

**Resource Recovery from Organic Waste through an
Anaerobic Fermentation Platform**

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Abstract

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Resource recovery strategies have increasingly been employed nowadays due to the increasing waste generation, strict waste disposal regulations and increased public awareness. Anaerobic fermentation is a common method used for resource recovery of waste streams. This study focused on recovery of volatile fatty acids (VFA) from anaerobic co-fermentation of food waste and primary sludge and sequential conversion to polyhydroxyalkanoate (PHA). The ultimate objective was to maximize the VFA and PHA production by manipulating the fermentation operating conditions (substrate; food waste FW vs mixture of primary wastewater sludge and food waste PS+FW, organic loading rate OLR 10 and 25 kg-COD/m³/d, pH 7, 7.5, 8, and 9, hydraulic retention time HRT 2, 4, and 6 days) for VFA production and the substrate (feeding pattern; one-pulse vs multi-pulse, composition and source; fermentation-derived vs synthetic acids) for PHA production. The factors that were evaluated for the VFA and PHA optimization were the VFA yield, concentration and speciation and PHA yield and speciation.

Results showed that VFA production was higher at the higher of the two OLR, and the FW reactor showed higher concentrations of VFA than the PS+FW reactor, which showed that food waste are able to improve the fermentation process. Experiments showed that the preferred conditions for max VFA and PHA production were co-fermentation of primary sludge and food waste of OLR 25 kg-COD/m³/d, at alkaline conditions of pH 9 and HRT 6d and then feeding of fermentation derived VFA in one-pulse in PHA accumulating reactor. Maximum PHA

accumulation achieved was 0.38 ± 0.05 g-PHA/g-VSS from fermentate produced at pH 9.0 and HRT 6d.

Polyhydroxyalkanoates (PHAs) are biopolymers synthesized by microorganisms. This study also focused on the microbial ecology of the two processes involved in PHA production; (1) acidogenic co-fermentation of primary sludge and food waste, and (2) enrichment of PHA accumulating microorganisms. The analysis regarding the fermentation reactor included comparison of microbial ecology between the different fermentation conditions tested (pH 7, 8 and 9 and HRT 2, 4 and 6 days). Most of the bacterial families present in the fermentation reactor belonged to the phylum of Firmicutes, Bacteroidetes and Proteobacteria. The microbial species in that condition were *Tepidimicrobium xylanilyticum*, *Tepidimicrobium ferriphilum*, *Vagococcus lutrae*, and *Vagococcus acidifermentans*. Regarding the PHA reactor, the analysis included monitoring of the shift in microbial ecology throughout the enrichment process. The maximum PHA accumulation achieved was $38\% \pm 5\%$, g-PHA/g-VSS, and the representative microbial species were *Acinetobacter radioresistens*, *Pseudoxanthobacter liyangensis*, and *Xanthobacter agilis*. The objective of this study was to connect the VFA and PHA production with the microbial ecology of each process. Study of the microbial ecology of the combined system of VFA production with further conversion to PHA through mixed microbial cultures could contribute to an efficient process design with possible application into Water Resource Recovery Facilities (WRRF) and decrease of the cost of PHA production, therefore increase their market value. Retrofitting of the existing anaerobic digestion infrastructure could create a biorefinery platform that uses wastes to produce biodegradable plastics instead of methane.

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Εκ γαίης γαρ πάντα και εις γην πάντα τελευτά
Everything comes from earth and everything ends in Earth

Xenophanes, 570-480 BC, Ancient Greek Philosopher

Chapter 1: Introduction and Research Scope

1.1 Waste Streams and Anaerobic Digestion

Environmental sustainability is of major importance nowadays as the existing waste management infrastructure continues to face huge pressure due to increasing waste generation coupled with stringent waste disposal regulations and increased public awareness. (A. Liu et al. 2015). Generation of municipal solid waste (MSW) globally is 1.3 billion tons per year (1.2 kg per capita per day). (Tyagi et al., 2018) The main methods of disposal of MSW are landfilling, recycling, composting and combustion with energy recovery. In the United States, 52.5% of MSW is disposed in the landfill (“Advancing Sustainable Materials Management: 2015 Fact Sheet.” 2018). Landfills are often associated with contamination of adjacent water and solid bodies due to leachate containing heavy metals and microbial pathogens. Also, landfills can cause air pollution from greenhouse gases (GHG) and volatile organic compounds (VOC). The organic fraction of MSW (OFMSW) accounts for 46% of MSW and includes food scraps, yard waste and process residues. Specifically, food waste (FW) accounts for 15.1% of MSW (~40 million tons). Most of the food waste (75%) is disposed in the landfills, as only 25% of generated food waste goes into recycling and composting and combustion with energy recovery. (“Advancing Sustainable Materials Management: 2015 Fact Sheet.”, 2018)

Anaerobic digestion (AD) is the most commonly used technique for energy recovery from OFMSW as it creates two valuable products; biogas which can be used for electricity and heat production, and a digestate rich in nutrients which can be used in agriculture as fertilizer. AD is also used for sewage sludge treatment. Co-digestion of those two substrates can provide

several benefits such as positive synergistic effect on process efficiency, increase of biodegradable content, dilution of toxic or inhibitory compounds, increase of nutrients availability and moisture balance. OFMSW is highly biodegradable and it has high moisture content due to the large fraction of FW in it. FW consists of food processing and kitchen waste. The primary component of OFMSW is carbohydrates (53% by weight), and the primary component of sewage sludge is protein (30% by weight) with lower carbon to nitrogen ratio compared to OFMSW (Romero Aguilar et al. 2013). AD of protein increases the ammonia concentration and that could cause process inhibition because of the toxicity of ammonia (H. B. Nielsen and Angelidaki 2008). Therefore, co-digestion of carbohydrate-rich and protein-rich substrates can help the C:N ratio balance while reducing the risk of ammonia inhibition (Tyagi et al. 2018)

Wastewater treatment plants (WWTP) have been adding food waste as co-digestion component in the treatment process of sewage sludge in order to increase the fermentable content and improve productivity. This way, wastewater treatment facilities offer the opportunity to divert large amounts of food waste from the landfill and promote recycling. Also, less food waste in the landfills leads to fewer methane release in the atmosphere.

1.2 Wastewater Treatment Plants and Anaerobic Digestion

Wastewater treatment includes the following steps: preliminary, primary, secondary, tertiary, and biosolids stabilization. Preliminary step uses screens and a grit chamber to remove big objects and sand, grit or small stones that might clog or damage pipes in downstream processes. The water that leaves the grit chamber still contains dissolved organic and inorganic components and suspended solids. During the primary process, water enters a sedimentation tank

where suspended solids sink at the bottom, creating the primary sludge (PS). Approximately 25 - 50% of the incoming COD (Chemical Oxygen Demand), 50 - 70% of the total suspended solids (TSS), and around 65% of the oil and grease are removed during primary treatment. (Metcalf, 2005) The next process is a biological treatment process where microorganisms break down the organic matter still contained in the wastewater, metabolizing it using oxygen. This step includes an aeration tank where microorganisms use the oxygen in order to grow and rapidly metabolize the organic matter, and a secondary sedimentation tank where the sludge containing microorganisms (activated sludge) settles and then is recycled or removed. The last step provides a final treatment stage; filtration and disinfection (chlorine, ozone and ultraviolet radiation) to further improve the effluent quality before it is discharged to the receiving environment. The primary and secondary sludge (biosolids) produced in the wastewater treatment process are being stabilized to control odor and reduce disease-causing organisms, and then they can be used as fertilizers, get incinerated or landfilled. (Metcalf, 2005) Anaerobic digestion is a form of stabilization.

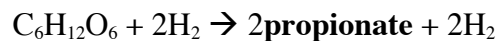
Conventional anaerobic digestion treatment of biosolids produces biogas that is usually used for electricity and heat demands internally in the wastewater treatment plant. However, AD can be an efficient method of resource recovery in a broader spectrum. (Zhou et al. 2018) Anaerobic digestion consists of 4 stages: 1. Hydrolysis, 2. Acidogenesis, 3. Acetogenesis, and 4. Methanogenesis. Volatile fatty acids (VFA) are produced in steps #2 and #3 through a series of biochemical reactions that are catalyzed by acidogenic and acetogenic bacteria. VFA have great added value potential as they have many applications, such as biopolymers (polyhydroxyalkanoates, PHA), biofuels, bulk chemicals, and biological nutrient removal in

wastewater treatment. (F. C. Silva et al. 2013). WWTP that can produce a variety of value-added products are referred as Water Resource Recovery Facilities (WRRF).

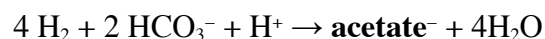
1.3 Anaerobic Digestion and Acidogenic Fermentation

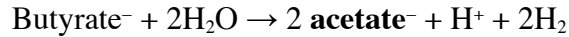
Anaerobic digestion is the biodegradation of complex biological macromolecules by microorganisms in the absence of oxygen with final products that include mainly methane and carbon dioxide. This biological process consists of four stages (Figure 1- 1); hydrolysis, acidogenesis, acetogenesis and methanogenesis. (Tom Fenchel, 2012)

- During hydrolysis, enzymes produced by hydrolytic bacteria such as *Streptococcus* and *Enterobacterium* hydrolyze macromolecules such as carbohydrates, proteins and lipids into their monomers; monosaccharides, amino acids, and long chain fatty acids. Those enzymes are amylase, protease and lipase. (Tom Fenchel, 2012)
- During acidogenesis, acidogenic bacteria convert the products of hydrolysis into volatile fatty acids and alcohols (formic acid, acetate, propionate, butyrate, lactic acid, methanol and ethanol). Some acidogenic bacteria that participate in this stage are *Pseudomonas*, *Bacillus*, *Clostridium*, *Micrococcus*, and *Flavobacterium*. (Tom Fenchel, 2012)



- During acetogenesis, acetogenic bacteria, such as *Syntrophomonas* and *Syntrophobacter*, convert the products of acidogenesis into acetate, carbon dioxide and hydrogen. (Tom Fenchel, 2012)





- During methanogenesis, methane is produced either from acetate splitting or carbon dioxide reduction. The first process occurs by acetoclastic methanogens such as *Methanosarcina* and *Methanosaeta*, and the second process occurs by hydrogenotrophic methanogens. (Tom Fenchel, 2012)



Acidogenic fermentation refers to the production of VFA during anaerobic digestion involving the processes of hydrolysis, acidogenesis and acetogenesis.

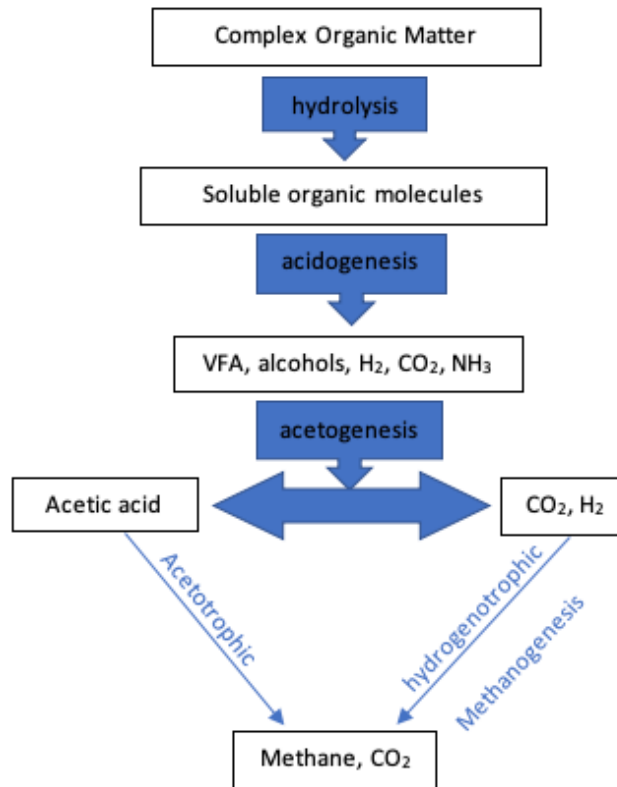


Figure 1- 1: Stages of anaerobic digestions. Adapted from (Tom Fenchel 2012)

1.4 Metabolic Pathways of Acidogenic Fermentation

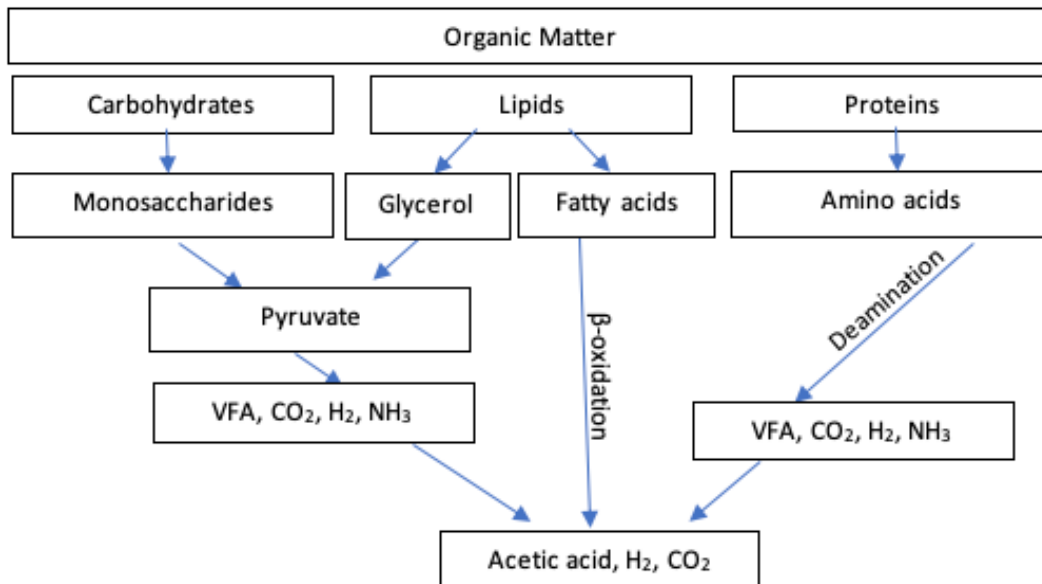


Figure 1- 2: The fate of organic matter during acidogenic fermentation. Adapted from (Sikora et al. 2019)

As shown in

Figure 1- 2 organic matter consists of 3 main carbonaceous components; carbohydrates, lipids and proteins. The microorganisms secrete hydrolytic enzymes to the bulk liquid where they absorb to a macromolecule or microorganisms attach to the macromolecules and secrete hydrolytic enzymes at the vicinity of the macromolecules. (Sander, 1999) Then, the hydrolytic enzymes; generally called hydrolases are responsible for the degradation of organic matter by acting on ester bonds (esterases), glycoside bonds (glycosidases), cellulose (cellulase; endo-1,4-beta-D-glucanase, cellulose 1,4-beta-cellobiosidase, etc), peptide bonds (peptidases) and other hydrolases acting on carbon-nitrogen bonds, ether bonds, carbon-carbon bonds, phosphorus-nitrogen bonds, sulfur-nitrogen bonds, carbon-phosphorus bonds, sulfur-sulfur bonds, carbon-sulfur bonds, acid anhydrides. After the macromolecules have broken down during hydrolysis,

the monomers (monosaccharides, glycerol, fatty acids and amino acids) are going through the acidogenic stage. The key intermediate of this stage is pyruvate. Pyruvate is then further converted to short-chain fatty acids.

1.4.1 Carbohydrates

Carbohydrates are converted to pyruvate with transformation of glucose to pyruvate through glycolysis (the Embden-Meyerhof-Parnas pathway) as shown in Table 1- 1.

Table 1- 1: Reactions for pyruvate production from carbohydrates. (Sikora et al. 2019)

(table continues at the next page)

Reactions		Enzymes
Reactants	Products	
D-glucose + ATP	\rightleftharpoons D-glucose-6-phosphate + ADP	Hexose kinase
D-glucose-6-phosphate	\rightleftharpoons D-fructose-6-phosphate	Phosphoglucose isomerase
ATP + D-fructose-6-phosphate	\rightleftharpoons ADP + D-fructose-1,6-bisphosphate	Phosphofructose kinase
Fructose-1,6-bisphosphate	\rightleftharpoons dihydroxyacetone phosphate + glyceraldehyde-3-phosphate	Fructose-bisphosphate aldolase
Glyceraldehyde-3-phosphate	\rightleftharpoons dihydroxyacetone phosphate	Triose phosphate isomerase
D-glyceraldehyde-3-phosphate		Glyceraldehyde-3-phosphate dehydrogenase

phosphate + phosphate + NAD ⁺	\rightleftharpoons 1,3-bisphosphoglycerate + NADH + H ⁺	phosphate dehydrogenase
1,3- bisphosphoglycerate + ADP	\rightleftharpoons 3-phosphoglycerate + ATP	Phosphoglycerate kinase
3-phosphoglycerate	\rightleftharpoons 2-phosphoglycerate	Phosphoglycerate mutase
2-phospho-D- glycerate	\rightleftharpoons phosphoenolpyruvate + H ₂ O	Enolase
Phosphoenolpyruvate + ADP	\rightleftharpoons pyruvate + ATP	Pyruvate kinase

1.4.2 Lipids

Lipids are converted to pyruvate with transformation of glycerol to pyruvate through an oxidative pathway of glycerol transformations; and fatty acids are converted to acetate through acyl-CoA and acetyl-CoA via β -oxidation (Figure 1- 3). Glycerol transformation is shown in Table 1- 2.

Table 1- 2: Reactions for production of pyruvate from lipids. (Sikora et al. 2019)

Reactions		Enzymes
Reactants	Products	
Glycerol + NAD ⁺	⇌ glycerone	Glycerol dehydrogenase
ATP + glycerone	⇌ ADP + dihydroxyacetone phosphate	Dihydroxyacetone
Glyceraldehyde-3- phosphate	⇌ dihydroxyacetone phosphate	Triose phosphate isomerase
D-glyceraldehyde-3- phosphate + phosphate + NAD ⁺	⇌ 1,3-bisphosphoglycerate + NADH + H ⁺	Glyceraldehyde-3- phosphate dehydrogenase
1,3- bisphosphoglycerate + ADP	⇌ 3-phosphoglycerate + ATP	Phosphoglycerate kinase
3-phosphoglycerate	⇌ 2-phosphoglycerate	Phosphoglycerate mutase
2-phospho-D- glycerate	⇌ phosphoenolpyruvate + H ₂ O	Enolase
Phosphoenolpyruvate + ADP	⇌ pyruvate + ATP	Pyruvate kinase

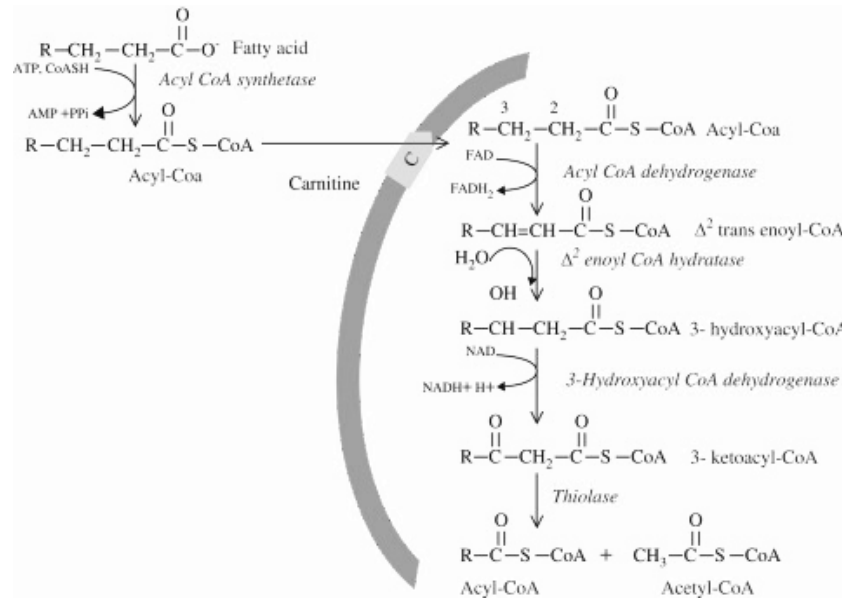


Figure 1- 3: Pathway of β-oxidation. Adapted from Kumari (2018)

Figure 1- 3 shows the pathway of beta-oxidation. The first step is the oxidation of the fatty acid by the enzyme acyl-CoA-dehydrogenase. The enzyme catalyzes the formation of a double bond between the 2nd and 3rd carbon atoms. The next step is the hydration of the bond by enoyl-CoA-hydratase. The third step is the oxidation of 3-hydroxyacyl-CoA by NAD⁺ with conversion of the hydroxyl group into a keto group. The final step is the cleavage of 3-ketoacyl-CoA by the thiol group of another molecule of Coenzyme A. The thiol is inserted between the 2nd and 3rd carbon atom. This cycle repeats, in every cycle the Acyl-CoA unit is shortened by two carbon atoms and the continues until all of the carbons in the fatty acid are turned into acetyl CoA.

1.4.3 Proteins

The amino acids from the proteins are converted to short-chain fatty acids, carbon dioxide and hydrogen with or without syntrophy with H₂-scavenging microorganisms.

The case of syntrophy with H₂-scavenging microorganisms, involves deamination of amino acids to the corresponding a-keto acid by amino acid dehydrogenases and further conversion of a-keto acids to fatty acids through oxidative decarboxylation:

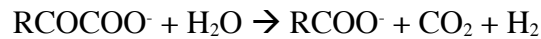
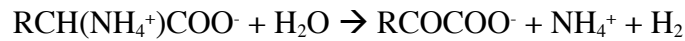


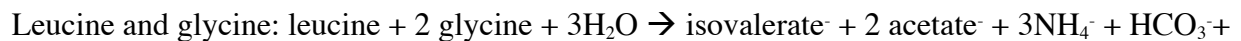
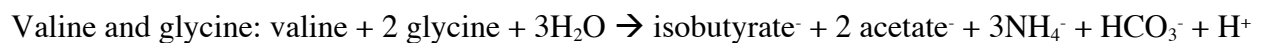
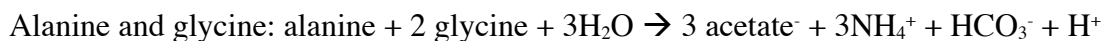
Table 1- 3: Examples of amino acid dehydrogenases catalyzing deamination of amino acids to the corresponding a-keto acids. (Schink and Stams 2013)

Reactions		Enzymes
Reactants	Products	
L-aspartate + H ₂ O + NAD(P) ⁺	⇌ oxaloacetate + NH ₃ + NAD(P)H + H ⁺	Aspartate dehydrogenase
L-valine + H ₂ O + NADP ⁺	⇌ 3-methyl-2-oxobutanoate + NH ₃ + NADPH + H ⁺	Valine dehydrogenase
L-alanine + H ₂ O + NAD ⁺	⇌ pyruvate + NH ₃ + NADH + H ⁺	Alanine dehydrogenase
L-leucine + H ₂ O + NAD ⁺	⇌ 4-methyl-2-oxopentanoate + NH ₃ + NADH + H ⁺	Leucine dehydrogenase

Table 1- 4: Serine and threonine pathways. (Schink and Stams 2013)

Reactions		Enzymes
Reactants	Products	
L-serine	\rightleftharpoons pyruvate + NH₃	Serine dehydratase
L-serine	\rightleftharpoons 2-aminoprop-2-enoate + H ₂ O	
2-aminoprop-2-enoate	\rightleftharpoons 2-iminopropanoate	
2-iminopropanoate + H ₂ O	\rightleftharpoons pyruvate + NH₃	
L-threonine	\rightleftharpoons 2-oxobutanoate + NH₃	Threonine dehydratase
L-threonine	2-aminobut-2-enoate + H ₂ O	
2-Aminobut-2-enoate	2-iminobutanoate	
2-Iminobutanoate + H ₂ O	2-oxobutanoate + NH₃	

The case of syntrophy with H₂-scavenging microorganisms, involves coupled deamination of amino acids; one amino acid is oxidized (dehydrogenated) and the other is reduced (hydrogenated). This type of reaction is called Stickland Reaction (Nisman 1954).



H⁺

Table 1- 5: Glycine pathway. (Nisman 1954)

Reactions			Enzymes
Reactants		Products	
Glycine + phosphate +	\rightleftharpoons	Acetyl-phosphate + NH ₃ + oxidized	Glycine reductase
reduced thioredoxin +		thioredoxin + H ₂ O	
H ⁺			
Acetyl-phosphate +	\rightleftharpoons	acetate + ATP	Acetate kinase
ADP			

1.4.5 Pyruvate to VFA

Pyruvate produced from the pathways explained above is converted to volatile fatty acids, carbon dioxide and hydrogen. The reaction involved in these pathways are shown in Table 1- 6.

Table 1- 6: Reactions involved in the conversion of pyruvate to VFA. (Berg et al. 2002)(Angenent et al. 2004)

(table continues at the next page)

Reactants	Reactions		Enzymes
		Products	
Pyruvate + NADH	\rightleftharpoons	lactate + NAD ⁺	Lactate dehydrogenase
Pyruvate + CoA + oxidized Fd	\rightleftharpoons	acetyl-CoA + reduced Fd + CO ₂ + H ⁺	Pyruvate:ferredoxin oxidoreductase
CoA + acetyl phosphate	\rightleftharpoons	acetyl-CoA + phosphate	Phosphotransacetylase
ATP + acetate	\rightleftharpoons	ADP + acetyl phosphate	Acetate kinase
Acetaldehyde + NADH + H ⁺	\rightleftharpoons	ethanol + NAD ⁺	NAD ⁺ -dependent ethanol dehydrogenase
Acetaldehyde + CoA + NAD ⁺	\rightleftharpoons	acetyl-CoA + NADH + H ⁺	Acetaldehyde dehydrogenase
2-acetyl-CoA	\rightleftharpoons	CoA + acetoacetyl-CoA	Acetyl-CoA acetyltransferase
3-Acetoacetyl-CoA + NADPH + H ⁺	\rightleftharpoons	3-hydroxybutanoyl-CoA + NADP ⁺	3-Hydroxybutyryl-CoA dehydrogenase
3-Hydroxybutanoyl-CoA	\rightleftharpoons	crotonoyl-CoA + H ₂ O	Crotonase 3-OH-butyryl-CoA dehydratase
2NADH+ oxidized Fd + crotonyl-CoA	\rightleftharpoons	2 NAD+ reduced Fd + butyryl-CoA	butyryl CoA dehydrogenase/electron-transfer flavoprotein complex

Butanoyl-CoA + 2 NAD ⁺ + 2 reduced Fd	\rightleftharpoons Crotonoyl-CoA + 2 NADH + 2 oxidized Fd	Butyryl-CoA dehydrogenase
Butanoyl-CoA + phosphate	\rightleftharpoons CoA + butanoyl phosphate	Phosphotransbutyrylase
Butanoyl phosphate + ADP	\rightleftharpoons butanoate + ATP	Butyrate kinase
Pyruvate + CoA	\rightleftharpoons acetyl-CoA + formate	Pyruvate formate lyase
ATP + pyruvate + HCO ₃ ⁻	\rightleftharpoons ADP + phosphate + oxaloacetate	Pyruvate carboxylase
Malate + NAD ⁺	\rightleftharpoons oxaloacetate + NADH + H ⁺	Malate dehydrogenase
Malate	\rightleftharpoons fumarate + H ₂ O	Fumarate hydratase
Fumarate + NADH	\rightleftharpoons succinate + NAD ⁺	Fumarate reductase
GTP + succinate + CoA	\rightleftharpoons GDP + phosphate + succinyl-CoA	Succinyl-CoA synthetase
Succinyl-CoA	\rightleftharpoons (R)-methylmalonyl-CoA	Methylmalonyl-CoA mutase
(R)-methylmalonyl-CoA	\rightleftharpoons (S)-methylmalonyl-CoA	Methylmalonyl-CoA epimerase
(S)-methylmalonyl-CoA	\rightleftharpoons propanoyl-CoA + CO ₂	Methylmalonyl-CoA dextraxylase
Acetate + propanoyl-CoA	\rightleftharpoons acetyl-CoA + propanoate	Priopionate-CoA transferase

1.5 Factors Affecting Acidogenic Fermentation

1.5.1 Hydrolysis Rate

Hydrolysis is the rate limiting step of AD (Schink and Stams 2013), cell wall and extracellular polymeric substances impose physical and chemical barriers to the hydrolysis of intracellular organic matter. Improving the hydrolysis stage could increase the VFA production. Pretreatment of the substrate could enhance solubilization and increase the sCOD produced during hydrolysis (Fdez.-Güelfo et al. 2011), and further improve the downstream process of conversion of the sCOD to VFA. There are several methods of pretreatment such as chemical and physical (Table 1- 7). Chemical methods use acid or alkaline conditions, ozone or hydrogen peroxide, and physical methods include thermal, microwave or ultrasound for the pretreatment of the substrate.

Acid and alkaline conditions increase the solubilization of the extracellular polymeric substances with ionization that causes strong repulsion between them resulting in the breakdown of the bonds (Devlin et al. 2011). That happens with ionization of the charged groups. However, when such extreme values of pH are applied, there is a higher risk for corrosion of the equipment, which increases the operational cost. Ozone, as an oxidizing agent, is able to decompose into hydroxyl radicals that can react with the substrate, leading to solubilization, but ozone production is also costly. As an alternative, hydrogen peroxide could be used, however it is less reactive, hence less efficient than ozone (Eskicioglu et al. 2008).

Microwave method uses heat that is generated from the rotation of the dipolar molecules such as water in the electromagnetic field along with the alignment of the polarized side chains

of macromolecules such as lipids and proteins with the poles of the electromagnetic field in order to break the hydrogen bonds and destabilize the molecules structure (Ahn et al., 2009).

Ultrasound method creates cavitation bubbles that when they burst, they generate mechanical shear forces that break down the macromolecules of the substrate. During that process, the temperature and pressure increase which leads to the formation of reactive hydroxyl radicals and thermal decomposition of the substrate. (Tiehm et al. 2001)

Table 1- 7: Pretreatment methods for hydrolysis enhancement.

(table continues at the next two pages)

Pretreatment method	Pretreatment conditions	Waste type	Effect of pretreatment	References
Acid	HCl, pH 3, 4°C, 24 h	Food waste	sCOD increased by 28%	(Elbeshbishy et al. 2011a)
Acid	HCl, pH 1, 24 h	Waste Activated Sludge (WAS)	sCOD increased by 4 times	(Devlin et al. 2011)
Alkaline	Ca(OH) ₂ , dosage 2.3 g/L, 6 h	OFMSW	sCOD increased by 1.4 times	(González-Sánchez and Revah 2007)
Alkaline	NaOH, pH 12.5, 30 min	WAS	sCOD increased by 66 times	(J. Kim et al. 2003)
Ozone	Ozone dosage	OFMSW	sCOD increased by	(Cesaro and

	0.16 g/g-TS		56%	Belgiorno 2013)
Ozone	Ozone dosage 0.16 g/g-TS	WAS	COD solubilization of 22% ^a	(Bougrier et al. 2006)
Hydrogen peroxide	Hydrogen peroxide dosage 0.66g/g-TS	OFMSW	sCOD increased by 1.4 times	(Shahriari et al. 2012)
Hydrogen peroxide	Hydrogen peroxide dosage 1 g/g- TS	WAS	sCOD increased by 3.9 times	(Eskicioglu et al. 2008)
Microwave	Microwave(24 50 MHz), 20.6 min, 7.8°C/min, heating from 22°C to 175°C	Kitchen waste	sCOD increased by 2.5 times	(Marin et al., 2010)
Microwave	Microwave (800 W), 3.5 min, final temp. 80°C	Sewage sludge	sCOD increased by 3.1 times	(Appels et al. 2013)

Ultrasound	Frequency 20 kHz, specific energy 90,692 J/g initial	OFMSW+sew age	sCOD variation of 71.8%	(Cesaro et al. 2012)
Ultrasound	Frequency 20 kHz, specific energy 79 kJ/g-TS	Food waste	sCOD increased by 25%	(Elbeshbishy et al. 2011b)
Ultrasound	Frequency 20 kHz, specific energy input 45,000 kJ/Kg-TS	WAS+PS	sCOD/tCOD ratio increased by 5.7 times	(D.-H. Kim et al. 2010)

1.5.2 pH

The pH affects the VFA productions because it affects the microbial ecology. Acidogens do not survive in extreme acidic or alkaline condition of pH lower than 3 and higher than 11 (H. Liu et al. 2012). Based on the different types of substrates the optimal pH values for VFA production are 5 to 11. In addition, the concentration of VFA is influenced by the presence of methanogens that consume VFA for methane production. Alkaline conditions are not favorable for methanogens, thus at these conditions VFA consumption prevention is achieved.

Table 1- 8: Studies on pH conditions optimization during fermentation.

Type of waste	Optimal pH	pH studied	Reactor operating conditions	VFA production	References
Primary sludge (PS)	10	3-11	Batch reactor, 25°C, 5 d	60 mg-COD/g-VSS/d	(Wu et al. 2009)
Waste Activated Sludge (WAS)	9	4-11	Batch reactor, 35°C, 5 d	298 mg-COD/g-VSS	(P. Zhang, Chen, and Zhou 2009)
WAS	11	8-12	Batch reactor, 25°C, 4 d	1558 mg-COD/L	(Yu et al. 2013)
Kitchen waste	7	5-11	Batch reactor, 35°C, 4 d	36,000 mg/L	(B. Zhang et al. 2005)

1.5.3 Temperature

Temperature affects the production of VFA because it increases the solubilization of the substrate and also affects the microbial ecology of the system in terms of growth of microorganisms and activities of enzymes. (M. Kim et al. 2003). The temperatures that have been used are psychrophilic (4-20°C), mesophilic (20-50°C), thermophilic (50-60°C) and

extreme/hyper-thermophilic (60–80°C) (Zhuo et al. 2012). Mesophilic condition is the most efficient in terms of VFA yield and energy requirements. (J. Jiang et al. 2013). However, when we take into account the composition of VFA the optimal temperature can vary. The dominant microbial species vary depending on the temperature, hence the metabolic pathways followed and the final VFA products change accordingly. According to literature, fermentation of food waste at temperature of 35°C and 45°C resulted in acetic and propionic acids, while at 55°C temperature the prevailing acid was butyrate accounting for 81%, followed by acetic and propionic acids (J. Jiang et al. 2013). Similar results were found in the study of He et al. (2012); butyric acid prevailed at temperature 55°C compared to the other two temperatures studied (35°C and 70°C).

1.5.4 HRT and SRT

Retention time is the time that the waste and microorganisms spend in the fermenter. Hydraulic retention time (HRT) refers to the waste stream, while solids retention time (SRT) refers to the microorganisms.

Longer HRT means that the microorganisms have longer time to react with the substrate and produce VFA, given that there is available substrate. When the substrate is consumed, extending the reaction time will not result in increased VFA production. (Bengtsson et al. 2008). Increase of HRT from 4 to 8 days in acidogenic fermentation of food waste showed increase of the VFA yield (Lim et al. 2008). Another study showed that the VFA concentration increased when the HRT increased from 1 to 2 days but remained the same when the HRT increased from 3 to 4 days (Dinsdale et al. 2000).

Regarding the SRT, lower SRT can prevent the dominance of methanogens in the fermenter as acidogenic microorganisms have greater growth rate than methanogens, so methanogens require more time. (Ferrer et al., 2010) Methanogenesis typically occurs an SRT greater than 8 days.(Miron et al. 2000)

1.5.5 Organic Loading Rate

Higher loading rate can increase the VFA production as the availability of the substrate increases. Higher VFA production can lead to drop of the pH in the fermenter which could inhibit methanogenesis, resulting in further increase of the VFA. Studies on the effect of loading rate have shown three main trends:

- VFA concentration increases as OLR increases (Jian Yu, 2001),
- VFA concentration increases till it reaches a maximum and then decreases as OLR increases (Oktem et al., 2008),
- VFA concentration increases but VFA yield decreases as OLR increases. (Jiang et al, 2013)

1.5.6 Headspace Gas Pressure

Hydrogen is produced during acidogenic fermentation. As hydrogen is built up in the headspace of the reactor, the electron flow in the metabolic pathways of bacteria might change, affecting the VFA production (Valdez-Vazquez et al., 2006), hence maintaining a low level of hydrogen concentration is important for the VFA production.

1.6 Chemical Structure and Physical Properties of PHA

Polyhydroxyalkanoates (PHA) are biodegradable biopolymers. PHA polymerization is performed naturally by bacteria. Microorganisms use VFA as substrate and create granular shaped inclusions for energy storage purposes in the cells. They were first discovered by the French scientist Lemoigne in 1925. More specifically, PHA were found in *Bacillus megaterium* in the form of poly (3-hydroxybutyrate) (PHB). To date, more than 300 bacterial species have been identified as PHA producers. (Raza et al. 2018). There is a variety of PHA composed of 3-hydroxy fatty acids. The monomer is shown in Figure 1- 4. The R group can vary in length from methyl (C1) to tridecyl (C13). The *n* value is typically between 100 and 30,000. PHA synthase creates an ester bond between the carboxyl group of one monomer with the hydroxyl group of the following monomer during the polymerization reaction. The most common polymers are shown in Table 1- 9 . (S. Y. Lee 1996). When mixed substrates are used copolymers are produced; examples of copolymers are poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) or poly(3-hydroxybutyrate-co-4-hydroxybutyrate) (PHB4B) (Yan et al. 2005). Physical properties of some copolymers compared to polypropylene (PP) are shown in

Table 1- 10.

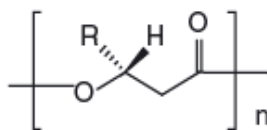


Figure 1- 4: Monomer of poly(3-hydroxyalkanoates) (Verlinden et al. 2007)

Table 1- 9: Examples of R-groups in PHA. (Verlinden et al. 2007)

R group	Name	Abbreviation
CH ₃	Poly(3-hydroxybutyrate)	PHB
CH ₂ CH ₃	Poly(3-hydroxyvalerate)	PHV
CH ₂ CH ₂ CH ₃	Poly(3-hydroxyhexanoate)	PHH _x

Table 1- 10: Properties of PHA and polypropylene (PP). (Tsuge 2002)

Parameters	PHB	PHBV	PHB4B	PHBH _x	PP
Melting temperature (°C)	177	145	150	127	176
Glass transition temperature (°C)	2	-1	-7	-1	-10
Crystallinity (%)	60	56	45	34	50-70
Tensile strength (MPa)	43	20	26	21	38
Extension to break (%)	5	50	444	400	400

PHBV contains 20% 3HV-monomer, PHB4B contains 16% 4HB-monomer, PHBHx contains 10% 3HHx-monomers

1.7 PHA Production from Pure Cultures

Different bacterial cultures are able to produce PHA. *Cupriavidus necator* is the most studied. Other strains that have been studied are: *Bacillus spp.*, *Pseudomonas spp.*, *Halomonas boliviensis*, *Alcaligenes spp.*, *Burkholderia sacchari*, *Rhodopseudomonas palustris*, *Aeromonas hydrophila*. Table 1- 11 gives an overview of the PHA producing strains.

Table 1- 11: Bacterial strains used for PHA production.

(table continues at the next two pages)

Bacterial strains	Carbon sources	PHA produced	References
<i>Aeromonas hydrophila</i>	Lauric acid, oleic acid	mcl-PHA	(S. H. Lee et al. 2000)
<i>Alcaligenes latus</i>	Malt, soy waste, milk waste, vinegar waste, sesame oil	PHB	(Wong et al. 2005)
<i>Bacillus cereus</i>	Glucose, sugarbeet molasses	PHB	(Labuzek and Radecka 2001)
<i>Bacillus spp.</i>	Nutrient broth, glucose, alkanoates, soy molasses	PHB, PHBV	(Katırcıoğlu et al. 2003)

<i>Burkholdenia sacchari sp. nov.</i>	Adonitol, arabinose, arabitol, cellobiose, fructose, fucose, lactose, maltose, melibiose, raffinose, rhamnose, sorbitol, sucrose, trehalose, xylitol	PHB, PHBV	(Bramer et al. 2001)
<i>Burkholdenia cepacian</i>	Palm olein, palm stearin, crude palm oil, palm kernel oil, oleic acid, xylose, levulinic acid, sugar beet molasses	PHB, PHBV	(Keenan et al. 2004)
<i>Caulobacter crescentus</i>	glucose	PHB	(Qi and Rehm 2001)
<i>Halomonas boliviensis</i>	Starch hydolysate, maltose, meltotetraose, maltohexaose	PHB	(Quillaguamán et al. 2005)
<i>Legionella pneumophila</i>	Nutrient broth	PHB	(James et al. 1999)
<i>Methylocystis sp.</i>	Methane	PHB	(Wendlandt et al. 2005)
<i>Microlunatus phosphovor</i>	Glucose, acetate	PHB	(Akar et al. 2006)
<i>Pseudomonas aeruginosa</i>	Glucose, oleic acid	mcl-PHA	(Hoffmann and Rehm 2004)

<i>Pseudomonas oleovorans</i>	Octanoic acid	mcl-PHA	(Durner, Witholt, and Egli 2000)
<i>Pseudomonas putida</i>	Glucose, octanoic acid, undecenoic acid	mcl-PHA	(Tobin and O'Connor 2005)
<i>Pseudomonas stutzeri</i>	Glucose, soybean oil, alcohols, alkaotes	mcl-PHA	(Xu et al. 2005)
<i>Rhizobium meliloti</i> , <i>R. viciae</i> , <i>Bradyrhizobium japonicum</i>	Glucose, sucrose, galactose, mannitol, trehalose, xylose, raffinose, maltose, dextrose, lactose, pyruvate, sugar beet molasses, whey	PHB	(Mercan and Beyatli 2005)
<i>Rhodopseudomonas palustris</i>	Acetate, malate, fumarate, succinate, propionate, malonate, gluconate, butyrate, glycerol, citrate	PHB, PHBV	(Mukhopadhyay, Patra, and Paul 2005)
<i>Spirulina platensis</i> (cyanobacterium)	Carbon dioxide	PHB	(Jau et al. 2005)
<i>Cupriavidus necator</i>	Glucose, sucrose, fructose, valerate, octanoate, lactic acid, soybean oil	PHB, copolymer	(Kichise et al. 1999) (Volova and Kalacheva 2005)

mcl-PHA: medium-chain-length polyhydroxyalkanoates, PHB: poly(3-hydroxybutyrate),

PHBV: poly(3-hydroxybutyrate-co-valerate)

1.8 PHA Production from Mixed Microbial Cultures (MMC)

In order to reduce the cost of PHA production, mixed microbial cultures have been used instead of pure strains. Mixed microbial culture systems use non-sterile conditions, reducing the energy cost, and the microorganisms' selection is achieved with enrichment techniques by applying different operational conditions to the system. The production consists of two steps (Figure 1- 5); the first step includes enrichment in microbial species that are able to produce PHA in high capacity by applying dynamic conditions. The second step includes the PHA production from the enriched culture of the previous step and sequential harvesting of cells for extractions and purification of PHA.

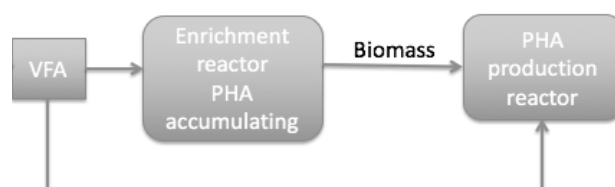


Figure 1- 5: Two-step process for PHA production.

PHA accumulation that can be achieved by mixed microbial cultures fed with fermented food waste, fermented waste activated sludge (Y. Jiang, Chen, and Zheng 2009), fermented sugar cane molasses (M. G. E. Albuquerque, Torres, and Reis 2010) and fermented paper mill effluent (Jiang Y 2012) ranges from 26% to 80% (g-PHA/g-VSS). M. Zhang et al. (2014) reported use of waste activated sludge from wastewater treatment plant and food waste as substrates for VFA production and further conversion to PHA (22-30%). Furthermore, the highest biomass PHA content has been achieved by enrichment cultures with *Plasticicumulans acidivorans* as

dominant species (77-89% per cell dry weight) by using pure VFA or fermented VFA-rich feedstocks (Johnson et al. 2009).

Current research trends on the operational parameters optimization focus mostly on solids retention time (SRT), nutrient level, and feeding pattern. Chua et al. (2003) showed that SRT of 3 days resulted in better accumulation results than SRT of 10 days. Valentino et al. (2017) reported that optimal ranges of N and P levels (N/COD in the range 2.0- 15.0 mg/g, and P/COD 0.5-3.0 mg/g) resulted in improved PHA productivity. Popular feeding patterns that have been studied are single-pulse (Beccari et al. 2009), multi-pulse (Campanari et al. 2014), and continuous (Morgan-Sagastume et al. 2015). Multi-pulse feeding is often tested because it could avoid potential substrate inhibition problems (M. G. E. Albuquerque et al. 2007)

1.9 Enrichment Methods

1.9.1 Anaerobic/Aerobic Enrichment (AN/AE)

Phosphate accumulating organisms (PAO) and glycogen accumulating organisms (GAO) have been found to accumulate PHA under anaerobic conditions, and then consume it during aerobic conditions. Hence, by applying alternating cycles of anaerobic and aerobic phases could result in enrichment of microorganisms that produce PHA in high capacity.

1.9.2 Aerobic Dynamic Feeding (ADF)

Aerobic dynamic feeding entails alternating cycles of substrate availability and substrate limitation in the form of carbon source under aerobic conditions, these cycles are called feast and famine (FF) cycles (Reis et al. 2003). Bacteria that are able to convert carbon to PHA, do so

during the feast phase, while during the famine phase they use the produced PHA as carbon and energy source allowing them to grow in contrast with the non-PHA producing microorganism that ultimately are washed out (M. G. E. Albuquerque et al. 2007). PHA production using ADF (up to 90% dry cell weight using pure cultures) has been reported to be higher compared to AN/AE (Salehizadeh and Van Loosdrecht 2004).

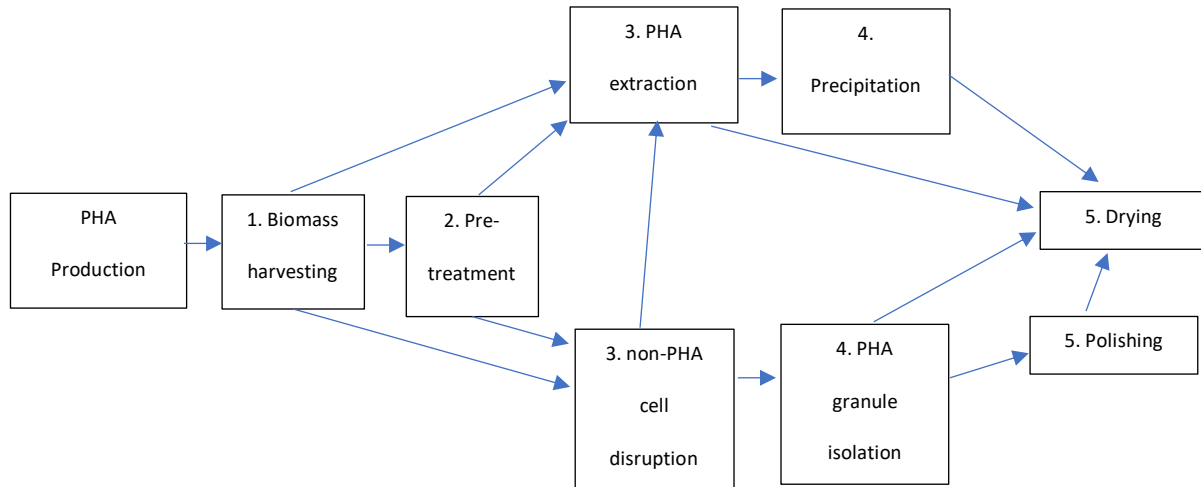
1.10 Recovery and Purification

PHA recovery and purification consists of five steps (Figure 1- 6):

1. biomass-harvesting,
2. pretreatment,
3. PHA retrieval,
4. PHA accumulation, and
5. polishing and drying.

Biomass harvesting (step 1) is achieved through filtration or centrifugation. Then, there is an optional pretreatment step (step 2) which includes lyophilization, thermal drying, mechanical, chemical, enzymatic or biological methods. These techniques are meant to facilitate the next step which is PHA retrieval from the microbial biomass (step 3). PHA retrieval includes two main methods: (a) PHA solubilization/extraction and (b) disruption of non-PHA cells. PHA extraction is achieved using non-halogen and halogen solvents, or supercritical fluids followed by a precipitation step (step 4) using methanol or ethanol or direct drying. The disruption of non-PHA cells is performed using mechanical, chemical, enzymatic, biological or osmotic methods and the recovery of PHA granules (step 4) is achieved by centrifugation, filtration or flotation. Then

PHA can be polished for removal of residues using washing methods (water, detergent, alcohols) or direct drying (step 5).



1. biomass-harvesting, 2. pretreatment, 3. PHA retrieval, 4. PHA accumulation, and 5. Polishing and drying.

Figure 1- 6: PHA recovery and purification stages. Adapted from Kourmentza et al. (2017)

1.11 PHA Applications and Commercialization

PHA have a broad range of end-use applications; such as packaging and coating (Bucci, Tavares, and Sell 2005), toners for printing, electronic products, agricultural applications (encapsulation of seeds, encapsulation of fertilizers for slow release, biodegradable plastic films for crop protection), medical applications due to its biocompatibility (sutures, repair patches, orthopedic pins, adhesion barriers, stents, nerve guides and bone marrow scaffolds).

However, worldwide PHA production accounts for only 0.5% of total plastic production. This is due to its cost, which ranges from 1.1 to 2.5 \$/lbs compared to 0.5 \$/lbs of petroleum-based plastics. (Valentino et al. 2017). The high cost of PHA production is attributed to the purity of substrates and sterilization requirements for pure cultures, and the amount of solvents required for the downstream processes.

To date, most of the research on PHA production has been performed in lab scale but also some efforts have been devoted to the implementation of that 3-step process in pilot scale. Table 1- 12 shows some pilot-scale studies. Most pilot-scale studies focus on incorporating the PHA production into the existing processes in wastewater treatment plants in order to reduce the production cost.

Table 1- 12: PHA production in pilot-scale.

(table continues at the next page)

Pilot plant	Feedstock	Origin of MMC	Enrichment strategy	Yield (g-COD/g-COD)	PHA % (%mol HB: %mol HV)	Reference
	Pre-fermented					
Nagpur (India)	milk, ice cream processing wastewater	Activated sludge		0.425	39-43	(Tamis et al. 2014)
Lucun WWTP	Hydrolyzed/aci	Activated	aerobic	0.044-0.29		(Jia et al.

