ISOLATION AND CHARACTERIZATION OF CELLULOLYTIC BACTERIA FOR IMPROVING BIOGAS PRODUCTION FROM CROP RESIDUES THROUGH BIOLOGICAL PRETREATMENT AND CODIGESTION

By

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ABSTRACT

Biofuel production from renewable resources is a rising concern due to depletion of fossil fuels, increasing fuel prices and green house gas emissions in the world. Zimbabwe is renowned for its agricultural industry, which generates millions of tonnes of crop residues, such as straw, husks, cobs, stover and hulls. Crop residues contain large amounts of organic matter that can be converted into biofuels. Anaerobic digestion (AD) is a technology which combines biogas production and sustainable waste management in a circular bioeconomy. Biogas is a clean renewable energy carrier with numerous benefits. The main objective of the study was to isolate, identify and characterize cellulolytic bacteria that can be used to enhance biogas production from crop residues through biological pretreatment and codigestion.

The recalcitrant nature of crop residues makes pretreatment an essential step towards sustainable biogas production. In order to select the most suitable pretreatment strategy for crop residues, a systematic study was conducted using the PRISMA method. Biological pretreatment was found to be the best-fit option for improving the hydrolysis of crop residues. It was regarded as an ecofriendly technology with low capital and energy needs, and no generation of toxic compounds. As a result, there was need to promote biological pretreatment as a technology that enhances biogas production from crop residues.

The focus was on isolating cellulolytic bacteria from hot springs for potential pretreatment agents. Of all the strains screened from hot springs, only three strains designated as LB-4, LB-6 and LB-8 had high cellulolytic activity. The strains were preliminarily identified as rod shaped, Gram positive and appeared to belong to a group of motile bacillus through morphological and biochemical identification. Homology analysis against the NCBI GenBank showed strains LB-4, LB-6 and LB-8 to be 99.13%, 98.26% and 98.91% related to *Bacillus subtilis*, *Bacillus* sp., and *Bacillus licheniformis*, respectively. Using submerged fermentation, the optimum cellulase activity of the strains were observed after 24 hours at pH 7 and 40ºC while utilizing 1% CMC as a carbon source and 1% yeast extract as a nitrogen source.

Comprehensive analysis of maize stover, wheat straw and soybean straw for proximate composition showed a significant variability among the three crop residues. All the crop residues were reported to contain more than 30% cellulose and revealed high potential for biogas production. Cellulose, hemicelluloses and lignin content in crop residues ranged from 34.6 - 37.8%, 19.7 - 28.2% and 16.2 - 23.5%, respectively. Wheat straw had a higher cellulose (37.8%) and hemicellulose content (28.2%) than the other crop residues. Soybean straw reported the highest lignin content of 23.5%. However, the acidic nature (pH 5.3 - 5.5) and high total nitrogen content (3.1 - 8.2%) of the crop residues highlighted the need for codigestion with other organic substrates.

Strains *B. subtilis* LB-4, *Bacillus* sp. LB-6 and *B. licheniformis* LB-8 were used to construct a hot spring cellulolytic microbial consortium (HSCMC). The HSCMC consortium was applied for biological pretreatment of crop residues. The pretreated feedstocks were characterized for total reducing sugar, ash, total solids (TS) and volatile solids (VS). Significant variation between pretreated crop residues and control conditions was observed in relation to chemical

characteristics. The highest decline in VS (69.2%) content was reported from maize stover, whereas wheat straw showed maximum reduction of 83.9% in TS content. The TRS concentrations for pretreated hydrolysates of maize stover, wheat straw and soybean straw were significantly enhanced by 60.9, 96.3 and 84.7%, respectively. The biomethane potential assay was performed using batch fermentation to evaluate the feasibility of pretreating crop residues using the HSCMC consortium. Results established that pretreatment of crop residues using HSCMC can significantly improve the cumulative methane yield of maize stover, wheat straw and soybean straw by 50.2%, 50.6% and 56.6%, respectively. Cattle manure with pH, ash, VS and TS content of 7.12, 15.10%, 47.20% and 51.34%, respectively, was selected as a cosubstrate for this study. Codigestion of pretreated crop residues with cattle manure increased methane yield in the range of 13.3 - 25.1% compared to unpretreated groups.

