, the contribution of each compartment to COD removal (Figure 4-6a) and the biogas production of each compartment (Figure 4-6b) changed with RR considerably. As can be seen in Figure 4-6a, the COD removal contribution of different compartments became closer together at higher RR compared to the lower RR. In fact, increasing the RR decreased the COD gradient along the reactor which caused the performance of hybrid ABR approaches a completely mixed system (Barber and Stuckey, 1999) and therefore the COD removal efficiency for each compartment became closer together. Based on the Monod kinetic, the substrate (COD)

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consumption rate by microorganism is proportional to the substrate concentration. At higher RR, the later compartments in hybrid ABR received higher concentration of COD (Figure 4-6a) and consequently had higher contribution to total COD removal (21% and 16% in 3rd and 4th compartment at RR of 20) in comparison with lower RR (17% and 9% in 3rd and 4th compartment at RR of 10). At the lower RR (e.g. 10), the performance of ABR was more similar to a plug flow reactor. Moreover, the recycle ratio had the highest impact on the COD removal contribution of 1st and last (4th) compartment. At the RR of 20, the 1st and 2nd compartment had almost the same contribution to COD removal while the difference between the contribution of those compartments increased in response to the decrease in RR. The decrease in COD gradient along ABRs in response to an increase in RR was also shown in the study of Saritpongteeraka and Chaiprapat (2008a) on treatment of rubber latex wastewater in an ABR when they changed the RR from 0 to 0.5 at the fixed HRT of 1.25d. The operating condition such as RR can be adjusted to optimize the reactor design. For example, if the 4th compartment of the ABR is removed at RR of 20 and OLR of 3.5 kg COD m^{-3} d^{-1}, the reactor will lose 16% of its capability to remove COD. However, this lost will be less (9%) at RR of 10 and OLR of 3.5 kg COD m^{-3} d^{-1} (mass balances for COD are provided in Appendix B).

The biogas production rate in each compartment (Figure 4-6b) also followed the same trend as COD removal in each compartment (Figure 4-6a) so that the biogas production rate in the 1st compartment increased with a decrease in RR. However, the CH_4 yield was almost constant at 0.30 L CH_4 g^{-1} $COD_{removed}$ in all RRs, studied.

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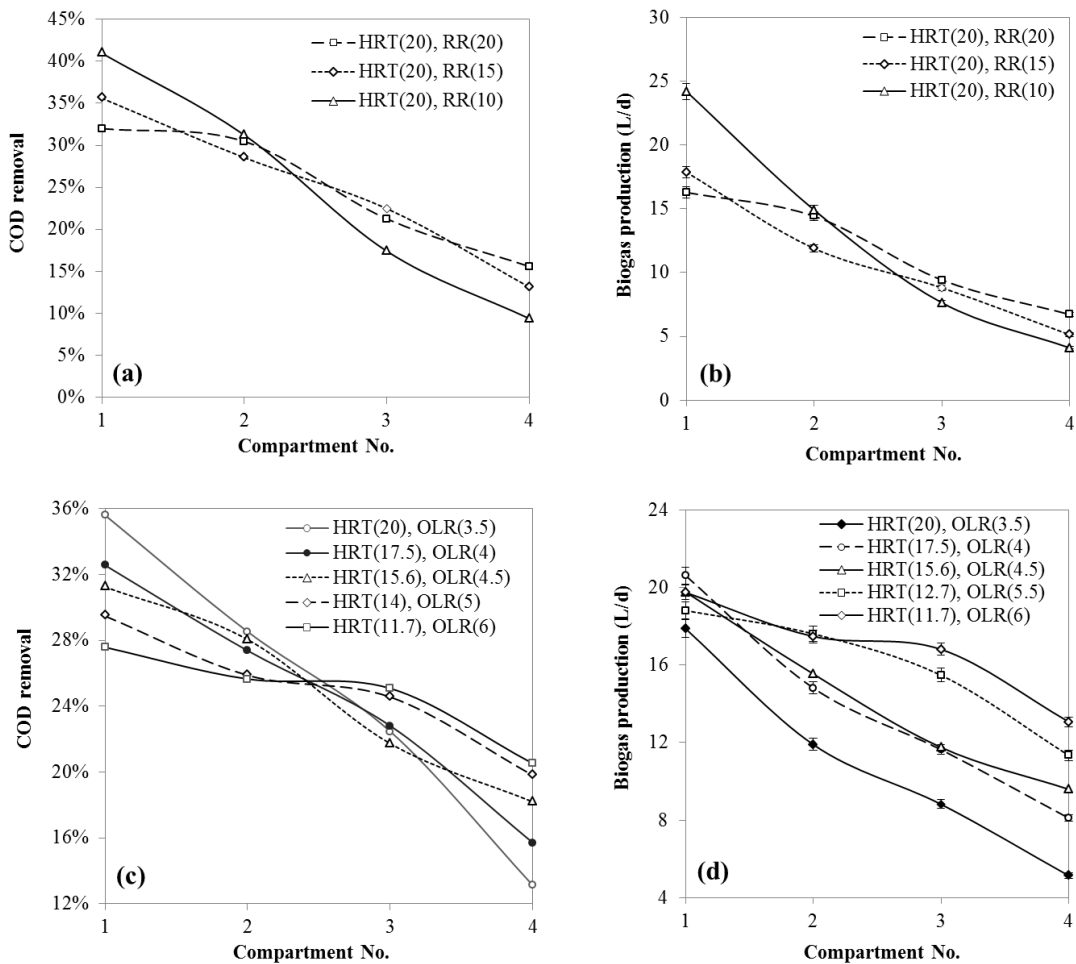
Figure 4-6: (a) The COD removal and (b) biogas production rate at different RR. (c) The COD removal and (d) biogas production rate at different HRTs (days) and OLR (kg COD m^{-3} d^{-1})

The effect of HRT on the profile of COD removal and biogas production is also shown in Figure 4-6c and Figure 4-6d, respectively. As can be seen, the contribution of compartments to total COD removal became closer together when HRT decreased. The contribution 1st and 4th compartment to COD removal was 36% and 13%, respectively at the HRT of 20d while it changed to 28% and 21% at the HRT of 11.7d. The same observation is reported in the study of Cao and Mehrvar (2011) on slaughterhouse

wastewater treatment using a 5 compartment-ABR. In their study, the highest COD and total organic carbon (TOC) removal happened in the first compartment in all HRTs. In their study, the contribution of last three compartments at higher HRT (HRT of 3.8d and OLR of 0.2 kg TOC m^{-3} d^{-1}) was insignificant while the role of the compartments in COD removal increased when the HRT decreased to 0.9d (OLR of 1.1 kg TOC m^{-3} d^{-1}).

Thin stillage has been treated in different digesters. Table 4-2 compares various anaerobic digesters and their performances treating thin stillage. According to Table 4-2, the AFBR was able to handle the highest OLR of thin stillage (29 kg COD $m^{-3}d^{-1}$) at HRT of 3.5d among other digesters (Andalib et al., 2012) at the expense of high energy consumption due to high recirculation ratio (Hamza et al., 2016). The highest COD removal from thin stillage (99%) was obtained in an AnMBR (Dereli et al., 2014) nevertheless, membrane fouling and associated operating costs are the main barriers for widespread application of AnMBRs for wastewater treatment (Lin et al., 2013). In the mentioned works for AnMBR and AFBR, the effect of operating condition such as HRT and RR on COD removal efficiency was not studied. The lowest COD removal efficiency among different digesters belongs to CSTRs. In the mesophilic anaerobic digestion of corn-thin stillage in a CSTR, the OLR of range of 2.6 to 4.5 kg COD $m^{-3}d^{-1}$ (HRT of 40 to 20) was applied (without any recycling) and the COD removal efficiency of 84-86% was obtained (Lee et al., 2011). Also, in a thermophilic CSTR (Schaefer and Sung, 2008), a similar COD removal efficiency (83-84%) to the mesophilic CSTR at the OLR range of 3.4-6.4 kg COD $m^{-3}d^{-1}$ (HRT of 30-15 days) was achieved. However, the digester failure occurred for the thermophilic CSTR at HRT of 12d and OLR of 7.3 kg COD $m^{-3}d^{-1}$ when

the VFA concentration and VFA/TA ratio reached 4200 mg L^{-1} and 0.84, respectively. ASBR achieved the COD removal efficiency and methane yield of 90% and 0.254 L CH$_4$ g^{-1} COD$_{fed}$, respectively, at OLR of 9.5 kg COD m^{-3}d^{-1} and HRT of 10d under thermophilic condition (Agler et al., 2008).

Table 4-2: Comparison of anaerobic treatment various feedstock with different reactors

Feedstock	Reactor	Volume (L)	Pre-treatment	Influent COD (g L^{-1})	OLR (kg COD m^{-3}d^{-1})	COD removal (%)	HRT (d)	RR	CH$_4$ yield	Ref
Thin stillage	ABR[M]	27	Addition of NaHCO$_3$, filtration and dilution	70	3.5-6	79-94	11.7-20	10-20	0.27-0.31 [b]	Present study
Thin stillage	CSTR[M]	18	Addition of trace element	105-131	2.9-4.5	84-86	25-40	0	0.68-0.77[a]	(Lee et al., 2011)
Thin stillage	CSTR[T]	10	Ultrasonic pre-treatment and Addition of trace element	96-102	3.4-6.4	83-84	15-30	0	0.73-0.69[a]	(Schaefer and Sung, 2008)
Thin stillage	ASBR[T]	5	Addition of trace element	74-100	9.5	90	10	0	0.28[b]	(Agler et al., 2008)
Thin stillage	AFBR[M]	17.6	No pretreatment.	130	29	88	3.5	105	0.34[b]	(Andalib et al., 2012)
Thin stillage	AnMBR[M]	10	Dilution	72	8.3	99	10-12	N.A.	0.26[b]	(Dereli et al., 2014)
Soybean protein processing	ABR[M]	80	Dilution with tap water and Addition of trace element	2-10	1.2-6	92-97	1.7	0	-	(Zhu et al., 2008)
Whisky distillery	ABR[M]	35	Dilution with tap water and neutralizing with NaOH	9.5	1-4.8	80-92	2-10	0	-	(Akunna and Clark, 2000)
Pulp and Paper Mill Black Liquor	ABR[M]	10	Dilution with tap water and neutralizing with HCL	4	2-5	68-70	2-5	0	0.10-0.13 [b]	(Grover et al., 1999)
baker's yeast manufacturing wastewater	ABR	14.5	No pretreatment.	16	2.5-7.5	78-94	2-6	22-140	0.46-0.39[b]	(Pirsaheb et al., 2015)
Rubber latex wastewater	ABR	23	pH adjustment with NaOH	6	0.7-4.7	83-67	1.25-10	0-0.5	0.29 [c, b]	(Saritpong teeraka and Chaiprapat, 2008a)
PVA-containing desizing wastewater	ABR	79	pH adjustment to 6.5-8	13.5	1.9-4.5	18-42	3-7	94-215	0.3 [d, b]	(Rongrong et al., 2011)
Palm oil mill wastewater	ABR	20	No pretreatment.	16	1.6-5.3	95-87	3-10	30	0.42-0.32 [b]	(Faisal and Unno, 2001)

[a] L CH$_4$ g^{-1} VS $_{removed}$, [b] L CH$_4$ g^{-1} COD $_{removed}$, [c] at the HRT of 10d, [d] at HRT of 5d, [M] Mesophilic, [T] Thermophilic

Unlike mentioned digesters above, conventional ABRs are able to provide two phase system allowing the acidogens and methanogens grow in their desirable condition. This causes enhanced protection against toxic materials as well as organic/hydraulic shocks (Zhu et al., 2015). The design of hybrid ABR in this study augments the phase separation due to the incorporation of solid/liquid/gas in each compartment and consequently lower rate of biomass washout. In the present study, the OLR range of 3.5 to 6 kg COD $m^{-3}d^{-1}$ (HRT of 11.7 to 20) was applied to a hybrid ABR as well as the RR of 10-20d and COD removal range of 79-94% was obtained which is comparable with the performance of other reactors for treating thin stillage. The effect of RR on the performance of various reactors treating thin stillage was not studied in the literature as shown in Table 4-2. However, in the most of mentioned reactors, an increase in OLR (decrease in HRT) resulted in a decrease in COD removal efficiency. In order to compare the results of current work with the other studies with respect to the operation of ABRs, the application of different ABRs for various wastewaters and their performance are also provided in Table 4-2. As can be seen in the table, the COD removal efficiency in ABRs decreased when the HRT decreased (OLR increased); however, the extent of response to a change in HRT is different depending on the type of wastewater. For example, when the HRT decreased from 10 to 3d (OLR of 1.6-5.3 kg COD m^{-3} d^{-1}) in the treatment of palm oil mill wastewater, the COD removal efficiency changed from 95 to 87% (Faisal and Unno, 2001) but when HRT was reduced from 7 to 3d (OLR of 1.9-4.5 kg COD m^{-3} d^{-1}) for treating PVA-containing desizing wastewater, the COD removal efficiency decreased from 42 to 18% (Rongrong et al., 2011). The decrease in the COD removal as a result of increase in RR is also mentioned

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in the literature (Pirsaheb et al., 2015; Rongrong et al., 2011; Saritpongteeraka and Chaiprapat, 2008a). Moreover, acceptable range of COD removal (68%-97%) with ABR was achieved in different studies without recycling the effluent (Akunna and Clark, 2000; Grover et al., 1999; Zhu et al., 2008).

4.4.4 Sulfate removal

Thin stillage contains a considerable amount of sulfate (2936±76 mg L^{-1}) which is originated from the addition of sulfuric acid for pH adjustment in bioethanol plants (Bajpai, 2013; Sayedin et al., 2018). In the present work, the sulfate removal efficiency in the hybrid ABR remained almost constant (97%) at different RRs, indicating that RR had no significant effect on sulfate removal efficiency at the RR range of 10-20 and sulfate loading rate (SLR) of 0.16 g L^{-1} d^{-1} and HRT of 20d (OLR of 3.5 kg COD m^{-3} d^{-1}). This behavior was also observed in digestion of rubber latex wastewater in an ABR in which the sulfate removal efficiency remained in the narrow range of 86.0–87.3% at RRs of 0, 0.3 and 0.5 and SLR of 1.34 g L^{-1} d^{-1} (HRT of 1.25d and OLR of 4.7 kg COD m^{-3} d^{-1}) (Saritpongteeraka and Chaiprapat, 2008a).

In the hybrid ABR, sulfate removal efficiency decreased from 97% to 93% in response to the decrease in HRT from 20 to 11.7d (SLR from 0.16 to 0.26 g L^{-1} d^{-1}). A higher HRT results in an increase in the sulfate removal efficiency (Saritpongteeraka and Chaiprapat, 2008a). This shows that the system can handle higher load of sulfate. The change in the profile of sulfate along the reactor (Figure 4-7) is also noticeable in which at the higher HRT (e.g. 20d) almost all the sulfate was removed in the first compartment while at lower HRTs (e.g. 11.7d), the other compartments started to play more role in sulfate removal.

The fact was also reported by different studies for treatment of sulfate containing wastewater in ABRs (Barber and Stuckey, 2000; Vossoughi et al., 2003). The effect of HRT on the sulfate removal from wastewater using ABR was also investigated in the study of Saritpongteeraka and Chaiprapat (2008a) in which sulfate was removed from a rubber latex wastewater (with approximate sulfate concentration of 1800 mg L^{-1}). They achieved the sulfate removal efficiency of 97 to 87% for the SLR of 0.2 to 1.34 g L^{-1} d^{-1} (OLR range of 0.66 to 4.43 kg COD m^{-3} d^{-1} and HRT of 10 to 1.25d). The effect of HRT and RR on the sulfate removal efficiency from thin stillage has not been addressed to the best of our knowledge in the prior literature.
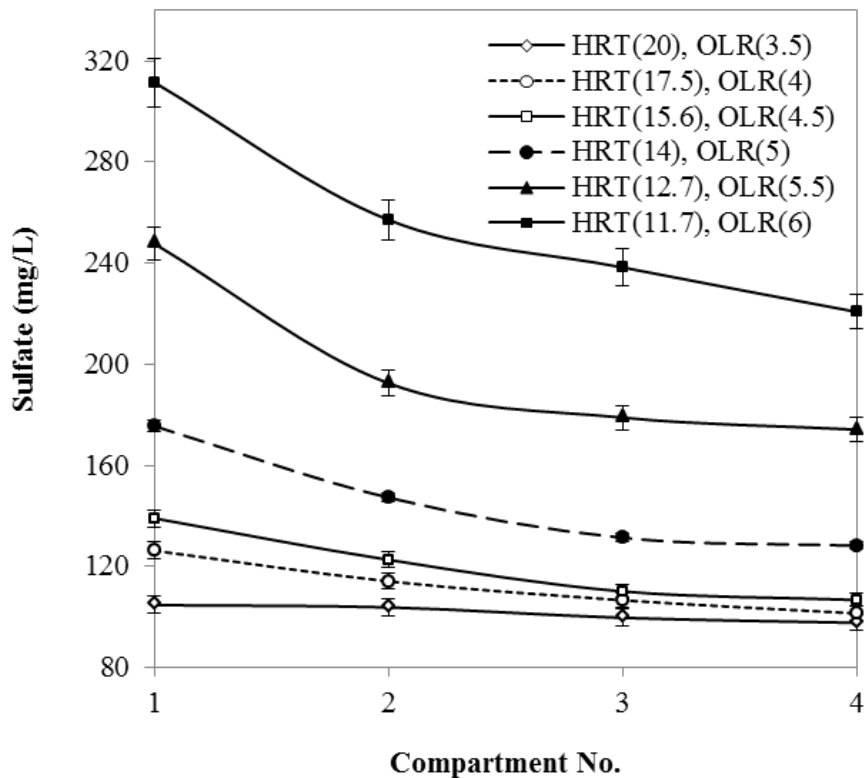
Figure 4-7: The profile of sulfate in the hybrid ABR at different HRT (days) and OLR (kg COD m^{-3} d^{-1}) and fixed RR of 15

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4.4.5   Nitrogen and phosphorus removal

The nutrient such as nitrogen and phosphorus cannot be effectively removed from thin stillage with anaerobic digestion (Wilkie et al., 2000). However, the formation and precipitation of struvite can result in the removal of nitrogen and phosphorus from the digestate, generating valuable fertilizer and reducing the environmental impact (Agler et al., 2008; Dereli et al., 2014; Jia et al., 2017). During the operation of hybrid ABR, the precipitates was taken and analyzed for the content of Mg, nitrogen and phosphorus. The Mg, nitrogen and phosphorus content of the precipitant were 10.8%, 5.8% and 12.4%, respectively which is roughly similar to the structure of struvite (Mg=9.7%, TN=5.7% and TP=12.6%). The phosphorus removal in this study (49%) can be compared with phosphorus removal (68%) in the study of Andalib et al. (2012) on treatment of thin stillage in an AFBR. Analysis of output effluent of ABR (initial concentration in Table 4-3) showed that the limiting species for further formation of struvite and removal of nutrient in the hybrid ABR is Mg. Therefore, to evaluate the possibility of further removal of nitrogen and phosphorus with struvite precipitation, Mg in the form of $Mg.SO_4.7H_2O$ was added to the digestate in a batch system. The results of experiments are shown in Table 4-3. As can be seen, almost 81% of TP and 44% of ammonia was removed from the effluent of ABR, which shows the high potential of nutrient recovery in the form of struvite. Moreover, the N:P mass ratio in digestate changed from 0.94:1 to 2.71:1.

