

Potential of Crassulacean Acid Metabolism (CAM) Plants as Biomass for Bioenergy and Biorefinery



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Bioenergy crops have been exploited in the past decades as a mean to combat climate change and address energy security issues (IEA, 2014). However, the food vs fuel dilemma of employing energy crops has prompted scientists to search for alternative sustainable biomass options. Recently, Crassulacean Acid Metabolism (CAM) plants such as agave and prickly pear have gained research interest as alternative energy crops. Having evolved a specialised photosynthesis mode, CAM plants possess key features that enable them to thrive in arid and semi-arid regions where water is scarce and soil quality is poor. Stimulated by this interest, this study initially focused on evaluating the potential for methane production from CAM plants via anaerobic digestion and subsequently as a means of generating high-value products such as bioplastics.

With over 16,000 CAM species from which to select, the first study screened a range of CAM plants, which were representative of 5 genera (*Agave*, *Ananas*, *Euphorbia*, *Kalanchoe*, and *Opuntia*) that encompass a variety of features typical of CAM plants. The biochemical methane potential (BMP) assay was employed to determine methane yields from the selected CAM plants. The BMP test was carried out using 250 ml glass bottles with 150 ml working volume and operating conditions of 39.0 ± 1.0 °C with constant agitation of 120 rpm. The results demonstrated that the BMPs of the CAM plants investigated ranged between 281 and 382 L_{CH₄} kgVS⁻¹, the highest of which was obtained for *Agave angustifolia*. The methane yields from the CAM plants tested were equivalent to that of maize, suggesting that they could have the potential as AD feedstocks and be highly attractive as a substrate in the biorefinery industry. Based on the results obtained from the screening experiment, *Euphorbia tirucalli* and *Opuntia ficus-indica* were selected as model CAM crops for further study.

Euphorbia tirucalli was selected as it is also known as the petroleum plant with hydrocarbon components detected in their toxic latex resembling crude oil. Further, the effect of the inoculum to substrate (I/S) ratio, operating temperature, pH, and mixing (E:M) ratio via co-digestion on methane yields from *E. tirucalli* was investigated. The results demonstrated that the highest methane yields of *E. tirucalli* obtained in mesophilic and thermophilic conditions were 206 and 266 L_{CH₄} kgVS⁻¹, respectively, and an improvement of methane yields by 19–29% was observed under thermophilic conditions (55.0 ± 1.0 °C). The influence of the initial pH was observed but the results were not consistent. However, based on the energy balance assessment, mesophilic temperature (38.0 ± 1.0 °C) and pH 7.0 were found

to provide optimal conditions for methane production. Methane yields obtained from the co-digestion of *E. tirucalli* and chicken manure were similar to those achieved in mono-digestion, regardless of the mixing (E:M) ratio.

The second CAM crop selected, *Opuntia ficus-indica*, has previously been reported to have low lignin content but be rich in soluble sugars, which are desirable as an AD feedstock. A comprehensive study of *O. ficus-indica* was conducted using the BMP assay, to examine the influence of different factors such as substrate processing technique and plant age on methane production. The cladodes of *O. ficus-indica* were processed into 3 distinctive substrate types (ground cladode, liquid extract, and solid peel), which were demonstrated to produce the specific methane yields of 241, 222 and 271 L kgVS⁻¹, respectively. Based on these findings, the processed *O. ficus-indica* cladode could be employed as an AD feedstock without the need for further processing. The liquid extract, which produced the highest methane yield, also had potential as a substrate for the production of higher-value biochemical products. A significant decrease in methane yields was detected in cladodes aged 3 years and older ($p < 0.001$), and this was attributed to their higher lignin composition. The results of the 90-day semi-continuous digestion of *O. ficus-indica* study revealed that the highest achievable organic loading rate (OLR) was 5.52 gVS L_R⁻¹ day⁻¹, and the specific methane yield obtained at this OLR was 269 L kgVS⁻¹ day⁻¹.

The concept of biorefinery was applied to this study. *O. ficus-indica* was selected as an example of biomass that could be systematically exploited firstly to a higher-added value product and subsequently to generate methane. The liquid extracted from *O. ficus-indica* (OLE) was found to be rich in sugars and organic acids. The liquid could potentially be directly exploited as a substrate for bio-fermentation, bypassing the conventional route of biomass conversion to volatile fatty acids (VFAs) via anaerobic digestion. Subsequently, proof-of-concept research was conducted using OLE as a substrate for polyhydroxyalkanoate (PHA) production employing the bacterium *Cupriavidus necator* H16. GC-MS metabolite analysis confirmed that the main compounds assimilated by this strain for their growth and PHA synthesis were fructose, citric/isocitric acid, and malic acid. *C. necator* H16 was able to produce polyhydroxybutyrate (PHB) of up to 2.12 g L⁻¹, accounting for 45.5% of the cell dry weight. This study demonstrated for the first time that the synthesis of polyhydroxybutyrate (PHB) by *C. necator* using *O. ficus-indica* can be achieved.

Keywords: anaerobic digestion, CAM plants, *Opuntia*, *Euphorbia*, energy crop, biogas, biorefinery, PHA

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Nomenclatures

AD	Anaerobic Digestion
ADF	Acid Detergent Fibre
ADL	Acid Detergent Lignin
BMP	Biochemical Methane Potential
CAM	Crassulacean Acid Metabolism
C/N	Carbon Nitrogen Ratio
COD	Chemical Oxygen Demand
CSTR	Continuously Stirred Tank Reactor
DI	Deionised
DM	Dry Matter
FM	Fresh Matter
FT-IR	Fourier-transform Infrared
GC	Gas Chromatography
GC-MS	Gas Chromatography – Mass Spectrometry
HRT	Hydraulic Retention Time
LCFA	Long Chain Fatty Acid
L _R	Litre (unit of a reactor volume)
NDF	Neutral Detergent Fibre
OLR	Organic Loading Rate
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
SBY	Specific Biogas Yield
SMY	Specific Methane Yield
SRT	Solid Retention Time
STP	Standard Temperature and Pressure
VFA	Volatile Fatty Acid
TKN	Total Kjeldahl Nitrogen
TS	Total Solid
TVFA	Total Volatile Fatty Acid
VBP	Volumetric biogas production
VFA	Volatile Fatty Acid
VS	Volatile Solid

Chapter 1

Introduction

1.1 Background to research

Bioenergy is a form of renewable energy derived from biomass such as agricultural wastes and energy crops. In the past decade, bioenergy has been employed as one of the strategies to mitigate climate change and increase energy security (IEA, 2014). Energy crops are specifically grown for bioenergy but there is a growing concern over competition for water and land resources between food and fuel crops. This ‘food vs fuel’ dilemma will become progressively intense over the next few decades due to the increasing demand for food in response to the rapidly growing global population. The use of energy crops will eventually become a less desirable option, which stimulates the research community to investigate alternative crops (Hess et al., 2009; Sawatdeenarunat et al., 2015).

Crassulacean Acid Metabolism (CAM) plants have recently attracted attention as alternative energy crops (Borland et al., 2009; Cushman et al., 2015; Mason et al., 2015; Yan et al., 2011). Their specialised mode of photosynthesis helps CAM plants reduce transpirational water loss by closing their stomata during the daytime and opening them during the cooler, more humid nighttime for carbon dioxide uptake. CAM plants are therefore able to thrive in semi-arid regions where water supplies are erratic and soil nutrients are limited. Another key characteristic of CAM plants such as agave, aloe and prickly pear is that they have low lignin content due to them being succulents, which possess a high water-storage capacitance associated with the swelling and shrinking of their leaves and stems, storing water during the rainy season and utilising this internal water reserve during the dry season. CAM

plants also have low lignin content so they could potentially offer an advantage in terms of substrate hydrolysis over conventional lignocellulosic biomass. However, the few studies to date that have focused on the bioethanol or biogas production from CAM plants have investigated just two CAM genera, *Agave* and *Opuntia*, from the families Asparagaceae and Cactaceae. *Opuntia ficus-indica* and *Agave sisalana* are amongst a few examples of CAM crops that have been exploited for biogas production via anaerobic digestion (Calabrò et al., 2018; Ramírez-Arpide et al., 2018).

Anaerobic digestion (AD) is one of the technologies that have a positive net energy production rate and can provide clean, reliable and renewable energy. The AD process is the biotransformation of organic matters in the absence of oxygen by a group of microorganisms to produce biogas, which can then be used as a fuel. AD of biomass such as energy crops and lignocellulosic agricultural residues has been commonly practised over the past decades. CAM plants have been viewed as a viable option as AD feedstocks but few studies have been carried out on the anaerobic digestion of CAM plants. Several unknown factors such as cultivation, characteristics, and digestibility of CAM crops need to be further investigated in order to assess whether they could be used as promising AD substrates, particularly at an industrial scale.

Anaerobic digestion has been a matured technology for biomass utilisation for decades, offering several benefits from producing renewable energy in the form of biogas to improving the living quality of the community around the biogas digesters. However, the added value of biogas as an AD product has been viewed as limited and an alternative biomass valorisation approach to anaerobic digestion should also be further explored. The principle of “Cascading use of biomass” has been proposed

to maximise the efficiency of resource use whilst reducing the emission of greenhouse gases (Keegan et al., 2013). This includes anaerobic bioconversion for the generation of high-value products and bioenergy and is also referred to as the concept of biorefinery. The strategy was to prioritise the use of biomass for high-value products prior to exploiting it for energy production. There are various platform chemicals that can be produced from lignocellulosic biomass in biorefinery processes such as C5 and C6 sugars, ethanol, furfural, glycerol, succinic and lactic acids, sorbitol, and Polyhydroxyalkanoates (PHAs) (Isikgor and Becer, 2015; Takkellapati et al., 2018). In recent years, PHAs have been viewed as one of the solutions to address the plastics accumulation issue and improve plastic waste management. PHAs are non-petrochemical based plastics, 100% biodegradable, have good processability, and possess similar thermo-chemical characteristics to those of polypropylene (PP) (Isikgor and Becer, 2015). PHA is therefore another promising product worth exploring. Since the biorefinery concept, particularly the production of PHAs, has never been applied to Crassulacean Acid Metabolism (CAM) plants to the best of the author's knowledge, further investigation on the potential use of CAM plants for other applications apart from AD should be explored.

1.2 Overall aim and objectives

Therefore, the overall aim of this research was to explore the potential to generate bioenergy and biorefinery products via anaerobic digestion and other microbial fermentation technologies using Crassulacean acid metabolism (CAM) plants as substrates. Strategies to achieve this aim included using a biochemical methane potential (BMP) assay, which has been widely employed by researchers to determine the methane potential of new feedstock materials. Parameters that

influence the anaerobic digestion of CAM plants such as inoculum to substrate (I/S) ratio, operating temperature, pH, co-substrate mixing ratio were also investigated. A semi-continuous digestion study of a selected CAM biomass was also undertaken to assess its suitability to be applied at a larger scale. In addition, the selected biomass was tested for bioplastic production and the economic feasibility of the project was also evaluated. The specific objectives of this study were to:

- Screen and identify suitable CAM families and species as viable AD feedstock based on their methane potentials.
- Investigate the influence of different operating conditions on the specific methane yields (SMYs) of the selected CAM plants (*Euphorbia tirucalli* and *Opuntia ficus-indica*).
- Assess other parameters affecting the biomass characteristics such as processing technique and harvesting age.
- Evaluate the performance of a semi-continuous digester using *Opuntia ficus-indica* as a substrate.
- Establish the fermentation process for PHA bioplastics production using *Opuntia ficus-indica* as a raw material.

1.3 Thesis outline

This thesis is divided into six chapters. Chapter 1 begins with a brief introduction describing the rationale behind the use of CAM plants as feedstocks for the production of bioenergy and biorefinery products. Chapter 2 presents the literature review of CAM plants, the anaerobic digestion process and microbial fermentation for biopolymer production. Chapter 3 describes the materials and methods

employed in the thesis. Chapter 4 presents the results and discussion for the anaerobic digestion studies, which include 1) the biochemical methane potential study of five different CAM species and maize, 2) the investigation of the effect of inoculum to substrate (I/S) ratio, pH, operating temperature and co-digestion mixture on the SMY of *Euphorbia tirucalli*, and 3) the batch and semi-continuous studies of *Opuntia ficus-indica*. Chapter 5 details the results and discussion of the study on the potential of *Opuntia ficus-indica* as a raw material for biopolymer production via microbial fermentation. A summary of the key findings, conclusions, experimental issues, and suggestions for future work is reported in Chapter 6.

Chapter 2

Literature Review

2.1 What is Crassulacean Acid Metabolism (CAM)?

Crassulacean Acid Metabolism or CAM is a specialised mode of photosynthesis, which has been developed from the C₃ mode (Winter and Smith, 1996a). This elaboration features nocturnal CO₂ uptake, increased water use efficiency (WUE) and the ability to survive and thrive in a semi-arid or arid environment. Examples of commonly known CAM plants are cactus, agave, orchid, pineapple and jade plant.

2.1.1 Potential of CAM plants as bioenergy crops

In recent years, a number of species such as *Ananas comosus* (pineapple), *Agave sisalana* (sisal), *Agave tequilana* (blue agave) and *Opuntia ficus-indica* (prickly pear) have attracted increasing attention as potential bioenergy crops (Borland et al., 2009; Mason et al., 2015; Yan et al., 2011). These four plants share several physiological attributes favourable for cultivation as a bioenergy crop but the most important one is their CAM photosynthesis. The CAM mode allows the plants to conduct their gas exchange (CO₂ and O₂) at night when the atmosphere is cooler and more humid compared to during the daytime. This condition, therefore, helps CAM plants to minimise their transpirational water loss and dramatically improve their water use efficiency (WUE).

2.1.2 Biochemistry of CAM plants

The CAM biochemical process can be described as a four-phase model of gas exchange that occurs both during the daytime and at night (Osmond, 1978). The metabolic steps involved in the CAM cycle are illustrated in **Fig. 2.1**.

Phase I is the nocturnal metabolism where the production of malic acid in the cytosol occurs. The process begins with the opening of the stomata which allows CO_2 (C1 compound) into the cytosol to be converted to bicarbonate (HCO_3^-). The bicarbonate ion is then captured by the enzyme phosphoenolpyruvate carboxylate (PEPC) along with phosphoenolpyruvate (PEP), which is the product of the glycolytic breakdown of starch in the chloroplast. The carboxylation of PEP and HCO_3^- produces oxaloacetate (OAA), a C4 compound, which is then converted to malate (C4 compound). Malate is transported to the cell vacuole and is stored in the form of malic acid.

Phase II occurs briefly before the start of the photoperiod when a surge of CO_2 uptake occurs. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO), which is generally known as the key enzyme for CO_2 fixation in photosynthesis, is activated and CO_2 can then be fixed by enzymes PEPC and RUBISCO.

Phase III is the diurnal metabolism where the decarboxylation of malic acid occurs. Malic acid is exported out of the vacuole into the cytosol. The decarboxylation process involves one or several enzymes, depending on the plant species, which converts malate (C4 compound) to pyruvate and PEP (C3 compounds). During the same period, a high concentration of CO_2 is generated and saturated in the intercellular space behind the closed stomata. This high internal pressure of CO_2 suppresses the photorespiration process, which typically competes with the photosynthesis process via the Calvin cycle.

Phase IV occurs when the level of internal CO_2 concentration drops, and the supply of malic acid is exhausted later in the photoperiod. During this process, the stomata may re-open to allow CO_2 to enter and the direct fixation of the atmospheric CO_2 via RUBISCO can proceed. The manifestation of the four phases of CAM

biochemical processes is highly plastic and dependent on environmental conditions, plant species and leaf development (Winter et al., 2008).

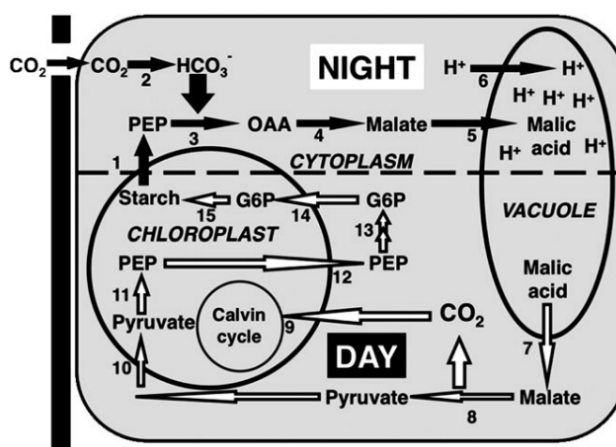


Fig. 2.1 The crassulacean acid metabolism (CAM) pathway in a mesophyll cell. Dark reactions which occur at night are indicated by filled arrows and light reactions during the daytime are indicated by open arrows. The leaf epidermis is represented by the black box on the left and the gap which permits the CO₂ in represents the stomatal pore. This diagram is adopted (Borland et al., 2009).

In comparison to the standard C₃ pathway found in barley, rice and wheat, CAM photosynthesis incurs a higher additional energy cost of up to 10% due to the transportation of malic acid from the cytosol into the vacuole and the storage of pyruvates produced during Phase III in the form of starch (Winter and Smith, 1996a). However, the suppression of photorespiration due to high internal CO₂ concentration can reduce the energy cost of net CO₂ fixation by at least 25%, and this cost in CAM species is significantly lower than that in C₃ crops.

2.1.3 Desirable characteristics of CAM plants as a bioenergy crop

CAM plants have distinctive growth attributes compared to C₃ and C₄ plants and these characteristics such as their drought tolerance and exceptionally efficient use of water render CAM plants as potential contenders for bioenergy crops that can be cultivated on marginal, semi-arid lands. The traits of agronomic relevance and potential benefits of CAM plants are summarised in **Table 2.1**.

Table 2.1 Growth characteristics of CAM plants desirable for the potential application as a bioenergy crop in marginal semi-arid areas. Based on (Borland et al., 2009) with modifications

Trait	Comments and examples
High water-use efficiency (WUE)	WUE is defined as the ratio of CO ₂ fixed (mmol) to H ₂ O lost (mol). The WUE values are 0.5–2.0 for typical C ₃ and C ₄ plants and 4.0–10.0 for CAM plants (Nobel, 1991). This implies that CAM plants have water consumption during the growth of up to 20 times less than typical energy crops. The use of plants with high WUE for bioenergy contributes to the more sustainable production of food, fodder and biomass (Gerbens-Leenes et al., 2009)
High potential growth rate	For <i>Opuntia ficus-indica</i> , the annual biomass productivity was predicted to be up to 50 tonnes of dry biomass per hectare (de Cortázar and Nobel, 1991). The recent data showed that the biomass productivity of <i>O. ficus-indica</i> from two sites with different agronomic management in Mexico was between 18.5 and 29.1 dry mass tonnes ha ⁻¹ year ⁻¹ (Ramírez-Arpide et al., 2018)
High contents of non-structural carbohydrates and sugar and low lignin content	Monocotyledons such as Bromeliaceae (pineapple) can have up to approximately 20% of non-structural carbohydrate content per total dry weight (Borland et al., 2009). The non-structural carbohydrates can be readily biodegraded by microorganisms with minimal pretreatment or hydrolysis. Some CAM plants such as <i>O. ficus-indica</i> consists of up to 95% water (Ginestra et al., 2009). This succulent maintains its rigidity through turgor pressure and therefore becomes less reliant on lignin for structural support. One of the

	latest studies on the biomass composition of <i>O. ficus-indica</i> reported an exceptionally low value of lignin content of 2.89%TS (Krümpel et al., 2020). The lignin contents of <i>Agave</i> spp. were between 4.9–15.3%TS (Li et al., 2012), relatively lower than those of maize stover, switchgrass and miscanthus, which were reported to be higher than 20%TS (Escamilla-Treviño, 2012)
Resilience to environmental extremes (drought, high temperature, UV and PPFD)	CAM plants have a high tolerance to both seasonal and unpredicted droughts, which are frequent in semi-arid areas. CAM succulents store water in parenchyma tissue which allows them to maintain water homeostasis during drought season (Owen et al., 2016). <i>Agave</i> spp., for example, adapts to drought season by hydraulic isolation. The plant roots shrink to minimise the contact with soil to reduce plant dehydration (Davis et al., 2011). CAM plants can endure up to 70°C whereas C₃ plants can tolerate up to 55°C (Borland et al., 2009). Cultivation of typical energy crops will likely be affected by increasing ambient temperatures and higher intensity of radiation due to climate change.
Tolerance to low-nutrient, degraded soil	CAM crops require minimal fertilisation and irrigation. The study on the rate of photosynthesis showed that <i>Agave</i> spp. and <i>Opuntia</i> spp. were able to achieve up to 97% of the total daily net CO₂ at night despite the plants being cultivated on semi-arid lands without the addition of water and nutrients (Nobel et al., 2002)

PPFD - Photosynthetic photon flux density

2.1.4 Examples of CAM species examined in this research

In this thesis, a variety of CAM plants were screened for their suitability and potential as AD substrates. The plants investigated were Caribbean agave (*Agave angustifolia*), pineapple (*Ananas comosus*), pencil cactus (*Euphorbia tirucalli*), Gifboom or poison tree (*Euphorbia virosa*), Mother of thousands (*Kalanchoe daigremontiana*), prickly pear (*Opuntia ficus-indica*), and brittle prickly pear (*Opuntia fragilis*). Two species were selected for further study after the initial screening experiment and therefore a brief description of these two plants is provided as follows.

2.1.4.1 *Euphorbia tirucalli*

2.1.4.1.1 Taxonomy, morphology, and distribution

Euphorbia tirucalli, also known as the pencil tree, is one of the 8,000 species under the family *Euphorbiaceae* (Mwine and Damme, 2011). The origin of *E. tirucalli* has been traced to East Africa, and it was believed that the plants were spread by Portuguese travellers to other parts of the world where the plants were introduced for their medicinal uses. The medicinal and pesticidal features of *E. tirucalli* could be attributed to the composition of the latex found in the plant. All parts of the *Euphorbia* plant, in particular the laticifers, contain white poisonous latex, the majority of which are resin-soluble substances and rich in hydrocarbon compounds such as sterols and triterpenes and has the properties resembling those of crude oil. (Calvin, 1980).

A shrub or a small tree of *E. tirucalli* can grow up to 12 m in height and the diameter of the full-grown trunk is between 15–20 cm (Mwine and Damme, 2011). The evergreen branches are about 7 mm in thickness and the shape of the branch

inspired the name ‘pencil tree’ (**Fig. 2.2**). *E. tirucalli* is propagated by cuttings, which root very easily. The plant can grow rapidly and produce a high yield of biomass despite growing on marginal soil under extreme drought environments. *E. tirucalli* also has parenchyma, like other succulents, for extra water storage. The photosynthesis system of *E. tirucalli* is unique as the stem uses CAM mode whereas the leaves have C₃ mode. The biomass composition of *E. tirucalli* from various studies is presented in **Table 2.2**. The cellulose content of *Euphorbia* (19.8–34.4%TS) is considerably higher compared to the contents of hemicellulose (4.5–12.1%TS) and lignin (4.1–7.9%TS).



Fig. 2.2 *E. tirucalli* plants from Morocco. Sourced from (Mwine and Damme, 2011)

2.1.4.1.2 Biomass productivity

The literature on the biomass productivity of *E. tirucalli* is quite limited with only two articles found reporting the biomass yields of this plant. The biomass yield of *E. tirucalli* from field research in Kenya was reported to be 20 dry tonnes per year at densities of over 80,000 plants per hectare (Declerck et al., 1985). Another study in Israel found that the yield was 2.2 dry tonnes per year with much lower densities of 500 plants per hectare compared to Declerck’s field study (Eshel et al., 2011).

Table 2.2 Biomass composition of *E. tirucalli* reported in the literature. The values are the fraction of dry biomass weight (%TS)

Biomass composition (%TS)					References
Cellulose	Hemicellulose	Lignin	Ash	Protein	
19.8	16.9	5.8	-	-	(Gebbru, 2014)
26.07	12.1	4.2	3.20	-	(Girmay, 2014)
30.1	-	6.6	13.3	8.8	(Gemed and Hassen, 2015)
28.1–34.4	4.5–6.6	4.1–6.1	12.1–13.6	4.6–7.6	(Krümpel et al., 2020)
32.3	10.9	7.9	4.1	-	(Dagne, 2020)

2.1.4.2 *Opuntia ficus-indica*

2.1.4.2.1 Taxonomy, morphology, and distribution

Opuntia ficus-indica is one of the 300 species under the genus *Opuntia*, and one of over 1,500 members of the *Cactaceae* family (Shedbalkar et al., 2010). It is one in 12 *Opuntia* spp. that their fruits and cladodes have been utilised for food and fodder production (FAO, 2013). The cladodes have several other applications such as medicinal ointment (Galati et al., 2003), as a dietary fibre supplement (Saenz and Sáenz, 1997) and as an additive in construction materials (Hernández-Zaragoza et al., 2008). Moreover, the tender stems or nopalitos of *O. ficus-indica* harbour some insects such as cochineal, which, despite being a pest, can be harvested for red colourant production.

Opuntia ficus-indica, commonly known as prickly pear, has long been domesticated as an important agricultural crop in semi-arid and arid regions. The genetic evidence revealed that *O. ficus-indica* originates from the central and southern parts of Mexico, where the domestication of this species began (Griffith, 2004). It is likely that *O. ficus-indica* was introduced from central Mexico to Cuba and other Caribbean islands, where the plant was first known to the European explorers (Donkin, 1977). The spread of *O. ficus-indica* through trade via European travellers

led to the introduction of this plant to the Mediterranean countries and subsequently other arid and semi-arid parts of Africa.

A mature *O. ficus-indica* plant can grow up to 2 m high and approximately 3 m wide (**Fig. 2.3**). The shape of the stem or cladode is oval or elliptical with an average weight of 1.0 kg with a length of up to 50 cm (Paiva et al., 2016). The cladodes have epicuticular wax covering the surface to minimise water loss through evaporation for maximum water storage. The thickness and chemical composition of the wax layer is varied among different cultivars of *O. ficus-indica* (Ben Salem-Fnayou et al., 2014). Despite most species from the *Opuntia* genus having thorns on the cladodes, *O. ficus-indica* is an exception, however, some cultivars still have spines on the cladode surface.



Fig. 2.3 *Opuntia ficus-indica* farm (Pepe Aromas) in Evora, Portugal

O. ficus-indica is a xerophyte like other CAM plants so their physiological characteristics allow them to grow under extreme conditions of arid and semi-arid regions (Paiva et al., 2016). *O. ficus-indica* and other succulents such as *Agave* spp. have a water storage tissue called parenchyma, which serves as a buffer system

against the water-deficit stress during the extended drought period (Goldstein et al., 1991; Owen and Griffiths, 2014).

Propagation of *O. ficus-indica* can be achieved via cladodes vegetatively or via seeds. Similar to other *Opuntia* species, the successful rate of germination of *O. ficus-indica* is very low as the seeds have a hard lignified integument (an outer protective layer), which prevents the protrusion of the radicle tip (Shedbalkar et al., 2010). Therefore, vegetative propagation is often preferred by using seedlings, which are the healthy cladodes from the central part of the adult plant (Paiva et al., 2016). The lignified cladodes at the stem base should be avoided as sprouting from these cladodes can be difficult.

The biomass characteristics of *O. ficus-indica* reported in the literature is presented in **Table 2.3**. The literature values of lignin content of *O. ficus-indica* were 1.8–13.8%TS, comparable to those of *E. tirucalli* (4.1–7.9%TS) and *Agave* spp. (7.4–16%TS) (Escamilla-Treviño, 2012). Unlike *E. tirucalli* with cellulose content, *Opuntia* cladodes consist of a higher proportion of hemicellulose compared to cellulose and lignin.

Table 2.3 Composition of *Opuntia ficus-indica* cladodes reported in the literature. The values are the fraction of dry biomass weight (%TS)

Biomass composition (%TS)					References
Cellulose	Hemicellulose	Lignin	Ash	Protein	
6.8	9.1	11.8	22.5	5.5	(Mciteka, 2008)
24.3	42	7.9	16.8	7.5	(Kuloyo, 2012)
13.1	18.5	12.3	23.7	7.4	(Yang et al., 2015)
-	-	6.8–13.8	7.8–10.7	-	(Santos et al., 2016)
12.8	1.6	2.8	-	-	(Valenti et al., 2018a)
7.7	8.6	8.3	-	-	(Calabrò et al., 2018)
8.4	33.1	-	-	-	(Myovela et al., 2019)
14.1–19.0	7.0–8.1	1.8–3.9	20.0–22.6	4.6–12.9	(Krümpel et al., 2020)

2.1.4.2.2 Biomass productivity

The estimated biomass productivity of *Opuntia ficus-indica* in Chile using the Environmental Productivity Index (EPI) could be over 30 dry tonnes ha⁻¹ year⁻¹ under optimal growth conditions (de Cortázar and Nobel, 1991). In other areas such as western South America, the yields could be up to 24 tonnes ha⁻¹ year⁻¹ whereas, in Africa, they could be lower at approximately 12–18 tonnes ha⁻¹ year⁻¹. One of the reviews on the potential of CAM crops for bioenergy reported the data from the unpublished trial suggesting that the biomass yields of *O. ficus-indica* in Laikipia, Kenya were estimated to be up to 40 dry tonnes ha⁻¹ year⁻¹ (Mason et al., 2015). However, these high biomass productivity values could be overestimated as there are several factors governing the plant growth rate such as climate, the application of irrigation and fertiliser, the field location and the plant varieties (FAO, 2013).

There are a few recent studies, which carried out field experiments of *O. ficus-indica* and reported actual biomass productivity data. The socio-economic assessment of *O. ficus-indica* as a potential crop for bioethanol and biogas production reported the average biomass productivity of three varieties of *O. ficus-indica* in North-eastern Brazil (Santos et al., 2016). The biomass yields were 7.3–8.4 dry tonnes ha⁻¹ year⁻¹, which were relatively low compared to the predicted values of 12–40 dry tonnes ha⁻¹ year⁻¹. However, the results also suggested that the yield difference among the three varieties was relatively small. More recent studies of *O. ficus-indica* production at a farm-scale in Mexico reported considerably higher figures of dry biomass productivity (Ramírez-Arpide et al., 2019, 2018). The fresh biomass yields were found to be between 264 and 417 tonnes ha⁻¹ year⁻¹ depending on the type of agronomic management. Based on the value of total solid (TS) content of 7% presented in the same literature, the dry biomass yields could be calculated to be

18.5–29.2 dry tonnes ha⁻¹ year⁻¹, which were the closest to the predicted value of 30 dry tonnes ha⁻¹ year⁻¹ mentioned earlier in this section.

2.1.5 Current perspectives on the biomass productivity of CAM plants

Climate change has resulted in a warmer and drier climate, which has a significant effect on the biomass productivity of several bioenergy crops in the world. Compared to other current biomass species, CAM plants have an adaptive photosynthetic system, which allows them to thrive in a hotter and drier environment, are more likely to survive. As mentioned in the previous section, CAM plants possess several benefiting traits such as high water use efficiency (WUE) and high tolerance to extreme environmental conditions. However, these characteristics are subjected to several factors.

One of the desirable characteristics of CAM plants such as *O. ficus-indica* is their high growth rate. Plant growth is governed by several parameters such as water and nutrient availability in soil, amount of sunlight and CO₂ availability. The productivity of *O. ficus-indica* of 50 tonnes of dry biomass per hectare was likely overpredicted (de Cortázar and Nobel, 1991). Although there have not been many field studies of CAM plants in the past, the available data showed that the crop productivity of *O. ficus-indica* was likely about between 7 and 40 tonnes of dry biomass per hectare, depending on the agronomic management and climate (Mason et al., 2015; Ramírez-Arpide et al., 2019, 2018; Santos et al., 2016). The latest field study on the effect of water input on the growth of *Opuntia* spp. showed that the number and the dry weight of cladodes increased linearly by 1.88 and 1.53-fold, respectively when the rainfall availability increased from 300 to 716 mm per year (Neupane et al., 2021). This means that high biomass productivity could be achieved given that there is sufficient irrigation. In the semi-arid or arid region where

precipitation level is lower than 300 mm per year, the productivity is expected to be lower, still, the chance that these CAM plants can survive compared to other conventional crops is very likely.

The prickly pear farm in Portugal (**Fig. 2.3**), which supplied the materials for use in the studies in this thesis, produces approximately 10–20 tonnes of fresh biomass per hectare per year, equivalent to 1–4 tonnes of dry biomass depending on the moisture content of the cladodes. Despite the benefit of higher precipitation levels offered by the Mediterranean climate in Portugal than those in the semi-arid or arid region, the biomass productivity is still much lower than the predicted number. More long-term field studies of CAM plants are therefore necessary to determine the effect of different irrigation, sunlight, soil condition, nutrient availability and cultivation strategies on the biomass yields of these plants.

Several CAM plants as mentioned in the earlier sections such as *Opuntia ficus-indica*, *Euphorbia tirucalli* and *Agave* spp. are perennial crops, which offer several ecological and economic benefits compared to annual bioenergy crops such as sugarcane and maize. For example, CAM perennial crops do not require annual cultivation and have a more extensive root system and better ground coverage, which helps prevent soil erosion and improve the soil quality on the degraded land (Zhang et al., 2011). Biomass from these CAM crops can be harvested by coppicing, but information on rotation length and number of coppices of CAM crops, which also determine the annual productivity of CAM plants, is very scarce. Thus, CAM plants, despite their several desirable characteristics, need further studies not only on their bioenergy potentials but also on the crop cultivation possibilities.

CAM plant cultivation for biomass is still in development and will take many years to establish optimal cultivating and harvesting strategies for each plant species.

Therefore, it is probable that the current biomass productivity of CAM crops obtained is still quite low. Other approaches to utilise CAM plants would need to be adopted. For example, in an anaerobic digestion study, a co-digestion strategy has been successfully implemented for several types of feedstocks to improve methane yields and the project's economic viability or to alleviate the issue arising from low biomass productivity from monoculture farming (Rabii et al., 2019). One co-substrate that has been extensively studied is animal manure (Dechrugsa et al., 2013; Huang et al., 2016; Moset et al., 2015a; Valenti et al., 2018b). Animal manure offers various benefits as a co-substrate. It can be easily sourced, provides a nutrient balance in the system, and enhances synergism. Thus, the co-digestion strategy is an attractive approach to study the potential use of CAM plants to mitigate the initial lack of information on crop cultivation and crop yield estimation.

2.1.6 Previous works on anaerobic digestion of CAM plants

There have been previous studies reporting that CAM plant biomass can be employed as an AD feedstock and the investigation of *Opuntia ficus-indica* first appeared in Chile in the early 1980s (Contreras and Toha, 1984). In this study, the batch fermentation experiment was carried out using cladodes of *Opuntia ficus-indica* fermented with an active digestate. Other researchers continued working on the AD of *O. ficus-indica*, however, it was often found that the authors did not provide sufficient information for further study on both experimental procedure and the gas yield (Uribe et al., 1992; Varnero et al., 1992). During the same period, the AD study of other CAM plants such as *Euphorbia tirucalli* and *Agave sisalana* were published (Oudshoorn, 1995; Rajasekaran et al., 1989; Sow et al., 1989). *E. tirucalli*, in particular, was discovered as potential bioenergy and a solution to combat the 1979 oil crisis (Calvin, 1980). The latex of *Euphorbia* contains hydrocarbon

materials, the physico-chemical properties of which are similar to petroleum. However, by the end of the year 2000, there were less than 10 publications, known to the author, on AD of these three CAM plants.

The summary of experimental results on AD of CAM plants focusing on the three most studied genera, *Agave*, *Euphorbia* and *Opuntia*, are summarised in **Table 2.4**. Based on this summary, the number of CAM species used as AD substrates was extremely limited (< 10 species), given that there are over 16,000 CAM species known to date (Winter and Smith, 1996b). The majority of the studies of *Euphorbia* spp. and *Opuntia* spp. in **Table 2.4** used the whole biomass (leaves and stems) as AD substrate whereas, for the AD of *Agave* spp., all the substrates investigated were wastes derived from either the tequila industry (bagasse) or sisal fibre production (pulp or decortication residue).

Most anaerobic digestion studies on CAM plants were carried out under batch conditions. There were some studies on semi-continuous digestion system, which was mostly carried out using the continuously stirred tank reactors (CSTR) and some conducted via anaerobic sequencing batch reactors (AnSBR) (Arreola-Vargas et al., 2015; Breton-Deval et al., 2018). Regarding the operating temperatures, most studies were carried under mesophilic conditions (30–40 °C). There was only one experiment investigating the AD of *E. tirucalli*, which was carried out at a temperate thermophilic temperature (48°C) (Sow et al., 1989).

Approaches such as substrate pre-treatment and co-digestion have also been employed in the study of CAM plants as AD feedstocks. The bagasse of *Agave* spp., for example, typically known to be fibrous, was pre-treated in some studies prior to being further investigated for anaerobic digestion. There were several pre-treatment techniques employed for *Agave* spp. such as enzymatic pretreatment

(Mshandete et al., 2005), mechanical pretreatment via grinder (Mshandete et al., 2006), and steam explosion (Weber et al., 2020, 2019). In contrast, for *Opuntia ficus-indica*, it was found that only acid pretreatment increased the methane yield of *O. ficus-indica* by 16% (Calabrò et al., 2018). Other techniques such as thermal and alkali pretreatment, in fact, negatively impacted methane production. Thus, pretreatment of *Opuntia* spp. may not be necessary as a number of previous studies reported that the *Opuntia* cladodes typically have lignin content < 10% of the total dry weight (Ginestra et al., 2009; Kuloyo, 2012; Rodrigues et al., 2016). Instead, several studies focused on the co-digestion of *Opuntia* spp. with animal manure (Ramírez-Arpide et al., 2019; Varnero et al., 1992), slaughterhouse waste (Panizio et al., 2020), and agricultural residues (Valenti et al., 2018b).

There was still a dearth of information regarding anaerobic digestion of CAM crops, and this is reflected in a limited number of publications shown in **Table 2.4**. The grey area refers to the AD study of CAM plants before 2016, prior to the current research presented in this thesis. Of all the three CAM genera, *Agave* spp. had the highest number of publications, followed by *Opuntia* spp. and *Euphorbia* spp. However, several previous studies of these plants, particularly *E. tirucalli*, reported only the biogas yield but other key information such as substrate characteristics, essential test condition, or biogas composition were not included. Some protocols employed for AD investigation of CAM plants in these previous studies were also not up to the current standard of the latest BMP guidelines (Angelidaki et al., 2009; Holliger et al., 2016). Due to the limitations and weaknesses of the past studies, it is justifiable that some experiments on the AD of *E. tirucalli* should be reinvestigated and further study on improving the AD of *O. ficus-indica* should be conducted.

Table 2.4 Summary of previous studies on batch and semi-continuous anaerobic digestion of CAM species

Substrate	Species	Digester modes and volume (l)	Temperature (°C)	CH ₄ yield (unit)	Sources
Agave	<i>A. tequilana</i> (bagasse)	AnSBR (3.6)	32	260 (L kgCOD ⁻¹)	(Arreola-Vargas et al., 2015)
	<i>A. tequilana</i> (bagasse)	AnSBR (1.2)	35	280 (L kgCOD ⁻¹)	(Breton-Deval et al., 2018)
	<i>A. tequilana</i> (bagasse)	Batch (0.70)	35	324 (L kgVS ⁻¹)	(Gómez-Guerrero et al., 2019)
	<i>A. tequilana</i> (pretreated)	BMP (0.125)	36	260–290 (L kgCOD ⁻¹)	(Weber et al., 2019)
	<i>A. tequilana</i> (pretreated)	BMP (0.50)	36	235 (L kgVS ⁻¹)	(Weber et al., 2020)
	<i>A. sisalana</i> (decortication residue)	-	-	400 (L kgVS ⁻¹)	(Oudshoorn, 1995)
	<i>A. sisalana</i> (pulp)	BMP (0.60)	27	320 (L kgVS ⁻¹)	(Mshandete et al., 2004)
	<i>A. sisalana</i> (pulp co-digested with fish waste)	BMP (0.60)	27	310–620 (L kgVS ⁻¹)	(Mshandete et al., 2004)
	<i>A. sisalana</i> (pre-treated pulp)	BMP (0.30)	37	120–240 (L kgVS ⁻¹)	(Mshandete et al., 2005)
	<i>A. sisalana</i> (pre-treated pulp)	BMP (0.61)	33	180–220 (L kgVS ⁻¹)	(Mshandete et al., 2006)
	<i>A. sisalana</i> (decortication residue)	Semi-continuous (2.0)	35–37	250–310 (L kgCOD ⁻¹)	(Mshandete et al., 2008)
	<i>A. sisalana</i> (decortication residue)	BMP (0.20)	28	292 (L kgVS ⁻¹)	(Muthangya et al., 2009)
	<i>A. sisalana</i> (decortication residue)	Batch (1.00)	30–40	157–368 (L _{biogas} kgVS ⁻¹)	(Rajabu and Manyele, 2015)
	<i>A. angustifolia</i> (bagasse)	Batch (0.70)	35	193 (L kgVS ⁻¹)	(Gómez-Guerrero et al., 2019)
Euphorbia	<i>E. tirucalli</i>	Batch (2.5 L)	28	19.2 (L _{biogas})	(Rajasekaran et al., 1989)
	<i>E. tirucalli</i>	Batch (2.0 L)	37–48	0.38 and 0.77 L L ⁻¹ d ⁻¹	(Sow et al., 1989)
	<i>E. tirucalli</i>	BMP (1.25 L)	38	114–637 (L _{biogas} kgVS ⁻¹)	(Hastilestari et al., 2013)
	<i>E. tirucalli</i>	Batch (-)	Ambient	18.1–55.7 L _{biogas}	(Girmay, 2014)

<i>E. tirucalli</i>	BMP (-)	-	215–234 L kgVS ⁻¹	(Krümpel et al., 2020)	
Opuntia	<i>O. ficus-indica</i>	-	-	300 L kgTS ⁻¹	(Contreras and Toha, 1984)
	<i>O. ficus-indica</i>	-	30	-	(Uribe et al., 1992)
	<i>O. ficus-indica</i> (co-digested with goat manure)	-	30	-	(Varnero et al., 1992)
	<i>O. ficus-indica</i>	Batch (2.80)	-	6.18 L _{biogas}	(Jigar et al., 2011)
	<i>O. ficus-indica</i> (co-digested with agricultural waste residue)	Batch (1.54)	38	237-260 (L kgVS ⁻¹)	(Valenti et al., 2018a)
	<i>O. ficus-indica</i> (pretreated)	Batch (1.0)	35	289-604 (L kgVS ⁻¹)	(Calabrò et al., 2018)
	<i>O. ficus-indica</i> (pretreated)	Batch (0.50)	31	286–702 (L kgVS ⁻¹)	(Myovela et al., 2019)
	<i>O. ficus-indica</i> (co-digested with cow manure)	Semi-continuous (10.0)	-	420 (L _{biogas} kgVS ⁻¹)	(Ramírez-Arpide et al., 2019)
	<i>O. ficus-indica</i> (co-digested with slaughterhouse wastes)	Batch (8.0)	38	87 L _{biogas}	(Panizio et al., 2020)
	<i>O. ficus-indica</i>	BMP (-)	-	280–295 (L kgVS ⁻¹)	(Krümpel et al., 2020)
<i>O. heliabravoana</i>	Semi-continuous (1.54)	20	154-257 (L kgVS ⁻¹)	(Quintanar-Orozco et al., 2018)	
<i>O. maxima</i> (co-digested with <i>Scenedesmus</i> spp.)	Batch (1.0)	37	233.6 (L kgVS ⁻¹)	(Ramos-Suárez et al., 2014)	
	Semi-continuous (3.0)	37	282–321 (L kgVS ⁻¹)		

2.2 Introduction of anaerobic digestion

Anaerobic digestion (AD) is a series of degradation processes of complex organic material in the absence of oxygen by a community of microorganisms. AD can be manipulated to obtain desired products such as biogas (a mixture of methane, CH_4 , carbon dioxide, CO_2 , water vapour and other trace gases), hydrogen gas, volatile fatty acids (VFAs), and digestate. AD process is usually divided into four biological stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (**Fig. 2.4**).

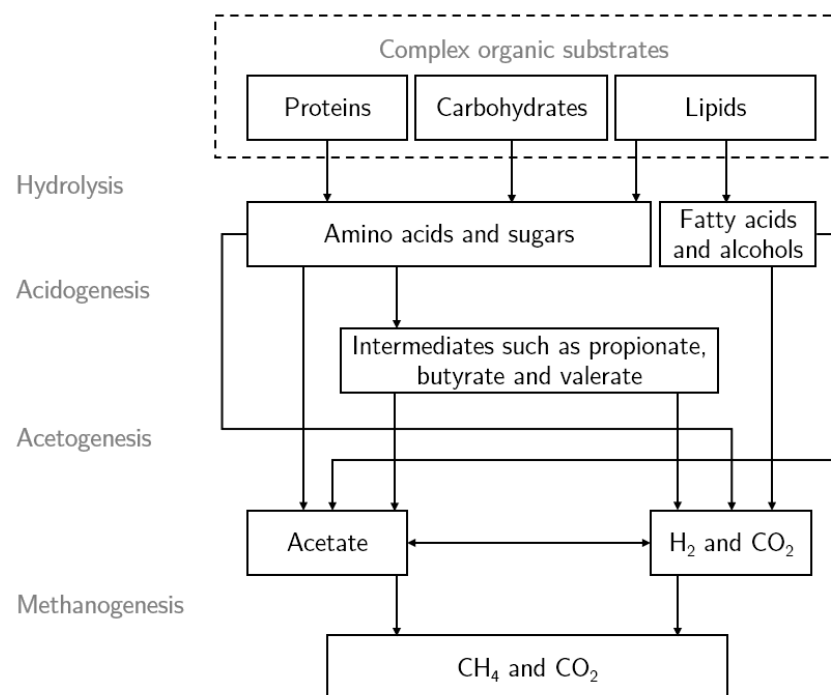


Fig. 2.4 A schematic pathway of the four biochemical stages of anaerobic digestion of biomass. Adapted from (Mara and Horan, 2003)

2.2.1 Biochemistry of anaerobic digestion

2.2.1.1 Hydrolysis

This is the first step in which the complex insoluble organic matters such as carbohydrates, fats and proteins are degraded by hydrolytic microorganisms. Cellulose, for example, is hydrolysed by exoenzymes released by *Cellulomonas*, one of the hydrolytic microbes, into glucose molecules, which can then be absorbed by

microorganisms (Gerardi, 2003). The degradation of the complex insoluble substrate often acts as a bottleneck during anaerobic digestion and the hydrolysis rate is commonly referred to as the rate-limiting step (Nopharatana et al., 2007).

2.2.1.2 Acidogenesis

Once soluble compounds from the hydrolysis step enter microbial cells, they are further degraded into hydrogen gas, carbon dioxide, alcohols and organic acids (Gerardi, 2003). Other organic nitrogen and organic sulphur compounds are also found at this step as the products of protein degradation. Acidogenesis is typically considered the fastest of the four stages within the AD process. Some of the acidogenic microbes are facultative and thus can reduce the presence of oxygen in the system, creating an optimal environment for methanogenic microorganisms (Mara and Horan, 2003).

2.2.1.3 Acetogenesis

This is the step where acetic acid, which is a precursor in methanogenesis, is produced. The activity of the acetogenesis step is controlled by two groups of microorganisms. The first group is the proton reducing acetogens, which are responsible for the conversion of fatty acids and alcohols into acetic acid, carbon dioxide and hydrogen via β -oxidation (Mara and Horan, 2003). The growth of this type of acetogens is governed by the concentration of hydrogen, which is maintained by methanogens. Acid accumulation in the AD system typically inhibits the activity of methanogens. It could also impact the syntrophic relationship between these two microbe groups (proton reducing acetogens and methanogens) and therefore indirectly inhibits the activity of acetogens. The other acetogen group is called homoacetogens, which are strict anaerobes. They synthesise acetic acid from hydrogen and carbon dioxide.

2.2.1.4 Methanogenesis

Methanogenesis is the last stage where methane is produced from acetic acid, carbon dioxide and hydrogen by methanogens, which are strict anaerobes belonging to the kingdom of *Archaea*. There are two groups of methanogens responsible for methane production in this stage: acetoclastic and hydrogenotrophic methanogens. The first group produces up to 70% of methane in the entire process by utilising acetic acid from the previous stage (Mara and Horan, 2003). Hydrogenotrophic methanogens or hydrogen-utilising microbes are chemolithotrophic and therefore able to produce methane by utilising only hydrogen and carbon dioxide. Methanogens work actively in the pH between 6.5 and 8.0, thus they are susceptible to changes in acid concentrations in the system.

2.2.2 Factors affecting the AD performance

There are several factors that can influence the overall performance of an anaerobic digestion system. Some of these parameters are operating temperature, pH, and biomass composition (lignin content and carbon to nitrogen ratio). Inoculum to substrate ratio, inoculum type and inoculum activity also play an important role in the BMP study whereas the performance of continuous digesters is influenced by the organic loading rate and retention time

2.2.2.1 Inoculum

The inoculum used in the BMP study should be as fresh as possible from the active anaerobic digester to ensure that they consist of a diverse microbial community capable of digesting a variety of substrates (Angelidaki et al., 2009). Degassing of the inoculum may also be performed to reduce endogenous methane production. It was recommended that the inoculum should have endogenous methane lower than 50 L kgVS⁻¹ (Holliger et al., 2016). The inoculum should also be tested for its

methanogenic activity, which indicates the quality of the inoculum. The activity can be tested using different substrates such as acetic acid and cellulose (Angelidaki et al., 2009). For an active inoculum, it was advised that the activity, measured as the BMP of cellulose, should be between 352 and 414 L kgVS⁻¹, which is 85–100% of the theoretical BMP value of cellulose (Holliger et al., 2016). Another essential factor that needs to be taken into consideration is the inoculum to substrate (I/S) ratio. It is recommended that the organic matter proportion of inoculum should be higher than that of the substrate. For most applications, I/S ratios should be approximately 2–4. However, for unknown substrates, several I/S ratios should be tested to prevent acidification or possible inhibition from the substrate (Holliger et al., 2016).

2.2.2.2 Temperature

The operating temperature not only has a direct influence on the growth and metabolism of the microorganisms but also affects the physicochemical properties of the contents in the anaerobic digesters (Appels et al., 2008). Although methane-forming microorganisms can work at a wide temperature range between 5°C and 65°C, the majority of them optimally work under two operating temperature conditions: mesophilic (30–42 °C) and thermophilic (50–60 °C) (**Fig. 2.5**) (Gerardi, 2003; Weiland, 2010). Mesophilic digesters offer better temperature stability and require less energy input whereas thermophilic temperatures improve the methane production rate and the growth rate of methanogens (Lettinga et al., 2001; Weiland, 2010). Thermophilic digesters are able to accept higher loading due to an increase in the growth and metabolic rate of microbes (Mara and Horan, 2003).

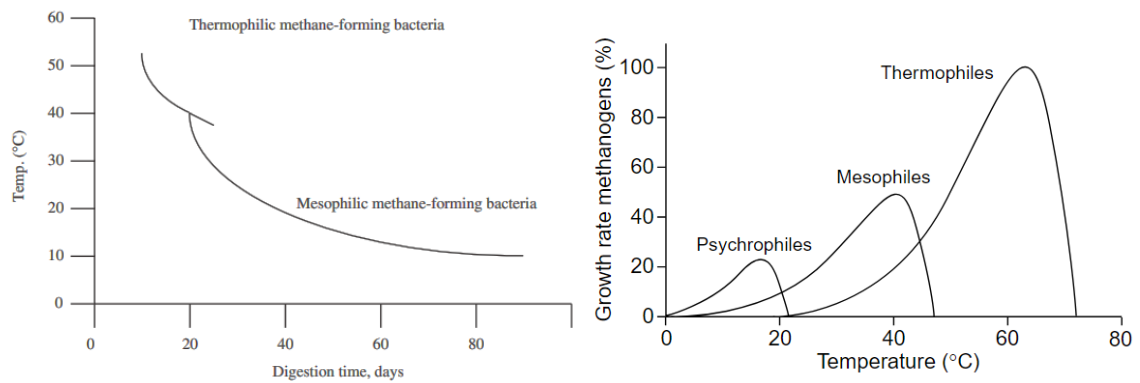


Fig. 2.5 The relationship between two operating temperatures range and duration of anaerobic digestion (Gerardi, 2003) (**left**). Relative growth rates of methanogens under three different operating conditions (Lettinga et al., 2001) (**right**).

2.2.2.3 pH

The ideal pH range for AD microorganisms should be between 6.5 to 7.8 and specifically, the pH values of 6.8–7.2 are optimal for methanogens (Appels et al., 2008; Mara and Horan, 2003). Acidogenic bacteria, however, are able to work at a slightly lower pH range (above 5.0). The AD process can be severely inhibited if the values of pH are below 6.0 or above 8.0 (Gerardi, 2003).

Digester pH is essentially dependent on the concentrations of carbon dioxide, volatile fatty acids (VFAs) and ammonia in the system. The release of carbon dioxide produced from the acidogenesis, acetogenesis and methanogenesis steps to the system results in the production of carbonic acid, which can then be dissociated into carbonate ions (CO_3^{2-}) and bicarbonate ions (HCO_3^-). Ammonia production from protein degradation is dissociated into ammonium ions (NH_4^+). The balance of these ions determines the digester pH (Gerardi, 2003). In contrast, volatile fatty acids (VFAs) cause the inhibition in the undissociated form and therefore the inhibitory effect of VFA amplifies at low pH conditions. (Weiland, 2010).

2.2.2.4 Carbon to nitrogen (C/N) ratio

Several CAM species have been shown to have high nitrogen use efficiency and some were able to grow effectively under nitrogen-deficient conditions (Lüttge,

2004). For this reason, the C/N ratio of CAM substrates is predicted to be relatively low compared to other C₃ energy crops such as wheat. Since the C/N ratio of biomass is one of the important factors affecting bacterial growth and microbial activity, this parameter should be carefully monitored in order to maintain process stability.

The recommended range for the optimum C/N ratio in the biomass is between 20/1 and 30/1 (Hagos et al., 2017). A substrate with a low C/N ratio can contribute to an increase in ammonia in the system. Ammonia generally increases the alkalinity of the digester but the presence of ammonia in high concentrations can lead to ammonia toxicity (Gerardi, 2003; Parkin and Owen, 1986).

One way to overcome nitrogen deficiency in plant feedstocks is to co-digest them with a substrate high in nitrogen content. Several studies have found that the use of cattle and chicken manures combined with agricultural residues (Li et al., 2013a; Wang et al., 2012) and wastewater (Angelidaki and Ahring, 1997) resulted in an increase methane yield and improved AD performance.

2.2.2.5 Organic loading rate (OLR)

The organic loading rate is the amount of organic dry matter loaded per unit digester volume per unit time. Methane production typically increases with OLR to such an extent that methanogenic microbes are unable to degrade volatile fatty acids (Gerardi, 2003). A high OLR is therefore preferable in order to achieve a greater volume of methane produced. However, overloading or shock loading of organic matter will cause a build-up of volatile fatty acids, which creates instability of the AD system (Lyberatos and Skiadas, 1999). Severe inhibition by VFA accumulation may lead to digester failure if remedy measures such as increasing hydraulic retention time (HRT) fail. Overloading can also cause biomass washout before it is degraded, resulting in a lower methane yield (Rajeshwari et al., 2000).

