UNIVERSIDADE DE SÃO PAULO
ESCOLA DE ENGENHARIA DE SÃO CARLOS

FIAZ AHMAD

PRODUÇÃO DE METANO EM RESPOSTA AO PRÉ-TRATAMENTO HÍDROTERMICO COM ÁCIDO SULFÚRICO E PERÓXIDO DE HIDROGÊNIO DO BAGAÇO DE CANA-DE-AÇUCAR

METHANE PRODUCTION IN RESPONSE TO SULFURIC ACIDIC AND HYDROGEN PEROXIDE ASSISTED HYDROTHERMAL PRETREATMENT OF SUGARCANE BAGASSE

SÃO CARLOS/SP
2017

FIAZ AHMAD

METHANE PRODUCTION IN RESPONSE TO SULFURIC ACID AND HYDROGEN PEROXIDE ASSISTED HYDROTHERMAL PRETREATMENT OF SUGARCANE BAGASSE

Thesis submitted to Engineering School of São Carlos – University of São Paulo as a partial requirement to obtain the title of Doctor of Science: Hydraulics Engineering and Sanitation

Area of Study: Hydraulics and Sanitation

Supervisor: Prof. Assoc. Maria Bernadete Amâncio Varesche

Corrected version

São Carlos/SP

2017

Ahmad, Fiaz
A286p PRODUÇÃO DE METANO EM REPOSTA AO PRÉ-TRATAMENTO HÍDROTERMICO COM ÁCIDO SULFÚRICO E PERÓXIDO DE HIDROGÊNIO DO BAGAÇO DE CANA-DE-AÇUCAR / Fiaz Ahmad; orientadora Maria Bernadete Amâncio Varesche; coorientador Edson Luiz Silva. São Carlos, 2017.

Tese (Doutorado) - Programa de Pós-Graduação em Engenharia Hidráulica e Saneamento e Área de Concentração em Hidráulica e Saneamento -- Escola de Engenharia de São Carlos da Universidade de São Paulo, 2017.

1. Biogás. 2. Comunidade microbiana. 3. Lignina. I. Título.

FOLHA DE JULGAMENTO

Candidato: Bacharel **FIAZ AHMAD.**

Título da tese: "Produção de metano em resposta ao pré-tratamento hidrotérmico com ácido sulfúrico e peróxido de hidrogênio do bagaço de cana de açúcar ".

Data da defesa: 09/06/2017.

Comissão Julgadora:	**Resultado:**
Profa. Associada **Maia Bernadete Amâncio Varesche (Orientadora)** (Escola de Engenharia de São Carlos/EESC)	Aprovado
Prof. Titular **Marcelo Zaiat** (Escola de Engenharia de São Carlos/EESC)	Aprovado
Profa. Dra. **Sandra Imaculada Maintinguer** (Universidade Estadual Paulista "Júlio de Mesquita Filho"/UNESP- Rio Claro)	Aprovado
Profa. Dra. **Katia Sivieri** (Universidade Estadual Paulista "Júlio de Mesquita Filho"/UNESP-Araraquara)	Aprovado
Prof. Dr. **Antonio José Gonçalves da Cruz** (Universidade Federal de São Carlos/UFSCar)	Aprovado

Coordenadora do Programa de Pós-Graduação em Engenharia Hidráulica e Saneamento:
Profa. Associada **Maria Bernadete A. Varesche Silva**

Presidente da Comissão de Pós-Graduação:
Prof. Associado **Luis Fernando Costa Alberto**

ACKNOWLEDGEMENTS

This work has been conducted in the Laboratorio de Processos Biológicos, Engineering School of Sao Carlos - University of Sao Paulo for the duration 2014-2017. First of all, I would like to thank my supervisor Prof. Dr. Maria Bernadete Amâncio Varesche for giving me this opportunity and for all his invaluable advice during these years. Besides, I am very grateful to my co-supervisor, Prof. Dr. Edson Luiz Silva for all the conversations, statistical design of my experiments, and guidance. Moreover, I have been lucky to have fellow-researchers standing by me and sharing all the good and bad moments with experimental devices as well as during courses and seminars.

I am grateful to Dr. Isabel Kimiko Sakamoto, who helped me with the molecular biology experiments and her valuable time and suggestions for the write up of molecular biology. Without her sincere efforts, I could not have come this long. I also extend my gratitude to Dr. Eloisa Pozzi Gianotti for her help in preparation of Electron microscopy samples, Dr. Carolina Sabatini for her valuable help in sample analysis, Juliana for her kind assistance in 'monitoramento PAE', and Dr. Maria Angela Talarico who performed HPLC analyses and assist in GC analysis of methane measurement.

I humbly thankful to Vanessa Cristina da Silva, Kiemi Murata, Clara Faria for their invaluable company during my stay in Brazil; hanging out and festas ☺. I would like to extend my gratitude to Dr. Marcus Vinicius F Andrade for his kind help, share good and bad moments in and outside of the laboratory.

I am thankful for Dr. Maria Angela Talarico and Dr. Fabrício Montteran for their suggestion in qualification presentation.

I am humbly thankful for all my lab fellows at laboratorio de processos biologicos; Carla Diniz, Lívia Botta, Lucas Fuess, Clara Faria, Kiemi Murata, Vanessa Silva, Dagoberto Okada, Tiago Palladino, Thais Zaninetti, Rodrigo Carneiro, Adriana Maluf, Juliana Kawanishi, Fabrício Montteran, Raíssa Mazzareli, Alejandra Vila, Leonardo Gerosa, Alana Moura, Vinicius Masquetti, Carol Granatto and Leandro.

I am thankful for CAPES to fund this study, USP, EESC, SHS, and LPB.

Finally, family member; Riaz Ahmad, Ejaz Ahmad, lovely sisters; Khizra, Sidra and Iqra. Finally yet importantly, Mom and Dad, you all are my sunshine!

São Carlos, 2017 **Fiaz Ahmad**

Ahmad, F. **Produção de metano em reposta ao pré-tratamento hídrotermico com ácido sulfúrico e peróxido de hidrogênio do bagaço de cana-de-açucar.** 2017. 224 p. Tese (Doutorado) - Escola de Engenharia de São Carlos, Universidade de São Paulo, São Carlos, 2017.

O objetivo deste estudo foi otimizar a produção de metano investigando as condições do pré-tratamento hidrotérmico assistido do bagaço da cana de açúcar sob impregnação de ácido (H_2SO_4) e álcali (H_2O_2) utilizando-se a razão substrato (g kg^{-1}) – inóculo (g kg^{-1}) de 1:2. Os reatores em batelada foram mantidos em condições mesofílicas (37 ºC). Para otimizar as condições de pré-tratamento hidrotérmico, o design de composto central rotacional (DCCR) foi realizado utilizando três fatores: temperatura (ºC), tempo (min) e concentração do composto químico (H_2O_2 (%v/v) e H_2SO_4 (% p/v)). Trinta e dois pré-tratamentos hidrotérmicos foram realizados de acordo com a concepção do DCCR. O pré-tratamento hidrotérmico assistido do bagaço, com H_2O_2 resultou em maior recuperação de sólidos (93,13%), elevado percentual de glicana (139,52%) e menor recuperação de lignina (76,48%) da fração sólida pré-tratada, se comparada aquele com H_2SO_4. Nesse último caso, observou-se menor rendimento de sólidos (12,27%) e glucanas (187,01%) e maior recuperação de lignina (358,85%). No líquido hidrolisado do pré-tratamento hidrotérmico assistido do bagaço com H_2SO_4 foi observada elevada solubilização de DQO (25,20 g L^{-1}), menor teor de fenóis totais (658,13 mg L^{-1}), elevado sulfato (7240 mg L^{-1}), furfural (925,77 - 2216,47 mg L^{-1} e 5-hidroximetilfurfural (70,95 - 970,08 mg L^{-1}). Enquanto, foi registrado menor solubilização de DQO (17,27 g L^{-1}), maior teor de fenóis totais (3005,63 ppm), e menor concentração de furfural (0 - 56.91 mg L^{-1}), 5-hidroximetilfurfural (2,56 - 50,60 mg L^{-1}) com H_2O_2. Em relação ao a produção de metano nas condições com H_2O_2, observou-se 5.59 Nmmol g^{-1} STV (2%v/v H_2O_2) a 13.49 Nmmol g^{-1} STV (6%v/v H_2O_2). No tratamento com 7.36% de H_2O_2 observou-se 14,43 Nmmol g^{-1} STV que foi 118.16% maior se comparado com o bagaço não-tratado (6,60 Nmmol g^{-1} STV). Inibição metanogênica foi observada na maioria dos reatores pré-tratados com H_2SO_4 (1 – 3% p/v), e a produção mínima observada foi de 0.58 Nmmol g^{-1} TVS no pré-tratamento com 2% p/v de H_2SO_4. Ácido acético foi o principal ácido orgânico volátil observado somente no reatores por tratamento de H_2O_2.. Por meio da A análise da comunidade microbiana, para o domínio Bacteria, foi observado prevalência dos gêneros *AUTHM297*, *Clostridium* e *Treponema* nos reatores cujo substrato foi pré-tratado com H_2O_2. Gêneros relacionados à degradação de compostos aromáticos foram identificados e estiveram em maior abundância nos reatores cujo substrato foi pré-tratado com H_2SO_4. *Methanolinea*, *Methanobacterium*, e *Methanosaeta* foram os microrganismos do domínio Archaea mais abundantes e identificados em ambos os pré-tratamentos. O pré-tratamento hidrotérmico assistido com H_2O_2 foi a melhor opção em relação ao H_2SO_4, devido a maior solubilização de lignina, maior recuperação de glucano e baixa produção de compostos furânicos.

Palavras-chave: biogás, comunidade microbiana, lignina

Ahmad, F. **Methane production in response to acidic and alkaline hydrogen peroxide assisted hydrothermal pretreatment of sugarcane bagasse.** 2017. 224 p. Thesis (PhD) – Engineering School of Sao Carlos, University of Sao Paulo, Sao Carlos, 2017.

The aim of this study was to optimize methane production by investigating hydrothermal pretreatment of sugarcane bagasse impregnated with acid (H_2SO_4) and alkaline H_2O_2 using substrate (g kg^{-1}) -inoculum (g kg^{-1}) ratio of 1:2. Batch reactors were realized under mesophilic conditions (37 °C). A central composite design (CCD) involving three factors; temperature (°C), time (min), and chemical compound concentration (H_2O_2 (%v/v) and H_2SO_4 (%w/v)) was utilized to optimize hydrothermal pretreatment. Thirty-two hydrothermal pretreatments were conducted according to CCD. H_2O_2 assisted hydrothermal pretreatment resulted in higher solid recovery (93.13%), higher percent glucan increase (139.52%), and lower lignin recovery (76.48%) in pretreated solid fraction in comparison to H_2SO_4 impregnated hydrothermal pretreatment. In the latter case, lower solid yield (12.27%), glucan recovery (187.01%) and higher lignin recovery (358.85%) was recorded. Higher COD solubilization (25.20 g L^{-1}), lower total phenolic (content 658.13 ppm), higher sulfate (7240 mg L^{-1}), furfural (925.77-2216.47 mg L^{-1}) and 5-hydroxymehtylfurfural (70.95-970.08 mg L^{-1}) were observed in liquid hydrolysate of H_2SO_4 assisted hydrothermal pretreatment. While lower COD solubilization (17.75 mg L^{-1}), higher total phenolic content (3005.63 ppm), lower concentration of furfural (0 - 56.91 mg L^{-1}) and 5-hydroxymethylfurfural (2.56 - 56.60 mg L^{-1}) was recorded with H_2O_2 assisted hydrothermal pretreatment. Concerning methane production for H_2O_2 assisted conditions, 5.59 Nmmol g^{-1} TVS (2% H_2O_2) to 13.49 Nmmol g^{-1} TVS (6% H_2O_2) was recorded. However, pretreatment with 7.36% H_2O_2 resulted in 14.43 Nmmol g^{-1} TVS, which was 118.16% higher comparing to untreated sugarcane bagasse (6.60 Nmmol g^{-1} TVS). Methanogenic inhibition was recorded for most of the H_2SO_4.pretreated reactor (1 – 3 %w/v H_2SO_4). Minimum CH_4 production observed was 0.58 Nmmol g^{-1} TVS in pretreatment O-HSO (2% w/v H_2SO_4). Acetic acid was the predominant volatile fatty acid observed in digestion process of H_2O_2 treated batch reactor however was not recorded in H_2SO_4 treated batch reactors. Microbial community analysis indicated the prevalence of unclassified *AUTHM297, Clostridium,* and *Treponema* related genera in H_2O_2 treated reactors. Genera related aromatic compound degradation were identified and abundant in H_2SO_4 treated reactors. *Methanolinea, Methanobacterium, and Methanosaeta* were abundant methanogens in both pretreatments. Hydrogen peroxide assisted hydrothermal pretreatment was verified as a better choice for methane production comparing to sulfuric acid assisted hydrothermal pretreatment primarily on account of higher lignin solubilization, higher glucan recovery, and lower furanic compounds production

Keywords: biogas, microbial community, lignin

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

31-PNMR	Phosphorus-31 nuclear magnetic resonance
AD	Anaerobic digestion
AIL	Acid insoluble lignin
Aka	Also known as
ARDRA	Amplified ribosomal DNA restriction analysis
ASL	Acid soluble lignin
ATR-FTIR	Attenuated total reflectance Fourier transform spectroscopy
BMP	Biomethane potential
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CCD	Central composite design
COD	Chemical oxygen demand
CTC	Cane technology center
DGGE	Denaturing gradient gel electrophoresis
DM	Dry matter
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
FISH	Fluorescence in situ hybridization
FTIR	Fourier transform infrared spectroscopy
5-HMF	5-hydroxymethylfurfural
HPLC	High performance liquid chromatography
H_2SO_4	Sulfuric acid
H_2O_2	Hydrogen peroxide
K_w	Water autohydrolysis constant
LPB	Laboratório de processos Biológicos
NCBI	National center for biotechnology information
NMR	Nuclear magnetic resonance
NREL	National renewable energy laboratory

OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
pH	Potential of hydrogen
RISA	Ribosomal intergenic spacer analysis
RNA	Ribonucleic acid
SCB	Sugarcane bagasse
SEM	Scanning electron microscopy
SRB	Sulfate reducing bacteria
SUS	Steel used stainless
TCD	Thermal conductivity detector
TEM	Transmission electron microscopy
TGGE	Temperature gradient gel electrophoresis
TPC	Total phenolic content
TRFLP	Terminal restriction fragment length polymorphism
TS	Total solids
UNICA	Brazilian sugarcane industry association
VFA	Volatile fatty acids
TVS	Total volatile solids
USP	University of São Paulo
WIS	Water insoluble solid
XRD	X-ray powder diffraction

LIST OF SYMBOLS

%	Percentage
°C	Degree Celsius
D	Dominance
g L^{-1}	Gram per liter
g 100 g^{-1} TS	Gram per hundred gram of total solids
h	Hour
H′	Shannon-Wiener index
HT	Hydrothermal pretreatment
min	Minute
mg L^{-1}	Milligram per liter
Nmmol g^{-1} TVS	Normal millimoles per gram of total volatile solids
Nmmol g^{-1} TVS h^{-1}	Normal millimoles per gram of total volatile solids per hour

TABLE OF CONTENTS

1 INTRODUCTION

Industrial revolution after Second World War, the rapid increase in world population, urbanization, and lavish lifestyle triggered the fossil fuel consumption worldwide in the twentieth century. Nowadays, fossil fuels serve up to 80% of world's energy demand (Braun *et al.*, 2010). United States, China, and Japan consume ca. 40% of world's total oil consumption (DOE, 2006). Fossil fuels are finite, nonrenewable, and potential source of environmental pollution even though a global certainty has not yet been established between fossil fuel usage and environmental quality but it is certain that fossil fuel consumption, in metropolitan cities, is a source of localized air pollution, global climate change, and greenhouse effect (Ramanathan and Feng, 2009). In addition, waste production and its accumulation is an irrefutable fact in human society. Consequently, its safe disposal is one of the great challenges to cope with in the 21st century (Taherzadeh and Keikhosro, 2008).

In view of these challenges, global research theme has been shifted in the last couple of decades to seek biotechnological routes that could deal with waste stabilization issue, even, make them available to renewable and clean energy carriers (biofuels). Besides, interest in biofuels production is increasing due to expected shortage of conventional fuels, emission of greenhouse gasses, increased energy demand for transportation, electricity, industrial processes, and an upsurge in oil prices. It could safely be assumed that in a couple of decades, wind energy, solar energy, and bioenergy i.e., biogas, ethanol, and butanol would be in our energy portfolio. World Wildlife Fund reported that by 2050 world would be fueled 100% by renewable energy (WWF, 2011)

Lignocelluloses, carbon-based-polymers, are attaining global attraction owing to mass availability, low cost, easy transportation, high quantity, and renewable nature (Kaparaju *et al.*, 2009). Lignocellulosics produce in enormous amount, ca. 2×10^{11} tons annually in comparison to 1.5×10^{8} tons of synthetic polymers (Mohanty *et al.*, 2000). Furthermore, with the agricultural advancement, millions of tons of agricultural residues are available every year (Somerville *et al.*, 2010). Moreover, it is the only feedstock that could be biologically transformed into liquid and gaseous fuels, ethanol and biogas, respectively by exploiting simple microbial routes (Hatti-Kaul *et al.*, 2007) and could replace the petroleum-based fuels utilizing the same infrastructure. The

potential lignocellulosic feedstocks comprise on agricultural residues including sugarcane bagasse (SCB), wheat straw and corn stover, forest residues, dedicated energy crops, waste sludge from pulp and paper industry, switch grass and algae (Sims and Bassam, 2004; Sjöde *et al.*, 2007; Sassner *et al.*, 2008; Vergara-Fernández *et al.*, 2008; Wingren *et al.*, 2008).

Lignocellulosic material is composed predominantly of carbohydrate fractions (cellulose, hemicellulose) and non-carbohydrate fractions (lignin, pectin, protein, extractives and ash (Henning *et al.*, 2007; Ahmad, 2013). Cellulose, a glucose polysaccharide, forms highly dense and ordered groups with a degree of polymerization (DP) in the 10,000s (before any treatment). Hemicellulose is a mixture of hexoses and pentoses with a much lower degree of polymerization (100-200), but can physically prohibit enzymatic access to the cellulose. Lignin is the largest non-carbohydrate portion of plant matter. Composition and quantification of bio-fibers are dependent primarily on plant age, plant type, cultivation practices, growth conditions, soil type, soil conditions, and environmental conditions (Henning *et al.*, 2007). In native lignocellulose, cellulose and hemicellulose are cemented together by lignin, which is responsible for the integrity, structural rigidity, and prevention of fiber swelling (Ramirez *et al.*, 2013).

Sugarcane (*Saccharum* spp.) is an indigenous crop that originates from New Guinea (southeast Asia) and is grown in tropical and subtropical regions, on both sides of the equator (Lisboa *et al.*, 2011). Sugarcane, C_4 plant, is very efficient in converting solar radiation into biomass with unique ability within the *Poaceae* family to accumulate up to 60% of its mature stem dry weight (Casu *et al.*, 2007; Anuj *et al.*, 2012)

Brazil is the world leader in sugarcane production. The Cane Technology Center (CTC) in consultation with The Brazilian Sugarcane Industry Association (UNICA) speculated that sugarcane harvest in Brazil could reach up to 590 million tons (UNICA, 2015). According to Ministry of Agriculture, livestock and farming of Brazil (MAPA, 2016) sugar export of Brazil may reach up to 32.6 million tons.

Sugarcane bagasse is the most abundant lignocellulosic residue among agricultural residues in tropical countries. Sugarcane bagasse (SCB) is a valuable byproduct of ethanol distilleries and sugar mills with ca. 270-280 kg (50% moisture content) per metric ton of sugarcane (Karp *et al.*, 2013) with a global production of 250 million tons per year (Kanokratana *et al.*, 2013). Around 5.4×10^{8} tons of dry sugarcane bagasse is a processed annually worldwide. It usually

finds its way (50%) in boilers to satisfy the energy requirements of the distilleries (Aita and Kim, 2010) due to its higher (1800 kcal kg^{-1}) calorific value (Zanin *et al.*, 2000). It is an inexpensive carbohydrate source (60-70%) and a potential feedstock for anaerobic digestion to produce biogas (Badshah *et al.*, 2012), and ethanol (Rabelo *et al.*, 2011). In addition, based on lower ash content (2-3%) it offers advantages over the wheat straw and rice straw (Basso *et al.*, 2013). However, being a recalcitrant material its utilization as a feedstock for anaerobic digestion is a challenging task.

Anaerobic digestion (AD) has been recognized as the most efficient technology for waste stabilization with associated benefits of clean energy (Muhammad *et al.*, 2012). Anaerobic digesters were primarily designed to digest sewage sludge and animal excreta. However, substrates are of less biodegradable potential in comparison to agricultural residues, food wastes, and municipal solid wastes (Forster-Carneiro *et al.*, 2012). It is a less energy demanding rather energy generating process. The major obstacle in the successful operation of AD process is inefficient hydrolysis, a rate-limiting step in digestion process, owing to recalcitrant nature of lignocellulosic material (Valo *et al.*, 2004; Fernandes *et al.*, 2009) as in the case of sugarcane bagasse, whilst methanogenesis is considered a rate-limiting step for easily degradable compounds (Gavala *et al.*, 2003; Skiadas *et al.*, 2005). In order to disrupt the bio-fibers arrangement, enhancing enzymatic attack of fermentative bacteria of microbial consortium, thus overcoming hydrolysis rate-limiting step, a pretreatment step is inevitable. Pretreatment constitutes 30% of overall processing cost (Aita and Kim, 2010).

With technological advancement, a number of pretreatment strategies have been developed to unlock the lignocellulosic complex in the last couple of decades. However, due to intrinsic variety in lignocellulosics chemical composition owing to aforementioned factors, not a single pretreatment method can be recommended for different biofuels. In addition, every pretreatment has its own pros and cons. A suitable pretreatment process should be of following characteristics: (a) simple; (b) cost effective; (c) highly efficient in terms of sugar release; (d) less energy intensive; (e) non-corrosive to reactor material; (f) minimum process side products; and (g) minimal inhibitory product formation (Taherzadeh and Keikhosro, 2008).

Sugarcane bagasse is a coherent lignocellulosic feedstock; efficient utilization of this feedstock for anaerobic digestion necessitates a pretreatment step. Hydrolysis is considered a

bottleneck owing to lignocellulosic feedstock recalcitrance (Wang *et al.*, 2015). It is further enhanced by macroscopic factors; tissue compositional heterogeneity, mass transfer limitations, and microscopic factors; cellulose crystallinity, criss-cross nature of lignin-carbohydrate complex (Chundawat *et al.*, 2011). Acid pretreatment has been reported to increase the hemicellulose solubilization (Yi *et al.*, 2014) while, alkaline oxidative pretreatment proved to be a good option for lignin removal (Zheng *et al.*, 2010). Hydrothermal pretreatment has been reported to open up the lignocellulosic structure and higher temperature and sudden release of pressure further break down the structural components. In the present study, an attempt is made to combine the three processes; acid, alkaline and hydrothermal processing. Sugarcane bagasse was impregnated in dilute H_2SO_4 and alkaline H_2O_2 separately in two phases before introduction to the hydrothermal reactor.

The current study focused on a promising pretreatment, hydrothermal processing, to pretreat sugarcane bagasse, to rupture its complex structure, as a tool to enhance methane production. To best of our knowledge, no work has been reported on a comparison of acidic (sulfuric acid) and alkaline oxidative (hydrogen peroxide) assisted hydrothermal pretreatment of sugarcane bagasse for anaerobic digestion. Furthermore, microbial consortium was studied in response to pretreatment effect by state of the art denaturing gradient gel electrophoresis (DGGE) and Illumina MiSeq Sequencing

2 OBJECTIVES

2.1 General objective

The general objective of this study was to evaluate the methane production of sugarcane bagasse after hydrothermal pretreatment assisted with dilute H_2SO_4 and alkaline H_2O_2 impregnation

2.2 Specific objectives

The specific objectives of the research were:

a) To analyze sugarcane bagasse morphologically and physicochemically for anaerobic digestion;
b) To correlate pretreatment severity factor with inhibitory products (furfural and 5-hydroxymethylfurfural) formation;
c) To compare both pretreatments for highest methane and organic acids yield in batch reactors under mesophilic conditions (37 °C);
d) To evaluate the impact of pretreatments on the microbial community structure.

3 REVIEW OF LITERATURE

To design an optimum pretreatment process for anaerobic digestion, fundamental knowledge of lignocellulosic material, impact of pretreatment on structural and chemical composition of lignocellulosic substrate, biochemistry and microbiology of digestion process, processes understanding of inhibitory product formation during pretreatment, and microbial response to pretreatment side products in terms of kinetics, is an imperative to adopt a pretreatment on industrial scale. Pretreatment is the primary step to open up the coherent lignocellulosic structure, making the components accessible to enzymatic action of microorganisms for the release of monomeric sugars (Mosier *et al.*, 2005). Furthermore, it has a pervasive impact on downstream processing.

In this chapter, a brief review of the chemical composition of lignocellulosic biomass is presented supported with references from the literature. Hydrothermal pretreatment mechanism, its impact on the chemical composition of solid and liquid fractions, a brief overview of anaerobic digestion process and impact of hydrothermal pretreatment on subsequent digestion process are discussed.

3.1 Chemistry of lignocellulosic biomass

To obtain desired results and optimum pretreatment efficiency, it is important to get a clear picture of the lignocellulosic composition to visualize what is going on with lignocellulosic components during pretreatment. 31 P NMR (phosphorus 31 – Nuclear Magnetic Resonance) is a direct analysis tool to quantify hydroxyl groups in lignin (El Hage *et al.*, 2010). Real-time monitoring of lignocellulosic components during pretreatment may pave the way to understand pretreatment impact better and to optimize maximum sugar recovery. Physical properties of the lignocellulosic material; water-holding capacity, specific porosity, specific surface area and crystallinity index" change with each kind of pretreatment applied but to a different extent depending on pretreatment type, severity, and lignocellulosic composition.

Lignocellulosic biomass irrespective of their physical appearance shares the same chemical make-ups; cellulose (30-70%), hemicellulose (15-30%), and lignin (10-25%), and minute quantity of extractives (Monlau *et al.*, 2013). Extractives are those compounds that are not accounted for integral parts of biomass structure. Common components encountered in the lignocellulosic

composition of different feedstocks are presented in Table 3.1. While chemical composition of sugarcane bagasse by various researchers is given in Table 3.2. Graphical representation of the various components of lignocellulosic biomass originating from plant biomass is shown in Fig 3.1. Lignocellulosic composition, structural features and factors affecting recalcitrance have been reviewed elsewhere (Himmel *et al.*, 2007; Zhao *et al.*, 2012).

Cellulose was first separated in 1839 by Anselme Payen (Chen, 2014). Cellulose, [$C_{6n}H_{10n+2}O_{5n+1,}$ n – degree of polymerization of glucose] the most abundant polysaccharide, is a constituent of anhydro-glucan units linked together by β, 1-4 glycosidic linkages in a linear fashion (Edward, 2008). A number of glucan units in a polymeric chain terms into the degree of polymerization (DP). Chain length ranges from hundreds to over tenths of thousands. The hydrogen bonds between glucan units determine cellulose crystallinity. Furthermore, some chains are irregularly arrayed rendering amorphous regions intertwined with crystalline cellulose (Taherzadeh and Keikhosro, 2008). Chain length is inversely proportional to hydrolysis efficiency (Karimi and Taherzadeh, 2016). It is insoluble in water and dilute acids and alkaline solutions at room temperature. Cellulose in its amorphous form is most susceptible to microbial degradation (Monlau *et al.*, 2013).

Hemicellulose $(C_5H_8O_4)_n$ is a linear and highly branched-heteropolymer composed primarily of D-xylose, L-arabinose (members of C_5 sugar family), D-glucose, D-mannose, D-galacturonic acid, D-galactose, and glucuronic acid (members of C_6 sugar family) and C_7 sugar 4-O-methyl glucuronic acid (Soccol *et al.*, 2011 b). Individual sugars may be methylated or acylated. This group contains three pentoses (D-xylose, L-arabinose, and D-ribose) and two pentitols (D-arabitol, and ribitol) (Singh and Mishra, 1995). The composition is heavily dependent upon the source whether it is derived from angiosperm (hardwood) or gymnosperm (softwood). Xylose is the principle sugar for angiosperms and agricultural wastes while glucomannan for softwood (Ebringerová and Heinze, 2000). Hemicelluloses have lower molecular weight, lower DP, and less crystallinity with random amorphous structure compared to highly packed cellulose rendering it more susceptible to hydrolysis than cellulose (Yoshida *et al.*, 2008; Heather and Charles, 2013).

Table 3.1 - Composition of agricultural and other lignocellulosic residues on dry matter basis (Saini *et al.*, 2015)

Feedstock	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**	**Ash (%)**	**Extractives (%)**
Algae (green)	20-40	20-50	-	-	-
Cotton, flax	80-95	5-20	-	-	-
Grasses	25-40	25-50	10-30	-	-
Hardwoods	45±2	30±5	20 ± 4	0.6 ± 0.2	5 ± 3
Hardwood barks	22-40	20-38	30-55	0.8±0.2	6±2
Softwoods	42±2	27±2	28±3	0.5±0.1	3±2
Softwood barks	18-38	15-33	30-60	0.8±0.2	-
Cornstalk	39-47	26-31	3-5	12-16	-
Wheat straw	37-41	27-32	13-15	11-14	-
Newspaper	40-55	25-40	18-30	-	-
Chemical pulp	60-80	20-30	2-10	-	-
Sorghum stalks	27	25	11	-	-
Corn stover	38-40	28	7-21	3.6-7.0	-
Coir	36-43	0.15-0.25	41-45	2.7-10.2	-
Sugarcane Bagasse	32-48	19-24	23-32	1.5-5	-
Rice straw	28-36	23-28	12-14	14-20	-
Wheat straw	33-38	26-32	17-19	6-8	-
Barley straw	31-45	27-38	14-19	2-7	-
Sorghum straw	32	24	13	12	-
Sweet sorghum bagasse	34-45	18-28	14-22	-	-

Lignin, the most abundant highly branched natural non-carbohydrate polymer next to cellulose and hemicellulose; 40% of dry biomass weight (Effendi *et al.*, 2008), is an important component in plant cell wall providing structural strength. Moreover, to counter it from biotic and abiotic stresses (Weng and Chapple, 2010). It is composed of phenylpropane alcohols; coniferyl,

sinapyl, and to a lesser extent *p*-coumaryl which form guaiacyl (G), sinapyl (S), and *p*-hydroxyphenyl units linked through the (β-O-4) β-aryl ether and 5-O-4 (biphenyl ether). C-C ("condensed") bonds such as 5-5 (biphenyl) linkages or a combination of C-C and ether linkages (Adler, 1977; Boerjan *et al.*, 2003; Rowel, 2005; Zeng *et al.*, 2014). Furthermore, lignin frequently forms a covalent bond with surrounding carbohydrate mainly hemicellulose (Saake and Lehnen, 2007).

Table 3.2 - Chemical composition of sugarcane bagasse on DM basis

Cellulose (%)	**Hemicellulose (%)**	**Lignin (%)**	**Ash (%)**	**References**
45.5	27	21.1	2.2	(De Moraes *et al.*, 2011)
41.1	22.7	31.4	2.4	(Pitarelo, 2007)
38.8	26	32.4	2.8	(da Silva *et al.*, 2010)
45.0	25.8	19.1	1.0	(Canilha *et al.*, 2011)
42.4	25.2	19.6	1.6	(Brienzo *et al.*, 2009)
38.4	23.2	25.0	1.5	(Rabelo *et al.*, 2011)
32 – 44	27 – 32	19 – 24	4.5 – 9	(Soccol *et al.*, 2010)
30.2	56.7	13.4	1.9	(Bharathiraja et al., 2014

DM = Dry matter

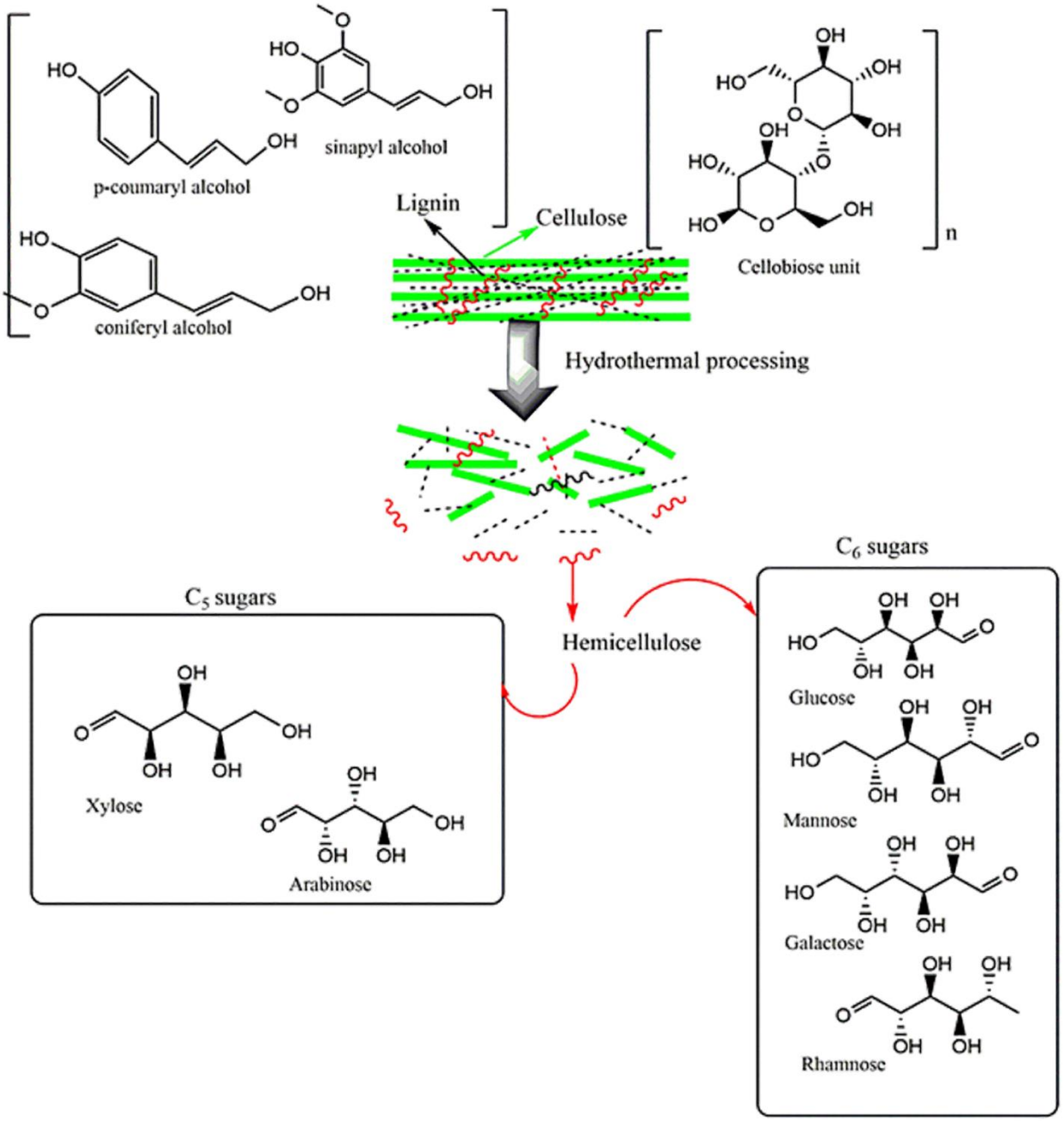

Figure 3.1 - The components and structure of lignocellulosic biomass (modified after (Edward, 2008))

The relative portion of each component varies by plant species. Coniferyl alcohol is the principal monomer in softwood lignin while both sinapyl and coniferyl monomers are the building blocks for hardwood lignin. Others “non-canonical” aromatic monomers also known to be incorporated into lignin framework (Ralph *et al.*, 2004; Bonawitz and Chapple, 2010). It provides the protective cover around the cellulose and hemicellulose as a hydrophobic sheet and has high

significance for anaerobic digestion since lignin concentration varies inversely to methane production (Kobayashi *et al.*, 2004; Ahring *et al.*, 2015). While lignin's role in cell wall recalcitrance is universally accepted, the precise set of factors that contribute to this recalcitrance is not generally acknowledged.

Factors specific to lignin's role in recalcitrance have been proposed to include the total lignin abundance (Soest, 1994; Chen and Dixon, 2007; Studer *et al.*, 2011), lignin location within the cell wall (Yang and Wyman, 2004), and the properties of lignin such as hydrophobicity (Nakagame *et al.*, 2011), as well as indirect impacts such as lignin's ability to bind enzymes (Rahikainen *et al.*, 2011).

3.2 Hydrothermal pretreatment mechanism

Pretreatment is the initial step to overcoming the recalcitrance property of lignocellulosic material. Pretreatment techniques are divided into four broad categories; physical, physicochemical, chemical and biological. There are several methods within each category. A bunch of reviews in the literature can be found specifically on pretreatment of lignocellulosic biomass for biogas production (Yi *et al.*, 2014; He *et al.*, 2015), biological pretreatment (white rot fungi) (Rouches *et al.*, 2016), and pretreatments used for high-fat cattle slaughterhouse wastewater (Harris and McCabe, 2015). Understanding the pretreatment process itself is as important as its impact on the lignocellulosic biomass composition.

Hydrothermal pretreatment (aka., liquid hot water pretreatment, hot compressed water pretreatment, hydrothermolysis, un-catalyzed solvolysis, aqueous fractionation or aquasolv, hydrothermal carbonization or wet torrefaction (Lynam *et al.*, 2014) is a process in which lignocellulosic biomass is cooked with water in liquid state at higher temperature (140 – 300 °C) with corresponding pressure for several minutes to few hours. Water at high-temperature acts as an acid owing to autoionization with hydrogen ions generation (Equation 3.1), thus significantly reduces the pH to an acidic level.

$$K_w = \frac{[H^+]\,[OH^-]}{[H_2O]} \tag{3.1}$$

Where; K_w = water autohydrolysis constant

$[H^+]$ = Hydrogen ion

$[OH^-]$ = Hydroxyl ion

$[H_2O]$ = Water

Pure water exhibits pH of 5 at 250°C that enhances the catalytic ability of water 25 times to room temperature conditions (Lloyd and Wyman, 2004). In this process, water (subcritical state) enters the lignocellulosic biomass to hydrate cellulose, solubilize a significant portion of hemicellulose and partly remove lignin (Pérez *et al.*, 2007; Chandra *et al.*, 2012). Higher kinetic energy available in the system at high temperature increases the rate of polymerization reactions, thereby, increasing the breakdown of cell wall polymers. Furthermore, it causes swelling (nonchemical) of cellulose, thereby, increasing the surface area of cellulose, essential for enzymatic action.

Water, under pressure, helps in cleaving the acetyl and uronic acid groups of hemicellulose producing acetic acid and other organic acids making oligosaccharides further solubilize (Garrote *et al.*, 1999; Aita and Kim, 2010). Hydronium ions generated by acetic acid are of greater importance than of water as indicated by Garrote et al. (Garrote *et al.*, 1999). It results in opening up the structure, expansion of specific surface area, and pore size of lignocellulosic biomass (Zakaria *et al.*, 2015). Since water is the reaction medium, it voids neutralization step afterward for bioethanol production due to lower inhibitory products formation.

The effectiveness of the pretreatment is a function of substrate composition and pH in addition to temperature employed and retention time (Taherzadeh and Keikhosro, 2008; Hendriks and Zeeman, 2009). Hydrothermal pretreatment has been reported a better choice for biomasses with higher acetyl content (Nitsos *et al.*, 2013). Hydrothermal pretreatment is aimed to balance the fermentable sugar yield delivered by acid pretreatment with lower operational cost (Eggeman and Elander, 2005).

Hydrothermal pretreatment is carried out in three types of reactors; cocurrent (biomass and liquid water cooked together), counter current (movement of biomass and liquid water in the opposite direction), and flow-through reactors (hot water flow over a stationary bed of biomass) (Liu and Wyman, 2005). Hydrothermal pretreatment combat against recalcitrance of

lignocellulosic biomass under heat and pressure at different reactor configurations to break strong bonding between carbohydrate-lignin matrix (Guilliams *et al.*, 2016).

Hydrothermal processing is a thermochemical conversion process. It is further categorized into three processes depending upon the severity conditions applied; hydrothermal carbonization (< 247 °C), hydrothermal liquefaction (247 – 373 °C), and hydrothermal gasification (>373 °C). Each process results in a distinct main product. Hydrochar is the main product of hydrothermal carbonization which has properties similar to low-rank coal (Heilmann *et al.*, 2011). Biocrude, liquid fuel, is the product of hydrothermal liquefaction. Synthetic fuel gas is the product of hydrothermal gasification. Hydrothermal liquefaction followed by gasification results into higher carbon efficiencies (Brown *et al.*, 2010).

The severity factor is a measure of the pretreatment intensity in hydrothermal processes and it allows comparing the results of experiments carried out under different conditions (Rocha *et al.*, 2013). It has extensively been used to report pretreatment severity in the literature (Overend *et al.*, 1987; Chum *et al.*, 1990; Bouchard *et al.*, 1991; Abatzoglou *et al.*, 1992; Jollez *et al.*, 1994; Montané *et al.*, 1994; van Walsu, 2001; Yourchisin and Peter Van Walsum, 2004; Kabel *et al.*, 2007; Pedersen and Meyer, 2010; Agbor *et al.*, 2011; Lee and Jeffries, 2011; Temiz and Akpinar, 2016). The generalized impact of pretreatment time and temperature on lignocellulosic composition is first proposed by Overend et al (Overend *et al.*, 1987) in terms of severity factor (Equation 3.2).

$$\log R_0 = \log\left\{t.\exp\left(\frac{T-100}{14.75}\right)\right\} \tag{3.2}$$

Where

t = the holding time

T = the reaction temperature

100 = the reference temperature

The fitted value (14.75) is based on activation energy, assuming pseudo first order kinetics (Carvalheiro *et al.*, 2009). The Equation 3.2 is not valid under variable pH conditions, therefore, to understand the influence of pH on pretreatment severity, Equation 3.3 has been derived by

having acidic and alkaline pretreatment impact on biomass digestibility (Pedersen and Meyer, 2010).

The severity factor is quite important in modeling individual stages of the processing; (a) to describe the behavior of xylan removal during reactions (Overend and Chornet, 1989; Heitz *et al.*, 1991; Saska and Ozer, 1995), (b) biomass solubilization (Bouchard *et al.*, 1991; Abatzoglou *et al.*, 1992), and c) lignin removal (Bouchard *et al.*, 1991; Montané *et al.*, 1994; Trajano *et al.*, 2013).

$$\log R_0' = \log R_0 - pH \tag{3.3}$$

Where

$\log R_0'$ = Severity factor at variable pH

$\log R_0$ = Severity factor without considering pH

pH = Potential of hydrogen

3.3 Impact of hydrothermal pretreatment on the lignocellulosic biomass composition on solid fraction

Hydrothermal pretreatment has a significant impact on solid yield predominantly due to hemicellulose solubilization and partial lignin dissociation. Furthermore, solid recovery indicates extraction pattern of components into the liquid stream based on chemical composition of lignocellulosics. Since hemicellulose solubilization is in direct relation to temperature, therefore, solid yield has a direct link, in decreasing order, with an increase in temperature (Yang *et al.*, 2004). Organic matter solubilization at higher temperature mainly targets carbohydrates followed by protein in the lignocellulosic structure. Mendez et al. (Mendez *et al.*, 2014) reported a 4-6-fold increase in carbohydrate dissolution in comparison to 1-2 fold for protein.

The solid yield of *Eucalyptus camaldulensis* declined to 54.2% from 72.7% when ammonium chloride catalyzed hydrothermal pretreatment temperature increased from 140 to 180 °C (Shen *et al.*, 2016). Similarly, Deng et al. (Deng *et al.*, 2015) reported a decrease in the solid residue of corncob from 96.5% to 60.67% when pretreatment temperature was increased from 170 °C, (0 min) to 190 °C (60 min). Sun et al. (Sun *et al.*, 2014) reported a progressive decrease in

solid residues of corncob 69.4% to 48.7% when temperature increased from 170 °C to 190 °C. Further, it reached to the lowest value of 47.0% at 200 °C.

Hydrothermal pretreatment optimization was carried out by Lu et al (Lu *et al.*, 2009) to evaluate the impact of H_2SO_4 addition, pretreatment time and solid content on rapeseed straw, solid recoveries were in the range of 55% to 88%, the lowest recovery was obtained at highest acid concentration studied (2%), at 5% solid loading for a retention time of 20 min. Moutta et al. (Moutta *et al.*, 2013) reported recovery of 24.5 g, 22.0 g, and 20.8 g of insoluble solid on the introduction of 30.0 g bagasse, straw and mixture of bagasse and straw (1:1), respectively. Ko et al. (Ko *et al.*, 2015) reported a decrease in solid recovery up to 71.1% from 93.9% in response to increasing the temperature from 180 °C to 210 °C.

Perez et al. (Pérez *et al.*, 2007) reported lower solid recovery on wheat straw within the range of 57.5% to 90% depending upon pretreatment severity. The lowest value was indicated at 200 °C and 40 min. Li et al. (Li *et al.*, 2013) performed liquid hot water pretreatment at different severities on suspended and precipitated solid of *Miscanthus lutarioriparious* and reported lowest solid recovery at high severity (4.71); 65.46% ± 0.16 and 64.85± 0.46 for suspended and precipitated solid, respectively. Solid yield is an important parameter for consideration with reference to anaerobic digestion; higher pretreatment temperature leads to an increase in lignin content in the solid fraction thus resulting in lower overall methane yield as reported by Fernandez et al. (Fernandez-Cegri *et al.*, 2012).

3.3.1 Cellulose

Hydrothermal pretreatment has a minimal direct impact on cellulose dissolution in comparison to hemicellulose and lignin due to its thermal stability, highly packed structure, and encapsulation by the lignin sheath (Mosier *et al.*, 2005). Cellulose structural rearrangements can occur at high pretreatment temperature (Mok *et al.*, 1992). C-O-C bond cleavage mechanism in cellulose involves protonation of glycoside bonds. The proton can attack the oxygen bond in between two glucose monomers or cyclic oxygen. The mechanism involves the rapid formation of an intermediate complex with the oxygen and proton followed by slow splitting of glycosidic bonds by water molecules (Fan *et al.*, 1987).

Cellulose undergoes crystalline to amorphous transformation at 320 °C, 25 MPa (Deguchi *et al.*, 2006). In contrast, Sakaki et al. (Sakaki *et al.*, 2002) reported degradation of cellulose started into hexoses and oligosaccharides above 230 °C and almost all decomposition at 295 °C. Since cellulose has a high degree of polymerization (DP); higher hydrogen bonding within cellulose fibers resists its breakdown and pose a major hindrance to hydrolyze the biomass (Karimi and Taherzadeh, 2016). Lower DP may increase the susceptibility of cellulose hydrolysis (Zhang and Lynd, 2005), a clear-cut relationship between DP and hydrolysis has not yet been established (Yang *et al.*, 2011). Though having little impact on cellulose dissolution, hydrothermal pretreatment substantially increases cellulose reactivity with hydrolytic enzymes, predominantly, due to hemicellulose removal and disruption of lignin-hemicellulose-cellulose meshwork thus making cellulose accessible.

Lignocellulosic solubilization has been reported to increase in direct relation to an increase in temperature (Ruiz *et al.*, 2012). Cellulose content, as a whole, tends to increase after hydrothermal pretreatment owing to the dissolution of hemicellulose and lignin. Nitsos et al. (Nitsos *et al.*, 2013) reported an increase in cellulose content up to 63% w/w in comparison to an untreated sample, 42% w/w. In similar fashion, 83% increase in glucan yield reported by Lilis et al. (Lilis *et al.*, 2009) in a pretreated solid fraction of switchgrass. Ruiz et al. (Ruiz *et al.*, 2012) reported 63.7% cellulose on glucan content basis in the pretreated solid fraction. In another study, Fang et al. (Fang *et al.*, 2015) reported 31.63% increase in glucan content of date palm leaflet at 210 °C while 60.12% increase in pretreated rachis at 200 °C. Sun et al. (Sun *et al.*, 2014) reported an increase in cellulose content from 45.01% to 59.69%. Perez et al. (Pérez *et al.*, 2007) reported 45% to 65% increase in the cellulose in a pretreated solid fraction on a dry weight basis in comparison to untreated wheat straw (37.4%). Pretreated solid obtained from hydrothermal pretreatment of *Eucalyptus globulus* reported containing almost 98.5% cellulose (Romaní *et al.*, 2010).

3.3.2 Hemicellulose

Liquid hot water pretreatment primarily makes available hydronium ions by water fractionation at elevated temperature. Hydronium ions are highly reactive, having a significant impact on xylan depolymerization and acetyl group cleavage from hemicellulose backbone (Rocha

et al., 2013). It has shown to solubilize hemicellulose up to 89% as reported by Xiao et al. (Xiao *et al.*, 2011) at 200 °C for 3 h. on woody biomass *Tamarix ramosissima*.

Temperature is an important parameter with respect to hemicellulose dissolution; lower temperature has no impact on hemicellulose solubilization as Nitsos et al. (Nitsos *et al.*, 2013) reported on beech wood that hydrothermal pretreatment has virtually no impact on hemicellulose dissolution at a mild temperature (130 °C, 15 min). Removal of hemicellulose began at (150 °C, 15 min) with almost complete removal at 220 °C for 15 min corresponding to log R_0 4.69. Similar results are reported by (Hendriks and Zeeman, 2009; Fernandez-Cegri *et al.*, 2012).

Senila and fellows (Senila *et al.*, 2014) reported complete hemicellulose solubilization of silver fir wood at 200 °C with a holding time of 10 min. Sun et al. (Sun *et al.*, 2014) reported a significant decrease in hemicellulose from 19.51% to 1.69% with a corresponding increase in pretreatment temperature from 100 °C to 180 °C for 60 min. Perez et al. (Pérez *et al.*, 2007) reported a decrease in xylan content in the wheat straw by reporting a recovery of xylan content in the water-insoluble solid fraction in the range of 0.3% to 25.4% depending upon conditions studied comparing to 26.3% hemicellulose in the untreated sample.

Hydrothermal pretreatment has been proven to be of lower impact when applied to softwood materials owing to lower acetyl content of softwood (Alvira *et al.*, 2010). On the other hand, Nitsos et al. (Nitsos *et al.*, 2013) reported hydrothermal pretreatment as a viable option for lignocellulosics having higher acetyl content.

3.3.3 Lignin

It is generally believed that hydrothermal pretreatment increases the lignin content in solid fraction primarily due to solubilization of hemicellulose in the liquid fraction. Long pretreatment time is another significant factor to increase klason lignin in the solid fraction due to recombination reaction between lignin and holocellulose (Kobayashi *et al.*, 2004). Lignin recovery is in direct proportion to the pretreatment temperature (Hansen *et al.*, 2004). The process could solublize, depending upon pretreatment severity, all hemicellulose in addition to 4-22% cellulose and 35-60% lignin (Aita and Kim, 2010). In contrast Iwona et al. (Iwona *et al.*, 2013) reported minimal removal of lignin due to subsequent repolymerization on cellulose fibers. Lignin, hydrophobic in nature, on exposure to biomass surface, contributes in two ways to biomass recalcitrance; (a)

impedes cellulases accessibility to biomass, (b) unproductive diffusion of cellulose enzymes to active sites on a biomass surface. The process sufficiently changes the lignin structure by melting and coagulation. Syringil units have been reported the most susceptible to hydrothermal degradation (Garrote *et al.*, 1999).

Lignin solubilization involves the breaking of lignin-carbohydrate bonds and depolymerization reactions (Garrote *et al.*, 1999). The fraction of solubilized lignin depends upon the raw material (Wallis and Wearne, 1985) and operational conditions (Shimizu *et al.*, 1989). Lignin during hydrothermal pretreatment undergoes various chemical reactions, phase transitions; solid and liquid phase, and morphological changes. Various researchers observed droplets on cellulose in response to pretreatment.

It determined to be lignin as a result of phase transition; glassy to a rubbery state, coalescence, and extrusion from cell wall (Donaldson *et al.*, 1988; Donohoe *et al.*, 2008). Repolymerization counteracts lignin removal, although ether bonds cleaved, delignification rate was reported close to zero (Li *et al.*, 2007). In addition, an increase in molecular weight of lignin after pretreatment is a clear indication of repolymerization of lignin (Li *et al.*, 2007). Furthermore, sugar oligomers and solubilized lignin are reported to inhibit cellulose digestion by reattaching to the surface of pretreated fibers. (Liu and Charles, 2005).

Garrote and co-workers (Garrote *et al.*, 2007) reported an increase in lignin content at high temperature owing to a combination of lignin and protein. The higher pretreatment severity led to an increase in lignin recovery yield, which might be caused by the condensation reactions of lignin with other degradation products. Hydrothermal pretreatment of date palm leaflets and rachis substantially increase lignin content up to 64.95 % and 49.45%, respectively, at 210 °C. Sun et al. (Sun *et al.*, 2014) reported an increase in lignin from 25.84% to 54.35. Mittal et al. (Mittal *et al.*, 2009) reported an initial solubilization of lignin (15%), thereafter, on prolonged pretreatment (175 °C, 8 h) lignin value reached a maximum value of 103% on initial lignin content basis.

Romani and colleagues (Romaní *et al.*, 2010) reported recovery of 85.4% lignin on average in the pretreated solid of *Eucalyptus globulus* while studying autohydrolysis at 195, 205, 220 and 230 °C. Nitsos and fellows (Nitsos *et al.*, 2013) reported 26%-35% increase in lignin content in pretreated beach wood comparing to native at the severity of log R_0 4.69. Ko et al. (Ko *et al.*, 2015) studied the fate of lignin on hardwood chips and reported an increase in lignin from 29.3% to

40.3% for a severity range of 8.25 to 12.51 with a maximum of 90% recovery at severity log R_0 > 11.39. Authors observed a change in the ratio of acid insoluble lignin (AIL) to acid-soluble lignin (ASL), reporting that higher severity lead to increase in acid insoluble lignin with the corresponding decrease in acid soluble lignin.

The percentage of lignin increased from 29.3 to 40.3% with increasing pretreatment severity mainly due to the solubilization of xylan while the cellulose and lignin remain in the solids recovered after pretreatment. In the range of pretreatment severity of log R_o ¼ 8.25–12.51, 75–85% of the lignin initially present was recovered in pretreated solids. However, at the severity of log R_o > 11.39, the apparent lignin recovery increased to 90% (Ko *et al.*, 2015).

Kristensen et al. (Kristensen *et al.*, 2008) reported re-localization of lignin in response to hydrothermal pretreatment that subsequently makes cellulose susceptible to enzymatic hydrolysis. Fernandez et al. (Fernandez-Cegri *et al.*, 2012) indicated that lignin tends to concentrate in the pretreated solid primarily owing to solidification and re-deposition on cooling, authors observed 33% lignin in pretreated solid at severing pretreatment conditions of 200 °C in comparison to 14% of untreated sunflower oil cake. Sun et al. (Sun *et al.*, 2014) reported an increase in klason lignin up to a maximum value of 41.7% in a corncob.

3.4 Impact of hydrothermal pretreatment on hydrolysate on liquid fraction

Thermal and thermochemical pretreatment of lignocellulosic biomass results into two fractions: (a) solid fraction rich in cellulose, lignin, and residual organic acids and (b) liquid fraction mainly containing xylose, organic acids released, phenolic compounds; vanillin, syringaldehyde and other lignin derived phenolics, and furanic compounds; furfural, 5-hydroxymethylfurfural (5-HMF) (Hendriks and Zeeman, 2009; Du *et al.*, 2010; González-Fernández *et al.*, 2012; Sambusiti *et al.*, 2013). Furanic compounds are known to be detrimental for archaea and bacteria by inhibiting cell growth, damaging DNA, and inhibiting enzymes of glycolysis pathway. (Palmqvist and Hahn-Hägerdal, 2000; Almeida *et al.*, 2009). Furfural found to be more toxic than 5-HMF owing to its lower molecular weight that facilitates easy penetration into microbial cell membrane (Quéméneur *et al.*, 2012). Toxicity of Lignin-derived phenolic inhibitors directly related to molecular weight; low molecular weight phenolic compounds are more lethal than high molecular weight phenolics (Jönsson and Martín, 2016).

The degradation products; furfural, 5-HMF (5-hydroxymethylfurfural), acetic acid, and formic acid are an undeniable fact during chemical or physicochemical pretreatment; hydrothermal pretreatment is not an exception. Furfural is a pentose, while 5-HMF is a hexose dehydration product. Former can be converted to 2-furoic acid through hydraulic fission of aldehyde groups, while latter can be transformed to equimolar amounts of levulinic acid and formic acid under acid conditions at elevated temperatures (Clark and Mackie, 1984; Ulbricht *et al.*, 1984; Nitsos *et al.*, 2013).

Mechanism of inhibitory products formation during hydrothermal pretreatment is presented in Fig. 3.2. 5-HMF and furfural, under anaerobic fermentation condition, are mainly converted to their corresponding alcohols, furan dimethanol, and furfuryl alcohol, respectively, while aerobic metabolism in *Saccharomyces cerevisiae* converts furfural to furoic acid (Sárvári Horváth *et al.*, 2003; Liu *et al.*, 2004).

A lower concentration of 5-HMF has been reported in hydrolysate due to limited hexose degradation in comparison to furfural (Chandel *et al.*, 2011). Phenolic compounds production is related mostly to lignin degradation. Production of these undesirable compounds may be prevented by careful optimization of process parameters of pretreatments. Maintaining pH between 4 - 7 minimizes the risk of inhibitory products formation (Hendriks and Zeeman, 2009). Production of these undesirable inhibitory products is widely dependent upon nature of lignocellulosic biomass, reaction conditions, and reaction time of hydrolysis (Palmqvist and Hahn-Hägerdal, 2000). El Hage et al. (El Hage *et al.*, 2010) studied autohydrolysis on *Miscanthus x giganteus*, reported that furans increase steadily at mild conditions (130-140 °C), while the substantial increase was observed to a maximum of 5.5% at 150 °C for 20 h.

Hydrothermal pretreatment of sweet sorghum was conducted by Sun and colleagues (Sun *et al.*, 2015). The authors reported that the concentrations of acetic acid (4.03 g L^{-1}) and furfural (4.53 g L^{-1}) were significantly higher in the severest pretreatment condition (230 °C, 30 min). Furthermore, it was noted that degradation products closely associated with pretreatment temperature and time; increasing temperature at a constant time or vice versa. The authors observed lower 5-HMF concentration in comparison to furfural indicating limited cellulose solubilization and consequent glucose formation. Table 3.3 presents various possibilities of hydrolysate composition in response to hydrothermal pretreatment of various feedstocks.

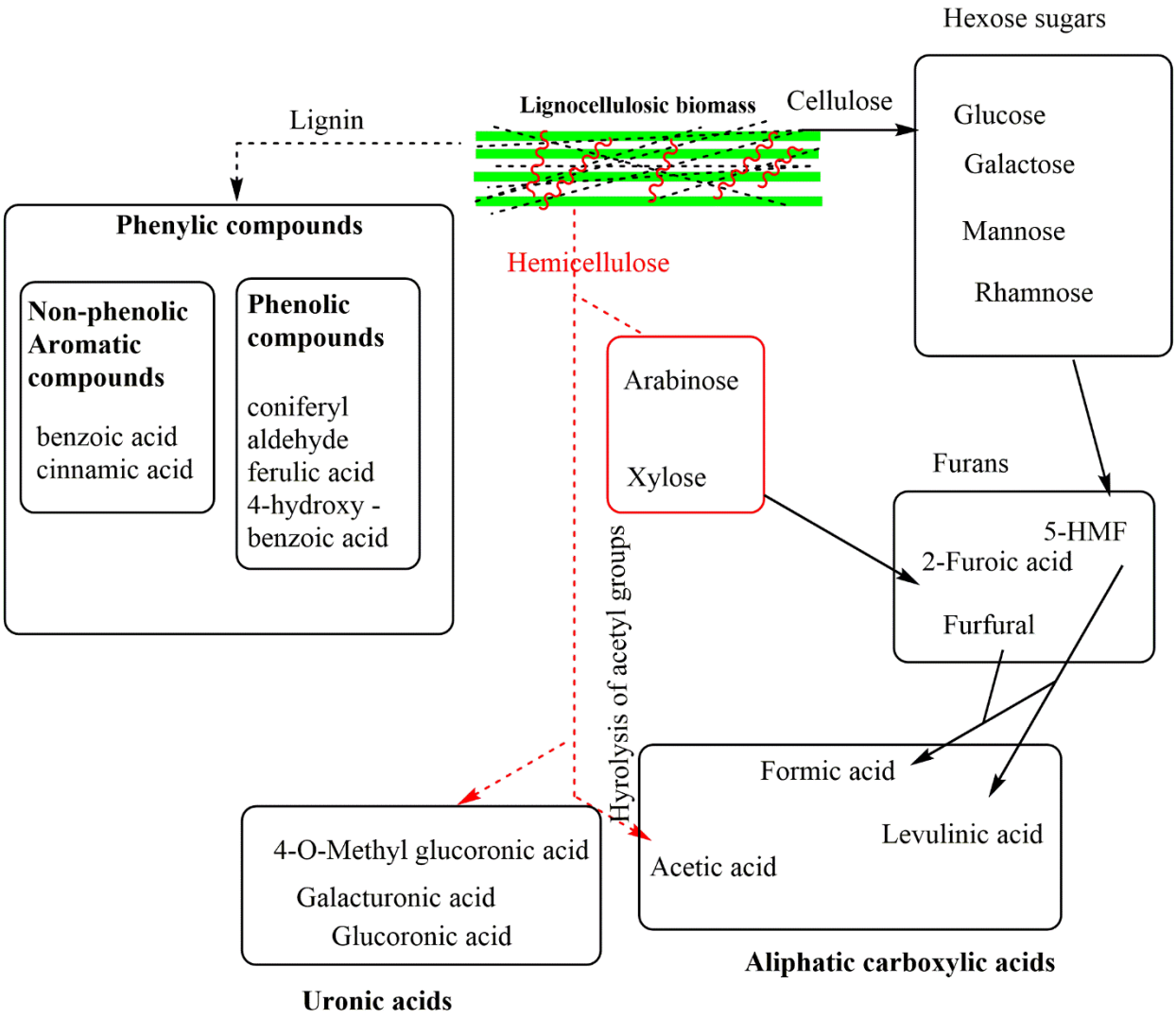

Figure 3.2 - Mechanism of inhibitory compounds production during hydrothermal pretreatment modified after (Jönsson and Martín, 2016)

Table 3.3 - Hydrolysate composition in response to hydrothermal pretreatment of various feedstocks

Feedstock	Chemical addition	Pretreatment conditions	Furfural g L^{-1}	5-Hydroxymethylfurfural g L^{-1}	Acetic acid g L^{-1}	Reference
Sugarcane bagasse	-	195 °C, 10 min	0.59 ±0.10	0.03±0.01	2.03±0.40	(de Moutta *et al.*, 2013)
Straw	-	195 °C, 10 min	0.81±0.15	0.33±0.01	1.93±0.10	(de Moutta *et al.*, 2013)
Yard waste	-	WS[a], AIS[b], AcS[3] 180°C, 30 min	0.08, 0.00, 0.87 (mg mL^{-1})	0.09, 0.00, 0.90	2.75, 2.35, 1.80 (mg mL^{-1})	(Wangliang *et al.*, 2014)
Beach wood	-	130°C - 220°C, 15 - 180 min	0.03 - 2.73 (mg mL^{-1})	0.03 - 0.84	0.08 - 3.41 (mg mL^{-1})	(Nitsos *et al.*, 2013)
Corn stover	-	200°C, 5 min – 60 min	4.8 – 21.5 mg g^{-1}	0.42 – 3.2 mg g^{-1}	-	(Saha *et al.*, 2013)
Wheat straw	-	170°C – 200°C 0 min – 40 min	<0.1 – 3.32 g 100 g^{-1} untreated material	<0.1 – 0.50 g 100 g^{-1} untreated material	0.51 – 3.86 g 100 g^{-1} untreated material	(Pérez *et al.*, 2007)
Eucalyptus urophylla	-	100°C – 240°C 15 min – 60 min	ND[d] – 1.45	ND – 1.27	ND – 3.28	(Sun *et al.*, 2014)
Switchgrass	-	190°C – 210°C 10 min – 20 min	<0.2 – 0.91	<0.15 – 0.79	-	(Lilis *et al.*, 2009)
Sugarcane bagasse	-	180°C – 295°C	2.8 - 18.2	0.9 – 3.9	1.1 – 2.6	(Rocha *et al.*, 2013)
Corn Stover	Acetic Acid (0 – 400 g Kg^{-1} DM)	195 °C 15 min	0.19 – 1.74	0.02 – 0.23	-	(Xu *et al.*, 2009)
Sugarcane press mud	-	140 – 210 °C 2 – 23 min	1.21	0.034 – 0.362	6132 mg L^{-1}	(González *et al.*, 2014)
Corn cob	-	170 – 230 °C	5.13	1.27	-	(Sun *et al.*, 2014)
Eucalyptus camaldulensis	Aqueous $AlCl_3$ (0.02 M)	140 – 180 °C 1 h	1.31 – 3.05	0.53 – 1.53	2.63 – 5.23	(Shen *et al.*, 2016)

[a] Hot compressed water, [b] 2.1g NaOH, [c] 1.4 mL H_2SO_4 [d] Not detected

3.5 Impact of hydrothermal pretreatment on morphological structure of biomass

Morphological structure of lignocellulosic biomass is an important aspect of studying the chemical components distribution and their inter-linkages (Hon and Shiraishi, 2001). The in-depth morphological study would help to broaden the knowledge about physiochemical properties of lignocellulosics in question. Furthermore, it would help to enhance lignocellulosic bioconversion and understand enzyme accessibility during downstream processing

A number of techniques have widely been employed to visualize structural changes, including Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR), X-ray powder diffraction (XRD), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). The collective use of FTIR and NMR can provide detailed structural elucidation of pretreatment impacts on lignocellulosic, SEM is used to visualize morphological changes (Wi *et al.*, 2015), while XRD is used to estimate crystallinity index (Zhang *et al.*, 2014).

Structural disruption of the lignocellulosic matrix is a complex process to understand at the molecular level. Xylan dissolution mechanism during hydrothermal pretreatment has recently been reported by transmission electron microscopy at a ultra-structural level that will advance our understanding of reaction mechanisms impact on the structural morphology of biomass (Ma *et al.*, 2015).

Amorphous cellulose is relatively easy to solubilize than of crystalline cellulose. Deconstruction of cellulose involves swelling under concentrated acid condition and breakage of glycosidic bonding. Cellulose chain cleavage started when temperature slightly exceeds to 150 °C during hydrothermal pretreatment (Ma *et al.*, 2013). Decomposition of cellulose occurs within a temperature range of 250-400 °C with maxima at 334 °C, while hemicellulose and lignin have maximum deconstruction rate at 375 °C and 296 °C, respectively (Nitsos *et al.*, 2013). Moreover, authors reported that at mild temperature conditions no significant morphological changes were observed while at higher severity log $R_0 \geq 3.5$ significant morphological changes were observed under SEM microimages indicating lignin removal and recondensation in droplets forms on the surface of particles.

To visualize the impact of hydrothermal pretreatment on ultrastructure and molecular organization of biomass, Kristensen et al. (Kristensen *et al.*, 2008) reported studies on wheat straw in comparison to SO_2 impregnated steam explosion.

A hypothesis was put forward by Donohoe et al. (Donohoe *et al.*, 2008) for the appearance of lignin droplets on the surface of cell wall after hydrothermal pretreatment based on their study on corn stover after comparing several techniques; FTIR, NMR analysis, antibody labeling, and cytochemical staining. The authors hypothesized that whenever thermochemical pretreatment temperature goes above the lignin phase transition temperature range, it causes lignin to coalesce into larger molten bodies and make it migrate within and out of the cell wall and it can redeposit on the surface of the cell wall. Kumar et al. (Kumar *et al.*, 2013) proposed another hypothesis that spherical droplets are pseudo-lignin derived from carbohydrate like xylan components at high severity hydrothermal pretreatments.

The degree of polymerization and chain break in bamboo as a result of hydrothermal pretreatment were studied by Ma et al. (Ma *et al.*, 2013). The authors observed that cellulose degradation is favored at a higher temperature as indicated by Xiang and co-workers (Xiang *et al.*, 2003). Hardwood was pretreated hydrothermally at different severities by Ko et al. (Ko *et al.*, 2015) to study its impact on enzymatic cellulose hydrolysis and lignin properties. The authors compared SEM micro images of the untreated and pretreated sample and observed fragmentation and disruption in the morphology of treated samples as compared to the flat and smooth surface of untreated hardwood. Spherical droplets of lignin were noticed on the surface even on the corners of the disrupted cell thus might be a source of a physical barrier for impeding cellulose hydrolysis.

Date palm (*Phoenix dactylifera L.)* leaflets and rachis were pretreated with hydrothermal pretreatment at four temperature levels; 180, 190, 200, and 210 °C for a holding time of 10 min to evaluate pretreatment impact on enzymatic digestibility and bioethanol potential in addition to assessing pretreatment severity effect on morphological changes (Fang *et al.*, 2015). By SEM images, a significant difference between untreated and pretreated samples was observed. Rachis surface was found to be more irregular in contrast to leaflets at same severity condition. Furthermore, the structure of leaflets and rachis showed a variety of smaller fragments in response to hydrothermal pretreatment. Pretreatment at 210 °C depicted significant morphological and structural changes than 180 °C for both leaflets and rachis. High severity results in significant cell

wall destruction thereby increasing surface area for enzymatic hydrolysis and improve lignocellulosic bio-convertibility (Ding *et al.*, 2012; Li *et al.*, 2014).

Untreated and liquid hot water pretreated (170 – 230 °C) corncob samples were visualized for SEM images to look into morphological characteristics. A substantial cell wall disruption was observed for pretreated samples comparative to compact surface structure and intact morphology of untreated ones. Authors noted minimal structural disruption at lower temperature (170 – 180 °C), fiber length reduction at moderate temperature (190 °C) while complete cell wall disruption at high temperature (above 190 °C), lignin droplets were also observed on cell wall surface for samples pretreated at 220 °C (Sun *et al.*, 2014)

A comparison of hydrothermal and wet explosion pretreatment was studied to map and characterize changes in lignin by Kaparaju and Felby (Kaparaju and Felby, 2010) with the help of microscopy (atomic force microscopy, scanning electron microscopy), and ATR-FTIR (Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy). The authors reported substantial removal of hemicellulose in both pretreatments, but the values observed slightly higher for hydrothermal than wet explosion pretreatment. This might be due to the combined effect of counter-current water flow system and partial hydrolysis of cellulose during hydrothermal pretreatment

3.6 Anaerobic Digestion: process and affecting parameters

Anaerobic digestion is a series of processes governed by several bacterial and archaeal guilds to break down organic matter in the absence of oxygen to produce clean energy, methane, along with carbon dioxide plus hydrogen, hydrogen sulfide and new bacterial biomass (Kavuma, 2013; De Vrieze and Verstraete, 2016). A comprehensive review of the biochemical reactions and microbiological perspective of the digestion process is beyond the scope of this text.

The readers are referred to Adekunle and Okolie (Adekunle and Okolie, 2015) for in-depth study of the biochemical process of anaerobic digestion and to Vrieze and Verstraete (De Vrieze and Verstraete, 2016) for microbiological perspectives. A brief overview of anaerobic digestion process and affecting parameters is presented in this text. Hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Fig 3.3) are the key steps of the digestion process, based on the chemical reactions undertaken by various obligatory and facultative anaerobes (Yi *et al.*, 2014). The

products of one-step are taken up as substrate by microbial community of the subsequent step. Therefore, the imbalance between chemical reactions of microbes will influence microbial kinetics, substrate consumption rate, final product formation, and finally could lead to system failure (Franke-Whittle *et al.*, 2014; Adekunle and Okolie, 2015).

Several factors including physical, chemical, and biological could affect the digestion process. Alteration in substrate characteristics, organic loading rate, temperature fluctuation, pH, nutrients, and mixing conditions can have an influencing impact on biodegradation rate, biogas compositions, substrate utilization, and specific microbial growth rate.

The biogas yield, composition, and success of digestion process are strongly dependent upon the feed characteristics in terms of its constituents protein, fat (Schnürer and Jarvis, 2010), and carbohydrates; cellulose, hemicellulose, and lignin (Hartmann and Ahring, 2006). Moreover, substrate particle size, biodegradability, total solids, and volatile solids play a vital part in anaerobic digestion.