Table 4-3: The characteristics of thin stillage digestate before and after struvite precipitation

Initial concentration (mg L^{-1})			Struvite production (mg L^{-1})	Final concentration (mg L^{-1})			TP removal	N-NH$_3$ removal
N-NH$_3$	TP	Mg		N-NH$_3$	TP	Mg		
478±11	508±5	0.7±0.01	3440±53	267±6	97±2	113±1	80.9±0.4%	44.2±1.3%

The mass of species present in the chemical structure of struvite is shown in Table 4-4. 50 mL of digestate was examined for its potential for struvite recovery and the total mass of precipitant was 171 mg. Based on the elemental analysis and the mass balance, it was confirmed that the majority of materials in the precipitant was struvite.

Table 4-4: Mass balance analysis of struvite precipitation

	$N-NH_3$	TP	Mg
Mass in 50 mL of digestate (mg)	23.9	25.4	0.03
Added Mg mass (mg)	-	-	22.2
Mass in 50 mL of filtrate (mg)	13.3	4.8	5.7
Mass in 171 mg precipitant (mg)	9.8	20.1	16.6
Mass in (filtrate+ precipitant) (mg)	23.1	25.0	22.3

The solids were also submitted for XRD analysis and based on the results, the precipitants were identified as struvite (Figure 4-8).

Moreover, the mass balance for the ammonia was done since the experiment occurred in a close system and the ammonia loss because of stripping was insignificant (Jia et al., 2017). Table 4-4 shows that the mass of input of elements are consistent with the mass of output (filtrate and precipitant). For example from 22.2 mg added mass of Mg, 16.6 mg (75%) recovered in the form of struvite and 5.7 mg (25%) remained in the filtrate. The Mg, TP and N contents of precipitant are consistent with those of struvite.

As shown in Table 4-3, the molar ratio of nitrogen to phosphorus (N:P) in effluent of ABR is 2.1:1 and the molar ratio in struvite is 1:1, therefore even with 100% removal of phosphorus from the effluent in the form of struvite, 248 mg L^{-1} $N-NH_3$ will remain in the digestate. It shows that further removal of ammonia is required. Also, the largest cost of chemical in this experiment and in conventional methods for struvite precipitation from

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wastewaters is the cost of Mg chemical (Lahav et al., 2013). Thus, cheaper sources of Mg such as seawater can be used for struvite recovery from the effluent of hybrid ABR (Lahav et al., 2013). A wide range of phosphorus removal efficiency (70-90%) from various anaerobic digestate with struvite precipitation is reported in the literature (Desmidt et al., 2015; Kataki et al., 2016; Wrigley et al., 1992).
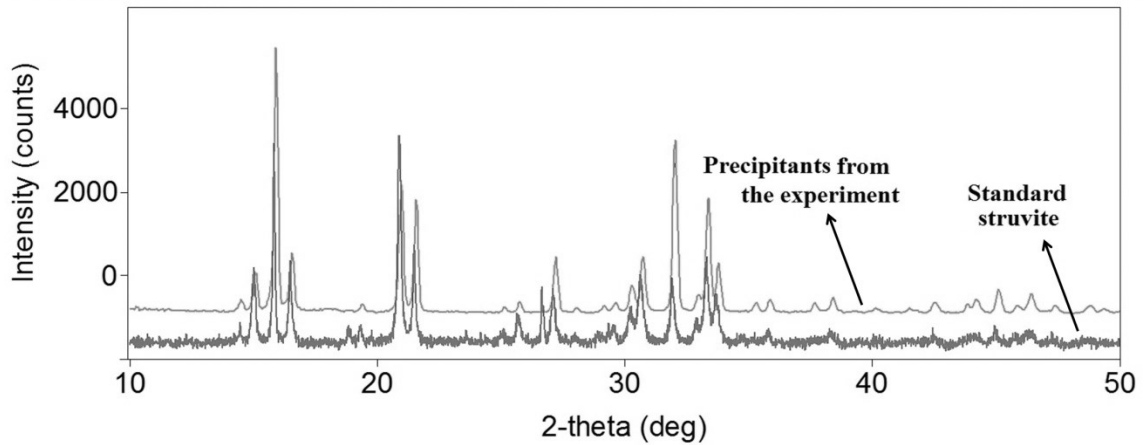
Figure 4-8: XRD pattern of the precipitants as compared to standard struvite

Different parameters such molar ratio of Mg:P and pH can affect the rate of precipitation. For example within the pH range 7.5 to 9.5, the higher rate of precipitation happens at pH range of 8.9-9.25 (Nelson et al., 2003) while the pH in this experiment was 6.9. Also, the effect of different ranges of Mg:P ratio such as 1:1-1.6:1 (Rahaman et al., 2008) and 1.5:1-3.6:1 (Quintana et al., 2005) is studied so that the higher ratio of Mg:P increases the precipitation rate whereas the molar ratio of Mg:P was adjusted to 1:1 at the beginning of this experiment. Therefore, the phosphorus removal efficiency can be increased by adjusting the pH of digestate to 9 using NaOH or by increasing the molar ratio

of Mg:P. However, both methods impose the cost of additional chemicals on the process of struvite precipitation.

4.5   Conclusions

In this study, the effect of RR and HRT on the operation and performance of a hybrid ABR was investigated, with respect to biomass washout rate, phase separation, COD and sulfate removal efficiency and biogas production. The results showed that the hybrid ABR could effectively treat thin stillage under different HRTs and RRs. Under the experimental conditions in this study, the highest COD and sulfate removal were 94% and 97% respectively at HRT of 20d and RR of 10. Decreasing the HRT and increasing the OLR, reduced the COD and sulfate removal efficiency of the hybrid ABR as well as methane yield. Even though increasing the RR slightly decreased the COD removal efficiency, it reduced the average concentration of VFA inside the reactor resulting in enhanced robustness of process. On the other hand, both RR and HRT could change the contribution of each compartment to COD removal. Removal of nitrogen and phosphorus up to 37% and 49% mainly in the form of struvite was observed in the reactor and the results showed that Mg was the limiting element for further formation of struvite and removal of nitrogen and phosphorus in the hybrid ABR. Further removal of nitrogen (44%) and phosphorous (81%) from effluent of hybrid ABR with Mg addition was achieved in a batch system. Recovery of struvite, which has application as fertilizer from the digestate of thin stillage will improve the economics of the corn bioethanol production process.

In order to identify the optimum operating conditions for the novel ABR, mathematical models such as anaerobic digestion model No. 1 (ADM1) can be applied to describe the

performance of system. The parameters of the model can be obtained through calibration with a set of experimental data from the ABR. For model validation, an independent set of experimental data should be compared with predicted values from the model. Moreover, the hydrodynamic flow patterns for the novel ABR as a function of RR and HRT can be studied by computational fluid dynamics (CFD) simulation.

Future research must focus on microalgae cultivation in digestate for nutrient recycling as the next step, following struvite recovery. Struvite recovery will result in reducing the concentration of ammonia and its subsequent inhibitory effect on microalgae. Moreover, the struvite recovery improved the nitrogen to phosphorus ratio for the growth of microalgae. For the future studies, a pilot scale of the novel ABR can be built in order to examine the effect of scale up on the performance of this reactor. The feasibility of using cheaper Mg sources such as seawater can be investigated, however, its effect on downstream process such as microalgae cultivation needs to be examined.

4.6 Acknowledgements

CHAPTER 5   MICROALGAE CULTIVATION IN THIN STILLAGE DIGESTATE FOR NUTRIENT RECOVERY AND BIOFUEL PRODUCTION

This chapter will be submitted to a journal to be considered for publication.

5.1 Abstract

Anaerobically-digested thin stillage, which is rich in nitrogen-ammonia (478±11 mg L^{-1}) and phosphorus (508±5 mg L^{-1}), offers great potential to be utilized as a source of nutrients for microalgae cultivation. However, the high concentration of ammonia is inhibitory to microalgal growth. In this study, ammonium present in the thin stillage digestate was partially recovered in the form of struvite to reduce the ammonia concentration to 267±13 mg L^{-1} and to improve the nitrogen to phosphorus ratio from 2.1 to 14.4 for microalgae cultivation. *Chlorella sorokiniana* in two times dilution of struvite-removed-digestate achieved a biomass concentration of 1.62±0.11 g L^{-1} and nutrient removal efficiencies of 95.3±1% (nitrogen) and 78.3±1.1% (phosphorous) at day 18. Protein, starch and lipid contents of *C. sorokiniana* biomass were 37.8±3.4%, 17.8±0.8% and 8.9±0.3% of dry weight, respectively at day 18. Moreover, a dramatic increase in genera of *Alcaligenes* and *Acinetobacter* (known as nitrifying bacteria) was observed in bacterial populations during algal cultivation.

5.2 Introduction

Microalgae as feedstocks for bioethanol production have gained a lot of attention due to increasing demand for clean energy sources. However, the production of biofuels from microalgae is currently not economically viable or sustainable due to the high-energy demand associated with their cultivation, harvesting and processing (Kermanshahi-Pour et al., 2013; Peng et al., 2018; Yeong et al., 2018). Another challenge with regard to microalgae cultivation, particularly in an open pond systems is the collapse of culture due to bacterial contamination. Process integration is considered as an opportunity to enhance the sustainability of microalgal biomass and product production (Kermanshahi-Pour et al., 2013). Integration of a microalgae-bioethanol production system within an existing corn-bioethanol plant may significantly reduce the capital and operating costs, enabling a more sustainable biofuel production process by nutrient recovery and bioproduct production through microalgae cultivation. In the integrated biorefinery, first, thin stillage, which is a liquid nutrient-rich byproduct of corn-ethanol plants is processed in an anaerobic digester to release the nutrients to soluble forms and to produce energy in the form of methane gas. The resulting digestate of thin stillage is still rich in organic carbon, nitrogen, and phosphorus, which can be used to cultivate microalgae. Starch-rich microalgae grown on digestate can be recycled back into the front-end of the plant without the need for an energy-intensive harvesting process to reduce reliance on corn as a feedstock. Mixing corn and starch-rich microalgae within an existing chemical plant eliminates the need for additional infrastructure for product recovery from microalgal biomass, and may result in significant savings in the capital and operating cost.

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The proposed process integration addresses the challenge associated with energy-intensive product recovery processes; another area considered to limit the commercialization of algal bioproducts (Alhattab et al., 2019). The high turbidity and ammonia concentration of digestate can adversely affect the growth of microalgae. The capability of microalgae species to grow on various digestates and their corresponding nutrient removal efficiency are different. Moreover, the tolerance of microalgae to toxic components in digestates, such as ammonia, is species-specific. A wide range of nutrient removal efficiencies from various digestates and wastewaters have been reported for different microalgae species and strains, indicating the importance of screening the optimum microalgae with respect to nutrient removal efficiency and growth rate for each specific wastewater (Cai et al., 2013a). Many studies used dilution to decrease the dosage of anaerobic digestate to reduce ammonia concentration and turbidity (Bohutskyi et al., 2016; Lizzul et al., 2014; Singh et al., 2011). However, the use of water for dilution must be minimized for economic and environmental reasons (Marcilhac et al., 2014). On the other hand, the unbalanced nitrogen to phosphorus (N/P) ratio (2.1) in the thin stillage digestate (based on thin stillage characteristics in chapter 3) might result in a low phosphorus removal efficiency by microalgae (Cai et al., 2013a; Marcilhac et al., 2015). The ratio should be close to its optimum (16) in the digestate to support microalgal growth and to ensure the highest removal efficiency of both nitrogen and phosphorus (Cai et al., 2013a; Xin et al., 2010). Applying pre-treatment methods such as filtration or autoclaving for removing bacterial contamination are costly, energy-intensive and complex; especially on a large scale (Zhu et al., 2013). On the other hand, the relationship between bacteria and

microalgae can be symbiotic or antagonistic and this relationship may be exploited to achieve higher removal efficiencies compared to the processes that rely solely on the utilization of single microalgae species (Ryu et al., 2014).

The main objective of this study was to examine the potential of thin stillage digestate as a microalgal growth media to remove nitrogen, phosphorus and organic carbon as well as to evaluate the potential of biomass for biofuels and bioproducts production. It was hypothesized that recovery of struvite from digestate would result in a more suitable media for microalgal growth due to a reduced ammonia concentration and subsequent reduction in the required dilution of digestate as well as an adjustment of the nitrogen to phosphorus ratio close to an optimum ratio for microalgal growth. A secondary objective was to develop insight into the evolution of the bacterial community present in the digestate during algal cultivation. To the best of our knowledge, thin stillage digestate has not yet been evaluated for microalgae cultivation and limited work has been done on the effect of struvite removal prior to nutrient recovery from digestate using microalgae. The significance of this work is the integration of microalgae cultivation within a corn-ethanol plant, which may offer great potential to reduce the environmental impact at the water-energy nexus.

5.3 Materials and Methods

5.3.1 Microalgae strains and pre-cultivation

At the preliminary screening stage, various microalgae strains, including *Tetraselmis suecica*, *Dunaliella tertiolecta, Spirulina platensis*, *Ankistrodesmus falactus* and

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Micractinium sp were cultivated in the anaerobic digestate of thin stillage but they were not able to survive. On the other hand, different strains of *Chlorella* and *Scenedesmus* are used in literature for nutrient removal from wastewaters and biofuel production (Abinandan and Shanthakumar, 2015; Arita et al., 2015; Chiu et al., 2015). Microalgae strains of *Chlorella sorokiniana* (UTEX 1230), *Scenedesmus obliquus* (UTEX 393) and *Chlorella saccharophila* (UTEX 27) were received from the Department of Oceanography at Dalhousie University, Halifax, NS, Canada and cultivated in sterilized Fritz freshwater f/2 medium (Fritz Industries, Inc.) in a growth chamber at 18 °C and 60 μmol m^{-2} s^{-1} light intensity with a 13/11 light/dark cycle.

5.3.2 Collection and characterization of anaerobic digestate

Anaerobic digestate (AD) was collected from the effluent of an anaerobic baffled reactor treating thin stillage. The characteristics of thin stillage and effluent and also the configuration and operating conditions of the anaerobic baffled reactor are discussed in detail in chapter 3 and chapter 4. In order to remove sediments, the digestate was centrifuged at 1800×g for 5 min.

5.3.3 Procedure for pretreatment and cultivation

Due to the high concentration of ammonia in the AD and its high turbidity, different pre-treatment methods including centrifugation, dilution and struvite recovery were applied to make the AD a more suitable medium for microalgae cultivation. For the struvite removal process, 4.23 g MgCl$_2$.6H$_2$O (Sigma–Aldrich) was added to each liter of digestate and after ten hours of mixing at 200 rpm, the mixture was centrifuged at 1800×g for 10

min to remove the struvite crystals from the digestate. Then, six different media were prepred: AD, two times dilution of AD (AD2X), five times dilution of AD (AD5X), struvite removed AD (SRD), two times dilution of struvite removed AD (SRD2X) and five times dilution of struvite removed AD (SRD5X). A series of screening experiments was performed by cultivating each microalgae strain in open 50 mL glass tubes using the media at room temperature (23±2 °C) and 210 μmol m^{-2} s^{-1} light intensity with a 13/11 light/dark cycle under mixing at 400 rpm. The cultivation conditions (light intensity and dark/light period) were chosen based on the literature in order to provide enough light for microalgae growth in digestate (Bohutskyi et al., 2016; Franchino et al., 2013; Marcilhac et al., 2014). The purpose of the screening phase was to identify the optimum microalgae strain and medium with respect to final biomass concentration and nutrient removal capacity. Since *C. sorokiniana* in SRD2X achieved the highest biomass concentration compared to other microalgae and media, *C. sorokiniana* and SRD2X were selected for further studies.

In another experiment, the color of SRD2X was further removed with chitosan flocculation and high speed centrifugation before the cultivation of *C. sorokiniana*. For high-speed centrifugation, the SRD2X was centrifuged at 15000×g and the supernatant was used for cultivation. Chitosan stock solution was prepared by dissolving 500 mg of chitosan (medium molecular weight, Sigma-Aldrich) in 50 mL of 0.1 M HCL. After 1 h, 50 mL of deionized water was added to the solution. The stock solution was added to the SRD2X until the chitosan dosage reached 200 mg L^{-1} followed by a rapid mixing at 200 rpm for 5min and a slow mixing step at 60 rpm for 55 min (Amuda and Amoo, 2007; Rizzo et al., 2008). Afterwards, the SRD2X was centrifuged at 1800×g and the supernatant was

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collected to be used as a medium for cultivation of *C. sorokiniana*. Considering the cost of color removal and a slight increase in the final biomass concentration in the color removed media, SRD2X without color removal was selected for the next phase.