2.2.2.6 Retention time

Retention time is one of the most important operational conditions, which directly affect the conversion of volatile solids to methane. There are two terms of retention time used in the AD process: hydraulic retention time (HRT) and solid retention time (SRT). The first term is the average time that the digester content remains in the digester while SRT is the average time the solids (microorganisms) spend in the digester (Gerardi, 2003). For a conventional, continuously mixed anaerobic digester (CSTR), there is no recycling stream, which means that the amount of time that both digestate and solids stay in the digester is the same and hence HRT is equal to SRT (Mara and Horan, 2003). The retention time of fewer than 10 days is not recommended as a significant proportion of methanogenic microbes would be washed out, leading to VFA accumulation (Appels et al., 2008). The doubling times of aerobic and facultative anaerobic organotrophs responsible for hydrolysis, acidogenesis and acetogenesis steps, are approximately 15–30 minutes, which is significantly faster compared to methanogens (5–10 days) (Gerardi, 2003; Mara and Horan, 2003). However, increasing retention time results in a larger digester size, which contributes to a higher investment cost.

2.2.3 Biochemical Methane Potential (BMP) Study

In the BMP test, a substrate is mixed with an active anaerobic inoculum retrieved from a full-scale digester. The vial containing the mixture is incubated at either mesophilic or thermophilic conditions and constantly shaken. The test is run until the cumulative biogas production has reached a plateau and the daily gas production is less than 1% of the total accumulated gas production.

There are a number of BMP guidelines that have been used widely in the research community (Angelidaki et al., 2009; Holliger et al., 2016; VDI, 2006). These

protocols provide different information on experimental conditions and setups, which could affect the accuracy of the BMP results.

2.2.3.1 Factors affecting the BMP results

2.2.3.1.1 Substrate

The substrate is one of the important parameters affecting the BMP values so the characterisation of the substrate should always be considered. The content of cellulose, hemicellulose and lignin, in particular, determines the BMP of the substrate. The methane potential of organic material can also be estimated from its characteristics using the Buswell equation (**Equation 2.1**), which gives a theoretical value, assuming 100% material breakdown or analysed by using the biochemical methane potential (BMP) assay, giving an experimental yield.

$$\begin{aligned}
 C_c H_h O_o N_n S_s + \frac{1}{4}(4c - h - 2o + 3n + 2s)H_2O \\
 \rightarrow \frac{1}{8}(4c - h + 2o + 3n + 2s)CO_2 + \frac{1}{8}(4c + h - 2o - 3n - 2s)CH_4 \\
 + nNH_3 + sH_2S \quad (2.1)
 \end{aligned}$$

The fibre content of the plants is typically dependent on plant maturity. Thus, the plant harvesting period is a key factor that also influences methane production from plant feedstocks. There have been several studies on the effect of harvesting age on the performance of anaerobic digestion of energy crops such as napier grass (Pizarro-Loaiza et al., 2020; Surendra and Khanal, 2015), giant reed (Ragaglini et al., 2014), switchgrass (Massé et al., 2010), and miscanthus (Mangold et al., 2019; Wahid et al., 2015). The studies on these perennial grasses revealed that harvest time significantly affects their biomass yields and characteristics. Despite an increase of agronomic yield with the harvesting age, the highest methane yield was obtained from younger biomass and the digestibility of cellulose and hemicellulose was found to be the lowest for the crop of the highest maturity. However, biomass

harvested at early ages does not always guarantee the highest net energy yield per hectare, a life cycle analysis (LCA) is recommended to ensure that parameters such as substrate storage, age, and processing techniques are also taken into consideration (Surendra and Khanal, 2015).

In terms of sample preparation, it was recommended the preparation procedure should be minimal to avoid altering the physico-chemical properties of the substrate and its degradability. The size of the substrate also needs to be considered as it affects the kinetics of the BMP study but not the actual BMP value (Angelidaki et al., 2009). Standardisation of the particle size is recommended and one of the BMP guidelines suggested that substrate particles should be smaller than 1 centimetre in all dimensions (Holliger et al., 2016). However, the guideline warned that the size reduction process needs to be carefully monitored, heat generated during the process could result in a loss of volatile organic compounds (VOCs). The loss of VOCs can occur during the drying process of the substrate, so the drying temperature and type of feedstock used need to be carefully selected.

Substrate storage is also a key factor that needs to be considered. It was suggested that the substrate used should be as fresh as possible. If storage is required, the substrate should be refrigerated at 4°C but not more than five days (Holliger et al., 2016). Freezing the substrate at -20°C is also an option but this may alter the properties of the substrate. Ice crystals formed within the cells can rupture the plant cell walls of the frozen plant materials, making them more readily biodegradable by microorganisms (Liu et al., 2008). Some research suggests that the freezing/thawing technique can be used as mechanical pretreatment for the substrate to improve its digestibility (Kinnunen et al., 2014).

2.2.3.1.2 Inoculum

All the guidelines mentioned earlier recommended that the inoculum should be fresh from an active anaerobic digester to ensure a broad microbial community (Angelidaki et al., 2009; Holliger et al., 2016; VDI, 2006). Pre-incubation of the inoculum may be conducted if the endogenous methane production is high. All the guidelines suggested that the inoculum concentration should be higher than that of the substrates. Specifically, the inoculum to substrate ratio recommended should be at least 2 (Holliger et al., 2016; VDI, 2006).

2.2.3.1.3 Experimental setups

The setups for the BMP test vary across laboratories and this variation contributes to a difference in BMP results. There are several types of BMP settings, but the two main ones are the conventional bottle test and the automatic BMP test.

The automated BMP setup has been widely used recently and one of the most popular options is developed by Bioprocess Control (Lund, Sweden) called the Automated Methane Potential Test System II (AMPTS II). The AMPTS II offers automatic, real-time measurements and data logging of biogas production, as seen in several studies (Breton-Deval et al., 2018; Wang et al., 2015, 2014; Zhao et al., 2018). The tool monitors biogas by using a gas counter. Biogas is passed through an alkali solution which traps carbon dioxide and hydrogen sulphide gases before it reaches the gas counter.

The automated system offers several advantages over the manual bottle test. For example, using the automated system is time-saving and less labour intensive (Wang et al., 2014). The authors analysed the time spent performing the BMP test using different experiment setups and found that the use of the AMPTS II system

eliminated the workload on following up experimental data such as the analysis of gas volume and composition during the incubation period. The software that comes with the system also manages the data and interpret it for the users. The automatic system also eliminates systematic errors committed by humans while injecting gas samples to the gas chromatograph for biogas composition analysis or reading the water column to determine the gas volume. However, the automated system also has its drawbacks, one of which is that the system such as AMPTS II has been designed specifically for biogas determination only. Measurement of volatile fatty acid (VFA) and pH is currently not possible with the current version of the kit. The conventional bottle test can be conducted with additional sacrificial bottles for the experiment specifically designed to study the profiles of volatile fatty acids (VFAs) and pH. Another advantage of the conventional test is that the number of bottles tested is less limited compared to the AMPTS II system, which can only hold 15 reactors for each kit. The inexpensive bottle test is therefore suitable for studying the effect of several different parameters on the BMP simultaneously.

2.3 Alternative use of biomass via biorefinery

The International Energy Agency (IEA) Bioenergy Task 42 defined the concept of biorefinery as *“the sustainable processing of biomass into a spectrum of marketable products (food, feed, materials, chemicals) and energy (fuels, power, heat)”* (de Jong et al., 2011). Biorefinery is the centre of the biobased economy with the main goal of achieving the highest efficiency and sustainability in converting biomass into chemical, food, and energy. Thus, integrated biorefinery has been considered an efficient method for sustainable biomass valorisation where the production of bio-based materials is facilitated by the side-stream of bioenergy products (Stegmann et al., 2020)

Another concept similar to biorefinery is the principle of cascading use of biomass. This principle necessitates the systematic use of biomass by first exploiting for higher-added-value products before using it as an energy source (Keegan et al., 2013). The term ‘added value’ can be referred to in both environmental and economic senses (Olsson et al., 2016). The cascading concept implies linear, multiple successive uses of biomass through a series of substrate conversion, reuse, recycling and lastly energy extraction and is often illustrated as a biobased pyramid in **Fig. 2.6**. The most valuable products such as pharmaceuticals and biomaterials should be prioritised or isolated first from the biomass. The residue from the biomass isolation process could be further used as food and fodder ingredients. The remaining, which can no longer be utilised, should be recovered for energy such as biogas via anaerobic digestion.

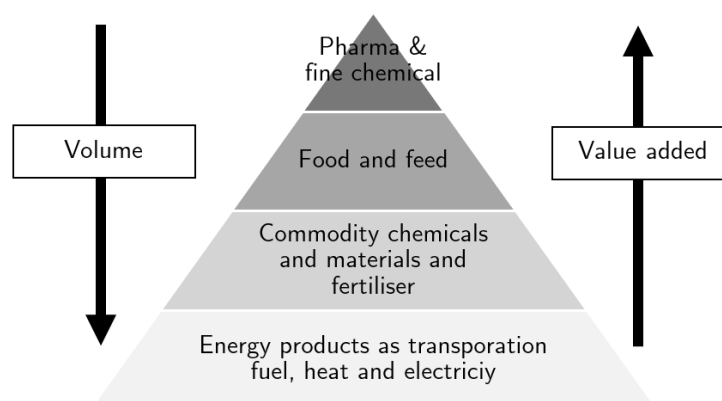


Fig. 2.6 biomass value pyramid adapted from the biobased pyramid (Hagman et al., 2018)

The two concepts ‘biorefinery’ and ‘cascading use of biomass’ are complementary and can potentially be applied to the context of CAM plants in this research, for example,

- ‘Cascading concept’ – CAM substrates are mechanically pretreated into more readily biodegradable materials, from which the valuable compounds such as organic acids and amino acids could be first extracted. The residue from

the pretreatment step can be processed into animal feed ingredients whereas the waste from the earlier extraction step is fermented for biogas.

- ‘Integrated biorefinery’ – biogas from anaerobic digestion of the waste from the extraction process is combusted to provide heat and electricity which can be used to facilitate the production stream of higher added value products (organic acids and amino acids)

One of the biobased products, which currently has a fast-evolving economy is bioplastics. The consumption of bioplastics worldwide has increased from 0.9 million tonnes in 2012 to approximately 3.0 million tonnes in 2020, and over the same period, the economy of bioplastics in Europe has grown from 420 million to 1.4 billion euros (Scarlat et al., 2015). The share of bioplastics on the market in 2018 was still below 1% but this number was expected to increase up to 70–85% in the future (EEA, 2018). The emerging bioplastic sector hence deserves closer attention.

There are some issues associated with bioplastics that need to be addressed. For example, many biobased plastics are not biodegradable. It was reported that in 2019, of all the biobased plastics used, less than 20% were expected to be biodegradable (EEA, 2018). Examples of biodegradable plastics include polylactic acid (PLA) and polyhydroxyalkanoate (PHA), which have already been commercially available and have the potential to replace petroleum-based plastics for various applications (Wu et al., 2017). PHA, which also holds higher economic and environmental values compared to methane, was then considered in this study as a potential higher value-added product that could be produced from CAM plants.

2.3.1 Polyhydroxyalkanoates (PHAs)

PHAs are a family of natural polyesters that can be synthesised by a wide range of microorganisms. The polyester contains the monomer units of 3-hydroxyalkanoic acid (3-HA). PHAs are biopolymers with some of the most diverse structures as there have been over 150 different types of PHA monomers reported (Chen, 2009a). Therefore PHAs have become one of the most promising biopolymers completely synthesised by microorganisms as they have mechanical properties such as tensile strength and melting temperature comparable to petroleum-based plastics (Sudesh et al., 2000). Apart from their versatility, PHAs are completely biodegradable. It has been demonstrated that PHB packaging can be totally degraded in soil within 3 weeks (Altaee et al., 2016; Shin et al., 1997).

PHAs can be generally classified into three different types according to the chain length (**Fig. 2.7**). Short-chain-length (SCL) polymers consist of 3 to 5 monomers while medium-chain-length (MCL) polymers consist of up to 16 monomers. The last category is a copolymer of combined short and medium-chain length polymers (Jung et al., 2010). The structure of the polymer, which in this case is the polymer chain length, determines the mechanical properties of the material. For example, SCL-PHAs are thermoplastics, which are stiff crystalline polymers with properties resembling that of polypropylene. MCL-PHAs are thermoplastic elastomers (TPE), which are semi-crystalline materials. The last polymer type, SCL-MCL PHA, has the combined features of short and medium-chain length polymers, which makes the mechanical properties of this material, similar to those of low-density polypropylene (LDPE).

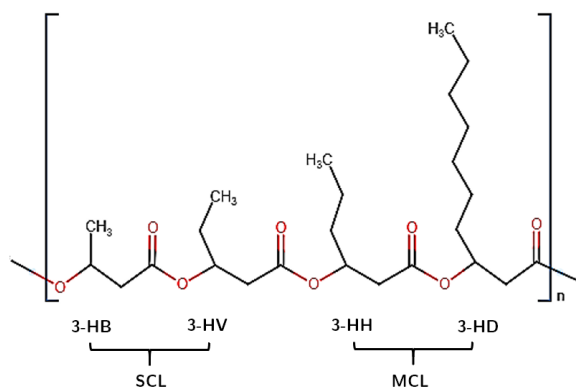


Fig. 2.7 The structure of PHA consisting of different types of monomer units: 3-hydroxybutyrate (3-HB), 3-hydroxyvalerate (3-HV), 3-hydroxyhexanoate (3-HH), and 3-hydroxydecanoate (3-HD). These units can be categorised into two different groups based on their carbon atoms: short-chain length (SCL) polymers and medium-chain length polymers (MCL).

There are two crucial factors regulating the composition of PHA that need to be taken into consideration: the specificity of PHA synthase and the metabolic capacity of the bacteria (Rehm, 2003). To manufacture a specific type of PHA, the production process requires synthetic biology tools such as metabolic engineering and key enzyme engineering to optimise the microorganisms used (Jung et al., 2010). The yield could also be maximised through the optimisation of fermentation.

2.3.2 *Cupriavidus necator* H16 for PHA production

Cupriavidus necator, formerly known as *Ralstonia eutropha*, *Wautersia eutropha* and *Alcaligenes eutrophus*, is one of the most common strains used for the study of PHA production (Chen, 2009b). *C. necator* is a Gram-negative, coccoid rod-shaped bacterium (**Fig. 2.8**) and a lithotroph by oxidising hydrogen aerobically as an energy source (Cramm, 2008; Pohlmann et al., 2006). It is also facultative and can undergo denitrification in the absence of O_2 by using nitrate (NO_3^-) as an electron acceptor.

C. necator H16 is a versatile wild-type strain, which has an optimum growth temperature of 30°C (Raberg et al., 2018) and an optimum growth pH of 6.8. (Lu et al., 2013). The *C. necator* H16 strain is capable of utilising a broad range of compounds as carbon sources such as carbon dioxide, fructose, organic acids, lignin derivatives and glycerol (Volodina et al., 2016). Some sugars such as glucose,

galactose and lactose cannot be metabolised by the wild-type strain due to a lack of gene expression for glucose-utilising enzymes. Other strains such as DSM545 have been reported to be able to utilise glucose as a carbon source (Chen, 2009b).

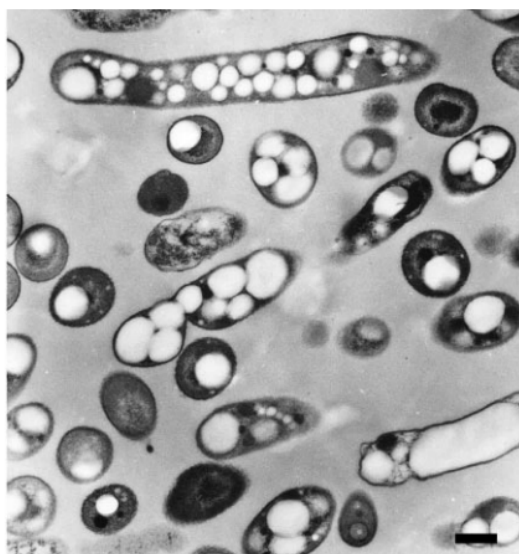


Fig. 2.8 TEM images of *C. necator* H16 after 48 hours of cultivation. The black bar indicates the size of 0.5 μm . The image was sourced from (Tian et al., 2005).

2.3.2.1 Polyhydroxybutyrate (PHB) synthesis in *C. necator* H16

The simplified process of PHB synthesis in *C. necator* H16 is illustrated in **Fig. 2.9**. The diagram depicts the synthesis of poly-3-hydroxybutyrate or P(3HB) from four different compounds, which are fructose, acetic acid, fatty acids and butyric acid. The synthesis of PHB from fructose and acetic acid as a carbon source are relatively straightforward. It involves three key enzymes which are β -ketothiolase, NADPH-dependent acetoacetyl-CoA reductase, and PHA synthase (Sudesh et al., 2000). The first step begins with the condensation reaction of acetoacetyl-CoA from two molecules of acetyl-CoA via β -ketothiolase. This enzyme, however, can be inhibited by coenzyme A (CoA), which will channel acetyl-CoA toward the TCA cycle under nutrient-rich conditions. In contrast, under nitrogen or phosphorus-limiting conditions, acetoacetyl-CoA from the second step is then reduced into (R)-3-hydroxybutyryl-CoA via NADPH-dependent acetoacetyl CoA dehydrogenase. The last step is the polymerisation of (R)-3-hydroxybutyryl-CoA monomers using PHA

synthase. This enzyme in *C. necator* has been reported to be preferentially active towards monomers of 3–5 carbon atoms, and thus the PHB synthesised by this bacterium tends to be a short-chain length polymer (Doi et al., 1987).

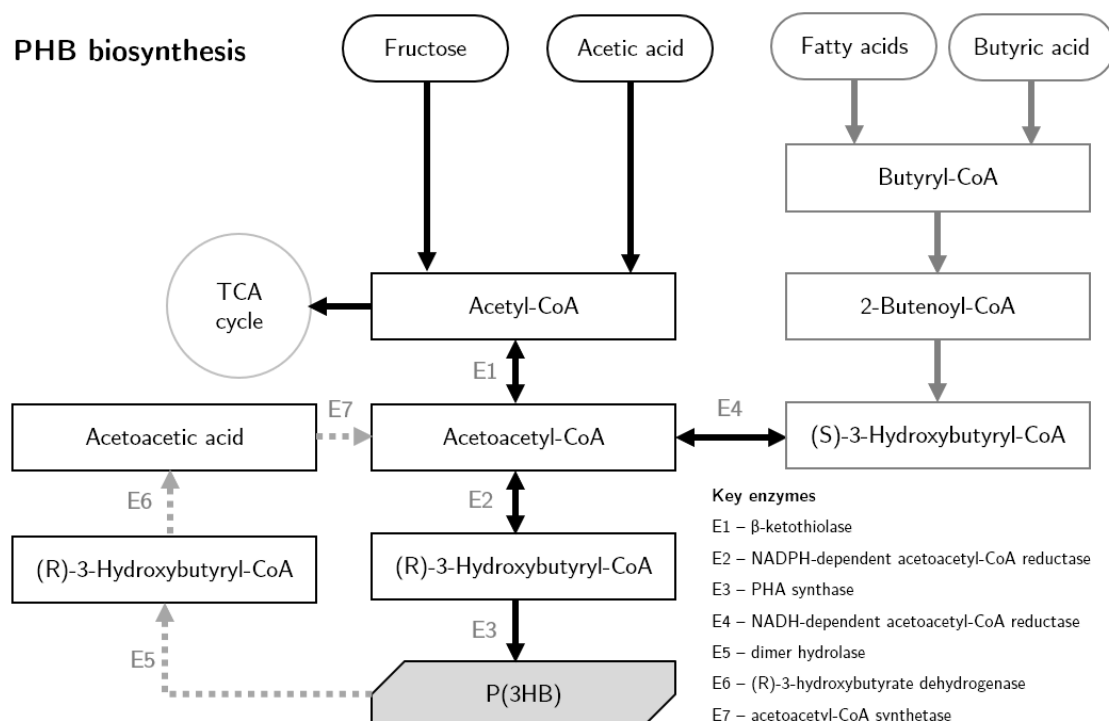


Fig. 2.9 The biosynthesis pathway of poly-3-hydroxybutyrate, P(3HB), from 4 different types of carbon sources in *C. necator* H16. The diagram was adapted from (Sudesh et al., 2000)

2.3.3 Challenges of PHA production

The main challenge of PHA production is the market price, which is high compared to that of petroleum-based plastic. The high price of PHB is largely attributed to the cost of raw materials such as sugars (Koller et al., 2010). Alternative substrates such as organic wastes, agricultural residues, and lignocelluloses are promising low-cost carbon sources, which need further investigation. Other factors that could make PHA less competitive compared to conventional plastics are the high downstream processing cost and the inconsistency of PHA quality (Chen, 2009b). Also, the mechanical properties of PHA are still not equivalent to those of typical plastics. As well as making an effort to minimise the production costs, another solution is to search for other applications of PHA with high market values.

2.3.4 Alternative substrates as carbon sources for PHA production

The cost of raw materials, particularly the cost of carbon sources is one of the most important factors affecting the price of the final PHA product. To lower the cost of PHA, alternative inexpensive raw materials should be investigated whether they are utilised as carbon sources for PHA production. Examples of these raw materials are wastes from the agro-product industry and lignocellulosic feedstocks. The utilisation of waste materials as substrates for PHA production not only offers cost-benefit but also an effective waste management solution. **Table 2.5** shows a previous study of various raw materials used as carbon sources for PHA production.

In one study, the compositional analysis of the juice pressed from *Opuntia ficus-indica cladodes* and the remaining bagasse was performed (Yang et al., 2015). The results showed that the major constituents of the juice were carboxylic acids and sugars, whereas the remaining bagasse, after juice extraction, contained high amorphous cellulose fractions which suggested that the biomass would be less recalcitrant to biotransformation compared to lignocellulosic feedstocks. According to these compositional analysis data, *Opuntia* juice and bagasse may also hold the potential as a substrate for PHA production.

Table 2.5 Summary of PHB production by different strains of *C. necator* using various agro-product/waste-derived carbon sources

Carbon source	Strain	Fermentation type, culture volume (L)	Concentration (g L ⁻¹)		%PHB (w/w)	Reference
			Biomass	PHB		
Hydrolysate from sugarcane bagasse, 4.7 g L ⁻¹ of glucose	H16	Shake flask, -	8.00	4.29	53.7	(Yu and Stahl, 2008)
Orange juice waste , 22.2 g L ⁻¹ of fructose	H16	Shake flask, 0.1	9.58	7.31	76.3	(Guzman Lagunes and Winterburn, 2016)
Oil extracted from date seed, 10.8 g L ⁻¹ of fructose	H16	Shake flask, 0.1	6.3	4.6	73.0	(Yousuf and Winterburn, 2016)
Hydrolysate from alligator weed, 34 g L ⁻¹ of carbon source	H16	Shake flask, 0.1	8.5	4.8	56.5	(Zhang et al., 2020)
Rapeseed oil , 20 g L ⁻¹ in salt medium	H16	Shake flask, 0.25	2.0	0.55	27.5	(Verlinden et al., 2011)
Hydrolysate from wheat, 19.5 g L ⁻¹ of sugar	H16	Shake flask, -	10.5	7.0	66.7	(Xu et al., 2010)
Hydrolysate from Miscanthus, 9 g L ⁻¹ of sugars	5119	Shake flask, 0.20	4.6	2.02	44.0	(Bhatia et al., 2019)
Sweet sorghum juice , 40 g L ⁻¹ of total sugars	TISTR-1095	Shake flask, -	4.67	1.84	39.4	(Kaewkannetra et al., 2008)
Sugarcane juice , 50 g L ⁻¹ of total sugars	TISTR-1095	Shake flask, 0.1	6.01	1.84	30.6	(Suwannasing et al., 2011)
Hydrolysate from water hyacinth, 35 g L ⁻¹ of reducing sugar	MTCC-1472	Shake flask, 0.1	12.0	7.0	58.3	(Radhika and Murugesan, 2012)
Hydrolysate from aquatic invasive weeds, 10 g L ⁻¹ of hexose sugar	MTCC-8320	Shake flask, 0.2	4.44	0.96	21.6	(Pradhan et al., 2017)
Sugar beet juice , 20 g L ⁻¹ of carbon source	ATCC-29714	Shake flask, 0.1	10.30	4.01	38.7	(Wang et al., 2013)
Hydrolysate from sugarcane molasses, 20 g L ⁻¹ of reducing sugar	DSM 545	Fed batch, 4.0	20.9	11.7	56.0	(Dalsasso et al., 2019)

2.4 Significance of research

Anaerobic digestion of CAM plants is still in its infancy and requires further research into exploring potential CAM candidates, which could ultimately become viable bioenergy crops. The current understanding of CAM plants as an AD feedstock is also inadequate as there have been less than ten species of CAM plants to date that have been investigated as a substrate in AD studies. There is a lack of continuity in the AD research of CAM plants, which is reflected by a limited number of publications. Most of the research has focused on BMP tests or batch systems and therefore the feasibility and practicality of utilising CAM crops as a substrate in the continuous AD system need to be further explored. There are several factors that could affect anaerobic digestion, particularly of CAM plants, but they have never been investigated fully. Moreover, the investigation of CAM plants as biomass has mainly focused on the production of bioethanol and biogas. With the recent rapid development of biorefineries, CAM plants also deserve new attention for their potential to produce higher added value products.

Chapter 3

Materials and Methods

This chapter presents the materials and the experimental methods that were employed for the studies in this thesis. It is divided into two main parts: **Section 3.1** describes the experimental setup for AD study and **Section 3.2** summarises the procedures for PHA production experiments.

3.1 Experimental procedure for AD experiments

3.1.1 Materials

3.1.1.1 Feedstocks

None of the CAM plants investigated in this study are native to the UK, so it was necessary to import fresh biomass from outside the country. The import of plant materials from outside EU countries requires a Phyto certificate via the PEACH system issued by the Department for Environment, Food and Rural Affairs (Defra). None of the UK botanical gardens were able to supply sufficiently large quantities of CAM materials. Therefore, it was impossible to perform different studies on a substrate from a large single batch.

Throughout the course of this research, AD feedstocks had been collected from different sources, as shown in **Table 3.1**. The plants investigated in **Chapter 4.1 (Fig. 3.1a-c)** were sourced from a laboratory glasshouse and a local botanic garden but the quantity of sample per species provided by the two sources was very limited. The plants were cultivated in a glasshouse under natural irradiation with supplementary heating to maintain minimum temperatures $> 10\text{ }^{\circ}\text{C}$. For each species, the materials were harvested from at least three plants to ensure good sample representation. The harvested materials were coarsely chopped to 5.0 ± 1.0

cm employing a knife and dried in a ventilated oven at 80 °C until the mass was constant. The dried samples were then ground to pieces of approximately 1–2 mm using a commercial blender (Waring Pro® Stainless Steel Blender, 550 W).

Table 3.1 A summary of plant materials used as substrates in each chapter of this thesis

Chapter	Plant species	Common name	Sources
4.1	<i>Ananas comosus</i> (L.) Merr.	Pineapple	Glasshouse at the Department of Plant Sciences, University of Oxford, and the Oxford Botanic Garden, UK
	<i>Agave angustifolia</i> Haw.	Agave	
	<i>Opuntia fragilis</i> (Nutt.) Haw.	Brittle prickly pear	
	<i>Kalanchoe daigremontiana</i> Raym.-Hamet & H.Perrier	Kalanchoe	
	<i>Euphorbia virosa</i> Willd.	Euphorbia	
	<i>Zea mays</i> L.	Maize	
4.2	<i>Euphorbia tirucalli</i> L.	Pencil cactus	Royal Botanic Garden, Kew, UK
4.3 and 5	<i>Opuntia ficus-indica</i> (L.) Mill.	Prickly pear	Pepe Aromas farm, Portugal

In **Chapter 4.2**, approximately 5 kg of fresh *Euphorbia tirucalli* was collected from the Royal Botanic Garden, Kew, UK. The plant material was coarsely chopped using a knife and ground to pieces with the same blender. The material was then stored in an airtight container and frozen at -20 °C until use when the substrate was thawed overnight at room temperature. Fresh chicken manure was obtained from Willowbrook farm, Oxfordshire and employed in the co-digestion study with *Euphorbia tirucalli*.

The cladodes of *Opuntia ficus-indica* were sourced from Pepe Aromas farm in Portugal (38.740000, -7.815028). The cladodes were sliced into pieces of approximately 3–5 cm and ground using a food processor (Magimix® Cuisine 5200, 1,100 W). The ground material was then stored in an airtight container and frozen at -20 °C until use when the substrate was thawed overnight at room temperature.

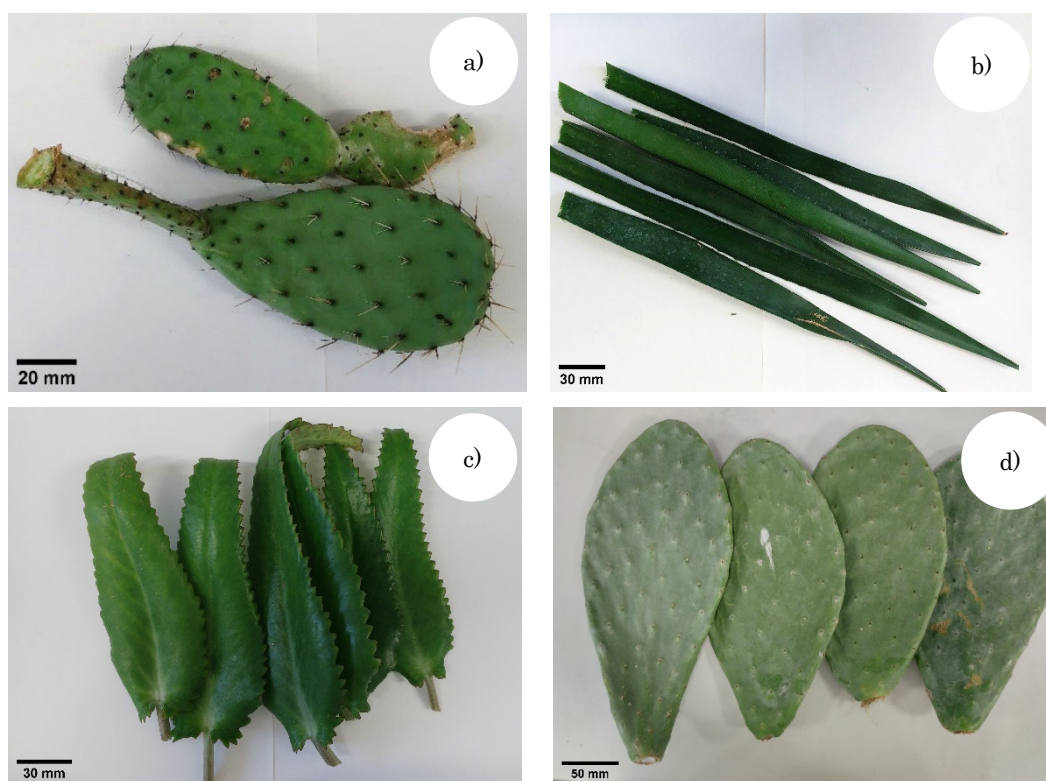


Fig. 3.1 Examples of plant materials used in the research. **a)** Brittle prickly pear cladodes (*Opuntia fragilis*). **b)** Agave leaves (*Agave angustifolia*). **c)** Kalanchoe leaves (*Kalanchoe daigremontiana*). **d)** Prickly pear cladodes (*Opuntia ficus-indica*) from Pepe Aroma farm.

3.1.1.2 Inoculum

Digestate was obtained from mesophilic digesters at Severn Trent Green Power AD facility in Cassington, Oxford, UK. The digesters operate at 39.0 ± 1.0 °C processing food wastes collected in Oxfordshire. The inoculum was always used on the collection day.

3.1.1.3 Trace element medium solution

The culture media used were based on the recipe previously developed (Angelidaki and Sanders, 2004). This contained macronutrients, micronutrients, trace minerals, vitamin mixtures, and buffer solution necessary for the growth of microorganisms.

3.1.2 Biochemical Methane Potential (BMP) study

The BMP assay employed was performed in 250 ml (150 ml working volume) clear glass bottles sealed with rubber stoppers and aluminium caps (**Fig. 3.2a**). The bottles were placed in a shaking incubator with temperature control at 39.0 ± 1.0

°C and a constant agitation at 100 rpm. The inoculum-to-substrate (I/S) ratio employed was 2:1 on a volatile solid (VS) basis. According to the literature review (**Section 2.2.3**), the organic matter proportion of inoculum should be higher than that of the substrate and for most applications, I/S ratios should be approximately 2–4. The I/S ratio of 2 has been used widely for the BMP tests of different lignocellulosic crops and agricultural residues and was therefore selected for the study in this thesis (Buitrón et al., 2019; Li et al., 2019; Sun et al., 2015). Trace element medium solution was also added to the mixture. Biogas was collected using a eudiometer, a glass burette filled with an acidified 75% (v/v) saturated sodium chloride solution (pH 2), minimising methane loss through dissolution (**Fig. 3.2c**). The solution heights in the burette were recorded for biogas displacement. The gas volume was normalised by taking into account standard temperature and pressure (STP) at 0 °C and 101.325 kPa (Walker et al., 2009). Gas composition analysis was performed together with the gas collection process. Blanks (inoculum) and positive controls (microcrystalline cellulose powder, Avicel®, Sigma-Aldrich, US) were run alongside the samples. All the tests were performed in triplicate and were terminated when cumulative biogas production has reached a plateau and the daily value was less than 1% of the total. Volatile fatty acid (VFA) concentration and pH were also measured throughout the test. Liquid samples were collected from the BMP bottles for monitoring volatile fatty acid (VFA) concentration and pH. One ml sample was collected per bottle daily during the first five days and then every five days until day 30. Approximately 10 ml of liquid samples in total were collected from each bottle, accounting for 6.7% of the original working volume (150 ml).



Fig. 3.2 **a)** BMP apparatus showing a set of 250 ml glass bottles sealed with rubber stoppers and aluminium caps. **b)** A rack of BMP bottles to be incubated in an orbital shaking incubator. **c)** A eudiometer for biogas volume measurement.

3.1.3 Semi-continuous digestion experiment

Four 5 L continuously stirred-tank reactors (CSTR) used in this study were kindly gifted by the Water and Environmental Engineering Group, University of Southampton. They were made of PVC cylinders with a gas-tight seal at the top and bottom (**Fig. 3.3b**). The base plate was fitted with a liquid outlet port for digestate removal and the lid was fitted with an inlet port and a gas outlet port. The stirrer inserted in the middle of the top plate was connected to an 80 rpm, 24 V motor (RS Components, UK). The reactors were placed in the water heating coil connected to a water bath for temperature control at 37 °C.



Fig. 3.3 Laboratory-scale CSTR digesters used in the research. **a)** a 5 L digester. **b)** A set of four 5 L digesters in a heated water jacket connected to a temperature-controlled water bath.

The digesters were started as a batch operation. The reactors were operated in duplicate, digesters 1 and 2 (R1 and R2) running in the same condition, as were digesters 3 and 4 (R3 and R4). The inoculum to substrate ratios for R1 R2 and R3 R4 at the beginning of the experiment were 2:1 and 1:1 on a VS basis. The batch system was run for 18 days until biogas yields began to plateau. Subsequently, the digesters were fed daily and digestate was removed simultaneously to ensure a constant working volume was maintained. Substrate and digestate were also weighed. The pH and VFA of digestate were recorded daily whereas its TS and VS were determined periodically. Biogas volume was measured using an automatic gas counter (MilliGascounter MGC-1 PMMA, Ritter, Germany). The gas counter outlet port was connected to a gas sampling bag for gas composition determination. The gas counters were also calibrated using gas bags attached to the vents of the gas counters before use. When the gas measurement cell tilted, this was equal to 1 ml of gas as per the factory standard. After calibration, one tilt was found to be equal to 0.88, 0.88, 1.14, 1.23 ml of gas for digesters R1, R2, R3, and R4, respectively.

The semi-continuous digesters were fed daily with a specific amount of feedstock on a VS basis. The following operational parameters such as organic loading rate, OLR

(gVS L⁻¹ day⁻¹) and hydraulic retention time, HRT (day) were taken into consideration and determined:

$$\text{OLR} = \frac{\text{VS}_F \times W_F}{V_R} \quad (3.1)$$

where VS_F is the feed volatile solid content (%), W_F is the daily fresh feed weight (g day⁻¹), and V_R is the working reactor volume (L)

$$\text{HRT} = \frac{V_R}{Q} \quad (3.2)$$

where Q is the material flow rate, i.e. the volume of substrate added/ digestate removed per day, (L day⁻¹). The density of the feed and the digestate was assumed to be the density of water (1,000 kg m⁻³) for flow rate calculation.

3.1.4 Analytical Methods

3.1.4.1 Total solid (TS) and volatile solid (VS) analysis

TS and VS of the materials were determined according to standard procedures (APHA, 1998). Approximately 10 ± 0.001 g of homogeneously mixed sample was transferred into a weighed dried crucible by spatula and weighed using a balance (Acculab ATILON analytical balance, UK). The crucibles were then placed in a drying oven (Hotbox oven with fan size 1, Gallenkamp, UK) overnight at 105 ± 1 °C. The samples were transferred to a desiccator to cool down for an hour and then weighed with the same balance. Subsequently, the crucibles with samples were reduced to ash in a muffle furnace (Carbolite RWF 1200, Hope Valley, UK) heating at 550 ± 10 °C for 2 hours. The crucibles were rested in a desiccator again to cool down for an hour. The final weight of each crucible with ash was recorded. Total and volatile solids were calculated according to the following equations:

$$\%TS = \frac{W_3 - W_1}{W_2 - W_1} \times 100 \quad (3.3)$$

$$\%VS(FS) = \frac{W_3 - W_4}{W_2 - W_1} \times 100 \quad (3.4)$$

$$\%VS(TS) = \frac{W_3 - W_4}{W_3 - W_1} \times 100 \quad (3.5)$$

where W_1 is the weight of an empty dried crucible (g), W_2 is the weight of a crucible and a fresh sample (g), W_3 is the weight of a crucible and a sample after drying (g), and W_4 is the weight of a crucible and a sample after ashing (g)

3.1.4.2 pH

The sample pH was measured using a benchtop pH meter (Orion Star™ A211, Thermo Scientific, UK) with a glass electrode, calibrated in buffer solutions at pH 4.01, 7.01 and 10.01. Samples were thoroughly mixed to homogeneity during measurements. The pH probe was rinsed with deionised water and blotted with lint-free wipes in between each measurement. The sample pH was measured directly after collection to avoid pH changes through loss of dissolved CO_2 .

3.1.4.3 Volatile fatty acid (VFA) determination via gas chromatography (GC)

Liquid samples were prepared for VFA analysis by centrifugation at $30,000 \times g$ for 15 minutes and supernatant was transferred by pipette into a 1.5 ml GC vial and acidified with 3% (v/v) formic acid. The vials were then loaded onto the GC autosampler. A set of standard solutions containing acetic, propionic, isobutyric, *n*-butyric, isovaleric, *n*-valeric and caproic acids, was also prepared in the vials using the same procedure described above (**Fig. 3.4**). Ten dilutions of the acids provided individual concentrations of 19.5, 39.6, 78.1, 156.3, 312.5, 625, 1,250, 2,500, 5,000, and 10,000 mg l⁻¹.

VFA quantification was conducted by using gas chromatography (Shimadzu GC-2010, Japan) equipped with a flame ionisation detector (GC-FID) and a 30 m × 0.25 mm × 0.25 μm fused-silica capillary column (ZB-FFAP). Helium was used as a

carrier gas at a flow rate of 1.68 ml min⁻¹. The temperatures of the detector and the injector were 350 °C and 250 °C, respectively. The oven temperature was set at 100 °C for 2 minutes and then increased at a rate of 8 °C min⁻¹.

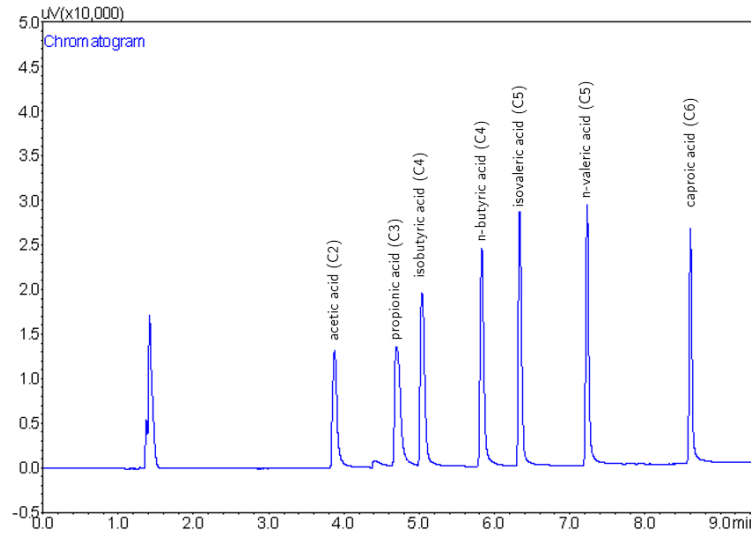


Fig. 3.4 Gas chromatogram showing the analysis of standard VFA solution containing C2 – C6 named acids. The x and y axes represent retention time and signal intensity, respectively.

3.1.4.4 Biogas volume from semi-continuous digesters

Biogas volume in a gas sampling bag (Supelco 30227-U, Sigma-Aldrich, UK) was determined by using a water displacement gasometer (**Fig. 3.5**). Biogas was released from the bag into a water column from the top. The initial and final heights of water in the column were recorded before and after releasing the biogas. The displaced volume of the column calculated was determined to be the biogas volume in the gas sampling bag. The ambient temperature (T) and pressure (P) were also noted on the day of measurement for reporting the normalised biogas volume under standard temperature and pressure (STP) at 0 °C and 101.325 kPa using the following equation (Walker et al., 2009):

$$V_{B,N} = \frac{AT_{STP}}{P_{STP}T_{atm}} \left(P_{atm} - P_{water,T_{atm}} - \rho_s g(h_i - h_f) \right) h_i \quad (3.6)$$

where $V_{B,N}$ is the normalised biogas volume (m³), A is the cross-sectional area of the water column (m), P_{STP} is the standard pressure at 101,325 Pa, P_{atm} is the ambient pressure (Pa), $P_{water,T_{atm}}$ is the saturated water vapour pressure at T_{atm} (Pa), T_{STP} is

the standard temperature at 273.15 K, T_{atm} is the atmospheric temperature (K), ρ_s is the density of the water (kg m^{-3}), g is the gravitational acceleration, 9.81 m s^{-2} , h_i is the initial height of the liquid in the column (m), and h_f is the final height of the liquid in the column (m).

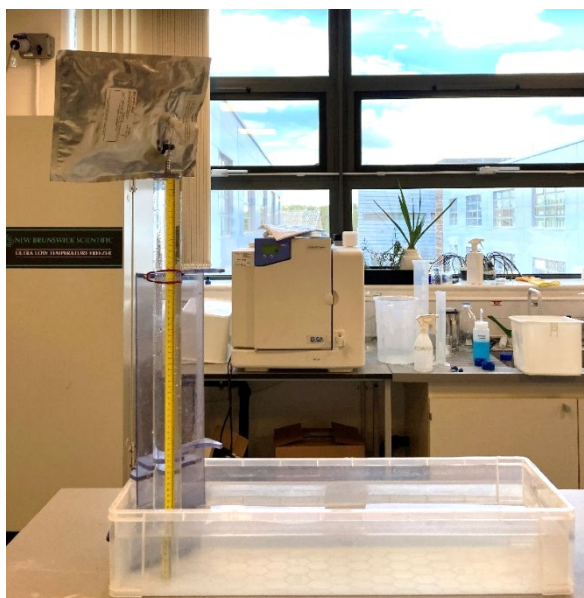


Fig. 3.5 A water displacement gasometer connected to a gas sampling bag for biogas volume measurement.

3.1.4.5 Gas composition determination using gas chromatography

For BMP tests, a sample of 2 ml was taken directly from the headspace of the bottle using a syringe and injected into the GC through a gas sampling valve. For semi-continuous experiments, a sample of 2 ml was taken from a gas sampling bag previously connected to the vent port of the gas counter connected to the digester.

Quantification of biogas composition was performed by gas chromatography (Shimadzu GC-2010, Japan) using a thermal conductivity detector (GC-TCD) and a $2.0 \text{ m} \times 0.53 \text{ mm I.D}$ (**Fig. 3.6**). mesh 80/100 Micropacked column (ShinCarbon ST, Restek, UK). The temperatures of the detector, injector and oven were 200°C , 200°C , and 175°C , respectively. The carrier gas was helium at a flow rate of 3.43 ml min^{-1} . The biogas composition was calibrated against 4 standard gases, 100% CH_4 , 100% N_2 , 100% CO_2 and 80%/20% CO_2/H_2 (v/v).

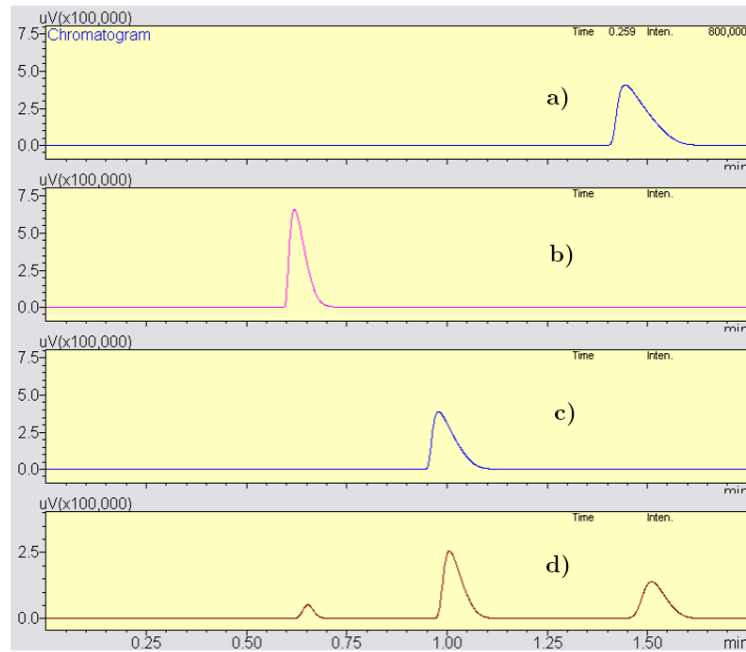


Fig. 3.6 Gas chromatogram showing the essential peaks obtained for **a)** carbon dioxide **b)** nitrogen **c)** methane and **d)** biogas sample. The x and y axes represent retention time and signal intensity, respectively.

3.1.5 Data and statistical analysis

3.1.5.1 Specific biogas and methane production

The digester performance was expressed in terms of specific biogas and methane yields (SBY and SMY, respectively), which were determined using the following equations:

$$SBY = \frac{V_B}{OLR \times V_R} \quad (3.7)$$

$$SMY = \frac{\%CH_4 \times V_B}{OLR \times V_R} \quad (3.8)$$

where V_B is the daily biogas volume (L), OLR is the organic loading rate ($gVS L^{-1} day^{-1}$), and V_R is the working reactor volume (L)

3.1.5.2 Kinetic modelling

3.1.5.2.1 First-order model

First-order kinetics was employed to assess the hydrolysis rate of the substrate. The kinetic constant can be determined using equation 3.9.

$$Y = Y_0[1 - e^{-kt}] \quad (3.9)$$

where Y is cumulative methane yield at time t (L kgVS⁻¹), Y_0 is the ultimate methane yield (L kgVS⁻¹), t is the digestion period (day), and k is the first-order rate constant or the hydrolysis rate constant.

3.1.5.2.2 Modified Gompertz model

A modified Gompertz equation was used to model the methane production profile to determine the methane production potential, maximum methane production rate and lag phase for each condition.

$$Y = A \exp \left\{ - \exp \left[\frac{\mu_m e}{A} (\lambda - t) + 1 \right] \right\} \quad (3.10)$$

where Y is cumulative methane yield at time t (L kgVS⁻¹), A represents methane production potential (L kgVS⁻¹), μ_m is maximum methane production rate (L kgVS⁻¹ day⁻¹), λ refers to lag phase time (day), e is equal to $\exp(1)$ and t is the digestion period (day). The parameters in both equations were estimated by the Solve system of nonlinear equations and best-fitting with the experimental data using MATLAB R2018a.

3.1.5.3 Energy balance assessment

The analysis of energy balance was performed based on the experimental data (specific methane yield, SMY) and 1 kg of dried biomass. All equations, constants and assumptions were based on the previous studies on the co-digestion of agriculture residues (Paranhos et al., 2020; Shirzad et al., 2019; Valenti et al., 2018b). The energy potential of methane was calculated using the following equation:

$$\text{Energy potential of methane} = \frac{\text{SMY(TS)} \times \text{LHV}}{3,600} \quad (3.11)$$

where the energy potential of methane is the estimated heat energy released from methane after combustion (kWh-e kgTS^{-1}), SMY is the specific methane yield ($\text{m}^3 \text{kgTS}^{-1}$), 3,600 is the number of seconds in an hour (a conversion factor for kJ to kWh), and LHV is the low heating value of methane at STP ($35,800 \text{ kJ m}^{-3}$). The low heating value of methane was used as it represents the amount of heat available from the fuel (methane) after the latent heat of vaporisation to the gas engine. The unit kWh-e is defined as kilowatt-hour equivalent, representing the unit of kJh s^{-1} . Heat and electricity as energy outputs were assumed to be generated from methane gas by using a CHP unit. These outputs can be calculated using the following equations:

$$\text{Heat output} = \frac{M \times \text{HHV} \times \eta_{\text{CHP}}}{3,600} \quad (3.12)$$

$$\text{Electricity output} = \frac{M \times \text{HHV} \times \eta_{\text{G}}}{3,600} \quad (3.13)$$

$$M = \frac{\text{SMY}(\text{TS}) \times 16}{T \times 0.082} \quad (3.14)$$

where M is methane production ($\text{kg}_{\text{CH}_4} \text{kgTS}^{-1}$), 16 is the molecular weight of methane (kg kmol^{-1}), 0.082 is a gas constant ($\text{m}^3_{\text{atm}} \text{K}^{-1} \text{kmol}^{-1}$), T is the temperature of methane gas (K), 3,600 is the number of seconds in an hour (a conversion factor for kJ to kWh), HHV is the high heating value of methane ($55,600 \text{ kJ kg}^{-1}$), η_{CHP} is the thermal efficiency of the CHP (0.39), and η_{G} is the electrical efficiency of the gas engine (0.41). The efficiency values were quoted for the reciprocating engine CHP installation (DOE, 2016).

The energy inputs such as heat for maintaining the digester temperature and electricity for powering the equipment were calculated from the following equations:

$$\text{Heat input} = \frac{\frac{m}{\%TS_D} \times C_p \times (T_D - T_A) \times 1.3}{3,600} \quad (3.15)$$

$$\text{Electricity input} = \frac{m}{\%TS_D} \times 0.00788 \quad (3.16)$$

where m is the weight of the dried biomass, which, in this case, is assumed to be 1 kg for energy balance analysis, $\%TS_D$ is the total solid content of the material (for *Euphorbia tirucalli*, the $\%TS_D$ is 15.37), C_p is the specific heat capacity of the fresh material ($4.12 \text{ kJ kg}^{-1} \text{ K}^{-1}$), 3,600 is the number of seconds in an hour (a conversion factor for kJ to kWh), T_D is the targeted digester temperature (K), T_A is the ambient temperature of the fresh material, which is assumed to be 20°C or 293 K. Previous studies of the energy balance assessment of anaerobic digestion system assumed that the extra heat required to maintain the AD system was approximated 20–30% (Bustamante and Liao, 2017; Valenti et al., 2018b; Yue et al., 2013). Therefore, 1.3 was used in this study refer to the additional heat required to maintain the digester temperature in order to compensate heat loss. 0.00788 is the average value of the electricity demand for digester operation (kWh kg^{-1}). The value was previously quoted in the literature but further reference could not be traced (Valenti et al., 2018b). A lower value of electricity consumption was found in another source in the literature for biogas system of separated slurry (Hamelin et al., 2010). The value was 5.60 kWh per 1,000 kg of the mixed biomass or $0.00560 \text{ kWh kg}^{-1}$. The higher factor values (1.3 and $0.00788 \text{ kWh kg}^{-1}$) were used to obtain an upper limit of the energy input and hence a lower limit of the net energy output.

The energy generation efficiency can be calculated from the equation below:

$$\text{Energy generation efficiency (\%)} = \frac{\text{Net energy output}}{\text{Energy potential of methane}} \times 100 \quad (3.17)$$

3.1.5.4 Statistical analysis

All data for BMP experiments are presented as means $\pm$ standard deviation of triplicate measurements. Analysis of variance (ANOVA) and Tukey HSD tests were performed using the software Origin 2017 to test the statistical significance of the data. Values of p lower than 0.001 (***), between 0.001 and 0.01 (**), and between 0.01 and 0.05 (*) were considered to be statistically significant.

3.2 Experimental procedures for PHA production experiments

3.2.1 Materials

3.2.1.1 Substrate

Immature *Opuntia ficus-indica* cladodes (approximately 0.5-year-old) were purchased from Pepe Aromas farm, Portugal and delivered to the laboratory at Oxford.

As shown in **Fig. 3.7**, The cladodes were sliced into pieces of approximately 3–5 cm by knife and were ground using a food processor (Magimix® Cuisine 5200, 1,100 W). The homogeneously ground material was then centrifuged at 12,000 $\times g$ to separate liquid from the pulp. The *Opuntia* extract liquid (OLE) was immediately stored in an airtight container and frozen at -20 °C until use when the substrate was thawed overnight at room temperature. To sterilise the substrate, the extracted liquid was autoclaved at 120 °C for 20 minutes and left to cool down at room temperature. Precipitates formed during autoclaving were removed by centrifugation at 4,000 $\times g$ for 15 minutes. The liquid was transferred aseptically to a glass bottle and stored at room temperature for use on the following day.

Sterile standard fructose solution (5 g L⁻¹) was used as a reference compound. Fructose was selected in this study as a comparative carbon source to assess the effectiveness of OLE for *C. necator* growth and PHA production. Fructose has also

been widely accepted as an appropriate carbon source for *C. necator* in the literature and at an industrial scale (Baei et al., 2011; Suwannasing et al., 2011; Yousuf and Winterburn, 2016).

