EFFECT OF ENZYMATIC HYDROLYSIS PRETREATMENT ON BATCH ANAEROBIC DIGESTION OF WASTEWATER GENERATED IN DESICCATED COCONUT PROCESSING PLANTS

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Abstract

Enzymes are widely used to accelerate the biochemical reactions in biological wastewater treatment processes. Commercially developed enzymes such as amylases, proteases and lipases improve microbial biodegradation by accelerating the hydrolysis rate of carbohydrates, proteins and lipids. In this study, wastewater generated in desiccated coconut processing plants were pre-treated with lipase originated from porcine pancreas and then anaerobic digestion was performed to evaluate the effect of enzymatic hydrolysis pre-treatment. Hydrolysis pre-treatment was performed using three different concentrations of lipase i.e. 0% enzyme, 0.01% (w/v) enzyme and 0.1% (w/v) enzyme. Following hydrolysis pre-treatment, anaerobic batch digestion was performed in twenty-four 50 ml reactors under two scenarios i.e. scenario 1: twelve reactors under pH adjusted condition and Scenario 2: 12 were pH not adjusted. Under scenario 1, hydrolyzed pre-treated wastewater samples with three separate enzyme concentrations i.e. 0%, 0.01% and 0.1% were used as substrates and at each enzyme concentration four different inoculum to substrate ratio in volume basis of 1:4, 2:3, 3:2 and 4:1 were used. Similar procedure was followed under Scenario 2 as well. All anaerobic batch experiments were conducted under atmospheric temperature of 31±1°C and pressure of 0.997±0.002atm. Batch experiment was conducted for 60 days and during this period, samples were analyzed. The highest initial biogas production rate of 25.43 ml/day and highest average gas production rate during first 10 days of 7.16 ml/day were achieved for the sample with 0.1% lipase at inoculum to substrate ratio in volume basis of 2:3 under scenario 1. Following 60 days of complete degradation, for the same sample, experimental bio-methane yield of 42.75 mlCH4/gVS substrate added, cumulative biogas production of 95ml, cumulative methane production of 81.55ml, TS reduction of 51.77% and VS reduction of 67.68% were also achieved. The wastewater generated in desiccated coconut processing plants consists of high concentrations of medium chain saturated triglycerides. These triglycerides were hydrolyzed quickly when enzyme was added during enzymatic pre-treatment, resulting higher initial biogas production rate in the beginning as well as higher daily biogas production rate during first 10 days. The initial biogas production rate and daily biogas production rate during first 10 days of the initially pH-adjusted samples into pH 7.0 showed higher biogas production rate than pH not adjusted samples because it was the most favorable pH value for the methanogens for their optimal growth. The bio-methane potential increased when the inoculum to substrate ratio decreased because quantity of hydrolyzed triglycerides available for the anaerobic microorganisms to convert into methane gas was higher. The bio-methane yield of enzyme added samples were much higher than the enzyme

not added samples because enzymes accelerated the hydrolysis of lipids. According to this research study, it can be concluded that adding enzymes will improve the batch anaerobic digestion process of wastewater generated in desiccated coconut processing plants.

Keywords: Anaerobic digestion; enzymatic hydrolysis; lipase; desiccated coconut industry; lipid degradation

DEDICATION

Dedicated with gratitude to my loving **PARENTS** for being the greatest pliers of my life.

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LIST OF ABBREVIATIONS

Abbreviation	Description
DC	Desiccated coconut
DCWW	Desiccated coconut wastewater
COD	Chemical oxygen demand
sCOD	Soluble chemical oxygen demand
BOD	Biochemical oxygen demand
APHA	American public health association
TS	Total solid
VS	Volatile solid
O&G	Oil and grease
VSS	Volatile suspended solid
CWW	Desiccated coconut wastewater
LP	Lipase

1. INTRODUCTION

Wastewater generated in desiccated coconut industry typically consists of coconut kernel water, coconut water and sterilization water [1]. It consists of high concentration of biodegradable organic compounds such as carbohydrates, lipids and fatty acids.

Wastewater generated in desiccated coconut processing plants, is typically treated via combined effect of physical, chemical and biological wastewater treatment processes. Major part of the biodegradable organic matter is degraded via the anaerobic wastewater treatment process. Biological hydrolysis of biodegradable organic compounds has been identified as the rate-limiting step in anaerobic digestion of wastewater containing lipids and fatty acids [2]. Presence of these lipids and fatty acids led into inhibition of anaerobic digestion according to the previous research studies [3].

Inhibition caused by lipids in anaerobic digestion was addressed for different types of wastewater that consisted of high concentrations of lipids and fatty acids via different strategies such as enzymatic hydrolysis pre-treatment, saponification pre-treatment and optimizing the substrate to inoculum ratio. The effect of enzymatic hydrolysis pre-treatment combined with saponification pre-treatment and optimizing the substrate to inoculum ratio has not been studied for the anaerobic treatment of wastewater generated in desiccated coconut processing plants so far. Therefore, in this research study, optimization of the combined effect of these three factors will be conducted using experimental approach.

1.1. Research problem

The major problem in anaerobic treatment process of wastewater generated in desiccated coconut processing plants is presence of higher concentration of biodegradable organic matter and presence of inhibitory compounds such as lipids and fatty acids.

The conceptual diagram shown in Figure 1.1 identifies the research problem in this study.

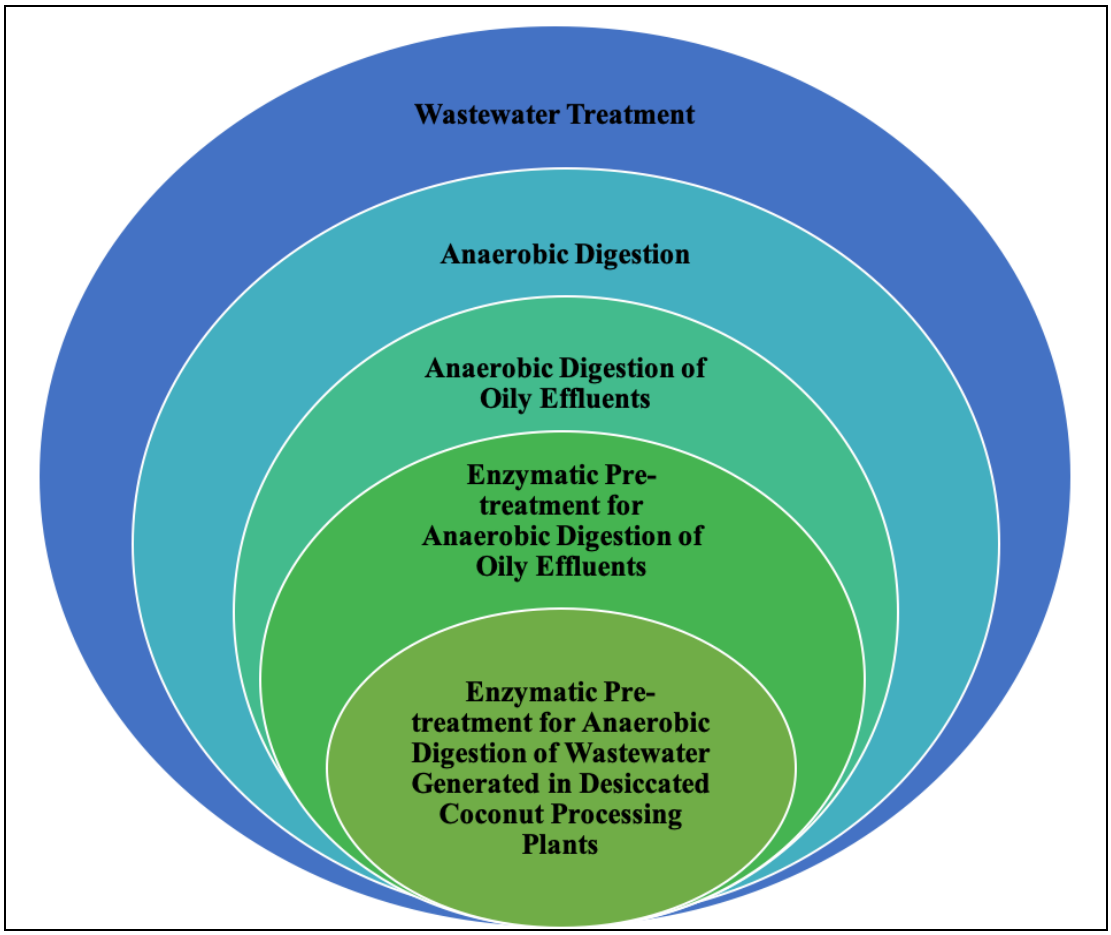

Figure 1.1: Conceptual diagram for enzymatic pretreatment strategy to overcome inhibition caused by lipids in desiccated coconut processing plants

Therefore, the focus of this research study is to identify the inhibitory conditions in anaerobic digestion and optimize the anaerobic digestion process via enzymatic hydrolysis pre-treatment combined with initial pH adjustment of the substrate and optimizing the substrate to inoculum ratio.

1.2. Research objectives

- Detail characterization of wastewater generated in desiccated coconut processing plants and identification of different constituents containing lipids

- Conducting micro scale experiments to monitor the process performance
- Develop new pre-treatment strategy to overcome inhibition caused by lipids in anaerobic digestion

1.3. Thesis outline

The thesis begins with extensive literature review on the characteristics of wastewater generated in desiccated coconut processing plants, inhibition caused by lipids and fatty acids on anaerobic treatment process and different strategies used to overcome the inhibition in Chapter 2. In Chapter 3, materials and methods, which were used throughout this research, are described. In Chapter 4, experimental results and discussion are presented. Finally, conclusions and recommendations are given in Chapter 5.

2. LITERATURE REVIEW

In this chapter, extensive literature review on pre-treatment strategies used to overcome inhibition and improve the anaerobic digestion are discussed.

2.1. Anaerobic digestion

Anaerobic digestion is a sustainable waste management strategy, which had been used for the management of different types of wastes, which consist of high concentrations of biodegradable substances. These biodegradable substances undergo a series of biochemical reactions such as hydrolysis, acidogenesis, acetogenesis and methanogenesis by syntrophic interactions between anaerobic microorganisms as shown in Figure 2.1. These syntrophic anaerobic microorganisms convert these complex biodegradable substances such as carbohydrates, proteins and lipids into smaller substances and reduce the eutrophication capacity of the wastes.

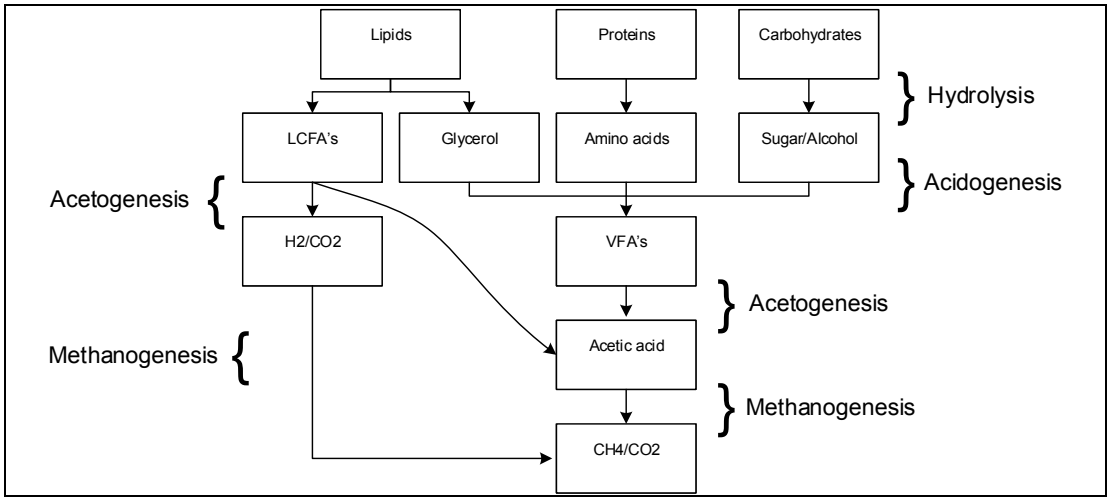

Figure 2.1: Metabolism pathway in anaerobic digestion

In hydrolysis process, carbohydrates, proteins and lipids are converted into dissolved compounds such as sugars, alcohols, amino acids, fatty acids and glycerol by the exoenzymes excreted by anaerobic hydrolytic microorganisms. Those hydrolytic products are converted into volatile fatty acids by anaerobic acidogenic microorganisms. The volatile fatty acids and long chain fatty acids are converted into

acetic acid, carbon dioxide gas and hydrogen gas by anaerobic acetogenic microorganisms. The final products of acetic acid, carbon dioxide gas and hydrogen gas are converted into methane and carbon dioxide gas by anaerobic methanogenic microorganisms.

2.2. Lipid degradation in anaerobic digestion

Lipid generates in various industries such as olive oil mills, slaughterhouses, diary product industries, edible oil factories, fish processing plants and poultry factories [2]. Lipids are triglycerides, which consists of trans-esterified three fatty acid units with one glycerol unit. In anaerobic digestion, lipids are degraded into glycerol and fatty acids by exoenzymes excreted by anaerobic hydrolytic microorganisms as shown in Figure 2.2. These exoenzymes are known as lipases.

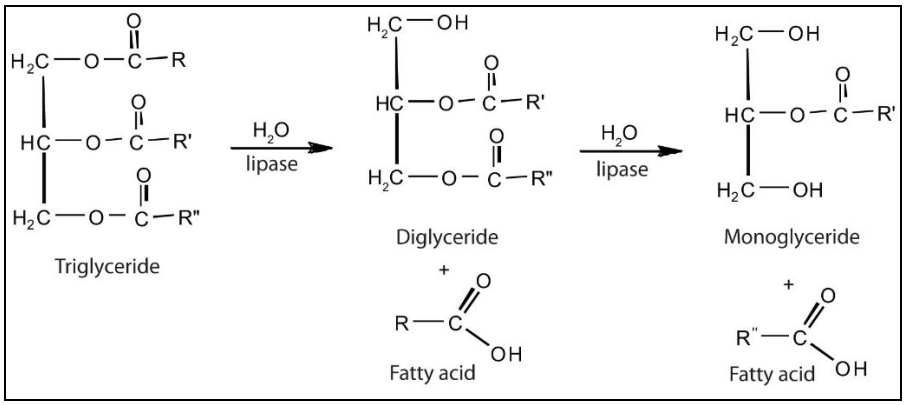

Figure 2.2: Hydrolysis of lipids.

Glycerol is easily degraded by anaerobic microorganisms but fatty acids undergo a complex degradation pathway called as "β-oxidation" [4]. β-oxidation occurs inside the cells of fatty acid degrading microorganisms. The final products of the biochemical reactions in β-oxidation are acetic acid, carbon dioxide gas and hydrogen gas.

Considering the fatty acid catabolism in "Krebs Cycle",

- Saturated fatty acids are broken down in pairs.
- For unsaturated sites, the unsaturated bond is isomerized as saturated fatty acids for the normal β-oxidation.

- The branched fatty acids require α-oxidation inside the peroxisome.

Hydrogen gas is converted into methane gas by hydrogenotrophic anaerobic microorganisms and acetic acid is converted into methane gas by aceticlastic anaerobic microorganisms [5]. The biochemical reactions of methane formation in anaerobic digestion are mentioned below.

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad \Delta G°_f = -52.36 \quad \Delta H°_f = (+)18.16$$

$$CO_2 + 4\ H_2 \rightarrow CH_4 + 2H_2O \quad \Delta G°_f = -130.5 \quad \Delta H°_f = (-)252.7$$

Considering the above thermodynamic data, $\Delta G°_f > 0$ and therefore the reactions are spontaneous but for the breakdown of acetic acid into methane gas require energy. Therefore, a syntrophic corporation between anaerobic methanogens and other anaerobic microorganisms should be there to occur these reactions.

2.3. Inhibition caused by lipids in anaerobic digestion

According to the previous research conducted on anaerobic digestion of oily effluents, it has been identified that lipid is one of the potential inhibitory substances in anaerobic digestion. In large-scale continuous anaerobic reactors, which treat oily effluents, inhibition occurs manly due to,

- When higher input loading rate of lipids into the anaerobic bioreactors occurs than the average inhibitory concentration of lipids (1 g/l – 5 g/l) [6]
- Beyond inhibitory concentrations of lipids, the lipids will attach around the cell walls of the anaerobic microorganisms causing mass transfer limitations in their metabolism process. [6]
- pH drop occurs inside anaerobic bioreactors because of rapid acidogenesis due to higher growth rate of acidogenic anaerobic microorganisms. Normally desirable pH range for the growth of anaerobic methanogens is 6.8 – 7.2 [6]

The biomass growth phases of anaerobic microorganisms are shown in Figure 2.3 [7]. The lag phase is the time required for microorganisms to acclimate to their new environment. The exponential growth phase is the phase where microorganisms multiplying at their maximum rate when there are no limitations in substrate or nutrients. In stationary phase, number of microorganisms remains constant where

amount of cell growth is offset by the cell death. In death phase, the substrate has depleted and the number of microorganisms reduces due to their death.

