Enhancement of biogas production from rice straw by co-digestion and pretreatment techniques

A Thesis

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By

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CERTIFICATE

This is to certify that the thesis entitled **"Enhancement of biogas production from rice straw by co-digestion and pretreatment techniques"** submitted by **Jyoti Kainthola** (Registration No. 166152003) to the Indian Institute of Technology Guwahati, for the award of the degree of Doctor of Philosophy is a record of bonafide research work carried out by her under our supervision and guidance. The thesis work, in our opinion, has reached the requisite standard fulfilling the requirement for the degree of Doctor of Philosophy. The results contained in this thesis have not been submitted to other university or institute for award of any degree or diploma to the best of my knowledge and belief.

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STATEMENT

I do hereby declare that the work contained in this thesis is original and has been done by me under the general supervision of Prof. Ajay Kalamdhad (supervisor) and Prof. Vaibhav V. Goud (co-supervisor).

The work reported herein has not been submitted to any other Institute for any degree or diploma. Whenever I have used materials (concepts, ideas, text, expressions, data, graphs, diagrams, theoretical analysis, results, etc.) from other sources, I have given due credit by citing them in the text of the thesis and giving their details in the references. Elaborate sentences used verbatim from published work have been clearly identified and quoted.

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ABSTRACT

Due to rapid economic development over the past few decades, global energy consumption has intensified continuously, causing not only greenhouse gas emissions, but also energy shortage, and in some areas even energy crises. This imminent energy consumption and demand have been the motivation for world scientists to explore alternative energy sources that could replace fossil fuels. Agricultural residues (i.e., wheat straw, rice straw, corn straw, etc.) are the most abundant resource of lignocellulosic wastes, and contribute a major role in producing low-cost and sustainable forms of energy via anaerobic digestion. Anaerobic digestion is a realistic approach to concurrently manage rice straw and harness renewable energy. Inoculum plays a major role in the process of anaerobic digestion; selecting appropriate inoculum is a crucial factor to initiate the anaerobic digestion process. It not only validates the several biochemical and microbial processes, but also enhances the overall methane yield**.** In order to select the appropriate inoculum for rice straw, biochemical methane potential (BMP) assay of anaerobic digestion of rice straw with digested cow dung and fresh cow dung revealed methane yields of 125.77 and 72 mL/g-VS_{added}, respectively. The 16S metagenomics sequencing revealed that DCD is enriched with majority of anaerobes.

Initial characterization of rice straw expressed that it has high potential for energy recovery in the form of biogas but biodegradability is restricted by improper nutritional and recalcitrance structure. To balance the improper nutritional structure of rice straw and recompense the nitrogen deficiency of rice straw, rice straw was digested with other nitrogen-rich co-substrates (*Hydrilla verticillata* and food waste) to advance its characteristics in anaerobic co-digestion study (AcoD). A central composite design – response surface methodology (CCD-RSM) was used for defining the experimental design for AcoD of rice straw with *Hydrilla verticillata* and food waste and results of this study showed significant interaction of carbon/nitrogen (C/N) ratio, food/microrganism (F/M) ratio and pH on methane yields (output response). The optimum condition for anaerobic co-digestion of *Hydrilla verticillata* (C/N-29.18, F/M:2.45 and pH 7.37) and food waste (C/N-30, F/M:1.87 and pH 7.32), showed methane yield of 287.6 mL/g-VS_{added} and 323.78 mL/g-VS_{added}, 38.9% and 49.55% higher than the mono-digestion of rice straw, respectively.

The recalcitrant behaviour of rice straw marks pretreatment an important step to facilitate the transformation into renewable energy. Therefore, different pretreatment

techniques i.e. thermal (hot air oven, microwave, autoclave, water bath), electrohydrolysis and fungal (three fungal strains i.e. *Pleurotus ostreatus, Phanerochaete chrysosposrium, Ganoderma lucidum*) were used for accelerated hydrolysis in anaerobic digestion of rice straw. BMP assay were assessed corresponding to the maximum solubilization rates obtained in pretreatment study and specific methane yields were obtained as 367.68 mL/g-VS_{added} (F/M:2.5) (thermal), 319.03 mL/g-VS_{added} (F/M:2) (electrohydrolysis) and 339.31 mL/g-VS_{added} (F/M:2) (fungal) for different pretreatment. The physicochemical characteristics of pretreated rice straw were investigated by Fourier transform infrared spectroscopy (FTIR) and Field emission scanning electron microscopy (FESEM). FESEM analysis showed the increased porosity of the rice straw, FTIR analysis showed the omission of hemicellulose and lignin from the matrix.

In order to check the feasibility of optimum conditions (maximum methane yields) obtained from batch study in continuous mode, single phase continuous anaerobic reactor (SPCAR) was operated in three phases with untreated, pretreated (microwave) and co-digested (food waste). The results of continuous study suggested that pretreated and co-digested rice straw produced significantly enhanced methane production as compared to untreated rice straw. The reactor showed superior process performance for pretreatment (microwave) at 8.3 kg-VS/m^3/d OLR progressively, with maximum 33.43% VS degradation, co-digested rice straw showed 24.02% VS degradation obtained for an OLR of 6.5 kg-VS/m^3/d, whereas, untreated rice straw showed the VS degradation of 9.92% for an OLR of 3.5 kg-VS/m^3/d.

Keywords: *Anaerobic digestion, rice straw, pretreatment, co-digestion, biochemical methane potential, food waste, Hydrilla verticillata.*

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LIST OF ABBREVIATION AND SYMBOL

ABE	Acetone–butanol–ethanol
AcoD	Anaerobic co-digestion
AD	Anaerobic digestion
ADM1	Anaerobic digestion model no. 1
AWAO	Alkaline wet air oxidation
BMP	Biochemical methane potential
C/N	Carbon/Nitrogen
CSTR	Continuous stirred tank-reactor
DCD	Digested cow dung
F/M	Food/microorganisms
FA	Free ammonia
FCD	Fresh cow dung
FESEM	Field Emission Scanning Electron Microscopy
FTIR	Fourier Transform Infrared spectroscopy
FW	Food waste
GL	*Ganoderma lucidum*
HRT	Hydraulic retention time
H.verticillata	*Hydrilla verticillata*
LCC	Lignin carbohydrates complex
LFD	Liquid fraction of digestate
LFM	Logarithm function model
LS-AD	Liquid-state anaerobic digestion
M	Methane production potential
M.C.	Moisture content
MGM	Modified Gompertz model
NDF	Neutral detergent fibre
OLR	Organic loading rate
PC	*Phanerochaete chrysosposrium*
PO	*Pleurotus ostreatus*
R_m	Maximum methane production rate
RS	Rice straw
sCOD	Soluble chemical oxygen demand

SPCAR	Single phase continuous anaerobic reactor
SS-AD	Solid-state anaerobic digestion
TFM	Transference function model
TS	Total solids
VFA	Volatile fatty acids
VS	Volatile solids
Z	Cumulative methane production rate
λ	Lag phase constant

CHAPTER 1

INTRODUCTION

This chapter consists of a brief discussion about the production of agricultural residues and the problems associated with its management. The overview, objectives and the need of the study have also been included in this chapter.

1.1 OVERVIEW

The major global challenges that exist today involve climate change, energy crisis and food security for a burgeoning population. If the ongoing fossil fuel exploitation continues at the current pace, the proven reserves of oil, coal and gas shall lapse after 100 years (Singh et al., 2014). However, it is estimated that the global energy demand, irrespective of the compounding factors like climate change, urbanization, industrialization and increasing affluence, will increase by 50%, by 2030, based on the forecasted population surge. Apart from the scarcity of fossil fuels, other disadvantages associated with the usage of fossil fuels, such as global warming due to greenhouse gases emission, pollution, resource depletion, geopolitics due to concentration of fossil fuels in some countries and unbalanced demand-supply relations, have led to increasing need for the search of alternate source of energy to satisfy the global energy demand (Report, 2018). This needs environmentally sustainable solutions that simultaneously tackle each of these challenges, while at the same time maintain cost-effective approach, which is practical for large scale applications (Dong et al., 2018). This imminent energy consumption and demand have been the motivation for world scientists to explore alternative energy sources that could replace fossil fuels. Use of crop residue as a possible source of feedstock for bioenergy production must be critically and objectively assessed because of its impact on reducing global greenhouse gas emissions and making valuable land resources available for food production. Agricultural residues (i.e. wheat straw, rice straw (RS), corn straw etc.) are the most abundant resource of lignocellulosic wastes, and contribute a major role in producing low-cost and sustainable forms of energy. Agriculture is a source of energy through its production of biomass, which can be used as biofuel and is a renewable resource. The energy content of residue varies among different crop. The annual residue production is estimated to be 2.8 billion tonne of cereals, 305 million tonne of legumes, 108 million tonne of oil crops, 373 million tonne of sugar crops and 170 million Mg of tubers. The total crop residue production in the world is estimated to be 3.8 billion tonne, of which 74% are of cereals, 8% of legumes, 3% of oil

crops, 10% of sugar crops and 5% of tubers (Lal, 2012). The Crop residue managed predominantly through burning leads to significant air pollution. India is the second largest producer of wheat and rice in the world. According to Government of India (GoI) Annual Report 2016-17, 54.6% of the population is engaged in agriculture and allied activities and it contributes 17% to the country's Gross Value Added. Thus, agriculture plays a pivotal role in India's economy (Soam et al., 2017).

Ministry of New and Renewable Energy (Hiloidhari et al., 2014), GOI estimated that about 500 Mt of crop residue is generated every year. In terms of availability, RS is the major agricultural residue in the world, rice crop alone contributes 34% of crop residues (Croce et al., 2016). Jain et al. (2014) reported that for every ton of rice harvested, approximately 1.35 tons of RS remain in the field with energy potential. RS, composed mainly of cellulose, hemicellulose and lignin, remains in the fields after harvest and is costly to gather as it is bulky and difficult to transport (Gu et al., 2014). To clear the field for the next crop, farmers apply the illegal practice of burning of straw leading to harmful effects on the environment and leads to the loss of nutrients such as Nitrogen (N), Phosphorous (P) and Sulphur (S) (Soam et al., 2017). Therefore, to avoid the deleterious effects of burning and to take advantage of the huge energy potential of straw, the utilization of straw for various other activities should be promoted. However, some shortcomings were observed in direct utilization of RS, such as long assimilation time due to low cellulose conversion rate, and excessive Carbon/Nitrogen (C/N) ratio. Excessive C/N ratio can inhibit the methane production due to the changes occurred in the microbial composition and metabolic pathway transfer. There are several productive techniques that can be used for straw management such as composting, recycling in soil, production of electricity and animal fodder. In addition, RS is also a promising feedstock for ethanol and biogas production (Sawatdeenarunat et al., 2015; Zhu et al., 2016).

AD is considered to be one of the most efficacious technologies for sustainable alternative energy recovery on both small and large scales. During the AD, organic waste (i.e. RS) is biologically decomposed and converted into biogas. Pretreatment is used to improve the degradability of RS and accelerate the AD process. Induced delignification process by pretreatment increases the rate of biomass decomposition as well as accelerated biogas production. Whereas, anaerobic co-digestion (AcoD) is a process in which more than one waste are treated in same digester that results in improved balance of nutrients (C/N ratio); due to synergistic effects of microorganisms. Therefore, advanced AD may be a promising alternative approach to deal with RS disposal problems in concentrated rice production regions.

Biological processes (i.e. AD) require much less energy input and can accommodate either wet or dry feedstock economically on both small and large scales. During the anaerobic digestion, organic waste (i.e. RS) is biologically decomposed and converted into biogas (Abbasi et al., 2012; Mao et al., 2015). However, RS mainly consists of cellulose, hemicellulose and lignin and is denoted by a complex recalcitrant structure to decomposition (Caroline et al., 2018; Wang et al., 2018). The lignin percentage in substrate determines the degradation capacity of the substrate, as this component prevents the effectiveness of microbial action. Pretreatment is used to improve the degradability of RS and accelerate the AD process. A wide range of processes have been studied, concerning their application as pretreatment methods, resulting into enhanced biogas production. These methods can be classified as physical (e.g. milling, freezing, grinding), chemical (e.g. acid, oxidative, alkaline), physico-chemical (steam explosion, liquid hot water, CO_2 explosion) and biological, combination of these pretreatment have also been tried (Li et al., 2018). In order to be considered as efficient method; pretreatment techniques not only disrupt the structure of biomass but also have less energy demand need and less treatment cost (economical). Various studies have been performed using crop residue as mono-substrate for biogas production (Khalid et al., 2011; Wang et al., 2014) but the direct utilization of RS by microorganism in anaerobic digestion process is complicated because of unstable nutritional properties (high C/N ratio) (Janke et al., 2015; Guan et al., 2018). Therefore, co-digestion is assumed to be cost-effective enhancement techniques for compensating the imbalanced parameters. Anaerobic co-digestion is a process in which more than one wastes are treated in same digester that results in improved biogas yield, improved balance of nutrients (C/N ratio); due to synergistic effects of microorganisms and increased organic load. For efficient co-digestion, it is required that manure has high total nitrogen contents because it decreases C/N ratio of RS. Agricultural residues such as RS are produced annually in large quantities throughout the world. Since agricultural wastes are a plentiful source of organic matter, these can be used as a valuable alternative feedstock for biogas production (Li et al., 2011). Furthermore, these wastes also have a considerable amount of carbon that may be beneficial for anaerobic co-digestion with animal manure. Therefore, advanced anaerobic digestion may be a promising alternative approach to deal with RS disposal problems in concentrated rice production regions.

Financial incentives and legislation are growing in support of this technology and it is ideal way to recover costs from excess agricultural waste in a farm-scale system. Historically, RS has not been a selected substrate for energy production because of its complex, lignocellulose structure that makes it difficult to decompose (Dehghani et al.,

2015; Soam et al., 2017). But several emerging factors including the abundance of RS, discoveries on appropriate inoculant and pre-treatment strategies, and the depleted tolerance for wasted biomass contributing to greenhouse gas emissions, support the perspective that RS can no longer be overlooked as a viable renewable energy source that must be utilized.

1.2 OBJECTIVES OF THE STUDY

The main objective of the study was to enhance the biogas production of RS by co-digestion strategy that utilizes nitrogen rich co-substrate and pretreatment, where lignocellulosic substrate was subjected to pretreatment (thermal, electrohydrolysis and fungal), as a means of introducing disruption to its structures, and consequently enhancing its degradability. The scope of the present study was limited to:

- BMP (1 L) test of rice straw with fresh cow dung (FCD) and digested cow dung (DCD) as inoculum followed by 16 S rRNA Metagenomics study of best inoculum.
- BMP test for co-digestion of rice straw with *Hydrilla verticillata* and food waste (nitrogen rich co-substrate) followed by optimization of operational parameters (C/N ratios, F/M ratios and pH) by central composite design – response surface methodology (CCD-RSM).
- Pretreatment studies i.e. thermal (hot air oven, microwave, autoclave, water bath), electrohydrolysis and fungal (three fungal strains) for accelerated hydrolysis in anaerobic digestion.
- Design, fabrication and operation of lab scale single phase continuous reactor and its feasibility study for untreated, pretreated and co-digested rice straw.

1.3 NEED OF THE STUDY

Biogas has become an alternative clean source of energy. Agricultural residues being renewable and abundant resources could be efficiently used as a feed for methane production. Production of rice continues to rise in order to provide a stable food source for over half of the world population, consequently, RS produced will be an abundant and available agricultural waste. The collection and treatment of RS through anaerobic digestion is not only a viable option for producing clean, renewable energy, but it will also eliminate a major source of greenhouse gas emissions from common practices of open burning or tilling the straw back into the fields. The specific methane potential of RS ranges from 92 to 404 L/kg-VS_{added}, depending on the digestion parameters and pretreatment methods. RS has very less amount of moisture and nitrogen content, so using

another substrate and appropriate balance and buffering capacity of the system can be maintained. One of the several significant challenges with the digestion of RS is the lignocellulosic structure that makes bacterial decomposition difficult. This challenge can be overcome by pretreatment strategies such as thermal (i.e. hot air oven, microwave, autoclave and water bath), electrohydrolysis and fungal; which are successful in increasing biogas potential and accelerating the degradation process of RS.

1.4 SCOPE OF THE STUDY

In order to enhance the biogas yield, pre-treatment process and co-digestion are the prerequisite to disrupt the carbohydrate-lignin complex that weakens the accessibility of enzymes and microbial population and balance the improper nutritional structure that weakens the growth rate of microbes. The scope of the present study is limited to characterize the RS, fresh and digested cow dung, *Hydrilla verticillata* (*H.verticillata*) and food waste (FW) collected from the Amingoan village near Indian Institute of Technology Guwahati (IITG) campus and Deepor Beel, which is a Ramsar wetland site close to IITG. To conduct the biochemical methane potential (BMP) test (1 L capacity) studies for untreated RS with fresh and digested cow dung, nitrogen rich co-substrates for different C/N ratio and pretreated RS and optimizing the best result from the study. Effects of different pretreatment techniques on the hydrolysis of RS with respect to pH, soluble chemical oxygen demand (sCOD), volatile fatty acids (VFA), volatile solids (VS) etc. and its characterization using Field Emission Scanning Electron Microscopy (FESEM) and Fourier Transform Infrared spectroscopy (FTIR) will be studied to determine the best pretreatment technique. Based on the above studies, batch study was done (20 L capacity) with the best C/N ratio and best pretreatment technique. Finally, design, fabrication and operation of lab scale single phase continuous anaerobic reactor for biogas production with untreated, pretreated and co-digested rice straw optimum parameters.

1.5 THESIS ORGANIZATION

The present thesis covers eight chapters with appropriate sections and subsections and also contains references and visible research outputs. A brief description of these chapters is mentioned as follows:

- **Chapter 1** gives the brief discussion about the RS production and problems related to its improper management, proposed anaerobic digestion technique, objectives, need of the study and scope of the thesis.
- **Chapter 2** includes the detailed literature review of anaerobic digestion of RS, challenges in anaerobic digestion, pretreatment techniques, co-digestion, enhancement in inoculation efficiency, optimum parameters in anaerobic digestion process and various biogas reactors.
- **Chapter 3** consists of experimental design of different phases I, II, III and IV and methodologies. The detailed procedures of biochemical and physio-chemical analysis and instrumental analysis are provided.
- **Chapter 4** presents the BMP assay to check the efficacy of digested and fresh cow dung as inoculum and optimum F/M ratio for study.
- **Chapter 5** includes the anaerobic co-digestion of RS with *H.verticillata* and FW.
- **Chapter 6** deals with the effect of different pretreatment techniques i.e. thermal, electrohydrolysis and fungal pretreatment on solublisation of RS for enhanced methane yields.
- **Chapter 7** deals with the design and operation of single phase anaerobic reactor operated in for different operational optimum parameters obtained from phase chapter 4, 5 and 6.
- **Chapter 8** lists the conclusion obtained from different phases and recommendation for future work.

CHAPTER 2

LITERATURE REVIEW

This chapter covers the detailed literature review on the AD of RS, pretreatment of lignocellulosic biomass and co-digestion with different substrate to increase the performance of AD. This chapter also includes the effect of these techniques on lignocellulosic degradation, synergistic interaction with high C/N ratio feedstock and improving the buffering capacity by inoculum ratio optimization.

2.1 BIOCHEMISTRY OF ANAEROBIC DIGESTION

AD is a biological process for conversion of organic substrate into biogas by microorganisms in the absence of oxygen. It is the process, which not only generates renewable biogas energy (methane ≈ 50-75% and carbon dioxide (CO_2) ≈ 25-50%), but also contributes in reduction of emission of greenhouse gases, eutrophication, depletion of dissolved oxygen etc. AD is affected by various operational parameters such as temperature, pH, C/N ratio, alkalinity, organic loading rate, hydraulic retention time and concentration of VFA (Mussoline et al., 2017). Composition and heating value of the produced biogas depends upon the utilized substrate and provided digestion conditions. Biogas mostly constitute of methane and carbon dioxide, with minor amount of other gases viz. hydrogen sulphide, nitrogen, hydrogen, ammonia and water vapours.

AD process comprises of four stages namely hydrolysis, acidogenesis, acetogenesis and methanogenesis (Fig. 2.1), which are carried out by different set of microorganisms i.e. acidogens, methanogens etc. Hydrolysis is the first step of AD in which conversion of complex biopolymers such as lipids, carbohydrates, polysaccharides, proteins and nucleic acid into soluble compounds such as monomers, sugar, amino acid, fatty acids, purines and pyrimidine's takes place by the hydrolytic bacteria. In the next step, i.e. acidogenesis, conversion of simplified monomers, sugar, amino acids and fatty acids into intermediate propionic, butyric acid etc. is carried out by fermentative bacteria. Acetogenesis is the process of conversion of product of acidogenesis into acetate by homoacetogens. At the final stage, methanogenesis takes place, in which methane (biogas) is produced from acetate and carbon dioxide, by two groups of methanogens; i.e. acetoclastic (acetate consumers) and hydrogen utilizing methanogens (carbon dioxide reducing methanogens). Acetoclastic methanogens convert acetate into methane and carbon dioxide, while hydrogen - utilizing methanogens produce methane using carbon dioxide and hydrogen as electron acceptor and

donor respectively (Shah et al., 2015). The different functional microbial interactions are complex and, imbalance between microbial groups affects the reaction rate and causes the accumulation of inhibitory substances. Among all microbial groups, methanogens are considered to have the slowest growth rate, thus growth of methanogens are supposed to be rate limiting stage in AD process. However, in case of lignocellulosic substance hydrolysis is the rate limiting step. AD process is classified into solid and liquid-state AD (LS-AD) depending upon on TS content, AD consisting TS% more than 15% are classified as solid-state AD (SS-AD) and less than 15% as LS-AD (Zhao et al., 2014; Zheng et al., 2018). LS-AD usually has greater reaction intensity and shorter retention times, whereas SS-AD system has lesser reactor capacity, less energy necessity, which are required to control the floating of lignocellulosic material can be solved easily.

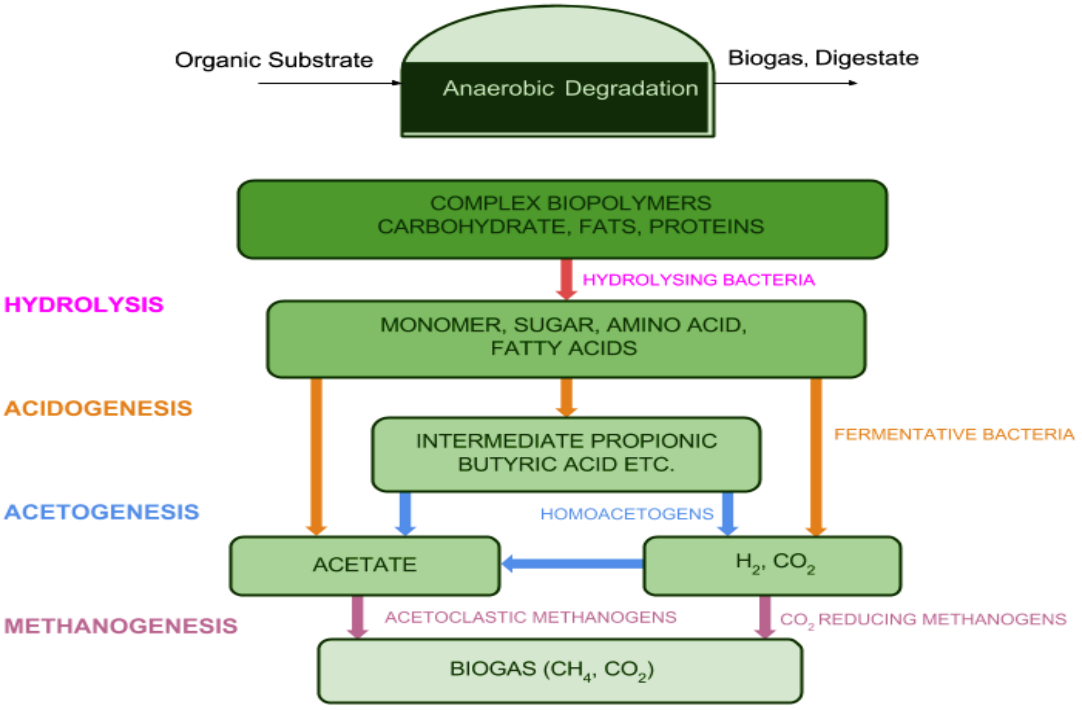


Fig. 2.1. Process flow diagram of anaerobic digestion of organic substrate

2.2 OPERATING PARAMETERS FOR EFFICIENT ANAEROBIC DIGESTION

AD process is very intricate in nature that degrades all organic matter in the absence of oxygen. This process is governed by various parameters such as pH, trace elements, micro-macro nutrients etc. It is thus particularly significant to maintain these parameters within the desired level for long term operability of AD process. These parameters are described in the subsequent section.

2.2.1 Temperature

Temperature is one of the important parameter for survival of microbes in AD process. Temperature range can be used to categorize different degradation process. Three operational temperature range exists in which AD can be carried out; psychrophilic (below 20ºC), mesophilic (20-45ºC), thermophilic (55-70ºC) (Appels et al., 2008; Divya et al., 2015). Thermophilic range has the benefits above mesophilic range, such as its earlier degradation and larger organic loading rate, consequently shows greater efficiency, but fatty acids accumulation; which causes acidification, occurs in thermophilic range. This acidification causes the inhibition of biogas production. Reduced stability, poor quality effluent, toxicity increase, deprived methangonesis are some of the other disadvantages during AD in thermophilic range. Mesophilic AD shows greater process stability and better richness in anaerobic bacteria, but they provide lesser biogas yield in comparison to thermophilic AD process. Thus optimal conditions for AD would be hydrolysis in thermophilic range and methanogenesis in mesophilic range (Hedegaard and Jaensch, 1999; Ward et al., 2008). Seasonal/ambient temperature digestion is also used to degrade organic substrates. This AD processes does not entail additional heat quantity but expresses lower biogas yield and lesser stability than thermophilic and mesophilic range. AD microbes are very sensitive to temperature gradient; reduction in temperature results in reduction of VFA generation rate, feedstock utilization rate and metabolism of microbes and also results in reduced biogas yields.

2.2.2 pH and buffer capacity

The operating pH directly affects the AD progress and the intermediate products. The appropriate pH range for the process is described to be 6.8-7.4 (Mao et al., 2015). Organic acids in greater amount are produced during the initial days of digestion, which decreases the pH of the reactor in the initial days. But as the process progresses the amount of ammonia production enhances, and pH value increases to 7-7.2. When pH reaches to 7.2-8.2, the methane generation stabilizes in the system. During the degradation of agriculture residue, fermentation/acetogenesis stage is very rapid; this inhibits the process by the reduced pH value. In this case pH drop is avoided by the addition of lime (Ramasamy and Abbasi, 2001; Abbasi and Abbasi, 2012). In general, drop of pH is the indicator of generation of higher amount of carbon dioxide. The growth rate is significantly influenced by the pH change. The abundant microbial population is observed from pH 4-7.5. *Clostridium butyricum* bacteria population exists at pH 6, however at pH 8, *Propionibacterium* community prevails during acidogenesis phase in the chemostat culture

(Zhang et al., 2009). A substantial correlation existed between pH and hydrolysis. Thus, the hydrolysis rate is deliberated to be pH dependent. Acidogenic and methanogenic bacteria have optimum pH range for operation; 5.5-6.5 and 6.5-8.2 respectively, this is why anaerobic reactor separating the hydrolysis and methanogenesis is the favored mode of action.

Buffering capacity is also considered one of the indicators for measuring AD process imbalance than directly measuring the pH in the system, as buffering capacity will decrease suddenly with the accumulation of fatty acids before the drop of pH is observed (Mussoline et al., 2012). It is often described as alkalinity in AD, thus it is proportional to quantity of bicarbonate ions. Increasing the buffering capacity is mainly accomplished by decreasing the influent loading rate, though directly adding bicarbonate is more precise as addition of carbon dioxide will entail time lag for equilibrium of gas to occur, which resulted into over-dosing in the system (Ward et al., 2008). S/M ratio can also be used to retain high buffering capacity and constant pH in the system.

2.2.3 Organic loading rate (OLR) and hydraulic retention time (HRT)

OLR is defined as amount of volatile matter fed into the anaerobic digester/day during continuous feed. Addition of high concentration of fresh feed daily resulted into alteration in the reactor environment and temporarily inhibited the microbial activity during the initial phases of fermentation. This microbial inhibition occurred due to large OLR causing to greater hydrolysis/fermentation bacterial activity than methanogens activity, thus leading to accumulation of acids. Biogas production rate is proportional to OLR and its yield was found to rise with reducing organic loading rate. Optimum feed rate exits for every particular size of plant, which produces maximum amount of biogas. On the basis of biogas plant studies, maximum gas yield was obtained for an OLR of 0.24 g/m^3, though 66.67% of VS reduction was obtained in the digester (Nagao et al., 2012; Zhou et al., 2012). Thermophilic and digestate recirculation system have higher potential to release the overloading inhibitory effects. Bacterial community also varies with OLR, *Firmicutes* exists at low OLR and *Actinobacteria*, *Deferribacteres* and *Bacteroidetes* have been perceived at large OLR. Hydraulic retention time (HRT) or hydraulic loading rate (HLR) is the average time spent by the substrate in the reactor. Lesser retention time surfaces the risk of active biomass washout, whereas longer retention time needs a larger volume of the reactor; hence less economical design. In the thermophilic and mesophilic temperature ranges fermentation/acidogenesis can occur at less HRT without stressing the other process (Leitão et al., 2006).

2.2.4 Accelerator in biogas production

The addition of accelerants in the AD process increases the localized surface area and desirable conditions for the adsorption of microbes on substrate, which leads to higher biogas yield. Different accelerant like green biomass, biological additives and inorganic additives exists in the nature. Greenery biomass consists of plant extracts, weeds residue, lignocellulosic biomass and ensilage material. These extract contains naturally existing steroids that acts as metabolic stimulator for microbes. Teresan and aquasan stimulants have been used in previous studies to improve AD. Leaves of some legumes and plants in powered form have been found to increase biogas yield by 1.18 to 1.4 times (Nagao et al., 2012; Zhou et al., 2012). Biological additives such as fungal, microbial consortium, enzymes etc. enhances the availability of cellulose and hemicellulose, thus contributing to greater digestibility. The addition of hetero and homo hydrolysing strains has depicted positive influence on AD, but the mixture of these strains with bacteria or fungus have shown even better performance. An enzyme depicts high degradation results by biochemical catalytic interaction. Enzyme as a microbial supplement ensures the optimum growth rate and activity of different type of microbes by making them more resilient to shock loading. Inorganic additives like chemical, alkali and oxidative reagents etc. are largely used for treatment of recalcitrant lignocellulosic material due to their low cost and higher effectiveness. These additives results in the disruption of hydrogen bonds, covalents bonds of the substrate, which consequently increases the degradability of substrate. These additives are not suitable for easily degradable substrate having high concentration of carbohydrate, due to its accelerated digestion and subsequent accumulation of fatty acids

2.2.5 C/N ratio

The composition of carbon to nitrogen in an organic substrate is denoted in terms of C/N ratio. It is important to maintain C/N ratio in the optimal range for efficient AD. C/N ratio in the range of 16-25 or 20-30 or 20-35 has been shown to optimum for balanced AD. It reveals the nutrient levels of the digestive process, thus system is sensitive to C/N ratio. Low protein solubilisation rate occurs in terms of in terms of high C/N ratio, which leads to low FA and total ammonical nitrogen (TAN) in the system. High C/N ratio also ceases the availability of nitrogen that is required to maintain the desirable microbial flux in the reactor, which leads to decreased biogas generation and vice versa. Feedstock with extremely low C/N ratio augments the risk of ammonia inhibition in the system, this high accumulated ammonia is toxic to methanogens and inhibits the biogas production (Gupta et al., 2012; Kondusamy and Kalamdhad, 2014). Few studies reported that C/N ratio diverges

with temperature. Ammonia inhibition observed at 35ºC and 55ºC with C/N ratio of 15 and 20, respectively (Chen et al., 2016). The maximum methane yield of 341 L/ (Kg of VS added) was achieved from co-digestion of straw and manure at 25 C/N ratios. Equally 25 and 30 C/N ratios delivered the highest cumulative methane production levels, 3 times equated to C/N ratio of 15 (Mao et al., 2015; Mussoline et al., 2017).

2.2.6 Mixing/agitation condition

Mixing or agitation is necessary to impart efficient interaction of active microbes and organic matter, to prevent settling of coarser material, to achieve slurry homogeneity, to avoid development of temperature gradient etc. in the AD system (Gómez et al., 2006; Rizwan et al., 2015). Mixing occurs in continuous or intermittent mode; numerous times in an hour or numerous times in a day, with an energy input from 10-100 W-h/m^3. High speed mixing reduces the biogas production whereas; low speed mixing allows digester to absorb the disruption due to shock loading. The extent of agitation also depends upon solid concentration in the reactor. Formation of anaerobic granules has been shown to be the reason for low yield of biogas. Efficiency of a mixing in a reactor is measured by its hydrodynamic test study, which determines if a rector is operational to its full capacity. Hydrodynamic study uses Li^+ as detectable tracer in AD; this detector is non-toxic to the system in low concentration, and likely to be present in most of the feedstock (Ward et al., 2008). Recirculation of digestate or biogas with the pump through the bottom of the digester can also be used to provide desired mixing in the reactor.

2.2.7 Toxicity

Toxic substances either pre-exist in the system or are produced during the degradation of substrate. The non-dissociated concentration of hydrogen sulphide is lethal for sulphate reducers and methanogens (Naji et al., 2016; Zhou et al., 2016). This form can easily diffuse through the membrane of cell, so it is considered to be the most toxic form. It causes the denaturation of protein and also interferes with the assimilatory metabolism of bacteria. Concentration, lesser than 0.003 and 0.002 mole/L for sulphur, H_2S respectively are considered to be inhibitory. Some studies suggested that toxicity is related to the unionised concentration of sulphide in pH range of 6.8-7.2 (Weiß et al., 2010; Anjum et al., 2016) Detergents and mineral ions of heavy metals are some other toxic substances that inhibit the growth of microbes in the reactor. Smaller concentration of these substances stimulates the growth but larger concentrations could be inhibitory for the bacteria's growth. Termination of feeding in the reactor and flushing the contents to reduce the concentration of these

substances below the toxic level is the recovery method for the digester (Appels et al., 2008).

2.2.8 Effect of trace elements on performance

Trace elements such as iron (Fe), cobalt (Co), nickel (Ni), zinc (Zn) etc. present in the digestion system influences the multistage AD process. Trace elements should be supplied in adequate amount to support the metabolism of microbial population, else. the performance of the AD process will cease (Choong et al., 2016). Studies revealed that trace elements Co, Fe, Cu, Zn, Ni concentration lesser than 30, 1.32, 0.12, 1.13, 4.8 g/L respectively inhibited the growth of methanogens in the system (Zhang et al., 2003). Fe was found to be significant in stimulating heme protein ferroxins (Fd) and cytochromes; responsible for energy metabolism in cell (Yenigün and Demirel, 2013). Trace element occurrence and supplementation are interrelated and have different effect in the various stages of AD process. The addition of micronutrients influences the methanosarcina and archeal population and subsequently enhances the AD performance. The supply of optimum dosage of trace elements results includes the efficient organic matter degradation, low fatty acids accumulation, higher digester stability, which in turn leads to the enhanced biogas production. Supplementations of multiple elements have positive impact on digestibility of substrate (Shitophyta and Fuadi, 2016).

2.3 CROP RESIDUE GENERATION

Over the centuries, agriculture is the backbone of economic as well as social development in India. As agricultural productivity of the country is good, crop residues production is also huge, it is estimated that approximately 500-550 Mt of crop residues are produced per year in the country. The cereal crops (rice, wheat, maize, millets) contribute 70%, while rice crop alone contributes 34% to the crop residues, wheat ranks second with 22% of the crop residues, whereas fiber crops contribute 13% to the crop residues generated from all crops (Sawatdeenarunat et al., 2015; Nguyen et al., 2016).

Current disposal methods for crop residue management have caused widespread environmental concerns. The open burning of agricultural residue is one of the significant global source of greenhouse gas emissions and has a significant impact on climate change and human health (Sharma et al., 2010). Field burning of crop residues reduces total N and C in the upper soil layer. Besides, this elevated soil temperature due to field burning causes death of active beneficial microbial population. Therefore, recycling of nutrients in the soil–plant ecosystem is essential for soil fertility enhancement, through incorporation of crop

residues in the field (Soam et al., 2017). Use of agricultural residues as a feedstock as energy source is gaining importance to support and stimulates the India's eco-agricultural growth (Parawira, 2004). In India, estimated amount of surplus residue available is between 84 and 141 Mt year^{-1} of which nearly 70 MTs (44.5 Mt RS and 24.5 Mt wheat straws) are burned annually (Pellera and Gidarakos, 2018). Therefore, AD of RS is a prominent technique to convert valuable nutrients like N, P, C, K etc. present in straw into methane rich biogas and avoid onsite burning of RS. This methane gas can be further used to produce electricity, to run fuel cells, for cooking and heating purpose and for water boilers in industries. AD is considered to be one of the most environmentally friendly processes for converting agricultural biomass into renewable energy (Singh and Sidhu, 2014).

2.4 LIGNOCELLULOSIC BIOMASS RECALCITRANCE

Lignocellulosic substrates, i.e. woody biomass, energy crops and agricultural residues, are an abundant organic resource. Activities like agriculture, forestry, municipal and others contribute huge quantities of lignocellulosic biomass. Lignocellulosic biomass; essentially comprising of cellulose (a homopolymer of D-glucose unit), hemicellulose (a heteropolymer of D-pentoses and L-sugars units) and lignin (a cross linked polymer of phenyl propanoid); are carbohydrate-rich (55-75% dry basis) and can be used to harness bioenergy forms like bioethanol and biogas by AD (Akobi et al., 2016; Rouches et al., 2016). This composition and its proportion vary amid different lignocellulosic biomasses. Lignocellulosic substrate is suitable for biofuels generation by the fermentability of cellulose and hemicellulose fraction of biomass after hydrolysis. But, the innate physical and chemical features of lignocellulose mark it resilient to degradation for the attack of microbes and enzymes. Cellulose is considered to be the most important constituent of plant cell walls. Cellulose consists of a straight chain polysaccharides comprising of D-glucose subunits connected by β (1→4)-glycosidic bonds forming the disaccharide cellobiose, which further joined via H-bonds and Van der Waals forces forms lengthy chains of several units. A different orientation of cellulose molecules leads to varied level of crystallinity. Cellulose generally exists in crystalline form (high crystallinity) but small amount is found as amorphous (low crystallinity), former being more resistant to hydrolysis, whereas latter more susceptible to enzymatic degradation (Pe, 2002). Crystallinity index represents the crystallinity of cellulose; higher the crystallinity index, more difficult it is to biodegrade cellulose. Hemicellulose, besides D-glucose, comprises of D-mannose, L-arabinose, D-xylose, D-glucuronic, D-galactose, 4-O-methyl-glucuronic and D-galacturonic acids, which are joined together to form short chains by (1→4)-β- and occasionally by (1→3)-β-

glycosidic bonds (Sánchez, 2009). Unlike cellulose, hemicellulose consists of branched polymer of shorter chains and easily hydrolysable by hemicellulase enzymes as well as dilute acid/base, while cellulose is an unbranched polymer of long chains and is resistant to hydrolysis. In nature, lignin is found to be the second largest biotic compound (after cellulose). Lignin enmeshed with hemicellulose and cellulose forms an impassable barrier in the plant cell wall. Lignin acts like a cementing agent for cross-linkage connection of cellulose and hemicellulose, thereby forming a stiff three-dimensional structural matrix. Presence of lignin in plants provides the structural rigidity, impermeability as well as endurance against the attack of microbes and oxidative stresses. Lignin is an amorphous cross-linked polymer, water-insoluble and optically inert, that is formed from joining of phenyl propane units by nonhydrolyzable linkages. Three monolignol monomers, linked together by aryl-ether and C-C linkages, are the precursors for the synthesis of lignin, all of which are methoxylated to different degrees: coniferyl alcohol, sinapyl alcohol and p-coumaryl alcohol. Nevertheless, it is well-known that the presence of lignin restricts the accessibility for cellulose degrading microorganisms and thereby biodegradability of lignocellulosic biomass. It has been observed that lignin dissolves in water at a very high temperature (180°C) and neutral pH or sometimes acid/alkaline conditions that depends as per the antecedents of the lignin (Grabber, 2005). This property of lignin makes it the most refractory part of the lignocellulosic residues. Higher the lignin component, greater is the resistance of the lignocellulosic biomass to the biological and thermo-chemical degradation. This recalcitrant nature of the biomass is also responsible for reduced methane yields in SS-AD. Lignin is irrepressible to bioconversion under anaerobic environments and higher lignin percentage has the ability to inhibit digestion. It is difficult to hydrolyze lignin. It is only after hydrolysis, the lignocellulosic biomass shall be ready for conversion. Lignin is enmeshed to cellulose and hemicellulose constituting Lignin carbohydrates complex (LCC) making it highly recalcitrant and obstructing any enzymatic or chemical pre-treatment. Besides lignin, other parameters like cellulose crystallinity intensifies cell wall toughness and checks its degradation (Shirkavand et al., 2017). In order to increase the methane yield by enhancing rate of hydrolysis, percentage of lignin contents need to be broken down and the holocellulose (cellulose and hemicellulose) need to be disintegrated by this the accessibility of substrate is increased (Zhang et al., 2015). Therefore, an efficient and cost-effective pre-treatment is necessary in order to modify crystallinity of holocellulose and delignify the lignocellulosic biomass.

