Renewable Energy-Assessment of Pre-treatment and Co-digestion Strategies to Optimize Methane Production from Microalgae

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Abstract

Anaerobic digestion (AD) of microalgae is an option to generate renewable energy in the form of methane-rich biogas. However, the high resistance of the cell wall, and unbalanced carbon to nitrogen (C/N) ratios can cause low methane production and an unstable AD process when using microalgae as the feedstock. Therefore, this research aims to optimize methane production from microalgae following pre-treatment and co-digestion strategies. Pre-treatment experiments were conducted in batch biochemical methane potential (BMP) tests, and the results showed that enzymatic hydrolysis offered a higher solubilisation of organic matters and methane yields compared to low-temperature thermo-alkaline pre-treatment. However, both pre-treatments were considered energetically efficient in pre-treating microalgae. Further BMP tests evaluated the feasibility of using potato processing waste (PPW) as a co-substrate with microalgae. The results showed that the addition of PPW to microalgae increased C/N ratios, methane production rates and final methane yields. BMP tests found that the addition of relatively high proportions of PPW could reduce the concentration of free ammonia nitrogen (FAN), and improve digestion performance and stability by reducing the likelihood of ammonia toxicity. The feasibility of using PPW as a co-substrate for co-digestion with microalgae was further evaluated in the semi-continuous co-digestion studies. The start-up strategy with immediate feeding enhanced methane yields significantly, and reduced the risk of ammonia toxicity for the methanogens. Co-digestion of microalgae with potato discarded parts (PPW_{dp}) produced higher methane yields than co-digestion with potato peel (PPW_{p}). This start-up strategy and co-digestion of microalgae with PPW_{dp} supported a high relative abundance of *Methanosarcina*. A second semi-continuous co-digestion study was carried out to optimize methane production from microalgae by co-digestion with PPW and glycerol. Results showed that glycerol added to mixtures of 25:75 microalgae: PPW enhanced methane production significantly. Glycerol dosage of 1% v/v could be the optimal dosage with highest specific methane production and stable digestion process. However, glycerol dosage of 2% v/v was more likely to accumulate volatile fatty acids (VFA), leading to an unstable digestion process. *Methaneosaeta* was abundant in the digesters employing co-digestion of microalgae and PPW_{dp} with or without glycerol. *Methaneosaeta* also predominated in the digester of co-digestion microalgae and PPW_{p} with glycerol, however a higher relative abundance of *Methanosarcina* was detected due to the accumulation of VFA. Overall, this research shows that pre-treatment and co-digestion strategies can improve methane production from microalgae, and the results are encouraging for the future use of microalgae as a sustainable AD feedstock.

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Journal Publications

A journal paper based on results of Chapter 4 of this thesis has been pubilised.
Zhang Y, Caldwell GS, Sallis PJ. Renewable energy: evaluation of low energy demand pre-treatments to optimize methane production from microalgae. IET Renewable Power Generation, 2019, *13 (10)*, *1701–1710*.

A journal paper based on results of Chapter 5 of this thesis has been pubilised.
Zhang Y, Caldwell GS, Zealand AM, Sallis PJ. Anaerobic co-digestion of microalgae Chlorella vulgaris and potato processing waste: Effect of mixing ratio, waste type and substrate to inoculum ratio. Biochemical Engineering Journal, 2019, 143, 91–100.

A journal paper based on results of Chapter 6 of this thesis has been pubilised.
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A paper manuscript based on results of Chapter 7 entitled '*Co-digestion of microalgae with potato processing waste and glycerol: effect of glycerol addition on methane production and the microbial community*' has been submitted and currently under review.

Conferences

An oral presentation entitled '*Assessment of co-digestion strategies to optimize methane production from microalgae*' has been presented at the Anaerobic Digestion Network: a BBRSC NIBB annual early career research conference. Birmingham, UK. 3-4 July, 2017.

An oral presentation entitled *'Co-digestion of microalgae with potato processing waste and glycerol: the effect of glycerol dosage on methane production and microbial diversity*' has been presented at the Anaerobic Digestion Network: a BBRSC NIBB annual early career research conference. York, UK. 16-17 July, 2018.

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Abbreviations

AD	Anaerobic Digestion
ANOVA	Analysis of Variance
BBM	Bold’s Basal Medium
BCA	Bicinchoninic Acid
BMP	Biochemical Methane Potential
BSA	Bovine Serum Albumin
CHP	Combined Heat and Power
C/N	Carbon to Nitrogen
COD_t	Total Chemical Oxygen Demand
COD_s	Soluble Chemical Oxygen Demand
CSTR	Continuously Stirred Tank Reactor
DW	Dry Weight
FAN	Free Ammonia Nitrogen
GHG	Greenhouse Gas
HRT	Hydraulic Retention Time
NMDS	Non-Metric Dimensional Scaling
OLR	Organic Loading Rate
OTU	Operational Taxonomic Unit
PBR	Photo Bio-reactor
PPW	Potato Processing Waste
PPW_{dp}	Potato Discarded Parts
PPW_p	Potato Peel
SIR	Substrate to Inoculum ratio
STP	Standard Temperature and Pressure
TA	Total Alkalinity
TS	Total Solids
TSS	Total Suspended Solids
VFA	Volatile Fatty Acids
VS	Volatile Solids

Chapter 1. Introduction

Global energy consumption is expected to grow by 28% from 2015 to 2040 (IEO, 2017), and fossil fuels including coal, oil and natural gas still remain the primary source for the energy sector. However, the utilisation of fossil fuels in the energy sector has led to increase levels of greenhouse-gas (GHG) emissions that represent nearly 66% of total anthropogenic GHG emissions (OECD/IEA, 2015). GHG warm the atmosphere, seriously affect rainfall patterns, cause the retreat of glaciers and sea ice, and raise sea levels due to climate change (Ramanathan and Feng, 2009). Therefore, in order to avoid further escalation of climate change, effective action should be taken in the energy sector, particularly using clean and new energy sources instead of conventional fossil fuels. Among other alternative energy sources, the fastest growing source predicated for the next few decade is renewable energy (Figure 1.1) (BP, 2018)

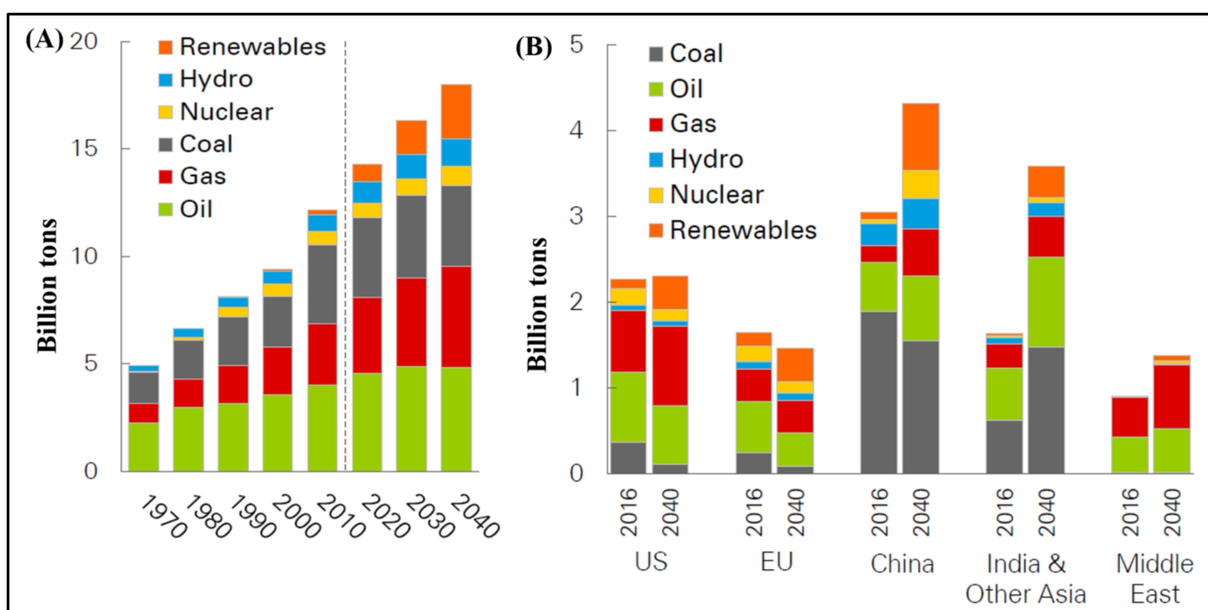


Figure 1.1 Projected energy demand by type of energy source: (A) global overview; (B) regional demand, adapted from BP (2018).

Renewable energy sources include wind, marine, solar, hydro, geothermal and biomass energy, among them biomass energy represents the largest proportion of 14% out of 18% of renewables in the 2016 energy mix (WEC, 2016). Biomass energy in the forms of heat, electricity and liquid fuels (biofuels) can be produced from a wide range of biomass sources such as virgin wood, energy crops, agricultural residues and food and industrial waste streams via various conversion processes (Ellabban *et al.*, 2014). Biomass energy shares similar

characteristics to that from fossil fuels, although it belongs to the renewable energy category. For example, biomass can be burned to obtain heat energy, and can also converted to various liquid and gas biofuels, which can be transported and stored for heat and power generation (Ellabban *et al.*, 2014). Therefore, biomass energy will play an important role in future energy scenarios, and a recently emerging strategy is to convert biomass into clean energy fuels via biorefinery and biotransformation technologies (Ellabban *et al.*, 2014).

Biorefinery is a sustainable process to obtain biofuels, energy and high value products via processes and equipment used for biomass transformation (Chew *et al.*, 2017). It has been identified as the most promising way to generate a biomass-based industry (González-Delgado and Kafarov, 2011). Biorefinery is classified according to the type of biomass feedstock (Gorry *et al.*, 2018). First-generation biorefineries are based on energy crops such as sugar cane, corn or soybean to produce biofuels including ethanol, butanol and biodiesel, chemicals (e.g. lactic acid, itaconic acid and 1,3-propanediol) as well as other valued products for feed and food applications (Gorry *et al.*, 2018). However, the main concern in using energy crops for the bioenergy market is that their production on agricultural land may detrimentally affect global food supplies (Dębowski *et al.*, 2017).

Lignocellulosic materials are used as the feedstock for second-generation biorefineries, where the lignocellulose can be converted to biofuels such as cellulosic ethanol and syngas, including CO, CO_2, H_2 and CH_4 (Gorry *et al.*, 2018). Compared to energy crops, using lignocellulosic biomass for biofuel production has advantages such as its abundance and a relatively cheap production process (González-Delgado and Kafarov, 2011). However, lignocellulosic biomass comprises a strong structural matrix formed by digestible polymers (cellulose and hemicellulose) being embedded within the relatively recalcitrant lignin component, and therefore an additional treatment is required for it to be broken down completely into simple sugars (Gabhane *et al.*, 2014).

Third-generation biorefineries are the most advanced process that can utilise a combination of technologies to convert a mixture of biomass sources into multiple valued products (Gorry *et al.*, 2018). Microalgae are considered to be a potential feedstock for third-generation biorefineries due to their cell characteristics that are capable of producing a multitude of products (Chew *et al.*, 2017). The interest in a microalgae biorefinery has four main advantages. In comparison with energy crops, microalgae may be grown independently of arable land, and their productivity may potentially reach 100 – 150 tonnes/ha/year, which is

10 – 15 times higher than that of conventional agricultural crops (Chinnasamy *et al.*, 2010). Secondly, the lignin component of microalgae biomass represents less than 2% (Ververis *et al.*, 2007), and this low lignin fraction can facilitate enzymatic access, leading to the enhancement of hydrolysis rates in microalgae biorefineries. Moreover, some microalgae strains can grow under saline conditions or in wastewater treatment systems, which reduces the need for freshwater (Pittman *et al.*, 2011; Arashiro *et al.*, 2018; Maeda *et al.*, 2018). More importantly, microalgal photosynthesis allows CO_2 fixation, which may mitigate the elevated CO_2 concentrations present in the atmosphere (Blair *et al.*, 2014).

Microalgae are considered as potential feedstocks for the generation different types of biofuels such as biodiesel, biogas, bioethanol, biohydrogen and bio-oil (Harun *et al.*, 2011; Ebrahimian *et al.*, 2014; Zhao *et al.*, 2014) via various conversion technologies including: thermochemical conversion, biochemical conversion, transesterification and photosynthetic microbial fuel cells (Naik *et al.*, 2010; Chew *et al.*, 2017). Currently, biogas and biodiesel are the most common types of biofuel because they can be used to replace natural gas and petroleum diesel, respectively (Alaswad *et al.*, 2015). Therefore, the two crucial techniques of microalgae biorefinery downstream processing are transesterification and anaerobic digestion (AD) which convert the microalgal biomass into biodiesel and biogas (Figure 1.2).

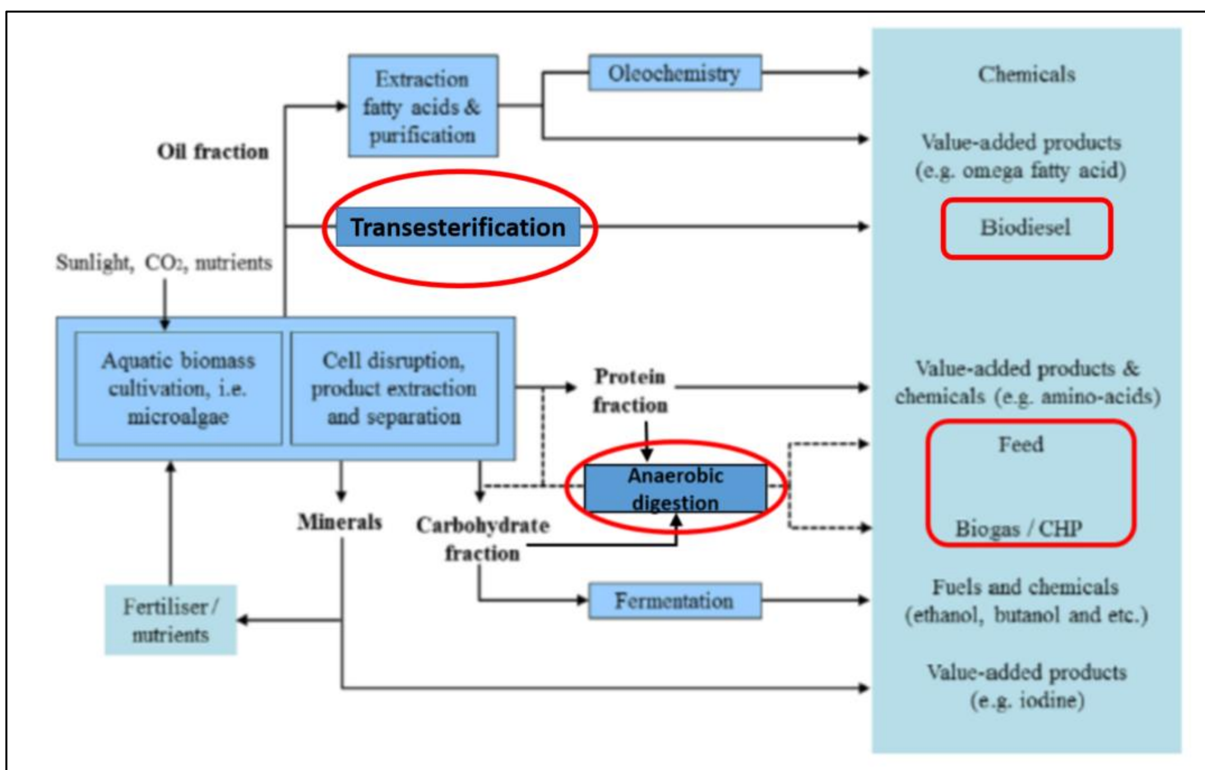


Figure 1.2 Flow diagram of a microalgae biorefinery (Chew et al., 2017).

The rapid growth rate of microalgae results in high oil productivity, ranging from 58,700 to 136,9000 L/ha, which is significantly higher than for other oil crops such as corn, soybean, coconut and oil palm (Chisti, 2007). Therefore, biodiesel production from microalgae remains a primary research focus. In addition to the lipid content, microalgal biomass contains carbohydrate, protein and other nutrients (Sialve *et al.*, 2009), and in order to improve the economic balance, after lipid extraction, microalgal residues could be converted to methane through AD (Chisti, 2007). Therefore, a simple scenario was proposed by Torres *et al.* (2013) in which, the lipid, protein and carbohydrate components of microalgae were assumed to be 30, 45 and 35% of volatile solids (VS), respectively. First, lipids were extracted from microalgal biomass, and used to produce biodiesel; and the residues of carbohydrate and proteins were used for methane production (Figure 1.3 A). In this conceptual process, biodiesel production was estimated to produce an energy yield of 11.7 MJ/kg, and an energy yield of 17.3 MJ/kg was produced by AD of the residues. However, microalgae biodiesel production is associated with substantial energy consumption, including the drying of biomass, lipid extraction and oil transesterification, with the energy required for drying accounting for nearly 80% of total energy consumption (Lardon *et al.*, 2009). The cultivation step is another area of concern when using microalgae as the feedstock for biodiesel production. Generally, closed photobioreactors are used for the cultivation of specific microalgae strains in order to avoid contamination, but this cultivation system requires high energy consumption for mixing the culture and pumping (Scott *et al.*, 2010). Moreover, the composition of growth medium needs to be controlled in order to improve the cell lipid content. For example, for most microalgae strains, a nitrogen-limited growth medium enhances their lipid content, but also limits their growth rate (Sialve *et al.*, 2009). Therefore, an alternative is using whole microalgae for biofuel production via AD. Lipids have the highest theoretical methane yield of 1.014 L CH_4/g VS, which is higher than the values of 0.415 and 0.851 L CH_4/g VS for carbohydrates and proteins, respectively (Montingelli *et al.*, 2015). In another conceptual process proposed by Torres *et al.* (2013) (Figure 1.3 B), AD of whole microalgae could potentially produce a total energy yield of 28.2 MJ/kg, which is similar to the level produced by the combined biodiesel/biogas production process (29 MJ/kg). Compared to biodiesel and bioethanol production, AD is a straightforward biological process where most microalgal macromolecules can be fermented to generate methane-rich biogas. Moreover, microalgal biomass can be used for the AD process without drying, and biogas generated by AD of microalgae can be used to produce both electricity and heat (Torres *et al.*, 2013).

In conclusion, the selection of production scenario depends on the lipid content in the microalgal biomass. Sialve *et al.* (2009) suggested that, if a microalgal cell lipid content is less than 40%, the AD of whole microalgae would be most favourable, whereas a combined biodiesel and biogas production process would be the preferred option when lipid levels are higher than 40%.

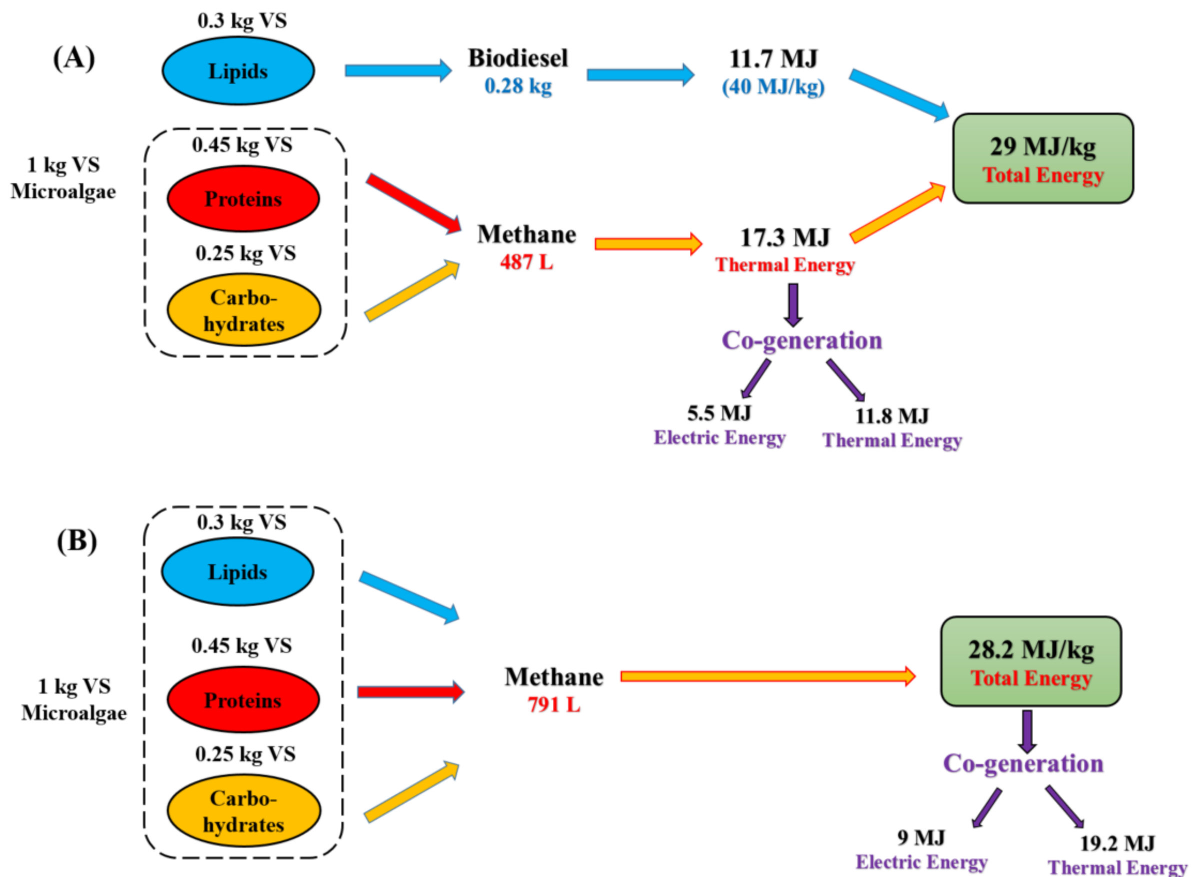


Figure 1.3 Potential alternatives for microalgae AD: (A) Biodiesel production and AD of microalgal residues to produce methane; (B) AD of whole microalgae for biogas production (Torres et al., 2013).

Studies of microalgae AD started in the 1960s, but there are still significant problems associated with the use of whole microalgae as a feedstock for AD. The theoretical methane yields of AD microalgae were estimated to range from 0.48 to 0.80 L CH_4/g VS (Sialve *et al.*, 2009). However, the experimental yields based on previous investigations are substantially lower at 0.05 – 0.39 L CH_4/g VS (Gonzalez-Fernandez *et al.*, 2012). Previously, several key factors have been identified that affect methane production, such as biomass moisture content,

concentration of digestible substrate, cell wall digestibility and the composition of cell macromolecules (Torres *et al.*, 2013; Montingelli *et al.*, 2015), and experimentation into how to break down microalgal cell walls and rebalance the cell composition remains a current research focus.

Previous investigations have tested a wide range of pre-treatments involving high energy demand, including mechanical (such as ultrasound), thermal and high-temperature thermo-chemical methods to enhance methane production from microalgae (González-Fernández *et al.*, 2012; Mendez *et al.*, 2013). However, these pre-treatments are associated with high energy inputs, which can be higher than the amount of energy recovered in the form of additional methane. These pre-treatments are energetically unbalanced, although they have been reported to improve methane yields efficiently. Therefore, research interest has been directed towards the use of low-energy demand pre-treatments for microalgal biomass. Enzymatic pre-treatment requires a mild pre-treatment temperature, and therefore it is an energetically competitive method. Moreover, the use of enzymes can reduce the possibility of releasing by-products that might inhibit the subsequent AD process (Mahdy *et al.*, 2014c). However, further research is still required to identify an effective enzymatic pre-treatment for microalgae prior to AD. Another energetically competitive method is chemical pre-treatment, and this also requires low energy inputs compared to thermal and ultrasonic pre-treatments (Cho *et al.*, 2013). However, in order to improve the pre-treatment efficiency, chemical pre-treatments are usually combined with high temperatures in pre-treating microalgal biomass (Cho *et al.*, 2013; Bohutskyi *et al.*, 2014). Therefore, from the energy viewpoint, there is a need to investigate the effect of low-temperature thermal-chemical pre-treatment of microalgal biomass for AD.

Microalgae without rigid cell walls could also represent a potential feedstock for AD (Mussgnug *et al.*, 2010), but most microalgae strains have high protein content that may lead to imbalanced carbon-to-nitrogen (C/N) ratios. Such an imbalance becomes an inhibitory factor when using microalgae as the feedstock for either the AD of whole cell or the combined biodiesel and biogas production process. Anaerobic co-digestion of microalgae with other carbon-rich feedstocks is the most commonly used strategy to rebalance the C/N ratio and to enhance methane production. Therefore, to further support the economic case for digesting microalgae, research must focus on assessing carbon-rich feedstocks already available as waste-streams, rather than feedstocks produced specifically for bioenergy production.

1.1 Aim

The aim of this research is to enhance methane production from microalgae through pre-treatment and anaerobic co-digestion strategies.

1.2 Objectives

The specific research objectives are:

1. To evaluate the effect of low energy pre-treatments on the degree of solubilisation of the microalgal biomass, and the subsequent methane yields achieved in batch biochemical methane potential (BMP) tests.

2. To evaluate the feasibility of using potato processing waste as a co-substrate for co-digestion with microalgae in batch BMP tests.

3. To evaluate the feasibility of using potato processing waste as a co-substrate for co-digestion with microalgae in semi-continuous reactor studies.

4. To evaluate the effects of glycerol supplementation on the co-digestion of microalgae and potato processing waste in semi-continuous reactor studies.

1.3 Thesis Structure

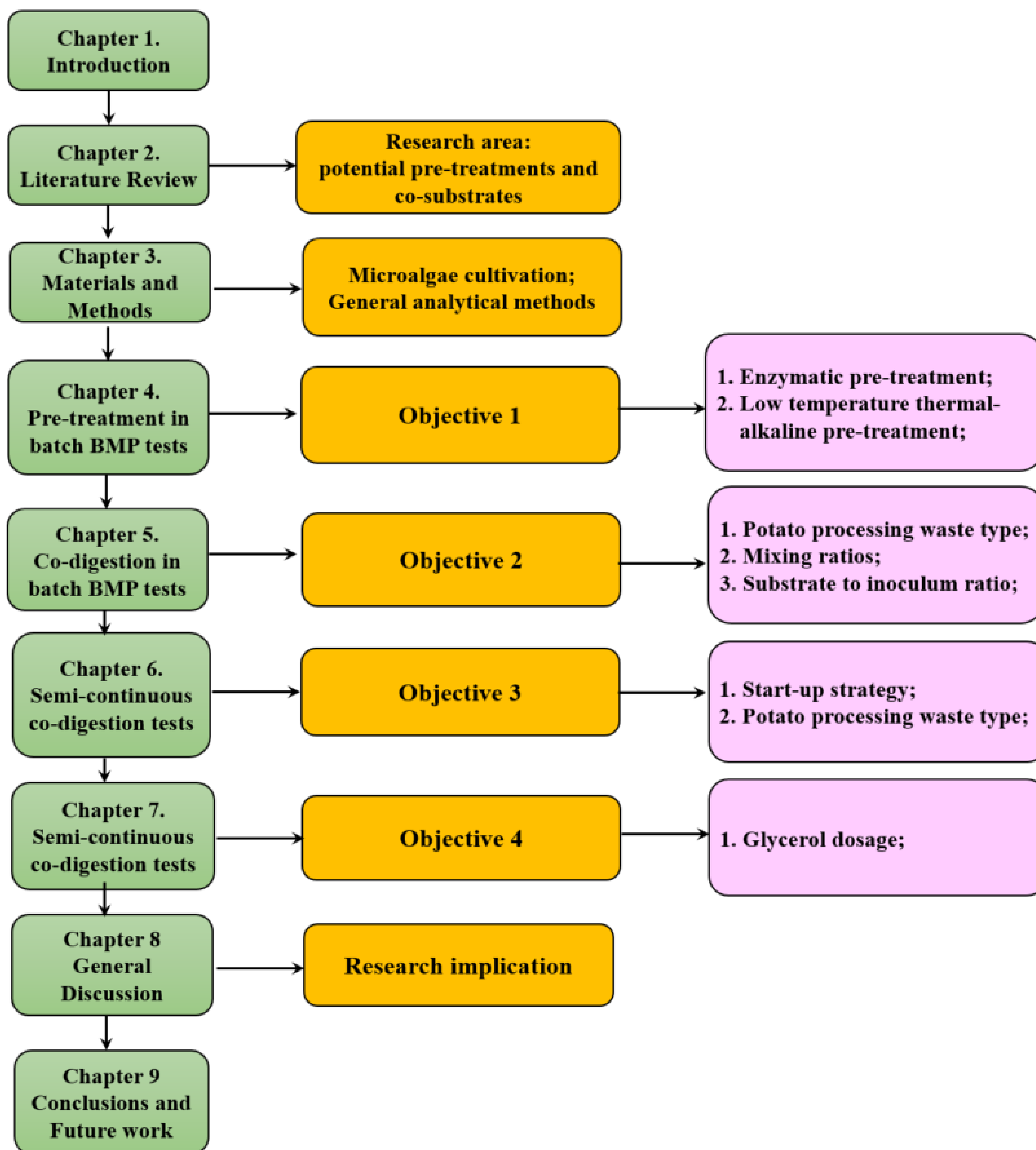


Figure 1.4 Flow diagram of structure of thesis

Chapter 2. Literature Review

2.1 Microalgae

2.1.1 *General characteristics and composition*

Algae are a diverse group of prokaryotic and eukaryotic organisms and, like terrestrial plants, most contain chlorophyll that can convert light energy into chemical energy via photosynthesis (Rocca *et al.*, 2015). The classification of algae is based on their properties such as cell structure, cell wall composition, pigmentation, storage products for photosynthesis and the lifecycle (González-Delgado and Kafarov, 2011). The 11 algae divisions are Cyanobacteria (blue-green algae), Glaucophyta, Rhodophyta (red algae), Chlorophyta (green algae), Charophyta, Haptophyta, Cryptophyta, Ochrophyta, Chlorarachniophyceae, Dynophyceae and Euglenophyceae (Barsanti and Gualtieri, 2014). Algae are classified in two general groups of microalgae and macroalgae based on their size, and microalgae are the focus of this research and detailed information about them is provided below.

Microalgae are a group of single-cell photosynthesising microorganisms that grow in both marine and fresh water environments (González-Delgado and Kafarov, 2011; Maeda *et al.*, 2018). Microalgae are very small plant-like organisms around 1 – 50 micrometres in diameter. They share similar photosynthetic mechanisms with land-based plants, but can more efficiently access carbon dioxide (CO_2) and nutrients due to their simple cellular structure. Moreover, microalgae live submerged in an aqueous environment where they can gain access to water easily (Carlsson *et al.*, 2007). Microalgae are a heterogeneous group of prokaryotic (Cyanbacteria) and eukaryotic organisms (Carlsson et al., 2007; Maeda et al., 2018). Based on their chemical and morphological characteristics, microalgae can be classified as green algae (Chlorophyceae), golden-brown algae (Chryophyceae), diatoms (Bacillariophyceae) and blue-green algae (Alaswad *et al.*, 2015; Rocca *et al.*, 2015). Microalgae exhibit an enormous diversity with more than 50,000 species, but only about 30,000 of them have been studied and analysed (González-Delgado and Kafarov, 2011).

Protein, carbohydrate and lipid are the main chemical components of microalgae, and the proportions of these macromolecules are strongly affected by species differences and environmental conditions such as light source and intensity, temperature, growth conditions and nutrients (Sialve *et al.*, 2009). Based on data provided in the literature (Rocca *et al.*, 2015), the major component is protein, with concentrations ranging from 30 – 71% of dry weight (DW) (Figure 2.1). The concentrations of carbohydrate varies between 4 – 58% DW. Lipid concentrations are reported at between 2 and 45 % DW (Rocca *et al.*, 2015), but this may increase up to 63% DW under certain growth conditions such as low nitrogen (Sialve *et al.*, 2009).

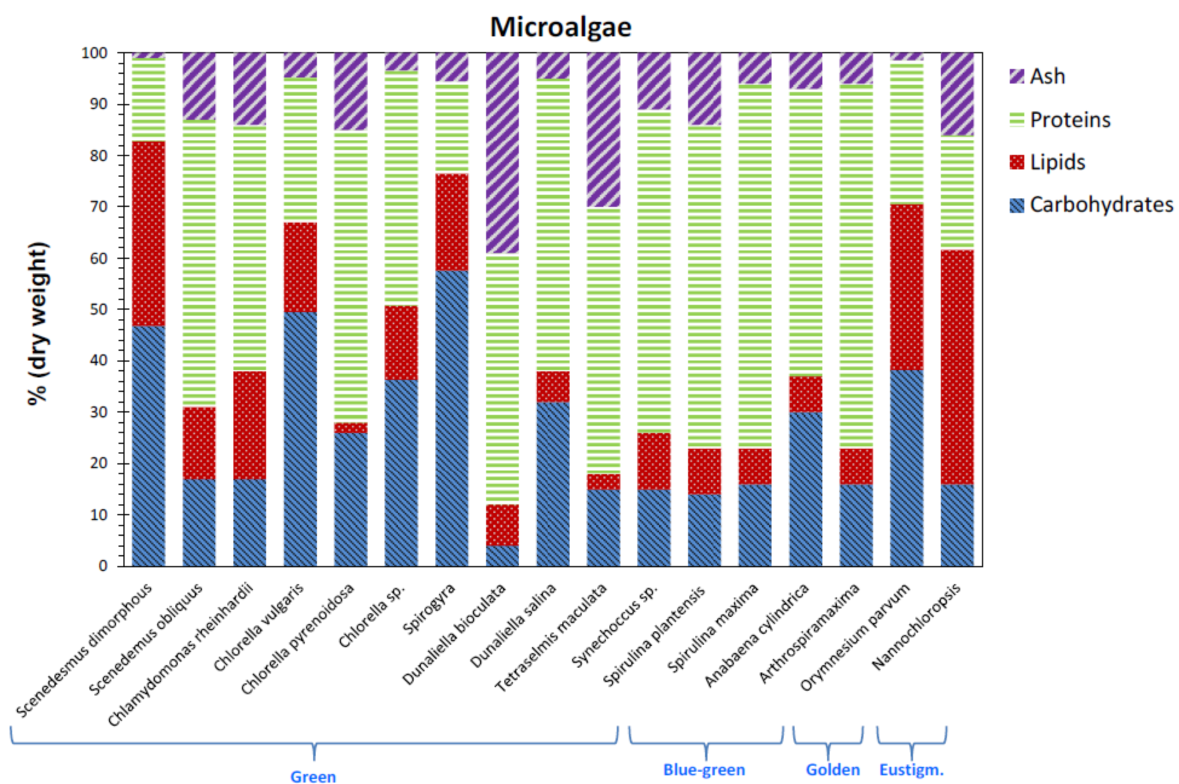


Figure 2.1 Chemical composition of microalgae based on different species, adopted from Rocca et al. (2015).

2.1.2 *Microalgae cultivation and harvesting*

In comparison with conventional agricultural crops, microalgae can be cultivated in various environmental conditions such as freshwater, seawater and wastewater. Table 2.1 compares the biomass productivity of microalgae and conventional land-based crops.

Table 2.1 Biomass productivity of land-based plants and microalgae, adapted from Uggetti et al. (2014).

	Biomass Productivity (dry tons/ha.y)
Rapa seeds	2.7
Corn grain	7
Mixed grasses	3.6 – 15
Woody biomass	10 – 22
Sugarcane	73 – 87
Microalgae (*Tetraselmis suecica*)	10 – 22
Microalgae (*Arthrospira*)	27 – 70

The commercial large-scale cultivation of microalgae started in the early 1960s, where the microalgae strain *Chlorella* was cultivated in Japan (Borowitzka, 1999). The culture system for cultivating microalgae depends on the species to be cultivated and products to be harvested, and recent cultivation technologies include open and closed systems (Ozkan *et al.*, 2012) (Table 2.2). Open systems include large shallow ponds, tanks, circular ponds and race-way ponds, which are relatively simple and inexpensive to set-up and operate, and sunlight can be used as the light source (Borowitzka, 1999; Ozkan *et al.*, 2012). However, a large pond is difficult to monitor in comparison with a closed system. Therefore, this type of culture system is not suitable for cultivating fast-growing species. Closed systems such as flat and tubular photo bio-reactors (PBRs) are commonly used for high value products such as pigments and vitamins (Ozkan *et al.*, 2012). Compared to open systems, most closed systems are operated indoors using artificial lighting, which is associated with high energy costs (Borowitzka, 1999). However, unlike open systems, the intensity and time of lighting, temperature, culture medium and environment can be controlled; and also the potential risk of predators, parasites and competitive algae species gaining access to systems can be reduced (Dębowski *et al.*, 2013).

Table 2.2 Comparison of culture systems for microalgal biomass production

Culture system	Biomass concentration (kg/m^3)	Productivity (g/m^2.day)	Photosynthetic efficiency (%)	References
Open	0.1 – 0.5	4.0 – 21.0	1.0 – 4.0	Borowitzka (1999) Ozkan *et al.* (2012) Hase *et al.* (2000) Stephens *et al.* (2010)
Closed	2.0 – 8.0	10.2 – 22.8 (Flat PBRs) 13.0 – 47.7 (Tubular PBRs)	2.3 – 9.6	Pulz (2001) Brennan and Owende (2010)

When a microalgae culture has reached the stationary phase, the microalgal biomass needs to be recovered from the water medium prior to downstream processing. The selection of harvesting technology is dependent on the characteristics of microalgae such as size, density and the value of the target products (Brennan and Owende, 2010). In general, microalgae harvesting involves two steps of bulk harvesting (or separation) and thickening (or dewatering) (Brennan and Owende, 2010; Rocca *et al.*, 2015). Bulk harvesting aims to separate the microalgal biomass from the bulk suspension, and the concentration of biomass after this step will reach to 6 – 20% of the total suspended solids (TSS) (Rocca *et al.*, 2015). Flocculation, flotation or gravity sedimentation can be used in this step, and the selection of technology depends on the initial biomass concentration. Thickening is a more energy intensive step that aims to further concentrate the slurry via centrifugation, filtration or ultrasonic aggregation technologies (Brennan and Owende, 2010). After this step, the concentration of microalgal biomass will reach 10 – 25% TSS (Rocca *et al.*, 2015).

2.1.3 *Applications and algal biofuels*

Microalgae are capable of accumulating high-value products used to produce pigments such as β-carotene and chlorophyll, vitamins including A, B, B_2, B_6 and C, minerals like iron, potassium, calcium and magnesium, antioxidants and nutraceuticals (de Souza *et al.*, 2018; Khanra *et al.*, 2018; Rizwan *et al.*, 2018). The bio-products from microalgae can be used in commercial, industrial, environmental and medical areas (de Souza *et al.*, 2018; Rizwan *et al.*, 2018).

Microalgae are also considered to be potential sources to generate different types of biofuels such as biodiesel, biogas, bioethanol, biohydrogen and bio-oil through various conversion technologies (Harun *et al.*, 2011; Ebrahimian *et al.*, 2014; Zhao *et al.*, 2014) (Figure 2.2). Conversion technologies include thermochemical or biochemical conversion, transesterification and photosynthetic microbial fuel cells (Naik *et al.*, 2010; Chew *et al.*, 2017). The selection of conversion technology depends on the microalgae strain, economic factors, process specifications and the final products. Among the latter, biogas and biodiesel are the most common types of biofuels because they can be used to replace natural gas and petroleum diesel, respectively (Alaswad *et al.*, 2015). Indeed, most contemporary studies focus using microalgae to produce biodiesel. However, when compared to the biodiesel production process, AD is a more straightforward biological process used to produce bioenergy without biomass concentration and drying, lipid extraction and fuel conversion. Moreover, compared to biodiesel and bioethanol production, most microalgae macromolecules can be fermented via AD to generate methane-rich biogas. Additionally, the microalgae cultivation process can be integrated with the AD process. In this integrated process, not only can the liquid phase of the digestate generated by the AD process be used as a nutrient and inorganic carbon source for the cultivation of microalgae, but also the generated energy such as heat and electricity can be used for cultivation and downstream processes (Gonzalez-Fernandez *et al.*, 2015). Therefore, the microalgae AD process for bioenergy production may be less complex.

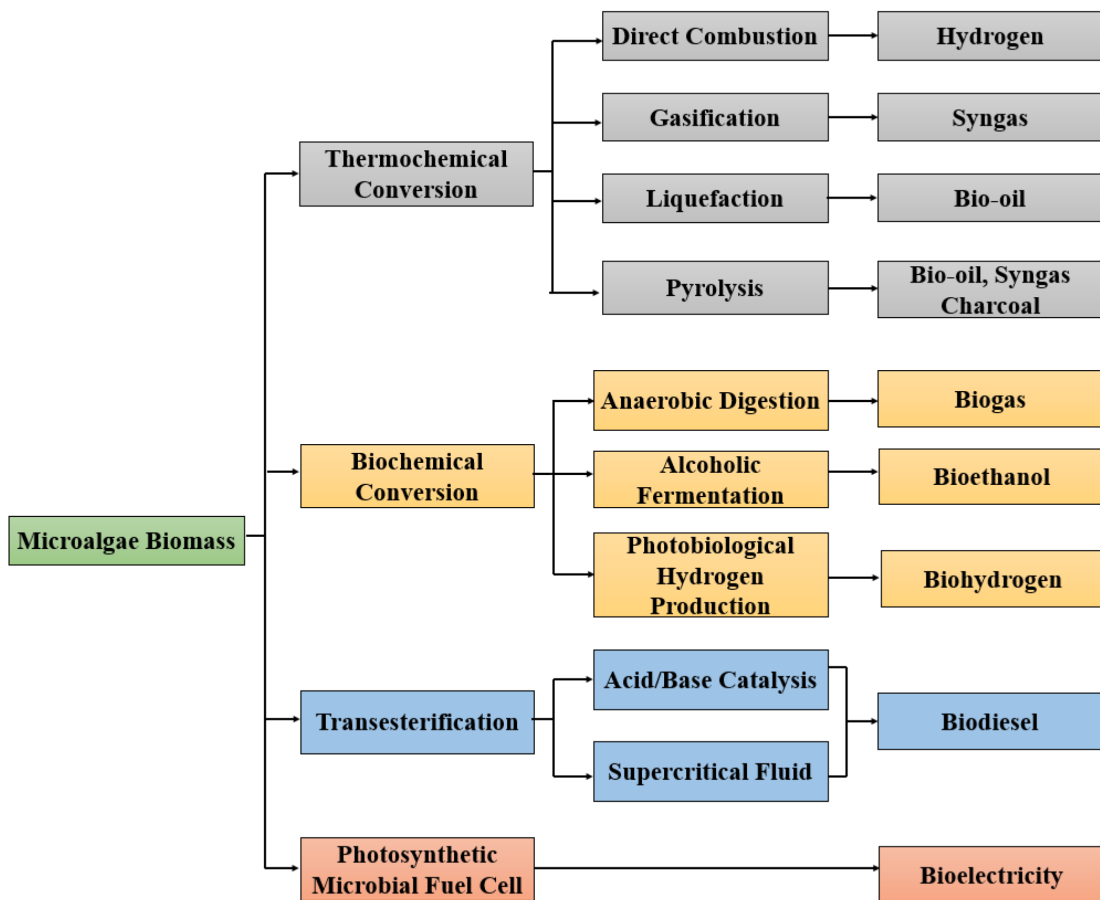


Figure 2.2 Conversion processes of microalgal biomass for biofuel production, adapted from Naik et al. (2010) and Chew et al. (2017).

2.2 Anaerobic digestion

Anaerobic digestion (AD) is a robust biochemical conversion process whereby macromolecules such as carbohydrates, proteins and lipids can be degraded by anaerobic microorganisms to generate biogas in an oxygen-free environment, with the digestate potentially used as a nutrient fertilizer in agriculture (Ward *et al.*, 2008; Appels *et al.*, 2011). The main composition of biogas is around 40% carbon dioxide (CO_2) and 60% methane (CH_4), and the percentages vary depending on the digested feedstocks (Maeda *et al.*, 2018). Methane is the simplest hydrocarbon, and produces more heat per unit of mass (55.7 kJ/g) than other more complex hydrocarbons. Moreover, the burning of methane produces less CO_2 for each unit of heat than other hydrocarbon fuels. Therefore, methane is a promising valuable biofuel generated from the AD process. Moreover, methane could be used in combined heat and power (CHP) systems to produce heat and electricity (Wang *et al.*, 2016).

2.2.1 *Anaerobic digestion process*

AD occurs in four key biological and chemical steps, including hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 2.3).

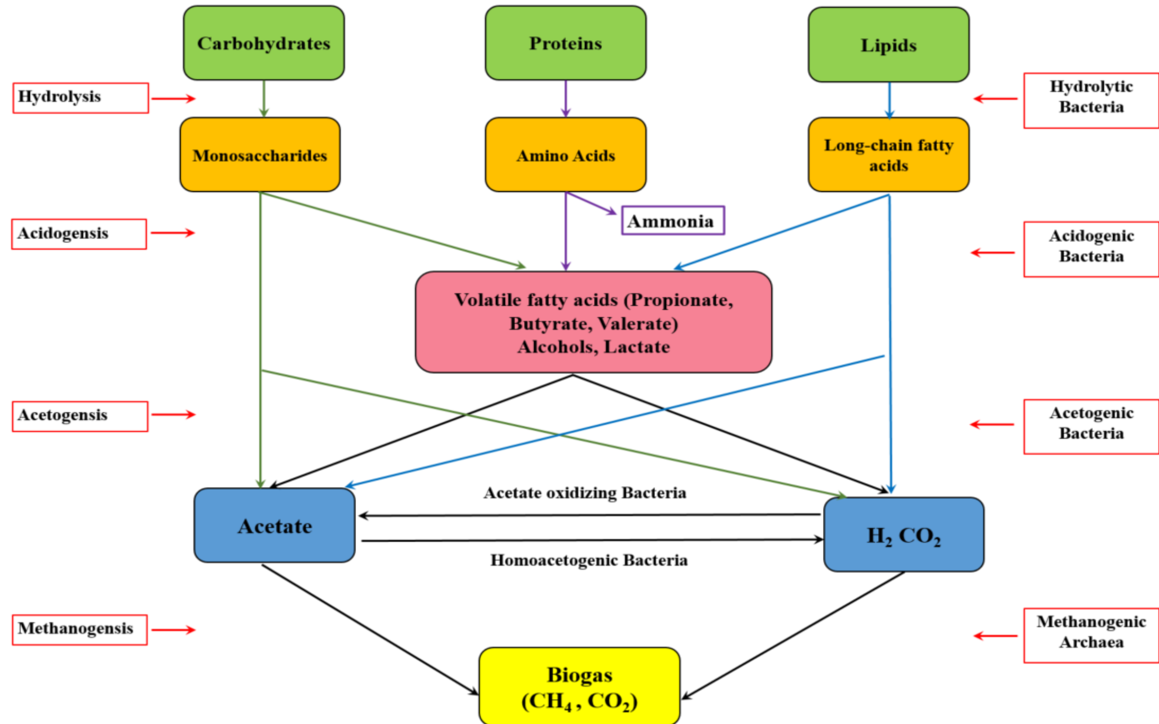
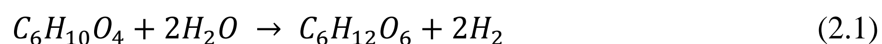


Figure 2.3 Pathway of anaerobic digestion, adopted from Kangle et al. (2012) and Christy et al. (2014).

2.2.1.1 Hydrolysis

The initial stage in AD is hydrolysis, where complex organic polymers such as polysaccharides, proteins and lipids are catabolized into simple sugars, amino acids and long-chain fatty acids and other simple organic compounds (Kangle *et al.*, 2012). An example of a hydrolysis reaction of catabolize organic waste into the simple sugar such as glucose is shown in Equation 2.1 (Christy *et al.*, 2014).



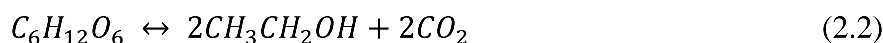
$$C_6H_{10}O_4 + 2H_2O \rightarrow C_6H_{12}O_6 + 2H_2 \qquad (2.1)$$

This step is crucial in the AD process due to the large size of organic polymers which cannot be directly consumed by microorganisms. Therefore, a group of hydrolytic microorganisms such as *Bacteroides*, *Butyrivibrio*, *Clostridia*, *Fusobacterium*, *Micrococci* and *Streptococcus*

secrete different extracellular enzymes, including cellulase, xylanase, amylase, protease and lipase, to assist in the degradation of these polymers (Christy *et al.*, 2014). However, the AD of substrates containing more structural stable compositions such as cellulose and hemi-cellulose requires longer for hydrolysis than the digestion of proteins (Adekunle and Okolie, 2015). Therefore, this step has been reported to be a rate limiting stage in the AD process (Adekunle and Okolie, 2015).

2.2.1.2 Acidogenesis

The second stage is acidogenesis, where sugars, amino acids and long-chain fatty acids are further catabolized by fermentative microorganisms such as *Bacillus*, *Escherichia coli*, *Lactobacillus*, *Streptococcus* and *Salmonella* to become acetate, propionate, butyrate and other short-chain fatty acids as well as ethanol (Dioha *et al.*, 2013; Christy *et al.*, 2014). The typical acidogenesis reactions are shown in Equations 2.2 – 2.4 (Christy *et al.*, 2014).