(China)	dified raw excess sludge	sludge	feast/famine			2014)
		Pre-				
Eslov (Sweden)	Beet process water 38% VFA content	fermented effluent of Procordia Foods			60 (85:15)	(Anterrieu et al. 2014)
Brussels North WWTP (Belgium)	Pre-hydrolyzed and fermented WWTP sludge	Sludge from municipal wastewater	aerobic feast/famine	0.25-0.38	27-38 (66- 74:26- 34)	(Morgan- Sagastume et al. 2015b)
	Fermented				34-42	
Leeuwarden WWTP (Netherlands)	residual from greenhouse tomato production	Sludge from municipal wastewater	Anoxic feed/aerobic famine	0.30-0.39	(51- 58:42- 49)	(Bengtsson et al. 2017)
Mars company (Netherlands)	Fermented wastewater from candy bar factory	Activated sludge from WWTP	aerobic feast/famine	0.30	70-76 (84:16)	(Tamis et al. 2014)

1.12 Metabolic Pathways of PHA Production

In pathway I, carbon in the form of sugars is converted to acetyl-CoA. When entry of acetyl-CoA into the Krebs cycle is restricted during nutrient limitation, the surplus acetyl-CoA is channeled into PHA biosynthesis (Colin Ratledge, 2001) (Figure 1- 7). Two units of acetyl-CoA are condensed to acetoacetyl-CoA by 3-ketothiolase (PhaA). Then, acetoacetyl-CoA reductase (PhaB) converts the product to (R)-3-hydroxybutyryl-CoA, and finally PHA synthase (PhaC) polymerizes the (R)-isomers. Sugars such as glucose and fructose are mostly processed via pathway I, yielding PHB homopolymer (Aldor and Keasling 2003).

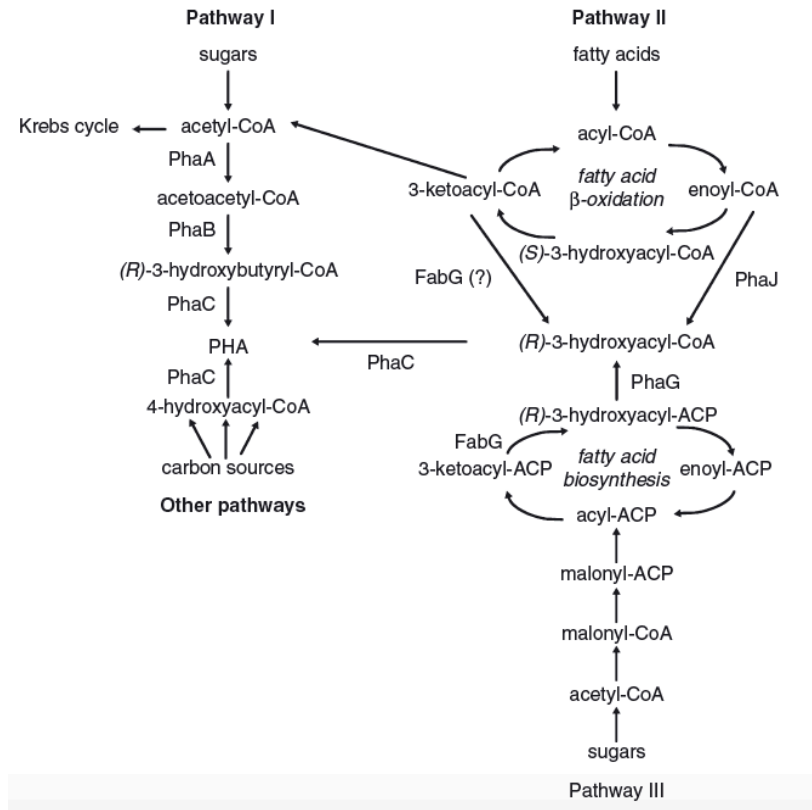
3-ketothiolase is a member of the family of β -ketoacyl-CoA thiolases and they are divided into two groups based on their substrate specificity. The first group has a broad specificity range of chain specificity; C4 to C16 and they are involved in the degradation of fatty acids, located in the cytoplasm of prokaryotes. The second group has a narrow range of chain specificity; C3 to C5 and these thiolases are involved in ketone body formation, steroid and isoprenoid biosynthesis, and PHA synthesis. The ones involved in PHA synthesis have specificity for acetoacetyl-CoA (Madison and Huisman 1999). For example, *Cupriavidus necator* contains two β -ketothiolase that are involved in PHA synthesis. The first one is a homotetramer of 44-kDa subunits and converts acetoacetyl-CoA and 3-ketopentanoyl-CoA, while the second one is a homotetramer of 46-kDa subunits, has a broader substrate specificity and cleaves acetoacetyl-CoA as well as 3-ketopentanoyl-CoA, 3-ketohexanoyl-CoA, 3-ketoheptanoyl-CoA, 3-ketooctanoyl- CoA, and 3-ketodecanoyl-CoA. (Madison and Huisman 1999) Acetoacetyl-CoA reductase is an (R)-3-hydroxyacyl-CoA dehydrogenase. In *Zoogloea ramigera*, that enzyme is a homotetramer of 25-kDa subunits and has been classified as an NADPH-dependent reductase PHA polymerases have molecular masses of about 63,000 Da,

with the exception of the polymerases in *Chromatium vinosum*, *Tulbaghia violacea*, and *Synechococcus* spp. that are composed of two subunits of 40 and 45 kDa. It was also proposed that two cysteines participate in the process; one holding the incoming monomer and the other holding the growing PHA polymer. (Madison and Huisman 1999)

In pathways II and III, intermediate products of fatty acids β -oxidation and intermediate products of fatty acids biosynthesis, respectively, are used for PHA synthesis. Several fluorescent pseudomonades; *Pseudomonas putida*, *P. oleovorans*, and *P. aeruginosa*, follow these pathways for production of copolymers from fatty acids or sugars. The product of these pathways is (R)-3-hydroxyacyl-Co-A monomers that are then polymerized into PHA by PhaC (PHA synthase). More specifically, in pathway II (R)-specific enoyl-CoA hydratase (PhaJ) provides enoyl-Coa from the fatty acid β -oxidation for conversion to (R)-3-hydroxyacyl-Co-A and in pathway III, (R)-3-hydroxyacyl-ACP-CoA transferase (PhaG) provides (R)-3-hydroxyacyl-ACP from the fatty acid biosynthesis for conversion to (R)-3-hydroxyacyl-Co-A. It should be noted that 3-ketoacyl-ACP reductase (FabG), which participates in the fatty acid biosynthesis pathway, accepting acyl-ACP, there is evidence that can also acyl-CoA as a substrate from the fatty acid β -oxidation. (Taguchi et al. 1999).

Several bacterial species can produce PHA. The most studied one is *Cupriavidus necator* (formerly known as *Ralstonia eutropha* or *Alcaligenes eutrophus*). Other strains that have been studied for PHA production include: *Bacillus* spp., *Alcaligenes* spp., *Pseudomonas* spp. (*Pseudomonas oleovorans*, *Pseudomonas putida*, *P. fluorescens*, *P. jessenii*, *Pseudomonas stutzeri*), *Aeromonas hydrophila*, *Rhodopseudomonas palustris*, *Caulobacter crescentus*, *Burkholderia sacchari*, *Burkholderia cepacia*, *Halomonas boliviensis*, *Legionella pneumophila*, *Methylocystis* sp., *Micrococcus phosphovorans*, *Rhizobium* spp. (*Rhizobium meliloti*, *R. viciae*,

Bradyrhizobium japonicum), *Spirulina platensis* (cyanobacterium), *Staphylococcus epidermidis*, *Alcaligenes latus*, and *Comamonas acidovorans*. (Tsuge 2002)



PhaA, 3-Ketothiolase; **PhaB**, NADPH-dependent acetoacetyl-CoA reductase; **PhaC**, PHA synthase; **PhaG**, 3-hydroxyacyl-ACP-CoA transferase; **PhaJ**, (R)-specific enoyl- CoA hydratase; **FabG**, 3-ketoacyl-ACP reductase.

Figure 1- 7: Metabolic pathways for PHA production. Adapted from (Tsuge, 2002)

1.13 16S rRNA Gene Sequencing for Bacterial Identification

Metagenomics is the study of all the genes present in mixed microbial communities (DNA level), and metatranscriptomics, the study of transcribed genes (RNA level), employing high-throughput DNA-sequencing. 16S-targeted metagenomics show the structure of mixed

microbial communities; what are the different microbial species present in the community. 16S rRNA gene sequences are present in all bacteria and the length of the gene is large enough for informatics purposes (Patel, 2001), thus it has been used for identification of bacterial taxonomy. Whole-genome metagenomics show the available metabolic pathways in the communities, while metatranscriptomics (mRNA-sequencing) is the study of the function and activity; what genes are expressed under specific environmental conditions.

The first step of the sequencing process includes DNA extraction from biomass samples and amplification of 16S rRNA gene targets using specific primers and PCR. Multiple samples are sequenced simultaneously by attachment of barcodes on the end of the amplified fragments for each sample. Then these fragments are ligated to adaptor sequences that adhere to semiconductor chip for use on the sequencing platform. Ion Torrent (Thermo Fisher) uses the prepared single-stranded DNA libraries and promotes the elongation of the complementary DNA strands for each fragment. When a nucleotide is incorporated into the DNA strand, a hydrogen ion is released, which will affect the pH of the solution and can be detected by the ion sensor that converts the chemical information into digital information. The Ion Personal Genome Machine™ (PGM™) sequencer introduces one nucleotide after the other onto the chip. When the nucleotide matches with the complementary base, hydrogen ion is released and voltage signal appears, while when a nucleotide does not match, there is no signal. Then, pH-based signals are converted into readable sequences of nucleotides for each fragment by signal processing. Finally, the reads are identified by comparison with reference databases for 16S rRNA sequences.

1.14 Research Objectives

Our project focused on recovery of VFA from anaerobic fermentation of waste and sequential conversion to polyhydroxyalkanoate using mixed microbial culture, with ultimate objective to maximize the VFA and PHA production.

The work is divided in three parts:

- The effect of substrate and loading rate on the production of volatile fatty acids during anaerobic fermentation of organic feedstock.
- Optimization of volatile fatty acids and polyhydroxyalanotes production from fermentate generated from primary sludge and food waste.
- Microbial ecology associated with volatile fatty acids and polyhydroxyalkanoates production through co-fermentation of waste streams.

Part I

In this part of the project, we investigated the potential of food waste for enhancing VFA production via fermentation, by examining two different substrate composition; (1) food waste and (2) primary sludge from wastewater treatment plant mixed with food waste. The purpose for that was to determine if the co-fermentation would result is better VFA production, as food waste are often being incorporated in the digestion processing of biosolids in wastewater treatment plants. Two loading rates were tested: (1) 10 kg-COD/m³/d, and (2) 25 kg-COD/m³/d in order to determine the conditions that improve the fermentation process.

The hypothesis was that VFA concentration will increase as loading rate increases; by providing more substrate to the system, the fermentation product (VFA) is expected to increase,

assuming that the productivity of the system will remain unchanged. The specific objective was to assess the effect of OLR on VFA concentration, productivity, and speciation.

Part II

In this part of the project, VFA production and further conversion to PHA was achieved by anaerobic co-fermentation of primary sludge and food waste, and sequential enrichment in PHA-producing microorganisms through aerobic dynamic feeding of feast and famine cycles. The goal was to maximize the VFA and PHA production, thus, attempts were made towards improving both processes by manipulating the fermentation operational parameters such as organic loading rate (OLR), pH, and hydraulic retention time (HRT), and by manipulating the PHA production operational parameters such as the feeding pattern, and substrate (VFA) source.

The hypotheses were that: **(1)** PHA yield will increase with: (i) multi-pulse feeding; when providing the substrate in multiple smaller doses (multi-pulse feeding) instead of one big dose (single-pulse feeding), the system could increase its productivity, if high concentrations of substrate (VFA) during single-pulse feeding cause substrate inhibition, (ii) synthetic VFA as substrate; acids are the preferred substrate for PHA production compared to other soluble compounds, thus providing pure acids as substrate should result in increased PHA concentrations. **(2)** VFA speciation can affect the PHA speciation; since PHA (products) are synthesized by VFA (substrate), different VFA species could lead to different PHA species.

The specific objectives were to: **(1)** Compare the PHA production when: (i) applying the two different feeding patterns (single-pulse vs multi-pulse), determine if the system shows substrate inhibition, and select the feeding pattern that results in better PHA production, (ii) providing two different substrate sources; synthetic VFA (pure acids) vs. fermentate effluent

VFA (mixture of VFA and other soluble compounds), and that way, evaluate the PHA-storing capacity of the system. (2) Examine the correlation of VFA species and PHA species.

Part III

In this part of the project, 16S-rRNA gene sequencing was used to identify the dominant microbial species of the cultures in the fermenter and in the PHA enrichment reactor. The objective was to investigate the connection of the VFA production and the fermentation microbial ecology with the PHA production; in terms of concentration and speciation, and PHA producers. The reasoning behind that research objective lied in the fact that in order to have an in depth understanding of the PHA production process, we need to have an understanding of the microorganisms of each step of the process, because ultimately, they are responsible for the system's efficiency. Without in depth knowledge of the microbial ecology, any system can be represented by a "black box" where we only know the reactants and products without any insight into the factors that caused the conversion of the reactants into products.

Chapter 2: The Effect of Substrate and Loading Rate on the Production of Volatile Fatty Acids during Anaerobic Fermentation of Organic Feedstock

2.1 Introduction

Resource recovery from waste is becoming more popular as it provides a great waste management solution while wastes are converted to high added value bio-products through a bio-refinery platform. (Owusu-Agyeman, et al. 2018) Anaerobic digestion is an efficient strategy for resource recovery. (Zhou et al. 2018) Volatile fatty acids (VFA) are intermediate products of anaerobic fermentation with great added value potential as they have many applications, such as biopolymers (polyhydroxyalkanoates, PHA), biofuels, bulk chemicals, and biological nutrient removal in wastewater treatment. (F. C. Silva et al. 2013). Volatile fatty acids are valued as 0.45 \$/lb of biomass, while biomethane as 0.027-0.068 \$/lb of biomass, and compost as 0.0023-0.0045 \$/lb of biomass. (Colombo et al. 2017)

Anaerobic fermentation consists of 4 stages: 1-Hydrolysis, 2-Acidogenesis, 3-Acetogenesis, and 4-Methanogenesis (Figure 2- 1). Volatile fatty acids (VFA) are produced in steps #2 and #3 through a series of biochemical reactions that are catalyzed by acidogenic and acetogenic bacteria. There are distinct co-existing metabolic pathways, but in all of them, pyruvate is the key intermediate that can be converted into different products. These metabolic pathways are the following: acetic acid/ethanol type, propionic acid type, butyric acid type, mixed-acid type and lactate type. (Zhou et al. 2018)

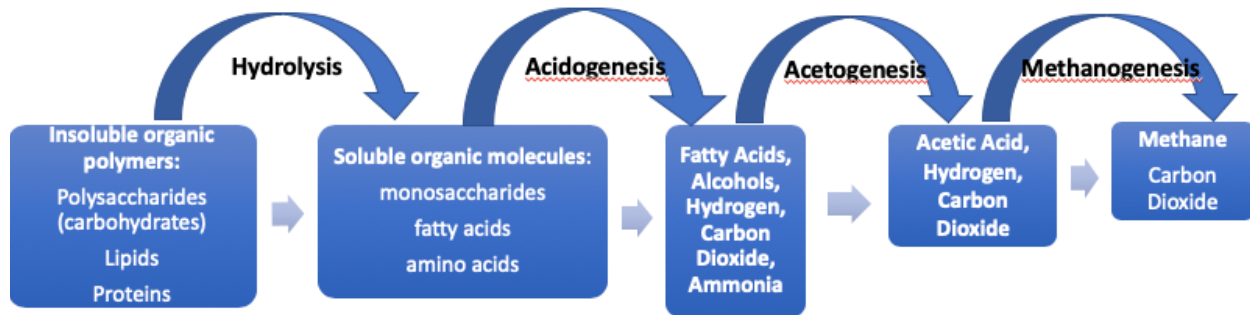


Figure 2- 1: Stages of anaerobic digestion.

Different types of solid and liquid wastes have been studied for the production of volatile fatty acids (VFA). Among them, the most investigated ones are sludge, food waste, organic fraction of municipal solid waste, wastewaters generated from agricultural, dairy, paper industries and mixtures of those. (W. S. Lee et al. 2014) According to current research it is not clear which type of waste is the most effective for volatile fatty acids (VFA) production. Even though there are a lot of studies on this area, there are not solid conclusions because of the different operating conditions and VFA production expression units used in each study (Table 2-1). For this reason, in this study we focused on two main components; primary sludge (PS) and food waste (FW). Both of these waste types are massively generated hence there is the need for efficient disposal and also are rich in organic matter which gives them a great fermentative potential for VFA production. The COD of PS ranges from 14,800 mg/L to 23,000 mg/L (Ji, et al. 2010 , Zhang et al. 2009), and the COD of food waste from 91,900 to 166,180 mg/L (Hee Jun Kim et al. 2006). The soluble COD of PS is 10 to 100 times lower than its total COD which slows down the production of VFA, making hydrolysis the rate limiting step of anaerobic fermentation (Ji, et al. 2010). By adding FW, the increased fermentable content can improve the

VFA production. In fact, wastewater treatment plants have been implementing this concept during the digestion of biosolids produced during the wastewater treatment (Nghiem et al., 2017). Thus, the study of co-fermentation of PS and FW could have a great impact on the improvement of anaerobic digestion in wastewater treatment plants.

Table 2- 1: Various wastes and condition used for volatile fatty acids (VFA) production.

(table continues at the next three pages)

Type of Waste	Organic content (mg COD/L)	Reactor type and operating conditions	VFA production performance	References
Waste Activated Sludge	5,470 ^a	Batch reactor, pH 11, 60°C, 7d, 0.02g SDBS ^b /g VSS	2561 mg TOC/L	(Mengmeng et al. 2009)
Waste Activated Sludge	18,657	Batch reactor, pH 9, 35°C, 5 d	298 mg-COD/g-VSS	(P. Zhang, Chen, and Zhou 2009)
Waste Activated Sludge	18,657	Batch reactor, pH 8, 55°C, 9 d	368 mg-COD/g-VSS	(P. Zhang, Chen, and Zhou 2009)
Waste Activated Sludge	14,878	Batch reactor, 21°C, 6 d	339 mg-COD/L	(S. Jiang et al. 2007)

Sludge				
Primary Sludge	22,838	Batch reactor, 21°C, 6 d	85 mg-COD/g-VSS	(Ji, Chen, and Chen 2010)
Primary Sludge	20,631	Batch reactor, pH 10, room temp., 5 d	60 mg-COD/g-VSS/d	(Wu et al. 2009)
Primary Sludge	343 ^c	Continuous-flow completely mixed reactor, 25°C, HRT 1.25 d, SRT 10 d	31 mg/g-VSS/d	(Maharaj and Elefsiniotis 2001)
Food Waste	Not available	Semi-continuous reactor (once-a-day feeding and drawoff), pH 6, 35°C, HRT 8 d, OLR 9 g/L/d	25,000 mg/L	(Lim et al. 2008)
Food Waste	91,900	Batch reactor, 37°C, initial pH 5.5	8950 mg-COD/L	(Elbeshbishy et al. 2011a)
Food Waste	146,100	Batch reactor, 35°C, 5 d, enzymatic pretreated food waste	5610 mg-COD/L	(Hee Jun Kim et al. 2006)
Kitchen Waste	166,180	Batch reactor, pH 7, 35°C, 4 d	36,000 mg/L	(B. Zhang et al. 2005)

Primary Sludge + Waste Activated Sludge	22,256	Batch reactor, 21°C, 6 d, mixing ratio 1:1 (on VSS basis)	118 mg-COD/g- VSS	(Ji, Chen, and Chen 2010)
Primary Sludge + Waste Activated Sludge	15,480	Semi-continuous reactor, 37°C, HRT = SRT 5 d	114 mg-COD/g- VSS	(Ucisik and Henze 2008)
Food Waste + Sludge	Not available	Semi-continuous reactor (once-a- day draw off and feeding), pH 6.99, 35 °C, HRT = SRT 8.92 d, OLR 8.31 g VSS/ L/d, 88% food waste + 12% dewatered sludge (on VSS basis)	29,100 mg- COD/L	(Hong and Haiyun 2010)
Food Waste + Sludge	22,125	Batch reactor, pH 8, 20°C, 4 d	8237 mg-COD/L	(Leiyu Feng et al. 2011)
Food Waste + Sludge	29,050	Continuous upflow reactor, pH 5.5–5.9, 18°C, HRT 1 d, 25% food waste + 75% primary sludge (on	3,610 mg/L	(Min et al. 2005)

weight basis)

^a mg TOC/L.

^b Sodium dodecylbenzene sulfonate.

^c sCOD after dilution.

^d mg COD/kg.

Apart from the type of substrate, there are other factors that influence the VFA production and those are the operation conditions of the fermentation process. More specifically, the operation conditions that affect the concentration, yield and composition of VFA are the pH, temperature, retention time (hydraulic and solid), organic loading rate (ORL) and additives. In this part of the study we examined the organic loading rate (OLR) because literature review has showed inconsistent trends. Those trends are:

(A) VFA concentration increases as OLR increases,

(B) VFA concentration increases till it reaches a maximum and then decreases as OLR increases,

(C) VFA concentration increases but VFA yield decreases as OLR increases.

As shown in Table 2- 2 the organic loading rate has inconsistent effect on VFA production. Therefore, in this study we examined the effect of organic loading rate (OLR) to provide more knowledge on this topic and also determine how our system responded to the increase of OLR under the specific pH and HRT condition applied. In addition, we investigated the effect of the mixture of food waste and primary sludge as fermentation substrate, which is a topic that has not been studied in regard to the different organic loading rates (OLR) for volatile fatty acids (VFA) production.

Table 2- 2: Effect of OLR increase on VFA production.

OLR increase & Feedstock type	VFA concentration	Reference
1 to 32 g-COD/L/d starchy wastewater	Linear increase (A)	Jian Yu (2001)
7 to 13 g-COD/L/d pharmaceutical wastewater	Linear increase till OLR 14 g-COD/L/d, 40% drop with further OLR increase (B)	Oktem et al. (2008)
3.2 to 15.1 g-COD/L/d olive oil mill solid residue	Linear increase till OLR 12.9 g-COD/L/d (B)	Rincón et al. (2008)
5 to 13 g/L/d food waste	Linear increase till OLR 13 g/L/d. Unstable reactor operation due to high viscosity with further OLR increase (B)	Lim et al. (2008)
5, 11, and 16 g TS/L/d food waste	Linear increase. VFA yield decrease (C)	Jiang et al. (2013)

(A) VFA concentration increases as OLR increases.

(B) VFA concentration increases till it reaches a maximum and then decreases as OLR increases.

(C) VFA concentration increases but VFA yield decreases as OLR increases.

The goal of this part of the project was to evaluate the potential of food waste in improving the fermentation process since ultimately we were aiming to investigate the co-fermentation of primary sludge and food waste, as it is the current trend in digestion components in wastewater treatment plants (primary sludge with addition of food waste to improve the fermentable content). In that process we investigated two loading rates (OLR10: 10 kg-COD/m³/d, and OLR25: 25 kg-COD/m³/d) and two feedstocks (FW: food waste, and FW+PS: mix of food waste and primary sludge). The hypothesis was that VFA concentration will increase as loading rate increases; by providing more substrate to the system, the fermentation product (VFA) should increase, assuming that the productivity of the system will remain unchanged. The specific objective was to assess the effect of OLR on VFA concentration, productivity, and speciation.

2.2 Material & Methods

2.2.1 Fermentation Reactor Description

Anaerobic fermentation was performed in a 6.0 L glass reactor (Bellco glass, Vineland, NJ). The hydraulic retention time (HRT) was set to 2 days to limit methanogenesis (D. Liu, 2008). The fermenters were operated in sequencing batch reactor (SBR) mode, 3L of fermentate was pumped out and 3L of fresh feed was added to the reactor once every day. The temperature in the reactor was maintained at 37°C using heating tapes (BriskHeat, Columbus, OH). The pH of the reactors was set to 6.5±0.1 through automated addition of 1:1 1M NaHCO₃ and 1M NaOH solutions. Mixing (relative centrifugal force, 8xG) was provided using magnetic stir plates.

2.2.2 Feed Source and Organic Loading Rate

Two fermenters (“PS+FW” and “FW”) were operated in parallel. The feed of “PS+FW reactor” was a 1:1 COD based mixture of primary sludge (PS) and food waste (FW) while “FW reactor” was fed with 100% FW. Primary sludge was obtained monthly from Loudoun Water Broad Run Water Reclamation Facility and stored at 4°C. The food waste was obtained from Columbia University cafeteria and was blended in a mixer and suitably diluted using de-ionized (DI) water. The organic loading rates that were tested in both fermenters were 10 kg-COD/m³/d and 25 kg-COD/m³/d. Both reactors ran for over 50 days in each condition.

2.2.3 Analytical Procedures

30 mL of fermentate sample was withdrawn two or three times a week using the syringe sampling port assembly. Fermentate was thoroughly mixed and appropriately diluted for total COD measurement. The fermentate was centrifuged (10,000 rpm, 9133 x g, 10 min, 4°C) and the supernatant was filtered through a 0.22 µm syringe filter and then appropriately diluted for soluble COD, total carboxylic acids (TCA), soluble carbohydrate (sCARB) and ammonia (NH₃-N) measurement.

The chemical parameters including the total and soluble COD (tCOD and sCOD), ammonia and TCA were determined using colorimetry (Hach kits 2125915, 2606945 and 2244700, Loveland, CO). The total suspended solid (TSS) and volatile suspended solid (VSS) content in the influent and effluent was determined gravimetrically by filtering 5mL sample through a Millipore glass fiber filter (1.6µm, 47 mm diameter, 90% porosity) (“ESS Method 340.2: Total Suspended Solids, Mass Balance Volatile Suspended Solids”, 1993). The filter was dried in a furnace (Thermoscientific, Asheville, NC) at 105°C and 550°C for TSS and VSS respectively until no further decrease in the weight was observed. The volatile fatty acids (VFA) composition in the fermentate was determined using ion chromatography (IC) (Dionex ICS-2100) using a Dionex IonPac™ column (AS11-HC-4µm, RFIC™ & HPIC™ 2 X 250 mm). Analytical method of IC was Anion method 2 to 27mM (20mA), and IC processing method was 2mM to 10mM (20mA). (Manning and Bewsher 1997)

2.3 Results & Discussion

2.3.1 Feed Characteristics

Table 2- 3 summarizes the characteristics of the feeds used in the two fermenters. The characteristics that we focused on in our study were:

- tCOD and sCOD (detailed data in Appendix Figure 2- 14,Figure 2- 15,Figure 2- 16,Figure 2- 17);
- TCA and VFA (detailed data in Figure 2- 3, Figure 2- 5, Figure 2- 7, Figure 2- 9 and Appendix Figures Figure 2- 20,Figure 2- 23,Figure 2- 26,Figure 2- 29);
- Ammonia (detailed data in Appendix Figure 2- 30,Figure 2- 31,Figure 2- 32,Figure 2- 33);
- TSS and VSS (detailed data in Appendix Figure 2- 35,Figure 2- 37,Figure 2- 39,Figure 2- 41);
- sCARB (detailed data in Appendix Figure 2- 44, Figure 2- 45).

Table 2- 3: Feed characteristics at OLR 10 and 25 kg-COD/m³/d.

(table continues at next two page)

	PS+FW / OLR 10	FW / OLR 10	PS+FW / OLR 25	FW / OLR 25
tCOD	11,000 ± 1,000	9,700 ± 1,200 (n=17)	24,700 ± 1,000 (n=34)	24,300 ± 2,200
(mg L⁻¹)	(n=17)			(n=33)
sCOD	1,900 ± 160	2,100 ± 1,400 (n=17)	5,300 ± 170	9,700 ± 900
(mg L⁻¹)	(n=17)		(n=34)	(n=31)
TCA (mg	970 ± 180 (n=5)	550 ± 110 (n=10)	1,200 ± 130 (n=20)	670 ± 120 (n=6)

COD L⁻¹				
TCA (mg L⁻¹)	900 ± 130 (n=5)	500 ± 90 (n=10)	1,100 ± 130 (n=20)	630 ± 110 (n=6)
AA (mg L⁻¹)	170 ± 130 (n=15) (18% ± 14%)	47 ± 28 (n=8) (9% ± 5.6%)	600 ± 340 (n=8) (55% ± 31%)	450 ± 300 (n=8) (71% ± 49%)
PA (mg L⁻¹)	97 ± 65 (n=15) (11% ± 7.6%)	0.0 ± 0.0 (n=8) (0.0% ± 0.0%)	310 ± 35 (n=8) (28% ± 4.4)	0.0 ± 0.0 (n=8) (0.0% ± 0.0%)
FA (mg L⁻¹)	4.1 ± 1.3 (n=15) (0.5% ± 0.2)	0.0 ± 0.0 (n=8) (0.0% ± 0.0%)	0.02 ± 0.0 (n=8) (0.002% ± 0.0002)	0.0 ± 0.0 (n=8) (0.0% ± 0.0%)
BA (mg L⁻¹)	44 ± 12 (n=15) (4.9% ± 1.6%)	0.0 ± 0.0 (n=8) (0.0% ± 0.0%)	65 ± 20 (n=8) (5.9% ± 1.9%)	0.0 ± 0.0 (n=8) (0.0% ± 0.0%)
VA (mg L⁻¹)	29 ± 13 (n=15) (3.2% ± 1.5%)	0.0 ± 0.0 (n=8) (0.0% ± 0.0%)	43 ± 12 (n=8) (3.9% ± 1.2%)	0.0 ± 0.0 (n=8) (0.0% ± 0.0%)
SA (mg L⁻¹)	0.0 ± 0.0 (n=15) (0.0% ± 0.0%)	0.0 ± 0.0 (n=8) (0.0% ± 0.0%)	21 ± 0.0 (n=8) (1.9% ± 0.2%)	9.8 ± 0.0 (n=8) (2.0% ± 0.35%)
Other LCA (mg L⁻¹)	556 ± 175 (n=15) (62% ± 22%)	453 ± 150 (n=8) (91% ± 35%)	60.98 ± 10 (n=8) (5.2% ± 1.0%)	170 ± 20 (n=8) (27% ± 5.8%)
Ammonia (mg L⁻¹)	120 ± 4.5 (n=14)	83 ± 4.0 (n=10)	138 ± 7.6 (n=19)	53 ± 15 (n=20)
TSS	6,100 ± 820	5,800 ± 1,700 (n=12)	10,800 ± 1,100 (n=13)	11,000 ± 1,400

(mg L ⁻¹)	(n=11)			(n=12)
VSS	6,000 ± 840	4,800 ± 2,100 (n=12)	10,200 ± 900	10,800 ± 1,500
(mg L ⁻¹)	(n=10)		(n=13)	(n=10)
VSS/TSS				
(%)	98 ± 1	83 ± 2	95 ± 2	98 ± 2
sCARB	650 ± 200 (n=13)	1,200 ± 80 (n=5)	1,600 ± 93 (n=16)	3,600 ± 170
(mg L ⁻¹)				(n=16)

*AA: acetic acid, PA: propionic acid, FA: formic acid, BA: butyric acid, VA: valeric acid, SA: succinic acid, LCA: long-chain acids.

2.3.2 Effluent Characteristics

Table 2- 4: Comparison of effluent of PS+FW reactor and FW reactor, OLR 10 and 25 kg-COD/m³/d. summarizes the characteristics of the effluent of the two fermenters. The characteristics that we focused on in our study were:

- tCOD and sCOD (detailed data in Appendix Figure 2- 10, Figure 2- 11, Figure 2- 12, Figure 2- 13, Figure 2- 14, Figure 2- 15, Figure 2- 16, Figure 2- 17);
- TCA and VFA (detailed data in Figure 2- 2, Figure 2- 3, Figure 2- 4, Figure 2- 5, Figure 2- 6, Figure 2- 7, Figure 2- 8, Figure 2- 9, and Appendix Figure 2- 18, Figure 2- 19, Figure 2- 21, Figure 2- 22, Figure 2- 24, Figure 2- 25, Figure 2- 27, Figure 2- 28);
- Ammonia (detailed data in Appendix Figure 2- 30, Figure 2- 31, Figure 2- 32, Figure 2- 33);
- TSS and VSS (detailed data in Appendix Figure 2- 34, Figure 2- 36, Figure 2- 38, Figure 2- 40);

- sCARB (detailed data in Appendix Figure 2- 42, Figure 2- 43, Figure 2- 44, Figure 2- 45).

Table 2- 4: Comparison of effluent of PS+FW reactor and FW reactor, OLR 10 and 25 kg-COD/m³/d. (table continues at next two pages)

	PS+FW / OLR 10	FW / OLR 10	PS+FW / OLR 25	FW / OLR 25
tCOD (mg L⁻¹)	12,000 ± 300 (n= 17)	11,000 ± 490 (n=17)	25,100 ± 1000 (n= 28)	25,400 ± 800 (n=24)
sCOD (mg L⁻¹)	4,500 ± 150 (n=17)	5,400 ± 73 (n=17)	9,400 ± 120 (n=31)	12,000 ± 200 (n=32)
TCA (mg-COD L⁻¹)	3,300 ± 240 (n=17)	3,600 ± 280 (n=17)	4,700 ± 230 (n=27)	5,800 ± 300 (n=29)
TCA (mg L⁻¹)	3,200 ± 200 (n=17)	3,400 ± 260 (n=17)	4,400 ± 230 (n=27)	5,500 ± 300 (n=29)
sCOD_{Produced}/pC OD_{in}	32 ± 9.4% (n=15)	48 ± 9.5% (n=16)	30 ± 7.6% (n=29)	41 ± 11% (n=9)
TCA- COD_{Produced}/sC OD_{eff}	52 ± 5.3% (n=13)	57 ± 7.0% (n=13)	39 ± 2.5% (n=15)	44 ± 4.0% (n=28)
AA (mg L⁻¹)	910 ± 200 (n=17) (29% ± 6.7%)	1,100 ± 210 (n=15) (32% ± 6.6%)	2,600 ± 900 (n=8) (59% ± 20%)	2,900 ± 900 (n=8) (53% ± 17%)
PA (mg L⁻¹)	990 ± 280 (n=17) (32% ± 9.3%)	750 ± 220 (n=15) (22% ± 6.7%)	820 ± 200 (n=8) (19% ± 4.7%)	920 ± 120 (n=8) (17% ± 2.4%)
FA (mg L⁻¹)	260 ± 180 (n=17)	140 ± 100	36 ± 14 (n=8)	170 ± 100 (n=8)

		(8.2 % ± 5.7%)	(n=15)	(0.8% ± 0.3%)	(3.0% ± 1.8%)
			(4.2% ± 3.0%)		
BA (mg L⁻¹)		190 ± 10 (n=17)	370 ± 140	290 ± 190 (n=8)	590 ± 65 (n=8)
		(6.0% ± 0.5%)	(n=15)	(6.7% ± 4.4%)	(11% ± 1.3%)
			(11% ± 4.2%)		
VA (mg L⁻¹)		150 ± 90 (n=17)	260 ± 130	250 ± 12 (n=8)	400 ± 120 (n=8)
		(4.9% ± 3.0%)	(n=15)	(5.7% ± 0.4%)	(7% ± 2.1%)
			(7.8 % ± 3.9%)		
SA (mg L⁻¹)		0.0±0.0 (n=17)	0.0±0.0 (n=15)	250 ± 0.0 (n=8)	280 ± 130 (n=8)
		(0.0% ± 0.0%)	(0.0% ± 0.0%)	(5.7%±0.3%)	(5% ± 2.3%)
Other (mg L⁻¹)	LCA	700± 165 (n=17)	780± 170	154 ± 25 (n=8)	240 ± 70 (n=8)
		(19.9% ± 4.9%)	(n=15)	(3.1% ± 0.5%)	(4% ± 1.2%)
			(23% ± 5.3%)		
Ammonia (mg L⁻¹)		340 ± 9.3 (n=10)	190 ± 25(n=10)	540 ± 16 (n=18)	160 ± 14 (n=18)
TSS (mg L⁻¹)		4,300 ± 490 (n=11)	3,900 ± 430 (n=12)	8,700 ± 850 (n=13)	7,400 ± 1,500 (n=15)
VSS (mg L⁻¹)		3,800 ± 450 (n=11)	3,800 ± 330 (n=10)	7,700 ± 840 (n=13)	6,900 ± 1,400 (n=15)
VSS/TSS (%)		88 ± 5	97 ± 2	88 ± 2	93 ± 2
sCARB (mg L⁻¹)		140 ± 9 (n=9)	150 ± 7 (n=8)	120 ± 18 (n=16)	94 ± 11 (n=16)

2.3.3 Hydrolysis Efficiency and COD

As shown in Table 2- 4, for the PS+FW reactor, after increasing the OLR from 10 to 25 kg-COD/m³/d the hydrolysis efficiency (pCOD conversion to sCOD) did not change significantly ($p=0.47$, $\alpha=0.05$), same for the FW reactor, after increasing the OLR from 10 to 25 kg-COD/m³/d the hydrolysis efficiency ($p=0.34$, $\alpha=0.05$). Nonetheless, the effluent concentrations of soluble COD (sCOD) were higher at the higher OLR for each reactor; $9,400 \pm 120$ and $12,000 \pm 200$ mg-COD/L for PS+FW reactor and FW reactor respectively. Hence, it can be concluded that even though the hydrolysis efficiencies of PS+FW reactor and FW reactor did not improve with increasing the OLR, the amount of available COD for further conversion to acids in the reactor was higher. Thus, the 25 kg-COD/m³/d OLR condition was preferred. It should be noted that the discrepancy of the values of tCOD between the feed and the effluent (Table 2- 3 and Table 2- 4) is attributed to the fact that the feed contains bigger particles and analysis errors are much more common in that case. While in the effluent samples, the heavier particles have been broken down to smaller ones through hydrolysis, resulting in more homogenized samples.

Rybicki (2014) reported 20% hydrolysis of PS at 15°C and SRT=2.5days. Our findings for PS+FW reactor (ranging from $30\% \pm 7.6\%$ to $32\% \pm 9.4\%$ at 37°C) showed higher hydrolysis, which may be a result of higher temperature or/and it might be an indication of a positive synergy of food waste and primary sludge regarding hydrolysis. Tembhurkar, A. R., and V. A. Mhaisalkar (2007) reported that the hydrolysis efficiency of food waste depends on the temperature and the biodegradability of the substrate and for 40°C it was 24 – 47%. Vajpeyi and Chandran (2015) on their study on food waste fermentation, at 37°C, pH 6, and HRT 2 days reported $53 \pm 11\%$ conversion to sCOD. Our findings regarding food waste ranged $41\% \pm 11\%$ to

48% \pm 9.5%. The differences in efficiency can be explained by differences in biodegradability of substrate as food waste composition (carbohydrates, proteins, lipids content) can vary. In fact, proteins are less biodegradable than carbohydrates and lipids because of their tertiary (three-dimensional folding patterns) and quaternary structure (if they are composed of two or more polypeptide chains), making them less susceptible to protease action. (Strazzera et al., 2018)

2.3.4 Fermentation Efficiency and TCA, VFA

In PS+FW reactor, the fermentation efficiency (sCOD conversion to TCA), for OLR 10 kg-COD/m³/d was 52 \pm 5.3% (n=13). While for OLR 25 kg-COD/m³/d was 39 \pm 2.5% (n=15) (p=0.023*10⁻², α =0.05). Also, the carboxylic acid yield (mg-TCA/mg-pCOD hydrolyzed) dropped from 0.15 to 0.11. Thus, we can conclude that as the OLR increased, the ability of the microorganisms to convert sCOD into VFA dropped, which can be attributed to the maximum capacity of microorganisms to assimilate substrate and the kinetics of their metabolic pathways. That restrained us from increasing the OLR any further. However, given that at high OLR there is more COD available by definition, the VFA concentration was higher at the higher OLR. In FW reactor, the fermentation efficiency also dropped between the two OLR (from 57 \pm 7.0% to 44 \pm 4.0%, p=0.017*10⁻², α =0.05) and the carboxylic acid yield (mg-TCA/mg-pCOD hydrolyzed) dropped from 0.34 to 0.10.

Han and Shin (2004) in their study on biohydrogen production by fermentation of food waste at 35°C and uncontrolled pH starting from 6.5, showed that the fermentation efficiency in terms of VFA production ranged from 30.9% to 36.5%. Also, Vajpeyi and Chandran (2015) on their study on food waste fermentation, at 37°C, pH 6, and HRT 2 days, at OLR 27 kg-COD/m³/d, reported 38.7 \pm 12.9% conversion of sCOD to VFA. Our results were higher (FW

reactor: $44 \pm 4.0\%$ at OLR25 to $57 \pm 7.0\%$ at OLR10, 37°C , pH 6.5, and HRT 2 days). Difference in sCOD conversion can be possibly attributed to different composition of the feedstock. For example, lipids are less suitable than carbohydrates and proteins, because of their slower biodegradation kinetics. Furthermore, the long-chain fatty acids produced from the hydrolysis of lipids can adhere to the cell wall inhibiting the nutrients transportation and ultimately affecting the bacterial metabolism. (Strazzera et al., 2018).

In PS+FW reactor, after increasing the OLR from 10 to 25 kg-COD/m³/d, the TCA concentration increased from $3,300 \pm 240$ to $4,700 \pm 230$ mg-COD/L ($p=0.009*10^{-3}$, $\alpha=0.05$) For both OLR the most abundant VFA were acetic and propionic acid ($29\% \pm 6.7\%$ and $32\% \pm 9.3\%$ respectively at OLR 10 kg-COD/m³/d and $59\% \pm 20\%$ and $19\% \pm 4.7\%$ respectively at OLR 25 kg-COD/m³/d, more detailed data in Figure 2- 2 and Figure 2- 4. VFA concentrations are shown in Figure 2- 3, Figure 2- 5. In FW reactor, after increasing the OLR from 10 to 25 kg-COD/m³/d, the TCA concentration increased from $3,600 \pm 280$ mg-COD/L to $5,800 \pm 300$ mg-COD/L ($p=0.007*10^{-7}$, $\alpha=0.05$) The most abundant VFA in FW reactor for both OLR were again acetic and propionic acid ($32\% \pm 6.6\%$ and $22\% \pm 6.7\%$ at OLR 10 kg-COD/m³/d and $53\% \pm 17\%$ and $17\% \pm 2.4\%$ at OLR 25 kg-COD/m³/d, more detailed data in Figure 2- 6, Figure 2- 8). VFA concentrations are shown in Figure 2- 7, Figure 2- 9. It should be noted that the TCA concentration at OLR 25 kg-COD/m³/d in FW reactor was higher than the TCA concentration in PS+FW reactor (for the same OLR value). So, we can conclude that pure food waste lead to better TCA yield than the combination of food waste and primary sludge, which indicates that food waste have greater potential for VFA production.

A study by Wijekoon et al.(2011) on VFA profile variation with ranging OLR (5 to 12 kg-COD/m³/d, molasse wastewater) in thermophilic conditions showed that acetate and n-

butyrate were identified as the most abundant components and that increasing the OLR shifts the predominant VFA type from acetic acid to n-butyric acid and the total VFA concentration increased. In our study, the predominant VFA types are acetate and propionate and the increase in OLR resulted in increase of the abundance of acetate over propionate and the rest of VFA. The difference could be explained based on the different temperature conditions applied between this and our study, as VFA speciation depends on the conditions where the microorganisms grow (Owusu-Agyeman, et al., 2018). Another factor affecting the VFA composition is the feedstock; molasses is comprised by carbohydrates, while food waste and primary sludge are comprised by carbohydrates, proteins and fats. Carbohydrates are converted to butyric, propionic and acetic acids in that order of abundance, while proteins are converted to mainly acetic acid. (Strazzera et al., 2018) Also, differences have been noticed between the source of proteins (vegetable or animal), as vegetable proteins are converted to acetic, valeric, propionic and valeric acids, in that order of abundance, while animal proteins lead to equal abundance of these VFA species. (Strazzera et al., 2018) Rybicki (2014) in their study regarding fermentation of PS at 15°C with 2.5 days SRT reported that the composition of short-chain fatty acids was 30% acetate and 29% propionate. These results are within the ranges of abundance of acetic and propionic acids in PS+FW reactor presented in our study (acetate ranged from $29 \pm 6.7\%$ to $59 \pm 20\%$ and propionate from $19 \pm 4.7\%$ to $32 \pm 9.3\%$), however the temperature and the feedstock differ. Another study on waste activated sludge (WAS) and food waste co-fermentation by Leiyu Feng et al. (2011) showed that at ambient temperature, HRT 4 days and varying pH values, the short-chain fatty acids production was maximum at pH 8.0 (8,200 mg-COD/L) followed by pH 7.0 (6,500 mg-COD/L), while when there was no food waste addition, at pH 8.0 the concentration of acids was 970 mg-COD/L. This clearly shows and supports our findings that the combination of

FW and sludge improves the acid production. The study by Vajpeyi and Chandran (2015) using food waste as substrate at OLR 27 kg-COD/m³/d, showed that the most common VFA in the effluent were acetate (52±0.6%), butyrate (35±0.1%) and propionate (14±1.4%). These results validate the results of our study (59% ± 20% acetate, and 19% ± 4.7% propionate, for OLR 25 kg-COD/m³/d).

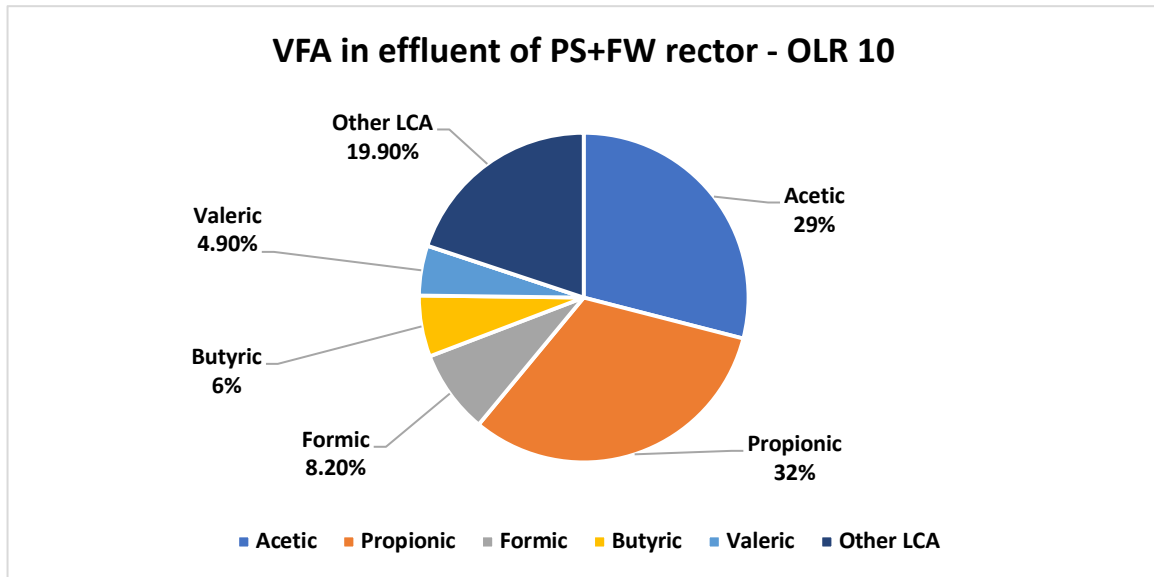


Figure 2- 2: Distribution of VFA in effluent of PS+FW reactor, OLR 10 kg-COD/m³/d.

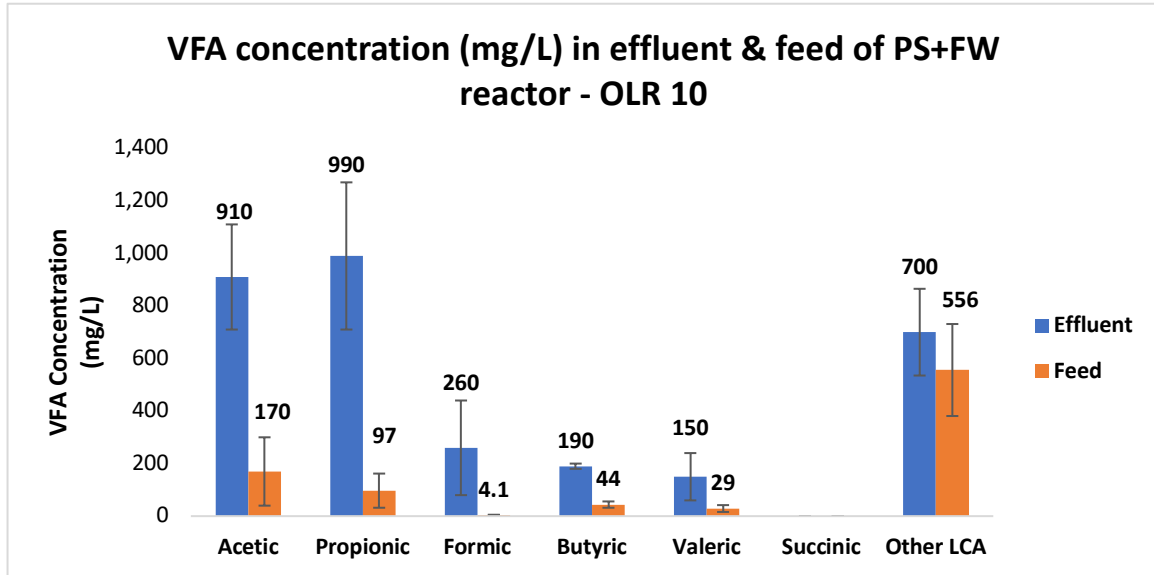


Figure 2- 3: Concentrations of VFA in effluent and feed of PS+FW reactor, OLR 10 kg-COD/m³/d.

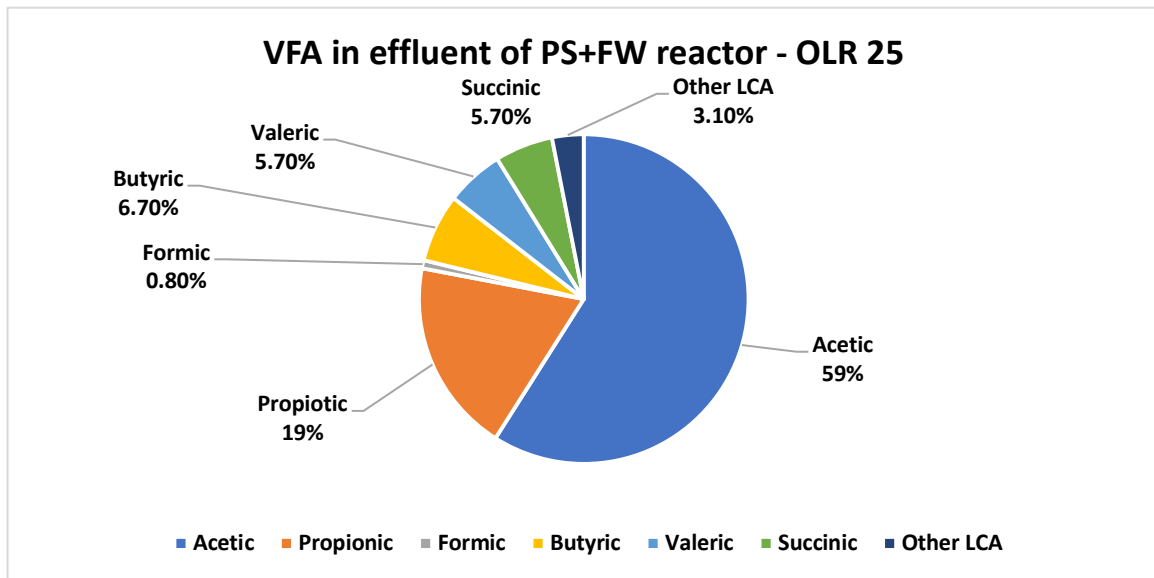


Figure 2- 4: Distribution of VFA in the effluent of PS+FW reactor, OLR 25 kg-COD/m³/d.