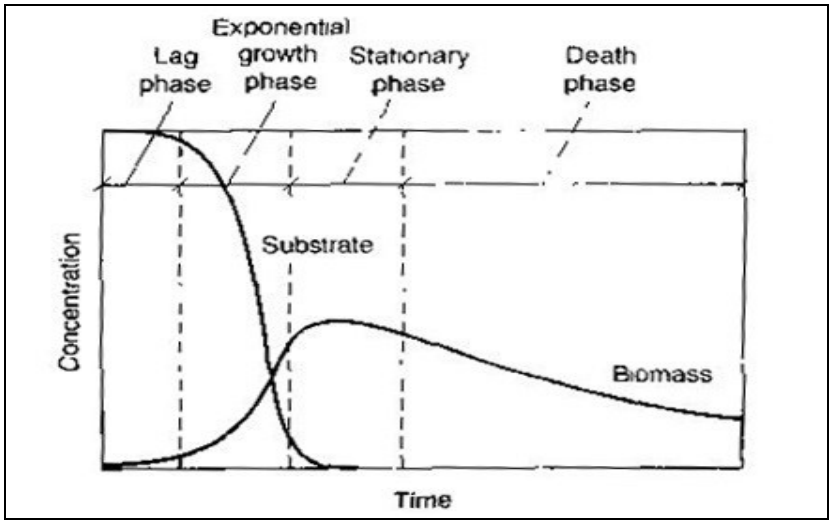

Figure 2.3: Biomass growth phases

Lipids are hydrophobic and high-energy nutrients than carbohydrates and proteins. Therefore, anaerobic microorganisms tend to degrade lipids in the end. Even though the effect of inhibition caused by lipids is not permanent, long recovery times may be required for anaerobic microorganisms for the degradation of lipids [2]. The anaerobic bioreactors which treat oily effluents mainly fail because of increase in lag phase and unable to treat oily effluents up to the expected level. Sometimes those bioreactors completely fail due to permanent inhibition caused by higher input loading rate of lipids.

2.4. Inhibition caused by lipids in anaerobic digestion of wastewater generated in desiccated coconut processing plants

Wastewater generated in desiccated coconut processing plants mainly consists of wash water, coconut water and condensate loss in flow rate ratio of 16:4:1.

- Wash water generates during paring and washing process
- Coconut water generates during the splitting process
- Condensate loss generates during the sterilization process

According to previous studies, typical characteristics of wastewater generated in desiccated coconut processing plants is shown in Table 2.1[1].

Table 2.1: Typical characteristics of wastewater generated in desiccated coconut industries

Parameter	Range
pH Value	4.0 – 5.5
Chemical Oxygen Demand – COD (mg/l)	4000 – 8000
Biochemical Oxygen Demand – BOD (mg/l)	1000 – 5000
Total Lipids (mg/l)	4000

The fatty acid composition of wastewater generated in desiccated coconut industries is shown in Figure 2.4 [8].

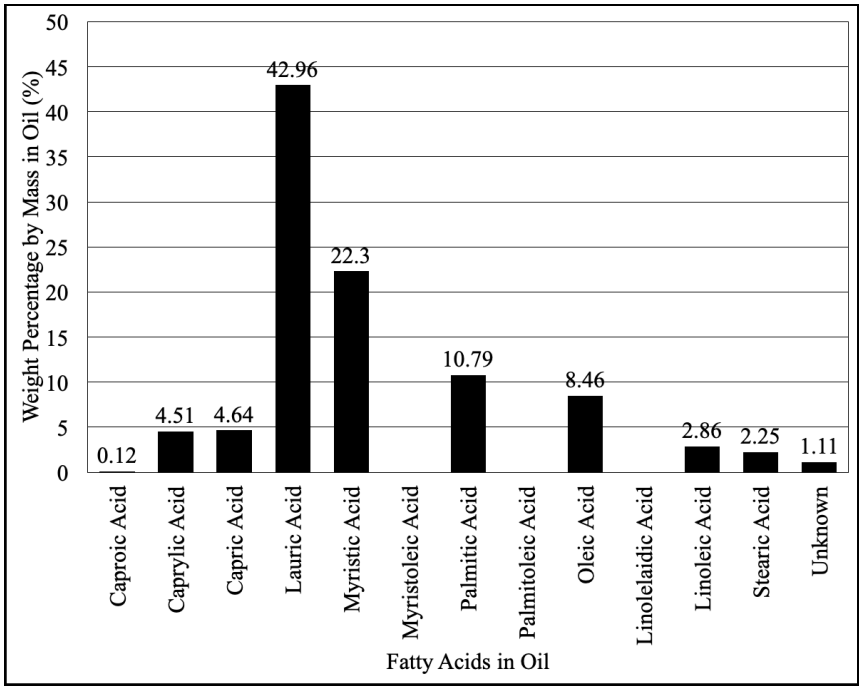

Figure 2.4: Fatty acid composition in wastewater generated in desiccated coconut industries

The presence of high concentrations of oil and grease typically leads towards the inhibition in anaerobic digestion process. It will cause adverse effects such as,

- Instability of the anaerobic reactors
- Biomass washout
- Clogging of the pipelines
- Eutrophication
- Odor problems

- Reduction of the treatment efficiency
- Reduction of the methane yield

2.5. Strategies used to overcome inhibition caused by lipids in anaerobic digestion of various oily effluents

Different strategies were conducted in previous research studies in order to overcome inhibition caused by lipids in anaerobic digestion as shown in Table 2.2.

Table 2.2: Strategies used to overcome inhibition caused by lipids in anaerobic digestion

Pre-treatment Strategy	Used wastewater	Used Chemicals for Pre-treatment	Varied Process Conditions	References
Enzymatic pretreatment	Dairy wastewater	Lipase originated from Penicillium Sp.	With enzyme and without enzyme	[9]
	Dairy wastewater	Lipase originated from Porcine Pancreas	With enzyme and without enzyme	[10]
			Hydrolysis pre-treatment time	
	Lipid rich solid waste from oil refining industry	Lipase originated from Staphylococcus Haemolyticus strain	Concentration of waste	[11]
	Effluents from a poultry processing plant	Enzymes produced from fungi Penicillium Simplicissimum and Penicillium Brevicompactum	Type of enzyme	[12]
			Concentration of enzyme	
	Tannery wastewater	Steapsin lipase	Concentration of enzyme	[13]
Addition of oil absorbent	Cattle waste and Bentonite bounded oil mixture	Bentonite	Concentration of waste	[14]
			Concentration of absorbent	
			Anaerobic batch reactor residence time	
	Slaughterhouse waste	Zeolite	Anaerobic batch reactor residence time	[15]
Ultrasound sonication	Dairy wastewater	Lipase Z (lipase from Candida rugosa-free	Ultrasonic sonication time	[16]

		form)	Concentration of enzyme	
			Concentration of waste	
			Operational temperature	
	Landfill leachate	No chemicals required for this pre-treatment	Specific energy input of ultrasonic sonication	[17]
			Ultrasonic sonication time	
Two-stage anaerobic digestion	Oily food waste	Alkaline supplement – Calcium Carbonate	Two sequential anaerobic reactors operational temperature	[18]
			Two sequential anaerobic reactors hydraulic retention times	
	Olive oil mill effluents	NA	Two sequential anaerobic reactors	[19]
Anaerobic co-digestion	Pure and slaughterhouse carbohydrate, protein, and lipid substrates were tested	NA	Carbohydrate, Protein and Lipid composition in waste	[20]
	Organic fraction of municipal solid waste and FOG waste from a sewage treatment plant	NA	Co-digestion ratio	[21]
Alkaline saponification	Solid slaughterhouse fatty waste	Sodium Hydroxide	Saponification combined with recirculation of anaerobic sludge	[22]
	Dairy waste activated sludge	Sodium Hydroxide Potassium Hydroxide Calcium Hydroxide	Type of alkaline solution	[23]
			Homogenizing pre-treatment time	
	Organic fraction of municipal solid waste	Calcium Hydroxide	Concentration of alkaline solution	[24]
			Solubilizaton time	

- Enzyme addition – Extracellular enzymes that aid to hydrolyze and transfer the substrates from the liquid phase to the anaerobic microorganisms for the digestion.
- Absorbent addition – Attract soluble fatty acids towards their surfaces and reduce the mass transfer limitations of anaerobic microorganisms' due to high concentrations of lipids.
- Ultrasound Sonication – Reducing the particle size of the organic substances and increase the sCOD in wastewater for the ease the digestion.
- Process parameters – Maintaining proper process parameters such as temperature, feeding sequence will improve the growth rate of anaerobic microorganisms and enhance the anaerobic digestion.
- Co-digestion – Providing a suitable nutrient supply for anaerobic microorganisms to improve their microbial growth and enhance the anaerobic digestion.
- Saponification – Alkaline hydrolysis of triglycerides.

2.6. Identification of a pre-treatment strategy to overcome inhibition caused by lipids

The wastewater generated in desiccated coconut processing plants consists of high concentrations of lipids. Therefore, above mentioned strategies such as enzymatic pretreatment, addition of oil absorbent, ultrasound sonication, two-stage anaerobic digestion, anaerobic co-digestion and alkaline saponification could be used to overcome from lipid inhibition in anaerobic digestion of wastewater generated in desiccated coconut processing plants [6]. Among those strategies, it was concluded that enzymatic pretreatment is a promising solution to overcome inhibition caused by lipids because,

- Enzymes do not interrupt anaerobic microbial growth because they are biological catalysts. Enzyme loading does not cause any harm to anaerobic microorganisms.

- Enzymes enhance the catabolism of organic matter by reducing activation energies of biological reactions. Therefore, enzymes have a direct correlation with substrate utilization by anaerobic microorganisms.
- Enzyme addition into the anaerobic bioreactors with input feed is very easier process than other strategies.
- Enzymes are commercially expensive but with mass production using new advanced technologies, they are becoming commercially cheaper.

The first biochemical reaction in anaerobic digestion is hydrolysis. Hydrolysis is typically catalyzed by the exoenzymes excreted by anaerobic microorganisms. But, whenever there is a higher substrate loading, those anaerobic microorganisms are not capable of excreting exoenzymes in that rate. Therefore, addition of enzymes will ease their catabolism and help to overcome from inhibition. Enzymes such as lipases increase the reaction rate by reducing the activation energy as shown in the Figure 2.5 and Figure 2.6 [25].

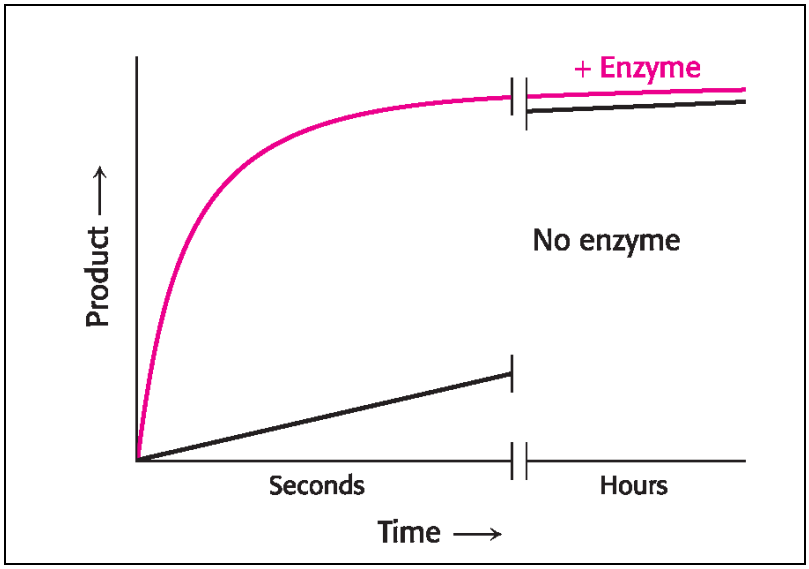

Figure 2.5: Enzymes increase the rate of reaction

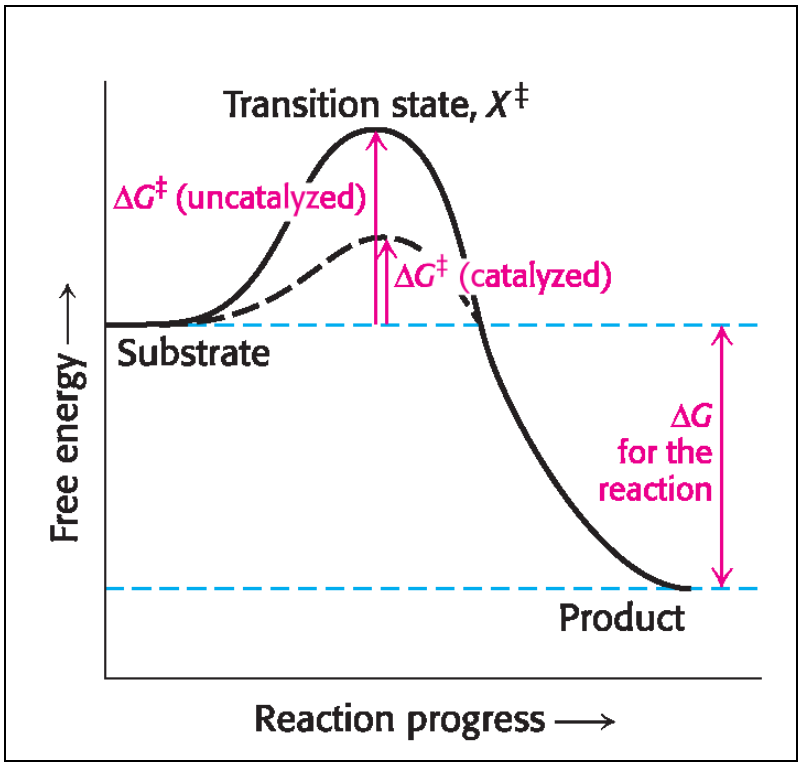

Figure 2.6: Enzymes decrease the activation energy

2.7. Enzymes used hydrolysis various oily effluents

There are three major types of enzymes used for the hydrolysis of organic matter. They are amylase, which catalyzes the breakdown of carbohydrates, protease which catalyzes the breakdown of proteins and lipase which catalyzes the breakdown of lipids. In order to overcome lipid inhibition in oily effluents, different lipases produced from different sources are used as shown in Table 2.3.

Table 2.3: Lipases and their applications on treating oily effluents in anaerobic digestion

Type of Lipase	Wastewater	Operating Conditions	References
Candida rugosa	Dairy wastewater	Pretreatment time 8h – 16h, Optimum conditions pH 6.6 and Temperature 40^{o}C	[26]
	Animal fat, vegetable oil and floatable grease skimmed from food waste	5.0% (w/w) Lipase concentration, Pretreatment time 0 – 36h, Operating temperature 40^{o}C, pH 7.0, Mixing speed 200 RPM	[27]

Geothricum candidum	Dairy wastewater	Pretreatment time 8h – 16h, Optimum conditions pH 6.6 and Temperature 40^{o}C	[26]
Porcine pancreatic	Animal fat, vegetable oil and floatable grease skimmed from food waste	5.0% (w/w) Lipase concentration, Pretreatment time 0 – 36h, Operating temperature 40^{o}C, pH 7.0, Mixing speed 200 RPM	[27]
Aspergillus oryzae	Animal fat, vegetable oil and floatable grease skimmed from food waste	5.0% (w/w) Lipase concentration, Pretreatment time 0 – 36h, Operating temperature 40^{o}C, pH 7.0, Mixing speed 200 RPM	[27]
Lipase produced by a Penicillium sp. during solid-state fermentation	Dairy semi synthetic wastewater	0.1% (w/v) Lipase concentration, Pretreatment time 24h, Operating temperature 30^{o}C, Mixing speed 120 RPM	[9]
	Dairy semi synthetic wastewater	0.1% (w/v) Lipase concentration, Pretreatment time 24h, Operating temperature 30^{o}C, pH 7.0, Mixing speed 120 RPM	[28]
Enzymes produced by immobilized fungus spores of Rhizopus oryzae F-814	Starch processing wastewater, Meat processing wastewater, slaughterhouse wastewater, mixed dairy processing wastewater, soybean processing factory wastewater	Pretreatment time 30h, Operating temperature 28^{o}C, Mixing speed 180 RPM	[29]
Staphylococcus haemolyticus	By product of sunflower oil refining industry	Pretreatment time 48h, Operating temperature 37^{o}C, Mixing speed 200 RPM	[11]
Lipase (Steapsin) ex. Microorganism	Fleshings waste	0 – 13% (w/VS) Lipase concentration	[13]
Lipase produced by solid-state fermentation of the fungus P. simplicissimum	Fish processing industry wastewater	0.2 – 0.5% (w/v) Lipase concentration, Pretreatment time 8h, Operating temperature 30^{o}C, Mixing speed 150 RPM	[30]
	Poultry wastewater	0 – 0.5% (w/v) Lipase concentration, Pretreatment time 4h, Operating temperature 30^{o}C, Mixing speed 150 RPM	[12]
An enzyme additive (Septaid PowderTM	Primary sludge of a wastewater treatment plant	0 – 3.125 g/l Lipase concentration	[31]

Bio-Cat Inc,Troy, VA, USA)			
Lipase 80,000 from Rhizopus oryzae	Synthetic lipid rich wastewater	3.6, 61.0 and 120.8 IU/g VSadded Lipase concentrations, Operating temperature 30^{o}C, Mixing speed 150 RPM	[2]

The cost of the enzymes depends on their sources and technology used to manufacture them. The list of current pricing of commercially available lipases is shown in Table 2.4.