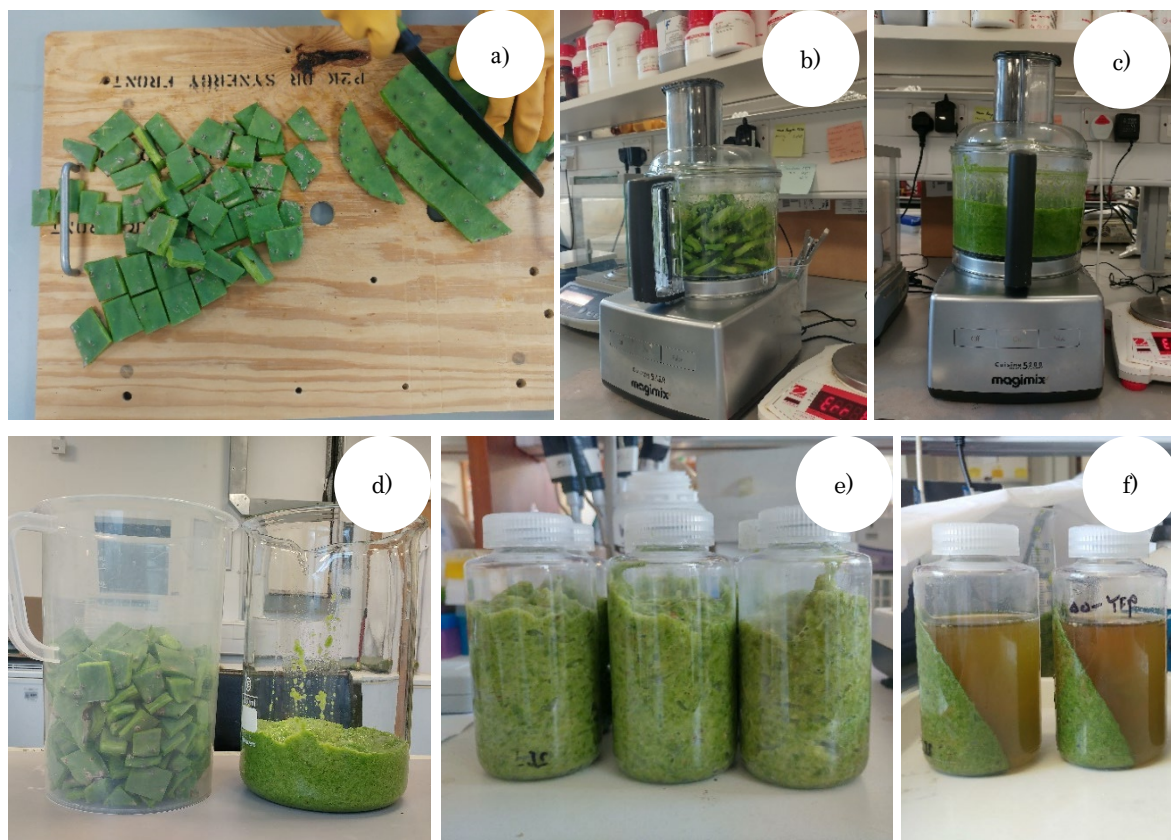


Fig. 3.7 Preparation process of *Opuntia* liquid extract (OLE). **a)** Slicing *Opuntia* cladodes before grinding. **b) – d)** Grinding prickly pear pieces until the material became homogeneous. **e) – f)** Using high-speed centrifugation to separate *Opuntia* liquid from the solid pulp.

3.2.1.2 Seed culture

The strain of *Cupriavidus necator* was re-activated from the agar plate (**Fig. 3.8a**). A single colony was inoculated into 50 ml of nutrient LB broth to ensure cell growth after overnight culture. After 24 hours, the culture was centrifuged at 2,000xg and 4 °C for 10 minutes to harvest the cells. The supernatant was discarded, and the cells were resuspended in 20 ml of the culture medium (**Section 3.2.1.3**). This was used as the seed culture for the experiment.

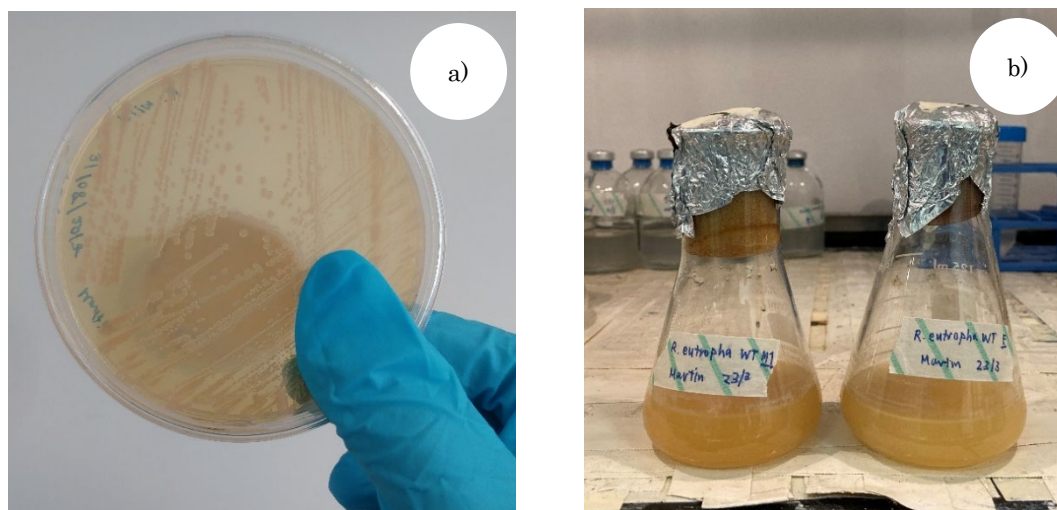


Fig. 3.8 a) *C. necator* H16 on an LB agar plate b) Flasks of *C. necator* H16 in LB broth for preparation of seed culture after 24 hours.

3.2.1.3 Culture medium

The culture medium was prepared according to the recipe listed in **Table 3.2** and autoclaved. Solutions A, C, D and E were prepared in separate bottles and autoclaved at 120 °C (**Table 3.3**). Vitamin solution (B) was sterilised by using a 0.02 µm membrane filter (Millipore, US). The medium bottle was left to cool down in a 50 °C water bath and subsequently mixed aseptically with 5 ml of solution B and 20 ml of solution C. Buffer solutions (D and E) were added to the culture medium before inoculation. For 100 ml of the medium, 5 ml of solution D and 2 ml of solution E were required.

Table 3.2 Composition of the culture medium for *C. necator* H16 fermentation

Compound	Amount (g)
NH ₄ Cl	1.000
MgSO ₄ 7H ₂ O	0.500
CaCl ₂ 2H ₂ O	0.010
MnCl ₂ 4H ₂ O	0.005
NaVO ₃ H ₂ O	0.005
Trace element solution (Solution A)	5 ml
Deionised water	915 ml

The recipes for necessary solutions required for the preparation of the culture medium are presented in the following table.

Table 3.3 Chemicals used in the preparation of different solutions for mixing the culture medium

Solution type	Compound	Amount (g L ⁻¹)
Solution A	ZnSO ₄ 7H ₂ O	0.100
Trace element solution	MnCl ₂ 4H ₂ O	0.030
	H ₃ BO ₃	0.300
	CoCl ₂ 6H ₂ O	0.200
	CuCl ₂ 2H ₂ O	0.010
	NiCl ₂ 6H ₂ O	0.020
	Na ₂ MoO ₄ 2H ₂ O	0.030
Solution B	Riboflavin	0.100
Standard vitamin solution	Thiamine-HCl 2H ₂ O	0.500
	Nicotinic acid	0.500
	Pyridoxine-HCl	0.500
	Ca-pantothenate	0.500
	Biotin	0.001
	Folic acid	0.002
	Vitamin B12	0.010
Solution C	Ferric ammonium citrate	2.500
Solution D	KH ₂ PO ₄	46.00
Phosphate buffer solution	Na ₂ HPO ₄ 2H ₂ O	58.00
Solution E	NaHCO ₃	100.0
Carbonate buffer solution		

3.2.2 Initial experiments

Initial experiments were performed to gain information on the growth of *C. necator* H16 and its kinetics using *Opuntia* liquid extract (OLE) as a substrate under different culture conditions. The liquid culture was prepared by mixing 10 ml of the medium and 10 ml of the substrate. It was then pH-adjusted to 6.8 with HCl and NaOH solutions and transferred into a 50 ml centrifuge tube before inoculation. The seed culture volume was calculated to ensure that the starting optical density (OD) of the culture was 0.05. After inoculation, the tubes were placed in a rack and incubated at 30 °C with a stirring speed of 150 rpm. Samples were taken at times for measurement of OD and pH. All experiments were performed in triplicate.

3.2.3 Flask fermentation experiment

The experiment was performed in triplicate of 250 ml Erlenmeyer flasks with 50 ml working volume. Based on the results obtained from the initial experiments, deionised water was used instead of the medium solution but the ratio between water and the substrate was reserved at 1:1. Thus, each flask contained 25 ml of water and 25 ml of substrate. The culture pH was adjusted to 6.8 before inoculation. The volume of the seed culture was calculated to achieve the starting culture OD of 0.05. The flasks were placed in an orbital shaking incubator set at 30 °C and 150 rpm. Samples were taken at 3-hour intervals and measurements of OD, pH, metabolite concentration, biomass concentration and PHB content were performed.

3.2.4 Analytical methods for PHA production

3.2.4.1 Optical density (OD₆₀₀) measurement

The optical density (OD₆₀₀) is an indirect method to measure bacterial growth by determining cell density from the amount of light absorbed by cells proportional to the cell concentration. The OD of *C. necator* cells was measured using a UV-1800 spectrophotometer (Shimadzu, Milton Keynes, UK). The sample was pipetted into a polystyrene microcuvette (Fisher Scientific, UK). The scan was performed at a wavelength of 600 nm. To ensure the accuracy of the measurement according to the Beer-Lambert law, the OD readings were kept between 0.05 and 0.9. Samples with absorbance > 0.9 were diluted with water and the reading was multiplied by the dilution factor to obtain an actual OD result.

3.2.4.2 Biomass determination

Biomass content was determined by means of cell dry weight. Two ml sample of the liquid culture taken from the tube or flask was transferred into an Eppendorf

microcentrifuge tube. The samples were centrifuged at 4°C and 13,000xg for 20 minutes. The supernatant was removed and stored in a separate microcentrifuge tube for metabolite analysis. The remaining biomass was freeze-dried for 24 hours and the dried sample was weighed.

3.2.4.3 PHB extraction

Cells need to be disrupted in order to extract PHB, which is an intracellular product, from the biomass. The cell disruption process was achieved by cell lyophilisation as described in **Section 3.2.4.3**. The dried biomass was then transferred into a Teflon-stoppered glass tube and chloroform (100 ml g_{cell}⁻¹) was added to the tube in order to extract PHB from the lysed cells. The tube was closed tightly and left at room temperature for 48 hours. Afterwards, the liquid was filtered to remove cell debris via vacuum filtration. The filtrate was transferred to a glass beaker in a fume hood where it was left for chloroform to completely evaporate. A 10-fold volume of pre-cooled methanol was later added to the beaker to precipitate PHB overnight. The PHB was subsequently dried at room temperature and finally weighed (**Fig. 3.9**).

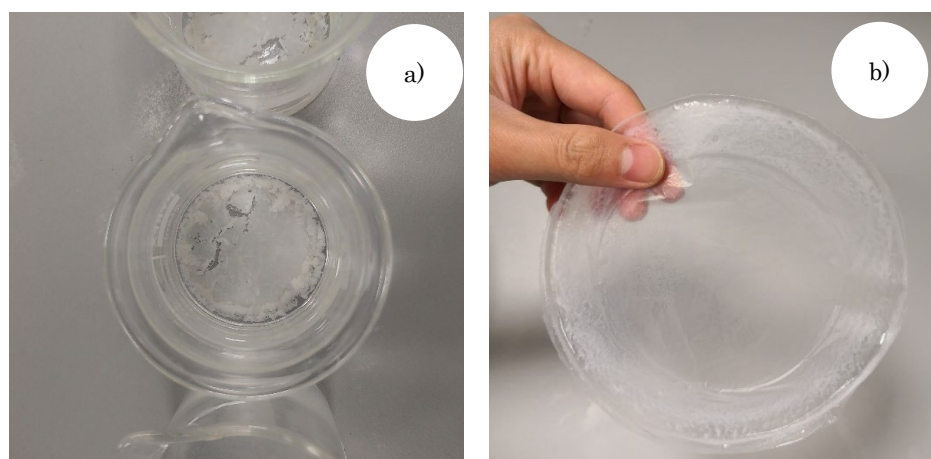


Fig. 3.9 PHB extraction process **a)** Precipitation of PHB after adding methanol in the final step. **b)** Dried PHB extracted from 300 ml of culture using fructose as a substrate.

3.2.4.4 PHB identification

Fourier-transform infrared (FTIR) spectroscopy was employed to characterise the PHB sample. Sample preparation was not required for this analysis. Infrared spectroscopy was carried out using a Digilab Excalibur Fourier Transform Spectrometer (Agilent, USA). The dried PHB samples obtained from **Section 3.2.4.3** were directly compressed and flatten to a uniform thickness on the diamond window, which was used to measure the sample IR spectrum.

3.2.4.5 PHB quantification

Gas chromatography (GC) was employed for PHB quantification according to the previously developed method (Jung et al., 2019). After lyophilisation, the dried biomass was ground to ensure sample homogeneity by using a pestle and mortar. Ten mg of each sample was weighed in a Teflon-stoppered glass tube and chloroform (1.00 ml), methanol (0.85 ml) and sulphuric acid (0.15 ml) were added to the sample. The tube was then incubated at 105°C for 2 hours, cooled to room temperature, and incubated on ice for 10 minutes. 0.5 ml of deionised water was added to the tube. The sample was vortexed for 1 minute and then transferred to a microcentrifuge tube. The tube was centrifuged at 2,000xg for 5 minutes and the organic phase (bottom layer) was extracted by a pipette into a GC vial.

PHB quantification was conducted by using gas chromatography (Shimadzu GC-2010, Japan) equipped with a flame ionisation detector (GC-FID) and a 30 m × 0.25 mm × 0.25 µm fused-silica capillary column (ZB-FFAP). Helium was used as a carrier gas at a flow rate of 1.74 ml min⁻¹. The temperatures of the detector and the

injector were 350 °C and 250 °C, respectively. The oven temperature was set at 80 °C for 4 minutes and then increased at a rate of 16 °C min⁻¹ to 160 °C.

A standard calibration curve of PHB was constructed (**Fig. 3.10**). Five known concentration solutions were prepared by weighing 1.0, 2.0, 3.0, 5.0, and 10.0 mg of pure poly-3-hydroxybutyric acid powder (Sigma Aldrich, UK) in Teflon-stoppered glass tubes. The following steps followed the same procedure for sample preparation as described above.

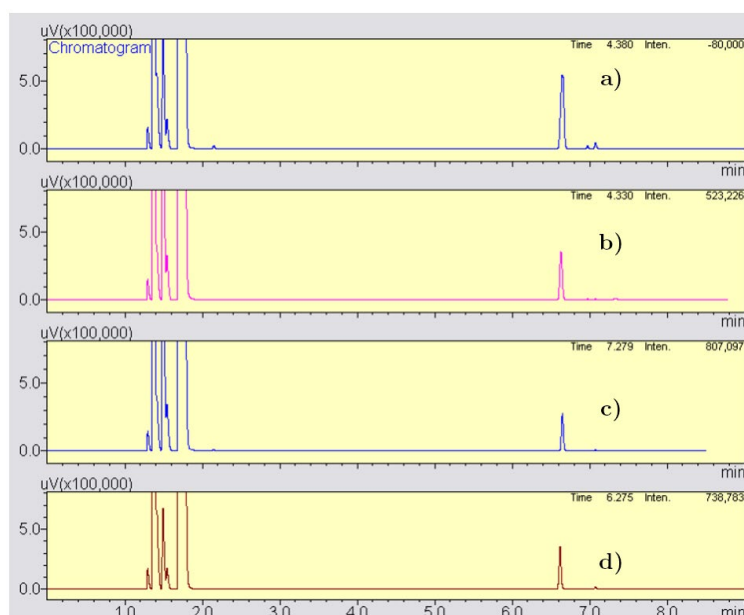


Fig. 3.10 GC chromatogram showing the peaks corresponding to PHB at 6.6 minutes. **a) – c)** Pure PHB solutions at concentrations of 10.0, 5.0, and 3.0 g L⁻¹, respectively. **d)** PHB extracted from the culture of *Opuntia* liquid extract using *C. necator* H16.

3.2.4.6 Metabolite analysis

3.2.4.6.1 Sample preparation for GC-MS

Two ml samples of the liquid culture taken from the flask were transferred into an Eppendorf microcentrifuge tube. Each was centrifuged at 4 °C and 13,000xg for 20 minutes. The 100 µl supernatant was diluted to 1.0 ml with deionised water and the remaining supernatant was pipetted into a separate microcentrifuge tube for storage. The diluted sample was mixed using a vortex mixer for 30 seconds and 10 µl of liquid was pipetted into another microcentrifuge tube containing 10 µl of 0.1%

(w/v) ribitol solution as an internal standard. The sample was then freeze-dried overnight.

3.2.4.6.2 Gas chromatography—Mass spectroscopy

Dried pellets containing metabolites were dissolved in 30 µl of pyridine and incubated at 37 °C and 900 rpm for 120 minutes. The tubes were briefly spun afterwards to ensure all liquid was at the bottom of the tube. Then, 70 µl of N-Methyl-N-(trimethylsilyl)trifluoroacetamide, MSTFA (HiChrom, Berkshire, UK) was added to each tube and the samples were incubated at 37 °C with shaking at 900 rpm for 30 minutes. Again, the tubes were briefly spun and the derivatised samples were transferred to a 0.2 ml conical crimp neck glass vial (Macherey-Nagel, Düren, Germany). The metabolite analysis was conducted by GC-MS (Agilent 7890A GC coupled with Agilent 5975C quadrupole MS, USA) equipped with a 30 m × 0.25 µm Agilent HP 5-ms column. The procedure for the analysis was previously described by (Williams et al., 2010).

3.2.4.6.3 Chemical Oxygen Demand (COD)

The COD measurement of *Opuntia* liquid extract was conducted on the Hach Lange DR2800 spectrophotometer using the Hach Lange COD test, LCK 914 (Hach-Lange, Germany) The samples were analysed in triplicate, following the recommended procedures in the kit protocol

Chapter 4

Results and Discussion: Anaerobic Digestion Studies

Chapter 4 reports on the results of the anaerobic digestion studies of CAM plants. It includes several studies on the biochemical methane potential of CAM plants, anaerobic digestion of *Euphorbia tirucalli*, and anaerobic digestion of *Opuntia ficus-indica*.

4.1 Biochemical Methane Potential (BMP) of Crassulacean Acid Metabolism (CAM) plants¹

4.1.1 Aim of the study

The focus of this work was to determine the biomass characteristics of different types of CAM plants and their biochemical methane potentials. The few studies to date that focused on the bioethanol or biogas production from CAM plants have investigated just two genera, *Agave* and *Opuntia*, from the families *Asparagaceae* and *Cactaceae*. Thus, this study aimed to broaden this perspective and evaluate the potential of five genera of CAM plants from different families, specifically *Agave* (*Asparagaceae*), *Euphorbia* (*Euphorbiaceae*), *Kalanchoe* (*Crassulaceae*), *Ananas* (*Bromeliaceae*) and *Opuntia* (*Cactaceae*) as AD feedstocks using a BMP assay.

4.1.2 Biomass Characteristics of CAM plants and inoculum

The total solid (TS), volatile solid (VS), crystalline cellulose and lignin contents, and matrix polysaccharide composition of the plant biomass and inoculum are presented in **Table 4.1**. *Euphorbia*, pineapple, and maize contained the highest dry mass content (TS). While the TS percentages of these substrates were $17.55 \pm 0.48\%$,

¹ Parts of this chapter have been published in (Lueangwattanapong et al., 2020)

17.52 ± 0.41%, and 15.63 ± 0.57%, respectively, *Agave*, *Opuntia* and *Kalanchoe* had TS contents in the range 8.15–9.96%. On a dry matter (TS) basis, the VS contents of CAM plants ranged between 67.19 ± 0.54% and 88.32 ± 0.22%. Maize contained the highest organic content (VS) of 95.05 ± 0.60% and the highest crystalline cellulose of 39.92 ± 4.32% among all the substrates. The lowest percentages of crystalline cellulose were detected in *Kalanchoe* and *Opuntia* (8.09 ± 2.83% and 8.26 ± 2.50%, respectively).

Lignin content is considered to be one of the key factors that determine the methane yield of biomass since it is difficult to degrade and tends to inhibit the activity of hydrolytic enzymes (Salter, 2007). Despite having the lowest cellulose composition, *Kalanchoe* and *Opuntia* were found to contain relatively lower lignin content compared to the other plant species studied, especially maize (18.81 ± 2.19%). The lignin content of *Opuntia fragilis* (8.26 ± 2.50%) was congruent with the literature values of 7.95–13.8% of *Opuntia ficus-indica* (Kuloyo, 2012; Santos et al., 2016). The lignin content of *Agave angustifolia* (14.42 ± 3.85%) investigated in this study was also similar to the reported data (15.6 ± 0.8%) (Pérez-Pimienta et al., 2018).

Pectin and hemicellulose can be referred to collectively as matrix polysaccharides of the cell wall biomass. These polysaccharides can be degraded into monosaccharides such as arabinose, rhamnose, glucose and xylose, the percentages of which are shown in **Fig. 4.1**. Glucose and galactose accounted for the majority of sugars detected in all substrates (15.6–30.0% and 10.9–20.9%, respectively). Pineapple and maize contained the highest content of xylose (36.5% and 31.6%, respectively). Three out of five CAM substrates (*Agave*, *Euphorbia* and pineapple) had relatively higher total matrix polysaccharides (1.14%, 1.21% and 1.01%, respectively) than maize (0.95%).

The inoculum employed in this study had TS and VS contents of $4.50 \pm 0.02\%$ and $60.10 \pm 0.22\%$, respectively. This VS(%TS) value is in agreement with literature VS values of between 55.6% and 76.5% (Gonzalez-Fernandez et al., 2018; Li et al., 2013b; Wall et al., 2013). The initial pH of the inoculum was moderately alkaline (8.02). The methanogenic activity of the inoculum was tested using microcrystalline cellulose to ensure the suitability of the inoculum for BMP assay. The specific methane yield of the cellulose was $355 \pm 20.5 \text{ L}_{\text{CH}_4} \text{ kgVS}^{-1}$, which was within the expected range of 352 to 414 $\text{L}_{\text{CH}_4} \text{ kgVS}^{-1}$, according to the established guideline (Holliger et al., 2016). However, the relative standard deviation (RSD) of the BMP value for cellulose in this study was $\pm 5.77\%$, slightly higher than the recommended value of 5% mentioned in the guideline mentioned earlier. The raw values ranged from 311–412 $\text{L}_{\text{CH}_4} \text{ kgVS}^{-1}$ without an outlier identified when a statistical test (Dixon's) was performed. This means that the data values were widely spread out from the mean and therefore not very precise.

Table 4.1 Biomass characteristics of inoculum and plant feedstocks. Values are means of triplicate samples $\pm$ standard deviations

Materials	Characteristics ¹				
	TS (%FM)	VS (%TS)	Cellulose (%TS)	Lignin (%TS)	MP (%TS) ²
Inoculum	4.50 ± 0.02	67.22 ± 0.01	-	-	-
Substrates					
Maize	15.63 ± 0.57	95.05 ± 0.60	39.92 ± 4.32	18.81 ± 2.19	0.95
<u>CAM species</u>					
<i>Agave</i>	8.51 ± 0.04	67.19 ± 0.54	27.39 ± 6.04	14.42 ± 3.85	1.14
<i>Euphorbia</i>	17.55 ± 0.48	88.32 ± 0.22	27.63 ± 1.04	13.89 ± 3.64	1.21
<i>Kalanchoe</i>	9.96 ± 0.06	80.23 ± 0.80	20.28 ± 2.18	8.09 ± 2.83	0.24
<i>Opuntia</i>	8.48 ± 0.05	91.53 ± 0.12	16.51 ± 1.34	8.26 ± 2.50	0.87
<i>Pineapple</i>	17.52 ± 0.41	85.68 ± 0.65	32.56 ± 3.71	17.35 ± 2.28	1.01

¹Cellulose, lignin and MP content were determined by the biology lab, University of York. The data are also included in the recent article published by the author (Lueangwattanapong et al., 2020). ²MP – Total sugar composition of matrix polysaccharide

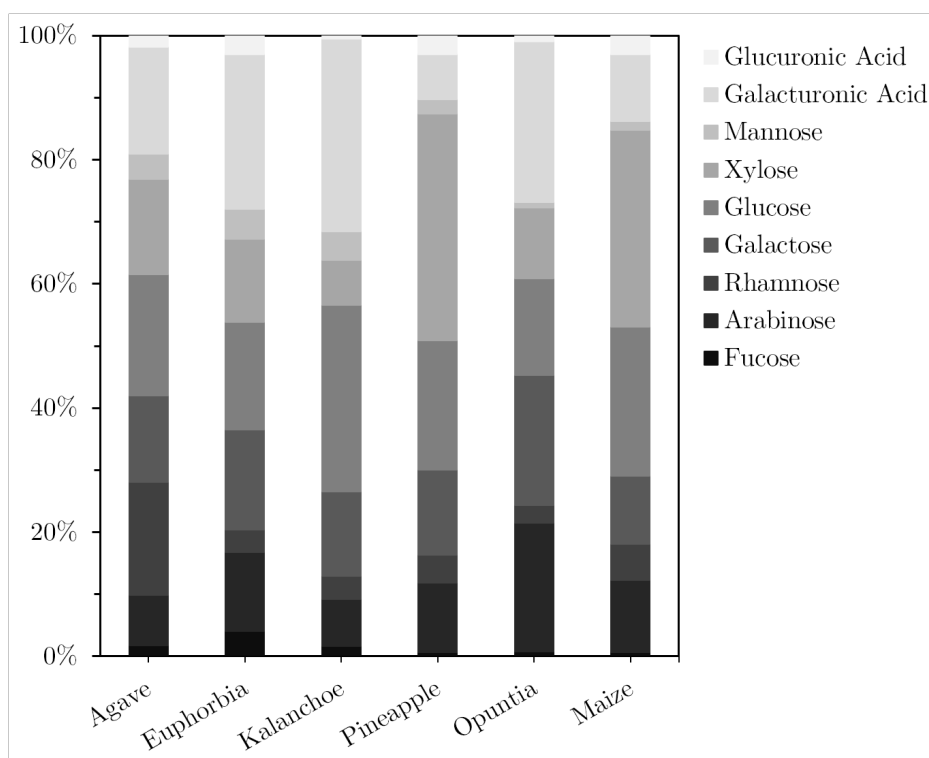


Fig. 4.1 Composition of matrix polysaccharides (MP) from different plant substrates

4.1.3 Biochemical methane potential of CAM plants

4.1.3.1 Methodology

A biochemical methane potential (BMP) assay was carried out to determine the methane potential of five different CAM plant samples and maize. The detailed description of the BMP setup is described in **Chapter 2, Section 2.1.2**. The inoculum to substrate (I/S) ratio of 2:1 volatile solid (VS) weight was used, and the test was run against blank and positive control (microcrystalline cellulose). Biogas and liquid samples were collected at regular intervals to determine gas volume, gas composition, pH and VFA content.

4.1.3.2 Results

The BMP curves of anaerobic digestion of six different substrates are presented in **Fig. 4.2**. Similar curves of cumulative methane yields were observed for all feedstocks. The experiment was terminated on day 38 when the cumulative methane production has reached a plateau and the daily methane production was

less than 1% of the total. Maize, which was used as a reference plant, produced a final cumulative methane yield of 308 ± 22 L kgVS⁻¹. The specific methane yields of CAM plants were comparable to that of maize; the values in ascending order were 281 ± 14 , 298 ± 28 , 301 ± 19 , 338 ± 22 and 382 ± 19 L kgVS⁻¹ for *Euphorbia*, pineapple, *Opuntia*, *Kalanchoe* and *Agave*, respectively. During the first 10 days, *Agave* and *Kalanchoe* were the two substrates observed to give biomethane yields exceeding 200 L kgVS⁻¹ (205 and 221 L kgVS⁻¹, respectively). Their steeper slopes during this initial period suggested that their hydrolysis rates were potentially higher than other plants. In **Section 4.1.3.4**, the correlation between the hydrolysis rate and the plant composition was further discussed.

There was virtually no difference in methane compositions among different substrates. The average methane content was $56.4 \pm 1.5\%$ after 120 hours of digestion. The percentages of methane for all substrates ranged between 61.9% and 62.9% on day 15 and remained constant until the end of the experiment. By day 38, the mean percentage of methane for all substrates was $63.3 \pm 1.1\%$. Methane and carbon dioxide composition can also be estimated using the Buswell equation if the chemical composition of an organic material is known ($C_cH_hO_oN_nS_s$) (Buswell and Mueller, 1952). Although the elemental analysis of the CAM substrates used in this study was not performed, CAM plants have been claimed to have low nitrogen content. This means that, according to the Buswell equation, methane and carbon dioxide were mainly produced from carbohydrate (cellulose) and their composition should be close to 50%, regardless of the CAM substrates. However, the average values of the methane content in this study were higher than 60%. A eudiometer filled with an acidified 75% (v/v) saturated sodium chloride solution (pH 2) was used to determine the biogas volume. The solution was prepared once at the beginning of the experiment and was used for the course of this experiment. Dissolution of

CO₂ may have occurred when the solution has been reused several times and hence the composition of CO₂ was underestimated, leading to an apparent increase in methane concentration in the biogas. However, methane dissolution is assumed to be low and therefore the BMP value is still considered to be accurate. Another reason could be that the headspace pressure was still slightly positive compared to the atmospheric pressure, which increased the amount of CO₂ dissolved in the solution. The gas sample collected from the vial headspace could potentially consist of a lower percentage of CO₂.

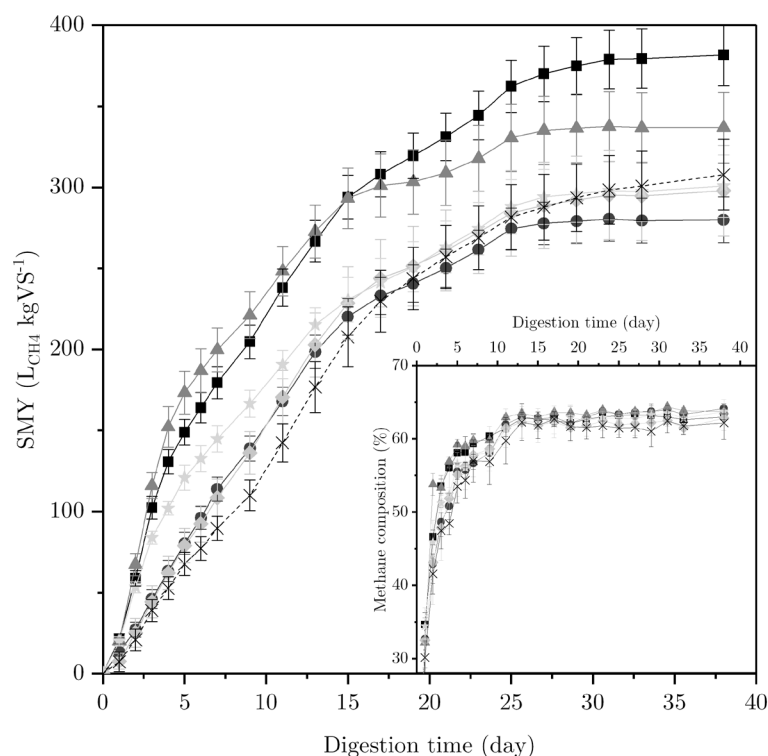


Fig. 4.2 BMP profiles showing cumulative specific methane yield (SMY) from six different substrates at 39°C. (■), (●), (▲), (◆), (★) and (×) denote the six substrates which are *Agave*, *Euphorbia*, *Kalanchoe*, pineapple, *Opuntia* and maize, respectively. Methane composition is shown in the inset on the bottom right of this graph. Error bars represent standard deviations of triplicate samples.

One-way ANOVA was used to determine whether there are any statistical differences between the BMP values on day 38 from CAM substrates and that obtained from maize. It was found that there was no significant difference ($p = 0.347$) in methane yields between the CAM plants tested and maize (i.e. $p > 0.05$). This suggests that the CAM species used in this study could compete with maize,

which is a conventional energy crop, in terms of methane production. This study therefore demonstrates that CAM crops could potentially be utilised as viable biomass for anaerobic digestion, particularly in semi-arid areas where conventional bioenergy crops such as maize struggle to grow.

Liquid samples were collected on the same day as the gas was sampled for pH and volatile fatty acid (VFA) determination. Total volatile fatty acid (TVFA) concentration was estimated from the sum of six acids which were acetate, propionate, isobutyrate, *n*-butyrate, isovalerate, and *n*-valerate. Caproic acid (C6) was not present in any of the samples analysed or might be present under the detection limit and therefore the TVFA only accounted for the six acids mentioned earlier. In all batches, acetate and propionate were the predominant acids detected with *n*-butyrate present in a very low concentration. The sum of these three acids also accounted for 99% of the TVFA concentrations.

TVFA concentrations increased in all substrates after 24 hours of incubation (**Fig. 4.3a**). The highest change in TVFA was seen in *Kalanchoe*; the concentration increased from $99 \pm 25 \text{ mg L}^{-1}$ to $343 \pm 34 \text{ mg L}^{-1}$. The TVFA concentrations after 24 hours in ascending order were 134 ± 20 , 177 ± 32 , 202 ± 34 , 239 ± 14 and $272 \pm 19 \text{ mg L}^{-1}$ for *Euphorbia*, maize, pineapple, *Opuntia* and *Agave*, respectively. VFAs were subsequently metabolised rapidly, which was seen as a significant drop in TVFA concentrations for all substrates by day 3. After 120 hours, the levels of TVFA for all substrates were below 10 mg L^{-1} . Rapid substrate hydrolysis was assumed to occur between 24 and 72 hours, whilst more easily biodegradable substrates were biotransformed in the absence of acid accumulation. In all cases, the TVFA concentrations remained lower than 100 mg L^{-1} until day 25 onwards, when the acids in the liquid samples were undetectable. The inoculum to substrate ratio of

2:1 on a VS basis selected for this study was therefore considered to appropriate, as acid accumulation, which could hinder biogas production, was not observed at any point during the experiment (Holliger et al., 2016).

Fig. 4.3b represents the pH profiles of different substrates over the 38-day incubation. There was a close link between TVFA and culture pH in anaerobic digestion. Increasing TVFA production resulted in a decline in pH and vice versa (Li et al., 2017). The initial pH of the inoculum was 8.02, however, after mixing with the medium solution, the final pH of the mixture at the beginning of incubation was 8.50. The pH values of all substrates then decreased to between 7.98 and 8.25 after 24 hours of digestion, when TVFA concentrations increased accordingly (**Fig. 4.3b**). Even though TVFA concentrations were below 100 mg L⁻¹ and there was no VFA accumulation detected after 120 hours of digestion, the pH of samples continued to decrease. This suggests that the pH drop could be attributed to the accumulation of other organic acids or long-chain fatty acids (LCFAs), which were not detected by the analytical methods but may have been present. After 38 days, the final pH values of all substrates were between 7.58 ± 0.10 and 7.70 ± 0.05 . It has been reported that methanogenic microorganisms are sensitive to a change in pH and the optimal range for biogas production was 6.8–7.2 (Ward et al., 2008). In this study, moderately alkaline pHs of the samples did not have an apparent detrimental effect on methane production. Therefore, different optimal pH values could be expected, depending on the substrate type and source of inoculum employed.

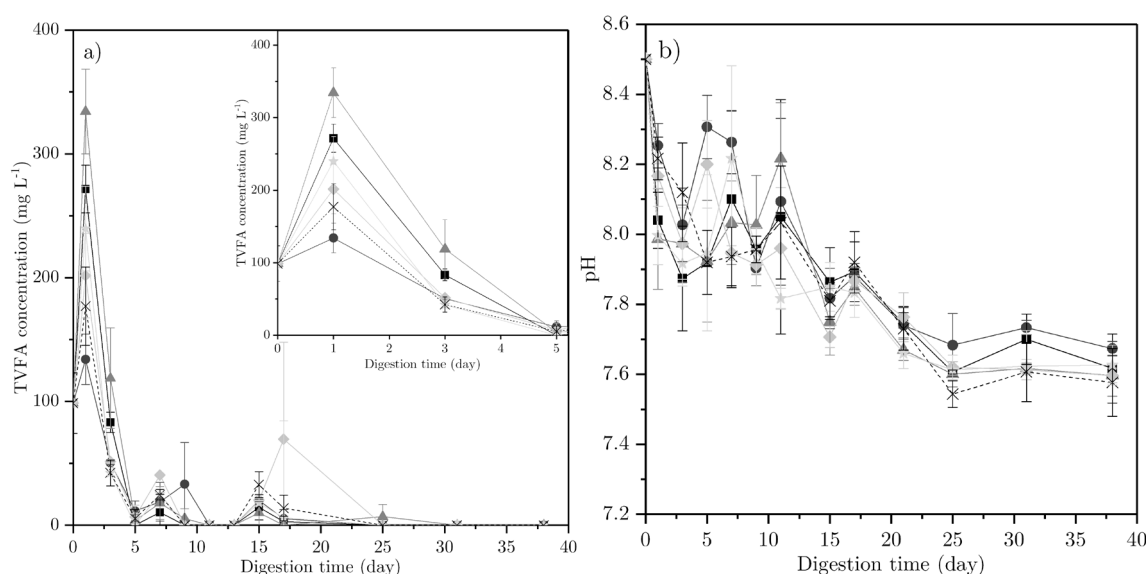


Fig. 4.3 a) Total volatile fatty acid (TVFA) concentration and **b)** pH profiles from six different substrates at 39°C. (—■—), (—●—), (—▲—), (—◆—), (—★—) and (—×—) denote the six substrates, which were *Agave*, *Euphorbia*, *Kalanchoe*, pineapple, *Opuntia* and maize, respectively. Error bars represent standard deviations of triplicate samples.

4.1.3.3 Comparison with published studies

The specific methane yields (SMYs) obtained from the 38-day anaerobic digestion of individual CAM substrates (Table 4.2) were compared with CAM species previously reported. *Agave angustifolia* Haw. in this study gave relatively higher methane yield (382 L kgVS^{-1}) than the value reported for *Agave angustifolia* (193 L kgVS^{-1}) (Gómez-Guerrero et al., 2019) and *Agave sisalana* ($176\text{--}320 \text{ L kgVS}^{-1}$) (Mshandete et al., 2006, 2004). *Agave angustifolia* is known for the production of alcoholic beverages, as well as *Agave tequilana*. Other applications of *Agave angustifolia* are as an additive, medicine and prebiotic (Stewart, 2015). It is thus a plant species, which has already been grown commercially and at scale, so clearly has the potential to be scaled-up and exploited as a bioenergy source. *Euphorbia tirucalli* has long been used as an AD substrate for the study of biogas production, but the published studies only reported biogas production without methane yields obtained from this plant (Rajasekaran et al., 1989; Sow et al., 1989). There have been no reports of methane production from *Euphorbia virosa*, but a few studies on AD of *E. tirucalli* could be found. The methane yield of *E. virosa* investigated in this

study was 281 L kgVS⁻¹, which is within the data range of 77–319 L kgVS⁻¹ (Hastilestari et al., 2013) reported in the literature for *E. tirucalli*.

The cumulative methane yield from *Kalanchoe daigremontiana* was 337 L kgVS⁻¹, which was relatively higher than the other substrates listed in **Table 4.2**. This study is the first to report on the methane production from a plant of the genus *Kalanchoe* and hence there is no comparison of the gas yields from the same species. *Kalanchoe daigremontiana*, commonly known as the ‘mother of thousands’, is cultivated horticulturally and is not widely grown for commercial purposes. The plant however is easy to propagate vegetatively from plantlets, is easy to harvest, and has a low lignin content in its cell walls. These characteristics are attributes that are favourable in feedstock for bioenergy generation.

Opuntia species, commonly known as prickly pears, have gained increasing interest as a bioenergy feedstock for bioethanol and biogas production (Mason et al., 2015). The prickly pears are easy to propagate from pads, and the spineless varieties of *Opuntia* can be easily harvested and coppiced. In addition, the cladodes are low in lignin. These characteristics are desirable features of biomass for anaerobic digestion. *Opuntia fragilis* investigated here produced a comparable methane yield (301 L kgVS⁻¹) to the values previously reported for other *Opuntia* spp.: 423 L kgVS⁻¹ for *O. ficus-indica* (Calabrò et al., 2018) and 143 L kgVS⁻¹ for *O. maxima* (Ramos-Suárez et al., 2014)

Table 4.2 Comparison of specific methane yields between the current study and those in the literature

Substrate	Species	Reactor modes	Digester/ working volume (l)		Inoculum type	TS (%FM)	VS (%TS)	CH ₄ yield (unit)	Sources
Agave	<i>A. tequilana</i>	AnSBR	1.2	/ -	AGS	-	-	280 (L kgCOD ⁻¹)	(Breton-Deval et al., 2018)
	<i>A. tequilana</i>	UASB	3.6	/ -	AGS	-	-	260 (L kgCOD ⁻¹)	(Arreola-Vargas et al., 2015)
	<i>A. sisalana</i>	-	-	/ -	Digestate	14.66	81.99	292 (L kgVS ⁻¹)	(Muthangya et al., 2009)
	<i>A. sisalana</i>	Semi-continuous	2	/ -	WB	-	-	250–310 (L kgCOD ⁻¹)	(Mshandete et al., 2008)
	<i>A. sisalana</i>	Batch	1	/ 0.61	WB	91.6	92	176–216 (L kgVS ⁻¹)	(Mshandete et al., 2006)
	<i>A. sisalana</i>	Batch	1	/ 0.60	WB	9	87.5	320 (L kgVS ⁻¹)	(Mshandete et al., 2004)
	<i>A. angustifolia</i>	Batch	0.25	/ 0.15	FD	8.51	67.19	382 (L kgVS⁻¹)	This study
Euphorbia	<i>E. tirucalli</i>	Batch	2.50	/ -	CDS	-	-	19.2 (L _{biogas})	(Rajasekaran et al., 1989)
	<i>E. tirucalli</i> L.	Batch	1.25	/ -	SL	-	-	77–319 (L kgVS ⁻¹)	(Hastilestari et al., 2013)
	<i>E. virosa</i>	Batch	0.25	/ 0.15	FD	17.55	88.32	281 (L kgVS⁻¹)	This study
Kalanchoe	<i>K. daigremontiana</i>	Batch	0.25	/ 0.15	FD	9.96	80.23	338 (L kgVS⁻¹)	This study
Pineapple	<i>A. comosus</i>	Semi-continuous	-	/ -	-	0.99–1.61	10.9–60.1	114 (L kg ⁻¹)	(Azouma et al., 2018)
	<i>A. comosus</i>	Batch	5	/ 2	SL	-	-	430 (L kg ⁻¹)	(Jehan et al., 2017)
	<i>A. comosus</i>	Continuous	12	/ -	FD	-	-	110–430 (L kgCOD ⁻¹)	(Namsree et al., 2012)
	<i>A. comosus</i>	Fed-batch	6.5	/ 4	CDS	-	-	210–470 (L kgVS ⁻¹)	(Rani and Nand, 2004)
	<i>A. comosus</i>	Batch	0.25	/ 0.15	FD	17.52	85.68	298 (L kgVS⁻¹)	This study
Opuntia	<i>O. ficus-indica</i>	Batch	2.2	/ 1.54	Digestate	3.8–4.0	67.5–69.3	237–260 (L kgVS ⁻¹)	(Valenti et al., 2018a)
	<i>O. heliabravoana</i>	Semi-continuous	-	/ 1.54	RM	9.75	85.23	154–257 (L kgVS ⁻¹)	(Quintanar-Orozco et al., 2018)
	<i>O. ficus-indica</i>	Batch	1.1	/ 1	AID	6.2–7.5	-	289–604 (L kgVS ⁻¹)	(Calabrò et al., 2018)
	<i>O. maxima</i>	Batch	1	/ -	AGS	14.1	81.7	143 (L kgVS ⁻¹)	(Ramos-Suárez et al., 2014)
	<i>O. maxima</i>	Semi-continuous	-	/ 3	AGS	10.9	79.8	282–321 (L kgVS ⁻¹)	(Ramos-Suárez et al., 2014)
	<i>O. fragilis</i>	Batch	0.25	/ 0.15	FD	9.46	85.75	301 (L kgVS⁻¹)	This study

AGS – Anaerobic Granular Sludge, AID – Agro-industry digestate, CDS – Cow Dung Slurry, FD – Food waste digestate, RM – Rabbit manure, SL – Slurry, WB – Wastewater Biosolids

4.1.3.4 Kinetic analysis

4.1.3.4.1 First-order model

The first-order model gave a very good fit to experimental data obtained in this study with R^2 values between 0.97 and 0.99 (**Fig. 4.4**). The first-order rate constants or the hydrolysis rate constants (k) for *Agave*, *Euphorbia*, *Kalanchoe*, pineapple and *Opuntia* were 0.098, 0.078, 0.150, 0.071 and 0.099 day⁻¹, respectively. Compared to CAM plants, the hydrolysis rate of maize was the slowest; the k value was 0.053 day⁻¹. There was a negative correlation between the lignin composition of the substrate and the hydrolysis rate ($R^2 = 0.72$); specifically, the rates decreased with increasing lignin content (inset to **Fig. 4.4**). The results therefore imply substrates with higher lignin contents are more difficult to degrade. Recalcitrant chemicals such as lignin have been known to resist biodegradation (Gerardi, 2003). The degradation of lignin is generally difficult in anaerobic condition as it was found that several lignin degradation processes obligately requires oxygen as a final electron acceptor. The rate constants for *Kalanchoe* and *Opuntia*, which had the lowest lignin contents (8.09% and 8.06% of dry biomass, respectively), were the highest of all the substrates investigated. *Euphorbia* had lignin content of 13.89%TS and its hydrolysis rate constant was the second-lowest, slightly higher than that of pineapple. However, the low BMP of *E. virosa* was possibly not only influenced by its lignin content. The plant contains milky latex, which is the most poisonous among *Euphorbia* spp. (Kontsiwe, 2013). This toxic compound may have been harmful to the microbial community thus negatively affecting the digestion performance, which led to its lower BMP.

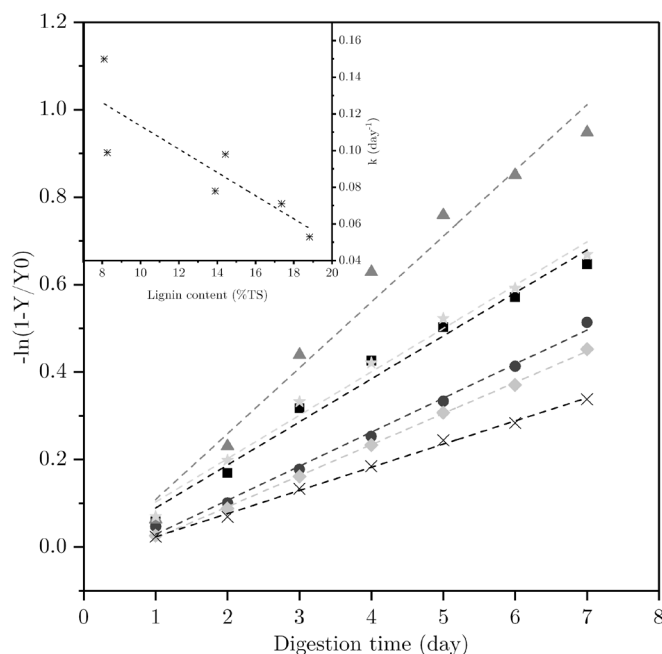


Fig. 4.4 First order kinetic plot of cumulative methane yields at time t , (Y) and ultimate methane yield (Y_0) from six different substrates during the first 7 days of digestion. (■), (●), (▲), (◆), (★) and (×) denote the six substrates, which were *Agave*, *Euphorbia*, *Kalanchoe*, pineapple, *Opuntia* and maize, respectively. The correlation between lignin composition percentages and hydrolysis rate constants (k) are shown in the top left of this graph. The constant k was estimated from the first-order rate equation (Equation 3.9, Section 3.1.5.2.1).

4.1.3.4.2 Modified Gompertz model

The modified Gompertz model parameters determined from the experimental methane yields (using Equation 2.12, Section 2.1.5.5) are presented in **Table 4.3**. The experimental data as the cumulative methane yields for all substrates had a strong fit to this model with exceptionally high R^2 values of 0.97–1.00.

The lag phase values, λ , for CAM substrates were calculated to be 0–1.02 days, which were slightly lower than that for maize (1.45 days). The relatively longer lag phase observed in the digestion of maize could be due to the plant having the highest lignin content among all the feedstocks investigated. The lag phase values for *Agave*, *Kalanchoe*, and *Opuntia* were shown as zero (-), even though the estimated values obtained from the modified Gompertz model were negative, which is mathematically possible. However, the negative numbers for the lag phase do not bear any biological meaning and hence are indicated as none (-). The highest

maximum methane production rates, μ , belonged to *Agave* and *Kalanchoe* (26.5 and 21.2 L kgVS⁻¹ d⁻¹, respectively). In contrast, maize had the lowest rate of 15.7 L kgVS⁻¹ d⁻¹, which is in agreement with literature values which ranged between 8.5 and 26.7 L kgVS⁻¹ d⁻¹ (Li et al., 2015).

The differences between the experimental specific methane yields, shown as SMY, and the methane production potential, A , ranged from 0.13% to 1.53% for the feedstocks investigated. The percentage difference between SMY and A was less than 10% and considered marginal, indicating that the modified Gompertz model accurately predicted the methane production profiles.

Table 4.3 Modified Gompertz parameters obtained from anaerobic digestion of different CAM substrates and maize as a reference material

Plant material	SMY (L kgVS ⁻¹)	Parameters from modified Gompertz model ¹			R^2
		A (L kgVS ⁻¹)	μ (L kgVS ⁻¹ d ⁻¹)	λ (d)	
Maize	307.8	312.5	15.71	1.45	1.00
<i>Agave</i>	381.7	376.9	21.18	-	0.98
<i>Euphorbia</i>	280.5	283.4	17.08	0.67	1.00
<i>Kalanchoe</i>	337.6	326.1	26.54	-	0.97
<i>Pineapple</i>	297.9	298.3	17.75	1.02	1.00
<i>Opuntia</i>	300.8	296.9	16.81	-	0.98

¹A – Methane production potential; μ_m – Maximum methane production rate; λ – Lag phase time

4.1.4 Conclusion

The results of this study confirm that CAM substrates were able to generate methane yields (298–382 L kgVS⁻¹) as high as that obtained for maize (308 L kgVS⁻¹), which is one of the typical AD commercial feedstocks. *Agave angustifolia* and *Kalanchoe daigremontiana* generated the highest methane yields. This suggests that CAM plants may become viable energy crops with yields equivalent to maize, but with the added advantage of being able to grow in the areas where rainfall is low or erratic and soil quality is poor.

4.2 Anaerobic digestion of *Euphorbia tirucalli*

4.2.1 Aim of the study

The focus of this study was to determine the suitability of *Euphorbia tirucalli* as an anaerobic digestion feedstock. The objectives of the research reported in this section were to investigate key factors affecting the specific methane yield (SMY) of *E. tirucalli* such as inoculum to substrate (I/S) ratio, pH, and operating temperature, and to study the effect of mixing ratio on the anaerobic co-digestion of *E. tirucalli* and chicken manure.

4.2.2 Characterisation of biomass and inoculum

The characteristics of the substrates and inoculum such as total solids (TS), volatile solids (VS), pH and total Kjeldahl nitrogen (TKN) were determined as shown in **Table 4.4**. The values of TS and VS content of *Euphorbia tirucalli* were $15.37 \pm 1.66\%$ and $84.16 \pm 0.60\%$, respectively. There has been only one previous study of *E. tirucalli*, which reported on the TS and VS contents of this species (Girmay and Berhe, 2014). They reported values of TS and VS, 23.87% and 86.59%, higher than those determined in this study.

The fibre analysis performed was reported in terms of neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL). Higher ratios of NDF and ADF to ADL indicate a larger proportion of cellulose and hemicellulose as compared to lignin. In this study, the values of NDF, ADF, and ADL per dry mass content of *E. tirucalli* were 27.90%, 25.99%, 19.40%, respectively, which are lower than 42.42%, 30.31% and 4.24% previously reported for this species (Girmay and Berhe, 2014). In comparison, *E. tirucalli* employed in this study had a relatively lower digestible fibre proportion (NDF and ADF) but contained substantially higher

lignin content (ADL). Since plant growth conditions, harvesting practices and plant ages all directly influence crude fibre content, but to date not previously included with these data, a clear comparison of biomass characteristics between different studies could not be made.

The total Kjeldahl nitrogen (TKN) of chicken manure (4.45%TS) in this study was 2.5 times greater than it was for *E. tirucalli* (1.79%TS). The addition of chicken manure to the substrate mixture may help improve methane production by adjusting the carbon to nitrogen (C:N) ratio of the mixture to the optimum range between 20 and 30, as previously reported in the study of batch co-digestion of sisal pulp and fish wastes (Mshandete et al., 2004).

Table 4.4 Biomass characteristics of *Euphorbia tirucalli*, chicken manure and inoculum. Values are means of replicates $\pm$ standard deviations ($n = 3$)

Parameters	<i>E. tirucalli</i>	Chicken manure	Inoculum
TS (%FM)	15.37 $\pm$ 1.66	28.91 $\pm$ 2.06	5.22 $\pm$ 0.00
VS (%TS)	84.16 $\pm$ 0.60	73.19 $\pm$ 1.66	69.99 $\pm$ 0.09
pH	-	-	8.03
NDF* (%)	27.90	35.50	-
ADF* (%)	25.99	19.77	-
ADL* (%)	19.40	10.77	-
TKN* (g/kg)	17.9	44.5	-
NH ₃ -N* (mg/kg)	-	826	-

* The analysis of these parameters was performed by an external laboratory (see **Appendix A**)

The inoculum used in this study had higher TS and VS contents (5.22 $\pm$ 0.00% and 69.99 $\pm$ 0.09%, respectively) compared to that used in the batch investigation reported in **Chapter 4, Section 4.1.2** (4.50 $\pm$ 0.02% for TS and 60.10 $\pm$ 0.22% for VS). The specific methane yield of the cellulose, referred to as inoculum activity, was 286.7 $\pm$ 20.3 L_{CH₄} kgVS⁻¹, which accounted for 69% of the theoretical yield of cellulose. This value was lower than that of the inoculum obtained from the same

source previously reported ($355.3 \pm 20.5 \text{ L}_{\text{CH}_4} \text{ kgVS}^{-1}$). The lower inoculum intrinsic activity may have affected the SMYs, especially at lower I/S ratios.

4.2.3 Experiment I – Investigation of the effect of I/S ratio, initial pH, and operating temperature on the mono-digestion of *E. tirucalli*

4.2.3.1 Objective

To determine the effect of inoculum to substrate (I/S) ratio, pH, and operating temperature on the methane production from *Euphorbia tirucalli*.

