Anaerobic co-digestion of fish sludge originating from a recirculating aquaculture system

by

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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DEDICATION

I dedicate this work to my mother, Phophi Grace Netshivhumbe for her guidance, courage, and support which gave me the strength to grow in various aspects of my career life.

ABSTRACT

Recirculation aquaculture systems (RAS) are considered as sustainable and environmentally friendly aquaculture systems capable of meeting the growing demand of seafood for human consumption. However, RAS produce large quantities of waste sludge from uneaten feed and fish faecal matter, which need to be removed from the recirculating water and treated to prevent adverse environmental impacts. Anaerobic digestion (AD) has been considered as an alternative method to stabilize the amount of organic waste in the environment before its disposal, with the simultaneous production of bio-methane that can serve as a source of energy within RAS. However, there are some drawbacks in the mono-digestion process of fish sludge (FS) such as process inhibition, unbalanced nutrient contents, and low methane yields. The biomethane production from FS, food waste (FW), and fruit & vegetable waste (FVW) was optimized during anaerobic co-digestion using a mixture design. The synergistic and antagonistic interaction effects of the three substrates on specific methane yield, volatile solids reduction, and process stability were evaluated in both batch and semi-continuous mode. A mixture design was used to determine the best mixture compositions of FS, FW, and FVW for specific methane yield and volatile solids removal during the anaerobic co-digestion process based on biomethane potential (BMP) measurements. The results showed that the optimum mixture proportions of FS, FW, and FVW were 63 %, 18 %, and 19 %, respectively. The results showed the maximum methane production and VS removal of 401 mL CH_4/gVS and 64%, respectively under the optimum mixture. Anaerobic co-digestion of FS with FW and FVW enhanced the methane yields by 8 folds compared with mono-digestion of FS.

The optimum mixture proportions obtained from batch BMP tests were further evaluated in 50 L batch and 30 L semi-continuous pilot-scale digesters to evaluate the effect of organic loading rate (OLR) on biogas production and process performance stability. The methane yield obtained from the batch pilot-scale digester was 272 NmLCH_4 /gVS. This was 71 % of the methane yield obtained from the BMP test under the same optimum mixture condition. The batch digester showed no substantial inhibition of the system due to its strong buffering capacity. In semi-continuous mode, the digester was conducted under different OLRs of 1, 2, and 3 $gVSL^{-1}d^{-1}$ to investigate the impacts of OLR on biogas and methane production, and process performance stability of the anaerobic co-digestion of FS, FV, and fruit and FVW. The highest total biogas and methane production of 388 L/gVS and 67 L/gVS, with a methane content of 66.8% obtained at an OLR of 2 $gVSL^{-1}d^{-1}$ compared to OLRs of 1 and 3 $gVSL^{-1}d^{-1}$. The digester showed instabilities or failure at an OLR of 3 $gVSL^{-1}d^{-1}$ due to acid crash and accumulation of VFA of

11g/L. An OLR of 1- 2 $gVSL^{-1}d^{-1}$ is recommended for anaerobic co-digestion of FS, FW, and FVW in semi-continuous digesters because of less inhibitor indicators observed.

Keywords: biomethane production, fish sludge, anaerobic co-digestion, OLR, Mixture design

OPSOMMING

Hersirkuleringsakwakultuursisteme (RAS) word beskou as volhoubare en omgewingsvriendelike akwakultuursisteme in staat om aan die groeiende aanvraag van seekos vir menslike gebruik te voldoen. RAS produseer egter groot hoeveelhede afvalslyk van ongeëete voer en visfekalieë, wat verwyder moet word uit die hersirkulerende water en behandel moet word om nadelige omgewingsimpak te verhoed. Anaerobiese vertering (AD) is oorweeg as ’n alternatiewe metode om die hoeveelheid organiese afval in die omgewing te stabiliseer voor dit verwyder word, met die gelyktydige produksie van biometaan wat as ’n bron van energie binne RAS kan dien. Daar is egter sommige nadele in die monoverteringsproses van visslyk (FS) soos prosesinhibisie, ongebalanseerde nutriëntinhoud, en lae metaanopbrengs. Die biometaanproduksie uit FS, voedselafval (FW), en vrugte-en-groente-afval (FVW) is geoptimaliseer gedurende anaerobiese kovertering deur ’n mengselontwerp te gebruik. Die sinergistiese en antagonistiese interaksie-effek van die drie substrate op spesifieke metaanopbrengs, vlugtige vastestofreduksie, en prosesstabiliteit is geëvalueer in beide lot- en semi-aaneenlopende modes. ’n Mengselontwerp is gebruik om die beste mengselkomposisies van FS, FW, en FVW vir spesifieke metaanopbrengs en vlugtige vastestofverwydering gedurende die anaerobiese koverteringproses gebaseer op biometaanpotensiaal (BMP) mates, te bepaal. Die resultate het gewys dat die optimale mengselproporsies van FS, FW, en FVW 63%, 18% en 19% onderskeidelik was. Die resultate het getoon dat maksimum metaanproduksie en VS-verwydering van 401 mL CH_4/gVS en 64%, onderskeidelik onder die optimale mengsel was. Anaerobiese kovertering van FS met FW en FVW het die metaanopbrengste 8 keer verhoog in vergelyking met monovertering van FS.

Die optimale mengselproporsies verkry van lot BMP-toetse is verder geëvalueer in 50 L-lot en 30 L-semi-aaneenlopende loodskaalverteerders om die effek van organiese ladingstempo (OLR) op biogasproduksie en prosesdoeltreffendheidstabiliteit te evalueer. Die metaanopbrengs verkry uit die lotloodskaalverteerder was 272 NmLCH/gVS. Dit was 71% van die metaanopbrengs verkry uit die BMP-toets onder dieselfde optimale mengselkondisies. Die lotverteerder het geen substansiële inhibisie van die sisteem getoon nie as gevolg van sy sterk bufferkapasiteit. In semi-aaneenlopende mode, is die verteerder onder verskillende OLRe van 1, 2, en 3 $gVSL^{-1}d^{-1}$ uitgevoer om die impak van OLR op biogas en metaanproduksie, en prosesdoeltreffendheidstabiliteit van die anaerobiese kovertering van FS, FV, en vrugte en FVW, te ondersoek. Die hoogste totaal biogas en metaanproduksie was 388 L/gVS en 67 L/gVS, met ’n metaaninhoud van 66.8% verkry by ’n OLR van 2 $gVSL^{-1}d^{-1}$ in vergelyking met OLRe van 1 en 3 $gVSL^{-1}d^{-1}$. Die verteerder het onstabiliteite of mislukking getoon by ’n OLR van 3 $gVSL^{-1}d^{-1}$ as gevolg van suurineenstorting en akkumulasie van VFA van 11 g/L. ’n OLR van 1-

2 $gVSL^{-1}d^{-1}$ word voorgestel vir anaerobiese kovertering van FS, FW en FVW in semi-aaneenlopende verteerders as gevolg van minder inhiberende indikators waargeneem.

Sleutelwoorde: biometaanproduksie, visslyk, anaerobiese kovertering, OLR, mengselontwerp

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LIST OF ABBREVIATIONS

AD	Anaerobic digestion
AMPTS II	Automatic Methane Potential Test System II
AnMBR	Anaerobic Membrane Bioreactor
ASBR	Anaerobic Sequential Batch Reactor
BMP	Bio-methane potential
BOD	Biochemical oxygen demand
C/N	Carbon to Nitrogen ratio
CDI	Co-digestion index
COD	Chemical Oxygen Demand
CPI	Co-digestion Performance Index
CSTR	Continuous Stirred Tank Reactor
FS	Fish Sludge
FVW	Fruit and Vegerable waste
FW	Food Waste
GHG	Greenhouse gas emissions
HRT	Hydraulic retention time
ISR	Inoculum to substrate ratio
LCFA	Long Chain Fatty Acids
MC	Moisture content
OLR	Organic Loading Rate
PVSR	Percentage of Volatile Solids Removal
RAS	Recirculating Aquaculture Systems
RSM	Response Surface Methodology
SBY	Specific Biogas Yield
SCMD	Simplex Centroid Mixture Design
SMY	Specific Methane Yield
SRT	Solids Retention Time

STP	Standard conditions of Temperature and Pressure
TBMP	Theoretical Biomethane Potential
TKN	Total Kjehdahl nitrogen
TN	Total Nitrogen
TOC	Total organic carbon
TP	Total Phosphorus
TS	Total Solids
TSS	Total Suspended Solids
tVFA	Total Volalite Fatty Acids
UASB	Upflow Anaerobic Sludge Blanket
VFA	Volatile Fatty Acids
VS	Volatile Solids
VS%	Volatile solids concentration
VSS	Volatile Suspended Solids
WSP	Water Stabilization pound

1 CHAPTER 1: INTRODUCTION

1.1 Background and Motivation

In 2016, global aquaculture production reached about 171 million tons out of which 80 million tons (43%) were used for human consumption and 53% of non-food uses (FAO, 2018). Globally, fisheries production has increased due to the need to meet and satisfy the growing demand of seafood consumption as the world population increases (FAO, 2018). However, due to the fast-growing of aquaculture production in the world, the large amounts of polluting waste from recirculation aquaculture system (RAS) discharge are produced; this waste contains a high organic matter concentration, which is problematic due to environmental impacts such as eutrophication associated with their disposal (Rijn, J. Van,1996; Timmons *et al.*,2002; Quinn *et al.*, 2016; Zhang *et al.*, 2016).

Fish sludge produced from an aquaculture system is primarily composed of suspended matter originating from fish faeces, and a small amount of uneaten feed, which contains organic content such as nitrogen and phosphorus (Gebauer, 2004; Pillay, 2004; Mirzoyan *et al.*, 2010; Quinn *et al.*, 2016). Fish sludge is usually expelled from the recirculating system into receiving aquatic bodies such as a local sewer and a decentralised treatment (stabilisation pond) (Timmons and Ebeling, 2007). The accumulation of fish faeces often increases the ammonium nitrogen and phosphate-phosphorus concentrations in the surrounding water (Pillay, 2004). Furthermore, the high nutrients in organic wastes may cause algal bloom, oxygen depletion of the aquatic organism, water pollution, and transmission of diseases (Pillay, 2004; X. Zhang *et al*., 2013).

The sludge generated from the aquaculture system requires proper management strategies to prevent environmental impacts such as water pollution and eutrophication. Currently, the methods used for fish sludge disposal are primarily by flocculation/coagulation processes to reduce waste sludge volume, with subsequent composting for land dispersal (Mirzoyan *et al.*, 2008). However, the disposal of fish waste sludge in landfill or by composting have adverse impacts such as emission of unpleasant odour, and formation of leachates with high polluting potential due to high organic matter content (Luo *et al.*, 2013; Serrano, Angel and Lopez, 2014). Furthermore, the high salinity of the sludge, especially from saline aquaculture, limits fish sludge management options for land application as fertilizer and landfilling, as discharge into these environments can lead to

salination of soils and water as a result of leaching (Mirzoyan and Gross, 2013; Zhang *et al.*, 2016). The disposal of aquaculture sludge into aquatic bodies is generally restricted due to the high organic load and salinity, which could hinder the treatment process (Mirzoyan *et al.*, 2010). Anaerobic digestion (AD) has been suggested as an alternative approach for the treatment of organic waste such as fish wastes sludge, in order to recover renewable energy in the form of biomethane, due to its environmental benefits (Mirzoyan *et al.*, 2008; Quinn *et al.*, 2016; Zhang *et al.*, 2016). Anaerobic digestion is a biological process in which organic matter is decomposed by different microbial groups in the absence of oxygen to produce biogas and digestate (bio-fertilizer) (Sol and Lansing, 2013; Hadiyarto *et al.*, 2015). Anaerobic digestion over the years has been applied to stabilise and reduce wastewater, although only recently for aquaculture waste sludge, due to its simplicity of operation with the capability to handle high loading rates, reduction in sludge generation, and the production of renewable energy and profit (Appels *et al.*, 2008). AD process have the potential to reduce the volume of the organic contents for the production of renewable energy (Eiroa *et al.*, 2012). The biomethane generated through the anaerobic digestion process can be utilized as a source of heat or electricity, which can cover some of the energy demands of the RAS (Gebauer, 2004). Furthermore, renewable energy could also reduce the utilization of fossil fuels for energy resources, thereby reducing environmental impacts, including carbon dioxide emission and global warming (Quinn *et al.*, 2016).

Fish sludge from an aquaculture system is an attractive feedstock for the production of biogas through the anaerobic digestion process due to its readily biodegradable organic matter content, high nitrogen source, and high moisture content (Mirzoyan *et al.*, 2008; Kafle and Kim, 2012; Quinn *et al.*, 2016; Zhang *et al.*, 2016). However, there are some drawbacks in the mono-digestion process of fish sludge characterized by the unbalanced nutrients content, particularly the low carbon to nitrogen ratio due to high contents of nitrogen resulting in low methane yields. Also, the high amounts of free ammonia produced by the degradation of protein rich substrate can lead to process failure due to microbial inhibition during the anaerobic digestion (Mirzoyan *et al.*, 2008; Zhang *et al.*, 2013).

Co-digestion is the anaerobic digestion of two or more wastes simultaneously in one bioreactor to balance the nutrients, resulting in an enhanced methane yield and stable performance of the process (Li *et al.*, 2013; Mata-alvarez *et al.*, 2014; Kashi *et al.*, 2017). Therefore, in order to overcome the limitation of nutrient imbalance, possible ammonia accumulation inhibition, and process instability with the mono-digestion of fish sludge, it is crucial to co-feed the fish sludge

with carbon-rich co-substrates such as food waste, fruit & vegetable waste, or agricultural waste residues that will balance the carbon to nitrogen ratios, limit the accumulation of ammonia inhibition and thereby increase the degradation efficiency and methane production (Masih-das and Tao, 2018; Vats *et al.*, 2019). Several studies have been conducted in order to evaluate the effect of two or three substrates during the anaerobic co-digestion on methane or biogas production (Wang *et al.*, 2013; Pagés Díaz, 2014; Rahman *et al.*, 2019).

A few studies have been done on methane production of fish sludge from RAS through anaerobic digestion (Gebauer, 2004; Quinn *et al.*, 2016; Zhang *et al.*, 2016). Hence it has been suggested that the substrate mixture ratios of fish sludge with other co-substrates must be optimized in order to improve the digestibility, avoid inhibition and increase biogas yields (Mirzoyan and Gross, 2013; Zhang *et al.*, 2016). Therefore, a substrate such as fish sludge, which has high contents of nitrogen and low total solids, can be co-digested with other co-substrates characterized by high concentrations of carbon such as fruit and vegetable waste (Pavi *et al.*, 2017), and food waste. Moreover, there is little knowledge of understanding the co-digestion of fish sludge together with other substrates in order to optimize methane production. This study aims to investigate the biomethane production potential of fish sludge from RAS and the effects of co-digestion of the different co-substrate mixture ratios (i.e., food waste, fruit & vegetable waste) in both laboratory-scale and pilot-scale digesters to obtain optimal methane yield through an anaerobic co-digestion process.

1.2 Scope of the study

This study primarily focuses on the production of methane from fish sludge originating from a recirculating aquaculture system. The study aimed to evaluate the effects of three waste mixtures on biogas yield and methane production in an anaerobic co-digestion process. Therefore, experiments were conducted using the RSM simplex centroid mixture design to study the effects of co-substrate proportions. BMPs tests were performed to determine the optimal mixing ratio of anaerobic digestion co-substrates: This study will demonstrate if the co-digestion of fish waste sludge with food waste and fruit & vegetable waste as co-substrates produces better methane yield than individual digestion. The optimum substrate ratios were further verified in the batch pilot-scale digester (50 L) and semi-continuous digester (30 L) to evaluate the biogas and methane production, as well as the process's stability under different organic loading rates.

OUTLINE OF THE THESIS

This thesis is organized as follows.

Chapter 1: Presents the background and study rationale

Chapter 2: Literature Review

Chapter 3: Research Aim and Objectives of the study

Chapter 4: Describes the experimental set-up of BMP tests, batch and semi-continuous tests, and the analytical methods using the study.

Chapter 5: Discuss the results of the characteristics of the substrates, mono-digestion, and co-digestion BMP assays, optimisation of the methane yields, validation results, and results of the up-scaled batch and semi-continuous digesters, together with the digestate.

Chapter 6: This chapter outlined the conclusions and recommendations for future work.

Chapter 7: References

2 CHAPTER 2: LITERATURE REVIEW

2.1 Recirculating aquaculture systems

Recirculating aquaculture system (RAS) is defined as the farming of aquatic organisms such as finfish, shellfish, mulluscs, and aquatic animals/plants in a sustainable aquatic environment, with some form of monitoring and continuously controlling system to maintain the feeding, water, temperature, oxygen, waste removal and protection from predators in order to enhance production (FAO, 2018; Ahmad *et al.*, 2021). Aquaculture systems are relatively closed systems that require little make-up (fresh) water input into the fish tank, compared to open culture systems such as a pond or cage-based aquaculture (Zhang *et al.*, 2013; Quinn *et al.*, 2016; Zhang *et al.*, 2016). Recirculating aquaculture systems have been developed to reduce the environmental impacts of open systems such as pond culture, raceway culture, and pet aquaculture (Pillay, 2004). RAS provides many advantages compared to a raceway culture or, traditional aquaculture technologies, such as reduced water usage, reduction of pathogenic bacteria, and disease outbreak, high control of operational parameters to reduce pollution, and reduced nutrients emission (Pillay, 2004; Zhang *et al.*, 2016), while supporting the sustainable development of the fast-growing seafood economy (Monsees *et al.*, 2017).

Water use with RAS is reduced through the efficient filtration of the faeces, unconsumed feed, and waste compounds to maintain an appropriate level of accumulating sludge (X. Zha*ng et al*., 2013; Zhang *et al*., 2016). In addition, water quality is maintained at an adequate level via the biological treatment of wastewater (bio-filtration) to neutralize harmful metabolic waste, and the application of UV sterilization, ozonation, and oxygen injection (Rijn, 1996). Fish sludge that accumulates in the system is removed through mechanical filtration (e.g. drum filters) that are commonly used as solids separation units in RAS; these wastes contain soluble metabolites such as inorganic or organic nitrogenous compounds, minerals, and sediment solid wastes (Pillay, 2004). Nitrogen removal within the system is essential since the fish excrete ammonia from their gills and ammonia is toxic at high concentrations. The removal of the organic matter from the effluents of the aquaculture system is often required with strict regulations to reduce the effect of the discharge on the environment (Summerfelt *et al.*, 1999). Uncontrolled discharge or disposal of the waste sludge will negatively impact the environment, health, and society, hence the need to mitigate risks (Mirzoyan *et al.*, 2008).

2.2 Fish sludge production in aquaculture

Aquaculture sludge is mainly formed by faeces, fish excretion, and a small amount of uneaten feed (Mirzoyan *et al.*, 2010), including, total suspended solids deterioration during bio-filtration (Chen *et al.*, 1997). It has been reported that between 36-40% of the fish feeding pellets introduced into the fish tanks accumulate in the form of sludge within the aquaculture system (Rijn, 1996; Mirzoyan *et al.*, 2008; Luo *et al.*, 2013) while solid wastes produced in the aquaculture system originate from the excretion of faeces by the cultured fish (Chen *et al.*, 1997). A study by Mirzoyan et al. 2008 reported that sludge produced from an aquaculture system is enriched with organic matter, micronutrients, phosphorus, and the metabolic by-products of fish such as ammonia-nitrogen.

Phosphorus, nitrogen, and calcium are the main dissolved substances generated in the aquaculture system, and can cause oxygen depletion and eutrophication when discharged (Zhang *et al.*, 2013). The accumulation of fish solid faeces may increase ammonium nitrogen and phosphate-phosphorus concentrations in the surrounding water, which can cause water pollution problems not only affecting the environment but also disrupt the production of fish in the aquaculture system by causing depletion of dissolved oxygen (Pillay, 2004). This waste must be treated before its disposal to prevent water pollution and allow proper fish growth (Mirzoyan *et al.*, 2008).

Fish sludge characteristics are important for the design and operation of sludge treatment and disposal methods (Chen *et al.*, 1997). The production and composition of sludge from RAS mainly depends on the feed used, fish species, feeding rate, and the water treatment process (Van Rijn, 2013; Chen *et al.*, 1997). Typically, aquaculture sludge is characterized by its low carbon to nitrogen (C/N) ratio, low total solids (1.5-3%), and biochemical oxygen demand (BOD) compared to other organic waste such as sewage sludge, animal manure, or wastewater from industrial processes (Gebauer, 2004; Mirzoyan *et al.*, 2008). A study by Opurum et al. (2015) reported that fish sludge has a low C/N ratio of 4:1, whereas a C/N ratio of 9:1 was reported by (Mirzoyan *et* al., 2008). Fish sludge is also characterized by dissolved and particulate organic matter, total suspended solids (TSS), and high nutrients such as phosphorus, nitrogen, and other organic or inorganic compounds emanating from the fish droppings (Piedrahita, 2003). Fish sludge often has a high digestion efficiency removal (92-98 %) after anaerobic digestion process, resulting from a typical composition of low total solids of 1.5-3% and a volatile organic fraction ranging from 78.6-86.9% (Gebauer and Eikebrokk, 2006; Mirzoyan, Tal and Gross, 2010).

2.3 Fish aquaculture production in South Africa

According to the Department of Agriculture, Forestry and Fisheries (DAFF), it has been reported that South Africa aquaculture industry has a high growth potential of commercial aquaculture production due to increasing demand for fish production, human consumption, and South Africa's abundance of freshwater and marine resources (DAFF, 2019). However, South Africa aquaculture entrepreneurs are still facing challenges in the development and expansion of sustainable fish aquaculture systems/industries (FAO, 2018). This is due to the lack of necessary resources such as farming land, infrastructure, financial support. In South Africa, Western Cape and Eastern Cape provinces are the two major fish/aquaculture farming producers of freshwater aquaculture species such as trout, tilapia, and catfish (Madibana *et al.*, 2020; Moyo and Rapatsa, 2021). Even though the aquaculture production industry in SA is still underdeveloped, the current running industrial aquaculture system/ small fish farmers in South Africa are facing some problems such as handling the aquaculture effluent sludge, poor water quality, and high cost of production (Moonsamy *et al.*, 2020). However, there is still lacking of data about waste sludge generated within the aquaculture per annual/month/day in general in South Africa. The disposal/treatment of the wastewater is often expensive.

2.4 Aquaculture sludge disposal and treatment

The fish sludge generated from the aquaculture system needs proper treatment before disposal into the environment. Therefore, different disposal options have been reported for sludge treatment and stabilization depending on the sludge characteristics (Chen *et al.*, 1997). These options include anaerobic digestion, aerobic treatment via aerobic digestion or in an aerobic lagoon, incineration, landfilling, or composting (Ruffino *et al.*, 2015). However, due to the high moisture content, and low caloric value of the organic material, the treatment of aquaculture sludge by compositing or incineration will not be an economical option because incineration requires high external energy to burn waste containing large amounts of water while compositing will require dewatering of the sludge (Mirzoyan *et al.*, 2010). Disposal of organic waste into landfills is prohibited, as it usually involves high volumes with high organic matter content and leads to negative environmental impacts.

The use of the aerobic treatment process requires aeration facilities which result in high operational cost of treatment together with the high energy demand that is required for oxygenation (Chen *et al.*, 1997; Zhang *et al.*, 2013). Dumping organic waste into landfills is not

allowed due to the contamination of groundwater pollution and causing odours. The treatment and stabilization of waste sludge is more environmentally friendly when sludge is stabilized by reducing the high volume of toxic materials that have a negative impact on the environment (Opurum *et al.*, 2015).

Among all the different conventional treatment methods that have been proposed, anaerobic digestion is an appropriate method for the stabilization and degradation of sludge under anaerobic conditions by microbes (Mirzoyan *et al.*, 2008; Kuusik *et al.*, 2014). Anaerobic digestion of organic wastes is preferred among the disposal options because of lower operating costs effective and with the relatively low initial investment, the production of renewable energy in the form of biomethane, reduction of nuisances odours, reduction of pathogenic microorganism content in the digestate as well as improved waste management before disposal (Rahman *et al.*, 2019). The other advantages of using anaerobic treatment include low nutrients requirements, no oxygen requirements, stabilising high organic waste with a lower energy requirement for treatment process, and reduced greenhouse gas emissions compared to conventional waste treatment methods such as aerobic processing (Li *et al.*, 2013; Sol and Lansing, 2013; Ruffino *et al.*, 2015). Therefore, because of the advantages aforementioned above, anaerobic digestion is currently gaining attention for the treatment of aquaculture sludge, therefore it is worth to do further investigation.

2.5 Anaerobic Digestion (AD) process

Generally, anaerobic digestion is defined as a biological process in which anaerobic microbial consortium breaks down biodegradable organic material in the absence of oxygen to produce biogas and a nutrient-rich digestate (Hadiyarto *et al.*, 2015; Cadavid-Rodríguez *et al.*, 2019). Typically, biogas composition derived from the AD process of organic substrates is a mixture of methane, carbon dioxide, and a small amount of other gases as shown in **Table 1** (Seadi *et al.*, 2008; Jekayinfa *et al.*, 2015). However, the composition of biogas production may differ based on the biodegradability and chemical composition of the substrate used, and also depends on the process operating parameters such as organic loading rate, temperature, and pH (Rodriguez-chiang and Dahl, 2015; Bharati *et al.*, 2019).

The valorization of organic waste via AD for biogas production is considered suitable for use as an alternative energy source because it is environmentally friendly and can be used to substitute the use of coal, butane, and other materials derived from fossils fuel leading to reduction of carbon

dioxide emissions (Hadiyarto *et al.*, 2015; Ivanovs *et al.*, 2018). In recent years, anaerobic digestion has gained momentum as an alternative method that can be used to recover bio-energy in the form of biomethane from different kinds of organic wastes such as animal wastes, wastewater sewage waste, industrial wastes, agricultural residues wastes, and aquaculture wastes (Ward *et al.*, 2008; Cadavid-Rodríguez *et al.*, 2019).

Table 1: Composition of biogas (adapted from Jekayinfa *et al.*, 2015)

Compound	Chemical formula	Volume percentage of biogas (%)
Methane	CH_4	55 – 70
Carbon dioxide	CO_2	35 – 45
Nitrogen	N_2	0-3
Hydrogen	H_2	<1
Oxygen	O_2	<0.4
Hydrogen sulphide	H_2S	<1

2.6 The microbial mechanisms of the anaerobic digestion (AD) process

Biogas production in the AD process of organic waste takes place through four different sequential steps namely: hydrolysis (1), acidogenesis (2), acetogenesis (3), and methanogenesis (4) (Appels *et al.*, 2008; Zhang *et al.*, 2014; Meegoda *et al.*, 2018). The degradation process of the AD starts with hydrolytic bacteria to break down complex organic polymers such as carbohydrates, proteins, and lipids into monomers for acid forming bacteria, which ferments the sugars and amino acids to carbon dioxide, hydrogen, and organic acids (VFAs). These intermediate products are further converted into hydrogen, and acetic acid by acetogenic bacteria (Awosusi *et al.*, 2021). Finally, the methanogenic bacteria convert the acetogenic products into carbon dioxide and biomethane, known as biogas. A flow diagram of the different organic compounds and the four steps of the AD stages are illustrated in **Figure 1** below. As shown in **Figure 1** below one can see that the anaerobic digestion process is carried out by diverse groups of microorganisms depending on each phase nutrients products, growth factors and organic matter, and sensitivity to the environment. Therefore, it is crucial to understand the microbial metabolic pathway of the anaerobic digestion process.

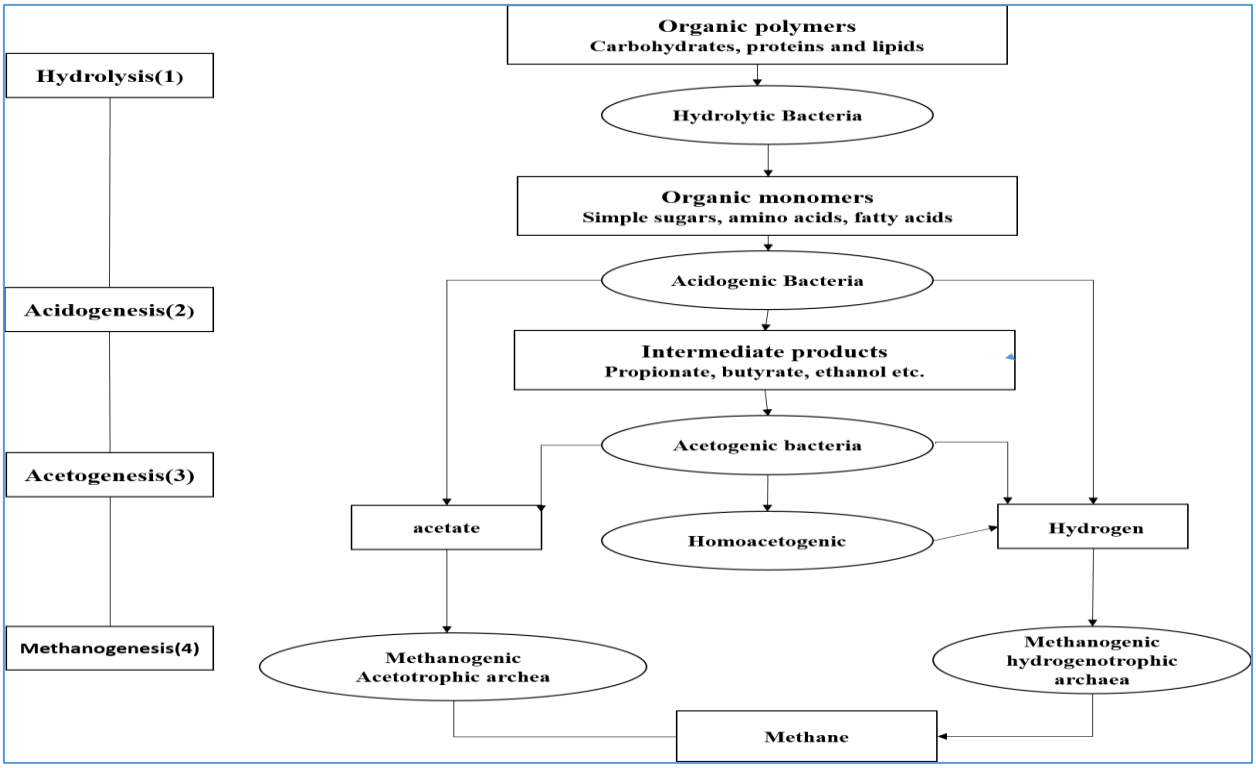


Figure 1: Schematic representation of the anaerobic digestion process (adopted and modified from Curry and Pillay, 2012; Roopnarain and Adeleke, 2017; Kumar *et al.*, 2019).

Step: 1 Hydrolysis

During hydrolysis process, the complex organic compounds (polymers) such as polysaccharides, lipids, proteins, and carbohydrates from organic biomass are enzymatically broken down into simple and soluble monomers such as amino acids, long-chain fatty acids, sugars, and glycerol by hydrolytic bacteria exo-enzymes. (Seadi *et al.*, 2008; Rajagopal and Choudhury, 2019). Therefore, the biodegradation of large organic molecules (carbohydrates, proteins, and lipids) involves different groups of extracellular hydrolytic enzymes such as cellulase, amylase, protease, and lipase, which are secreted by hydrolytic bacteria to break down and solubilize polymers to be easily degradable for acidogenesis and acetogenesis as shown in **Table 2** (Seadi *et al.*, 2008; Adekunle and Okolie, 2015). The hydrolysis step is considered as the rate-limiting step in the anaerobic digestion of large-complex solid substrates, such as cellulose, lignocellulose, lignin, and hemicellulose (Rajagopal *et al.*,2019) because the rate of degradation during the hydrolysis process is influenced by the chemical composition of the substrate, the size particles and the rate of the enzyme production by the hydrolytic bacteria (Adekunle and Okolie, 2015; Arif *et al.*, 2018). The hydrolysis rate can also be influenced by several factors such as pre-treatment, pH,

temperature, and the concentration of substrate loading rate (Rajagopal *et al.*, 2019). However, the rate of hydrolysis can be improved by the anaerobic co-digestion or pre-treatment depending on the type of the organic waste.

Table 2: Hydrolytic enzymes and their functions (Seadi *et al.*, 2008; Adekunle and Okolie, 2015)

Polymers	**Exo-enzymes**	**Hydrolysis products**
Carbohydrates (Polysaccharides)	Cellulases, Hemicellulases, amylases	Sugars (Monosaccharide)
Lipids	Lipases	Fatty acids, glycerol
Proteins	Proteases	Amino acids

Step: 2 Acidogenesis

In this step, the small soluble compounds (monomers) produced from the hydrolysis are being further broken down by acid-forming obligatory and facultative acidogenic (fermentative) bacteria to produce intermediate short-chain organic acids known as volatile fatty acids (butyric, propionic, and acetic acids), alcohols, hydrogen, and carbon dioxide (Seadi *et al.*, 2008; Lim *et al.*, 2019). During this step, fatty acids, amino acids, and simple sugars such as glucose, xylose, mannose are converted into fermentation products such as organic acids and alcohols (Arif *et al.*, 2018). However, it is also important to note that high production of VFAs has been also reported to be an inhibitor of the anaerobic digestion process, especially if the organic waste is rich in carbohydrates or operating at digester at lower pH (Meegoda *et al.*, 2018). More information about the effect of pH and VFAs are elaborated in section 2.7.6 and 2.8.2.