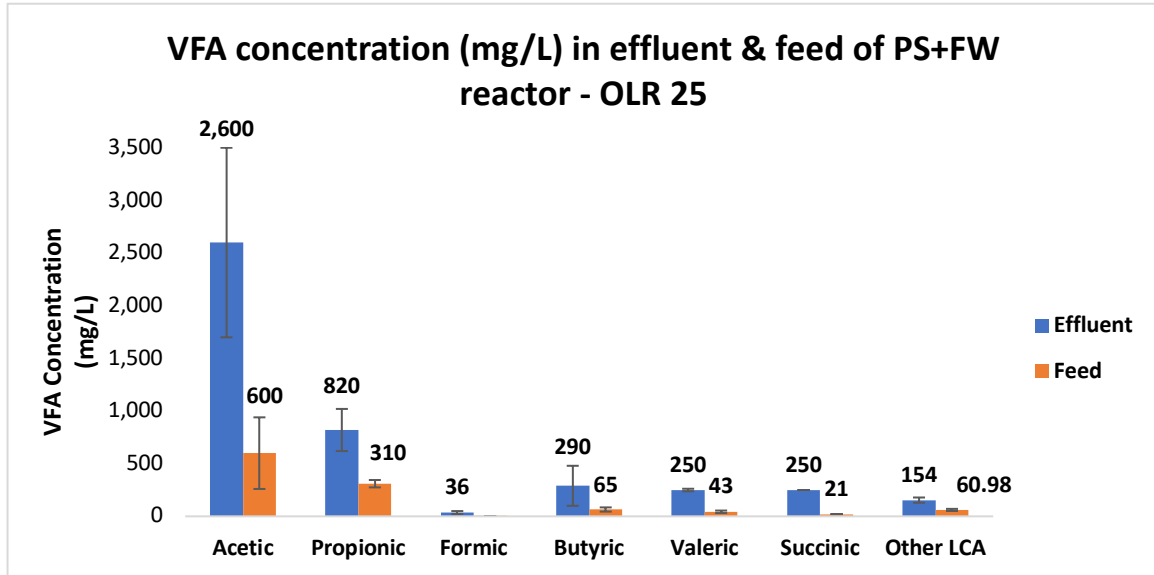


Figure 2- 5: Concentrations of VFA in effluent and feed of PS+FW reactor, OLR 25 kg-COD/m³/d.

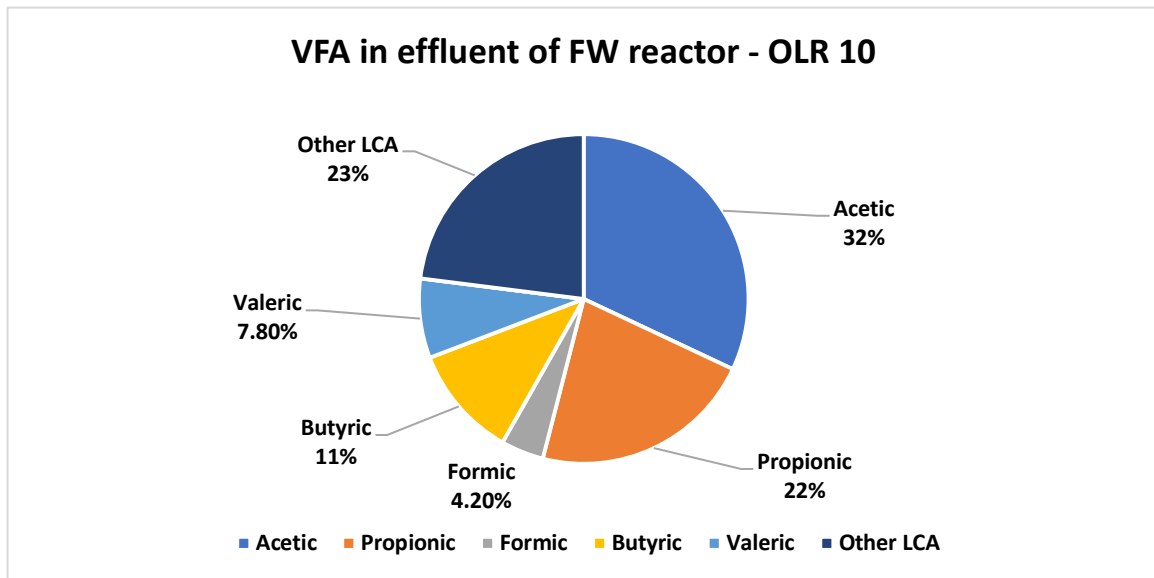


Figure 2- 6: Distribution of total VFA in effluent of FW reactor, OLR 10 kg-COD/m³/d.

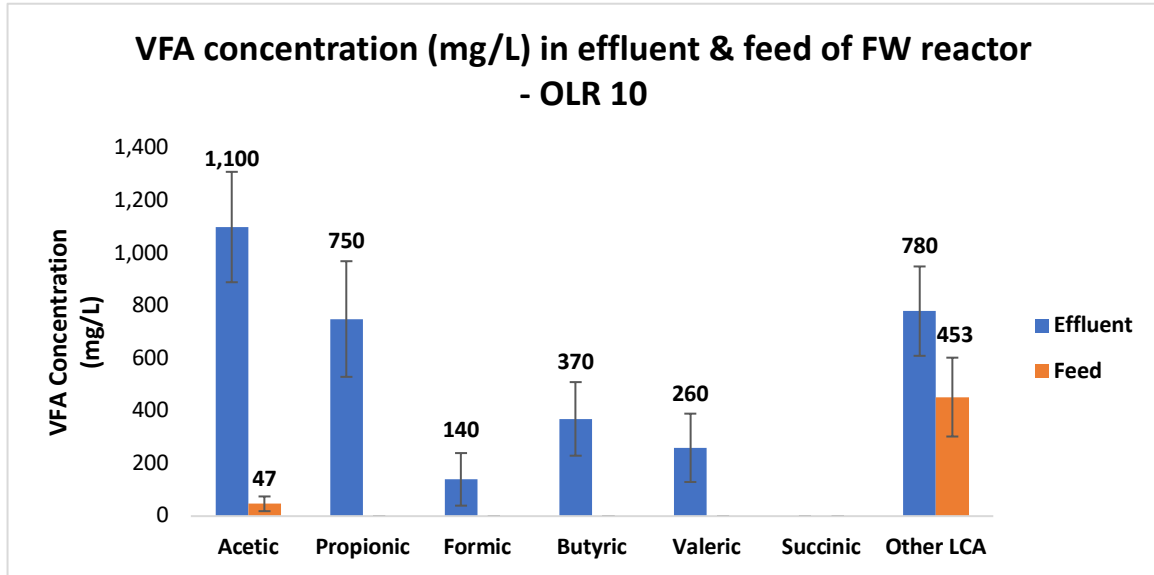


Figure 2- 7: Concentrations of VFA in effluent and feed of FW reactor, OLR 10 kg-COD/m³/d.

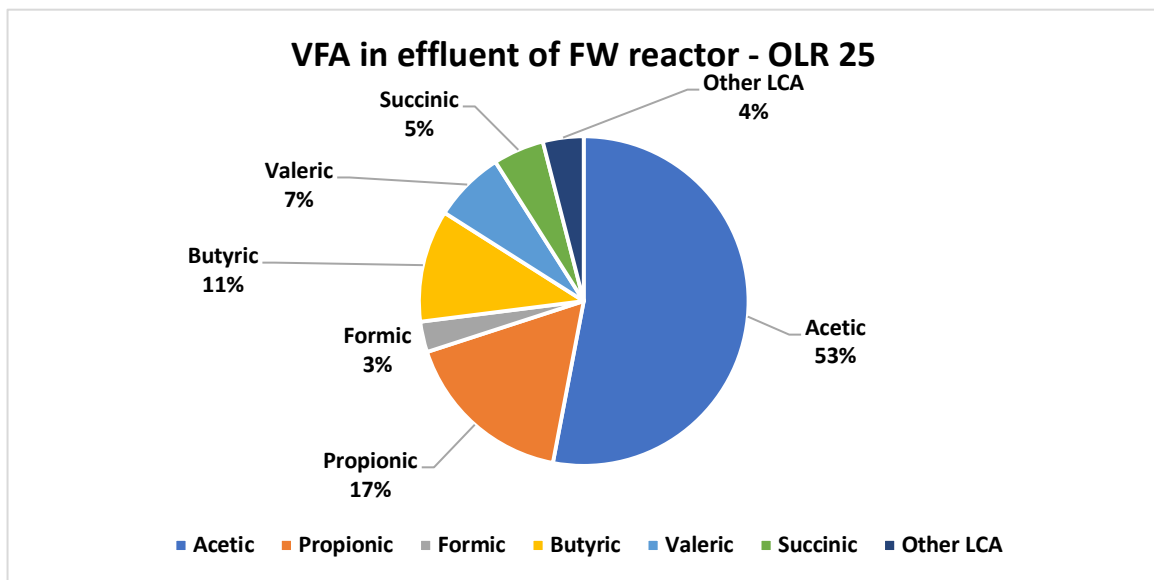


Figure 2- 8: Distribution of total VFA in effluent of FW reactor, OLR 25 kg-COD/m³/d.

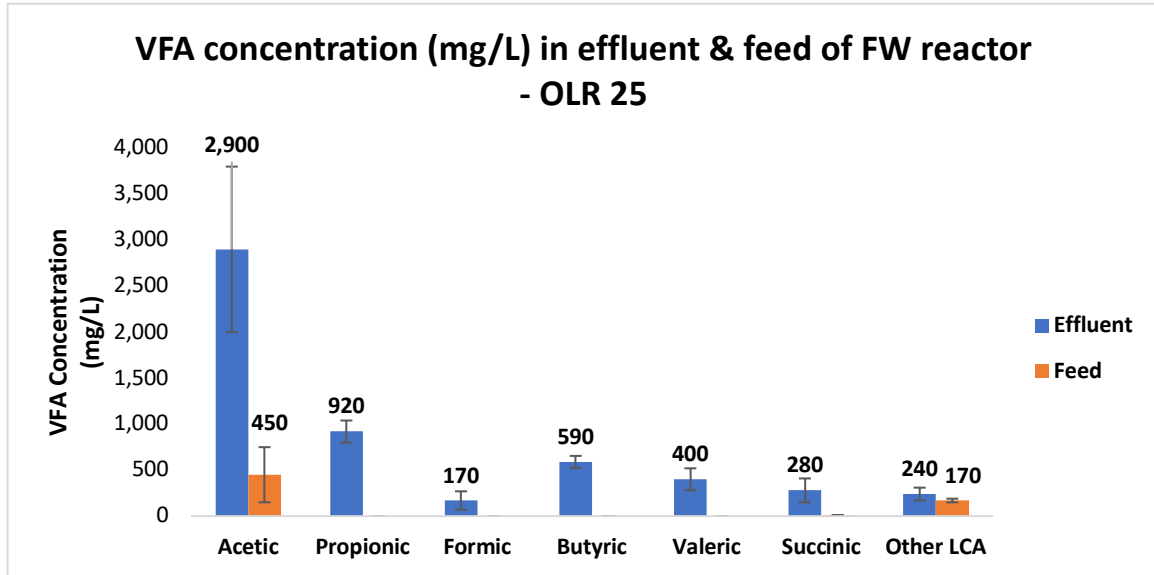


Figure 2- 9: Concentrations of VFA in effluent and feed of FW reactor, OLR 25 kg-COD/m³/d.

2.3.5 Ammonia, Solids, and Soluble Carbohydrates

As shown in Table 2- 4, in the effluent the concentration of ammonia was higher than in the feed. At OLR 10 kg-COD/m³/d the effluent concentration was 340 ± 9.3 mg/L ($p=0.0025$, $\alpha=0.05$) in PS+FW reactor, and 190 ± 25 mg/L ($p=0.0124$, $\alpha=0.05$) in FW reactor. While at OLR 25 kg-COD/m³/d the concentration in PS+FW reactor was 540 ± 16 mg/L ($p= 0.015 \cdot 10^{-6}$, $\alpha=0.05$), and in FW reactor 160 ± 14 mg/L ($p=0.00067$, $\alpha=0.05$). More detailed data can be found in Appendix Figure 2- 30, Figure 2- 31, Figure 2- 32, Figure 2- 33. The increase in the concentration of ammonia is caused by the degradation of nitrogen-containing matter in the feedstock. (Babson et al., 2013) High concentrations of free ammonia (NH₃); over 2g/L can inhibit hydrolysis, acidogenesis and methanogenesis. (Hong Chen et al., 2016) Inhibition by ammonia has been explained by several mechanisms; increase of intracellular pH or inhibition of enzyme reactions. Therefore, it was important to monitor the ammonia concentration to avoid

inhibition of the fermentation process. In fact, the maximum ammonia concentration in our study was 540 ± 16 mg/L which is lower than the lower limit of 2g/L for inhibition (Hong Chen et al., 2016).

The solids in the feed were higher than the solids in the effluent (Table 2- 3 and Table 2- 4). More specifically, at OLR 10 kg-COD/m³/d, the solids in PS+FW reactor in the effluent were $4,300 \pm 490$ mg/L ($p=0.03 \cdot 10^{-4}$, $\alpha=0.05$) and in FW reactor $3,900 \pm 430$ mg/L ($p=0.038$, $\alpha=0.05$). Also, at OLR 25 kg-COD/m³/d, solids in PS+FW reactor were $8,700 \pm 850$ mg/L ($p=0.01 \cdot 10^{-3}$, $\alpha=0.05$) and in FW reactor $7,400 \pm 1,500$ mg/L ($p=0.009 \cdot 10^{-4}$, $\alpha=0.05$). The effluent contained fewer solids than the feed due to the breaking down of substrate during hydrolysis. Regarding the ratios of volatile solids over total solids, U.S. EPA report by Donald M.D. Gray, et al., (2008) showed that VS/TS of food waste in the feed was 86%-90%, while VS/TS of municipal wastewater solids in the feed was 77%. In our study, food waste in the feed ranged from $83 \pm 2\%$ to $98 \pm 2\%$ and the mixture of primary sludge and food waste feed was $95 \pm 2\%$ to $98 \pm 1\%$. That shows that the combination of food waste and primary sludge increases the organic/biodegradable fraction of the feed. More detailed data can be found in Appendix Figure 2- 35, Figure 2- 37, Figure 2- 39, Figure 2- 41.

For PS+FW reactor and FW reactor, after increasing the OLR from 10 to 25 kg-COD/m³/d the sCARB did not change significantly ($p=0.64$ and $p=0.41$, $\alpha=0.05$, for PS+FW reactor and FW reactor respectively). The sCARB in the feed was higher than in the effluent (Table 2- 3 and Table 2- 4). More specifically, at OLR 10 kg-COD/m³/d, the effluent of PS+FW reactor contained 140 ± 9 mg/L ($p=0.004$, $\alpha=0.05$) (Figure 2- 42) and the effluent of FW reactor contained 150 ± 7 mg/L ($p=0.003$, $\alpha=0.05$) (Figure 2- 44). At OLR 25 kg-COD/m³/d, the effluent of PS+FW reactor contained 120 ± 18 mg/L ($p=0.013 \cdot 10^{-4}$, $\alpha=0.05$) (Figure 2- 43) and the

effluent of FW reactor contained 94 ± 11 mg/L ($p=0.024 \times 10^{-5}$, $\alpha=0.05$) (Figure 2- 45). The decrease in the sCARB concentration during fermentation occurs because of the breakdown of carbohydrates during that process. Complex polysaccharides undergo hydrolysis and fermentation in the fermenter while the soluble polysaccharides and sugars directly undergo fermentation. (Dhanalakshmi Sridevi V., et al. 2012)

2.4 Conclusions

In sum, we demonstrated the effect of substrate type and loading rate on the production of VFA (concentration and speciation) using anaerobic fermentation, as VFA are intermediates of the fermentation process with a range of different applications and potential for conversion to other value-added end products. More specifically, increase of organic loading rate did not show statistically significant decrease of hydrolysis for both reactors. However, the fermentation efficiency decreased in both reactor with increasing organic loading rate. That showed that under the environmental conditions applied (37°C, pH 6.5 and HRT 2 days) the microorganisms reached their maximum productivity at a loading rate lower than 25 kg-COD/m³/d. However, given the higher available COD at the higher OLR, both reactors showed highest VFA concentrations at OLR 25 kg-COD/m³/d. In addition, FW reactor showed higher VFA concentration than the PS+FW reactor. That showed that the food waste can improve the fermentation process, making it a good co-substrate candidate for VFA production. As food waste are being incorporated in the anaerobic digestion treatment of biosolids in wastewater treatment plants, this study of the effect of organic loading rate on VFA production could advance the field of anaerobic digestion from primary sludge and food waste, as well as show the

capability of conventional wastewater treatment plants to redirect their products towards a variety of alternative to methane products, promoting resource recovery.

Chapter 3: Optimization of Volatile Fatty Acids and Polyhydroxyalkanoates (PHA) Production from Fermentate Generated from Primary Sludge and Food Waste

3.1 Introduction

The existing waste management infrastructure continues to face huge pressure due to increasing waste generation coupled with stringent waste disposal regulations and increased public awareness (A. Liu et al. 2015). According to US EPA report of July 2018, 262 million tons of municipal solid waste (MSW) were generated in 2015 and only 91 million tons were processed for recovery (recycling, composting and combustion). Specifically, food waste accounted for 15.1% of MSW (~ 40 million tons) (“Advancing Sustainable Materials Management: 2015 Fact Sheet.” 2018). Most of the food waste (75%) was disposed in the landfills, as only 25% of generated food waste went into recycling and composting and combustion with energy recovery. Another important waste stream is the wastewater from domestic, industrial and commercial sources, as well as storm water, that is being collected in the sewers and delivered to treatment facilities. Wastewater contains pathogens and nutrients like carbon, phosphorus and nitrogen from human waste, food scraps, oils, soaps and chemicals. In order to comply with water quality regulations, it needs to be treated. The clean water effluent can return to the water cycle or be reused.

Wastewater Treatment Plants (WWTP) also referred as Water Resource Recovery Facilities (WRRF) apply physical, chemical and biological processes to remove soluble and particulate compounds with potential risk for the public health and the environment (Ronald L.

Droste 2018). WRRF have been adding food waste as co-digestion component in the treatment process of sewage sludge in order to increase the fermentable content and improve productivity. This way, wastewater treatment facilities offer the opportunity to divert large amounts of food waste from the landfill and promote recycling. Also, less food waste in the landfills leads to fewer methane release in the atmosphere.

Steps towards achieving electrical neutrality in WRRF require effective organic carbon management. Energy recovery from biomethane can be used directly towards the energy requirements of the facilities. However, channeling organic carbon through an anaerobic fermentation platform allows recovery of a wider spectrum of commodity chemicals. More specifically, VFA are a valuable substrate to a variety of applications such as production of biodegradable plastics (polyhydroxyalkanoate, PHA), generation of bioenergy (biodiesel and biohydrogen), or other chemicals (pharmaceuticals, detergents etc.) and biological nutrient removal (F. C. Silva et al. 2013). *Our project focused on recovery of VFA from anaerobic fermentation and sequential conversion to polyhydroxyalkanoate (PHA), with ultimate objective to maximize the VFA and PHA production.*

3.1.1 VFA Production and Optimization

VFA are short-chain fatty acids consisting of six or less carbon atoms and they can be produced by manipulating the hydraulic retention time (HRT) of the anaerobic digestion (AD) process in WRRF. AD in wastewater treatment plants has an HRT of about 20 days. That process includes 4 steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. By shortening the HRT to about 2-4 days, the fermentation process stops before the methanogenesis step. (Eddy & Metcalf 2005) At this stage the VFA are in maximum concentration and can be collected and

used in further applications, instead of being converted to biogas. (Eddy & Metcalf 2005) In more detail, the production of VFA from waste through AD involves hydrolysis and acidogenesis, also known as acidogenic fermentation (Bengtsson et al., 2008). In hydrolysis, complex organic polymers in waste are broken down into simpler organic monomers by the hydrolytic enzymes excreted from the microorganisms. Subsequently, acidogens ferment these monomers into mainly VFA. Both processes involve a complex consortium of obligate and facultative anaerobes, such as *Bacterioides*, *Clostridia*, *Bifidobacteria*, *Streptococci* and *Enterobacteriaceae* (Weiland, 2010).

Global research on maximizing the production of VFA has been focusing on different types of waste substrates and on different operating conditions of fermentation. The most investigated wastes are sludge (Wu et al. 2009), (P. Zhang, Chen, and Zhou 2009), (Maharaj and Elefsiniotis 2001), food waste and organic fraction of municipal solid waste (B. Zhang et al. 2005), (Bolzonella et al. 2005). Also, wastewaters generated from the agricultural, dairy, pulp and paper industries are frequently utilized for VFA production (S.K. Hong 2010), (Jiang Y 2012). The operational pH, temperature, retention time, and organic loading rate are the parameters that it is shown to have great effects on the yield and the composition of VFA produced from waste. (W. S. Lee et al. 2013). pH and temperature are directly related to the microbial growth and metabolism as each microorganism has an optimum range of pH and temperature for growth. Hence, changes in the pH or temperature values would cause changes in the microbial community involved in acidogenic fermentation. Retention time is the duration that the fermentation liquid stays in the reactor, hence the time that the substrate is allowed to react affects the product (VFA) yield. The organic loading rate indicates the amount of organic matter fed into the reactor per day and reactor volume. (Strazzera et al. 2018)

3.1.2 PHA Production and Optimization

Polyhydroxyalkanoates (PHA) are biopolymers that can be synthesized by microorganisms using VFA as substrate. The most common species of microbial produced PHA are polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) (X. Tang et al. 2014). Acetic and butyric acid are associated with PHB production, while propionic and valeric acid with PHV production (Jiang Y 2012). PHA have a broad range of applications in various industries (Philip, Keshavarz, and Roy 2007) and is environmental-friendly. However, its substitution over the conventional petrochemical-based plastic is limited by the high production cost because of the use of synthetic VFA and pure microbial cultures that require sterile conditions. Hence, low-cost substrate such as waste-derived VFA combined with mixed cultures is a promising option. To that end, the process that has been mainly used consists of 3 stages: (A) acidogenic fermentation to obtain a VFA-rich stream, (B) enrichment in PHA-storing microorganisms, and (C) a PHA accumulation stage where (A) and (B) outputs are combined in a final biopolymer production process. PHA accumulation that can be achieved by mixed microbial cultures fed with fermented food waste, fermented waste activated sludge (Y. Jiang, Chen, and Zheng 2009), fermented sugar cane molasses (M. G. E. Albuquerque, Torres, and Reis 2010) and fermented paper mill effluent (Jiang Y 2012) ranges from 26% to 80% (g-PHA/g-VSS). M. Zhang et al. (2014) reported use of waste activated sludge from wastewater treatment plant and food waste as substrates for VFA production and further conversion to PHA (22-30%). To date, most of the research on PHA production has been performed in lab scale but also some efforts have been devoted to the implementation of that 3-step process at pilot scale, by the integration of mixed culture microbial PHA production to municipal wastewater treatment plants with the potential benefits of sludge

minimization (Morgan-Sagastume et al. 2015) and to wastewater treatment processes of food processing industries (Anterrieu et al. 2014). Furthermore, the highest biomass PHA content has been achieved by enrichment cultures with *Plasticicumulans acidivorans* as dominant species (77-89% per cell dry weight) by using pure VFA or fermented VFA-rich feedstocks (Johnson et al. 2009).

Current research trends on the operational parameters optimization focus mostly on solids retention time (SRT), nutrient level, and feeding pattern. Chua et al. (2003) showed that SRT of 3 days resulted in better accumulation results than SRT of 10 days. Valentino et al. (2017) reported that optimal ranges of N and P levels (N/COD in the range 2.0- 15.0 mg/g, and P/COD 0.5-3.0 mg/g) resulted in improved PHA productivity. Popular feeding patterns that have been studied are single-pulse (Beccari et al. 2009), multi-pulse (Campanari et al. 2014), and continuous (Morgan-Sagastume et al. 2015). Multi-pulse feeding is often tested because it could avoid potential substrate inhibition problems (M. G. E. Albuquerque et al. 2007).

The goal of this part of the project was to maximize the VFA production through anaerobic co-fermentation of primary sludge and food waste by manipulating parameters such as organic loading rate (OLR), pH, and hydraulic retention time (HRT), as a preliminary step for optimizing PHA production, and then, to maximize the PHA production by manipulating the operational parameters such as the feeding pattern, and substrate (VFA) source.

The hypotheses were that: **(1)** PHA yield will increase with: (i) multi-pulse feeding; when providing the substrate in multiple smaller doses (multi-pulse feeding) instead of one big dose (single-pulse feeding), the system could increase its productivity, if high concentrations of

substrate (VFA) during single-pulse feeding cause substrate inhibition, (ii) synthetic VFA as substrate; acids are the preferred substrate for PHA production compared to other soluble compounds, thus providing pure acids as substrate should result in increased PHA concentrations. **(2)** VFA speciation can affect the PHA speciation; since PHA (products) are synthesized by VFA (substrate), different VFA species could lead to different PHA species.

The specific objectives were to: **(1)** Compare the PHA production when: (i) applying the two different feeding patterns (single-pulse vs multi-pulse), determine if the system shows substrate inhibition, and select the feeding pattern that results in better PHA production, (ii) providing two different substrate sources; synthetic VFA (pure acids) vs. fermentate effluent VFA (mixture of VFA and other soluble compounds), and that way, evaluate the PHA-storing capacity of the system. **(2)** Examine the correlation of VFA species and PHA species.

Even though global research on PHA production has mainly been focusing on the use of mixed cultures and residuals as feedstock, mainly consisting of industrial process effluent waters and wastewaters or food waste (Valentino et al. 2017), food waste and sludge combined, which is the co-fermentation substrate in our study, has not been studied thoroughly according to current literature review. M. Zhang et al., (2014) is one study on PHA production that used food waste and dewatered excess sludge as substrates for PHA production. That study focused on the effect of even-numbered and odd-numbered VFA on PHA speciation (PHB and PHV) (M. Zhang et al., 2014). In addition, recently, a pilot-scale study on the production of PHA from the organic fraction of municipal solid waste and sewage sludge was carried out by Valentino et al. (2019).

In our study, we decided to use food waste and primary sludge as primary sludge is less treated than waste activated sludge (WAS) and activated sludge (AS), which as mentioned above have mainly been used in other related studies. Primary sludge being less treated, has more

fermentable content which can lead to potentially higher VFA yields. Also, as shown in the previous chapter, food waste can improve VFA production in the fermentation process. These points addressed in our project can expand the current global research on PHA production from primary sludge and food waste, while reshaping the boundaries of conventional wastewater treatment plants, targeting on more attractive end products than methane.

3.2 Material & Methods

A two-reactor strategy was employed. The first reactor served as a continuous enrichment system. This reactor was fed fermentate generated using the OLR25 (25 kg-COD/m³/d) operational strategy as studied in Chapter 2. The goal of this system was to generate a seed culture with a high propensity for carbon storage. Biomass from the enrichment reactor was harvested and used within a dedicated production reactor. The production reactor was fed fermentate generated using the OLR25 operational strategy. Employing a two-reactor strategy allowed for decoupling of enrichment and production, while also ensuring that sufficient enriched seed culture is available.

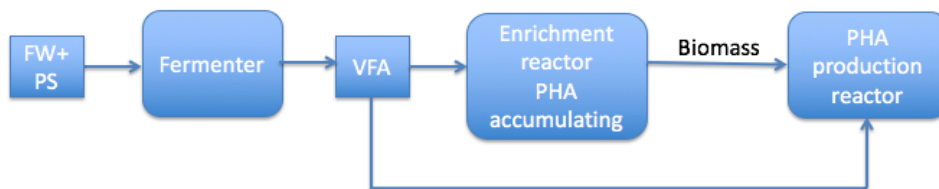


Figure 3- 1: Reactors configuration.

The overall goal was to maximize PHA production using fermentate generated from primary sludge (PS) and food waste (FW). From a practical perspective, this means that maximizing VFA production during fermentation would benefit PHA production. Along these lines, fermentation studies were

performed with PS and FW to determine the conditions that would maximize production of VFA that could be used in a PHA production system.

3.2.1 Fermentation Reactor Description & VFA Production Optimization Experiments (OLR, pH, HRT)

Anaerobic fermentation was performed in a 6.0 L glass reactor (Bellco glass, Vineland, NJ). The hydraulic retention time (HRT) was set to 2 days to limit methanogenesis (D. Liu, Zeng, and Angelidaki 2008). The fermenters were operated in sequencing batch reactor (SBR) mode, 3L of fermentate was pumped out and 3L of fresh feed was added to the reactor once every day. The temperature in the reactor was maintained at 37°C using heating tapes (BriskHeat, Columbus, OH). The pH of the reactors was set to 6.5±0.1 through automated addition of 1:1 1M NaHCO₃ and 1M NaOH solutions. Mixing (relative centrifugal force, 8xG) was provided using magnetic stir plates. The feed of the reactor was a 1:1 COD based mixture of primary sludge (PS) and food waste (FW) at organic loading rate of 25 kg-COD/m³/d. Primary sludge was obtained monthly from Loudoun Water Broad Run Water Reclamation Facility and stored at 4°C. The food waste was obtained from Columbia University cafeteria and was blended in a mixer and suitably diluted using de-ionized (DI) water. The reactor ran for over 50 days.

At OLR 25 kg-COD/m³/d, the “PS+FW” fermenter was operated in alkaline conditions, increasing the pH from 6.5 to 7.0, 7.5, 8.0 and 9.0, while the HRT was 2 days. While the pH was maintained at 9.0, we examined the effect of HRT by increasing the HRT to 4 and 6 days. During testing, the fermentation efficiency was monitored by examining particulate COD hydrolysis efficiency and total carboxylic acid yield. It should be noted that the efforts to

optimize the VFA production include the investigation of the effect of OLR which was studied in Chapter 2; where two different OLR values were tested; 10 and 25 kg-COD/m³/d.

3.2.2 Polyhydroxyalkanoates (PHA) Enrichment/Accumulation Reactor & PHA Production Optimization Experiments (Feeding Pattern, Substrate Composition)

Enrichment of a PHA accumulating culture was performed using a 6.0 L glass reactor (Bellco glass, Vineland, NJ), using working volume of 4L. The HRT and SRT of the PHA reactor were 1 and 2.1 ± 0.2 days respectively. The enrichment reactor was operated in SBR mode, with a 12-hour cycle applying feast and famine conditions (Table 3- 1). Profiles of TCA and VFA consumption, dissolved oxygen concentration, and total suspended and volatile solids can be found in Appendix Section (Figure 3- 9, Figure 3- 10, Figure 3- 11). An anaerobic reaction phase was included to help minimize growth of filamentous organisms and avoid sludge bulking. Influent of the PHA reactor comprised of effluent from the PS+FW fermenter that had been centrifuged to remove solids. The organic loading rate was 3,000 mg-sCOD/L/cycle. The temperature in the PHA reactor was maintained at 25°C using heating tapes (BriskHeat, Columbus, OH). pH was maintained at 7.0 ± 0.2 through automated addition of 1M NaHCO₃ and 1 M NaOH solutions, or 2N HCl solution. Mixing was provided using magnetic stir plates.

Table 3- 1: PHA reactor cycle configuration.

Phase	Duration (hr)	Cumulative time (hr)
Anaerobic (feast)	0.67	0.67
Aerobic (feast)	5.00	5.67
Aerobic (famine)	6.00	11.67
Settle and Decant	0.33	12.00

Biomass from the PHA reactor was used for multiple batch experiments that were focused on determining conditions that might increase PHA production.

- Substrate Feeding Pattern Experiments:** In these experiments, the impact of substrate feeding pattern on PHA production was examined. Two feeding patterns were tested in this work. The first pattern involved applying 100% of the organic substrate load in a single dose at the beginning of the cycle (single pulse). The second pattern involved applying organic substrate at three intervals in the cycle (multi-pulse), thereby stimulating three consecutive feast famine mini cycles. All batch experiments were operated such that the initial soluble COD concentration to the reactor was equivalent (~1,500 mg-sCOD/L).
- Substrate Composition Experiments:** In these experiments, the impact of substrate composition on PHA production was examined. The first batch production experiments were performed with fermentate generated by the PS+FW reactor at OLR 25. The second phase of batch experiments were performed using a substrate mixture comprised of

synthetic VFA that matched the profile of VFA observed in the fermentate. The third set of experiments involved running batch experiments with fermentate generated from alkaline fermentation experiments. The fourth set of experiments were performed using fermentate generated by PS+FW reactor running at HRT 4d and 6d.

3.2.3 Volatile Fatty Acids (VFA) and Total Carboxylic Acids (TCA) Quantification

Fermentate sample was withdrawn two or three times a week using the syringe sampling port assembly. The fermentate was centrifuged (10,000 rpm, 9133 x g, 10 min 4°C) and the supernatant was filtered through a 0.22 µm syringe filter and then appropriately diluted for soluble COD, and total carboxylic acids (TCA). The chemical parameters including the total and soluble COD (tCOD and sCOD) and TCA were determined using colorimetry (Hach kits 2125915 and 2244700, Loveland, CO). The VFA composition in the fermentate was determined using ion chromatography (Dionex ICS-2100) using a Dionex IonPac™ column (AS11-HC-4µm, RFIC™ & HPIC™ 2 X 250 mm). Analytical method of IC was Anion method 2 to 27mM (20mA), and IC processing method was 2mM to 10mM (20mA). (Manning and Bewsher 1997)

3.2.4 PHA Quantification

Review of several methods of quantification, revealed a preference for Gas Chromatographic analysis of esterified samples collected from the reactor over other methods including ion-exchange chromatography (Hesselmann et al. 1999) and high-performance liquid chromatography (Giin-Yu Amy Tan et al. 2014) (Andreas Grubelnik et al. 2008). Protocol was adapted from Johnson et al. (2009), Brandl H, et al. (1988) and Timm and Steinbüchel (1990).

The methyl esters were assayed using gas chromatography coupled with flame ionization detector (GC-FID, SRI 8610C) and MXT-Wax column (30 m 0.53 mmID 0.5 μ mdf) (Restek, Bellfonte, PA). Pure Sigma poly[(R)-3-hydroxybutyric acid] (PHB) and poly(3-hydroxybutyric-co-3-hydroxyvaleric acid) (PHBV, 12% mol HV) with weights between 1 and 10 mg were esterified and analyzed along with reactor samples to be used as reference standards. In addition, Nile Blue A staining was used for observation of PHA accumulation within bacterial cells (M. Oshiki, H. Satoh and T. Mino 2011)

3.3 Results & Discussion

3.3.1 VFA Production Optimization: Impact of OLR, pH and HRT on Co-fermentation

3.3.1.1 Impact of OLR on Co-fermentation

When the OLR of the fermented increased from 10 kg-COD/m³/day (OLR10) to 25 kg-COD/m³/day (OLR25), the sCOD yield showed non statistically significant decreased ($p=0.47$, $\alpha=0.05$) from 0.32 ± 0.09 to 0.30 ± 0.08 g-sCOD_{produced}/g-pCOD_{in}, the TCA yield decreased ($p=0.005$, $\alpha=0.05$) from 0.25 ± 0.08 to 0.22 ± 0.09 g-COD-TCA_{produced}/g-pCOD_{in}, however the VFA yield remained constant at 0.22 ± 0.08 g-COD-VFA_{produced}/g-pCOD_{in} (Figure 3- 2). The effluent VFA concentration at OLR25 was higher than the VFA concentration at OLR10. Results showed that VFA concentration between OLR 10 and 25 increased from $2,500 \pm 600$ mg-COD/L to $4,246 \pm 580$ mg-COD/L ($p=0.004 \times 10^{-3}$, $\alpha=0.05$) (Figure 3- 3). Cumulatively, these results indicated that operating at the higher OLR would be favorable for VFA production.

Consequently, all further fermentation experiments were performed using the OLR25 loading condition.

Other studies showed that increasing the OLR, the VFA concentration increased (Peilin Yang et al. 2007). However, depending on the substrate composition, and other operating conditions (pH, HRT, temperature) the concentrations of VFA can vary (Leiyu Feng et al., 2011). Our results showed increase of VFA concentration between OLR 10 and 25, from $2,500 \pm 600$ mg-COD/L to $4,246 \pm 580$ mg-COD/L ($p=0.004 \times 10^{-3}$, $\alpha=0.05$) (Figure 3- 3) (FW+PS, HRT 2d, pH=6.5, T=37°C, batch reactor), while Peilin Yang et al. (2007) reported increase from 2,500 to 3,400 mg-COD/L between OLR 10 and 15 respectively (FW, HRT 1d, pH 4-5, T=37°C, batch reactor). Most abundant VFA species was acetic acid (76% and 65% for OLR 10 and 15 respectively). Our study also showed that acetic acid was the prevailing VFA species for OLR 10 ($32\% \pm 6.6\%$) and OLR 25 ($53\% \pm 17\%$).

3.3.1.2 Impact of pH on Co-fermentation

Increasing the pH from 6.5 to 9.0 in the fermenter did not show significant change in sCOD ($p=0.5$, $\alpha=0.05$), TCA ($p=0.6$, $\alpha=0.05$) and VFA yield ($p=0.06$, $\alpha=0.05$) (Figure 3- 2). The concentration of TCA peaked at pH 8.0 ($5,300 \pm 600$ mg-COD/L) (Figure 3- 3).

Our results were consistent with findings from prior researchers as summarized by Gilmore, K., et al. (2017). Another study on activated sludge and food waste co-fermentation by Leiyu Feng et al. (2011) showed that at ambient temperature, HRT 4 days and varying pH values, the short-chain fatty acids production was maximum at pH 8 (8,200 mg-COD/L) followed by pH 7 (6,500 mg-COD/L), while when there was no food waste addition, at pH 8 the concentration of acids was 970 mg-COD/L. This clearly shows that the combination of FW and

sludge improves the acid production. Also, the higher max VFA concentration achieved by Leiyu Feng et al. (2011) can be attributed to the higher HRT applied in that study compared to the HRT of 2 days used in ours. Moreover, Dahiya et al. (2015) in their study, they used FW as substrate and tested pH from 5 to 11 (T=28°C, HRT 2d, OLR 15 kg-COD/m³/d, batch fermentation reactor). Maximum VFA concentration was reported at pH 10 (6,300 mg-COD/L). That concentration is higher than what we reported in our study; however, we did not investigate pH values greater than 9. In addition, another study by P. Zhang et al. (2009) using waste activated sludge (WAS) as substrate and examining different pH from 4 to 11 during fermentation (T=35°C, HRT 7d, OLR 2.6 kg-COD/m³-d, batch reactor) reported highest VFA concentration again at pH 10 (3,900 mg-COD/L). That concentration is lower than what was reported by the other studies mentioned above including our study, which supports that the co-fermentation is favorable for VFA production compared to using the substrates individually, hence max VFA production from co-fermentation of food waste and sludge can be achieved in less alkaline conditions than what described in the study by P. Zhang (2009).

In our study, the distribution of VFA in the fermenter effluent change as the pH increased. Between pH 6.5 and 8.0, acetic acid comprised the majority of the carboxylic acids produced (43±5 to 59±8%) (p=0.002). At pH 9.0, propionic acid was the dominant carboxylic acid produced (69±14%) (p=0.04). These results confirmed findings from other researchers that indicated that altering fermentation pH can impact the species of VFA produced. Dahiya et al. (2015) also reported change in VFA species; at pH 7 and 11, butyric acid was the most abundant, while at pH 6 and 8 – 10 the most abundant acid was acetic. However, Zhang et al. (2009) reported no change in VFA speciation throughout the pH values tested (acetic acid was the most abundant for pH 4 to 11). Leiyu Feng et al. (2011) also reported no change in VFA speciation

(pH 6 to 9) but in that study the most abundant acid was propionic. The differences in the dominant species of each of these studies depend on the operational conditions of each system and their microbial communities.

3.3.1.3 Impact of HRT on Co-fermentation

TCA ($p=0.3$, $\alpha=0.05$) and VFA ($p=0.23$, $\alpha=0.05$) yield did not show significant change when hydraulic retention times (HRT) increased to 4 days and 6 days at pH 9 (Figure 3- 2). TCA concentration increased as HRT increased and reached a maximum at pH 9 and HRT 6d ($6,500 \pm 400$ mg-COD/L) (Figure 3- 3). Increasing the HRT at pH 9.0 showed change in the VFA profile; acetic acid was the predominant species with $75 \pm 4\%$ (HRT 4d) and $78 \pm 11\%$ (HRT 6d).

D. Liu et al. (2008) in their study (FW, pH=7, T=70°C, batch reactor) report that VFA concentration increased when HRT increased from 2d to 4d and then decreased when HRT increased to 6d. The reported VFA concentrations were 7,900, 8,500, and 8,400 mg-COD/L for HRT 2d, 4d, and 6d respectively. Higher VFA concentrations compared to our results can be attributed to higher temperature and different substrate composition (100% FW) (Leiyu Feng et al., 2011). In our study the maximum VFA concentration was achieved at HRT 6d. This can be explained by the presence of alkaline conditions (pH 9) in comparison to neutral conditions (pH 7) in the study by D. Liu et al. (2008). Alkaline conditions inhibit methanogens, preventing the decrease of acids concentrations. In the study by D. Liu et al. (2008) the most abundant VFA species was acetic acid (82%) at all tested HRT (2d, 4d, 6d) which supports our findings (acetic acid: $75 \pm 4\%$ at 4d HRT and $78 \pm 11\%$ at 6d HRT).

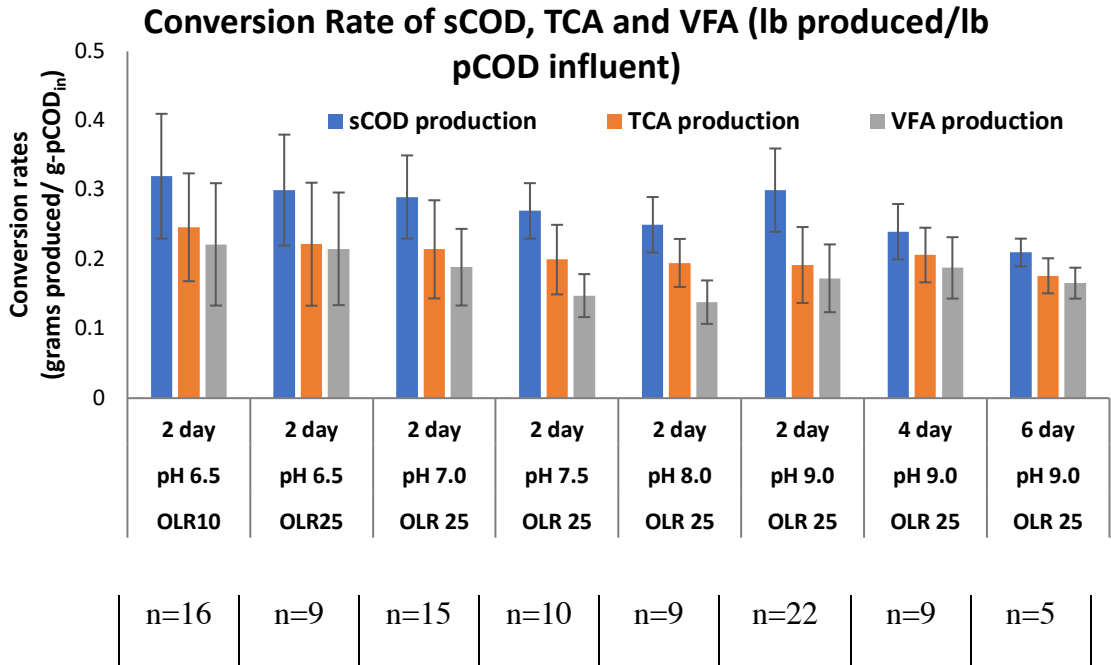


Figure 3- 2: Fermenter sCOD, TCA and VFA yield under varied OLR, pH and HRT.

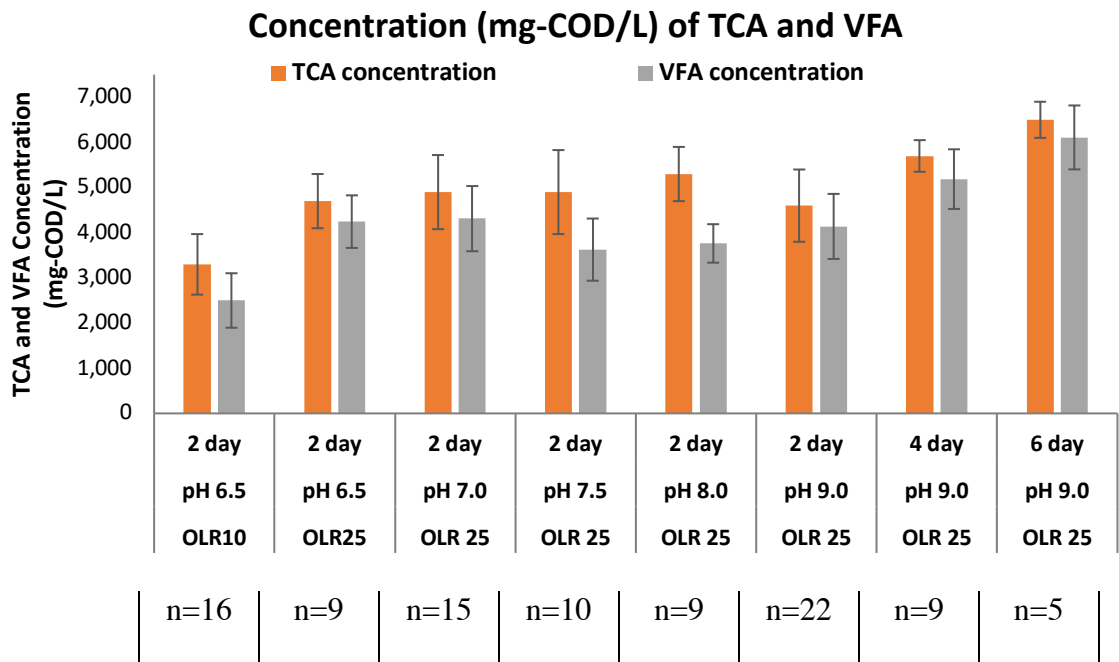


Figure 3- 3: Fermenter TCA and VFA concentration under varied OLR, pH, and HRT.

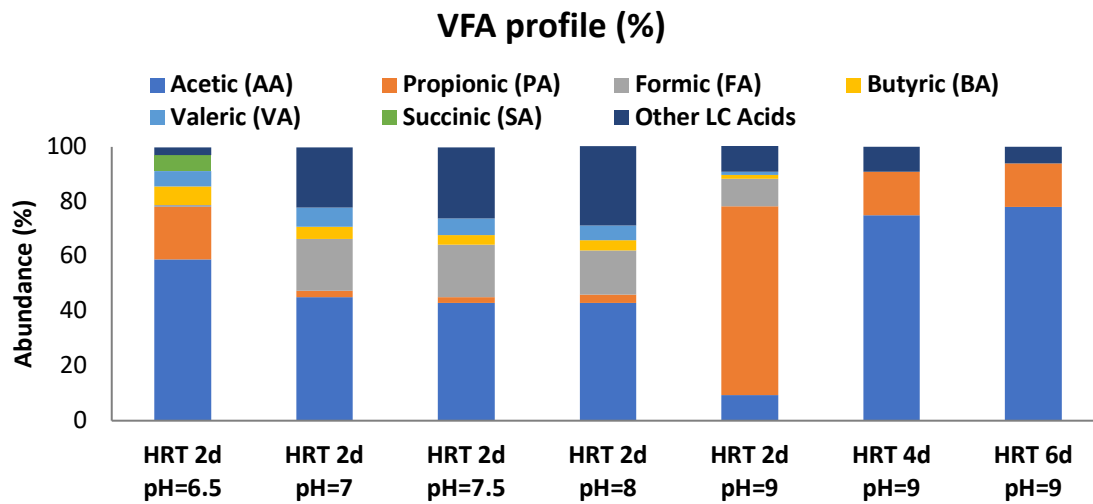


Figure 3- 4: Distribution of VFA species in fermenter effluent as a function of pH and HRT for OLR25.

3.3.2 PHA Accumulation

Preliminary results from enrichment of PHA accumulating organisms indicated that some PHA accumulation was occurring during the feast phase and visualization of the PHA granules was performed using Nile Blue A staining (Figure 3- 5)

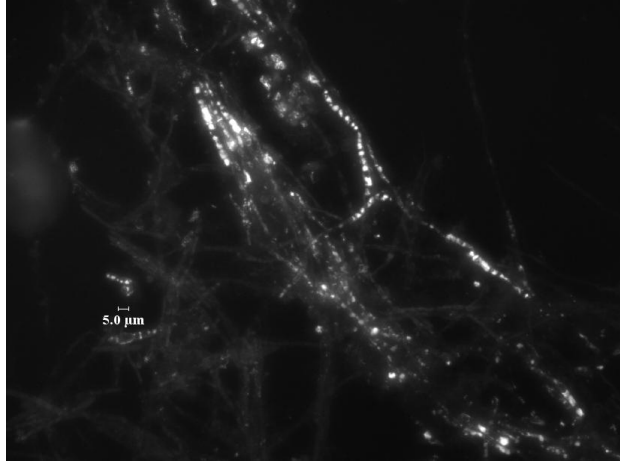


Figure 3- 5: PHA granules visualized using Nile Blue A Stain (40x fluorescence).

3.3.3 PHA Production Optimization: Impact of Substrate Feeding Pattern and Composition on PHA Production

As described in detail in section “3.2.2 Polyhydroxyalkanoates (PHA) Enrichment/Accumulation Reactor & PHA Production Optimization Experiments (Feeding Pattern, Substrate **Composition**)”, in order to optimize the PHA production, the follow scenarios were investigated:

A) substrate feeding patterns:

- i) *single pulse* (100% of the organic substrate load was applied in a single dose at the beginning of the cycle)
- ii) *multiple pulses* (organic substrate was applied at three intervals in the cycle)

B) substrate compositions:

- i) *fermentate* (generated by the PS+FW reactor at OLR 25, pH 7 and HRT 2 days)

- ii) *synthetic VFA of same distribution* (the profile of VFA matching the fermentate VFA profile)
- iii) *fermentate* (generated by the PS+FW reactor at OLR 25 in *alkaline conditions*; pH 7, 7.5, 8, 9)
- iv) *fermentate* (generated by the PS+FW reactor at OLR 25 in *HRT 4 and 6 days*)

3.3.3.1 Impact of Substrate Feeding Pattern on PHA Production

Experiments with varied feeding regimes indicated that single-pulse and multi-pulse feeding with fermentate did not show significant increase ($p=0.199$, $\alpha=0.05$) in PHA yield (Figure 3- 6). Hence, there was no substrate inhibition when the substrate was fed altogether in one pulse and as a result the single pulse feeding pattern was preferred.

