INVESTIGATION ON ANAEROBIC DIGESTION OF CHEESE WHEY FOR ENHANCED METHANE GENERATION

A Thesis

Submitted in partial fulfilment of the requirements

for the award of the degree of

Doctor of Philosophy

by

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JULY 2023

Declaration

This is to certify that the work presented in the thesis entitled **"INVESTIGATION ON ANAEROBIC DIGESTION OF CHEESE WHEY FOR ENHANCED METHANE GENERATION"** is a bonafide work done by me under the supervision of **Dr. P. Venkateswara Rao** and was not submitted elsewhere for the award of any degree. I declare that this written submission represents my ideas in my own words and where others' ideas or words have been included, I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that any violation of the above will be a cause for disciplinary action by the institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

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This is to certify that the thesis entitled **"INVESTIGATION ON ANAEROBIC DIGESTION OF CHEESE WHEY FOR ENHANCED METHANE GENERATION"** submitted by Ms. Bella K to the National Institute of Technology, Warangal, for the award of the degree of Doctor of Philosophy in Civil Engineering is a bonafide research work carried out by her under my supervision and guidance. The results contained in this thesis have not been submitted in part or full to any other University or Institute for award of any degree or diploma.

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Acknowledgements

Words can hardly convey the depth of gratitude that I feel towards my esteemed supervisor, **Dr. P Venkateswara Rao**, Associate Professor, Department of Civil Engineering, National Institute of Technology, Warangal. In the pursuit of my academic journey, he has been a profound source of inspiration, a pillar of strength, and a beacon of unwavering support. I am deeply grateful for his patience and encouragement during the challenging moments, as well as for his ability to inspire and motivate me to strive for excellence in every aspect of my work. I wholeheartedly acknowledge his full co-operation that I received from the beginning of the work to the completion of this thesis.

I express my deepest sense of reverence and indebtedness to Dr. P. Sridhar, Assistant Professor, Department of Civil Engineering, National Institute of Technology, Warangal for his invaluable contributions, support, and mentorship throughout my work.

I am also thankful to my doctoral scrutiny chairman *Prof. Gunneswara Rao T.D***,** Head of Civil Engineering Department and other committee members *Prof. N.V. Umamahesh*, Department of Civil Engineering, *Prof. R. Satish Babu*, Department of Biotechnology and *Dr.Sumanth Chinthala,* Assistant professor, Department of Civil Engineering for their invaluable contributions and guidance throughout the journey of this thesis.

The provision of samples by NSR dairy, located in Warangal and Sanitation Park, located in Ammavaripet were crucial in conducting the experimental work, and I am immensely grateful for their collaboration in this aspect. Their cooperation and prompt response to queries have been truly commendable, making this research possible.

I would like to thank all the lab assistants from Environmental Engineering lab especially Mr. Naveen Kumar for his constant help in the lab which made me work very smoothly considering all the hinderance.

I would like to express my deepest gratitude to our team including my friend Ms. Sneha Korpe and Mrs. Sagarika Mothe for all the useful insights and assistance offered during the course of work. Throughout the ups and downs, there are some close friends who have stood by my side, offering words of motivation, understanding my moments of stress, and celebrating each milestone achieved. I would like to express my deepest gratitude to *Ms. Parvathy M L, Mr. Aravind Takkallapally, Ms. Samhita Ankareddy, Mr. Vipin K P, Ms Sneha Korpe, Mr. G V Sai Krishna Reddy, Mr. Sudheekar Reddy, Mr. Prasanta Majee, Mr M. Sagar Kumar* and *Mr.Uppala Lavakumar* for their constant support.

I would not have undertaken this journey without the support, love and encouragement by my parents- *Mr. Sivadasan K* and *Mrs. Sreeja T .U* whose belief in my abilities have inspired me to pursue my dreams and never settle for anything less than my best. I am also thankful to my in-laws family, *Mr. Mohan Das K.R ,Mrs. Sheeba O.P, Mr. Sumesh Mohan K R* for their support and prayers.

Throughout this challenging journey, my partner *Mr. Minosh Mohan K.R* have been my rock, providing a constant source of motivation and belief in my abilities. Your patience, love, and understanding have sustained me during the long hours of research and writing, making this achievement possible. I want to express my heartfelt appreciation for your unconditional love and encouragement, which have been instrumental in overcoming the challenges faced during this academic journey. I am so grateful to have you by my side on this remarkable journey.

Finally, I dedicate this thesis to my beloved parents.

Bella K

Abstract

Whey residues or whey wastewater, generated during cheese or paneer manufacturing from dairies, pose significant pollution challenges due to their high organic content, containing milk solids, proteins, fats, and lipids. The levels of organic matter, as indicated by the chemical oxygen demand and biochemical oxygen demand, can reach up to 60,000mg/l and 46,000mg/l, respectively, in whey wastewater. Due to the financial and technological constraints imposed by the treatment of whey wastes, small-scale dairy producers have long had difficulty in managing these wastes. Anaerobic digestion (AD), a promising biological treatment approach that provides opportunities for energy recovery, has come to light as a solution to this problem. Although several researchers have already looked into this subject, there are still some significant gaps. By applying suitable solutions including co-digestion, pre-treatment techniques, and the addition of external materials, challenges experienced during the AD of cheese whey wastewater, such as quick acidity, sludge flotation, and insufficient buffering, can be slightly alleviated. This study helps to identify a suitable co-substrate to enhance the digestibility and methane productivity of whey, to adopt appropriate pre-treatment method conducting detailed energy and cost analysis, and to understand the effect of additive like biochar in stabilising AD process.

Objective 1- The present study investigated the possibilities of improving the digestibility from anaerobic digestion of lipid rich dairy by-product, cheese whey using septage as the co-substrate with different inoculum. Biochemical methane potential assays were conducted under mesophilic temperature conditions and results were validated using Modified Gompertz Model. Two sets of BMP tests were done; to assess the individual and combined digestion abilities of septage in anaerobic co-digestion of whey and to assess the ability of 3 inoculum sources (cattle manure, sewage sludge, and acclimatized anaerobic sludge) in the co-digestion process. The results indicated that septage is an excellent co-substrate that has better adaptability with CW and the optimum mix ratio was found as 40:60 (CW: SP). BMP tests were also conducted with inoculum at S/I ratio of 1 and statistical analysis was performed to study the synergistic effect of both co-digestion and inoculum. The tests revealed that the cattle manure resulted in the highest biogas production (342.22mL/gVS) at 60% whey fraction. Modified Gompertz model fitted the experimental data well and identified an increase in lag phase times when whey fraction is increased. Comparatively higher lag phase times ranging from 1.98 to 4.35 days were obtained for sewage sludge inoculated samples. The maximum methane production (P_{max}) was

obtained at 60% whey fraction (369.63 ± 4.05mL/gVS) at a very short lag time of 0.76 ± 0.17days for cattle manure inoculated mixture.

Objective 2- Lactose in cheese whey wastewater makes it difficult to degrade under normal conditions. The effect of ultra-sonication (US), ozonation and enzymatic hydrolysis on increasing the bioavailability of organic matter in CW and biogas production were evaluated. The pre-treatment conditions were: specific energy input varied from 2130 to 8773kJ/kgTS for a sonication time of 4.5–18.5 min, Ozone (O_3) dosages ranging from 0.03 to 0.045gO_3/gTS were applied for 4–16 min, pH (3.8–7.1), temperature (35◦C–55◦C), enzyme dosage (0.18–0.52%), was operated from 7.75 to 53 min for enzymatic hydrolysis by β-galactosidase. The results of the US reported a maximum sCOD solubilisation of 77.15% after 18.5 min of operation, while the corresponding values for ozonation and enzymatic methods were 64.8% at 16 min and 54.79%, respectively. The organic matter degradation rates evaluated in terms of protein and lactose hydrolysis were 68.78%,46.03%; 47.83%,16.15% and 54.22%,86.2%respectively, for US, ozonation and enzymatic methods. The cumulative methane yield for sonicated, ozonised and enzymatically hydrolysed samples were 412.4 ml/g VS, 361.2 ml/g VS and 432.3mlCH_4/ gVS, respectively. Regardless of the lower COD solubilisation rates attained, enzymatic pre-treatment showed maximum methane generation compared to US and ozonation. This could be attributable to the increased activity of β-galactosidase in hydrolysing whey lactose. The energy calculations revealed that the pre-conditioning of organic-rich CW with enzymatic hydrolysis is more effective and efficient, yielding a net energy gain (gross output energy-input energy) of 9166.7 kJ and an energy factor (ratio of output to input energy) of 6.67. The modified Gompertz model well simulated all experimental values.

Objective 3- The addition of septage-derived biochar helped in increasing the methane yield in all mixtures. The maximum cumulative methane yield was obtained at 50 g/l of biochar loading at 10 % TS content, 486 ml/g VS. The lowest methane yield was reported at 5% TS concentration with 6.25 g/l of biochar loading as 243.2 ml/g VS. The daily methane yield was lowered from 25th day onwards for mixtures with biochar loadings 25 g/l and 50 g/l at TS concentrations > 10%. The biochar dosage was found to be more significant than total solids concentrations. Undesirable biochemical changes observed in the digestion mixture at higher total solid content due to increased viscosity and reduced diffusion coefficient might have resulted in lower methane production.

Objective 4- The AD of CW and septage with cattle manure as inoculum was successfully executed in a 2 stage lab-scale anaerobic digester. The highest biogas yield was obtained when acidogenic reactor was operated at an organic loading rate of 85.8 gCOD/ld and HRT 1 day. Complete inhibition of methanogens was found since biogas was exclusively composed of hydrogen and carbon dioxide. The microbial population shift and the complexity of the feed medium could be potential causes for the observed inhibition, which may be attributed to the extended operational time. The steady-state conditions for methanogenic reactor was obtained at HRT 14d and OLR 6.12gCOD/ld. Highest biogas and methane yield obtained was 1.81L/L_rd and 1.02L/L_rd respectively. Based on the lab scale results obtained, an industrial scale anaerobic digester of total 26m^3 volume was designed having a bioenergy generation potential of 274.7kwh.

Summary: Anaerobic co-digestion of cheese whey with the nitrogen-rich septage waste enhanced methane productivity by providing essential nutrients and balancing carbon-to-nitrogen ratio. Methane productivity and efficiency of digestion were further enhanced by introducing a mature and active microbial community containing inoculum-cattle manure. Among various physico-chemical pre-treatment techniques, enzymatic hydrolysis was identified as the most efficient, feasible, and cost-effective method to hydrolyse whey lactose and reduce substrate complexity. Further, septage-derived biochar with a buffering capacity and essential nutrients contributed to reactor stability. The two-stage lab-scale digester was operated successfully, and the data on real-time whey wastewater generation rates were used to design an industrial-scale anaerobic digester. The design confirmed a bioenergy potential of 697.1 Wh, with the potential for generating 487.9 Wh of heat and 209 Wh of electricity. A brief summary would be that this study aimed to eliminate the problems encountered during AD of CW by identifying a suitable co-substrate for co-digestion, choosing an inexpensive, efficient pre-treatment method, and selecting an appropriate additive which can be scaled further for adoption at dairy industries.

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List of Acronyms

AD	Anaerobic digestion
ACoD	Anaerobic Co-digestion
Alk	Alkalinity
AnMBR	Anaerobic Membrane Bioreactor
ANOVA	Analysis of variance
AS	Anaerobic sludge
BC	Biochar
BEP	Bioenergy potential
BET	Brunauer-Emmett-Teller surface area
BMP	Biochemical methane potential
BOD	Biochemical oxygen demand
CCD	Central composite design
C/N	Carbon-to Nitrogen ratio
CM	Cattle manure
COD	Chemical oxygen demand
CSTR	Continuously stirred tank reactor
CW	Cheese whey
DNSA	Di-nitro salicylic acid
DO	Dissolved oxygen
DWW	Dairy wastewater
EGSB	Expanded granular bed reactor
FA	Free ammonia
FTIR	Fourier Transform Infrared Spectroscopy
HC	Hydrochar
HHV	Higher heating value
HRT	Hydraulic retention time
NEG	Net energy gain
OFMSW	Organic fraction of municipal solid waste
OLR	Organic loading rate
RSM	Response surface methodolgy
sCOD	Soluble chemical oxygen demand
SEM	Scanning Electron microscope

SMY	Specific methane yield
SS	Sewage sludge
SSA	Specific surface area
SP	Septage
tVFA	Total volatile fatty acids
TKN	Total Kjeldahl Nitrogen
TAN	Total ammonia nitrogen
TS	Total solids
TSS	Total suspended solids
UASB	Upflow anaerobic sludge blanket reactor
US	Ultra-sonication
VFA	Volatile fatty acids
VS	Volatile solids
XRD	X-Ray Diffraction

Chapter 1

Introduction

1.1 Overview

In this chapter, there is a concise examination of the worldwide and Indian milk and milk product production statistics in recent years. Subsequently, the focus shifts to the discussion of the significant dairy product residues that contribute to environmental pollution. Following that, the chapter delves into the production process of cheese, as well as the various waste management solutions for the cheese whey (CW) wastewater that is generated during cheese production. Additionally, a review is provided on the recent literature works that explore the anaerobic digestion (AD) of CW, along with their key findings. Other topics covered in this chapter encompass strategies for improving the digestibility of whey by addressing the obstacles faced during anaerobic digestion. This includes exploring the feasibility of using different organic wastes as co-substrates, as well as examining the impact of pre-treatment techniques and the use of additives in AD. The concluding section provides a clear overview of the research objectives and outlines the structure of the dissertation.

1.2 Global milk production

India occupies first position in milk production globally since 1998. Other major milk producers were United states, China, Pakistan and Brazil. According to the OECD-FAO Agricultural Outlook 2022-29; India produced 221.1 million tons of milk which comprises about 23% of that year's total milk production in year 2021-22 (FAO, 2022). Also per capita availability has increased from 178 gm/day in 1991–1992 to 444 grams/day in 2021-22. Figure 1.1 shows the year wise milk production of India during last 10 years. Over the years, milk production in India has been consistently rising, and this has resulted in a significant contribution of the dairy industry towards the rural economy and livelihoods in the country. Top milk-producing states in India are; Uttar Pradesh (16.3%), Rajasthan (12.6%), Madhya Pradesh (8.5%), Andhra Pradesh (8%), and Gujarat (7.7%) which altogether contributes around 53.1% of the total milk produced in the country (B.A.H.S, 2019).

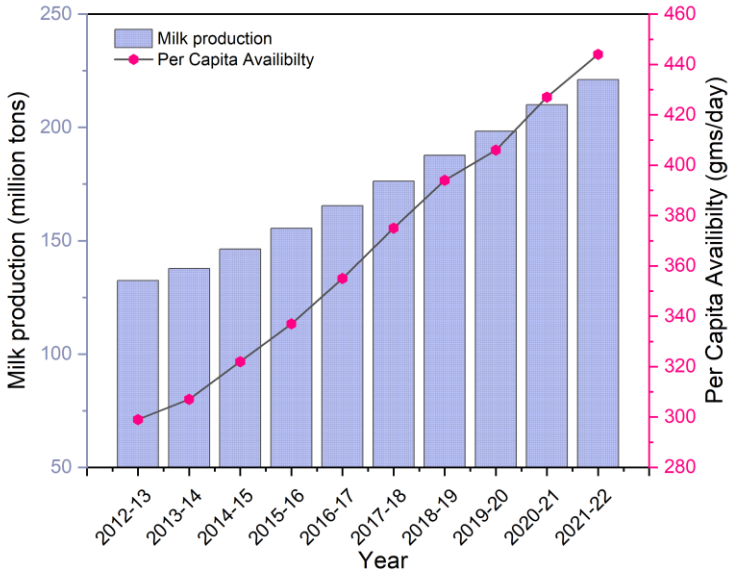

Figure 1. 1 Milk production statistics of India

1.3 Milk processing and wastes generated

A growing population throughout the world is challenging dairy industry producers to meet the growing demand for milk and milk-products. This rise in demand led to the establishment of more and more dairy units in urban and rural settlements. Milk production units are mostly located in rural areas and urban centres were sources for the location of milk processing units where different types of products are manufactured (Kolhe et al., 2002). From milk production unit to collection and processing steps different streams of wastewater are generated in the dairy industry. Dairies have relocated to more desirable climates, lands, and water availability due to technological advancements in milk handling and processing.

Apart from milk, value-added products like butter, curd, ghee, cheese, paneer, yogurt, flavoured milk, etc., and many more products are made. Different types of wastewater are generated during the production of a wide range of products. Generally, wastewater generated in a dairy industry can be categorized into 3 different streams; cleaning wastewater, processing wastewater, and sanitary wastewater (Kolev Slavov, 2017). As the rate of water consumption is high in dairies, the quantity of water generated as waste effluent is also large. It is estimated that around 2-2.5litres of water generates as effluent after processing 1litre of milk. Gathering and processing raw milk into a variety of pasteurised and condensed goods, including cottage cheese, yoghurt, cream, butter products, drinks, lactose, whey powder, and a variety of sweets, is the main process in a dairy. (Nadais et al., 2010; Trevor J.Britz et al., 2005).

Before selecting the best treatment method, it is crucial to be aware of the many waste streams generated by the dairy industry. Different forms of waste effluents are produced as a result of

the manufacture of a wide variety of dairy products. Small-scale industries primarily concentrate on producing pasteurized milk and ghee from scoured milk, whereas larger-scale industries, benefiting from abundant milk supply, manufacture a diverse array of dairy products. This includes milk powder, butter, yogurt, cheese, casein, buttermilk, ice cream, as well as a variety of traditional Indian sweets like khoa, paneer, srikhund, regular milk cake, and others. (Kolhe et al., 2002). A layout of different steps involved in milk-processing is depicted in Figure 1.2.

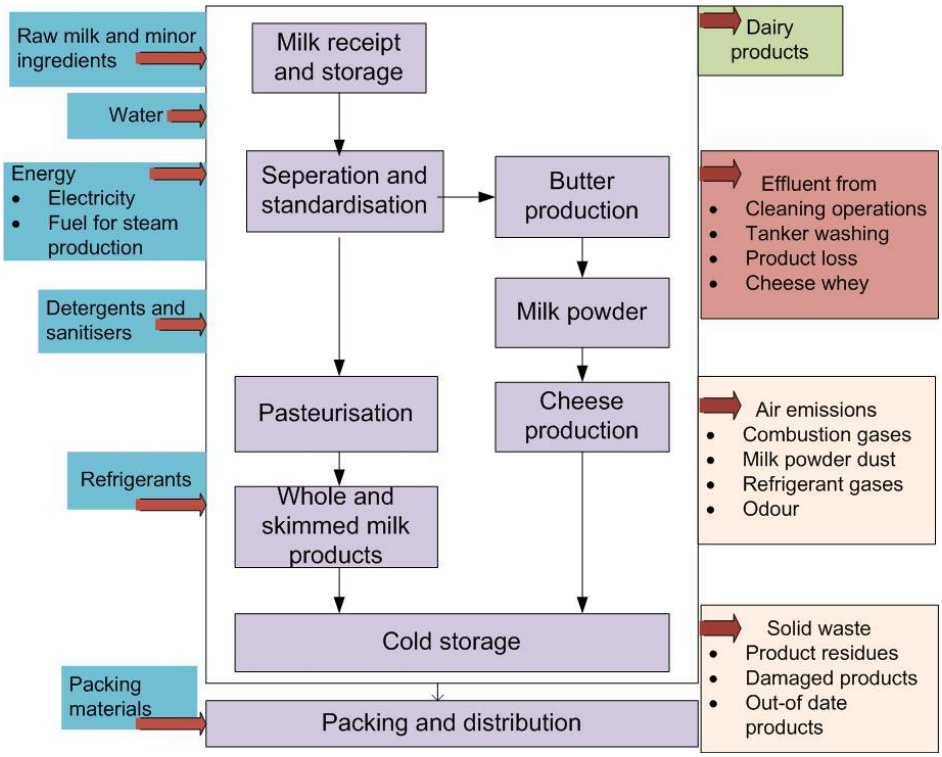

Figure 1. 2 Flowchart of dairy industry milk processing steps

The production processes of typical dairy products can vary between different dairy industries. As a consequence, this leads to the creation of distinct waste streams, each exhibiting considerable variations in their characteristics. Dairy industries require more water than other food sectors do; typically, 2 to 5 litres of water are needed to process 1 litre of milk, depending on the size and technology used. Based on their origin and composition, the waste streams generated in the dairy sector can generally be divided into processing fluids, cleaning wastewaters, and sanitary wastewater (Trevor J.Britz et al., 2005). After pasteurization, milk undergoes various processing methods to produce other dairy products such as butter, cheese, ice-cream, yogurt, and more. Table 1.1 outlines the key features of residues found in major milk products, providing a clear understanding of the pollution potential of dairy effluents.

Table 1. 1 Characteristics of different dairy effluents

Type of waste	pH	COD (g/L)	BOD (g/L)	Solids (g/L)	Nitrogen (mg/L)	Phosphorous (mg/L)	Reference
Cheese whey	4.9	68.6	7.71	1.95	1120	500	(Vidal et al., 2000)
Hard cheese whey	5.8	73.45	29.48	NA	NA	NA	(Janczukowicz et al., 2008)
Cottage cheese whey	5.35	58.55	26.76	NA	NA	NA	(Janczukowicz et al., 2008)
Ice-cream wastewater	5.2	5.2	2.45	3.9	60	14	(Rafael Borja and Charles J.Banks, 1995)
Milk processing wastewater	4-7	5-10	3-5	3-7	20-150	50-70	(Bezerra RA et al., 2007)
Yogurt wastewater	4.53	6.5	NA	NA	NA	NA	(Tezcan Un and Ozel, 2013)
Milk permeate	5.5-6.52	55.2-63.8	NA	2.67-3.80	300-400	350-450	(Wang et al., 2009)
Fresh cream wastewater	8-11	2-6	1.2-4	NA	NA	NA	(Danalewich et al., 1998)

1.4 Cheese whey generation and utilization in India

In the dairy industry, the production of cheese results in the generation of CW, or whey for short, which contributes a significant organic load to the combined dairy effluent. The raw milk after collection, pasteurisation and testing was undergone coagulation by adding enzymes, vinegar or acid-like substances which thickens milk and curd is formed. These curds are heated, stirred and filtered to remove whey. Then salting and moulding is done. The extraction step in this process vary for different type of cheese (Trevor J.Britz et al., 2005). The various steps in production of cheese was depicted in Figure 1.3. The global production of CW is around 160 million tons per year. In India, the main sources of whey were chhana and paneer production. Around 5 million tons of whey was estimated to produce in India annually (Singh and Rani, 2019). CW, due to its high nutritional content can be processed into many edible food products

and protein powders. Due to technological and economical constraints, small and medium-sized dairies are unable to process CW further and are forced to discard large amounts of effluent generated during the manufacturing process.

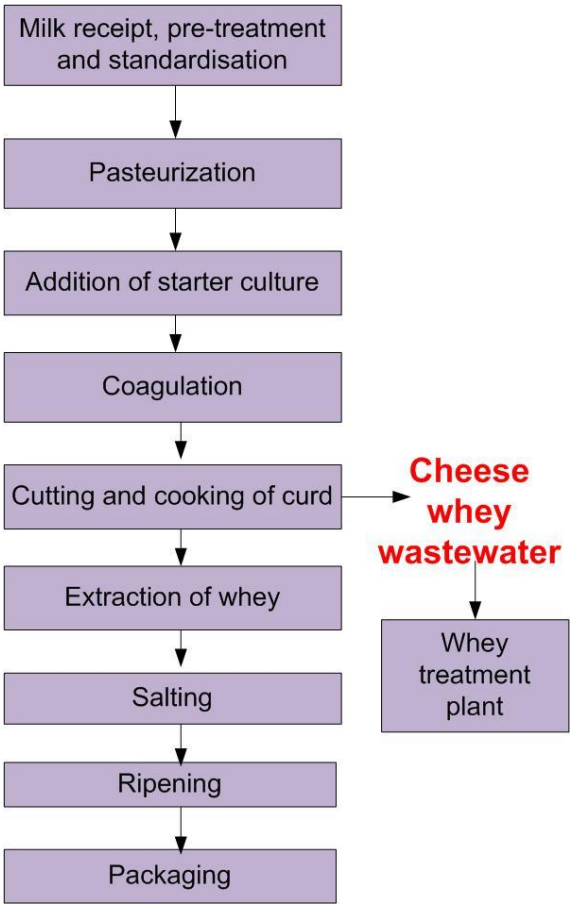

Figure 1. 3 Schematic steps of cheese production process

1.5 Characteristics of cheese whey wastewater

Whey residues are the main liquid by-product of the cheese manufacturing process, which contributes about 85–95% of the milk volume and contain about 55% of the milk contents. Whey retains a significant portion of the whole milk's proteins, fats, lactose, water-soluble minerals, and other nutrients. The characteristics of whey depend on many factors like the type of cheese manufactured, operating conditions chosen for production, process technology used for cheese production etc. Whey, whether acidic or sweet, contains lactose concentrations varying from 3.3-6%, fat 0.15-1%, proteins 0.32-0.7% and salt traces (Gelegenis and Georgakakis, 2007; Tsakali et al., 2010). COD concentration varies significantly for DWW which reaches up to 60,000 mg/l for whey processing water (Gannoun et al., 2008). Similarly, BOD values are also critically high for whey processed waters (0.56–40 g/l) (Dareioti and Kornaros, 2015; Gannoun et al., 2008; Trevor J.Britz et al., 2005). The cheese whey effluents are characterised by high organic content because of the presence of lactose (0.15–60 kg/m^3), fats (0.08–10.58 kg/m^3), and proteins (1.4– 33.5kg/m^3). Other constituents include minerals

(0.46–10 kg/m^3) total suspended solids (0.1–22 kg/m^3), phosphorous (0.006–0.5 kg/m^3), and Total Kjeldahl nitrogen (0.01–1.7 kg/ m^3) (Erguder et al., 2001). The presence of chlorides is seen in salty whey solutions and brines. If effluents having chlorides > 400 mg/ l is disposed into streams, this will lead to chronic toxicity.

1.6 Anaerobic digestion of cheese whey

AD, a well-established waste-to-energy method, involves the biological conversion of organic matter into usable energy sources. This process holds significant potential for stabilizing manure, controlling odors, reducing sludge volume, and producing energy. In addition to these benefits, AD utilizes a simpler and more cost-effective technology compared to aerobic treatment systems, requiring less energy and space. (Cantrell et al., 2008). Moreover, it employs simpler and relatively inexpensive technology which requires less energy and space compared with aerobic treatment systems. Effluents from industries such as sugar, wood, and dairy, which contain high levels of organic content, can be effectively utilized for energy generation in the form of biogas through AD. The produced biogas primarily consists of methane (CH4) and carbon dioxide (CO2) and can be employed as a combustion gas to operate a generator, generating both heat and electricity. Moreover, biogas has various other applications, including being used as a cooking gas alternative to natural gas, as a fuel source (bio-methane) for vehicles and other purposes, and as a raw material for chemical synthesis processes. (Vasudevan et al., 2019). This versatility makes AD an environmentally friendly and sustainable solution for organic waste management and energy production. Four steps of AD are hydrolysis, acidogenesis, acetogenesis, and methanogenesis. The general pathway of various stages of anaerobic digestion is shown in Figure 1.4.

AD is widely recognized as an environment-friendly option to treat highly organic and biodegradable industrial wastes like dairy effluents. The application of anaerobic methods for treating dairy effluents is not only energy conserving but also helps in the generation of energy in the form of biogas. AD of CW can be a 3-way process; energy recovery, pollution reduction, and nutrient recovery (Kataki et al., 2016). AD has become a more attractive and sustainable option as a result of the constant increase in the amount of surplus cheese whey. AD allows for the simultaneous recovery of organic carbon and bioenergy in the form of bio-methane (CH_4). Many laboratory and pilot scale studies have been reported on the anaerobic treatment of CW with potential methane generation values ranging from 0.32 – 0.85mL/g VS (Dreschke et al., 2015; Labatut et al., 2011).

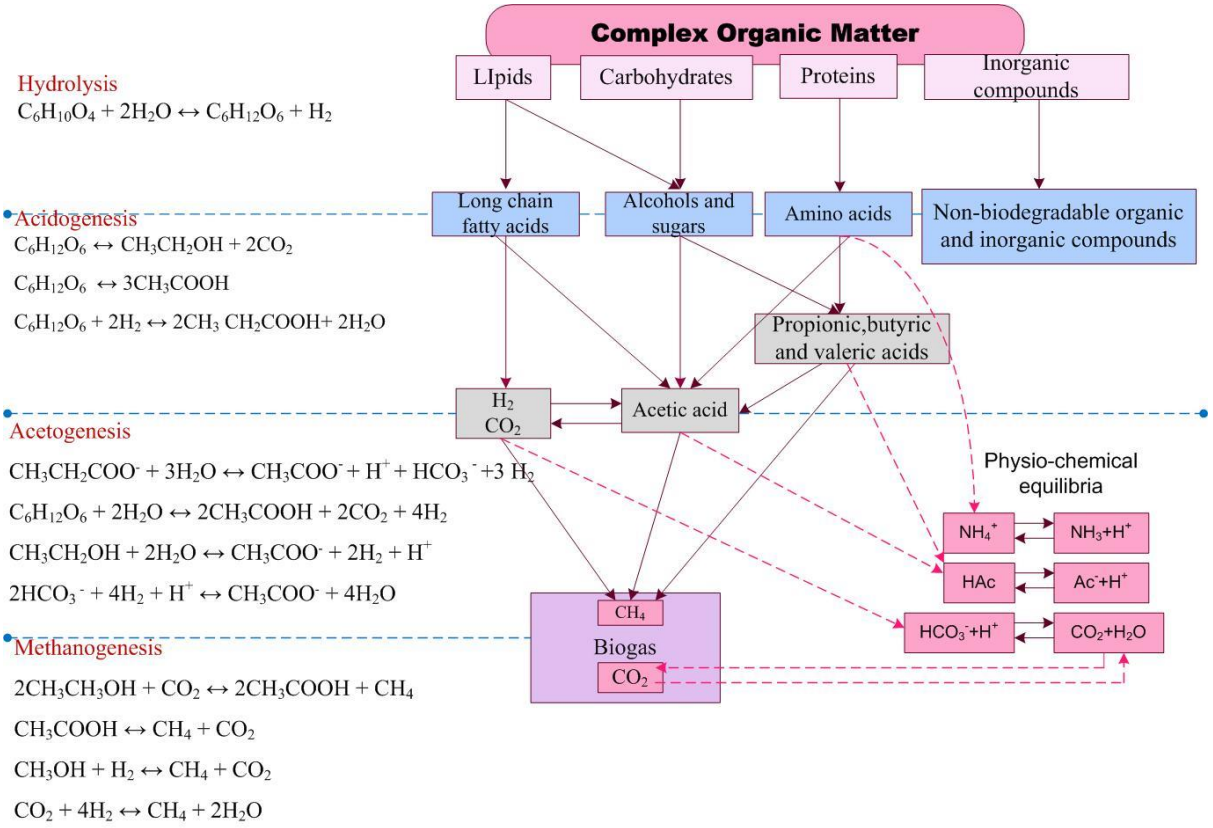

Figure 1. 4 Stages of Anaerobic digestion

1.7 Limitations of anaerobic digestion of whey

The AD of CW faces various challenges due to its compositional characteristics as well as difficulty in maintaining operation parameters during digestion process. These challenges include rapid acidification, lack of alkalinity, difficulty in attaining granulation, etc. which may eventually lead to digester failure. Although CW is rich in biodegradable organic content, biogas generation often get inhibited due to volatile fatty acid accumulation caused by lactose fermentation. Due to this, the build-up of acids can result in a pH decrease, promote the proliferation of acetogenic bacteria, and hinder the activity of methanogenic microorganisms (Yang et al., 2003). Being an acidic substrate, mono digestion of CW may result in the release of inhibitory substances like ammonia, long chain fatty acids, etc. during the hydrolysis, which may inhibit the process.

CW is a surprisingly concentrated organic substrate and has high levels of both chemical and biochemical oxygen demand (BOD and COD). This accumulation of organic debris has the potential to cause process instability and the potential suppression of anaerobic microbes if not properly handled. Another difficulty arises due to the abundance of organic matter found in cheese whey, having potential to overwhelm the anaerobic digestion system, resulting in process inefficiencies or even complete failure if not appropriately handled. Fats, lipids, and certain organic acids, among other components found in CW, might hinder the growth of

anaerobic microbes, which could lead to a reduction in biogas output or system failure. Successfully digesting CW often requires longer retention durations in the digester due to its complex makeup. This results in bigger reactor volumes and increased operational costs. The proteins in cheese whey have the potential to cause excessive foaming, which obstructs the separation of gas and liquid and can damage or clog equipment. Additionally, scum build-up on the digester's surface can make things more difficult overall.

Despite these drawbacks, AD of CW is still a workable and environmentally beneficial waste management method as long as the right pre-treatment and process optimisation are used to address the problems at hand.

1.8 Motivation

Despite the economic gains in the amount of whey being processed, a large amount of whey produced is still disposed of as raw whey. Disposal options include treatment at municipal sewage plants, spreading on local farmlands, direct discharge to surface waters, supply as cattle feeds etc. Some portions of whey like acid whey used to remain unutilised due to its mineral content and low pH. Biological methods are widely used for treating wastewaters having organic content since the process is based on maintaining a biological environment for the growth of microorganisms. These microbes convert the organic matter into new cells, several gaseous and dissolved products. The technology of anaerobic digestion of whey has been recognised as the most viable option for treating highly organic wastes like whey with efficient energy generation.

Among the processing techniques discussed earlier, anaerobic methods are found viable for treating highly organic wastes like whey wastewater. There has been growing interest in employing anaerobic methods for treating dairy wastewater due to the known benefits like energy generation, high rate of biodegradation, no requirement of energy for aeration, operation at high organic loads etc. But the anaerobic process involves complex reactions and a group of unidentified microbes that control the biochemical reactions. The process lacks many deficiencies in terms of uncertainties related to operational parameters, reactor instabilities, poor start-up etc. Many of these instabilities can be effectively solved if appropriate pre-treatment and co-digestion substrates were chosen. The studies on effective digestion of whey wastes is little or none.

1.9 Aim and objectives of the thesis

The overall aim of the work is to enhance the degradability of cheese whey to successfully reduce its polluting potential and to obtain energy through AD of whey in a sustainable manner. The specific objectives can be listed out as follows:

- To evaluate the biogas generation potential of Cheese Whey using septage as a co-substrate in anaerobic digestion.
- To compare the effectiveness of pre-treatment technologies in the anaerobic digestion of Cheese Whey.
- To study the feasibility of biochar addition in the anaerobic digestion of Cheese Whey.
- To establish process parameters for a 2 stage lab-scale anaerobic digester for the selected substrates.

1.10 Organization of thesis

The present thesis covers 8 chapters properly explained in sections and sub-sections containing visible results and cited references. A brief outline of each chapter is given below.

Chapter 1 gives a concise introduction regarding the significant dairy products that contribute to pollution, along with statistics on the annual generation of whey in India and worldwide. The chapter also explains various whey waste management options available and the advantages of anaerobic digestion method over other treatment methods.

Chapter 2 includes a detailed discussion on recent papers published in the domain of anaerobic digestion of whey. Various challenges faced during digestion of complex whey proteins reported in literature are noted. The chapter is explained under various sections like co-digestion of whey, pre-treatment methods adopted and additives used in anaerobic digestion of organic wastes.

Chapter 3 consists of the details about the substrates, inocula and other materials used in the study. The procedures followed for conducting physico-chemical analysis as well as information regarding instruments are mentioned. The detailed experimental plan adopted for the study is also explained.

Chapter 4 discusses the experimental outputs obtained after conducting co-digestion batch tests on whey and septage. The effect of inocula in the digestion process is also evaluated and the optimum mix ratio for attaining maximum productivity is noted.

Chapter 5 deals with the pre-treatment studies conducted in this study. The efficiency of each pre-treatment on organic matter degradation and lactose hydrolysis was mentioned. The chapter also compares the efficiency, energy and cost requirements for each method. The optimum conditions for obtaining maximum lactose hydrolysis using β-galactosidase enzyme was derived and discussed in detail.

Chapter 6 deals with the effect of septage-derived biochar on anaerobic digestion of whey and septage. The characteristics of biochar influencing the rates of biogas production like alkaline

pH, increased specific surface area and pore volume, presence of O-containing functional groups etc. are discussed.

Chapter 7 presents the operational performance of 2 stage lab scale digester with whey and septage as feeds. The steady state conditions were derived for each reactor. The design details of the proposed industrial scale digester were revealed.

In **Chapter 8**, the entire study is summarized by highlighting the key findings and outcomes obtained from each of the aforementioned investigations. It also includes future scope of the study.

Chapter 2

Review of Literature

2.1 General

As a result of economic reforms and liberalisation, India's dairy industry has penetrated the large international cheese market in recent years. There has been much growth in India's cheese production, and by 2027, it is expected to increase to 2059 million kilograms. Whey is more difficult to degrade because of its complexity, and CW can pollute the environment due to excessive oxygen consumption, impermeabilization, eutrophication, toxicity, and other factors. Most of these are generated from small and medium-scale dairies in rural regions. Considering the high investment costs associated with whey processing and environmental concerns related to land application, anaerobic and aerobic treatment could be viable options for dairy plants. Among the biological methods employed, AD is a widely utilised treatment method for highly organic wastewater like whey.

The problems faced during AD of highly organic wastes like rapid acidification, lack of alkalinity, etc. can be alleviated to an extent by co-digestion with suitable waste. Also, pre-treatment methods help to reduce the substrate complexity and more soluble fraction of organic matter can be made available for the growth of microbes, which eventually result in generation of biogas. Use of additives are also found to be a method enhancing AD process. In the following sections, some recent studies on codigestion and pre-treatment of CW, as well as additive studies, will be reviewed.

2.2 Factors affecting anaerobic digestion of dairy effluents

The efficiency of a biological treatment process is highly dependent on various factors like operating pH, hydraulic loading rate, hydraulic retention time, solids retention time, biomass growth rate, temperature, nutrient availability, etc. These factors constitute the pre-requisites for the design and operation of a full-scale anaerobic digester. Almost all industrial wastewaters which are highly polluting in nature are preferably suitable to treat by anaerobic mode due to their high organic load, energy production capabilities, and less sludge production rate. But coming to practical application, the AD method possesses several process instabilities due to the low growth rate of microorganisms, improper digester operation conditions (pH, temperature, organic loading rate, hydraulic retention time, etc.), and the presence of inhibitors like ammonia. Problems like accumulation of VFAs and ammonia, pH drop, alkalinity depletion

occur because of a lack of knowledge and control over the chemical reactions in AD (Anukam et al., 2019). Some of the other factors having an influence on AD are feedstock moisture content flow patterns (stratified and unstirred fluid flows) and types of reactor configurations and their influence. Major factors influencing AD process are discussed below.

2.2.1 Temperature

Temperature is an important factor influencing the rate of biogas production, the content of methane in biogas as well as system heat requirements. AD can be done at 3 different temperature ranges, namely; psychrophilic (15–25 °C), mesophilic (35–40 °C), and thermophilic (50–60 °C). conditions. This is because different genera of microbes involved in the digestion process multiply at different temperatures (29-41 °C or 49-60 °C) (Bharati et al., 2017). Generally, a 35–37 °C temperature range is found to be ideal for smooth digestion operations (Arikan, 2015; Bohn et al., 2007). Care should be taken in maintaining the selected temperature range constant as much as possible. Because in thermophilic digestion, ± 2 °C variations in temperature can result in almost 30% less methane production. As mesophiles are less sensitive, fluctuations of ± 3°C are tolerable without affecting methane production (Zupancic and Ros, 2003). Studies are being conducted in different temperature ranges to reach an acceptable COD removal rate.