Step: 3 Acetogenesis

During the acetogenesis, the primary purpose is to break down the products, such as volatile fatty acid and any long-chain fatty acids (LCFAs) obtained in the acidogenesis reaction step, and which cannot be utilized by the methanogenic microbial for biomethane production (Adekunle and Okolie, 2015). These organic acid products from acidogenesis are then transformed into acetic acid, hydrogen (H_2) and carbon dioxide (CO_2) by the acetogenic bacteria for subsequent methanogenesis (Seadi *et al.*, 2008; Arif *et al.*, 2018). The acetogenesis step in anaerobic digestion is one of the more complex stages and very sensitive to environmental factors such as pH, temperature, and the organic load rate at which the digester is fed (Hagos *et al.*, 2017).

Step: 4 Methanogenesis

Methanogenesis is the last step in the anaerobic digestion process, where the intermediate products are consumed by the methanogenic bacteria to produce methane in the absence of oxygen. In this step, the monomers such as acetate, alcohols, and gases (CO, H_2, and CO_2) that were formed during the acetogenesis are converted into methane (CH_4), CO_2 and water by the methanogens (Kumar *et al.*, 2019). However, this formation can occur in two reactions by the hydrogenotrophic methanogens or acetoclastic methanogens. In the first case, the hydrogenotrophic methanogens convert $H_2$and CO_2 to form methane and water as shown by Equation (1), whereas acetoclastic methanogens mainly use acetic acid to form methane and carbon dioxide as shown in Equation (2) (Arif *et al.*, 2018). The rate of the methanogenesis degradation step has been reported being to be significantly slower compared to the acetogenesis as the amount of biogas produced depends on several changes in the operation and environmental parameters like pH, feeding rate, temperature, and absence of oxygen (Seadi *et al.*, 2008; Mao *et al.*, 2015). In addition, the process could be terminated if the digester is overloaded or if there is a high concentration of ammonia or VFAs, or oxygen in the reactor (Seadi *et al.*, 2008).

Hydrogen + carbon dioxide ⟶ methane + water **(1)**

Acetic acid ⟶ methane + carbon dioxide **(2)**

2.7 Factors affecting the anaerobic digestion process.

The efficiency of the anaerobic digestion process of organic material depends on different operating parameters for stable performance. The various groups of microorganisms involved in the anaerobic degradation process such as hydrolytic bacterial, facultative acidogenic, and acetogenic, methanogens, are sensitive and their metabolism depends on a wide range of operational and environmental conditions (Seadi *et al.*, 2008; Mao *et al.*, 2015). To achieve a stable AD process, different operational and environmental conditions for the microorganisms such as carbon to nitrogen (C/N) ratio, pH, temperature, organic loading rate (OLR), hydraulic retention time, total solids accumulation of VFAs, and other inhibitors such as ammonia and heavy metals should be maintained and monitored regularly with the optimum ranges of the anaerobic digestion (Kesharwani and Bajpai, 2021). These are important parameters that influence the process stability and the amount of the total biogas that can be produced from a substrate during the anaerobic digestion process (Li *et al.*, 2013; Kumar *et al.*, 2019). Changes in one of these

parameters can cause the process to be inhibited, which may eventually be leading to low biogas yield (Seadi *et al.*, 2008). Therefore, it is important to maintain these parameters of the AD process within the optimum range to optimize methane production and avoid inhibition or instability (Krishna and Kalamdhad, 2014; Kumar *et al.*, 2019). However, the optimum range of the operational parameters differs for each substrate characteristic, type of the biodigester, and this is the reason one needs to optimize the process for each substrate. The effect and optimum ranges of these parameters in the AD process are elaborated below:

2.7.1 Temperature

In AD, the temperature of the reactor is one of the main critical operating parameters as it has a significant effect on the microbial growth community, biodegradability, solubility of a substrate, process kinetics, metabolism, stability, enzyme production, and methane yield (Kothari *et al.*, 2014; Krishna and Kalamdhad, 2014; Awosusi *et al.*, 2021). The effects of temperature in anaerobic digestion have been widely reported by many authors (Bouallagui *et al.*, 2004; Kim *et al.*, 2006; Kothari *et al.*, 2014). The formation of methane in the AD process can take place in three different primary temperature ranges, including psychrophilic (10-20℃), mesophilic (35-37℃), and thermophilic (50-55℃) conditions (Seadi *et al.*, 2008; Hagos *et al.*, 2017). Therefore, for AD to occur in any of these ranges, it is important to develop/obtain an inoculum (a consortium of microbes for start-up) that is acclimated to the desired operating temperature range to ensure the proper growth and metabolism of the microbial community. However, among these three different temperature ranges, mesophilic and thermophilic temperatures are often used for anaerobic digestion processes depending on the type of digester, inoculum source, substrate composition, and optimization of parameters (Kothari *et al.*, 2014). At lower temperatures, the digestion process presents a lower substrate utilization rate, lower microbial growth, lower biodegradation efficiency, and produces less biogas (Kim *et al.*, 2006).

In the literature, mesophilic temperatures ranging from 35℃ to 37℃ have been reported to be the most favorable for methanogenesis, with improved and more stable methane production than thermophilic conditions. This is because the methanogenic bacteria can withstand sudden fluctuations in pH and feeding rate at mesophilic conditions, and require less energy costs than thermophilic temperatures (Kumar *et al.*, 2019; Xu *et al.*, 2018). However, operating a digester in the thermophilic condition also provides several benefits such as higher degradation rate, lower quantities of sludge, destruction of pathogenic bacteria, enhanced solids removal efficiency, lower

retention time, but at the same time, higher energy demands to maintain the thermophilic process temperature compared to the mesophilic process (Appels *et al.*, 2008; Hagos *et al.*, 2017; Xu *et al.*, 2018). Thermophilic bacteria are more sensitive to environmental changes and even relatively small fluctuations of pH due to the production of the high volatile fatty acid and accumulations of ammonia can lead to the inhibition of the methanogenesis, depending on the activeness of the adapted inoculum under the same condition (Kim *et al.*, 2006). Most anaerobic digesters are designed to operate in the mesophilic temperature range 35-37℃, which is considered suitable for supporting methanogenic biological activities and reducing the risk of process failure.

2.7.2 Retention Time

Retention time is defined as the duration the organic matter required for biodegradation in the digester during the anaerobic digestion process before removing it as effluent (Matheri *et al.*, 2017; Kesharwani and Bajpai, 2021) There are two types of retention times in the anaerobic digestion process: the time which the liquids spend in the digester is known as the hydraulic retention time (HRT), and the time in which solids spends in the digester is called solid retention time (SRT) (Khalid *et al.*, 2011; Matheri *et al.*, 2017; Kesharwani and Bajpai, 2021). The SRT depends on the type of substrate, organic loading rate, volume of the digester, and the temperature of the bioreactor in which the anaerobic digestion system is being operated (Kumar *et al.*, 2019). In addition, the production of biogas also depends on the retention time the solids remain in a digester: The longer the SRT, the higher the methane production and reduction of biodegradable material from the substrate (Luo *et al.*, 2013; Mirzoyan and Gross, 2013; Meegoda *et al.*, 2018).

The retention time for anaerobic digestion of organic matter under mesophilic conditions varies from 20 to 40 days, whereas under thermophilic conditions may require less time to digest (Kesharwani and Bajpai, 2021). A study by Mirzoyan and Gross (2013) showed that a low HRT decreased methane production rates and the stability of the AD process. The operational retention time decreases with an increase in temperature conditions.

2.7.3 Organic loading rate (OLR)

The organic loading rate (OLR) is an important parameter especially in semi-continuous operation mode as it determines the amount of organic solids (feedstock) which can be fed in the AD digester. The OLR is a control parameter in the anaerobic digester because overloading beyond a specific level can result in a low biogas yield due to the accumulation of inhibitory compounds

such as volatile fatty acids (VFA) and the formation of scum layers in the digester (Antonio et al., 2014; Kumar et al., 2019). Although the production of methane significantly depends on the percentage of the carbon component in the organic waste material, adding feedstock at an organic loading rate that is more than the rate of degradation in the digester can cause imbalances in the anaerobic digestion process, decreases the pH and results in lower methane production (Mao *et al.*, 2015). Overloading a digester can cause the organic waste to be hydrolysed and acidified faster if the rate of utilisation of the aforementioned products is slower than the rate of their production, which leads to the accumulation of volatile fatty acids and the inhibition of the methanogens (Franke-Whittle *et al.*, 2014; Meegoda *et al.*, 2018). The optimal OLR mainly depends on the volume of the digester, type of organic waste, and HRT. Elsayed et al. (2021) conducted a semi-continuous co-digestion of multi-substrates at an OLR of 0.5, 1, and 1.5 $gVSL^{-1}d^{-1}$ using 50 L digester under mesophilic conditions for HRT of 25 days. They obtained the highest biogas and methane production at an OLR of $1gVSL^{-1}d^{-1}$, while the lowest biogas production was obtained at an OLR of 1.5 $gVSL^{-1}d^{-1}$ due to overfeeding the digester and acidification of the medium. Browne et al. (2014) achieved the highest methane production of 560 $CH_{4\ g/VS}$ at an OLR of 2 kgVS/m^3 with a HRT of 30 days but when an OLR was increased to 4 kgVS/m^3, low methane production reported due to accumulation of ammonia levels of 7000 mg/L (Browne *et al.*, 2014).

2.7.4 Carbon to nitrogen ratio

The carbon to nitrogen (C/N) ratio present in the organic materials is considered one of the most important parameters to determine the performance of the anaerobic mono-digestion or co-digestion processes with mixtures of substrates (Rizwan *et al.*, 2015; Hagos *et al.*, 2017). The C/N ratio of the substrates should be in the optimum range of 20-30:1 for suitable growth of microbes, nutrient balance, maintenance of the stable environment, and functioning of the anaerobic digestion process (Chen *et al.*, 2008; Krishna and Kalamdhad, 2014). Although an optimum C/N ratio range for the AD process is still not clear in the literature, many researchers considered an optimum C/N ratio of 20:1-30:1 (Angelidaki and Sanders, 2004; Kothari *et al.*, 2014) but other studies found that the AD performed well at lower C/N ratio range of 17-20 (Kothari *et al.*, 2014; Zhang *et al.*, 2014).

An optimum C/N ratio will benefit biogas production from various substrates due to proper nutrient balance (Yen and Brune, 2007). Organic substrate such as fish sludge with a lower C/N

ratio between 6-15, increases the production of ammonia within the system, which may lead to an increase in the pH above 8.5 leading to a decrease in microbial activity (Chen *et al.*, 2008; Mirzoyan *et al.*, 2008; Kothari *et al.*, 2014).

Lower biogas production was reported due to a low C/N ratio of 15.3, probably due to inhibition caused by the release of ammonia during the digestion process (Balaji *et al.*, 2018). However, for such substrates with insufficient nutritional composition, it has been reported that co-digestion with suitable co-substrates such as fruits & vegetable waste or food waste that have C/N ratio ranging from 17-40 can be used to adjust the C/N ratios for microbial growth, improve the process stability and to prevent the inhibition of the methanogenesis (Kothari *et al.*, 2014; Hagos *et al.*, 2017; Kumar *et al.*, 2019).

2.7.5 Mixing

During anaerobic digestion, substrate mixing is essential for anaerobic digesters as it prevents the sedimentation of denser particulate material in the digester and foaming (scum) (McCarty, 1964; Xie *et al.*, 2016). Appropriate mixing influences the contact between the substrates, input feedstock, and active microbial population for an effective AD process preventing the formation of dead zones and floating layers that can reduce the effective volume of the digester (Ward *et al.*, 2008; Yang *et al.*, 2021). However, inadequate mixing or excessive mixing can decrease the efficiency of the digestion rate by disrupting the formation of bacteria and essential components required for microbial growth especially methanogen. (Chen *et al.*, 2008; Yang *et al.*, 2021).

2.7.6 pH

The pH value is a crucial parameter in the anaerobic digestion process, which significantly affects the microbial growth, rate of microbial activity, and process stability (Krishna and Kalamdhad, 2014). However, microbes such as the hydrolytic and fermentative bacteria are less affected by acid concentration, whereas acetogenic and methanogenic bacteria are very sensitive to changes in pH, and their growth can be inhibited under such conditions (Hagos *et al.*, 2017). The pH values for hydrolysis, acidogenesis, acetogenic, and methanogenesis are different for each step. The pH less than 6.6 and above 8.2 have been reported to lower methane production (Mao *et al.*, 2015) due to the inhibitory effect of the methanogens (Chen *et al.*, 2008). In addition, increases in the pH above the optimal level of 7.0 may lead to an increase in the concentrations of the alkalinity, delays the methanogenic rate, thus inhibiting the anaerobic digestion process (McCarty, 1964).

An excess concentration of volatile fatty acids can cause a drastic reduction of pH value below 5, and affect the growth rate of methanogens (McCarty, 1964). The most preferred pH value for anaerobic digestion is considered to be near neutral ranging from 6.8 to 7.2 and has been recommended as a suitable condition for methanogenesis microorganisms to maintain reactor stability (Mirzoyan *et al.*, 2008; Kumar *et al.*, 2019) with an optimum pH 7.0-7.2 (Krishna and Kalamdhad, 2014; Hagos *et al.*, 2017). A reviewed study by Krishna and Kalamdhad (2014) reported that sodium bicarbonate ($NaHCO_3$), sodium hydroxide ($NaOH$), and hydrochloric acid (HCl) can be used to maintain pH within the optimum range of the sludge in digesters to control stability parameters such as VFA, bicarbonate, and alkalinity concentration to achieve the maximal biogas yield during the AD process.

2.8 Inhibitory factors

Microorganisms involved in anaerobic digestion are different in nutrient requirements, physiology, growth kinetics, and sensitivity to environmental conditions. Inhibition during the anaerobic digestion is usually indicated by the decrease in biomethane production, accumulation of compounds such as organic acids or ammonia, and failure of the anaerobic reactor (Appels *et al.*, 2008). Many organic and inorganic compounds can cause inhibition of the anaerobic digestion process. These may include ammonia (NH_3), ammonium ions (NH_4+), heavy metals, light metals, and alkalinity cations (Ca^{2+}, Mg^{2+}, K^+, and Na^+,) and excess volatile fatty acid (VFAs) (Appels *et al.*, 2008).

2.8.1 Ammonia

Ammonia is one of the most common compounds that cause inhibition during the anaerobic biodegradation of substrates rich in proteins and nitrogen content (Kayhanian, 1999; Pagés-Díaz *et al.*, 2015) as fish sludge. Substrates rich in nitrogen contents reduce the carbon source fast during the AD process and thus can lead to process inhibition from ammonia which may be present in the form of the ammonium ion (NH_4+) and free ammonia (NH_3) (McCarty, 1964). Free ammonia is considered as a stronger inhibitor than its ammonium ion since it is highly membrane-permeable and can diffuse into microbial cells (Kayhanian, 1999). The formation of ammonia and its concentration have also been reported to increase with increasing pH and temperature (Mirzoyan and Gross, 2013). However, it has been reported that a proper control of pH from 8 to an optimum range of 7.2-7.4, and reduction of temperature to mesophilic conditions

reduced the formation of ammonia toxicity concentrations and improved methane yields (Kayhanian, 1999; Pagés-Díaz *et al.*, 2015). Furthermore, it is found that an ammonia concentration below 200mg/L is suitable for anaerobic bacteria as it is an essential nutrient (Appels *et al.*, 2008), but could inhibit an unaclimatised inoculum (Mirzoyan and Gross, 2015).

A review by Appels et al. (2008) showed that inhibition of methanogenesis by ammonia occurs when the total ammonia nitrogen (TAN) concentration was between 1500- 3000 mg/L, and the pH is above 8.3. Ammonia nitrogen concentrations above 3 000 mg/L during the AD process will lead to a decrease in biogas production due to strong inhibition of the methanogenesis, which can also cause the digestion process to be terminated, and make the digestate less suitable as a fertilizer (Appels *et al.*, 2008; Morales-polo and Soria, 2018). It has been reported that the problem of inhibition by ammonia can be overcome by either dilution, co-digesting of substrates to balance the nutrients content (C/N ratio), increasing the buffering capacity of the system (Gebauer, 2004; Mirzoyan *et al.*, 2008; Kumar *et al.*, 2019) or removal of the source of ammonia-nitrogen from the substrates by chemical, physical and biological techniques (McCarty, 1964).

2.8.2 Volatile fatty acid (VFA)

Volatile fatty acids are important intermediates products such as propionic, acetic, butyric, and isobutyric acid that are produced by acidogenic and acetogenic bacteria in anaerobic digestion, these are typically used to indicate the digester performance and anaerobic digestion process stability (Franke-Whittle *et al.*, 2014). The accumulation of VFAs to concentrations of 3500 mg/L or above may cause inhibition of the biogas production yield and rate because it decreases the pH values in the digester, which may contribute to the failure of the anaerobic digestion system (Bouallagui *et al.*, 2004; Krishna and Kalamdhad, 2014; Mehariya *et al.*, 2018). It is also reported that the accumulation of VFA concentrations in the anaerobic bioreactor increases with increasing the organic loading rates in the digester (Franke-Whittle *et al.*, 2014; Rizwan *et al.*, 2015). Different researchers have reported the accumulation of VFAs as an indicator for process instability and imbalance, associated with low buffering capacity and reduction in pH of the system, which can affect the microbial activity and hinder the growth of methanogens (Franke-Whittle *et al.*, 2014; Mehariya *et al.*, 2018).

2.8.3 Toxic trace metals

Metals (macro and micro) concentration in the substrate is an important indicator of sludge suitability activities as it affects the bacterial growth enzyme synthesis activity and the anaerobic digestion (Appels *et al.*, 2008; Krishna and Kalamdhad, 2014). However, excessive concentrations of heavy metals such as copper, zinc, nickel, and light metals such as sodium, magnesium, and calcium can lead to inhibition of anaerobic digestion (Appels *et al.*, 2008). The concentrations of metals such as sodium (10520 mg/L), magnesium (1610 mg/L), and calcium (1050 mg/L), in aquaculture sludge were found to be high in the ranges where AD could be inhibited (Gebauer, 2004). Calcium (Ca) and Magnesium (Mg) ions have been shown to affect the growth of several strains of methanogens and may cause changes in the composition and morphology of microbial population activities in a bio-digester (Singh *et al.*, 1999). Both Ca^{2+} and Mg^{2+} ions were reported to cause a reduction in methanogenic activity in digester if their concentration in the raw material is more than 800 and 243 mg/L, respectively, resulting from their inhibition of granulation (McCarty, 1964). However, some researchers have noted that there was no inhibition found even when the Ca concentration was high up to 7000 mg/L and the optimum Ca concentration range is 150–300 mg/L (Mirzoyan and Gross, 2013).

A study done by Gebauer (2004) on anaerobic digestion of fish sludge from aquaculture showed that the concentration of Ca^{2+} (4640 mg/L), Mg^{2+} (1759 mg/L), and Na^{+} (10 200 mg/L) ions were higher than the inhibitory level for anaerobic digestion. Trace metals, such as zinc were found in very low concentrations in the sludge, suggesting no potential inhibitory effects on sludge digestion or methanogenesis (Appels *et al.*, 2008). If toxic materials are introduced into the biodigester in high concentrations, the process may be inhibited. McCarty (1964) reported that substrate must be free from inorganic compounds toxic materials to have successful anaerobic digestion. **Table 3** below summarizes toxic inorganic compounds reported to be inhibitory for anaerobic digestion at high concentrations (Appels *et al.*, 2008).

Table 3: Inhibitory concentration of inorganic in anaerobic digestion process (Appels *et al.*, 2008)

Compounds	**Moderate toxic concentration (mg/L)**	**Strong toxic concentration (mg/L)**
Sodium ($\mathbf{Na^{+}}$)	500-5500	8000
Potassium ($\mathbf{K^{+}}$)	2500-4500	12000
Calcium ($\mathbf{Ca^{2+}}$)	2500-4000	8000
Magnesium ($\mathbf{Mg^{2+}}$)	1000-1500	3000
Ammonia (NH4+)	1500-3000	3000
Sulfide ($\mathbf{S^{2-}}$)	200	200
Copper ($\mathbf{Cu^{2+}}$)	-	0.5 (soluble) 50-70 (total)
Nickel ($\mathbf{Ni^{2+}}$)	-	30 (total)
Zinc ($\mathbf{Zn^{2+}}$)	-	1.0 (soluble)

2.9 Anaerobic digestion of fish sludge from aquaculture

Although many studies have focused on the potential of fish waste for biogas production through the process of anaerobic digestion, few studies have reported the biomethane potential of fish sludge from aquaculture systems. Fish sludge as a sole substrate for AD poses major problems, such as low biogas yield and process failure due to low C/N ratio and ammonia inhibition. **Table 4** below summarizes the biogas yields, methane content, volatile solids removal, and digestion efficiency of anaerobic digestion of fish sludge from previous studies.

Gebauer (2004) studied the anaerobic digestion of fish sludge with Total Solids (TS) of 8.2 to 10.2 % in 15 L continuous stirred tank reactors (CSTR) at a temperature (35°C), and 55-60 days hydraulic retention time (HRT). In this study, the anaerobic digestion process was strongly inhibited by high free ammonia concentration and salinity in the undiluted fish sludge. However, dilution of the fish sludge with deionized water reduced the inhibition by salinity and free ammonia inhibition. Furthermore, the methane content of biogas yield was reported to be 49–55%, methane yield was 0.14–0.15 Lg^{-1}COD added, and up to 53.5% of COD removal for the

sludge. The high sodium concentration of 10 200 mg/l was found unfavorable for the growth of mesophilic anaerobes (beyond the limit range as shown in **Table 4**) which was also the cause of inhibition of the digestion process. The author concluded that the treated sludge might not be suitable to use as a fertilizer, due to the high content of VFA 18-28 g/L and salinity (Gebauer 2004). However, besides methane production from anaerobic digestion other authors have reported the transformation of organic wastes into other added-value products, such as volatile fatty acids, and oils (Cadavid-Rodríguez *et al.*, 2019).

The anaerobic digestion of brackish aquaculture sludge was conducted in an Upflow Anaerobic Sludge Blanket (UASB) reactor by Mirzoyan *et al.* (2008) with an HRT of 15 days. The reduction of sludge mass in the bioreactor was 35 to 70%. Moreover, the average of 0.012 Lg^{-1}COD added of biogas production was achieved and the concentration of the methane yield in the biogas was between 30-60%. Mirzoyan *et al.* (2008) reported that the concentration of calcium and magnesium was higher than the limit of the inhibitory levels although the inhibition of methanogenesis was not reported. Therefore it is reported that these might be attributed to the alkaline pH in the sludge, which may cause some precipitation with carbonates that can reduce the high active concentration of the elements (Mirzoyan *et al.*, 2008).

A study by Mirzoyan and Gross (2013) conducted an experiment using a UASB reactor for brackish aquaculture sludge digestion under different conditions (C/N ratio, temperatures, and HRT). The study reported that high removal efficiencies of over 80% of volatile solids, chemical oxygen demand, and total suspended solids in all reactors. The methane yield achieved was very low at 0.001-0.075 Lg^{-1}COD added, which may be due to the low C/N ratio of 15.4, which is lower than the optimum C/N ratio of 20 to 30 for anaerobic digestion (Angelidaki and Sanders, 2004; Kothari *et al.*, 2014). However, the highest methane concentration was observed with co-digestion with cotton wool to increase the C/N ratio of nutrients in the feed. In all reactors, no inhibition was reported because all inhibitors compounds were lower than inhibitory concentrations. The authors concluded that the methane produced was not enough to be used as an energy source (Mirzoyan and Gross, 2013). Co-digestion can provide a better nutrient balance of the C/N ratio to enhance methane production (Mata-Alvarez *et al.*, 2014).

Lanari and Franci 1998 investigated the potential of biogas production by fish-farm effluents from freshwater aquaculture under different feeding ranges with 1.3 to 2.4% TS at 25℃ with HRT of 22-38 days. The results of this study showed that biomethane production was between 0.198-

0.250 Lg^{-1}COD, respectively. The highest biogas and methane production were reported at the high loading rate, while the high qualities of more than 80% biogas methane content were produced in a bioreactor with a lower loading rate. In addition, the digestion efficiencies of volatile solids (93–97%), suspended solids (SS) (96–99%), and total ammonia nitrogen (TAN) content (59–70%) in the anaerobic digester were also reported. In this study, a high ammonia nitrogen concentration (1500-1900 mg/l) was reported, but no inhibition was reported due to the addition of zeolite column in the digestion process which allowed the removal of nitrogen from the sludge (Lanari and Franci 1998).

Opurum et al. (2015) co-digested fish sludge with cow manure in a batch bioreactor system under a temperature (26-35°C) to produce biogas. The highest biogas production potential for fish sludge effluents obtained was 111.15 mL/g VS. The pH levels of all the bioreactors were maintained at the optimum range of 6.8 to 7.2 for 33 days of hydraulic retention time (HRT). They concluded that anaerobic digestion could be adopted in the treatment of fish sludge effluent by converting the organic matter content into methane gas and bio-fertilizer (Opurum *et al.*, 2015).

Table 4: Methane yields, methane content, VS removal, and digestion efficiency of the fish sludge from previous studies.

Sludge source	**Methane yield** ($L\ COD\ g^{-1}$)	**VS removal (% VS)**	**Methane (%)**	**Inhibition**	**References**
Brackish	0.114-0.184	47-62	49-54	Na	Gebauer, (2004)
Brackish	0.140-0.154	74-79	59-61	NH_3& LCFA	Gebauer and Eikebrokk (2006)
Freshwater	0198-0.250	93-97	>80	-	Lanari and Franci 1998.
Brackish	0.02	-	30-60	-	Mirzoyan *et al.* (2008)
Brackish	0.015	94-97	15-53.0	-	Mirzoyan and Gross (2013)
Freshwater	0.013-0.022	91-96	28-57	-	Luo *et al.* (2013)

(-) not detected

2.10 Anaerobic Co-digestion

Anaerobic co-digestion is defined as the simultaneous digestion process of two or more different biodegradable substrates fed into the same digester at a particular time, to overcome undesirable constraints associated with mono-digestion such as ammonia inhibition, lack of micronutrients,

and imbalanced C/N ratio to achieve improvements in total biogas yield, methane content, and process stability (Álvarez *et al.*, 2010; Kashi *et al.*, 2017; Castro-molano *et al.*, 2018). Co-digestion is employed to enhance the biotransformation efficiency, biodegradability of organic matter content, buffering capacity, and maximize methane production as different types of waste are being co-digested (Álvarez *et al.*, 2010; Awosusi *et al.*, 2021). Additional benefits of using anaerobic co-digestion process include reducing the concentrations of toxic compounds such as inorganic salts or pathogens in the digested substrates, and it also improves the composition of the digestate to ensure that it can be utilized as a bio-fertilizer in agricultural without chemical treatment (Kumar *et al.*, 2019). However, proper selection of co-digestion substrates ratio should be done carefully to avoid process imbalances, instability, and reduction of biogas potential yields due to antagonistic effect, accumulation of volatile fatty acids, and toxic (ammonia) compounds (Sol and Lansing, 2013; Mata-Alvarez *et al.*, 2014; Masih-das and Tao, 2018). The mixing ratio of substrates during anaerobic co-digestion is an important parameter for process optimisation (Lian *et al.*, 2021)

The performance of the anaerobic co-digestion of organic wastes depends on the fractions of the components, the amount of the mixture substrates, and other parameters such as C/N ratios, temperature, nutrients of the substrate, total solids, and biodegradability (Pagés-díaz *et al.*, 2015). Co-digesting multiple organic wastes can improve biogas production compared to mono-digestion due to the synergistic interactions (Rao and Baral, 2011).

Few studies have reported the use of fish sludge in anaerobic co-digestion with other substrates. Kuusik et al. (2014) studied the anaerobic co-digestion of sewage sludge with fish sludge in batch and continuous reactors under 35°C. The methane production yields were observed to increase from 63.3 to 74.6% after co-digestion of fish waste sludge with sewage compared to mono-digestion (Kuusik *et al.*, 2014). Another study by Mirzoyan and Gross (2013) found that the mixture of cotton wool with fish sludge significantly increased methane production from 2% to 53% and a higher removal of organic matter, as compared to a digester with mono-substrates of fish sludge.

A study by Aragaw et al. (2013) showed that co-digestion of different substrates increased the biogas production by 24 to 47% than the organic waste and cow manure alone. Callaghan *et al.* (1999) observed an improvement in the methane yield from 230 to 450 L CH_4/kg VS after increasing the proportion of the fish offal sludge to 50% from 20% in co-digestion with cow

manure. **Table5** summarises the advantages that can be achieved when fish sludge cooperated with substrates such as food waste or fruit & vegetable waste during the co-digestion process and its limitations depend on the operational conditions.

Table 5: Advantages and disadvantages of co-digestion (Khalid *et al.*, 2011; Mata-Alvarez *et al.*, 2014; Kashi *et al.*, 2017; Kumar *et al.*, 2019)

Advantages	**Disadvantages**
Improve nutrient balance for an optimal digestion	Increased digester effluent H_2S
Improvement of process stabilization	Additional pre-treatment methods requirements
Additional biogas production	Increased mixing requirements
Increasing the total amount of organic matter loaded	Restrictions of land use of digestate due to high VFA build-up
High economic feasibility benefits	Transport costs for collection of co-substrates
Dilution of potential inhibitory substances (ammonia)	
Balancing buffering capacity system	
Optimum C/N ratio can be achieved	

Co-digestion is used as an alternative way to maximize biogas production, methane yield and to control the anaerobic digestion process stability. In order to have a successful co-digestion process, it is necessary to balance the C/N ratio of the substrate and co-substrate. Different types of organic waste such as fruit and vegetable waste, food waste, and animal manure have been reported as co-substrate for anaerobic digestion of organic waste such as the fraction of municipal solid waste (OFMSW), corn straw, animal manure, sewage sludge, and solid slaughterhouse wastes (Pagés-díaz *et al.*, 2015; Gomes *et al.*, 2017; Lüdtke *et al.*, 2017)

2.10.1 Fruit and vegetable waste as co-substrate

The anaerobic co-digestion of fruit & vegetable waste (FVW) could improve the characteristics of substrates such as fish sludge by balancing the macro and micro-nutrients of the system, providing a better C/N ratio, and facilitating the adjustment of total solids and moisture content. Fruit and vegetable wastes can be used to overcome the rapid accumulation of ammonia in the bioreactors due to its high C/N ratio, relatively high hemicellulose, and cellulose content (Shen *et*

al., 2013). However, substrates with lower hemicellulose are recommended to obtain methane production in a short retention time during the anaerobic co-digestion process (Li *et al.*, 2021).

Fonoll et al. (2015) reported that co-digestion of sewage waste and fruit wastes resulted in higher methane production, with approximately 110% to 180% improvement depending on the co-substrates' biodegradability. Anaerobic digestion of fruit and vegetable waste as a single substrate has also presented some limitations due to high simple sugars content, which promotes the accumulation of volatile fatty acids (VFA), long-chain fatty acids, fast acidification, which hinders methanogenic activity (Gomes *et al.*, 2017).

The co-digestion of organic fraction of municipal solid waste (OFMSW) with fruit and vegetable waste (FVW) has been studied by Gomes et al. (2017) in a batch experiment under mesophilic conditions, with different mixing ratios of OFMSW: FVW (VS basis) of 1:0, 1:1, 1:3, and 0:1. According to the authors, the co-digestion of OFMSW and FVW increased the cumulative biogas yield and methane yield by 141% and 43%, respectively, compared with the mono-digestion of OFMSW. They reported that the optimal mixing proportion of OFMSW/ FVW was 1:3.

Bres et al. (2018) co-digested fruit & vegetable waste with poultry manure at mesophilic range using semi-continuous anaerobic reactors at a bench scale. The results of their study showed that biogas and methane production from a mixture of poultry manure (50%) and fruit & vegetable waste (50%) at a 1:1 wet weight ratio was increased by 32% compared to anaerobic digestion of poultry manure alone. They noted that the higher methane yields were obtained in anaerobic co-digestion because the process was more stable and faster than mono digestion.

2.10.2 Food waste as co-substrate

Food waste has been used as a substrate as well as a co-substrate for anaerobic digestion due to its heterogeneous composition. The advantages of using food waste as a co-substrate during anaerobic co-digestion include its abundant availability, its high biochemical compositions regarding organic components like lipids , protein, carbohydrates, its high buffer capacity, and high organic content (Lin *et al.*, 2011). Furthermore, food waste is characterised by high total solids content which can be used to adjust the main substrates total solids and nutrients necessary for optimal bacterial growth (Angeriz-campoy *et al.*, 2015). The substrate with higher solids concentration can help to increase the solids retention time of the digester *(Lee et al.*, 2019).

According to morales review, the C/N ratio of food waste ranged from 12-24 depending on the type of food waste compositions (Morales-polo and Soria, 2018) .