Euphorbia tirucalli is endemic in central Africa countries such as Angola, Rwanda, Senegal, Sudan, and Uganda where commercial biogas plants are still at an early stage (Kemausuor et al., 2018). Inoculum could be difficult to source in this area and become a limiting factor for setting up and operating a full-scale AD plant. Therefore, the study focused on the use of low inoculum to substrate ratios to evaluate its effect on the methane production of *Euphorbia tirucalli*.

4.2.3.2 Methodology

The bottle test based on the BMP assay was carried out to determine the specific methane yield of *Euphorbia tirucalli* at different conditions. The plant was tested at five inoculum to substrate ratios (0.5, 0.67, 1.00, 1.50, and 2.00), two pH values (7.0 and 9.0) and two operating temperatures (38°C and 55°C). Biogas and liquid samples were collected at regular intervals to determine gas volume and composition, pH, and VFA content. The modified Gompertz model was used to fit the data of cumulative methane yields obtained from the experiment to estimate the ultimate methane yield, maximum methane production rate and the lag phase. Further details of the experimental setup are presented in **Chapter 3, Section 3.1.2**.

4.2.3.3 Results

4.2.3.3.1 Effect of inoculum to substrate ratio and initial pH on methane production, total volatile fatty acid (TVFA) and sample pH

The methane curves of anaerobic digestion of *Euphorbia tirucalli* under mesophilic conditions (38°C) at two different initial pH values (7.0 and 9.0) are depicted in **Fig. 4.5a** and **Fig. 4.5d**, respectively. Similar curves of cumulative methane yields were observed for all experimental conditions. With an initial pH 7.0, the overall results showed that specific methane yields (SMYs) increased with I/S ratios (**Fig. 4.5a**). The maximum SMYs were 167, 178, 194, 183, and 206 L kgVS⁻¹, at I/S ratios of 0.5, 0.67, 1.0, 1.5 and 2.0, respectively. In **Fig. 4.5d**, there was less variation in final methane yields across different I/S ratios tested at the initial pH of 9.0. The maximum methane yields were 171, 175, 187, 186, and 188 L kgVS⁻¹, for I/S ratios of 0.5, 0.67, 1.0, 1.5 and 2.0, respectively (**Table 4.5**). Comparing methane production between two different initial pHs at the same I/S ratios, the SMY results were relatively similar, except at the I/S ratio of 2.0, where it decreased from 205 L kgVS⁻¹ (pH 7.0) to 188 L kgVS⁻¹ (pH 9.0).

The rates of methane production for the initial pH 7.0, shown as the slopes of the BMP curves, were very similar at different I/S ratios in the first 10 days (**Fig. 4.5a**). The difference in methane production rates became noticeable between days 10 and 20 when steeper slopes were observed at higher I/S ratios, with the highest rate of methane production corresponding to the I/S ratio of 2.0. A different profile of methane production rates at the initial pH 9.0 was observed (**Fig. 4.5d**). The slope became steeper with decreasing I/S ratios in the first 15 days, when the I/S ratio of 0.5 gave the highest production rate of methane. Subsequently, the rates of methane production at higher I/S ratios (1.0–2.0), rapidly increased after day 20 and surpassed those at I/S ratios of 0.5 and 0.67 by day 30.

The change in TVFA concentrations for the initial pH 7.0 over 30 days is presented in **Fig. 4.5b**. No accumulation of TVFA was detected after 30 days of digestion in any of the scenarios investigated. However, TVFA was produced very rapidly at the beginning of the experiment. The TVFA concentrations peaked on day 5, rising from 0.09 g L⁻¹ on day 0 to 3.68, 2.77, 1.82, 1.34, and 0.95 g L⁻¹ for I/S ratios of 0.5, 0.67, 1.0, 1.5 and 2.0, respectively. Acid accumulation during the first 5 days resulted in an almost complete halt of methane production. Similar results for the initial pH 9.0 are illustrated in **Fig. 4.5e**, where the TVFA peaked after 4 days of incubation. The highest TVFA concentrations were 3.57, 2.76, 1.96, 1.52, and 0.95 g L⁻¹ for I/S ratios of 0.5, 0.67, 1.0, 1.5 and 2.0, respectively.

An increase in the initial pH from 7.0 to 9.0 led to a lower consumption rate of TVFA at higher I/S ratios (**Fig. 4.5b** and **Fig. 4.5e**). For example, the TVFA concentrations for I/S ratio of 2.0 and at the initial pH 7.0 decreased from 0.95 g L⁻¹ on day 5 to 0.22 g L⁻¹ on day 15. During the same period, the TVFA concentrations slightly dropped from 0.95 g L⁻¹ to 0.86 g L⁻¹, for the same I/S ratio but at the initial pH 9.0. In contrast, the rate of acid consumption at lower I/S ratios accelerated. By day 20, the TVFA levels at I/S ratios of 0.5 and 0.67 were lower for the initial pH 9.0 (0.24 and 0.16 g L⁻¹, respectively) compared to 0.54 and 0.33 g L⁻¹ for the initial pH 7.0.

As shown in **Fig. 4.5c**, the pH values of the samples ranged from 6.42 to 7.43 over 30 days of the digestion period. The lowest pH value (6.42) corresponded to the I/S ratio of 0.5 on the fifth day after incubation when the highest concentration of TVFA was also detected (3.68 g L⁻¹). The pH values at different I/S ratios on day 5 were between 6.42 and 6.95. A subsequent increase in pH was observed and the values were between 7.22 and 7.26 by day 15, when the pH became relatively stable afterwards. The final pH values after 30 days ranged from 7.25 to 7.43. However,

the change in pH was more considerable for the samples initiated at pH 9.0 (**Fig. 4.5f**). The pH values decreased from 9.0 at the start of the study to between 6.78 and 7.58 after 5 days of incubation, the lowest of which corresponded to the I/S ratio of 0.5. The pH values then increased to between pH 7.05 and 7.65 by day 10 and to 7.26–7.63 by day 15.

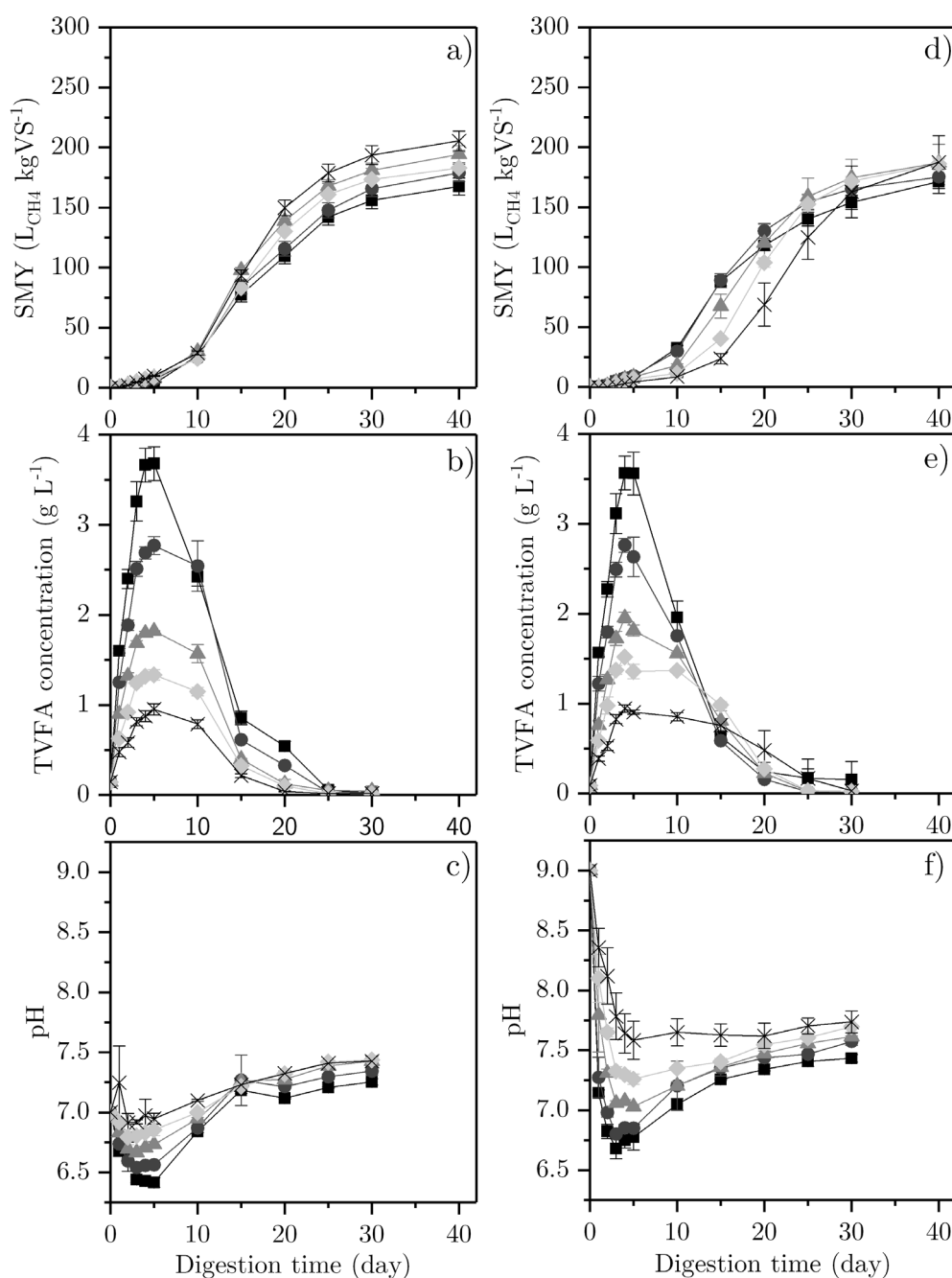


Fig. 4.5 Specific methane yield (SMY), total volatile fatty acid (TVFA) concentration and pH data obtained from anaerobic digestion of *Euphorbia tirucalli* at 38°C (mesophilic condition). The effect of different inoculum to substrate (I/S) ratios and starting pH 7.0 (**a–c**) and pH 9.0 (**d–f**) on cumulative methane yield was investigated. (—■—), (—●—), (—▲—), (—◆—), and (—×—) denote the I/S ratios of 0.5, 0.67, 1.0, 1.5, and 2.0, respectively. Error bars represent $\pm$ standard deviations ($n = 3$).

A summary of specific methane yields (SMYs) of *Euphorbia tirucalli* in mesophilic conditions (38°C) at different I/S ratios and initial pHs is shown as a bar chart in **Fig. 4.6**. At the initial pH 7.0, the I/S ratio significantly affected the methane yields ($p = 0.02$), especially for I/S ratios of 0.5 and 2.0. In contrast, there was no significant difference in methane yields between different I/S ratios tested at the initial pH 9.0 ($p > 0.05$). This suggests that the I/S ratio lower than 0.67 may not be suitable for anaerobic digestion of *Euphorbia tirucalli* when the initial pH is adjusted to 7.0.

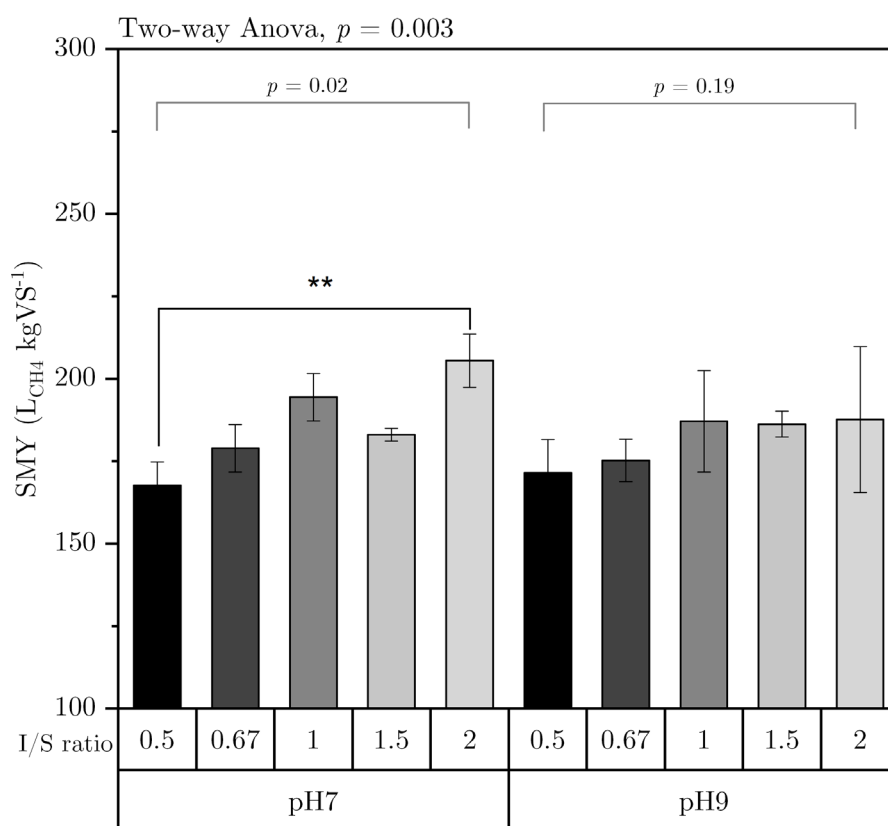


Fig. 4.6 Bar plot summarising the specific methane yields (SMY) of *Euphorbia tirucalli* in mesophilic conditions at different starting pHs and inoculum to substrate (I/S) ratios. One-way ANOVA analysis was performed for the effect of inoculum to substrate ratio. A two-way ANOVA test was employed for the comparison of mean methane yields between two different pH values. ****** denotes a significant difference ($p \leq 0.01$). Error bars represent $\pm$ standard deviations of triplicate samples.

4.2.3.3.2 Effect of I/S ratio and temperature on methane production, total volatile fatty acid (TVFA) and sample pH

The cumulative specific methane yields (SMYs) for *Euphorbia tirucalli* under different I/S ratios and operating temperatures are presented in **Fig. 4.7**. Under thermophilic conditions (55°C), methane production began immediately after 24 hours of digestion, but the cumulative yields were still below 15 L kgVS⁻¹ by day 5 (**Fig. 4.7d** and **Fig. 4.7g**). The difference in cumulative SMYs at different I/S ratios was first noticeable as early as day 10 when a faster rate of methane production was observed for higher I/S ratios. This however was not observed in mesophilic conditions until day 20. The methane yields continued to increase until day 30, after which they gradually levelled off by day 40. Approximately 90% of the final methane yields were obtained within 25 days of digestion for thermophilic conditions and within 30 days for mesophilic conditions (**Fig. 4.7a**). At 55°C, the final methane yields at the initial pH 7.0 were 159, 189, 192, 237, and 246 L kgVS⁻¹ for I/S ratios of 0.5, 0.67, 1.0, 1.5, and 2.0, respectively (**Table 4.5**). Higher methane yields of 195, 244, 266, 244, and 230 L kgVS⁻¹ were obtained at the initial pH 9.0 for the I/S ratios of 0.5, 0.67, 1.0, 1.5, and 2.0, respectively.

TVFA concentration profiles at three different conditions, 38°C and pH 7.0, 55°C and pH 7.0, and 55°C and pH 9.0 over 30 days of batch digestion are presented in **Fig. 4.7b**, **Fig. 4.7e**, and **Fig. 4.7h**, respectively. On day 5, the highest TVFA concentrations of 3.56, 2.49, and 2.62 g L⁻¹, respectively were observed for an I/S ratio of 0.5. This accounted for approximately 30% and 26% decrease in TVFA concentrations for the initial pH values 7.0 and 9.0, respectively, as the operating temperature increased from 38°C to 55°C. The rates of VFA consumption were also faster under thermophilic conditions; the TVFA concentrations obtained at the

thermophilic temperature on day 15 were 0.16–0.41 g L⁻¹, compared to 0.33–0.86 g L⁻¹ obtained at mesophilic temperature.

The TVFA concentrations were relatively lower for thermophilic conditions throughout 30 days of digestion. A slightly higher accumulation of TVFA was seen at the initial pH 9.0 compared to pH 7.0 (**Fig. 4.7e** and **Fig. 4.7h**). Similar results were reported in a previous study on the effect of different pH on VFA accumulation by waste activated sludge (Jie et al., 2014). They found that high concentrations of VFA accumulated at the pH values between 9.0 and 10.0. Under strongly alkaline conditions, the hydrolysis rate increased but the methanogenesis rate decreased, leading to a lower VFA consumption rate and a higher level of acid accumulation.

The pH values for all conditions were measured temporally and are presented in **Fig. 4.7c**, **Fig. 4.7f**, and **Fig. 4.7i**. At the initial pH 7.0, a decrease in pH after 24 hours was negligible or not observed under thermophilic conditions (**Fig. 4.7f**), which contrasted with the initial pH drop under mesophilic conditions shown in **Fig. 4.7c**. At 55°C, the pH values after 120 hours were 7.07, 7.04, 7.16, 7.16, and 7.26, for I/S ratios of 0.5, 0.67, 1.0, 1.5, and 2.0, respectively. The change in pH values was marginal until day 25 when the pH rose to between 7.69 and 7.98.

A substantial decrease in pH was seen at the beginning of the incubation (**Fig. 4.7i**), however, the pH values remained above 7.0 throughout the digestion period. On day 5, the values of pH decreased from 9.0 at the start to 7.21, 7.36, 7.43, 7.60, and 7.94 for I/S ratios of 0.5, 0.67, 1.0, 1.5, and 2.0, respectively. These pH values were at their lowest on day 10, ranging between 7.03 and 7.54. On day 15, the pH levels increased with decreasing TVFA concentrations and then remained relatively stable until day 30.

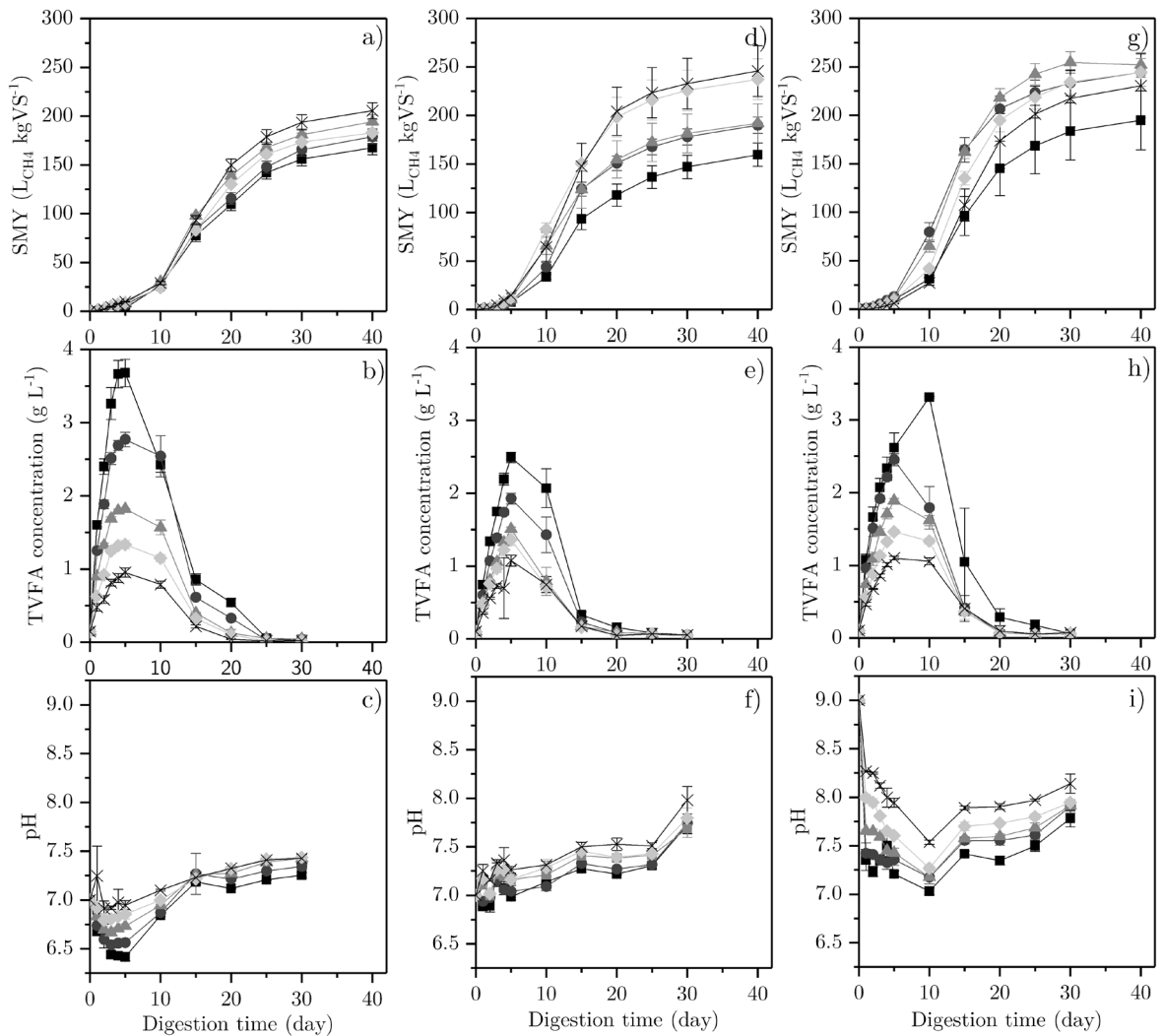


Fig. 4.7 Specific methane yield (SMY), total volatile fatty acid (TVFA) concentration and pH data obtained from anaerobic digestion of *Euphorbia tirucalli*. The effect of different inoculum to substrate ratios, starting pH and operating temperatures, 38°C and pH 7.0 (a–c as also shown in Fig. 4.5) and 55°C and pH 7.0 (d–f), and 55°C and pH 9 (g–i) on cumulative methane yield were investigated. (■), (●), (▲), (◆), and (×) denote the inoculum to substrate ratios of 0.5, 0.67, 1.0, 1.5, and 2.0, respectively. Error bars represent $\pm$ standard deviations ($n = 3$).

The SMYs from anaerobic digestion of *Euphorbia tirucalli* (pH 7.0) at different I/S ratios and operating temperatures are presented as bar plots in Fig. 4.8. It can be seen that both parameters, I/S ratio and operating temperature, had a significant effect on the methane yields, especially in thermophilic conditions where the effect was more pronounced ($p < 0.001$). The SMYs at I/S ratios of 1.5 and 2.0 were significantly higher than those at I/S ratios of 0.5 and 0.67. The highest methane yield of 246 L kgVS⁻¹ obtained at I/S ratio of 2.0 and 55°C, was significantly greater than any other conditions presented, with the exceptions of I/S ratio of 2.0 and 38°C ($p = 0.073$) and I/S ratio of 1.5 and 55°C ($p = 0.99$). There was a strong interaction

between the I/S ratio and operating temperature ($p = 0.005$), which led to a synergistic effect promoting an increase in methane yield.

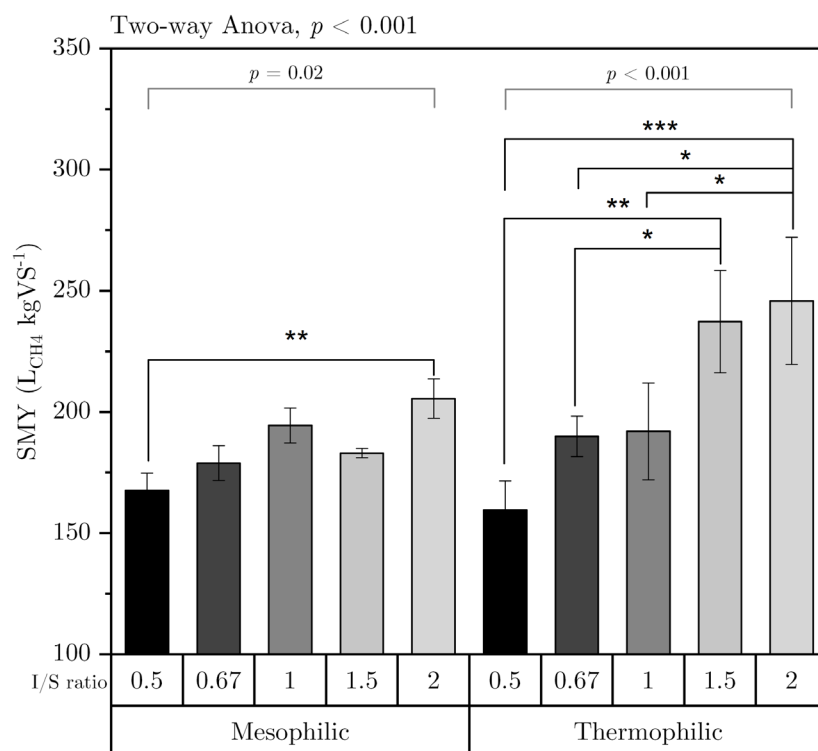


Fig. 4.8 Bar plot summarising the specific methane yields (SMY) of *Euphorbia tirucalli* at two operating temperatures (38°C and 55°C) and inoculum to substrate (I/S) ratios. The culture pH was adjusted to 7.0 at the start. The results obtained under mesophilic conditions are also presented in **Fig. 4.6**. A one-way ANOVA test was performed for the effect of the inoculum to substrate ratio. A two-way ANOVA test was employed for the comparison of mean methane yields between two different operating temperatures. *, **, and *** denote significant differences ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively). Error bars represent $\pm$ standard deviations of triplicate samples.

4.2.3.3.3 Comparison with published studies

The specific methane yield (SMY), the maximum TVFA concentration, and the final pH for different operating conditions are summarised in **Table 4.5**. The highest specific methane yields obtained in this study for *E. tirucalli* were 206 and 266 $L \text{ kgVS}^{-1}$, for mesophilic and thermophilic conditions, respectively. Since most of the past research publications have only reported their biogas data with/without methane percentages, their methane yields are estimated in order to compare them with the results from the current study.

Table 4.5 Specific methane yield (SMY), maximum total volatile fatty acid (TVFA_m) concentration, and sample pH from anaerobic digestion of *E. tirucalli* at different operating conditions. The values are shown as means of triplicate samples $\pm$ standard deviations ($n = 3$)

Operating conditions			SMY	TVFA _m (g/l)	Final pH
Temperature	pH	I/S ratio	(L kgVS ⁻¹)		
Mesophilic	7.0	0.5:1.0	167 $\pm$ 7	3.68 $\pm$ 0.19	7.25 $\pm$ 0.03
		0.67:1.0	179 $\pm$ 7	2.77 $\pm$ 0.09	7.34 $\pm$ 0.03
		1.0:1.0	194 $\pm$ 7	1.82 $\pm$ 0.05	7.43 $\pm$ 0.03
		1.5:1.0	183 $\pm$ 2	1.34 $\pm$ 0.06	7.43 $\pm$ 0.03
		2.0:1.0	206 $\pm$ 8	0.95 $\pm$ 0.06	7.43 $\pm$ 0.04
Mesophilic	9.0	0.5:1.0	172 $\pm$ 10	3.57 $\pm$ 0.19	7.43 $\pm$ 0.03
		0.67:1.0	175 $\pm$ 6	2.76 $\pm$ 0.08	7.57 $\pm$ 0.03
		1.0:1.0	187 $\pm$ 15	1.96 $\pm$ 0.06	7.61 $\pm$ 0.01
		1.5:1.0	186 $\pm$ 4	1.52 $\pm$ 0.02	7.74 $\pm$ 0.02
		2.0:1.0	188 $\pm$ 22	0.95 $\pm$ 0.04	7.74 $\pm$ 0.09
Thermophilic	7.0	0.5:1.0	159 $\pm$ 12	2.49 $\pm$ 0.07	7.72 $\pm$ 0.09
		0.67:1.0	189 $\pm$ 8	1.93 $\pm$ 0.07	7.74 $\pm$ 0.01
		1.0:1.0	192 $\pm$ 20	1.51 $\pm$ 0.03	7.69 $\pm$ 0.09
		1.5:1.0	237 $\pm$ 21	1.36 $\pm$ 0.08	7.79 $\pm$ 0.10
		2.0:1.0	246 $\pm$ 26	1.07 $\pm$ 0.07	7.98 $\pm$ 0.14
Thermophilic	9.0	0.5:1.0	195 $\pm$ 31	2.62 $\pm$ 0.20	7.78 $\pm$ 0.09
		0.67:1.0	244 $\pm$ 14	2.45 $\pm$ 0.08	7.90 $\pm$ 0.07
		1.0:1.0	266 $\pm$ 13	1.89 $\pm$ 0.03	7.91 $\pm$ 0.06
		1.5:1.0	244 $\pm$ 15	1.46 $\pm$ 0.03	7.95 $\pm$ 0.03
		2.0:1.0	230 $\pm$ 33	1.09 $\pm$ 0.03	8.14 $\pm$ 0.09

In an earlier investigation with *E. tirucalli*, biogas production was found to be 21.9 L per 750 g of a fresh substrate over the course of a 9-week digestion period, equivalent to 29.2 L kgFM⁻¹ (Rajasekaran et al., 1989). That study did not determine the TS and VS content of *Euphorbia* along with the biogas data, but assuming the same values of TS and VS of *E. tirucalli* used in the present study, the biogas yield can be estimated to be 225 L kgVS⁻¹. In another study, biogas production from different genotypes of *E. tirucalli* was reported to be between 114 and 637

L kgVS⁻¹ (Hastilestari et al., 2013). The average methane contents ranging from 43% to 69%, depending on the genotype, were reported instead of individual methane percentages over time. Thus, the estimated methane yields from these data were calculated to be between 79 and 319 L kgVS⁻¹. A recent study investigated the use of *Euphorbia tirucalli* harvested from three fields of different plantation densities as an AD substrate (Krümpel et al., 2020); the specific methane yields of *E. tirucalli* from these three fields ranged from 215 to 234 L kgVS⁻¹.

From the limited published literature, the methane yields of *E. tirucalli* were between 79 and 238 L kg VS⁻¹, with one data of exceptionally high methane yield (319 L kgVS⁻¹) reported for *E. tirucalli* grown in Kenya. These published results were comparable to the range of methane yields from *E. tirucalli* determined in this study under mesophilic conditions (167–206 L kgVS⁻¹).

4.2.3.3.4 Kinetic models

First-order model

The first-order model was employed to estimate the hydrolysis rate constant (k) of *E. tirucalli* biomass undergoing anaerobic digestion at different operating conditions. The kinetic parameter was estimated from the experimental specific methane yields (SMY) at time t using **Equation 3.9, Section 3.1.5.2.2. Fig. 4.9** presents the k values of triplicate samples for each condition. Despite an initial lag period during the production of methane, the R^2 values between 0.823 and 0.979 indicated a good fit of the model to the experimental data for all scenarios. Since k represents the rate of degradation of substrate, a higher k value is indicative of a faster hydrolysis rate.

One-way ANOVA was performed to test the significant difference of the hydrolysis rate constant (k) obtained for each condition. At mesophilic temperature (38°C), the maximum k value of 1.06 day⁻¹ was recorded for an I/S ratio of 2.0 at the initial pH 7.0. This k value was significantly higher than those at I/S ratios of 0.5, 0.67, and 1.0 ($p = 0.004$). In contrast, the k value at the I/S ratio of 2.0 at the initial pH 9.0 was significantly lower than those at I/S ratios of 0.5 and 0.67 ($p = 0.017$). At thermophilic temperature (55°C), the influence of the I/S ratio and initial pH on the hydrolysis rate constants was not observed as the difference between the k values was deemed insignificant ($p > 0.05$).

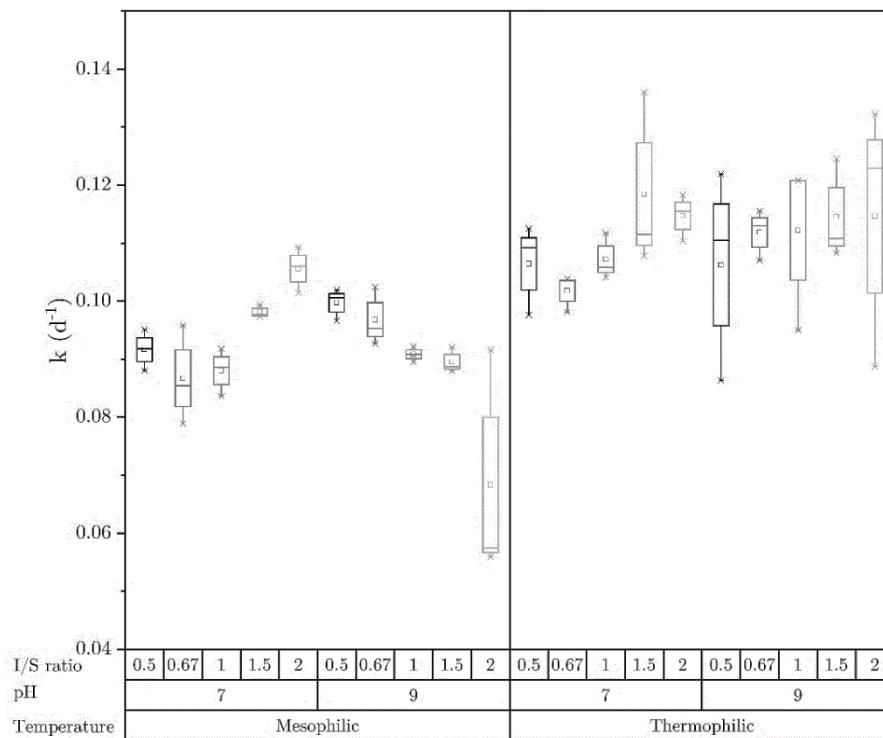


Fig. 4.9 Box plots summarising the hydrolysis rate constants, k (day⁻¹), obtained from anaerobic digestion of *Euphorbia tirucalli* at different I/S ratios, pHs, and operating temperatures using the first-order model. Mean values of triplicate samples are represented by a hollow square dot. Median is presented as the bold horizontal line crossing each box. The maximum and minimum values are shown by the whiskers.

Two-way ANOVA analysis was carried out to evaluate the significance of the interaction of any two parameters. It was found that the k values were relatively higher at thermophilic conditions regardless of starting pH and I/S ratios. At the initial pH 7.0, the k values ranged from 0.087–0.106 day⁻¹ for mesophilic and 0.102–

0.118 day⁻¹ for thermophilic conditions. The difference in k values at two different temperatures was significant ($p < 0.001$). Similarly, at the initial pH 9.0, the values of k obtained for thermophilic temperature were 0.106–0.115 day⁻¹, significantly higher than those for mesophilic temperature, 0.068–0.099 day⁻¹ ($p = 0.005$).

Modified Gompertz model

The methane production potential (A), the maximum methane production rate (μ), and the lag phase time (λ) are presented in **Table 4.6**. These values were estimated from the experimental SMY values using **Equation 3.10, Section 3.1.5.2.2**. The values of λ for mesophilic conditions at pH 7.0 were between 6.94 and 7.78 days. The range of λ values at pH 9.0 was however much wider, from 5.79 to 13.6 days, the lowest of which corresponded to the I/S ratio of 0.5.

It was found that, under mesophilic conditions, there is a negative correlation between λ and k values, suggesting that the lag phase decreased with an increasing hydrolysis rate constant. However, this correlation between λ and k values was only observed at pH 9.0. The λ values for thermophilic conditions at pH 7.0 and pH 9.0 were 4.91–6.37 days, and 5.25–8.51 days, respectively, which were in a similar range to those obtained in mesophilic conditions and pH 7.0 (6.94–7.78 days). This suggests that increasing temperature did not significantly reduce the lag time.

The clear trend of variation in maximum methane production rate (μ) with I/S ratios was observed in both operating temperature and pH conditions. It was found that the μ values increased with I/S ratios. For example, at mesophilic temperature and pH 7.0, the values of μ were 9.28, 9.54, 11.6, 11.4, and 12.9 L kgVS⁻¹ d⁻¹, for I/S ratios of 0.5, 0.67, 1.0, 1.5, and 2.0, respectively. The highest μ value of 19.6 L kgVS⁻¹ d⁻¹ was obtained at an I/S ratio of 1.0, pH 9.0, and under thermophilic conditions.

The ultimate methane potentials (A) of 245 and 257 L kgVS⁻¹ were obtained under thermophilic conditions for pH 7.0 and I/S ratio of 2.0, and for pH 9.0 and I/S ratio of 1.0, respectively. Under mesophilic conditions, the highest A value of 208 L kgVS⁻¹, was obtained at an I/S ratio of 2.0 and pH 7.0. Increasing operating temperature and I/S ratio resulted in the higher values of the ultimate methane potential and maximum methane production rate, however, both parameters were not seen to be influenced by the initial pH condition.

Table 4.6 Kinetic parameters of a modified Gompertz model obtained from anaerobic digestion of *E. tirucalli* with different I/S ratios, starting pH values and operating temperatures.

Operating conditions			SMY (L kgVS ⁻¹)	Parameters from the model ¹			R ²
Temperature	pH	I/S ratio		A (L kgVS ⁻¹)	μ (L kgVS ⁻¹ d ⁻¹)	λ (d)	
Mesophilic	7.0	0.5:1.0	168	171	9.28	7.12	0.999
		0.67:1.0	179	183	9.54	6.95	0.998
		1.0:1.0	194	196	11.6	6.94	0.998
		1.5:1.0	183	186	11.4	7.78	0.998
		2.0:1.0	206	208	12.9	7.72	0.998
	9.0	0.5:1.0	172	172	8.86	5.79	0.998
		0.67:1.0	175	177	10.6	6.75	0.998
		1.0:1.0	187	192	11.4	9.00	0.998
		1.5:1.0	186	190	12.8	11.7	0.998
		2.0:1.0	188	199	11.3	13.6	0.998
Thermophilic	7.0	0.5:1.0	159	157	9.64	5.99	0.997
		0.67:1.0	189	184	13.7	6.37	0.997
		1.0:1.0	192	188	12.5	4.91	0.999
		1.5:1.0	237	234	15.9	5.04	0.999
		2.0:1.0	246	245	16.2	5.72	0.999
	9.0	0.5:1.0	195	196	11.9	7.09	0.999
		0.67:1.0	244	241	17.3	5.25	0.999
		1.0:1.0	266	257	19.6	6.51	0.999
		1.5:1.0	244	242	17.8	7.51	0.999
		2.0:1.0	230	229	16.4	8.51	0.999

¹ A – Methane production potential; μ - maximum methane production rate; λ – lag time.

4.2.3.3.5 Energy balance assessment

The energy balance of the experiments at different operating conditions was evaluated to compare their performances of anaerobic digestion of *E. tirucalli*. The analysis was conducted based on 1 kg of dried biomass; all equations, constants and assumptions were based on the previous studies on the energy analysis of the co-digestion of agriculture residues (Paranhos et al., 2020; Shirzad et al., 2019; Valenti et al., 2018b). The equations used for the calculation of energy balance are described in **Chapter 3, Section 3.1.5.4. Table 4.7** shows the estimated values of energy balance and net energy output for three different experimental conditions. Examples of the energy balance calculations can be found in **Appendix B. Condition I** was a combination of mesophilic temperature, initial pH 7.0 and I/S ratio of 2.0. The other two conditions included thermophilic temperature, but the initial pH and I/S ratio were 7.0 and 2.0, respectively, for **Condition II** and 9.0 and 1.0, respectively, for **Condition III**.

Condition III produced the highest methane yield (224 L kgTS^{-1}) compared to the other two conditions (171 and 207 L kgTS^{-1}). The energy inputs for heat were $0.174 \text{ kWh-e kgTS}^{-1}$ for **Condition I** (38°C) and $0.339 \text{ kWh-e kgTS}^{-1}$, the same value for **Condition II** and **III** due to them being operated at the same thermophilic temperature (55°C). Methane produced from anaerobic digestion can be combusted for the generation of heat and electricity via a CHP unit. Overall, positive net energy outputs were observed in all scenarios, which implies that all of the selected operating conditions were deemed suitable for methane production. However, **Condition II** had the worst performance in terms of both net heat outputs and energy generation efficiency.

Table 4.7 Energy balance of anaerobic digestion of *Euphorbia tirucalli* at selected conditions

Parameters	Unit ¹	Experimental conditions ²		
		Condition I	Condition II	Condition III
Specific methane yield, SMY	m ³ kgVS ⁻¹	0.206	0.246	0.266
SMY(TS)	m ³ kgTS ⁻¹	0.173	0.207	0.224
Energy potential of methane	kWh-e kgTS ⁻¹	1.724	2.059	2.226
Energy balance				
Heat input	kWh-e kgTS ⁻¹	0.174	0.339	0.339
Electricity input	kWh-e kgTS ⁻¹	0.051	0.051	0.051
Heat output	kWh-e kgTS ⁻¹	0.695	0.831	0.898
Electricity output	kWh-e kgTS ⁻¹	0.731	0.873	0.944
Net energy output				
Net heat output	kWh-e kgTS ⁻¹	0.521	0.491	0.559
Net electricity output	kWh-e kgTS ⁻¹	0.680	0.822	0.833
Energy generation efficiency	%	69.7	63.8	65.2

¹kWh-e is kilowatt-hour equivalent; ²**I** – 38°C, pH7, and I/S ratio of 2.0; **II** – 55°C, pH7 and I/S ratio of 2.0; **III** – 55°C, pH9 and I/S ratio of 1.0

4.2.3.3.6 Discussion – Experiment I

In this experiment, the highest SMYs of *E. tirucalli* were obtained with an I/S ratio of 2.0 in most cases, except at 55°C and initial pH 9.0. However, statistically, there was no significant difference within SMYs at I/S ratios of 0.67, 1.0, 1.5, and 2.0 for mesophilic conditions. This means that even a slightly lower proportion of inoculum compared to the substrate could still be used for establishing or operating the anaerobic digester for *E. tirucalli*. However, higher I/S ratios are still recommended if inoculum can be sourced. The effect of the I/S ratio on methane production was more pronounced under thermophilic conditions where the methane yields at I/S ratios of 1.0 or below were significantly lower compared to those at 1.5 and 2.0.

The methane production rate was slow initially but began to increase after day 5 when the TVFA level reached the highest point (**Fig. 4.5**). The delay in methane production after the peak of TVFA at an initial stage of digestion was also observed and attributed in earlier studies to the effects of inoculum to substrate ratio on

biogas production from grass and pig manure (Dechrugsa et al., 2013). The rates of hydrolysis and acidogenesis were higher than methanogenesis at first and this was due to the conversion of easily biodegradable substrates to VFA. Acclimatisation of methanogens was required at least 24–48 hours after the peak of TVFA to metabolise and convert VFA to biogas. One of the key indicators that the system had a good buffering system to handle the TVFA peak was the pH value. A continuous drop of pH after the highest TVFA concentrations would imply that the I/S ratio was too low, leading to acid accumulation and inhibition.

Increasing the initial pH from 7.0 to 9.0 reduced the effect of acid accumulation in the sample with low I/S ratios (0.5 and 0.67) but negatively affected the methane production for high I/S ratio (2.0) (**Fig. 4.5c** and **Fig. 4.5f**). The hydrolysis and acidogenesis processes occurring within the first 24–120 hours resulted in a drop in pH. With a high I/S ratio and initial pH, the sample pH stayed above 7.5 throughout the experiment. This pH was too high for the hydrolysis and acidogenesis steps for microorganisms that function optimally at a pH between 5.5 and 6.5 (Yu and Fang, 2002). This pH inhibition slowed down the production of acid intermediates, which subsequently resulted in a lower expected methane yield. In contrast, employing a high starting pH (9.0) prevented the sample pH from decreasing too rapidly at a low I/S ratio (0.5). Under this condition, the sample pH was 6.78, close to the ideal pH range of 6.8–7.2 for methanogens (Mosey and Fernandes, 1989).

The results of the study of the impact of operating temperature on methane production from *Euphorbia tirucalli* are in agreement with those reported in the 1980s. The study of biogas production of the same species inoculated with rumen fluid as an inoculum confirmed that thermophilic conditions (48°C and 55°C) accelerated methane production by 5–15 days (Sow et al., 1989). Better methane

production at higher operating temperatures of other substrates has also been reported in several studies (Moset et al., 2015b; Suhartini et al., 2014; Vindis and Mursec, 2009). In addition to improvement in methane yield, this study demonstrated that the level of volatile fatty acid (VFA) accumulation during the first 5 days was lower at thermophilic temperature (55°C) and the VFA concentration decreased more rapidly to below 0.5 g L⁻¹ by day 15. Previous studies reported that in batch digestion under thermophilic conditions, the rate of VFA consumption, which was a function of temperature, was faster, indicating that the system was able to rapidly degrade the substrate (Martínez et al., 2016; Riggio et al., 2017). It was reported that the Gibbs free energy values of the oxidation reactions of propionic and butyric acids become less positive when the operating temperature increases from 25°C to 55°C, making the reactions more thermodynamically feasible (Amani et al., 2011).

However, an increase in methane production from *Euphorbia tirucalli* under thermophilic conditions may be suboptimal. Despite a faster rate observed under thermophilic conditions compared to mesophilic ones, still, there was an initial lag in methane production observed over a shorter period. Taking the lag parameter (λ) obtained from the modified Gompertz model into consideration, it was found that increasing operating temperature did not significantly reduce the lag phase. This lag period may have been reduced or eliminated if a fully adapted inoculum had been used (Ortega et al., 2008). The inoculum in this study was sourced from a mesophilic biogas digester and used without proper acclimatisation. The duration of this experiment was 40 days, which may have been too short for the microbial consortium to shift and significantly affect biomass degradability. Methanogens grow very slowly and are sensitive to temperature change and therefore need a longer acclimatisation period (Gerardi, 2003).

4.2.4 Experiment II – Co-digestion of *E. tirucalli* with chicken manure

4.2.4.1 Objective

To study the effect of mixing ratio on the anaerobic co-digestion of *E. tirucalli* and chicken manure at two different temperatures.

As previously discussed in the literature (**Chapter 2, Section 2.1.5**), there is a lack of information regarding the cultivation of CAM plants such as *E. tirucalli* for use as biomass. In addition, the biomass yields of these plants are still low. One strategy is to utilise this plant as an AD substrate in co-digestion applications. Chicken manure has been widely used as a co-substrate for anaerobic co-digestion study and therefore selected in this study.

4.2.4.2 Methodology

The bottle test based on the BMP assay was performed to investigate co-digestion of *E. tirucalli* and chicken manure, which were examined at 6 different mixing ratios (0:1, 1:4, 2:3, 3:2, 4:1, and 1:0). Biogas and liquid samples were collected at regular intervals to determine gas volume, gas composition, pH and VFA content. The modified Gompertz equation was used to fit the data of cumulative methane yields obtained from the experiment to estimate the ultimate methane yield, maximum methane production rate and the lag phase duration. The detailed description of the experimental setup is described in **Chapter 3, Section 3.1.2**.

4.2.4.3 Results

The specific methane yields (SMYs) obtained in this study varied between 186 and 251 L kgVS⁻¹ (**Table 4.8**). Under mesophilic conditions, the highest SMY was obtained from chicken manure at 210 L kgVS⁻¹, which was comparable with the literature data (127–258 L kgVS⁻¹) previously reported (Kafle and Chen, 2016;

Rahman et al., 2017; Wang et al., 2012). The methane yield obtained from the mono-digestion of *Euphorbia tirucalli* in this study was 206 L kgVS⁻¹. By mixing the two substrates into 4 different *Euphorbia* to manure (E:M) ratios of 1:4, 2:3, 3:2, and 4:1, the cumulative methane production profiles of these mixtures including pure substrates of *E. tirucalli* and chicken manure over 40 days of digestion are illustrated in **Fig. 4.10**. The methane yields for E:M ratios of 1:4, 2:3, 3:2, and 4:1, were 199, 186, 187, and 193 L kgVS⁻¹, respectively. The methane production rates were determined from the slope of the methane yield over digestion time. As can be seen in **Fig. 4.10a**, on days 10 and 15, the addition of chicken manure progressively reduced the methane production rates. The SMY values for E:M ratios of 0:1, 1:4, 2:3, 3:2, 4:1, and 1:0 on day 15 were 9.9, 9.9, 11.0, 12.2, 18.6, and 28.8, respectively.

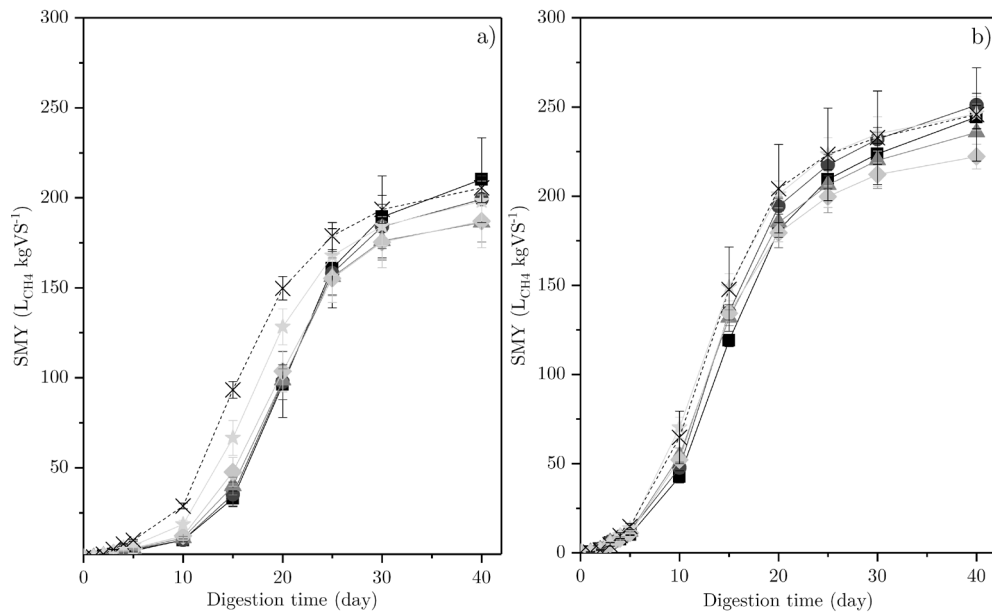


Fig. 4.10 BMP curve showing specific methane yields (SMY) for different ratios of *Euphorbia tirucalli* and chicken manure at **a)** 38°C and **b)** 55°C. The following symbols (■), (●), (▲), (◆), (★), and (×) denote the mixing ratios of *Euphorbia tirucalli* and chicken manure at 0:1, 1:4, 2:3, 3:2, 4:1, and 1:0, respectively. Error bars indicate ± standard deviations of triplicate samples.

The statistical analysis of the specific methane yields from different mixtures of *E. tirucalli* and chicken manure is illustrated as bar charts in **Fig. 4.11**. One-way ANOVA was performed on the SMY results at each operating temperature and it was found that there was no significant difference in SMYs between different

mixtures of *E. tirucalli* and chicken manure ($p > 0.05$). However, the p -value obtained from the Two-way ANOVA test was 0.006, which indicated that, overall, there was a significant difference in the cumulative methane yield values. Since the earlier statistical results showed that E:M ratio did not affect the SMYs, an improvement in the SMYs was attributed solely to operating temperature.

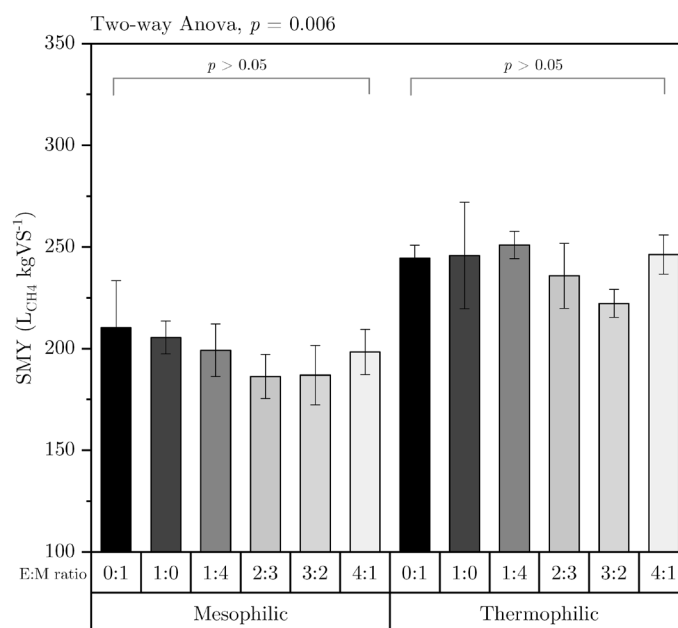


Fig. 4.11 Bar plot summarising the specific methane yields (SMY) of co-digestion of *Euphorbia tirucalli* and chicken manure at different mixing ratios of *Euphorbia* and manure (E:M). The culture was initially adjusted to pH7 at the start. A one-way ANOVA test was performed to determine the effect of substrate mixing ratio. A two-way ANOVA test was employed for comparison of mean methane yields between two different operating temperatures. Error bars represents $\pm$ standard deviations ($n = 3$).

4.2.4.3.1 Comparison of the predicted and experimental SMYs from the mono- and co-digestion of *E. tirucalli* and chicken manure

The experimental yields obtained from the co-digestion of two or more feedstocks can be predicted from the methane yields of the individual substrate. The predicted yield in this study was then calculated proportionally from the SMYs of mono-digestion of *Euphorbia tirucalli* and chicken manure. The difference between the methane yields from the co-digestion samples and the predicted yields is shown in Table 4.8.

Overall, regardless of the mixing ratio, the experimental methane yields were lower than the predicted ones and the difference ranged between 4 and 12% for mesophilic temperature. The greatest difference between the experimental SMY and the corresponding predicted yield was $-22.4 \text{ L kgVS}^{-1}$ at an E:M ratio of 2:3, which accounted for 60% of chicken manure in the substrate mixture.

In contrast, under thermophilic conditions, the E:M ratios of 1:4 and 4:1 gave higher experimental yields than the predicted values by 2% and 0.1%, respectively. At mixing ratios of 2:3 and 3:2, the experimental yields were lower by 4% and 11%, compared to the predicted yields. It was previously reported that the co-digestion of grass silage and slurry from a dairy farm resulted in a 4–11% decrease in methane yields from the predicted data (Wall et al., 2013). The greatest differences in yields were seen for the substrate mixtures with high slurry content (60–80%).

Co-digestion of two substrates in this study did not result in higher methane yields compared to the mono-digestion of the individual substrate. Also, statistically, there was no significant difference between methane yields from different mixing ratios. This means that *E. tirucalli* and chicken manure could potentially be used as co-substrates for anaerobic digestion at any mixing ratios. However, this conclusion needs to be considered carefully since the batch co-digestion test is inoculum-influenced (Regalado et al., 2021). No differences between experimental and predicted yields and between methane yields of different mixing ratios could stem from other causes. There could be synergy between the two substrates, but it was not shown in this study potentially due to the confounding effect of the inoculum masking the interaction of the co-substrates.