Then, the optimum microalgae strain and medium (SRD2X) with respect to nutrient removal and biomass concentration (selected from the screening phase) was used in a 1 L glass bottle. The operating conditions (temperature, light intensity, mixing rate and light/dark cycle) for the 1 L experiment were as same as screening experiments, except that a continuous aeration rate of 0.01 vvm with 2% CO_2 was applied. The output gas from the photobioreactors was collected in 25 L Tedlar$^{®}$ gas sampling bags. In order to determine the volatilization of volatile fatty acids (VFAs) in the photobioreactor, a simulated digestate with the same concentration of VFA in SRD2X was prepared by adding 1.51 g acetic acid (Fisher, ACS reagent grade), 0.54 g propionic acid (Sigma, 99.5%), 0.37 g butyric acid (Alfa Aesar, 99%) and 7 g sodium bicarbonate (VWR Chemicals BDH, +97%) to 1 L of deionized water and tested for the same period of time. All experiments were performed in triplicate and un-inoculated medium for each experiment was used as controls.

5.3.4   Microalgal growth

Algal growth was determined using optical density (OD) at the wavelength of 680 nm. The OD of algae culture was calculated by subtracting the OD of a control (medium with no algae) from the OD of algae culture in the medium to account for the color of medium and the presence of bacteria. Moreover, the biomass concentration was measured using total suspended solids (TSS) according to standard methods (Eugene et al., 2012). Microalgal cell density was also determined under a light microscope (Helmut Hund

GmbH, Wetzlar, Germany) with a Neubauer haemocytometer (Bright-Line[TM] from Sigma-Aldrich).

5.3.5 Microbial identification

Biomass samples from the photobioreactors were collected during different stages of cultivation and processed by Integrated Microbiome Resource (IMR) at Dalhousie University (Halifax, Canada) for DNA extraction, sequencing, and analysis according to the procedure of IMR (www.cgeb-imr.ca). The detailed procedure can be found in chapter 3.

5.3.6 Analytical methods

The elemental analysis of biomass was performed at the Minerals Engineering Center at Dalhousie University (Halifax, Nova Scotia, Canada) using inductively coupled plasma optical emission spectrometry (ICP-OES) where 6 mL of HCl and 2 mL of HNO_3 were added to the biomass and digested at 80 °C for 30-60 minutes. For determining metals in the liquid samples using ICP-OES, 6 mL HCl and 2 mL HNO_3 were added to 1 mL of sample and digested *in situ* in hotblocks at 80 °C for 15 minutes. The NH_3-N, NO_2-N, NO_3-N, total phosphorus, sulfate and chemical oxygen demand (COD) were measured using HACH analysis kits, and a UV−VIS spectrophotometer (DR6000, HACH). The pH and dissolved oxygen of samples were determined by a VWR symphony pH meter and a Vernier Dissolved Oxygen Probe (DO-BTA), respectively.

The carbohydrate composition of microalgae was determined using the National Renewable Energy Laboratory (NREL) method for total carbohydrates in algal biomass

(Van Wychen and Laurens, 2016). In the method, the algal biomass was digested in 72% H_2SO_4 for 1 h at 30 °C followed by autoclaving of samples in 4% H_2SO_4 at 121 °C for 1 h. Then, it was neutralized and filtered through a 0.2 μm syringe filters. Different sugars in the filtrate were determined using a HPLC equipped with a refractive index detector (RID) and a Hi-Plex H column (300 × 7.7 mm; particle size, 8 μm, Agilent Technologies). In the method, the mobile phase was 5 mM H_2SO_4 and the flowrate was 0.4 mL min^{-1}. Additionally, the temperature of the column and detector was 35 °C according to Zaky et al. (2017). The same HPLC method and column were used to determine the concentrations of acetic, propionic and butyric acid in the media during the cultivation period.

For starch analysis, around 9 mg of freeze-dried biomass was added to 6 mL of sodium acetate buffer along with 15 μL of α-amylase. The temperature was kept at 55 °C while stirring. The glucose concentration of samples were measured everyday using 3,5-dinitrosalicylic acid (DNS) to determine reducing sugars until it reached a plateau (Kermanshahi-pour et al., 2014).

Lipid in the algal biomass was extracted using methanolic HCl *in-situ* transesterification (Tibbetts et al., 2017) and the resulting fatty acid methyl esters (FAMEs) were identified and quantified by a GC-FID equipped with a DB-23 capillary column (30 m × 320 μm). In the method, the carrier gas was helium and the flow rate was 3 mL min^{-1}. The inlet temperature was 250 °C and the oven temperature program was 110°C for 1 min, then increasing by 5 °C min^{-1} to 250 °C where the program was held for 20 min (total run time of 49 min). The detector temperature was 300 °C and the split ratio was 15.

Total nitrogen (N) content of biomass was determined on each sample by elemental analysis (950 °C furnace) using a Leco N analyzer (model FP-528, Leco Corporation, St. Joseph, MI) with ultra-high purity oxygen as the combustion gas and ultra-high purity helium as the carrier gas. Crude protein contents of the samples were then calculated using the generalized microalgae N-to-P conversion factor (N×4.78) (Tibbetts et al., 2015).

The CO_2 concentration in the output gas of the photobioreactors was determined by a gas chromatography (GC, 490 Micro GC, Agilent Technologies) including thermal conductivity detectors (TCDs) and a 10-metre PPU column. In the analysis, the carrier gas was helium, the columns temperature was 80 °C and the temperature of the injector was 110 °C.

5.4 Results and Discussion

5.4.1 Struvite recovery from digestate

The digestate samples were collected from the effluent of an anaerobic baffled reactor treating thin stillage (explained in chapter 4). The characteristics of digestate and struvite removed digestate (SRD) are shown in Table 5-1. The results show that the struvite recovery decreased ammonia, total phosphorus (TP), orthophosphate concentration and OD_{680} and increased the magnesium concentration, but did not have a significant effect on other parameters. In the process, the N/P molar ratio changed from 2.1 to 14.4, which became closer to the optimum ratio (16) for microalgae growth (Cai et al., 2013a; Li et al., 2011; Xin et al., 2010).

Table 5-1. Characteristics of digestate and struvite removed digestate (SRD)

Parameters	Digestate (mg L^{-1})	SRD (mg L^{-1})
NH$_3$-N	478±11	267±13
NO$_3$-N	N.D.	N.D.
NO$_2$-N	N.D.	N.D.
TP	508±5	41±3
PO$_4$-P	493±8	39±2
COD	9290±362	9130±329
Mg	31±0.4	174±2.3
Na	1151±13.7	1156±13.8
S	35±2	36±2
Ca	6.4±0.1	7.6±0.1
K	1771±25	1723±24
OD$_{680}$	1.437	1.213
pH	6.9±0.08	7.1±0.1

N.D. not detected

5.4.2 Microalgae screening

The growth of *Chlorella sorokiniana, Chlorella sacchrophila* and *Scenedesmus obliquus* in different media including two times and five times dilution of anaerobic-digestate (AD) and struvite-removed-digestate (SRD) are shown in Figure 5-1. The initial NH$_3$-N concentrations from each culture was measured and indicated for each culture in mg L^{-1} in Figure 5-1. None of the species could grow in AD (478±11 mg L^{-1} N-NH$_3$) or SRD (267±13 mg L^{-1} N-NH$_3$) without dilution, which is probably due to a high ammonia concentration and dark background color (data not shown). The lowest growth rate was observed in the case of AD2X (252±11 mg L^{-1}). The three media (AD, AD2X and SRD) with ammonia concentration higher than 252±11 mg L^{-1} did not show any considerable growth, indicating growth inhibition due to the high concentration of ammonium.

Bohutskyi et al. (2016) cultivated various species (*Chlorella sorokiniana, Chlorella vulgaris, Scenedesmus acutus f. alternans* and *Scenedesmus dimorphus*) in different dosages of the anaerobic digestion centrate in primary and secondary wastewater. They showed that when the dosage of anaerobic digestion centrate in secondary effluent was increased from 0 to 5-10%, the growth rate increased from 0.2-0.3 d^{-1} to 0.7-0.9 d^{-1}. However, when the dosage increased to 20%, the growth rate reduced to 0.6 d^{-1}. Therefore, the growth rate of each species depends on the dosage of anaerobic digestate in the medium and ammonia concentration (Singh et al., 2011; Uggetti et al., 2014). Uggetti et al. (2014) cultivated a mixed microalgal culture dominated by *Scenedesmus* sp. in anaerobic digestate from a wastewater treatment plant. They increased NH_4-N concentration from 50 to 260 mg L^{-1} by increasing the dosage of digestate and, as a result, the growth rate of microalgae decreased so that the highest growth rate was observed at the NH_4-N concentration of 50 mg L^{-1}. For *C. sorokiniana*, no inhibition was observed up to an NH_4–N concentration of 85 mg L^{-1} in anaerobic digested effluent from cattle manure (Kobayashi et al., 2013). Likewise, the biomass concentration of *C. sorokiniana* increased from 650 to 1950 mg L^{-1} when the dosage of anaerobic digestate of palm oil mill effluent decreased from 100% to 20% (Khalid et al., 2018) as shown in Table 5-2. Among the species in the present work, *Chlorella sorokiniana* showed the highest biomass concentration in all media (Figure 5-1). In order to reduce the inhibitory effects of digestate and make it more suitable for microalgae cultivation, various pre-treatments were applied to the digestate. As can be seen in Figure 5-1(a), (b) and (c), the struvite removed digestate was more suitable for microalgae growth compared to digestate with respect to biomass concentration, which can

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be due to the lower concentration of ammonia and optimum N/P ratio for microalgal growth. Of the examined microalgae species in this study, *C. sorokiniana* was the optimum candidate for the growth in these media and the optimum medium for its growth was SRD2X (statistical analysis is shown in Appendix E).
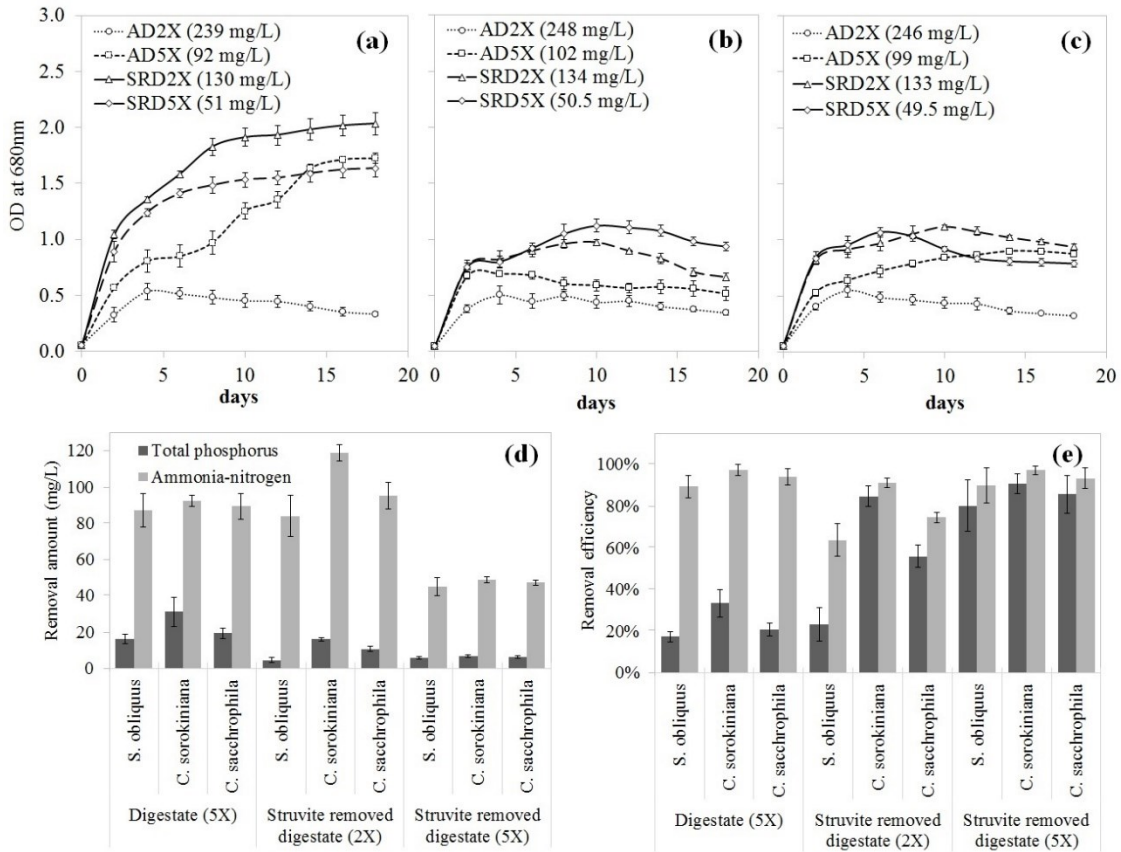
Figure 5-1. The optical density of (a) *C. sorokiniana* (b) *S. obliquus* (c) *C. Saccharophila* cultures during the cultivation time and their (d) ammonia and phosphorus removal amount and (e) removal efficiency

Figure 5-1(d) and Figure 5-1(e) show the nutrient removal amount and efficiency in different media. The comparison between AD2X and AD5X showed that all microalgae species had higher nutrient removal efficiency in more diluted digestates, which is probably

due to lower ammonia concentration and higher light penetration. On the other hand, comparing the nutrient removal efficiency between all three species in AD5X (N/P ratio 2) and SRD5X (N/P ratio of 14.5), shows that the microalgae could achieve higher removal efficiency in SRD5X. Since the same dilution is used for AD5X and SRD5X, the only difference is the pre-treatment by struvite recovery, which reduced the nitrogen-ammonia concentration and optimized the N/P ratio.

Besides the ammonia concentration, the N/P ratio also affects the growth rate and nutrient removal efficiency (Xin et al., 2010). To ensure a high growth rate and simultaneous removal of nitrogen and phosphorus, the N/P ratio should be within a proper range (Cai et al., 2013a). The Redfield ratio of N/P, based on microalgae cell composition, of 16 shows the faster removal rate of nitrogen than phosphorus (Cai et al., 2013a). Karapinar Kapdan and Aslan (2008) found a N/P optimal ratio of 17.7 for *Chlorella vulgaris* with respect to nutrient removal efficiency. The same optimal N/P range (11.1-17.7) is reported for *Scenedesmus* sp. for nutrient removal efficiency (Xin et al., 2010). Lee et al. (2013) used a consortium of microalgae including *Scenedesmus*, *Chlorella, Nitzschia*, and other filamentous microalgae and applied the optimum N/P ratio of 14 in the medium. The optimal N/P ratio of 12-17.7 is reported for freshwater microalgal growth in the literature (Cai et al., 2013a; Karapinar Kapdan and Aslan, 2008; Lee et al., 2013; Xin et al., 2010). The lower N/P ratio decreases the removal efficiency of phosphorus (Cai et al., 2013a; Marcilhac et al., 2015). This explains why the phosphorus removal efficiency of all species (17±3% for *S. obliquus*, 33±6% for *C. sorokiniana* and 21±3% for *C. saccharophila*) in AD5X (N/P of 2) was lower than the removal efficiency (80±6% for *S.*

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obliquus, 91±5% for *C. sorokiniana* and 86±4% for *C. saccharophila*) in SRD5X (N/P of 14.5).

Among microalgae species in this study, the highest and lowest nutrient removal efficiency from SRD2X were observed in the cases of *C. sorokiniana* and *S. obliquus*, respectively. The highest removal efficiency for all microalgae species with respect to ammonia and phosphorus was observed in SRD5X due to the lower initial concentration of both nitrogen and phosphorus and also the optimum N/P ratio (Marcilhac et al., 2015). However, to ensure the process is economical and feasible, the amount of water used for the process should be as small as possible (Marcilhac et al., 2014). Therefore, considering that the highest biomass concentration and highest removal amount of nutrient were achieved in the case of SRD2X amongst the experimental conditions evaluated and the fact that targeting lowest dilution is desirable, *C. sorokiniana* in SRD2X was selected for further investigation in a scaled-up process.