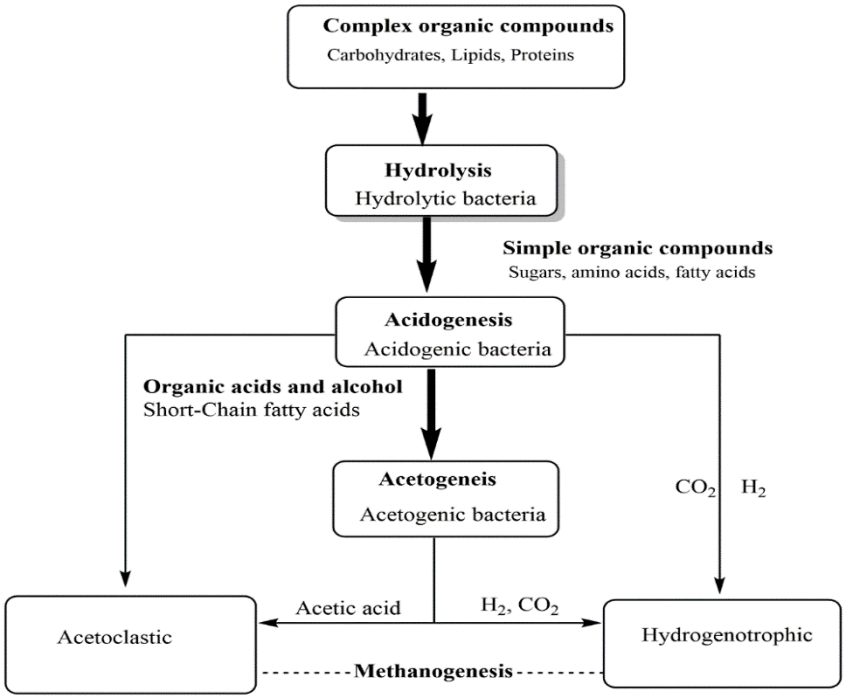

Figure 3.3 - Schematic conversion of macromolecules into biomethane in anaerobic digestion process (modified after (Yi et al., 2014))

The pH value is an important parameter and indicator of process stability. It has a significant impact on the microbial growth (Yadvika *et al.*, 2004). Since a consortium of

microorganisms is involved in biogas production, their pH requirements vary widely. Microorganisms can be divided into two groups based on pH optima for anaerobic digestion. Acidogenic bacteria prefer a pH range of 5.5 – 6.5, while methanogens thrive best at 7.8 – 8.2 (Khanal, 2008). However, methanogens have been reported to remain active outside this range (Whitman *et al.*, 2006). pH control is vital to the bacterial growth and conversion processes in digestion.

In single stage digestion reactors, pH is maintained at neutral since methanogens are very susceptible to pH change since microorganisms of both groups are present at this stage. Methane generation rate may decrease if pH is lower than 6.3 or higher than 7.8 (Stronach *et al.*, 1986). However, studies are reported for acidophilic (Bräuer *et al.*, 2006) and alkaliphilic methanogens (Sorokin *et al.*, 2015).

Microorganisms, like other organisms, require nutrients for their growth, activity, and to perform metabolic processes. A balanced anaerobic digestion system necessitates mix of nutrients, both macro and micro, to fulfill the microbial needs involved in the process (Goodwin *et al.*, 1990; Takashima *et al.*, 1990). Nutrients fall into two categories based on their requirement; a) macronutrients, required in substantial amount, carbon, nitrogen, hydrogen, phosphorus, potassium, and sulphur (Kayhanian and Rich, 1995), and micronutrients; cobalt, zinc, molybdenum, nickel, iron, copper, selenium, and tungsten and also vitamins, required in minute quantities but essential for growth (Rivard *et al.*, 1989; Kayhanian and Rich, 1995). The trace elements requirement by methanogens, both acetoclastic and hydrogenotrophic, is not entirely understood. Therefore, it presents toxic or inhibitory effects during digestion if exceed a threshold level of microbial requirement.

The anaerobic process requires lower macronutrients than aerobic process owing to lower biomass yield while degrading equal amount of waste. A single nutrient recipe is not available, as substrates having different chemical composition require specific nutrients for the digestion process. Nutrient requirements can be determined with COD (Chemical Oxygen Demand) of the substrate, COD:N ratio. A COD:N ratio of 350:7 and 1000:7 is recommended for highly and low loaded system (Henze and Harremoës, 1983). Most authors prefer to cite as C/N ratio in literature. C/N ratio lies in the range of 10-30 with an optimum value of 25-30:1 for the digesters to work at full potential (Mital, 1996; Yadvika *et al.*, 2004). In the case of high C/N ratio digester will be

deficient in nitrogen impeding microbial growth (Alvarez and Lidén, 2008) while in the other circumstances degradation leads to ammonia inhibition (Hartmann and Ahring, 2006), VFA (Volatile Fatty Acids) accumulation, and ultimately reactor failure (Franke-Whittle *et al.*, 2014).

3.7 Hydrothermal pretreatment in relation to subsequent methanogenesis

Hydrothermal pretreatment parameters; temperature, reaction time, total solids, and pH are aspects of paramount importance in relation to pretreatment efficiency and subsequently on anaerobic digestion. Furanic (sugar monomers degradation products) and phenolic compounds (lignin degradation products) are undeniable products during pretreatment. These products are toxic and can possibly inhibit growth mechanism of bacteria and methanogenic archaea (Ghasimi *et al.*, 2016). Whereas Barakat et al. (Barakat *et al.*, 2012) reported contradictory results by demonstrating the adaptability of microbial consortia to phenolic and furanic compounds. Lignin-derived phenolics specifically inhibit hydrolytic enzymes cellulase, glucanase, and xylanase (Berlin *et al.*, 2006).

Massive volatile fatty acids, ammonia nitrogen and amino acids released into liquid hydrolysate at a higher severity. Higher VFAs; propionic acid 900 mg L^{-1} could stop the methanogenesis (Wang *et al.*, 2009). Pretreatment parameters can be optimized to a lower production of inhibitory compounds, maximizing sugar yield, and ultimately higher digestion efficiency.

Hydrothermal pretreatment recently gets more attraction in lignocellulosic pretreatment as it did not require particle size reduction resulting in cost reduction at large scale (Weil *et al.*, 1997; Taherzadeh and Keikhosro, 2008). Use of hydrothermal pretreatment for biogas production is not only limited to energy crops (Koponen, 2010) and agricultural residues (Grimaldi *et al.*, 2015), but also expands to algal biomass (Passos and Ferrer, 2015). Qiao et al. (Qiao *et al.*, 2011) reported an increase of 67.8, 18.5, 13.3, and 7.8% biogas in hydrothermally pretreated (170 ºC, 1 h) municipal sewage sludge, fruit/vegetable waste, cow manure, and pig manure, respectively. The authors also observe 3.5% decrease in biogas with 6.9% reduction in methane content for food waste under same pretreatment conditions; this might be a result of valorization of readily degradable feedstock during hydrothermal pretreatment.

In another study, hydrothermal pretreatment was carried out on sugar beet pulp to enhance biogas production. Zieminski et al. (Zieminski *et al.*, 2014) investigated the pretreatment of sugar beet pulp (100 g TS) conducted at 120, 130, 140, 150, 160, 170, and 200 ºC in a 600 mL thermostatic reactor for 20 min under varying pressure range. Batch digestion experiments of pretreated hydrolysate were carried out in glass chambers of 1 L working volume. The authors observed the highest cumulative methane yield of 502.50 L CH_4 kg^{-1} VS from 160 ºC pretreated pulp. The highest free glucose release of 3.29 mg mL^{-1} was observed at 160 ºC, 4 folds higher than 120 ºC.

Rice straw, a potential AD (Anaerobic Digestion) feedstock, was studied by Chandra et al. (Chandra *et al.*, 2012) using two different pretreatments: (1) NaOH, and (2) hydrothermal followed by NaOH addition. NaOH pretreatment was given at 3% NaOH for 120 h at 37 ºC in a temperature controlled incubator at 10% TS (20 g). Hydrothermal pretreatment was conducted at 200 ºC with a residence time of 10 min under pressure of 1.55 MPa in a SUS (Steel Used Stainless) 316 austenitic steel reactor followed by 5% NaOH to adjust the pH of pretreated slurry for subsequent AD experiments. The pretreated material with both pretreatments was subjected to digestion for 60 days under mesophilic conditions (37 ºC) with a C/N ratio of 25. The study results revealed a biogas production of 140, and 184 L kg^{-1} VS a for untreated and 3% NaOH pretreated, wheat straw while highest biogas production (315.9 L kg^{-1} VSa) was observed for hydrothermal pretreatment followed by 5% NaOH addition.

Poplar leaves, considering yard waste, were primarily screened to remove plastics, sticks, and metals. These were then hydrothermally pretreated using three catalysts; hot compressed water, acidic solution, and alkaline solution, to investigate the effect on anaerobic digestion in batch reactors. The batch reactors were maintained at mesophilic conditions (37 ºC) with an inoculum to substrate ratio of 1 mL g^{-1} for a period of 32 days. The highest concentration of glucose and xylose were observed in the acidic pretreated sample. Biogas yield for samples pretreated with an alkaline solution, acidic solution, and hot compressed water was increased by 364, 107, and 79%, respectively in comparison to the untreated sample. Furthermore, authors noted a decrease in the COD in liquid phase followed the same order as of biogas yield (Wangliang *et al.*, 2014).

Sunflower oil cake was studied by Fernandez et al. (Fernandez-Cegri *et al.*, 2012) for hydrothermal pretreatment. The study was aimed at to elucidate the impact of different

temperatures (25, 100, 150, and 200 ºC) of hydrothermal pretreatment on the chemical composition of cake and subsequent biomethane potential tests. Batch digestion reactors were conducted for pretreated solids and hydrolysate samples obtained after separation of pretreatment slurry at inoculum to substrate ratio of 2 (2 g inoculum / 1g substrate) on VS basis at mesophilic conditions (35 ± 1 ºC). The authors reported hemicellulose decrease in solid content from 13 to 6% while an increase was observed in lignin content by 16%, for pretreatment temperature range of 25 – 200 ºC. The pretreatment conducted at 100 ºC reported representing highest methane yield, 6.5% higher, based on combined methane yields from solid and hydrolysate.

A dedicated crop Giant reed (*Arundo donax)* was subjected to a hydrothermal pretreatment by Giuseppe and co-workers (Giuseppe *et al.*, 2013) to evaluate its potential for biogas production. Three conditions were tested for hydrothermal pretreatment; uncatalyzed, 24 h impregnation of the substrate with 2% (w/w) H_2SO_4, and immediate addition of 2% (w/w) H_2SO_4 before steam cooking for pretreatment parameters; temperature (150 and 180 ºC) and time (10 and 20 min). Results of subsequent batch digestion tests, conducted with 4 g VS L^{-1} under thermophilic conditions (53 ºC) for a period of 39 days, revealed into methane yield of 273 mL g^{-1} VS added for untreated biomass. Reactors with uncatalyzed biomass achieved a yield of 23% at temperature/time combination of 180 ºC/10 min. Conversely, the reactors fed with catalyzed biomass were observed methanogenic inhibition. The inhibition might be due to competition with sulfate reducing bacteria (SRB) as sulfate concentration was observed in the hydrolysates of acid-catalyzed pretreatments.

Jorn and his team (Jörn *et al.*, 2014) conducted thermobarical pretreatment on solid cattle manure and liquid cattle manure obtained from two different location in Germany to evaluate its efficiency on the biomethanation. Pretreatment was performed in a mini reactor (600 mL) at a temperature range of 140, 160, 180, 200 & 220 °C for a retention time of 5 min. The authors observed the high concentration of inhibitory compounds (furfural, 5-HMF, and phenolic compounds) at a temperature of 220 °C; resulting into lower degradability and lower methane output. Authors claimed 58% increase in the methane yield with pretreatment conducted at 180 °C. Furthermore, reported an optimum temperature of 164 °C based on regression analysis of methane generation rate and methane yield.

3.8 Microbial community structure of anaerobic digestion and molecular fingerprinting techniques

Anaerobic digestion is a microbial mediated process involving bacterial and archaeal communities working in close relation, as both communities need each other to degrade the complex organic compounds and for their own survival. The process is initiated by the hydrolytic bacteria by converting complex organic compounds into simpler ones. Carbohydrates, proteins and lipids are converted into simple sugars, amino acids and fatty acids, respectively as depicted in Fig 3.3. Input material dictates the bacterial community composition within the reactor for the hydrolytic stage, whilst, the temperature is the key driver for microbial community diversity and evenness (Leven *et al.*, 2007). Digesters treating cellulosic material are dominant with *Clostridium* spp. along with *Bacteroidetes* and *Proteobacteria* but at lower abundance (Carballa *et al.*, 2015). *Clostridium* spp. and other members of *Firmicutes* are abundant in lipid-rich digesters (Zakrzewski *et al.*, 2012). Whilst, *Bacillus* spp. *Clostridia* spp., and representatives of *Proteobacteria* were found in protein-rich reactors (Kovács *et al.*, 2015).

Identification of specific organism about specific biochemical-stage of digestion process has been a challenging task due to culture restraints. It is quite impossible to generate a pure culture from syntrophic interactions. Recent advancements in the molecular biology, specifically regarding DNA sequencing technology has made an in-depth study of microbial community structure possible. Furthermore, microbial community characterization and monitoring is currently the theme of research to increase methane output and capacity of digesters (Vanwonterghem *et al.*, 2014).

The emergence of 16S rRNA as universal target led to a new era in taxonomic classification of microorganisms based on DNA sequencing information (Woese and Fox, 1977). Sequence divergence of the ribosomal small subunit RNA (16S rRNA) is the basis for culture-independent techniques (Nayak *et al.*, 2009). This approach classified methanogens into a new Archaeal Domain. Recently, other universal targets have also been developed based on bacterial type I *cpn60 (Hill et al., 2004)* and archaeal type II thermosome chaperonins (Chaban and Hill, 2012). These chaperone proteins are highly conserved for both archaea and bacteria and help in protein folding (Hemmingsen *et al.*, 1988). Groups of microorganisms with a similar taxonomic relationship based

on DNA sequences put together in an operational taxonomic unit (OTU). Phylogenetically, OTU permits greater resolution of closely related microorganisms (Links *et al.*, 2012).

A number of fingerprinting techniques have been developed to study the microbial diversity and structure; amplified ribosomal DNA restriction analysis (ARDRA), single strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (TRFLP), and ribosomal intergenic spacer analysis (RISA). Fluorescence in situ hybridization (FISH), DNA microarray, and sequencing are the tools for microbial identification. DGGE could give a snapshot of microbial community, but it is not suitable to identify changes in microbial community at specie level (Ercolini, 2004)

DGGE technique is generally applied to separates complex mixtures of 16S rRNA gene amplicons of the same length having different sequences. The mixture of 16S rRNA amplicons is applied on a polyacrylamide gel with a linearly increasing gradient of denaturant (formamide or urea). An electric current is applied and the amplicons migrate though the gel. First, the fragments travel according to their molecular weight and as they are exposed to the increasing concentration of denaturant, the DNA strands begin to denature. At their specific melting point their migration stops. Therefore, separation of the fragments is the consequence of different melting temperatures according to their DNA sequence variations. The separated strands could be excised and directly utilize for sequencing after following proper protocol.

4 MATERIALS & METHODS

The Study was planned according to the following flow-chart diagram Fig 4.1

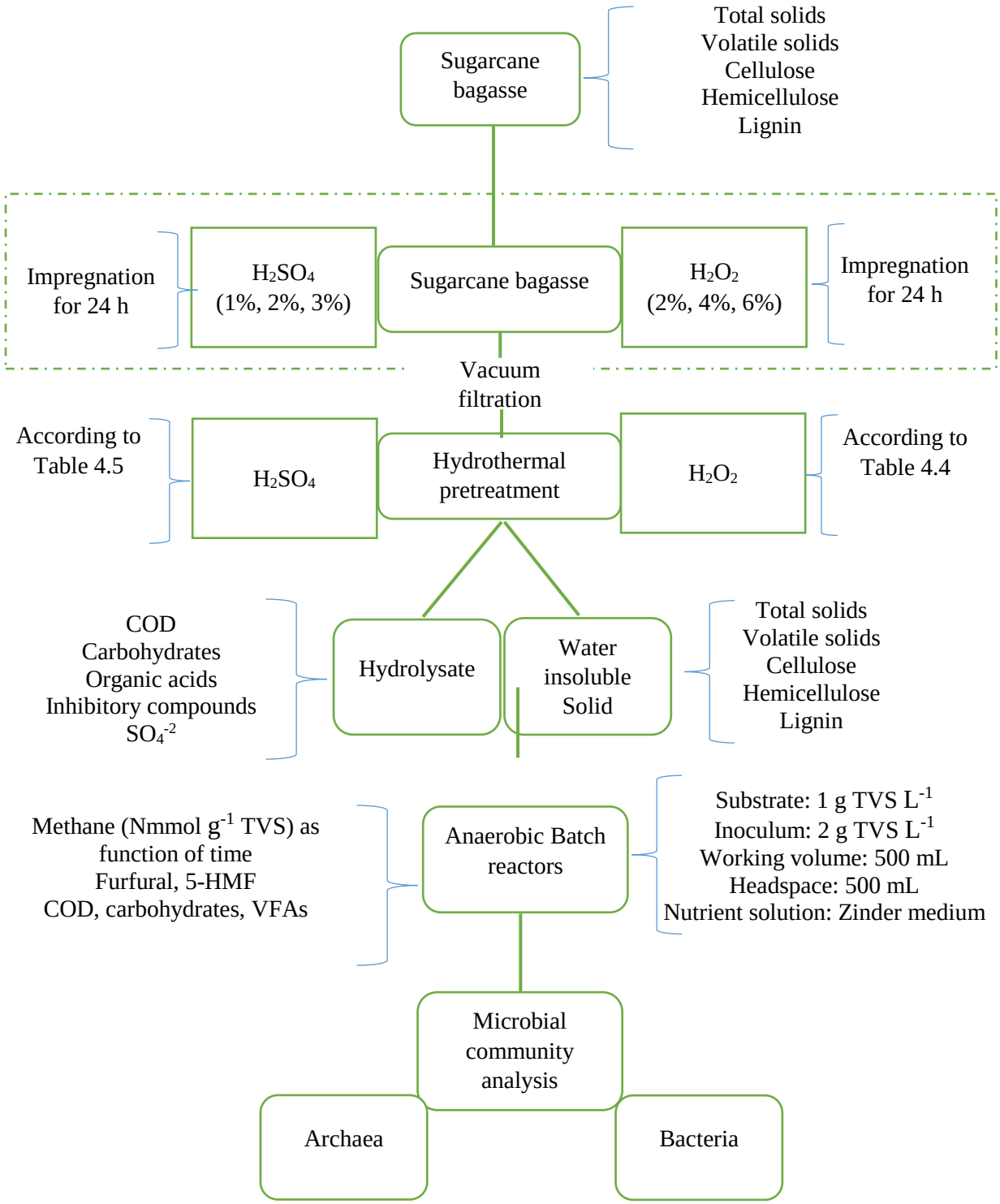

Figure 4.1 - Flow-chart diagram of experiments

4.1 Substrate

Sugarcane bagasse was generously provided by Laboratório Nacional de Ciência e Tecnologia do Bioetanol, Campinas - Brazil. It was dried at 37 ºC in an incubator and afterward stored in plastic bags at ambient temperature until further use for pretreatment and compositional analysis.

4.2 Hydrothermal pretreatment

4.2.1 Substrate impregnation

Sugarcane bagasse was impregnated with sulfuric acid and hydrogen peroxide at solid to liquid ratio of 1:10 in plastic beakers according to pretreatment index presented in Table 4.4 and 4.5. The beakers were sealed with paper film strengthened by rubber wires. The beakers were left for 24 h at room temperature under static conditions. Sugarcane bagasse was separated by vacuum filtration, afterward. In the case of hydrogen peroxide, pH was raised to 11.0 with 5 M NaOH before impregnation. Acid pretreatment will be indexed as A-HSO, B-HSO, C-HSO, and so on till P-HSO. On the other hand, hydrogen peroxide pretreatment will be indexed as A-HO, B-HO, C-HO, and so on till P-HO".

4.2.2 Description of the hydrothermal reactor

The hydrothermal reactor was built in-house by Departamento de Engenharia de Mecânica, Universidade de São Paulo, São Carlos – Brazil. A brief description of the reactor is presented as follow: The reactor was made of steel with a capacity of 150 mL. The reactor (Fig 4.2) consists of a temperature control panel, pressure gauge to measure the inside reactor pressure, pressure valve to release the pressure, and a cyclone-shaped receiver to receive the pretreated biomass.

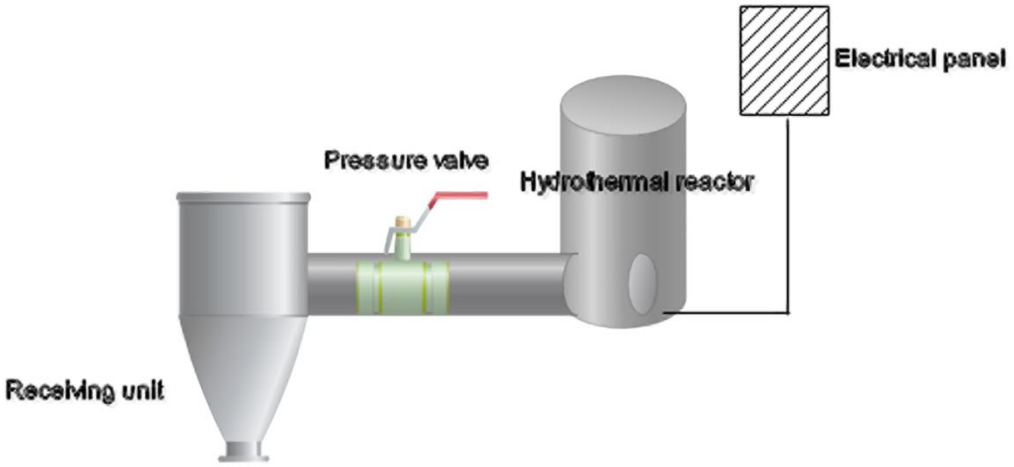

Figure 4.2 - Schematic diagram of hydrothermal reactor

4.2.3 Pretreatment process

Before each pretreatment, the reactor was flushed with water and preheated. Approx 12 g of impregnated bagasse was loaded to hydrothermal reactor according to the Tables 4.4 and 4.5 with 110 mL distilled water. After substrate loading, the heating system was initiated. The countdown started after the temperature was reached to required pretreatment temperature of 160, 180, 200, 146.4 and 213.6 °C (Table 4.4 & 4.5). Pressure (bar) was recorded. At the same time, the countdown started. After defined time of 5, 12, 19, 0.24, and 23.76 min for different pretreatments (Table 4.4 & 4.5), the heating system was switched off and pressure valve was released at once to sudden decrease the pressure inside the reactor that further dismantle the lignocellulosic structure. Pretreated biomass was recovered from the wall of the cyclone while hydrolysate was obtained from the plastic basket placed under the cyclone. Furthermore, the solid fraction was separated from the liquid (hydrolysate) via filter paper. A part of the solid fraction was transferred to pre-weighed crucibles to dry at room temperature for structural analysis. Total recovered solid fraction after each pretreatment was recorded to calculate solid recovery. Both fractions were stored at 4 °C until further analysis and batch digestion.

4.3 Inoculum

The inoculum was granular sludge obtained from Upflow Anaerobic Sludge Blanket (UASB) reactor treating poultry wastewater – Dacar, Tiete, São Paulo. Inoculum was transported

from the facility to Laboratório de Processos Biológicos (LPB) USP Campus 2 in 30 L plastic bottles and subsequently stored in cold storage room, temperature maintained at 4 °C. Inoculum was macerated in household juicer followed by TS and VS characterization.

4.4 Characterization of solid biomass

Pretreated solid recovered after filtration of pretreatment slurry was equilibrated to moisture content (4.2 – 6.6%), and solid recovery (%) was determined by equation 4.1 according to de Vasconcelos et al. (de Vasconcelos *et al.*, 2013)

$$Solid\ recovery\ (\%) = \ (Solid\ recovery\ (g)\ after\ pretreatment\ on\ dry\ matter\ basis)\ /\ (Raw\ bagasse\ \ (g)\ \ on\ DM\ bais) \times 100 \qquad (4.1)$$

Total solids and volatile solids of untreated bagasse and WIS (water insoluble solid) fraction of pretreatment slurry were determined according to Standard Methods procedures 2540 B and 2540 E (APHA *et al.*, 2005).

4.4.1 Compositional analysis

Chemical characterization of biomass (sugarcane bagasse) is very important in order to determine its biomethane potential. Sugarcane bagasse was milled using household juice maker and passed through 2.5 mm sieve for compositional analysis. Cellulose (glucan) and hemicellulose (xylan+arabinan) were determined following Sluiter protocol (Sluiter *et al.*, 2008) available online (http://www.nrel.gov/docs/gen/fy08/42623.pdf). Briefly, 0.16 g solid was mixed with 1.5 mL H_2SO_4 (72 % w/w) in digestion tube placed in a water bath pre-adjusted to 30 °C for 1 h. The contents were mixed at intervals of 0, 20, and 40 min. The reaction was terminated by adding 42 mL distilled water to make H_2SO_4 concentration to 4 % w/w. The contents of the tube were autoclaved for 1 h at 120 °C. Afterwards, the contents were filtered through 0.2 µm glass fiber filter (Sartorius stedim Biotech®) in pre-ashed porcelain crucibles. The solids obtained were dried at 105 °C and reported as % Klason lignin (Equation 4.6) corrected to ash (550 °C, 3 h). The filtrate obtained was stored at -20 °C after neutralization with $CaCO_3$ for HPLC. Glucose and xylose were separated on Aminex HPX-87H (Bio-Rad, Hercules, USA) column (300 mm × 7.8 mm). All samples were passed through Sep-Pak C18 cartridges and filtered through 0.2 µm syringe filter. Ultrapure water with 0.005 N H_2SO_4 was used as mobile phase with a flow rate of 0.5 mL/min, and the oven temperature was maintained at 43 °C.

Glucan, xylan, and arabinan in the untreated and pretreated solid fraction were determined according to formulas described in NREL protocol. Sugar recovery standards after dilute acid hydrolysis was calculated according to Equation 4.2

$$\%\, R\_sugar = \ (concentration\ detected\ by\ HPLC, mg/L)\ / \ (known\ concentration\ of\ sugar\ before\ hydrolysis\ \times 100) \quad (4.2)$$

Sugars in hydrolyzed samples were calculated using the Equation 4.3

$$C\_X = \ (C\_HPLC \times Dilution\ factor)\ /\ (\%R\_sugar/100) \quad (4.3)$$

Where

C_HPLC = Concentration of sugars determined by HPLC

$\%R\_sugar =$ Recovery of specific sugar component

$C\_X =$ Concentration of sugar (mg L^{-1}) in hydrolyzed sample after correction for loss on 4% hydrolysis

Polymeric sugars concentrations were determined using anhydro correction factor; 0.88 for xylan and arabinan while 0.90 for glucose, as indicated by Equation 4.4

$$C_{anhydro} = C_X \times Anhydro\ Correction \quad (4.4)$$

Where

$C_{anhydro}$ = Concentration of polymeric sugar mg L^{-1}

C_X = Sugar concentration after 4% hydrolysis

$Anhydro\ Correction$ = Correction factor

Ash was determined based on Equation 4.5

$$\%\ Ash = {Weight_{Crucible\ plus\ ash} - Weight_{Crucible}} \Big/ {Oven\ Dry\ Weight_{Sample}} \times 100 \quad (4.5)$$

Klason lignin was determned according to Equation 4.6

$$\%\ Klason\ lignin = (Weight_{Acid\ insoluble\ residue} - Weight_{Ash}) \Big/ {Oven\ dry\ weight\ of\ sample} \times 100 \quad (4.6)$$

Recovery and removal for sugar monomers were determined by following formulas equation 4.7, and 4.8, respectively according to (Su *et al.*, 2015). While delignification was determined by equation 4.9

$$Recovery\ (\%) = {Weight\ (g)\ pretreated\ solid}/{Initial\ weight\ (g)\ in\ raw\ biomass} \times 100 \quad (4.7)$$

$$Removal\ (\%) = 1 - \left({weight\ (g)\ pretreated\ solid}/{initial\ weight\ (g)\ in\ raw\ biomass}\right) \times 100 \quad (4.8)$$

$$Delignification\ (\%) = {Initial\ lignin\ (g) - residual\ lignin\ (g)}/{Initial\ lignin\ (g)} \times 100 \quad (4.9)$$

4.5 Morphological characterization

Morphology of raw and selected pretreated solid fractions was visualized with field emission Zeiss sigma™ scanning electron microscope at Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos – SP, Brazil. The samples were mounted on aluminum stubs and coated with gold prior to analysis.

4.6 Chemical Characterization of liquid hydrolysate

Liquid hydrolysate obtained from pretreatment slurry was characterized for sugar monomers, organic acids, total phenolic content, total carbohydrate, and chemical oxygen demand (COD)

4.6.1 Sugar monomers determination

Briefly, 20 mL liquid sample was taken in digestion tubes followed by addition of predetermined amount of H_2SO_4 (72 % w/w) based on pH value. The tubes were then submitted to the autoclave for 1 h. Liquid aliquots obtained were neutralized with $CaCO_3$ and stored at -20 °C until further analysis. Samples were extracted with Sep-Pak C-18 cartridges following 0.2 µm glass filters before submitted to HPLC. HPLC analysis was conducted as described in section 4.4.1.

4.6.2 Total carbohydrate analysis

Total carbohydrate analysis of the hydrolysate fraction was determined by the phenol-sulfuric acid method proposed by (DuBois *et al.*, 1956). Briefly, 1 mL of sample was transferred to 10 mL glass vials followed by 1 mL of 5% (w/w) phenol solution and 5 mL 96% (w/w) H_2SO_4. Glass vials were screw capped and slightly inverted twice and left for 10 min at room temperature. Afterward, placed in cold water for 15 min. The total carbohydrates (mg L^{-1}) were determined using spectrophotometer (Hach® model no. DR-14000) at 420 nm. The analysis was performed under a fume hood.

4.6.3 Organic acids determination

Organic acids in liquid hydrolysate produced during hydrothermal pretreatment and during digestion process were determined using high performance liquid chromatography (HPLC) equipped with a UV diode array detector (SPD-M10Avp), a refraction index detector (RID-10A), a CTO-20A oven, a LC-10ADvp Pump, a SCL10 Avp controller and Aminex HPX-87H column, 300mm×7.8mm (BioRad). The mobile phase consisted of H_2SO_4 (0.01 N) at 0.5 mL/min flow rate (Penteado *et al.*, 2013). Samples were passed through 0.2 µm glass filter before introduction to HPLC.

4.6.4 Total phenolic content

Total phenolic content in the hydrolysate was determined with protocol explained by Caza et al . (Caza *et al.*, 1999). Briefly, 2 mL sample was supplemented with 2 mL deionized water, 200 µl ferricyanide solution and 200 µl 4-aminoantipyrine solution. The contents were mixed and left for 7 minutes afterward absorbance was detected at 500 nm against predetermined calibration curve.

4.6.5 Chemical oxygen demand

Chemical oxygen demand of hydrolysate fraction was determined by potassium dichromate-ferrous ammonium sulfate method according to Standards Methods (APHA *et al.*, 2005).

4.7 Anaerobic digestion assays preparations

4.7.1 Zinder basal medium

To avoid inhibition owing to the nutritional requirement, Zinder basal medium (Zinder *et al.*, 1984) was employed as a medium of batch reactors instead of distilled water. The medium was prepared according to chemicals presented in Table 4.1 in ultrapure water.

Table 4.1 - Composition of Zinder medium

Chemical	**Quantities - q.s.p[a]. 1000 mL of Ultrapure water**
NH_4Cl	0.5 g
KH_2PO_4	0.4 g
$MgCl_2.6H_2O$	0.1g
$CaCl_2.2H_2O$	0.05 g
Trace metal solution	10 mL
Vitamin solution	0.1 mL

[a] quantity of solute per volume

Trace metal solution and vitamin solution were prepared according to Table 4.2. The both solutions were vacuum filtered through 0.22 µm glass filter, subsequently flushed with N_2 (100%) gas for 20 min via gas distribution system to provide anaerobic conditions and stored in a refrigerator at 4 °C under dark conditions.

4.7.2 Batch digestion reactors

Digestion experiments for the pretreatments (in duplicate reactors) were conducted in three sets. Batch experiments were conducted in 1000 mL glass bottles with a working volume of 500 mL and 500 mL of headspace. Defined amount of substrates (solid fraction of pretreatment A-HO, B-HO, …, P-HO, and A-HSO, B-HSO, …, P-HSO) on (1 g kg^{-1}) TVS basis were introduced into glass reactor followed by zinder nutrient solution. Reactor composition for each pretreatment is presented in Table 9.1 and 9.2 (Appendix). The inoculum was introduced on 2 g kg^{-1} TVS basis. The pH of reactors was adjusted to around neutrality (7.0 – 7.1) with 5 M NaOH and where necessary with 1M H_2SO_4. Thereafter, reactors were flushed with pure N_2 (100%) gas for 10 min

via gas distribution system to ensure anaerobic conditions and immediately sealed with rubber septum followed by plastic caps. The reactors were placed in an incubator programmed at 37 °C.

Three points were selected for COD, carbohydrates, and volatile fatty acids determination; a) time zero (reactor initiation), b) approximate exponential phase, c) reactor terminations. Each time 10 mL sample was collected into 13 mL plastic vials. The vials were stored in a freezer until further analysis. All experiments were performed in duplicate except blank and positive control (glucose). The results presented are the average values of duplicate reactors.

Table 4.2 - Composition of trace metal solution and vitamin solution – Zinder medium

Trace Metal Solution	
Chemical	**Quantities - q.s.p. 1000 mL of ultrapure water**
Tritriplex III	4.5 g
$FeSO_4.7H_2O$	0.556g
$MnSO_4.H_2O$	0.086g
$CoCl_2.6H_2O$	0.17g
$ZnSO_4.7H_2O$	0.21g
H_3BO_3	0.19g
$NiCl_2$	0.02g
Na_2MoO_4	0.01g
Vitamin Solution	
Biotin	0.002g
Folic Acid	0.002g
Thiamin	0.005g
Riboflavin	0.005g
Nicotinic acid	0.005g
Calcium pentothenate	0.005g
Pyridoxin.HCl	0.010g

4.8 Determination of methane

The reactors were manually shaken for approx. 1 min to avoid thermal stratification, before taking a gas sample. The pressure in the reactors was recorded by inserting syringe into the septum, linked with a pressure transducer (T&S equipamentos electronicos). Each time 0.5 mL gas sample was taken for GC analysis. Methane content was determined via Shimadzu® gas chromatography (GC-2010) equipped with thermal conductivity detector (TCD). The column was Carboxen 1010 PLOT, 30 m x 0.53 mm. Injector temperature, oven temperature, and detector temperature were 220 °C, 130 °C, and 230 °C, respectively. Argon gas was used as carrier gas with a flow rate of 5.66 mL/min with makeup volume of 12 mL/min according to Motteran e al. (Motteran *et al.*, 2014). CH_4 concentration is reported in this text in normal mmol - Nmmol g^{-1} TVS, i.e., at standard temperature and pressure conditions (273 K, 101.325 KPa). The cumulative methane content (Nmmol g^{-1} TVS) as a function of time was determined according to Equation 4.5

$$X_{STP} = X_m \cdot \frac{T_{Standard} \cdot P_m}{T_m \cdot P_{Standard}} \quad (4.5)$$

Where

X_{STP} = Methane content at standard temperature and pressure

X_m = Methane content at room temperature and pressure

$T_{Standard}$ = Standard temperature - 273 K

P_m = Pressure of the reactor determined

T_m = Actual temperature - 37 °C

$P_{Standard}$ = Standard pressure – 101.325 KPa

Assuming CH_4 production is proportional to microbial activity. The experimental data (average values of duplicate reactors) obtained was adjusted using modified Gompertz equation 4.6 (Zwietering *et al.*, 1990) with Origin software package version

$$H = P \cdot \exp\left\{-\exp\left[\frac{R_m \cdot e}{P}(\lambda - t) + 1\right]\right\} \quad (4.6)$$

Where

P = CH_4 production potential (Nmmol g^{-1} TVS)

R_m = CH_4 production rate (Nmmol g^{-1} TVS /h)

t = incubation time of reactors (h)

e = Euler number (2.71828)

λ = lag phase before CH4 production starts (h)

4.9 Experimental design

A 3^3 full factorial design was selected to optimize the hydrothermal pretreatment in order to test the pretreatment time, temperature and concentration of sulphuric acid (H_2SO_4) and Hydrogen peroxide (H_2O_2). Coded values and real values for central composite design are presented in Table 4.3. The matrix of factors and levels of pretreatments applied to sugarcane bagasse for H_2O_2 and H_2SO_4 are presented in Table 4.4 and Table 4.5, respectively.

Table 4.3 - Coded and uncoded values for central composite design for H2SO4 and H2O2 assisted hydrothermal pretreatment

Coded values	***X1*** **(temperature, °C)**	***X2*** **(time, min)**	***X3*** ***(H_2SO_4 % w/v)***
+ 1.68	213.6	23.76	3.68
1	200	19	3
0	180	12	2
-1	160	5	1
-1.68	146.4	0.24	0.32
Coded values	***X1*** **(temperature, °C)**	***X2*** **(time, min)**	***X3*** **(H_2O_2, % v/v)**
+ 1.68	213.6	23.76	7.36
1	200	19	6
0	180	12	4
-1	160	5	2
-1.68	146.4	0.24	0.64

Table 4.4 - Central composite design matrix for H_2O_2 assisted hydrothermal pretreatment

	Coded values			Real values		
Pretreatment run	**Temperature X1**	**Time X2**	**H_2SO_4 X3**	**Temperature X_1**	**Time X_2**	**H_2O_2 X_3**
A-HO	1	1	1	200	19	6
B-HO	1	1	-1	200	19	2
C-HO	1	-1	1	200	5	6
D-HO	1	-1	-1	200	5	2
E-HO	-1	1	1	160	19	6
F-HO	-1	1	-1	160	19	2
G-HO	-1	-1	1	160	5	6
H-HO	-1	-1	-1	160	5	2
I-HO	0	0	0	180	12	4
J-HO	0	0	0	180	12	4
K-HO	0	0	1.68	180	12	7.36
L-HO	0	0	-1.68	180	12	0.64
M-HO	0	1.68	0	180	23.76	4
N-HO	0	-1.68	0	180	0.24	4
O-HO	1.68	0	0	213.6	12	4
P-HO	-1.68	0	0	146.4	12	4

Table 4.5 - Central composite design matrix for H_2SO_4 assisted hydrothermal pretreatment

	Coded values			Real values		
Pretreatment run	**Temperature X1**	**Time X2**	**H_2SO_4 X3**	**Temperature X_1**	**Time X_2**	**H_2SO_4 X_3**
A-HSO	1	1	1	200	19	3
B-HSO	1	1	-1	200	19	1
C-HSO	1	-1	1	200	5	3
D-HSO	1	-1	-1	200	5	1
E-HSO	-1	1	1	160	19	3
F-HSO	-1	1	-1	160	19	1
G-HSO	-1	-1	1	160	5	3
H-HSO	-1	-1	-1	160	5	1
I-HSO	0	0	0	180	12	2
J-HSO	0	0	0	180	12	2
K-HSO	0	0	1.68	180	12	3.68
L-HSO	0	0	-1.68	180	12	0.32
M-HSO	0	1.68	0	180	23.76	2
N-HSO	0	-1.68	0	180	0.24	2
O-HSO	1.68	0	0	213.6	12	2
P-HSO	-1.68	0	0	146.4	12	2

4.10 Microbial community characterization by molecular techniques

Samples for microbial community characterization were taken at the end of batch digestion reactors. Samples were analyzed by denaturing gradient gel electrophoresis for the relative similarity of bacterial and archaeal domains. PCR/DGGE analysis was conducted for all the batch reactors of both pretreatments except pretreatment 15 (213.6 °C) and 16 (146.4 °C) in both cases (H_2O_2 and H_2SO_4) due to extreme temperature conditions applied.

Selected samples were characterized by bacterial and chemical community structure using next-gen Illumina MiSeq Sequencing. Seven samples were sent for next gen Illumina MiSeq Sequencing; 3 from H_2O_2 impregnated and 3 from H_2SO_4 impregnated pretreatments. Samples selected from H_2O_2 pretreatments were B-HO, K-HO and P-HO. While D-HSO, I-HSO and N-HSO were chosen from H_2SO_4 assisted pretreatments. Sample from positive control (glucose) was also characterized to determine the difference in archaeal and bacterial diversity in digestion reactors of pretreated lignocellulose substrate and pure glucose.

4.10.1 Sample collection and DNA extraction

Samples were collected by centrifugation at 4 °C at 6000 rpm for 10 min followed by cleaning with phosphate buffer, thereafter were stored at -20 °C. Genomic DNA was collected by cell lysis with glass beads (Sigma) followed by a phenol-chloroform method utilizing modified Griffith's protocol (Griffiths *et al.*, 2000).

4.10.2 PCR/DGGE

Polymerase chain reaction (PCR) was conducted with bacterial primer set 968FGC (5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACCTTAC-3′) /1401R (5′-CGGTGTGTACAAGACCC-3′) (Nubel *et al.*, 1996) and archaeal primer set 1100FGC (5′-AACCGTCGACTCAGGYAACGAGCGAG-3′) and 1400R (5′ CGGCGAATTCGTGCAAGGAGCAGGGGA3′) (Kudo *et al.*, 1997). The PCR products were checked for purity by 1.2% agarose gel electrophoresis. In the current study, DGGE was conducted under denaturants 45% and 65% (100% denaturant – 7M Urea and 40% (v/v) deionized formamide) for bacterial and archaeal domains. Running conditions of the apparatus DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) were; 75V, 16

h at a constant temperature of 60 °C (Muyzer *et al.*, 1993). Ethidium bromide was utilized as a fluorescent solution. Gels were visualized with Foto documentation L. PixTouch (Loccus Biotechnology) under 254 nm UV exposure.

Multivariate analysis of DGGE band profiles was conducted using BioNumerics 3.5 software package (Applied Maths, Belgium) with Pearson correlation. Dendrograms were constructed with UPMA method. The Shannon-Wiener diversity index (H), and Dominance (D) were calculated with Past 3.0 software package.

4.10.3 Illumina MiSeq Sequencing

The V4 variable region of 16S rRNA gene was PCR amplified with a barcoded 515F/806R primer set (Table 4.6); universal for bacteria and archaea (Table 4.6) (Walters *et al.*, 2011). HotStarTaq Plus Master Mix Kit (Qiagen, USA) was used for amplification by PCR. Amplicons were checked for the success of amplification and relative intensity of bands with 2% agarose gel electrophoresis. Multiple samples were pooled based on their molecular weight and DNA concentration and were purified using calibrated Ampure XP beads. Purified amplicons were used to prepare Illumina DNA library. Sequencing was performed at MR DNA (Shallowater, TX – USA) on a MiSeq following manufacturer's protocol.

Table 4.6 - Bacterial and Archaeal primers and sequences for Illumina MiSeq Sequencing

Primers	**Sequences 5′ - 3′**	**References**
515F	5'-GTGCCAGCMGCCGCGGTAA-3′	(Walters *et al.*, 2011)
806R	5'-GGACTACHVHHHTWTCTAAT-3′	

MR DNA pipeline was used to process sequence data using three steps;

a) Joining of paired-end reads.
b) Removal of barcodes and sequences <150 bp were depleted in addition to sequences with ambiguous base calls.
c) Operational taxonomic units (OUTs) were assigned to a most relevant taxonomic level based on BLASTn similarity percentage (97%).

Final OTUs were taxonomically classified using BLASTn against a curated database derived from RDPII and NCBI (http://rdp.cme.msu.edu, www.ncbi.nlm.nih.gov).

5 RESULTS AND DISCUSSION

5.1 Compositional analysis of untreated sugarcane bagasse

The composition of lignocellulosic biomass is important to determine the potential for methane production. Polymeric and non-polymeric compounds encountered in raw sugarcane bagasse are presented in Table 5.1

Table 5.1 - Compositional analysis of sugarcane bagasse

Composition	%DM basis*
Glucan	31.4
Xylan	36.6
Arabinan	5.0
Lignin	24.8
Ash	1.2

* On percent Dry Matter (DM) basis

As indicated in Table 5.1, sugarcane bagasse was characterized for sugar monomers; glucan (cellulose), xylan and arabinan (hemicellulose), Klason lignin, and ash content according to equations mentioned in section 4.4.1. Glucan, xylan, arabinan, and lignin content of sugarcane bagasse in the current study were 31.4, 36.6, 5.0, and 24.8 g 100 g^{-1} of dry matter, respectively. Other researchers documented approx. similar results for sugarcane bagasse; Da Silva et al. (da Silva *et al.*, 2010) reported 38.8, 26 and 32.4% cellulose, hemicellulose and lignin content on percent dry matter basis. While De Moraes et al. (De Moraes *et al.*, 2011) reported slightly different composition of sugarcane bagasse; 45.5% cellulose, 27% hemicellulose, and 21.1% lignin on dry matter basis. In another study, Bharathiraja et al (Bharathiraja *et al.*, 2014) reported 56.7% hemicellulose content, 30.2% cellulose and 13.4% lignin in sugarcane bagasse.

Ash content (1.2%) was also in accordance with other reported studies for sugarcane bagasse; 1% (Canilha *et al.*, 2011), 1.5% (Rabelo *et al.*, 2011) and 1.6% (Brienzo *et al.*, 2009). Lower ash content is desirable for biofuels production and directly affects the heating value of the plant biomass (Demirbas, 2009). The compositional analysis of sugarcane bagasse in the current study is in confirmation with already reported studies. Differences in composition among reported studies and current results are due to cultivar type, soil characteristics, and process differences of processing plants where sugarcane is being processed.

5.2 Impact of H_2O_2 assisted hydrothermal pretreatment on chemical composition of sugarcane bagasse

Impact of H_2O_2 (0.64 – 7.36 % v/v) impregnation for 24 h was observed in 16 pretreatment runs of HT pretreatment according to CCD matrix (Table 4.4). Solid yield, glucan, xylan, klason lignin (%), and lignin solubilization (%) in raw sugarcane bagasse and pretreated solid residues are presented in Table 5.2

Table 5.2 - Chemical characterization of solid fraction of H_2O_2 pretreated sugarcane bagasse; Solid yield (%), Glucan (g 100 g^{-1} TS), Xylan (g 100 g^{-1} TS), Klason lignin (%), Glucan increase %), Xylan removal (%), and lignin solubilization (%)

Run	Designation	Solid yield (%)	Glucan (g 100 g^{-1} TS)	Xylan (g 100 g^{-1} TS)	Klason lignin (g 100 g^{-1} TS)	Glucan increase (%)	Xylan removal (%)	Lignin solubilization (%)
1	A-HO	54.41	68.20	10.12	9.60	117.19	72.36	61.39
2	B-HO	62.55	55.52	16.74	23.2	76.81	54.28	6.86
3	C-HO	59.36	63.21	15.60	7.4	101.31	57.39	70.33
4	D-HO	71.58	49.15	28.63	16.2	56.53	21.81	34.97
5	E-HO	58.26	47.50	12.74	9.3	51.27	65.21	62.51
6	F-HO	74.21	42.61	29.64	15.0	35.70	19.05	39.72
7	G-HO	66.98	45.75	18.39	10.2	45.70	49.78	58.82
8	H-HO	89.20	39.65	32.18	14.9	26.27	12.11	40.08
9	I-HO	71.08	50.02	25.60	11.9	59.29	30.08	52.00
10	J-HO	73.11	49.15	26.60	12.8	56.52	27.35	48.55
11	K-HO	64.99	60.42	8.85	6.7	92.42	75.83	73.09
12	L-HO	93.13	39.17	31.41	22.4	24.74	14.22	9.93
13	M-HO	49.75	56.19	24.33	12.9	78.94	33.55	48.02
14	N-HO	73.30	46.14	29.14	10.6	46.94	20.42	57.47
15	O-HO	36.57	75.21	18.74	19.0	139.52	48.82	23.52
16	P-HO	76.49	33.12	27.15	12.0	5.47	25.85	51.62

As indicated in Table 5.2, the lowest solid yield (36.57 %) was observed at pretreatment O-HO (213.60 °C, 12 min, 4 % H_2O_2), while the highest solid yield of 93.13 % was observed at pretreatment L-HO (180 °C, 12 min, 0.32% H_2O_2). Solid yield increased (9.09%) from 54.41% to

59.6% when pretreatment time was decreased from 19 min (PRT A-HO) to 5 min (PRT C-HO) at a temperature of 200 °C and H_2O_2 concentration of 6 (% v/v). However, 14.43% increase was observed in solid yield from 62.55% (PRT B-HO) to 71.58% (PRT D-HO) when the reaction time was 5 min at conditions of 200 °C and 2% H_2O_2. A reduction in pretreatment temperature to 160 °C favored an increase in solid yield of 7.07% and 18.64%, 14.43%, and 24.61% in pretreatment E-HO, F-HO, G-HO, and H-HO in comparison to pretreatment A-HO, B-HO, C-HO, and D-HO, respectively at similar pretreatment conditions. At pretreatment temperature of 180 °C, an increase in H_2O_2 concentration from 4% (PRT I-HO) to 7.36% (PRT K-HO) at pretreatment time of 12 min resulted in a 9.3% decrease in solid yield. While, at the similar condition (180 °C, 12 min), a decrease in H_2O_2 concentration from 7.3% to 0.64% resulted in 43.29 % increase in solid yield. On the other hand, a decrease in pretreatment time from 23.7 min to 0.24 min did not show a significant difference in solid yield at similar H_2O_2 concentration and pretreatment temperature.

Hydrothermal pretreatment has a significant impact on solid yield predominantly due to hemicellulose solubilization and partial lignin dissociation. Since hemicellulose solubilization is in direct relation to temperature, therefore, solid yield has a direct relation, in decreasing order, with an increase in temperature (Yang *et al.*, 2004). The solid yield of *Eucalyptus camaldulensis* declined to 54.2% from 72.7% when ammonium chloride catalyzed hydrothermal pretreatment for temperature increased from 140 to 180 °C (Shen *et al.*, 2016). Similarly, Deng et al (Deng *et al.*, 2015) reported a decrease in the solid residue of corncob from 96.5% to 60.67% when pretreatment temperature was increased from 170 °C, (0 min) to 190 °C (60 min). In similar fashion, Sun and his team (Sun *et al.*, 2014) reported successive decrease in solid residues of corncob 69.4% to 48.7% when temperature increased from 170 °C to 190 °C, further it reached to the lowest value of 47.0% at 200 °C. In the current study, solid yield increased with a decrease in H_2O_2 concentration applied as evidenced in pretreatment L-HO where a maximum solid yield of 93.13% was observed with lowest H_2O_2 (0.64 % v/v) was studied. An increase in solid yield with a decrease in reaction temperature under similar reaction time is in line with the already reported studies

Glucan and xylan content in the pretreated solid fraction indicated proportional increase and decrease, respectively, in response to pretreatment conditions applied. Glucan content in literature is reported to increase in the pretreated solid fractions due to the removal of hemicellulose content and partial lignin solubilization to liquid stream. Glucan content was

variable between 49.15 – 68.20 g 100 g^{-1} TS for pretreatment set (A-HO – D-HO) conducted at 200 °C. Higher glucan content was noted for higher H_2O_2 loading. A decrease in glucan content in the range of 39.65 – 47.50 g 100 g^{-1} TS was seen with a decrease in reaction temperature 160 °C (pretreatment set E-HO – H-HO) in comparison to pretreatment temperature 200 °C. Although similar H_2O_2 loading was applied to this pretreatment set (E-HO – H-HO), however, glucan increase was not as consistent as was seen at 200 °C, indicating that reaction temperature played a significant role.

Glucan content was in the range of 39.17 – 60.42 g 100 g^{-1} TS for pretreatments at reaction temperature 180 °C. Lowest glucan content (33.12) was seen at lowest reaction temperature studied (pretreatment run P-HO, 146.4 °C). Percent glucan increase in solid fraction was in the range of 5.47 – 139.52% in the pretreatments conducted in this phase of the study. The percent glucan increase was quite higher in the current study than in comparison to Zhao et al. (Zhao *et al.*, 2016). The authors reported up to 97.6% glucan in H_2O_2-ammonia fiber explosion for corn stalk, with lignin removal in the range of 2.4 – 28.8%. However, authors reported results for an H_2O_2 loading of 0.1 – 0.7 g g^{-1} dry biomass and lower reaction temperature range 90 – 130 °C.

Shao and co-workers reported (Shao *et al.*, 2013) 1.5 % glucan increase after hydrogen peroxide (0.7%) ammonia fiber explosion at reaction temperature of 150 °C. Shen et al (Shen *et al.*, 2011) reported an increase in reducing sugars up to 347.2% after enzymatic hydrolysis of tobacco stem that could indirectly relate to higher glucan recovery after pretreatment with H_2O_2 (0.6%) for 9 h at 60 °C. Higher percent glucan increase in current study could relate to (Yu *et al.*, 2013). The authors reported that hydrogen peroxide combined with sodium carbonate and sodium hydroxide (green liquor) resulted in the selective removal of lignin leaving behind approx. complete glucan for higher enzymatic hydrolysis.

Xylan content was in the range of 10.12 – 31.41 g 100 g^{-1} TS for the H_2O_2 assisted hydrothermal pretreatments corresponding to xylan recovery in pretreated solid fractions in the range of 24.17 – 85.78%. Zhao et al. (Zhao *et al.*, 2016) reported maximum of 97.4% xylan recovery in their study for corn stalk employing H_2O_2 in the range of 0.1 – 0.7 % at reaction temperature in the range of 90 – 130 C. Xylan removal in the current study were in the range of 12.11 – 75.83%, respectively. Arabinan recovered in the solid fraction was in the range of 0.46 –

3.97%. The results of the current study could not be compared to the literature owing to different pretreatments, pretreatment conditions and variability in the feedstock.

Second-order polynomial equation was derived from the experimental data of glucan and xylan contents to determine the impact of pretreatment parameters; reaction temperature, reaction time, and H_2O_2 concentration on their availability in the solid fraction in response to H_2O_2 assisted hydrothermal pretreatment. Equation 5.1 and 5.2 represent the second order polynomial equations, respectively, for glucan and xylan.

$$Glucan\ (\text{g } 100\ \text{g}^{-1}\ TS) = 49.62 - 9.62X_1 + 2.41X_2 + 5.38X_3 + 1.53{X_1}^2 + 0.47{X_2}^2 - 0.02{X_3}^2 + 0.83X_1X_2 + 1.97X_1X_3 - 0.32X_2X_3 \quad \text{Equation 5.1}$$

It was observed that all the studied parameters; temperature (X_1), time (X_2) and H_2O_2 concentration (X_3) showed significant impact on the glucan (g 100 g^{-1} TS) content. The model described that all the linear model terms X_1, X_2, and X_3 showed the significant positive impact on the glucan content with magnitude values of 9.62, 2.41, and 5.38, respectively. It indicated that temperature (X_1) had a higher positive impact followed by H_2O_2 concentration (X_3), and reaction time (X_2) implying that reaction temperature is an important parameter for glucan recovery under the studied conditions.

The interaction of studied parameters revealed that not a single interaction among three parameters was significant. One-way analysis of variance (Table 5.3) indicated that interaction of parameters was insignificant i.e., *p*-value for the interaction of all the parameters; X_1X_2, X_1X_3, and X_2X_3 was more than 0.05.

Equation 5.2 indicates the second-order polynomial equation for xylan (g 100 g^{-1} TS) content. The model equation showed that all the three linear terms of temperature (X_1), time (X_2), and H_2O_2 concentration (X_3) were significant with magnitude values of 2.63, 2.46, and 6.46, respectively. However, it can be observed that all the linear terms are negative significant. Furthermore, temperature and time had approx. similar negative impact while H_2O_2 concentration had higher negative impact implying that H_2O_2 concentration was more important in xylan content as indicated in one-way analysis of variance (Table 5.4)

$$Xylan\ (\text{g } 100\ \text{g}^{-1}\ TS) = 26.35 - 2.63X_1 - 2.46X_2 - 6.46X_3 - 1.72{X_1}^2 - 0.38{X_2}^2 - 2.71{X_3}^2 - 1.15X_1X_2 + 1.38X_1X_3 + 0.41X_2X_3 \quad \text{Equation 5.2}$$

Table 5.3 - Analysis of variance (ANOVA) for glucan content (g 100 g-1 TS) in pretreated solids impregnated in H2O2.

Source	Sum of square	Degree of freedom	Mean Square	F-value	P-value (prob > F)
X_1 (T)$^{\alpha}$	1263.437	1	1263.437	3338.451	0.011017*
X_1^2	21.651	1	21.651	57.209	0.083683
X_2 (t)$^{\beta}$	79.616	1	79.616	210.375	0.043822*
X_2^2	2.032	1	2.032	5.369	0.259371
X_3 (H_2O_2 Conc.)$^{¥}$	395.227	1	395.227	1044.332	0.019693*
X_3^2	0.002	1	0.002	0.006	0.948823
X_1X_2	5.528	1	5.528	14.606	0.162922
X_1X_3	31.008	1	31.008	81.934	0.070047
X_2X_3	0.839	1	0.839	2.216	0.376598
Lack of fit	93.100	5	18.620	49.200	0.107800
Pure Error	0.378	1	0.378		
Total SS	1895.691	15			

R-square = 0.9506, Adjusted R-square = 0.876, MS pure error = 0.37, * represents significant factors, $^{\alpha}$ (T) represents Temperature, $^{\beta}$ (t) represents time, and $^{¥}$ represents H_2O_2 concentration

Sugarcane bagasse impregnation into H_2O_2 before introduction to hydrothermal pretreatment resulted in significant decrease in Klason lignin as shown in Table 5.2. Klason lignin varied from 7.4 to 23.2 g 100 g^{-1} TS for pretreatment run A-HO to D-HO conducted at 200 °C. While, a decrease in temperature to 160 °C indicated a relative decrease in Klason lignin in the range of 9.3 to 14.9 g 100 g^{-1} TS for pretreatment E-HO to H-HO. Hansen et al (Hansen *et al.*, 2004) reported that lignin recovery is in direct relation to pretreatment temperature. Pretreatment B-HO presented maximum lignin (23.2 g 100 g^{-1} TS) and correspondingly lowest lignin solubilization (6.86%) in the pretreated solid possibly due to lower H_2O_2 (2%), higher temperature 200 °C and longer retention time (19 min). Minimum Klason lignin (6.7 g 100 g^{-1} TS) with correspondent highest lignin dissolution (73.09%) was observed in pretreatment K-HO conducted at 7.36% H_2O_2, 180 °C, and 12 min. Complete degradation of lignin during the hydrothermal process is not possible due to recondensation process (Alvira *et al.*, 2010).

Table 5.4 - Analysis of variance (ANOVA) for xylan content (g 100 g-1 TS) in pretreated solids impregnated in H2O2

Source	Sum of square	Degree of freedom	Mean Square	F-value	P-value (prob > F)
X_1 (T)$^{\alpha}$	94.9178	1	94.9178	189.836	0.046124*
X_1^2	27.3930	1	27.3930	54.786	0.085492
X_2 (t)$^{\beta}$	82.9096	1	82.9096	165.819	0.049339*
X_2^2	1.3349	1	1.3349	2.670	0.349638
X_3 (H_2O_2 Conc.)$^{¥}$	570.6716	1	570.6716	1141.343	0.018838*
X_3^2	68.2786	1	68.2786	136.557	0.054346
X_1X_2	10.5341	1	10.5341	21.068	0.136563
X_1X_3	15.2352	1	15.2352	30.470	0.114092
X_2X_3	1.3612	1	1.3612	2.722	0.346871
Lack of fit	43.7950	5	8.7590	17.518	0.179350
Pure Error	0.5000	1	0.5000		
Total SS	902.7186	15			

R-square = 0.9509, Adjusted R-square = 0.877, MS pure error = 0.5, * represents significant factors, $^{\alpha}$ (T) represents Temperature, $^{\beta}$ (t) represents time, and $^{¥}$ represents H_2O_2 concentration

Degradation mechanism of lignin with H_2O_2 during hydrothermal pretreatment could be explained with Equations 5.3 and 5.4 proposed by Gould (Gould, 1985). Kadla and Chang (Kadla and Chang, 2001) reported that hydrogen peroxide reacts readily with lignin to produce low molecular weight degradation compounds.

$$H_2O_2 \rightarrow H^+ + HOO^- \quad (5.3)$$

$$H_2O_2 + HOO^+ \rightarrow .OH + O_2.- + H_2O \quad (5.4)$$

Hydroxyl radical acts as a powerful lignin oxidant, which lead to delignification of lignocellulosic biomass by oxidation and degradation. While perhydroxy anion cleaves the diferulate linkages which cross-link polysaccharides resulting in depolymerization of polysaccharides (Kim and Lee, 1996).

Zhao et al (Zhao *et al.*, 2016) studied the impact of H_2O_2 (0.1, 0.4, and 0.7 mass ratios to dry biomass) presoaking before ammonia fiber expansion on physical and chemical characteristics

of the corn stalk. The authors observed 2.4 - 28.8% lignin removal that increased with increase in pretreatment temperature and H_2O_2 loading. In another study, Shen et al (Shen *et al.*, 2011) reported 66.5 ± 1.6% lignin removal for tobacco stem pretreated with H_2O_2 (0 1.2% w/v) at 90 °C for 24 hrs. Song et al (Song *et al.*, 2013) reported 12.5% decrease in lignin content of rice straw after pretreatment with H_2O_2 (1, 2,5 and 4%) at 25 ± 2 °C for 7 days. Higher lignin dissolution (74.2%) in the current study was might be due to direct introduction of impregnated bagasse into the hydrothermal reactor after H_2O_2 removal whereas Shen et al (Shen *et al.*, 2011) dried tobacco after H_2O_2 impregnation pretreatment. Another reason for higher lignin removal is higher H_2O_2 (v/v %) loading.

A Second-order polynomial equation (Equation 5.5) was derived from the experimental data of lignin solubilization (%) to explain the impact of studied factors. It was observed that all the studied parameters; temperature (X_1), time (X_2) and H_2O_2 concentration (X_3) showed significant impact on lignin dissolution. The model described that linear model terms X_1 and X_2 showed the significant negative impact on lignin dissolution with magnitude values of 5.48 and 3.63, respectively, while X_3 showed the highest positive significant impact on lignin dissolution with magnitude value of 17.40. It implied that H_2O_2 concentration proved to be a parameter of significant interest for lignin dissolution.

The interaction of studied parameters indicated that X_1X_2 showed a negative impact on magnitude value of 5.05 while X_1X_3 showed a positive impact on magnitude value of 6.05. It implied that to remove lignin content from sugarcane bagasse interaction between temperature (X_1) and H_2O_2 (X_3) are more important than reaction time (X_2). One-way analysis of variance (Table 5.5) indicated that X_1, X_2, X_3, X_1X_2, and X_1X_3 were significant with *p*-values of 0.009, 0.047, 0.000, 0.038, and 0.019, respectively (Table 5.5).

$$\%\ lignin\ dissolution = 49.96 - 5.48X_1 - 3.63X_2 + 17.40X_3 - 3.72{X_1}^2 + 1.64{X_2}^2 - 2.34{X_3}^2 - 5.05X_1X_2 + 6.05X_1X_3 + 2.90X_2X_3 \quad (5.5)$$

Table 5.5 - Analysis of variance (ANOVA) for percent lignin dissolution in pretreated solids impregnated in H_2O_2.

Source	Sum of square	Degree of freedom	Mean Square	F-value	P-value (prob > F)
X_1 (T)$^{\alpha}$	409.99	1.00	409.99	14.20	0.009*
X_1^2	128.70	1.00	128.70	4.46	0.079
X_2 (t)$^{\beta}$	180.15	1.00	180.15	6.24	0.047*
X_2^2	24.81	1.00	24.81	0.86	0.390
X_3 (H_2O_2 Conc.)$^{¥}$	4135.19	1.00	4135.19	143.23	0.000*
X_3^2	50.57	1.00	50.57	1.75	0.234
X_1X_2	203.82	1.00	203.82	7.06	0.038*
X_1X_3	292.43	1.00	292.43	10.13	0.019*
X_2X_3	67.37	1.00	67.37	2.33	0.177
Error	173.22	6.00	28.87		
Total SS	5742.97	15.00			

R-square = 0.969, Adjusted R-square = 0.925, MS error = 28.87, * represents significant factors, $^{\alpha}$ (T) represents Temperature, $^{\beta}$ (t) represents time, and $^{¥}$ represents H_2O_2 concentration

5.2.1 Morphological characteristics of H_2O_2 assisted hydrothermally pretreated solid fraction

Scanning electron microscopy is a well-documented technique to study the morphology of lignocellulosic biomass and has been proven useful to study structural characteristics at the molecular level (Trajano *et al.*, 2013). Native sugarcane bagasse and solid fractions of selected pretreatments, based on CH_4 yield, were compared for the ultrastructural differences by scanning electron microscopy. The impact of H_2O_2 assisted pretreatment on visual appearance of sugarcane bagasse at the lowest (146.4 °C) and highest (213.6 °C) studied temperature is depicted in Fig 5.4

The SEM micrographs of native Sugarcane bagasse and BOH, and KOH pretreatments are shown in Fig 5.1 (A, B, and C). The SEM images show a distinct difference between the morphology of raw Fig 5.1 (A) and pretreated samples Fig 5.1 (B, C). Raw sample (Fig. 5.1 A) shows a smooth surface, rigid, and compact rather highly organized morphological structure. Cellulosic fibers Fig 5.1 (B) are present in stripes (higher magnification) as tightly packed

structures and encapsulated within lignin sheath, in addition, covered with extractives, superficially. This kind of structure makes cellulosic microfibrils virtually inaccessible for enzymatic action, and poses challenges to hydrolysis process.