Table 4.8 Specific methane yield (SMY), predicted methane yields, maximum total volatile fatty acid (TVFA_m) concentration, and pH from co-digestion of *E. tirucalli* and chicken manure at different operating conditions. The values are presented as means of triplicate $\pm$ standard deviations

Operating conditions		SMY	Predicted yield ¹	Difference ²		TVFA _m (g/l)	Final pH
Temperature	E:M ratio	(L kgVS ⁻¹)	(L kgVS ⁻¹)	(L kgVS ⁻¹)	(%)		
Mesophilic	0:1	210 $\pm$ 23	-	-	-	0.85 $\pm$ 0.12	7.49 $\pm$ 0.01
	1:4	199 $\pm$ 13	209.2	-10.2	-5%	0.79 $\pm$ 0.05	7.45 $\pm$ 0.04
	2:3	186 $\pm$ 11	208.4	-22.4	-12%	0.82 $\pm$ 0.01	7.46 $\pm$ 0.01
	3:2	187 $\pm$ 15	207.6	-20.6	-11%	0.81 $\pm$ 0.01	7.46 $\pm$ 0.05
	4:1	198 $\pm$ 11	206.8	-8.8	-4%	0.87 $\pm$ 0.01	7.44 $\pm$ 0.02
	1:0	206 $\pm$ 8	-	-	-	0.95 $\pm$ 0.06	7.43 $\pm$ 0.04
Thermophilic	0:1	245 $\pm$ 7	-	-	-	0.97 $\pm$ 0.02	7.94 $\pm$ 0.05
	1:4	251 $\pm$ 7	245.2	5.8	2%	1.01 $\pm$ 0.02	7.92 $\pm$ 0.10
	2:3	236 $\pm$ 16	245.4	-9.4	-4%	0.99 $\pm$ 0.04	7.80 $\pm$ 0.04
	3:2	222 $\pm$ 7	245.6	-23.6	-11%	0.98 $\pm$ 0.04	7.69 $\pm$ 0.09
	4:1	246 $\pm$ 9	245.8	0.2	0.1%	0.98 $\pm$ 0.04	7.79 $\pm$ 0.03
	1:0	246 $\pm$ 26	-	-	-	1.07 $\pm$ 0.07	7.98 $\pm$ 0.14

1 – Predicted methane yield calculated in proportion to SMY values for E:M of 1:0 and 0:1, respectively

2 – Difference between experimental yields (SMY) and predicted yields

4.2.4.3.2 Kinetics parameters

The hydrolysis rate constant (k) obtained from the first-order model and the lag time obtained from the modified Gompertz model for all operating conditions are presented as box plots in **Fig. 4.12**. The fitting accuracy calculated as R^2 values for k constants and lag time were 0.85–0.97 and 0.97–0.99, respectively, demonstrating the two models were very well fitted.

The k values obtained from 6 different E:M ratios were 0.079–0.099 day⁻¹ at 38°C, and 0.109–0.138 day⁻¹ at 55°C. A one-way ANOVA test was performed on the k values at different E:M ratios under each temperature condition. The p -values for both conditions were greater than 0.05 (0.077 for 38°C and 0.133 for 55°C), indicating that the differences within the hydrolysis constants were not significantly affected by the co-digestion of the two substrates. However, k values under thermophilic conditions were significantly higher than those under mesophilic conditions (Two-way ANOVA, $p < 0.001$). It can be concluded from this that increasing operating temperature significantly improved the hydrolysis rates by 16–64%, particularly at higher E:M ratios.

Lag phase time obtained from the modified Gompertz model reflected the microbial response to acclimatise to the new system (Mao et al., 2017). The values of lag time obtained for E:M ratios of 0:1, 1:4, 2:3, 3:2, 4:1 and 1:0 at 38°C were 12.8, 12.6, 12.0, 10.9, 9.4, and 7.4 days, respectively. The lag phase time significantly decreased with increasing E:M ratios ($p < 0.001$). At 55°C, the shortest lag time of 5.35 days was obtained for E:M ratio of 4:1 and found to be significantly shorter than those for E:M ratios of 0:1 (6.74 days) and 1:4 (6.52 days), the two highest proportion of manure in the substrate mixture ($p = 0.004$). Evidently, thermophilic co-digestion

of *E. tirucalli* and chicken manure could significantly reduce the lag phase times of the experiment up to half (22–51%) of those under mesophilic conditions ($p < 0.001$).

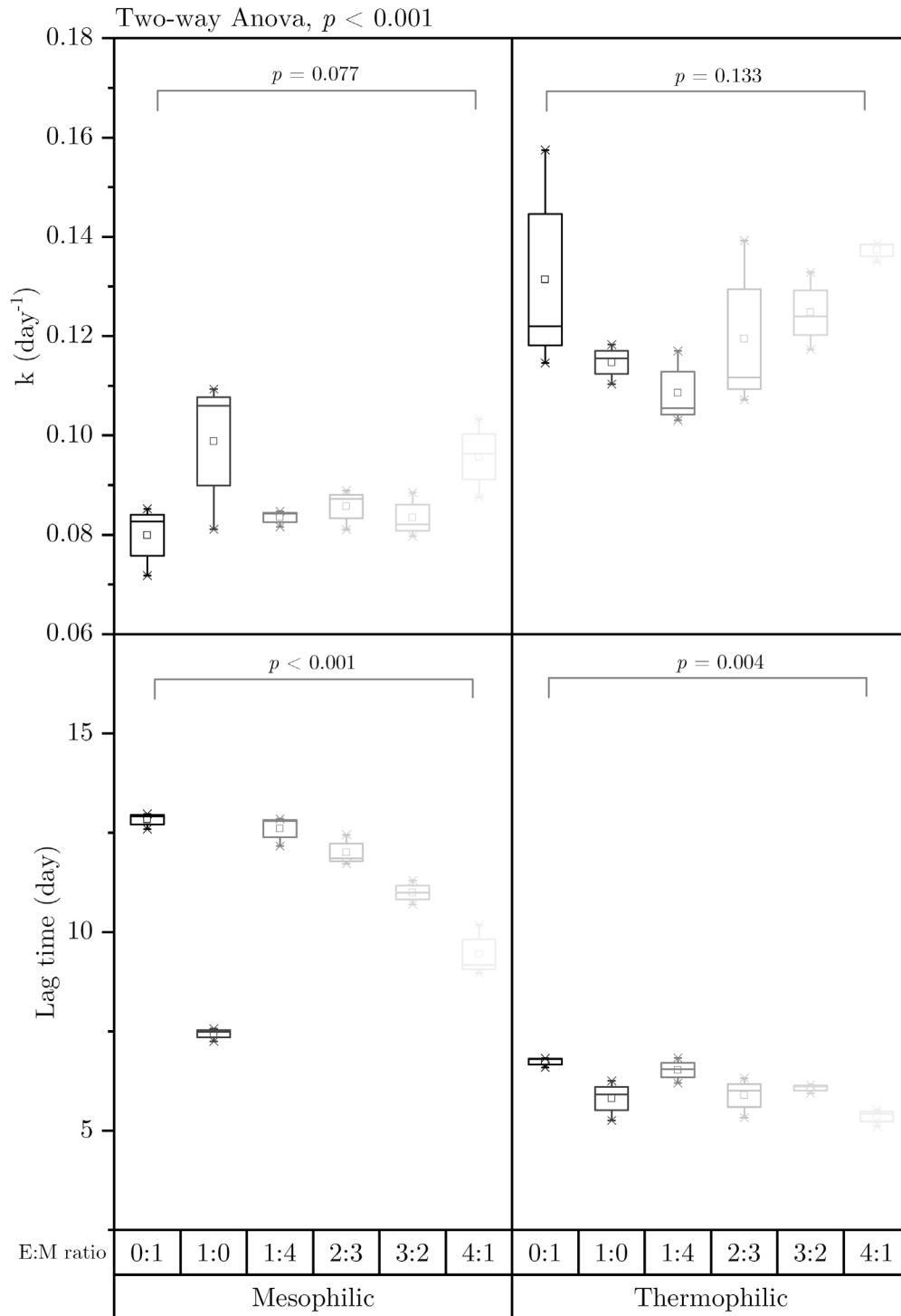


Fig. 4.12 Box plots summarising the hydrolysis rate constants, k (day⁻¹), and lag time (day) obtained from anaerobic digestion of *E. tirucalli* at different substrate mixtures and operating temperatures using the first-order and modified Gompertz models. Mean values of triplicate samples are represented by a hollow square dot. Median is presented as the bold horizontal line crossing each box. The maximum and minimum values are shown by the whiskers.

4.2.5 Conclusion

This study confirms that *Euphorbia tirucalli* has the potential to be employed as an AD feedstock for both mono- and co-digestion with other substrates such as chicken manure.

The results demonstrated that the highest methane yields of *E. tirucalli* obtained for mesophilic and thermophilic conditions were 206 and 266 L kgVS⁻¹, respectively. Under mesophilic conditions, the use of I/S ratios of 0.67, 1.0, 1.5 and 2.0 resulted in comparable methane yields, implying that low inoculum proportion could be used for setting up and operating the anaerobic digestion of *E. tirucalli*. An I/S ratio of 0.5 is not recommended as the proportion of inoculum is too small compared to the substrate for effective AD processes. Operating at a thermophilic temperature significantly improved the final methane yields by 19–29%, but this was also dependent on the I/S ratio and initial pH. In addition, approximately 90% of methane yield was obtained 5 to 10 days earlier under thermophilic conditions. The lag was still observed at thermophilic temperature, and this could be due to the use of unacclimatised inoculum. The initial pH did not have a consistent effect on methane production, however, higher initial pH (9.0) enhanced the hydrolysis rate and reduced the inhibitory effect of acid accumulation, especially at I/S ratio of 0.5.

Based on the assessment of energy balance, the optimal condition for methane production and best energy generation efficiency was at mesophilic temperature with an initial pH of 7.0 (**Condition I**). Increasing operating temperature to 55°C (**Condition II**) resulted in a 6% lower energy generation efficiency. Even though, adjusting the initial pH to 9.0 at 55°C (**Condition III**) resulted in an increase in the energy generation efficiency, the process would incur an additional cost of the

alkaline solution required to adjust the system pH to 9.0 and would raise health and safety concerns.

The results obtained from the co-digestion of *E. tirucalli* and chicken manure showed that regardless of the mixing ratio, the methane yields were similar to those obtained from the mono-digestion of each substrate. A significant improvement in methane yields was achieved under thermophilic conditions; the highest methane yield obtained from the co-substrates of an E:M ratio of 4:1 was 251 L kgVS⁻¹.

4.3 Anaerobic digestion of *Opuntia ficus-indica*

4.3.1 Aim of the study

The aim of the work carried out in this section was to determine the biomass characteristics of *Opuntia ficus-indica*, its biochemical methane potential (BMP), and the feasibility or performance of the long-term anaerobic digestion of *O. ficus-indica* under semi-continuous operations. Factors such as substrate processing method and harvesting age were also tested for their effects on the BMP.

4.3.2 Experiment I – Effect of I/S ratio and substrate processing technique on the methane production from *O. ficus-indica*

4.3.2.1 Objective

To determine the biochemical methane potential of *O. ficus-indica* and the effects of I/S ratio and substrate processing technique on the BMP.

4.3.2.2 Methodology

A biochemical methane potential (BMP) assay was carried out to determine the methane potential of *O. ficus-indica*. *Opuntia* cladodes were processed into three different types of samples using the procedures as outlined in **Section 3.2.1**. The three sample types (ground cladode, liquid extract, and solid peel) from different processing steps were tested at six I/S ratios (0.35, 0.71, 1.04, 1.39, 2.08, 2.78) for their methane potentials. Biogas and liquid samples were collected at regular intervals to determine gas volume, gas composition, pH and VFA content. The modified Gompertz equation was used to fit the data of cumulative methane yields obtained from the BMP experiment to estimate the ultimate methane yield, maximum methane production rate and the lag phase duration. The detailed description of the BMP setup is described in **Chapter 3, Section 3.1.2**.

4.3.2.3 Biomass characterisation of feedstock and inoculum

The biomass composition of the three different types of substrates and the inoculum is shown in **Table 4.9**. The ground cladodes of *O. ficus-indica* had the total solid (TS) and volatile solid (VS) percentages of $12.84 \pm 0.15\%$ and $87.98 \pm 0.16\%$, respectively. The ground material was then centrifuged to separate the liquid from the solid peel. The TS contents of *Opuntia* liquid extract and solid peel were $9.94 \pm 0.08\%$ and $18.89 \pm 0.32\%$, respectively. An additional processing step did not alter the organic matter content of the materials as all substrates still had similar VS contents (87.9%, 88.2%, and 88.6% for the ground cladode, solid peel, and liquid extract, respectively).

The lignin content of *Opuntia* cladode in this study was 9.15%TS, which was relatively lower compared to other fruit wastes previously studied as substrates for biogas and bioethanol production. The lignin contents of banana peel, orange peel, and sugar beet pulp were 12.17–27.59% (Palacios et al., 2017), 5.80–12.0% (Orozco et al., 2014), and 36.0–43.0% (Ahmadi et al., 2015), respectively.

The inoculum used in this study had similar TS and VS compositions ($5.15 \pm 0.03\%$ and $62.73 \pm 0.06\%$, respectively) compared to the inoculum obtained from the same source used in the batch studies previously reported in **Sections 4.1 and 4.2** (4.50–5.22% for TS and 60.1–69.9% for VS). The inoculum had a slightly alkaline pH of 7.90. The inoculum was tested for its methanogenic activity by determining the methane potential of cellulose. It was found the BMP of cellulose from this study was $310 \pm 39 \text{ L kgVS}^{-1}$. The value was slightly higher than that of the previous batch ($287 \pm 20 \text{ L kgVS}^{-1}$) but was still lower than the recommended BMPs of cellulose of 352–414 L kgVS^{-1} (Holliger et al., 2016). The underperformed inoculum may have affected the BMPs obtained in this study.

Table 4.9 Biomass characteristics of the inoculum and plant feedstocks. Values are means of samples $\pm$ standard deviations (n = 3)

Parameters ¹	<i>Opuntia ficus-indica</i>			Inoculum
	Ground cladode	Solid peel	Liquid extract	
TS (%FM)	12.84 $\pm$ 0.15	18.89 $\pm$ 0.32	9.94 $\pm$ 0.08	5.15 $\pm$ 0.03
VS (%TS)	87.98 $\pm$ 0.16	88.20 $\pm$ 0.18	88.63 $\pm$ 0.98	62.73 $\pm$ 0.06
pH	-	-	5.03	7.90
NDF ² (%TS)	38.40	-	-	-
ADF ² (%TS)	23.76	-	-	-
ADL ² (%TS)	9.15	-	-	-

¹TS – total solid content; VS – volatile solid content; FM – fresh matter weight; NDF – neutral detergent fibre; ADF – acid detergent fibre; ADL – acid detergent lignin. ²The analyses of these parameters were performed by an external laboratory (see **Appendix A**)

4.3.2.4 Results

In this experiment, three different types of processed *Opuntia* material, namely ground cladodes, solid peel, and liquid extract were digested with food waste digestate used as an inoculum at 6 different I/S ratios (0.35, 0.71, 1.04, 1.39, 2.08, and 2.78). **Fig. 4.13** depicts the cumulative methane yields of different types of *Opuntia ficus-indica* at different inoculum to substrate (IS) ratios. Methane production was recorded daily between day 1 and day 5 and then at intervals of 5 days until the experiment was terminated. Overall, the specific methane yields of all the substrates increased with increasing I/S ratios from 0.35 to 2.78. The lowest methane yields were 169 ± 22 , 162 ± 11 , and 196 ± 15 L kgVS⁻¹, at I/S ratios of 0.35 for the ground cladodes, solid peel, and liquid extract, respectively. The highest SMY was 289 ± 20 L kgVS⁻¹ obtained from *Opuntia* liquid extract (OLE) at the I/S ratio of 2.08. In general, methane production started from the first day at all I/S ratios, irrespective of the substrate type (**Fig. 4.13**). Strong inhibition of methane production was observed with the I/S ratio of 0.35 where the production of methane almost stopped after 3 days of digestion, and this impact was independent of the

substrate type (**Fig. 4.13a**). The lag phase at the I/S ratio of 0.35 lasted the longest but became shorter with increasing I/S ratios. The lag was unnoticeable at the ratio of 2.78. The rate of methane production can be assessed from the slope of the cumulative methane curves and in most cases, the production rates were the highest between days 15 and 25 where the slopes were steepest. The curves began to level off thereafter and the experiment was terminated at day 50 when the cumulative methane production had reached a plateau and the daily methane value was <1% of the total.

Of the three substrates, solid peel produced the lowest methane yields and was the substrate where methane production terminated the fastest regardless of the I/S ratios. In contrast, the liquid extract yielded the highest methane out of the three substrate types, however, the lag periods observed for liquid extract were the longest compared to those of ground cladodes and solid peel; the difference was noticeable particularly at lower I/S ratios (**Fig. 4.13a and b**).

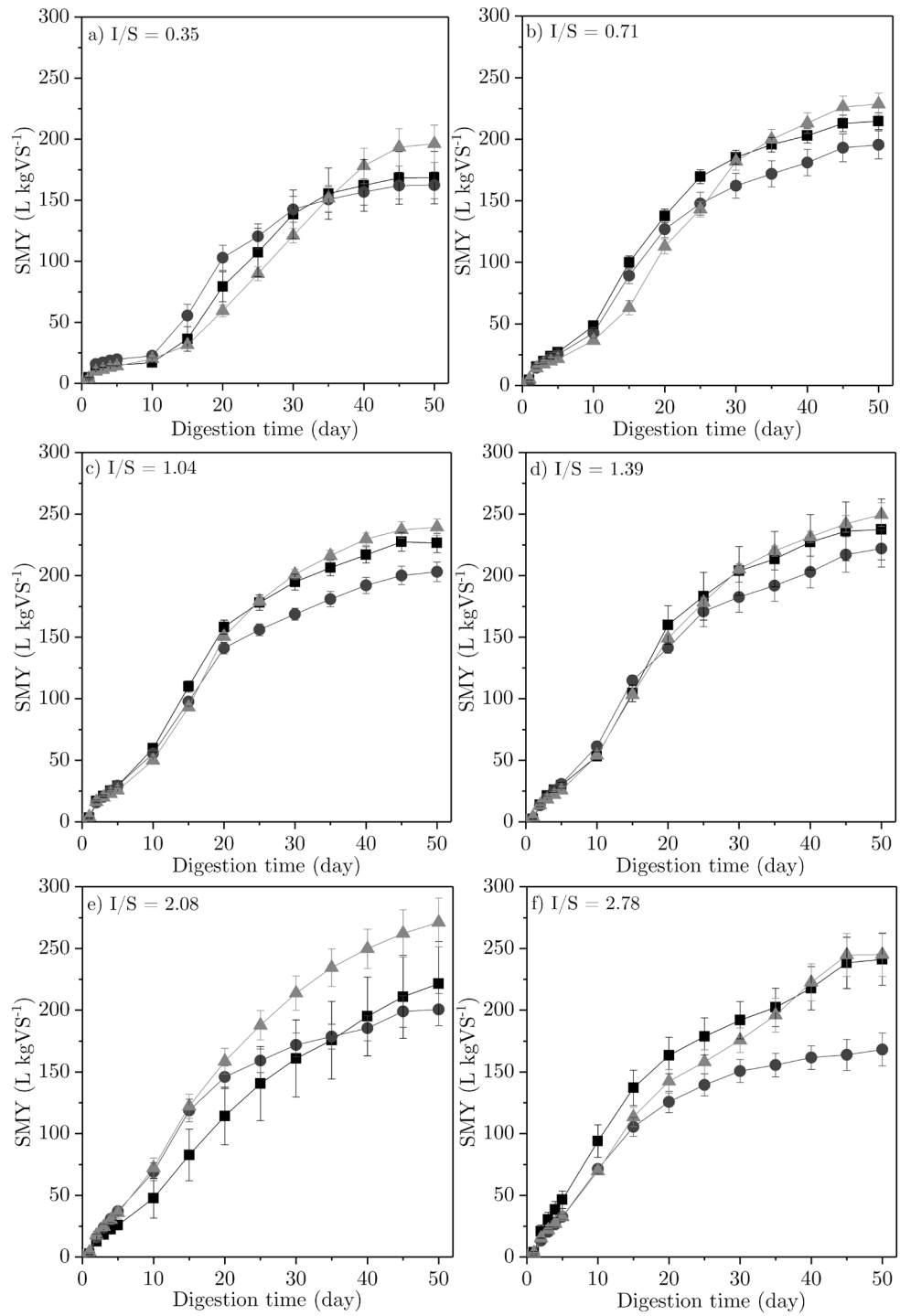


Fig. 4.13 BMP profiles showing specific methane yield, SMY, from six different inoculum to substrate (i/S) ratios: **a)** 0.35, **b)** 0.71, **c)** 1.04, **d)** 1.39, **e)** 2.08, and **f)** 2.78. (■), (●), and (▲) denote three different types of *Opuntia ficus-indica*, ground cladode, solid peel, and liquid extract, respectively. Error bars represent $\pm$ standard deviations ($n = 3$).

The results from the one-way ANOVA test (**Fig. 4.14**) showed that there were significant differences in methane yields among the six I/S ratios regardless of the substrate type ($p = 0.028$ for ground cladode and $p < 0.001$ for both liquid extract and solid peel). Tukey's test was then used to further analyse the difference between each pair of mean SMYs from two different I/S ratios. For the ground cladode, the I/S ratio of 0.35 significantly affected the methane yields when compared to the ratios of 1.39, 2.08, and 2.78 (p values = 0.018, 0.048, and 0.005, respectively). Similarly, for liquid extract, the I/S ratio of 0.35 yielded significantly lower methane compared to the rest of the I/S ratios tested. In contrast, the results obtained from solid peel showed a slightly different pattern compared to the other two substrate types. The highest methane yield was observed at the I/S ratio of 1.39 and was significantly higher than the other two extreme I/S ratios tested, 0.35 and 2.78 (p values = 0.014 and 0.015, respectively).

It can be concluded that the I/S ratios of 0.71 and above were optimal for anaerobic digestion of *O. ficus-indica* regardless of the substrate types tested. There was one exception where the I/S ratio of 2.78 could be marginally suboptimal for methane production from the solid peel. Therefore, the recommended I/S ratios for *O. ficus-indica* were between 0.71 and 2.08.

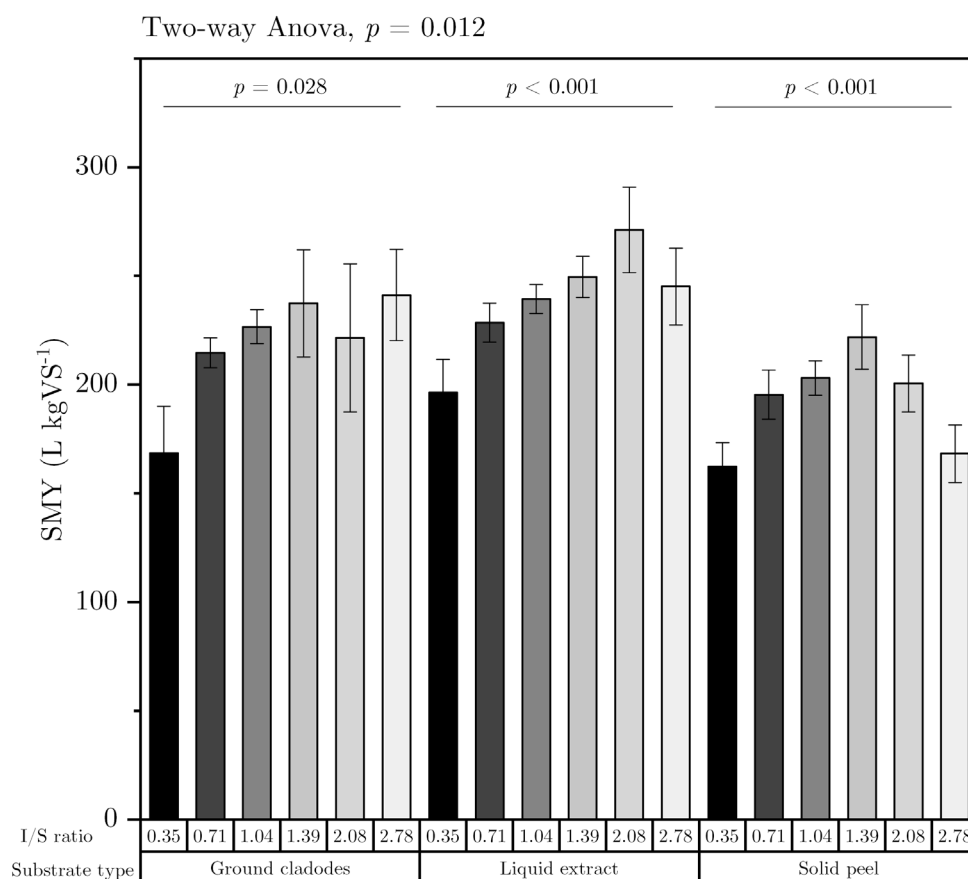


Fig. 4.14 Bar plot summarising the specific methane yields (SMY) of three different types of *Opuntia ficus-indica* in mesophilic conditions at different inoculum to substrate (I/S) ratios. One-way ANOVA analysis was performed for the effect of inoculum to substrate ratio. A two-way ANOVA test was employed for the comparison of mean methane yields between three different processing techniques. Error bars represent standard deviations of triplicate samples.

Volatile fatty acids were produced as an intermediate product and their concentrations along with pH were used to assess the stability of the AD system. The change in total volatile fatty acid (TVFA) concentrations at six different I/S ratios for three different substrate types was monitored and the results are presented in **Fig. 4.15**. In general, there was a clear relationship between TVFA and pH where a considerable increase in TVFA concentrations after 24 hours of fermentation resulted in a sudden decrease in pH values. Higher TVFA concentrations were observed at lower I/S ratios, especially at 0.35 and 0.71. Detailed VFA analysis showed that acetic acid was the main acid present followed by propionic acid and butyric acid. Neither I/S ratio nor substrate type had an influence on the VFA composition and hence only the TVFA values are presented.

At the I/S ratios of 2.08 and 2.78, the peak concentrations of TVFA were observed on the first day whereas those at lower I/S ratios were seen later on day 6. The highest TVFA concentrations were observed at an I/S ratio of 0.35 and the values were 7.95 ± 0.34 , 8.77 ± 0.09 , and $7.86 \pm 0.43 \text{ g L}^{-1}$, for ground cladodes, solid peel and liquid extract, respectively. After day 6, rapid VFA consumption was observed at all I/S ratios and the final concentrations of TVFA in most cases were detected to be below 0.1 g L^{-1} by day 30. The liquid extract at an I/S ratio of 0.35 was one exception where the final VFA concentration remained high at $1.60 \pm 0.22 \text{ g L}^{-1}$.

The starting pH of the BMP culture was 8.10 (**Fig. 4.15**). The pH in all conditions decreased rapidly after 24 hours of digestion and the largest decrease in pH was observed at an I/S ratio of 0.35. For the I/S ratios above 1.04, the pH values remained in a mild alkaline range between 7.0 and 7.5. The values of pH fluctuated slightly during the first 10 days then stay almost constant until the end of the experiment. The average final pH values for all I/S ratios were 7.35 ± 0.08 , 7.40 ± 0.09 , and 7.30 ± 0.10 , for ground cladodes, solid peel, and liquid extract, respectively. These near-neutral pH implied that the volatile fatty acids were efficiently converted into methane (Cremonez et al., 2019).

In contrast, a substantial decrease in pH to below 7.0 was observed at the I/S ratios below 1.04. At an I/S ratio of 0.35, the pH steadily decreased from day 1 and was the lowest on day 8 at 6.40 ± 0.16 and 6.46 ± 0.24 , for ground cladode and solid peel, respectively. The sample pH subsequently increased in response to a drop in TVFA level. By day 20, when the TVFA concentrations were lower than 3.0 g L^{-1} , the pH values increased to above 7.0. For the liquid extract at an I/S ratio of 0.35, the sample was under acidic pHs for a longer period as opposed to the other two

substrates. The pH continued to decrease from day 8 and was the lowest at 6.24 ± 0.07 on day 15.

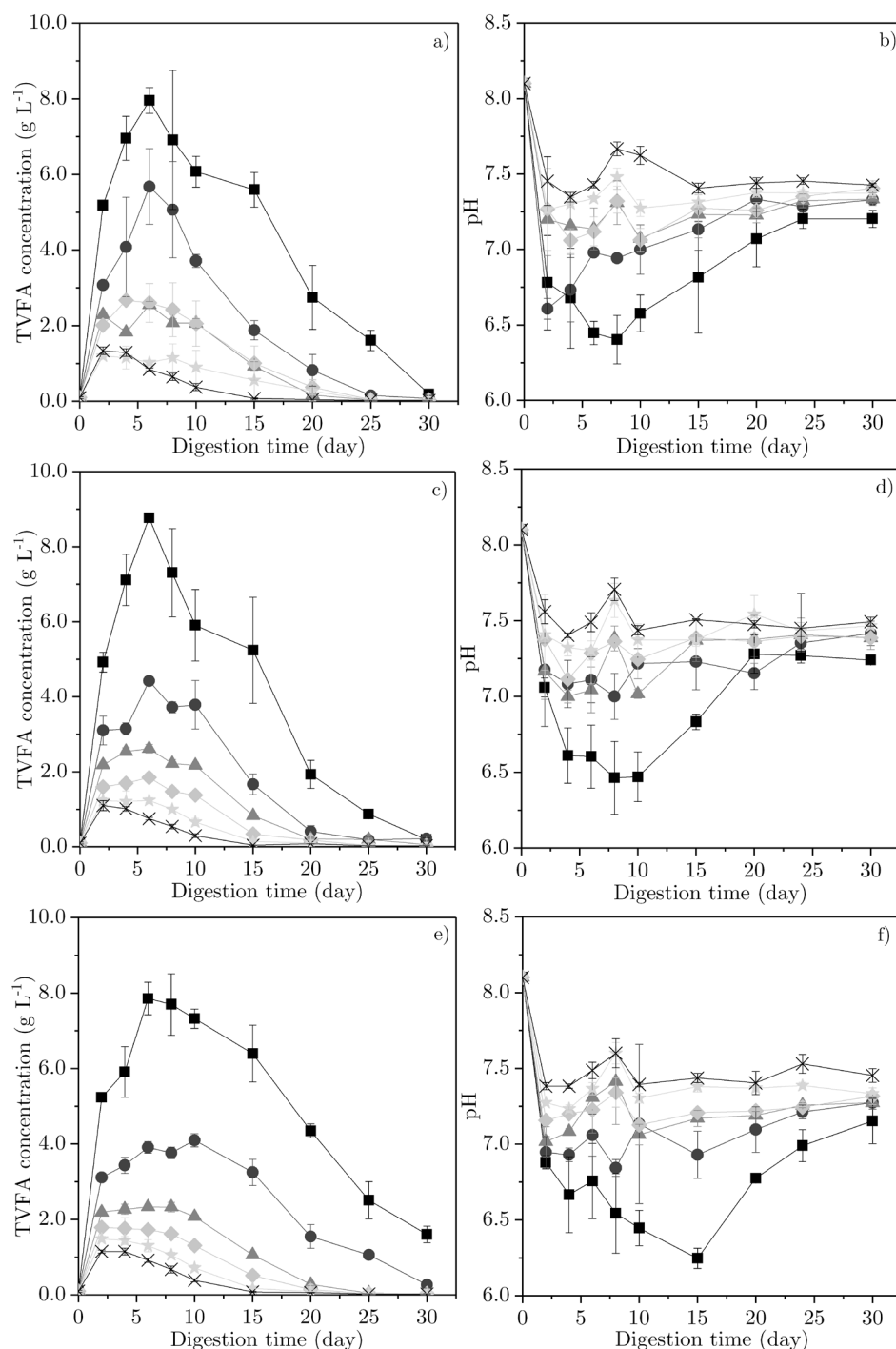


Fig. 4.15 Total volatile fatty acid (TVFA) concentration and pH evolution from three different types of *Opuntia ficus-indica* substrate, **a-b**) ground cladodes, **c-d**) solid peel, and **e-f**) liquid extract. The effect of different inoculum to substrate ratios and substrate types on cumulative methane yield were investigated. (—■—), (—●—), (—▲—), (—◆—), (—★—) and (—×—) denote the inoculum to substrate ratios of 0.35, 0.71, 1.04, 1.39, 2.08, and 2.78, respectively. Error bars represent $\pm$ standard deviations ($n = 3$).

4.3.2.4.1 Modified Gompertz model

The methane production kinetics for all samples are presented in **Table 4.10**. The results obtained from the model indicated that the experimental and predicted values were in very good agreement with R^2 values between 0.988 and 0.999. For all three substrate types, the lag periods became shorter with increasing inoculum to substrate (I/S) ratios. The lowest lag time (0.47 days) was observed with ground cladode at I/S ratios of 2.78, implying that the methane production started from the first day. Solid peel had the shortest lag time compared to ground cladode and liquid extract in all I/S ratios. For example, at the I/S ratio of 2.04, the λ values were 2.70, 1.21, and 2.08 days, for the ground cladode, solid peel, and liquid extract, respectively. Despite having the longest lag phase, the liquid extract also had the highest ultimate methane potential (A) among the three substrates, irrespective of the I/S ratios. The ranges of A values were 182–241 L kgVS⁻¹, 172–218 L kgVS⁻¹, and 250–276 L kgVS⁻¹, for the ground cladode, solid peel, and liquid extract, respectively. The A values also increased with increasing I/S ratios from 0.35 to 2.08 in substrate types. Lastly, no clear correlation was found between the μ values and substrate types or I/S ratios.

Table 4.10 Kinetic parameters of a modified Gompertz model obtained from anaerobic digestion of three different types of *Opuntia ficus-indica* with different inoculum to substrate (I/S) ratios.

Operating conditions		SMY	Parameters from Modified Gompertz model ¹			
Substrate type	I/S	(L kgVS ⁻¹)	A (L kgVS ⁻¹)	μ (L kgVS ⁻¹ d ⁻¹)	λ (d)	R ²
Ground cladodes	0.35	169 ± 22	182	6.51	7.64	0.989
	0.71	215 ± 7	218	8.39	3.01	0.997
	1.04	227 ± 8	229	9.15	2.70	0.997
	1.39	237 ± 25	241	9.36	3.23	0.996
	2.08	222 ± 34	233	6.49	2.70	0.996
	2.78	241 ± 21	232	8.96	0.47	0.989
Solid peel	0.35	162 ± 11	172	6.14	4.12	0.988
	0.71	195 ± 11	197	7.29	2.77	0.996
	1.04	203 ± 8	203	7.87	2.21	0.996
	1.39	222 ± 15	218	8.46	2.25	0.996
	2.08	201 ± 13	195	8.42	1.34	0.995
	2.78	168 ± 13	164	7.82	1.29	0.996
Liquid extract	0.35	196 ± 15	250	5.83	8.90	0.994
	0.71	229 ± 9	250	7.51	5.00	0.996
	1.04	239 ± 7	247	9.02	3.68	0.997
	1.39	250 ± 10	254	9.07	3.54	0.999
	2.08	271 ± 20	276	9.05	2.15	0.999
	2.78	245 ± 18	254	7.41	1.53	0.999

¹ A – Methane production potential; μ - maximum methane production rate; λ – lag time.

4.3.2.5 Discussion

The BMPs of the ground cladode of *O. ficus-indica* in this study ranged between 169 and 241 L kgVS⁻¹. The yields obtained from this study were relatively lower compared to the values reported in a recently reported study, in which the BMPs of *O. ficus-indica* from different plantations and different harvesting seasons were found to be 280–295 L kgVS⁻¹ (Krümpel et al., 2020). The difference in methane yields between the current and previous studies partly could have been due to the variation in the substrate characteristics. The lignin composition of *Opuntia* in this study was 9.15%TS which was considerably higher compared to 1.80–3.88%TS reported for their *Opuntia* substrates (Krümpel et al., 2020). Another point to

consider was substrate selection. Their study selected relatively younger cladodes (between two and three-year-olds) as substrates and the average TS (dry matter) content for their cladodes was 10.5% of fresh weight, lower than that of *Opuntia* cladode used in this study (12.8%). The higher TS and lignin content of the cladodes investigated in the current study implied that the cladodes were most likely older than 3 years old and thus was more rigid and lignified, which made the substrate more difficult for microorganisms to degrade. Inoculum activity was also another factor to consider when the BMP of a substrate was relatively low. The methanogenic activity of the inoculum used in this study was 310 L kgVS⁻¹ which was under the recommended values of between 352 and 414 L kgVS⁻¹ (Holliger et al., 2016). Thus, the BMPs of *Opuntia* cladodes in this study could have been underestimated.

There was a significant difference in methane yields obtained from different substrate types, particularly between the solid peel and the liquid extract. Cladodes of *O. ficus-indica* have been reported in several studies to contain sugars, organic acids, amino acids, and essential minerals and vitamins (Ayadi et al., 2009; El-Mostafa et al., 2014; Santos et al., 2016). After substrate processing, the liquid obtained contained the majority of these compounds whereas the remaining solid from the extraction process was mostly the cladode peel. The liquid extract did not contain lignin and other recalcitrant constituents and thus was significantly more biodegradable relative to the solid peel. For more easily degradable substrates, the I/S ratio of equal or greater than 4.0 was recommended to ensure that rapid VFA production would not lead to process inhibition (Holliger et al., 2016), however, the I/S ratio for *Opuntia* liquid was optimum at 2.08 in this study. The solid pulp, which mostly contained lignocellulosic components, would not require as a high I/S ratio as the liquid extract and thus the highest SMY was found at the I/S ratio of 1.39.

The lowest I/S ratio of 0.35 was found to be impractical to be used for the BMP study of *O. ficus-indica*. At this ratio, the peak concentrations of acetic acid for all three substrates were between 5.27 and 6.56 g L⁻¹. A surge in the production of volatile fatty acids (VFAs) led to a substantial drop in pH to between 6.24 and 6.46 and methane production was halted at this stage. These pH values have been reported to be inhibitory to methane production as the optimum pH values suitable for methanogens to function were 6.5–7.8 (Li et al., 2014). The use of low I/S ratios could also negatively affect the process stability and was therefore not recommended, as suggested by other previous studies (Raposo et al., 2009, 2006).

4.3.3 Experiment II – Effect of cladode age on anaerobic digestion of *O. ficus-indica*

4.3.3.1 Objective

The objective of this experiment was to determine the effect of the harvesting age of cladodes on the BMP of *Opuntia ficus-indica*.

4.3.3.2 Methodology

Opuntia cladodes of five different ages (approximately 0.5, 1.0, 2.0, 3.0, and 4.0 years) were selected for the BMP test to investigate the effect of cladode age on methane production. The cladodes from each age group were subsequently homogenised by grinding, according to the procedures described in **Section 2.2.1**. The homogeneous material was then used as the substrate for this experiment. Biogas and liquid samples were collected at regular intervals to determine gas volume, gas composition, pH and VFA content. The modified Gompertz equation was used to fit the data of cumulative methane yields obtained from the BMP experiment to estimate the ultimate methane yield, maximum methane production

rate and the lag phase duration. The detailed description of the BMP setup is described in **Chapter 3, Section 3.1.2**.

4.3.3.3 Biomass and inoculum characteristics

The biomass characteristics of *Opuntia* cladodes of different ages and the inoculum employed were investigated. The results of this analysis are shown in **Table 4.11**. As can be seen, the total solid (TS) contents decreased with the increasing age of *Opuntia* cladodes and the highest TS percentage was $10.21 \pm 0.05\%$ obtained for 0.5-year-old cladodes. However, there was no clear correlation between the volatile solid (VS) content and the plant age; the VS contents of *Opuntia* cladodes aged 0.5–4 years old ranged between 79.7% and 87.5%. *Opuntia* cladodes used in this experiment had relatively lower TS contents (8.01–10.21%) compared to that of the previous batch (12.8%) presented in **Section 4.3.2.3**. **Table 4.11** also presents the differences in length, thickness, and weight of the cladodes at different ages. An increase in size and weight of the cladodes was noticeable with ages from 0.5 to 3.0 years but this change was less evident between the ages of 3 and 4 years.

The TS and VS contents ($5.11 \pm 0.03\%$ and $66.10 \pm 1.19\%$, respectively), and pH (8.04) of the inoculum used in this experiment were comparable to those of the previous batches. The positive control (microcrystalline cellulose) was used in this study to determine the specific methanogenic activity of the inoculum. It was observed that the cellulose control had a specific methane yield of $359 \pm 9 \text{ L kgVS}^{-1}$. This value showed reasonable agreement with the recommended guideline and indicated that an inoculum was active and suitable for BMP assay (Holliger et al., 2016).

Table 4.11 Biomass characteristics of inoculum and *Opuntia* samples at different ages. Values are means of replicates $\pm$ standard deviations (n = 3).

Parameters	Age of <i>Opuntia</i> cladodes (years)					Inoculum
	0.5	1.0	2.0	3.0	4.0	
TS (%FM)	10.21 $\pm$ 0.05	9.58 $\pm$ 0.10	9.42 $\pm$ 0.12	8.01 $\pm$ 0.11	9.74 $\pm$ 0.17	5.11 $\pm$ 0.03
VS (%TS)	84.63 $\pm$ 0.09	87.48 $\pm$ 0.19	85.88 $\pm$ 0.39	79.66 $\pm$ 0.35	86.40 $\pm$ 0.39	66.10 $\pm$ 1.19
pH	-	-	-	-	-	8.04
Maximum length (cm) ¹	28.1 $\pm$ 1.5	31.5 $\pm$ 1.6	35.5 $\pm$ 1.9	44.0 $\pm$ 2.9	41.8 $\pm$ 1.8	-
Maximum width (cm) ¹	13.5 $\pm$ 1.6	15.3 $\pm$ 0.1	17.5 $\pm$ 0.8	17.9 $\pm$ 2.6	20.8 $\pm$ 3.3	-
Thickness (mm) ¹	6.5 $\pm$ 0.6	9.6 $\pm$ 0.1	11.7 $\pm$ 0.7	13.2 $\pm$ 0.5	12.8 $\pm$ 2.8	-
Vascular bundle diameter at node (mm) ¹	9.0 $\pm$ 1.0	12.2 $\pm$ 0.8	16.0 $\pm$ 1.1	20.0 $\pm$ 0.8	22.9 $\pm$ 2.4	-
Weight (g) ¹	280.9 $\pm$ 57.5	466.7 $\pm$ 76.5	513.0 $\pm$ 84.8	729.2 $\pm$ 52.5	1,027.4 $\pm$ 30	-

¹ Mean values of 5 samples

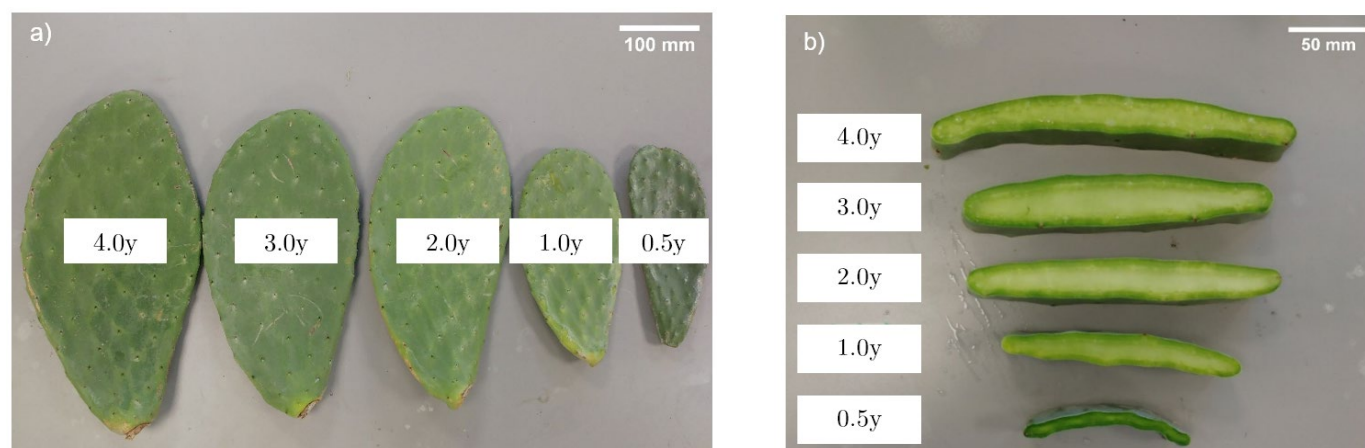


Fig. 4.16 a) *Opuntia ficus-indica* cladodes of five different ages b) Transverse-section of *Opuntia* cladode of different ages

4.3.3.4 Results

The influence of cladode age on methane yields of *O. ficus-indica* was investigated by examining cladodes whose ages range from 0.5, 1.0, 2.0, 3.0, and 4.0 years. The cumulative methane yields, methane percentages and pH values over the digestion period of 40 days are shown in **Fig. 4.17**. Similar patterns of methane production were observed for all five samples, where methane production started slowly for the first 10 days, then accelerated afterwards and stopped after 40 days of digestion. The specific methane yields (SMY) were 352 ± 9 , 363 ± 9 , 360 ± 19 , 319 ± 10 , and 323 ± 11 L kgVS⁻¹, for cladodes aged 0.5, 1.0, 2.0, 3.0, and 4.0 years old, respectively. According to the BMP results, the cladode samples could be categorised into two different groups: young cladodes (ages between 0.5 and 2.0-year-old) and lignified cladodes (ages between 3.0 and 4.0-year-old).

The specific methane yields (SMYs) of *Opuntia* cladodes at different ages are illustrated as bar plots in **Fig. 4.18**. The one-way ANOVA test suggests that the age of prickly pear cladode had a significant effect on the methane yield ($p < 0.001$). The SMYs obtained from 0.5, 1.0 and 2.0-year-old cladodes were significantly higher than those at 3.0 and 4-year-old ones. The three highest methane yields obtained from the younger cladodes (age below 2 years) were very similar (350 ± 9.4 , 354 ± 10.1 , and 354 ± 20.2 L kgVS⁻¹ for 0.5, 1.0 and 2.0-year-old cladodes, respectively). There was no significant difference in methane composition among different cladode ages ($p > 0.05$) (**Fig. 4.17b**). The average methane contents between day 15 and day 40 were $57.9 \pm 0.6\%$, $57.9 \pm 0.6\%$, $58.3 \pm 1.1\%$, $58.4 \pm 0.6\%$, and $58.2 \pm 0.5\%$ for 0.5, 1.0, 2.0, 3.0 and 4.0-year-old cladodes, respectively.

The change in pH was also monitored in this experiment and the results are reported in **Fig. 4.17c**. The starting pH for all samples was 7.50, which then

decreased after 24 hours to the lowest pHs between 6.91 and 7.06. Subsequently, there was a constant increase in pH values, which became relatively steady after day 5 throughout the digestion period. The average pH values between day 5 and 30 were 7.29 ± 0.05 , 7.24 ± 0.03 , 7.24 ± 0.02 , 7.26 ± 0.02 , and 7.23 ± 0.01 , for 0.5, 1.0, 2.0, 3.0 and 4.0-year-old cladodes, respectively.

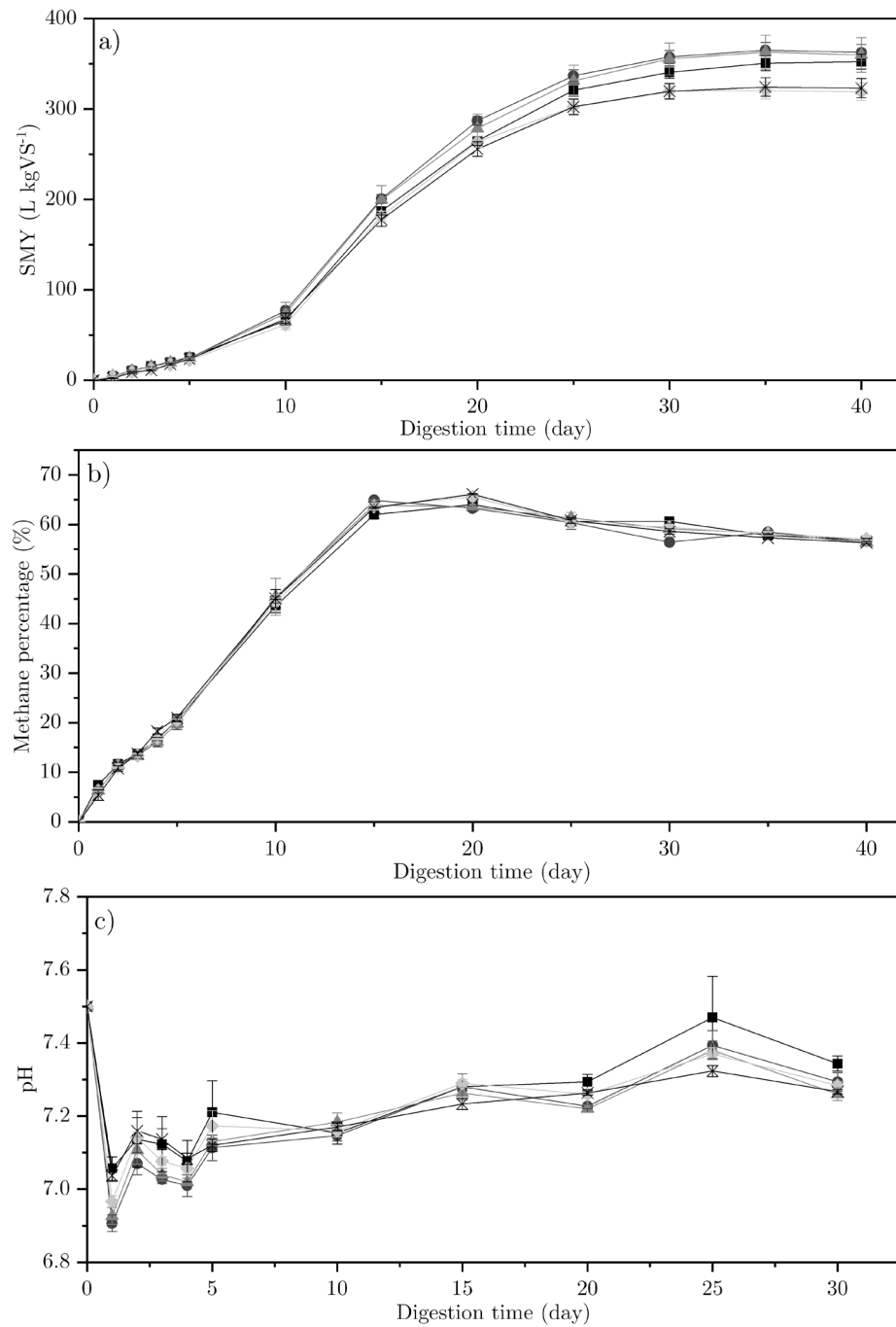


Fig. 4.17 BMP profiles showing **a)** specific methane yield, SMY, **b)** methane percentage and **c)** pH from five different ages of *Opuntia ficus-indica* cladodes. (■), (●), (▲), (◆), and (×) denote different cladode ages, 0.5, 1.0, 2.0, 3.0 and 4.0 years old, respectively. Error bars represent $\pm$ standard deviations of samples ($n = 3$).

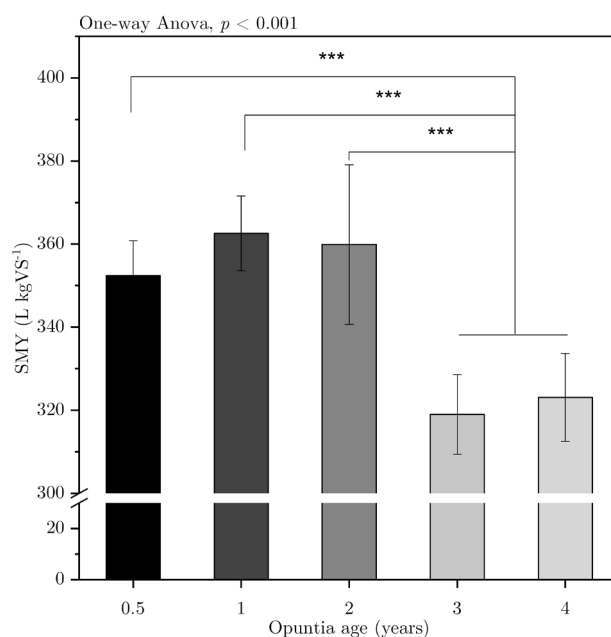


Fig. 4.18 Bar plot summarising the mean specific methane yields (SMY) of the cladodes of *Opuntia ficus-indica* at different ages. One-way ANOVA analysis was performed for the effect of cladode age on the methane yields. *** denotes the significant difference ($p \leq 0.001$). Error bars represent $\pm$ standard deviations of samples ($n = 3$)

The starting TVFA concentration for all substrates was 0.037 g L^{-1} , which increased rapidly after 24 hours and peaked between 1.75 and 2.01 g L^{-1} on day 5 (**Fig. 4.19**). Afterwards, a steady decline in TVFA concentrations was observed between day 10 and day 30 when the rate of methane production increased. The final concentrations of TVFA were below 0.100 g L^{-1} after 30 days of digestion. The most abundant volatile fatty acids detected were acetate, propionate, and butyrate. Acetate was the most abundant during the first 10 days of digestion and its concentration was the highest on day 5 at between 1.24 – 1.39 g L^{-1} . Most of the acetic acid was consumed by day 15 when the concentration dropped to between 0.061 and 0.203 g L^{-1} . No change in the concentration of propionic acid was observed between day 3 and day 15 when the acetate concentration predominated. From day 15, the propionic acid concentration began to drop, and the acid was completely consumed by day 30. The concentration of butyric acid was the lowest compared to acetic acid and propionic acid. They were relatively constant at around 0.100 g L^{-1} between day 2 and day 10 but eventually dropped to zero by day 15.