Table 2.4: Prices of commercially available lipases (2017)

Type of Lipase	Activity	Price (USD/g)	Manufacturer
Candida rugosa	Type VII, ≥700 unit/mg solid	4.91	Sigma Aldrich L1754 SIGMA
Porcine pancreatic	Type II, 100-500 units/mg protein (using olive oil (30 min incubation)), 30-90 units/mg protein (using triacetin)	0.55	Sigma Aldrich L3126 SIGMA
Aspergillus oryzae	Lyophilized, powder, white, ~50 U/mg	1196	Sigma Aldrich 62285 SIGMA
Rhizopus oryzae	Powder, light brown, ≥30 U/mg (Sigma)	6.18	Sigma Aldrich 80612 SIGMA
Lipase (Steapsin) ex. Microorganism	40-70 units/mg protein	0.31	Sisco Research Laboratories Pvt. Ltd. 60770 (124549)
Rhizopus oryzae	Powder, light brown, ≥30 U/mg	6.18	Sigma Aldrich 80612 SIGMA

Among those lipases, lipase from porcine pancreatic and lipase (Steapsin) ex. Microorganism are the cheapest commercially available lipases currently and they have been successfully applied to pretreat oily effluents in previously conducted research.

- Lipase from porcine pancreatic: Animal fat, vegetable oil and floatable grease skimmed from food waste

- Lipase (Steapsin) ex. Microorganism: Tannery fleshing waste

Therefore, these two lipases were selected to overcome inhibition caused by lipids in anaerobic digestion of wastewater generated in desiccated coconut processing plants.

2.8. Development of an improved pre-treatment strategy with combined effect of enzymatic pre-treatment, initial pH adjustment in feed and changing substrate to inoculum ratio

2.8.1. Enzymatic hydrolysis

Enzymes are biological catalysts, which increase the rate of the reactions. The enzymatic pre-treatment was a well-known strategy because it was a scientifically proven fact that enzymes increase the rate of reaction by reducing the activation energy and improve the hydrolysis reaction rate. It has been reconfirmed that the lipase pre-treatment enhance methane production rate and organic matter removal in effluents from poultry slaughterhouses using lipase produced by solid-state fermentation of the fungus P. restrictum [32] and using porcine pancreatic lipase [33]. Similarly, for the diary effluents, the lipase pre-treatment improved the biogas generation and organic matter removal while using porcine pancreas lipase [10], using enzyme produced through solid-state fermentation of penicillium sp. fungus [9] and using enzymatic extract preparation from pseudomonas aeruginosa KM110 [34]. Anaerobic digestion of swine slaughterhouse waste with enzymatic hydrolysis pre-treatment from porcine pancreas lipase also enhanced the biomethane generation [35].

2.8.2. Saponification and initial pH adjustment

Saponification is alkaline hydrolysis of triglycerides. According to a recent study conducted using slaughterhouse waste, pH adjustment in feed using sodium hydroxide [22] on anaerobic digestion enhanced the methane production rates and substrate degradation efficiency. Similarly, solubilization led to an increased sludge biodegradability in pH adjustment in feed applied for the dairy wastewater using sodium hydroxide, potassium hydroxide and calcium hydroxide [23].

Longer recovery times were observed in anaerobic digestion of oily effluents consists of high concentration of oil and grease ≥ 1 mg/l [2]. The excess lipids attached around the microbial hydrophobic cell walls and cause adverse effects such as mass transfer limitations between anaerobic microorganisms and substrates, biomass washout, and reduction in the treatment efficiency. Therefore, performing the anaerobic digestion at optimum substrate to inoculum ratios also improve the biogas production rate and treatment efficiency in anaerobic bioreactors.

Wastewater generated in desiccated coconut (DC) industries also consists of higher concentration of oil and grease [6] ≥ 1 mg/l which was known to be inhibitory for the anaerobic microorganisms [8]. Therefore, it causes adverse effects such as instability of the anaerobic reactors, biomass washout, clogging of the pipelines, eutrophication, odor problems and reduction of the treatment efficiency. Enzymatic pre-treatment, pH adjustment in feed and performing anaerobic digestion at a proper substrate to inoculum ratio were used to improve the treatment efficiency of anaerobic reactors in previous research studies. The effect of enzymatic hydrolysis pre-treatment combined with initial pH adjustment and suitable substrate to inoculum ratio on anaerobic digestion of DC wastewater has not been studied so far. Therefore, the novelty of this research study is to provide an alternative improved enzymatic pre-treatment strategy for the anaerobic digestion of DC wastewater via combined effect of enzymatic pre-treatment, initial pH adjustment in feed and a proper substrate to inoculum ratio. The lipid composition of desiccated coconut wastewater is different from the wastewater investigated before.

3. MATERIALS AND METHODS

In this chapter, characterization of wastewater, experimental system, reagents used in this research and standard analytical methods used to analyze the physicochemical parameters are discussed.

3.1. Process flow diagram of an existing desiccated coconut processing plant

In order to remove lipids, oil separators are used prior to the anaerobic digesters to reduce oil and grease content in wastewater as shown in Figure 3.1 [6].

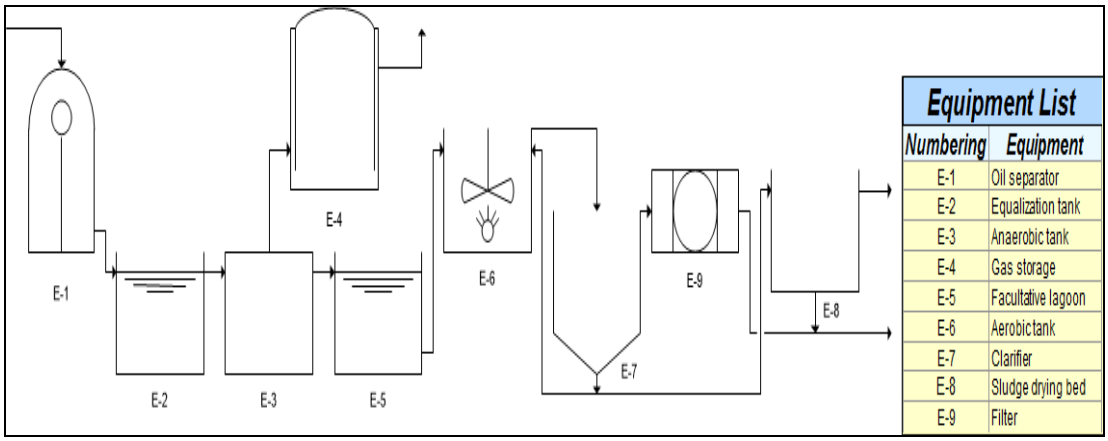

Figure 3.1: Process flow diagram of a typical desiccated wastewater treatment plant

In order to evaluate the effect of the combined pre-treatment strategy on anaerobic digestion, wastewater was obtained from the equalization tank, which comes after the oil separators.

3.2. Characterization of wastewater

Wastewater used in this research was collected from a large-scale desiccated coconut processing plant, which was located at Giriulla, Sri Lanka. The physicochemical characteristics of wastewater were determined by using standard analytical measuring techniques (APHA) at the laboratory as shown in Table 3.1. Collected wastewater was stored in a refrigerator at 4°C.

Table 3.1: Physicochemical characteristics of wastewater used for batch experiments

Parameter	Value
Total Chemical Oxygen Demand (mg/l)	6462.32 ± 608.22
Soluble Chemical Oxygen Demand (mg/l)	5261.81 ± 246.33
pH Value	4.86
Total Solid (g/l)	5.49 ± 0.042
Volatile Solid (g/l)	3.25 ± 0.124
Oil and Grease (g/l)	3.748

The fatty acid composition of the wastewater, which was measured by gas chromatography, is given below in Figure 3.2.

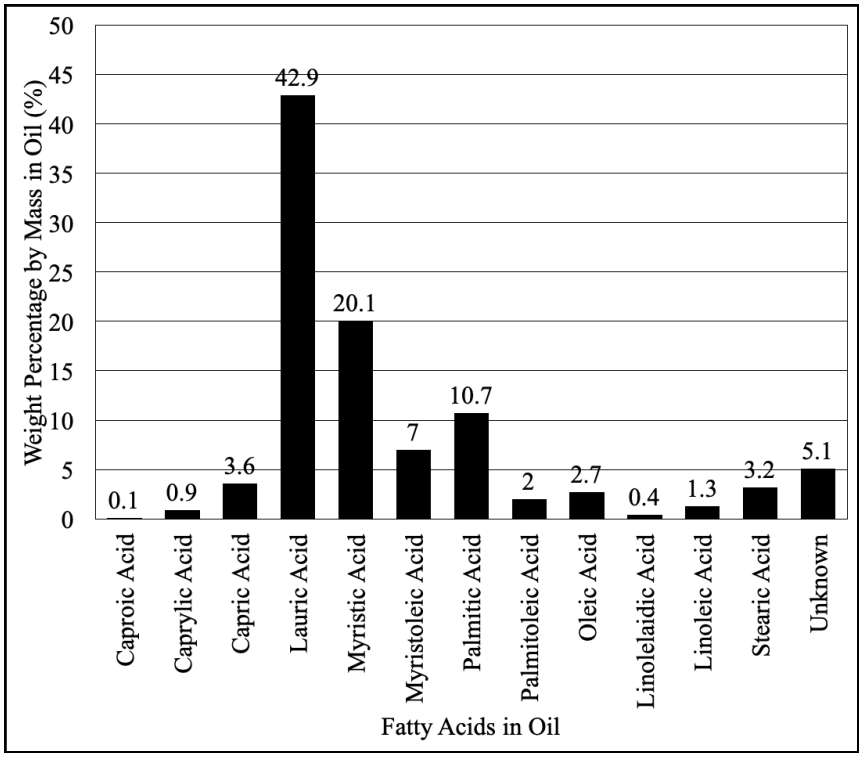

Figure 3.2: Fatty acid composition of wastewater used for batch experiments

3.3. Seed anaerobic granular sludge

The seed anaerobic granular sludge was obtained from an active up-flow anaerobic sludge bed (UASB) anaerobic reactor of a dairy wastewater treatment plant. The physicochemical characteristics of sludge were determined by using standard analytical measuring techniques at the laboratory and given in Table 3.2.

Table 3.2: Physicochemical characteristics of seed anaerobic granular sludge

Parameter	Value
Soluble Chemical Oxygen Demand (mg/l)	5415.07 ± 44.24
Total Solid (g/l)	78.86 ± 2.561
Volatile Solid (g/l)	66.60 ± 2.380
Volatile Suspended Solids (g/l)	67.23 ± 0.892

3.4. Enzyme

The enzyme used in this research study was Sigma-Aldrich lipase originated from porcine pancreas –Type II. It had activity of 100-500 units/mg protein (using olive oil (30 min incubation)), 30-90 units/mg protein (using triacetin). (Source – Sigma Aldrich Website)

3.5. Enzymatic hydrolysis pre-treatment

The lipase used in this study was lipase originated from porcine pancreas – Type II (Sigma-Aldrich). Three different concentrations of this lipase were used in this hydrolysis experiment, i.e. wastewater without lipase 0% (w/v), wastewater with 0.01% (w/v) lipase and wastewater with 0.1% (w/v) lipase. Enzyme was added at the beginning of the pre-hydrolyzing experiment. The wastewater was pre-hydrolyzed inside closed beakers (1L beakers containing 1L of wastewater) placed inside temperature controlled hot water bath operated at 37°C with magnetic stirring of 100 rpm for 24 hours as shown in Figure 3.3. Hydrolyzed effluents were removed after 24 hours and stored at 4°C inside a refrigerator until they were transferred to the batch anaerobic digestion tests.

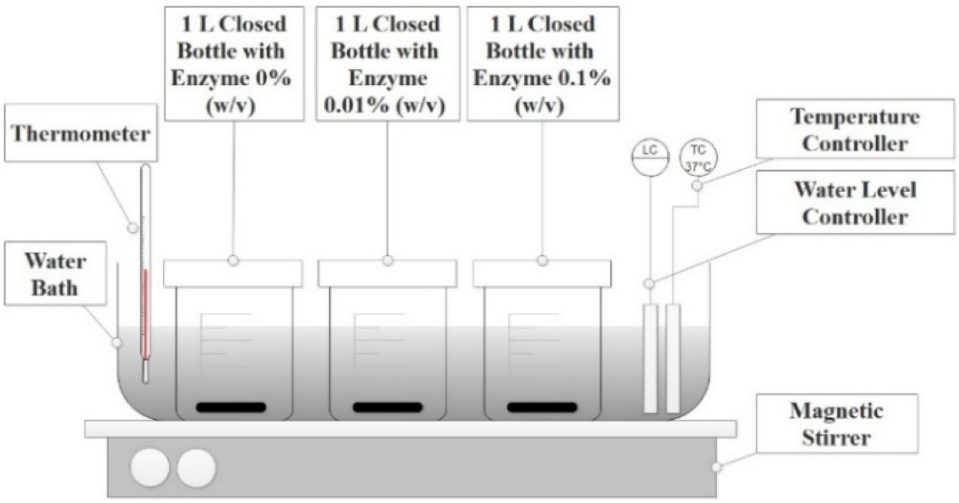

Figure 3.3: Schematic diagram for the experimental setup of enzymatic hydrolysis pre-treatment

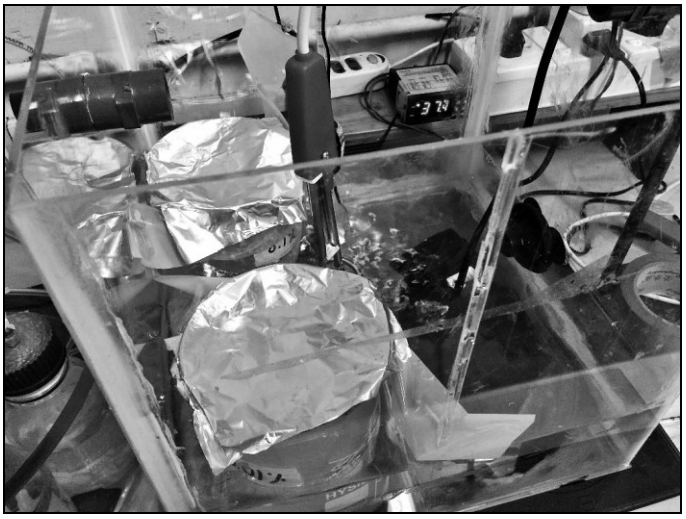

Figure 3.4: Fabricated hydrolysis experimental setup

3.6. Initial pH adjustment in feed

The initial pH adjustment in feed was conducted by adding sodium hydroxide after the enzymatic hydrolysis pre-treatment before the samples were subjected to the anaerobic digestion. The initial pH value of all enzymatic pre-treated and enzymatic untreated wastewater samples was adjusted to pH 7.0±0.2 using sodium hydroxide pellets.

3.7. Batch experimental setup

A single anaerobic batch reactor consisted of two sterilized 50 ml and 25 ml syringes as shown in the Figure 3.5. The 50 ml syringe was filled with 50 ml substrate and inoculum mixture and 25 ml syringe was kept empty in the beginning. Two syringes were interconnected using intravenous tubing. The biogas was collected and measured daily using the 25 ml syringe by the volume displacement. Therefore, maximum amount of biogas that could be collected in a single reactor was 25 ml. When the biogas volume in each bioreactor was getting closer to 25 ml, methane composition was analyzed. Following analysis, the empty 25 ml syringe was connected back to the 50 ml syringe. Mininert luer-tip syringe valves were used for maintaining anaerobic conditions and gas sampling.