2.5 ENHANCEMENT TECHNIQUES

To efficiently improve the degradation process, it is of significant importance to be aware of substrate characteristics and possible metabolic mechanism during AD. Due to unreachability of lignocellulosic substrates lower methane yield is observed, which also enhances the accumulation of inhibitory compounds in the process. Thus, different enhancement techniques are used, which are mentioned further, that increase the degradability of the substrate and also improve the hydrolysis rate and methanogens metabolism.

2.5.1 Pretreatment of rice straw

The lignocellulosic material pretreatment has been recognized to be an essential step prior to its degradation. The digestibility of the RS is mainly affected by the lignin percentage, interlinkage of cellulose and hemicellulose association in the structure, crystallinity of the cellulose, inaccessible surface area for microorganisms. Pretreatment employed must meet the following conditions; avoidance of formation of inhibitory compounds, loss of carbohydrate on degradation and it should be cost effective. The pretreatment methods include two processes separation of lignin from structure and exposing the rest matrix to degrading enzymes and disruption of lignocellulosic matrix into cellulose, hemicellulose and lignin (Sun and Cheng, 2002; Vivekanand et al., 2012). Several pretreatment methods are used for accelerating the hydrolysis of recalcitrant agriculture residues. The description of different treatment method is explained further.

2.5.1.1 Physical pretreatment

Grinding (size reduction) is preliminary technique of destroying the structure of lignocellulosic structure. Sieving helps in collecting the desired fine powder of RS. The influence of grinding is directly on the altering the polymerization degree, porosity, increasing surface area and decreasing the crystallinity of the substrate. High moisture content (M.C.) increases the power consumption of the machine, whereas low M.C. of RS is bliss for reducing the size by grinding. Maryanty et al. (2017) studied the effect of particle size on biogas generation of RS. It was observed that lesser size of particle leads to higher potential of RS degradation. Particle size of 0.038, 0.053, 0.112 mm of RS showed the cellulose degradation of 71.96%, 50.15% and 24.03%, respectively in lab scale reactor (100 mL). Menardo et al. (2012) observed that on undergoing size reduction less than 50 mm in lab scale reactor (2 L), it was observed that particle size upto 5 mm, resulted in high methane yield and increased electric energy balance. The pretreatment result indicates the

efficacy of physical pretreatment for improving accessible surface area, breakdown of lignin-hemicellulosic complex, and enhancing the available cellulosic content.

2.5.1.2 Chemical pretreatment

Chemical agents are used as catalyst for delignification and disrupting the bond of lignocellulosic matrix in RS (Boonterm et al., 2016). Major effects of chemical pretreatment are shown in Table 2.1. Acid pretreatment involves usage of H_2SO_4, H_2O_2, HCl, HNO_3 etc. Acid pretreatment enhances the biogas production by altering the biodegradability of RS by dissolving the hemicellulose. On pretreatment of RS by using H_2SO_4 (6%), Qin et al. (2011) reported maximum biogas production of 150 mL/g-TS, which was 99.8% higher than control. Due to strong oxidizability of H_2O_2 and almost no secondary residues, H_2O_2 pretreatment of RS with 4% of H_2O_2 was reported as optimum for digestion with biogas production of 327.5 mL/g-VS, 115.4% being more than control (Song et al., 2012). Dai and Dong (2018) on pretreatment of RS with different concentrations of HCl (2, 4, 6 and 8%) revealed that pretreatment reduced hemicellulose content and only 2% HCl pretreated substrate produced 3% higher biogas yield with respect to control, whereas others resulted in decrease in biogas production. Alkali pretreatment involves usage of NaOH, Na_2CO_3, lime, ammonia, etc. Alkali pretreatment methods can increase RS accessibility for the microorganisms by disrupting the lignocellulosic structure with enhanced surface area and porosity and decreased cellulose crystallinity. Pretreatment of RS using NaOH has been studied in recent years due to its shortened fermentation time and increased accessible area resulting in higher biogas production. During NaOH pretreatment of RS, Guodong and Ronghou, (2011) revealed that optimal NaOH concentration is 6% that resulted in biogas yield of 246.6 mL/g-TS, 450.4% more than control. Furthermore, Dai and Dong, (2018) also reported 6% as optimum NaOH concentration with biogas yield of 273.8 mL/g VS due to improved biodegradability. However, NaOH pretreatment has also been reported as expensive for large-scale applications. Morone et al. (2018) reported alkaline wet air oxidation (AWAO) to be relatively efficient for RS pretreatment during the bio-digestion process. AWAO functioned with minimal chemical inputs of 6.5 g/L Na_2CO_3; and enhanced enzymatic cellulose convertibility to 69.1 ± 4.9% consistent with 401.3 ± 2.9 g sugars/kg untreated RS, with absence of potential inhibitors. On investigation of different bio-chemical pretreatment methods of RS, CaO-LFD (liquid fraction of digestate) achieved the best effect with methane production of 274.65 mL/g VS which was 57.56% more than control (Guan et al., 2018). Also, T_{80} (time required for 80% methane production) was found to be 42.86% lower than control indicating shortened time and economic

affordability. Pretreatment of the RS with 75% (v/v) aqueous ethanol containing 1% (w/w) sulphuric acid at 180°C for 30 min resulted in glucose yield of 46.2% and the highest Acetone–butanol–ethanol (ABE) concentration and productivity (10.5 g/L and 0.20 g/L h, respectively) (Amiri et al., 2014). Thus, organosolv pretreatment can be efficiently applied for the efficient production of solvents from RS. Ebrahimi et al. (2017) on pretreatment of rice husk with 20% ammonium carbonate solution reported improved enzymatic hydrolysis to 67.7% and ethanol concentration of 10.61 g/L (47.78%). Chang et al. (2016) observed synergistic effects of surfactant-assisted ionic liquid pretreatment RS with enhanced delignification up to 49.48%, high total reducing sugar yield and substantial alteration of cellulose crystallinity and surface morphology. Chemical pretreatment is simple in operation and it improves biodegradation resulting in enhanced biogas yield, nevertheless, sometimes it produces inhibitory compounds that may obstruct the AD. Some methods may cause secondary residues which needs further recovery.

Table 2.1. Pretreatment agents and its effect on lignocellulosic waste

Pretreatment	Chemical/Catalyst	Major Effect	References
Acid	H_2SO_4 (6%)	Biogas yield of 150 mL/g-TS, 99.8% more than control, Increased accessible surface area, improved buffering capacity	Qin et al. (2011)
	H_2O_2 (4%)	Biogas yield of 327.5 mL/g-VS, 115.4% more than control. No secondary residues, expensive for industrial-scale application	Song et al. (2012)
	HCl (2, 4, 6 and 8%)	3% higher biogas production and higher TS and VS removal efficiency, hemicellulosic fractions removed	Dai and Dong (2018)
Alkaline	NaOH (6%)	Biogas yield of 246.6 mL/g-TS, 450.4% more than control, Increased accessible surface area, shortened	Guodong and Ronghou (2011)

		fermentation time, expensive for industrial-scale application	
	NaOH (2, 4, 6 and 8%)	Biogas yield of 273.8 mL/g-VS, highest TS (53.8%) and VS (36.8%) removal efficiencies with 6% concentration, improved biodegradability	Dai and Dong (2018)
	Na_2CO_3 (alkaline wet air oxidation)	Increased enzymatic cellulose convertibility to 69.1% corresponding to 401.3g sugars/kg untreated RS, improved cellulose accessibility and digestibility	Morone et al. (2018)
Bio-chemical	CaO-LFD (liquid fraction of digestate)	methane yield of 274.65 mL/g-VS, 57.56% more than control, T_{80} was 42.86% lower than control indicating affordability	Guan et al. (2018)
Organic Solvents	Ethanol (75% v/v) + H_2SO_4 (1% w/w)	glucose yield of 46.2, highest ABE concentration and productivity (10.5 g/L and 0.20 g/L h, respectively)	Amiri et al. (2014)
	Ammonium carbonate ($(NH_4)_2CO_3$) (20% v/v)	Improved enzymatic hydrolysis to 67.7%, glucan content of 40.9%, ethanol concentration of 10.61 g/L (47.78%)	Ebrahimi et al. (2017)
Surfactants-ionic liquid	1-butyl-3-methylimidazolium chloride (ionic liquid) + 1% sodium dodecyl sulfate (surfactant)	Increased lignin removal to 49.48%, lower cellulose crystallinity, less ordered cellulose structure, porous and disordered RS structure.	Chang et al. (2016)

2.5.1.3 Thermo-physical and Thermo-chemical pretreatment

Pretreatment at accelerated temperature are commonly used for increasing the porosity of the surface and enhancing the delignification. Liquid at elevated temperature hydrolyze the lignocellulosic contents of the substrate. At higher temperature and pressure water molecules dissociate into H_3O^+ and OH^- ions that helps in catalytic conversion of lignocellulosic structures. Pretreatment of lignocellulose at higher temperatures and short reaction time (i.e. dilute sulfuric acid, ammonia recycle percolation (APR), or steam explosion) could effectively improve the porosity and delignification efficiency. The treated biomass has higher cellulosic content. Some hemicellulosic content is also removed which increases the available area for enzymatic attack. Ma et al. (2009) showed that under the optimal microwave intensity of 680W and irradiance time of 24 min, the cellulose and hemicellulose removal efficiencies of 30.6% and 43.3% was obtained (100 mL). Zhu et al. (2005) revealed that microwave pretreatment of 700 W (30 min) achieved the lignin removal of 6%, which consequently improved the hydrolysis rate. However, these processes also generate some inhibitory compounds (i.e. Furan derivatives, phenolic substances etc.) that hamper the anaerobic fermentation efficiency. Hence, removal of the anaerobic fermentation inhibitors is also a crucial step for bioconversion of lignocellulosic biomass to biogas. The selection of appropriate pretreatment process is highly required for the sustainable conversion into renewable energy source.

2.5.1.4 Biological pretreatment

There are few literatures available regarding the effect of ensiling pre-treatment. It comprises of lactic fermentation (anaerobic); converting sugars into lactic acids, acetic acids etc. Lignin, therefore, is not digested which is normally less than 6% without adding NaOH, while high losses of holocellulose can be observed, being measured from 1-13% (Herrmann et al., 2011). These losses depend upon duration of ensiling varying from 10-365 d, feedstock (wheat straw, RS, maize, etc.) and chemicals such as sodium nitrite ($NaNO_2$) and methenamine or biological agents such as Lactobacillus buchneri, Lactobacillus plantarum. The enhanced methane yield of 17% was obtained but it leads to very less increase in specific methane yield (per g VS). Some researchers have used complex microbial consortia for the purpose of pre-treatment viz. Zhong et al. (2011) used a 0.01% mixture of complex consortium comprising of lignin degrading white-rot fungus i.e. *Pleurotus florida sp., cellulolytic bacteria, Bacillus licheniformis sp., Pseudomonas sp., and Bacillus subtilis sp.,* yeasts and the other acid degrading bacteria *Lactobacillus deiliehii sp.* for 15 days pre-treatment on corn straw which led to a 75% enhancement in methane yield

(Fig. 2.2). Taha et al. (2015) have reported seven fold increases in saccharification rate by using fungal consortium. On comparison of the impact of fungal pre-treatment (*Trametes versicolor*) on corn silage over ensiling, a 41.3% increase in methane production was obtained along with positive effect on pH stability (Ti et al., 2018). The most repeatedly investigated biological pre-treatment of lignocellulosic biomass is fungal pre-treatment prior to its AD which uses less energy and impacts fewer harm to the environment. In this process, the required number of steps is least and it does not necessitate any additional feedstock for the production of fungi. Moreover, reduced waste streams, low downstream costs and by-products produced during fungal pre-treatment, usually, would not inhibit subsequent AD processes since the pre-treatment is carried out under mild conditions are other advantages associated with it. Fungi, capable of degrading lignocellulosic biomass, can be classified as soft-, brown- and white-rot fungi. Compared to white-rot and brown-rot fungi, not much information is available about the lignin degradation behavior of soft-rot fungi. However, Sánchez (2009) has reported that soft-rot fungi is able to degrade lignin and they typically attack materials with lower lignin content and higher moisture. Soft-rot fungi belong to Ascomycetes, while brown-rot and white-rot fungi associated to Basidiomycetes. The depolymerization of holocellulose while only modifying the lignin is rapidly done by brown-rot fungi, thus, lignin survives as a main component after fungal pre-treatment (Sánchez, 2009). However, white-rot fungi, with more than 1500 diverse classes have the unique tendency to most effectively destruct the structure of lignin, the most recalcitrant component of lignocellulosic biomass, to CO_2 (Miiller and Trfisch, 1986; Wan and Li, 2012). Lower substrate utilization specificity and robust oxidative activity of its ligninolytic enzymes is responsible for the unique degradative capability of white-rot fungi. Apart from lignin, white-rot fungi also have the ability of degrading variety of environmental pollutants such as heterocyclic aromatic hydrocarbons, chlorinated aromatic compounds, various dyes and synthetic polymers (Bermek et al., 1998). Therefore, white-rot fungi find its use in various soil and water remediation techniques. However, free phenoxy and aromatic radicals formed by peroxidases are smaller enough to penetrate cell wall causing initial cracking reaction which starts decomposition of lignin polymers and radicals are finally depredated. The involvement of radicals in lignin degradation is an indicator that it is not a highly specific process. The activities of ligninolytic enzymes are not always interconnected with the degradation of lignin indicating insufficient understanding of ligninolytic enzyme complex, also these phenolic compounds have inhibitory effect on AD process and thus fungal pre-treatment is suitable prior AD of lignocellulosic biomass.

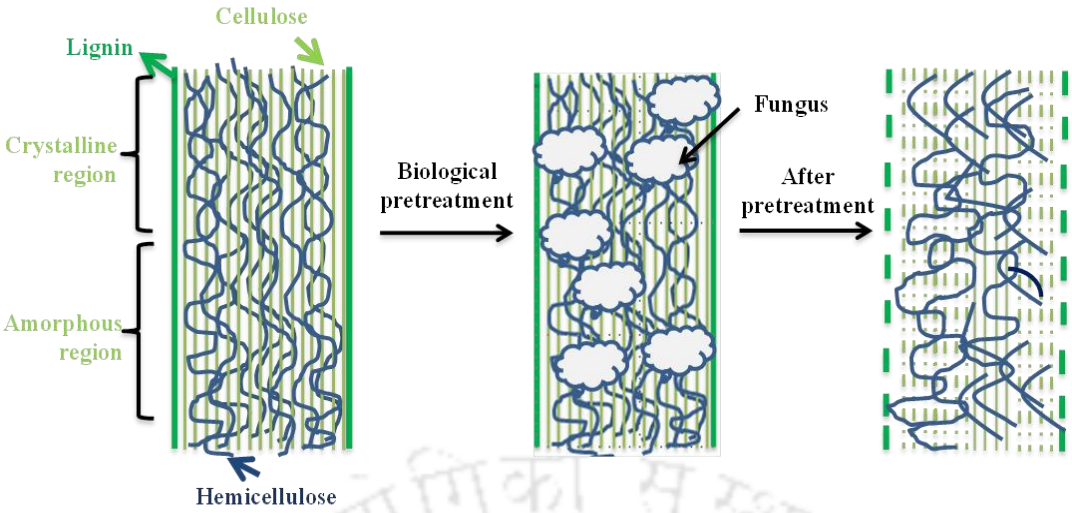


Fig. 2.2. Schematic diagram of biological pre-treatment of lignocelluloses

LCC makes lignocellulosic biomass highly recalcitrant by restricting the accessibility for enzymes to holocellulose and thus limiting the biomass conversion into biofuels. Contemplating the delignification and resultant increase in biomass digestibility, white-rot fungi manage a huge potential to increase methane yield. Although biological pre-treatments using white-rot fungi have many advantages, however, it is also fraught with various challenges such as long incubation time (weeks to months), substrate colonization by the inoculum and few studies related to selective delignification of lignocellulosic biomass (especially RS) using white-rot fungi. Fungal pre-treatment during storage can solve the issue of long incubation time (Cui et al., 2012) and further research needs to be taken to improve fungal pre-treatment.

2.5.2 Anaerobic co-digestion

2.5.2.1 Anaerobic digestion with co-substrate

Lignocellulosic biomass have improper nutrient structure, high organic load, deficiency of diversified microbes and low nitrogen content; all these factors are the potential inhibitors for the AD process . Agriculture residues deprived of pre-treatment and premixing with other co-substrate effects into low biogas yield. High C/N ratio, lignin percentage and contamination with pesticides influenced the process dynamics. Most of these kinds of problems are resolved by the addition of co-substrate in reactor, and this process is called as AcoD. AcoD is the simultaneous mixing of two or more substrate to overcome the drawbacks of mono-digestion and enhance the economic feasibility of AD

process. The main benefits of the co-digestion process are (i) to enhance process stabilization (ii) diluting the inhibitory effects (iii) attainment of required M.C. in the digester (iv) sharing of apparatus and funds (v) increasing the organic loading rate (vi) positive synergism in the digester (vii) macro-micro nutrients balance (Griffin et al., 1998; Mata-Alvarez et al., 2000; Zheng et al., 2014). During the co-digestion, two or more organic material is managed properly to enhance the biogas yield up to 25-400% comparative to mono-digestion of feedstock (Table 2.2). As co-digestion can create the respectable synergism in the digester, it is an economically viable option. Within the last decades, co-digestion research has focused mainly on mixing of substrates; which dilute the inhibitory effects of mono-substrates. Agriculture uses the most co-substrate; suitable substrate for co-digestion is nitrogen rich easily degradable co-substrate (Herrmann et al., 2016; Shitophyta and Fuadi, 2016) even in continuous stirred tank-reactor (CSTR). The ratio for selection of waste depends on optimization of the C/N ratio (Donoso-bravo and Fdz-polanco, 2013), but other parameters such as pH, alkalinity etc. also play important role in co-digestion. Some researches expressed that the best C/N ratio for co-digestion is 20; and when cassava pulp is co-digested with pig manure, reported the maximum yield, when the C/N ratio was 33 (Mata-Alvarez et al., 2014). Thus, agricultural residues are the most important substrate for co-digestion, and the issue with availability and seasonality can be overcome by improving the digester volume with other biodegradable organic waste. The AcoD can enhance the digestibility of agriculture residues (cellulose, hemicellulose) and buffering capacity.

Table 2.2. Summary of co-digestion studies, operational parameters in the AcoD process

Substrates: co-substrates	**Digester condition**	**Temperature (ºC)**	**Methane yield (mL /g-VS)**	**Increment (%)**	**Reference**
Energy crops: cow manure	CSTR	37/55	210	60	Ma et al. (2009)
Energy crops: cow manure, fruit and vegetable waste	CSTR	37/55	250	91	Zheng et al. (2014), Zhang and Banks, (2013)
Agriculture	CSTR	35	350	67	Zhang et al.

waste: sewage sludge					(2014)
Agricultural residue: FW, pig manure	Batch	37	674.4 (biogas yield)	71.67	Menardo et al. (2012)
Agricultural residue: FW, anaerobic sludge	Batch		580		Chang et al. (2016)

2.5.2.2 Microbial population dynamics in AcoD process

Microbial population in AD process is categorized into four different metabolic consortium, which can also be divided from functionality of microbial domain i.e. archaea and bacteria. Bacteria have the capacity to take part into hydrolysis; acetogenesis; acidogenesis etc., therefore bacteria catabolizes a huge variety of substrate. The most important phyla involved in the co-digestion process are firmicutes, clostridiales and actinobacteria (Gupta et al., 2012; Liotta et al., 2016; Siddique and Wahid, 2018). The archaeal consortium holds a special place in AD process, as they are responsible for methane generation. Temperature is one of the important parameter that affects the consortia in AD. The temperature shift leads to significant reduction of methanogenic bacteria. Methanogenic bacteria in co-digestion can be classified into two categories (i) the acetoclastic methanogenic bacteria (ii) the hydrogentrophic methanogenic bacteria consisting of *methanobacteriales, methanococcales* etc. Methanogens are most susceptible to operating conditions and environmental factors (Xing et al., 2008; Yin et al., 2016). The alteration in the substrate concentration can modify the digester environment and can lead to VFA accumulation and ammonia inhibition. For instance, the substrate with low C/N ratio increases the ammonia level in AD process and shifts the phyla towards *methanococcales* methanogens (Mata-Alvarez et al., 2014). *Crenarcheota* is detected to be in high concentration in AcoD process (Ziganshin et al., 2013).

2.5.2.3 Modeling of AcoD process

Modeling of AD system is required i) to reduce time and extent resources utilization in the system ii) to transform lab to industrial scale iii) designing of system for optimum operational parameters. The necessity of models has become a normal practice in anaerobic

digester design, control, prediction and monitoring of the process. Mostly, dynamic models of the fundamental processes are used for conducting modeling. The scopes of complexity in physico-chemical conditions convoluted in AcoD make it perplexing for the practitioner to understand the intricate details of the model. Depending on these parameters, various models have been designed and developed and are used for simulation tools. The AD model no.1 (ADM1) developed by IWA group for waste treatment, is easiest framework in AcoD models (Hagos et al., 2017; Mata-Alvarez et al., 2014). It is the most advanced, easily extensible and flexible model, which is suitable for predicting and monitoring biogas generation involved in the reactor. The modified ADM1 can envisage biogas yield, effect of OLR, buffering capacity (alkalinity) and free ammonia (FA) concentration in order to augment the process stability and efficiency of the reactor. MATLAB/SIMULINK® is the most used platform to solve algebraic differential equation of the modified ADM1 model. The hydrolysis phase should be considered as limiting step and feedstock input concentration should be given with respect to carbohydrate, protein and lipids etc. ADM1 model considers the first order kinetics, which depends on both nature of solid substrate and particle size. Few other options to include soluble substrate which has not been described by the ADM1 model are suggested by some other researchers. They developed a technique to convert soluble compounds into glucose yields. Zhou et al. 2012 expressed the possibility of some substrates in variable conditions, under different operational conditions (OLR and HRT). ADM1 and modified ADM1 model could not distinguish the performance of microbes in the process. Methanogenic and anaerobic reduction-oxidation taking place during the AD are categorized by a Gibbs energy change (ΔG), due to the absence of external electron acceptor (Turgay, 2007). This low ΔG makes changes in the system to obtain thermal equilibrium in the system. This equilibrium shows the tendency of achieving the active bioprocess in the system. Therefore, considering the thermodynamic aspects of AcoD and adding it into dynamic ADM1 model improves the monitoring mechanism of AD process.

2.5.3 Enhancement in inoculation efficiency

To efficiently improve the degradation process, it is of significant importance to be aware of substrates characteristics and possible metabolic mechanism during AD. Due to unreachability of lignocellulosic substrates lower methane yield is observed, which also enhances the accumulation of inhibitory compounds in the process. Thus, different enhancement techniques are used, which are mentioned further, these techniques increase

the degradability of the substrate and also improve the hydrolysis rate and methanogens metabolism.

2.5.3.1 Inoculum selection and leachate recirculation

Selecting suitable inoculum is of huge importance in the process, as it not only provides trace elements, M.C., nutrients (macro and micro) but also provides the buffering capacity in the system. Effluent (finished materials) collected from LS-AD and SS-AD is generally better than other types of inoculum such as rumen fluid, activated sludge (El-Mashad et al., 2006; Xi et al., 2014) etc. Using effluent from reactor as inoculum creates the system more sustainable, more total solids (TS) percentage and supplies active microbial community (acidogens, methanogens) acclimatized to the substrate and operational situations (Jeihanipour et al., 2011). In one study lag phase of AD declined from 25-30 days to 3-5 days, when the inoculum was substituted form fresh to LS-AD digestate (Yang et al., 2015). Varieties of lignocellulosic organic wastes have been inoculated successfully by using digestate. To minimize the cost of transportation of LS-AD and SS-AD system collocation is a prerequisite. An integrated pilot plant situated in Zanesville, Ohio, is a supporting case for this viable option (Forster-Carneiro et al., 2007). Hydrolytic microbes and methanogenic microbes supplementation to improve methane yield is also employed (Tsavkelova et al., 2018). It increases the xylanase activity by 1.62 times and methane yield by 1.53 times (Weiß et al., 2010). The optimum ratio of methanogens and hydrolytic microbes (μ) in AD was suggested to be 24, μ lesser than this value makes the hydrolysis rate limiting step and higher value of μ marks the methanogenesis a rate limiting step (Ma et al., 2013). Leachate circulation is also used to enhance the mass transfer in the reactor between inoculum and substrate, specifically when fractional mixing is provided in the reactor. Leachate recirculation is combined with water to dilute the inhibitory effect of accumulated ammonia, VFA and other metabolites in leachate (Chen et al., 2008; Wang et al., 2017).

2.5.3.2 Inoculation ratio optimization

The inoculation ratio in AD is described as the substrate-to-microbe (S/M), feedstock-to-inoculum (F/I), F/M, or substrate-to-inoculum (S/I). It can be calculated on the basis of VS, total solids or loading rate. A greater inoculation ratio can lessen the start-up time and enhance the methane yield. The higher inoculation ratio also increases the microbial populations, buffering capacity and in some processes balances the C/N ratios (El-Mashad et al., 2006; Xu et al., 2014). Conversely, excessive inoculation decreases the volumetric efficiency and occupies the space in the reactor. High substrate concentration reduces the

mass transfer rate between inoculum and substrate. To optimize the methane yield and volumetric efficiency, S/M ratio of 2-3 is selected for lignocellulosic biomass under mesophilic condition (Liew et al., 2011; Zhu et al., 2014). Lower inoculation size is selected at thermophilic conditions. Higher temperature surges the accretion of FA, which inhibits microbes. Optimum range of F/I for thermophilic AD is, in range of 4-6 (El-Mashad et al., 2006; Stabnikova et al., 2008; Xu et al., 2016).

2.6 THE STEP EN ROUTE FOR BIOGAS PLANT DIGESTER

The simple needs of an anaerobic digester are: to continuously maintain large organic loading rate, less hydraulic retention time (to reduce rector size) and to harvest the maximum yield of methane. While selecting the shape of reactor, mixing and heat loss should be taken into consideration. As in case of rectangular and square shape, suboptimal mixing allows the building of refractory matter at the corner, consequently leading to reduction of rector volume. There is various type of reactor in practice today, and working design is associated with the matter to be digested. Three main types of reactor commonly exist: batch digesters are the most common one. Batch reactors are filled with the slurry and left for digestion time (hydraulic digestion time) after which they are vacated. The second kind is single stage continuous reactor, where all the reactions take place in one reactor. Third category is multistage (or two stage) continuous digester, where hydrolysis/acetogenesis/methanogenesis takes place in separate compartments (Griffin et al., 1998; Mata-Alvarez et al., 2000). As the optimum environment for their growth varies for all processes, multistage system advances the stability of the reactions inside the reactor, mainly by using simply hydrolysable substrate. Fluctuations in organic loading rate, heterogeneous waste, presence of excess inhibitors also causes instability in the reactor. Multistage system provides the stability against shock loading or high organic loading rate, as the buffering capacity of the system gets increased. The digested material passing from one stage to another gets homogenized and become more stable, but the construction of multistage digester is expensive to maintain. Specific operational parameters and characteristics of different digester with different operating condition are shown in Table 2.3. In case of one stage and two stage digester for treating cattle manure, it was observed that, specific methane yield was 6 to 8% more in later case (Nelson et al., 1964). An increase of 21% was found when, municipal solid waste was treated with multistage rector (Liu et al., 2004). One stage reactor suffers short circuiting (due to shorter retention of passage) due to the improper operational conditions in the reactor. Use of prechamber in case of single phase decreases the consequence of short circuiting.

Table 2.3. Comparison of operational parameters of anaerobic reactor in different conditions

Standards	**One phase vs two phase anaerobic reactor**		**Continuous vs batch anaerobic reactor**	
	One phase	**Two phase**	**Batch**	**Continuous**
Biogas yield	Unbalanced and discontinuous	Larger and stable production	Unbalanced and discontinuous	Larger and highly stable
% solid content	10-45	2-45	25-45	2-15
Economical aspect	less	more	less	more
VS% reduction	less	more	40-75	40-75
HRT (day)	15-65	10-15	30-65	30-65
Organic loading rate (OLR) (VS kg/m^3-day)	0.8-15	5-10 (one stage) 10-15 (second stage)	12-15	0.7-1.4
Shock absorbance	low	more	more	high

2.7 CONCLUDING REMARKS

As production of rice increases continuously in order to accomplish stable food requirements of half of the world's population, consequently RS will be an abundant and accessible agricultural residue generated. The treatment and collection of RS through AD is not only a feasible alternative for production of conventional source of energy but also eradicates a chief source of greenhouse gas release from collective practices of straw burning in fields and tilling it back into fields. The philosophy and technology for biogas generation are matured and well established; the future research needs to be focused on optimization of parameters influencing environment for efficient growth of microorganisms. Mono-digestion of RS results in nutrient disproportion as RS has very low nitrogen content and high C/N; therefore, optimization of the operational parameters like C/N ratio, pH, F/M ratio etc. for co-digestion is most cost-effective approach for decreasing the pertinent

toxicity and is also easy to install. Though published literature on practicable loading rate is still insufficient, reducing HRT is a feasible option to obtain optimum OLR. The addition of accelerants in the anaerobic process enhances the performance by adsorbing the substrate on the surface of the accelerants. Green materials are naturally available in environment and create no pollution; therefore this type of co-substrate is considered as capable accelerants. With further advancements on the progress of AD process, the authors recommend that focus has to be given to the arrangement of factors as mentioned in the article. Research should also focus on construction of microbial consortium and stimulating degradation of RS. Additionally, due to huge investment cost and operative cost, the expansion of biogas plant in domestic scale would be obstructed. The authors commend that practicality of technological, economic inputs and theoretical research on small-scale experiments should be emphasized. Consequently improved operability, stability and global logistics of implementation need to be steered for anaerobic process.

CHAPTER 3

MATERIALS AND METHODS

Different experiments were performed to accomplish the objectives. The research work was carried out in different phases using RS as a substrate and DCD and FCD as an inoculum. The detailed methodology is summarized below.

3.1 EXPERIMENTAL FLOW CHART

In order to achieve the desired goals, the proposed research work was accomplished in different phases as shown in Fig. 3.1.

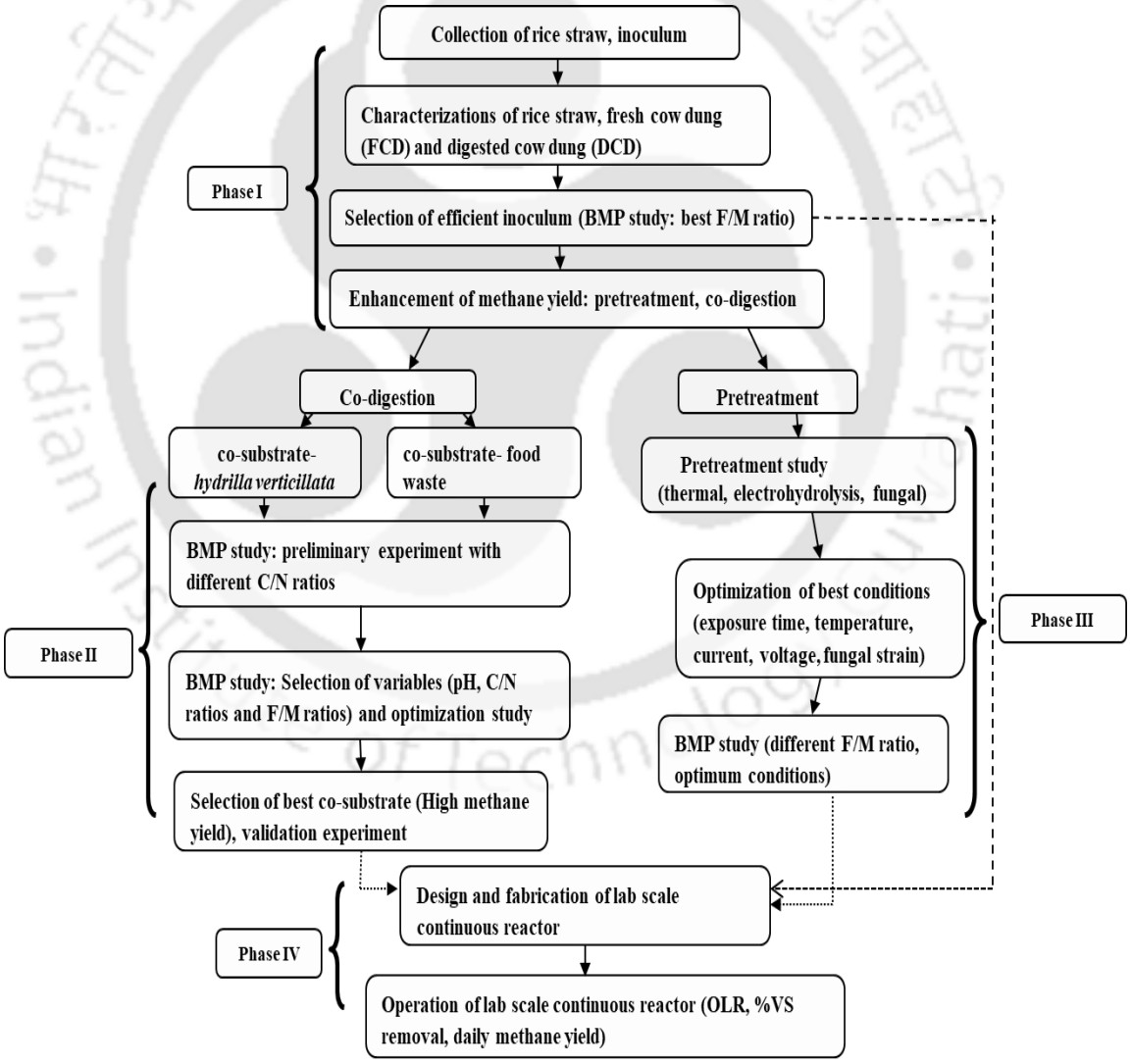


Fig. 3.1. Experimental flow chart of research work

In phase I, the BMP test was conducted to check the efficacy of fresh and digested (degassed) cow dung as an inoculum and exploring the optimum F/M ratio. In phase II, the effect of co-digestion with different co-substrates (*H.verticillata*, FW) on methane yields was evaluated. In phase III, effect of pretreatment study on lignocellulosic degradation and enhanced methane yields from RS were determined. In phase IV, lab scale continuous reactor was operated in for different operational optimum parameters obtained from phase I, II and III.

3.2 SUBSTRATE, CO-SUBSTRATE AND INOCULUM

RS was collected from a field near the IITG campus, in Assam (India). At the end of the harvesting period, air-dried RS was collected and processed into a smaller size (5-10 mm) with the aid of a high cutting speed grinder (Bajaj, SKU-410025, India). The reduction of particle size increases the surface area, porosity and potential degradability of the substrate. The milled RS sample was stored in air-tight plastic bags at room temperature until treatment. Co-substrate *H.verticillata* was collected from Deepor Beel, which is located in south-west of IITG campus, which is a Ramsar wetland site. Food waste (co-substrate) was collected on daily basis from the canteen of the Lohit hostel of IITG institute. Active inoculum with well-proportioned microbial communities is an essential parameter for reducing the primary start up time for stable and efficient performance of AD. Fresh cow dung was collected from a Gaushala in Amingaon (Guwahati) India, whereas, pre inoculated, digested cow dung to be fed as an inoculum was collected from fixed dome digester located in Maligaon, Guwahati, India and used as a seed inoculum for the BMP study. Acclimatization to new operating state was not strictly needed for degassed inoculums. Degassed cow dung was stored at 4°C in the refrigerator before use. The physico-chemical characterizations of substrate, co-substrate and inoculum tested in the current study are shown in Table 4.1 and Table 5.1.

3.3 PHASE I-ANAEROBIC BMP SETUP FOR EFFICIENT INOCULUM STUDY

The inoculum study emphasizes on the comparative exploration of RS for efficient biogas production with two different inoculums: fresh cow dung and digested cow dung, under different food to microorganism (F/M) ratios.

3.3.1 Biochemical methane potential experimental design

To study the rate and extent of AD of RS, batch reactors in the form of 1000 mL reagent glass bottles were prepared. Rubber cork and Teflon tape were used to seal the openings. Each Bottle was connected to specific aspirator bottle containing 1.5 N NaOH solution through long silicon tubing (Fig. 3.2). Methane production was quantified daily using water displacement method (Stroot et al., 2001). The experiments were carried out individually with two different inoculums i.e. FCD and DCD. The different F/M ratios of 0.25, 0.375, 0.5, and 0.75 were considered based on their VS content. RS was used as a substrate with the addition of essential micro and macro nutrients. A control setup with inoculum was also established without addition of RS. Finally, 10 mL of phosphate buffer was added in each bottle for maintaining the pH within the limits of AD process. The anaerobic conditions were maintained by purging Nitrogen gas (N_2) for 3 min into the reactors. The pH was also maintained as per the process suitability. To evaluate the stability of the AD process, the BMP experiments were run in triplicate.

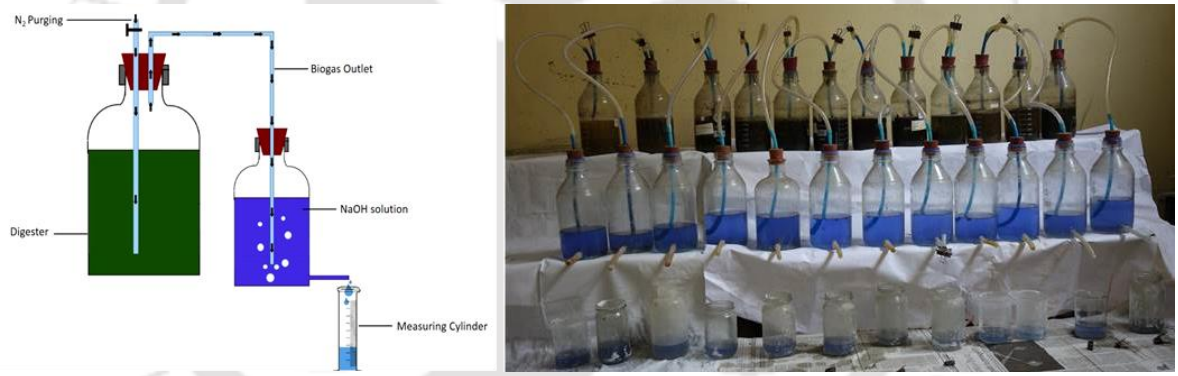


Fig. 3.2. Experimental diagram of anaerobic BMP setup

3.3.2 16S Metagenome sequencing of inoculum

Sample of efficient inoculum from BMP study was sent for 16S metagenomics profiles. The isolation of genomic DNA from the sample was carried out using modified Xcelgen soil gDNA Kit. Quality of genomic DNA was checked on 1% agarose gel (laden 3 µl) for the single intact band. The gel was run at 110 V for 30 mins. 1µl of each sample was used for measuring the concentration using Qubit® 2.0 Fluorometer. The amplicon library was organized using Nextera XT Index Kit (Illumina inc.) as per the 16S metagenomics sequencing library preparation protocol. Primers for the amplification of the V3-V4 hyper-variable region of 16S rDNA gene of bacteria and archaea were designed in-house by Xcelris Labs Limited. The bacterial community was analysed by paired-end sequencing,

which permits the template fragments to be sequenced in both the forward and reverse directions on Illumina platform.

3.4 PHASE II- CO-DIGESTION STUDY

The aim of phase was to investigate the anaerobic co-digestion of rice straw with *H.verticillata* and FW as an external nitrogen source for enhanced methane generation and effect of different operating parameter on methane production during anaerobic co-digestion.

3.4.1 Co-digestion of rice straw with *Hydrilla verticillata*

The C/N ratios of RS and *H.verticillata* were 43 and 6.85 (Table 5.1), respectively, which were clearly outside the optimal range. Five C/N ratios i.e. 15, 20, 25, 30, 35 and control were selected for the experiment (Table 3.1). RS and *H.verticillata* were added according to C/N ratios selected in the screening experiment before selecting range for optimization. The BMP test was performed in 1 L glass bottle closed by rubber cork at the mouth with a connection for collecting gas by water displacement method and purging of N_2 for imparting anaerobic condition in the reactor, mixing was done manually two times a day. All the reactors were placed in duplicate; by placing the reactors in a mesophilic environment. Greater bacterial and archaeal diversified population was found at mesophilic temperature (30-40ºC) than thermophilic temperature (50-60ºC) (Liu et al., 2009). Subsequently, acclimatized inoculum was collected from the digester and 20 experimental runs (co-digestion) with designated C/N ratios, F/M ratios and pH value were placed. The F/M ratio in this study was determined as a ratio of VS in the substrate and co-substrate to VS content in the inoculum. C/N ratios were determined based on C (%) and N (%) in the substrate and co-substrate. Different amount of substrate, co-substrate and inoculum were added to the reactor to give required C/N ratios (14.89, 20, 27.5, 35 and 40.11), F/M ratios (0.15, 1, 2.25, 3.5 and 4.35) and pH (6.16, 6.5, 7, 7.5 and 7.84) as suggested by the experimental matrix. The mixing and homogenization were done after which sample became paste. Specifically, sampling was done every seventh day for a period of total 50 days. The slurry sample was taken from the reactors and analyzed for process parameters.