Dennehy *et al.* (2016) studied anaerobic co-digestion of food waste and pig manure in batch mode experiments under mesophilic temperature to identify the synergistic effects of co-digestion on the methane yield. The results showed that the co-digestion of PM and FW at mixing ratio 1:4 (based on dry weight) improved the specific methane yield from 260 to 521 $mLCH_4$/gVS added, respectively, compared to the mix with the 1: 0 ratio. The addition of FW to PM also had synergistic effects on reaction kinetics, increasing the C/N ratios within the optimal range for anaerobic digestion and reducing the potential inhibitory ammonia levels.

Although several studies have reported the advantages of co-digestion using fruit & vegetable waste or food waste as co-substrates with other organic waste in a batch experiment, the preferred mixture proportions of fish sludge with fruit & vegetable waste and food waste have not been well described in the literature.

2.11 Use of digestate as an organic fertilizer in agricultural practice

The end product of anaerobic digestion known as the digestate has been considered as a bio-fertilizer to improve soil fertility and increase crop production (Insam *et al.*, 2015). The digestate residue may obtain solids or liquids after the anaerobic digestion process, which are rich in nitrogen, potassium, and phosphorus and can therefore be useful as a fertilizer on agricultural land (Chen *et al.*, 1997). The use of digestate as fertilizer in agriculture will reduce the use of chemical fertilizers and greenhouse gas emissions resulting from their production (Mata-alvarez *et al.*, 2014). However, it is essential to know the parameters such as nutrient content, salinity, heavy metals, pH, chemical toxicity, and feasible pathogens of the digestate before applying on land (Apruzzese *et al.*, 2017). The anaerobic digestion process results in mineralization of organic nutrients, reduction of odours, and a potential inactivation of pathogenic bacteria depending on the process temperature and the retention time of the sludge in the digester improving suitability of digestate for application as fertilizer (Wieland, 2010).

The suitability of the digestate derived from anaerobic digestion of fish sludge for agricultural application as bio-fertilizers is dependents on the extent of hygienization and, sanitization, its chemical composition, stability, and the concentrations of heavy metal in digestate, which should be lower than the recommended levels for bio-fertilizers (Gebauer, 2004). Digestates obtained

from the digester operated under a shorter hydraulic retention time or high organic loading rate may contain a large amount of non-degraded organic matter (Mata-alvarez *et al.*, 2014). The use of quickly removed digestate from the digester may cause immobilization and increase microbial activity in the environment, which might inhibit plant growth and soil fertility (Mata-alvarez *et al.*, 2014). The suitability of the digestate, for application as a fertilizer, must be assessed to avoid nutrients leaching and the spread of pollutants on land (Mata-Alvarez *et al.*, 2014).

2.12 Types of anaerobic digesters

The anaerobic digestion experiments are mainly carried out in three different modes/types of digesters and scales: batch and semi-continuous applied to laboratory scale (100 mL to 1000 mL), pilot-scale (5 L -300 L), and full scale (Lüdtke *et al.*, 2017; Ruffino *et al.*, 2015).

2.12.1 Biochemical methane potential (BMP) test

The biochemical methane potential (BMP) test is an analytical method (batch laboratory scale) commonly used to evaluate the suitability and bio-digestibility of different organic substrates and also to provide information about the kinetics and potential inhibitory compounds that can be detected (e.g. VFA), and potential methane production that can be derived from a given substrate performed under batch anaerobic digestion experiments (Ward *et al.*, 2008; Sol and Lansing, 2013; Regalado *et al.*, 2021). In addition, this batch test also helps in process optimization (co-digestion), modeling, and simulations as well as the designing and functioning of the semi-continuous pilot-scale or full-scale AD plant to establish the profitability of the anaerobic digestion plant in terms of methane yield and quality of methane content in the biogas that can be produced from organic materials (Ferreira and Carlos, 2012; Holliger *et al.*, 2017; Lüdtke *et al.*, 2017). The procedure of the BMP assay is performed by incubating a mixture of active anaerobic seed (inoculum) and substrate at a laboratory scale in a batch reactor under a specific temperature (mesophilic or thermophilic) and measuring the biogas production until it stabilizes (e.i. a small amount of less than 1% of methane content is produced) (Holliger *et al.*, 2017; Lüdtke *et al.*, 2017). The amount of inoculum in the reactor must be enough to provide microorganisms (microbial consortium), buffering capacity, and to cope with the build-up of volatile fatty acids and ammonia (Angelidaki *et al.*, 2018). The traditional BMP assays following the above principle require daily manual sampling of biogas and determination of biogas volume released and analysis of biogas composition using gas chromatography. The BMP value after the experimental operation is reported as the total volume of methane produced by the substrate during the

anaerobic digestion processes over the amount of organic matter added in the bioreactor (mL CH_4g/VS or CH_4g/COD as commonly used in literature) (Lüdtke *et al.*, 2017; Angelidaki *et al.*, 2018).

2.12.2 Scaled-up anaerobic batch and semi-continuous digesters

The semi-continuous or pilot-scale experiments are performed in laboratory digesters to evaluate the process performance stability, variability, organic loading rate with higher amounts of heterogeneous substrates, and optimizes the biogas and methane production, changes of operational parameters (pH, temperature, and alkalinity), the impact of solids or hydraulic retention time, the influence of mixing digester due to larger-scale (Ruffino *et al.*, 2015; Lüdtke *et al.*, 2017). Ruffino et al. (2015) evaluated the effect of AD in laboratory scale and pilot scale (300 L) digesters under mesophilic conditions. They reported that the methane yield obtained from the pilot-scale mode closely resembled the methane yield from the laboratory scale.

Lüdtke et al. (2017) investigated the replicability of the methane yield from batch and semi-continuous laboratory scale to the full-scale continuous digester. They reported that the results of the batch and semi-continuous laboratory experiments could be used to estimate the specific methane production significantly ($p< 5\%$) of the full-scale anaerobic digestion. Different studies concluded that laboratory scales BMP tests overestimate the methane yield compared to the methane yield obtained from the pilot-scale digesters (Ruffino *et al.*, 2015; Lüdtke *et al.*, 2017).

2.13 Conclusion and research gaps

According to the literature review, it was discovered that excessive amounts of fish sludge are being generated around the recirculation aquaculture system. The use of anaerobic digestion will help as a disposing method of the fish sludge as well as producing energy from it. Biogas production from anaerobic digestion of fish sludge can be used as an alternative and renewable source of energy to produce heat, clean electricity, and other valuable sources of energy compared to fossil fuels that affect the global climate due to the emission of greenhouse gases. However, it was found that there are some disadvantages of anaerobic digestion, but it is a better way of disposing of waste as compared to landfilling, incineration, and aerobic digestion.

Various studies in literature using different substrates such as food waste, animal manure, activated sludge, agricultural biomass, municipal and industrial wastes have been performed and it was found that they can be digested successfully and efficiently together. Few studies have also

been conducted using fish waste sludge originating from the aquaculture system as well as fish sludge with sewage sludge, and it was found that co-digestion enhances methane production. However, there is limited literature regarding the co-digestion of fish wastes sludge from aquaculture with other substrates such as food wastes, animal manure, algae, agricultural residues in a single study as co-substrates. The previous studies illustrate the benefits of using food waste or fruit & vegetable waste as co-substrates with other organic wastes (main substrate) for the improvement in cumulative biogas and methane production. Co-digesting fish sludge with food waste/ fruit &vegetable waste can reduce problematic of substrate characteristics/compositions such as low solid content, high ammonia concentrations, and balancing nutrients that may lead to enhanced nutrient availability for microorganisms, improving a better carbon/nitrogen ratio (C/N) in the feedstock, and enhance the process performance by overcoming inhibitory compounds such as ammonia or VFA inhibition which mostly occur in mono-digestion of substrates. Most of the studies of anaerobic co-digestion were conducted in laboratory batch scale reactors. To the best of our knowledge, no studies has conducted an optimization of methane production from anaerobic co-digestion of fish sludge with food waste and fruit & vegetable waste using mixture design and validated to large scale batch and semi-continuous digesters. Therefore, the objective of the study was to fulfill the existing gap which is to obtain the optimal methane yield from a mixture of different waste combinations on the anaerobic co-digestion process. In addition, there are few studies reported in the literature on the effect of organic loading rate on biogas and methane production, and process performance in batch and semi-continuous pilot-scale digesters. In this study, laboratory batch (500 mL) tests will be scaled up to pilot (30 &50 L) digesters to investigate the effect of organic loading rate and process stability in large digesters.

3 CHAPTER 3: AIM AND OBJECTIVES

3.1 Research Aim and Objectives

This research aims to optimize the biogas and biomethane production from fish sludge originating from RAS using anaerobic co-digestion.

In order to achieve the main aim of this research, the following objectives are set:

- To determine the chemical compositions of fish sludge from a RAS and its suitability for anaerobic digestion.
- To determine the biochemical methane potential of fish sludge
- To evaluate the effects of co-digestion on methane production from the digestion of fish sludge and suitable co-substrates
- To determine the optimum co-substrate ratios that will maximize biomethane production during anaerobic co-digestion
- To evaluate the effect of organic loading rates on biogas production, methane yield, and process stability of anaerobic co-digestion in a pilot-scale semi-continuous digester, using the preferred substrate ratios.

3.2 Research questions

- How do the characteristics of fish waste sludge affect the anaerobic digestion process?
- What is the biomethane potential of the fish sludge?
- What is the appropriate co-substrate proportion that will result in optimal specific methane yield and volatile solids removal?
- Does the anaerobic digestion of fish sludge with selected food waste and fruit & vegetable waste improve biogas and VS removal?
- What is the effect of OLR on the process performance of the semi-continuous digester?

4 CHAPTER 4 : MATERIALS AND METHODS

This chapter discussed materials and analytical methods used during the anaerobic co-digestion experimental, including the experimental designs applied during small-scale of BMPs, as well as testing in batch and semi-continuous pilot-scale digesters. All the experimental tests were carried out at the Department of Process Engineering laboratory.

4.1 Substrates and inoculum collection

4.1.1 Inoculum

The anaerobic seed sludge (inoculum) used in this study was collected from the Uilenkraal dairy farm biogas plant treating cow manure in Darling, Malmesbury, Western Cape, South Africa. After collection, it was re-adapted to the mesophilic (37ºC) conditions in 50 L pilot-scale anaerobic digester by feeding with cow manure, running in the Department of Process Engineering Laboratory, Stellenbosch University. Before commencement of the experiment, the inoculum was taken out in a container for 2-3 days to minimize the amount of biogas and residual organic materials present inside (degassing) (Gomes *et al.*, 2017; Lim *et al.*, 2021). After degassing, the inoculum was analysed for physico-chemical properties such as pH, alkalinity, moisture content, total solids, volatile solids, and ash. After characterization, the inoculum was immediately used for the anaerobic digestion tests.

4.1.2 Substrate and co-substrates

The primary substrate used in the present study was fish sludge, while food waste and fruits & vegetable wastes were used as co-substrates. The fish sludge used was obtained from Karoo Catch fish farm in Eastern Cape, South Africa, and consisted of fish faeces and uneaten feed. It was collected in a tight container and transported to the biogas laboratory.

The food waste and fruit & vegetable waste used in the study were obtained from local supermarkets (Food Lovers and Pick n Pay) in Stellenbosch, South Africa. These wastes were discarded into a waste storage facility. The food waste used is composed of cooked foods, such as rice, meat, fish, noodles, bread, and cooked vegetable salads. The fruit and vegetable wastes used in this study were a mixture of spoiled fruits such as apple, pear, pineapple, banana, watermelon, papaya, and vegetables such as cabbage, lettuce, spinach, and potatoes. The particles such as seeds, plastic bags, and bones were hand-sorted and removed from the food waste and

fruits & vegetable waste before being homogenized. The fruit & vegetable waste and food waste were then individually crushed, macerated, and homogenized into small particles size using a bowl cutter. Then, the substrates samples were aliquoted into small zip-lock plastic bags and stored at -20ºC to prevent repeated thawing and biodegradation until used for the experiment. The substrates were characterised for parameters of AD such as pH, moisture content (%), volatile solids (%), total solids (%), carbon to nitrogen (C/N) ratio as discussed below. Prior to any experiment, the substrates samples were taken out of the freezer and thawed at 4°C.

4.2 Substrates characterization

The biomethane potential of the fish sludge, co-substrates, and inoculum used in this study was analysed based on their physico-chemical parameters that mostly affect the anaerobic digestion process. These include TS, VS, pH, moisture content, proximate and ultimate analyses which were conducted as follows:

4.2.1 Total solids (TS), volatile solids (VS), and moisture contents (MC)

The total solids (TS), volatile solids (VS), and moisture contents (MC) of the substrates and inoculum before and after digestion were determined according to the APHA standard methods for the examination of water and wastewater 2540B, 2540E, and 2320, respectively (APHA, 1998). To determine the TS content of the feedstocks, a 2g of fresh feedstock samples (C_b) were weighed in an empty ceramic crucible (C_a) and then the crucibles containing samples were dried in an oven at 105 °C for 12-24 hours (C_c) until constant weight is observed. After drying at 105 °C, the samples were cooled in a desiccator and subsequently weighed on a digital balance. The TS and moisture contents of the samples were determined by calculating the difference (by gravity) between the fresh sample and the oven-dried sample according to Equation (3-4). The volatile solids content (VS) was performed by placing the dried samples in a muffle furnace oven and igniting the dried samples at 550 °C for 2 hours (C_d). The crucibles were removed from the muffle furnace and cooled in a desiccator after which the samples were weighed again. The VS concentration was determined using Eq.(5) respectively as shown below.

$$\text{TS (\%)} = \frac{C_c - C_a}{C_b} \times 100 \qquad (3)$$

Where, C_a is the weight of the empty crucible (g), C_b is the weight of the sample (g), C_c is the weight of the crucible containing sample dried matter after drying at 105°C (g)

$$MC\ (\%) = 100 - TS\ (\%) \quad (4)$$

$$VS\ (\%) = \frac{C_c - C_d}{C_b} \times 100 \quad (5)$$

Where, C_b is the weight of the sample (g), C_d is the weight of the crucible containing the sample dried matter after heating at 550 ℃ (g)

4.3 pH, Alkalinity, and VFA

4.3.1 pH

A laboratory Hanna (HI 5221) pH meter with a combined electrode was used for measuring the pH of the substrates and inoculum before and after the digestion process. The pH meter was calibrated before using it by dipping the probe into commercial standards (buffer) solutions of pH 4.0, 7.0, and 10.0. After calibration, the pH probe was subsequently dipped into the sample, and the pH value was recorded. During the experimental setup preparations, 3 M sodium hydroxide ($NaOH$) and 3 M Hydrochloric acid (HCl) were used to adjust the pH of the inoculum and substrate to an optimum range (7.0-7.2), which support the growth of methanogens (Opurum *et al.*, 2015; Lian *et al.*, 2021).

4.3.2 VFA

The volatile fatty acids content of the samples was determined using the Spectroquant® Volatile Organic Acids Cell Test kit (Merck, Germany). All analyses were performed in duplicate and conducted according to the manufacturer's manual. Prior to the analysis, the digested liquid samples were centrifuged at 10 000 rpm for 30 minutes, and the obtained supernatant was then filtered with 0.22 µm pore size FilterBio® CA syringe filters before analysis. A volume of 1 mL of the supernatant liquid sample was pipetted into a reagent vial; the vial was closed and then mixed by shaking. Then the vials were placed into a heater reactor and were cooled at room temperature followed by the absorbance measurement using a Spectrophotometer.

4.3.3 Alkalinity and Ammonium

Alkalinity concentrations were measured using the Acid Capacity cell (total alkalinity) Spectroquant® test kit (range 20-400 mg/L$CaCO_3$), and ammonium nitrogen concentrations were determined using the Ammonium Spectroquant® cell test kit (range 2.0-150 mg/NH_4N). The

procedure of acid capacity and Ammonium cell tests kits were performed according to the instruction of the manufacturer guide (Merck, Germany).

4.3.4 Carbon-to-nitrogen (C/N) ratio

The elemental compositions such as carbon (C), hydrogen (H), nitrogen (N), and Sulphur (S) content were combusted through a CHN analyzer in order to determine the C/N ratio. The samples were prepared for analysis by milling the dried samples using a porcelain mortar until the sample was reduced to a fine powder. The dried samples approximately (2g) were then taken to Central Analytical Facility (CAF), Stellenbosch University for the CHNS analysis. The samples were measured by Elemental Vario EL cube Elemental Analyzer at Central Analytical Facility based on ASTM D4239 and ASTM D5373 standard methods.

4.4 Biomethane Potential Test (BMP)

4.4.1 Experimental procedure and set up

Automatic Methane Potential Test System

The biochemical methane potential test for the fish sludge sample of the study was conducted using the automatic methane potential test system (AMPTS II) and the Gas Endeavour system (Bioprocess Control AB, Sweden) as depicted in **Figure 2**. The AMPTS II system consists of four component units which include the incubation (thermostatic water bath) (unit A), Gas Endeavour (unit B), CO_2 absorption fixing (pH indicator) (unit C), and gas flow cell tripping (unit D). The incubation unit contains a maximum of 15 (600 mL total volume) glass bottle bioreactors, each bioreactor equipped with an agitator motor that can be set up to mix continuously or intermittently either clockwise or anticlockwise for the substrate and inoculum during the anaerobic digestion process. The biogas produced in each bioreactor counted first by the gas Endeavour system (consist of 15 flow cells) before moving to the CO_2 absorption unit which consists of bottles containing 80 mL 3 M sodium hydroxide ($NaOH$) solution and 0.4% thymophalein as a pH indicator solution. The CO_2 absorption unit retains any non-methane gases such as carbon dioxide and hydrogen sulphide, while allowing methane only to pass over to the gas flow cell tripping unit D.

The gas flow cell (unit D) measures the methane produced from each reactor by using the wet gas flow measuring device. The biogas counter flow cell unit was filled up with deionized water up

to the recommended level. This device measured the biogas production according to the standard method of liquid displacement and buoyancy enabling to monitor ultra-low gas flows. The accumulated biogas and methane volume yields (NmL) are quantified and recorded either hourly or daily flow rate by the computer software. The amount of gas volume generated by the AMPTS program is normalized to standard temperature (0 °C), standard atmospheric pressure (1Atm), and moisture content. More detailed information about the AMPTS system can be found in Bioprocess Control Operation and Maintenance Manual (Bioprocess Control, Sweden, 2017).

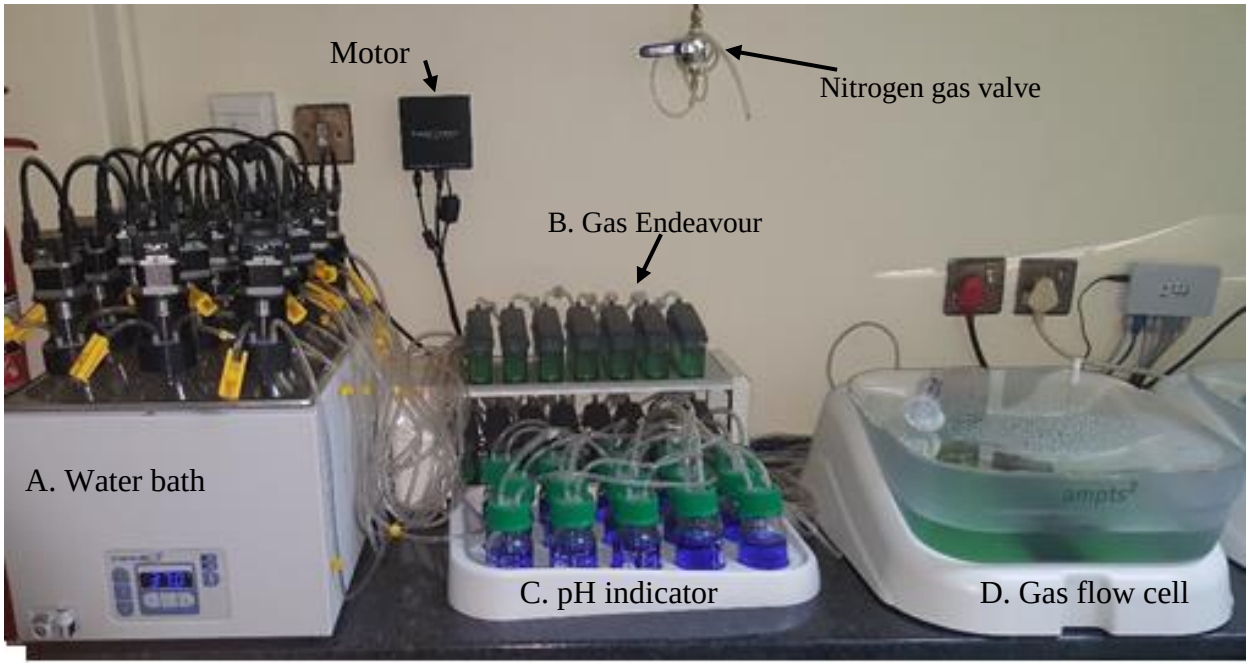


Figure 2: Automatic methane potential test system (AMPTS) II and Gas Endeavour system used for laboratory batch assay.

Preparation of 3 M sodium hydroxide (NaOH) solution for CO_2 absorption unit

The 3 M sodium hydroxide solution was prepared by adding 240 g of pure NaOH dissolved in 2 L of distilled water to make a 3 M NaOH solution. Secondly, 0.4% thymolphthalein pH indicator solution was prepared by dissolving 40 mg of thymolpthalein in 9 mL ethanol (99.5%) followed by the addition of 1 mL distilled water. Then, a 10 mL of Thymolphthalein pH indicator solution was added to 2 L of 3 M NaOH solution to prepare the CO_2 absorption fixing solution with blue colour. However, the Thymolphthalein solution can change from blue to colourless during the anaerobic digestion process. The changes in colour indicate an excessive absorption of CO_2 by

NaOH solution. Therefore, it is crucial to replace it with a new pH indicator solution to prevent the CO_2 gas to pass to the gas volume measuring device.

4.5 Anaerobic batch experiments I: Biochemical methane potential (BMP) tests of Fish sludge.

The BMP tests were conducted and set up on a laboratory scale using the AMPTS II and Gas Endeavour system according to Angelidaki et al.(2009) and Raposo et al. (2011). The preliminary tests were performed to determine the biomethane potential, anaerobic biodegradability rate, suitability of the fish sludge at bench scale. The BMP tests were performed using 500 mL glass bottle bioreactors with a working volume of 400 mL. The amount of inoculum and substrates required in each reactor were determined based on VS of substrate and inoculum following as stated below (Eq.6-8). The recommended inoculum to substrate ratio (ISR) of 2:1 was applied in reactors based on a VS of inoculum and substrate for the experiments. ISR 2 was chosen in this study in order to reduce or overcome any inhibitory problems associated with the build-up of VFA and ammonia (Holliger *et al.*, 2016; Elsayed *et al.*, 2021).

The BMP tests were performed in triplicates, including fish sludge as the substrate, positive control tests (Avicel® pH-101 cellulose, Sigma Aldrich, South Africa), and blank experiments containing inoculum only (without substrate) to correct the biomethane coming from the inoculum. Each reactor bottle was inoculated with the appropriate inoculum and substrate concentration based on Eq. 6-8. After which, the pH of the reactors was measured and adjusted to pH 7.0 (Krishna and Kalamdhad, 2014) using 3 M sodium hydroxide ($NaOH$) and Hydrochloric acid (HCl) solutions. All the reactors were then sealed with a rubber stopper to prevent gas leak and were placed in the thermostatic water bath filled up with distilled water and maintained at 37°C. The agitator motor systems of each reactor were connected in sequence with the motor cables followed by the flushing of the headspaces with gas mixture (60% N_2, 40% CO_2) for 2 minutes in order to provide anaerobic conditions (Koch *et al.*, 2017; Panigrahi *et al.*, 2020). After purging with nitrogen, the Tygon tubes and rubber stopper hose were then closed with a tube clamp. The reactors were then connected corresponding with the lid of the CO_2 fixing absorption using Tygon plastic tubes. The agitation system was set to intermittently mix at 10 minutes mixing and 5 minutes resting by a slow rotation agitator (100 rpm) in order to homogenize material between the inoculum, substrates, nutrients, and the microorganisms (Alkanok *et al.*, 2014). The BMP assays were operated for 28 days, and the experiments were also terminated when the daily

biogas production was less than 1% of the cumulative biogas production per day or when the biogas production ceased to form (Holliger *et al.*, 2016; Koch *et al.*, 2017).

The total biogas produced in each reactor passes through a CO_2 fixing absorption bottle containing $NaOH$ solution with 0.4% thymophalein as a pH indicator which allows only methane gas to pass through to the gas flow tripping unit which records the volume of methane produced from each vial through a wet gas flow measuring device. The CO_2 absorption fixing unit was monitored daily for changes in colour from blue to colourless. The daily biogas flow rate, the volume of methane produced with normalized values (NmL), and biogas volume were automatically recorded by a data acquisition system by Bioprocess Control software™. At the end of the experiment, the recorded data of accumulated biogas and methane were transferred into an MS Excel ™ file for statistical analysis.

Biogas and methane produced from inoculum (blank) reactors were subtracted from each of the reactors to correct the amount of biogas produced by the substrate. During the digestion process, biogas samples (15 mL) were taken from the headspaces of the reactors three times per week using a 50 mL pressure-tight syringe, and the samples were immediately analyzed for gas composition.

Calculation of the amount of inoculum and substrate required in each reactor.

The amounts of inoculum and substrate required in each bioreactor were determined according to Equations 6, 7 & 8. The inoculum to substrate ratio of 2:1 based on VS was chosen in the study (Angelidaki *et al.*, 2009; Holliger *et al.*, 2016).

$$M_{inoculums} = \frac{800 \times VS_{substrate}}{Vs_{inoculums} + (2 \times VS_{substrate})} \quad (6)$$

$$\frac{M_{inoculums} \times VS_{inoculums}}{M_{substrate} \times VS_{substrate}} = 2 \quad (7)$$

$$M_{substrate} + M_{inoculums} = 400 \quad (8)$$

Where, $M_{inoculums}$ is the amount of the inoculums (g), $M_{substrate}$ is the amount of the substrate (g), $VS_{substrate}$ is the VS percentage of the substrate (%), $Vs_{inoculums}$ is the VS percentage of the inoculum (%).

BMP calculation.

The biochemical methane potential generated was calculated by subtracting accumulated methane production from the substrate from the accumulated methane from the inoculum divided by the mass of substrate VS content according to equation 9 (Bioprocess Control, Sweden, 2017).

$$BMP = \frac{V_{Substrate\ \&inoculums} - V_{inoculums\ (Blank)} \frac{M_{inoculums,,Sample}}{M_{inoculums,Blank}}}{M_{VS,substrate}} \quad (9)$$

BMP- is the normalised amount of the cumulative methane produced per gram VS of substrate added (NmL/gVS), $V_{Substrate\ \&inoculums}$ is the total accumulated volume of methane produced from the bioreactor containing sample and inoculum, $V_{inoculums\ (Blank)}$ is the total accumulated volume of methane produced from the blanks bioreactors (containing inoculum only), $M_{inoculums,,Sample}$ is the total amount of inoculum in the sample (g), $M_{inoculums,Blank}$is the total amount of inoculum in the blank, $M_{VS,substrate}$ is the amount of organic content of substrate contained in the sample

4.6 Anaerobic Co-digestion study with BMP measurements

4.6.1 Determination of optimum substrate ratio

In the second set of BMP experiments, different co-digestion tests of fish sludge with mixed food waste, and fruit & vegetable waste as co-substrates were carried out in laboratory batch bioreactors (AMPTS II) to determine the best mixture combination and ratio for optimum biomethane yield.

4.6.2 Design of experiments and statistical analysis

Response surface methodology (RSM) was utilized to determine the optimum co-substrate mixture ratio using a mixture design. A mixture design was used to evaluate the effects of co-substrates ratio and their interactions during the anaerobic co-digestion of fish sludge (FS), food waste (FW), and fruit & vegetable waste (FVW) to improve biomethane production. The RSM mixture design is a design in which the independent variables are the proportions of the two or more components in a mixture (Shuang *et al.*, 2017; Rahman *et al.*, 2019).

In this study, fourteen (14) different substrate combinations with three replicates were generated using simplex centroid mixture design (SCMD), as shown in **Table 6**. Response methodology is usually applied to optimize the performance of the bioprocess, evaluate the interaction effects of

substrates and minimize the number of experimentations runs in order to determine the best mixture proportions which maximize the response variables (Rao and Baral, 2011; Shuang *et al.*, 2017; Balaji *et al.*, 2018; Rahman *et al.*, 2019). The mixture design of the present study contained a pure substrate, combinations of two substrates (binary), and mixtures of all three (trinary) components of the substrates at wet weight proportions (% ww) to a total concentration of 100%. The two response variables were specific methane yield (SMY) and Percentage Volatile Solids removal (PVSR). The PVSR is used to quantify the amount of organic waste converted into biogas production during the anaerobic digestion process (Jha *et al.*, 2021).

Table 6: Simplex centroid mixture design of three substrates of the batch anaerobic co-digestion sets.

Run	**Mixture proportion of substrate volume (%)**		
	FS	**FW**	**FVW**
1	33.33	33.33	33.33
2	0	100	0
3	0	50	50
4	16.67	66.67	16.67
5	100	0	0
6	100	0	0
7	50	50	0
8	0	100	0
9	0	0	100
10	0	0	100
11	50	0	50
12	50	50	0
13	66.67	16.67	16.67
14	16.67	16.67	66.67

The statistical analysis and graphs were done to present the findings using MS Excel 2016 and Design Expert 11 software (Stat-Ease Minneapolis, USA). An analysis of variance (ANOVA) was performed to identify whether the co-digestion process led to any significant difference in the

methane yield potential of the BMP tests. The three-dimensional surface response and contour plots showing the interactive effects on each response were generated.

4.6.3 Anaerobic co-digestion BMP test procedure

BMP tests were conducted to obtain the optimal co-substrate ratio and waste combinations that improve the biomethane production of fish sludge. The fish sludge was co-digested with co-substrates according to the experimental mixture design presented in **Table 6**. A total of thirteen batch anaerobic co-digestion of various mixture proportions were set up, and biogas yield was recorded for 25 days.

The experiment tests were conducted in a 600 mL glass batch bioreactor of AMPTS II with a working volume of 400 mL. All the experiments were performed in triplicate under the same conditions described in section 4.5. The ISR of 2:1 based on VS was applied in all the runs, and the AMPTS II system setup was carried out as described previously in the first experiment in section (4.4.1).

The proportions of all different substrates compositions in each mixture were based on the VS (%). In this study, the TS concentration was kept at 10% as it is reported to be the optimum substrate loading for wet anaerobic co-digestion (Abbassi-Guendouz *et al.*, 2012). During the substrate mixtures preparation, food waste and fruits & vegetable waste were diluted with appropriate volume of distilled water to attain the desired total solids content (~10%) of the wet anaerobic digestion process (Abbassi-Guendouz *et al.*, 2012; Shuang *et al.*, 2017). The TS of the fish sludge was not diluted because it was very low at 1.9%. The mixtures were characterized for the TS, VS content, C/N in relation to the resulting contribution % VS of the total VS in the calculated mixtures.

Control assays containing pure cellulose, and blank assays containing only the inoculum were used to estimate methane production from the substrate. During the experiment, biomethane yields, biogas yields, cumulative biogas, and biomethane production were monitored daily. Samples were taken after the experiment to analyse the pH, VS removal (%), VFA, and alkalinity to evaluate anaerobic digestion efficiency.

4.7 Evaluation of synergistic and antagonistic effects in BMP co-digestion measurements

The synergistic and antagonistic effects of the mixtures substrates were determined to evaluate the performance of the anaerobic co-digestion process with regards to biomethane production.

The synergistic or antagonistic interaction effects were obtained by comparing the experimental biomethane potential of the co-digested substrate with the weighted average biomethane potential (BMP_w) of the pure substrate (Ebner *et al.*, 2016; Castro-molano *et al.*, 2018). The weighted biomethane potential of the individual substrates was calculated using Eq.(10) (L. M. Cárdenas-Cleves and Al, 2018).

$$BMP_w = BMP * FS\,(\%) + BMP * FW(\%) + BMP * FVW(\%) \quad (10)$$

Where, BMP_w refers to the weighted biochemical methane potential, BMP is the experimental BMP value obtained from the mono-digestion of FS (100%), FW (100%), and FVW (100%) respectively. Synergistic and antagonistic effects were determined using Eq. (11) (Awosusi *et al.*, 2021) as follows:

$$CDI = {}^{BMP_{ACoD}}/_{BMP_w} \quad (11)$$

Where BMP_{ACoD} refers to the experimental BMP obtained from binary or trinary ratio during co-digestion. If the co-digestion index (CDI) > 1, the mixture indicated synergistic interaction effects. If the CDI <1, the mixture indicated antagonistic interactions effects. If the CDI =1, the interaction effects of the mixture combinations of the co-digestion of the substrates were undefined or no interactions.