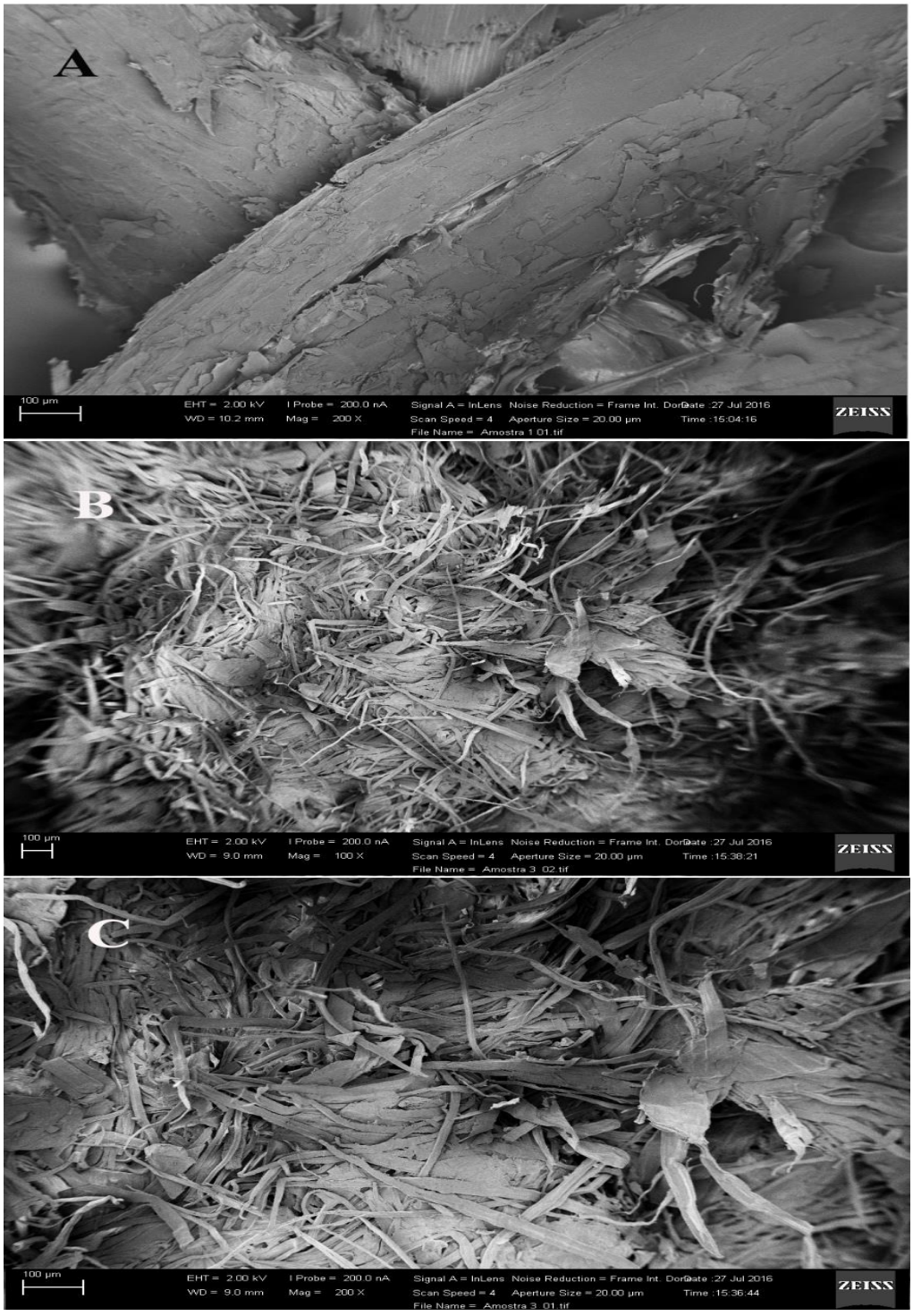

Figure 5.1 – Comparison of SEM images; Raw (A), B-HO (B), and K-HO (C)

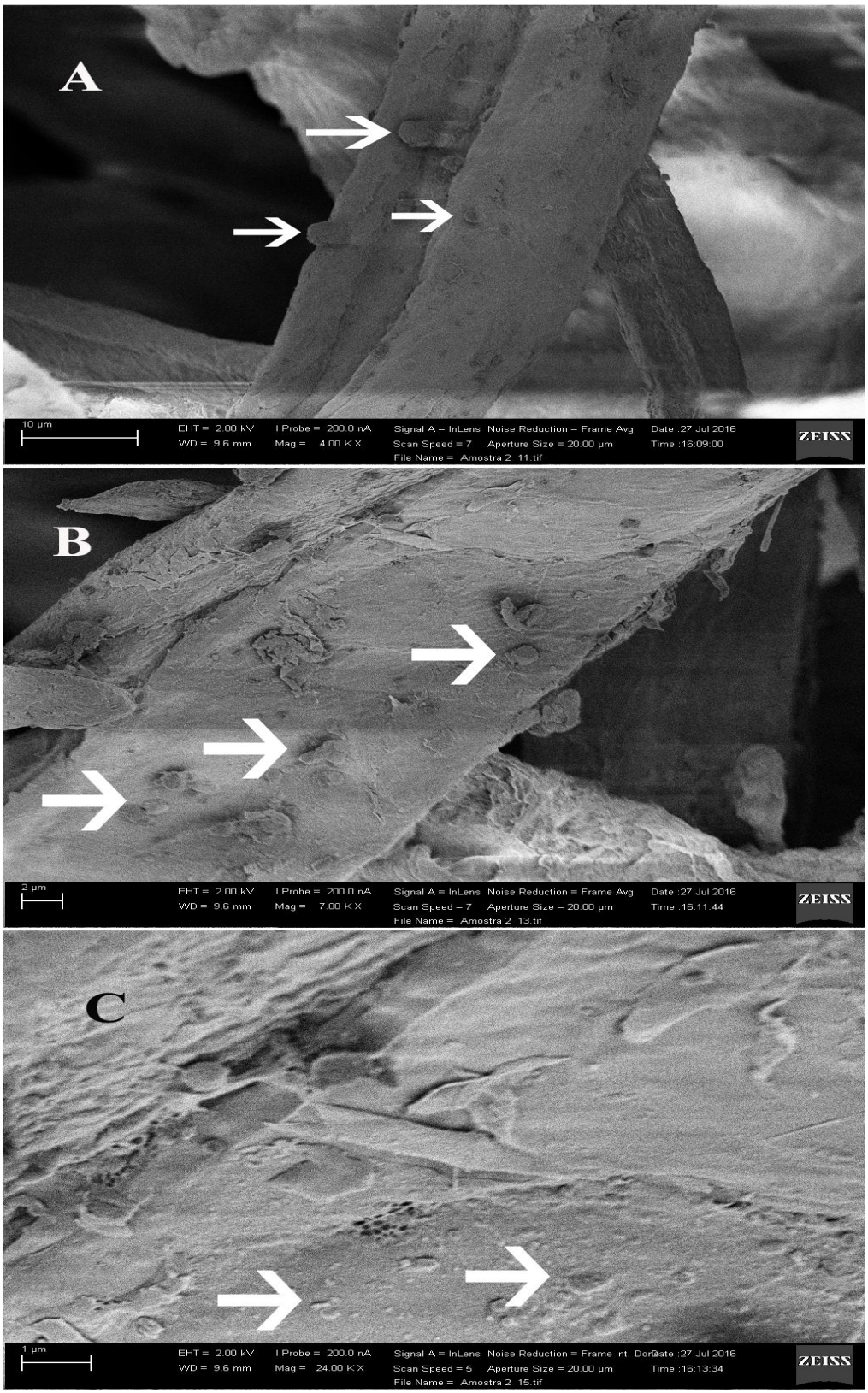

Figure 5.2 – SEM images of B-HO indicating pseudo-lignin droplets on cell wall surface

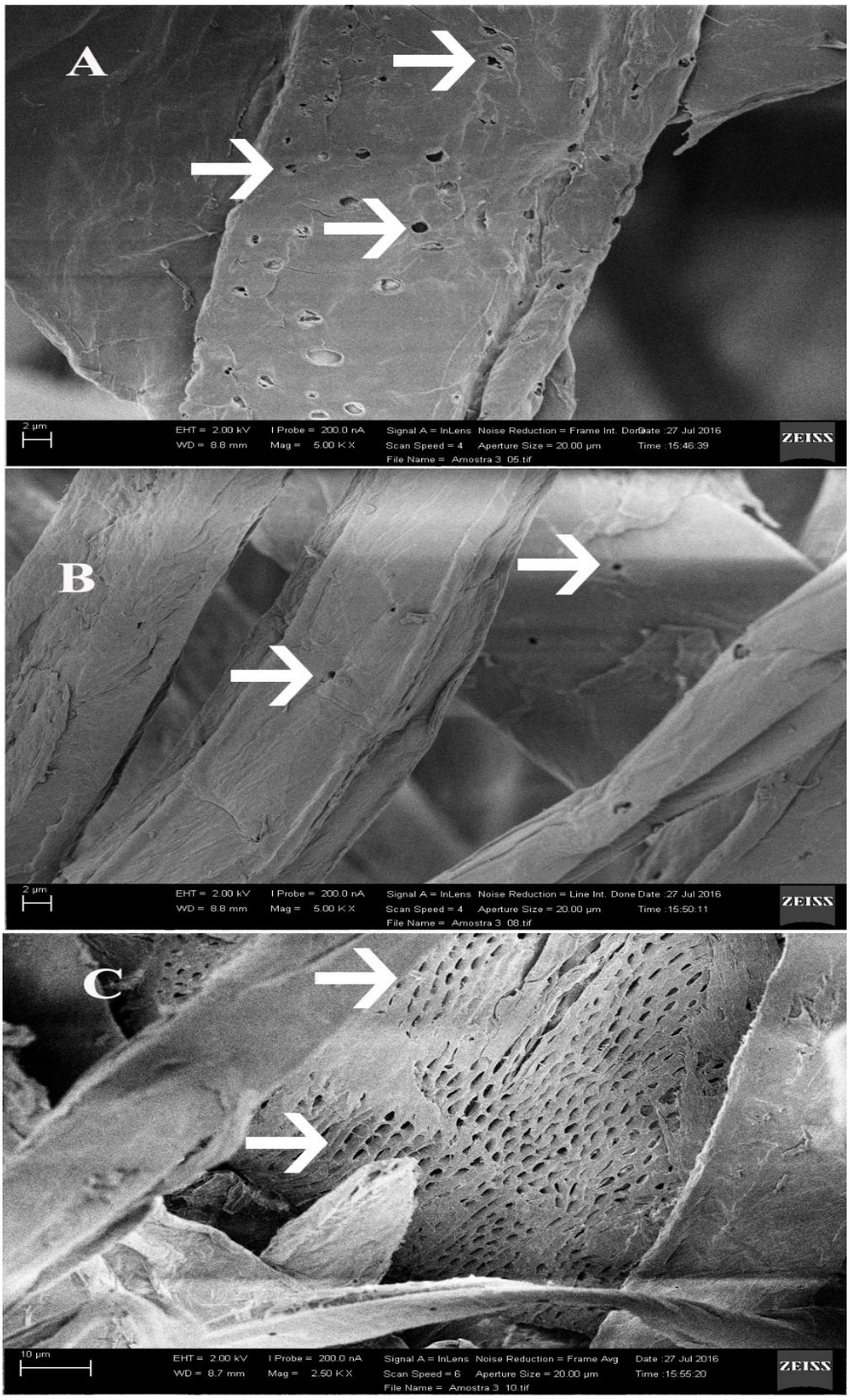

Figure 5.3 – SEM images of K-HO indicating porous surface due to lignin removal

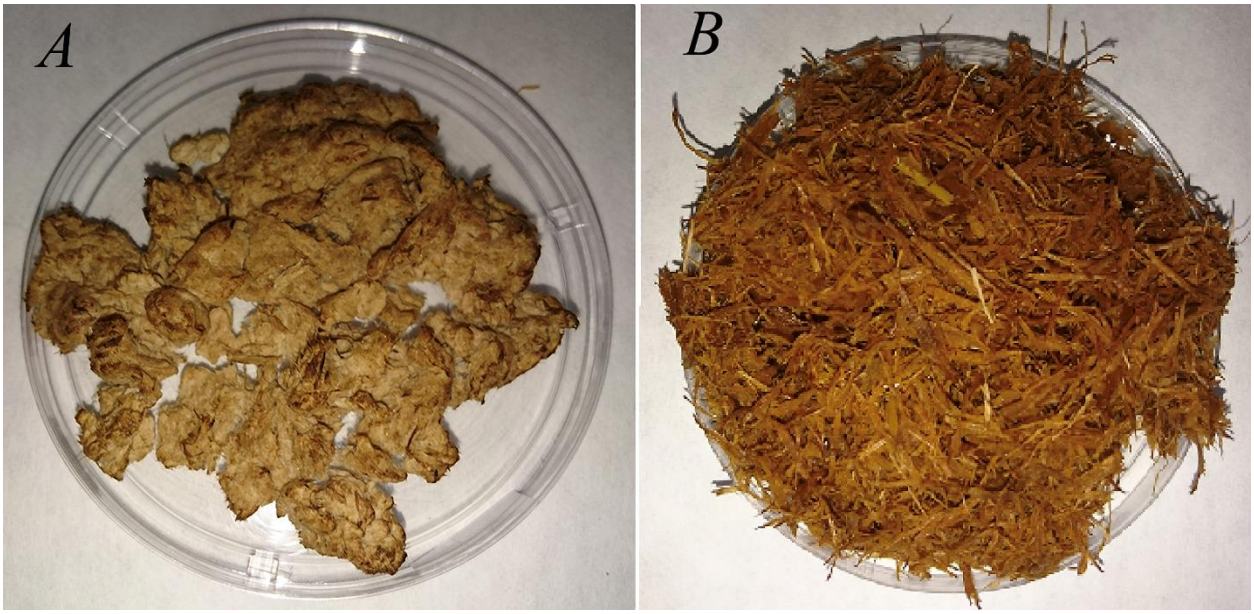

Figure 5.4 - Impact of H_2O_2 assisted hydrothermal pretreatment on visual appearance of sugarcane bagasse; (A) Highest studied temperature (pretreatment run O-HO – 213.6 °C), (B) Lowest studied temperature (pretreatment run P-HO – 146.4 °C)

A significant difference between pretreated samples cannot be seen at lower magnification, a distinct difference can be observed at a higher resolution (Fig 5.3 & Fig 5.4 A, B, and C). Both pretreated samples showed disorganized structure, characterized with scattered microfibrils; bundles appear flexible because of twisted appearance (Fig. 5.1 B, C). Heather et al (Heather *et al.*, 2013) reported that presence of carbohydrates in the biomass influence the solubility of lignin during pretreatment process. Cellulosic fibers are more exposed to the pretreated sample, which allows better access to enzymes for higher hydrolysis yield (Martín *et al.*, 2012; Cao and Aita, 2013). Complex agglomerated structures appeared on the surface (Fig 5.2 A, B, C) in the form of droplets of varying sizes in B-HO. These structures have been reported as pseudo-lignin in several other studies (Donohoe *et al.*, 2008; Kumar *et al.*, 2013; Nitsos *et al.*, 2013; Sun *et al.*, 2014). It appeared that lower concentration of H_2O_2 (2 % v/v) did not help in removing the lignin, in spite higher temperature (200 °C) showed significant impact on lignin phase transition and coalescence and pseudo-lignin formation. Furthermore, pores were not visible on the surface that are characteristic to lignin removal. The higher lignin content (23.2%) in B-HO solid fraction correlated well with the lower CH_4 yield (5.59 Nmmol g^{-1} TVS). Fig 5.3B depicts higher order dismantled surface of biomass for K-HO pretreatment.

Impregnation of bagasse at 7.36 (% v/v) H_2O_2 resulted in 73.09% delignification leading to the perforated surface (Fig 5.3 C). It demonstrated that impregnation in higher H_2O_2 concentration facilitated the degradation reactions, thereby, breaking down the lignin structure and evaporation of volatile compounds might have been resulted into the porous surface. The highly dispersed and porous surface represents a loss of physical and morphological integrity due to lignin removal owing to attack of degradation products; hydroxyl (HO^-) and superoxide radical anions (O_2^-) of H_2O_2 on lignin (Damaurai *et al.*, 2014). The porous surface structure might helpful for bacterial contact and further improve the degradation process during fermentation. Rezende et al (Rezende *et al.*, 2011) observed that delignification increased the fragility and holes in the cell wall structure of sugarcane bagasse. Authors further observed detachment of fibers and dismantling of vascular bundles as an impact of NaOH pretreatment on sugarcane bagasse. Limiting residence time of depolymerized lignin moieties during pretreatment could significantly improve lignin removal. Delignification promotes biomass swelling, which increases internal surface area and median pore volume (Zhu *et al.*, 2008).

Chandel and co-workers (Chandel *et al.*, 2014) reported comparable results while studying sodium hydroxide pretreatment on cellulignin obtained after dilute acid hydrolysis. Authors reported substantial removal of lignin in response to alkali pretreatment and visibility of pores on the surface. The morphological structure of sugarcane bagasse in the current study is further strengthened by the finding of Corrales et al (Corrales *et al.*, 2012) for the presence of round elongated shaped lignin particles on the biomass surface.

5.3 Chemical composition of liquid hydrolysate obtained from H_2O_2 assisted hydrothermal pretreatment

Liquid hydrolysate obtained from hydrothermal pretreatment was characterized by chemical oxygen demand (COD), total carbohydrate content, total phenolic content, organic acids and monomeric sugar compounds; glucose, and xylose.

Hydrothermal pretreatment results in breakage of chemical bonds within the lignin-cellulosic complex, thereby degrading structural compounds that make their route to liquid stream. Hence, resulted into increase in chemical oxygen demand and carbohydrate content.

Table 5.6 - Characterization of liquid hydrolysate obtained from H_2O_2 assisted hydrothermally pretreated sugarcane bagasse

Pretreatment	Temperature (°C)	Time (min)	H_2O_2 (v/v %)	COD (g L^{-1})	Total Carbohydrate (g L^{-1})	Phenolic content (ppm)
A-HO	200	19	6	17.75	4.14	3,005.63
B-HO	200	19	2	9.98	3.17	1,578.75
C-HO	200	5	6	12.98	3.32	2,068.13
D-HO	200	5	2	12.53	3.99	1,316.25
E-HO	160	19	6	10.28	3.27	1,991.25
F-HO	160	19	2	10.10	2.48	941.25
G-HO	160	5	6	13.49	2.24	1,316.25
H-HO	160	5	2	9.84	3.92	741.25
I-HO	180	12	4	9.63	3.88	2,028.75
J-HO	180	12	4	11.94	3.39	2,053.75
K-HO	180	12	7.36	17.21	4.18	1,468.13
L-HO	180	12	0.64	8.07	2.85	1,116.25
M-HO	180	23.76	4	13.44	3.35	1,411.88
N-HO	180	0.24	4	9.87	3.03	1,303.75
O-HO	213.6	12	4	12.84	1.96	1,828.75
P-HO	146.4	12	4	10.34	3.00	1,478.75

As indicated in Table 5.6, approx. 2-fold decrease in chemical oxygen demand (COD) was observed when H_2O_2 was decreased from 6 to 2% under similar conditions of temperature (200 °C) and time (19 min) for pretreatment A-HO and B-HO, respectively. On the other hand, 42% decrease (17.75 to 10.28 g L^{-1}) was noted when retention time (19 min) and H_2O_2 (6%) was held constant and pretreatment temperature was decreased from 200 to 160 °C for pretreatment A-HO and E-HO, respectively. A retention time of 5 min did not affect much on COD values with the decrease in H_2O_2 (6% to 2%) at 200 °C, though it was higher from COD values at 160 °C except for pretreatment G-HO, where it was bit higher (13.49 g L^{-1}), than pretreatment C-HO, under similar reaction time and H_2O_2 concentration. Pretreatment K-HO and L-HO showed 53.11% decrease (17.21 to 8.07 g L^{-1}) in COD with a decrease in H_2O_2 from 7.36 to 0.64% under similar

conditions of temperature (180 °C) and reaction time (12 min). It implicates that H_2O_2 applied had a notable impact on the release of organics into the liquid stream.

A maximum reaction time (23.76 min) studied in this study resulted in 26.56% decrease (13.44 to 9.87 g L^{-1}) in COD when compared to lowest (0.24 min) reaction time at pretreatment temperature of 180 °C and 4 (% v/v) H_2O_2. While only 19.47% decrease (12.84 to 10.34 g L^{-1}) was observed between pretreatments with highest (213.6 °C) and lowest (146.4 °C) studied temperature, keeping reaction time and H_2O_2 held constant. A decrease in COD solubilization at highest temperature could be due to the formation of humic substances or maximum achievable organic compounds degradation (Ahuja, 2015).

González-Fernández et al. (González-Fernández *et al.*, 2012) reported that higher temperature results in more solubilization of organic matter into liquid stream than lower temperature. Hao et al (Hao *et al.*, 2009) reported that COD of municipal solid waste increased from 1.8 to 7.5 g L^{-1} when temperature was increased from 90 °C to 190 °C. Authors further observed substantial increase in COD from 5.9 g L^{-1} to 12 g L^{-1}, when NaOH was incorporated from 1 g to 4 g 100 g^{-1} of biomass. Kaukuri et al. (Katukuri *et al.*, 2017) reported an increase in COD up to 6 g L^{-1} at 1% H_2O_2 for 24 h pretreatment and speculated that increase in COD with an increase in H_2O_2 loading is a direct indication of degradation of organic compounds. Ana et al (Anna *et al.*, 2010) reported 4.22 g L^{-1} COD for paper tube residuals treated with 2% H_2O_2 at 190 °C for 10 min in comparison to 0.026 g L^{-1} for untreated ones. Higher COD in the current study is a result of higher reaction temperature and higher H_2O_2 loading. The combination with treatments of temperatures, times and H_2O_2 should be assessed in further studies for optimum COD solubilization, however, was not the focus of this study.

Total carbohydrates content was in the range of 3.17 to 4.14 g L^{-1} at 200 °C for pretreatment A-HO – D-HO. The decrease in temperature to 160 °C under a similar condition of retention time and H_2O_2 resulted in a decrease in carbohydrate content in the range of 2.24-3.92 g L^{-1} for pretreatment E-HO – H-HO. Lower total carbohydrates content in the liquid slurry is a result of dehydration reactions of sugars to acids at a higher temperature. The argument is supported with the lowest total carbohydrate content (1.96 g L^{-1}) for the maximum temperature (pretreatment O-HO – 213.6 °C) applied in this study. Maximum total carbohydrate content (4.18 g L^{-1}) at maximum H_2O_2 applied (180°C, 12 min, 7.36% H_2O_2) indicated maximum lignin removal

(73.09% solubilization) making cellulose and hemicellulose readily available thereby increasing overall carbohydrate content. Lucas et al (Lucas *et al.*, 2012) reported that oxidative pretreatment like H_2O_2 results into the extreme degradation of hemicellulose thereby making it unavailable for the downstream fermentation process.

Since lignin is a complex phenolic polymer (Lange *et al.*, 2013), alkaline oxidative H_2O_2 pretreatment resulted in the appreciable amount of phenolic components release into the liquid stream. As presented in Table 5.6, phenolic compounds decreased (47.47%) with a decrease in H_2O_2 (6 to 2 %) from 3,005.63 to 1,578.75 ppm at the similar reaction temperature (200 °C) and time (19 min). When reaction time (19 min) and H_2O_2 (6% v/v) were held constant, a decrease in pretreatment temperature from 200 to 160 °C resulted in 33.74% decrease in phenolic content (3,005.63 to 1,991.25 ppm). A substantial decrease in phenolic contents was recorded at 160 °C with a decrease in H_2O_2; phenolic content decreased 52.73% (1,991.25 to 941.25 ppm) when H_2O_2 decreased from 6 to 2% at 19 min reaction time while at reaction time of 5 min 43.68% (1,316.25 to 721.25 ppm) decrease was noted between pretreatment run 7 and 8.

A 23.97% decrease in phenolic content was observed between pretreatments with maximum and minimum H_2O_2 employed at reaction temperature of 180 °C for 12 min. 19.4% difference in phenolic content was seen between pretreatments with highest and lowest temperature employed. The phenolic contents data in the hydrolysate indicated that pretreatment temperature and H_2O_2 concentration utilized had effectively broken down the ester linkages between lignin and hemicellulose thereby degrading lignin and release of phenolic monomers into liquid hydrolysate. Katukuri et al (Katukuri *et al.*, 2017) reported an increase in total phenolic content with an increase in H_2O_2 concentration for *Miscanthus floridulus* (a perennial grass). Harmsen et al (Harmsen *et al.*, 2010) reported that phenolic contents are more toxic to fermenting bacteria due to their capability to disintegrate microbial enzymes and speculated as more toxic than furfural and 5-hydroxymehtylfurfural. However, studies are available representing the consumption of phenolic compounds by several microbial guilds especially gut microbiota (Mosele *et al.*, 2015). This indicates that impact of phenolic compounds on microbial communities is genera dependent.

Sugarcane bagasse was introduced to the hydrothermal reactor at approx. pH of 12 after discarding H_2O_2. pH values of resulting liquid streams of different pretreatments are presented in

Table 5.7. The decrease in pH is a direct indication of production of organic acids during pretreatment due to degradation of structural components and break down of linkages within the lignin-carbohydrate complex. Lowest pH 4.66 was noted for the pretreatment B-HO (200 °C, 19 min, 2% H_2O_2). However, a minimal decrease in pH (9.68) was observed for pretreatment P-HO (146.4 °C, 12 min, 4% H_2O_2).

Table 5.7 - Selected organic acids, furanic compounds, and sugar monomers in liquid hydrolysate obtained from H_2O_2 assisted hydrothermal pretreatment of sugarcane bagasse

Pretreatment	**pH**	**Glucose (mg mL^{-1})**	**Xylose (mg mL^{-1})**	**Acetic acid (mg L^{-1})**	**Formic acid (mg L^{-1})**	**Furfural (mg L^{-1})**	**5-HMF (mg L^{-1})**
A-HO	8.23	0.19	2.63	201.23	884.07	0.00	5.25
B-HO	4.66	0.61	2.25	450.11	839.35	56.91	37.45
C-HO	8.78	0.34	5.90	409.56	851.00	0.00	56.60
D-HO	5.35	0.25	8.55	581.02	736.67	0.00	8.01
E-HO	9.85	0.44	3.93	280.00	654.83	0.00	7.47
F-HO	8.65	0.18	4.45	255.78	54.95	0.00	12.69
G-HO	9.38	0.17	3.64	377.13	482.13	0.00	15.03
H-HO	8.64	0.20	4.52	550.94	144.43	0.00	32.00
I-HO	8.89	0.17	4.03	367.66	509.08	0.00	27.46
J-HO	8.19	0.17	3.84	189.84	504.06	0.00	18.31
K-HO	9.49	0.50	6.56	494.86	952.31	0.00	2.56
L-HO	5.2	1.29	9.60	199.93	222.69	7.62	39.00
M-HO	8.92	0.26	1.27	305.39	532.47	0.00	8.91
N-HO	8.91	0.78	7.48	107.42	151.77	0.00	21.78
O-HO	4.85	0.82	4.95	474.54	1108.02	27.46	46.44
P-HO	9.68	0.38	7.92	339.90	416.76	0.00	23.74

Low-molecular-weight carboxylic acids are produced during hydrothermal pretreatment due to degradation mechanism of polymeric carbohydrates at high temperature. Selected acids (Acetic and formic), furanic compounds (furfural and 5-hydroxymethylfurfural) as well as sugar monomers produced in the current study are presented in Table 5.7. Acetic acid released from O-acetyl groups in hemicellulose results into lowering the pH during hydrothermal pretreatment that

further facilitates the removal of sugar monomers (Chen *et al.*, 2010; Shen *et al.*, 2016). Another possible reason for a decrease in pH during hydrothermal pretreatment could be hydrothermal oxidation of different minerals present in sugarcane bagasse (Chandra *et al.*, 2012).

Acetic acid varied in the range of 107.42 to 581.02 mg L^{-1} for H_2O_2 pretreated samples. Concentration pattern of acetic acid in the liquid hydrolysate of sixteen pretreatments indicated that pretreatment parameters had an effect on its production in relation to pH of the liquid hydrolysate. Acetic acid is reported to be produced from the oxidation of levulinic and lactic acid during hydrothermal pretreatment (Jin *et al.*, 2005; Fang *et al.*, 2012). Lower acetic acid concentration could be attributed to its further degradation.

Quitain et al (Quitain *et al.*, 2002) reported that formic and acetic acid are intermediate products before their complete degradation to volatile carbon and water. Higher concentrations of formic acid were observed at a higher temperature (200 °C) irrespective of the reaction time and H_2O_2 concentration. However, Jin et al (Jin *et al.*, 2008) reported that higher yields of formic acids could be attained by adding base catalyst or H_2O_2 during hydrothermal pretreatment. The current study revealed that formic acid could be produced in higher yields even at lower H_2O_2 at a higher temperature; however, higher formic acid at lower temperature required higher H_2O_2 (pretreatment K-HO – 7.36% H_2O_2, 12 min, 180 °C). Highest formic acid (1,108.02 mg L^{-1}) was observed in current study at pretreatment O-HO (213.6 °C, 12 min, 4% H_2O_2).

C_5 sugars degrade into furfural while decomposition of C_6 sugars results into 5-HMF, both are further degraded into formic acid (Shen *et al.*, 2016). Furfural was not observed in 13 of 16 pretreatments primarily due to higher pH. Furfural can be degraded into formic acid via hydrolytic fission of aldehyde groups. Furfural was only observed in pretreatments where liquid hydrolysate pH was approx. closer to 5.

5-HMF concentrations were ranging from 2.56 to 56.60 mg L^{-1}. The lower furanic compounds in H_2O_2 impregnated pretreatments are in agreement with Teixeira et al. (Teixeira *et al.*, 2014). The authors suggested that pH of reaction mixture must be kept between 4-7 to avoid the formation of inhibitory compounds because this pH disfavors the formation of monomers, carbohydrates retained in oligomeric form resulting into lower furanic compounds production. However, Du et al (Du *et al.*, 2010) presented another viewpoint that under alkaline conditions, degradation of sugars might occur through some alternate pathway.

Glucose and xylose in the liquid fraction were determined after Sluiter protocol (Sluiter *et al.*, 2011). Lower values of glucose were seen in liquid hydrolysate indicating that most of the cellulose was retained in the solid fraction. On the other hand, relatively higher values of xylose in the range of 1.27 – 9.60 mg mL^{-1} indicated removal of xylose as indicated by higher xylan removal (Table 5.2). Su et al. (Su *et al.*, 2015) conducted fractional pretreatment on corncob with H_2O_2 to characterize its major components. Authors observed no xylose and arabinose in the liquid fraction. However, the pretreatment strategy was different comparing to the present study. The authors conducted pretreatment in a shaking water bath at 50 °C at pH 11. However, the present study was realized in a hydrothermal reactor solubilizing xylan content leading to higher pentose sugars in the liquid hydrolysate. The authors speculated that hemicellulose removal during hydrogen peroxide is a dissolution mechanism and not reaction mechanism.

5.4 Methane production from solid fractions of H_2O_2 assisted hydrothermal pretreatment

Solid fractions of H_2O_2 impregnated hydrothermal pretreatments were tested for methane production. Batch reactors were set up based on total volatile solids (TVS) according to CCD matrix (Table 4.4). The composition of each reactor is presented in Table 9.1 (Appendix). Batch reactors were supplemented with 1 g TVS of the pretreated substrate and inoculated with 2 g TVS of inoculum. The actual substrate (g) and inoculum (g) values are presented in Table 9.1 (Appendix). The reactors were incubated in a neutral starting pH.

Total time of incubation was variable (600 to 1200 h) across different pretreatments based on stable methane production in different reactors. Sugarcane bagasse is a heterogeneous material, it requires a consortium of hydrolytic microorganisms to break down its polymeric structure for the release of fermentable sugars for fermenting microbial. Methane production data (Nmmol g^{-1} TVS) was processed with modified Gompertz model (Zwietering *et al.*, 1990). Modified Gompertz model has been utilized to describe the progress of cumulative CH_4 production in batch experiments taking into account relationship between microbial growth and metabolism (Ghasemian *et al.*, 2016; Siripatana *et al.*, 2016; Li *et al.*, 2017).

In the first set of experiments, six pretreatments were tested for digestion experiments; B-HO, C-HO, D-HO, E-HO, F-HO and H-HO besides control without substrate addition i.e., only inoculum to subtract indigenous CH_4. Pretreatment B-HO had presented longest lag phase of 88.45

h (Table 5.9) before the methane production started. Higher lag phase in B-HO could be a result of higher lignin (23.2%) content in the pretreated solid besides higher furanic compounds (furfural 0.06 g L^{-1} and 0.04 g L^{-1} 5-HMF) in the pretreatment slurry.

Lignin structure resulting due to hydrothermal pretreatment is inhibitory to microbial cellulase (Ko *et al.*, 2015). Microorganisms need to acclimatize to the reactor conditions and inhibitory compounds like furfural and 5-HMF (Monlau *et al.*, 2014). Anna et al (Anna *et al.*, 2010) reported a lag phase of 5 days (120 h) for paper tube residuals treated at 190 °C with 2% H_2O_2 for 10 min. Methane reached to a maximum level of 5.59 Nmmol g^{-1} TVS in 250 h afterward its production started to decrease that could possible due to unavailability of fermentative products due to higher lignin (23.2%) content in this pretreatment. Lignin makes it difficult for the microbial cellulases to degrade polysaccharides due to their unproductive adsorption.

For other pretreatments in this set, lag phase varied in the range of 37.32 – 86.80 h. A rapid increment in CH_4 was seen in C-HO and E-HO after the lag phase in comparison to other pretreatments. The CH_4 value was 5.75 and 6.00 Nmmol g^{-1} TVS for pretreatment C-HO and E-HO, respectively while for B-HO, D-HO, F-HO and H-HO it was 1.67, 2.28, 4.52, 4.22 Nmmol g^{-1} TVS, respectively at approx. log phase of 132 h. Thereafter, CH_4 production was observed in all the pretreatments at relatively lower rate until it reached to plateau at approx. 348 h. Overall methane production rate for this set was in the range of 0.03 – 0.12 Nmmol g^{-1} TVS h^{-1} (Table 5.9). The reactors were terminated at 600 h assuming stable production. However, as appeared in Fig 5.5, if the reactors could let for further few days CH_4 production might continue and there might be a higher value of CH_4 in the first set of experiments documented in this text. Positive control (glucose) was also tested for methane production (Fig 5.5). Maximum methane 6.8 Nmmol was recorded in 288 h afterward its production decreased and reactor was terminated after 408 h. As indicated in Fig 5.5, methane production rate was high enough that in 24 h of incubation methane production almost reached to its maximal point.

In the second set of experiments, I-HO, M-HO, N-HO, and O-HO were tested. The lag phase of -35.73 h in I-HO indicated an abrupt production of CH_4 in this pretreatment while in other three pretreatments lag phase varied from 28.77 – 35.56 h. In 108 h, CH_4 production reached to 5.9, 3.2, 3.06 and 4.21 Nmmol g^{-1} TVS (approx. log phase) for I-HO, M-HO, N-HO and O-HO, respectively. However, a rapid increase in CH_4 was observed in O-HO and M-HO afterward.

Methane production continued to increase at a lower rate until it reached to stable phase in 659 h. The reactors were discarded after observing not a significant increase in CH_4 at 779 h. The overall methane production rate for this set of experiments was ranging from 0.03 – 0.04 Nmmol g^{-1} TVS h^{-1}.

In the third set of experiments, six pretreatments namely A-HO, G-HO, J-HO, K-HO, L-HO and P-HO were set up for digestion. A lower lag phase was observed in pretreatment P-HO (-37.59 h) followed by G-HO (0.50 h). While, for other pretreatments lag phase varied from 14.35 – 24.44 h. After the lag phase, the instant increment in CH_4 production was observed for A-HO, G-HO, J-HO, and K-HO. CH_4 reached to 6.18, 4.51, 3.0 and 4.45 Nmmol g^{-1} TVS in 108 h. On the other hand, a relatively slower increment was seen for L-HO and P-HO even though minimum lag phase was observed in P-HO. Relatively slower production of CH_4 in L-HO and P-HO could be justified with the pretreatment conditions; L-HO was pretreated with lowest H_2O_2 (0.64% v/v) at 180 °C and 12 min, P-HO was pretreated at lowest pretreatment temperature 146.4 °C for 12 min and 4% (v/v) H_2O_2. These conditions might have not able to degrade sugarcane bagasse structure for the action of microbial enzymes thereby resulting in the slower activity of hydrolytic microbial consortia leading to a relatively slower increment in CH_4.

As depicted in Fig 5.5, after a steep slope for CH_4 around 108 h, CH_4 production continued to increase until it reached a maximum value. The reactors were discarded at 1200 h after verifying no CH_4 increment in overall production. Methane production rate for this set of experiments was in the range of 0.01 – 0.05 Nmmol g^{-1} TVS/h. Lowest CH_4 production rate for L-HO and P-HO further strengthen the assumption that pretreatment conditions for these had barely impacted the sugarcane bagasse structure.

A comparison of the current study with literature is not possible because of pretreatment conditions and feedstocks differences. Song et al. (Song *et al.*, 2013) reported 88% (288 mL $.g^{-1}$ VS, 12.8 $mmol.g^{-1}$ VS) higher methane production in rice straw, however, the pretreatment conditions are different from the current study. The authors conducted pretreatment at room temperature 25 ± 2 °C by impregnating rice straw into H_2O_2 (1 – 4%) for 1-7 days.

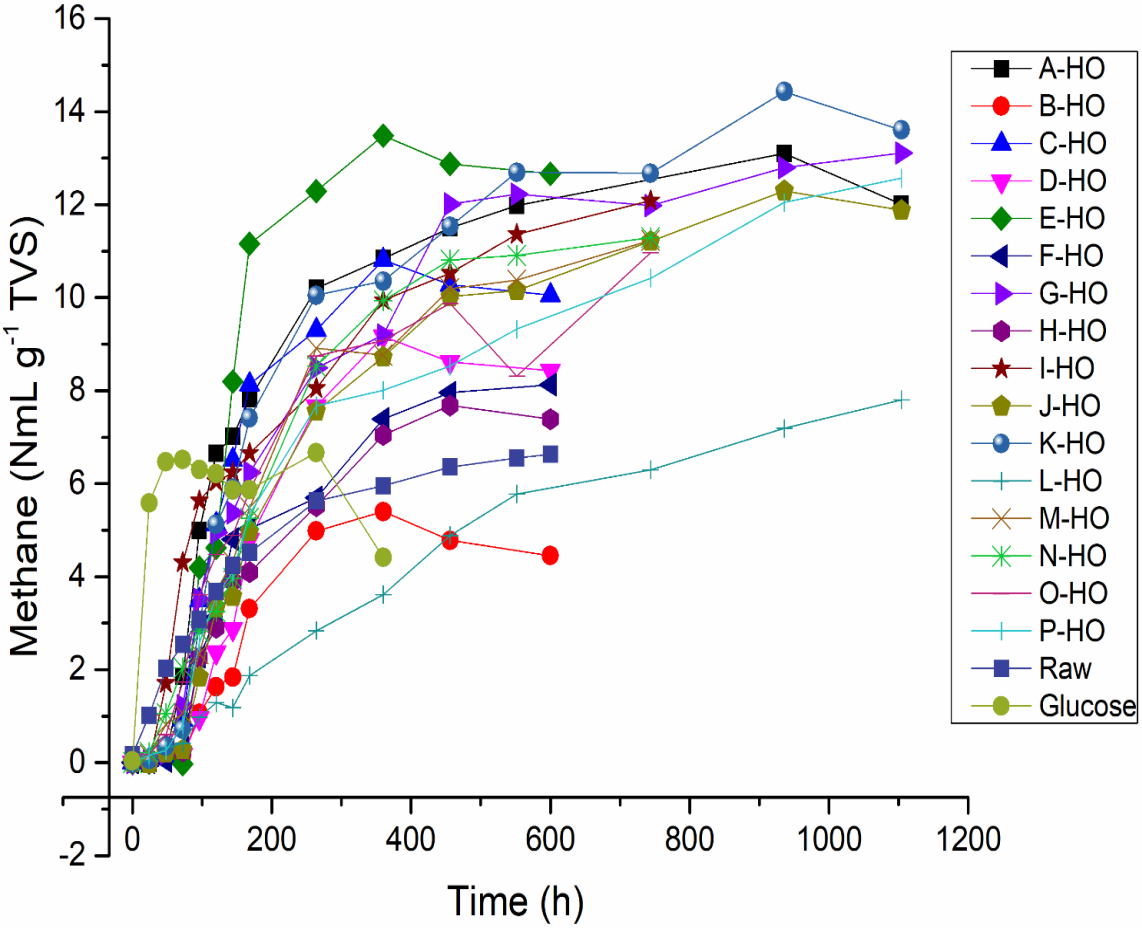

Figure 5.5 - Time-course profile of cumulative methane production for solid fraction of H_2O_2 impregnated hydrothermal pretreatment

In another study, Michalsk and Ledakowiz (Michalsk and Ledakowicz, 2014) reported better methane production at 5% H_2O_2 for 24 h impregnation at 25 °C. Rabelo et al (Rabelo *et al.*, 2011) conducted alkaline hydrogen peroxide pretreatment of sugarcane bagasse at 4 – 15 % DM with 7.36% H_2O_2 at 25 °C for 1 h under 150 rpm and reported a production of 72.1 L CH_4 per kg of bagasse. Song et al (Song *et al.*, 2012) conducted hydrogen peroxide impregnation study on rice straw for 7 days at 4% and 3% H_2O_2 under room temperature conditions (25 °C) and reported 327.5 and 319.7 mL biogas per gram of VS. Anna et al (Anna *et al.*, 2010) conducted explosive

pretreatment on paper tube residuals at 220 °C for 10 min with the aid of 2% NaOH and 2% H_2O_2 and reported 493 Nml g^{-1} VS (22.0 Nmmol g^{-1} VS) methane, which was 107% higher than untreated residuals. The current study revealed that higher concentration of H_2O_2 employed resulted in higher methane production from the pretreated solid fraction owing to the higher amount of contained glucan and lignin removal. Pretreatment 11 (180 °C, 12 min, 7.36 (% v/v) H_2O_2) resulted in maximum methane production of 14.43 Nmmol g^{-1} TVS.

Microbial acclimatization to the reactor conditions where directed to methane production also caused a simultaneous increase in COD due to the production of organic acids and sugar monomers by the action of fermentative bacteria. As shown in Fig 5.6 COD values for the first set of experiments in the start of digestion process was in the range of 121 ± 10.61 – 194.5 ± 10 $mg.L^{-1}$ depending upon pretreatment conditions.

Inoculum sludge could also influence initial COD value but since the same volume of inoculum was supplemented on total volatile solids (2 $g.kg^{-1}$) basis, its contribution to COD assumed to be constant in all the pretreatments. A 2-fold increase in COD value was observed in 132 h ranging from 215.38 ± 7.06 to 329.5 ± 21.92 mg L^{-1}. B-HO presented the lowest increase in COD in 132 h from 194.5 ± 10.61 to 200.38 ± 7.06 mg L^{-1}. This low increase justifies the presence of higher lignin that inhibited bacterial cellulase from degrading the polymeric substrate.

COD was in the range of 122 ± 14.14 to 248.15 ± 18.17 mg L^{-1} at reactor termination. Initial COD for the second set of experiments (Fig 5.6) was in the range of 129.5 ± 24.75 to 236.50 ± 30.41 mg L^{-1}, which subsequently increased to 302.0 ± 26.57 to 344.0 ± 29.70 $mg.L^{-1}$ at approx. log phase in 108 h. High variation in initial COD was due to pretreatment conditions. Highest initial COD was for O-HO, which was conducted at 213.6 °C, while other pretreatments in this set were realized at 180 °C.

Final COD for the second set of experiments was in the range of 238.50 ± 31.82 to 273.50 ± 23.33 mg L^{-1}. COD values for the third set of experiments (Fig 5.6*)* increased to 208.50 ± 10.68 to 312.60 ± 18.10 mg L^{-1} in 108 h and 228 h (L-HO and P-HO) from initial COD, which was in the range of 189.86 ± 6.43 to 240.48 ± 21.21 mg L^{-1}.

An increase in COD was noted with an increment in fermentation time due to the production of organic acids. A maximum increase in COD was verified in pretreatment K-HO up

to 396.75 mg L^{-1}, however, subsequently reduced to 186.85 mg L^{-1} at the end of digestion indicating consumption of organic acids produced during the process. However, minimal increase in COD was observed in pretreatment run 2 indicating minimal activity of fermenting bacteria owing to higher lignin content resulting in lower organic acids production and ultimately lower methane production.

Total carbohydrates (Fig 5.7) at the start of digestion experiments were in the range of 16.83 ± 4.4 to 38.55 ± 3.83 mg L^{-1} depending upon pretreatment conditions applied and carbohydrates left in solid fraction after separation from the liquid hydrolysate. A subsequent decrease in the total carbohydrate content (8.21 ± 1.64 – 30.59 ± 0.50 mg L^{-1}) was observed during the digestion process that is normal due to the action of acetogenic bacteria utilizing available carbohydrates for the production of organic acids. The minimal decrease was observed in B-HO from 37.25 to 30.59 mg L^{-1} that justify the higher klason lignin in solid fraction.

On the other hand, an increase in total carbohydrate content was noted for three pretreatments; C-HO (27.63 – 33.55 mg L^{-1}), E-HO (24.7 – 27.69 mg L^{-1}), and K-HO (24.91 – 28.39 mg L^{-1}). An increase in total carbohydrate content in these pretreatments could be justified with the argument that since these were pretreated with higher H_2O_2 and resulted in efficient lignin removal giving an opportunity for hydrolytic bacteria to act rapidly. The lowest total carbohydrate consumption (45.10%) was observed in B-HO (37.25 – 20.45 mg L^{-1}). On the other hand, approx. 100% consumption was seen in G-HO and J-HO where total carbohydrate was below the detection limit. Lowest carbohydrate consumption in B-HO seems obvious due to methanogenic inhibition owing to higher klason lignin content.

pH decrease is a direct indication of the release of organic acids, it decreased from neutrality to a value in the range of 6.37 to 6.67 for different pretreatment conditions at 108 h, at 132 h, and at 228 h (Fig 5.8). These points are at around approx. log phase of methane curve for respective pretreatments. pH remained lower for the entire digestion period. Final pH for reactors was in the range of 6.15 to 6.61 (Fig 5.8). The optimal range for methanogenesis is reported to be in the range of 6.8 – 7.5 (Alrawi *et al.*, 2011).

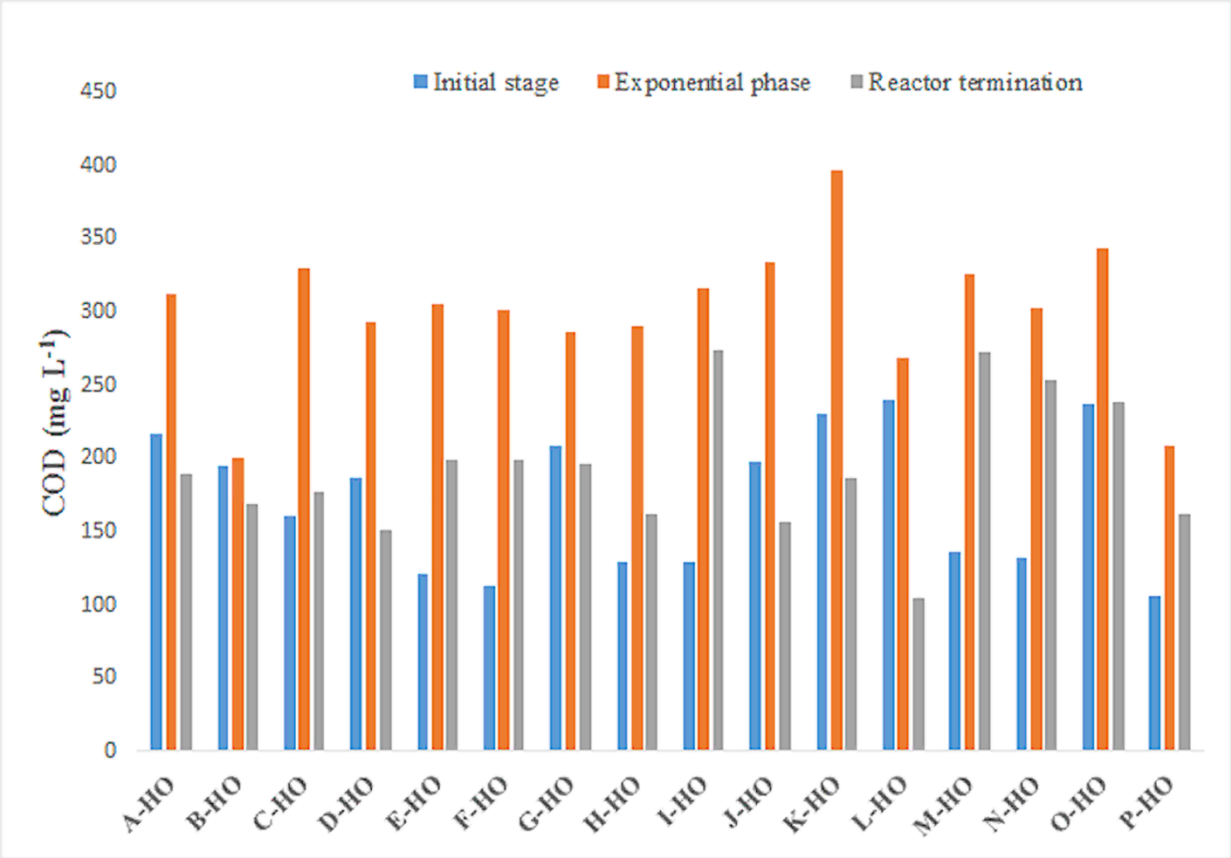

Figure 5.6 - COD variation during digestion experiments

As depicted in Fig 5.9, acetic acid was the most abundant organic acid encountered at the initiation of batch digestion experiments in different pretreatment reactors ranging from 57.2 to 145.6 mg L^{-1} followed by iso-butyric acid in the range of 8.39 to 105.6 mg L^{-1}. Propionic acid was only observed in pretreatment E-HO (16.27 mg L^{-1}). Butyric, valeric, isovaleric, and formic acid were also found in low quantities ranging from 10.3 – 51.3, 3.0 – 15.3, 4.2 – 21.8, and 2.10 – 10.0 mg L^{-1}, respectively. The relatively higher concentration of acetic acid, in addition to other factors, might have helped in improved acclimatization of microbial biomass as indicated by lower lag phase (14.35 h) for pretreatment run K-OH containing 145.26 mg L^{-1} acetic acid. The polymeric carbohydrates contained in lignocellulosic biomass are degraded into glucose then to pyruvate, which further converted to lactic acid or two molecules of acetyl-CoA and either formic acid or CO_2. With minor differences, lactate, acetate, ethanol, formate or CO_2 production from pyruvate is similar in Gram-positive and Gram-negative bacterial strains (Nielsen *et al.*, 2011)

Cumulative VFAs production at the approx. log phase (108, 132 and 228 h) for different pretreatments was in the range of 110.05 – 228.04 mg L^{-1}. Lower production of organic acids at

approx. log phase could be due to efficient utilization of organic acids, since bagasse is a heterogeneous material and is not readily available for the microbial enzymatic action of hydrolytic bacteria for the release of sugars to be consumed by fermentative bacteria producing organic acids.

It means methanogenic archaea are scavenging organic acids at a rate much higher than are being produced by the acidogenic bacteria. Zhang et al (Zhang *et al.*, 2011) reported similar situation for total VFAs production at approx. log phase during digestion of cassava residue. O'Sullivan and Burrel (O'Sullivan and Burrell, 2007) reported that lignocellulosics composition and degree of lignification have a direct impact on the release and type of organic acids detected in an acidification system.

Type and concentration of organic acids showed significant change at log phase comparing with initiation phase. The concentration of formic acid reduced substantially at log phase as it is directly taken up by methanogens besides acetic acid, methanol, methylamines, carbon dioxide, methyl-sulfide and H_2 (Li *et al.*, 2016). Efficient utilization of acetic acid is reflected by the higher acetotrophic methanogens community in comparison to lower hydrogenotrophic methanogens.

Acids produced in the digestion process are converted to the ultimate substrate for methanogens; acetate, H_2, CO_2 and other substrates by various degradation pathways. Isomerization of butyric acid into normal and iso- form of butyric acid is reported into anaerobic digestion where propionic acid is an intermediate product (Lin and Hu, 1993). Since the samples for VFAs were taken within 200 h of digestion, lower VFAs concentrations (Fig 5.9)were encountered; 65.72 – 159.24 mg L^{-1} acetic acid.

Lower propionic acid concentrations at reactor termination (2.15 to 15.74 mg L^{-1}) is an indication of stable digestion process as the accumulation of propionic acid is reported to be a sign of process disturbance (Bjornsson *et al.*, 2000; Murto *et al.*, 2004). Propionic acid concentration about 1-2 g L^{-1} is considered toxic to digestion process (Wijekoon *et al.*, 2011), while, Wang et al (Wang *et al.*, 2009) reported that 900 mg L^{-1} propionic acid is enough to inhibit methanogens. The low concentration of propionic acid accumulated during the initial phase of fermentation will benefit subsequent anaerobic digestion because propionate-assimilating microorganisms are among the slowest growing organisms due to low free-energy gain from the conversion of propionate to acetate. Furthermore, their complicated syntrophic relation to hydrogen-utilizing methanogens (Zhang *et al.*, 2011).

Butyric acid was observed in the range of 2.96 to 34.97 mg L^{-1}). While, ethanol was only observed at the end of digestion experiments in low concentrations 3.02 – 9.8 mg L^{-1}) in some reactors. Relatively higher acetic acid (13.53 - 103.30 mg L^{-1}) at the end of digestion is an indication that organic acids produced during the digestion process were converted to the principal substrate for methanogenesis. Wang et al (Wang *et al.*, 2009) reported that even higher concentrations of acetic acid (2400 mg L^{-1}) and butyric acid (1800 mg L^{-1}) had not exerted inhibition effect on methanogens.

Relatively higher concentrations of isovaleric acid were found at the end of digestion experiments in the range of 7.5 to 15.4 mg L^{-1}. Hill and Bolte (Hill and Bolte, 1989) reported that isovaleric acid more than 15 mg L^{-1} is an indication of a problem in the system. However, opinions vary in the literature regarding VFAs concentration for better digestion performance and inhibition indicators. Angelidaki et al (Angelidaki *et al.*, 1993) reported that different systems have their own levels of VFAs being considered 'normal', furthermore, conditions considered abnormal in one system might not be the same for the other system.

A higher production of acetic acid (159.24 mg L^{-1}) at approx. log phase was recorded in pretreatment run 3. However, it reached to methane value of 10.81 Nmmol g^{-1} TVS. Methane production rate for this pretreatment was a bit higher (0.08 Nmmol g^{-1} TVS h^{-1}) in comparison to pretreatment 11 with a production rate of 0.04 Nmmol g^{-1} TVS h^{-1} even though acetic acid for this pretreatment at log phase was 110.4 mg L^{-1}.

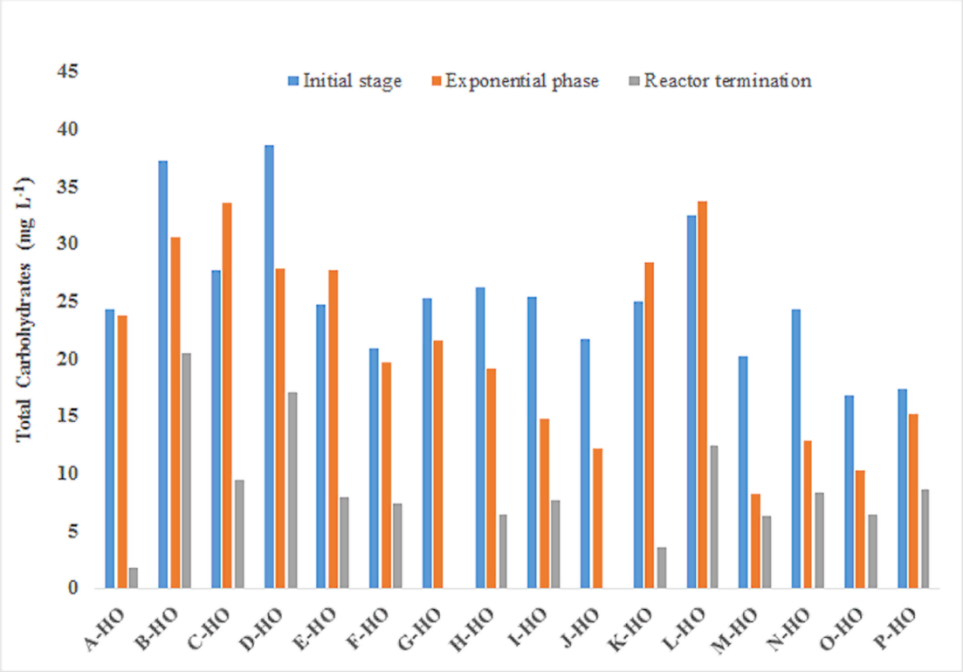

Figure 5.7 - Total carbohydrates consumption during digestion experiments

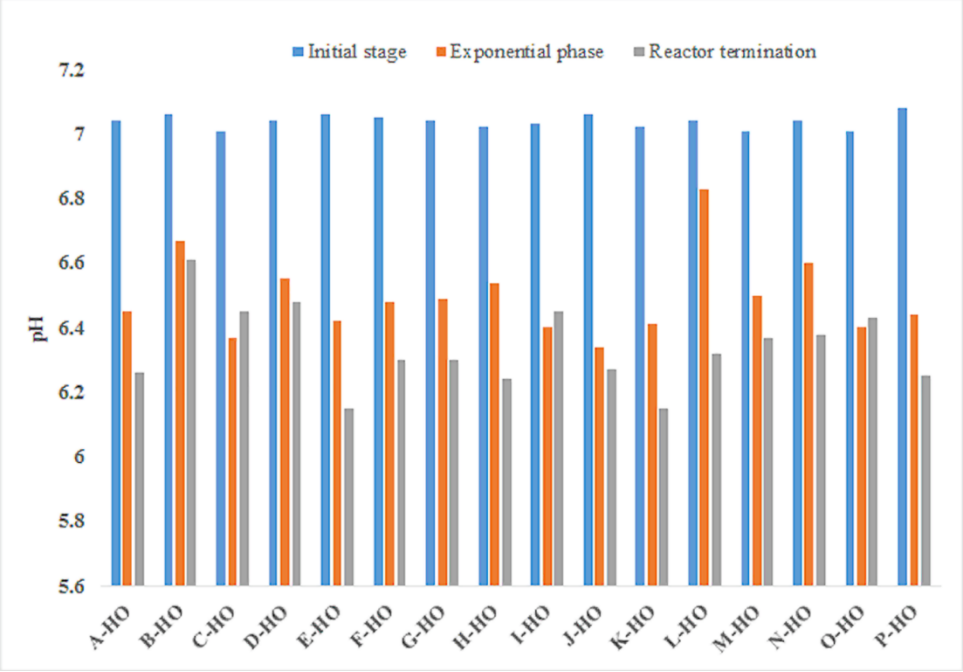

Figure 5.8 - pH variation in digestion experiments

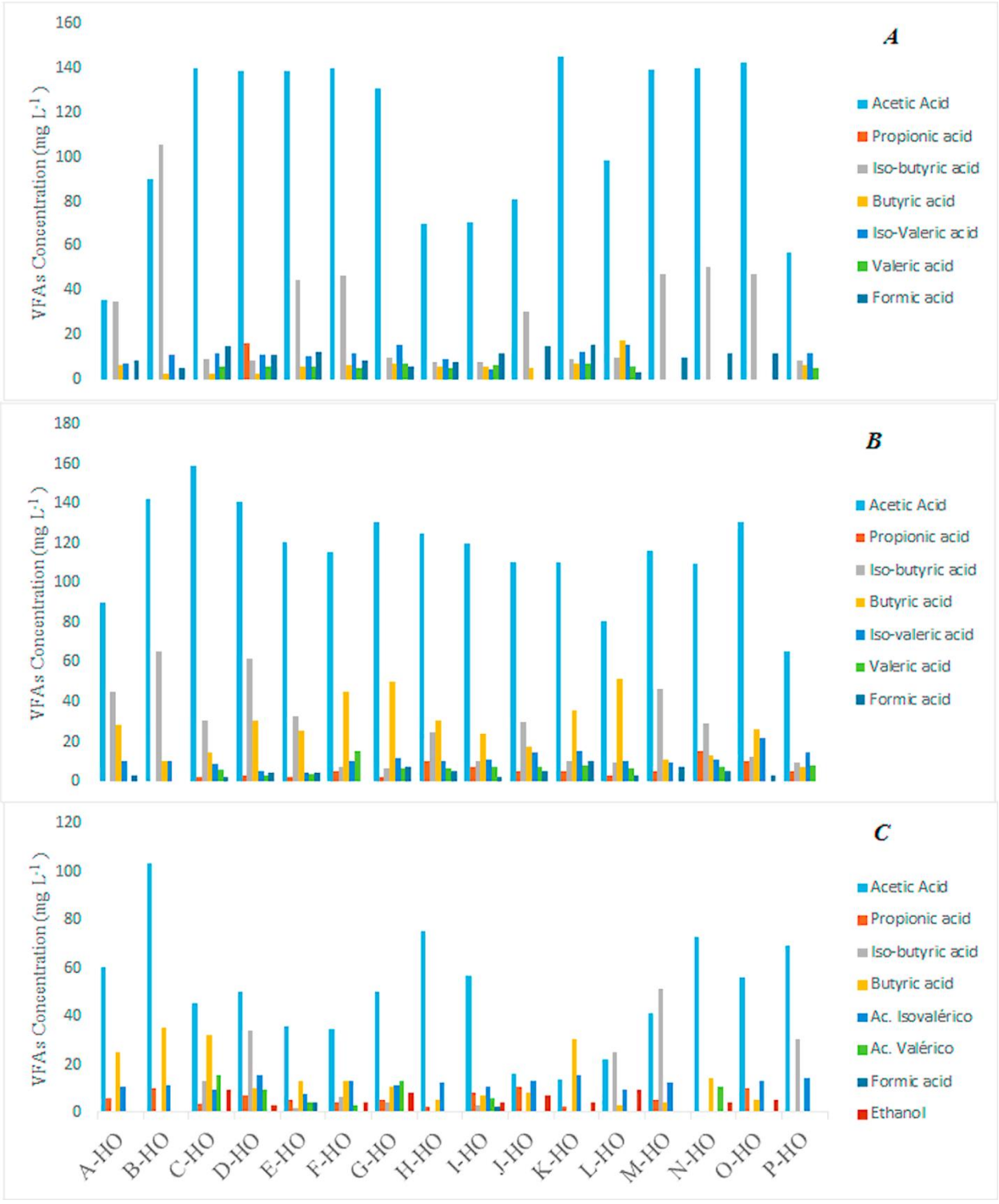

Figure 5.9 - Organic acids at three selected position on methane production curve; A (Initial stage of reactor), B (approx. exponential phase), C (reactor termination)

5.5 Statistical methane optimization with H_2O_2 assisted HT pretreatment

The proposed influential parameters; temperature (°C), time (min), and H_2O_2 (% v/v) were explored with response surface methodology for individual impact and their interaction on CH_4 yield (Nmmol g^{-1} TVS) from pretreated sugarcane bagasse in comparison to raw bagasse (Table 5.9). Quadratic regression analysis was conducted on the experimental data to explain CH_4 production. Second-order polynomial equation was derived for the parameters is presented in Equation 5.6

$$CH_4\ Yield\ = 12.34 - 0.48X_1 - 0.01X_2 + 2.28X_3 - 0.46{X_1}^2 - 0.55{X_2}^2 - 0.67{X_3}^2 - 0.26X_1X_2 - 0.21X_1X_3 + 0.73X_2X_3 \quad (5.6)$$

Where X_1, X_2, and X_3 are coded values for temperature (°C), time (min), and H_2O_2 (% v/v), respectively.

The statistical significance of the model was found by F-test (ANOVA) (Table 5.8). Correlation coefficient (R^2) value was found to be 0.8903. The R-square value indicated a measure of variability in the observed response values. It implied that the model could explain 89.03% of the sample variation and the model cannot explain only 10.97%. For a good statistical model R^2 value should be in the range of 0.75 – 1.0 (Niladevi *et al.*, 2009).

The magnitude and sign are important in the polynomial equation to explain the importance of variables and their interaction. The model Equation indicated that linear model term of time (X_2) besides quadratic model term of temperature (${X_1}^2$) were insignificant ($p>0.05$) along with the interaction of (X_1X_2) and (X_1X_3). While linear model terms of temperature ($X1$), H_2O_2 concentration (X_3) and quadratic model terms of time (${X_2}^2$) and H_2O_2 concentration (${X_3}^2$) and interaction of X_2X_3 showed significant impact ($P<0.05$) on CH_4 yield. The model coefficient of linear model term X_3 (2.28) and interaction of X_2X_3 (0.73) exerted positive effect while model coefficients of quadratic model terms ${X_2}^2$ and ${X_3}^2$ exerted a negative impact on the model. The model coefficients explained that H_2O_2 concentration has higher magnitude impact (2.28) in comparison to the interaction of H_2O_2 concentration (X_3) and time of pretreatment (X_2) (0.76).

Table 5.8 - Regression analysis for the experimental methane production for quadratic response surface model fitting (ANOVA)

Source	Sum of square	Degree of freedom	Mean Square	*F* value	P-value (prob. > *F)*
X_1 (T) $^{\alpha}$	3.15	1.00	3.15	194.62	0.0456*
X_1^2	1.95	1.00	1.95	120.48	0.0578
X_2 (t) $^{\beta}$	0.00	1.00	0.00	0.13	0.7775
X_2^2	2.84	1.00	2.84	175.44	0.0480*
X_3 (H_2O_2 conc.) $^{¥}$	70.91	1.00	70.91	4376.88	0.0096*
X_3^2	4.21	1.00	4.21	259.84	0.0394*
X_1X_2	0.54	1.00	0.54	33.42	0.1090
X_1X_3	0.34	1.00	0.34	20.83	0.1373
X_2X_3	4.22	1.00	4.22	260.35	0.0394*
Lack of Fit	10.37	5.00	2.07	127.97	0.0670
Pure Error	0.02	1.00	0.02		
Total SS	94.68	15			

R-square = 0.8903, Adjusted R-square = 0.7258, MS pure error = 0.0162, * represents significant factors, $^{\alpha}$ (T) represents Temperature, $^{\beta}$ (t) represents time, and $^{¥}$ (Conc.) represents H_2O_2 concentration

The interaction between temperature (°C) and time (min) on the methane yield (Nmmol g^{-1} TVS) is presented in Fig 5.10. It can be seen from the curvature of the surface, an increase in time and temperature up to 180 °C resulted in a maximum response. It might be due to the fact that higher pretreatment temperature for longer exposure time results in more dissolution of lignin into liquid hydrolysate resulting from the higher availability of carbohydrates in the solid biomass. Higher temperature beyond 180 °C results into degradation products of sugars; furfural, 5-HMF that have inhibitory effects on methanogenesis

The interaction between hydrogen peroxide and temperature showed that concentration of H_2O_2 had a higher impact on the methane yield (Fig 5.11). Methane yield increased with increasing the H_2O_2 concentration in the studied range. It seems obvious as being an oxidizing agent H_2O_2 has a large influence on lignin dissolution. Lignin content has a negative correlation with the

methane yield (Kobayashi *et al.*, 2004; Ahring *et al.*, 2015). Fig 5.11 demonstrated that maximum response could be obtained with higher H_2O_2 applied even at a lower temperature.

The interaction between time (min) and hydrogen peroxide (% v/v) on the methane yield is shown in Fig 5.12. The curvature of the surface depicted that an increase in pretreatment time coupled with an increase in H_2O_2 concentration resulted in a maximum response. Longer the contact time of lignocellulosic biomass with H_2O_2 helped in an increase in delignification and consequently higher methane yield.

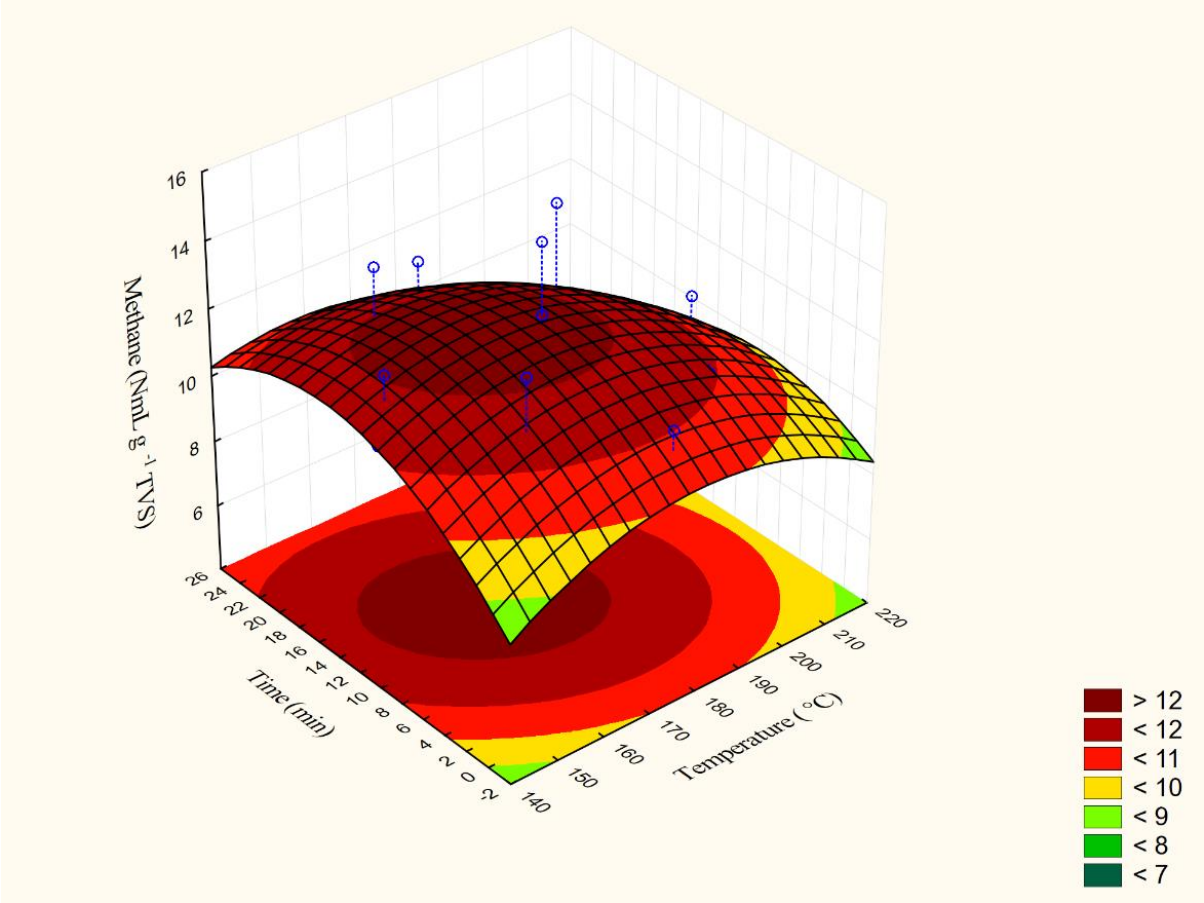

Figure 5.10 - Interaction between temperature (°C) and time (min) on the methane concentration

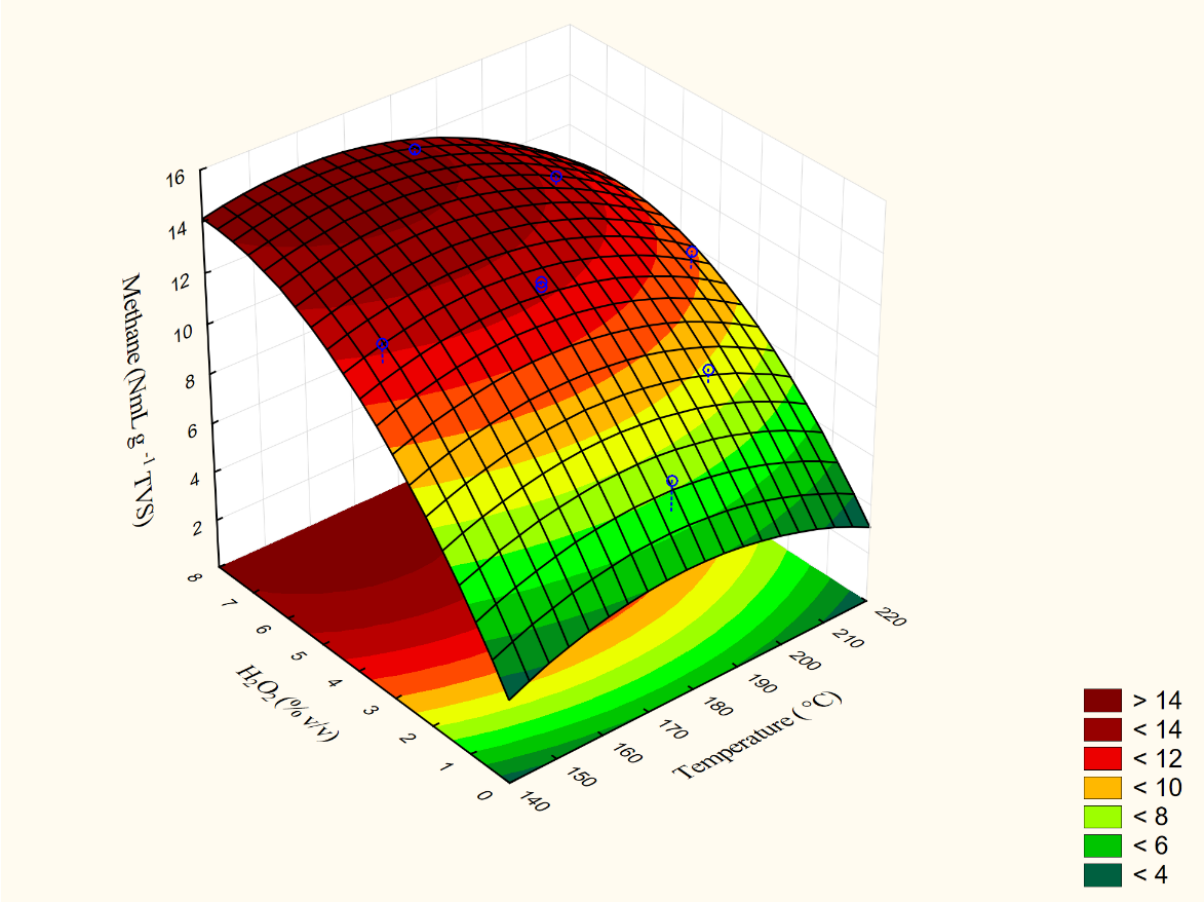

Figure 5.11 - Interaction between temperature (°C) and hydrogen peroxide (% v/v) on methane concentration

5.6 Kinetics analysis of methane production

Table 5.9 shows the experimental methane (Nmmol g^{-1} TVS), percent variation in methane over raw bagasse, and kinetics data (predicted methane volume, CH_4 production rate, lag phase and the goodness of fit; R^2) obtained from experimental methane data of modified Gompertz equation for H_2O_2 impregnated and hydrothermally pretreated sugarcane bagasse. The lowest value obtained for percent variation was observed for pretreatment B-HO (-15.26 %) while the highest was observed for pretreatment K-HO (118.16 %).

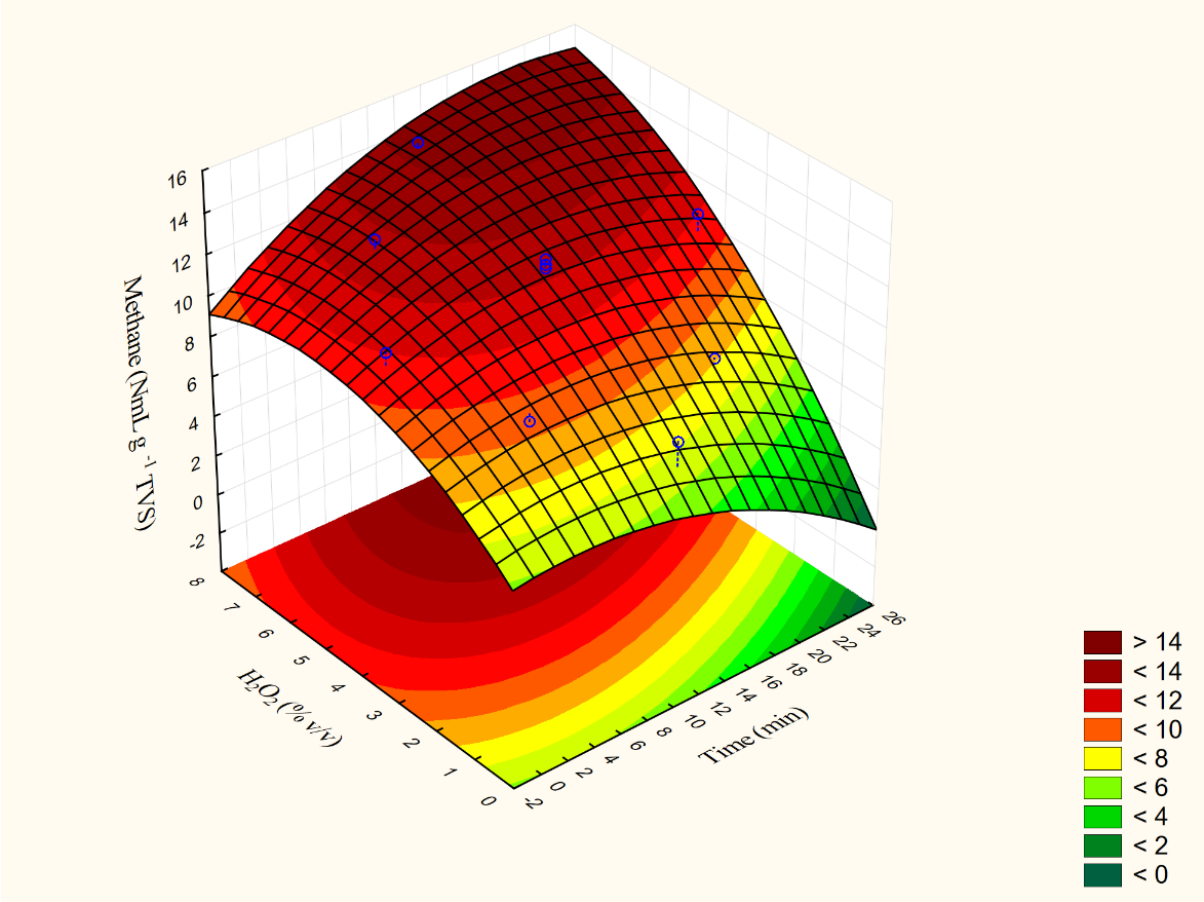

Figure 5.12 - Interaction between hydrogen peroxide (% v/v) and time (min) on the methane yield

The rate of methane production was not much affected by the pretreatment parameters. *Xc* was limited in the range of 0.02 to 0.12 Nmmol g^{-1} TVS h^{-1}. The highest lag phase of 82 h was observed for pretreatment D-HO (200 °C, 5 min, 2% H_2O_2), while abrupt methane production was observed for pretreatment I-HO (180 °C, 12 min, 4% H_2O_2) as evident from the lowest lag phase of 0 h. It indicated that methane production started at once. Similar was noted (*Xc 0 h)* for pretreatment P-HO (146.4 °C, 12 min, 4% H_2O_2). The goodness of fit (R^2) values was observed in the range of 0.94-1.00 for the 16 pretreatments. Graphs with fitted curves are presented in Appendix.