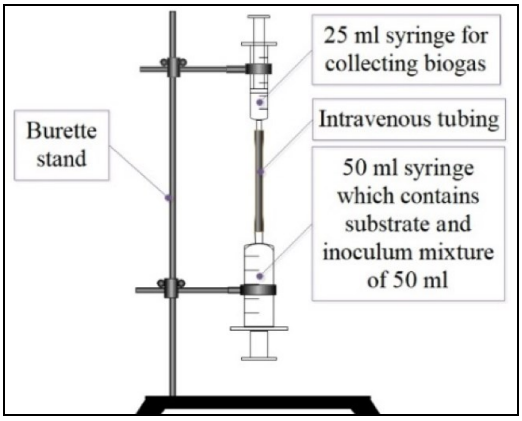

Figure 3.5: Experimental setup of a single batch reactor

All the anaerobic batch experiments were conducted under atmospheric temperature and pressure. During 60 days of complete anaerobic digestion, ambient temperature of the environment was in between 30°C - 32°C and ambient pressure of the environment was in between 0.995 atm. - 0.999 atm. The anaerobic biodegradability tests were performed in 24 identical anaerobic batch reactors using the similar batch experimental setups as shown in the Figure 3.6. The 24 identical anaerobic reactors were fabricated and performed under different process conditions as given by the Table 3.3.

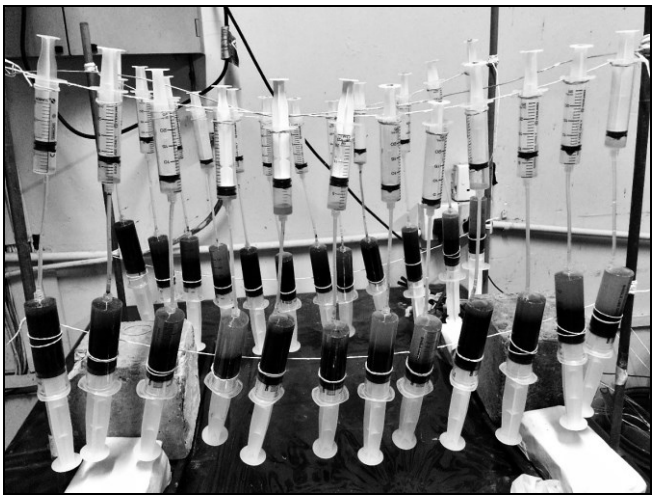

Figure 3.6: Fabricated batch experimental setups

Table 3.3: Process conditions of different anaerobic batch reactors

	a (no lipase)	**b** (0.01 (w/v) % lipase)	**c** (0.1 (w/v) % lipase)
A (pH adjusted)	Aa(I), Aa(II), Aa(III), Aa(IV)	Ab(I), Ab(II), Ab(III), Ab(IV)	Ac(I), Ac(II), Ac(III), Ac(IV)
B (pH not adjusted)	Ba(I), Ba(II), Ba(III), Ba(IV)	Bb(I), Bb(II), Bb(III), Bb(IV)	Bc(I), Bc(II), Bc(III), Bc(IV)

Definitions to four different inoculum to substrate ratios in volume basis used in this study are,

(I) 1:4 ratio (Oil and grease in mixture = 0.150g, sCOD in mixture = 5292.46 mg/l)
(II) 2:3 ratio (Oil and grease in mixture = 0.112g, sCOD in mixture = 5323.11 mg/l)
(III) 3:2 ratio (Oil and grease in mixture = 0.075g, sCOD in mixture = 5353.77 mg/l)
(IV) 4:1 ratio (Oil and grease in mixture = 0.037g, sCOD in mixture = 5384.42 mg/l)
(a) Wastewater pre-treated without lipase
(b) Wastewater pre-treated with 0.01 (w/v)% lipase
(c) Wastewater pre-treated with 0.1 (w/v)% lipase
(A) Initial pH adjusted into pH 7.0±0.2 by sodium hydroxide
(B) Initial pH not adjusted

The parameters monitored to evaluate anaerobic biodegradability were daily biogas production, methane composition of biogas, biogas production rate, TS reduction efficiency, VS reduction efficiency and bio-methane potential.

The above experiment was also conducted for the scenarios defined below as shown in Table 3.4.

Table 3.4: Process conditions of different anaerobic batch reactors under two different enzymes

	1 0.1% (w/v) Lipase 1	**2** 0.1% (w/v) Lipase 2
A (Initially pH adjusted)	A1(V), A1(VI), A1(VII), A1(VIII)	A2(V), A2(VI), A2(VII), A2(VIII)

Definitions to four different inoculum to substrate ratios in volume basis used in this study are,

(V) Substrate : Inoculum ratio (VS/VS%) = 1.3
(VI) Substrate : Inoculum ratio (VS/VS%) = 0.66
(VII) Substrate : Inoculum ratio (VS/VS%) = 0.33
(VIII) Substrate : Inoculum ratio (VS/VS%) = 0.16

1. Wastewater pre-treated with 0.1 (w/v)% lipase (Steapsin) ex. Microorganism (Sisco Research Laboratories Pvt. Ltd. – activity 40 to 70 Units/mg)
2. Wastewater pre-treated with 0.1 (w/v)% lipase from porcine pancreas Type II (Sigma Aldrich – Activity 30-90 Units/mg)

(A) Initial pH adjusted into pH 7.0±0.2 by sodium hydroxide

3.8. Sampling and preservation

After every sampling, the liquid phase samples were also placed inside refrigerator (except gas samples) at 4°C to reduce the microbial activities. Whenever possible, each and every sample were analyzed immediately after withdrawn from the anaerobic reactors. The gas samples were analyzed immediately after withdrawn from the anaerobic reactors.

3.9. Measured parameters and analytical methods

The measured parameters in this study were,

- pH
- Daily biogas production
- Methane composition
- Chemical oxygen demand (COD)
- Total solid (TS)
- Total volatile solid (TVS)
- Volatile suspended solid (VSS)
- Bio-methane yield
- Oil and grease content
- Fatty acid content

3.9.1. pH measurement

The pH measurement was conducted using YSI 1200 laboratory pH instrument. The pH instrument was calibrated daily with two-point calibration using buffer solutions of pH = 7.0 and pH = 4.0.

3.9.2. Daily biogas production

The daily biogas production was measured through the volume displacement occurred within syringes at ambient temperature 30°C - 32°C and ambient pressure of 0.995 atm. - 0.999 atm.

3.9.3. Methane composition

The methane composition was analyzed using the syringe protocol published by Paul Harris on 6th of September 2010. In that method, 9N potassium hydroxide solution was used to remove carbon dioxide gas in the biogas and evaluate the methane composition.

3.9.4. Chemical oxygen demand

The chemical oxygen demand was determined by closed reflux digestion and titration method according to the ASTM D 1252-00. In soluble COD (sCOD) analysis, samples were centrifuged using Eppendorf 5804 series centrifuge at 1200g for 10 minutes to separate solids and filtered with 0.45μm nylon syringe filter prior to the standard COD analysis. According to the standard COD analysis, the samples were diluted using dilution factor of 25 or 10. For the 2.5 ml of diluted sample, 1.5 ml of dichromate digestion solution and 3.5 ml of sulfuric acid solution were added. Then the samples were digested in Lovibond RD 125 COD reactor at 150°C for 2 hours. Then, samples were taken out and cooled down into room temperature. Then, each sample was titrated using ferroin indicator and ferrous ammonium sulfate titrant until the color change from blue-green to reddish brown. The standard COD value was calculated by the procedure mentioned in the standard procedure and the final COD value was multiplied by the dilution factor in order to calculate the actual COD value.

3.9.5. Total solid (TS), total volatile solid (TVS) and volatile suspended solid (VSS) analysis

The TS, TVS and VSS analysis were conducted according to the method 1684, which was developed by (APHA) U.S. Environmental Protection Agency for the determination of total, fixed, and volatile solids in water, solids, and bio solids. TS analysis was conducted using Remi Laboratory oven at 105°C - 110°C. TVS and VSS analysis were conducted using Lenton muffle furnace at 550±10°C.

3.9.6. Bio-methane yield

The bio-methane yields in batch experimental setups were analyzed by the evaluating volume of methane gas produced per unit weight of volatile solids added from the substrate.

3.9.7. Oil and grease content

The amount of oil and grease content was analyzed according to the hexane extractable gravimetric method 10056 which was developed by U.S. Environmental Protection Agency for the determination of oil and grease content [36]. First 500 ml of sample was taken and extracted all oil and grease by hexane with a 1-liter separatory funnel. After the separation, the oil and hexane mixture was filtered through a 10g sodium sulfate layer to absorb all the water in it. Then the extract was distilled at 85±2°C by model YCW-010E Gemmy Industrial water bath with a previously dried and weighted boiling flask. Then with weight difference, amount of oil and grease content was measured.

3.9.8. Fatty acid content

Oil and grease extracted from the wastewater using hexane extractable gravimetric method 10056 was converted into fatty acid methyl esters using a mixture of methanol-BF3. The fatty acid composition was evaluated by the Agilent 7890A, gas chromatograph equipped with a DB-23 column of 30m length and 0.25 mm internal diameter where nitrogen gas was used as carrier gas along with hydrogen and air for the flame.

4. RESULTS AND DISCUSSION

4.1. Results from the case study

According to the case study conducted using the wastewater samples taken prior to the anaerobic reactors in wastewater treatment plant of a typical desiccated coconut processing plant, the inlet COD value of the anaerobic reactor showed considerable deviations during operation. Conversely, the COD removal efficiency of the anaerobic reactor varied and at a certain threshold point of COD level between 6,500 - 8,000 mg/l, it reduced drastically as shown in Figure 4.1 and Figure 4.2 [6].

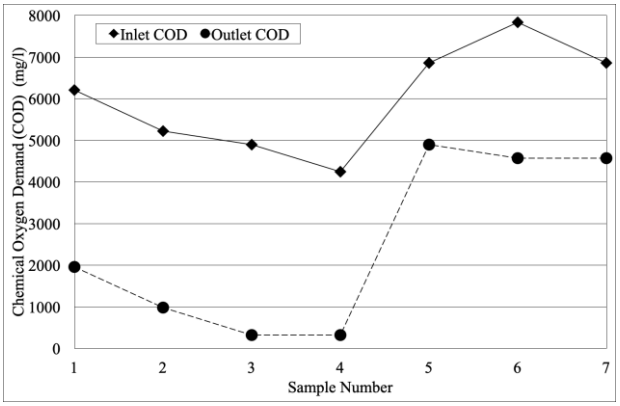

Figure 4.1: COD variations of anaerobic reactor in a large-scale desiccated coconut processing plant

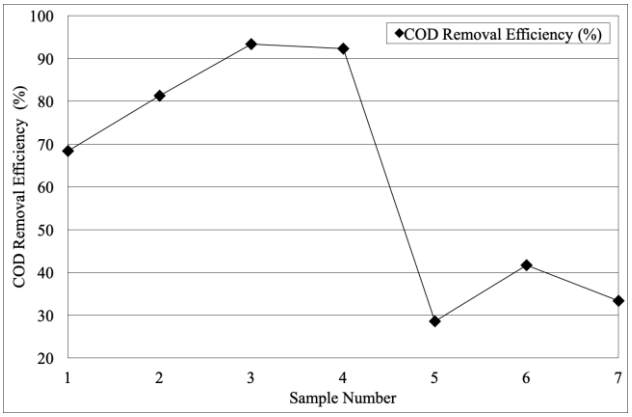

Figure 4.2: COD removal efficiency of anaerobic reactor in a large-scale desiccated coconut processing plant

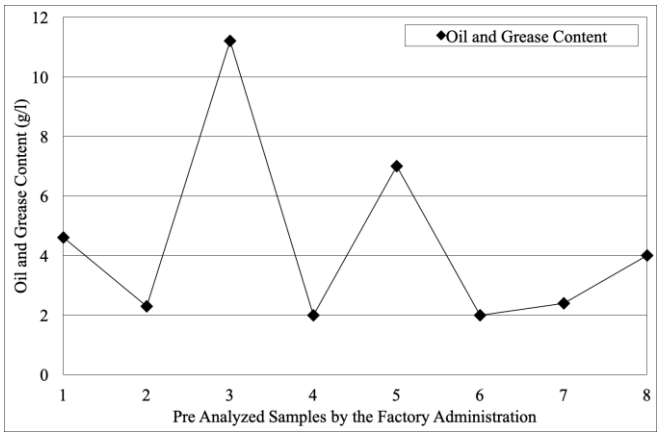

Figure 4.3: Oil and grease variation of wastewater generated in desiccated coconut processing plant prior to the oil separators (Source: Data from the factory)

According to Figure 4.3, oil and grease content of the input of the anaerobic reactor could be varied above 2 mg/l and the inhibitory concentration is ≥ 1 mg/l for the anaerobic microorganisms. Reduction of COD removal efficiencies observed in this plant was due to this lipid inhibition. Therefore, the below pre-treatment strategy was applied as a possible strategy to overcome the inhibition and improve the treatment efficiency of anaerobic reactors.

4.2. Identification of the methanogens in inoculum

To confirm the availability of active methanogens in inoculum, microscopic observation was performed using Zeiss Axio Lab.A1 microscope with digital imaging system. Rod shaped Methanosaeta were observed by light microscopy under the magnification of 100x as shown in Figure 4.4 below. These results indicate the presence of active methanogens in inoculum used for subsequent experiments.

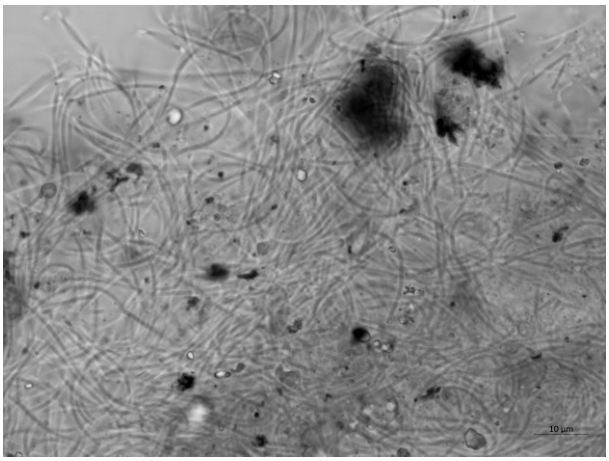

Figure 4.4: Microscopic image of anaerobic microorganisms in anaerobic granular sludge

4.3. Cumulative biogas production during the first 10 days

4.3.1. Effect of substrate to inoculum ratio

The cumulative biogas production during the first 10 days of all anaerobic reactors in ascending order were under category (I), (IV), (III), (II) as shown in the Figure 4.5. The sCOD concentrations were almost similar in every reactor. According to the analysis conducted, oil and grease content of the input substrate was similar but the organic loading rate varied because of addition of different volumetric ratios. According to this, the oil and grease added from wastewater in ascending order were under category (IV), (III), (II), (I). When the organic loading rate was increased in terms of oil and grease, it could be observed that cumulative biogas production increased according to the ascending order under category (IV), (III) and (II) but in the category (I) it was deviated because it might have exceeded the inhibitory lipid concentration for the anaerobic microorganisms.

4.3.2. Effect of pH adjustment

Even under this inhibition conditions, all pH adjusted reactors (A) showed the higher biogas production than pH not adjusted (B). Cumulative biogas production in the reactors under category (IV) is higher than the category (I), because of no inhibition and due to desirable substrate to inoculum ratio for the anaerobic microorganisms.

According to the Figure 4.5, all reactors under category (II), pH adjusted reactors showed the highest cumulative biogas production during first 10 days.

When considering initial pH adjustment in feed, the biogas production rate in the beginning was higher in category (A) reactors than category (B) reactors. According to a previous research study conducted on pH adjustment in feed using sodium hydroxide applied for the anaerobic digestion of slaughterhouse waste [22], the pH adjustment in feed has enhanced the methane production rates and improved the COD degradation efficiencies. In another research study conducted on pH adjustment in feed using sodium hydroxide, potassium hydroxide and calcium hydroxide applied for the anaerobic digestion of dairy wastewater [37], the pre-treatment has improved the COD solubilization, suspended solid reduction and bio-methane potential.