Campanari et al. (2014) in their study using olive oil mill wastewater showed lower PHA accumulation using multi-pulse feeding (0.30 g-PHA/g-VSS, VFA content 0.42-0.50 g-COD/g-COD) compared to the study of Beccari et al. (2009) where the same substrate with single pulse feeding was used and PHA accumulation was 0.80 g-PHA/g-VSS (VFA content 0.22 g-COD/g-COD). Also, Morgan-Sagastume et al. (2015) reported 0.27 to 0.38 g-PHA/g-VSS using multi-pulse feeding of VFA from fermentation of waste activated sludge, while Erik Coats et al. (2007) and Mengmeng et al. (2009) reported 0.53 to 0.57 g-PHA/g-VSS using single pulse feeding of VFA produced from municipal wastewater and excess sludge fermentation, with a VFA content of 0.37 to 0.50 g-COD/g-COD. In addition, studies from Duque et al. (2014) and Oehmen et al. (2014) reported 0.56 to 0.58 g-PHA/g-VSS (VFA content 0.65-0.80 g-COD/g-COD) using multi-pulse feeding of VFA produced from fermentation of molasses, and Bengtsson et al.

(2010) reported 0.32 to 0.37 g-PHA/g-VSS (VFA content 0.75 g-COD/g-COD) using multi-pulse feeding of VFA produced from fermentation of sugar cane/molasses, while using single-pulse feeding of VFA produced from fermentation of sugar cane/molasses showed higher accumulation of max 0.61 g-PHA/g-VSS (VFA content 0.70 g-COD/g-COD) (Albuquerque et al., 2010). These results agreed with our conclusion that the single pulse feeding pattern is preferred.

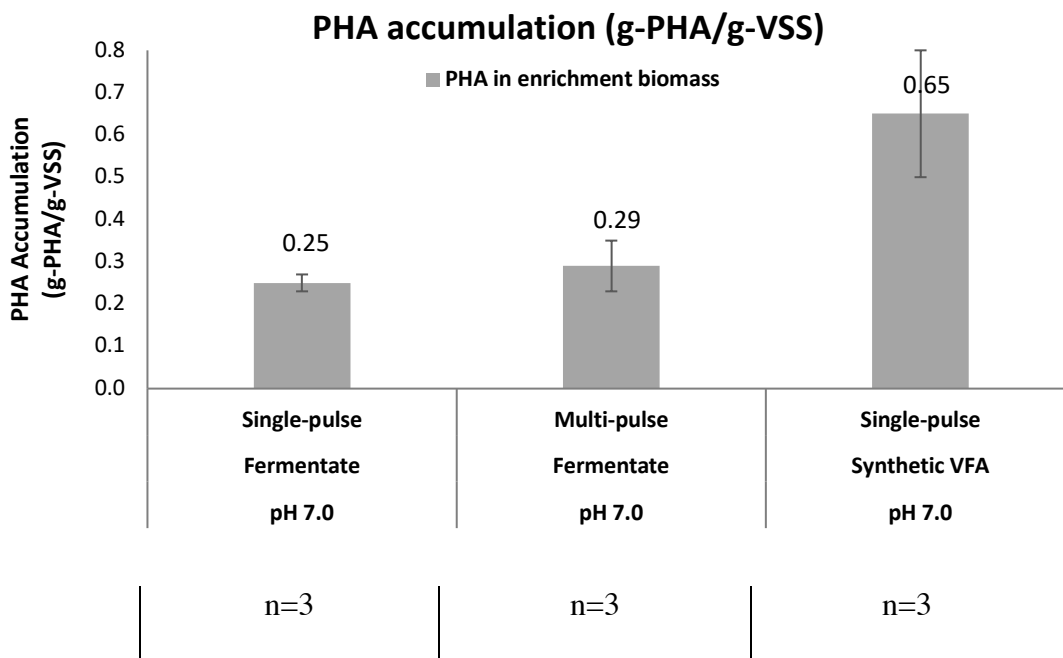


Figure 3- 6: PHA content of biomass from enrichment reactor for different feeding patterns and VFA sources (*Fermentate produced from PS+FW reactor, HRT 2d, 37°C).

3.3.3.2 Impact of Substrate Composition on PHA Production

PHA yield was significantly higher in experiments with synthetic VFA (0.65 ± 0.15 g-PHA/g-VSS, $p=0.04 \times 10^{-3}$, $\alpha=0.05$) vs. fermentate generated from food waste and primary sludge

(Figure 3- 6). Also, the substrate consumption rate was significantly higher too when synthetic VFA were used (0.45 ± 0.01 g-PHA/g-VSS, $p=0.03$, $\alpha=0.05$) (Figure 3- 7 and Appendix Figure 3- 12). That result confirmed that PHA production in combined fermentation/PHA production systems is limited by the mass and purity of VFA available. The profiles of substrate consumption and PHA accumulation during one cycle can be found in Appendix Section (Figure 3- 13, Figure 3- 14)

Villano et al. (2010) reported 0.23 to 0.38 g-PHA/g-VSS using synthetic substrate of VFA (mixture), while Valentino et al. (2014) reported a broader range of 0.14 to 0.51 g-PHA/g-VSS. These values were lower than our results of 0.65 g-PHA/g-VSS which might be a result of the different composition of VFA mixture or different microbial ecology with different substrate assimilation capacity. Comparing those results to the PHA accumulation using VFA from fermentate mentioned earlier [Beccari et al. (2009), Jiang Y (2012), Erik Coats et al. (2007), Mengmeng et al. (2009)] , we can see that the PHA accumulation in the latter case (fermentate VFA) is higher. That contradicts with our findings that showed that synthetic VFA gave higher PHA accumulation than fermentate VFA. That could be due to the lack of nutrients naturally present in organic waste that are important for the growth of PHA producing microorganisms. (M. Reddy, S. Mohan, 2012)

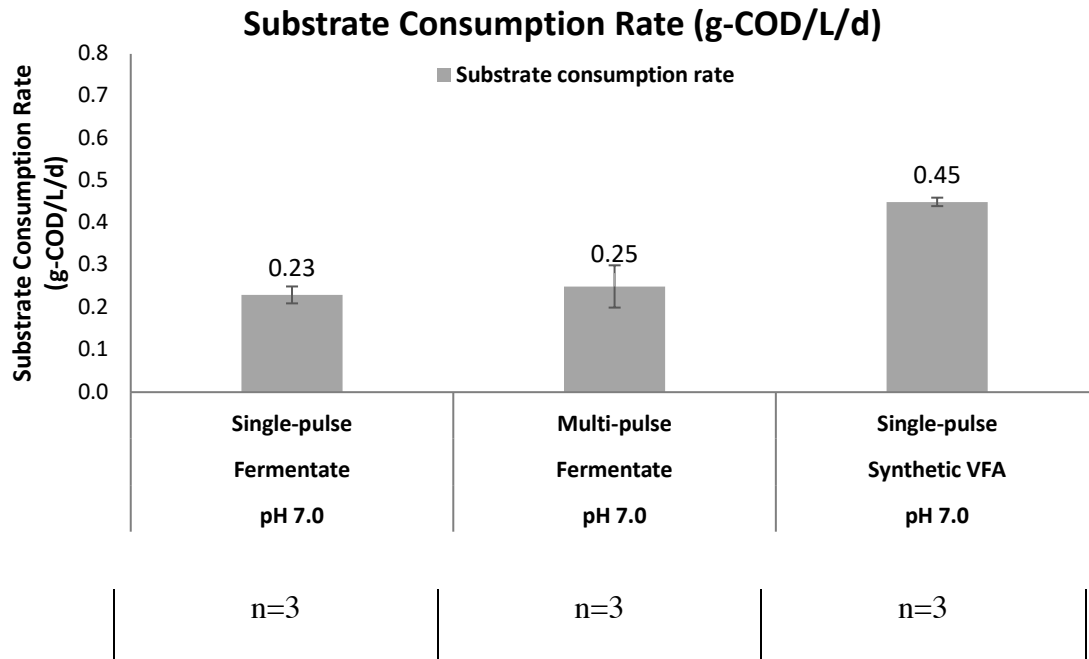


Figure 3- 7: Substrate consumption rates and max PHA accumulation from batch experiments with different feeding patterns and acids (VFA) sources.

Figure 3- 8 shows that the PHA content of the biomass in the enrichment reactor ranged from 0.25 ± 0.02 to 0.38 ± 0.05 g-PHA/g-VSS ($p=0.03$, $\alpha=0.05$), as the fermentate (substrate) production conditions changed (pH and HRT). The maximum PHA accumulation was achieved from fermentate produced at pH 9 and HRT 6d. Also, from Figure 3- 8 it can be noticed that accumulation is proportional to TCA concentration. The drop at pH 9 and HRT 2d is not statistically significant ($p=0.077$, $\alpha=0.05$)

Erik Coats et al. (2007) and Mengmeng et al. (2009) reported 0.53 to 0.57 g-PHA/g-VSS using VFA produced from municipal wastewater and excess sludge fermentation, with a VFA content of 0.37 to 0.50 g-COD/g-COD. VFA produced from fermentation of other substrates,

such as sugar cane molasses, olive oil mill wastewater and paper mill wastewater give a higher range of PHA content 0.33 to 0.61 g-PHA/g-VSS (VFA content 0.70 g-COD/g-COD) (Albuquerque et al., 2011), 0.80 g-PHA/g-VSS (VFA content 0.22 g-COD/g-COD) (Beccari et al., 2009) and 0.77 to 0.84 g-PHA/g-VSS (VFA content 0.60-0.65 g-COD/g-COD) (Jiang Y., 2012) respectively. This shows that these substrates can also be good candidates for co-fermentation with primary sludge as an alternative to food waste.

In addition, in our study there was a shift in the PHA speciation throughout the experiments. At the conditions pH 7, 7.5, and 8 (HRT 2 days) the average PHB content was $88\% \pm 6.2\%$ and the average PHV content was $12\% \pm 3.1\%$, while at the conditions HRT 4 and 6 days (pH 9) the average PHB content increased to $95\% \pm 6\%$ and average PHV content decreased to $5\% \pm 0.6\%$ ($p=0.031$, $\alpha=0.05$). (Table 3- 3). Also, it can be observed that as the even-numbered VFA increased, the PHB content also increased ($p=0.031$, $\alpha=0.05$), while when the odd-numbered VFA increased, the PHV content also increased ($p=0.041$, $\alpha=0.05$). (Table 3- 3) Hence, it can be concluded that even-numbered VFA lead to PHB synthesis and odd-numbered VFA to PHV synthesis. Studies by M. Zhang et al. (2014) and Wang et al. (2007) support these findings.

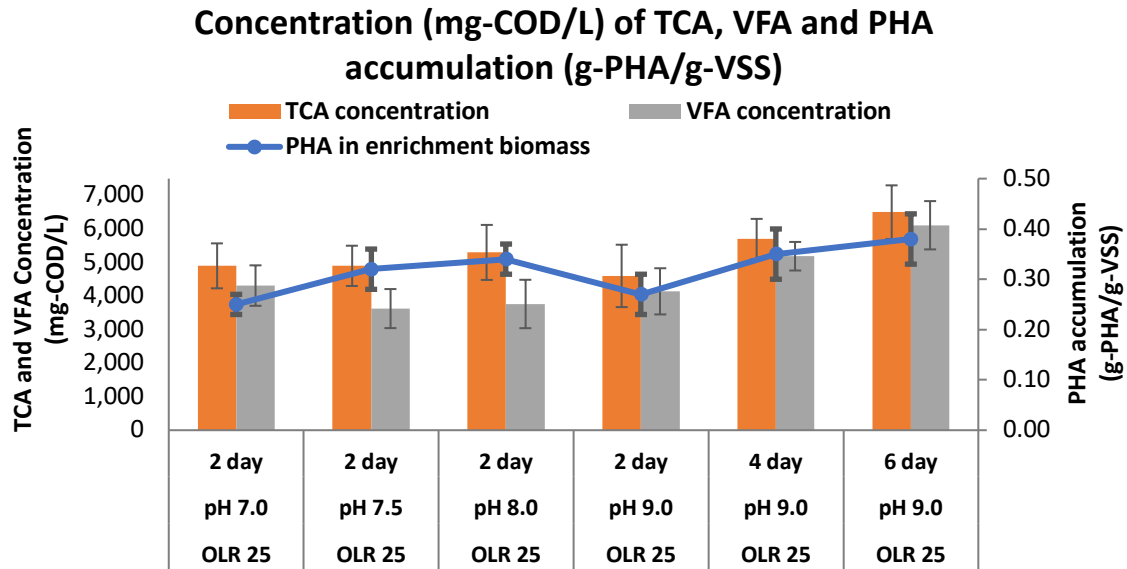


Figure 3- 8: Concentrations of TCA and VFA at different fermentation conditions (HRT and pH) and the corresponding accumulation of Polyhydroxyalkanoates.

3.4 Conclusions

In conclusion, we determined the optimum conditions to recover carbon in the form of volatile fatty acids (VFA) through anaerobic co-fermentation of primary sludge and food waste, and we explored further uses of such VFA for the production of other high-value products such as polyhydroxyalkanoates (PHA). Two organic loading rates (OLR); 10 kg-COD/m³/d and 25 kg-COD/m³/d, and various pH; 6.5, 7.0, 7.5, 8.0, 9.0, and HRT; 2 days, 4 days, and 6 days, were tested in order to maximize the VFA production and proceed in the enrichment of PHA accumulating microorganisms for production of PHA. For the optimization of PHA production we tested the substrate feeding pattern (single-pulse and multi-pulse) and the substrate source (fermentate VFA produced under several pH and HRT conditions and synthetic VFA). Based on the experiments of this study the preferred conditions for max VFA and PHA production were co-fermentation of primary sludge and food waste of OLR 25 kg-COD/m³/d, at alkaline

conditions of pH 9 and HRT 6d and then feeding of fermentation derived VFA in one-pulse in PHA accumulating reactor. The maximum PHA accumulation achieved was 0.38 ± 0.05 g-PHA/g-VSS. That process of producing waste-derived PHA can lead to a more sustainable way to produce biodegradable plastics, while having the benefit of being applicable to WRRF.

Chapter 4: Microbial Ecology Associated with Volatile Fatty Acids (VFA) and Polyhydroxyalkanoates (PHA) Production through Co-Fermentation of Waste Streams

4.1 Introduction

Polyhydroxyalkanoates (PHA) are biopolymers synthesized by microorganisms. They are granular shaped inclusions, formed naturally inside the cells for energy storage purposes. They were first discovered by the French scientist Lemoigne in 1925. More specifically, PHA were found in *Bacillus megaterium* in the form of poly (3-hydroxybutyrate) (PHB). To date, more than 300 bacterial species have been identified as PHA producers. (Raza et al. 2018)

PHA are fully biodegradable and biocompatible plastics with comparable mechanical properties to those of conventional petrochemically derived plastic, such as polypropylene and polyethylene. (Mengmeng et al. 2009) However, worldwide PHA production accounts for only 0.5% of total plastic production. This is due to its cost, which ranges from 1.1 to 2.5 \$/lbs compared to 0.5 \$/lbs of petroleum-based plastics. (Valentino et al. 2017) Commercially available PHA are produced by pure microbial cultures and through fermentation of synthetic acids with high market price. (Maria G. E. Albuquerque et al. 2013). Hence, the main factors leading to the higher price of PHA are the energy cost for sterilization of fermentation equipment to maintain the pure cultures, the cost of the substrates and the efficiency of the downstream processes (extraction, purification, etc.) (Fernández-Dacosta et al. 2015)

Therefore, efforts have been applied towards the utilization of mixed microbial cultures (MMC) and waste/residual feedstock that do not compete with primary food crops and have low

cost. Diverse microbial cultures are able to produce PHA and become enriched in microorganisms with PHA accumulating capability, under repeated cycles of dynamic conditions of excess external carbon (feast) and carbon starvation (famine). The produced PHA can be used for bacterial growth, which ultimately determines the survival of PHA producers and the washout of non-PHA producers. (Fradinho et al. 2014). Figure 4- 9 in Appendix shows the metabolic pathway during feast and famine conditions. Mixed microbial cultures with PHA storing microorganisms are naturally present in activated sludge in wastewater treatment plants. Consequently, research on PHA production by mixed microbial cultures has been focusing on biological wastewater treatment using process residuals and wastes. (Valentino et al. 2017) (Maria G. E. Albuquerque et al. 2013) Additionally, significant amounts of wastewater sludge and food waste are generated daily worldwide. Handling, treatment, and disposal of these wastes create a cost that could be avoided if those resources were utilized further for value-added products, such as PHA. (Mengmeng et al. 2009). More specifically, to date, most of the research in PHA production by mixed microbial culture from waste has been performed at laboratory scale and the process is divided in three steps: i) acidogenic fermentation of waste to obtain a VFA-rich stream, ii) enrichment of PHA-storing microorganisms, iii) PHA accumulation by combining the VFA-rich stream from step (i) and the PHA producers from step (ii) for PHA production. (Salehizadeh and Van Loosdrecht 2004)

As shown in detail in the previous two chapters, research on VFA and PHA production focuses mainly on optimization of system's operating conditions. Those parameters, for VFA production, are: pH, temperature, HRT, loading rate, substrate type, (W. S. Lee et al. 2014) and for PHA: HRT, pH, temperature, nutrients, dissolved oxygen concentration, cycle length,

feast/famine ratios, substrate type and concentration (Valentino et al. 2017) (Campanari et al. 2014) (Chua et al. 2003) (Villano et al. 2010) (Venkateswar Reddy and Venkata Mohan, 2012).

Studies of microbial ecology in anaerobic fermentation are mainly focused on methane production, instead of VFA, and the conditions used in those systems have steady parameters such as pH and HRT [(Jabari et al. 2016) (Y. Tang et al. 2005) (L. Li et al. 2016) (Shin et al. 2010) (W.Q. Guo et al. 2008)]. To the best of our knowledge, there is no study that examines the shift in microbial ecology through different conditions of pH and HRT, even though these parameters affect the dominance of microbial species in mixed microbial cultures. Furthermore, studies of microbial ecology in PHA production systems are mainly focused on pure cultures using synthetic acids or fermented food waste as substrates (Reddy et al. 2003) (C. Nielsen et al. 2017) (Hafuka et al. 2011). There is limited research describing the microbial ecology of MMC utilizing complex fermented substrates; such as dairy manure (Coats et al., 2016), molasses (Carvalho et al. 2014), paper mill effluent (Bengtsson et al., 2008) and food waste (Maria G. E. Albuquerque et al., 2013). However, no study explains the microbiology of the full process; starting from acidogenesis all the way to PHA production. By studying the connected system of VFA production (fermentation) and PHA production we could provide a complete overview of the system, which can be extended to economical and sustainable pilot-scale studies or even future full-scale projects. In addition, it is documented that the VFA distribution affects the PHA monomer composition (M. Zhang et al., 2014). Hence, looking into the ecology of the combined system can give engineers directions for adjusting the fermentation conditions and ultimately shifting the microbial ecology in such way that produces target VFA species that could control the PHA species along the process. Therefore, in our study, we focused on the holistic study of the ecology of acidogenic co-fermentation of primary sludge from wastewater treatment process

enhanced with food waste, and comparison between different fermentation conditions (pH and HRT) and the ecology of PHA producers, monitoring the shift in microorganisms throughout the enrichment process.

The hypotheses were that: **(1)** microbial ecology in the fermentation reactor will shift with increasing pH and HRT; as microorganisms have optimum growth conditions, if the system's conditions are altered, the dominant microorganisms are expected to change, **(2)** the presence of methanogens in longer than 4 days HRT will be avoided due to the alkaline fermentation conditions; methanogens are unwanted in our system because they consume the acids available for PHA production, and they require longer retention times than acidogenic microorganisms in order to grow (D. Liu et al., 2008), however alkaline conditions inhibit methanogens growth (Yuan et al. 2019). **(3)** microbial ecology in the PHA enrichment reactor will shift with time as the enrichment process progresses due to the applied dynamic conditions of feast and famine; by combining periods of substrate availability (feast) with longer periods of substrate deficiency (famine), PHA producing microorganisms are enriched over non-PHA producing ones, as the first ones use the PHA that was produced during the feast phase in order to survive during the famine phase (Kourmentza et al. 2017).

The objective of this study was to investigate the connection of the VFA production (concentration and speciation) and the fermentation microbial ecology with the PHA production (concentration and speciation) and PHA producers. The reasoning behind that research objective lied in the fact that in order to have an in depth understanding of the PHA production process, we need to have an understanding of the microorganisms of each step of the process, because ultimately, they are responsible for the system's efficiency. Without in depth knowledge of the microbial ecology, any system can be represented by a "black box" where we only know the

reactants and products without any insight into the factors that caused the conversion of the reactants into products. Further study of those factors can lead to better understanding of the mechanisms that took place. Study of the microbial ecology of the combined system of VFA production with further conversion to PHA could contribute to an efficient process design with possible application into WRRF. Retrofitting of the existing anaerobic digestion infrastructure could create a biorefinery platform that uses wastes to produce biodegradable plastics instead of methane.

4.2 Metabolic Pathways of Acidogenic Fermentation and PHA Production and Related Microbial Ecology

4.2.1 Metabolic Pathways of Acidogenic Fermentation

Acidogenic fermentation is a complex process involving a series of chemical reactions and enzymes. That intricate network of metabolic pathways involves numerous microorganisms playing a crucial role in performance of the system. The main intermediate in the acidogenic metabolic pathway is pyruvate, that can be converted into a variety of products as shown in Figure 4- *I*. The metabolic pathways of fermentation are categorized as follows: **(I)** acetate-ethanol type (AET), **(II)** propionate type (PTF), **(III)** butyrate type (BTF), **(IV)** mixed-acid type (MAF), and **(V)** lactate type (LTF), **(VI)** Homoacetogenic type.

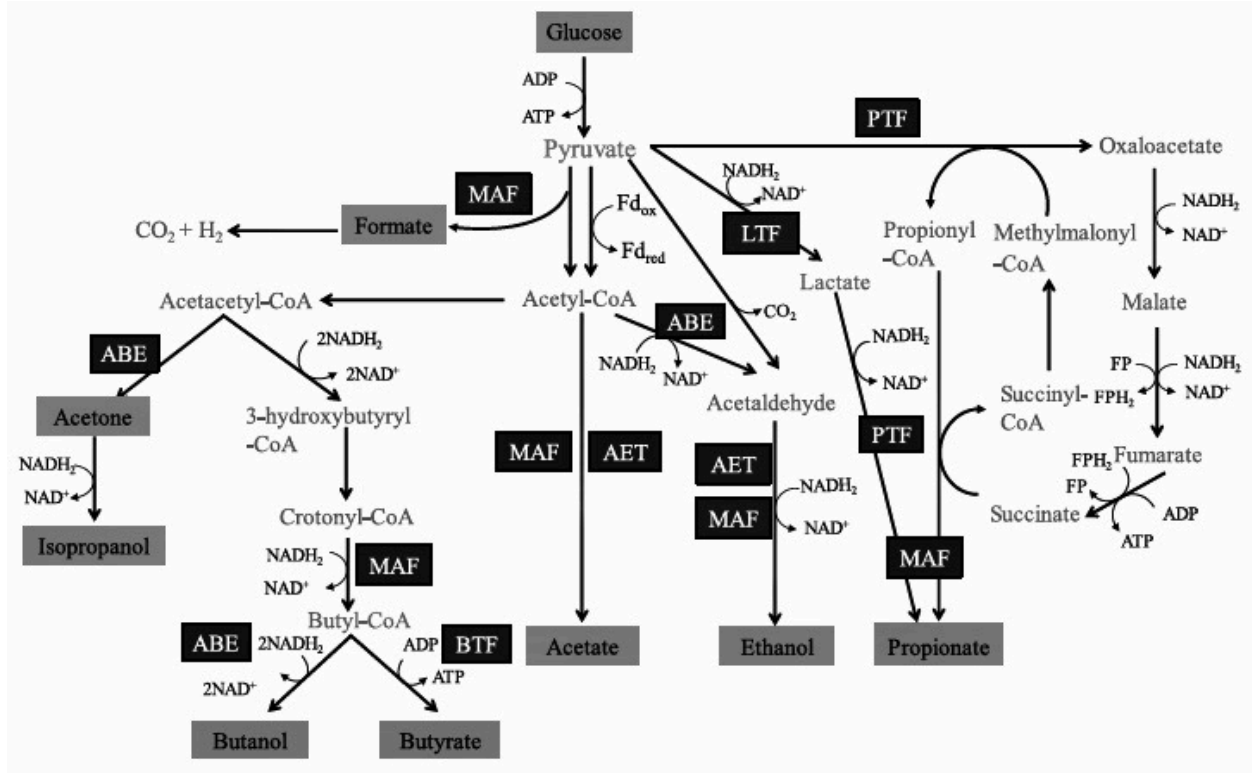
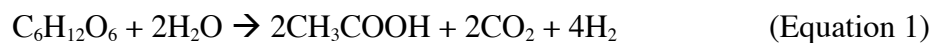


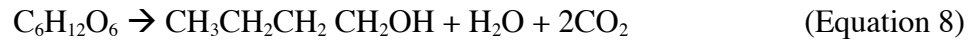
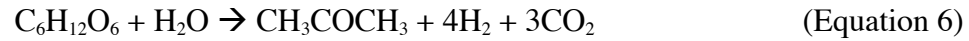
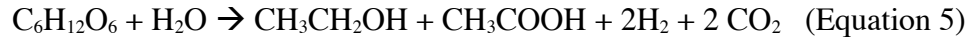
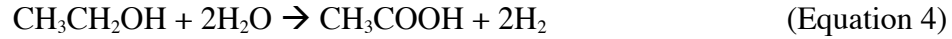
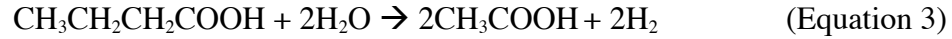
Figure 4- 1: Metabolic pathways in acidogenic fermentation. Adapted from Zhou et al. (2018)

(*AET*, acetate-ethanol type fermentation; *ABE*, acetone-butanol-ethanol; *PTF*, propionate type fermentation; *BTF*, butyrate type fermentation; *MAF*, mixed-acids type fermentation; *LTF*, lactate type fermentation. *Fd_{ox}* and *Fd_{red}*, oxidized and reduced form of ferredoxin.)

4.2.1.1 Acetate-ethanol Type Metabolic Pathway

Acetate-ethanol type (AET) of fermentation metabolic pathways has acetate and ethanol, as main products. The reactions related to this pathway are the following:





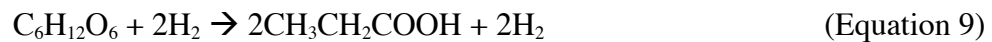
Acetate can be produced from pyruvate through acetyl-CoA (Eq.1) or from syntrophic oxidation of other volatile fatty acids; such as propionate (Eq.2) and butyrate (Eq.3), or from ethanol (Eq.4) (Nicolai Müller et al., 2010). Ethanol is another product that is produced from fermentation of glucose or other organic matter (Eq.5). There are two pathways; one pathway includes three steps with acetyl-CoA and acetaldehyde as the intermediates (Chaganti et al., 2011), and the other pathway includes two steps with conversion of pyruvate to acetaldehyde and then reduction to ethanol (Bensaid et al., 2015). *Enterobacteriaceae* follow the three-step pathway. *Aspergillus awamori*, and *Saccharomyces cerevisiae* have been identified as species that follow the acetate-ethanol type (AET) metabolic pathways (Zhou et al. 2018).

Acetone and butanol can be produced from glucose during ethanol production by some microorganisms such as *Clostridium acetobutylicum* and *Clostridium beijerinckii*. (Zhou et al.

2018) This pathway is the acetone-butanol-ethanol (ABE) type of fermentation pathway. (Eq.6 and Eq. 8) In that pathway glucose is converted to acetyl-CoA through pyruvate, with a following conversion to acetacetyl-CoA for production of acetone and butanol. (Figure 4- 1) At the acetone pathway can be further converted to isopropanol. *Clostridium beijerinckii* follow that pathway. In the butanol pathway, acetacetyl-CoA is converted to butanol via the intermediates 3-hydroxybutyl-CoA, crotonyl-CoA and butyl-CoA.

4.2.1.2 Propionate Type Metabolic Pathway

Propionate type of fermentation (PTF) metabolic pathways has propionate, as main product. The reactions related to this pathway are the following:

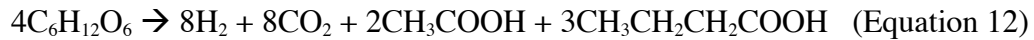
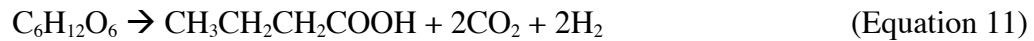


Propionate can be produced from two pathways as shown in Figure 4- 1. One pathway includes conversion of glucose to pyruvate and then reduction of pyruvate to lactate by the enzyme lactate dehydrogenase; then lactate is reduced to propionate by the propionate dehydrogenase (H.-S. Lee, et al., 2008). *Clostridia*, *b-Proteobacteria*, and *Bacteroidetes* follow this propionate-type metabolic pathway (Feng, Chen, and Zheng 2009). The other pathway includes a transcarboxylase cycle and it is performed by acidogenic bacteria such as *Corynebacteria*, *Propionibacterium acidipropionici* and *Bifidobacterium*. Intermediates in that

pathway are oxaloacetate, malate, fumarate, succinate, succinyl-CoA, methylmalonyl-CoA, and propionyl-CoA. (Zhou et al. 2018)

4.2.1.3 Butyrate Type Metabolic Pathway

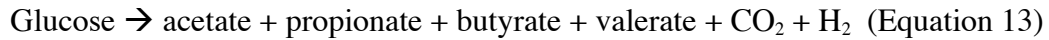
Butyrate type of fermentation (BTF) metabolic pathway has butyrate and acetate, as main products and hydrogen as by-product. The reactions related to this pathway are the following:



Butyrate is produced with conversion of pyruvate to acetyl-CoA by pyruvate dehydrogenase, then acetyl-CoA is converted to butyryl-CoA, with several intermediates; acetoacetyl-CoA, 3-hydroxybutyryl-CoA and crotonyl-CoA. The enzymes that catalyze these reactions are thiolase, 3- hydroxybutyryl-CoA dehydrogenase and butyryl-CoA dehydrogenase (Chaganti et al., 2011). Butyryl-CoA is sequentially converted to butyrate by enzymes phosphotransbutyrylase and butyrate-kinase or by the butyryl-CoA: acetate CoA-transferase (Vital et al., 2014). In this pathway, two NADH₂ are consumed to reduce the intermediates. *Clostridium Tyrobutyricum* has been identified as species that follows the butyrate type (BTF) metabolic pathway. (Zhou et al. 2018)

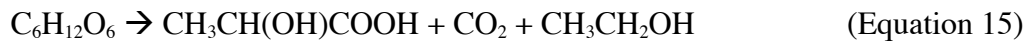
4.2.1.4 Mixed-acid Type Metabolic Pathway

In mixed-acid type of fermentation (MAF) metabolic pathway the products are a mixture of acids such as acetate, propionate, butyrate, and valerate, and biogas (CO₂ and H₂) as it is shown in Eq.13.



4.2.1.5 Lactate Type Metabolic Pathway

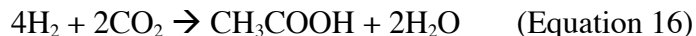
Lactate-type of fermentation (LTF) metabolic pathway has lactate as main product. The reactions related to this pathway are the following:



Lactate is produced with glucose conversion to pyruvate through glycolysis and then pyruvate is converted to lactate by lactate dehydrogenase with the transformation of NADH₂ to NAD⁺. There are two types of reactions; homolactate (Eq.14) and heterolactate (Eq.15). During homolactate fermentation one mole of glucose is converted to two moles of lactate, while during heterolactate fermentation, one mole of glucose is converted to one mole of lactate along with carbon dioxide and ethanol. (Castillo Martinez et al. 2013). Bacteria that follow that metabolic pathway are *Lactobacillus acidophilus*, *Lactobacillus casei*, *Streptococcus thermophilus*. (Zhou et al. 2018)

4.2.1.6 Homoacetogenic Type Metabolic Pathway

Homoacetogenic metabolic pathway is performed by homoacetogens, which is a group of obligate anaerobes that use hydrogen as electron donor to reduce carbon dioxide to acetate (homoacetogenesis) (Saady 2013). *Acetobacterium woodii* has been identified as species that follows the homoacetogenic metabolic pathway. (Zhou et al. 2018). This pathway is also called Wood-Ljungdahl pathway (Drake et al., 2006). The reaction related to this pathway is the following:



This pathway has two parts as shown in Figure 4- 2. The one part reduces CO_2 to formate by formate dehydrogenase. Then sequential reactions are taking place with participating enzymes formate dehydrogenase, methylene-H₄F reductase and methetyltransferase, in order to synthesize a methyl group. That synthesis takes up 6 mol of reducing equivalents. The other part reduces CO_2 to carbon monoxide (CO) by acetyl-CoA synthase, and this step consumes 2 mol of reducing equivalents. Then the methyl group and the carbon monoxide are reacting to create Acetyl-CoA which is then converted into acetate with ATP synthesis during catabolism or to cell carbon during anabolism (X. M. Guo et al. 2010). All in all, this pathway consumes energy; 1 ATP is produced when 2 mol of CO_2 are reduced to acetate, one ATP is consumed in the reaction of formate in the first part, and in the second part 1/3 ATP is consumed in the reduction of CO_2 to CO (Drake 1994).

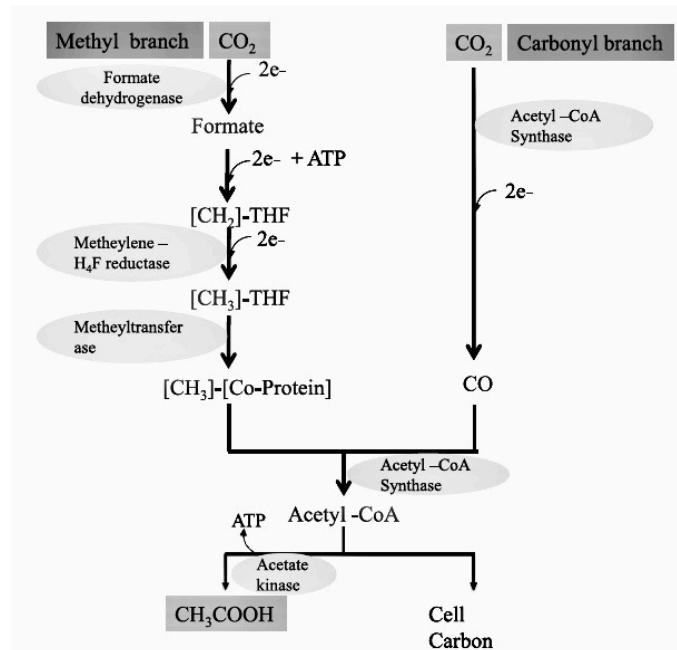


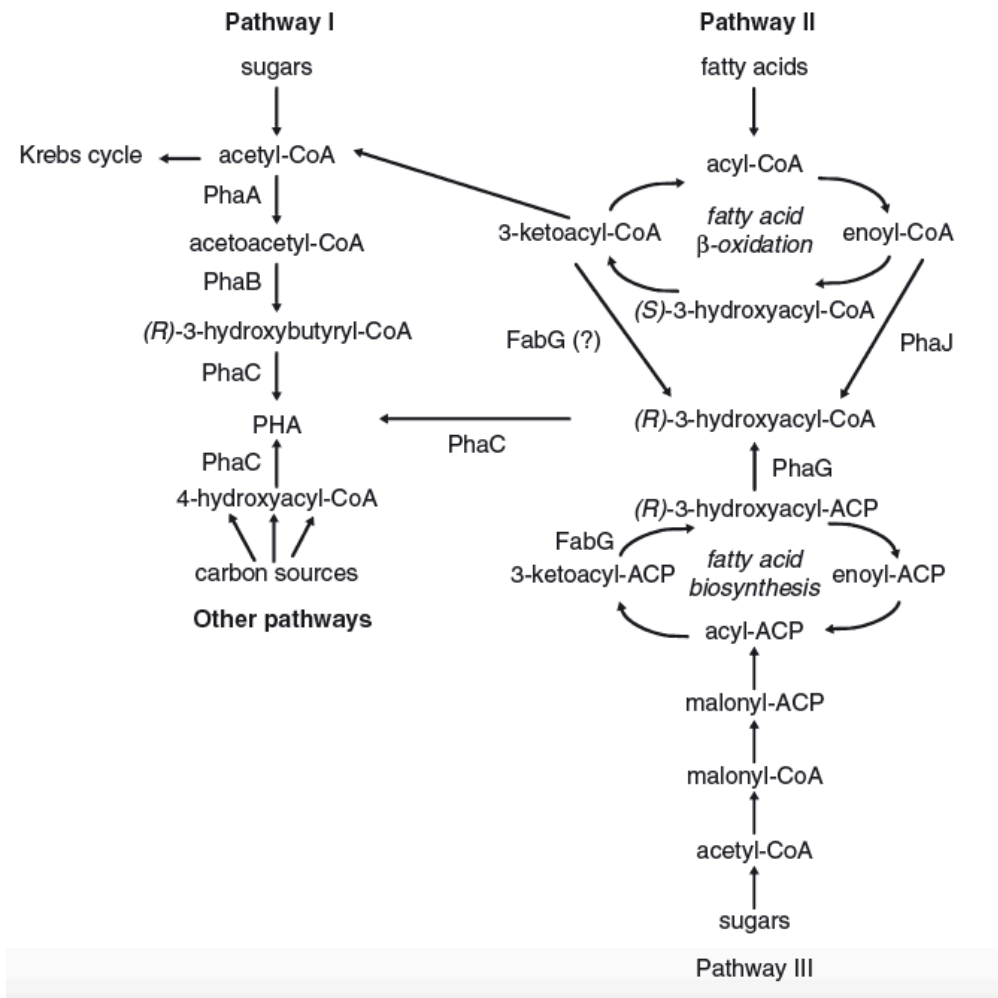
Figure 4- 2: Homoacetogenic metabolic pathways of acetate production from CO₂ (THF: tetrahydrofolate; [Co-Protein]: corrinoid enzyme). Adapted from (Zhou et al. 2018)

4.2.2 Metabolic Pathway of PHA production

There are three most-studied pathways for naturally occurring PHA biosynthesis (Figure 4- 3). In pathway I, carbon in the form of sugars is converted to acetyl-CoA. When entry of acetyl-CoA into the Krebs cycle (Figure 4- 10) is restricted during nutrient limitation, the surplus acetyl-CoA is channeled into PHA biosynthesis (Colin Ratledge, 2001). Two units of acetyl-CoA are condensed to acetoacetyl-CoA by 3-ketothiolase (PhaA). Then, acetoacetyl-CoA reductase (PhaB) converts the product to (R)-3-hydroxybutyryl-CoA, and finally PHA synthase (PhaC) polymerizes the (R)-isomers. Sugars such as glucose and fructose are mostly processed via pathway I, yielding PHB homopolymer (Aldor and Keasling 2003). In pathways II and III, intermediate products of fatty acids β -oxidation and intermediate products of fatty acids

biosynthesis, respectively, are used for PHA synthesis. Several fluorescent pseudomonades; *Pseudomonas putida*, *P. oleovorans*, and *P. aeruginosa*, follow these pathways for production of copolymers from fatty acids or sugars. The product of these pathways is (R)-3-hydroxyacyl-CoA monomers that are then polymerized into PHA by PhaC (PHA synthase). More specifically, in pathway II (R)-specific enoyl-CoA hydratase (PhaJ) provides enoyl-CoA from the fatty acid β -oxidation for conversion to (R)-3-hydroxyacyl-CoA and in pathway III, (R)-3-hydroxyacyl-ACP-CoA transferase (PhaG) provides (R)-3-hydroxyacyl-ACP from the fatty acid biosynthesis for conversion to (R)-3-hydroxyacyl-CoA. It should be noted that 3-ketoacyl-ACP reductase (FabG), which participates in the fatty acid biosynthesis pathway, accepting acyl-ACP, there is evidence that can also acyl-CoA as a substrate from the fatty acid β -oxidation. (Taguchi et al. 1999).

Several bacterial species can produce PHA. The most studied one is *Cupriavidus necator* (formerly known as *Ralstonia eutropha* or *Alcaligenes eutrophus*). Other strains that were studied for PHA production include: *Bacillus* spp., *Alcaligenes* spp., *Pseudomonas* spp. (*Pseudomonas oleovorans*, *Pseudomonas putida*, *P. fluorescens*, *P. jessenii*, *Pseudomonas stutzeri*), *Aeromonas hydrophila*, *Rhodopseudomonas palustris*, *Caulobacter crescentus*, *Burkholderia sacchari*, *Burkholderia cepacia*, *Halomonas boliviensis*, *Legionella pneumophila*, *Methylocystis* sp., *Microlunatus phosphovoratus*, *Rhizobium* spp. (*Rhizobium meliloti*, *R. viciae*, *Bradyrhizobium japonicum*), *Spirulina platensis* (cyanobacterium), *Staphylococcus epidermidis*, *Alcaligenes latus*, and *Comamonas acidovorans*. (Tsuge 2002)



PhaA, 3-Ketothiolase; ***PhaB***, NADPH-dependent acetoacetyl-CoA reductase; ***PhaC***, PHA synthase; ***PhaG***, 3-hydroxyacyl-ACP-CoA transferase; ***PhaJ***, (R)-specific enoyl-CoA hydratase; ***FabG***, 3-ketoacyl-ACP reductase.

Figure 4- 3: Metabolic pathways for PHA production. Adapted from (Tsuge, 2002)

4.3 Material & Methods

4.3.1 Fermentation Reactor

Anaerobic fermentation was performed in a 6L glass reactor (Bellco glass, Vineland, NJ). The fermenter was operated in sequencing batch reactor (SBR) mode, 3L of fermentate was pumped out and 3L of fresh feed was added to the reactor once every day. The temperature in the reactor was maintained at 37°C using heating tapes (BriskHeat, Columbus, OH). Mixing (relative centrifugal force, 8xG) was provided using magnetic stir plates. The feed was a 1:1 COD based mixture of primary sludge (PS) and food waste (FW). PS was obtained monthly from Loudoun Water Broad Run Water Reclamation Facility and stored at 4°C. The food waste was collected from a canteen at Columbia University and was blended in a mixer and suitably diluted using de-ionized (DI) water. The feed composition was kept constant and the organic loading rate was 25 kg-COD/m³/d. The hydraulic retention time (HRT) values tested were 2, 4 and 6 days. The pH of the reactor was controlled through automated addition of 1:1 1M NaHCO₃ and 1M NaOH solutions and the pH values tested were 7, 7.5, 8, and 9.

4.3.2 Polyhydroxyalkanoates (PHA) Accumulation Reactor

Enrichment of PHA accumulating culture was performed in a 6L glass reactor (Bellco glass, Vineland, NJ), using a working volume of 4L. The HRT was 1 day and the solids retention time (SRT) was 2.1 ± 0.2 days. The enrichment reactor was operated in SBR mode, at a 12-hour cycle applying feast and famine conditions (decant 1L, settling, decant 1L, feeding 2L, anaerobic zone, reaction). The organic loading rate was 3,000 mg-sCOD/L/day. The temperature in the reactor was maintained at 25°C using heating tapes (BriskHeat, Columbus, OH). The pH of the

reactor was set at 7.0 ± 0.2 through automated addition of 1:1 1M NaHCO₃ and 1M NaOH solutions, and 2N HCl solution. Mixing was provided using magnetic stir plates. Air supply was set at 1vvm.

4.3.3 Volatile Fatty Acids (VFA) and Total Carboxylic Acids (TCA) Quantification

Fermentate sample was withdrawn two or three times a week using the syringe sampling port assembly. The fermentate was centrifuged (10,000 rpm, 9133 x g, 10 min 4°C) and the supernatant was filtered through a 0.22 µm syringe filter and then appropriately diluted for soluble COD, and total carboxylic acids (TCA). The chemical parameters including the total and soluble COD (tCOD and sCOD) and TCA were determined using colorimetry (Hach kits 2125915 and 2244700, Loveland, CO). The VFA composition in the fermentate was determined using ion chromatography (Dionex ICS-2100) using a Dionex IonPac™ column (AS11-HC-4µm, RFIC™ & HPIC™ 2 X 250 mm). The analytical method of IC was Anion method 2 to 27mM (20mA), and IC processing method was 2mM to 10mM (20mA). (Manning and Bewsher 1997)

4.3.4 PHA Quantification

Review of several methods of quantification, revealed a preference for Gas Chromatographic analysis of esterified samples collected from the reactor over other methods including ion-exchange chromatography (Hesselmann et al. 1999) and high-performance liquid chromatography (Giin-Yu Amy Tan et al. 2014) (Andreas Grubelnik et al. 2008). The protocol was adapted from Johnson et al. (2009), Brandl H, et al. (1988) and Timm and Steinbüchel (1990). (More details in Appendix section 6.1) The methyl esters were assayed using gas

chromatography coupled with flame ionization detector (GC-FID, SRI 8610C) and MXT-Wax column (30 m 0.53 mmID 0.5 μ mdf) (Restek, Bellfonte, PA). Pure Sigma poly[(R)-3-hydroxybutyric acid] (PHB) and poly(3-hydroxybutyric-co-3-hydroxyvaleric acid) (PHBV, 12% mol HV) with weights between 1 and 10 mg were esterified and analyzed along with reactor samples to be used as reference standards. (detailed protocol of PHA quantification is included in Appendix)

4.3.5 DNA Extraction, Library Preparation and Next-Generation Sequencing

DNA was extracted manually according to the manufacturer's protocol (Qiagen). The quantity and purity of DNA were evaluated using NanoDrop™ (Thermo Fisher). PCR (Bio-Rad) for 16S rRNA sequencing was conducted by modifying protocols from previous studies (de Almeida Fernandes et al. 2018) (Nehmé et al. 2009) using the following primers: (i) for Bacteria: EUB1055F (5'-ATGGCTGTCGTCAGCT-3'), and EUB1392R (5'-ACGGGCGGTGTGTAC-3') with experimentally determined optimal melting temperature 57°C, and (ii) for Archaea: ARC787F (5'-ATTAGATACCCSBGTAGTCC-3'), and ARC1059R (5'-GCCATGCACCWCTCT-3') with experimentally determined optimal melting temperature 56.4°C, and was normalized to 100 pM using the Bioanalyzer (Agilent). Quantitative analysis of bacteria and archaea was performed using qPCR (Bio-Rad) with SYBR® Green I fluorescent detection method, where one sample was used for each HRT/pH condition in the fermentation reactor, and one sample for each month of operation of the PHA reactor. DNA templates were prepared using Ion PGM Hi-Q View OT2 Kit (Thermo Fisher) and were loaded onto the 318-chip in the PGM sequencer according to manufacturer's protocol (Thermo Fisher). Raw sequencing data were stored at Ion Torrent Server (Thermo Fisher) and were downloaded as bam

files and trimmed based on the amplicon size (300-400 bp for bacteria, 200-300bp for archaea) and quality score using default values in Galaxy. Also, the chimera reads were checked using Geneious (version 11.0.4). Geneious (version 11.0.4) was also used for microbial identification and quantification at phylum, class, order, family and genus level, while NCBI Genome Database was used for species level identification using the highest identification score and ID>99%. Ion Reporter (Thermo Fisher) was used for biodiversity analyses (alpha and beta diversity) (Magurran, A. E 2004) (Whittaker 1960). Alpha diversity showed how many different types (such as families) there were in the microbial community. Shannon index accounted for both number (richness) and relative abundance (dominance or evenness) of the different types of families present. Typical values are generally between 1.5 and 3.5. The Shannon index increases as both the richness and evenness of the community increase. (Magurran, A. E 2004). Beta diversity was introduced by Whittaker in 1960. He defined beta diversity as “the extent of change in community composition, or degree of community differentiation, in relation to a complex-gradient of environment, or a pattern of environments” (Whittaker 1960).

4.4 Results & Discussion

4.4.1 FERMENTATION REACTOR

4.4.1.1 Microorganisms at Family Level in Fermentation Reactor

Table 4- 1 and Figure 4- 4 show the bacterial families in the fermentation reactor that were identified with over 90% confidence level, based on 16s rRNA sequencing.

- At fermentation conditions HRT 2 days – pH 7 the most abundant bacterial families belonged to the phylum of Firmicutes (*Veillonellaceae*: 48.7% ± 30.2%, *Clostridiales Incertae Sedis XI*: 19.3% ± 15.4%), and Bacteroidetes (*Porphyromonadaceae*: 11.5% ± 6.4%).
- At HRT 2 days – pH 8 the most abundant bacterial families belonged to the phylum of Proteobacteria (*Enterobacteriaceae*: 46.7% ± 6.4%), Bacteroidetes (*Porphyromonadaceae*: 23.5% ± 3.5%), and Firmicutes (*Clostridiales Incertae Sedis XI*: 20.8% ± 7.4%).
- At HRT 2 days – pH 9 the most abundant bacterial families belonged to the phylum of Firmicutes (*Clostridiales Incertae Sedis XI*: 26.4% ± 16.6%, *Bacillaceae*: 25.8% ± 16.9%, *Enterococcaceae*: 20.8% ± 15.5%), Proteobacteria (*Rhodobacteraceae*: 15.6% ± 13.5%), and Bacteroidetes (*Porphyromonadaceae*: 12.7% ± 2.5%).
- At HRT 4 days – pH 9 the most abundant bacterial families belonged to the phylum of Firmicutes (*Enterococcaceae*: 32.5% ± 21.3%, *Clostridiales Incertae Sedis XI*: 23.0% ± 3.6%), Bacteroidetes (*Porphyromonadaceae*: 12.0% ± 2.8%), and Proteobacteria (*Rhodobacteraceae*: 9.7% ± 5.7%).
- At HRT 6 days – pH 9 the most abundant bacterial families belonged to the phylum of Firmicutes (*Enterococcaceae*: 44.3% ± 13.0%, *Clostridiales Incertae Sedis XI*: 16.5% ± 5.3%), and Proteobacteria (*Rhodobacteraceae*: 10.3% ± 3.5%).