Table 3.1. Composition for different C/N ratios for BMP assay

C/N ratio	Rice straw (g)	Hydrilla verticillata (g)	Digested cow dung (g)	Water (g)
Control	5	-	100	595
C/N-15	5	272	100	323
C/N-20	5	139	100	456
C/N-25	5	79	100	516
C/N-30	5	45	100	550
C/N-35	5	23	100	572

3.4.2 Co-digestion of rice straw with food waste

In this study, the screening and optimization experiment were performed in two phases, in phase I preliminary experiment was performed in order to select the independent variables (C/N, F/M and pH) and range of level for designing the experimental matrix for phase II, and investigating the degradation and reactor performance in co-digestion. Five different C/N ratios 15, 20, 25, 30 and 35 (co-digestion) and control (mono-digestion) were chosen for setting the BMP for phase I (Table 3.2), without adding any external nutrients. 100 g inoculum (DCD) was added to all the reactors at the start of BMP test. C/N ratio was calculated on the basis of total composition of C and N in RS and FW on dry weight basis. Control consisted of RS and DCD (mono-digestion) with no co-substrate (food waste) in it. The BMP test was conducted with 1000 mL anaerobic digester connected to aspirator bottle (biogas collector) of same capacity and 250 mL Erlenmeyer flask for collecting the displaced 1.5 N NaOH solution (equivalent to methane production). To initiate the anaerobic condition, N_2 was purged in the reactor for 3 min and all reactors were tested for possible leakage before sealing with rubber stoppers. Monitoring of reactor during BMP test was done by collecting sample after every 7^{th} day, and collected samples were analysed for pH, ORP, VS, VFA and sCOD. In phase II, two independent variables were considered for designing the experimental matrix, as from the results of phase I it was clear C/N ratio 20 25, 30 and 35 showed the same cumulative methane yield (mL/g-VS_{added}). pH and F/M ratios were chosen as two independent input variables and range of pH was chosen from 6.6-7.4 and F/M ratio was chosen from 1-3.5 as per the result of phase I. Different F/M ratios such as 0.48, 1, 2.25 and 4.02 and initial pH 6.43, 6.6, 7, 7.4 and 7.57 were selected with constant C/N ratio of 30 for phase II BMP assay. BMP with scale up capacity of 20 L was used for validation experiment to confirm the results from models. All the reactors

were placed in duplicates with manual mixing of 2-3 times a day. Batch setup (large scale of BMP assay) was an extended study of BMP assay to find the maximum methane production time and retention time. Batch study was conducted in a 20 L plastic bottle with the working volume of 14 L capacity (20 times of BMP). Batch experiment was studied for best F/M ratio of both control (without pretreatment) and pretreatment BMP assay and validation experiment of model for co-digestion study.

Fig. 3.3. Batch study setup

Table 3.2. Composition of the material for biochemical methane potential assay

F/M ratio	Rice straw (g)	Food waste (g)	Digested cow dung (g)	Water (g)
Control	5		100	595
C/N-15	5	131	100	464
C/N-20	5	46	100	549
C/N-25	5	23	100	572
C/N-30	5	12	100	583
C/N-35	5	6	100	589

3.4.3 Experimental design for statistical analysis and optimization

RSM was chosen for the optimization of the selected parameters. A three-level-three (three factors n = 3, ± α = 1.682) CCD-RSM was used to obtain optimization of design. This run was basically a complete 2^3 factorial plan expanded by six axial sets coded to ± α and six duplications of central set points, amounting to twenty experimental data points in

co-digestion with *H.verticillata*. Evaluation of lack of fit and experimental inaccuracy was measured by center run. The levels of the independent variable for dependent methane yield are represented in Table 3.3. The levels of X_1, X_2, X_3 were computed with the following equations: $x_1 = (X_1 - 27.5)/7.5$, $x_2 = (X_2 - 2)/1.5$, $x_3 = (X_3 - 7)/0.5$. Functional relationship was developed between the dependent or response variable (Z) and set of independent variables (X_1, X_2 and X_3). The output response was the methane yield (mL/g-VS_{added}). Independent parameters and its interaction were evaluated to confirm the reasonable hypothesis with estimation with 0.05 confidence level. The data point normality was also proved by the plotting of residual normality plots.

$$Z = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{23} X_2 X_3 + \beta_{13} X_1 X_3 \quad (3.1)$$

Where, Z is the dependent or response variable (methane yield, mL/g-VS_{added}); β_0, β_1, β_2, β_3 are coefficients of linear expressions; β_{11}, β_{22}, β_{33} are quadratic coefficients; β_{12}, β_{23}, β_{13} are interaction coefficients; X_1, X_2, X_3 represents the independent variables, viz., C/N ratio, F/M ratio and pH.

Table 3.3. Level of factors used for optimization of methane production

Independent variable		**Variable Level**				
		-1.682 (-α)	**-1**	**0**	**1**	**+1.682 (+α)**
X_1	C/N	14.89	20.0	27.5	35	40.11
X_2	F/M	0.15	1.0	2.0	3.5	4.35
X_3	pH	6.16	6.5	7.0	7.5	7.84

The two-level CCD was used for determining the optimizations of the output response for co-digestion with FW. 13 runs were performed for this experiment design. The levels for this study is expressed in Table 3.4, and this value is calculated using the equation as mentioned; $x_1 = (X_1 - 7)/0.4$, $x_2 = (X_2 - 2.25)/1.25$. To uphold the rotatability of the model, α value is calculated using; $\alpha = (2N)^{1/4}$, where N is the number of factors in an experimental run. The methane yield (output response) (mL/g-VS_{added}) Y, and independent parameters X_1, X_2 functional association was derived with 5% confidence interval. The normality of outlier points were drawn by the normality residual plots. The relationship between Y (dependent response variable) and independent variable is explained by the equation as,

$$Y = \varphi_0 + \varphi_1 X_1 + \varphi_2 X_2 + \varphi_{11} X_1^2 + \varphi_{22} X_2^2 + \varphi_{12} X_1 X_2 \quad (3.2)$$

Where, φ_0, φ_1 are linear coefficients, φ_{11}, φ_{22} are quadratic coefficients and φ_{12} is the coefficient of interaction

Table 3.4. Level of factors used for optimization of methane production

Independent variable		**Variable level**				
		-1.414 (-α)	**-1**	**0**	**1**	**+1.414 (+α)**
X_1	pH	6.43	6.6	7.0	7.4	7.57
X_2	F/M	0.48	1.0	2.25	3.5	4.02

The effect of the variables (X_1, X_2 and X_3) and their relationship with response variable (methane yield, Z) was analyzed by performing analysis of variance (ANOVA) and significance experiments on dependent response to check adequacy of the model. The value of the modeling equation is denoted by regression coefficients (R^2) and adjusted regression coefficients (R_{adj}^2). The statistical analysis was established by the Fisher test (F-test) dependent on the p of 95% confidence level. The optimized process parameters were selected through the dependent response analyzed functional parameters of the MINITAB software. Three-dimensional interface plotting and two-dimensional (2D) plotting of contour was done on Design Expert 7.0.

3.5 PHASE III- PRETREATMENT STUDY

3.5.1 Sample preparation

The collected individual RS samples were cleaned manually, grinded and sieved for removal of particulate matter. The water-soluble extract was prepared by the following procedure: samples were prepared by mixing 5 g of grinded RS with 45 ml of distilled water in a conical flask (glassware) to make a solid:liquid ratio of 1:10. Then, conical flasks containing substrates were kept for mechanical shaking for 2 h at 100 rpm in order to make sample consistent (Fig. 3.3).

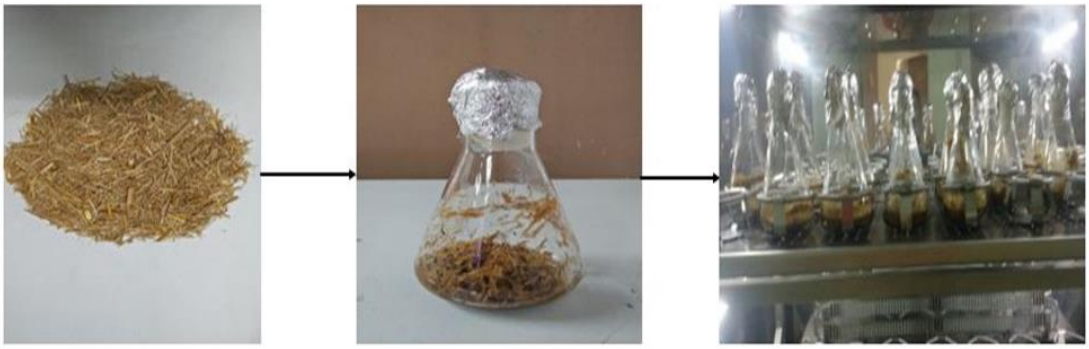


Fig. 3.4. Sample preparation for pretreatment experiment

3.5.2 Thermal pretreatment

In this study, the comparative effect of different thermal pretreatment techniques (hot air oven, hot water bath, microwave and autoclave) on the solublisation and degradability of RS was studied. This study is divided into two parts as temperature study and time study, for different pretreatment conditions obtained from literatures (Kim et al., 2003; Eskicioglu et al., 2006; Ennouri et al., 2016). The influence of treatment on degradability was assessed by measuring the accelerated methane potential of substrate by BMP setup for different F/M ratios (0.5, 1.5, 2, 2.5, 3).

3.5.2.1 Hot air oven

RS samples were prepared in sealed conical flasks and allowed to stand in hot air oven ((Fisher Scientific, Isotemp 637G Oven, USA) (3.5 a)), which works upon conduction and convection principle for the transfer of heat energy throughout the sample for pretreatment purpose. Previous study done by Veluchamy and Kalamdhad (2017) for pretreatment of pulp and paper mill sludge (lignocellulosic material) using hot air oven suggested pretreatment temperatures 70, 80, 90, 100, 110 and 120°C for 45 min as exposure time. Best temperature was selected based on optimum conditions obtained in temperature study of RS and further a trial has been made to obtain best exposure time from series of exposure times i.e. 30, 45, 60, 90 and 120 min.

3.5.2.2 Hot water bath

Sealed conical flasks containing RS were kept in hot water bath ((Fisher Scientific, Isotemp 3016H, USA) (Fig. 3.5 b)), in which conduction and convection principles were responsible for heat transfer throughout the RS samples and hydrating the complex structure

of RS. Thus, this process is also called hydrothermolysis. RS samples were allowed to treat at different pretreatment temperatures like 60, 70, 80, 90 and 100°C for 60 min. Best pretreatment temperature was selected based on maximum solubilisation expressed as sCOD and in second phase RS samples were allowed to treat at different exposure times: 30, 60, 90 and 120 min at the same pretreatment temperature obtained from first experiment.

3.5.2.3 Autoclave

In this pretreatment, conical flasks containing RS were kept in an autoclave in which water vapour (steam) works as media to transfer heat through conduction and convection to rupture recalcitrant matrix of RS, thus process also known as autohydrolysis (Fig. 3.5 c)). Pretreatment temperatures were considered as 80, 90, 100, 110 and 120°C, out of which pretreatment temperature showing the maximum solublisation (sCOD) was selected as best temperature and RS samples were replicated for further treatment at different exposure times i.e. 15, 20, 35, and 45 min at the optimum temperature obtained in temperature study.

3.5.2.4 Microwave

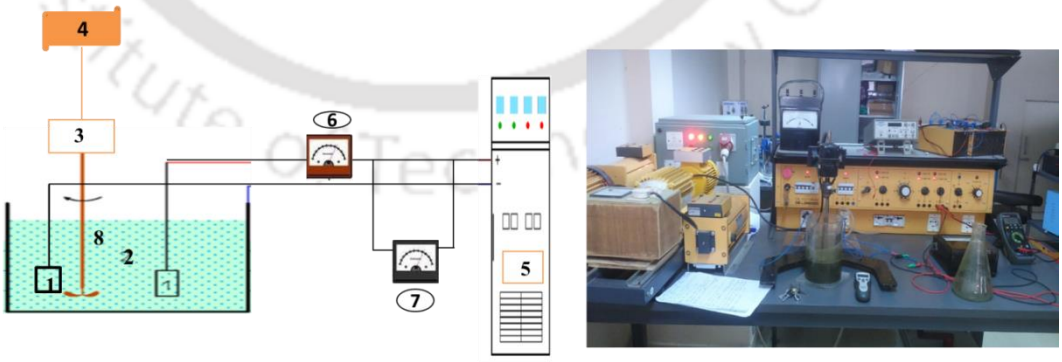
Sealed conical flasks having RS samples were allowed to keep inside the microwave ((Samsung CE 118KF) (Fig. 3.5 d)), which works on radiation principle, by virtue of which energetic electrons or photons were responsible for heat transfer throughout the sample to breakdown the complex structure of RS. For each experimental run, 5 g of the air dried sample was taken in Erlenmeyer flask (250 mL) with 2.45 GHz frequency, with predetermined time and temperature. After the pretreatment is done it is cooled down to room temperature inside a desiccator. Samples were placed in the centre of the plate in microwave. The sample rotates inside the electromagnetic field formed in the oven, allowing constant absorption of energy in the sample. Pretreatment temperatures i.e. 130, 150, 170, 190, 210 and 230°C for 3 min were selected on the basis of previous literature (Sapci, 2013), out of which temperature corresponding to optimum results (maximum sCOD) was considered as best temperature at which again experiments has been done for different exposure times: 2, 3, 4 and 5 min.

a) b) c) d)

Fig. 3.5. a) Hot air oven b) hot water bath c) autoclave and d) microwave oven

3.5.3 Electrohydrolysis pretreatment

Fig. 3.5 shows the schematic diagram and experimental setup of electrohydrolysis pretreatment. The set up consists of cylindrical plastic feed tank with volume 2 L having 10 cm diameter and 26 cm height, DC power supplier, RPM regulator, flash mixer, graphite electrodes, ammeter, multimeter and tachometer. Tachometer was used to measure the rotation of flash mixture. Multimeter and ammeter were used to measure applied voltage and current respectively and also to close the electric circuit so that current easily flow through the circuit. The feed tank was half filled with sample and electric current was allowed to pass through the sample with the help of graphite electrodes connected to DC power supply. The distance between two electrodes was 8 cm, and two-third part of electrodes (cathode and anode) was immersed in the mixture without touching the flash mixture. It was controlled by RPM regulator (200 rpm) to ensure homogeneity of mixture throughout the treatment process.



1-Graphite electrodes 2-Electrolyte 3-Motor 4-Speed Control Unit 5-DC Power Supply 6-Ammeter 7-Voltmeter 8-Flash mixture

Fig. 3.6. Schematic and experimental setup illustration of electrohydrolysis pretreatment

In voltage study, the effect of applied DC voltage on RS was studied at different voltages i.e. 10, 15, 20, 25 and 30 V for a fixed exposure time of 30 min (Zhen et al., 2013). An untreated sample of RS was kept as control. In time study, the effect of exposure time on RS was studied for dissimilar pretreatment time intervals i.e. 15, 30, 45, 60 and 80 min at constant applied voltage obtained in voltage study and one sample was kept as control (Veluchamy et al., 2017). Finally, optimum pretreatment conditions in terms of applied voltage and exposure time were chosen based on the maximum sCOD and VFA in reactors fed with DCD (inoculum containing microorganisms) and RS (substrate) pretreated at best pretreatment conditions obtained in voltage and time studies, corresponding to five F/M ratios (0.5, 1.5, 2, 2.5, and 3) based on VS content. One reactor was loaded with untreated RS and DCD, considered as control. Table 3.5 depicts the composition of substrate and inoculum (untreated/pretreated RS, DCD) taken in an anaerobic reactor.

Table 3.5. Composition of different materials taken in anaerobic reactors for BMP assay

F/M ratio	Rice straw (g)	Inoculum (g)	Water (g)
Control	5	100	595
F/M:0.5	2.67	100	597.33
F/M:1.5	8	100	592
F/M:2	10.67	100	589.33
F/M:2.5	13.33	100	586.7
F/M:3	16	100	584

3.5.4 Fungal pretreatment

3.5.4.1 Inoculation of fungal strain into substrate

Pure culture of *Pleurotus ostreatus* (PO) (NRRL 3526), *Phanerochaete chrysosposrium* (PC) (NRRL 6370), and *Ganoderma lucidum* (GL) (NRRL 66208) were purchased from the National Center for Agricultural Utilization Research (NCAUR), in Peoria, Illinois, USA. All fungi strains were cultured on potato dextrose agar (PDA) plates in an incubator at 30°C for 7 days (pH ≈ 5-5.5). After that, 3-4 pieces of agar medium (1.0 x 1.0 cm) containing fungal hyphae were transferred to an Erlenmeyer flask (250 mL) containing 50 mL of potato dextrose broth. The Erlenmeyer flask was subsequently capped with sterilized cotton

plugs and incubated at 30°C for 7 days in orbital shaker (100 rpm). The freshly-grown fungal hyphae were separated from the liquid broth by centrifuging (Beckman Coulter, Allegra 64 R Centrifuge, USA) at 5000 rpm for 10 min followed by suspending it into autoclaved deionized water corresponding to moisture requirements (PC 75%, PO 70%, GL 65%) (Wang et al., 2001; Shi et al., 2008; Nishitoba et al., 2014) for different fungal strains on the basis of TS contents. These suspensions of fungal mycelium were further used as an inoculum for hydrolysis of RS. Further, 5g of RS sample was put in an Erlenmeyer flask with cotton plugs, and after sterilizing at 120°C for 30 min (to check microbial growth). A control was also set at 75% moisture. All the reactors were placed in an incubator at 30°C, 70% relative humidity for five weeks on continuous shaking (150 rpm). Characteristics of pretreated RS were measured weekly to check different parameters, as mentioned in section 2.3. Hydrolysis was continued until we achieved constant VS% in the substrate (5 weeks).

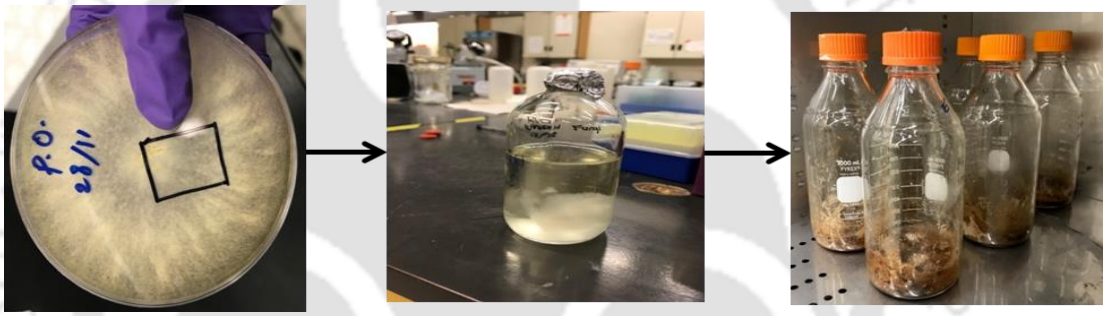


Fig. 3.7. Inoculation of RS with different fungal strain

3.5.4.2 BMP setup

After fungal pretreatment, to check the biodegradability of the hydrolyzed substrate, a BMP test was done. The BMP setup consisted of a 1000 mL glass reactor (working volume 700 mL), a 500 mL aspirator bottle for displacement of liquid (1.5 N NaOH), and a 100 mL glass beaker for collection of displaced liquid. The quantification of methane was done by the water displacement method (Stroot et al., 2001), where, the methane produced displaces the liquid and CO_2 dissolves in the liquid, making a precipitate (with thymol blue indicator). Therefore, the displaced NaOH solution gives the estimation of methane produced daily. Mixing was done manually 2-3 times a day. Fifteen BMP reactors were set up, containing pretreated and untreated substrate in triplicate. Nine reactors consisted of PO, PC and GL pretreated RS (10 g) with cow dung (25 g) with F/M ratio of 2. Control 1 (C1) was fed with cow dung (25 g) only to determine the actual enhanced methane produced from pretreatment of RS (value is deducted from all reactors' yields). Control 2 (C2) reactors was

fed with RS (untreated) (10 g) and cow dung (25 g) (F/M:2). Macro (phosphate buffer) and micro nutrients (iron (0.35 mg/L, $FeCl_3 \cdot 7H_2O$), magnesium (20.5 g/L, $MgSO_4 \cdot 7H_2O$), calcium (27.5 g/L, $CaCl_2 \cdot 2H_2O$), cobalt (0.05 mg/L, $Co(NO_3)_2 \cdot 6H_2O$, nickel (0.05 mg/L, $NiCl_2 \cdot 2H_2O$)) were added in the reactors, after which all reactors were expunged with N_2 gas for 3 min, so as to have anaerobic state. In the end, all reactors were sealed with rubber cork drilled with pipe for gas collection.

3.6 PHASE IV- DESIGN AND OPERATION OF SINGLE PHASE CONTINUOUS ANAEROBIC REACTOR (SPCAR)

3.6.1 Design of single phase continuous anaerobic reactor

The key process stages of AD include hydrolysis, acidogenesis, acetogenesis and methanogenesis. The design of anaerobic digester affects the microbial health of these processes and the overall performance of the digester. Therefore, the goal of this stage was to develop and commission a lab scale SPCAR. Dimension of the SPCAR has a volume of 20 L, (working volume, 14 L). All details about the position of inlet, outlet and mixing shaft are shown in Fig. 3.5. Dimension of the preliminary batch scale study helped to determine baseline biogas production values and expected retention time.

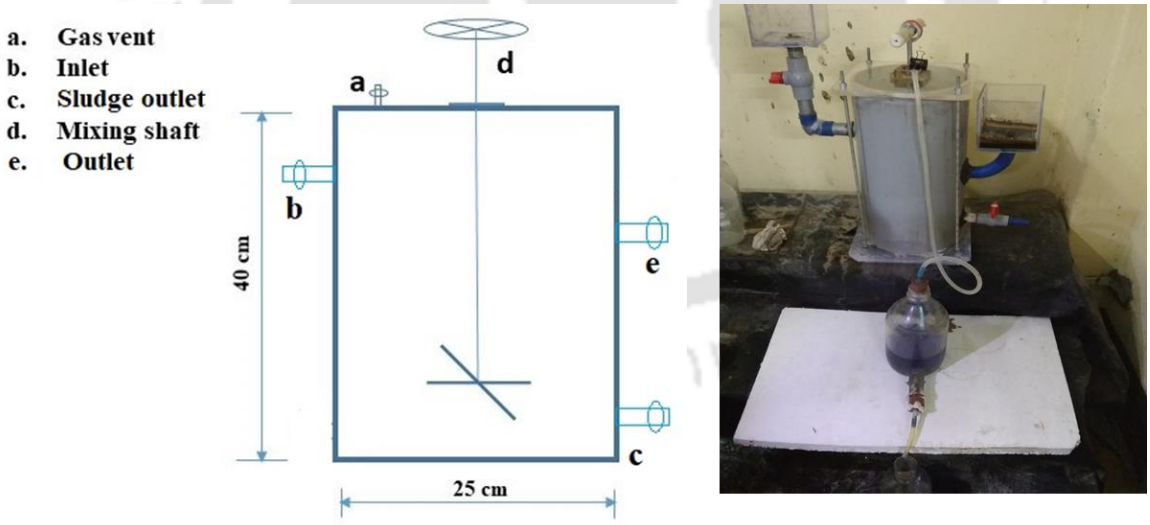


Fig. 3.8. a) Schematic and b) pictorial representation of SPCAR

3.6.2 Operation of single phase continuous anaerobic reactor

The continuous reactor was operated for 150 days in three stages. Stage I includes the feeding of SPCAR with substrate (untreated) with optimum F/M ratio from inoculum study.

Stage II includes the microwave pretreated RS with optimum F/M ratio from pretreatment study and stage III consists of the RS co-digested with FW with optimum parameters. During initial 30 days (stage I) of digestion time after acclimatization period, SPCAR was fed with untreated RS with OLR of 3.5 kg-VS/m^3/d. During next 60 days (stage II) of digestion time, SPCAR was fed with pretreated RS obtained as best in batch study with 8.3 kg-VS/m^3/d and last 60 days were fed in the reactor co-digested with FW for the optimum condition obtained from the batch study with 6.5 kg-VS/m^3/d OLR. Daily methane production rate, pH, sCOD and VS% degradation were calculated to quantify the operation of SPCAR in all stages and to check relevance of different enhancement techniques.

3.7 ANALYSIS OF DATA THROUGH KINETIC MODELS

In this study, methane yield was modelled using three well-established kinetic models i.e. Modified Gompertz Model (MGM), Logistic Function Model (LFM) and Transference Function Model (TFM) (Deepanraj et al., 2015; Ware and Power, 2017) for BMP assay results. Modeling of methane yield curve done by many researchers was useful in determining the various parameters, such as specific growth curve (methanogens), antimicrobials impact, lag phase etc. The MGM model is usually applied to feedstock generating methane and hydrogen, and follows the L-shape curve. The feedstock containing high concentration of fats, lipids etc. follow a S-shape degradation curve (cumulative methane yield) (LFM, TFM). A nonlinear regression (least-square) analysis was used for 'SQP' function in MATLAB R2015a, for fitting the nonlinear modeling equations as expressed in Table 3.6.

Table 3.6. Equation of the model used for analysis

Model used	Equation of the model
MGM	$Z = M \cdot \exp\{-\exp[\frac{Rm \cdot e}{M}(\lambda - t) + 1]\}$
LFM	$Z = \frac{\mathrm{M}}{1 + \exp\{\frac{4 \cdot Rm \cdot (\lambda - t)}{M} + 2\}}$
TFM	$Z = M\{1 - \exp(-\frac{Rm \cdot (t - \lambda)}{M})\}$

Where, Z is cumulative methane production rate (mL/d), M is the methane production potential (L/kg-VS_{added}), R_m is the maximum rate of methane production (L/kg-VS_{added}/d) and λ is the lag phase constant (days). This check was done to investigate the correctness in describing the degradation profile linked with the substrate. The average cumulative methane yield (BMP assay) with respect to time from triplicate samples was analysed for model constants (λ, M, R_m). The measured methane yields were fitted with predicted methane yields to determine the graphical fit. Coefficient of correlation (R^2) was also determined by considering the regression tool (Mu et al., 2007; Deepanraj et al., 2015). To check the goodness of fit of the predicted graph 95% confidence interval was chosen. Visual check of fitted predicted and measured value in addition to regression value was used into consideration to investigate the feasibility of the model.

3.8 ANALYTICAL METHODS AND SPECTROSCOPIC CHARACTERIZATION FOR SAMPLE ANALYSIS

Samples were taken on weekly basis from the reactor and analysed for various parameters such as TS, VS, M.C. and sCOD were determined according to standard methods (APHA, 2005). Samples were centrifuged at 10,000 rpm for 10 minutes and supernatant was filtered through a 0.45-μm membrane filter for the analysis of VFA. pH value of the sample was measured by making a mixture of substrate and deionized water in 1:10 ratio and mixing for 2 h at 150 rpm in horizontal shaker. Portable pH meter was used for determining the pH of the sample. pH value of the sample was measured by making a mixture of substrate and deionized water in 1:10 ratio and mixing for 2 h at 150 rpm in horizontal shaker. FA concentration (NH_3-N) was calculated from the total ammonical nitrogen (NH_4-N) concentration, pH and temperature (Hansen et al., 1998). pH titration method by DiLallo and Albertson (1961) was used for quantifying the VFA concentration in the reactor. The lignin content (acid soluble and insoluble lignin) was determined by the National Renewable Energy Laboratory (NREL) protocol (Sluiter et al., 2012). RS sample was used to determine acid insoluble lignin content by gravimetric technique. Acid soluble lignin was determined by UV spectrophotometer through colorimetric technique at 205 nm with the hydrolysate obtained after filtration of cooled insoluble lignin and 4% H_2SO_4 solution was set as a reference blank. For cellulose analysis, 3 mL of acetic/nitric reagent was added to 0.5 g of RS sample and hydrated in water bath and was centrifuged. After cooling, supernatant was discarded and residue was infused with 67% H_2SO_4. Further, anthrone reagent was added to diluted sample and boiled for 10 min. Finally, cellulose content was measured through colorimetric technique at 603 nm by using

spectrophotometer and anthrone reagent was set as reference blank. (Goering and Van (1975) method was used in determining hemicellulose content by finding the difference between neutral detergent fibre (NDF) and acid detergent fibre (ADF). Oxidation reduction potential (ORP) was measured by pH and platinum ORP sensor (Hanna Instruments Hi98194). Potassium ferricyanide with an ORP value of +234 mV was used for calibration. The C/N analysis was executed by elemental analyzer (Euro Vector, EuroEA3000, Italy) in Biotech Park, Assam, India. The experimental data were investigated and plotted using Origin software (version 8.5).

FESEM (Germany, Sigma, Zeiss) micrographs of pretreated and untreated RS showed the visual characterization of the structural morphology of RS sample. Double coated gold samples were used for the imaging (UK, Quorum, SC7620), to put off degradation and for the charge to build up. The presence of modified functional groups and microcrystalline structure signifies the lignocellulosic component alteration of the RS after pretreatment. The FTIR fingerprint spectrums (Japan, SHIMADZU, IR Affinity-1) were recorded from 4000 to 500 cm^{-1} with 16 scans at a resolution of 4 cm^{-1}. 1 mg of dried RS was mixed with 300 mg of KBr in a mortar, and then the mixture was compressed for 3 min at 10 MPa to set up the sample disc.

3.9 INSTRUMENTS USED

Different instruments were used for analysis of the samples and understanding the change of process parameters and morphological changes in substrates. Table 3.7 shows the list of different instruments that were used during different experiments.

Table 3.7. Instrument used during experimental analysis

Parameter	Instrument/ Protocol
Centrifugation	Centrifuge machine
Chemical oxygen demand	COD digester
Volatile fatty acids	DiLallo and Albertson, 1961
Topographic details on the surface (Imaging)	Field emission electron microscopy
Infrared spectrum of absorption of a sample	Fourier Transform Infrared spectroscopy
Size reduction	Grinder

Oxidation reduction potential	HM Digital ORP-200
Total solids	Hot air oven
Thermal treatment	Hot water bath, Microwave, Autoclave
Elemental composition	CHNS analyzer
Shaking incubator	Mixing
Volatile solids	Muffle Furnace
pH	pH meter
Absorbance of sample	Spectrophotometer
Autoclave	Sterilization

CHAPTER 4

INOCULUM STUDY

4.1 INITIAL CHARACTERIZATION OF THE RS AND INOCULUM

Table 4.1 summarizes the initial characterization of RS, FCD and DCD. Initial TS of RS was 94.03% with 80.5% of VS shows its higher organic matter content. The substrate and inoculum in this study had a wide range of VS/TS content along with sCOD suggesting its suitability for AD. The initial nearly neutral pH and VFA of RS and the inoculums also support AD process.

Table 4.1. Characterizations of the RS and different inoculum

Parameters	RS	DCD	FCD
pH	6.85 ± 0.50	7.75 ± 0.20	7.08 ± 0.15
TS (%)	94.03 ± 0.45	11.98 ± 0.78	19.8 ± 1.8
VS (% TS)	80.50 ± 1.40	32.62 ± 1.32	65.028 ± 0.47
sCOD (g/L)	5.91 ± 0.70	7.84 ± 0.73	8.13 ± 0.46

4.2 BIOCHEMICAL METHANE POTENTIAL SETUP

To evaluate the feasibility of DCD and FCD with RS as a substrate, four different F/M ratios varying from 0.25 to 0.75 were tested for a period of 48 days. For anaerobic biodegradation, F/M is considered as an important parameter that helps in the determination of biodegradability pattern along with the bio-activity of different inoculums. It also affects the cumulative methane yield and the process kinetics. Inoculum plays a key role in initiating the fermentation by balancing the microbial population of syntrophobacter and methanogens. The inoculum also sustains the syntrophic metabolism for the thermodynamic feasibility of reactor.

Initially, the hydrolytic bacteria break down the complex organics into a more soluble form by releasing extracellular enzymes by the process called hydrolysis. This happens to be the rate limiting step in the overall digestion process. The hydrolysis product acts as a substrate for acidogenic bacteria. Subsequently, the intermediate products are transformed into simpler compounds such as acetate, carbon dioxide, hydrogen and formate by secondary fermentation with a final decrease in the pH of the system. The production of

acid can be visualized by increased VFA production. Finally, methanogenic archaea, which are highly specific to these substrates, converts them to biogas. The breakdown or degradation of lignocellulosic substrate leads to increase in organics which subsequently causes an increase in the soluble COD.

4.2.1 Biomethane production

The main aim of AD is to break the complex structures of organic compounds into smaller units which is eventually converted to biomethane. Various physiological parameters such as inoculum characteristics, pH, VS, VFA and temperature affect the overall biomethane production by affecting the degradation process. Fig. 4.1 a) and b) showed the daily methane production for various F/M ratios for FCD and DCD. In the early days, gas production data was very uncertain; it was difficult to predict which F/M ratio produces higher biogas. In the existing experiment, it was observed that initially biogas production was comparatively same with all the F/M ratios. It can therefore, be perceived that the initial methane yield may be due to readily available organics present within the inoculums. The solubilization rate of RS was very slow and took approximately 20 days to start but was consistent up to 40th day.

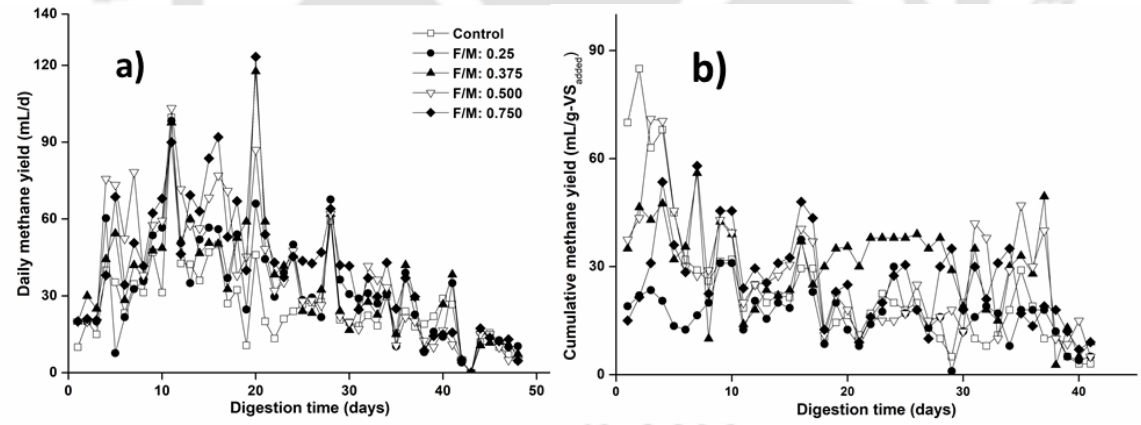


Fig. 4.1. Daily methane production at different F/M ratios for a) FCD and b) DCD

In case of FCD as inoculum, from the 18th day onwards the F/M ratio of 0.5 and 0.75 were most productive. The maximum cumulative methane production of 1939 mL was achieved in the case of F/M ratio 0.75. With DCD as inoculum, the methane gas production was consistent throughout the process and found to be maximum (1127 mL) for F/M ratio of 0.375 and ceased after 40th days Fig. 4.2 a) and b).

The digested sludge is considered to have higher nutrients concentration and comprises essential micro and macro nutrients that may aid to improve the microbial activity on the other hand, due to lower degradable organics present in the DCD, the bacterial inoculum cannot further sustain due to unavailability of simpler carbon source for initial acclimatization under the recalcitrant atmosphere of RS. In contrast, the FCD inoculum contains enough amount of degradable organics (can be perceived by higher initial VS content of FCD) for the initial acclimatization of microbial population.

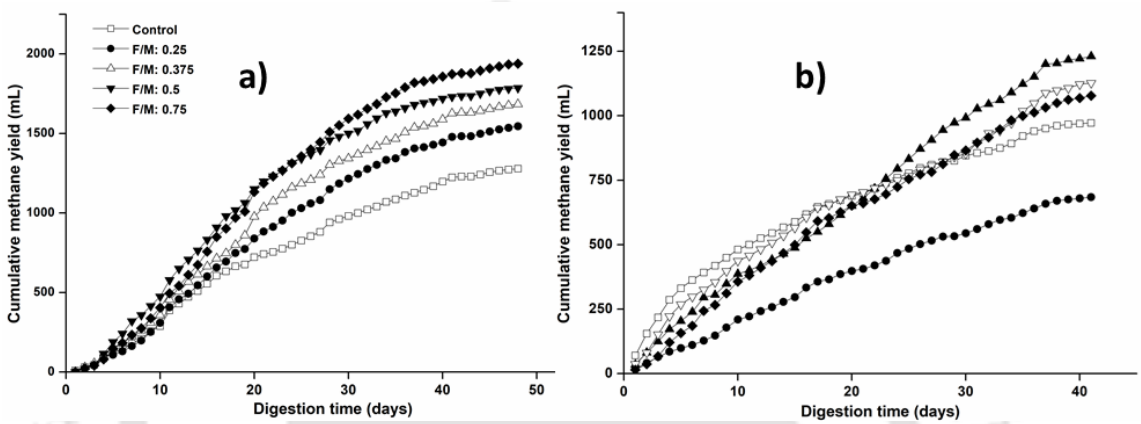


Fig. 4.2. Cumulative methane production at different F/M ratios for a) FCD and b) DCD

Control produced the lowest amount of gas of 1278 mL for FCD and 972.5 mL for DCD setup. Cumulative methane yield on the basis of VS added in the reactor came out to be 72.25 and 125.767 mL/g-$VS_{added,}$ for FCD and DCD respectively.

4.2.2 Effect on VS% degradation

Mass loss in AD is indication of fall of VS, and can be directly co-related with the biogas production. Higher degradation refers to higher solubilization. In general, the reduction of VS is indication of microbial activity for the conversion of complex organics to simpler molecules which can be further converted to biogas. Initially at day zero, the solubility of RS with inoculum appeared to be very less because almost all the substrate was settled at the bottom of the reactor but after the solubilization of substrate with inoculums, the definite VS was being measured. The analysis of the results of BMP studies has been shown in Fig. 4.2 a) and b) respectively for FCD and DCD samples. Clearly, VS is reduced during the process for both FCD and DCD respectively as shown in Fig. 4.3 a) and b).

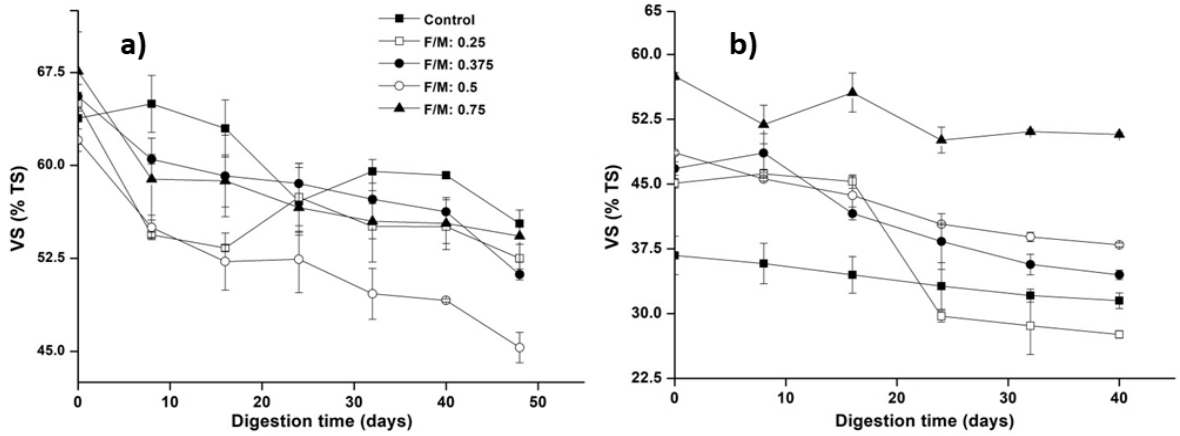


Fig. 4.3. VS % at different F/M ratios for a) FCD and b) DCD

In case of DCD as inoculum source the degradation is more due to lowered activity of bacteria. This may be due to the reduction less quantity of organics present in DCD with initial VS of 37.49%. While in case of FCD as inoculum, the availability of organics was adequate for initial adaptation of bacterial population. Therefore, reduction in case of FCD was comparatively higher with respect to DCD as inoculum source.

4.2.3 Effect of degradation on change in pH

In AD process, solubilization or digestion of organics is an indication of change in pH. For a reliable anaerobic process, appropriate pH-values in the range of 6.8-7.2 are required. The methanogens are most sensitive to changes in the pH. The VFA that are produced by acetogenic bacteria reduce the pH, which affects the overall activity of methanogens and subsequently decrease the biogas yield. Therefore, maintaining pH to optimum range is crucial.

For maintaining the buffering capacity in an acceptable range, dominant buffer systems, such as bicarbonate are required. In the present BMP-experiments, pH-values fell proximately. Therefore, on 7^{th} day, the addition of sodium bicarbonate ($NaHCO_3$) to the digester was done to maintain the pH in a neutral (pH~7) range. Fig. 4.4 (a) and (b) demonstrate the changes in pH for FCD and DCD respectively.