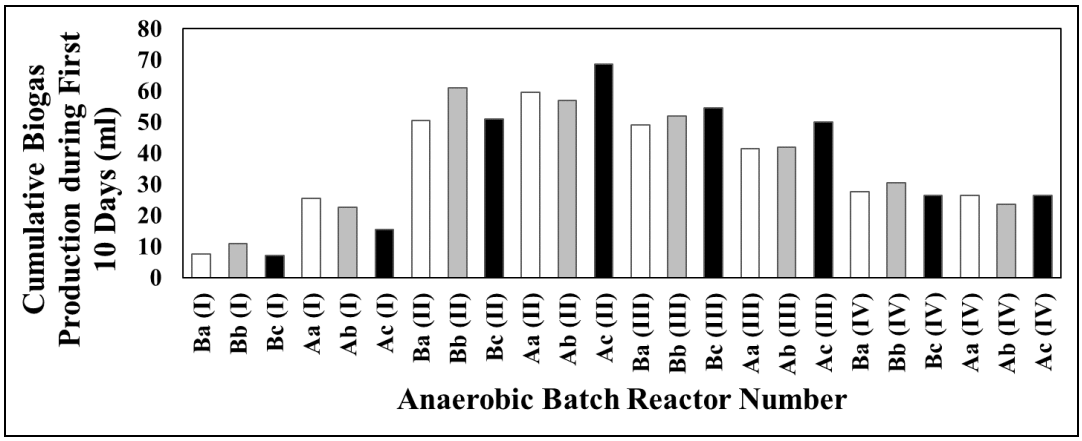

Figure 4.5: Cumulative biogas production during first 10 days of different anaerobic batch reactors

A similar phenomenon was observed in a previous research study conducted on anaerobic digestion of soap stock from sunflower oil refining pre-treated with lipase generated from staphylococcus haemolyticus [11]. The cumulative biogas production during the first 10 days was higher in category (IV), (III) and (II) reactors, which contained higher oil and grease concentration. But the category (I) reactors which contained highest amount of lipids deviated from other reactors because, both lipase pre-treated and untreated reactors had shown a lower cumulative biogas production during the first 10 days than the other reactors [11].

The highest cumulative biogas production during the first 10 days of 68.5 ml was observed in Ac(II) reactor where the oil and grease content of 0.112g was added from the substrate.

4.3.3. Effect of enzymatic hydrolysis pre-treatment

Similar to the cumulative biogas production during first 10 days, the highest biogas production rate in the beginning was also reported from anaerobic reactors in category (II), and other reactors in ascending order were (I), (IV), (III) as shown in Figure 4.6. This occurred because substrate to inoculum ratio in category (II) reactors was much favorable for anaerobic microorganisms for their rapid growth. During the pre-treatment particulate matter hydrolysis and produce more soluble substrates and it was observed in the phase 1 in which gas production rate decreases and in phase 2 it increases. During the anaerobic conversion process, solubilized components first degraded within phase 1 and then rest of the particulate matter degraded. This is the reason for increased gas production rate.

Considering the enzymatic pre-treatment, the enzymatically pre-treated Ac(II) reactor has shown the highest biogas production rate in the beginning than all the other reactors.

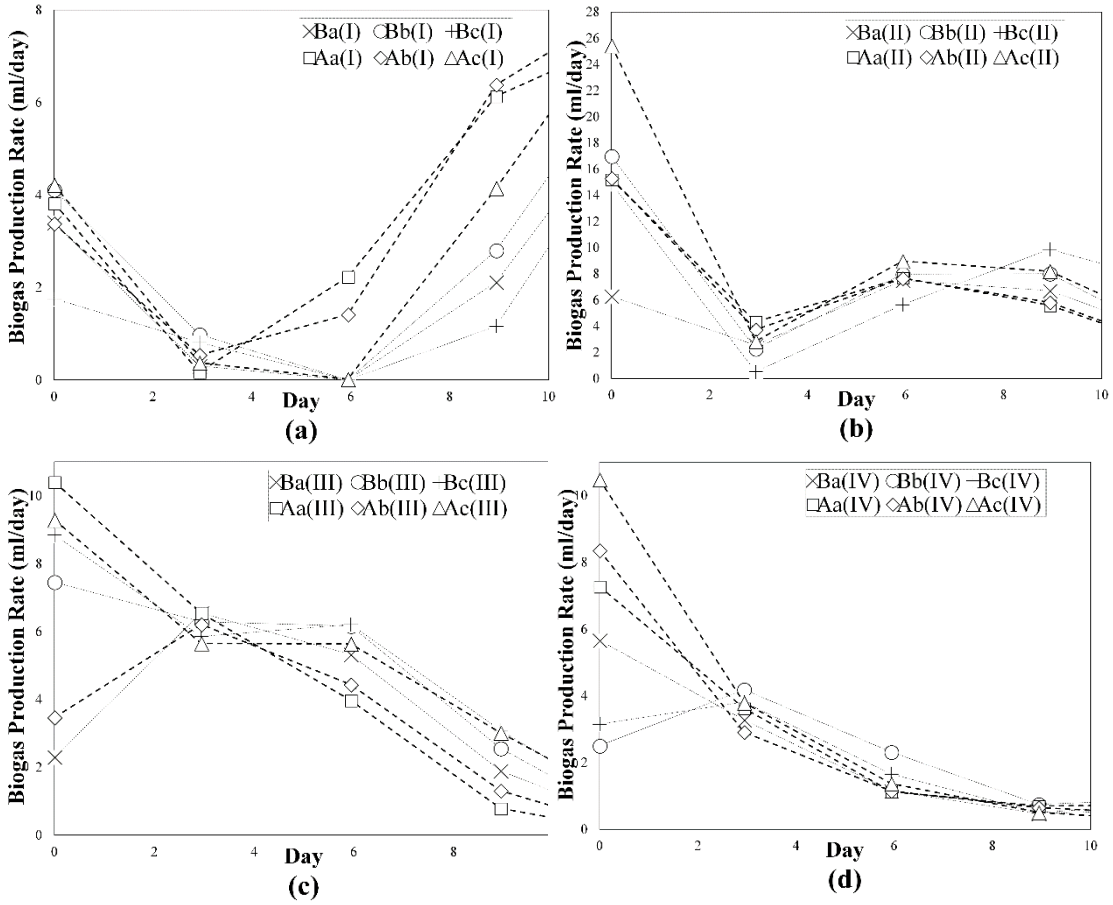

Figure 4.6: Biogas production rate graphs during first 10 days of category (I), (II), (III) and (IV) anaerobic batch reactors.

According to a previous research study conducted on enzymatic pre-treatment using Steapsin lipase applied for the anaerobic digestion of fleshings and sludge in a tannery industry [38], it was observed that the biogas production rate during the first 10 days was higher in the reactors which contained higher lipase concentration where the lipid concentration of the mixture was 3±1.4 g/l. It concluded that the enzymatic hydrolysis pre-treatment has the potential to improve the anaerobic digestion process. In a similar research study conducted on enzymatic pre-treatment using porcine pancreas lipase applied for the anaerobic digestion of dairy wastewater where the oil

and grease concentration was 3.1± 0.15 g/l [39], hydrolysis of lipids and cumulative biogas production improved due to the enzymatic hydrolysis pre-treatment. This happened probably because; the enzymatic pre-treatment enhanced the hydrolysis of lipids and eased the metabolism of anaerobic digestion process.

The initial biogas production rates of the enzymatic pre-treated reactors were higher than the untreated reactors. This also shows that enzymatic pre-treatment increases the rate of substrate utilization and biogas production. The highest initial biogas production rate of 25.43 ml/day was observed in Ac(II) reactor where the oil and grease content of 0.112g was added from the substrate.

4.4. Cumulative biogas production and percentage of VS reduction after complete degradation in 60 days

At the end of 60 days batch cycle in all reactors, comparatively there was not much difference in cumulative biogas production between I, II, III and IV reactors as shown in the Figure 4.7. Similarly, there was not much difference in the percentage of VS reduction as shown in the Figure 4.8.

According to the characterization, the volatile solid consists of not only soluble substrate but also non-biodegradable material also present. All reactors had similar soluble COD concentrations. It was assumed that this soluble COD contributes for the cumulative gas production. Therefore, similar cumulative gas production was observed. This is further confirmed from the VS reduction efficiencies observed in Figure 4.8.

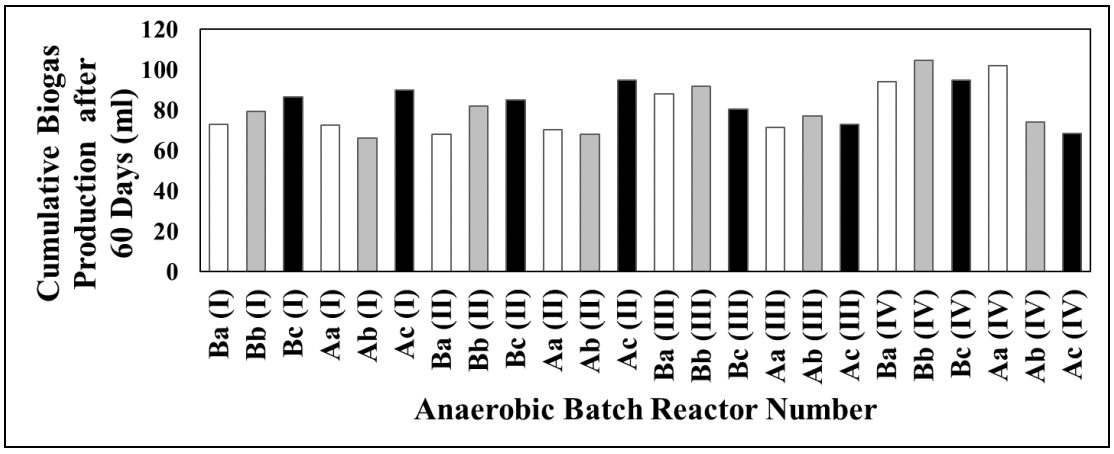

Figure 4.7: Cumulative biogas production after 60 days of complete substrate degradation in different anaerobic batch reactors

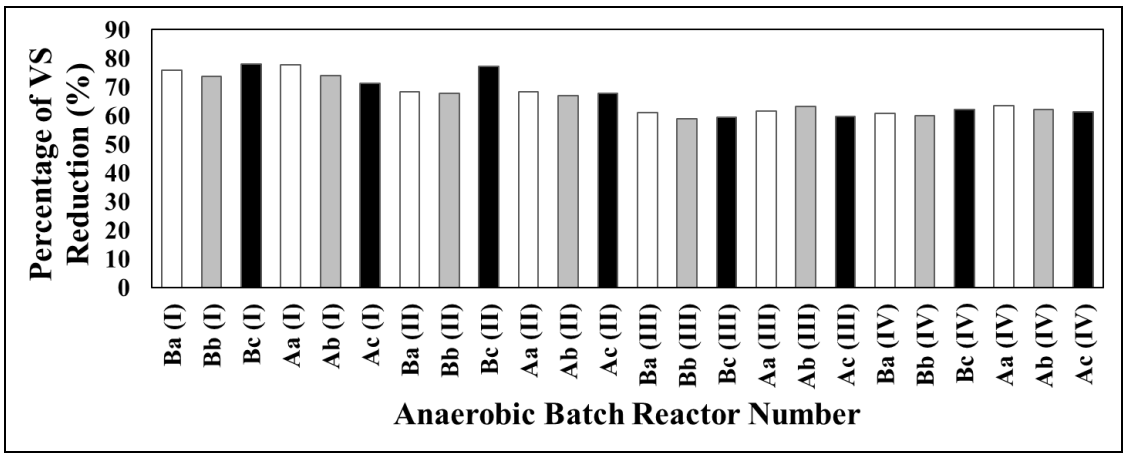

Figure 4.8: Percentage of VS reduction after 60 days of complete substrate degradation in different anaerobic batch reactors

According to a study conducted on enzymatic pre-treatment using lipase 80,000 from Rhizopus oryzae applied for the anaerobic digestion of lipid rich waste [2], it was concluded that the inhibition caused by lipids was not permanent even though there was a higher oil and grease content in the substrate. This shows that the inhibition caused by lipids towards anaerobic digestion of DC wastewater is only a temporary inhibition, which has the potential to delay the degradation of substrates.

Considering the dynamics of biogas production, in category (I), longer lag phase of 6 days in cumulative gas production and 24 days of complete degradation time was

taken as shown in Figure 4.9. This longer lag phase is also the reason for the temporary inhibition.

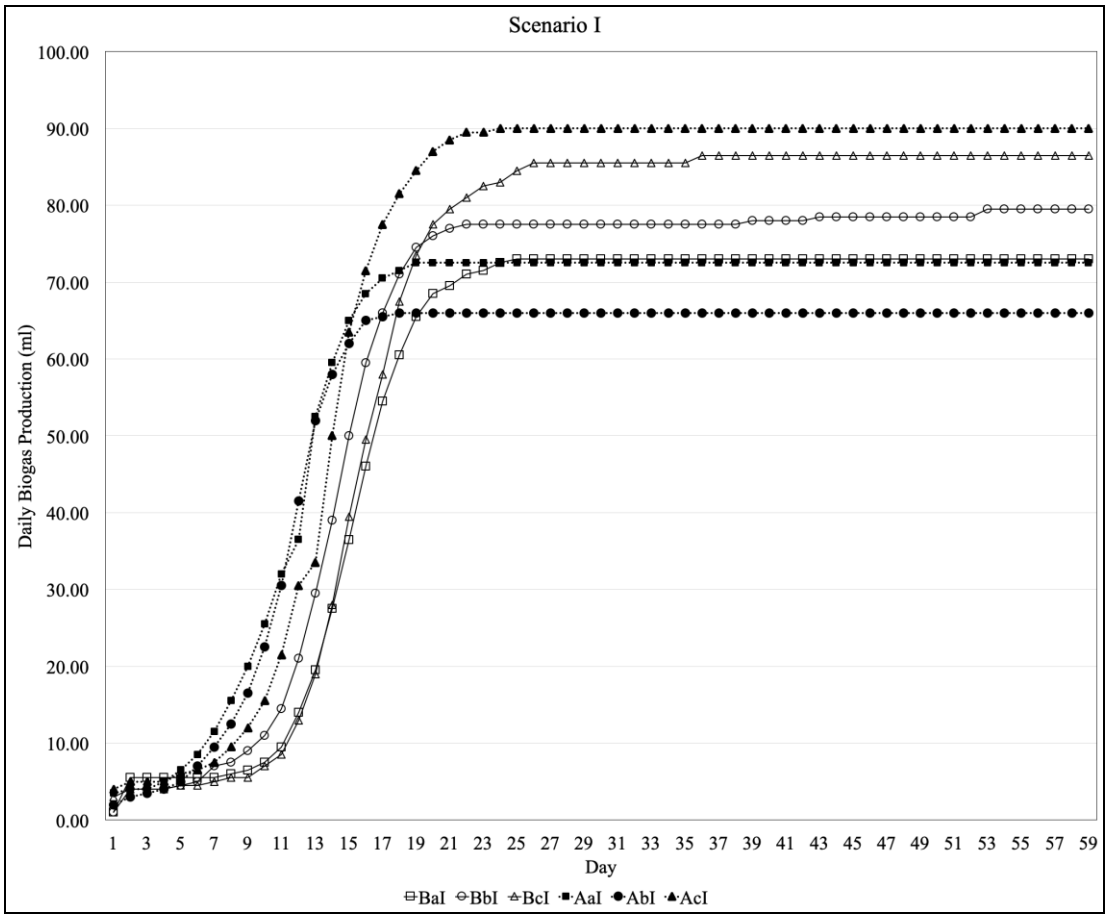

Figure 4.9: Daily biogas production in category (I) all reactors

Considering the dynamics of biogas production, in category (II), there was no lag phase in cumulative gas production and 45 days of complete degradation time was observed. The cumulative biogas production of 60 ml – 80 ml were observed during time of first 11 days as shown in Figure 4.10. From 11 days to 30 days, there was no biogas production but after 30 days, there was slight increase in biogas generation. The best performance was observed in category (II) reactors, which also confirm from the zero lag phase.