In conclusion, bacteria with high cellulolytic ability were successfully isolated from hot springs and an effective microbial consortium, HSCMC was developed. The HSCMC consortium enhanced methane yield of crop residues after pretreatment and codigestion with cattle manure. This study provides useful information and original contribution that could validate the up-scaling of bench-scale findings to pilot- and demonstration-scale towards commercialization.

DECLARATION

I, the undersigned, declare that the work contained herein this thesis submitted to the Chinhoyi University of Technology for the Degree of Doctor of Philosophy is my original work unless acknowledged in text. This work has not been submitted to any other university for a degree or for the purpose of publication. I am conscious that the incorporation of material from other studies or paraphrase of such material will be treated as plagiarism subject to the custom and usage of the subject, according to the University Regulations on Conduct of Examinations. Therefore, I authorize Chinhoyi University of Technology to allow other institutions, organizations or individuals to access this report for academic purposes.

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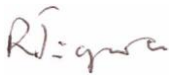
Principal Supervisor:  Date: 02/08/2023

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DEDICATION

This work is dedicated to my wife, children and family for their courage and support throughout the period of study.

GOD BLESS YOU

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LIST OF PUBLICATIONS ASSOCIATED WITH THIS STUDY

This thesis is supported by the work covered in the following publications:

Peer-reviewed Journal Articles

1. Kamusoko, R., Jingura, R.M., Chikwambi, Z., & Parawira, W. (2021b). Strategies for valorization of crop residues into biofuels and other value-added products. *Biofuels, Bioproducts & Biorefining, 15*(6), 1950-1964.
2. Kamusoko, R., Jingura, R.M., Parawira, W., & Sanyika W.T. (2019). Comparison of pretreatment methods that enhance biomethane production from crop residues: A systematic review. *Biofuel Research Journal, 24*, 1080-1089.
3. Kamusoko, R., Jingura, R.M., Parawira, W., & Chikwambi, Z. (2023). Isolation and characterization of cellulolytic bacteria from Lubimbi hot springs in Binga, Zimbabwe, *Journal of Bioscience & Biotechnology, 12*(1), 33-39.
4. Kamusoko, R., Jingura, R.M., Parawira, W., & Chikwambi, Z. (2022a). Characterization of lignocellulosic crop residues for potential biogas production in Zimbabwe. *Biofuels, Bioproducts & Biorefining, 16*(5), 1165-1171.

Book Chapters

1. Kamusoko, R., Jingura, R.M., Parawira, W., & Chikwambi, Z. (2022b). Biogas: Microbiological research to enhance efficiency and regulation. In Sahay, S (Ed.), *Handbook of biofuels* (pp. 485-497). Elsevier.
2. Kamusoko, R., Jingura, R.M., Parawira, W., & Chikwambi, Z. (2021a). Purification and amplification of DNA from cellulolytic bacteria: Application for biogas production from crop residues. In Basu, C (Ed.), *Biofuels and biodiesel. Methods in molecular biology*, vol 2290 (pp. 187-201). Humana, New York, NY.