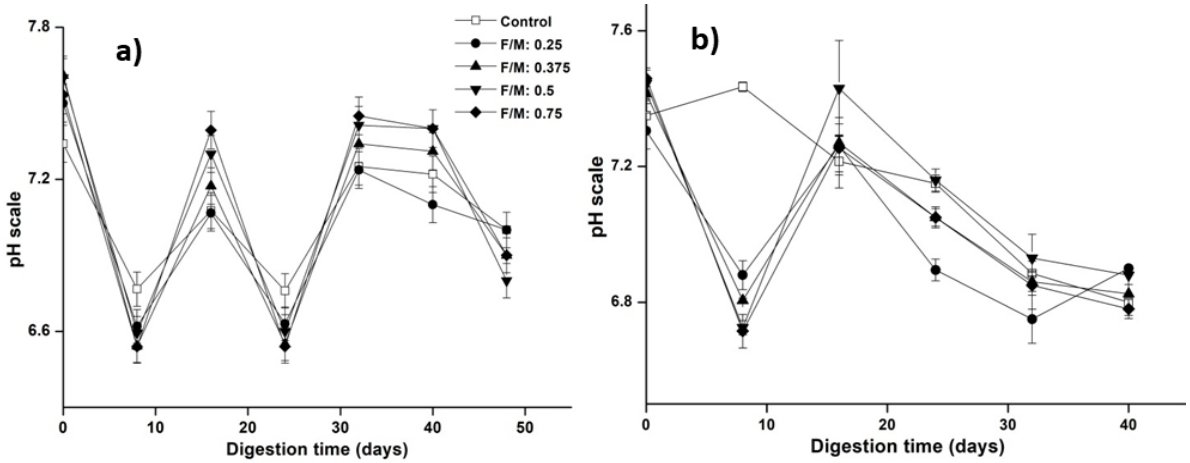


Fig. 4.4. pH at different F/M ratios for a) FCD and b) DCD

4.2.4 VFA

In AD of RS, different VFA profiles were revealed for F/M ratios varying from 0.25 to 0.75. In case of FCD as inoculum, initially the VFA concentration was moderately low due to lower availability of the solubilized substrate, while in case of DCD as inoculums; the higher accumulated VFA in inoculum gives higher values. Later, with hydrolytic and acetogenic activities of bacterial consortium, there was a gradual increase in the VFA content as observed for both reactors. In case of DCD as inoculum VFA production was reasonably very low, this reflects the lower activity of inoculum for RS.

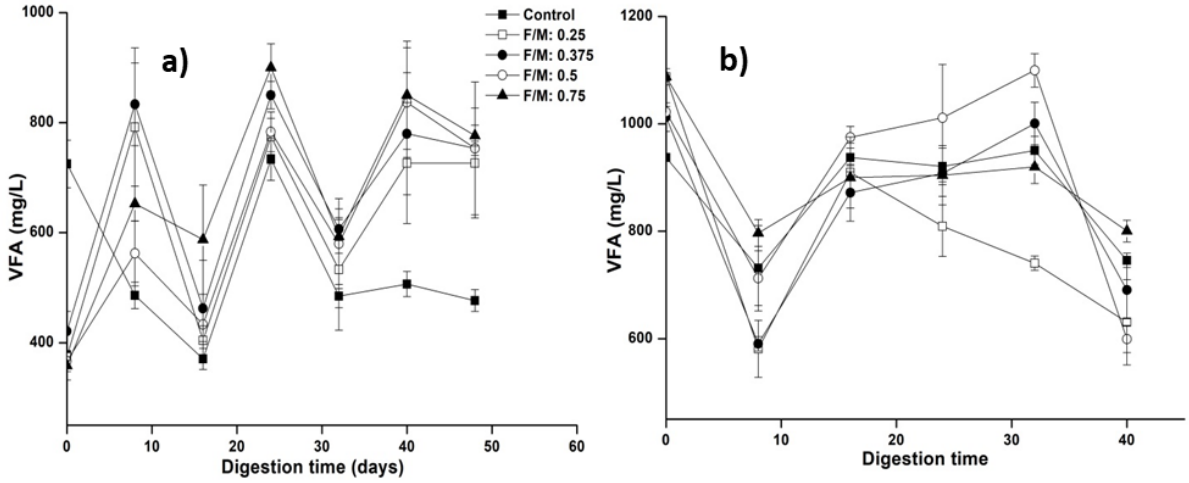


Fig. 4.5. Change in VFA at different F/M ratios for a) FCD and b) DCD

The acidic monomers thereby produced were affected and therefore seems to oscillate in the overall production of VFA. After the organic matters are transformed to VFA, the removal can be interpreted in terms of gas production. Fig. 4.5 a) and b) indicate that the VFA concentrations of BMP-experiments increase on the early days, during that time proteins as well as carbohydrates and fats are converted into acids by hydrolysis and acidogenesis.

4.2.5 sCOD

With the information on the sCOD, the hydrolysis and solubilization of substrate can be evaluated. Hydrolytic bacteria that are present in inoculum convert the complex organics to simpler monomer units by secreting extracellular enzymes which lead to the increase in the sCOD. Later, the simpler monomers are then consumed by another class of microorganisms such as homoacetogens and methanogens which simultaneously convert it to biogas under anaerobic conditions which subsequently decreases the sCOD of the reactor. The sCOD for both FCD and DCD is shown in Fig. 4.6 a) and b) respectively. Different F/M ratios provides varied substrate removal rate in the form of sCOD. Addition of 1N $NaHCO_3$ for pH balance is responsible for the twisted curve during the process, which ultimately affects the sCOD concentration by disturbing the VFA concentration of the AD process.

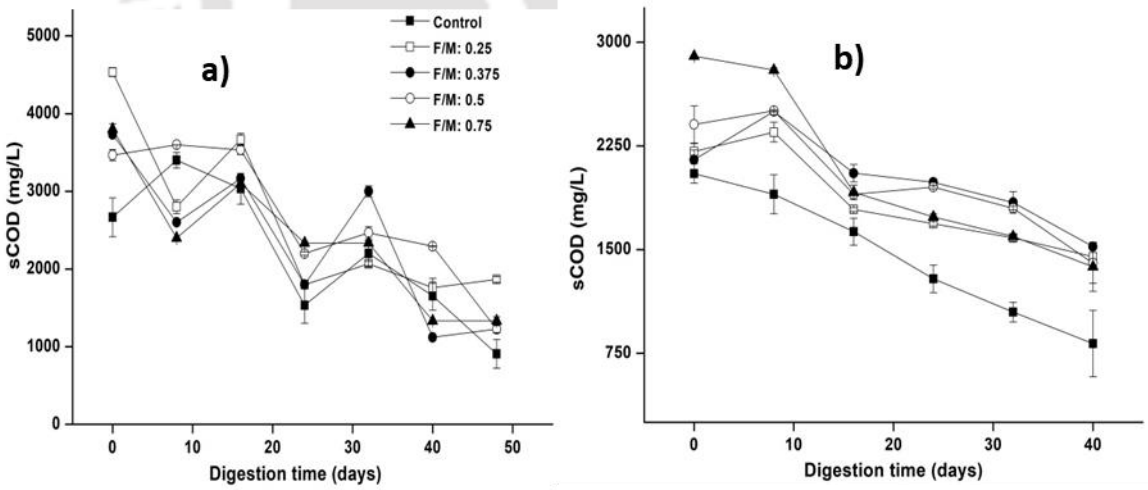


Fig. 4.6. sCOD at different F/M ratios for a) FCD and b) DCD

4.3 16S METAGENOME SEQUENCING OF INOCULUM

The main aim of this study was to focus on the identification of different bacterial and archaeal communities present in the inoculum for carrying out the anaerobic degradation of

RS in co-digestion and pretreatment studies. The DCD was analyzed by 16S Metagenome sequence method.

4.3.1 Isolation, qualitative and quantitative analysis of gDNA and preparation of libraries for 2 x 250 bp run chemistry

The isolation of gDNA from the sample was carried out using Xcelgen soil gDNA isolation kit. Quality of genomic DNA was checked on 0.8% agarose gel (loaded 3µl) for the single intact band. The gel was run at 110 V for 30 mins. 1µl of each sample was used for determining the concentration using Qubit® 2.0 Fluorometer. The amplicon library was prepared using Nextera XT Index Kit (Illumina inc.) as per the 16S metagenomics sequencing library preparation protocol. Primers for the amplification of the V3-V4 hyper-variable region (Table 4.2) of 16S rDNA gene of bacteria and archaea were designed in-house by Xcelris Labs Limited. These primers were synthesized in Xcelris PrimeX facility**.** The amplicon with the Illumina adaptors were amplified by using i5 and i7 primers that add multiplexing index sequences as well as common adapters required for cluster generation (P5 and P7) as per the standard Illumina protocol. The amplicon libraries were purified by 1X AMpureXP beads, checked on Agilent DNA1000 chip on Bioanalyzer 2100 and quantified by Qubit Fluorometer 2.0 using Qubit dsDNA HS Assay kit (Life Technologies).

Table 4.2. Primers used in the present study

S.No.	Oligo name	Oligo Sequence (5' to 3')	Primer length	Product size (Approx.)
1	V3 - Forward	CCTACGGGNBGCASCAG	17	~460 bps
	V4 - Reverse	GACTACNVGGGTATCTAATCC	21	

4.3.2 Cluster Generation and Sequencing

After obtaining the Qubit concentration for the library and the mean peak size from Bioanalyser profile, library was loaded onto Illumina platform at appropriate concentration (10-20 pM) for cluster generation and sequencing. Paired-End sequencing allows the template fragments to be sequenced in both the forward and reverse directions on Illumina platform. The kit reagents were used in binding of samples to complementary adapter oligos on paired-end flow cell. The adapters were designed to allow selective cleavage of the

forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragment.

4.3.3 Quality control (QC) of DNA on Agarose Gel

The library was prepared from analysis of sample after amplifying V3-V4 region of 16S segment. The 16S mean library size is 629 bp and the library was sequenced using the Illumina 2 x 250 bp sequencing chemistry to generate ~150Mb of data per library. The bands on polyacrylamide gels shows the conformation polymorphism and bands in gel revealed the reproducibility of DNA from PCR amplification (Fig. 4.7).

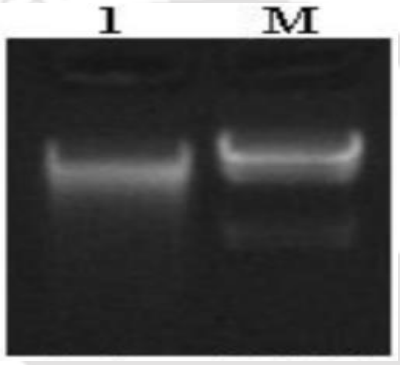


Fig. 4.7. QC of gDNA on 0.8% Agarose gel

4.3.4 Taxonomic distribution

The pie charts below illustrate the distribution of taxonomic domains, phyla and orders for the annotations. Each slice indicates the percentage of reads with predicted proteins and ribosomal RNA genes annotated to the indicated taxonomic level.

4.3.4.1 Taxonomic hits distribution at Phylum Level

The methane yield from the batch process totally depends upon the microbial community present in the reactor (Fig. 4.8). The taxonomic hits distribution at phylum level expressed that inoculum (DCD) is enriched with majority of *Proteobacteria, Bacteroidetes, Firmicutes* and *Chloroflexi* in the starting of reactor, which are helpful for the AD of substrate. The addition of first four phyla level comprises the 77.4% of the total reads. Phylum *Proteobacteria* (37%) was the highest among all. These can easily survive in fermentative and obligate stage in the reactor. *Firmicutes* (20.3%) is the facultative bacteria, which synthesize lignocellulosic degrading enzymes i.e. cellulase, proteases etc.

Chloroflexi (6.8%), which has the ability of degrading macromolecule was also found in abundant.

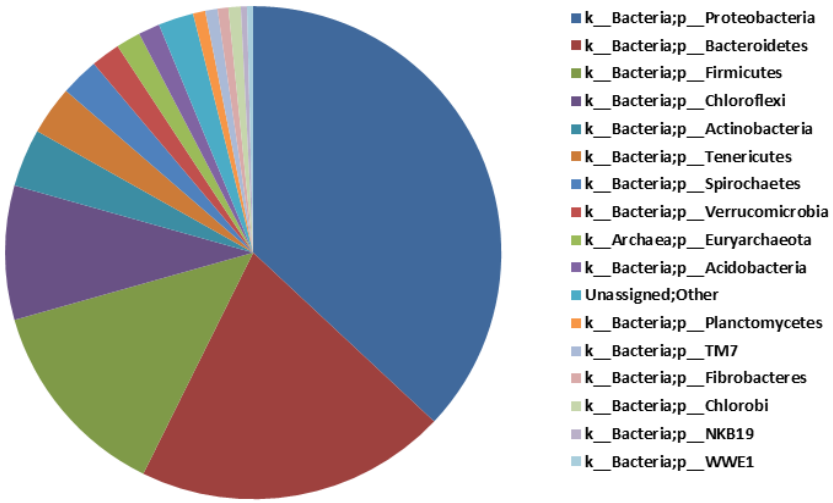


Fig. 4.8. Taxonomic hits distribution at phylum level

4.3.4.2 Taxonomic hits distribution at Class Level

Taxonomic hits distribution at class level shows that DCD has 16.2% *Bacteroidia*, 13.1% *Betaproteobacteria*, 11.6% *Gammaproteobacteria* and 10.6% *Clostridia* etc. as represented in the Fig. 4.9. In general, the dominance of *Bacteroidia* in biogas reactors fed with livestock manure has been dominant member of the thermophilic microbial community during co-digestion, with 14% of relative abundance.

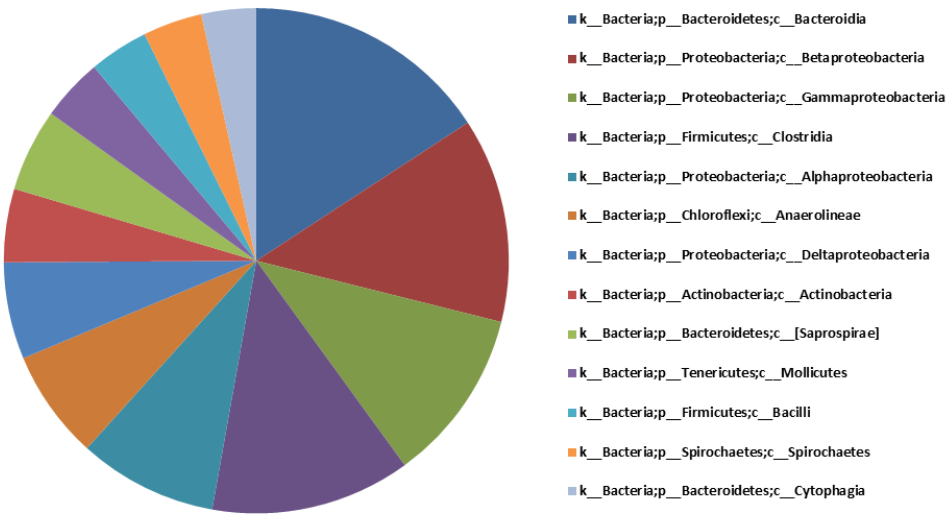


Fig. 4.9. Taxonomic hits distribution at class level

4.3.4.3 Taxonomic hits distribution at Order Level

Taxonomic hits distribution at order level shows that sample of DCD has 15.8% *Bacteroidales* and 12.6% *Clostridiales*, 9.1% *Rhodocyclales* and 7.2% *Rhizobiales* etc. as represented in the Fig. 4.10. These bacterial taxa were previously found to represent a significant fraction of the entire biogas community (Fykse et al., 2016; Treu et al., 2019). Concerning *Clostridiales*, an 87% similarity was found in NCBI database with *Ercella succinigenes*, which is an austerely anaerobic mesophilic bacterium able to ferment carbohydrates, generating H_2 and acetate as main product.

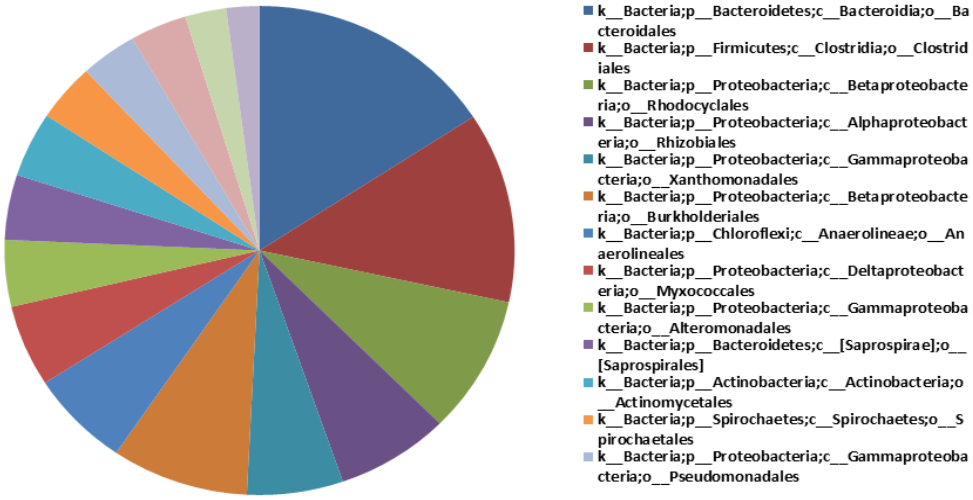


Fig. 4.10. Taxonomic hits distribution at order level

4.4 CONCLUSION

The BMP study makes it evident that RS is viable source for biogas production with DCD as an inoculum source than with FCD. The microbial profile of the reactors (DCD) was adequately correlated with the recorded biochemical parameters from DCD with majority of *Proteobacteria*. At order level, DCD is enriched with *Bacteroidales* and *Clostridiales* highlighting the main microbial player of microbial consortium for AD. It is predicted that low methane yield of 125.767 mL/g-VS_{added} from RS may be lower degradability of RS. This calls for a need of an extensive study on co-digestion and pretreatment studies, which are discussed in chapter 5 and chapter 6.

CHAPTER 5

CO-DIGESTION STUDY

This chapter includes the co-digestion of RS (substrate) with *H.verticillata* and FW (co-substrates), focused mainly on the individual and interactive effect of various parameters (C/N ratio, F/M ratio and pH) on methane yield from co-digestion and methane yield obtained for mono-digestion.

5.1 CHARACTERIZATION OF SUBSTRATE AND CO-SUBSTRATES

Co-digestion is assumed to be more cost effective for nutritional supplementation in comparison to pretreatment. However, the stability and effectiveness of mono-digestion of RS was low due to low buffering capacity, which causes acidification. Due to this reason, the co-digestion of RS with aquatic weed *H.verticillata* and FW not only balanced the improper C/N ratio but also improved the stability of fermentation process. The feedstocks characteristics are summarized in Table 5.1. The slightly neutral, alkaline and acidic pH values of 6.85, 7.4 and 5.4 were found for RS, *H.verticillata* and FW. C/N ratios of all substrates RS (43), *H.verticillata* (6.85) and FW (11.35) were individually not in desired range for AD.

Table 5.1. Characterization of substrate (RS) and co-substrates (*H.verticillata*, RS)

Parameters	**RS**	***H.verticillata***	**FW**
pH	6.85 ± 0.50	7.40 ± 0.20	5.4 ± 0.10
M.C. (%)	5.91 ± 0.30	90.82 ± 3.20	72.4 ± 3.60
sCOD (g/L)	5.91 ± 0.70	3.40 ± 0.60	7.4 ± 0.40
VS (%/TS)	80.50 ± 1.40	68.88 ± 2.70	46.31 ± 3.70
C (%/TS)	31.39	16.72	24.06
N (%/TS)	0.73	2.44	2.12
C/N	43.00	6.85	11.35

C/N ratio and VS% are the most significant parameters to give consideration in AD. RS is characterized by significantly high TS (94.09%) and VS (80.5%) content, more than co-substrates (*H.verticillata*, FW). Low VS% in inoculum shows its non-utilizable organic content; therefore it is used as a source of microbial flux only (Table 4.1).

5.2 CO-DIGESTION OF RS WITH *H.verticillata*

AD is a proven efficient bioenergy technology that generates methane-rich biogas from degradation of plant biomass, animal manure and crop residues. It helps in recycling range of feedstock into manures/fertilizers and production of bio-methane, a carbon-neutral source of energy. Methane generation from AD of RS is a promising alternative. Several studies have been accomplished using RS as mono-substrate for methane generation. However, some shortcomings were observed in direct utilization of RS, such as long assimilation time due to low cellulose conversion rate and excessive C/N ratio due to nitrogen scarcity (Mussoline et al., 2013). Excessive C/N ratio can inhibit the methane production due to the changes occurred in the microbial composition and metabolic pathway transfer, less nitrogen content also conciliates the cell growth. Some researchers (Yong et al., 2015; Zhu et al., 2014) reported the research work pertaining to co-digestion with FW, using additional nitrogen source (aqueous ammonia treatment) but no reports were found regarding addition of an aquatic weed. As the geographical location of the both the substrate and co-substrate are same, which increases its usefulness on larger a scale.

H.verticillata is a perennial, underwater, aquatic weed. Due to its adaptability and strong adsorption capacity of heavy metals (Lu et al., 2017) and nutrients (Takahashi and Asaeda, 2014), it is used for many treatment purposes i.e. removal of heavy metals, therapeutical treatment (Pal and Nimse, 2006). However, large spatial spread of *H.verticillata* is also a source of river pollution as it causes stagnation, reduction in dissolved oxygen content, foul odor and change in ecosystem. Dead *H.verticillata* further adds to river pollution and releases pollutants. Given the high harvesting cost, AD technique seems to be an environmental and economic approach for regulating extremely propagating macrophytes. However, low C/N ratio of *H.verticillata* causes the inhibition of methanogenesis due to release of high concentration of FA on degradation and accumulation of VFA. Due to its huge untapped potential for AD, it deserves more attention for co-digestion. It needs a lesser energy inputs and a lesser initial investment cost (Esposito et al., 2012; Zhang et al., 2013). Co-digestion brought in the innovative prospect for digestion of these two substrates for

improved yield and also diluting the inhibitory effect of mono-digestion and increasing the utilization efficiency.

5.2.1 Biogas generation from co-digestion of rice straw and *Hydrilla verticillata* for different C/N ratios

The variation in the cumulative methane and daily methane production during 50 days digestion time is expressed in Fig. 5.1 a) and b). The ratio of carbon to nitrogen is considered to be the main indicator of co-digestion treatment. High C/N ratios mean limitation of nitrogen rapidly, whereas low C/N causes the inhibition to methanogens. Here, methane production elevated and subsequently decreased with a series of different peaks. C/N ratio plays an important role in co-digestion; therefore, it is necessary to optimize the C/N ratio in the digestion of multiple substrates. By increasing C/N ratio cumulative methane production also increased reaching its peak at C/N-25 (Fig. 5.1 a)). C/N-25 and C/N-30 had higher methane potential about 2.5 fold more in comparison to C/N-15. FA is known to be very noxious form of nitrogen, which inhibits growth of methanogens. Literature had reported that FA value above 99-150 mg/L is inhibitory for the methanogenesis (Murto et al., 2004; Yen and Brune, 2007). Therefore, it can be suggested that lesser methane yield at C/N-15 in this work resulted from inhibition by increasing concentration of FA as on 30th day; it was 315 mg/L. Previous study showed that optimal ratio in fermentation process is in range C/N-15 to 25 (Amirta et al., 2006) and C/N-25 to 30 (Yen and Brune, 2007), which is also consistent with the results of this study. In contrast to this, high C/N-35 also showed low methane production. This low value was attributed to rapid utilization of nitrogen by methane producing bacteria. Higher methane production was achieved from co-digestion than mono-digestion. Up to 40% increase was observed without investing any extra effort (in terms of energy and chemical). Methane yield was higher due to synergistic effects occurring within the system. Fig. 5.1 b) showed the daily methane production peak and valley was observed at the end of 17th day for C/N-25 with 68.45 ± 0.25% (3 times higher than control), 21st day for control, 16th day for C/N-30 and at 20th for C/N- 35. However, for C/N-15 peak was observed at 10th day and after 28th day it suddenly dropped and stopped completely after 31st day. It can be clearly seen that the co-digestion process reduced the peak and valley difference, which ensured steady methane generation supply. This experiment was carried out in mesophilic range and this temperature range has an effective control on mass transfer during degradation process. It enhances the reduction of organic substance during the process for achieving high metabolic flux. The liquid displacement method has some disadvantages due to evaporation of the barrier solution. So,

results give underestimation of the methane production, therefore, correction is applied in cumulative methane production. However, known volume of the barrier solution is kept in measuring cylinder and difference in level (+35 mL) is added in the cumulative methane value as shown in Fig. 5.1 a). Methane production rate were achieved in this order; C/N-25 > C/N-30 > C/N-35 > C/N-20 > control > C/N-15.

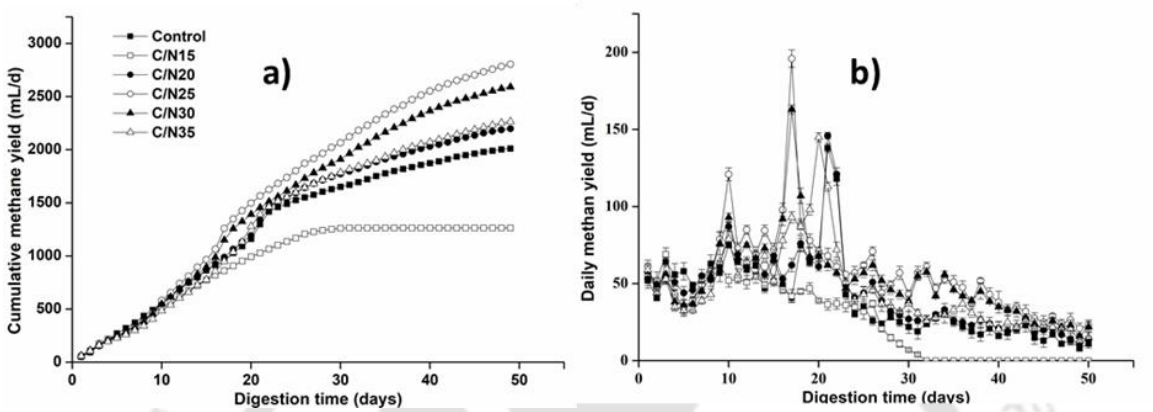


Fig. 5.1. a) Cumulative methane yield and b) daily methane yield for different C/N ratios

5.2.2 Influence of C/N ratios on change in pH during degradation

As the degradation stages are dependent on each other, a disparity in operational parameters can deteriorate the performance of the reactor. pH is a significant parameter for controlling the efficiency of the AD process. Enzymatic response of concerned microorganisms depends on pH. The optimal pH for high methane yield was 6.8-7.2 (O'Flaherty et al., 2010; Yin et al., 2016). Accumulation of VFA drops the pH below optimal range and descends the methane yield. In case of co-digestion, the high buffering capacity of the system shields the methanogenic activity. Initial pH governs the process stability, as on the start of degradation, pH was observed from 6.71-7.04 (optimum level). C/N-25 and C/N-30 had stable value around pH 7.0. On 7th day, pH for control (mono-digestion) reduced to 6.59, which was slightly less than the optimum value (Fig. 5.2 a)). pH in all the reactors was in the range of 6.7-7.3, throughout the digestion period, except in the case of C/N-15 ratio. On 21st day, pH decreased to 6.3 and at the end of 28th day, it reduced to 5.9, much below the optimum value. Lesser C/N ratios have high amount of ammonia on degradation, FA concentration increased to four times more than the threshold limit. Acetate-utilizing methanogens, hydrogen-utilizing methanogens are mostly influenced by ammonia production; however, the inhibitory amount was different for various trials (Zhang et al., 2016). Process failure due to low pH and FA also resulted in accumulation of VFA

and methane production started decreasing after this point. Sometimes, interaction between pH, FA and VFA resulted in an inhibited steady state, a situation where course of process looks stable but yield is negligible.

5.2.3 VFA

Anaerobic degradation of lignocellulosic material essentially includes hydrolysis (rate limiting step), acidogenesis, acetogenesis and methanogenesis. The efficiency of the hydrolysis process and acidogensis can be evaluated by accumulated concentration of VFA, respectively. The amount of VFA is the indicator of metabolic condition, though indicative value could not be explained absolutely as it depends on operating condition and composition of material. The VFA concentration in the beginning of the process ranged between 800-1100 mg/L (Fig. 5.2 b)), which were extensively influenced by the variable proportion of *H.verticillata* (co-substrate). For C/N-15, it increased up to 3400 mg/L and got accumulated in the reactor, which led to the failure of the process. In case of other ratios, VFA value first increased to its maximum value and then continually decreased, whereas C/N-15 showed the antagonist effect. On 14^{th} day, VFA concentration was around 3500 mg/L but methane production peak was delayed (Fig. 5.1 b)) which showed the accumulation of the VFA, that was later rapidly consumed by the acidogens. This was obtained due to the imbalance of slight biochemical balance among the acidogenic and methanogenic microorganisms. Healthy conditions of the reactor expressed that good nutritional supplementation (C/N) and sufficient micronutrients were present for the functioning of the process.

5.2.4 sCOD

The aim of obtaining optimal methane production from anaerobic co-digestion is challenged by method instability, process parameters like VS degradation, pH, VFA are used as online indicators for producing high volume of methane (Murto et al., 2004; Yen and Brune, 2007). However, several researchers have pointed out the demerits of these process variables due to less sensitivity and consistency. Instead sCOD measurement as shown in Fig. 5.2 a) is used as a product of the indication of improved hydrolysis. It is accepted as reliable indicators for the efficient AD process, as it accumulates and shows the metabolic imbalance in the system on occurrence of inhibition. Methanogenesis steps leads to reduction in sCOD. The reactor with different C/N ratios showed variable substrate hydrolysis rate. The value of sCOD initially showed a sudden increase but after 14^{th} day it showed a sudden increase, biomethane production also increased during this duration (Yen

and Brune, 2007; Herrmann et al., 2015). During initial days hydrolysable organic fraction is utilized by acidogens, which leads to decrease of pH also. The decrease in sCOD is directly proportional to the increase in yield of methane. Co-digestion improved the solubilisation rate as compared to control. Some researchers (Ennouri et al., 2016; Pellera and Gidarakos, 2017) also perceived that during the initial time of co-digestion of lignocellulosic agricultural residue, sCOD value enhanced in the initial days of the digestion time and afterwards decreased. These variations were subjected to the advanced degradation of recalcitrant organic matter. On the other hand some observed decrease in HRT which was related with a sCOD reduction (Comino et al., 2010; El-mashad and Zhang, 2010). The obtained value was showed less fluctuation during maximum yield duration. Furthermore, similar to other process variables, there is level of alteration in this parameter.

5.2.5 Effect of oxidation reduction potential on process stability

ORP reactions could directly signify its role in the stability of enzymes and synthesis of bacteria (Bier and Specialist, 2009; Chen et al., 2015; Zhen et al., 2016). It occurs within cells and makes ORP values to show electron transport and redox equilibrium in intracellular metabolism. ORP value indicates the change in metabolic flux, which is responsible for the existence of different extracellular enzymes secretion. The microbial cell growth yield is dependent upon the potential difference amongst the electron acceptor and donor, which renders into the energy accessible for the microorganisms. This potential or energy difference is calculated by ORP difference between reduced and oxidized compounds, yet bacteria could not always utilize the actual potential, as losses occur between degrading compounds. These enzymes are responsible for the hydrolysis in AD process. To explore the effects of ORP value on degradation efficiency, ORP level was measured at definite interval (Fig. 5.2 d)). After 7 days, the ORP value decreased swiftly with a maximum change of -312.8mV for C/N-25, -305.4 mV for C/N-30 and -288.4mV for C/N-20. The ORP level reached to stable value between 14 and 28 days (-228.1 ± 29.52 to -263.73 ± 30.58). This trend was absolutely consistent with maximum methane production. This (14-28 days) was also a zone with maximum production of biomethane, around 55% of the methane was produced during this time. The value of ORP was the lowest when methane yield was the highest (Fig. 5.1 b)), and C/N-25, 30, 35 showed the lowest reducing potential as compared to control and C/N-15, 20. The lowest value of ORP was reached on 21st day and maximum methane generation peak was also apparent during the same time. Control without any co-substrate showed the low reducing potential during the process. The

termination of the degradation process is followed by high oxidation potential. At the end of degradation stage, ORP of the entire reactors varied from +12.98 to +45.09 mV.

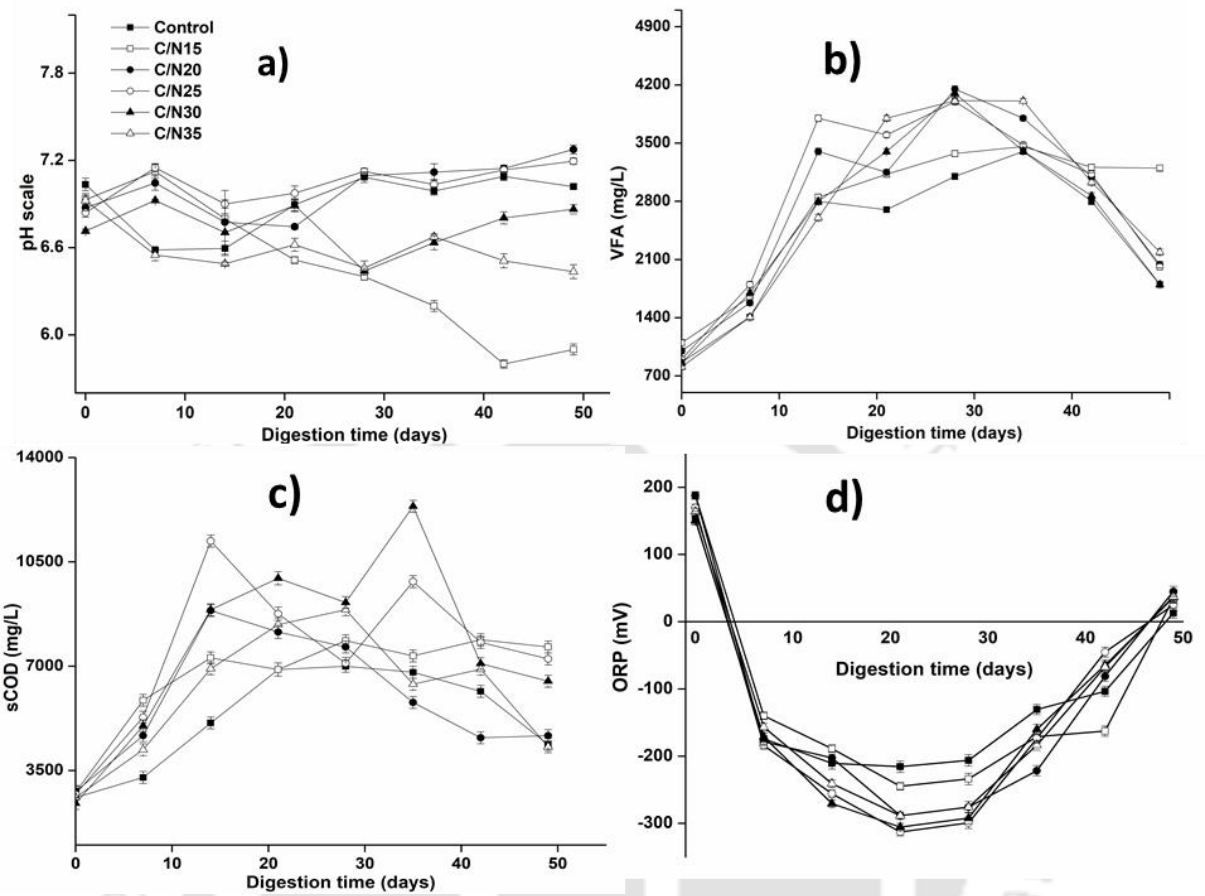


Fig. 5.2. Effect of different C/N ratios on a) pH b) VFA c) sCOD and d) ORP

5.2.6 Optimization of methane production using response surface methodology

Co-digestion is considered as cost-effective process for balancing the nutritional parameters; the addition of nitrogen-rich co-substrate *H.verticillata* balances the unstable C/N ratio of RS and improves the process stability by supplementing required nutrients in the process. Different factors influence AD, mainly including pH, C/N ratio and F/M ratio. The study, which focuses only on single dimensional effect of parameters on the AD, is incapable of reaching the optimum conditions. To modify this problem three parameters (a) F/M ratio (b) C/N ratio (c) pH were chosen in this study. Optimization of parameters imparts significant role in volumetric biogas production. Recently different optimization methods i.e. Artificial neural network, Genetic algorithm, Taguchi techniques and RSM are broadly used for optimizing the experiments variables. Applications of these methods are effectively used in physical, biological and chemical fields. These processes help in saving

the working outlay and time, as well as gives more efficient output response. Reliable and accurate results can be obtained by using design of experiment approach. The prime reason for selecting RSM in this study is that it allows assessment of several parameters in the same duration with less experimental sets and offers quantitative results. The F/M ratio is a significant parameter while determining the biogas potential of a substrate or operating a large size batch reactor for poor selection of F/M ratio in the AD system, the methane generation could reduce or even ceases. The optimum F/M ratios for different substrate degradation process are variable, for fine grinded RS it was found to be high. The optimal pH range for efficient AD is 6.8-7.2 (Zhang et al., 2013). The growth rate of methanogens can be reduced significantly when the pH value is well below 6.6 and high alkaline pH can cause disintegration of anaerobic flux and succeeding failure of the degradation process. The C/N ratio is a significant parameter for operating an AD process. The effect of operational process variables on AD is repeatedly studied individually and the results observed in the literature showed the interaction of these process parameters on enhanced biogas production. Therefore, in order to determine the optimum C/N ratio, F/M ratio and pH, the RSM design of co-digestion of substrate is important, particularly when finding a maximum yield of biogas and analyzing the interactive effect of operational parameters (C/N ratio, F/M ratio and pH) and finding the optimum condition by RSM-CCD.

5.2.6.1 Interactive effect of C/N ratios and F/M ratios on methane yield

The three-dimensional surface plots and contour plots were created keeping one variable at central level of plot and distinguishing the others inside the selected experimental range. Statistical difference was found to be significant ($P < 0.01$) for interactive effect of C/N ratio and F/M ratio. This shows that these two parameters have better control on AD and direct correlation on digester performance. C/N ratio quantifies the performance of AD, carbon acts as source of energy for microorganisms, whereas nitrogen effects in the formation of microbial population. C/N must be in the range 20-30 and to maintain this ratio, RS of high C/N ratio is mixed with *H.verticillata* of low C/N ratio. The F/M ratio is an important term considering the balance of inoculums and substrate (Liu et al., 2009). The methane yield is influenced by the F/M ratio: higher the F/M ratios lesser is the methane yield. This converse relation might be because of low number of methanogens or inefficient methanogenic activity, in the reactor, which could affect in the increase of VFA accumulation, in the reactor leading to low methane yield. F/M ratio was increased from 1 to 3.5 and showed maximum value for F/M:2.25 (Jiménez et al., 2015; Li et al., 2015). Increasing the value of C/N ratio affected into increased methane yield (mL/g-VS_{added}) (pH

value was retained at its central level). However, the methane yield (mL/g-VS_{added}) decreases promptly when C/N ratio increased above 27.5 (Fig. 5.3 a)). High C/N ratios indicate the exhaustion of N by microorganisms that utilizes N to fulfill their protein needs, consequently resulting in low methane yield. The C/N-20 and C/N-27.5 had higher protein needs than the lower C/N ratio and control. With the increasing C/N ratio, the methane yield initially increased to maximum value for C/N ratio 27.5 and then declined for higher C/N ratios as shown in Fig. 5.3 a). Methane yield reached the value of 205.68 mL/g-VS_{added} at high C/N ratio. Low C/N ratio showed very low methane yield (108.51 mL/g-VS_{added}), it is mainly due to higher concentration of inhibitory compounds especially FA on degradation, which are toxic for the anaerobic microorganisms. *H.verticillata* has higher proportion of nitrogenous organic matter, which on degradation accumulates higher amount of ammonia and inhibits the process. The optimum co-digestion ratio gives the methane yield of 279.43 mL/g-VS_{added} higher than the predicted value due to balance of nutrient and synergistic effect in the reactor. Combined effect showed that C/N-27.5 and F/M 0.15 gave methane yield of 231.17 mL/g-VS_{added} and C/N-27.5 and F/M:2.25 gave methane yield of 279.43 mL/g-VS_{added}, this difference might be due to the disturbance in the equilibrium of microbial flux. The highest methanogenic activity was observed at the C/N-27.5, F/M:2.25 and C/N-35, F/M:1. At C/N-14.89 and F/M:2.25, methane yield was the lowest due to inhibition of the process. Previous studies have also showed the similar effect by co-digestion of mixed substrate on performance of the process by diluting the inhibitory compounds (Esposito et al., 2012; Liotta et al., 2016). Co-digestion on balanced ratio decreased the TAN and FA amounts within the reactor, which showed any toxicity and potential inhibition to methane yield. It also enhanced the nutrients availability and improved the bacterial diversity in the reactor. The obtained methane yield (279.43 mL /g-VS_{added}) was larger than that from mono-digestion (156.32 mL/ g-VS_{added}) due to synergism established in the medium.