Table 5.9 - Kinetic parameters of methane production from H_2O_2 pretreated sugarcane bagasse

Designation	**Experimental Methane (Nmmol g^{-1} TVS)**	**% variation over SCB**	**Modified Gompertz Kinetic Data**			
			$A^{¥}$ (Nmmol g^{-1} TVS)	Xc^{A} Nmmol g^{-1} TVS h^{-1}	K^{B} h	R^2
Raw	6.59	-	6.63	0.02	0.00	0.988
A-HO	13.11	98.14	12.22	0.05	24.44	0.971
B-HO	5.59	-15.26	4.94	0.05	88.45	0.945
C-HO	10.81	63.51	10.25	0.08	57.22	0.992
D-HO	9.17	38.75	8.76	0.06	86.80	0.993
E-HO	13.49	103.85	12.94	0.12	73.24	0.989
F-HO	8.12	7.87	7.89	0.03	34.97	0.965
G-HO	13.12	98.36	12.69	0.03	0.50	0.972
H-HO	7.69	5.32	7.55	0.03	37.32	0.971
I-HO	12.11	83.10	12.01	0.03	0.00	0.959
J-HO	12.29	85.82	11.67	0.03	19.21	0.968
K-HO	14.43	118.16	13.36	0.04	14.35	0.959
L-HO	7.80	18.14	7.84	0.01	21.09	0.989
M-HO	11.55	74.70	10.89	0.04	33.30	0.981
N-HO	11.36	71.85	11.19	0.04	35.56	0.997
O-HO	10.89	64.66	9.89	0.04	28.77	0.954
P-HO	12.57	89.99	11.47	0.02	0.00	0.936

¥ Predicted value obtained from Gompertz equation, A Rate of methane production (Nmmol g^{-1} TVS h^{-1}), B time elapsed before CH_4 production started (lag phase),

5.7 Microbial community analysis

Microbial community structure was determined with fingerprinting technique DGGE for bacterial and archaeal domains. Selected pretreatment runs were also analyzed with next generation high throughput Illumina MiSeq sequencing.

5.7.1 Denaturing Gradient Gel Electrophoresis

Microbial community structure for bacterial and archaeal domains in response to pretreatment parameters i.e., temperature (°C), time (min) and H_2O_2 concentration (% v/v) was determined by DGGE at the end of batch reactors. Pretreatment runs 15 (O-HO) and 16 (P-HO) were excluded from the microbial community analysis based on extreme limits of temperature applied during pretreatment.

Similarity coefficient (S_c) between pretreatment runs was calculated by Pearson correlation. PCR-DGGE band profiles for bacterial and archaeal communities are presented as a dendrogram in Fig 5.13 and 5.14, respectively. For bacterial community, S_c decreased with a decrease in pretreatment time (19 – 5 min) at temperature of 200 °C from 83% (A-HO and B-HO) to 44% (C-HO and D-HO), while under same conditions, but at lower pretreatment temperature (160 °C) S_c increased from 68% (E-HO and F-HO) to 83% (G-HO and H-HO).

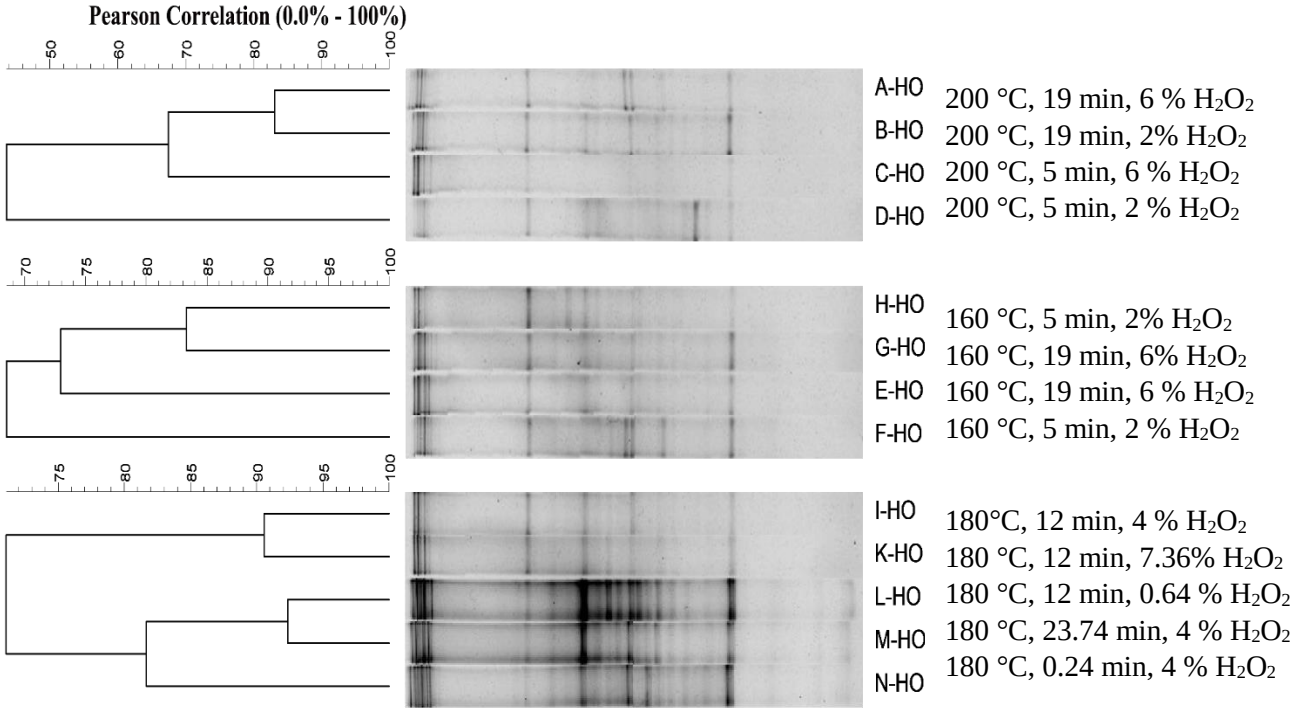

Figure 5.13 - Pearson Similarity Coefficient (S_c) of DGGE band patterns for bacterial communities

A decrease in H_2O_2 concentration from 6% to 2% resulted in a decrease in *Sc* from 67% (A-HO and C-HO) to 44 % (B-HO and D-HO) at pretreatment temperature of 200 °C. Similar

trend was observed at 160 °C where only 7.3% decrease in *Sc* was recorded with a decrease in H_2O_2 concentration.

Fig 5.13 depicted that the highest *Sc* of 92% was noted surprisingly between L-HO and M-HO though both were pretreated at significantly different conditions; L-HO was pretreated for 12 min with 0.64 % (v/v) H_2O_2 and M-HO was pretreated with 4% (v/v) H_2O_2 at 23.7 min, though both conditions met a similarity of temperature, 180 °C. Methane yield of both samples is also quite different; 174.78 and 258.79 NmL g^{-1} TVS for L-HO and M-HO, respectively. It could possibly mention that only the pretreatment temperature influenced such a high *Sc* between the two samples.

Second highest similarity 91% was observed between I-OH and K-OH both pretreated at 180 °C and 12 min. it showed that change in concentration did not influence the bacterial populations. Furthermore, it can also be seen bacterial populations thrive best when sugarcane bagasse was pretreated at a temperature of 180 °C and 12 min irrespective of change in H_2O_2 concentration employed as indicated by the highest *Sc*.

Archaeal community bands on the PCR-DGGE gel as indicated in Fig 5.14 showed not much variation across all the pretreatment runs. *Sc* varied from 67% to 98% from A-OH to N-OH. The lowest *Sc* 67% was noted at between two pretreatment sets A-HO and B-HO, and A-HO and C-HO at pretreatment temperature 200 °C, while the highest 98% *Sc* was observed between K-HO and L-HO at pretreatment temperature of 180 °C. A comparison of archaeal community with the volume of CH_4 produced showed that the volume of CH_4 produced was lowest (5.59 Nmmol g^{-1} TVS) for B-HO whereas 13.11 and 10.81 Nmmol g^{-1} TVS was recorded for A-HO and C-HO, respectively. Even though the highest similarity was recorded between K-HO and L-HO, however, the CH_4 value was approx. 2-fold in K-HO (14.43 Nmmol g^{-1} TVS) in comparison to L-HO (7.80 Nmmol g^{-1} TVS).

Table 5.10 - Microbial diversity (Shannon-Wiener and Dominance) for Bacteria and Archaea of H_2O_2 assisted anaerobic digesters

Designation	**Temperature**	**Time**	**H_2O_2**	**Bacteria**		**Archaea**	
	(°C)	(min)	(% v/v)	Dominance	Shannon-Wiener	Dominance	Shannon-Wiener
A-HO	200	19	6	0.11	2.26	0.08	2.72
B-HO	200	19	2	0.07	2.67	0.07	2.78
C-HO	200	5	6	0.14	2.10	0.07	2.79
D-HO	200	5	2	0.06	2.91	0.07	2.79
E-HO	160	19	6	0.08	2.57	0.07	2.78
F-HO	160	19	2	0.05	3.11	0.07	2.76
G-HO	160	5	6	0.07	2.70	0.07	2.78
H-HO	160	5	2	0.07	2.72	0.08	2.75
I-HO	180	12	4	0.08	2.72	0.07	2.83
K-HO	180	12	7.36	0.08	2.64	0.07	2.77
L-HO	180	12	0.64	0.04	3.18	0.06	2.86
M-HO	180	23.76	4	0.04	3.22	0.07	2.78
N-HO	180	0.24	4	0.04	3.18	0.07	2.76

Shannon-Wiener index (H′) and Dominance (D) for bacterial communities computed from the DGGE profile patterns is presented in Table 5.10. It indicates a decrease (6 – 2) in H_2O_2 concentration at 200 °C tends to increase the bacterial diversity and consequently lowers the dominance. Dominance is approximately similar to 200 °C for same H_2O_2 concentrations employed but a noticeable increase in the diversity was seen from 2.26 (A-HO) to 2.91 (D-HO) with a decrease in H_2O_2 (% v/v) from 6 to 2 and pretreatment time (19 – 5 min).

Similar conditions of H_2O_2 and pretreatment time but a decrease in temperature to 160 °C resulted in an increase in bacterial diversity. The decrease in H_2O_2 at pretreatment time of 5 min did not affect the bacterial diversity indicated by approximate H′ values of 2.70 and 2.72 for G-HO and H-HO, respectively. Interestingly, pretreatment time of 19 min induced a reasonable increase in bacterial diversity as evident from H′ values of 2.57 (E-HO) and 3.11 (F-HO). The

highest H′ value of 3.22 was noted for M-HO at pretreatment conditions of 180 °C, 23.76 min and 4 % (v/v) H_2O_2.

Surprising results were observed at 180 °C, an increase in H_2O_2 from 0.64 to 4 % (v/v) and decrease in pretreatment time from 12 to 0.24 min did not impact bacterial diversity (H′ - 3.18), while an increase in H_2O_2 (% v/v) from 4 to 7.36 resulted in negligible decrease in H′ value (2.72 – 2.64) at pretreatment time of 12 min. It could say that pretreatment temperature of 180 °C solely impacts the bacterial diversity of batch reactors. Higher Shannon diversity implicates lower dominance of community. H′ values are generally found between 1.5 – 3.5, while D value mostly occurs between 0 – 1 in most ecological studies (Magurran, 2004).

Archaeal Shannon diversity (H′) was approximately similar across all the pretreatment runs in the range of 2.72 to 2.86. Table 5.10 indicates a change in temperature, pretreatment time, and change in H_2O_2 did not much affect the archaeal community diversity. Even the H′ value of pretreatment B-HO (2.78) didn't differ much from the K-HO (2.77) where the former produced the lowest methane (125.17 NmL g^{-1} TVS) and the later exhibited maximum methane 323.33 NmL g^{-1} TVS.

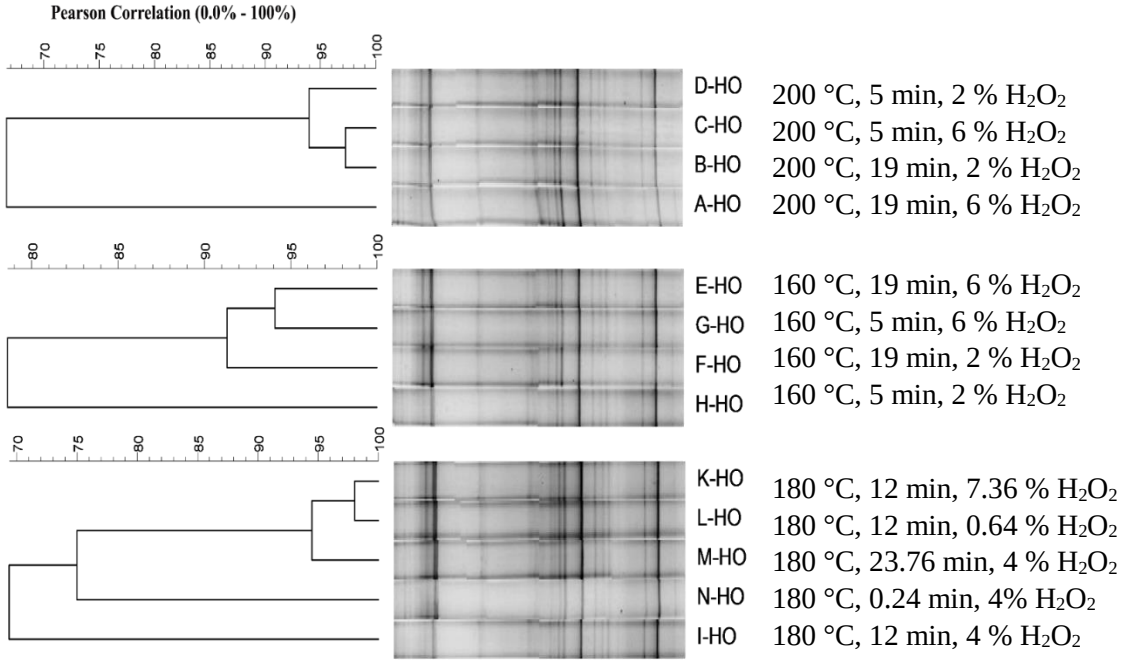

Figure 5.14 - Pearson Similarity Coefficient (S_c) of DGGE band patterns for archaeal communities

5.7.2 Illumina MiSeq Sequencing

Pretreatments B-HO, K-HO, and P-HO were selected for Illumina MiSeq sequencing to determine the microbial community structure. The community structure was explored for bacteria and archaea domains. In total, 35,128 sequences were retrieved after RDP classifier. Reads were classified into operational taxonomic units (OTUs) based on 3% distance level for phylogenetic estimation. Bacterial and archaeal OTUs obtained were 1,025 and 176, respectively. A total of 31, 32, and 33 phyla were noted for bacterial composition for pretreatment B-HO, K-HO, and P-HO, respectively, where only *Euryarchaeota* was predominant phylum for the archaeal community. Bacterial community in the selected samples was similar but abundance was quite different. Pretreatment P-HO presented a higher Shannon (6.24) and Chao-1 (1314.1) than pretreatment B-HO and K-HO.

5.7.2.1 Bacterial Diversity in H_2O_2 pretreated samples

A heterogeneous bacterial community was observed between pretreated samples. The selected pretreated samples after phylogenetic analysis revealed relatively similar bacterial diversity but quite different abundance at phylum level possibly because of the impact of pretreatment conditions applied (Fig 5.15). Main phyla observed were *Bacteroidetes*, *Thermotogae*, *Firmicutes*, *Proteobacteria*, *Spirochaetes*, and *Chloroflexi*. While *Synergistetes*, *Hyd24-12* lineage, and *WWE1* lineage were in lower abundance.

Bacteroidetes was the dominant phylum with the relative abundance of 27.32, 48.66, and 28.96% for B-HO, K-HO and P-HO, respectively (Fig 5.15) The phylum *Bacteroidetes* consists of four recognized classes *Bacteroidia, Flavobacteria, Sphingobacteria and Cytophagia (Krieg et al., 2010).* However, only one class *Bacteroidia* was found in the current study with the relative abundance of 27.05, 31.23, and 28.52% for pretreatment B-HO, K-HO, and P-HO, respectively. Members of *Bacteroidia* are Gram-stain negative, non-motile, anaerobic, saccharolytic, chemoorganotrophic and weakly proteolytic with acetate and succinate as major fermentation products (Song *et al.*, 2015).

Members of *BLVii28* belonging to family *Rikenellaceae* root to phylum *Bacteroidetes* are a rod or coccus-shaped bacteria producing extracellular hydrolase, supposed to contribute to anaerobic degradation (Hirakata *et al.*, 2016). *BLVii28*-related bacteria are previously reported in

granular sludge in UASB reactor and anaerobic wastewater treatment facilities (Kaksonen *et al.*, 2004; Narihiro *et al.*, 2009). Relatively higher *BLVii28* abundance was recorded in pretreatment K-HO (3.50 %) followed by pretreatment run B-HO (2.74 %) and pretreatment run P-HO (1.72 %). Relatively higher abundance of *BLVIii28* in pretreatment K-HO might be due to higher removal of lignin leaving behind easily accessible degraded cellulose

Thermotoage was the most abundant phylum in pretreatment B-HO (26.58%) followed by pretreatment K-HO (13.06%) and P-HO (9.05%) (Fig 5.15). *Thermotogae* members are anaerobic thermophilic gram-negative bacteria surrounded by sheath-like envelope called "toga". *Thermotogae* contains 10 genera with 41 validated species belonging to order *Thermotogales* that roots to single class *Thermotogae* (Bhandari and Gupta, 2014). *Thermotogales* members are anaerobic heterotrophs able to ferment a range of substrates including glucose and xylose. H_2 severely inhibits the *Thermotogales* growth (Reysenbach, 2015).

Huber et al (Huber *et al.*, 1986) reported that acetic acid, CO_2 and H_2 are the fermentation products of *Thermotogales*. Phylogenetic analysis indicated two genera of *Thermotogaceae* for this study; uncultured *AUTHM297* and *Kosmotoga*, where former was abundant with the relative abundance of 8-26% (Fig 5.17) and later was less than 1%.

Lower hydrogenotrophic activity in pretreatment run B-HO could be a reason for higher *AUTHM297* lineage abundance (26.58%) in comparison to pretreatment K-HO (13.06%) and P-HO (9.05%) as indicated by the lower abundance of *Methanobacterium* (18.22%) for pretreatment run 2. Briones et al (Briones *et al.*, 2007) reported that *AUTHM297* is capable of utilizing elemental sulfur and/or thiosulfate as an electron acceptor to form H_2S. Since, an H_2S determination is beyond the scope of the current study so the author unable to comment due to unavailability of data for H_2S and sulfates. Syntrophic activity of *Thermotogales* with *Methanobacteriaceae* is reported by Balk et al (Balk *et al.*, 2002) that only one member can serve as a syntrophic member with *Thermotogales.*

Since, the current study was conducted under mesophilic conditions (37 °C) the presence of *Thermotogae* was surprising. However, recently other studies (Nesbo *et al.*, 2006; Briones *et al.*, 2007) have also been reported the presence of *Thermotogae* related members in mesophilic conditions in anaerobic reactors treating sulfate-rich waste streams. Though digestion experiments were conducted under mesophilic conditions (37 °C), interestingly *AUTHM297* dominance

followed the order of pretreatment temperature; 26.13% (pretreatment B-HO – 200 °C), 12.63% (pretreatment K-HO - 180 °C), and 8.55% (pretreatment P-HO – 146.4 °C).

Firmicutes was abundant in pretreatment P-HO (19.47%) followed by pretreatment B-HO (15.59%) and K-HO (7.89%). It is a renowned group for hydrolytic activity, most members stain Gram-positive. *Firmicutes* contain three classes *Bacilli, Clostridia,* and *Erysipelotrichia*.

The current study revealed the presence of one major Class *Clostridia* with the relative abundance of 15.31, 7.84, and 19.37%, respectively, for pretreatment B-HO, K-HO and P-HO. *Clostridia* members perform hydrolysis and acidogenesis, in addition to acetogenesiss and syntrophic acetate oxidation (Wirth *et al.*, 2012; Ziganshin *et al.*, 2013).

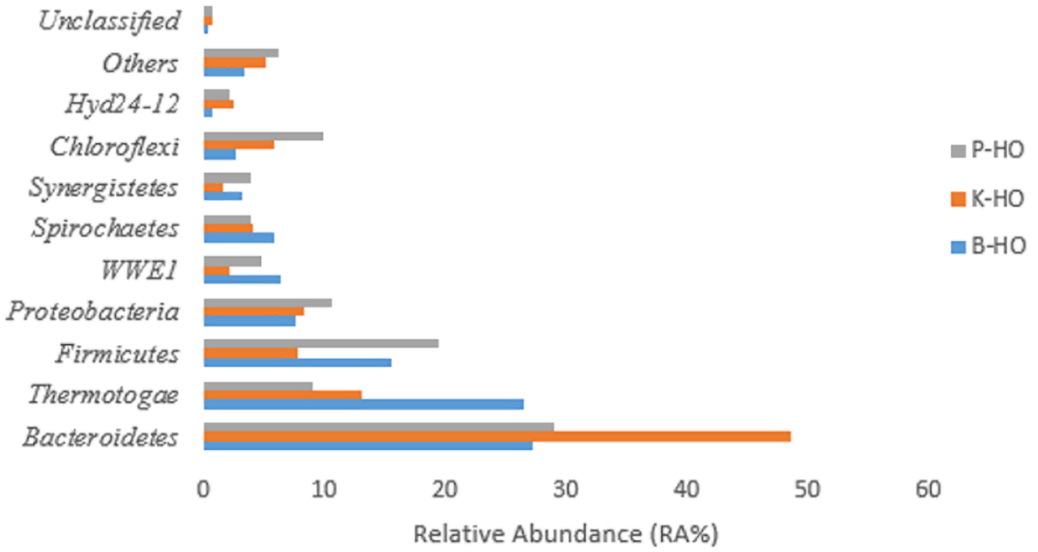

Figure 5.15 - Bacterial community classification at Phylum level. Relative abundance (RA%) less than 1 in the samples was accumulated and presented as Others.

Clostridia are also efficient hydrogen producers. *Clostridia* may be saccharolytic, proteolytic or bearing both metabolic pathways. *Clostridium* may metabolize carbohydrates, amino acids, purines, steroids or other organic compounds (Rainey *et al.*, 2015). *Clostridia* represent the most prevalent bacterial class in full-scale mesophilic biogas plants fed with plant biomass (Krause *et al.*, 2008). Some species of the order *Clostridiales* are known to create cellulosomes, which are intensively involved in the anaerobic digestion of recalcitrant cellulose, supporting acetogens and methanogens with compounds necessary for their growth (Ziganshin *et al.*, 2013).

Members of the clostridial family *Ruminococcaceae* are known to hydrolyze a variety of polysaccharides by different mechanisms, e.g., the production of a cellulosome enzyme complex and cellulose adhesion proteins (Morrison and Miron, 2000).They are able to ferment hexoses as well as pentoses, which are the hydrolysis products from cellulose and hemicelluloses.

Clostridium genus was negligibly found in the pretreatment B-HO (0.63%) and K-HO (0.28%). However, relatively higher abundance was recorded in pretreatment P-HO (5.23%). *Clostridium* are rod-shaped Gram-stain-positive, endospore forming obligate anaerobes. *Clostridium spp.* are extensively reported in biogas plants treating cellulosic biomass (Herbel *et al.*, 2010; Hanreich *et al.*, 2013; Sun *et al.*, 2015). The relatively higher abundance of *Clostridium* in the pretreatment 16 could be justified by the lower pretreatment temperature (146.4 °C) applied rendering lower glucan content 33.12 g 100 g^{-1} TS. Higher pretreatment temperature of 200 and 180 °C, respectively, for pretreatment B-HO and K-HO might have altered significantly the lignocellulosic structure breaking lignin-carbohydrate matrix though leaving behind higher glucan content 55.52 and 60.42 g 100 g^{-1} TS glucan content in the pretreated solids, However, in-depth study is required to determine the behavior of clostridia under anaerobic conditions in response to H_2O_2 impregnated substrates, as the glucan content was reasonably higher for K-HO (60.42%) in comparison to P-HO (33.12%).

Proteobacteria is considered to play an important role in methane production in syntrophic relation with archaea. Bacterial community of the tested samples at phyla rank showed higher abundance for pretreatment P-HO (10.58%) followed by pretreatment K-HO (8.27%) and B-HO (7.64%).

Class *Deltaproteobacteria* was dominant with the relative abundance of 6.55% in P-HO followed by 4.98% (K-HO), and 3.61% (B-HO). Other classes *alpha*, *beta*, *epsilon*, and *Gammaproteobacteria* were also found but in lower abundance. *Syntrophus (Deltaproteobacteria)* was the only *proteobacteria* phylotype was above than 1% in relative abundance. *Syntrophus* are Gram-negative, strictly anaerobic, chemoorganotrophic rod-shaped bacteria. Kuever and Schink (Kuever and Schink, 2015) reported that *Syntrophus* could oxidize fatty acids only in the presence of H_2/formate-utilizing methanogenic archaea or sulfate-reducing bacteria. Furthermore, incomplete oxidation could lead to acetate formation. *Syntrophomonas*-related bacteria are fatty-acid-oxidizing bacteria, able to convert various organic acids to H_2 and acetate for subsequent

hydrogenotrophic methanogenesis (Baserba *et al.*, 2012; Li *et al.*, 2013). The lower but approx. similar relative abundance of *Syntrophus* in the tested samples (1.08 – 1.88 %) reflected the occurrence of the syntrophic relationship during methanogenesis.

Comparatively similar *Synergistetes* abundance was observed for B-HO and P-HO 3.29 and 3.92%, respectively. Members of *Synergistetes* phylum are gram-negative with various morphologies and metabolism. It was recently characterized into phylum (Jumas-Bilak *et al.*, 2009). *Synergistia* was the only class encountered in the current study with relative abundance between 1.6-3.92%. *Aminiphilus* (family *Aminiphilaceae*) was the only genus in the phylum *Synergistetes* above than 1% relative abundance in pretreatment B-HO and P-HO

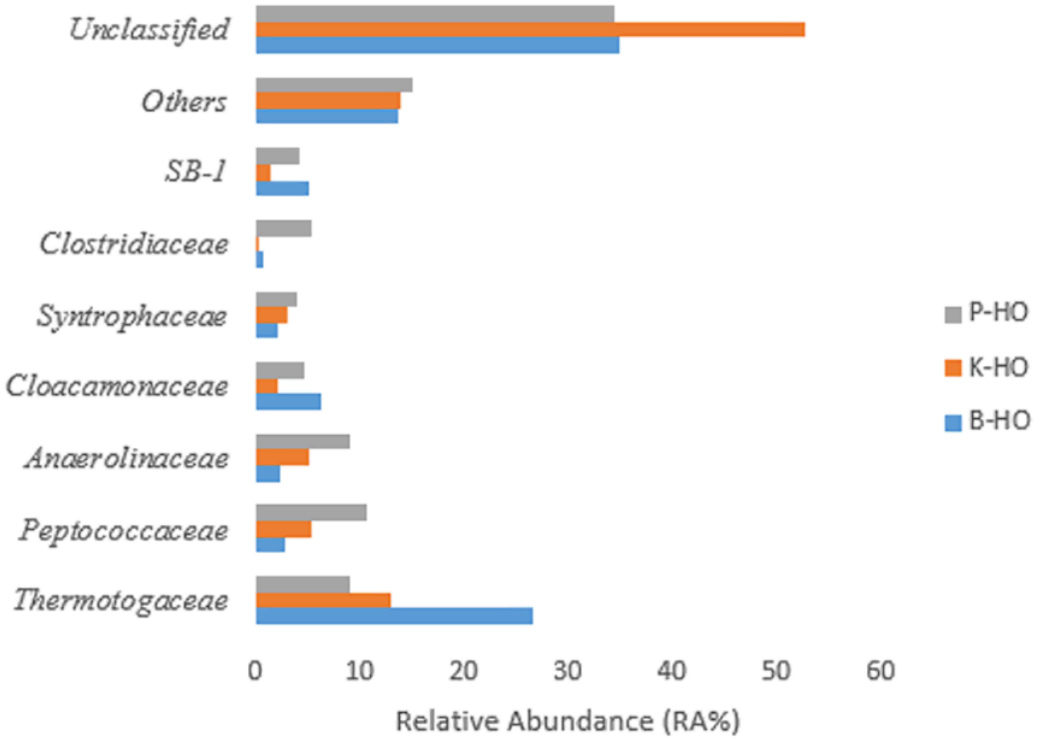

Figure 5.16 - Bacterial community classification at Family-level. Relative abundance (RA%) less than 1 in the samples was accumulated and presented as Others

The candidate division *WWE1* (Waste Water of Evry 1), an uncultured bacterium, was also found in relatively higher proportion (6.38 - 4.77%) in pretreatments B-HO and P-HO, respectively. *WWE1* members are reported to act as amino acid degraders and capable of propionate and butyrate oxidation as well as cellulose degradation (Sieber *et al.*, 2012; Limam *et al.*, 2014). *Cloacamonae* was the only class with relative abundance between 2.1-6.4% was

observed with two distinct phylotypes; uncultured *W22* and *Candidatus cloacamonas. W22* was observed in relatively higher abundance in pretreatment B-HO (5.33 %) than pretreatment P-HO (2.17 %) while negligibly found in pretreatment K-HO (0.48 %).

W22 related species are reported to degrade phenolic compounds under methanogenic conditions (Ju and Zhang, 2014). Pretreatment B-HO had a higher lignin content. Lignin is a highly complex phenolic polymer (Lange *et al.*, 2013). Higher abundance (5.53%) of *W22* in pretreatment B-HO could be attributed to degradation of lignin. *W22* related species metabolize amino acids and butyrate during phenol metabolism (Ju and Zhang, 2014). Esquivel-Elizondo et al. (Esquivel-Elizondo *et al.*, 2016) reported that *W22* might responsible for propionate and butyrate fermentation to H_2, CO_2, and acetate in the presence of hydrogenotrophic and acetoclastic methanogens. Wong et al. (Wong *et al.*, 2016) reported that *W22* might contain unique enzyme system and biochemical pathway relevant to lignocellulose conversion.

Candidatus cloacamonas was found in negligible abundance in pretreatment B-HO (0.67 RA%), K-HO (1.45 RA%) and P-HO (2.16 RA%). *In silico* Proteome analysis indicated that *C.clocomonas* might attain energy from sugars in the Embden-Meyerhof pathway and from the fermentation of amino acids, thereby, produces hydrogen and carbon dioxide. The occurrence of the oxidative propionate degradation pathway in *C. cloacamonas* as well as the capability to produce hydrogen via fermentation and its Fe-only hydrogenases are the reasons; the bacterium is assigned as a syntrophic bacterium (Pelletier *et al.*, 2008).

Phylum *Spirochaetes* found in less abundance with $RA_{\%}$ of 5.79, 4.03, and 3.91, respectively for pretreatment B-HO, K-HO, and P-HO. *Spirochaetes* generally unable to degrade cellulose but are abundantly reported in anaerobic digesters (Liu *et al.*, 2009; Lee *et al.*, 2012). Rainey et al. (Rainey *et al.*, 1991 1490) isolated only one known cellulose degrading *Spirochaetes* species. Though members of *Spirochaetes* unable to degrade cellulose they can ferment carbohydrates and amino acids with major fermentation products; ethanol, acetate, CO_2, and H_2. *Spirochaetes* interaction with cellulose degraders might be the reason of their abundance in the anaerobic reactors (Azman *et al.*, 2015). Only one notable class observed in the current study was *Spirochaetes. Spirochaetes* class was higher for pretreatment B-HO with the relative abundance of 5.27% in comparison to pretreatment K-HO (2.8%) and P-HO (3.11%).

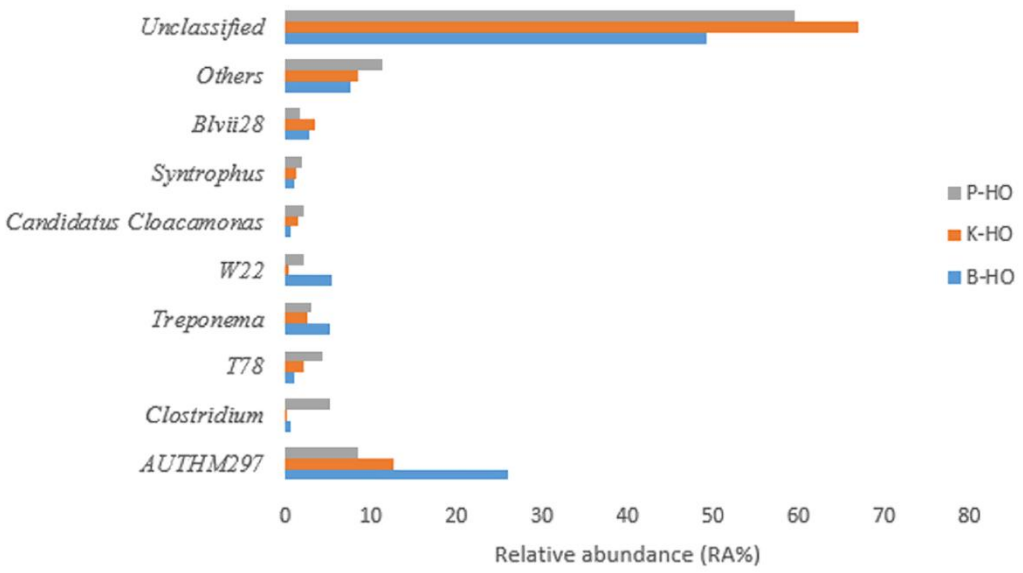

Figure 5.17 - Bacterial community classification at Genus-level. Relative abundance less than 1 in the samples was accumulated and presented as 'Others'

Treponema was the only phylotype significant in the current study with the relative abundance of 5.23% while approx. similar abundance was noted for pretreatment K-HO (2.69%) and P-HO (3.03%). *Treponema* members being homoacetogens produce acetate by consuming H_2 and CO_2 (Wang *et al.*, 2013). Homoacetogenesis is typically observed under psychrophilic conditions, as homoacetogens have a better ability to adapt to low temperatures compared with hydrogenotrophic methanogens (Siriwongrungson *et al.*, 2007).

It has been reported that homoacetogenesis cannot compete with hydrogenotrophic methanogenesis under mesophilic or thermophilic conditions because of its lower energy yield. The lower abundance of *Treponema* in this study might be a reason for the higher competing interest of *Treponema* with hydrogenotrophic methanogenesis. Li et al. (Li *et al.*, 2016) reported that *Treponema* proliferation in anaerobic reactors could be used as a potential indicator of process instability. Reactor failure for pretreatment B-HO can be justified with higher *Treponema* abundance in comparison to pretreatment K-HO and P-HO with the argument reported by Li et al. (Li *et al.*, 2016). The genus *Treponema* consists of various species typically found in animal and human gastrointestinal flora (Nordhoff and Wieler, 2005).

Chloroflexi (green non-sulfur bacteria) is one of the largest phyla with members having various metabolic features. Filamentous morphology is the characteristic feature of its class.

Members of *Chloroflexi* are Gram-negative, exhibiting gliding motility and do not contain lipopolysaccharide containing outer membrane (Garrity and Holt, 2015). Class *Anaerolineae* lineage in *Chloroflexi* has been reported in anaerobic digesters (Riviere *et al.*, 2009; Narihiro *et al.*, 2012). Some researcher speculated *Anaerolineae* as semi-syntrophic having the capability of reverse electron transfer via coupled mutualistic interaction with methanogens (Narihiro *et al.*, 2012). However, such interaction is not yet validated (Xia *et al.*, 2016).

A significant difference was observed for *Chloroflexi* diversity at phylum level in the current study. Maximum abundance was noted for pretreatment P-HO (10.02%) followed by K-HO (5.87%) and B-HO (2.77%). Higher abundance of *Chloroflexi* in the pretreatment P-HO could possibly support the argument that cellulose structure might not severely disturb owing to lowest temperature 146.4 °C that might have helped to develop a higher population of *Chloroflexi*. This argument could be strengthened by a recent study where Podosokorskaya et al. (Podosokorskaya *et al.*, 2013) reported a discovery of cellulolytic strain *Ornatilinea apprima* of class *Anaerolineae.*

Uncultured genus *T78* of family *Anaerolinaceae* was noted in relatively higher abundance in pretreatment P-HO (4.45 %) than pretreatment B-HO (1.09 %) and pretreatment K-HO (2.10 %). *T78* is previously reported in co-digestion of whey permeate and cow manure. It is suggested as carbohydrate utilizer (Miura and Okabe, 2008; Yamada and Sekiguchi, 2009). It also acts as anaerobic degradation of oil hydrocarbons (Parnadi *et al.*, 2007). The lowest abundance of the *T78* population in pretreatment B-HO further strengthen the fact that higher lignin content resulted into lesser development of carbohydrate degrader bacterial populations.

Uncultured *Hyd24-12* members are globally distributed but morphological, ecological, and physiological information about this phylum is not available in the literature. This phylum harbors under mesophilic conditions. Genomic annotation and metabolic reconstruction implicate that members of this phylum are Gram-negative. The *Hyd24-12* lineage is delineated into four clades, designated B-1AC, zEL51, Hyd-32 and B9.18 (Kirkegaard *et al.*, 2016). The current study revealed a lower abundance of *Hyd24-12* lineage with $RA_{\%}$ of 0.73, 2.48, and 2.22 for pretreatment B-HO, K-HO, and P-HO, respectively.

As depicted in Fig 5.17, 49-67% reads at genus-level were not belong to any known phylum indicating the potential for the new discovery of bacterial taxonomic groups for the samples treated with H_2O_2. Such a higher proportion of unclassified reads could be justified by the reason that a

huge number of bacteria are uncultured and are not classified into taxonomic groups. Furthermore, it also indicated the potential for novel bacterial taxonomic groups that have not yet been discovered.

5.7.2.2 Archaeal Diversity in H_2O_2 pretreated samples

Archaeal diversity in H_2O_2 pretreated samples at phylum-level indicated the prevalence (99%) of phylum *Euryarchaeota*, while *Crenarchaeota* was found in less than 1%. *Euryarchaeota* are rods, cocci, spiral-shaped, triangular, or squares shaped methanogens exhibiting Gram-positive or Gram-negative characteristics (Garrity and Holt, 2015).

As depicted in Fig 5.18 at Class-level, that archaeal community was not as diverse as it appeared for the bacterial population. Phylum *Euryarchaeota* contains eight classes (Gargaud and Irvine, 2015). However, classes *Methanomicrobia* and *Methanobacteria* were predominant in the current. Class *Thermoplasmata* was also observed in less than 1% in pretreatment 11 (0.77%) and pretreatment 16 (0.69%). Its abundance was slightly higher in pretreatment 2 (1.19%). However, Class *Thermoplasmata* is of no interest for methane production because it is a non-methanogenic archaeal class (Bapteste *et al.*, 2005). Uncultivated class *MCG* (Miscellaneous Crenarchaeota Group) was observed in less than 1%. This taxon is a prominent archaeal group for dehalogenation of aromatic compounds (Meng *et al.*, 2014). The role of *MCG* in anaerobic digestion is largely unknown; however, Collins et al (Collins *et al.*, 2005) reported that *MCG* collocate with *Methanosaeta* cells in granular digester biofilm suggesting a syntrophy interaction with methanogens.

Class *Methanomicrobia* was predominant (76.26-79.52%) in the tested samples with the prevalence of two families *Methanoregulaceae* (44.73-62.33%) and *Methanosaetaceae* (15.94-30.42%). Members of *Methanoregulaceae* are hydrogenotrophic, however, each specie exhibits different physiological features (Imachi and Sakai, 2015). *Methanoregulaceae* members are rod and coccoid-shaped, Gram-negative strict anaerobes that require energy by CO_2 reduction to form methane utilizing H_2 or formate as electron donors. Two phylotypes within family *Methenoregulaceae* were observed in the current study (Fig 5.19); *Methanolinea* (24.52-34.86%) and *Candidatus methanoregula* (4.43-4.77%).

Higher abundance (34.86%) of *Methanolinea* in pretreatment B-HO (Fig 5.20) in comparison to approx. similar abundance in pretreatment run K-HO and P-HO (24.52-24.75%) implicates higher hydrogenotrophic activity in pretreatment B-HO that is supported with lower *Methanosaeta* (15.94%) where *Methanosatea* abundance was two-fold in pretreatment K-HO and P-HO (26.7-30.4%). *Methanosaeta* are rod-shaped obligate anaerobes utilizing the only acetate as an energy source while CO_2 and acetate serve as a carbon source. *Methanosaeta* are unable to utilize H_2/CO_2, formate, methanol, and methylamine for growth or methane production (Zinder *et al.*, 1987; KAMAGATA and MIKAMI, 1991). *Methanolinea* are reported previously in digestion of grass *Pennisetum* (Pengyu *et al.*, 2017) and food waste (Wilkins *et al.*, 2015)

Class *Methanobacteria* was lower with the relative abundance of (18.56-22.14%). *Methanobacterium* was the dominant (18.21-21.95%) genus while *Methanobrevibacter* was encountered in less than 1%. *Methanobacterium* are Gram-positive strict anaerobes with optimum growth temperature 37 – 45 °C. Although, some strains use formate, secondary alcohols, and CO, CO_2 reduction coupled to H_2 oxidation is the major growth mechanism (Boone, 2015). *Methanospirillum, Methanomethylovorans, Methanomassiliicoccus, Methanocella* were found in less than 1%. 18.25-25.42% read could not be categorized into archaeal taxonomic groups at the genus level (Fig 5.20).

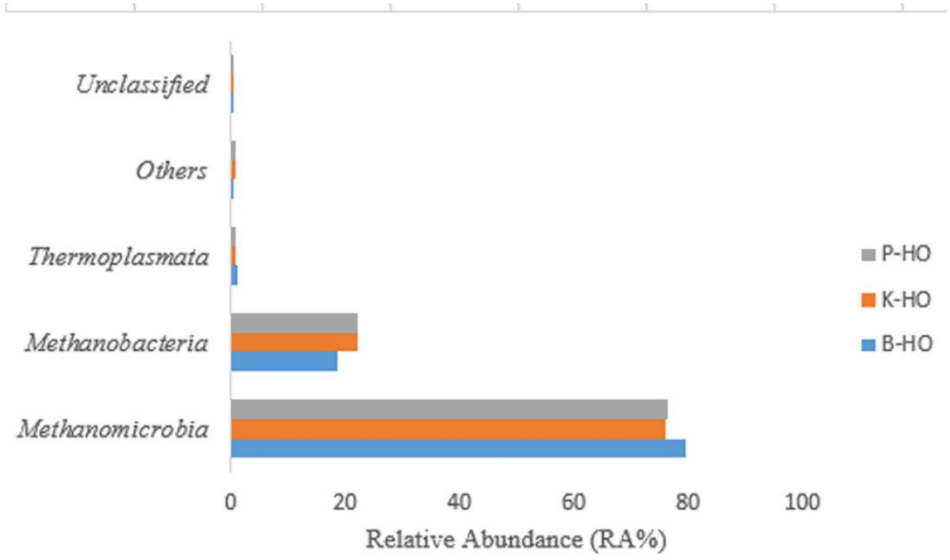

Figure 5.18 - Archaeal community classification for selected H_2O_2 pretreated samples at Class-level. All taxonomic groups with relative abundance less than 1% are accumulated and presented as others

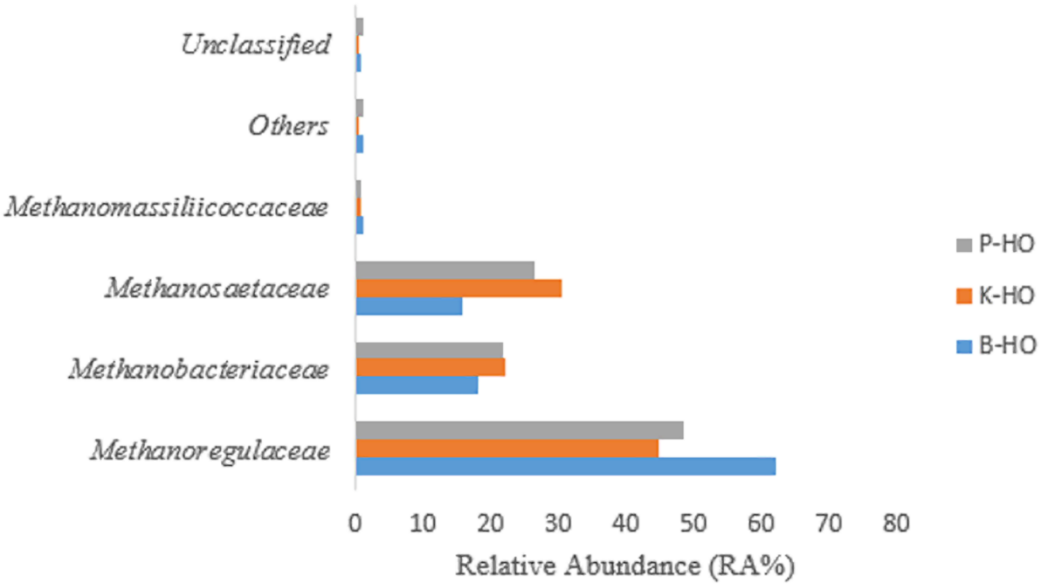

Figure 5.19 - Archaeal community classification for selected H_2O_2 samples at Family-level. All taxonomic groups with relative abundance less than 1% are accumulated and presented as others

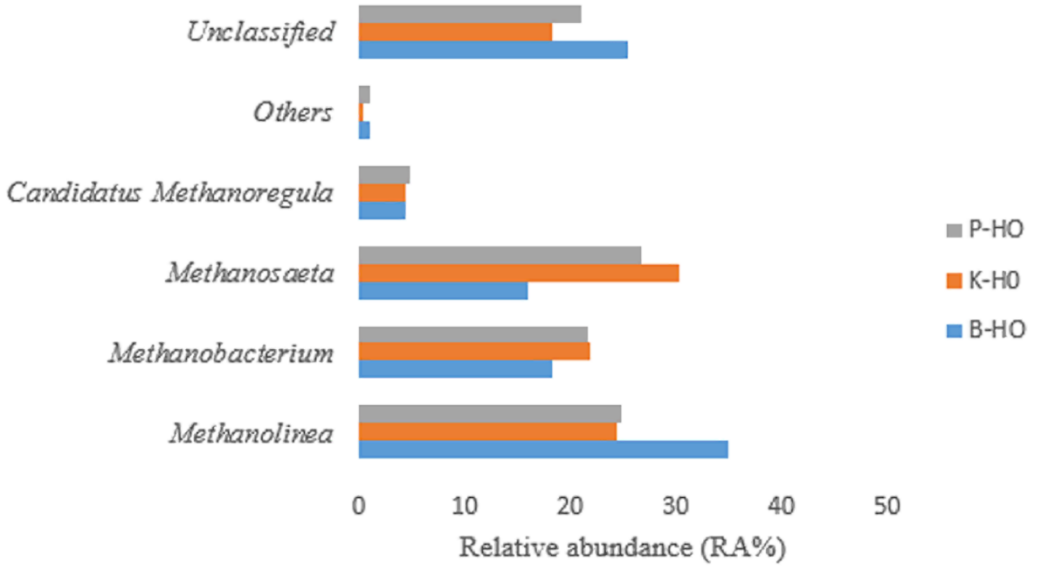

Figure 5.20 - Archaeal community classification for selected H_2O_2 samples at Genus-level. All taxonomic groups with relative abundance less than 1% are accumulated and presented as others.

5.8 Impact of sugarcane bagasse impregnation in H_2SO_4 before hydrothermal pretreatment on pretreated solid characteristics

In the second phase of the study, sugarcane bagasse was impregnated with H_2SO_4 for 24 h before hydrothermal pretreatment according to CCD (Table 4.5). The recovered solid after the pretreatment was characterized for carbohydrate polymers (glucan, xylan), Klason lignin and lignin recovery (Table 5.11).

Table 5.11 - Impact of H_2SO_4 impregnation of sugarcane bagasse before hydrothermal pretreatment on Solid yield (%), Glucan (g 100 g^{-1} TVS), Xylan (g 100 g^{-1} TVS), Klason Lignin (%), Glucan increase (%), xylan removal (%), and Lignin Recovery (%)

Run	Designation	Solid yield (%)	Glucan (g 100 g^{-1} TS)	Xylan (g 100 g^{-1} TS)	Klason lignin (%)	Glucan increase (%)	Xylan removal (%)	Lignin recovery (%)
1	A-HSO	12.27	14.34	0.00	84.61	-54.33*	100.00	340.08
2	B-HSO	17.83	44.15	0.00	53.20	40.60	100.00	213.84
3	C-HSO	22.04	20.98	0.00	76.74	-33.18	100.00	308.47
4	D-HSO	34.95	57.09	5.40	33.96	81.81	85.25	136.49
5	E-HSO	32.83	40.29	6.80	51.11	28.31	81.43	205.43
6	F-HSO	63.00	53.60	10.50	33.05	70.70	71.32	132.83
7	G-HSO	40.69	39.28	14.15	44.65	25.09	61.35	179.49
8	H-HSO	75.28	46.81	20.80	30.87	49.07	43.19	124.08
9	I-HSO	46.48	49.48	10.60	37.69	57.58	71.05	151.50
10	J-HSO	49.04	51.10	8.10	37.33	62.74	77.88	150.04
11	K-HSO	34.03	42.54	4.30	52.12	35.47	88.26	209.51
12	L-HSO	41.28	51.00	8.80	37.30	62.42	75.97	149.91
13	M-HSO	51.44	58.80	4.80	35.72	87.26	86.89	143.59
14	N-HSO	57.10	51.14	12.40	34.11	62.86	66.13	137.10
15	O-HSO	20.02	8.12	0.00	89.28	-74.14	100.00	358.85
16	P-HSO	64.52	41.80	16.90	39.80	33.12	53.84	159.99

Where * -ve sign indicates decrease in glucan content

As indicated in Table 5.11, solid recovery increased with a decrease in reaction temperature and H_2SO_4 concentration employed for impregnation of sugarcane bagasse. Solid recovery increased from 12.27 to 17.83 (45.31%) when pretreatment temperature (200 °C) and reaction time (19 min) held constant, and H_2SO_4 concentration decreased from 3 to 1% (w/v). While, when the reaction time was decreased to 5 min for pretreatment C-HSO and D-HSO at the same reaction temperature of 200 °C, a decrease in H_2SO_4 concentration resulted in 58.57% (22.04 to 34.95%) increase in solid recovery.

It showed that a decrease in reaction time increased the solid recovery when reaction temperature and H_2SO_4 concentrations were held constant. On the other hand, at a reaction temperature of 160 °C, a decrease in H_2SO_4 from 3 to 1% (w/v) resulted into approx 2-fold increase in solid recovery (32.83 to 63.00%) While, 85% increase (40.69 to 75.28%) was observed between pretreatment G-HSO (160°C, 5 min, 3% H_2SO_4) and pretreatment H-HSO (160°C, 5 min, 1%w/v H_2SO_4)

A comparison indicated that when reaction temperature was decreased from 200°C (pretreatment A-HSO) to 160°C (pretreatment E-HSO) keeping reaction time (19 min) and H_2SO_4 concentration (3%) held constant, solid recovery improved 167.56% (12.27 to 32.83%). Similarly, a decrease in temperature to 160°C resulted in 253.3% (17.83 to 63.00%), 84.61% (22.04 to 40.69), and 115.39% (34.95 to 75.28%) increase for pretreatment runs F-HSO, G-HSO, and H-HSO, respectively in comparison to pretreatment runs B-HSO, C-HSO, and D-HSO.

Solid recovery comparison for pretreatment runs K-HSO (3.68% w/v H_2SO_4) and L-HSO (0.32% w/v H_2SO_4) indicated 116.69% (19.05 to 41.28%) increase where reaction temperature (180 °C) and reaction time (12 min) held constant. Lesser increase (11%) was observed for maximum (23.76 min) and minimum (0.24 min) reaction time studied at conditions; reaction temperature of 180 °C and H_2SO_4 concentration (2%). Furthermore, a decrease in reaction temperature from 213.6 to 146.4 °C resulted into 222.27% (20.02 to 64.52%) increase in solid yield.

Lu et al (Lu *et al.*, 2009) conducted H_2SO_4-catalyzed hydrothermal pretreatment of rapeseed straw for ethanol conversion and reported maximum solid yield of 88.7% at H_2SO_4 loading of 0.5 (%w/w), 5 min reaction time, 20% (w/w) solid loading and reaction temperature of 180 C. In another study, Yang et al. (Yang *et al.*, 2012) reported 92.4% solid yield for triploid

poplar chips at reaction temperature of 100°C for 2 hrs reaction time and H_2SO_4 loading of 0.5 (% v/v). In the current study, maximum solid yield (64.52%) was achieved at the lowest pretreatment temperature (146.4 °C, 12 min, 2 % v/v H_2SO_4). Low solid yield in comparison to referenced studies was due to higher H_2SO_4 concentration employed.

Glucan content was affected notably due to pretreatment parameters as represented in Table 5.11. Glucan content in pretreatment run A-HSO and C-HSO were 14.34 and 44.15 g 100 g^{-1} TS indicating cellulose solubilization owing to lower than raw sugarcane bagasse 31.4 g 100 g^{-1} TS for pretreatment A-HSO. However, pretreatment run B-HSO resulted in an increase in glucan content showing lower H_2SO_4 concentration was unable to affect the cellulose crystallinity even at 200 °C.

Cellulose is a very coherent and stabilized structure that reported to start to solubilize above 230 °C and completely decompose at 295 °C (Sakaki *et al.*, 2002). However, the current study revealed that cellulose degradation could be initiated at a lower temperature (200 °C) if substrate solubilized with 3% H_2SO_4 before hydrothermal pretreatment. Pretreatments conducted at 200 °C indicated that utilization of 3% H_2SO_4 resulted into cellulose solubilization.

Glucan content was in the range of 14.34 – 57.09 g 100 g^{-1} TS for the pretreatment set conducted at 200 °C, lower H_2SO_4 concentration resulted in percent increase in glucan complemented with xylan and lignin solubilization. While, glucan content was in the range of 39.28 – 53.60 g 100 g^{-1} TS for pretreatments conducted at 160 °C. Glucan content of 8.12 g 100 g^{-1} TS for pretreatment run O-HSO (213.6 °C, 12 min 2% H_2SO_4) indicated that even 2% H_2SO_4 could result into cellulose solubilization at temperature 213 °C complemented with 100% xylan removal and 358.85% klason lignin recovery in pretreated solid. However, 146.4 °C (pretreatment run P-HSO) could not dissociate cellulose structure with 2% H_2SO_4 as indicated by higher glucan content 41.80 g 100 g^{-1} TS. Glucan recovery in the pretreated solid fraction was in the range of 25.83 – 187.01%.

Lu et al. (Lu *et al.*, 2009) reported maximum of 105.1 % glucan recovery in the water-insoluble solid fraction of H_2SO_4-catalyzed rapeseed straw at 1% H_2SO_4, 10 min reaction time with solid content of 15% at 180 °C. However, current study could not be compared on the grounds of different pretreatment conditions, H_2SO_4 employed and variation in feedstock composition.

Xylan content indicated that most of the xylose was solubilized during the pretreatment as it was not observed at higher severity (200 °C, 19 min 3% H_2SO_4). Hydrothermal pretreatment is aimed at the removal of hemicellulose (xylan and arabinan) by xylan depolymerization and cleavage of acetyl groups from hemicellulose as reported by (Xiao *et al.*, 2011; Rocha *et al.*, 2013; Senila *et al.*, 2014). H_2SO_4 further catalyze the process by lowering the pH. Xylan content was in the range of 4.3 – 20.80 g 100 g^{-1} TS for various pretreatment conditions. Minimal xylan removal was noted for pretreatment run H-HSO conducted at 160 °C, 5 min and 1% H_2SO_4 (% w/v). Xylan removal was recorded in the range of 43.19 to 100%. Even though minimal temperature (146.4 °C) was studied for pretreatment P-HSO, xylan solubilization was lower on account of higher H_2SO_4 concentration employed.

Yang et al. (Yang *et al.*, 2012) reported 100% xylan removal for hydrothermal pretreatment of triploid poplar at 200 °C catalyzed with 0.5 H_2SO_4. In another study, Lu and colleagues (Lu *et al.*, 2009) observed a decrease in xylan content from 19.6% to 1.4% for hydrothermal pretreatment of rapeseed straw at 180 °C, 2% w/w H_2SO_4, 20 min reaction time and with a solid content ratio of 5% (w/w).

Second-order polynomial equation was derived from the experimental data of glucan content (g 100 g^{-1} TS) to determine the impact of pretreatment parameters; reaction temperature, reaction time, and H_2SO_4 concentration on their availability in the solid fraction in response to H_2SO_4 assisted hydrothermal pretreatment. Equation 5.7 represents the second order polynomial equations for glucan (g 100 g^{-1} TS). The model equation presented that linear terms of temperature (X_1), H_2SO_4 concentration (X_3), quadratic term of temperature X_1^2, and interaction between temperature and concentration X_1X_3 were negatively significant with magnitude values of 7.32, 7.39, 9.46, and 5.63, respectively. While, linear term of reaction time (X_2), quadratic terms of reaction time (X_2^2) and H_2SO_4 concentration (X_3^2), and interaction terms of X_1X_2 and X_2X_3 were insignificant as indicated in ANOVA (Table 5.12).

$$Glucan\ (\text{g } 100\ \text{g}^{-1}\ TS) = 50.49 - 7.32X_1 + 0.08X_2 - 7.39X_3 - 9.46{X_1}^2 + 1.15{X_2}^2 - 1.75{X_3}^2 - 3.42X_1X_2 - 5.63X_1X_3 + 0.06X_2X_3 \quad \text{Equation 5.7}$$

Table 5.12 - Analysis of variance (ANOVA) for glucan content (g 100 g^{-1} TS) in pretreated solids impregnated in H_2SO_4

Source	Sum of square	Degree of freedom	Mean Square	*F* value	P-value (prob. > *F)*
X_1 *(T)*$^{\alpha}$	733.206	1	733.2064	558.8102	0.026915*
X_1^2	829.291	1	829.2913	632.0410	0.025309*
X_2 *(t)*$^{\beta}$	0.091	1	0.0913	0.0696	0.835781
X_2^2	12.208	1	12.2079	9.3042	0.201680
X_3 *(H_2SO_4 Conc.)*$^{¥}$	746.821	1	746.8206	569.1862	0.026669*
X_3^2	28.354	1	28.3536	21.6096	0.134893
X_1X_2	93.717	1	93.7170	71.4260	0.074979
X_1X_3	254.002	1	254.0018	193.5865	0.045677*
X_2X_3	0.033	1	0.0332	0.0253	0.899570
Lack of Fit	379.358	5	75.8717	57.8253	0.099496
Pure Error	1.312	1	1.3121		
Total SS	3340.561	15			

R-square = 0.886, Adj. R-square = 0.715 MS pure error =1.31, $^{\alpha}$ (T) represents Temperature, $^{\beta}$ (t) represents time, and $^{¥}$ (Conc.) represents H_2O_2 concentration

Klason lignin increased with a successive increase in pretreatment temperature and H_2SO_4 concentration (Table 5.11). Klason lignin was in the range of 33.96 to 84.61% for pretreatment set A-HSO – D-HSO conducted at 200 °C with varying retention time and H_2SO_4. A decrease in H_2SO_4 (3 to 1%) resulted in 37.12% decrease in klason lignin keeping reaction time (19 min) and reaction temperature 200 °C held constant. A predominant influence of decreasing reaction temperature was observed on klason lignin where it was in the range of 30.57 to 51.11% for pretreatments E-HSO – H-HSO.

Reaction temperature directly influence on the release of hemicellulosic sugar thereby have a direct impact on klason lignin. Hydrothermal pretreatment not only increases the lignin content but it also effectively modify the structure of lignin due to simultaneous de- and re-polymerization reactions of lignin (Chang and Holtzapple, 2000; Li *et al.*, 2007). Yang et al (Yang *et al.*, 2012) reported lignin redeposition on the polymeric structure during dilute acid pretreatment under severe condition resulting into a physical barrier to enzymatic action.

It was observed in the current study that a decrease in reaction temperature (200 to 160 °C) resulted in 40% (84.61 to 51.11%) decrease in klason lignin for pretreatment run E-HSO in comparison to pretreatment run A-HSO, where reaction time and H_2SO_4 conc held constant. It was observed that decrease in reaction time from 23.76 to 0.24 min had virtually no impact on klason lignin where pretreatment temperature (180 °C) and H_2SO_4 (2 % w/v) held constant. It indicated that reaction temperature was a more important factor in comparison to reaction time for the release of sugars and subsequently for klason lignin content.

In comparison, when reaction temperature was decreased from 213.6 to 146.6 °C, 55.42% decrease in klason lignin was observed between pretreatment runs O-HSO and P-HSO. Yang et al (Yang *et al.*, 2012) reported an increase in klason lignin from 20.78 to 50.35% with increasing severity for triploid poplar wood chips. A possible reason for an increase in klason lignin is explained by (Hu and Ragauskas, 2012), that xylose depolymerized into furfural during hydrothermal pretreatment. Repolymerization of furfural and other aromatic compounds produced during pretreatment may turn into pseudo-lignin, which results in an increase in klason lignin. Ramos et al (Ramos, 2003) reported that C_5 sugars are the principal source of pseudo-lignin formation.

Second-order polynomial equation (Equation 5.8) was derived from the lignin recovery experimental data to determine the impact of studied parameters. Maximum lignin recovery (358.85%) was observed for the maximum temperature employed (213.6 °C) for pretreatment run O-HSO. The model explained that studied parameters; temperature X_1, time X_2, and H_2SO_4 concentration X_3 showed significant impact as presented in ANOVA (Table 5.13).

The model presented that linear model terms X_1, X_2, X_3 and quadratic model terms ${X_1}^2$ and ${X_3}^2$ showed positive significant effect with magnitude values of 50.63, 11.32, 38.55, 40.59 and 12.41, respectively while quadratic model term ${X_2}^2$ showed negative non-significant impact with magnitude value of 1.51. The interaction of studied parameters indicated that X_1X_2 and X_1X_3 showed positive significant impact whilst X_2X_3 showed the negative non-significant effect on lignin recovery in pretreated solids.

$$\%\ lignin\ Recovery = 149.87 + 50.63X_1 + 11.32X_2 + 38.55X_3 + 40.59{X_1}^2 - 1.51{X_2}^2 + 12.41{X_3}^2 + 9.28X_1X_2 + 21.28X_1X_3 - 3.57X_2X_3 \quad (5.8)$$

Table 5.13 – Analysis of variance for percent lignin recovery in pretreated solids impregnated in H_2SO_4

Source	Sum of square	Degree of freedom	Mean Square	*F* value	P-value (prob. > *F)*
$X_1 (T)^{\alpha}$	35012.71	1	35012.71	33048.67	0.003502*
X_1^2	15264.82	1	15264.82	14408.54	0.005303*
$X_2 (t)^{\beta}$	1749.63	1	1749.63	1651.49	0.015662*
X_2^2	21.02	1	21.02	19.84	0.140605
X_3 $(H_2SO_4\ Conc.)^{¥}$	20295.47	1	20295.47	19157.00	0.004599*
X_3^2	1427.36	1	1427.36	1347.30	0.017340*
X_1X_2	689.28	1	689.28	650.61	0.024946*
X_1X_3	3621.49	1	3621.49	3418.35	0.010888*
X_2X_3	101.92	1	101.92	96.20	0.064683
Lack of Fit	6511.21	5	1302.24	1229.19	0.021651*
Pure Error	1.06	1	1.06		
Total SS	87149.55	15			

R-square = 0.925, Adj. R-square = 0.813.726, MS pure error =1.06, $^{\alpha}$ (T) represents Temperature, $^{\beta}$ (t) represents time, and $^{¥}$ (Conc.) represents H_2O_2 concentration

5.8.1 Morphological characteristics of H_2SO_4 assisted hydrothermally pretreated solid fraction

Only one pretreatment O-HSO was selected to determine the morphological characteristics in response to H_2SO_4 impregnation before hydrothermal pretreatment because this pretreatment resulted in the lowest methane 0.58 Nmmol g^{-1} TVS in this phase of the study. The surface of untreated sugarcane bagasse showed smooth intact fibril symmetry rather compact structure (Fig 5.21 *A*) in comparison to an extremely shattered structure for O-HSO (Fig 5.21 *B*) that could be attributed to cellulose disintegration and disturbance of lignin-carbohydrate meshwork. It further describes a decrease in particle size that could play a significant role in the availability of larger surface area for microbial action, but lignin deposition could severely affect enzymatic action.

Fig 5.21 *B* further indicated that hydrothermal pretreatment at higher severity (213.6 °C, 12 min, 2% H_2SO_4) significantly altered the structure at ultra-molecular level. A huge number of drop-like structures are quite visible on the surface of pretreated residue (Fig 5.21 *C*). These structures are reported as lignin droplets in literature. Several researchers reported about the

presence of lignin droplets on the surface of pretreated residues, which have a negative impact on cellulose hydrolysis in downstream processing (Donohoe *et al.*, 2008; Li *et al.*, 2014).

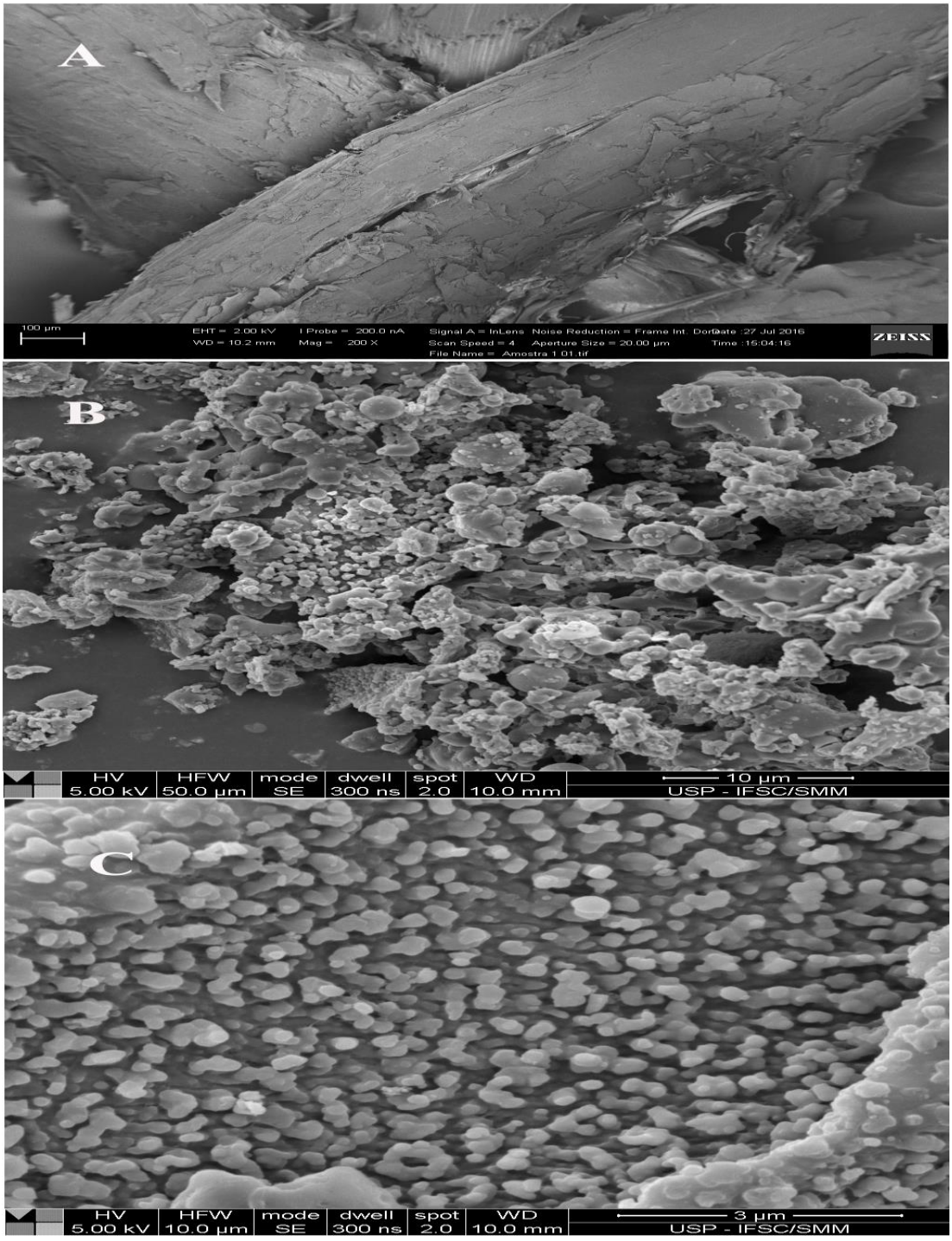

Figure 5.21 - Comparison between SEM images of untreated sugarcane bagasse (A) and O-HSO (B, C)

Selig et al (Selig *et al.*, 2007) speculated that at higher temperature lignin mass turns to fluid (phase change) that could transfuse through cell wall matrix into the liquid stream and redeposit on the surface, as spherical droplets (Fig 5.21 *C*). Commercial cellulase preparations are reported to bind to lignin unproductively (Berlin *et al.*, 2006; Yang and Wyman, 2006), thereby impeding cellulose hydrolysis. This could be the reason that digestion experiments of O-HSO resulted in methanogenic inhibition on the basis of unavailability of cellulosic material for hydrolytic bacteria to initiate the chain process. The impact of H_2SO_4 assisted pretreatment on visual appearance of sugarcane bagasse is depicted in Fig 5.22.

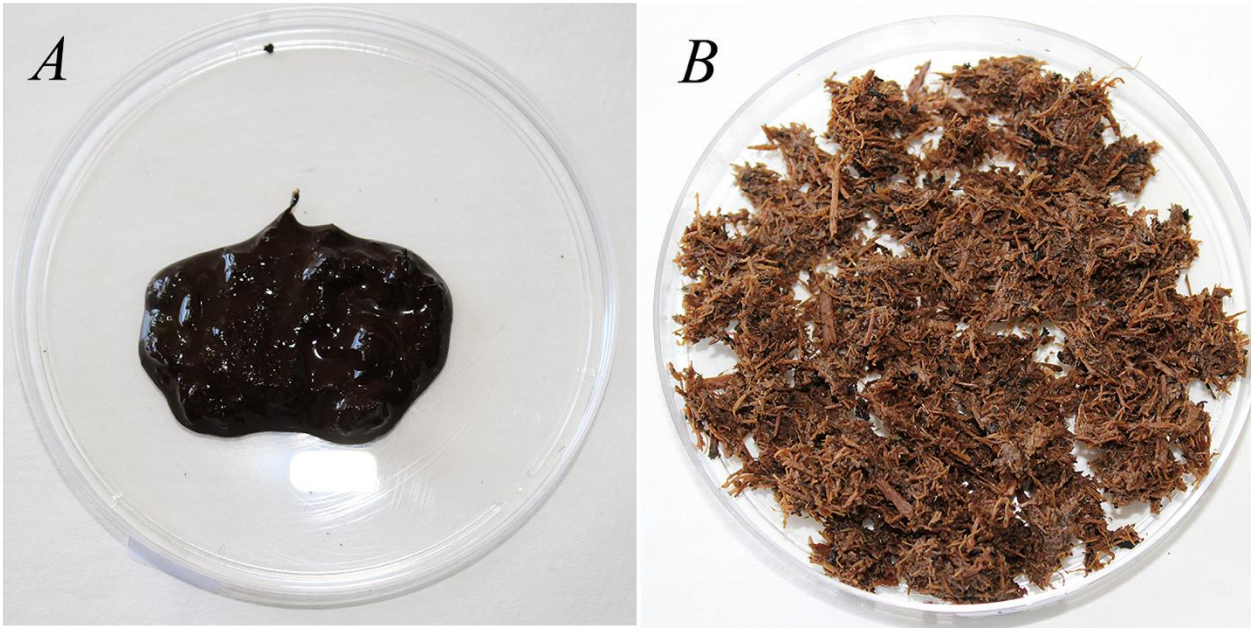

Figure 5.22 – Impact of H_2SO_4 assisted hydrothermal pretreatment on visual appearance of sugarcane bagasse; (A) Highest studied temperature (pretreatment run O-HSO – 213.6 °C), (B) Lowest studied temperature (pretreatment run P-HSO – 146.4 °C)

5.9 Chemical composition of liquid hydrolysate obtained from H_2SO_4 assisted Hydrothermal pretreatment

Liquid hydrolysate fraction separated from pretreated slurry was characterized for following parameters; chemical oxygen demand, total carbohydrate content, total phenolic content (TPC), organic acids, sulfate (SO_4^{-2}) content, and monomeric sugars; glucose, xylose and arabinose and furanic compounds; furfural and 5-hydroxymethylfurfural (Table 5.14 and 5.15).