5.4.3   Color removal from struvite removed digestate

The results are shown in Figure 5-1, indicated that *C. sorokiniana* and SRD2X were the optimum species and medium, respectively, due to higher nutrient removal and biomass concentration. However, SRD2X medium is still dark (OD of 0.544 at 680 nm), which reduces the light availability for autotrophic growth of microalgae. On the other hand, more dilution with water for increasing light penetration brings about environmental and economic concerns (Marcilhac et al., 2014). Hence, other common practices for color removal in wastewater such as centrifugation or coagulation/flocculation can be used to

reduce the color of the medium. To investigate the effect of centrifugation on the color of medium and microalgae growth, the g-force of 15000 was selected. For coagulation/flocculation, different reagents such as metallic salts (as aluminium sulfate (alum), ferric chloride and ferric sulfate) are commonly used in wastewater treatment and color removal but the metal ions react with phosphate in the medium and precipitate and consequently alter the chemical composition of the medium (Verma et al., 2012). Therefore, using metal salts for coagulation/flocculation is not beneficial in the case of SRD2X medium in which the N/P ratio has been optimized by struvite recovery. Chitosan as a bioflocculant has gained a lot of attention since it is non-toxic, non-corrosive and does not change the chemical properties of medium (Verma et al., 2012). Therefore, chitosan was chosen as a bioflocculant reagent. The growth of *C. sorokiniana* in non-treated SRD2X, high-speed-centrifuged SRD2X, and chitosan-treated SRD2X are illustrated in Figure 5-2(a) and Figure 5-2(b). According to Figure 5-2, the final biomass concentrations in chitosan-treated-SRD2X and high-speed-centrifuged SRD2X were slightly higher than the concentration in non-treated-SRD2X. This was expected since the color of chitosan-treated-SRD2X (OD_{680} of 0.023) and high-speed-centrifuged-SRD2X (OD_{680} of 0.045) are much lower than the color of non-treated-SRD2X (OD_{680} of 0.544), which allows for better light penetration and reduces the effect of microalgal self-shading at high biomass concentrations. In all the measurements (the final biomass concentration, OD_{680} and cell count), the difference between color-removed-SRD2X and none-treated-SRD2X was significant (P-value<0.05). However, the difference between chitosan-treated-SRD2X and high-speed-centrifuged-SRD2X in the case of final biomass concentration or OD680 was

not significant (P-value>0.05). On the other hand, the final cell count for the chitosan-treated-SRD2X was higher than the count for the high-speed-centrifuged-SRD2X (P-value<0.05), which can be attributed to lower initial color of chitosan-treated-SRD2X (statistical analysis is shows in Appendix E). Cell counting is a better method in measuring the biomass growth when there is a change in the color of medium (Marcilhac et al., 2014). The ammonia-nitrogen removal efficiency in chitosan-treated-SRD2X, high-speed-centrifuged SRD2X and non-treated-SRD2X was 95.6±1.1%, 94.7±2.2% and 91.3±2.4%, and the phosphorus removal was 84.1±4.2%, 85.2±3.5% and 84.8±5%, respectively (Table 5-2).
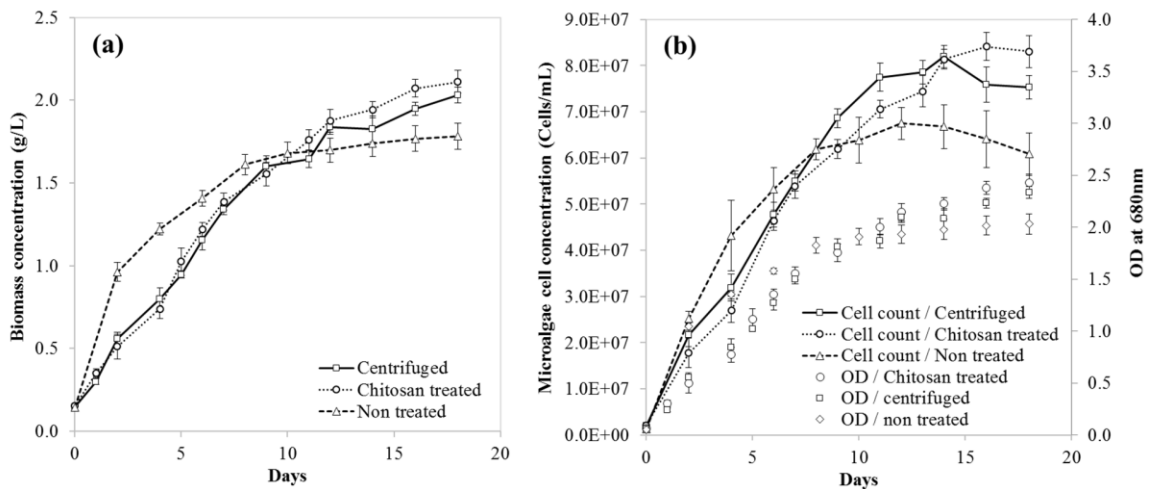
Figure 5-2. Changes in the cultures during the cultivation time (a) biomass concentration (b) cell concentration and OD$_{680}$

5.4.4   Cultivation of *C. sorokiniana* in struvite removed digestate

Considering the slightly improved biomass concentration of *C. sorokiniana* in high-speed-centrifuged and chitosan treated SRD2X compared to untreated SRD2X and the cost of color removal, the untreated SRD2X was selected for the 1 L experiment. Figure 5-3(a)

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and Figure 5-3(b) show the biomass concentration, OD and cell density during the course of the experiment. The biomass concentration of *C. sorokiniana* culture reached 1630 ± 110 mg L^{-1} at day 18. The culture was pale green after 1 day and became dark green after three days. The biomass concentration in this study is comparable with the reported values in the literature. As shown in Table 5-2, Ramsundar et al. (2017) cultivated *C. sorokiniana* on anaerobic centrate of municipal wastewater and reached the biomass concentration of 1080 mg L^{-1}. Kobayashi et al. (2013) grew *C. sorokiniana* CS-01, *C. sorokiniana* UTEX 1230, and *C. sorokiniana* UTEX 2714 in 10% anaerobic digestate of cattle manure and obtained biomass concentrations of 280, 280 and 150 mg L^{-1}, respectively.
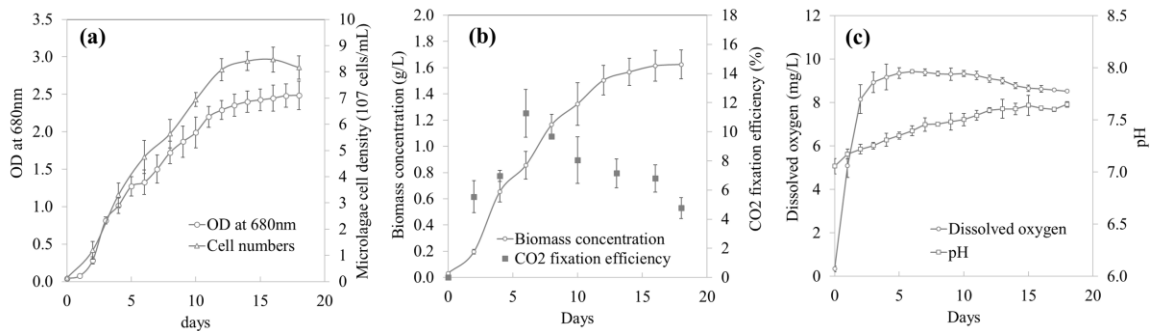
Figure 5-3. (a) Optical density and cell count (b) biomass concentration and CO$_2$ fixation efficiency (c) pH and dissolved oxygen during cultivation in two times dilution of struvite removed medium over 18 days

Figure 5-3(b) shows the CO$_2$ fixation efficiency during the experiment. Microorganisms such as microalgae uses CO$_2$ as a carbon source for photosynthesis. The CO$_2$ fixation by microalgae reduces the atmospheric CO$_2$ and it is advantageous for the human ecosystem. The CO$_2$ fixation efficiency can be determined with the following equation (Ho et al., 2013b):

$$\frac{Influent\ of\ CO_2 - Effluent\ of\ CO_2}{Influent\ of\ CO_2} \times 100$$

The efficiency is a function of various parameters such as CO_2 concentration, microalgae species, operating conditions and photobioreactor configuration (De Morais and Costa, 2007). In our study, the daily CO_2 fixation efficiency increased from 5.5±1.1% at day 2 to 11.3±1.6% at day 6 and then decreased again to 4.8±0.7% at day 18. Ho et al. (2013b) studied the CO_2 fixation efficiency by *Scenedesmus obliquus* CNW-N in which the efficiency increased to the maximum of 12% at day 4 and decreased again until the end of experiment. They fed 2.5% CO_2 into the culture continuously. De Morais and Costa (2007) cultivated different microalgae species in a 2 L vertical tubular photobioreactor with intermittent aeration at a rate of 0.3 VVM for 15 min every hour with 6% CO_2. They reported the CO_2 fixation efficiency of 9.3, 5.0, 6.3 and 5.5% for *Spirulina* sp., *S. obliquus*, *C. vulgaris* and *Chlorella kessleri*, respectively. The efficiency for these species decreased to 2.5, 1.0, 0.6 and 2.5% when the CO_2 concentration increased to 18%. Moreover, the growth rate in all species except *C. kessleri* decreased when the CO_2 concentration in the input increased from 6% to 18%.

The evolution of pH during the cultivation time is shown in Figure 5-3(c). The pH usually increases slightly during the growth process (Lizzul et al., 2014) which is attributed to the uptake of dissolved carbon species such as CO_2 by *C. sorokiniana* (Ramanna et al., 2014). This behavior is reported by various studies (Lizzul et al., 2014; Ramanna et al., 2014). Zheng et al. (2013) examined the effect of pH from 5 to 9 on the growth rate of *C. sorokiniana* and the optimum result was obtained at pH 7, which is close to the range of pH in the present work.

The change in the dissolved oxygen is illustrated in Figure 5-3 (c). High concentration of dissolved oxygen (100-400% air saturation) can inhibit photosynthesis but the inhibitory concentration is species dependent (Peng et al., 2013). The study of Ugwu et al. (2007) showed that the inhibition for *C. sorokiniana* happened at 200% air saturation while the maximum observed dissolved oxygen in the present work was 110% of air saturation at 22 °C. As can be seen in Figure 5-3(c), the initial amount of dissolved oxygen in the medium (Day 0) is insignificant, however, it started to increase and then decrease through the course of cultivation to reach an almost constant value (98% of air saturation). The amount of dissolved oxygen is linked to the rate of photosynthesis in which higher growth rate and photosynthesis increases the dissolved oxygen concentration (Peng et al., 2013). This phenomenon is also observed by Li et al. (2003) during the cultivation of *Dunaliella salina* in a synthetic medium.

The profile of nutrient removal is shown in Figure 5-4. As can be seen, most of N-NH$_3$ and TP removal happened during the exponential phase of growth and then reached a plateau. In the medium, ammonia was the main source of nitrogen since the concentration of nitrate and nitrite was below 0.3 and 0.6 mg L^{-1}, respectively. The growth of culture removed 95.3±1.0% of N-NH$_3$. During the experiment, the concentration of nitrate fluctuated from 0.778 to 1.73 mg L^{-1}. Due to the pH level (around 7), ammonia stripping was considered negligible (Marcilhac et al., 2014). Moreover, the mass balance between the nitrogen removal and the nitrogen content of biomass shows that most of nitrogen ended up in the biomass.

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The microalgae removed 78.3±1.1% of TP. Most of the TP is in the form of orthophosphate (as shown in Table 5-1) which makes the phosphorus available to microalgae compared to phosphorus pentoxide (Kobayashi et al., 2013). The high TP removal is because of the optimum N/P ratio in the medium. The pH of medium did not exceed 8, which reduces the possibility of TP removal due to precipitation (Li et al., 2011).

The concentration of COD decreased from 4540±135 to 736±50 mg L^{-1} and the removal efficiency was 83.8±0.6%. Gupta et al. (2016) cultivated *C. sorokiniana* on raw sewage (COD of 320 mg L^{-1}) and obtained the COD removal of 69.4% (Table 5-2). It can be assumed that *C. sorokiniana* consumed organic carbon. Some studies have shown the mixotrophic behavior of *C. sorokiniana* (Gupta et al., 2016; Lizzul et al., 2014). However, it is not clear that *C. sorokiniana* or bacteria consumed the COD. Therefore, in order to confirm the assumption of COD by *C. sorokiniana*, the time course concentration of volatile fatty acids was monitored in cultures (algae-bacteria) and control (bacteria) which is shown in Figure 5-4. The figure shows the synergy between bacteria and algae for removal of volatile fatty acids (organic carbon). According to the literature, in low nutrient media, bacteria and algae compete but in nutrient-rich environments they co-exist and support the growth of each other (Gupta et al., 2016). Figure 5-4 shows that the mixed culture of *C. sorokiniana* and bacteria removed all the acetic acid and propionic acid in 5 days and butyric acid in 4 days. However, in control (only bacteria), it took 8 days to consume all the volatile fatty acids. This shows the role of *C. sorokiniana* in the removal of volatile fatty acids and COD.
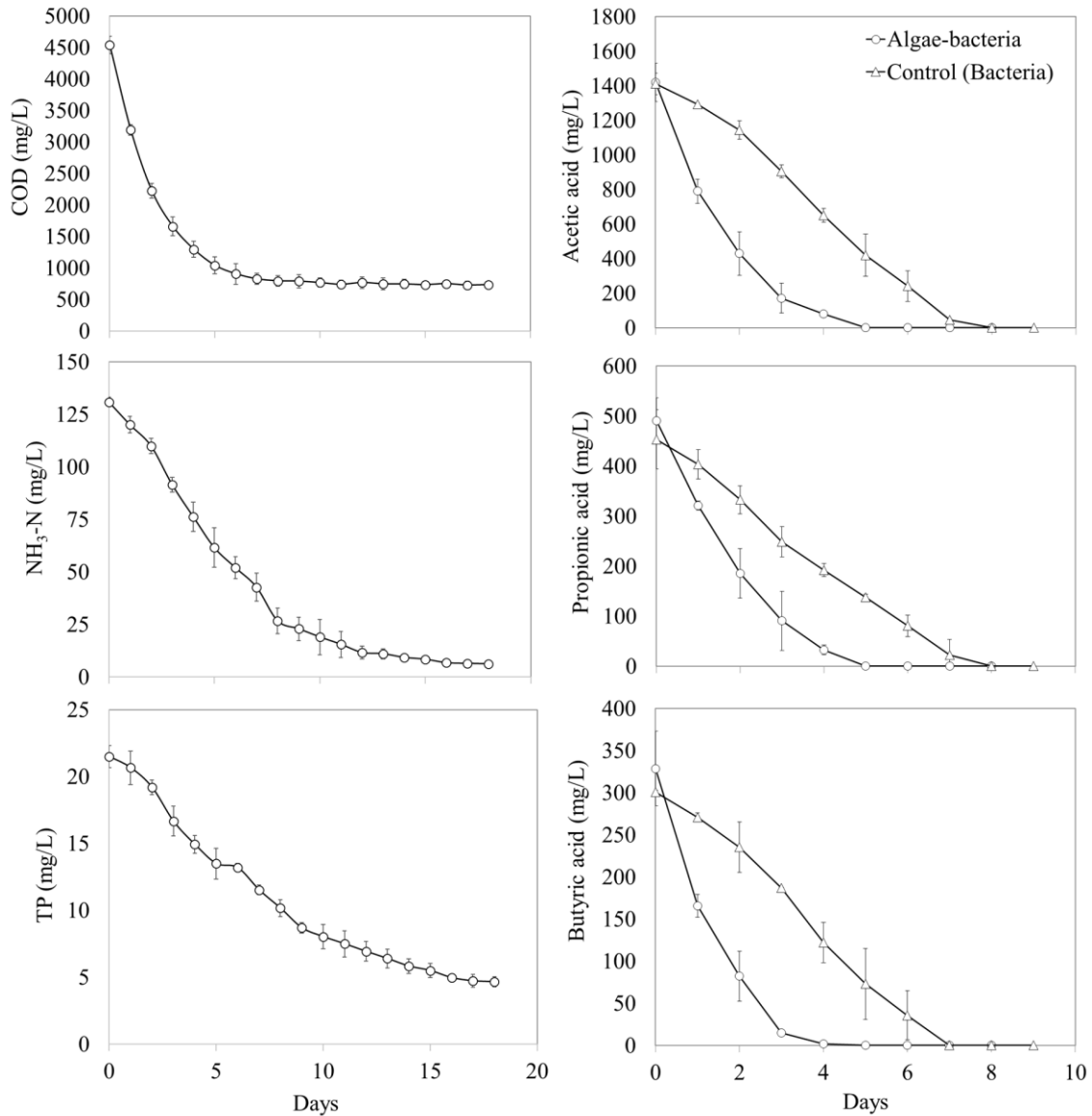
Figure 5-4. Nutrient removal profile during the cultivation of *C. sorokiniana*

Based on Canada's Wastewater Systems Effluent Regulations (WSER), the maximum concentration of un-ionized ammonia in the effluent should not exceed 1.25 mg L^{-1} as nitrogen. However, no regulation for total nitrogen, ammonium or phosphorus is mentioned in the regulations. On the other hand, some provinces such as British Columbia and Manitoba have implemented the limit of 1 mg L^{-1} total phosphorus and 15 mg L^{-1} total

nitrogen for discharge into freshwater environments (Schmidt, 2016). In the photobioreactor of this study, the total nitrogen and phosphorus were reduced from 130.9 ± 2.3 and 21.5 ± 0.8 mg L^{-1} to 6.2 ± 1.4 and 4.8 ± 0.4 mg L^{-1}, respectively. This means the nitrogen concentration in the effluent of the photobioreactor meets the regulation while the phosphorus concentration still needs to be further reduced.

5.4.5 Biomass composition

To evaluate the microalgae cultivation for bioproducts potential, the protein, starch and lipid contents of biomass were measured and shown in Figure 5-5. The starch in the biomass increased from $13.2\pm1.0\%$ to $17.8\pm0.8\%$ of dry weight during the cultivation. The carbohydrate composition of biomass at day 18 was also analyzed in which $13.8\pm1.6\%$ of biomass was glucose and $2.6\pm0.5\%$ was galactose. de Souza et al. (2017) cultivated *C. sorokiniana* (UTEX1663) in BBM medium and harvested it after 30 days and freeze-dried it. They obtained the glucose and galactose content of 28% and 4% of dry weight biomass, respectively. In another experiment, Hernández et al. (2015) cultivated *C. sorokiniana* in mineral salt medium (MSM) enriched with a sterile solution of glucose, peptone and yeast extract and they obtained 18.2% carbohydrate in *C. sorokiniana* in which the majority of it was glucose.