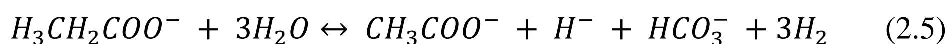
$$C_6H_{12}O_6 \leftrightarrow 2CH_3CH_2OH + 2CO_2 \quad (2.2)$$

$$C_6H_{12}O_6 + 2H_2 \leftrightarrow 2CH_3CH_2OH + 2H_2O \quad (2.3)$$

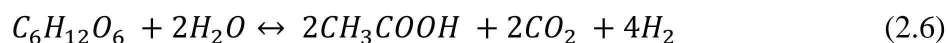
$$C_6H_{12}O_6 \rightarrow 3CH_3CH_2OH \quad (2.4)$$

2.2.1.3 Acetogenesis

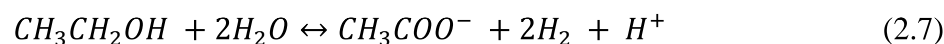
The third step is acetogenesis or acetogenic oxidation, where propionate, butyrate, valerate and simple molecules such as ethanol produced through acidogenesis stage are further catabolised by acetogenic bacteria to produce acetate and hydrogen (Dioha *et al.*, 2013). Equations 2.5 – 2.7 represent three pathways for the conversion of propionate, glucose and ethanol to acetate by actogenic bacteria (Christy *et al.*, 2014).



$$H_3CH_2COO^- + 3H_2O \leftrightarrow CH_3COO^- + H^- + HCO_3^- + 3H_2 \quad (2.5)$$



$$C_6H_{12}O_6 + 2H_2O \leftrightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (2.6)$$



$$CH_3CH_2OH + 2H_2O \leftrightarrow CH_3COO^- + 2H_2 + H^+ \quad (2.7)$$

As strict anaerobes, acetogenic bacteria have an optimum pH value around 6, and they have relatively slow growth rates, and are usually sensitive to environmental changes (Christy *et al.*, 2014). Acetogenic bacteria include *Sporomusa silvacetia*, *Acetobacterim woodii*, *Moorella thermoacetica* and *Thermoanaerobacter kibui* (Karnholz *et al.*, 2002).

2.2.1.4 Methanogenesis

The final step is methanogenesis, where methanogens use either acetate or hydrogen to produce methane (Alaswad *et al.*, 2015). Hydrogenotrophic methanogens can use CO_2 as the substrate to produce methane as shown in Equation 2.8, whereas acetotrophic methanogens utilise acetate as substrate (Equation 2.9) (Liu and Whitman, 2008; Christy *et al.*, 2014). Moreover, other methylotrophic methanogens and *Methanosarcina* belonging to the acetotrophic methanogen group can also use methylated C1 compounds as substrates to produce methane (Liu and Whitman, 2008).

$$CO_2 + H_2 \rightarrow CH_4 + 2H_2O \quad (2.8)$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (2.9)$$

In conclusion, AD is a promising technology for both waste treatment and the production of renewable energy. Generally, the AD process will be affected by operational factors such as temperature, pH, organic loading rate (OLR) and hydraulic retention time (HRT) as well as the characteristics of feedstock (Chen *et al.*, 2008; Mao *et al.*, 2015). The following sections discuss the applications and limitations when using microalgae as a substrate for AD.

2.3 Anaerobic digestion of microalgae

Studies of microalgae AD started in the 1960s, and a wide range of microalgae species have been studied for methane production in laboratory conditions (Table 2.3). By using the Buswell equation, the specific methane yields of carbohydrates, lipids and proteins are 0.415, 1.014 and 0.851 L CH_4/g VS, respectively (Sialve *et al.*, 2009). Generally, microalgae contain 4 – 58% of carbohydrates, 2 – 45% lipids and 30 – 71% proteins, therefore, the estimated theoretical methane potential of microalgae ranges from 0.47 – 0.80 L CH_4/g VS (Sialve *et al.*, 2009). However, the experimental methane yields have been reported from 0.05 – 0.44 L CH_4/g VS (Table 2.3). Previously, several key factors have been identified that affect microalgae methane yields, such as special biomass features, the configuration of the AD reactor and operating parameters such as HRT and OLR (Gonzalez-Fernandez *et al.*, 2015). Studies of how the features of microalgal biomass affect methane yields are the most common area of research.

Methane production is strongly dependent on the microalgae strains used (Mussgnug *et al.*, 2010). Therefore, in order to maximize methane production, the selection of ideal microalgae strains for AD should consider the following factors: (1) the microalgae has a thin or no cell wall (Torres *et al.*, 2013); (2) it has fast growth rate even a in non-sterile medium (Mussgnug *et al.*, 2010; Torres *et al.*, 2013); (3) it can be cultured in a waste-based system (Cho *et al.*, 2013); (4) it has high resistance against natural contaminants, and (5) it can be harvested from various natural environments (Cho *et al.*, 2013; Torres *et al.*, 2013).

Freshwater microalgae have been reported to produce 0.05 – 0.34 L CH_4/g VS of methane via AD (Table 2.3). In comparison with marine microalgae, freshwater microalgae have been previously studied the most for methane production due to their fast growth rate and, more importantly, they can be cultured using wastewater or AD digestate which may improve the overall economics of the process (Lim *et al.*, 2010; Gonzalez-Fernandez *et al.*, 2015). However, freshwater microalgae have been reported to exhibit hard cell walls that protect the cells from attack by anaerobic microorganisms, and consequently this limits methane yields (Cho *et al.*, 2013; Bohutskyi *et al.*, 2014; Gonzalez-Fernandez *et al.*, 2015). Moreover, the cell composition of organic macromolecules is directly linked to the acidogenesis step in the AD process, but high proteins composition in microalgae may lead to the production of more ammonia, and this may become an inhibitor for the AD process and consequently limit methane production (Torres *et al.*, 2013; Montingelli *et al.*, 2015).

Table 2.3 shows that 0.22 – 0.44 L CH_4/g VS of methane can be generated by AD of marine microalgae strains. The toxicity of compounds in terms of high levels of sodium still needs to be considered when utilizing marine microalgae as feedstock for AD, but this issue has been discussed and the use of a salt-adopted inoculum seems more efficient for the AD of saline waste (Sialve *et al.*, 2009). Therefore, studies have proven that it is feasible to use marine microalgae for methane production. Additionally, marine microalgae can be cultured using seawater as a culture medium, and this may reduce the cost of overall culturing (Santos *et al.*, 2014). In contrast to freshwater microalgae, some marine strains such as *Dunaliella salina* and *Isochrysis* have a simple cell membrane (D'Hondt *et al.*, 2018), and therefore they may have the potential to produce more methane. Indeed, Mussgnug *et al.* (2010) observed that AD of the marine green microalgae *Dunaliella salina* obtained higher methane yield than from the other freshwater microalgae strains *Chlorella kessleri*, *Euglena gracilis* and *Scenedesmus obliquus*. However, a marine microalgae like *Isochrysis galbana* lack of a rigid cell wall, as with freshwater strains their methane production potential is also limited due to high protein compositions (Caporgno *et al.*, 2015).

Table 2.3 Previous studies on the anaerobic digestion of different microalgae strains.

Microalgae Species	Fresh (F) or Marine (M) starins	Reactor Type	Temperature (℃)	Methane Yield (L CH_4/g VS)	References
Senedesmus obliquus	F	BMP	38	0.26	Ometto *et al.* (2014)
Chlorella sorokiniana	F	BMP	38	0.27	Ometto *et al.* (2014)
Arthrospira maxima	M	BMP	38	0.18	Ometto *et al.* (2014)
Dunaliella salina	M	BMP	38	0.32	Mussgnug *et al.* (2010)
Chlorella sp. and Scenedesmus sp.	F	BMP	37	0.34	Cho *et al.* (2013)
Nannochloropsis gaditana	M	BMP	35	0.30 – 0.33	Alzate *et al.* (2014)
Chlorella sp.	F	BMP	35	0.34	Bohutskyi *et al.* (2014)
Nannochloropsis sp.	M	BMP	35	0.36	Bohutskyi *et al.* (2014)
Chlorella vulgaris	F	BMP	35	0.14[a]	Mendez *et al.* (2013)
Isochrysis galbana	M	BMP	33	0.34	Caporgno *et al.* (2015)
Isochrysis galbana	M	BMP	50	0.22	Caporgno *et al.* (2015)
Dunaliella	M	BMP	35	0.44	Gonzalez-Fernandez *et al.* (2012)
Nannochloropsis salina	M	BMP	40	0.22	Schwede *et al.* (2013)
Scenedesmus sp.	F	CSTR	35	0.10 – 0.11[a]	González-Fernández *et al.* (2013)
Chlorella sp.	F	CSTR	37	0.12	Solé-Bundó *et al.* (2017b)
Chlorella vulgaris	F	CSTR	35	0.15 – 0.24	Gonzalez-Fernandez *et al.* (2012)
Chlorella and Scenedesmus	F	CSTR	35	0.09 – 0.14	Gonzalez-Fernandez *et al.* (2012)
Spirulina maxima	F	CSTR	35	0.09 – 0.15	Gonzalez-Fernandez *et al.* (2012)
Spirulina maxima	F	CSTR	55	0.05 – 0.11	Gonzalez-Fernandez *et al.* (2012)

[a] values correspond to L CH_4/g COD

2.3.1 *Degradability of the microalgae cell wall*

Low biodegradability is a common issue in the AD of different feedstocks such as sludge, lignincellulose and photosynthetic microorganisms (Bougrier *et al.*, 2006; Gabhane *et al.*, 2014; Magdalena *et al.*, 2018). During the AD process, the feedstock's cell walls are firstly degraded by the extracellular enzymes of hydrolytic bacteria, but the duration and rates of hydrolysis process strongly depend on the feedstock type (Magdalena *et al.*, 2018). For microalgal biomass, some strains which are able to thrive in wastewater effluents or severe environments such as high salinity have chemically and structurally robust cell walls (Safi *et al.*, 2014).

The microalgal cell wall represents 12.8 – 41.0% of the total cell mass, where carbohydrates and proteins are the main components representing 30.0 – 80.0 and 1.7 – 37.3% of the cell wall, respectively (Torres *et al.*, 2013). The carbohydrates in microalgae are complex, which consist of a mixture of neutral sugars, amino sugars and uronic acids (Templeton *et al.*, 2012). The composition of microalgal carbohydrates vary according to species and growth conditions. For example, the predominate monosaccharide in *C. vulgaris* is galactose, while mannose and glucose are the major sugars in *P. tricornutum* and *Nannochloropsis sp.* (Templeton *et al.*, 2012). Generally, microalgal cell walls have outer and inner layers. The outer layer of the microalgal cell wall is composed of specific matrix polysaccharides such as pectin, chitin agar or algaenan, while the inner layer contains microfibrillar cellulose as well as hemicellulose and glycoproteins (Demuez *et al.*, 2015). Similar to plants, the proportions of different compositions in microalgal cell wall are 25 – 30% of cellulose, 15 – 25% of hemicellulose, 35% pectin and around 5 – 10% glycoproteins (Mahdy *et al.*, 2014a). Cellulose is a complex, water-insoluble polysaccharide with a rigid linear structure (Festucci-Buselli *et al.*, 2007), and this structurally stable cellulose together with hemicellulose, proteins and other compounds forms a rigid cell wall that protects the microalgae against attack from hydrolytic bacteria, and consequently prevents efficient biodegradability and lows methane yields (Mahdy *et al.*, 2014c). Therefore, Mussgnug *et al.* (2010) reported that the presence of the cell wall and its composition are the main reasons affecting microalgae biodegradability and biogas production. Their results suggested that microalgae strains without a cell wall or with a protein-based cell wall should be preferred in terms of the efficiency of methane production. Moreover, energy consuming pre-treatment can potentially be avoided when digesting these types of microalgae. However, they also pointed out that microalgae without rigid cell walls in some respects may not be ideal substrates for AD because they may produce harmful

compounds which inhibit bacterial biocenosis, for example, inhibit the methanogenic archaea. Therefore, for those microalgae strains which have a robust cell wall, cell wall disruption in pre-treatment is needed to facilitate the access of extracellular enzymes that assist the anaerobic microorganisms in using organic matter of the microalgae (Magdalena *et al.*, 2018). More detailed information concerning microalgae pre-treatments is provided in Section 2.4 below.

2.3.2 *Carbon-to-nitrogen ratio*

The digestion potential of a feedstock is determined by its cell macromolecular composition, but the high proportions of proteins in microalgae may lead to an unbalanced carbon-to-nitrogen (C/N) ratio which is unsuitable for AD. The optimum C/N ratios for AD range from 20/1 to 30/1, but values for microalgae have been reported from 4.65/1 to 17/1 (Caporgno *et al.*, 2016; Rétfalvi *et al.*, 2016; Wang *et al.*, 2016; Li *et al.*, 2017). This imbalance leads to the release of extra nitrogen in the form of ammonia during AD, which may become an inhibitory to methanogens (Sialve *et al.*, 2009). Compared to other anaerobic bacteria, methanogens exhibit lower growth rates and low tolerance to toxic compounds, especially ammonia nitrogen (Magdalena *et al.*, 2018). The total ammonia nitrogen (TAN) exists in two forms of ammonium (NH_4^+) and free ammonia (FAN), and the proportions of these two forms depend on temperature and pH (Akindele and Sartaj, 2018). Generally, the methanogenesis stage could be inhibited by TAN and FAN at 1700 – 1800 and 150 mg/L, respectively (Yenigün and Demirel, 2013). The ammonia, are especially FAN, diffuses through the cell membrane of methanogens and neutralizes the membrane potential by changing the cytoplasmic pH; and at high concentrations may cause cell death (Martinelle *et al.*, 1996). Moreover, the activities of microorganisms which degrade propionic acids were reduced by higher ammonia concentrations, leading to an accumulation of propionic acids that further inhibits the methanogens, and consequently increases in volatile fatty acids (VFA) resulted in an imbalance in the AD system (Angelidaki and Ahring, 1993; Banks *et al.*, 2012). Therefore, different strategies have been reported to avoid ammonia inhibition, such as the cultivation of microalgae in a low-nitrogen medium, and sludge bioaugmentation including the use of an ammonia tolerant inoculum for the AD of microalgae. However, the most interesting strategy is the anaerobic co-digestion of microalgae with a high-carbon feedstock used to rebalance the C/N ratio and consequently to enhance the methane yields already reported in many previous studies, and detailed information concerning microalgae co-digestion is discussed in Section 2.5.

2.4 Microalgae pre-treatment

The mechanical strength, shape and rigidity of the microalgae cell wall mainly depend on the diversity of cell wall composition and its polymer arrangement (Demuez *et al.*, 2015). Therefore, effective pre-treatment must be optimized for the targeted cell wall to be disrupted. Different microalgae pre-treatments, namely mechanical (ultrasound), thermal, chemical (alkaline and acid hydrolysis), thermo-chemical and biological (enzymatic) processes have been reported (Cho *et al.*, 2013; Mahdy *et al.*, 2014b; Santos *et al.*, 2014). Based on the energy inputs involved, pre-treatments are classified into two groups of high- and low-energy demand pre-treatments (Table 2.4).

2.4.1 *Ultrasound pre-treatment*

Ultrasound treatment is the most common mechanical pre-treatment that has been applied to disrupt microalgae cell walls for methane production (Alzate *et al.*, 2012; González-Fernández *et al.*, 2012). The efficiency of ultrasound pre-treatment is strongly dependent on the specific supplied energy (E_s), and this can be calculated from the ultrasonic power, ultrasonic time, volume of treated solution and initial total solids (TS) of the raw biomass (Alzate *et al.*, 2012; González-Fernández *et al.*, 2012). For example, González-Fernández *et al.* (2012) found that *Scenedesmus sp.* pre-treated with the highest E_s of 128.9 MJ/kg TS increased methane yield by 87%, whereas the two low E_s values of 35.5 and 47.2 MJ/kg TS did not enhance methane yields compared to untreated microalgae. However, the high E_s is associated with high input energy, and in the most previous studies the additional methane production was not sufficient to balance the energy required for the pre-treatment of the microalgae (Ometto *et al.*, 2014). Therefore, there is some argument about whether or not a lower E_s has a positive effect on the energy balance. Passos *et al.* (2014) investigated the effect of low E_s (16.0 – 67.2 MJ/kg TS) on the AD of microalgae. In their studies, methane yields after pre-treatment were increased by 6 – 33% compared to untreated microalgal biomass. However, the energy input still cannot be balanced by the extra methane production. Therefore, the high input energy required is the main limitation of ultrasound pre-treatment when compared to thermal, chemical and biological methods (Magdalena *et al.*, 2018).

Table 2.4 Summary of previous studies on pre-treatment of microalgae for methane production.

	Pre-treatment	Microalgae Strains	Conditions	Methane yield increase (%)	References
High energy demand	Ultrasound	*Scenedesmus sp.*	35.5 – 128.9 MJ/kg TS for 15 – 30 min	up to 87.0	González-Fernández *et al.* (2012)
		Mixed culture	16.0 – 67.2 MJ/kg TS for 10 – 30 min	6.0 – 33.0	Passos *et al.* (2014)
	Thermal (>100 °C)	*Chlorella sp.*, *Nanochloropsis sp.*, *Thalassiosira weissflogii*, *Tetraselmis sp.*, *Pavlova_cf sp.*	121 °C for 30 min	up to 30.0	Bohutskyi *et al.* (2014)
		Mixed culture (*Chlamydomonas*, *Scendesmus*, *Nannochloropsis*)	110 – 170 °C for 15 min	19.0 – 46.0	Alzate *et al.* (2012)
	Thermo-chemical (>100 °C)	*Chlorella vulgaris*	4 M NaOH at 120 °C for 20 – 40 min	71.2 – 73.2	Mendez *et al.* (2013)
		Chlorella vulgaris	4 M H_2SO_4 at 120 °C for 20 – 40 min	59.7 – 64.7	Mendez *et al.* (2013)
Low energy demand	Enzymatic	*Chlorella vulgaris*	Protease at 0.146 – 0.585 AU/g DW	40.0 – 60.0	Mahdy *et al.* (2014c)
		Chlorella vulgaris *C. reinharidtii*	Cellulase at 0.3 mL/g DW	-3.0 – 14.0	Mahdy *et al.* (2014b)
		Scenedesmus obliquus, *Chlorella sorokininan*, *Arthrospira maxima*	Cellulase mixed with pectinase at 150 U/mL	324 – 690[a]	Ometto *et al.* (2014)
		Scenedesmus obliquus, *Chlorella sorokininan*, *Arthrospira maxima*	Esterase mixed with protease at 150 U/mL	218 – 735[a]	Ometto *et al.* (2014)
	Thermal (<100 °C)	Mixed culture (*Chlorella sp.* and *Scenedesmus sp.*)	50 and 80 °C for 30 min	4.5 – 14.3	Cho *et al.* (2013)
		Scenedesmus sp.	70 and 80 °C for 20 min	9.2 – 57.3	González-Fernández *et al.* (2012)
	Thermo-chemical (<100 °C)	*Chlorella vulgaris* *Scenedesmus sp.*	0.05 – 5% w/w NaOH at 50 °C for 24 – 48 h	up to 20.0	Mahdy *et al.* (2014a)

[a] values correspond to biogas enhancement.

2.4.2 Thermal pre-treatment

Thermal pre-treatment is able to break down the biomass cell wall, releasing both extracellular and intercellular materials, and it has been used to pre-treat different types of feedstocks such as sludge, manure and lignocellulosic materials (Carrere *et al.*, 2008; González-Fernández *et al.*, 2008). Thermal pre-treatment has also been applied to treat the microalgal biomass at temperatures ranging from 50 to 170 °C with different pre-treatment durations from minutes to hours. In terms of the energy demand of pre-treatments, thermal pre-treatment can be classified in two groups at low temperatures below 100 °C and high temperatures above 100 °C (Passos *et al.*, 2013). High-temperature thermal pre-treatment shows better hydrolysis efficiency and higher methane production compared to low-temperature methods. For example, a mixture of the microalgae *Chlamydomonas*, *Scendesmus sp*. and *Nannocloropsis* was pre-treated at high temperature ranging from 110 to 170 °C for 15 minutes, and methane production increased by 19 to 46 % (Alzate *et al.*, 2012). Similar results were found when using low-temperature thermal pre-treatment, where the mixed microalgae species *Chlamydomonas* and diatoms *Nitzchia* were pre-treated by various temperatures from 55 to 95 °C, and methane production was improved by 12 – 61% (Passos *et al.*, 2013). However, in their study, the pre-treatment time was set from 5 to 15 hours, which is longer than for high-temperature pre-treatment. Cho *et al.* (2013) tried to pre-treat a mixture of microalgae *Chlorella sp.* and *Scendesmus sp.* at the low-temperature of 50 and 80 °C within a short reaction time of 30 minutes. However, methane production only increased by 4.5 – 14.3% after pre-treatment. Moreover, in some respects, low-temperature thermal pre-treatment may not be efficient for the enhancement of methane production from microalgae. For instance, the methane production was not significantly enhanced when *Chlorella vulgaris* and *Scendesmus sp.* were treated at 55 °C for 24 and 48 hours of pre-treatment, respectively (Mahdy *et al.*, 2014a).

2.4.3 ***Chemical pre-treatment***

Compared to thermal pre-treatment, chemical pre-treatment requires low input energy (Cho *et al.*, 2013). Chemical pre-treatment, including ozonolysis, oxidative delignification (wet oxidation), acid and alkaline hydrolysis, is commonly used to treat various biomass such as lignocellulosic materials, sludge and solid waste (Genç *et al.*, 2002; Sun and Cheng, 2002; Torres and Lloréns, 2008; Kumar *et al.*, 2009). Among these pre-treatment methods, ozonolysis and oxidative delignification are used to degrade the lignin content in biomass

(Kumar *et al.*, 2009). Since the lignin composition in microalgal biomass is less than 2% (Ververis *et al.*, 2007), these two methods are not commonly used to treat microalgal biomass.

Several studies have reported the use of acid or alkaline hydrolysis of microalgae for the production of bioethanol, biogas and bio-hydrogen (Harun *et al.*, 2011; Yang *et al.*, 2011; Scholz *et al.*, 2013; Mahdy *et al.*, 2014a). For acid hydrolysis, concentrated acids such as H_2SO_4 or HCl are generally used to treat the biomass. Although they are powerful agents for degradation of cellulose, the drawbacks of using concentrated acids include the fact that corrosive and hazardous acids may damage the reactors, and, more importantly, toxic by-products may be formed that could inhibit the downstream process (Kumar *et al.*, 2009; Mendez *et al.*, 2013). Several studies consider the use of dilute acids instead of concentrated acids to treat the biomass (Mendez *et al.*, 2013). However, the use of dilute acids in the pre-treatment process requires high temperatures above 100 ℃, which means that the process may not be economically viable (Kumar *et al.*, 2009). Therefore, acid hydrolysis is not generally used to pre-treat microalgal biomass for methane production via the AD process. Alkaline hydrolysis causes less sugar degradation, and most of the caustic salts formed can be recovered and/or regenerated compared to with acid hydrolysis (Kumar *et al.*, 2009). Moreover, an alkaline pre-treatment of microalgae can enhance the solubilisation of both carbohydrates and proteins, whereas acid pre-treatment only enhances the solubilisation of carbohydrates (Mendez *et al.*, 2013). In order to enhance the efficiency of hydrolysis and methane production, the use of alkaline is normally combined with different pre-treatment temperatures in what is called thermo-alkaline pre-treatment (Mahdy *et al.*, 2014a).

2.4.4 *Thermo-alkaline pre-treatment*

Thermo-alkaline pre-treatment increases the surface area of the cellulose by causing the swelling of the biomass, and this decreases the degree of polymerization and cellulose crystallinity (Agbor *et al.*, 2011). Therefore, thermo-alkaline pre-treatment of microalgae is more effective in terms of hydrolysis efficiency and methane production than thermal or alkaline pre-treatments alone (Bohutskyi *et al.*, 2014). In thermo-alkaline pre-treatment, the temperature used is one of the critical factors that affect the pre-treatment efficiency. Based on the energy demand concerned, thermo-alkaline pre-treatment can also be divided into two groups of high-temperature (>100 ℃) and low-temperature (<100 ℃) pre-treatment. Several studies have used the high-temperature (>100 ℃) thermo-alkaline pre-treatment of the microalgal biomass to enhance methane production through AD. For instance, Bohutskyi *et*

al. (2014) used a high temperature of 121 ℃ combined with different concentrations from 0 to 20 g NaOH /L of sodium hydroxide (NaOH) to pre-treat five microalgae strains. They found that for the microalgae strains except *T. weissflogii*, the solubilisation of COD was significantly increased by up to 60% after high-temperature thermo-alkaline compared to alkaline pre-treatment. Moreover, at the end of digestion process, the biogas and methane yields for all microalgae strains were enhanced by up to 30 – 40% compared to untreated microalgae biomass. Mendez *et al.* (2013) applied high-temperature thermo-alkaline pre-treatment using 4M NaOH at 120 ℃ to *C. vulgaris* for 20 – 40 minutes, and found that a 73.2% increase in methane yields was achieved.

Previous studies have also investigated the effect of low-temperature (<100 ℃) thermo-alkaline pre-treatment on biomass solubilisation and methane production. For instance, Tsapekos *et al.* (2016) used different concentrations of 2, 4 and 6% w/w TS of NaOH to pre-treat digested manure biofibres at three temperatures of 55, 90 and 121 ℃. In their studies, rather than high-temperature thermo-alkaline pre-treatment, using 6% w/w NaOH at 55 ℃ was identified as the most efficient method, and enhanced methane production by 26%. Indeed, pre-treatment at low temperature below 100 ℃ has attracted close attention in recent research interests because high-temperature thermo-alkaline pre-treatment may involve organic losses due to the degradative reactions (Mahdy *et al.*, 2014a). Moreover, although the methane yields of microalgae are improved by high-temperature thermo-alkaline pre-treatment, the additional methane yield is still not enough to balance the energy required for the pre-treatment (Ometto *et al.*, 2014). Therefore, low-temperature thermo-alkaline pre-treatment could be considered as a suitable method to reduce the energy demand and improve the energy balance of an AD system (Passos *et al.*, 2013). However, compared to high-temperature thermo-alkaline pre-treatment, few investigations have used low-temperature techniques to pre-treat microalgal biomass for methane production.

The type of alkaline used is another factors that affect the efficiency of pre-treatment. Suitable alkaline pre-treatment agents include sodium, calcium, potassium and ammonium hydroxides and, of these four, NaOH has been studied the most for the pre-treatment of microalgal biomass for methane production (Cho *et al.*, 2013; Mendez *et al.*, 2013; Mahdy *et al.*, 2014a). However, drawbacks in using NaOH as the pre-treatment reagent should be considered, such as the high cost and the complex subsequent regeneration process (Kumar *et al.*, 2009). Moreover, applying NaOH may increase the concentrations of Na^+ in the digestate, which could be potentially be toxic to the methanogens (Feijoo *et al.*, 1995). Calcium hydroxide

($Ca(OH)_2$) is less expensive than other types of alkaline and can be regenerated using established lime kiln technology (Kumar *et al.*, 2009). Previously, lime has been used to pre-treat other substrates such as solid wastes and smooth cordgrass to improve their biodegradability and methane production via the AD process (Torres and Lloréns, 2008; Liang *et al.*, 2013). However, few investigations have used lime to pre-treat microalgae, especially at lower pre-treatment temperatures.

Other parameters such as the dosage of alkaline and pre-treatment time also need to be considered when applying thermo-alkaline pre-treatment to microalgae. Previously, it has been reported that methane yields are gradually enhanced by increasing the NaOH dosage at the same pre-treatment temperature when pre-treating manure (Tsapekos *et al.*, 2016). Similar results were found for thermo-alkaline pre-treatment of microalgae. For example, Mahdy *et al.* (2014a) found when pre-treating the microalgae *Scenedesmus* using NaOH at 50 ℃, the solubilisations of both COD and carbohydrate were increased with NaOH dosage from 0.05 to 5% w/w. Moreover, Solé-Bundó *et al.* (2017a) found that when using lime to pre-treat the microalgae *C. vulgaris* at the same pre-treatment temperature, the best hydrolysis efficiency and highest methane yield were observed with the highest dosage. Mahdy *et al.* (2014a) also investigated the effect of pre-treatment time of thermo-alkaline pre-treatment on microalgae methane production. In their study, NaOH was used to pre-treat *C. vulgaris* with two pre-treatment time of 24 and 48 hours. However, the results found that pre-treatment time had no significant effect on methane yields. Compared to NaOH, $Ca(OH)_2$ requires a relatively longer time for catalysis to occurs especially at lower temperature (Kumar *et al.* (2009). Previously, Liang *et al.* (2013) studied the use of lime for the pre-treatment of the smooth cordgrass of *Spartina alterniflora* at mild temperatures of 25 – 55 ℃. In their study, pre-treatment time was set at 7 – 28 days, and the highest methane yield was obtained by pre-treatment conditions of 28 days at 45 ℃. Since using $Ca(OH)_2$ to pre-treat microalgae has been little studied, it is also worth investigating the effect of pre-treatment time on methane production when using $Ca(OH)_2$ in the pre-treatment of microalgae.

2.4.5 ***Enzymatic pre-treatment***

Enzymatic pre-treatment requires a mild pre-treatment temperature and reduces the possibility of the release of by-products which inhibit the AD process (Mahdy *et al.*, 2014c). This pre-treatment may also have the potential to avoid the corrosion of equipment due to the low and neutral pH reaction conditions, thereby reducing maintenance costs (Demuez *et al.*, 2015). Consequently, enzymatic cell wall disruption methods are considered environmentally-friendly and less energy consuming than mechanical, thermal and chemical pre-treatments. This type of pre-treatment has been reported to be a promising strategy to break microalgal cell walls, thereby enhancing methane yields (Mahdy *et al.*, 2014b; Mahdy *et al.*, 2014c). Ometto *et al.* (2014) determined that the efficiency of enzymatic pre-treatment is linked to the composition of the microalgae cell wall. Therefore, the characteristics of the microalgal cell wall should be taken into consideration in the selection of enzymes.

Generally, microalgal cell walls are mainly composed of carbohydrates, and an efficient pre-treatment can be designed to hydrolyse both the cell wall and storage carbohydrates into simple sugars using a carbohydrase (Demuez *et al.*, 2015). Carbohydrase including cellulase, pectinase, amylase and β-glucanase have been reported to pre-treat the microalgal biomass, converting polysaccharides into simple sugars. Passos *et al.* (2016) used 1.0% w/w of cellulase to pre-treat microalgal biomass and compared to untreated biomass, biomass solubilisation in terms of values of soluble volatile solids (VS) increased by 110% and the methane yield was enhanced by 8%. Ometto *et al.* (2014) used pectinase to pre-treat the three microalgae strains *S.obliquus*, *C. sorokiniana* and *A. maxima*; however, the results showed that concentrations of soluble COD were only enhanced for *S. obliquus*, suggesting that this enzyme targeted a specific cell wall component. In the same study, α-amylase was also used to pre-treat microalgae and as with pectinase, it was particularly active on *C. sorokiniana*. Mahdy *et al.* (2014b) used the commercial enzyme Viscozyme L to pre-treat the microalgae *C. vulgaris* and *C. reinhardtii*. Viscozyme L is a commercial cocktail including the main activity of β-glucanase and other side activities of arabanase, cellulose, b-glucanase, hemicellulase and xylanase. In their study, *C. vulgaris* pre-treated by carbohydrolase at 0.3 ml/g dry weight (DW) increased methane production by 14% compared to untreated microalgae. However, for the pre-treatment of *C. reinhardtii*, the results showed that methane production was not improved significantly.

Protease is able to hydrolyze peptides into amino acids, and since proteins are also components of the microalgae cell wall, therefore the use of protease to degrade the protein fraction in microalgae has been reported. Mahdy *et al.* (2014c) used protease to pre-treat *C. vulgaris*, and after enzymatic hydrolysis the methane yields were enhanced by 50 – 70% in batch BMP tests. In the semi-continuous AD process, the reactor feeding with pre-treated *C. vulgaris* by protease could enhance methane yield 2.6-fold compared to the reactor feeding with untreated microalgae (Mahdy *et al.*, 2015).

A single enzyme may have limited hydrolytic activity, whereas mixtures of enzymes can exhibit a synergistic effect, improving the efficiency of hydrolysis (Yang *et al.*, 2010; Alvira *et al.*, 2011). Therefore, previous studies have investigated using mixtures of enzyme to treat feedstocks for methane production. For example, Yang *et al.* (2010) investigated the effect of enzymatic hydrolysis on sludge solubilisation in AD conditions. In their study, the highest solubilisation of sludge observed was 68.43% using a mixture of protease and amylase, which was higher than the values obtained by only protease (39.70%) or amylase (54.24%). Yu *et al.* (2013) also applied enzymatic hydrolysis to waste activated sludge for biogas production via AD. In their study, the final biogas production was improved by 20.2% when sludge was treated by amylase mixed with protease, whereas it was enhanced by 18.6 and 15.6% treated by amylase or protease alone, respectively. For the pre-treatment of microalgal biomass, Ometto *et al.* (2014) investigated that the use of a mixture of cellulase and protease at 150 u/mL to pre-treat the three microalgae strains *Scenedesmus obliquus*, *Chlorella sorokiniana* and *Arthrospira maxima*. In their study, after enzymatic hydrolysis, the maximum biogas improvement occurred with *A. maxima* yielding 1996 ± 254 mL/gVS_{added} (+ 898%) followed by *S. obliquus* (1669 ± 63 mL/gVS_{added}, + 485%) and *C. sorokiniana* (1292 ± 148 mL/gVS_{added}, + 387%). Moreover, compared to other methods such as ultrasound and thermal pre-treatments, enzymatic pre-treatment is a most promising method with low energy demand. However, previous studies have mostly focused on the pre-treatment of microalgae using single enzymes, and only a few investigations have considered the effect of mixed enzymes on microalgae AD. Therefore, there is a need to further investigate the effect of mixed enzyme on microalgae solubilisation and methane production.

2.5 Microalgae anaerobic co-digestion

Anaerobic co-digestion is applied to digest a homogeneous mixture of two or more substrates simultaneously, aiming to overcome the disadvantages of mono-digestion (Hagos *et al.*, 2017). Moreover, the economic viability of AD plants is further enhanced because of their higher methane production. Co-digestion in most cases may cause a positive synergistic effect on the biodegradability of both substrates, resulting in enhanced methane production (Oliveira *et al.*, 2014). Other advantages include the dilution of inhibitory compounds and increased diversity of the anaerobic microorganism species that help establish a stable AD process (Nielfa *et al.*, 2015). Moreover, co-digestion provides economic advantages, such as increasing the economic scale by increasing the quantity of wastes used (Mata-Alvarez *et al.*, 2000; Nielfa *et al.*, 2015).

Microalgae have been reported to co-digest effectively with carbon-rich feedstocks such as waste paper, energy crops, sewage and animal manures, thereby rebalancing the C/N ratio and increasing methane production (Yen and Brune, 2007; Schwede *et al.*, 2013; Rétfalvi *et al.*, 2016). A study carried out by Yen and Brune (2007) demonstrated that the C/N ratio and methane productivity were improved by the co-digestion of mixed algal sludge (*Scenedesmus spp.* and *Chlorella spp.*) and waste paper. In their study, adding 50% (on the basis of VS) of waste paper in algal sludge achieved the highest methane production of 1170 ± 75 mL/L day, which was double that from the mono-digestion of algal sludge.

Maize silage is another type of common co-substrate, which has been mixed with the microalgae. *C. vulgaris* was used for co-digestion with maize silage in semi-continuously anaerobic digesters as reported by Rétfalvi *et al.* (2016). In their study, adding maize silage to *C. vulgaris* could increase the C/N ratio to 16, and from the co-digestion of these two substrates the volumetric methane yield was 1.19 ± 0.08 L/L, which is significantly higher than for the mono-digestion of *C. vulgaris* (0.38 ± 0.07 L/L). Anaerobic co-digestion of *Nannochloropsis saline* with maize silage has been used to enhance the stability of the process due to the balanced nutrient supply, especially under higher organic loading rates (OLR) as reported by Schwede *et al.* (2013). In their study, in batch BMP tests, a ratio of 1/6 of *Nannochloropsis saline* mixed with maize silage produced the highest methane yield of 0.66 m^3/kg VS, which was significantly higher than for the mono-digestion of microalgae (0.28 m^3/kg VS).

Sewage sludge has also been introduced for the co-digestion with microalgae, and Caporgno *et al.* (2016) investigated sewage sludge co-digestion with microalgae *Nannochloropsis oculata* in batch BMP tests. They found that adding sewage sludge to microalgae slightly enhanced methane yields, but the co-digestion showed no synergistic effect. A similar study was reported by Caporgno *et al.* (2015), who found that biogas production between batch reactors did not differ significantly either when marine microalgae *Isochrysis galbana* and sewage sludge were digested together or separately. Similar results were achieved when using animal manure for co-digestion with microalgae. These types of feedstock are not popular either due to their relatively low C/N ratios and because ultimate methane yields are not significantly improved with increasing dosage (Wang *et al.*, 2016; Li *et al.*, 2017).

In conclusion, anaerobic co-digestion of microalgae with carbon-rich feedstocks is a promising strategy in terms of balanced C/N ratios and improved methane production. When considering to choose an ideal co-substrate for co-digestion with microalgae, in order to further support the economic case for digesting microalgae, any carbon-rich feedstocks should come as waste-streams rather than be produced specifically for bioenergy production. Detailed information concerning potential co-substrates for co-digestion with microalgae is discussed in Sections 2.5.1 and 2.5.2.

2.5.1 *Potato processing waste*

Potato processing waste (PPW) is a main by-product of industrial potato processing such as in the manufacturing of French fries, canned foods and starch products (Liang and McDonald, 2015). Generally, PPW consists of potato discarded parts (PPW_{dp}), which are whole or cut potatoes discarded due to size, blemishes or failure to meet standard quality for human food, and potato peel (PPW_{p}) (Pistis *et al.*, 2013; Schalchli *et al.*, 2017). The annual global production of potatoes reached 377 million tonnes in 2016, with around 50% of harvested potatoes being processed into industrial products (Liang and McDonald, 2015; FAOSTAT, 2018). Typical manufacturing losses of approximately 8% of potato weight generated 15.08 million tonnes of waste in 2016 (Liang and McDonald, 2015). Therefore, there is growing interest in strategies to treat these waste streams, particularly as they represent zero value waste from the manufacturing process as well as high perishability, and food companies have to dispose of this waste quickly, leading to high disposal costs (Arapoglou *et al.*, 2010; Schalchli *et al.*, 2017). Furthermore, potatoes contain high levels of nutrients and decomposing potato waste has the potential to contaminate both ground and surface water

(Olsen *et al.*, 2001). Given these factors, there is a need for an integrated, environmentally-friendly solution for PPW treatment.

The carbohydrate content of PPW is typically around 55.6 – 68.7% of DW, primarily as starch (Zhu *et al.*, 2008; Arapoglou *et al.*, 2010; Pistis *et al.*, 2013), which is easily broken down into monomers or simple sugars (Hess *et al.*, 2007). Moreover, the total moisture content of PPW is around 85% (Zhu *et al.*, 2008; Arapoglou *et al.*, 2010; Pistis *et al.*, 2013), and it is thus eligible for AD. However, because of its high biodegradability, the mono-digestion of PPW can lead to rapid and strong acidification, resulting in the accumulation of VFAs, which consequently inhibit the activity of methanogens with an unstable AD process (Pistis *et al.*, 2013). Therefore, previous studies have suggested that the use of a high temperature (thermophilic) process or a two-stage AD could overcome this problem when mono-digestion of PPW (Zhu *et al.*, 2008; Pistis *et al.*, 2013). The C/N ratios of potato waste range from 12.1/1 to 30.0/1 (Pistis *et al.*, 2013; Lucas, 2014), and therefore it is a promising feedstock for anaerobic co-digestion with other low-carbon substrates.

Yadanaparthi *et al.* (2014) studied the co-digestion of potato waste with dairy manure in both mesophilic and thermophilic conditions. They found that adding 10% (based on VS) of potato waste to 90% dairy manure increased methane production by around 12 – 26% under mesophilic conditions. For thermophilic anaerobic co-digestion, the final methane yield was enhanced to 157 – 158% when co-digestion occurred with 20% potato waste and 80% dairy manure. Parawira *et al.* (2004) demonstrated that co-digestion of potato waste and sugar beet leaves in batch BMP tests. In their study, the results showed that methane yields improved by 6 – 31%, and the co-digestion of 24% (based on TS) of potato with 16% sugar beet leaves gave the highest methane yield. Mu *et al.* (2017) investigated that co-digestion of cabbage waste and potato waste in both batch and semi-continuous modes. For batch BMP tests, they found the mixing ratio of 1:1 by VS was optimal for the co-digestion of cabbage waste and potato waste. Co-digestion enhanced methane yields by 18.4% as compared to the mono-digestion of potato waste in semi-continuous tests. However, to date, there is little information available about the anaerobic co-digestion of microalgae with PPW. Therefore, in the current work, PPW can be considered as a potential feedstock for co-digestion with microalgae.

2.5.2 *Glycerol*

For the microalgae anaerobic co-digestion process, reliance on a single waste stream could pose some operational risks to an AD plant (Nges *et al.*, 2012), and therefore additional levels of feed need to be considered as a secondary co-digestion feedstock. Glycerol is the main by-product of the biodiesel production process, representing 10% w/w of the total product stream (González-Pajuelo *et al.*, 2004; Mu *et al.*, 2006; Da Silva *et al.*, 2009). This means that the production of 100 kg of biodiesel could generate 10 kg of glycerol. By 2020, the total production of biodiesel will reach 42 million tonnes that would generate 4.2 million tonnes of glycerol (OECD/FAO, 2011). The rapid growth of the biodiesel industry has led to the overproduction of crude glycerol with its lowest price at $0.05/lb, and the purification costs of crude glycerol are around $0.20/lb (Chi *et al.*, 2007). Moreover, the disposal of glycerol is associated with environmental concerns (Fernando *et al.*, 2007). An alternative to disposal, which would concomitantly mitigate its surplus production, is to convert the glycerol into other valuable products.

The high concentration of COD of crude glycerol ranges from 1000 to 1900 g/L (Sell *et al.*, 2011; Larsen *et al.*, 2013), and it is easy to degrade by acidogenic bacteria to produce organic acids (Viana *et al.*, 2012). Therefore, it is regarded as one promising co-substrate with other organic wastes to produce biogas via AD. For instance, anaerobic co-digestion of glycerol with pig manure could improve biogas production by 100 – 400% (Wohlgemut *et al.*, 2011; Astals *et al.*, 2012). A study by Serrano *et al.* (2014) reported that the addition of glycerol to mixtures of strawberry and fish waste could accelerate the hydrolytic stage leading to the highest biodegradability measured at 96.7% with 308 L/kg VS of methane production. Larsen *et al.* (2013) investigated the co-digestion of glycerol with starch industry effluent, and found that biogas production increased by 69% when co-digested with 2% v/v glycerol.

Glycerol was also introduced to co-digest with algal biomass in order to boost methane production. Oliveira *et al.* (2014) designed a batch mode experiment aiming to optimise biogas production via the co-digestion of macroalgae *Gracilaria vermiculophylla* with glycerol, and the addition of 2% w/w glycerol to macroalgal biomass was found to increase the biomethane potential by 18%. Another study carried out by Oliveira *et al.* (2015) conducted the co-digestion of macroalgae *Sargassum sp.* with glycerol and waste oil to improve biogas production in batch BMP test. Their results showed that co-digestion with

glycerol increased methane yields and methane production rates by 56% and 38%, respectively.

Unlike macroalgae, microalgae contains a high proportion of lipid that can be used as raw biomass to produce biodiesel. The protein-rich microalgae residues after lipid extraction for the biodiesel production process are treated as waste and need to disposal. Therefore, previous studies have reported that co-digestion of lipid-spent microalgae with glycerol in order to enhance biogas production. Neumann *et al.* (2015) investigated that anaerobic co-digestion of 90% lipid-spent *Botryococcus braunii* with 10% glycerol in batch BMP tests, it was found that methane yields slightly increased compared to digestion with mono-substrate. However, the methane production rates were not significantly enhanced. AD of whole microalgae seems to be an optimum strategy in terms of energy balance if the microalgal cell-lipid composition is less than 40% (Sialve *et al.*, 2009). Glycerol is also considered to be a potential feedstock for microalgae co-digestion although there is little information available about co-digestion of whole microalgae with glycerol.

2.6 Conclusion

Although there have been some studies evaluating the potential of microalgae as a feedstock for AD, some gaps in knowledge still needed to be filled. In order to achieve a more energetically balanced AD system, the effect of low-energy demand enzymatic and low-temperature thermo-alkaline pre-treatments on microalgae AD needs to be evaluated. Moreover, in order to improve the feasibility and economic of methane production from microalgae, potential waste-stream co-substrates of PPW and glycerol also needed to be fully investigated.

Chapter 3. Materials and Methods

3.1 Microalgae cultivation and harvesting

3.1.1 *Microalgal growth conditions and medium*

The *Chlorella vulgaris* strain (CCAP 211/63) was obtained from Culture Collection of Algae and Protozoa, UK. *C. vulgaris* was pre-cultivated in 200 mL flasks for 22 days using sterilized Bold's Basal medium (BBM) (Ilavarasi *et al.*, 2011; Sharma *et al.*, 2016) in laboratory conditions at 19 ℃ under artificial light with a 16:8 light dark photoperiod. The composition of BBM is shown in Table 3.1.

Table 3.1 Chemical composition of BBM (Ilavarasi et al., 2011; Sharma et al., 2016)

	Chemical name	Concentration (g/L)	Quantity to add per L of medium (mL)
Macronutrients[a]	$NaNO_3$	25	10.0
	$CaCl_2.2H_2O$	2.5	10.0
	$MgSO_4.7H_2O$	7.5	10.0
	K_2HPO_4	7.5	10.0
	KH_2PO_4	17.5	10.0
	NaCl	2.5	10.0
Micronutrients[a]	Na_2EDTA	0.75	
	$FeCl_3.6H_2O$	0.097	
	$MnCl_2.4H_2O$	0.041	6.0
	$ZnCl_2$	0.005	
	$CoCl_2.6H_2O$	0.002	
	$Na_2MoO_4.2H_2O$	0.004	
Vitamins[b]	Vitamin B_1 (Thiaminhydrochloride)	1.2	1.0
	Vitamin B_{12} (Cyanocobalamin)	0.01	1.0

[a] Macro- and micronutrients solutions were sterilized in an autoclave at 121 ℃ for 15 minutes, and stored at 4 ℃ cold room;
[b] Vitamin solutions were sterilized by using 0.2 μm nylon filter (VWR, UK), and stored at 4 ℃ cold room.

The pre-cultivated *C. vulgaris* was then transferred to medium size (1 to 4 L) photo-bioreactors (PBRs). After 20 days of cultivation in the medium size PBRs, the *C. vulgaris* was transferred to 10 L PBRs for continuous microalgae cultivation. The cultivation process is shown in Figure 3.1 A. For PBRs, aeration was supplied by an air pump through Hepa-Vent filters (VWR, UK). The cultivation process was undertaken in the algal culture room, which is located in the Ridley Building, School of Nature and Environmental Sciences, Newcastle University (Figure 3.1 B).

Figure 3.1 Laboratory scale microalgae cultivation: (A) cultivation process; (B) culture room

3.1.2 *Growth analysis*

In order to evaluate the efficiency of cultivation and to determine the optimal time to harvest the microalgae, the growth of the microalgal biomass was evaluated by measurements of optical density from the first day until the 57th day at 540 nm (Ilavarasi *et al.*, 2011) using a spectrophotometer (Pharo 100, Merck, UK). Based on the growth curve (see Appendix A, Figure A.1), the microalgae are within the stationary phase after 25 – 30 days of culture, and the microalgae were first harvested manually through the sampling port of 10 L PBRs using a sterile plastic tube, and drained into 5 L plastic containers.

Due to the limited working volume of the laboratory centrifuge, the harvesting method employed in this study was a combination of sedimentation and centrifugation. Microalgae were first concentrated by sedimentation for 48 hours at 4 ℃, and then the supernatant was discarded. The remaining microalgae were centrifuged at 3392×g for 10 minutes in 8×45 mL sterilized centrifuge tubes (VWR, UK). The supernatant was discarded and the pellet was washed with distilled water to remove the culture medium. After that, the concentrated microalgae were stored at 4 ℃ up to 2 weeks prior to AD tests.