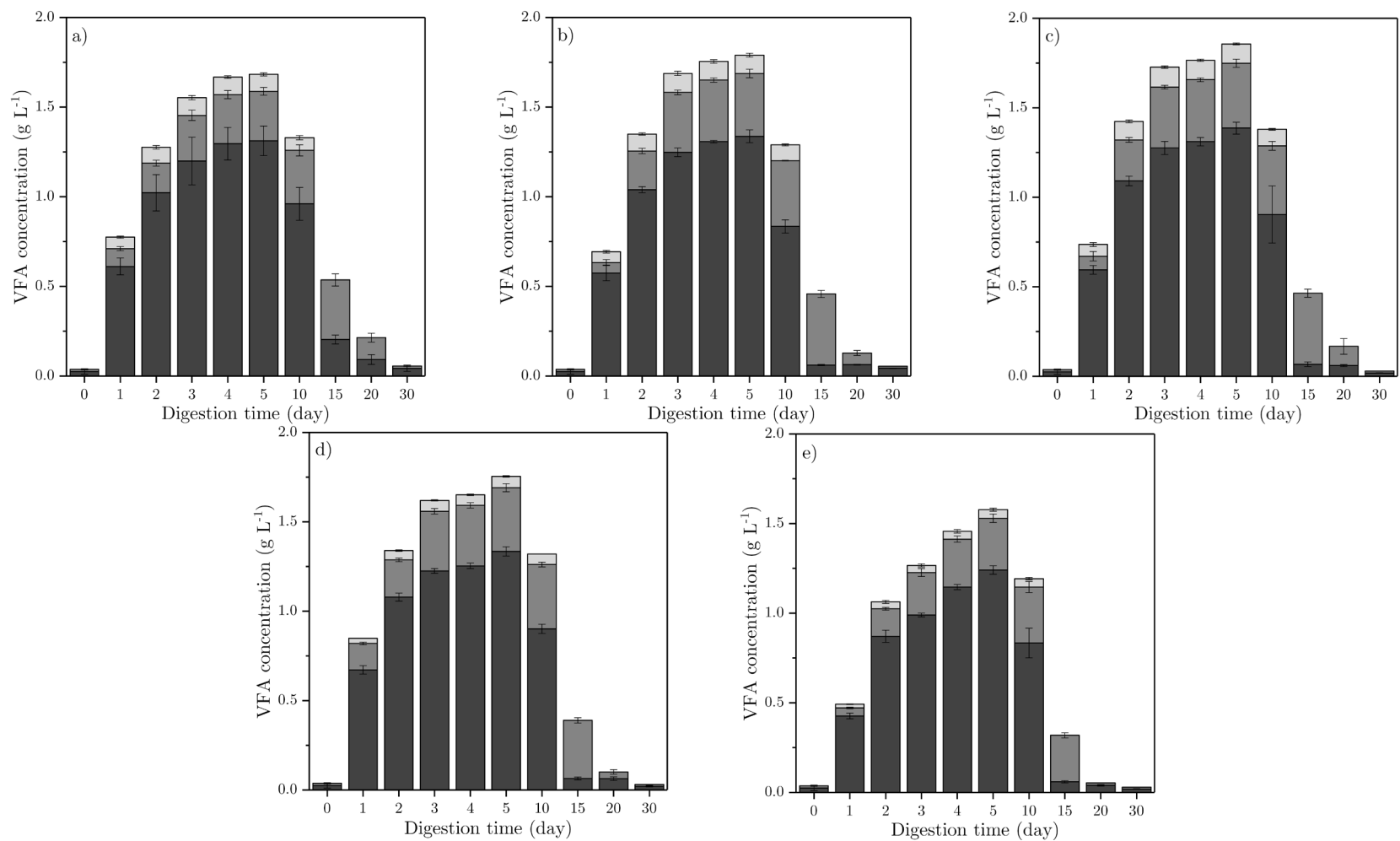


Fig. 4.19 VFA concentration profiles obtained from anaerobic digestion of *O. ficus-indica* at five different ages: **a)** 0.5 years **b)** 1.0 year **c)** 2.0 years **d)** 3.0 years and **e)** 4.0 years. (■), (▒), and (░) denote acetic acid, propionic acid, and butyric acid, respectively. Error bars represent ± standard deviation ($n = 3$).

4.3.3.4.1 Kinetic model

Modified Gompertz model parameters were determined from the SMYs and are presented in **Table 4.12**. The R^2 values of 0.995–0.996 in all cases revealed that the modified Gompertz model was a good fit for the experimental data. There was no detected influence of age of *Opuntia* cladodes on the maximum methane production rate (μ) and the lag period (λ). The μ values were between 21.0 and 23.9 L kgVS⁻¹ d⁻¹ and the values of lag time were 6.23–7.24 days. In contrast, lower ultimate methane yields (A) were associated with older cladodes. The A values were 361, 370, 368, 325, and 331 for the cladode ages of 0.5, 1.0, 2.0, 3.0 and 4.0 years.

Table 4.12 Kinetic parameters of a modified Gompertz model obtained from anaerobic digestion of *Opuntia ficus-indica* cladode of five different ages.

Cladode age (year)	SMY (L kgVS ⁻¹)	Parameters from modified Gompertz model ¹			
		A (L kgVS ⁻¹)	μ (L kgVS ⁻¹ d ⁻¹)	λ (d)	R^2
0.5	352 ± 9	361	21.2	6.26	0.996
1.0	363 ± 9	370	23.5	6.29	0.996
2.0	360 ± 19	368	22.7	6.23	0.996
3.0	319 ± 10	325	23.9	7.24	0.995
4.0	323 ± 11	331	21.0	6.37	0.996

¹ A – Methane production potential; μ - maximum methane production rate; λ – lag time

4.3.3.5 Discussion

The SMYs of *O. ficus-indica* at five different ages ranged from 319 to 363 L kgVS⁻¹. The results revealed that the age of the cladode significantly impacted the methane potential. Cladodes aged 3 and 4-year-olds, in particular produced 11–12% less methane compared to the one-year-old cladodes. Although fibre analysis of the cladodes used in this study was not performed, it has previously been reported that the percentage dry matter (TS) and crude fibre content increased with the cladode age (Nefzaoui and Ben Salem, 2002; Pimienta-Barrios et al., 1993; Rodriguez-Felix and Cantwell, 1988). The crude fibre content increased from 8 g per 100 g of dry

biomass in young cladodes (0.5 years) to 17.5 g in 4-year old lignified cladodes (Pimienta-Barrios et al., 1993). Older cladodes with higher crude fibre content consisted of a relatively higher proportion of lignin percentage, and therefore these cladodes were more difficult to degrade.

The effect of crop maturity on methane production has also been studied in more conventional energy crops such as Napier grass (Surendra and Khanal, 2015). The authors found that the lignin percentages of the biomass nearly doubled within 6 months between the first and last harvest. Napier grass at the age of 2 months produced significantly higher methane yields than the 8-month-old substrate. Also, they found that size reduction improved the digestibility of the materials, however, the impact of processing on methane yields was less significant than plant maturity, which suggests that younger crops should be used regardless of the biomass processing technique employed.

Apart from the crude fibre content, other chemical constituents of *Opuntia* cladodes such as the contents of protein, lipid and ash have also been previously reported (Nefzaoui and Ben Salem, 2002; Pimienta-Barrios et al., 1993; Rodriguez-Felix and Cantwell, 1988). The results were in agreement that the crude protein content decreased as age and dry matter of the cladodes increased. According to these findings, the carbon to nitrogen (C/N) ratio was likely to be relatively lower in younger cladodes compared to the lignified stem of *Opuntia*. One anaerobic digestion study of *O. ficus-indica* reported the BMP value of 424 ± 14 L kgVS⁻¹ for one-year-old cladodes, which had a C/N ratio of 26.7 (Calabrò et al., 2018). Thus, the use of younger cladodes (ages between 0.5 and 2.0 years) was recommended as they were likely to contain the C/N ratios of 20/1–30/1, which have been considered

optimum for growth and enzymatic activities of AD microorganisms (Hagos et al., 2017).

Nevertheless, the BMPs of *O. ficus-indica* in this experiment (319–363 L kgVS⁻¹) were considerably higher than the BMP of the ground cladode observed in Experiment I (241 L kgVS⁻¹). Although, as discussed in **Section 4.3.3.4**, the cladodes investigated in Experiment I were deemed to be older than three years old and had become lignified, they produced considerably lower methane yield (25.4% reduction), compared to the 4-year-old cladodes used in this experiment. The difference in yields of *Opuntia* between the two experiments could be attributed to two main factors: the substrate characteristics and the inoculum performance. It was apparent that the BMP of the ground cladode in Experiment I could have been underestimated, and this was due to the relatively low activity of the inoculum used in the previous experiment.

4.3.4 Experiment III – Semi-continuous digestion of *O. ficus-indica*

4.3.4.1 Objective

The objective of this experiment was to determine the methane potential of *O. ficus-indica* under mesophilic conditions at different loading rates in a semi-continuous experiment to assess the operational stability of the digesters

4.3.4.2 Methodology

The study employed 4 x 5-litre digesters run in duplicate (R1 R2 and R3 R4). Detailed reactor design and operation procedures are described in **Section 3.1.3**. The four digesters were previously run as a batch process for a comparative BMP study at a larger scale to Experiment I. The digesters were divided into two sets of duplicates with the starting I/S ratios of 2:1 and 1:1 for the digesters R1 R2 and the digesters R3 R4, respectively. When the biogas production started to plateau, the digesters were fed daily and the semi-continuous study began.

For the semi-continuous study, all digesters were operated at the hydraulic retention time (HRT) of 30 days and TS content of 5% initially (**Phase I**). The loading rate was adjusted every 4 weeks of operation. In Phase II, the effect of total solid content (TS) on biogas production was studied. The TS was increased from 5% to 7% for digesters R1 and R2 and from 5% to 10% for digesters R3 and R4. In the last phase (Phase III), the effect of hydraulic retention time (HRT) on biogas production was studied. Since digesters R3 and R4 were already operated at 10%TS and HRT of 30 days in Phase II, the author decided that in Phase III all digesters were operated at the same TS content (10%), which was approximately the %TS of *O. ficus-indica* used as a substrate in this study. Instead, the HRTs were varied by reducing from 30 days in Phase II to 20 days for digesters R1 and R2 and 15 days for digesters R3 and R4. Detailed digester operation is presented in **Table 4.13**.

Table 4.13 Test conditions of digesters in Experiment III

Digesters	Phase	Duration (days)	TS (%)	HRT (days)	OLR (gVS L ⁻¹ day ⁻¹)
R1 & R2	batch	18	-	-	-
	I	28	5	30	1.38
	II	28	7	30	1.93
	III	28	10	20	4.14
R3 & R4	batch	18	-	-	-
	I	28	5	30	1.38
	II	28	10	30	2.76
	III	28	10	15	5.52

4.3.4.3 Characterisation of biomass and inoculum

Biomass characteristics of inoculum and substrate are presented in **Table 4.14**. The total solid and volatile solid contents of the ground *Opuntia* cladodes investigated in this experiment were comparable to those of the previous batches used in Experiment I and II (TS and VS values were 8.01–12.8% and 79.7–87.9%, respectively). However, the fibre analysis results showed that the lignin content (ADL) of the cladodes used in this experiment was substantially lower than that of the more mature cladodes in Experiment I (9.15%). The 50-day BMP of the ground cladode in this study was 339 ± 22 L kgVS⁻¹, comparable to the average BMP result obtained from cladodes of different ages in Experiment II (343 ± 20 L kgVS⁻¹).

Table 4.14 Detailed characteristics of *Opuntia ficus-indica* and digestate. Values are means of replicates $\pm$ standard deviations (n = 3)

Parameters ¹	<i>Opuntia ficus-indica</i>	Digestate
TS (%FM)	10.57 $\pm$ 2.90	4.86 $\pm$ 0.29
VS (%TS)	82.74 $\pm$ 4.77	64.51 $\pm$ 0.29
pH	-	7.87
NDF² (%)	22.40	-
ADF² (%)	10.89	-
ADL² (%)	5.61	-

¹TS – total solid content; VS – volatile solid content; FM – fresh matter weight; NDF – neutral detergent fibre; ADF – acid detergent fibre; ADL – acid detergent lignin. ²The analyses of these parameters were performed by an external laboratory (see **Appendix A**).

4.3.4.4 Results

4.3.4.4.1 A summary of reactor history

A summary of the reactor history is shown in **Table 4.15**. The experiment demonstrated a smooth run between Phase 0 (batch) and Phase II (semi-continuous), despite some operational issues while setting up the digesters such as gas leakage and malfunction of gas metre caused by the initial high flow rate of biogas. Some deviations from the original plan were made during Phase III in response to changes in biogas production rate, digester pH and TVFA concentration. For example, the motor of digester R3 stopped on day 63 overnight. It was observed on the following day that upon restarting the DC power supply, the motor was able to work again but it ran intermittently and eventually stopped for the second day. The problem was initially assumed to occur from the digester being charged with higher OLR which may increase the viscosity of the content inside the digester. However, it was realised that the issue directly came from the motor as this was resolved after it was replaced on day 65. Another issue was observed on day 69 when the DC power supply failure occurred overnight, causing all the motors to stop working. The system was reset the next morning. The performance of digester R3 deteriorated very rapidly during Phase III so feeding was suspended from day 74 till day 80. Feeding to R3 resumed on day 81 at a lower OLR ($2.62 \text{ gVS L}^{-1} \text{ day}^{-1}$ instead of $3.55 \text{ gVS L}^{-1} \text{ day}^{-1}$) to allow the system to gradually re-adjust to the substrate. Other digesters (R1, R2 and R4) also showed a sign of stress from day 70. The digester performance continued to drop so feeding was interrupted on day 81 for two days and resumed on day 83 at their corresponding OLRs applied in Phase II.

Table 4.15 Summary of reactor history for digesters R1–R4

Phase	Date	Day	R1	R2	R3	R4	
0	31/08/2019	-17	Digester set-up as batch experiment for 15 days				
	1-3/09/2019	-16-14	Gas leakage found and gas metre malfunctioned				
I	17/09/2019	0	Started feeding at OLR of 1.38 gVS L ⁻¹ day ⁻¹ (5% TS and HRT of 30 days)				
II	15/09/2019	28	Increased OLR to 1.96 gVS L ⁻¹ day ⁻¹ (7% TS, HRT of 30 days)		Increased OLR to 2.62 gVS L ⁻¹ day ⁻¹ (10% TS, HRT of 30 days)		
III	13/11/2019	57	Increased OLR to 2.65 gVS L ⁻¹ day ⁻¹ (10% TS, HRT of 20 days)		Increased OLR to 3.55 gVS L ⁻¹ day ⁻¹ (10% TS, HRT of 15 days)		
	18- 19/11/2019	63-64			R3 Motor stopped overnight		
	20/11/2019	65			R3 Motor replaced		
	24/11/2019	69	DC Power supply failure overnight (R1–R4)				
	29/11/2019	74			Feed suspension for 7 days		
	06/12/2019	81	Feed suspension for 2 days		Decrease OLR to 2.62 gVS L ⁻¹ day ⁻¹	Feed suspension for 2 days	
	08/12/2019	83	Decreased OLR to 1.96 gVS L ⁻¹ day ⁻¹			Decreased OLR to 2.62 gVS L ⁻¹ day ⁻¹	
	16/12/2019	91	Feeding ceased (experiment was terminated)				

4.3.4.4.2 A comparative BMP study

The experiment was set up as a batch system initially to re-test the conditions in Experiment I, which was conducted to study the effect of the I/S ratio on the BMP of *O. ficus-indica*, at a larger laboratory scale. According to the results from Experiment I, there was no significant difference in methane yields when the

system was initiated at the I/S ratios of between 1.0 and 2.0. Thus, digesters R1 and R2 were set up at an I/S ratio of 2.0 while R3 and R4 were at an I/S ratio of 1.0.

Fig. 4.20a shows the cumulative biogas yields obtained from 4 digesters running in duplicate as a batch system. All digesters experienced a leakage problem during the first 24 hours and therefore, biogas data were not accurately recorded. Besides, the gas metre for R2 encountered a gas overflowing issue, which caused it to malfunction. The metre for the R2 digester was fixed by day 3. Due to these unprecedented issues, biogas data obtained for the duplicate R1 and R2 were considerably different. In contrast, there was a very good agreement in biogas yields between the replicate (R3 and R4). The biogas yields on day 18 obtained for digesters R3 and R4 were 403 and 431 L kgVS⁻¹, respectively.

The change in total volatile fatty acid (TVFA) concentrations and pH in different digesters on selected days are illustrated in **Fig. 4.20b and c**. Similar TVFA profiles were observed between the duplicate samples (R1 R2 and R3 R4). The TVFA levels peaked between day 5 and day 10 where the average concentrations were 2.80 and 4.47 g L⁻¹ for digesters R1 R2 and R3 R4, respectively. The TVFA concentrations then decreased by day 15 to 2.12, 1.82, 3.27, and 3.35 g L⁻¹ for digesters R1, R2, R3 and R4, respectively. The pH values of the four digesters over the 15 days varied between 7.12 and 7.84 (**Fig. 4.20c**). After 5 days of digestion, the pH values decreased from 7.87 to 7.20, 7.31, 7.05, and 7.15, for digesters R1, R2, R3 and R4, respectively. The relatively lower pH observed in digesters R3 and R4 were due to their lower I/S ratio, which resulted in a higher degree of acid accumulation. By day 15, the average pH values for digesters R1 R2 and R3 R4 were 7.72 and 7.68, respectively. The high pH values throughout the batch digestion period indicated

that both I/S ratios (1.0 and 2.0) were able to provide all digesters with sufficient buffering capacity despite the presence of VFA at high concentrations.

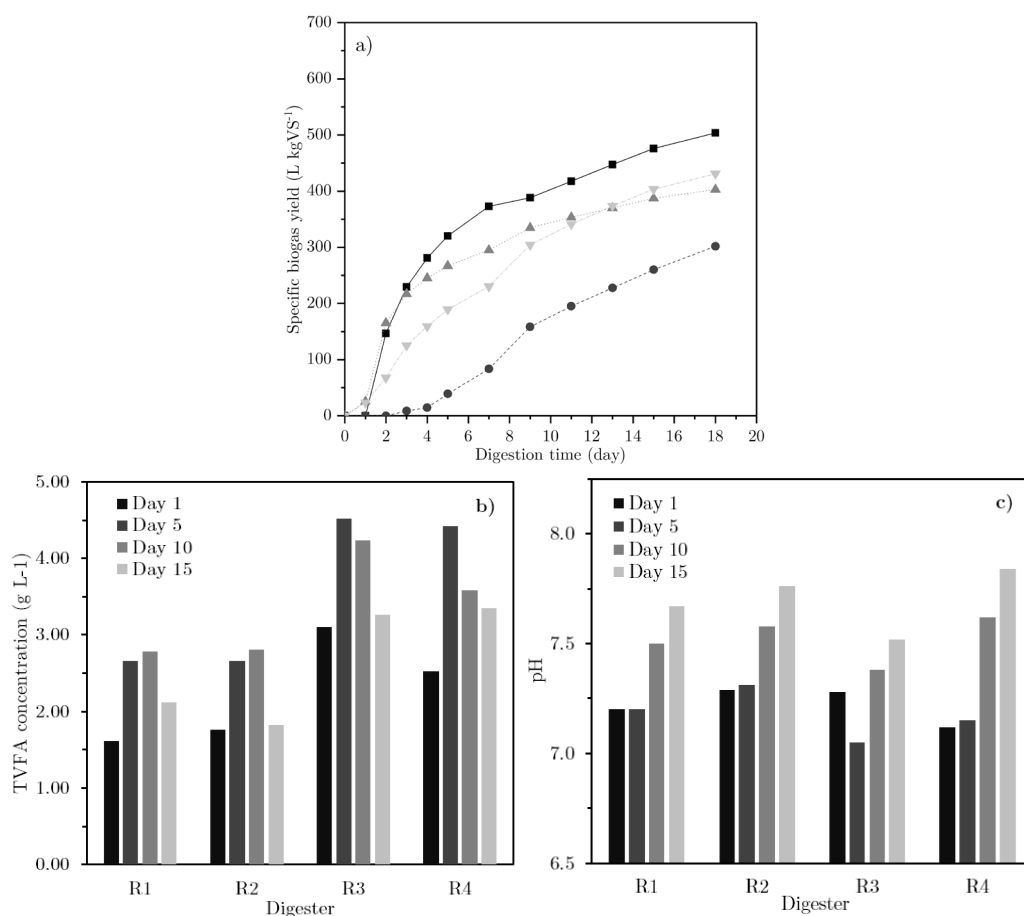


Fig. 4.20 a) Cumulative biogas yields from four different anaerobic digesters during the start-up period. (—■—), (—●—), (---▲---) and (---▼---) denote digesters R1, R2, R3, and R4, respectively. **b)** Total volatile fatty acid (TVFA) and **c)** pH profiles of four batch digesters on selective days 1, 5, 10 and 15.

4.3.4.4.3 The performance of semi-continuous digesters R1 and R2 between Phase I and Phase III

The daily biogas production from the semi-continuous digesters, R1 and R2, and their corresponding specific biogas yields (SBY) are shown in **Fig. 4.21**. In Phase I, both digesters R1 and R2 were fed daily to achieve the conditions of 5% digester TS at the hydraulic retention time (HRT) of 30 days. Both digesters demonstrated a similar pattern as they had shown a stable biogas production rate. The ranges of the daily biogas production were 0.71–0.86 L L_R⁻¹ day⁻¹ and 0.72–0.85 L L_R⁻¹ day⁻¹, for R1 and R2, respectively, between day 6 and day 28. The average methane

contents were $52.2 \pm 5.3\%$ and $53.6 \pm 2.5\%$ for R1 and R2, respectively. Gas data obtained at the initial stage of the experiment (the first 5 days) highly fluctuated and were excluded. The biogas production stabilised as early as day 5 at the rate of 0.63 and $0.73 \pm 0.20 \text{ L L}_R^{-1} \text{ day}^{-1}$, for R1 and R2, respectively. The dotted line shown in **Fig. 4.21b** represents the ultimate biogas yield of *O. ficus-indica* obtained from the BMP experiment at 640 L kgVS^{-1} . The biogas yields of R1 and R2 for Phase I were around $512\text{--}621 \text{ L kgVS}^{-1} \text{ day}^{-1}$ and $522\text{--}617 \text{ L kgVS}^{-1} \text{ day}^{-1}$, respectively, which accounted for over 80% of the ultimate biogas yield. Results from the paired *t*-test indicated that the difference in biogas yields obtained from both digesters was not statistically significant ($p = 0.25$), suggesting that the digesters R1 and R2 had a stable and comparable performance throughout Phase I.

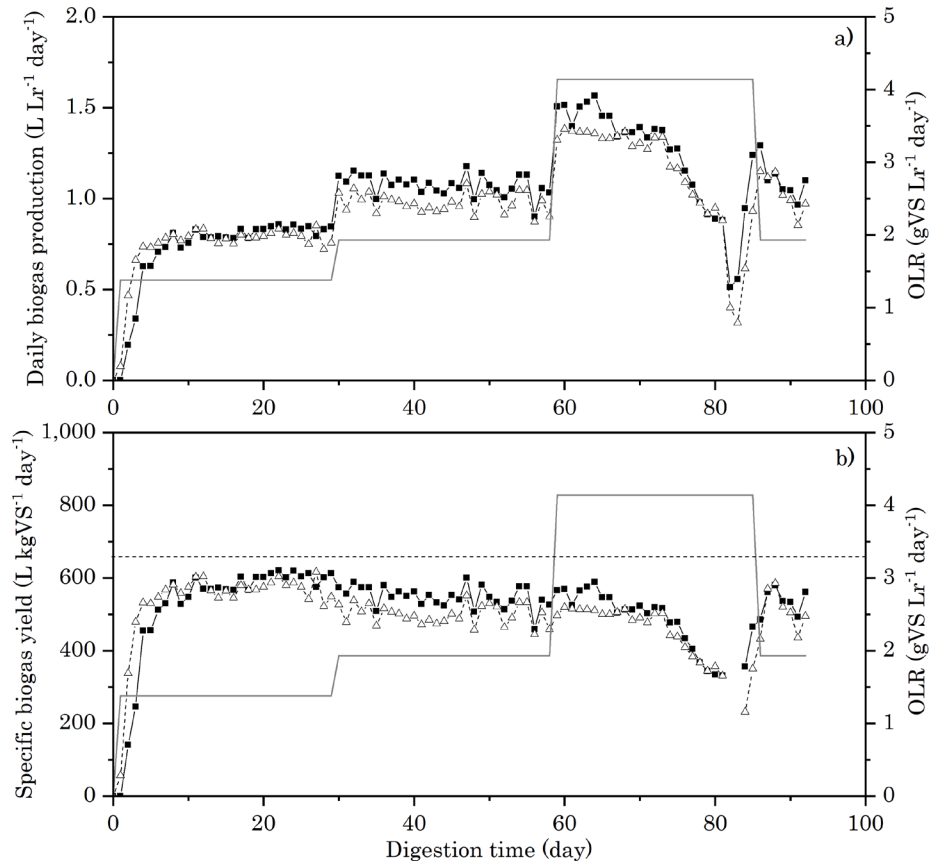


Fig. 4.21 a) Daily biogas production rate and b) specific biogas yield obtained from the duplicate digesters over 91 days of semi-continuous digestion experiment. (—■—), (---△---), (—) and (---) denote digester R1, digester R2, organic loading rate (OLR), and ultimate biogas potential of *Opuntia ficus-indica*, respectively.

After 28 days, feeding was adjusted to increase the percentage TS content for both digesters from 5% to 7% but the HRT was set to remain at 30 days, which resulted in an increase in organic loading rate (OLR) from 1.38 to 1.93 gVS L_R⁻¹ day⁻¹. During Phase II, both digesters became relatively less stable compared to Phase I and the difference in the daily biogas production rates and specific biogas yields between R1 and R2 were tested to be significant ($p < 0.001$). The daily biogas production for R1 and R2 in Phase II was around 0.90–1.18 L L_R⁻¹ day⁻¹ and 0.87–1.08 L L_R⁻¹ day⁻¹, respectively. The specific biogas yields slightly decreased compared to Phase I with an increasing OLR in Phase II. The specific biogas yields were 459–600 L kgVS⁻¹ day⁻¹ and 445–552 L kgVS⁻¹ day⁻¹ for digesters R1 and R2, respectively. The effect of OLR on the specific methane yields was also discussed in **Section 4.3.4.4.6**.

In Phase III, feeding was further adjusted to achieve the digester TS content of 10% while the HRT was also changed from 30 days to 20 days. This resulted in an increase in OLR to 4.14 gVS L_R⁻¹ day⁻¹. The biogas production from both digesters was relatively stable for approximately 15 days. During this period, the biogas production rates were around 1.27–1.57 L L_R⁻¹ day⁻¹ and 1.17–1.38 L L_R⁻¹ day⁻¹ for R1 and R2, respectively. The biogas yields continued to decrease with increasing OLR in Phase III. The values were 477–589 L kgVS⁻¹ day⁻¹ for R1 and 438–520 L kgVS⁻¹ day⁻¹ for R2. Due to an operational issue on day 69, the system was disturbed, causing the rate of biogas production to substantially decrease to below 0.5 L L_R⁻¹ day⁻¹ by day 80. After feeding was suspended for two days, both digesters were fed again on day 83 at the previous loading used in Phase II (1.93 gVS L_R⁻¹ day⁻¹). The lower loading rate would allow the system to recover from inhibition whereas delayed the rate of VFA production. There was a huge fluctuation in biogas production after the feeding was resumed after day 83. Although it seemed that both digesters rapidly responded to the lower loading and the biogas production

rate resumed to nearly the same production level observed in Phase II, the recovery might not last very long. Had the experiment not been terminated, the daily biogas production would very likely drop. This was because the total volatile fatty acid (TVFA) level, in particular the propionate level, remained very high after feed suspension. The relationship between the TVFA level and the biogas production is further discussed in **Section 4.3.5.3**.

4.3.4.4.4 The performance of semi-continuous digesters R3 and R4 between Phase I and Phase III

Similar patterns of daily biogas production from digesters R3 and R4 are illustrated in **Fig. 4.22**. Both digesters were treated using the same operating conditions as the digesters R1 and R2 in Phase I (5% TS content and HRT of 30 days). The daily biogas production rate of the digester R3 was relatively stable after 2 days of feeding, which was relatively faster than that of the digester R4, which took around 5 days. The biogas production rate was stable until day 20 when some instability was observed between the duplicates. The biogas production rates for digesters R3 and R4 were 0.57–0.79 L L_R⁻¹ day⁻¹ and 0.60–0.87 L L_R⁻¹ day⁻¹, respectively. Both digesters were able to achieve at least 65% of the ultimate biogas yield of *O. ficus-indica*, corresponding to the specific biogas yields of 416–573 L kgVS⁻¹ day⁻¹ and 437–628 L kgVS⁻¹ day⁻¹ for digester R3 and R4, respectively. Despite some fluctuation in biogas yields towards the end of Phase I, the results from the paired *t*-test suggested that the difference in biogas yields between R3 and R4 was not statistically significant.

Upon increasing the OLR from 1.38 and 2.76 gVS L_R⁻¹ day⁻¹ in Phase II, the daily biogas production for R3 and R4 increased to 1.30–1.74 and 1.24–1.57 L L_R⁻¹ day⁻¹, respectively. Similar to Phase I, the specific biogas yields of digesters R3 and R4 in Phase II were 496–662 L kgVS⁻¹ day⁻¹ and 473–601 L kgVS⁻¹ day⁻¹, respectively,

which accounted for at least 69% of the ultimate biogas yield from the BMP experiment. However, biogas yields obtained from R3 and R4 during Phase II were significantly different ($p < 0.001$), implying that the two digesters began to behave differently from Phase II.

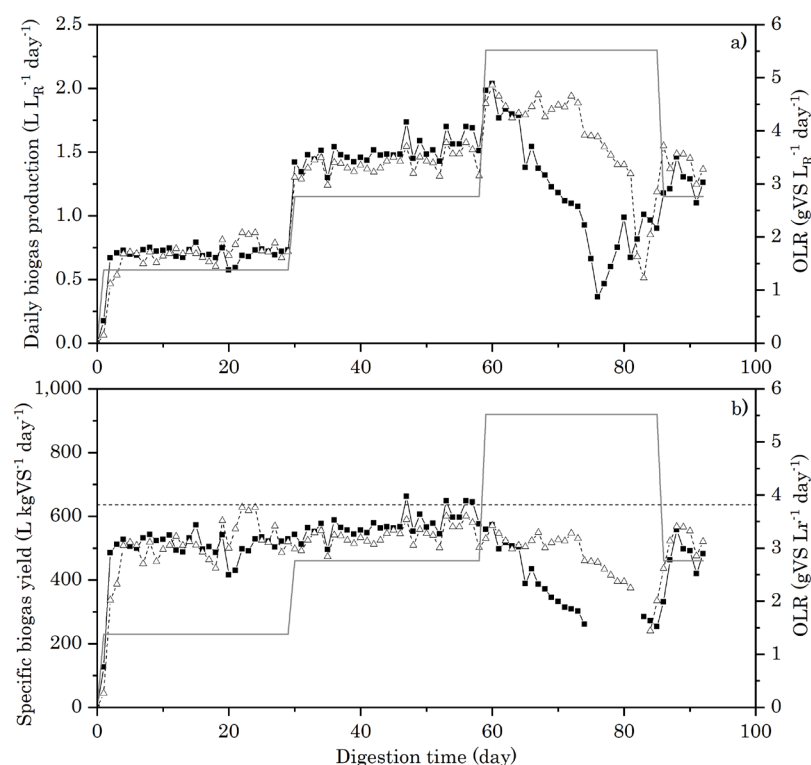


Fig. 4.22 a) Daily biogas production rate and b) specific biogas yield obtained from the duplicate digesters over 91 days of semi-continuous digestion experiment. (—■—), (—△—), (—) and (---) denote digester R3, digester R4, organic loading rate (OLR), and ultimate biogas potential of *Opuntia ficus-indica*, respectively.

HRT was reduced from 30 to 15 days during the final phase while maintaining the percentage TS content of the digesters at 10%. Decreasing HRT resulted in a lower specific biogas yield for both digesters. Steady biogas production was observed from digester R4 for approximately 15 days and during this period the biogas production rate and the specific biogas yield of digester R4 were 1.63–2.03 L L_R⁻¹ day⁻¹ and 458–571 L kgVS⁻¹ day⁻¹, respectively. Subsequently, there was a continuous decline in the biogas production rates and yields attributed to the acid build-up in the digester. Feeding was temporarily halted on day 81 for two days.

There was not enough time for the biogas production from digester R3 to become stable due to the motor malfunctioning issue 5 days after the feeding was adjusted in Phase III. The daily biogas production rate dropped to the lowest point at $0.36 \text{ L L}_R^{-1} \text{ day}^{-1}$ on day 74. Feed suspension was introduced at this stage for seven days to rescue the digestion system in R3. The feeding was resumed on day 81 at a previous loading used in Phase II ($2.76 \text{ gVS L}_R^{-1} \text{ day}^{-1}$). As was the case for R1 and R2, the biogas yields of R3 and R4 returned to close to the level achieved in Phase II. The final daily biogas production rates of digesters R3 and R4 were 1.26 and $1.37 \text{ L L}_R^{-1} \text{ day}^{-1}$, respectively, which were 84% and 95% of the rates achieved in Phase II at the same OLR.

4.3.4.4.5 Total volatile fatty acid (TVFA) and pH evolution in four semi-continuous digesters (R1–R4)

Daily biogas production was not the only parameter used for monitoring digestion stability. TVFA concentration and pH were also monitored during the experimental period. Overall, there was a very good agreement in TVFA concentration and pH level between duplicate samples, R1 R2 and R3 R4, especially in the first two operating phases **Fig. 4.23**. The TVFA peaks were observed at beginning of Phase I at the concentrations of 3.83 , 2.88 , 3.90 , and 3.63 g L^{-1} , for digesters R1, R2, R3 and R4, respectively. The level of TVFA dropped very rapidly during the first 15 days to below 0.5 g L^{-1} and continued to decrease to the end of Phase I. During Phase II, TVFA concentration levels had stabilised in all digesters, although, minor fluctuation in TVFA was observed between day 45 and day 55. The average TVFA concentrations during this period were 0.17 , 0.15 , 0.16 , and 0.17 g L^{-1} , for digesters R1, R2, R3 and R4, respectively. The decrease in hydraulic retention time (HRT) in Phase III did not affect the TVFA concentrations in digesters R1, R2 and R4 until day 69 when the DC power supply failed. The TVFA concentrations increased

considerably afterwards and peaked on day 80 at 1.61, 1.74, and 1.19 g L⁻¹ for R1, R2, and R4, respectively. Feeding was temporarily halted for two days to reduce the effect of acid accumulation. The TVFA concentrations decreased slightly but then rose very quickly once the feeding was resumed on day 83.

The concentrations of individual VFAs (acetate, propionate, and butyrate) during the period of acid accumulation between day 70 and day 85 are shown in **Fig. 4.24**. For digesters R1, R2 and R4, the acid accumulation was observed as early as day 71, when the concentrations of acetate and particularly propionate started to increase. The ratio of acetate to propionate decreased from 4.29, 5.78, and 2.32 in R1, R2, and R4 on day 70 to 0.96, 2.05, and 0.51 on day 81, respectively. The degradation of propionate was likely to occur during this period as the acetate concentrations were greater than 0.5 g L⁻¹ (Gorris et al., 1989; Mawson et al., 1991).

Although two-day suspension of feed between days 81 and 83 resulted in a decrease in the concentration of acetic acid and butyric acid, the level of propionic acid remained consistently high. After the feed was re-introduced, the acetate and propionate concentrations rapidly increased to a level higher than those before the feed suspension was imposed. A similar phenomenon was also observed in digester R3 (**Fig. 4.24c**), but the effect of acid accumulation was more severe. VFAs started building up after the motor failure incident and the concentrations of TVFA reached 1.39 g L⁻¹ by day 70. Feeding was halted on day 74 but the TVFA level continued to rise and peak on day 75 at 3.01 g L⁻¹. Between day 74 and day 80, the acetic acid level dropped considerably from 2.23 to 0.17 g L⁻¹, whereas the concentration of propionic acid decreased slightly from 0.92 to 0.59 g L⁻¹. As the digester was re-fed on day 81, the build-up of acetic acid was observed in the following day.

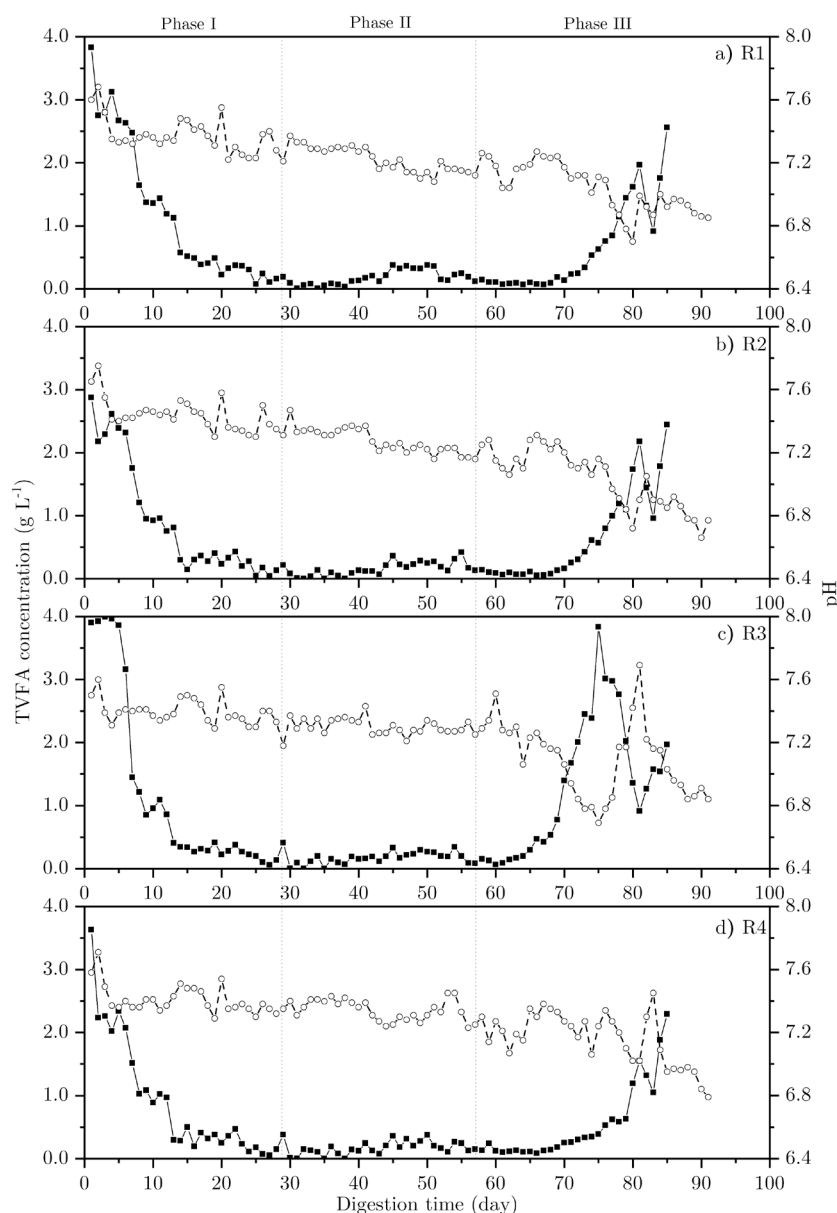


Fig. 4.23 TVFA concentration (—■—) and pH (---○---) evolutions of four digesters R1 and R2 (a and b) and R3 and R4 (c and d) for 90 days of semi-continuous digestion of *Opuntia ficus-indica*.

The pH in all digesters generally decreased over time but fluctuated substantially towards the end of Phase III (**Fig. 4.23**). The starting pH values on day 1 were relatively similar at 7.60, 7.65, 7.50, and 7.58 for digesters R1, R2, R3 and R4, respectively. The pH values then marginally decreased due to the increase in TVFA concentration at the beginning but soon stabilised between 7.33–7.40 after the first 5 days. The average pH values during Phase I were 7.37, 7.43, 7.39, and 7.41 for R1, R2, R3 and R4, respectively. Compared to Phase I, a lower degree of pH fluctuation in Phase II was observed and the average pHs in all digesters were 7.22–

7.33. As the HRTs were reduced from 30 days to 20 and 15 days for digesters R1 R2 and R3 R4, respectively, during Phase III, the pH values in two sets of digesters began to slightly vary. Particularly in R3, the pH dropped drastically from 7.30 immediately after day 63 when the motor stopped for almost two days to 6.69 by day 75. Feeding was then suspended for R3 accordingly. The performance of the other digesters started to be affected after the DC power supply failure on day 69. Even though the variation in pH values was not initially noticeable, as soon as the TVFA concentration increased above 1 g L^{-1} , the pH decreased to the lowest point on day 80 at 6.70, 6.72, and 7.02 for digesters R1, R2, and R4, respectively. Temporary feed suspension was imposed but the digesters had failed to counter the effect of TVFA accumulation. Once they were recharged with feed, and the pH further decreased to 6.77–6.85.

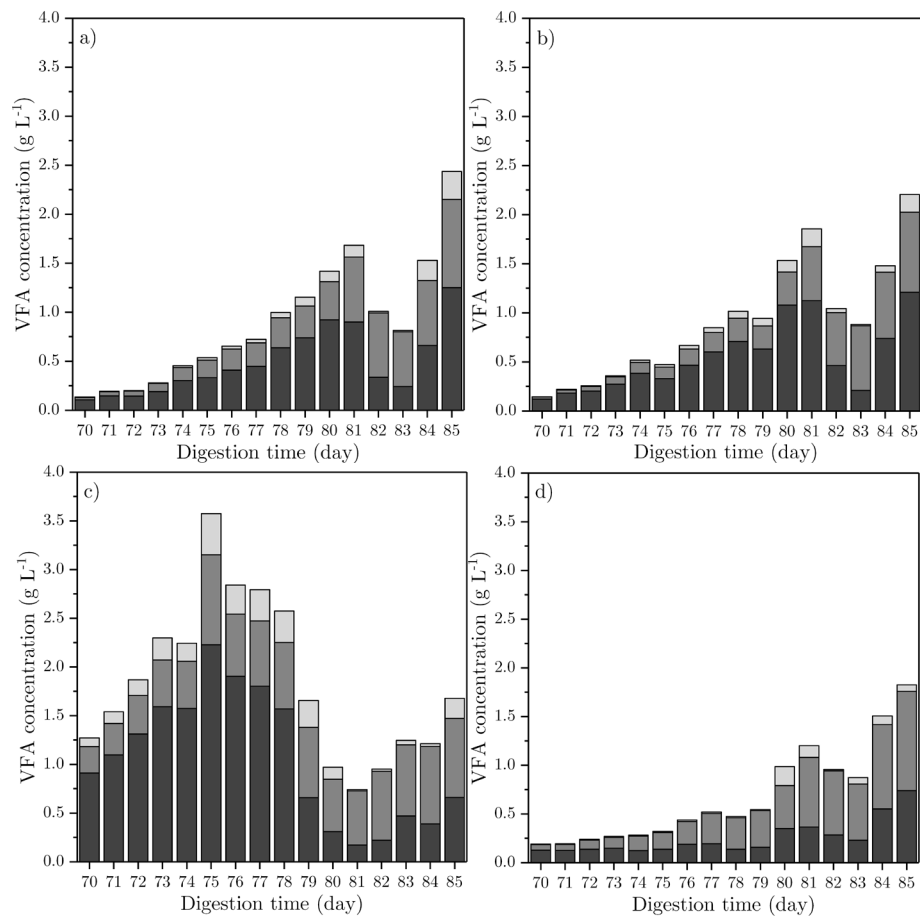


Fig. 4.24 VFA concentration profiles obtained from four semi-continuous digesters, **a)** R1 **b)** R2 **c)** R3 and **d)** R4 during the unstable period between day 70 and day 85. (■), (▒), and (□) denote acetic acid, propionic acid, and butyric acid, respectively.

4.3.4.4.6 Specific methane yield (SMY) and solid parameters

The specific methane yield (SMY) is an indicator of digester stability. As well as the specific biogas yield, the value of specific methane yield should not be affected by an increase in organic loading rate (OLR) unless the high throughput leads to inhibition from acid accumulation which causes the system to fail. Therefore, SMY was used in this study to determine the highest possible OLR that the system was able to function without a significant drop in the methane yield.

Fig. 4.25a shows the SMY values of all digesters at three different phases and the horizontal dotted line represents the ultimate methane yield of *O. ficus-indica* (339 L kgVS⁻¹). The SMY values were relatively stable across the three phases. The results show that during the most favourable condition (Phase I) where the lowest OLR was applied (1.38 gVS L_R⁻¹ day⁻¹), the SMYs were above 80% of the ultimate methane yield in all the digesters. The highest SMY was 330 L kgVS⁻¹ day⁻¹ obtained from digester R1 on day 15. In Phase II, the total solid content was increased from 5% to 7% in digesters R1 and R2, and from 5% to 10% in digesters R3 and R4. The yields fluctuated over time but overall were quite similar to those observed in Phase I for all digesters. After the two experimental accidents occurred, the digester R3 suffered from the shock as the SMY substantially decreased from 312 L kgVS⁻¹ day⁻¹ to 125 and 107 L kgVS⁻¹ day⁻¹ by day 70 and 75, respectively. The final methane yields on day 75 for digesters R1, R2 and R4 before the system failure were 271, 251 and 284 L kgVS⁻¹ day⁻¹, respectively.

As seen in **Fig. 4.25b and c**, the continuous drop of TS and VS contents indicated that the system had not achieved steady state yet. The TS contents of the digestate in all the digesters decreased constantly over the three phases of the digestion period. The TS values of the digestate were initially between 5.8% and 6.7% and

then continued to decrease until about 2.4%–3.0% after 75 days of operation. The high values of %TS at the beginning was because these digesters were previously used for the batch experiment studying the effect of inoculum to substrate ratio on the BMP, as described in **Section 4.3.4.4.2**. The decrease in %TS also corresponded with the appearance of the digestate, which became watery but still slimy towards the end of the operation. The decrease in solids may have implied a better rate of substrate degradability, however, this was not translated into an increase in SMYs. Hydrolysis was likely occurring at a high rate, leading to a greater rate of VFA accumulation. Since the conversion rate of VFA to methane may not be as rapidly as the rate of VFA production, this resulted in acid buildup occurred as the organic loading rate increased in Phase III as shown in **Fig. 4.23**.

The VS contents also decreased from about 66% to 55% between day 0 and day 60 for all digesters. This suggested that the substrates may have become more degradable over time. This could have been due to the fact that the ground *Opuntia* used as substrate was frozen at -20°C. As described in the literature chapter (**Section 2.2.3.1.1**), freezing plant materials would make the materials more digestible by the microorganisms because the ice crystals can destruct the plant's cell walls. Also, some *Opuntia* samples were taken out of the freezer multiple times as the remaining sample was put back in the freezer. Thawing and freezing the sample repeatedly may also amplify the cell wall destruction effect. After day 70 when all digesters experienced some stress, the digester performance dropped, which led to a rise in %VS.

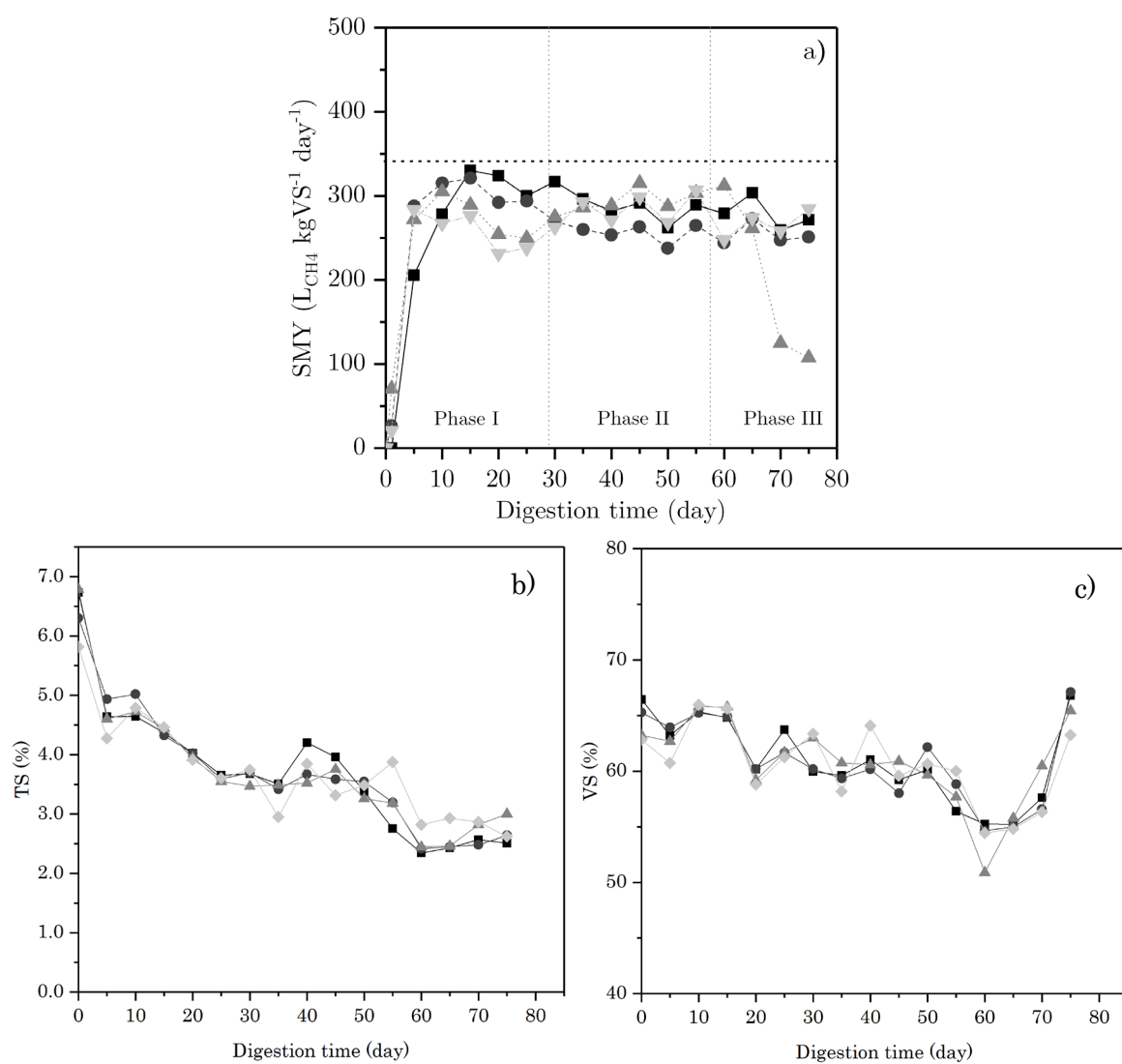


Fig. 4.25 a) Specific methane yield, SMY and b and c) total solid (TS) and volatile solid (VS) contents over the semi-continuous digestion period of 75 days. (■), (●), (▲) and (▼) denote digesters R1, R2, R3, and R4, respectively. The horizontal dotted line indicates the BMP of *Opuntia ficus-indica* ($339 \pm 22 \text{ L kgVS}^{-1}$).

Table 4.16 Performance indicators of semi-continuous digestion of *O. ficus-indica* at different operating conditions for duplicate digesters.

Digester	Phase	Duration (days)*	%TS	HRT (days)	OLR (gVS L _R ⁻¹ day ⁻¹)	VBP (L L _R ⁻¹ d ⁻¹)	SBY (L kgVS ⁻¹)	%CH ₄	%TS of digestate	%VS of digestate
R1	I	28	5	30	1.38	0.71–0.86	512–621	40.9–57.1	3.7–6.7	60.2–66.5
	II	28	7	30	1.93	0.90–1.18	459–600	50.1–54.7	2.8–3.7	56.4–61.0
	III	28	10	20	4.14	1.27–1.57	477–589	50.5–53.0	2.3–3.1	55.2–66.8
R2	I	28	5	30	1.38	0.72–0.85	522–617	46.6–56.8	3.6–6.3	60.2–65.3
	II	28	7	30	1.93	0.87–1.08	445–552	50.5–55.5	3.2–3.7	58.0–62.2
	III	28	10	20	4.14	1.17–1.38	438–520	48.1–53.2	2.4–2.6	54.6–67.1
R3	I	28	5	30	1.38	0.57–0.79	416–573	52.3–57.8	3.5–6.8	59.2–65.8
	II	28	10	30	2.76	1.30–1.74	496–662	51.9–56.0	3.2–3.8	57.7–63.0
	III*	28	10	15	5.52	-	-	-	2.4–2.8	50.9–65.4
R4	I	28	5	30	1.38	0.60–0.87	437–628	46.5–54.7	3.6–5.8	60.7–66.0
	II	28	10	30	2.76	1.24–1.57	473–601	45.8–56.8	3.0–3.9	58.2–64.1
	III**	28	10	15	5.52	1.63–2.03	458–571	49.4–55.0	2.6–2.9	54.4–63.3

*Gas data were not reported for R3 during Phase III due to the failure of the digesters. **The results reported for R4 in Phase III were collected over 15 days before the digester experienced stress instead of 28 days in the original plan. VBP – volumetric biogas production; SBY – specific methane yield

4.3.5 General discussion

4.3.5.1 Effect of I/S ratio on the BMP results at a larger scale

In this study, four digesters were divided into two sets where the first two digesters R1 and R2 were started at the I/S ratio of 2.0 whereas the other two digesters R3 and R4 were at 1.0. It was difficult to assess the effect of the I/S ratio on methane production as the gas data were missing for the first few days of the batch digestion due to leakage issue. The digesters R1, R2, R3, and R4 produced 296, 302, 312, 276 L kgVS⁻¹ of methane, respectively. Although these methane yields seemed to imply that the two I/S ratios resulted in comparable methane production, it could not be accurately compared to the findings found in Experiment I that the I/S ratios of 1.04 and 2.08 resulted in similar BMPs. In **Fig. 4.20**, the BMP curves started levelling off, but the experiment may have been halted prematurely to perform a semi-continuous study. This, including the leakage issue that occurred initially, resulted in lower methane yields than the actual values for all digesters. This means that the results from the current study did not provide concrete evidence to support the earlier claim that there was no significant difference between the methane yields obtained from the digesters of I/S ratios 1.04 and 2.08.

As previously mentioned, the semi-continuous experiment was performed immediately after the batch experiment was terminated. This may not have been an appropriate way to start the study of semi-continuous digestion. During the start-up period, it was recommended to gradually feed the substrate/ increase the OLR until it met the target value (Schnürer et al., 2016; Usack et al., 2012). This was to allow acclimatisation to the new feedstock and prevent shock loading. Another start-up strategy could have been to feed smaller proportions of *Opuntia* with other typical and easily more biodegradable substrates. This is to allow the microorganisms to augment very quickly at the beginning while adapting to the

new substrate like *Opuntia*. Then the proportion of *Opuntia* is gradually increased until the digester can be fed by using *Opuntia* as a sole substrate. This strategy could also be adapted in the case of inoculum limitation. In certain parts of the world where AD technology is still not well established, a lack of inoculum could be an issue. The digesters with a low inoculum proportion at the beginning may be fed with known, easily digestible substrates to augment and enrich the versatile microbial consortia until they are ready to use. Then, the digesters are fed with CAM feedstocks instead.

4.3.5.2 Effect of total solid content (TS) on digester performance

Increasing the total solid content between Phase I and Phase II, from 5% to 7% and 10% for digesters R1 R2 and R3 R4, respectively, resulted in higher biogas production rates with some slight fluctuations (**Table 4.16**). Biogas and methane yields slightly decreased but still stayed comparable to previous levels at a solid content of 5%TS. However, an increasing %TS did not cause acid accumulation in any digesters as the level of TVFAs was below 0.5 g L⁻¹ throughout 28 days of Phase II. The TS and VS contents of the digestate collected from all digesters constantly decreased throughout Phase I and II. The continuous drop of TS and VS then indicated that after two phases of approximately 60-day operation, all the digesters have still not reached a steady state. By the end of Phase II, approximately 73% of the original content in the digesters had been replaced, as shown in **Table 4.17**.