4.8 Model validation experiments for BMP co-digestion tests

The additional anaerobic co-digestion experiment of the selected optimum mixture compositions obtained in this study was conducted to validate the reliability of the special quartic regression model generated by the simplex centroid mixture design. To validate the predicted SMY and PVSR values by the models, the validation experiment tests of the optimum mixture compositions (63% FS: 18 % FW: 19% FVW) and (40 % FS: 41 % FW: 19% FVW) were performed using 600 mL AMPTS laboratory scale bioreactors. The AMPTS system setup of the validation runs was carried out as described previously in section (4.4.1). After that, the laboratory scale experiment tests of the optimum mixtures were up-scaled to 50 L batch and 30 L semi-continuous digesters.

4.9 Batch scale-up experiment (50 L) anaerobic digester study

A batch scale-up experiment was carried out to validate the optimum co-substrate ratio achieved at the bench scale and evaluate the synergistic effect of the co-substrates on the process

performance efficiency when the volume of digester increased. Therefore, for the up-scaling capacity, the study was conducted using 50 L anaerobic digester (**Figure 3**) with a working volume of 35 L, which corresponds to 70% of the digester capacity. The digester was equipped with an inlet and outlet ports for feeding and effluent discharge, biogas sampling port, hot water jacket for temperature control, stirrer motor, biogas sensor, and gas effluent port to the gas collecting system. The bioreactors were connected to the gas collecting system, which records the daily biogas production rate via the manometer-based online gas measurement system. The digesters were filled with active inoculum and mixture ratios of the substrates based on VS content. The digesters were flushed with nitrogen gas for 3-5 minutes to initiate the anaerobic condition. The experiment was conducted for 30 days at a mesophilic temperature of 37 ℃ and a low-speed mixing of 150 rpm. Experiments were conducted in duplicates.

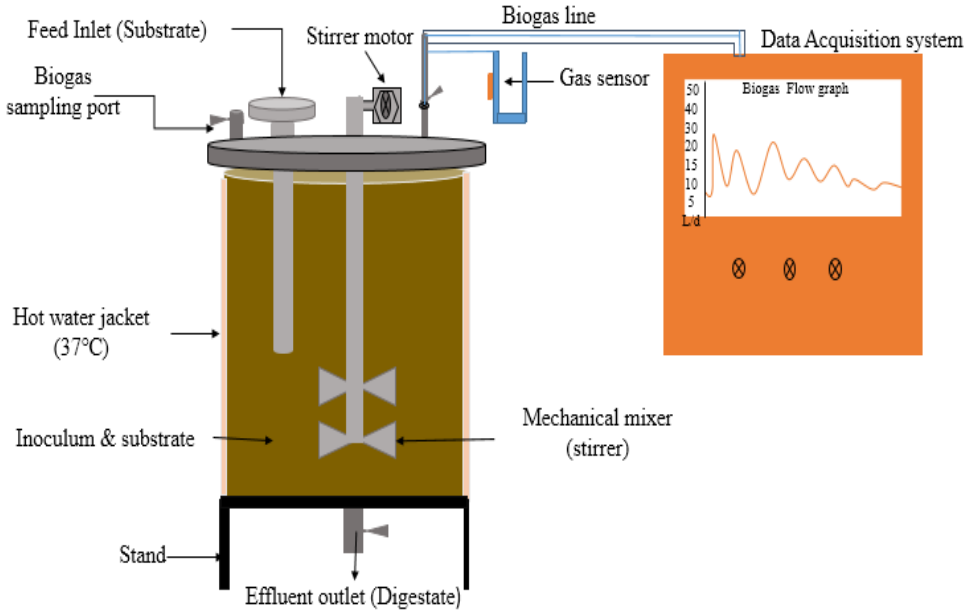


Figure 3: Schematic view of 50 L batch pilot-scale digester of anaerobic co-digestion of fish sludge, food waste, and fruit and vegetable wastes.

The biogas samples were collected daily in Tedlar bags for gas composition analysis. The biogas production, methane content, alkalinity, VFA, ammonium nitrogen, and pH were measured every two days to evaluate the performance and stability of the digesters, using the same analytical methods as described in section 4.3. The performance of the anaerobic co-digestion process was

evaluated based on the biogas and biomethane yields, organic matter efficiency (VS removal %), stability indicators of VFA, alkalinity, and ammonium nitrogen.

4.9.1 Analysis and Calculations

Gas Chromatography (GC)

The biogas composition such as methane content (%) was measured using a Compact GC 4.0 Gas chromatography (Global Analyser Solution) with a built-in thermal conductivity detector (TCD) and a 3 m stainless column packed with porapak Q (60-80). The operational temperatures of the injection port, column oven, and detector were fixed at 60°C, 50°C, and 110°C, respectively. The carrier gases used in this Compact GC were helium and argon at flow rates of 5 mL/min. The GC instrument used was composed of three channels namely channel 1, 2, and 3. Each channel was contained a sample loop, column, and detector. For biogas composition (H_2 O_2, N_2, and CH_4) analysis channels 2 & 3 were used with a TCD detector. Before switching on the detectors, the columns (channel 2 & 3) were allowed (approximately 20 minutes) to increase from 50°C until reaching the required conditions of FID temperature (160^{o}C) and TCD temperature (110°C). The detectors for channels 2 & 3 were switched on and autozero after the required conditions were attained. Before starting the analysis of the samples, an air test was also performed to check the nitrogen percentage (75%). A nitrogen percentage of air test less than the optimal range of 74-75% indicates that the GC machine is not yet stable. Biogas of ~2 mL of the filtered sample was directly injected with a syringe into the tube at the front of the Compact GC. The chromatograph calibration curves were used to determine the primary biogas standard with H_2 O_2, N_2, and CH_4composition. Chromatographic Data Acquisition System Chromeleon chromatography studio was used for data processing.

The biogas composition was analysed throughout the digestion process period three days per week. For laboratory-scale experiments, 50 mL vaclok vacuum pressure syringes were used to collect the biogas samples within the headspace of the AMPTS bioreactors. Biogas sample of ~15 mL from each reactor was collected from the headspace of the bioreactors. For pilot scale digesters, biogas samples were collected within the headspace of the digesters through the gas sampling port using 50 mL vaclok vacuum pressure syringes. All measurements were performed in duplicate or triplicate for greater reliability and validating of the results. The average results were reported.

BIOGAS 5000 analyzer

Due to technical problem experienced with Compact GC equipment during the anaerobic co-digestion study. The biogas compositions were not measured in BMP tests. The biogas composition was analysed during the up-scaled batch and semi-continuous pilot-scale studies. The biogas compositions (H_2S, O_2, CO_2, N_2, and CH_4) of the digesters were measured by a BIOGAS 5000 analyzer (Geotech, UK) (**Figure 4**). The biogas composition production from the digesters was analysed daily.

Figure 4: 5000 Biogas analyzer

Biomethane yield

The biomethane methane yield (SMY_{CH4}, LCH_4/g VS) was calculated according to equation (12) based on the organic loading rate added to the digester.

$$SMY_{CH4} = \frac{CM}{gVS} \quad (12)$$

Where: SMY= biomethane yield in g/L VS; CM= Cumulative methane production (L); gVS Volatile solids concentration added to each reactor (g)

Theoretical Methane Potential (Buswell Equation)

The theoretical methane potential of the fish sludge, food waste, fruit & vegetable waste, and their mixtures was determined from the elemental composition of the substrates by using Buswell equations (13).

$$C_cH_hO_oN_n + \left(c - \frac{h}{4} - \frac{o}{2} + \frac{3n}{4}\right)H_2O \rightarrow \left(\frac{c}{2} + \frac{h}{8} - \frac{o}{4} - \frac{3n}{8}\right)CH_4 + \left(\frac{c}{2} - \frac{h}{8} + \frac{o}{4} + \frac{3n}{8}\right)CO_2 + n.NH_3$$

$$\text{TBMP} = \frac{1000 \times 22.4 \times \left(\frac{c}{2} + \frac{h}{8} - \frac{o}{4} - \frac{3n}{8}\right)}{12c + h + 16o + 14n} \frac{\text{mLCH}_4}{\text{gVS}} \quad (13)$$

Anaerobic biodegradability index

The anaerobic biodegradability index (ABI %) was calculated according to Eq. (14):

$$\text{ABI (\%)} = \frac{BMP}{TBMP} \times 100 \quad (14)$$

Where: TBMP is the theoretical methane determined using the equation as presented by the Buswell equation.

Daily methane production

The amount of daily methane production rate (L/day) was calculated by multiplying the methane content with the biogas production rate using Eq. (15) shown below:

$$\text{CH}_4\ \text{(L)} = \frac{\text{CH}_4\ \%}{100} \times biogas\ flow\ rate\ (l/d) \quad (15)$$

The methane production rate was determined using Eq. (16) presented below:

$$Methane\ production\ rate = \frac{L\ of\ methane\ per\ day}{V_{digester}} = \frac{\text{CH}_4\ \%}{100} \times \frac{biogas\ flow\ rate}{V_{digester}} \quad (16)$$

VS Reduction

The volatile solids reduction indicates the percentage removal of VS during the anaerobic digestion process. The VS reduction efficiency was determined using the following eq.(17) (Rao and Baral, 2011; Browne *et al.*, 2014).

$$VS\ reduction = \frac{VS_{in} - VS_{out}}{VS_{in}} \times 100\% \quad (17)$$

Where: VS_{in} is the amount of volatile solids initially added (g); and VS_{out} is the amount of volatile solids taken out (g) at the end of the anaerobic digestion.

4.10 Semi-Continuous co-digestion experiment: 30 L CSTR anaerobic digester

The pilot-scale semi-continuous co-digestion experiment was performed in order to evaluate the performance of anaerobic co-digestion at the optimal co-substrate ratio combination and to explore the continuous operating conditions such as the organic loading rate (OLR) and impacts on the stability of the process, biogas production rate, the reduction of organic matter, and monitoring stability indicators (VFA, pH, alkalinity), biogas production rate, the reduction organic matter. Based on the results of the BMP batch co-digestion experiments, the semi-continuous test was carried out using a 30 L digester with a 21 L working capacity, which consisted of a gas sampling port, feed inlet, and a sampling digestate outlet, mechanical agitator, and biogas effluent port to the gas collecting system. **Figure 5** illustrates the schematic view of one of the pilot-scale digesters used to setup the semi-continuous co-digestion experiment. The digester temperature was maintained at a mesophilic condition of 37 °C with intermittent mixing at a low stirring rate of 150 rpm for a minimum of 45 minutes per day controlled by programming system. The digester was fed with the validated optimum mixture ratios of fish sludge and co-substrates. The organic loading rate was evaluated from 1-3 gVS/L day. The feeding pattern of the digester was done manually once a day. However, the loading rate was also depending on the health condition of the digester system to avoid an acid crash. The semi-continuous co-digestion study of a mixture (63 % FS+ 18% FW+ 19% FVW) was conducted for 52 days. The digester was operated at an HRT of 15 days for each OLR based on the retention time obtained from previous batch digester results. The digester headspace was flushed with nitrogen gas for 3-5 minutes to ensure anaerobic conditions. The digesters were connected to the gas collecting system, which records the daily biogas production via the manometer-based online gas measurement system. The cumulative biogas production, methane content, and biogas yield were analysed. The biogas compositions and pH of the digester were measured every day. Different parameters such as total VFA concentration, ammonium, and alkalinity were analyzed twice per week by collecting samples analysis before feeding the digester with a fresh substrate to assess the health and process stability of the reactor using the same methods as described in section 4.3. When one of the parameters is out of range for AD, it can affect the microbial growth activity, resulting in lower biogas and methane production (Pagés-díaz *et al.*, 2015).

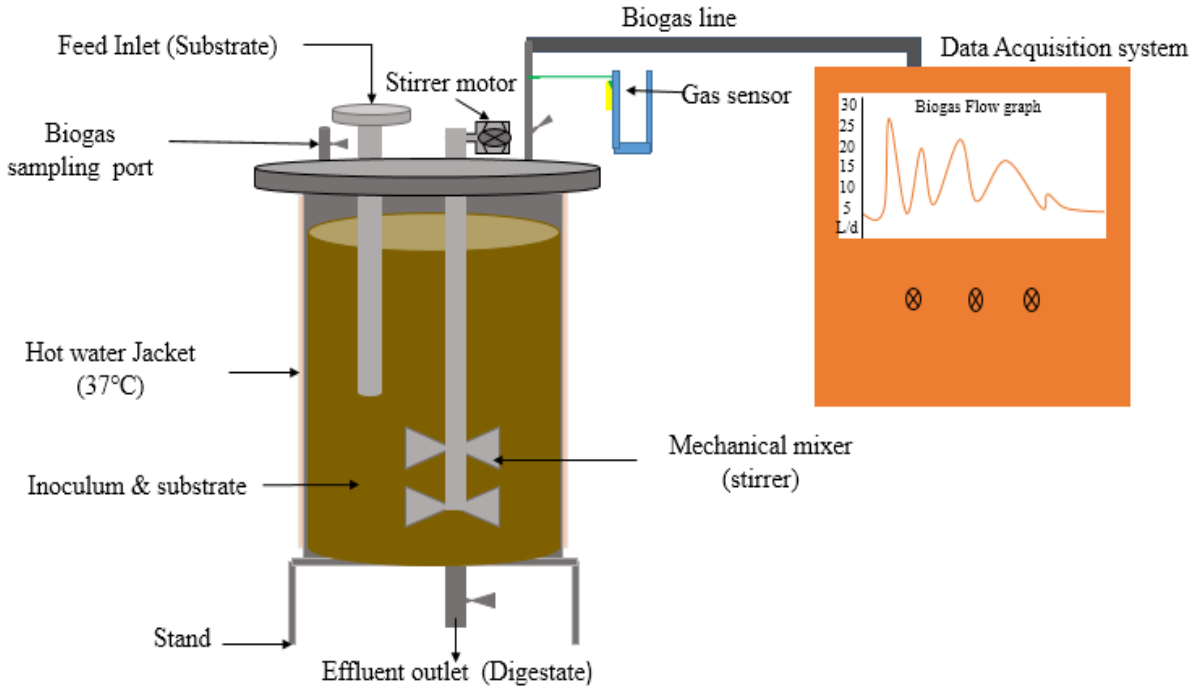


Figure 5: Schematic view of 30 L pilot-scale semi-continuous digester used during anaerobic co-digestion of fish sludge, food waste, and fruits and vegetable waste.

The **organic loading rate** (gVS/L.day) was determined as the amount of organic matter fed to the digester per day using equation (18):

$$\boldsymbol{OLR} = \frac{\boldsymbol{Q.S}}{\boldsymbol{V}} \qquad (18)$$

Where: Q is the daily flow rate of the substrate (L/d); V is the volume of the digester (L), S is the substrate concentration (gVS/L).

The **hydraulic retention time (HRT)** for an OLRs was determined using Eq. (19)

$$\boldsymbol{HRT} = \frac{\boldsymbol{V}}{\boldsymbol{Q}} \qquad (19)$$

5 CHAPTER 5: RESULTS AND DISCUSSION

5.1 Characterization of the substrates

The compositions of the fish sludge, food waste, and fruit & vegetable waste used in this study are presented in **Table 7**. As shown in **Table 7**, the highest total solids content was obtained in the food waste (38.97%), followed by fruits &vegetables waste (16.38%), with the lowest in fish sludge (1.97%). The TS content represents both organic and inorganic dry matter either in percentage (%) or concentration, whereas the volatile solids denote the organic matter of the feedstock (Meegoda *et al.*, 2018). The percentages of volatile solids in fish sludge, food waste, and fruit & vegetable waste were 1.48%, 15.3%, and 36.7%, respectively. Moreover, based on the total dry matter content, the VS/TS ratios of all the substrates chosen in this study ranged from 75-94.32%, indicating the potential for high biodegradability and suitability for the anaerobic digestion (Wang *et al.*, 2013; Gomes *et al.*, 2017; Blasius *et al.*, 2020). The high biodegradable content of food waste and fruit & vegetable waste observed in this study was comparable to the VS/TS ratio of 85% and 94.01% reported by (Zhang *et al.*, 2007; Lee and Jahng, 2011). The C/N ratio, pH, moisture content of the food waste, and fruit and vegetable waste reported in this study were also consistent with previous studies (Zhang *et al.*, 2013; Tian *et al.*, 2015). The food waste and fruits & vegetable waste fell under the acidic range of pH 4.5-5.3, which was typical below the optimum range (7.0-7.2) indicated as ideal for the growth of the microorganism, involved in the anaerobic digestion process, particularly the methanogens. Blasius *et al.* (2020) observed a similar pH of 4.73 during the performance of anaerobic digestion of food waste.

As expected, fruit and vegetable waste had a higher C/N ratio (40.04) than food waste and fish sludge (17.04 and 6.11, respectively), but it lacked acid buffering capacity (alkalinity). Fish sludge had the lowest C/N ratio compared to the other substrates, indicating a higher potential for ammonia formation during anaerobic digestion. This suggests that combining fruits and vegetable waste and food waste as co-substrates with fish sludge can optimize the C/N ratio to an optimum range for the AD process.

Previous studies suggested that the C/N ratios of the substrates should be in the range of 20-30:1 for optimal biogas production (Gomes *et al.*, 2017; Blasius *et al.*, 2020). The fish sludge used in this study had similar characteristics in terms of TS, pH, VS/TS ratio, and C/N ratio to the fish sludge reported by (Mirzoyan *et al.*, 2008), which had TS between 1.5-2.2%, pH 7.5, and VS/TS

of 75.6% except for the C/N ratio (15.4), which was higher than the C/N ratio reported in the current study. This could be due to the difference in the compositions of feed and the fish.

Table 7: Characteristics of the raw substrates used in this study.

Parameters	**Fish sludge (FS)**	**Food waste (FW)**	**Fruits & vegetable waste (FVW)**
pH	8.4	4.7	4.9
TS (% w/w)	1.97 ± 0.04	38.97±0.2	16.38±0.9
VS (% w/w)	1.48 ± 0.04	36.76±0.4	15.38±0.02
VS/TS ratio (% w/w)	75 ± 0.04	94.32±0.00	93.93±0.1
Moisture content (% w/w)	98.03±0.1	61.03±0.23	83.62±0.3
Ash (% w/w)	0.30±0.03	4.81 ±0.13	6.99±0.03
Carbon, C (% of TS)	36.62	53.0	45.6
Hydrogen, H (% of TS)	6.27	6.1	6.7
Nitrogen, N (% of TS)	5.99	3.1	1.1
Sulphur, S (% of TS)	1.57	-	
C/N ratio	6.11±0.02	17.04±0.01	40.04±0.02
VFA (mg/L)	573 9±06	7680 ±0.02	4204 ±0.06
Carbohydrate (% w/w)	< 1	24.1±07	14.2±0.05
Fat (% w/w)	<0.50 ± 003	8.21±0.3	0.51±0.6
Total protein (% w/w)	<0.25 ± 0.7	6.61±0.23	1.49±0.01
Crude Fibre (% w/w)	<0.5 ± 0.04	<0.5±0.06	3.38±0.04
Alkalinity (mg $CaCO_3$/L)	1773 ± 31	11.5±1.35	324±12.75
Hemicellulose (%)	-	-	7.75±0.25
Cellulose (%)	-	36.70±0.38	21.14±0.24
Lignin (%)	-	30.68±0.04	33.23±0.02

WW: wet weight. Values reported are the average and standard deviation of triplicate.

Fish sludge had a C/N ratio that is lower than the adequate range, indicating a high excess of nitrogen and a lack of carbon. Lack of carbon sources within the feedstock affects the microbial growth communities and biomethane production rate (Hegde, 2019). The carbon contents of food waste and fruit and vegetable waste were relatively high, at 53.0 % and 45.6 %, respectively. Substrates rich in carbonaceous require additional nitrogen sources for the growth of methanogens, and to stabilise the pH and VFA concentrations so that the intermediate metabolites produced from hydrolysis and acidogenesis process can be consumed by methanogens (Mei *et al.*, 2016). These results suggested that mixtures of food waste, and fruit and vegetable waste could be good carbon sources during anaerobic co-digestion with high nitrogen content in the fish sludge. This indicates that the excess carbon from FVW could compensate for the lack of carbon in the FS.

The low total alkalinity and pH in fruits & vegetable waste and food waste indicated a low buffering capacity of the system during the anaerobic digestion process (Blasius *et al.*, 2020). As a result of VFAs accumulation, the anaerobic digestion process may become unstable or inhibited. The fish sludge used in this study had the highest pH (8.4), consistent with a high total alkalinity content of 1773 mg/L **(Table 7**), indicating high buffering capacity. Food waste used in the present study had higher carbohydrate, fat, and total protein compared to fish sludge and fruits & vegetable waste used as shown in **Table 7** above. The cellulose content in the food waste was higher than in the fish sludge and fruits & vegetable waste. However, fruit &vegetable had the highest contents of lignin (30.68%), crude protein, and hemicelluloses (7.75%) (**Table 7**). Hemicellulose (7.75%) of fruits &vegetable waste observed is similar with 9.24 % cited by (Bharati *et al.*, 2019). Biomass containing cellulose and hemicellulose is considered suitable for co-digestion with waste sludge for enhanced biogas and methane production (Li *et al*., 2021). This indicated that the three different substrates combination could enhance the biodegradability, biogas production, and stability of the anaerobic digestion process significantly compared to mono digestion.

5.2 Anaerobic co-digestion study results: BMP results

5.3.1 Model fitting and analysis

The experimental data obtained from the anaerobic co-digestion after the retention time of 26 days based on the simplex centroid mixture design (**Table 8**) were used to find the best mixture proportions of fish sludge, food waste, and fruits & vegetable waste on the target response of specific methane yield and volatile solids removal response.

Table 8: The simplex centroid design method used to determine the mixture ratio of the three samples based on 100% volume.

		Mixture proportion (%)			Responses	
Standard run/Reactor	**Run**	**FS**	**FW**	**FVW**	**SMY (NmL CH_4/gVS)**	**PVSR Reduction (%)**
7	1	33.33	33.33	33.33	435.28	50
2	2	0	100	0	438.30	40
6	3	0	50	50	452.43	63.27
9	4	16.67	66.67	16.67	508.38	40
11	5	100	0	0	48.45	50
1	6	100	0	0	51.43	60
14	7	50	50	0	429.28	44.44
12	8	0	100	0	441.32	40
13	9	0	0	100	369.74	60
3	10	0	0	100	394.16	60
5	11	50	0	50	287.70	60
4	12	50	50	0	433.28	44.44
8	13	66.67	16.67	16.67	379.17	64.14
10	14	16.67	16.67	66.67	323.18	57.35

Fish sludge (FS), Food waste (FW), Fruit & Vegetable waste (FVW).

The independent parameters and the response variables were fitted to linear, quadratic, special cubic, and special quartic models to evaluate the interactive effects on the three substrates on

specific methane yield and VS removal (Rao and Baral, 2011; Shuang *et al.*, 2017). The models were subjected to regression analysis of variance to assess the fitness and adequacy. The parameters are summarized in **Table 9**. The best model was chosen based on the highest statistical parameters values of R^2 (coefficient of determination) and adjusted R^2 with $p < 0.05$, coefficient of variation, the lowest value of the standard deviation of the residuals, and lack of fit (Rao and Baral, 2011; Borhan *et al.*, 2014; Shuang *et al.*, 2017; Sukhesh, Muske and Rao, 2019).

As shown in **Table 9**, the special quartic model was suggested as the best-fitted model with the coefficient of determination R^2 (0.9997), predicted R^2 (0.9627), adjusted R^2 (0.9992), and the lowest value of standard deviation (4.04). The R^2 and predicted R^2 of the special quartic model were close to 1, indicating the accuracy and robustness of the model (Gunes *et al.*, 2021). A study by Rao and Baral, (2011) on the response optimization of mixture proportion for anaerobic co-digestion of cow dung, sewage waste, fruit juice wastewater, and garden waste reported an R^2 and adjusted R^2 of 0.9643 and 0.9384, respectively, which is similar to the results obtained in the current study. Therefore, the special quartic model was further used to justify the model and predict the specific methane yield values under the optimum mixture proportions.

Table 9: Summary of model statistics for methane production.

Source	Standard deviation	Regression R^2 (%)	Adjusted regression R^2 (%)	Predicted R^2 (%)	PRESS
Linear	86.78	0.6831	0.6255	0.4607	1.410E+05
Quadratic	42.30	0.9452	0.9110	0.8962	27125.45
Special Cubic	44.59	0.9468	0.9011	0.6510	91241.24
Special Quartic∗	**4.04**	**0.9997**	**0.9992**	**0.9627**	**9756.08**

R^2: coefficient of determination; PRESS predicted residual sum of squares; ∗ was considered as best-fitted model.

5.3.2 Model analysis on specific methane yield (SMY) from BMP tests

The results of the ANOVA analysis for a quartic model on specific methane yield are shown in **Table 10.** The model terms adequacy and significance were determined by the p-value ($p<0.05$) and F-value. The p-value and F-value of the special quartic model observed were 0.0001 and

2000.58, respectively. The $p < 0.05$ observed in this study indicated that the model terms are significant and could explain 99% of the variation observed in the response (Sukhesh, Muske and Rao, 2019). The model F-value indicates that there is only a 0.01% chance that a "F-value" this large could occur due to noise. The adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Therefore, the ratio of 140.527 obtained by analysis of variance in this study indicates an adequate signal of the model. This also indicates that the model can be used to navigate the design space. The coefficient of variance of the model was observed to be 1.13% (**Table 10**), indicating that the experimental data results are accurate and consistent with the predicted values obtained in the present study.

Table 10: Analysis of variance of the special quartic model evaluated for SMY response.

Source of Variation	Sum of Squares	DF	Mean Square	F-value	p-value	
Model	2.613E+05	8	32665.12	2000.58	< 0.0001	significant
Linear Mixture	1.786E+05	2	89284.03	5468.21	< 0.0001	
AB	47568.11	1	47568.11	2913.32	< 0.0001	
AC	4326.27	1	4326.27	264.96	< 0.0001	
BC	1418.03	1	1418.03	86.85	0.0002	
A^2BC	7725.71	1	7725.71	473.16	< 0.0001	
AB^2C	689.47	1	689.47	42.23	0.0013	
ABC^2	10079.28	1	10079.28	617.31	< 0.0001	
Residual	81.64	5	16.33			
Lack of Fit	81.64	1	81.64			
C.V. (%)	1.13					
Adeq Precision	140.5269					
Std. Dev.	4.04					

DF=Degree of freedom; $p > 0.05$ assumed not significant; $p < 0.05$ assumed significant. A: Fish sludge; B: Food waste; C: Fruits & Vegetable waste; C.V: Coefficient of Variance

The regression coefficients estimate represented the expected change in response per unit change in factor value when all remaining factors are held constant. The positive sign of estimated

regression coefficients represents the synergistic effects, while the negative sign of coefficients represents an antagonistic interaction effect between the independent variables and the responses (Pagés Díaz, 2014; Shuang *et al.*, 2017; Rahman *et al.*, 2019). The estimated regression coefficients and p-values result of the special quartic model for specific methane yield response are shown in **Table 11**.

All the model terms were significant (linear, quadratic, and quartic) with *p*-values of less than 0.05. The coefficient of teams ABC^2 was antagonistic, although there was significant interaction because of its $p < 0.05$. The mixture component of A^2BC had the highest coefficient (8686.48) indicates a strong synergistic interactive effect of the three substrates compared to linear and quadratic terms on the response parameters. In essence, a high synergistic effect on specific methane yield was observed when the three substrates were co-digested simultaneously in a mixture proportion containing higher fish sludge with an equal amount of food waste and fruits & vegetable waste. Generally, the interactive effects of the combination of three substrates were found to be more significant than mono-digestion and binary mixtures in terms of methane yield. This means that the proper mixture combination of the three substrates with different chemical compositions is required to enhance the specific methane yields. Co-digesting different substrate proportions with different chemical characteristics such as fats, proteins, carbohydrates, and C/N ratios can supply all the required nutrients by the methanogens in the digester (Pagés Díaz, 2014). Anaerobic co-digestion process has previously been proved to be a good option for treating different substrates simultaneously in order to improve methane production (Pagés Díaz, 2014).

In order to develop the regression coefficients equation from the model, all the non-significant effects must be eliminated (Rahman *et al.*, 2019). Based on the ANOVA results generated by the mixture design (**Table 11**), the second-order polynomial regression equation of the special quartic model for specific methane yield ($NmLCH_4/gVS$) in terms of coded factors value from the coefficient estimation is presented as **Equation 20**. The mathematical model regression equation developed by the simplex centroid mixture design was used to predict the specific methane yield under the co-substrate ratios of fish sludge, food waste, fruits& vegetable waste. **Figure 12** shows the correlation between the predicted values vs actual values of the specific methane yield. The actual specific methane yield and the predicted values were close to the diagonal line indicating no serious deviation. This proves that the developed model analysis was adequate by showing good predictions of the results for response (Gunes *et al.*, 2021).

SMY= 49.27A + 441.32B + 382.2C + 754.90AB + 292.83AC + 167.65 BC + 8683.48 A^2BC + 2594.08 AB^2C −10060.21 ABC^2. (20)

Where, SMY is the methane yield ($NmLCH_4/gVS$), A represents fish sludge, B represents food waste, and C represents fruits and vegetable waste proportions in the mixture.

Table 11: Regression coefficients in terms of Coded Factors for Specific methane yield

Component	**Coefficient Estimate**	**p-value**
A-Fish Waste	49.27	< 0.0001
B-Food waste	441.32	< 0.0001
C-Fruit and Veg	382.27	< 0.0001
AB	754.90	< 0.0001
AC	292.83	< 0.0001
BC	167.65	0.0002
A^2BC	8683.48	< 0.0001
AB^2C	2594.08	0.0013
ABC^2	-10060.21	0.0001

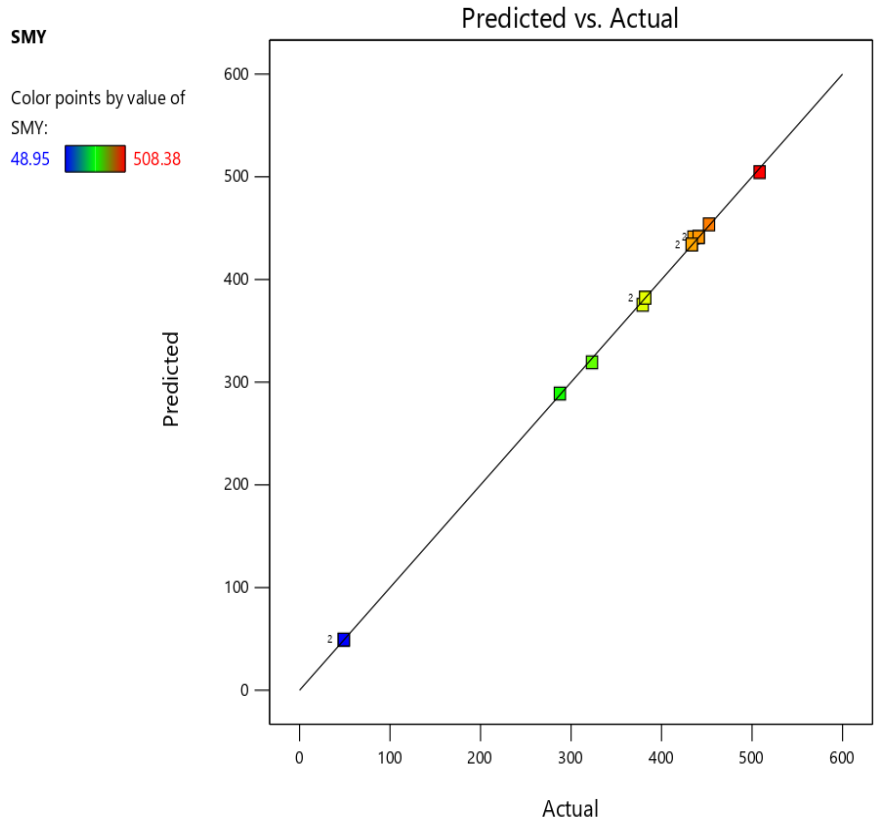


Figure 6: Plot of predicted vs actual values of specific methane yield (SMY) response.

5.3 Model analysis for VS removal (PVSR)

The experimental results of PVSR as a response variable were fitted to the linear, quadratic, special cubic, cubic, and special quartic models (**Table 12**) to obtain the suitable one. As presented in **Table 12,** the special quartic model was also suggested as the best suited for VSR response, with the highest values of R^2, predicted R^2, adjusted R^2, and low standard deviation compared to the linear, quadratic, special cubic models. The best-fitted model was selected based on statistical significance parameters such as coefficient of determination, adjusted R^2, lower standard error, and p-value (Luna-Avelar *et al.*, 2021). For the PVSR response, the R^2, adjusted R^2, and standard deviation of the special quartic model were 0.994, 0.986, and 1.06, respectively. The lower standard deviation value of the model represents the excellent reproducibility of the experimental data (Chakraborty and Uppaluri, 2018). This indicates that the obtained model can further predict the optimum substrate composition for an enhanced VS removal (Luna-Avelar *et al.*, 2021).

The ANOVA result of the special quartic model for VS removal obtained in this study are similar to the results of Rao and Baral (2011), which reported the R^2 of 0.992, Adjusted R^2 of 0.979, and

a lower standard deviation of 0.66 for VS reduction in the optimisation of mixture proportions for the co-digestion of cow dung, sewage sludge, garden waste, and fruit juice wastewater. The significance of the special quartic model and the interactive effects of the variables on VSR response were further evaluated to determine the variability of the model.