Most of the families present in the fermentation reactor belonged to the phylum of Firmicutes, Bacteroidetes and Proteobacteria. These phyla play a major role in the two main processes taking place in anaerobic digestion; hydrolysis and acidogenesis. Hydrolytic bacteria are diverse, but the most known species are Firmicutes and Bacteroidetes. They grow rapidly, can withstand changes in pH and temperature and break down polysaccharides, lipids, and proteins to their monomers using amylase, cellulase, lipase and other extracellular enzymes. The

most common acidogenic bacteria are Bacteroidetes, Firmicute, Proteobacteria and Chloroflexi. They convert the hydrolysis products to VFAs, alcohols, carbon dioxide and hydrogen. (Venkiteshwaran et al. 2015)

Several studies on the microbial ecology of anaerobic digestors using wastewater and/or food waste have identified microorganisms of the phylum of Firmicutes, Bacteroidetes, and Proteobacteria as they are involved in hydrolysis and acetogenesis. (Jabari et al. 2016) (Y. Tang et al. 2005) (L. Li et al. 2016) (Shin et al. 2010) Another study, by Guo et al. 2015 on microbial community structure of anaerobic reactor digesting activated sludge from wastewater also identified Proteobacteria, Firmicutes and Bacteroidetes as the main species, accounting for 41.2%, 12.5% and 9.6% of the total Bacteria, respectively. Aphaeobacteria were the most abundant class of Proteobacteria (36.4%). Clostridia and Bacilli were the most abundant classes of Firmicutes; 72.5% and 22.6% respectively, and *Streptococcus* and *Halothermothrix* were the most abundant genera of Firmicutes. Bacteroidia, Cytophagia, Flavobacteriia and Shingobacteriia were the most abundant classes of Bacteroidetes, with Bacteroidia being significantly higher than the rest of the classes. *Bacteroidaceae* was the most abundant family belonging to the phylum of Bacteroidetes. Overall it was concluded from the study by Guo et al. that the order of Halanaerobiales (mainly the genus *Halothermothrix*) were responsible for the hydrolysis process, and the Clostridia class and the *Bacteroidaceae* family were responsible for the acidogenic process and their results suggest that *Clostridium*, *Treponema*, *Eubacterium*, *Thermoanaerobacter* and *Moorella* were the dominant species (J. Guo et al. 2015)

Comparing the microbial ecology throughout the different pH and HRT conditions, based on the results shown in Table 4- 1/Figure 4- 4, we can observe that *Clostridiales Incertae Sedis XI* was present at all conditions examined, *Porphyromonadaceae* was present at HRT 2 – pH 7, 8

and 9 and HRT 4 – pH 9, *Enterococcaceae* was present only at pH 9 and its abundance increased with increased HRT (2, 4, and 6 days). *Rhodobacteraceae* was also present only at pH 9 at HRTs 2, 4, and 6 days. While *Veillonellaceae*, *Enterobacteriaceae* and *Bacillaceae* were only present in one condition each; at HRT 2 and pH 7, 8, and 9 respectively. It should be noted that at conditions HRT 2 days – pH 7, HRT 4 days – pH 9, and HRT 6 days – pH 9 there were more families that account for less than 5% of total bacteria. These species were considered having a non-significant effect in the system because of their small abundance, thus they are not represented in Table 4- 1/ Figure 4- 4. It should be noted that the findings are based on 16s rRNA sequencing with a confidence level greater than 90%, which means that the results of bacterial families presented were created by identifying the 16s gene sequences of these microorganisms with 90% or more DNA sequence similarity, leaving some room for uncertainty. Also, at all conditions of pH and HRT, Bacteria accounted for more than 99.999% (Table 4- 9), hence Archaea analysis was omitted.

Table 4- 1: Abundance of bacterial families in fermentation reactor, across different reactor's operational conditions (HRT, pH).

Bacterial Families	HRT 2 days	HRT 2 days	HRT 2 days	HRT 4 days	HRT 6 days
	pH 7 (n=6)	pH 8 (n=6)	pH 9 (n=8)	pH 9 (n=4)	pH 9 (n=4)
<i>Acidaminococcaceae</i>	8.3% ±				
	4.2%				
<i>Bacillaceae 2</i>			25.8% ±		
			16.9%		
<i>Carnobacteriaceae</i>					6.0% ±
					1.4%
<i>Christensenellaceae</i>		14.5% ±			
		3.5%			
<i>Clostridiales Incertae</i>	19.3% ±	20.8% ±	26.4% ±	23.0% ±	16.5% ±
<i>Sedis XI</i>	15.4%	7.4%	16.6%	3.6%	5.3%
<i>Enterobacteriaceae</i>		46.7% ±			
		6.4%			
<i>Enterococcaceae</i>			20.8% ±	32.5% ±	44.3% ±
			15.5%	21.3%	13.0%
<i>Porphyromonadaceae</i>	11.5% ±	23.5% ±	12.7% ±	12.0% ±	
	6.4%	3.5%	2.5%	2.8%	
<i>Rhizobiaceae</i>			5.0% ±		
			0.0%		
<i>Rhodobacteraceae</i>			15.6% ±	9.7% ±	10.3% ±
			13.8%	5.7%	3.5%
<i>Veillonellaceae</i>	48.7% ±				

The table does not show data of abundance < 5%.

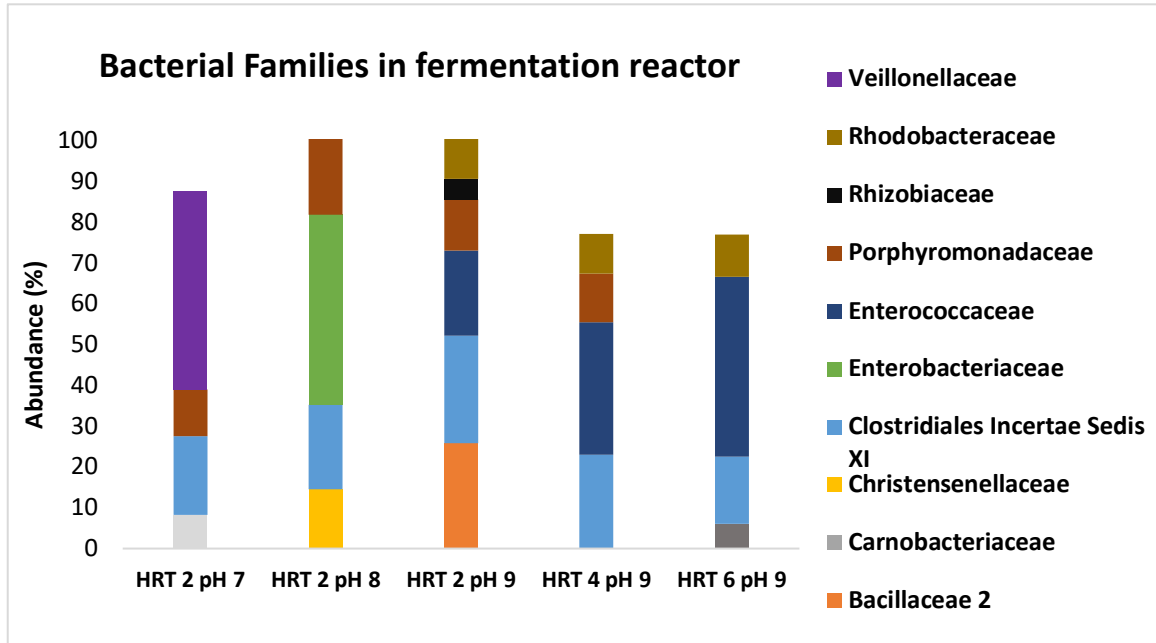


Figure 4- 4: Bacterial families in fermentation reactor, across different reactor's operational conditions (HRT in days, pH).

4.4.1.2 Biodiversity of Microorganisms at Family Level in Fermentation Reactor (alpha and beta diversity)

Figure 4- 5 shows that there was no specific trend of alpha diversity at the family level among the different fermentation conditions (HRT, pH), which means that the abundance and number of different families did not change in a specific trend when the pH increased neither when the HRT increased in the system. Data analysis showed that using different samples belonging in the same condition did not result in different trend, so each line represents one sample in the condition stated above for simplicity.

Beta diversity statistical analysis at the family level (Figure 4- 11, Appendix) showed that the community composition between the samples from different conditions (HRT, pH) was different, except for the conditions of HRT 4 days – pH 9 and HRT 6 days – pH 9. This can be supported by the results shown in Figure 4- 4. Samples at the conditions of HRT 4 days – pH 9 and HRT 6 days – pH 9 have the following families in common: *Enterococcaceae*, *Clostridiales Incertae Sedis XI*, and *Rhodobacteraceae*, and about the same amount of families that account for less than 5% abundance each, while the samples of the rest of the conditions differ more in composition.

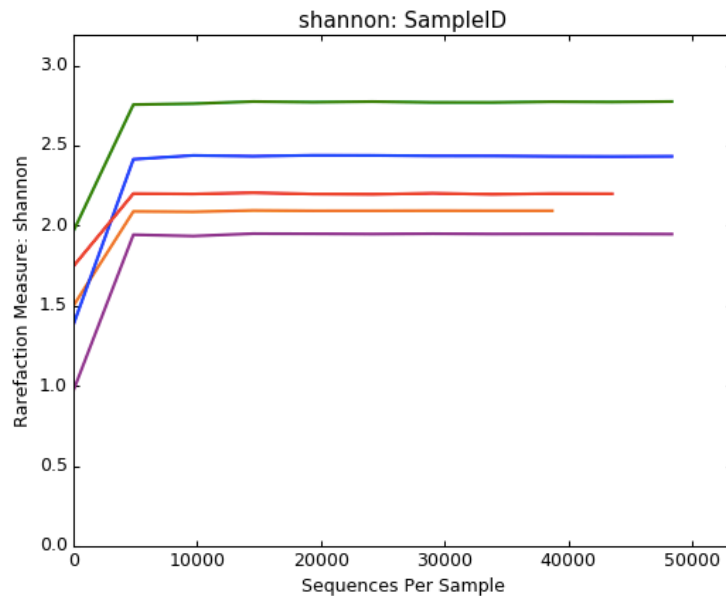


Figure 4- 5: Alpha diversity of microorganisms in fermentation reactor at family level.

Lines from top to bottom represent the conditions in the following order: HRT 2 days - pH 7 (green), HRT 4 days - pH 9 (blue), HRT 2 days - pH 9 (red), HRT 2 days - pH 8 (orange), HRT 6 days - pH 9 (purple).

4.4.1.3 Microorganisms at Species Level in Fermentation Reactor

Table 4- 2 shows the most dominant species that they appear at each of the fermentation conditions tested. It should be clarified that the dominant species were identified using 16s rRNA sequencing and the NCBI Genome database by selecting the highest identity score (95-100%), which again leaves some room for uncertainty.

- At HRT 2 days – pH 7: *Mitsuokella jalaludinii*, *Petrimonas mucosa*, *Sporanaerobacter acetigenes*, and *Tepidiphilus margaritifer*.
- At HRT 2 days – pH 8: *Christensenella minuta*, *Escherichia marmotae*, *Petrimonas mucosa*, *Salmonella bongori*, and *Sporanaerobacter acetigenes*.
- At HRT 2 days – pH 9: *Amphibacillus xylanus*, *Enterococcus saigonensis*, *Paracoccus aestuariivivens*, *Proteiniphilum saccharofermentans*, *Tepidimicrobium ferriphilum*, and *Tepidimicrobium xylanilyticum*.
- At HRT 4 days – pH 9: *Tepidimicrobium ferriphilum* and *Vagococcus lutrae*.
- At HRT 6 days – pH 9: *Tepidimicrobium ferriphilum*, *Vagococcus acidifermentans* and *Vagococcus lutrae*.

Also, it can be observed that some species were present only at one condition while others at a range of conditions of HRT and pH. More specifically, results showed that *Mitsuokella jalaludinii* and *Tepidiphilus margaritifer* were only present at HRT 2 days – pH 7, *Christensenella minuta*, *Escherichia marmotae*, *Petrimonas sulfuriphila*, and *Salmonella bongori* were only present at HRT 2 days – pH 8, *Amphibacillus xylanus*, *Enterococcus saigonensis*, *Paracoccus aestuariivivens*, *Proteiniphilum saccharofermentans* and *Tepidimicrobium xylanilyticum* were only present at HRT 2 days – pH 9 and *Vagococcus acidifermentans* only at HRT 6 days – pH 9. While, *Petrimonas mucosa* and *Sporanaerobacter*

acetigenes were present at both HRT 2 days – pH 7 and HRT 2 days – pH 8, *Vagococcus lutrae* was present at both HRT 4 – pH 9 and HRT 6 days – pH 9, and *Tepidimicrobium ferriphilum* at all three conditions of HRT 2 days – pH 9, HRT 4 days – pH 9, and HRT 6 days – pH 9.

Table 4- 2: Dominant species of microorganisms at each fermentation condition (table continues at next page)

Species	HRT 2 days pH 7	HRT 2 days pH 8	HRT 2 days pH 9	HRT 4 days pH 9	HRT 6 days pH 9
<i>Amphibacillus xylanus</i>			X		
<i>Christensenella minuta</i>		X			
<i>Enterococcus saigonensis</i>			X		
<i>Escherichia marmotae</i>		X			
<i>Mitsuokella jalaludinii</i>	X				
<i>Paracoccus aestuariivivens</i>			X		
<i>Petrimonas mucosa</i>	X	X			
<i>Petrimonas sulfuriphila</i>		X			
<i>Proteiniphilum saccharofermentans</i>			X		
<i>Salmonella bongori</i>		X			
<i>Sporanaerobacter acetigenes</i>	X	X			
<i>Tepidimicrobium ferriphilum</i>			X	X	X
<i>Tepidimicrobium</i>			X		

xylanilyticum

<i>Tepidiphilus margaritifer</i>	X	
<i>Vagococcus acidifermentans</i>		X
<i>Vagococcus lutrae</i>	X	X

Table 4- 3 shows the description of characteristics of all species found in fermentation reactor; oxygen requirements, morphology, gram-stain, motility, sporulation, optimum temperature and pH for cell growth, and fermentation end products. Table 4- 4 shows the concentrations of total carboxylic acid (TCA) and volatile fatty acids (VFA) and VFA speciation for each fermentation condition along with the dominant microorganisms present. Based on the information of these two tables we made the following observation:

- At HRT 2 days – pH 7 the average TCA concentration was 4,900 mg-COD/L, the average VFA concentration was 4,300 mg-COD/L and the most abundant VFA species were 45% ± 4% acetic acid and 19% ± 2% formic acid. (Table 4- 4) The dominance of acetic acid could be explained by the dominant microbial species and their metabolic end products presented in Table 4- 3. The dominant species were: *Mitsuokella jalaludinii*, *Petrimonas mucosa*, *Sporanaerobacter acetigenes*, and *Tepidiphilus margaritifer*, and their main end products are acetic, propionic and succinic acid (Table 4- 3).
- At HRT 2 days – pH 8 the average TCA concentration was 5,300 mg-COD/L, the average VFA concentration was 3,800 mg-COD/L and the most abundant VFA species were 43% ± 8% acetic acid and 16% ± 3.1% formic acid. (Table 4- 4) The dominance of acetic and formic acid could be explained by the dominant microbial species; *Christensenella minuta*, *Escherichia marmotae*, *Petrimonas mucosa*, *Salmonella bongori*, and *Sporanaerobacter*

acetigenes. The main fermentation products of these microorganisms are acetic, butyric, formic, propionic and succinic acid (Table 4- 3).

- At HRT 2 days – pH 9 the average TCA concentration was 4,600 mg-COD/L, the average VFA concentration was 4,100 mg-COD/L and the most abundant VFA species were 69% ± 14% propionic acid, 10% ± 5.2% formic acid and 9.3% ± 4.7% acetic acid. (Table 4- 4) The dominance of these VFA species could be explained by the presence of the dominant microbial species and their metabolism; the dominant microbial species of this conditions were: *Amphibacillus xylanus*, *Enterococcus saigonensis*, *Paracoccus aestuariivivens*, *Proteiniphilum saccharofermentans*, *Tepidimicrobium ferriphilum*, and *Tepidimicrobium xylanilyticum*, and their main fermentation products are acetic, butyric, formic and propionic acid. However, *Paracoccus aestuariivivens* assimilates acetic acid, so at this condition the system is under two counteracting activities (producing and consuming acetic acid). The lower concentrations of TCA and VFA and the lower percentage of acetic acid compared to the previous conditions could be explain by the presence of *Paracoccus aestuariivivens* that consumes acetic acid (Park et al. 2016). (Table 4- 3)
- At HRT 4 days – pH 9 the average TCA concentration was 5,700 mg-COD/L, the average VFA concentration was 5,200 mg-COD/L and the most abundant VFA species were 75% ± 11% acetic acid and 16% ± 1.8% propionic acid. (Table 4- 4) The dominance of acetic acid could be explained by the presence of the following microbial species in this condition; *Tepidimicrobium ferriphilum*, *Vagococcus acidifermentans* and *Vagococcus lutrae*. The main fermentation products of these species are acetic and butyric acid(Wang et al. 2011) (Lawson et al. 1999). (Table 4- 3)

- At HRT 6 days – pH 9 the average TCA concentration was 6,500 mg-COD/L, the average VFA concentration was 6,100 mg-COD/L and the most abundant VFA species were $78\% \pm 4\%$ acetic acid and $16\% \pm 1.4\%$ propionic acid. (Table 4- 4) The dominance of acetic acid could be explained by the dominant microbial species and their metabolisms (Table 4- 3). In this condition the dominant microbial species were: *Tepidimicrobium ferriphilum*, *Vagococcus acidifermentans* and *Vagococcus lutrae*. The main fermentation products of these species are acetic and butyric acid. (Table 4- 3)

By connecting results of the dominant microorganisms with the volatile fatty acids produced in the fermentation process, we can make two main observations:

- A)** VFA concentration had an increasing trend ($p=0.007$, $\alpha=0.05$) with increased pH and increased HRT, with a drop ($p=0.0005$, $\alpha=0.05$) at the condition HRT 2 days – pH 9.
- B)** At all condition the most abundant acid was acetic acid, except for the condition HRT 2 days – pH 9 where the most abundant acid was propionic acid.

An explanation for that drop and change in VFA speciation ($p=0.04$) could be the shift in the microbial community at this condition and the presence of *Paracoccus aestuariivivens* which consumes acetic acid. The dominant species at this condition were: *Amphibacillus xylanus*, *Enterococcus saigonensis*, *Paracoccus aestuariivivens*, *Proteiniphilum saccharofermentans*, *Tepidimicrobium ferriphilum*, and *Tepidimicrobium xylanilyticum*. All these species, except for *Tepidimicrobium ferriphilum*, were present only at that particular fermentation condition. Although these species produce acetic acid, they also produce other VFA like formic acid, propionic acid and butyric acid, so it is likely that the conditions were favorable towards the

production of other VFA species instead of acetic acid, hence the concentration of acetic acid was lower than the other conditions. More research is needed towards understanding the metabolic pathways of these microorganisms that would reveal more information on the specific reaction taking place and the end products of fermentation. The general trend of increasing concentration of VFA can be attributed to the combination of alkaline fermentation condition along with the increased reaction time (increased HRT), which are conditions favorable for acidogenic fermentation. (Wu et al. 2010), (D. Liu et al. 2008)

Table 4- 3: Description of species of microorganisms in fermentation reactor.

(table continues at the next three pages)

Species	O ₂ requirement	Morphology	Gram stain	Motility	Citation
<i>Amphibacillus xylandus</i>	facultative anaerobe	rods	positive	motile	(Niimura, Y. et al. 1990)
<i>Christensenella minuta</i>	strictly anaerobe	rods	negative	non-motile	(Morotomi et al. 2012)
<i>Enterococcus saigonensis</i>	facultative anaerobe	spherical/ ovoid cocci	positive	non-motile	(Harada et al. 2016) (Ramsey et al. 2014)
<i>Escherichia marmotae</i>	facultative anaerobe	rods	negative	non-motile	(S. Liu et al. 2015)

					(Clark 1989)
<i>Mitsuokella jalaludinii</i>	strictly anaerobe	rods	negative	non-motile	(Lan et al. 2002)
<i>Paracoccus aestuariivivens</i>	strictly anaerobe	coccoid/rod	negative	non-motile	(Park et al. 2016)
<i>Petrimonas mucosa</i>	facultative anaerobe	rods	negative	non-motile	(Hahnke et al. 2016)
<i>Petrimonas sulfuriphila</i>	facultative anaerobe	rods	negative	non-motile	(Grabowski et al. 2005)
<i>Proteiniphilum saccharofermentans</i>	facultative anaerobe	rods	negative	motile	(Hahnke et al. 2016)
<i>Salmonella bongori</i>	facultative anaerobe	rods	negative	motile	(Fookes et al. 2011)
<i>Sporanaerobacter acetigenes</i>	strictly anaerobe	rods	positive	motile	(Hernandez-Eugenio et al. 2002)
<i>Tepidimicrobium ferriphilum</i>	strictly anaerobe	rods	positive	motile	(Slobodkin et al. 2006)
<i>Tepidimicrobium xylanilyticum</i>	strictly anaerobe	rods	positive	motile	(Niu et al. 2009)
<i>Tepidiphilus margaritifera</i>	facultative anaerobe	rods	negative	motile	(Manaia et al. 2003)
<i>Vagococcus</i>	facultative	coccus	positive	non-	(Wang et

<i>acidifermentans</i>	anaerobe			motile	al. 2011)
<i>Vagococcus</i>	Facultative				(Lawson et
		coccus	positive	motile	
			Optimum		
Species	Motility	Sporulation	Temp & pH for growth	Fermentation end products	Citation
<i>Amphibacillus xylanus</i>	motile	spore-forming	32°C pH 8-10	FA, AA, ethanol	(Niimura, Y. et al. 1990)
<i>Christensenella minuta</i>	non-motile	non-spore-forming	37-40°C pH 6-9	AA BA	(Morotomi et al. 2012)
<i>Enterococcus saigonensis</i>	non-motile	non-spore-forming	37°C pH 8	AA, FA, ethanol	(Harada et al. 2016) (Ramsey et al. 2014)
<i>Escherichia marmotae</i>	non-motile	non-spore-forming	37°C pH 7	AA, FA, Ethanol, SA	(S. Liu et al. 2015) (Clark 1989)
<i>Mitsuokella jalaludinii</i>	non-motile	non-spore-forming	42°C pH 7	AA, SA, Lactic acid	(Lan et al. 2002)
<i>Paracoccus aestuariivivens</i>	non-motile	non-spore-forming	30°C pH 6.5-7.5	N/A (*)	(Park et al. 2016)
<i>Petrimonas</i>	non-	non-spore-	37-40°C	AA, PA, CO ₂	(Hahnke et

<i>mucosa</i>	motile	forming	pH 7		al. 2016)
<i>Petrimonas sulfuriphila</i>	non-motile	non-spore-forming	37-40°C pH 7	AA, Lactic acid, H ₂	(Grabowski et al. 2005)
<i>Proteiniphilum saccharofermentans</i>	motile	non-spore-forming	35-40°C pH 7.5	AA, PA, CO ₂ , H ₂	(Hahnke et al. 2016)
<i>Salmonella bongori</i>	motile	non-spore-forming	35-37°C pH 7	H ₂ S Other gases	(Fookes et al. 2011)
<i>Sporanaerobacter acetigenes</i>	motile	spore-forming	40°C pH 7.5	AA (glucose) BA (peptone) VA (peptone)	(Hernandez -Eugenio et al. 2002)
<i>Tepidimicrobium ferriphilum</i>	motile	non-spore-forming	50°C pH 7.5-8	AA, BA, Ethanol CO ₂ , H ₂	(Slobodkin et al. 2006)
<i>Tepidimicrobium xylanilyticum</i>	motile	spore-forming	60°C pH 8.5	AA, BA, Ethanol CO ₂ , H ₂	(Niu et al. 2009)
<i>Tepidiphilus margaritifera</i>	motile	spore-forming	50°C pH 7	N/A	(Manaia et al. 2003)
<i>Vagococcus acidifermentans</i>	non-motile	non-spore-forming	37°C pH 8-9	Lactic acid, AA CO ₂ , ethanol	(Wang et al. 2011)
<i>Vagococcus lutrae</i>	motile	non-spore-forming	37°C pH 8-9	Lactic acid, AA CO ₂ , ethanol	(Lawson et al. 1999)

AA: Acetic acid, PA: Propionic acid, VA: Valeric Acid, SA: Succinic acid, FA: Formic acid, BA: Butyric acid

(* *Paracoccus aestuariivivens* uses L-Arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, D-mannose, sucrose, D-trehalose, D-xylose, **acetate**, citrate, L-malate, pyruvate and **succinate as carbon and energy sources**, but doesn't use maltose, benzoate, formate, L-glutamate and salicin.

Table 4- 4: Average Total Carboxylic Acids (TCA), Volatile Fatty Acids (VFA) and VFA speciation for each fermentation condition along with dominant families and species.

(table continues at the next three pages)

Conditions	Dominant Families	Dominant Species	TCA	VFA	VFA
	(% of bacteria) (*)	(**)	(***) (mg-COD/L)	(***) (mg-COD/L)	speciation (***)
HRT 2 days	<i>Veillonellaceae</i> (48.7% ± 30.2%)	<i>Mitsuokella jalaludinii</i>	4,900 ± 800 (n=25)	4,300 ± 700 (n=25)	AA 45 ± 4% ,
	<i>Clostridiales Incertae Sedis XI</i> (19.3% ± 15.4%)	<i>Petrimonas mucosa</i>			PA 2.4 ± 1%
pH 7	<i>Porphyromonadaceae</i> (11.5% ± 6.4%)	<i>Tepidiphilus margaritifera</i>			FA 19 ± 2%, BA 4.4 ± 3%

					VA 7.1 ±
					1%,
					other 22
					± 15%
					(n=4)
					AA 43 ±
					8%,
		<i>Christensenella</i>			PA 3.1 ±
		<i>minuta</i>			1.6%,
	<i>Enterobacteriaceae</i>	<i>Escherichia</i>			FA 16 ±
	(46.7% ± 6.4%)	<i>marmotae</i>			3.1%,
HRT 2	<i>Porphyromonadaceae</i>	<i>Petrimonas mucosa</i>	5,300 ±	3,800 ±	BA 3.9 ±
days	(23.5% ± 3.5%)	<i>Petrimonas</i>	600	400	0.3%,
pH 8	<i>Clostridiales Incertae</i>	<i>sulfuriphila</i>	(n=10)	(n=10)	VA 5.2 ±
	<i>Sedis XI</i> (20.8% ±	<i>Salmonella bongori</i>			0.9%,
	7.4%)	<i>Sporanaerobacter</i>			other 29
		<i>acetigenes</i>			± 14%
					(n=2)
	<i>Clostridiales Incertae</i>	<i>Amphibacillus</i>			AA 9.3 ±
HRT 2	<i>Sedis XI</i> (26.4% ±	<i>xylanus</i>	4,600 ±	4,100 ±	4.7%,
days	16.6%)	<i>Enterococcus</i>	800	700	PA 69 ±
pH 9	<i>Bacillaceae</i> (25.8% ±	<i>saigonensis</i>	(n=18)	(n=18)	14%,
	16.9%)	<i>Paracoccus</i>			FA 10 ±

<i>Enterococcaceae</i>	<i>aestuariivivens</i>	5.2%,
(20.8% ± 15.5%)	<i>Proteiniphilum</i>	BA 1.4 ±
<i>Rhodobacteraceae</i>	<i>saccharofermentans</i>	0.5%,
(15.6% ± 13.5%)	<i>Tepidimicrobium</i>	VA 1.3 ±
<i>Porphyromonadaceae</i>	<i>ferriphilum</i>	0.7%,
(12.7% ± 2.5%)	<i>Tepidimicrobium</i>	other
	<i>xylanilyticum</i>	9.4±9%
		(n=5)

	<i>Enterococcaceae</i>				AA 75 ±
	(32.5% ± 21.3%)				11%,
	<i>Clostridiales Incertae</i>				PA 16 ±
HRT 4	<i>Sedis XI</i> (23.0% ±	<i>Tepidimicrobium</i>	5,700 ±	5,200 ±	1.8%,
days	3.6%)	<i>ferriphilum</i>	350	300	FA 0%,
pH 9	<i>Porphyromonadaceae</i>	<i>Vagococcus lutrae</i>	(n=9)	(n=9)	BA 0%,
	(12.0% ± 2.8%)				VA 0%,
	<i>Rhodobacteraceae</i>				other 9 ±
	(9.7% ± 5.7%)				7.2%
					(n=3)
	<i>Enterococcaceae</i>	<i>Tepidimicrobium</i>			AA 78 ±
HRT 6	(44.3% ± 13.0%)	<i>ferriphilum</i>	6,500 ±	6,100 ±	4%,
days	<i>Clostridiales Incertae</i>	<i>Vagococcus</i>	400	400	PA 16 ±
pH 9	<i>Sedis XI</i> (16.5% ±	<i>acidifermentans</i>	(n=10)	(n=10)	1.4%,
	5.3%)	<i>Vagococcus lutrae</i>			FA 0%,

<i>Rhodobacteraceae</i>	BA 0%,
(10.3% ± 3.5%)	VA 0%,
	other 6 ±
	5%
	(n=5)

(*) Rest of the families were <5% of bacteria, average values. (**) Highest identity shown in NCBI Genome database (95-100%). (***) average of all values/samples of each condition HRT - pH at steady state.

Examining the microorganisms in relation to the pH and HRT conditions in our study, it can be observed that some bacterial species were present only at one condition while others at a range of conditions of HRT and pH. More specifically, results showed that:

- *Mitsuokella jalaludinii* and *Tepidiphilus margaritifer* were only present at HRT 2 days – pH 7. Optimum growth conditions for these species are 42°C/pH 7 and 50°C/pH 7 respectively (Table 4- 3).
- *Christensenella minuta*, *Escherichia marmotae*, *Petrimonas sulfuriphila*, and *Salmonella bongori* were only present at HRT 2 days – pH 8. Optimum growth conditions for these species are 37-40°C/pH 6-9, 37°C/pH 7, 37-40°C/pH 7, 35-37°C/pH 7 respectively (Table 4- 3).
- *Amphibacillus xylanus*, *Enterococcus saigonensis*, *Paracoccus aestuariivivens*, *Proteiniphilum saccharofermentans* and *Tepidimicrobium xylanilyticum* were only present at HRT 2 days – pH 9 and *Vagococcus acidifermentans* only at HRT 6 days – pH 9. Optimum

growth conditions for these species are 32°C/pH 8-10, 37°C/pH 8, 30°C/pH 6.5-7.5, 35-40°C/pH 7.5, 60°C/pH 8.5, 37°C/pH 8-9 respectively (Table 4- 3)

- *Petrimonas mucosa* and *Sporanaerobacter acetigenes* were present at both HRT 2 days – pH 7 and HRT 2 days – pH 8. Optimum growth conditions for these species are 37-40°C/pH 7, and 40°C/pH 7.5 respectively (Table 4- 3).
- *Vagococcu lutrea* was present at both HRT 4 – pH 9 and HRT 6 days – pH 9. Optimum growth conditions for that species is 37°C/pH 8-9 (Table 4- 3).
- *Tepidimicrobium ferriphilum* at all three conditions of HRT 2 days – pH 9, HRT 4 days – pH 9, and HRT 6 days – pH 9. Optimum growth conditions for that species is 50°C/pH 7.5-8 (Table 4- 3).

That shows that even though fermentative bacteria have optimum growth conditions, they can survive in a range of conditions which gives researchers the option to use a range of conditions in order to optimize the fermentation process. However, the optimum growth conditions of each one of them should be taken under consideration when it is desired to enrich in certain species in order to control the VFA species produced through the metabolism of the microbial species.

The highest concentration of VFA was 6,100 mg-COD/L at HRT 6 days – pH 9, and the most abundant VFA species were 78% ± 4% acetic acid and 16% ± 1.4% propionic acid. The microbial species in that condition were *Tepidimicrobium xylanilyticum*, *Tepidimicrobium ferriphilum*, *Vagococcus lutrae*, and *Vagococcus acidifermentans*. These species belong to the families of *Clostridiales Incertae Sedis XI* (*Tepidimicrobium xylanilyticum*, *Tepidimicrobium*

ferriphilum) and *Enterococcaceae* (*Vagococcus lutrae*, *Vagococcus acidifermentans*). Thus, it can be concluded that based on our study, the species of the families of *Clostridiales Incertae Sedis XI* and *Enterococcaceae* are preferred species for acidogenic fermentation and that the preferred condition for acidogenic fermentation were HRT 6 days – pH 9. Moreover, it should be noted that the system could have operated in even higher HRT condition (HRT > 6), and that should be taken into account in future research. Methanogens could appear during fermentation as HRT increases; they consume acids to produce methane and belong to the domain of archaea. However, the archaea species were negligible throughout all conditions tested (Table 4- 9 in Appendix). Hence, increasing the HRT further than 6 days could have improved VFA production and the efficiency of downstream processes (conversion to PHA).

4.4.2 POLYHYDROXYALKANOATES (PHA) REACTOR

4.4.2.1 Microorganisms at Family Level in PHA Reactor

Figure 4- 6 and Table 4- 5 show the bacterial families in PHA reactor across the timeline of reactor's operation, starting from the seed sample taken from a bio-P reactor and going all the way along the reactor's operation lifetime sampling every few months in order to monitor the enrichment process. The graph shows the families that accounted for over 5% of total bacteria. We considered families of less than 5% abundance to have a negligible effect on the system, hence that data is not represented in this graph. It can be noticed that the number of families accounting for less than 5% of total bacteria is decreasing with time which is an indication of enrichment. Looking at Figure 4- 6, we can make the following observations:

- The three main bacterial families in the PHA reactor were *Comamonadaceae*, *Moraxellaceae* and *Xanthobacteraceae* and the abundance of the sum of the three families increased over time, on average accounting for $28 \pm 8\%$ during the first semester, $40 \pm 17\%$ during the second semester, and $76 \pm 16\%$ during the last four months.
- The number of different families identified over time decreased, which indicates enrichment in the reactor due to the dynamic pressure of feast and famine cycles applied to the system.

It should be noted that the findings are based on 16s rRNA sequencing with a confidence level greater than 90%, which means that the results of bacterial families presented were created by identifying the 16s gene sequences of these microorganisms with 90% or more similarity, leaving some room for uncertainty. Also, throughout the entire time of reactor's operation, Bacteria accounted for more than 99.999% (Table 4- 10, Appendix), hence Archaea analysis was omitted.

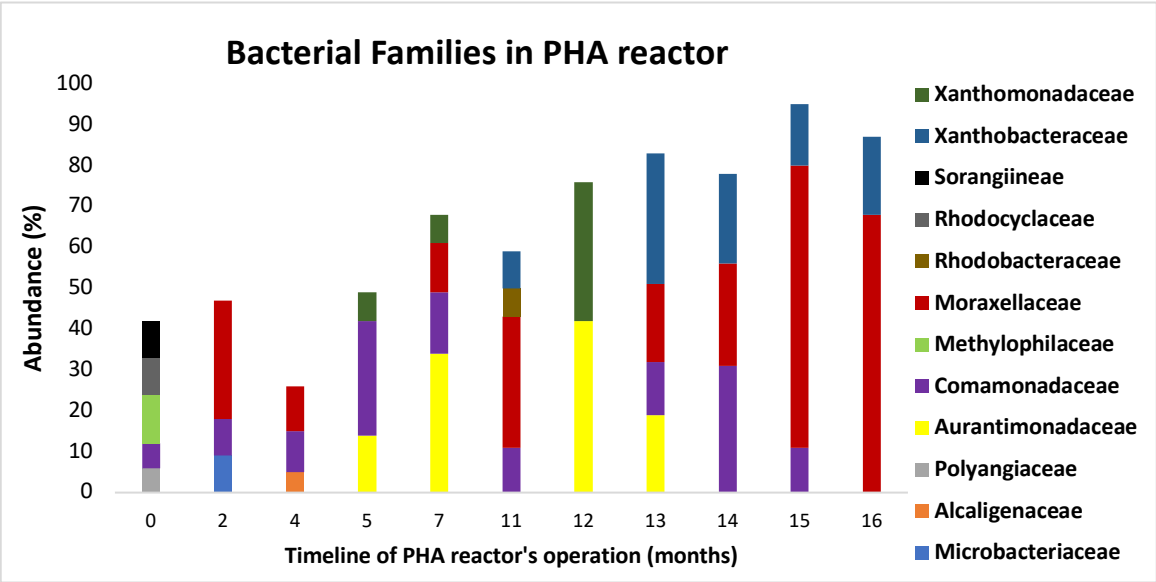


Figure 4- 6: Bacterial families in PHA reactor, across the timeline of reactor's operation.

Table 4- 5: Bacterial families in PHA reactor, across the timeline of reactor's operation.

Bacterial Families	Months of reactor's operation (one sample per month)											
	0	2	4	5	7	11	12	13	14	15	16	
<i>Alcaligenaceae</i>			5									
<i>Aurantimonadaceae</i>		15 *	49 *	1	34		42	19				
<i>Comamonadaceae</i>	6	9	10	2	15	11		13	31	11		
<i>Methylophilaceae</i>	1			8								
<i>Microbacteriaceae</i>	2											
<i>Moraxellaceae</i>		9										
<i>Polyangiaceae</i>		29	11		12	32		19	25	69	68	
<i>Rhodobacteraceae</i>	6											
<i>Rhodocyclaceae</i>						7						
<i>Sorangineae</i>	9											
<i>Xanthobacteraceae</i>							9	32	22	15	19	
<i>Xanthomonadaceae</i>				7	7		34					

The table does not show data of abundance < 5%.

(*) low confidence level 33-43%. Rest of the data had confidence level over 90%

4.4.2.2 Biodiversity of Microorganisms at Family Level in PHA Reactor (alpha and beta diversity)

Figure 4- 7 shows that alpha diversity at family level was getting lower through time, which means that the abundance and number of families decreased with time indicating enrichment of the system.

Beta diversity statistical analysis at the family level (Figure 4- 12, Appendix) showed that the community composition throughout the reactor's lifetime was different, which means it kept changing. That can be attributed to the families that accounted for less than 5% since the rest of the families seemed to repeat among the samples. That gives an unpredictability to the system in terms of efficiency since ideally an enrichment reactor should result in a homogenous community of species that perform a desired function; in this case the ability to accumulate intracellular PHA.

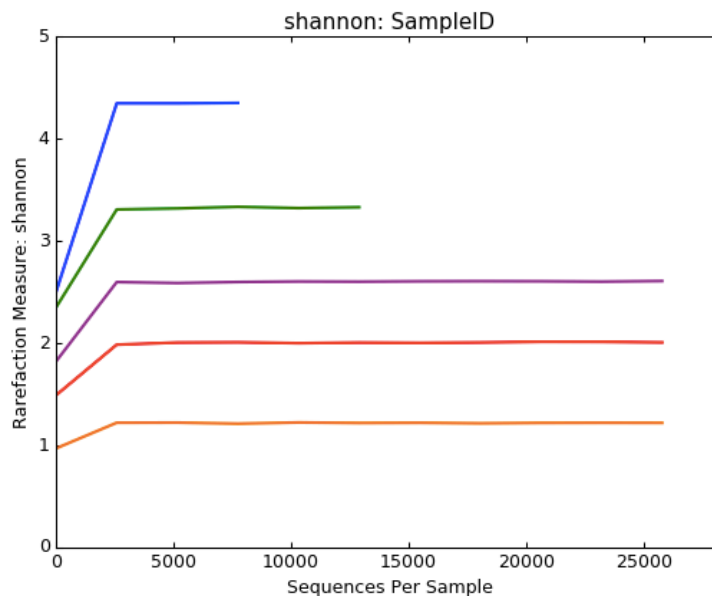


Figure 4- 7: Alpha diversity of microorganisms in PHA reactor at family level. Lines from top to bottom represent samples along the timeline of the reactor’s operation in the following order: seed (blue), 3 months (green), 7 months (purple), 11 months (red), 15 months (orange).

4.4.2.3 Microorganisms at Species Level in PHA Reactor

Table 4- 6 shows the most dominant species that they appeared throughout the timeline of PHA reactor’s operation (symbol “X”). It should be clarified that the dominant species were identified using 16s rRNA sequencing and the NCBI Genome database by selecting the highest identity score (95-100%), which again leaves some room for uncertainty.

it can be observed that some species were present only once while others at a prolonged period. More specifically, *Methylovorus mentalis*, *Acinetobacter vivianii*, *Acinetobacter tandoii*, *Acinetobacter populi* appeared as most abundant species only the first and the last months, while *Acinetobacter radioresistens* appeared during the second semester of reactor’s

operation and during the last two months as well. *Aurantimonas endophytica* was present at the end of first and second semesters. *Comamonas jiangduensis* was identified as dominant species during the middle of the first semester till almost the end of the reactor's operation, and *Pseudoxanthobacter liyangensis* only appeared the last four months.

Table 4- 6: Dominant species of microorganisms along the timeline of PHA reactor's operation.

Bacterial Species	Months of reactor's operation											
	0	2	4	5	7	11	12	13	14	15	16	
<i>Acinetobacter populi</i>									X			
<i>Acinetobacter radioresistens</i>			X		X	X				X		X
<i>Acetivobacter tandoii</i>								X				
<i>Acinetobacter vivianii</i>		X										
<i>Aurantimonas endophytica</i>				X	X		X	X				
<i>Comamonas jiangduensis</i>			X	X	X	X		X		X	X	
<i>Methylovorus mentalis</i>	X											
<i>Pseudoxanthobacter liyangensis</i>								X	X	X	X	
<i>Xanthobacter agilis</i>							X					X

Table 4- 7 shows the description of characteristics of all species found in PHA reactor; oxygen requirements, morphology, gram-stain, motility, sporulation, optimum temperature and pH for cell growth, PHA accumulation capability, and VFA as carbon/energy source.

Methylovorus menthalis does not accumulate PHA and it assimilates methanol. (Doronina, Kaparullina, and Trotsenko 2011) *Acinetobacter vivianii*, *Acinetobacter radioresistens* and *Acinetobacter tandoii* accumulate PHA. *Acinetobacter vivianii* and *Acinetobacter tandoii* use acetic and succinic acids as carbon and energy sources and *Acinetobacter radioresistens* assimilates acetic and succinic acid, fumarate, DL-lactate, L-malate, malonate, hexadecane, heptadecane, octadecane, eicosane, ethanol, n-butanol, L-alanine, L-glutamate, L-leucine, and L-proline. (Y. Li et al. 2015) (Nishimura, Ino, and Iizuka 1988) *Comamonas jiangduensis* accumulates PHB and PHV and assimilates propionic acid, valeric acid, adipic acid, malate, L-proline, itaconic acid, suberic acid, sodium lactic acid and sodium acetate. (Sun et al. 2013) (Yee et al. 2012) *Aurantimonas endophytica* does not accumulate PHA and it assimilates malic acid, mannitol, mannose, L-Serine, L-Glutamic acid, D-Sorbitol, D-Mannitol, D-Arabitol, myo-Inositol, Glycerol, L-Rhamnose, D-Fucose, D-Fructose, Cellobiose, Gentiobiose. It does not assimilate acetic, propionic acids or other VFA. (B.-B. Liu et al. 2016) (A.-Z. Li et al. 2017) *Xanthobacter agilis* accumulates PHB (PHB can constitute up to 600 mg per g dry weight of cells) and assimilates acetic, succinic, citric, lactic, malic, fumaric, gluconic, glutaric, and glutamic acids. (Wiegel 2006) (MALIK and CLAUS 1979) *Pseudoxanthobacter liyangensis* accumulates PHB and it assimilates acetic acid, rhamnose, D-ribose, inositol, glycogen, mannitol, L-glucose, D-glucose, arabinose, D-sorbitol, L-histidine, L-fucose, urea, arginine, malic acid, D-xylose, fructose, mannose, trehalose, N-acetylglucosamine, xylitol, L-proline and D-lyxose. (X.-M. Liu et al. 2014)

From results at Table 4- 6 it can be observed that the two species that do not produce PHA (*Methylovorus menthalis* and *Aurantimonas endophytica*) were either present at the seed sample which was taken from a wastewater treatment bio-P reactor or at samples where other species that did produce PHA were also present (months 5, 7, 12, and 13). This means that the reactor started without any significant amount of PHA accumulators and eventually it became enriched and the most abundant species throughout the entire time of operation of the PHA reactor was capable of assimilating the VFA that were provided by the fermentation reactor, and ultimately produce PHA. Also, it should be highlighted that the ability of microorganisms to assimilate VFA and produce PHA is one important factor, meaning the microorganisms' genes that correspond to the enzymes that take part in PHA synthesis are present in the microorganisms genetic code, but another important factor is the conditions that would enable the expression of those genes for production of the necessary enzymes.

Table 4- 7: Description of species of microorganisms in PHA reactor.

(table continues at the next four pages)

Species	Sporulation	Optimum Temp & pH for growth	PHA accumulator	VFA as Carbon/ Energy source	Citation
<i>Acinetobacter populi</i>	Non-spore forming	10-41°C pH 6-8	Yes (1*)	AA SA	(Y. Li et al. 2015)
<i>Acinetobacter</i>	Non-spore	27-31°C	Yes (1*)	AA	(Nishim

<i>radioresistens</i>	forming	pH 7		SA Ethanol (2*)	uret al. 1988) (Y. Li et al. 2015)
<i>Acetobacter tandoii</i>	Non-spore forming	30°C pH 7	Yes (1*)	AA SA	(Nishim ura, Ino, and Iizuka 1988) (Y. Li et al. 2015)
<i>Acinetobacter vivianii</i>	Non-spore forming	30°C pH 7	Yes (1*)	AA SA	(Nishim ura, Ino, and Iizuka 1988)
<i>Aurantimonas endophytica</i>	Non-spore forming	25°C pH 7	N/A	No (3*)	((B.-B. Liu et al. 2016) (A.-Z. Li et al.

2017)

<i>Comamonas jiangduensis</i>	Non-spore forming	37°C pH 7	Yes PHB PHV PHVB	PA VA (4*)	(Sun et al. 2013) (Yee et al. 2012)
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(Doroni

Methylovorus

menthalis

(seed, from non-PHA accumulating reactor)

Non-spore forming
25°C
pH 8.5-9

N/A

No
(6*)

na,
Kaparullina, and Trotsenko
(2011)

<i>Pseudoxanthobacter liyangensis</i>	Non-spore forming	30-37°C pH 7-8	Yes PHB	AA (5*)	(X.-M. Liu et al. 2014)
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(Wiegel 2006)

Xanthobacter agilis

Non-spore forming
5-34°C
pH 5-8.5

Yes PHB
(7*)

AA
SA
(8*)

(MALIK and CLAUS 1979)

Species	O ₂ requirement	Morphology	Gram stain	Motility	Citation
<i>Acinetobacter populi</i>	Obligate aerobe	rod	negative	Non-motile	(Y. Li et al. 2015)
<i>Acinetobacter radioresistens</i>	Obligate aerobe	rod	negative	Non-motile	(Nishimura et al. 1988)
<i>Acinetobacter tandoii</i>	Obligate aerobe	rod	negative	Non-motile	(Y. Li et al. 2015) (Nishimura, Ino, and Iizuka 1988)
<i>Acinetobacter vivianii</i>	Obligate aerobe	rod	negative	Non-motile	(Y. Li et al. 2015) (Nishimura, Ino, and Iizuka 1988)
<i>Aurantimonas endophytica</i>	Obligate aerobe	rod	negative	motile	((B.-B. Liu et

					al. 2016) (A.-Z. Li et al. 2017) (Sun et al. 2013) (Yee et al. 2012)
<i>Comamonas jiangduensis</i>	Obligate aerobe	rod	negative	motile	(Doroni na, Kaparull ina, and Trotsen ko 2011) (X.-M. Liu et al. 2014) (Wiegel 2006) (MALI
<i>Methylovorus menthalis</i> (seed, from non-PHA accumulating reactor)	Obligate aerobe	rod	negative	motile	
<i>Pseudoxantho- bacter liyangensis</i>	Obligate aerobe	rod	negative	Non- motile	
<i>Xanthobacter agilis</i>	Obligate aerobe	rod	negative	motile	

AA: Acetic acid, PA: Propionic acid, VA: Valeric Acid, SA: Succinic acid

N/A: no findings in the literature.

(1*) PHA, no speciation (e.i. PHB, PHV, etc) specified.

(2*) Assimilates acetate, fumarate, DL-lactate, L-malate, malonate, succinate, hexadecane, heptadecane, octadecane, eicosane, ethanol, n-butanol, L-alanine, L-glutamate, L-leucine, and L-proline.

(3*) Assimilates malic acid, mannitol, mannose, L-Serine, L-Glutamic acid, D-Sorbitol, D-Mannitol, D-Arabitol, myo-Inositol, Glycerol, L-Rhamnose, D-Fucose, D-Fructose, Cellobiose, Gentiobiose. No AA, or PA.

(4*) Assimilates adipic acid, malate, propionic acid, valeric acid, 3-hydroxybutyric acid, Lproline, itaconic acid, suberic acid, sodium lactic acid and sodium acetate.

(5*) Assimilates rhamnose, D-ribose, inositol, acetate, glycogen, mannitol, L-glucose, D-glucose, arabinose, D-sorbitol, L-histidine, L-fucose, urea, arginine, malic acid, D-xylose, fructose, mannose, trehalose, N-acetylglucosa- mine, xylitol, L-proline and D-lyxose.

(6*) Assimilates methanol.

(7*) PHB can constitute up to 600 mg per g dry weight of cells

(8*) Assimilates acetic, citric, lactic, succinic, malic, fumaric, gluconic, glutaric, and glutamic acids

Even though the species that were identified in our reactor were able to assimilate VFA and produce PHA, these species were not identified by any other study, except for species of the family *Comamonadaceae* (Tsuge 2002). Reddy et al. (2003) in their overview on the production of PHA by various bacteria, reported studies that used pure cultures of the following microorganisms: *Alcaligenes eutrophus*, *Bacillus megaterium*, *Methylobacterium rhodesianum*, *Methylobacterium extorquens*, *Pseudomonas aeruginosa*, *Pseudomonas denitrificans*, *Pseudomonas oleovorans*, *Pseudomonas putida*, *Sphaerotilus natans*. The carbon sources were gluconate, propionate, octanoate, glucose, fructose, methanol, and pentanol. Another study by Hafuka et al. (2011) reported the production of PHA from a pure culture of *Cupriavidus necator* using food waste as a carbon source. There are very few studies of the microbial communities of PHA producing systems by mixed microbial cultures (MMC) and even less that are using food waste or wastewater as a carbon sources. Moita and Lemos (2012) in their study used MMC and food waste as carbon source for PHA production. The four species identified were: *Thauera spp.*, *Azoarcus cluster beta*, *Zooglea ramigera* and *Amaricoccus*. Maria G. E. Albuquerque et al. (2013) in their study used MMC and fermented molasses as a carbon source for PHA production. The genera of species identified were: *Azoarcus*, *Thauera* and *Paracoccus*. *Paracoccus* was also identified by Kumar and Kim (2018) in their study of PHA production from glycerol and by Wen et al. (2018) in their study of PHA production from food waste using MMC. That species (*Paracoccus*) possesses several advantages: a) is able to denitrify which is useful for wastewater treatment, utilizing different substrates; to mention a few: methanol, n-pentanol, lignocellulosic hydrolysates, CO₂, b) is both autotrophic and heterotrophic, and c) PHA synthesis does not require necessarily nutrient limitation.

Differences in microbial ecology between different studies can be explained by the effect of the operating conditions applied to the system on the microorganisms as well as the enrichment strategy, along with the methods, parameters and databases used to identify microbial species. Also, it should be taken into account the factor of uncertainty in identification of microorganisms, since the identification is based on the 16S rRNA gene sequencing and a certain confidence level.