LIST OF ABBREVIATIONS

ABP	Anaerobic biogasification potential
AD	Anaerobic digestion
AcoD	Anaerobic codigestion
AMPTS	Automatic Methane Potential Test System
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
BMP	Biomethane potential
BOD	Biological oxygen demand
CMC	carboxymethyl cellulose
C/N	Carbon to nitrogen ratio
COD	Chemical oxygen demand
CSTR	Continuously stirred tank reactor
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic acid
EU	European Union
FAO	Food and Agricultural Organization of the United Nations
GHG	Greenhouse gas
HRT	Hydraulic retention time
HSCMC	Hot spring cellulolytic microbial consortium
IEA	International Energy Agency
LB	Luria Bertani
MSW	Municipal solid waste
NCBI	National Center for Biotechnology Information
OECD	Organization for Economic Cooperation and Development
OLR	Organic loading rate
PCR	Polymerase chain reaction
PRISMA	Preferred reporting items for systematic reviews and meta-analyses
rRNA	Ribosomal ribonucleic acid
SmF	Submerged fermentation
SSAD	Solid-state anaerobic digestion
TA	Total alkalinity
TN	Total nitrogen
TRS	Total reducing sugar
TS	Total solids
VDI	Verein Deutscher Ingenieure
VFAs	Volatile fatty acids
VM	Volatile matter
VS	Volatile solids
WBA	World Bioenergy Association

CHAPTER 1: THE PROBLEM AND ITS CONTEXT

1.8 Introduction

There has been intensifying pressure to increase production of renewable energy carriers, such as biofuels, geothermal resources, solar, hydropower and wind power due to population growth in the world (Mollahoseini et al., 2015). The mantra is to replace depleting fossil fuels and mitigate the global climate change (Malode et al., 2021; Huerta et al., 2023). Currently, fossil fuels are the major suppliers of energy contributing about 85% of the world's energy (International Energy Agency (IEA), 2021). Oil reserves are anticipated to exhaust by 2050 as consumption of fossil-fuels is more than 100 times faster than its natural source (Ali et al., 2017). In addition, burning of fossil fuels leads to severe adverse effects, which can be life-threatening. For example, fossil fuels emit carbon dioxide (CO_2) and other greenhouse gases (GHGs) that promote environmental pollution and global warming (Li & Haneklaus, 2021).

Biofuels can be a suitable alternative to fossil fuels in heat and power generation, and transportation. The global market share of biofuels is forecasted to accelerate by 28% from 2021 to 2026 (IEA, 2021). Biofuels are energy-rich chemicals derived from biochemical processes or living organisms, such as plants, algae and bacteria (Datta et al., 2019; Radionova et al., 2017). The most common types of biofuels are biogas, biodiesel and bioethanol. Approximately 90% of bioethanol comes from fermentation of starches and sugars from maize, wheat, sugarcane, cassava and sugar beet (OECD & FAO, 2022). Around 75% and 20% of biodiesel originates from edible vegetable oils and used cooking oils, respectively (OECD & FAO, 2022). This may pose serious threat to food security and trigger a rise in world food prices.

Large amounts of crop residues are generated from farming systems in the world. Crop residues are cheap and sustainable sources of cellulosic material for energy production (Zhong et al., 2011; Kamusoko et al., 2022a). However, modern technologies based on cellulosic feedstocks do not provide large shares to the global biofuel production (OECD & FAO, 2022). Anaerobic digestion (AD) is an efficient method for managing crop residues in a sustainable way. It transforms organic waste (i.e crop residues) into biogas whilst producing a biofertilizer (Sukhesh & Rao, 2018; Aravani et al., 2023; Huerta et al., 2023). The limitation of crop residues in AD is that they do not degrade easily to release fermentable sugars (Sukhesh & Rao, 2018; Aravani et al., 2023). The use of crop residues for AD needs to be objectively and practically analyzed with the view to minimize GHG emissions and food insecurity.

This Chapter provides a concise discussion on production of crop residues and their conversion into biogas through AD. Strategies (i.e pretreatment and codigestion) that can enhance the AD of crop residues are also summarized. The background, problem, justification, objectives and hypotheses of the study as well as the structure of the thesis have also been presented.

1.8 Background of the Study

Biogas is a renewable energy carrier that has a wide range of applications (Achinas et al., 2017; Alhassan et al., 2019). It can be used as an alternative source of fuel for combined heat and power generation, and for vehicles (Weiland, 2010). Biogas is likely to lower GHG emissions by 42 - 82% (Aparicio et al., 2020). The world produces more than 62.3 billion m^3 of biogas per year. This corresponds to an energy content of 1.43 EJ (World Bioenergy Association (WBA), 2021). Table 1.1 summarizes the supply of biogas in different parts of the world. Europe is the leading continent in biogas generation followed by Asia. Africa accounts for only 0.02% of the

annual global biogas mix (WBA, 2021). In Zimbabwe, statistics for biogas production are not well documented. However, more than 400 anaerobic digesters with a technical biogas potential of 5000 m^3 have been reported (Jingura et al., 2013).