5.2.6.2 Interactive effect of F/M ratios and pH on methane yield

Fig. 5.3 b) illustrates the interactive effect of F/M ratios and pH on methane yield. Methane yield increased with rise of F/M ratio and pH and then dropped on increasing it further. The contour plot showed that the interaction of F/M ratio and pH was insignificant. However, these factors have individual significant effect on methane yield (F/M ratio $p < 0.038$, pH $p < 0.0002$), whereas p for $X_2X_3 > 0.5389$. Optimization of the pH in the reactor was selected to be ranging from 6.5 to 7.5, when pH value is highly greater than this value it promotes a lethal condition for the methanogenic microorganisms. Co-digestion in this

study not only balances the nutrition for microbial population but also adjusted the pH in the reactor by improving the buffering capacity of reactor during the whole process. The acidification and methanation process causes the failure of fermentation leading to low methane yield. The insufficient buffering capacity leads to drop of pH in mono-digestion whereas in co-digestion pH was stable. The change of pH has inhibitory effect on the function of methanogenic archaea, which disrupts the generation of biogas. Therefore, pH is the general indicator to maintain the AD process (Lindmark et al., 2014; Xi et al., 2014). The value of initial and final pH after 50 days of degradation is shown in Fig. 5.4. The initial pH value was lower in the reactor for higher F/M ratio in design of experiment for co-digestion. The initial and final pH value of the control in the reactor was 7.03 and 7.0, respectively. The reduction in the pH from initial to final pH value was almost the same (0.24, 0.25, 0.28) at most F/M ratios. The abrupt pH change (0.55, 0.61, 0.7, 0.72 and 0.73) was seen in some reactors. Considerable pH change was seen as 1.4, which showed the instability in the process. The highest methane yield was obtained at neutral pH (7, 7.5 and 7.84) level, lowest yield was obtained at pH 6.5. This low value inhibited the growth of methanogens and process stopped completely at pH 5.6 and the lowest yield attained here. Maximum methane yield considering F/M ratios was obtained at 2.25, 3.5. The effluent pH generally would increase in the denitrification supported C/N balanced system due to the high alkalinity of the system. The pH value slightly decreased during the digestion period for high initial pH value reactors (Xu et al., 2018). A decreasing pH value showed more production of acidic matter, which would lead to less methane yield, whereas increasing effluent pH revealed higher stability of the system. Consequently, it could be accomplished that reactor pH close to neutral condition showed higher methane yield. The best F/M ratio and pH value were 2.25, 7 respectively. Results of this study showed that methane yield can be enhanced by optimizing initial pH and F/M ratios in the process. So, it can be clearly seen that different initial pH value and F/M ratio resulted in the different methane yield because these factors not only affect the microbial activities and growth rates, but similarly changed the metabolic pathway. If co-digestion of different substrates is taken, the microbial structure of community would change as shown in previous study (Matheri et al., 2017).

5.2.6.3 Interactive effect of C/N ratios and pH on methane yield

Effect of C/N ratio and pH was statistically insignificant ($p > 0.05$) in this model but its individual effects are significant (Fig. 5.3 c)). Although many researchers have suggested the different range of optimal ratio for co-digestion, this depiction of C/N ratios could be

influenced by the various operating conditions. An increase in pH value resulted in high methane yield but above certain value it showed further inhibition of the process (Fig. 5.4). C/N ratios below 20 showed inhibitory effect in the process (Liu et al., 2009; Yang et al., 2015). Previous studies revealed that there is an interactive effect of pH and C/N ratio. On increasing C/N ratio, digestion efficiency increases effectively and methane yield of 279.43 mL/g-VS_{added} was obtained (Pan et al., 2008). Methane yield initially increased and then decreased after obtaining the optimum value in this study. pH values were between 6.89-7.45 with maximum yield combination. Stable pH values around 7.24 were obtained for C/N ratio 27.5, when C/N ratio was 14.89, pH values dropped from 7 to 5.6. Co-digestion conditions showed stable reactor performance than mono-digestion. It was observed that a decrease in pH value resulted in decrease of methane yield due to accumulation of the VFA in initial days of the degradation process.

5.2.7 Statistical checking and diagnostic analysis of the model

Design matrix with experimental results and predicted value is presented in Table 5.2 and 5.3. The methane yield (mL/g-VS_{added}) was determined by dividing the cumulative methane production by the total amount of VS added to each reactor. The experimental results were then themed to response surface analysis to assess the interactive effect between C/N ratios (X_1), F/M ratios (X_2) and pH (X_3). After applying several regression investigations, the results were fitted into a second-order polynomial equations. Hence, the regression model for the methane yield (mL/g-VS_{added}) in terms of coded and uncoded factors was attained as follows:

$$\text{Methane yield} = 278.99 + 24.21X_1 - 12.11X_2 + 17.93X_3 - 40.35X_1^2 - 19.91X_2^2 - 6.74X_3^2 + 9.91X_1X_2 + 2.69X_2X_3 + 1.31X_1X_3 \quad (5.1)$$

Or

$$\text{Methane yield} = -1766.22 + 37.86X_1 - 11.52X_2 + 393.86X_3 - 0.72X_1^2 - 12.74X_2^2 - 26.94X_3^2 + 1.06X_1X_2 + 4.30X_2X_3 + 0.35X_1X_3 \quad (5.2)$$

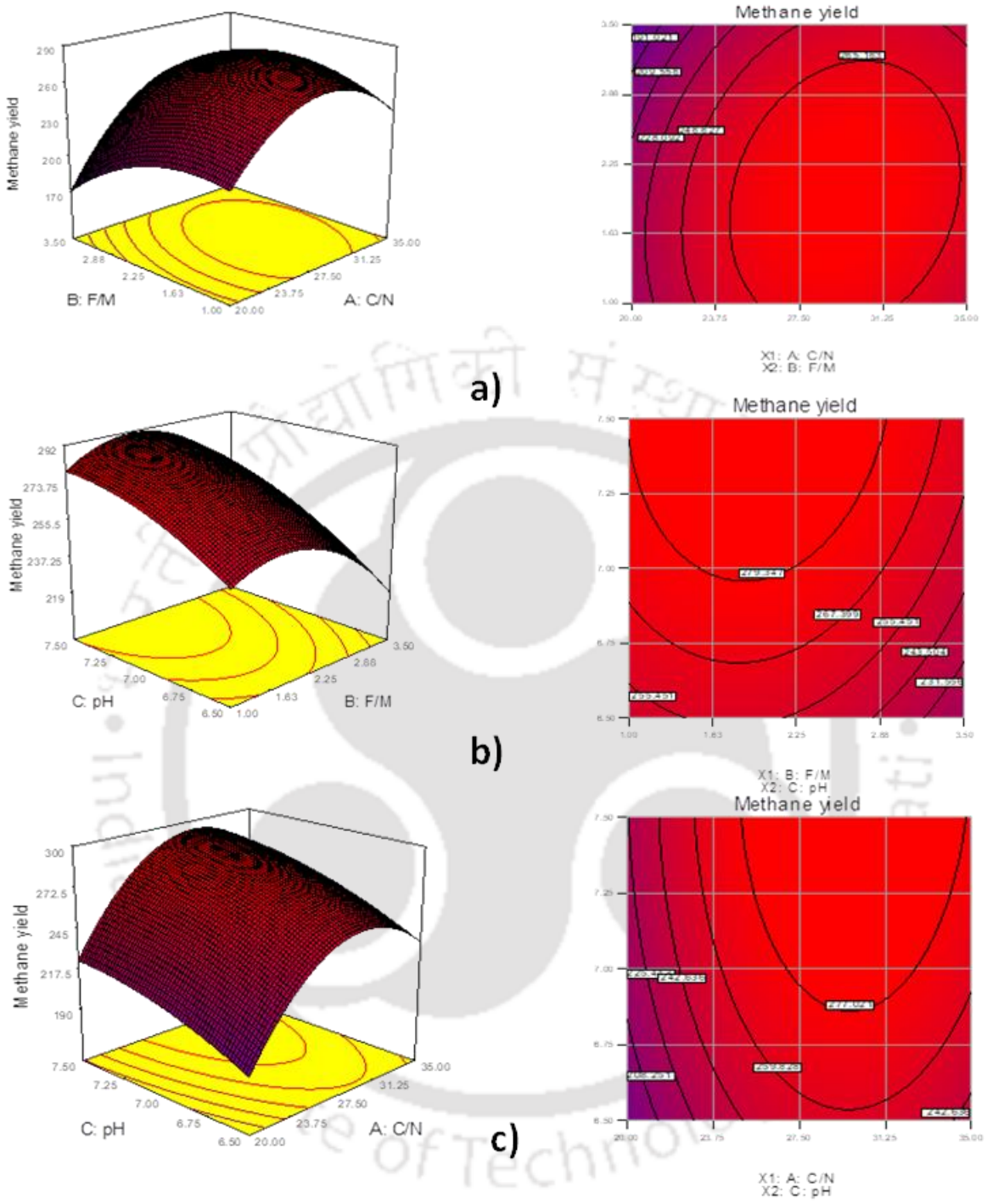


Fig. 5.3. Response surface and contour plots of different parameters interaction on methane yield (a) C/N and F/M ratio (b) F/M ratio and pH (c) C/N ratio and pH

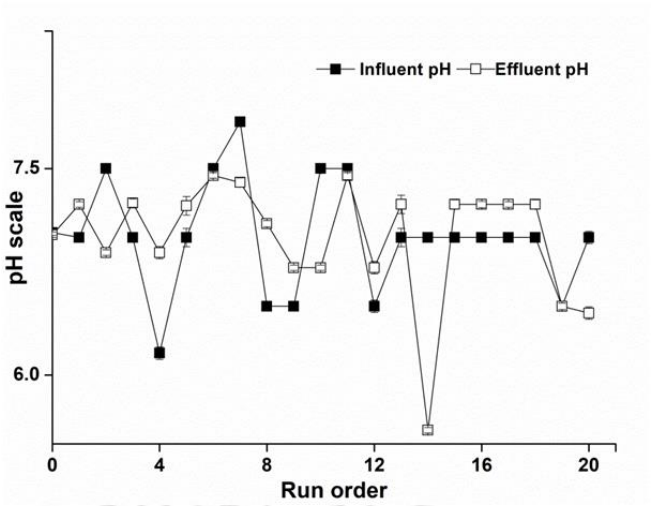


Fig. 5.4. Variation of pH at start and end of digestion time in all reactors

ANOVA is a very important tool in finding the best fitted mathematical model. The result of the ANOVA is shown in Table 5.3. The F-value of the model as 33.04 implies that the model is significant; F-value of such high magnitude may be due to the noise and signal, its prediction possibility for this high value is around 0.01%. The lack of fit value of 1427.02 implies that it is significant relative to the pure error. The value of coefficient of the variance is 5.12, which is very low; this low value shows the high degree of accuracy and high reliability of the experiments data. The R^2 value of 0.9675 revealed that this mathematical model could explain the 96.75 % variability in the methane yield response. A good statistical model should have R^2 value in the range of 0.75-1, which directs a best fit of the model (Reungsang et al., 2012). Adequate precision of the model reveals the signal to noise ratio and a ratio more than 4 is predictable. The regression model analysis of the design indicated that the linear model value (X_1, X_2 and X_3), Quadratic model term (X_1^2, X_2^2 and X_3^2) and interactive model term X_1X_2 are mathematically significant ($p< 0.05$); the interactive model term X_1X_3, X_2X_3 $p > 0.05$ are insignificant. To check the model adequacy, diagnostic model terms were calculated, standardized residuals value showed the slight difference between the response model terms and hypothesized model terms. Each observed value with residuals greater than 3 was neglected as outlier, indicating the negligible errors in observing the experimental value. The Fig. 5.5 a) and b) illustrate the normal probability plot of standardized residuals and actual and predicted methane yield plot. These plots showed that there was no abnormality in this experimentation and model was successful for obtaining enhanced methane yield.

Table 5.2. ANOVA for response surface by quadratic model

Source	DF	Sum of squares	Mean square	F value	P-value
Model	9	42432.40	4714.71	33.04	0.0001
X_1	1	8007.52	8007.52	56.11	0.0001
X_2	1	2004.11	2004.11	14.04	0.0038
X_3	1	4391.21	4391.21	30.77	0.0002
X_1X_2	1	785.82	785.82	5.51	0.0409
X_1X_3	1	13.69	13.69	0.096	0.7631
X_2X_3	1	57.75	57.76	0.40	0.5389
X_1^2	1	23467.40	23467.37	164.45	0.0001
X_2^2	1	5711.91	5711.91	40.03	0.0001
X_3^3	1	654.07	654.07	4.58	0.0579
Lack of fit	5	1427.02	285.40		
Pure Error	5	0.00	0.00		
R^2	-	0.9675			
Adj-R^2	-	0.94			
CV^p	-	5.12			
Adequate precision	-	19.65			
Predicted R-Squared	-	0.75			

5.2.8 Validation of model and optimum conditions for maximum yield

Finally, in order to check the validity of the models suggested, confirmation experiment in 20 L batch reactor was performed considering optimal conditions (C/N ratio 29.18, F/M ratio 2.45 and pH 7.37) for maximum methane yield and the second order quadratic model was obtained for the dependent input parameters.

Table 5.3. Experimental design matrix of the model for the maximum methane yield

Run order	Code values (C/N, F/M, pH)			Real Values (C/N,F/M, pH)			Exp. methane yield (mL/g-VS_{added})	Predicted Methane yield (mL/g-VS_{added})
	X_1	X_2	X_3	X_1	X_2	X_3		
1	0.00	0.00	0.00	27.50	2.25	7.00	279.43	278.99
2	-1.00	1.00	1.00	20.00	3.50	7.50	198.42	185.06
3	0.00	1.68	0.00	27.50	4.35	7.00	198.67	202.30
4	0.00	0.00	-1.68	27.50	2.25	6.16	234.68	229.77
5	1.68	0.00	0.00	40.11	2.25	7.00	205.68	205.57
6	1.00	1.00	1.00	35.00	3.50	7.50	260.72	255.93
7	0.00	0.00	1.68	27.50	2.25	7.84	269.67	290.09
8	1.00	-1.00	-1.00	35.00	1.00	6.50	219.46	221.85
9	-1.00	-1.00	-1.00	20.00	1.00	6.50	202.04	195.86
10	-1.00	-1.00	1.00	20.00	1.00	7.50	238.61	223.73
11	1.00	-1.00	1.00	35.00	1.00	7.50	265.17	254.96
12	1.00	1.00	-1.00	35.00	3.50	6.50	208.17	212.07
13	0.00	0.00	0.00	27.50	2.25	7.00	279.43	278.99
14	-1.68	0.00	0.00	14.89	2.25	7.00	108.51	124.13
15	0.00	0.00	0.00	27.50	2.25	7.00	279.43	278.99
16	0.00	0.00	0.00	27.50	2.25	7.00	279.43	278.99
17	0.00	0.00	0.00	27.50	2.25	7.00	279.43	278.99
18	0.00	0.00	0.00	27.50	2.25	7.00	279.43	278.99
19	-1.00	1.00	-1.00	20.00	3.50	6.50	147.19	146.44
20	0.00	-1.68	0.00	27.50	0.15	7.00	231.17	243.05

The optimum conditions were observed to be same as C/N-29.18, F/M:2.45 and pH 7.37. The rate of degradation in this experiment follows first-order ($P = P\mu^* (1-e^{-kt})$) reaction kinetics ($R^2 > 0.97$), where P denoted cumulative biomethane generation at time t; Pμ

denotes maximum biomethane yield; k denotes first order reaction kinetics (Fig. 5.5 a) and b)). The validation experiment obtained methane yield of 287.6 mL/g-VS_{added}, which is very close to 290.471 mL/g-$VS_{added;}$ (Pμ) predicted value obtained from the model. The first order reaction kinetic coefficient value 0.09 d^{-1} indicates that co-digestion with *H.verticillata* increased the hydrolysis of RS in initial stages, thus enhancing the efficiency of methane production.

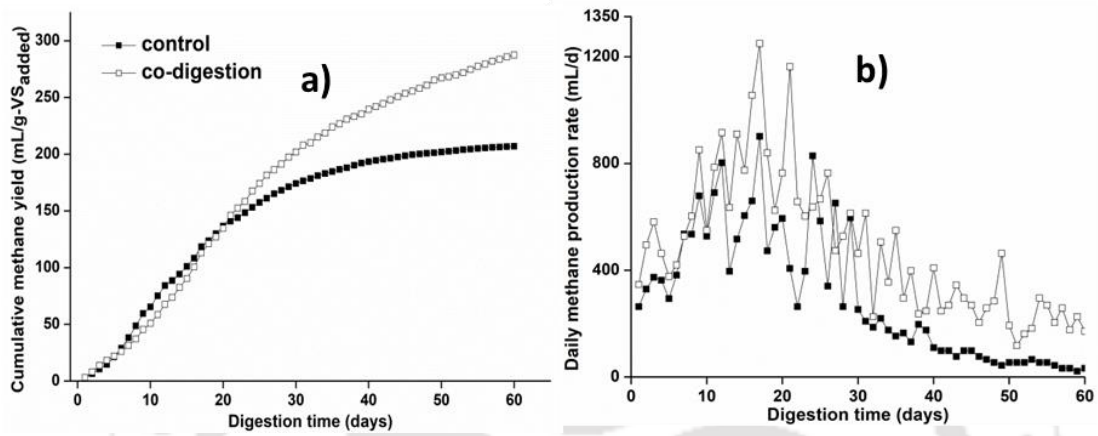


Fig. 5.5. Model validation for optimum response condition a) cumulative methane yield and b) daily methane yield

The higher nonlinear regression fit value proved that the model follows the first order kinetics. The student t-test was conducted to find out the link between observed methane yield and predicted yield. Therefore, we could accept the null hypothesis as there was no significant variation between p (0.045).

5.3 CO-DIGESTION OF RICE STRAW WITH FOOD WASTE

Co-digestion has several benefits as it overcomes the possible limitations in mono-digestion and enhances efficiency of bio-energy recovery with co-disposal and management of several wastes at the same time. Min et al. (2015) documented the expediency of co-digestion by achieving the biogas production of 316.11 L /kg-$COD_{removed}$ and highest organic matter removal (77%) by addition of 75% FW to activated sludge. Switch grass co-digested with dairy manure is beneficial in the improvement of buffering capacity and consequent fermentation efficiency, and this has been validated by Zheng et al. (2015), the importance of which can be understood by 39% increase in production of methane as compared with mono-digestion. However, there were few previous literature (Zheng et al., 2015; Neshat et al., 2017; Silva et al., 2018) focusing on co-digestion of RS and FW, but

those studies have not focused on the different factors affecting anaerobic co-digestion and their interactive effects on methane yield.

Co-digestion has brought in a new opportunity for the processing of RS and FW. 1.3 billion tons of the FW (1/3rd of human consumption) is wasted yearly as reported by Food and Agriculture Organization of the United Nation in 2011 report (Kondusamy and Kalamdhad, 2014). The main disposal method for the generated FW is landfilling, which subsequently produces methane having high global warming potential (21 times of CO_2) (Wilkie and Evans, 2010). High volatile fraction and moisture in FW is considered to be suitable for AD, but due to low C/N ratio and readily degradable organic matter, acidification occurs in the reactor, leading to decrease in utilization efficiency of FW.

To address the problem of mono-digestion of RS and FW, RS was co-digested with FW. This work mainly focused on the individual and interactive effect of various parameters (C/N ratio, F/M ratio and pH) on methane yield from co-digestion and mono-digestion.

5.3.1 Biogas generation from co-digestion of rice straw and food waste for different C/N ratios

There is a definite requirement of C and N for efficient growth of microbial community in the reactors. Single substrate (mono-digestion) digestion often ceases the methane yield of the organic substrate and causes the digester instability. High C/N ratio of RS (43) leads to the nitrogen scarcity for microbial growth, which ultimately decreases the methane yield (inactivity of methanogens). Whereas, low C/N ratio of FW (11.35) showed the carbon scarcity for methanogens, causing VFA accumulation in the reactor. To alleviate the problems, co-digestion provides the balance of C/N for anaerobic degradation. The experimental results of BMP are shown in Fig. 5.6. Fig. 5.6 a) shows the variation of pH during degradation, with direct impact on efficiency of anaerobic digester. pH variation depicted the enzymatic reactions involved in the degradation, the intital pH in the reactor varied from 6.85-6.95, which was in optimum range as suggested in literature (Reungsang et al., 2012). As the degradation proceeds, the pH increased to 7.1 ± 0.5, in all the reactor, except control (mono-digestion), which showed the lower value due to poor buffering capacity. After 21st day pH reduced to 6.7 ± 1.55 in reactor with C/N-25, C/N-30 and C/N-35. A study carried by Cheng and Zhong (2014), revealed that the optimum pH for the co-digestion was found to be 6.5, due to high concentration of produced VFA, in case of mono-digestion. Therefore the optimum pH for phase two was kept to be 6.6-7.4, as high initial pH protects the reactor from acidification failure of the reactor. The Fig. 5.6 b) and c)

showed change in VFA and sCOD, it was clear that during the production of maximum daily yield of methane both the value was higher. From 14-35 days duration VFA and sCOD varies 2870 ± 756, 5150 ± 1573 mg/L respectively, at the end of digestion this value is reduced by 83% than its value during the period of high methane yield zone. There was no inhibition of VFA as the biomethane yield was directly proportional to the amount of VFA produced and it was directly consumed by the methanogens (high methane yield during this period). High VFA concentration showed improved hydrolysis rate, it also increases the degradation of recalcitrant lignocellulosic structure of RS, which enhances the biochemical condition of the reactor and improves the biodegradability. Fig. 5.6 d) displays the results of ORP variation with digestion period; ORP value shows the redox equilibrium and electron transfer in cellular mechanism, difference in metabolix flux rate clearly indicates the secretion of enzymes for AD. At 0^{th} day, ORP value varied from 72.25 ± 3.93, as the degradation progressed this value decreased to -179.42 ± 9.78, this swift decrease showed the high reduction potentail in the reactor. On 28^{th} day, the lowest ORP value was obtained, which was quite evident from the high daily methane yield during that period. Control showed the less redox potential or metabolic flux as compared to co-digestion.The cumulative methane yield results showed that the C/N-15 received the cumulative methane yield of 258.84 mL/g-VS_{added}, and C/N-25, C/N-30 and C/N-35 almost showed the similar methane yield (294.17 ± 3.78 mL/g-VS_{added}), i.e. 296.33, 297.32 and 288.85 mL/g-VS_{added} respectively as shown in Fig. 5.6 e) and f). As in the previous study (Yan et al., 2015), it was found that different C/N-15, C/N-24 and C/N-33 expressed 170.9, 341.3 and 219.8 mL/g-VS_{added} methane yield, respectively. Therefore in the present study variation in C/N ratio range is not considered to be a significant factor in optimizing the methane yields, whereas in phase II for futher study C/N ratio 30 was kept for optimization, as it showed the maximum yield.

5.3.2 Optimization of methane production using response surface methodology

To evaluate and optimize the different parameters on methane yield, BMP potential study for finding the initial range was followed by CCD-RSM designed experimental matrix. Though, it was imperative to define the parameters i.e. C/N ratio, F/M ratio and pH, as imbalance can disturb the stability of AD process. RSM can be used for designing the experimental matrix to enhance the methane yield from degradation without perceptive relation between the input parameters.

5.3.2.1 Interactive impact of pH and F/M ratios on accelerating cumulative methane yield

A 3D plot of dependent response variable was generated by assuming one variable at its centre, while keeping the other variable within its experimental range. In Fig. 5.7 methane yield over two numerical variables of pH and F/M ratio is appraised by 3D response surface (Fig. 5.7 a)) and 2D contour lines (Fig. 5.7 b)). Fig. 5.7 a) shows that methane yield increased with increase in pH and F/M ratio until reaching its optimum value and then decreased subsequently with a further change in pH (< 6.43) and F/M ratio (> 2.25). The cumulative methane yield of co-digested RS with FW was shown in Table 5.4 (P-value < 0.05). Furthermore, X_1X_2 had a low P-value (< 0.05) signifying the synergistic effect of pH and F/M ratio on the methane yield. Predicted methane yield (by RSM) in peak point (pH 7, F/M ratio 2.25) was 304.68 mL/g-VS_{added}. For the optimisation of the pH in the reactor, the pH was designated to be in the range of 6.6 to 7.4, as toxic condition for the methanogenic microbes is produced when pH value is much beyond this range.

The cumulative methane yield of co-digested RS with FW was shown in Table 5.5, whereas, control (mono-digested RS) had a cumulative methane yield of 170.49 mL/g-VS_{added} in the 50 d observation duration. Co-digestion with FW not only stabilizes the nutrition for microorganisms but also maintains the neutral pH in the reactor as a result of improved buffering capacity of reactor, thereby ensuring AD stability besides high methane yield. In mono-digestion, the insufficient buffering capacity leads to acidification causing fermentation failure leading to low methane.

pH is stable in co-digestion despite much higher concentration of VFA as compared to mono-digestion. Moreover, unstable pH inhibits the functioning of methanogenic microorganisms thereby disrupting the methane production. Thus, pH is a common indicator of the successful AD process. The impact of pH and F/M ratio on RS co-digested with FW was studied by varying pH from 6.43 to 7.57 and F/M ratio from 0.48 to 4.02 in five levels. In the experiment, the highest methane yield (318.45 mL/g-VS_{added}) was achieved at a pH of 7.57 and F/M ratio of 2.25, whereas, the lowest methane yield (185.37 mL/g-VS_{added}) was attained at a pH of 7 and F/M:4.02. Also, a methane yield of 208.87 mL/g-VS_{added} was obtained at pH 6.6 and F/M:1. This low yield values indicate that both upper and lower F/M ratio reduced methane yield and a lower pH inhibited the growth of acidogenic microorganisms resulting in accumulation of VFA, causing lower methane yield. Consequently, it can be accomplished that the high methane yield can be obtained by optimizing the pH above neutral value in the range of 7 to 7.57, while F/M ratio of 1.5 to

2.5 as indicated in the Fig. 5.7. The best pH and F/M ratios were observed to be 7.57 and 2.25, respectively. Thus, data of these experiments revealed that optimization of both pH and F/M ratio is of significance to achieve optimum methane yield. It can be clearly observed that different methane yield was obtained due to different pH value and F/M ratio, since these factors not only have an effect on the microbial activities and rate of growth but also affects the metabolic pathway. Co-digestion of various substrates not only enhances the buffering capacity and balances the nutrition for microbes but also modifies the microbial structure of community as revealed in the earlier studies (Li et al., 2014; Roslina et al., 2014).

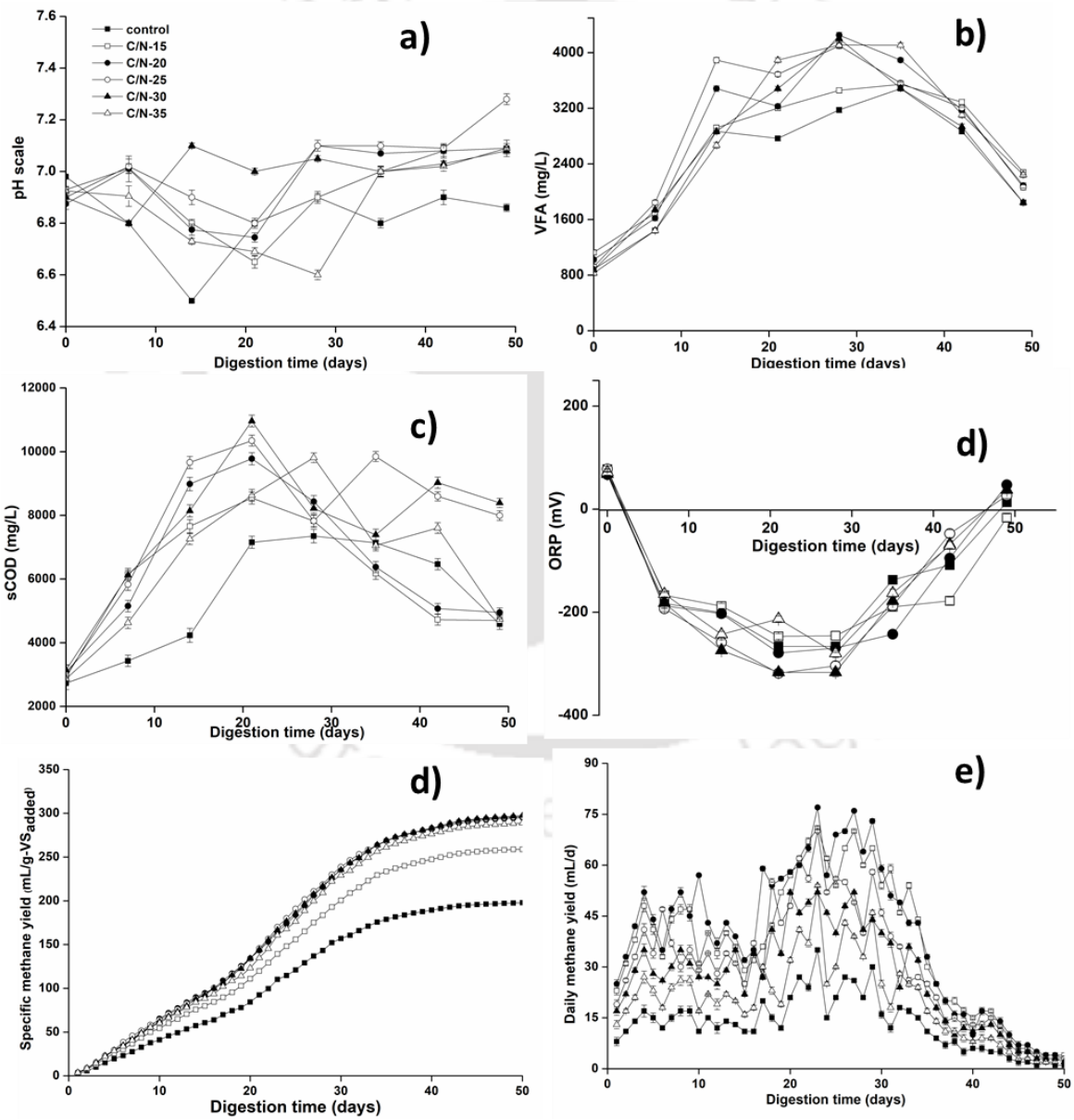


Fig. 5.6. Effect of different C/N ratios on a) pH b) VFA c) sCOD d) ORP e) daily methane yield and f) specific cumulative methane yield

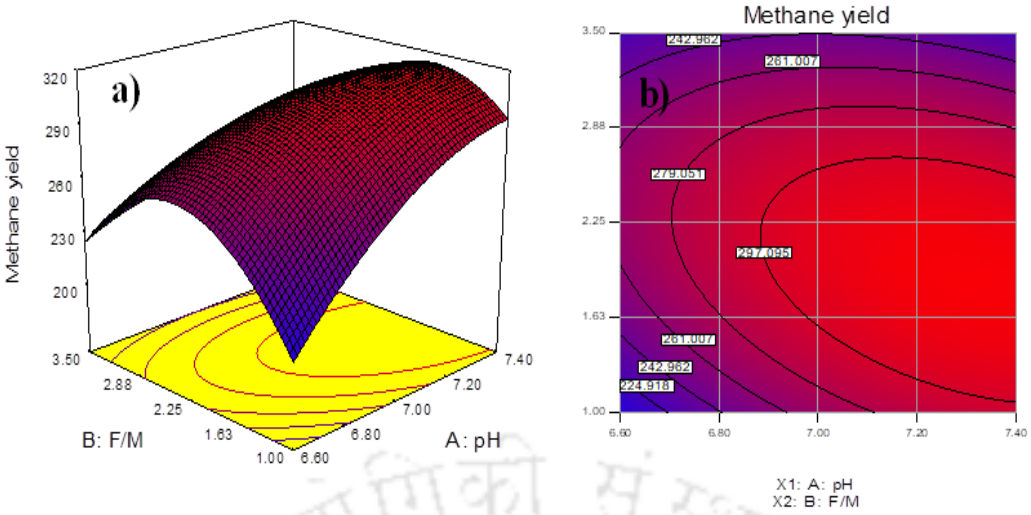


Fig. 5.7. a) Response surface and b) contour plots of interactive effect of F/M ratio and pH

Table 5.4. Experimental design matrix of the model for the maximum methane yield by quadratic

Run order	Coded values (pH, F/M)		Real Values (pH, F/M)		Experimental methane yield (mL/g-VS_{added})	Predicted Methane yield (mL/ g-VS_{added})
	X_1	X_2	X_1	X_2		
1	0.00	-1.41	7.00	0.48	234.57	224.14
2	1.00	1.00	7.40	3.50	213.66	223.98
3	0.00	0.00	7.00	2.25	304.68	304.68
4	0.00	0.00	7.00	2.25	304.68	304.68
5	0.00	0.00	7.00	2.25	304.68	304.68
6	0.00	0.00	7.00	2.25	304.68	304.68
7	-1.00	1.00	6.60	3.50	235.67	224.80
8	-1.00	-1.00	6.60	1.00	208.87	206.87
9	0.00	1.41	7.00	4.02	185.37	187.48
10	1.00	-1.00	7.40	1.00	274.56	293.75
11	0.00	0.00	7.00	2.25	304.68	304.68
12	1.41	0.00	7.57	2.25	318.45	299.31
13	-1.41	0.00	6.43	2.25	227.65	238.47

5.3.3 Statistical checking and diagnostic analysis of the model

Table 5.5 represents the experimental values of the design matrix. In order to evaluate the interactive effect between two variables pH (X_1) and F/M ratio (X_2), the experimental results were subjected to ANOVA. After the application of various regression investigations, a quadratic polynomial equation was developed in order to predict the methane yield as a function of X_1 and X_2 and their interactions, which is described below in terms of coded and uncoded factors:

$$\text{Methane yield} = +304.68 + 21.51X_1 - 12.96X_2 - 17.90X_1^2 - 49.43X_2^2 - 21.92X_1X_2 \quad (5.3)$$

Or

$$\text{Methane yield} = -6379.59 + 1718.25X_1 + 438.95X_2 - 111.84X_1^2 - 31.63X_2^2 - 43.85X_1X_2 \quad (5.4)$$

Table 5.5. Analysis of Variance of the model for the maximum methane yield by quadratic

Source	DF	Sum of squares	Mean square	F value	P-value
Model	5	24896.18	4979.24	29.21	0.0002
X_1	1	3701.90	3701.90	21.72	0.0023
X_2	1	1343.67	1343.67	7.88	0.0262
X_1X_2	1	1922.82	1922.82	11.28	0.0121
X_1^2	1	2227.69	2227.69	13.07	0.0086
X_2^2	1	17000.48	17000.48	99.73	0.0001
Lack of fit	3	1193.20	397.73		
Pure Error	4	26089.39	0.00		
R^2	-	0.9543			
Adj-R^2	-	0.9216			
CV^p	-	4.96			
Adequate precision	-	13.21			
Predicted R-Squared	-	0.6748			

In order to determine the optimum value for each independent parameter chosen so as to optimize the methane production in the anaerobic degradation process, the equation (iii) or (iv) was solved. The Model F-value (29.21) suggests that the model is significant. P-value less than 0.05 imply selected model is mathematically significant and the model is adequate for optimizing the methane yield from co-digestion. In this case, the regression model analysis indicated that the X_1, X_2, X_1X_2, X_1^2 and X_2^2 were significant model terms for methane yield, but there are none insignificant model terms. The model value of coefficient of the variance (CV) is 4.96%, which is very less indicating the higher degree of accuracy and reliability of the experimental data. The best fit of the model was also stated by the R^2 value (0.9543), which is within the range. The R^2 value of model signified that the model could elucidate up to 95.43% of the variability in the methane yield response. The Predicted R^2 model value of 0.6748 is not as close to the Adjusted-R^2 model value of 0.9216, as one might normally expect indicating a possible problem with the model or data. This might be possibly due to the fact that only two variables were used in the analysis. Adequate Precision of the model gauges the signal to noise ratio and the desirable value of it is greater than 4. Therefore, the ratio of 13.21 indicated a suitable signal for the model to be used to traverse the design space. To examine the suitability of the model, calculation of diagnostic model terms was done and standardised residuals value revealed an insignificant variance between the response and hypothesised model terms. Every stated value with residuals more than 3 was disregarded as an outlier, signifying the negligible inaccuracies in noting the experimental models value.

5.3.4 Validation of model and optimum conditions for maximum yield

Finally, in order to ensure the reliability of the optimized data from the contour plots, confirmatory experiment was conducted considering optimal conditions given by the model (pH 7.32 and F/M ratio 1.87) (0.975 variability) for maximum methane yield in 20 L batch reactor. For the dependent input factors, a second order quadratic polynomial model was attained in this study (equation (iii) or (iv)). Furthermore, the estimation of optimum response output was done by computing the partial derivatives and optimal settings were identical as pH 7.32 and F/M ratio 1.87. The degradation rate in the experiment ensues first order reaction kinetics ($P = P_\mu(1-e^{-kt})$, $R^2 > 0.94$), where P denotes cumulative methane production at time t; P_μ represents maximum methane production and k denotes first ordered reaction kinetic coefficient (Fig. 5.8). The difference between maximum methane yield of confirmatory experiment (323.78 mL/g-VS_{added}) and theoretically predicted (P_μ) value achieved from the model (315.14 mL/g-VS_{added}) was very less confirming the validity

of RSM. Experimental value was 49.55% higher than the control, the enhanced hydrolysis of RS due to co-digestion with FW was implied through the value of coefficient of first order reaction kinetic (k = 0.037 d^{-1}), thereby enhancing the methane production efficiency.

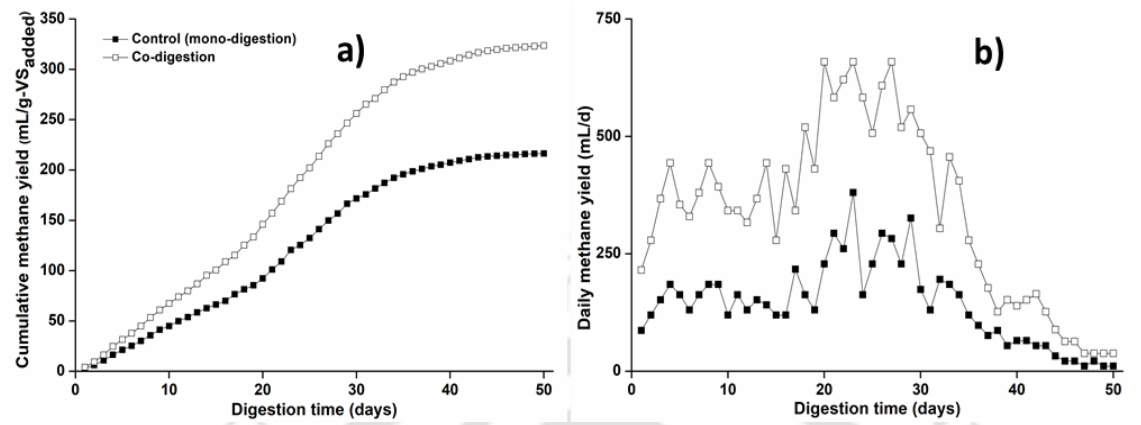


Fig. 5.8. Model validation for optimum response condition a) cumulative methane yield and b) daily methane yield

5.4 CONCLUSION

The results of study demonstrated that co-digestion of RS with *H.verticillata* and FW (low C/N) balanced the C/N ratio in the AD leading to high methane yield. Co-digestion of RS with *H.verticillata* showed the maximum methane yield for the optimal condition i.e. C/N ratio 29.18, F/M ratio 2.45 and pH 7.37. At central level, methane yield was enhanced by improving C/N ratio and F/M ratio in co-digestion it reduced the inhibitory effect due to ammonia inhibition and VFA accumulation and balanced the improper nutritional structure (high C/N) of RS. A synergistic effect of pH and F/M ratio was observed on the methane yield on co-digestion with FW. The highest experimental methane yield of 323.78 mL/g-VS_{added}, 49.55% higher than the control was achieved at pH 7.32 and F/M ratio 1.87 and it was revealed that optimization of pH (7-7.57) and F/M (1.5-2.5) is of significance so as to attain maximum methane yield for co-digestion with.

CHAPTER 6

PRETREATMENT STUDY

This chapter deals with the influence of different pretreatment techniques i.e. thermal, electrohydrolysis and fungal methane yield.

6.1 THERMAL PRETREATMENT

Pretreatment techniques have been proven to be more effective method for improving the digestibility of lignocellulosic materials, leading to increase in biogas yield. Several physical, chemical, biological and thermal pretreatment methods exist for AD (Chen et al., 2014; Dehghani et al., 2015; Brémond et al., 2018). Most chemical pretreatment methods use a huge quantity of chemicals and liquids to infuse solid substrate, this process generates a large quantity of toxic effluents, which causes high investment in facility, huge treatment price and impending environment contamination. Thermal pretreatment of lignocellulosic feedstock is gaining huge importance in the 21st century due to no requirement of additional chemicals and corrosion resistant tools. Moisture under high temperature and pressure can infiltrate into the substrate, can hydrate cellulose and detach the hemicellulose and partial lignin concentration in the process. The objective of the current study is to study the comparative effect of different thermal pretreatment techniques (hot air oven, hot water bath, microwave and autoclave) on the solublisation of RS. This study has proven that thermal pretreatment could enhance the digestibility and lead to enhanced biogas production. It enhances the accessible available surface area of the biomass and makes it more reachable to hydrolytic enzymes. The current study comprehended the hydrolysis pattern of untreated and thermally treated RS with various thermal pretreatment techniques to present the aptness and comparative usefulness of the different mode of heat transfer (hot water bath, hot air oven, autoclave and microwave) for enhanced methane production. This study also focused on the correlation between the compositional changes and physical alterations with optimal residence time and temperature. This study was conducted in two stages to determine the optimum conditions required for solubilizing RS. They are as follows; a) temperature and b) temporal pretreatment studies of RS.