3.2 General analytical methods

3.2.1 *Total and volatile solids*

Total solids (TS) and volatile solids (VS) were determined according to APHA standard methods (APHA, 2005). Briefly, clean crucibles (VWR, UK) were prepared and heated at 550 ℃ in a muffle furnace for 1 hour. Then, the heated crucibles were cooled and stored in a desiccator (VWR, UK). Each crucible was weighted prior to use, and the weight recorded as "W_{dish}". Approximately 10 – 20 mL of samples was added to the crucible, and the weight taken again as "$W_{dish+sample}$". After that, the samples were dried at 103 ℃ oven for a minimum 12 hours, and cooled down in a desiccator. Then, the weight was recorded as "W_{104}". The residues were then heated at 550 ℃ in the muffle furnace for 1 hour, and cooled them to equilibrium temperature in a desiccator, and the weight recorded as "W_{550}". Calculations of TS and VS based were on the Equations 3.1 and 3.2.

$$TS\ (g/L) = \frac{W_{104} - W_{dish}}{W_{dish+sample} - W_{dish}} \times 1000 \quad (3.1)$$

$$VS\ (g/L) = \frac{W_{104} - W_{550}}{W_{dish+sample} - W_{dish}} \times 1000 \quad (3.2)$$

3.2.2 *Total and soluble chemical oxygen demand*

Concentrations of total chemical oxygen demand (COD_t) and soluble oxygen demand (COD_s) were measured using Merck COD cell test kits (VWR, 1.14541.0001, UK) based on the standard methods of APHA 5220 D (APHA, 2005). For sample preparation, the measurement range of the test kits was from 25 to 1500 mg/L COD, and therefore both COD_t and COD_s samples with high concentrations needed to be diluted using distilled water. For the measurement of COD_s, samples were centrifuged at 3392×g for 10 minutes in a centrifuge (Sigma 3-16P, UK), and then the supernatant was filtered using a 0.2 μm nylon filter (VWR, UK). The filtrate was then analysed for COD_s using COD cell test kits.

3.2.3 *Ammonium and free ammonia nitrogen*

Concentrations of ammonium nitrogen ($NH4^+$-N) were measured using Merck ammonium cell test kits (VWR,1.00683.0001, UK) based on the standard methods of APHA 4500-NH_3 F (APHA, 2005). For the preparation of test samples, the maximum measurement range of the test kits was from 5 to 150 mg/L NH_4-N, and so samples containing more than 150 mg/L $NH4^+$-N needed to be diluted with distilled water. Raw or diluted samples were first centrifuged at 3392×g for 10 minutes in a centrifuge (Sigma 3-16P, UK), and then the supernatants were filtered by a 0.2 μm nylon filter (VWR, UK) before measurement of $NH4^+$-N concentrations.

The concentrations of free ammonia nitrogen (FAN) were calculated based on Equation 3.3 (Jaroszynski *et al.*, 2012).

$$FAN = \frac{{NH_4}^+ - N \times 10^{pH}}{e^{6344/(273+T)} + 10^{pH}} \quad (3.3)$$

3.2.4 *pH*

pH was measured using a Jenway 3010 pH-meter (Jenway, UK) equipped with a double junction electrode (VWR, UK). Calibration was needed using two commercially certified standards (pH 4 and pH 7) prior to measure the samples.

3.2.5 *Carbohydrates analysis*

Total and soluble carbohydrate contents were measured via the phenol-sulphuric acid method (Nielsen, 2010). Briefly, 1.0 mL of sample was diluted with 1.0 mL of distilled water and then reacted with 0.05 mL of phenol (80% w/v) and 5 mL of concentrated sulphuric acid (98%) for 30 minutes at room temperature (25 ℃). After that, the absorbance was measured at 490 nm using a spectrophotometer (Pharo 100, Merck, UK). To determine the sample concentration, a standard curve using D-glucose (100 mg/L) was prepared in the same way.

3.2.6 *Proteins analysis*

Measurements of the total and soluble protein contents were based on the bicinchoninic acid (BCA) protein assay (Kralj *et al.*, 2014). The measurements were carried out using a protein assay kit (Thermo Sciencitific Pierce, 23227, UK), with bovine serum albumin (BSA) as the standard.

This reaction is based on the chelation of two molecules of BCA with one cuprous ion (Cu^{2+}), which produces a purple-coloured reaction product that can be measured using a spectrophotometer. The standard BSA curve was prepared following the manufacturer's instruction, and testing samples with high concentrations needed to be diluted using distilled water due to the working range of this assay kit being between 20 to 20,000 ug/mL. Each standard and unknown sample (0.1 mL) was reacted with 2.0 mL of working reagent at 37 ℃ for 30 minutes, and then cooled down at room temperature. The working reagent was prepared following the manufacturer's instruction, where 50 parts of reagent A were mixed with 1 part of reagent B. Analysis was undertaken using a spectrophotometer (Pharo 100, Merck, UK) at 562 nm.

3.2.7 *Elemental analysis*

For elemental analysis, samples were first oven dried at 60 ℃ until their weight was constant. Then 50 mg of powdered samples were placed in a clean (pre-combusted) ceramic crucibles (VWR, UK), and analysed for carbon (C) and nitrogen (N) using an Elementar VarioMAX CNS analyzer (Elementar, Germany). Based on the manufacturer's instruction, the analysis of samples involved combustion at 1145 ℃ in an oxygen-enriched helium atmosphere with the presence of tungsten oxide as a catalyst. The calibration standard of sulfadiazine (% C = 47.99, % N = 22.37 and % S = 12.81) needed to be analyzed at the start and end of the sample sequence, and after 5 – 10 samples. The amounts of C and N were calculated by reference to the calibration standard during the analysis using Elementar software.

3.2.8 *Alkalinity analysis*

The total alkalinity (TA) of samples from the semi-continuous digester was determined using the titration method as described by Lossie and Pütz (2008). Samples were first centrifuged at 3392×g for 30 minutes, and 20 mL of supernatant was put in a 50 mL flask. The flask was then placed on a magnetic stirrer and homogenised continuously during the titration process. Titration was conducted using 0.1 N sulphuric acid (H_2SO_4) to pH 5, and the volume of H_2SO_4 added was recorded. The concentration of TA was calculated based on Equation 3.4.

$$TA\ (mg/L) = (V_1 - V_2) \times 250 \qquad (3.4)$$

where V_1 is the starting volume of H_2SO_4 in mL, and V_2 is the volume of H_2SO_4 added from the start to pH 5.0 in mL.

3.2.9 *Volatile fatty acids analysis*

The total volatile fatty acids (total VFA) of samples from the semi-continuous digesters was also determined by titration methods followed by the measurement of TA (Lossie and Pütz, 2008). The continuous titration of test samples using 0.1 N H_2SO_4 from pH 5.0 to pH 4.4, and the volume of H_2SO_4 added was recorded. The concentration of total VFA was calculated based on Equation 3.5.

$$Total\ VFA\ (mg/L) = ((V_2 - V_3) \times 1.66 - 0.15) \times 500 \qquad (3.5)$$

where V_2 is the volume of H_2SO_4 at pH 5.0 mL, and V_3 is the volume of H_2SO_4 added from pH 5.0 to pH 4.4 in mL.

The measurement of individual VFA values was carried out using chromatography methods. Briefly, samples were first diluted and then filtered using a 0.2 μm nylon filter (VWR, UK). Then, the filtered sample was mixed with 0.1 M octane sulphonic acid at a ratio of 1:1 before sonicating for 40 minutes (Zealand *et al.*, 2017). Ion Chromatography Dionex Aquion system equipped with an AS-AP auto sampler with Chameleon 7 Software was applied to analyse the sonicated samples. The detection range of this method is between 5 to 500 ppm without dilution, and concentrations over this range needed to be diluted with deionised water.

3.2.10 *Methane concentration analysis*

The methane composition of the biogas was determined using a GC-FID instrument (Carlo-Erba 5160 GC) with hydrogen as the carrier gas and the injector at 150 ℃ and FID at 300 ℃. Methane standards (10% or 80% CH_4 balanced with CO_2; Scientific and Technical Gases Ltd., UK) were used in triplicate injections of 50, 40, 30, 20 and 10 μL of standard gas to make a calibration standard. A minimum calibration coefficient (R^2) of 0.99 is required before the analysis of test gas samples. After that, triplicate injections of a 50 μL sample of biogas, taken from the headspace of the BMP bottles or gas bags of the semi-continuous digesters using a 100 μL gastight syringe (SGE, 100R-V-GT), were quantified by reference to the standard curve. The volume of methane was calculated under standard temperature and pressure (STP) conditions (0 ℃, 1atm).

3.3 Microbial community analysis

3.3.1 *DNA extraction*

For microbial community analysis, genomic DNA from samples of the semi-continuous digesters and the negative control were extracted using an isolation kit (DNeasy PowerSoil kit, QIAGEN, UK) following the manufacturer's instructions. After extraction, the quality of DNA samples were determined using a DeNovix spectrophotometer (DeNovix, US) measuring the absorbance at 260 and 280 nm.

3.3.2 *Sequencing*

The extracted total DNA samples were sent to Northumbria University for paired-end Illumina MiSeq sequencing the V4 hypervariable region of 16S rRNA. The basic processes for Illumina MiSeq sequencing are library preparation, cluster amplification, sequencing and alignment and data analysis. The universal primers set 515F and 806R were used to allow amplification of the V4 region of both bacteria and archaea (Nelson *et al.*, 2014). The amplicon libraries were sequenced on the Illumina MiSeq platform using the Wet Lab SOP as described by Kozich *et al.* (2013). At the end, FASTQ files with quality score encoding were generated.

3.3.3 *Sequencing data processing*

The raw Illumina FASTQ files were demultiplexed and quality filtered using QIIME 2 with plugin wraps DADA 2 (https://docs.qiime2.org/2018.4/). Sequences presenting at 99% similarity were grouped into one operational taxonomic unit (OTU), and assigned taxonomy from the SILVA 119 reference database. Methanogens have relatively low growth rates than other anaerobic microorganisms and they are more sensitive to the environmental changes. Therefore, in this research, the processed sequencing data were further analysed, and focusing on the analysis of archaeal community.

3.4 Performance calculation

3.4.1 *Biochemical methane potential (BMP) test calculations*

The volume of dry biogas under STP conditions was calculated based on Equation 3.6 (VDI/4630., 2006).

$$V_0 = \frac{V*(P-P_W)*T_0}{P_0*T} \quad (3.6)$$

where V_0 is the volume of dry biogas under STP conditions (0 ^{0}C, 1atm) (mL), V is the volume of biogas produced (mL), P is the pressure of the gas phase at the time of reading (hPa), P_W is the vapour pressure of water as a function of the temperature of the ambient space (hPa), T_0 is the normal temperature, T_0=273K, P_0 is the normal pressure, P_0=1013 hPa and T is the temperature of the fermentation gas, T=37 ^{0}C (310K).

The methane content of dry gas in the BMP tests was calculated based on Equation 3.7 (VDI/4630., 2006).

$$CH_4{}^D = CH_4{}^H * \frac{P}{P-P_W} \quad (3.7)$$

where $CH_4{}^D$ is the methane content of dry biogas in % by volume, $CH_4{}^H$ is the methane content in humid gas in % by volume, P is the pressure of the gas phase at the time of reading (hPa), and P_W is the vapour pressure of water as a function of temperature of the ambient space (hPa).

The volume of methane (V_{CH_4}) produced under STP conditions was calculated based on Equation 3.8 (Edward *et al.*, 2015).

$$V_{CH_4} = CH_4^D \times (V_H + V_S - V_{H_0}) \quad (3.8)$$

where V_H is the calculated daily biogas production in the headspace (mL), V_S is the daily measured biogas in the syringe (mL), and V_{H_0} is the volume of biogas produced from the headspace on the previous day (mL).

The experimental methane yields (BMP_{exp}) under STP conditions can be calculated using Equation 3.9 (Edwards, 2015).

$$BMP_{\exp(STP)}(\text{mL } CH_4/\text{g VS}) = \frac{V_{Substrate} - V_{blank}}{VS_{added}} \qquad (3.9)$$

where $V_{substrate}$ is the methane volume from the substrate calculated based on equation 3.8 (mL), V_{blank} is the methane volume from inoculum (mL) produced in the blank bottle, VS_{added} is the value of VS of the substrate was added to the substrate bottle (g).

3.4.2 *Semi-continuous test calculations*

The volume of dry biogas under STP conditions and methane content in dry gas can be calculated in the same way as in the BMP tests according to equations 3.6 and 3.7. However, the final methane composition in biogas for the semi-continuous tests needs to be subjected to headspace correction due to the biogas component being diluted by the inert gas in the headspace at the beginning of the digestion process. The corrected methane concentration can be calculated as in Equation 3.10 (VDI/4630., 2006).

$$CH_4{}^C = CH_4{}^D_{t2} + (CH_4{}^D_{t2} - CH_4{}^D_{t1}) * \frac{V_H}{V_B} \qquad (3.10)$$

where $CH_4{}^C$ is the corrected final methane content in % by volume, $CH_4{}^D_{t2}$ and $CH_4{}^D_{t1}$ are the methane contents (%) in dry biogas calculated using equation 3.7, t_2 and t_1 are the two different measurement times ($t_2 > t_1$), V_H is the headspace volume (mL), and V_B is the produced biogas volume in the gas bag at t_2 (mL).

The organic loading rate (OLR) is defined as the amount of VS or COD if fed to the digester per day per unit digester volume (Montingelli *et al.*, 2015). For the semi-continous digester, the OLR can be calculated using Equation 3.11 (Edwards, 2015).

$$OLR(g/L.d) = \frac{g\ OM_{added}}{V_{digester}.d} \qquad (3.11)$$

where $g\ OM_{added}$ is the quantity of organic material in terms of VS or COD added to the digester, $V_{digester}$ is the digester volume (L), and d is day.

The hydraulic retention time (HRT) can be calculated using Equation 3.12 (Edwards, 2015).

$$HRT(d) = \frac{V_{digester}}{Q} \quad (3.12)$$

where $V_{digester}$ is the digester volume (L) and Q is the influent flow rate (L/d).

Chapter 4. Evaluation of enzymatic and low-temperature thermo-alkaline pre-treatments on anaerobic digestion of microalgae

4.1 Introduction

Biochemical methane potential (BMP) tests have been widely used in previous studies to quantify the production rates and yields of biogas, and also to characterise the biodegradability of various substrates (Carrère *et al.*, 2009; Costa *et al.*, 2012; Nielfa *et al.*, 2015). Compared to continuous reactor experiments, BMP tests are relatively quick, cheap and require less laboratory space. Therefore, BMP tests are a good starting point for microalgae AD study in this research.

The rigid cell walls of microalgae protect them against the attack from hydrolytic bacteria, and consequently prevent efficient biodegradability (Mahdy *et al.*, 2014c). Mechanical, physical, chemical and enzymatic cell wall disruption methods have been reported to pre-treat microalgae (Cho *et al.*, 2013; Mahdy *et al.*, 2014b; Santos *et al.*, 2014). For enzymatic pre-treatment, previous studies mostly focus on the pre-treatment of microalgae using single enzymes, and only few investigation have considered the effect of mixed enzymes on microalgae AD, especially cellulase mixed with α-amylase. Alkaline pre-treatment is usually combined with high temperature (Cho *et al.*, 2013; Bohutskyi *et al.*, 2014), but few studies have investigated the effect of low temperature thermo-alkaline on microalgae AD, especially using $Ca(OH)_2$ as the catalytic reagent.

This Chapter aimed to investigate the effect of low energy demand enzymatic and low-temperature thermo-alkaline pre-treatments on the degree of solubilisation of the microalgal biomass, and the subsequent methane yields in batch BMP tests.

1. For enzymatic pre-treatment, the effects of different dosages and types of enzymes were investigated.

2. For low-temperature thermo-alkaline pre-treatment, the effects of $Ca(OH)_2$ dosages, pre-treatment temperature and pre-treatment time were investigated.

4.2. Materials and methods

4.2.1 *Microalgae and inoculum*

The *C. vulgaris* strain (CCAP 211/63) was cultured in Bold's Basal Medium in 10 L Nalgene carboys (Ilavarasi *et al.*, 2011; Sharma *et al.*, 2016) at 19 ℃ under artificial light with a 16:8 light dark photoperiod. The detailed procedures of cultivation and harvesting of microalgae are described in Section 3.1. The microalgal biomass was characterised by its TS, VS, COD_t and COD_s, carbohydrate and protein content (Table 4.1).

The anaerobic seed inoculum was collected from a manure-based farm anaerobic digester located at Cockle Park Farm, Northumberland, UK. The TS and VS of the anaerobic inoculum were 12.2 ± 0.1 and 6.6 ± 0.1 g/L, respectively.

Table 4.1 Characterisation of microalgae *Chlorella vulgaris*

Parameters	Value
TS (g/L)	2.0 ± 0.2[a]
VS (g/L)	1.9 ± 0.2
VS/TS (%)	91.9 ± 1.9
COD_t (g/L)	2.7 ± 0.9
COD_s/COD_t (%)	2.7 ± 2.1
Total Protein (% VS)	39.2 ± 4.8
Total Carbohydrate (% VS)	27.6 ± 3.2

[a] Mean ± SD, n=4.

4.2.2 *Experimental design*

4.2.2.1 Enzymatic pre-treatment

Enzymatic pre-treatment was designed to evaluate the effect of concentrations and types of enzyme on hydrolysis efficiency in terms of solubilisations of COD, carbohydrate and protein; and methane yields. Three commercial enzymes (Table 4.2) were used alone or in combination at two different concentrations of 0.5 and 1.0 % v/w. The enzyme concentrations were selected based on previous studies (Choi *et al.*, 2010; Mahdy *et al.*, 2014b).

Table 4.2 Characterisation of three type of commercial enzymes

Enzymes No.	Commercial Name	Composition	Specific Activity (Purity)	Supplier
C	Carezyme 1000L	Cellulase	≥1000 U/g	
P	Flavourzyme@ 500L	Protease	≥500 U/g	Sigma-Aldrich, UK
A	BAN™ 240L	α-Amylase	≥250 U/g	

The types of enzyme tested in this study are cellulase (C), protease (P), amylase (A), mixtures of cellulase plus protease (CP) and cellulase plus amylase (CA). For mixed enzymes, the mixture ratio of the two enzymes was set at 1:1. In order to catalyze the activity of the enzymes, pre-treatment conditions were set at 55 ℃ for 24 hours (Choi *et al.*, 2010; Lin *et al.*, 2011; Ometto *et al.*, 2014). After 24 hours pre-treatment, the samples were heated in an oven at 75 ℃ for 15 minutes to deactivate the enzymes (Mahdy *et al.*, 2014c). A summary of experimental design of enzymatic pre-treatment is shown in Table 4.3.

Table 4.3 Summary and coding of the experimental design for enzymatic pre-treatment trials. Treatment coding: 1-2 corresponds to the concentrations of enzymes, C=cellulase, P= protease, A= α-amylase, CP = cellulase mixed with protease, CA= cellulase mixed with α-amylase

Enzyme concentration (% v/w)	**Experimental groups**				
0.5	C1	P1	A1	CP1	CA1
1.0	C2	P2	A2	CP2	CA2

4.2.2.2 Low-temperature thermo-alkaline pre-treatment

The low-temperature thermo-alkaline pre-treatment was designed to use lime ($Ca(OH)_2$) to pre-treat *C. vulgaris* at two low temperatures of 37 and 55 ℃ at different dosages (0, 10 and 15% w/w). These dosages were selected based on previous studies (Harun *et al.*, 2011; Gabhane *et al.*, 2014; Mahdy *et al.*, 2014a). The effect of pre-treatment time at 24 or 48 hours on hydrolysis efficiency and methane yields was also evaluated. The experimental design is shown in Table 4.4.

Table 4.4 Summary and coding of the experimental design for low-temperature thermo-alkaline pre-treatment trials. Treatment coding: 1-3 corresponds to the dosage of $Ca(OH)_2$, L= low pre-treatment temperature of 37 ℃, and H=high pre-treatment temperature of 55 ℃; A=24 hours of pre-treatment time, B=48 hours of pre-treatment time

$Ca(OH)_2$ dosage (% w/w)					
0		10		15	
L1A	L1B	L2A	L2B	L3A	L3B
H1A	H1B	H2A	H2B	H3A	H3B

4.2.3 *Evaluation of hydrolysis efficiency*

The solubilisation of COD is a measure widely used to determine the efficiency of pre-treatments (Alzate *et al.*, 2014; Mahdy *et al.*, 2014c), and the solubilisation of COD (% COD) was calculated according to Equation 4.1.

$$\% \, COD = \frac{COD_{sp} - COD_{so}}{COD_t - COD_{so}} \times 100\% \qquad (4.1)$$

where COD_{sp} is the concentration of the soluble COD of pre-treated microalgae, COD_{so} is the concentration of the soluble COD of untreated microalgae, and COD_t is the total concentration of COD of microalgae.

C. vulgaris is characterised by a carbohydrate-based cell wall, which is mainly composed of cellulose (25 - 30%) and hemicellulose (15 - 25%) (Abo-Shady *et al.*, 1993; Yamamoto *et al.*, 2005). Moreover, since microalgae are rich in protein contents; therefore, in order to further evaluate the efficiency of pre-treatments, solubilisations of carbohydrate (% CH) and proteins (% PT) were also calculated based on the Equations 4.2 and 4.3.

$$\% \, CH = \frac{CH_{sp} - CH_{so}}{CH_t - CH_{so}} \times 100\% \qquad (4.2)$$

$$\% \, PT = \frac{PT_{sp} - PT_{so}}{PT_t - PT_{so}} \times 100\% \qquad (4.3)$$

where CH_{sp} and PT_{sp} are the concentrations of soluble carbohydrate and protein of pre-treated microalgae, CH_{so} and PT_{so} are the concentrations of soluble carbohydrate and protein of untreated microalgae, and CH_t and PT_t are the total concentrations of carbohydrate and protein of microalgae.

4.2.4 *Biochemical methane potential (BMP) test*

Batch BMP tests were performed based on the guidelines recommended by Angelidaki *et al.* (2009), using glass bottles with a capacity of 160 mL and closed with butyl rubber seals and aluminium caps. The addition of 10% v/v (9 mL) of $NaHCO_3$ (5g/L) solution was made to each test bottle to maintain the pH value (Dechrugsa *et al.*, 2013). The untreated or pre-treated *C. vulgaris* and anaerobic inoculum were mixed to achieve a ratio of 0.5:1 on the basis of VS (Lee *et al.*, 2013; Ometto *et al.*, 2014). Quantities were calculated to obtain 90 mL of final liquid volume and to allow 43.75% of headspace.

The daily biogas production was measured volumetrically, and on each measurement day, a 10 mL syringe (VWR, UK) was connected to the top of the BMP bottle to measure the daily biogas production before measuring the methane percentage, and also to make sure that the internal pressure was equal to atmospheric pressure (Bohutskyi *et al.*, 2014). Each BMP assay was performed in triplicate for each individual substrate in order to identify the biogas production level and percentage of methane, and a blank test containing only inoculum was also performed and subtracted from the treatment bottles. The volume of methane was calculated under STP conditions (0 ℃, 1atm), and details of the calculations can be found in Section 3.4.1. All BMP bottles were placed in a 37 ℃ incubator at 70 rpm for 25 or 26 days of reaction.

4.2.5 *Analytical methods*

Measurements of TS, VS, COD_t, COD_s, carbohydrate and protein were conducted according to the methods are described in Section 3.2.

4.2.6 ***Kinetics of anaerobic digestion***

The modified Gompertz equation has been used in many previous studies, and also assumes that methane production is proportional to microbial activity, which indicates the growth of microorganisms (Lay *et al.*, 1997; Patil *et al.*, 2012; Nielfa *et al.*, 2015). The kinetic data obtained from all digesters were checked for the fitness of the modified Gompertz equation using Equation 4.4.

$$M = P \times \exp\left\{-\exp\left[\frac{R_m \times e}{P}(\lambda - t) + 1\right]\right\} \quad (4.4)$$

where M is the cumulative methane production (ml/gVS) at time t, P is the methane yield potential (ml/gVS), R_m is the maximum methane production rate (ml/gVS/d), λ is the duration of lag phase (d), and t is the digestion time (d).

4.2.7 ***Energy requirement consideration***

An energy requirement study was carried out to estimate whether the energy requirement for pre-treatment can be balanced by the extra methane produced. To evaluate the energy balance of each pre-treatment method, the ratio between energy input (E_i) and energy output (E_o) is used as an indicator (Passos *et al.*, 2013; Ometto *et al.*, 2014).
The E_i was estimated using Equation 4.5, where the main energy input was determined by the heat required to raise the microalgal biomass from the initial temperature (T_o) to the pre-treatment temperature (T_p).

$$E_i = \frac{\rho \times V \times \gamma \times (T_p - T_o) \times (1 - \emptyset)}{VS} \quad (4.5)$$

where ρ is microalgae specific density, which was assumed to be equal to that of water of 1g/mL, V is the sample volume (mL), γ is the specific heat values of 4.18×10^3 kJ/g.°C, T_p is the pre-treatment temperatures of 37 and 55 °C for thermo-alkaline pre-treatment and, 55 and 75 °C for enzymatic pre-treatment, T_o is the initial temperature for microalgal biomass, which was assumed to be equal to the ambient temperature of 20 °C, $\emptyset$ is the heat recovery

efficiency, which was assumed to be equal to 85%, and VS is the volatile solids content in pretreated samples (g).

The energy required for pre-treatment should at least be covered by the extra methane produced; therefore, the E_o was calculated from the difference in methane yields between pre-treated and untreated microalgae using Equation 4.6.

$$E_o = \frac{\Delta P_{CH_4} \times LCP}{10^6} \quad (4.6)$$

where ΔP_{CH_4} is the enhanced methane yield after pre-treatment (mL/g VS), and LCP is the lower calorific power (LCP) of methane, which has been reported to be approximately 35.8×10^3 kJ/m^3 CH_4 under standard conditions (STP) of 1atm and 0 ℃ (Serrano *et al.*, 2014).

4.2.8 *Statistical analysis*

For enzymatic pre-treatment, experimental data for the solubilisations of COD, carbohydrate and protein as well as methane yields were analysed by a two-way mixed analysis of variance (ANOVA) with the Bonferroni post hoc test used to determine the significance of statistical differences between the concentrations of enzymes (Field, 2009). The Turkey post hock test was used to determine the differences between types of enzymes.

Experimental data for thermo-alkaline pre-treatment were analysed by a three-way mixed ANOVA with the Bonferroni post hoc test used to determine the statistical differences between the dosages of $Ca(OH)_2$ (Field, 2009).

A Pearson correlation coefficient analysis was run to determine the relationship between the solubilisations of COD, carbohydrate or protein and methane yields (Field, 2009). A confidence interval of differences of 95% ($p<0.05$) was chosen to define statistical significance. All statistical analyses were conducted using IBM SPSS statistics, Version 23. MATLAB, R2015a was used to calculate P, R_m and λ for each digester.

4.3 Results

4.3.1 *Hydrolysis efficiency*

4.3.1.1 Enzymatic pre-treatment

Figure 4.1 shows the hydrolysis efficiency after the enzymatic pre-treatment. COD solubilisation after enzymatic pre-treatments ranged from 41 to 67% (Figure 4.1a), and the concentrations of enzymes had a significant effect on COD solubilisation ($F(1.104,30)=6500.00$, $p<0.001$). The type of enzyme also had a significant effect on COD solubilisation ($F(4,15)=60.26$, $p<0.001$). Enzyme mixtures showed a higher COD solubilisation than single enzymes. Cellulase plus α-amylase (CA) exhibited higher COD solubilisations, which were significantly higher than those of cellulase ($p<0.001$), protease ($p=0.001$) and α-amylase ($p<0.001$). Similarity, cellulase plus protease (CP) also showed significantly higher COD solubilisation than those of cellulase ($p<0.001$), protease ($p=0.004$) and α-amylase ($p<0.001$). However, COD solubilisations were not significantly different between cellulase plus protease (CP) and cellulase plus α-amylase (CA) ($p=1.000$). The significant effect on COD solubilisation was also qualified in terms of an interaction effect between concentration and type of enzymes ($F(4.42,15)=40.14$, $p<0.001$). This interaction effect can be seen where the COD solubilisations from single enzymes and mixed enzymes were not significantly different with an enzyme dosage at 0.5% v/w. However, the COD solubilisations observed for mixed enzymes were significantly higher than the values observed for single enzymes when the enzyme dosage was set at 1.0% v/w.

Carbohydrate solubilisation after enzymatic hydrolysis ranged from 13 to 44% (Figure 4.1b), and was significantly increased with increasing enzyme concentrations ($F(1.10,30)=1400.00$, $p<0.001$). The enzyme type also significantly affected carbohydrate solubilisation ($F(4,15)=3840.00$, $p<0.001$). For single enzymes, cellulase released significantly more carbohydrates than did protease ($p<0.001$) and α-amylase ($p<0.001$). Enzyme mixtures performed well compared to single enzymes in terms of carbohydrate solubilisation, where cellulase plus protease (CP) showed significantly higher carbohydrate solubilisation than cellulase ($p<0.001$), α-amylase ($p<0.001$) and protease ($p<0.001$). Similarity, cellulase plus α-amylase (CA) also showed significantly higher carbohydrate solubilisation than cellulase ($p<0.001$), α-amylase ($p<0.001$) and protease ($p<0.001$).

Protein solubilisation was observed to range from 28 to 72% after enzymatic pre-treatment (Figure 4.1c). The concentration of enzyme had a significant effect on protein solubilisation ($F(1.32,30) = 4750.00$, $p<0.001$), and mixed enzymes exhibited significantly higher solubilisation of protein than single enzymes ($F(4,15)=657.00$, $p<0.001$). The significant effect on protein solubilisation was also qualified by an interaction effect between concentration and type of enzymes ($F(5.29,30)=106.39$, $p<0.001$). This interaction effect can be seen where the cellulase plus protease (CP) and single protease (P) were the most effective enzymes for protein solubilisation, and they both showed higher values than other types of enzyme.

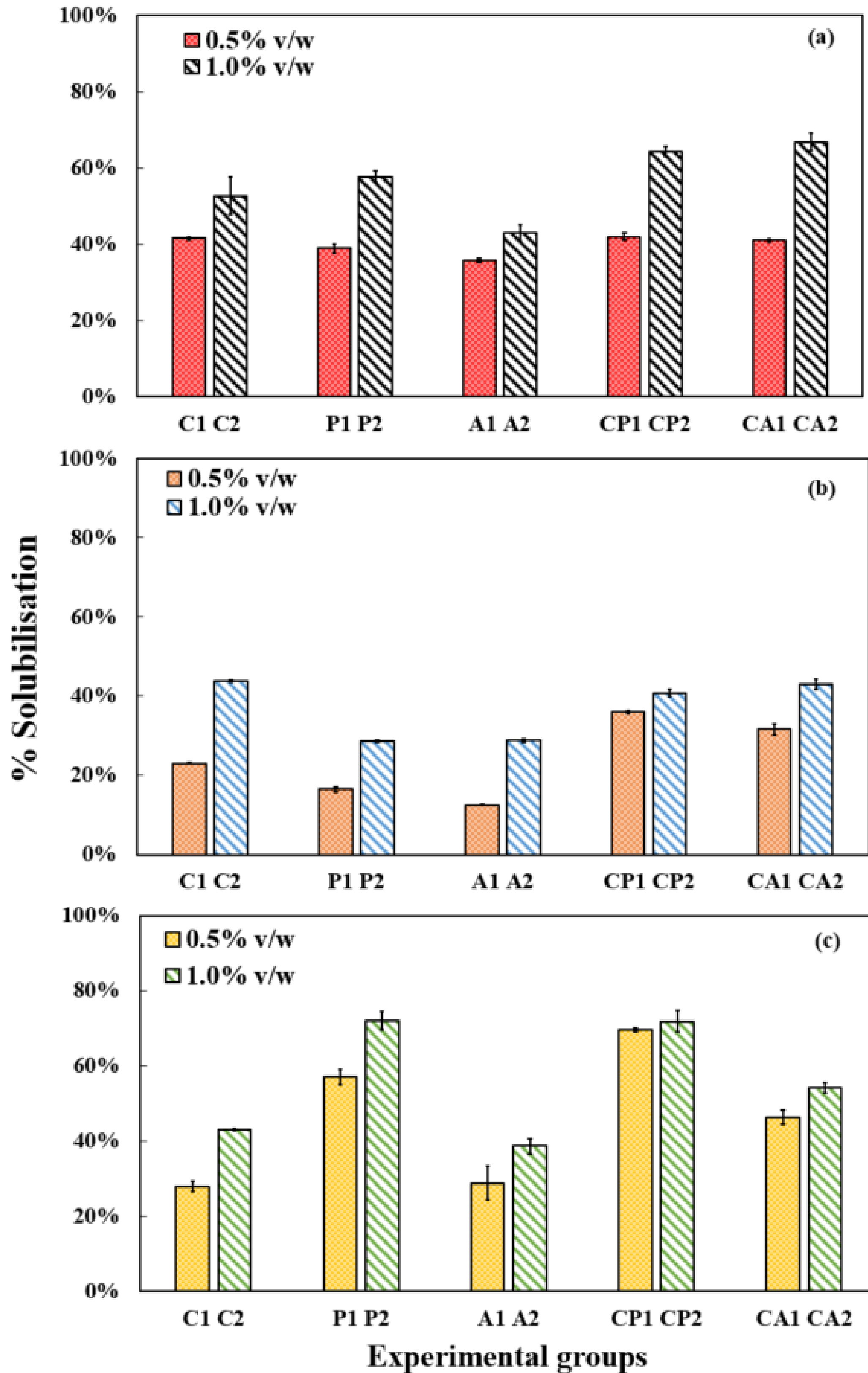


Figure 4.1 Solubilisation of (a) COD, (b) carbohydrate and (c) protein after enzymatic pre-treatment: C=cellulase, P= protease, A= α-amylase, CP = cellulase plus protease mix, CA= cellulase plus α-amylase mix. Error bar=mean ± SD, n=4.

4.3.1.2 Low-temperature thermo-alkaline pre-treatment

Figure 4.2 shows the hydrolysis efficiency after low-temperature thermo-alkaline pre-treatment. COD solubilisation was observed to range from 18 to 28% (Figure 4.2 a), and $Ca(OH)_2$ dosage had a significant effect on COD solubilisation ($F(3,12)=2838.00$, $p<0.001$). The highest values were obtained with a dosage of 15% w/w, and were significantly higher than those with 0% w/w dosage ($p=0.001$). However, there was no significant difference between the results with 10 and 15% w/w dosages ($p=1.000$). The pre-treatment temperature also had a significant effect on the solubilisation of COD ($F(1,4)=10.99$, $p=0.030$), with values observed at 55 ℃ higher than those of at 37 ℃. However, pre-treatment time had no significant effect on COD solubilisation ($F(1,4)=0.00$, $p=0.983$).

Carbohydrate solubilisation was observed to range from 27 to 50% after low-temperature thermo-alkaline pre-treatment (Figure 4.2 b). $Ca(OH)_2$ dosage had a significant effect on carbohydrate solubilisation ($F(3,12)=955.08$, $p<0.001$). The highest values were obtained with a dosage of 15% w/w, and were significantly higher than those for 0% w/w dosage ($p=0.013$). However, there was no significant difference between the results with 10 and 15% w/w dosage ($p=0.475$). Pre-treatment time and temperature both had significant effects on the solubilisation of carbohydrate ($F(1,4)=20.60$, $p=0.011$ and $F(1,4)=42.26$, $p=0.003$, respectively).

Between 14 – 26% of proteins were solubilized after low-temperature thermo-alkaline pre-treatment (Figure 4.2 c), and $Ca(OH)_2$ dosage had a significant effect on protein solubilisation ($F(3,12)=1452.42$, $p<0.001$). The highest values were observed with 15% w/w dosage, and were significantly higher than those with 0% w/w ($p=0.001$) and 10% w/w dosages ($p=0.023$). The solubilisation of protein observed at 55 ℃ were significantly higher than those of at 37 ℃ ($F(1,4)=34.15$, $p=0.004$). Pre-treatment time also had a significant effect on protein solubilisation ($F(1,4)=29.28$, $p=0.006$).

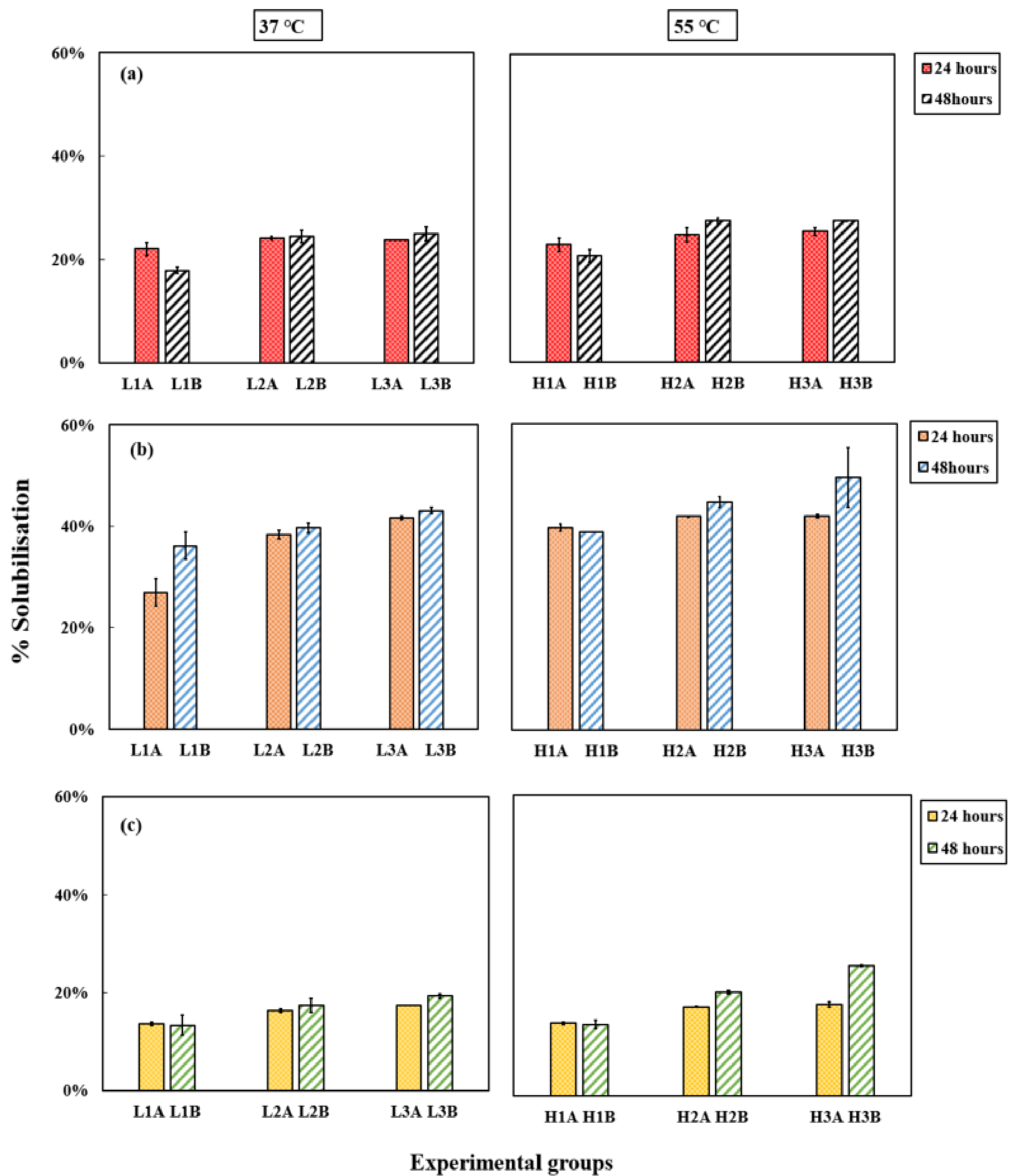


Figure 4.2 Solubilisation of (a) COD, (b) carbohydrate and (c) protein after low-temperature thermo-alkaline pre-treatment: L= low pre-treatment temperature of 37 °C, H=high pre-treatment temperature of 55 °C; A=24 hours pre-treatment time, B=48 hours pre-treatment time. Error bar=mean ± SD, n=2.

4.3.2 *Methane yields and BMP kinetic model*

4.3.2.1 Enzymatic pre-treatment

Figure 4.3 shows the cumulative amounts of methane produced by digestion of untreated and enzymatic pre-treated *C. vulgaris*. The lowest methane yield of 120 mL CH_4/g VS was produced by digestion of untreated *C. vulgaris*, and methane yields were improved by 22 – 162% after enzymatic pre-treatments. Methane yields were significantly improved after enzymatic pre-treatment ($F(2,50)=647.79$, $p<0.001$). The methane yields were significantly higher for the 1.0% v/w dosage than for 0.5% v/w dosage ($p<0.001$) and untreated microalgae ($p<0.001$). The type of enzyme also had a significant effect on methane yields ($F(4,25)=127.95$, $p<0.001$). The mixed enzymes showed higher methane yields compared to single enzymes, and the highest methane yields were achieved by cellulase plus protease (CP), which were significantly higher than those for cellulase ($p<0.001$), protease ($p<0.001$), α-amylase ($p<0.001$) and cellulase plus α-amylase ($p=0.002$).

Gompertz kinetics data obtained by digestion of untreated and enzymatic pre-treated *C. vulgaris* are summarized in Table 4.5. The values of R_m were significantly increased after enzymatic hydrolysis ($F(2,10)=166.33$, $p<0.001$). The values obtained by 0.5 and 1.0% v/w dosages were all significantly higher than for untreated *C. vulgaris* ($p<0.001$ and $p<0.001$, respectively). Enzyme type also had a significant effect on the values of R_m ($F(4,5)=11.37$, $p=0.010$). The highest R_m values were obtained by cellulase plus protease, which were significantly higher than those for cellulase plus α-amylase ($p=0.034$). However, there was no significant difference between results for cellulase plus protease and cellulose ($p=1.000$), protease ($p=1.000$) and α-amylase ($p=0.074$). The duration of lag phase (λ) were shorter than those for untreated microalgae in some pre-treatment conditions (A1, CA1 and CA2). However, there were no significant effects of enzyme dosage and type on the time values of λ ($F(2,10)=2.56$, $p=0.127$ and $F(4,5)=3.28$, $p=0.112$, respectively).

Table 4.5 Summary of modified Gomerptz kinetics data for AD of untreated and enzymatic pre-treated *C. vulgaris*

No.	R_m ($mLCH_4/gVS/d$)	λ (d)	R^2
Untreated	18.88	0.70	0.9925
C1	33.32	0.96	0.9971
C2	38.82	1.14	0.9974
P1	35.61	1.06	0.9945
P2	43.17	0.95	0.9977
A1	26.31	0.58	0.9958
A2	35.92	0.91	0.9975
CP1	43.40	1.00	0.9859
CP2	36.52	0.74	0.9906
CA1	30.57	0.57	0.9885
CA2	28.69	0.44	0.9893

4.3.2.2 Low-temperature thermo-alkaline pre-treatment

Figure 4.4 shows the cumulative methane yields after low-temperature thermo-alkaline pre-treatment. After 25 days of reaction, final methane yield of 129 mL CH_4/g VS was achieved by digestion of untreated *C. vulgaris*. The methane yields were improved by 4 – 19% (expect for L1B) compared to untreated *C. vulgaris* when the pre-treatment temperature was 37 ℃ (Figures 4.4 a and 4.4 b), and the highest yield of 154 mL CH_4/g VS was produced by L2B (10% w/w $Ca(OH)_2$ dosage, and 48 hours pre-treatment). When the pre-treatment temperature was 55 ℃ (Figures 4.4 c and 4.4 d), the methane yields were improved by 8 – 26% compared to untreated *C. vulgaris*, and the highest yield of 162 mL CH_4/g VS was produced by H2A (10% w/w $Ca(OH)_2$ dosage, and 24 hours pre-treatment). Overall, the dosage of $Ca(OH)_2$ had a significant effect on methane yield ($F(3,12)=11.34$, $p=0.001$). Pre-treatment temperature also had a significant effect on methane yield ($F(1,4)=9.79$, $p=0.035$), with yields obtained at 55 ℃ higher than those of at 37 ℃. However, pre-treatment time had no significant effect on methane yield ($F(1,4)=0.56$, $p=0.495$).

Table 4.6 shows the Gompertz kinetics data of the low-temperature thermo-alkaline pre-treatment. $Ca(OH)_2$ dosage had no significant effect on the values of R_m ($F(3,12)=3.31$, $p=0.057$), but pre-treatment temperature had a significant effect on R_m ($F(1,4)=9.90$, $p=0.035$). Moreover, there was a significant interaction effect between $Ca(OH)_2$ dosage and pre-treatment temperature ($F(3,12)=9.68$, $p=0.002$). This interaction effect can be seen where at the high pre-treatment temperature of 55 °C, the values of R_m were increased compared to untreated microalgae. However, the effect of pre-treatment time on R_m was not significant ($F(1,4)=0.17$, $p=0.698$). The time values of λ were significantly increased after thermo-alkaline pre-treatment compared to untreated microalgae ($F(3,12)=57.07$, $p<0.001$). The time values of λ at 37 °C were significantly shorter than at 55 °C ($F(1,4)=159.15$, $p<0.001$). However, the values of λ did not significantly differ between 24 and 48 hours of pre-treatment time ($F(1,4)=0.92$, $p=0.392$).

Table 4.6 Summary of modified Gomerptz kinetics data for AD of untreated and thermo-alkaline pre-treated *C. vulgaris*

No.	**R_m ($mLCH_4/gVS/d$)**	**λ (d)**	**R^2**
Untreated	16.62	1.45	0.9850
L1A	9.46	1.60	0.9825
L1B	8.53	1.55	0.9868
L2A	13.85	2.35	0.9746
L2B	15.18	2.09	0.9685
L3A	17.21	2.70	0.9681
L3B	16.79	2.90	0.9793
H1A	17.20	5.96	0.9783
H1B	20.97	6.14	0.9685
H2A	22.82	2.78	0.9632
H2B	16.62	3.87	0.9833
H3A	19.66	4.17	0.9789
H3B	16.62	3.87	0.9833

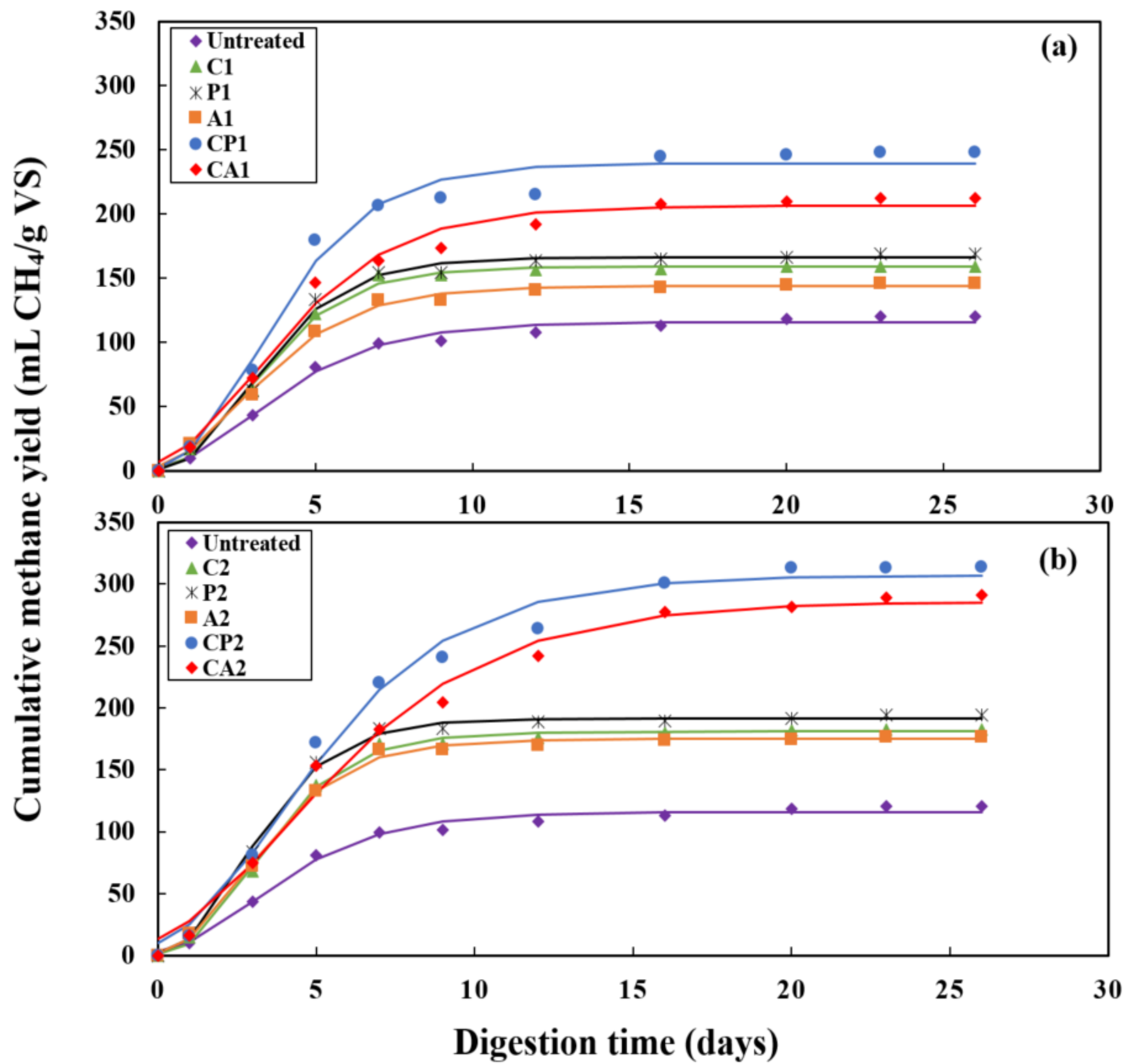


Figure 4.3 Cumulative methane yields after enzymatic pre-treatment: (a) enzyme dosage at 0.5% v/w, and (b) enzymatic dosage at 1.0 % v/w.