H_2SO_4 impregnation of sugarcane bagasse facilitated the removal of hemicellulose partially removing lignin and hydrating cellulose, therefore, resulting in the release of monomeric

sugars, oligomers, and organic acids from cell wall matrix into hydrolysate. This resulted in an overall increase in COD of resulting hydrolysate stream. In the current study, COD value of hydrolysate affected by the joint impact of pretreatment parameters, H_2SO_4 concentration, reaction time, and reaction temperature.

Table 5.14 - Characterization of liquid hydrolysate obtained from H_2SO_4 aided hydrothermal pretreatment

Pretreatment run	Temperature (°C)	Time (min)	H_2SO_4 (w/v %)	COD_s (g L^{-1})	Total Carbohydrate (g L^{-1})	Phenolic content (ppm)	$SO4^{-2}$ mg L^{-1}
A-HSO	200	19	3	16.81	2.96	1014.38	4702.5
B-HSO	200	19	1	14.97	6.25	1101.88	1250
C-HSO	200	5	3	25.20	13.26	1399.69	5655
D-HSO	200	5	1	18.61	6.05	683.13	1445
E-HSO	160	19	3	21.79	11.19	1674.22	5245
F-HSO	160	19	1	15.02	12.98	708.13	1765
G-HSO	160	5	3	19.64	20.90	1008.13	5210
H-HSO	160	5	1	16.58	17.06	689.38	1520
I-HSO	180	12	2	8.91	5.74	808.13	4328.5
J-HSO	180	12	2	8.33	4.96	826.88	3657.5
K-HSO	180	12	3.68	20.09	12.56	1369.53	7240
L-HSO	180	12	0.32	11.77	8.20	596.72	360
M-HSO	180	23.76	2	9.24	7.26	901.88	3569.5
N-HSO	180	0.24	2	18.83	16.31	801.88	3811.5
O-HSO	213.6	12	2	24.07	8.55	1615.31	3338.5
P-HSO	146.4	12	2	17.01	13.06	658.13	3954.5

For a reaction temperature of 200 °C, COD value varied from 14.97 to 25.20 g L^{-1}. Higher reaction time (19 min) resulted in lower COD value (14.97-16.87 g L^{-1}) for pretreatment runs A-HSO and B-HSO in comparison to lower reaction time of 5 min., where it was in the range of 18.61-25.20 g L^{-1} for pretreatment runs C-HSO and D-HSO. Lower COD value at higher retention time could be justified by the argument some organics converted into volatiles and water. Quitain

et al (Quitain *et al.*, 2002) reported that formic and acetic acids transformed to water and volatiles thereby decreasing COD value.

For a reaction temperature of 160 °C, COD varied from 15.02 to 21.79 g L^{-1}. Higher reaction time (19 min) resulted in 31.06% (21.79 to 15.02 g L^{-1}), while lower reaction time (5 min) resulted in 15.58% (19.64 to 16.58 g L^{-1}) decrease in COD value when H_2SO_4 concentration decreased from 3 to 1% at a reaction temperature of 160 °C.

COD values at central points (pretreatment runs I-HSO and J-HSO) were not much differed from each other as expected. A decrease in H_2SO_4 concentration from 3.68 to 0.32 % (w/v) resulted into 41.41% (20.09 to 11.77 g L^{-1}) decrease in COD, while a decrease in reaction time from 23.76 to 0.24 min resulted in 103.78% (9.24 to 18.83 g L^{-1}) increase in COD. It implicated that H_2SO_4 and reaction time notably contributed to the release of organics into the liquid stream to aid in COD value. The decrease in reaction temperature from 213.6 to 146.4 °C indicated 29.33% decrease in COD value of hydrolysate.

Total carbohydrates are the amount of sugar monomers and oligomers released in response to pretreatment. Table 5.14 indicated that pretreatment parameters collectively affected the total carbohydrate content in the hydrolysate. Total carbohydrates varied between 2.96 and 13.26 g L^{-1} for pretreatments conducted at 200 °C implicating that reaction time and H_2SO_4 concentration had an effect on the release of sugars.

Higher reaction time (19 min) resulted in lower total carbohydrates (2.96 to 6.25 g L^{-1}) in comparison to 5 min reaction time where it was higher 6.05 (pretreatment run D-HSO) and 13.26 g L^{-1} (pretreatment run C-HSO). It was seen that higher reaction time coupled with higher H_2SO_4 concentration resulted into lower carbohydrates released possibly due to degradation to organic acids.

Pretreatments conducted at a reaction temperature of 160 °C and 5 min reaction time presented higher carbohydrate content than at 19 min reaction time. Furthermore, pretreatment G-HSO (3% w/v H_2SO_4) showed highest (20.90 g L^{-1}) carbohydrate content in the tested conditions. A comparison between pretreatment conducted at 200 and 160 °C, while keeping reaction time and H_2SO_4 concentration constant, 278.04, 107.68, 57.62 and 181.98% increase was observed for pretreatment runs E-HSO, F-HSO, G-HSO, and I-HSO, respectively, in comparison to

pretreatment runs A-HSO, B-HSO, C-HSO, and D-HSO. Central points differed only 13.58 % from each other.

Total carbohydrate content in comparison to decrease in H_2SO_4 concentration from 3.68 to 0.32 (% w/v) indicated 34.71% (12.56 to 8.20 g L^{-1}) decrease with other parameters held constant at 180 °C and 12 min. On the other hand, a decrease in reaction time from 23.76 to 0.24 min indicated 124.66% (7.26 to 16.31 g L^{-1}) increase in carbohydrate content. Similarly, reduction in reaction temperature showed 52.75% (8.55 to 13.06 g L^{-1}) increase in total carbohydrate content for pretreatment run P-HSO. Higher carbohydrate content at lower reaction time (0.24 min) in comparison to pretreatment run M-HSO demonstrated a higher sugar degradation that longer the sugarcane bagasse remain in contact with reaction medium higher degradation of sugars will result and vice versa.

Total phenolic content varied from 683.13 to 1101.88 ppm for pretreatments conducted at 200 °C. Much lower phenolics were observed in hydrolysate of H_2SO_4 pretreatment in comparison to H_2O_2 pretreatment where it was in the range of 1316.25 to 3005.63 ppm at 200 °C. This much difference can be justified that H_2SO_4 primarily aimed to remove hemicellulose and partially degrade lignin (Kim *et al.*, 2005), while H_2O_2 principally act to delignify lignocellulosic biomass (Chaturvedi and Verma, 2013).

Phenolic contents for pretreatments conducted at 160 °C varied between 689.38 to 1674.22 ppm. It was observed that decrease in H_2SO_4 from 3 to 1% resulted in 57.70% (1674.22 to 708.14 ppm) and 31.62% (1008.13 to 689.38 ppm) decrease in phenolic content for a reaction temperature of 160 °C, 19 min and 5 min, respectively. A comparison between pretreatment conducted at 200 and 160 °C showed 65.05% (1014.38 to 1674.22 ppm) increase for pretreatment run E-HSO in comparison to pretreatment run A-HSO. However, 35.73% and 27.96% decrease was noted in phenolic contents for pretreatment runs F-HSO and G-HSO in comparison to pretreatment runs B-HSO and C-HSO, respectively. Central points presented 2.32% difference in at reaction temperature 180 °C, for 12 min and 2% (w/v) H_2SO_4. A decrease in H_2SO_4 from 3.68 to 0.32% resulted in 56.43% (1369.53 to 596.72 ppm) decrease in phenolic content. On the other hand, a decrease in reaction time from 23.76 to 0.24 min and decrease in reaction temperature (213.6 to 146.4 °C) resulted in 11.09% (901.88 to 801.88 ppm) and 59.26% (1615.31 to 658.13 ppm) decrease in phenolic contents of liquid hydrolysate, respectively.

Since sugarcane bagasse was impregnated in H_2SO_4 prior to hydrothermal pretreatment, the appearance of sulfate in the liquid filtrate was inevitable; sulfate concentration in the liquid was proportional to H_2SO_4 concentration employed for the impregnation. However, reaction temperature and reaction time of hydrothermal pretreatment had an effect on sulfate concentration in the liquid hydrolysate. Minimal concentration (360 mg L^{-1}) was seen in pretreatment run L-HSO, where 0.32 (% w/v) H_2SO_4 was used for impregnation, similarly impregnation in higher concentration (3.68 %w/v) of H_2SO_4 resulted in maximum (7240 mg L^{-1}) sulfate concentration for pretreatment 11.

Sulfate concentration was in the range of 1250 – 5655 mg L^{-1}) for pretreatment set A-HSO – D-HSO following a similar pattern as described above. Higher reaction temperature (pretreatment run O-HSO – 213.6 °C) and longer reaction time (pretreatment M-HSO – 23.76 min) resulted into dissociation of sulfate thereby leading to lower concentration in the liquid filtrate.

Monomeric sugars concentration in the liquid filtrate of H_2SO_4 assisted hydrothermal pretreatment are presented in Table 5.15. Relatively higher glucose concentration was seen in comparison to xylose concentration. It demonstrated that xylose was much solubilized and further degraded into organic acids and furanic compounds under higher severity conditions as evidenced from higher furfural concentration (Table 5.15).

Highest glucose (13.53 mg mL^{-1}) was noted at pretreatment run G-HSO (160 °C, 5 min, 3% H_2SO_4). It indicated that higher pretreatment temperature combined with higher H_2SO_4 concentration though resulted into more solubilization of hemicellulose and solubilization of cellulose. However, monomeric sugars further degraded into organic acids and furanic compounds. Higher contact time also resulted into lower glucose 1.93 mg mL^{-1} (pretreatment run M-HSO – 180 °C, 23.76 min, 2% H_2SO_4) in comparison to higher glucose 6.65 mg mL^{-1} for less reaction time (pretreatment run N-HSO – 180 °C, 0.24 min 2% H_2SO_4). Longer the contact time, higher will be the degradation of monomeric sugar to organic acids and furanic compounds. Similar is true for highest reaction temperature studied (213.6 °C). Pretreatment run O-HSO resulted into lower glucose (2.51 mg mL^{-1}) in comparison to pretreatment run P-HSO (146.4 °C) it is strengthened by the almost 2-fold production of formic acid (3611.28 mg L^{-1}) in pretreatment 15 comparing to 1405.49 mg L^{-1}) for pretreatment 16.

Though most of the xylose was solubilized at 200 °C (pretreatment set A-HSO – D-HSO), lesser recovery in liquid filtrate indicated that it was further degraded into organic acids. Higher recovery of xylose was recorded at 160 °C irrespective of H_2SO_4 concentration employed (pretreatment set E-HSO – H-HSO). Higher xylose (13.87 mg mL^{-1}) in liquid filtrate was noted at pretreatment run H-HSO (160 °C, 5 min, 1% H_2SO_4), followed by pretreatment run N-HSO (180 °C, 0.24 min, 2% H_2SO_4) and pretreatment run P-HSO (146.4 °C, 12 min, 2% H_2SO_4).

Table 5.15 - Selected organic acid and furanic compounds in liquid hydrolysate obtained from H_2SO_4 assisted hydrothermal pretreatment of sugarcane bagasse

Pretreatment run	pH	Glucose (mg mL^{-1})	Xylose (mg mL^{-1})	Acetic acid (mg L^{-1})	Formic acid (mg L^{-1})	Furfural (mg L^{-1})	5-HMF (mg L^{-1})
A-HSO	1.12	2.34	1.03	1,892.73	1,093.66	1,402.19	147.36
B-HSO	1.48	4.86	1.44	1,114.61	834.35	1,080.94	471.52
C-HSO	1.22	4.40	0.43	2,205.76	2,425.46	1,321.73	493.70
D-HSO	1.76	2.54	2.06	1,435.21	688.31	3,013.14	342.74
E-HSO	1.19	4.00	4.92	2,080.82	2,668.62	2,216.47	970.08
F-HSO	1.65	5.23	9.30	2,031.71	1,463.78	1,839.84	174.94
G-HSO	1.20	13.53	8.09	1,927.23	1,977.09	2,181.24	713.26
H-HSO	1.60	3.02	13.87	1,408.34	1,003.07	925.77	70.95
I-HSO	1.50	2.77	2.56	1,608.05	799.22	1,239.33	178.80
J-HSO	1.49	2.36	2.03	1,435.73	729.84	1,122.86	168.93
K-HSO	1.38	7.81	1.97	2,198.89	2,384.15	1,774.14	788.62
L-HSO	2.86	2.83	2.66	1,888.45	788.04	1,674.15	285.99
M-HSO	1.42	1.93	1.49	1,445.42	796.93	939.89	245.89
N-HSO	1.36	6.65	12.69	1,685.85	1,763.46	1,283.79	130.57
O-HSO	1.65	2.51	0.73	2,191.82	3,611.28	1,135.85	296.11
P-HSO	1.81	3.26	10.29	1,750.05	1,405.49	1,178.81	106.38

A higher concentration of organic acids (acetic and formic) and sugar degradation products (furfural and 5-HMF) were noted in hydrolysate fraction of H_2SO_4 impregnated hydrothermal pretreatment. Acetic acid was in the range of 1,114.61 – 2,198.89 mg L^{-1}. Furthermore, it was observed that higher H_2SO_4 conc. resulted in the higher acetic acid release into hydrolysate

implicating higher removal of the hemicellulosic fraction. Formic acid was in the range of 729.84 – 3,611.28 mg L^{-1}, it also demonstrated a similar trend to acetic acid release. However, highest formic acid release (3,611.28 mg L^{-1}) was noted for O-HSO (213.6 °C, 12 min, 2% H_2SO_4). Yang et al (Yang *et al.*, 2012) reported highest acetic acid and formic acid release of 109.96 and 130.15 mg L^{-1}, respectively, at 200 and 130 °C for triploid poplar wood chips.

The relatively higher concentration of furfural (925.77 – 3,013.14 mg L^{-1}) was observed comparing to 5-HMF (70.95 – 970.08 mg L^{-1}). A lower concentration of 5-HMF was expected based on lower hexose content in hemicellulose, higher hexose resistance and higher 5-HMF reactivity as reported by Mussatto et al. (Mussatto and Roberto, 2004). Another reason of lower 5-HMF concentration is its conversion into levulinic acid and formic acid under acidic hydrothermal conditions (Nitsos *et al.*, 2013). Yang et al (Yang *et al.*, 2012) reported that furfural and 5-HMF appeared in hydrolysate when pretreatment temperature was increased above 140 °C. Similarly, Marasabessy et al (Marasabessy *et al.*, 2012) reported that pretreatment temperature above 160 °C did not result in an increase in pentose release due to degradation into furfural. A lower concentration of pentose sugar in the hydrolysate of current study verify the above said findings of Marasabessy and colleagues (Marasabessy *et al.*, 2012) since reaction temperature employed was much higher 180 °C up to 213.6 °C.

5.10 Impact of H_2SO_4 impregnation before hydrothermal pretreatment on methane production

H_2SO_4 impregnation of sugarcane bagasse for 24 h before hydrothermal pretreatment according to CCD matrix (Table 4.5) was studied on the methane production (Nmmol g^{-1} TVS) for obtained water-insoluble solid fractions. Reactor composition for each tested pretreatment is presented in Table 9.2 (Appendix). Digestion experiments were realized at neutral pH supplemented with 1g TVS substrate with 2 g TVS inoculum under mesophilic conditions (37 °C). Total digestion time was 800 h. Experimental data of methane production was processed with modified Gompertz model (Zwietering *et al.*, 1990).

Table 5.16 presents the methane values obtained after incubation and percent increase or decrease in comparison to methane (Nmmol g^{-1} TVS) obtained from raw sugarcane bagasse (6.60 Nmmol g^{-1} TVS). In the first set of experiments, eight pretreatments namely A-HSO, and B-HSO were realized for biomethane potential. Cumulative methane production was 1.22 and 3.97 Nmmol

g^{-1} TVS which was less than untreated sample (5.59 Nmmol g^{-1} TVS). In the second set, D-HSO, F-HSO, H-HSO, I-HSO, J-HSO, M-HSO, and N-HSO were realized for biomethane potential. Cumulative methane production for this set of experiments was in the range of 1.73 – 7.14 Nmmol g^{-1} TVS. Cumulative methane value of the pretreated samples was less than untreated sugarcane bagasse except for H-HSO, I-HSO, and N-HSO.

Fig 5.23 indicated that CH_4 appeared after a lag phase of 150.75 h (Table 5.16) for A-HSO but only reached to a maximum of 1.22 Nmmol g^{-1} TVS in 306 h thereafter the production was ceased. An abrupt increase in methane was noted for pretreatment I-HSO, it reached to 4.2 Nmmol g^{-1} TVS in 168 h afterward a slower increase was observed until it reached a maximum value of 7.14 Nmmol g^{-1} TVS. A similar trend was noted in rest of the pretreatments in this set of experiments.

In the third set of experiments, C-HSO, E-HSO, G-HSO, K-HSO, L-HSO, O-HSO, and P-HSO were realized for biomethane potential. Cumulative methane production was in the range of 0.55 – 5.77 Nmmol g^{-1} TVS. All the pretreatments in this set exhibited lower methane than untreated sugarcane bagasse (6.60 Nmmol g^{-1} TVS). The lowest methane value 0.55 Nmmol g^{-1} TVS for pretreatment O-HSO (213.6 °C, 12 min, 2% H_2SO_4 (% w/v)) could be justified with the highest klason lignin recovery (358.85%) in solid fraction of this pretreatment.

Since sugarcane bagasse was H_2SO_4 impregnated, the presence of SO_4^{-2} is inevitable. Lowest methane production might be a result of competition between methanogens and sulfate reducers. Sulfate reducers could outcompete methanogens under higher SO_4^{-2} concentrations. Sulfate reducers compete with methanogens for acetate and H2/CO_2 (MIZUNO *et al.*, 1998). Sulfate reducers outcompete methanogens and homoacetogens when H_2 is deficient in the system with higher SO_4^{-2} availability (Muyzer and Stams, 2008).

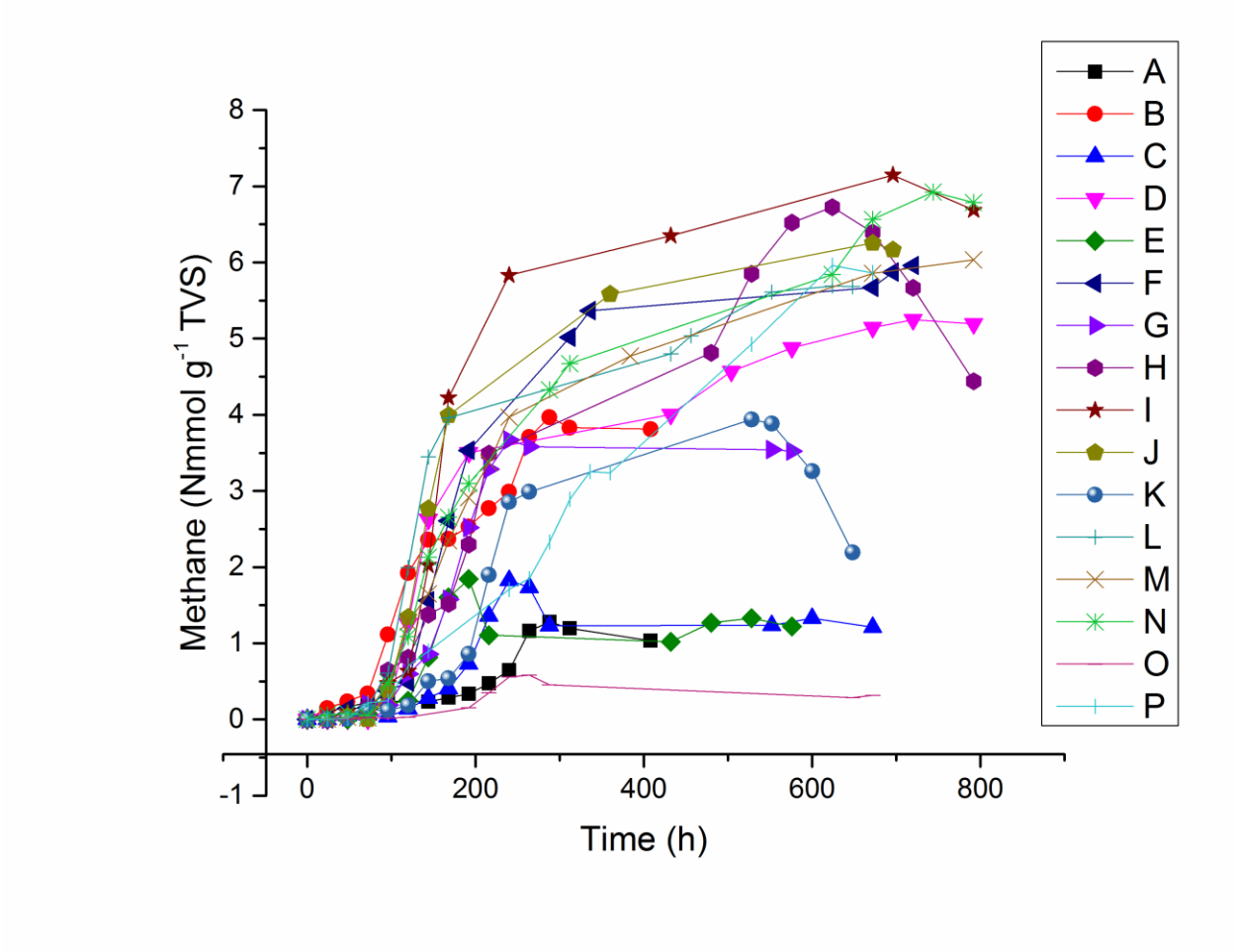

Figure 5.23 - Time-course profile of Methane production for solid fraction of H_2SO_4-catalyzed hydrothermal pretreatment

Table 5.16 demonstrated that fermentation of solid fraction obtained from H_2SO_4 impregnated hydrothermal pretreatment resulted in methanogenic inhibition when compared with the raw bagasse. Maximum production (7.15 Nmmol g^{-1} TVS) was observed at pretreatment I-HSO corresponds to 8.18% increase over raw bagasse (6.59 Nmmol g^{-1} TVS). Maximum percent decrease of 91.67% was noted at pretreatment O-HSO followed by 81.52% for pretreatment A-HSO. It was observed that higher temperatures were chosen during HT pretreatment with higher H_2SO_4 conc. resulted in methanogenic inhibition from solid fraction as evident from O-HSO (213.6 °C, 2 % H_2SO_4) and A-HSO (200 °C, 3% H_2SO_4).

The possible reason for the lower methane production is the transfer of fermentable sugar into liquid hydrolysate as indicated by higher glucose and xylose contents obtained in the liquid

hydrolysate fraction (Table 5.15) resulting in a higher fraction of klason lignin content. Pretreatment O-HSO exhibited the highest klason lignin value 89.28% followed by A-HSO 84.61%. Methane yield and lignin content are reported to have an inverse linear relationship i.e., higher the lignin content lower will be the methane and vice versa (Kobayashi *et al.*, 2004; Liew *et al.*, 2012; Ahring *et al.*, 2015). Current study reported the similar trend (Fig 5.24).

In contrast, a number of studies have been reported that lignin can be degraded anaerobically (Yue *et al.*, 2008; Ko *et al.*, 2009) even into methane (Healy and Young, 1979; Barakat *et al.*, 2012). However, *P*-coumaric acid, a lignin monomer reported to severely inhibit methane production. Den Camp et al (den Camp *et al.*, 1988) reported that higher concentrations (30 mM) of *p*-coumaric acid completely inhibit cellulose degradation and in turn methane production. In another study, Schroyen et al (Schroyena *et al.*, 2016) reported that 2000 mg L^{-1} *p*-coumaric acid resulted in 33% methanogenic inhibition.

Since, the impact of pretreatment on the release of lignin monomers was beyond the scope of current study; therefore, further study is required to determine the impact of H_2SO_4 impregnated hydrothermal pretreatment on sugarcane bagasse for the availability of *p*-coumaric acid in solid fraction regarding its effect on the digestion process. The results of the current study could be strengthened by the Giuseppe et al (Giuseppe *et al.*, 2013). The authors observed methanogenic inhibition for 24 h impregnated H_2SO_4 (2% w/w) pretreated *Arundo donax* (giant cane). In another study, Jorn et al (Jörn *et al.*, 2014) observed that a temperature of 220 °C led to lower degradability and lower methane output for dairy cattle waste.

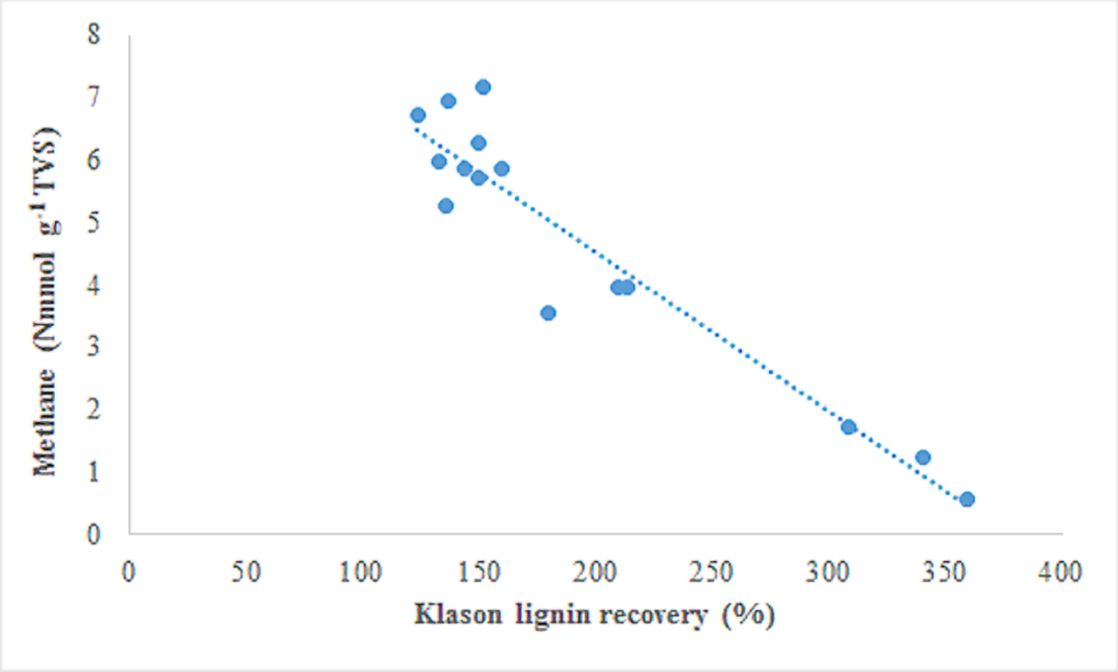

Figure 5.24 - Relationship between klason lignin (%) and methane production

Fig 5.25 depicts the change in COD in time-course fashion. COD was in the range of 97.45 ± 1.77 – 183.45 ± 12.52 mg L^{-1} for all the pretreatments at the start of digestion depending upon pretreatment conditions applied. Minimal COD was observed for pretreatment L-HSO (180 °C, 12 min, 0.32% H_2SO_4). An increase in COD values was observed as the digestion proceeds. At approx. log phase, COD values increased to 194 ± 7.95 – 316.5 ± 10.61 mg L^{-1} with a simultaneous decrease in pH (Fig 5.27) implicating an increase in VFAs production. Final COD values at the end of digestion process were in a range of 175.9 ±16.12 to 440.30 ±14.28 mg L^{-1}. An increase in COD was recorded at the end of digestion process in all the pretreatments comparing to initial COD that might be justified by the fact that methane production was inhibited.

Total carbohydrates content were not notably reduced (Fig 5.26) during second-phase of study i.e., digestion of H_2SO_4 pretreated solid fraction. Total carbohydrates were in the range of 39.65 ± 1.48 – 106.1 ± 10.04 mg L^{-1} depending upon pretreatment conditions at the initial stage of digestion. Total carbohydrate contents decreased to 28.15 ± 1.34 – 90.6 ± 3.39 mg L^{-1} on approx. log phase at respective pretreatments. Since hemicellulosic and cellulosic fractions are enclosed within lignin cover as indicated in (Fig 5.21 *C*). It is difficult for the microbial enzymes of hydrolytic bacteria to degrade cellulosic fractions. Acetogenic bacteria might have degraded the freely available sugars present in the solid fraction after separation from liquid hydrolysate to

VFAs. Since SRB (sulfate reducing bacteria) do not metabolize natural biopolymers; starch, protein and lipid. Therefore, they have to depend upon the action of hydrolytic bacteria for their degradation products. The SRB do not compete with fast-growing fermentative bacteria (Muyzer and Stams, 2008)

Igarashi and fellows (Igarashi *et al.*, 2011) reported that cellulase started cellulose hydrolysis layer by layer from the surface by sliding in unidirectional form, therefore, when cellulose surface is blocked by lignin droplet (Fig 5.21 *C*) it stopped cellulase access to inner layers of cellulose. Two approaches are reported in the literature to address this situation to start cellulose hydrolysis "traffic jam" approach and "enzymatic deinking". In the former approach, accumulation of enzyme molecules exerts a push to eliminate the lignin droplets to start cellulose hydrolysis (Igarashi *et al.*, 2011). In Enzymatic deinking approach, lignin droplets believed to be 'peeled off' from the cellulose surface due to loosening by enzymatic action. In the current study, it seemed to be true as total carbohydrate content started to increase in P-HSO and L-HSO. It could also be a reason of lower consumption of total carbohydrates that cellulose hydrolysis stopped and started due to traffic jam approach resulting in production and consumption of fermentable sugars. However, due to only one point selection during the digestion process this hypothesis could not be verified.

A significant decrease in pH was seen as depicted in Fig 5.27 at approx. log phase except for O-HSO, where it was interestingly increased to 7.1, but afterward decreased to 6.98 suggesting the lowest production of organic acids supported with a lower decrease in total carbohydrate content. After an initial decrease, pH appears to increase due to the buffering capacity of the system.

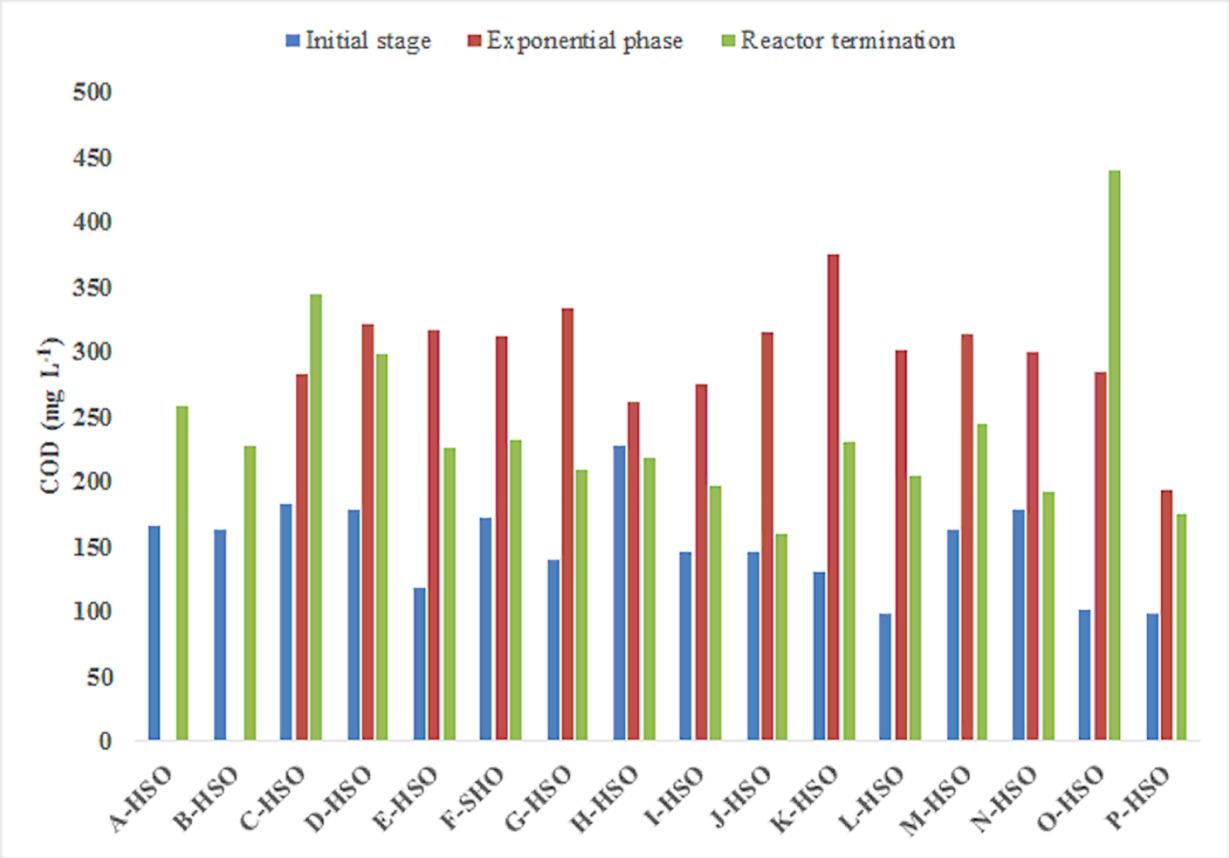

Figure 5.25 - COD (mg L^{-1}) variation during digestion process of H_2SO_4 pretreated samples

Acetic acid was the predominant volatile fatty acid encountered at reactor initiation in the range of 71.42 to 153.35 mg L^{-1}. The formic acid, butyric acid, propionic acid, ethanol and furanic compounds were observed in lower concentration. Only 4 mg L^{-1}. 5-HMF was encountered at initial digestion in pretreatment run H-HSO, while for other reactors its concentration was below detection limit. Furfural concentration was also below the detection limit. Therefore, it could be stated that methanogenic inhibition was solely the result of higher Klason lignin in solid fraction and competition between SRB and methanogens. Even though a significant decrease in pH was noted, VFAs appeared to below detection limit at the end of digestion period.

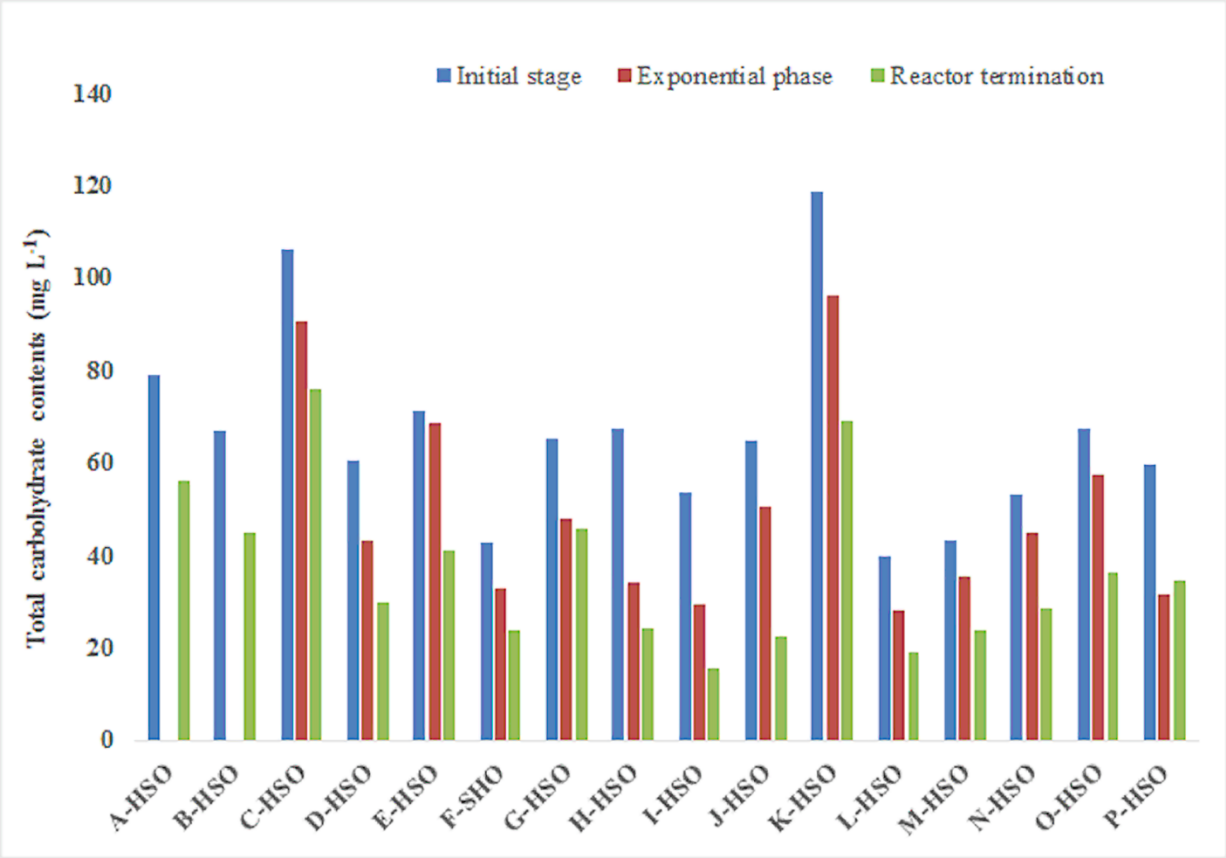

Figure 5.26 - Total carbohydrate content (mg L^{-1}) during digestion process of H_2SO_4 pretreated samples

It could be speculated that VFAs produced are taken up by sulfate-reducing bacteria as indicated by their higher microbial flora at the end of digestion process (Fig 5.34). Therefore, VFAs concentration at the approx. log phase was below detection limit. Since only one point was selected during the digestion process. It is difficult to report VFAs production and consumption along the digestion process. SRB show higher affinity for H_2 than methanogens, which gives them an additional competitive advantage (Colleran *et al.*, 1995).

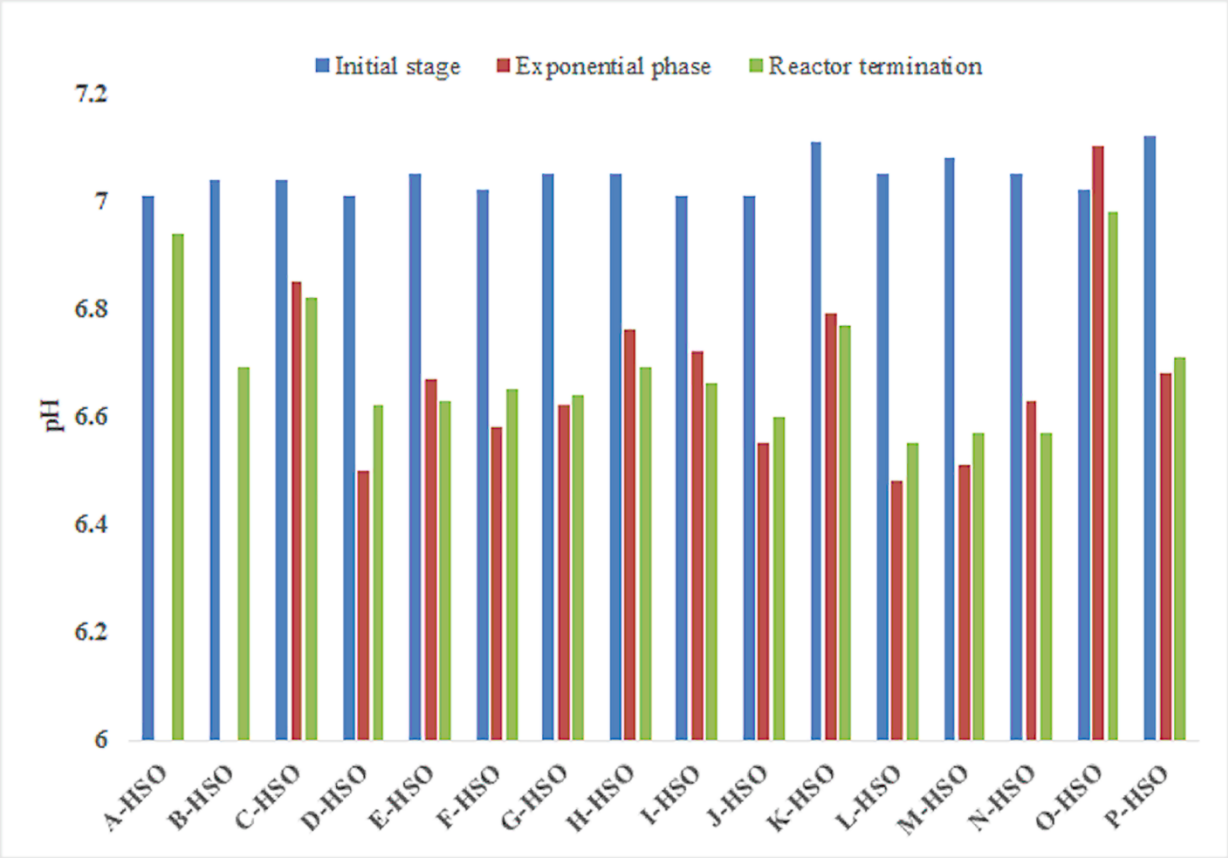

Figure 5.27 - pH variation during digestion process

Kinetic data for digestion obtained from anaerobic reactors supplemented with H_2SO_4 pretreated sugarcane bagasse is presented in Table 5.16. Maximum predicted the value of methane (Nmmol g^{-1} TVS) 6.87 was observed for I-HSO while the lowest was for O-HSO 0.54 (Nmmol g^{-1} TVS). Methanogens are slow-growing microorganisms. Van Haandel and Lettinga (van Haandel and Lettinga, 1994) reported a low specific growth rate of 0.0167 h^{-1} for methanogens though it increased with temperature. A general rule for microbial growth is explained by Arrhenius equation i.e., growth rate doubles with every 10 °C increase (Connors, 1990). For the current study, the rate of methane production (*Xc*) ranges from 0.01 to 0.066 Nmmol g^{-1} TVS.

Table 5.16 – Experimental value of methane (Nmmol g^{-1} TVS) and kinetic analysis data obtained from Gompertz equation

Designation	Experimental Methane (Nmmol g^{-1} TVS)	Percent variation over SCB	Kinetics analysis data			
			$A^{¥}$ (Nmmol g^{-1} TVS)	Xc^{A} Nmmol g^{-1} TVS h^{-1}	K^{B} h	R^2
Raw	6.60	-	6.63	0.027	0	0.988
A-HSO	1.22	-81.52	1.15	0.010	150.75	0.86
B-HSO	3.97	-41.06	3.99	0.020	41.42	0.97
C-HSO	1.73	-72.58	1.63	0.024	153.45	0.92
D-HSO	5.26	-22.12	4.83	0.044	91.58	0.97
E-HSO	1.33	-71.82	1.54	0.039	118.40	0.88
F-HSO	5.96	-14.24	5.71	0.039	102.98	0.99
G-HSO	3.54	-44.70	3.69	0.039	120.84	0.98
H-HSO	6.73	1.36	6.03	0.025	94.22	0.96
I-HSO	7.15	8.18	6.87	0.066	110.03	0.99
J-HSO	6.25	-12.27	6.01	0.059	97.08	1.00
K-HSO	3.94	-43.18	3.88	0.032	156.17	0.99
L-HSO	5.69	-14.09	5.36	0.057	85.15	0.99
M-HSO	5.86	-17.73	5.74	0.026	80.45	0.99
N-HSO	6.93	1.67	6.54	0.022	66.96	0.98
O-HSO	0.58	-91.67	0.54	0.012	180.13	0.96
P-HSO	5.86	-12.58	6.82	0.013	109.39	0.99

¥ Predicted value obtained from Gompertz equation, A Rate of methane production (Nmmol g^{-1} TVS h^{-1}), B time elapsed before CH_4 production started (lag phase),

Similar *Xc* values of 0.012 and 0.013 Nmmol g^{-1} TVS h^{-1} were observed for the lowest methane value (pretreatment run O-HSO, 0.54 Nmmol g^{-1} TVS) and second highest methane production (pretreatment run P-HSO, 6.82 Nmmol g^{-1} TVS), respectively. It suggests that rate of production does not depend upon the maximum methane harvest during the study. However, maximum production rate (0.066 Nmmol g^{-1} TVS h^{-1}) was noted for the highest methane produced (6.87 Nmmol g^{-1} TVS).

Lag phase observed was too high ranging from 41.42 h (pretreatment run B-HSO – 200 °C, 1% H_2SO_4, 5 min) to 180.13 h (pretreatment run O-HSO – 213.6 °C, 2% H_2SO_4, 12 min). Table 5.16 indicates that at 200 °C, 3% H_2SO_4 (pretreatment A-HSO and C-HSO) resulted in higher lag phase compared to 1% H_2SO_4 (pretreatment B-HSO and D-HSO). While, change in

pretreatment time from 19 to 5 min showed no impact on lag phase when compared with H_2SO_4 concentration at 200 °C. A decrease in pretreatment temperature to 160 °C (pretreatment run E-HSO – H-HSO) resulted in a corresponding decrease in lag phase as compared to 200 °C, but with similar trend i.e., 3% H_2SO_4 showed higher lag phase of 118.40 and 120.84 h for pretreatment E-HSO and G-HSO, respectively.

Pretreatment temperature of 180 °C (pretreatment run I-HSO-N-HSO) showed lower lag phase compared to 200 °C it could be explained with lower H_2SO_4 employed except pretreatment K-HSO where highest 3.68 % H_2SO_4 was utilized resulting into a lag phase of 156.17 h. It is also worth noting that pretreatment time of 23.76 h (pretreatment M-HSO) showed lower lag phase 80.45 h indicating that it is the pretreatment temperature that affects the lag phase as indicated by 180.13 h of lag phase exhibited by pretreatment run O-HSO (213.6 °C).

5.10.1 Response surface methodology

The second-order polynomial equation was derived from the experimental methane data (Nmmol g^{-1} TVS) to interpret the effects of pretreatment parameters and their interaction on the methane production. The equation is presented in Equation 5.9.

$$Methane\ production\ \ (Nmmol\ g^{-1}\ TVS) = 6.54 - 1.068\ X_1\ - 0.499\ X_2 - 1.177\ X_3 - 1.354\ {X_1}^2 - 0.325\ {X_2}^2 - 0.806\ {X_3}^2 + 0.123\ X_1X_2 + 0.105\ X_1X_3 - 0.013\ X_2X_3 \quad (5.9)$$

The model Equation indicated that linear term of temperature (X_1) and H_2SO_4 concentration (X_3) while the quadratic term of temperature X_1^2 showed the significant negative impact on the methane production. Linear term of time X_2, quadratic terms of time X_2^2, and concentration X_3^2, and interaction between temperature and time (X_1X_2), temperature and H_2SO_4 concentration (X_1X_3), and time and concentration (X_2X_3) did not show significant impact on the methane yield. Magnitude values 1.068 and 1.177 of linear terms of temperature (X_1) and H_2SO_4 concentration (X_3), respectively as presented in equation 5.6 implied that pretreatment temperature and H_2SO_4 concentration must be decreased to get a higher methane.

Table 5.17 – Multiple-regression analysis for experimental methane production for quadratic response methodology

	Sum of square	Degree of freedom	Mean square	F Value	*p*-value Prob>F
X_1 $(T)^{\alpha}$	15.56	1.00	15.56	10.23	0.02*
X_1^2	16.98	1.00	16.98	11.17	0.02*
X_2 $(t)^{\beta}$	3.40	1.00	3.40	2.23	0.19
X_2^2	0.98	1.00	0.98	0.64	0.45
X_3 $(H_2SO_4\ Conc.)^{¥}$	18.91	1.00	18.91	12.43	0.01*
X_3^2	6.02	1.00	6.02	3.96	0.09
X_1X_2	0.12	1.00	0.12	0.08	0.79
X_1X_3	0.09	1.00	0.09	0.06	0.82
X_2X_3	0.00	1.00	0.00	0.00	0.98
Error	9.13	6.00	1.52		
Total SS	66.04	15.00			

R-square = 0.862, Adj. R-square = 0.654, MS residual = 1.521, * represents significant factors, $^{\alpha}$ (T) represents Temperature, $^{\beta}$ (t) represents time, and $^{¥}$ (Conc.) represents H_2O_2 concentration

Table 5.17 presents the one-way analysis of variance (ANOVA) for the quadratic response model presented in the equation in 5.6. Significant model parameters were determined at 5% confidence interval. ANOVA described that linear terms of temperature (X_1), H_2SO_4 concentration (X_3) and quadratic term of temperature (X_1^2) were significant with a *p*-value less than 0.05. The goodness of model fit was determined with F-value. R^2 value of 0.862 indicated that model was significantly supported with lower F tabulated value of 4.76 compared to the higher F-calculated value of 11.27. R^2 indicated that there is 86.2 % variation in the response data could be explained by the model and only 13.8% could not be explained with proposed model.

The interaction between temperature X_1 and time X_2 on methane production in a response surface curve (Fig 5.28) depicted a plateau for methane production for variable time X_2 implicating that changing the time variable had no impact on methane production after a specified temperature limit. Maximum response was seen for X_1 at around 180 °C, while changing the X_2 value from 0

to 16 min showed no increase in the in the response value. It could be explained by the fact that at 180 °C maximum sugars might have been released into liquid hydrolysate making solid fraction sugar deficient with higher klason lignin.

The interaction between temperature (X_1) and H_2SO_4 concentration (X_3) is depicted in Fig 5.29. A critical analysis of the Fig 5.29 demonstrated that there is a significant effect on methane production when H_2SO_4 conc. and pretreatment time is compared. A concurrent increase in the response value (methane production) was observed with an increase in pretreatment temperature and H_2SO_4 concentration up to a certain limit.

Maximum response value was observed at 180 °C for 1% (w/v) H_2SO_4 utilized for bagasse impregnation. A further increase in in pretreatment temperature above 180 °C and higher H_2SO_4 concentration resulted in lower methane production as depicted in the response curve. Furthermore, response surface revealed that maximum response point was located within the experimental region.

Effect on methane production in relation to the interaction between pretreatment time and H_2SO_4 conc. is presented in Fig 5.30. The interaction of both parameters depicted that response value had a plateau for reaction time; increase in pretreatment time above 12 min had a similar impact on response value at 1% H_2SO_4 concentration. Furthermore, the visual inspection of the graph depicted that maximum point was located within the experimental region.

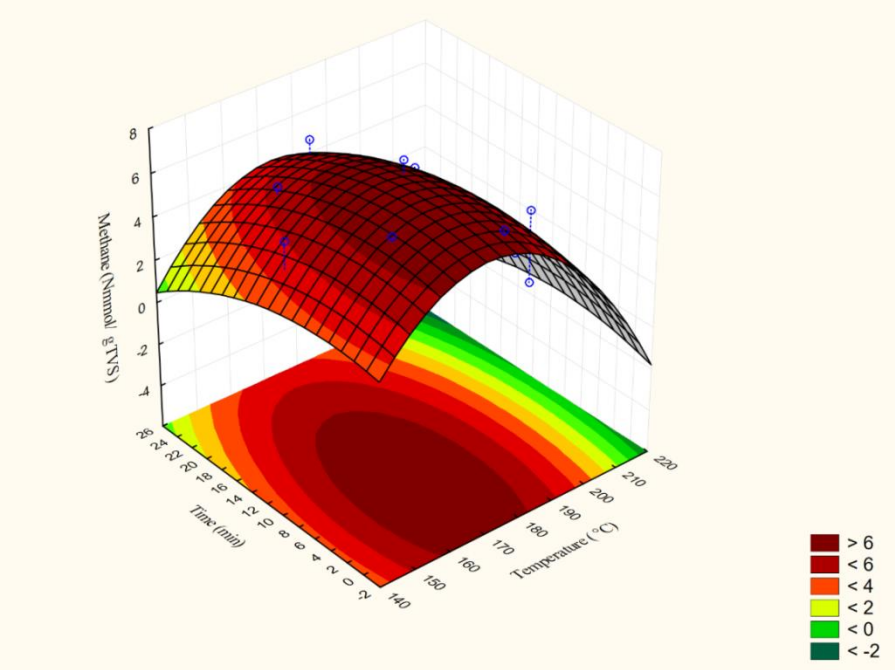

Figure 5.28 - Interaction between temperature (°C) and time (min) variable on Methane (Nmmol g^{-1} TVS) production

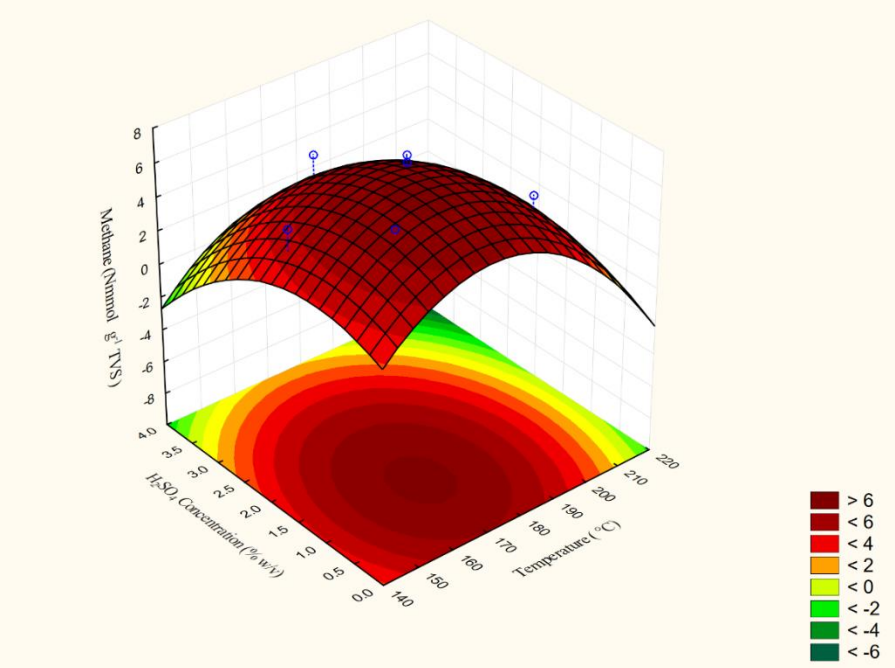

Figure 5.29 - Interaction between temperature (°C) and H_2SO_4 (%w/v) variable on Methane (Nmmol g^{-1} TVS) production

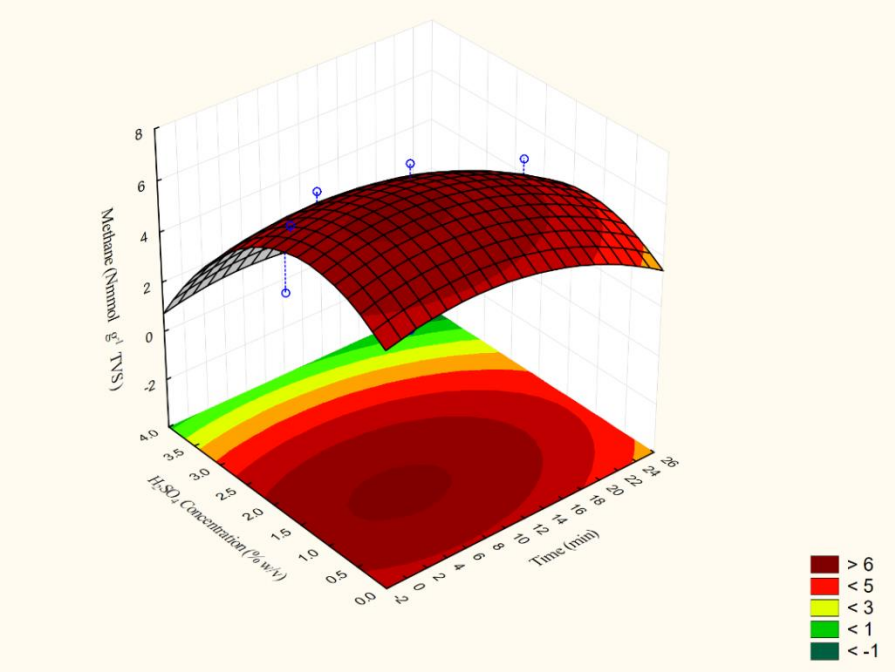

Figure 5.30 - Interaction between Time (min) and H_2SO_4 (%w/v) variable on Methane (Nmmol g^{-1} TVS) production

5.11 Microbial community Structure of H_2SO_4 impregnated sugarcane bagasse

5.11.1 Denaturing gradient gel electrophoresis for H_2SO_4 impregnated anaerobic reactors

Dendrogram for the bacterial community of anaerobic reactors supplemented with H_2SO_4 assisted hydrothermally pretreated sugarcane bagasse is depicted in Fig 5.31. The band pattern showed notable variability in bacterial community structure among anaerobic reactors. Similarity coefficient varied from 16% (K-HSO – L-HSO) to 88% between M-HSO and N-HSO. A decrease in pretreatment time (19 – 5 min) with similar H_2SO_4 conditions resulted in a decrease in *Sc* from 69% (A-HSO – B-HSO) to 38% (C-HSO – D-HSO) at 200 °C. Similar trend was observed for 160 °C under similar condition where *Sc* decreased from 79% (E-HSO – F-HSO) to 61% (G-HSO – H-HSO). It also indicated that a decrease in temperature from 200 °C to 160 °C resulted in increase in bacterial similarity i.e., from 69% (A-HSO – B-HSO) to 79% (E-HSO –F-HSO)

An increase in similarity coefficient from 38% (A-HSO – C-HSO) to 77% (B-HSO – D-HSO) was noted with a decrease in H_2SO_4 concentration (3 – 1 %w/v) with constant pretreatment time at pretreatment temperature of 200 °C. While a decrease in pretreatment temperature to 160 °C resulted in a decrease in *Sc* value from 79% (E-HSO – G-HSO) to 61% (F-HSO – H-HSO) at similar pretreatment time conditions. It can be seen when comparing H_2SO_4 concentrations a

decrease in temperature (200 -160 °C) resulted in an increase in bacterial similarity (38% - 79%) at 3% H_2SO_4, while a decrease in *Sc* value (77 – 61%) was observed for 1% H_2SO_4.

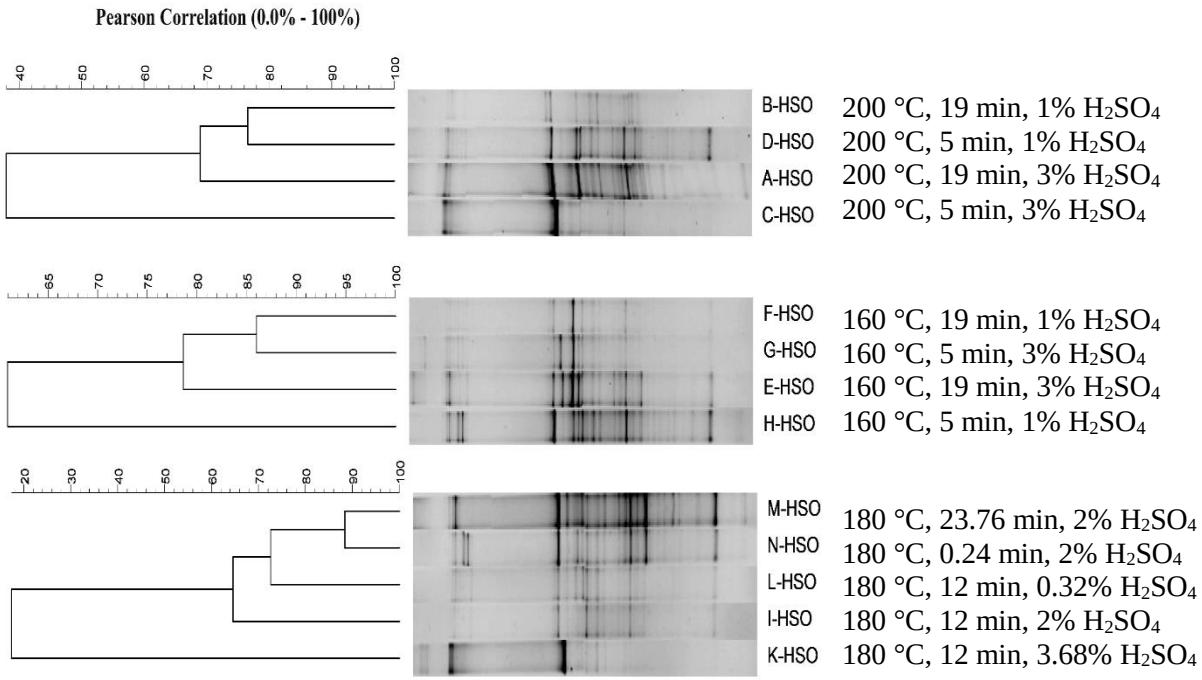

Figure 5.31 - PCR-DGGE band profile for bacterial community structure for H_2SO_4 impregnated anaerobic reactors

It is interesting to note that lowest and maximum similarity between reactors was observed at 180 °C. It was noted at similar pretreatment time of 12 min a decrease in H_2SO_4 conc. from 2% to 0.32% and an increase in H_2SO_4 conc. from 2% to 3.68% had no impact on bacterial composition as evident from 16% *Sc* value between I-HSO – L-HSO and I-HSO – K-HSO, respectively. Similarly, at similar H_2SO_4 conc. increase or decrease in pretreatment time did not affect the bacterial community as represented by *Sc* value of 65% for I-HSO – M-HSO and I-HSO – N-HSO. However, it was noted that change in pretreatment time resulted in higher bacterial similarity value (65%) than a change in H_2SO_4 conc. (16%).

Archaeal PCR-DGGE profile for anaerobic reactors supplemented with H_2SO_4 impregnated sugarcane bagasse is shown in Fig 5.32. A critical look of DGGE gel indicated that archaeal community is not much affected by the pretreatment parameters. *Sc* value for archaeal community varied from 35% to 93% as indicated in Fig 5.32. It is indicated that a decrease in

pretreatment temperature (19 -5 min) resulted in a decrease in *Sc* value from 93% (A-HSO – B-HSO) to 83% (C-HSO – D-HSO) at similar H_2SO_4 conc. values under pretreatment temperature of 200 °C. Keeping H_2SO_4 and pretreatment time values constant, a decrease in pretreatment temperature resulted in a slight decrease in *Sc* value to 90% for E-HSO – F-HSO at 19 min while a slight increase (92% *Sc* value) was observed for G-HSO – H-HSO at pretreatment time of 5 min. It is shown that a decrease of 40 °C from 200 °C to 160 °C resulted in a minimal decrease in archaeal similarity value at 19 min while the minimal increase was observed at 5 min pretreatment time.

A notable increase in the archaeal *Sc* value was observed from 56% to 83% when H_2SO_4 conc. value decreased (3 – 1%) at pretreatment temperature of 200 °C. Conversely, at 160 °C keeping pretreatment time and H_2SO_4 values same as at 200 °C, the archaeal similarity between reactors was unaffected being 72% for E-HSO – G-HSO and F-HSO – H-HSO. A comparison of *Sc* value at 200 °C and 160 °C for similar pretreatment times indicated approx. negligible decrease in *Sc* values at 19 min (93 % – 90 %) and increase in *Sc* value from 83 to 92% at 5 min. A similar comparison for H_2SO_4 conc. values indicated a decrease in temperature resulted in an increase in archaeal similarity from 56 to 72% for 3% H_2SO_4 while a decrease in archaeal *Sc* value from 83 to 72% for 1% H_2SO_4 conc.

The lowest *Sc* values were noted between three pretreatment sets I-HSO – K-HSO, I-HSO – M-HSO, and I-HSO – N-HSO where I-HSO, M-HSO, N-HSO were pretreated at 2% H_2SO_4 and pretreatment time of 12, 23.7, and 0.24 min, respectively. While, K-HSO was pretreated for 12 min with 3.68 (%w/v) H_2SO_4. The lowest *Sc* value was satisfied owing to significant different pretreatment conditions. The high *Sc* value of 88% between K-HSO and L-HSO at 180 °C indicated that pretreatment time could be a possible reason for such a high archaeal similarity between two pretreatments as both were pretreated at significant different H_2SO_4 conc. but at similar pretreatment time of 12 min.

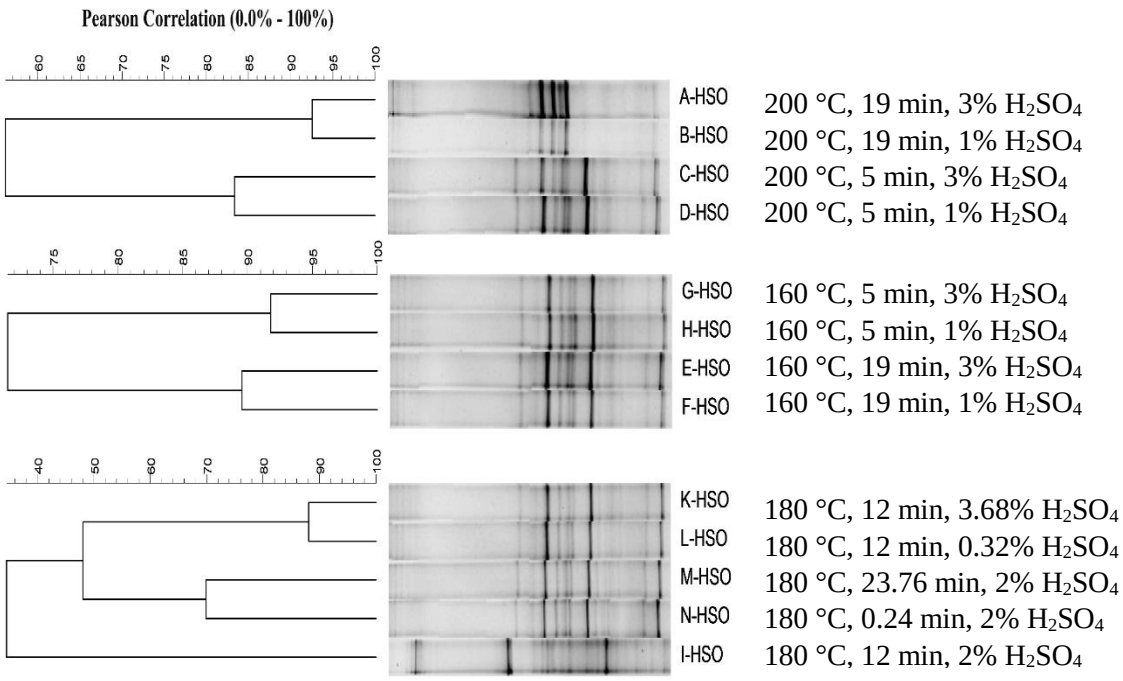

Figure 5.32 - PCR-DGGE band profile for archaeal community of H_2SO_4 impregnated anaerobic reactors

Shannon diversity (H′) and dominance (D) for bacterial and archaea communities are presented in Table 5.18. Approximately negligible effect of pretreatment parameters was observed on Shannon diversity (H′) and dominance (D). H′ value ranged from 2.59 to 3.25 for pretreatment temperature of 200 °C for Bacteria. Approx. similar values 3.12 and 3.25 were noted for A-HSO and D-HSO, respectively though both were pretreated at distinctly different pretreatment conditions. It indicated that change in pretreatment conditions did not have a significant effect on bacterial diversity. A similar trend was observed for bacterial Shannon diversity at 160 °C. Approx. values of 3.17 and 3.23 were recorded for E-HSO (19 min, 3% H_2SO_4) and H-HSO (5 min, 1% H_2SO_4), respectively.

On the other hand, a distinct pattern was observed for archaeal diversity for pretreatment time; decrease in pretreatment time resulted into increase in Shannon diversity. In other words, at similar pretreatment time, change in H_2SO_4 conc. showed no impact on archaeal diversity at 200 °C.

Table 5.18 – Microbial diversity (Shannon-Wiener and Dominance) for Bacteria and Archaea of H_2SO_4 assisted anaerobic reactors

Designation	Temperature	Time	H_2SO_4	Bacteria		Archaea	
	(°C)	(min)	(% w/v)	Dominance	Shannon-Wiener	Dominance	Shannon-Wiener
A-HSO	200	19	3	0.05	3.12	0.13	2.14
B-HSO	200	19	1	0.07	2.66	0.14	2.09
C-HSO	200	5	3	0.08	2.59	0.09	2.51
D-HSO	200	5	1	0.04	3.25	0.10	2.43
E-HSO	160	19	3	0.05	3.17	0.09	2.50
F-HSO	160	19	1	0.07	2.80	0.10	2.49
G-HSO	160	5	3	0.07	2.80	0.10	2.49
H-HSO	160	5	1	0.04	3.23	0.09	2.58
I-HSO	180	12	2	0.05	2.92	0.06	2.87
K-HSO	180	12	3,68	0.08	2.58	0.10	2.48
L-HSO	180	12	0.24	0.06	2.87	0.10	2.49
M-HSO	180	23.76	2	0.04	3.23	0.09	2.51
N-HSO	180	0.24	2	0.05	3.03	0.11	2.39

5.12 Illumina MiSeq Sequencing of selected H_2SO_4 pretreatments

5.12.1 Bacterial diversity for H_2SO_4 pretreated samples

Microbial community structure for H_2SO_4 pretreated samples was conducted for 3 selected pretreatments D-HSO, I-HSO, and N-HSO to have an idea how pretreatment parameters impacted the microbial diversity. RDP-classifier after Illumina MiSeq sequencing resulted into 220569, 189756, 206848, reads for D-HSO, I-HSO, and N-HSO, respectively. Phylogenetic estimation at 3% distance level resulted into 2700 and 2667 OTUs for Bacteria and Archaea domains, respectively. Bacterial diversity was characterized into 23 phyla; however, only *Bacteroidetes*, (16.82-34.16%), *Firmicutes* (18.40-19.83%), *Chloroflexi* (13.68-19.71%), *Proteobacteria* (11.92-19.31%), *Cloacimonates* (5.25-6.52%), *Thermotogae* (6.24-9.17%), and *Spirochaete* (2.80-5.53%) were abundant. Furthermore, phyla with relative abundance less than 1% were *Actinobacteria, Planctomycetes, Caldiserica, Chlamydiae, Fusobacteria, Cyanobacteria, Nitrospinae, Chlorbi,* and *Nitrospirae. Euryarchaeota* (98.05-99.13%) was the only predominant archaeal phylum.

As depicted in Fig 5.33 bacterial community was not as diverse as the relative abundance that might be due to pretreatment conditions. Bacterial diversity at genus-level indicated that it was rich in lignin degrading (aromatic compound degraders) and sulfate-reducing phylotypes with lower abundance of polysaccharide degrading microbial communities. *Bacteroidetes* was the dominant phylum with 34.16% in pretreatment N-HSO where its abundance was almost half in D-HSO (16.82%) and I-HSO (18.20%). Three of the four recognized classes in the tested sample were *Bacteroidia* (9.04-14.80%), *Flavobacteria* (0.31-22.83%), and *Cytophagia* (1.54-2.22%). *Bacteroides* and *Anaerophaga* were the predominant genera in class *Bacteroidia* while *Cytophaga* and *Flavobacterium* were dominant of class *Cytophagia* and *Flavobacteria,* respectively. *Bacteroides* abundance was approx. similar in D-HSO (10.64%) and I-HSO (11.54%), while it was lower in N-HSO (7.14%). *Bacteroides* are known to metabolize cellobiose and xylose (Pobeheim *et al.*, 2010). *Bacteroides* is one of the bacterial populations involved in hydrolysis step of digestion process (Anderson *et al.*, 2003).

Anaerophaga was in lower abundance in D-HSO and I-HSO (2.56-2.83%) while was not identified in N-HSO (0.85%). *Anaerophaga* are chemoorganotrophic, nonphotosynthetic strict rod-shaped Gram-negative anaerobes bearing a fermentative type of metabolism, using organic compounds as substrates. *Anaerophaga* members are unable to utilize inorganic electron acceptors (Schink, 2015). Denger et al (Denger *et al.*, 2002) reported that some strains of *Anaerophaga* ferment hexoses and pentoses to equal molar amounts of acetate, propionate, and succinate. Lower abundance of *Anaerophaga* in current study indicated lower fermentative activity. Furthermore, complete removal of *Anaerophaga* in N-HSO might be justified by the competition among bacterial strains for metabolites.

Members of *Cytophagia* are Gram-negative, microaerobic to strict anaerobic, chemoorganotrophs with the capability to metabolize proteins, lipids, starch, and cellulose (Nakagawa, 2015). Lower abundance (1.53-2.12%) of *Cytophaga* members indicated inaccessibility of cellulose due to higher lignin sheath that has resulted owing to pretreatment. Pandit et al (Pandit *et al.*, 2016) reported *Cytophaga* as cellulose and hemicellulose degraders.