The protein content of biomass first increased from $39.4\pm3.9\%$ at day 3 to 47.7 ± 0.7 of dry weight at day 10 and then decreased to $37.8\pm3.4\%$ at day 18. The same pattern of change in protein was observed in the study conducted by Kobayashi et al. (2013) on cultivation of *C. sorokiniana* on Bold's Basal Medium (BBM) standard medium. The decrease in protein synthesis and protein content of biomass during the cultivation time as

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a result of a decrease in nitrogen is observed in *C. sorokiniana* as well as other microalgae species (Breuer et al., 2012; Ho et al., 2013b). As shown in Table 5-2, a wide range of protein content (14.1-38.5%) is reported for *C. sorokiniana.*
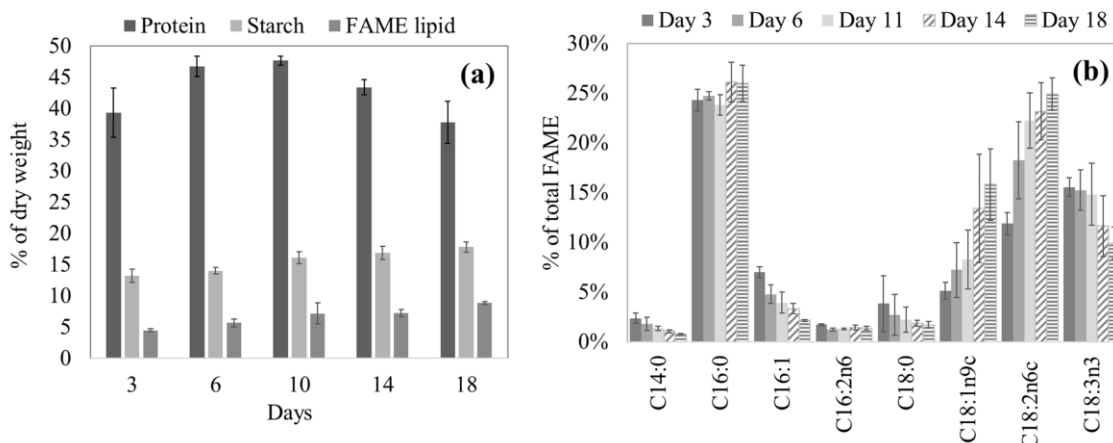
Figure 5-5. (a) Protein, total carbohydrate, lipid and (b) lipid composition during the cultivation of *C. sorokiniana* in struvite removed digestate

The FAME lipid content of biomass changed from 4.5±0.3% at day 3 to 8.9±0.3% of dry weight at day 18. The FAME lipid level in the culture of *C. sorokiniana* is in agreement with the reported range (4.5%-23.7%) (Bohutskyi et al., 2016; Kobayashi et al., 2013; Ramsundar et al., 2017). Bohutskyi et al. (2016) cultivated *C. sorokiniana* UTEX B 3010 in primary and secondary wastewater using different inoculum sizes and the obtained lipid content varied from 4.9 to 8.1% of ash-free dry biomass. The main fatty acids in FAME were C16:0, C16:3n-4, C18:1n-9c, C18:2n-6c and C18:3n-3, which is in close agreement with the fatty acid profile of the highly related species *Chlorella vulgaris* (Tibbetts et al., 2017; Tibbetts et al., 2015) and the presence of these fatty acids in *C. sorokiniana* are also reported in the literature (Kobayashi et al., 2013; Ramanna et al., 2014). In this study, the

level of C16:0, C18:1 and C18:2 increased while the concentration of C16:3 and C18:3 decreased during the cultivation time. The same trend of change in the composition of fatty acid is reported in the study of Kobayashi et al. (2013). According to EN 14214 biodiesel standard, the content of C18:3 and fatty acids with more than four double bonds must be less than 12% and 1%, respectively (Breuer et al., 2012). In this study, no fatty acids containing more than four double bonds were observed. However, the C18:3 content of oil at day 3 (15.6±1%) or 6 (15.2±2%) does not meet the standard while oil content of biomass at day 18 (9.9±1.6% C18:3) can be considered a suitable biodiesel feedstock. Breuer et al. (2012) showed that a deficiency of nitrogen can trigger the reduction in C18:3.

Table 5-2. Nutrient removal and bioproduct production by various strains of *C. sorokiniana* in batch mode

Condition	Pre-treatment	Strain	Volume (L)	Cultivation time (d)	Biomass (mg/L)	Initial			Removal %			Lipid (%)	Starch (%)	Protein (%)	Ref
						COD	TN (N-NH₃)	TP	COD	TN (N-NH3)	TP				
10% anaerobic digested effluent from cattle manure	-	CS-01	80	21	279	-	231.3 (89.3)	11.9	-	85.5 (74.7)	61.3	7.8 [a]	20.8 [a]	30.2 [a]	(Kobayashi et al., 2013)
	-	UTEX 1230	80	21	268	-	231.3 (89.3)	11.9	-	88.7 (65)	65	11.9 [a]	22.8 [a]	33.9 [a]	
	-	UTEX 2714	80	21	153	-	231.3 (89.3)	11.9	-	87.4 (72.2)	64.1	12.8 [a]	22.2 [a]	24.0 [a]	
100% Anaerobic digestate of palm oil mill effluent	autoclave	UKM3 (CS-N)	-	20	950 [b]	2359	350 (190)	22.4 [d]	-	(96.0)	35.9	-	-	-	(Khalid et al., 2018)
20 % Anaerobic digestate of palm oil mill effluent + 80% final effluent	autoclave	UKM3 (CS-N)	-	20	~ 1900 [b]	665 [c]	108.6 [c] (49.6 [C])	18.5 [d]	-	~100	59	-	-	-	
Influent after primary screening	Filtration (0.45μm)	-	1	14	1015	474.4	(50.5)	19.7	36.7	-(89.1)	87.2	19.5	24.1	14.1	(Ramsundar et al., 2017)
Anaerobic tank centrate	Filtration (0.45μm)	-	1	14	1080	185.5	(35)	24	44	(94.3)	83.3	22.3	13	15.3	
100% raw sewage	Filtration (0.25 mm)	AB731602.1	2	15	-	320	(52)	8.5	69.4	86.9	68.2	22.7	-	-	(Gupta et al., 2016)
25% raw sewage	Filtration (0.25 mm)	AB731602.1	2	15	-	80 [C]	(13 [C])	2.1 [C]	55.2	88.9	77.0	27.7	-	-	
4% poultry litter anaerobic digester (AD) effluent	-	-	0.25	12	366	-	76	6	-	-	-	12.3	21.4	37.9	(Singh et al., 2011)
8% poultry litter anaerobic digester (AD) effluent	-	-	0.25	12	313	-	152	12	-	-	-	12.4	18.8	38.5	
Winery wastewater	Filtration (0.2 μm)	UTEX 2805	0.2	4	-	154	- (89)	14	8	(~100)	(~100)	-	-	-	(Higgins et al., 2018)
	-	UTEX 2805	0.2	4	-	154	- (89)	14	70	(~100)	(~100)	-	-	-	
10% Final effluent supplemented with 12% CO2	autoclave	UTEX1230	1	4	250	-	8	2.6	-	(~100)	N.S	12.8	-	-	(Lizzul et al., 2014)

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Condition	Pre-treatment	Strain	Volume (L)	Cultivation time (d)	Biomass (mg/L)	Initial			Removal %			Lipid (%)	Starch (%)	Protein (%)	Ref
						COD	TN (N-NH₃)	TP	COD	TN (N-NH3)	TP				
10% Final effluent supplemented	autoclave	UTEX1230	1	4	220	-	8	2.6	-	(~100)	N.S	7.3	-	-	
10% anaerobic digester centrate 10% supplemented with 12% CO2	autoclave	UTEX1230	1	4	320	-	53	9.4	-	(~100)	N.S	5[b]	-	-	
10% anaerobic digester centrate	autoclave	UTEX1230	1	4	170	-	53	9.4	-	65	N.S	7.1[b]	-	-	
Two times dilution of anaerobic digestate of thin stillage	Struvite recovery	UTEX 1230	1	18	1630	4540	(130.9)	21.5	83.8	(95.3)	78.3	8.9	17.8	37.8	This study
Two times dilution of anaerobic digestate of thin stillage	Struvite recovery + high speed centrifugation	UTEX 1230	0.05	18	2030	4495	(132)	19.2	82.5	(94.7)	85.2	-	-	-	
Two times dilution of anaerobic digestate of thin stillage	Struvite recovery + Chitosan flocculating	UTEX 1230	0.05	18	2110	4420	(130.7)	19.7	81.3	(95.6)	84.1	-	-	-	
Two times dilution of anaerobic digestate of thin stillage	Struvite recovery	UTEX 1230	0.05	18	1780	4540	(130.9)	21.5	84.2	(91.3)	84.8	-	-	-	
Five times dilution of anaerobic digestate of thin stillage	Struvite recovery	UTEX 1230	0.05	18	1250	1721	(50.2)	7.3	73.6	(97.4)	90.7	-	-	-	
Five times dilution of anaerobic digestate of thin stillage	-	UTEX 1230	0.05	18	1330	1759	(94.7)	92.5	71.8	(97.5)	33.4	-	-	-	

[a] Ash-free dry weight, [b] Denotes values estimated from graphical representations in figures, [c] calculated based on dilution, [d] PO₄ is converted to TP, N.S. not significant

The elemental analysis of biomass at day 18 is shown in Table 5-3. The concentration of phosphorus in the biomass was 0.95±0.05%. According to the literature, algal biomass usually contain 0.1 to 3.3% phosphorus (Li et al., 2011; Tibbetts, 2018). The N/P ratio in biomass is 18.4 which is close to the ratio in the medium (14.5). This was expected since the N/P ratio in biomass was approximately similar to the ratio in the medium (Marcilhac et al., 2015).

Table 5-3. Elemental analysis of algal biomass of *C. sorokiniana* cultivated on SRD2X in a 1L PBR

Elements	% DW biomass
N	7.91 ± 0.71
P	0.95 ± 0.05
Ca	0.12 ± 0.01
K	1.40 ± 0.10
Mg	0.51 ± 0.03
Na	0.94 ± 0.08
S	0.50 ± 0.03

As shown in Table 5-2, for each liter of SRD2X, 95.3% of ammonia-nitrogen (127.2±1.3 mg) and 78.3% of phosphorus (16.1±0.2 mg) is removed. On the other hand, based on Table 5-3, 7.91% of the recovered biomass (1630 mg per each liter of SRD2X) is nitrogen (128.9±11.6 mg) and 0.95% is phosphorus (15.5±0.8 mg). It shows that almost all removed nitrogen and phosphorus were recovered in the form of biomass (mass balances for nitrogen and phosphorus are shown in Appendix B).

5.4.6 Evolution of microbial population

To gain insight into the evolution of the bacterial population, the composition of bacteria during the cultivation of *C. sorokiniana* on SRD2X in the 1 L PBR is shown in Figure 5-6. The other influencing factor on the microalgae growth and nutrient removal is the synergy between microalgae and bacteria which can improve the efficiency of treatment as well as the growth rate of both microorganisms (Borde et al., 2003; Higgins et al., 2018; Su et al., 2012). Microalgae can utilize the CO_2 which is produced by bacterial respiration and synthesize organics such as sugars, acetate, and glycerol for heterotrophic growth of bacteria. Moreover, the growth of many photosynthetic microalgae requires vitamins such as B_{12} and vitamin B_{12} auxotrophs are widespread among microalgae and only prokaryotes can synthesize this vitamin (Subashchandrabose et al., 2011). Higgins et al. (2018) demonstrated that culturing *C. sorokiniana* with native wastewater, the microbial community improved the nutrient removal ability of all the microorganisms as well as their growth rate compared to when those algae or bacteria were cultivated separately in wastewater. They also showed that the cultivation of microalgae and operating conditions changed the microbial population during the cultivation period. Su et al. (2012) studied the cooperation between wastewater-born algae and activated sludge for wastewater treatment. They tested different algae/sludge ratios (10:1, 5:1, 1:1 and 1:5) as an inoculum and the highest nitrogen and phosphorus removal efficiencies were observed at algae/sludge of 5:1.
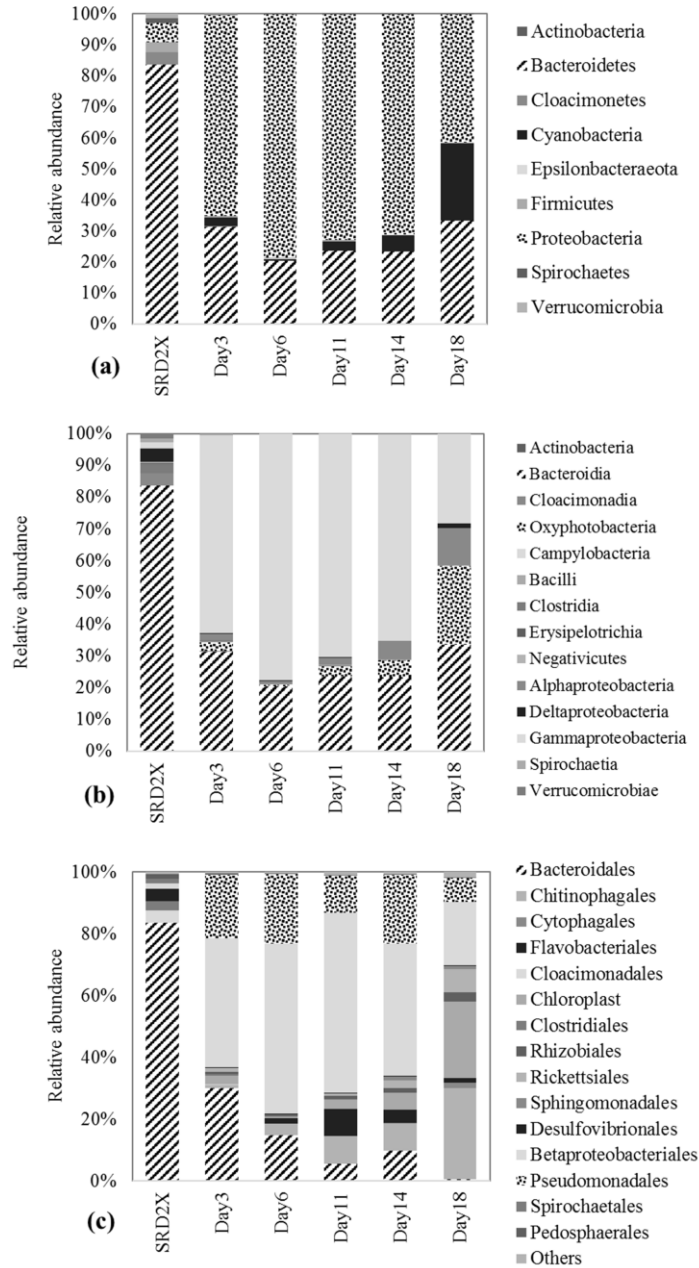
Figure 5-6. The evolution of bacterial community structures of SRD2X at (a) phylum, (b) class and (c) order level in response to the growth of *C. sorokiniana* in 1L PBR

Despite mentioned studies, many aspects of the relationship between algae and bacteria is still unclear. It includes the effect of environmental conditions (light and nutrient) on microalga-bacteria interaction and the change in microalgae composition and

cell wall in response to presence of bacteria (Lenneman et al., 2014; Ramanan et al., 2016). The relationship can also be competitive or antagonistic. Therefore, the relationship depends on the environmental conditions and population of wastewater-borne bacteria (Ma et al., 2014).

In this work, the native microbial populations were mainly from the phyla of *Bacteroidetes* (83.6%), *Cloacimonetes* (4%), *Firmicutes* (3.2%) and *Proteobacteria* (6.3%), which are common in anaerobic wastewater treatment systems (Higgins et al., 2018; McGarvey et al., 2007). About 72% of the native populations are from the genus of *Macellibacteroide* (Phylum of *Bacteroidetes*). The aeration of anaerobic digestate in photobioreactors can change the microbial population significantly (Higgins et al., 2018). After three days of aeration in this work, the composition of phylum in digestate had a dramatic decrease in *Bacteroidetes* (31.1%), *Cloacimonetes* (0.1%), *Firmicutes* (0.6%) and an increase in *Proteobacteria* (64.5%). The main change in genus composition was the reduction in relative abundance of *Macellibacteroide* from 72.0% in digestate to 0.2% in the medium at day 18. This might be due to growth inhibition of obligate anaerobes by dissolved oxygen concentration (McGarvey et al., 2007). The same phenomenon was observed in the study of Higgins et al. (2018) when they aerated an anaerobic digestate from winery wastewater. The presence of *Mesorhizobium* genus, which is able to synthesize vitamin B_{12}, was identified in the bacterial population. The positive impact of the species from this genus on algal growth promotion is reported in the literature (Fuentes et al., 2016). The interaction between microalgae and bacteria depends also on microalgae and bacteria species (Subashchandrabose et al., 2011). The symbiotic relationship between

C. sorokiniana and genera of *Microbacterium*, *Pseudomonas*, *Ralstonia* and *Acinetobacter* has been shown in different studies (Subashchandrabose et al., 2011). In the present research, these genera were found in the bacterial community after co-cultivation with *C. sorokiniana* on SRD2X in the PBR.

The net nitrogen removal in wastewaters is an accumulative effect of nitrification/denitrification, stripping and its assimilation by algae (Delgadillo-Mirquez et al., 2016). Other than microalgae growth, the other processes can be responsible for more than 50% of nitrogen removal in some cases (De Godos et al., 2009). Nitrification can reduce the nitrogen loss due to ammonia volatilization and decrease the toxicity of high ammonia concentration on microalgae growth (Abdel-Raouf et al., 2012; Collos and Harrison, 2014). Nitrification inhibition is reported in the literature because of intense competition between nitrifiers and microalgae for CO_2 (de Godos et al., 2010). Moreover, the studies show that the microalgae outcompeted nitrifier bacteria when phosphorus was limiting (Marcilhac et al., 2014). Various microbial population can be responsible for nutrient removal. In this study, the degradation of organic matter was likely done by the phylum of *Proteobacteria*, *Actinobacteria* and *Firmicutes* (García et al., 2017). *Alcaligenes*, *Acinetobacter* and *Pseudomonas* are known as heterotrophic nitrifying bacteria (Lee et al., 2016). The total population of these three genera in the struvite removed digestate (SRD2X) was less than 1.8%. However, the abundance of *Alcaligenes* and *Acinetobacter* reached 53.7% and 22.6%, respectively in the PBR at day 6. The presence of nitrifying bacteria was expected in the culture since the nitrate concentration in the medium was insignificant at the beginning but it increased and started to fluctuate between

0.778 to 1.73 mg L^{-1} during the cultivation. The denitrification was considered negligible due to the level of dissolved oxygen (average of 8.7 mg L^{-1}) in the medium (Marcilhac et al., 2014). Combination of microalgae growth and nitrification/denitrification can be applied by technologies such as photo-sequencing batch reactor in which oxygen is provided by microalgae for nitrification during light period and the lack of dissolved oxygen during dark period promotes denitrification and removal of nitrogen. Oxygenation by microalgae to support the growth of nitrifiers was also applied by Karya et al. (2013). In their study, an open photobioreactor was inoculated with a culture of *Scenedesmus sp.* and nitrifiers. The *Scenedesmus sp.* provided oxygen for nitrification and full nitrification was obtained without aeration. In the system, the majority of ammonium removal (81-85%) was achieved by nitrification. With these systems, a higher removal efficiency for nitrogen can be achieved with lower demand for aeration (Wang et al., 2015; Wang et al., 2016). However, optimum environmental conditions such as light intensity, nutrient balance and mixing condition need to be determined to promote the growth and activity of these three microbial communities and prevent them from outcompeting each other (de Assis et al., 2017; de Godos et al., 2010; Delgadillo-Mirquez et al., 2016; Marcilhac et al., 2014). Coppens et al. (2016) used a two-stage method for nutrient recovery from urine. In the first stage, the urine was stabilized through nitrification. The second stage included the cultivation of a microalgae species (*Arthrospira platensis*) on the nitrified urine, which resulted in a protein content of 62%. Even though different studies have evaluated microalgal–bacteria consortia systems for nutrient removal, more attention should be paid to wastewaters with high ammonia concentration (Jia and Yuan, 2016).