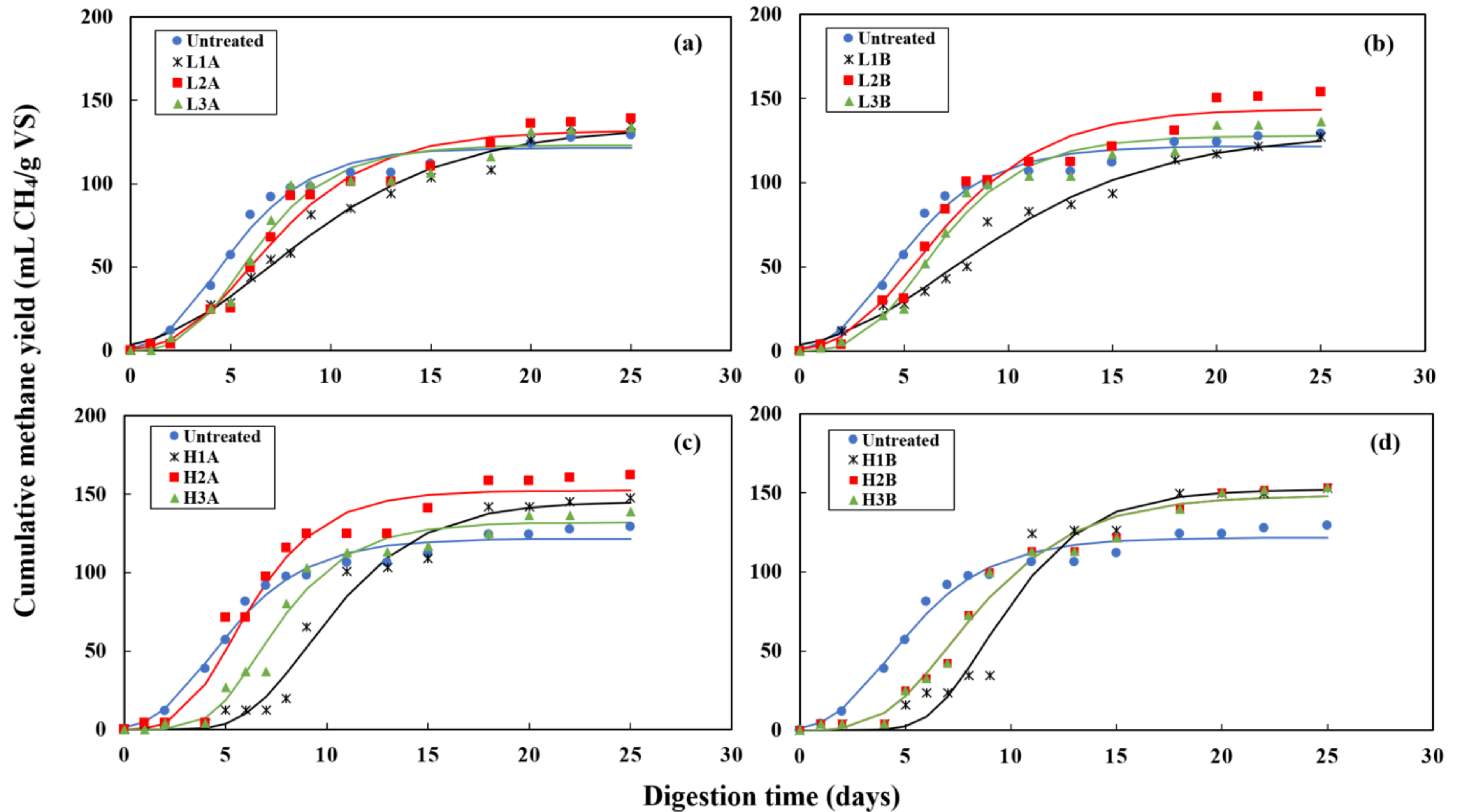


Figure 4.4 Cumulative methane yields after low-temperature thermo-alkaline pre-treatment: (a) pre-treatment at 37 °C for 24 hours, (b) pre-treatment at 37 °C for 48 hours, (c) pre-treatment at 55 °C for 24 hours, (d) pre-treatment at 55 °C for 48 hours

4.3.3 *Effects of hydrolysis efficiency on methane yield*

A Pearson correlation was used to determine the relationship between hydrolysis efficiency (solubilisations of COD, carbohydrate and protein) and final methane yields. For enzymatic pre-treatment, there was a positive correlation between the solubilisation of COD and methane yield ($r(22)=0.755$, $p<0.001$) (Figure 4.5 a). The solubilisation of carbohydrate also was also positively correlated with methane yield ($r(22)=0.787$, $p<0.001$) (Figure 4.5 b). Similarly, there was a positive correlation between the solubilisation of protein and methane yield ($r(22)=0.733$, $p<0.001$) (Figure 4.5 c).

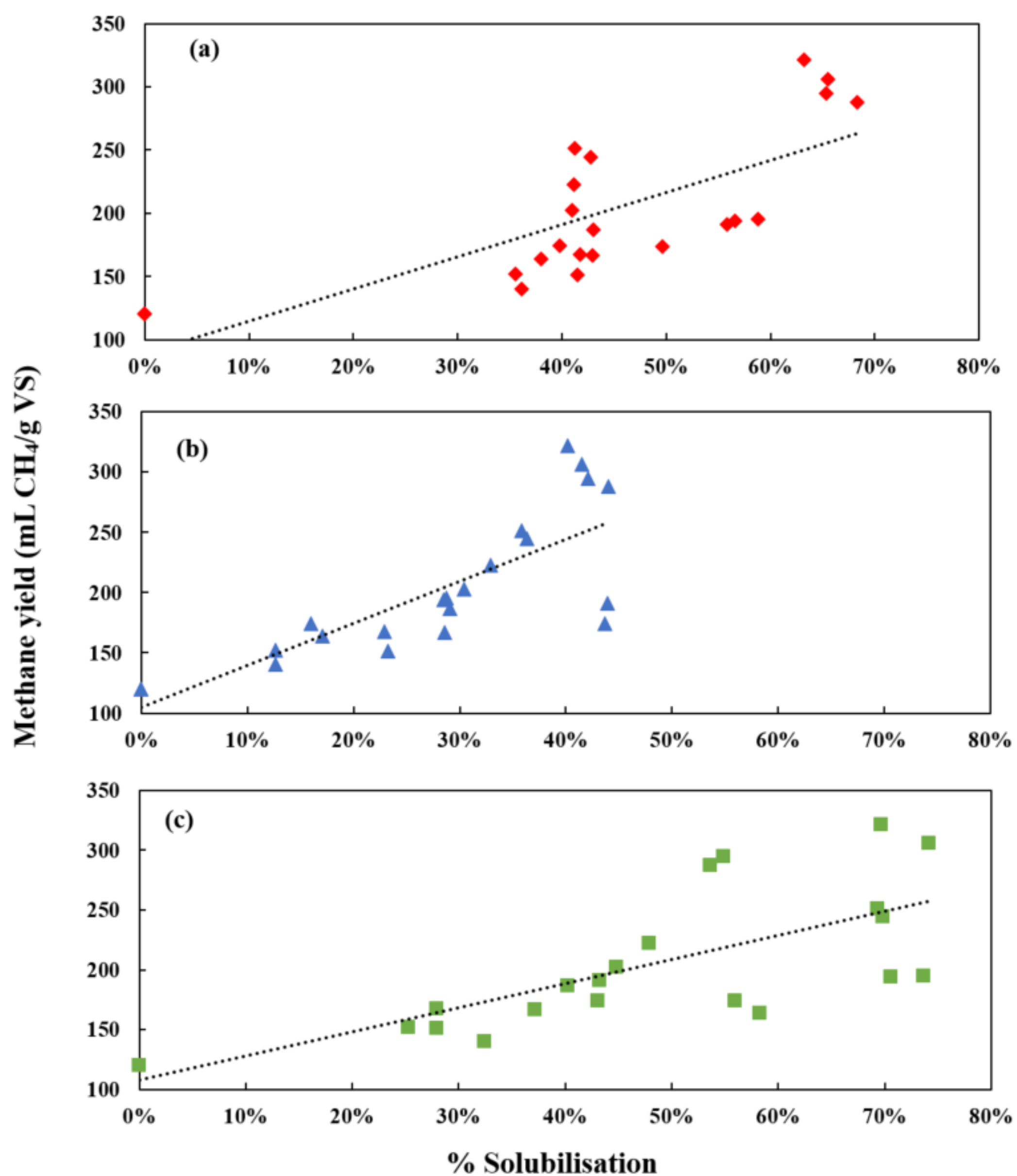


Figure 4.5 Correlation between methane yield and the solubilisation of (a) COD, (b) carbohydrate and (c) protein for the enzymatic pre-treatment.

For low-temperature thermo-alkaline pre-treatment, the solubilisations of COD and carbohydrate were both positively correlated with methane yield (r(26)=0.476, p=0.014 and r(26)=0.457, p=0.019, respectively) (Figures 4.6 a and 4.6 b). Moreover, there was also a positive correlation between the solubilisation of protein and methane yield (r(26)=0.489, p=0.011) (Figure 4.6 c).

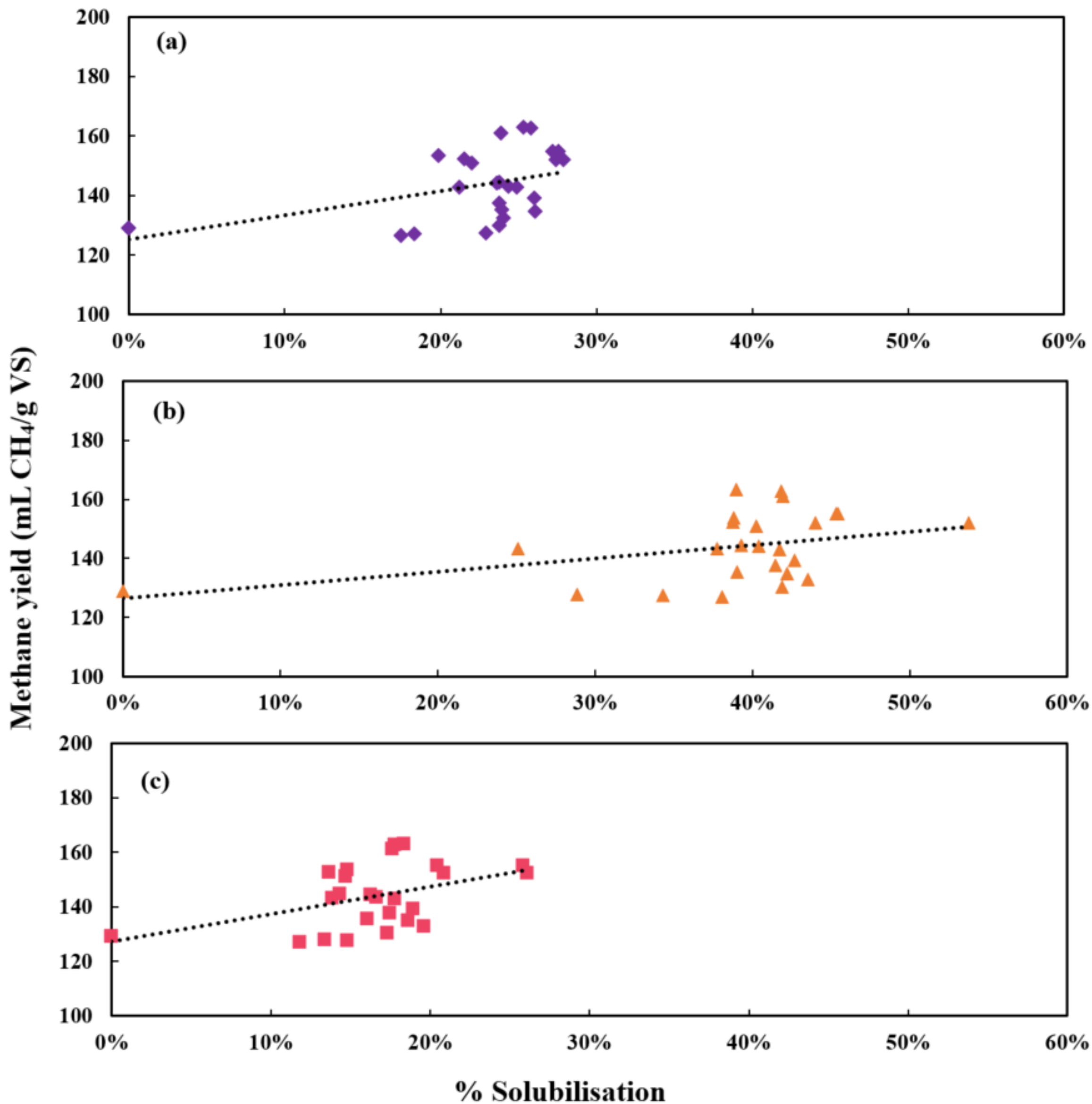


Figure 4.6 Correlation between methane yield and the solubilisation of (a) COD, (b) carbohydrate and (c) protein for thermo-alkaline pre-treatment.

4.3.4 *Energy balance*

In the current study, the methane yields were enhanced by the tested pre-treatment methods, and the energy output (E_o) and the energy input (E_i) to energy output (E_o) ratios of E_i/E_o are summarized in Table 4.7. The value of E_i for enzymatic hydrolysis was estimated at 1.25 kJ/g VS, and this was calculated based on the temperature used to catalyst (55 ℃) and deactivate (75 ℃) the enzymes. The values of E_o increased with enzyme dosage and mixed enzymes obtained higher values than single enzymes (Table 4.7). The ratios of E_i/E_o were all lower than one (except A1), which indicates there were the positive energy balances for enzymatic hydrolysis.

For low-temperature thermo-alkaline hydrolysis, values of E_i were estimated at 0.28 and 0.58 kJ/g VS for 37 and 55 ℃, respectively. Higher values of E_o were achieved by high pre-treatment temperature of 55 ℃ compared to 37 ℃. For 55 ℃ pre-treatment, the ratios of E_i/E_o at most conditions (except for H3A) were lower than one, whereas only L2A and L2B (10% $Ca(OH)_2$ dosage) obtained positive energy balances for 37 ℃ pre-treatment.

Table 4.7 Methane yields, energy output (E_o) and energy ratios (E_i/E_o) of enzymatic and thermo-alkaline pre-treatments

No.	Methane yield	E_o (kJ/gVS)	E_i/E_o	No.	Methane yield	E_o (kJ/gVS)	E_i/E_o
Untreated	120 ± 15[a]	/	/	**Untreated**	129 ± 13	/	/
C1	159 ± 11	1.41	0.89	**L1A**	135 ± 11	0.22	1.25[b]
P1	169 ± 7	1.76	0.71	**L1B**	127 ± 0	/	/
A1	146 ± 9	0.93	1.34[b]	**L2A**	139 ± 6	0.37	0.77
CP1	248 ± 5	4.58	0.27	**L2B**	154 ± 13	0.88	0.32
CA1	213 ± 14	3.32	0.38	**L3A**	134 ± 5	0.18	1.58[b]
C2	183 ± 12	2.24	0.56	**L3B**	136 ± 5	0.25	1.11[b]
P2	194 ± 1	2.67	0.47	**H1A**	148 ± 5	0.67	0.86
A2	177 ± 14	2.03	0.62	**H1B**	153 ± 1	0.86	0.68
CP2	314 ± 11	6.94	0.18	**H2A**	162 ± 1	1.18	0.49
CA2	291 ± 5	6.13	0.20	**H2B**	154 ± 2	0.88	0.66
				H3A	139 ± 6	0.35	1.66[b]
				H3B	154 ± 2	0.88	0.66

[a] Error bar= mean ± SD, n=3;

[b] The ratio of E_i/E_o >1, which indicates the negative energy balance.

4.4 Discussion

4.4.1 *Effect of pre-treatments on hydrolysis efficiency*

4.4.1.1 Enzymatic pre-treatment

The efficiency of enzymatic pre-treatment is linked to the composition of the microalgal cell wall (Ometto *et al.*, 2014). In the current study, the tested microalgae strain is *C. vulgaris*, which belongs to the glucosamine-type *Chlorella* genus, and the main components of its cell wall are cellulose (25 – 30%) and hemicellulose (15 – 25%) (Abo-Shady *et al.*, 1993; Yamamoto *et al.*, 2005). Therefore, in agreement with previous studies, single cellulase at higher dosage (1.0% v/w) performed well in the solubilisation of carbohydrate compared to protease and α-amylase (Choi *et al.*, 2010; Mahdy *et al.*, 2014b). In the present study, the total protein content in *C. vulgaris* was observed at 39.2% of VS, and Safi *et al.* (2014) reported that 20% of the total protein in *C. vulgaris* is bound to the cell wall. Therefore, protease could also be an optimizing enzyme for the pre-treatment of *C. vulgaris* as reported by Mahdy *et al.* (2014b), Mahdy *et al.* (2014c) and Ometto *et al.* (2014). In agreement with previous studies, *C. vulgaris* pre-treated by protease performed well in the solubilisations of both COD and protein. After 24 hours pre-treatment, the maximum solubilisation of protein with a 1.0% v/w dosage of protease was observed to be 72%. α-Amylase is targeted to break down the starch content in microalgae, whereas the starch content in *C. vulgaris* is generally located in the chloroplast and is composed of amylase and amylopectin together with sugars to store the energy for the cells (Safi *et al.*, 2014). The cell wall composition of *C. vulgaris* lacks starch, and therefore, this is likely to explain why the hydrolysis efficiency obtained by pre-treatment with α-amylase alone are lower than for cellulase and protease pre-treatments.

However, higher degrees of the solubilisations of COD and carbohydrate were observed when α-amylase was mixed with cellulase. The single enzyme may have limited hydrolytic activity, whereas mixtures of enzymes can exhibit a synergistic effect, improving the hydrolysis efficiency (Yang *et al.*, 2010; Alvira *et al.*, 2011). Therefore, the higher hydrolysis efficiency achieved by enzymes mixtures were probably a result of synergistic effects caused by the presence of two type of enzymes. Similarly, cellulase mixed with protease also performed well compared to single enzymes, especially in protein solubilisation. This finding corroborates the findings of Mahdy *et al.* (2014b) and Ometto *et al.* (2014). In their studies,

cellulase mixed with protease or pectinase led to higher hydrolysis efficiency compared to single enzymes.

4.4.1.2 Low-temperature thermo-alkaline pre-treatment

The combination of alkaline and temperature increases the surface area of cellulose due to the swelling of the biomass, and the degrees of polymerization and cellulose crystallinity are decreased (Agbor *et al.*, 2011). In the current study, for low-temperature thermo-alkaline pre-treatment, the solubilisations of COD, carbohydrates and proteins increased significantly with $Ca(OH)_2$ dosage and pre-treatment temperature. This finding agrees with those of Solé-Bundó *et al.* (2017a) that the highest hydrolysis efficiency was observed with the highest alkali dosage and temperature.

Alkaline usually combined with high temperature (60 – 120 ℃) to pre-treat microalgae, as reported by Harun *et al.* (2011), Bohutskyi *et al.* (2014) and Solé-Bundó *et al.* (2017a), and the cellulose may not solubilized by the alkaline at low temperatures between 25 and 55 ℃ (Kumar *et al.*, 2009). However, in the current study, 27 – 50% of the carbohydrate solubilised, which is higher than the levels reported in previous studies by Mahdy *et al.* (2014a) and Solé-Bundó *et al.* (2017a). In their studies, NaOH or CaO were combined with low temperatures of 25, 50 or 55 ℃ to pre-treat microalgae, and the solubilisation of carbohydrate observed to range from 9.8 – 30.0%. It should be noticed that, in the current study, the highest $Ca(OH)_2$ dosage was 15% w/w, which is higher than the dosages used in those previous studies, which were from 0.05 to 10% w/w. Solé-Bundó *et al.* (2017a) found that increasing the alkaline dosage could release more of the cell wall structural sugars of glucose, xylose and arabinose at the same pre-treatment temperature. Therefore, the results of the current study indicate that increasing $Ca(OH)_2$ dosage may have the potential to break down the microalgae cell wall, and solubilisation of the cell wall constituents can then occur at low pre-treatment temperatures.

From the current study, it was found that the effect of pre-treatment time had no significant effect on COD solubilisation, and this finding agrees with the results reported by Mahdy *et al.* (2014a). Their study showed that incubation time (24 and 48 hours) barely affected COD solubilisation when using NaOH to pre-treat microalgae *C. vulgaris* and *Scenedemus sp.*. Kumar *et al.* (2009) reported that the contact time should be increased with decreasing temperatures when using lime for biomass pre-treatment. In the current study, the pre-

treatment time was set at either 24 or 48 hours, which is shorter than the in previous study of Liang *et al.* (2013). In their study, smooth cordgrass of *Spartina alterniflora* was pre-treated by lime at mild temperatures of 25 – 55 °C for 7 – 28 days. However, in comparison with the lignocellulosic biomass of cordgrass, microalgae are lignin-free substrate, and therefore the pre-treatment time may not need to be that long. Moreover, González-Fernández *et al.* (2013) found that pre-treatment temperature is the key factor that affects the sufficient hydrolysis of the microalgael cell wall rather than pre-treatment time. In their study, microalgae strain *Scenedesmus sp.* was thermally pre-treated by three temperatures of 70, 80 or 90 °C. The values of soluble COD significantly increased with temperature. These values were also significantly enhanced after 15 minutes pre-treatment, but afterwards only increased by 8% from 15 minutes to 4 hours.

4.4.2 *Effect of pre-treatment on methane yield*

In the current study, the ultimate methane yields were enhanced by both enzymatic and low-temperature thermo-alkaline pre-treatments. The improvements in methane yields were possibly proportional to levels of COD solubilisation (Cho *et al.*, 2013). In the current study, for both pre-treatments, COD solubilisation was positively correlated with methane yield. This probably explains why methane yields were enhanced compared to untreated *C. vulgaris*. Moreover, methane yields were also positively correlated with levels of carbohydrate and protein solubilisation. This agree with the findings of Solé-Bundó *et al.* (2017a) that the highest solubilisation of both carbohydrate and protein resulted in the highest methane yield. For enzymatic pre-treatment, cellulase mixed with protease released more protein than other types of enzymes, and consequently produced the highest methane yield. This result agrees with the findings of Ometto *et al.* (2014), who demonstrated that effective enzymatic hydrolysis should take into account proteic and polysaccharidic components. They found that the highest methane yield was produced by a protease plus esterase mix.

The rigid cell wall of microalgae protects them against attack from anaerobic microorganisms. Mahdy *et al.* (2014c) reported that effective pre-treatment not only aims to improve the ultimate methane yield but also to increase methane production rate. In the current study, production rates for enzymatic pre-treatment were significantly increased compared to untreated microalgae. After low-temperature thermo-alkaline pre-treatment, the production rates were also significantly increased at the high pre-treatment temperature of 55 °C. These results may suggest that the initial hydrolysis stage of AD was enhanced by the tested pre-

treatment methods. The lag phase for some conditions of enzymatic pre-treatment were shorter than for untreated microalgae; however, in low-temperature thermo-alkaline pre-treatment the increased methane yields were accompanied by an extended lag phase, especially for the high concentration of 15% w/w $Ca(OH)_2$. This was probably due to the generation of phenolic compounds when using a relatively high concentration of alkaline (Antonopoulou *et al.*, 2015).

In the current study, low-temperature thermo-alkaline pre-treatment released more carbohydrates, and this may indicate that this pre-treatment is more efficient in the degradation and solubilisation of cell wall components. However, enzymatic pre-treatment released more COD and proteins than thermo-alkaline pre-treatment, and this may suggest that the microalgae cell wall is broken down more efficiently via enzymatic pre-treatment, releasing more intercellular organic matters (Ometto *et al.*, 2014). Therefore, this likely explains why enzymatic pre-treatment produced more methane than low-temperature thermo-alkaline pre-treatment. However, Yen and Brune (2007) and Cho *et al.* (2013) reported that the biological decomposition of microalgal biomass via the hydrolysis of anaerobic microorganisms acts more strongly than pre-treatments. Therefore, the methane production of microalgae might be enhanced by other factors, such as the substrates to inoculum ratio, or operational conditions such as AD temperature rather than pre-treatments (Passos *et al.*, 2013; Caporgno *et al.*, 2015). Moreover, the high protein composition in microalgal biomass results in unbalanced carbon-to-nitrogen (C/N) ratios that may cause low methane yields (Schwede *et al.*, 2013; Li *et al.*, 2017).

4.4.3 *Effects of pre-treatment on energy balance*

The energy balance of microalgae pre-treatment was estimated in order to gain insights into the possibility of full-scale implementation (Passos *et al.*, 2013). In the current study, the extra methane production was sufficient to balance the energy required for the pre-treatment of microalgal biomass in most of tested conditions. The enzymatic pre-treatment showed the most energetically balanced conditions compared to low-temperature thermo-alkaline pre-treatment, and this result agrees with the finding of Ometto *et al.* (2014). In their study, after enzymatic pre-treatment, microalgal biomass produced more methane and leading to a more positive energy balance compared to thermal and ultrasound pre-treatments.

For low-temperature thermo-alkaline pre-treatment, higher energy inputs were associated with higher pre-treatment temperature, but more methane was also produced resulting in a more positive energy balance. This finding corroborates those of Passos *et al.* (2013), who found that for microalgae after thermal pre-treatments, the E_i/E_o ratios were decreased with increasing pre-treatment temperature from 55 to 95 °C; and a positive energy balance was obtained at temperatures over 75 °C. It should be noted that, in the current study, the temperatures used of 37 or 55 °C are lower than in the previous study. However, the combination of $Ca(OH)_2$ with low temperature was able to enhance methane yields leading to a positive energy balance, especially at an optimal dosage (such as 10% w/w). Therefore, the results suggest that low-temperature thermo-alkaline pre-treatment could also be an energetically efficient method in the pre-treatment of microalgae for methane production.

4.5 Conclusion

In this chapter, the effect of enzymatic and low-temperature thermo-alkaline pre-treatments on microalgae solubilisation and methane yield was investigated. For enzymatic pre-treatment, the methane yields were significantly enhanced after pre-treatment, where the enzyme concentration at 1.0% v/w dosage performed the best. Moreover, mixed enzymes gave better hydrolysis efficiency and produced more methane than the use of single enzymes.

For thermo-alkaline pre-treatment, the solubilisation of organic matters and methane yields increased with the pre-treatment temperature and $Ca(OH)_2$ dosage. However, increasing $Ca(OH)_2$ dosage did not significantly improve the maximum methane productions. The solubilisations of carbohydrates and proteins significantly increased with the pre-treatment time, but it had no significant effect on methane yields.

Overall, enzymatic pre-treatment could break down the microalgae cell wall efficiently, and may releasing more intercellular components, and producing more methane than low-temperature thermo-alkaline pre-treatment. The results also showed that both enzymatic and low-temperature thermo-alkaline pre-treatments were considered to be energetically efficient methods to pre-treat microalgae. However, unbalanced C/N ratios in microalgal biomass can cause low methane yields and an unstable AD process when using microalgae as a mono-digestion feedstock. Therefore, follow-up studies should carried out using co-digestion strategies to enhance methane production from microalgae.

Chapter 5. Anaerobic co-digestion of microalgae *Chlorella vulgaris* and potato processing waste in biochemical methane potential (BMP) tests: effect of mixing ratio, waste type and substrate to inoculum ratio

5.1 Introduction

An unbalanced carbon-to-nitrogen (C/N) ratio in microalgae can cause low methane yields and an unstable AD process when using microalgae as a mono-digestion feedstock. Co-digestion of microalgae with other carbon-rich substrates, such as waste paper and maize can balance the C/N ratios and increase methane yields as shown by Yen and Brune (2007), Schwede *et al.* (2013) and Rétfalvi *et al.* (2016).

Potato processing waste (PPW) consists of potato discarded parts (PPW_{dp}) and potato peel (PPW_{p}) (Pistis *et al.*, 2013; Schalchli *et al.*, 2017), and the C/N ratios of PPW ranges from 12.1/1 to 30.0/1 (Pistis *et al.*, 2013; Lucas, 2014), and therefore it is a promising feedstock for anaerobic co-digestion with other low carbon substrates such as dairy manure and sugar beet leaves (Parawira *et al.*, 2004; Yadanaparthi *et al.*, 2014). However, there is little information available about the anaerobic co-digestion of microalgae with PPW.

The substrate to inoculum ratio (SIR) is crucial during BMP tests as it ensures a balance of the bacteria and archaea that carry out the acidification and methanogenic process (Raposo *et al.*, 2009; Eskicioglu and Ghorbani, 2011; Fagbohungbe *et al.*, 2015)

The aim of this Chapter was to investigate the feasibility of using PPW as a co-substrate for co-digestion with microalga *Chlorella vulgaris* in batch BMP tests:

1. To evaluate the effects of the mixing ratios between *C. vulgaris* and PPW on methane yield;

2. To evaluate the effects of different types of PPW- discarded parts (PPW_{dp}) and peel (PPW_{p}) on methane yield;

3. To evaluate the effect of SIR on methane yield.

5.2 Materials and methods

5.2.1 *Experimental design*

The current study utilised a 5×2×2 mixed factorial design, including one within-independent variable, i.e. the mixing ratios between *C. vulgaris* and PPW, with five levels: 100:0, 75:25, 50:50, 25:75 and 0:100 on the basis of VS. The proportions were selected based on previous microalgae co-digestion studies (Yen and Brune, 2007; Wang *et al.*, 2016). The first between-independent variable was PPW type, either PPW_{dp} or PPW_{p}. The second between-independent variable was the substrate to inoculum ratio (SIR), where substrate and anaerobic inoculum were mixed to achieve a ratio of 0.5:1 or 1:1 on the basis of VS (Lee *et al.*, 2013; Ometto *et al.*, 2014). A summary of the experimental design is shown in Table 5.1. The dependent variables were biomenthane potential (experimental final methane yields and BMP kinetic results), and process stability that measured as the concentrations of soluble COD (COD_{s}) and FAN.

Table 5.1 Summary and coding of the experimental design for microalgae co-digestion trials. Treatment coding: D = potato discarded parts (PPW_{dp}), P = potato peel (PPW_{p}), 1-5 corresponds to the mixing ratio, A = SIR of 1.0, B = SIR of 0.5.

***C. vulgaris* : PPW (based on VS)**									
100:0		**75:25**		**50:50**		**25:75**		**0:100**	
D1A	P1A	D2A	P2A	D3A	P3A	D4A	P4A	D5A	P5A
D1B	P1B	D2B	P2B	D3B	P3B	D4B	P4B	D5B	P5B

5.2.2 *Microalgae and potato processing waste*

The *C. vulgaris* strain (CCAP 211/63) was used in this study and detailed cultivation and harvesting methods are described in Section 3.1. The simulated PPW was made in two groups of PPW_{dp} and PPW_{p} as shown in Figure 5.1. The waste, with 2 L of distilled water, was homogenized using a kitchen blender.

The feedstocks were characterised by their TS, VS, COD_t, carbohydrate and proteins content, as well as carbon (C) and nitrogen (N) content are summarised in Table 5.2. The anaerobic seed inoculum was collected from a manure-based farm anaerobic digester located at Cockle Park Farm, Northumberland, UK.

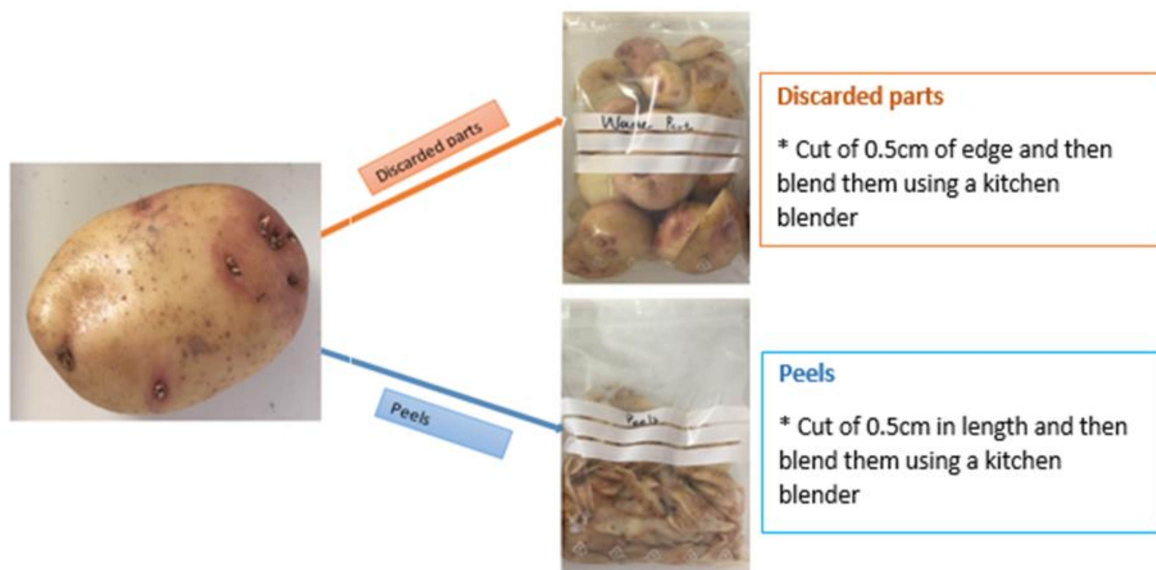


Figure 5.1 Preparation of potato processing waste

5.2.3 *Biochemical methane potential (BMP) test*

Detailed methods of set up batch BMP tests are described in Section 4.2.4. The volume of methane was calculated under STP conditions (0 °C, 1atm), and detailed methods can be found in Section 3.4.1. All BMP bottles were placed in a 37 °C incubator at 70 rpm for 25 or 27 days of reaction.

5.2.4 *Analytical methods*

General analytical procedures are described in Section 3.2.

5.2.5 *Kinetics of anaerobic digestion*

The modified Gompertz kinetics model is described in Section 4.2.6.

5.2.6 *Synergistic effect*

The synergistic effect is the inner reaction produced by the co-digestion of different feedstocks (Nielfa *et al.*, 2015). This effect can be calculated as Equation 5.1.

$$\alpha = \frac{Experimental\ Yield}{Theoretical\ Yield} \quad (5.1)$$

where "Experimental Yield" is the methane yield obtained from BMP test for each co-digestion mixture, "Theoretical yield" was calculated from the experimental yields of the individual substrates taking into account of VS values of each substrate.

5.2.7 *Statistical analysis*

Experimental data (final methane yield, kinetic data, COD_s, and FAN) were analysed by a 3-way mixed analysis of variance (ANOVA) with the Bonferroni post hoc test (Quinn and Keough, 2002; Field, 2009) using IBM SPSS statistics, version 23. The 95% confidence interval of differences ($p<0.05$) was chosen to define the statistical significance. MATLAB, R2015a was used to calculate P, R_m and λ for each digester.

5.3 Results

5.3.1 *Substrate and inoculum characterisation*

C. vulgaris had a C/N ratio of 6.43/1 (Table 5.2). The addition of PPW_{dp} enhanced the C/N ratio to within a range of 8.03/1 to 22.77/1. The C/N ratios were also increased by adding PPW_p (ranging from 7.99/1 to 19.86/1). For these co-digestion mixtures, the carbohydrates content increased whereas the protein content decreased.

The seed inoculum had TS of 15.2 ± 0.1 g/L, VS/TS of 63.0 ± 1.0%, a pH of 7.96 and concentrations of NH_4^+-N and FAN of 4100 ±141 and 433 ± 15 mg/L, respectively.

Table 5.2 Characterisation of microalgae *Chlorella vulgaris*, potato discarded parts (PPW_{dp}) and peel (PPW_{p}), and co-digestion mixtures.

	TS (g/L)	**VS (g/L)**	**VS/TS (%)**	**COD (g/L)**	**COD/VS**	**Protein (% VS)**	**Carbohydrate (% VS)**	**C (% DW)**	**N (%DW)**	**C/N**
Chlorella	3.6 ± 0.6[a]	3.5 ± 0.7	97.0 ± 1.2	5.6 ± 1.0	1.6 ± 0.1	35.9 ± 3.1	20.0 ± 3.3	52.55	8.17	6.43
PPW_{dp}	11.5 ± 8.2[b]	10.0 ± 6.9[b]	90.1 ± 5.8	20.5 ± 15.6	1.8 ± 0.5	16.4 ± 2.3	77.0 ± 1.3	39.76	0.97	40.78
PPW_{p}	8.1 ± 5.9	6.9 ± 4.8	88.2 ± 5.3	10.4 ± 6.9	1.6 ± 0.2	17.8 ± 1.3	63.3 ± 10.6	40.25	1.40	28.59
25% PPW_{dp}	4.6 ± 1.3	4.4 ± 1.2	95.0 ± 1.0	6.7 ± 1.8	1.5 ± 0.1	27.1 ± 3.3	44.4 ± 2.5	49.47	6.16	8.03
50% PPW_{dp}	5.4 ± 1.8	5.0 ± 1.6	92.7 ± 2.6	7.4 ± 2.3	1.5 ± 0.0	25.7 ± 3.1	51.7 ± 3.4	46.96	4.16	11.24
75% PPW_{dp}	8.8 ± 5.1	7.9 ± 4.4	91.5 ± 3.7	11.5 ± 5.7	1.5 ± 0.2	21.0 ± 1.2	67.2 ± 2.3	44.25	1.94	22.77
25% PPW_{p}	3.9 ± 1.1	3.6 ± 1.0	93.9 ± 0.9	5.5 ± 1.6	1.5 ± 0.1	25.2 ± 2.8	42.4 ± 4.3	50.03	6.26	7.99
50% PPW_{p}	4.1 ± 1.2	3.7 ± 1.0	90.0 ± 3.9	5.1 ± 1.7	1.4 ± 0.2	23.6 ± 1.5	51.8 ± 5.2	46.22	4.13	11.19
75% PPW_{p}	6.3 ± 3.5	5.4 ± 2.8	88.7 ± 5.3	7.0 ± 3.8	1.3 ± 0.2	21.0 ± 0.1	54.0 ± 9.0	42.65	2.15	19.86

[a] Mean = ± SD, n = 4.

[b] the large variations in TS and VS content was due to different amounts of raw substrate having been diluted with distilled water.

5.3.2 *Biomenthane potential of mono- and co-digestion*

5.3.2.1 Experimental BMP

Figure 5.2 shows the cumulative methane produced by mono-digestion of *C. vulgaris*, and co-digestion with PPW_{dp} (Figure 5.2 A) or PPW_p (Figure 5.2 B) for 1.0 SIR. The methane yields were inhibited during the first 7 days for all experimental conditions, but after 9 days the methane yields increased linearly with time. Mono-digestion of *C. vulgaris* produced 158 mL CH_4/g VS, compared with the yields of 232 and 340 mL CH_4/g VS by mono-digestion of PPW_p and PPW_{dp}, respectively.

Figure 5.3 shows the cumulative methane produced by mono-digestion of *C. vulgaris*, and co-digestion with PPW_{dp} (Figure 5.3 A) or PPW_p (Figure 5.3 B) for 0.5 SIR. After 3 days, the methane yields increased linearly with time for all treatments. The lowest yield was 176 mL CH_4/g VS by mono-digestion of *C. vulgaris*, while the high methane yields were 439 and 348 mL CH_4/g VS by mono-digestion of PPW_{dp} and PPW_p, respectively.

For both SIRs, co-digestion of the mixtures showed methane yields between those of the two mono-substrates, and the mixing ratios between *C. vulgaris* and PPW had a significant effect on the final methane yields ($F(4,32)=100.68$, $p<0.001$). A greater PPW introduction relative to *C. vulgaris* led to an improvement of the methane yields. The yields were achieved by co-digestion of 25:75 *C. vulgaris* and PPW were significantly higher than the mixing ratios of 100:0 ($p<0.001$), 75:25 ($p<0.001$) and 50:50 ($p=0.031$). Moreover, the type of PPW also had a significant effect on yields ($F(1,8)=52.94$, $p<0.001$), with PPW_{dp} giving significantly higher methane values than both mono-digestion or co-digestion with PPW_p. To access the impact of SIR on methane yields, it was found that the overall methane yields were significantly higher for 0.5 SIR than for 1.0 SIR ($F(1,8)=54.82$, $p<0.001$).

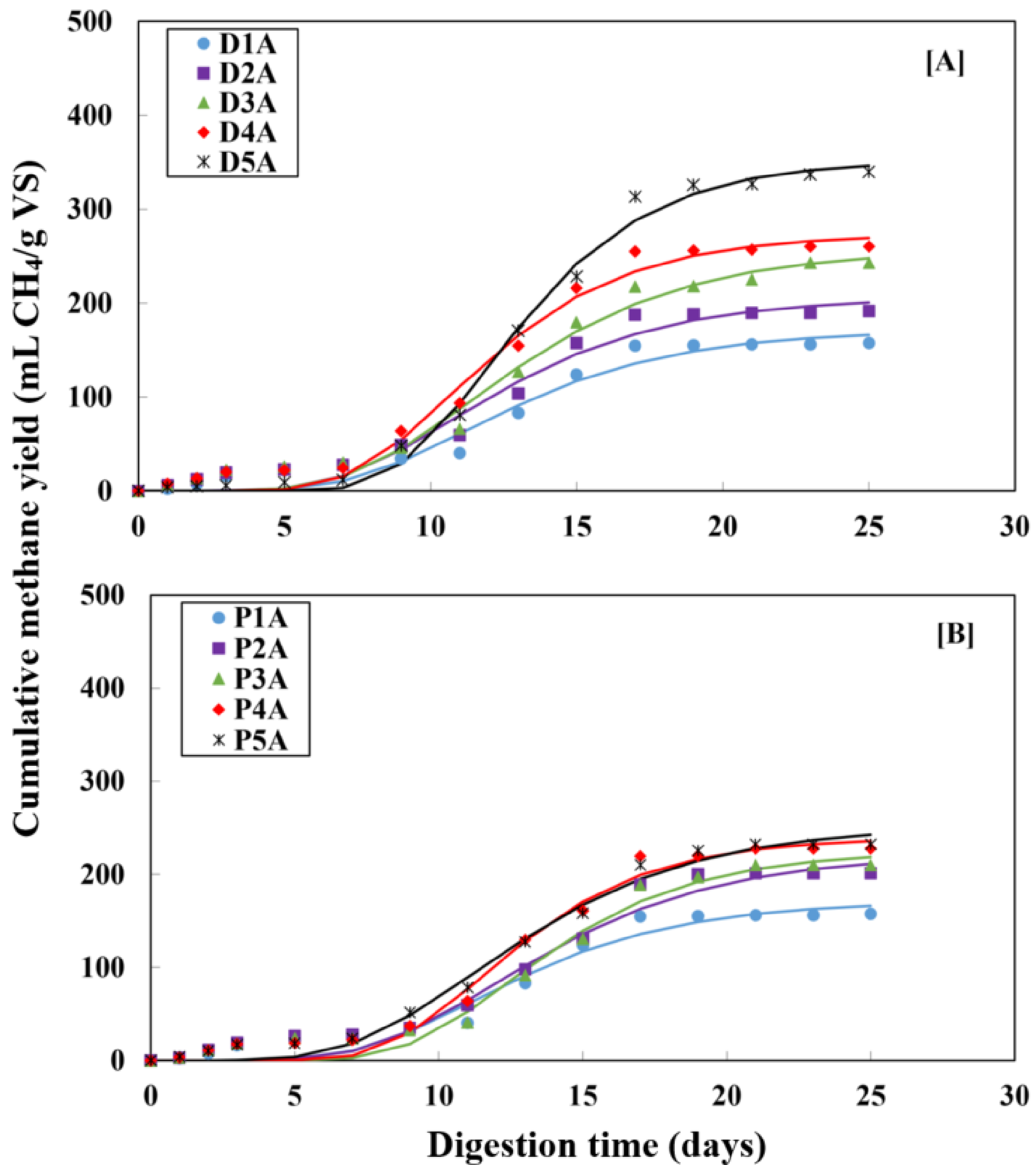


Figure 5.2 Cumulative methane yield of *Chlorella vulgaris* co-digested with PPW for 1.0 SIR with [A] potato discarded parts (PPW_{dp}) and [B] potato peel (PPW_p). The solid line represents the Gompertz model fit data. Co-digestion with PPW_{dp} at 1.0 SIR (D1A-D5A), co-digestion with PPW_p at 1.0 SIR (P1A-P5A).

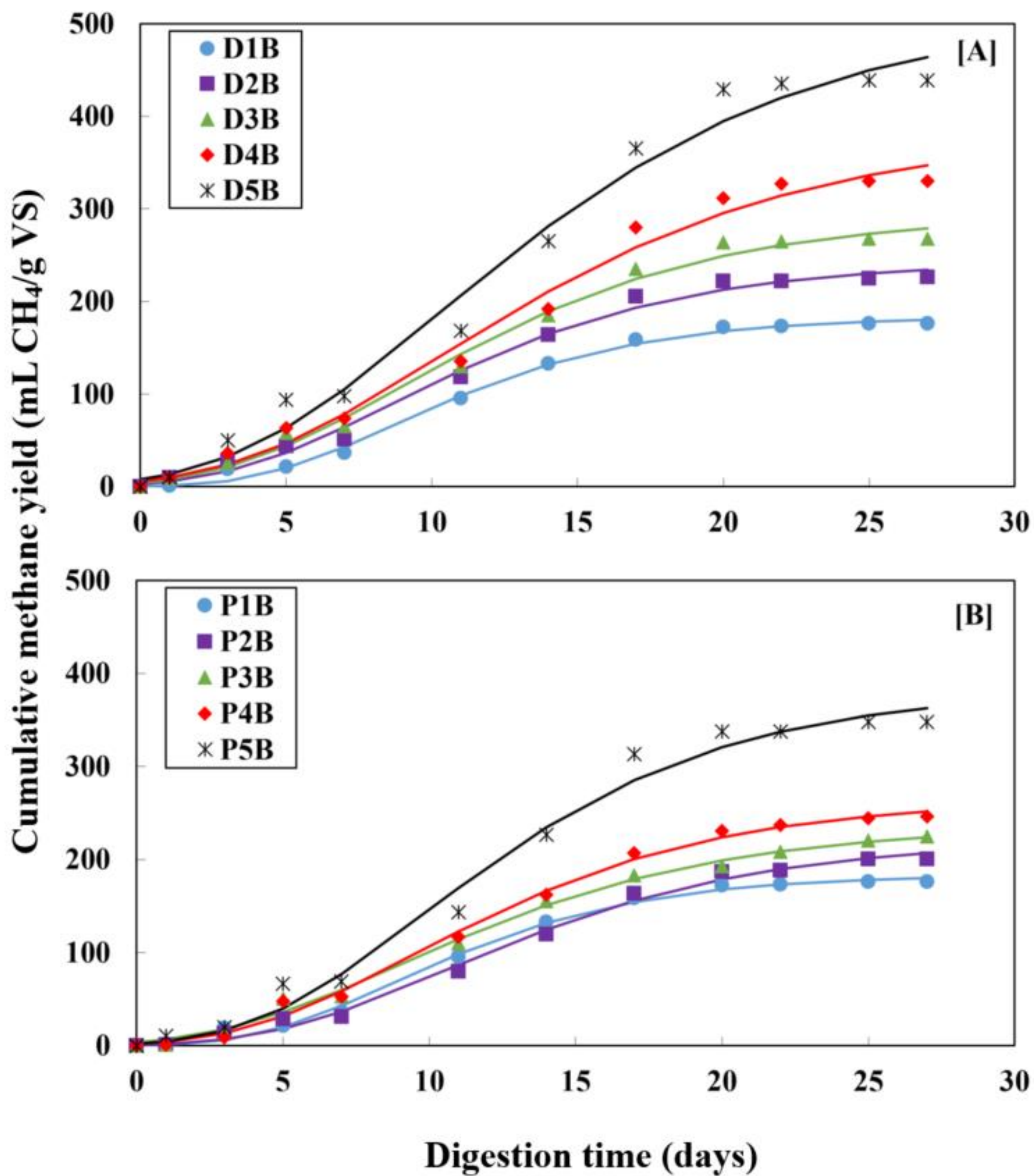


Figure 5.3 Cumulative methane yield of *Chlorella vulgaris* co-digested with PPW for 0.5 SIR with [A] potato discarded parts (PPW_{dp}) and [B] potato peel (PPW_p). The solid line represents the Gompertz model fit data. Co-digestion with PPW_{dp} at 0.5 SIR (D1B-D5B); co-digestion with PPW_p at 0.5 SIR (P1B-P5B).

5.3.2.2 BMP kinetic model

The modified Gompertz model was applied to the experimental BMP data and used to determine the maximum methane production rate (R_m) and lag phase (λ) for each substrate (Table 5.3). The values of R_m obtained by co-digestion with PPW were gradually improved as the proportions of PPW_{dp} or PPW_p were increased, and the mixing ratios between *C. vulgaris* and PPW had a significant effect on R_m ($F(1.736,32)=18.52$, $p<0.001$). The PPW type also had a significant effect on R_m ($F(1,8)=17.89$, $p=0.003$). Co-digestion or mono-digestion with PPW_{dp} gave higher production rates than with PPW_p. It was also found that the values of R_m for 0.5 SIR were significantly lower than those produced by 1.0 SIR ($F(1,8)=58.28$, $p<0.001$). The time values of λ for 0.5 SIR were significantly shorter than at 1.0 SIR ($F(1,8)=177.59$, $p<0.001$). The effect of mixing ratios on time values of λ was not significant ($F(4,32)=0.54$, $p=0.711$). Also, the values of λ did not significantly differ between PPW_{dp} and PPW_p ($F(1,8)=1.76$, $p=0.221$). However, there was a significant interaction effect between the mixing ratios, type of PPW and SIR ($F(4,32)=3.58$, $p=0.016$). This interaction effect can be seen as at 0.5 SIR, when the type of PPW is PPW_{dp}, the values of λ being reduced as increasing the proportions of PPW.