Flavobacterium (Family *Flavobacteriaceae*) astonishingly identified with maximum abundance (22.82%) in N-HSO, while in other studied pretreatments it was (0.29 – 0.44%). Koga et al. (Koga *et al.*, 1999) reported *Flavobacterium meningosepticum* to grow on phenolic

compounds. Some *Flavobacterium* spp. can degrade carboxymethylcellulose (Bernardet and Bowman, 2015).

Desulfosporosinus (8.85-11.20%) and *Clostridum* (2.32-7.28%) belonging to Class *Clostridia* of *Firmcutes* were abundant in the current study. *Desulfosporosinus* is a member of sulfate-reducing bacteria; the majority of them belong to δ–proteobacteria (Moestedt, 2015). *Desulfosporosinus* members are Gram-positive strict anaerobes with three documented species (Hippe and Stackebrandt, 2015). Syntrophic relation of *Desulfosporosinus* with methanogens has not been validated (Kato *et al.*, 2015). Higher abundance of *Desulfosporosinus* could be justified with the presence of higher lignin content (aromatic compounds) in the pretreated solids. *Desulfosporosinus* members are frequently recovered in microbial consortia aimed to degrade aromatic compounds (Fowler *et al.*, 2014; Kuppardt *et al.*, 2014). *Clostridium* population was in the range of 2.32 to 7.23%, the lower population could be related to lower hydrolytic activity.

Geotoga (Family *Thermotogaceae*), a newly defined genus, was the only genus encountered in the current study with relative abundance in the range of 6.13 – 9.09%. *Geotoga* are Gram-negative, fermentative, strict anaerobes. They are moderate thermophilic i.e., grow in a range of 30 to 60 °C (Davey *et al.*, 2015). *Geotoga* could metabolize mannose, glucose, galactose, maltose, sucrose as carbon and energy source, but could not grow on xylose. Furthermore, *Geotoga* members are unable to utilize ethanol, lactate, acetate, and formate (Davey *et al.*, 2015).

Chloroflexi categorized into two classes *Anaerolinea* and *Dehalococcoidia* were observed in H_2SO_4 pretreated samples. Genera reported in this study under *Anaerolinea* were *Bellilinea* (5.10-7.73%), *Levilinea* (1.60-4.03%), and *Longilinea* (1.75-2.02%) while *Dehalococcoides* (3.35-4.89%) was an only abundant genus in class *Dehalococcoidia. Bellilinea, Levilinea,* and *Longinilea* members are Gram-negative, nonsporulating, strict anaerobes. *Bellilinea* is newly classified genus that is reported to grow under thermophilic conditions. The type strain *Bellilinea caldifistuale* sp. nov. is reported to grow optimally at 55 °C (Yamada *et al.*, 2007). In contrast, relatively higher abundance was observed under mesophilic condition (37 °C). Furthermore, its growth is supported with hydrogenotrophic methanogens. *Levilinea* spp. was identified in this study even without supplementation of yeast extract. Yeast extract is reported to be a primary growth factor for *Levilinea* spp. (Yamada, 2006). It exhibits optimum growth at 37 °C at pH 7. Yamada et al (Yamada *et al.*, 2007) reported that *Levilinea* members could not utilize sulfate,

sulfite, thiosulfate and elemental sulfur as electron acceptors. Since these are the newly classified genera literature is unable to furnish much information for discussion and comparison with other studies.

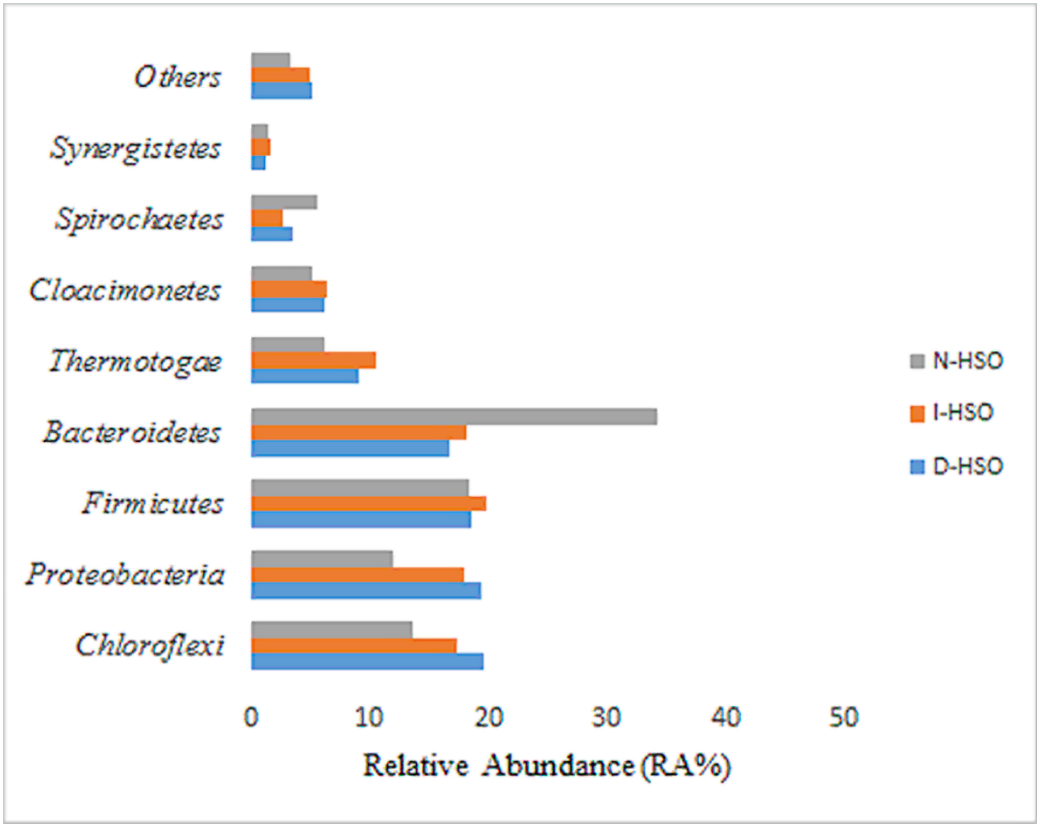

Figure 5.33 - Bacterial community classification at Phylum-level. Relative abundance less than 1% of the samples was accumulated and presented as 'Others'

Dehalococcoides were found in relatively lower abundance (3.35 – 4.88%) might be due to the presence of higher lignin. *Dehalococcoides* are slow-growing, strictly anaerobic, disk-shaped bacteria. 16S rRNA phylogeny revealed their cell-wall resemblance to archaea than to Gram-positive or Gram-negative (Koukkou, 2011). Hydrothermal pretreatment resulted in phase transition of lignin; solid to liquid to glass transition phase. It could happen over a range of temperature 80 – 193 °C (Irvine, 1985; Trajano *et al.*, 2013). Lignin is composed of methoxylated derivatives of benzene called monolignols (coniferyl, sinapyl, and coumaryl alcohols) (Moore *et al.*, 2011). Lignin depolymerization and recondensation during hydrothermal pretreatment are reported by Trajano et al (Trajano *et al.*, 2013). *Dehalococcoides* identified in the three selected samples could be attributed to degrade benzene monomers (Kublik *et al.*, 2016).

Proteobacteria is a diverse phylum harboring bacteria of varied physiological functions and metabolic pathways. The genera identified in this study belonging to *Proteobacteria* were sulfate-reducers and aromatic compound degraders. Bugg et al (Bugg *et al.*, 2011) reported that potential lignin-degrading bacteria are mostly derived from guts of wood-eating insects and include *Alphaproteobacteria*, *Gammaproteobacteria* and *Actinomycetes*. *Syntrophus* (2.52-3.78%), *Syntrophorhabdus* (0.97-1.30%) and *Geobacter* (1.06-1.85%) root to class *Deltaproteobacteria* while *Pseudomonas* (0.49-2.83%) and *Sulfurovum* (0.96-1.53%) belong to class *Gammaproteobacteria* and *Epsilonproteobacteria.* Despite belonging to the same class, each genus root to a different family.

Syntrophus (Family *Syntrophaceae*) were identified in lower abundance with approx. similar abundance (2.52 - 3.78%) in tested samples. Members are strictly anaerobic, having the fermentative type of metabolism and grow only in the presence of H_2/formate-utilizing partners in syntrophic associations. Substrates are oxidized either incompletely to acetate or completely to carbon dioxide. *Syntrophorhabdus* are Gram-negative, nonsporulating, nonmotile strict anaerobe having an obligate syntrophic metabolism. It is an aromatic compound degrader that exhibits obligate syntrophic relation with H_2 scavenging methanogen (Qiu *et al.*, 2008). Lower abundance (0.97-1.30%) of *Syntrophorhabdus* in the tested samples indicated unavailability of H_2 for their growth. *Syntrophorhabdus* members are previously reported in sulfate-reducing co-cultures from sewage sludge treatment plants (Qiu *et al.*, 2004).

Pseudomonas spp. recently reported to produce novel cellulolytic enzymes consisting of an endoglucanase, exoglucanase, β-glucosidase and xylanase that could effectively hydrolyze alkaline-pretreated bagasse, achieving over 95% monosaccharide yield (Cheng and Chang, 2011), *Pseudomonas* identified in the current study for H_2SO_4 pretreated samples are in the range of 0.85-2.83%. *Pseudomonas* spp. reported to degrade cellulose and hemicellulose (Zhong *et al.*, 2011). Tian et al (Tian *et al.*, 2014) reported that *Pseudomonas* could utilize biphenyl components of lignin, which are nearly 10% constituents of total lignin depending upon the source of lignin. Some strains of *Pseudomonas* reported exhibiting Beta-ketoadepate pathway, which is significant for lignin degradation (Bugg *et al.*, 2011). *P. fluorescens*was reported producing extracellular lignin peroxidase for lignin degradation (Kong *et al.*, 2010). The difference in *Pseudomonas* abundance

(0.49-2.83%) in the tested samples could be related to lignin degradation and release of phenolic monomers depending upon pretreatment temperature employed.

Geobacter another *Deltaproteobacteria* member with similar abundance (1.06-1.85%) was identified in tested samples. Members are non-fermenting, chemoorganotrophic mesophilic anaerobes with the capability to oxidize acetate, C_1-C_5 organic acids and C_2-C_4 alcohols. Species of this genus are reported to perform versatile physiological functions. Rotaru et al (Rotaru *et al.*, 2014) reported that *Methanosaeta* spp. directly accepts an electron from *Geobacter* spp. to reduce CO_2 into methane via direct electron transfer process instead of H_2 inter-specie transfer. In another study Childers et al (Childers *et al.*, 2002) reported aromatic compounds degradation in *Geobacter metallireducens* with Fe^{3+} as electron acceptor under anaerobic conditions suggesting the existence of specific enzymes. Some species are also reported to oxidize monoaromatic hydrocarbons like benzene and phenol (Roling, 2014).

Sulfurovum (Class *Epsilonproteobacteria*) were identified in lower abundance (0.95-1.53%). This genus was first described in 2004 after its discovery from hydrothermal vents. *Sulfurovum* members are Gram-negative, non-motile, coccoid-shaped, mesophilic, chemolithoautotrophic facultative anaerobes utilizing elemental sulfur or thiosulfate as an electron donor (Inagaki *et al.*, 2004). Organic acids, alcohols, sugars and hydrogen do not support the growth of *Sulfurovum lithotrophicum.*

Treponema (Class *Spirochaetia*) abundance was in the range of 1.06 to 3.92% in tested samples. *Treponema* species have been shown to interact with cellulolytic bacteria and increase the degradation rate of cellulose. Bacteria from this genus likely are able to metabolize exopolysaccharides produced by the primary cellulolytic bacteria, functioning as fermenters (Warnecke *et al.*, 2007).

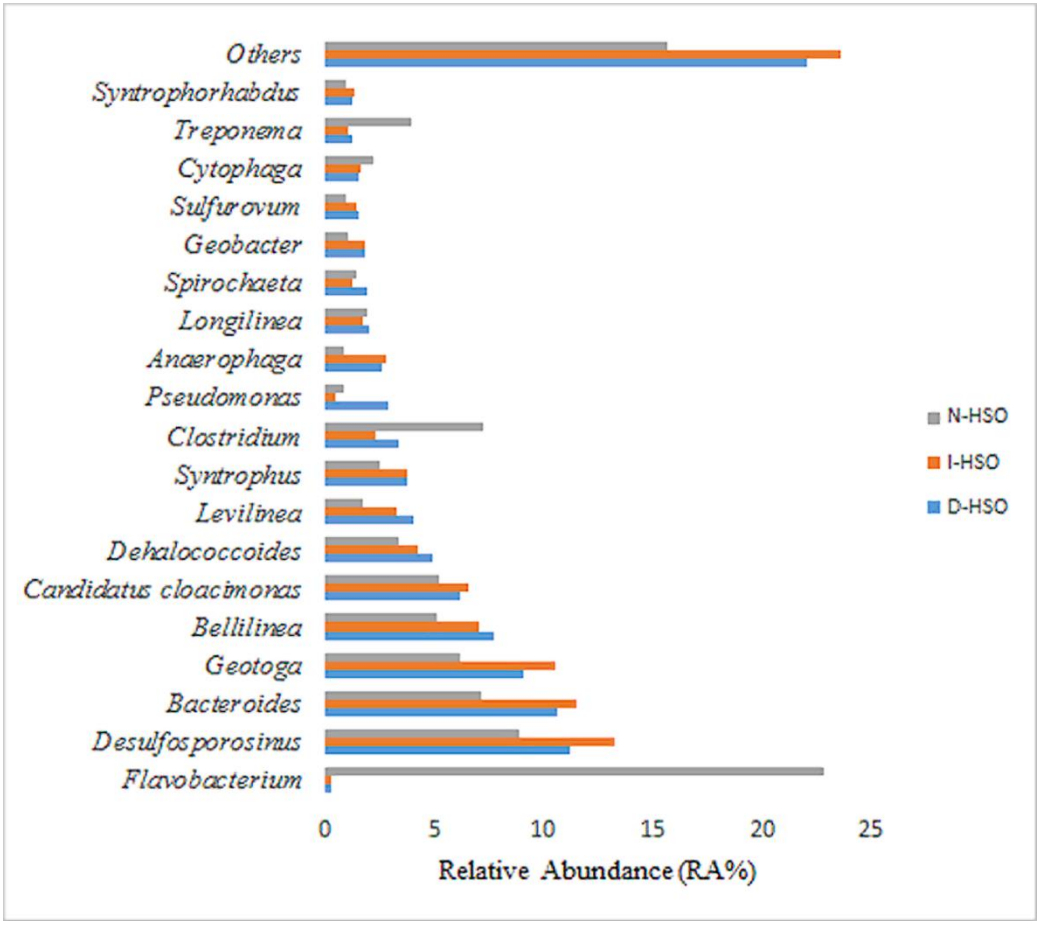

Figure 5.34 Bacterial community classification for H_2SO_4 pretreated samples at Genus-level. Relative abundance less than 1% of the samples was accumulated and presented as 'Others'

5.12.2 Archaeal diversity in selected H_2SO_4 pretreated samples

Archaea belonging to Phylum *Eurarychaeota* were predominant in sequences with relative abundance in the range of 98.05 to 99.22% of the tested samples. While, *Crenarchaeota* abundance was between 0.86 to 1.94%. Class *Methanomicrobia* was the most dominant (71.43 – 83.22%) followed by *Methanobacteria* (13.40 – 25.28%) and *Thermoplasmata* (1.29 – 2.43%) (Fig. 5.35).

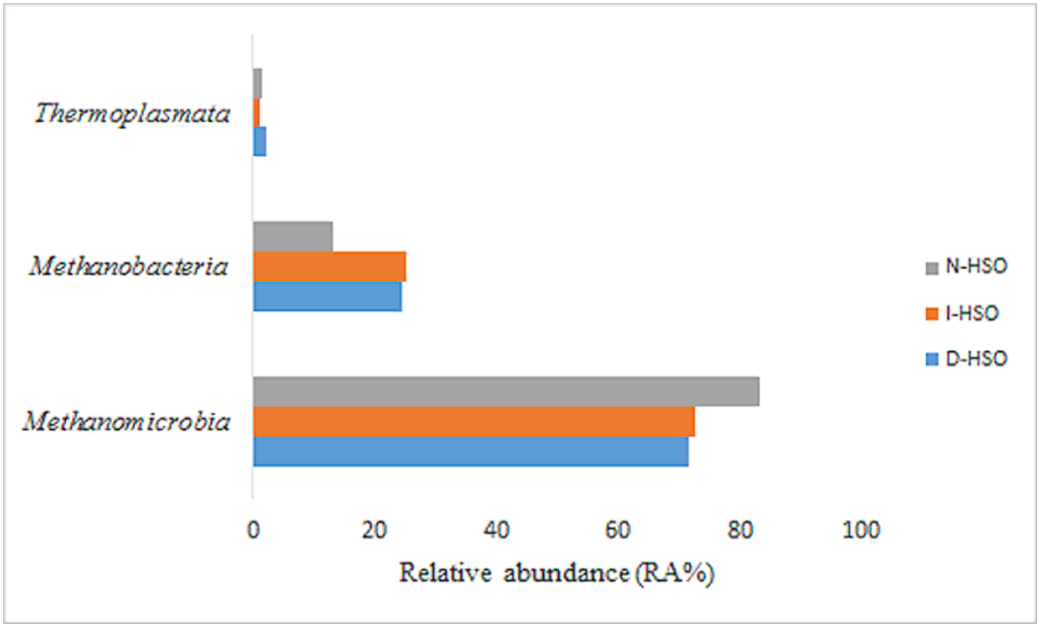

Figure 5.35 - Archaeal community classification for selected H_2SO_4 pretreated samples at Class-level.

Representatives belonging to Family *Methanoregulaceae* (57.3 – 68.3%), *Methanobacteriaceae* (13.4 – 25.3%) and *Methanosaetaceae* (11.7 – 14.4%) were the most prevalent in pretreatments D-HSO, I-HSO, and N-HSO (Fig 5.36). The family *Methanoregulaceae* currently consists of three genera: *Methanoregula*, *Methanolinea*, and *Methanosphaerula*. The family is affiliated with the order *Methanomicrobiales*, which were identified in D-HSO, I-HSO, and N-HSO reactors at 59.4%, 58% and 68.9%, respectively. The members are slow-growing, rod-shaped or coccoid, nonmotile methanogens that can use H_2/CO_2 and sometimes formate as substrates for methanogenesis. The members are neutrophilic or slightly acidophilic mesophiles. Some species of *Methanoregulaceae* were isolated in diverse habitats including reactors for the anaerobic treatment of organic waste, and oil fields (Garcia *et al.*, 2006) The most abundant genus of *Methanoregulaceae* family, in current study using sulfuric acid, was *Methanolineae* that grouped 51,9%, 51,2% and 63,1% in reactors D-HSO, I-HSO, and N-HSO, respectively. *Methanolineae* is a methanogen that uses H_2/CO_2 and requires acetate for growth (Imachi *et al.*, 2008; Sakai *et al.*, 2012)

Representatives belonging to Family *Methanobacteriaceae* comprehend morphologically diverse microorganism with cell shape varying from cocci or short rods to long filamentous rods. All species are strict anaerobes, and most members of the family obtain energy for growth from

the reduction of CO_2 with H_2. Members of the family are widely distributed in anaerobic environments including aquatic sediments, sewage treatment systems, gastrointestinal tracts of animals, and in geothermal areas (Bonnin and Boone, 2006; Tabatabaei *et al.*, 2010). In acid pretreatment, the representative genus of *Methanobacteriaceae* was *Methanobacterium* (13.8 – 24.7%). Some species of *Methanobacterium* use formate, secondary alcohols, and CO, CO_2 reduction coupled to H_2 oxidation is the major growth mechanism (Boone, 2015).

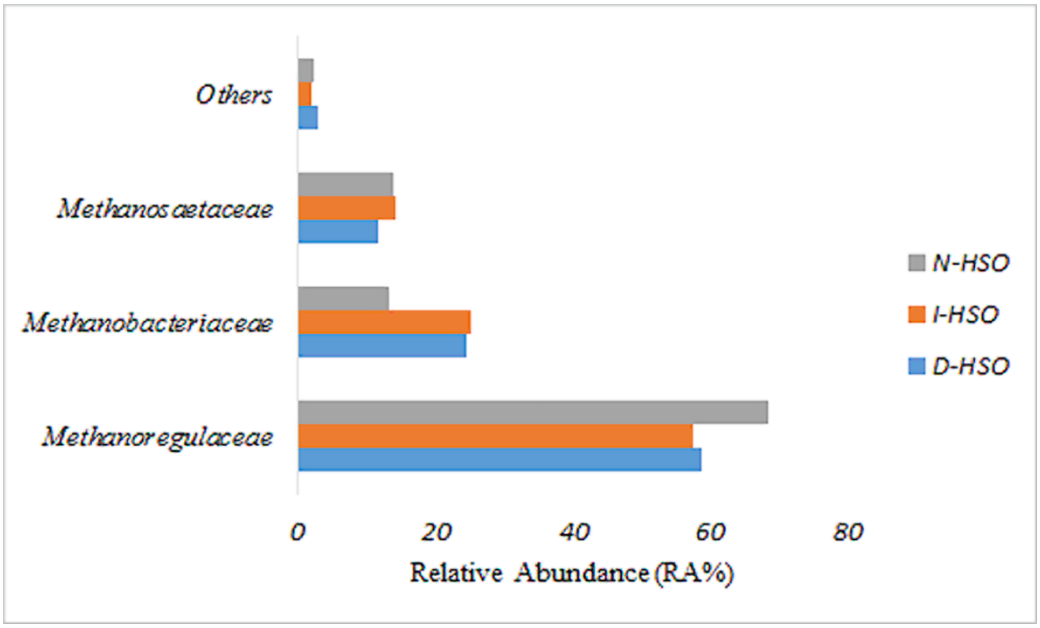

Figure 5.36 - Archaeal community classification for H_2SO_4 pretreated samples at Family-level. Relative abundance less than 1% of the samples was accumulated and presented as 'Others'

The most abundant genera identified, in reactors under acid pretreatment, were *Methanolinea* (51.15 – 63.06%), *Methanobacterium* (13.08 – 24.67%), *Methanosaeta* (11.77 – 13.99%), and *Methanoregula* (5.22 – 6.79%), while, *Thermogymnomonas, Candidatus nitrosocaldus* and *Methanomassiliicoccus* grouped less than 3% (Fig 5.37)

Methanoregula represented 6.79% (D-HSO), 6.11% (I-HSO) and 5.22% (N-HSO), respectively, of total sequences. Some species of this genus have been isolated from anaerobic reactors (Brauer *et al.*, 2011; Yashiro *et al.*, 2011). The microorganism can use H_2/CO_2 and formate in methane production, growth occurs at pH 7.0 – 7.6 and temperature range between 10 – 40 ºC.

Methanomassiliicoccus members are regular or coccoid shaped Gram-stain positive obligate mesophilic anaerobes and slightly alkaliphilic. *Methanomassiliicoccus luminyensis*

produces methane by methanol reduction with H_2 as an electron donor. It is unable to produce methane with methanol when H_2 or methanol is the only energy source. Specie cannot produce methane from a formate, acetate, trimethylamine, 2-butanol, 2-propanol, cyclopentanol, 2-pentanol, and ethanol (Dridi *et al.*, 2012). *Candidatus nitrosocaldus* is an autotrophic crenarchaeota and not relevant to methane production; the major metabolism of this genus is ammonia oxidation (De La Torre *et al.*, 2008).

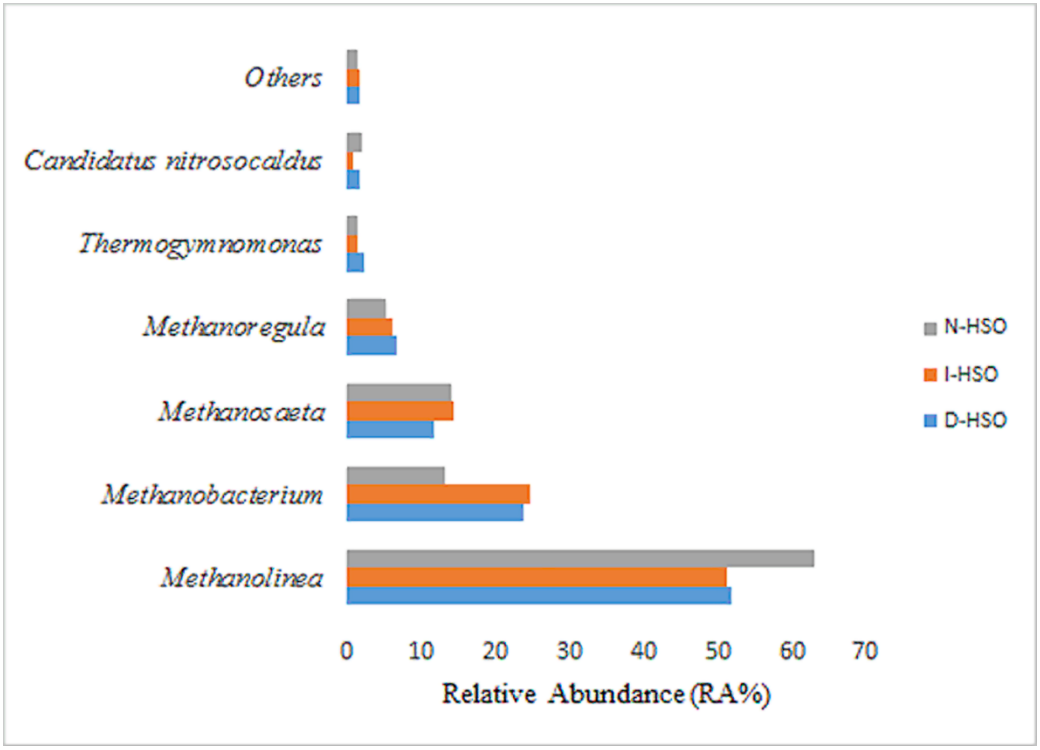

Figure 5.37 - Archaeal community classification for H_2SO_4 pretreated samples at Genus-level. Relative abundance less than 1% of the samples was accumulated and presented as 'Others'

5.13 Microbial community structure difference between positive control and pretreated samples

The microbial community of positive control (glucose) was also determined at the end of the digestion by Illumina MiSeq sequencing. RDP-classifier resulted into 165901 reads that were characterized into 2700 OTUs. Bacterial and archaeal diversity for the positive control (glucose) indicated a distinct difference in comparison to the microbial community of H_2O_2 and H_2SO_4 pretreated samples.

Firmicutes (66.0%) and *Proteobacteria* (23.29%) were the dominant bacterial phyla along with a lower abundance of *Bacteroidetes* (3.34%) and *Chloroflexi* (2.04%) in the positive control.

Clostridia (63.12%) and *Gammaproteobacteria* (19.15%) were identified as the dominant classes in the positive control whereas *Chloroflexi, Firmicutes, Bacteroiedetes, Thermotogae, Cloacimonetes, Spirochaetes* were the dominant classes in H_2O_2 and H_2SO_4 samples. The Most abundant bacterial genera (Fig 5.38 *A*) identified in the positive control were *Clostridium* (55.68%), *Aeromonas* (13.37%), *Tolumonas* (5.48%), and *Bacteroides* (2.60%), whereas *Methanobacteirum* (95.90%) was the sole abundant archaeal genus (Fig 5.38 *B*).

Since *Clostridia* bears saccharolytic, proteolytic or both metabolic pathways. *Clostridium* encountered in the positive control could be of saccharolytic nature because glucose was the only substrate provided. *Clostridia* are also efficient hydrogen producers. Kim et al. (Kim *et al.*, 2014) reported *Clostridial* proliferation in their study suggesting excessive H_2 production. *Clostridial* community (55.68%) in positive control supports higher *Methanobacterium* abundance (95.90%), a hydrogenotrophic bacterium, suggesting that hydrogenotrophic methanogenesis might be the sole mechanism for methane production in the positive control.

Aeromonas and *Tolumonas* of family *Aeromonadaceae* were abundant genera in the positive control next to *Clostridium.* However, these genera were not identified in H_2O_2 pretreated (B-HO, K-HO, and P-HO) and H_2SO_4 pretreated (D-HSO, I-HSO, and N-HSO) samples. *Aeromonas* are Gram-negative, chemoorganotrophic, facultative anaerobes exhibiting fermentative metabolism of *D*-glucose with optimum growth temperature in the range of 22 – 37 °C. *Aeromonas* exhibits versatile metabolic growth to utilize amino acids, carboxylic acids, and carbohydrates for organic acid production (Brenner *et al.*, 2004) that results in its rapid growth and absolute abundance during reactor startup (Kim, 2007). Easy availability of substrate (glucose) might be the reason of *Aeromonas* abundance in positive control as reported by (Kim, 2007) for its rapid growth during the digestion process.

Tolumonas is facultative anaerobic, Gram-negative *Gammaproteobacteria* only identified in the positive control (5.48%). It is reported to be isolated from wastewater and anoxic sediments of freshwater (Fischer-Romero *et al.*, 1996; Caldwell *et al.*, 2011). Ethanol, acetate, formate, and short chain organic acids are metabolic products when grown on glucose (Caldwell *et al.*, 2011). *Lactobacillus, Ethanoligenens,* and *Bacteroides* were also found in similar abundance in the positive control with the relative abundance of 2.53%, 2.46%, and 2.31%, respectively.

Lactobacillus and *Ethanoligenens* were not identified in pretreated samples, whereas *Bacteroides* was observed in less than 1% abundance in H_2O_2 and 7.14 -10.64% in H_2SO_4 pretreated samples. *Lactobacillus* (Family *Lactobacillaceae*) is Gram-positive, non-spore-forming bacteria with an optimum growth temperature of 30 – 40 °C. *Lactobacillus* generally possess complex nutritional requirement; vitamins, salts, fatty acid esters, and fermentable carbohydrates. Lactic acid is considered the sole product of carbohydrate metabolism by *Lactobacillus* (Walter, 2008).Oude et al. (Oude Elferink *et al.*, 2001) reported that *Lactobacillus buchneri* could convert lactic acid to acetic acid; a principal substrate for acetoclastic methanogenesis. The lower abundance of *Lactobacillus* further confirmed that hydrogenotrophic methanogenesis was favored in positive control.

Ethanoligenens (Family *Ruminococcaceae)* are Gram-positive, non-spore-forming, chemo-organotrophic, obligate anaerobes with optimum temperature and pH range, respectively, 20 – 44 °C, and 3.5 – 9.0. *Ethanoligenens* can ferment several mono-, di-, and oligosaccharides. Major fermentation products of glucose metabolism include ethanol, H_2, CO_2 and acetate (Xing *et al.*, 2006).

Methanobacterium was the only abundant genus in archaeal diversity (95.89%) where *Methanomassiliicoccus* and *Methanosaeta* were present in lower abundance 1.57% and 1.3%, respectively. It suggested that hydrogenotrophic methanogenesis was the only mechanism for methane production in positive control.

To sum up, a distinct difference in the microbial community structure was observed between positive control (Fig 5.38) and both pretreated samples; H_2O_2 (Fig 5.39) and H_2SO_4 (Fig 5.40) primarily because of difference in the substrate provided. Sugarcane bagasse is a heterogeneous feedstock so a number of bacterial strains belonging to different taxonomic groups needed to work together for the release of fermentable sugars to initiate the digestion process chain whereas it was not the case for pure glucose; readily available for the microbial

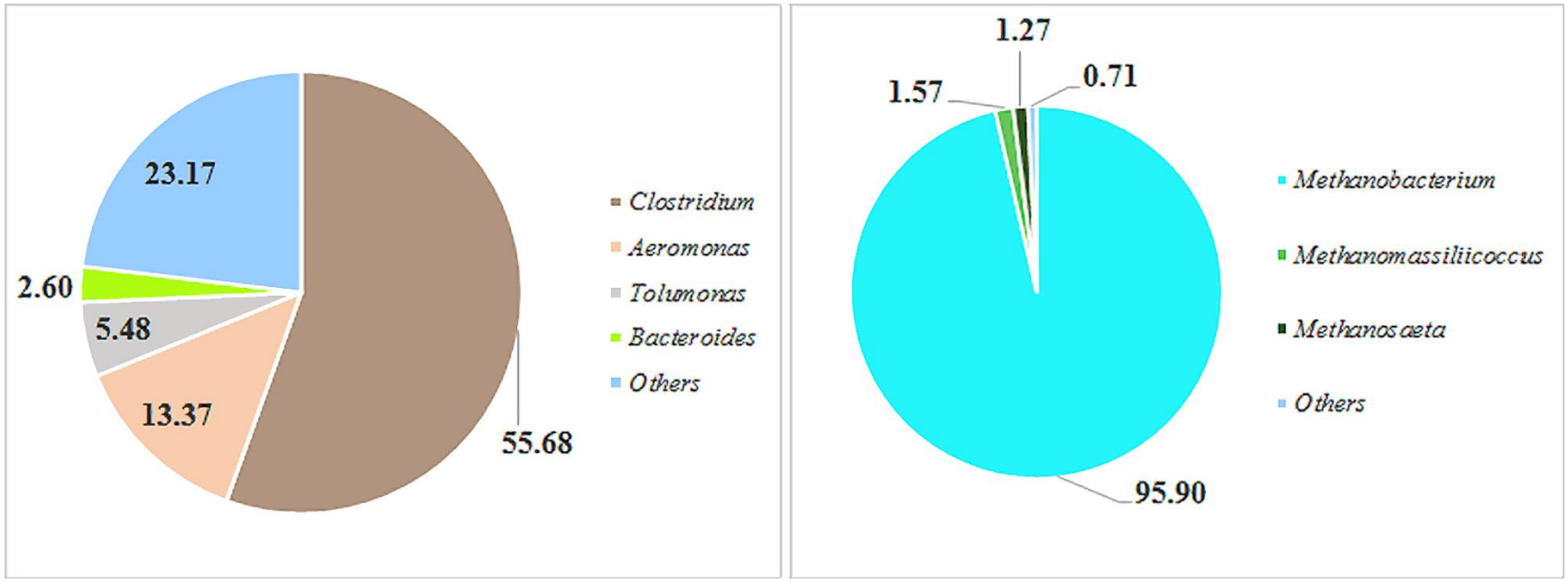

Figure 5.38 - Microbial community structure of positive control at Genus-level for bacteria (A) and archaea (B). All the taxonomic groups less than 1% are accumulated and represented as 'Others'

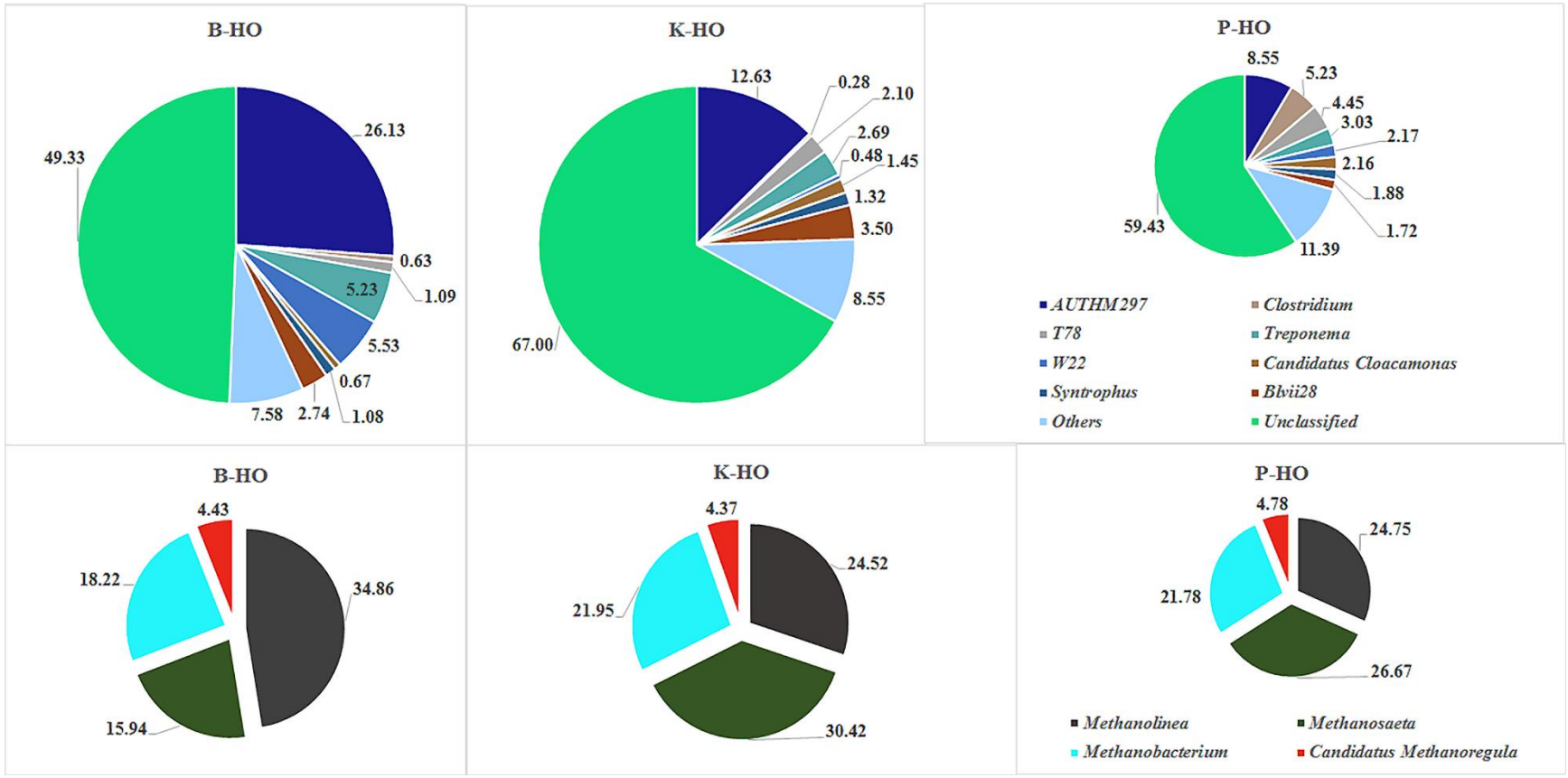

Figure 5.39 - Microbial community structure of bacteria (upper row) and archaea (lower row) at Genus-level for selected H_2O_2 treated batch digestion reactors. All the taxonomic groups less than 1% are accumulated and represented as 'Others'

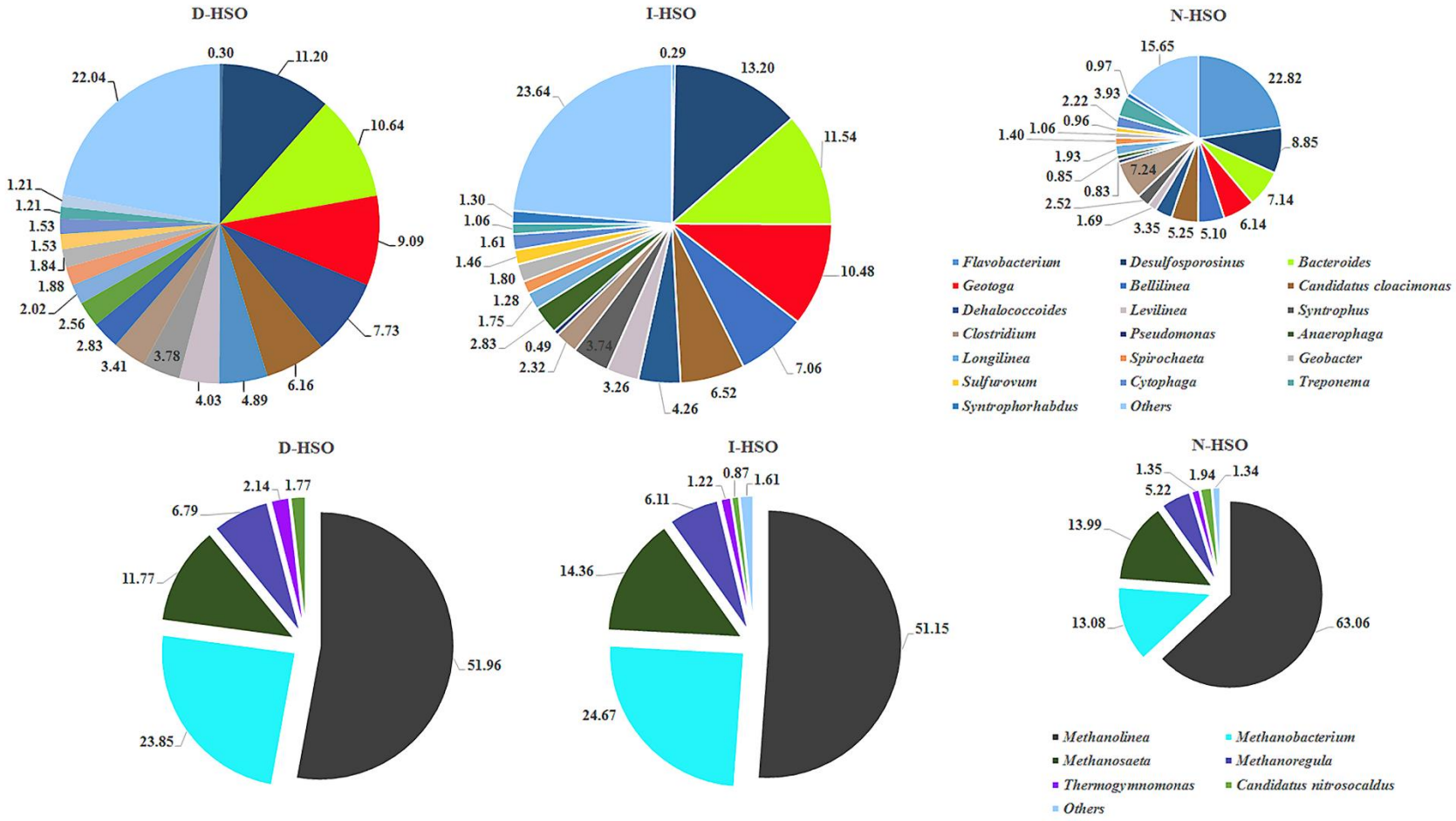

Figure 5.40 - Microbial community structure of bacteria (upper row) and archaea (lower row) at Genus-level for selected H_2SO_4 treated batch digestion reactors. All the taxonomic groups less than 1% are accumulated and represented as 'Others'

6 COMPARISON BETWEEN SULFURIC ACID AND HYDROGEN PEROXIDE ASSISTED HYDROTHERMAL PRETREATMENT

Methane production from lignocellulosic feedstocks appears a favorable renewable energy option to lower the world's dependence on fossil fuels while at the same time mitigating Food vs. Fuel dilemma. However, the recalcitrance of lignocellulosic material needs to be reduced to utilize this valuable resource at its optimum potential. Pretreatment step is a mandatory requirement to fractionate lignocellulosic material for downstream processing. The thesis focused on a novel pretreatment strategy applied to the sugarcane bagasse; sugarcane bagasse was impregnated with H_2O_2 and H_2SO_4 separately before hydrothermal pretreatment employing a central composite design of three factors; temperature (°C), time (min), and chemical compounds concentration. The notable results were obtained from the both pretreatments, which are contrasting to each other primarily due to the difference in chemical compound utilized for the impregnation of sugarcane bagasse. Chemical composition of solid and liquid fraction, morphological characteristics of solid fraction, impact on subsequent digestion experiments, and impact on microbial community structure will be briefly compared for the both pretreatments in this subsection.

A notable impact on the solid recovery after hydrothermal pretreatment was verified for both pretreatments. Solid yield tends to decrease with an increase in temperature and chemical compound (H_2O_2, H_2SO_4) utilized for sugarcane bagasse impregnation. H_2O_2 resulted in significant lignin solubilization while H_2SO_4 effected hemicellulose thereby leading to breakdown of inter-molecular bonding between polymeric sugars and lignin meshwork resulting in lower solid recovery. The lowest solid yield (12.27 %) was observed for H_2SO_4 assisted hydrothermal pretreatment (pretreatment run A-HSO – 200 °C, 19 min, 3% w/v H_2SO_4) in comparison to H_2O_2 assisted pretreatment where maximum solid yield (93.13 %) was noted for the lowest H_2O_2 employed (pretreatment run L-HO – 180 °C, 12 min, 0.64% v/v H_2O_2). Compositional analysis of solid fraction revealed significant lignin solubilization (73.09 %) for H_2O_2. On the other hand, maximal lignin recovery (358.85 %) was recorded for H_2SO_4 assisted hydrothermal pretreatment. A higher glucan content up to 75.21 g 100 g^{-1} TS was recorded in the H_2O_2 assisted hydrothermal pretreatment (pretreatment run O-HO – 213.6 °C, 12 min, 4 % v/v H_2O_2) primarily due to lignin solubilization and xylan removal to the liquid stream. Whilst, glucan degradation was observed in the H_2SO_4 assisted hydrothermal pretreatment at higher reaction temperature (200 °C), reaction time (19 min), and 3 % w/v H_2SO_4.

Xylan content was also decreased in H_2O_2 assisted pretreatment. The lowest xylan removal (12.11 %) was noted in pretreatment run H-HO (160 °C, 5 min, 2 % v/v H_2O_2). In contrast, H_2SO_4 assisted pretreatment indicated significant impact on xylan removal up to 100 % removal in a number of pretreatments (A-HSO, B-HSO, C-HSO and O-HSO) depending upon the joint impact of reaction temperature, reaction time and H_2SO_4 concentration.

Morphological characterization by scanning electron microscopy verified significant differences in structural characterization of sugarcane bagasse in response to both studied pretreatments besides raw sugarcane bagasse. Micrographs depicted compact and smooth surface for raw sugarcane bagasse. However, selected H_2O_2 assisted pretreatment (pretreatment run K-HO – 180 °C, 12 min 7.36 % v/v H_2O_2) displayed porous surface indicating lignin removal whereas pretreatment run O-HSO (213.6 °C, 12 min, 2 % w/v H_2SO_4) resulted in repolymerization of klason lignin (pseudo lignin) in droplets form on the cell wall surface.

Liquid hydrolysate characterization of both pretreatments resulted significant differences in their chemical composition. Lower concentration of furfural and 5-HMF in the range of 0 – 56.91 mg L^{-1} and 2.56 – 56.60 mg L^{-1}, respectively, were verified in liquid hydrolysate of H_2O_2 assisted hydrothermal pretreatment besides lower concentrations of glucose (0.50 – 1.29 mg L^{-1}) and xylose (1.27 – 9.60 mg L^{-1}) and higher concentrations of acetic acid (107.42 – 581.02 mg L^{-1}) and formic acid (54.95 – 1108.02 mg L^{-1}), COD (8.07 – 17.75 g L^{-1}), total carbohydrate (1.96 – 4.14 g L^{-1}), and phenolic content (741.25 – 3,005.63 ppm). In contrast, liquid hydrolysate of H_2SO_4 assisted hydrothermal pretreatment presented higher furanic compounds concentration; furfural and 5-HMF in the range of 939.89 – 2,181.24 mg L^{-1} and 70.95 – 970.08 mg L^{-1}, respectively. Higher concentration of organic acids was also verified; acetic acid (1,114.61 – 2,198.89 mg L^{-1}), formic acid (688.31 – 3,611.28 mg L^{-1}). Chemical oxygen demand was in the range of 8.33 – 25.20 g L^{-1}, total carbohydrate content (2.96 – 20.90 g L^{-1}). However, lower phenolic content 596.72 – 1674.22 ppm) was noted in comparison to H_2O_2 assisted pretreatment. Sulfate (360.0 – 7,240.0 mg L^{-1}) was only determined for H_2SO_4 pretreatment. Furthermore, relatively higher concentrations of glucose (1.93 – 13.53 mg L^{-1}) and xylose (0.43 – 13.87 mg L^{-1}) was verified when compared to H_2O_2 assisted hydrothermal pretreatment for glucose (0.19 – 1.29 mg L^{-1}) and xylose (1.27 – 9.60 mg L^{-1}), respectively.

Biomethane potential assays for the solid fraction of both pretreatments indicated significant differences in the methane yield. H_2O_2 assisted hydrothermal pretreatment resulted into notably higher methane production primarily due to lignin solubilization and higher glucan content in the solid fraction. Maximum methane production (14.43 Nmmol g^{-1} TVS) was recorded in the pretreatment run K-HO (180 °C, 12 min, 7.36 % v/v H_2O_2), which was 118.16% higher than the methane production of raw sugarcane bagasse 6.59 Nmmol g^{-1} TVS. However, H_2SO_4 assisted hydrothermal pretreatment resulted in significant methanogenic inhibition in most of the studied pretreatments. It could be explained with the logic that most of the fermentable sugars were released into liquid fraction coupled with high klason lignin content in the solid fraction.

Microbial community structure determined with DGGE revealed that reaction temperature, reaction time, and H_2O_2 concentration had a notable impact on Pearson similarity coefficient for the bacterial community; similarity coefficient decreased with a decrease in reaction time and H_2O_2 concentration. However, increased with a decrease in reaction temperature. On the other hand, for an archaeal community much difference in the Pearson similarity coefficient was not observed. Shannon-Wiener (H′) values were in the range of 2.10 – 3.18 and 2.72 – 2.86, respectively for Bacteria and Archaea while Dominance values were in the range of 0.04-0.14 and 0.06-0.08, respectively for Bacteria and Archaea.

DGGE band profile of H_2SO_4 assisted batch digestion reactors depicted notable variability in Pearson similarity coefficient for bacteria; decrease in reaction time resulted in a decrease in similarity coefficient value while a decrease in reaction temperature resulted in an increase in similarity coefficient value. On the other hand, an increase in similarity coefficient value was observed with a decrease in H_2SO_4 concentration. The archaeal population was not much affected by pretreatment parameters. Shannon-Wiener (H′) values were in the range of 2.59 – 3.23 and 2.09 – 2.51, respectively for Bacteria and Archaea. While Dominance values were in the range of 0.04 – 0.08 and 0.06 – 0.14, respectively for Bacteria and Archaea.

Illumina MiSeq sequencing of selected pretreatments identified the predominance of uncultured *AUTHM297* genera belonging to phylum *Thermotogae* in H_2O_2 assisted samples whereas *Flavobacterium, Desulfosporosinus, Bacteroides* and *Geotoga* were abundant in H_2SO_4

treated samples. However, *Methanolinea* was the abundant archaeal genus in H_2O_2 and H_2SO_4 treated samples.

7. CONCLUSIONS

The experimental studies are concluded with the following observations according to specified objectives:

a) Compositional analysis of sugarcane bagasse verified that it is a suitable lignocellulosic material for methane production owing to its higher polymeric sugar content (73%) and lower ash content (1.2%). Morphological characterization depicted that its structure was compact and smooth that necessitate a pretreatment step for further down processing.

b) Pretreatment severity of H_2O_2 assisted hydrothermal pretreatment did not influence the formation of furfural and 5-hydroxymethylfurfural formation because of high pH value. Furthermore, it indicated that pH value of resulting hydrolysate dictate the formation of furanic compounds. On the other hand, H_2SO_4 assisted hydrothermal pretreatment indicated that pretreatment severity resulted in high production of furfural and 5-hydroxymethylfurfural formation. Increase in severity resulted in increased furanic compounds concentration and vice versa.

c) Highest methane production 14.43 Nmmol g^{-1} TVS was obtained in H_2O_2 assisted hydrothermal pretreatment for pretreatment run K-HO (180 °C, 12 min, 7.36 % v/v H_2O_2). Whereas, methanogenic inhibition was encountered in most of H_2SO_4 assisted hydrothermal pretreatment. Cumulative organic acids at approx. log phase was in the range of 110.05 – 228.04 mg L^{-1} for batch anaerobic reactors treated with H_2O_2 assisted hydrothermal reactors where acetic acid was the major organic acid observed. However, in batch anaerobic reactors of H_2SO_4 assisted reactors organic acids were below the detection limit.

d) Microbial community structure of selected pretreatments in both pretreatment conditions indicated difference verified by Illumina MiSeq sequencing. Uncultured *AUTHM297* was predominant in batch reactors of H_2O_2 assisted pretreatments while *Flavobacterium, Desulfosporosinus, Bacteroides* and *Geotoga* were abundant in batch reactors of H_2SO_4 assisted pretreatment. However, *Methanolinea* was abundant in both H_2O_2 and H_2SO_4 assisted hydrothermal pretreatments.

8 FUTURE RECOMMENDATIONS

a) There is a need for improved understanding of the complex chemical reactions taking place in the lignin and polysaccharide phases, which lead to the formation of acidic, furanic and phenolic compounds

b) Enzymatic hydrolysis of pretreated solid fraction of H_2O_2 assisted HT pretreatment is needed to be explored in future for enhanced glucose yield for further downstream processing

c) H_2O_2 assisted HT pretreatment resulted into maximal lignin solubilization (73.09%) into the liquid stream. Lignin is a valuable compound for various products including activated carbon, binders, carbon fibers, and sorbents. Further research is needed to develop methods to extract and purify lignin from solubilized liquid fraction for its potential application. Therefore, H_2O_2 assisted HT could be used for the production of an array of products in a biorefinery perspective

d) Author recommend a lower concentration of H_2SO_4 (% w/v) as an impregnation agent before HT with minimal impregnation period. Hydrolysate of H_2SO_4-HT could further study for digestion owing to higher COD and lower total phenolic contents with possible longer lag phase due to appreciable furanic compounds.

e) A dedicated study is required with lower H_2SO_4 concentration to optimize HT pretreatment for minimal monomeric sugar dehydration into furanic compounds.

f) H_2O_2 assisted HT would be a good option to scale-up for continuous methane production but further study is required to minimize impregnation time to determine optimum lignin solubilization

9 REFERENCES

ABATZOGLOU, N. et al. Phenomenological kinetics of complex systems: the development of a generalized severity parameter and its application to lignocellulosics fractionation. **Chemical Engineering Science,** v. 47, n. 5, p. 1109-1122, 1992/04/01 1992. ISSN 0009-2509. Available at: < http://www.sciencedirect.com/science/article/pii/0009250992802355 >.

ADEKUNLE, K. F.; OKOLIE, J. A. A review of biochemical process of anaerobic digestion. **Advances in Bioscience and Biotechnology,** v. 6, p. 205-212, 2015.

ADLER, E. Lignin chemistry-past, present and future. **Wood Science and Technology,** v. 11, n. 3, p. 169-218, 1977. ISSN 0043-7719. Available at: < http://dx.doi.org/10.1007/BF00365615 >.

AGBOR, V. B. et al. Biomass pretreatment: Fundamentals toward application. **Biotechnology Advances,** v. 29, n. 6, p. 675-685, 11// 2011. ISSN 0734-9750. Available at: < http://www.sciencedirect.com/science/article/pii/S0734975011000607 >. Accessed on: 2011/12//.

AHMAD, F. "Plant cell wall structure-pretreatment" the critical relationship in biomass conversion to fermentable sugars. In: SHARMA, S. K. (Ed.). **Green biomass pretreatment for biofuels production**: Springer, 2013. p.1 - 30.

AHRING, B. K. et al. Making lignin accessible for anaerobic digestion by wet-explosion pretreatment. **Bioresource Technology,** v. 175, p. 182188, 2015. ISSN 0960-8524. Available at: < http://dx.doi.org/10.1016/j.biortech.2014.10.082 >.

AHUJA, N. **Impact of Operating Conditions on Thermal Hydrolysis Pre-Treated Digestion Return Liquor**. 2015. 132 (Master of Science). Civil and Environmental Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA.

AITA, G. M.; KIM, M. Pretreatment technologies for the conversionof lignocellulosic materials to bioethanol. In: EGGLESTON, G. (Ed.). **Sustainability of the Sugar and Sugar−Ethanol Industries**: American Chemical Society, 2010. p.117-145.

ALMEIDA, J. R. M. et al. Metabolic effects of furaldehydes and impacts on biotechnological processes. **Applied Microbiology and Biotechnology,** v. 82, n. 4, p. 625-638, 2009. ISSN 1432-0614. Available at: < http://dx.doi.org/10.1007/s00253-009-1875-1 >.

ALRAWI, R. A. et al. Anaerobic co-digestion of palm oil mill effluent with rumen fluid as a co-substrate. **Desalination,** v. 269, n. 1–3, p. 50-57, 3/15/ 2011. ISSN 0011-9164. Available at: < http://www.sciencedirect.com/science/article/pii/S0011916410007563 >.

ALVAREZ, R.; LIDÉN, G. Semi-continuous co-digestion of solid slaughterhouse waste, manure, and fruit and vegetable waste. **Renewable Energy,** v. 33, n. 4, p. 726-734, 4// 2008. ISSN 0960-1481. Available at: < http://www.sciencedirect.com/science/article/pii/S0960148107001309 >.

ALVIRA, P. et al. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. **Bioresource Technology,** v. 101, n. 13, p. 4851-4861, 7// 2010. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852409015983 >.

ANDERSON, K.; SALLIS, P.; UYANIK, S. 24 - Anaerobic treatment processes. In: (Ed.). **Handbook of Water and Wastewater Microbiology**. London: Academic Press, 2003. p.391-426. ISBN 978-0-12-470100-7.

ANGELIDAKI, I.; ELLEGAARD, L.; AHRING, B. K. A mathematical model for dynamic simulation of anaerobic digestion of complex substrates: focusing on ammonia inhibition. **Biotechnol Bioeng,** v. 42, n. 2, p. 159-66, Jun 20 1993. ISSN 0006-3592 (Print)

0006-3592.

ANNA, T. et al. Pretreatment of paper tube residuals for improved biogas production. **Bioresource Technology,** v. 101, n. 4, p. 12061212, 2010. ISSN 0960-8524. Available at: < http://dx.doi.org/10.1016/j.biortech.2009.09.029 >.

ANUJ, K. C. et al. Sugarcane bagasse and leaves: foreseeable biomass of biofuel and bio-products. **Journal of Chemical Technology and Biotechnology,** v. 87, n. 1, p. 11-20, 2012. ISSN 1097-4660. Available at: < http://dx.doi.org/10.1002/jctb.2742 >.

APHA; AWWA; WEF. **Standard methods for the examination of water and wastewater**. Washington DC: American Public Health Association, 2005.

AZMAN, S. et al. Presence and Role of Anaerobic Hydrolytic Microbes in Conversion of Lignocellulosic Biomass for Biogas Production. **Critical Reviews in Environmental Science and Technology,** v. 45, n. 23, p. 2523-2564, 2015/12/02 2015. ISSN 1064-3389. Available at: < http://dx.doi.org/10.1080/10643389.2015.1053727 >.

BADSHAH, M. et al. Use of an Automatic Methane Potential Test System for evaluating the biomethane potential of sugarcane bagasse after different treatments. **Bioresource technology,** v. 114, p. 262-269, 2012. ISSN 0960-8524. Available at: < http://dx.doi.org/10.1016/j.biortech.2012.02.022 >.

BALK, M.; WEIJMA, J.; STAMS, A. J. Thermotoga lettingae sp. nov., a novel thermophilic, methanol-degrading bacterium isolated from a thermophilic anaerobic reactor. **Int J Syst Evol Microbiol,** v. 52, n. Pt 4, p. 1361-8, Jul 2002. ISSN 1466-5026 (Print)

1466-5026.

BAPTESTE, E.; BROCHIER, C.; BOUCHER, Y. Higher-level classification of the Archaea: evolution of methanogenesis and methanogens. **Archaea,** v. 1, n. 5, p. 353-63, May 2005. ISSN 1472-3646 (Print).

BARAKAT, A. et al. Effect of lignin-derived and furan compounds found in lignocellulosic hydrolysates on biomethane production. **Bioresource Technology,** v. 104, p. 90-99, 1// 2012. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852411015264 >.

BASERBA, M. G.; ANGELIDAKI, I.; KARAKASHEV, D. Effect of continuous oleate addition on microbial communities involved in anaerobic digestion process. **Bioresource Technology,** v. 106, p. 74-81, 2// 2012. ISSN 0960-8524. Available at: < //www.sciencedirect.com/science/article/pii/S0960852411017664 >.

BASSO, T. P. et al. Towards the production of second generation ethanol from sugarcane bagasse in Brazil. In: MATOVIC, M. D. (Ed.). **Biomass Now - Cultivation and Utilization**: InTech, 2013.

BERLIN, A. et al. Inhibition of cellulase, xylanase and beta-glucosidase activities by softwood lignin preparations. **J Biotechnol,** v. 125, n. 2, p. 198-209, Sep 1 2006. ISSN 0168-1656 (Print)

0168-1656.

BERLIN, A. et al. Inhibition of cellulase, xylanase and β-glucosidase activities by softwood lignin preparations. **Journal of Biotechnology,** v. 125, n. 2, p. 198-209, 9/1/ 2006. ISSN 0168-1656. Available at: < http://www.sciencedirect.com/science/article/pii/S0168165606001714 >.

BERNARDET, J.-F.; BOWMAN, J. P. Flavobacterium. In: (Ed.). **Bergey's Manual of Systematics of Archaea and Bacteria**: John Wiley & Sons, Ltd, 2015. ISBN 9781118960608.

BHANDARI, V.; GUPTA, R. S. The Phylum Thermotogae. In: ROSENBERG, E.;DELONG, E. F., *et al* (Ed.). **The Prokaryotes: Other Major Lineages of Bacteria and The Archaea**. Berlin, Heidelberg: Springer Berlin Heidelberg, 2014. p.989-1015. ISBN 978-3-642-38954-2.

BHARATHIRAJA, B. et al. Insights on Lignocellulosic Pretreatments for Biofuel Production- SEM and Reduction of Lignin Analysis. **International Journal of ChemTech Research,** v. 6, n. 9, p. 4334 - 4444, 2014. ISSN 0974-4290. Available at: < http://sphinxsai.com/2014/RTBCE/6/(4434-4445)%20014.pdf >.

BJORNSSON, L.; MURTO, M.; MATTIASSON, B. Evaluation of parameters for monitoring an anaerobic co-digestion process. **Appl Microbiol Biotechnol,** v. 54, n. 6, p. 844-9, Dec 2000. ISSN 0175-7598 (Print)

0175-7598.

BOERJAN, W.; RALPH, J.; BAUCHER, M. LIGNIN BIOSYNTHESIS. **Annual Review of Plant Biology,** v. 54, n. 1, p. 519-546, 2003. Available at: < http://www.annualreviews.org/doi/abs/10.1146/annurev.arplant.54.031902.134938 >.

BONAWITZ, N. D.; CHAPPLE, C. The Genetics of Lignin Biosynthesis: Connecting Genotype to Phenotype. **Annual Review of Genetics,** v. 44, n. 1, p. 337-363, 2010. Available at: < http://www.annualreviews.org/doi/abs/10.1146/annurev-genet-102209-163508 >.

BONNIN, A. S.; BOONE, D. R. The order Methanobacteriales. In: DWORKIN, M.;FALKOW, S., *et al* (Ed.). **The Prokaryotes: Archaea. Bacteria: Firmicutes, Actinomycetes**. Singapore: Springer, 2006.

BOONE, D. R. Methanobacterium. In: (Ed.). **Bergey's Manual of Systematics of Archaea and Bacteria**: John Wiley & Sons, Ltd, 2015. ISBN 9781118960608.

BOUCHARD, J. et al. Analytical methodology for biomass pretreatment. Part 2: Characterization of the filtrates and cumulative product distribution as a function of treatment severity. **Bioresource Technology,** v. 36, n. 2, p. 121-131, 1991/01/01 1991. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/096085249190169K >.

BRAUER, S. L. et al. Methanoregula boonei gen. nov., sp. nov., an acidiphilic methanogen isolated from an acidic peat bog. **Int J Syst Evol Microbiol,** v. 61, n. Pt 1, p. 45-52, Jan 2011. ISSN 1466-5026.

BRÄUER, S. L. et al. Characterization of acid-tolerant H2/CO2-utilizing methanogenic enrichment cultures from an acidic peat bog in New York State. **FEMS Microbiology Ecology,** v. 57, n. 2, p. 206-216, 2006. ISSN 1574-6941. Available at: < http://dx.doi.org/10.1111/j.1574-6941.2006.00107.x >.

BRAUN, R.; WEILAND, P.; WELLINGER, A. Biogas from energy crop digestion. **IEA Bioenergy Task 37,** <**www.IEA-Biogas.net/**>, 2010.

BRENNER, D.; KRIEG, N.; STALEY, J. **Bergey's Manual of Systematic Bacteriology: Proteobacteria**. 2nd. New York: Springer, 2004.

BRIENZO, M.; SIQUEIRA, A. F.; MILAGRES, A. M. F. Search for optimum conditions of sugarcane bagasse hemicellulose extraction. **Biochemical Engineering Journal,** v. 46, n. 2, p. 199-204, 10/1/ 2009. ISSN 1369-703X. Available at: < http://www.sciencedirect.com/science/article/pii/S1369703X09001569 >.

BRIONES, A. M. et al. Microbial diversity and dynamics in multi- and single-compartment anaerobic bioreactors processing sulfate-rich waste streams. **Environ Microbiol,** v. 9, n. 1, p. 93-106, Jan 2007. ISSN 1462-2912 (Print)

1462-2912.

BROWN, T. M.; DUAN, P.; SAVAGE, P. E. Hydrothermal Liquefaction and Gasification of Nannochloropsis sp. **Energy & Fuels,** v. 24, n. 6, p. 3639-3646, 2010/06/17 2010. ISSN 0887-0624. Available at: < http://dx.doi.org/10.1021/ef100203u >.

BUGG, T. D. et al. The emerging role for bacteria in lignin degradation and bio-product formation. **Curr Opin Biotechnol,** v. 22, n. 3, p. 394-400, Jun 2011. ISSN 0958-1669.

CALDWELL, M. E. et al. Tolumonas osonensis sp. nov., isolated from anoxic freshwater sediment, and emended description of the genus Tolumonas. **International Journal of Systematic and Evolutionary**

Microbiology, v. 61, n. 11, p. 2659-2663, 2011. Available at: < http://ijs.microbiologyresearch.org/content/journal/ijsem/10.1099/ijs.0.023853-0 >.

CANILHA, L. et al. A study on the pretreatment of a sugarcane bagasse sample with dilute sulfuric acid. **Journal of Industrial Microbiology & Biotechnology,** v. 38, n. 9, p. 1467-1475, 2011/09/01 2011. ISSN 1367-5435. Available at: < http://dx.doi.org/10.1007/s10295-010-0931-2 >.

CAO, S.; AITA, G. M. Enzymatic hydrolysis and ethanol yields of combined surfactant and dilute ammonia treated sugarcane bagasse. **Bioresource Technology,** v. 131, n. 0, p. 357-364, 3// 2013. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852412020287 >.

CARBALLA, M.; REGUEIRO, L.; LEMA, J. M. Microbial management of anaerobic digestion: exploiting the microbiome-functionality nexus. **Current Opinion in Biotechnology,** v. 33, p. 103-111, 6// 2015. ISSN 0958-1669. Available at: < http://www.sciencedirect.com/science/article/pii/S0958166915000178 >.

CARVALHEIRO, F. et al. Wheat straw autohydrolysis: process optimization and products characterization. **Applied biochemistry and biotechnology,** v. 153, n. 1-3, p. 84-93, 2009. Available at: < http://dx.doi.org/10.1007/s12010-008-8448-0 >.

CASU, R. E. et al. Identification of transcripts associated with cell wall metabolism and development in the stem of sugarcane by Affymetrix GeneChip Sugarcane Genome Array expression profiling. **Funct Integr Genomics,** v. 7, n. 2, p. 153-67, Apr 2007. ISSN 1438-793X (Print)

1438-793x.

CAZA, N. et al. Removal of phenolic compounds from synthetic wastewater using soybean peroxidase. **Water Research,** v. 33, n. 13, p. 3012-3018, 9// 1999. ISSN 0043-1354. Available at: < http://www.sciencedirect.com/science/article/pii/S0043135498005259 >.

CHABAN, B.; HILL, J. E. A 'universal' type II chaperonin PCR detection system for the investigation of Archaea in complex microbial communities. **Isme j,** v. 6, n. 2, p. 430-9, Feb 2012. ISSN 1751-7362.

CHANDEL, A. K. et al. Multi-scale structural and chemical analysis of sugarcane bagasse in the process of sequential acid–base pretreatment and ethanol production by Scheffersomyces shehatae and Saccharomyces cerevisiae. **Biotechnology for Biofuels,** v. 7, n. 1, p. 1-17, 2014. ISSN 1754-6834. Available at: < http://dx.doi.org/10.1186/1754-6834-7-63 >.

CHANDEL, A. K.; SILVA, S. S. R. D.; SINGH, O. V. Detoxification of Lignocellulosic Hydrolysates for Improved Bioethanol Production. In: BERNARDES, M. A. D. S. (Ed.). **Biofuel Production-Recent Developments and Prospects**: In Tech, 2011. ISBN 10.5772/16454.

CHANDRA, R.; TAKEUCHI, H.; HASEGAWA, T. Hydrothermal pretreatment of rice straw biomass: A potential and promising method for enhanced methane production. **Applied Energy**, 2012. ISSN 0306-2619. Available at: < http://dx.doi.org/10.1016/j.apenergy.2012.01.027 >.

CHANG, V. S.; HOLTZAPPLE, M. T. Fundamental factors affecting biomass enzymatic reactivity. **Appl Biochem Biotechnol,** v. 84-86, p. 5-37, Spring 2000. ISSN 0273-2289 (Print)

0273-2289.

CHATURVEDI, V.; VERMA, P. An overview of key pretreatment processes employed for bioconversion of lignocellulosic biomass into biofuels and value added products. **3 Biotech,** v. 3, n. 5, p. 415-431, 09/05

05/27/received

08/13/accepted 2013. ISSN 2190-5738. Available at: < http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3781263/ >.

CHEN, F.; DIXON, R. A. Lignin modification improves fermentable sugar yields for biofuel production. **Nature Biotechnology,** v. 25, n. 7, p. 759-761, 2007. ISSN 1087-0156. Available at: < http://dx.doi.org/10.1038/nbt1316 >.

CHEN, H. **Biotechnology of lignocellulose: Theory and practice**. Chemical Industry Press, Springer, 2014.

CHEN, X.; LAWOKO, M.; HEININGEN, A. V. Kinetics and mechanism of autohydrolysis of hardwoods. **Bioresource Technology,** v. 101, n. 20, p. 7812-7819, 10// 2010. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852410008175 >.

CHENG, C.-L.; CHANG, J.-S. Hydrolysis of lignocellulosic feedstock by novel cellulases originating from Pseudomonas sp. CL3 for fermentative hydrogen production. **Bioresource Technology,** v. 102, n. 18, p. 8628-8634, 9// 2011. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S096085241100410X >.

CHILDERS, S. E.; CIUFO, S.; LOVLEY, D. R. Geobacter metallireducens accesses insoluble Fe(III) oxide by chemotaxis. **Nature,** v. 416, n. 6882, p. 767-9, Apr 18 2002. ISSN 0028-0836 (Print)

0028-0836.

CHUM, H. L. et al. Pretreatment-Catalyst effects and the combined severity parameter. **Applied Biochemistry and Biotechnology,** v. 24, n. 1, p. 1-14, 1990. ISSN 1559-0291. Available at: < http://dx.doi.org/10.1007/BF02920229 >.

CHUNDAWAT, S. P. S. et al. Multi-scale visualization and characterization of lignocellulosic plant cell wall deconstruction during thermochemical pretreatment. **Energy & Environmental Science,** v. 4, n. 3, p. 973-984, 2011. ISSN 1754-5692. Available at: < http://dx.doi.org/10.1039/C0EE00574F >.

CLARK, T. A.; MACKIE, K. L. Fermentation inhibitors in wood hydrolysates derived from the softwood Pinus radiata. **Journal of Chemical Technology and Biotechnology. Biotechnology,** v. 34, n. 2, p. 101-110, 1984. ISSN 1935-1828. Available at: < http://dx.doi.org/10.1002/jctb.280340206 >.

COLLERAN, E.; FINNEGAN, S.; LENS, P. Anaerobic treatment of sulphate-containing waste streams. **Antonie van Leeuwenhoek,** v. 67, n. 1, p. 29-46, 1995. ISSN 1572-9699. Available at: < http://dx.doi.org/10.1007/BF00872194 >.

COLLINS, G. et al. Distribution, Localization, and Phylogeny of Abundant Populations of Crenarchaeota in Anaerobic Granular Sludge. **Applied and Environmental Microbiology,** v. 71, n. 11, p. 7523-7527, November 1, 2005 2005. Available at: < http://aem.asm.org/content/71/11/7523.abstract >.

CONNORS, K. A. **Chemical Kinetics: The Study of Reaction Rates in Solutions**. New York: VCH Publishers, 1990.

CORRALES, R. C. N. R. et al. Structural evaluation of sugar cane bagasse steam pretreated in the presence of CO2 and SO2. **Biotechnology for Biofuels,** v. 5, 2012.