Table 1.1 Biogas supply in the world

Continent	Biogas supply (EJ)	Biogas supply (Billion m^3)
Africa	0.00	0.01
Americas	0.19	8.44
Asia	0.50	21.8
Europe	0.72	31.2
Oceania	0.02	0.85

Source: World Bioenergy Association (2021)

Biogas is produced by AD of organic matter using consortia of bacteria (Hagos et al., 2017; Kumar et al., 2018; Mulat & Horn, 2018). The AD is a complex multi-stage process involving hydrolysis, acidogenesis, acetogenesis and methanogenesis (Zheng et al., 2014; Kumar et al., 2018). Hydrolysis is often the rate-limiting step in AD of lignocellulosic feedstocks, such as crop residues, due to their recalcitrant nature (Zheng et al., 2014). The AD is mainly affected by substrate composition, reactor design and operational conditions (Jingura & Kamusoko, 2017). Organic substrates that can be digested for biogas production include animal manure, municipal solid waste (MSW), sewage sludge, food waste, energy crops and crop residues (Achinas et al., 2017; Rabii et al., 2019).

The potential of disparate substrates to produce biogas is evaluated using the biomethane potential (BMP). The BMP is the maximum volume of methane produced per gram of volatile

solids (VS) in a substrate. It is an indicator of the biodegradability and quality of a substrate for AD. The BMP test is a convenient method that can determine the BMP of AD substrates (Jingura & Kamusoko, 2017).

Crop residues are the most predominant agricultural biomass on earth for biofuel production. Over 200 billion tons of crop residues are produced annually in the world (Horváth et al., 2016; Patinvoh et al., 2016). About 7.8 Mt per year of crop residues are produced in Zimbabwe (Jingura & Matengaifa, 2008). This represents around 81.5 PJ of the annual energy potential. The energy potential of the main types of crop residues in Zimbabwe is shown in Table 1.2. Stover and stalks are the most prevalent crop residues in Zimbabwe (Jingura & Matengaifa, 2008). Crop residues are mainly disposed of by burning, roof thatching, fencing, used as stock feed or buried into the soil to improve fertility (Sukhesh & Rao, 2018; Kamusoko et al., 2021b). Although, some authors report on value-addition of crop residues through energy and biochemical production, the potential of crop residues to produce biogas is still undervalued.

Biogas substrates can be classified and placed on a spectrum according to BMP values (Das & Mondal, 2016). Substrates at the lower end of the BMP continuum are resistant to microbial degradation (Patinvoh et al., 2016). Crop residues occupy the lower end of the BMP spectrum because they are highly lignocellulosic. This limits the use of crop residues for biogas production. The actual methane yield of crop residues is less than 60% of the predicted value (Almansa, 2015). This variation can be reduced by use of suitable techno-economic strategies such as pretreatment, codigestion, bioaugmentation, microbial community monitoring and optimizing reactor operations (Kamusoko et al., 2022b).

Table 1.2 Energy potential of crop residues produced in Zimbabwe

Crop	Type of residue	Total residues (Mt)	Energy potential (PJ)
Maize	Stover, cobs	2.37	30.9
Sorghum	Stover, threshed heads	0.12	1.5
Wheat	Straw	0.22	3.1
Sugarcane	Tops, bagasse	4.26	32.8
Soybean	Straw	0.20	3.3
Cotton	Hulls, husks, stalks	3.89	6.2
Sunflower	Heads, hulls	0.04	0.6
Tea	Spent leaf	0.02	0.2
Groundnuts	Halms, hulls	0.18	2.9

Source: Jingura & Matengaifa (2008)