4.4.3 Connection Between Microorganisms in PHA Reactor, PHA Accumulation and Fermentation Reactor's Performance

Table 4- 8 shows the PHA accumulation at the PHA reactor along with the most dominant microorganisms present throughout the timeline of PHA reactor's operation, combined with the VFA content and dominant VFA species in fermentation reactor, for each condition (pH, HRT), which is also the influent of PHA reactor.

During the months 4, 5 and 7 the PHA accumulation was $14\% \pm 5\%$ g-PHA/g-VSS, $24\% \pm 6\%$ g-PHA/g-VSS, and $27\% \pm 1\%$ g-PHA/g-VSS respectively and the VFA content in the influent of PHA reactor was $49\% \pm 8\%$ (g-VFA/g-sCOD) with most abundant VFA being acetic acid ($45\% \pm 4\%$). The VFA were produced by the fermentation reactor which was operating at HRT 2 – pH 7 during that period. The PHA reactor species at that period were *Acinetobacter vivianii*, *Acinetobacter radioresistens*, and *Comamonas jiangduensis*. These species assimilate mainly acetic and succinic acid, and less propionic and valeric acids for PHA production (Y. Li et al. 2015) (Nishimura, Ino, and Iizuka 1988) (Sun et al. 2013) (Yee et al. 2012).

During the months 11, 12, 13 and 14 the PHA accumulation was $28\% \pm 0.5\%$ g-PHA/g-VSS, $25\% \pm 3\%$ g-PHA/g-VSS, $28\% \pm 0.5\%$ g-PHA/g-VSS and $27\% \pm 1\%$ g-PHA/g-VSS respectively and the VFA content in the influent of PHA reactor was $45\% \pm 8.8\%$ (g-VFA/g-sCOD) with most abundant VFA being propionic acid ($69\% \pm 14\%$). The VFA were produced by the fermentation reactor which was operating at HRT 2 – pH 9 during that period. The PHA reactor species at the period were *Acinetobacter radioresistens*, *Xanthobacter agilis*, *Pseudoxanthobacter liyangensis*, *Acetobacter tandoii*, *Aurantimonas endophytica*, *Acinetobacter populi*, *Comamonas jiangduensis*. These species assimilate mainly acetic and succinic acids and less propionic and valeric acids for PHA production. Also, should be noted that *Aurantimonas endophytica* does not produce any PHA (Y. Li et al. 2015) (Nishimura, Ino, and Iizuka 1988) (Wiegel 2006) (MALIK and CLAUS 1979) (X.-M. Liu et al. 2014) (B.-B. Liu et al. 2016) (A.-Z. Li et al. 2017) (Sun et al. 2013) (Yee et al. 2012).

At the 15th month the PHA accumulation was $35\% \pm 5\%$ g-PHA/g-VSS, and the VFA content in the influent of PHA reactor was $57\% \pm 4.2\%$ (g-VFA/g-sCOD) with most abundant VFA being acetic acid ($75\% \pm 11\%$). The VFA were produced by the fermentation reactor which was operating at HRT 4 – pH 9 during that period. The PHA reactor species at the period were *Acinetobacter radioresistens*, *Pseudoxanthobacter liyangensis*. *Acinetobacter radioresistens* assimilates acetic and succinic acids. (Y. Li et al. 2015) (Nishimura, Ino, and Iizuka 1988) (X.-M. Liu et al. 2014)

At the 16th month the PHA accumulation was $38\% \pm 0.5\%$ g-PHA/g-VSS, and the VFA content in the influent of PHA reactor was $63\% \pm 7.4\%$ (g-VFA/g-sCOD) with most abundant VFA being acetic acid ($78\% \pm 4\%$). The VFA were produced by the fermentation reactor which was operating at HRT 6 – pH 9 during that period. The PHA reactor species at the period were

Acinetobacter radioresistens, *Pseudoxanthobacter liyangensis*, *Xanthobacter agilis*. *Acinetobacter radioresistens* and *Xanthobacter agilis* assimilate acetic and succinic acids, while *Pseudoxanthobacter liyangensis* only acetic acid. (Y. Li et al. 2015) (Nishimura, Ino, and Iizuka 1988) (X.-M. Liu et al. 2014) (Wiegel 2006) (Malik et al., 1979)

Table 4- 8: Families & species of microorganisms in the PHA reactor, PHA accumulation, VFA content and VFA speciation for each fermentation condition.

(table continues at the next four pages)

Sample # (timeline of reactor's operation)	Dominant Families (% of bacteria) (*)	Dominant Species (**)	VFA as Carbon/ Energy source (Table 4- 7)	PHA Accumula tion (g-PHA/g- VSS)	Fermenter Conditions	VFA content g-VFA/g- sCOD in fermenter effluent (= PHA reactor influent)	Dominant VFA In fermenter effluent (= PHA reactor influent) (***)
Seed (Sample #1)	<i>Methylophilaceae</i> (12%) <i>Rhodocyclaceae</i> (9%) <i>Sorangiiineae</i> (9%) <i>Comamonadaceae</i> (6%) <i>Polyangiaceae</i> (6%)	<i>Methylovorus menthalis</i>	No	N/A	HRT 2 pH 7	49%±8%	AA 45± 4% [PA 2.4± 1%, FA 19± 2%, BA 4.4± 3%, VA 7.1± 1%, other 22±

							15%] (n=4)
2 Months (Sample #2)	Moraxellaceae (29%) <i>Microbacteriaceae</i> (9%) <i>Comamonadaceae</i> (9%)	<i>Acinetobacter vivianii</i>	AA SA	N/A	HRT 2 pH 7	49%±.8%	AA 45± 4% [PA 2.4± 1%, FA 19± 2%, BA 4.4± 3%, VA 7.1± 1%, other 22± 15%] (n=4)
4 Months (Sample #3)	Moraxellaceae (11%) Comamonadaceae (10%) <i>Alcaligenaceae</i> (5%)	<i>Acinetobacter radioresistens</i> <i>Comamonas jiangduensis</i>	AA SA PA VA	14%±5%	HRT 2 pH 7	49%±.8%	AA 45± 4% [PA 2.4± 1%, FA 19± 2%, BA 4.4± 3%, VA 7.1± 1%, other 22± 15%] (n=4)
5 Months (Sample #4)	Comamonadaceae (28%) Aurantimonadaceae	<i>Comamonas jiangduensis</i> <i>Aurantimonas</i>	PA VA	24%±6%	HRT 2 pH 7	49%±.8%	AA 45± 4% [PA 2.4± 1%, FA

	(14%)	<i>endophytica</i>					19± 2%, BA 4.4± 3%, VA 7.1± 1%, other 22± 15%] (n=4)
7 Months (Sample #5)	<i>Aurantimonadaceae</i> (34%)	<i>Aurantimonas</i> <i>endophytica</i>	AA SA PA	27%±5%	HRT 2 pH 7	49%±8%	AA 45± 4% [PA 2.4± 1%, FA 19± 2%, BA 4.4± 3%, VA 7.1± 1%, other 22± 15%] (n=4)
	<i>Comamonadaceae</i> (15%)	<i>Comamonas</i> <i>jiangduensis</i>	VA				
	<i>Moraxellaceae</i> (12%)	<i>Acinetobacter</i> <i>radioresistens</i>					
	<i>Xanthomonadaceae</i> (7%)						
11 Months (Sample #6)	<i>Moraxellaceae</i> (32%) (11%)	<i>Acinetobacter</i> <i>radioresistens</i> <i>Comamonas</i> <i>jiangduensis</i>	AA SA PA VA	28%±0.5%	HRT 2 pH 9	45%±8.8%	PA 69± 14% [AA 9.3± 4.7%, FA 10± 5.2%, BA 1.4± 0.5%, VA 1.3± 0.7%, other 9.4± 9%] (n=5)
	<i>Rhodobacteraceae</i> (7%)						
	<i>Xanthobacteraceae</i> (9%)						

12	<i>Aurantimonadaceae</i>	<i>Aurantimonas</i>	AA	25%±3%	HRT 2	45%±8.8%	PA 69±
Months	(42%)	<i>endophytica</i>	SA				
(Sample							[AA 9.3±
#7)	<i>Xanthomonadaceae</i>	<i>Xanthobacter</i>					4.7%, FA
	(34%)	<i>agilis</i>					10± 5.2%,
							BA 1.4±
							0.5%, VA
							1.3± 0.7%,
							other 9.4±
							9%]
							(n=5)
13	<i>Xanthobacteraceae</i>	<i>Pseudoxantho</i>	AA	28%±0.5%	HRT 2	45%±8.8%	PA 69±
Months	(32%)	<i>-bacter</i>	SA				
(Sample		<i>liyangensis</i>	PA				[AA 9.3±
#8)	<i>Moraxellaceae</i> (19%)		VA				4.7%, FA
		<i>Acetobacter</i>					10± 5.2%,
		<i>tandoii</i>					BA 1.4±
	<i>Aurantimonadaceae</i>						0.5%, VA
	(19%)	<i>Aurantimonas</i>					1.3± 0.7%,
		<i>endophytica</i>					other 9.4±
	<i>Comamonadaceae</i>						9%]
	(13%)	<i>Comamonas</i>					(n=5)
		<i>jiangduensis</i>					
14	<i>Comamonadaceae</i>	<i>Comamonas</i>	PA	27%±1%	HRT 2	45%±8.8%	PA 69±
Months	(31%)	<i>jiangduensis</i>	VA				
(Sample			AA				[AA 9.3±
#9)	<i>Moraxellaceae</i> (25%)	<i>Acinetobacter</i>					4.7%, FA
		<i>populi</i>					10± 5.2%,
							BA 1.4±
	<i>Xanthobacteraceae</i>	<i>Pseudoxantho</i>					0.5%, VA

	(22%)	<i>-bacter</i> <i>liyangensis</i>					1.3± 0.7%, other 9.4± 9%] (n=5)
15	Moraxellaceae (69%)	<i>Acinetobacter</i>	AA	35%±5%	HRT 4	57%±4.2%	AA 75±
Months		<i>radioresistens</i>	SA		pH 9		11%
(Sample	Xanthobacteraceae		PA				[PA 16±
#10)	(15%)	<i>Pseudoxantho</i>	VA				1%,
		<i>-bacter</i>					FA 0%, BA
	Comamonadaceae	<i>liyangensis</i>					0%,
	(11%)						VA 0%,
		<i>Comamonas</i>					other 9±
		<i>jiangduensis</i>					13%] (n=5)
16	Moraxellaceae (68%)	<i>Acinetobacter</i>	AA	38%±0.5%	HRT 6	63%±7.4%	AA 78 ±
Months		<i>radioresistens</i>	SA		pH 9		4%
(Sample							[PA 16 ±
#11)	Xanthobacteraceae	<i>Pseudoxantho</i>					2%, FA
	(19%)	<i>-bacter</i>					0%, BA
		<i>liyangensis</i>					0%,
		<i>Xanthobacter</i>					VA 0%,
		<i>agilis</i>					other 6 ±
							5%] (n=5)

sCOD = soluble COD

(* Rest of the families were <5% of bacteria, (** Highest identity shown in NCBI Genome database, (96-100%). (***) average of all values/samples of each condition.

Figure 4- 8 shows the TCA and VFA concentration at each fermentation condition (HRT, pH) and PHA accumulation:

- At HRT 2 days – pH 7 VFA concentration was $4,300 \pm 720$ mg-COD/L and PHA accumulation was $25\% \pm 2\%$ g-PHA/g-VSS ($85\% \pm 3.0\%$ PHB, $15\% \pm 1.3\%$ PHV).
- At HRT 2 days – pH 7.5 VFA concentration was $3,600 \pm 690$ mg-COD/L and PHA accumulation was $32\% \pm 4\%$ g-PHA/g-VSS ($90\% \pm 4.8\%$ PHB, $10\% \pm 2.1\%$ PHV).
- At HRT 2 days – pH 8 VFA concentration was $3,800 \pm 420$ mg-COD/L and PHA accumulation was $34\% \pm 3\%$ g-PHA/g-VSS ($89\% \pm 2.5\%$ PHB, $11\% \pm 1.9\%$ PHV).
- At HRT 2 days – pH 9 VFA concentration was $4,100 \pm 720$ mg-COD/L and PHA accumulation was $27\% \pm 4\%$ g-PHA/g-VSS ($81\% \pm 15\%$ PHB, $19\% \pm 5.4\%$ PHV).
- At HRT 4 days – pH 9 VFA concentration was $5,200 \pm 660$ mg-COD/L and PHA accumulation was $35\% \pm 5\%$ g-PHA/g-VSS ($96\% \pm 2.0\%$ PHB, $4.0\% \pm 0.5\%$ PHV).
- At HRT 6 days – pH 9 VFA concentration was $6,100 \pm 700$ mg-COD/L and PHA accumulation was $38\% \pm 5\%$ g-PHA/g-VSS ($94\% \pm 5.7\%$ PHB, $6.0\% \pm 0.3\%$ PHV).

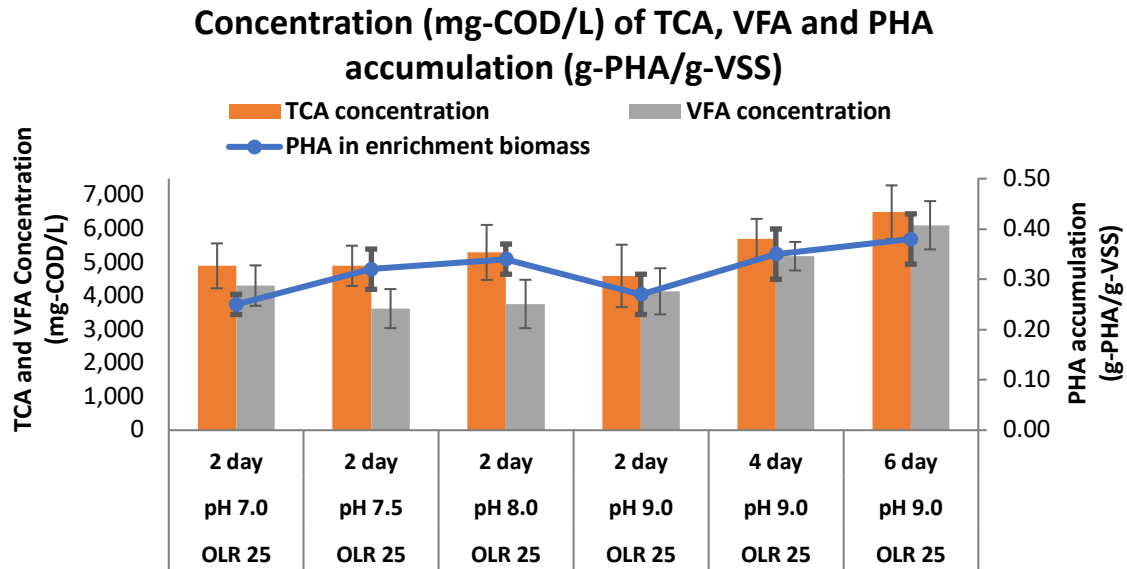


Figure 4- 8: Concentrations of TCA and VFA at different fermentation conditions (HRT and pH) and the corresponding accumulation of Polyhydroxyalkanoates.

The PHA accumulation trend followed the TCA and VFA concentration trend. More specifically, as the VFA concentration and content (% VFA/sCOD) increased, the PHA accumulation increased (Figure 4- 8). Thus, it can be concluded that the PHA accumulation was proportional to the amount of VFA that were introduced into the PHA production system as substrate.

Another factor influencing the PHA accumulation amount and composition is the ability of the microorganisms to assimilate VFA that varies with time depending on the different microbial species and their abundance in each one of them (Table 4- 8). It was observed that the higher the range of VFA species a microorganism was able to assimilate, the higher the PHA accumulation achieved. However, the storage capacity of each identified species was not examined. Hence, it cannot be concluded whether all species have similar storage capacities or

only certain ones produce most of the PHA. Future research focusing on the study of storage capacity would enable researchers to select the reactor's operating condition in favor of the microorganisms with the higher storage capacity.

In addition, as the acetic acid (prevailing acid) abundance in the substrate increased (from $45\% \pm 8.8\%$ to $75\% \pm 11\%$) – between 7th and 15th month in Table 4- 8 – , the PHA accumulation also increased (from $27\% \pm 1\%$ to $35\% \pm 5\%$, g-PHA/g-VSS), while when propionic acid became the most abundant VFA species ($69\% \pm 14\%$) – 14th month in Table 4- 8 – with the total VFA content remaining constant $\sim 47\%$ g-VFA/g-COD, the PHA accumulation did not change ($27\% \pm 1\%$ g-PHA/g-VSS). This means that microorganisms were assimilating both acetic and propionic acids for the production of PHA, and that acetic acid was more preferred substrate than propionic acid, since the higher maximum abundance of propionic acid (PA $69\% \pm 14\%$ compared to AA $45\% \pm 4\%$) did not result in higher PHA accumulation.

4.5 Conclusions

In conclusion, we identified the ecology of acidogenic co-fermentation of primary sludge from wastewater treatment process and food waste, and comparison between different fermentation conditions (pH and HRT) and the ecology of PHA producers, monitoring the shift in microorganisms throughout the enrichment process.

The highest concentration of VFA was 6,100 mg-COD/L at HRT 6 days – pH 9, and the most abundant VFA species were $78\% \pm 4\%$ acetic acid and $16\% \pm 1.4\%$ propionic acid. The microbial species in that condition were *Tepidimicrobium xylanilyticum*, *Tepidimicrobium ferriphilum*, *Vagococcus lutrae*, and *Vagococcus acidifermentans*. These species belong to the

families of *Clostridiales Incertae Sedis XI* (*Tepidimicrobium xylanilyticum*, *Tepidimicrobium ferriphilum*) and *Enterococcaceae* (*Vagococcus lutrae*, *Vagococcus acidifermentans*). Thus, it can be concluded that based on our study, the species of the families of *Clostridiales Incertae Sedis XI* and *Enterococcaceae* are preferred species for acidogenic fermentation and that the preferred condition for acidogenic fermentation were HRT 6 days – pH 9.

The three main bacterial families in the PHA reactor were *Comamonadacea*, *Moraxellaceae* and *Xanthobacteracea*; the species that belong to these families (*Comamonas jiangduensis*, *Acinetobacter populi*, *Acinetobacter radioresistens*, *Acetivibrio tandoii*, *Acetivibrio vivianii*, *Pseudoxanthobacter liyangensis*, *Xanthobacter agilis*) are all PHA accumulators that use VFA as carbon and energy source. The maximum PHA accumulation achieved was $38\% \pm 5\%$ g-PHA/g-VSS, the influent of PHA reactor was produced by fermentation reactor which was operating at HRT 6d – pH 9 during that period. The dominant species in the PHA reactor at that period were *Acinetobacter radioresistens*, *Pseudoxanthobacter liyangensis*, and *Xanthobacter agilis*.

By studying the connected system of VFA production (fermentation) and PHA production we could provide a complete overview of the system. Findings of this study could contribute in economical and sustainable pilot-scale studies or even future full-scale projects. Study of the microbial ecology of the combined system of VFA production with further conversion to PHA through MMC could contribute to an efficient process design with possible application into WRRF and decrease of the cost of PHA, therefore increase their market value. Retrofitting of the existing anaerobic digestion infrastructure could create a biorefinery platform that uses wastes to produce biodegradable plastics instead of methane. However, to better understand the microbial metabolism and how VFA are being utilized to produce PHA, the

current research needs to be expanded. Future research should include metatranscriptome sequencing to provide insight into the metabolic pathways of microorganisms. That would allow to obtain whole gene expression profiles of the complex microbial communities involved, quantify their expression level and monitor how they change in different environmental conditions.

Epilogue

This dissertation represents efforts towards deeper understanding of resource recovery from organic waste, using the nutrient content of organic waste for conversion into biochemical value-added products with commercial potential. Deeper understanding of system's operation and related microbial ecology could lead to better design of engineered waste treatment systems. Specifically, we investigated the potential of food waste for enhancing VFA production via fermentation, by examining two different substrate composition (pure food waste, and mixed primary sludge with food waste) in order to determine if the co-fermentation would result in better VFA yields, as food waste are often being incorporated in the digestion processing of biosolids in wastewater treatment plants. Then, VFA production and further conversion to PHA was achieved by anaerobic co-fermentation of primary sludge and food waste, and sequential enrichment in PHA-producing microorganisms through aerobic dynamic feeding of feast and famine cycles. Attempts were made towards improving both processes by manipulating fermentation operational parameters such as organic loading rate, pH, and hydraulic retention time, and manipulating PHA production operational parameters such as the feeding pattern, and substrate (VFA) source, in order to maximize the VFA and PHA production. Finally, 16S-rRNA gene sequencing was used to identify the dominant microbial species of the cultures in the fermenter and in the PHA enrichment reactor, as a preliminary step towards investigating possible metabolic pathways involved in the production of VFA and PHA; laying the foundation for future directions towards expanding the ability of engineers to utilize microbial communities more effectively by understanding the microorganisms involved in the production of VFA and PHA and their metabolic capabilities.

Key Findings and Significance

- The incorporation of food waste in fermentation of primary sludge improved the VFA production. The study of the OLR impact on the VFA production can provide some insight on the area of co-fermentation of primary sludge and food waste, as there were no other reported findings on the effect of OLR of co-substrate of primary sludge and food waste. Even though these substrates have been previously studied separately in regard to the organic loading rate, they have not been studied combined before. The importance of the combination of the two substrates lies in the potential synergistic effects of the two components. The findings provided in this study can have a great impact on the design of digesters in wastewater treatment plants that use primary sludge and food waste as substrates for the stabilization of biosolids.
- The overall preferred conditions for maximum VFA and PHA production from primary sludge and food waste, included co-fermentation at OLR 25 kg-COD/m³/d, pH 9 and HRT 6d and then feeding of fermentation-derived VFA in one-pulse in PHA accumulating reactor applying consecutive feast and famine cycles. This work aimed to expand the currently very limited research related to the use of primary sludge and food waste as co-substrates in combined VFA-PHA production system in regard to operational conditions. These findings have the potential to extend the knowledge of the field of resource recovery from organic waste in regard to optimization of VFA and PHA production, and especially from primary sludge and food waste which is a feedstock massively produced and with great synergistic effects on fermentation. Also, it should be noted that most conditions of pH and HRT tested, acetic acid was the prevailing acid, however at pH 9 and HRT 2 days, propionic acid was the

dominant carboxylic acid produced ($69\pm 14\%$). That finding could give direction to future research that is targeted in VFA species-specific production, along with methods of isolation of acids from liquid mixture.

- Other parameters that were examined for VFA production optimization were the pH and HRT. The highest concentration of VFA (6,100 mg-COD/L) was achieved at conditions of HRT 6 days – pH 9, and the most abundant VFA species were acetic acid ($78\% \pm 4\%$) and propionic acid ($16\% \pm 1.4\%$). The most abundant species belonged to the families of *Clostridiales Incertae Sedis XI* (*Tepidimicrobium xylanilyticum*, *Tepidimicrobium ferriphilum*) and *Enterococcaceae* (*Vagococcus lutrae*, *Vagococcus acidifermentans*). Dominant species were identified for all different fermentation conditions tested. The findings of this work can provide a better understanding of the shift in microbial ecology through different conditions of pH and HRT, which is an area that literature is lacking any related reports. Understanding the relation between the change of dominant microbial species in complex systems and environmental conditions could provide useful insight on the optimum fermentation conditions when designing anaerobic digesters. Also, provides the first layer of information towards discovering the potential metabolic pathways and the enzymes involved.
- The maximum PHA accumulation achieved was $38\% \pm 5\%$ g-PHA/g-VSS, the influent of PHA reactor was produced by fermentation reactor which was operating at HRT 6d – pH 9 during that period. The dominant species in the PHA reactor at the time belonged to the families of *Moraxellaceae* (*Acinetobacter radioresistens*), and *Xanthobacteraceae*

(*Pseudoxanthobacter liyangensis* and *Xanthobacter agilis*). As there is limited research describing the microbial ecology of mixed microbial cultures utilizing complex fermented substrates for PHA production, these findings can contribute in expanding the knowledge of the microbial species involved in PHA production in mixed microbial culture systems. By identifying and studying of the PHA-producing microorganisms, engineers can apply conditions favorable for growth of the PHA-producing microbial species to enhance the enrichment process. In addition, potential identification of new species can expand the field of PHA production from fermentation derived substrates by mixed microbial cultures.

Broader Impact and Future Research Recommendations

The process described in this dissertation addresses a major environmental problem that societies currently face; waste management. It is an alternative and sustainable method that cities can implement in order to reduce the footprint of organic waste disposal. Wastewater treatment facilities present a great advantage for application, as the digestion infrastructure and expertise already exist, and they are usually located in populated areas which facilitates the transportation of food waste. However, several factors should be taken into account, such as the requirement for larger digestion reactors, which increases the cost of the operation taking also into account the transportation requirements of food waste to the wastewater treatment facilities.

By studying the connected system of VFA production and PHA production we provided a complete overview of the system, which can be extended to economical and sustainable pilot-scale studies or even future full-scale projects. In that direction, future work could focus on using the scenarios studied in this dissertation and apply them for modeling purposes using BioWin or other environmental simulators. BioWin is a wastewater treatment process simulator and it is

used for designing, upgrading and optimizing wastewater treatment plants. Furthermore, as it is documented that the VFA distribution affects the PHA monomer composition, looking into the ecology of the combined system can give engineers directions towards adjusting the fermentation conditions and ultimately shifting the microbial ecology in such way that produces target VFA species that could control the PHA species in downstream process. Further extraction, purification and evaluation of the properties of the bioplastics produced would determine applications that could be appropriate for, hence future research could extend the research performed in this dissertation and focus on the extraction and purification of the polymer. Another important direction for future research would be the study of storage capacity of each identified PHA producing species for better understanding of whether all species have similar storage capacities or only certain ones produce most of the PHA. That would enable engineers to select the reactor's operating condition in favor of the microorganisms with the highest storage capacity. Metatranscriptome sequencing would provide insight on the metabolic pathways of microorganisms and explain specific reactions taking place and the end products of fermentation. Metatranscriptomics would provide information on the functional profile of genes expressed in the complex microbial communities involved in VFA and PHA production while quantifying their expression level and monitoring how they change in different environmental conditions.

In order to address global challenges such as increasing waste production and decreasing availability of non-renewable resources, the economy needs to shift towards a circular economy where multidimensional growth is achieved towards environmental, economic, technical, and social directions. Wastewater treatment facilities can be reoriented to function as water resource recovery facilities (WRRFs) so that economic development is combined with environmental sustainability. Innovative technologies must be accompanied by business models for

implementation. In that direction, future research should also focus on developing strong resource recovery business cases that could influence the industry and government.

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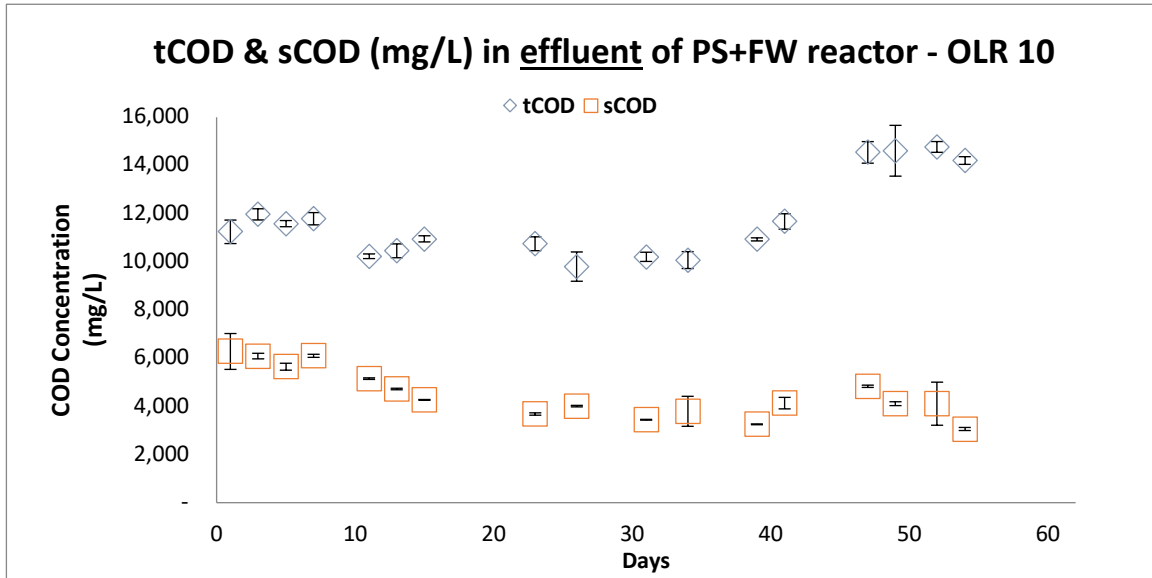
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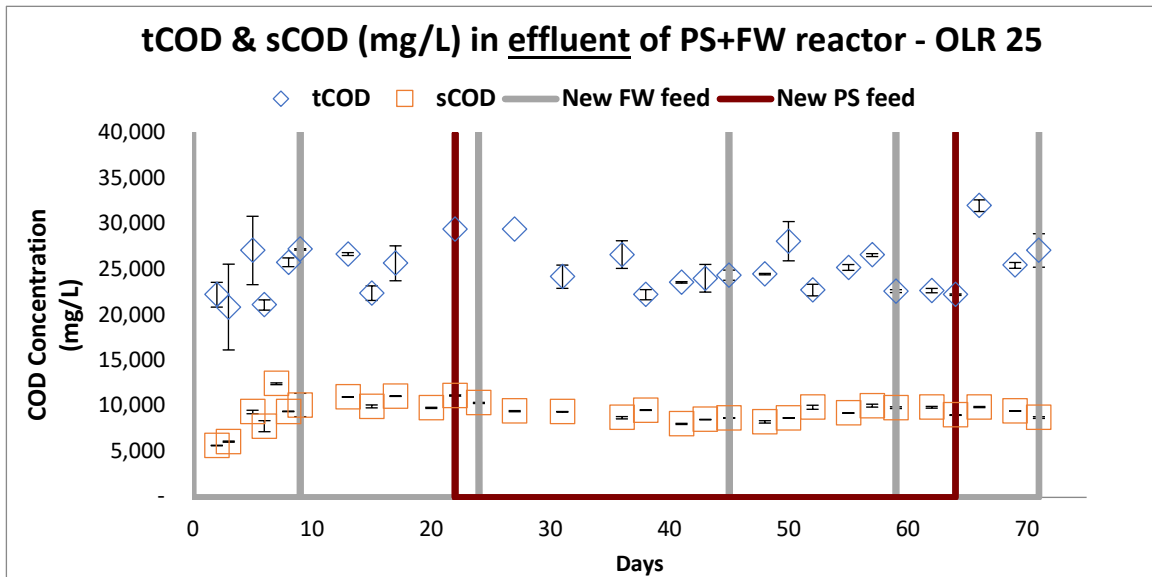
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Appendix A (in reference to Chapter 2)



**Figure 2- 10: Concentration of tCOD and sCOD in effluent of PS+FW reactor at OLR 10
kg-COD/m³/d.**



**Figure 2- 11: Concentration of tCOD and sCOD in effluent of PS+FW reactor at OLR 25
kg-COD/m³/d.**

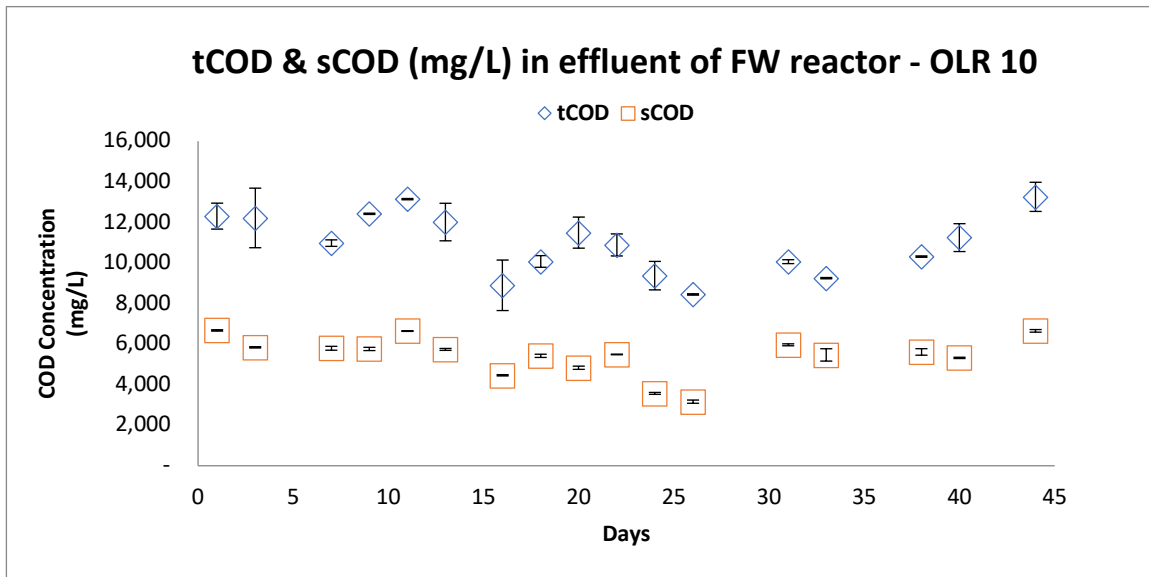


Figure 2- 12: Concentration of tCOD and sCOD in effluent of FW reactor at OLR 10 kg-COD/m³/d.

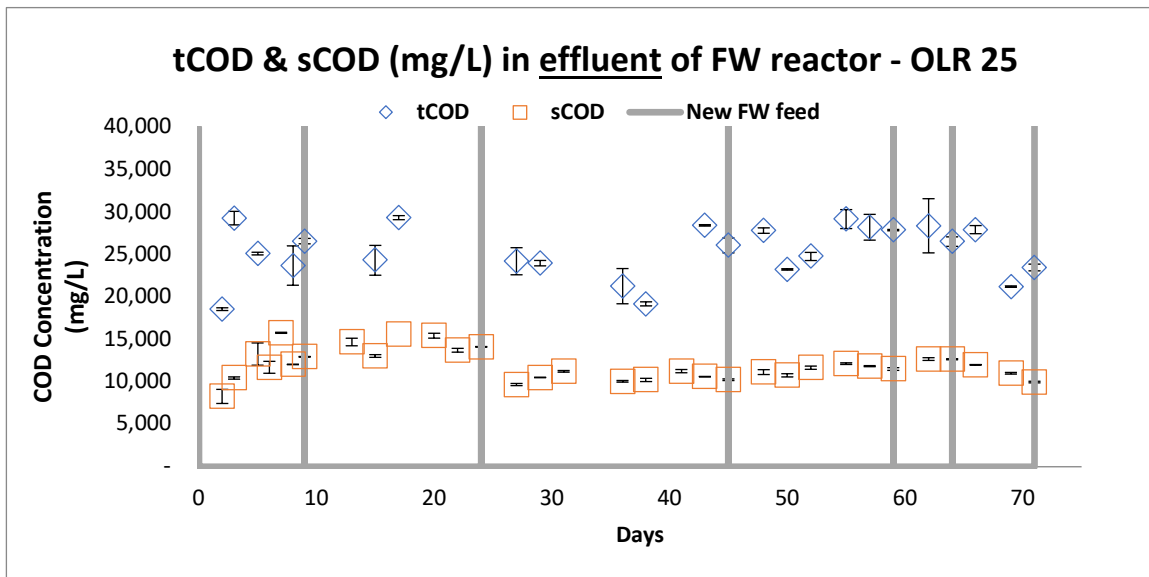


Figure 2- 13: Concentration of tCOD and sCOD in effluent of FW reactor at OLR 25 kg-COD/m³/d.

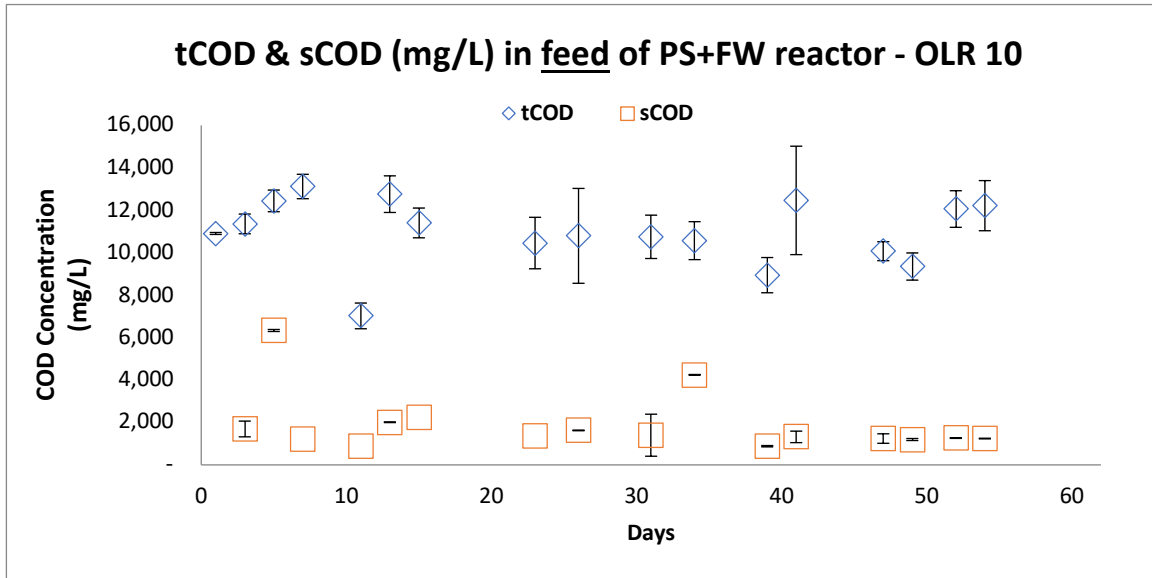


Figure 2- 14: Concentration of tCOD and sCOD in feed of PS+FW reactor at OLR 10 kg-COD/m³/d.

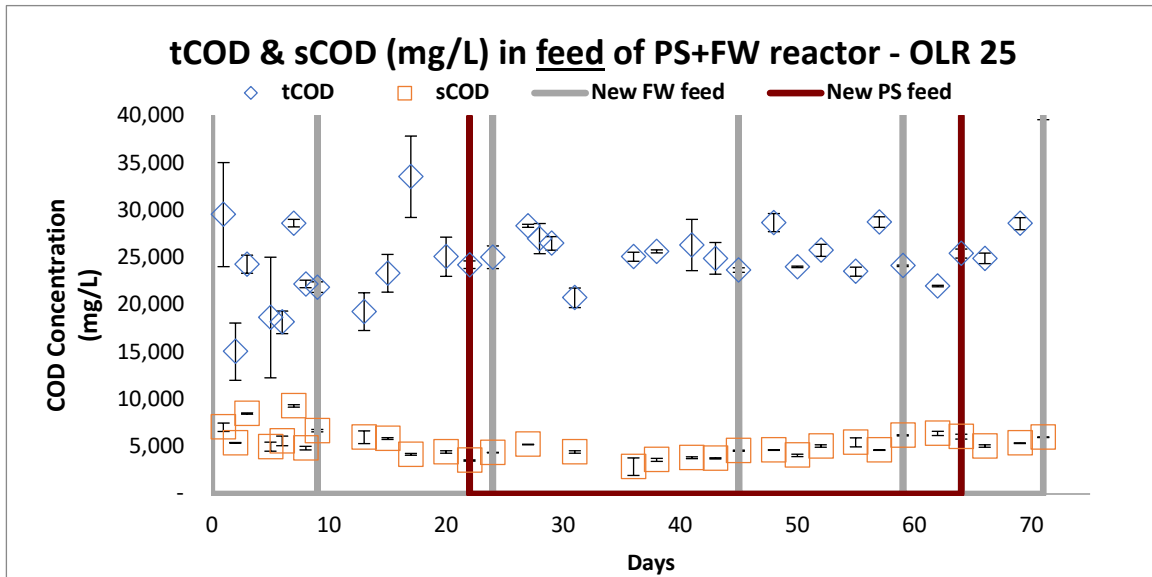


Figure 2- 15: Concentration of tCOD and sCOD in feed of PS+FW reactor at OLR 25 kg-COD/m³/d.

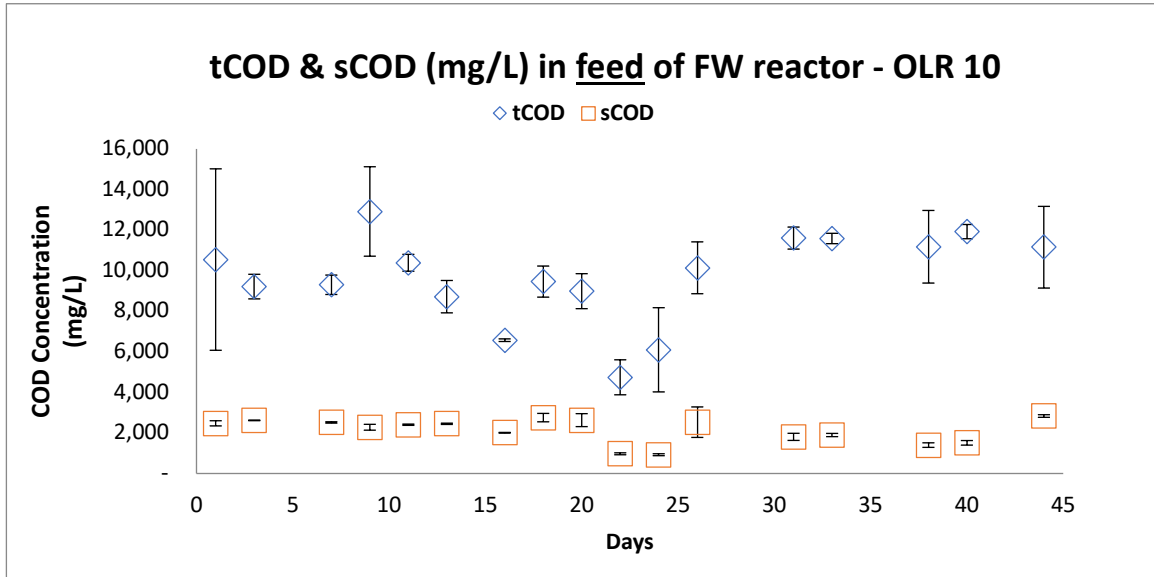


Figure 2- 16: Concentration of tCOD and sCOD in feed of FW reactor at OLR 10 kg-COD/m³/d.

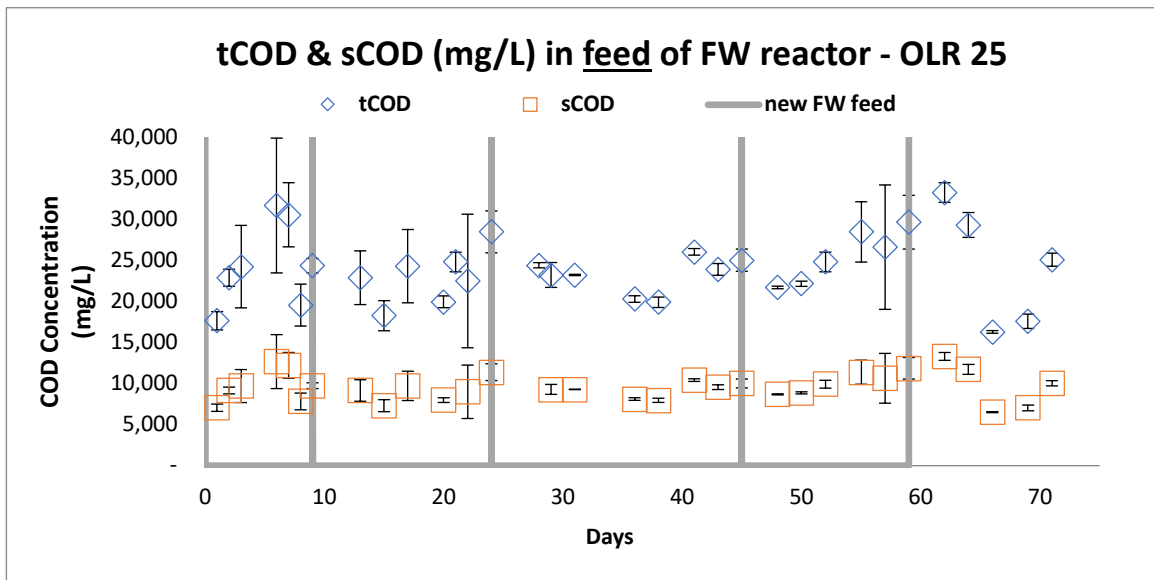


Figure 2- 17: Concentration of tCOD and sCOD in feed of FW reactor at OLR 25 kg-COD/m³/d.

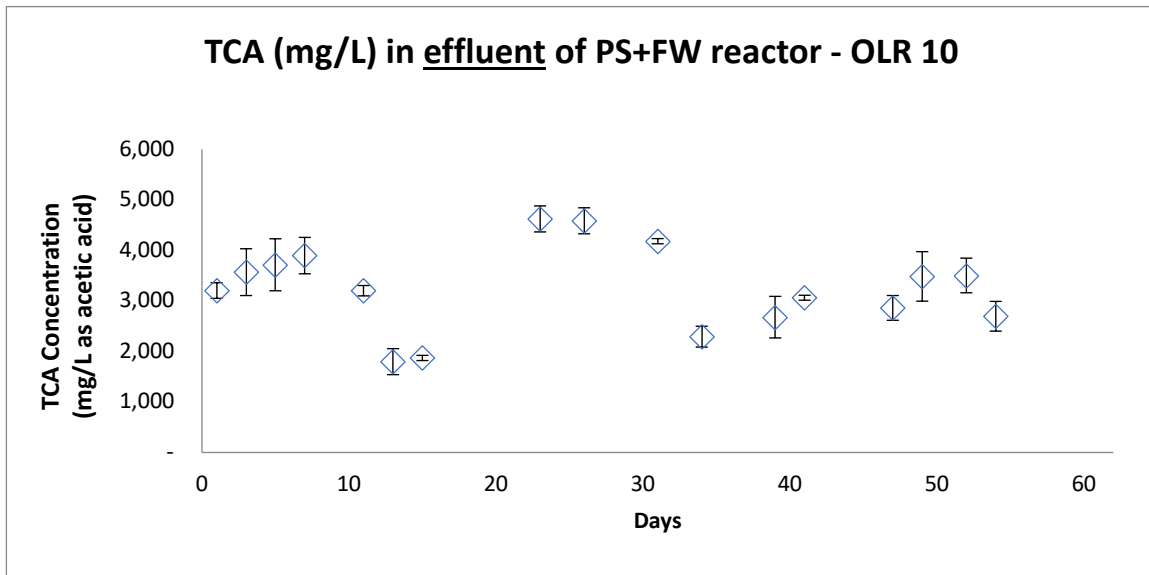


Figure 2- 18: Concentration of TCA in effluent of PS+FW reactor expressed as mg-acetate/L, OLR 10 kg-COD/m³/d.

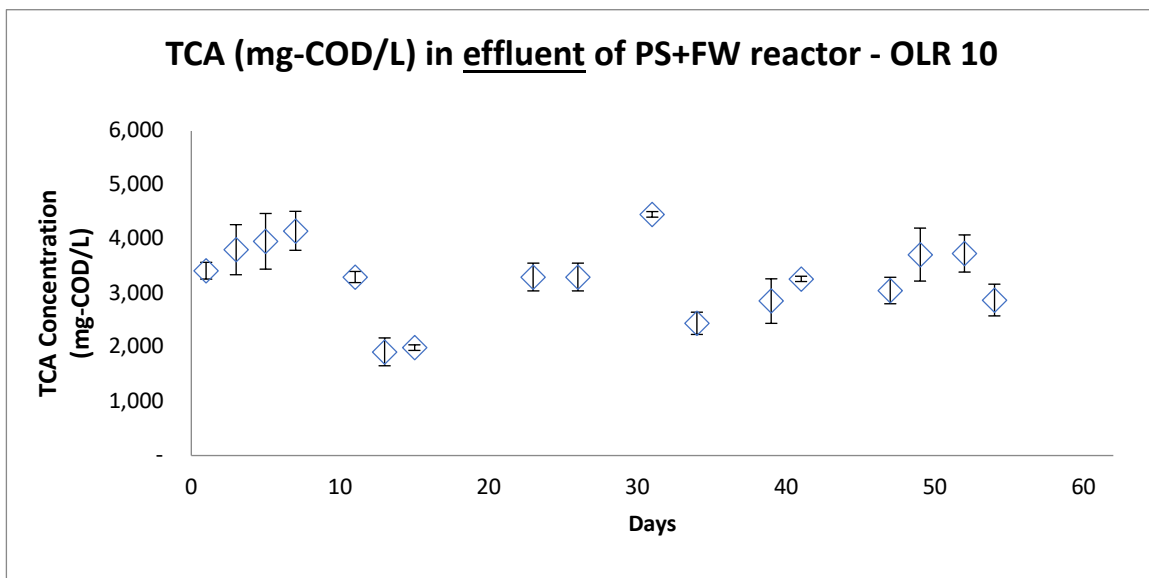


Figure 2- 19: Concentration of TCA in effluent of PS+FW reactor expressed as mg-COD/L, OLR 10 kg-COD/m³/d.

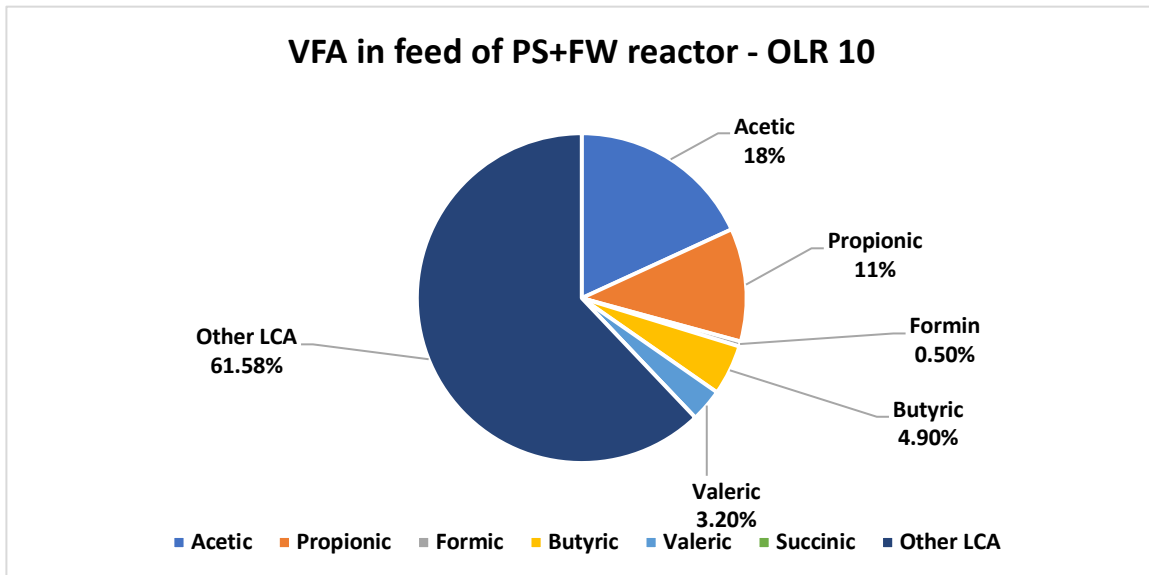


Figure 2- 20: Distribution of VFA in feed PS+FW reactor at OLR 10 kg-COD/m³/d.