DA SILVA, A. et al. Milling pretreatment of sugarcane bagasse and straw for enzymatic hydrolysis and ethanol fermentation. **Bioresource technology,** v. 101, n. 19, p. 7402-7409, 2010. ISSN 0960-8524. Available at: < http://dx.doi.org/10.1016/j.biortech.2010.05.008 >.

DAMAURAI, J.; CHAMPREDA, V.; LAOSIRIPOJANA, N. Optimization of alkaline peroxide pretreatment of rice straw. **Journal of Natural Sciences Research,** v. 4, n. 13, p. 29-33, 2014.

DAVEY, M. E.; MACGREGOR, B. J.; STAHL, D. A. Geotoga. In: (Ed.). **Bergey's Manual of Systematics of Archaea and Bacteria**: John Wiley & Sons, Ltd, 2015. ISBN 9781118960608.

DE LA TORRE, J. R. et al. Cultivation of a thermophilic ammonia oxidizing archaeon synthesizing crenarchaeol. **Environmental Microbiology,** v. 10, n. 3, p. 810-818, 2008. ISSN 1462-2920. Available at: < http://dx.doi.org/10.1111/j.1462-2920.2007.01506.x >.

DE MORAES, R. G. J. et al. Dilute mixed-acid pretreatment of sugarcane bagasse for ethanol production. **Biomass and Bioenergy,** v. 35, 2011. ISSN 0961-9534. Available at: < http://dx.doi.org/10.1016/j.biombioe.2010.10.018 >.

DE MOUTTA, R. et al. Comparative Response and Structural Characterization of Sugarcane Bagasse, Straw and Bagasse-Straw 1:1 Mixtures Subjected to Hydrothermal Pretreatment and Enzymatic Conversion. **Journal of Microbial & Biochemical Technology,** v. 01, n. S12, 2013. Available at: < http://dx.doi.org/10.4172/1948-5948.S12-005 >.

DE VASCONCELOS, S. M. et al. Diluted phosphoric acid pretreatment for production of fermentable sugars in a sugarcane-based biorefinery. **Bioresource Technology,** v. 135, p. 46-52, 5// 2013. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852412015854 >.

DE VRIEZE, J.; VERSTRAETE, W. Perspectives for microbial community composition in anaerobic digestion: from abundance and activity to connectivity. **Environmental Microbiology,** v. 18, n. 9, p. 2797-2809, 2016. ISSN 1462-2920. Available at: < http://dx.doi.org/10.1111/1462-2920.13437 >.

DEGUCHI, S.; TSUJII, K.; HORIKOSHI, K. Cooking cellulose in hot and compressed water. **Chemical Communications**, n. 31, p. 3293-3295, 2006. ISSN 1359-7345. Available at: < http://dx.doi.org/10.1039/B605812D >.

DEMIRBAS, A. **Biorefineries: for biomass upgrading facilities**. New York: Springer, 2009.

DEN CAMP, H. J. M. O. et al. Effects of lignin on the anaerobic degradation of (ligno) cellulosic wastes by rumen microorganisms. **Applied Microbiology and Biotechnology,** v. 29, n. 4, p. 408-412, 1988. ISSN 1432-0614. Available at: < http://dx.doi.org/10.1007/BF00265827 >.

DENG, A. J. et al. Corncob lignocellulose for the production of furfural by hydrothermal pretreatment and heterogeneous catalytic process. **Rsc Advances,** v. 5, n. 74, p. 60264-60272, 2015. ISSN 2046-2069. Available at: < <Go to ISI>://WOS:000358230400056 >.

DENGER, K. et al. Anaerophaga thermohalophila gen. nov., sp. nov., a moderately thermohalophilic, strictly anaerobic fermentative bacterium. **Int J Syst Evol Microbiol,** v. 52, n. Pt 1, p. 173-8, Jan 2002. ISSN 1466-5026 (Print)

1466-5026.

DING, S.-Y. et al. How Does Plant Cell Wall Nanoscale Architecture Correlate with Enzymatic Digestibility? **Science,** v. 338, n. 6110, p. 1055-1060, 2012-11-23 00:00:00 2012. Available at: < http://science.sciencemag.org/sci/338/6110/1055.full.pdf >.

DOE, U. **Breaking the Biological Barriers to Cellulosic Ethanol: A Joint Research Agenda. Report from the December 2005 Workshop**. U.S. Department of Energy Office of Science 2006

DONALDSON, L. A.; WONG, K. K. Y.; MACKIE, K. L. Ultrastructure of steam-exploded wood. **Wood Science and Technology,** v. 22, n. 2, p. 103-114, 1988. ISSN 1432-5225. Available at: < http://dx.doi.org/10.1007/BF00355846 >.

DONOHOE, B. S. et al. Visualizing lignin coalescence and migration through maize cell walls following thermochemical pretreatment. **Biotechnology and Bioengineering,** v. 101, n. 5, p. 913-925, 2008. ISSN 1097-0290. Available at: < http://dx.doi.org/10.1002/bit.21959 >.

DRIDI, B. et al. Methanomassiliicoccus luminyensis gen. nov., sp. nov., a methanogenic archaeon isolated from human faeces. **Int J Syst Evol Microbiol,** v. 62, n. Pt 8, p. 1902-7, Aug 2012. ISSN 1466-5026.

DU, B. et al. Effect of varying feedstock–pretreatment chemistry combinations on the formation and accumulation of potentially inhibitory degradation products in biomass hydrolysates. **Biotechnology and**

Bioengineering, v. 107, n. 3, p. 430-440, 2010. ISSN 1097-0290. Available at: < http://dx.doi.org/10.1002/bit.22829 >.

DUBOIS, M. et al. Colorimetric Method for Determination of Sugars and Related Substances. **Analytical Chemistry,** v. 28, n. 3, p. 350-356, 1956/03/01 1956. ISSN 0003-2700. Available at: < http://dx.doi.org/10.1021/ac60111a017 >.

EBRINGEROVÁ, A.; HEINZE, T. Xylan and xylan derivatives – biopolymers with valuable properties, 1. Naturally occurring xylans structures, isolation procedures and properties. **Macromolecular Rapid Communications,** v. 21, n. 9, p. 542-556, 2000. ISSN 1521-3927. Available at: < http://dx.doi.org/10.1002/1521-3927(20000601)21:9<542::AID-MARC542>3.0.CO;2-7 >.

EDWARD, M. R. Genomics of cellulosic biofuels. **Nature,** v. 454, n. 7206, p. 841-845, 2008. ISSN 0028-0836. Available at: < http://dx.doi.org/10.1038/nature07190 >.

EFFENDI, A.; GERHAUSER, H.; BRIDGWATER, A. V. Production of renewable phenolic resins by thermochemical conversion of biomass: A review. **Renewable and Sustainable Energy Reviews,** v. 12, n. 8, p. 2092-2116, 10// 2008. ISSN 1364-0321. Available at: < http://www.sciencedirect.com/science/article/pii/S1364032107000652 >.

EGGEMAN, T.; ELANDER, R. T. Process and economic analysis of pretreatment technologies. **Bioresource Technology,** v. 96, n. 18, p. 2019-2025, 12// 2005. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852405000702 >.

EL HAGE, R. et al. Effect of autohydrolysis of Miscanthus x giganteus on lignin structure and organosolv delignification. **Bioresource Technology,** v. 101, n. 23, p. 9321-9329, 12// 2010. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S096085241001148X >.

ERCOLINI, D. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. **J Microbiol Methods,** v. 56, n. 3, p. 297-314, Mar 2004. ISSN 0167-7012 (Print)

0167-7012.

ESQUIVEL-ELIZONDO, S. et al. Archaea and Bacteria Acclimate to High Total Ammonia in a Methanogenic Reactor Treating Swine Waste. **Archaea,** v. 2016, p. 10, 2016. Available at: < http://dx.doi.org/10.1155/2016/4089684 >.

FAN, L. T.; GHARPURAY, M. M.; LEE, Y. H. **Cellulose hydrolysis**. Berlin: Springer, 1987. 57.

FANG, C. et al. Hydrothermal Pretreatment of Date Palm (Phoenix dactylifera L.) Leaflets and Rachis to Enhance Enzymatic Digestibility and Bioethanol Potential. **BioMed Research International,** v. 2015, p. 216454, 08/12

02/03/received

03/18/revised

03/28/accepted 2015. ISSN 2314-6133

2314-6141. Available at: < http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4549489/ >.

FANG, C. J. et al. Hydrothermal Pretreatment of Date Palm (Phoenix dactylifera L.) Leaflets and Rachis to Enhance Enzymatic Digestibility and Bioethanol Potential. **Biomed Research International**, 2015. ISSN 2314-6133. Available at: < <Go to ISI>://WOS:000360209500001 >.

FANG, Y. et al. An Acidic Two-Step Hydrothermal Process To Enhance Acetic Acid Production from Carbohydrate Biomass. **Industrial & Engineering Chemistry Research,** v. 51, n. 12, p. 4759-4763, 2012/03/28 2012. ISSN 0888-5885. Available at: < http://dx.doi.org/10.1021/ie202506c >.

FERNANDES, T. V. et al. Effects of thermo-chemical pre-treatment on anaerobic biodegradability and hydrolysis of lignocellulosic biomass. **Bioresource Technology,** v. 100, n. 9, p. 2575-2579, 5// 2009. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852408010705 >.

FERNANDEZ-CEGRI, V. et al. Effect of hydrothermal pretreatment of sunflower oil cake on biomethane potential focusing on fibre composition. **Bioresource Technology,** v. 123, p. 424-429, Nov 2012. ISSN 0960-8524. Available at: < <Go to ISI>://WOS:000310401100063 >.

FISCHER-ROMERO, C.; TINDALL, B. J.; JUTTNER, F. Tolumonas auensis gen. nov., sp. nov., a toluene-producing bacterium from anoxic sediments of a freshwater lake. **Int J Syst Bacteriol,** v. 46, n. 1, p. 183-8, Jan 1996. ISSN 0020-7713 (Print)

0020-7713.

FORSTER-CARNEIRO, T. et al. Anaerobic Digestion: Pretreatments of Substrates. In: (Ed.). **Biogas Production**: John Wiley & Sons, Inc., 2012. p.1-25. ISBN 9781118404089.

FOWLER, S. J. et al. Identification of toluene degraders in a methanogenic enrichment culture. **FEMS microbiology ecology,** v. 89, n. 3, p. 625-636, 2014. ISSN 0168-6496. Available at: < http://dx.doi.org/10.1111/1574-6941.12364 >.

FRANKE-WHITTLE, I. H. et al. Investigation into the effect of high concentrations of volatile fatty acids in anaerobic digestion on methanogenic communities. **Waste Management,** v. 34, n. 11, p. 2080-2089, 11// 2014. ISSN 0956-053X. Available at: < http://www.sciencedirect.com/science/article/pii/S0956053X14003250 >.

GARCIA, J.; OLLIVIER, B.; WHITMAN, W. B. The Order Methanomicrobiales. In: DWORKIN, M.;FALKOW, S., *et al* (Ed.). **The Prokaryotes: Archaea. Bacteria: Firmicutes, Actinomycetes**. Singapore: Springer, 2006.

GARGAUD, M.; IRVINE, W. M. **Encyclopedia of Astrobiology**. 2nd. Springer, 2015.

GARRITY, G. M.; HOLT, J. G. Chloroflexi phy. nov. In: (Ed.). **Bergey's Manual of Systematics of Archaea and Bacteria**: John Wiley & Sons, Ltd, 2015. ISBN 9781118960608.

_____. Euryarchaeota phy. nov. In: (Ed.). **Bergey's Manual of Systematics of Archaea and Bacteria**: John Wiley & Sons, Ltd, 2015. ISBN 9781118960608.

GARROTE, G.; DOMÍNGUEZ, H.; PARAJÓ, C. J. Hydrothermal processing of lignocellulosic materials. **Holz als Roh- und Werkstoff,** v. 57, n. 3, p. 191-202, 1999. ISSN 1436-736X. Available at: < http://dx.doi.org/10.1007/s001070050039 >.

GARROTE, G.; DOMÍNGUEZ, H.; PARAJÓ, J. C. Hydrothermal processing of lignocellulosic materials. **Holz als Roh- und Werkstoff**, 1999. ISSN 0018-3768. Available at: < http://dx.doi.org/10.1007/s001070050039 >.

GARROTE, G. et al. Autohydrolysis of agricultural residues: Study of reaction byproducts. **Bioresource Technology,** v. 98, n. 10, p. 1951-1957, 7// 2007. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852406004019 >.

GAVALA, H. N. et al. Mesophilic and thermophilic anaerobic digestion of primary and secondary sludge. Effect of pre-treatment at elevated temperature. **Water Res,** v. 37, n. 19, p. 4561-72, Nov 2003. ISSN 0043-1354 (Print)

0043-1354.

GHASEMIAN, M.; ZILOUEI, H.; ASADINEZHAD, A. Enhanced Biogas and Biohydrogen Production from Cotton Plant Wastes Using Alkaline Pretreatment. **Energy & Fuels,** v. 30, n. 12, p. 10484-10493, 2016/12/15 2016. ISSN 0887-0624. Available at: < http://dx.doi.org/10.1021/acs.energyfuels.6b01999 >.

GHASIMI, D. S. M. et al. Impact of lignocellulosic-waste intermediates on hydrolysis and methanogenesis under thermophilic and mesophilic conditions. **Chemical Engineering Journal,** v. 295, p. 181-191, 7/1/ 2016. ISSN 1385-8947. Available at: < http://www.sciencedirect.com/science/article/pii/S1385894716302753 >.

GIUSEPPE, D. G. et al. Effects of hydrothermal pre-treatments on Giant reed (Arundo donax) methane yield. **Bioresource Technology,** v. 147, p. 152159, 2013. ISSN 0960-8524. Available at: < http://dx.doi.org/10.1016/j.biortech.2013.08.006 >.

GONZÁLEZ-FERNÁNDEZ, C. et al. Thermal pretreatment to improve methane production of Scenedesmus biomass. **Biomass and Bioenergy,** v. 40, p. 105-111, 5// 2012. ISSN 0961-9534. Available at: < http://www.sciencedirect.com/science/article/pii/S0961953412000803 >.

GONZÁLEZ, L. et al. Effect of liquid hot water pre-treatment on sugarcane press mud methane yield. **Bioresource Technology,** v. 169, 2014. ISSN 0960-8524. Available at: < http://dx.doi.org/10.1016/j.biortech.2014.06.107 >.

GOODWIN, J. A. S.; WASE, D. A. J.; FORSTER, C. F. Effects of nutrient limitation on the anaerobic upflow sludge blanket reactor. **Enzyme and Microbial Technology,** v. 12, n. 11, p. 877-884, 11// 1990. ISSN 0141-0229. Available at: < http://www.sciencedirect.com/science/article/pii/014102299090026M >.

GOULD, M. J. Studies on the mechanism of alkaline peroxide delignification of agricultural residues. **Biotechnology and Bioengineering,** v. 27, n. 3, p. 225-231, 1985. ISSN 1097-0290. Available at: < http://dx.doi.org/10.1002/bit.260270303 >.

GRIFFITHS, R. I. et al. Rapid Method for Coextraction of DNA and RNA from Natural Environments for Analysis of Ribosomal DNA- and rRNA-Based Microbial Community Composition. **Applied and Environmental Microbiology,** v. 66, n. 12, p. 5488-5491, December 1, 2000 2000. Available at: < http://aem.asm.org/content/66/12/5488.abstract >.

GRIMALDI, M. P. et al. Evaluation of lime and hydrothermal pretreatments for efficient enzymatic hydrolysis of raw sugarcane bagasse. **Biotechnology for Biofuels,** v. 8, p. 205, 12/02

07/01/received

11/16/accepted 2015. ISSN 1754-6834. Available at: < http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4667421/ >.

GUILLIAMS, A. et al. Physical and chemical differences between one-stage and two-stage hydrothermal pretreated hardwood substrates for use in cellulosic ethanol production. **Biotechnology for Biofuels,** v. 9, n. 1, p. 1-13, 2016. ISSN 1754-6834. Available at: < http://dx.doi.org/10.1186/s13068-016-0446-9 >.

HANREICH, A. et al. Metagenome and metaproteome analyses of microbial communities in mesophilic biogas-producing anaerobic batch fermentations indicate concerted plant carbohydrate degradation. **Syst Appl Microbiol,** v. 36, n. 5, p. 330-8, Jul 2013. ISSN 0723-2020.

HANSEN, T. L. et al. Method for determination of methane potentials of solid organic waste. **Waste Management,** v. 24, n. 4, p. 393-400, // 2004. ISSN 0956-053X. Available at: < http://www.sciencedirect.com/science/article/pii/S0956053X0300223X >.

HAO, W. et al. Digestibility Improvement of Sorted Waste with Alkaline Hydrothermal Pretreatment. **TSINGHUA SCIENCE AND TECHNOLOGY,** v. 14, n. 3, p. 378-382, 2009.

HARMSEN, P. et al. **Literature review of physical and chemical pretreatment processes for lignocellulosic biomass**: Tech. Rep. 1184, Biosynergy, Wageningen UR Food & Biobased Research 2010.

HARRIS, P. W.; MCCABE, B. K. Review of pre-treatments used in anaerobic digestion and their potential application in high-fat cattle slaughterhouse wastewater. **Applied Energy,** v. 155, p. 560-575, 10/1/ 2015. ISSN 0306-2619. Available at: < http://www.sciencedirect.com/science/article/pii/S0306261915007849 >.

HARTMANN, H.; AHRING, B. K. Strategies for the anaerobic digestion of the organic fraction of municipal solid waste: An overview. **Water Science and Technology,** v. 53, n. 8, p. 7-22, 2006.

HARTMANN, H.; AHRING, B. K. Strategies for the anaerobic digestion of the organic fraction of municipal solid waste: an overview. **Water Sci Technol,** v. 53, n. 8, p. 7-22, 2006. ISSN 0273-1223 (Print)

0273-1223.

HATTI-KAUL, R. et al. Industrial biotechnology for the production of bio-based chemicals--a cradle-to-grave perspective. **Trends in biotechnology,** v. 25, n. 3, p. 119-124, 2007. ISSN 0167-7799. Available at: < http://dx.doi.org/10.1016/j.tibtech.2007.01.001 >.

HE, L. L. et al. A Review of Hydrothermal Pretreatment of Lignocellulosic Biomass for Enhanced Biogas Production. **Current Organic Chemistry,** v. 19, n. 5, p. 437-446, 2015. ISSN 1385-2728. Available at: < <Go to ISI>://WOS:000352166300006 >.

HEALY, J. B.; YOUNG, L. Y. Anaerobic biodegradation of eleven aromatic compounds to methane. **Applied and Environmental Microbiology**, 1979. Available at: < http://aem.asm.org/content/38/1/84.short >.

HEATHER, L. T.; CHARLES, E. W. Fundamentals of biomass pretreatment at low pH. In: CHARLES, E. W. (Ed.). **Aqueous pretreatmet of plant biomass for biological and chemical conversion to fuels and chemicals**: John Wiley & Sons, 2013. ISBN 978-0-470-97202-1.

HEATHER, L. T. et al. The fate of lignin during hydrothermal pretreatment. **Biotechnology for Biofuels**, 2013. ISSN 1754-6834. Available at: < http://dx.doi.org/10.1186/1754-6834-6-110 >.

HEILMANN, S. M. et al. Hydrothermal carbonization of microalgae II. Fatty acid, char, and algal nutrient products. **Applied Energy,** v. 88, n. 10, p. 3286-3290, 10// 2011. ISSN 0306-2619. Available at: < http://www.sciencedirect.com/science/article/pii/S0306261910005611 >.

HEITZ, M. et al. Fractionation of Populus tremuloides at the pilot plant scale: Optimization of steam pretreatment conditions using the STAKE II technology. **Bioresource Technology,** v. 35, n. 1, p. 23-32, 1991/01/01 1991. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/096085249190078X >.

HEMMINGSEN, S. M. et al. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. **Nature,** v. 333, n. 6171, p. 330-334, 05/26/print 1988. Available at: < http://dx.doi.org/10.1038/333330a0 >.

HENDRIKS, A. T. W. M.; ZEEMAN, G. Pretreatments to enhance the digestibility of lignocellulosic biomass. **Bioresource technology,** v. 100, n. 1, p. 10-18, 2009. ISSN 0960-8524. Available at: < http://dx.doi.org/10.1016/j.biortech.2008.05.027 >.

HENNING, J.; JAN BACH, K.; CLAUS, F. Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. **Biofuels, Bioproducts and Biorefining,** v. 1, n. 2, p. 119-134, 2007. ISSN 1932-1031. Available at: < http://dx.doi.org/10.1002/bbb.4 >.

HENZE, M.; HARREMOËS, P. Anaerobic Treatment of Wastewater in Fixed Film Reactors — A Literature Review. **Water Science and Technology,** v. 15, n. 8-9, p. 1-101, 1983.

HERBEL, Z. et al. Exploitation of the extremely thermophilic Caldicellulosiruptor saccharolyticus in hydrogen and biogas production from biomasses. **Environmental Technology,** v. 31, n. 8-9, p. 1017-1024, 2010/07/01 2010. ISSN 0959-3330. Available at: < http://dx.doi.org/10.1080/09593330.2010.484075 >.

HILL, D. T.; BOLTE, J. P. Digester stress as related to iso-butyric and iso-valeric acids. **Biological Wastes,** v. 28, n. 1, p. 33-37, 1989/01/01 1989. ISSN 0269-7483. Available at: < http://www.sciencedirect.com/science/article/pii/0269748389900475 >.

HILL, J. E. et al. cpnDB: A Chaperonin Sequence Database. **Genome Research,** v. 14, n. 8, p. 1669-1675, 04/01/received

05/28/accepted 2004. ISSN 1088-9051. Available at: < http://www.ncbi.nlm.nih.gov/pmc/articles/PMC509277/ >.

HIMMEL, M. E. et al. Biomass Recalcitrance: Engineering Plants and Enzymes for Biofuels Production. **Science,** v. 315, n. 5813, p. 804-807, February 9, 2007 2007. Available at: < http://www.sciencemag.org/content/315/5813/804.abstract >.

HIPPE, H.; STACKEBRANDT, E. Desulfosporosinus. In: (Ed.). **Bergey's Manual of Systematics of Archaea and Bacteria**: John Wiley & Sons, Ltd, 2015. ISBN 9781118960608.

HIRAKATA, Y. et al. Effects of Predation by Protists on Prokaryotic Community Function, Structure, and Diversity in Anaerobic Granular Sludge. **Microbes and Environments,** v. 31, n. 3, p. 279-287, 07/12

04/04/received

05/20/accepted 2016. ISSN 1342-6311

1347-4405. Available at: < http://www.ncbi.nlm.nih.gov/pmc/articles/PMC5017804/ >.

HON, D.; SHIRAISHI, N. **Wood and cellulosic chemistry**: CRC Press 2001.

HU, F.; RAGAUSKAS, A. Pretreatment and Lignocellulosic Chemistry. **Bioenergy Research,** v. 5, n. 4, p. 1043-1066, Dec 2012. ISSN 1939-1234. Available at: < <Go to ISI>://WOS:000310377600022 >.

HUBER, R. et al. Thermotoga maritima sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90 oC. **Arch. Microbiol.,** v. 144, p. 324-333, 1986.

IGARASHI, K. et al. Traffic Jams Reduce Hydrolytic Efficiency of Cellulase on Cellulose Surface. **Science,** v. 333, n. 6047, p. 1279-1282, 2011. ISSN 0036-8075. Available at: < http://dx.doi.org/10.1126/science.1208386 >.

IMACHI, H.; SAKAI, S. Methanoregulaceae. In: (Ed.). **Bergey's Manual of Systematics of Archaea and Bacteria**: John Wiley & Sons, Ltd, 2015. ISBN 9781118960608.

IMACHI, H. et al. Methanolinea tarda gen. nov., sp. nov., a methane-producing archaeon isolated from a methanogenic digester sludge. **INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY,** v. 58, n. 1, p. 294-301, 2008. ISSN 1466-5026. Available at: < http://dx.doi.org/10.1099/ijs.0.65394-0 >.

INAGAKI, F. et al. Sulfurovum lithotrophicum gen. nov., sp. nov., a novel sulfur-oxidizing chemolithoautotroph within the ε-Proteobacteria isolated from Okinawa Trough hydrothermal sediments. **International Journal of Systematic and Evolutionary Microbiology,** v. 54, n. 5, p. 1477-1482, 2004. Available at: < http://ijs.microbiologyresearch.org/content/journal/ijsem/10.1099/ijs.0.03042-0 >.

IRVINE, G. M. The significance of the glass transition of lignin in thermomechanical pulping. **Wood Science and Technology,** v. 19, n. 2, p. 139-149, 1985. ISSN 1432-5225. Available at: < http://dx.doi.org/10.1007/BF00353074 >.

IWONA, C.; GRZEGORZ, B.; HANWU, L. Hydrothermal Pretreatment of Lignocellulosic Biomass. In: GU, T. (Ed.). **Green Biomass Pretreatment for Biofuels Production**. Dordrecht Heidelberg New York London: Springer 2013. ISBN 978-94-007-6052-3 (ebook).

JIN, F. et al. Hydrothermal conversion of carbohydrate biomass into formic acid at mild temperatures. **Green Chemistry,** v. 10, n. 6, p. 612-615, 2008. ISSN 1463-9262. Available at: < http://dx.doi.org/10.1039/B802076K >.

JIN, F. et al. Controlling Hydrothermal Reaction Pathways To Improve Acetic Acid Production from Carbohydrate Biomass. **Environmental Science & Technology,** v. 39, n. 6, p. 1893-1902, 2005/03/01 2005. ISSN 0013-936X. Available at: < http://dx.doi.org/10.1021/es048867a >.

JOLLEZ, P.; CHORNET, E.; OVEREND, R. Steam-aqueous fractionation of sugar cane bagasse: An optimization study of process conditions at the pilot plant level. In: BRIDGEWATER, A. V. (Ed.). **Advances in thermochemical biological conversion**, 1994. p.1659-1669.

JÖNSSON, L. J.; MARTÍN, C. Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. **Bioresource Technology,** v. 199, p. 103-112, 1// 2016. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852415014042 >.

JÖRN, B. et al. Effects of thermobarical pretreatment of cattle waste as feedstock for anaerobic digestion. **Waste Management**, 2014. ISSN 0956-053X. Available at: < http://dx.doi.org/10.1016/j.wasman.2013.10.023 >.

JU, F.; ZHANG, T. Novel microbial populations in ambient and mesophilic biogas-producing and phenol-degrading consortia unraveled by high-throughput sequencing. **Microb Ecol,** v. 68, n. 2, p. 235-46, Aug 2014. ISSN 0095-3628.

JUMAS-BILAK, E.; ROUDIERE, L.; MARCHANDIN, H. Description of 'Synergistetes' phyl. nov. and emended description of the phylum 'Deferribacteres' and of the family Syntrophomonadaceae, phylum 'Firmicutes'. **Int J Syst Evol Microbiol,** v. 59, n. Pt 5, p. 1028-35, May 2009. ISSN 1466-5026 (Print)

1466-5026.

KABEL, M. A. et al. Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. **Bioresource Technology,** v. 98, n. 10, p. 2034-2042, 7// 2007. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852406004032 >.

KADLA, J. F.; CHANG, H. M. The Reactions of Peroxides with Lignin and Lignin Model Compounds. In: (Ed.). **Oxidative Delignification Chemistry**: American Chemical Society, v.785, 2001. chap. 6, p.108-129. (ACS Symposium Series). ISBN 0-8412-3738-7.

KAKSONEN, A. H. et al. Culturable Diversity and Community Fatty Acid Profiling of Sulfate-Reducing Fluidized-Bed Reactors Treating Acidic, Metal-Containing Wastewater. **Geomicrobiology Journal,** v. 21, n. 7, p. 469-480, 2004/10/01 2004. ISSN 0149-0451. Available at: < http://dx.doi.org/10.1080/01490450490505455 >.

KAMAGATA, Y.; MIKAMI, E. Isolation and Characterization of a Novel Thermophilic Methanosaeta Strain. **International Journal of Systematic and Evolutionary Microbiology,** v. 41, n. 2, p. 191-196, 1991. Available at: < http://ijs.microbiologyresearch.org/content/journal/ijsem/10.1099/00207713-41-2-191 >.

KANOKRATANA, P. et al. Phylogenetic analysis and metabolic potential of microbial communities in an industrial bagasse collection site. **Microb Ecol,** v. 66, n. 2, p. 322-34, Aug 2013. ISSN 0095-3628.

KAPARAJU, P.; FELBY, C. Characterization of lignin during oxidative and hydrothermal pre-treatment processes of wheat straw and corn stover. **Bioresource Technology,** v. 101, n. 9, p. 3175-3181, 5// 2010. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852409016368 >.

KAPARAJU, P.; SERRANO, M.; ANGELIDAKI, I. Effect of reactor configuration on biogas production from wheat straw hydrolysate. **Bioresource Technology,** v. 100, n. 24, p. 6317-6323, 12// 2009. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852409007822 >.

KARIMI, K.; TAHERZADEH, M. J. A critical review of analytical methods in pretreatment of lignocelluloses: Composition, imaging, and crystallinity. **Bioresource Technology,** v. 200, p. 1008-1018, 1// 2016. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852415015485 >.

KARP, S. G. et al. Pretreatment strategies for delignification of sugarcane bagasse: a review. **Brazilian Archives of Biology and Technology,** v. 56, p. 679-689, 2013. ISSN 1516-8913. Available at: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1516-89132013000400019&nrm=iso >.

KATO, S. et al. Methanogenic degradation of lignin-derived monoaromatic compounds by microbial enrichments from rice paddy field soil. **Scientific Reports,** v. 5, p. 14295, 2015. Available at: < http://dx.doi.org/10.1038/srep14295 >.

KATUKURI, N. R. et al. Enhanced methane production of Miscanthus floridulus by hydrogen peroxide pretreatment. **Fuel,** v. 199, p. 562-566, 7/1/ 2017. ISSN 0016-2361. Available at: < http://www.sciencedirect.com/science/article/pii/S0016236117302879 >.

KAVUMA, C. **Variation of Methane and Carbon dioxide Yield in a biogas plant**. 2013. 46 (MSc). Department of Energy Technology, Royal Institute of Technology, Stockholm, Sweden.

KAYHANIAN, M.; RICH, D. Pilot-scale high solids thermophilic anaerobic digestion of municipal solid waste with an emphasis on nutrient requirements. **Biomass and Bioenergy,** v. 8, n. 6, p. 433-444, // 1995. ISSN 0961-9534. Available at: < http://www.sciencedirect.com/science/article/pii/0961953495000437 >.

KHANAL, S. K. **Anaerobic Biotechnology for Bioenergy Production: Principles and Applications**. KHANL, S. K.: Wiley-Blackwell 2008.

KIM, J. **Characterization population dynamics in acidogenesis of various substrates by use of 16 S rRNA gene quantification: diversity and biokinetics.** . 2007. (PhD). POSTECH, Pohang - South Korea.

KIM, K. H.; TUCKER, M.; NGUYEN, Q. Conversion of bark-rich biomass mixture into fermentable sugar by two-stage dilute acid-catalyzed hydrolysis. **Bioresour Technol,** v. 96, n. 11, p. 1249-55, Jul 2005. ISSN 0960-8524 (Print)

0960-8524.

KIM, S. et al. A pilot scale two-stage anaerobic digester treating food waste leachate (FWL): Performance and microbial structure analysis using pyrosequencing. **Process Biochemistry,** v. 49, n. 2, p. 301-308, 2// 2014. ISSN 1359-5113. Available at: < //www.sciencedirect.com/science/article/pii/S1359511313006247 >.

KIM, S.; LEE, Y. Y. Fractionation of herbaceous biomass by ammonia-hydrogen peroxide percolation treatment. **Applied Biochemistry and Biotechnology,** v. 57-58, n. 1, p. 147-156, 1996. ISSN 0273-2289. Available at: < http://dx.doi.org/10.1007/BF02941695 >.

KIRKEGAARD, R. H. et al. Genomic insights into members of the candidate phylum Hyd24-12 common in mesophilic anaerobic digesters. **ISME J**, 04/08/online 2016. ISSN 1751-7370. Available at: < http://dx.doi.org/10.1038/ismej.2016.43 >.

KO, J. J. et al. Biodegradation of high molecular weight lignin under sulfate reducing conditions: Lignin degradability and degradation by-products. **Bioresource …**, 2009. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852408007943 >.

KO, J. K. et al. Effect of liquid hot water pretreatment severity on properties of hardwood lignin and enzymatic hydrolysis of cellulose. **Biotechnology and Bioengineering,** v. 112, n. 2, p. 252-262, 2015. ISSN 1097-0290. Available at: < http://dx.doi.org/10.1002/bit.25349 >.

KOBAYASHI, F. et al. Methane production from steam-exploded bamboo. **Journal of Bioscience and Bioengineering,** v. 97, n. 6, p. 426428, 2004. ISSN 1389-1723. Available at: < http://dx.doi.org/10.1016/S1389-1723(04)70231-5 >.

KOGA, S. et al. Novel bacterial peroxidase without catalase activity from Flavobacterium meningosepticum: purification and characterization. **Biochim Biophys Acta,** v. 1435, n. 1-2, p. 117-26, Nov 16 1999. ISSN 0006-3002 (Print)

0006-3002.

KONG, L. et al. Preliminary purification and characterization of extracellular lignin peroxidase from Pseudomonas fluorescens GcM5-1A. **Journal of Beijing Forestry University,** v. 32, n. 3, p. 112-116, 2010. ISSN 1000-1522.

KOPONEN, H. **Biogas production from ensiled maize with and without hydrothermal pretreatment**. 2010. 24 (Masters). Department of Biological and Environmental Science, UNIVERSITY OF JYVÄSKYLÄ

Finland.

KOUKKOU, A.-I. **Microbial Bioremediation of Non-metals: Current Research | Book**. Greece: Caister Academic Press, 2011. ISBN 978-1-904455-83-7.

KOVÁCS, E. et al. Augmented biogas production from protein-rich substrates and associated metagenomic changes. **Bioresource Technology,** v. 178, p. 254-261, 2// 2015. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852414012279 >.

KRAUSE, L. et al. Taxonomic composition and gene content of a methane-producing microbial community isolated from a biogas reactor. **Journal of Biotechnology,** v. 136, n. 1–2, p. 91-101, 8/31/ 2008. ISSN 0168-1656. Available at: < //www.sciencedirect.com/science/article/pii/S0168165608002629 >.

KRIEG, N. R. et al. Phylum XIV. Bacteroidetes phyl. nov. In: KRIEG, N. R.;PARTE, A., *et al* (Ed.). **Bergey's Manual of Systematic Bacteriology**. 2nd, v.4, 2010. p.25.

KRISTENSEN, J. B. et al. Cell-wall structural changes in wheat straw pretreated for bioethanol production. **Biotechnology for Biofuels,** v. 1, n. 1, p. 1-9, 2008. ISSN 1754-6834. Available at: < http://dx.doi.org/10.1186/1754-6834-1-5 >.

KUBLIK, A. et al. Identification of a multi-protein reductive dehalogenase complex in Dehalococcoides mccartyi strain CBDB1 suggests a protein-dependent respiratory electron transport chain obviating quinone involvement. **Environmental Microbiology,** v. 18, n. 9, p. 3044-3056, 2016. ISSN 1462-2920. Available at: < http://dx.doi.org/10.1111/1462-2920.13200 >.

KUDO, Y. et al. Methanogen flora of paddy soils in Japan. **FEMS Microbiology Ecology,** v. 22, n. 1, p. 39-48, 1997. ISSN 1574-6941. Available at: < http://dx.doi.org/10.1111/j.1574-6941.1997.tb00354.x >.

KUEVER, J.; SCHINK, B. Syntrophus. In: (Ed.). **Bergey's Manual of Systematics of Archaea and Bacteria**: John Wiley & Sons, Ltd, 2015. ISBN 9781118960608.

KUMAR, R. et al. Carbohydrate derived-pseudo-lignin can retard cellulose biological conversion. **Biotechnol Bioeng,** v. 110, n. 3, p. 737-53, Mar 2013. ISSN 0006-3592.

KUPPARDT, A. et al. Phylogenetic and Functional Diversity Within Toluene-Degrading, Sulphate-Reducing Consortia Enriched from a Contaminated Aquifer. **Microbial Ecology,** v. 68, n. 2, p. 222-234, 2014. ISSN 1432-184X. Available at: < http://dx.doi.org/10.1007/s00248-014-0403-8 >.

LANGE, H.; DECINA, S.; CRESTINI, C. Oxidative upgrade of lignin – Recent routes reviewed. **European Polymer Journal,** v. 49, n. 6, p. 1151-1173, 6// 2013. ISSN 0014-3057. Available at: < http://www.sciencedirect.com/science/article/pii/S0014305713001183 >.

LEE, J. W.; JEFFRIES, T. W. Efficiencies of acid catalysts in the hydrolysis of lignocellulosic biomass over a range of combined severity factors. **Bioresour Technol,** v. 102, n. 10, p. 5884-90, May 2011. ISSN 0960-8524.

LEE, S.-H. et al. Monitoring bacterial community structure and variability in time scale in full-scale anaerobic digesters. **Journal of Environmental Monitoring,** v. 14, n. 7, p. 1893-1905, 2012. ISSN 1464-0325. Available at: < http://dx.doi.org/10.1039/C2EM10958A >.

LEVEN, L.; ERIKSSON, A. R.; SCHNURER, A. Effect of process temperature on bacterial and archaeal communities in two methanogenic bioreactors treating organic household waste. **FEMS Microbiol Ecol,** v. 59, n. 3, p. 683-93, Mar 2007. ISSN 0168-6496 (Print)

0168-6496.

LI, A. et al. A pyrosequencing-based metagenomic study of methane-producing microbial community in solid-state biogas reactor. **Biotechnology for Biofuels,** v. 6, n. 1, p. 3, 2013// 2013. ISSN 1754-6834. Available at: < http://dx.doi.org/10.1186/1754-6834-6-3 >.

LI, C. et al. Assessment of regional biomass as co-substrate in the anaerobic digestion of chicken manure: Impact of co-digestion with chicken processing waste, seagrass and Miscanthus. **Biochemical Engineering Journal,** v. 118, p. 1-10, 2/15/ 2017. ISSN 1369-703X. Available at: < http://www.sciencedirect.com/science/article/pii/S1369703X1630314X >.

LI, H.-Q. et al. Pretreatment on Miscanthus lutarioriparious by liquid hot water for efficient ethanol production. **Biotechnology for Biofuels,** v. 6, n. 1, p. 1-10, 2013. ISSN 1754-6834. Available at: < http://dx.doi.org/10.1186/1754-6834-6-76 >.

LI, H. et al. Investigation of lignin deposition on cellulose during hydrothermal pretreatment, its effect on cellulose hydrolysis, and underlying mechanisms. **Biotechnology and Bioengineering,** v. 111, n. 3, p. 485-492, 2014. ISSN 1097-0290. Available at: < http://dx.doi.org/10.1002/bit.25108 >.

LI, J.; HENRIKSSON, G.; GELLERSTEDT, G. Lignin depolymerization/repolymerization and its critical role for delignification of aspen wood by steam explosion. **Bioresource Technology,** v. 98, n. 16, p. 3061-3068, 11// 2007. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852406005724 >.

LI, L. et al. A mesophilic anaerobic digester for treating food waste: process stability and microbial community analysis using pyrosequencing. **Microbial Cell Factories,** v. 15, n. 1, p. 65, 2016// 2016. ISSN 1475-2859. Available at: < http://dx.doi.org/10.1186/s12934-016-0466-y >.

LI, X. et al. Evaluation biogas production performance and dynamics of the microbial community in different straw. **J Microbiol Biotechnol**, Nov 04 2016. ISSN 1017-7825.

LIEW, L. N.; SHI, J.; LI, Y. Methane production from solid-state anaerobic digestion of lignocellulosic biomass. **Biomass and Bioenergy,** v. 46, p. 125-132, 11// 2012. ISSN 0961-9534. Available at: < http://www.sciencedirect.com/science/article/pii/S0961953412003546 >.

LILIS, S. et al. Effect of hydrothermolysis process conditions on pretreated switchgrass composition and ethanol yield by SSF with Kluyveromyces marxianus IMB4. **Process Biochemistry**, 2009. ISSN 1359-5113. Available at: < http://dx.doi.org/10.1016/j.procbio.2009.01.011 >.

LIMAM, R. D. et al. Members of the uncultured bacterial candidate division WWE1 are implicated in anaerobic digestion of cellulose. **Microbiologyopen,** v. 3, n. 2, p. 157-67, Apr 2014. ISSN 2045-8827.

LIN, C.-Y.; HU, Y.-Y. Mesophilic degradation of butyric acid in anaerobic digestion. **Journal of Chemical Technology & Biotechnology,** v. 56, n. 2, p. 191-194, 1993. ISSN 1097-4660. Available at: < http://dx.doi.org/10.1002/jctb.280560212 >.

LINKS, M. et al. The Chaperonin-60 Universal Target Is a Barcode for Bacteria That Enables De Novo Assembly of Metagenomic Sequence Data. **PLoS One,** v. 7, n. 11, p. e49755, 2012.

LISBOA, C. C. et al. Bioethanol production from sugarcane and emissions of greenhouse gases – known and unknowns. **GCB Bioenergy,** v. 3, n. 4, p. 277-292, 2011. ISSN 1757-1707. Available at: < http://dx.doi.org/10.1111/j.1757-1707.2011.01095.x >.

LIU, C.; CHARLES, E. W. Partial flow of compressed-hot water through corn stover to enhance hemicellulose sugar recovery and enzymatic digestibility of cellulose. **Bioresource Technology**, 2005. ISSN 0960-8524. Available at: < http://dx.doi.org/10.1016/j.biortech.2005.01.012 >.

LIU, C.; WYMAN, C. E. Partial flow of compressed-hot water through corn stover to enhance hemicellulose sugar recovery and enzymatic digestibility of cellulose. **Bioresour Technol,** v. 96, n. 18, p. 1978-85, Dec 2005. ISSN 0960-8524 (Print)

0960-8524.

LIU, F. H. et al. The structure of the bacterial and archaeal community in a biogas digester as revealed by denaturing gradient gel electrophoresis and 16S rDNA sequencing analysis. **J Appl Microbiol,** v. 106, n. 3, p. 952-66, Mar 2009. ISSN 1364-5072.

LIU, Z. L. et al. Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran. **Journal of Industrial Microbiology and Biotechnology,** v. 31, n. 8, p. 345-352, 2004. ISSN 1476-5535. Available at: < http://dx.doi.org/10.1007/s10295-004-0148-3 >.

LLOYD, T. A.; WYMAN, C. E. Predicted Effects of Mineral Neutralization and Bisulfate Formation on Hydrogen Ion Concentration for Dilute Sulfuric Acid Pretreatment. In: FINKELSTEIN, M.;MCMILLAN, J. D., *et al* (Ed.). **Proceedings of the Twenty-Fifth Symposium on Biotechnology for Fuels and Chemicals Held May 4–7, 2003, in Breckenridge, CO**. Totowa, NJ: Humana Press, 2004. p.1013-1022. ISBN 978-1-59259-837-3.

LU, X. B.; ZHANG, Y. M.; ANGELIDAKI, I. Optimization of H2SO4-catalyzed hydrothermal pretreatment of rapeseed straw for bioconversion to ethanol: Focusing on pretreatment at high solids content. **Bioresource Technology,** v. 100, n. 12, p. 3048-3053, Jun 2009. ISSN 0960-8524. Available at: < <Go to ISI>://WOS:000265335400024 >.

LUCAS, M. et al. Evidence for room temperature delignification of wood using hydrogen peroxide and manganese acetate as a catalyst. **Bioresource Technology,** v. 119, p. 174-180, 9// 2012. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852412008395 >.

LYNAM, J. G. et al. Hydrothermal carbonization of various lignocellulosic biomass. **Biomass Conversion and Biorefinery,** v. 5, n. 2, p. 173-181, 2014. ISSN 2190-6823. Available at: < http://dx.doi.org/10.1007/s13399-014-0137-3 >.

MA, J. et al. The mechanism of xylans removal during hydrothermal pretreatment of poplar fibers investigated by immunogold labeling. **Planta,** v. 242, n. 1, p. 327-337, 2015. ISSN 1432-2048. Available at: < http://dx.doi.org/10.1007/s00425-015-2313-5 >.

MA, X. J. et al. Hydrothermal pretreatment of bamboo and cellulose degradation. **Bioresource technology,** v. 148, p. 408-413, 2013. ISSN 0960-8524. Available at: < http://dx.doi.org/10.1016/j.biortech.2013.09.021 >.

MAGURRAN, A. E. **Measuring Biological Diversity**. Blackwell Publishing Company, 2004.

MAPA. Ministério da Agricultura, P. e A. 2016. Avaialble at: < http://www.agricultura.gov.br/vegetal/culturas/cana-de-acucar >.

MARASABESSY, A. et al. Dilute H2SO4-catalyzed hydrothermal pretreatment to enhance enzymatic digestibility of Jatropha curcas fruit hull for ethanol fermentation. **International Journal of Energy and Environmental Engineering,** v. 3, n. 1, p. 15, 2012. ISSN 2251-6832. Available at: < http://dx.doi.org/10.1186/2251-6832-3-15 >.

MARTÍN, C. et al. Enzyme loading dependence of cellulose hydrolysis of sugarcane bagasse. **Química Nova,** v. 35, p. 1927-1930, 2012. ISSN 0100-4042. Available at: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0100-40422012001000007&nrm=iso >.

MENDEZ, L. et al. Effect of high pressure thermal pretreatment on Chlorella vulgaris biomass: Organic matter solubilisation and biochemical methane potential. **Fuel,** v. 117, p. 674-679, 2014. ISSN 0016-2361. Available at: < http://dx.doi.org/10.1016/j.fuel.2013.09.032 >.

MENG, J. et al. Genetic and functional properties of uncultivated MCG archaea assessed by metagenome and gene expression analyses. **Isme j,** v. 8, n. 3, p. 650-9, Mar 2014. ISSN 1751-7362.

MICHALSK, K.; LEDAKOWICZ, S. Alkaline hydrogen peroxide pretreatment of energy crops for biogas production. **Chemical Papers,** v. 68, n. 7, p. 913-922, 2014. ISSN 1336-9075. Available at: < http://dx.doi.org/10.2478/s11696-013-0531-5 >.

MITAL, K. M. **Biogas Systems: Principles and Applications**. New Delhi, India: New Age International (P) Limited Publishers, 1996.

MITTAL, A. et al. Modeling xylan solubilization during autohydrolysis of sugar maple wood meal: Reaction kinetics. **Holzforschung,** v. 63, p. 307–314, 2009.

MIURA, Y.; OKABE, S. Quantification of Cell Specific Uptake Activity of Microbial Products by Uncultured Chloroflexi by Microautoradiography Combined with Fluorescence In Situ Hybridization. **Environmental Science & Technology,** v. 42, n. 19, p. 7380-7386, 2008/10/01 2008. ISSN 0013-936X. Available at: < http://dx.doi.org/10.1021/es800566e >.

MIZUNO, O.; LI, M. Y. Y.; NOIKE1, M. T. THE BEHAVIOR OF SULFATE-REDUCING BACTERIA IN ACIDOGENIC PHASE OF ANAEROBIC DIGESTION. **Water Res,** v. 32, n. 5, p. 1626-1634, 1998.

MOESTEDT, J. **Biogas production from thin stillage**. 2015. (PhD). Department of Microbiology, University of Uppsala, Uppsala - Sweden.

MOHANTY, A. K.; MISRA, M.; HINRICHSEN, G. Biofibres, biodegradable polymers and biocomposites: An overview. **Macromolecular Materials and Engineering,** v. 276-277, n. 1, p. 1-24, 2000. ISSN 1439-2054. Available at: < http://dx.doi.org/10.1002/(SICI)1439-2054(20000301)276:1<1::AID-MAME1>3.0.CO;2-W >.

MOK, W. S.; ANTAL, M. J.; VARHEGYI, G. Productive and parasitic pathways in dilute acid-catalyzed hydrolysis of cellulose. **Industrial & Engineering Chemistry Research,** v. 31, n. 1, p. 94-100, 1992. ISSN 0888-5885. Available at: < http://dx.doi.org/10.1021/ie00001a014 >.

MONLAU, F. et al. Lignocellulosic Materials Into Biohydrogen and Biomethane: Impact of Structural Features and Pretreatment. **Critical Reviews in Environmental Science and Technology,** v. 43, n. 3, p. 260-322, 2013. ISSN 1064-3389. Available at: < http://dx.doi.org/10.1080/10643389.2011.604258 >.

MONLAU, F. et al. Do furanic and phenolic compounds of lignocellulosic and algae biomass hydrolyzate inhibit anaerobic mixed cultures? A comprehensive review. **Biotechnology advances,** v. 32, n. 5, p. 934-951, 2014. ISSN 0734-9750. Available at: < http://dx.doi.org/10.1016/j.biotechadv.2014.04.007 >.

MONTANÉ, D. et al. Phenomenological kinetics of wood delignification: application of a time-dependent rate constant and a generalized severity parameter to pulping and correlation of pulp properties. **Wood Science and Technology,** v. 28, n. 6, p. 387-402, 1994. ISSN 1432-5225. Available at: < http://dx.doi.org/10.1007/BF00225458 >.

MOORE, D.; ROBSON, G. D.; TRINCI, A. P. J. **21st century guidebook to fungi**. Cambridge, UK: Cambridge University Press, 2011.

MORRISON, M.; MIRON, J. Adhesion to cellulose by Ruminococcus albus: a combination of cellulosomes and Pil-proteins?1. **FEMS Microbiology Letters,** v. 185, n. 2, p. 109-115, 4/15/ 2000. ISSN 0378-1097. Available at: < //www.sciencedirect.com/science/article/pii/S0378109700000409 >.

MOSELE, J. I.; MACIÀ, A.; MOTILVA, M.-J. Metabolic and microbial modulation of the large intestine ecosystem by non-absorbed diet phenolic compounds: a review. **Molecules,** v. 20, n. 9, p. 17429-17468, 2015.

MOSIER, N. et al. Features of promising technologies for pretreatment of lignocellulosic biomass. **Bioresource Technology,** v. 96, n. 6, p. 673-686, 4// 2005. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852404002536 >.

MOTTERAN, F. et al. Methanogenic potential of an anaerobic sludge in the presence of anionic and nonionic surfactants. **International Biodeterioration & Biodegradation,** v. 96, p. 198-204, 12// 2014. ISSN 0964-8305. Available at: < http://www.sciencedirect.com/science/article/pii/S0964830514002893 >.

MOUTTA, R. et al. Comparative Response and Structural Characterization of Sugarcane Bagasse, Straw and Bagasse-Straw 1:1 Mixtures Subjected to Hydrothermal Pretreatment and Enzymatic Conversion.

Journal of Microbial & Biochemical Technology, v. 01, n. S12, 2013. Available at: < http://dx.doi.org/10.4172/1948-5948.S12-005 >.

MUHAMMAD, N. I.; MOHD GHAZI, T. I.; OMAR, R. Production of biogas from solid organic wastes through anaerobic digestion: a review. **Appl Microbiol Biotechnol,** v. 95, n. 2, p. 321-9, Jul 2012. ISSN 0175-7598.

MURTO, M.; BJÖRNSSON, L.; MATTIASSON, B. Impact of food industrial waste on anaerobic co-digestion of sewage sludge and pig manure. **Journal of Environmental Management,** v. 70, n. 2, p. 101-107, 2// 2004. ISSN 0301-4797. Available at: < http://www.sciencedirect.com/science/article/pii/S0301479703001981 >.

MUSSATTO, S. I.; ROBERTO, I. C. Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: a review. **Bioresource Technology,** v. 93, n. 1, p. 1-10, 5// 2004. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852403002827 >.

MUYZER, G.; DE WAAL, E. C.; UITTERLINDEN, A. G. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. **Applied and Environmental Microbiology,** v. 59, n. 3, p. 695-700, 1993. ISSN 0099-2240

1098-5336. Available at: < http://www.ncbi.nlm.nih.gov/pmc/articles/PMC202176/ >.

MUYZER, G.; STAMS, A. J. M. The ecology and biotechnology of sulphate-reducing bacteria. **Nat Rev Micro,** v. 6, n. 6, p. 441-454, 06//print 2008. ISSN 1740-1526. Available at: < http://dx.doi.org/10.1038/nrmicro1892 >.

NAKAGAME, S. et al. Enhancing the enzymatic hydrolysis of lignocellulosic biomass by increasing the carboxylic acid content of the associated lignin. **Biotechnology and Bioengineering,** v. 108, n. 3, p. 538-548, 2011. ISSN 1097-0290. Available at: < http://dx.doi.org/10.1002/bit.22981 >.

NAKAGAWA, Y. Cytophagaceae. In: (Ed.). **Bergey's Manual of Systematics of Archaea and Bacteria**: John Wiley & Sons, Ltd, 2015. ISBN 9781118960608.

NARIHIRO, T. et al. Comparative analysis of bacterial and archaeal communities in methanogenic sludge granules from upflow anaerobic sludge blanket reactors treating various food-processing, high-strength organic wastewaters. **Microbes Environ,** v. 24, n. 2, p. 88-96, 2009. ISSN 1342-6311 (Print)

1342-6311.

NARIHIRO, T. et al. Quantitative detection of previously characterized syntrophic bacteria in anaerobic wastewater treatment systems by sequence-specific rRNA cleavage method. **Water Research,** v. 46, n. 7, p. 2167-2175, 5/1/ 2012. ISSN 0043-1354. Available at: < http://www.sciencedirect.com/science/article/pii/S0043135412000541 >.

NAYAK, B. S. et al. Microbial population dynamics in laboratory-scale solid waste bioreactors in the presence or absence of biosolids. **J Appl Microbiol,** v. 107, n. 4, p. 1330-9, Oct 2009. ISSN 1364-5072.

NESBO, C. L. et al. Evidence for existence of "mesotogas," members of the order Thermotogales adapted to low-temperature environments. **Appl Environ Microbiol,** v. 72, n. 7, p. 5061-8, Jul 2006. ISSN 0099-2240 (Print)

0099-2240. Available at: < https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1489369/pdf/0342-06.pdf >.

NIELSEN, J.; VILLADSEN, J.; LIDEN, G. **Bioreaction Engineering Principles**. Springer, 2011. ISBN 978-1-4419-9688-6.

NILADEVI, K. N. et al. Optimization of laccase production from a novel strain—Streptomyces psammoticus using response surface methodology. **Microbiological Research,** v. 164, n. 1, p. 105-113, 1// 2009. ISSN 0944-5013. Available at: < http://www.sciencedirect.com/science/article/pii/S0944501306001303 >.

NITSOS, C. K.; MATIS, K. A.; TRIANTAFYLLIDIS, K. S. Optimization of Hydrothermal Pretreatment of Lignocellulosic Biomass in the Bioethanol Production Process. **Chemsuschem,** v. 6, n. 1, p. 110-122, Jan 2013. ISSN 1864-5631. Available at: < <Go to ISI>://WOS:000313702200016 >.

NITSOS, C. K. et al. The role of catalytic pretreatment in biomass valorization toward fuels and chemicals. In: TRIANTAFYLLIDIS, K. S.;LAPPAS, A. A., *et al* (Ed.). **The role of catalysis for the sustainable production of biofuels and biochemicals**. Great Britain: Elsevier, 2013. ISBN 978-0-444-56330-9.

NORDHOFF, M.; WIELER, L. H. [Incidence and significance of treponemes in animals]. **Berl Munch Tierarztl Wochenschr,** v. 118, n. 1-2, p. 24-36, Jan-Feb 2005. ISSN 0005-9366 (Print)

0005-9366.

NUBEL, U. et al. Sequence heterogeneities of genes encoding 16S rRNAs in Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. **J Bacteriol,** v. 178, n. 19, p. 5636-43, Oct 1996. ISSN 0021-9193 (Print)

0021-9193.

O'SULLIVAN, C. A.; BURRELL, P. C. The effect of media changes on the rate of cellulose solubilisation by rumen and digester derived microbial communities. **Waste Manag,** v. 27, n. 12, p. 1808-14, 2007. ISSN 0956-053X (Print)

0956-053x.

OUDE ELFERINK, S. J. W. H. et al. Anaerobic Conversion of Lactic Acid to Acetic Acid and 1,2-Propanediol by Lactobacillus buchneri. **Applied and Environmental Microbiology,** v. 67, n. 1, p. 125-132, 05/15/received

10/11/accepted 2001. ISSN 0099-2240

1098-5336. Available at: < http://www.ncbi.nlm.nih.gov/pmc/articles/PMC92530/ >.

OVEREND, R. P.; CHORNET, E. Steam and aqueous pretreatments: are they prehydrolyses? In: CHICHESTER, E. H. L. (Ed.). **Wood processing and utilization**. New York: Elsevier, 1989. p.395-400.

OVEREND, R. P.; CHORNET, E.; GASCOIGNE, J. A. **Fractionation of Lignocellulosics by Steam-Aqueous Pretreatments [and Discussion]**. 1987. 523-536 Available at: < http://rsta.royalsocietypublishing.org/roypta/321/1561/523.full.pdf >.

PALMQVIST, E.; HAHN-HÄGERDAL, B. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. **Bioresource Technology,** v. 74, n. 1, p. 25-33, 8// 2000. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852499001613 >.

PANDIT, P. D. et al. Mining of hemicellulose and lignin degrading genes from differentially enriched methane producing microbial community. **Bioresource Technology,** v. 216, p. 923-930, 9// 2016. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852416308380 >.

PARNADI, P. S. et al. Improving the Effectiveness of Crude-Oil Hydrocarbon Biodegradation Employing Azotobacter chroococcum as Co-Inoculant. **Microbiol Indones,** v. 1, p. 5-10, 2007.

PASSOS, F.; FERRER, I. Influence of hydrothermal pretreatment on microalgal biomass anaerobic digestion and bioenergy production. **Water Research,** v. 68, p. 364-373, Jan 1 2015. ISSN 0043-1354. Available at: < <Go to ISI>://WOS:000347756900032 >.

PEDERSEN, M.; MEYER, A. S. Lignocellulose pretreatment severity - relating pH to biomatrix opening. **New biotechnology,** v. 27, n. 6, p. 739-750, 2010. ISSN 1871-6784. Available at: < http://dx.doi.org/10.1016/j.nbt.2010.05.003 >.

PELLETIER, E. et al. "Candidatus Cloacamonas acidaminovorans": genome sequence reconstruction provides a first glimpse of a new bacterial division. **J Bacteriol,** v. 190, n. 7, p. 2572-9, Apr 2008. ISSN 0021-9193.

PENGYU, D. et al. Comparison of dry and wet milling pre-treatment methods for improving the anaerobic digestion performance of the Pennisetum hybrid. **RSC Advances,** v. 7, n. 21, p. 12610-12619, 2017. Available at: < http://dx.doi.org/10.1039/C6RA27822A >.

PENTEADO, E. D. et al. Influence of seed sludge and pretreatment method on hydrogen production in packed-bed anaerobic reactors. **International Journal of Hydrogen Energy,** v. 38, n. 14, p. 6137-6145, 5/10/ 2013. ISSN 0360-3199. Available at: < http://www.sciencedirect.com/science/article/pii/S0360319913001535 >.

PÉREZ, J. A. et al. Effect of process variables on liquid hot water pretreatment of wheat straw for bioconversion to fuel-ethanol in a batch reactor. **Journal of Chemical Technology & Biotechnology,** v. 82, n. 10, p. 929-938, 2007. ISSN 1097-4660. Available at: < http://dx.doi.org/10.1002/jctb.1765 >.

PITARELO, A. P. **Avaliac¸˜ ao da susceptibilidade do bagac¸o e da palha de cana-de-ac¸´ ucar` a bioconvers˜ ao via pr´e-tratamento a vapor e hidr´ olise enzim´ atica (Evaluation of susceptibility of sugarcane bagasse and straw on the bioconversion by steamexplosion and enzymatic hydrolysis)**. 2007. (M.Sc.). Federal Univesity of Paran, Paran, Brazil.

POBEHEIM, H. et al. Characterization of an anaerobic population digesting a model substrate for maize in the presence of trace metals. **Chemosphere,** v. 80, n. 8, p. 829-836, 8// 2010. ISSN 0045-6535. Available at: < http://www.sciencedirect.com/science/article/pii/S0045653510006582 >.

PODOSOKORSKAYA, O. A. et al. Ornatilinea apprima gen. nov., sp. nov., a cellulolytic representative of the class Anaerolineae. **International Journal of Systematic and Evolutionary Microbiology,** v. 63, n. 1, p. 86-92, 2013. Available at: < http://ijs.microbiologyresearch.org/content/journal/ijsem/10.1099/ijs.0.041012-0 >.

QIAO, W. et al. Evaluation of biogas production from different biomass wastes with/without hydrothermal pretreatment. **Renewable Energy,** v. 36, n. 12, p. 3313-3318, Dec 2011. ISSN 0960-1481. Available at: < <Go to ISI>://WOS:000293424400011 >.

QIU, Y.-L. et al. Syntrophorhabdus aromaticivorans gen. nov., sp. nov., the First Cultured Anaerobe Capable of Degrading Phenol to Acetate in Obligate Syntrophic Associations with a Hydrogenotrophic Methanogen. **Applied and Environmental Microbiology,** v. 74, n. 7, p. 2051-2058, April 1, 2008 2008. Available at: < http://aem.asm.org/content/74/7/2051.abstract >.

QIU, Y.-L. et al. Identification and Isolation of Anaerobic, Syntrophic Phthalate Isomer-Degrading Microbes from Methanogenic Sludges Treating Wastewater from Terephthalate Manufacturing. **Applied and Environmental Microbiology,** v. 70, n. 3, p. 1617-1626, March 1, 2004 2004. Available at: < http://aem.asm.org/content/70/3/1617.abstract >.

QUÉMÉNEUR, M. et al. Inhibition of fermentative hydrogen production by lignocellulose-derived compounds in mixed cultures. **International Journal of Hydrogen Energy,** v. 37, n. 4, p. 3150-3159, 2// 2012. ISSN 0360-3199. Available at: < http://www.sciencedirect.com/science/article/pii/S0360319911025316 >.

QUITAIN, A. T. et al. Low-molecular-weight carboxylic acids produced from hydrothermal treatment of organic wastes. **Journal of Hazardous Materials,** v. 93, n. 2, p. 209-220, 7/22/ 2002. ISSN 0304-3894. Available at: < http://www.sciencedirect.com/science/article/pii/S0304389402000249 >.

RABELO, S. et al. Production of bioethanol, methane and heat from sugarcane bagasse in a biorefinery concept. **Bioresource technology,** v. 102, n. 17, p. 7887-7895, 2011. ISSN 0960-8524. Available at: < http://dx.doi.org/10.1016/j.biortech.2011.05.081 >.

RABELO, S. C. et al. Ethanol production from enzymatic hydrolysis of sugarcane bagasse pretreated with lime and alkaline hydrogen peroxide. **Biomass and Bioenergy,** v. 35, n. 7, p. 2600-2607, 7// 2011. ISSN 0961-9534. Available at: < http://www.sciencedirect.com/science/article/pii/S0961953411001176 >.

RAHIKAINEN, J. et al. Inhibition of enzymatic hydrolysis by residual lignins from softwood—study of enzyme binding and inactivation on lignin-rich surface. **Biotechnology and Bioengineering,** v. 108, n. 12, p. 2823-2834, 2011. ISSN 1097-0290. Available at: < http://dx.doi.org/10.1002/bit.23242 >.

RAINEY, F. A.; HOLLEN, B. J.; SMALL, A. M. Clostridium. In: (Ed.). **Bergey's Manual of Systematics of Archaea and Bacteria**: John Wiley & Sons, Ltd, 2015. ISBN 9781118960608.

RAINEY, F. A. et al. Isolation and characterization of an obligately anaerobic, polysaccharolytic, extremely thermophilic member of the genus Spirochaeta. **Archives of Microbiology,** v. 155, n. 4, p. 396-401, 1991. ISSN 1432-072X. Available at: < http://dx.doi.org/10.1007/BF00243461 >.

RALPH, J. et al. Lignins: Natural polymers from oxidative coupling of 4-hydroxyphenyl- propanoids. **Phytochemistry Reviews,** v. 3, n. 1, p. 29-60, 2004. ISSN 1572-980X. Available at: < http://dx.doi.org/10.1023/B:PHYT.0000047809.65444.a4 >.

RAMANATHAN, V.; FENG, Y. Air pollution, greenhouse gases and climate change: Global and regional perspectives. **Atmospheric Environment,** v. 43, n. 1, p. 37-50, 1// 2009. ISSN 1352-2310. Available at: < http://www.sciencedirect.com/science/article/pii/S1352231008008583 >.

RAMIREZ, R. S.; HOLTZAPPLE, M.; PIAMONTE, N. Fundamentals of biomass pretreatment at high pH. In: WYMAN, C. (Ed.). **Aqueous pretreatment of plant biomass for biological and chemical conversion to fuels and chemicals**. Noida, India: John Wiley & Sons, Ltd., 2013. p.145-167.

RAMOS, L. P. The chemistry involved in the steam treatment of lignocellulosic materials. **Quimica Nova,** v. 26, n. 6, p. 863-871, Nov-Dec 2003. ISSN 0100-4042. Available at: < <Go to ISI>://WOS:000186718500015 >.

REYSENBACH, A.-L. Thermotogales ord. nov. In: (Ed.). **Bergey's Manual of Systematics of Archaea and Bacteria**: John Wiley & Sons, Ltd, 2015. ISBN 9781118960608.

REZENDE, C. et al. Chemical and morphological characterization of sugarcane bagasse submitted to a delignification process for enhanced enzymatic digestibility. **Biotechnology for Biofuels,** v. 4, n. 1, p. 1-19, 2011/11/28 2011. Available at: < http://dx.doi.org/10.1186/1754-6834-4-54 >.

RIVARD, C. J. et al. Waste to energy: Nutrient requirements for aerobic and anaerobic digestion. **Journal Name: Journal of Environmental Health; (USA); Journal Volume: 52:2**, p. Medium: X; Size: Pages: 96-101, 1989.

RIVIERE, D. et al. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. **Isme j,** v. 3, n. 6, p. 700-14, Jun 2009. ISSN 1751-7362.

ROCHA, G. J. M. et al. Effect of Xylan and Lignin Removal by Hydrothermal Pretreatment on Enzymatic Conversion of Sugarcane Bagasse Cellulose for Second Generation Ethanol Production. **Sugar Tech,** v. 15, n. 4, p. 390-398, Dec 2013. ISSN 0972-1525. Available at: < <Go to ISI>://WOS:000327935800007 >.

ROLING, W. F. M. The Family Geobacteraceae. In: ROSENBERG, E. (Ed.). **The Prokaryotes: Deltaproteobacteria and Epsilonpoteobacteria**. 4th. New York: Springer, 2014. p.157-172.

ROMANÍ, A. et al. Experimental Assessment on the Enzymatic Hydrolysis of Hydrothermally Pretreated Eucalyptus globulus Wood. **Industrial & Engineering Chemistry Research,** v. 49, n. 10, p. 4653-4663, 2010. ISSN 0888-5885. Available at: < http://dx.doi.org/10.1021/ie100154m >.

ROTARU, A.-E. et al. Direct Interspecies Electron Transfer between Geobacter metallireducens and Methanosarcina barkeri. **Applied and Environmental Microbiology,** v. 80, n. 15, p. 4599-4605, August 1, 2014 2014. Available at: < http://aem.asm.org/content/80/15/4599.abstract >.

ROUCHES, E. et al. Improvement of anaerobic degradation by white-rot fungi pretreatment of lignocellulosic biomass: A review. **Renewable and Sustainable Energy Reviews,** v. 59, p. 179-198, 6// 2016. ISSN 1364-0321. Available at: < http://www.sciencedirect.com/science/article/pii/S1364032115017001 >.

ROWEL, R. M. **Handbook of wood chemistry and wood composites**. CRC press, 2005.

RUIZ, H. A. et al. Bioethanol production from hydrothermal pretreated wheat straw by a flocculating Saccharomyces cerevisiae strain – Effect of process conditions. **Fuel,** v. 95, p. 528-536, 5// 2012. ISSN 0016-2361. Available at: < http://www.sciencedirect.com/science/article/pii/S0016236111006788 >.

SAAKE, B.; LEHNEN, R. Liginin. In: BOHNET, M.;BELLUSSI, G., *et al* (Ed.). **Ullmann's Encyclopedia of Industrial Chemistry**. New Yoark: Wiley, 2007.

SAHA, B. C. et al. Hydrothermal pretreatment and enzymatic saccharification of corn stover for efficient ethanol production. **Industrial Crops and Products,** v. 44, p. 367-372, Jan 2013. ISSN 0926-6690. Available at: < <Go to ISI>://WOS:000315659400053 >.

SAINI, J. K.; SAINI, R.; TEWARI, L. Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments. **3 Biotech,** v. 5, n. 4, p. 337-353, 08/21

05/19/received

08/05/accepted 2015. ISSN 2190-572X

2190-5738. Available at: < http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4522714/ >.

SAKAI, S. et al. Methanolinea mesophila sp. nov., a hydrogenotrophic methanogen isolated from rice field soil, and proposal of the archaeal family Methanoregulaceae fam. nov. within the order

Methanomicrobiales. **International Journal of Systematic and Evolutionary Microbiology,** v. 62, n. Pt 6, p. 1389-1395, 2012. ISSN 1466-5026. Available at: < http://dx.doi.org/10.1099/ijs.0.035048-0 >.

SAKAKI, T. et al. Saccharification of Cellulose Using a Hot-Compressed Water-Flow Reactor. **Industrial & Engineering Chemistry Research,** v. 41, n. 4, p. 661-665, 2002/02/01 2002. ISSN 0888-5885. Available at: < http://dx.doi.org/10.1021/ie010614s >.

SAMBUSITI, C. et al. A comparison of different pre-treatments to increase methane production from two agricultural substrates. **Applied Energy,** v. 104, p. 62-70, 4// 2013. ISSN 0306-2619. Available at: < http://www.sciencedirect.com/science/article/pii/S0306261912007799 >.

SÁRVÁRI HORVÁTH, I. et al. Effects of Furfural on the Respiratory Metabolism of Saccharomyces cerevisiae in Glucose-Limited Chemostats. **Applied and Environmental Microbiology,** v. 69, n. 7, p. 4076-4086, July 1, 2003 2003. Available at: < http://aem.asm.org/content/69/7/4076.abstract >.

SASKA, M.; OZER, E. Aqueous extraction of sugarcane bagasse hemicellulose and production of xylose syrup. **Biotechnology and Bioengineering,** v. 45, n. 6, p. 517-523, 1995. ISSN 1097-0290. Available at: < http://dx.doi.org/10.1002/bit.260450609 >.

SASSNER, P.; GALBE, M.; ZACCHI, G. Techno-economic evaluation of bioethanol production from three different lignocellulosic materials. **Biomass and Bioenergy,** v. 32, n. 5, p. 422-430, 5// 2008. ISSN 0961-9534. Available at: < http://www.sciencedirect.com/science/article/pii/S0961953407001997 >.

SCHINK, B. Anaerophaga. In: (Ed.). **Bergey's Manual of Systematics of Archaea and Bacteria**: John Wiley & Sons, Ltd, 2015. ISBN 9781118960608.

SCHNÜRER, A.; JARVIS, Å. **Microbiological handbook for biogas plants, Swedish waste management**. Swedish gas centre. Sweden. 2010

SCHROYENA, M. et al. Inhibitory effects of p-coumaric acid on the biogas production process, and the interaction with an enzymatic pretreatment. International Conference on Sustainable Solid Waste Management, 2016, Cyprus.

SELIG, M. J. et al. Deposition of Lignin Droplets Produced During Dilute Acid Pretreatment of Maize Stems Retards Enzymatic Hydrolysis of Cellulose. **Biotechnology Progress,** v. 23, n. 6, p. 1333-1339, 2007. ISSN 1520-6033. Available at: < http://dx.doi.org/10.1021/bp0702018 >.

SENILA, L. et al. The Autohydrolysis of Albies Alba Wood Using Adaptive Neural Fuzzy Interference System Mathematical Modeling. **International Journal of Green Energy,** v. 11, n. 6, p. 611-624, 2014/07/03 2014. ISSN 1543-5075. Available at: < http://dx.doi.org/10.1080/15435075.2013.777907 >.

SHAO, Q. et al. Hydrogen peroxide presoaking of bamboo prior to AFEX pretreatment and impact on enzymatic conversion to fermentable sugars. **Bioresource Technology,** v. 142, p. 26-31, 8// 2013. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852413007712 >.

SHEN, G. et al. EFFECT OF HYDROGEN PEROXIDE PRETREATMENT ON THE ENZYMATIC HYDROLYSIS OF CELLULOSE. **Journal of Food Process Engineering,** v. 34, n. 3, p. 905-921, 2011. ISSN 1745-4530. Available at: < http://dx.doi.org/10.1111/j.1745-4530.2009.00518.x >.