High-temperature requirement for optimum operation of the anaerobic digester is a major challenge. Because diluted liquid wastes are not always promising to generate methane sufficient for waste heating. This limitation leads to the researches on low-temperature applicability for operating anaerobic digester (Collins et al., 2007) and some are discussed here. An EGBR is used to treat dilute DWW to check the feasibility of operation at 10 °C which also aimed to study the microbial composition and bioreactor dynamics at this low temperature (Bialek et al., 2013). Retention of biomass at this low temperature is a challenge. To overcome this, a higher H: D (Height: Diameter) ratio is used. A COD removal of 85% is achieved in the above study, which promises that even the low-temperature AD process is applicable in temperature climate zones to save heating energy requirements and improve energy balance. A recent study on treating DWW at lower temperature using 2 reactor configurations; Upflow anaerobic sludge blanket (UASB0 reactor and Expanded granular sludge bed (EGSB) reactor showed that at low temperatures (15°C), diversity of available microbial consortium has reduced, but the reactors still continued to perform well and UASB out-performed EGSB (Mcateer et al., 2020).

2.2.2 pH

Normally microorganisms are sensitive to too acidic or too alkaline media. Three groups of bacteria in AD namely, acidogenic, acetogenic, and methanogenic bacteria prefer 3 different pH ranges for their growth. Fast-growing acidogens grow in optimum pH range 5.2–6.5 while acetogens and slow-growing methanogens prefer 6.6–7.6 and 7.5–8.5 pH ranges respectively (Demirer and Chen, 2004). pH and OLR require special control in one phase digester due to these variable requirements. An optimum pH of 6.6–7.6 is recommended for anaerobic degradation of organic waste. The pH of the substrate also affects the performance of the digester. For example, lactose-rich wastes like dairy waste promote the growth of acidogens under anaerobic conditions (Bharati et al., 2017). This leads to over-production and accumulation of volatile fatty acids(VFA), resulting in a rapid drop in pH. An example is the digestion of cheese whey. Due to its low pH and high biodegradability, CW generates a high amount of VFA through lactose degradation which may get accumulated in the system dropping the pH. This pH drop along with low bicarbonate alkalinity (50 meq/L) leads to acid inhibition of methanogens and leads to digester failure (Charalambous et al., 2020). Thus, the composition of feed material is also important.

2.2.3 Alkalinity

Neutral pH around 6.7–7.4 is preferred for the effective operation of an anaerobic reactor which is attained from the buffering ability of various contents inside the reactor (Bharati et al., 2017). When organic materials are degraded, carbon dioxide is released resulting in the formation of carbonic acid, carbonate alkalinity, and bicarbonate alkalinity. Dairy wastewaters are found to have alkalinity less than 1000 mg $CaCO_3$/L in most cases (Demirel and Yenigun, 2006, 2004), which is not enough to sustain an anaerobic process. At neutral pH, bicarbonate alkalinity will be a major source of alkalinity. At least 500–900 mg/L as CaCO3 of bicarbonate alkalinity is needed to maintain a pH greater than 6.3 (Bharati et al., 2017). If it is absent, external alkalinity adding substances like lime ($CaCO_3$)), sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), etc. should be added. Some studies reported that NaOH has got better buffering capacity than Na_2CO_3 and $NaHCO_3$ during municipal solid waste digestion (Chen et al., 2015). When the wastewater contains no whey contamination there was no necessity to add bicarbonate alkalinity to maintain the stability once the digester gets mature. But it was found that for wastes containing whey bicarbonate alkalinity is not high enough to and VFA gets accumulated disturbing the digester stability and methanogenic growth (Gutirrez, 1991).Under ideal pH conditions, the alkalinity helps in controlling possible VFA accumulation

and enhances digester stability. Alkalinity above 2500 mg/L and small pH variations favored the development of a buffer effect in digesters.

2.2.4 Organic loading rate

Organic loading rate (OLR) refers to the number of volatile solids loaded into the digester each day per unit volume of the digester (volatile solids are a portion of organic matter that can be degraded, the remaining part is fixed solids that are non-digestable). At varying influent feed concentration and flow rate, the loading rate can be altered. Actual loading depends on the composition of wastes used to feed to the digester because biodegradability or level of biochemical activity occurs based on this loading rate (Bharati et al., 2017). Feeding the digester above its maximum limit will lead to failure of the digester because OLR is an indication of the biological conversion capacity of the digester. A two-stage continuously stirred tank reactor ,CSTR-UASB reactor study was done at OLR ranging from 6.7 to 23.4 kg COD/m^3 day at HRT 9.5 h to analyse the corresponding COD removal rate (Diamantis et al., 2014). The acidifying biomass is allowed to recirculate in this study which resulted in 87% COD removal which is lower compared with other studies (Antonopoulou et al., 2008; Gavala et al., 1999). In a single-stage methanogenic anaerobic digester for the treatment of whey waste, 95% COD removal efficiency is obtained at an OLR lower than 10kg COD/m^3 day (Anderson and Yang, 1992). The author suggested that whether the digester is single or 2 staged, cheese whey waste gets completely fermented at low OLR as it is an easily degradable substance. In anaerobic membrane bioreactors (AnMBRs), biomass retention is guaranteed (with the use of a membrane) for treating lipid-rich wastes like dairy effluents at OLRs up to 8 kg COD/m^3 (R K Dereli et al., 2013). Major drawbacks like sludge floatation, biomass washout, and in-sufficient sludge granulation can be solved in AnMBRs.

2.2.5 Carbon-to-nitrogen ratio

C/N ratio is an important parameter of the digestion process as it is too low or too high value either slow down the process or even stop. If nitrogen is present in a high amount, methanogens will consume it rapidly and result in low gas production; whereas low nitrogen value leads to ammonia inhibition (Kangle K.M et al., 2014). For the successful operation of AD, a C/N ratio of 25 to 30 is suggested expecting that even the largest percentage of carbon can degrade (Marchaim and Krause, 1993; Yen and Brune, 2007). As these ratios are not always available, it is desirable to mix with other suitable substrates. Generally, feedstocks with a C/N ratio less than 40 are suggested to mix with dairy wastes to avoid reactor instability and for

proper nutrient balance. Optimum C/N ratio can be obtained by mixing high and low C/N containing feed materials, for example mixing municipal waste with manure. Carbon-to-nitrogen ratios of some substrates are illustrated in Fig. 5 that are suitable to digest along with dairy wastes. In a study conducted by mixing cheese whey waste, poultry waste, and cattle manure (3:2:1), 62% CH_4 was obtained (Desai et al., 1994). It is identified that cheese whey promotes the growth of acid formers due to high carbohydrate content. On the other hand, poultry waste increases nitrogen content which helps in reducing the inhibitory effect of acid-forming microbes on methanogens.

2.2.6 Hydraulic retention time

Acidogenic and methanogenic bacteria grow at different rates. Methanogenic bacteria are fast-growing microbes compared with acidogenic ones. Therefore, in single stage reactors, it is important to control the growth time of both these types of microbes. Because the acidogenic group prefers less HRT and low pH which is inhibitory to methanogenic microbes (Demirer and Chen, 2004). Conventional anaerobic digesters require long HRT ranging from 20 to 200 days and a large area, also biogas gets directly emitted into the atmosphere contributing to greenhouse gas (Liew et al., 2019). This problem can be overcome by using high rate digesters as they are having less area of footprint and low retention time. Some researchers have stated steady-state conditions in anaerobic bioreactors as multiples of HRT duration. Steady-state conditions are defined or established by evaluating the standard deviations in values of CH4 production and organic matter removal efficiency. In some studies, 5 times HRT (Goblos Sz et al., 2008) or 7–17 HRT duration (Cotta-Navarro et al., 2011) or 2 times HRT (Kundu et al., 2013) were used. In the study conducted by Cotta-Navarro et al., (2011) with whey residues as substrate, the author found out that by slowly reducing HRT at frequent intervals keeping constant substrate concentration will help in the development of microbial communities. Drastic reduction in HRT has resulted in washing out of biomass in some cases.

2.3 Anaerobic co-digestion of cheese whey

Numerous studies on anaerobic treatment of dairy wastewater were conducted, as pilot-, bench-, and large-scale efforts during the last decades. Low-rate, single-phased, and high-rate digesters are the two varieties of anaerobic digesters used for industrial waste treatment. Mono-digestion of dairy wastes sometimes faces problems due to their insufficient microbial composition, alkalinity, and presence of nitrogen compounds. The addition of another organic matter to digest along with dairy waste helps in remediating the problems encountered during the mono-digestion of dairy wastes. Anaerobic co-digestion (ACoD) indicates simultaneous digestion of

2 or more substrates mixed in specific proportions for biogas production. ACoD is found to be enhancing methane production by providing space for proper interaction among micro-organisms and supplying nutrients necessary for the digestion process.

Several problems associated with anaerobic digestion of whey alone can be solved to an extent with co-digestion technique. Mono-digestion of whey is a challenge in many cases due to low pH, insufficient microbial composition, low alkalinity, rapid acidification etc. During ACoD, two or more substrates are digested simultaneously in a definite proportion, increasing biogas production and thereby improving digester stability. Various literatures have been reported about different organic wastes which can be used as co-substrate with whey. A variety of organic materials represent the co-substrates, such as the organic fraction of municipal solid waste, as well as cattle manures, pig manures, poultry farm wastes, sewage sludge and food wastes (Abdallah et al., 2022; Almeida et al., 2023; Hallaji et al., 2019; Iglesias-Iglesias et al., 2021). Co-digestion with organic substrates having complimentary characteristics may help in balancing C/N ratio, ensure buffering capacity and enough supply of nutrients.

The co-substrate to be selected for ACoD with CW should have some characteristics like alkaline range of pH, presence of trace elements, less organic load, easy mixing properties, locally available and cheap. Cattle manure is being used most commonly for co-digestion with carbon-rich substrates at ratios ranging from 15-45% generating more biogas (Jaimes-Estévez et al., 2022; Rico et al., 2015). In cattle manure, high alkalinity, a fresh supply of microorganisms, and trace elements are used to ensure bacterial and archaeal growth. Besides conventional wastes, rare organic wastes like herm herds, coffee pulp wastes, crude glycerol, etc., are being used for co-digestion. The residues left from hemp cultivation which includes seeds and fibres rich in lignin were used in a study of co-digestion with CW (Papirio et al., 2020). The bio-methane production has increased by 10.7% at a mix ratio of 70:30 (CW: hemp wastes). Similarly, the coffee pulp generated after processing coffee berries was co-digested with CW which contains high amounts of carbohydrates, minerals and proteins (Gonzalez-Piedra et al., 2021). The study resulted in a methane yield of around 77.54ml/gVS at equal proportions of whey and coffee pulp. In a study by Almeida et al., (2022), ACoD of crude glycerol (a by-product of diesel production) with CW yielded 253 ml/gCOD_{remov} with 87% reduction in organic matter concentration. Table 2.1 shows summarised observations on many organic substrates used for anaerobic co-digestion with whey.

Table 2. 1 Co-digestion studies conducted on cheese whey with various substrates

Co-digestion substrate	Scale of study & Type of reactor	Maximum Methane yield	Reference
Dairy cattle manure (CM)	Lab scale study conducted in 50 ml serum bottles	400L $CH_4.Kg^{-1}VS$	(Adghim et al., 2020)
Crude glycerol (CG)	Lab scale study conducted in 1litre serum bottles	225 mL/g-V_{load}	(Chou and Su, 2019)
Hemp waste (HW)	Lab scale study conducted in 100ml serum bottles	446 mL $CH_4 \cdot g\ VS^{-1}$	(Papirio et al., 2020)
Sewage sludge (SS) & food waste (FW)	Field scale study employing a single stage mesophilic digester of volume 2400m^3	87000m^3 of CH_4 per month	(Sembera et al., 2019)
Fish ensilage(F) and Cow Manure(CM)	Lab scale study conducted in 550ml serum bottles	566 mL CH_4 g^{-1} VS	(Vivekanand et al., 2017)

2.4 Use of septage as a co-substrate in anaerobic digestion

Septage (SP), the anaerobic domestic waste sludge collected from septic tanks and other authorized faecal treatment units are being used as an energy recovery option by many researchers over the years. Majority of Indian cities are yet to be provided with sewer systems and people are mainly dependant on conventional septic tanks. According to the USAID estimates of 2010 reports, around 148 million Indian urban households have septic tanks (Wankhade, 2015). With the advent of the Swach Bharath Mission in India, which facilitates safe sanitation to citizens and reinforces urban centres with solid waste handling systems lots of faecal waste management units have been installed all over the country. Despite, the National Urban Sanitation Policy (NUSP), 2008 forbidding the need for collection, treatment, and disposal of septage from onsite installations, SP is being dumped anywhere and everywhere, polluting water and soil environment leading to severe health problems (Luthra et al., 2017). Presently, the possibility of bio-methane recovery from septage through AD was being utilized as the partially digested SP possesses many nutrients and anaerobic microbes desirable for the digestion process. Sometimes, the high ammonia concentration in SP inhibits the methanogens which can be minimized to a level by co-digesting it with some complementary substrates.

Septage, which is rich in nitrogen and nutrients sometimes show low biodegradability due to its complex nature and due to the presence of stabilized solids (Park and Li, 2012). On the other hand, CW, carbon rich compound has more volatile solids and lacks nitrogen content. Hence, the chemical and physical properties of both substrates (CW and septage) are found to be complementary to each other which increases their applicability in co-digestion. Studies have shown that resulting C/N ratio and buffer capacities of substrates after employing nitrogen rich wastes like cattle manure (Rico et al., 2015), poultry manure (Wang et al., 2012), as a co-substrate in ACoD with many organic wastes have helped in improving the end performance both by increasing biogas production and pollution reduction. Only a few studies have been reported for the co-digestion of SP with other organic wastes. Mixing SP with lipid-rich CW may help in increasing the lipid solubility and help in attaining optimal C/N values.

2.5 Pre-treatment methods

Pre-treatment methods were mostly employed to increase the biodegradability of complex substrates by disintegrating complex proteins and sugars of substrate in AD. Even if pre-treatment methods enhance AD performance, they may still not be sustainable in terms of environmental footprints. Physical, chemical and biological methods were the main divisions of pre-treatment methods widely used. Combined pre-treatment methods were also applied for more complex wastes. The effect of a particular pre-treatment method was dependent on factors like substrate complexity, type and operation of the method used etc. Sonication, thermal, chemical, microfiltration and enzymatic methods are found in some literature used for pre-treating whey effluents (Gannoun et al., 2008; Kazimierowicz et al., 2022; Mainardis et al., 2019). Details regarding those are shown in Table 2.2.

Table 2. 2 Details of some pre-treatment studies on AD of cheese whey

Method used	Operational conditions	Remarks	Reference
Ultrasound method	352.8–437.3 NmL CH_4/g VS_{added}	simplicity, ambient conditions for reaction, faster degradation kinetics, reduced toxic by-products formation and good compatibility with conventional advanced oxidation processes	(Hogan et al., 2004)
Enzymatic	346 mL CH_4.g VS^{-1}	Utilisation of enzymes enhanced hydrolysis stage Biogas production was increased by 70-76%	(Liew et al., 2019)
Enzymatic	Lactic acidification by *Lactobacillus paracasei*, Temp 32ºC	Methane yield obtained around 280 l/kg $COD_{removed}$ 50% COD removal and 60% TSS removal achieved	(Gannoun et al., 2008)
Enzymatic	@30ºC,180rpm, 48hrs	168.4 and 56.7ml CH_4 at STP were obtained with *Candida rugose* & *Geothricum candidum respectively*	(Domingues et al., 2015a)

CW is a complex substrate having highly complex components like lipids, fats, proteins, etc. which are resistant to biodegradation. Hence an efficient pre-treatment method is necessary to apply before its anaerobic digestion. Among the various methods reviewed, biological pre-treatment methods like enzymatic methods are found economical, reliable and efficient in application. Biological pre-treatments may include aerobic and anaerobic pre-treatments, although these treatments are generally not applied to municipal wastes. Pre-treatment with aerobic organisms such as composting or micro-aeration can be an effective method to achieve better hydrolysis of substrate complexes through the increased production of hydrolytic enzymes that are induced by increased microbial population. Enzymes produced by industrial fermentation processes can also be used as an accelerant for the pre-treatment of lignocellulosic wastes.

2.6 Effect of additives in anaerobic digestion

Additives influence the process of anaerobic digestion in many ways. They serve benefits like improved buffering capacity, reduce ammonia and other inhibitions, balances pH, supplies nutrients necessary for the growth of microbes, etc. Anaerobic digestion can be enhanced by using enzymes or surfactants to break down complex organic solids. Biogas production and solids reduction are improved with their enhanced hydrolysis and conversion of organic matter into readily biodegradable compounds. Biological agents and inorganic substances can be used as additives. It is possible to add enzymes to facilitate the solubility of particulate organic matter using biological additives, such as bio-augmentation and the dosage of microbial inoculum with high hydrolytic or methanogenic activity. A variety of inorganic additives, such as chemical reagents, minerals, and waste materials, can provide micronutrients and/or promote biomass immobilization (Romero-Güiza et al., 2016).

Biochar, nanomaterials and electroactive microorganisms are also used to promote anaerobic degradation and bio-methane production (Ma et al., 2020; Xiao et al., 2020). Several works have been presented to date that consider various supporting materials to enhance methanogenesis. In recent times, there has been a growing interest in carbon-based materials such as graphite, graphene, activated carbon, biochar, carbon cloth, carbon nanotube, and their composites (Capson-Tojo et al., 2018; S. Chen et al., 2014; Lee et al., 2016; Zhang et al., 2018; Zhao et al., 2015). This is due to the recognition that their incorporation can improve the effectiveness of AD .The best way to achieve this goal is to use biochar (BC), and recent utilisation ideas focus on integrating thermochemical processes with AD to achieve maximum economic and environmental efficiency (Codignole Luz et al., 2018). In recent years, a number of authors have established the potential for increased CH_4 production through the addition of BC, putting forth various potential mechanisms, including the following: (1) improvement of the AD system's buffering capacity; (2) reduction of inhibition events or materials; (3) assist medium for biomass immobilization; (4) encouragement of syntrophic catabolisms; (5) improvement of digestate quality; and (6) cleaning and improving the biogas (Chiappero et al., 2020). When compared to other carbon-based materials, BC demonstrates superior economic effectiveness (such as single-walled carbon nanotubes and graphene) since it can be produced from waste biomass. Furthermore, high surface area, significant porosity, abundant presence of functional groups, and excellent electron transferring ability were key advantages of BC over other materials in enhancing anaerobic methane production (Zhao et al., 2021).

The pyrolysis of biomass results in generation of biochar and is normally applied as a soil conditioner. Methane production can be increased in low-solids digesters that are ammonia-stressed by biochar, reducing lag times before methane production begins and increasing peak daily methane yields (Lü et al., 2016). The properties of biochar like buffering capacity, high adsorption rate due to large specific surface area, presence of trace elements, biofilm formation etc. impose positive influence on rate of biogas production during anaerobic digestion. Particular studies on the effect of biochar addition on biogas production from anaerobic digestion of dairy wastes have not been conducted so far.

2.7 Summary of literature review

AD of whey has attracted much attention in the past. Compared to other waste treatment methods, this process offers significant benefits including cost-effectiveness, high energy efficiency, and process simplicity. Cheese as an exclusive product can significantly increase COD concentration to 70 g/l when whey is present in effluent. Nevertheless, many cheese producers, especially small and medium-sized ones, do not have the necessary resources or means to invest in technologies that allow them to reuse the cheese whey produced during production, so they dispose of their effluents in the waterways without treating them.

For the effective functioning of digestion of whey anaerobically, several operating conditions like pH, HRT (hydraulic retention time), OLR (organic loading rate), temperature, substrate to inoculum ratio etc. have to be proposed. Along with this suitable pre-treatment method and co-substrate for co-digestion have to be decided. Effect of enzymatic pre-treatment methods in lipid degradation is not studied so far in terms of oil and fat reduction, lipase activity and time of pre-treatment duration. From the literature reviewed it was found that SP has been used as an excellent substrate in anaerobic digestion over years. Although its adaptability with CW in ACoD has not been explored yet. Sewage sludge, food waste, garden waste, OFMSW are some of the organic wastes used in digestion with SP so far. Sparse studies were found in employing dairy wastes for co-digestion with septage and no particular studies on whey waste has been reported yet. Also, the effect of biochar addition in AD of CW has not been studied. Optimum dosage of biochar has to be evaluated when feedstock and digestion conditions are changed.

Chapter 3

Materials and Methods

The objective of this chapter is to provide a comprehensive overview of the tools, materials, and methodologies employed in addressing the research questions and objectives established in the preceding chapters. The materials employed in this study includes the substrates, inocula, enzymes and additives. The source of collection of these materials and detailed characterization methods used are also explained. The experimental methods used as well as procedures followed are discussed thereafter. The statistical tools and models used in this work are added at the end of the chapter.

3.1 Materials

3.1.1 Substrates and inocula

The cheese whey for the study was obtained from the NSR dairy plant located in Atmakur, Warangal district of Telangana [18º03'55"N 79º42'12"E].. The samples of whey (sweet whey) were collected from the coagulation tank in 5 litre plastic cans and were stored at 4°C until analysis. The septage samples were brought from the Faecal sludge treatment plant (Sanitation Resource park) located in Ammavaripet, Warangal [17º56'15"N 79º33'31"E]. The septic tank sludge collected was around 2-3 years old and was obtained as a slurry having 92.56% water content. The Sanitation park established in the year 2009 was focused on faecal waste management in Warangal city where around 5000 litres of septage was being converted into biochar through a very clean and scientific process.

Three inocula are used in this study viz, sewage sludge, anaerobic digestate, and cattle manure. The sewage sludge was collected from the sewage treatment plant located inside the campus of the National Institute of Technology (NIT), Warangal which treats the wastewater generated from the hostels and cafeteria associated. The sludge was specifically collected from the digestion tank connected to the clarifier. The acclimatized anaerobic digestate was obtained from the biogas plant located in the NIT campus which uses the food waste (mainly cooked rice, vegetables, fruits, and meat) as the primary substrate generated from hostel messes. The third inocula, cattle manure was collected from a local dairy farm located 4km away from the campus. All the 3 inocula were prepared according to the method suggested by Rajput et al., (2018) Both the substrates and inocula are undergone preliminary test analysis for getting basic characteristics as soon as they were brought to the lab and were kept

at a temperature of 4°C to minimize the loss in COD during feeding and storage. The sample images of substrates and inocula are shown in Figure 3.1.

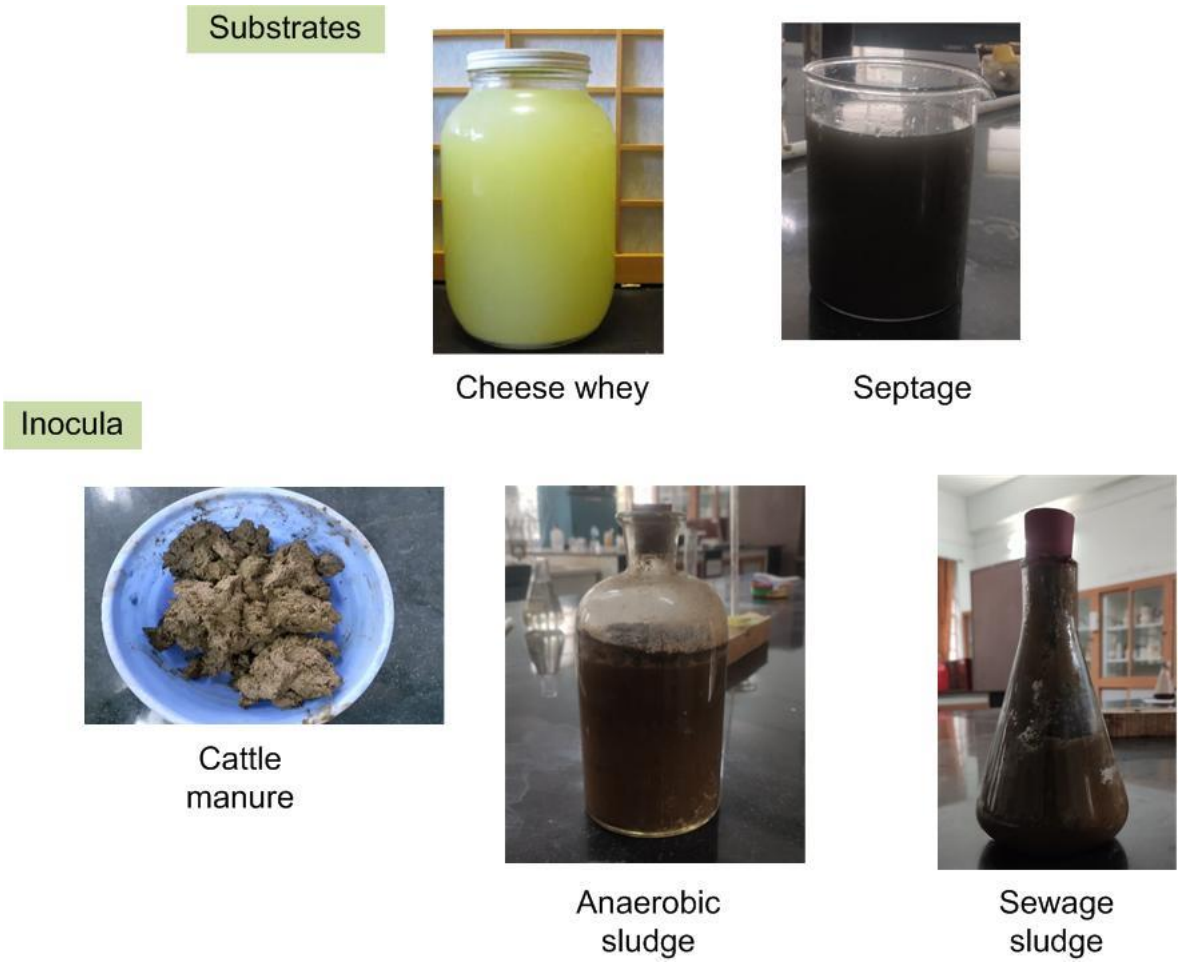

Figure 3. 1 Substrates and inocula used in the study

3.1.2 Biochar

The biochar was collected from the Sanitation Park located at Ammavaripet, Warangal where septage is processed through pyrolysis to make biochar. The biochar manufacturing steps are depicted in Figure 3.2. The septage was collected and transported to the Sanitation park by trucks from various urban parts of Warangal. After screening and grit removal, the septage was passed into mechanical sludge dryer unit and sludge is made to dry. The sludge from dryer was undergone pyrolysis at a temperature of 850ºC and biochar was produced.

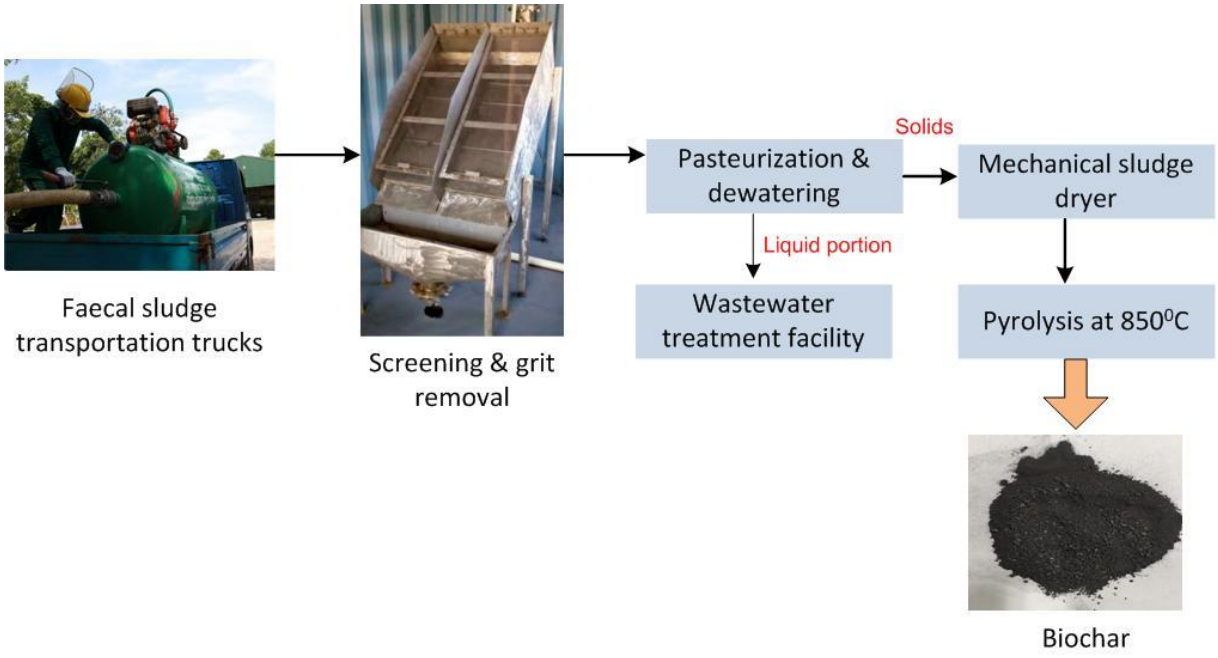

Figure 3. 2 Various steps of processing of septage producing biochar

3.1.3 Enzyme

The commercial food grade enzyme, β-Galactosidase derived from the fungus *Aspergillus oryzae*, supplied by Sigma Aldrich (bearing lot BCBV3825 with a nominal activity), was used for hydrolysing whey lactose into β-D galactose and α-D glucose. The enzyme activity with lactose is defined as the amount of enzyme that hydrolyses one µmole of lactose per minute under the given assay conditions.

3.2 BMP experimental set-up for co-digestion studies

The batch mode of experiments using Biochemical Methane Potential (BMP) assays is adopted for conducting the study. The BMP assays were planned by following the principles explained by Owen et al., (1979) which were later revised by Hansen et al., (2004). The pyrex glass bottles of volume 120ml with a working volume of 80ml were used as batch reactors. The mixing was done manually to each reactor for 2-4 minutes twice a day. Initial pH was measured and glass bottles were sealed immediately with rubber septa and aluminum crimp caps. Triplicate bottles were kept for each mix and were incubated at 37±2°C for 35 days. No external nutrients, external alkalinity, or inocula were added to the bottles. The volume of gas was taken using the water displacement method and a glass syringe was used to collect gas samples in alternative days for determining gas composition.

3.3 Methodology adopted for pre-treatment study

Ozonation, ultra-sonication and enzymatic methods were the pre-treatment methods chosen for the study. The detailed methodology employed for each method are explained below.

3.3.1 Ozonation

The device used was an AQUAZONE ozonator with a nominal O_3 generation power of 6.12g/h for varying O_2 flow rates of oxygen ranging from 1-5LPM. The ozonator used O_2 as feed gas generated from a separate O_2 concentrator. An illustration of the ozonation pre-treatment process is depicted in Figure 3.3. 1000mL of CW was treated with O_2 at 4 time intervals at a room temperature of 37ºC and atmospheric pressure. After ozonation, deozonation was performed to remove excess O_3 from whey by stirring it for 60 minutes at 40ºC.

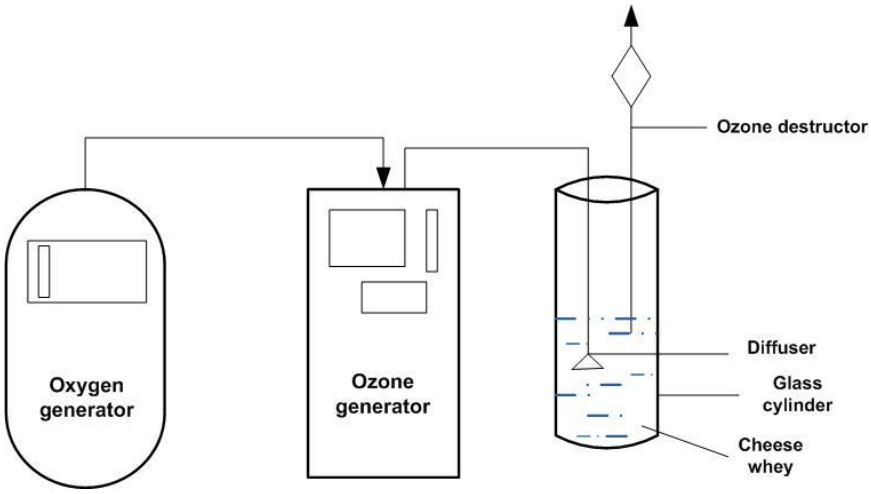

Figure 3. 3 Scheme of ozonation pre-treatment of cheese whey

3.3.2 Ultra-Sonication

The US equipment used for pre-treatment was PS1200LCD probe sonicator, equipped with a titanium alloy probe 10mm and 20mm in size. The maximum operating frequency is 20kHz and the maximum power is 1200W. An illustration of the US pre-treatment process is represented in Figure 3.4. During each pulse, a five minutes' pause was given to avoid overheating. The specific energy of US was calculated as per equation 3.1. The specific energy can be defined as the energy used by the sonicator during US. In a glass cylinder, 500mL of the sample was placed inside the cabinet of the sonicator and the probe tip was immersed in the sample to a depth of 2cm.

$$S_E = \frac{P \times t}{V \times TSS} \quad \text{..... Eqn (3.1)}$$

Where E_S is the specific energy input (KJ/Kg TS), P is the ultrasonic power (kW), t is the duration of US (seconds), V is the sample volume (litres) and TSS is the initial total solids content (g/L).

The degree of solubilisation (S_d) after pre-treatment was evaluated using the equation developed by Appels et al., (2010); in which change in soluble chemical oxygen demand was determined. S_d is given by following equation.

$$S_d(\%) = \frac{sCOD_{pre} - sCOD_{un}}{TCOD_{un}} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots \text{Eqn (3.2)}$$

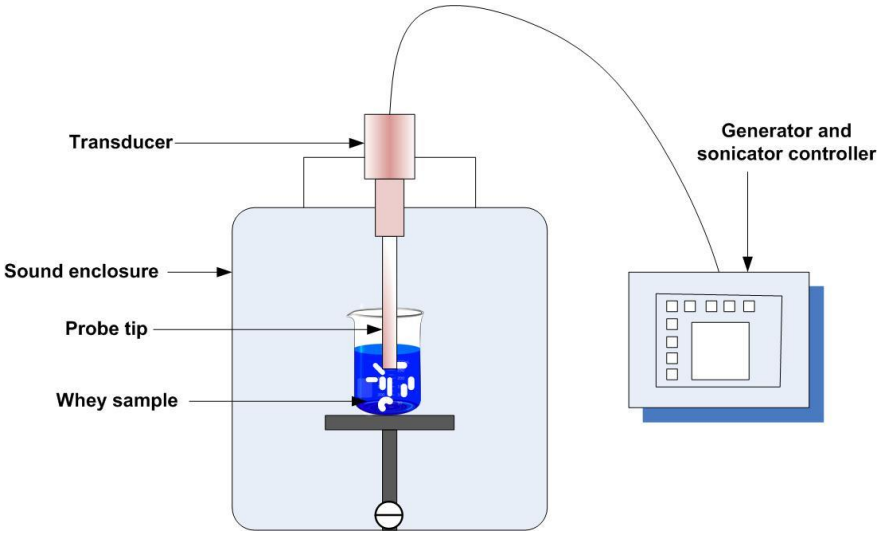

Figure 3. 4 Scheme of Ultra-sonication pre-treatment

3.3.3 Enzymatic pre-treatment

The commercial food grade enzyme, β-galactosidase derived from the fungus *Aspergillus oryzae* was used for performing enzymatic hydrolysis of CW. The enzymatic pre-treatment of whey was aimed to hydrolyse whey lactose into β-D galactose and α-D glucose. Based on the procedure developed by Ghosh et al., (2017), the hydrolysis experiments were performed. After pasteurisation of whey at 65ºC, enzyme hydrolysis was done at different combinations of operating variables like temperature, enzyme dose, pH and time period of hydrolysis. The suitable combinations are chosen by using design expert software. The samples after hydrolysis are kept at 4ºC for lactose and reducing sugar analysis. The efficiency of lactose hydrolysis was evaluated by means of degree of lactose hydrolysis, calculated by equation 3.3.

$$E_h = \frac{C_G \times m_L}{C_L \times m_G} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{Eqn (3.3)}$$

Where E_h is the degree of enzymatic hydrolysis (%), C_G is the glucose concentration (g/L), m_L and m_G are the molar masses of lactose and glucose respectively (g/mole) and C_L is the initial lactose concentration (g/L).

3.3.4 Anaerobic digestion experiments

The biochemical methane potential (BMP) test set-ups were used to perform AD experiments. 120mL glass pyrex bottles with working volume of 70mL was used. The substrate to inoculum ratio was fixed as 1 based on the results obtained from earlier study. The mix ratio of substrates was chosen as 60:40 (CW: SP) in terms of volatile solids. The pre-incubated inoculum and substrates are added in equal proportions. Duplicate bottles were kept for each measurement at 37ºC for 35 days. No nutrients and external alkalinity agents were added. Control bottles are kept for substrates and inoculum in order to evaluate the specific methane yield (SMY).

3.3.5 Energy and cost analysis

The energy analysis was done to assess the amount of energy recovered through AD of pre-treated whey after the application of pre-treatment methods. The output energy to input energy is defined as energy ratio (E_r), was evaluated to assess the biogas energy performance. The techno-economic feasibility of application of pre-treatment methods was calculated using equation (3.4).

$$E_r = \frac{E_{out}}{E_{inp}} \quad \text{..... Eqn (3.4)}$$

where E_{out} is the total energy produced by biogas in kJ and calculated using equation (3.5)

$$E_{out} = \sum_{i=1}^{35} M_d M_{per} M_{hv} \text{..... Eqn (3.5)}$$

where M_d is the daily biogas yield in litres, M_{per} is the percentage of methane present in biogas, and M_{hv} is the lower heating value of methane, 36.4kJ/l (Armstrong, 1966).

Input energy (E_{inp}) is the total energy consumed during the application of US, ozonation and enzymatic methods, and were calculated as follows in each case.

Ultra-sonication

$$E_{inp} = E_{US} + E_{hum} + E_{heat} \text{..... Eqn (3.6)}$$

where E_{US} was the specific energy used by US (kJ/kgTS), calculated using eqn (3.1), E_{hum} was the manual labor energy spent chosen as 0.27MJ/h as per Kitani et al., (2006). E_{heat} is calculated using eqn (3.7).

$$E_{heat} = Q \times \left(T°C_{dig} - T°C_{in}\right)\delta_{CW} C_{cw} \quad \text{.....Eqn (3.7)}$$

Where Q is initial mass of solids (kg/m^3), T_{dig} is digester temperature (37ºC) and T_{in} is sample temperature (28ºC), δ_{CW} is density of whey sample (1030kg/m^3) and C_{cw} is specific heat of whey (2480J/KgºC).

Ozonation

$$E_{inp} = O_3 dose \times m_s \times Ele_{O_3} + E_{heat} \quad \text{.....Eqn (3.8)}$$

Where m_s is the solids contents of sample in kg, Ele_{O3} is the electrical energy consumption by ozonator in kWh, calculated as product of power supplied (230W) and time of operation (hours).