Pretreatment of crop residues is a promising strategy that can enhance biogas production (Mustafa et al., 2018; Venturin et al., 2018; Dahunsi, 2019; Liu et al., 2019). It can be broadly classified into physical, chemical and biological methods. Physical and chemical pretreatment methods have been widely reported in various studies. They have limited utility at industrial scale due to high operational and energy costs, and production of inhibitory compounds (Cater et al., 2014; Munoz et al., 2014; Jain et al., 2015). Biological pretreatment is a new area that still needs rigorous investigation (Kumar et al., 2018). However, the method is often regarded as inexpensive and ecofriendly (Chatuverdi & Verma, 2013; Vasmara et al., 2015). Despite the limited growth of fungi, white-rot fungi are the most widely studied biological agents for pretreatment of AD feedstocks (Rodriguez et al., 2016; Amin et al., 2017).

Bacteria with cellulolytic potential have been isolated from multifarious habitats, such as sewage, soil, hot springs, and the guts of ruminants and insects (Martin-Ryals, 2012). Cellulolytic bacteria possess suitable enzyme expression systems, grow fast in low-cost medium and resist extreme conditions (Martin-Ryals, 2012; Seo et al., 2013). Pretreatment of different substrates with cellulolytic bacteria was previously demonstrated by Munoz et al. (2014), Poszytek et al. (2016), Kavitha et al. (2017) and Patinvoh (2017).

Codigestion of crop residues with other substrates of low carbon to nitrogen (C/N) ratio, such as sewage sludge and animal manure is another strategy that can improve biogas yield (Sun et al., 2015). There has been an increase in the number of publications on anaerobic codigestion (AcoD) from 2000 to 2015 (Hagos et al., 2017). This indicates continued research efforts on the subject matter. Codigestion creates synergism in the digester, reduce inhibition, and improve C/N balance, buffering capacity and biogas yield (Shu et al., 2015; Singh & Chaudhary, 2016).

1.8 Statement of the Problem

Zimbabwe is endowed with vast amounts of crop residues, yet their potential to solve energy security issues remains untapped. These crop residues have an estimated annual energy potential of 81.5 PJ (Jingura & Matengaifa, 2008). This value corresponds to around 28% of the total amount of energy consumed in the country each year. Generally, crop residues do not enter the energy supply chain as they are disposed off as stock feed or as soil amendment (Jingura & Matengaifa, 2008). When used as energy carriers, they are converted via inefficient processes, such as combustion in open fires. Conversion efficiencies of biomass in open fires vary between 5% and 10% (Jingura et al., 2013). The rest of the energy is lost.

The energy potential of crop residues in Zimbabwe needs to be exploited, especially for biogas production. Although information on BMP of crop residues can be extrapolated from extant literature, the BMP of locally available crop residues is not yet fully understood and exploited. Despite, the actual BMP of crop residues may vary widely as it is affected by biomass type and geographical location.

Traditionally, valorization of crop residues has faced some bottlenecks because they are highly lignocellulosic. This limits the use of crop residues for biogas production as their polymerization restrains microbial decomposition. The cost of biogas production from crop residues is moderately high due to reduced glucose yield and increased cost of hydrolysis. Physical and chemical pretreatment of crop residues have not been fully utilized due to high operational and energy costs, and production of inhibitory compounds (Yang et al., 2014; Kamusoko et al., 2019). Alternatively, fungi have slow growth rate and have been touted to consume some useful carbohydrates such as cellulose and hemicelluloses during pretreatment (Yang et al., 2014). On the other hand, enzymatic pretreatment method has limited utility in AD due to high cost of enzymes (Zheng et al., 2014). Thus, the need for alternative approaches is high.

Bacteria are ubiquitous with fast growth rate, simple cultivation and short generation time; hence they can be suitable alternatives for pretreatment of crop residues (Yang et al., 2014). Not much work has been done to characterize the bacterial pretreatment of crop residues for biogas production in as much as many reports have reported the isolation of cellulolytic bacteria. In addition, bacteria pretreatment using a sole microorganism has not been investigated on a full-scale. Furthermore, there is limited information on how specific cellulolytic bacteria consortia perform with different substrates of crop residues.