6.1.1 Hot air oven pretreatment

Fig. 6.1 a) depicts temperature pretreatment study of RS, where the substrate was allowed to pretreat in hot air oven. Increase in temperature leads to degradation of

recalcitrant matrix of RS into monosaccharides showing greater solubilisation in terms of sCOD and VFA. VFA showed a direct correlation with sCOD as the soluble products formed in the first step again convert into short chain VFA in the second step of AD. Thus, VFA and sCOD showed peak values at 80°C which is considered as best temperature. Further increase in temperature showed reduction in the both the parameters probably due to loss of VFA through vaporization and formation of inhibitory phenolic compounds. Fig. 6.1 b) depicted temporal pretreatment study of RS conducted at best pretreatment temperature (80°C). Increase in exposure time revealed higher solubilisation in terms of VFA and sCOD because of severe structural destruction of RS into soluble products. At 90 min of exposure time, VFA and sCOD showed maximum values due to solubilisation of oligosaccharides into liquid fraction of RS at higher exposure time. Finally, sCOD showed an increment of 25% with respect to control/untreated RS sample in temperature study whereas VFA increased by 2.12 times as compared to control in the study.

6.1.2 Hot water bath pretreatment

Temperature pretreatment study of RS was performed at various temperatures by using hot water bath expressed in Fig. 6.1 c), which showed continuous increment in sCOD and VFA. Rise in sCOD might be caused because of O-acetyl and uronic acid degraded product from hemicellulose, which help catalyze the hydrolysis of polysaccharides into soluble monomeric sugars (Durak, 2019). The maximum sCOD was obtained in thermally treated RS sample at 90°C with respect to control. Temperature rise also leads to increment in the VFA concentration of liquid fraction of RS and reached to maximum value at 90°C with respect to control probably because of fermentative bacteria ferment the soluble products formed in first step (hydrolysis) to a mixture of long chain fatty acids. Thus, 90°C was considered to be a best pretreatment temperature. Further, increase in the temperature resulted in reduction of sCOD and VFA concentrations because too severe conditions promote the formation of inhibitory compounds like furfural and 5-hydroxymethy 1-2-furaldehyde (HMF) which are non-degradable at this range of temperature. Fig. 6.1 d) shows the temporal pretreatment study of RS conducted at 90°C pretreatment temperature for different exposure times. Increment in pretreatment exposure time showed a progressive pattern in sCOD and VFA curves which reached to greater values at 90 min of exposure time whereas, the higher time of exposure leads to reduction in the concentrations of both the parameters. Finally, we noticed 26.4% increase in sCOD with respect to the untreated RS sample whereas VFA concentrations got doubled with respect to the concentration of untreated RS sample at pretreatment temperature of 90°C for an exposure time of 90 min.

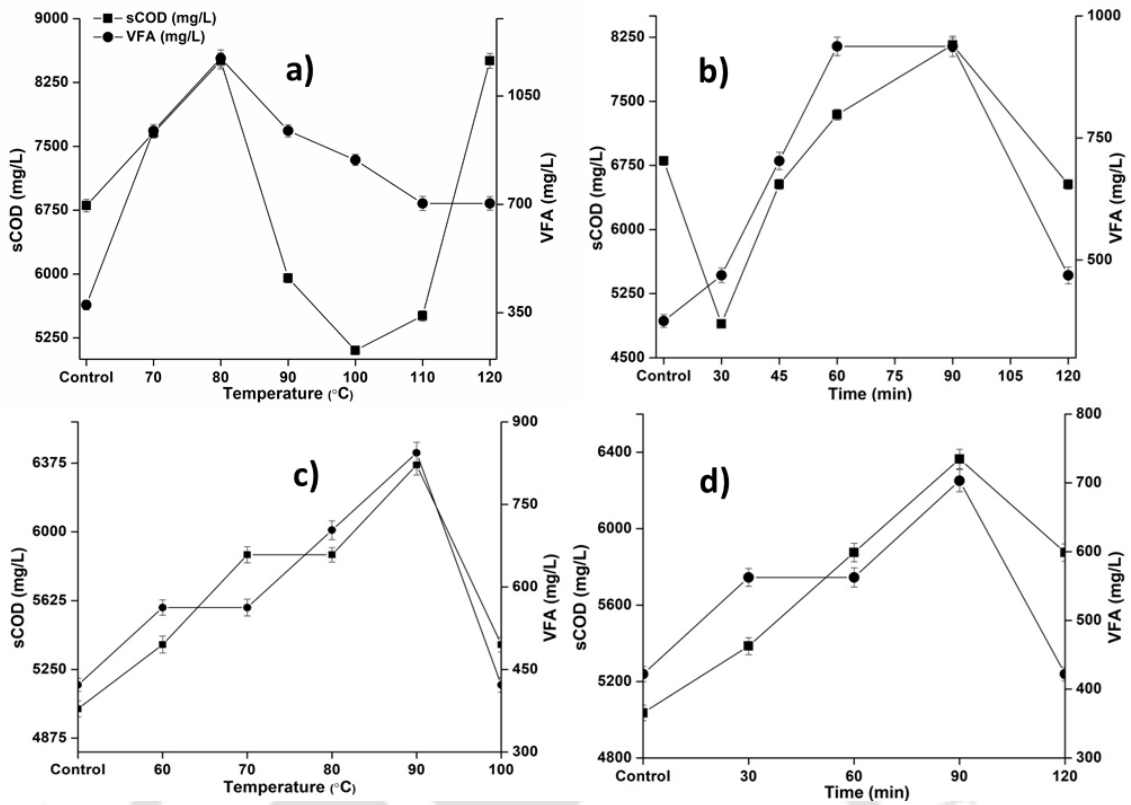


Fig. 6.1. Temperature and temporal pretreatment effect on RS in terms of sCOD and VFA a) hot air oven pretreatment (45 min) b) hot air oven pretreatment (80°C) c) hot water bath pretreatment (60 min) d) hot water bath pretreatment (90°C)

6.1.3 Autoclave pretreatment

Fig. 6.2 a) shows the effect of temperature pretreatment on the RS sample. This study showed that sCOD and VFA concentrations first increased and then became constant as soluble products present in the liquid fraction of sample broke down into long chain fatty acids. Further increase in the temperature gave rise to higher solubilisation of monomeric sugars, which are represented in the form of sCOD along with VFA because during autoclaving of RS, parts of hemicellulose hydrolyzed and formed fatty acids. Thus, sCOD and VFA increased at pretreatment temperature of 110°C, which is considered as best temperature for this study but beyond this temperature; sCOD and VFA showed a decreasing nature probably due to formation of phenolic and heterocyclic compounds like vanillin, vanillin alcohol, furfural and HMF. Fig. 6.2 b) reveals the characteristics of temporal pretreatment study of RS by using autoclave in which RS samples were allowed to treat at different exposure times at constant temperature of 110°C. On increasing autoclaving exposure time, sCOD and VFA progresses towards higher value and became highest at 20 min of exposure time due to degradation of complex polymers into monomers. As time reached beyond 20 min. sCOD and VFA showed fluctuations in their

concentrations due to formation of non-degradable compounds (Hahn-Hägerdal, 2000). Finally, we obtained 44.4% hike in the sCOD of pretreated sample of RS with respect to the untreated sample of RS in both the temperature and temporal studies whereas we have noticed 1.5 and 0.66 times higher VFA than untreated sample of RS in temperature and temporal study respectively.

6.1.4 Microwave pretreatment

Fig. 6.2 c) well represents the pretreatment study of RS by using microwave (irradiation method) in which concentrations of sCOD and VFA increased continuously with temperature and became maximum at 190°C indicating degradation of oligosaccharides into monomeric sugars giving rise to higher solublization.

During thermal pretreatment, temperatures of 160°C and higher causes the solubilisation of hemicellulose and lignin. The amount of soluble sugars present in the suspension degrade into mixture of long chain fatty acids or organic acids, which resulted in the higher concentration of VFA at 190°C. But at temperature (>200°C) led to reduction in the concentrations of sCOD and VFA, this may be because of pretreatment process tend towards the pyrolysis (Sapci, 2013). Thus, pretreatment has been done at 190°C provided the optimum results. Fig. 6.2 d) showed temporal study of microwave pretreatment of RS. As the exposure time for pretreatment increases hemicellulose start degrading into organic acids and soluble sugars, which caused increase of sCOD and VFA with an increasing pattern and reached to maximum values at 4 min of exposure time of pretreatment after that we have found reduction in sCOD and VFA probably due to formation of phenolic and heterocyclic compounds like vanillin, vanillin alcohol, furfural, HMF etc. at higher exposure time for pretreatment. Thus, 4 min of exposure time in case of microwave irradiation method was considered to be the best residence time for pretreatment. Finally, we estimated 66.6% hike in the concentration of sCOD of pretreated sample of RS with respect to the untreated sample whereas, VFA concentration was increased by 0.66 times as compared to the untreated sample of RS in this study.

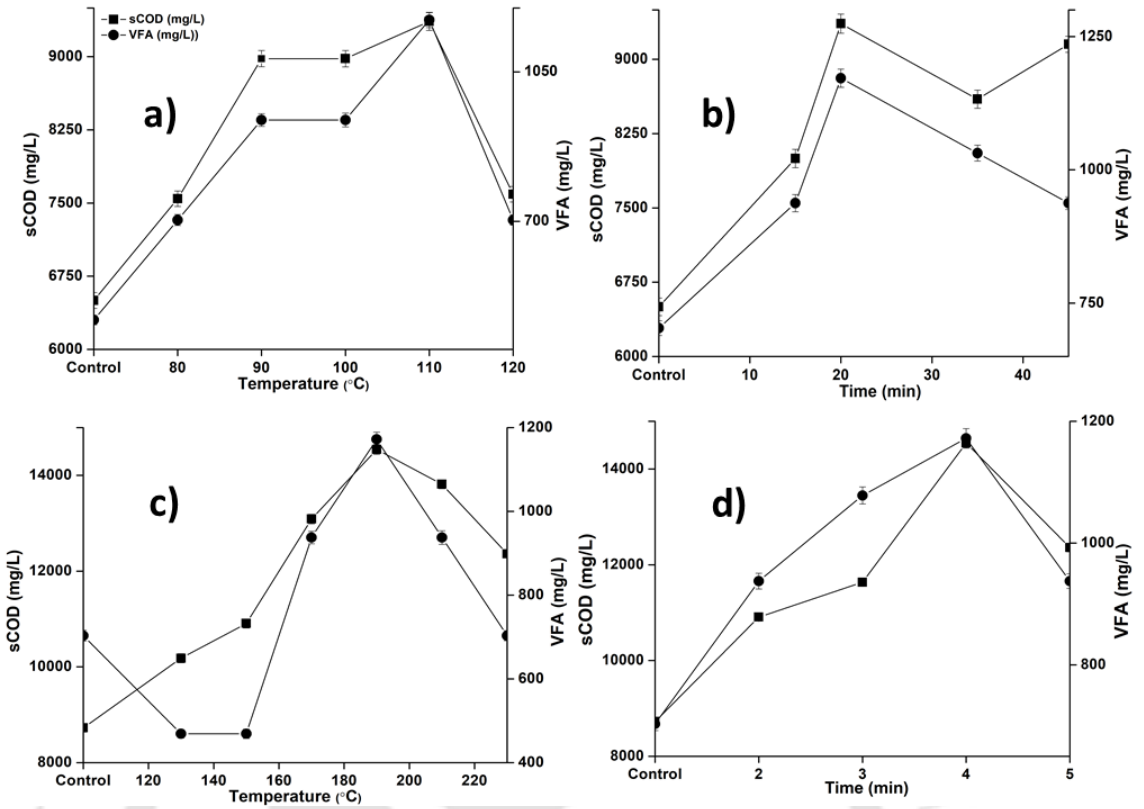


Fig. 6.2. The temperature and temporal pretreatment effect on RS in terms of sCOD and VFA (a) autoclave pretreatment (20 min) (b) autoclave pretreatment (110°C) (c) microwave pretreatment (3 min) (d) microwave pretreatment (190°C)

6.1.5 Microstructure analysis

6.1.5.1 FESEM

FESEM micrographs of untreated and pretreated RS sample under different conditions by using hot air oven, hot water bath, autoclave, and microwave pretreatments are presented in Fig. 6.3. The structural morphology of untreated sample of RS clearly defined by Fig. 6.3 a), it showed that a regular and complex, crystalline and rigid structure of untreated sample of RS. Whereas Fig. 6.3 b), depicted ruptured and amorphous structure of pretreated RS which showed the impact of hot air oven pretreatment. Fig. 6.3 c) indicated more chiselled outline of RS sample pretreated by using hot water bath which increased the surface area of RS. Fig. 6.3 d) represented more ruptured and destroyed structure of RS pretreated by autoclave pretreatment which gave rise to more accessible area for the attack of microbes. Fig. 6.3 e) concluded severe effect of microwave pretreatment on RS sample which inferred completely collapsed and highly structural breakdown at higher temperatures. So, we can easily distinguish the structural morphology of untreated and pretreated sample of RS and

moreover, can select best pretreatment method out of four methods with the help of these images taken at the magnification of 1000 times.

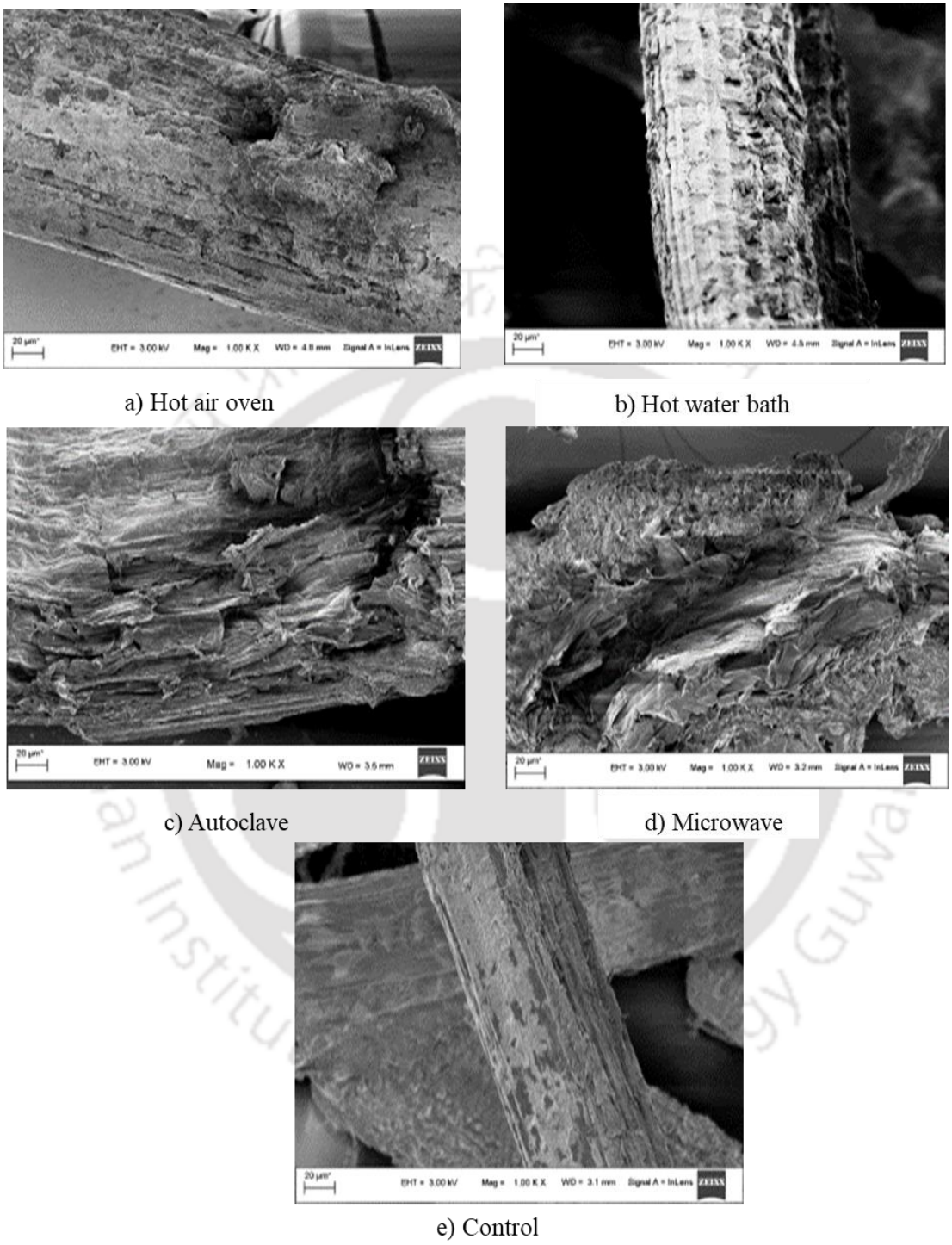


Fig. 6.3. FESEM micrographs of pretreated RS by using a) Hot air oven (80°C, 90 min) b) Hot water bath (90°C, 90 min) c) Autoclave (110°C, 20 min) d) Microwave (190°C, 4 min) e) Control

6.1.5.2 FTIR

FTIR spectra were used to analyze the bonds transformation in lignin-carbohydrate complexes. Lignin is associated with polysaccharides (cellulose and hemicellulose) by phenyl glycosidic, α-ether and ester bonds (Li et al., 2014). The FTIR spectra of the different thermal treatment study are shown in Fig. 6.4. Thermal pretreatment can dissociate lignin-carbohydrate matrix by hydrolyzing bonds and thus improving the degradability of RS. The intermolecular and intramolecular changes are represented by declining absorbance pattern of treated and untreated sample. It was perceived that absorbance at 1167 cm^{-1}, which revealed presence of ester bonds vanished after pretreatment. Thermal pretreatment destroyed the linkage and released cellulose from lignin-carbohydrate complexes. The absorbance at 1718 cm^{-1}, which is assigned to carbonyl (C=O) group also changed. This disappearance of carbonyl group indicated that the lignin was broken in the treatment. The change in band of 898 cm^{-1} absorbance (glycosidic linkage) also showed the breakages in the hydrogen bonding in cellulose. Appearance of peak at 1017 cm^{-1}, denotes aromatic ring destruction in C-H surface.

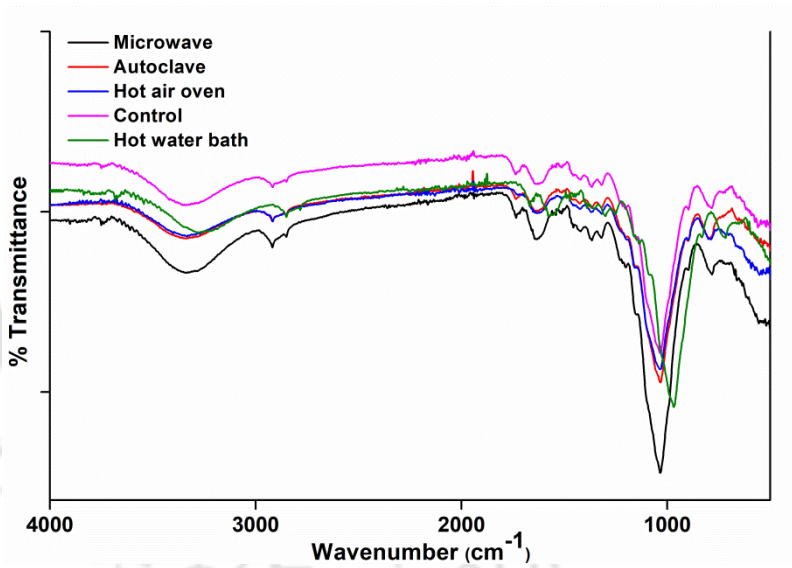


Fig. 6.4. FTIR spectra of untreated and thermal pretreated RS

6.1.6 Compositional analysis

The properties of lignocellulosic biomass like RS render it resistant to biodegradation. Several structural and compositional properties were found to have impacts on the biodegradability of RS, including cellulose crystallinity, accessible surface area, degree of cellulose polymerization, presence of lignin and hemicellulose, and degree of hemicellulose acetylation (Rusanen et al., 2019; Tran et al., 2019). The goal of pretreatment is to alter such properties to improve RS amenity to enzymes and microbes. The effects of different

pretreatment techniques on the chemical composition and physical characteristics of RS are summarized in Table 6.1. In general, different pretreatment methods affected these properties to different degrees; however, all methods had a major effect on the accessible area of RS. Experiments conducted on solid fraction of pretreated RS sample provided noteworthy results which showed that maximum 34.13% and 23.63% reduction in the acid insoluble lignin and acid soluble lignin, respectively. Apart from this, we obtained 27.4% increase and 22.93% reduction in the cellulose and hemicellulose content respectively in case of microwave pretreated RS sample as compared to the untreated RS (control). Such a drastic reduction in these parameters confirmed that microwave pretreatment not only gave rise to maximum solubilisation in the form of soluble sugars in the liquid fraction of RS sample but also removed lignin cover and made cellulose easily available to microbes for enzymatic hydrolysis (Demirbas and Ozturk, 2015). Generally, decreased lignin content also leads to increased biogas yield. The biodegradability of lignocellulosic biomass increased with decreasing lignin content (Fernandes et al., 2009).

Table 6.1. Lignocellulosic contents of RS before and after different pretreatment conditions

Pretreatment condition	**Optimum Temperature (°C)**	**Optimum Time (min)**	**Acid insoluble lignin (%)**	**Acid soluble lignin (%)**	**Hemicellulose (%)**	**Cellulose (%)**
Control	_	_	1.67 ± 0.13	12.44 ± 0.06	27.9 ± 0.57	36.3 ± 2.10
Hot air oven	80	90	1.38 ± 0.11	11.12 ± 0.22	25.87 ± 0.63	39.7 ± 1.36
Hot water bath	90	90	1.27 ± 0.07	10.57 ± 0.07	25.1 ± 0.76	38.2 ± 1.23
Autoclave	110	20	1.16 ± 0.13	9.84 ± 0.13	24.3 ± 1.03	42.9 ± 1.37
Microwave	190	4	1.10 ± 0.23	9.50 ± 0.13	21.5 ± 1.79	46.3 ± 1.57

6.1.7 BMP study of thermally pretreated RS

6.1.7.1 Daily biogas/methane production

Fig. 6.5 depicts the variation of pH, sCOD, VFA and daily methane production rate with respect to digestion time. In this work, the following methane yield was achieved 230.52 mL/g-VS_{added} for control, 265.72 mL/g-VS_{added} for F/M:0.5, 286 mL/g-VS_{added} for F/M:1.5, 290.66 mL/g-VS_{added} for F/M:2, 325.76 mL/g-VS_{added} for F/M:2.5 and 275.72 mL/g-VS_{added} for F/M:3 (Fig. 6.6 a)). These findings can be well compared with results of Marin et al. (2010) and Zhou et al. (2013). The reason for enhanced biomethane production was due to accelerated hydrolysis of the substrate, enhanced cell membrane fragility, desirable cellular disruption and efficient release of soluble compounds. In AD process lignocellulosic biomass needs an intensive pretreatment to accelerate the hydrolysis step (rate limiting step). Thermal pretreatment de-structure the lignin; the main conferring structural support unit of the biomass; as a result, the accessibility of the cellulolytic enzymes gets improved (oxidative stress decreases). Due to decrease in cellulose crystallinity and rise in available area for hydrolysis, the specific methanogenic activity is also increased. The interactive effect amid exposure time and microwave temperature significantly enhances the biomass digestibility. Similar use of microwave for upgrading the bio-digestibility was used by many researchers (Ethaib, 2015; Kaur and Phutela, 2016; Li et al., 2016). Thermal treatment above 160°C initiates the solubilization of lignin. Hydrolysis of lignin produces the reactive compounds, which causes inhibition of the biodegrading enzymes. Therefore, for higher temperature we need to reduce the exposure time. pH has a significant effect on AD. Microorganisms have inclination towards a pH range of 6.8-7.2 (Savoo and Mudhoo, 2018) as microbes cannot survive in highly acidic or alkaline conditions (Fig. 6.5 a)). During initial days of digestion a large amount of fatty acids (acidogenesis is faster than methanogenesis) are produced and pH of the reaction dropped at 7^{th} day. In general decrease in pH is the indication of the imbalance in the process, in this situation pH was managed by the addition of lime or sodium bicarbonate. The pH variation in the course of digestion process was due to variation in metabolism of acidogens and methanogens. The major factor influencing the drop in the digestion process is the VFA concentration, as system becomes more acidic. The annihilation of substrate cell membrane affected due to microwave treatment led to the attack of extracellular hydrolytic enzymes (improved degradability). From Fig. 6.5 b), it can be observed that as the fraction of VFA increases, pH of the reactor falls if it is not consumed by methanogens immediately. After attaining maximum value VFA start decreasing till quasi state of equilibrium. Steady concentration

achieved showed the equilibrium amid hydrolysis, acidogenesis, acetogenesis and methanogenesis. Fig. 6.5 c) showed the change in sCOD for BMP assay (all F/M ratios). Initial high sCOD value indicates the high biodegradability of the BMP assays. This high value of sCOD reduced with time with the conversion of organic matter into methane by anaerobic microorganisms. Maximum sCOD in initial hydrolysis was achieved with microwave heating of @190°C; 4 min, which was used for hydrolysis of BMP assays. Similar results was achieved by Marin et al. (2010), who treated kitchen waste (KW) by microwave heating @175°C and observed an increase of 17.98% with high rate of methane yield. sCOD of different F/M ratios varies from 1560 ± 200 mg/L to 3780 ± 190 mg/L, this value rise steadily and achieved maximum value on the 14th day, whereas, untreated sample showed the highest value after 21st day. This was probably due to the pretreatment stimulating breakdown of the lignocellulosic feedstock and enhanced solubilization. Microwave pretreated RS had greater biomethane potential as compared to the untreated one, which was evidently clear from the well-defined trend.

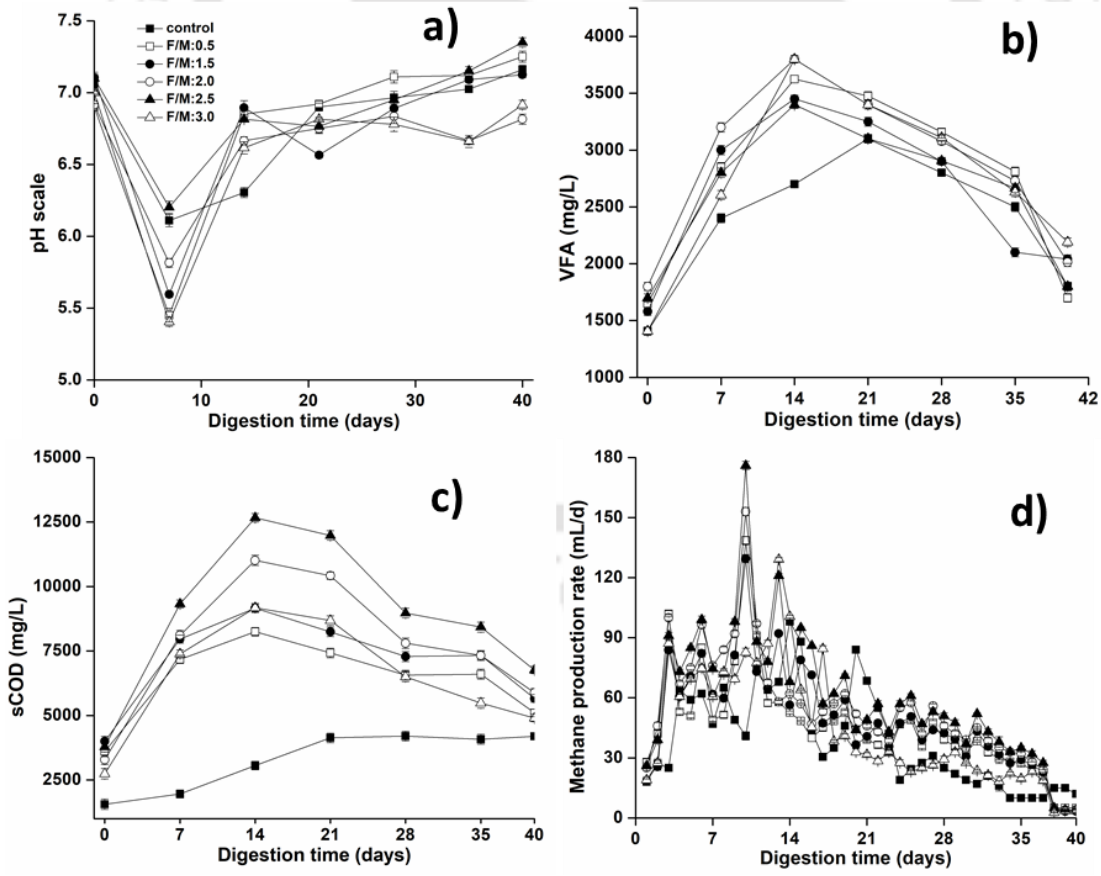


Fig. 6.5. Influence of F/M ratios on different parameters a) pH b) VFA c) sCOD and d) daily methane production rate

6.1.7.2 Kinetic modeling

Fig. 6.6 showed the fit of the three different models from the experimental results of AD of microwave-treated RS samples. On equating the performance of the model in terms of biomethane production potential (M mL/g-VS_{added}/d), it followed the trend; MGM>TFM>LFM (for all F/M ratios).

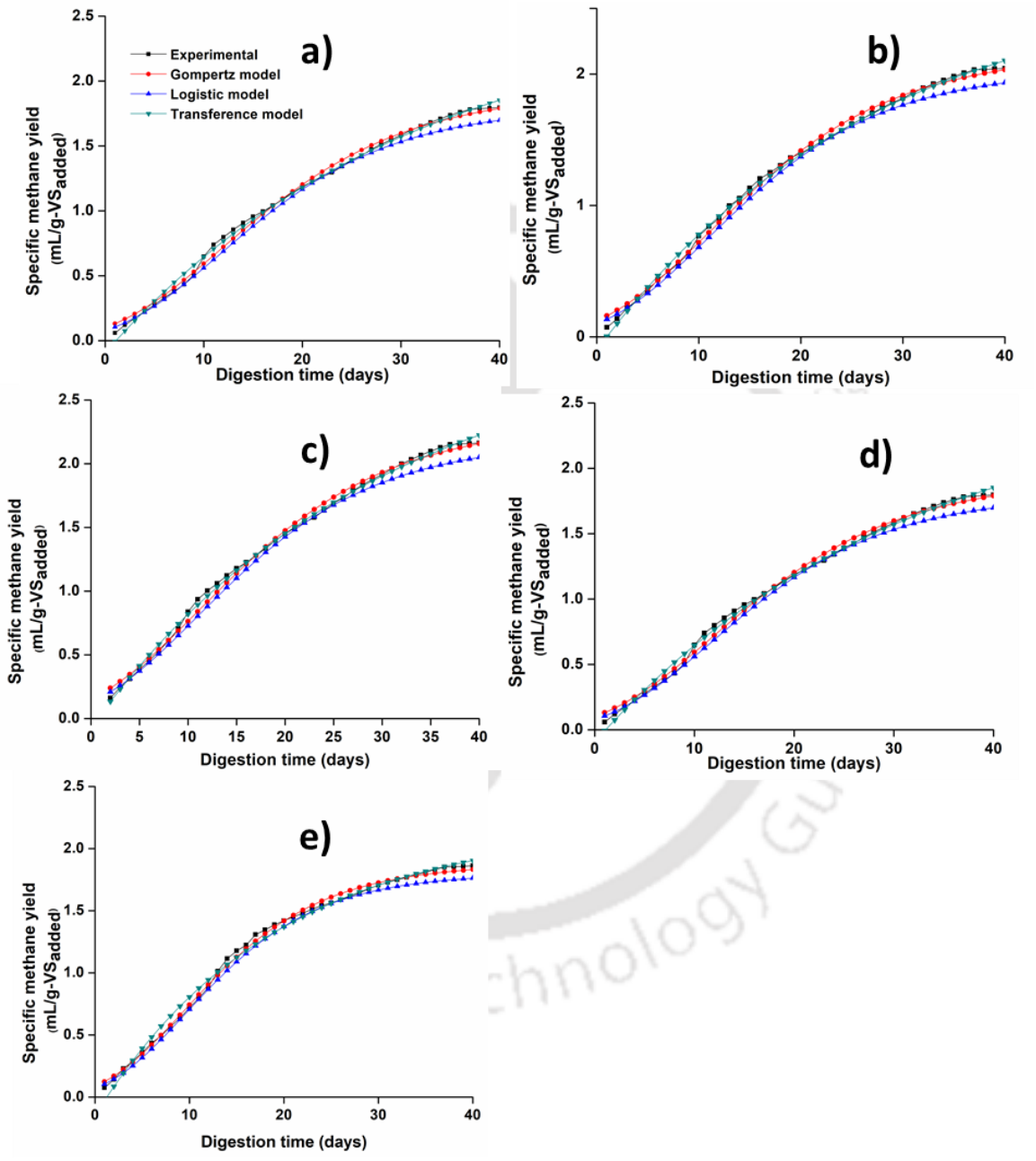


Fig. 6.6. Model fit for cumulative methane yield using microwave pretreatment for a) F/M:0.5 b) F/M:1.5 c) F/M:2 d) F/M:2.5 and e) F/M:3

Maximum value of M was obtained for F/M:2.5 (TFM (3.328), MGM (2.6059) and LFM (2.4545)) with correlation coefficient value of 0.981. Maximum specific methane production (R_m mL/g-VS_{added}/d) was obtained in different order for all the F/M ratios, no fixed pattern was observed. Maximum value of R_m was obtained for F/M:2.5 (TFM (0.1219)), which was 33.07% higher than the MGM and LFM. The lag time (λ) was negligible in case of MGM, whereas in cases of LFM and TFM this value ranged between 0.0005-0.0014 days. Lag phase value of less than 1 indicates that biodegradable fraction was readily consumed by the microbes. The maximum cumulative methane generation is observed for F/M:2.5 followed by F/M:2. Therefore, including the results of these kinetic studies, we can interfere that check of the parameters and operations factors is necessary in terms of monitoring and operation of AD process and reactors performance.

6.2 ELECTROHYDROLYSIS PRETREATMENT

Hydrolysis is considered to be a rate limiting step in AD of RS. Usually, pre-hydrolysis prior to AD is usually required to decrease the existing structural hindrance of lignocellulose biomass and to make cellulose and hemicellulose readily available to microbial breakdown. Induced delignification process by pretreatment increases the rate of biomass decomposition as well as accelerated methane production. This neoteric study is about the electrohydrolysis pretreatment of RS to accelerate hydrolysis step of AD. The principle of electrohydrolysis pretreatment is based on ohmic heating, electrophoresis, and electro-osmosis, which causes the disruption of complex lignocellulose structure of RS and finally triggers the hydrolysis. The process of heating substrate by passing electric current through it is defined as ohmic heating. Electrical resistance heating lets the substrate to heat at the same rate by dissipation of electrical energy and fasten heating with high solid fractions (Varghese et al., 2014). The electrical conductivity is a function of the structure of the material which varies on application of electric current through the materials (Camargo et al., 2010). Electrophoresis is a phenomenon by virtue of which movement of solid particles present in suspension occurs under the impact of electric field developed across two electrodes connected to direct current (DC) supply. The process of polarization (charging) of solid particles takes place by inducing external electric field, which allows complex polymers to break down into charged monomers. This process is called as electro-osmosis. On connecting the electrodes to DC power supply, one converts to positively charged electrode (anode) and another converts to negatively charged electrode (cathode). The ionized soluble monomers (electrolytes) move towards the electrodes i.e. cations transfer towards the cathode and anions transfer towards the anode. The aim of this novel

study is to investigate the impact of electrohydrolysis pretreatment conditions (applied voltage and time) on physico-chemical changes that occurred in the RS before and after the pretreatment in terms of compositional alteration. As for lignocellulosic substrate hydrolysis is a rate limiting effect but after pretreatment methanogenesis become the rate limiting step. Therefore, to achieve the maximum yield and to avoid the inhibition due to instability in acidogenesis phase. Second phase includes the BMP assay of hydrolysate (obtained from pretreatment) to optimize the F/M ratio. The performance of the BMP assay was evaluated from increased methane yield. In addition to that, energy assessment and kinetic modeling using three models were applied to determine the performance on this process.

6.2.1 Change in sCOD and VFA with applied voltage

The sCOD and VFA are considered as the main parameters for estimation of the bioavailability of soluble organic matter (monosaccharides). Induced pretreatment is responsible in the disruption of bonds in lignocellulosic substrate and subsequently enhanced the intracellular organic matter and extracellular substances. Increased sCOD and VFA can be readily used as substrate for methane production in AD (Rani et al., 2012). Fig. 6.7 a) shows the increment in the sCOD and VFA with the increase in applied DC voltage. The sCOD significantly increased upto 25 V for constant exposure time of 30 min due to a breakdown of polymeric matrix into monosaccharides, and cell lysis prompted by ohmic heating (Gharibi et al., 2013). Due to polarization of substrates from DC current disruption in the structure of microfibrils occurs, that improves the availability of the cellulose to enzymes. RS sample at 25 V depicted an improvement of 0.45 and 1.5 times in sCOD and VFA respectively with respect to the untreated sample. The amount of electrons released at 25 V was adequate to counterbalance the available protons (H^+) provided by the substrate. At higher voltage (>25 V), sCOD and VFA followed a decreasing trend; which might be due to the extra release of electrons by the DC to neutralize the limited amount of protons liberated by the substrate (Kargi et al., 2010). Beyond 25 V, electrical energy was directly dissipating into substrate, which resulted into decline in sCOD and VFA concentration. At 25 V the percentage of soluble inerts i.e. silica and dissolved organic carbon concentration increased in the system, which decreased the conductivity of the substrate. Thus, 25 V was selected to be the optimum pretreatment voltage for RS. High availability of silica enhances the reducing condition in the system, which promotes the rapid mobilization of nutrients N, P, K etc. in the system (Reithmaier et al., 2017).

6.2.2 Change in sCOD and VFA with time

The applied DC voltage was kept constant at 25 V and RS samples were pretreated at different exposure intervals. Fig. 6.7 b) depicts that sCOD and VFA concentrations increased upto 60 min and then decreased at higher exposure time (>60 min). At exposure time of 60 min, hike of 0.55 and 1.62 times in sCOD and VFA, respectively were observed as compared to untreated sample probably due to removal of lignin which made cellulose component available for further disintegration into soluble monomers. During electrohydrolysis pretreatment, fall in sCOD was observed after 60 min due to loss of VFA caused by excessive ohmic heating (Varghese et al., 2014). Electrohydrolysis pretreatment at 25 V for 60 min ensured destruction of lignin layer which allowed release of extracellular and intracellular biopolymers into soluble phase along with increase in sCOD and VFA concentration (Eskicioglu et al., 2006).

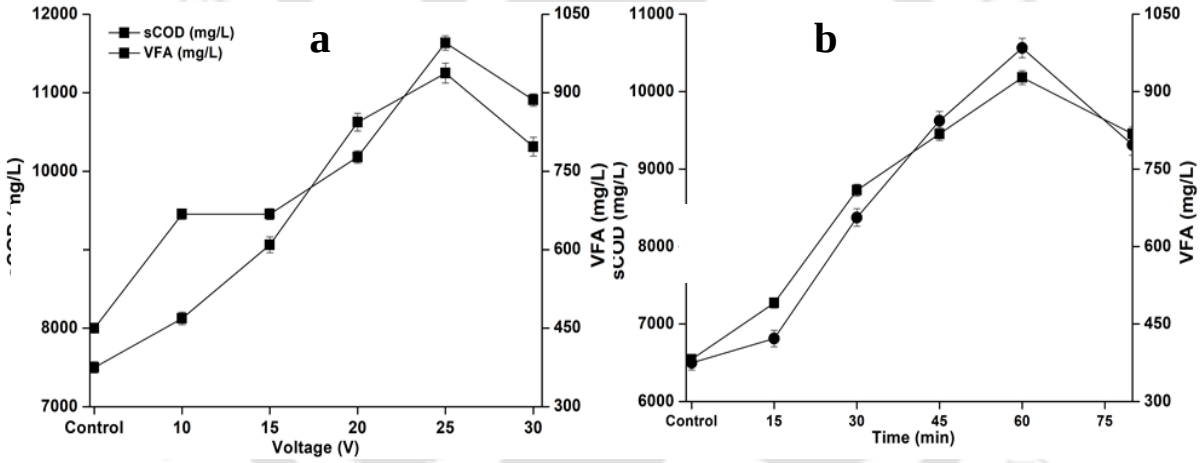


Fig. 6.7. The effect of applied DC voltage and exposure time on sCOD and VFA with (a) applied voltage for exposure time of 30 min (b) exposure time at 25 V applied voltage

Hence, electrohydrolysis pretreatment at 25 V for 60 min allowed maximum disintegration of substrate into simpler monomers (sugars), which made them easily accessible to microorganisms during AD.