The net energy gain (NEG) is defined as the difference between gross energy output and input.

Enzymatic

Enzymatic pre-treatment method requires low or no energy for performing the hydrolysis step as enzyme can be derived from microbes or commercially procured. Here, E_{hum} and E_{heat} were considered as the only the input energy. In this case, as the enzyme was procured commercially the total cost for conducting enzymatic hydrolysis can be evaluated using following equation.

Cost requirement for enzymatic pre-treatment = Dosage of enzyme * Unit cost of enzyme.

The unit cost of food grade enzyme Lactase from *A.orizae* with lot number BCBV3825 supplied from sigma Aldrich was 110.72$/mg of solid.

3.4 Design of experiments for optimisation studies

Whey hydrolysis with lactase enzyme was tested under different conditions to get optimum values of essential parameters like enzyme load, operating pH, temperature and time of reaction. Response surface methodology (RSM) was used to evaluate the effect of 4 parameters chosen on enzymatic hydrolysis of lactose by β–galactosidase. The relationship between the 'X' set of independent parameters, pH (X_1), enzyme load (X_2), the time course of reaction (X_3), and temperature (X_4) and dependent variable, degree of lactose hydrolysis (Y_1), was derived. The coded and un-coded levels of independent variables are found first. The experiment model is designed using central composite design method (CCD) with five levels,

and the quadratic model is used (Rodrigues et al., 2014). Multiple parameter optimisation can be carried out with Response Surface Methodology (RSM) by analysing the combined effect of all parameters on a particular response (Kishore and Kayastha, 2012). The Central Composite Design (CCD) is the standard method chosen in RSM by many researchers (Abubakar IK and and Ibrahim A, 2021; Dima et al., 2020). Enzyme concentrations were chosen to vary from 0.18% to 0.52%, temperature from 35ºC to 55º C, time from 7.5 minutes to 53.5 minutes and pH from 3.81 to 7.18. The number of runs is calculated using the equation 2^k+2k+n_c, where k is the number of variables and n_c is the number of repetitions around the central point. The model significance was tested by ANOVA. The statistical parameters like lack of fit, multiple correlation coefficients, coefficient of variance, etc., were compared between different polynomial models to obtain the best fit model. Fisher's F test was also conducted in the same program to assess statistical significance. The combined and individual effects of parameters on lactose hydrolysis were understood using the response plots generated. All the runs were done in duplicate.

3.5 Experimental set-up for studies on biochar addition

The AD experiments on studying the effect of BC were conducted in batch mode. The BMP set-up utilised 120ml glass serum bottles with a working volume of 80ml. The substrates; CW and SP was mixed in 60:40 ratio. The substrate volumes are adjusted to fix the total solids content in range of 5%, 7.5%, 10%, 12.5% and 15%. The biochar dosages were 0, 0.5g, 1g, 2g and 4g corresponding to the biochar loadings 0,6.25,12.5,15, 25 and 50g/l respectively. The bottles are flushed with N_2 gas to maintain anaerobic conditions and kept in oven at a temperature of 37±1ºC for a period of 45 days till the biogas production ceases. Each experiment was done thrice to increase the accuracy of results. The measurement of biogas was initially conducted on a daily basis for the first 15 days, after which it was performed every other day. The lab set-up was shown in Figure 3.6.

Figure 3. 5 Biochemical methane potential experimental set-up

3.6 Estimation of theoretical methane yield and degradation rate

The theoretical methane yield of whey and septage is found out using the Buswell formula (A. M. Buswell, 1936). The Buswell formula (Eqn 3.9) is based on the assumption that the only products of anaerobic digestion are CH_4 and CO_2. By knowing the chemical composition of the substrate, the quantity of methane generated can be calculated from a stoichiometric formula (Eqn 3.10).

$$C_aH_bO_c + \left(a - \frac{b}{4} - \frac{c}{2}\right)H_2O \rightarrow \left(\frac{a}{2} + \frac{b}{8} - \frac{c}{4}\right)CH_4 + \left(\frac{a}{2} - \frac{b}{8} + \frac{c}{4}\right)CO_2 \quad \text{..... Eqn (3.9)}$$

$$\text{Theoretical methane yield, } X_{CH_4} = \frac{22.4\,X\left(\frac{a}{2}+\frac{b}{8}-\frac{c}{4}-\frac{3d}{8}\right)}{12a+b+16c+14d} \quad \text{..... Eqn (3.10)}$$

The degree of degradation (f_d)is defined as the proportion of organic matter present in the substrate that gets converted into biogas through anaerobic digestion. 'f_d' is calculated using the equation (3.11);

$$f_d\,(\%) = \frac{Experimental\ methane\ yield}{Theoretical\ methane\ yield} * 100 \quad \text{..... Eqn (3.11)}$$

3.7 Estimation of Bioenergy potential

The bioenergy generation potential (BEP) was calculated based on the amount of VS present in the substrates (Rosas-Mendoza et al., 2021). The volume of generated methane was calculated by equation 3.12.

$$V_{CH_4} = V_R \times VS_i \times VS(\%) \times Y_{CH_4} \times 10^{-5} \quad \text{..... Eqn (3.12)}$$

Where V_R is the volume of the reactor in which CW and SP are added as feed (in liters), VS_i is the initial volatile solids concentration (in g/l), VS (%) is volatile solids removal efficiency (in %), and Y_{CH4} is the experiment methane yield at STP/$gVS_{removed.}$

1×10^{-5} serves as a conversion factor for expressing the methane produced through AD in cubic meters (m^3) at standard temperature and pressure (STP).

BEP is calculated by equation 3.13.

$$BEP = V_{CH_4} \times HP \quad \text{..... Eqn (3.13)}$$

Where HP is the heating power at STP; 9.94 kWh/m^3.

Assuming electricity energy and heat energy conversion efficiencies as 30% and 70% respectively, they are calculated as follows.

$$Electricity = BEP \times 0.3 \times 10^{-2} \quad \text{..... Eqn (3.14)}$$

$$Heat = BEP \times 0.7 \times 10^{-2} \quad \text{..... Eqn (3.15)}$$

3.8 Kinetic study

The cumulative biogas production and fermentation time are closely related to each other. The modified Gompertz model can be used to simulate the experimental biogas production values obtained for different whey proportions and different inoculum types (Jiunn-Jyi et al., 1997). The model equation is written as: (Eqn 3.16)

$$Y(t) = P_{max} * exp\left\{-\exp\left[\frac{R*e}{P_{max}}(\lambda - t) + 1\right]\right\} \quad \text{..... Eqn (3.16)}$$

Where Y(t) (mL/gVS) is the cumulative methane production at time t, P_{max} (mL/gVS) is the maximum methane potential, R (mL/gVS d) is the methane production potential, λ (days) is the lag phase time and e is Euler's constant of value 2.7182. The above equation is fitted with cumulative methane production curves using OriginPro 2018 software.

3.9 Biogas measurement

The volume of biogas was measured manually using a 120mL glass syringe equipped with a stopcock. Methane content of biogas was analysed using a gas chromatograph of YL Instruments Model 6500 comprising a steel column of length 15 feet, Porapak Q (80–100 mesh) and a thermal conductivity detector. Hydrogen was the carrier gas used and the temperatures maintained at injection port, column oven and detector were 40ºC, 50ºC and 100ºC respectively. A standard mixture of biogas comprising 51.65% of CO_2 and 48.35% of CH_4 was used for obtaining the biogas composition. The volume of biogas was measured daily, while the biogas composition was analysed in every two days.

3.10Analytical methods

The physical and chemical characteristics of substrates (S_{CW} and S_{SP}) and inocula (I_{CM}) are determined using methods described in 'Standard methods for examination of water and wastewater' (APHA, 2005). The soluble COD (sCOD) was measured using open reflux method after filtering the sample through 0.45µm to remove all suspended matter. The total kjeldahl nitrogen (TKN) was evaluated using distillation method and total nitrogen (TN) was calculated by multiplying TKN with a factor of 6.25. Total carbon content was determined using a TOC analyser. The elemental composition of both substrates was determined using Carlo Erba EA 1108 CHNS-O analyser. Free ammonia concentration was measured for digestate using the following equation (Olsen et al., 1985).

$$\frac{[NH_3]}{[NH_3]_T} = \left(1 + \frac{10^{-pH}}{10^{-\left(0.09018+\frac{2729.12}{T(K)}\right)}}\right) \quad \text{..... Eqn (3.17)}$$

where, $[NH_3]$- free ammonia concentration, $[NH_3]_T$ – Total ammonia concentration, and

T(K)- the temperature in kelvin units.

The free volatile fatty acids were analysed using Nordmann method (Jobling Purser et al., 2014), in which the digestate samples collected after AD were centrifuged at 6000rpm for 15 minutes and then filtered using 0.22m filters.

The Fourier Transform Infrared spectrometer (BRUKER, Alpha) analysis was done to analyse the possible bond cleavage in raw and hydrolysed whey after biological pre-treatment.

The lactose concentration of CW before and after hydrolysis was estimated using phenol-sulphuric method (Dubois et al., 1951). Protein was evaluated by using Lowry method measuring the absorbance at 660nm (Waterborg and Matthews, 1994). Bovine serum albumin (BSA) was used as protein standard and calibration curve was prepared at concentrations varying from 0 to 16µg/100µl. The reducing sugar method using DNSA reagent was used for assessing glucose and galactose employing UV-VIS double beam spectrophotometer (Lasany International, LI 2800) at wavelengths 540nm and 490nm respectively.

3.11 Methodology adopted

The step-by-step process of the entire work is summarised in Figure 3.7 below.

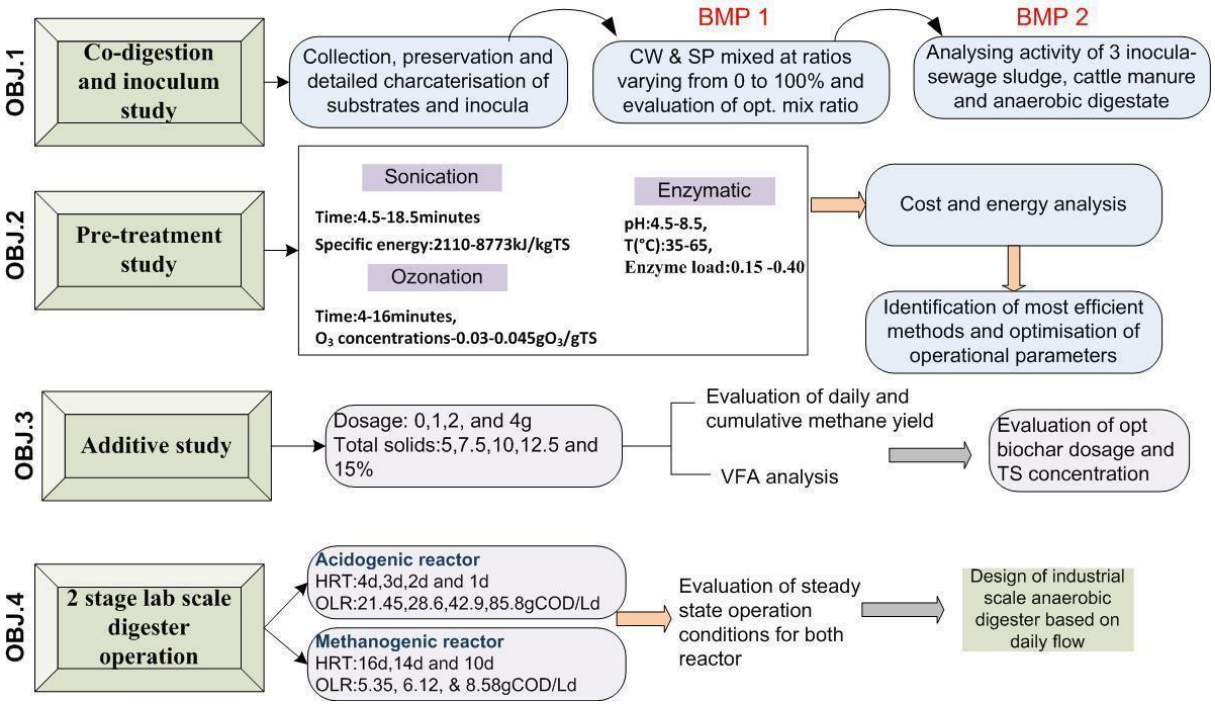

Figure 3. 6 Experimental plan for the study

Chapter 4

Anaerobic co-digestion of cheese whey and septage

4.1 General

The mono digestion of CW may result in the release of inhibitory substances like ammonia, long chain fatty acids, etc. during the hydrolysis, which may inhibit the process. This has warranted choosing a suitable co-digesting substrate that can balance the composition, improve the stability and maximize the biogas yield. Many studies have reported the wide usage of livestock wastes (manures) as co-substrates for anaerobic co-digestion of CW. Septage (SP), the anaerobic domestic waste sludge collected from septic tanks and other authorized faecal treatment units are being used as an energy recovery option by many researchers over the years. Presently, the possibility of bio-methane recovery from SP through AD was being utilized as the partially digested SP possesses many nutrients and anaerobic microbes desirable for the digestion process. Sometimes, the high ammonia concentration in SP inhibits the methanogens which can be minimized to a level by co-digesting it with some complementary substrates. SP, which is rich in nitrogen and nutrients sometimes show low biodegradability due to its complex nature and due to the presence of stabilised solids. On the other hand, CW, carbon rich compound has more volatile solids and lacks nitrogen content. Hence, the chemical and physical properties of both substrates (CW and septage) are found to be complementary to each other which increases their applicability in co-digestion. Mixing SP with lipid-rich CW may help in increasing the lipid solubility and help in attaining optimal C/N values.

A good source of inoculum can provide an extra methane-producing microbial consortium which can enhance the anaerobic biodegradability and increase methane production. In addition, inoculum helps in reducing lag time, provides essential micronutrients, and makes digestion more stable. Moreover, there exists a relation between substrates and inoculum based on the amount of inoculum used, rate of methane generation, and ability of the system to overcome possible inhibitions created due to the presence of organic and inorganic toxicants like long chain volatile fatty acids, free ammonia, sulphides, etc.(J. L. Chen et al., 2014; Soto et al., 1993). Hence it is important to study the inoculum type and its composition before it is used for the digestion of a specific substrate or mix of different substrates. Therefore, the specific contents of this work includes; a) To study the effect of the composition of CW on the biodegradability of the mixture and find out specific methane yield of both substrates b) To optimize the co-digestion of CW and SP waste by determining the best co-digestion mix ratio

which ensures high biogas production and reactor stability, c) To study the influence of inoculum on biogas production and digestion, and d) To validate the results obtained from BMP tests using Modified Gompertz equation and to evaluate the kinetic parameters(P_{max}, R, and λ)

4.2 Substrates characterization

The physicochemical characteristics of substrates and inocula used in this study are shown in Table 4.1. The composition of both substrates was different mainly because of the amount of organic matter present and pH. Whey has an acidic pH range, while septage and all inocula are found to be alkaline. The TS content of S_{cw} was almost twice the septage, while the VS content of whey is found slightly higher than that of septage. Similar values of TS of whey were reported by Bertin et al., (2013). Unlike in whey, solids present in septage are more stabilized. The TS fraction of I_{CM} was comparatively higher than that of I_{SS} and I_{AS}. TS and VS values of I_{CM} reported in this study are slightly higher than values reported by Bertin et al., (2013). It is observed that the organic content of CW is higher than that reported by Gelegenis and Georgakakis, (2007). The C/N ratio of whey is higher than that of septage, which depicts the low nitrogen content of whey. Co-digestion of nitrogen-rich septage with low and moderate nitrogen containing whey may help in generating more optimal C/N ratios.

4.3 Specific methane production rates of cheese whey and septage

The first set of BMP assays was intended to determine the specific methane yield (SMY) of CW and SP. Mono and co-digestion experiments were carried out to evaluate the compatibility of using septage with cheese whey for increasing its biodegradability and rate of biogas production. Mono-digestion of substrates was conducted at a concentration of 1gVS/l which resulted in cumulative methane yields of 66.1 ± 0.8 mL_{CH4}/gVS and 148 ± 3 mL_{CH4}/gVS for S_{CW} and S_{SP} respectively. The depletion in the rate of methane production was noted from the 15^{th} day onwards for S_{CW} which showed its organic complexity. The rapid rise in BMP values during the initial 10 days indicates the fast hydrolysis of whey. A C/N ratio of 22.3 and ammonia inhibition (1800mg/l) was also observed during mono-digestion of whey. The obtained methane yield for CW was slightly lower than that reported by other authors (Bertin et al., 2013; Malaspina et al., 1996) . Methane yield of 274 mL_{CH4}/gVS was reported in a study by Fernández-Rodríguez et al., (2021) when whey was digested alone.The possible reasons for the low biogas yield observed in the present study might be the accumulation of volatile fatty acids followed by inhibition in the growth of methanogens. In the present study, the anaerobic digestate was analyzed for volatile fatty acids and pH was found to be dropped below 4.1

Table 4. 1 Physico-chemical characteristics of substrates and inoculums used in experiments (Mean ± Standard deviation)

Parameter	Substrates		Inocula		
	S^a_{CW}	S^b_{SP}	I^c_{CM}	I^d_{AS}	I^d_{SS}
pH	4.87	6.9	6.81	7.1	7.21
COD (g/l)	67.6 ± 3.1	32.40 ± 2.1	43.01 ± 2.76	10.52 ± 0.41	13.62 ± 0.32
TS (g/l)	51.50 ± 2.3	26.90 ± 1.4	36.60 ± 2.21	19.30 ± 2.2	18.60 ± 1.85
VS (% of TS)	82.13	64.79	57.45	62.69	51.62
C/N	22.3	9.33	41.43	28.1	24.2
TKN (g/l)	0.49 ± .08	0.25 ± 0.05	2.03 ± 0.36	0.68 ± 0.07	0.39 ± 0.85
TAN (g/l)	0.21 ± 0.01	0.87 ± 0.21	1.31 ± 0.18	0.53 ± 0.02	0.28 ± 0.05
TN (g/l)	0.48 ± 0.02	2.1 ± 0.2	3.83 ± 0.21	0.78 ± 0.02	0.46 ± 0.04
TP (g/l)	0.34 ± 0.07	0.63 ± 0.02	0.72 ± 0.06	0.63 ± 0.05	0.17 ± 0.03

[a] S_{CW} : Cheese whey

[b] S_{SP} : Septage

[c] I_{CM} : Cattle manure

[d] I_{AS} : Anaerobic sludge

[e] I_{SS} : Sewage sludge

Methane yield of SP was found almost three times that of whey. Since SP has undergone half digestion in septic tanks, it consists of an active anaerobic group of microbes which actually helps in further digestion and thereby more biogas production. All bottles fed with SP performed well without having a longer startup time and other inhibitions. Anaerobic digestion of SP alone has reported higher biogas production by other authors too (Lu and Zhang, 2016; Rajagopal et al., 2013). The theoretical methane yield of both substrates is calculated separately using the elemental composition method (Table 4.2). The extend of applicability of a substrate in anaerobic digestion can be evaluated by analyzing its elemental composition. Organic matter in S_{CW} and S_{SP} is represented by the chemical formula $C_{20.61}H_{16.24}O_{10.83}N_{2.35}$ and $C_{14.67}H_{19.04}O_{9.55}N_{4.28}$ respectively. The obtained values are compared to evaluate the degree of biodegradability of both substrates. Theoretical and experimental methane yield values for S_{SP} were not varying much, indicating around 48% of organic carbon conversion to methane. The results have shown higher digestibility of S_{SP} demonstrating its potential to use as a major substrate in anaerobic digestion.

Table 4. 2 Elemental composition, theoretical and experimental CH4 yield of CW and SP

Substrate	Elemental composition (%,dry w/w)				$X^{a}_{(CH4)theor}$ (mL_{CH4}/gVS)	$X^{b}_{(CH4)exp}$ (mL_{CH4}/gVS)	f_d[c] (%)
	C	H	O	N			
S_{CW}	45.8	5.8	32.1	6.1	417	66	15.82
S_{SP}	32.6	6.8	28.3	11.1	314	148	47.13

[a] Theoretical methane yield is obtained from the Buswell formula

[b] Experimental methane yield is the total methane yield after 45 days of mono-digestion of cheese whey and septage

[c] Degree of degradation (f_d) is calculated based on Eqn (3) using the theoretical methane yield calculated using Eqn.2 and experimental methane yield.

Digestion of CW, when compared to that of SP, showed weaker degradation. In contrast, a study by Fernández-Rodríguez et al., (2021) showed 99.6% biodegradability during mono-digestion of goat CW. The experimental methane yield was only 66.1 ± 0.8 mL_{CH4}/gvs^{-1} while theoretical methane yield was obtained as 417 mL_{CH4}/gvs^{-1} ie; 15.82% of conversion of organic matter into biogas (Figure 4.1). Low methane yield observed in whey digestion can be attributed to the unbalance in pH and C/N ratio. Acid range of pH values is reported for whey which has not undergone any pretreatment techniques and no inoculum was supplied to provide adequate alkalinity. Estimation of reduction in VS has helped in obtaining the rate of organic matter degradation.

4.4 Co-digestion of cheese whey and septage without inoculum

The two substrates are mixed at proportions ranging from 0 to 100% (v/v) at progressive variations of 10%. Figure 4.2 shows the cumulative biogas production from each co-digestion set of S_{CW} and S_{SP} mixed at proportions ranging from 0 to 100% without any inoculum. The BMP results are normalized with the value of VS added to the bottles. In co-digestion mixtures, when the S_{SP} fractions are increased to 60% higher biogas production rates are noticed. The highest methane yield was obtained as 469.54 ±10.8 L_{CH4}/gVS in 7th run with 60% septage fraction. This is attributable to the utilization of readily available and easily hydrolysable substrate fractions which are composed of different levels of degradable components. Previous studies have also reported the adaptability of septage as a co-substrate with other organic wastes like food waste (Abunde Neba et al., 2020; Kujawa-Roeleveld et al., 2006; Rajagopal et al., 2013), microalgae present on surface waters (Lu and Zhang, 2016), raw dairy waste (Luostarinen and Rintala, 2005), municipal solid waste (Valencia et al., 2009), etc. in the process of anaerobic digestion. The presence of acclimatized methanogenic or hydrolytic

bacteria in septic sludge helped in adapting to the anaerobic conditions that prevailed in the bottles with S_{CW}+S_{SP}. In contrast, a recent study by Merlin et al., (2021) reported that co-digestion of whey with grease and septage, with septage fractions>30% showed a strong negative effect on biogas production. The trajectory line showing the variations in pH values throughout experiment duration was also depicted in figure 2. The desirable pH conditions are also achieved by the addition of suitable quantity of septage to the whey mixture. Initial acidic pH (4.69) was changed to a neutral range (6.98) and then to alkaline (8.21) when septage fractions are increased above 70%. High pH values noted towards the end of experiment duration could be attributed to the high ammonia concentration in septage and dissolution of those salts into the substrate mixture. A similar type of increase in pH values was reported in a study conducted by Lin et al., (1999) using SP and landfill leachate as substrates.

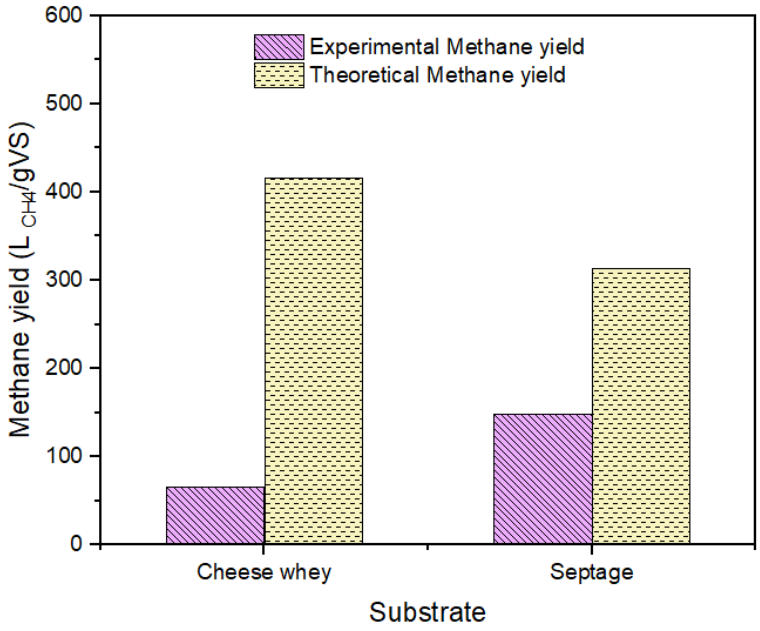

Figure 4. 1 Theoretical and experimental methane yields of CW and SP

Co-digestion samples with whey fractions above 50% showed a significant reduction in biogas production. The lowest biogas yield was reported at 100% whey fraction(98.01±0.89ml/gVS). These results are comparable to the earlier inferences made during mono digestion of CW. A major portion of this was reported within the first 15 days of operation and thereafter a lag phase of little or no methane production was observed. A similar kind of low biogas yield was reported by Jasko and Dubrovskis, (2014) which was in the range of 136-216mL/gVS. Even lower biogas yield values more similar to our observations were

reported by Gameiro et al., (2020), a gas volume of 66cm^3. The pH values for the first 4 runs of mixtures ranged from 4.69 to 5.98, which indicates an acidic environment. Undesirable pH values have a negative impact on the methanogenic activity in AD. The alkalinity of those mixtures having CW fractions of more than 40% was found below the desirable limit of alkalinity, ie. 1000mg $CaCO_3$/l. The drop in pH values has led to a decrease in alkalinity. To ensure the effectiveness of the digestion system, alkalinity is suggested to be between 1000-3000 mg/l $CaCO_3$. Below this level, the system has to be supplied alkalinity externally. Figure 2 shows the evolution of alkalinity increase when SP contents are increased which in turn increased the buffering capacity also.

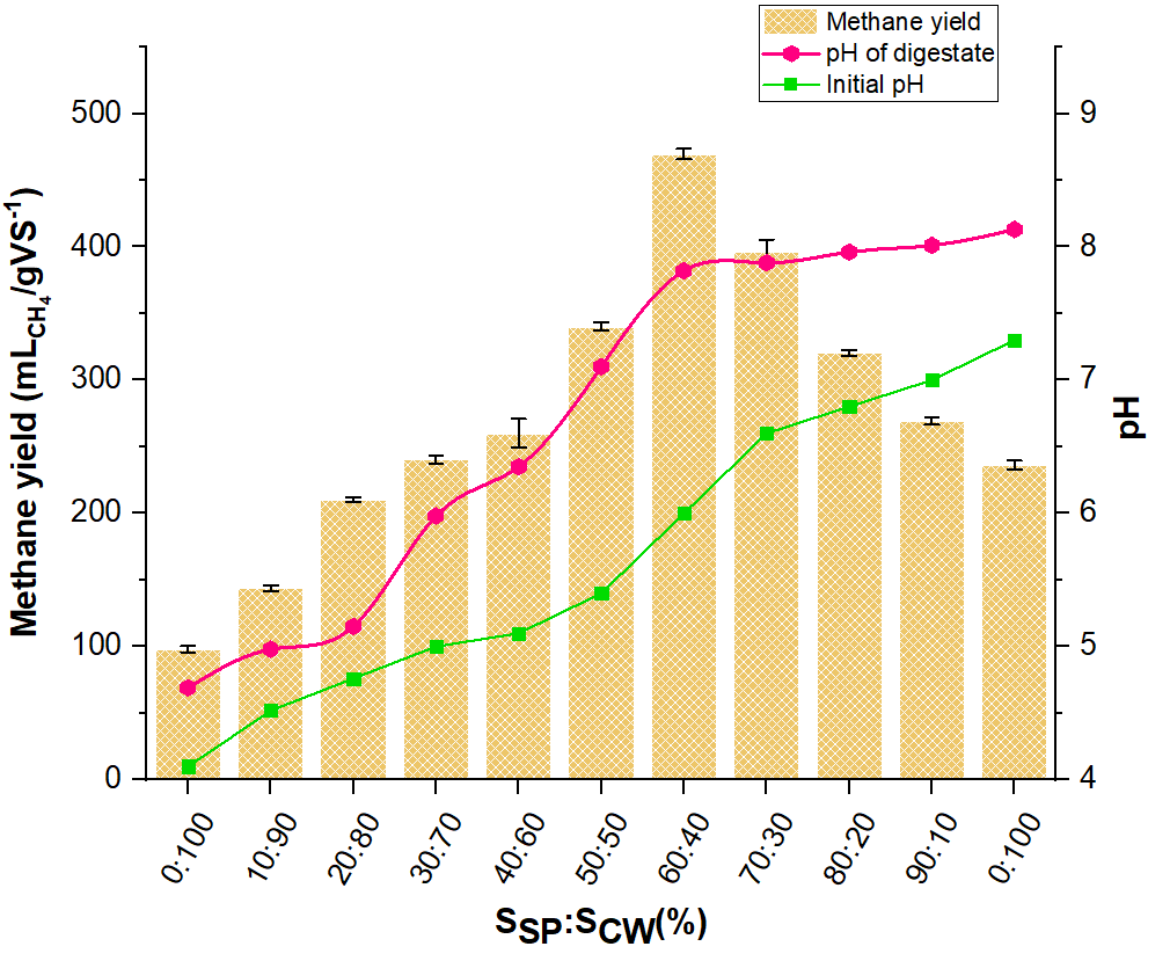

Figure 4. 2 Methane yields measured for co-digestion of CW and SP at different mix ratios during 35 days

Regarding VFAs, at initial pH 4.8 for 100% whey mixture, valeric, butyric, and caproic acids production were reported. For mixtures with whey fractions less than 60%, the production of propionic and acetic acids is favoured. This indicates that VFA production is profoundly influenced by pH values and whey fraction in the co-digestion mix. Similar results were reported by authors (Cheah et al., 2019; Slezak et al., 2020) that at pH>7 acetic and propionic acid production and pH<6 butyric acid production is favoured. Lower VFA concentration was observed in reactors containing septage alone. The drop in alkalinity previously mentioned is correlatable with higher VFA values. The VFA to alkalinity ratio was found as < 0.2 which indicates poor stability and requires more feeding. Therefore, it is essential to use an external buffering agent or a proper inoculum to accomplish the anaerobic digestion process. In addition, whey wastewater contained high concentrations of SO_4^{2-} (280-320mg/l) and oil and grease (2812-3270mg/l) in each trial. It has been reported that oil and grease particles can cause sludge floatation problems in lipid rich dairy wastewaters adversely affecting CH_4 yield (Vidal et al., 2000).

4.5 Kinetic modeling of co-digestion studies

For the batch tests (without inoculum) conducted, the cumulative methane production curves are fitted with the Gompertz model. Figure 4.3 depicts the results of model fitting. The model parameters; maximum cumulative methane production(P_{max}), methane production potential rate(R), and lag phase times were obtained and plotted against whey % (Fig.4.3b-d). The maximum cumulative methane production curve showed its peak value at 40% whey fraction which is 530.95 ± 5.9 ml/gVS. All curves are fitted with best-fitting models and R^2 values are also shown in respective graphs. The lag phase times are found to be highly fluctuating. The lag phase times increased first with increasing whey content, reached a peak value, and decreased again. The highest lag phase time is obtained at 40% whey fraction which is 4.21 days. When septage content is mounted to 80,90 and 100%, lag phase times were 1.09, 0.83, and 0.34 days respectively which shows less time is required for methanogenesis to start normally (Table 4.3).

The possible causes for increased lag phase time might be related to the complexity in degradation of whey proteins and lack of starter microbial communities. Normally some inoculum sources are used to initiate the digestion process in the case of dairy wastes (M.Chartrain, L.Bhatnagar, 1987). Even though the methane generation potential of lipids is higher than that of carbohydrates, the hydrolysis stage of lipids is highly dependent on the acclimatization of the micro-organisms (Yu and Fang, 2001). The pH values were between 4.5

and 5.7 when whey fractions are above 60% in the co-digestion mixes (Fig 4.2). Lack of acclimatized microbes along with reduced buffering capacity might have led to lower methane production and higher lag phase times. The difference between observed and predicted methane yield by the Gompertz model ranged between 4.36-28.23%.

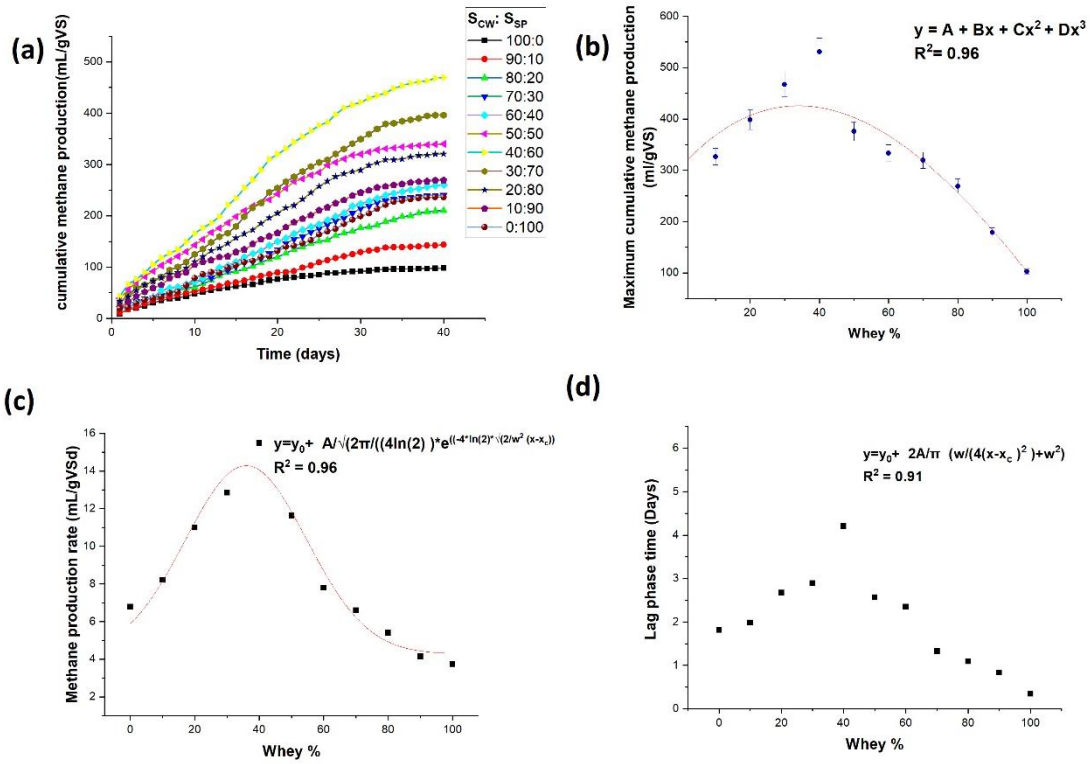

Figure 4. 3 (a)) Measured and simulated methane production values for different co-digestion mixtures of whey and septage (whey % is shown in legend) (b) Relation between maximum cumulative methane production and whey % from model fit (c), methane production rate from model fit (d), and lag phase times from model fit. Points indicate measure data, and lines indicate simulated results. Error bars represent the standard deviation of triplicate tests.

Table 4. 3 Comparison of experimental and kinetic model results on anaerobic digestion of whey and septage without inoculum

Experimental		Gompertz model parameters		
S_{CW}/S_{SP}[a]	X_{CH4}[b] (mL_{CH4}/gVS)	P[c] (mL_{CH4}/gVS)	R[d] ($mL_{CH4}/gVSd$)	Λ[e] (days)
1	98.03	102.50	3.731	0.34
0.9	143.65	178.86	4.148	0.83
0.8	210.32	277.55	5.870	1.09
0.7	240.21	319.56	7.381	1.33
0.6	260.01	333.22	7.795	2.35
0.5	340.08	375.82	11.638	2.57
0.4	469.54	530.95	15.490	4.21
0.3	396.43	467.08	12.850	2.89
0.2	320.32	383.54	10.113	2.67
0.1	269.23	326.69	8.210	1.98
0	236.21	329.12	6.786	1.81

[a] S_{CW}/S_{SP}: Fraction of cheese whey to septage,
[b] X_{CH4}: experimental methane yield (mL_{CH4}/gVS),
[c] P: maximum cumulative methane production(mL_{CH4}/gVS),
[d] R: methane production potential rate($mL_{CH4}/gVSd$),
[e] λ: lag phase time(days)

4.6 Effect of inoculum on methane productivity

Three inocula I_{CM}, I_{SS}, and I_{BS} were used for the study. All the three inocula were degassed through pre-incubation for 3 days as described by Angelidaki et al., (2009). Three separate BMP assays comprising 3 sets of 30 bottles each, were prepared by mixing S_{CW} and S_{SP} at varying fractions ranging from 0 to 100% and one inoculum out of three in each set. The working volume of bottles was fixed as 80 ml in 100 ml glass bottles. The volume of substrates and inocula were maintained with a substrate to inoculum ratio (SIR) of 1:1. Same proportions of whey and septage were used to make co-digestion mixtures. Blank assays or control bottles are kept additionally for each inoculum in triplicate (SIR=0). Details of setup are illustrated in Table 4.4. All bottles are flushed with N_2 gas and closed immediately after adding contents with rubber stoppers. The reactors were placed in incubators at 35ºC for 35 days and daily gas measurement was taken by the water displacement method.

Table 4. 4 Substrate and inoculum fractions for the second set of BMP tests

Mix ratio (S_{CW}: S_{SP})	Volume of substrates		The volume of inoculum (I_{CM}/I_{SW}/I_{AS})	Total working volume (ml)	No of replications
	S_{CW} (ml)	S_{SP} (ml)			
100:0	50	0	30	80	3
90:10	45	5	30	80	3
80:20	40	10	30	80	3
70:30	35	15	30	80	3
60:40	30	20	30	80	3
50:50	25	25	30	80	3
40:60	20	30	30	80	3
30:70	15	35	30	80	3
20:80	10	40	30	80	3
10:90	40	45	30	80	3
0:100	0	50	30	80	3
Control	0	0	80	80	3

Nb - S_{CW}: Cheese whey, S_{SP}: Septage, I_{CM}: Cattle manure, I_{SW}: Sewage sludge, I_{AS}: Anaerobic sludge

Figure 4.4 and Figure 4.5 show the cumulative biogas and methane values for the BMP experiments conducted using cattle manure, sewage sludge, and anaerobic digestate respectively. The curve pattern, peak values, and initial lag time were the main differences. I_{CM} showed promising results in terms of biogas production with S_{CW} and S_{SP} when compared to I_{SS}, and I_{BS}. Although I_{CM} had the highest biogas yield than others, the initial lag time was longer than that of both I_{SS} and I_{BS}. In Figure 4.3 (a), the biogas production gradually increased as whey fractions decreased and the highest biogas yield was found to be obtained at 40% whey fraction with I_{CM} as inoculum. In previous experiments, more biogas production was reported in samples having 60% septage fraction and the optimum C/N ratio for that particular mix was 32.3. With the use of inoculum; I_{CM} having more nitrogen content and S_{CW} having more carbon content, the desired C/N ratio of 34.01 was achieved early. Cattle manure has the highest total biogas and methane content ratio (68.35%) which led to a specific methane yield of 130.08 mL/gVS. Cattle manure has shown a similar kind of methanogenic activity and better adaptability with other organic wastes in other studies too. The S/I ratio maintained in our experiments is within the range suggested by Moller et al., (2004) for I_{CM}. As per this study, cattle manure having an average VS concentration of 895.23g/Kg of dry matter yielded around 148L CH_4/gVS. The percentage of VS in a substrate affects the digestibility and the quantity of methane generation capacity (Angelidaki and Ahring, 2018). I_{CM} was found to have VS around 21,000mg/l which contributed to achieving higher methane yields.