Table 4.17 The contents of the digesters at different operational phases

Digesters	Phase	Duration (days)	TS (%)	HRT (days)	OLR (gVS L ⁻¹ day ⁻¹)	Digestate removed (L)	Remaining content* (L)	%
R1 & R2	I	28	5	30	1.38	0.167	1.93	38.7
	II	28	7	30	1.93	0.167	1.38	27.6
	III	15	10	20	4.14	0.250	1.24	24.7
R3 & R4	I	28	5	30	1.38	0.167	1.93	38.7
	II	28	10	30	2.76	0.167	1.38	27.6
	III	15	10	15	5.52	0.333	1.19	23.8

*Remaining original digester content at the end of the operational phase

4.3.5.3 Effect of hydraulic retention time (HRT) on digester performance

From Phase II to Phase III, the HRTs of digesters R1 and R2 were reduced from 30 days to 20 days, while, for digesters R3 and R4, the HRTs were reduced from 30 days to 15 days. All digesters were operated at a solid content of 10%. However, for digesters R1 and R2, this TS was increased from 7% in Phase II, so the effect observed during this period may have been contributed by both changes.

Overall, the decrease of HRT improved the biogas production rates but not the specific biogas/ methane yields. The TS and VS contents of the digestate continued to decrease in Phase III until the point that the digesters experienced stress where the %TS and %VS started to increase. A decrease in HRT may have contributed to the shock-loading condition, leading to a sharp decrease in pH and VFA concentrations. At the lowest HRT of 15 days (OLR = 5.52 gVS L_R⁻¹ day⁻¹), the SMY of 269 L kgVS⁻¹ was obtained from the digester R4 before the digester fails.

However, apart from TVFA concentration and pH, digester alkalinity should have also been tested in order to assess the digester performance. VFA accumulation did not necessarily cause a decrease in pH (Weiland, 2010). There were two periods where transient peaks of TVFA were observed: at the beginning of the semi-continuous digestion study (day 1) and during Phase III (day 70 onwards). The

TVFA concentrations in all digesters on day 1 were above 3.0 g L^{-1} but the pH values were in a slightly alkaline range (7.58–7.65). This level of TVFA however did not inhibit the digestion system. In contrast, the pH values in all digesters were the lowest between day 75 and day 79, ranging from 6.69–7.02 at the TVFA concentrations under 2.0 g L^{-1} . Based on these results, despite high TVFA concentrations during the first period, the system might have had high concentration of ammonium ions, NH_4^+ , and bicarbonate ions, HCO_3^- , which are known to be a major source of a good buffering capacity (Parkin and Owen, 1986). As the digesters had been fed with *Opuntia ficus-indica* for a long period, the system may have been low in ammonium ions, which are the product of the protein degradation. *Opuntia* generally has low nitrogen contents due to their succulent growth form, and thus, there may not be sufficient buffer to neutralise the acids during the second period of VFA accumulation.

The suspension of feed was introduced in this experiment to ease the inhibition of VFA accumulation. During the non-feeding period, the concentrations of acetate and butyrate decreased whereas the propionate level was almost constant. The degradation of propionic acid may have been inhibited when the concentration of acetic acid was above 0.5 g L^{-1} , as were reported in other studies (Gorris et al., 1989; Mawson et al., 1991). Toward the end of the feed suspension period, the concentrations of acetate were between 0.17 and 0.24 g L^{-1} , which became a suitable condition for propionic acid degradation. However, in this study, the feeding was re-introduced too early when a decrease in propionic acid concentration was not yet observed and the acetic acid concentration began to rise. It has been reported that when the accumulation of longer chain VFAs such as propionate and butyrate occurred, an increase in acetic acid would continue for a longer period (Raposo et

al., 2006). If the experiments were to continue for an extended period, increasing VFAs would soon become inhibitory to methane production.

Accumulation of propionic acid in this study may have stemmed from the deficiency of trace elements in the digesters. It was reported that insufficient trace elements could lead to VFA build-up and specific micronutrients such as Se and Mo are related to propionate accumulation (Banks et al., 2012). Washout of critical trace elements may have occurred in this experiment. As shown in **Table 4.17**, over 75% of the original content of all the digesters would have been replaced by the end of Phase III before the digesters failed. The digesters were fed only ground *Opuntia* without supplementation of inoculum or nutrients. Washout of critical trace elements including Se and Mo may have occurred, which led to propionate and acetate accumulation, resulting in digester instability. In addition, the digesters were run for a batch study for 18 days before the semi-continuous experiment commenced using the same digesters. A lot of nutrients may have also been used during the batch operation, which supposedly contributed to the depletion of essential nutrients during Phase III of the semi-continuous operation.

The other possible cause of instability of the digesters was the accumulation of possible unknown toxic compounds. The digesters had been fed a single substrate for an extended period. An unknown compound in *O. ficus-indica* may have gradually accumulated to a certain point that it became toxic to the bacteria community in the digester. However, this explanation seemed less likely compared to the depletion of trace elements.

Another observation from the current study data was that the pH dropped below 7.0 after the TVFA concentration had increased above 1 g L⁻¹. There seemed to be a delay of pH response to the change in acid level, which was also reported in the

literature. A decrease in pH occurred after a well-established imbalance of the carbonate ions, bicarbonate ions, ammonium ions and volatile fatty acid level and as a result, pH should not be used as a warning indicator of acid accumulation (Gerardi, 2003; Switzenbaum et al., 1990).

4.3.6 Conclusion

The SMY results obtained from the study of the effect of I/S ratio and substrate processing technique revealed that the ground cladodes, liquid extract, and solid peel produced the highest methane yields of 241, 271, and 222 L kgVS⁻¹, respectively. Despite being processed differently, the three substrates still had similar organic dry matter contents (VS) of 87.9–88.6%. The liquid extract was readily biodegradable and less recalcitrant compared to the other two substrates and hence produced the highest methane yield. However, a longer period of VFA accumulation was observed with liquid extract as the substrate, especially at low inoculum to substrate (I/S) ratios. The lowest methane yields were obtained from the solid peel irrespective of the I/S ratio used in the experiment. The range of the optimum I/S ratios for all substrate types was between 0.71 and 2.08 and the lowest I/S ratio of 0.35 was found to significantly reduce methane productivity and therefore unsuitable for the BMP study. Another factor affecting methane production from *O. ficus-indica* was the age of cladode. It was found that methane yields decreased significantly for the cladodes with an age > 3 years.

The results from the semi-continuous trial indicated that *O. ficus-indica* was a promising AD substrate. The SMY of *O. ficus-indica* at the highest OLR of 5.52 gVS L_R⁻¹ day⁻¹ was 269 L kgVS⁻¹, which accounted for approximately 80% of the BMP. However, at this loading rate, the digesters began to experience some stress. Therefore, the highest loading rate recommended for semi-continuous digestion of *O. ficus-indica* was 2.76 gVS L_R⁻¹ day⁻¹ because, at this OLR, there was no sign of acid accumulation nor a significant drop in methane production.

Chapter 5

Results and Discussion: CAM Plants in Biorefinery

In the past decade, the concept of ‘cascading use of biomass’ has been proposed, where the approach aimed to maximise the efficiency of biomass use in order to minimise the greenhouse gas emission (Keegan et al., 2013). The cascading use of biomass is aimed at creating a system in which a feedstock could be allocated to a higher-value use as such occurs in biorefinery approaches, before being used for energy extraction. This alternative concept can also be applied to *Opuntia ficus-indica*, which has been demonstrated in this study as a promising feedstock for bioconversion processes. Therefore, an alternative route, which *O. ficus-indica* could be exploited before being used as an AD substrate, is proposed in this chapter. Chapter 5 thus reports on the results from the study of a CAM plant as a potential feedstock for the biorefinery industry specifically on the use of *O. ficus-indica* as a raw material for bioplastic production.

5.1 PHB production from the *Opuntia* liquid extract (OLE)

5.1.1 Aim of the study

The focus of this work was to demonstrate for the first time that *Opuntia* liquid extract or OLE, which has never been reported to be used as a substrate or a carbon source for any fermentation processes, could be exploited as a sole substrate for the production of PHB by the bacterial strain *C. necator* H16.

5.1.2 Characterisation of *Opuntia* liquid extract (OLE)

Opuntia liquid extract (OLE) used in this study was obtained from the ground material of young cladodes aged approximately 0.5-year-old. The preparation

process of OLE is described in **Section 3.2.1.1**. The extraction efficiency was determined to be about 67% where the remaining one-third of the ground material was the solid pulp, which was oven-dried and stored for further use as an AD feedstock. The liquid obtained from younger cladodes was preferred as they tended to have a lower content of mucilage, which exhibited gel-like properties. The OLE obtained from cladodes older than 1-year old with higher mucilage content (**Fig. 5.1b**) was less transparent and more viscous compared to the liquid obtained from the 6-month old cladodes (**Fig. 5.1a**). The viscous OLE was found to be immiscible with the culture medium and therefore was not recommended as a substrate for PHA fermentation process.



Fig. 5.1 *Opuntia* liquid extract (OLE) after centrifugation obtained from **a)** 6-month-old cladodes and **b)** 2-year-old cladodes

The measurements of TS, VS, pH, chemical oxygen demand (COD) of OLE were performed and the results are presented in **Table 5.1**. *Opuntia* liquid extract (OLE) was high in organic content, which was reflected by the values of COD and VS(%TS). COD is the measurement of the mass of oxygen consumed per volume of the solutions and this test could be indirectly used to refer to the concentration of organic compounds in the liquid, while VS(TS) is the ratio of organic content to a dry weight of the material. The pH and VS(TS) of OLE were 4.53 ± 0.02 and 88.63

$\pm 0.98\%$, respectively, which are similar to literature values of 4.66 ± 0.25 and $92.7 \pm 2.1\%$, respectively (Santos et al., 2016). However, the crude protein of OLE, 0.4% (w/w), was relatively low compared to a range of $7.38\text{--}7.52\%$ (w/w) for *Opuntia* cladodes previously reported (Rodrigues et al., 2016). It should be noted that the lower value of crude protein of OLE only represented the composition of the liquid extract and not the entire cladode as reported in the literature. The amount of crude protein per 100 grams of the dry mass of OLE was 0.4 g , which was equivalent to the nitrogen concentration of $0.00064\text{ g g}_{\text{substrate}}^{-1}$, according to the standard method which describes the calculation of crude protein by multiplying the Kjeldahl nitrogen by 6.25 (APHA, 1998). Since the dry content of OLE per fresh weight, TS(%FM), was 9.94% , assuming the specific gravity of OLE is equivalent to 1.0, the nitrogen concentration in fresh liquid of *Opuntia* extract was estimated to be 0.0636 g L^{-1} . Generally, the medium solution containing NH_4Cl was added to the culture to ensure sufficient growth of *C. necator* H16. The standard concentration of nitrogen used was 1 g L^{-1} . Further study is necessary to evaluate whether the concentration of nitrogen of OLE (0.06 g L^{-1}) is sufficient to support microbial growth.

Table 5.1 Characterisation of *Opuntia* liquid extract (OLE). Values are means of three samples $\pm$ standard deviations

Parameters	<i>Opuntia</i> liquid extract (OLE)
TS (%FM)	9.94 ± 0.08
VS (%TS)	88.63 ± 0.98
VS (%FM)	8.81 ± 0.10
pH	4.53 ± 0.02
COD (g L^{-1})	44.93 ± 0.37
Crude protein (%w/w)*	0.4

%TS (FM) – the percentage of dry biomass per fresh mass of biomass; %VS (TS) – the percentage of organic content mass per dry biomass; %VS (FM) – the percentage of organic content mass per fresh mass of biomass. * The analysis of crude protein was performed by an external laboratory (see **Appendix A**)

GC-MS was employed to qualitatively identify organic compounds in OLE that could potentially be utilised by *C. necator*. **Table 5.2** presents a list of organic compounds detected in OLE using GC-MS analysis. The GC-MS chromatogram indicating peaks corresponding to each compound at different retention times is shown in **Fig. 5.2**. There are several peaks, for example, at the retention times of 11.2, 14.2, 14.9 minutes that could not be identified by the available in-house database and therefore they are not shown in **Table 5.2**.

All organic compounds detected were separated into three different groups: soluble sugars, organic acids, and amino acids. There were 11 soluble sugars detected, most of which were pentose and hexose sugars. Some disaccharides such as maltose and trehalose were also detected. Of all the sugars present, d-glucose and d-fructose were estimated to be most abundant as is shown in **Fig. 5.2** where the peaks corresponding to glucose and fructose at retention times between 24 and 25 minutes were the most prominent. It was assumed that the sum of these two sugars accounted for the majority of soluble sugars detected in OLE. This is congruent with a previous study, which reported on the potential of hydrolysed sugars from *Opuntia ficus-indica* cladodes for bioethanol production (Santos et al., 2016). In their study, glucose was the most abundant sugar ($25.4 \pm 1.3\%$) obtained from the dry prickly pear cladodes. In another study, it was reported that glucose and fructose were the major available sugars, and fructose accounted for 90.33% of total soluble sugar in the spineless variety of *O. ficus-indica* cladodes (Ayadi et al., 2009).

The main organic acids detected by GC-MS analysis were lactate, pyruvate, succinate, fumarate, GABA, malate and citrate. All other acids except malate and citrate were present in relatively low concentrations in OLE as the peaks for these acids in **Fig. 5.2** were small or not shown. There were two prominent peaks at

retention times of 18.228 and 23.407 minutes, which corresponded to malic acid and citric acid, respectively. These strong responses were assumed to be proportional to high acid concentrations accumulated by *O. ficus-indica*. The current finding was in agreement with data on *O. ficus-indica* previously published (Yang et al., 2015). They reported the presence of malic and citric acids at 10.9 and 3.9 g L⁻¹, respectively as major organic acids found in the cladode juice along with other sugars such as fructose, glucose, and galactose. Traces of amino acids were also detected, but their signal intensity was not as strong as other organic compounds, and hence are not present on the chromatogram (Fig. 5.2).

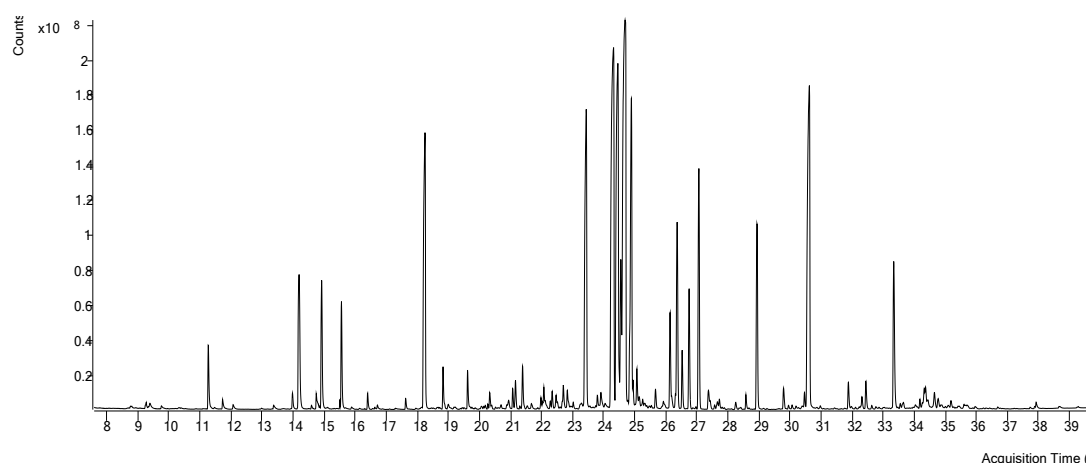


Fig. 5.2 GC-MS spectrum of liquid extract from *Opuntia ficus-indica*

Table 5.2 Metabolites detected in the *Opuntia* liquid extract (OLE) employing GC-MS analysis

Type of Compounds	Acquisition time (min)	Compound name
Soluble sugars	21.068, 21.155	D-Xylose
	21.387	D-Ribose
	22.106	D-Rhamnose
	23.426	2-Deoxyribose
	24.264, 24.409	D-Fructose
	24.558	D-Galactose
	24.677, 24.859	D-Glucose
	27.054	Myo-Inositol
	29.800	D-Mannose
	34.373	Maltose
	34.442	Trehalose
Organic acids	9.358	Lactic acid
	10.352	Pyruvic acid
	15.500	Succinic acid
	15.557	Fumaric acid
	18.228	Malic acid
	18.822	GABA
	23.407	Citric/isocitric acid
Amino acids	10.383	L-Alanine
	11.253, 14.925	Glycine
	14.155, 14.931, 15.888	L-Serine
	14.787, 16.389	L-Threonine
	17.327, 18.728	L-Aspartic acid
	18.700	L-Proline
	19.491	Cysteine
	20.692	L-Phenylalanine

The characterisation of OLE confirms its potential as a carbon source for PHA production. It was rich in sugars, fructose in particular the could be used as a culture liquid for *C. necator* growth and PHB production. However, OLE was different from hydrolysates previously studied in terms of composition, which were mainly composed of sugars. OLE was a complex substrate that comprised sugars, organic acids, proteins, lipids, and other unidentified compounds. Despite the presence of fructose, which, as confirmed by literature, can be utilised by *C. necator* H16, other components in the OLE may affect the growth of microbes and the accumulation of PHB.

The crude protein of OLE was 0.4%, which is relatively lower compared to those of date seed extract (4.8%) (Yousuf and Winterburn, 2016) and orange juice waste (7.2%) (Guzman Lagunes and Winterburn, 2016) investigated in previous studies. In their studies, additional nitrogen was not used in the medium. Since OLE had a considerably lower protein content, it should be tested whether it could promote the microbial growth and activity or nitrogen supplementation was required.

The culture of PHB usually requires mineral and vitamin medium, which is necessary for the growth of microorganisms, particularly at the initial stage. It has been reported that *Opuntia* spp. cladodes contained various minerals and vitamins, including some phosphate and carbonate ions, which act as buffering agents, in the plant tissue (Corrales-García et al., 2004; El-Mostafa et al., 2014). Therefore, it could be assumed that *Opuntia* liquid extract (OLE) used in this study may also contain some minerals and vitamins essential for bacterial growth. However, these compounds were not characterised nor identified, OLE needed to be tested whether it could be used solely as a liquid culture or required addition of medium solution.

5.1.3 Experiment I – Suitability of *Opuntia* liquid extract (OLE) for *C. necator* H16 growth

5.1.3.1 Objectives

To study the feasibility of using OLE as a carbon source for the growth of *C. necator* H16 in three media of differing nitrogen concentrations.

5.1.3.2 Methodology

The liquid culture was prepared by mixing 10 ml of the medium and 10 ml of the substrate (OLE). Fructose was also used as a control. Three media of differing nitrogen concentrations (0.25, 0.50, and 1.00 g L⁻¹) were tested in this study. Samples were taken at times for measurement of OD and pH. All experiments were performed in triplicate. The detailed description of the initial experimental procedure is described in **Chapter 3, Section 3.2.2**.

5.1.3.3 Results

C. necator H16 was able to grow and utilise OLE, as the sole substrate, as well as fructose (5 g L⁻¹), with the maximum growth (OD₆₀₀) observed for fructose of 9.56 ± 0.36 (**Fig. 5.3a**). No typical S-curve pattern was obtained as growth did not reach a stationary phase after 6 days, so the experiment was terminated. Overall, similar final cell growth (OD₆₀₀) of 8.67, 10.19 and 10.74 were obtained from the cultures of OLE at nitrogen concentrations of 0.25, 0.50 and 1.00 g L⁻¹, respectively. ANOVA test on these results gave a borderline *p*-value ($p = 0.0552$), which indicated that the difference in cell growth among cultures tested at different nitrogen concentrations were not statistically significant ($p > 0.05$). Therefore, this suggests that OLE was suitable to support growth as well as fructose despite being a complex substrate with several unknown compounds. In addition, different nitrogen concentrations did not impact bacterial growth in this study. This implies that

although the crude protein of OLE was much lower compared to other substrates previously studied as mentioned in **Section 5.1.2.** the amount of nitrogen in OLE was sufficient to support microbial growth.

During fermentation, pH was not regulated but was monitored. Two different pH profiles were observed from the cultures growing in OLE and fructose (**Fig. 5.3b**). Starting at 6.80, the pH values of OLE cultures rose to between 6.99 and 7.00 after 24 hours and steadily increased over the incubation period. The final pH values recorded for the OLE cultures with nitrogen concentrations at 0.25, 0.50 and 1.0 g L⁻¹ were 7.55, 7.59 and 7.48, respectively. In contrast, the pH of the fructose culture dropped from 6.80 to 6.58 overnight but subsequently remained constant throughout the fermentation.

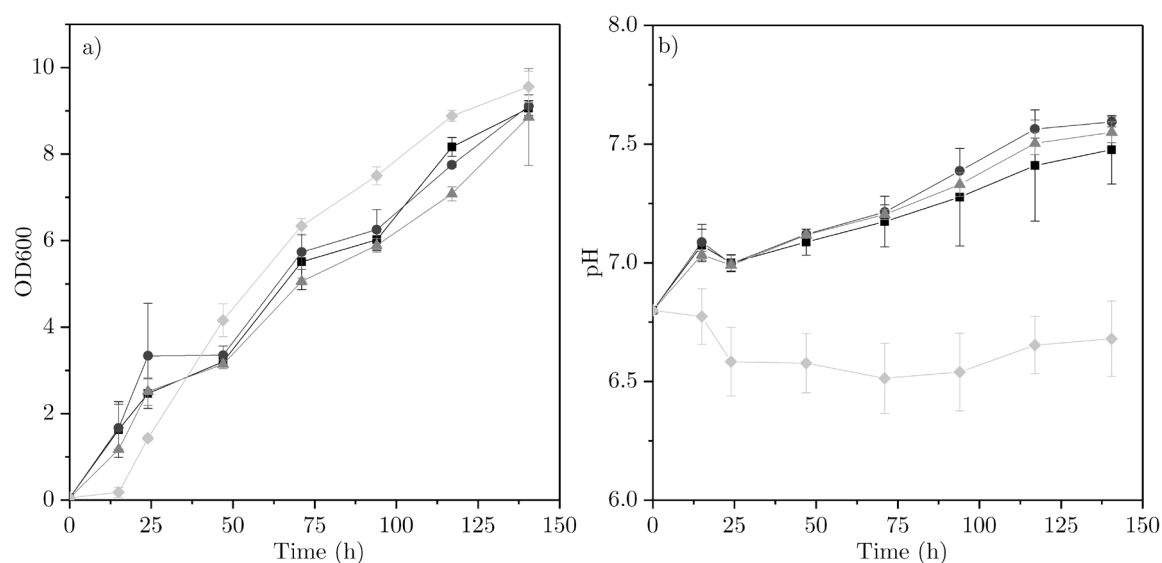


Fig. 5.3 a) Growth curve of *C. necator* H16 over 148 hours in *Opuntia* liquid extract (OLE) supplemented with medium solutions composed of different nitrogen concentrations. **b)** pH evolution of *C. necator* cultures with varying concentrations of nitrogen in the medium solution. Fructose was used as the reference carbon source for comparison. (—■—), (—●—), and (—▲—) denote OLE mixed with a medium solution with nitrogen concentrations of 0.25, 0.50 and 1.00 g L⁻¹, respectively. (—◆—) refers to fructose mixed with a medium solution with a nitrogen concentration of 1.00 g L⁻¹. Error bars denote $\pm$ standard deviations ($n = 3$).

5.1.4 Experiment II – Effect of nutrient supplementation on *C. necator* H16 growth

5.1.4.1 Objective

To examine the effect of nutrients (nitrogen, vitamin and mineral, and phosphate and carbonate) in the medium on the growth of *C. necator* H16.

5.1.4.2 Methodology

Five different liquid cultures were prepared by mixing media and substrate (OLE) as described in **Table 5.3**. Samples were taken at times to determine OD and pH. All experiments were performed in triplicate. The detailed description of the preliminary experimental procedure is described in **Chapter 3, Section 3.2.2**.

Table 5.3 Experimental design for studying the influence of medium composition on *C. necator* growth

Condition	Name	Medium ¹	Medium volume (ml)	Substrate (OLE) volume (ml)	Total liquid culture volume (ml)
0	O	-	-	20	20
1	O+M1	Water only	10	10	20
2	O+M2	Water and buffer solution (KH ₂ PO ₄ , Na ₂ HPO ₄ and NaHCO ₃)	10	10	20
3	O+M3	Water and mineral and vitamin solutions	10	10	20
4	O+M4	Water, buffer solution	10	10	20

¹The composition of buffer, mineral (trace element) and vitamin solutions can be referred to **Table 3.3** in **Chapter 3**

5.1.4.3 Results

The growth curve of *C. necator* grown in OLE with different media supplements over 123 hours is shown in **Fig. 5.4a**. In general, *C. necator* were able to grow in all of the different media tested. The best performance was observed with the final OD₆₀₀ of 6.04 ± 0.08 for the culture with the medium solution containing vitamins,

minerals, and buffer solution (**M4**). The final OD₆₀₀ values at 123 hours for the cultures using media **M1** (water only), **M2** (water and buffer solution) and **M3** (water and vitamin solution) were 4.79, 4.79, and 4.43, respectively. However, one-way ANOVA analysis revealed that there was no significant difference ($p > 0.05$) in cell growth between the culture with the medium (**M4**) and those with other media, **M1**, **M2** and **M3** ($p = 0.21$, 0.21 , and 0.08 , respectively). This suggests that supplementing OLE with vitamins and minerals, or with buffer solutions, did not significantly promote cell growth.

However, using OLE solely as a substrate without dilution (**O**) negatively impacted the growth of *C. necator* ($p = 0.03$), and this was attributed due to its high concentrations of organic acids when compared to the culture with **M1** medium (water). A lag phase was then observed within the first 24 hours with this condition (**O**), which was thought to be the result of high acid concentration inhibition.

A gradual increase in pH in all cultures with OLE as the substrate were observed (**Fig. 5.4b**). However, the medium that lacked the buffer solution (**M1** and **M3**) showed a greater rise in pH after 24 hours, from 6.80 to 7.04 and 7.08, respectively. The pH of these two cultures remained higher than those with buffer solution in the media (**M2** and **M4**) throughout the experiment. Conversely, a transient decrease in pH from 6.80 to 6.66 was seen in the culture using OLE as a liquid substrate without a medium (**O**), but only over the first 24 hours.

The results from this experiment therefore suggested that OLE with **M1** medium (water) was sufficient to support growth. Supplementation of nutrients was not necessary to promote bacterial growth and activity. Using OLE solely without dilution however was not feasible in this study since the concentrations of some compounds might be too high, suppressing the growth of the microorganisms.

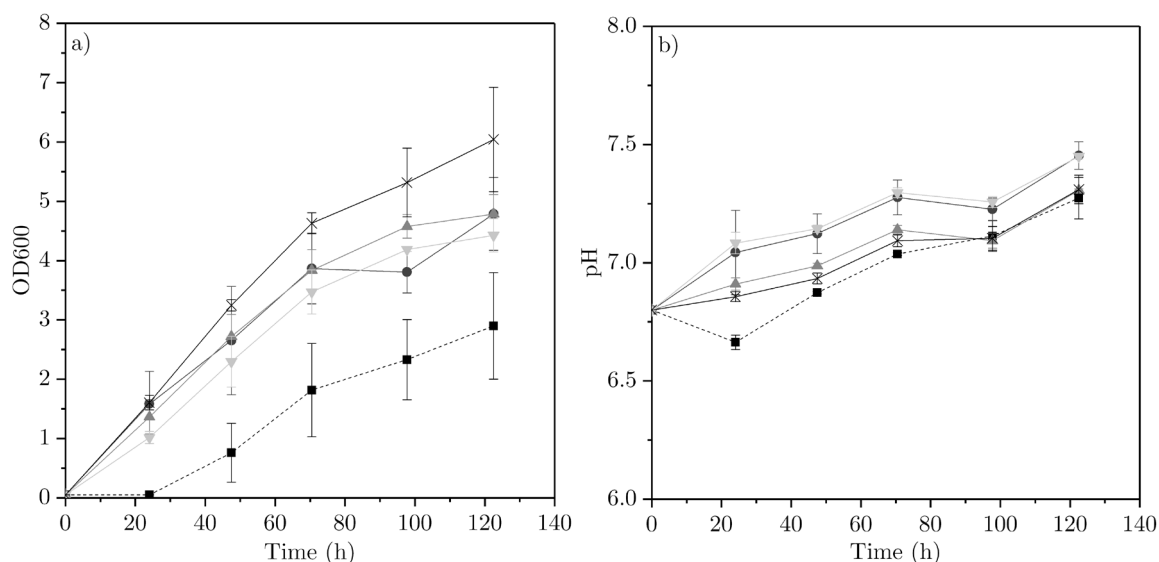


Fig. 5.4 (a) Growth curve of *C. necator* H16 using *Opuntia* liquid extract (OLE) as a carbon source in different medium compositions over 123 hours of cultivation period. **(b)** pH evolution of fermentation of *C. necator* H16 in different medium compositions **(b)**. (—■—) denotes OLE without additional medium solution or O. (—●—), (—▲—), (—◆—), and (—×—) refer to OLE mixed with the following components in a ratio of 1:1, **M1** water, **M2** water and buffer solution, **M3** water and mineral and vitamin solution, and **M4** the original medium recipe, respectively. Error bars indicate ± standard deviations ($n = 3$).

5.1.5 Experiment III - PHB production from *Opuntia* liquid extract

5.1.5.1 Objective

To evaluate the feasibility of batch fermentation of OLE as a sole substrate for PHB production by *C. necator* H16.

5.1.5.2 Methodology

The cultures of *C. necator* H16 using OLE as a sole substrate in 250 ml shake flasks were studied in triplicate, using the experimental procedures described in **Section 3.2.3**. Analytical methods for determining the essential parameters such as OD₆₀₀, pH, substrate concentration, biomass concentration, PHB content and PHB identification are described in **Section 3.2.4**.

5.1.5.3 Results

The suitability of using *Opuntia* liquid extract (OLE) as the substrate for *C. necator* H16 growth and PHB production was investigated. Based on the results of the

initial studies (**Experiments I and II**), the medium solution, as described in **Table 3.3, Section 3.2.1**, for *C. necator* culture was deemed to be dispensable and could be replaced with just water. Optical density (OD₆₀₀) of samples were taken at 2–3-hour intervals in order to determine cell concentration and the profile of these OD₆₀₀ values is presented in **Fig. 5.5a**. Sample pH was also recorded simultaneously. Typically, an S-curve pattern of cell growth was observed, starting with a lag phase, where there was little or no growth during the first 6 hours (OD₆₀₀ = 0.10). It was then followed by an exponential phase where cell concentration increased significantly; OD₆₀₀ increased to 13.67 ± 2.07 after 25 hours. Then, the culture entered a stationary phase where growth slowly occurred and the final OD₆₀₀ achieved at the end of fermentation (35 hours) was 12.79 ± 0.73 .

Correspondingly, the culture pH followed the shape of the growth curve but reached a plateau more swiftly (**Fig. 5.5a**). Initially, the pH of the culture was adjusted to 6.80 but it increased to 7.28 ± 0.05 after 6 hours of cultivation during the lag phase. The pH substantially increased to 8.34 ± 0.05 observed at 15 hours around the mid-exponential phase. Subsequently, a change in pH was more gradual, pH increased to 8.48 ± 0.06 at 20 hours and steadily rose to 8.75 ± 0.05 by the end of cultivation (35 hours).

Opuntia liquid extract (OLE) contained various organic compounds that could be utilised by *C. necator* H16 for cell growth and PHB accumulation. To understand the effect of individual compounds on biomass growth and PHB production, the liquid culture from each flask was sampled at regular intervals for metabolite analysis. Eight out of 14 sampling points on the growth curve shown in **Fig. 5.5a** were selected for the metabolite study, covering three phases of bacterial growth (lag, exponential and stationary).

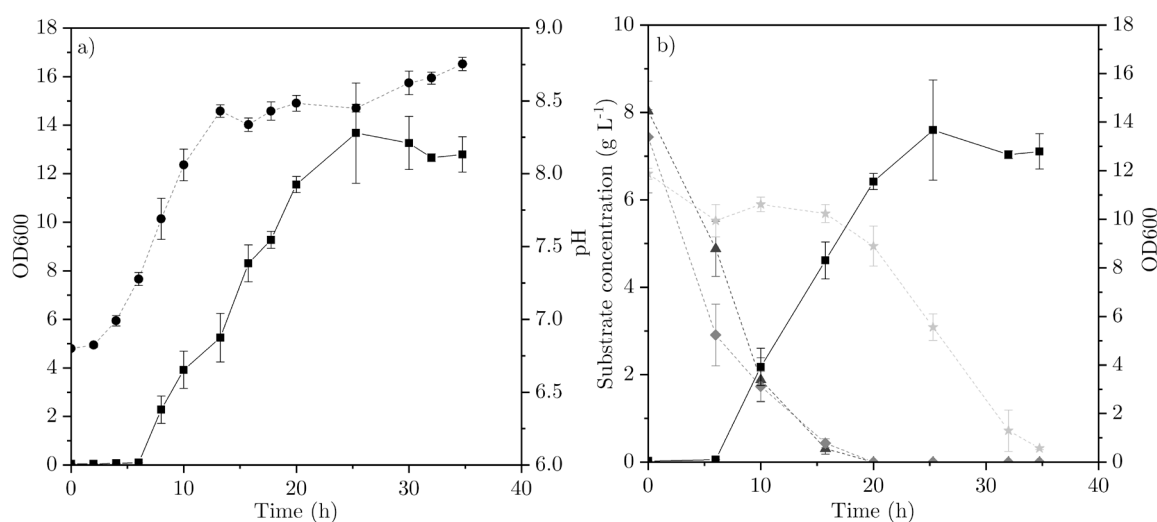


Fig. 5.5 (a) Growth curve (shown as OD) and pH evolution of *C. necator* H16 in *Opuntia* liquid extract (OLE) over 35 hours of cultivation. **(b)** Comparison of cell growth and key compound consumption by *C. necator* H16. (—■—), (—●—), (—▲—), (—◆—), and (—★—) denote OD600, pH, malic acid, citric acid, and fructose, respectively. Error bars represent $\pm$ standard deviations ($n = 3$).

Metabolite analysis by GC-MS revealed that there was a change in the quantity detected of at least three compounds, which was shown by the difference in peak area between the two samples (**Fig. 5.6**). These peaks corresponded to citric/isocitric acid (RT = 23.407 minutes), malic acid (RT = 18.228 minutes), and fructose (RTs = 24.264 and 24.409). As shown in **Fig. 5.6**, fructose, malic acid and citric acid had the largest change in responses between 6 and 32 hours compared to other compounds. There was no change in chromatogram peaks for ribose, galactose and glucose, as confirmed by literature that these sugar compounds, except fructose and N-acetylglucosamine, cannot be metabolised by *C. necator* H16 (Pohlmann et al., 2006). Other organic acids such as succinic and fumaric acids (RTs = 15.500 and 15.557 minutes, respectively) were likely to be consumed by *C. necator* H16, but their responses were not as strong as malic and citric acids. Therefore, they were not included in this quantification analysis. From henceforth, this study focused specifically on malic acid, citric acid and fructose and their effects on bacterial activity. Standard solutions of malic acid, citric acid and fructose were run along with the samples in order to quantify each compound. Detailed chromatograms

demonstrating a change in concentrations of malic acid, citric acid and fructose over 8 different incubation times are presented in **Fig. 5.7**.

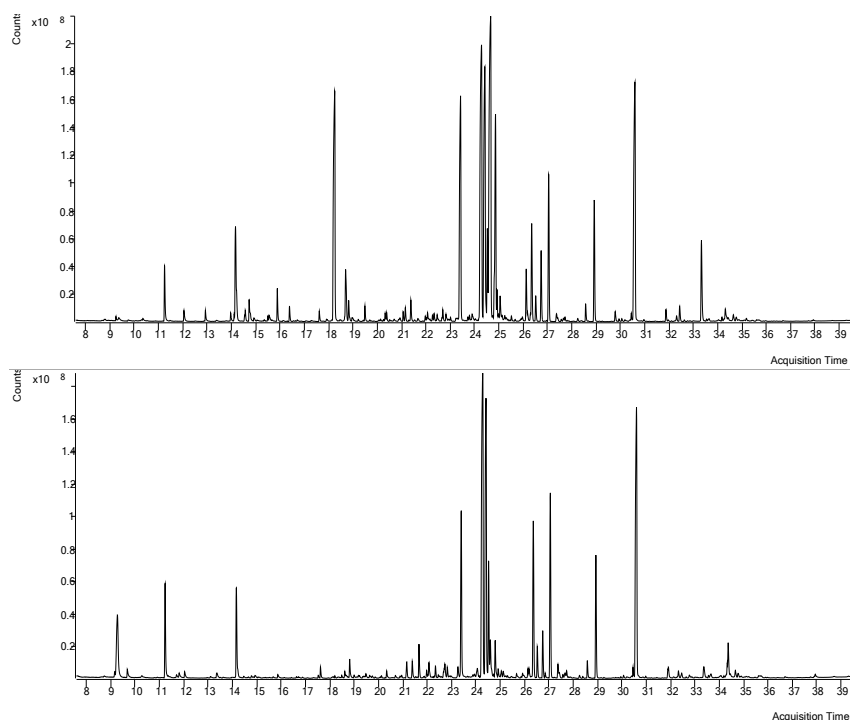


Fig. 5.6 GC-MS chromatogram of metabolites from liquid samples collected at 6 hours (**top**) and 32 hours (**bottom**)

The starting concentration of the total carbon source (OLE) used in this study, combining fructose (6.6 g L^{-1}), malic acid (8.0 g L^{-1}) and citric/isocitric acid (7.4 g L^{-1}) was assumed to be 22.0 g L^{-1} . Overall, the three compounds were completely utilised by *C. necator* within 35 hours, but the consumption rate of each compound was different at various times. The concentrations of the three compounds (malic acid, citric/isocitric acid, and fructose) plotted against the growth curve of *C. necator* H16 over 35 hours are presented in **Fig. 5.5b**. In the first 6 hours, fructose concentration decreased from $6.59 \pm 0.12 \text{ g L}^{-1}$ to $5.52 \pm 0.38 \text{ g L}^{-1}$. The change was relatively subtle compared to those of organic acids. The concentrations of malic acid and citric acid rapidly decreased from $8.03 \pm 0.04 \text{ g L}^{-1}$ to $4.88 \pm 0.63 \text{ g L}^{-1}$ and from $7.44 \pm 1.28 \text{ g L}^{-1}$ to $2.91 \pm 0.71 \text{ g L}^{-1}$, respectively.

The switch of lag to exponential phase occurred after 6 hours. A steady decline in concentrations of citric and malic acids was observed during this period and both acids were completely consumed within 20 hours. Conversely, fructose concentration slightly decreased from 5.52 ± 0.37 to 4.94 ± 0.46 g L⁻¹. After exhaustion of organic acids, the concentration of fructose swiftly decreased to 3.09 ± 0.30 g L⁻¹ at 25 hours and continued to decline at the same rate. The final concentration of fructose was 0.32 ± 0.03 g L⁻¹ at the end of fermentation.

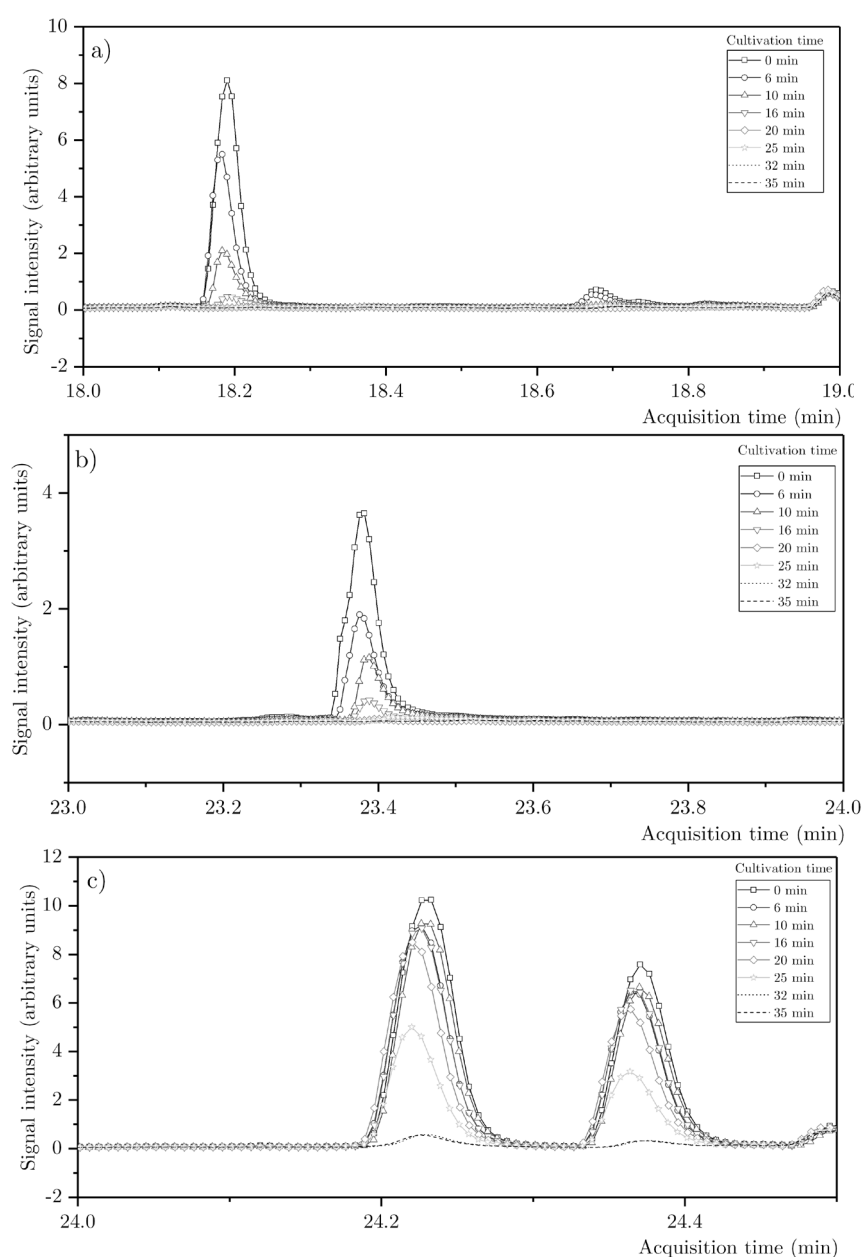


Fig. 5.7 GC-MS chromatograms demonstrating a change in the signal intensity of the main three compounds: **a)** malic acid, **b)** citric/isocitric acid, and **c)** fructose, from liquid samples collected at 8 different cultivation times over 35 hours

The change in concentrations of individual compounds, biomass and PHB over time are presented in **Fig. 5.8a**. Despite a rapid decrease of organic acids during the lag phase (6 hours), the concentration of biomass obtained was still low at 0.525 g L^{-1} . A substantial increase in biomass was observed at 20 hours when the cell concentration was $4.55 \pm 0.33 \text{ g L}^{-1}$ and all acids were completely depleted. The maximum concentration of biomass was achieved at 32 hours ($5.25 \pm 0.16 \text{ g L}^{-1}$) but later dropped to $5.00 \pm 0.07 \text{ g L}^{-1}$ by 35 hours. **Fig. 5.8b** presents a percentage change in PHB accumulation against concentration profiles of biomass and PHB over 8 different sampling points. No PHB production was detected in the first 6 hours during lag phase. PHB concentration of $0.26 \pm 0.04 \text{ g L}^{-1}$ was firstly detected at 10 hours when it accounted for 10.2% of the total biomass. Even though PHB production occurred soon after the lag phase, the majority of PHB accumulated at the later stages of the exponential phase. After 15 hours of fermentation, the concentration of PHB quadrupled to $1.09 \pm 0.04 \text{ g L}^{-1}$. The PHB concentration peaked at $2.12 \pm 0.21 \text{ g L}^{-1}$ at 25 hours when the biomass concentration was $4.65 \pm 0.21 \text{ g L}^{-1}$. At this point, the highest percentage of PHB accumulation was achieved at 45.6%. Afterwards, a decrease of PHB concentration was observed from $2.01 \pm 0.08 \text{ g L}^{-1}$ at 32 hours to $1.83 \pm 0.04 \text{ g L}^{-1}$ at 35 hours.

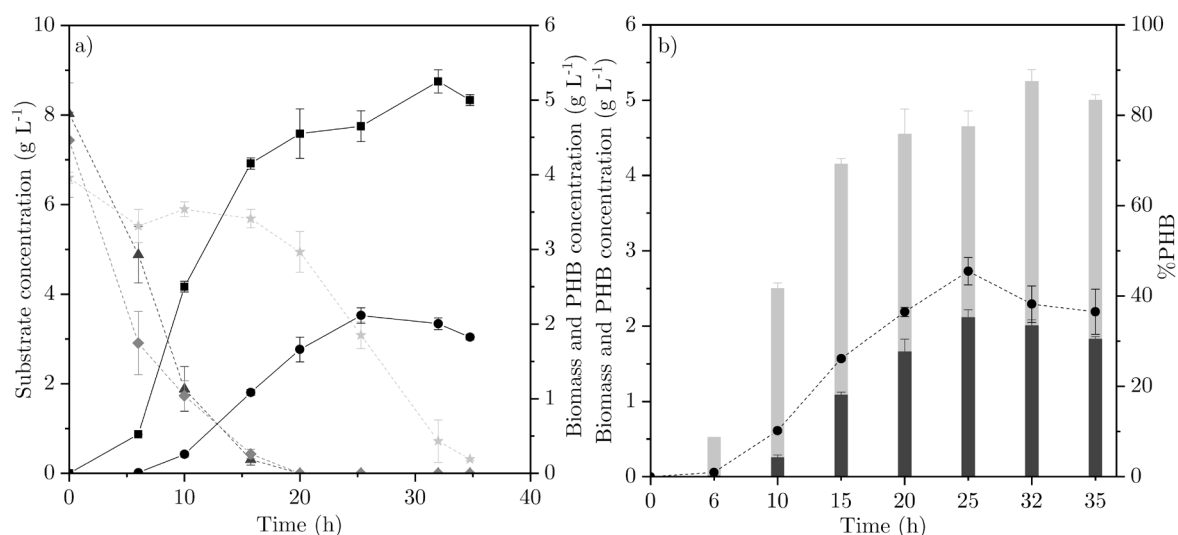


Fig. 5.8 (a) Comparison of biomass and PHB accumulation and consumption of key compounds for 35 hours. (—■—), (—●—), (—▲—), (—◆—), and (—★—) denote concentrations of biomass, PHB, malic acid, citric/isocitric acid, and fructose, respectively. **(b)** The percentage change of accumulated PHB content in the cells of *C. necator* H16 over time. (■), (●), (●) refer to biomass concentration, PHB concentration, and percentage of PHB per dry cell biomass, respectively. The error bars represent the standard deviation of triplicates.

In general, bacteria in a liquid culture containing mixed substrates can exhibit different patterns of growth behaviour, which is determined by the characteristics and concentration of the compounds present. The absence of a curve for diauxic growth (double growth of bacteria in the presence of two substrates) of *C. necator* in this study, suggests that there was no growth interference due to the presence of other unknown compounds as shown in **Fig. 5.5a**. This implies that more than one compounds in OLE were utilised as a carbon source simultaneously. Both malic and citric/isocitric acids were consumed as the main carbon source in the first 20 hours whilst fructose was simultaneously used but at a much slower rate. A recent study confirmed that fructose and citric acid in banana juice extract can be metabolised by *C. necator* in parallel without interfering with one another (Poltronieri et al., 2017). Fructose was converted to acetyl-CoA via glycolysis while citric acid and presumably directly entered the tricarboxylic cycle (TCA), also known as the citric acid cycle (CAC). Citric acid present in the mixed substrate could be used directly in the CAC for the production of biosynthetic precursors vital for protein and nucleic acid synthesis. Generally, acetyl-CoA as an intermediate combines with

oxaloacetate, forming citric acid for the CAC (Raberg et al., 2014). Acetyl-CoA is also an intermediate compound for PHB production. In the presence of citric acid, the process of converting acetyl-CoA to citrate will therefore be shifted towards the PHB synthesis pathway, increasing the final yield of accumulated PHB. Other acids found in OLE such as malic, succinic and fumaric acids could behave similarly to citric acid, directly entering the citric acid cycle.

As can be seen in **Fig. 5.8a**, the study of biomass growth and PHB accumulation during the exponential phase between 6 and 25 hours could be split into two phases:

- 1) **Phase I** (between 6 and 15 hours) – During this time, the growth of bacteria was rapid, as reflected by a steep increase of biomass concentration from 0.53 to 4.15 g L⁻¹. PHB concentration and percentage accumulation of PHB increased from 0.26 to 1.09 g L⁻¹ and from 1.0% to 26.1%, respectively.
- 2) **Phase II** (between 15 and 25 hours) – Compared to **Phase I**, an increase in biomass concentration was considerably lower, from 4.15 to 4.65 g L⁻¹, whereas the PHB concentration increased from 1.09 to 2.12 g L⁻¹ and the percentage of PHB content increased from 26.1% to 45.5%, accordingly.

Comparing these results between the two periods, a change in PHB concentration was similar, 0.83 g L⁻¹ (**Phase I**) and 1.03 g L⁻¹ (**Phase II**). However, a 7.8-fold increase in biomass concentration was observed during **Phase I** whereas the biomass in **Phase II** only grew by 12%. A considerable change in biomass between the two phases could imply that the culture may have been nitrogen-limited after 15 hours of cultivation. During **Phase I**, an abundance of nitrogen was favoured over PHB synthesis. A subsequent lack of nitrogen (**Phase II**) combined with unlimited availability of fructose then resulted in a high carbon to nitrogen ratio of the liquid culture, which was a desirable condition (nitrogen-limiting) for PHB synthesis by

C. necator. Thus, the maximum PHB percentage was obtained at the end of this phase (45.6%).

When the concentration of fructose became low (0.72 g L^{-1}) at 32 hours, there was a slight decrease in PHB concentration from 2.12 g L^{-1} to 2.01 g L^{-1} and the decline continued to 35 hours when the final concentration of PHB was 1.83 g L^{-1} . Towards the end of the stationary phase, fructose became limiting, so bacteria were presumed to utilise intracellular PHB as an alternative nutrient source. This phenomenon was previously reported when *C. necator* consumed PHB granules in the absence of usable exogenous substrate for cell growth and survival (Handrick et al., 2000).

5.1.5.3.1 Identification of PHB product using FTIR spectroscopy

PHB was extracted (the extraction method is described in **Section 3.3.3**) from *C. necator* H16 cells and characterised for its properties using FTIR analysis. The FTIR spectrum of the PHB sample extracted from the biomass (**Fig. 5.10**) in this study was similar to the PHB previously reported (Nurbaş and Kutsal, 2004; Pradhan et al., 2017; Zhang et al., 2020).

FTIR spectra of the triplicated samples (**Fig. 5.10**) were almost identical, confirming that the three cultures of *C. necator* H16 with OLE produced a similar quality of polymer, PHB. There were two main FTIR peaks confirming the feature of PHB molecules (**Fig. 5.9**). The first adsorption band at around $1,277\text{--}1,279\text{ cm}^{-1}$ corresponds to ester bonding (--C--O). Another strong adsorption peak at $1,720\text{--}1,721\text{ cm}^{-1}$ corresponds to --C=O stretching of the ester group. The presence of this C=O stretching vibration region indicates that there was a highly ordered crystalline structure in the molecular chain of the polymer. The extracted polymer from this study may not be entirely pure, and trace of solvent might still be present. This was shown as a --CH band at $2,976\text{--}2,9777\text{ cm}^{-1}$, which corresponds to chloroform and/or the tail end of the polymer chain.

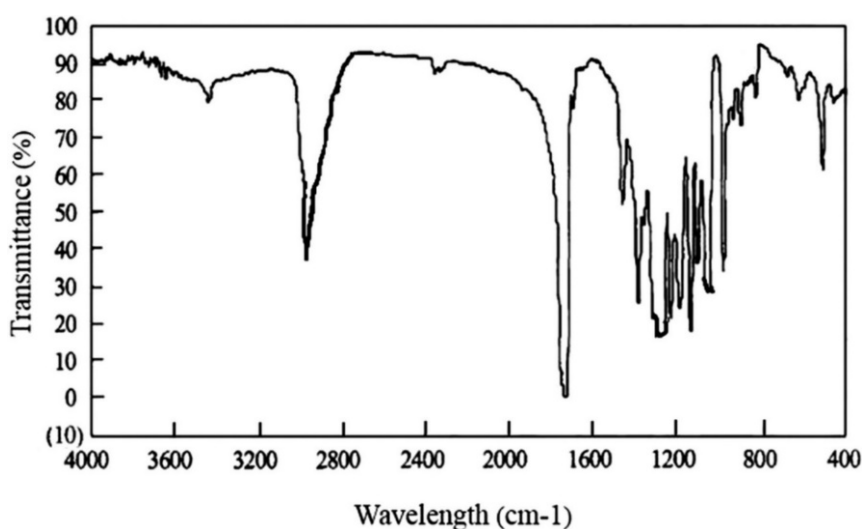


Fig. 5.9 FT-IR spectra of PHB from *C. necator* H16 (Zhang et al., 2020).