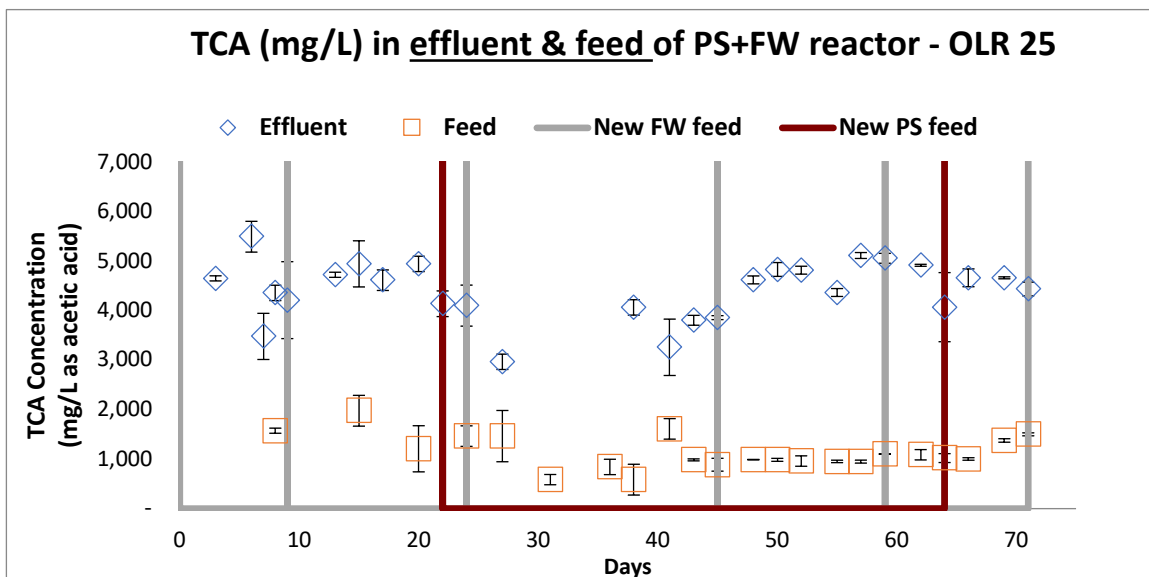


Figure 2- 21: Concentration of TCA in effluent and feed of PS+FW reactor expressed as mg-acetate/L, for OLR 25 kg-COD/m³/d.

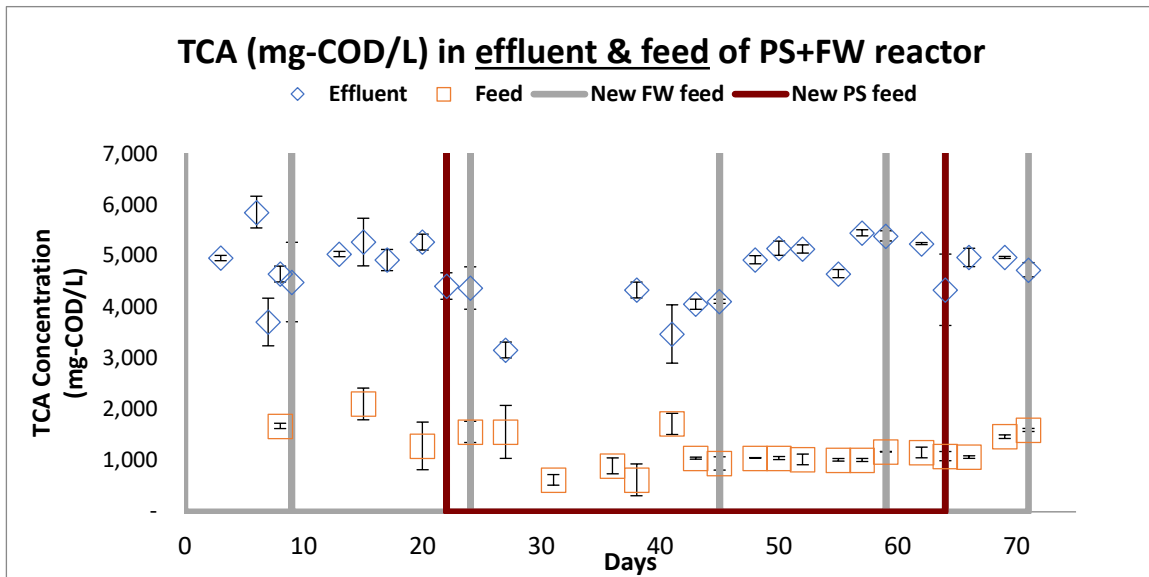


Figure 2- 22: Concentration of TCA in effluent and feed of PS+FW reactor expressed as mg-COD/L, for OLR 25 kg-COD/m³/d.

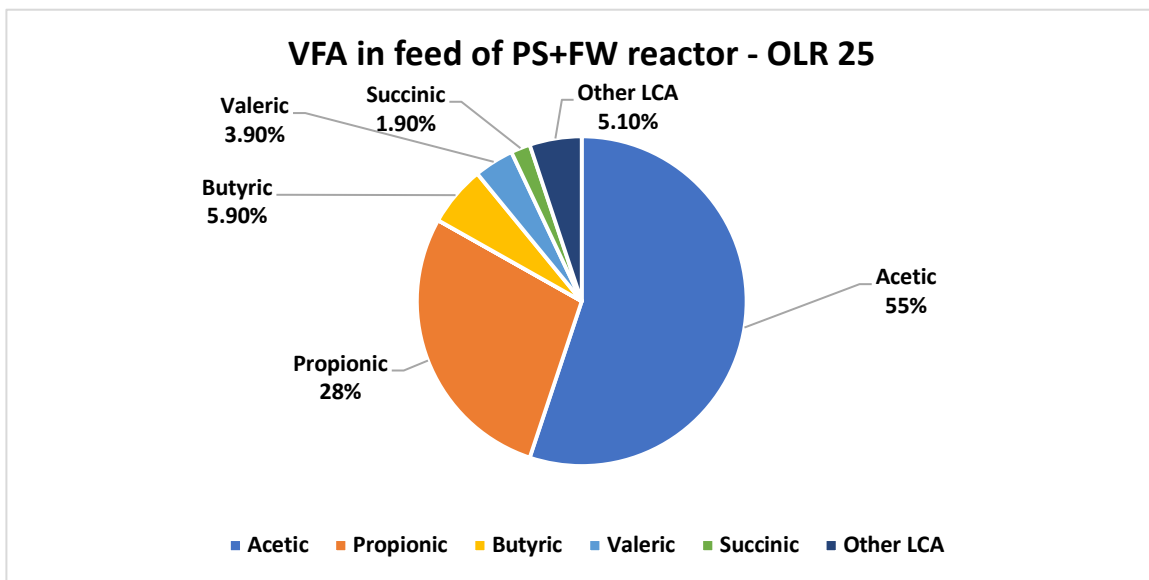


Figure 2- 23: Distribution of VFA in feed PS+FW reactor at OLR 25 kg-COD/m³/d.

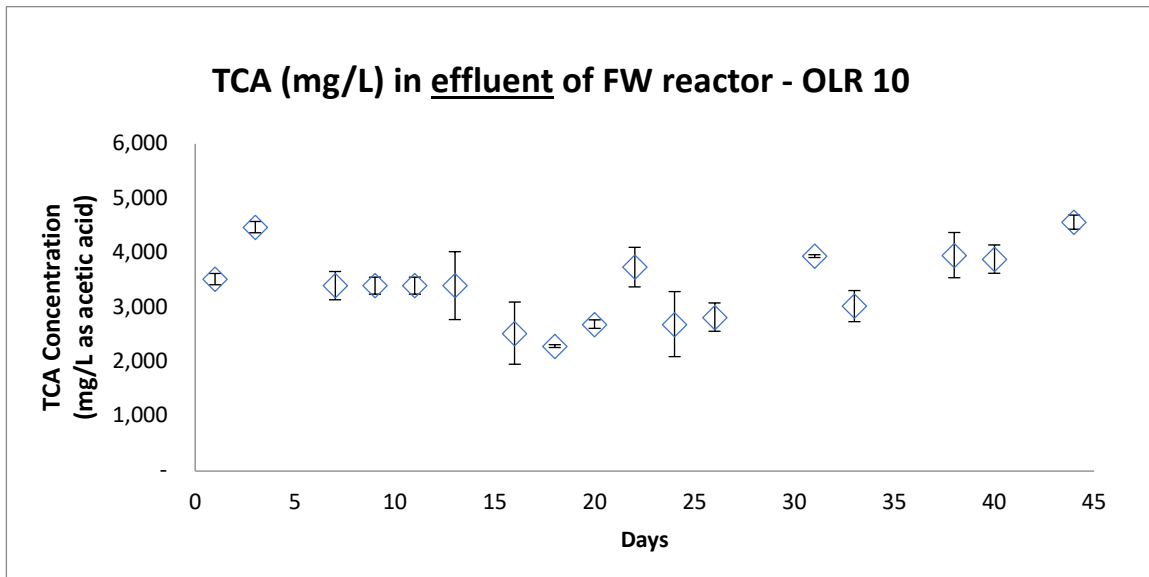


Figure 2- 24: Concentration of TCA in effluent of FW reactor expressed as mg acetate/L, OLR 10 kg-COD/m³/d.

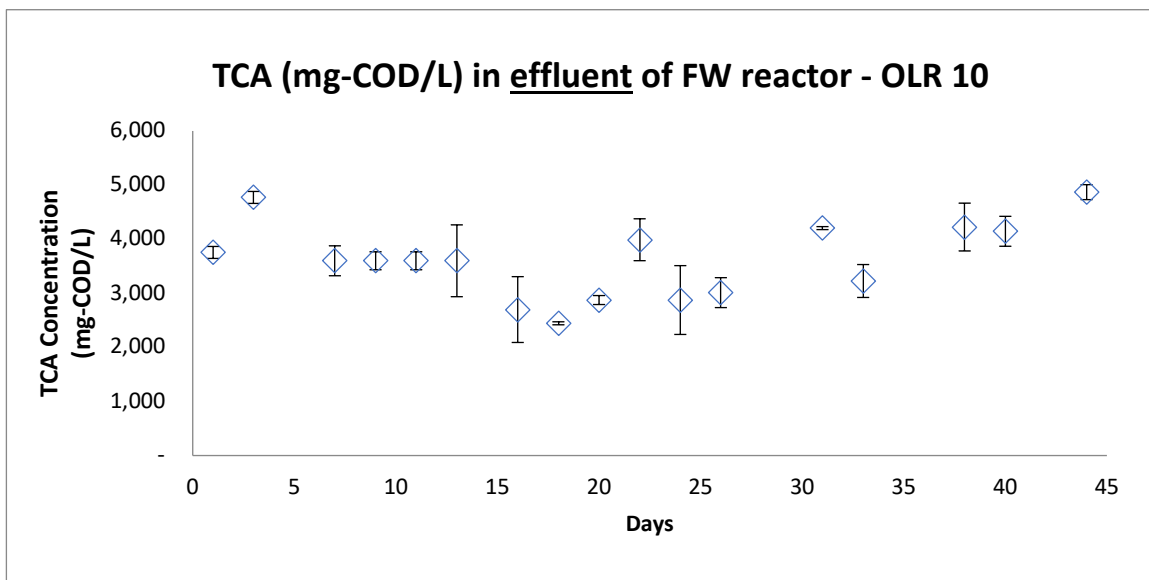


Figure 2- 25: Concentration of TCA in effluent of FW reactor expressed as mg-COD/L, OLR 10 kg-COD/m³/d.

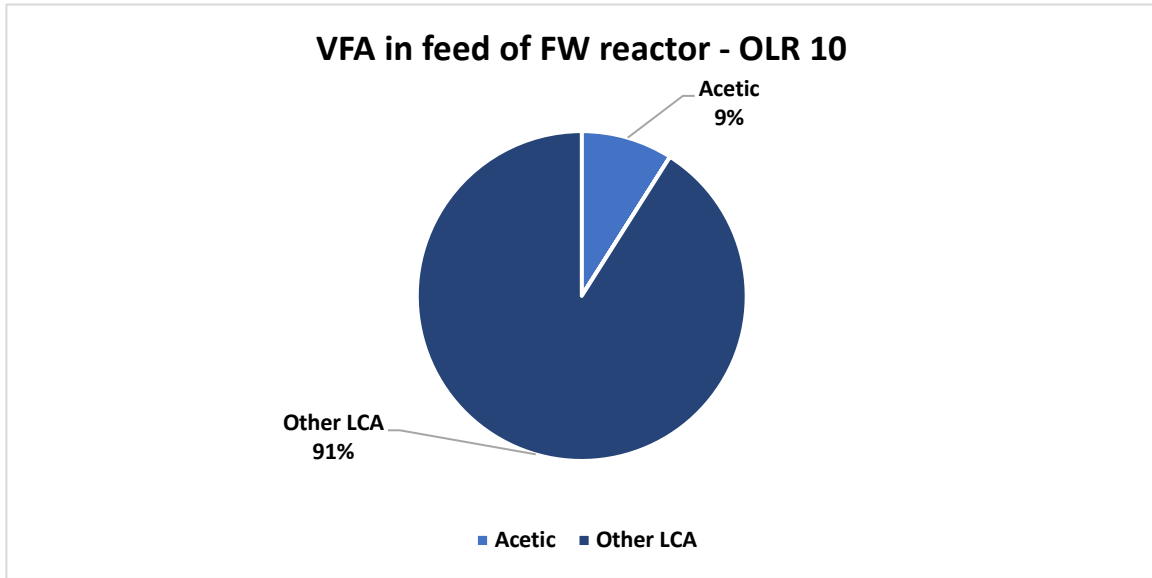


Figure 2- 26: Distribution of total VFA in feed of FW reactor at OLR 10 kg-COD/m³/d.

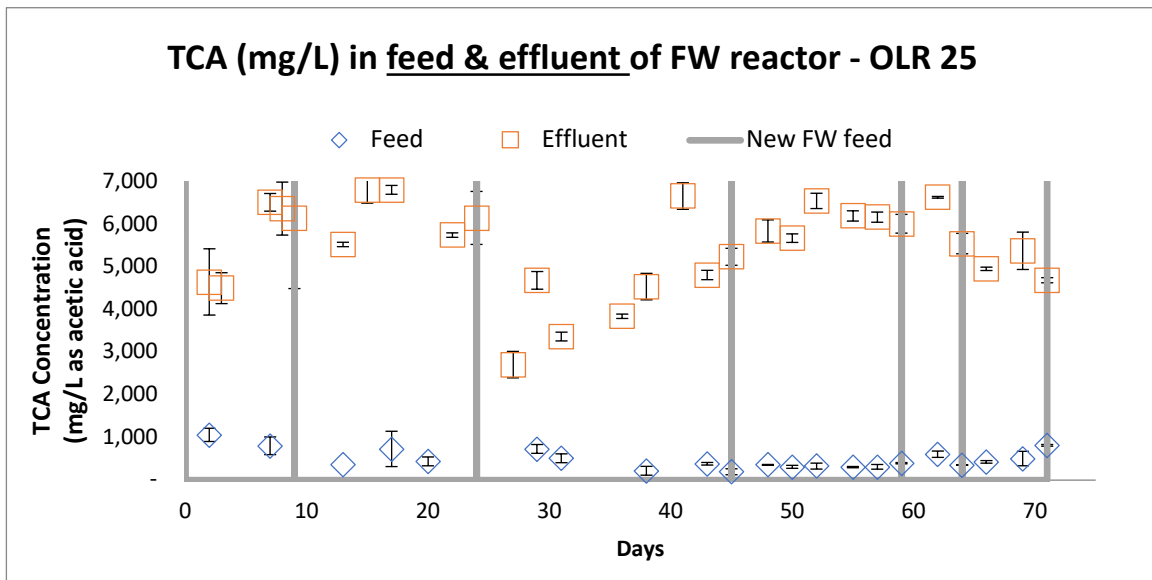


Figure 2- 27: Concentration of TCA in feed and effluent of FW reactor, expressed as mg-acetate/L, OLR 25 kg-COD/m³/d.

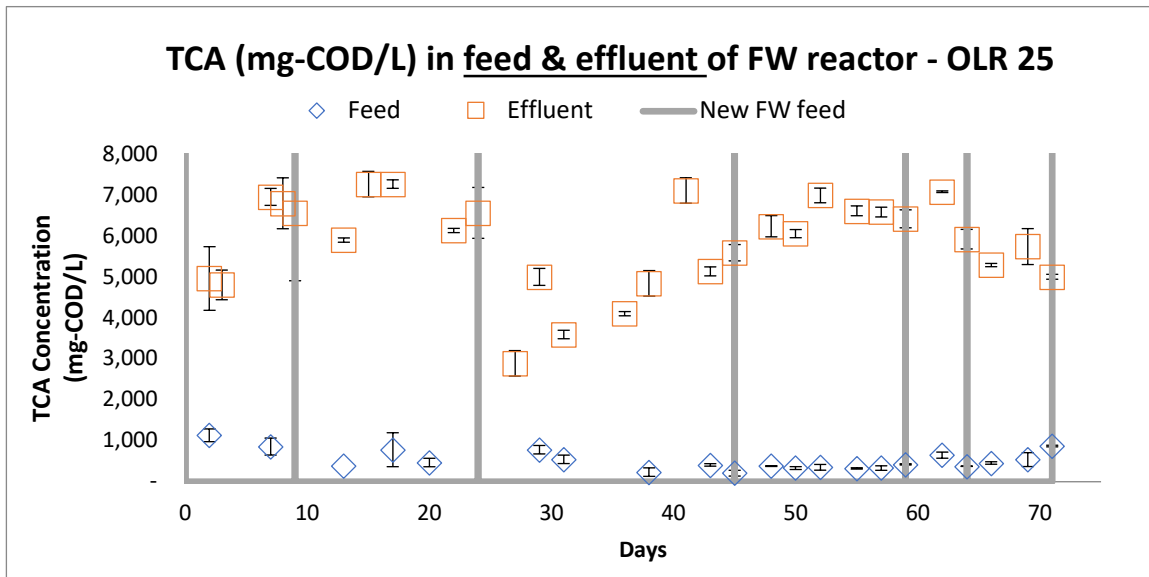


Figure 2- 28: Concentration of TCA in feed and effluent of FW reactor expressed as mg-COD/L, OLR 25 kg-COD/m³/d.

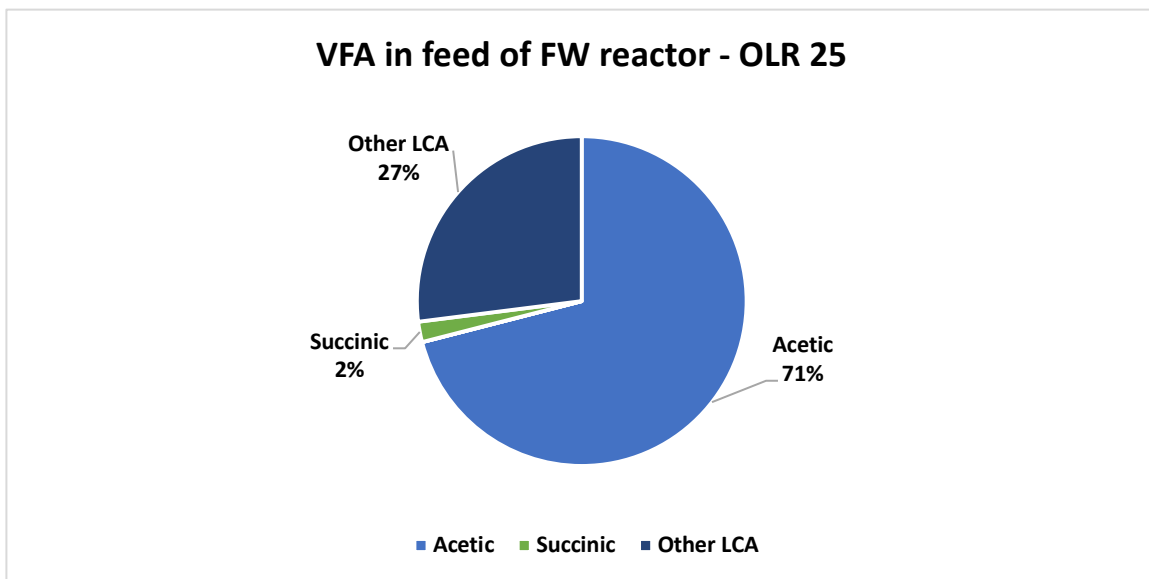


Figure 2- 29: Distribution of total VFA in feed of FW reactor at OLR 25 kg-COD/m³/d.

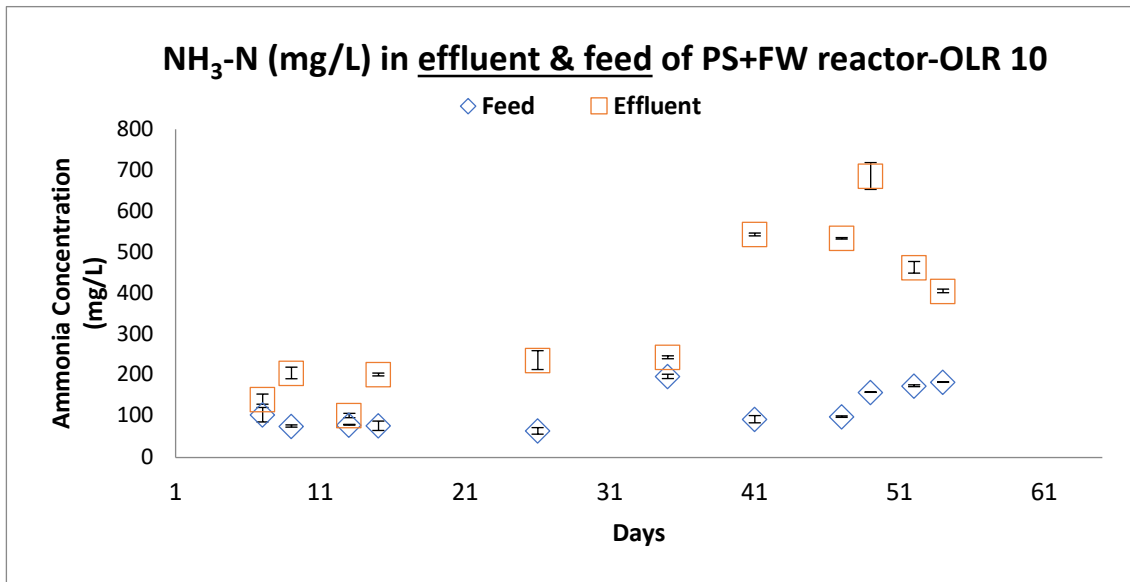


Figure 2- 30: NH₃-N concentration in effluent and feed of PS+FW reactor, OLR 10 kg-COD/m³/d.

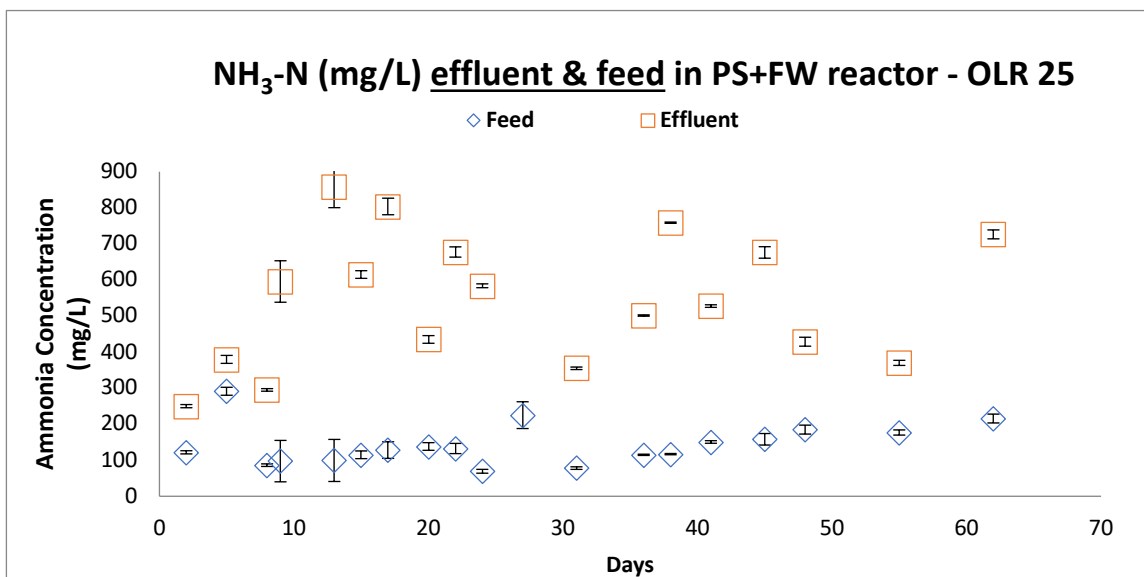


Figure 2- 31: NH₃-N concentration in effluent and feed of PS+FW reactor, OLR 25 kg-COD/m³/d.

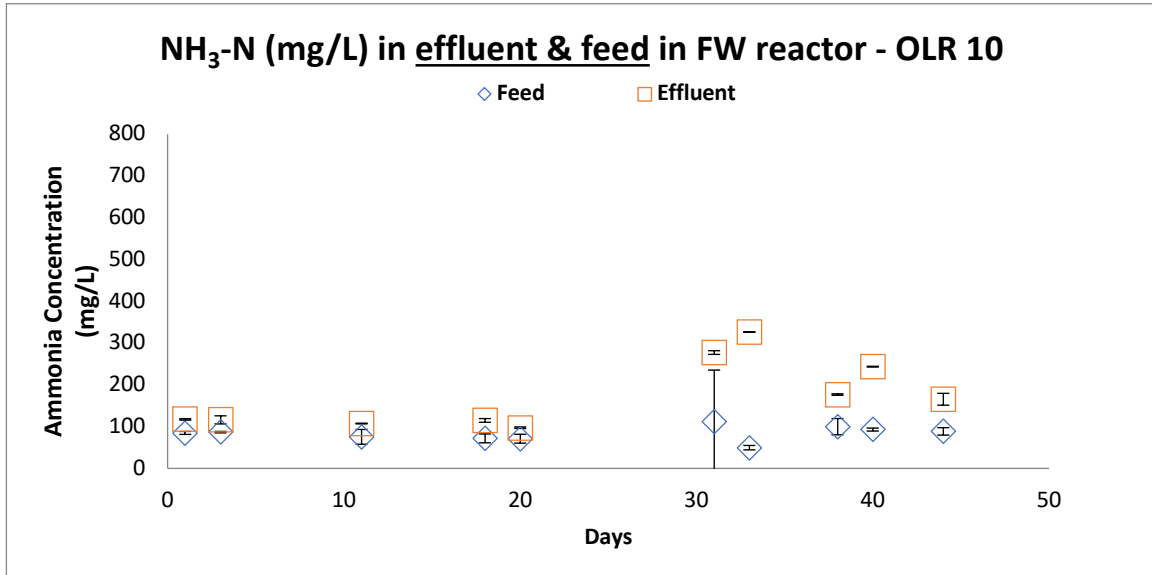


Figure 2- 32: NH₃-N concentration in effluent and feed of FW reactor, OLR 10 kg-COD/m³/d.

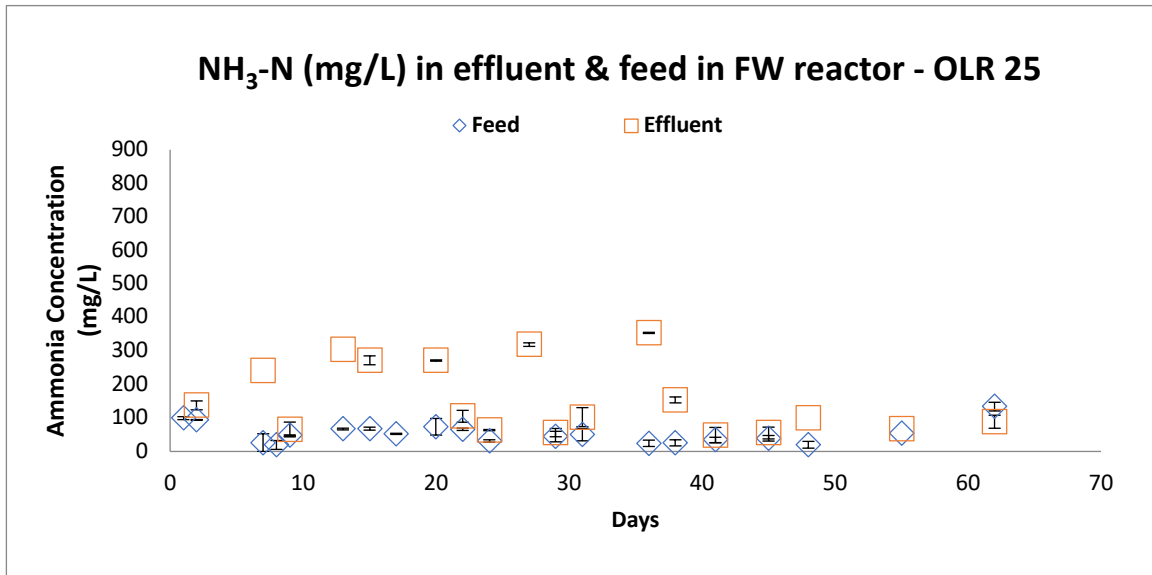


Figure 2- 33: NH₃-N concentration in effluent and feed of FW reactor, OLR 25 kg-COD/m³/d.

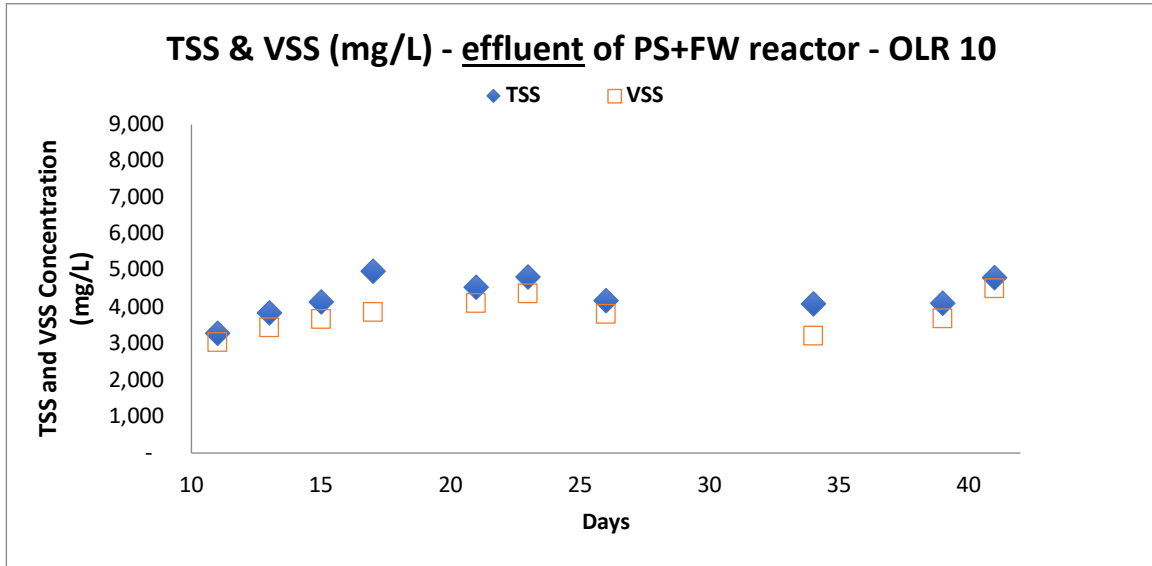


Figure 2- 34: Concentration of TSS and VSS in the effluent of PS+FW reactor, OLR 10 kg-COD/m³/d.

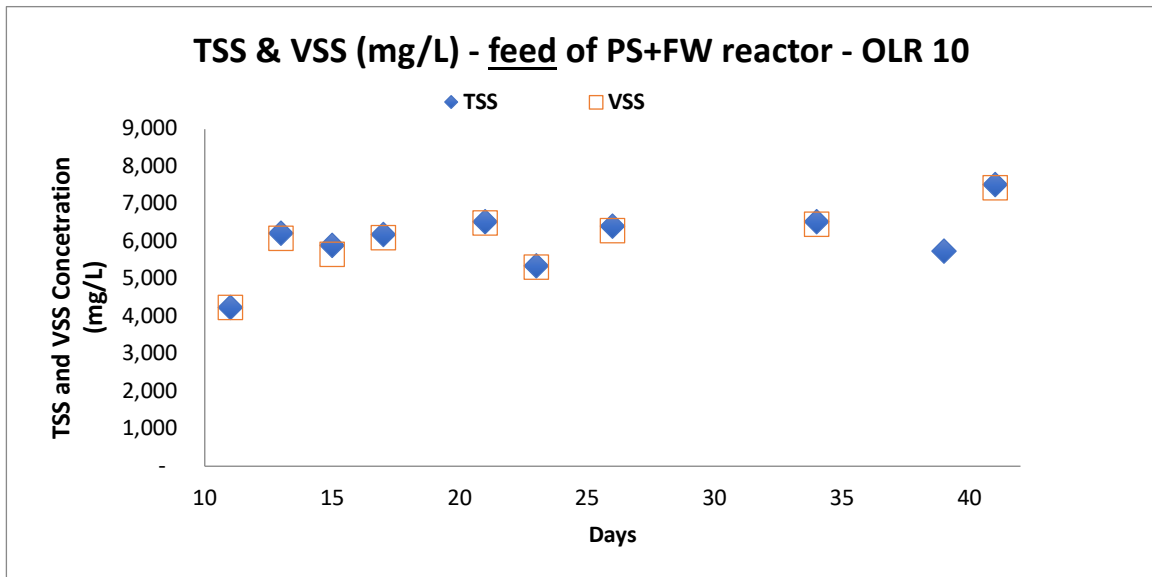


Figure 2- 35: Concentration of TSS and VSS in the feed of PS+FW reactor, OLR 10 kg-COD/m³/d.

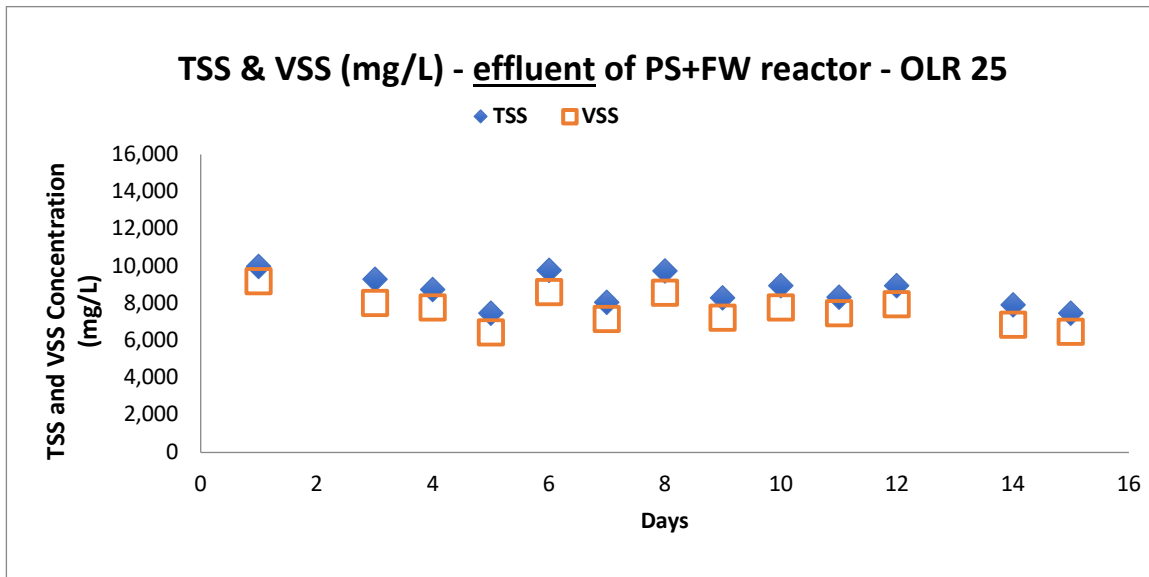


Figure 2- 36: Concentration of TSS and VSS in the effluent of PS+FW reactor, OLR 25 kg-COD/m³/d.

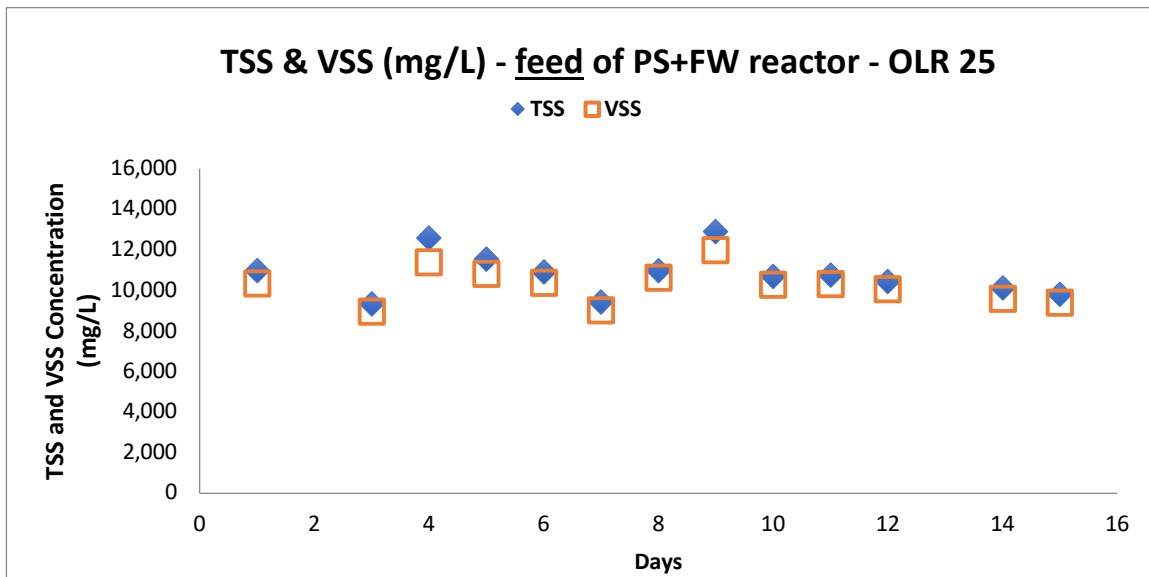


Figure 2- 37: Concentration of TSS and VSS in the feed of PS+FW reactor, OLR 25 kg-COD/m³/d.

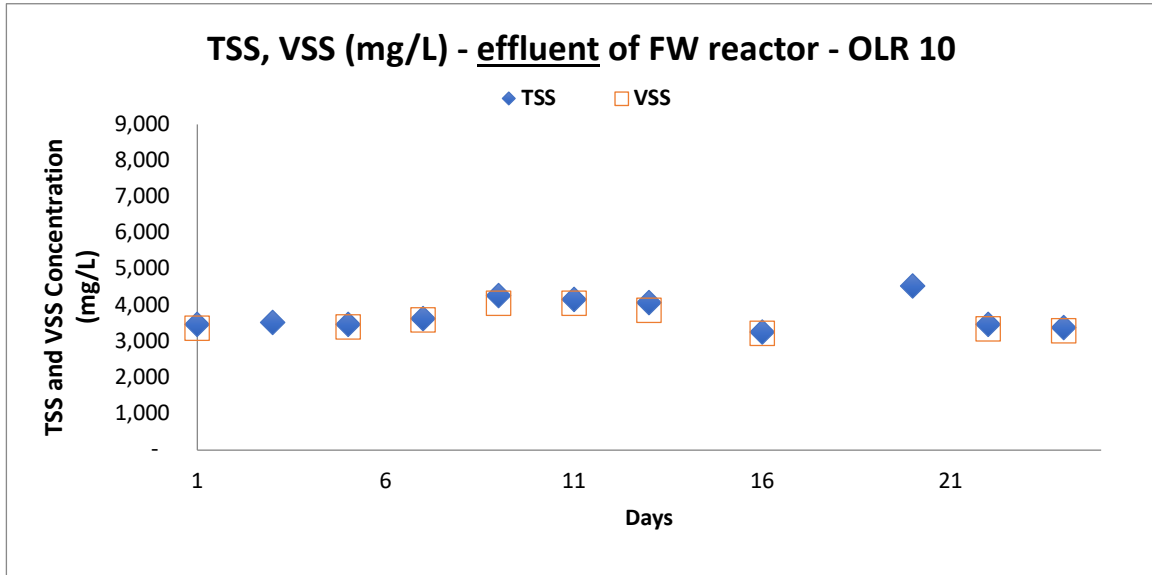


Figure 2- 38: Concentration of TSS and VSS in the effluent of FW reactor, OLR 10 kg-COD/m³/d.

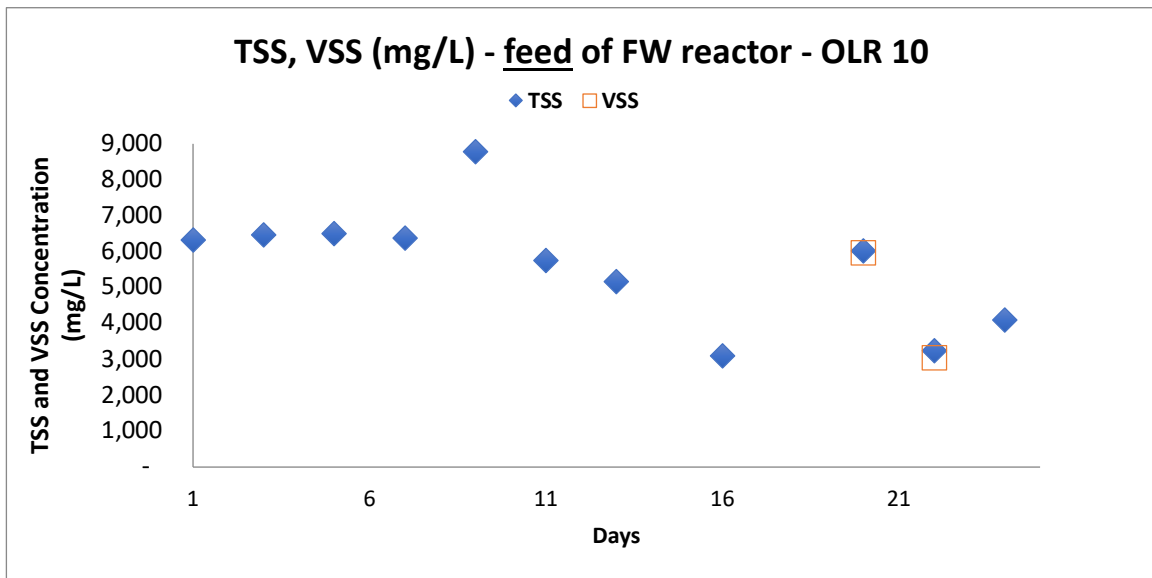


Figure 2- 39: Concentration of TSS and VSS in the feed of FW reactor, OLR 10 kg-COD/m³/d.

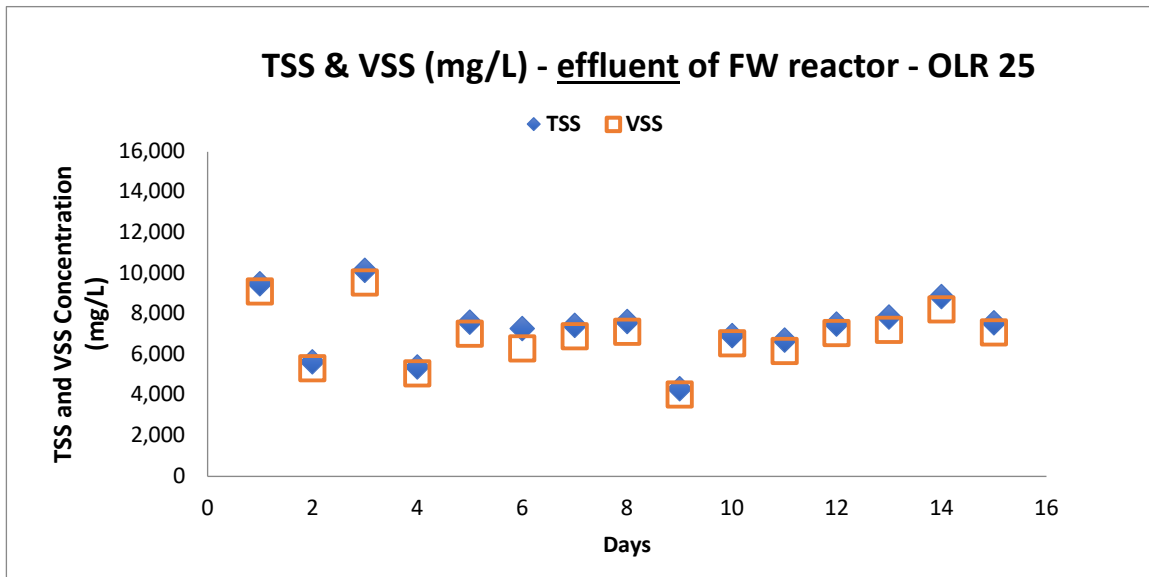


Figure 2- 40: Concentration of TSS and VSS in the effluent of FW reactor, OLR 25 kg-COD/m³/d.

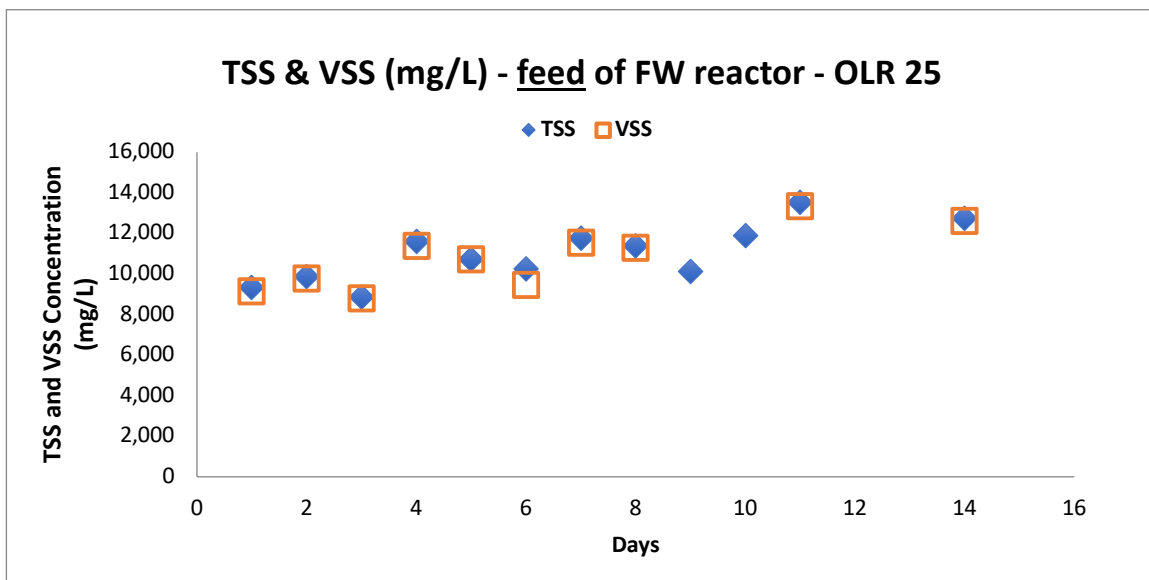


Figure 2- 41: Concentration of TSS and VSS in the feed of FW reactor, OLR 25 kg-COD/m³/d.

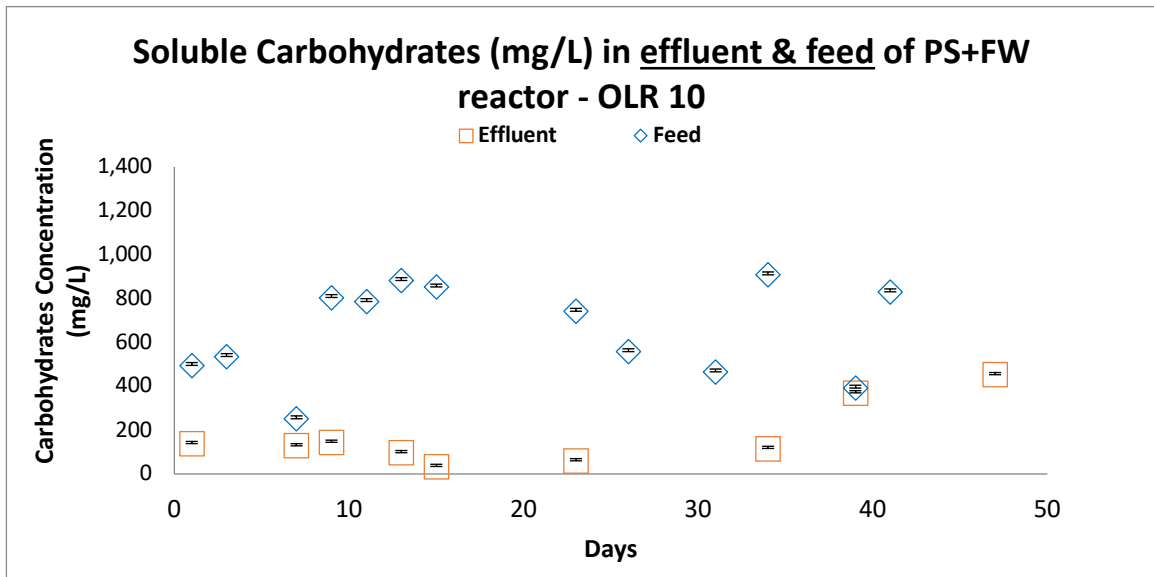


Figure 2- 42: Concentration of sCARB in PS+FW reactor, OLR 10 kg-COD/m³/d.