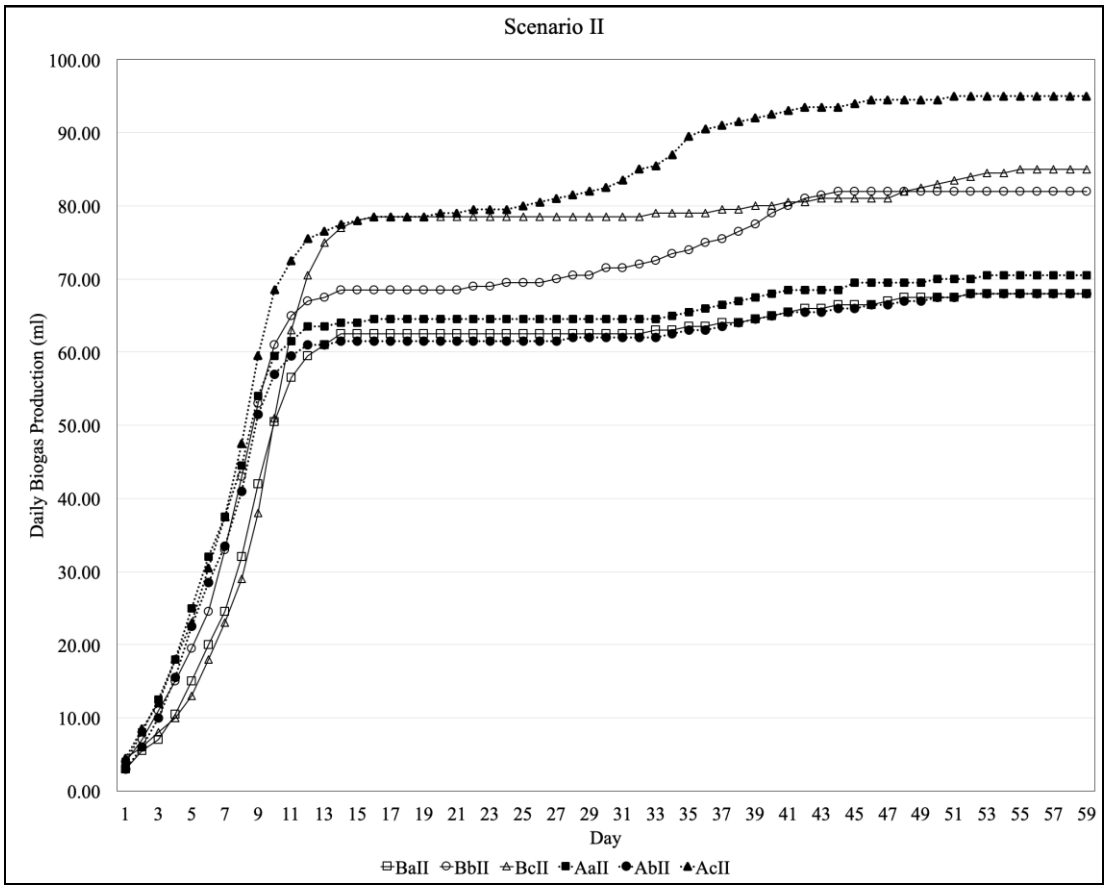

Figure 4.10: Daily biogas production in category (II) all reactors

Considering the dynamics of biogas production, in category III, there was no lag phase in cumulative gas production and 45 days of complete degradation time was taken. The cumulative biogas production of 40 ml – 65 ml were observed during first 9 days as shown in Figure 4.11. From 9 days to 25 days, there was no biogas production but after 25 days, there was also a considerable increase in biogas generation. In category (III) reactors, amount of long-term biodegradable material was higher because of addition of higher inoculum content and amount of short-term biodegradable compounds were less. Therefore, it took longer time to complete the degradation than category (II) and (I) reactors.

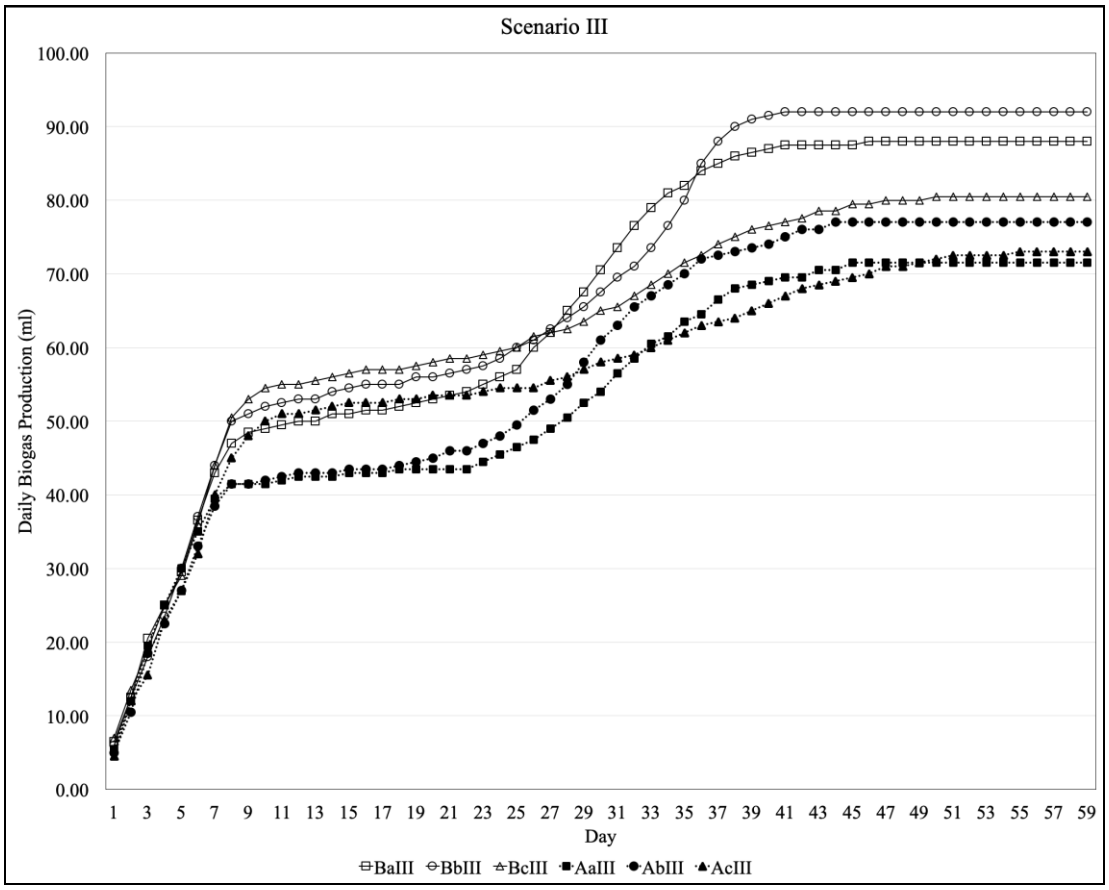

Figure 4.11: Daily biogas production in category (III) all reactors

Considering the dynamics of biogas production, in category IV, there was no lag phase in cumulative gas production and 45 days of complete degradation time was taken. The cumulative biogas production of 20 ml – 30 ml were observed during first 6 days as shown in Figure 4.12. From 6 days to 10 days, there was no biogas production but after 10 days, there was also a considerable increase in biogas generation. In category (IV) reactors, amount of long-term biodegradable material was higher because of addition of higher inoculum content and amount of short-term biodegradable compounds were less. Because of presence of more long-term biodegradable substrate, the intermediate period of degradation was higher than category (III) reactors. Therefore, it takes longer time to complete the degradation than category (III), (II) and (I) reactors.

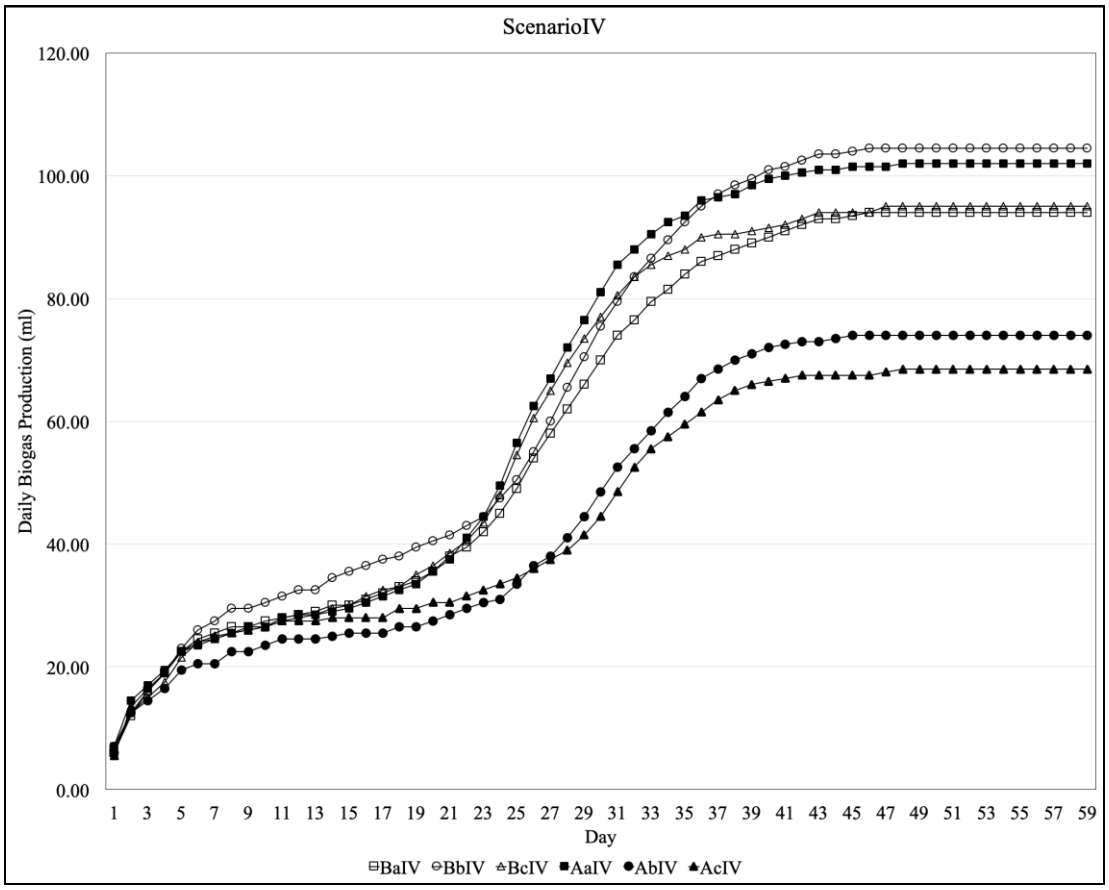

Figure 4.12: Daily biogas production in category (IV) all reactors

4.5. Biogas production rate during 60 days of complete degradation

Considering category "I", in both pH adjusted and pH not adjusted samples, there was a sudden increase in biogas production rate and it dropped after first 3 – 5 days. Previous literature denoted that this happened due to degradation of easily biodegradable substances in the beginning. Then there was no biogas production for 3 – 5 days. Then biogas production rate increased and decreased until 24 days of complete degradation. This happened due to degradation of long term biodegradable substances. The graph of biogas production rate at inoculum: substrate ratio of 1:4 was shown in Figure 4.13. In pH adjusted samples, the curves of gas production rate were shifted to left side resulting an increase in the rate of biogas production.

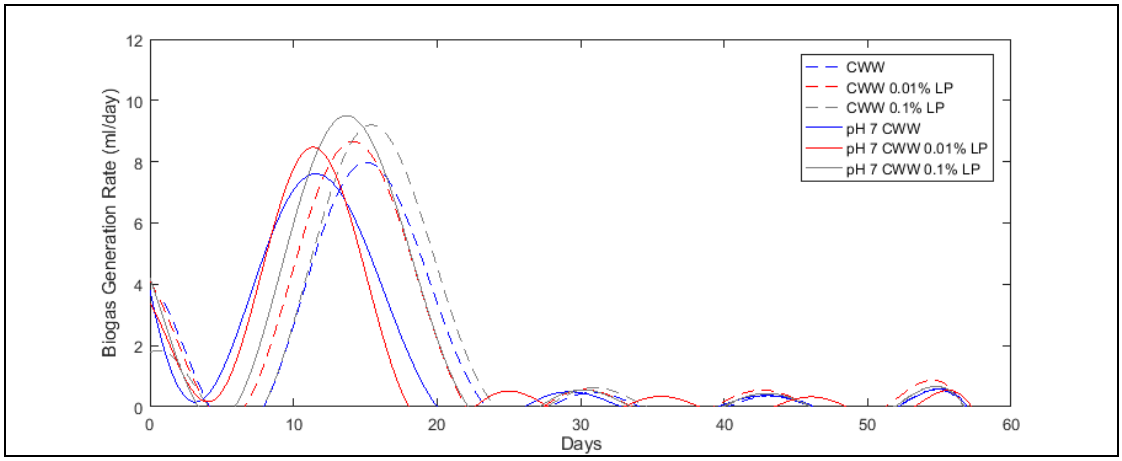

Figure 4.13: Biogas generation rate during 60 days in category (I) reactors

Considering category II all reactors, in both pH adjusted and pH not adjusted samples, there was a sudden increase in biogas production rate and it dropped after first 2 – 4 days. This happened due to degradation of easily biodegradable substances in the beginning. Then there was very low biogas production rate for 1 – 2 days. Then biogas production rate increased and decreased until 11 days of major degradation. This happened due to degradation of long term biodegradable substances. The graph of biogas production rate at inoculum: substrate ratio of 2:3 was shown in Figure 4.14. In pH adjusted samples, the curves of gas production rate were shifted to left side resulting an increase in the rate of biogas production.

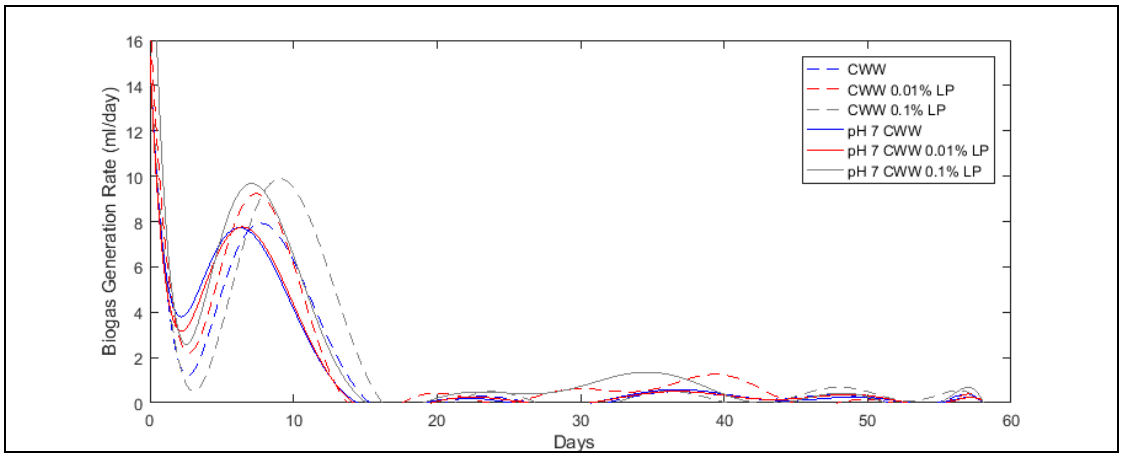

Figure 4.14: Biogas generation rate during 60 days in category (II) reactors

Considering category III reactors, in both pH adjusted and pH not adjusted samples, there was a sudden increase in biogas production rate and it dropped after first 10 – 15 days. This happened due to degradation of easily biodegradable substances in the beginning. Then there was very low biogas production rate for 15 – 20 days. Then biogas production rate increased and decreased until 45 days of major degradation. This happened due to degradation of long term biodegradable substances. The graph of biogas production rate at inoculum: substrate ratio of 3:2 was shown in Figure 4.15. In pH adjusted samples, the curves of gas production rate were shifted to left side resulting an increase in the rate of biogas production.

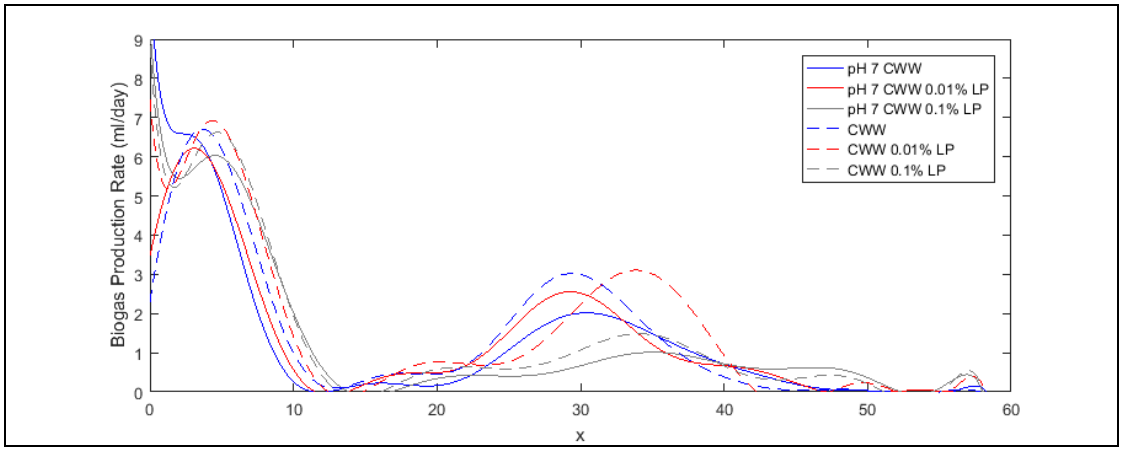

Figure 4.15: Biogas generation rate during 60 days in category (III) reactors

Considering category IV reactors, in both pH adjusted and pH not adjusted samples, there was a sudden increase in biogas production rate and it dropped after first 10 – 15 days. This happened due to degradation of easily biodegradable substances in the beginning. Then there was very low biogas production rate for 15 – 18 days. Then biogas production rate increased and decreased until 45 days of major degradation. This happened due to degradation of long term biodegradable substances. The graph of biogas production rate at inoculum: substrate ratio of 4:1 was shown in Figure 4.16.