Table 5.3 Summary of modified Gomerptz kinetics data for *Chlorella vulgaris* co-digestion with PPW_{dp} or PPW_p at two substrate to inoculum ratios (SIR). Co-digestion with PPW_{dp} at 1.0 SIR (D1A-D5A) or 0.5 SIR (D1B-D5B); co-digestion with PPW_p at 1.0 SIR (P1A-P5A) or 0.5 SIR (P1B-P5B).

1.0 SIR	**R_m (mLCH_4/gVS/d)**	**λ (d)**	**R^2**	**0.5 SIR**	**R_m (mLCH_4/gVS/d)**	**λ (d)**	**R^2**
D1A	15.75	7.14	0.9659	**D1B**	14.33	4.06	0.9957
D2A	21.45	6.85	0.9899	**D2B**	23.91	3.90	0.9879
D3A	18.97	6.76	0.9730	**D3B**	15.74	2.95	0.9923
D4A	22.77	7.17	0.9817	**D4B**	17.60	2.82	0.9924
D5A	40.90	8.73	0.9942	**D5B**	26.15	3.11	0.9845
P1A	15.75	7.14	0.9659	**P1B**	14.33	4.06	0.9957
P2A	29.41	7.22	0.9865	**P2B**	19.67	3.15	0.9882
P3A	18.77	7.57	0.9739	**P3B**	13.32	4.48	0.9945
P4A	22.94	8.76	0.9775	**P4B**	13.85	2.70	0.9953
P5A	21.45	6.85	0.9899	**P5B**	23.91	3.90	0.9879

5.3.2.3 Synergistic effects of co-digestion

To study the synergistic effects produced by co-digestion of *C. vulgaris* with PPW_{dp} or PPW_p, the theoretical yields of co-substrates were calculated from the experimental yields of the sole substrates taking into account of VS values of each substrate. Figure 5.4 shows that the synergistic effects (experimental yields higher than theoretical yields) were only found in co-digestion of *C. vulgaris* with PPW_p for 1.0 SIR.

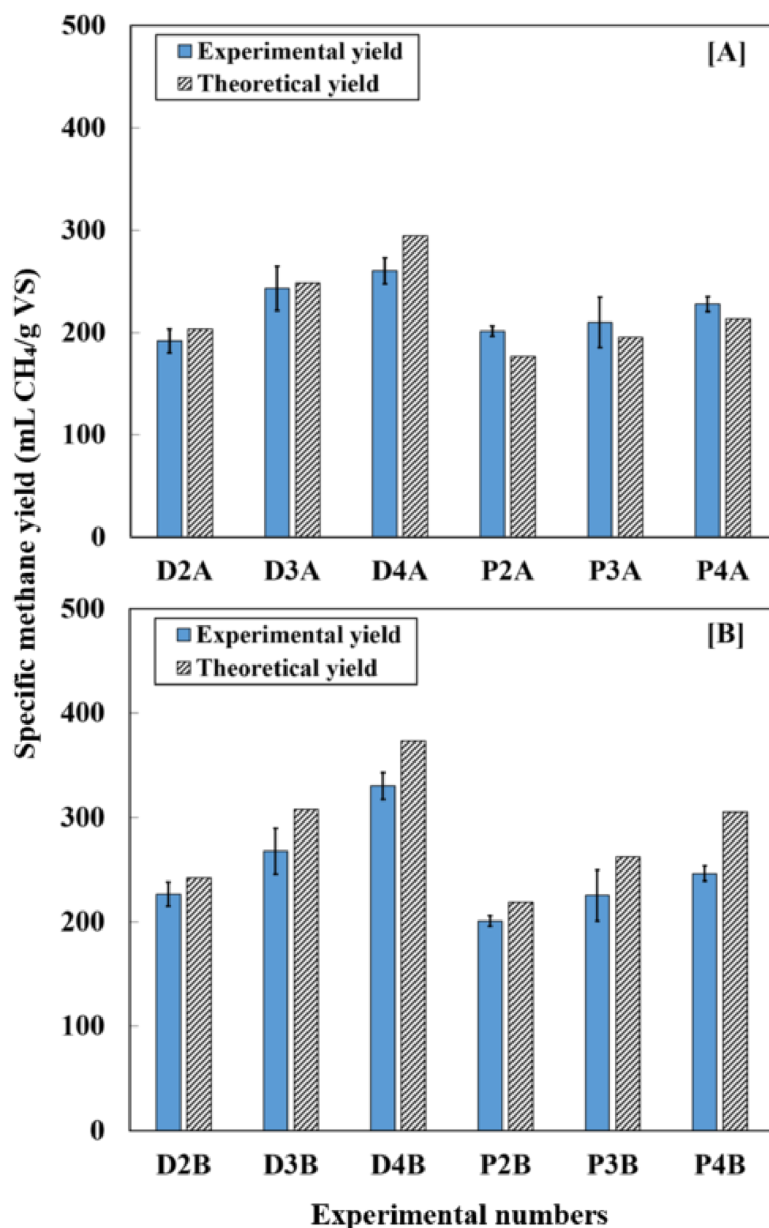


Figure 5.4 Experimental and theoretical methane yields for the co-digestion of *Chlorella vulgaris* with PPW at [A] 1.0 SIR and [B] 0.5 SIR. Potato discarded parts (PPW_{dp}) at 1.0 SIR (D2A-D4A) and 0.5 SIR (D2B-D4B); or with potato peel (PPW_p) at 1.0 SIR (P2A-P4A) and 0.5 SIR (P2B- P4B). Error bars = mean ± SD, n=2.

5.3.3 *Process stability*

In the current study, concentrations of COD_s, NH^{+}_{4}-N and pH were measured at the end of the BMP tests. The concentrations of FAN were calculated based on the values of pH and NH^{+}_{4}-N. Figure 5.5 shows that the highest COD_s present at the end of the BMP tests was 3210 mg/L and was produced by mono-digestion of PPW_{dp} at 1.0 SIR. The mixing ratios had a significant effect on COD_s ($F(4,16)=38.26$, $p<0.001$). The overall COD_s obtained by digestion with PPW_{dp} were significantly higher than with PPW_p ($F(1,4)=122.51$, $p<0.001$). The concentrations of COD_s improved with increasing the SIR ratio, and the values obtained by 0.5 SIR were significantly lower than by 1.0 SIR ($F(1,4)=1473.68$, $p<0.001$). The significant effect on COD_s was also qualified by an interaction effect between mixing ratios, type of PPW and SIR ($F(4,16)=33.49$, $p<0.001$). This interaction effect can be seen as the similar amounts of COD_s being produced by co-digestion of *C. vulgaris* with PPW_p for both SIRs. During co-digestion of *C. vulgaris* with PPW_{dp}, similar amounts of COD_s were obtained at 0.5 SIR; however, for 1.0 SIR the concentrations of COD_s increased significantly with increasing proportions of PPW_{dp}. Specifically, mono-digestion of *C. vulgaris*, and co-digestion with 25% and 50% PPW_{dp} produced similar amounts of COD_s. However, the concentrations of COD_s increased significantly when the proportions of PPW_{dp} were at 75% and 100%.

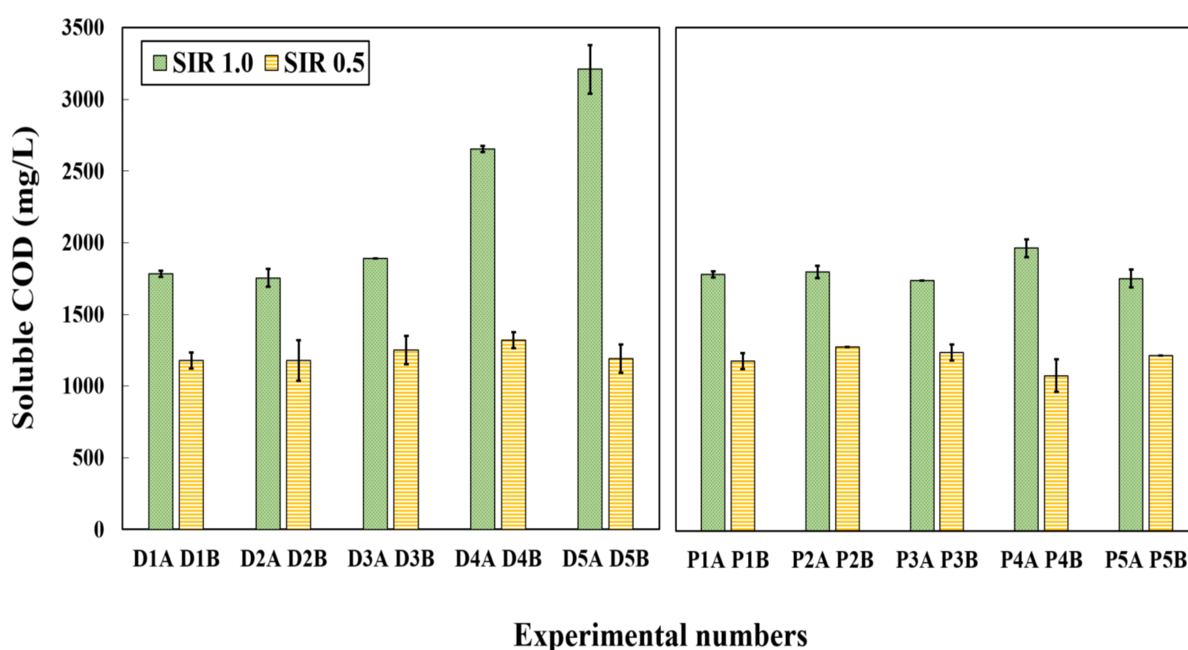


Figure 5.5 Concentrations of soluble COD obtained at the end of co-digestion of *Chlorella vulgaris* with potato discarded parts (PPW_{dp}) at 1.0 SIR (D1A-D5A) and 0.5 SIR (D1B-D5B); or with potato peel (PPW_p) at 1.0 SIR (P1A-P5A) and 0.5 SIR (P1B- P5B). Error bars = mean ± SD, n=2.

Figure 5.6 shows that the highest FAN of 123 mg/L was obtained by mono-digestion of *C. vulgaris* at 0.5 SIR, and the lowest value of 20 mg/L was obtained by mono-digestion of PPW_{dp} at 1.0 SIR. The mixing ratios of PPW had a significant effect on FAN ($F(4,16)=20.70$, $p<0.001$). Also, the concentrations of FAN were significantly increased at lower SIR ($F(1,4)=626.42$, $p<0.001$). The main effects of mixing ratios of PPW and SIR on concentrations of FAN can also qualified by a significant interaction effect between these two factors ($F(4,16)=15.44$, $p<0.001$). This interaction effect can be seen as, for 0.5 SIR, the concentrations of FAN showed a clear decreasing trend as increasing the proportions of PPW_{dp} or PPW_p. The type of PPW also had a significant effect on concentrations of FAN ($F(1,4)=13.86$, $p=0.020$).

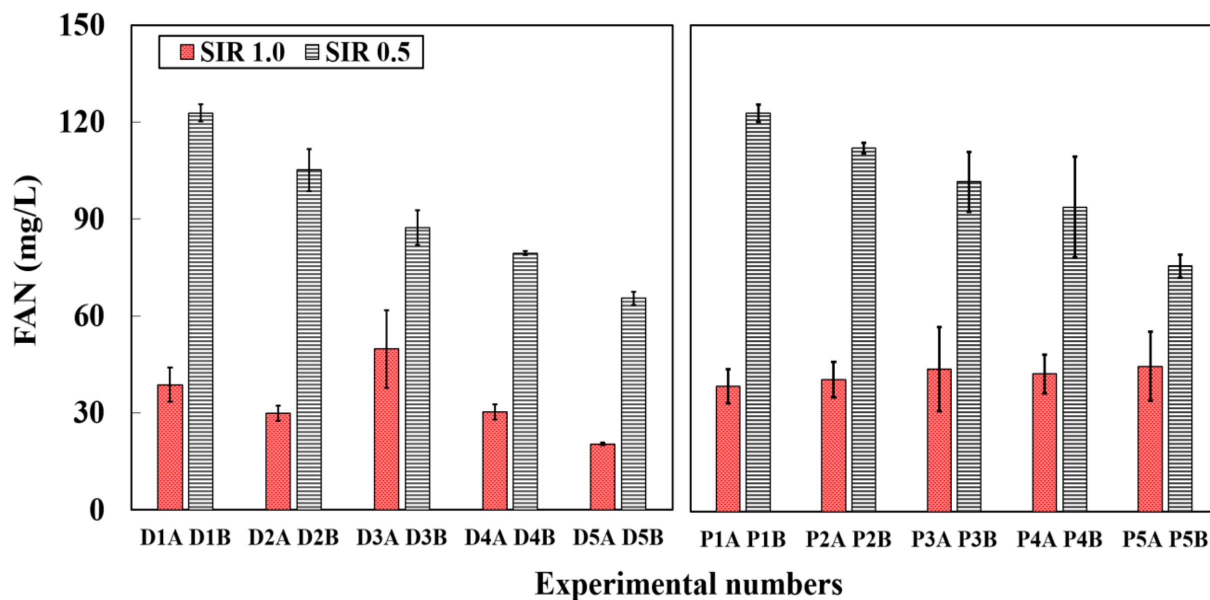


Figure 5.6 Concentrations of free ammonia nitrogen (FAN) obtained at the end of co-digestion of *Chlorella vulgaris* with potato discarded parts (PPW_{dp}) at 1.0 SIR (D1A-D5A) and 0.5 SIR (D1B-D5B); or with potato peel (PPW_p) at 1.0 SIR (P1A-P5A) and 0.5 SIR (P1B- P5B). Error bars = mean ± SD, n=2.

5.4 Discussion

5.4.1 *Effect of co-digestion on biomethane potential and process stability*

In the current study, 158 and 176 mL CH_4/g VS were produced by mono-digestion of *C. vulgaris*, which is lower than previously reported by Wang *et al.* (2016). Their results showed that 250 mL CH_4/g VS was produced by mono-digestion of *Chlorella sp.* and a possible reason for this is that the growth media they applied to cultivate the microalgae was a mixed media containing synthetic and real AD swine effluent. Consequently, the mature *Chlorella sp.* was harvested with a C/N ratio at 17/1, which is much higher than in the current study (6.43/1).

The benefits of co-digestion of microalgae with carbon-rich feedstocks are to rebalance the C/N ratio, reduce the concentration of inhibitory compounds affecting methanogens, and provide a stable AD process (Yen and Brune, 2007). In the current study, the addition of PPW_{dp} or PPW_p to *C. vulgaris* both resulted in an increase in the C/N ratio, and the results indicate that PPW has the potential to be an effective co-substrate for microalgae co-digestion in terms of generating more balanced C/N ratios. Consequently, the co-digestion of *C. vulgaris* with PPW_{dp} increased methane yields by 22 – 47% above that of *C. vulgaris* mono-digestion, while co-digestion with PPW_p increase it by 12 – 32%. Solé-Bundó *et al.* (2017b) investigated the co-digestion of wheat straw with mixed microalgae in batch BMP tests. Their results showed that the final methane yield increased by only 5 – 9% compared to microalgae mono-digestion, which is lower than the current study. Wheat straw is a lignocellulosic biomass consisting of 40.8 – 49.8% of cellulose, 26.4% of hemicellulos and 19.6 – 22.9% of lignin (Beltrame *et al.*, 1992; Zimbardi *et al.*, 1999; Kasmani and Samariha, 2011). Lignocellulosic biomass comprises a strong structural matrix formed by the digestible polymers (cellulose and hemicellulose) being embedded within the relatively recalcitrant lignin component, and therefore requires additional treatment to be broken down completely into simple sugars. However, 65.0 – 85.0 % of the carbohydrate in potato waste is present as starch (Arapoglou *et al.*, 2010; Leonel *et al.*, 2017), and unlike lignocellulosic biomass, it is easily broken down into sugars (Hess *et al.*, 2007). Therefore, this suggests that PPW could be more efficient than lignocellulose biomass as a co-substrate with microalgae.

Improved kinetics data is another benefits of co-digestion of two feedstocks had reported by Ramos-Suárez *et al.* (2014) and Solé-Bundó *et al.* (2017b). In the current study, the addition of PPW to *C. vulgaris* significantly increased the values of R_m, while a significant reduction the values of λ was seen for the co-digestion with PPW_{dp} at 0.5 SIR. The improved kinetics may also suggest that PPW could be a useful co-substrate in co-digestion with microalgae. Moreover, the concentrations of FAN were significantly reduced by co-digestion with PPW, and FAN is regarded as the active component leading to ammonia inhibition for AD process (Yenigün and Demirel, 2013). Therefore, in the current study, the results indicating that co-digestion of microalgae with PPW brings further benefits by reducing the risk of ammonia toxicity.

The synergistic effects would be an additional benefits provided by co-digestion of different feedstocks (Nielfa *et al.*, 2015). However, in the current study, synergistic effects in final methane yields were only found for the co-digestion of *C. vulgaris* with PPW_p at 1.0 SIR. Research to identify the possible mechanisms leading to the improvement of co-digestion performance has not focused entirely on the balancing of C/N ratios in feedstock. Some studies reported that the synergistic effect of co-digestion of microalgae with other co-substrates was attributed to certain micronutrients and essential trace elements provided to the microorganisms, and this may hiding the true benefits of the co-digestion (Schwede *et al.*, 2013; Olsson *et al.*, 2014). However, in the current study, additional nutrients were not supplied in BMP bottles, and therefore the presence of potentially toxicity components during digestion was thought to influence the synergistic effects seen in co-digestion studies (Cheng and Zhong, 2014). In the current study, the seed inoculum was collected from a manure-based anaerobic digester with high concentrations of ammonia nitrogen, especially FAN which was measured at over 400 mg/L. This inoculum was used without diluting and washing, and could have provided extra nitrogen in BMP bottles, masking the true benefits of the co-digestion mixtures in batch BMP tests. Consequently, for 0.5 SIR, the concentrations of FAN were significantly higher than for 1.0 SIR because of the relatively higher proportion of inoculum that was added into the BMP bottles. Therefore, this likely explains why the synergistic effects of the co-digestion substrates were only found for 1.0 SIR.

5.4.2 *Effect of mixing ratios on biomethane potential and digester stability*

The co-digestion of *C. vulgaris* with PPW, the methane yields also affected by the mixing ratios between *C. vulgaris* and PPW proportions. In the current study, the best performance was found at ratio of 25:75 *C. vulgaris* and PPW compared to ratios of 75:25 and 50:50. Wang *et al.* (2016) found that the C/N ratio of swine manure was 35/1, and compared to mono-digestion with *Chlorella sp.*, the methane yields improved by around 13 – 28% after co-digestion with these two substrates. This improvement is similar to that achieved by co-digestion of PPW_p with *C. vulgaris*, however, in the previous study the highest yield of 348 mL CH_4/g VS was obtained from co-digestion with 6% *Chlorella sp.* and 94% swine manure on the basis of VS. Since the 25:75 ratio was the highest co-digestion ratio investigated in the current study that contained *C. vulgaris*, it is possible that higher methane yields might have been found at ratios containing greater proportions of PPW (e.g a 10:90 ratio). However, in the current study, the results showed that the concentrations of COD_s increased with increasing proportions of PPW to microalgae, and the highest COD_s observed by mono-digestion of PPW. According to González-Fernández and García-Encina (2009), the COD_s produced in an anaerobic process corresponds mainly to oxidation produced volatile fatty acids (VFA). Moreover, potato waste has high degree of soluble components and high biodegradability resulting in rapid and strong acidification, and consequently accumulated more VFA which may have inhibited the activity of methanogens (Kaparaju and Rintala, 2005; Pistis *et al.*, 2013).Therefore, the results may indicate that adding higher proportions (> 75%) of PPW to microalgae increased the possibility of generating high VFA concentrations that might inhibit the AD process. Similarly, the mono-digestion of PPW creates a possibility that the AD process might inhibited due to VFA accumulation, although it obtained highest methane yields in the current batch BMP tests. In the current study, the C/N ratios in the mixtures of *C. vulgaris* with PPW_{dp} or PPW_p at a mixing ratio of 25:75 were 22.77/1 and 19.86/1, respectively, both within the optimal range quoted for AD process (Yen and Brune, 2007; Weiland, 2010). Therefore, the current study suggests that a mixing ratio of 25:75 might provide more optimal conditions for the co-digestion of microalgae and PPW.

5.4.3 *Effect of type of PPW on biomethane potential and digester stability*

The addition of PPW_{dp} or PPW_{p} to *C. vulgaris* increased the C/N ratios and the final methane yields. Therefore, both of these sources of PPW could be used as co-digestion substrates with microalgae. Methane yields during mono- or co-digestion with PPW_{dp} were significantly higher than during mono- or co-digestion with PPW_{p}. PPW_{dp} consisting of 54.3 – 76.8 % of dry matter as starch (Leonel *et al.*, 2017), while Arapoglou *et al.* (2010) and Liang *et al.* (2014) observed for PPW_{p} that between 34.3 – 52.1 % of dry matter is starch. In the present study, the total carbohydrates content for PPW_{dp} was 77.0 % of dry weight, and 63.3 % of dry weight for PPW_{p}, suggesting that PPW_{dp} contained higher amounts of starch (around 85% of total carbohydrates) than the values (65% of total carbohydrates) for PPW_{p}, although the starch analysis was not determined directly. Spets *et al.* (2010) reported that starch is easily broken down into monosaccharides, and the higher starch contents in anaerobic feedstocks may improve their anaerobic biodegradability (Kaparaju and Rintala, 2005). Therefore, in the current study, the higher methane yields achieved by mono- or co-digestion with PPW_{dp} was probably a result of their higher starch content with respect to PPW_{p}. Moreover, increased methane production rates (R_{m}) and shorter lag phase (λ) were seen during mono- or co-digestion with PPW_{dp} compared to PPW_{p}. These results suggest that the PPW_{dp} contained greater concentrations of soluble components than PPW_{p}.

5.4.4 *Effect of SIR on biomethane potential and digester stability*

In the current study, the results showed that methane yields were increased at lower SIR, and agree with previous studies using different substrates (Raposo *et al.*, 2009; Eskicioglu and Ghorbani, 2011; Fagbohungbe *et al.*, 2015). An optimum SIR in the digester is considered to contain the balanced amount of anaerobic microorganisms for digestion of both primary and intermediate products (Raposo *et al.*, 2009; Eskicioglu and Ghorbani, 2011; Fagbohungbe *et al.*, 2015). However, the lag phase (λ) in the present study significant reduced at higher SIR, indicating that the activity of methanogens was limited for 1.0 SIR compared to 0.5 SIR; supported by the findings of Li *et al.* (2014b) and Fagbohungbe *et al.* (2015).

The concentrations of COD_{s} decreased at the lower SIR, which agree with a previous study reported by Fagbohungbe *et al.* (2015). And higher COD_{s} concentrations were measured at the end of digestion for 1.0 SIR, compared to 0.5 SIR, and hence it is likely that much of this was from VFA. In the current study, a significant interaction effect was observed between the

mixing ratios, type of PPW and SIR, and may indicate that the co-digestion of *C. vulg*aris with higher proportions (> 75% VS) of PPW has the potential to accumulate more VFAs in terms of COD_s under higher SIR. In the present study, the concentrations of FAN were significantly increased at lower SIR, contrasting the results obtained by González-Fernández and García-Encina (2009). In their study, it was found that the final concentrations of FAN was reduced with a decreasing SIR. The high concentrations of FAN present in the seed inoculum could have been responsible for the observed effects in the current study.

5.5 Conclusion

This Chapter has demonstrated the possibility that potato processing waste (PPW) could be used effectively as a feedstock co-digestion with microalgae. In batch BMP tests the methane production rates and final methane yields were all increased significantly as the proportion of PPW in the mixed waste was increased. Addition of relatively high proportions of PPW could decrease the concentrations of FAN, and improve digestion performance and stability by reducing the likelihood of ammonia toxicity. The PPW_{dp} and PPW_p co-digestion feedstocks both show good potential for co-digestion with microalgae. Co-digestion of *C. vulgaris* with PPW_{dp} increased the methane yields the most, by 22 – 47%, whilst co-digestion of *C. vulgaris* with PPW_p enhanced the methane yields by 12 – 32%. Methane yields and duration of lag phase were both affected significantly by the variation of the SIRs. The residual level of COD_s present at the end of BMP tests was greater at the higher SIR, and may limit observed methane yields.

Overall, the investigation suggests that PPW_{dp} and PPW_p are both promising feedstocks for co-digestion with microalgae. The enhanced methane yields resulting from co-digestion can be attributed mainly to the balanced C/N ratios. However, the presence of relatively high concentrations of ammonia in seed inoculum could have hidden the true benefits of the co-digestion. Therefore, follow-up studies should carried out using continuously fed anaerobic digesters to verify the potential of PPW as a feedstock for co-digestion with microalgae.

Chapter 6. Semi-continuous anaerobic co-digestion of marine microalgae with potato processing waste: the effects of start-up strategy and waste type on methane production and archaeal community

6.1 Introduction

In Chapter 5, it has demonstrated that potato processing waste (PPW) can be used an effective feedstock for co-digestion with freshwater microalgae *C. vulgaris* in batch BMP tests. However, the presence of relatively high concentrations of ammonia in seed inoculum could have hidden the true benefits of the co-digestion. Moreover, methane production is also dependent on the microalgae strains used (Mussgnug *et al.*, 2010). Some marine strains such as *Isochrysis* have a simple cell membrane (D'Hondt *et al.*, 2018), but as with freshwater strains their methane production potential is also limited due to high protein compositions (Caporgno *et al.*, 2015). Therefore, this chapter was carried out using semi-continuously fed anaerobic digesters to verify the potential of PPW as a feedstock for co-digestion with marine microalgae.

The start-up stage of a continuous anaerobic digester is an important step in the AD process, the success of which is related to a number of factors such as initial OLR, HRT, and the source and initial amounts of the seed inoculum (Angelidaki *et al.*, 2006). Successful start-up of an anaerobic digester also aims to develop an appropriate microbial community to shorten the period of acclimatization as reported by Benabdallah *et al.* (2007) and Westerholm *et al.* (2016). Moreover, changes in microbial communities during the AD process have been reported that link to the performance of anaerobic digesters (Moset *et al.*, 2014). However, microbial community information during the AD of microalgae is still limited.

The aim of this Chapter was to investigate the feasibility of using potato processing waste as a co-substrate for co-digestion with microalgae in semi-continuous co-digestion studies:

1. To evaluate the effects of start-up strategy on methane yields, digester performance and digester microbial communities.

2. To evaluate the effects of PPW type on methane yields, digester performance and digester microbial communities.

6.2 Materials and methods

6.2.1 *Substrates and inoculum*

A commercial marine microalgae strain *Tisohrysis lutea* (CCMP 1324) (also frequently referred to as *Isochrysis*), and marketed as 'Instant Algae', was obtained from Varicon Aqua Solutions Ltd, UK. Approximately 0.4 L of algae concentrate (117.8 g TS/L) was resuspended in 2 L of distilled water. Two types of simulated PPW were prepared as PPW_{dp} and PPW_p. The PPW was homogenized using a kitchen blender with the appropriate addition of distilled water, and the detailed information about the preparation of PPW have been described in Section 5.2.2. The *T. lutea*, PPW_{dp} and PPW_p feedstocks were characterised by their total solids (TS), volatile solids (VS), total chemical oxygen demand (COD_t), and carbohydrate and protein content as well as carbon (C) and nitrogen (N) content, as summarized in Table 6.1.

The TS and VS of inoculum were 14.8 ± 0.1 and 8.3 ± 0.1 g/L, respectively. pH value of inoculum was 7.94, and concentrations of NH_4^+-N and FAN were 4100 ±141 and 416 ± 14 mg/L, respectively.

Table 6.1 Feedstock characterisation: *Tisochrysis lutea*, potato discarded parts (PPW_{dp}) and potato peel (PPW_p).

	T. lutea	**PPW_{dp}**	**PPW_p**
TS (g/L)	22.1 ± 0.1[a]	20.4 ± 0.7	17.0 ± 0.5
VS (g/L)	10.5 ± 0.2	18.4 ± 0.1	15.0 ± 0.4
VS/TS (%)	47.6 ± 1.0	90.2 ± 0.1	88.1 ± 0.1
COD_t (g/L)	18.2 ± 0.6	19.4 ± 0.4	15.3 ± 0.3
Protein (% VS)	63.2 ± 2.3	18.2 ± 0.1	22.0 ± 1.0
Carbohydrate (% VS)	13.6 ± 1.3	75.6 ± 0.1	68.7 ± 5.5
pH	8.10	5.92	5.91
C/N	9.50	40.78	28.59

[a] Mean ± SD, n=2.

6.2.2 *Semi-continuous anaerobic digester conditions and operations*

Eight identical one litre Duran bottles (VWR, UK) were used as the semi-continuous co-digestion digesters, with working volume of 0.8 L (Figure 6.1). Each digester was sealed with a rubber bung with two holes, of which one hole was connected with a gas bag to determine gas production and the other was a closed port (silicon tube with tube-clamp) for sampling/feeding. At the beginning of the experiment, all digesters were filled with 0.8 L of seed inoculum collected from Cockle Park Farm, and flushed with N_2 to ensure anaerobic conditions. All digesters were placed in a temperature-controlled water-bath at an operating temperature of 37 °C. The digester was mixed by hand mixing before and after feeding.

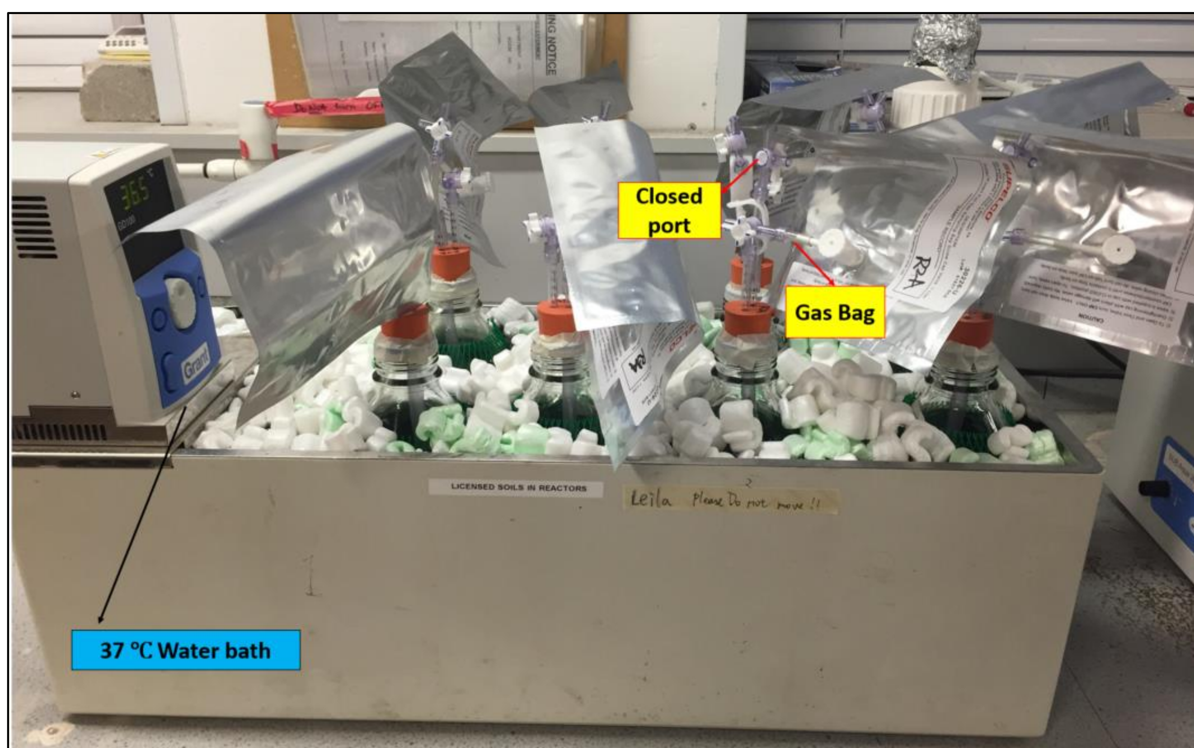


Figure 6.1 Schematic of the semi-continuous anaerobic digesters used for microalgae co-digestion

This study utilised a 2×2 independent factorial design, and the first between-independent variable was start-up strategy, where two start-up strategies were evaluated, namely fed start-up strategy: the digester immediate feeding with 100% PPW after inoculation, and unfed start-up strategy: the digester delayed feeding, which means no feed being supplied during first 5 days. The second between-independent variable was PPW type, either PPW_{dp} or PPW_p.

The whole experiment was divided into three phases, and detailed information of variation of OLR and feedstock composition over the co-digestion process is summarized in Table 6.2. Period I (day 1 – 5) is the initial period, where digesters D2 and D4 were fed every day with 100% PPW_{dp} or PPW_p at an OLR of 0.5g VS/L/d, and digesters D1 and D3 were unfed. Period II (day 6 – 25) is the substrate change period, where all digesters were start to feed a mixture of 25% *T. lutea* and 75% PPW_{dp} or 75% PPW_p based on the proportion of VS. A 25:75 ratio of *T. lutea* and PPW also had the potential to provide an optimum C/N ratio and produce higher methane yields compared to other tested mixing ratios (e.g. 75:25 and 50:50). The detailed results are discussed in Appendix A, Section A.3. Period III (day 26 – 64) is the experimental period, and same with Period II, digesters D1 and D2 were fed with the mixtures of 25:75 *T. lutea* and PPW_{dp}, and digesters D3 and D4 were fed with the mixtures of 25:75 *T. lutea* and PPW_p. All digesters were fed every two days at an average OLR of 1.0 g VS/L/d during Periods II and III. The HRT of 25 days for Periods I and II, Period III had a HRT of 20 days.

Table 6.2 Start-up strategy, feedstock composition and OLR for co-digesting *T. lutea* and PPW

No.	Start-up Strategy	Period	Operation time (days)	OLR (g VS/L/d)	Feed Composition (% VS)	
					T. lutea	**PPW[a]**
		I	1 – 5	/	0	0
D1	Unfed start-up	II	6 – 25	1.0	25	75
		III	26 – 64	1.0	25	75
		I	1 – 5	0.5	0	100
D2	Fed start-up	II	6 – 25	1.0	25	75
		III	26 – 64	1.0	25	75
		I	1 – 5	/	0	0
D3	Unfed start-up	II	6 – 25	1.0	25	75
		III	26 – 64	1.0	25	75
		I	1 – 5	0.5	0	100
D4	Fed start-up	II	6 – 25	1.0	25	75
		III	26 – 64	1.0	25	75

[a] Digesters D1 and D2 were fed with potato discarded parts (PPW_{dp}); Digesters D3 and D4 were fed with potato peel (PPW_p).

During the semi-continuous co-digestion process, biogas production, methane content and pH values were measured every feeding day. Digestate samples removed during feeding were analysed weekly for TS, VS, and COD_t, COD_s NH_4^+-N, FAN and VFAs concentrations.

6.2.3 *Analytical methods*

The general analytical procedures have been described in Section 3.2.

6.2.4 *Microbial community analysis*

For microbial community analysis, genomic DNA was extracted from digesters on days 0, 27, 46 and 64 using an isolation kit (DNeasy PowerSoil kit, QIAGEN, UK) following the manufacturer's instructions. Detailed information of DNA sequencing and processing sequencing data are described in Section 3.3.

6.2.5 *Energy analysis*

The energy yields (MJ/kg VS) of the mixtures of *T. lutea* with PPW_{dp} or PPW_p were estimated based on experimental data (see Section 6.3.3), and calculated using Equation 6.1 (Serrano *et al.*, 2014):

$$Energy\ yield = Y_{CH_4/S} \times LCP \qquad (6.1)$$

where $Y_{CH_4/S}$ (m^3_{STP}/kg VS) is the methane yield coefficient, and LCP is the lower calorific power (LCP) of methane, which has been reported to be approximately 35.8 MJ/m^3 under standard conditions (STP) of 1atm and 0 °C (Serrano *et al.*, 2014).

6.2.6 *Statistical analysis*

Semi-continuous experimental data for the concentrations of VFA, concentrations of NH_4^+-N and FAN, pH values, specific methane yields and relative abundance of methanogenic archaea were analysed by two-way independent ANOVA (Field, 2009). A Spearman's rank-order correlation was run to determine the relationship between the relative abundance of methanogenic archaea and digester operating parameters (VFA concentrations and specific methane yields) (Field, 2009). A confidence interval of differences of 95% ($p<0.05$) was

chosen to define statistical significance. All statistical analyses were conducted using IBM SPSS statistics, Version 23.

6.3 Results and discussion

6.3.1 *Digester performance - VFAs production*

The initial stage in AD is hydrolysis, where complex organic polymers are catabolized into simple sugars, amino acids and long-chain fatty acids (Kangle *et al.*, 2012). The acidogenesis stage is the following step, where these components are further catabolized by acidogenic bacteria (Dioha *et al.*, 2013). In this step, the important intermediate products of VFA can accumulate. Successful start-up of an anaerobic digester should avoid the accumulation of VFA that could inhibit the activity of acetogens and methanogens (Angelidaki *et al.*, 2006).

Figure 6.2 shows the concentrations of VFA during the semi-continuous co-digestion process, reaching its maximum values in the digesters (except digester D3) at day 15. The start-up strategy had a significant effect on the production of total VFA ($F(1,12)=24.13$, $p<0.001$), with fed start-up digesters (D2 and D4) producing more VFA than unfed start-up digesters (D1 and D3). The type of PPW also significantly affected the production of VFA ($F(1,12)=76.63$, $p<0.001$), with co-digestion of *T. lutea* with PPW_{dp} producing more total VFA than with PPW_{p}.

Acetate and propionate were the two major VFA produced, and the observed concentrations of acetate and propionate in all digesters also reached their maximum values at day 15 (Figure 6.2). The start-up strategy also had a significant effect on the concentrations of acetate ($F(1,12)=11.18$, $p=0.006$) and propionate ($F(1,12)=127.08$, $p<0.001$), with digesters D2 and D4 producing more acetate and propionate than unfed start-up digesters (D1 and D3). The type of PPW also significantly affected the production of acetate ($F(1,12)=73.47$, $p<0.001$) and propionate ($F(1,12)=18.55$, $p=0.001$). Co-digestion of *T. lutea* with PPW_{dp} produced more acetate and propionate than co-digestion with PPW_{p}.

Zhou *et al.* (2013) found that the production of VFA is affected by the proportion of feedstocks when co-digesting with two different feedstocks. In the current study, the digesters D2 and D4 were immediate feeding with 100% PPW_{dp} or PPW_p during the first five days, and Kaparaju and Rintala (2005) and Pistis *et al.* (2013) reported that AD of potato waste could cause rapid and strong acidification due to its high concentration of biodegradable components. Therefore, in the current study, the observed effect probably resulted from the high proportion of PPW added to the fed start-up digesters (D2 and D4); these results agree with the findings of Banerjee *et al.* (1999) and Elefsiniotis *et al.* (2005). Their studies demonstrated that the addition of starch-rich potato processing wastewater to primary sludge or municipal wastewater could stimulate acidogenesis and generate high amounts of VFA.

PPW_{dp} consists of 54.3 – 76.8% of dry matter as starch (Leonel *et al.*, 2017), whereas Arapoglou *et al.* (2010) and Liang *et al.* (2014) observed values of 34.3 – 52.1% for PPW_p. In the current study, the total carbohydrate content of PPW_{dp} was 75.6% VS, and 68.7% of VS for PPW_p, suggesting that PPW_{dp} contained higher amounts of starch (around 87 % of total carbohydrate) than the values (63% of total carbohydrate) for PPW_p, although starch content was not determined directly. Starch is readily catabolized into simple sugars (Spets *et al.*, 2010), and the higher amount of VFA produced by co-digestion with PPW_{dp} was probably a result of the higher starch content with respect to PPW_p.

Efficient degradation of VFA is critical to the success of the AD process (Onoh *et al.*, 2017). Propionate degrading microorganisms have lower specific growth rates than acetate or butyrate degrading microorganisms (Griffin *et al.*, 1998; Moset *et al.*, 2014). Therefore, Nielsen *et al.* (2007) suggested that the accumulation of propionate could be one of the major parameters that indicates the instability of an anaerobic system. However, in the present study, propionate did not accumulate in all digesters (Figure 6.2). From day 15 to 27, propionate was degraded more efficiently in fed start-up digesters than unfed start-up digesters. There were reductions of 71 and 84% of propionate concentrations in digesters D2 and D4, respectively, which were higher than those in digesters D1 and D3 (53 and 56%, respectively). Therefore, on day 27 the propionate concentrations in fed start-up digesters were significantly lower than unfed start-up digesters ($F(1,12)=5.74$, $p=0.034$). This finding corroborates Moset *et al.* (2014), who found that the residual concentrations of propionate in digesters with fed during start-up were significantly lower than in unfed digesters. During Period III, VFAs concentrations reduced in all digesters, and there were no excessive accumulations of VFAs caused by co-digestion of *T. lutea* with PPW_{dp} or PPW_p.

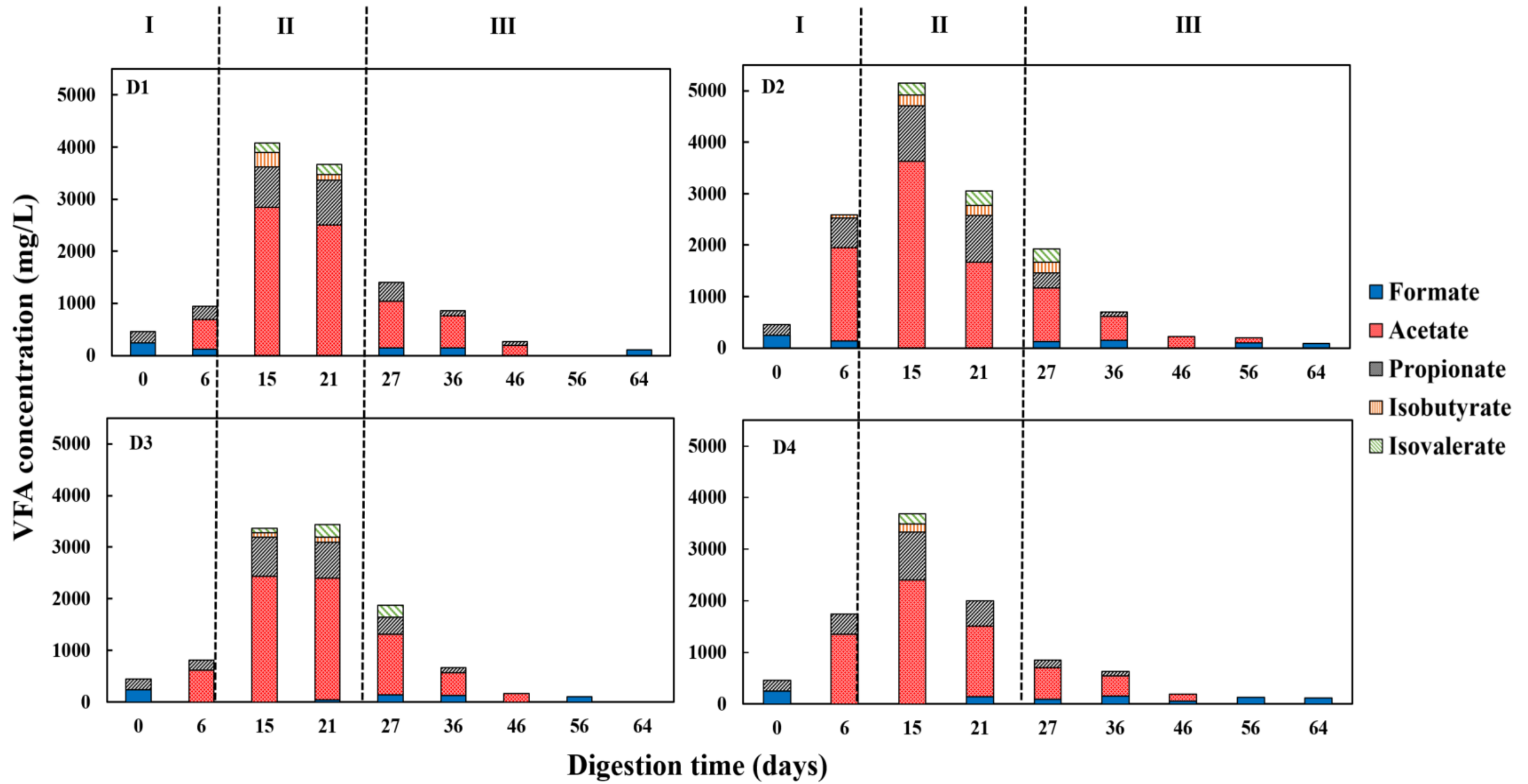


Figure 6.2 Concentrations of VFAs for semi-continuous co-digestion of *T. lutea* with PPW_{dp} (digesters D1 and D2), or with PPW_p (digesters D3 and D4) under different start-up strategies

6.3.2 *Other indicators of digester performance*

Figure 6.3a shows that the pH values started to decrease after feeding, and at day 6 pH values of 7.33 and 7.48 were observed in fed start-up digesters (D2 and D4) because of the high amounts of VFAs produced after feeding with 100% PPW_{dp} or PPW_p, whereas the pH values in unfed start-up digesters (D1 and D3) remained at 7.81.

Microalgal biomass is characterised by high protein content, leading to ammonia inhibition when used as a mono-digestion feedstock. One major advantage of co-digesting microalgae with high carbon feedstocks is to reduce ammonia concentrations (Yen and Brune, 2007). Figure 6.3b presents the variation in FAN concentrations during semi-continuous co-digestion of *T. lutea* with PPW_{dp} or PPW_p. On day zero, high concentrations of FAN (416 mg/L) were observed in all digesters since the seed inoculum was collected from a manure-based anaerobic digester. During Period I (day 0 to 6), the FAN concentration showed a decreasing trend in all digesters, and at day 6 the observed concentrations in fed start-up digesters (D2 and D4) were significantly lower than in unfed start-up digesters (D1 and D3) ($F(1,4)=508.20$, $p<0.001$). FAN is regarded as the active form of ammonia that leads to ammonia inhibition in the AD process (Yenigün and Demirel, 2013). Therefore, the results show that feeding 100% PPW during start-up can reduce ammonia toxicity at the beginning of semi-continuous co-digestion.

During Period III, the pH values of all digesters ranged from 6.88 to 7.05, which are within the optimum range for AD (Ward *et al.*, 2008). Concentrations of FAN showed a declining trend in all digesters (Figure 6.3b). After 46 days of operation, FAN concentrations of approximately 50 mg/L were observed in all digesters, which is less than the previously reported methanogenic toxicity level of 80 – 150 mg/L (Nielsen and Angelidaki, 2008; Wang *et al.*, 2016). Therefore, the results show that co-digestion of *T. lutea* with PPW_{dp} or PPW_p at a ratio of 25:75 avoids ammonia toxicity for methanogens.