5.5 Conclusions

In this study, thin stillage digestate was pre-treated with struvite recovery which is a value-added product with the application as a fertilizer. Cultivation of *C. sorokiniana* on the treated digestate was able to remove chemical oxygen demand, ammonia-nitrogen and total phosphorus up to $83.8\pm0.6\%$, $95.3\pm1\%$, and $78.3\pm1.1\%$, respectively. Moreover, the produced microalgal biomass had a significant content of potential bioproducts such as protein ($37.8\pm3.4\%$), starch ($17.8\pm0.8\%$) and lipid ($8.9\pm0.3\%$), which can be integrated into an existing corn ethanol plant to reduce the corn consumption and increase the protein content of the dried distiller's grain and corn-oil yield. Additionally, identification of the microbial community in the photobioreactor revealed that the potential for nitrification of ammonium can be employed as a means to reduce the concentration of ammonium and its subsequent inhibitory effect to microalgae. This biorefinery concept can be the basis for environmentally and economically sustainable bioethanol industry.

5.6 Acknowledgement

CHAPTER 6   CONCLUSION

The objective of this study was to evaluate the feasibility of integrating an anaerobic digestion-microalgae cultivation system in an existing corn bioethanol plant. Instead of conventional energy intensive evaporation and drying of thin stillage in bioethanol plants, anaerobic digestion of thin stillage can produce energy in the form of methane which can be utilized in power plants. The anaerobic digestion of thin stillage was performed in lab scale conventional and novel anaerobic baffled reactors. The design of the novel anaerobic baffled reactor reduced the biomass washout and allowed for better performance compared to the conventional ABR with respect to COD and sulfate removal and methane production. Moreover, the novel ABR could handle higher OLR than conventional ABR. In addition to design, the operating parameters of ABR should be optimized to enhance the treatment efficiency and methane production. Therefore, the changes in those parameters in response to a change in recycle ratio and OLR was monitored which showed a decrease in treatment efficiency and methane yield when the OLR increased. The RR did not have a significant effect on the overall performance of the reactor but it affected the balance between the compartments of the hybrid ABR so that the performance of all compartments in COD removal was almost similar at higher RRs, whereas at lower RRs, the first compartments had the highest contribution to overall COD removal. These findings helped to achieve one of the objectives of this study, which was to develop an understanding on the strengths and challenges of two-phase systems and to overcome those limitations. The limitations with respect to biomass washout, phase separation, COD and sulfate removal as well as methane yield for anaerobic digestion of thin stillage in the conventional ABR were identified and

the innovate system design of the novel ABR was applied to overcome the mentioned challenges and to improve the performance of the system. Moreover, investigating the effect of operating parameters on performance of the novel ABR provided a better understanding of reactor's behavior and a useful tool for optimizing the operational and design characteristics of the reactor.

During the operation of the ABR, the natural removal of nitrogen and phosphorus occurred in the form of struvite precipitation by 37% and 49%, respectively. The limiting nutrient for further formation of struvite was magnesium. This hypothesis was proven by addition of magnesium to the digestate effluent and removal of nitrogen (44%) and phosphorus (81%) by struvite recovery. Struvite can be utilized as a source of fertilizer and it is a value-added product. Even with struvite recovery nitrogen can not be removed effectively.

The digestate can be used for microalgae cultivation. However, the digestate is not an optimized medium for microalgae cultivation due to its dark color, high ammonia concentration and unbalanced nitrogen to phosphorus ratio. Therefore, various microalgae species with different growth rates and tolerance to ammonia were cultivated in untreated and treated (i.e. dilution and struvite recovery) digestate mediums. The microalgae could not grow in two times dilution of digestate, while two times dilution of struvite removed digestate and *C. sorokiniana* were the best medium and microalgae species with respect to microalgae growth rate and nutrient removal efficiency. The optimum medium and microalgae species was used in a 1 L photobioreactor. In the photobioreactor, biomass concentration of 1620 ± 110 mg L^{-1} and the nitrogen and phosphorus removal efficiencies

of $95.3\pm1\%$ and $78.3\pm1.1\%$ were achieved at the end of cultivation time. The protein, starch and lipid content of biomass were $37.8\pm3.4\%$, $17.8\pm0.8\%$ and $8.9\pm0.3\%$, respectively. The bioproducts and nutrient can be recycled to add value to corn ethanol processing. Another goals of this work were to reduce dilution of digestate and to identify robust algae species capable of growing on thin stillage digestate. The lowest dilution of digestate, in which the microalgae could grow, was five times and struvite recovery helped to reduce the dilution to two times. On the other hand, the results of screening experiments revealed that *C. sorokiniana* was the most robust algae species among the tested microalgae for growing on the thin stillage digestate.

Another objective of this study was to investigate the synergy of bacterial population and microalgae for nutrient and carbon conversion. The results showed that the microalgae cultivation caused a dramatic change in the population of native bacteria so that the relative abundance of anaerobic bacteria decreased and the abundance of other groups such as nitrifiers increased. Moreover, the presence of nitrate in the culture due to activity of nitrifiers was observed. The nitrification reduces the toxicity of ammonia to microalgae growth and prevent nitrogen loss due to ammonia striping. Moreover, bacterial species, which are able to produce vitamin B_{12}, required by microalgae, were identified in the culture. Also, the comparison between the consumption rate of volatile fatty acids in the culture (algae-bacteria) and the control (only bacteria), showed that the algae-bacteria culture could remove volatile fatty acids as a major part of COD much faster than only bacteria. This shows the synergy between microalgae and bacteria for removing organic carbon.

6.1 Recommendations for Future Work

The formation of granule in novel ABR can significantly improve the capability of reactor in treating thin stillage. Therefore, as a future study, the effect of liquid hydrodynamic, biogas and mixing condition on formation of granule, rate of mass transfer of nutrients from bulk liquid to microorganisms and reactor's efficiency with respect to COD and sulfate removal and methane production can be investigated through computational fluid dynamic (CFD) and kinetic modeling. Moreover, the operating parameters such as recycle ratio can change the contribution of each compartment in COD removal and methane production. As a result, the number of compartments and size of the reactor can be reduced by optimizing the recycle ratio. Modeling can describe the behavior of the ABR with different design configurations and operating conditions. The model can be used to predict the performance of the system at larger scales regarding methane production and COD removal.

Recovery of non-renewable nutrients such as phosphorus is critical for food security. The phosphorus is partially removed in ABR due to struvite precipitation. However, the deposits cause clogging of the reactor. In order to solve the problem, a struvite crystallizer can be designed and installed to remove struvite from the recycle stream before it enters the reactor. However, the crystallizer will be different from commercial crystallizers. In commercial crystallizers, pH of digestate is increased to about 8.5 by aeration and addition of chemicals, while pH of 8.5 and presence of oxygen are toxic to anaerobic bacteria. Therefore, the struvite crystallizer prior to the ABR must be functional without pH adjustment and addition of chemicals. Moreover, the struvite recovery process will change

the nutrients ratio (COD:N:P) in the input of reactor. Thus, the feasibility of installing a struvite crystallizer and its effect on the efficiency of the ABR regarding COD removal and methane production need to be investigated.

For future works, a mixed sample of microalgae biomass in struvite removed digestate and corn can be used for ethanol production after liquefaction and fermentation to study the potential of bioethanol production from the mixed feedstock. Dilution of the digestate and cost of Mg addition are two disadvantages of the proposed system. However, a cheaper source of Mg such as seawater can be used to achieve both dilution and struvite recovery, but the effect of increased salinity on the downstream processes such as microalgae cultivation, liquefaction and fermentation need to be studied.

On the other hand, the configuration of photobioreactor can be optimized to allow for higher availability of light to microalgae and longer retention time for CO_2 absorption into the medium. In this regard, the height, diameter and shape of photobioreactor can be changed to optimize the illuminated area to culture volume, light distribution and gas-liquid mass transfer. These parameters are important, since an excessive or low CO_2 transfer to liquid can reduce the microalgae growth. On the other hand, a strong light intensity can create a large dark zone inside the reactor and a high intensity zone near the surface of reactor, which both are not appropriate for microalgae. In addition to photobioreactors, microalgae cultivation on struvite removed digestate should be performed in open pond systems to address the limitations and challenges of scale up.

Moreover, to evaluate the effect of native bacteria on the microalgae biomass production and nutrient removal, microalgae can be cultivated on the sterilized digestate.

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The comparison between achieved biomass concentration, composition and nutrient removal in sterilized and unsterilized digestate will reveal the influence of bacteria.

Finally, in order to investigate the effect of the proposed integrated system on the economic, sustainability and energy balance of an existing corn-bioethanol plant, a life cycle assessment and technoeconomic analysis are necessary.

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APPENDIX A   COPYRIGHT AGREEMENTS

APPENDIX B   MASS BALANCES
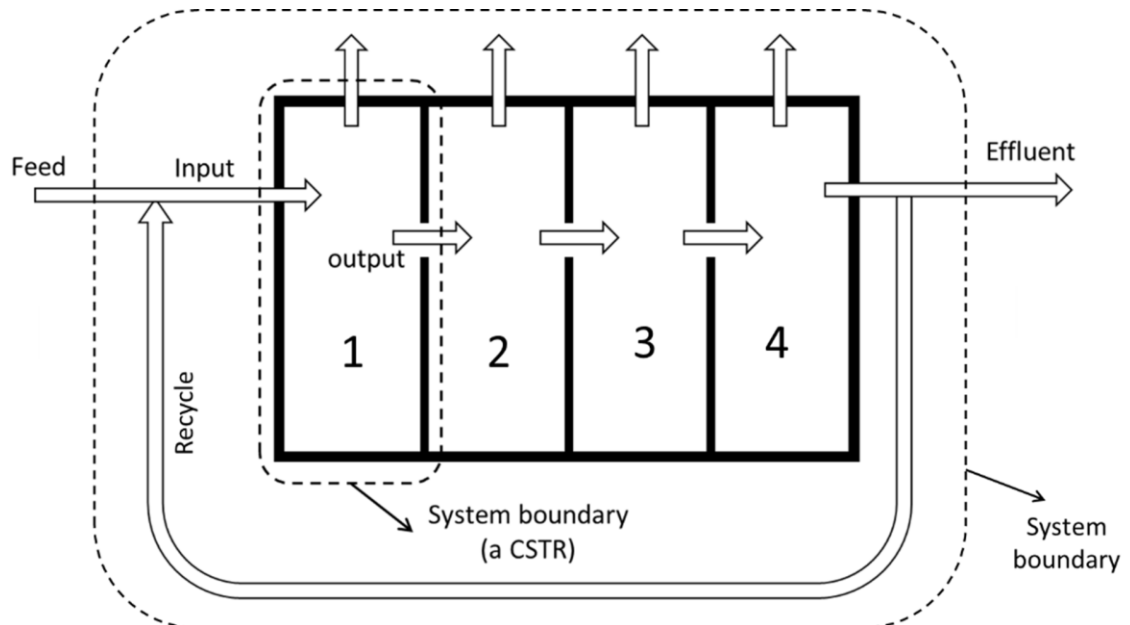
Figure S1. The schematic view of ABR and system boundaries for mass balances

In the top figure, the whole reactor or one compartment can be considered as a CSTR for performing a mass balance.

COD mass balances for the hybrid ABR in Chapter 3:

Sample calculation for OLR of 1 kg COD/m^3.d in the following table:

Measured COD in the input = COD_{input} = 11.14 g/L

Feeding flow rate = Input flow rate = Q = 2.52 L/d

Mass flow of input $=$ $COD_{input} \times Q = 11.14$ g/L $\times 2.52$ L $= 28.7$ g/d

Measured COD in the output = COD_{output} = 0.36 g/L

Output flow rate = Input flow rate = Q = 2.52 L/d

Mass flow of output = $COD_{output} \times Q$ = 0.36 g/L $\times$ 2.52 L = 0.9 g/d

 Biomass yield = 0.08 g VSS/g COD for low-yield anaerobic processes (Liang et al., 2007)

g COD converted to biomass = Biomass yield $\times$ (g COD input $-$ g COD output)

g COD converted to biomass = 0.08 $\times$ (28.7 g/d $-$ 0.9 g/d) = 2.17 g/d

Measured output CH_4 = 7.1 L/d

g COD equivalent of each liter CH_4 gas$\rightarrow$ 0.350 L CH_4 g^{-1} COD

$$\text{g COD of output } CH_4 = \frac{L\ CH_4\ output}{0.35\ L/g\ COD} = \frac{7.1\ L/d}{0.35\ L/g\ COD} = 20.17\ \text{g COD/d}$$

$$\text{Mass balance } = \frac{(\text{g COD of output } CH_4) + (\text{g COD converted to biomass})}{(\text{g COD input} - \text{g COD output})} \times 100$$

$$\text{Mass balance } = \frac{(20.17\ g/d) + (2.17\ g/d)}{(28.07\ g/d - 0.9\ g/d)} \times 100 = 82.2\%$$

Table B1. Overall COD mass balance for the hybrid ABR

OLR (kg COD/m³.d)	COD$_{input}$		COD$_{output}$		COD for Cell growth (g/d)	CH$_4$ output (g COD/d)	Mass balance (%)
	(g/L)	(g/d)	(g/L)	(g/d)			
1	11.14	28.07	0.36	0.90	2.17	20.17	82.2%
1.5	16.34	41.18	0.67	1.70	3.16	34.11	94.4%
2	20.32	51.21	0.92	2.32	3.91	42.66	95.3%
2.5	27.42	69.10	1.44	3.62	5.24	55.15	92.2%
3	32.52	81.95	1.89	4.76	6.18	64.42	91.5%
3.5	38.32	96.57	2.41	6.07	7.24	74.26	90.1%

Table B2. Mass balance for the 1ˢᵗ compartment of hybrid ABR

OLR (kg COD/m³.d)	COD$_{input}$		COD$_{output}$		COD for Cell growth (g/d)	CH$_4$ output (g COD/d)	Mass balance (%)
	(g/L)	(g/d)	(g/L)	(g/d)			
1	1.28	35.40	0.62	17.10	1.46	11.37	70.1%
1.5	2.01	55.72	1.13	31.37	1.95	22.22	99.3%
2	2.63	72.97	1.69	46.80	2.09	23.18	96.6%
2.5	3.74	103.69	2.59	71.86	2.55	26.85	92.3%
3	4.60	127.48	3.32	92.16	2.83	30.09	93.2%
3.5	5.59	154.85	4.12	114.18	3.25	32.71	88.4%

Sample calculation for OLR of 2 kg COD/m³.d in the following table:

Measured COD in the input =Output of previous compartment= COD$_{input}$ = 1.69 g/L

Input flow rate = Feeding flow rate + Recycle flow rate =2.52+25.2 = Q = 27.72 L/d

Mass flow of input $=$ COD$_{input}$ $\times$ Q = 1.69 g/L $\times$ 27.72 L $=$ 46.8 g/d

Measured COD in the output = COD$_{output}$= 1.11 g/L

Output flow rate = Input flow rate = Q = 27.72 L/d

Mass flow of output $=$ COD$_{output}$ $\times$ Q = 1.11 g/L $\times$ 27.72 L $=$ 30.87 g/d

g COD converted to biomass $=$ Biomass yield $\times$ (g COD input $-$ g COD output)

g COD converted to biomass $=$ 0.08 $\times$ (46.8 g/d $-$ 30.87 g/d) = 1.27 g/d

Measured output CH$_4$ = 5.03 L/d

g COD equivalent of each liter CH$_4$ gas$\rightarrow$ 0.350 L CH$_4$ g^{-1} COD

$$\text{g COD of output } CH_4 = \frac{L \ CH_4 \ output}{0.35 \ L/g \ COD} = \frac{5.03 \ L/d}{0.35 \ L/g \ COD} = 14.38 \text{ g COD/d}$$

$$\text{Mass balance} = \frac{(\text{g COD of output } CH_4) + (\text{g COD converted to biomass})}{(\text{g COD input} - \text{g COD output})} \times 100$$

$$\text{Mass balance} = \frac{(14.38 \text{ g/d}) + (1.27 \text{ g/d})}{(46.8 \text{ g/d} - 30.87 \text{ g/d})} \times 100 = 82.2\%$$

Table B3. Mass balance for the 2nd compartment of hybrid ABR

OLR (kg COD/m³.d)	COD$_{input}$		COD$_{output}$		COD for Cell growth (g/d)	CH$_4$ output (g COD/d)	Mass balance (%)
	(g/L)	(g/d)	(g/L)	(g/d)			
1	0.62	17.19	0.45	12.39	0.38	6.72	148.2%
1.5	1.13	31.43	0.79	21.95	0.76	8.07	93.1%
2	1.69	46.80	1.11	30.87	1.27	14.38	98.3%
2.5	2.59	71.86	1.76	48.74	1.85	20.16	95.2%
3	3.32	92.16	2.30	63.84	2.27	24.21	93.5%
3.5	4.12	114.18	3.02	83.75	2.43	26.53	95.2%

Table B4. Mass balance for the 3rd compartment of hybrid ABR

OLR (kg COD/m³.d)	COD$_{input}$		COD$_{output}$		COD for Cell growth (g/d)	CH$_4$ output (g COD/d)	Mass balance (%)
	(g/L)	(g/d)	(g/L)	(g/d)			
1	0.45	12.39	0.41	11.23	0.09	1.21	112.1%
1.5	0.82	22.79	0.74	20.40	0.19	2.24	101.9%
2	1.12	30.94	1.00	27.61	0.27	3.17	103.3%
2.5	1.76	48.84	1.52	42.19	0.53	5.94	97.4%
3	2.31	63.98	2.00	55.55	0.67	7.37	95.4%
3.5	3.03	83.94	2.63	72.85	0.89	9.68	95.3%