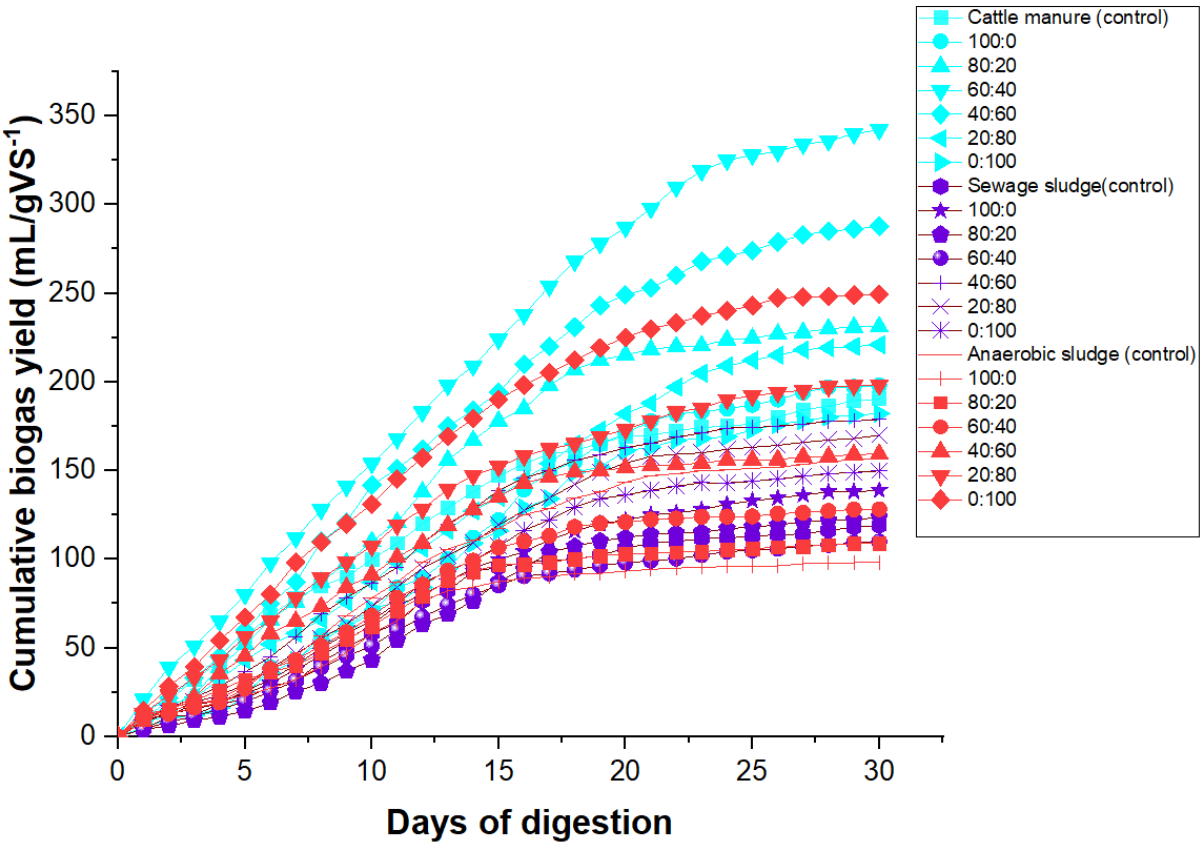

Figure 4. 4 Cumulative biogas production values for various mixtures of whey(CW) and septage(SP) inoculated with 3 different inocula (ICM: cattle manure, ISS: sewage sludge, I_{AS}: anaerobic sludge). Curves are labelled by percentage mix ratios of CW: SP on a VS basis. Curve and symbol colours indicate the inoculum used; blue for I_{CM}, Violet for I_{SS}, and red for I_{AS}.

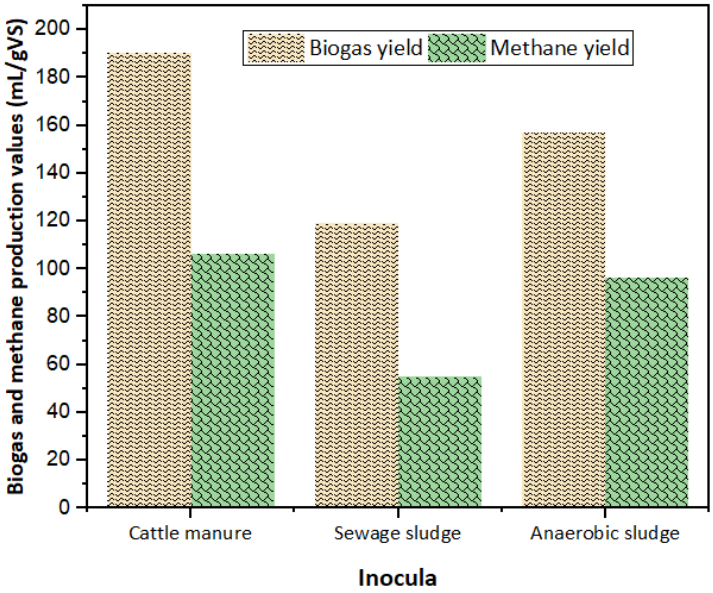

Figure 4. 5 Total biogas and Specific methane value for I_{SS}, I_{AS}, and I_{CM}

Sewage sludge has shown its better adaptability with septage than with cheese whey. Because a higher quantity of biogas around 178.8mL/gVS was obtained at 40% whey fraction (Figure 4.4). The specific methane yield of I_{SS} was found to be 118.92mL/gVS which is the lowest among other inocula. Córdoba et al., (2017) studied the activity of sewage sludge as inoculum for digesting CW, which resulted in lower biogas production at a higher SIR ratio(3-6). The presence of more amount of non-biodegradable portion might be the reason for the low SMY of I_{SS} (Kavitha et al., 2017; Xiao et al., 2018). In the studies focused on the digestion of sewage sludge, several pre-treatment studies are suggested to increase its methanogenic activity(Cano et al., 2015; Pilli et al., 2015; Zhen et al., 2017). Compared to I_{CM} curves, slopes are less steep for I_{SS} curves. In co-digestion mixtures with more CW fractions (100%,80%, and 60%), overall biogas yield was less. Poor hydrolysis rates of complex sludge flocs and complexity of whey particles may have led to poor methane yield. (Moestedt et al., 2019) has reported that hydrolysis and acidogenesis stages were found slow and difficult to achieve in the digestion process of sewage sludge. Also, the pH was found to increase from 6.2 to 7.8 at the end of experiments, which indicates that acidification was harder to accomplish without initial pH correction and pre-treatment.

The anaerobic sludge from the biogas plant, I_{AS} was thickened in lab under gravitational force prior to its use. Using I_{AS} as inoculum, a maximum biogas production of 249.33mL/gVS was obtained at 0% whey (100% septage) (Fig 5). Both S_{SP} and I_{AS} are sludges rich in anaerobic

groups of microbes and other nutrients which will help in reducing the initial acclimation period. The specific methane yield of I_{BS} was obtained as 156.9 mL/gVS. The biogas production curves for mixtures having higher septage fractions are steep compared to that of mixtures having more whey fractions. Also for 100,80 and 60% whey mixtures, curves touched plateau shape within the first 15 days. This means that almost 80% of the ultimate biogas yield was achieved within 15 days of experiment.

Alkalinity values for inoculated mixtures were reported between 1950-2300mg$CaCO_3$/l except for the control which is 1289mg$CaCO_3$/l. Inoculum sources and co-substrate provided adequate buffering capacity. In the case of VFA production, the concentration of acetic acid was found high (2430mg/l) for I_{CM} inoculated mixtures having whey fraction >40%. Butyric and propionic acids were found at about 1200 and 480mg/l respectively. VFA/TA quotients ranged from 0.89-1.08 which showed a maximum range for AD with VFA accumulation risk. In contrast, Kim M and Kim S, (2018) reported maximum biogas production at VFA/TA ratio 1.131-1.870. VFA concentration was 18% more than that produced during individual substrate digestion. VFA accumulation lead to a drastic drop in pH which was depicted in biogas production after 25 days. Average distribution of VFA for inoculated co-digestion mixtures was; butyric acid (16.92-19.7%), acetic acid (20.1-31%), propionic acid (18.1-23%), valeric acid (9.6-11.8%) and others (13.5-15.4%). However, these values did not reach till toxic threshold limit ie;3000mg/l of acetic acid.

The results obtained so far indicates that the activity of 3 inocula was different with substrates, whey, and septage. Cattle manure showed better adaptability with S_{CW} and S_{SP} and biogas values were comparatively higher for all I_{CM} inoculated reactors. Since whey contains a higher amount of easily degradable carbon than septage, there is a chance of more acidification and eventually, lower biogas yield will result from mixtures having more whey fraction. It can be concluded from the aforementioned results that compared to the sludges (I_{BS} and I_{SS}) used as inoculum, I_{CM} exhibited better activity.

4.7 Ammonia inhibition

CW having high organic content and biodegradability are also capable of limiting the AD process due to its high protein content. The protein degradation leads to the generation of ammonia which results in process inhibition and reactor instability. The pH reduction observed in the present study need not be due to VFA accumulation alone but instead is probably due to high NH_4 concentration (Protein hydrolysis) (Costamagna et al., 2020). The pH of the final

effluent was checked for assessing the digester stability and simultaneously free ammonia concentration and TAN were also calculated using equation 3.17. Reactor pH ranged between 4.69-8.21 for effluents of 1st co-digestion set (without inoculum) and 5.25-7.6 for effluents of 2nd set (with inoculum). TAN value of whey sample during its mono digestion was obtained as 1800mg/l. During codigestion with septage, as whey fractions increased TAN values also raised. For mixtures (without inoculum); 80% S_{SP}, 20% S_{CW} TAN was 1428mg/l, 60% S_{SP} 40% S_{CW} TAN was 1683mg/l, which is near to the value reported by Yenigün and Demirel, (2013) as the toxic limit for methanogenic activity due to ammonia.

Ammonia inhibition encountered can be reduced to a level with the use of septage rich in several minerals like K^+, Ca^{2+}, $Mg^{2+,}$ etc. This fact is validated by the low TAN value observed in the reactor containing 100% septage alone (1240mg/l). Free ammonia(FA) concentration was similar in all reactors except for the reactor containing whey as the sole substrate. For I_{CM} inoculated mixture with 100% septage fractions, the concentration of FA was 48.6mg/l. For mixtures with whey fractions varying from 10 to 90%, FA concentrations were obtained in the range of 28.9 to 37.6mg/l. I_{CM} inoculated samples showed more ammonia inhibition than I_{SS} and I_{AS} inoculated mixture. For 100% whey, FA concentration reached 54.6mg/l, which is lower than that reported by (Yenigün and Demirel, 2013) which is 150mg/l. This indicates that FA values are not inhibiting the methanogenic activity.

4.8 Kinetic modelling of inoculum studies

Figure 4.6 depicts the simulated results of the Gompertz fit model on different co-digestion mixes of 3 inocula along with their respective correlation coefficient values. The estimated model parameters (P_{max}, R, and λ) are summarized in Table 4.5. The predicted methane production values showed a good correspondence with the experimentally determined values. The R^2 values ranged between 0.996-0.998 for tests with cattle manure as inoculum, while it was between 0.995-0.998 and 0.985-0.997 for inocula sewage sludge and anaerobic sludge respectively. Lag phase times were ranging between 0.76 – 3.59 days for I_{CM} added tests which show faster adaptation of biomass to the substrate mix. The maximum cumulative methane production (P_{max}) was obtained at 60% whey fraction (I_{CM} inoculated) which is 369.63 ± 4.05mL/gVS. The highest methane production rate was also obtained at the same mix proportion (17.41 ± 0.32 mL/gVSd). The P_{max} values for I_{SS} and I_{AS} inoculated sets were 185.40 ± 1.23mL/gVS and 259.23 ± 1.91mL/gVS which were obtained at 40% and 0% whey fractions respectively.

Higher lag phase times are reported for mixtures having more whey content. The untreated whey contains a high amount of complex proteins which are hard to degrade and thus lag phase times are found a bit longer. A lower λ value indicates that the substrate is more comfortable with the methanogenic bacteria. Decomposition of an acidic substrate will generate more non-dissociated acids which will directly penetrate to the bacterial cell and denature the protein of bacteria (Deublein, D., Steinhauser, 2011). The abundant release of organic acids can disturb the activity of methanogenic bacteria and reduce biogas generation. Pre-treatment of lipid rich substrates like whey might help in reducing lag phase times and increase hydrolysis rates (Diamantis et al., 2021). The lag phase times for I_{AS} inoculated samples (0.44-2.04d^{-1}) were found less compared to that of I_{CM} and I_{SS} which might be because of the presence of more adapted microbes present in the inoculum medium.

Estimation of kinetic parameters by Gompertz model helped in the evaluation of digester performance and reactor stability. Results from BMP tests are best fitted by the Gompertz model, which alone will not guarantee good digester performance in a continuous mode of operation. In the batch mode of operation, reactor stability was observed. The presence of fats and lipids in the main substrate has caused a rise in lag time. Both lab scale and pilot scale anaerobic digesters can be operated by concerning the lipid percentages in whey, as combining it with strong organic wastes will result in higher biogas production. However, higher lipid concentration can lead to potential ammonia inhibition also. Hence organic load supplied should adapt with the buffering capacity of the medium (VFA/TA 0.2-0.5) (Alessio Siciliano et al., 2019). Whey pre-treatment or microbial acclimatization is recommended to avoid this inhibition in continuous mode operation. Although values for I_{CM} inoculated mixtures are more compared to I_{AS}, improved buffering capacity along with nutrient balance (C/N ratio 34.1) were observed for I_{CM} inoculated samples.

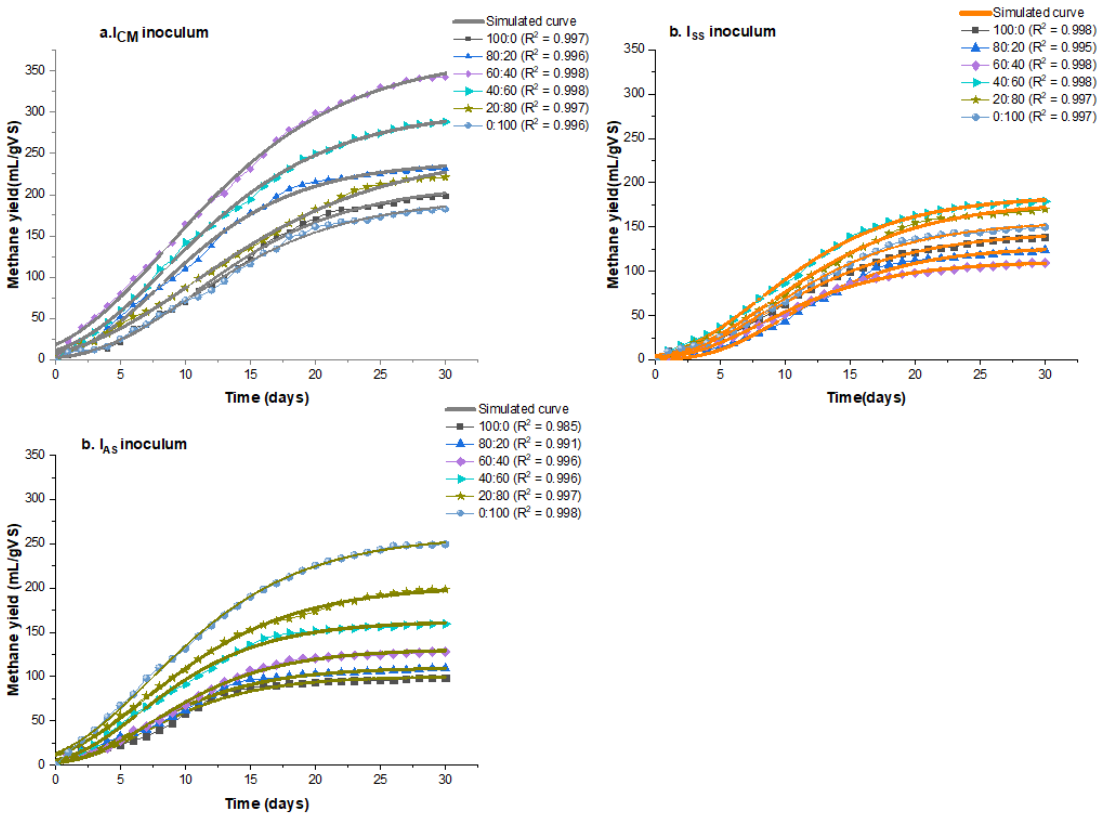

Figure 4. 6 Cumulative methane yield plotted against time using Gompertz kinetic model for 3 inocula (a) I_{CM}: Cattle manure, (b) I_{SS}: Sewage sludge, (c) I_{AS}: Anaerobic sludge, for different whey percentages. (Mix ratios are shown as CW: SP, R^2 represent correlation co-efficient)

4.9 Combined effect of co-digestion and inoculum

From the mono and co-digestion experiments conducted on whey and septage, it is evident that the addition of septage waste up to a certain extent helped in improving the digestibility and biogas generation capacity of whey. A clear antagonistic effect was visible only after 50% septage additions. Septage, being an anaerobically rich naturally available organic waste is an excellent co-substrate for whey digestion. Studies conducted with septage as co-substrate with dairy wastes in the anaerobic digestion domain are scarce. Luostarinen & Rintala (2005) operated an onsite UASB-septic tank for studying the co-digestion of black water and dairy parlor wastewater. The study aimed at organic matter removal rather than biogas production. The results of the study stressed the potential applicability of septage as a co-substrate with dairy wastes. The present study is found to be in line with the previous one in which septage was found well adapted to whey waste by supplying lacking nutrients, balancing the C/N ratio, balancing pH, and providing anaerobic microbes.

In addition to the co-digestion, inoculum sources used helped in supplying the buffering capacity also. The synergistic effect could also be explained by observing the increased stability of reactors and methane yield due to the benefits of adding inoculum which helped to compensate for low alkalinity, low C/N ratio, and low nutrient levels. The synergistic effect of both can be stated with the help of statistical studies. According to the analysis of variance conducted with two variables (co-digestion and inoculum type) the model had a significant fitting ($p<0.01$), depicting the significant effect of both variables on biogas generation.

Table 4. 5 Values of the kinetic parameters obtained from Gompertz fit model analysis on BMP set 2 tests. Pmax : the maximum accumulative methane production (mL/gVS), R: the maximum methane production rate (ml/gVS.d), λ: the lag phase time (days). Standard deviation values represent results of triplicate tests

Inoculum: Cattle Manure				**Whey %**		
Model Parameters	**100%**	**80%**	**60%**	**40%**	**20%**	**0%**
P_{max}	213.72 ± 3.06	240.88 ± 2.42	369.63 ± 4.05	303.44 ± 2.74	252.35 ± 3.79	196.17 ± 2.79
R	11.42 ± 0.28	14.73 ± 0.37	17.41 ± 0.32	15.57 ± 0.26	10.72 ± 0.19	10.34 ± 0.25
λ	3.59 ± 0.21	2.01 ± 0.19	0.76 ± 0.17	1.39 ± 0.15	1.88 ± 0.18	1.13 ± 0.21
Error (%)	7.9	4.17	8.01	5.39	6.2	7.66
Inoculum: Sewage Sludge				**Whey %**		
Model Parameters	**100%**	**80%**	**60%**	**40%**	**20%**	**0%**
P_{max}	144.55 ± 1.18	127.96 ± 1.76	111.17 ± 0.79	185.40 ± 1.23	179.11 ± 1.77	156.72 ± 1.50
R	8.19 ± 0.14	8.55 ± 0.29	7.29 ± 0.14	11.33 ± 0.18	10.06 ± 0.21	9.31 ± 0.20
λ	2.13 ± 0.14	4.35 ± 0.24	2.52 ± 0.14	1.98 ± 0.12	2.40 ± 0.17	2.59 ± 0.17
Error*(%)	4.05	3.28	1.15	3.65	5.37	4.56
Inoculum: Anaerobic Sludge				**Whey %**		
Model Parameters	**100%**	**80%**	**60%**	**40%**	**20%**	**0%**
P_{max}	100.04 ± 1.56	110.79 ± 1	131.10 ± 1.1	162.58 ± 1.3	202.45 ± 1.6	259.23 ± 1.9
R	7.52 ± 0.40	7.71 ± 0.30	9.06 ± 0.23	11.22 ± 0.28	11.52 ± 0.23	14.45± 0.25
λ	1.84 ± 0.35	1.13 ± 0.28	2.04 ± 0.18	1.21 ± 0.18	0.63± 0.16	0.44± 0.14

Error*(%)	2.07	1.43	2.26	2.03	2.13	3.97

4.10 Novelty, scientific significance and future research

The current work explored the chances of utilizing septage for improving the anaerobic biodegradability of CW. As septage treatment facilities are not developed in many Indian cities, AD offers a scientific way to process it further. Instead of disposing it unscientifically on agricultural lands or drains, the desludging units of septic tanks can transport it to digestion plants installed in dairy farms. Implementing AD technology in small-scale dairy industries is not economical as continuous availability of CW throughout the year cannot be ensured. Thus AD plants can be installed in dairy farms where cattle manure is available for inoculum and whey can be transported from cheese making units of dairies.

Although this study evaluated the volume of biogas and its composition, it was limited in studying the characteristics of digestate produced. Therefore, it provides little information on how digestate can be further processed for utilization or safe disposal. Inoculum acclimation to TN is relevant to this study as all inocula used are rich in Nitrogen content. Studies show that batch tests are sensitive to inoculum characteristics (Raposo et al., 2020). Since one-third of the substrate used is lipids, the inoculum supplied should be able to degrade VFA. More studies need to be conducted on evaluating the activity of inoculum acclimation to TN to confirm the positive interactions observed between TN and VFA. In addition, the use of continuous digesters will provide more insights into the observed results. The observed results indicate that the co-digestion of CW with septage could be implemented on a larger scale, which offers dual benefits-pollution reduction and energy recovery. In such cases, the addition of more lipids may help in higher biogas production by alleviating inhibition due to TN.

4.11 Important findings

- Mono-digestion of whey and septage resulted in methane yield of f 66.1 ± 0.8 ml/gVS and 148 ± 3 ml/gVS respectively.
- Co-digestion with septage increased the methane productivity of cheese whey by 117% when 10% of septage fractions are added.
- Co-digestion of CW with SP showed highest methane yield at 60% septage fraction (469.54±10.8ml/gVS).
- The specific methane yield values for I_{CM}, I_{SS} and I_{AS} were 169.43mL/gVS, 118.92ml/gVS and 156.92mL/gVS respectively.

- Maximum methane yield was obtained for co-digestion mix of 60:40 (CW:SP) in presence of cattle manure as inoculum (352.22mL/gVS)
- Activity of inocula on digestion process of cheese whey and septage is found in order
    - Sewage sludge < Anaerobic digestate < Cattle manure
- Modified Gompertz model fitted the experimental data well and identified an increase in lag phase times when whey fraction is increased.

4.12 Conclusion

The results of the present study demonstrated the suitability of using septage as a co-substrate in the anaerobic digestion of cheese whey. The mono digestion of CW and Septage was found to be giving only 15.82 and 47.13% of the theoretical yield respectively. Septage is an excellent source of valuable anaerobic microbes and is also rich in Nitrogen helped in attaining the optimum C/N ratio for the co-digestion mix. The optimum mix ratio for co-digestion of whey and septage (without inoculum and pre-treatment) was obtained as 40:60. Visible antagonistic effects are observed when the whey fraction is approximately above 50%. Therefore, keeping a whey fraction less than half of the total substrate mixture is found to be improving methane yield and also biodegradability. However, the use of an appropriate inoculum (CM) has improved the fraction of CW to 60% from 40%, giving more scope to utilize the waste effectively to generate more biogas. Experimental data from BMP tests were well described by the modified Gompertz model. The model stated that the maximum methane production rate decreased when whey content is increased beyond 40%. The overall results state that anaerobic co-digestion of CW with SP by supplying inoculum sources can be a viable CW management option, although further studies are required in the variations of concentrations of wastes on process stability and resilience is needed in batch mode for practical implementation. Pre-treatment such as hydrolysis of whey proteins may be carried out before digestion, which may improve the methane yield.

Chapter 5

Pre-treatment study

5.1 General

Complex wastes like whey also require pre-treatment methods along with co-digestion to enhance hydrolysis. Different pre-treatment methods have been used to reduce substrate complexity for increasing organic matter solubility as well biogas production in AD. Majorly the changes like reduction in particle size, biodegradability enhancement, refractory compound formation and organic matter solubilisation are likely to occur due to pre-treatment. Many of the pre-treatment methods have not been investigated for the production of biogas from CW that contains milk protein or lactose components. It is essential to examine and compare the existing pre-treatment methods in order to hydrolyse complex proteins in CW. Only few studies have been conducted in this domain. The treatment of CW includes physical degreasing, as well as chemical and biological degradation. Physical and chemical pre-treatment methods are costlier and utilises high energy, whereas biological methods are energy saving. Even though biological methods are energy-saving, they have some drawbacks, such as longer exposure times and difficult pH and temperature controls.

In light of these shortcomings, the main purpose of this chapter was to evaluate how ultrasonication, ozonation, and enzymatic pre-treatment methods improved organic matter separation and biogas production in AD. Furthermore, detailed study on energy and cost analysis of each pre-treatment method is carried out to know about net effect on profitability and energy yield.

5.2 Performance of pre-treatment methods

The efficiency of the pre-treatment methods used in this study in transforming complex whey particles into more readily available substrate was evaluated on the basis of parameters like degree of solubilisation, increase in sCOD concentrations, degree of lactose hydrolysis, and reduction in TSS concentrations. In Table 5.1, operating conditions for ozonation and sonication are listed The effect of each pre-treatment is discussed below separately.

Table 5. 1 Operating conditions for US and ozonation pre-treatment

Ozonation				Sonication		
O_2 flow (LPM)	**O_3 concentration (gO_3/gTS)**	**Duration (minutes)**	**O_3 generation power (g/hr)**	**Duration (minutes)**	**Specific energy (KJ/KgTS)**	**Initial TS (g/l)**
1	0.045	4	2.70	4.5	2130.36	58.3
2	0.038	8	4.56	8.5	4044.82	58
3	0.034	12	6.12	12.5	6000	57.5
4	0.03	16	7.20	15.5	7505.26	57
				18.5	8773.19	58.2

5.2.1 Effect of sonication

After US studies at different specific energy (S_E) levels and time intervals, noticeable changes in the fraction of soluble organic matter was observed. The average sCOD increased from 24.33mg/l to 48.96 mg/l after 18.5 minutes of sonication with increase in specific energy from 2130.36KJ/KgTS to 8773.19KJ/KgTS. As a result of US, the mixture could undergo physical and chemical changes, increasing the amount of soluble organic matter. The S_d value denotes the percentage value of organic fraction that is transferred from solid to aqueous phase (Grübel and Suschka, 2015). US pre-treatment in terms of S_d (%) at various specific energy and US times is presented in the Table 5.2. For the same power, with increasing S_E and operation time, the solubilisation rates were increased. This could be possibly due to the more acoustic cavitation generated when temperature of the sample is raised after prolonged US (S. Şahinkaya et al., 2012). Figure 5.1 shows the effect of S_E on COD and degree of solubilisation rates. The applied S_E was found to be directly proportional to the degree of solubilisation. The sCOD concentration increased linearly with S_E (sCOD=0.00354S_E-16.29). Similarly, when the S_E increased S_d values also increased linearly from 46% to 77%. Similar disintegration rates have been reported by other researchers during US of whey (Mainardis et al., 2019; Marcin Debowski et al., 2020; Pilli et al., 2016). High solid concentration of whey can be attributed to high sCOD solubilisation rates. Pilli et al., (2016) found 1.12% increase in sCOD for sonicated sludge at 40g/l of TS concentrations. Kazimierowicz et al., (2022) harnessed low frequency ultrasonics of 24kHz and 400W for the pre-treatment of acid whey and found considerable COD increase (31.4g/l to 53.6g/l). Mainardis et al., (2019) reported a 14.5% increase in sCOD concentrations

after US of skimmed whey at an operating frequency of 20kHz and 80W power. Higher ultrasound intensity and longer US treatments have been reported to enhance organic matter solubilisation (Ladero et al., 2000; Naddeo et al., 2009).

Table 5. 2 US pre-treatment conditions and corresponding results in terms of sCOD and S_d

Power	***W***	230	230	230	230	230
Time	***Minutes***	4.5	8.5	12.5	15.5	18.5
Energy applied	***J/ml***	124.2	234.6	345	427.8	510.6
	Kwh/m^3	34.5	65.17	95.83	118.83	141.83
	Kwh/KgTS	0.61	1.12	1.67	2.08	2.43
sCOD release	***g/l***	29.6	31.1	34.68	42.19	48.76
S_d	***%***	46.83	49.21	54.87	66.75	77.15

Major components of whey are protein and lactose. Hence, the sCOD increase of whey can be mainly attributed to change in protein and lactose concentrations. US enhanced lactose hydrolysis. The residual lactose concentration was considerably reduced with increased exposure time and S_E. The initial concentration of lactose was around 53g/l which got reduced to 28.6 g/l showing around 46.03% degree of lactose hydrolysis. Similarly, the concentration of soluble protein in sonicated samples were also found increased with increase in time and SE. Figure 5.2 shows the effect of SE on residual lactose and soluble protein concentrations.

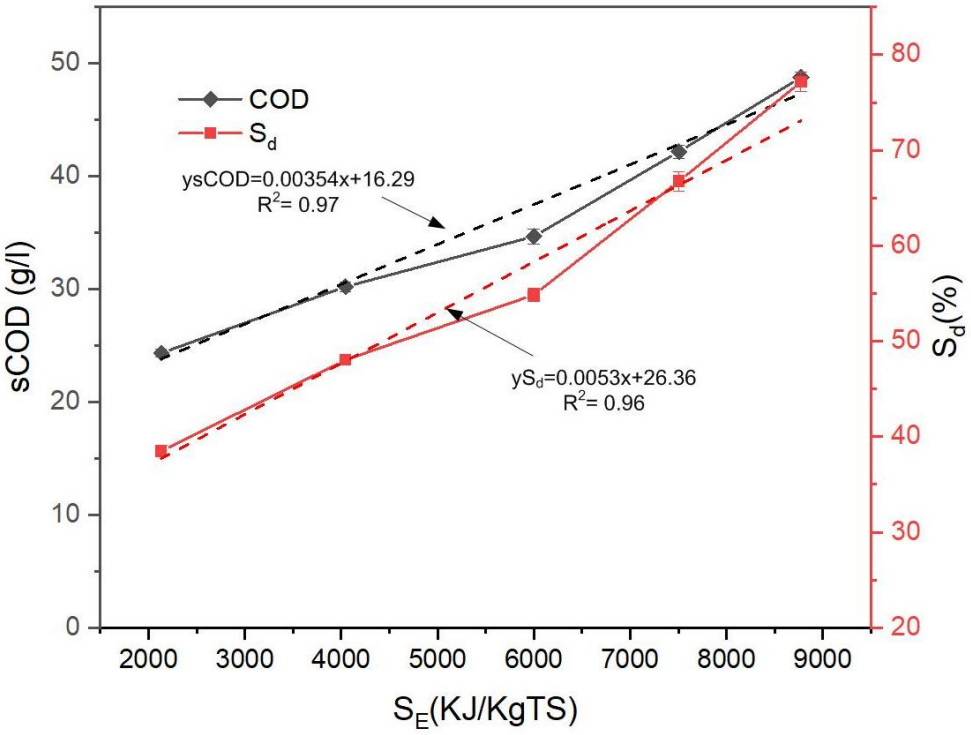

Figure 5. 1 Effect of SE on COD solubilisation and degree of solubilisation. (Error bars represent standard deviations of duplicate results)

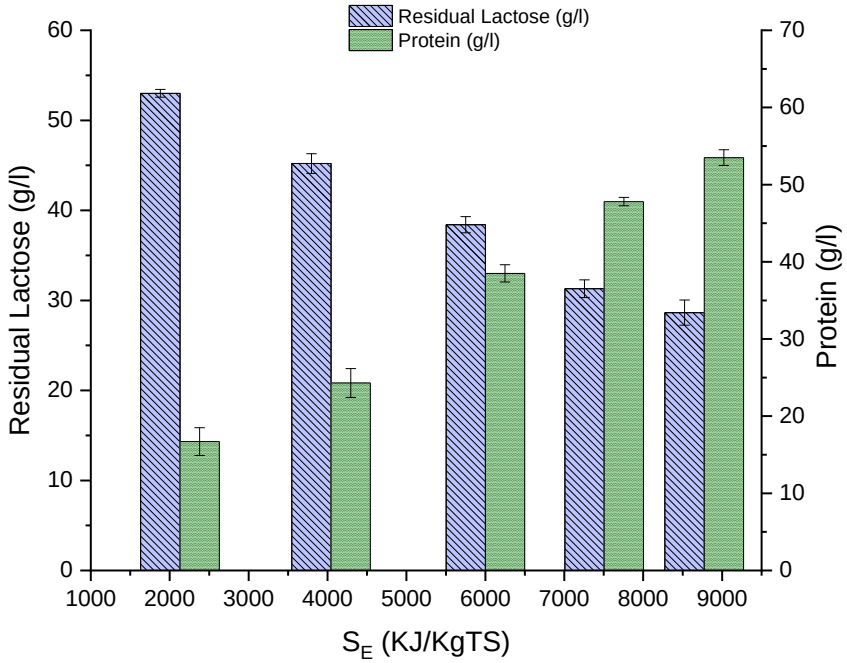

Figure 5. 2 Residual lactose and protein concentrations at different specific energy

5.2.2 Effect of ozonation

The effect of ozonation on CW were studied through monitoring the change in the sCOD concentration as well as organic matter (non-soluble) concentration. Compared to US, a lower degree of solubilisation was obtained for ozonation. Table 5.3 shows the results of ozonation pre-treatment of whey and graphical representation of same was shown in Figure 5.3. The S_d value ranged from 44.38% to 64.8% when ozone dosages were increased from 0.03 to 0.045g/gTS. Both solubilisation and mineralisation were noticed when O_3 exposure time was increased. Higher S_d values were observed for low O_3 dose and longer ozonation time. For example, the degree of solubilisation was only 44% for an O_3 dose of 0.038g/gTS applied for 8 minutes, while it increased to 64% when ozonation was continued for 16 minutes. Lower solubilisation rates observed at higher O_3 dosage might be due to increased mineralisation. In contrast to US, there was considerably less hydrolysis of protein and lactose. Studies have pointed out that O_3 pre-treatment might facilitate organic matter solubilisation by producing non-selective and highly oxidizing free radicals (Pei et al., 2015).

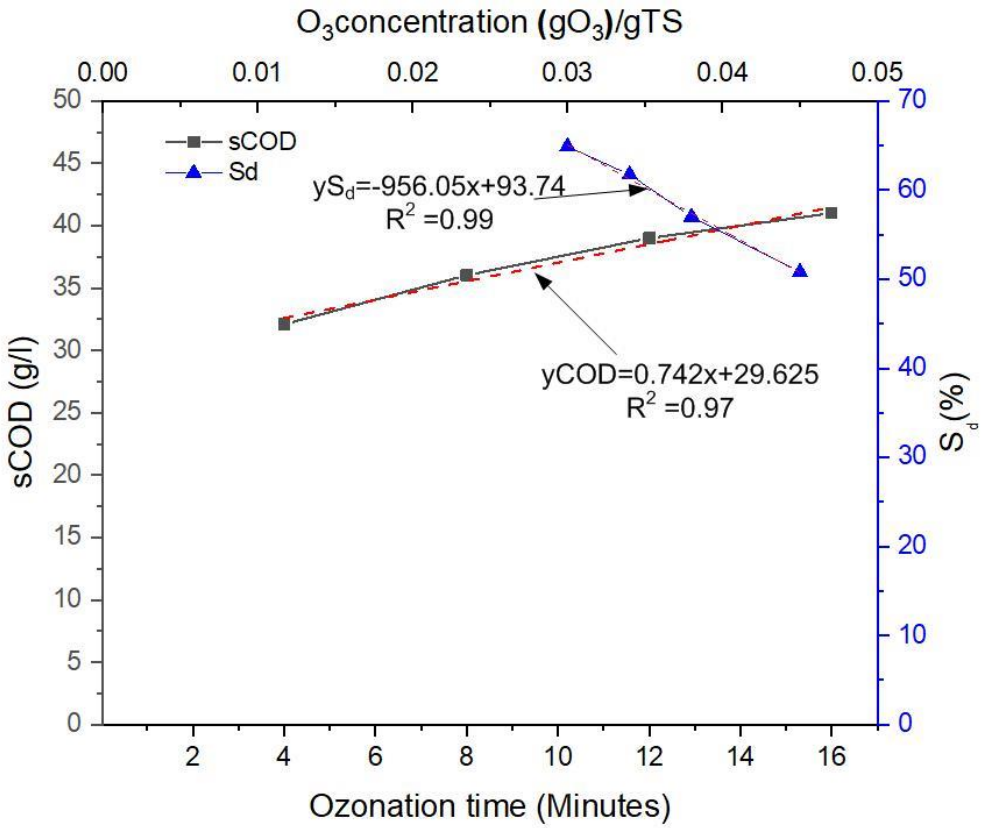

Figure 5. 3 Effect of ozonation on degree of solubilisation and change in sCOD concentrations

Table 5. 3 Effect of ozonation pre-treatment on cheese whey organics

O_3 concentration (gO_3/gTS)	Ozonation time (Minutes)	sCOD (g/l)	S_d (%)	Residual Lactose (g/l)	Protein (g/l)
0.045	4	32.11	50.80	52.6	16.9
0.038	8	36.04	57.02	48.3	24.3
0.034	12	39.02	61.74	48	27.4
0.03	16	41.01	64.88	44.1	32.4

5.2.3 Effect of enzymatic hydrolysis

The independent variables chosen for performing enzymatic pre-treatment using β-galactosidase were pH (3.81-7.18), enzyme load (0.18-0.52%), temperature (35ºC-55ºC) and time of operation (7.5- 53.5minutes). The efficiency of enzymatic pre-treatment was evaluated in terms of lactose hydrolysis (E_h) rates and change in sCOD concentrations (Table 5.4). Figure 5.4 shows the change in sCOD concentrtions and E_h rates at different runs. The E_h values ranged from 52.7% to 86.21% and maximum increase in sCOD was obtained as 54.8%. Among the 4 parameters, pH was found to be highly influencing the activity of β-galactosidase enzyme. The findings are consistent with the manufacturer's instructions for the usage of A. oryzae enzyme, which state that acidic conditions favour lactose hydrolysis. In the range of 3.5-5.5, the ideal pH value is attained. The findings from prior research shows similar range of pH values (4-4.5) for attaining maximum lactose hydrolysis (Czermak et al., 2004; Mlaik et al., 2019). The activity of β-galactosidase declines at a pH of 6.5, and the hydrolysis rate was at lowest value (52.7%) at pH 7.5 during 30.3 minutes of time and at a temperature of 45°C. Since substrate composition, enzyme usage and reaction mode (batch or continuous) differ between investigations, it is challenging to compare findings with other studies. For example, the continuous mode of enzyme hydrolysis performed by using free or immobilised mode of β-galactosidase enzyme at fixed lactose concentration of 50g/l showed maximum hydrolysis at pH 6.7, enzyme dose 6.5g/l and temperature 36°C (Das et al., 2015).