1.8 Justification of the Study

Biogas production is a clean technology that can improve the conversion efficiency of biomass in sustainable bioenergy systems. The process of biogas production needs to be supported and enhanced in Zimbabwe. The country has produced biogas at only 8% of its potential (Jingura et al., 2013). It is more likely to surpass this by diversifying the feedstock streams. Crop residues being abundant, renewable and rich in cellulose, could potentially widen the resource base of feedstocks for biogas production. Their energy potential needs to be fully exploited as they are highly resistant to degradation.

It is known that pretreatment and codigestion are efficient and cost-effective strategies that can enhance biogas production from crop residues. These technologies need to be supported through innovation. One way to do so is by taking biological pretreatment as a techno-economic strategy that can improve the biodegradability of crop residues. This method is cheap and does not generate toxic inhibitory compounds (Abraham et al., 2020; Awogbemi & Kallon, 2022). This calls for continuous research and innovation efforts to isolate and characterize microbes that have the potential to delignify crop residues rapidly and efficiently so as to maximize the ability to extract bioenergy from them.

Bacteria grow rapidly on cheap media, tolerate harsh conditions and have convenient gene expression systems with improved bioconversion rate (Yang et al., 2014). Thus, they are suitable candidates to degrade crop residues. Pretreatment using bacteria consortium is considered to be more effective than a single microorganism in enhancing the degradation of agricultural wastes (Abraham et al., 2020). Rigorous research needs to be conducted on novel bacterial strains with

higher cellulolytic potential and better stability against environmental conditions to enhance their degradation efficiency. This is the focus of this study.

1.8 Objectives of the Study

1.5.1 Main Objective

The main objective of the study was to isolate, identify and characterize cellulolytic bacteria that can be used to enhance biogas production from crop residues through biological pretreatment and codigestion.

1.5.2 Specific Objectives

The specific objectives of the study were to:

(a) Isolate and identify cellulolytic bacteria from hot springs.

(b) Determine optimum fermentation conditions for cellulase production by the isolated bacteria.

(c) Determine the proximate composition of crop residues.

(d) Assess the BMP of crop residues pretreated with a consortium of cellulolytic bacteria.

(e) Assess the effect of codigestion of the pretreated crop residues and animal manure.

1.8 Research Hypotheses

The hypotheses tested in this study were:

(a) Highly active cellulolytic bacteria can be isolated from local hot springs.

(b) An efficient microbial consortium can be developed from the isolated bacteria.

(c) The BMP of crop residues is enhanced by pretreatment with cellulolytic bacteria consortium.

(d) Proximate composition of crop residues differs depending on biomass type and geographical location.

(e) Codigestion of crop residues pretreated with cellulolytic bacteria consortium and animal manure improves biogas yield.

1.7 Scope of the Study

It is a fundament to deploy innovative strategies (i.e pretreatment and codigestion) so as to delignify crop residues that are resistant to biodegradation, and complement nutrients during biogas production. In view of this scenario, the scope of the present study was limited to screen cellulolytic bacteria from hot springs as biological agents for pretreatment of crop residues. The study focused on three types of crop residues (wheat straw, maize stover and soybean straw) as feedstocks for biogas production. The study was restricted to cattle manure as a cosubstrate for AcoD with crop residues. Feedstocks were collected from local farms surrounding Chinhoyi. The inoculum in form of rumen solid waste was sourced from a local abattoir. Feedstocks and the inoculum were examined for biochemical composition using Standard Methods for Examination of Water and Wastewater, and spectrophotometry. The study involved monodigestion and AcoD of crop residues pretreated with cellulolytic bacteria consortium and cattle manure to enhance methane production. The AD was conducted in 500 mL batch digesters at lab-scale study. The BMP assay was confined to liquid displacement using the NaOH absorption method.