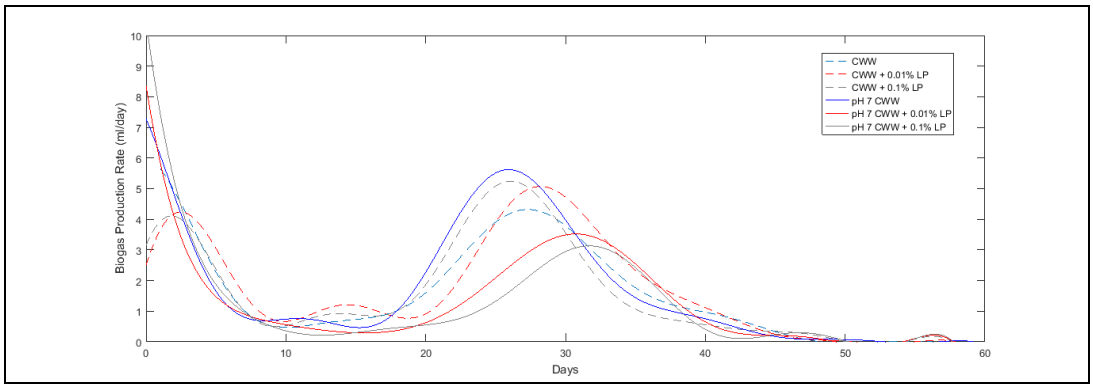

Figure 4.16: Biogas generation rate during 60 days in category (IV) reactors

4.6. Bio-methane yield after 60 days of complete degradation

The bio-methane yield after the complete substrate degradation in anaerobic reactors in ascending order were IV, III, II, I as shown in the Figure 4.17. The oil and grease content added from wastewater in ascending order were IV, III, II, I as mentioned before. Even though the sCOD concentration in IV, III, II and I were almost similar, the bio-methane yield of reactors under the category (I) were higher because they contained higher organic loading. The enzymatic pre-treated reactors under category (c) have shown higher bio-methane yield than the other reactors under category (b) and category (a). Under category (I) reactors, the bio-methane yield was higher in the reactors in which enzymatically pretreated with 0.1% (w/v) lipase. It was also apparent that the bio-methane yield was higher in all enzymatic treated reactors.

According to a research study conducted on enzymatic pre-treatment using a commercial lipase isolated from pig pancreas applied for the anaerobic digestion of swine slaughterhouse waste [35], it was concluded that lipase pre-treatment prior to the anaerobic digestion significantly improved bio-methane yield. Similarly, lipase pre-treatment in this research study also improved the bio-methane yield.

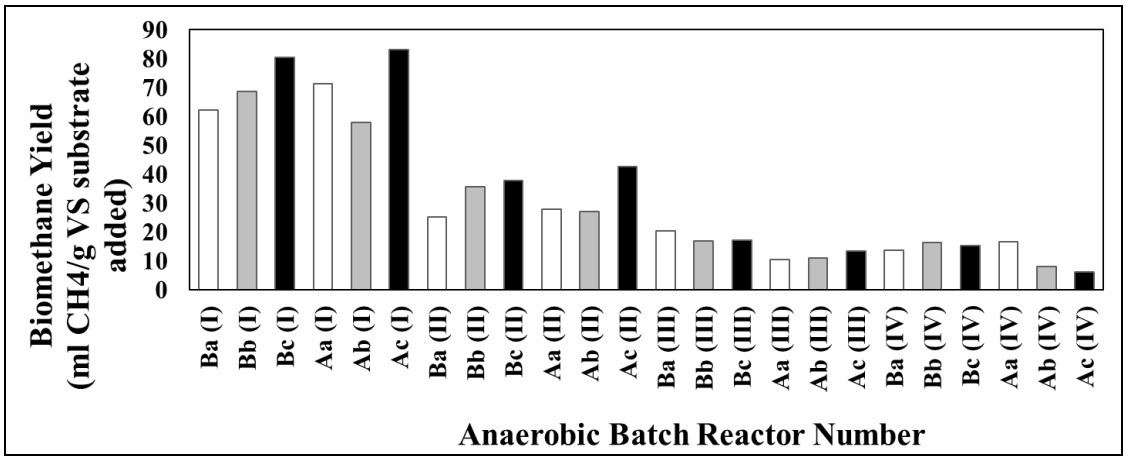

Figure 4.17: Bio-methane yield after 60 days of complete substrate degradation in different anaerobic batch reactors

4.7. Optimization of initial biogas production rate, initial pH adjustment in wastewater and inoculum to substrate ratio

The independent variables considered for this optimization were; inoculum to substrate ratio in volume basis, initial pH adjustment in wastewater and amount of enzymes added in each reactor. The dependent variables considered for this optimization were; initial biogas production rate, cumulative biogas production during first 10 days and bio-methane potential.

The three dimensional surface plotting was conducted using MATLAB R2018a version. By using the code written, the following graphs were generated and optimized the effect of enzymatic pre-treatment, initial pH adjustment and inoculum to substrate ratio on anaerobic digestion.

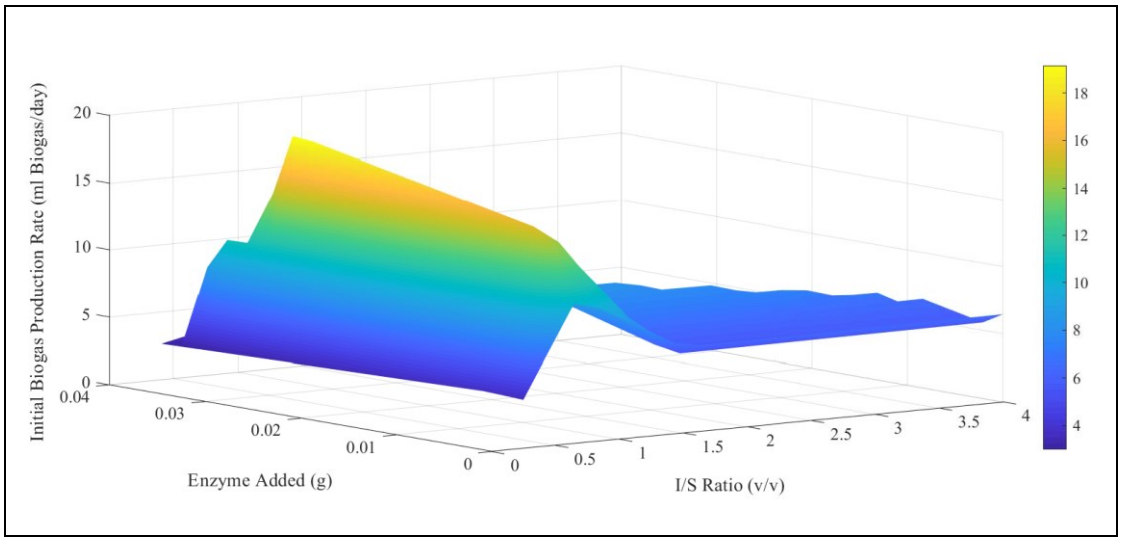

Figure 4.18: Effect on initial biogas production rate by amount of enzyme added and inoculum to substrate ratio

The dependent variable in the Figure 4.18 was the initial biogas production rate and the independent variables were amount of enzyme added into the reactors and inoculum to substrate ratio in volume basis.

According to the Figure 4.18, initial biogas production got higher when the amount of enzyme used for the pre-treatment got higher within the specific range of 0.5 – 2.0 of inoculum to substrate ratio in volume basis. This happened because; the hydrolysis process in anaerobic digestion was enhanced by enzyme addition within that specific range of inoculum to substrate ratio. Further deep scientific analysis should be conducted in order to evaluate this scenario clearly.

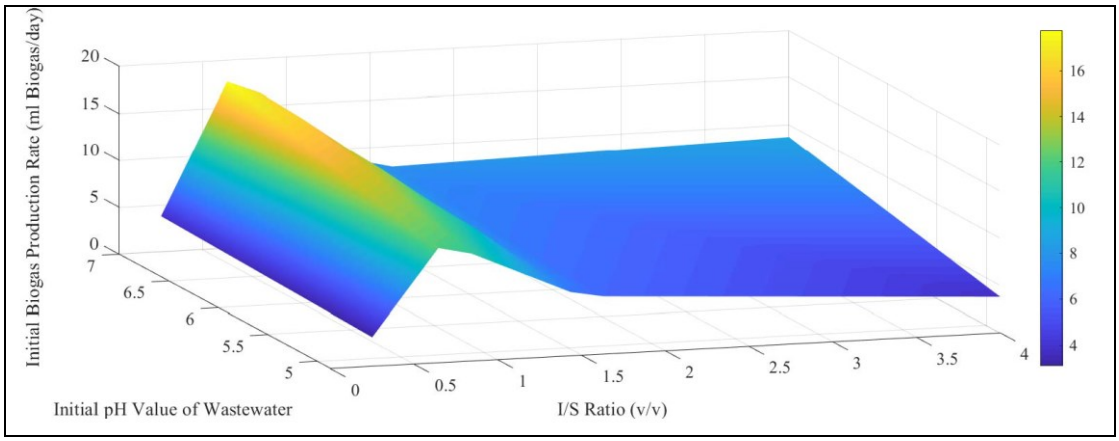

Figure 4.19: Effect on initial biogas production rate by initial pH value of wastewater and inoculum to substrate ratio

The dependent variable in the Figure 4.19 was the initial biogas production rate and the independent variables were initial pH value of the wastewater and inoculum to substrate ratio in volume basis in reactors.

According to the Figure 4.19, initial biogas production was higher when the initial pH value of the wastewater in the beginning was higher within the specific range of 0.5 – 1.5 of inoculum to substrate ratio in volume basis. This occurred because; the hydrolysis process in anaerobic digestion was enhanced by the initial pH adjustment of the wastewater in the beginning within that specific range of inoculum to substrate ratio.

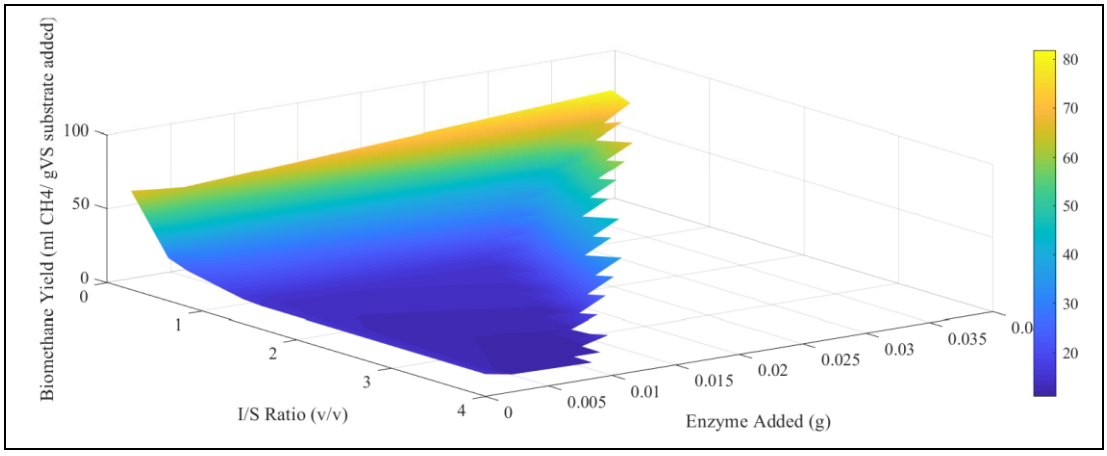

Figure 4.20: Effect on bio-methane yield by the amount of enzyme added and the inoculum to substrate ratio

The dependent variable in the Figure 4.20 was the bio-methane yield and the independent variables were amount of enzyme added and inoculum to substrate ratio in volume basis in anaerobic reactors.

According to the Figure 4.20, bio-methane yield was higher when the amount of enzyme added was higher within the specific range of 0.0 – 1.5 of inoculum to substrate ratio in volume basis. This occurred because; the hydrolysis process in anaerobic digestion was enhanced by the enzyme addition of the wastewater throughout the complete anaerobic degradation process, which occurred for 60 days. This implies that enzyme was active from the beginning to the end of anaerobic digestion by helping degradation process of organic matter into methane gas by anaerobic microorganisms.

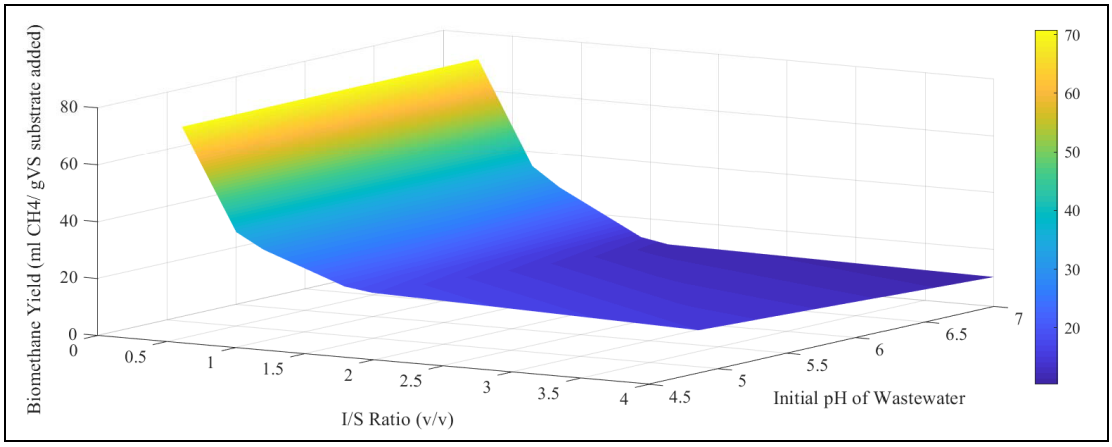

Figure 4.21: Effect on bio-methane yield by initial pH value of the wastewater and the inoculum to substrate ratio

The dependent variable in the Figure 4.21 was the bio-methane yield and the independent variables were initial pH of the wastewater in the beginning and inoculum to substrate ratio in volume basis in anaerobic reactors.

According to the Figure 4.21, bio-methane yield had no effect when the initial pH value of wastewater in the beginning was varied. This happened because; the hydrolysis process in anaerobic digestion was enhanced by the initial pH adjustment

only in the beginning. Hence, sodium hydroxide did not act as a buffer solution; the chemical hydrolysis was not occurred throughout the complete anaerobic degradation process, which occurred for 60 days. This implies that initial pH adjustment supports the initial biogas production but it did not improve the bio-methane yield by helping the anaerobic microorganisms for their methane generation process throughout the complete degradation.

4.8. Optimum performances in anaerobic digestion by enzymatic pre-treatment

Considering all above analysis, the highest process performance and economically applicable reactor conditions were shown with the corresponding control reactor condition in the Table 4.1.

Table 4.1: Process conditions of best-performed anaerobic reactors

Scenario	Highest Process Performance	Highest Process Performance + Economically Applicable
Most Suitable Reactor	Ac(II)	Aa(II)
Reactor Condition	I:S (v/v)% Ratio 2:3, pH not adjusted and 0.1 (w/v)% lipase pre-treated reactor	I:S (v/v)% Ratio 2:3, pH adjusted and 0.1 (w/v)% lipase pre-treated reactor
Control Reactor	Ba(II)	Ba(II)
Compared Reactor Condition	I:S (v/v)% Ratio 2:3, pH not adjusted and enzymatically untreated reactor	I:S (v/v)% Ratio 2:3, pH not adjusted and enzymatically untreated reactor

Considering the above two reactors Ac(II) and Aa(II), the following process improvement could be observed relative to their control reactor Ba(II) in Table 4.2.

Table 4.2: Process performance of best-performed anaerobic reactors

Reactor	Ac(II) vs. Ba(II)	Aa(II) vs. Ba(II)
Increase in initial biogas production rate	+(305)%	+(142)%
Increase in cumulative biogas	+(36)%	+(18)%

production during first 10 days		
Increase in cumulative biogas production after complete degradation	+(40)%	+(4)%
Increase in bio-methane potential	+(69)%	+(11)%
Increase in percentage of volatile solid reduction	(1)%	(0)%
Increase in percentage of total solid reduction	-(12)%	-(10)%
Chemical cost USD/ liter of treated wastewater (for enzyme addition and pH adjustment)	0.55 USD/l	0.0000178 USD/l
Income USD/ liter of treated wastewater (from conversion of methane gas into electricity)	0.0001 USD/l	0.0000919 USD/l

Considering the above two reactors Ac(II) and Aa(II), the best process performance was observed in Ac(II) reactors in which both enzymatic hydrolysis and initial pH adjustment was performed with I/S (v/v) ratio of 2:3. Both economically viable and better process performance was observed in Aa(II) reactors in which only the initial pH adjustment was performed with I/S (v/v) ratio of 2:3.