Table 5. 4 Effect of operational variables on Eh and sCOD concentration in enzymatic pre-treatment

Run No	pH	Temperature (ºC)	Enzyme load (%)	Time (minutes)	Lactose hydrolysis, E_h (%)	sCOD concentration (g/l)
1	6.5	50	0.25	19	62.1	28.31
2	5.5	45	0.35	30.5	80.5	31.05
3	7.5	45	0.35	30.5	52.7	26.8
4	4.5	40	0.45	42	84.2	30.3
5	5.5	45	0.55	30.5	86.21	34.63
6	4.5	50	0.25	19	78.3	29.03

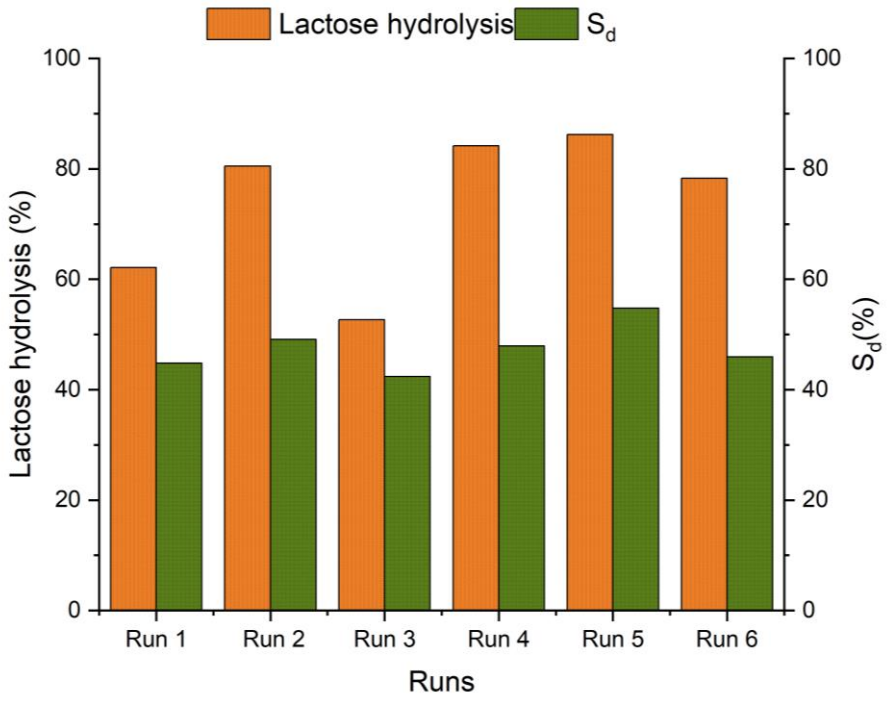

Figure 5. 4 Level of degree solubilisation and lactose hydrolysis at different runs during enzymatic pre-treatment

Higher thermal stability for the enzyme was observed between 35ºC-45ºC. At temperatures > 50ºC the activity of enzyme was reduced. An optimum value of temperature 37ºC was suggested for maximum hydrolysis in this study. Unlike disparities in factor at pH, more studies support this optimum range of temperature for lactose hydrolysis (Haider and

Husain, 2009; Mattar et al., 2010). Warmerdam et al., (2013) observed higher stability of enzyme at higher enzyme concentration. It is important to study the interactions between these parameters to derive at an optimum range of values for attaining maximum lactose hydrolysis. Another influence of temperature can be seen in the change in conformations of enzyme. Due to the temperature sensitivity of protein, its activity starts to decline at about 45°C and nearly ends at 60°C. The amino acids composing the same enzyme's active site are highly conserved across species because substrate binding is so selective. Substrates do not attach to enzyme surfaces with altered conformation upon protein conformation at optimal temperature (Das et al., 2015).

Enzyme activity was directly correlated with both temperature and enzyme concentration. At temperatures above 50°C, enzyme activity declines regardless of enzyme concentration. An increase in the concentration of reducing sugars (galactose and glucose) was used to assess how time affected hydrolysis rates. The initial 30 minutes of hydrolysis showed a 46% increase in the concentration of reducing sugar. The time related enzymatic activity was found to lesser time only. An optimum hydrolysis rate was observed in first 19-30 minutes of hydrolysis at acidic pH range. This study suggests an ideal range for the operating factors, ranging from pH 3.5 to 5.5, time 19 to 30 minutes, enzyme dose 0.25-0.45% and temperature 35 to 45ºC, in order to achieve maximum lactose hydrolysis.

5.2.4 Specific methane yield of cheese whey and septage

Mono-digestion of CW and SP without any pre-treatment was carried out to evaluate their individual methane generation capacity and compared with the theoretical yield. The AD of CW showed fewer methane yield (68.6ml/gVS) compared to that of septage (143.6ml/gVS). Due to the presence of rich anaerobic culture media and more stabilised solids, the degradation of septage by AD was found easier. In past research, a similar range of methane yield levels was also recorded (Lu and Zhang, 2016; Rajagopal et al., 2013). The rapid hydrolysis of whey (acidic in nature) followed by pH reduction due to accumulation of volatile fatty acids might have contributed to lower biogas yield in CW. Additionally, the challenge of hydrolysing complex milk proteins and lipids worsens the predicament. Using eqn (4) and (5), the theoretical methane yield of CW and SP was evaluated. The chemical formula of both whey and septage were obtained as $C_{20.47}H_{4.5}O_{6.04}N_{0.36}$ and $C_{10.18}H_{4.08}O_{4.1}N_{3.98}$ respectively. The theoretical methane yield of whey was much lower than the experimental value, which shows only 11.77%

of degradation rate. Septage has resulted in 51.48% of degradation rate, might be due to the presence of rich anaerobic bacteria and partially stabilised solids.

5.3 Anaerobic co-digestion of pre-treated CWW and septage

The cumulative methane production values evaluated during AD of ozonated, sonicated and enzymatically pre-treated CW with SP at best pre-treatment conditions was shown in figure 5.5. The pre-treatment conditions were chosen based on maximum solubilisation rates and organic matter dissolution rates obtained. They are; ozonation at O_3 concentration of $0.03gO_3/gTS$ for 16 minutes, US at S_E of 510.6J/ml for 18.5 minutes and enzymatic pre-treatment at pH 4.63, time 25.9minutes, enzyme load 0.49% and temperature 40.5ºC. Enzymatic method was found to be most effective in terms of methane generation (432.2ml/gVS), around 70.7% increase compared to non-pre-treated whey. Due to the slow hydrolysis step in the untreated samples, methane percentages increased inside the digester over a longer period of time. Sonicated and ozonised samples showed an increase of 66.3% and 64.18% in methane yield respectively. Enzymatic pre-treatment showed better methane yield mainly due to the increase in concentration of soluble organics, in particular to soluble proteins and monosaccharides- glucose and galactose released after lactose hydrolysis. Enzymatic hydrolysis helped microorganisms to quickly metabolize lactose hydrolysates, glucose, and galactose. Previous studies have reported similar kind of increased biogas yield rates by performing enzymatic hydrolysis of high fat dairy effluents (Cammarota and M.G.Freire, 2001; Domingues et al., 2015b; Gannoun et al., 2008). In a study by Mobarak-Qamsari et al., (2012)enzymatic hydrolysis of dairy wastewater using a lipase produced from *Pseudomonas aeruginosa* enhanced biogas production (4719ml) and attained 90% COD removal efficiency. Volatile solids removal was found to be 58.9% in this study, lower than the removal rates in the ozonised mixtures (72.7%) and sonicated mixtures (68.1%).

Mainardis et al., (2019) reported a study, in which AD of sonicated samples of skimmed and fat whey showed highest methane yield at low power of 40W applied for 10minutes duration. The same study claimed that fat whey exhibited a stronger sonication effect when compared to other types of whey. This kind of non-linear behaviour between applied sonication power and methane yield was earlier reported by Zielinski et al., (2012), they pointed out that larger US energy doesn't lead to higher biogas yield. A higher US power than a longer sonication time appears to be advantageous according to these results.

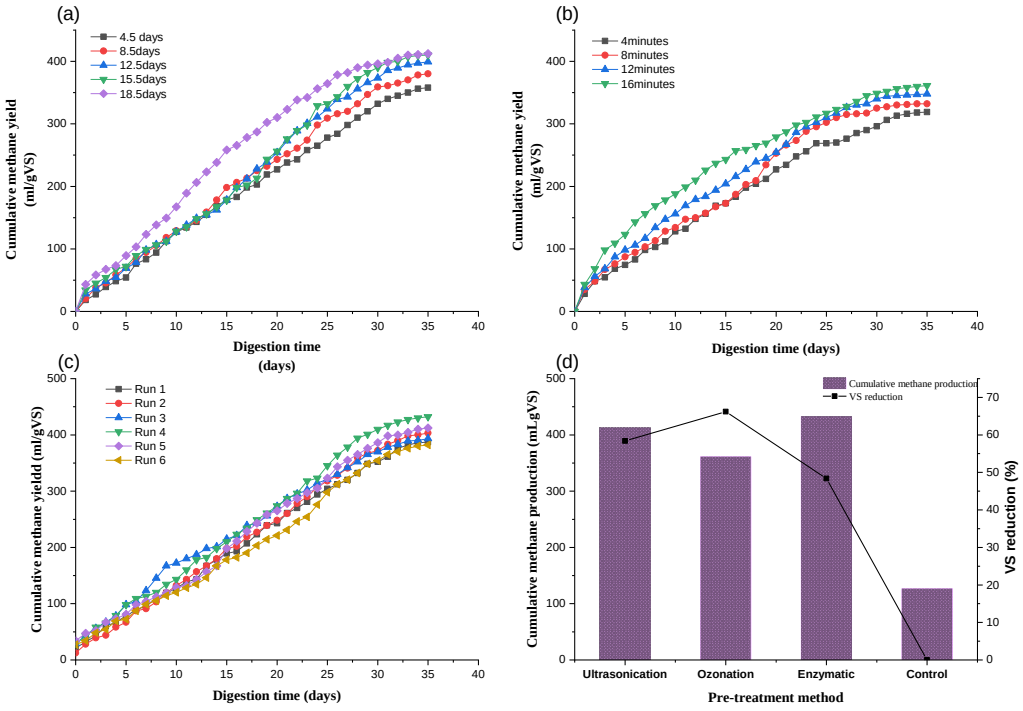

Figure 5. 5 Cumulative methane yield values for; a) sonicated samples, b) Ozonised samples, c) enzymatically hydrolysed samples and, d) Methane yield and VS reduction rates for different pre-treatment methods

The cumulative methane yield from sonicated, ozonised and enzymatically hydrolysed mixtures were 412.4ml/gVS,361.2ml/gVS and 432.3ml/gVS respectively. Although high rates of COD solubilisation were achieved for those samples, methane yield was lower than that obtained with enzymatic pre-treatment. The possible reason for this reduction might be insufficient protein and lactose hydrolysis in sonicated and ozonised samples. Kazimierowicz et al., (2022) demonstrated effect of ultrasound sonication on organic matter degradation in acid whey and found that 15minutes of sonication resulted in 0.203 $dm^3/gCOD_{in}$ of methane generation. In addition, neither biogas production nor organic removal rates changed significantly above 15 minutes of exposure (Kazimierowicz et al., 2022). Similarly, no large increase in methane production values was observed after 15.5 minutes of sonication in this study. The ozonation study carried out by Skripsts et al., (2011) at low dose of 0.037 gO_3/gTS imparted no visible change in methane production during AD of cheese whey. In contrast, this study reported that 12minutes exposure to 0.034gO_3/gTS have resulted in 361.2ml/gVS of methane generation. This might be due to longer exposure time provided. In another study, an increased dose of 0.1 $gO_3/gCOD$ application on sludge, methane production values have increased by factor 1.8 (Chiavola et al., 2019). According to this study, prolonged exposure to low doses of O_3 has resulted in a greater degradation of organic matter. Hence, increasing the

dose of O_3 application was less recommended as it increases the cost of pre-treatment as well as the energy consumption for ozone production.

Among the 3 methods, enzymatic method was found more effective in terms of increased biodegradability and methane productivity. Similar kind of results were obtained for Gannoun et al., (2008), in which a combined lime treatment of whey with acidification using *L. paracasei* caused faster conversion of lactose into lactic acid and helped in attaining better anaerobic degradability. A COD removal rate of 98% was achieved with this combined system at HRTs varying from 2 to 5 days and a COD loading rate of 4 g.COD/Ld, while operating under stable conditions throughout the experiment. The main obstacles to widespread use of biological pre-treatment using enzymes appears to be the cost and lifetime of enzyme activity after addition at greater scales. It is also essential to emphasize that enzymatic pre-treatment is a versatile technology that cannot only be evaluated economically through the lens of a higher biogas yield.

5.4 Kinetic modelling

The experimental and simulated values obtained after performing modified gompertz modelling are presented in Table 5.5. The Gompertz model fitted curve for all pre-treated samples was shown in Figure 5.6. The maximum methane production potential (P_{max}) values were obtained for enzymatically hydrolysed AD samples (473.55-632.39ml/gVS) with regression co-efficient value greater than 0.98 in all cases. The corresponding values of methane production rates (R) ranged between 11.95 to 13.77 ml/gVS.d with lag phase time between 0.10 to 2.23 days. Comparatively lower lag phase times were obtained in case of enzymatically hydrolysed samples. Increased lactose hydrolysis might have helped in reducing the complexity of whey proteins in degradation, which resulted in lower λ values. The λ values obtained for sonicated samples were less than that of ozonation showing positive correlations with experimental values. Similar range of λ values were reported by Chu et al., (2021) during sonolysis combined aerobic pre-treatment of corn straw.

The P_{max}, R and λ values obtained for control group were 188.62 ± 3.06 ml/gVS and 7.98 ±0.32 ml/gVS/d, 2.68days respectively. The kinetic results showed that lag phase time has reduced significantly in all pre-treated mixtures. Λ indicated the time needed for methanogens to adapt to the substrate before producing methane. A longer lag phase time (2.98days) and lower methane value (375.09 ± 8.53ml/gVS) was reported for ozonation, which indicates that the digester's response time to produce was lower compared to other samples. The maximum

deviations from experimental values reported were 4%, which highly proves the suitability of applying Gompertz model for the AD studies conducted. From the kinetic model results, it is apparent that enzymatic pre-treatment of CW helped achieve maximum biogas yield at the shortest digestion time possible.

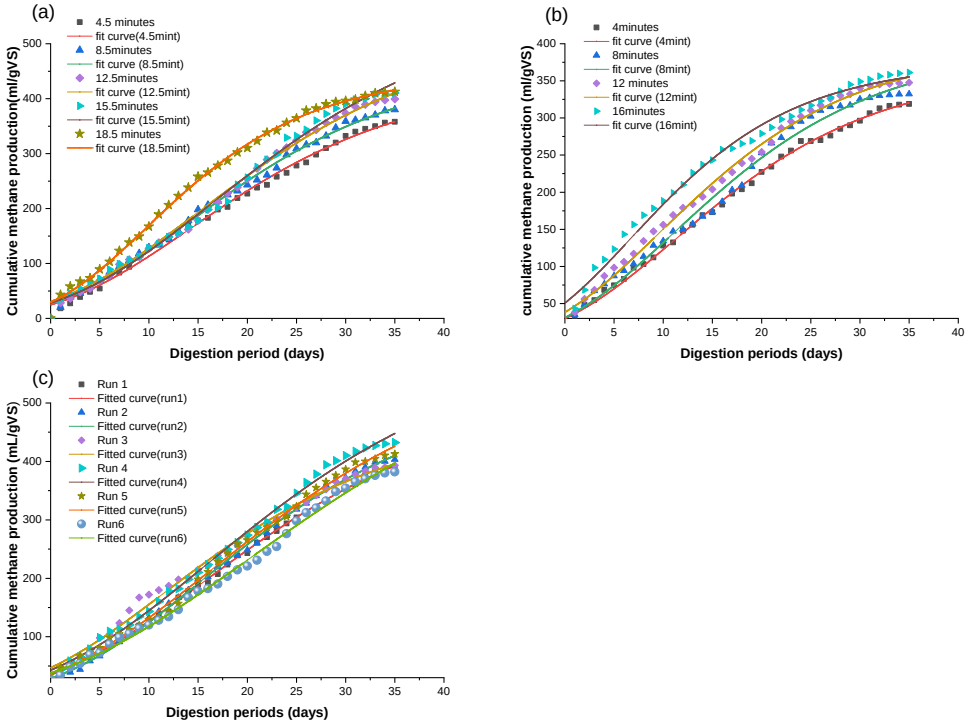

Figure 5. 6 Cumulative methane production values obtained after Gompertz modelling of a) Ultra-sonicated sample, b) ozonated samples, and c) enzymatically hydrolysed samples

Table 5. 5 Values of the kinetic parameters obtained from Gompertz fit modelling analysis of digestion studies

Sonication	**Pre-Treatment Conditions**				
Model Parameters	**4.5minutes**	**8.5minutes**	**12.5minutes**	**15.5minutes**	**18.5minutes**
P^a_{max}	440.26 ± 14.30	467.89 ± 13.36	527.21± 43.59	573.71 ± 26.77	446.80 ± 15.13
R^b	12.20 ± 0.27	13.07 ± 0.27	13.46 ± 0.62	14.07 ± 0.30	17.02 ± 0.31
Λ^c	0.82 ± 0.31	0.64 ± 0.29	0.63 ± 0.69	1.49 ± 0.33	0.03 ± 0.212
AdJ R^2	0.990	0.990	0.974	0.982	0.991
Ozonation	**Pre-Treatment Conditions**				
Model Parameters	**4 minutes**	**8 minutes**	**12 minutes**	**16 minutes**	
P_{max}	367.86 ± 8.48	396.86 ± 14.26	395.61 ± 9.77	375.09 ± 8.53	
R	11.21 ± 0.27	12.22 ± 0.47	12.81 ± 0.41	14.21 ± 0.65	
λ	0.93 ± 0.32	0.80 ± 0.51	1.73 ± 0.42	2.98 ± 0.56	
AdJ R^2	0.994	0.987	0.991	0.984	
Enzymatic	**Pre-Treatment Conditions**				
Model Parameters	**Run 1**	**Run 2**	**Run 3**	**Run 4**	**Run 5**	**Run 6**
P_{max}	518.54 ± 11.8	525.76 ± 13.1	473.55 ± 14.0	611.61 ± 21.8	584.33 ± 21.79	632.39 ± 38.19
R	12.37 ±0.14	13.58 ±0.19	12.70 ±0.32	13.77 ±0.21	13.29 ±0.20	11.95 ±0.16
λ	0.10 ± 0.18	0.91 ± 0.21	2.23 ± 0.37	0.38 ± 0.25	0.21 ± 0.25	0.65 ± 0.32
AdJ R^2	0.998	0.997	0.993	0.996	0.996	0.995

[1] $P_{max:}$ Maximum methane production potential in ml/gVS

[1] R: Methane production rate in ml/gVS/d

[1] Λ: Lag phase time in days

5.5 Optimisation studies for whey lactose hydrolysis

The enzymatic pre-treatment of whey proved to be cost effective, highly efficient, and highly effective in hydrolyzing whey into simpler compounds for enhanced biogas production. The enzyme β-Galactosidase having animal, plant and microbial (yeast, fungi and bacteria) origin, is highly productive in microbial forms. Enzymes derived from fungi (Aspergillus niger and Aspergillus oryzae) and yeasts (Kluyveromyces fragilis and Kluyveromyces lactis) show high commercial potential. However, the activity of these enzymes is greatly affected by pH, temperature, pressure, the concentration of reactants and the presence of metal ions. When optimal operating conditions are developed for an enzyme, enzyme wastage can be reduced, resulting in higher hydrolysis rates and shorter hydrolysis times.

Precisely, two enzymatic hydrolysis experiments were planned; one to optimise lactose hydrolysis and the other to prepare hydrolysed samples to be tested using a BMP set-up. Enzymatic hydrolysis was carried out as per the procedure described by Ghosh et al., (2017). The homogenised whey was pasteurised at 65º C for 20 minutes. Afterwards, enzyme solutions (40 ml) of varying concentrations ranging from 0.18% to 5.8% were added to 60 ml of whey. The desired pH values are adjusted using NaOH and H_2SO_4. The hydrolysis was carried out at a definite time and temperature by incubation in a water bath. After hydrolysis, the enzyme activity was deactivated by incubating the sample at 85º C for 7 minutes. After that, the system is cooled to room temperature. The hydrolysed samples were centrifuged at 3000 rpm for 20 minutes to separate the enzyme from the whey permeate. The unequal volumes of permeate due to pH corrections by NaOH or H_2SO_4 were equalised by adding the required quantity of distilled water. The hydrolysed samples are stored at 4ºC for lactose analysis and quantified for reduced sugar concentrations. The detailed methodology is explained in Figure 3.5 and the degree of lactose hydrolysis is calculated using Eqn (3) mentioned in section 3.3.3. Table 3.2 shows detailed experimental conditions designed for enzymatic hydrolysis of whey lactose using Stat-Ease-Design Expert software (version 6.0.11, Stat-Ease Inc, Minneapolis, MN).

Whey hydrolysis with lactase enzyme was tested under different conditions to get optimum values of essential parameters like enzyme load, operating pH, temperature and time of reaction. Enzyme concentrations were chosen to vary from 0.18% to 0.52%, temperature from 35°C to 55°C, time from 7.5 min to 53.5 min and pH from 3.81 to 7.18. The optimised values of these parameters were later used for enzymatic hydrolysis of whey for the preparation

of whey samples for anaerobic digestion. Table 5.6 shows detailed experimental conditions designed for enzymatic hydrolysis of whey lactose using Stat-Ease-Design Expert software (version 6.0.11, Stat-Ease Inc, Minneapolis, MN) and Figure 5.7 shows the experimental procedure followed for carrying out enzymatic hydrolysis.

Table 5. 6 Independent variables with their symbols and levels proposed for hydrolysis experiment

Independent variables	**Symbol**	**Level**				
		-α	-1	0	+1	+α
pH	X_1	3.81	4.5	5.5	6.5	7.18
Enzyme load (%)	X_2	0.18	0.25	0.35	0.45	0.52
Time course of reaction (min)	X_3	7.5	19	30.5	42	53.5
Temperature (ºC)	X_4	35	40	45	50	55

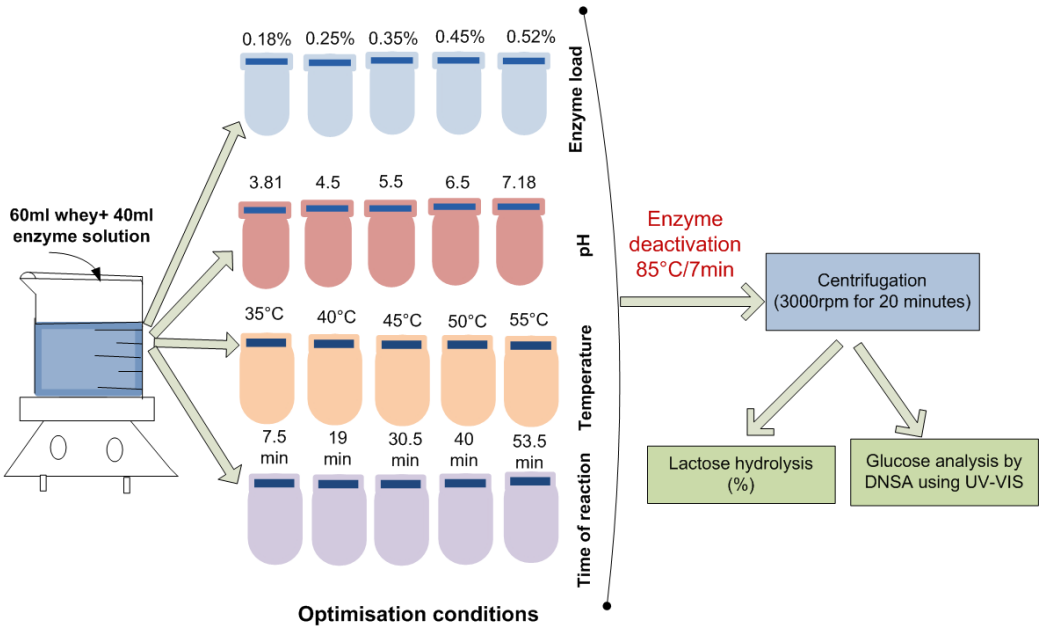

Figure 5. 7 Different steps of enzymatic hydrolysis assays

5.6 Results of optimisation study

Using CCD, the data were analysed based on their combined effect and individual effect on lactose hydrolysis and the following results were obtained.

5.6.1 Effect of independent variables on lactose hydrolysis

The interactions among the selected parameters were evaluated using CCD and optimum values were determined. CCD has assisted in reducing the number of runs to be conducted and making the process more efficient. The experimental conditions for independent variables in each run and corresponding response variable values (experimental and predicted) are shown in Table 5.7.

pH values profoundly influenced the activity of β-galactosidase. The results show that lactose hydrolysis is favoured by acidic conditions and is in agreement with the specifications given by the manufacturer regarding the use of enzyme from A. oryzae. The optimum pH value is obtained in the range of 3.5–5.5. The results obtained are in line with literature studies, i.e. Mlaik et al., (2019) and Czermak et al., (2004) reported a pH range of 4.5–5.0 as the optimal pH for obtaining maximum β-galactosidase activity, while Das et al., (2015) reported a pH range from acidic to neutral (5-7). Beyond 6.5 pH, the activity of β-galactosidase decreases, and the lowest hydrolysis rate is obtained at 7.5 (52.7%) at an intermediate time of 30.3 min and at 45°C. The higher value of lactose hydrolysis was obtained as 84.73% at pH 4.5, 19 min, 0.45% enzyme load, and 40° C temperature. Next higher value of lactose hydrolysis (82.1%) was obtained at a comparatively lower pH (3.5), lower enzyme load (0.35%) and a temperature of 45°C.

The comparison with other literature is difficult since substrate composition, reaction mode (batch or continuous), and free or immobilised enzyme usage vary between the studies. For example, Das et al., (2015) conducted a study on free and immobilised mode of β-galactosidase hydrolysis of lactose with fixed initial lactose concentration (50 g/l). (Das et al., 2015) reported an optimum pH value of 6.7, temperature of 36.5°C, and enzyme concentration of 6.7 mg/L, which differ from those reported here. Low pH and high temperature seem to favour lactose hydrolysis in our study. The initial lactose concentration and the mode of enzyme usage can affect hydrolysis rates. The kinetic behaviour of free and immobilised enzymes can differ due to conformational and diffusional effects (Ladero et al., 2003). Inhibitions due to high lactose concentration were reported in a study by (Ladero et al., 2000). It was found that

immobilised enzyme stabilised at low lactose concentrations was less stable than free enzyme at higher concentrations.

The thermal stability of β-galactosidase was assessed at temperatures ranging from 35°C to 55°C. A higher degree of enzyme activity was observed between 35 and 45 °C, but at 50° C and 55° C, the activity was decreased. Mattar et al., (2010) reported that optimum activity of β-galactosidase was observed around 37°C. Similarly, Haider and Husain, (2009) evaluated whey hydrolysis using β-galactosidase obtained 70% lactose hydrolysis at 37°C. 60% of the enzyme activity is lost when the temperature reached 50°C. Although the rate of hydrolysis increases as the reactor temperature increases, the deactivation rate also increases. The reaction temperatures can reach higher values than the enzyme's stable ranges in aqueous solutions or be equal to or close to their optimal values at high substrate concentrations (Song et al., 2011). Free enzymes are less resistant to temperature than immobilised ones (Peterson et al., 1989). In this study, the denaturation of enzyme is observed at a temperature range of 45-50°C. Higher thermal stability was observed for β-galactosidase at high substrate concentration in a study by Warmerdam et al., (2013)

Table 5. 7 Full experimental design conditions and response values

Standard order	Run order	Independent variables				Lactose hydrolysis(%)	
		pH (A)	Enzyme load (B,%)	Time (C,min)	Temperature (D,ºC)	Experimental value	Predicted value
10	1	6.5	0.25	19	50	62.1	62.92
26	2	5.5	0.35	30.5	45	80.5	82.71
4	3	6.5	0.45	19	40	69.9	71.01
18	4	7.5	0.35	30.5	45	52.7	50.76
12	5	6.5	0.45	19	50	63	64.14
7	6	4.5	0.45	42	40	84.2	85.41
1	7	4.5	0.25	19	40	79	79.56
8	8	6.5	0.45	42	40	69.9	71.64
11	9	4.5	0.45	19	50	79.8	81.46
29	10	5.5	0.35	30.5	45	81	82.71
20	11	5.5	0.55	30.5	45	86.21	82.49
28	12	5.5	0.35	30.5	45	82.45	82.71
15	13	4.5	0.45	42	50	81.2	82.24
22	14	5.5	0.35	53.5	45	80	78.06
17	15	3.5	0.35	30.5	45	82.1	80.09
19	16	5.5	0.15	30.5	45	75	74.56
25	17	5.5	0.35	30.5	45	84.4	82.71

5	18	4.5	0.25	42	40	77.9	78.69
14	19	6.5	0.25	42	50	64.2	64.51
30	20	5.5	0.35	30.5	45	84	82.71
27	21	5.5	0.35	30.5	45	83.9	82.71
16	22	6.5	0.45	42	50	64.6	66.07
23	23	5.5	0.35	30.5	35	80	77.84
24	24	5.5	0.35	30.5	55	73	71.21
21	25	5.5	0.35	7.5	45	79	76.99
13	26	4.5	0.25	42	50	78	78.92
6	27	6.5	0.25	42	40	66.3	66.67
2	28	6.5	0.25	19	40	65.5	66.39
9	29	4.5	0.25	19	50	78.3	78.49
3	30	4.5	0.45	19	40	84.32	85.93

A higher enzyme concentration has a greater impact on lactose hydrolysis rate at a specific range of maximum hydrolysis efficiency (86.21%). Additionally, authors Horner et al., (2011) and Akgül et al., (2012) also observed that enzyme concentration directly impacts lactose hydrolysis; a fourfold increase in enzyme concentration has doubled the concentration of hydrolysed lactose. Figure 5.7 (a, b, c) illustrates the interactions between the independent variables and response variables. 3- D surface plots were generated by using the response surface method. Figure 5.7 (a) shows change in lactose hydrolysis rates concerning temperature and pH. Initially, an increase in temperature caused an increase in lactose hydrolysis rates. Temperature changes the conformation of the enzyme. This protein has a sensitive temperature profile, due to which the activity decreases after 45°C and almost stops the activity at approximately 60°C.

As substrate binding is so specific, the amino acids forming the active site for the same enzyme are highly conserved from one species to another. Upon protein conformation at optimum temperature, substrates cannot adhere to enzyme surfaces that have been altered (Das et al., 2015). Denaturation of the enzyme at 45°C may have occurred following the enzyme conformation. As a result, the lactose hydrolysis rate didn't differ much beyond 45°C. Similarly, with an increase in pH, lactose hydrolysis rates are also increased. Beyond 6.5, the activity of enzyme gets reduced. Figure 5.7(a) shows maximum hydrolysis at pH 4.5 and temperature 40°C.

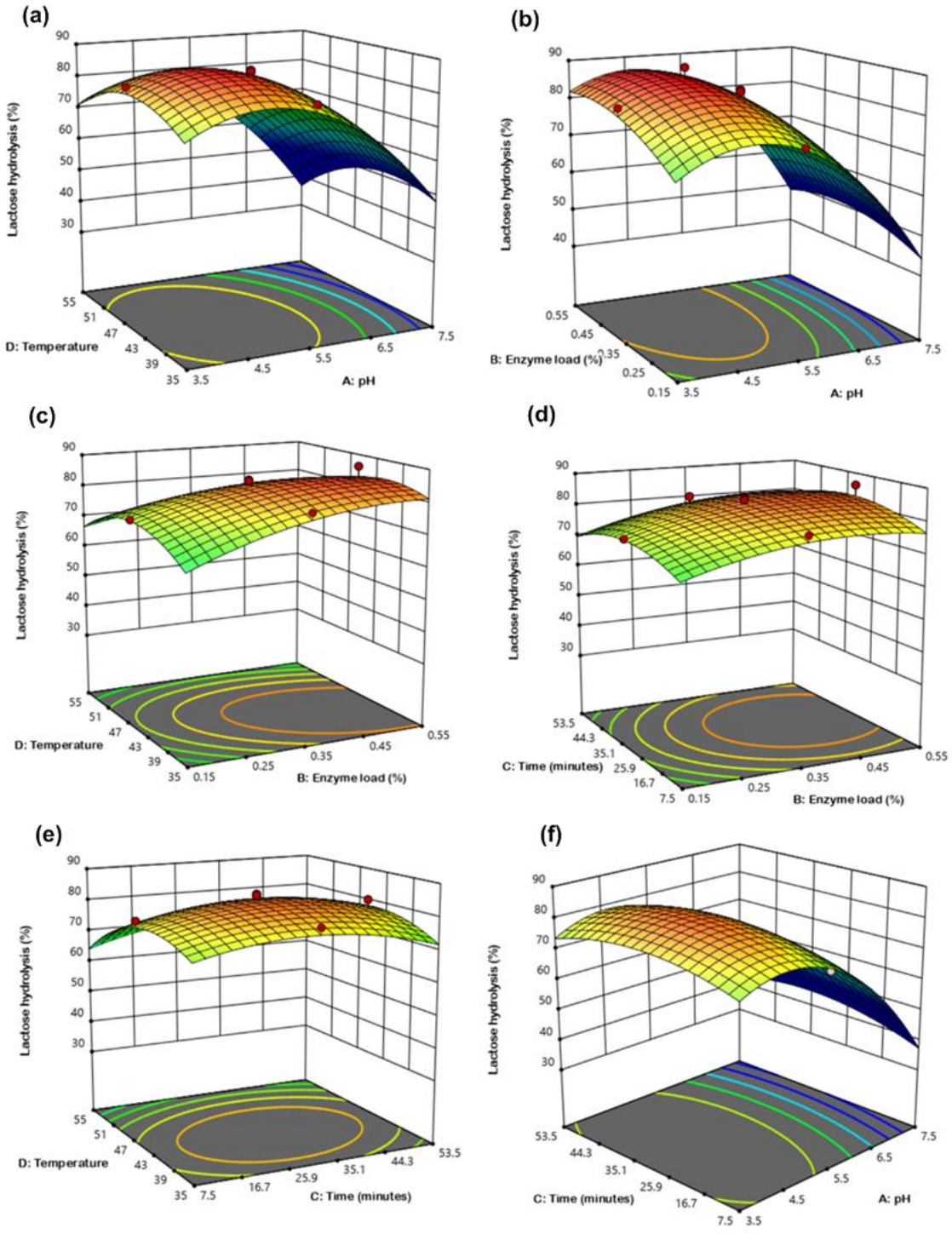

Figure 5. 8 D Response surface model graphs showing interactions between different variables (a) effect of temperature and pH on lactose hydrolysis (b) effect of enzyme load and pH on lactose hydrolysis (c) effect of temperature and enzyme load on lactose hydrolysis (d) effect of time and enzyme load on lactose hydrolysis (e) effect of temperature and time on lactose hydrolysis (f) effect of time and pH on lactose hydrolysis.

Figure 5.7 (b) shows the effect of temperature and enzyme load on lactose hydrolysis rates. As enzyme concentration increases, lactose hydrolysis is also increased to a particular

temperature (45°C). By increasing the enzyme concentration from 0.25% to 0.45% at 40°C, the hydrolysis rates increased from 66.3% to 84.32%. At 45°C, the hydrolysis rate increases by approximately 37.26% when the enzyme load is increased from 0.35% to 0.55%. Demirhan et al., (2010) reported a similar rise in residual lactose concentration (66.3–85.8%) and enzyme stability when enzyme concentration is raised from 0.5 to 3.0 mL/L. Beyond 45°C, no noticeable changes were observed in the hydrolysis rate. A possible reason for this might be due to the formation of the enzyme-lactose complex when enzyme concentration is increased beyond 0.45% at high temperature (>45°C). The formation of enzyme-protein complexes sometimes inhibits the stabilisation of multimeric enzymes, as the dissociation of subunits results in the inactivation of enzymes (Bhaskara and Srinivasan, 2011). As in Figure 5.7(c), enzyme activity was higher at higher enzyme concentrations and temperatures. Both temperature and enzyme concentration is directly proportional to enzyme activity. Regardless of enzyme concentration, enzyme activity is less at temperatures above 50°C.

Figure 5.7(d, e, f) shows that time has a lesser effect on lactose hydrolysis than the other 3 parameters; enzyme load, temperature and pH. The impact of time on hydrolysis rates was evaluated in terms of an increase in reducing sugars concentration (glucose & galactose) and a change in sCOD. Within the first 30 min of hydrolysis, the reducing sugar concentration increased by 46% and the sCOD release increased by 28%. At pH > 6.5 and a temperature of 50°C, the hydrolysis rates did not noticeably increase when the time increased. However, reducing sugar concentration rose to 54% after 53 min, with no change in sCOD was noticed. Therefore, time-related increases in hydrolysis rates occurred only to a lesser extent. The optimum enzyme activity was observed for 19–30.5 min at an acidic range of pH and temperature between 35°C and 45°C.

5.6.2 Fitting the model

Response surface methodology has been applied to evaluate the interactions between independent variables and to build an appropriate model using statistical, theoretical and mathematical techniques (Homayoonfal et al., 2015). The multiple regression analysis was done to evaluate the optimum values of independent variables. The fit summary of the model obtained has shown that the quadratic model is the best fit model. The estimated regression model and regression coefficient (R^2) obtained through ANOVA are shown in Table 5.9. The coefficient of the quadratic model equation was evaluated from the experimental data to predict

the response variable values. The obtained regression equation for lactose hydrolysis (%) is given in equation (5.1).

$$Lactose\ hydrolysis\ = -240.08 + 46.35A + 191.49B + 0.20C + 8.11D - 4.38AB + 0.02AC - 0.12AD + 0.07BC - 1.70BD + 0.01CD - 4.32A^2 - 104.56B^2 - 0.01C^2 - 0.08D^2 \quad \textbf{Eqn (5.1)}$$

The results of statistical analysis revealed that quadratic model fits the experimental data well with a R^2 value of 0.96. The model F value 31.98 implies that the model is significant. Accordingly, the lack of fit resulted in insignificant errors in terms of pure error, indicating that our model is statistically accurate. The p-values for pH, temperature, and enzyme load are <0.01 or <0.05, which indicates they are significant. ANOVA reveals that pH (<0.0001) is the most significant factor followed by enzyme load (<0.0004) and temperature (<0.0018). The quadratic terms are also equally significant as all are having values less than 0.01 or <0.05. A higher F value and a lower p value suggest stronger impact of independent variables on the response variable. An experiment with a low CV value is considered to be highly reliable. IN the present study a CV of 2.83 shows that experiment is reliable. Adequate precision stands for the signal to noise ratio. For adequate precision, a value >4 is desirable, and here it is 23.22, indicating the signal is adequate. The normal plot of residuals shown in Figure 5.8 indicates that residual values follow a straight-line path. Figure 5.8 shows the plot of residual vs run, where residual values of each run were found lying on both sides of the centre line, with run no 11 showing a higher residual value.