6.2.3 Effect of electrohydrolysis pretreatment on electrogenic activity

The performance of electrohydrolysis pretreatment was assessed by monitoring variation of overall electrogenic activity in terms of current and resistance (ability to flow freely) with time for different applied DC voltages as well as variation in current and resistance with time at same applied voltage for the different exposure time. As listed in Table 6.2, current increased with time at particular applied voltage, although increment was very less at low

voltage but the results were favourable with increased DC voltage up to 25 V. Current increased by 10, 12.5 and 11.5% at 15 V, 25 V and 30 V, respectively, whereas resistance decreased by 9.1, 11.1 and 10.34% at 15 V, 25 V and 30 V, respectively. This is due to the self-induced electrogenic capacity in the substrate to bio-transform organic polymer to simple soluble monomers by catabolism. Considering exposure time, on increasing time of treatment similar values were obtained at two different time intervals like 0 and 5 min, since initial increment in exposure time from 0 to 5 min was not enough to produce electrogenic activity in the substrate. Substrate was not readily bio-transformed into monomer through charge induction at an increment of 5 min. Further, increment in time increased current, which became constant after 15 min of electric field across electrodes. Measuring current and resistance at every 5 min interval was done to get precise time of exposure for treatment of RS.

Table 6.3 depicts that at constant applied voltage of 25 V, on increasing time of exposure in electrohydrolysis pretreatment current increased by 4.4, 8.3, 11.5 and 14.7%, while resistance decreased by 4.2, 7.7, 10.34 and 12.8% for time interval of 15, 30, 45 and 60 min respectively. It supports the fact that when complex organic matter disintegrate into simpler forms, it releases large number of protons as an effect of increasing current and results into the degradation of recalcitrant structure of RS. Previously, Chandrasekhar and Venkata Mohan (2014) studied that oxidative catalytic current was perceived as 3.34 mA at 0^{th} h followed by a rise and was obtained to be 3.79 mA and 4.22 mA at 5^{th} and 10^{th} h, consequently during the bio-electrolysis of food wastes. Thus, this study marked that electrical energy was sufficient at applied voltage of 25 V, which illustrated higher solubilization in terms of sCOD and VFA and electrogenic activity (current and resistance) also confirmed the effectiveness of electrohydrolysis pretreatment.

Table 6.2. Variation of current and resistance with time upto 30 min for different applied voltages

Time (min)	Current (amp)	Resistance (ohm)	Current (amp)	Resistance (ohm)	Current (amp)	Resistance (ohm)	Current (amp)	Resistance (ohm)	Current (amp)	Resistance (ohm)
	(10 V)	(10 V)	(15 V)	(15 V)	(20 V)	(20 V)	(25 V)	(25 V)	(30 V)	(30 V)
0	0.05	200	0.1	150	0.15	133.33	0.32	78.125	0.26	115.38
5	0.05	200	0.1	150	0.15	133.33	0.32	78.125	0.26	115.38
10	0.05	200	0.1	150	0.16	125	0.34	73.52	0.27	111.11
15	0.055	181.81	0.1	150	0.16	125	0.34	73.52	0.28	107.14
20	0.055	181.81	0.11	136.36	0.17	117.64	0.36	69.44	0.28	107.14
25	0.055	181.81	0.11	136.36	0.17	117.64	0.36	69.44	0.29	103.44
30	0.055	181.81	0.11	136.36	0.17	117.64	0.36	69.44	0.29	103.44

Table 6.3. Variation of current and resistance with time at 25 V for different exposure times

Time (min)	**Current (amp)**	**Resistance (ohm)**	**Current (amp)**	**Resistance (ohm)**	**Current (amp)**	**Resistance (ohm)**	**Current (amp)**	**Resistance (ohm)**	**Current (amp)**	**Resistance (ohm)**
	at 15 min	at 15 min	at 30 min	at 30 min	at 45 min	at 45 min	at 60 min	at 60 min	at 80 min	at 80 min
0	0.23	108.69	0.24	104.16	0.26	96.15	0.34	73.52	0.30	83.33
5	0.23	108.69	0.24	104.16	0.27	92.59	0.35	71.42	0.30	83.33
10	0.23	108.69	0.25	100	0.27	92.59	0.35	71.42	0.30	83.33
15	0.24	104.16	0.25	100	0.27	92.59	0.35	71.42	0.31	80.64
20			0.25	100	0.28	89.28	0.36	69.44	0.31	80.64
25			0.26	96.15	0.28	89.28	0.36	69.44	0.31	80.64
30			0.26	96.15	0.28	89.28	0.37	67.56	0.31	80.64
35					0.28	89.28	0.37	67.56	0.32	78.13

Time (min)	Current (amp)	Resistance (ohm)	Current (amp)	Resistance (ohm)	Current (amp)	Resistance (ohm)	Current (amp)	Resistance (ohm)	Current (amp)	Resistance (ohm)
	at 15 min	at 15 min	at 30 min	at 30 min	at 45 min	at 45 min	at 60 min	at 60 min	at 80 min	at 80 min
40					0.29	86.20	0.37	67.56	0.32	78.13
45					0.29	86.20	0.38	65.78	0.32	78.13
50							0.38	65.78	0.32	78.13
55							0.39	64.10	0.33	75.75
60							0.39	64.10	0.33	75.75
65									0.33	75.75
70									0.34	73.52
75									0.34	73.52
80									0.34	73.52

6.2.4 Microstructure analysis

6.2.4.1 FESEM

FESEM analysis was conducted to observe morphological features of untreated and electrohydrolysis pretreated RS. FESEM micrographs (Fig. 6.8) depicted that untreated sample of RS have regular well-arranged and compact structure with some finer grains present on the surface correspond to silicon compounds which acts as masking layer to inner organic content and substantially decreased digestibility of RS, as studied by Karlsruhe and Berichte (2007).

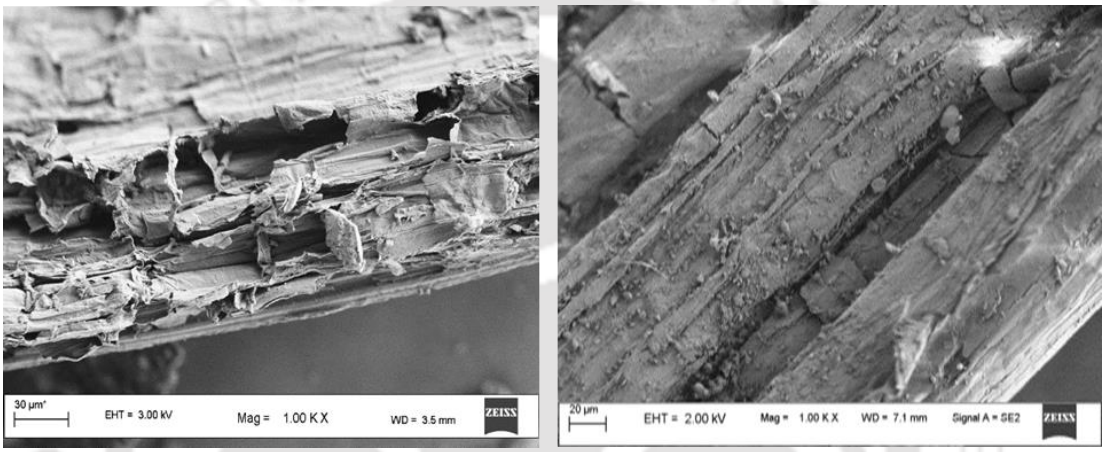


Fig. 6.8. FESEM micrographs a) Electrohydrolysis pretreated RS b) Untreated RS

On the other hand, pretreated sample have ruptured lignin cover, scattered bunch of grooves and grains, broken branched of hemicellulose and internal chiselled structure containing loosed cellulose microfibrils. This analysis demonstrates that electrohydrolysis pretreatment has the ability to break three dimensional matrix of cellulose-hemicellulose-lignin. Similar results were observed by Zheng et al. (2018) after pretreatment of wheat straw (lignocellulosic material).

6.2.4.2 FTIR

The FTIR spectra of untreated and electrohydrolysis pretreated RS are shown in Fig. 6.9. The sharp peak at 3338 cm^{-1} is linked with intermolecular and intramolecular hydrogen bonding in hydroxyl group of cellulose in case of untreated RS. Compressed band in electrohydrolysis pretreated RS suggest the breaking of hydrogen bonding in cellulose, which results in the formation of soluble monomers. The absorbance at 1733 cm^{-1}

corresponding to the ester group, indicating that functional group existed in lignin but these destructive spectra were removed after electrohydrolysis pretreatment.

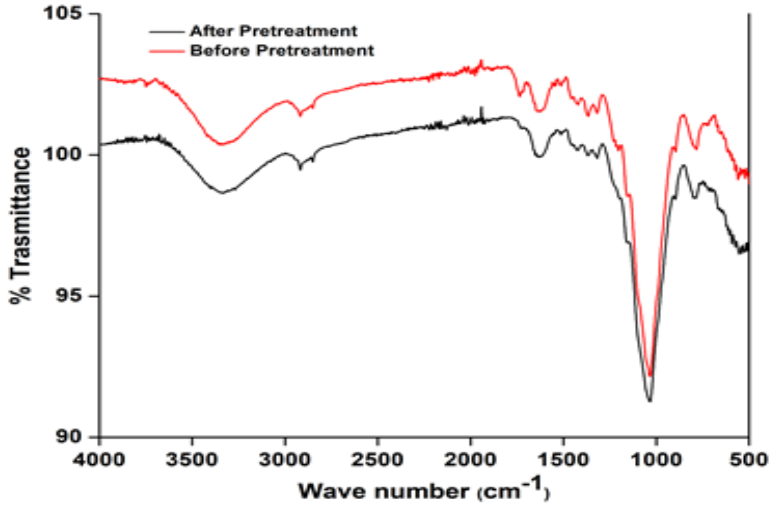


Fig. 6.9. FTIR spectra of untreated and electrohydrolysis pretreated RS

Comparable results were observed by Zhang et al. (2018) in lignocellulosic material like cotton stalk. The enlargement of C-H stretch absorbance at 1374 cm^{-1} for pretreated RS implies that electrohydrolysis pretreatment can eliminates the linkage between lignin and carbohydrate. The intensity of absorption peak at 691 cm^{-1} corresponding to β-D-cellulose glycosidic linkages was disappeared in pretreated RS, concluding the efficient conversion of crystalline cellulose to amorphous components.

6.2.5 Compositional analysis

Changes that occurred in the composition of RS due to electrohydrolysis pretreatment are listed in the Table 6.4. Pretreatment performed at 25 V for 60 min time interval showed 37.1, 54.8 and 22.4% reduction in acid soluble lignin, acid insoluble lignin and hemicellulose, respectively. An increment of 15.1% has been noticed in the cellulose content of RS in comparison to control. Decreasing lignin content of pretreated RS sample promotes the delignification and the reduction in hemicellulose percent ensured breaking of hemicellulose crosslinking across cellulose. Removal of lignin and hemicellulose made cellulose easily accessible to hydrolytic bacteria responsible for destruction of β-1, 4 glycosidic linkages to soluble D-glucose subunits, which could be readily available food for microbial flux in AD. Fernandes et al. (2009) and Liew et al. (2012) observed that the biodegradability of lignocellulosic biomass increased with reducing the lignin content,

greater the lignin content lower the biomethane production. This study has reported the correlation between higher lignocellulosic biomass disintegration (i.e. lignin, cellulose, and hemicellulose) and higher methane production. Thus, electrohydrolysis pretreatment has proved to be an effective pretreatment method, which reduce the obstacles and generates multiple necessary effects (e.g. lignin removal, breaking of hemicellulose interlocking and increase cellulose amenity to microorganisms), so that RS can be efficiently utilized for accelerated methane production.

Table 6.4. Compositional changes and characterization of RS before and after pretreatment at 25 V for different time of exposures

Parameters	Voltage (V)	Time (min)	Acid soluble lignin (%)	Acid insoluble lignin (%)	Cellulose (%)	Hemicellulose (%)
Control	_	_	12.76 ± 0.32	1.77 ± 0.25	38.37 ± 0.25	28.73 ± 0.15
	25	15	12.13 ± 0.31	1.57 ± 0.15	39.65 ± 0.14	27.65 ± 0.21
	25	30	11.33 ± 0.21	1.27 ± 0.15	41.92 ± 0.2	26.34 ± 0.3
Electrohydrolysis	25	45	10.1 ± 0.30	0.93 ± 0.15	43.45 ± 0.18	24.76 ± 0.25
	25	60	8.03 ± 0.21	0.8 ± 0.1	45.17 ± 0.25	22.3 ± 0.15
	25	80	7.63 ± 0.21	0.67 ± 0.15	45.89 ± 0.3	21.8 ± 0.23

6.2.6 BMP study electrohydrolysis pretreated RS

6.2.6.1 Daily biogas/methane production

Fig. 6.10 shows the variation of sCOD, VFA, pH and daily methane production rate with digestion time, which collectively quantified biodegradability of RS during AD (BMP test) after electrohydrolysis pretreatment. Organic components present in soluble phase were decomposed to produce monosaccharides and VFA during initial steps (hydrolysis and acidogenesis) of AD. The conversion of cellulose to monosaccharides was attributed by the

increase in sCOD concentration within 14 days of digestion time as shown in Fig. 6.12 a). Cellulose conversion was intensified during early days of digestion period because digesters were composed of hydrolytic bacteria, to which organic sugars were readily accessible after electrohydrolysis pretreatment. At 14th day, reactor with F/M:2 showed maximum sCOD concentration of 10544.4 ± 120 mg/L followed by F/M:2.5, F/M:3, and F/M:1.5 showed 9817.2 ± 145 mg/L, 9453.6 ± 176 mg/L and 8726.4 ± 190 mg/L sCOD concentration respectively. Untreated sample attained maximum sCOD concentration at 21st day due to low accessibility of cellulose covered by lignin layer, which was removed in electrohydrolysis pretreatment to accelerate hydrolysis (rate limiting step) of substrate. Further, sCOD was decreased, when acidogenic proteobacteria became active and degraded soluble organic matter to VFA. The methane (or biogas) yield for an AD is commonly stated as a function of reduction in sCOD (Eskicioglu et al., 2006).

The VFA concentration along with pH, affects many aspects of AD, such as the microbial communities dominance, hydrolysis rate of substrate, biogas and methane yield and AD inhibitors (Ma et al., 2016). Fig. 6.10 b) shows that during first 14 days of anaerobic process, VFA concentration was increased for different F/M ratios due to availability of readily digestible substrates (breakdown products of hydrolytic bacteria) to acidogenic bacteria, which produced organic acids of low molecular weight. Maximum VFA formation was achieved by F/M:2 as 1406.25 ± 36 mg/L after electrohydrolysis pretreatment, whereas untreated sample has minimum VFA concentration as 843.75 ± 24 mg/L at the end of 14 days of mesophilic digestion. Fig. 6.10 c) confirms VFA formation, which leads to drop in pH values for F/M ratios (0.5, 1.5, 2, 2.5 and 3). pH value of 6.4 was recorded as minimum for F/M:2 and F/M:3. Former steps of AD suggested decrease in the VFA concentration because acetogenic bacteria convert VFA into acetate, H_2 and CO_2, which are finally used by the methanogens. In methanogenesis step, the intermediates formed in acetogenesis step were converted into methane by methanogenic microorganisms. In the maximum yield stage of BMP test, a syntrophic relationship was established among acidogens, acetogens and methanogens microorganisms which maintained pH of reactor in neutral range for various F/M ratios as shown in Fig. 6.10 c). Fig. 6.10 d) present observed daily methane production from reactors digesting pretreated/untreated RS samples.

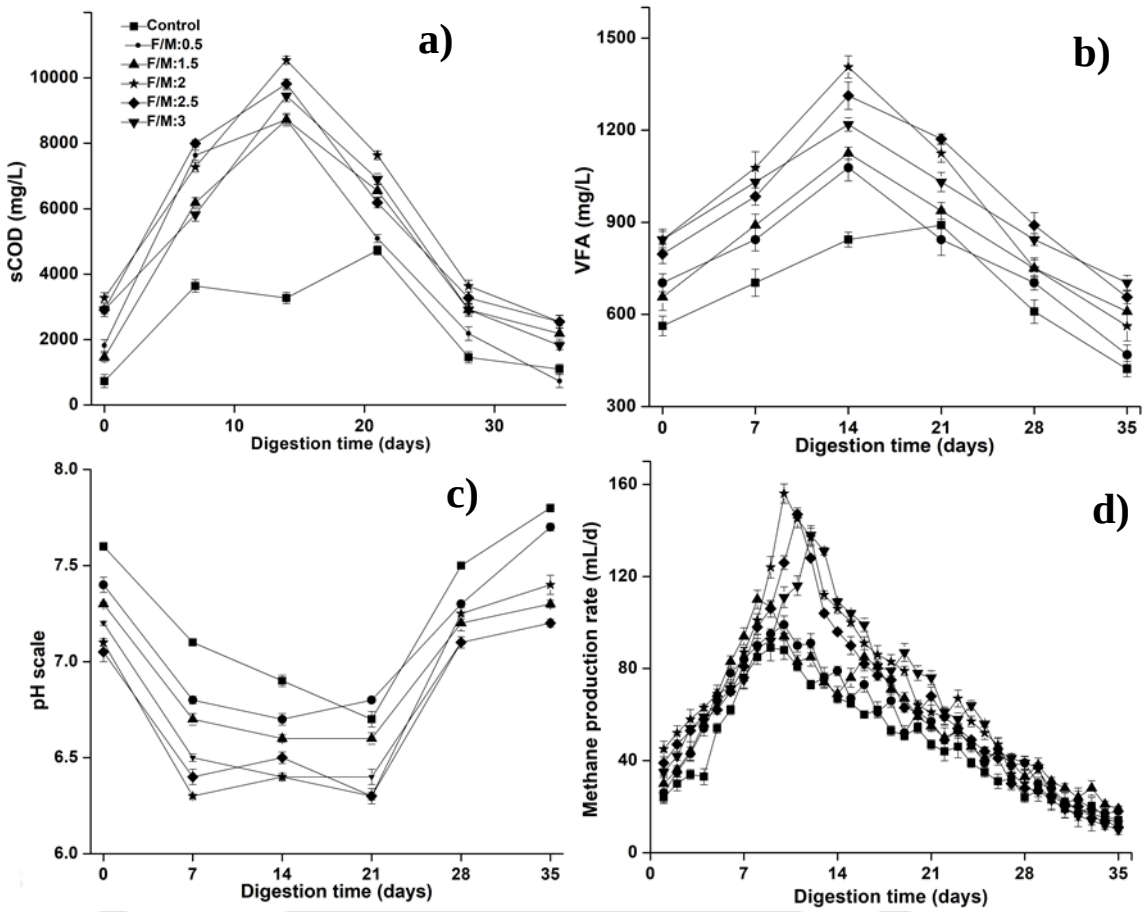


Fig. 6.10. Influence of different F/M ratios on a) sCOD b) VFA c) pH and d) daily methane rate

Increase in substrate utilization rate was attributed by increase in methane production rate for different F/M ratios upto 14 days of digestion period. Gradually decreasing trend was followed by methane production, when maximum amount of substrate was utilized by anaerobic microorganisms. In this study, maximum daily methane production was obtained for F/M:2 as 156 ± 4.2 mL/g-VS_{added} at 10^{th} day followed by F/M:2.5 as 147 ± 2.8 mL/g-VS_{added} at 11^{th} day, F/M:3 as 138 ± 4.1 mL/g-VS_{added} at 12^{th} day, F/M:1.5 as 110 ± 4 mL/g-VS_{added} at 8^{th} day, and F/M:0.5 as 99 ± 3.9 mL/g-VS_{added} at 10^{th} day. The BMP test suggested, F/M:2 produced maximum specific methane yield of 319 mL/g-VS_{added}, which was 42.4% greater than untreated sample. These increases in daily methane generation rate were due to the breakdown of organic matter during electrohydrolysis pretreatment, and made RS a more bioavailable substrate for anaerobic microorganisms and reducing the hydrolysis step duration during AD.

6.2.6.2 Kinetic modeling

The estimated parameters indicated the delayed response of microbial flux to alter the environment, and its consequent adaptation in the system.

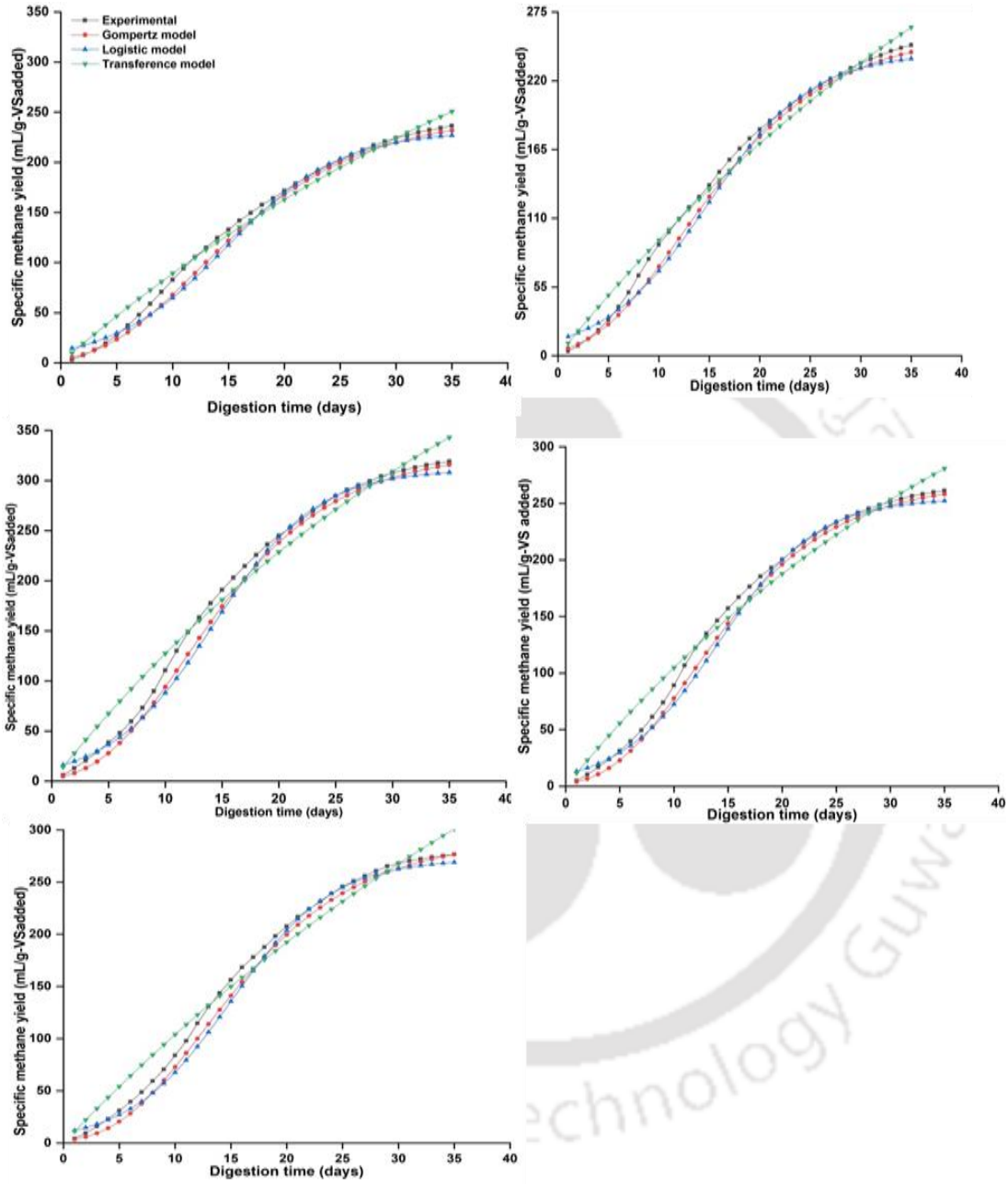


Fig. 6.11. Model fitting for accumulative methane yield by electrohydrolysis pretreatment for a) F/M:0.5 b) F/M:1.5 c) F/M:2 d) F/M:2.5 and e) F/M:3

Here, all parameters i.e. λ, R_m, M and Z, expressed the similar trend, it decreased initially and then increased (Fig. 6.11). The value of λ varies from 0-4.93 days and based on this value organic conversion tendency can be predicted. Greater λ, indicates the shorter

degradation rate and irretrievable process inhibition. This improvement in the process could be explained by the enhanced buffer capacity and enzyme activities. To further predict the soundness of the model, the expected values were plotted against the experimental results and R^2 values for different F/M ratios were found between 0.9078-0.9986. Similar results were also conveyed in other studies, in which different mathematical models were used for predicting the cumulative methane production (Li et al., 2012; Zhu et al., 2014; Veluchamy and Kalamdhad, 2017b). Results of this study indicated the positive correlation ($p < 0.05$) between experimental values and predicted values. The experimental values were similar to predicted value for MGM and LFM, whereas for TFM overestimation of the methane production was observed ($p>0.05$). The highest accumulative methane production is obtained for F/M:2 followed by F/M:3. Therefore, considering the kinetic study results we can conclude that examination of the parameters is essential in terms of monitoring and operation of AD process and performance of reactors.

6.2.7 Batch study

Conducted batch study was similar to BMP test (1 L), but it was scaled up to capacity of 20 L for digestion period of 60 days. Each such reactor was fed with DCD (inoculum) and pretreated RS (substrate) in best F/M ratio obtained from hydrolysis study as F/M:2.5 and F/M:2 in BMP study of microwave and electrohydrolysis pretreated RS, respectively on the basis of governing parameters like pH, VFA, sCOD, VS and maximum methane yield. Fig. 6.12 a) showed daily methane production rate of microwave and electrohydrolysis pretreated RS. Microwave pretreated and electrohydrolysis pretreated RS produced highest methane of 1.71 L and 1.66 L at the 17^{th} day. Microwave pretreated sample has higher peak than electrohydrolysis pretreated sample, whereas untreated RS produced methane of 0.9 L at 17^{th} day. Fig. 6.12 b) showed specific methane yield of microwave pretreated RS was higher than electrohydrolysis pretreated and untreated RS. Cumulative methane yield of microwave pretreated RS was predicted as 367.71 mL/g-VS_{added} followed by electrohydrolysis pretreated and untreated RS with 319.03 and 207 mL/g-VS_{added}, respectively during digestion period of 60 days.

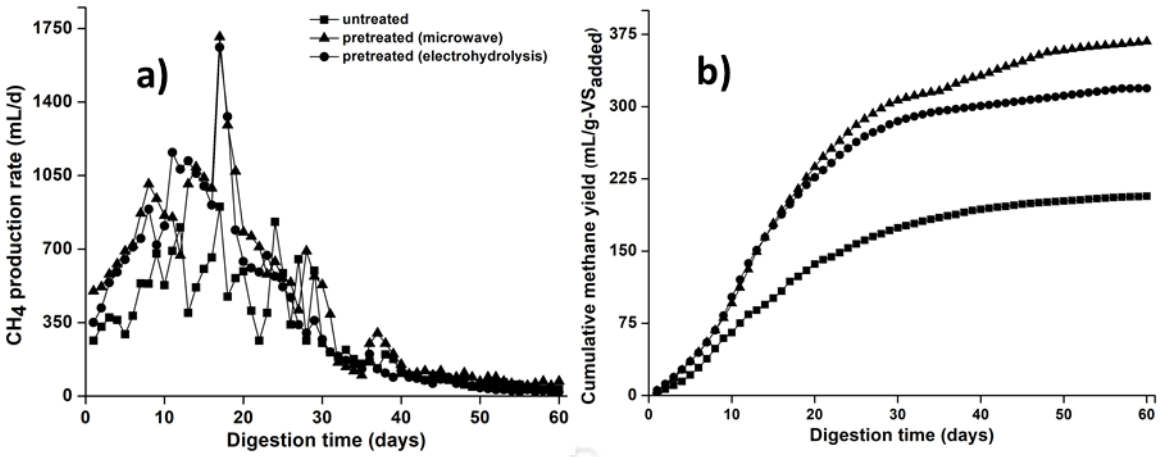


Fig. 6.12. a) Cumulative methane yield and b) daily methane yield for different C/N ratios

6.3 FUNGAL PRETREATMENT

White rot fungi are vital for successful biological pretreatment due to its high affinity of lignin degradation over cellulose. However, the cell wall deconstruction pattern by white rot fungi varies amid species and strains. Most white rot fungi (e.g. *Ceriporiopsis subvermispora, Trametes versicolor* etc.), simultaneously decompose lignin and holocellulose (cellulose and hemicellulose) that results in low cellulose recovery, while some species preferentially disintegrate lignin and part of hemicellulose, thereby leaving cellulose-rich residue. Some of the most efficient wood-decaying fungi are *Pleurotus ostreatus (PO), Phanerochaete chrysosposrium (PC), T. versicolor, Irpex lacteus (I. lacteus), Ganoderma lucidum (GL) Aspergillus niger (A. Niger),* etc., which selectively disintegrates lignin with very high cellulose residue (Pothiraj et al., 2006; Dashtban et al., 2010). Successful application of *Trichoderma reesei (T. reesei)* and *Pleurotus ostreatus* (PO) in improving the methane yield has been studied, but the measured methane yield (263 and 214 mL/g-VS_{added}) was very low (Mustafa et al., 2016). Mustafa et al. (2016) used the combination of milling and fungal (*Pleurotus ostreatus*) pretreatment to improve the degradability of RS and found a significant energy savings with a methane yield of 258 mL/g-VS_{added}. *A. Niger* and *Phanerochaete chrysosposrium* were used for increasing the delignification of RS, and results showed an increase of 15.01%. It also increases the susceptibility of the substrate for microbial degradation and enzyme attack (Chen et al., 2018). A mixed culture of GL and *Chlorella vulgaris* (C. vulgaris) were also used for upgrading the biogas production from digester slurry and maximum removal efficiency (COD) of 78.09% was observed. This study also enhanced the economic efficiency of the

biogas upgrading process (Cao et al., 2017). However, there have been very few reports studying RS subjected to microbial pretreatment by GL and PC. This study entails the application of these different white rot fungi (i.e., PO, PC) to solubilise RS with cellulolytic enzymes synthesized by fungi, and an attempt to appraise the BMP test of pretreated RS.

6.3.1 Effect on degree of solubilization

The white rot fungi have the ability of breaking down the lignocellulosic matrix and discharging the lower-metabolized components. The extracellular lignin-modifying enzymes (LME), mediators, and other compounds secreted by white rot fungi are responsible for the degradation of RS. LME secreted by white rot fungi are very oxidative and includes singlet oxygen radicals, which disrupts the lignocellulosic structure by demethylation, propyl cleavage and oxidation reactions mechanism. The effect of fungal pretreatment on solublization of RS was quantified by measuring different parameters (i.e. VFA, sCOD, VS degradation, and change in pH of the solid digestate). Different panels in Fig. 6.13 show the change in all stated parameters with pretreatment time. The degradation compounds obtained in pretreatment are successively transformed into VFA, which are degraded to acetate; this acetate is finally converted into biogas. VFA conserve all chemical energy of the substrate for anaerobic fermentation. The VFA profiles of pretreated (PC, PO, GL) and untreated RS (control) showed an increasing trend in incubation time for all three fungi. The VFA yield of PC pretreated fungi was approximately 4.44-fold higher than the control on the 7^{th} day of the hydrolysis period (Fig. 6.13 a)), whereas PO and GL showed an increase of 2.66 and 1.73-fold with respect to an untreated RS sample on the 7^{th} day. The maximum VFA production for PC (2991 mg/L) and GL (1984.5 mg/L) was obtained in week 4; after that it showed a decrease in VFA value. PO showed the maximum VFA yield on week 3, which was 8.6% less than the PC on the same pretreatment time. The high concentration of VFA revealed that pretreatment of RS is suitable for downstream process enhancement. Reduction in VS% (Fig. 6.13 d)) is part of the secondary metabolism of white rot fungi; it degrades RS co-metabolically for an essential carbon source requirement. The high C/N ratio of RS (Table 5.1) mimics the high production of LME by the subjected fungal strains and these oxidative enzymes have the ability to diffuse into the cell wall of lignin structure. Therefore, the LME production is directly proportional to the degradation of lignocellulosic substrate. Lignin peroxides (LiP), a hemeprotein secreted by the PC is responsible for the cleavage of non-phenolic bonds present in lignin. This was quite evident from the 27% decrease in VS degradation (an 11-fold increase in VFA) of RS in pretreatment. Manganese peroxides (MnP) is the other enzyme, which is also secreted by

PC; MnP catalyzes the degradation by oxidative cleavage of recalcitrant bonds in RS (Kuijk et al., 2015). PO and GL showed a VS degradation of 17.2% (7.2-fold increase in VFA) and 11.61% (5.7-fold increase in VFA), respectively. Hydrolytic enzymes easily support the degradation of RS as a carbon source for the fungal growth, which accelerates the growth rate and pretreatment. After week 4, percentage change in VS degradation was very less (0.94 ± 0.19%); this showed the utilization of an available carbon source as a food for fungal strains. The radical rise in the sCOD profile (Fig. 6.13 c)), showed a high rate of solublization of RS or the fungal strains.

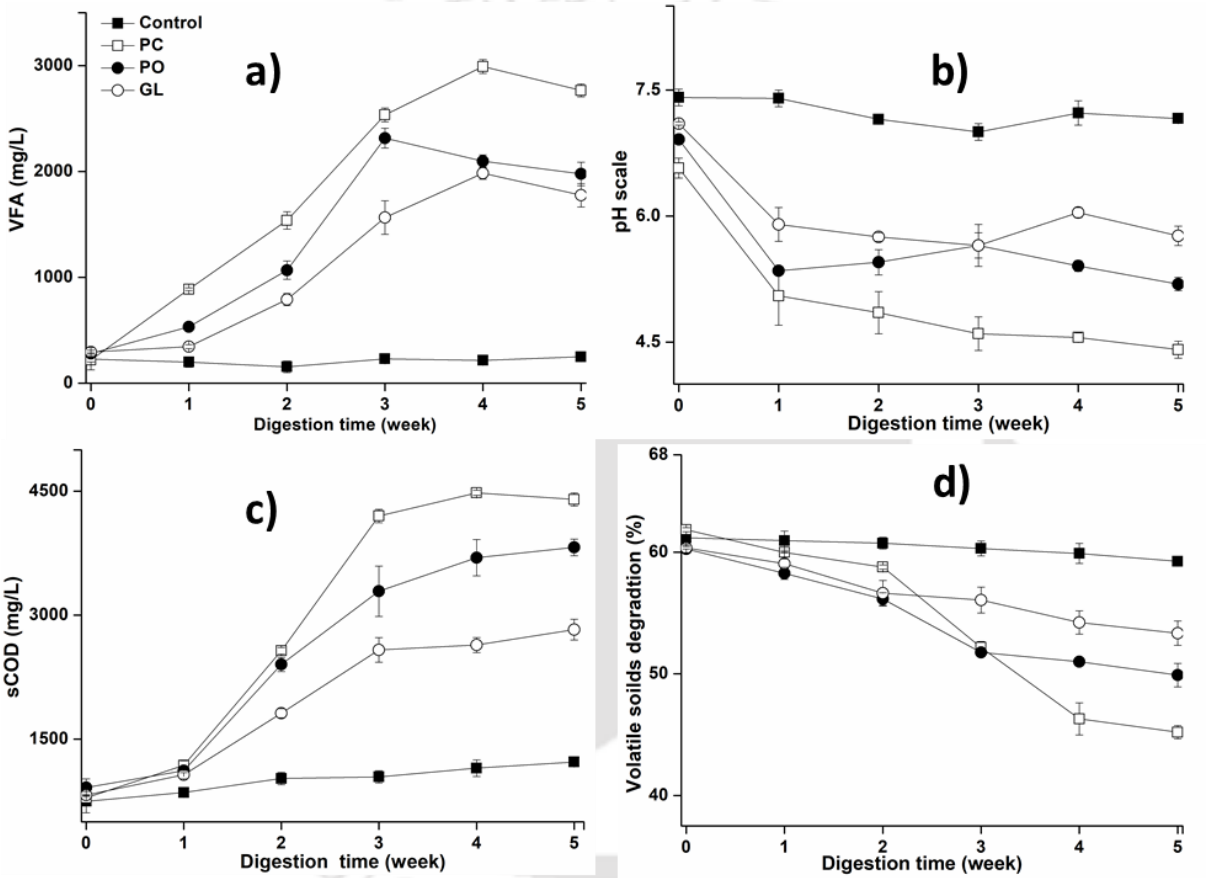


Fig. 6.13. Effect of fungal pretreatment on parameters a) VFA b) pH c) sCOD and d) VS% degradation

The rupture of the lignocellulosic cell wall showed the release of organic micro-molecules in the soluble phase. Therefore, the readily-available organic matter accelerates the biogas production from RS (Mustafa et al., 2016). pH is one of the vital parameters having a considerable impact on the growth of fungi and generation of non-specific extracellular enzymes (Arantes et al., 2007; Ku, 2015). The variation in the pH during

fungal pretreatment of the substrate is shown in the Fig. 6.13 b). In the present study, the initial pH of the substrate ranged from 6.5-7.5, which decreased drastically by approximately 1.5 unit during the first week. Subsequently, a slight fluctuation in the pH was observed for GL, which later stabilised to 5.5. The pH variation in this study was comparable to an earlier study of Fang et al. (2018), in which *Pleurotus sajor caju* and *T. Versicolor* were grown on the solid digestate for three weeks, and a decrease in pH was observed from 4.75-5.5. The formation of VFA (Fig 6.13 a)) from the delignification and hemicellulose degradation by white-rot fungi can be attributed to the drop in pH. The degradation of spruce wood lignin by PC was investigated by Chen et al. (1983), testifying that aromatic carboxylic acids comprised greater than 35% of the low molecular weight products. Thus, the variation in pH suitably showed the degradation of RS, by the action of extracellular enzymes secreted by white rot fungi.

6.3.2 Microstructure analysis

6.3.2.1 FESEM

The structural characteristics of untreated and pretreated substrates were shown by FESEM analysis. Despite the integrity maintained by the surface of a raw substrate, a condensed structure with small holes in the surface of raw substrate was observed that could have been the result of the autoclaving process. To some extent, a rough and cracked surface was observed during the autoclave process. A significant amount of mycelia penetration (GL) in the lignocellulosic biomass of solid substrate substantially damaged the surface of solid digestate (Fig. 6.14). Presumably, the structure of solid digestate was more efficiently dented through delignification by PC that led to an increased surface area due to an increase in the pore size of the substrate. During pretreatment, fungi have the ability of increasing the surface area and pore sizes of the substrate by penetration into its structure. The pretreatment by PO also exposed the secondary rigid walls for microbial accessibility to the cellulose, thereby improving the AD process. Nonetheless, the damage to the structure of solid digestate by GL was inferior to PC, PO as indicated (Fig. 6.14) and not enough surface area was exposed for anaerobic microbes, which probably is the rationale for lower VFA production by GL (Fig. 6.13 a)).

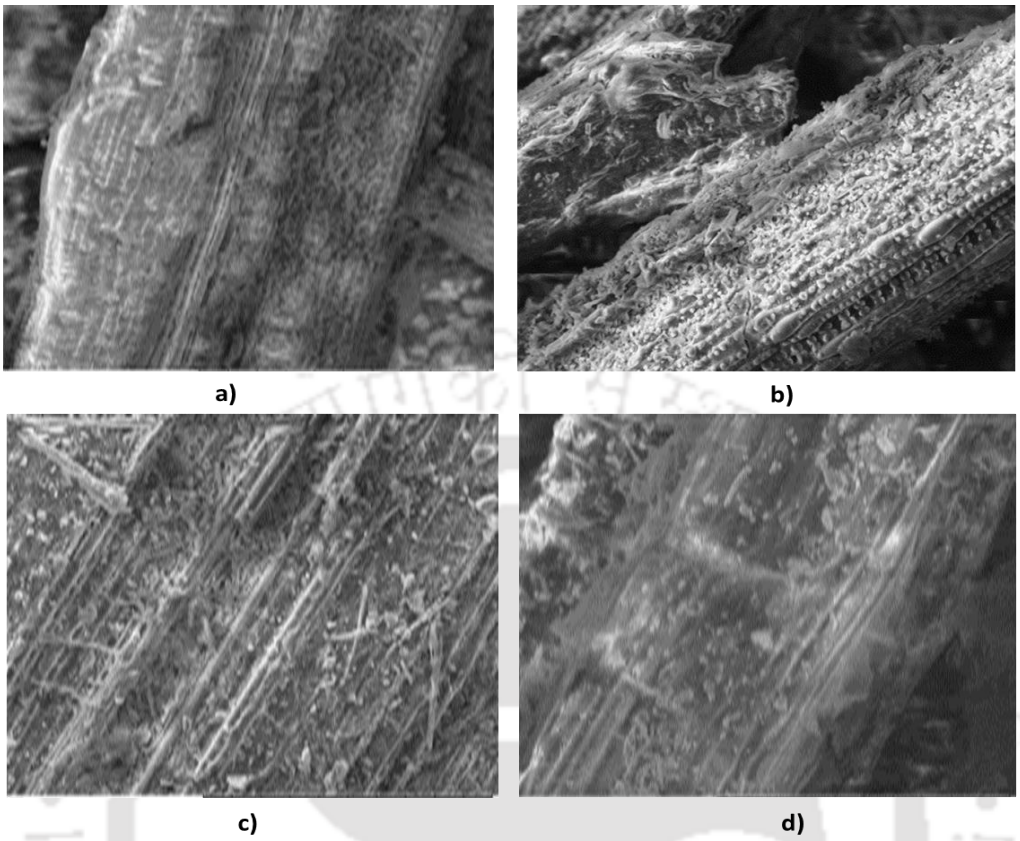


Fig. 6.14. FESEM micrographs a) Untreated rice straw, Pretreated b) GL c) PC d) PO

6.3.2.2 FTIR

As represented (Fig. 6.15) after five weeks of inoculation, the intensity of peaks at about 2922 cm^{-1} relating to the C-H stretch in methyl and methylene groups in lignin were reduced with PC. Significant changes in the peaks at about 1300 cm^{-1} relating to the C-O-C groups in holocellulose were observed for PC, PO, GL. As indicated by the results in the present study, the selective degradation of substrate during pretreatment implies the higher selectivity of PC. Moreover, selective lignin degradation using PC resulted in the disentangling of the cellulosic fibres, thereby enhancing the accessibility for the hydrolytic enzymes to the substrate during AD. These results observed were, in general, positive reinforcement for the enhancement of VFA production significantly from the fungal pretreated RS. From visual observation, it was revealed that peak at 1651 cm^{-1} (1700-1500 cm^{-1}, allocated to protein), was very protruding. The occurrence of peak at 1651 cm^{-1} was because of protein, which was formed by PC, PO, GL, during their metabolic degradation of RS. Wavenumber 1021 cm^{-1}, assigned to C-O, showed the higher vibration in PC, PO, and

GL, which was due to degradation of carbohydrate to lower molecules. The same FTIR pattern was also observed by Cornet et al. (2018) during pretreatment of poplar wood.