Table B5. Mass balance for the 4th compartment of hybrid ABR

OLR (kg COD/m³.d)	COD$_{input}$		COD$_{output}$		COD for Cell growth (g/d)	CH$_4$ output (g COD/d)	Mass balance (%)
	(g/L)	(g/d)	(g/L)	(g/d)			
1	0.41	11.23	0.36	9.92	0.10	0.87	74.6%
1.5	0.74	20.40	0.68	18.74	0.13	1.57	102.6%
2	1.01	27.61	0.92	25.61	0.16	1.92	104.4%
2.5	1.52	42.19	1.44	39.92	0.18	2.20	104.6%
3	2.00	55.55	1.89	52.45	0.25	2.76	96.9%
3.5	2.63	72.85	2.41	66.86	0.48	5.35	97.3%

Here is the COD mass balances for chapter 4:

Table B6. Overall COD mass balance for the hybrid ABR at different HRTs and RRs

Operation mode	OLR (kg COD/m³.d)	Q (L/d)	COD$_{input}$ (g/d)	COD$_{output}$ (g/d)	COD for Cell growth (g/d)	CH$_4$ output (g COD/d)	Mass balance (%)
HRT20d, RR20	3.5	1.38	95.08	8.36	6.94	73.62	92.9%
HRT20d, RR15	3.5	1.38	95.77	7.15	7.09	77.25	95.2%
HRT20d, RR10	3.5	1.38	97.01	6.29	7.26	78.78	94.8%
HRT17.5d, RR15	4	1.58	110.08	10.74	7.95	82.94	91.5%
HRT15.6d, RR15	4.5	1.77	127.04	14.92	8.97	96.64	94.2%
HRT14d, RR15	5	1.97	139.18	19.75	9.55	100.92	92.5%
HRT12.7d, RR15	5.5	2.17	153.75	27.13	10.13	101.96	88.5%
HRT11.7d, RR15	6	2.37	168.20	35.44	10.62	104.21	86.5%

Table B7. COD mass balance for the 1st compartment of hybrid ABR at different HRTs and RRs

Operation mode	OLR (kg COD/m³.d)	Q (L/d)	COD$_{input}$ (g/d)	COD$_{output}$ (g/d)	COD for Cell growth (g/d)	CH$_4$ output (g COD/d)	Mass balance (%)
HRT20d, RR20	3.5	28.98	273.25	245.46	2.22	23.17	91.4%
HRT20d, RR15	3.5	22.08	204.55	173.00	2.52	26.73	92.7%
HRT20d, RR10	3.5	15.18	161.31	124.15	2.97	31.41	92.5%
HRT17.5d, RR15	4	25.23	269.62	237.41	2.58	27.01	91.9%
HRT15.6d, RR15	4.5	28.39	349.60	314.65	2.80	29.87	93.4%
HRT14d, RR15	5	31.54	432.96	397.96	2.80	29.75	93.0%
HRT12.7d, RR15	5.5	34.70	558.47	524.62	2.71	22.79	75.3%
HRT11.7d, RR15	6	37.85	698.09	661.56	2.92	25.66	78.2%

Table B8. COD mass balance for the 2nd compartment of hybrid ABR at different HRTs and RRs

Operation mode	OLR (kg COD/m^3.d)	Q (L/d)	COD$_{input}$ (g/d)	COD$_{output}$ (g/d)	COD for Cell growth (g/d)	CH$_4$ output (g COD/d)	Mass balance (%)
HRT20d, RR20	3.5	28.98	245.46	219.01	2.12	22.73	93.9%
HRT20d, RR15	3.5	22.08	173.00	147.71	2.02	22.12	95.5%
HRT20d, RR10	3.5	15.18	124.15	95.82	2.27	24.91	95.9%
HRT17.5d, RR15	4	25.23	237.41	210.34	2.17	22.91	92.6%
HRT15.6d, RR15	4.5	28.39	314.65	283.27	2.51	27.26	94.9%
HRT14d, RR15	5	31.54	397.96	367.23	2.46	25.91	92.3%
HRT12.7d, RR15	5.5	34.70	524.62	491.44	2.65	28.21	93.0%
HRT11.7d, RR15	6	37.85	661.56	627.57	2.72	27.87	90.0%

Table B9. COD mass balance for the 3rd compartment of hybrid ABR at different HRTs and RRs

Operation mode	OLR (kg COD/m^3.d)	Q (L/d)	COD$_{input}$ (g/d)	COD$_{output}$ (g/d)	COD for Cell growth (g/d)	CH$_4$ output (g COD/d)	Mass balance (%)
HRT20d, RR20	3.5	28.98	219.01	200.56	1.48	15.90	94.2%
HRT20d, RR15	3.5	22.08	147.71	127.79	1.59	17.82	97.4%
HRT20d, RR10	3.5	15.18	95.82	80.03	1.26	14.49	99.7%
HRT17.5d, RR15	4	25.23	210.34	187.77	1.81	19.37	93.8%
HRT15.6d, RR15	4.5	28.39	283.27	258.95	1.94	21.36	95.9%
HRT14d, RR15	5	31.54	367.23	338.06	2.33	24.85	93.2%
HRT12.7d, RR15	5.5	34.70	491.44	460.34	2.49	26.89	94.5%
HRT11.7d, RR15	6	37.85	627.57	594.34	2.66	27.62	91.1%

Table B10. COD mass balance for the 4th compartment of hybrid ABR at different HRTs and RRs

Operation mode	OLR (kg COD/m^3.d)	Q (L/d)	COD$_{input}$ (g/d)	COD$_{output}$ (g/d)	COD for Cell growth (g/d)	CH$_4$ output (g COD/d)	Mass balance (%)
HRT20d, RR20	3.5	28.98	200.56	187.02	1.08	11.82	95.3%
HRT20d, RR15	3.5	22.08	127.79	116.15	0.93	10.58	98.9%
HRT20d, RR10	3.5	15.18	80.03	71.52	0.68	7.96	101.5%
HRT17.5d, RR15	4	25.23	187.77	172.23	1.24	13.65	95.8%
HRT15.6d, RR15	4.5	28.39	258.95	238.60	1.63	18.15	97.2%
HRT14d, RR15	5	31.54	338.06	314.54	1.88	20.42	94.8%
HRT12.7d, RR15	5.5	34.70	460.34	433.04	2.18	24.07	96.1%
HRT11.7d, RR15	6	37.85	594.34	567.16	2.17	23.05	92.8%

COD mass balances for Chapter 5:

$$Mass\ balance = \frac{Mass\ in\ the\ biomass}{Initial\ mass\ in\ the\ medium - final\ mass\ in\ the\ medium} \times 100$$

Table B11. Nitrogen and phosphorus mass balance in the 1 L photobioreactor

Element	Initial mass in medium (mg)	Final mass in medium (mg)	Mass in the biomass (mg)	Mass balance (%)
Nitrogen	130.9±2.3	6.2±1.4	128.6±4.0	103.1±4.0
Phosphorus	21.5±0.8	4.7±0.4	15.5±0.8	91.8±2.6

APPENDIX C   IMAGES AND SIZE DISTRIBUTION OF SLUDGE

During the operation of reactor in chapter 3, a significant change in appearance of sludge was observed. The original sludge loaded to the all compartments was black. However, after a while, the presence of grayish fluffy sludge in the first and second compartments (where the acidogenesis is the main process) was noticeable, while the sludge granules in the later compartments remained black, in which methanogens were the dominant bacteria groups. The difference between the physical characteristics of acidogens and methanogens was reported by Daffonchio et al. (1995). Akunna and Clark (2000) also observed this phenomenon in the treatment of whisky distillery wastewater using an ABR. The following figures show images and size distribution of sludge during the operation of the hybrid ABR in chapter 3.

Organic loading rate of 1 kg COD/m^3.d

1st compartment

2nd compartment

3rd compartment

4th compartment

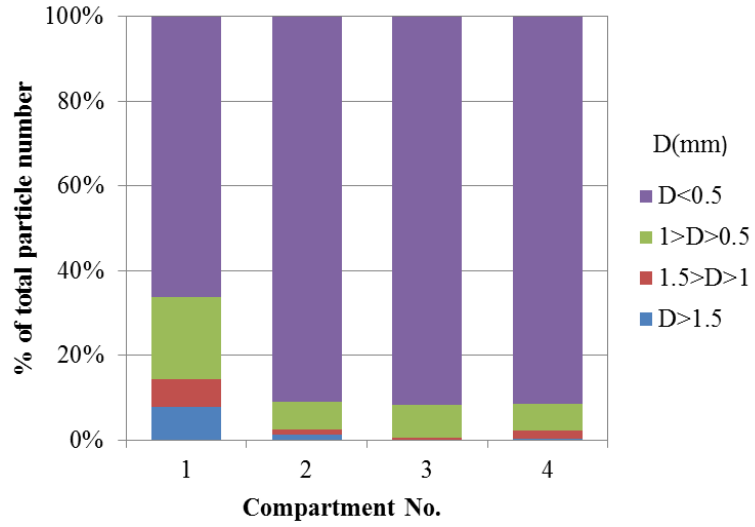**Figure C1. Picture of sludge from different compartments at OLR of 1 kg COD/m^3.d**

Figure C2. The size distribution of granules in each compartment in hybrid ABR at OLR of 1 kg COD/ m³.d

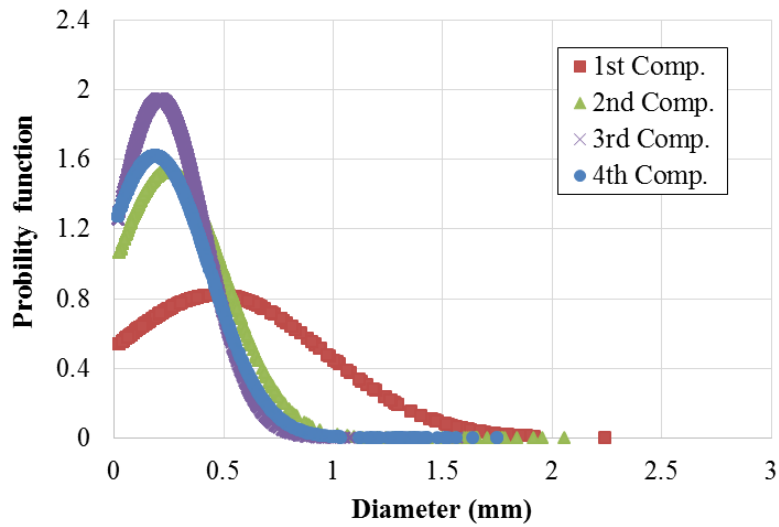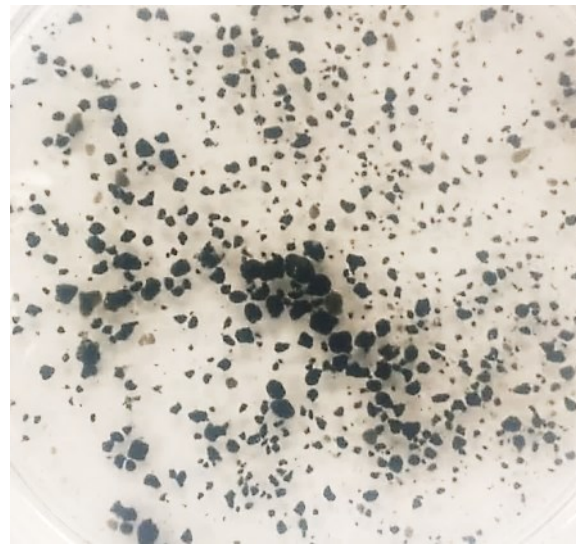**Figure C3. Normal size distribution of granules in each compartment in hybrid ABR at OLR of 1 kg COD/m³.d**

Organic loading rate of 1.5 kg COD/m³.d

1st compartment

2nd compartment

3rd compartment

4th compartment

Figure C4. Images of sludge from different compartments at OLR of 1.5 kg COD/m3.d
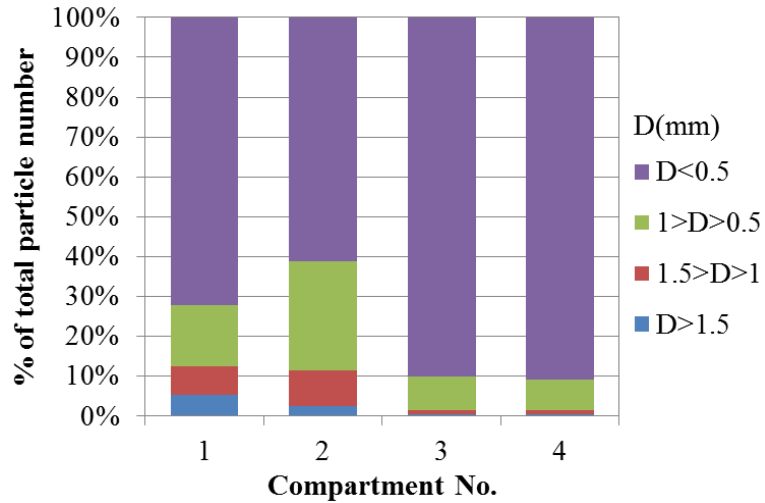
Figure C5. The size distribution of granules in each compartment in hybrid ABR at OLR of 1.5 kg COD/m3.d

Figure C6. Normal size distribution of granules in each compartment in hybrid ABR at OLR of 1.5 kg COD/m³.d

Organic loading rate of 2.5 kg COD/m^3.d

1st compartment

2nd compartment

3rd compartment

4th compartment

Figure C7. Images of sludge from different compartments at OLR of 2.5 kg COD/m^3.d

Figure C8. The size distribution of granules in each compartment in hybrid ABR at OLR of 2.5 kg COD/m^3.d

Figure C9. Normal size distribution of granules in each compartment in hybrid ABR at OLR of 2.5 kg COD/m^3.d