Table 5. 8 Analysis of variance and regression coefficients for the quadratic model obtained from experimental data

Source	**DF**	**Lactose hydrolysis (%)**		
		Coefficient	p-Value	F- Value
Model	14	82.71	<0.0001	31.98
Linear				
A-pH	1	-7.33	< 0.0001	281.41
B-Enzyme load	1	1.98	0.0004	20.60
C-Time	1	0.2658	0.5523	0.3697
D-Temperature	1	-1.66	0.0018	14.40
Interaction				
AB	1	-0.4387	0.4254	0.6714
AC	1	0.2888	0.5976	0.2908
AD	1	-0.5987	0.2811	1.25

BC	1	0.0863	0.8742	0.0259
BD	1	-0.8512	0.1327	2.53
CD	1	0.3263	0.5515	0.3712
Quadratic				
A^2	1	-4.32	< 0.0001	111.61
B^2	1	-1.05	0.0219	6.54
C^2	1	-1.30	0.0064	10.04
D^2	1	-2.05	0.0002	25.02
Residual	15			
Lack of Fit	10	Not significant	0.2315	1.99
Pure Error	5			
Total	29			

(A)

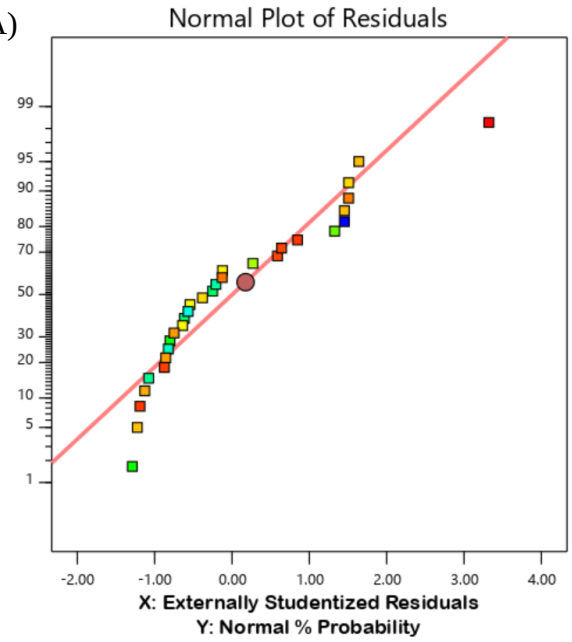

Figure 5. 9 Normal plot of residuals

(B

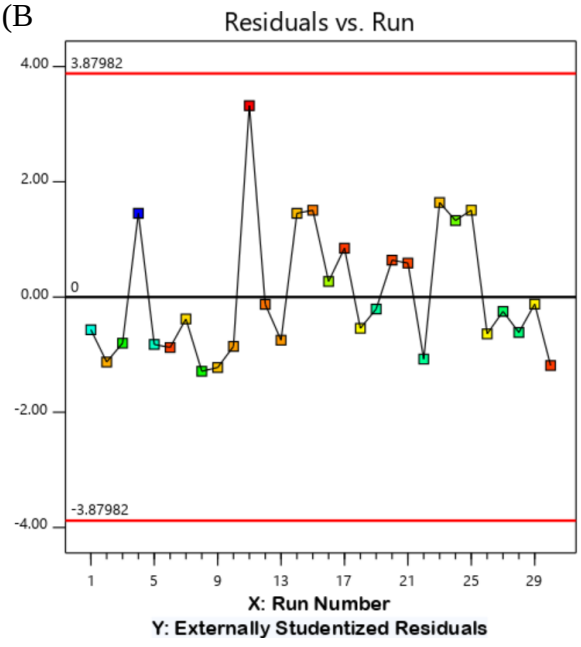

Figure 5. 10 Plot of residual vs run

5.6.3 Optimisation of parameters using desirability analysis

After analysing the effect of independent variables on the response variable, numerical optimisation was carried out using the desirability function. The criteria selected for obtaining maximum lactose hydrolysis were particular range levels of pH, temperature, enzyme load and time. It was considered optimal to set the parameter setting near 1 for the desirability value, and the geometric mean based on all responses is the simultaneous objective function. The objective was formulated to obtain maximum lactose hydrolysis. 95 solutions were obtained, and the adequate one was selected based on the maximum desirability value. The ramp plots (Figure 5.9) show the optimum input values for parameters and predicted output values: pH 4.63, enzyme load 0.49%, reaction time 25.96 min and temperature 40.47°C to obtain maximum lactose hydrolysis of 87.44%.

Figure 5.10 shows the desirability plot of the numerical estimation. A desirability value is a function that shows how closely the upper and lower limits are set to the actual optimum value. The desirability function determines the experimental conditions (factor levels) that will yield the optimal value for all variables evaluated simultaneously (Vera et al., 2014). Numerical optimisation aims to maximise the desirability function at a specific point. In the first step, individual desirability values are created for all factors using filled models and optimisation criteria. The desirability value always ranges from 0 to 1; 0 is an undesirable response, and 1 is the most desirable response. The optimisation procedure can also incorporate factor levels to prioritise certain suitable conditions within the experimental region. In this study, the overall desirability value of the combined objective is 0.94, which is a good measure because it is close to 1.

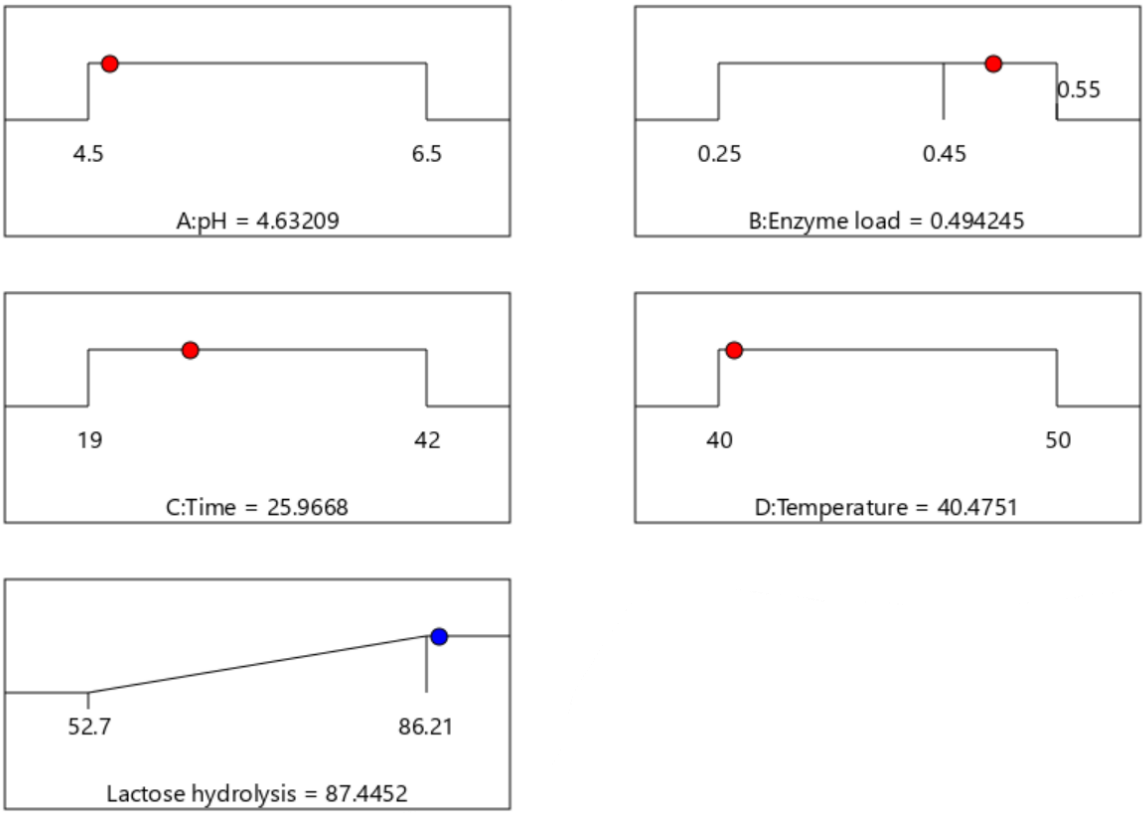

Figure 5. 11 Ramp Plot for desirability analysis

5.6.4 Validation of the model

The hydrolysed samples prepared under optimised parameter conditions were used for performing BMP tests. The optimised conditions were used to check the suitability of the model to predict the lactose hydrolysis rates. 86.21% of lactose was hydrolysed, which is not far off the value predicted (87.44%). Reducing sugar concentration and sCOD were obtained as 21.34 ± 3.1 g/l and 65.78 ± 2.76 g/l respectively. Change in sCOD concentration was found about 24.6%.

FTIR analysis was performed to compare the bond cleavages in raw and enzymatically hydrolysed whey (Figure 5.11 (a, b)). The highest peak was obtained at 3311.11 cm^{-1}, representing the stretching vibration of hydrogen bond groups; C-H and N-H bonds. The peak at 2679.73 cm^{-1} for raw whey and 2696.18 cm^{-1} for hydrolysed whey depicts the C-H stretching vibration in aliphatic compounds. The bands identified at 1920 cm^{-1} and 1758.38 cm−1 corresponds to C=O bonds in carboxylic acids (Manrique and Lajolo, 2002). Besides, for hydrolysed whey, the peak at 1752.21 cm^{-1} confirms the presence of peptide bond cleavage in protein (Ben Yahmed et al., 2017). The peak at 1124.95 cm^{-1} indicates the C-O stretching of carbohydrates or other polysaccharides (Li et al., 2013). A marked reduction in intensity was

noted at 3333.73 cm^{-1}, representing the C-H stretching and is caused due to enzymatic hydrolysis of lactose and other sugars in whey.

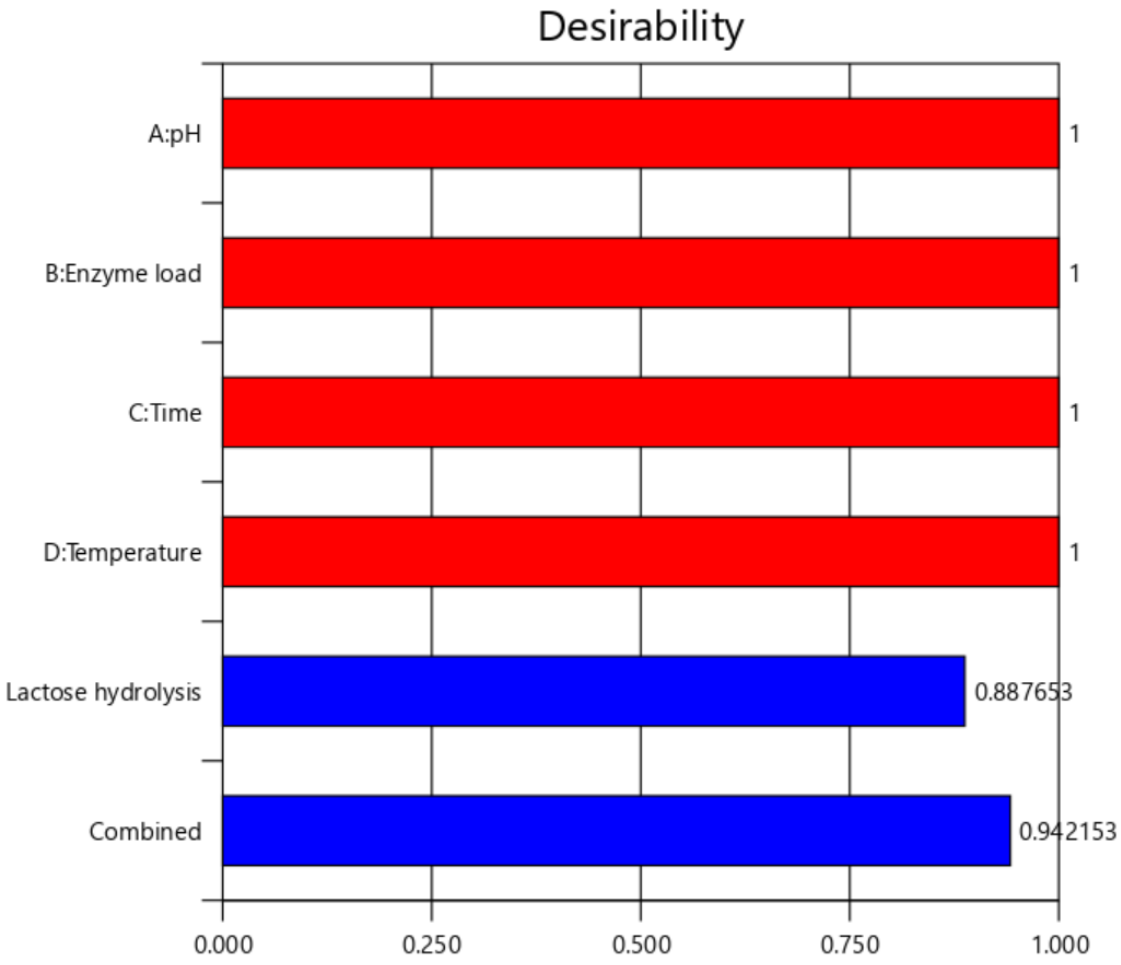

Figure 5. 12 Bar graph for Desirability analysis

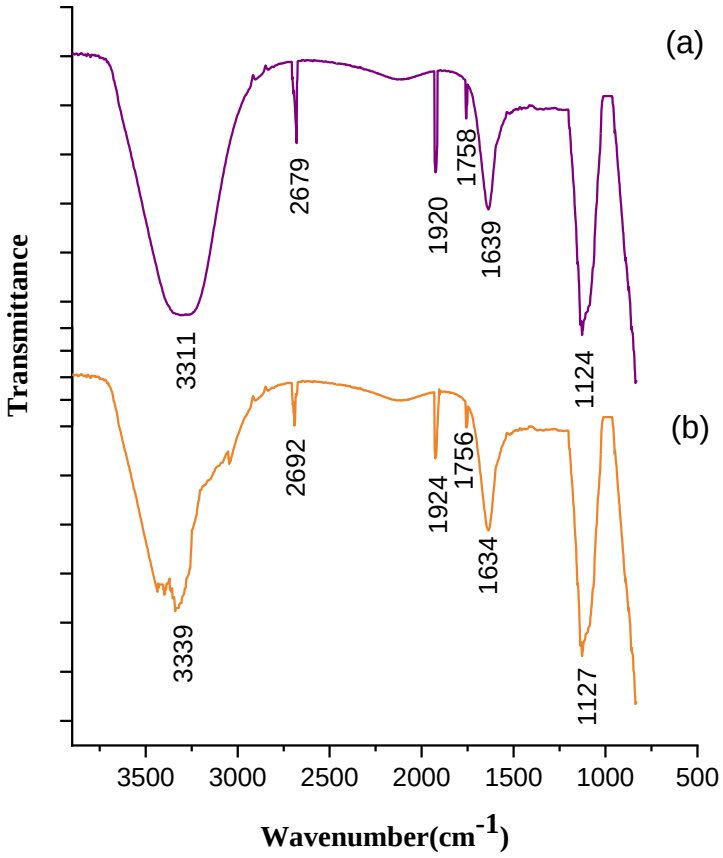

Figure 5. 13 FTIR spectra of (a) Raw whey (b) Enzymatically hydrolysed whey

5.6.5 Biogas production

Daily and cumulative methane production values of AD of raw CW, pre-treated whey and control are shown in Figure 5.12 (A, B). The biogas production from raw and pre-treated whey begins on the first day (Figure 5.12 (A)). An initial decline in biogas production was observed for raw whey from day 3 to 10. This shows the organic complexity of whey. Daily methane levels reached the maximum on the 13th day (35 mL/gVS). Enzymatically pre-treated whey showed a rapid rise in methane values from the 7th day onwards. Compared to raw whey, the hydrolysis rate was higher in pre-treated whey. Other authors reported similar low values of methane generated during mono-digestion of whey (Dubois et al., 1951; Malaspina et al., 1996). The possible reduction in anaerobic biodegradability of whey might be due to unbalanced pH, buffering capacity and accumulation of VFA followed by reduced activity of methanogens. For raw whey, methane production dropped from the 19th day and continued to release less methane till the end day of digestion. The cumulative methane yield resulting from the AD of untreated raw whey was around 132 ml/gVS (Figure 6.6(B)).

In the case of hydrolysed whey, a sharp rise in methane production was noticed after 6th day, and the maximum daily methane was reported as 95 mL/gVS (Figure 5.13(A)). This shows that the biodegradation rates in hydrolysed whey are faster than in raw whey. The cumulative methane yield was reported as 503 mL/gVS, which was about 3.6 times higher than that from raw whey (132 mL/gVS). A similar high methane value was reported by Cammarota and M.G.Freire, (2001), who carried out AD studies on fat-rich dairy wastewater, which is enzymatically pre-treated. A reduction in methane production was found once the peak value was reached, which might be caused due to possible ammonia inhibition. Total ammonia nitrogen was found as 1560 mg/l. Total volatile fatty acids and sCOD concentration before and after AD were presented in Table 5.9. An increase of 24.6% in sCOD concentration was observed after hydrolysis. AD of pre-hydrolysed whey with an initial sCOD concentration of 65.78 mg/l was reduced to 24.8 mg/l. Hence, around a 62.37% reduction in sCOD levels was obtained. The rate of VFA production in hydrolysed whey was found to be less than that of raw whey. Due to its low pH, CW encounters problems like volatile fatty acid accumulation during AD. Using hydrolysed whey in anaerobic digestion has solved this problem to some extent. Enzymatic pre-treated helped whey protein to biodegrade quickly. Anaerobic digesters' overall performance depends on a deep understanding of microbial dynamics and metabolic pathways (Kumar et al., 2022; Qin et al., 2021). Proper syntrophic relations between acetogens and methanogens will help recover more resources like valuable VFAs from products of AD (Lakshmi et al., 2021; Liu et al., 2021; Wainaina et al., 2019).

Table 5. 9 sCOD and VFA values of raw and hydrolysed whey before and after AD

Samples	**VFA (g/l)**		**sCOD (g/l)**	
	Initial	**Final**	**Initial**	**Final**
Raw whey	1.98 ± 0.52	4.39 ± 0.25	52.8 ± 2.80	68.2 ± 1.32
Pre-treated whey	1.57 ± 0.31	0.38 ± 0.01	65.78 ± 2.76	24.8 ± 1.80

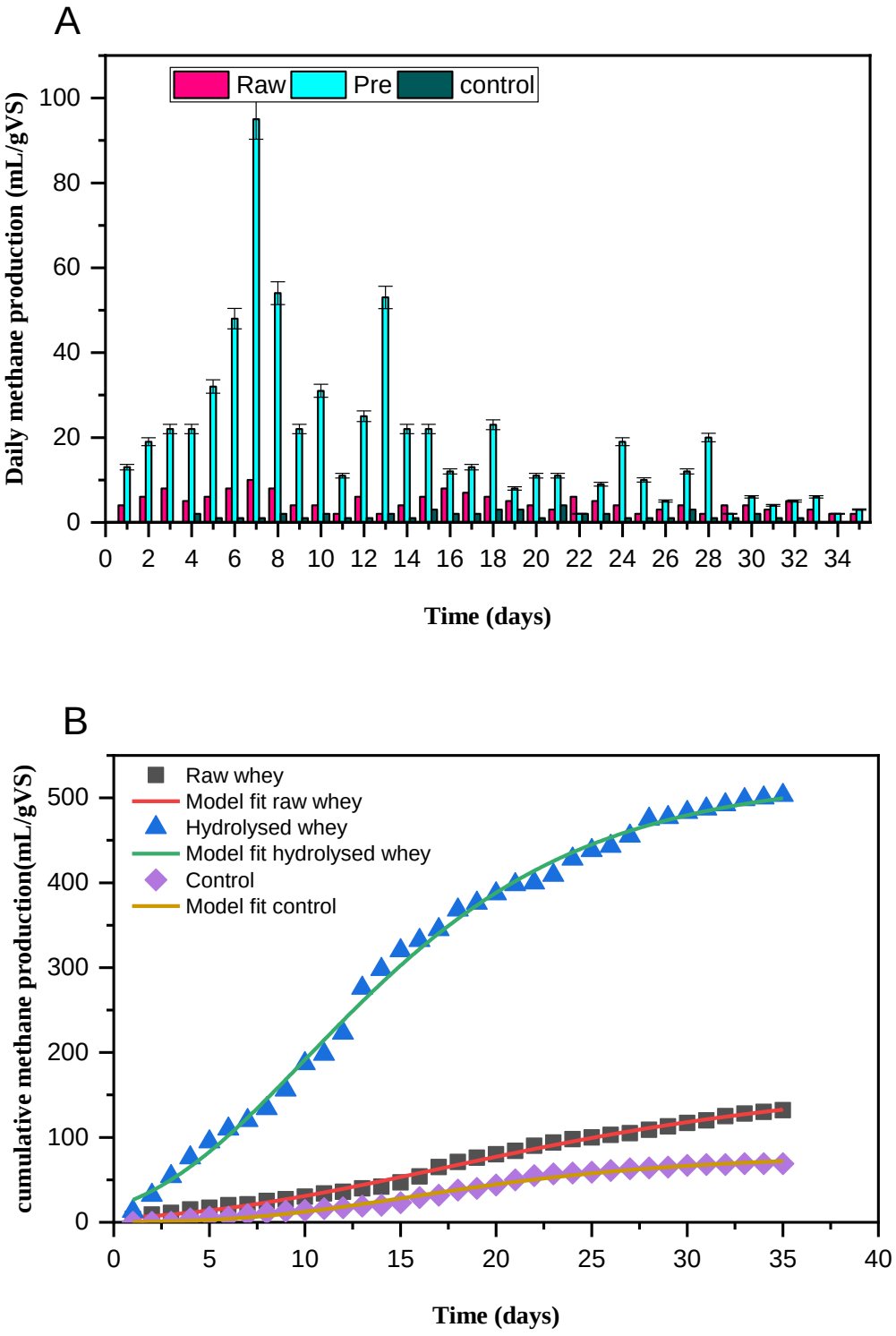

Figure 5. 14 (A) Daily methane production curve for raw whey, hydrolysed whey and control (B) Experimental and simulated curve for cumulative methane production values obtained for raw whey, hydrolysed whey and control.

5.6.6 Kinetic modelling

The effectiveness of enzyme hydrolysis of whey lactose was further assessed using the Modified Gompertz model . The model parameters include maximum cumulative methane production (P_{max}), Rate of methane production potential (R), and Lag phase times (λ). The cumulative methane production values are fitted well with the Gompertz equation (Figure 5.12 (B)), and the results are shown in Table 5.11. The maximum cumulative methane concentration for raw whey was 167.64 mL/gVS, whereas that of hydrolysed whey was 524.70 mL/gVS, three times greater than raw whey. It can be noticed that the lag phase time has reduced considerably for hydrolysed whey (Table 5.10). This can be validated by the high methane production potential rate obtained for hydrolysed whey. The R-value has increased from 4.85 ml/ gVS.dfor raw whey to 23.32 ml/gVS.d for hydrolysed whey.

Table 5. 10 Value of kinetic parameters determined using Gompertz model

Substrate	**P^a_{max} ($mLCH_4/gVS$)**	**R^b ($mLCH_4/gVSd$)**	**Λ^c (days)**	**R^{2d}**
Raw whey	167.64	4.85	3.96	0.996
Pre-treated whey	524.70	23.32	1.79	0.995
Control	77.07	3.54	6.50	0.986

P^a_{max} : Maximum cumulative methane production

R^b : Rate of methane production potential

Λ^c : Lag phase time

R^{2d} : Regression coefficient

5.7 Energy and cost analysis

Table 5.11 shows the evaluated input and output energy values of the digestion system examined after application of pre-treatment methods. Based on these values, the net energy gain (NEG) obtained for each method used were given in Table 5.12. Although maximum methane yield (412ml/gVS) was obtained at 18.5minutes of sonication application, the energy factor (0.99) reported was lesser. The NEG value decreased as time of operation increased in case of US. Maximum E_r value of 2.39 was reported at 4.5minutes of US application. The NEG increase and specific NEG were around 234.01% and 8.89KJ/gTS for US. Even though COD solubilisation and organic matter solubility were higher at longer application time, the NEG was higher at lower duration. This result shows that US was more beneficial to operate at lower

time range in terms of energy conservation. Methane production might be enhanced even further by longer hydraulic retention time and lower organic loading rate. At a loading rate of 1500 gVS/m^3day, Rasapoor et al., (2019) reported 13606KJ of NEG after pre-treating organic solid waste using US. The E_r value obtained for ozonation was around 2.26 slightly lower than that of US.

Ozonator and sonicator systems must be compared against the total cost for implementing them in order to determine their actual convenience. The specific cost required for ozonator provided by supplier (Aquazone) and sonicator (Ayyappa Scientific Sales).

- Cost of ozonator and O_2 concentrator equipment – 1420.73$
- Cost of sonicator, sound enclosure and probes - 3352.48$
- Cost of energy required – 0.074$/kwh

A cost analysis of ozone production was performed based on the amount of energy (kWh) and oxygen (kg) consumed in generating the ozone. Similarly, cost requirement for sonicator was calculated using energy consumption (kwh) and total operation hours.

Table 5. 11 Input and output energy values for each pre-treatment method

Sonication					Ozonation				Enzymatic			
Operating conditions	**E_{US} (KJ/KgTS)**	**E^{a}_{inp} (KJ)**	**E_{out} (KJ)**	**E^{b}_{r}**	**Operating conditions**	**E_{inp} (KJ)**	**E_{out} (KJ)**	**E_r**	**Operating conditions[c]**	**E_{inp} (KJ)**	**E_{out} (KJ)**	**E_r**
4.5 min	2130.3	3745.2	8962.3	2.39	4 min	3531	7997.8	2.26	(6.5,50,0.25%,19min)	1614.8	9527.3	5.89
8.5 min	4044.8	5659.6	9530.2	1.68	8 min	5018.1	8331.4	1.66	(5.5,45,0.35%,30.5min)	1614.8	10611.9	6.57
12.5min	6000	7614.8	10014.2	1.31	12 min	6232.4	8720.1	1.39	(7.5,45,0.35%,30.5min)	1614.8	9769.1	6.04
15.5 min	7505.2	9120	10287.6	1.12	16 min	7044.8	9058.7	1.28	(4.5,40,0.45%,42min)	1614.8	10255.4	6.35
18.5min	8773.2	10387	10342.8	0.99					(5.5,45,0.55%,30.5min)	1614.8	10781.5	6.67
-	-	-	-	-	-				(4.5,50,0.25%,19min)	1614.8	10001	6.19

[a]E_{inp} was calculated using eqn (8) considering E_{hum} as 270KJ/hr and E_{heat} calculated using eqn (9)

[b]E_r was calculated using eqn (7)

[c]Operating conditions were in order of pH, temperature, enzyme load and time of operation

Table 5. 12 Energy performance and net energy gain values for different pre-treatment methods

Energy parameter	Ultra-sonication	Ozonation	Enzymatic	Without any pre-treatment
Maximum energy ratio, Er	2.39	2.26	6.67	1.96
Increase in E_r (%)	21.94%	15.30%	240.36%	-
Net energy gain (KJ)	5217.1	4466.8	9166.7	-
Net energy gain without pre-treatment (KJ)	-	-	-	1552.66
Increase in NEG due to pre-treatment (%)	236.01%	187.68%	490.38%	-
Specific NEG with pre-treatment (KJ/KgTS)	8.98	7.66	15.80	
Specific NEG without pre-treatment (KJ/KgTS)	-	-	-	2.67
Net energy benefit[a]	6.31	4.99	13.13	-

[a] Net energy benefit is found as the difference between NEG of pre-treated and raw samples.

Compared to ozonation and US, enzymatic pre-treatment showed around 490.38% increase in NEG with an E_r value of 6.67. The input energy required was 131.4% lesser than ozonation and US. Although energy gain is high, the cost of enzyme is a critical factor. In this case, the cost enzyme required varied from 0.27 to 0.61$/ml of sample. It follows that enzymatic pre-treatment can be profitable only if enzymes can be synthesized. It is anticipated that enzyme costs will decrease because of advances in technology and the use of cheaper substrates (Parawira, 2012). Due to the lack of the need for uncontaminated enzymes, waste-based enzymes are a feasible option for enhancing biogas production. Sóti et al., (2018) reported around 60% cost reduction after using immobilised enzymes for hydrolysing sugars in lignocellulosic biomass. Other parameters that affect cost calculations include the operational conditions for pre-treatment, the energy conversion units, and methane market prices. This study has shown that enzymatic pre-treatment was energy-efficient, but enzyme production had a high cost. In order to resolve this issue, new technological advances in enzyme engineering, production of novel enzymes, and the use of immobilised enzymes were suggested.

5.8 Major findings of the study

The major observations made from the study are as follows;

- In sonication, increasing specific energy from 2130-38773.2 kJ/kgTS led to a 77.15% increase in sCOD solubilisation, as well as a 3.26-fold increase in methane yield.
- In case of sonication, maximum lactose hydrolysis was obtained at 9000kJ/kg TS which is around 46.03%.
- In case of ozonation, maximum sCOD solubilisation of 63.2% and methane yield 361.2mlCH_4/gVS were reported at lower O_3 dose and longer exposure time.
- Enzymatic hydrolysis by β-galactosidase showed a maximum lactose hydrolysis of 86.21% at optimised conditions; 4.63 pH, 26 minutes time, 0.49% enzyme dose and 40.5ºC temperature.
- The higher degree of lactose hydrolysis (85.1%) obtained showed that enzyme, β-galactosidase can be used as a biological agent for accelerating the hydrolysis step in AD of whey.
- The optimum conditions for enzymatic hydrolysis obtained as per desirability function analysis were 4.63 pH, 40ºC temperature, 25.96 minutes reaction time and 0.49% enzyme concentration.

- The validation experiments conducted showed that error obtained was only 2.68%.
- The bio-methane yield from pre-treated whey was 3.6 times higher than that of raw whey.
- A Net energy benefit of 13.13kJ/kgTS was obtained for enzymatic method compared to sonication (6.31 kJ/kgTS) and ozonation (4.99 kJ/kgTS).
- The order of increase in sCOD solubilisation rates was US>Ozonation>enzymatic, while methane production rates increased Enzymatic>US>Ozonation

5.9 Conclusion

This study assessed the effect of 3 different pre-treatment technologies-US, ozonation and enzymatic methods on reducing the complexity of whey proteins and enhancing the biogas production in AD of whey co-digested with septage. Results showed that as specific energy increased from 2130.3-8773.2 kJ/kgTS, sCOD solubilisation and methane yield increased by 77.15% and 3.26-fold respectively. In case of ozonation, maximum sCOD solubilisation of 63.2% and methane yield 361.2mlCH_4/gVS were reported at lower O_3 dose and longer exposure time. Enzymatic hydrolysis by β-galactosidase showed a maximum lactose hydrolysis of 86.21% at optimised conditions; 4.63 pH, 26 minutes time, 0.49% enzyme dose and 40.5ºC temperature. The order of increases in sCOD solubilisation rates was US>Ozonation>enzymatic, while methane production rates increased in order of enzymatic>US>Ozonation.

Enzymatic pre-treatment was used to reduce complexity and increase biodegradability in the anaerobic digestion of whey lactose for improved biogas production. The results have shown that it can achieve >95% of lactose hydrolysis when appropriate pH, temperature, and concentration combinations are used. The higher degree of lactose hydrolysis (86.21%) obtained in this study showed that the enzyme β-galactosidase could be used as a biological agent for accelerating the hydrolysis step in AD of whey. The optimum conditions for enzymatic hydrolysis were 4.63 pH, 40.47°C temperature, 25.96 min reaction time and 0.49% enzyme concentration. The change in sCOD levels at these optimum conditions was around 24.6%. Compared to raw whey, hydrolysed whey produced fewer VFAs. The biomethane yield from pre-treated whey was 3.6 times higher than that of raw whey. The BMP results were well fitted with the Gompertz model, and a substantial reduction in lag phase time was noticed for pretreated whey. Hence, the use of enzymes in the pretreatment of complex wastes like whey can be adopted to enhance AD. As commercial enzymes are expensive, the enzyme produced

from fermenting organic wastes is a better alternative to make the enzymatic process more cost-effective

Among 3 methods, enzymatic pre-treatment was suggested as the most efficient pre-conditioning method for CW degradation which resulted in maximum methane yield of 432.2mlCH_4/gVS and an energy factor of 6.67. Working under optimal conditions is essential to determining the enzymes' full potential for improving anaerobic digestion and biogas production. Hence, optimisation studies need to be conducted further. The experimental values showed better fitting with Gompertz model with average R^2 values 0.985, 0.989 and 0.995 respectively for US, ozonation and enzymatic methods.

Chapter 6

Study of additives

Biochar (BC) is a carbonaceous residue produced from the thermal conversion of biomass in an oxygen-free environment through a number of processes like, pyrolysis, hydrothermal carbonization, gasification and torrefaction. Literatures have pointed out the influence of biochar in promoting biogas production during AD of organic wastes. This chapter is intended to study the effect of BC addition on enhancing digestion performance in AD of CW and SP. The impact of biochar addition on the total solids content of the digester has yet to be determined through existing studies due to the use of diverse feed stocks and digester layouts.

6.1 Biochar collection

The BC utilized in this research was obtained from the Sanitation Park located in Ammavaripet, which processes Faecal sludge collected from urban areas within the Warangal corporation, Telangana. The biochar derived from septage was produced through pyrolysis at a temperature of 650°C, and the properties of the feedstock directly influenced the characteristics of the biochar. Studies showed that BC produced at higher temperature(>700ºC) has less positive influence in methane generation. This can be attributed to the lower presence of labile compounds on biochar surface, resulting in fewer microbial substrates available for fermentative and methanogenic bacteria (Bruun et al., 2011) .This is because of the fewer labile compounds at the surface of biochar particles indicating less microbial substrates for fermentative bacteria and methanogenic archaea. The operation conditions maintained during pyrolysis and feedstock type are two main factors influencing BC characteristics. Biochar compatibility in anaerobic digestion was evaluated beforehand by conducting detailed characterization studies, which will be discussed in the next section.

6.2 Biochar characteristics

Characterizing the physical and chemical properties of biochar is essential for understanding its fundamental structure and properties, as well as predicting its ability to act as a potential additive in AD process. The BC samples were undergone elemental analysis and proximate analysis, ultimate analysis, particle size distribution analysis, Brunauer–Emmett–Teller analysis (BET), Scanning electron microscope analysis (SEM), Fourier transform

infrared analysis (FTIR), X-ray diffraction spectroscopy (XRD) and thermo-gravimetric analysis (TGA) to get an overview on physical, chemical and structural properties of BC.

6.3 Proximate, ultimate and trace elemental analysis results

The results obtained after conducting proximate, ultimate and trace element analysis on BC and feed stock was shown in Table 6.1. The proximate analysis results comprising moisture content and ash content of BC samples were reported as 2.13% and 67.2% respectively. Higher ash content found in BC was correlatable to the ash content of its feed stock, SP (51.3%). A high ash content in faecal sludge, such as found in Warangal, is typically caused by inadequately lined containment structures that accumulate grit and sand (Niwagaba et al., 2014). Similar kind of observation was made by Krueger et al., (2020), who studied about characteristics of different samples of faecal sludge collected from faecal treatment units located at Warangal. The HHV of BC was only 15.3 MJ/kg owing to its high ash content. The HHV values obtained for feedstock and BC are in agreement with those BCs produced at temperatures between 450 and 750ºC (8.8-19.91MJ/kg) (Gold et al., 2018; Krueger et al., 2020). Elemental composition shows that BC has a carbon content of 23.22%, H 0.921%, N 1.22% and S 0.96%.

The pH of BC was found in alkaline range which is around 10.3, while that of SP was 8.2. Compared to the original feedstock, biochars often have an alkaline pH due to their higher concentration of inorganic elements (Steiner, 2016). Hence it can be assured that BC offers proper buffering in the media. Ca and Mg concentration were 90.3 and 10.2g/kg respectively. The ash fraction of biochars tends to concentrate heavy metals that were present in the feedstock. Total K in many biochars is equivalent to the available K (Schmidt et al., 2015). Total K in BC was around 21.2/kg which lies in the range reported by (Woldetsadik et al., 2018) which is 19-29g/kg. Immobilization is thought to occur due to entrapment of K within the carbon structure and subsequent bonding into more stable forms. The ash fraction of biochars tends to concentrate heavy metals that were present in the feedstock (Beesley et al., 2019). Aside from human excretion, industrial effluents, leachate infiltration from solid waste landfills, and inappropriate disposal of hazardous goods such as batteries into latrines are all sources of heavy metals in faecal sludge (FS). Major heavy metals detected in BC were Ni, Zn, Pb, Cu and Cr (Table 6.1). Studies have shown that some of the trace elements like Ni and Co were essential for the growth of acetogens and methanogens to support the some enzyme activity (Demirel and Scherer, 2011). Therefore, the presence of these trace elements in biochar could potentially have a positive impact on promoting microbial activity in anaerobic digestion.

Table 6. 1 Physico-chemical characteristics of feedstock and biochar

Parameter	Unit	Biochar	Septage
Moisture content	%	2.13	-
Volatile matter	%	14.2	45.3
Ash content	%	67.2	51.3
HHV	MJ/Kg	15.3	11.8
pH	-	10.3	8.2
C	%w/w	23.22	32.6
H	%w/w	0.921	6.8
N	%w/w	1.22	28.3
S	%w/w	0.96	1.54
Ca	g/kg	90.3	54.34
Mg	g/kg	10.1	4.86
K (total)	g/kg	21.2	4.6
C/N ratio	-	16.49	9.3
C/H	-	17.23	-
Zn	mg/kg	1120.3	-
Pb	mg/kg	226.3	-
Ni	mg/kg	162.3	-
Cu	mg/kg	283.1	-
Cr	mg/kg	48.1	-

6.3.1 Structural characterization

Figure 6.1 shows the SEM image of BC derived from septage. The compositional contrast that causes elements heavier than carbon to seem lighter allows for a clear differentiation between mineral and biological compounds. Some cylindrical shapes and honey-comb like structure was visible in the image. SEM images help to understand the porous nature and physical morphology of a material. The porous structures inherent in biochar, as well as uneven biochar forms that cause increased empty space between soil particles, are the primary processes underlying its adsorptive activity. The BET surface area, pore volume and pore size of BC obtained were 7.18m^2/g, 0.029cm^3/g and 46.418A°. It was observed that BC follows type II isotherm, which was characterized by wide range of pore sizes. Because the active sites on the biochar can attract and minimise the presence of inhibitors, biochar with a high porosity can be used as an adsorbent for removing inhibiting elements from AD media. Mineral matter is observed to be stuck to the surface of carbonaceous material and imprisoned within its structures, in addition to the obvious presence of bigger sand grains (Figure 6.1). The macroporous structure appeared mainly due to the decomposition of organic matter through

thermal degradation. The macropores observed in biochar are caused by the cellular structure of its predecessor material.