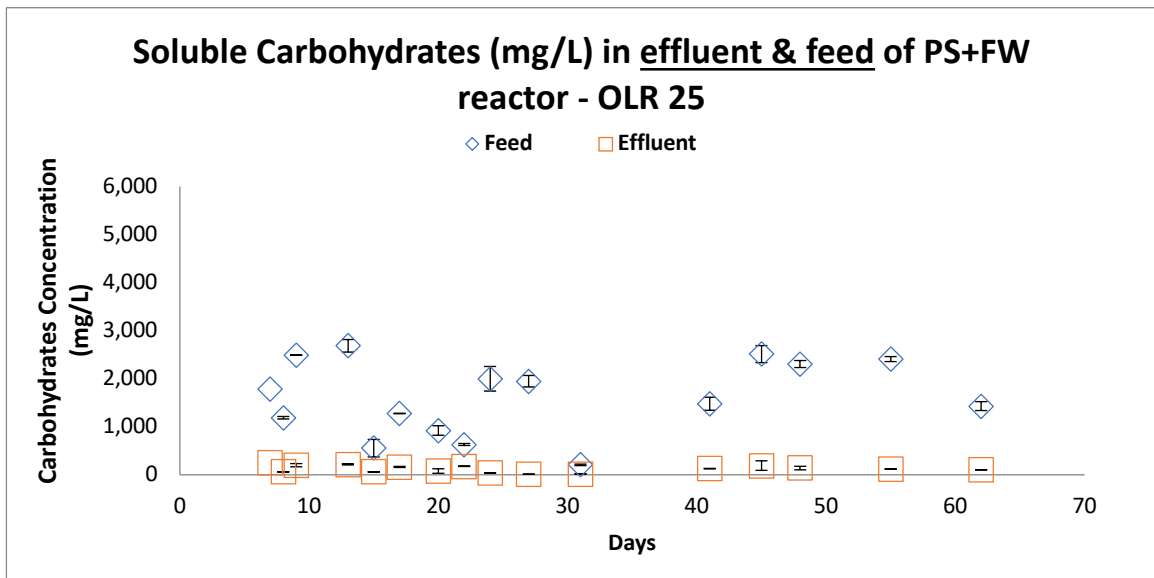


Figure 2- 43: Concentration of sCARB in PS+FW reactor, OLR 25 kg-COD/m³/d.

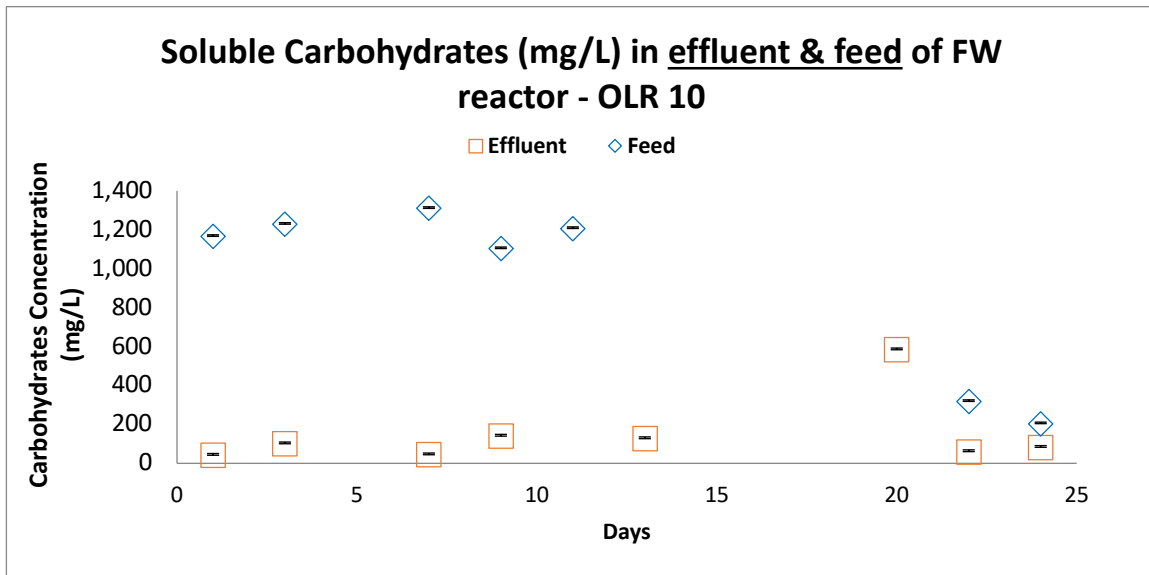


Figure 2- 44: Concentration of sCARB in FW reactor, OLR 10 kg-COD/m³/d.

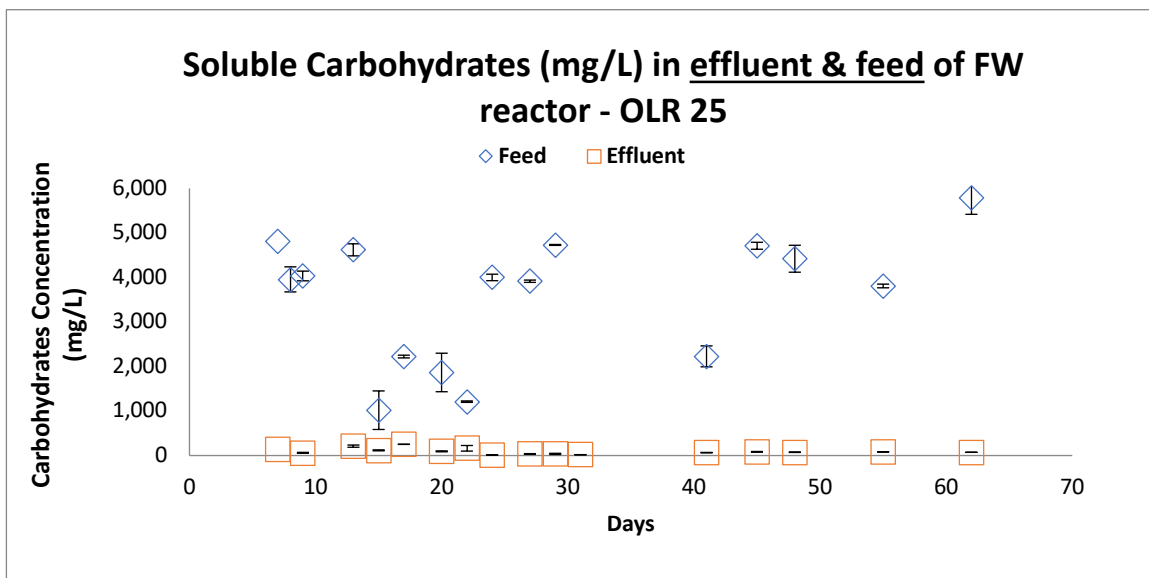


Figure 2- 45: Concentration of sCARB in FW reactor, OLR 25 kg-COD/m³/d.

Appendix B (in reference to Chapter 3)

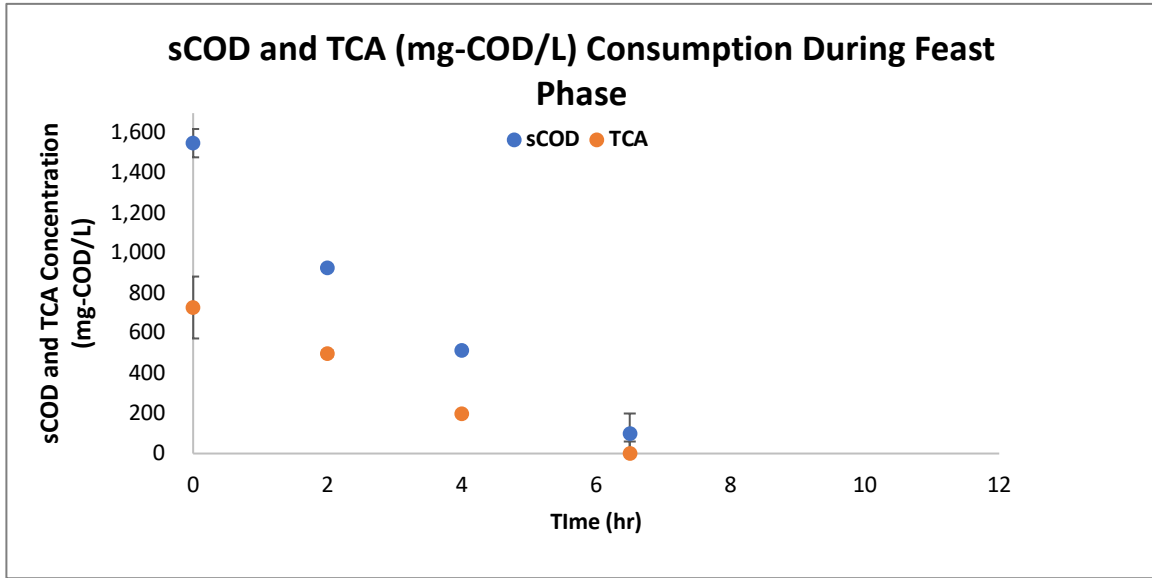


Figure 3- 9: sCOD and TCA concentrations, during 12-cycle, 1-pulse feeding, fermentate, in PHA reactor.

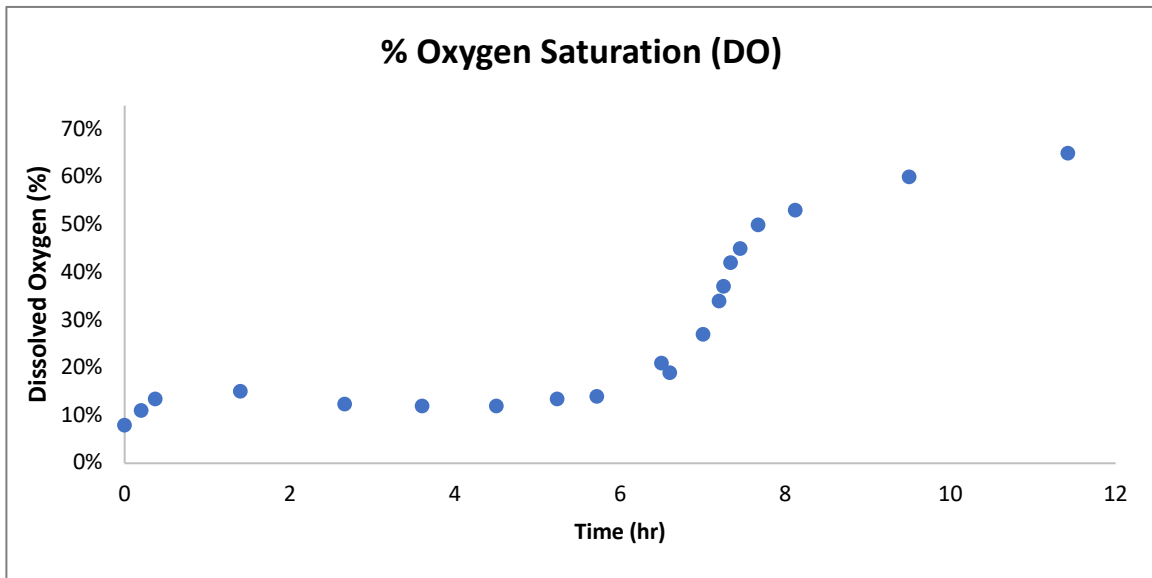


Figure 3- 10: Dissolved oxygen, during 12-hr cycle, in PHA reactor.

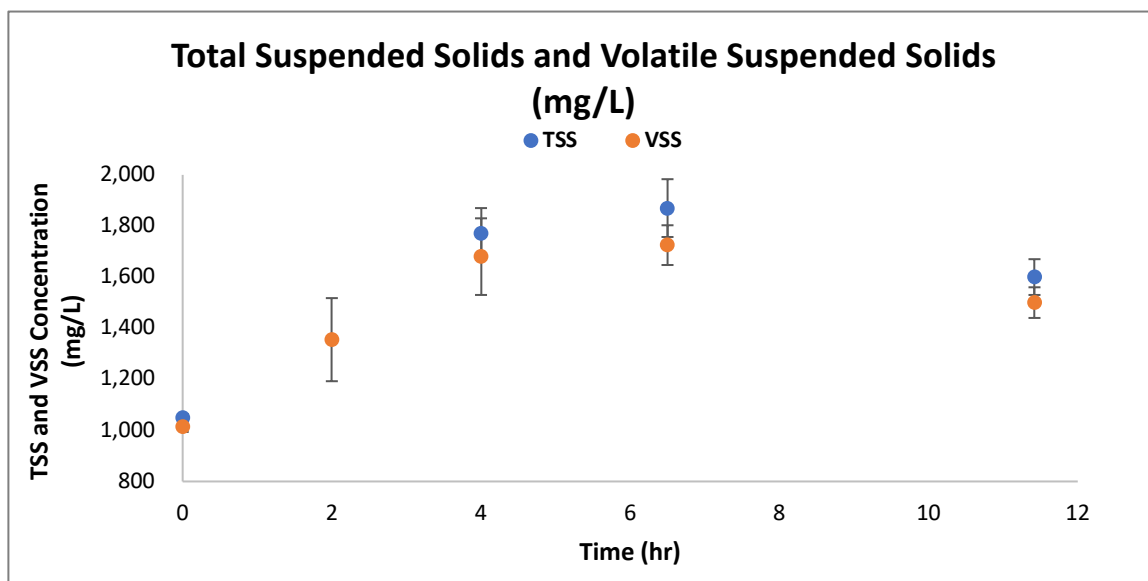


Figure 3- 11: TSS and VSS, during 12-hr cycle in PHA reactor.

Table 3- 2: VFA composition for pH values 6.5, 7, 7.5, 8, and 9 and HRT 2, 4 and 6 days in the fermenter and total concentration of TCA in the fermenter effluent.

pH	6.5		7		7.5		8		9	
Acids	Average (%)	St. dev, n=7	Average (%)	St. dev, n=2	Average (%)	St. dev, n=2	Average (%)	St. dev, n=2	Average (%)	St. dev, n=5
Acetic	59	8.0	45	4.0	43	5.0	43	8.0	9.3	4.7
Propionic	19	15.	2.4	1.0	2.2	1.0	3.1	1.6	69	14
Formic	0.8	0.0	19	2.0	19	1.0	16	3.1	10	5.2
Butyric	6.7	9.0	4.4	3.0	3.7	2.0	3.9	0.3	1.4	0.5
Valeric	5.7	0.0	7.1	1.0	6.0	1.0	5.2	0.9	1.3	0.7
Succinic	5.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Other LC Acids	3.	18.	22	15.	26	16	29	14	9.4	9.0
TCA _{effluent} (mg/L)	4,760	580	4,900	820	4,900	930	5,300	600	4,600	800

pH 9 / HRT	2 days		4 days		6 day	
Acids	Average (%)	St. dev, n=5	Average (%)	St. dev, n=3	Average (%)	St. dev, n=2
Acetic	9.3	4.7	75.1	4.3	78	11
Propionic	69	14	16.0	1.8	16	1.4
Formic	10.	5.2	0.0	2.0	0.0	1.0
Butyric	1.4	0.5	0.0	3.0	0.0	2.0

Valeric	1.3	0.7	0.0	1.0	0.0	1.0
Succinic	0.0	0.0	0.0	0.0	0.0	0.0
Other LC Acids	9.4	9.0	9.0	7.2	6.0	5.0
TCA_{effluent} (mg/L)	4,600	800	5,700	660	6,500	700

Table 3- 3: PHB and PHV content for each fermentate production condition.

Fermentate production conditions	Sum of even-numbered VFA	Sum of odd-numbered VFA	PHB content	PHV content
pH 7 - HRT 2 days	49.4% ± 12%	28.5% ± 17%	85% ± 3.0%	15% ± 1.3%
pH 7.5 - HRT 2 days	46.7% ± 5.0%	27.2% ± 2.4%	90% ± 4.8%	10% ± 2.1%
pH 8 - HRT 2 days	46.9% ± 8.0%	24.3% ± 3.6%	89% ± 2.5%	11% ± 1.9%
pH 9 - HRT 2 days	10.7% ± 4.7%	80.3% ± 14%	81% ± 15%	19% ± 5.4%
pH 9 - HRT 4 days	75.0% ± 4.0%	16.0% ± 2.0%	96% ± 2.0%	4.0% ± 0.5%
pH 9 - HRT 6 days	78.0% ± 11%	16.0% ± 1.0%	94% ± 5.7%	6.0% ± 0.3%

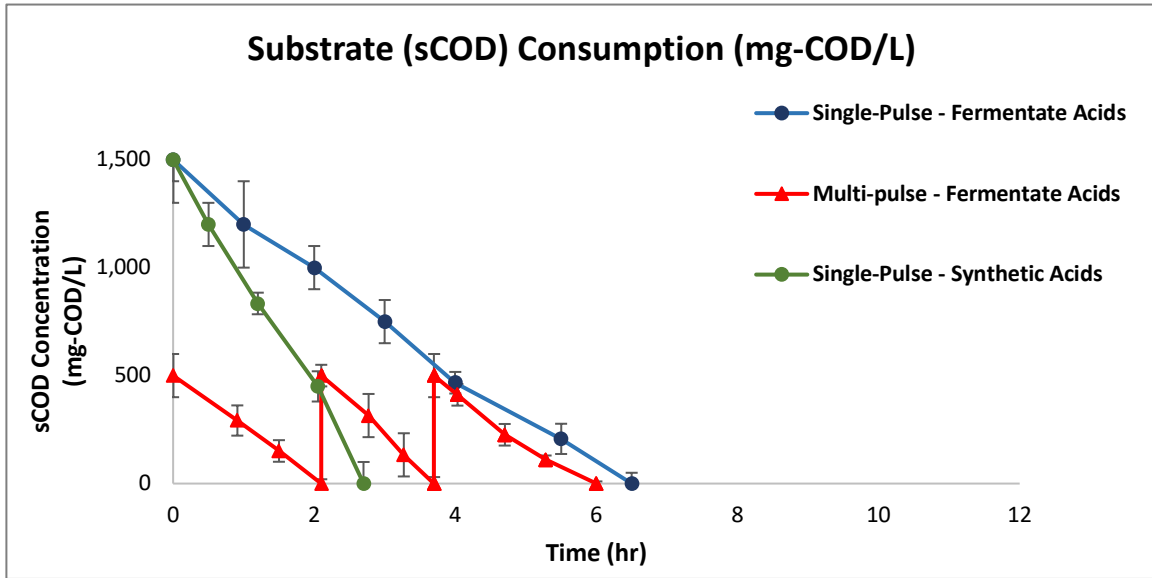


Figure 3- 12: Substrate (sCOD) concentration from batch experiments with different feeding patterns and acids (VFA) feedstocks in PHA reactor.

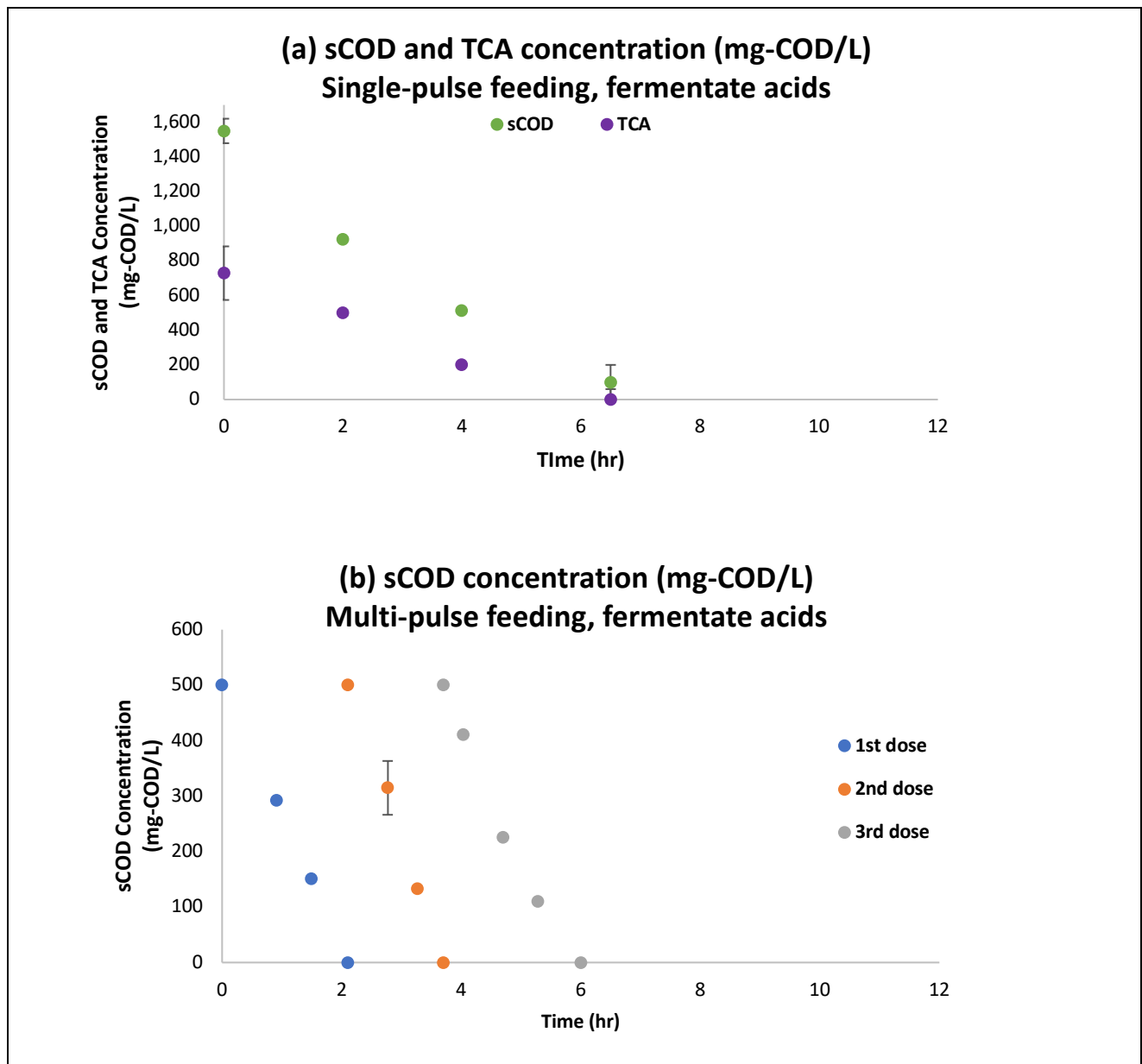


Figure 3- 13: (a) Substrate consumption in 1-pulse feeding system, (b) Substrate consumption in multi-pulse feeding system, in PHA reactor.

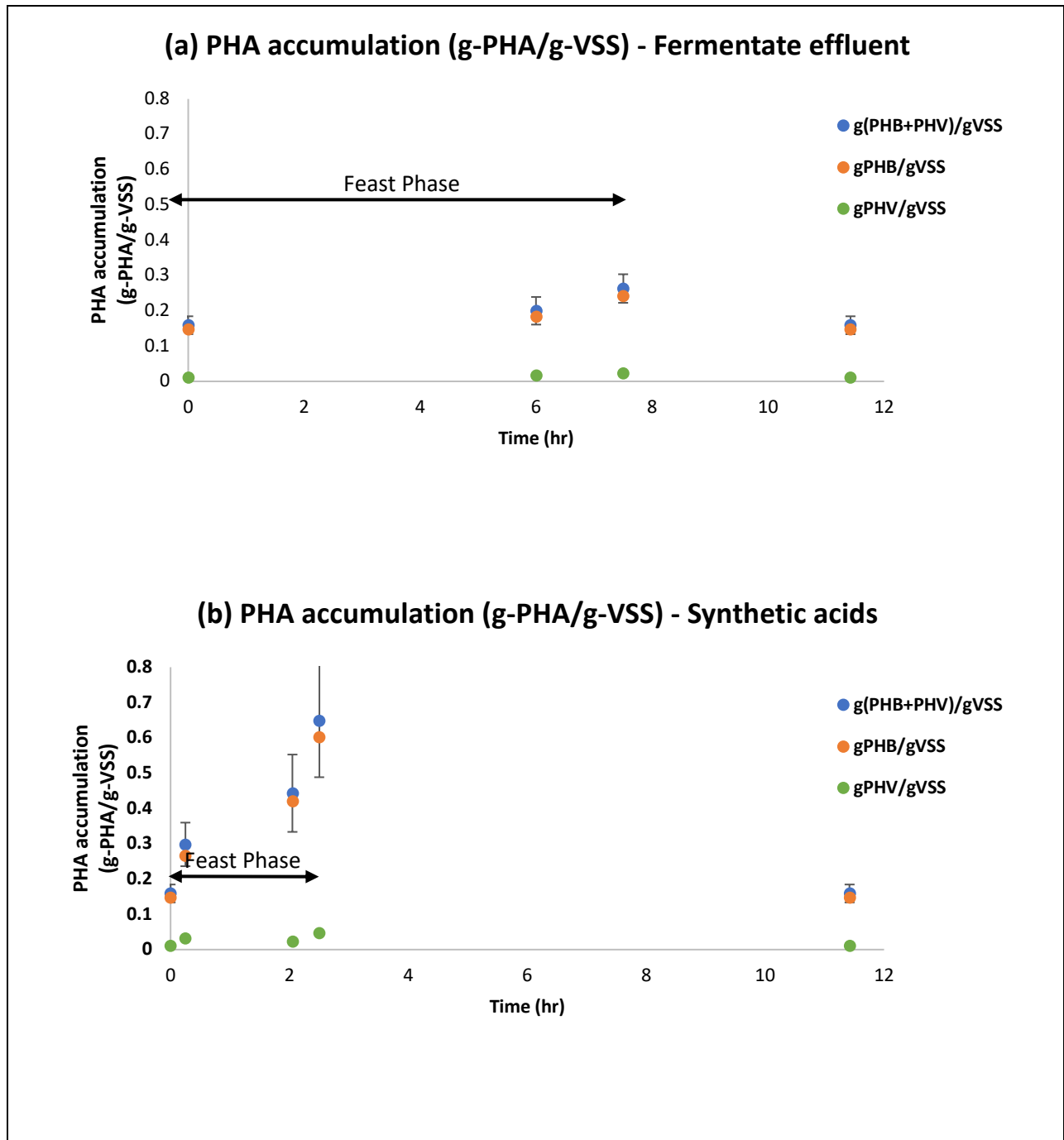


Figure 3- 14: Profile of PHA accumulation in (a) Fermentate effluent/acids feed and (b) Synthetic acids feed from batch experiments.

Appendix C (in reference to Chapter 4)

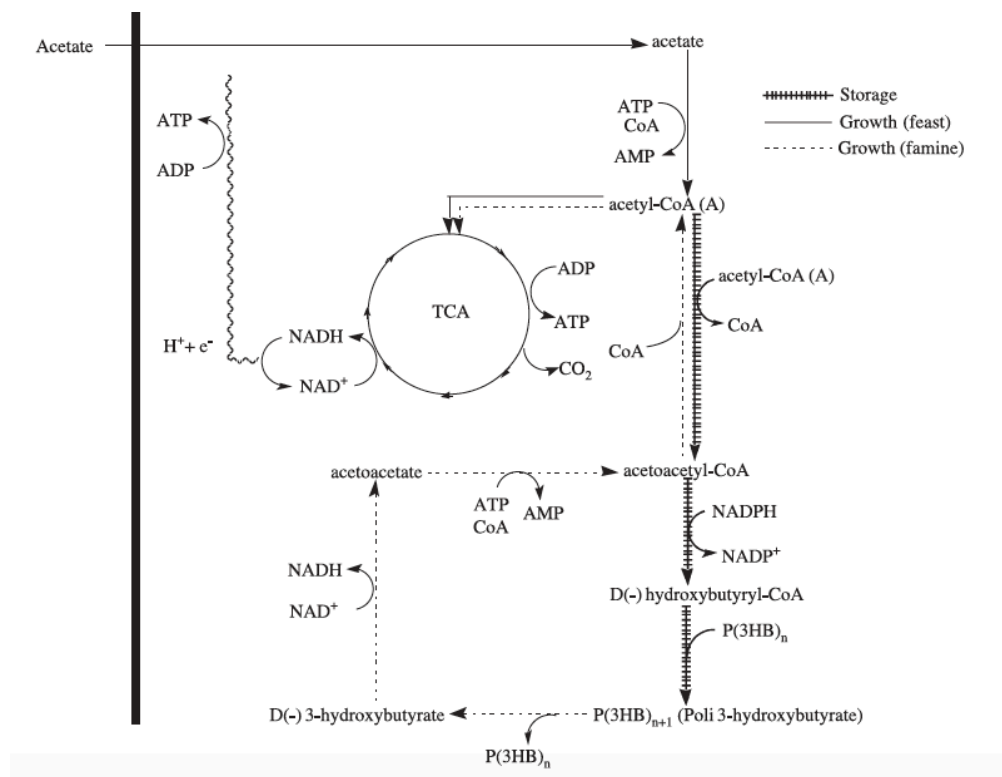


Figure 4- 9: PHA production pathway during feast and famine. Adapted from

Salehizadeh and Van Loosdrecht (2004)

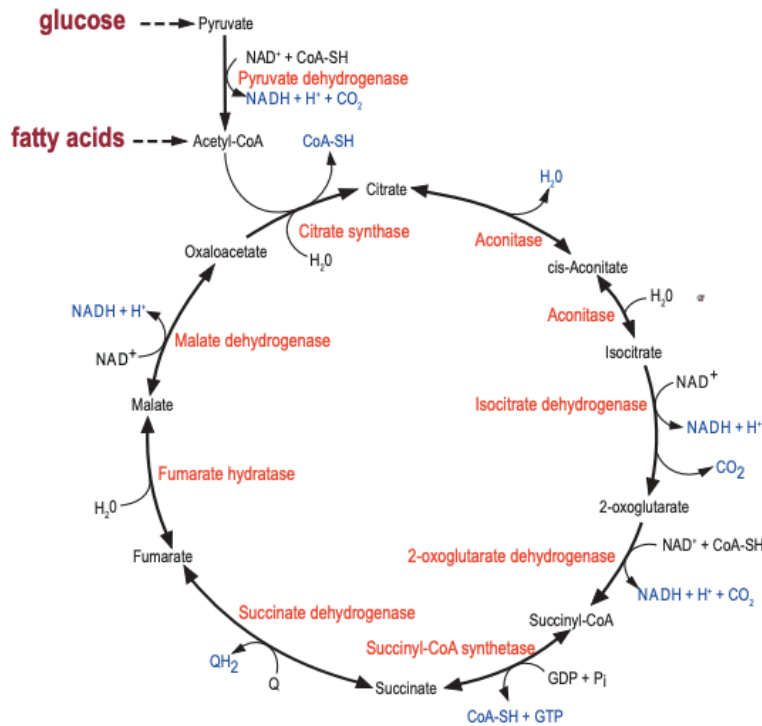


Figure 4- 10: Krebs cycle. Adapted from Czibik et al. (2014)

PHA Quantification Protocol: Samples were collected from the enrichment reactor at the end of the feast phase and end of the cycle. Five drops of formaldehyde were added for every 15mL of sample to stop biological activity. A biomass pellet was obtained after centrifuging (8133 rpm, 8000 x g, 10 min 4°C). Pellet was dried on a foil plate in a furnace for 1.5 hours at 105°C (Thermoscientific, Asheville, NC). 10-20 mg of dried biomass was reacted with 1.5 mL of 4:1 Propanol: HCl (37% conc) and 2 ml of chloroform at 100 °C for 120 minutes in a pre-programmed oven (HACH DRB 200). A total of 1mg benzoic acid in methanol was added before heating to serve as an internal standard. Samples were cooled to room temperature and 1.5mL deionized water added. The vials were vortexed for 5 seconds and centrifuged in 50mL Corning tubes to obtain separate phases (5,751 rpm, 4000 x g, 2 min 40C). The organic phase was

extracted and transferred to a microcentrifuge tube containing 50mg of anhydrous magnesium sulfate. The tubes were sealed, shaken and centrifuged (Eppendorf, Hauppauge, NY) (12,300 rpm, 14000 x g, 5 min 4°C). The supernatant was refrigerated at 4°C in glass scintillation vials for future analysis. The methyl esters were assayed using gas chromatography coupled with flame ionization detector (GC-FID, SRI 8610C) and MXT-Wax column (30 m 0.53 mmID 0.5 μ mdf) (Restek, Bellfonte, PA). Helium at 20 psi was used as carrier gas. The detector temperature was 220 °C. The oven temperature was 50°C for 5 minutes followed by a ramp to 195°C at 80C/min. Holding for longer at initial temperature allowed for the greatest separation of peaks at high temperatures. Pure Sigma poly[(R)-3-hydroxybutyric acid] (PHB) and poly(3-hydroxybutyric-co-3-hydroxyvaleric acid) (PHBV, 12% mol HV) with weights between 1 and 10 mg were esterified and analyzed along with reactor samples to be used as reference standards.

Table 4- 9: Abundance of Bacteria and Archaea in fermentation reactor.

	% of Bacteria	% of Archaea
HRT 2 days - pH 7	99.99939	0.0000061
HRT 2 days - pH 8	99.99975	0.0000025
HRT 2 days - pH 9	99.99912	0.0000088
HRT 4 days - pH 9	99.99996	0.0000004

HRT 6 days - pH 9	100.00000	0.0000000
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Table 4- 10: Abundance of Bacteria and Archaea in PHA reactor.

	% of Bacteria	% of Archaea
2mo	99.99993	0.0000007
4mo	99.99840	0.0000160
7mo	99.99940	0.0000060
11mo	99.99923	0.0000077
13mo	99.99984	0.0000016
16mo	99.99983	0.0000017

Table 4- 11: Dominant genera and species of microorganisms in fermentation reactor at HRT 2 days and pH 7.

HRT 2 days - pH 7	Dominant Genus (% of bacteria) (*)	Species (Identity %) (**)
Sample #1	<i>Tepidiphilus</i> (23%)	<i>Tepidiphilus margaritifer</i>
	<i>Sporanaerobacter</i> (11%)	(99%)
		<i>Sporanaerobacter acetigenes</i> (95%)
Sample #2	<i>Mitsuokella</i> (69%)	<i>Mitsuokella</i>
	<i>Petrimonas</i> (16%)	<i>jalaludinii</i> (99%)
		<i>Petrimonas mucosa</i> (97%)

Sample #3	<i>Mitsuokella</i> (82%)	<i>Mitsuokella jalaludinii</i> (99%)
Sample #4	<i>Sporanaerobacter</i> (22%) <i>Mitsuokella</i> (15%)	<i>Sporanaerobacter acetigenes</i> (95%) <i>Mitsuokella jalaludinii</i> (99%)
Sample #5	<i>Mitsuokella</i> (37%)	<i>Mitsuokella jalaludinii</i> (100%)
Sample #6	<i>Mitsuokella</i> (38%)	<i>Mitsuokella jalaludinii</i> (99%)

(*) Rest of the genera were <10% of bacteria, (**) Highest identity and score shown in NCBI Genome database. All samples are considered at steady-state, unless noted otherwise

Table 4- 12: Dominant genera and species of microorganisms in fermentation reactor at HRT 2 days and pH 8.

HRT 2 days - pH 8 (duration at this condition)	Dominant Genus (% of bacteria) (*)	Species (Identity %) (**)
Sample #1 (2 HRTs)	<i>Salmonella</i> (24%)	<i>Salmonella bongori</i> (99%)
Sample #2 (4 HRTs)	<i>Escherichia/Shigella</i> (20%) <i>Christensenella</i> (17%)	<i>Escherichia marmotae</i> (99%) <i>Christensenella minuta</i> (92%)
Sample #3 (5.5 HRTs)	<i>Escherichia/Shigella</i> (26%) <i>Salmonella</i> (23%)	<i>Escherichia marmotae</i> (99%)

		<i>Salmonella bongori</i>
		(99%)
Sample #4 (7.5 HRTs)	<i>Petrimonas</i> (25%)	<i>Petrimonas sulfuriphila</i>
	<i>Sporanaerobacter</i> (13%)	(92%)
		<i>Sporanaerobacter</i>
		<i>acetigenes</i> (98%)
Sample #5 (11 HRTs)	<i>Petrimonas</i> (19%)	<i>Petrimonas mucosa</i>
	<i>Sporanaerobacter</i> (15%)	(97%)
		<i>Sporanaerobacter</i>
		<i>acetigenes</i> (98%)

(*) Rest of the genera were <10% of bacteria, (**) Highest identity and score shown in NCBI Genome database. All samples are considered at steady-state, unless noted otherwise

Table 4- 13: Dominant genera and species of microorganisms in fermentation reactor at HRT 2 days and pH 9.

HRT 2 days - pH 9 (duration at this condition)	Dominant Genus (% of bacteria) (*)	Species (Identity %) (**)
Sample #1 (9.5 HRTs)	<i>Enterococcus</i> (45%)	<i>Enterococcus saigonensis</i>
	<i>Amphibacillus</i> (26%)	(99%)
		<i>Amphibacillus xylanus</i>
		(98%)
Sample #2 (23.5 HRTs)	<i>Amphibacillus</i> (16%)	<i>Amphibacillus xylanus</i>
	<i>Proteiniphilum</i> (15%)	(98%)
		<i>Proteiniphilum</i>
		<i>saccharofermentans</i> (99%)

Sample #3 (29 HRTs)	<i>Amphibacillus</i> (24%) <i>Tepidimicrobium</i> (18%)	<i>Amphibacillus xylanus</i> (99%) <i>Tepidimicrobium</i> <i>xylanilyticum</i> (93%)
Sample #4 (48.5 HRTs)	<i>Paracoccus</i> (26%) <i>Proteiniphilum</i> (13%)	<i>Paracoccus</i> <i>aestuariivivens</i> (99%) <i>Proteiniphilum</i> <i>saccharofermentans</i> (99%)
Sample #5 (53.5 HRTs)	<i>Tepidimicrobium</i> (28%) <i>Enterococcus</i> (13%)	<i>Tepidimicrobium</i> <i>ferriphilum</i> (94%) <i>Enterococcus saigonensis</i> (99%)
Sample #6 (65.5 HRTs)	<i>Enterococcus</i> (34%) <i>Tepidimicrobium</i> (14%)	<i>Enterococcus saigonensis</i> (99%) <i>Tepidimicrobium</i> <i>xylanilyticum</i> (93%)
Sample #7 (70.5 HRTs)	<i>Tepidimicrobium</i> (33%) <i>Enterococcus</i> (14%)	<i>Tepidimicrobium</i> <i>xylanilyticum</i> (93%) <i>Enterococcus saigonensis</i> (99%)
Sample #8 (74.5 HRTs)	<i>Amphibacillus</i> (52%)	<i>Amphibacillus xylanus</i> (99%)

(*) Rest of the genera were <10% of bacteria, (**) Highest identity and score shown in NCBI Genome database. All samples are considered at steady-state, unless noted otherwise

Table 4- 14: Dominant genera and species of microorganisms in fermentation reactor at HRT 4 days and pH 9.

HRT 4 days - pH 9 (duration at this condition)	Dominant Genus (% of bacteria) (*)	Species (Identity %) (**)
Sample #1 (0 HRTs) – transition period	<i>Enterococcus</i> (17%)	<i>Enterococcus</i>
	<i>Tepidimicrobium</i> (14%)	<i>saigonensis</i> (99%)
		<i>Tepidimicrobium</i> <i>ferriphilum</i> (94%)
Sample #2 (1.75 HRTs) – transition period	<i>Tepidimicrobium</i> (21%)	<i>Tepidimicrobium</i>
	<i>Proteiniphilum</i> (13%)	<i>ferriphilum</i> (94%)
		<i>Proteiniphilum</i> <i>saccharofermentans</i> (99%)
Sample #3 (4 HRTs)	<i>Vagococcus</i> (33%)	<i>Vagococcus lutrae</i> (98%)
Sample #4 (5.25 HRTs)	<i>Vagococcus</i> (62%)	<i>Vagococcus lutrae</i> (99%)
	<i>Tepidimicrobium</i> (16%)	<i>Tepidimicrobium</i>
		<i>ferriphilum</i> (94%)

(*) Rest of the genera were <10% of bacteria, (**) Highest identity and score shown in NCBI Genome database. All samples are considered at steady-state, unless noted otherwise

Table 4- 15: Dominant genera and species of microorganisms in fermentation reactor at HRT 6 days and pH 9.

HRT 6 days - pH 9 (duration at this condition)	Dominant Genus (% of bacteria) (*)	Species (Identity %) (**)
Sample #1 (1.6 HRTs) – transition period	<i>Vagococcus</i> (51%)	<i>Vagococcus lutrae</i>
	<i>Tepidimicrobium</i> (14%)	(99%) <i>Tepidimicrobium</i>
		<i>xylanilyticum</i> (95%)
Sample #2 (2.3 HRTs)	<i>Vagococcus</i> (42%)	<i>Vagococcus lutrae</i> (99%)
Sample #3 (3.8 HRTs)	<i>Vagococcus</i> (57%)	<i>Vagococcus lutrae</i>
	<i>Tepidimicrobium</i> (13%)	(99%) <i>Tepidimicrobium</i>
		<i>ferriphilum</i> (95%)
Sample #4 (4.6 HRTs)	<i>Vagococcus</i> (27%)	<i>Vagococcus</i> <i>acidifermentans</i> (99%)

(*) Rest of the genera were <10% of bacteria, (**) Highest identity and score shown in NCBI Genome database. All samples are considered at steady-state, unless noted otherwise

Table 4- 16: Dominant genera and species of microorganisms in PHA reactor.

Sample # (timeline of reactor’s operation)	Dominant Genus (% of bacteria) (*)	Species (Identity %) (**)
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Sample #1 (seed)	<i>Methylovorus</i> (11%)	<i>Methylovorus menthalis</i> (99%)
Sample #2 (2 months)	<i>Acinetobacter</i> (24%)	<i>Acinetobacter vivianii</i> (99%)
Sample #3 (4 months)	<i>Acinetobacter</i> (10%)	<i>Acinetobacter</i> <i>radioresistens</i> (99%)
Sample #4 (5 months)	<i>Comamonas</i> (15%)	<i>Comamonas jiangduensis</i> (100%)
Sample #5 (7 months)	<i>Aurantimonas</i> (19%) <i>Acinetobacter</i> (10%)	<i>Aurantimonas endophytica</i> (96%) <i>Acinetobacter</i> <i>radioresistens</i> (99%)
Sample #6 (11 months)	<i>Acinetobacter</i> (27%)	<i>Acinetobacter</i> <i>radioresistens</i> (100%)
Sample #7 (12 months)	<i>Xanthobacter</i> (23%) <i>Aurantimonas</i> (17%)	<i>Xanthobacter agilis</i> (99%) <i>Aurantimonas endophytica</i> (96%)
Sample #8 (13 months)	<i>Labrys</i> (21%) <i>Acinetobacter</i> (14%) <i>Aurantimonas</i> (11%)	<i>Pseudoxanthobacter</i> <i>liyangensis</i> (96%) <i>Acinetobacter tandoii</i> (99%) <i>Aurantimonas endophytica</i> (96%)
Sample #9 (14 months)	<i>Acinetobacter</i> (19%) <i>Labrys</i> (19%)	<i>Acinetobacter populi</i> (99%) <i>Pseudoxanthobacter</i>

	<i>Comamonas</i> (16%)	<i>liyangensis</i> (96%)
		<i>Comamonas jiangduensis</i> (99%)
Sample #10 (15 months)	<i>Acinetobacter</i> (61%)	<i>Acinetobacter</i>
	<i>Labrys</i> (12%)	<i>radioresistens</i> (99%)
		<i>Pseudoxanthobacter</i>
		<i>liyangensis</i> (97%)
Sample #11 (16 months)	<i>Acinetobacter</i> (60%)	<i>Acinetobacter</i>
	<i>Labrys</i> (19%)	<i>radioresistens</i> (99%)
		<i>Pseudoxanthobacter</i>
		<i>liyangensis</i> (96%)
Sample #12 (16.5 months)	<i>Xanthobacter</i> (20%)	<i>Xanthobacter agilis</i> (99%)

(*) Rest of the genera were <10% of bacteria, (**) Highest identity shown in NCBI Genome database

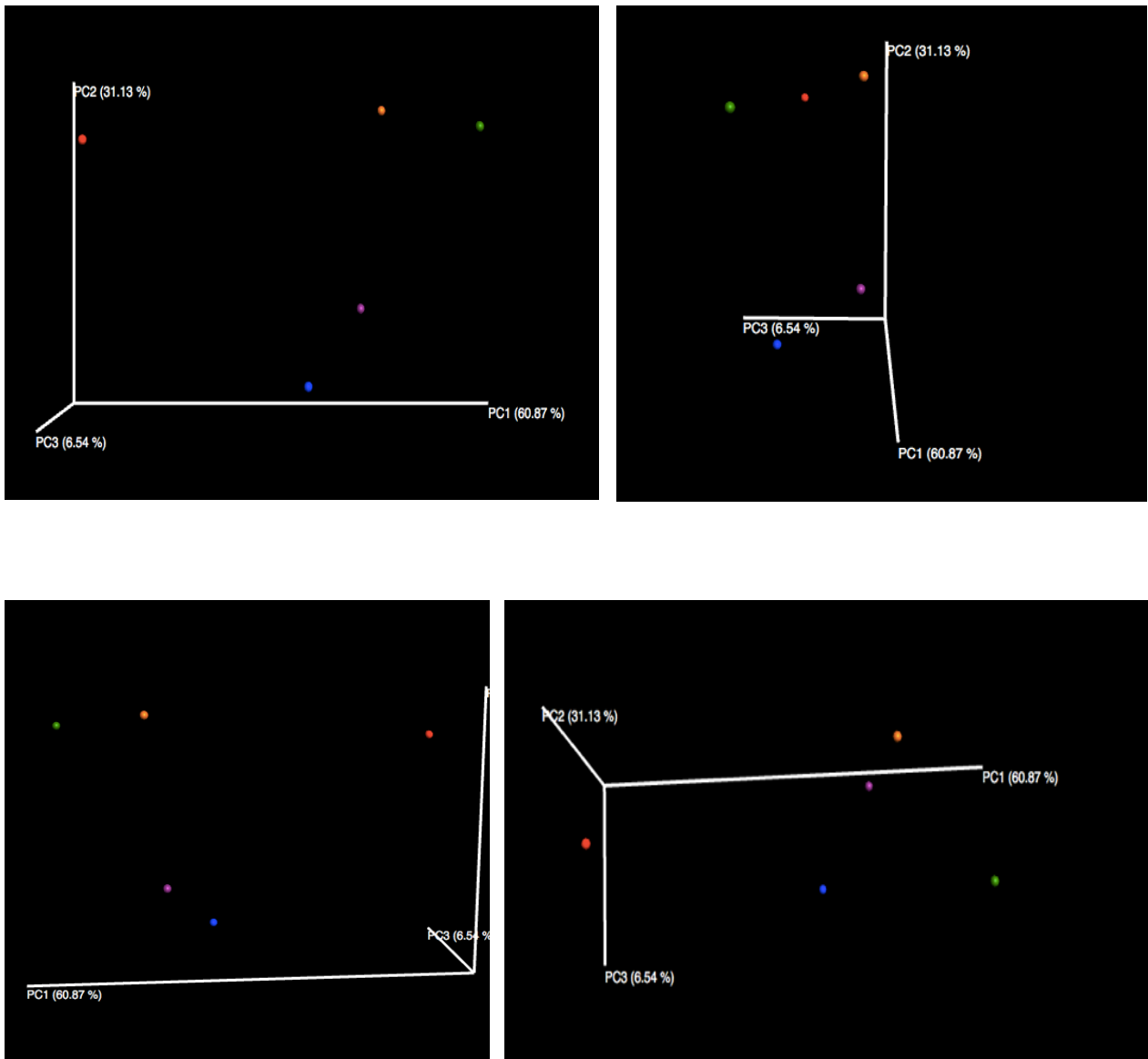


Figure 4- 11: Beta diversity in Family level (Bray Curtis) in Fermentation Reactor. Green: HRT 2 days – pH 7, Orange: HRT 2 days – pH 8, Red: HRT 2 days – pH 9, Blue: HRT 4 days – pH 9, Purple: HRT 6 days – pH 9.

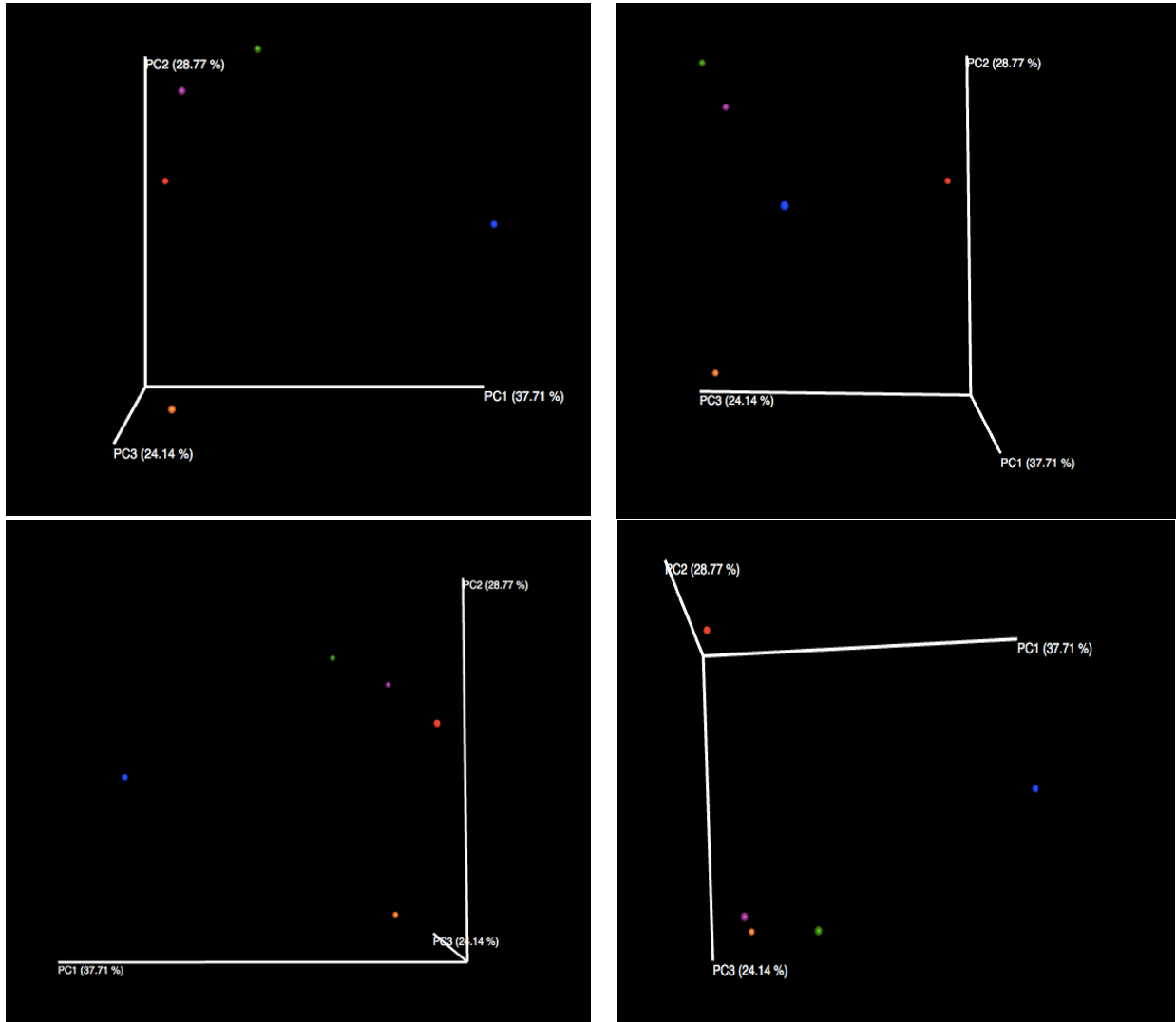


Figure 4- 12: Beta diversity in Family level (Bray Curtis) in PHA Reactor. Blue: seed, Green: 3 months, Purple: 7 months, Red: 11 months, Orange: 15 months.