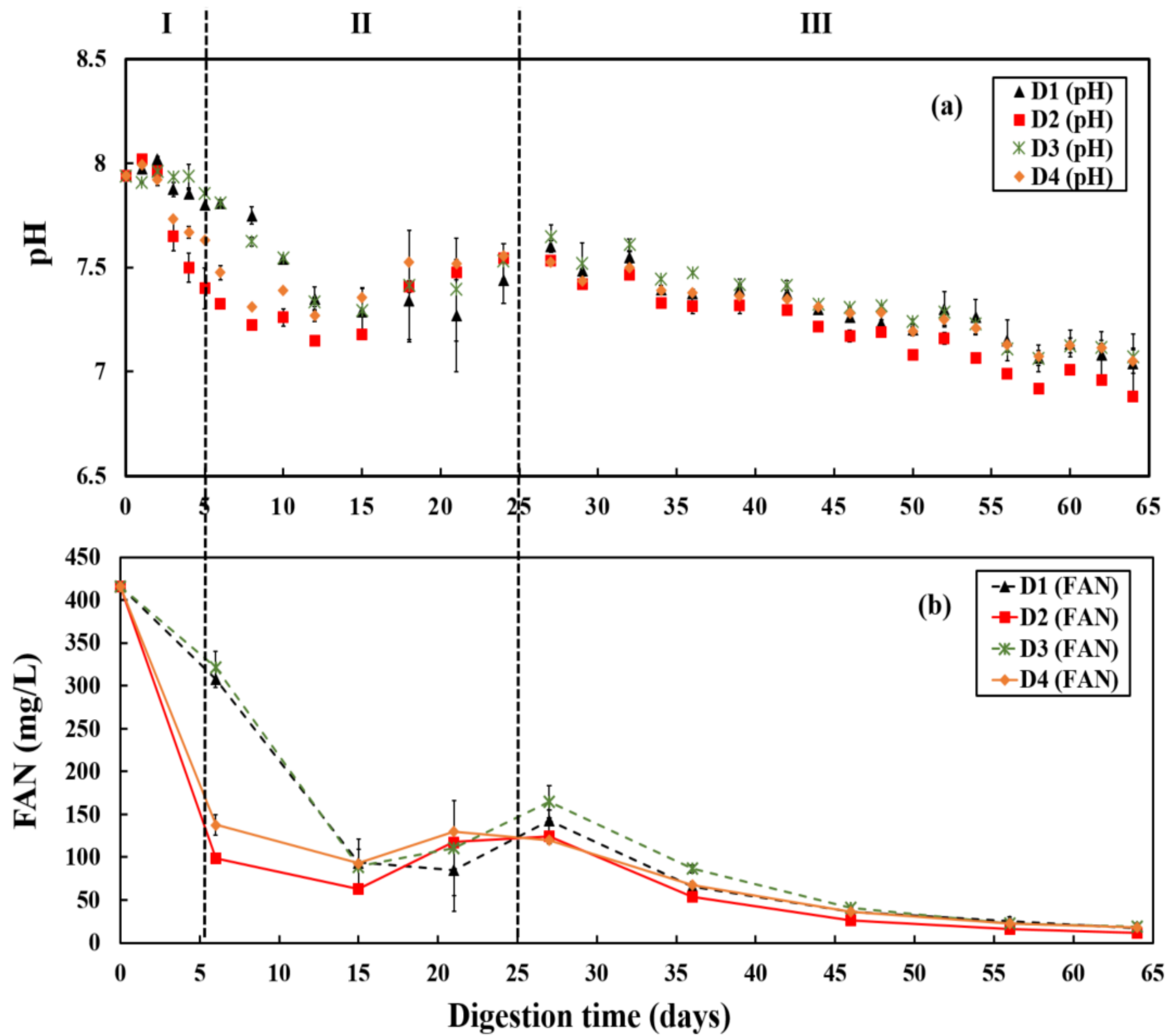


Figure 6.3 (a) pH values and (b) concentrations of FAN for semi-continuous co-digestion of *T. lutea* with PPW_{dp} (digesters D1 and D2), or with PPW_p (digesters D3 and D4) under different start-up strategies. Error bars= mean ± SD, n=2.

6.3.3 *Methane yield*

Methane yields increased steadily during Periods I and II for all digesters (Figure 6.4). Generally, the specific methane yields of fed start-up digesters (D2 and D4) were higher than those of unfed start-up digesters (D1 and D3). After 25 days of operation most of the seed inoculum had been washed out, and during Period III, the average specific methane yields produced by fed start-up digesters (D2 and D4) were 383 ± 46 and 343 ± 40 mL CH_4/g VS, respectively. For unfed start-up digesters (D1 and D3), the average methane yields were 332 ± 44 and 320 ± 38 mL CH_4/g VS, respectively. The start-up strategy had a significant effect on the specific methane yields ($F(1,148)=23.13$, $p<0.001$). This agrees with Moset *et al.* (2014) in which an easily biodegradable commercial broiler feed was used as the start-up feedstock before the addition of pig manure, and their results showed that more methane was produced following initial start-up. Li *et al.* (2014a) also found that an AD feedstock containing more readily degradable organic matter could enhance the activity of microorganisms during the start-up period. In the current study, during the first five days of start-up, PPW_{dp} or PPW_p were fed to digesters D2 and D4, resulting in a higher degradation efficiency of VFAs (especially propionate), and lower concentrations of FAN during subsequent operational phases compared to unfed start-up digesters (D1 and D3). These results suggest that the activity of microorganisms may be enhanced when using PPW as the initial feedstock, consequently yielding greater methane productivity in fed start-up digesters (D2 and D4) compared to unfed start-up digesters (D1 and D3).

PPW type also had a significant effect on specific methane yields ($F(1,148)=11.46$, $p=0.001$), with digesters co-digesting *T. lutea* with PPW_{dp} (D1 and D2) showing higher methane yields than digesters co-digesting *T. lutea* with PPW_p (D3 and D4). Kaparaju and Rintala (2005) reported that an AD feedstock containing more starch may improve its anaerobic biodegradability. In the current study, the high methane yields achieved by co-digestion with PPW_{dp} were probably a result of its higher starch content compared to PPW_p.

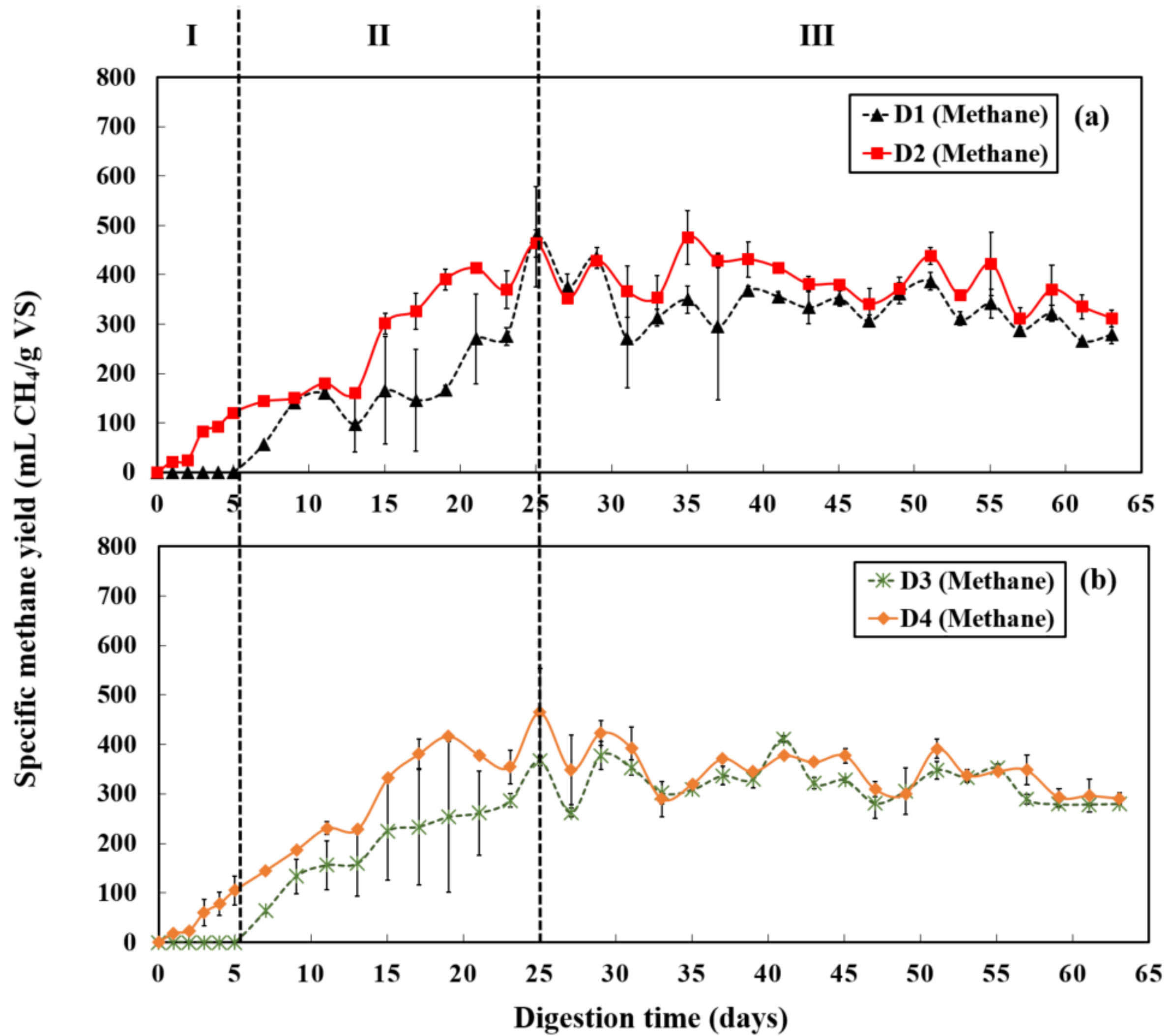


Figure 6.4 Specific methane yields for semi-continuous co-digestion of *T. lutea* with (a) potato discarded parts (PPW_{dp}) or (b) potato peel (PPW_p) under different start-up strategies. Error bars= mean ± SD, n=2.

6.3.4 *Characteristics of archaeal community*

To investigate the microbial communities present during the co-digestion process, samples were taken from digesters on days 0, 27, 46 and 64 for 16S rRNA gene sequencing analysis. Figure 6.5 shows that acetoclastic methanogens (*Methanosarcina* and *Methaneosaeta*) were more dominant than hydrogenotrophic methanogens. Initially, *Methaneosaeta* was the dominant genus (representing 56% of relative abundance), but was replaced by *Methanosarcina* by day 27. On day 27, the start-up strategy had a significant effect on the relative abundances of *Methanosarcina* ($F(1,12)=504.86$, $p<0.001$). *Methanosarcina* were detected at abundances of 82 and 77% in fed start-up digesters (D2 and D4), respectively. For unfed start-up digesters (D1 and D3), the relative abundances of *Methanosarcina* were 50 and 52%, respectively. However, PPW type had no significant effect on the relative abundances of *Methanosarcina* ($F(1,12)=1.09$, $p=0.318$).

From days 27 to 64, the relative abundances of *Methanosarcina* exhibited a decreasing trend in digesters D1, D3 and D4, but on day 46 *Methanosarcina* was still the predominant genus in all digesters. On day 46, the relative abundance of *Methanosarcina* in fed start-up digesters (D2 and D4) was significant higher than in unfed start-up digesters (D1 and D3) ($F(1,12)=15.22$, $p=0.002$). PPW type also had a significant effect on the relative abundance of *Methanosarcina*, and co-digesting *T. lutea* with PPW_{dp} supported a higher relative abundance of *Methanosarcina* than with PPW_p ($F(1,12)=47.55$, $p<0.001$).

At the end of the digestion process (on day 64), *Methanosaeta* was the dominant genus in digesters D1, D3 and D4, whereas *Methanosarcina* remained dominant in digester D2. The start-up strategy had a significant effect on the relative abundance of *Methanosarcina* ($F(1,12)=59.00$, $p<0.001$). *Methanosarcina* were detected at abundances of 90 and 28% in digesters D2 and D4, which were higher than in digesters D1 and D3, 30 and 21%, respectively. The start-up strategy also had a significant effect on the relative abundance of *Methanosaeta* ($F(1,12)=45.08$, $p<0.001$), with *Methanosaeta* the predominant genus in unfed start-up digesters (D1 and D3). PPW type significantly affected the relative abundances of both *Methanosarcina* and *Methanosaeta* at the end of the experiment ($F(1,12)=50.59$, $p<0.001$ and $F(1,12)=32.38$, $p<0.001$, respectively).

Methanosarcina and *Methaneosaeta* compete for acetate as a substrate (Zinder, 1993). In the current study, there was a positive correlation between the concentrations of acetate and the relative abundance of *Methanosarcina* (r_s (32) = 0.649, p<0.001), whereas the relative abundance of *Methaneosaeta* had a negative correlation with acetate concentration (r_s (32) = -0.711, p<0.001). Kobayashi *et al.* (2009) reported that *Methanosarcina* had higher growth rates than *Methaneosaeta* for digesters with high acetate concentrations. Therefore, the current study shows that digesters immediate feeding with 100% PPW not only significantly enhanced the production of acetate, but also supported a greater relative abundance of *Methanosarcina*. Competition between *Methanosarcina* and *Methanosaeta* is also affected by the operating conditions (feeding rates and stirring intensity) and feedstock type (Liu and Whitman, 2008; Lin *et al.*, 2012). In the current study, the results assumed that PPW_{dp} contained higher amounts of starch than PPW_p; therefore, co-digestion of *T. lutea* with PPW_{dp} could potentially produce higher amounts of acetate as well as support a higher relative abundance of *Methanosarcina* than co-digestion with PPW_p.

The relative abundance of *Methanosarcina* was also positively correlated with specific methane yields (r_s (32) = 0.769, p<0.001), whereas there was a negative correlation between the relative abundance of *Methanosaeta* and specific methane yields (r_s (32) = -0.649, p<0.001). Liu and Whitman (2008) reported that *Methanosarcina* is a relative generalist that can use acetate, CO_2, methyl-group containing compounds or H_2 as substrate to produce methane, whereas *Methanosaeta* is a specialist that only utilizes acetate. Therefore, the high methane yields achieved by immediate feeding with PPW, or during co-digestion with PPW_{dp}, were probably a result of the high relative abundances of *Methanosarcina* present in the digesters. Kobayashi *et al.* (2009) also reported that *Methanosaeta* has a competitive advantage over *Methanosarcina* in low acetate (<100 mg/L) environments. In the current study, from day 46 to 64, the observed concentrations of acetate for all digesters were less than 100mg/L, and consequently *Methanosaeta* became the dominant genus in digesters D1, D3 and D4 by the end of experiment. However, *Methanosarcina* remained dominant in digester D2 at the end of the digestion process. *Methanosacina* is tolerant to environmental stresses such as high salt and VFA concentrations (De Vrieze *et al.*, 2012). As a marine strain, *T. lutea* may contain high concentrations of salt, and this is likely to explain why a large *Methanoscarina* population was found in digester D2.

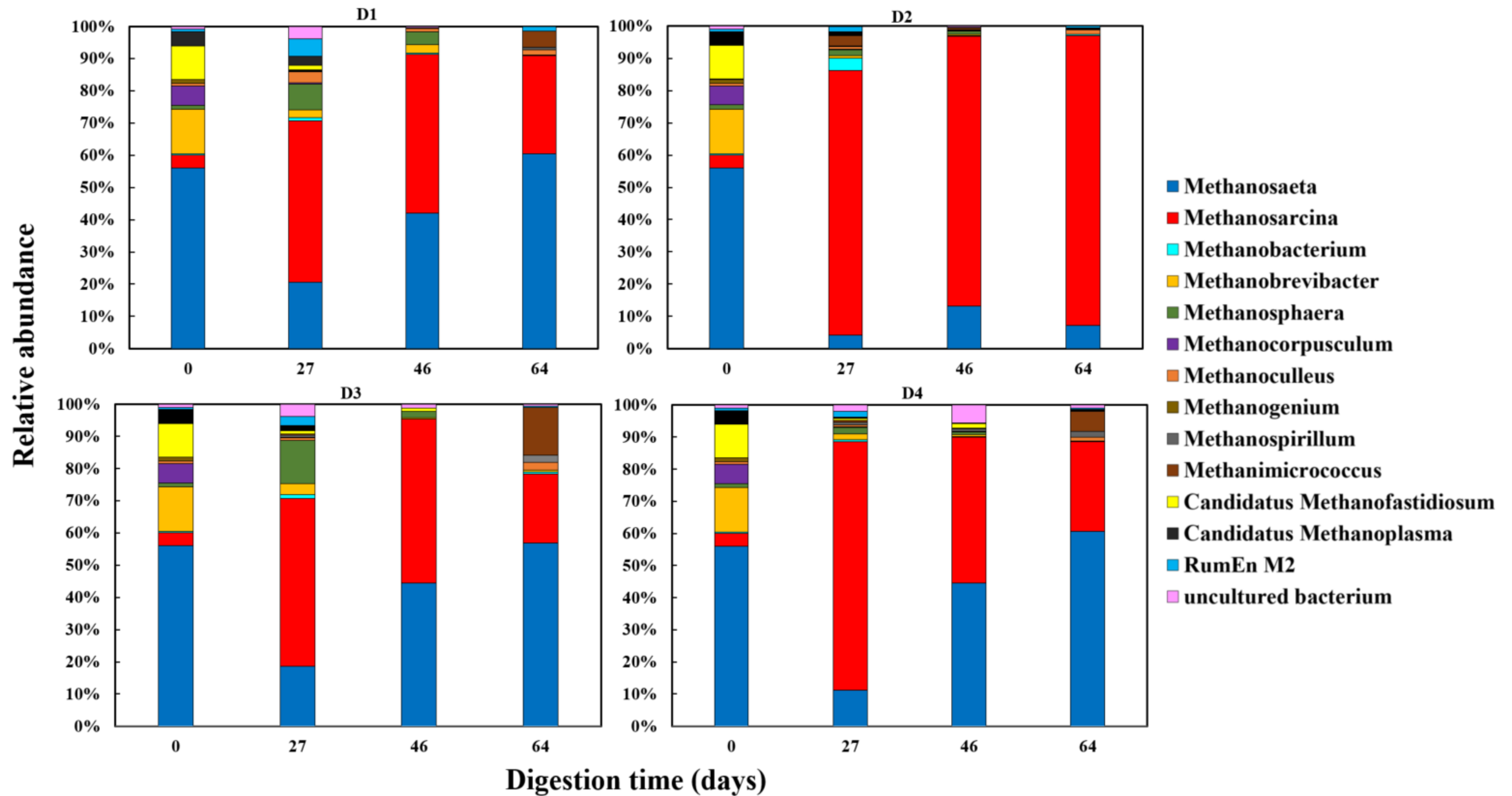


Figure 6.5 Relative abundance of methanogenic archaea (genus level) for semi-continuous co-digestion of *T. lutea* with PPW_{dp} (digesters D1 and D2), or with PPW_p (digesters D3 and D4) under different start-up strategies.

6.3.5 *Benefits of co-digestion and energy implications*

Anaerobic co-digestion of microalgae with high carbon feedstocks facilitates balanced C/N ratios, provides a favourable organic or inorganic nutriment composition, and supports a stable AD process (Yen and Brune, 2007). In the current study, *T. lutea* had a C/N ratio of 9.50/1, whereas PPW_{dp} and PPW_p had ratios of 40.78/1 and 28.59/1, respectively. The C/N ratios of feedstocks were balanced by the addition of PPW_{dp} or PPW_p to *T. lutea*. The C/N ratios in the mixtures of *T. lutea* with PPW_{dp} or PPW_p at a mixing ratio of 25:75 were 24.24/1 and 19.33/1, respectively (see Appendix A, Section A.3, Table A.3). These C/N ratios both within the optimal range for AD process (Yen and Brune, 2007; Weiland, 2010). Therefore, PPW_{dp} and PPW_p are both promising feedstocks for co-digestion with microalgae. Moreover, a mixing ratio of 25:75 of *T. lutea*: PPW_{dp} or *T. lutea*: PPW_p, enabled the co-digestion process to operate successfully without VFAs and ammonia inhibition.

In the current study, the energy yields of the mixtures of *T. lutea*: PPW_{dp} or *T. lutea*: PPW_p were estimated from experimental data taken from Period III of the semi-continuous digesters, and calculated using Equation (6.1). The energy yields for the 25:75 mixture of *T. lutea*: PPW_{dp} ranged from 11.90 to 13.70 MJ/kg, and from 11.50 to 12.30 MJ/kg for the 25:75 mixture of *T. lutea*: PPW_p. Using a conversion factor of 3.6 MJ per KWh (Cuéllar and Webber, 2008), potential energy yields of 3306 – 3806 KWh/tonne can be estimated for a 25:75 mixture of *T. lutea*: PPW_{dp}, and 3194 – 3417 KWh/tonne for a 25:75 mixture of *T. lutea* : PPW_p. The energy produced by co-digestion of *T. lutea* with PPW could be used in combined heat and power (CHP) systems, assuming a conversion efficiency for heat and electrical energy of 50 and 35%, respectively (Wang *et al.*, 2016), the energy that could be generated by co-digestion of the mixture of *T.* lutea: PPW_{dp} would be equivalent to 1157 – 1332 kWh/tonne of electricity and 1653 – 1903 kWh/tonne of heat. Equivalent values for the mixture of *T. lutea*: PPW_p would be 1118 – 1196 kWh/tonne of electricity and 1597 – 1709 kWh/tonne of heat. Currently, the challenges in using microalgal biomass exclusively for biogas production include high production costs and low production capacity (Schenk *et al.*, 2008). Therefore, co-digestion of microalgae with PPW could be a possible step to enhance the feasibility of biogas production from microalgae. However, energy consumptions from digester heating, mixing, pumping, digestate treatment and production should be considered, and therefore, further deep energy analysis is needed to in order to address the feasibility of anaerobic co-digestion.

6.4 Conclusion

In this Chapter, the feasibility of using potato processing waste as a potential feedstock for co-digestion with marine microalgae *T. lutea* in the semi-continuous anaerobic co-digestion tests was investigated.

The digesters were immediate feeding with 100% PPW (fed start-up strategy) significantly enhanced methane yields, and also reduced the potential risk of ammonia toxicity to the methanogens compared to the digesters delayed feeding (unfed start-up strategy). Co-digestion of *T. lutea* with PPW_{dp} produced higher methane yields than co-digestion with PPW_{p}, but both feedstocks exhibited good potential for co-digestion with *T. lutea* with balanced C/N ratios and stable digestion performance.

Microbial analyses showed that acetoclastic methanogens were abundant. *Methaneosaeta* was the predominant genus, but the fed start-up strategy and *T. lutea* co-digesting with PPW_{dp} supported high relative abundances of *Methanosarcina.* However, *Methaneosaeta* became dominant in some of digesters because of the acetate concentrations were observed to be less than 100 mg/L at the end of digestion process.

Chapter 7. Semi-continuous anaerobic co-digestion of microalgae with potato processing waste and glycerol: effect of glycerol addition on methane production and microbial diversity

7.1 Introduction

In Chapters 5 and 6, it has demonstrated stable co-digestion of a freshwater (*Chlorella vulgaris*) and a marine microalga (*Tisochrysis lutea*) with PPW in batch and semi-continuous modes. However, reliance on a single waste-stream could pose some operational risks to an AD plant (Nges *et al.*, 2012), and also in order to improve the economics of the process, secure co-feedstock supply (ideally as a waste-stream) is important. Glycerol is the main by-product of biodiesel production representing 10% w/w of the total product stream (Mu *et al.*, 2006; Da Silva *et al.*, 2009). The rapid growth of the biodiesel industry has led to overproduction of crude glycerol (OECD/FAO, 2011). Glycerol has high concentration of COD and is easily degraded by acidogenic bacteria to produce organic acids, which is now readily available as a cheap waste material (Sell *et al.*, 2011; Viana *et al.*, 2012; Larsen *et al.*, 2013). Therefore, glycerol is a promising potential anaerobic feedstock for co-digestion with low C/N ratios feedstocks such as microalgae.

This Chapter aimed to investigate the effects of glycerol supplementation on the co-digestion of microalgae and PPW (PPW_{dp} and PPW_p) in semi-continuous tests:

1. To evaluate the effect of glycerol dosage on methane production and process stability.

2. To evaluate the effect of glycerol dosage on microbial diversity/structural dynamics.

7.2 Materials and methods

7.2.1 *Microalgae and co-substrates*

Chlorella vulgaris strain (CCAP 211/63) was used in this study, and detailed information of cultivation and harvesting are described in Section 3.1. The detailed information of preparation of two PPW feedstocks of PPW_{dp} and PPW_p were described in Section 5.2.2. The feedstocks of *C. vulgaris*, PPW_{dp} and PPW_p were characterised for their TS, VS, COD_t, and carbohydrate and protein content as well as C and N content are summarised in Table 7.1.

A glycerol solution (Sigma-Aldrich, 4978, UK) with a purity of 86 – 89% was used as a co-substrate. The reason why using this solution is because the glycerol with this purity was essentially methanol free produced in industry biodiesel units (Zhang *et al.*, 2003). The glycerol solution had a COD_t of 1888.0 ± 2.8 g/L and density of 1.25 kg/L.

The seed inoculum had TS and VS of 18.6 ± 1.2 and 10.4 ± 0.9 g/L, respectively. pH value of inoculum was 7.89 ± 0.02, and concentrations of NH_4^+-N and FAN were 2905 ± 83 and 274 ± 18 mg/L, respectively.

Table 7.1 Feedstock characteristics: *Chlorella vulgaris*, potato processing waste of discarded parts (PPW_{dp}) and peel (PPW_p).

	C. vulgaris	**PPW_{dp}**	**PPW_p**
TS (g/L)	2.7 ± 0.2[a]	16.0 ± 0.4	15.7 ± 0.1
VS (g/L)	2.4 ± 0.1	14.8 ± 0.4	13.9 ± 0.1
VS/TS (%)	88.9 ± 3.2	92.2 ± 0.0	88.7 ± 0.0
COD_t (g/L)	3.5 ± 0.3	13.8 ± 0.2	12.5 ± 0.1
Proteins (% VS)	37.6 ± 4.0	13.0 ± 0.2	13.7 ± 1.1
Carbohydrates (% VS)	23.8 ± 3.3	74.8 ± 0.1	69.0 ± 3.7
C/N	6.4	40.8	28.6

[a] mean ± SD, n = 2.

7.2.2 *Operation of semi-continuous anaerobic digesters*

Eight identical one litre Duran bottles (VWR, UK) with a working volume of 0.8 L were used as the semi-continuous co-digestion digesters, and detailed information of the digester configuration is described in Section 6.2.2. At the beginning of the experiment, all digesters were filled with 0.8 L of anaerobic inoculum that collected from Cockle Park Farm, and flushed with N_2 to ensure anaerobic conditions. The semi-continuous digesters were kept at constant temperature of 37 °C by a temperature-controlled water-bath. The digester was mixed by hand mixing before and after feeding.

The semi-continuous digesters were studied at four feeding conditions: the digesters were fed with mixtures of *C. vulgaris*: PPW_{dp} and glycerol (C1); the digesters were fed with mixtures of *C. vulgaris*: PPW_{dp} without glycerol (C2); the digesters were fed with mixtures of *C. vulgaris*: PPW_p and glycerol (C3); the digesters were fed with mixtures of *C. vulgaris*: PPW_p without glycerol (C4). Detailed information of variation of OLR and feedstock composition over the co-digestion process is summarized in Table 7.2.

During Period I, all digesters were fed with 100% PPW_{dp} or 100% PPW_p. In Period II, all digesters were start to feed a mixture of 25% *C. vulgaris* and 75% PPW_{dp} or 75% PPW_p based on the proportion of VS. A 25:75 ratio of *C. vulgaris* and PPW had the potential to provide an optimum C/N ratio and produce high methane yields compared to other tested mixing ratios (e.g. 75:25 and 50:50) as discussed in Section 5.3.2. Digesters C1 and C3 were also fed with glycerol, and the dosage of glycerol in the mixture was progressively increased from 1 to 2% v/v over Period II. The glycerol dosages of 1 and 2% v/v were selected based on previous studies on anaerobic co-digestion of glycerol with other low carbon feedstocks (Sell *et al.*, 2011; Larsen *et al.*, 2013).

Periods III and IV are the experimental phases for digesters C1 and C2, where digester C1 was supplemented with different glycerol dosages, i.e., 2 and 1% v/v for Periods III and IV, respectively. Digester C2 was used as control digesters, and fed with 25:75 *C. vulgaris*: PPW_{dp} without glycerol supplementation. Overall, digesters C1 and C2 were operated 76 days. For digesters C3 and C4, the operating phases start from Periods III to VI. During Periods III and IV, digester C3 was supplemented with 2% v/v of glycerol, but had no glycerol supplementation in Phase V. Glycerol was back to add to digester C3 at 1% v/v

during Period VI. Control digester C4 was only fed with 25:75 *C. vulgaris*: PPW_p. Overall, digesters C3 and C4 were operated 132 days.

During the semi-contiguous co-digestion process, biogas yield, methane content, and pH values were measured every feeding day. Digestate samples removed during weekly and analysed for TS, VS, the concentrations of COD_t, COD_s, TAC, VFAs, NH_4^+-N and FAN.

Table 7.2 Organic loading rate (OLR) and feedstock composition for co-digesting *C. vulgaris* and PPW with or without glycerol.

Feeding regime	Period	Operation time (days)	OLR (g COD/L/d)	Feed Composition		
				C. vulgaris (% VS)	PPW[a] (%VS)	Glycerol (% v/v)
C1	I	1 - 3	0.14	0	100	0
		4 - 8	0.28	0	100	0
	II	9 - 15	0.47	25	75	1.0
		16 - 24	0.80	25	75	2.0
	III	25 - 56	1.20	25	75	2.0
	IV	57 - 76	0.70	25	75	1.0
C2	I	1 - 3	0.14	0	100	0
		4 - 8	0.28	0	100	0
	II	9 - 24	0.40	25	75	0
	III+IV	25 - 76	0.60	25	75	0
C3	I	1-3	0.12	0	100	0
		4 - 8	0.25	0	100	0
	II	9- 15	0.45	25	75	1.0
		16 - 24	0.75	25	75	2.0
	III+IV	25 - 56	1.12	25	75	2.0
	V	57 - 94	0.50	25	75	0
	VI	95 - 132	0.67	25	75	1.0
C4	I	1 - 3	0.12	0	100	0
		4 - 8	0.25	0	100	0
	II	9 - 24	0.25	25	75	0
	III+IV+V	25 – 76[b]	0.50	25	75	0
	VI	95 - 132	0.50	25	75	0

[a] Digesters C1 and C2 were fed with potato discarded parts (PPW_{dp}); Digesters C3 and C4 were fed with potato peel (PPW_p).

[b] During the recovery stage (from days 57 to 94), the digester C4 was unfed for a period (days 77 to 94).

7.2.3 *Analytical methods*

The general analytical procedures have been described in Section 3.2.

7.2.4 *Microbial community analysis*

For microbial community analysis, genomic DNA was extracted from all digesters on days 0, 23, 54, 76 and 132 using an isolation kit (DNeasy PowerSoil kit, QIAGEN, UK) following the manufacturer's instructions. Detailed information of DNA sequencing and processing sequencing data are described in Section 3.3. The non-metric dimensional scaling (NMDS) ordination plot was plotted based on the code described by Torondel *et al.* (2016).

7.2.5 *Statistical analysis*

The independent samples t-test and one-way ANOVA were utilized to test the effects of glycerol dose on the significance of methane production and digester performance by co-digestion of *C. vulgaris* and PPW (Field, 2009). A Spearman's rank-order correlation was run to determine the relationship between the relative abundance of methanogenic archaea and digester operating parameters (Field, 2009). A confidence interval of differences of 95% ($p<0.05$) was chosen to define statistical significance. All statistical analyses were conducted using IBM SPSS statistics, Version 23. The NMDS plots were conducted using RStudio software.

7.3 Results and discussion

7.3.1 *Effect of co-digesting C. vulgaris and PPW_{dp} with glycerol on AD performance*

During Period III (days 25 to 56), the glycerol feed was maintained at 2 % v/v with a high OLR at 1.20 g COD/L/day for digester C1 (Figure 7.1 A), whereas the OLR of digester C2 was kept at 0.60 g COD/L/day (Figure 7.2 A). The glycerol dosage significantly enhanced volumetric methane production ($F(2,61)=319.67$, $p<0.001$). Digester C1 (2% v/v dosage) had an average methane production of 0.59 ± 0.08 L $CH_4/L_{digester}$ (Figure 7.1 A), which was significantly higher than C2 (0.19 ± 0.02 L $CH_4/L_{digester}$; $p<0.001$) (Figure 7.2 A). Moreover, the addition of glycerol also significantly enhanced specific methane yields ($F(2,61)=213.67$, $p<0.001$). During Period III, digester C1 had an average specific methane yield of 0.49 ± 0.07 L CH_4/g COD, which was significantly higher than C2 (0.32 ± 0.03 L CH_4/g COD; $p<0.001$). Methane production and OLR of digester C1 rapidly increased with glycerol addition, likely due to catabolism of the readily biodegradable soluble COD in glycerol. This agrees with Wohlgemut *et al.* (2011) in which glycerol doubled methane production with a four times higher OLR when used as a co-substrate with pig manure.

The maximum concentrations of TA and VFA were observed on day 8 for both digesters, after which they decreased continuously (Figures 7.1 C and 7.2 C). During Period III, from days 31 to 48, the average VFA/TA ratio of digester C1 was 0.24, whereas digester C2 had a lower ratio of 0.14. By day 54, VFA concentrations increased slightly to 929 ± 117 mg/L in digester C1 causing the VFA/TA ratio to peak at 0.45; Ciotola *et al.* (2014) and Pontoni *et al.* (2015) suggested that digesters are overloaded when this ratio exceeded 0.40. pH values for digester C1 also showed a declining trend (6.94 ± 0.01 by day 54) (Figure 7.1 B), although this remained within the optimum range of 6.8 – 7.2 for the AD process (Ward *et al.*, 2008). These results agree with the work of Ciotola *et al.* (2014), who found that during AD of dairy manure with a high OLR (1.8 kg VS/m^3/day), the digester failed at a high VFA/TA ratio of 0.65, despite pH remaining at 6.92. This may have been due to the accumulation of short chain fatty acids leading to a significant reduction of buffering capacity before the pH dropped, as reported by Ward *et al.* (2008). Glycerol is rapidly consumed by acidogenic bacteria which generate large amounts of organic acids (Sell *et al.*, 2011; Larsen *et al.*, 2013); therefore, balanced alkalinity is important for AD of glycerol (Viana *et al.*, 2012). However, Astals *et al.* (2012) found that co-digesting pig manure with crude glycerol reduced the alkalinity because glycerol provides negligible alkalinity, resulting in the VFA/TA ratio

exceeding 0.60. Therefore, in their study, the crude glycerol dosage was reduced from 5 to 4% w/w which decreased the VFA/TA ratio to less than 0.4 after 2 days. In the current study, Figures 7.1 C and 7.2 C show that on day 54, digester C1 had a lower TA concentration (2062 ± 18 mg/L) than digester C2 (2800 ± 35 mg/L). Therefore, on day 57, the glycerol dosage was reduced to 1% v/v, consequently the OLR decreased to 0.70 g COD/L/day (Figure 7.1 A). Consequently, on day 60, the VFA/TA ratio of digester C1 decreased to 0.34, within the optimum range for stable AD. During Period IV (days 57 to 76), the average volumetric methane yield of C1 was 0.51 ± 0.05 L $CH_4/L_{digester}$ (Figure 7.1 A) being significantly higher than digester C2 (0.19 ± 0.03 L $CH_4/L_{digester}$; $p<0.001$) (Figure 7.2 A). The specific methane yield of C1 was 0.73 ± 0.07 L CH_4/g COD, which was also significantly higher than C2 (0.32 ± 0.05 L CH_4/g COD; $p<0.001$).

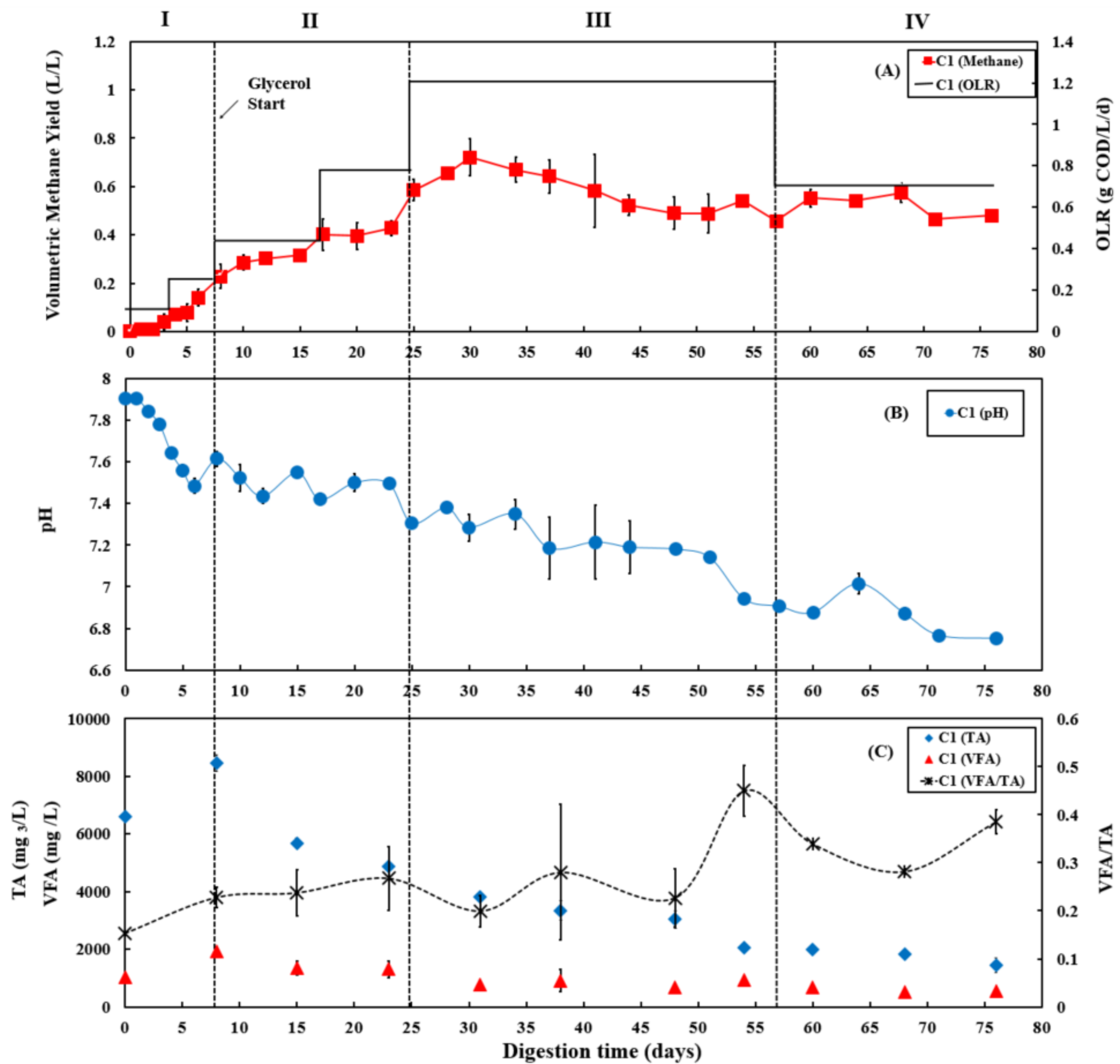


Figure 7.1 Variation in (A) volumetric methane yield, (B) pH, and (C) concentrations of total alkalinity (TA), volatile fatty acids (VFA) and ratio of VFA/TA during co-digestion of *C. vulgaris* and PPW_{dp} with glycerol (C1). Error bars = mean ± SD, n = 2.

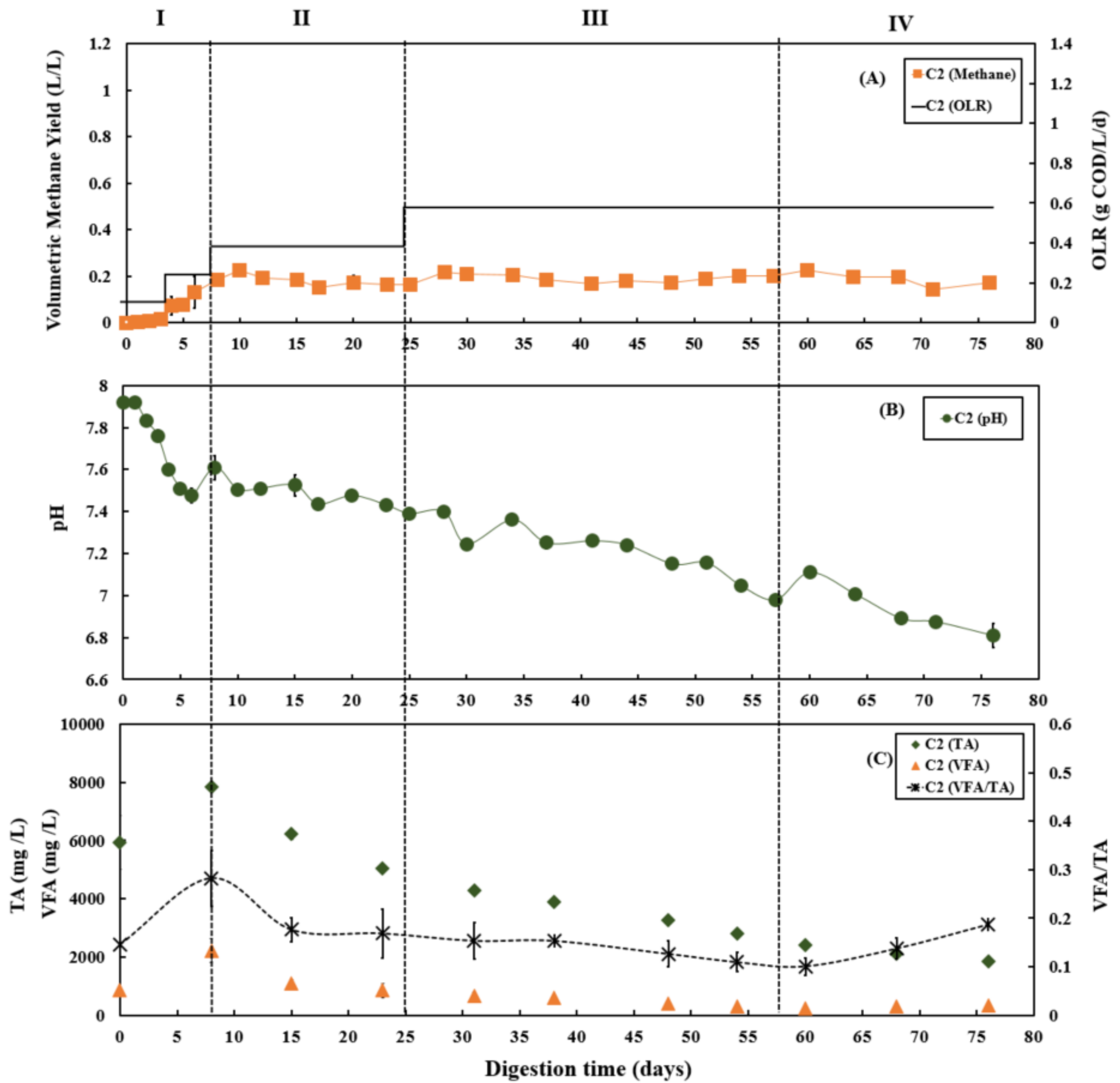


Figure 7.2 Variation in (A) volumetric methane yield, (B) pH, and (C) concentrations of total alkalinity (TA), volatile fatty acids (VFA) and ratio of VFA/TA during co-digestion of *C. vulgaris* and PPW_{dp} without glycerol (C1). Error bars = mean ± SD, n = 2.

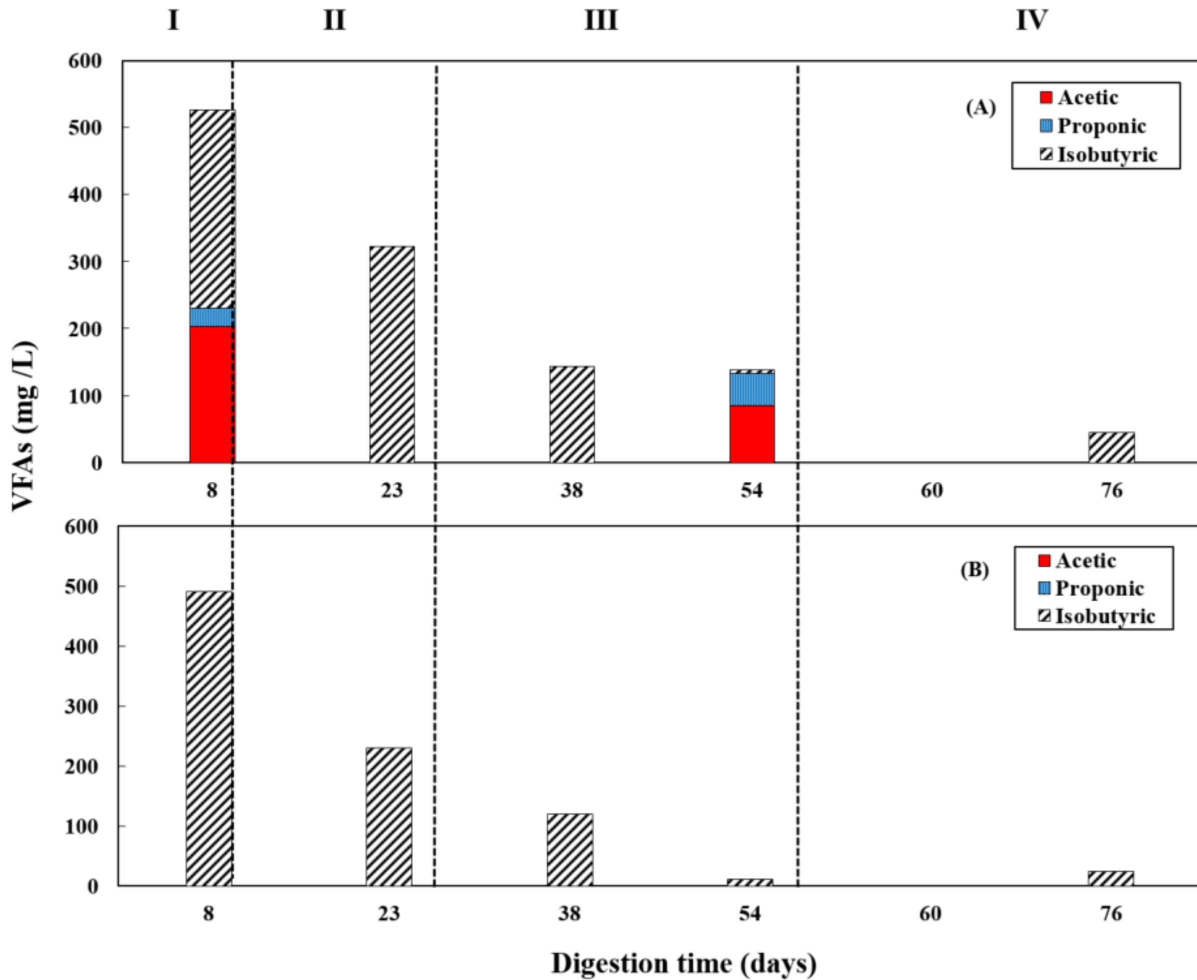


Figure 7.3 Concentrations of acetic, propionic and isobutyric acids in semi-continuous anaerobic co-digestion of *C. vulgaris* and PPW_{dp} (A) with glycerol (C1) and (B) without glycerol (C2). Note: acetic and proponic acids below detection in all digester C2, and some C1 periods.

7.3.2 *Effect of co-digesting C. vulgaris and PPW_p with glycerol on AD performance*

During Period III (from days 25 to 45), when the glycerol feed for digester C3 was increased to 2 % v/v with a high OLR at 1.12 g COD/L/day. Digester C4 was maintained at 0.50 g COD/L/day of OLR. Figures 7.4 B and 7.5 B show that there was a slow reduction of pH in both C3 and C4 during Period III. The glycerol dosage significantly affected the volumetric methane production ($F(2,65)=916.41$, $p<0.001$). During Period III, an average volumetric methane production of 0.60 ± 0.05 L $CH_4/L_{digester}$ in digester C3, which was significantly higher than the level of 0.15 ± 0.02 L $CH_4/L_{digester}$ in digester C4 ($p<0.001$). The average specific methane yield of 0.54 ± 0.04 L CH_4/g COD in C3, which was also significantly higher than the value of 0.31 ± 0.04 L CH_4/g COD in digester C4 ($p<0.001$).

The TA and VFA concentrations of digesters C3 and C4 also peaked after 8 days of operation, then decreased continuously until day 48 (Figures 7.4 C and 7.5 C). Subsequently, the TA and VFA concentrations continued to decrease in digester C4, with a relatively stable VFA/TA ratio. However, digester C3 started to accumulate VFA and experienced reduced TA concentrations, resulting in the VFA/TA ratio peaking at 1.89 on day 54, corresponding with a significant drop in pH of digester C3 to 6.22 and decreased methane production. VFAs are crucial intermediate products affecting methane production and AD process stability, and glycerol degradation by acidogenic bacteria produces VFA. Propionic acid degrading microorganisms have lower specific growth rates than acetic acid- or butyric acid consumers that require longer degradation times (Griffin *et al.*, 1998; Calli *et al.*, 2005; Moset *et al.*, 2014). Therefore, Nielsen *et al.* (2007) and Xiao *et al.* (2015) suggested that propionic acid accumulation could be one of the major parameters indicating AD process instability. The growth rates of both acidogenic bacteria and methanogens are inhibited when propionic acid concentrations exceed 900 mg/L, leading to reduced methane production (Wang *et al.*, 2009). On day 54 of the current study, the total propionic acid concentration in digester C3 was 1220 mg/L (Figure 7.6 A), which probably explains the reduction in methane yields in digester C3 at that time.

Lossie and Pütz (2008) suggested that the input of biomass to a digester should be reduced or stopped if the VFA/TA ratio exceeds 0.6. Therefore, on day 57, the feed of glycerol to digester C3 was stopped, with only the baseline feed of 25:75 *C. vulgaris*: PPW_p used to recover it during Period V. The recovery stage of Period V lasted for 37 days (from days 57 to 94), and the OLR of digester C3 was maintained at 0.50 g COD/L/day. During this recovery stage, pH, methane production and TA concentrations in digester C3 exhibited increasing trends (Figure 7.4), while VFAs, especially propionic acid, decreased continuously, reducing the VFA/TA ratio from 1.89 to 0.21 (Figure 7.4 C). Lossie and Pütz (2008) also suggested that biomass loading should be increased slowly when the VFA/TA ratio ranges from 0.2 to 0.3. Therefore, after recovery, during Period VI, glycerol feeding was restarted at 1% v/v to digester C3 on day 95, and the OLR was increased to 0.67 g COD/L/day (Figure 7.4 A). Period VI lasted 37 days (from days 95 to 132), the average volumetric methane production of 0.33 ± 0.02 L CH_4/L in digester C3, which was significantly higher than 0.17 ± 0.03 L CH_4/L produced by digester C4 ($p<0.001$). Moreover, the average specific methane yield of 0.55 ± 0.03 L CH_4/g COD in C3, which was significantly higher than the value of 0.34 ± 0.06 L CH_4/g COD in C4 ($p<0.001$).