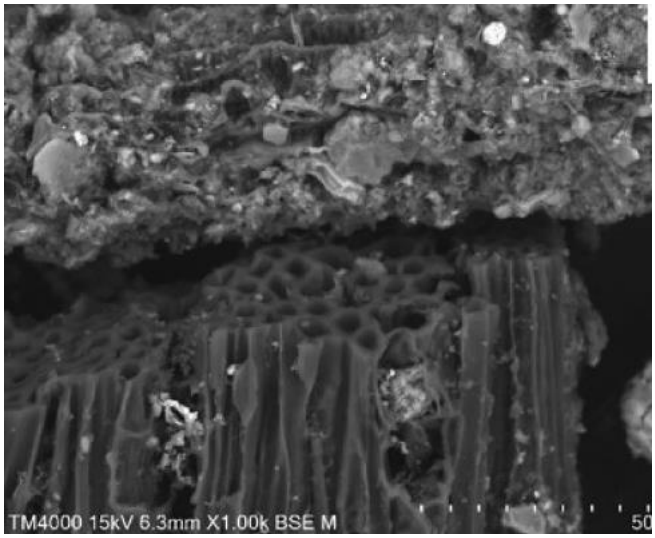

Figure 6. 1 SEM image of biochar derived from septage

6.3.2 X-ray diffraction (XRD) results

XRD results provide the information about crystallinity of the material. Usually, BC particles can have crystalline and non-crystalline or amorphous phases (Tsaneva et al., 2014). Figure 6.2 depicts the XRD pattern of BC. The sharp edged and narrow peaks indicated high crystalline nature of BC. The sharp peak at $2\theta = 26.55^{o}$ was identified as quartz. Studies have shown that the presence of quartz can affect the structural characteristics of BC. This phenomenon is usually found at a 2θ of 26^{o} in carbon materials that demonstrate long-term structural order. It is typically attributed to the loading of graphitic basal planes. The other peaks identified along the sides of highest peak were cinnabar and graphite. The other peak at $2\theta=29^{o}$ can be identified as Marshite. In addition to quartz, graphite and cinnabar, other crystal type minerals like marshite, periclase etc. are also present. The mineralogical composition of BC was found in agreement with the high ash content.

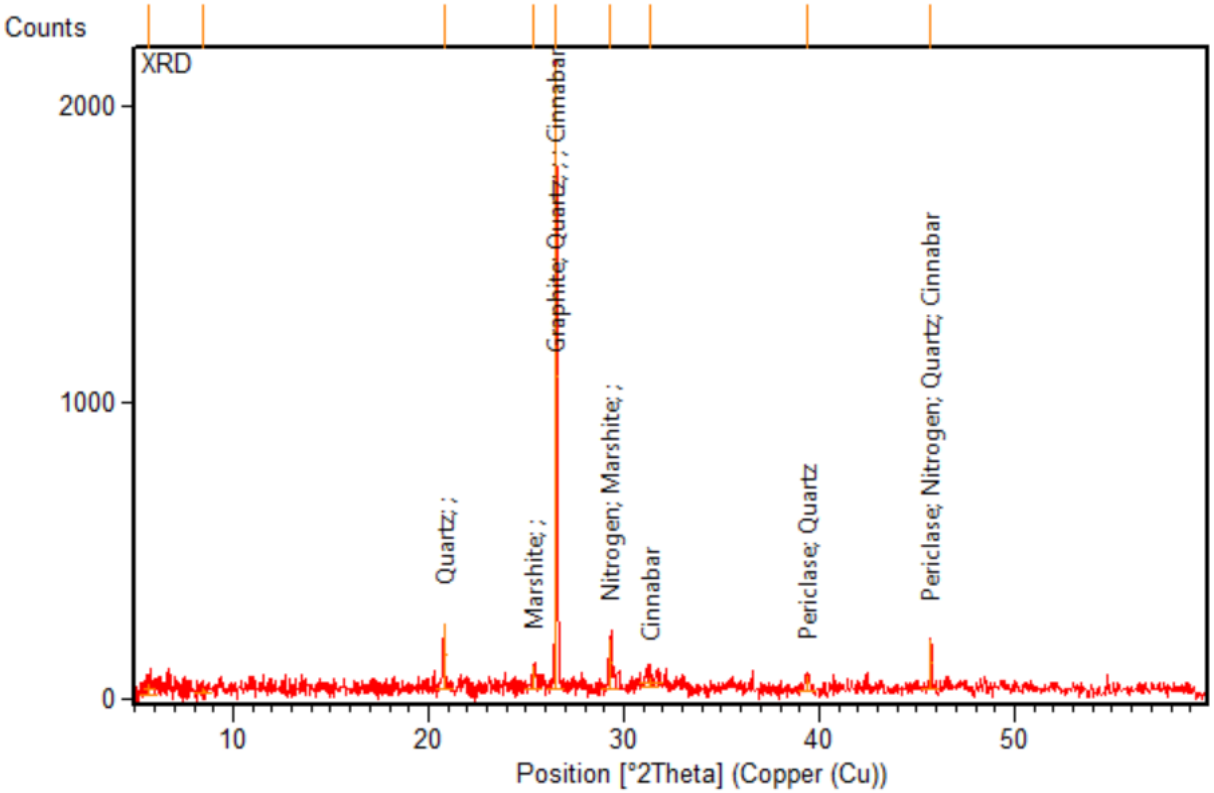

Figure 6. 2 XRD pattern of biochar samples

6.3.3 Fourier transform infrared analysis (FTIR)

The pyrogenic characteristics of biochar result in it containing a reservoir of aliphatic and aromatic structures, along with various functional groups containing oxygen, including ketones, quinones, carboxylic groups, and more. FTIR study helps to determine the major functional groups present on biochar surface. Figure 6.3 shows various bands of vibration present in FTIR spectra of biochar. The adsorption band observed at 3410cm^{-1} was due to the stretching vibrations of the hydroxyl (OH) group present and hydrogen bonding due to water adsorbed. The second peak in range 1540-1650cm^{-1} indicates the C-O stretching vibrations of amide groups and aromatic C=C stretching and carboxylate anionic vibrations. The peak between 1580-1600cm^{-1} shows the presence of C=C bonds. The peak at 1414cm^{-1} shows the asymmetric stretching of carbonate groups. The peak observed at 873cm^{-1} might be due to the presence of calcite ion ($CaCO_3$). The peak in between 1020-1030cm^{-1} indicates C-O stretching of ethers and primary amine C-N stretches. The vibrations of C–H bonds in the hetero-aromatic and aromatic compounds were observed at a frequency range of 603-876 cm-1, indicating their presence. The presence of functional groups in the biochar samples such as esters, ketones, aldehydes, carboxylic acids, ethers, and phenols shows their potential utility as adsorbents for pollutants (Stella Mary et al., 2016).

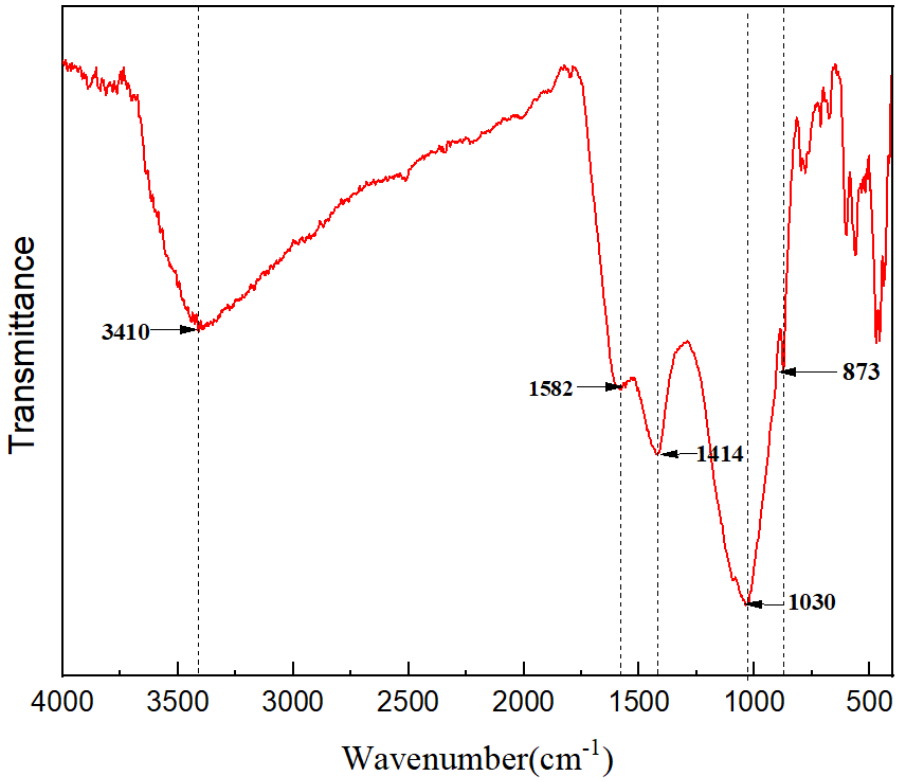

Figure 6. 3 FTIR spectra of biochar

6.3.4 Particle size distribution

The particle size distribution graph shown in Figure 6.4 shows that major fraction (47.6%) of BC material lies below 75µm. 31% of the fraction lies between 150-425µm. As per the guidelines of (Schmidt et al., 2015), the BC can be classified under fine powder category. The particle size of biochar is determined by both the feedstock parameters and the pyrolysis process. Fast pyrolysis at high temperatures, in particular, produces finer biochar particles (Bruun et al., 2012). The fine particle classes observed in the biochar production process are likely attributed to the combination of high heating rates in a continuous reactor and the particulate nature of the dried FS. Furthermore, the handling mechanisms involved in the process, such as auger feeds, can contribute to a smaller particle size by breaking up particles.

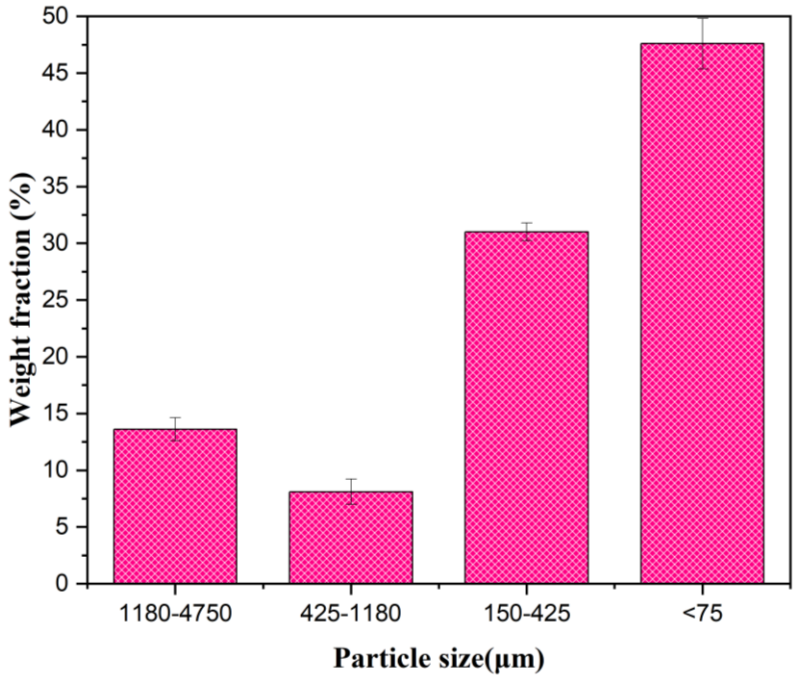

Figure 6. 4 Particle size distribution graph of biochar

6.4 Anaerobic digestion experiments

The BMP experiments were conducted on AD of CW and SP adding different doses of BC at various TS concentrations. The BC loadings were 6.25g/l, 12.5g/l, 25g/l and 50g/l at TS concentrations 5%, 7.5%, 10%, 12.5% and 15%. All BMP sets were done in duplicate. The digesters are kept at mesophilic temperature (37ºC) conditions for a span of 40 days. The biogas measurements were done using 500ml glass syringes on a daily basis. Uniform conditions were maintained by mixing the sample daily for 1 minute before biogas measurement. The substrates, inoculum and biochar were analysed for their physicochemical characteristics and observations are shown in Table 6.2. CW has a greenish white colour with unpleasant odour. CW was acidic in nature with pH 5.4, while septage was found to be alkaline. Wide variations were visible in organic strength of both substrates. The COD and TS contents for CW were almost double the value of that of SP indicating its organic complexity as well as pollution potential. The COD of CW was obtained as 69.8g/l, which falls in the range reported by Diamantis and Aivasidis (2018) and Pacheco et al., (2023). Low C/N ratio of SP shows its compatibility with CW as a co-substrate in AD.

Table 6. 2 Physico-chemical characteristics of substrates and inocula

Parameter	Substrates		Inoculum
	CW	SP	CM
pH	5.4	7.2	6.32
COD (g/l)	69.8 ± 2.51	29.5 ± 1.18	31.81 ± 1.28
TS (g/l)	52.1 ± 1.21	28.3 ± 1.06	31.81 ± 1.28
VS (% of TS)	71.3	60.8	52.1
TAN (g/l)	0.51 ± 0.03	0.83 ±0.21	1.18 ± 0.24
C (%w/w)	42.3	32.6	-
H (%w/w)	5.8	6.3	-
N (%w/w)	6.1	11.1	-
C/N	24.32	11.21	33.18

Nb: CW: Cheese whey, SP: Septage, CM: Cattle manure

6.5 Results and discussions

6.5.1 Daily and cumulative methane yield at different biochar dosages

The daily and cumulative methane yield of all cultures at different biochar dosage and TS contents is shown in Figure 6.5 and Figure 6.6 respectively. During initial days, all samples except control has shown increased methane production. It is evident that biochar addition has benefitted the methane yield in all mixtures. The lag phase time was not more than 1 day. With increasing biochar dosages, the methane production rate increased. The maximum cumulative methane yield was obtained at 50g/l of biochar loading at 10 % TS content, 486ml/gVS. The lowest methane yield was reported at 5%TS concentration with 6.25g/l of biochar loading as 243.2ml/gVS. At 5% TS content, the maximum cumulative methane yield was attained around 24^{th} day and thereafter steady state was maintained. The daily methane yield was lowered from 25^{th} day onwards for mixtures with biochar loadings 25g/l and 50g/l at TS concentrations > 10%. Hence the effect of biochar dosage on methane generation was dependent over the TS content in mixture. The concentration of divalent and monovalent cations increases with increase in biochar dosage (Linville et al., 2017). However excess dosage results in overlapping of adsorption sites and adsorption efficiency decreases.

The first peak was observed within 5 days for all mixtures added with biochar. As compared to digesters added with biochar, the daily and cumulative methane yield curve

observed in control was flatter and no pronounced peaks were observed. The peaks observed for all mixtures with biochar loading 50 g/l were higher than 25 g/l and 12.5 g/l. The total methane yield obtained for sample added with 50g/l of biochar at 10% TS was maximum. The increase in methane yield was 29.58% at 10% TS when biochar loading was increased from 12.5 to 50g/l. Wei et al., (2020) reported 17.8% increase in methane yield when corn stover biochar dose was increased to 3.06g/g TS in a batch scale study of AD of primary sludge. In contrast to this study, Sunyoto et al., (2016) reported maximum methane yield at a lower biochar dose of 8.3g/l during AD of municipal solid waste and sewage sludge.

The positive effect of biochar addition in digesters can be correlated with its characteristics and that of feedstock. Literatures have pointed out that feedstock types and pyrolysis temperatures affect the pore size distribution, pore structure and specific surface area (SSA) (Cantrell et al., 2012). The biochar used in this study has a SSA of 7.79m^2/g which was high enough to exhibit strong adsorption and immobilisation capacity. Another characteristic affecting digestion capacity is the ash content. The ash content of biochar used was 67.2% which indicates presence of more alkali elements having the ability to provide buffering capacity to the digester. Pyrolysis temperature and pH was reported to be linearly related (Fidel et al., 2017). Here the feedstock is alkaline in nature and pyrolysis temperature was 600ºC, which also contributes to alkaline behaviour of biochar. Biochar derived from nitrogen rich feedstock were likely to have high ash content and pH, imparting buffering nature to biochar (Ahmed and Hameed, 2020). Studies have shown that buffering capacity of biochar has helped in maintaining the neutral state of digester and helps in attaining stability (Maa et al., 2020). Lim et al., (2020) reported that pH of digester has increased to 8.15 after addition of biochar at loading of 15g/l to a semi-continuous digester containing food waste.

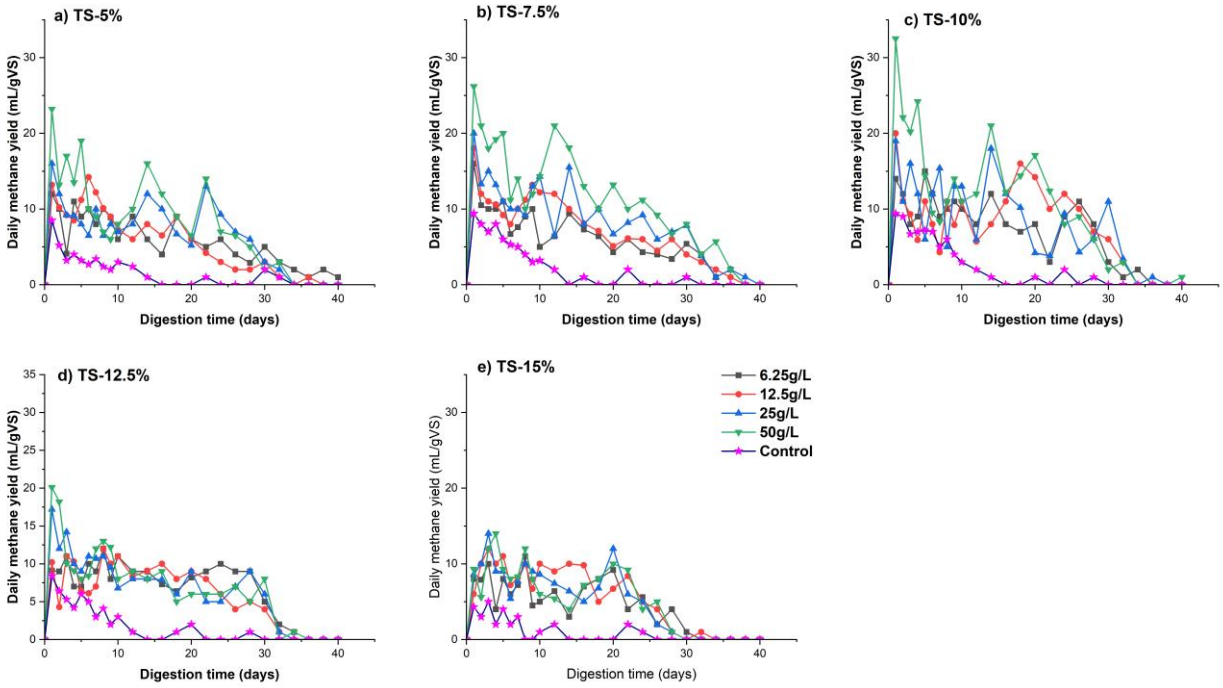

Figure 6. 5 Daily methane yield for biochar added and control mixture at different TS contents

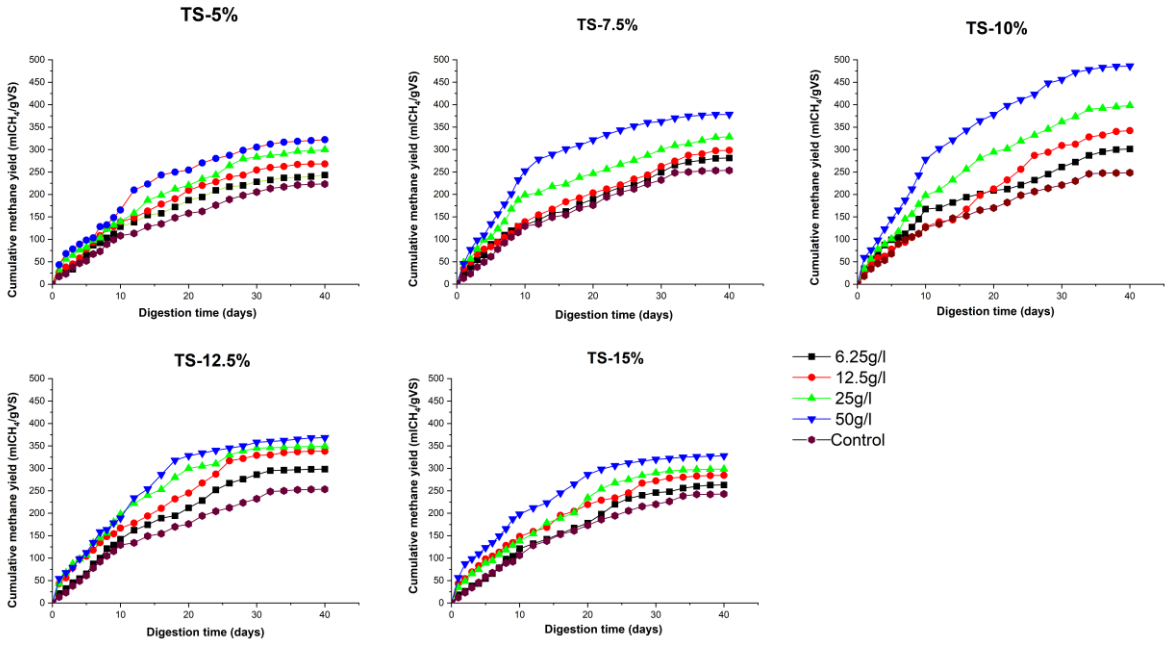

Figure 6. 6 Cumulative methane yield for biochar added and control mixture at different TS contents

6.5.2 Effect of total solids in methane generation

The cumulative methane yield for biochar loadings 6.25g/l, 12.5g/l, 25g/l and 50g/l increased in order of their increasing loading levels in all TS concentrations (Figure 6.6). But regardless of the biochar dosages, at TS>10% the methane yield reduced considerably. Increasing the TS concentration from 10 to 12.5% and to 15%, the cumulative methane yield has reduced by 24.27% and 32.7% respectively. The daily methane yield was higher for wet AD systems with 5, 7.5% and 10% TS concentrations reported on initial 7 days and then increased to maximum on 14^{th} day. Unlike this, dry AD systems (TS 12.5 and 15%), the daily methane yield was reported less after 20^{th} day. The depletion in methane production rates at high TS concentrations might be due to lower diffusion co-efficient caused by non-uniform mixing as well as due to undesirable biochemical changes of substrate (Duan et al., 2012). Hence, lag phase portrays a dominant behaviour in dry AD systems as maximum daily yield was achieved within 35 hours only.

The maximum cumulative methane yield at 5%,7.5%, 10% ,12.5% and 15% TS concentrations were 322.1, 378.2, 486, 368.3 and 327.2 ml/gVS respectively. The graph shows that in wet AD systems after 19-20 days maximum attainable methane production was achieved compared to dry AD systems. The hydraulic retention time for wet (5,7.5 and 10% TS) and dry (12.5 and 15% TS) AD systems were around 20 and 30days respectively. Regarding retention time, Chen et al., (2014) had similar kind of observation that wet AD systems can be completed in 25 days compared to dry AD systems taking prolonged time for digestion. Along with increased organic loading, biochar added might also cause improper mixing as well as increasing the viscosity of sludge. Hence, biochar addition at proper total solids content was crucial for maximising methane productivity. The daily methane yield at 5% TS with biochar addition wasn't as significant as that obtained at 7.5 and 10% TS. Similarly, at 15% TS content, the biochar addition has less pronounced effect. It is possible that smaller differences in percentage changes and biological variations can cause delay in achieving statistical significance.

6.5.3 Statistical significance and Gompertz model analysis

The effect of biochar dosage and total solid levels were studied using Two-way analysis of variance (ANOVA) in Microsoft Excel 2016 to know the statistical significance of the results obtained. The procedure followed to conduct ANOVA test was adopted from the methodology explained by Lin et al., (2016). Table 6.3 shows the values of the statistical parameters generated after analysis. The p-value of the interaction of TS content was obtained as 0.0136

which indicates less significant effect on cumulative methane yield at an alpha level of 0.05. Because statistically significant character is observed for samples having p-value less than 0.05. On the other hand, the p-value of biochar dosage was 5.08×10^{-21}, which shows very significant effect at an alpha level of 0.05. Also F value was quite higher than F_{crit}. This shows, unlike TS content, biochar addition has a consistent impact on cumulative methane yield. The biochar dosage is found more significant than TS content.

The modified Gompertz model parameters like maximum cumulative methane yield (Y_{max}), methane production potential rate (R) and lag phase time (λ) derived after simulating the BMP experimental results are shown in Table 6.4. The value of parameters for the sample (TS 10%) achieved maximum production are only shown. The percentage reduction in lag phase time with increase in biochar dosage was clearly visible from the values. Indren et al., (2020) found around 27% reduction in lag phase time at 10% TS content during AD of poultry litter using wood-pellet derived biochar as additive.The possible cause for the increased lag phase time in lower TS contents was discussed earlier. The Y_{max} value obtained for biochar loading 50g/l was close to the experimentally obtained one (486ml/gVS). The rate of methane production potential also increased with increasing biochar dosage.

Table 6. 3 Anova table for studying significance of biochar dose and total solids

Source of variation	**SS**	**df**	**MS**	**F**	**P-value**	**F-crit**
Total Solids*	61386.17	24	2557.75	2.52	0.013617	1.983
Biochar dose**	1001730	1	1001730	989.21	5.08×10^{-21}	4.259
Error	24303.54	24	1012.64			

*Significant at $p<0.05$

**Significant at $p<0.01$

Table 6. 4 Modified Gompertz model parameters derived for BMP experiments with and without biochar

Scenario	Maximum cumulative methane yield, Y_{max} (ml/gVS)		Rate of Methane production potential, R (ml/gVS.d)		Lag phase time, t (days)		R^2
	Mean	Sd	Mean	Sd	Mean	Sd	
TS-10%, Control	254.16	9.18	8.38	0.58	3.81	1.83	0.98
TS-10%, BC-6.25g/l	302.55	13.58	9.91	0.88	2.96	0.78	0.97
TS-10%, BC-12.5g/l	400.15	21.83	10.08	0.85	2.65	0.67	0.98
TS-10%, BC-25g/l	409.64	10.25	14.64	0.73	2.23	0.81	0.99
TS-10%, BC-50 g/l	438.11	11.83	16.13	0.78	1.45	0.54	0.97

6.5.4 Effect of biochar addition on acid-stress

The acid-stress on the digesters was studied by comparing the initial and finial pH of digestate and by evaluating the production of short chain volatile fatty acids (VFA). Figure 6.7 shows the VFA profiles of acetic, butyric, lactic and propionic acids measured for all mixtures after digestate analysis at end of digestion. It was clear that acetic, butyric and propionic acids were the dominant VFAs in all mixtures (Wang and Zhao, 2009). The lactic acid concentrations were comparatively low. The cultures with biochar addition degraded volatile fatty acids faster than those without. The concentration of propionic acid was found to be increasing with increasing biochar loading. As TS content increased, propionic acid accumulation became more noticeable (12.5% and 15%TS). This might be another reason for lower methane production at higher TS concentrations.

The concentration of propionic acid in 12.5 and 15% TS contents ranges from 322 to 720mg/l. Its concentration reached only 280mg/l at lower TS contents. In a study by Sunyoto et al., (2016), similar kind of propionic acid accumulation was observed at 25.1 and 33.3 g/l of

biochar loading. The energy required to oxidise propionate to acetate was around 76.1kJ/mol, almost double to the value required for butyrate (48.1kJ/mol) (Wang and Zhao, 2009). Consequently, propionic acid's acetogenic rate was slowed due to accumulation in the culture (Amani et al., 2011). The variations in pH of mixture with 10% TS are shown in Figure 6.8. Due to acidic nature of CW, the initial pH of sample without biochar addition was around 7.2 ± 0.2. The slightly alkaline behaviour was due to the presence of septage. The initial pH of samples added with biochar ranged from 7.7 to 8.9. Substantial reduction in pH was observed for all samples which can be correlated to the increased VFA production. The final pH of sample lied in range 7.2-7.5, indicating strong buffering capacity of the biochar added.

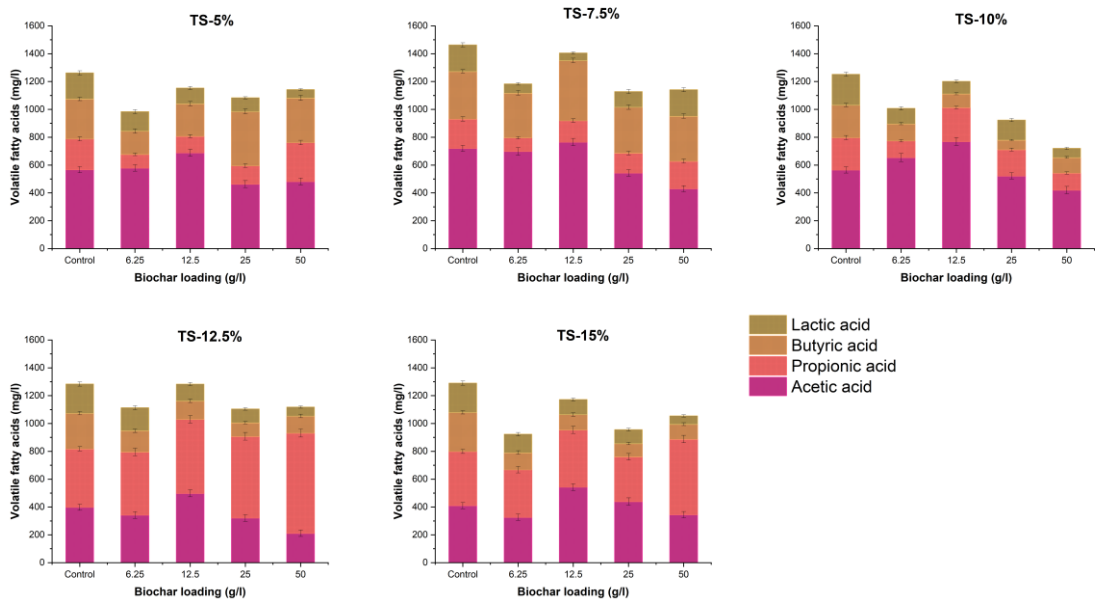

Figure 6. 7 Volatile fatty acid profiles of various mixtures at different TS contents and biochar loadings

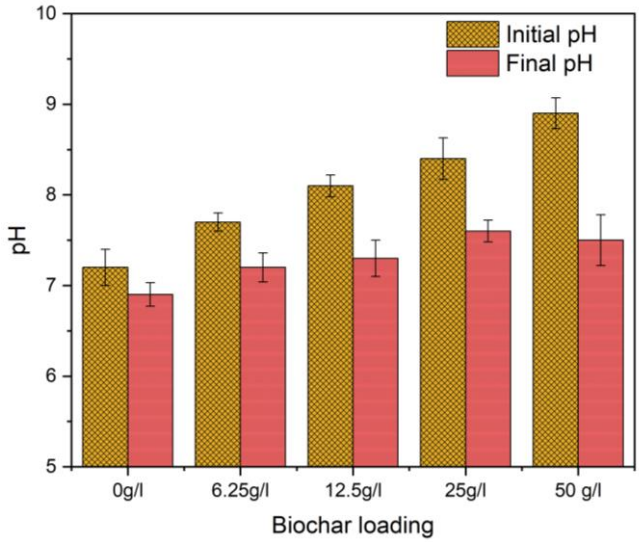

Figure 6. 8 Initial and final pH values of digestate at end of experiment

6.5.5 Mechanisms affecting biochar activity

Figure 6.9 shows the SEM images of digestate collected from control and 10% TS mixtures. The indication of possible formation of biofilms were observed in Figure 9(B). The Biofilms confirm the presence of active microbes or methanogens in the sample. Methanogens and bacteria found within these biofilms might be investigated in future studies. The cellular structure of a precursor material might have been responsible for the macropores present in a biochar (Parawira, 2004; Wildman and Derbyshire, 1991). Hence the septage-derived biochar is most likely to enhance the production of methane by serving as a good microbial carrier. The biochar used in this study was known to have characteristics like strong buffering capacity, immobilisation and adsorption ability, and presence of nutrients and trace elements. All these helped in reducing the lag phase time, better degradation of VFA, and maximising methane production.

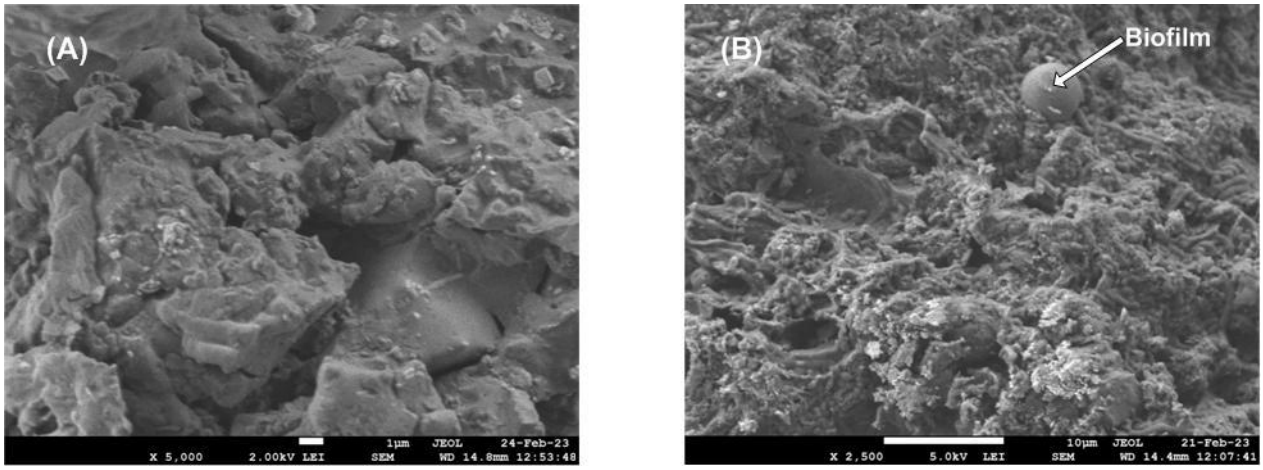

Figure 6. 9 SEM images of digestate collected from control and 10%TS digesters

6.6 Important findings

The major findings of this study were;

- The suitability of utilizing septage-derived biochar as an additive was checked by conducting detailed characterization studies.
- Biochar has an increased surface area of 7.19cm^2/g and microporous structure which helps in increasing the adsorption capacity.
- Absence of O and N containing functional groups make the biochar less hydrophobic, indicating presence of polar group.
- Since pyrolytic temperature>450ºC, biochar is more suitable to adsorb organic contaminants.
- Low H/C and O/C ratios indicate loss of O_2 and H_2, ensures the presence of fixed carbon making biochar more alkaline.
- Presence of -COO-(-COOH) and -O-(-OH) functional groups contained in the biochar helps to provide buffering capacity.
- The maximum cumulative methane yield was reported at 50g/l of BC loading and at 10% TS concentration, 486ml/gVS.
- The lowest methane production was obtained at 5% TS and 6.25g/l of BC loading, which was 243.2ml/gVS.
- A reduction in biogas production rates was observed at higher TS concentrations (TS>10%), which might be due to reduced diffusion rates.

- Statistical analysis results showed that TS contents has less effect compared to biochar dose in biogas production rates.
- Modified Gompertz model predicted the cumulative maximum methane yield as 438.11± 11.83, which is close to the experimentally obtained value.
- Acetic acid, propionic acid and butyric acid were the dominant VFAs identified in all digesters added with BC.
- Propionic acid accumulation caused inhibition to AD in digesters at higher TS concentrations.
- SEM images confirmed the presence of rich anaerobes in digesters added with BC.

6.7 Conclusion

The effect of adding biochar in the AD of CW and SP was studied at various total solids concentrations. Prior to the investigation, the suitability of septage-derived biochar as an additive was thoroughly assessed using extensive characterization studies. The biochar has remarkable properties such as increased surface area, a macroporous structure, and was classified as a fine powder. These characteristics lead to its high adsorption capability. Furthermore, the biochar provided an excellent source of key nutrients and trace elements required for the growth of certain methanogens. It is important to note that the biochar utilized in this study was derived from one of the co-substrates, namely septage. Biochar addition showed a 29.98% increase in biogas production when BC is added and maximum cumulative methane yield was reported at 10% TS concentration and 50g/l BC loading. Regardless of the BC dosage, biogas production at higher TS concentrations showed fewer methane productivity. Biochar is thought to have given temporary substrates for microbial metabolism and growth, as well as acting as a pH buffer in methane generation. Furthermore, biochar is believed to have aided in the formation of methanogenic biofilm, boosting methane generation.

Chapter 7

Design and operation of a continuous anaerobic digester

The preliminary batch-scale investigations in 120ml glass serum bottles produced promising findings, especially in terms of biogas and methane generation rates. The next step is to scale up the investigation to a lab-size pilot study to ensure the robustness of these findings and acquire insights towards large-scale implementation. While BMP studies provide useful baseline data for continuous anaerobic digester operation, they do not provide conclusive information about process stability, optimal organic loading rate (OLR), hydraulic retention time (HRT), or the operation of a two-stage AD system. The goal is to determine the best operating parameters for the acidogenic and methanogenic reactors, which will provide a thorough understanding of process efficiency and stability. Based on the daily whey wastewater flow, the findings were used to design an industrial scale anaerobic digester.

7.1 Design of 2-stage lab scale anaerobic digester

The anaerobic digester comprises of two upflow reactors and it is designed in such a way that it can be operated either in parallel or in series manner. A schematic diagram of two reactors with details is shown in Figure 7.1. The two reactors are in cylindrical shape of 5-liter capacity and made of stainless steel with double wall. An intermediary buffer tank is included between the reactors to allow excess flow from the first reactor to be discharged when the second reactor operates at a lower flow rate in series. Individual peristaltic pumps are installed in each packed bed reactor to establish and manage the liquid flow rate. Two heaters surround the reactor to warm the liquid passing through, and digital temperature controllers are allocated to each reactor to manage the temperature of the liquid. Two gas collecting vessels are installed to collect the produced gas. These vessels use a volumetrically calibrated water displacement collecting technology. A liquid seal mechanism with a constant head ensures that the reactor maintains a consistent gas pressure throughout the operation. During the run, the liquid seal can be replaced with water without affecting its effectiveness. Sampling of both liquid and gas can be done easily using dedicated sampling ports located in strategic locations. Non-return valves and syphon breaks are used to keep the volume of each reactor constant. This setup prevents accidental symphony activity while still assuring stable reactor functioning. The technical specifications of each unit are given in table 7.1.