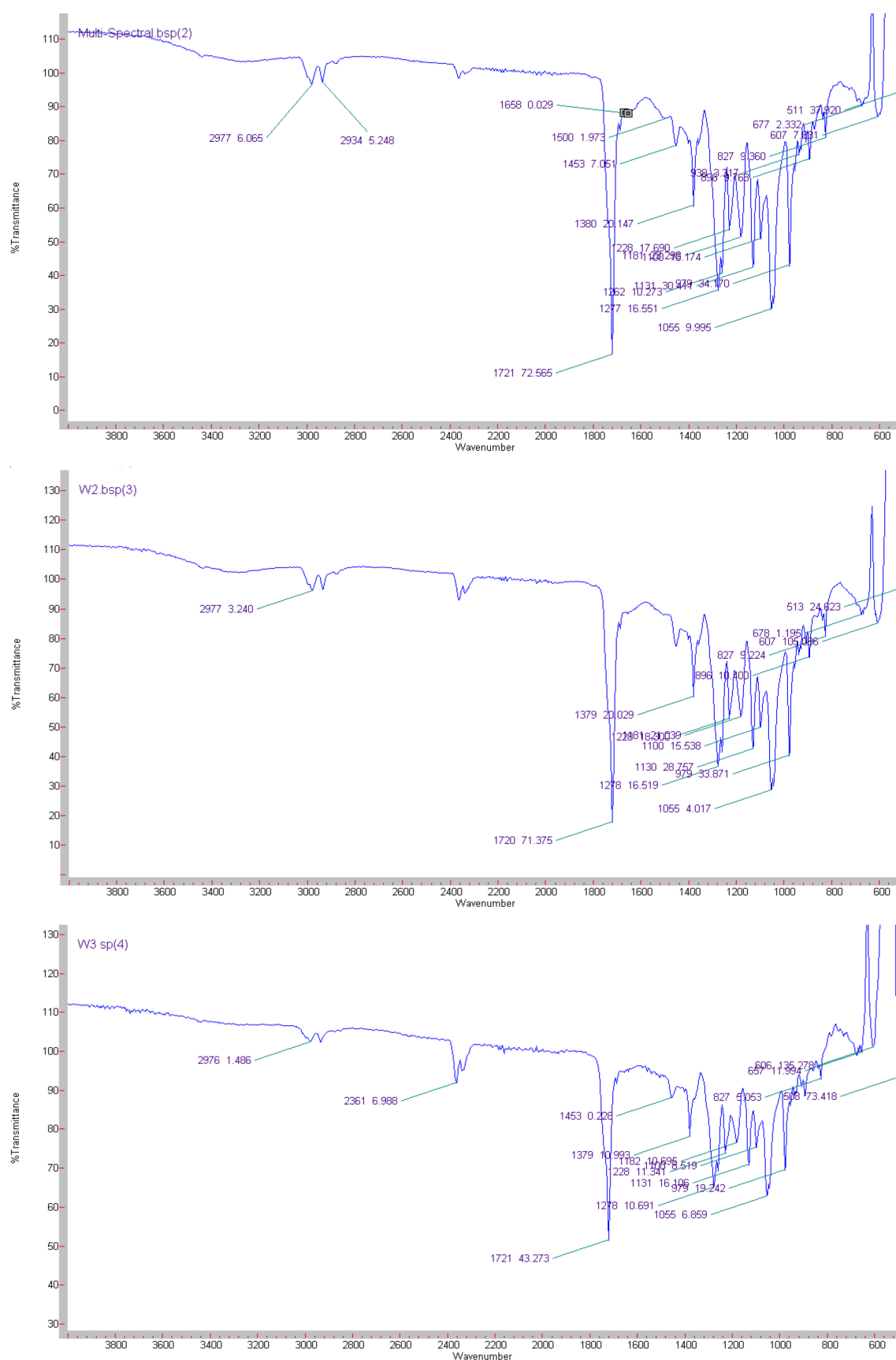


Fig. 5.10 FT-IR spectra of the PHB extracted from the triplicate fermentation of *C. necator* H16 using *Opuntia* liquid extract (OLE) as a substrate.

5.1.5.3.2 Comparison with literature studies

Opuntia liquid extract (OLE) was used as the sole substrate in this study for the production of PHB employing *C. necator* H16. The total initial concentration of carbon source used in this study, combining fructose, malic acid and citric acid, was 22.0 g L⁻¹, and the maximum biomass, PHB concentrations and PHB accumulation percentage obtained from were 5.25 ± 0.21 g L⁻¹, 2.12 ± 0.10 g L⁻¹, and 45.6%, respectively. The results were compared to the literature detailing the production of PHB using a variety of plant juices, extracts, or hydrolysates, as presented in Chapter x table x. In general, the dried biomass concentrations varied in previously published work from 4.44–20.9 g L⁻¹, depending on the strain type, initial substrate concentration, and operating conditions.

The most similar published results to this study focused on the production of PHB from sugar beet juice which had a starting concentration of 20 g L⁻¹ of total reducing sugars (Wang et al., 2013). Biomass and PHB concentrations from this study (5.25 and 2.12 g L⁻¹) were relatively lower than the figures in the literature (10.3 and 4.01 g L⁻¹ for sugar beet juice). However, the percentage of PHB accumulated achieved using OLE was slightly higher in this study at 45.6%, compared to 38.9% previously reported using sugar beet juice as the substrate.

Another similar study using 20 g L⁻¹ of sugarcane molasses as the main carbon source for PHB production by *C. necator* has more recently been published (Dalsasso et al., 2019). Despite their biomass concentration (20.9 g L⁻¹) being 4 times greater than the value obtained from this study (5.25 g L⁻¹), there are factors that need to be taken into consideration in order to make a reliable comparison. *C. necator* DSM 545 used in their study had an advantage over *C. necator* H16 as it is able to metabolise both fructose and glucose, the latter of which the wild-type

strain, H16, is not capable of metabolising. The experiment was also performed in fed-batch operation, which enabled optimisation of the fermentation process by implementing a pH control system and aeration for enhancing the product yield.

The highest percentage of PHB accumulation obtained from OLE (45.6%) in this study was comparatively higher than those previously reported using other types of plant extracts (39.4% for sweet sorghum juice and 30.6% for sugarcane juice) (Kaewkannetra et al., 2008; Suwannasing et al., 2011). Their studies also started the cultures with greater initial concentrations of substrate, 40 and 50 g L⁻¹, compared to 22 g L⁻¹ of the substrate in the current study. This means that OLE was able to provide the necessary carbon sources and nutrients essential for the growth and cellular activities of *C. necator* H16. However, the percentage of PHB accumulation could still be improved by process optimisation. For example, the fermentation of date seed oil at an initial substrate concentration of 10.8 g L⁻¹, half of that used in this study (22 g L⁻¹), was able to achieve an exceptional PHB accumulation percentage of 73.0% through reduction of nitrogen and oxygen availability in the culture flasks, which promoted PHB synthesis (Yousuf and Winterburn, 2016).

5.1.5.3.3 Economic feasibility of the PHB project

The economic viability of the PHB production should also be evaluated to assess the suitability of OLE as a substrate for PHB production. **Table 5.4** presents the calculation performed to estimate the cost of a carbon source for the annual PHB production of 100,000 kilograms. The scenario analysis was implemented for cost calculation in this study to demonstrate the influence of different cost options on the percentage of carbon source cost per total sale revenue. The descriptions of the four scenarios are as follows.

Scenario I – ‘The base case’. The cost of the carbon source was the actual cost of *O. ficus-indica* used in this study (£2.30 per kg of fresh cladodes). The extraction efficiency was 66.7% and the actual PHB yield as presented in Chapter 6 was 2.12 g L⁻¹. Based on the process developed in this study, the PHB production yield of 2.12 g L⁻¹ from 22 g L⁻¹ usable organic content in OLE was not economically viable as the cost of carbon source was enormously higher than the estimated sale revenue from the PHB. However, this fermentation process using OLE is still at the early stages and the following criteria should be considered to improve the process to be more cost-effective.

Scenario II – This scenario is an improvement from the base case by adjusting the cost of *O. ficus-indica* cladodes per kg from £2.30 to £0.006. The raw material cost is known to significantly contribute to production costs. *Opuntia ficus-indica* cladodes used in this experiment was sourced from Pepe Aromas farm in Portugal and the cost per kg fresh weight was EUR2.50 (£2.30). In practice, this raw material could be cultivated on-site and harvested to be used directly for PHB production. Based on the economic evaluation of cultivation of *Opuntia ficus-indica* in 1999, the average estimated production cost, including those of establishment, maintenance,

and production during the first six years was approximately \$0.05 per kilogram dry mass (Mondragon-Jacobo and Perez-Gonzalez, 2002). Assuming that the moisture content of *Opuntia ficus-indica* is 90% and the total inflation rate from 1999 to 2020 was 56.9%, the price per kilogram fresh weight today would be \$0.008 (£0.006). The percentage of carbon source costs per total sales revenue substantially decreases from 26,066% to 88.9%, which confirmed the fact that the raw material cost is the major production cost.

Scenario III – This scenario focuses on an improvement of PHB production. The current PHB yield of 2.12 could be increased to 11.7 g L⁻¹, which was achievable as reported in the literature. The current set-up employed for the fermentation was a batch process using an Erlenmeyer flask, which had several operating limitations. In a previously reported study, it was demonstrated that when 20 g L⁻¹ of hydrolysate from sugarcane molasses was used as the carbon source in a 4-L fed-batch reactor, a PHB concentration of 11.7 g L⁻¹ could be achieved (Dalsasso et al., 2019). Assuming the current process was able to achieve the same yield, the percentage of the cost of carbon source per the sale revenue would approximately decrease by 5 times.

Scenario IV – This scenario focuses on maximising the use of the raw material (cladodes). Currently, the extraction efficiency of OLE employing centrifugation was 66.7%. The moisture content of *Opuntia ficus-indica* cladodes were approximately 90% of the fresh biomass, but only two-thirds of liquid was extracted from the ground material via centrifugation. Further development of the extraction technique would be necessary to minimise the loss of unextracted OLE through the cladode peel. Increasing OLE extraction efficiency from 66% to 80% would save the raw material cost by 16%. In this study, OLE was extracted from ground *Opuntia*

using a laboratory centrifuge at 12,000xg. To operate an industrial-scale centrifuge at such a high G-force would be highly energy-intensive and hence unrealistic. However, there are several solid-liquid separation techniques that are currently used in the agro-product industry such as a basket centrifuge, a disk stack centrifuge or a membrane filter. One suitable option is a decanter centrifuge as it has been widely used in several production processes such as olive oil and cassava starch. The solids continuously removed by the screw conveyor from the centrifuge allows better separation between solid and liquid phases, which could compensate for the lower G-force that the decanter centrifuge can achieve (max. 4,000xg).

Other actions could be implemented to further reduce the production costs, for instance:

- One of the limitations of employing *C. necator* H16 was that this wild-type strain was not able to metabolise glucose, which, according to the GC-MS data in Chapter 6, was as equally as abundant as fructose. The biomass and PHB production yields may have been considerably enhanced if alternative strains, which could utilise a greater variety of substrates including both glucose and fructose, such as *C. necator* DSM 545 were used.
- The addition of nutrients was deemed unnecessary as OLE, which is a complex material, was able to sustain the growth of *C. necator* H16. The raw material costs could therefore be further saved.
- The results from Chapter 4 demonstrated that the remaining cladode peel from the liquid extraction process could be used as a substrate for biogas production. The CHP system can be operated with biogas to generate heat and electricity, which are then used in the PHB production process, saving the plant operating costs.

Table 5.4 The cost estimation of the PHB production based on the price of *Opuntia ficus-indica* cladodes

Parameters	Unit	Quantity			
		Scenario I	Scenario II	Scenario III	Scenario IV
PHB production yield	kg L ⁻¹	0.00212	0.00212	0.0117	0.0117
Assumed annual PHB production	kg	100,000	100,000	100,000	100,000
Estimated total production volume per annum	m ³	47,169	47,169	8,547	8,547
Total volume of OLE required (50% production volume)	m ³	23,585	23,585	4,274	4,274
OLE extraction efficiency	%	66.7	66.7	66.7	80.0
Density of OLE (assumed to be the density of water)	kg m ⁻³	1.00	1.00	1.00	1.00
Total fresh cladodes weight needed	t	35,359	35,359	6,407	5,341
Biomass productivity (fresh weight) ¹	t ha ⁻¹	73.5	73.5	73.5	73.5
Total land required for <i>O. ficus-indica</i> cultivation	ha	481	481	87.2	72.7
Price of cladodes per kg ²	£/kg	2.30	0.006	0.006	0.006
Total cost of total cladodes required per annum	m£	81.3	0.216	0.039	0.033
Global market price of PHB ³	£/kg	3.12	3.12	3.12	3.12
Annual sale of PHB (100,000 tonnes)	m£	0.312	0.312	0.312	0.312
Percentage of carbon source cost per total sale revenue	%	26,066	69.3	12.6	10.5

¹(Krümpel et al., 2020); ²Conversion rates \$1.0 = £0.78 and EUR1.0 = £0.92 as of 15/09/2020; ³Global market price of PHB obtained from Alibaba.com (\$4/kg)

5.1.6 General discussion

5.1.6.1 Characteristics of *Opuntia* liquid extract (OLE)

Opuntia liquid extract (OLE) is a complex substrate that contains many organic compounds, some at high concentrations. The OLE liquid substrate without medium solution or dilution may have contained inhibitory compounds that had an effect on *C. necator* and may explain the observed slow growth, which can be seen as the lag phase in **Fig. 5.3a**.

5.1.6.2 The effect of nitrogen and chemical compositions in the medium solution on the growth of *C. necator* H16

Results from the current study demonstrated that supplementary nitrogen was not necessary for the culture of *C. necator* using OLE as a sole substrate ($p > 0.05$) based on ANOVA statistical analysis, even though supplementary 1.0 g L^{-1} of nitrogen in the medium solution gave slightly higher OD_{600} . This concentration of nitrogen could be sufficient for the growth of *C. necator* H16.

Previous studies on metabolite characteristics of *O. ficus-indica* cladodes found that they contained amino acids, vitamin B and C, macro and micronutrients, fatty acids and mineral oils (Corrales-García et al., 2004; El-Mostafa et al., 2014). OLE obtained from the cladodes, which contained essential amino acids, were found to be able to support metabolic activities of *C. necator* H16. Particularly, aspartic acid and cysteine, detected in OLE in this study (**Table 5.2**), for example, are two of the three key amino acids of the catalytic triad on the active site required for PHA synthase enzyme production (Peplinski et al., 2010). Most of the vitamins, minerals and macronutrients of the medium solution used in this study were already present in OLE and therefore additional supplementation of these components was not necessary.

Based on the results presented in this section, for batch fermentation of OLE by *C. necator*, it is not necessary to add nitrogen, vitamins, or any other nutrients. The cost of such raw materials would thus be saved, which makes this fermentation process more economically feasible. However, the addition of buffer solution could be further investigated as the buffering capacity of a large-scale fermentation may differ from that of the current study. Other low-cost buffers can also be tested.

5.1.6.3 Using *Opuntia* liquid extract (OLE) as the sole substrate for PHB production by *C. necator* H16

The results of this study confirm that *C. necator* H16 was effective at synthesising PHB from *Opuntia* liquid extract (OLE) as a carbon source. The maximum PHB produced as a proportion of dried biomass using OLE (45.5%) in the present study was lower than those reported in earlier studies using the same initial concentration of the carbon source (Dalsasso et al., 2019; Wang et al., 2013). However, with OLE, there may still be scope for further improvement to achieve higher cell biomass and PHB concentrations. Simple batch fermentation such as flask studies tends to produce relatively low biomass, as the system is limited by the initial concentration of substrate and nutrients and the build-up of breakdown products. Also, as the cells grow and generate waste products, growth conditions become progressively less optimal. Fed-batch fermentation could negate these drawbacks by maintaining the concentrations of necessary compounds and pH that are optimal for bacterial growth. For example, in **Fig. 5.8**, the percentage of PHB accumulated decreased with the concentration of fructose after 25 hours. At this point, the additional substrate could be added to maintain a sufficient level of carbon source to promote further PHB synthesis.

Opuntia liquid extract (OLE) was found to be rich in glucose and the signal intensity from the peak corresponding to glucose was equally strong as fructose. Glucose, however, remained unutilised due to a wild-type strain (*C. necator* H16) not being able to metabolise it. Other strains such as DSM 545 could be employed instead of a wild type in order to take advantage of both glucose and fructose in the substrate. One recent study was able to generate 20.9 g L⁻¹ of biomass from a fed-batch fermentation of hydrolysates from sugarcane vinasse and molasses (Dalsasso et al., 2019). The hydrolysates consisted of fructose, glucose, lactic acid and acetic acid, all of which could be metabolised by *C. necator* DSM 545.

There have been several studies of PHB production using other recombinant strains such as *E. coli* which was genetically modified to harbour a plasmid carrying *C. necator* PHA synthesis genes (Lin et al., 2015; Poltronieri et al., 2017; Wu et al., 2016). These recombinant *E. coli* strains offered some advantages over *C. necator* H16 and a mutant strain DSM 545, for example, they were able to use various substrates as carbon sources and PHA productivity could be improved substantially. However, the use of these strains may encounter public resistance if bioplastics are used as organic food packaging, and the bioplastics produced from GM-strain and/or GM-crops as a carbon source could face governmental restriction particularly in Germany and the UK (Philp, 2014; Yeh et al., 2015).

In addition, the use of OLE as a natural substrate offers some advantages over hydrolysates employed in other previous studies (Pradhan et al., 2017; Radhika and Murugesan, 2012; Zhang et al., 2020). Pretreatment by acid hydrolysis usually requires strong acids such as H₂SO₄ or HCl as a catalyst and operates at high temperature and pressure, which leads to problems of health and safety, operational cost, and waste disposal. While enzymatic hydrolysis may offer a safer

operation, the extra cost of the enzymes used in the process contributes to material cost. The extraction of *Opuntia* liquid is simpler and requires only mechanical pretreatment without the addition of chemicals, solvents, or enzymes, reducing costs and minimising issues of health and safety. The only energy-intensive process is the separation of *Opuntia* liquid from the solid pulp via centrifugation. However, this energy input could be compensated by using biogas produced from the solid pulp via anaerobic digestion.

5.1.7 Conclusion

This study is the first to report the production of PHB derived from *Opuntia* liquid extract (OLE) employing a wild-type strain of *C. necator* H16. OLE was high in soluble sugars, particularly fructose, and organic acids and was confirmed its potential as a substrate for this fermentation process. The substrate also contained sufficient protein and essential amino acids which are vital for microbial growth. Therefore, supplementation of nitrogen, vitamin and mineral compounds to the liquid culture was not necessary. The current study demonstrated that OLE could be a viable substrate for PHB production when using *C. necator* H16 with a maximum PHB content of 45.6% (2.12 g L⁻¹). Furthermore, the biomass and PHB yields obtained from this experiment could be further improved using various strategies, including control of nitrogen and oxygen availability in the culture, which are known to directly affect PHB synthesis (Anderson and Dawes, 1990). Future studies should include the analysis of PHB properties such as polymer chain length, molecular weight and tensile strength as these properties, especially molecular weight, are essential for industrial processing of the polymer.

Chapter 6

General discussion and conclusions

The principal aim of this research was to investigate the potential of Crassulacean Acid Metabolism (CAM) plants as an alternative feedstock for the anaerobic digestion (AD). To potentially replace maize, which has become less acceptable as an energy crop because of the ‘food vs fuel’ controversy, CAM plants such as *Euphorbia tirucalli* and *Opuntia ficus-indica*, have previously been suggested as potential candidates for AD feedstocks (Mason et al., 2015). However, further investigations are required to provide practical evidence to demonstrate that these two species could represent viable bioenergy crops growing on marginal and semi-arid lands. Four main studies, which are presented in Chapters 4 and 5, were performed in order to achieve the thesis following objectives:

- Screen and identify suitable CAM species, which could potentially become viable AD feedstocks employing the biochemical methane potential (BMP) assay.
- Evaluate the influence of different process conditions such as inoculum to substrate (I/S) ratio, operating temperature, pH, and substrate mixing (E:M) ratio on the AD performance of selected species using BMP trials.
- Investigate other parameters such as substrate processing techniques and harvesting age, which directly affect the characteristics of the feedstocks.
- Assess the performance of the semi-continuous digestion of *O. ficus-indica* and identify the optimal achievable organic loading rate (OLR) and any potential inhibition.

- Demonstrate the use of *Opuntia ficus-indica* as a biomaterial for other applications, specifically, for the production of polyhydroxyalkanoate (PHA).

6.1 Summary of key thesis findings

6.1.1 Screening of potential CAM plants as AD feedstocks

The study summarised in **Chapter 4.1** focused on determining the methane productivity of different CAM species from five different families using a biochemical methane potential (BMP) assay.

- The investigation in **Chapter 4.1** demonstrated that *Agave angustifolia* produced the highest BMP (382 L kgVS⁻¹) of all the plant species tested including maize, a typical energy crop used in this study as a reference plant.
- In contrast, the BMPs of *Euphorbia virosa* and *Ananas comosus* (pineapple) were the lowest and this was related to the chemical composition of the biomass. Specifically, the lignin percentages of *E. virosa* and pineapple were the highest of the five CAM plant candidates investigated, which indicated that they could be more difficult to degrade microbiologically.
- There was a negative correlation between lignin content and the degradation rate ($R^2 = 0.72$). *Kalanchoe daigremontiana*, for example, had the lowest lignin content (8.09%) but the highest biodegradation rate and maximum methane production rate.
- Overall, CAM plants investigated in this study have demonstrated that they were able to produce methane yields as high as maize, a typical energy crop.

6.1.2 Anaerobic digestion of *Euphorbia tirucalli*

Euphorbia tirucalli was one of the two CAM species selected as a model crop that could potentially be used as an AD substrate. The work from **Chapter 4.2** focused

on determining the SMY of *Euphorbia tirucalli* at different inoculum to substrate (I/S) ratios and improving the methane yield of *E. tirucalli* through optimising operational conditions such as operating temperature, starting pH, and substrate mixing (E:M) ratio through a co-digestion study.

- The results revealed that the BMP of *Euphorbia tirucalli* was 206 ± 8 L kgVS⁻¹, which was obtained at an I/S ratio of 2.0, with starting pH 7.0, and at mesophilic temperature (38°C). The specific methane yield improved by 19.4% to 246 ± 26 L kgVS⁻¹ under thermophilic conditions (55°C). However, the highest specific methane yield of *E. tirucalli* (266 ± 13 L kgVS⁻¹) was obtained at thermophilic temperature (55°C), I/S ratio of 1.0 and an initial pH 9.0.
- Significant improvement in the methane yields was observed at higher operating temperature, 55°C ($p < 0.001$) and higher I/S ratios ($p = 0.02$). However, the effect of increasing an initial pH from 7.0 to 9.0 on methane production was inconclusive. There was no improvement in methane yield observed under mesophilic conditions but increasing the pH to 9.0 under thermophilic conditions improved the methane productivity of *E. tirucalli* at I/S ratios of 0.5–1.0.
- The hydrolysis rate constants obtained from the first-order model and maximum methane production rates from the modified Gompertz model increased with increasing operating temperature. However, based on the energy balance assessment in this study, lower energy generation efficiency was achieved at thermophilic temperature (55°C), irrespective of the initial pH. Operating the digestion of *E. tirucalli* under mesophilic conditions and at initial pH 7.0 was therefore deemed the best option in terms of energy efficiency.

- The study of co-digestion of *E. tirucalli* and chicken manure revealed that methane yields from 6 different mixing (E:M) ratios were comparable. However, thermophilic temperature (55°C) resulted in a significant increase in the methane yield of the co-digestion by 21.8% ($p = 0.006$).

6.1.3 Anaerobic digestion of *Opuntia ficus-indica*

The research undertaken in this study evaluated the effect of inoculum to substrate (I/S) ratios and plant age of *Opuntia* cladodes on methane production using a BMP assay. The feasibility of the semi-continuous digestion of *O. ficus-indica* was also tested at different organic loading rates. The studies reported in **Chapter 4.3** demonstrated that the BMPs of *O. ficus-indica* varied from 264–354 L kgVS⁻¹, depending on the substrate batch and the inoculum used at the time the BMP test was performed.

The first experiment assessed the impact of I/S ratios and substrate types on the methane yields of *O. ficus-indica*. Irrespective of the substrate type, the optimum range of I/S ratios for anaerobic digestion of *O. ficus-indica* was between 0.71 and 2.08. The I/S ratio of 0.35, which was the lowest ratio tested, resulted in significantly lower methane yields ($p < 0.001$) than those from other I/S ratios and therefore should be avoided for a BMP test.

- *Opuntia* cladodes investigated in this study were ground to a homogeneous mixture referred to as “the ground cladode”, which were subsequently centrifuged at a high speed to separate the “liquid extract” (pulp) from the “solid peel”. Of the three substrates, the liquid extract produced the highest methane yield of 289 ± 20 L kgVS⁻¹ at the I/S ratio of 2.0, whereas the cladode peel yielded the lowest methane compared to the ground cladode and the solid peel for all I/S ratios. The liquid extract or pulp typically contained sugar compounds,

which could potentially be readily metabolised by the microbial community. In contrast, the peel was mainly a fibrous structure and probably lacked other organic compounds such as fat and protein, which were necessary for AD microbial activities.

The second experiment was to investigate the effect of cladode age on methane production. Five different cladode ages (0.5, 1.0, 2.0, 3.0 and 4.0 years old) were selected for this study.

- The methane yields obtained from younger tender cladodes aged between 0.5 and 2.0 years old ($352\text{--}363\text{ L kgVS}^{-1}$) were significantly higher than the lignified cladodes aged 3.0 and 4.0 years old (319 and 323 L kgVS^{-1} , respectively ($p < 0.001$)). Despite a clear-cut difference in methane yields between the two groups, the maximum methane production rates and the lag times obtained from the modified Gompertz model were not affected by the age of the cladodes.
- It can be concluded from this study that *Opuntia* cladodes could be harvested every 6–12 months although the exact timing depended on the biomass growth rate. *Opuntia* pads or cladodes typically contain a hydrocolloid constituents known as mucilage (Sepúlveda et al., 2007). Pectin is one of the mucilages with gelling properties, which help the plant retain water within the pulp. The ground sample obtained from older cladodes tended to have a higher proportion of mucilage and thus exhibited a gel-like behaviour, which made the whole sample more resistant to agitation and processing. For laboratory experiments, the ground cladode with gel-like characteristics needed to be vigorously mixed to ensure that samples were uniformly homogeneous each time. In addition, the ground cladode samples, which were high in mucilage, may not have been

properly mixed in the digesters when being operated at high organic loading rates (OLRs) and low mixing intensity.

- The liquid extract from the older cladodes was very viscous and difficult to separate from the ground material via centrifugation. Therefore, younger cladodes, particularly 6-month-old, were preferred in order to obtain the liquid extract. This is an important consideration when assessing the potential to scale up to commercial applications.

The semi-continuous experiment was performed at different organic loading rates (OLRs) to evaluate the performance of the digester and determine the biodegradability of *O. ficus-indica* at the optimal feeding rate. It was also established to determine the potential of expanding the approach to a commercial scale.

- *O. ficus-indica* was successfully converted to methane via semi-continuous digestion and the specific methane yields achieved over 75 days were between 249–312 L kgVS⁻¹, which accounted for 73.5–92.0% of the BMP of the ground cladode sample. The highest achievable OLR was 5.52 gVS L⁻¹ day⁻¹, which was maintained for approximately 15 days during Phase III. Stable methane production from *O. ficus-indica* was observed during Phase I but became less stable with increasing OLRs in Phases II and III.
- There was no observable foaming blocking the gas outlet tubes in any of the digesters despite the small proportion of digester headspace (4% of the total digester volume).
- All four digesters exhibited initial signs of failures such as a decrease in biogas yields and a drop in digester pH values to an acidic range after 75 days of operation due to technical issues. Although temporary feed suspension slightly

improved the digester performance, propionic acid accumulation at high concentrations (above 0.5 g L⁻¹) inhibited the microbial activity. The VFA inhibition was more severe after feeding was resumed and the digesters could no longer be rescued.

6.1.4 CAM plants in biorefinery

Previously, the findings of Chapter 4.3 revealed that of the three different plant components of *O. ficus-indica* (the ground cladode, liquid extract and cladode peel), liquid extract (OLE) had the highest volatile solid contents (88.6%) and yielded the highest BMP of 289 ± 20 L kgVS⁻¹. The results provided evidence that OLE was rich in usable organic contents, which could be metabolised by the AD microbial community.

- The findings of Chapter 5 led to the conclusion that OLE was abundant in sugar compounds and organic acids, especially malic and citric/isocitric acids that CAM plants typically accumulated at night. The three main compounds present in the OLE which could be metabolised by *Cupriavidus necator* H16 were fructose (13.2 g L⁻¹), malic acid (16.0 g L⁻¹) and citric acid (14.8 g L⁻¹).
- The results from the first two initial studies revealed that OLE contained sufficient nitrogen and other nutrients necessary for the growth of *C. necator* H16 without the need for external addition of supplementary nutrients. The use of a 1:1 ratio of OLE and water as a liquid culture significantly improved the growth of bacteria, compared to a pure OLE as the values of OD₆₀₀ increased from 2.90 to 4.79 ($p = 0.03$). Therefore, this condition was later used in the flask experiment.
- The highest PHB concentration and PHB accumulation percentage attained from the flask experiment over 35 hours of fermentation were 2.12 ± 0.10 g L⁻¹

and 45.6%(w/w), respectively. This is the first known study to report the production of PHB from OLE using *C. necator* H16. However, this study is still at early stages and several process optimisation strategies could be implemented to improve the PHB yield and enhance the substrate competency.

6.2 General conclusions

- The results of this study confirm that the seven species of CAM plants from five distinct families investigated have the potential to be used as a bioenergy crop since their methane potentials were comparable to that of maize stover, a typical AD feedstock.
- The research evaluated the effect of inoculum to substrate (I/S) ratio, initial pH, and operating temperature on the anaerobic digestion of *Euphorbia tirucalli*. Experimental data showed that the combination of I/S ratio of 2.0, with an initial pH of 7.0 and temperature of 38°C was the best condition in terms of the optimal methane yield and the greatest energy generation efficiency.
- The findings from **Chapter 4** confirmed that the optimal inoculum to substrate (I/S) ratio for anaerobic digestion of the CAM species tested in this study was 2.0. The highest specific methane yields (SMYs) were achieved at this ratio with the lowest lag times. This ratio also worked best at both mesophilic and thermophilic conditions as demonstrated for the digestion of *E. tirucalli* in Chapter 4.2.
- The percentage lignin content of *O. ficus-indica* cladodes was relatively low (5.61–9.15%TS) compared to other CAM plants (8.09–17.35%TS) tested in this study. The cladodes could be processed into different substrate types such as ground cladode and liquid extract depending on the application of the substrate.

The highest BMP of *O. ficus-indica* of 354 L kgVS⁻¹ was obtained from the 1-year-old cladodes.

- Semi-continuous digestion of *O. ficus-indica* was feasible at the organic loading rate (OLR) of 5.52 gVS L_R⁻¹ day⁻¹, the highest OLR tested in this study. In this condition, the specific methane yield was 269 L kgVS⁻¹.
- The results of this study confirm that methane and polyhydroxybutyrate (PHB) could be produced from a liquid extract from *O. ficus-indica* (OLE). The highest methane yield achieved for OLE was 289 L kgVS⁻¹, while the highest PHB yield obtained from the fermentation of OLE using *Cupriavidus necator* H16 was 45.6% of the cell dry weight.

6.3 Contribution of research

- This research is the first reported comparative study of a representative range of various CAM plants in terms of their potential for bioenergy and biochemical production. The results from this thesis can serve as the foundation for the study of other CAM plants as viable biomass feedstocks.
- The biochemical methane potential of *Euphorbia tirucalli* and *Opuntia ficus-indica* was determined and several parameters influencing methane production such as inoculum to substrate ratio, operating temperature, initial pH and carbon to nitrogen ratio were investigated.
- This study is the first to apply the concept of “Cascading use of biomass” otherwise known as the concept of biorefining to maximally exploit *Opuntia ficus-indica*. This revealed *O. ficus-indica* could be separated into liquid and cladode peel. The liquid can potentially be used as a carbon source directly in the biorefinery industry whereas biogas could be produced from the cladode peel, providing energy to be used in operating the biorefinery process.

- This study is the first to investigate *Opuntia liquid extract* (OLE) as a substrate for both anaerobic digestion and microbial fermentation processes. OLE offers several advantages over conventional substrates used in the PHB production process. The two most significant costs in terms of bioplastics are sourcing carbon supplies and downstream separation processing. Previous researchers have demonstrated that low-cost substrates such as lignocellulosic biomass and agricultural wastes have been successfully hydrolysed to obtain fermentable sugars such as glucose, which can then be used as a substrate for the fermentation process. The hydrolysis method typically involves the use of acids or alkalis, which could lead to the production of inhibitory by-products that can negatively impact the subsequent fermentation. Furthermore, the extra hydrolysis step contributes to the raw material and operating costs and can lead to health and safety issues when dealing with hazardous chemicals. Therefore, OLE, which is naturally in a liquid form with no known or detected inhibitory compounds, could bypass this complicated and costly hydrolysis step and be used directly in the fermentation process.
- The exploitation of OLE was an improvement on conventional fermentation processes, which typically require a complex medium solution supplemented with nutrients to sustain bacterial growth. In the current experimental set-up, the use of the medium solution, which contained minerals and vitamins, was unnecessary and therefore removed, thus reducing the raw material cost.
- This research has established the technique of extracting liquid from *O. ficus-indica*, which involved mechanical processing and centrifugation. This could be applied to other succulent CAM plants with similar biomass structures and characteristics to *O. ficus-indica*.

6.4 Methodological impacts, issues, and limitations

This section provides a critical review of the experimental techniques used in this study.

6.4.1 Substrate supply and handling

CAM substrates employed in the studies detailed in thesis could not be sourced as a single batch as mentioned in the literature (**Chapter 3, Section 3.1.1**). The findings from **Chapter 4.3**, for example, revealed the variation in the BMPs from different batches of *O. ficus-indica* in three different studies (241, 339, and 354 L kgVS⁻¹).

The small sample size of the materials sourced for the screening experiment (**Chapter 3, Section 3.1.1**) may not truly represent the actual biomass characteristics of the species investigated. Another factor, which affected the characteristics of biomass was the growing conditions. A recent review concluded that plants growing in controlled conditions and the field had different growth rates and plant morphology due to a variation in the exposure of daily light and temperature (Poorter et al., 2016). Therefore, the biomass characteristics of the plants reported in this study may slightly differ from those of the same species grown in the field.

O. ficus-indica cladodes are typically large with high moisture content (approximately 90%). Fresh cladodes needed to be stored in a dry and airy environment that avoided stacking in order to prevent condensation which encourages the development of rots. Effective drying of the cladodes was not achieved due to a lack of a facility capable of handling a large number of plant materials. Air-drying of substrates with such high moisture content was not possible in the UK. Therefore, the fresh cladodes were mechanically ground to a

homogeneous material. The ground material could not be left at ambient temperatures and had to be stored in a freezer to prevent bacterial fermentation and fungi contamination. This required a large freezer space and a high energy input to preserve the plant material to be used for the experiments. However, these issues would become less problematic in countries with dry climates.

The handling of fresh *O. ficus-indica* was also a key factor in terms of bioprocessing. Due to the nature of the feedstock, which had a high moisture content and a gel-like behaviour, thorough mixing of the substrate was necessary to ensure a homogeneous substrate was fed to the digester each time.

6.4.2 Biomass characterisation

Biomass characteristics reported in this study were limited to only TS and VS contents, which are presented for all the substrates. Cellulose, hemicellulose and lignin percentages were also reported for most substrates, but the analysis was not performed in-house due to the unavailability of the specialised tools. The carbon to nitrogen ratio of the substrate and inoculum was also not determined, but this parameter would also be useful for interpreting the results from co-digestion experiments.

Another characterisation that should have been carried out was the elemental analysis of the substrate. The chemical composition of an organic substrate ($C_cH_hO_oN_nS_s$) could be used to estimate the biogas composition (methane and carbon dioxide) using the Buswell equation (Buswell and Mueller, 1952). Together with the carbon content and the total solid (%TS) of the material, the biogas composition obtained from the Buswell equation can be used to estimate biogas and methane production, assuming that a complete material breakdown occurs. The ratio between the experimental methane yield from the BMP test and the theoretical

methane yield obtained through the calculation mentioned earlier gives the anaerobic biodegradability of the substrate. This value is useful in terms of designing further experiments as it refers to how much the substrate in the system is converted in the system. Poor substrate biodegradability may indicate that pretreatment of the raw material is required prior to anaerobic digestion. One example from this study is *E. tirucalli*. Its BMP was 206 L kgVS⁻¹, which was relatively low compared to those of *O. ficus-indica*, ranging from 264–354 L kgVS⁻¹. However, without the elemental composition of *E. tirucalli*, it was difficult to evaluate how far this BMP result is compared to the theoretical value. Similarly, for *O. ficus-indica*, having the theoretical BMP value would help confirm or deny that *O. ficus-indica* could be easily degraded and did not require further pretreatment apart from grinding as the only substrate preparation step.

6.4.3 Biochemical methane potential (BMP) assay

6.4.3.1 Influence of the BMP measurement method

There are several BMP measurement methods, but the two most commonly used ones are the manual bottle test and the automatic system such as an AMPTS II unit. All the BMP experiments in this study were conducted via the manual approach and some of the experimental procedures may influence the BMP results.

- BMP bottles were removed from a shaking incubator for daily biogas measurement and biogas composition determination. Removing the BMP bottles from the temperature-controlled environment up to 30 minutes could have negatively affected the performance of the temperature-sensitive microorganisms, which may have resulted in a lower BMP.
- Liquid samples were collected from the BMP bottles for monitoring volatile fatty acid (VFA) concentration and pH. Based on this procedure, the BMP of a

substrate investigated in this study may have been slightly underestimated as some of the VFA were collected and not converted into methane. However, methane contribution from the liquid samples collected for VFA and pH measurement was not significant. The screening experiment (**Section 4.1**) demonstrated the highest total VFA collected over the course of the digestion was less than 0.001 g for *Kalanchoe*, which was equivalent to 0.0011 gCOD assuming that 0.937 g of acetic acid is equivalent to 1 g of COD (Rössle and Pretorius, 2001). The BMP of *Kalanchoe* was 338 L kgVS⁻¹ or 0.24 gCOD equivalent. Therefore, the contribution of COD lost through liquid samples collected was less than 1%. However, the number of liquid samples should be kept minimum to ensure minimise the error from this contribution.

6.4.3.2 Influence of inoculum quality

The inoculum used in all experiments was collected from the same source, the Severn Trent Green Power AD facility in Cassington and the characteristics of the inocula are presented in **Table 6.1**. A large variation in total solid (TS) and volatile solid (VS) contents were observed as the percentage differences between the minimum and maximum values of TS and VS were 37.3% and 12.1%. However, there was no clear correlation between TS and VS contents and the inoculum activity. The endogenous methane volume seemed to correspond to inoculum activity as those with higher activity such as sample numbers 1 and 6 produced higher methane volume in blank bottles than sample numbers 3 and 4 (**Table 6.1**), the inoculum activities of which were below 300 L kgVS⁻¹. According to the latest BMP assay guideline, the BMP of the positive control (microcrystalline cellulose in this case) should be between 352 and 414 L kgVS⁻¹. There were only two inocula samples which barely met the guideline. Inocula with lower microbial activities could have resulted in an underestimation of BMP of substrates.

Table 6.1 Summary of inoculum characteristics from all the experiments in this study. Values are means of triplicate samples $\pm$ standard deviations

Sample no.	Collection date	TS (%)	VS (%)	Blank CH ₄ volume (ml)	Inoculum activity (L kgVS ⁻¹)
1	27/07/2017	4.50 $\pm$ 0.02	67.2 $\pm$ 0.01	48.8 $\pm$ 2.3	355 $\pm$ 20
2	13/03/2018	5.15 $\pm$ 0.03	62.7 $\pm$ 0.03	57.7 $\pm$ 2.7	310 $\pm$ 39
3	30/04/2018	6.18 $\pm$ 0.02	69.8 $\pm$ 0.03	0.80 $\pm$ 0.1	276 $\pm$ 18
4	04/02/2019	5.22 $\pm$ 0.17	69.9 $\pm$ 0.04	5.4 $\pm$ 0.3	287 $\pm$ 20
5	30/08/2019	4.86 $\pm$ 0.29	64.5 $\pm$ 0.29	26.0 $\pm$ 1.8	308 $\pm$ 25
6	28/10/2019	5.11 $\pm$ 0.03	66.1 $\pm$ 1.19	37.4 $\pm$ 1.9	359 $\pm$ 9

6.4.3.3 Use of modified Gompertz model

The modified Gompertz model was used extensively in this thesis together with the BMP results to predict the ultimate methane yield, maximum methane production rate and the lag period. In this study, it was demonstrated that the model had an excellent fit with the SMY curves of CAM crops, particularly those with long lag phases. However, the application of the modified Gompertz model is quite limited to the BMP study or batch fermentation as it has been used to simulate the biogas production rate, assuming that it is a function of microbial growth rate in the anaerobic digester. In the BMP study, microorganisms are fed once at the beginning the substrate and other components that would help promote and sustain their growth. Microbes then progress through four stages of growth: lag, exponential, stationary, and death. The information that can be obtained from the model is the length of the lag period, the rate of exponential growth (methane production rate), the stationary period (ultimate methane yield potential). The BMP experiment is terminated when the curve reaches a plateau. The most useful information from the model is the ultimate BMP predicted from the experimental yields. This value represents the maximum possible SMY that can be obtained under non-limiting conditions. The value is then used as a baseline against the SMY obtained from the

semi-continuous/continuous trials. The length of the lag period could imply that the substrate requires pretreatment/processing, the inoculum needs a longer acclimatisation period, or the experimental setup is not in an optimal condition for microbial growth and activity. However, the lag period information was not useful for semi-continuous or continuous digestion studies because the microbial consortium exhibits a totally different behaviour under the different settings and operational conditions. Similarly, the methane production rate from a dynamic microbial community in the continuous digester is different from the maximum production rate predicted from the BMP results under batch operation. Therefore, the best application of the modified Gompertz model is to predict the ultimate methane yield of a substrate while the lag period and maximum production rate have very limited implications.

6.4.4 Semi-continuous digestion

The digester temperature was maintained by an external surrounding heating coil, which has water circulating through from the temperature-controlled water bath. The coils for each digester were connected in series and this led to a certain degree of temperature difference among the four digesters. The temperature of the digester with the shortest distance to the water bath would be approximately the same as the set-point temperature, however, a temperature gradient was detected between the four digesters as some of the heat in water was dissipated through the coil passing each digester. This temperature difference of 0.5–1.0°C may have affected the substrate degradation rate of the duplicate digesters.

During the study of semi-continuous digestion of *Opuntia ficus-indica* (**Chapter 4**), motor failure and electrical supply shortage caused a temporary shock to the digestion system. The digester stress resulted from a rise in total volatile fatty acid

(TVFA) concentration and a decrease in pH. Subsequent acid accumulation led to a digester failure during Phase III of digestion and feeding was suspended in response to the situation.

6.5 Recommendations and future works

- This study mainly focused on the two CAM species, *Euphorbia tirucalli* and *Opuntia ficus-indica*. Despite these two plants producing good biogas yields, their exploitation in countries where they are not indigenous could create problems. The introduction of alien species could attract negative perceptions from the public and face restrictions from national and local governments (Novoa et al., 2019; Shackleton et al., 2011). Future studies of new potential native CAM species should be encouraged as there are over 16,000 CAM species that could become successful energy crops (Winter and Smith, 1996a).
- Various CAM plants have different characteristics and physico-chemical properties. Storage and processing techniques such as freezing, grinding or drying would affect the properties of the biomass and therefore their influences on the biogas/methane yields of the plants should also be tested.
- There was limited information on biomass productivity and cultivation of *E. tirucalli* as it is not a widely domesticated crop. A life cycle assessment (LCA) of biogas production through anaerobic digestion of *Euphorbia tirucalli* should be undertaken in order to evaluate energy balance and environmental impacts associated with the process of using this species as an AD substrate.
- Investigations on anaerobic digestion of *E. tirucalli* should be repeated using an inoculum with superior methanogenetic activity ($>352 \text{ L kgVS}^{-1}$) to ensure the

BMP of this plant species was not restricted by the poor performance of the inoculum.

- The reason for low methane yields of *Euphorbia* spp. (*E. tirucalli* and *E. virosa*) may not only stem from using a poor inoculum. *Euphorbia* spp. were more fibrous and contained higher lignin content than *O. ficus-indica*. Pre-treatment of *E. tirucalli* could be investigated to determine whether this could improve the methane yields of these plants. Drying and grinding of *E. tirucalli* are recommended in order to obtain a better homogeneous sample, which ensures good replicability and comparability. The drying temperature should be lower than 80°C to avoid browning and loss of volatile organic compounds.
- Microbial community analysis of each batch of digestate obtained from the local commercial food waste digesters could have been performed to investigate the temporal variability in composition and dynamics of the inoculum.
- Data collection from the semi-continuous experiment should be improved. Analysis of total ammonia nitrogen (TAN) and alkalinity would provide more information on buffering capacity and digester stability. These data including VFA concentrations and pH values will give better and more thorough guidance when monitoring the digesters.
- A longer operational period of semi-continuous digesters should be investigated. The current study was limited to approximately 90 days largely due to time constraints at the end of the laboratory work and the limited supply of substrate. Future work on semi-continuous digestion of CAM plants such as *Opuntia ficus-indica* could be done outside the UK, particularly in southern Europe or northern African countries where the progress of the experiment will not be hindered by the unavailability of the substrate

- The processing techniques for the extraction of *Opuntia* liquid could be further explored. Centrifugation was a better technique compared to sterile microfiltration in terms of processing time and cost. Other solid-liquid separation techniques, which are more cost-effective and less energy-intensive at a larger scale compared to centrifugation should be tested.
- *Opuntia* liquid extract (OLE) needs to be sterilised before it was used as a carbon source in a pure-culture PHB fermentation. Autoclaving was the sterilisation technique used in this study as it was the simplest, fastest and most cost-effective compared to sterile microfiltration. However, vitamins, proteins and other organic compounds could decompose or become denatured from exposure to high heat. Metabolite analysis of OLE before and after autoclaving should be performed to determine the compounds, which could be affected by high heat. Alternative sterilisation techniques such as UV and Gamma irradiation should also be tested.
- Further studies on batch fermentation at a larger scale (5 L) and a fed-batch fermentation of *Cupriavidus necator* H16 for PHB production using OLE should be undertaken. Unfortunately, these experiments were not carried out due to a limited supply of *Opuntia* cladodes for liquid extraction and the site closure following the nationwide COVID-19 pandemic lockdown. Other bacteria strains, which are capable of assimilating a greater variety of substrates than the H16 strain such as DSM 545 should also be tested for PHB production from the OLE.

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Appendix A

Biomass characterisation

The analysis of biomass composition such as ADF, NDF, ADL, Crude protein and ammonium nitrogen was performed by an external laboratory. The results from the analysis are presented in **Figs. A1–A5**.



Certificate of Analysis

University of Oxford, Engineering Science Department
Abiodun Jegede
University of Oxford, Engineering §

Client Reference 2B
Details Euphorbia
Date Received 11-Oct-2019
Date Reported 23-Oct-2019
Lab Reference No 2048658
Order Number
Certificate No 2048658_2B_1.pdf
Product Miscellaneous - Feed Miscellaneous - Feed
Sampled Date
Sample Source

Method No		Test	Units	Result	Completed On
S1012		Neutral Detergent Fibre	%	27.9	18-Oct-2019
S1112	*	Acid Detergent Lignin	%	19.40	22-Oct-2019
S1113	*	Crude Protein (N X 6.25) (Kjeldahl)	-	11.2	16-Oct-2019
S1129	*	Acid Detergent Fibre	%	25.99	16-Oct-2019

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Alan Ash

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Technical Support Manager

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Fig. A1 Crude protein and fibre analysis of *Euphorbia tirucalli*. The results are also presented in **Table 4.4, Chapter 4**.



Certificate of Analysis

University of Oxford, Engineering Science Department
Abiodun Jegede
University of Oxford, Engineering Science Department

Client Reference 4B
Details Manure
Date Received 11-Oct-2019
Date Reported 23-Oct-2019
Lab Reference No 2048660
Order Number
Certificate No 2048660_4B_1.pdf
Product Miscellaneous Miscellaneous
Sampled Date
Sample Source

Method No	Test	Units	Result	Completed On
S1012 *	Neutral Detergent Fibre	%	35.5	18-Oct-2019
S1112 *	Acid Detergent Lignin	%	10.71	22-Oct-2019
S1113 *	Crude Protein (N X 6.25) (Kjeldahl)	-	27.8	16-Oct-2019
S1129 *	Acid Detergent Fibre	%	19.77	16-Oct-2019
S1162 *	Ammoniacal Nitrogen (mg/kg) AR	mg/kg	826	16-Oct-2019

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Fig. A2 Proximate analysis of chicken manure. The results are also presented in **Table 4.4**, **Chapter 4**.



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University of Oxford, Engineering Science Department

Client Reference 1B
Details Opuntia
Date Received 11-Oct-2019
Date Reported 23-Oct-2019
Lab Reference No 2048657
Order Number
Certificate No 2048657_1B_1.pdf
Product Miscellaneous - Feed Miscellaneous - Feed
Sampled Date
Sample Source

Method No		Test	Units	Result	Completed On
S1012		Neutral Detergent Fibre	%	38.4	18-Oct-2019
S1112	*	Acid Detergent Lignin	%	9.15	22-Oct-2019
S1129	*	Acid Detergent Fibre	%	23.76	16-Oct-2019

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Fig. A3 Fibre analysis of *Opuntia ficus-indica*. The results are also presented in **Table 4.9**, **Chapter 4**.



Certificate of Analysis

University of Oxford, Engineering Science Department
Abiodun Jegede
University of Oxford, Engineering Science Department

Client Reference 3B
Details Opuntia
Date Received 11-Oct-2019
Date Reported 23-Oct-2019
Lab Reference No 2048659
Order Number
Certificate No 2048659_3B_1.pdf
Product Miscellaneous - Feed Miscellaneous - Feed
Sampled Date
Sample Source

Method No		Test	Units	Result	Completed On
S1012		Neutral Detergent Fibre	%	22.4	18-Oct-2019
S1112	*	Acid Detergent Lignin	%	5.61	22-Oct-2019
S1129	*	Acid Detergent Fibre	%	10.89	16-Oct-2019

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Fig. A4 Fibre analysis of *Opuntia ficus-indica*. The results are also presented in **Table 4.14**, **Chapter 4**.



Certificate of Analysis

University of Oxford, Engineering Science Department
Abiodun Jegede

Client Reference 4
Details 4
Date Received 23-Dec-2019
Date Reported 7-Jan-2020
Lab Reference No 2098428
Order Number
Certificate No 2098428_4_1.pdf
Product Fats, Molasses & Liquids Liquid Miscellaneous
Sampled Date
Sample Source

Method No	Test	Units	Result	Completed On
S1113 *	Crude Protein (N X 6.25) (Kjeldahl)	-	0.4	30-Dec-2019
*	# Carbon	%	1.77	7-Jan-2020

Unless otherwise stated results are expressed on an 'as received' basis.

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Fig. A5 Crude protein analysis of *Opuntia* liquid extract (OLE). The results are also presented in **Table 5.1, Chapter 5.**

Appendix B

Energy balance calculations

This appendix presents the example calculation of energy balance analysis shown in Table 4.7. Key constants and factors used for the calculations are presented in Table B1.

Table B1 Parameters, constants and factors for energy balance assessment

Parameter	Unit	Symbol	Value
TS of <i>Euphorbia tirucalli</i>	%	%TS	15.37
VS of <i>Euphorbia tirucalli</i>	%	%VS	84.16
Molecular weight of methane	kg kmol ⁻¹	-	16
Gas constant	m ³ _{atm} K ⁻¹ kmol ⁻¹	-	0.082
Heat capacity of material	kJ kg ⁻¹ K ⁻¹	C _p	4.12
Reactor temperature	K	T _D	311 (mesophilic) 328 (thermophilic)
Feed temperature at ambient	K	T _A	298
Low heating value of methane	kJ m ⁻³	LHV	35,800
High heating value of methane	kJ kg ⁻¹	HHV	55,600
Electricity consumption by AD system	kWh kg ⁻¹	-	0.00788

The calculation of the energy balance was based on the specific methane yield of *E. tirucalli* at pH 7 and 38°C (206 L kgVS⁻¹ or 0.206 m³ kgVS⁻¹). The unit [kWh-e] is defined as kilowatt-hour equivalent, representing the unit [kJh s⁻¹].

- Energy potential of methane

$$\begin{aligned}
 \text{Energy potential of methane} &= \frac{\text{SMY}(\text{TS}) \times \text{LHV}}{3,600} \\
 &= \frac{0.206 \left[\frac{\text{m}^3}{\text{kgVS}} \right] \times 0.8416 \left[\frac{\text{kgVS}}{\text{kgTS}} \right] \times 35,800 \left[\frac{\text{kJ}}{\text{m}^3} \right]}{3,600 \left[\frac{\text{s}}{\text{h}} \right]} \\
 &= 0.172 \left[\frac{\text{kJ h}}{\text{kgTS s}} \right] \text{ or } \left[\frac{\text{kWhe}}{\text{kgTS}} \right]
 \end{aligned}$$

- Methane production (M)

$$\begin{aligned}
 M &= \frac{\text{SMY}(\text{TS}) \times 16}{T \times 0.082} \\
 &= \frac{0.206 \left[\frac{\text{m}^3}{\text{kgVS}} \right] \times 0.8416 \left[\frac{\text{kgVS}}{\text{kgTS}} \right] \times 16 \left[\frac{\text{kg}}{\text{kmol}} \right]}{293 [\text{K}] \times 0.082 \left[\frac{\text{m}^3}{\text{K kmol}} \right]} \\
 &= 0.115 \left[\frac{\text{kg}}{\text{kgTS}} \right]
 \end{aligned}$$

- Heat output

$$\begin{aligned}\text{Heat output} &= \frac{M \times \text{HHV} \times \eta_{\text{CHP}}}{3,600} \\ &= \frac{0.115 \left[\frac{\text{kg}}{\text{kgTS}} \right] \times 55,600 \left[\frac{\text{kJ}}{\text{kg}} \right] \times 0.39}{3,600 \left[\frac{\text{s}}{\text{h}} \right]} \\ &= 0.695 \left[\frac{\text{kJ h}}{\text{kgTS s}} \right] \text{ or } \left[\frac{\text{kWhe}}{\text{kgTS}} \right]\end{aligned}$$

- Electricity output

$$\begin{aligned}\text{Electricity output} &= \frac{M \times \text{HHV} \times \eta_{\text{G}}}{3,600} \\ &= \frac{0.115 \left[\frac{\text{kg}}{\text{kgTS}} \right] \times 55,600 \left[\frac{\text{kJ}}{\text{kg}} \right] \times 0.41}{3,600 \left[\frac{\text{s}}{\text{h}} \right]} \\ &= 0.731 \left[\frac{\text{kJ h}}{\text{kgTS s}} \right] \text{ or } \left[\frac{\text{kWhe}}{\text{kgTS}} \right]\end{aligned}$$

- Heat input

$$\begin{aligned}\text{Heat input} &= \frac{\frac{m}{\% \text{TS}_D} \times C_p \times (T_D - T_A) \times 1.3}{3,600} \\ &= \frac{\frac{1}{0.1537} \left[\frac{\text{kg}}{\text{kgTS}} \right] \times 4.12 \left[\frac{\text{kJ}}{\text{kg K}} \right] \times (311 - 293) [\text{K}] \times 1.3}{3,600 \left[\frac{\text{s}}{\text{h}} \right]} \\ &= 0.174 \left[\frac{\text{kJ h}}{\text{kgTS s}} \right] \text{ or } \left[\frac{\text{kWhe}}{\text{kgTS}} \right]\end{aligned}$$

- Electricity input

$$\begin{aligned}\text{Electricity input} &= \frac{m}{\% \text{TS}_D} \times 0.00788 \\ &= \frac{1}{0.1537} \left[\frac{\text{kg}}{\text{kgTS}} \right] \times 0.00788 \\ &= 0.051 \left[\frac{\text{kJ h}}{\text{kgTS s}} \right] \text{ or } \left[\frac{\text{kWhe}}{\text{kgTS}} \right]\end{aligned}$$

- Electricity generation efficiency

$$\begin{aligned}\text{Energy generation efficiency (\%)} &= \frac{\text{Net energy output}}{\text{Energy potential of methane}} \times 100 \\ &= \frac{(0.695 - 0.174) + (0.731 - 0.051)}{0.172} \times 100 \\ &= 69.7\%\end{aligned}$$