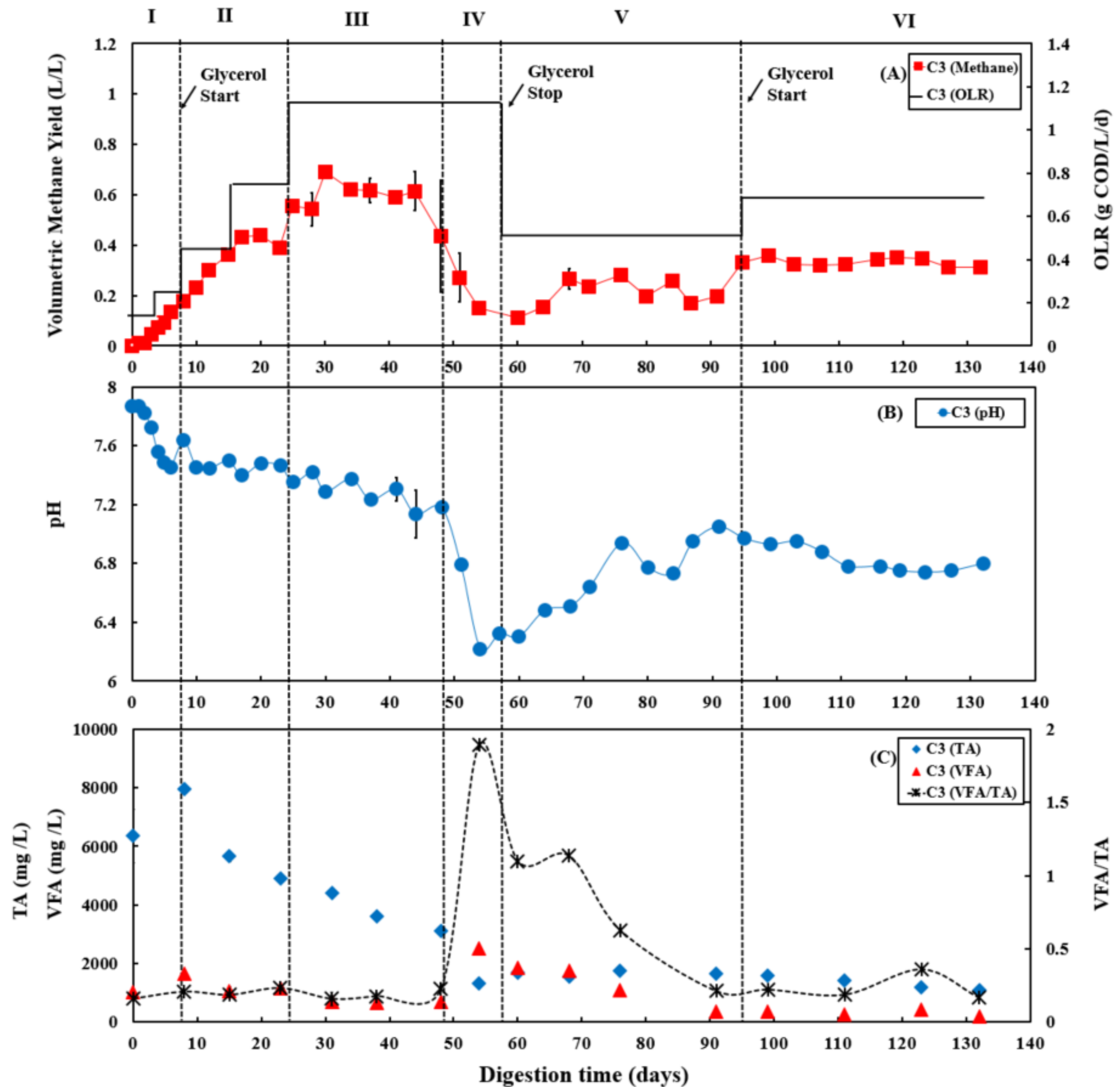


Figure 7.4 Variation in (A) volumetric methane yield, (B) pH, and (C) concentrations of total alkalinity (TA), volatile fatty acids (VFA) and ratio of VFA/TA during co-digestion of *C. vulgaris* and PPW_p with glycerol (C3). Error bars = mean ± SD, n = 2.

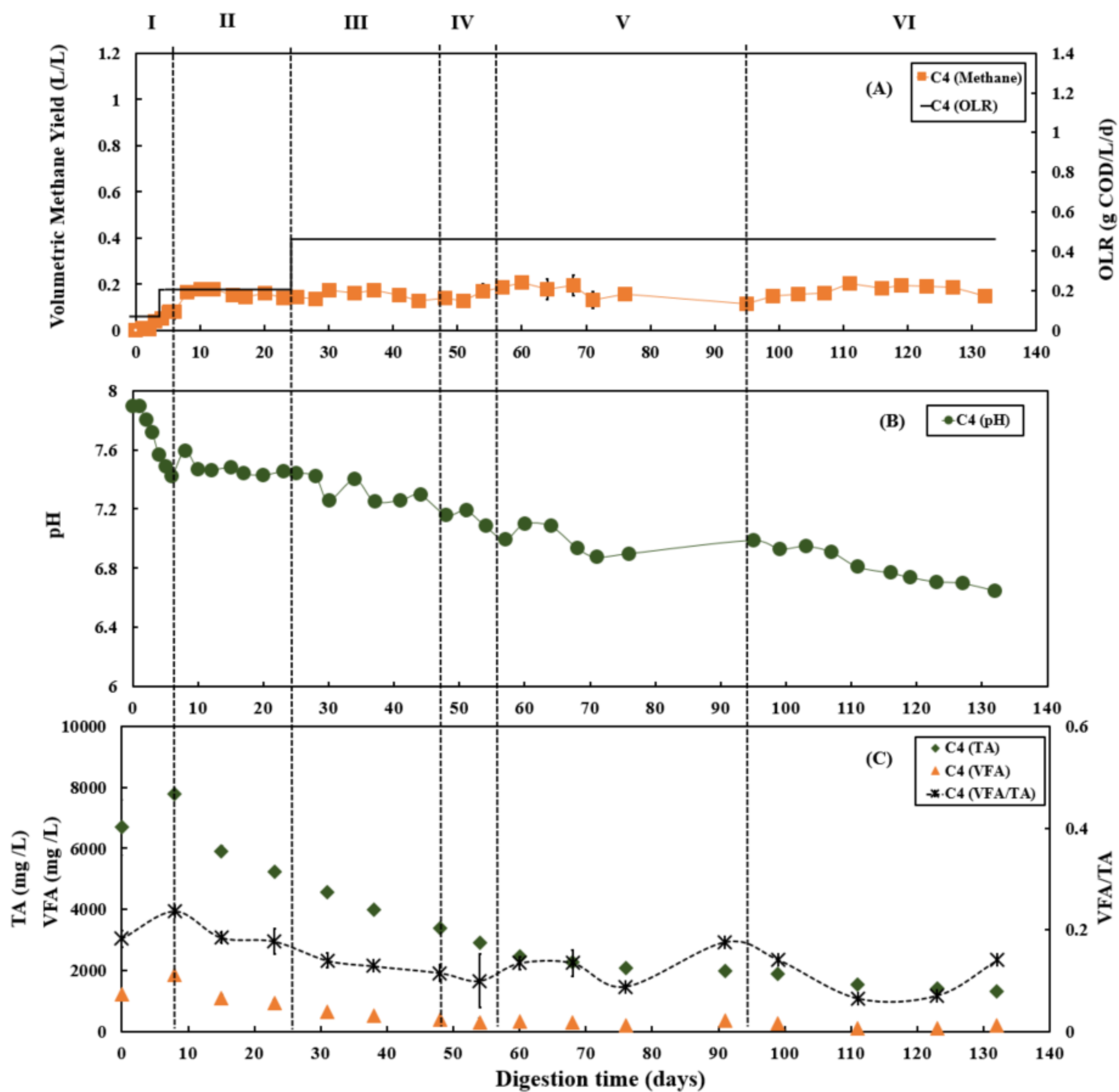


Figure 7.5 Variation in (A) volumetric methane yield, (B) pH, and (C) concentrations of total alkalinity (TA), volatile fatty acids (VFA) and ratio of VFA/TA during co-digestion of *C. vulgaris* and PPW_p without glycerol (C4). Error bars = mean ± SD, n = 2.

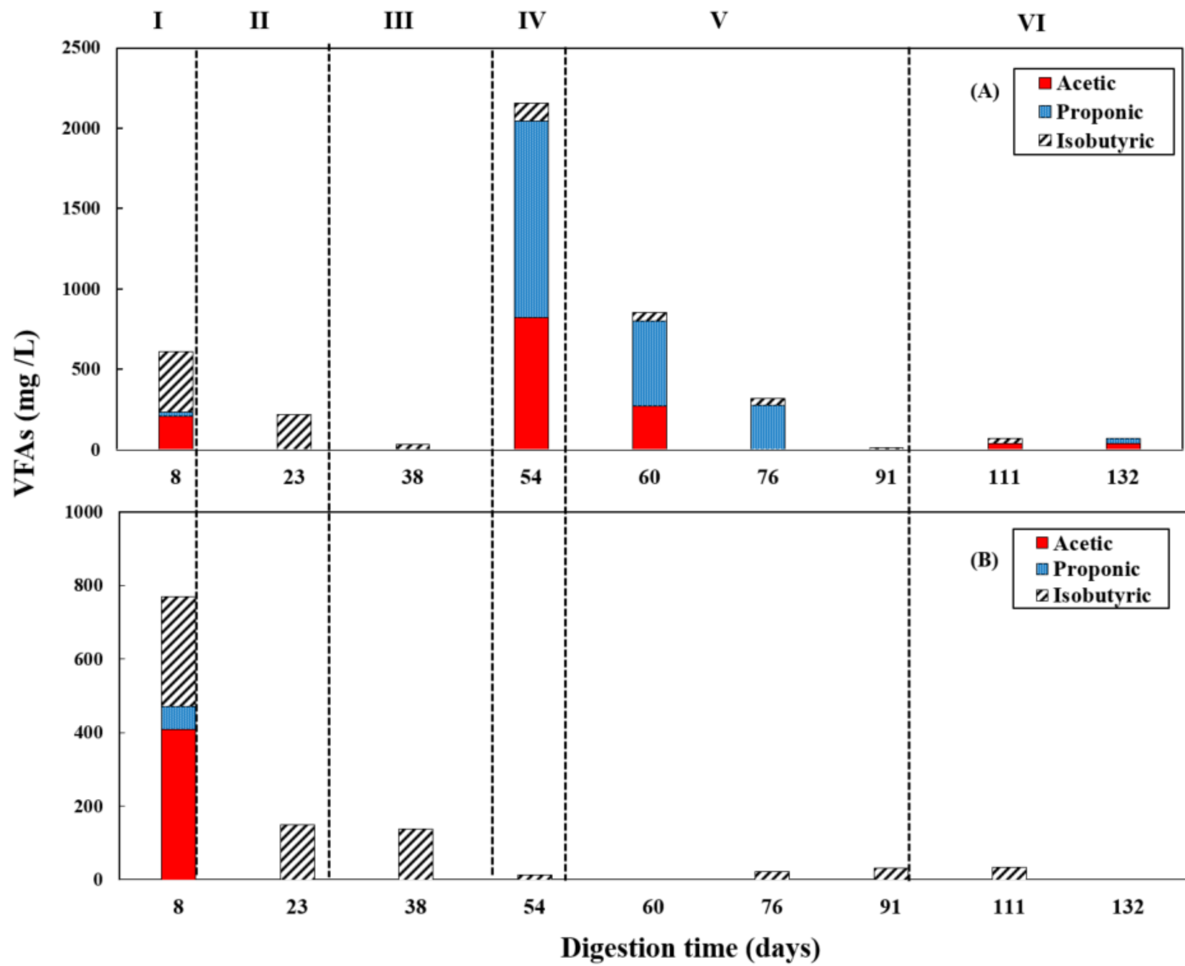


Figure 7.6 Concentrations of acetic, propionic and isobutyric acids in semi-continuous anaerobic co-digestion of *C. vulgaris* and PPW_p (A) with glycerol, digester C3 and (B) without glycerol, digester C4. Note: acetic and proponic acids below detection in some periods of digesters C3 and C4.

7.3.3 *Overall performance during co-digestion*

Tables 7.3 and 7.4 show the overall performance of co-digesting *C. vulgaris* and PPW (PPW_{dp} or PPW_p) both with and without glycerol during the different operating stages. At the end of Period III, the VS values increased when adding 2% v/v glycerol (C1 and C3), which were significantly higher than the digesters (C2 and C4) without glycerol ($t(6) = -7.799$, $p<0.001$). Similarly, the VS values in digesters C1 and C3 were significantly higher than C2 and C4 at the end of Period IV and Period VI ($t(6) = -3.243$, $p=0.018$). These results agree with the work of Fountoulakis *et al.* (2010) and Ma *et al.* (2008). Their studies demonstrated that the addition of glycerol to sewage sludge or potato processing wastewater could improve methane production due to enhanced growth of the active biomass in terms of increased VS values.

FAN is regarded as the active component leading to ammonia inhibition in an AD processes (Yenigün and Demirel, 2013), and microalgae biomass is characterised by having high protein content which can lead to high ammonia concentrations and inhibition when used as a mono-digestion feedstock. In the current study, the FAN concentrations of all digesters were less than 10 mg/L, lower than previously reported methanogenic toxicity levels of 80 – 150 mg/L (Nielsen and Angelidaki, 2008; Wang *et al.*, 2016). Therefore, the current study demonstrated that co-digestion of *C. vulgaris* and PPW, both with and without glycerol, helps avoid the development of ammonia toxicity.

The glycerol dosage level is a key factor affecting final methane production when using glycerol as a co-substrate because of its high COD concentration. Rapidly introducing high glycerol dosage would suddenly increase the OLR, and reduce digester performance by creating a "shock load" as described by Wohlgemut *et al.* (2011). In their study, the volumetric biogas/methane production stopped after 12 days because of VFAs accumulation (> 10,000 mg/L) when pig manure was co-digested with 4% v/v glycerol. Similarly, co-digestion with 2% v/v glycerol also accumulated VFAs (>7000 mg/L) after 25 days. During Period II in the current study, a slow and stepwise increase in glycerol dosage from 1 to 2% v/v was implemented successfully without creating any organic shock load.

Although PPW_{dp} and PPW_{p} are both promising feedstocks for stable microalgae co-digestion, the addition of small amounts (1 – 2% v/v) of glycerol significantly enhanced both volumetric methane yield and specific methane yield (PPW_{dp}: $F(2,61)=319.67$, $p<0.001$ and $F(2,61)=213.67$, $p<0.001$; PPW_{p}: $F(2,65)=916.41$, $p<0.001$ and $F(2,65)=207.72$, $p<0.001$ respectively). Although the higher glycerol dose led to enhanced volumetric methane production, the highest specific methane yield was achieved with the lower dose. When mixtures of *C. vulgaris*: PPW_{dp} co-digesting with 1% v/v glycerol, the specific methane yield was significantly higher than with 0% v/v dosage ($p<0.001$) and 2% v/v dosage ($p<0.001$) (Table 7.3). When co-digesting with mixtures of *C. vulgaris*: PPW_{p}, there was no significant difference between the specific methane yield at the 2 % and 1% v/v dosage ($p=0.687$) (Table 7.4). Moreover, the addition of 2% v/v glycerol was more likely to accumulate VFA, resulting in high VFA/TA ratios, leading to a potentially unbalanced system. Consequently, 1% v/v glycerol appears to be the better dosage when applied to 25:75 co-digestion mixture of *C. vulgaris*/PPW. This agrees with Fountoulakis *et al.* (2010) and Panpong *et al.* (2014) who applied 1% v/v glycerol during co-digestion with sewage sludge or canned seafood wastewater, doubling the volumetric methane production and specific methane yield. However, their systems also showed signs of organic overloading because of increased VFAs concentrations and decreased pH when the dosage exceeded 1% v/v.

Table 7.3 Performance of digesters co-digesting *C. vulgaris* and PPW_{dp} with or without glycerol during different experimental stages.

	Digester C1		**Digester C2**	
	Period III (day 24-56)	**Period IV (day 57-76)**	**Period III (day 24-56)**	**Period IV (day 57-76)**
OLR (g COD/L/d)	1.20	0.70	0.60	0.60
Process stability				
pH	6.94 ± 0.01[a]	6.75 ± 0.00	7.05 ± 0.01	6.81 ± 0.06
Total Alkalinity (g/L)	2.06 ± 0.02	1.45 ± 0.25	2.80 ± 0.04	1.86 ± 0.02
Total VFAs (g/L)	0.93 ± 0.11	0.56 ± 0.06	0.31 ± 0.06	0.35 ± 0.00
NH^{+}_{4}-N (g/L)	0.34 ± 0.00	0.32 ± 0.00	0.53 ± 0.02	0.35 ± 0.04
FAN (mg/L)	3.86 ± 0.21	2.35 ± 0.21	7.56 ± 0.60	2.86 ± 0.73
Digester effluent				
TS (g/L)	8.09 ± 0.19	5.67 ± 0.00	6.79 ± 0.35	4.70 ± 0.39
VS (g/L)	5.36 ± 0.05	3.81 ± 0.00	4.18 ± 0.32	2.77 ± 0.37
COD_t (g/L)	6.86 ± 0.06	6.04 ± 0.06	5.84 ± 0.00	4.43 ± 0.08
COD_s (g/L)	1.49 ± 0.02	1.01 ± 0.05	1.25 ± 0.04	0.72 ± 0.06
$VS_{removal}$ (%)	49 ± 7	64 ± 5	57 ± 1	71 ± 1
$COD_{removal}$ (%)	59 ± 7	64 ± 6	69 ± 2	77 ± 2
Methane production				
Volumetric CH_4 yield ($L/L_{digester}$)	0.59 ± 0.08	0.51 ± 0.05	0.19 ± 0.02	0.19 ± 0.03
CH_4 (%)	58 ± 6	62 ± 8	60 ± 5	65 ± 8
Specific CH_4 yield (L/g COD_{added})	0.49 ± 0.07	0.73 ± 0.07	0.32 ± 0.03	0.32 ± 0.05

[a] Mean ± SD, n = 2.

Table 7.4 Performance of digesters co-digesting *C. vulgaris* and PPW_p with or without glycerol during the different experimental stages.

	Digester C3		**Digester C4**	
	Period III (day 24-45)	**Period VI (day 95-132)**	**Period III (day 24-45)**	**Period VI (day 95-132)**
OLR (g COD/L/d)	1.12	0.60	0.50	0.50
Process stability				
pH	7.18 ± 0.00[a]	6.80 ± 0.00	7.16 ± 0.00	6.65 ± 0.00
Total Alkalinity (g/L)	3.10 ± 0.14	1.10 ± 0.00	3.39 ± 0.02	1.30 ± 0.00
Total VFAs (g/L)	0.70 ± 0.02	0.18 ± 0.00	0.39 ± 0.06	0.18 ± 0.00
NH^{+}_{4}-N (g/L)	0.53 ± 0.00	0.16 ± 0.00	0.57 ± 0.02	0.18 ± 0.04
FAN (mg/L)	10.4 ± 0.23	1.28 ± 0.03	10.5 ± 0.16	1.07 ± 0.04
Digester effluent				
TS (g/L)	8.75 ± 0.05	4.51 ± 0.00	7.22 ± 0.03	4.02 ± 0.00
VS (g/L)	5.70 ± 0.15	3.30 ± 0.00	4.34 ± 0.09	2.85 ± 0.00
COD_t (g/L)	10.7 ± 0.43	4.88 ± 0.01	7.67 ± 0.42	4.10 ± 0.00
COD_s (g/L)	3.02 ± 1.91	0.88 ± 0.00	1.07 ± 0.00	0.41 ± 0.02
$VS_{removal}$ (%)	48 ± 6	70 ± 3	56 ± 1	71 ± 1
$COD_{removal}$ (%)	50 ± 5	77 ± 1	58 ± 5	78 ± 2
Methane production				
Volumetric CH_4 yield ($L/L_{digester}$)	0.60 ± 0.05	0.33 ± 0.02	0.15 ± 0.02	0.17 ± 0.03
CH_4 (%)	58 ± 7	66 ± 2	60 ± 6	64 ± 5
Specific CH_4 yield (L/g COD_{added})	0.54 ± 0.04	0.55 ± 0.03	0.31 ± 0.04	0.34 ± 0.06

[a] Mean ± SD, n = 2.

7.3.4 *Microbial characteristics*

The microbial community from each digester was characterised on days 0, 23, 54 and 76 and extended to days 91 and 132 for digesters C3 and C4 during the recovery phase and the relative abundance of the methanogenic communities are presented in Figures 7.7 and 7.8. Among the methanogens, *Methanosaeta* had the highest relative abundance in the seed inoculum and remained dominant up to day 54. *Methanosaeta* abundance fluctuated somewhat in C2 but an overall decreasing trend was evident over the extended sampling periods for digesters C3 and C4. *Methanosaeta*, as a specialist acetate degrader, is expected to be favored in low acetic acid environments (<100 mg/L) (Liu and Whitman, 2008; Kobayashi *et al.*, 2009). From days 23 to 54, the total VFAs concentrations of digesters C1, C2 and C4 decreased steadily, with acetic acid concentrations remaining below 100 mg/L (Figures 7.3 and 7.6); acetic acid concentration correlating negatively with *Methanosaeta* abundance ($r_s(34) = -0.395$, $p=0.021$). Digester C3 experienced performance inhibition by day 54 through VFAs accumulation and low pH (Figure 7.6 A), and had to be recovered. During recovery, the high acetic acid concentration was effectively degraded, supporting a shift in dominance to *Methanosarcina* (up to 66% relative abundance).

Methanosarcina is a robust acetoclastic methanogen that can utilize acetate, CO_2, methyl-group containing compounds or H_2 as substrate (Liu and Whitman, 2008). It survives at pH 5 – 8 and is often associated with deteriorating digester performance (Kobayashi *et al.*, 2009; De Vrieze *et al.*, 2012). In C3, *Methanosarcina* abundance and methane production were negatively correlated ($r_s (34) = -0.487$, $p=0.004$), with abundance exhibiting a decreasing trend from day 91 to 132. The relative abundance of *Methanoculleus* increased from 6 to 41% over this period, becoming the dominant genus in digester C3 at the end of the digestion process. Furthermore, *Methaneosaeta* dominance was lost in digester C4 by day 132, yielding to *Methanoculleus* and the H_2-dependent methylotroph *Methanomassiliicoccales archaeon* RumEn M2 ((Lang *et al.*, 2015); Figure 7.8). *Methanoculleus* is a hydrogenotrophic methanogen that grows favourably at low acetate and hydrogen concentrations (Hori *et al.*, 2006) that were prevalent during the pseudo of Period VI. De Vrieze *et al.* (2012) reported that a robust methanogenic process can be established based on syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis by *Methanosarcina* under elevated OLR conditions. Therefore, during Period VI the high methane production achieved by digester C3 was probably a result of interactions between *Methanosarcina* and *Methanoculleus*.

The variations of methanogens abundance could significantly affect methane production. For example, the relative abundances of *Methanobrevibacter* and methane production were negatively correlated (r_s (34) = -0.392, p=0.022), with abundance exhibiting a decreasing trend in all digesters (Figures 7.7 and 7.8). *Methanobrevibacter* is a hydrogentrophic methanogen that generally produce methane using hydrogen as electron donor (Liu and Whitman, 2008). From day 0 to day 54, the concentrations of propionic and isobutyric acids in digesters C1, C2 and C4 were decreased continuously, indicating that the digesters may have low hydrogen levels. The efficient hydrogenotrophic methanogenesis are normally associated with low hydrogen levels resulting in the stable digestion process (Liu and Whitman, 2008). The elevated VFA concentrations leading to the unstable digestion process in digester C3 by day 54, and *Methanosarcina* becoming the dominant genus in digester C3 (Figure 7.8). It also found that the relative abundances of *Methanosarcina* and methane production had a negative correlation (r_s (34) = -0.487, p=0.004). During the recovery period (from days 57 to 94), the feed of glycerol to digester C3 was stopped, and the concentrations of VFA exhibited a decreasing trend. Methane production increased continuously, and was associated with the decreased *Methanosarcina* abundance.

Other notable community features included the maintenance of high relative abundances of the methyl-reducing syntroph, *Candidatus Methanofastidiosum* (formerly WSA2; (Nobu *et al.*, 2016)) in digesters without glycerol (C2 and C4), and the strong growth of the hydrogentrophic methanogen *Methanobacterium*, which can utilize H_2 (formate) to produce methane, in digesters C1 and C2 from day 54.

The extent of change observed within the methanogenic communities is illustrated in Figure 7.9, showing the influence of operation time and glycerol addition on the microbial population. Time had a greater influence on community development, with distinct clustering identifiable for each sampling date (Figure 7.9 A); this is unsurprising given the need for the manure-adapted seed inoculum to respond to a markedly different feedstock employed in this study. Only days 76 and 91 had substantial overlap (during the recovery phase), and with day 132 having only partial similarity with day 91. Continued methane production under sub-optimal operating conditions highlights the functional resilience of the communities, even during the recovery phase. Glycerol addition also shaped the methanogenic communities, but to a lesser degree (Figure 7.9 B). Glycerol amended and unamended treatments grouped within distinct clusters, but with substantial overlap; likely due to switching glycerol feed rates in response to digester instability.

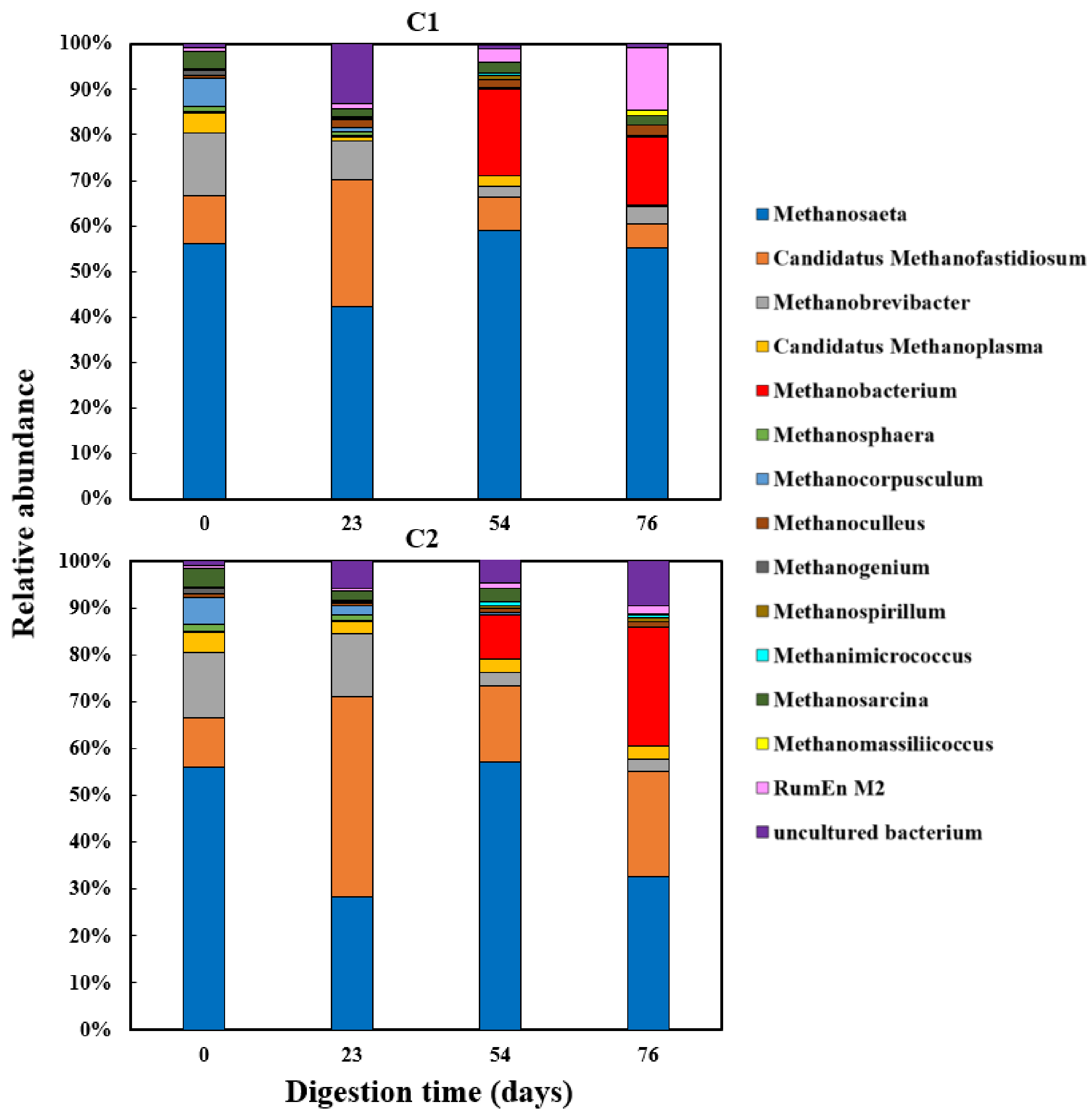


Figure 7.7 Relative abundance of methanogenic archaea (genus level) during co-digestion of *C. vulgaris* and potato discarded parts (PPW_{dp}) with (C1) or without (C2) glycerol addition.

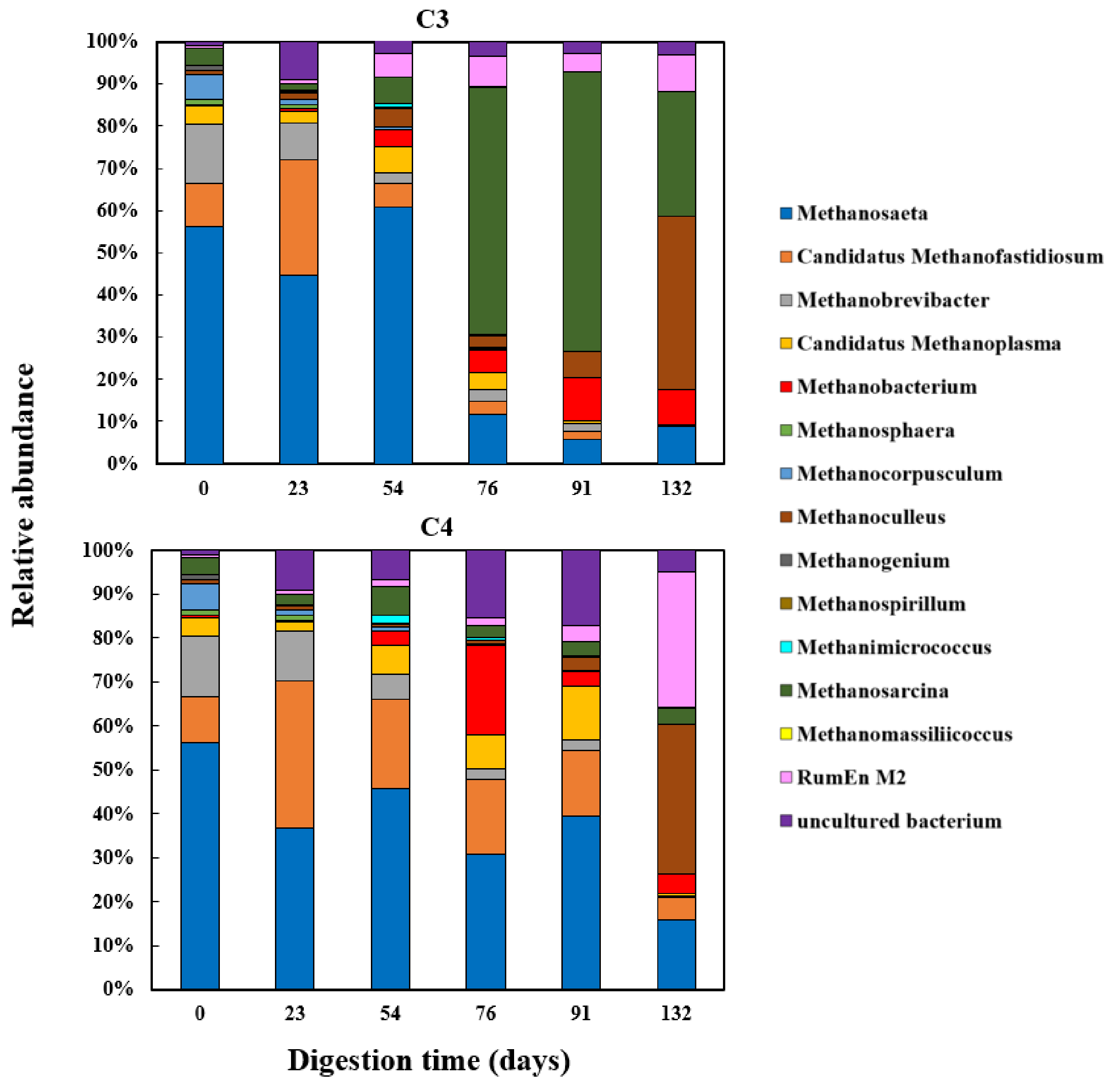


Figure 7.8 Relative abundance of methanogenic archaea (genus level) during co-digestion of *C. vulgaris* and potato peel (PPW_p) with (C3) or without (C4) glycerol addition.

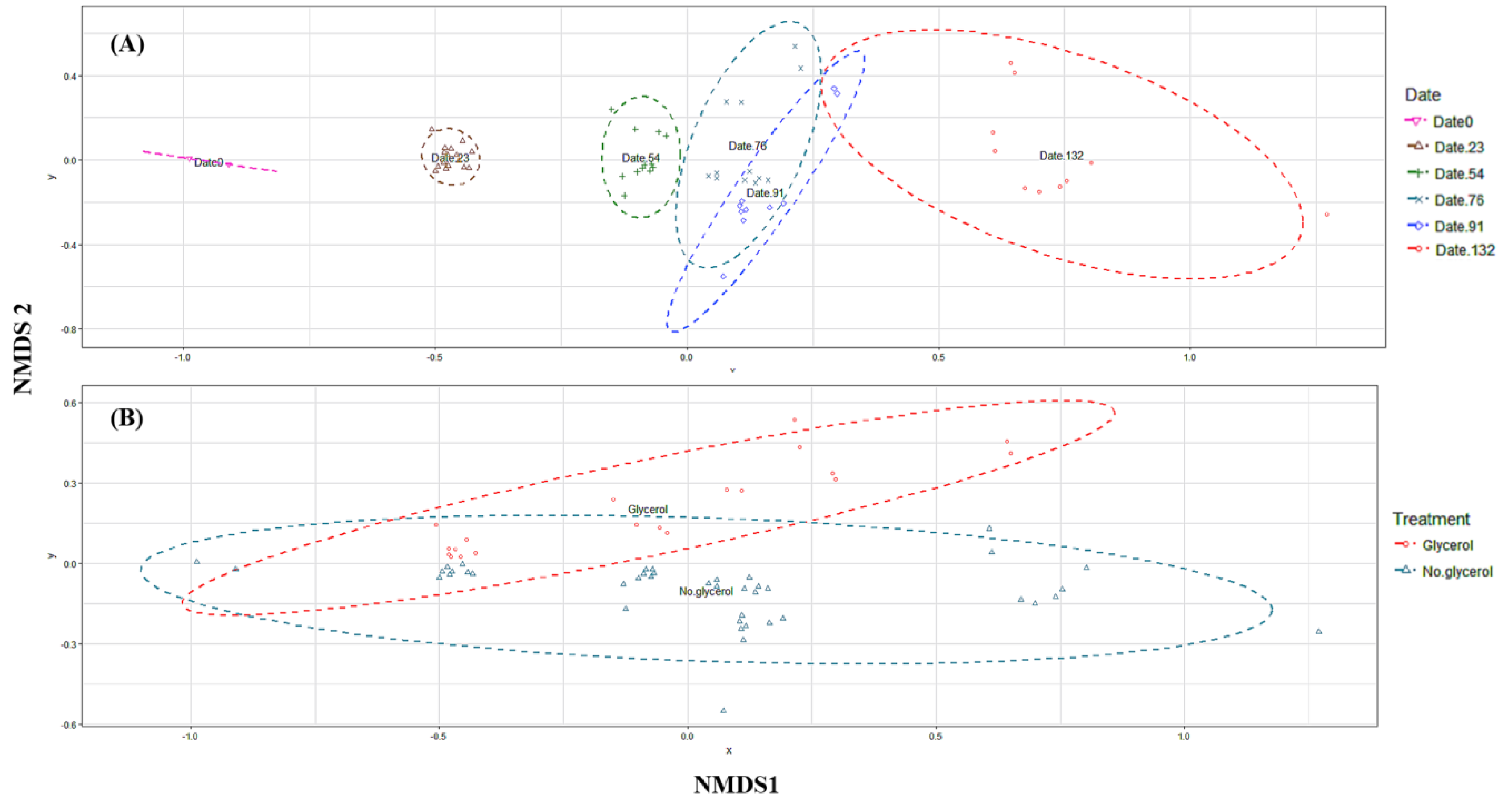


Figure 7.9 Non-metric multi-dimensional scaling ordination (NMDS) plot of all samples: (A) time-dependent clustering pattern; (B) feedstock-dependent (with or without glycerol) clustering pattern.

7.4. Conclusion

In this chapter, the feasibility of using glycerol as an additional co-substrate on the co-digestion of microalgae and PPW was evaluated in semi-continuous digester studies.

Results showed that the glycerol added to mixtures of *C. vulgaris*: PPW_{dp} enhanced the specific methane yields the most, by 53 – 128%, whilst co-digestion with mixtures of *C. vulgaris*: PPW_p enhanced the methane yields by 62 – 74%. When co-digesting with mixtures of *C. vulgaris*: PPW_{dp}, the highest specific methane yields of 0.73 ± 0.07 L CH_4 /g COD was achieved by 1% v/v glycerol dosage, which was significantly higher than 2% and 0% v/v dosage. When co-digesting with mixtures of *C. vulgaris*: PPW_p, the highest specific methane yields of 0.55 ± 0.03 L CH_4 /g COD was also achieved by 1% v/v glycerol dosage, which was significantly higher than 0% v/v dosage. However, there was no significant difference between 1 and 2% v/v dosage. Moreover, the 2% v/v dosage promoted the accumulation of VFA leading to an unstable process and requiring one treatment to be recovered.

The methanogenic communities diverged markedly over operational time, and to a lesser extent in response to glycerol addition. The acetoclast *Methaneosaeta* was abundant in all treatments but was replaced by *Methanosarcina* in the PPW_{dp} with glycerol treatment due to VFA accumulation.

Overall, this chapter demonstrate that the performance of microalgae co-digestion is substantially improved by the addition of glycerol as an additional co-substrate. The results suggested that 1% v/v could be the optimal dosage when co-digesting with mixtures of *C. vulgaris*: PPW to enhance methane production without organic overloading.

Chapter 8. Conclusions

8.1 Introduction

Microalgal biomass is an alternative to conversional terrestrial based energy crops for the future bioenergy market. AD of microalgae is a promising option not only in terms of the production of renewable energy but also in the reduction of greenhouse gas emissions. Moreover, AD of microalgae provides additional potential benefits by improving the economic case for microalgae biodiesel production through an integrated biorefinery process. The results reported in Chapters 4, 5, 6 and 7 have important implications for the development of an economically viable microalgae AD process. This chapter firstly discusses the potential implications of the findings of each investigation and highlights the future perspective for microalgae AD technology. Secondly, this chapter details the conclusions and proposes recommendations for the future work.

8.2 Implications

8.2.1 *Implication of microalgae pre-treatment*

Microalgae AD is a promising way to generate renewable energy, but the implementation of this process first requires the disruption of cell walls after harvesting microalgal biomass. Moreover, if integrating microalgae AD as a part of biorefinery process, a technique for mild cell disruption is required to ensure that cell components are released intact so that their subsequent extraction is easier (Vanthoor-Koopmans *et al.*, 2013). In order to ensure that the pre-treatments can be used in large-scale applications, the selection of suitable pre-treatments should consider their energy demand and overall cost. In Section 4.3.4, the results show that, in comparison with low-temperature thermo-alkaline pre-treatment, enzymatic pre-treatment was more energetically balanced, except when using 0.5% v/w of α-amylase. Moreover, in further considering the energy balance, enzymatic pre-treatment using a mixtures of enzymes may be favourable than single enzymes. Enzymatic pre-treatment is a mild cell disruption method which can break down the cell wall effectively, but the high cost of commercial enzymes could be an obstacle in applying this method at a large-scale. However, since the biofuel production process and pre-treatment efficiency are dependent on enzyme loading and, if this can be reduced, the enzyme costs will be proportionally lower (Liu *et al.*, 2016). Reducing the amount of enzyme consumed in hydrolysis process may reduce the enzyme

loading requirement, and consequently reduce the costs (Klein-Marcuschamer *et al.*, 2012). The results in Section 4.3.4 show that the AD of microalgae pre-treated with a 0.5% v/w dosage of a mixture of cellulase and protease could generate 248 mL CH_4/g VS of methane, which is higher than the values of 183 and 194 mL CH_4/g VS of methane produced by higher dosages of 1.0% v/w of cellulase and protease, respectively. Similar results were also found when using cellulase mixed with α-amylase to pre-treat microalgae. Based on these findings, enzymatic pre-treatment using mixed enzymes should consume lower amounts of enzyme compared to single enzyme pre-treatments, but should gave greater methane production. Therefore, microalgae pre-treatment using mixed enzymes may be a potential way to reduce enzyme costs, and improve the economic feasibility of the pre-treatment process prior to AD. However, other alternatives still need to be considered in order to further reduce the cost of enzymes. Information about potential alternatives are discussed in Section 9.2.1.

Chapter 4 also shows that the microalga *C. vulgaris* pre-treated by a low-temperature thermo-alkaline method produced less methane compared to enzymatic pre-treatment. However, as discussed above, one of the requirements in the selection of a suitable pre-treatment is that it should be less energy intensive. The results in Section 4.3.4 also showed that a combination of 10% w/w $Ca(OH)_2$ with low pre-treatment temperatures of 37 and 55 ℃ lead to a positive energy balance. In addition, the selection of pre-treatment temperature in the current work was based on the operational temperatures used in anaerobic digesters under mesophilic (37 ℃) and thermophilic (55 ℃) conditions. Therefore, when considering the utilisation of the low-temperature thermo-alkaline pre-treatment of microalgae prior to AD, the pre-treatment temperature could be set at the same as the subsequent AD process. Moreover, thermo-alkaline pre-treatment using $Ca(OH)_2$ has additional advantages, including inexpensive reagent costs and a safety process. Therefore, a low-temperature thermo-alkaline pre-treatment of microalgae with $Ca(OH)_2$ as an energetically balanced and a cost-effective method, has the potential for application beyond the laboratory scale investigations. However, some difficulties need to be considered before scaling up this pre-treatment. Firstly, an appropriate technique should be found to reduce the consumption of water used to wash the pre-treated biomass. Additionally, the pre-treated biomass may need neutralisation to rebalance the pH, and this process may increase the cost of downstream processing (Agbor *et al.*, 2011). Therefore, the selection of low-cost and environmentally-friendly chemicals for this neutralisation process is another area which requires further consideration.

8.2.2 *Implication of microalgae co-digestion*

Chapters 5, 6 and 7 focus on investigating the feasibility of using potato processing waste and glycerol as co-substrates for anaerobic co-digestion with microalgae. This section highlights the implications of the results reported in these three chapters for the development of an economically viable microalgae co-digestion process.

The results in Section 5.3.1 show that the concentrated microalgae *C. vulgaris* contained relatively low concentrations of TS and VS, and the addition of PPW could increase the load of biodegradable organic matter in terms of TS and VS. The most fundamental challenge that exists in proving the economic viability of converting microalgal biomass to methane, or alternative biofuels, is the relatively low concentration of harvested microalgal biomass generated after growing it (Ward *et al.*, 2014). This means that without the implementation of costly biomass concentration technologies, low OLRs, short HRTs or both would be experienced when using microalgal cultures directly as a substrate for a large-scale AD plant. Therefore, if the co-digestion of microalgae and PPW can be applied in large-scale AD, the OLRs may be potentially increased compared to the mono-digestion of microalgae. Moreover, increasing the OLRs can also reduce the size of digester, and subsequently reduce capital costs (Chandra *et al.*, 2012).

Currently, besides the low biomass concentration, high production costs are another challenge when using microalgae for biofuel production (Schenk *et al.*, 2008). Production costs are reported to be between $100,000 – 1,000,000/ha depending on the cultivation system used (Ghasemi *et al.*, 2012). The results in Chapter 5 show that the best co-digestion performance was achieved at a mixing ratio of 25:75 of microalgae and PPW compared to ratios of 100:0, 75:25 and 50:50. Therefore, when running a large-scale co-digestion digester, less input of microalgal biomass may be required, which consequently improves the economic feasibility of using microalgae for methane production.

When considering the use of microalgae as a feedstock for methane or other biofuels production, the water consumption for all processes is an important issue, especially for the cultivation process. Freshwater is a critical natural source, and thus the utilization of seawater or wastewater would be extremely beneficial for the cultivation of microalgae in order to reduce the demand for freshwater (Gonzalez-Fernandez *et al.*, 2012). However, very few investigations have utilised marine microalgae in the production of methane due to their high

salt concentrations. In the current study, Chapter 6 shows that successful co-digestion of the marine microalgae *T. lutea* and PPW without the production of compounds toxic to the methanogens. Therefore, co-digestion with PPW may also be a feasible method to reduce inhibition and enhance methane production when using marine microalgae as a feedstock for biogas production.

Chapters 5 and 6 show that PPW is a promising co-substrate to assist in the digestion of microalgae, and co-digestion with microalgae is also an environmentally-friendly and economical solution for PPW treatment/disposal. Work with batch BMP tests reported in Chapter 5 shows that the highest methane yields were obtained by the mono-digestion of PPW, but this increased the possibility of generating high VFA concentrations. The results in Chapter 6 identified further that semi-continuous digesters initially fed with 100% PPW could produce more VFA compared to the digesters not being fed this substrate. Methanogenesis was the rate-limiting step for the easily biodegradable PPW, whereas hydrolysis was the rate-limiting step for microalgae. Therefore, based on these findings, a two-stage anaerobic co-digestion process could be proposed in order to further increase methane production from microalgae.

As reported in Chapter 7, the supplementation of glycerol to the mixtures of microalgae and PPW allowed the operating OLR to be increased. Moreover, methane production was significantly enhanced when conducting co-digestion with glycerol at an optimum dosage of 1% v/v. When using microalgae as a substrate for biofuel production, one factor affecting financial viability is the transportation of the biomass to the operational site (Saratale *et al.*, 2018). In addition, feedstock availability is another important consideration when operating an AD plant. Therefore, when considering microalgae AD as part of a biorefinery concept, particularly where biodiesel production is one step in the value chain, glycerol will be produced on site thereby providing a readily available co-digestion feedstock and reducing the transportation costs. Another area which needs further consideration is the large amount of glycerol produced from biodiesel production that may require additional treatment, and this may potentially increase the cost of on-site waste management (Saratale *et al.*, 2018). Therefore, from an economic viewpoint, co-digestion with glycerol may be a technique that could be used to improve the overall efficiency of the AD process and the economics of the biorefinery plant. However, the utilisation of glycerol as a co-substrate requires a further economic viability analysis due to its high value as a chemical feedstock (Gonzalez-Fernandez *et al.*, 2012).

Changes in microbial communities during the microalgae AD process is a research area which needs further investigation (Ward *et al.*, 2014). *Methanosaeta* has previously been identified as the dominant archaea in the mesophilic AD of mixed microalgae harvested from a wastewater lagoon (Ellis *et al.*, 2012). However, microbial community information during the AD of specific microalgae strains is still limited. In the current work, the co-digestion process utilized specific microalgae strains, and the results in Section 6.4 showed that during the co-digestion of the marine microalgae *T. lutea* and PPW, *Methanosarcina* became the dominant genus present within the co-digestion process. Moreover, this type of methanogens remained dominant at the end of the digestion process in one treatment using *T. lutea* and PPW_{dp}. *Methanosarcina* is tolerant to environmental stresses such as high salt and VFA concentrations (De Vrieze *et al.*, 2012). As a marine strain, *T. lutea* may contain high concentrations of salt, and this is likely to explain why a large *Methanoscarina* population was found when *T. lutea* was co-digested with PPW. However, when investigating the co-digestion of the freshwater microalgae *C. vulgaris* and PPW in semi-continuous tests in Section 7.3.4, *Methanosaeta* had the highest relative abundance during the co-digestion process. Moreover, the microbial communities were also affected by the types of co-substrates, and the results in Section 7.3.4 show that when large amounts of glycerol were added to the mixtures of *C. vulgaris* and PPW, *Methanosarcina* also became the dominant genus due to the elevated concentrations of VFAs. Therefore, such information may provide insights at a molecular level to assist in the digestion of the microalgae when using specific microalgae strains and various co-substrates.