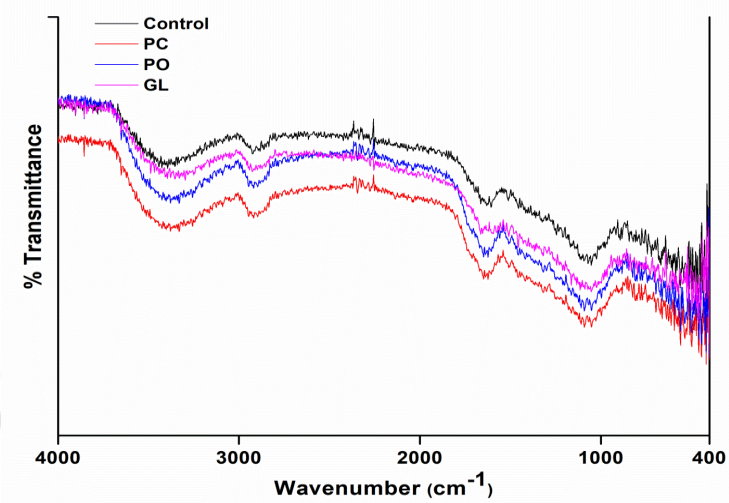


Fig. 6.15. FTIR spectra of RS in untreated (control) and pretreated (PC, PO, GL) conditions

6.3.3 Compositional analysis

The growth of fungi goes together with the decomposition of organic matter. Consequently, the investigation of the impact of fungal pretreatment on the chemical characteristics of substrate is displayed in Fig. 6.16. The substrate composition was significantly impacted by the autoclave process. A decrease in the holocellulose and lignin content of dry solid digestate from 4.32 ± 0.3% to 4.26 ± 0.23% after autoclaving indicated the removal or dissolution of some ash fractions in the substrate. Pretreatment by PC witnessed a lignin content of 8.9%, which was 36% and 38.74% less than autoclaved (control) and raw RS after five weeks of inoculation. Moreover, pretreatment of solid digestate by GL and PO resulted in the decline of lignin content to only 17.81% and 21.85%, respectively. Hence, it implies that PC had a robust lignin degradation efficiency compared to PO and GL. The breakage of the lignin-carbohydrate complex, especially by PC, could result in the increased activity of anaerobic microbes to the holocellulose component of solid digestate (Shirkavand et al., 2016). An appropriate parameter to appraise the biodegradability of solid digestate is the cellulose/lignin ratio. In this study, with pretreatment of the substrate by PC, the highest cellulose/lignin ratio of 4.84 was witnessed. As indicated by the results in the present study, the selective degradation of lignin over cellulose during pretreatment by PC presumably implies the higher selectivity of PC. The selective lignin degradation using PC resulted in the disentangling of the cellulosic

fibres, thereby enhancing the accessibility for the hydrolytic enzymes. Cellulose fraction of the RS pretreated by PC was increased to almost 18% with respect to the untreated RS sample. This enhancement in cellulose content could be due to the higher selectivity (ratio of lignin-to-cellulose degradation) of PC. Furthermore, the low cellulose/lignin ratio of substrate pretreated by GL (3.3) made this obvious that the simultaneous degradation of cellulose, along with lignin, resulted in the lowest recovery of cellulose. Conversely, lower selectivity by GL and PO in comparison with PC resulted in less increase in the methane yield of RS, as explained in section 6.3.4. From visual evaluation, RS was colonized by the all three fungi within week of inoculation. The total lignocellulosic fraction obtained was 78.11 ± 2.86, 73.27 ± 2.13, 73.42 ± 2.76 and 72.65 ± 2.46%, after recovery from pretreatment with control, PC, PO and GL, respectively. And ash fraction obtained from pretreatment was 19.85 ± 0.23, 25.85 ± 1.1, 26.23 ± 0.43 and 25.43 ± 0.36 from control, PC, PO and GL respectively. Some loss in fraction (1.54 ± 0.71) was accredited to the extraction and autoclaving process. The incubation of 5 weeks can be reduced in the further research by selecting the optimized strain of fungus from this study on wet storage of RS collected from the field. As from the study conducted by Mustafa et al. (2016), it is known that short-interval steaming in open atmosphere was sufficient for effective fungal growth on a large scale.

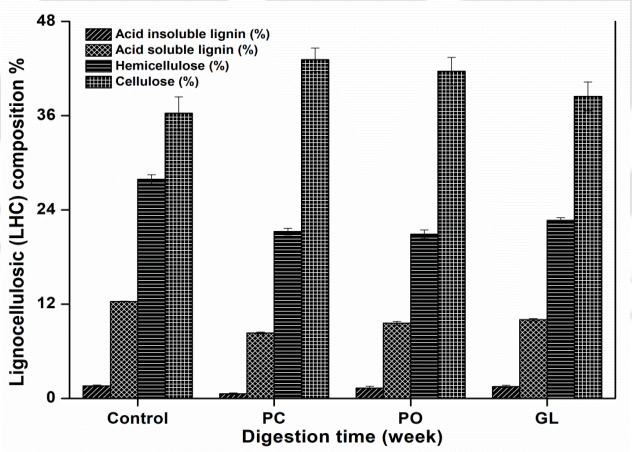


Fig. 6.16. Lignocellulosic degradation of RS before and after fungal pretreatment

6.3.4 Daily biogas/methane production

The impact of fungal pretreatment (PC, PO and GL) on the methane yield of RS was evaluated, as pretreatment increased the VFA production and degradability of RS for further microbial attacks in AD (Fig. 6.17). Methane production was observed for 35 days; after

that the yield from the entire reactor was negligible. Methane yield increased with time, and reached a plateau after obtaining maximum value. The methane yield obtained in this study was 339.31, 295.91, 269.99, 102.44 and 60.47 mL/g-VS_{added} for PC, GL, PO, C1, and C2, respectively (Fig. 6.19 a)). The pretreatment with PC resulted in a maximum yield with 2.22-fold higher than C2 (untreated RS). The GL and PO resulted in 1.88-fold, 1.64-fold higher than C2, respectively. The enhancement in the methane yield was because of cellular degradation and efficient release of soluble high-molecular compounds into low-molecular compounds for acidogens. The conferring supports (lignin) of RS microfibrils are disrupted by extracellular enzymes secreted by PC, PO and GL. As this process consumes less energy, it is considered to be most sustainable among all pretreatment processes. The maximum daily methane yields for PC and GC were obtained on the 13th (147.38 mL) and 14th (143.06 mL) days, respectively, whereas for PO, maximum daily yield was obtained on the 8th (121.63 mL) day (Fig. 6.19 b)). The untreated sample of RS showed the maximum yield on the 21st day. Therefore, pretreatment of RS reduced the retention time for maximum yield. The batch study of pretreated RS with PC showed that around 65% of the total yield was obtained on the 15th day, whereas, for PO and GL 70% and 60% yields were obtained on the same day. Therefore in order to shorten the total digestion process (increase the volumetric efficiency), PO seemed to be a suitable strain for pretreatment and achieving the higher-yield PC is suitable. Mustafa et al. (2016) in their study used the *T. reesei* and PO to enhance methane production from RS and obtained a biogas yield (approximately 42.5-72.5% methane yield) of 129-299 mL/g-VS_{added} and 234-367 mL/g-VS_{added} (20 day inoculation, 75% moisture), respectively, whereas the methane yield obtained in this study was about 50-60% higher than the previous study.

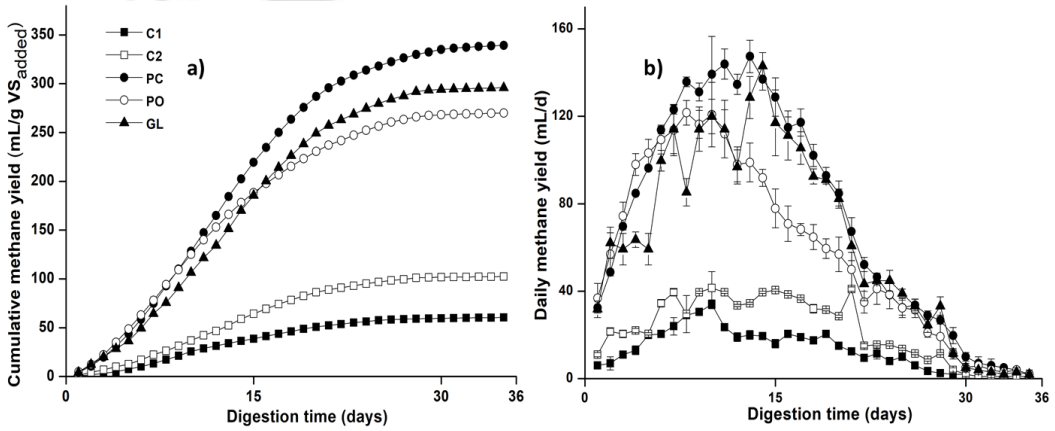


Fig. 6.17. a) Cumulative methane yield b) daily methane yield for untreated and pretreated RS

6.3.5 Kinetic modeling

Results obtained from fungal pretreatment of RS, followed by the BMP assay experiment and model simulation. The various parameters (λ, M, R_m), obtained in the kinetic analysis are expressed in Table 6.6. As per Kivak (2014), it was clear that the % error between the experimental and predicted values should be less than 25% for best fitting and analysing the results; it was observed that performance followed the trend MGM>LFM>TFM for C1, PC and PO, whereas C2, GL followed the trend for LFM>MGM>TFM for data of cumulative methane yield (mL/g-VS_{added}).

Table 6.5. Parameters and goodness of fit obtained from the evaluated model

Sample	Model used	M	R_m	λ (d)	Exp. data (L)	Q (L)	Δ
C1	MGM	62.36	3.53	3.23	60.47	61.09	3.13
	LFM	60.11	3.55	3.75		59.83	-0.60
	TFM	76.74	4.28	2.34		64.31	26.91
C2	MGM	107.43	5.85	3.60	102.44	104.67	4.87
	LFM	102.82	6.03	4.31		102.26	0.37
	TFM	139.21	6.71	2.33		110.38	35.89
PC	MGM	350.24	19.86	3.45	339.30	342.95	3.22
	LFM	336.68	20.30	4.09		335.25	-0.77
	TFM	438.37	23.41	2.35		361.68	29.20
PO	MGM	276.81	15.64	2.14	269.99	272.01	2.53
	LFM	267.70	15.55	2.54		266.65	-0.85
	TFM	321.11	20.90	1.87		283.95	18.93
GL	MGM	310.46	16.91	3.59	295.91	302.49	4.92
	LFM	297.15	17.43	4.31		295.52	0.42
	TFM	402.23	19.40	2.33		318.99	35.93

The maximum predicted value of M (mL/g-VS_{added}) from the model was obtained for PC, which was 438.37 mL/g-VS_{added} (TFM), approximately 30% (Δ) higher than the experimental value as shown in Fig. 4, and the highest and lowest Δ value was obtained for GL (TFM) and C2 (LFM) respectively. The lag phase constant, λ defines the time needed for bacteria to acclimatize in the system. The value of λ for this experiment varies from 2.33-4.31 days. The λ value proved that for PO pretreated batch reactor showed the lowest time (1.87) for acclimatization, which was also clear from the daily methane yield obtained by PC, PO and GL pretreated RS on 8^{th} day of the daily methane rate (Fig 6.17 b)).

In this study, the kinetic analysis by MGM fitted the predicted results with more accuracy in comparison with TFM and LFM. MGM followed the sigmoidal growth curve, with specific growth rate of methanogens proportional to the biodegradation of substrate and increase in in cumulative methane yield. Similar results were also Parameswaran and Rittmann (2012) and Huiliñir et al. (2014). Parameswaran and Rittmann (2012) revealed that MGM fit the batch methanogenic experimental data by Newtonian algorithm regression and hydrolysis constant followed the similar trend as lag constant. Huiliñir et al. (2014) also obtained that MGM best fitted the experimental results for biodegradation of paper and pulp sludge with the addition of natural zeolite (0.2-1 g/L).

6.4 CONCLUSION

In thermal pretreatment study, microwave pretreatment was obtained as best pretreatment method for RS with operating conditions (@190°C for 4 min exposure time) to accelerate hydrolysis step (367.68 mL/g-VS_{added}). Microstructure observations (FESEM and FTIR) of RS after thermal pretreatment also proposed microwave pretreatment as best method to destruct recalcitrant matrix of RS. Electrohydrolysis pretreatment study suggested 25 V DC voltage for exposure time of 60 min as best operating conditions to hydrolyze RS (mainly intracellular polymers) into soluble phase with methane yield of 319.03 mL/g-VS_{added}. Fungal pretreatment with PC showed a significant degradation of rice straw, and resulted in a 2.22-fold increase in methane yield (339.31 mL/g-VS_{added}). FESEM micrographs and FTIR spectra, also recommended complete destruction of lignin to release soluble sugars from RS after microwave, electrohydrolysis and fungal pretreatment performed at optimum conditions.

CHAPTER 7

DESIGN AND OPERATION OF A CONTINOUS ANAEROBIC REACTOR

This chapter deals with the operation of SPCAR in three different feed conditions. For initial days, reactor was fed with untreated RS and then microwave pretreated RS followed by the co-digested RS with FW.

7.1 ANAEROBIC REACTOR DESIGN

It is estimated that the global energy demand, irrespective of the compounding factors like climate change, urbanization, industrialization and increasing affluence, will increase by 2030, based on the forecasted population surge. Apart from the scarcity of fossil fuels, other disadvantages associated with the usage of fossil fuels, such as global warming due to greenhouse gases emission, pollution, resource depletion, geopolitics due to concentration of fossil fuels in some countries and unbalanced demand-supply relations, have led to increasing need for the search for alternate source of energy to satisfy the global energy demand (Report, 2018). Global crop production (e.g. rice) has clearly been on an increasing trend in the last decade. According to the Food and Agriculture Organization of the United Nations, a total of 679 million tons of crops were produced in 2014, which equates to approximately 916 million tons of residue. However, only 47% of the residues were used for cooking and heating purposes. This should definitely be appealing to both developed and developing countries (Cheng et al., 2014; Tian et al., 2018).

On a household scale, agricultural residues are just a biomass remains on the field after the crops have been harvested; which loses its moisture quickly (approximately 3-4 days) in appropriate weather conditions. Once the M.C. is below 25% it can be collected and baled that gives flat fibre (Ghaffar and Fan, 2013; Lindmark et al., 2014; Peces et al., 2015) with approximate dimensions of 0.5 cm in width and 20–60 cm in length .There are two processes to convert the waste biomass into fuel: Biological processes involve utilizing bacteria either through AD of organic matter generating methane or through saccharification and fermentation of sugars. When compared with other bioconversion and thermo-chemical conversion processes, AD process (energy output: input is 28:1) is more efficient process for energy generation (Frigon and Guiot, 2010). Since methane has approximately 20 times more global warming potential vis-a-vis CO_2, it is an added advantage to utilize the methane for energy production through AD instead of natural

methane emissions from the landfills or any other open environments. This technology can accommodate either wet or dry feedstock economically on both small and large scales. Further, as a by-product the anaerobic composting of rice straw, also known as methanization, gives fertilizer. It is considered to be one of the most environmentally friendly processes for converting biomass into renewable energy; however, its complex, lignocellulosic structure makes it difficult to decompose (Mussoline et al., 2013; Shitophyta and Fuadi, 2016). On the other hand thermochemical treatment processes, that also have higher energy input requirements, includes processes like pyrolysis, combustion and gasification. However, a few noteworthy concerns exist such as process instability and lesser biogas yield because of higher solid content and decreased biodegradability of the rice straw due to hydrophobic and cross-linking properties of the lignin. The effectiveness of the microbial action is inhibited by the percentage of lignin in substrate; which mainly governs the degradation capacity of substrate. These concerns can be dealt with by the usage of appropriate anaerobic digester. Shape of the anaerobic digester plays significant role and, among the three shapes of bio-digesters, cylindrical shaped anaerobic digester resulted in maximum biogas production vis-à-vis cubical and conical bio-digesters (Oloko-Oba et al., 2018). An anaerobic bio-digester should be simple, stable, efficient and economical. Mainly, there are two types of anaerobic bio-digesters; single-phase and two-phase bio-digesters.

The single phase reactor is a conventional reactor, which worked on low OLR and has the acidification and methanogenic reactor in single unit. The two phase reactor was capable of showing high VS degradation due to benefits of high buffering capacity, agreeing to constant feeding rate. The two processes was proficient in high performance, but material and energy balance showed that single phase was superior than two phase reactor. Intrinsic operational procedure showed that two phase reactor has lower hydrolysis efficiency and results in lower availability of organics for methanogenesis. Single phase processes are more economical in terms of energy yields as a low solid liquid separation occurs during the operation.

7.2 ACCLIMATIZATION OF SINGLE PHASE CONTINUOUS ANAEROBIC REACTOR

Microbial acclimatization is a multifaceted process, involving diverse bacterial consortium, and their physico-chemical and microbiological dependent interactions. Several aspects contribute in the process of acclimatization, considering DCD as seed materials, the VS% cannot be regarded as the ration for viability of microbial mass, as most of the content

is in recalcitrant forms. Therefore variation in sCOD and methanogenic activity is taken as a measure of the process. As DCD have more homogenous characteristics and less wash out potential, reactor was stable within two weeks of inoculation. Reactor was fed with 2.5 kg-VS/m^3/d of OLR and it found fluctuations in VFA yield from 1435 to 4600 mg/L, which became constant at an OLR of 3.5 kg-VS/m^3/d with a maximum sCOD of 7350 mg/L. Due to availability of large microbial flux in DCD, even a small retention time in the reactor permits the methanogenic bacteria to proliferate. During initial feeding pH was observed as 6.87 ± 0.76, which increased the physical characteristics of the reactor. Increase in pH resulted in enhanced methanogenic activity of anaerobic bacteria, however, the difference was insignificant as compared to the unacclimatized biomass.

7.3 METHANE PRODUCTION PROFILE FROM SPCAR

The lab scale single phase continuous reactor of capacity (20 L) was operated at different specified OLR (optimized results of batch study) for 150 days in three stages. Stage I include feeding with untreated RS (3.5 kg-VS/m^3/d) and stage II includes feeding with microwave pretreated RS at OLR of 8.3 kg-VS/m^3/d and stage III includes the feeding of reactor at an OLR of 6.5 kg-VS/m^3/d. Fig. 7.1 showed daily methane production profile of SPCAR fed with untreated RS during first 30 days of digestion period. Initially methane production increased upto 14 days of digestion because of higher initial activity of fermentative and methanogenic bacteria in AD. The extent of biodegradability is directly proportional to rate of biomethanation.

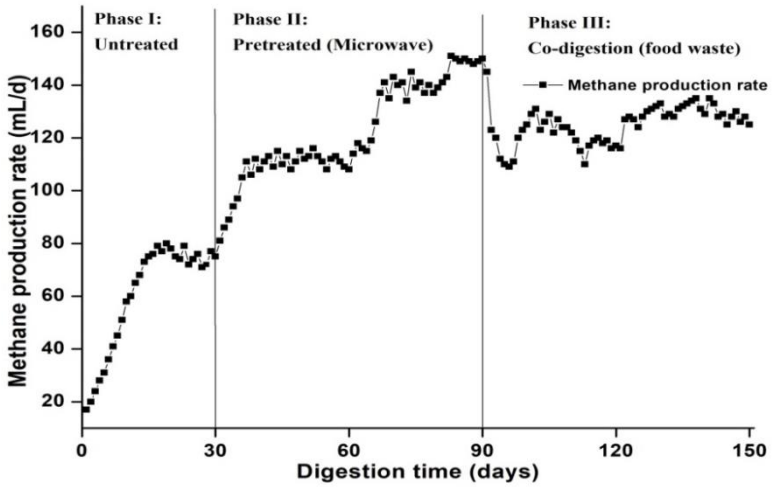


Fig. 7.1. Daily methane production rate for different feeding conditions

After 14 days, methane production became steady because anaerobic microorganisms felt resistance to access recalcitrant organic matter. In stage II, sudden increase in methane

production was observed because of readily digestible organics immediately accessed by the fermentative and methanogenic anaerobic bacteria, which was attributed by enhanced methane production as compared to stage I. During stage II, maximum methane production of 116 mL was obtained at 52nd day. During stage III for RS co-digested with FW, maximum daily methane was observed at 100-110th day with an average of 123 mL/d, after acclimatization of the stage.

7.4 pH, sCOD PROFILE AND VS% DEGRADATION

Fig. 7.2 showed the pH and sCOD variation during three stages operation of SPCAR fed with untreated RS in stage I and microwave pretreated RS in stage II and co-digested RS in stage III. In all stages, variation of pH lies within the acceptable limit (6.8-7.2) for AD as anaerobes are active in a very narrow pH range. To further elucidate the effect of change in OLR, pH profile of SPCAR was plotted, which showed steep profile during digestion period of 150 days. Low points in pH profile suggested formation of VFA, also drop in pH revealed the accumulation of VFA in the reactor, which is inhibitory for the AD process, while high points in pH profile suggested further improved degradation of VFA into organic acids (mainly acetic acid). Thus, pH was in neutral range throughout digestion period of 150 days because of syntrophic relation of fermentative bacteria and methanogenic bacteria. VFA formed during AD immediately used by acetogenic bacteria to convert them into acetic acid, which is finally converted into methane gas. sCOD increased during stage I of digestion because hydrolytic bacteria solubilized valuable component (cellulose) embedded in closed matrix. Decrease in sCOD attributed by degradation of soluble products into intermediates (low molecular VFA and organic acids). Increase in sCOD (7586 mg/L) suggested conversion of extracellular and intracellular biopolymers into soluble stage. Variation of sCOD during stage II showed well established synchronization among acidogenic, acetogenic and methanogenic bacteria. Organic matter was easily available for biodegradation by anaerobic microorganisms after microwave pretreatment. As for stage III, pH value increased to 7.34 and showed very less variation. Daily methane yield was mainly affected by the fluctuation in pH during stage III. Though, it was difficult to find the correlation between variations in pH in continuous mode, but pH was mostly in the optimum range due to obtained high buffering capacity of co-substrate. pH value was varied between 7-7.34, during stage III. sCOD of the effluent was also high, which showed the higher solublization during the degradation process. sCOD was 7767 mg/L, during the initial days, which decreased to 7474 mg/L and became constant after that. Along with that,

less fluctuation in VFA was also the indicator of the high hydrolytic efficiency of the reactor.

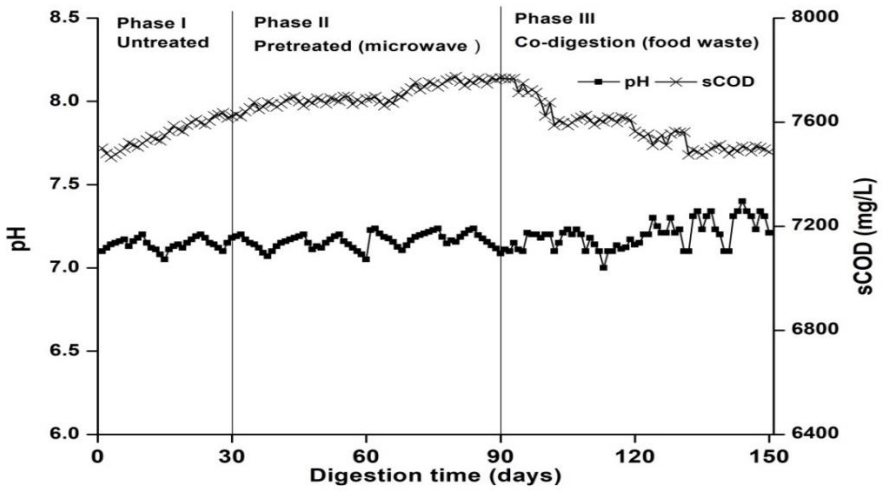


Fig. 7.2. Variation of pH and sCOD in SPCA

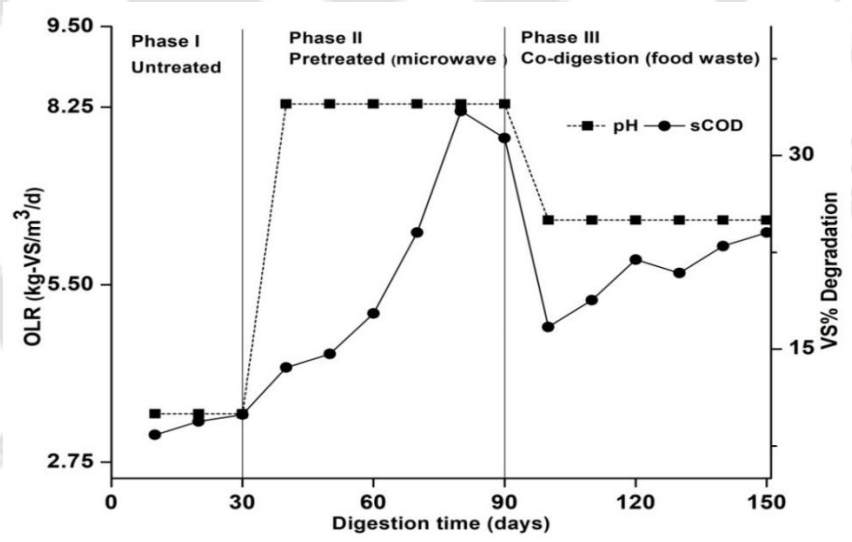


Fig. 7.3. Variation of VS% degradation with different OLR

Fig. 7.3 showed the varying of OLR with VS% degradation. VS% degradation of 33.43% was obtained at OLR of 8.3 kg-VS/m^3/d, for microwave pretreated RS in stage III, and in stage III, VS% degradation of 24.09% for an OLR of 6.5 kg-VS/m^3/d. As the OLR was increased the VS% degradation increased with improved enhancement techniques, as the operational condition was different in all the three stages, maximum value was obtained corresponding to more solubilized state of RS. Stage I, which consisted of untreated RS showed degradation of 9.92%, this low value was due to recalcitrance in the structure of RS.

As the optimum OLR is dependent upon the operating conditions and configuration of the reactor, therefore for SPCAR, optimum OLR of 8.3 kg-VS/m^3/d was obtained for microwave pretreatment conditions.

7.5 CONCLUSION

Challenges related to AD of RS are low biogas yield, poor buffering capacity, low quality end products and potential variability. These inadequacies are either because of low mass transfer (high solid contents, poor nutritional structure) or can be accredited to recalcitrant nature of the RS. Considering the characteristic of RS, AD in continuous state showed the stable performance for pretreatment and co-digestion of RS, i.e. maximum VS% degradation of 33.43% was obtained in stage II for pretreated RS at OLR of 8.3 kg-VS/m^3/d, in co-digested RS maximum VS% degradation was 24.02% obtained for an OLR of 6.5 kg-VS/m^3/d. Therefore, effective implementation of SPCAR can lead to the localized utilization of untapped potential of untreated RS.

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CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS

8.1 CONCLUSIONS

RS is a widely existing lignocellulosic waste with high potential for methane production. However, high C/N ratio of RS and recalcitrant nature causes the poor process stability, low methane yield and intensive digestion time. In inoculum study; the effects of inoculum DCD and FCD on AD and biogas production efficiency of RS were investigated using a BMP assay. The results evidenced that liquid digestate (DCD) is a more viable source for biogas production than FCD as an inoculum. This phase assessed the effects of different F/M ratios (0.25, 0.375, 0.5 and 0.75) on the degradation process with respect to continuous monitoring of pH, sCOD, VFA and VS% degradation. The highest methane production of 72.25 mL/g-VS_{added} with 24.47% of VS degradation at 48^{th} day was achieved for FCD (F/M:0.75), whereas, with DCD maximum of 125.77 mL/g-VS_{added} of methane production was achieved with 35.65% VS degradation (F/M:0.375). The taxonomic hit distribution at phylum level showed that DCD is enriched with majority of *Proteobacteria, Bacteroidetes, Firmicutes* and *Chloroflexi* in the start-up of the reactor, which was helpful for the AD of substrate. The addition of these phyla level comprises 77.4% of the total reads. These can easily survive in fermentative and obligate stage in the reactor. *Firmicutes* (20.3%) and *Chloroflexi* (6.8%), which have the ability of degrading macromolecules, were also found in large percentage. It may be concluded that addition of degradable organics may help to achieve degradation of lignocellulosic material but again an extensive study on pre-treatment and co-digestion technologies are needed due to low methane yield obtained from BMP.

In order to study the effect of AcoD of RS and co-substrates (*H.verticillata* and FW), two sets of experimental BMP studies were performed with various initial input parameters (C/N, F/M and pH) with level obtained from first set of experiments, which exhibits the necessity of co-digestion to improve physico-chemical and biochemical progression compared to mono-digestion. The additions of nitrogen-rich co-substrates balanced the unstable C/N ratio of RS and further enhanced the methane yield. The individual effect of C/N ratio, F/M ratio and pH, in addition to their interaction effects on methane yield (mL/g-VS_{added}) were explored. The results of co-digestion with *H.verticillata* suggested that there was substantial interaction between the selected input parameters and methane yield. For maximum methane yield the optimal conditions were C/N ratio 29.18, F/M ratio 2.45 and

pH 7.37. It reduced the inhibitory effect due to ammonia inhibition and VFA accumulation and balanced the improper nutritional structure (high C/N) of RS. Model validation proved the high adequacy of the model and methane yield is good output response variable for co-digestion study and it is necessary to optimize the transient variation in C/N ratio, F/M ratio and pH.

Co-digestion of RS with food waste (low C/N) balanced the C/N ratio in the AD leading to high methane yield. C/N ratio of 20, 25, 30 and 35 showed similar cumulative methane yields with C/N-30 showing maximum methane yield (297.32 mL/g-VS_{added}). Optimization of pH and F/M ratio with constant C/N ratio of 30, different F/M ratios i.e. 0.48, 1, 2.25 and 4.02 and initial pH 6.43, 6.6, 7, 7.4 and 7.57 were selected for optimization using CCD-RSM. A synergistic effect of pH and F/M ratio was observed on the methane yield. The highest experimental methane yield (318.45 mL/g-VS_{added}) was achieved at pH 7.57 and F/M:2.25 and it was revealed that optimization of pH (7-7.57) and F/M ratio (1.5-2.5) were of significance, so as to attain maximum methane yield.

The effect of different pretreatment techniques i.e. thermal, electrohydrolysis and fungal on the degradability of RS followed by methane production were studied to increase the degradability. These pretreatment methods resulted in reasonably high solubilisation of recalcitrant biomass. In thermal pretreatment maximum solublisation was attained by microwave (190°C; exposure time - 4 min) (66.6% higher) followed by autoclave, hot air oven and hot water bath methods. The results of this pretreatment study revealed that microwave pretreatment was illustrated to be the best pretreatment techniques. Microwave pretreatment offered an accelerated hydrolysis of hemicellulosic and cellulosic part of volatile solid matter during the digestion time. Direct interaction of the substrate molecules with electromagnetic field resulted into thermal and athermal mechanism, a two-fold action which causes the efficient solublisation of the substrate. High heating rate with low residence time decreases the concentration of inhibitory compounds, which was evident from the high specific methane yield (367.68 mL/g-VS_{added}) for F/M:2.5. The electrohydrolysis pretreatment was performed using graphite electrode with intermittent mode of input voltage fluctuating from 10 to 30 V (voltage study) and in continuous mode for 15 to 80 min (time study). The result implicated that electrohydrolysis pretreatment with 25 V and 60 min time achieved the maximum solublization (55%). The different F/M (0.5-3) ratios from BMP assay showed the cumulative methane production ranging from 236 to 319.03 mL/g-VS_{added} (maximum for F/M:2). Compositional characterization (FESEM and FTIR) studies also showed the efficacy in disrupting the lignin layer and reducing the cellulose crystallinity of RS. The comparative effect of three fungal strains on RS for the

production of biogas was studied. Enhancement in the methane yield after 5 weeks of inoculation time was obtained after pretreatment, which was 269.99, 295.91, and 339.31 mL/g-$VS_{added,}$ for PO, GL and PC respectively, 1.64-2.22-fold higher than the untreated one. Methane yield from this study showed that if this study is implemented on industrial scale the total global production of 769.65 million tons of RS, can potentially produce 159-200 billion cubic meters of biomethane with an increase of 140 billion cubic meters of biomethane. Kinetic modeling of cumulative methane yield showed that MGM, showed the best fit among all analysed models.

The lab scale single phase continuous reactor of capacity (14 L) was operated at OLR 3.5-8.3 kg-VS/m^3/d) for 150 days in three stages as shown. During stage I, initially methane production increased upto 14 days of digestion because of higher initial activity of fermentative and methanogenic bacteria in AD. After 14 days, methane production became steady because anaerobic microorganisms felt resistance to access digestible organic matter. The optimal OLR for pretreatment stage was 8.3 kg-VS/m^3/d (microwave pretreatment) with VS degradation of 33.43%, sudden increase in methane production was observed because of readily digestible organics immediately accessed by the fermentative and methanogenic bacteria, which was attributed by enhanced solublization as compared to stage I. Co-digestion with FW in continuous mode showed an optimal OLR of 6.5 kg-VS/m^3/d for accelerated methane production with 24.09% VS degradation.

8.2 RECOMMENDATIONS FOR FUTURE WORK

- Effects of changing parameters (OLR, pH, C/N ratio etc.) in AD on microbial species interactions and microbiome structure.
- The mechanism of AD process needs to be developed in more details in order to support the efficient digestion of RS.
- Combine effect of different pretreatment techniques on degradability of RS and economic feasibility on large scale application.
- Impacts of various inhibitory compounds (5-hydroxymethylfurfural (HMF), furfural, and phenolic compounds etc.) generated in pretreatment process and its removal to enhance cellulose hydrolysis.

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RESEARCH OUTPUT

JOURNAL PUBLICATIONS

Published article

- Kainthola, J., Kalamdhad, A.S., Goud, V.V., 2019. Optimization of methane production during anaerobic co-digestion of rice straw and *Hydrilla verticillata* using response surface methodology. ***Fuel***. 235, 92-99. DOI: 10.1016/j.fuel.2018.07.094.
- Kainthola, J., Kalamdhad, A.S., Goud, V.V., 2019. Enhanced methane production from anaerobic co-digestion of rice straw and *Hydrilla verticillata* and its kinetic analysis. ***Biomass and Bioenergy.*** 125, 8–16. DOI: 10.1016/j.biombioe.2019.04.011.
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- Kainthola, J., Shariq, M., Kalamdhad, A.S., Goud, V. V, 2019. Electrohydrolysis pretreatment methods to enhance the methane production from anaerobic digestion of rice straw using graphite electrode. ***Renewable Energy.*** 142, 1-10. DOI: 10.1016/j.renene.2019.04.083.
- Kainthola, J., Kalamdhad, A.S., Goud, V.V., 2019. A review on enhanced biogas production from anaerobic digestion of lignocellulosic biomass by different enhancement techniques. ***Process Biochemistry*** . https://doi.org/10.1016/j.procbio.2019.05.023**.**

Articles (submitted/ under review/ under preparation)

- Kainthola, J., Kalamdhad, A.S., Goud, V.V., 2019. Optimization of process parameters for accelerated methane yield from anaerobic co-digestion of rice straw and food waste. *Renewable Energy*. ***(Under review).***
- Kainthola, J., Kalamdhad, A.S., Goud, V.V., 2019. Comparative study of different thermal pretreatment of rice straw for enhanced biogas production and its physicochemical characterization. *Energy*. ***(Under review).***

- Kainthola, J., Kalamdhad, A.S., Goud, V.V., Goel. R. 2019. Fungal pretreatment of lignocellulosic biomass for improved biogas yield: An overview. *Renewable & Sustainable Energy and Reviews.* ***(Under review).***
- Kainthola, J., Shariq, M., Kalamdhad, A.S., Goud, V. V, 2019. Anaerobic digestion of rice straw – Performance of single phase continuous anaerobic reactor for pretreatment and co-digestion. ***(Under preparation)***.

International/ National conferences/ Webinar

- Kainthola, J., Kalamdhad, A.S., Goud, V.V., 2017. Enhanced biogas production from co-digestion technology. National Conference on Sustainable Advanced Technologies for Environmental Management (SATEM), Indian Institute of Engineering Science and Technology (IIEST), Shibpur, West Bengal, India.
- Kainthola, J., Kalamdhad, A.S., Goud, V.V., 2017. Environment life cycle assessment of rice straw. National Conference on Recent Advancements in Environmental Research (RAER) Indian Institute of Technology Guwahati, Assam, India.
- Kainthola, J., Kalamdhad, A.S., Goud, V.V., 2018. Improving methane yield via co-digestion of *Hydrilla verticillata and* rice straw and its kinetic analysis. International conference on waste management (RECYCLE-2018), Indian Institute of Technology Guwahati, India.
- Kainthola, J., Kalamdhad, A.S., Goud, V.V., 2018. Effect of open dumping on environment and public health – a case study of Ghazipur landfill. Winter school on "Sustainable solid waste management", SWIS-ISWA. University of Arlington, Texas, USA.
- Kainthola, J., Kalamdhad, A.S., Goud, V.V., 2018. International conference on "Effects of Improper Waste Management in South East Asia: Local Challenges, Global Solutions" with ISWA YPG. Webinar | *be Waste Wise*.
- Kainthola, J., Kalamdhad, A.S., Goud, V.V., 2019. Anaerobic co-digestion of rice straw and *Hydrilla verticillata*- Modeling and process parameter optimization study. Research Conclave, Indian Institute of Technology Guwahati, India.

Awards

- ***Bioenergy-Award for Cutting Edge Research (B-ACER)-2018***. Department of Biotechnology (DBT), Government of India and the Indo-US Science and Technology Forum (IUSSTF). Fellowship for six months in University of Utah, Salt Lake City, USA. (Aug'18- Feb'19).

The program is envisaged to:

- Provide an opportunity to the best and brightest Indian students and scientists to gain exposure and access to world class research facilities in leading US institutions;
- Promote research and capacity building in the frontline area of Biofuels and Bioenergy;
- Encourage and motivate outstanding students to take up research as a career path; and
- Pave the way for the next generation scientists and technologists from India to interact with American peers, thus helping to build long-term R&D linkages and collaborations

Bioenergy-Awards for Cutting Edge Research (B-ACER) 2018

The Department of Biotechnology (DBT), Government of India and the Indo-US Science and Technology Forum (IUSSTF) are pleased to announce the candidates* selected for the **Bioenergy-Awards for Cutting Edge Research (B-ACER) Program 2018**.

Candidates selected for B-ACER Internships

S. No.	Name of Applicant	Parent Institution
1.	Shubhasish Goswami	Indian Institute of Science Education and Research (IISER), Kolkata
2.	Ram Ji Dixit	Indian Institute of Technology (IIT) Delhi
3.	Paramvir Singh	National Institute of Technology Hamirpur, Himachal Pradesh
4.	Nilesh Kumar Sharma	Sardar Swaran Singh National Institute of Bio-Energy, Kapurthala
5.	Danish Eqbal	International Centre for Genetic Engineering and Biotechnology, New Delhi
6.	Jyoti Kainthola	Indian Institute of Technology (IIT) Guwahati
7.	Himanshu Karashanbhai Patel	CSIR-Central Salt and Marine Chemicals Research Institute

- ***Winner of Essay competition organized by ISWA-SWIS*** (15 days training on "Sustainable waste management landfill and landfill mining" at University of Arlington, Texas, USA. (Jan'18 – Feb'18).

- Certificate of reviewing by ***Energy Conversion and Management, Waste Management*** and ***Journal of Advanced Research*** (Elsevier), 2019.