Table 12: Summary of full model statistics for VSR

Source	Std. Dev.	Sequential p-value	R^2 (%)	Adjusted R^2 (%)	Predicted R^2 (%)	PRESS
Linear	5.73	0.0028	0.6565	0.5941	0.5087	516.82
Quadratic	5.72	0.4349	0.7513	0.5958	0.2317	808.28
Special Cubic	5.72	0.3511	0.7823	0.5957	-2.0659	3225.22
Special Quartic∗	**1.06**	**0.0001**	**0.9947**	**0.9861**	**0.3634**	**669.69**

R^2: Coefficient of determination; Std. Dev: Standard deviation; ∗ was considered as the best-fitted model.

The ANOVA result for the PVSR model response of fish sludge, food waste, and fruit & vegetable waste is shown in **Table 13**. The F-value of the model for VSR response was 116.70 implying that the model is significant. As shown in **Table 13**, the p-value (<0.0001) of the model was less than 0.05, which confirmed that the model was significant (Kim *et al.*, 2013). The p-values for the model terms AC, BC, A^2BC, AB^2C, and ABC^2 were found as significant terms ($p < 0.05$), however, the interactive model terms AB was not significant (p-value greater than 0.05). The results show that the interaction between the three substrates (quartic term) on VSR was more significant ($p < 0.05$) than the quadratic terms. All the model terms except AB (Fish sludge and food waste) (P =0.6252) showed a significant effect on VSR at the probability level with p-values of less than 0.05. The adequate precision was recorded as 28.99. Adequate precision of greater than 4 indicates the fitness and accuracy of the selected model (Chakraborty and Uppaluri, 2018). The regression analysis results demonstrated that the suggested special quartic model could predict the VSR under the optimum mixture conditions.

Table 13 shows the estimated regression coefficients and statistical significance results obtained by the quartic model in coded factors for VSR (%) response. The coefficient estimate represents

the expected change in response variable per unit change in factor value when all remaining factors are constant. The positive sign of the coefficients in the selected model indicates a synergistic interactive effect, while a negative sign indicates an antagonistic interactive effect of the substrates on the responses (Luna-Avelar *et al.*, 2021).

As shown in **Table 13**, the anaerobic co-digestion of fish sludge, food waste, and fruits & vegetables waste presented synergistic and antagonistic effects on PVSR response. The coefficient quartic term of A^2BC showed a positive effect on the PVSR response, while the quartic term of AB^2C showed the most negative effect on the PVSR response. The quartic terms AB^2C (-1165.33) and ABC^2 (-577.71) had antagonistic effects on the PSVR response but significantly contributed to VS removal with p-values of 0.0001 and 0.0028, respectively. In addition, the regression coefficients terms for AC, BC, and A^2BC demonstrated a positive (synergistic) effect between the interactions of binary or ternary substrates. The mixture component of A^2BC had the highest coefficient (1199.51). The positive coefficient effect in the PVSR response may be due to the synergistic effect of the co-digestion process. As it can be from **Table 14**, all the linear regression coefficients had a positive effect on this response. In contrast, the effect quadratic terms AB was showed an antagonistic effect on the PVSR response, which is also evident in the p-value (0.6252), corroborating the insignificant of the regression coefficients (Rahman *et al.*, 2019; Luna-Avelar *et al.*, 2021).

Table 13: ANOVA for the special quartic model for PVSR response

Source of variation	Sum of squares	Df	Mean Square	*F*-value	*p*-value	
Model	1046.36	8	130.80	116.70	< 0.0001	significant
Linear Mixture	690.66	2	345.33	308.11	< 0.0001	
AB	0.3031	1	0.3031	0.2705	0.6252	
AC	22.26	1	22.26	19.86	0.0067	
BC	147.57	1	147.57	131.67	< 0.0001	
A^2BC	147.42	1	147.42	131.53	< 0.0001	
AB^2C	139.14	1	139.14	124.14	0.0001	
ABC^2	33.24	1	33.24	29.66	0.0028	
Residual	5.60	5	1.12			
Lack of Fit	5.60	1	5.60			
Pure Error	0.0000	4	0.0000			
Cor Total	1051.96	13				
C.V.%	2.05					
Adeq Precision	28.9900					
Std. Dev.	1.06					

Based on the results of ANOVA (**Table 14**) generated using the simplex centroid mixture design, the second-order polynomial regression equation of the special quartic model for VSR (%) response in terms of coded factors values from the coefficient estimation is given in **Equation 20.** The predicted values of VSR were compared with actual values (**Figure 7**). The difference between the actual values of the VS removal percentage response and the predicted values was close to the diagonal line. This indicates that the developed mathematical model analysis was adequate shows good response predictions (Gunes *et al.*, 2021). Therefore, the simplex centroid mixture design can optimize and predict VSR response in the co-digestion of fish sludge, food waste, and fruit & vegetable waste. Only the significant model terms with $p < 0.05$ were used to predict VSR under the selected optimal mixture proportions of the substrates. After eliminating

the non-significant terms, the reduced regression coefficients model equation for the PVSR response variable was given as follows:

$$\boldsymbol{Y_{VSR}} = 50.08\ A + 40.08\ B + 60.08\ C + 21.00\ AC + 54\ .08\ BC + 1199.51\ A^2BC - 1165.33\ AB^2C - 577.71\ ABC^2. \quad \textbf{(21)}$$

Where, Y_{VSR} is the volatile solids removal after the anaerobic co-digestion process, A represents fish sludge, B represents food waste, and C represents fruits and vegetable waste proportions in the mixture.

Table 14: Estimated regression coefficients and statistical significance obtained by the special quartic model in terms of coded factors for VSR (%) response.

Component	**Coefficient Estimate**	***p*-value**
A-Fish Waste	50.08	< 0.0001
B-Food waste	40.08	< 0.0001
C-Fruit and Veg	60.08	< 0.0001
AB	-1.91	0.6252
AC	21.00	0.0067
BC	54.08	<0.0001
A^2BC	1199.51	< 0.0001
AB^2C	-1165.33	0.0001
ABC^2	-577.71	0.0028

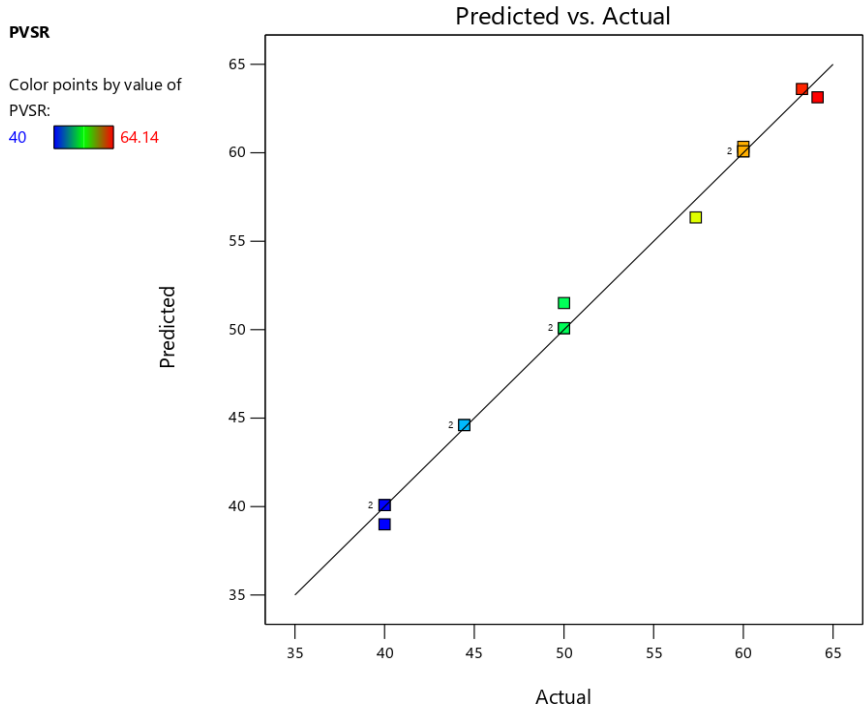


Figure 7: Plot of predicted vs actual values of VS removal response.

5.3.4 Effect of anaerobic co-digestion variables on biomethane yield

The contour and three-dimensional response surface plots were generated to determine the optimum proportion of mixture components and their interactive effects on methane yield, as presented in **Figures 8 and 9**. Contour and surface plots are useful in identifying desirable response values, proper mixture compositions, and studying the interactive effects between different independent substrates and their effects on the target response (Ranjan *et al.*, 2009; Pagés Díaz, 2014; Lian *et al.*, 2021).These plots demonstrate variations of the responses from the lowest values (Dark blue area) to the highest values (Dark red area) depending on the interaction effects between the three substrates (**Figure 8-9**).

The anaerobic co-digestion of the three substrates produced different amounts of specific methane yield depending on the effects of their interactions. The specific methane yields ranged from 48.95-508.38 NmL CH_4/gVS indicating that the mixture ratio of fish sludge, food waste, and fruits & vegetable waste has a great impact on methane production. It is evident from **Figure 8** that methane yield increased with the increase in food waste proportion in the mixtures. Based on the experimental results, the maximum amount of specific methane yield (508.58 NmL CH_4/gVS) was obtained in the mixture ratio consists of 16% FS: 67%FW: 16%FVW (Run 8). The lowest

SMY of 48. 95 NmL CH_4/gVS was obtained at the vertex of fish sludge (100% FS: 0%FW: 0% FWV). The results obtained in this study are similar to the results of Lee et al., (2019), who also obtained the maximum cumulative methane production when a high amount of food waste (46. %) in the mixture ratio was co-digested with sewage sludge (33.6%), and livestock manure (20.4%). The presence of food waste substrate in the mixture proportions plays an important role by balancing the nutrients of the system (Lee et al., 2019). As shown in **figures 8** and **9**, it can be concluded that the SMY range from 250-500 NmL CH_4/gVS could be achieved when three substrates co-digested together. This indicates that food wastes and fruits & vegetable wastes are good co-substrates for anaerobic co-digestion with fish sludge.

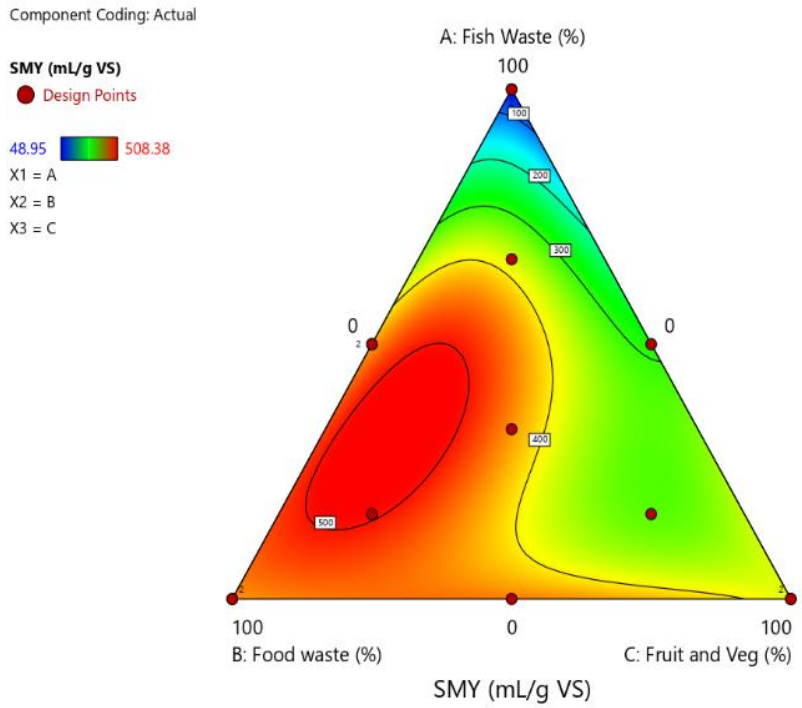


Figure 8: Contour plot showing the interactive effects on Specific methane yield (SMY).

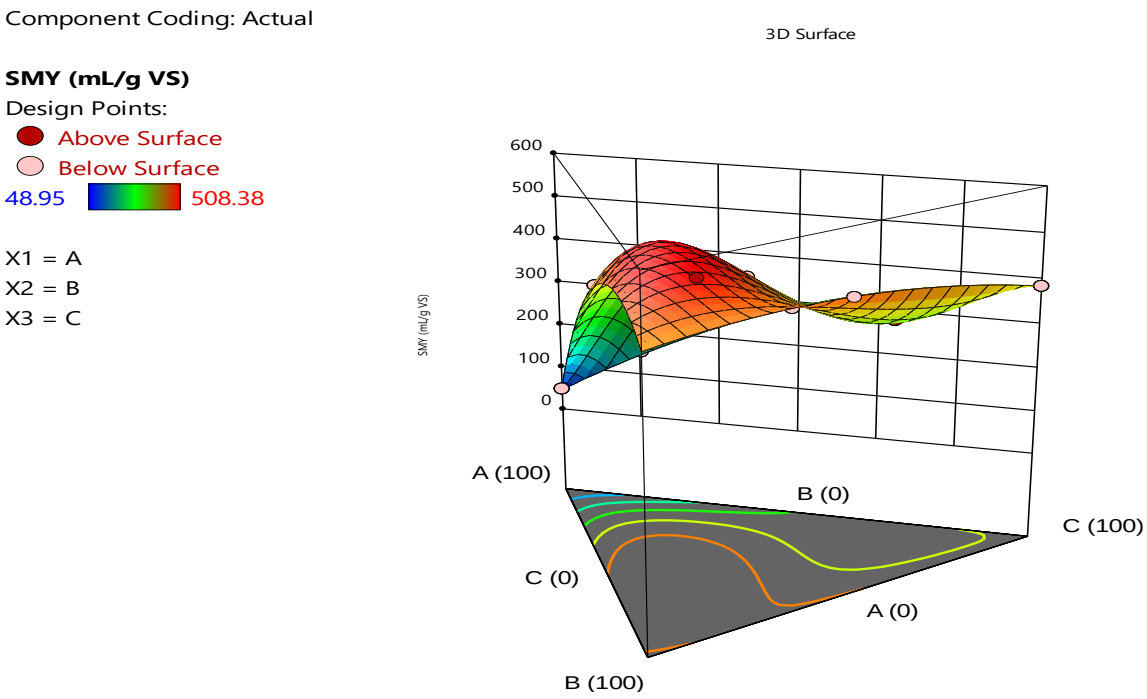


Figure 9: The three-dimensional surface response plot showing the interactive effects on Specific methane yield (SMY).

5.3.5 Effect of anaerobic co-digestion variables on PVSR response

The interactive effects of fish sludge, food waste, and fruits& vegetable waste on VS removal are shown in **Figure 10.** The volatile solids removal represents the efficacy of the substrate digestion (Wongarmat *et al.*, 2021). In contrast with the mono anaerobic digestion of fish sludge, the mixture of fruit & vegetable waste and food waste with the fish sludge significantly decreased the VS removal. However, the minimal effect was observed when food waste was added in high proportion in the mixture (**Figure 11**). The lowest percentage of VS removal of 40% was obtained with substrates proportion of FS: FW: FVW (0:100:0). The organic matter removal was observed to decrease when food waste was added in high proportion. This could be attributed to the presence of non-biodegradable fractions in food waste such as peels and lignocellulosic materials. As presented in Table 9, the food waste used in this study was characterized by high total solids and macromolecular compounds such as carbohydrate, protein, and cellulose compared to fish sludge and fruit & vegetable waste which could be responsible for the lower removal of the organic content (Lee et al., 2019b).

The co-digestion of binary or ternary substrates played a major role in VS removal compared with mono-digestion of food waste. A previous study by Lee et al. (2019b) suggested that the VS

removal of the feedstock can be improved by pretreating the food waste before the anaerobic co-digestion process. For instance, Lee et al 2019 reported They study showed that the pretreatment of food waste before anaerobic digestion improved VS removal by 45 % compared to the non-pretreated substrate (Lee et al., 2019b). It can be seen from **Figure 10** that the maximum VSR could be achieved towards the vertex containing the fruits and vegetables, and fish sludge in high proportion in the mixtures. The PVSR obtained in this study is contradicted with the results reported in the literature. It is cited that high PVSR removal will be directly proportional to high biogas and specific methane production (Lim *et al.*, 2021). This is attributed mainly due to the high organic matter of cellulose and lignin. As previously explained in section 5.1 (**Table 7**), food waste used in this study is composed of high cellulose and lignin content. Anaerobic digestion of feedstock such as food waste used in this study is not easily biodegraded due to high organic matter, especially cellulose and lignin. According to Peyrelasse et al. (2021), substrates characterized by high lignocellulosic, only 40-50% of the organic matter can be converted into biogas production (Peyrelasse *et al.*, 2021). Different studies used pretreatment methods such as physical, thermochemical, biological, alkaline, or both pretreatments in order to improve lignocellulose biodegradability, solubilize carbohydrates content, and improve the biogas and methane production, however, the use of pre-treatment is regarded as uneconomically (Peyrelasse *et al.*, 2021).

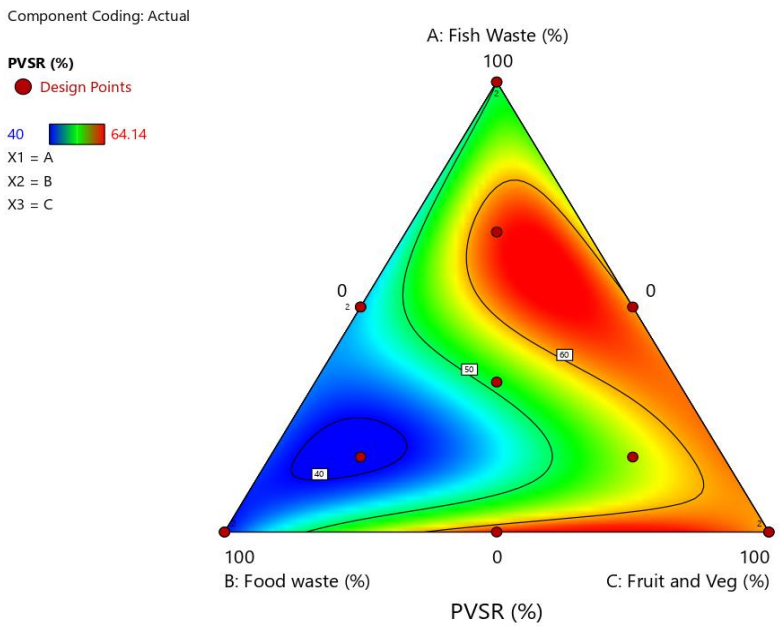


Figure 10: Contour plot showing the interactive effects on VS removal response.

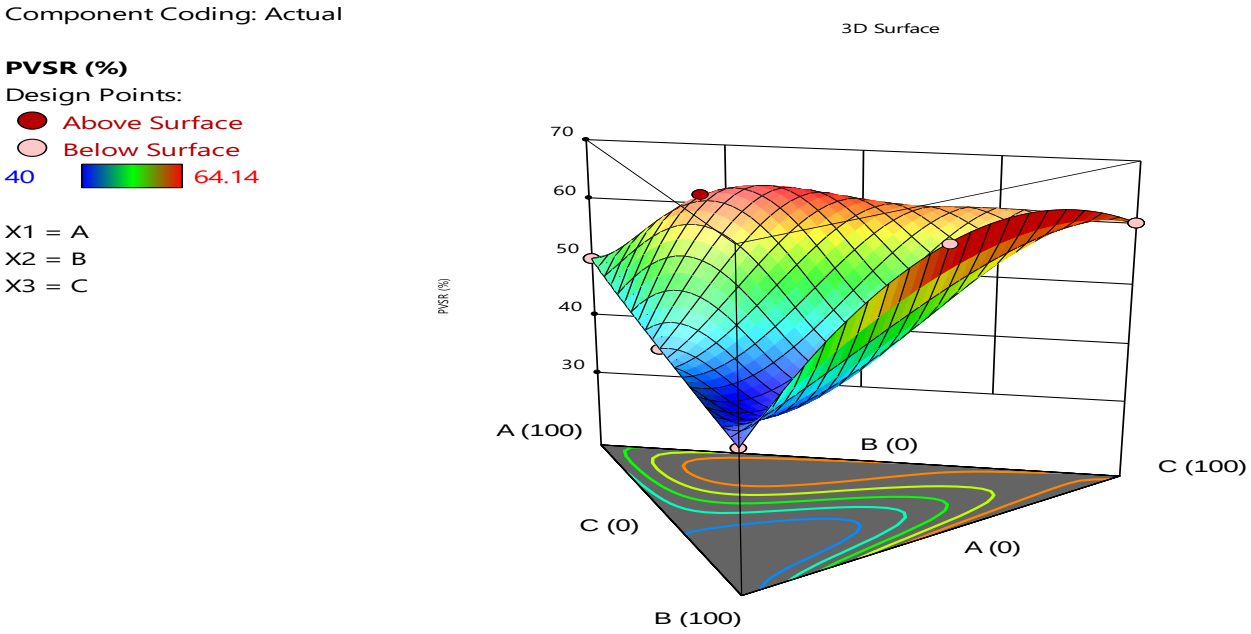
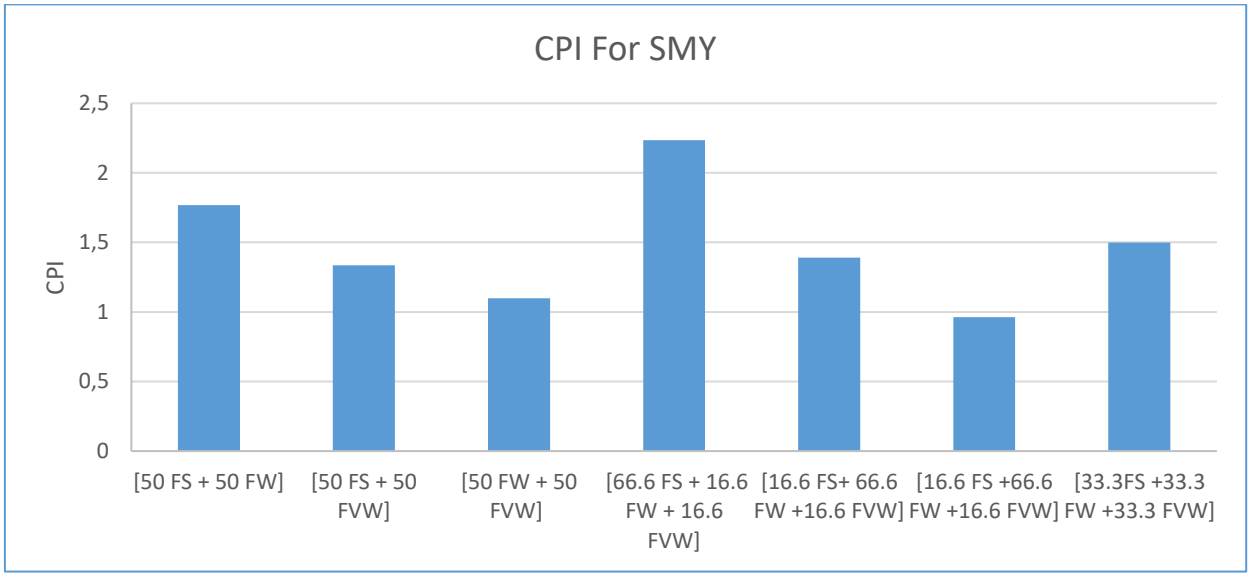


Figure 11: The three-dimensional surface response plot showing the interactive effects on VS removal (VSR).

5.4 Co-digestion Performance Index (CPI) of the substrates mixtures in BMP tests

The co-digestion performance index was also used to study the interactive effect of the substrates on the specific methane yield, as determined through BMP testing. The anaerobic co-digestion of different substrates can lead to synergistic or antagonistic effects. As presented in **Figure 18,** the co-digestion performance index of the co-digested substrates ranged between 0.96 and 2.3. This result shows that the co-digestion of fish sludge, food waste, and fruit & vegetable waste typically had positive synergistic and antagonistic effects on methane production. As previously described, a CPI ratio greater than 1 indicates that the mixture between the substrates had a synergistic effect, whereas, a CPI ratio less than 1 indicates that the mixture had an antagonistic effect (Nielfa *et al.*, 2015). The lowest CPI of 0.96 was obtained in the mixture containing high fruit and vegetable proportions (16 FS: 16 FW: 67 FVW). These results are similar to the one mentioned in section 5.3.1, which showed that although there was a negative impact in the substrate ratios (16 FS: 16 FW: 67 FVW) the interaction was statistically significant. This can be explained by the high C/N ratio and sugar content, leading to rapid accumulation of VFAs concentration and eventually process failure due to inhibition of the microorganisms activity during anaerobic digestion.

A synergistic effect occurs when there is an improvement of biodegradability and additional biogas and methane yield due to balance of C/N ratio, additional alkalinity, dilution of toxic inhibitors, or improvement of nutrients from co-digested substrates over single substrates (Awosusi *et al.*, 2021), while a mixture proportion that showed an antagonistic effect during anaerobic co-digestion resulted in the unstable performance of the digester due to VFA or ammonia accumulation, pH inhibition, corresponding with lower methane production (Pagés-díaz *et al.*, 2015; Bharati *et al.*, 2019).



CPI >indicates a synergistic effect; CPI > indicates an antagonistic effect; CPI = 1 indicates no effect

Figure 12: Co-digestion performance index of anaerobic co-digestion of different mixture proportions of fish sludge, food waste, and fruit & vegetable waste.

The highest CPI value of 2.3 was obtained in a mixture containing high fish sludge with lower food waste and fruit & vegetable waste proportion (**Figure 12**). This phenomenon indicates that the addition of food waste which was characterized by high cellulose, and fruit & vegetable (40 C/N) as co-substrates provides additional organic matter and carbon sources to balance the C/N ratio to the optimum range **(Table 15),** nutrients, and buffering capacity when co-digested with fish sludge (1.97% TS) which lead to improving the biomethane of fish sludge. Hou et al. (2020) reported that the co-digestion of rice straw and rice bran with food waste had a CPI value ranging from 1.03 to 1.24. The benefits of co-digesting two or more substrates simultaneously include increase methane yield, enhancing the VS removal, and reducing inhibitors substances that are detected during the mono-digestion due to synergistic effects that improve process stability (Wongarmat *et al.*, 2021). The experimental results of this study showed that co-digesting fish

sludge, food waste, and fruit and vegetable waste could be a good option to improve biomethane production and process stability for both substrates.

Table 15: Co-digestion evaluation of the synergistic and antagonistic effects

Mixture FS: FW: FVW	SMY ($NmLCH_4/gVS$)	TBMP (mL/gVS)	BMP_w ($NmLCH_4/gVS$)	CPI	C/N	Biodegradability
100 : 0: 0	48.94	554.88			6.11	8.81
0 : 100 : 0	440.99	785.88			17.04	56.11
0 : 100 : 0	381.95	796.22			40.04	47.971
50 : 50 : 0	433.27	812.68	244.97	1.8	16.18	53.31
50 : 0 : 50	287.69	766.69	215.45	1.3	30.68	37.52
0 : 50 : 50	452.43	830.41	411.47	1.1	18.24	54.48
67 :16 : 16	379.16	809.3	169.73	2.2	17.11	46.85
16 : 67 : 16	508.38	825.51	365.49	1.3	16.00	61.58
16 : 16 : 67	323.87	828.41	336.43	0.96	23.26	39.09
34 : 33 : 33	435.27	812.49	290.34	1.5	17.97	53.52

BMP_w was calculated from the SMY of the single substrates in each substrate contained in the mixture proportions, SMY represents the experimental methane yield obtained from each mixture proportion; TBMP: calculated based on chemical composition (C, H, O, N).

5.5 Optimisation of co-substrate ratio and model validation from BMP tests

The main objective of the response optimisation process using the experimental mixture design is to find the optimal combination of components that maximizes or predicts a single response or a set of responses variables (Ranjan *et al.*, 2009; Pagés Díaz, 2014). In the present work, the simplex centroid mixture design was used to find the best mixture proportions of the three substrates (using fish sludge as a major proportion) that optimizes both responses of the specific methane yield (SMY) and the volatile solids removal (VSR) simultaneously. The two optimum mixture solutions were selected in order to validate the model. The first solution was to maximize the use of fish sludge in the mixture composition with a minimum amount of food waste and fruits & vegetables waste to achieve higher biomethane yield and VS removal. As previously described, fish sludge

is used as the primary substrate while fruit & vegetable waste and food waste are used as co-substrates. The optimum mixture composition predicted by the mixture design consists of 63% FS: 18 % FW: 19 % FVW with the predicted SMY and VSR response of 401.5 NmL CH_4/gVS and 63.20%, respectively.

The second selected mixture was based on obtaining the best optimum mixture combination that maximizes both responses (SMY and VSR) simultaneously without limiting any substrate. The second optimum mixture composition was consists of 40 % FS: 41 % FW: 19% FVW with the predicted SMY and VSR values of 513 NmL CH_4/gVS and 50.3%, respectively.

The SMY of 384 $\pm$ 1.52 and 492.72 $\pm$ (NmL CH_4/gVS), and VSR of 60$\pm$ 1.3 % and 54 $\pm$2.1% were obtained from the validation experiments under optimal mixture compositions were close with the predicted values of the specific methane yields and VS removal, as shown in **Table 16**. The standard deviation between the experimental and predicted SMY values can be attributed to error from substrate homogenization during the preparation, changes in the inoculum.

As shown in **Table 16**, the relative error between the actual and predicted values of the responses SMY and VSR for solutions were found to be within -4.23-0.97 %, and -5.06-3.37% respectively, indicating that the model's data obtained were effectively accurate to predict the SMY and VSR responses under selected optimum mixture conditions of fish sludge, food waste, and fruits & vegetable waste The findings of solution 1 were consistent with our previous experimental results observed in Run 7 when fish sludge was used as a major proportion. This result indicates that co-digestion of fish sludge with food waste and fruit & vegetable waste could simultaneously enhance specific methane yield and VS removal. At the optimum mixture compositions, the specific methane yield was enhanced compared with mono-digestion of fish sludge.

The results in the present study indicate that methane production from fish sludge can be improved by the addition of food waste, fruit and vegetable waste proportions in anaerobic co-digestion process. It can be concluded that the simplex centroid mixture design model approach used to optimised specific methane yield and VS removal of fish sludge during the anaerobic co-digestion process was successful effective to predict the responses. The two selected optimum mixtures were further validated in pilot-scale digesters.

Table 16: Experimental and predicted response values obtained under selected optimum mixture proportions of fish sludge, food waste, fruit & vegetable waste.

Solutions	Optimal mixture compositions			Experimental results		Predicted values		RE of SMY	RE of PVSR
	FS (%)	FW (%)	FVW (%)	SMY (mL CH_4/gVS)	PVSR (%)	SMY (mLCH_4/gVS)	PVSR (%)	(%)	(%)
Solution 1	63	18	19	384	60	401.65	63.20	-4.23	-5.06
Solution 2	40	41	19	492	54	513	50.30	-4.05	3.37

RE: relative Error

5.6 Anaerobic co-digestion of selected optimal mixtures in batch pilot-scale digesters.

Based on the optimisation of co-substrates ratios obtained from previous section 5.6, the two selected optimal mixture proportions **(Table 16)** were further performed in batch pilot-scale in order to investigate the potential of total biogas and methane yields and also process stability on large volume digesters compare to the laboratory scale BMP results. Therefore, to investigate the performance of anaerobic co-digestion in pilot-scale mode, two batch digesters named MD 1 for mixture 1 (63 % FS: 18 % FW: 19 % FVW) and MD 2 for mixture 2 (40 % FS: 41 % FW: 19 % FVW) were conducted using 50 L digesters with a working capacity of 35 L. As previously described in section 4.9, the pilot-scale digesters were conducted under the same conditions as the AMPTS system for BMP tests at mesophilic temperature (37 ℃) and ISR of 2:1. The results obtained from batch pilot-scale digesters were compared to the results of the BMP tests and model-predicted values. The biogas production rate, pH, and biogas compositions were monitored daily to assess the process stability of the digesters.

Figure 13 shows the daily biogas flow rates observed from MD 1 and MD 2 during the anaerobic co-digestion process of fish sludge, food waste, and fruit &vegetable. As shown in **Figure 13**, the biogas flow rates started to produce on day 1 after inoculating the digesters with inoculum and mixtures. It can be seen from **figure 13** that there was no lag phase observed during anaerobic co-digestion of fish sludge, food waste, and fruit &vegetable waste in both digesters. This is because the substrates used in this study are easily biodegradable due to lower lignocellulosic organic matter, which rapid the hydrolysis process. Both digesters showed three peaks for daily biogas

production rates, which were detected on day 3, day 7, and day 10. It can be observed from **figure 17** that after day 10 of retention time, the biogas flow rates gradually decreased until it reaches a plateau. The digesters were shut down when the biogas flow rate was less than 1% in 3 consecutive days.