8.2.3 *Future perspectives*

Some potential implications of the present findings suggest that the economic feasibility of the use of microalgae for methane production can be improved, but the conversion of microalgal biomass exclusively for biogas production at an industrial level still faces challenges. The integration of microalgae production and AD based on a biorefinery approach seems to be a promising way forward as mentioned in literature (Uggetti *et al.*, 2014), and Figure 8.1 shows that a potential system can be proposed based on the current work.

Since high cultivation costs are the first challenge using microalgae for biofuel production, the integration with AD could be an opportunity for large-scale microalgae cultivation. In this integration process illustrated in Figure 8.1, digestate and CO_2 generated by the co-digestion process could be used as nutrients and inorganic carbon for microalgae cultivation. However,

information about the use of AD digestate to cultivate microalgae is limited. Future attention should focus on the evaluation of the characteristics of the digestate, and especially the lipid phase, before using it as a nitrogen source for microalgae cultivation.

Secondly, another issue with the use microalgae for biodiesel production is the requirement of extra energy for lipid extraction and oil transesterification. In the proposed systems, the heat and electricity generated from the co-digestion process could be used in the biodiesel production process. The glycerol generated by the biodiesel production process could also be used as the feedstock for anaerobic co-digestion. However, the complexity of these processes require further detailed environmental and economic assessments. In addition to microalgae biodiesel production, microalgal residues after lipid extraction could be used as substrates for co-digestion with PPW and glycerol. Since the current study focussed on using whole microalgal cells for the co-digestion process, the potential of using microalgal residues for co-digestion with PPW and glycerol is a research area which needs to further investigation.

The hydrolysis stage has also been reported as a rate-limiting step in the development of the feasibility of co-digestion systems (Hagos *et al.*, 2017). As discussed in Section 8.3, a two-stage co-digestion process could be a potential solution in the co-digestion of microalgae with PPW. This work found that enzymatic and low-temperature thermo-alkaline pre-treatments are energetically balanced methods, and therefore another alternative to solve this problem is to consider using a pre-treated microalgal biomass for the co-digestion process, as shown in Figure 8.1. Therefore, further research should extend the current co-digestion work by feeding pre-treated microalgal biomass, PPW and glycerol.

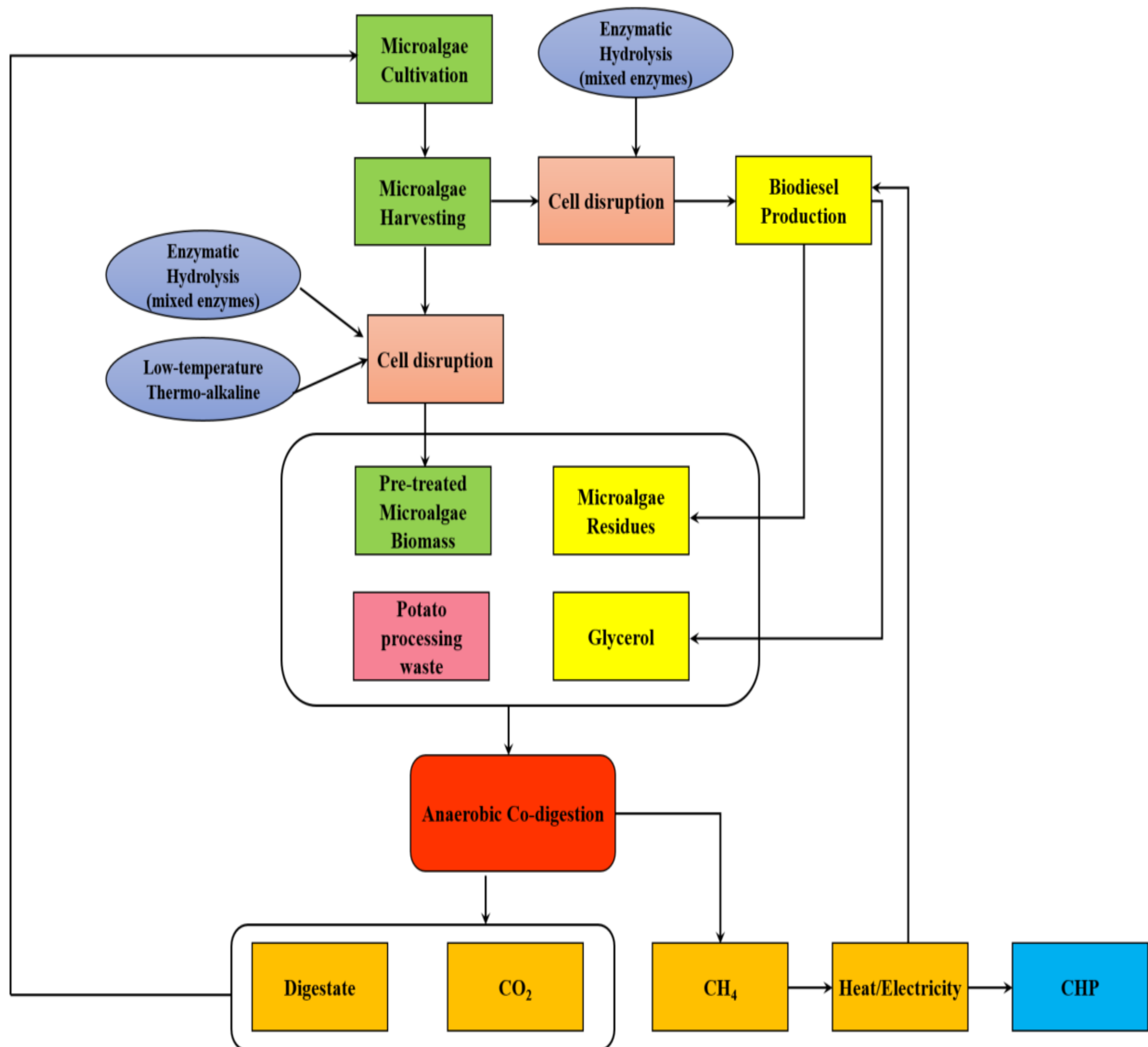


Figure 8.1 Proposed integration process of microalgae cultivation and AD based on a biorefinery concept.

8.3 Conclusions

The aim of this study was to evaluate how to enhance methane production from microalgae through pre-treatment and co-digestion strategies. The following four broad objectives of the research were designed to achieve this aim (further specific objectives within each of these are discussed in detail in Chapters 4 to 7):

1. To evaluate the effects of low energy pre-treatments on microalgae solubilisation and methane yields in batch BMP tests.
2. To evaluate the feasibility of using potato processing waste as a co-substrate for co-digestion with microalgae in batch BMP tests.
3. To evaluate the feasibility of using potato processing waste as a co-substrate for co-digestion with microalgae in semi-continuous co-digestion studies.
4. To evaluate the effects of glycerol supplementation on the co-digestion of microalgae and potato processing waste in semi-continuous co-digestion studies.

Results from Chapter 4 show that methane yields were significantly enhanced by both enzymatic and low-temperature thermo-alkaline pre-treatments. Microalgae *C. vulgaris* after enzymatic pre-treatment enhanced methane yields the most, by 22 – 162%, whilst *C. vulgaris* pre-treated by thermo-alkaline pre-treatment improved methane yields by 4 – 26%. For enzymatic pre-treatment, mixtures of enzymes may exhibit a synergistic effect and consequently improve the hydrolysis efficiency. Therefore, *C. vulgaris* pre-treated with mixed enzymes showed higher methane yields compared to single enzymes. In low-temperature thermo-alkaline pre-treatment, the level of enhancement in methane yields depended on the alkaline dosage and pre-treatment temperature, but the high alkaline dosages were associated with limitations such as a prolonged lag phase in the digestion process. In comparison with low-temperature thermo-alkaline pre-treatment, microalgae pre-treated enzymatically may release more intercellular components in terms of the concentrations of soluble COD and proteins, and these two parameters had a positive correlation with methane yields. Therefore, *C. vulgaris* pre-treated by enzymatic pre-treatment could produce more methane than low-temperature thermo-alkaline pre-treatment. From an energy viewpoint, both pre-treatments showed positive energy balances for the majority of experimental conditions, and therefore both pre-treatments are considered to be energetically efficient methods to pre-treat microalgae.

PPW could be used effectively as a feedstock in co-digestion with microalgae *C. vulgaris* in batch BMP tests as shown in Chapter 5. The addition of PPW (PPW_{dp} or PPW_{p}) to *C. vulgaris* both resulted in an increase in the C/N ratio and decreased the content of toxic components in terms of FAN concentrations, and consequently improved digestion performance and stability. Production rates and final yields of methane were significantly enhanced with increased proportions of PPW in the mixed waste, and the best performance was found at a ratio of 25:75 *C. vulgaris*: PPW compared to ratios of 75:25 and 50:50. The type of PPW also had a significant effect on methane yields, as *C. vulgaris* co-digested with PPW_{dp} increased the methane yield by 22 – 47%, versus a 12 – 32% enhancement with PPW_{p}. A lower substrate to inoculum ratio (SIR) is probably better for microalgae co-digestion with PPW, as a higher SIR of 1.0 led to an accumulation of soluble COD, resulting in decreased methane yield relative to digesters employing a SIR of 0.5.

The results of semi-continuous anaerobic co-digestion experiments reported in Chapter 6 also indicated that both PPW_{dp} and PPW_{p} are promising feedstocks for co-digestion with the marine microalga *T. lutea*. A mixing ratio of 25:75 of *T. lutea*: PPW gave the optimum C/N ratios, and enabled the semi-continuous co-digestion process to operate successfully without VFA and ammonia inhibition. The results also showed that the semi-continuous co-digestion of *T. lutea* with PPW_{dp} produced higher methane yields than co-digestion with PPW_{p}. Results also showed that methane yields were significantly affected by the start-up strategies used, with semi-continuous digesters that were immediate feeding with 100% PPW_{dp} or PPW_{p} showing significantly enhanced methane yields compared to the digesters that were not fed initially either of these substrates. Moreover, physiochemical variables were also affected by this 100% PPW start-up strategy, with concentrations of VFA and ammonia being decreased significantly, and consequently, this educed the potential risk to the methanogens. Moreover, digesters were immediate feeding with 100% PPW supported a high relative abundance of *Methanosarcina.*

The results of the semi-continuous anaerobic co-digestion experiments described in Chapter 7 suggest that the performance of microalgae co-digestion is substantially improved by the inclusion of glycerol as an additional feedstock. Supplementing glycerol into mixtures of *C. vulgaris*: PPW enhanced methane yield significantly. Co-digestion of glycerol with mixtures of *C. vulgaris*: PPW_{dp} enhanced the specific methane yields the most, by 53 – 128%, whilst co-digestion with mixtures of *C. vulgaris*: PPW_{p} enhanced the methane yields by 62 – 74%.

However, glycerol dosage is a key factor for the co-digestion process, and results showed a dosage of 2% v/v caused the accumulation of VFA, leading to an unstable process and requiring intervention to recover one of the digesters. Interestingly, at the beginning of the recovery period, the digester was dominated by *Methanosarcina*, and the relative abundance of this methanogen showed a negative correlation with methane yields.

8.4 Recommendations for future work

Overall, the methane yields obtained from microalgae were significantly enhanced due to the tested pre-treatments. Potato processing waste and glycerol are two promising waste materials that can be used as feedstocks for co-digestion with microalgae, and can rebalance C/N ratios, and improve process stability, leading to enhanced methane yields. However, the ideas developed in this work need to be examined further, and recommendations for the future work is provided below that should improve the feasibility of using microalgae as a feedstock for the production of bioenergy via AD.

8.4.1 *Recommendations for pre-treatment*

For enzymatic pre-treatment, the enzyme mixtures produced higher methane yields than single enzymes, but the mixture ratios of two enzymes were set at 1:1 in the current study. Therefore, future laboratory work could extend the enzymatic pre-treatment conditions by assessing the effect of other mixture ratios such as 1:3, 1:2, 2:1 and 3:1 on the solubilisation of microalgae and subsequent methane yields. Additionally, the pre-treatment time for enzymatic pre-treatment was set at 24 hours, and therefore, other reaction periods should be investigated to determine whether methane yields might be improved. For low-temperature thermo-alkaline pre-treatment, pre-treatment time had no effect on methane yields, and it appears that only short contact periods may be needed for microalgae compared to lignocellulosic biomass. However, future laboratory work still needs to investigate other the pre-treatment times to verify this. Moreover, the present pre-treatment work was all based on small batch BMP experiments, but in order to study the performance of anaerobic microorganisms in more depth by extended feeding with pre-treated microalgal biomass, a semi-continuously fed digester is required to evaluate the benefits, or possible long-term detrimental effects of both pre-treatments in terms of energy balance and overall costs.

Although the enzymatic pre-treatment of microalgae led to a higher solubilisation of organic matter and methane yields compared to low-temperature thermo-alkaline pre-treatment, a key difficulty with the use of this method in large-scale applications is the high cost associated with the purification of enzymes or the purchase of commercial enzymatic cocktails. Therefore, alternatives to these high cost sources of enzymes should be considered. The first alternative should consider on-site production using crude enzymes produced by microbes such as bacteria or fungi. For example, in order to reduce costs further and enhance enzymatic hydrolysis of the microalgae, bioaugmentation could be used whereby whole cells of enzyme-producing bacteria, or crude enzyme preparations from these bacteria, were added to suspensions of the microalgae prior to AD.

Microalgae contain intercellular enzymes associated with cell activities, and the second strategy would exploit the natural process of autolysis of microalgae to release its own digestive enzymes. Microalgae autolysis is a natural process that occurs during both asexual and sexual lifecycles, where autolysins (proteases) are capable of degrading microalgal cell wall. Microalgae autolysis is induced by various environmental stress conditions, including oxidative stress, darkness, heat-stress, high salinity and restricted CO_2. Therefore, the autolysis process might be a cheaper method to disrupt the microalgal cell wall when using microalgal biomass for the production of biofuels, and within a biorefinery process.

8.4.2 *Recommendations for co-digestion*

For the co-digestion experiments, reported in Chapter 6, the digesters were initially fed with 100% PPW showed better performance and higher methane yields than the digesters with delayed feeding. However, the period with 100% PPW feeding was set at five days, after which microalgae in the feedstock was set at a constant proportion of 25% based on VS. Therefore, further laboratory work should be conducted to determine whether a longer start-up period with 100% PPW, and/or a progressive increase in the proportion of microalgae from 0 to 25% VS, gives additional benefits compared to the current study.

To date, information about continuous digesters fed with microalgal biomass is still very limited compared to BMP tests. During the current work, as presented in Chapters 6 and 7, the semi-continuous anaerobic tests focused on an evaluation of the potential of the benefits of using carbon-rich waste materials as co-digestion substrates with microalgae. Since operating parameters such as OLR and HRT are known to affect the performance of the AD

process and influence methane production. Therefore, it is proposed that future work should investigate the effect of OLR and/or HRT on the semi-continuous co-digestion of microalgae with potato processing waste and/or glycerol.

Additionally, since digestion temperature is known to be key factor affecting methane production. Thermophilic AD (55 – 70 °C) giving potentially faster reaction rates, and higher biodegradability and productivity than mesophilic AD, but possibly causing detrimental effects as well, such as acidification and the accumulation of intermediate products. Future work could expand on the co-digestion experiments carried out to date by assessing the effect of temperature on digester performance and methane production.

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Appendix

Appendix A. Additional experimental data

A.1. Growth analysis of *C. vulgaris*

Figure A.1 shows the growth curve of cultivated *C. vulgaris*, and the stationary phase started from days 23 to 39 with the average optical density of 1.95 ± 0.01 at 540 nm.

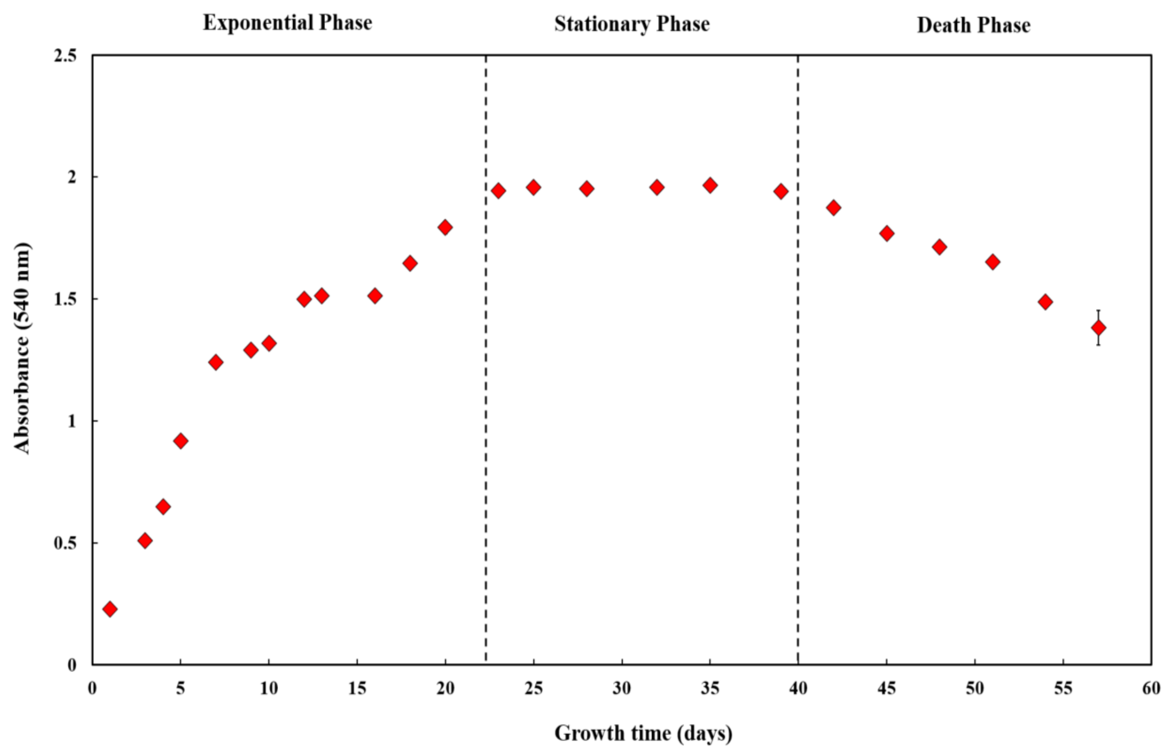

Figure A.1. *C. vulgaris* growth curve based on optical density at 540 nm.

A.2. Additional experimental data of *C. vulgaris* co-digestion with PPW in batch BMP studies

The theoretical maximum methane potential of C. vulgaris, PPW_{dp}, PPW_{p} and co-digestion mixtures can be calculated based on Equation A.1 (Nielfa *et al.*, 2015).

$$BMP_{thCOD} = \frac{n_{CH_4}RT}{PVS_{added}} \tag{A.1}$$

where BMP_{thCOD}- The theoretical production (ml CH_4/g VS added) at experimental conditions (1atm, 37 °C); R- Gas constant (R = 0.082 atm L/mol K); T -Temperature of the digestive bottle (37 °C=310K); P- Atmospheric pressure (1 atm); VS_{added}-the volatile solids (g/L) of the substrate; n_{CH_4}- The amount of molecular methane (mol) determined from Equation A.2.

$$n_{CH_4} = \frac{COD}{64(g/mol)} \tag{A.2}$$

Tables A.1 and A.2 show that the experimental and therotical methane yields produced by co-digestion of *C. vulgaris* and PPW.

Table A.1. Experimental, theoretical methane production and biodegradability of co-digestion of *C. vulgaris* and potato processing waste at 1.0 SIR

	SIR=1.0		
	BMP_{th} (ml CH_4/gVS)	**BMP_{exp} (ml CH_4/gVS)**	**BD_{exp} (%)**
C. vulgaris	563	158	28
PPW_{dp}	749	340	45
PPW_{p}	519	232	45
+ **25% PPW_{dp}**	532	207	36
+ **50% PPW_{dp}**	513	243	47
+ **75% PPW_{dp}**	486	260	53
+ **25% PPW_{p}**	532	201	38
+ **50% PPW_{p}**	506	210	41
+ **75% PPW_{p}**	462	228	49

Table A.2. Experimental, theoretical methane production and biodegradability of co-digestion of *C. vulgaris* and potato processing waste at 0.5 SIR

	SIR=0.5		
	BMP_{th} (ml CH_4/gVS)	**BMP_{exp} (ml CH_4/gVS)**	**BD_{exp} (%)**
C. vulgaris	539	176	33
PPW_{dp}	521	439	84
PPW_p	607	348	57
+ 25% PPW_{dp}	541	227	42
+ 50% PPW_{dp}	521	268	51
+ 75% PPW_{dp}	582	330	57
+ 25% PPW_p	532	201	38
+ 50% PPW_p	445	225	51
+ 75% PPW_p	411	246	60

A.3. Additional experimental data of *T. lutea* co-digestion with PPW in batch and semi-continous studies

Figure A.2 shows the cumulative methane produced by mono-digestion of *T. lutea*, and co-digestion with PPW_{dp} (Figure A.2a) or PPW_p (Figure A.2B) for o.5 SIR. Tables A.1 and A.2 show the summary of experimental data for *T. lutea* co-digestion with PPW_{dp} or PPW_p.

The mixing ratios between *T. lutea* and PPW had a significant effect on methane yields ($F_{(2.011, 16)}=91.59$, $p<0.001$). High content of PPW relative to *T. lutea* led to enhanced methane yields, and co-digestion of *T. lutea* and PPW at 25/75 produced significantly more methane than the ratios of 100/0 ($p<0.001$) and 75/25 ($p=0.012$). However, the methane yields from of ratios of 50/50 and 25/75 were not significantly different ($p=0.301$). The C/N ratios in the mixtures of *T. lutea* with PPW_{dp} or PPW_p at a mixing ratio of 25/75 were 24.24/1 and 19.33/1, respectively, both within the optimal range for AD process.

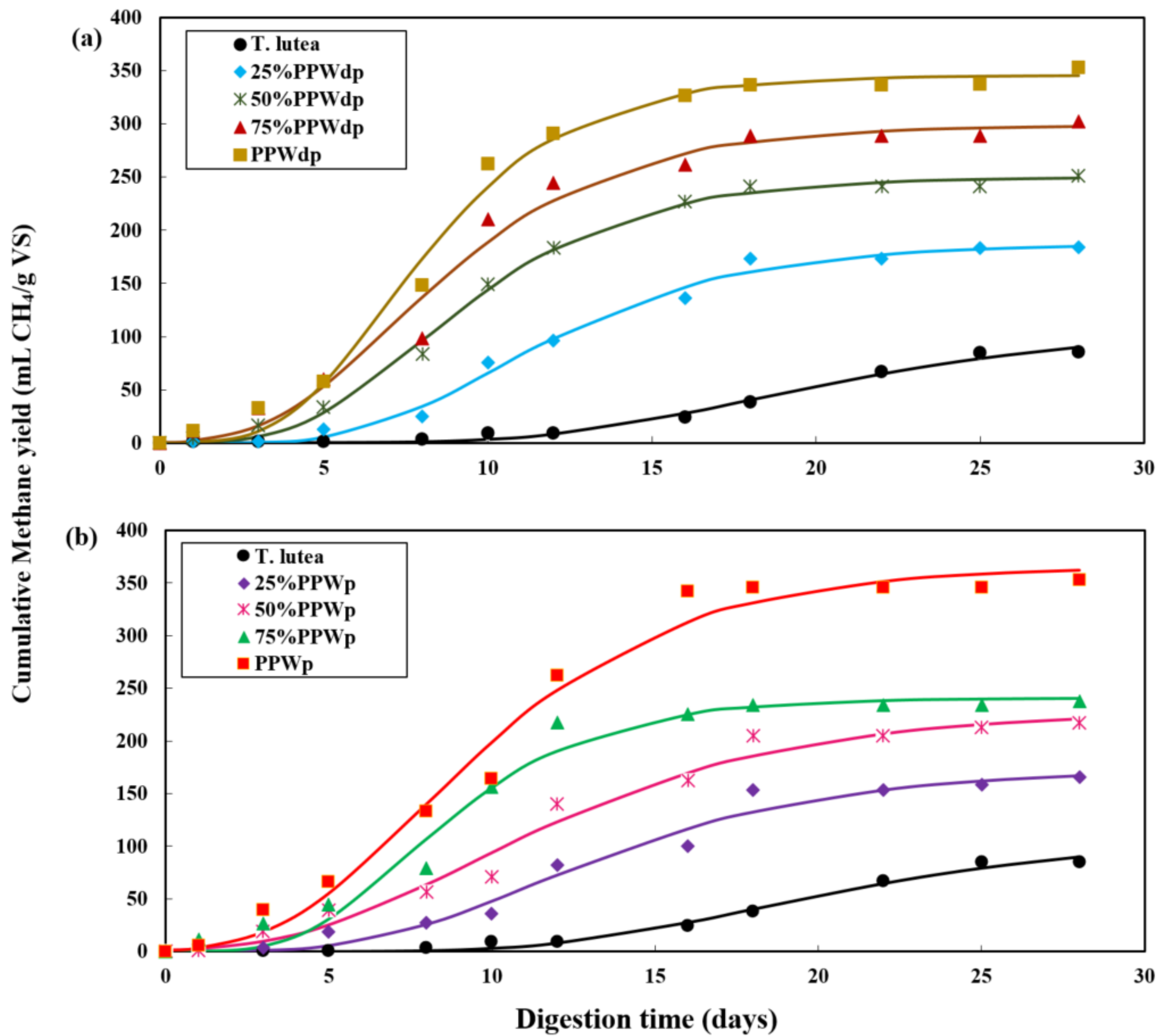

Figure A.2. Cumulative methane yield of *T. lutea* co-digested with PPW [a] potato discarded parts (PPW_{dp}) and [b] potato peel (PPW_p). The solid line represents the Gompertz model fit data.

Table A.3. Summary of C/N ratios and characterisation of digestate for *T. lutea* co-digestion with PPW_{dp} or PPW_p

	C/N	BMP_{exp} (mLCH₄/gVS)	pH	COD_S (g/L)	FAN (mg/L)
T. lutea	9.50	85 ± 10 [a]	7.79 ± 0.01	1.4 ± 0.1	80.7 ± 2.5
PPW_{dp}	40.78	353 ± 34	7.78 ± 0.04	1.7 ± 0.1	62.3 ± 18.8
PPW_p	28.59	353 ± 34	7.53 ± 0.00	1.5 ± 0.7	58.4 ± 1.8
+ 25% PPW_{dp}	13.60	184 ± 13	7.70 ± 0.01	1.5 ± 0.2	89.3 ± 1.4
+ 50% PPW_{dp}	17.85	251 ± 28	7.64 ± 0.09	1.3 ± 0.2	80.2 ± 0.4
+ 75% PPW_{dp}	24.24	302 ± 19	7.70 ± 0.14	1.4 ± 0.1	61.2 ± 0.4
+ 25% PPW_p	10.76	166 ± 30	7.78 ± 0.04	1.7 ± 0.1	97.6 ± 7.4
+ 50% PPW_p	17.08	217 ± 6	7.71 ± 0.03	1.4 ± 0.1	85.4 ± 4.3
+ 75% PPW_p	19.33	234 ± 26	7.73 ± 0.01	1.5 ± 0.1	88.8 ± 0.5

[a] Mean ± SD, n=3.

Table A.4. Summary of modified Gomerptz kinetics data for *T. lutea* co-digestion with PPW_{dp} or PPW_p

	Modified Gompertz Kinetics			
	P (ml CH_4 /gVS)	R_m (ml/gVS/d)	λ (d)	R^2
T. lutea	111.0	6.432	11.74	0.9902
PPW_{dp}	345.7	40.97	3.747	0.9919
PPW_p	366.2	30.43	3.382	0.9859
+ 25% PPW_{dp}	187.3	16.51	6.015	0.9920
+ 50% PPW_{dp}	250.0	25.13	4.167	0.9962
+ 75% PPW_{dp}	298.6	28.98	3.261	0.9825
+ 25% PPW_p	173.7	12.47	6.161	0.9788
+ 50% PPW_p	229.0	15.29	3.851	0.9807
+ 75% PPW_p	240.7	27.36	4.074	0.9792

Table A.5. Performance of digesters when co-digesting *T. lutea* and PPW during experimental period

	D1	D2	D3	D4
	Period III (day 26-64)	**Period III (day 26-64)**	**Period III (day 26-64)**	**Period III (day 26-64)**
OLR (g VS/L/d)	1.0	1.0	1.0	1.0
Process stability				
pH	7.04 ± 0.14[a]	6.88 ± 0.00	7.07 ± 0.04	7.05 ± 0.06
NH^{+}_{4}-N (g/L)	1.22 ± 0.04	1.15 ± 0.03	1.27 ± 0.03	1.26 ± 0.01
FAN (mg/L)	17.50 ± 5.81	11.19 ± 0.00	18.99 ± 1.51	18.06 ± 2.41
Digester effluent				
TS (g/L)	7.13 ± 0.59	7.63 ± 0.00	7.09 ± 0.19	7.07 ± 0.06
VS (g/L)	3.58 ± 0.80	4.15 ± 0.00	3.27 ± 0.36	3.23 ± 0.10
COD_t (g/L)	7.59 ± 0.02	8.36 ± 0.32	6.89 ± 0.73	6.33 ± 0.0.04
COD_s (g/L)	0.89 ± 0.17	0.75 ± 0.00	0.83 ± 0.10	0.77 ± 0.01
$VS_{removal}$ (%)	57 ± 10	58 ± 4	60 ± 4	61 ± 1
$COD_{removal}$ (%)	55 ± 3	50 ± 1	59 ± 2	62 ± 2
Methane production				
CH_4 (%)	62 ± 6	62 ± 7	66 ± 6	66 ± 7
Specific CH_4 yield (mL/g VS_{added})	332 ± 44	383 ± 46	320 ± 38	343 ± 40

[a] Mean ± SD, n=2

Appendix B. Statistical analysis of experimental data of Chapter 4

B.1. Enzymatic pre-treatment

EC stands for concentrations of enzymes, with 3 levels of 0, 0.5 and 1.0 %v/w; ET stands for enzymes type, with 5 levels: cellulase (C), protease (P), amylase (A), cellulase + protease (CP) and cellulase + amylase (CA).

Table B.1. Statistical analysis of solubilisation of COD after enzymatic pre-treatment

COD solubilisation	df.	F	p.
EC	1.104,30[a]	6500.00	<0.001***
ET	4,15	60.262	<0.001***
EC*ET	4.417,30	40.14	<0.001***

[a] "test of Sphericity" violated ($p<0.05$), and G-G Sphericity Epsilon estimates < 0.75, use Greenhouse-Geisser estimate;

*** The mean difference is significant at the 0.001 level

Table B.2. Statistical analysis of solubilisation of carbohydrates after enzymatic pre-treatment

Carbohydrates solubilisation	df.	F	p.
EC	1.099, 30 [a]	1400.00	<0.001***
ET	4, 15	3840.00	<0.001***
EC*ET	4.398, 30 [a]	252.14	<0.001***

[a] "test of Sphericity" violated ($p<0.05$), and G-G Sphericity Epsilon estimates < 0.75, use Greenhouse-Geisser estimate;

*** The mean difference is significant at the 0.001 level

Table B.3. Statistical analysis of solubilisation of proteins after enzymatic pre-treatment

Proteins solubilisation	df.	F	p.
EC	1.323,30 [a]	4750.00	<0.001***
ET	4,15	657.00	<0.001***
EC*ET	5.293,30 [a]	106.39	<0.001***

[a] "test of Sphericity" violated (p<0.05), and G-G Sphericity Epsilon estimates < 0.75, use Greenhouse-Geisser estimate;

*** The mean difference is significant at the 0.001 level

Table B.4. Statistical analysis of cumulative methane yields of enzymatic pre-treatment

Cumulative methane yield	df.	F	p.
EC	2,50	647.79	<0.001***
ET	4,25	127.95	<0.001***
EC*ET	8,50	45.95	<0.001***

*** The mean difference is significant at the 0.001 level.

Table B.5. Statistical analysis of maximum methane production rate of enzymatic pre-treatment

R_m	df.	F	p.
EC	2,10	166.33	<0.001***
ET	4,5	11.37	0.010**
EC*ET	8,10	7.17	0.003**

** The mean difference is significant at the 0.01 level;

*** The mean difference is significant at the 0.001 level;

Table B.6. Statistical analysis of lag phase of enzymatic pre-treatment

λ	df.	F	p.
EC	2,10	2.56	0.127
ET	4,5	3.28	0.112
EC*ET	8,10	2.49	0.089

Table B.7. Statistical analysis of Pearson correlation tests of enzymatic pre-treatment

	Methane yields
Solubilisation of COD (%)	r (22)=0.755, p<0.001***
Solubilisation of carbohydrates (%)	r (22)=0.787, p<0.001***
Solubilisation of proteins (%)	r (26)=0.733, p<0.001***

B.2 Thermo-Alkaline pre-treatment

CD stands for $Ca(OH)_2$ dosage at 3 levels of 0,10 and 15% w/w. PT stands for pre-treatment time with 2 levels of 24 and 48 hours, and PE stands for pre-treatment temperature with 2 levels of 37 and 55 °C. 00 level means untreated *C. vulgaris*.

Table B.8. Statistical analysis of solubilisation of COD after thermo-alkaline pre-treatment

COD solubilisation	df.	F	p.
CD	3,12	2838.00	<0.001***
PT	1,4	0.00	0.983
PE	1,4	10.99	0.030*
CD*PT	3,12	24.37	<0.001***
CD*PE	3,12	4.65	0.022*
PE*PT	1,4	2.11	0.220
CD*PE*PT	3,12	1.45	0.276

* The mean difference is significant at the 0.05 level;

*** The mean difference is significant at the 0.001 level;

Table B.9. Statistical analysis of solubilisation of carbohydrates after thermo-alkaline pre-treatment

Carbohydrates solubilisation	df.	F	p.
CD	3,12	955.08	<0.001***
PT	1,4	20.60	0.011*
PE	1,4	42.26	0.003**
CD*PT	3,12	2.48	0.111
CD*PE	3,12	5.72	0.011*
PE*PT	1,4	0.26	0.639
CD*PE*PT	3,12	6.83	0.006**

* The mean difference is significant at the 0.05 level;

** The mean difference is significant at the 0.01 level;

*** The mean difference is significant at the 0.001 level.

Table B.10. Statistical analysis of solubilisation of proteins after thermo-alkaline pre-treatment

Proteins solubilisation	df.	F	p.
CD	3,12	1452.42	<0.001***
PT	1,4	29.28	0.006**
PE	1,4	34.15	0.004**
CD*PT	3,12	24.63	<0.001***
CD*PE	3,12	11.91	0.001***
PE*PT	1,4	10.86	0.030*
CD*PE*PT	3,12	8.33	0.003**

* The mean difference is significant at the 0.05 level;

** The mean difference is significant at the 0.01 level;

*** The mean difference is significant at the 0.001 level.

Table B.11. Statistical analysis of cumulative methane yields of thermo-alkaline pre-treatment

Cumulative methane yield	df.	F	p.
CD	3,12	11.34	0.001***
PT	1,4	0.56	0.495
PE	1,4	9.79	0.035*
CD*PT	3,12	0.61	0.619
CD*PE	3,12	2.00	0.167
PE*PT	1,4	0.00	0.908
CD*PE*PT	3,12	2.30	0.130

* The mean difference is significant at the 0.05 level;

*** The mean difference is significant at the 0.001 level.

Table B.12. Statistical analysis of maximum methane production of thermo-alkaline pre-treatment

R_m	df.	F	p.
CD	3,12	3.31	0.057
PT	1,4	0.17	0.698
PE	1,4	9.90	0.035*
CD*PT	3,12	1.62	0.237
CD*PE	3,12	9.68	0.002**
PE*PT	1,4	0.28	0.625
CD*PE*PT	3,12	2.66	0.096

* The mean difference is significant at the 0.05 level;

** The mean difference is significant at the 0.01 level.

Table B.13. Statistical analysis of lag phase of thermo-alkaline pre-treatment

λ	df.	F	p.
CD	3,12	57.071	$<0.001^{***}$
PT	1,4	0.919	0.392
PE	1,4	159.151	$<0.001^{***}$
CD*PT	3,12	0.498	0.691
CD*PE	3,12	51.620	$<0.001^{***}$
PE*PT	1,4	0.784	0.426
CD*PE*PT	3,12	2.083	0.156

*** The mean difference is significant at the 0.001 level.

Table B.14. Statistical analysis of Pearson correlation tests of thermo-alkaline pre-treatment

	Methane yields
Solubilisation of COD (%)	r (26)=0.476, p=0.014*
Solubilisation of carbohydrates (%)	r (26)=0.457, p=0.019*
Solubilisation of proteins (%)	r (26)=0.489, p=0.011*

* The mean difference is significant at the 0.05 level.

Appendix C: Statistical analysis of experimental data of Chapter 5.

CPPW stands for the mixing ratios between *C. vulgaris* and PPW, with 5 levels of 100:0, 75:25, 50:50, 25:75 and 0:100. TPPW stands for PPW type, with 2 levels of PPW_{dp} and PPW_{p}. SIR is the substrate to inoculum ratio also with 2 levels of 0.5 and 1.0.

Table C.1. Statistical analysis of cumulative methane yields of co-digestion BMP tests

Cumulative methane yield	df.	F	p.
CPPW	4,32	100.68	$<0.001^{***}$
TPPW	1,8	52.94	$<0.001^{***}$
SIR	1,8	54.82	$<0.001^{***}$
CPPW*TPPW	4,32	9.51	$<0.001^{***}$
CPPW*SIR	4,32	8.72	$<0.001^{***}$
TPPW*SIR	1,8	2.03	0.193
CPPW*TPPW*SIR	4,32	1.10	0.373

*** The mean difference is significant at the 0.001 level.

Table C.2. Statistical analysis of maximum methane production of co-digestion BMP tests

R_m	df.	F	p.
CPPW	1.786,32[a]	18.52	$<0.001^{***}$
TPPW	1,8	17.89	0.003^{**}
SIR	1,8	58.28	$<0.001^{***}$
CPPW×TPPW	1.786,32	3.93	0.047^{*}
CPPW×SIR	1.786,32	1.53	0.249
TPPW×SIR	1,8	3.16	0.113
CPPW×TPPW×SIR	1.786,32	3.79	0.052

[a] "test of Sphericity" violated (p<0.05), and G-G Sphericity Epsilon estimates < 0.75, use Greenhouse-Geisser estimate

* The mean difference is significant at the 0.05 level;

** The mean difference is significant at the 0.01 level;

*** The mean difference is significant at the 0.001 level.

Table C.3. Statistical analysis of lag phase of co-digestion BMP tests

λ	df.	F	p.
CPPW	4,32	0.54	0.711
TPPW	1,8	1.76	0.221
SIR	1,8	177.59	<0.001***
CPPW×TPPW	4,32	2.77	0.044*
CPPW×SIR	4,32	5.46	0.002**
TPPW×SIR	1,8	0.29	0.603
CPPW×TPPW×SIR	4,32	3.58	0.016*

* The mean difference is significant at the 0.05 level;

** The mean difference is significant at the 0.01 level;

*** The mean difference is significant at the 0.001 level.

Table C.4. Statistical analysis of concentrations of COD_s at the end of co-digestion

COD_s	df.	F	p.
MPPW	4,16	38.26	<0.001***
TPPW	1,4	122.51	<0.001***
SIR	1,4	1473.68	<0.001***
MPPW×TPPW	4,16	39.99	<0.001***
CPPW×SIR	4,16	41.90	<0.001***
TPPW×SIR	1,4	98.95	0.001***
CPPW×TPPW×SIR	4,16	33.50	<0.001***

*** The mean difference is significant at the 0.001 level.

Table C.5. Statistical analysis of concentrations of FAN at the end of co-digestion

FAN	df.	F	p.
CPPW	4,16	20.70	<0.001***
TPPW	1,4	13.86	0.020*
SIR	1,4	626.42	<0.001***
CPPW×TPPW	4,16	1.996	0.144
CPPW×SIR	4,16	15.44	<0.001***
TPPW×SIR	1,4	0.03	0.862
CPPW×TPPW×SIR	4,16	1.64	0.212

* The mean difference is significant at the 0.05 level;
*** The mean difference is significant at the 0.001 level.

Appendix D. Statistical analysis of experimental data of Chapter 6

STM stands for start-up methods, and TPPW stands for type of potato processing waste.

D.1 Statistical analysis of experimental data on days 6, 15 and 27

Table D.1 Statistical analysis of concentrations of FAN on day 6

	df.	**F**	**p**
STM	1,4	508.20	<0.001***
TPPW	1,4	9.26	0.038*
STM*TPPW	1,4	2.14	0.218

* The mean difference is significant at the 0.05 level;

*** The mean difference is significant at the 0.001 level.

Table D.2 Statistical analysis of concentrations of total VFA on day 15

	df.	**F**	**p**
STM	1,12	24.13	<0.001***
TPPW	1,12	76.63	<0.001***
STM*TPPW	1,12	7.72	0.017*

* The mean difference is significant at the 0.05 level;

*** The mean difference is significant at the 0.001 level.

Table D.3. Statistical analysis of concentrations of acetic acids on day 15

	df.	**F**	**p**
STM	1,12	11.18	0.006**
TPPW	1,12	73.47	<0.001***
STM*TPPW	1,12	13.85	0.003**

** The mean difference is significant at the 0.01 level;

*** The mean difference is significant at the 0.001 level.

Table D.4. Statistical analysis of concentrations of propionate acids on day 15

	df.	F	p
STM	1,12	127.08	<0.001***
TPPW	1,12	18.63	0.001***
STM*TPPW	1,12	8.63	0.012*

* The mean difference is significant at the 0.05 level;

*** The mean difference is significant at the 0.001 level.

Table D.5. Statistical analysis of degradation efficiency of total VFAs from days 15 to 27

	df.	F	p
STM	1,12	5.57	0.036*
TPPW	1,12	0.17	0.691
STM*TPPW	1,12	5.08	0.044*

* The mean difference is significant at the 0.05 level.

Table D.6. Statistical analysis of concentrations of propionate acids on day 27

	df.	F	p
STM	1,12	5.74	0.034*
TPPW	1,12	3.83	0.074
STM*TPPW	1,12	1.23	0.290

* The mean difference is significant at the 0.05 level.

D.2 Statistical analysis of experimental data of biogas and methane yields during Period III

Table D.7. Statistical analysis of biogas production during Period III

	df.	F	p
STM	1,148	50.27	<0.001***
TPPW	1,148	68.60	<0.001***
STM*TPPW	1,148	9.30	0.003**

** The mean difference is significant at the 0.01 level;

*** The mean difference is significant at the 0.001 level.

Table D.8. Statistical analysis of specific methane yields during Period III

	df.	F	p
STM	1,148	23.13	<0.001***
TPPW	1,148	11.46	0.001***
STM*TPPW	1,148	3.17	0.077

*** The mean difference is significant at the 0.001 level.

D.3. Statistical analysis of relative abundance of archaea during the co-digestion process

Table D.9. Statistical analysis of relative abundance of *Methanosarcina* on day 27

	df.	F	p
STM	1,12	504.86	<0.001***
TPPW	1,12	1.09	0.318
STM*TPPW	1,12	7.13	0.020*

* The mean difference is significant at the 0.05 level;

*** The mean difference is significant at the 0.001 level.

Table D.10. Statistical analysis of relative abundance of *Methanosaeta* on day 27

	df.	F	p
STM	1,12	174.69	<0.001***
TPPW	1,12	7.82	0.016*
STM*TPPW	1,12	25.55	<0.001***

* The mean difference is significant at the 0.05 level;

*** The mean difference is significant at the 0.001 level.

Table D.11. Statistical analysis of relative abundance of *Methanosarcina* on day 46

	df.	F	p
STM	1,12	15.22	0.002**
TPPW	1,12	47.55	<0.001***
STM*TPPW	1,12	3.21	0.098

** The mean difference is significant at the 0.01 level;

*** The mean difference is significant at the 0.001 level.

Table D.12. Statistical analysis of relative abundance of *Methanosaeta* on day 46

	df.	F	p
STM	1,12	7.08	0.021*
TPPW	1,12	30.51	<0.001***
STM*TPPW	1,12	6.29	0.027*

* The mean difference is significant at the 0.05 level;

*** The mean difference is significant at the 0.001 level.

Table D.13. Statistical analysis of relative abundance of *Methanosarcina* on day 64

	df.	F	p
STM	1,12	59.00	<0.001***
TPPW	1,12	50.59	<0.001***
STM*TPPW	1,12	26.94	<0.001***

*** The mean difference is significant at the 0.001 level.

Table D.14. Statistical analysis of relative abundance of *Methanosaeta* on day 64

	df.	F	p
STM	1,12	45.08	<0.001***
TPPW	1,12	32.38	<0.001***
STM*TPPW	1,12	42.58	<0.001***

*** The mean difference is significant at the 0.001 level.

Appendix E. Statistical analysis of experimental data of Chapter 7

E.1 Statistical analysis of experimental data during the co-digestion process

Table E.1. Statistical analysis of the values of TS and VS between 0 and 2% v/v glycerol dosage at the end of Period III

	df.	t	p.
TS	6	-5.588	0.001***
VS	6	-7.799	<0.001***

*** The mean difference is significant at the 0.001 level.

Table E.2. Statistical analysis of the values of TS and VS between 0 and 1% v/v glycerol dosage at the end of Period IV and Period VI

	df.	t	p.
TS	6	-1.311	0.238
VS	6	-3.243	0.018*

* The mean difference is significant at the 0.05 level.

Table E.3. Statistical analysis of the volumetric and specific methane yields when microalgae co-digesting with PPW_{dp} with or without glycerol

	df.	F	p.
Volumetric methane yields	2,61	319.67	<0.001***
Specific methane yields	2,61	213.67	<0.001***

*** The mean difference is significant at the 0.001 level.

Table E.4. Statistical analysis of the volumetric and specific methane yields when microalgae co-digesting with PPW_p with or without glycerol

	df.	F	p.
Volumetric methane yields	2,65	916.411	<0.001***
Specific methane yields	2,65	207.720	<0.001***

*** The mean difference is significant at the 0.001 level.

Table E.5. Spearman correlation between relative abundance of methanogenic archaea and operating parameters [1]

	CH_4 yields	TS	VS	COD_t	COD_s
Methanosaeta	0.390	$r_s(34)=-0.416$ 0.015^{*}	$r_s(34)=-0.431$ 0.011^{*}	$r_s(34)=-0.360$ 0.036^{*}	$r_s(34)=-0.407$ 0.017^{*}
Candidatus Methanofastidiosum	0.831	$r_s(34)=0.563$ 0.001^{***}	$r_s(34)=0.539$ 0.001^{***}	$r_s(34)=0.465$ 0.006^{**}	$r_s(34)=0.504$ 0.002^{**}
Methanobrevibacter	$r_s(34)=-0.392$ 0.022^{*}	$r_s(34)=0.662$ $<0.001^{***}$	$r_s(34)=0.650$ $<0.001^{***}$	$r_s(34)=0.531$ 0.001^{***}	$r_s(34)=0.621$ $<0.001^{***}$
Methanobacterium	0.242	$r_s(34)=-0.753$ $<0.001^{***}$	$r_s(34)=-0.719$ $<0.001^{***}$	$r_s(34)=-0.623$ $<0.001^{***}$	$r_s(34)=-0.700$ $<0.001^{***}$
Methanoculleus	0.145	0.590	0.737	0.390	0.744
Methanosarcina	$r_s(34)=-0.487$ 0.004^{**}	0.644	0.302	0.903	0.418
RumEn M2	0.362	$r_s(34)=-0.627$ $<0.001^{***}$	$r_s(34)=-0.558$ 0.001^{***}	$r_s(34)=-0.498$ 0.003^{**}	$r_s(34)=-0.547$ 0.001^{***}

* The mean difference is significant at the 0.05 level;

** The mean difference is significant at the 0.01 level;

*** The mean difference is significant at the 0.001 level.

Table E.6. Spearman correlation between relative abundance of methanogenic archaea and operating parameters [2]

	FAN	pH	VFA	TA	VFA/TA
Methanosaeta	0.196	0.246	$r_s(34)=-0.395$ 0.021*	0.116	0.377
Candidatus Methanofastidiosum	$r_s(34)=0.350$ 0.042*	0.070	$r_s(34)=0.573$ <0.001***	$r_s(34)=0.428$ 0.012*	0.081
Methanobrevibacter	$r_s(34)=0.581$ <0.001***	$r_s(34)=0.562$ 0.001***	$r_s(34)=0.383$ 0.025*	$r_s(34)=0.649$ <0.001***	0.557
Methanobacterium	$r_s(34)=-0.622$ <0.001***	$r_s(34)=-0.595$ <0.001***	$r_s(34)=-0.505$ 0.002**	$r_s(34)=-0.673$ <0.001***	0.942
Methanoculleus	0.537	0.473	0.087	0.683	0.148
Methanosarcina	0.765	0.690	0.502	0.746	0.389
RumEn M2	$r_s(34)=-0.407$ 0.017*	$r_s(34)=-0.389$ 0.023*	$r_s(34)=-0.522$ 0.002**	$r_s(34)=-0.514$ 0.002**	0.251

* The mean difference is significant at the 0.05 level;

** The mean difference is significant at the 0.01 level;

*** The mean difference is significant at the 0.001 level.