1.8 Structure of the Thesis

This thesis consists of nine Chapters. The Chapters build on each other to address the research objectives of this study (Figure 1.1). The research work is supported by rigorous review of extant literature.

Chapter 1 is the main introduction to the study. The statement of problem, justification of the study, research objectives, hypotheses and the structure of the dissertation are provided.

Chapter 2 is the overall review of literature related to this study. It considers the biofuel industry on a global point of view, use crop residues as feedstocks for biogas production, the framework for AD, process parameters that affect AD, strategies to enhance AD of crop residues and the isolation of cellulolytic bacteria for potential application in biogas production.

Chapter 3 presents a systematic comparison of different pretreatment methods that can be used to enhance biomethane production from crop residues. It is within this context that biological pretreatment was chosen for this study.

Chapter 4 presents work on screening and identification of cellulolytic bacteria from local hot springs. Cellulolytic bacteria were utilized to construct a microbial consortium for pretreatment of crop residues prior to biogas production.

Chapter 5 reports data on proximate composition of different types of crop residues in Zimbabwe. The residues were subsequently tested as feedstocks to generate biogas after pretreatment using a consortium of cellulolytic bacteria.

Chapter 6 is a lab-scale attempt to improve biomethane production through biological pretreatment of crop residues with a consortium of cellulolytic bacteria.

Chapter 7 is a bench-scale attempt to enhance biomethane production through anaerobic codigestion (AcoD) of biologically pretreated crop residues with cattle manure.

Chapter 8 presents the general discussion of the study. This section reviews the findings of the study and puts them into context of the overall research.

Chapter 9 provides the general conclusion of the study and recommends for further research work for future developments.