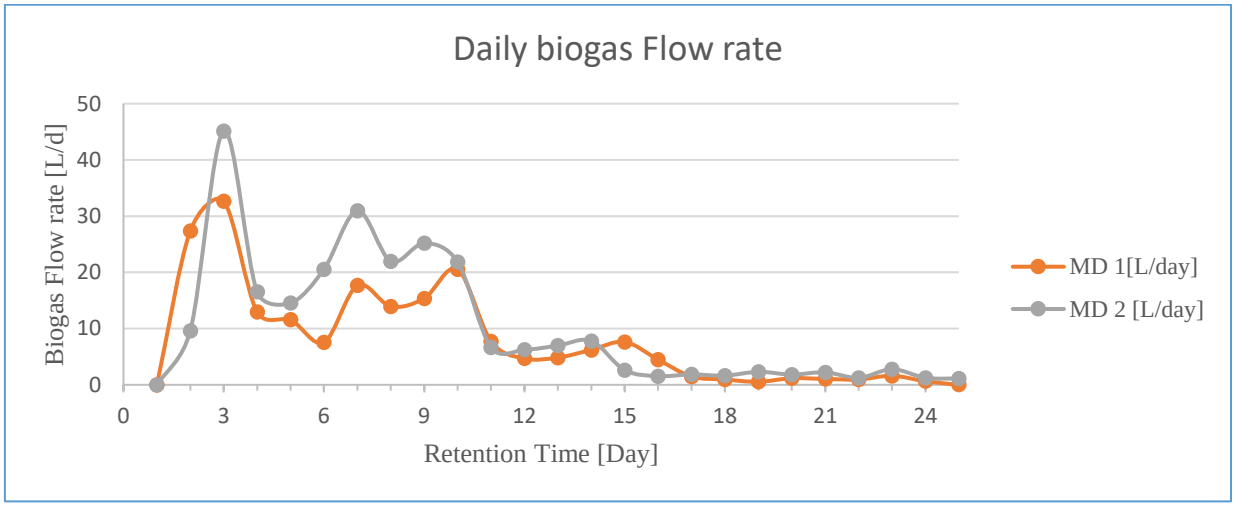


Figure 13: Daily biogas flow rate from batch pilot-scale digesters during the anaerobic co-digestion of the two optimal mixtures composition.

F**igure 14** shows the cumulative biogas production obtained from MD 1 and MD 2 after 25 days of retention time during the anaerobic co-digestion of fish sludge, food waste, and fruit &vegetable waste. The total biogas produced from MD 1 and MD 2 were found as 204 L and 258 L, respectively. **Table 17** summarizes the experimental results of the two batch pilot-scale digesters obtained after 25 days of the anaerobic co-digestion process of the two optimum mixture compositions. As expected, MD 2 accumulated the highest total biogas and methane production than MD 1. It can be seen from **Figure 14** that all the digesters reached a steady-state after 15 days of retention time. This was demonstrating the depletion of the organic matter and nutrients within the digesters required by microorganisms for growth activity.

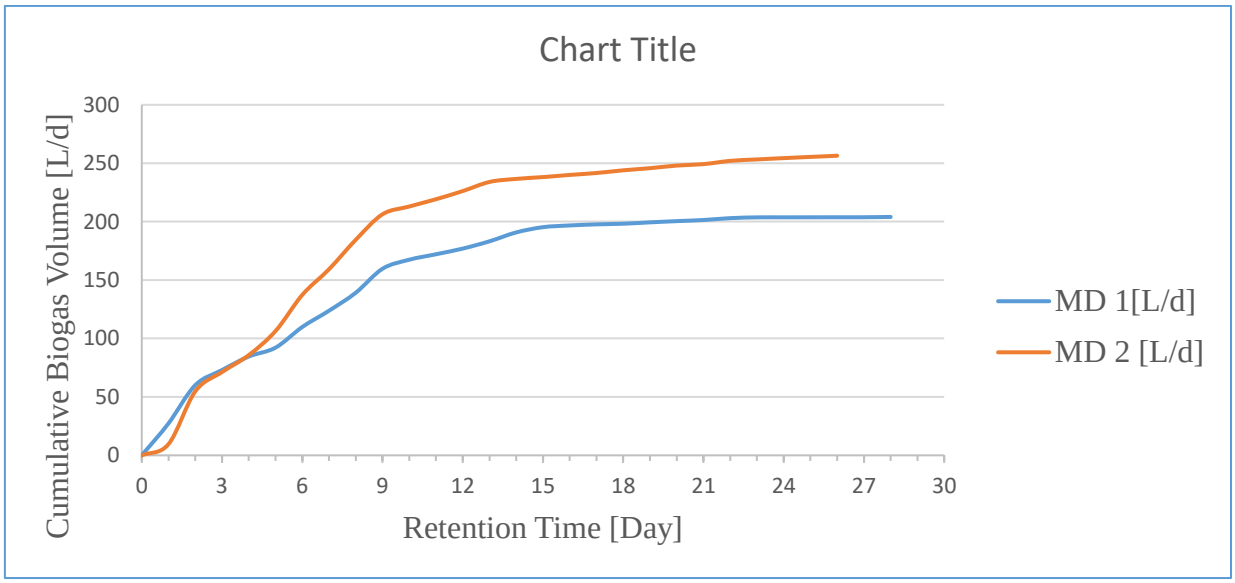


Figure 14: Cumulative biogas production from batch pilot-scale digesters during the anaerobic co-digestion of the two optimal mixtures composition.

Figure 15 demonstrates the biogas compositions (CH_4, CO_2, and H_2S) produced in each digester during the anaerobic co-digestion of the two optimal mixtures compositions. It can be seen from **Figure 15** that the methane content observed was in the range of 62%-76% and 60%-72%, for MD1 and MD2, respectively, corresponding with lower carbon dioxide which was below 40 % during the entire experiment **(Figure 15).** Although MD 1 accumulated low total biogas production compared to MD2 as observed from **Figure 14 above,** the methane content (%) was higher than MD 2 (**Figure 15**) throughout the experiment. The high methane content between 62%-76% attained in this study in both digesters indicates the quality of the biogas produced and good process stability of the anaerobic co-digestion process without inhibition. The reason for obtaining high methane content with lower carbon dioxide in batch pilot-scale mode is because digesters were not frequently opened when taking samples to avoid oxygen. This is another reason both digesters reached a steady state in terms of methane content and carbon dioxide. The hydrogen sulphide observed (**Figure 15**) from both digesters was below the inhibition threshold of 1000 ppm.

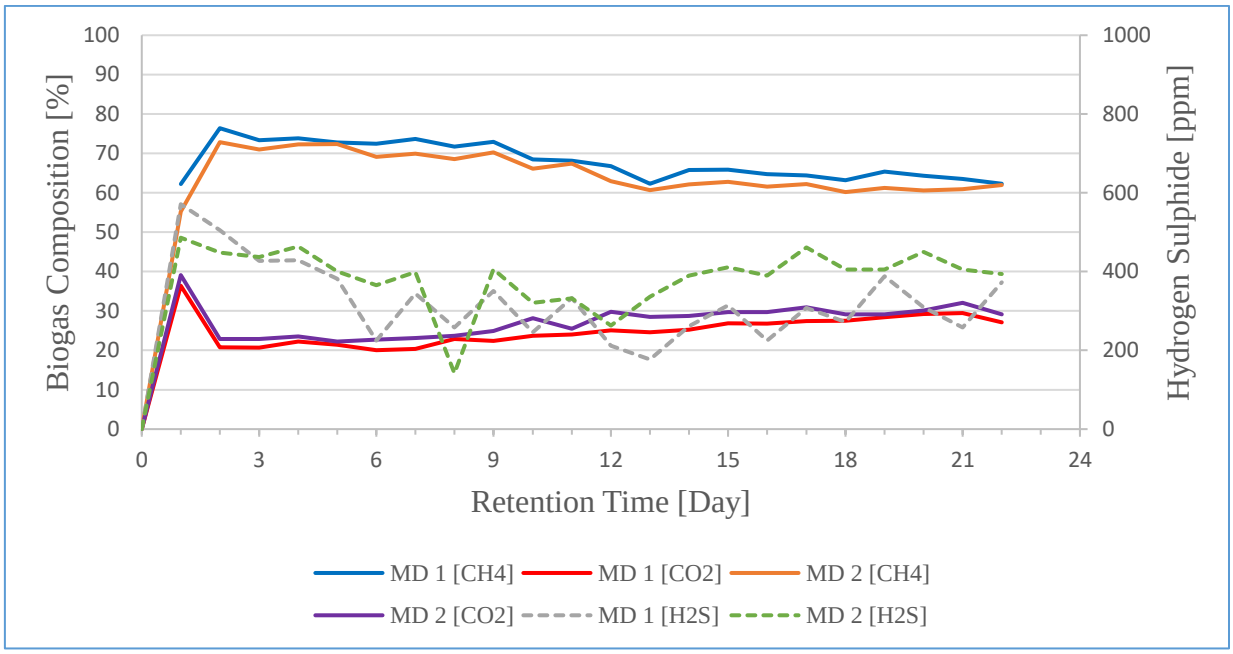


Figure 15: Biogas composition (%) observed from batch pilot-scale digesters during the anaerobic co-digestion of the two optimal mixtures composition.

The pH of the batch digesters observed during the experiment was remained relatively stable, with average values ranging from 6.9 to 8 **(Figure 16)** until the end of the experiment indicating a strong buffering capacity of the digestate within the digesters provided by ammonium nitrogen and high alkalinity concentrations. The total alkalinity obtained from MD1 and MD2 digesters after the digestion process was 6860 mg/L and 4162 mg/L (**Table 18)**, respectively, while the ammonium concentrations were 1001.6 mg/L and 979 mg/L for MD 1 and MD 2 (**Table 18)**. The high total alkalinity concentration observed indicated low tVFAs accumulation **or** lack of inhibitory problems during the digestion process of the batch pilot-scale digesters. The decrease of pH during anaerobic digestion is mainly associated with the accumulation of VFAs concentration which caused the inhibition of biogas and methane production, and methanogens (Ossa-Arias and González-Martínez, 2021). The VFA concentration during the batch pilot-scale runs was not detected. This is because of the strong buffering capacity (alkalinity) as previously mentioned above and the high pH observed which was within the optimal range (6.9-8) that favours the growth of methanogens throughout the whole experiment. It should be noted that there was no supplementation of calcium carbonate made to adjust the pH during the batch pilot-scale digesters runs since the system was naturally buffered by a combination of different substrates. It can be concluded that the anaerobic co-digestion of two selected optimal mixtures in batch pilot-

scale digesters reached a stable performance without substantial inhibition of biogas and methane production or methane content observed, indicating a good process performance stability.

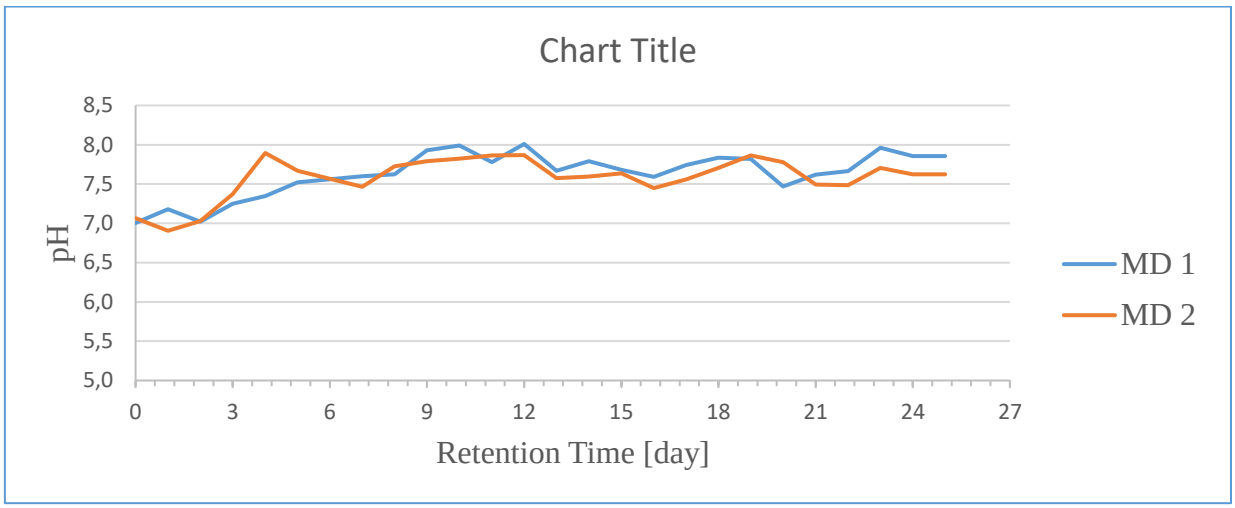


Figure 16: pH profile from batch pilot-scale digesters during the anaerobic co-digestion of the two optimal mixtures composition.

Table 17: Summary of pilot-scale batch digesters results during anaerobic co-digestion of two selected optimum mixtures.

Parameters	MD 1 [63 %FS :18%FW :19%FVW]	MD 2 [40 % FS :41 %FW : 19 %FVW]
Cumulative biogas (L)	204	258
SBY (mL/gVS)	540	740
Maximum daily biogas (L)	33	45
Avg. methane content (%)	70	66
Cumulative methane (L)	131	169
SMY, mL CH_4/gVS d^{-1}	272	410
Average CO_2 (%)	25	27.4
Total volatile acids, (g/L)	-	-
Total alkalinity ($CaCO_3$), (mg/L)	6860	4162
$NH_4 - N$ (mg/L)	1001.6	979
Average pH	7.6	7.6
Hydrogen Sulphide (ppm)	311.6	370

-: no detected; SBY: specific biogas yield; SMY: specific methane yield

5.6.1 Comparison between batch pilot-scale digesters and bench BMP tests

Figure 19 and 20 shows the comparison of results obtained from batch laboratory scale BMP tests, pilot-scale batch digesters, and predicted values by the model during the anaerobic co-digestion of the two selected optimal mixture compositions in terms of specific biogas yield (SBY) and specific methane yield (SMY). From **Figure 17-18**, it is clear that the SBY and SMY obtained from the batch pilot-scale digesters were lower compared to the laboratory batch-scale BMP results obtained using 500 mL AMPTS bioreactors and model values. The average SMY obtained from the batch pilot-scale digesters based on the mass of volatile solids (mLg/VS) added within the digesters were 272 mL/g VS and 408 mL/g VS for MD 1 and MD 2, respectively, representing 70.7 % and 82.81% of SMY obtained from BMP tests under the same optimum mixture

compositions. These results suggested that the laboratory scale BMP and model can be used to estimate the biogas and methane production of the pilot-scale digester.

The SBY obtained from the batch pilot-scale digester MD1 and MD 2 were 540 NmL /gVS and 735 NmL g/VS, respectively (**Figure 17**). This was 79.5 % and 88.2 % of the SBY obtained from BMP tests of the two optimal mixtures. The possible explanation behind this could be the abundance of the microorganisms in a large digester that might involve in the fast biodegradation of the organic waste. The main reason for obtaining less SBY and SMY in batch pilot-scale digesters in this study could be the less efficiency and accuracy of the quantification of the biogas production rate during the anaerobic digestion by the gas counter sensor or clogging of outlet biogas line of the pilot-scale digester compared to the AMPTS system. The AMPTS system used in this study is a more efficient and automated system, which recorded total biogas and methane production simultaneously and generate the datasheet with less labor of measuring compared to batch pilot-scale digesters.

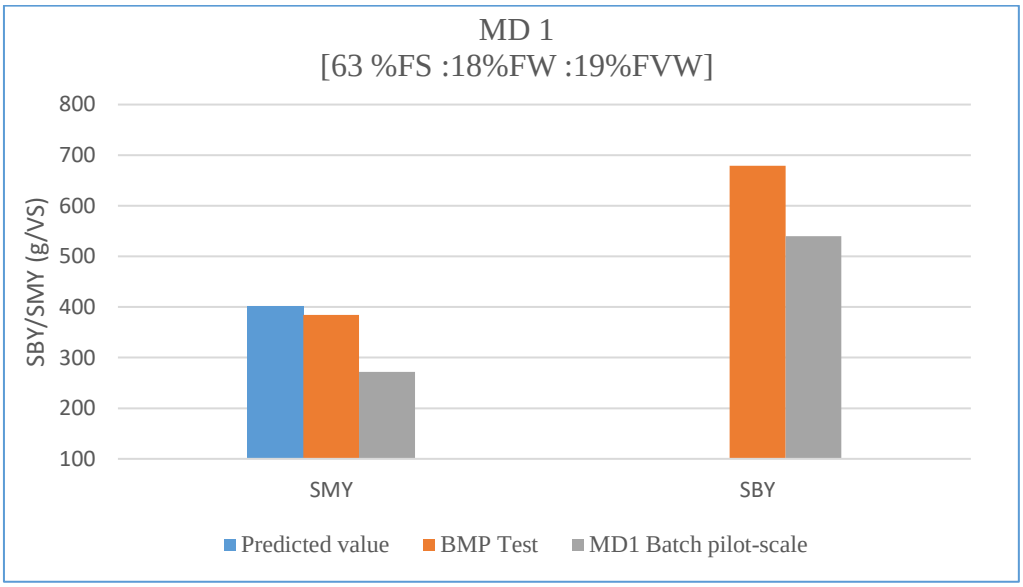


Figure 17: Comparison of specific methane and biogas yields of BMP test, batch pilot-scale, and predicted under the optimum mixture MD1.

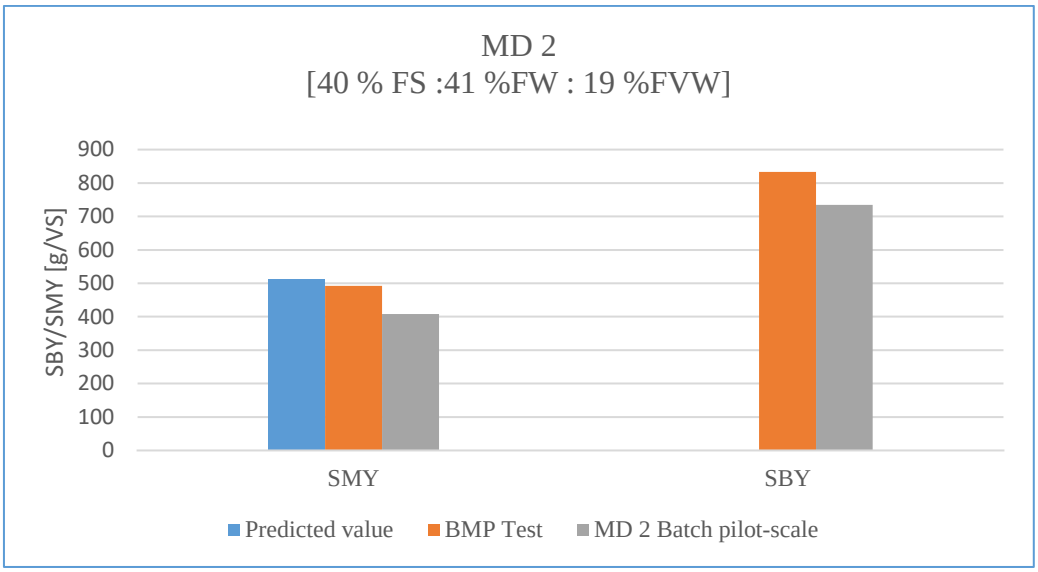


Figure 18: Comparison of specific biogas yield of BMP results, predicted value, and batch pilot-scale results under the optimum mixture MD 2.

In comparison, Peyrelasse et al. (2021) reported that the specific methane yields obtained from the batch pilot-scale (20 L) digester was 86-88% of the BMP potential obtained from the laboratory scale. This indicates that the BMP tests overestimated the results of the pilot-scale digesters. Another study also demonstrated that the batch BMP tests overestimated the biogas and methane production by approximately 20-30% more of the pilot-scale digesters (Ruffino *et al.*, 2015), which is similar to the results of the present study.

5.7 Anaerobic co-digestion performance in pilot-scale semi-continuous reactor system in response to organic loading rate (OLR)

This section aimed to evaluate the effects of organic loading rate (OLR) on the anaerobic co-digestion of fish sludge (FS), food waste (FW), and fruit & vegetable waste (FVW) based on the optimum co-substrate ratio obtained from statistical optimisation performed via BMP tests, and to assess the process performance/stability in a pilot-scale semi-continuous digester. In semi-continuous mode or full-scale AD plants, the organic loading rate is an important parameter for obtaining high biogas and methane yields while monitoring and maintaining stable process performance (Antonio *et al.*, 2014). Based on the results obtained from BMP and scaled-up batch digester studies (**Table 16**), a mixture consisting of 63% FS: 18% FW: 19% FVW composition was chosen for a semi-continuous pilot-scale study to evaluate the effects of OLRs on biogas and methane production, and process stability. It must be noted that this study was using fish sludge

as the main substrate. Therefore, only one mixture was further selected for semi-continuous study. This mixture was suggested to be utilized for semi-continuous study due to its good process performance stability **(Table 16),** and ability to use a high proportion of fish sludge (as the main substrate) in co-digestion with food waste and fruit & vegetable waste. The availability of co-substrates near the fish farm aquaculture also contributed to the selection of a mixture with a low proportion of food waste and fruit & vegetable waste.

Therefore, in order to evaluate the effects of the OLRs on the biogas and methane production as well as process performance of the anaerobic co-digestion of fish sludge, food waste, and fruit & vegetable waste, a 30 L semi-continuous pilot-scale mode with 21 L working capacity was conducted at the mesophilic conditions (37°C) (as described in section 4.10). The loading rate of the digester was done daily once per day. The OLR was gradually increased stepwise from 1 $gVSL^{-1}d^{-1}$ (day1 to 12) to 2 $gVSL^{-1}d^{-1}$ (day 22 to 37) and 3 $gVSL^{-1}d^{-1}$ (day 38 to 52) during the experimental operation with a substrates mixture (63% FS: 18% FW: 19% FVW).

5.7.1 Performance of semi-continuous digester at an OLR of 1 $gVSL^{-1}d^{-1}$.

Initially, the semi-continuous digester was fed with an OLR of 1 $gVSL^{-1}d^{-1}$ of the mixture (63 % FS: 18 % FW: 19 % FVW) from day 1 to day 12. **Figure 19-23** shows the biogas and methane production rate, methane content, pH, and VFAs observed during the anaerobic co-digestion of the mixture depending on OLRs. It can be seen from **Figure 19** that the daily biogas production, as well as daily methane production, were observed on the 1st day after the digester was inoculated with the substrates mixture and inoculum. This mainly occurs during the anaerobic digestion of easily biodegradable substrates, indicating the rapid of a soluble organic fraction by hydrolysis and acidogenesis process. The spikes in daily biogas and methane production rate (**Figure 19**) were observed mostly after feeding the digester with the fresh substrate due to the fast hydrolysis reaction of the easily biodegradable organic content (e.i. fruit &vegetable waste) as aforementioned. At an OLR of 1 $gVSL^{-1}d^{-1}$, the daily biogas and methane production reached a maximum of 25.1 L/d and 10.5 L/d, respectively, on day 10. During this stage when the digester was feeding with an OLR of 1 $gVSL^{-1}d^{-1}$, biogas and methane production showed fluctuations together with pH as presented in **(Figure 19-23).** The average methane content of the produced biogas was between 16-65%. As aforementioned above, the large variation of methane content was also influenced by the intermittent feeding of the digester. As shown in **figure 20**, the pH of the digester dropped slightly from 7.1 at the beginning to 6.8 on day two after feeding the digester

and then gradually increase to 7.5, corresponding with a low VFA concentration accumulation of 1 g/L observed on the same day **(Figure 21).** The pH range of 6.8-7.2 is primarily suitable for the growth of hydrolytic, acidogenic, and acetogenic microorganisms (Elsayed *et al.*, 2021). The decreased pH in the digester is primarily due to the accumulation of organic acids (Regalado *et al.*, 2021).

On day 12, the organic loading rate was gradually shifted upward from 1 to 2 $gVSL^{-1}d^{-1}$. However, after feeding, the digester became unstable (failure) from day 12 to day 17 (**Figure 19**). During these days, the pH declined from 7.0 to 6.2 **(Figure 20),** methane and biogas production rate also decreased **(Figure 19),** and a rapid accumulation of VFA concentration of 6.87 g/L was detected **(Figure 21).** In addition, the methane content dropped from 65% to 8 % on day 12 to day 17 **(Figure 22).** The fluctuations in the methane content were also used as an inhibition indicator to assess the process stability. This inhibition (failure) of the system was probably due to rapid VFA concentrations built up or wash out of essential nutrients for microbial metabolism by doubling the loading rate. The high content of cellulose, fats, and hemicellulose in food waste and fruits & vegetable waste may contribute to the accumulation of VFAs at this stage during the semi-continuous experiment in the present study. The initial stage can also be inhibited by VFA accumulation during the digestion process of readily biodegradable (carbon-rich) substrates such as fruit & vegetable or food waste with VS/TS of more than 70% (Ossa-Arias and González-Martínez, 2021). Methanogens are extremely sensitive to changes in digester conditions, such as lower pH or higher VFAs (Jan and Euverink, 2019). As a result, the digester feeding was stopped for a moment from day 15 to day 22 to recover the system.

In semi-continuous mode, it is recommended to stop feeding or reduce the loading rate of the digester for a short period to allow microorganisms to adapt to the new feeding substrate (Ossa-Arias and González-Martínez, 2021), to reduce the total VFA concentration by adding buffer solution (Tonanzi *et al.*, 2021) or when the pH falls below 6.8 to recover the system (Hegde, 2019). Many researchers have reported process failure during the semi-continuous system process during the initial stage of loading rate due to non-adaptation of the microorganisms with the fresh substrate added daily, overfeeding, insufficient methanogens population, or using non acclimatisation of the inoculum with the substrate (Pagés-Díaz *et al.*, 2015; Hegde, 2019; Tonanzi *et al.*, 2021).

According to Pagés Díaz. (2014), the optimal pH of the digester for methanogens microbial should be between 6.8 and 7.2, when the digester is normally fed by an optimal organic loading rate. Therefore, on day 15, the pH was adjusted to a neutral range (7-7.2) by adding an appropriate amount of 1% (w/v) calcium carbonite to the digester. After the digester was resuscitated with calcium carbonite, both biogas and methane production started to pick up gradually on day 17, indicating that the methanogenic bacteria had adapted to the environment. However, the addition of external solution (acid or base) in order to control the conditions (i.e. pH) in a pilot-scale digester is not recommended due to high operating costs (Garcia-peña *et al.*, 2011; Shuang *et al.*, 2017).

5.7.2 Performance of the semi-continuous digester at an OLR of 2 $gVSL^{-1}d^{-1}$

After the digester system was recovered by stopping feeding and adjusting the pH with calcium carbonite, the digester was then again attempted to be fed with an OLR of 2 $gVSL^{-1}d^{-1}$ on day 23 (**Figure 19**). When the digester was fed with an OLR of 2 $gVSL^{-1}d^{-1}$ for the second time, no inhibition of daily biogas and methane production rate was observed **(Figure 19).** This was indicating that the addition of calcium carbonite provided a sufficient buffering capacity (alkalinity) within the system, which supports the methanogens microbial growth. The highest daily biogas and methane production peaks of 45 L and 30.6 L, respectively, were observed on day 34 **(Figure 19)**.

The methane content of the produced biogas increased to approximately 73% on day 24 after recovery when the digester was fed with an OLR of 2 $gVSL^{-1}d^{-1}$. It can be seen from **Figure 22** that the methane percentage content was ranged between 48% and 73% from day 23 to 38 with an average methane content of 66.8 % of the produced biogas, which suggested that the methanogens bacteria were adapted to the substrate. At steady-state of the anaerobic digestion process, biogas composition is usually composed of methane content (50-70%) and carbon dioxide between 25% and 45% (Garcia-peña *et al.*, 2011; Angelidaki *et al.*, 2018), which indicate good stability of the anaerobic digestion process. The VFA and pH profile with 2 $gVSL^{-1}d^{-1}$ is presented in **Figure 20 & 21.** It can be seen from **Figure 21** that there was a gradual decrease trend in total VFA concentrations from 8.2 g/L to 3.5 g/L, corresponding with high pH, which was remains between 6.9 and 7.6 from day 22 to day 36. The tVFA concentrations ranging between 4 g/L and 3 g/L were observed when the digester was fed with an OLR of 2 $gVSL^{-1}d^{-1}$ from day 28 to 37 which was composed mainly of propionic acid concentration **(Figure 21**). The

VFA concentration detected in this stage showed no substantial inhibition of the digester system due to high pH (6.9-76) (**Figure 20**), which was remained between the optimum range that resist the inhibition of methanogens, and high biogas and methane production were still observed **(Figure 23**).

5.7.3 Performance of the semi-continuous digester at an OLR of 3 $gVSL^{-1}d^{-1}$

At the final stage of the semi-continuous digester operation, the loading rate gradually increased from 2 to 3 $gVSL^{-1}d^{-1}$ on day 38 as presented in **Figure 19-23**. It can be seen from **Figure 19** that after feeding the digester with an OLR of 3 $gVSL^{-1}d^{-1}$ on day 38, the daily biogas and methane production, as well as the methane content, gradually declined, corresponding with an increase of VFAs concentrations (**Figure 21)**, which also resulted in the decrease of the pH. The decline of daily biogas and methane production was mainly due to the accumulation of VFA observed, which hinder the growth of the methanogen's bacteria and their activity within the digester. The accumulation of VFAs in high concentration indicates that the four sequential reaction steps (e.g. hydrolysis, acidogenesis, acetogenesis, and methanogenesis) for anaerobic digestion process were not balanced. It should be noted that an appropriate amount of calcium carbonate (1 % w/v) was also supplemented into the digester on day 41 to resuscitate the system stability, but this was insufficient to prevent the inhibition of daily biogas and methane production due to the high accumulation of VFA and inhibition of the methanogenic. It can be seen from **Figure 21** that a process instability started when propionic acid concentrations started to rise in high concentrations, indicating the inhibition of the methanogens. The methane percentage content at OLR of 3 $gVSL^{-1}d^{-1}$ decreased gradually from 65.1 % on day 44 to 10% on day 52 (**Figure 21)**. During the last days of the HRT at OLR of 3 $gVSL^{-1}d^{-1}$ from day 45 to day 52, valeric acid, and cuproic acid were observed again after not detected at an OLR of 2 $gVSL^{-1}d^{-1}$, which was indicating the instability of the digester (**Figure 21**).

In comparison, Pagés-díaz et al. (2015) also observed a similar trend of decreased methane production, accompanied by an increase in the VFAs when the OLR was increased from 1.5 $gVSL^{-1}d^{-1}$ to 3 $gVSL^{-1}d^{-1}$ during the semi-continuous co-digestion of solid cattle slaughterhouse wastes with other organic wastes. The decrease of the biogas and methane levels on day 38 could also suggest that the digester did not stabilize due to the low activity of the methanogenic microbial community (Pagés-díaz *et al.*, 2015). At an OLR of 3 $gVSL^{-1}d^{-1}$ the VFA concentrations within the digester gradually increased up to 10 g/L (**Figure 21**). Zhang et

al. 2013 also detected severe acidification of the system at high OLR with the VFA of 14g/L during the semi-continuous AD of food waste. The accumulation of VFA concentration with propionic acid of 3.32 g/L caused a failure of the AD system (Mei *et al.*, 2016). The propionic acid observed at an OLR of 3 $VSL^{-1}d^{-1}$ in this study was above 3.5g/L of the total VFAs detected between days 39 and 47(**Figure 21)**. This resulted in the decrease of pH (from 7.4 to 6.2) on 42 day, indicating an upset of the digester system and inhibition of the methanogens (**Figure 20**). The VFAs concentrations observed at an OLR of 3 was high compared to the one detected at an OLR of 1 and 2. This showed that overloading the digester up to OLR of 3 or above could lead to severe accumulation of VFAs were inhibiting biogas production. The digester shut down due to the inhibitions of biogas and methane production by VFA accumulation.

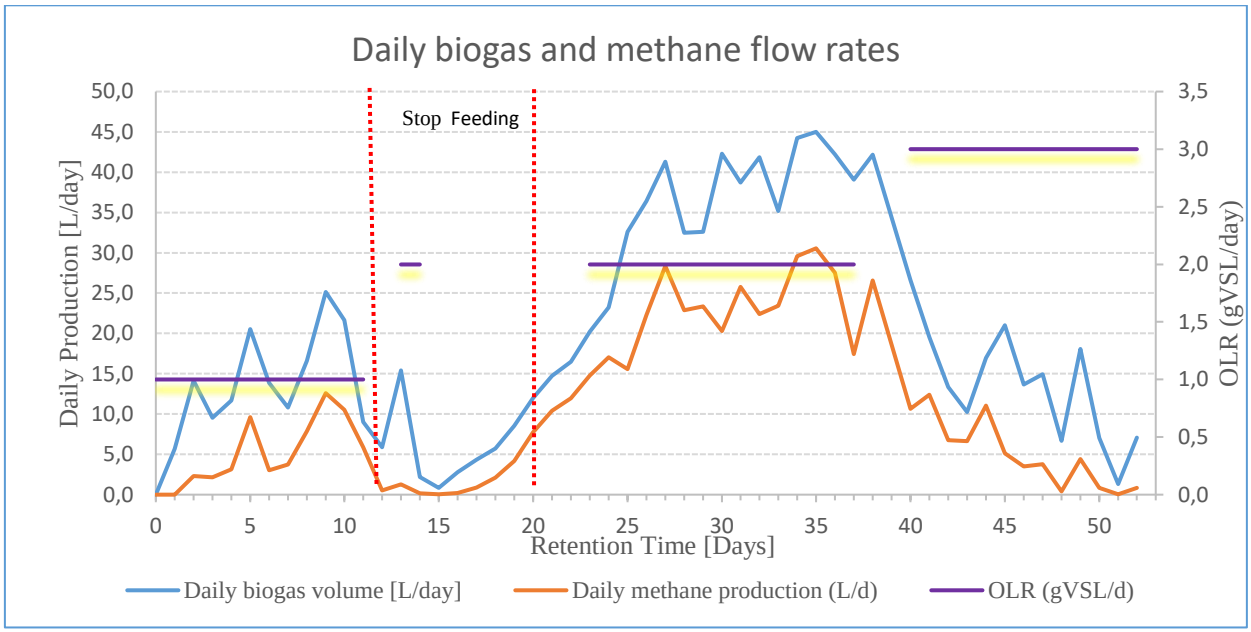


Figure 19: Daily biogas and methane production during the semi-continuous anaerobic co-digestion process under different OLR. The region between vertical lines is when the digester was stop feeding to recover the system.