Organic loading rate of 3.5 kg COD/m³.d

1ˢᵗ compartment

2ⁿᵈ compartment

3ʳᵈ compartment

4ᵗʰ compartment

Figure C10. Images of sludge from different compartments at OLR of 3.5 kg COD/m³.d
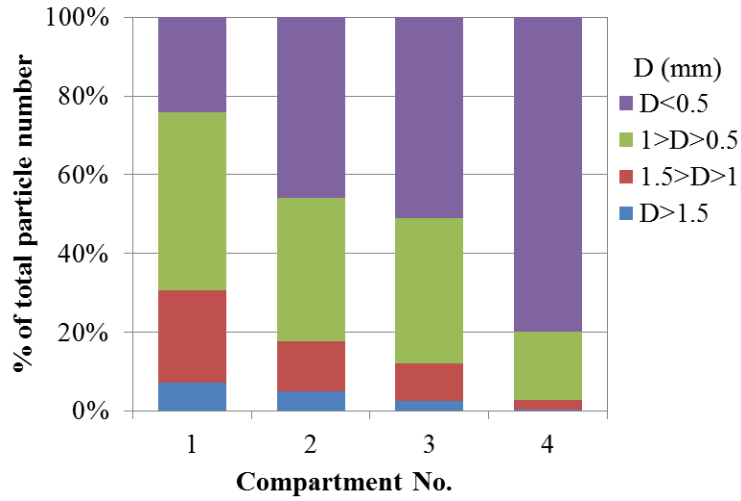
Figure C11. The size distribution of granules in each compartment in hybrid ABR at OLR of 3.5 kg COD/m^3.d
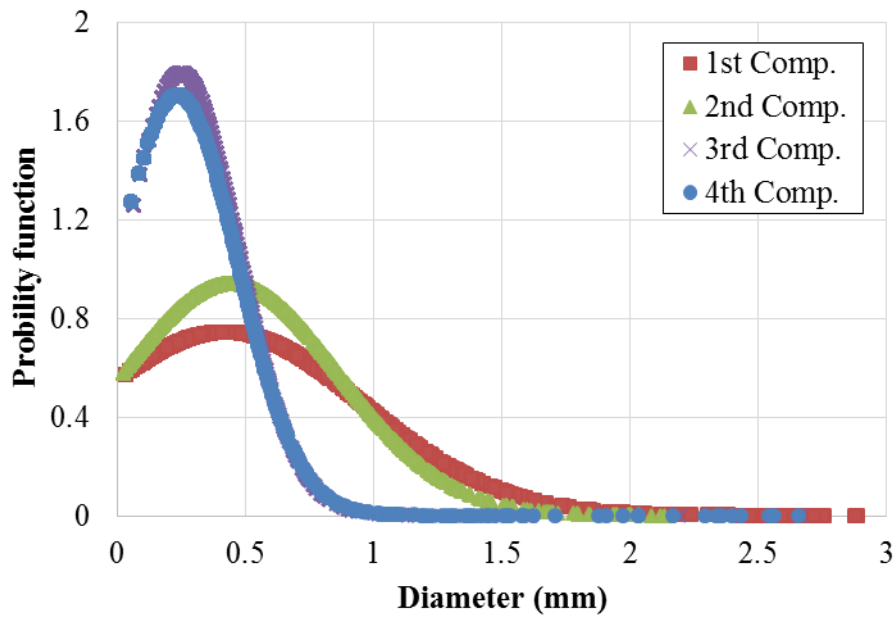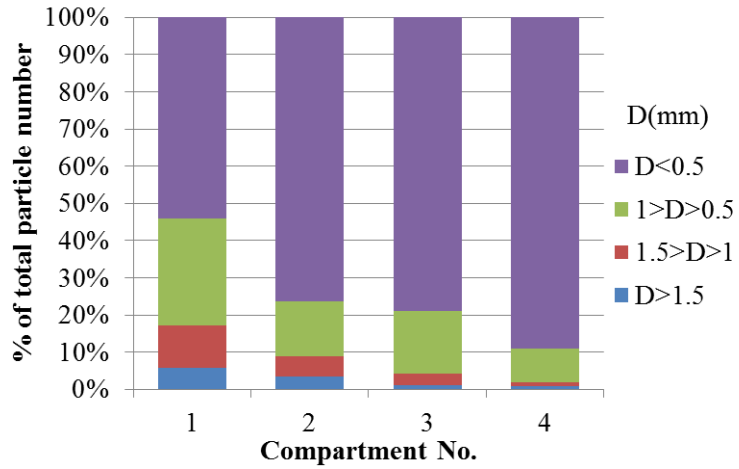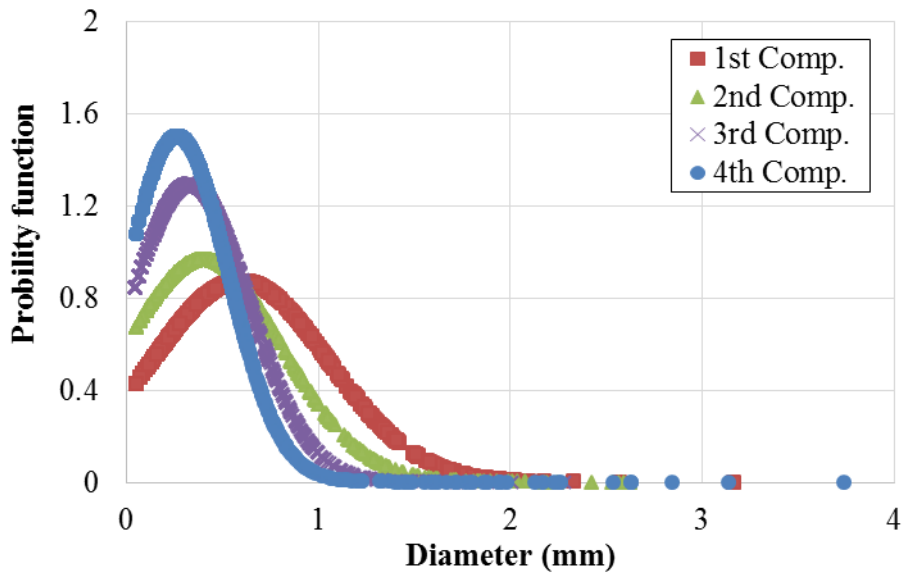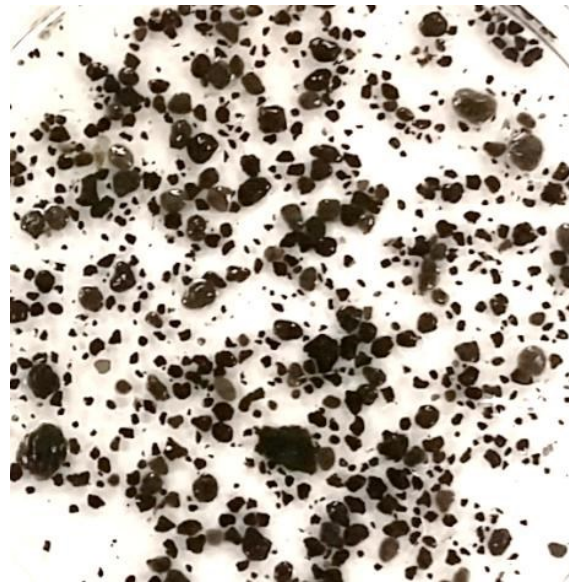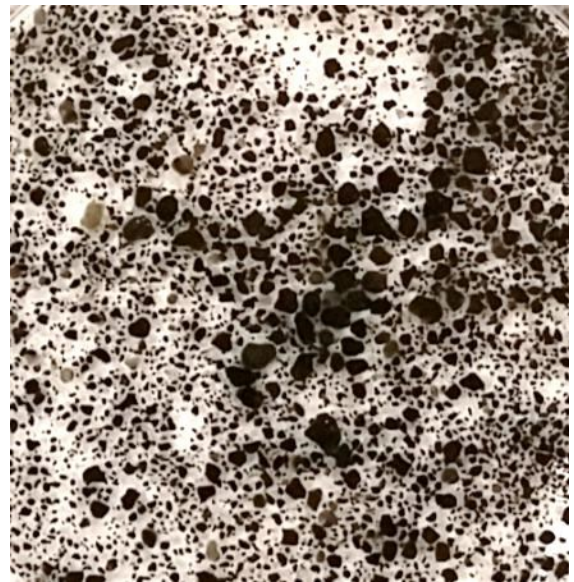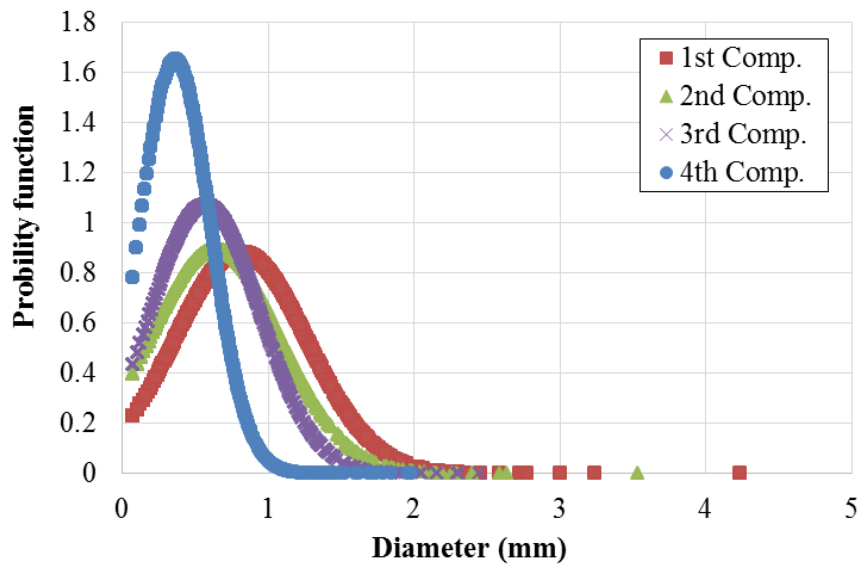

Figure C12. Normal size distribution of granules in each compartment in hybrid ABR at OLR of 3.5 kg COD/m^3.d

APPENDIX D   SAMPLE CALCULATION FOR SOLID RETENTION TIME

For the OLR of 6 kg COD/m^3.d in table 4-1, the calculations are as follows:

The samples were taken from different heights of sludge blanket.

Table D1. VSS values at various heights of sludge blanket in different compartments at the OLR of 6 kg COD/m^3.d

sample	Height (cm)	VSS (g/L)
1st Comp.	18	20.59
	9	20.31
	1	19.25
2nd Comp.	19	20.85
	9	21.19
	1	20.87
3rd Comp.	17	21.80
	8	20.70
	1	21.26
4th Comp.	17	20.88
	8	21.19
	1	20.90
Average		20.82
Standard deviation		0.59

The sludge blanket volume was 20.24 L.

Total amount of sludge $=$ Sludge blanket volume (L) $\times$ Average VSS (g/L)

Total amount of sludge $= 20.24$ L $\times 20.82$ g/L $= 421.34$ g VSS

Biomass concentration $=$ Total amount of sludge / Reactor volume

Biomass concentration $= 421.34$ g VSS/27.5 L $= 15.34$ g VSS/L

Table D2. Biomass washout from the hybrid ABR at the OLR of 6 kg COD/m³.d

Sample	Sample No.	VSS (mg/L)
Effluent	1	2925
Effluent	2	2955
Effluent	3	3089
Average		2989
SD		87

The effluent flow rate was 2.36 L/d

Biomass washout = effluent flow rate × average VSS in effluent

Biomass washout = 2.36 L/d × 2.99 g VSS/L = 7.05 g VSS/d

$$SRT = \frac{Reactor\ volume \times Biomass\ concentration}{Biomass\ washout}$$

$$SRT = \frac{27.5\ L \times 15.34\ g\ VSS/L}{7.05\ g\ VSS/d} = 59.83d\ or\ {\sim}60d$$

In this section, one-way analysis of variance (ANOVA) is used for statistical analysis. In the following tables, the means SS is sum-of-squares, df shows the degree of freedom, MS is mean squares, F is the F-statistic which shows significance.

The difference is considered statistically significant at $p < 0.05$ level

Statistical analysis for table 3-3 in chapter 3:

Table E1. ANOVA for comparison of "the biomass washout at OLR of 1.1 kg COD/m^3.d in the conventional ABR" and "the biomass washout at OLR of 1 kg COD/m^3.d in the hybrid ABR"

Source of Variation	SS	df	MS	F	P-value
Between Groups	0.1429	1	0.1429	171	0.0002
Within Groups	0.0033	4	0.0008		
Total	0.1462	5			

Table E2. ANOVA for comparison of "the biomass washout at OLR of 1.8 kg COD/m^3.d in the conventional ABR" and "the biomass washout at OLR of 2 kg COD/m^3.d in the hybrid ABR"

Source of Variation	SS	df	MS	F	P-value
Between Groups	1.3443	1	1.3443	2570	0.0000
Within Groups	0.0021	4	0.0005		
Total	1.3464	5			

Table E3. ANOVA for comparison of "the biomass washout at OLR of 2 kg COD/m^3.d in the hybrid ABR" and "the biomass washout at OLR of 3.5 kg COD/m^3.d in the hybrid ABR"

Source of Variation	SS	df	MS	F	P-value
Between Groups	1.5165	1	1.5165	326	0.0001
Within Groups	0.0186	4	0.0047		
Total	1.5351	5			

Statistical analysis for table 4-1 in chapter 4:

Table E4. ANOVA for comparison of "the biomass washout at RR of 20 in hybrid ABR" and "the biomass washout at RR of 10 in hybrid ABR"

Source of Variation	SS	df	MS	F	P-value
Between Groups	0.7333	1	0.7333	116	0.0004
Within Groups	0.0254	4	0.0063		
Total	0.7587	5			

Table E5. ANOVA for comparison of the biomass washout at HRTs of 20, 17.5, 15.6, 14, 12.7 and 11.7 days in hybrid ABR

Source of Variation	SS	df	MS	F	P-value
Between Groups	36.5483	5	7.3097	251	0.0000
Within Groups	0.3491	12	0.0291		
Total	36.8973	17			

Statistical analysis for Figure 5-1:

Table E6. ANOVA for comparison of final OD$_{680}$ of *C. sorokiniana*, *S. obliquus* and *C. Saccharophila* in AD5X (presented in Figure 5-1)

Source of Variation	SS	df	MS	F	P-value
Between Groups	2.3150	2	1.1575	133	0.0000
Within Groups	0.0522	6	0.0087		
Total	2.3672	8			

Table E7. ANOVA for comparison of final OD$_{680}$ of *C. sorokiniana*, *S. obliquus* and *C. Saccharophila* in SRD2X (presented in Figure 5-1)

Source of Variation	SS	df	MS	F	P-value
Between Groups	3.1417	2	1.5709	107	0.0000
Within Groups	0.0882	6	0.0147		
Total	3.2299	8			

Table E8. ANOVA for comparison of final OD$_{680}$ of *C. sorokiniana*, *S. obliquus* and *C. Saccharophila* in SRD5X (presented in Figure 5-1)

Source of Variation	SS	df	MS	F	P-value
Between Groups	1.2200	2	0.6100	58	0.0001
Within Groups	0.0636	6	0.0106		
Total	1.2836	8			

Statistical analysis for Figure 5-2:

Table E9. ANOVA for comparison of final biomass concentration of *C. sorokiniana* in "Chitosan treated", "high speed centrifuged" and "none treated" SRD2X (presented in Figure 5-2)

Source of Variation	SS	df	MS	F	P-value
Between Groups	0.1785	2	0.0893	20	0.0023
Within Groups	0.0273	6	0.0046		
Total	0.2059	8			

Table E10. ANOVA for comparison of final OD$_{680}$ of *C. sorokiniana* in "Chitosan treated", "high speed centrifuged" and "none treated" SRD2X (presented in Figure 5-2)

Source of Variation	SS	df	MS	F	P-value
Between Groups	0.2565	2	0.1282	8	0.0198
Within Groups	0.0952	6	0.0159		
Total	0.3517	8			

Table E11. ANOVA for comparison of final cell count of *C. sorokiniana* in "Chitosan treated", "high speed centrifuged" and "none treated" SRD2X (presented in Figure 5-2)

Source of Variation	SS	df	MS	F	P-value
Between Groups	7.68E+14	2	3.84E+14	29	0.0008
Within Groups	7.90E+13	6	1.32E+13		
Total	8.47E+14	8			

Table E12. ANOVA for comparison of final biomass concentration of *C. sorokiniana* in "Chitosan treated" and "high speed centrifuged" SRD2X (presented in Figure 5-2)

Source of Variation	SS	df	MS	F	P-value
Between Groups	0.0095	1	0.0095	3	0.1746
Within Groups	0.0139	4	0.0035		
Total	0.0234	5			

Table E13. ANOVA for comparison of final OD$_{680}$ of *C. sorokiniana* in "Chitosan treated" and "high speed centrifuged" SRD2X (presented in Figure 5-2)

Source of Variation	SS	df	MS	F	P-value
Between Groups	0.0134	1	0.0134	2	0.1935
Within Groups	0.0221	4	0.0055		
Total	0.0355	5			

Table E14. ANOVA for comparison of final cell count of *C. sorokiniana* in "Chitosan treated" and "high speed centrifuged" SRD2X (presented in Figure 5-2)

Source of Variation	SS	df	MS	F	P-value
Between Groups	9.07E+13	1	9.07E+13	10	0.0352
Within Groups	3.70E+13	4	9.26E+12		
Total	1.28E+14	5			

Statistical analysis for Figure 5-5:

Table E15. ANOVA for comparison of lipid content of biomass at day 3 and day 18 (presented in Figure 5-5)

Source of Variation	SS	df	MS	F	P-value
Between Groups	28.54	1	28.5366	407	0.0000
Within Groups	0.28	4	0.0702		
Total	28.82	5			

Table E16. ANOVA for comparison of starch content of biomass at day 3 and day 18 (presented in Figure 5-5)

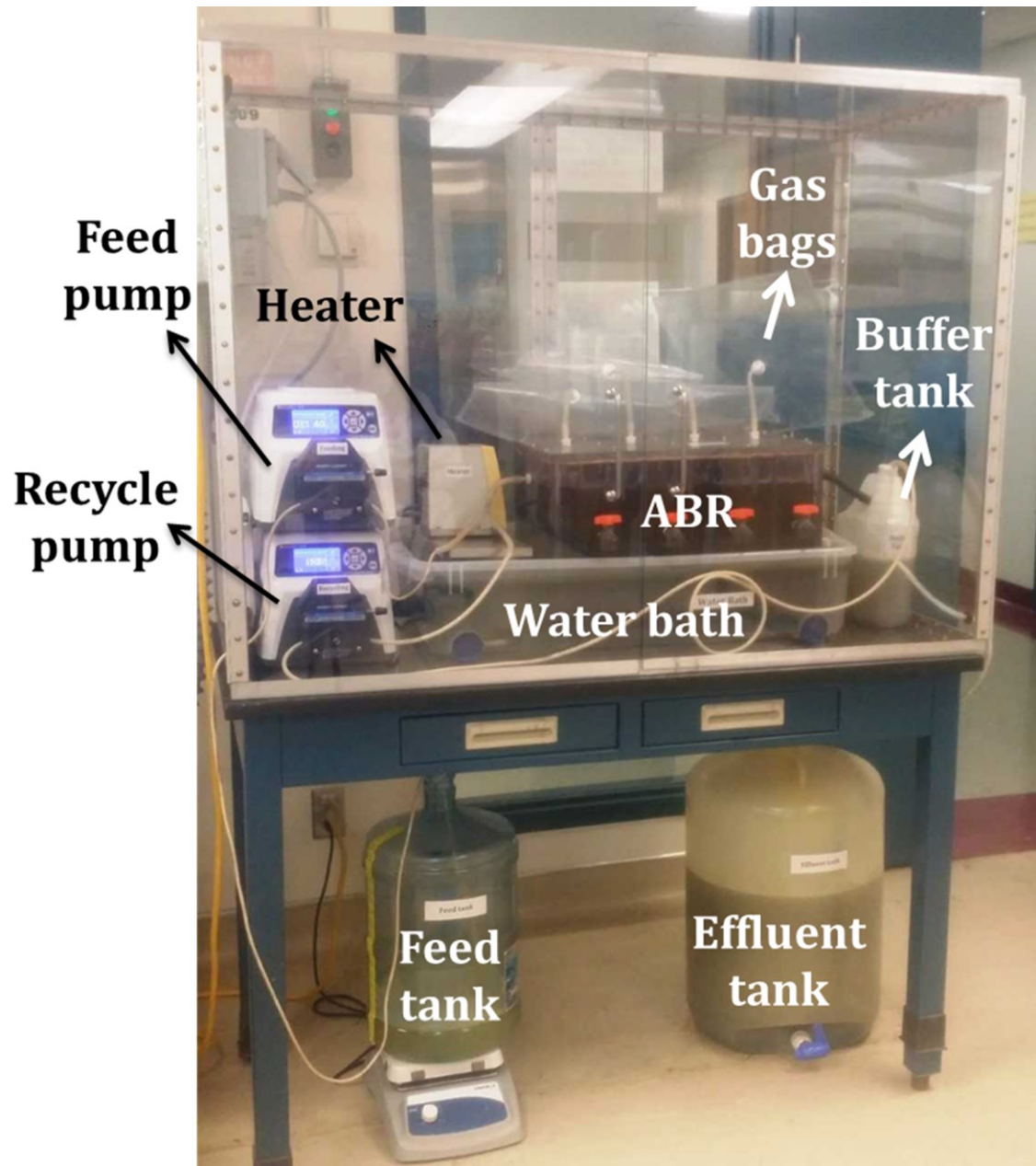| Source of Variation	SS	df	MS	F	P-value
Between Groups	31.7585	1	31.7585	37	0.0037
Within Groups	3.4158	4	0.8540		
Total	35.1743	5			

Figure F1. Picture of the ABR setup

Figure F2. Picture of 1 L photobioreactor in chapter 5