4.9. Effect of enzymatic hydrolysis pre-treatment using two different enzymes

A preliminary study was conducted to evaluate whether there is an effect of changing the type of enzyme of the modified pre-treatment process on anaerobic digestion of wastewater generated in desiccated coconut processing plants. According to that, the following results were obtained.

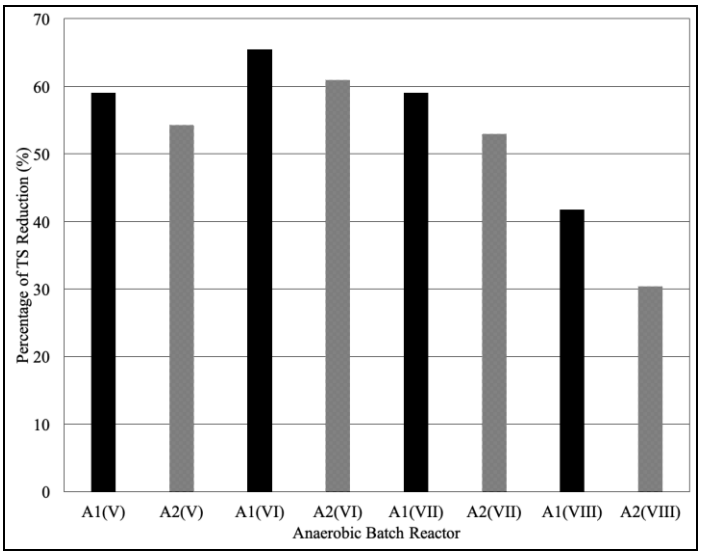

Figure 4.22: Percentage of TS reduction via two different enzymes

According to the Figure 4.22, the percentage of TS reduction at the end of the full degradation was highest in the Wastewater pre-treated with 0.1% (w/v) lipase (Steapsin) ex. Microorganism.

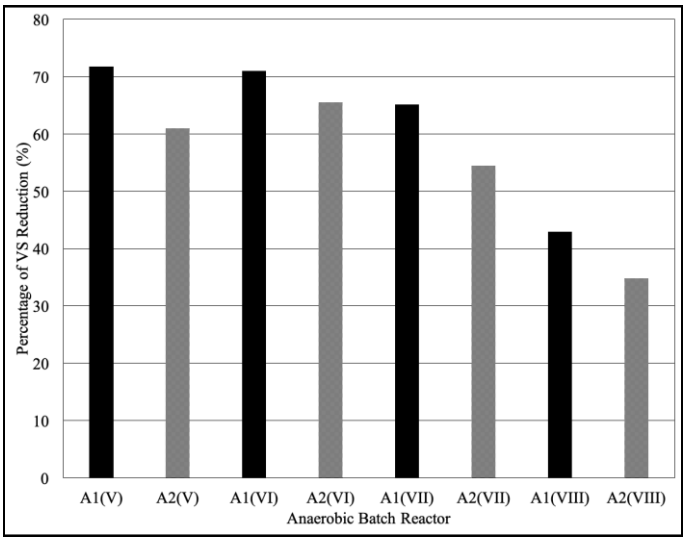

Figure 4.23: Percentage of VS reduction via two different enzymes

According to the Figure 4.23, the percentage of VS reduction at the end of the full degradation was also highest in the Wastewater pre-treated with 0.1% (w/v) lipase (Steapsin) ex. Microorganism.

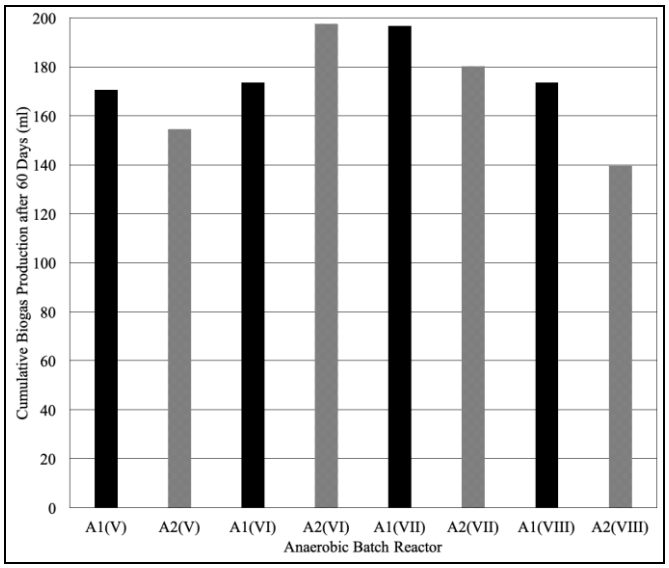

Figure 4.24: Cumulative biogas production via two different enzymes

According to the Figure 4.24, cumulative biogas production at the end of the full degradation was also highest in the Wastewater pre-treated with 0.1% (w/v) lipase (Steapsin) ex. Microorganism except in the category VI.

The above results show that wastewater pre-treated with lipase (Steapsin) ex. Microorganism had the potential to give much better outcome than the lipase from porcine pancreas. Therefore, further experiments should be conducted to optimize its process performance.

5. CONCLUSION AND RECOMMENDATIONS

Considering the results in which obtained from this research study, the following conclusions and further recommendations can be drawn.

5.1. Conclusions

Considering all the results above, the following conclusions can be drawn.

- Considering the overall degradation, the inhibition caused by lipids was not a permanent inhibition but it reduced the rate of biogas production during first few days in anaerobic digestion process.
- Lipase pre-treatment enhanced the initial biogas production rate and cumulative biogas production in the beginning as well as throughout the whole anaerobic degradation process. This happened because lipase existed within the batch anaerobic system and helped the biochemical degradation of lipids.
- Chemical hydrolysis pre-treatment also enhanced the initial biogas production rate and cumulative biogas production. This happened because the initial pH adjustment in feed of pH 7.0 created a growth supportive pH condition for the anaerobic microorganisms in the beginning.
- Cumulative biogas production during first 10 days was higher when initial biogas production rates were higher.
- Bio-methane yield was higher when enzyme concentration was higher. This clearly reveals that the lipases helped in biochemical degradation of substrate and methane generation. This happened because lipases existed within the system for 60 days and actively helped the biochemical degradation of lipids.
- Bio-methane yield did not change when increasing initial pH of wastewater. This shows that the initial pH value of wastewater does not effect on the biochemical degradation of substrate and methane generation. It happened because the hydroxyl ions in sodium hydroxide neutralized soon after the pre-treatment process.
- Percentage of TS and VS reduction after 60 days of complete degradation did not affect by lipase or chemical hydrolysis pre-treatment. This happened

because the enzyme addition and initial pH adjustment in feed only improved the reaction rate but not the degradation. In commercial scale applications, this was very important because this can improve the rate of the reaction.

5.2. Recommendations for the future work

The combined pre-treatment strategy improved the anaerobic digestion of wastewater generated in desiccated coconut processing plants. However, initial pH adjustment in feed and mixing inoculum and substrate in a proper ratio have shown a noticeable improvement in anaerobic digestion even though enzymatic hydrolysis pre-treatment also improved it. Therefore, different types of enzymes should be tested in order to clarify whether this improvement is same for the other lipases. Further studies should be conducted to clarify whether there is a lipase denaturing activity is happening here. The sequential batch experiments should be conducted using two different types of enzymes.

REFERENCES

[1] "Industrial Polution Control Guidelines : No. 3 - Desiccated Coconut Industry," 1992.

[2] D. G. Cirne, X. Paloumet, L. Björnsson, M. M. Alves, and B. Mattiasson, "Anaerobic digestion of lipid-rich waste-Effects of lipid concentration," *Renew. Energy*, vol. 32, no. 6, pp. 965–975, 2007.

[3] C. Ye, C. Jay J, and C. Kurt S., "Inhibition of anaerobic digestion process: A review Ye," *Bioresour. Technol.*, vol. 99, pp. 4044–4064, 2007.

[4] N. Rasit, A. Idris, R. Harun, W. Azlina, W. Ab, and K. Ghani, "Effects of lipid inhibition on biogas production of anaerobic digestion from oily effluents and sludges: An overview," *Renew. Sustain. Energy Rev.*, vol. 45, pp. 351–358, 2015.

[5] Carlos Augusto de Lemos Chernicharo, *Anaerobic Reactors*. IWA Publishing, 2007.

[6] B. K. T. Samarasiri, P. A. D. Mihiranga, and P. G. Rathnasiri, "Effect of Lipid Inhibition in Anaerobic Wastewater Treatment : A Case Study using Desiccated Coconut Wastewater Effect of Lipid Inhibition in Anaerobic Wastewater Treatment : A Case Study using Desiccated Coconut," in *Annual Sessions of IESL*, 2016, no. October, pp. 295–304.

[7] M. & E. Inc., G. Tchobanoglous, F. L. Burton, and H. D. Stensel, *Wastewater Engineering: Treatment and Reuse*, 4th Editio. McGraw Hill Higher Education, 2003.

[8] H. N. Chanakya, H. K. Khuntia, N. Mukherjee, R. Aniruddha, J. R. Mudakavi, and P. Thimmaraju, "The physicochemical characteristics and anaerobic degradability of desiccated coconut industry waste water," *Environ. Monit. Assess.*, vol. 187, no. 12, p. 772, Dec. 2015.

[9] D. R. Rosa *et al.*, "Performance and molecular evaluation of an anaerobic system with suspended biomass for treating wastewater with high fat content after enzymatic hydrolysis," *Bioresour. Technol.*, 2009.

[10] A. A. Mendes, E. B. Pereira, and H. F. de Castro, "Effect of the enzymatic hydrolysis pretreatment of lipids-rich wastewater on the anaerobic biodigestion," *Biochem. Eng. J.*, vol. 32, no. 3, pp. 185–190, Dec. 2006.

[11] S. Cherif, F. Aloui, F. Carrière, and S. Sayadi, "Lipase Pre-Hydrolysis Enhance Anaerobic Biodigestion of Soap Stock from an Oil Refining Industry," vol. 114, no. 2, pp. 109–114, 2014.

[12] P. Taylor, F. R. C. Damasceno, D. M. G. Freire, and M. C. Cammarota,

"Assessing a mixture of biosurfactant and enzyme pools in the anaerobic biological treatment of wastewater with a high-fat content," no. December 2014, pp. 37–41.

[13] K. Sri, B. Kameswari, C. Kalyanaraman, S. Porselvam, K. Thanasekaran, and E. T. Division, "Enhancement of Biogas Generation by Addition of Lipase in the Co-Digestion of Tannery Solid Wastes," vol. 39, no. 8, pp. 781–786, 2011.

[14] I. Angelidaki, S. P. Petersen, and B. K. Ahring, "Effects of lipids on thermophilic anaerobic digestion and reduction of lipid inhibition upon addition of bentonite," *Appl. Microbiol. Biotechnol.*, 1990.

[15] E. Nordell, A. B. Hansson, and M. Karlsson, "Zeolites relieves inhibitory stress from high concentrations of long chain fatty acids," *Waste Manag.*, vol. 33, no. 12, pp. 2659–2663, Dec. 2013.

[16] T. V. Adulkar and V. K. Rathod, "Ultrasound assisted enzymatic pre-treatment of high fat content dairy wastewater," *Ultrason. Sonochem.*, 2014.

[17] N. A. Oz and C. C. Yarimtepe, "Ultrasound assisted biogas production from landfill leachate," *Waste Manag.*, vol. 34, no. 7, pp. 1165–1170, Jul. 2014.

[18] L.-J. Wu, T. Kobayashi, Y.-Y. Li, and K.-Q. Xu, "Comparison of single-stage and temperature-phased two-stage anaerobic digestion of oily food waste," *Energy Convers. Manag.*, vol. 106, pp. 1174–1182, Dec. 2015.

[19] M. BECCARI, M. MAJONE, and L. TORRISI, "Two-reactor system with partial phase separation for anaerobic treatment of olive oil mill effluents," *Water Sci. Technol.*, vol. 38, no. 4–5, pp. 53–60, 1998.

[20] S. Astals, D. J. Batstone, J. Mata-Alvarez, and P. D. Jensen, "Identification of synergistic impacts during anaerobic co-digestion of organic wastes," *Bioresour. Technol.*, 2014.

[21] L. Martín-González, L. F. Colturato, X. Font, and T. Vicent, "Anaerobic co-digestion of the organic fraction of municipal solid waste with FOG waste from a sewage treatment plant: Recovering a wasted methane potential and enhancing the biogas yield," *Waste Manag.*, vol. 30, no. 10, pp. 1854–1859, Oct. 2010.

[22] R. Affes, J. Palatsi, X. Flotats, H. Carrère, J. P. Steyer, and A. Battimelli, "Saponification pretreatment and solids recirculation as a new anaerobic process for the treatment of slaughterhouse waste," *Bioresour. Technol.*, 2013.

[23] R. Uma Rani, S. Kaliappan, S. Adish Kumar, and J. Rajesh Banu, "Combined treatment of alkaline and disperser for improving solubilization and anaerobic biodegradability of dairy waste activated sludge," *Bioresour. Technol.*, vol. 126, pp. 107–116, Dec. 2012.

[24] M. López Torres and M. d C. Espinosa Llorens, “Effect of alkaline pretreatment on anaerobic digestion of solid wastes,” *Waste Manag.*, 2008.

[25] J. M. Berg, J. L. Tymoczko, and L. Stryer, *Biochemistry*, 7th Editio. Palgrave MacMillan, 2011.

[26] R. F. Domingues *et al.*, “Effect of enzymatic pretreatment on the anaerobic digestion of milk fat for biogas production,” *Food Res. Int.*, vol. 73, pp. 26–30, Jul. 2015.

[27] Y. Meng, F. Luan, H. Yuan, X. Chen, and X. Li, “Enhancing anaerobic digestion performance of crude lipid in food waste by enzymatic pretreatment,” *Bioresour. Technol.*, 2016.

[28] P. Taylor *et al.*, “The effect of enzymatic pre-hydrolysis of dairy wastewater on the granular and immobilized microbial community in anaerobic bioreactors,” no. December, pp. 37–41, 2013.

[29] E. Efremenko, O. Senko, D. Zubaerova, E. Podorozhko, and V. Lozinsky, “New Biocatalyst with Multiple Enzymatic Activities for Treatment of Complex Food Wastewaters,” vol. 9862, no. 2, pp. 208–212, 2008.

[30] V. M. F. Alexandre, A. M. Valente, M. C. Cammarota, and D. M. G. Freire, “Performance of anaerobic bioreactor treating fish-processing plant wastewater pre-hydrolyzed with a solid enzyme pool,” *Renew. Energy*, vol. 36, no. 12, pp. 3439–3444, 2011.

[31] K. J. Kennedy and J. D. B. O, “Effect of enzymes on anaerobic digestion of primary sludge and septic tank performance,” 2012.

[32] A. B. G. Valladão, D. M. G. Freire, and M. C. Cammarota, “Enzymatic pre-hydrolysis applied to the anaerobic treatment of effluents from poultry slaughterhouses,” *Int. Biodeterior. Biodegrad.*, 2007.

[33] G. Dors and A. A. Mendes, “Simultaneous enzymatic hydrolysis and anaerobic biodegradation of lipid-rich wastewater from poultry industry,” pp. 343–349, 2013.

[34] E. Mobarak-Qamsari, R. Kasra-Kermanshahi, M. Nosrati, and T. Amani, “Enzymatic pre-hydrolysis of high fat content dairy wastewater as a pretreatment for anaerobic digestion,” *Int. J. Environ. Res.*, vol. 6, no. 2, pp. 475–480, 2012.

[35] Z. Ning, J. Ji, Y. He, Y. Huang, G. Liu, and C. Chen, “E ff ect of Lipase Hydrolysis on Biomethane Production from Swine Slaughterhouse Waste in China,” 2016.

[36] *Oil and Grease, Hexane Extractable Gravimetric Method*. U. S. Environmental Protection Agency.

[37] R. Uma Rani, S. Adish Kumar, S. Kaliappan, I. T. Yeom, and J. Rajesh Banu, "Low temperature thermo-chemical pretreatment of dairy waste activated sludge for anaerobic digestion process," *Bioresour. Technol.*, vol. 103, no. 1, pp. 415–424, 2012.

[38] K. S. B. Kameswari, C. Kalyanaraman, S. Porselvam, and K. Thanasekaran, "Optimization of inoculum to substrate ratio for bio-energy generation in co-digestion of tannery solid wastes," pp. 241–250, 2012.

[39] A. A. Mendes, E. B. Pereira, and A. F. Jr, "Anaerobic Biodegradability of Dairy Wastewater Pretreated with Porcine Pancreas Lipase," vol. 53, no. December, pp. 1279–1284, 2010.