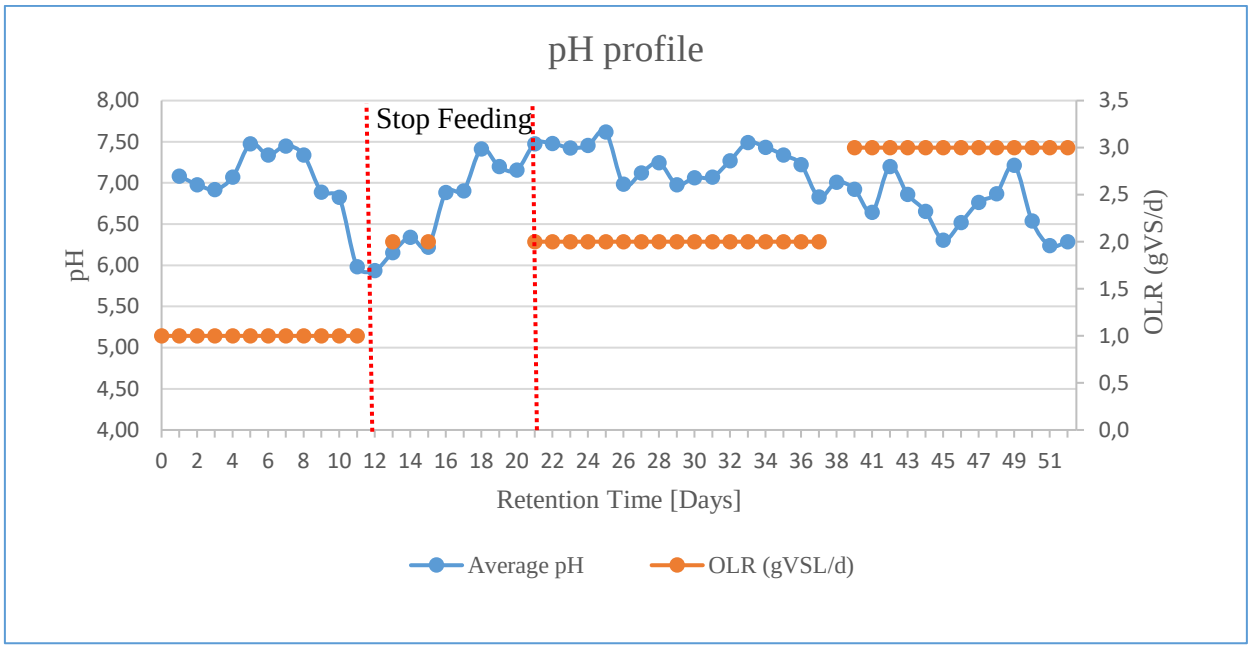


Figure 20: pH profile during the semi-continuous anaerobic co-digestion of fish sludge, food waste, and fruit & vegetable waste under different OLR.

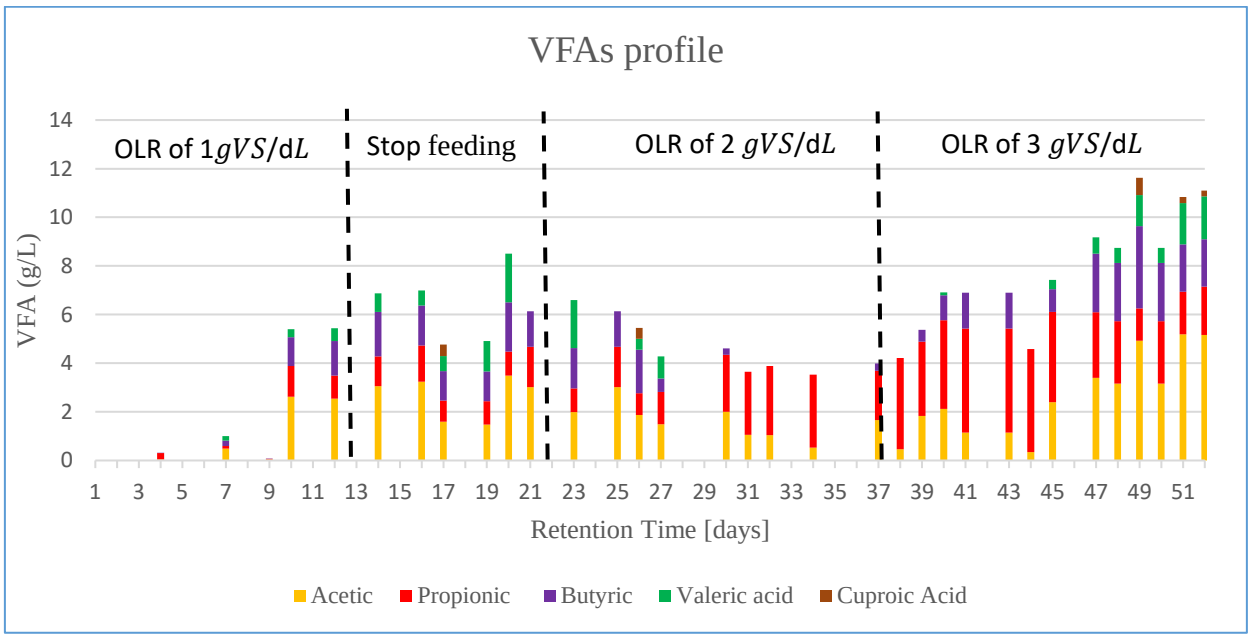


Figure 21: VFA profile during the semi-continuous co-digestion of fish sludge, food waste, and fruit and vegetable waste under different OLR.

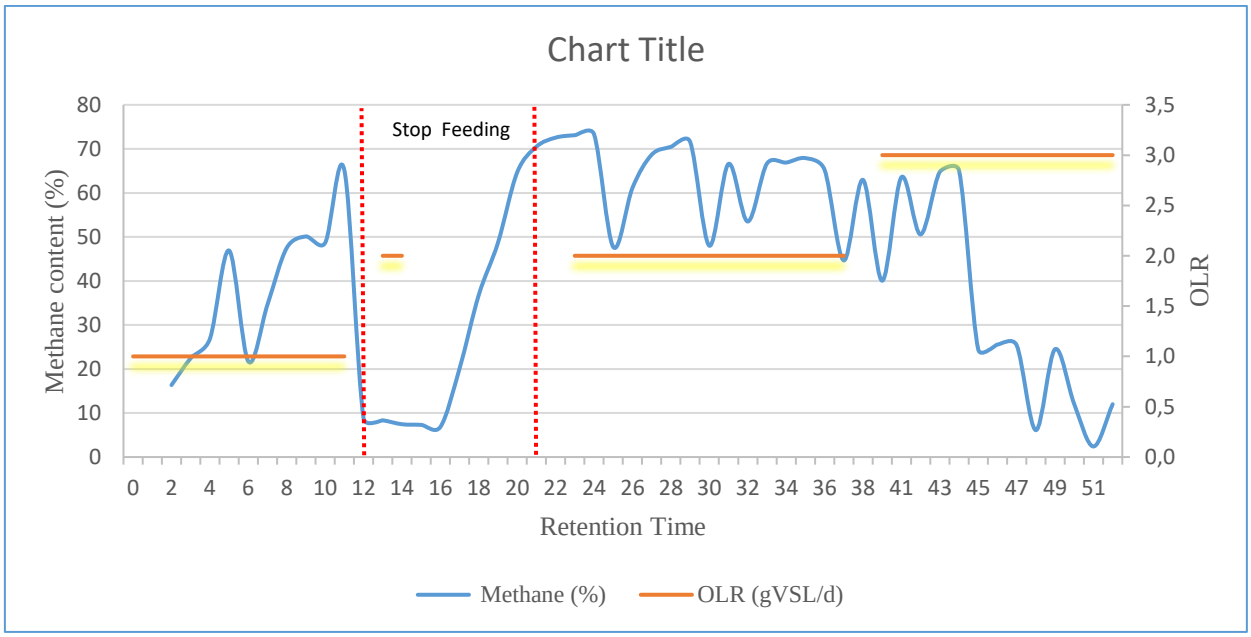


Figure 22: Average methane content during the semi-continuous anaerobic co-digestion under different OLRs.

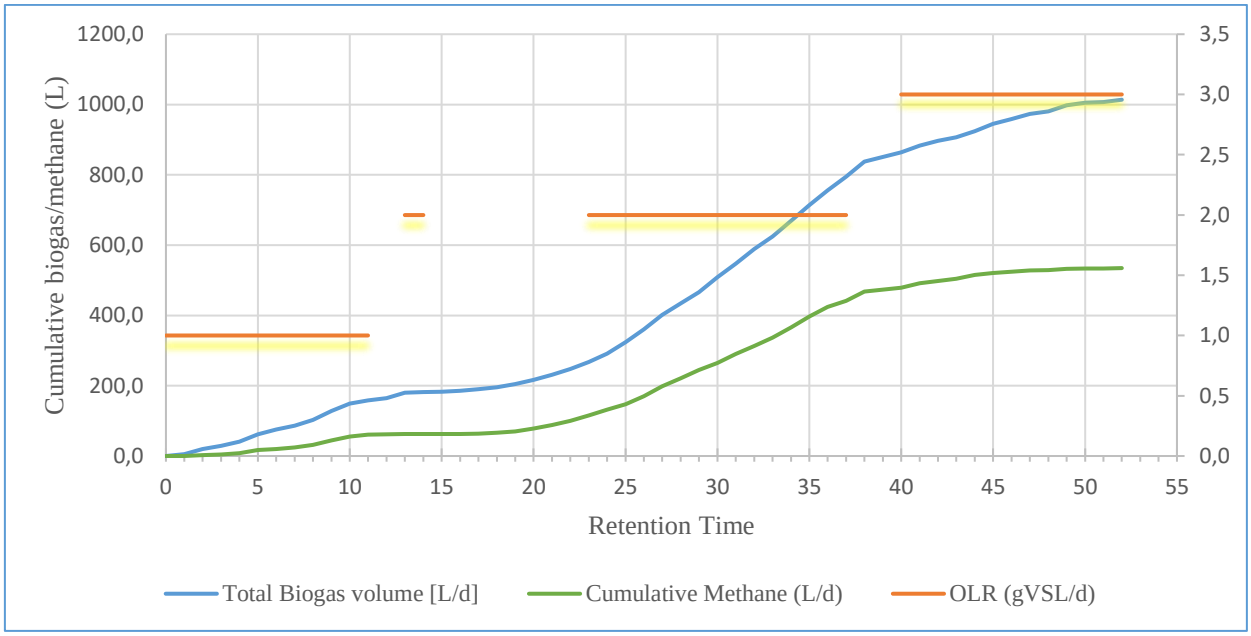


Figure 23: Cumulative biogas and methane production during the semi-continuous anaerobic co-digestion.

Other instabilities encountered during operation of the semi-continuous digester

It can also be reported that during the operation time of the semi-continuous digester of anaerobic co-digestion in this study, the formation of scum/foam layers was observed inside the digester. Overfeeding the digester or excessive mixing of the substrate may lead to the formation of scum in the present study. Foam formation during anaerobic digestion is mainly caused by substrates with high lipid and protein content, such as food waste (Pagés-díaz *et al.*, 2015).

SUMMARY

The instabilities of the methane content of the biogas produced, pH, biogas and methane production rate detected in semi-continuous digester mode were caused by increases in OLR, accumulation of VFA concentration, and non-acclimation of microorganisms with the new feeding in the digester. It can conclude that a semi-continuous system never reaches a steady-state, because of the daily intermittent feeding of the digester. Although the digester did not stabilise over the retention times in all OLRs tested in this study, it is possible to conclude that feeding the semi-continuous digester with an OLR of 2 $gVSL^{-1}d^{-1}$ produced better biogas and methane content with less instabilities and variations in pH and methane production compared to an OLRs of 1 and 3 $gVSL^{-1}d^{-1}$. The instability of the semi-continuous digester observed at an OLR of 1 and 3 with lower biogas production and methane content was due to inhibition of the system. Based on the findings from this study, it is recommended to feed the digester with an OLR of 1 $gVSL^{-1}d^{-1}$ up to 2 $gVSL^{-1}d^{-1}$ with a long retention time to avoid inhibition of biogas and methane production during anaerobic co-digestion of fish sludge, food waste, and fruit and vegetable waste. Due to time constraints, the retention time of the present study was short compared to previous studies operated in semi-continuous feeding mode digesters. The shorter the retention time, the higher the flow rate of the substrate inside the digester, which leads to overfeeding the digester and accumulation of inhibitory effects likeVFAs at an early phase. The longer the SRT, the higher the methane production and reduction of biodegradable material from the substrate.

6 CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

Fish sludge has the potential to be valorized through anaerobic digestion, due to the high organic matter (nitrogen-rich) and moisture content of the waste. However, using fish sludge as a single substrate during anaerobic digestion is not feasible due to low total biogas and methane production. The low total solids (1.9 %) content and C/N (6) ratio deficiency (nutrient imbalance) of the fish sludge resulted in lower methane production during the mono-digestion process. The best option to overcome the drawbacks of mono-digestion of fish sludge is through the anaerobic co-digestion process with other substrates with complementary compositions, such as food waste and fruit & vegetable waste, which can compensate for essential nutrients and improve the process stability. Co-digestion of fish sludge with food waste and fruit and vegetables may aid in overcoming the ammonification problem by supplying the necessary nutrients for stable biogas production.

The synergic and antagonistic interaction effects of fish sludge, fruits & vegetable waste, and food waste on specific methane yield and VS reduction were investigated with BMP measurements using a simplex centroid mixture design. Based on the BMP measurements in this study, an optimal specific methane yield of 384.67± 1.52 mL CH_4/gVS and VS reduction of 63.20% were obtained with the mixture composition of FS: FW: FVW (63 %: 18%: 19%). This represented an 8-fold improvement of anaerobic co-digestion BMP compared with the mono-digestion of fish sludge waste 48.95 mL CH_4/gVS. The experiment results of this study indicated that the co-substrates used with fish sludge had synergistic effects for biomethane production. The increase of methane production during anaerobic co-digestion may be influenced by the balance of nutrients of the mixture substrates composition. However, the addition of food waste in mixtures showed insignificant VS removal because it did not improve the VS removal due to high cellulose and hemicellulose content when compared to VS removal of fish sludge alone indicating the requirement of a pre-treatment method in order to improve the removal of organic matter.

The SBY and SMY obtained in batch pilot-scale digester of mixture [63% FS: 18 % FW: 19% FVW] were found as 540 NmL CH_4/gVS and 272 NmL CH_4/gVS, respectively. The SBY and SMY obtained in batch pilot-scale digesters were 70.7 % and 79.5 % of the BMP test results conducted under the optimal mixture compositions. The batch pilot-scale digesters showed a stable process performance in terms of methane content produced of biogas composition, which

was ranged between 62% and 76% in all digesters with no substantial inhibition of the system due to strong buffering capacity detected.

The semi-continuous pilot-scale digester of anaerobic co-digestion under optimal mixture of 63% FS: 18% FW: 19% FVW was conducted in order to investigate the effects of different OLRs on biogas and methane production, and also to assess the process stability. The highest total biogas, methane content, and methane production were obtained at an OLR of 2 $gVSL^{-1}d^{-1}$ after recovery from inhibition of the system. Overfeeding the digester up to OLR of 3 $gVSL^{-1}d^{-1}$ results in system failure again due to acid crash. The accumulation of VFA concentrations led to the inhibition of biogas and methane production at an OLR of 3 $gVSL^{-1}d^{-1}$. Therefore, an OLR of 2 $gVSL^{-1}d^{-1}$ is recommended to achieve a stable condition with higher biogas production and methane content of ~ 67 % in a semi-continuous digester. However, it can be concluded that the digester never reach the steady state in all OLRs tested due to the intermitted feeding of the digester and accumulation of VFA concentration. These results suggested that the fluctuations of biogas and methane production, pH, and methane content of the biogas composition observed during the semi-continuous run were affected by feeding the digester daily because in the batch pilot-scale digester no inhibition was observed.

The findings of this study showed that anaerobic co-digestion plant of fish sludge with food waste and fruit and vegetable waste could potentially be implemented within the fish production to produce biomethane, which can serve as source energy while simultaneously reducing the amounts of organic waste when performed under an optimum mixture of 62.05 %FS: 18.46% FW: 19.46% FVW. The co-substrates used in this study are biodegradable, available, and easily introduced into the aquaculture system.

6.2 RECOMMENDATIONS FOR FUTURE STUDIES

- Future research should investigate a large number of experiments using different inoculum and substrates in order to improve the biomethane production and process stability, and also compare to findings obtained in this study since there is a lack of literature in anaerobic co-digestion of fish sludge with other substrates.
- The semi-continuous study should be investigated for a long acclimation period for at least 100 days to assess different feeding strategies. This will help to prevent washout of microorganisms or inhibition of the digester system at an early stage of the anaerobic digestion process due to accumulation of VFAs concentration because in the present study the retention time for semi-continuous was short due to time constraints. A long-term period of semi-continuous operation increases the biogas and methane production due to acclimation of the feedstock and the methanogenic microbial (Garcia-peña *et al.*, 2011).
- For better quality biogas production and to prevent system instability in semi-continuous future studies should also focus on the removal of inhibitors such as VFA by extracting VFAs using in situ liquid-liquid extraction method (Tonanzi *et al.*, 2018, 2021; Morison, 2021)
- Future studies should include the pretreatment (chemical or physical) for the substrates characterized by high lignin and hemicellulose in order to improve the VS removal content.
- Future research work should also evaluate anaerobic co-digestion of fish sludge with other biowastes including animal manure that will provide buffer the pH of the system naturally in semi-continuous pilot-scale digester without adjusting it with external (calcium carbonate) solution for economical perspective, and avoid inhibition by VFA accumulation of the food waste and fruits & vegetable waste. This is because fish sludge in a mixture with food waste and fruit & vegetable waste was not providing enough buffering capacity to prevent the build-up of VFA
- The organic loading rates of the semi-continuous digesters should be investigated first in small reactors (e.g.1L to 5L) before up-scaled to pilot digesters. This will help to know the initial feeding rate of the digester and prevent an acid crash at an early stage. It is also recommended for future study that the loading rate should be gradually increased by lower OLR (e.g. 0.5 $gVSL^{-1}d^{-1}$) to avoid washout of the initial microbial population (e.g.

consortium) or built-up of VFAs inhibition at an early stage of the anaerobic digestion process.

- ❖ Future studies should further improve methane production by evaluating important parameters such as temperature, inoculum to substrate ratio, and VS of feedstock in pilot-scale digesters to provide more information about anaerobic digestion in large digesters.
- ❖ The future study should also investigate the effect of OLRs on the microbial (methanogenic) population activity developed during anaerobic co-digestion of the fish sludge, food waste, and fruit& vegetable waste in a semi-continuous digester under different loading rates using molecular methods (e.g. PCR). This will help to understand what types of microorganisms and quantities of microorganisms probably occur at lower and higher OLRs during the semi-continuous feeding mode.
- ❖ For a semi-continuous study, the mixture of substrates should first be acclimated to the anaerobic sludge (inoculum) to allow microorganisms to adapt to the new environment This will help to prevent the failure of the AD system at an early stage since methanogens require time to adapt.

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APPENDICES

APPENDIX A: EXPERIMENTAL MIXTURES RUN FOR ANAEROBIC CO-DIGESTION STUDY

Table 18: Experimental mixture proportions used for batch anaerobic co-digestion experiments generated by simplex centroid mixture design.

Run	Mixture proportion of substrate volume (%)		
	FS	FW	FVW
1	33.33	33.33	33.33
2	0	100	0
3	0	50	50
4	16.67	66.67	16.67
5	100	0	0
6	100	0	0
7	50	50	0
8	0	100	0
9	0	0	100
10	0	0	100
11	50	0	50
12	50	50	0
13	66.67	16.67	16.67
14	16.67	16.67	66.67

Table 19: Chemical compositions of different mixture runs that were used for the anaerobic co-digestion during the batch BMP tests.

Analysis composition	Run 1**	Run 2 &8	Run 3**	Run 4 **	Run 5 & 6	Run 7 & 12**	Run 9 & 10	Run 11**	Run 13**	Run 14**
Protein (%)	3	7	4	5	0	3	1	1	2	2
Fat (%)	3	8	4	6	1	4	1	1	2	2
C/N	18	17	18	16	6	16	40	31	17	23
Crude Fibre (%)	1	0	2	1	0	0	3	2	1	2
Carbohydrates (%)	13	24	19	19	0	12	14	7	7	14
Cellulose (%)	19	37	29	28	0	18	21	11	10	20
Hemicellulose (%)	3	0	4	1	0	0	8	4	1	5
Lignin (%)	21	31	32	26	0	15	33	17	11	27
Extractives (%)	34	51	51	42	0	25	51	25	17	42

**Data calculated based on the mixture ratios

APPENDIX B: EXPERIMENTAL RAW DATA FOR BATCH BIOCHEMICAL METHANE POTENTIAL (BMP) TRIALS GENERATED BY THE AMPTS II

Table 20: Raw data of accumulated methane production of mixture runs for anaerobic co-digestion runs during the BMP tests.

Time [Days]	Accumulated methane production volume [NmL]															
	Blank Assay (inoculum)	**Positive control (cellulose)**	**Run 1**	**Run 2**	**Run 3**	**Run 4**	**Run 5**	**Run 6**	**Run 7**	**Run 8**	**Run 9**	**Run 10**	**Run 11**	**Run 12**	**Run 13**	**Run 14**
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	7.8	55.6	263.4	244.25	286.25	307.3	48	50.4	389.65	240.4	463.9	407.75	294.4	402.9	315	286.8
2	39.2	261.5	275.9	263.65	300	320.5	84.4	89.2	399.3	259.6	491.4	428.5	311.9	418.7	383.1	321.75
3	80.7	351	298.6	287.15	327.25	346.8	128.3	135.8	431.25	281.8	511.7	449.7	335.65	473	528.55	353.2
4	141.9	440.6	368.8	359.8	404.85	420.45	163.6	169.95	505.3	353.4	581.9	521.45	405.15	574.2	751.75	419.15
5	215.3	589.8	495.3	484.2	543.25	555.65	186.5	193.6	694.9	477.1	709	650.1	518.15	734.8	1096.05	564.55
6	293.4	798.5	646.9	632.75	701.9	710.3	197.2	204.35	840.55	625.2	860.4	805.7	663.65	809.2	1281.35	740.15
7	375.8	989	840.9	815.35	904	907.05	200.9	207.25	1026.9	807.7	104.,5	993.55	838.25	1032.9	1448.4	989.35
8	445.3	1123.3	1026.3	987.6	1103.9	1115.5	201.3	202.1	1184.3	979.4	1256.5	1204.35	999.9	1188.1	1509.75	1145.35
9	505.1	1208.9	1205.4	1155.6	1296.85	1317.55	203.7	204.1	1254.5	1146.3	1442.2	1396	1143.35	1238.2	1589.5	1225.1
10	559.1	1267.7	1373	1327.3	1479.15	1517.15	**204.5**	**205.8**	1295.55	1317.5	1579.6	1531.75	1244.2	1293.8	1689.95	1308.8
11	595.5	1324.5	1521.9	1504.7	1640.2	1716.65			1380.7	1496.3	1637.9	1582.85	1294.45	1395.3	1784.65	1369.45
12	632.3	1394.5	1640	1670.25	1761.15	1881.05			1550.3	1664.9	1670.4	1614.8	1338.6	1552.7	1831.05	1441
13	669.6	1450.8	1736.7	1792.05	1853.65	2001.1			1725.25	1788.7	1717	1661.7	1385.15	1749.5	1865.25	1526.85
14	711.8	1505.5	1794.8	1857.25	1906.2	2062.55			1904.2	1853.4	1763.3	1708.7	1431.5	1916.8	1895.6	1616.4
15	727.6	1572.7	1843.5	1906.8	1954.5	2109.5			2001.8	1902.3	1814.2	1761.4	1489	2037.6	1914.4	1636.35
16	734.9	1662.7	1903.2	1967.4	2018.2	2166.85			2013.35	1962.3	1881.7	1832.6	1565	2141.4	1931.95	1657.4
17	738.7	1763.5	1974.4	2040.65	2095.7	2235.5			2191.05	2035.4	1959.4	1913.4	1639.9	**2225.3**	1949.75	1679.45

18	742.3	1872.8	2056.3	2121.65	2180	2317.5			**2245.25**	2116.9	2038.6	1993.05	1686.25		1960.55	1694.65
19	744.5	1989.1	2148.9	2204.6	2254.9	2403.55				2199.1	2087	2032.45	1699.45		1971.5	1707.95
20	745.9	2029.4	2190.7	2240.05	2281.6	2455.05				2234.5	2096.5	2040.65	1705.9		1977.55	1714.4
21	747.4	2036.8	2206.3	2252.85	2295.95	2479.2				2245.6	2105.8	2050.15	1712.65		1980.6	1716.7
22	748.7	2041.8	2222.7	2265.15	2308.7	2495.6				2258.4	2112.3	2057.4	1717.3		1983.65	1719
23	750.2	2045.8	2233	2270.7	2313.95	2504.9				2264.2	2115.5	2060.7	1720.9		1986.95	1721.7
24	752.3	2050.2	2240.1	2274.5	2318.9	2510.35				2268.6	**2118.2**	**2063.55**	1725		**1991.1**	**1725.1**
25	757.5	2058.7	2247.2	2277.65	**2323.35**	2515.65				2272.2			**1727**			
26	**749.5**	**2065.8**	**2254**	**2274.**7		**2518.65**				**2274.7**						

Table 20 (continued)

Table 21: Raw data of daily methane flow rate per day [NmL/day] for mixture runs from AMPTS II program of anaerobic co-digestion process

Time [Days]	Daily flow rate methane production [NmL/day]															
	Blank Assays (inoculum)	Positive control (cellulose)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Run 9	Run 10	Run11	Run 12	Run 13	Run 14
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	69	56	263	244	286	307	50	48	390	240.4	463.9	408	294	402.9	315	287
2	24	206	13	19	14	13	39	36.4	10	19.3	27.5	21	18	15.8	68	35
3	34	90	23	24	27	26	47	43.9	32	22.2	20.3	21	24	54.2	146	31
4	28	90	70	73	78	74	34	35.3	74	71.6	70.2	72	70	101.2	223	66
5	26	149	127	124	138	135	24	22.8	140	123.7	127.1	129	113	160.6	344	145
6	21	209	152	149	159	155	11	10.7	96	148.1	151.4	156	146	74.4	185	176
7	6	191	194	183	202	197	5	2.90	136	182.6	185.1	188	175	223.7	167	249
8	4	134	185	172	200	208	2	2	167	171.7	211	211	162	155.2	61	156
9	4	86	179	168	193	202	2	2	110	166.9	185.7	192	143	50.2	80	80
10	5	59	168	172	182	200	2	2	46	171.2	137.4	136	101	55.6	100	84
11	4	57	149	177	161	200			80	178.8	58.3	51	50	101.5	95	61
12	3	70	118	166	121	164			120	168.6	32.5	32	44	157.4	46	72
13	4	56	97	122	92	120			175	123.7	46.7	47	47	196.7	34	86
14	3	55	58	65	53	61			184	64.8	46.2	47	46	167.4	30	90
15	3	67	49	50	48	47			143	48.9	50.9	53	58	120.7	19	20
16	3	90	60	61	64	57			112	59.9	67.5	71	76	103.8	18	21
17	3	101	71	73	78	69			78	73.2	77.7	81	75	83.9	18	22
18	3	109	82	81	84	82			58	81.5	79.2	80	46	62.8	11	15
19	2	116	93	83	75	86				82.2	48.4	39	13		11	13

20	2.72	40	42	35	27	52				35.4	9.5	8	7		6	6
21	2.78	7	16	13	15	24				11.1	9.3	10	7		3	2
22	3.53	5	16	12	13	16				12.9	6.5	7	5		3	2
23	3.53	4	10	8	5	9				5.7	3.3	3	4		3	3
24	3.53	4	7	5	5	5				4.4	3.3	3	4		4	5
25		9		3	5	5				3.5			4			
26		9		3	4	6				3.5						

Table 21 (continued)

APPENDIX C: LABORATORY EXPERIMENTAL VALIDATION RAW DATA

Table 22: Raw data of daily flow rate methane production (NmL/day) for the optimum mixture (validation) runs from the AMPTS II system.

Time [Days]	Daily flow rate of methane production [NmL/day]											
	Blank Assay (inoculum)			Positive control (cellulose)			Optimum mixture solution 1 [63 % FS: 18 % FW: 19 % FVW]			Optimum mixture solution 2 [40 % FS: 41 % FW: 19% FVW]		
	A	B*	Average	A	B*	Average	A	B*	Average	A	B*	Average
1	59.1	63.4	61.25	81.6	72	76.8	305	313.2	309.1	338.7	414.2	376.45
2	34.3	33	33.65	207.1	208.7	207.9	71	67.2	69.1	49.3	52.8	51.05
3	41.9	41	41.45	137.2	131.3	134.25	151.7	150.9	151.3	100.1	107.7	103.9
4	42.7	40.6	41.65	170.2	165.4	167.8	215	219.6	217.3	188.4	183.7	186.05
5	32.8	30.6	31.7	191.9	191.6	191.75	204.6	212	208.3	239.7	240.9	240.3
6	28.2	26.5	27.35	118.3	159	138.65	165	162.2	163.6	204.1	202.4	203.25
7	22.2	21.5	21.85	65.5	65.8	65.65	105.3	98.5	101.9	169.4	172.6	171
8	17.3	14.7	16	67.3	67.4	67.35	72.2	61.2	66.7	116.5	118.4	117.45
9	10.8	7.7	9.25	78.6	72.4	75.5	65.2	53.9	59.55	72.1	83.5	77.8
10	10.1	8.1	9.1	103.8	98.1	100.95	74.5	62.5	68.5	66.8	68.1	67.45
11	10.5	9.7	10.1	132.9	126	129.45	91.1	86.7	88.9	70.4	70.5	70.45
12	10.2	7.4	8.8	109.6	146.5	128.05	88.5	81.1	84.8	81.2	77.8	79.5
13	11.9	7.8	9.85	13.1	29.7	21.4	25.4	19.7	22.55	105.4	98.9	102.15
14	11	8.1	9.55	8.6	10.4	9.5	18.4	15.9	17.15	70.8	93.2	82
15	13.9	10.4	12.15	9.1	10.6	9.85	18.7	16.5	17.6	21.6	23	22.3
16	10.2	10.2	10.2	6.8	8.8	7.8	15.2	15.1	15.15	15.6	16.9	16.25
17	7.5	5	6.25	9	10.9	9.95	12	10,5	11.25	14.3	15.8	15.05

18	7.4	5.2	6.3	12.3	13.9	13.1	10.6	11.7	11.15	17	18.8	17.9
19	7.7	5.5	6.6	9.6	12.3	10.95	13.5	12.1	12.8	12.6	14.3	13.45
20	4.8	2.6	3.7	8.6	8	8.3	10.9	8.6	9.75	9.4	10.3	9.85
21	4	1.9	2.95	6.2	4.3	5.25	8.9	8.5	8.7	7.6	7.9	7.75
22	2.1	1.9	2	4.4	5.3	4.85	7.8	7.5	7.65	5.5	6.3	5.9
23	2.1	1.9	2	5.6	8.3	6.95	12.1	11	11.55	9.2	9.4	9.3
24	2.1	3.3	2.7	7.6	8.7	8.15	11.8	13.9	12.85	10.7	9.8	10.25
25	3.4	6.4	4.9	7.2	7.3	7.25	6.4	6.7	6.55	11.4	15.5	13.45
26	6.7	4.5	5.6	3.8	3.8	3.8	4.8	5	4.9	14.3	16.7	15.5
27	2.2	4.5	3.35	3.8	3.7	3.75	4.6	4.9	4.75	6.3	7.5	6.9
28	2.2	3.7	2.95		1.7	1.7	4.5	4.7	4.6	2.8	4	3.4
29	2.2	3.7	2.95		1.7	1.7	4.3	4.1	4.2	2.8	4.1	3.45
30	2.3	3.7	3		1.7	1.7	3.8	3.5	3.65	2.8	4.1	3.45
31	2.7	3.7	3.2		1.7	1.7	3.7	3.4	3.55		4.1	4.1

*Duplicate values

Table 22 (continued)

Table 23: Raw data of accumulated methane yield volume for optimum mixture solutions, black assay, and positive control (cellulose).