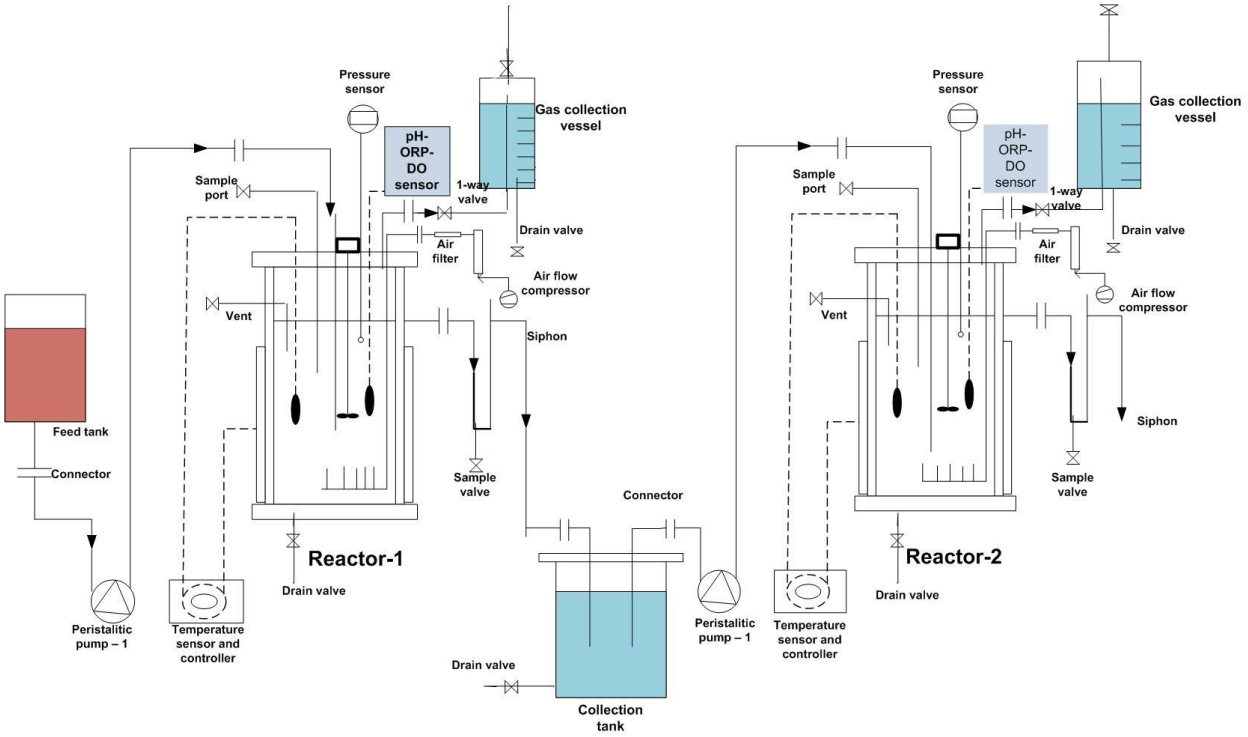

Figure 7. 1 Schematic diagram of 2-stage lab scale anaerobic digester

Table 7. 1 Technical specification of anaerobic digester

S.No.	Parts	Number	Specifications
1	Cylindrical reactor	2	Made of stainless steel, double cover, 5 liter capacity
2	Gas collection vessel	2	Linear scale, 5 liter capacity
3	Collection tank	1	2 liter capacity
4	Feed pump	2	Peristaltic, Two identical peristaltic pumps: variable speed flow rates from 1 to 150ml/min, provided with silicon tubes of different diameters for different speeds
5	Reactor Heater	2	200 W each ;heating jacket (electric heating mat) with PID control from a temperature sensor inserted into the reactor, set point within ambient to 55°C. Accuracy: +0.1 Different control for each reactor
6	Pressure sensor	1	Range: 0 – 3 bars
7	pH-ORP sensor	2	pH range: 0 to 14, Accuracy +0.01 ORP range: -300 to +300, Accuracy +0.1
8	Dissolved oxygen (DO) sensor	1	Range: 0 to 14mg/L, Accuracy +0.2mg/L
9	Air flow controller	2	Range: 0 to 1 LPM

7.2 Start-up and operation of digester

The digester operation can be divided into two sections. Stage 1 includes of inoculation, inoculum acclimatization, and digester stabilization with inoculum. The functioning of the digester under various OLR and HRT conditions covers stage 2. The two reactors were operated in a working volume of 4.5l, at pH 6.5 and temperature 37 ± 0.2ºC. The feed was mixture of CW and SP mixed at ratio 60:40 on the basis of volatile solids. CM was used as inoculum with substrate to inoculum ratio of 1:1.5. Initially, at the inoculation stage 3L of CM (around 2300g) was added to acidogenic reactor and operated in batch mode for a short period of 7 days. Thereafter, during start-up time 2L of CM was replaced with 3.5L of fresh CM, ensuring a working volume of 4.5L. The acidogenic reactor was operated in this condition for 20 days, replacing 3.5L of CM mix in every 10 days with fresh CM. During this time, no chemical was added to regulate pH, as neutral pH was already available. In stage 2, co-digestion mix of CW and SP was added as per the mix ratio mentioned earlier (1.8L).

7.3 Digester operating conditions

The acidogenic reactor was operated in batch mode for 48hr and then switched to continuous mode at HRT of 4 days and OLR of 14.45gVS/Ld, subsequently increased as the experiment proceeds. The acidogenic reactor was operated for a total duration of 82 days at HRT; 4,3,2 and 1 days and corresponding OLRs. The operating conditions for acidogenic and methanogenic reactors were listed in Table 7.2 and Table 7.3 respectively. The pH of acidogenic reactor was maintained as 6.5 ±0.2 as per the guidelines of Bouallagui et al., (2004). When the pH within the acidifier was not altered, it quickly plummeted to 5.0 0.2, especially near the conclusion of the first feed interval. The acidified effluent from first reactor was fed to the methanogenic reactor operated at 3 HRTs 16,14 and 10 days corresponding to equivalent OLRs 2.88, 3.29 and 4.33gVS/Ld. The two reactors are subsequently operated to obtain optimum HRT and OLR conditions for maximum methane production and organic matter removal. The parameters like VFA, COD, VS, TAN and total alkalinity were evaluated for the samples collected at the collection tank during feeding time. The volume and composition of biogas were evaluated daily or in alternate days.

Table 7. 2 Operating conditions for acidogenic and methanogenic reactor

HRT (d)	Flow rate (mL/d)	OLR (gVS/Ld)	OLR (gCOD/Ld)
4	1125	14.45	21.45
3	1500	19.26	28.6
2	2250	28.9	42.9
1	4500	42.9	85.8

Table 7. 3 Operating conditions for acidogenic and methanogenic reactor

HRT (d)	Flow rate (mL/d)	OLR (gVS/Ld)	OLR (gCOD/Ld)
16	281	2.88	5.35
14	321	3.29	6.12
10	450	6.12	8.58

7.4 Results from operation of acidogenic reactor

7.4.1 Biogas yield at different HRT

Following the start-up phases, the acidogenic reactor was run at HRT 4d, 3d, 2d, and 1d for 20, 22,20, and 20 days, for a total of 82 days to reach steady state condition. The biogas produced by the acidogenic reactor was mostly H_2 and CO_2, with traces of CH_4. Figure 7.2 shows the biogas production rates at different HRTs. Variations in biogas production can be ascribed to variations in microbial populations over a long period of operation. Furthermore, the feeding medium's complexity, notably the presence of several organic and inorganic chemicals, could have resulted in transient inhibitory effects. As HRT is reduced to 1d, biogas production increased. The mean biogas production rate at HRT 4d was only 0.64L/L_rd (L_r stands for unit volume of reactor), which systematically raised to 6.03L/L_rd at HRT 1d. The system under investigation has significant swings in biogas generation and other key characteristics. These changes occur not just during the transition from one phase to the next, but also from day to day, even within the same phase. Such instances were reported in other studies also which lead to the instability of the reactor (Mariakakis et al., 2011). In this study, the acidogenic reactor attained steady state at HRT 1d with an equivalent OLR of 57.8gVS/Ld.

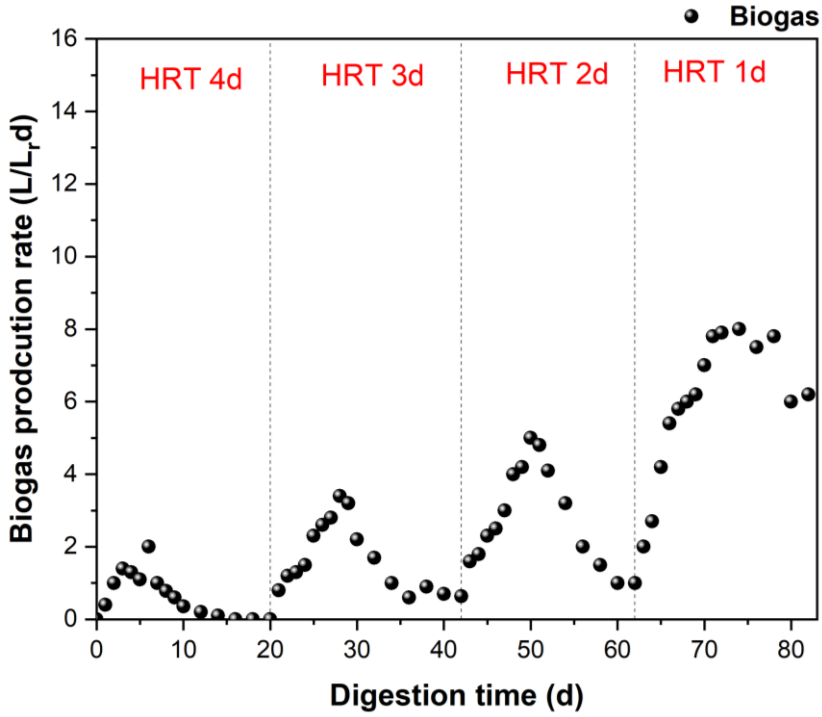

Figure 7. 2 Biogas production in acidogenic reactor at various HRTs

7.4.2 Volatile fatty acid production

The main aim of operating acidogenic reactor was to obtain maximum acidification rate, which points out to the conversion of high molecular organics into VFAs. Figure 7.3 depicts the major VFAs generated during hydrolysis and acidogenesis in first reactor. Acetic, propionic, iso-butyric, lactic and valeric acids were the main VFAs generated in acidogenic reactor. During whey fermentation, lactose in whey gets converted into lactic acid and other VFAs. The total VFA increased from 955.8mg/l to 2588mg/l when OLR was reduced with increasing HRT 1d to 4d. Valeric acid and ethanol concentrations were recorded, but at values less than 1000 ppm. In contrast, isovaleric acid was found in trace levels. The complicated composition of the whey and septage mix combination can be attributed to the intricate and varied distribution of end-products in this reactor. Major portion of total VFA was composed of acetic, butyric and isopropionic acid. Previously, Saddoud et al., (2007) studied AD of CW in a membrane reactor and found higher concentration of acetic acid, propionate and butyrate at HRT 1 d.

In comparison to others, the concentration of propionic acid in the reactor at HRT 3d was critically high. The energy required for the conversion of propionate to acetate and methane is substantial, which could explain why biogas output is lower at higher HRTs (Li and Wang, 2021). VFA buildup at higher HRTs is associated with reduced biogas generation. In a study by Yuan and Zhu, (2016), increase in propionic acid levels till 5.4g/l caused inhibition of the

system with reduction in methane yield. The breakdown of carbohydrates was blamed for the formation of soluble end products. The efficiency of carbohydrate utilization remained constant throughout all HRTs tested. These findings support prior studies suggesting that changes in HRT had little effect on the breakdown of carbohydrates in dairy wastewater (Dareioti and Kornaros, 2015; Fang and Yu, 2000). Attaining maximum solublisation of organic matter during acidogenegesis in first reactor was crucial for the operation of methanogenic reactor.

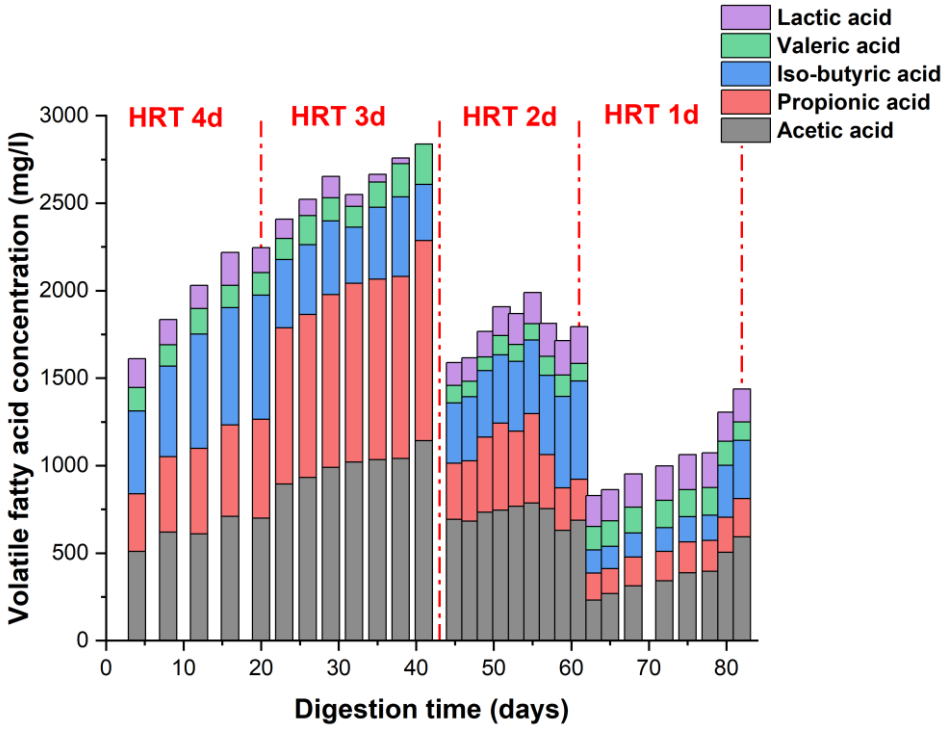

Figure 7. 3 Profile of main Volatile fatty acids generated in acidogenic reactor

7.4.3 Removal of organic matter

The removal of organic matter was evaluated in terms of TS,VS,TCOD and sCOD removal rates and illustrated in Figure 7.4 (A) and (B). No significant reduction COD levels was observed in the effluent from acidogenic reactor. Initial TCOD and sCOD values were 85.8g/l and 48.3g/l respectively. Similar kind of results were obtained for Dareioti and Kornaros, (2015), which studied the AD of CW, ensiled sorghum and CM in a 2-stage CSTR system. Figure 7.4(B) shows the concentrations of TS and VS of the acidified effluent from aciodegenic reactor, which showed a maximum of 20.1% of VS removal for all HRTs.

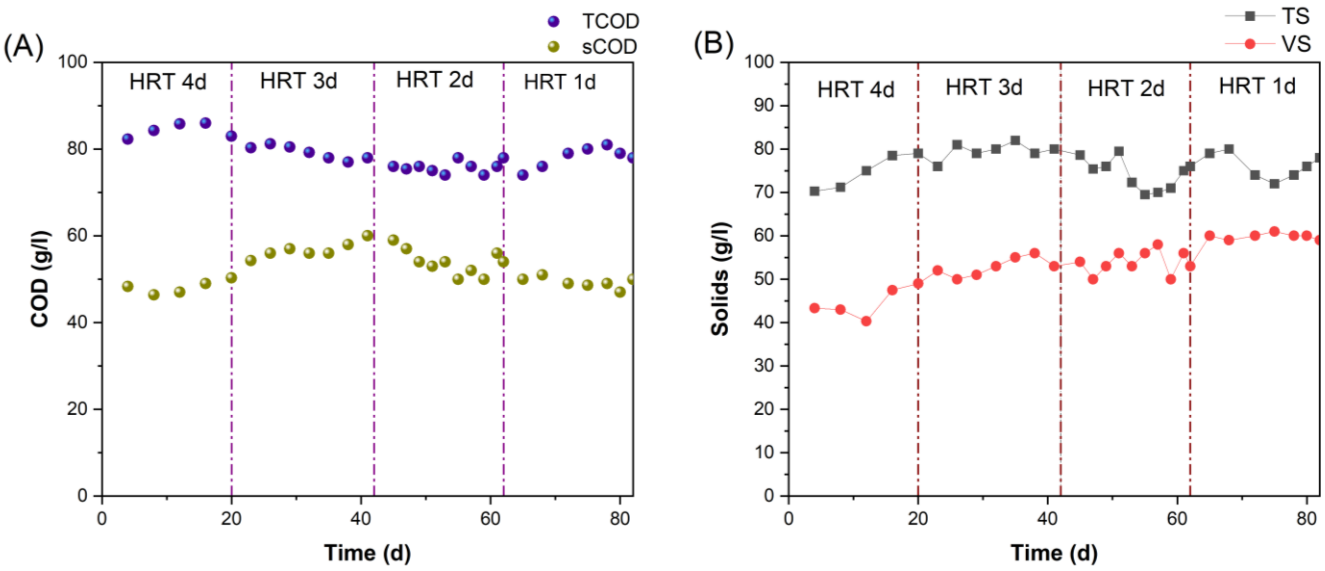

Figure 7. 4 (A)Total COD and soluble COD, (B) Total solids and volatile solids obtained from the acidogenic reactor

7.5 Results from operation of methanogenic reactor

7.5.1 Effect of HRT in methane generation

The methanogenic reactor was operated by treating the acidified effluent released from acidogenic reactor at 3 HRTs 16, 14 and 10 days. The steady state conditions obtained for operation of methanogenic reactor were highlighted in Table 7.4. Initially, when the reactor was started at HRT 16d, the mean biogas and CH_4 production rates were 0.9L/L_rd and 0.57L/L_rd respectively, which increased to 1.81L/L_rd and 1.13L/L_rd later at HRT 14d. This showed that switching to lower HRT from 16 to 14 days increased the CH_4 yield. The biogas and CH_4 production rates were shown in Figure 7.5. Thereafter, at 10d HRT the biogas production reduced drastically to 0.73L/L_rd with a 69.8% reduction in CH_4 yield. Dareioti and Kornaros, (2015) obtained the highest methane yield of 0.90 ± 0.12L/L_rd when the CSTR treating ensiled sorgum, CW and CM was operated at HRT 16d. Table 7.4 shows the steady-state average values of methane production rate, methane content, and methane yields for each HRT tested in this work. The methane yield was calculated by analysing the experimental data for each HRT, as shown in Table 7.3. It was determined using the volatile solids added (mL CH_4/g VS_{added}) and the chemical oxygen demand removed (mL CH_4/g $COD_{removed}$).

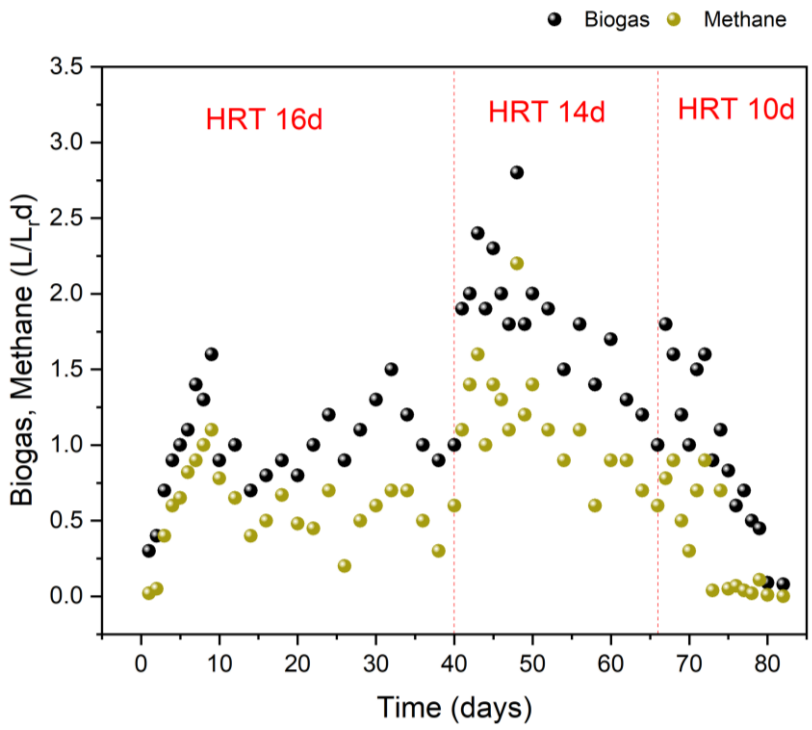

Figure 7. 5 Biogas and methane production in methanogenic reactor

Table 7. 4 Steady state conditions for operation of methanogenic reactor

Parameter	**HRT (days)**		
	16	**14**	**10**
pH	7.8 ± 0.08	7.5 ± 0.05	6.2 ± 0.02
Biogas (L/Ld)	0.96 ± 0.18	1.81 ± 0.32	0.41 ± 0.01
Methane (L/Ld)	0.63 ± 0.11	1.02 ± 0.23	0.22± 0.01
Methane (%)	55.21 ± 1.21	56.35 ± 1.02	47.33 ± 1.22
Yield CH_4 ($mLCH_4/gVS_{added}$)	350.51± 22.18	397.24 ± 28.18	190.81 ± 11.7
Yield CH_4 ($mLCH_4/gCOD_{consumed}$)	267.88 ± 17.04	283.55 ± 18.08	132.2 ± 9.22
TCOD removal (%)	84.33 ± 7.99	85.22 ± 7.33	38.44 ± 5.33
sCOD removal (%)	83.71 ± 7.65	86.05 ± 6.2	31.33 ± 4.06
VS removal (%)	62.33 ± 5.6	67.9 ± 5.98	43.63 4.22

7.5.2 Volatile fatty acid production

The lower values of methane yield obtained at HRTs 16 and 10 days can be correlated with the inhibition caused by production of VFAs in methanogenic reactor (Figure 7.6). The high concentration of acetic acid (up to 8.01 g/L) was principally responsible for the considerable increase in total VFA concentration seen during the 10-day HRT operation. There was also a moderate increase in propionic, butyric, and caproic acid concentrations (upto 2g/l, 3.05g/l and 0.98g/l respectively), albeit to a smaller level. Similar kind of inhibitions due to acetic acid accumulation was noticed in a study by Dareioti and Kornaros, (2015), which reported around 10.17g/l of acetic acid at 12d HRT. On the other hand, VFA at HRTs 16 and 14d was less (<0.6g/l) compared to that at HRT 10d. This indicates the process stability and more methane production. Hence reducing HRT beyond 14 days was not suggested for attaining process stability in a 2-stage digester operation during ACoD of CW and SP.

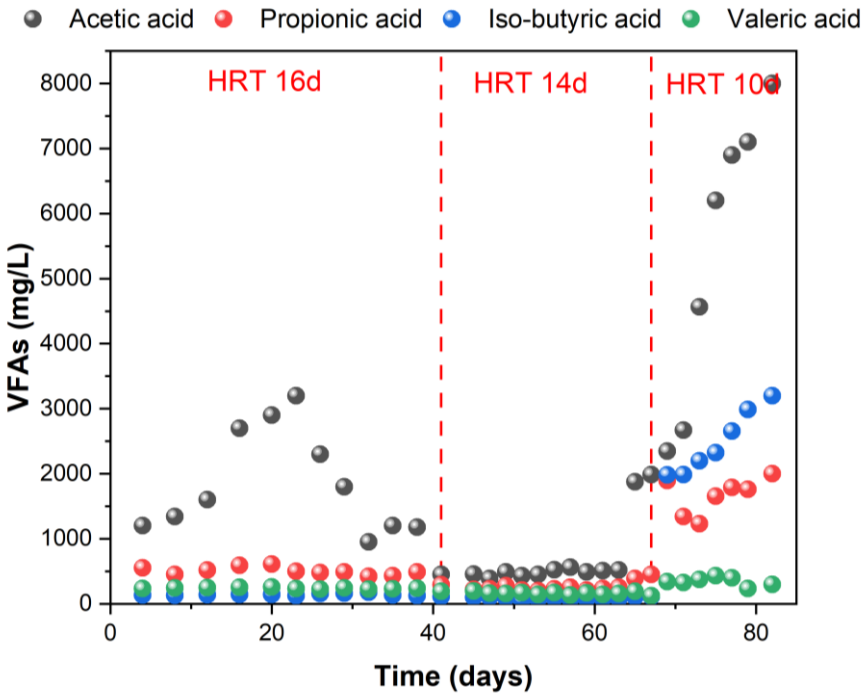

Figure 7. 6 Profile of main volatile fatty acids generated in methanogenic reactor

7.5.3 Removal of organic matter

The change in concentrations of TCOD, sCOD, tVFA and solids were shown in Figure 7.7 (A and B). A maximum of 85.22% of TCOD and 86.05% of sCOD removal rates were obtained at 14d HRT (Table 7.3). Almost similar levels of TCOD (84.33%) and sCOD (83.71%) removal rates were reported at 16d HRT also. But, switching to 10 day HRT showed an increase in TCOD and sCOD levels, with removal rates only 38.44% and 31.33% respectively. This was

reflected in the lower methane yields also. While the bulk of sCOD could be attributable to tVFAs at a HRT of 10 days, this was not the case at higher HRTs. This shows that other soluble by-products were present but were not discovered or accounted for in the analysis.

The highest TS and VS removal rates were obtained at optimum HRT of 14days, 41.39% and 67.9% respectively. The pH values remained within the neutral range for HRTs of 16 and 14 days, respectively. The pH dropped to 6.2 after a 10-day HRT, probably due to the formation of volatile fatty acids (VFAs). According to Callaghan et al., (2002), the ratio of tVFA to alkalinity (tVFA/Alk) can be used to predict process stability. When this ratio (equivalent acetic acid/equivalent calcium carbonate) is less than 0.3-0.4, the process is deemed stable and does not face the risk of acidification. The tVFA/Alk ratio was determined to be within acceptable limits during HRTs of 16 and 14 days, ranging from 0.04 to 0.15. These values were less than the suggested failure limit. However, after 12 days of HRT, the ratio increased significantly, reaching up to 2.56. This amount exceeded the safety level, owing to an increase in VFAs and a corresponding reduction in alkalinity to 6.53 g $CaCO_3$/l.

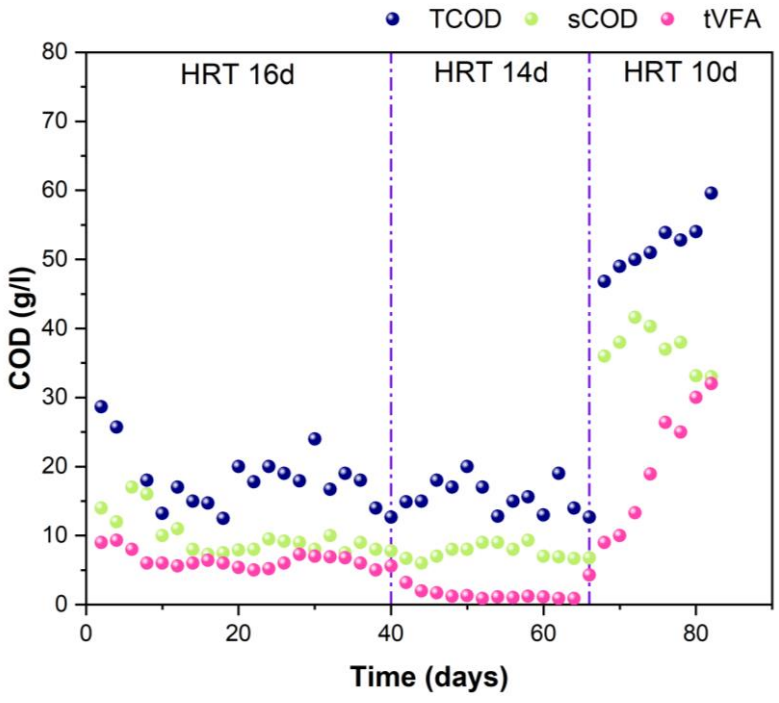

Figure 7. 7 Concentrations of TCOD, sCOD and tVFA for effluent from methanogenic reactor

7.6 Estimation of bioenergy potential

Estimating bioenergy potential (BEP) from methane yield includes determining the amount of methane that can be produced from a certain organic material or biomass. BEP was calculated using the equation (3.12) mentioned in chapter 3, under section 3.7 for all HRTs. Figure 7.8 shows the BEP values derived for all HRTs. It was evident from the results that maximum BEP was obtained for 14 day HRT around 697.1wh, while 564.83wh and 215.23wh were reported for HRTs 16d and 10d respectively. The biomethane produced by AcoD can be used to generate power and heat in a co-generator. The electricity and heat that can be generated were calculated using equation (3.13 1nd 3.14) and results are shown in Figure 7.8.

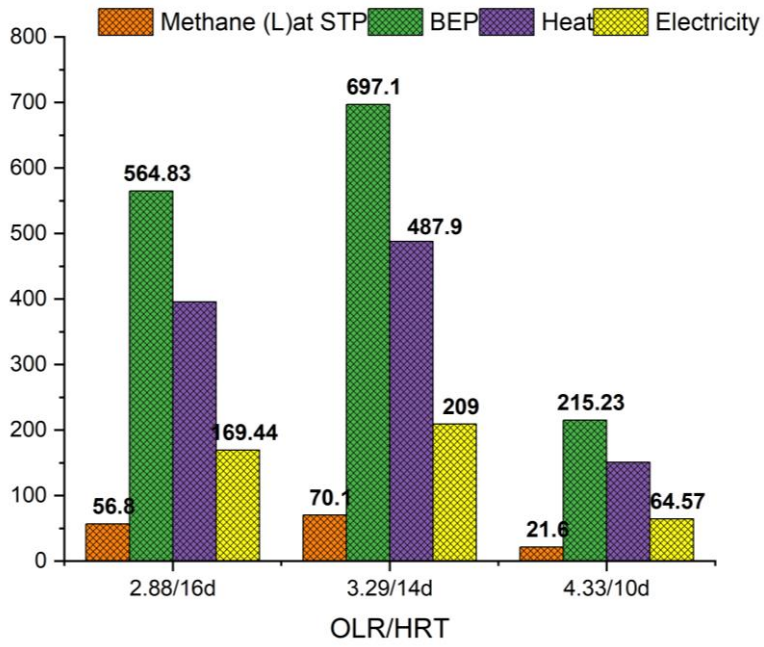

Figure 7. 8 8 Bioenergy potential derived from anaerobic co-digestion of whey and septage in lab scale digester

7.7 Design of industrial scale anaerobic digester

Based on the results obtained from the operation of lab-scale two-stage anaerobic digester treating whey and septage, an industrial scale digester can be designed. The quantity of whey wastewater generated at NSR dairy was considered for the design. The whole calculation was given below.

Estimation of daily whey wastewater flow

Assumption: 1Kg of cheese can be produced from 10L of milk

Quantity of milk used per cycle of cheese production = 500L

Number of cycles of cheese production per day=4

Total quantity of milk processed per day = 2000L

Daily cheese production = 200kg/d

Assuming that 85% of processed milk is generated as whey wastewater,

Quantity of whey wastewater produced = 1700L/d

Considering HRT of acidogenic reactor as 1 day, the volume of acidogenic reactor= 1700L

The volume of aciodegenic reactor can be approximately taken as $2m^3$ or 2000L

Considering HRT of methanogenic reactor as 10 days, volume=1700L/d * 14

= 23800L or 23.8m^3

Hence volume of methanogenic reactor can be taken as $24m^3$

Table 7. 5 Design details of industrial scale anaerobic digester

Acidogenic reactor (m^3)	**Methanogenic reactor (m^3)**	**OLR ($KgVS/m^3d$)**	**Obtainable methane yield (m^3/d)**	**Bioenergy potential (Kwh)**	**Obtainable electricity (Kwh)**	**Obtainable heat (Kwh)**
2	24	3.29	27.63	274.70	82.41	392.4

7.8 Major findings of the study

The significant findings of this study can be listed as;

- Anaerobic co-digestion of CW and SP (60:40) in presence of inoculum CM was efficiently demostrated in a 2-stage lab scale anaerobic digester.
- Acidogenic reactor attained steady state conditions when operated at HRT 1 day with an OLR of 57.8gVS/Ld.

- The mean maximum biogas production was obtained as 6.03L/L_rd at 1 day HRT with little or no methane content, showing complete inhibition of methanogens in acidogenic reactor.
- Among the VFAs generated in acidogenic reactor, acetic acid and propionic acid were identified as the major ones.
- The presence of increased propionic acid during a 3 day HRT led in VFA accumulation, which resulted in a decrease in biogas output.
- No change in COD levels was noticed in the effluent generated from acidogenic reactor, while volatiles solids showed 20% reduction.
- Switching to lower HRT (16 to 14d) has led to an increase in biogas and methane yield in methanogenic reactor.
- The average maximum biogas and methane yield were obtained at HRT 14d and OLR 3.29gVS/Ld
- Inhibition of VFA accumulation at HRT 10 days resulted in lower methane production in methanogenic reactor.
- A maximum BEP of 697.1wh was derived from methane yield at HRT 14d equivalent of heat and electrical energy of 487.9wh and 209wh respectively.
- An industrial scale anaerobic digester of total volume 26m^3 for treating whey was designed.

7.9 Conclusions

The performance of an upflow anaerobic digester, which treated a 60:40 mixture of CW and SP, produced favorable results in terms of methane production and organic matter degradation. It was discovered that maintaining appropriate operational conditions like as pH, temperature, alkalinity, and nutrient levels was critical to the digester's effectiveness. The two-stage system operation performed better than the single-stage system, especially for CW, which is ascribed to its high carbohydrate concentration. Furthermore, running the acidogenic reactor at a lower HRT reduced inhibitions produced by the formation of VFAs. Similarly, optimum value of HRT for operation of methanogenic reactor was obtained as 14d. The laboratory-scale digester's successful operation verified its potential for industrial-scale deployment, leading to the construction of an industrial-scale digester.

Chapter 8

Conclusions and recommendations

8.1 Conclusions

Numerous dairy enterprises have been established as a result of the rising demand for dairy products, and these sectors in turn generate wastewater with a significant potential for pollution. Whey residues, milk fat and proteins, dairy sludge, and liquid effluents from various cleaning, processing, and sanitation procedures are some of the main wastes produced by these sectors. Even though cheese whey dregs include a sizable amount of organic substance, primarily in the form of fats, lipids, and milk proteins, they were previously regarded as trash and discarded. Small and medium-sized dairies frequently dispose of these extremely toxic whey wastes in an improper manner due to financial restrictions. AD has been identified as a potential and eco-friendly method of treating highly organically loaded wastewaters, such as whey, to solve this environmental issue. AD of CW can offer three potential advantages, namely energy recovery, pollution reduction, and nutrient recovery. Although there has been numerous research on AD of whey wastewaters, there have also been issues with sludge flotation, acid accumulation, inadequate buffering, and nutrient imbalance in AD systems. This study makes some important recommendations for resolving these issues and enhancing the biodegradability and biogas generation of cheese whey wastes during AD.

The mono-digestion of whey resulted in fewer degradation rates with only 15.82% conversion of organic carbon to methane. An imbalance in pH and C/N ratio may explain whey digestion's low methane yield. The co-digestion studies with septage has shown that it served as an excellent co-substrate by providing essential nutrients, buffering and balancing C/N ratio. The maximum methane yield was reported for mixture with higher fraction of septage and whenever CW fractions are increased beyond 40% methane production rates reduced. When combined with active inoculum-cattle manure, the co-digestion mixture showed maximum methane yield at 60% whey fractions, indicating that this mix ratio is optimal. The results demonstrated that cattle manure exhibited higher methane production and better adaptability with CW and SP compared to the other two sewage sludge and anaerobic sludge. The experimental biogas production values were fitted to Gompertz model and all curves showed good fit with R^2 value lying between 0.996 and 0.998.

The study investigated three pre-treatment methods (sonication, ozonation, and enzymatic) for anaerobic digestion of whey. Results were analyzed based on sCOD solubilization, VS reduction, lactose hydrolysis, and methane production. Sonication led to increased sCOD release with longer treatment time, while ozonation decreases the sCOD solubilisation, but longer application time and less dosage increase the sCOD solubilisation. Enzymatic hydrolysis resulted in the highest lactose hydrolysis rates but lower sCOD solubilization. Among all, enzymatically hydrolyzed CW demonstrated the highest biogas production and shorter lag phase time, indicating faster digestion. Energy and cost analysis also favored the enzymatic method with a 490.8% increase in net energy gain compared to sonication and ozonation (236.01% and 187.68% respectively). The optimum operating conditions for carrying out enzymatic hydrolysis of whey was identified as; 4.63 pH, 40.47°C temperature,25.96 min reaction time and 0.49% enzyme concentration for achieving maximum lactose hydrolysis of 84.73%.

The effect of utilization of additives in AD of whey and septage was studied by adding septage-derived biochar at different TS concentrations (5-15%). The physico-chemical properties of biochar, such as elevated ash content, alkaline properties, greater porosity, increased surface area, and the inclusion of trace elements and essential nutrients, make it extremely well-suited for utilization as an additive in AD. The maximum cumulative biogas production was obtained at 50g/l of BC loading and 10% TS concentration, 486ml/gVS. With increase in biochar dosage, biogas production increased. Beyond 10% TS content, a reduction in methane yield was observed, which might be due to reduced diffusion co-efficient resulting from fewer mixing. The experimental and simulated values (using Gompertz model) showed least errors and R^2 value lied between 0.97 to 0.99.

In a lab-scale two-stage anaerobic digester, CW and SP were used as substrates under different organic loading rates (OLR) and hydraulic retention times (HRT). The acidogenic reactor produced a maximum daily biogas yield of 6.03 L/Lrd at HRT 1d, but this yield significantly decreased to 0.64 L/Lrd at HRT 4d. Higher HRTs (4d and 3d) resulted in increased volatile acid generation and propionic acid accumulation, indicating lower biogas production. The acidogenic reactor showed limited organic matter removal. In the methanogenic reactor, steady state conditions were achieved at HRT 14d, with an average methane yield of 1.02 ± 0.23 L/Lrd. Organic matter removal rates were reported as 85.22% (TCOD), 86.05% (sCOD), and 67.9% (VS). The calculated bioenergy potential at 14d HRT was approximately 697.1 Wh.

Based on these lab-scale results, a large-scale anaerobic digester with a volume of 26m^3 could be designed, assuming a daily whey wastewater generation of 1700L.

8.2 Significant findings of the study

- Co-digestion with septage increased the methane productivity of cheese whey by 117% when 10% of septage fractions are added.
- A co-digestion mix of 60:40 (CW: SP) in presence of cattle manure as inoculum resulted in a methane yield of 352.22 mL/g VS (mono-digestion of whey alone resulted in methane yield of only 66.1 ml/gVS) confirming the potential of septage as a co-substrate.
- Enzymatic hydrolysis by β-galactosidase showed a maximum lactose hydrolysis of 86.21% at optimised conditions; 4.63 pH, 26 minutes time, 0.49% enzyme dose and 40.5ºC temperature.
- A net energy benefit of 13.13 kJ/kg TS was obtained for the enzymatic method compared to sonication (6.31 kJ/kg TS) and ozonation (4.99 kJ/kg TS).
- The order of increase in sCOD solubilisation rates was US>Ozonation>enzymatic, while methane production rates increased Enzymatic>US>Ozonation.
- The bio-methane yield from enzymatically pre-treated whey was 3.6 times higher than that of raw whey.
- The energy calculations revealed that the pre-conditioning of organic-rich CW with enzymatic hydrolysis is more effective and efficient compared to physical and chemical pre-treatment methods.
- Septage derived biochar was found as a beneficial additive in AD on the basis of its structural, physical and chemical characteristics.
- Maximum cumulative methane production was reported for digester with 10% TS content added with 50g/l of biochar. (486.3 ml/gVS)
- Dominant VFA in digesters with TS content 5%,7.5% and 10% were acetic acid and butyric acid, whereas at TS-15% propionic acid accumulation was found.
- Acidogenic reactor attained steady state conditions when HRT is reduced from 4d to 1d with maximum biogas production rate of 6.03 L/L_rd.
- Switching to a lower HRT of 10 days from 14 days in methanogenic reactor resulted in reactor instability due to significant accumulation of VFA.

- Two stage anaerobic digester having capacities 2 m^3 and 24 m^3 can be designed for treating cheese whey wastewater for a local dairy having 1.7m^3 of daily wastewater flow.

8.3 Scope for the future work

- Studies on recovery of intermediates produced during AD of cheese whey wastewater like ethanol, butanol, hydrogen, can be done.
- Since whey and whey permeate are excellent sources of lactose, those can be effectively transformed into bioethanol under strict anaerobic conditions; which can be studied.
- More studies on immobilized enzyme activity during enzymatic pre-treatment can be conducted. Incorporation of by-products formed during other dairy product processing stages in anaerobic co-digestion with whey can be studied.

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