SHEN, X. J. et al. Effects of aluminum chloride-catalyzed hydrothermal pretreatment on the structural characteristics of lignin and enzymatic hydrolysis. **Bioresour Technol,** v. 206, p. 57-64, Apr 2016. ISSN 0960-8524. Available at: < http://ac.els-cdn.com/S0960852416000444/1-s2.0-S0960852416000444-main.pdf?_tid=88738638-7826-11e6-b800-00000aab0f6c&acdnat=1473601937_af227baceb9b5bcccdb3493b2b8f1f8d >.

SHIMIZU, K. et al. Total utilisation of wood components by steam explosion pretreatment. In: CHICHESTER, H. L. (Ed.). **Wood Processing an Utilization**, 1989. p.407-412.

SIEBER, J. R.; MCINERNEY, M. J.; GUNSALUS, R. P. Genomic insights into syntrophy: the paradigm for anaerobic metabolic cooperation. **Annu Rev Microbiol,** v. 66, p. 429-52, 2012. ISSN 0066-4227.

SIMS, R. E. H.; BASSAM, N. E. Chapter 1 - Biomass and Resources. In: SIMS, R. E. H. (Ed.). **Bioenergy Options for a Cleaner Environment**. Oxford: Elsevier, 2004. p.1-28. ISBN 978-0-08-044351-5.

SINGH, A.; MISHRA, P. **Microbial Pentose Utilization: Current Applications in Biotechnology**. Elsevier, 1995.

SIRIPATANA, C.; JIJAI, S.; KONGJAN, P. Analysis and extension of Gompertz-type and Monod-type equations for estimation of design parameters from batch anaerobic digestion experiments. **AIP Conference Proceedings,** v. 1775, n. 1, p. 030079, 2016. Available at: < http://aip.scitation.org/doi/abs/10.1063/1.4965199 >.

SIRIWONGRUNGSON, V.; ZENG, R. J.; ANGELIDAKI, I. Homoacetogenesis as the alternative pathway for H2 sink during thermophilic anaerobic degradation of butyrate under suppressed methanogenesis. **Water Res,** v. 41, n. 18, p. 4204-10, Oct 2007. ISSN 0043-1354 (Print)

0043-1354.

SJÖDE, A. et al. The Potential in Bioethanol Production From Waste Fiber Sludges in Pulp Mill-Based Biorefineries. In: MIELENZ, J.;KLASSON, K. T., *et al* (Ed.). **Applied Biochemistry and Biotecnology**: Humana Press, 2007. chap. 29, p.327-337. (ABAB Symposium). ISBN 978-1-60327-180-6.

SKIADAS, I. V. et al. Thermal pre-treatment of primary and secondary sludge at 70 degrees C prior to anaerobic digestion. **Water Sci Technol,** v. 52, n. 1-2, p. 161-6, 2005. ISSN 0273-1223 (Print)

0273-1223.

SLUITER, A. et al. **Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples**. National Renewable Energy Laboratory. 2008

SLUITER, A. et al. **Determination of structural carbohydrates and lignin in biomass**. National Renewable Energy Laboratory. DOE, USA 2011

SOCCOL, C. et al. Lignocellulosic bioethanol: current status and future perspectives. In: PANDEY, A.;LARROCHE, C., *et al* (Ed.). **Biofuels: Alternative Feedstocks and Conversion Processes**. San Diego: Academic Press, 2011 b. p.101-122.

SOCCOL, C. R. et al. Bioethanol from lignocelluloses: Status and perspectives in Brazil. **Bioresour Technol,** v. 101, n. 13, p. 4820-5, Jul 2010. ISSN 0960-8524.

SOEST, P. J. **Nutritional ecology of the ruminant**. Cornell University Press, 1994. Available at: < http://books.google.com/books?hl=en&lr=&id=-mwUu6PL1UgC&oi=fnd&pg=PR9&ots=DOUyB4GbOH&sig=dQwxX43S5hJiudOwsh38va5dUAM >.

SOMERVILLE, C. et al. Feedstocks for Lignocellulosic Biofuels. **Science,** v. 329, n. 5993, p. 790-792, August 13, 2010 2010. Available at: < http://www.sciencemag.org/content/329/5993/790.abstract >.

SONG, Y.; LIU, C.; FINEGOLD, S. M. Bacteroides. In: (Ed.). **Bergey's Manual of Systematics of Archaea and Bacteria**: John Wiley & Sons, Ltd, 2015. ISBN 9781118960608.

SONG, Z.-L. et al. Pretreatment of Rice Straw by Hydrogen Peroxide for Enhanced Methane Yield. **Journal of Integrative Agriculture,** v. 12, n. 7, p. 12581266, 2013. ISSN 2095-3119. Available at: < http://dx.doi.org/10.1016/S2095-3119(13)60355-X >.

SONG, Z. et al. Comparison of two chemical pretreatments of rice straw for biogas prodcution by anaerobic digestion. **Bioresources,** v. 7, n. 3, 2012 2012. Available at: < http://scholar.google.com/scholar?q=Comparison of two chemical pretreatments of rice straw for biogas prodcution by anaerobic digestion&btnG=&hl=en&num=20&as_sdt=0%2C22 >.

SOROKIN, D. Y. et al. Methanogenesis at extremely haloalkaline conditions in the soda lakes of Kulunda Steppe (Altai, Russia). **FEMS Microbiol Ecol,** v. 91, n. 4, Apr 2015. ISSN 0168-6496.

STRONACH, S. M.; RUDD, T.; LESTER, J. N. **Anaerobic Digestion Processes in Industrial Wastewater Treatment**. Springer, 1986. ISBN 978-3-642-71217-3.

STUDER, M. H. et al. Lignin content in natural Populus variants affects sugar release. **Proceedings of the National Academy of Sciences,** v. 108, n. 15, p. 6300-6305, 2011. ISSN 0027-8424. Available at: < http://dx.doi.org/10.1073/pnas.1009252108 >.

SU, Y. et al. Fractional pretreatment of lignocellulose by alkaline hydrogen peroxide: Characterization of its major components. **Food and Bioproducts Processing,** v. 94, p. 322-330, 4// 2015. ISSN 0960-3085. Available at: < http://www.sciencedirect.com/science/article/pii/S0960308514000479 >.

SUN, L. et al. Characterization of microbial community structure during continuous anaerobic digestion of straw and cow manure. **Microbial Biotechnology,** v. 8, n. 5, p. 815-827, 2015. ISSN 1751-7915. Available at: < http://dx.doi.org/10.1111/1751-7915.12298 >.

SUN, S.-N. et al. Structural characterization of residual hemicelluloses from hydrothermal pretreated Eucalyptus fiber. **International journal of biological macromolecules,** v. 69, p. 158-164, 2014. ISSN 0141-8130. Available at: < http://dx.doi.org/10.1016/j.ijbiomac.2014.05.037 >.

SUN, S. et al. Improving the enzymatic hydrolysis of thermo-mechanical fiber from Eucalyptus urophylla by a combination of hydrothermal pretreatment and alkali fractionation. **Biotechnology for Biofuels,** v. 7, Aug 20 2014. ISSN 1754-6834. Available at: < <Go to ISI>://WOS:000340914800001 >.

SUN, S. et al. Systematic evaluation of the degraded products evolved from the hydrothermal pretreatment of sweet sorghum stems. **Biotechnology for Biofuels,** v. 8, n. 1, p. 1-13, 2015// 2015. ISSN 1754-6834. Available at: < http://dx.doi.org/10.1186/s13068-015-0223-1 >.

SUN, T. S. et al. Hydrothermal treatment and enzymatic saccharification of corncobs. **BioResources,** v. 9, n. 2, p. 3000-3013, 2014. Available at: < http://scholar.google.com/scholar?q=Hydrothermal treatment and enzymatic saccharification of corncobs&btnG=&hl=en&num=20&as_sdt=0%2C22 >.

TABATABAEI, M. et al. Importance of the methanogenic archaea populations in anaerobic wastewater treatments. **Process Biochemistry,** v. 45, n. 8, p. 1214-1225, 8// 2010. ISSN 1359-5113. Available at: < http://www.sciencedirect.com/science/article/pii/S1359511310001984 >.

TAHERZADEH, M. J.; KEIKHOSRO, K. Pretreatment of Lignocellulosic Wastes to Improve Ethanol and Biogas Production: A Review. **International Journal of Molecular Sciences,** v. 9, n. 9, 2008. Available at: < http://dx.doi.org/10.3390/ijms9091621 >.

TAKASHIMA, M.; SPEECE, R. E.; PARKIN, G. F. Mineral requirements for methane fermentation. **Critical Reviews in Environmental Control,** v. 19, n. 5, p. 465-479, 1990/01/01 1990. ISSN 1040-838X. Available at: < http://dx.doi.org/10.1080/10643389009388378 >. Accessed on: 2015/09/08.

TEIXEIRA, R. S. et al. Biomass pretreatment: a critical choice for biomass utilization via biotechnological routes. **BMC Proceedings,** v. 8, n. 4, p. O34, 2014. ISSN 1753-6561. Available at: < http://dx.doi.org/10.1186/1753-6561-8-S4-O34 >.

TEMIZ, E.; AKPINAR, O. The Effect of Severity Factor on the Release of Xylose and Phenolics from Rice Husk and Rice Straw. **Waste and Biomass Valorization**, p. 1-12, 2016. ISSN 1877-265X. Available at: < http://dx.doi.org/10.1007/s12649-016-9608-z >.

TIAN, J. H. et al. Occurrence of lignin degradation genotypes and phenotypes among prokaryotes. **Appl Microbiol Biotechnol,** v. 98, n. 23, p. 9527-44, Dec 2014. ISSN 0175-7598.

TRAJANO, H. L. et al. The fate of lignin during hydrothermal pretreatment. **Biotechnology for Biofuels,** v. 6, Aug 1 2013. ISSN 1754-6834. Available at: < <Go to ISI>://WOS:000323166000001 >.

ULBRICHT, R. J.; NORTHUP, S. J.; THOMAS, J. A. A review of 5-hydroxymethylfurfural (HMF) in parenteral solutions. **Fundamental and Applied Toxicology,** v. 4, n. 5, p. 843-853, 1984/10/01 1984. ISSN 0272-0590. Available at: < http://www.sciencedirect.com/science/article/pii/0272059084901064 >.

UNICA. **SOUTH CENTRAL 2015/2016 HARVEST SHOULD REACH 590 MILLION TONS OF PROCESSED CANE, WITH PRIORITY ON ETHANOL PRODUCTION**: Brazilian Sugarcane Industry Association. 2015 2015.

VALO, A.; CARRÈRE, H.; DELGENÈS, J. P. Thermal, chemical and thermo-chemical pre-treatment of waste activated sludge for anaerobic digestion. **Journal of Chemical Technology & Biotechnology,** v. 79, n. 11, p. 1197-1203, 2004. ISSN 1097-4660. Available at: < http://dx.doi.org/10.1002/jctb.1106 >.

VAN HAANDEL, A. C.; LETTINGA, G. **Anaerobic Sewage Treatment: A Practical Guide for Regions with a Hot Climate.** Chichester, UK: Wiley, 1994. ISBN 0471951218.

VAN WALSU, G. P. Severity function describing the hydrolysis of xylan using carbonic acid. **Applied Biochemistry and Biotechnology,** v. 91, n. 1, p. 317-329, 2001. ISSN 1559-0291. Available at: < http://dx.doi.org/10.1385/ABAB:91-93:1-9:317 >.

VANWONTERGHEM, I. et al. Deterministic processes guide long-term synchronised population dynamics in replicate anaerobic digesters. **ISME J,** v. 8, n. 10, p. 2015-2028, 10//print 2014. ISSN 1751-7362. Available at: < http://dx.doi.org/10.1038/ismej.2014.50 >.

VERGARA-FERNÁNDEZ, A. et al. Evaluation of marine algae as a source of biogas in a two-stage anaerobic reactor system. **Biomass and Bioenergy,** v. 32, n. 4, p. 338-344, 4// 2008. ISSN 0961-9534. Available at: < http://www.sciencedirect.com/science/article/pii/S0961953407001912 >.

WALLIS, A. F. A.; WEARNE, R. H. Fractionation on the polymeric components of hardwoods by autohydrolysis-explosion-extraction. **Appita J.,** v. 38, p. 432-437, 1985.

WALTER, J. Ecological Role of Lactobacilli in the Gastrointestinal Tract: Implications for Fundamental and Biomedical Research. **Applied and Environmental Microbiology,** v. 74, n. 16, p. 4985-4996, August 15, 2008 2008. Available at: < http://aem.asm.org/content/74/16/4985.short >.

WALTERS, W. A. et al. PrimerProspector: de novo design and taxonomic analysis of barcoded polymerase chain reaction primers. **Bioinformatics,** v. 27, n. 8, p. 1159-61, Apr 15 2011. ISSN 1367-4803.

WANG, H. et al. An integrated approach for efficient biomethane production from solid bio-wastes in a compact system. **Biotechnology for Biofuels,** v. 8, n. 1, p. 62, 2015. ISSN 1754-6834. Available at: < http://www.biotechnologyforbiofuels.com/content/8/1/62 >.

WANG, W. et al. Performance and microbial community analysis of the anaerobic reactor with coke oven gas biomethanation and in situ biogas upgrading. **Bioresource Technology,** v. 146, p. 234-239, 10// 2013. ISSN 0960-8524. Available at: < //www.sciencedirect.com/science/article/pii/S0960852413011176 >.

WANG, Y. et al. Effects of volatile fatty acid concentrations on methane yield and methanogenic bacteria. **Biomass and Bioenergy,** v. 33, n. 5, p. 848-853, 5// 2009. ISSN 0961-9534. Available at: < http://www.sciencedirect.com/science/article/pii/S096195340900018X >.

WANGLIANG, L. et al. Anaerobic Digestion of Yard Waste with Hydrothermal Pretreatment. **Applied Biochemistry and Biotechnology**, 2014. ISSN 0273-2289. Available at: < http://dx.doi.org/10.1007/s12010-014-0724-6 >.

WARNECKE, F. et al. Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. **Nature,** v. 450, n. 7169, p. 560-565, 2007. ISSN 0028-0836. Available at: < http://dx.doi.org/10.1038/nature06269 >.

WEIL, J. et al. Pretreatment of yellow poplar sawdust by pressure cooking in water. **Applied Biochemistry and Biotechnology,** v. 68, n. 1-2, p. 21-40, 1997/10/01 1997. ISSN 0273-2289. Available at: < http://dx.doi.org/10.1007/BF02785978 >.

WENG, J.-K.; CHAPPLE, C. The origin and evolution of lignin biosynthesis. **New Phytologist,** v. 187, n. 2, p. 273-285, 2010. ISSN 1469-8137. Available at: < http://dx.doi.org/10.1111/j.1469-8137.2010.03327.x >.

WHITMAN, W. B.; BOWEN, T. L.; BOONE, D. R. The methanogenic bacteria. In: DWORKIN, M. (Ed.). **The Prokaryotes: An evolving electronic resource for the microbiological community**. New York: Springer, 2006.

WI, S. G. et al. Lignocellulose conversion for biofuel: a new pretreatment greatly improves downstream biocatalytic hydrolysis of various lignocellulosic materials. **Biotechnology for Biofuels,** v. 8, n. 1, p. 228, 2015// 2015. ISSN 1754-6834. Available at: < http://dx.doi.org/10.1186/s13068-015-0419-4 >.

WIJEKOON, K. C.; VISVANATHAN, C.; ABEYNAYAKA, A. Effect of organic loading rate on VFA production, organic matter removal and microbial activity of a two-stage thermophilic anaerobic membrane bioreactor. **Bioresource Technology,** v. 102, n. 9, p. 5353-5360, 5// 2011. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852410020663 >.

WILKINS, D. et al. Effects of sludge inoculum and organic feedstock on active microbial communities and methane yield during anaerobic digestion. **Frontiers in Microbiology,** v. 6, n. 1114, 2015-October-13 2015. ISSN 1664-302X. Available at: < http://journal.frontiersin.org/article/10.3389/fmicb.2015.01114 >.

WINGREN, A.; GALBE, M.; ZACCHI, G. Energy considerations for a SSF-based softwood ethanol plant. **Bioresource Technology,** v. 99, n. 7, p. 2121-2131, 5// 2008. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S096085240700466X >.

WIRTH, R. et al. Characterization of a biogas-producing microbial community by short-read next generation DNA sequencing. **Biotechnology for Biofuels,** v. 5, n. 1, p. 41, 2012. ISSN 1754-6834. Available at: < http://dx.doi.org/10.1186/1754-6834-5-41 >.

WOESE, C. R.; FOX, G. E. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. **Proc Natl Acad Sci U S A,** v. 74, n. 11, p. 5088-90, Nov 1977. ISSN 0027-8424 (Print)

0027-8424.

WONG, M. T. et al. Substrate-Driven Convergence of the Microbial Community in Lignocellulose-Amended Enrichments of Gut Microflora from the Canadian Beaver (Castor canadensis) and North American Moose (Alces americanus). **Frontiers in Microbiology,** v. 7, p. 961, 06/21

01/27/received

06/03/accepted 2016. ISSN 1664-302X. Available at: < http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4914502/ >.

WWF. **WWF - World Wildlife Fund for nature, The energy report 100% renewable energy by 2050**. 2011

XIA, Y. et al. Cellular adhesiveness and cellulolytic capacity in Anaerolineae revealed by omics-based genome interpretation. **Biotechnology for Biofuels,** v. 9, n. 1, p. 111, 2016// 2016. ISSN 1754-6834. Available at: < http://dx.doi.org/10.1186/s13068-016-0524-z >.

XIANG, Q. et al. Heterogeneous aspects of acid hydrolysis of alpha-cellulose. **Applied biochemistry and biotechnology,** v. 105 -108, p. 505-514, 2003. ISSN 0273-2289. Available at: < http://www.ncbi.nlm.nih.gov/pubmed/12721431 >.

XIAO, L.-P. et al. Impact of hot compressed water pretreatment on the structural changes of woody biomass for bioethanol prodution. **Biorsources,** v. 6, n. 2, p. 1576-1598, 2011.

XING, D. et al. Ethanoligenens harbinense gen. nov., sp. nov., isolated from molasses wastewater. **Int J Syst Evol Microbiol,** v. 56, n. Pt 4, p. 755-60, Apr 2006. ISSN 1466-5026 (Print)

1466-5026.

XU, J.; THOMSEN, M. H.; THOMSEN, A. B. Investigation of acetic acid-catalyzed hydrothermal pretreatment on corn stover. **Applied Microbiology and Biotechnology,** v. 86, n. 2, p. 509-516, 2009. ISSN 1432-0614. Available at: < http://dx.doi.org/10.1007/s00253-009-2340-x >.

YADVIKA et al. Enhancement of biogas production from solid substrates using different techniques--a review. **Bioresour Technol,** v. 95, n. 1, p. 1-10, Oct 2004. ISSN 0960-8524 (Print)

0960-8524.

YAMADA, T. Anaerolinea thermolimosa sp. nov., Levilinea saccharolytica gen. nov., sp. nov. and Leptolinea tardivitalis gen. nov., sp. nov., novel filamentous anaerobes, and description of the new classes Anaerolineae classis nov. and Caldilineae classis nov. in the bacterial phylum Chloroflexi. **Int J Syst Evol Microbiol,** v. 56, 2006// 2006. Available at: < http://dx.doi.org/10.1099/ijs.0.64169-0 >.

YAMADA, T. et al. Bellilinea caldifistulae gen. nov., sp. nov. and Longilinea arvoryzae gen. nov., sp. nov., strictly anaerobic, filamentous bacteria of the phylum Chloroflexi isolated from methanogenic propionate-degrading consortia. **Int J Syst Evol Microbiol,** v. 57, 2007// 2007. Available at: < http://dx.doi.org/10.1099/ijs.0.65098-0 >.

YAMADA, T.; SEKIGUCHI, Y. Cultivation of Uncultured Chloroflexi Subphyla: Significance and Ecophysiology of Formerly Uncultured Chloroflexi 'Subphylum I' with Natural and Biotechnological Relevance. **Microbes and Environments,** v. 24, n. 3, p. 205-216, 2009.

YANG, B. et al. Enzymatic hydrolysis of cellulosic biomass. **Biofuels,** v. 2, n. 4, p. 421-449, 2011/07/01 2011. ISSN 1759-7269. Available at: < http://www.tandfonline.com/doi/abs/10.4155/bfs.11.116 >.

YANG, B. et al. Unconventional Relationships for Hemicellulose Hydrolysis and Subsequent Cellulose Digestion. In: (Ed.). **Lignocellulose Biodegradation**: American Chemical Society, v.889, 2004. chap. 6, p.100-125. (ACS Symposium Series). ISBN 0-8412-3876-6.

YANG, B.; WYMAN, C. E. Effect of xylan and lignin removal by batch and flowthrough pretreatment on the enzymatic digestibility of corn stover cellulose. **Biotechnology and Bioengineering,** v. 86, n. 1, p. 88-98, 2004. ISSN 1097-0290. Available at: < http://dx.doi.org/10.1002/bit.20043 >.

_____. BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrates. **Biotechnology and Bioengineering,** v. 94, n. 4, p. 611-617, 2006. ISSN 1097-0290. Available at: < http://dx.doi.org/10.1002/bit.20750 >.

YANG, H. Y. et al. H2SO4-Catalyzed Hydrothermal Pretreatment of Triploid Poplar to Enhance Enzymatic Hydrolysis. **Industrial & Engineering Chemistry Research,** v. 51, n. 36, p. 11598-11604, Sep 12 2012. ISSN 0888-5885. Available at: < <Go to ISI>://WOS:000308575300002 >.

YASHIRO, Y. et al. Methanoregula formicica sp. nov., a methane-producing archaeon isolated from methanogenic sludge. **Int J Syst Evol Microbiol,** v. 61, n. Pt 1, p. 53-9, Jan 2011. ISSN 1466-5026.

YI, Z. et al. Pretreatment of lignocellulosic biomass for enhanced biogas production. **Progress in Energy and Combustion Science**, 2014. ISSN 0360-1285. Available at: < http://dx.doi.org/10.1016/j.pecs.2014.01.001 >.

YOSHIDA, M. et al. Effects of Cellulose Crystallinity, Hemicellulose, and Lignin on the Enzymatic Hydrolysis of Miscanthus sinensis to Monosaccharides. **Bioscience, Biotechnology, and Biochemistry,** v. 72, n. 3, p. 805-810, 2008/03/23 2008. ISSN 0916-8451. Available at: < http://dx.doi.org/10.1271/bbb.70689 >.

YOURCHISIN, D. M.; PETER VAN WALSUM, G. Comparison of microbial inhibition and enzymatic hydrolysis rates of liquid and solid fractions produced from pretreatment of biomass with carbonic acid and liquid hot water. **Applied Biochemistry and Biotechnology,** v. 115, n. 1, p. 1073-1086, 2004. ISSN 1559-0291. Available at: < http://dx.doi.org/10.1385/ABAB:115:1-3:1073 >.

YU, H.-L. et al. Improvement of the enzymatic hydrolysis of furfural residues by pretreatment with combined green liquor and hydrogen peroxide. **Bioresource Technology,** v. 147, p. 29-36, 11// 2013. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S096085241301242X >.

YUE, Z. B. et al. Enhanced anaerobic ruminal degradation of bulrush through steam explosion pretreatment. **Industrial & …**, 2008. Available at: < http://dx.doi.org/10.1021/ie800202c >.

ZAKARIA, M. R.; HIRATA, S.; HASSAN, M. A. Hydrothermal pretreatment enhanced enzymatic hydrolysis and glucose production from oil palm biomass. **Bioresource Technology,** v. 176, p. 142-148, 1// 2015. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852414016290 >.

ZAKRZEWSKI, M. et al. Profiling of the metabolically active community from a production-scale biogas plant by means of high-throughput metatranscriptome sequencing. **J Biotechnol,** v. 158, n. 4, p. 248-58, Apr 30 2012. ISSN 0168-1656.

ZANIN, G. M. et al. Brazilian bioethanol program. **Applied Biochemistry and Biotechnology,** v. 84, n. 1, p. 1147-1161, 2000. ISSN 1559-0291. Available at: < http://dx.doi.org/10.1385/ABAB:84-86:1-9:1147 >.

ZENG, Y. et al. Lignin plays a negative role in the biochemical process for producing lignocellulosic biofuels. **Current Opinion in Biotechnology,** v. 27, p. 38-45, 6// 2014. ISSN 0958-1669. Available at: < http://www.sciencedirect.com/science/article/pii/S0958166913006708 >.

ZHANG, J. et al. Understanding changes in cellulose crystalline structure of lignocellulosic biomass during ionic liquid pretreatment by XRD. **Bioresour Technol,** v. 151, p. 402-5, Jan 2014. ISSN 0960-8524.

ZHANG, Q. et al. Enhancement of methane production from cassava residues by biological pretreatment using a constructed microbial consortium. **Bioresource Technology,** v. 102, n. 19, p. 8899-8906, 10// 2011. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852411008881 >.

ZHANG, Q. et al. Optimization of thermal-dilute sulfuric acid pretreatment for enhancement of methane production from cassava residues. **Bioresource Technology,** v. 102, n. 4, p. 3958-3965, 2// 2011. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852410019619 >.

ZHANG, Y. H. P.; LYND, L. R. Determination of the Number-Average Degree of Polymerization of Cellodextrins and Cellulose with Application to Enzymatic Hydrolysis. **Biomacromolecules,** v. 6, n. 3, p. 1510-1515, 2005/05/01 2005. ISSN 1525-7797. Available at: < http://dx.doi.org/10.1021/bm049235j >.

ZHAO, C. et al. Physical and chemical characterizations of corn stalk resulting from hydrogen peroxide presoaking prior to ammonia fiber expansion pretreatment. **Industrial Crops and Products,** v. 83, p. 86-93, 2016. ISSN 0926-6690. Available at: < http://dx.doi.org/10.1016/j.indcrop.2015.12.018 >.

ZHAO, X.; ZHANG, L.; LIU, D. Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. **Biofuels, Bioproducts and Biorefining**, 2012. ISSN 1932-1031. Available at: < http://dx.doi.org/10.1002/bbb.1331 >.

ZHENG, M. et al. The Effects of Alkaline Pretreatment Parameters on Anaerobic Biogasification of Corn Stover. **Energy Sources, Part A: Recovery, Utilization, and Environmental Effects,** v. 32, n. 20, p. 1918-1925, 2010/08/13 2010. ISSN 1556-7036. Available at: < http://dx.doi.org/10.1080/15567030902937119 >. Accessed on: 2015/09/25.

ZHONG, W. et al. Effect of biological pretreatments in enhancing corn straw biogas production. **Bioresource Technology,** v. 102, n. 24, p. 11177-11182, 12// 2011. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S0960852411013654 >.

ZHU, L. et al. Structural features affecting biomass enzymatic digestibility. **Bioresource Technology,** v. 99, n. 9, p. 3817-3828, 6// 2008. ISSN 0960-8524. Available at: < http://www.sciencedirect.com/science/article/pii/S096085240700586X >.

ZIEMINSKI, K. et al. Effects of hydrothermal pretreatment of sugar beet pulp for methane production. **Bioresource Technology,** v. 166, p. 187-193, Aug 2014. ISSN 0960-8524. Available at: < <Go to ISI>://WOS:000338711100024 >.

ZIGANSHIN, A. M. et al. Microbial community structure and dynamics during anaerobic digestion of various agricultural waste materials. **Appl Microbiol Biotechnol,** v. 97, n. 11, p. 5161-74, Jun 2013. ISSN 0175-7598.

ZINDER, S. H.; ANGUISH, T.; LOBO, A. L. Isolation and characterization of a thermophilic acetotrophic strain of Methanothrix. **Archives of Microbiology,** v. 146, n. 4, p. 315-322, 1987. ISSN 1432-072X. Available at: < http://dx.doi.org/10.1007/BF00410928 >.

ZINDER, S. H. et al. Methanogenesis in a Thermophilic (58°C) Anaerobic Digestor: Methanothrix sp. as an Important Aceticlastic Methanogen. **Applied and Environmental Microbiology,** v. 47, n. 4, p. 796-807, April 1, 1984 1984. Available at: < http://aem.asm.org/content/47/4/796.abstract >.

ZWIETERING, M. H. et al. Modeling of the Bacterial Growth Curve. **Applied and Environmental Microbiology,** v. 56, n. 6, p. 1875-1881, 1990. ISSN 0099-2240

1098-5336. Available at: < http://www.ncbi.nlm.nih.gov/pmc/articles/PMC184525/ >.

10 APPENDIX

Table 10.1 – Batch reactor composition of H_2O_2 assisted hydrothermal pretreatment for anaerobic digestion

Index	Reactor Vol (ml)	Working Vol (ml)	Replication	Substrate (g L^{-1})	Inoculum (g L^{-1})	Nutrient Sol (mL)
A-HO	1000	500	2	2.44	21.75	475.81
B-HO	1000	500	2	2.94	21.75	475.31
C-HO	1000	500	2	3.52	21.75	474.73
D-HO	1000	500	2	2.49	21.75	475.76
E-HO	1000	500	2	2.41	21.75	475.85
F-HO	1000	500	2	2.23	21.75	476.02
G-HO	1000	500	2	2.83	21.75	475.42
H-HO	1000	500	2	2.39	21.75	475.87
I-HO	1000	500	2	2.54	21.75	475.72
J-HO	1000	500	2	2.37	21.75	475.89
K-HO	1000	500	2	2.85	21.75	475.41
L-HO	1000	500	2	2.97	21.75	475.28
M-HO	1000	500	2	2.51	21.75	475.75
N-HO	1000	500	2	2.33	21.75	475.92
O-HO	1000	500	2	2.50	21.75	475.75
P-HO	1000	500	2	2.34	21.75	475.91

Table 10.2 –Batch reactor composition of H_2SO_4 assisted hydrothermal pretreatment for anaerobic digestion

Index	Reactor Vol (ml)	Working Vol (ml)	Replication	Substrate (g L^{-1})	Inoculum (g L^{-1})	Nutrient Sol (mL)
A-HSO	1000	500	2	5.1	22.9	471.9
B-HSO	1000	500	2	4.0	22.9	473.0
C-HSO	1000	500	2	2.8	22.9	474.3
D-HSO	1000	500	2	3.3	22.9	473.8
E-HSO	1000	500	2	2.4	22.9	474.7
F-HSO	1000	500	2	2.2	22.9	474.9
G-HSO	1000	500	2	2.4	22.9	474.7
H-HSO	1000	500	2	2.2	22.9	474.9
I-HSO	1000	500	2	2.7	22.9	474.4
J-HSO	1000	500	2	2.6	22.9	474.5
K-HSO	1000	500	2	4.1	22.9	472.9
L-HSO	1000	500	2	2.1	22.9	475.0
M-HSO	1000	500	2	2.4	22.9	474.6
N-HSO	1000	500	2	2.4	22.9	474.7
O-HO	1000	500	2	2.50	21.75	475.75
P-HO	1000	500	2	2.34	21.75	475.91

Modified Gompertz fit curves for H_2O_2 assisted hydrothermally pretreated samples

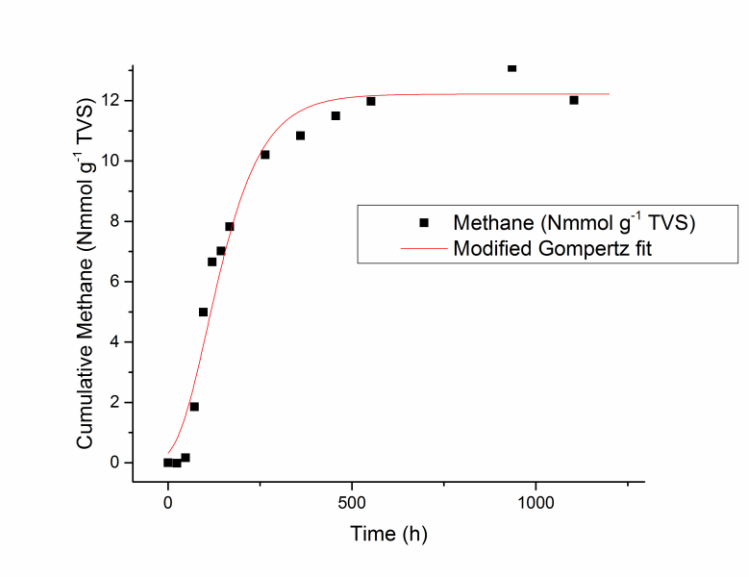

Figure 10.1 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for A-HO

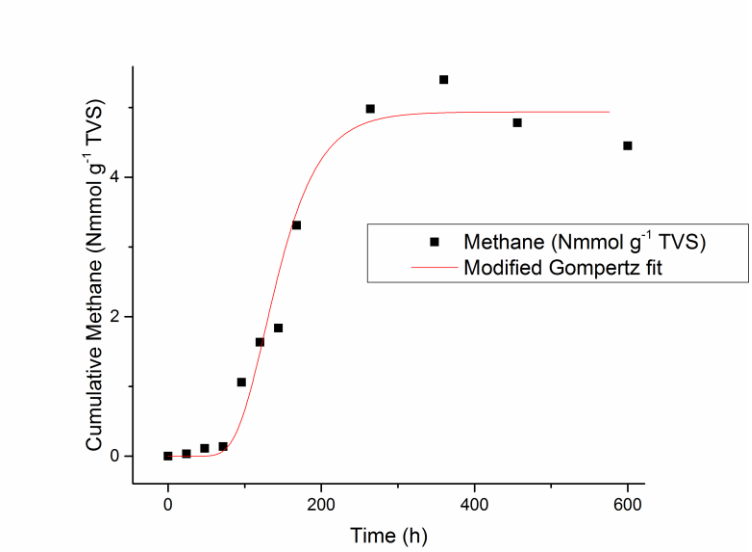

Figure 10.2 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for B-HO

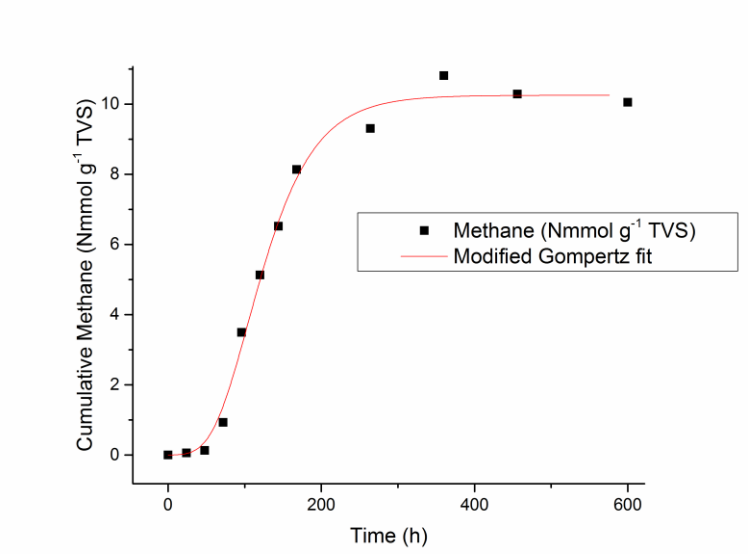

Figure 10.3 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for C-HO

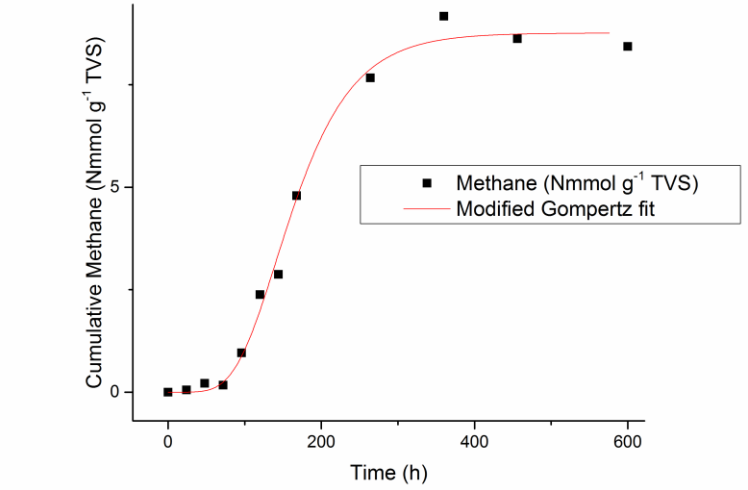

Figure 10.4 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for D-HO

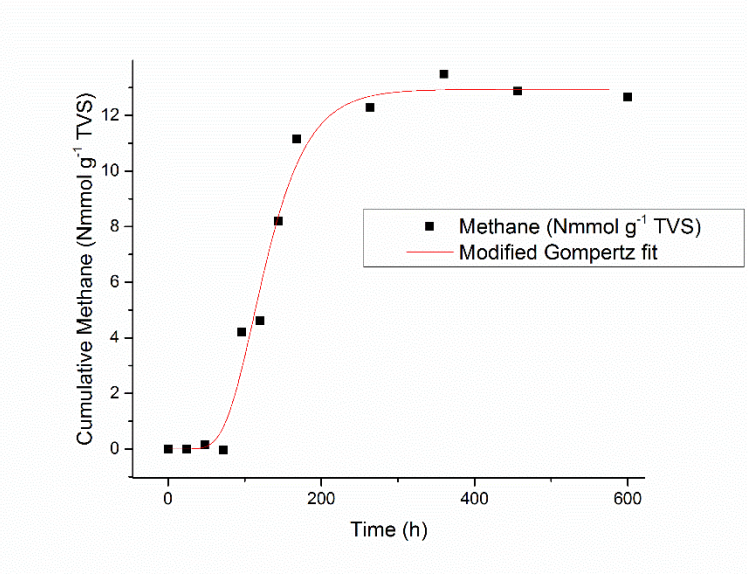

Figure 10.5 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for E-HO

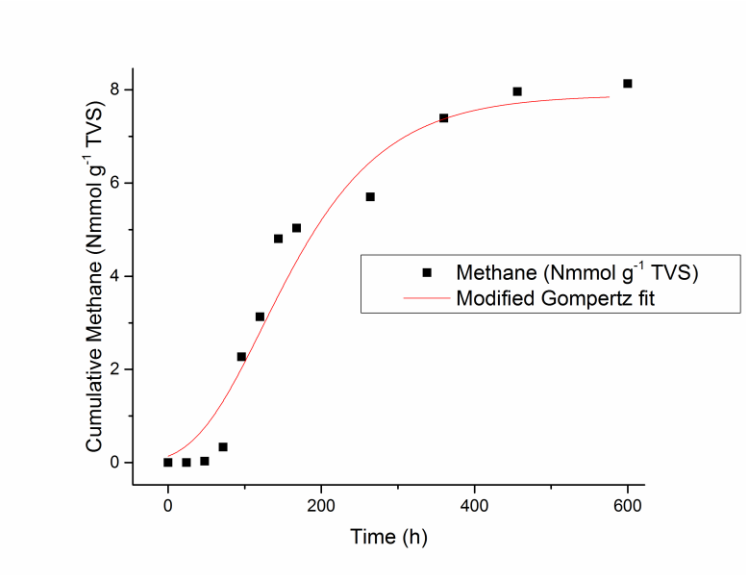

Figure 10.6 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for F-HO

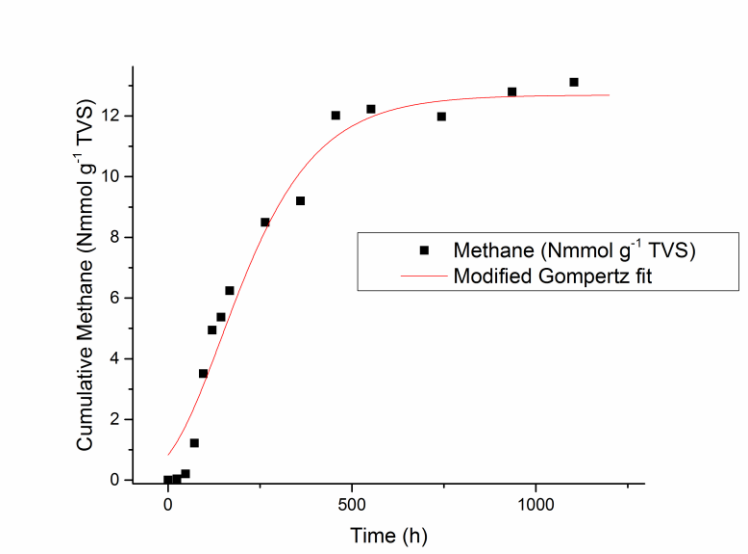

Figure 10.7 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for G-HO

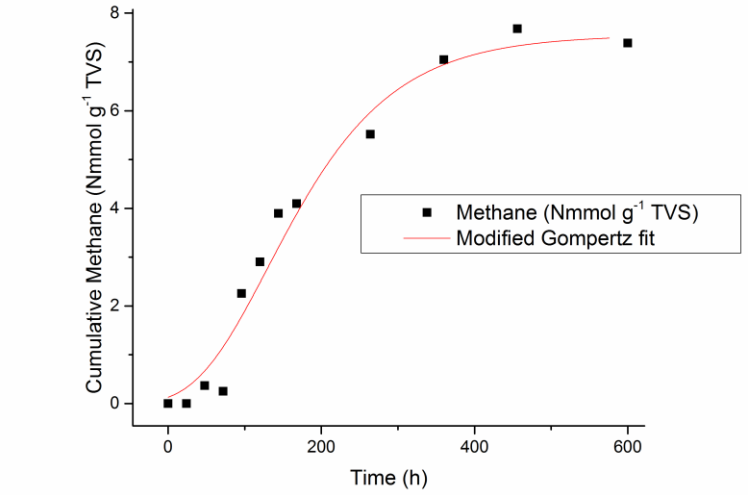

Figure 10.8 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for H-HO

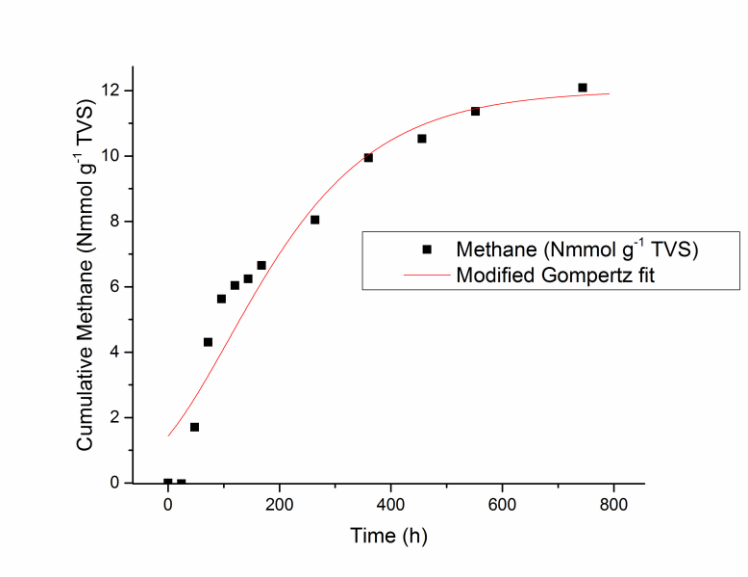

Figure 10.9 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for I-HO

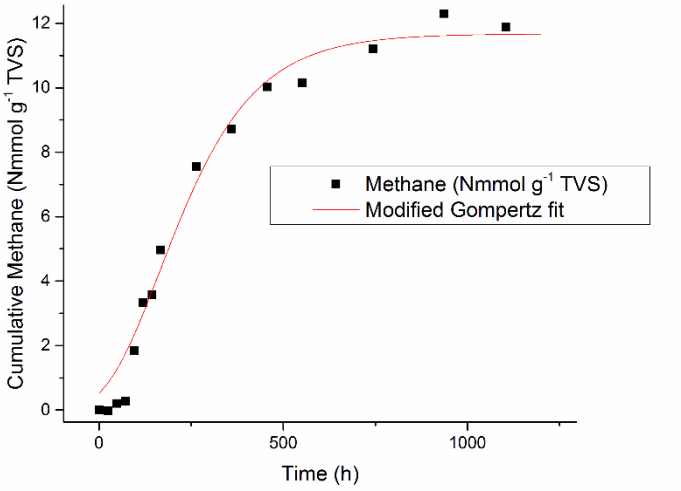

Figure 10.10 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for J-HO

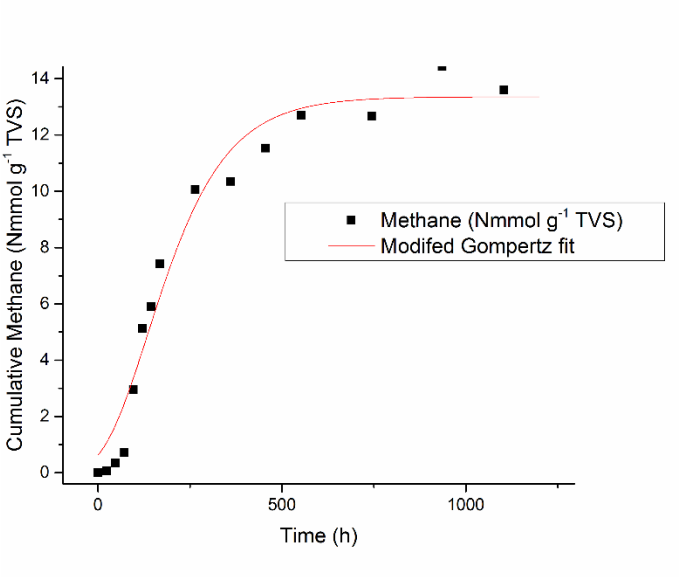

Figure 10.11 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for K-HO

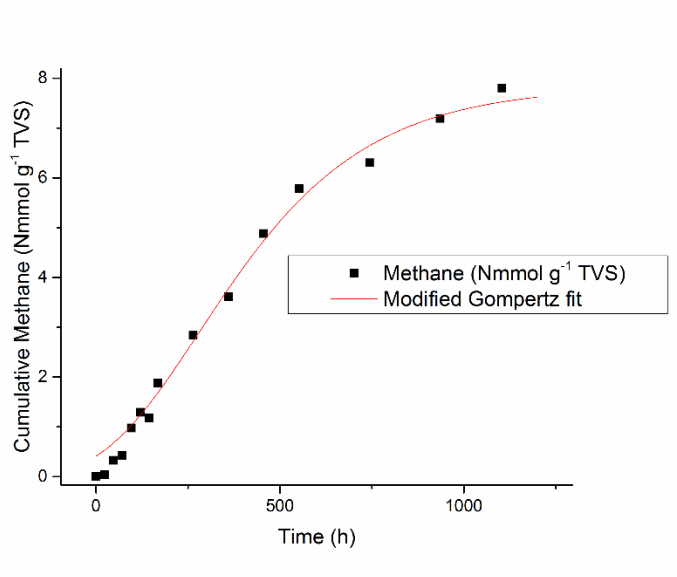

Figure 10.12 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for L-HO

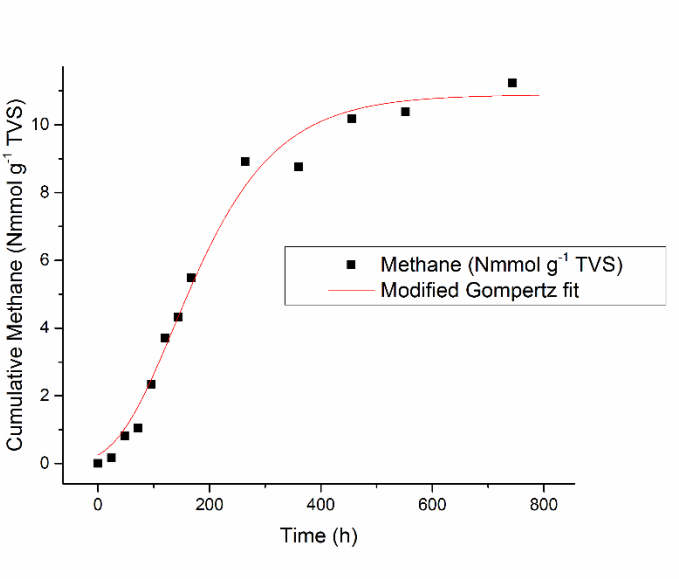

Figure 10.13 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for M-HO

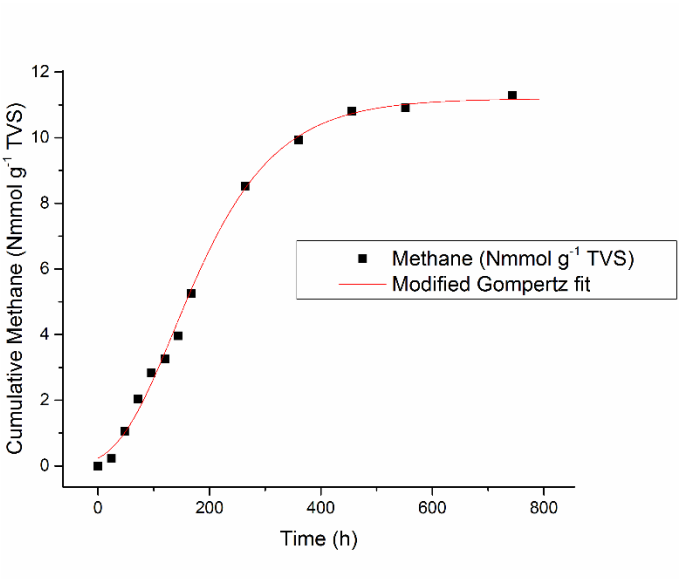

Figure 10.14 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for N-HO

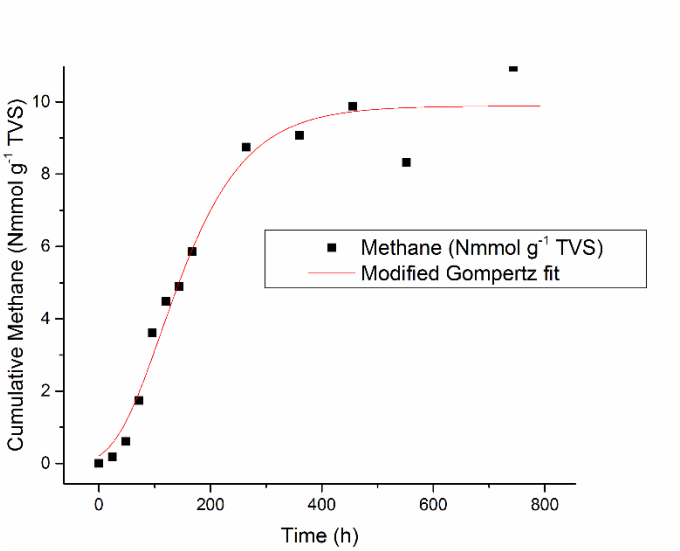

Figure 10.15 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for O-HO

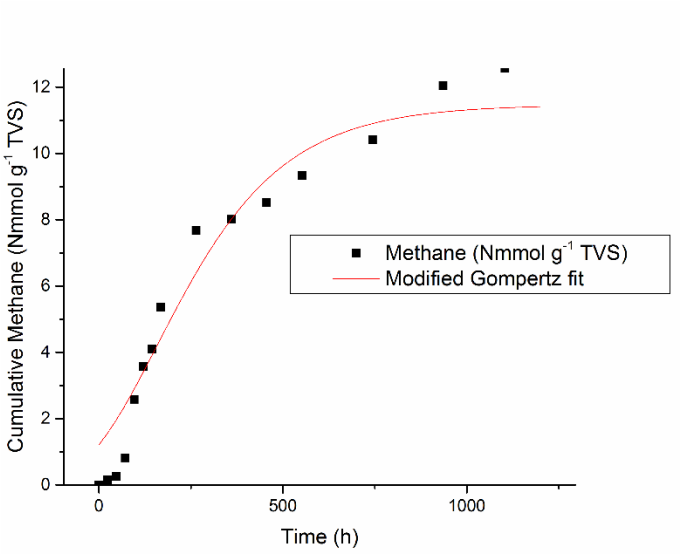

Figure 10.16 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for P-HO

Modified Gompertz fit curves of H_2SO_4 assisted hydrothermally pretreated reactors

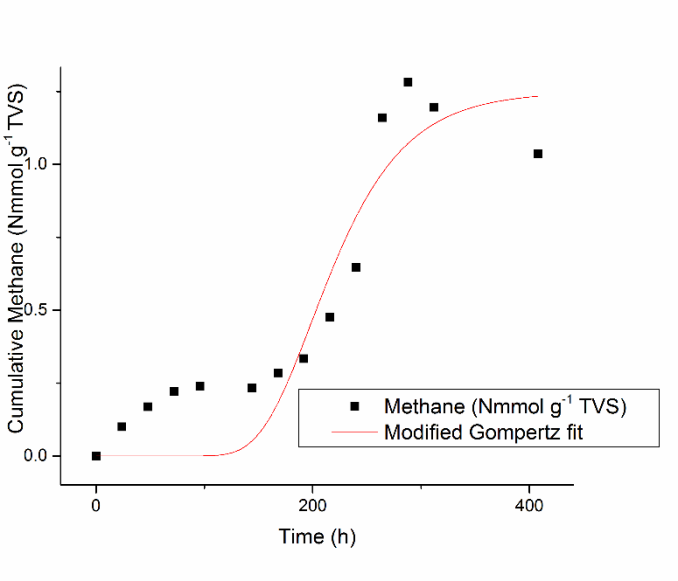

Figure 10.17 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for A-HSO

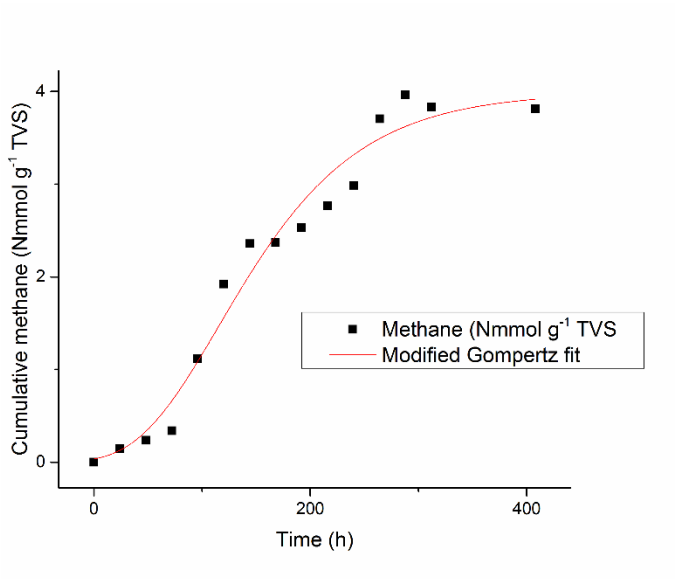

Figure 10.18 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for B-HSO

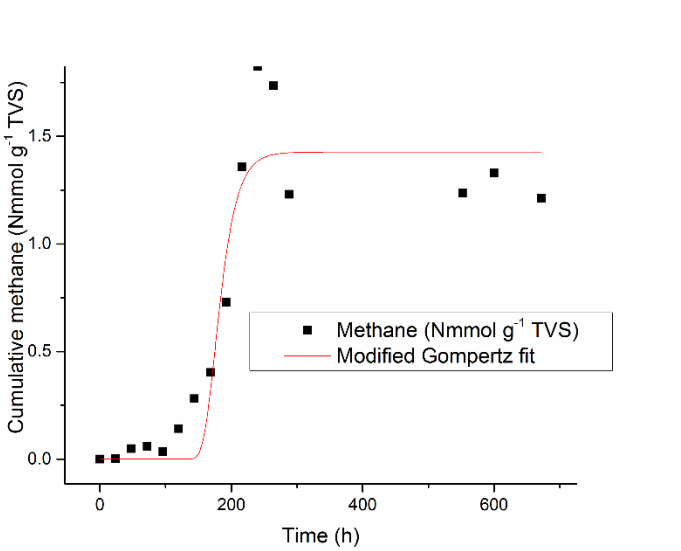

Figure 10.19 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for C-HSO

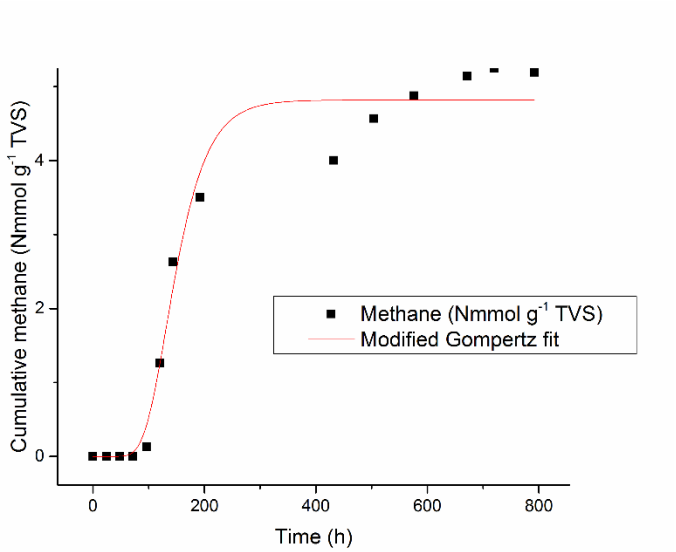

Figure 10.20 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for D-HSO

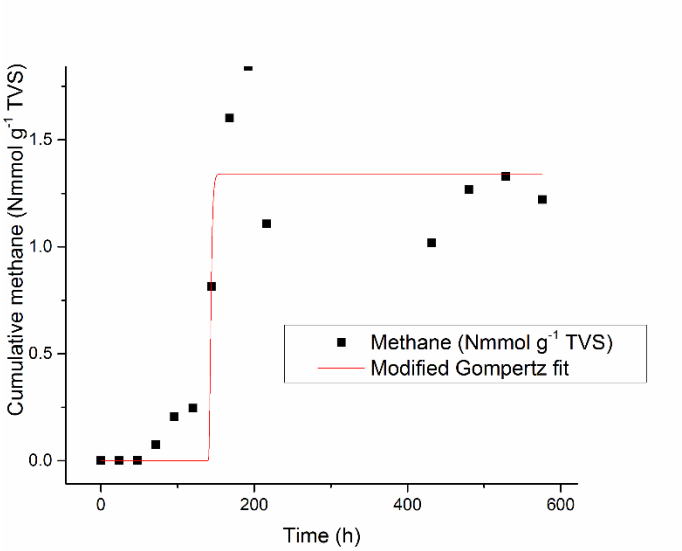

Figure 10.21 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for E-HSO

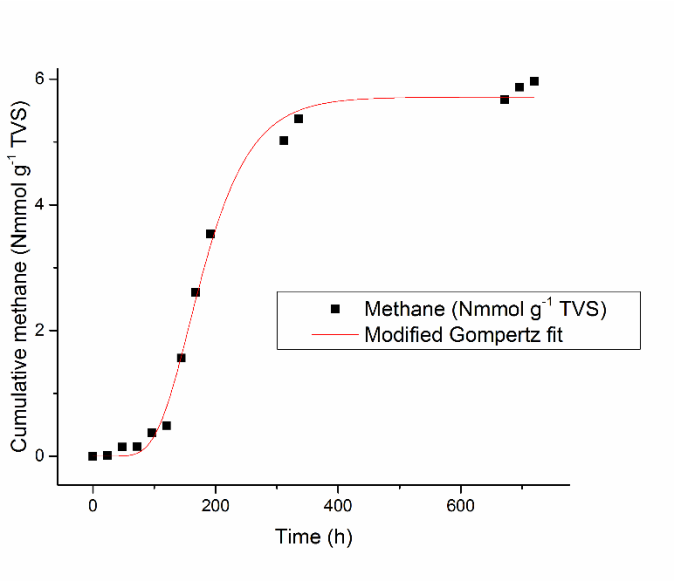

Figure 10.22 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for F-HSO

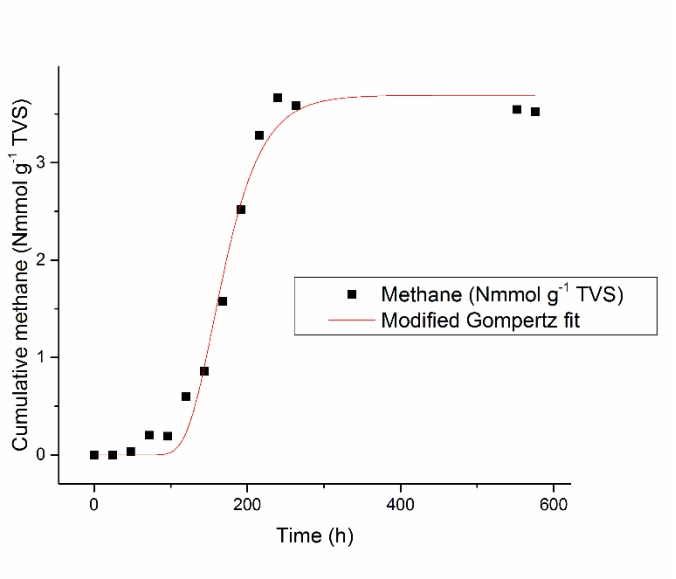

Figure 10.23 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for G-HSO

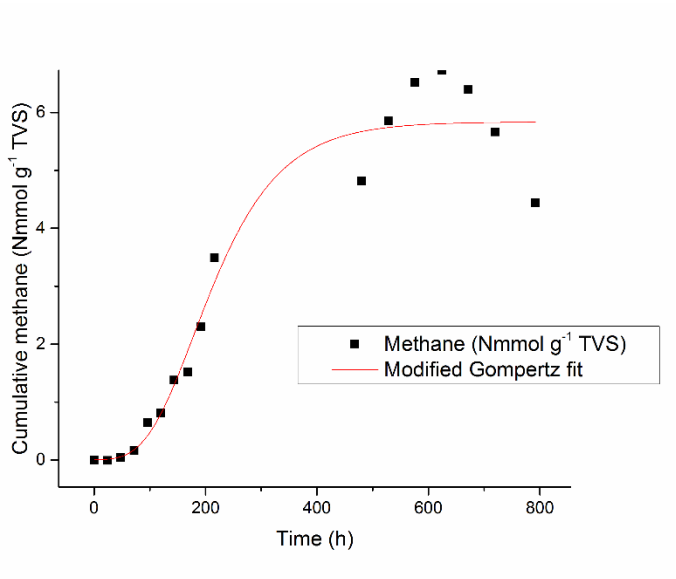

Figure 10.24 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for H-HSO

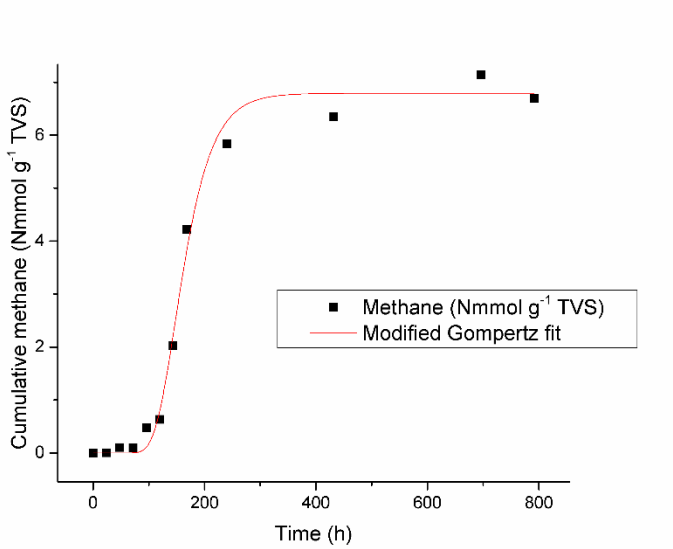

Figure 10.25 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for I-HSO

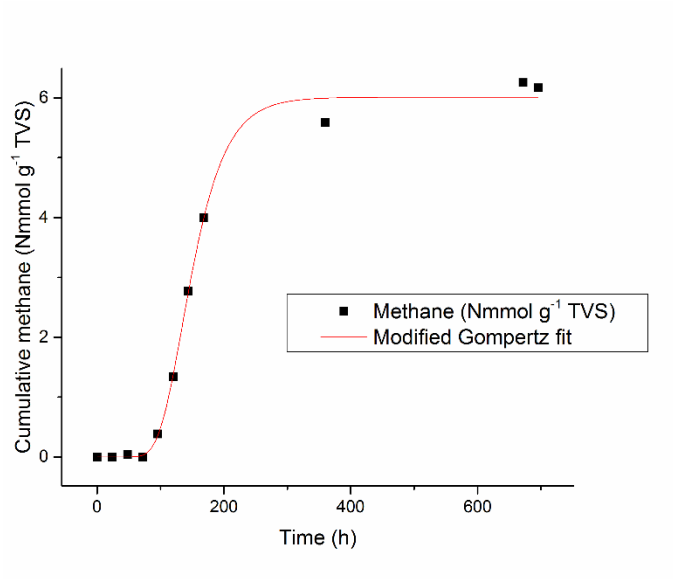

Figure 10.26 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for J-HSO

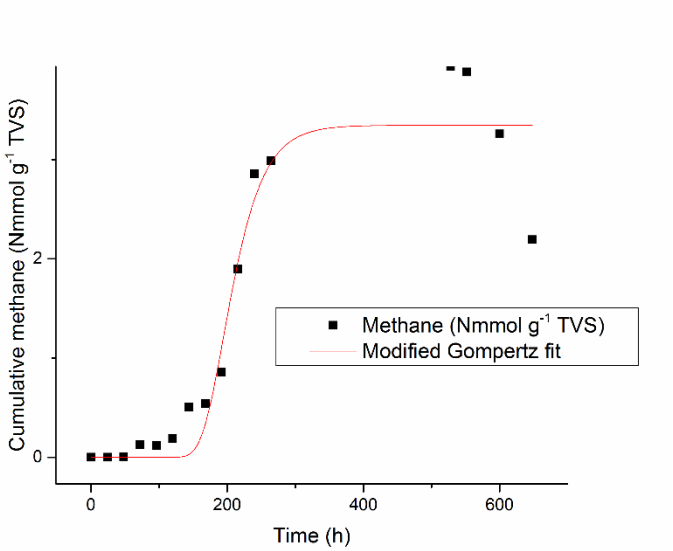

Figure 10.27 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for K-HSO

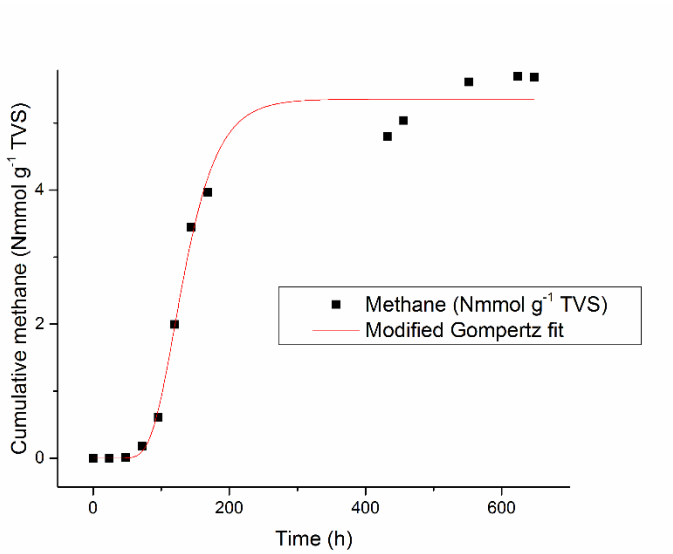

Figure 10.28 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for L-HSO

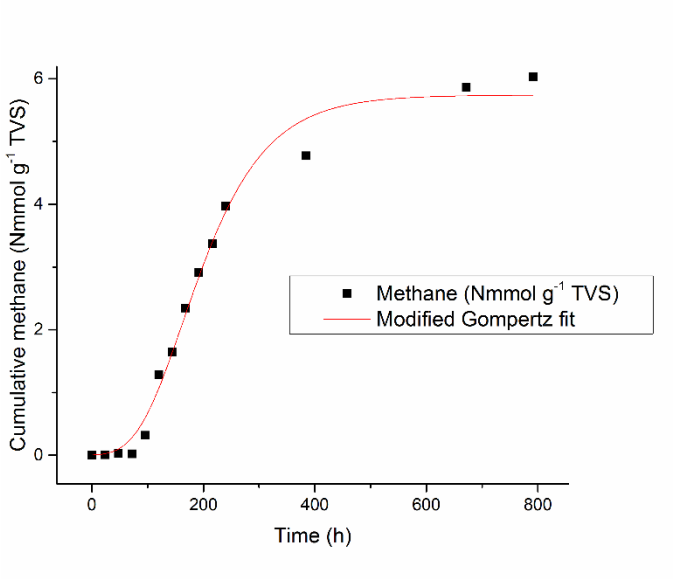

Figure 10.29 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for M-HSO

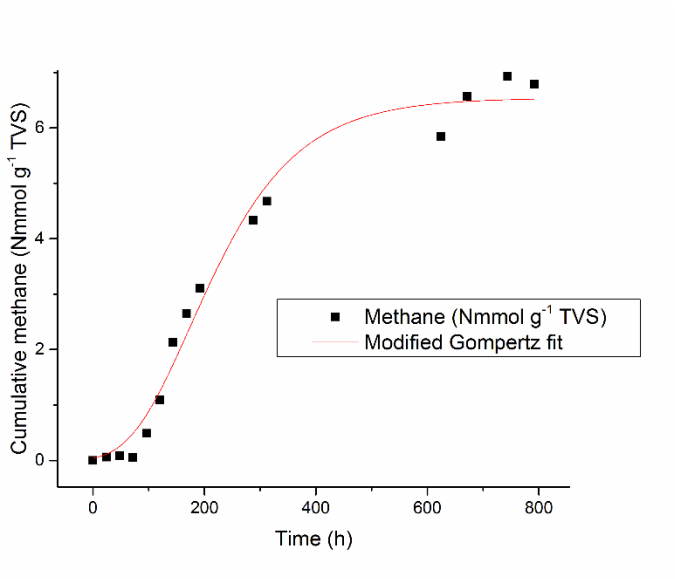

Figure 10.30 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for N-HSO

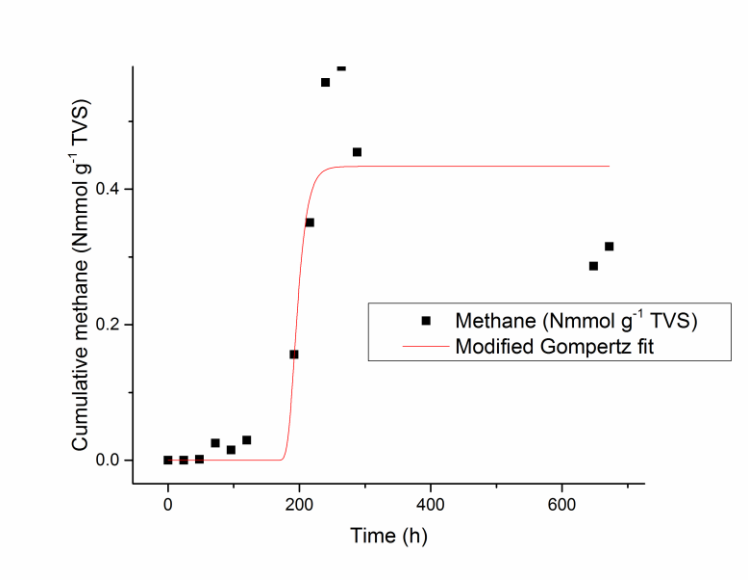

Figure 10.31 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for O-HSO

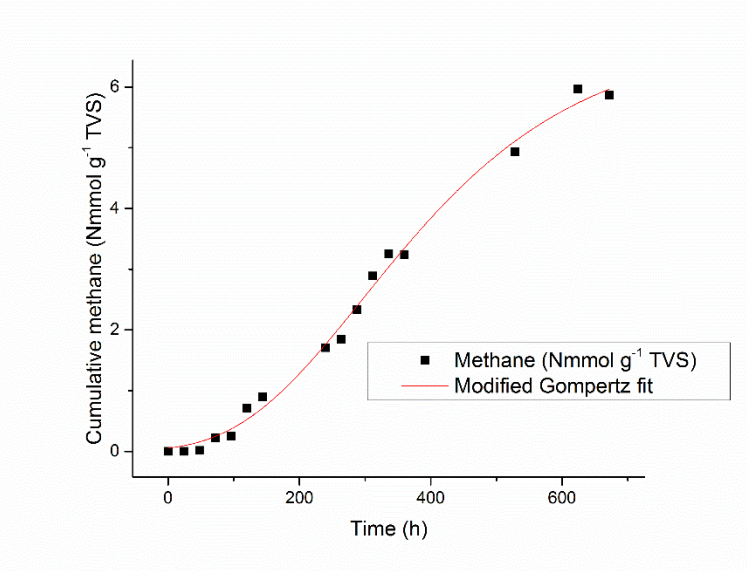

Figure 10.32 - Cumulative methane (Nmmol g^{-1} TVS) production with Modified Gompertz fit for P-HSO