Time [Days]	Accumulated Methane production Volume [NmL]																			
	Black assay (inoculum)				Positive control (cellulose)					Optimum mixture solution 1 [63 % FS: 18 % FW: 19 % FVW]					Optimum mixture solution 2 [40 % FS: 41 % FW: 19% FVW]				
	A	B*	Average	STD.V	A	B*	Average	STD.V	BMP	A	B*	Average	STD.V	BMP	A	B*	Average	STD.V	BMP
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1	62.9	63.4	63.1	0.3	76.8	72.0	74.4	2.4	2.7	305.0	313.2	309.1	4.1	67.8	395.3	414.2	404.8	9.4	95.5
2	96.0	96.4	96.2	0.2	284.7	280.7	282.7	2.0	46.8	376.0	380.4	378.2	2.2	77.7	447.3	467.1	457.2	9.9	100.9
3	137.1	137.4	137.3	0.1	419.0	412.0	415.5	3.5	69.9	527.7	531.3	529.5	1.8	108.1	553.1	574.8	564.0	10.8	119.2
4	178.0	178.0	178.0	0.0	586.7	577.4	582.1	4.7	101.6	742.7	751.0	746.9	4.1	156.9	738.0	758.5	748.3	10.2	158.7
5	208.9	208.6	208.7	0.1	778.5	769.0	773.8	4.8	142.1	947.2	962.9	955.1	7.8	205.9	978.6	999.4	989.0	10.4	216.7
6	235.6	235.1	235.3	0.2	917.2	928.0	922.6	5.4	172.9	1112.3	1125.1	1118.7	6.4	243.7	1181.5	1201.8	1191.6	10.2	265.3
7	255.9	256.6	256.3	0.3	982.8	993.8	988.3	5.5	184.2	1217.5	1223.6	1220.6	3.0	266.0	1353.2	1374.4	1363.8	10.6	307.0
8	272.2	271.3	271.8	0.5	1050.2	1061.2	1055.7	5.5	197.3	1289.8	1284.9	1287.4	2.4	280.2	1471.2	1492.8	1482.0	10.8	335.5
9	280.3	279.0	279.6	0.6	1125.7	1133.7	1129.7	4.0	213.9	1354.9	1338.8	1346.9	8.1	294.5	1551.8	1576.3	1564.1	12.3	356.2
10	288.6	287.1	287.9	0.8	1226.7	1231.8	1229.2	2.6	237.0	1429.4	1401.3	1415.4	14.1	311.2	1619.6	1644.4	1632.0	12.4	372.7
11	298.4	296.8	297.6	0.8	1356.1	1357.8	1357.0	0.9	266.7	1520.5	1488.0	1504.3	16.3	333.0	1690.1	1714.9	1702.5	12.4	389.5
12	306.3	304.3	305.3	1.0	1484.1	1504.3	1494.2	10.1	299.5	1609.0	1569.2	1589.1	19.9	354.4	1768.7	1792.7	178.	12.0	409.
13	314.6	312.1	313.3	1.2	1505.5	1534.0	1519.8	14.3	303.8	1634.3	1588.9	1611.6	22.7	358.4	1869.3	1891.7	1880.5	11.2	434.2
14	323.1	320.2	321.6	1.4	1515.0	1544.4	1529.7	14.7	304.2	1652.8	1604.8	1628.8	24.0	360.9	1956.9	1984.8	1970.8	14.0	457.3
15	333.8	330.5	332.2	1.7	1524.9	1555.1	1540.0	15.1	304.2	1671.5	1621.3	1646.4	25.1	362.8	1979.5	2007.8	1993.7	14.2	460.7
16	344.0	340.7	342.4	1.7	1532.7	1563.8	1548.2	15.6	303.6	1686.7	1636.4	1661.6	25.2	364.2	1996.1	2024.7	2010.4	14.3	462.5
17	349.3	345.7	347.5	1.8	1542.6	1574.7	1558.7	16.1	305.0	1698.7	1646.9	1672.8	25.9	365.9	2011.5	2040.5	2026.0	14.5	465.5
18	354.8	350.9	352.9	1.9	1555.8	1588.6	1572.2	16,4	307.0	1709.3	1658.6	1684.0	25.4	367.5	2029.9	2059.3	2044.6	14.7	469.2
19	360.6	356.4	358.5	2.1	1566.7	1600.9	1583.8	17.1	308.5	1722.8	1670.7	1696.8	26.1	369.5	2043.7	2073.6	2058.7	14.9	471.5
20	363.4	359.0	361.2	2.2	1575.0	1608.8	1591.9	16.9	309.9	1733.7	1679.3	1706.5	27.2	371,5	2053.8	2083.9	2068.9	15.1	473.6

21	365.6	360.9	363.3	2.4	1580.3	1613.2	1596.7	16.5	310.6	1742.6	1687.7	1715.2	27.4	373.3	2061.6	2091.8	2076.7	15.1	475.3
22	367.5	362.8	365.2	24	1585.1	1618.5	1601.8	16.7	311.3	1750.4	1695.2	1722.8	27.6	374.9	2067.7	2098.0	2082.8	15.2	476.4
23	369.5	364.7	367.1	24	1592.1	1626.8	1609.4	17.4	312.8	1762.5	1706.2	1734.4	28.2	377.6	2077.0	2107.4	2092.2	15.2	478.5
24	372.5	367.9	370.2	23	1600.2	1635.5	1617.9	17.7	314.1	1774.3	1720.2	1747.3	27.1	380.2	2087.0	2117.2	2102.1	15.1	480.4
25	378.7	374.4	376.5	2.1	1607.5	1642.8	1625.2	17.7	314.3	1780.8	1726.9	1753.9	26.9	380.3	2101.6	2132.8	2117.2	15.6	482.8
26	383.4	378.9	381.2	2.3	1611.3	1646.6	1629.0	17.7	314.1	1785.6	1731.9	1758.8	26.8	380.4	2117.6	2149.4	2133.5	15.9	486.1
27	387.1	383.1	385.1	2.0	1615.0	1650.3	1632.6	17.7	314.0	1790.2	1736.8	1763.5	26.7	380.5	2124.8	2156.9	2140.9	16.1	487.0
28	388.3	383.9	386.1	2.2	1615.9	1651.9	1633.9	18.0	314.1	1794.6	1741.4	1768.0	26.6	381.7	2128.6	2161.0	2144.8	16.2	488.
29	388.4	383.9	386.2	2.2	1616.6	1653.6	1635.1	18.5	314.4	1798.9	1745.5	1772.2	26.7	382.8	2132.3	2165.0	2148.6	16.4	489.1
30	392.2	388.7	390.5	1.8	1616.2	1655.2	1635.7	19.5	313.5	1802.8	1749.0	1775.9	26.9	383.3	2136.0	2169.2	2152.6	16.6	489.7
31	393.2	390.0	391.6	1.6	1617.9	1656.9	**1637.4**	19.5	313.6	1806.5	1752.4	**1779.5**	27.1	384.7	2142.5	2182.5	**2162.5**	20.0	492.7

Table 23 (continued)

APPENDIX D: BATCH PILOT-SCALE DIGESTERS [50 L] RAW DATA

Table 24: Raw data from batch pilot-scale digester for blank assay (inoculum only) and MD1 [63 % FS: 18 % FW: 19 % FVW] of the anaerobic co-digestion

Date	Time [Days]	Black assay [Inoculum]		MD1 [63 % FS: 18 % FW: 19 % FVW]						
		Daily biogas flow rate [L/d]	Total Biogas [L]	Daily biogas flow rate [L/day]	Accumulated Total Biogas volume [L]	Specific biogas yield [mL/gVS]	Methane content [%]	Daily methane flow rate [L/d]	Total methane [L]	Specific methane yield [mL/gVS]
	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0
20/10/2020	1	2.0	2.0	27.4	27.4	93.4	35.6	9.7	9.7	28.6
21/10/2020	2	11.5	13.4	32.7	60.1	171.2	56.9	18.6	28.3	54.7
22/10/2020	3	4.3	17.7	13.0	73.0	203.1	60.8	7.9	36.2	67.9
23/10/2020	4	3.5	21.2	11.6	84.6	232.9	66.9	7.7	44.0	83.6
24/10/2020	5	4.5	25.7	7.6	92.2	244.3	70.8	5.4	49.3	86.9
25/10/2020	6	6.5	32.2	17.7	109.8	285.2	70.8	12.5	61.8	108.9
26/10/2020	7	4.6	36.8	13.9	123.8	319.5	73.8	10.3	72.1	129.7
27/10/2020	8	5.3	42.1	15.4	139.2	356,5	72.4	11.1	83.3	151.1
28/10/2020	9	4.5	46.7	20.5	159.7	415.2	76.4	15.7	98.9	192.0
29/10/2020	10	1.4	48.0	7.7	167.4	438.6	73.4	5.7	104.6	207.9
30/10/2020	11	1.2	49.3	4.7	172.1	451.3	73.9	3.5	108.1	216.1
31/10/2020	12	1.3	50.6	4.8	177.0	464.2	73.0	3.5	111.6	224.2
01/11/2020	13	1.5	52.1	6.2	183.2	481.5	72.8	4.5	116.1	235.2
02/11/2020	14	0.5	52.6	7.6	190.8	507.6	72.8	5.6	121.7	253.7
03/11/2020	15	0.3	52.9	4.5	195.3	522.9	72.5	3.2	124.9	264.5
04/11/2020	16	0.4	53.3	1.5	196.8	526.9	73.7	1.1	126.0	267.1

05/11/2020	17	0.3	53.7	0.9	197.7	529.1	71.7	0.7	126.7	268.3
06/11/2020	18	0.5	54.1	0.5	198.2	529.3	72.9	0.4	127.1	268.0
07/11/2020	19	0.4	54.5	1.2	199.4	532.2	71.9	0.8	127.9	269.7
08/11/2020	20	0.5	55.0	1.0	200.5	534.3	71.0	0.7	128.7	270.7
09/11/2020	21	0.3	55.3	1.0	201.4	536.9	68.5	0.7	129.3	272.1
10/11/2020	22	0.6	55.9	1.6	203.0	540.6	68.2	1.1	130.4	274.0
11/11/2020	23	0.3	56.1	0.7	203.7	542.0	66.8	0.4	130.9	274.6
12/11/2020	24	0.2	56.4	0.0	203.7	541.3	62.3	0.0	130.9	273.8
13/11/2020	25	0.2	56.6	0.0	203.7	540.6	65.8	0.0	130.9	273.1
14/11/2020	26	0.2	56.8	0.0	203.8	539.9	65.9	0.0	130.9	272.3
15/11/2020	27	0.2	57.0	0.0	203.8	539.4	65.6	0.0	130.9	271.8
16/11/2020	28	0.1	57.1	0.3	204.0	539.9	65.9	0.2	131.1	272.0

Table 24 (continued)

Table 25: Raw data from batch pilot-scale digester for MD2 [40 % FS: 41 % FW: 19% FVW] of the anaerobic co-digestion.

Date	Time [Days]	Black assay (Inoculum)		MD2 [40 % FS: 41 % FW: 19% FVW]						
		Daily biogas flow rate [L/day]	Cumulative Biogas [L]	Daily biogas flow rate [L/day]	Cumulative Biogas volume [L/d]	Specific Biogas yield [mL/gVS]	Methane content (%)	Daily methane flow rate [L/d]	Cumulative Methane volume [L]	Specific methane yield [mL/gVS]
	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
20/10/2020	1	2.0	2.0	9.6	9.6	28.2	46.9	4.	4.5	9.4
21/10/2020	2	11.5	13.4	45.1	54.7	152.5	55.2	24.9	29.4	58.9
22/10/2020	3	4.3	17.7	16.5	71.3	197.7	64.4	10.6	40.1	82.3
23/10/2020	4	3.5	21.2	14.5	85.8	238.6	67.0	9.7	49.8	105.4
24/10/2020	5	4.5	25.7	20.5	106.4	298.0	71.6	14.7	64.5	143.1
25/10/2020	6	6.5	32.2	30.9	137.3	388.2	72.3	22.4	86.9	201.5
26/10/2020	7	4.6	36.8	22.0	159.3	452.3	71.0	15.6	102.5	242.0
27/10/2020	8	5.3	42.1	25.2	184.5	525.8	55.2	13.9	116.4	273.7
28/10/2020	9	4.5	46.7	21.8	206.3	589.7	72.9	15.9	132.3	315.6
29/10/2020	10	1.4	48.0	6.7	213.0	609.3	71.0	4.7	137.1	328.0
30/10/2020	11	1.2	49.3	6.2	219.2	627.8	74.2	4.6	141.7	340.5
31/10/2020	12	1.3	50.6	7.0	226.2	648.6	72.3	5.0	146.7	354.1
01/11/2020	13	1.5	52.1	7.8	234.0	671.8	72.3	5.6	152.4	369.3
02/11/2020	14	0.5	52.6	2.6	236.6	679.5	72.4	1.9	154.3	374.4
03/11/2020	15	0.3	52.9	1.6	238.2	684.1	69.1	1.1	155.3	377.2
04/11/2020	16	0.4	53.3	1.8	240.0	689.5	69.9	1.3	156.6	380.5
05/11/2020	17	0.3	53.7	1.6	241.6	694.3	68.6	1.1	157.8	383.4

06/11/2020	18	0.5	54.1	2.3	244.0	701.1	70.3	1,6	159.4	387.6
07/11/2020	19	0.4	54.5	1.8	245.8	706.4	66.1	1.2	160.6	390.6
08/11/2020	20	0.5	55.0	2.2	248.0	712.9	66.2	1.5	162.1	394.3
09/11/2020	21	0.3	55.3	1.2	249.3	716.5	66.1	0.8	162.9	396.4
10/11/2020	22	0.6	55.9	2.8	252.0	724.6	67.5	1.9	164.8	401.1
11/1/20201	23	0.3	56.1	1.3	253.3	728.2	63.0	0.8	165.5	403.0
12/11/2020	24	0.2	56.4	1.1	254.4	731.5	60.7	0.7	166.2	404.7
13/11/2020	25	0.2	56.6	1.0	255.4	734.5	62.2	0.6	166.9	406.2
14/11/2020	26	0.2	56.8	1.0	256.4	737.4	62.2	0.6	167.5	407.7
15/11/2020	27	0.2	57.0	0.7	257.2	739.5	62.0	0.5	167.9	408.8
16/11/2020	28	0.1	57.1	0.5	257.7	740.9	62.8	0.3	168.3	409.5
17/11/2020	29	0.1	57.2	0.5	258.1	742.3	61.6	0.3	168.5	410.2
18/11/2020	30	0.0	57.2	0.0	258.1	742.3	62.3	0.0	168.5	410.2
19/11/2020	31	0.0	57.2	0.0	258.2	742.3	60.2	0.0	168.6	410.3
20/11/2020	32	0.0	57.2	0.6	258.7	744.5	61.2	0.4	168.9	411.6
21/11/2020	33	0.0	57.2	0.4	259.1	745.8	61.3	0.2	169.1	412.4
22/11/2020	34	0.0	57.2	0.0	259.1	746.0	62.0	0.0	169.2	412.5
23/11/2020	35	0.0	57.2	0.0	259.2	746.0	60.6	0.0	169.2	412.5
24/11/2020	36	0.0	57.2	0.0	259.2	746.0	61.0	0.0	169.2	412.5

Table 25 (continued)

Table 26: Biogas composition for two optimum mixtures in batch pilot-scale digesters during anaerobic co-digestion of fish sludge, food waste, and fruit & vegetable waste

Date	Time [Days]	MD1 Digester [63 % FS: 18 % FW: 19 % FVW]						MD2 Digester [40 % FS: 41 % FW: 19% FVW]					
		CH_4 [%]	CH_4 [%]*	Average CH_4 [%]	CO_2[%]	o_2 [%]	HS_4 [ppm]	CH_4 [%]	CH_4 [%]*	Average CH_4 [%]	CO_2 [%]	O_2 [%]	H_2S [ppm]
26/10/2020	0	0	0				0	0	0	0			0
27/10/2020	1	48.2	47.9	62.25	36.4	4.3	571	55.22	55.22	55.2	39.1	7.6	486
28/10/2020	2	76.5	76.3	76.4	20.8	2.8	505	72.9	72.8	72.85	22.9	2.1	448
29/10/2020	3	73.1	73.6	73.35	20.7	5.4	427	70.7	71.2	70.95	22.9	1.9	437
30/10/2020	4	74.1	73.6	73.85	22.2	3.3	429	72.3	72.3	72.3	23.5	2.5	464
02/11/2020	5	73.6	72	72.8	21.4	5.2	381.3	72.3	72.4	72.35	22.2	3.2	400
03/11/2020	6	72.5	72.4	72.45	20	7.6	225	68.8	69.4	69.1	22.7	2.2	365
04/11/2020	7	73.6	73.7	73.65	20.4	7.8	344	70.6	69.2	69.9	23.1	2.3	399
05/11/2020	8	71.5	71.9	71.7	22.9	1.7	258	68.7	68.4	68.55	23.7	2.1	142
06/11/2020	9	73	72..8	72.9	22.4	5	351	69.2	71.3	70.25	24.9	2.4	406
09/11/2020	10	68.3	68.7	68.5	23.7	6.8	246	65.1	67.1	66.1	28.2	2.2	321
10/11/2020	11	68.2	68.1	68.15	24	4.2	333	67.7	67.2	67.45	25.5	1.5	332
11/11/2020	12	66.5	67	66.75	25.1	6	212	62.9	63	62.95	29.8	2.7	263
12/11/2020	13	61.9	62.7	62.3	24.6	7.6	177	61.6	59.8	607	28.5	2.3	336
13/11/2020	14	65.4	66.1	65.75	25.2	9.7	261	62.3	62	62.15	28.7	1.8	390
16/11/2020	15	65.9	65.8	65.85	26.9	3.8	314	62.4	63.2	62.8	29.7	1.8	411
17/11/2020	16	64.7	64.8	64.75	26.8	7	225	61.5	61.6	61.55	29.7	2.2	390
18/11/2020	17	64.2	64.6	64.4	27.4	4.1	308	62.3	62.2	62.25	30.9	1.7	461
19/11/2020	18	63.2	63.1	63.15	27.5	5.3	272	60.3	60	60.15	29.1	2.2	405

20/11/2020	19	65.5	65.3	65.4	28.4	1	388	61.1	61.3	61.2	29.1	2.2	405
23/11/2020	20	64.3	64.3	64.3	29.2	4.4	309	61.1	60	60.55	30.1	1.7	450
24/11/2020	21	63.4	63.7	63.55	29.5	5.6	258	60.8	61.1	60.95	32.1	1.5	405
25/11/2020	22	62.2	62.4	62.3	27.1	2.5	373	62	61.9	61.95	29.1	1.6	394
26/11/2020	23												
			Average	67.93	25.1		311.6		Average	65.10	27.52		386.81
			min	62.22	20		177		Min	60.15	22.2		142
			Max	76.4	36.4		571		Max	72.85	39.1		486

*Duplicate values

Table 26 (continued)

APPENDIX E: PROCESS PERFORMANCE FOR BATCH PILOT-SCALE DIGESTERS RA RAW DATA

Table 27: pH, total alkalinity, and ammonia nitrogen production raw data monitored during the anaerobic co-digestion of two optimum mixtures in batch pilot-scale digesters.

Date	Time [Days]	MD1 [63 % FS: 18 % FW: 19 % FVW]					MD2 [40 % FS: 41 % FW: 19% FVW]				
		pH	pH	Average pH	Final Alkalinity ($Caco_3$) [mg/L]	Ammonia [mg/L]	pH	pH	Average pH	Alkalinity ($Caco_3$) [mg/L]	Ammonia [mg/L]
20/10/2020	0	7	7.01	7.0			7.05	7.08	7.1		
21/10/2020	1	7.02	7.34	7.2			6.9	6.91	6.9		
22/10/2020	2	7.01	7.02	7.0			7.03	7.24	7.0		
23/10/2020	3	7.26	7.24	7.3		1100	7.34	7.4	7.4		1600
26/10/2020	4	7.34	7.35	7.3			7.94	7.85	7.9		
27/11/2020	5	745	7.59	7.5			7.56	7.78	7.7		
28/11/2020	7	7.61	7.59	7.6			7.45	7.48	7.5		
29/11/2020	8	7.62	7.63	7.6			7.72	7.73	7.7		
30/11/2020	9	7.92	7.94	7.9			7.79	7.79	7.8		
02/11/2020	10	8	7.98	8.0		1350	7.82	7.83	7.8		1300
03/11/2020	11	7.78	7.78	7.8			7.88	7.85	7.9		
04/11/2020	12	8.22	7.8	8.0			7.96	7.78	7.9		
05/11/2020	13	7.65	7.69	7.7			7.56	7.59	7.6		
06/11/2020	14	7.79	7.79	7.8			7.6	7.59	7.6		
09/11/2020	15	7.68	7.68	7.7		950	7.64	7.63	7.6		900
10/11/2020	16	7.58	7.6	7.6			7.44	7.46	7.5		
11/11/2020	17	7.73	7.75	7.7			7.58	7.54	7.6		
12/11/2020	18	7.84	7.83	7.8			7.81	7.6	7.7		

13/11/2020	19	7.83	7.81	7.8			7.87	7.86	7.9		
16/11/2020	20	7.47	7.47	7.5		900	7.78	7.78	7.8		1000
17/11/2020	21	7.62	7.62	7.6			7.49	7.5	7.5		
18/11/2020	22	7.66	7.67	7.7			7.48	7.49	7.5		
19/11/2020	23	7.95	7.97	8.0			7.7	7.71	7.7		
20/11/2020	24	7.85	7.86	7.9			7.62	7.63	7.6		
23/11/2020	25	7.85	7.86	7.9	4162	1050	7.62	7.63	7.6	6860	850

Note: Alkalinity was recorded at the end of the anaerobic co-digestion. It is also important to note that the VFA production was not detected during the anaerobic co-digestion in batch pilot-scale digesters.

Table27 (continued)

APPENDIX F: SEMI-CONTINOUS PILOT-SCALE DIGESTER [30 L] RAW DATA

Table 28: Raw data for daily biogas flow rate, total biogas yield, biogas composition, daily methane production, and methane yield during anaerobic co-digestion of fish sludge, food waste, fruit &vegetable waste at different OLR in semi-continuous digester.

Date	Time [Day]	Semi-continuous pilot scale digester [30 L] [63 % FS: 18 % FW: 19 % FVW]									
		OLR [$gVSL^{-1}d^{-1}$]	Daily biogas volume [L/day]	Total Biogas volume [L/d]	Methane content [CH_4] [%]	CO_2 [%]	O_2[%]	H_2S [ppm]	Daily methane production [L/d]	Cumulative Methane (L/d)	Cumulative methane per OLR
26/10/2020	1	1	5.7	5.7					0.0	0.0	
27/10/2020	2	1	14.1	19.8	16.4	50.7	1.6	787	2.3	2.3	2.3
28/10/2020	3	1	9.5	29.3	22.5	67.7	1.5	1390	2.1	4.4	4.4
29/10/2020	4	1	11.7	41.0	26.9	62.3	1.5	1455	3.1	7.6	7.6
30/10/2020	5	1	20.5	61.5	46.9	67.5	2	1064	9.6	17.2	17.2
31/10/2020	6	1	13.8	75.4	21.7				3.0	20.2	20.2
01/11/2020	7	1	10.8	86.1	34.7				3.7	23.9	23.9
02/11/2020	8	1	16.6	102.7	47.5	44.6	1.3	2228	7.9	31.8	31.8
03/11/2020	9	1	25.1	127.9	50.1	42.1	1.8	1522	12.6	44.4	44.4
04/11/2020	10	1	21.6	149.5	48.7	49.1	1.8	1033	10.5	55.0	55.0
05/11/2020	11	1	9.0	158.5	65.2	35.1	1.9	889	5.9	60.8	**60.8**
06/11/2020	12	2	5.9	164.4	8.8	66.9	1.6	735	0.5	61.3	61.3
07/11/2020	13	2	15.4	179.8	8.3				1.3	62.6	62.6
08/11/2020	14		2.2	182.0	7.5	68.8	2	1056	0.2	62.8	

09/11/2020	15		0.8	182.9	7.3	65.8	7.6	1092	0.1	62.9	0.1
10/11/2020	16		2.8	185.7	6.9	66.5	3.2	1110	0.2	63.0	0.3
11/11/2020	17	**Stop feeding**	4.3	190.0	20.2	36.5	2.2	135	0.9	63.9	1.1
12/11/2020	18		5.7	195.7	36.9	31.7	2.3	667	2.1	66.0	3.2
13/11/2020	19		8.6	204.3	48.7	30.0	1.4	1442	4.2	70.2	7.4
14/11/2020	20		12.0	216.3	64.7				7.8	78.0	15.2
15/11/2020	21		14.8	231.0	70.5				10.4	88.4	25.6
16/11/2020	22	2	16.5	247.5	72.6	20.8	1.5	879	12.0	100.3	37.5
17/11/2020	23	2	20.2	267.7	73.1	20.8	1.7	762	14.8	115.1	52.3
18/11/2020	24	2	23.2	291.0	73.3	20.72	1.4	690	17.0	132.1	69.3
19/11/2020	25	2	32.6	323.6	47.7	44	1.4	984	15.6	147.7	84.9
20/11/2020	26	2	36.4	360.0	61.2	32.3	3.7	821	22.3	170.0	107.2
21/11/2020	27	2	41.3	401.3	68.7				28.3	198.3	135.5
22/11/2020	28	2	32.5	433.8	70.5				22.9	221.2	158.4
23/11/2020	29	2	32.6	466.4	71.6	23.1	1.2	1061	23.3	244.5	181.7
24/11/2020	30	2	42.3	508.7	48.0	48.5	1.6	1641	20.3	264.8	202.0
25/11/2020	31	2	38.7	547.4	66.6	27.4	5.2	973	25.8	290.6	227.8
26/11/2020	32	2	41.9	589.3	53.5	35	1.8	1520	22.4	313.0	250.2
27/11/2020	33	2	35.2	624.4	66.7	27	8.8	837	23.4	336.4	273.7
28/11/2020	34	2	44.2	668.7	66.9				29.6	366.0	303.3
29/11/2020	35	2	45.0	713.7	67.9				30.6	396.6	333.8
30/11/2020	36	2	42.2	755.9	65.3	29.7	2.7	1785	27.5	424.1	361.3
01/12/2020	37	2	39.1	794.9	44.7	46.5	2.7	1720	17.4	441.6	**378.8**
02/12/2020	38	3	42.2	837.1	63.0	31.5	6.7	1420	26.6	468.1	405.3
03/12/2020	40	3	26.6	863.7	40.1	52.2	5	2005	10.7	478.8	405.3
04/12/2020	41	3	19.5	883.3	63.6	33.5	3.3	1374	12.4	491.2	417.7

05/12/2020	42	3	13.3	896.6	50.6				6.7	498.0	6.7
06/12/2020	43	3	10.3	906.9	64.8				6.6	504.6	13.4
07/12/2020	44	3	17.0	923.8	65.1	27	4.2	811	11.0	515.6	24.4
08/12/2020	45	3	21.0	944.9	24.5	66.5	5.9	1050	5.1	520.8	29.6
09/12/2020	46	3	13.7	958.5	25.6				3.5	524.3	33.1
10/12/2020	47	3	14.9	973.4	25.4				3.8	528.1	36.9
11/12/2020	48	3	6.7	980.1	6.1				0.4	528.5	37.3
12/12/2020	49	3	18.1	998.2	24.5				4.4	532.9	41.7
13/12/2020	50	3	7.0	1005.2	12.0				0.8	533.7	42.5
14/12/2020	51	3	1.3	1006.5	2.4				0.0	533.8	42.6
15/12/2020	52	3	7.1	1013.6	12.0				0.9	534.6	**43.4**

Note: The red shaded area represents the days where the digester was stopped feeding for 8 days due to the inhibition of the anaerobic digestion system by the accumulation of VFA. **Table 28** (Continued)

Table 29: Raw data for pH and VFA concentration production in semi-continuous pilot-scale digester during anaerobic co-digestion of fish sludge, food waste, and fruit & vegetable waste at different OLR.

Date	Semi-continuous pilot scale digester [30 L] [63 % FS: 18 % FW: 19 % FVW]										
	Day	OLR [$gVSL^{-1}d^{-1}$]	pH	pH	Average pH	Acetic acid	Propionic acid	Butyric acid	Valeric acid	Cuproic Acid	Total VFA [g/L]
26/10/2020	1	1	7.01	7.16	7.09						
27/10/2020	2	1	6.98	6.98	6.98						
28/10/2020	3	1	6.93	6.91	6.92	0.05	0.26	0.01			0.32
29/10/2020	4	1	7.06	7.08	7.07						
30/10/2020	5	1	7.48	7.47	7.48						
31/10/2020	6	1	7.34	7.34	7.34	0.49	0.10	0.2	0.18		1.00
01/11/2020	7	1	7.45	7.45	7.45						
02/11/2020	8	1	7.34	7.34	7.34	0.05	0.03	0.01			0.09
03/11/2020	9	1	6.89	6.89	6.89	2.62	1.27	1.18	0.33		0.09
04/11/2020	10	1	6.8	6.85	6.83						
05/11/2020	11		5.99	5.98	5.99	2.54	0.94	1.43	0.53		5.44
06/11/2020	12	2	5.91	5.96	5.94						
07/11/2020	13	2	6.10	6.21	6.16	3.06	1.22	1.82	0.76		6.87
08/11/2020	14	**Stop Feeding**	6.32	6.36	6.34						
09/11/2020	15		6.23	6.21	6.22	3.243	1.48	1.65	0.62		7.00
10/11/2020	16		6.88	6.89	6.89	1.589	0.87	1.22	0.62	0.48	4.77
11/11/2020	17		6.9	6.91	6.91						

12/11/2020	18		7.4	7.43	7.42	1.47	0.95	1.23	1.25		4.91
13/11/2020	19		7.2	7.2	7.20	3.49	0.99	2.03	2.01		8.50
14/11/2020	20		7.16	7.16	7.16	3.01	1.66	1.47			6.14
15/11/2020	21		7.48	7.47	7.48						
16/11/2020	22	2	7.49	7.47	7.48	1.98	0.97	1.66	1.97		6.59
17/11/2020	23	2	7.42	7.43	7.43						
18/11/2020	24	2	7.45	7.46	7.46	3.0	1.66	1.47			6.14
19/11/2020	25	2	7.62	7.62	7.62	1.85	0.89	1.78	0.45	0.44	5.45
20/11/2020	26	2	6.98	6.99	6.99	1.50	1.32	0.57	0.91		4.28
21/11/2020	27	2	7.12	7.12	7.12						
22/11/2020	28	2	7.26	7.23	7.25						
23/11/2020	29	2	6.98	6.98	6.98	2.01	2.33	0.27			4.61
24/11/2020	30	2	7.06	7.07	7.07	1.05	2.59				3.64
25/11/2020	31	2	7.07	7.07	7.07	1.04	2.84				3.88
26/11/2020	32	2	7.22	7.32	7.27						
27/11/2020	33	2	7.5	7.48	7.49	0.53	3.01				3.53
28/11/2020	34	2	7.45	7.42	7.44						
29/11/2020	35	2	7.34	7.34	7.34						
30/11/2020	37	2	7.22	7.23	7.23	1.67	2.02	0.31			4.00
01/12/2020	38	2	6.83	6.83	6.83	0.46	3.75				4.21
02/12/2020	39	3	7	7.02	7.01	1.83	3.05	0.47			5.37
03/12/2020	40	3	6.93	6.92	6.93	2.12	3.66	1.04	0.12		6.91
04/12/2020	41	3	6.65	6.64	6.65	1.15	4.27	1.48			6.90

05/12/2020	42	3	7.20	7.20	7.20	-	-	-	-	-	
06/12/2020	43	3	6.86	6.86	6.86	1.15	4.27	1.48		-	6.90
07/12/2020	44	3	6.66	6.65	6.66	0.34	4.24				4.58
08/12/2020	45	3	6.28	6.33	6.31	2.4	3.71	0.94	0.38		7.43
09/12/2020	46	3	6.52	6.52	6.52	-					
10/12/2020	47	3	6.76	6.77	6.77	3.41	2.70	2.40	0.67		9.18
11/12/2020	48	3	6.87	6.87	6.87	3.16	2.57	2.40	0.62		8.74
12/12/2020	49	3	7.20	7.23	7.22	4.93	1.33	3.38	1.28	0.7	11.62
13/12/2020	50	3	6.54	6.54	6.54	3.16	2.57	2.40	0.62		8.74
14/12/2020	51	3	6.23	6.25	6.24	5.19	1.76	1.94	1.70	0.25	10.83
15/12/2020	52	3	6.34	6.23	6.3	5.16	1.99	1.95	1.76	0.24	11.104

Table 29 (continued**)**