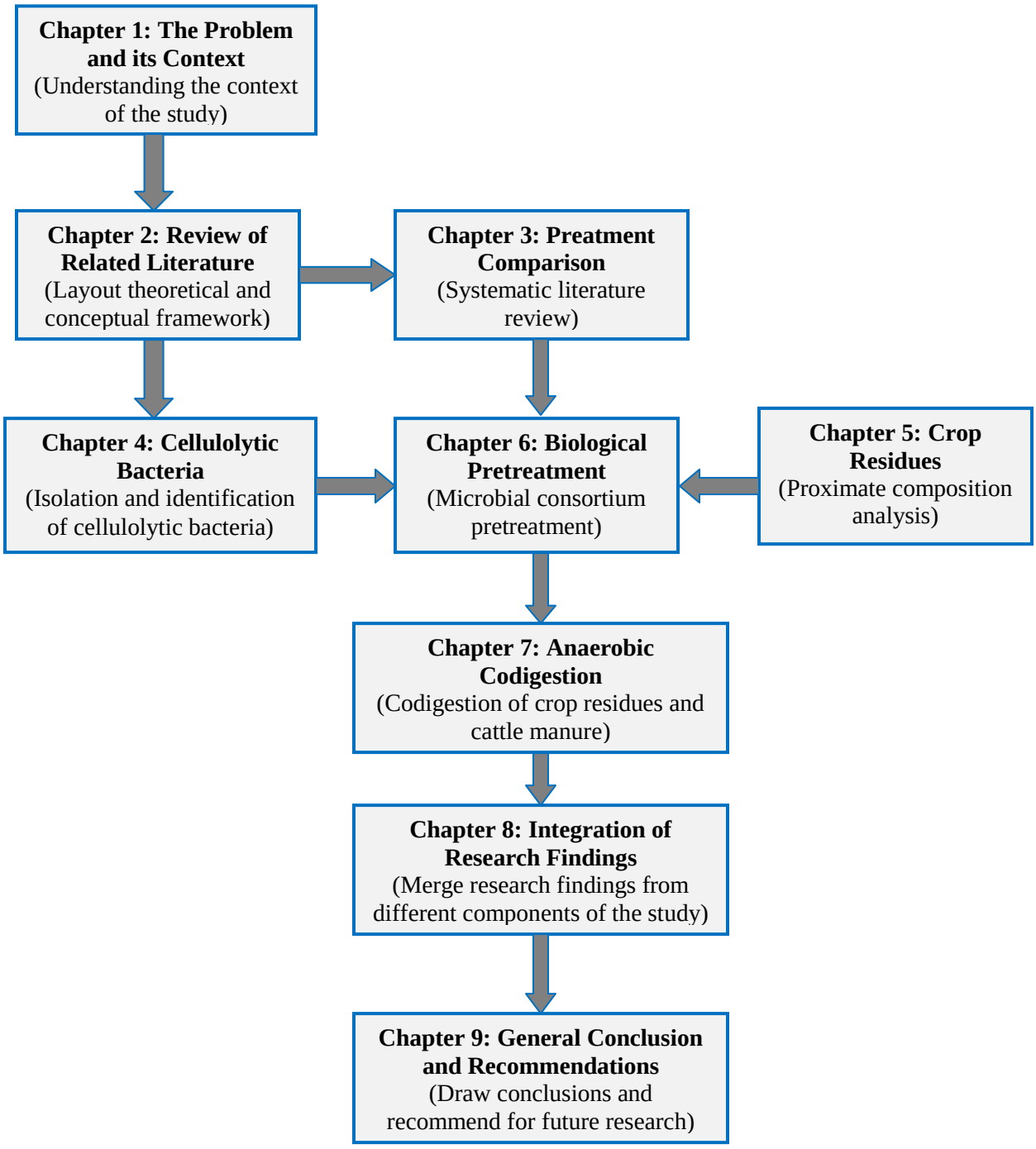

Figure 1.1 Schematic presentation of the work performed in this study

CHAPTER 2: REVIEW OF RELATED LITERATURE

2.1 Introduction

The overall aim of this Chapter is to lay out the theoretical and conceptual frameworks that underpin this study. The Chapter reviews extant literature on biofuel research with special focus on biogas technology. It reviews the state-of-art with application of various pretreatment methods and codigestion technology to improve biogas production from crop residues. The Chapter starts by introducing energy consumption in the context of biomass conversion to biofuels at a global perspective. It then looks at the different types of crop residues, their structure and composition as well as the estimated BMP values. The AD technology and the parameters that affect this process are then presented. The chapter provides a detailed account on pretreatment strategies that can be deployed to optimize the microbial efficiency of biogas production from crop residues. The impact of codigestion technology on BMP of crop residues is discussed. Lastly, it summarizes how specific cellulolytic bacteria for improving the hydrolysis efficiency of crop residues can be isolated from disparate sources.

2.2 Introduction to Biofuels

With more than 70% vehicles relying on petroleum fuel, a global shortage of the product is projected by 2070 - 2080. There is intensifying pressure to invest in renewable energy resources in an era of climate change and high energy demand (Malode et al., 2021). The total energy supply is calculated approximately at 606 EJ per year in the world (International Energy Agency (IEA), 2021). Fossil fuels, mostly petroleum, coal and natural gas contribute about 85% to the global energy supply (IEA, 2021). The main challenge of fossil fuels is that they release GHGs that may lead to climate change and global warming (Sambusiti, 2013; Kamusoko et al., 2022b).

The GHG emissions from petroleum are estimated to rise to about 43 billion Mt by 2040 (Malode et al., 2021). This calls for governmental and non-governmental organizations to intervene and seek for renewable alternatives that are sustainable and ecofriendly in the global energy matrix (Malode et al., 2021). China is the main producer of GHGs, contributing about 27% to the total global GHG emissions (Datta, 2022).

Plant biomass is a potential source of renewable energy. According to Datta (2022), the global biofuel production increased by 10 billion liters to 154 billion liters in 2018, and it is further projected to rise by 24% in 2024. The 2030 Agenda for Sustainable Development and the Paris Agreement on Climate Change and Sound Energy Statistics has put energy security as a priority area (United Nations (UN), 2022). Furthermore, the European Union (EU) set a target to reduce GHG emissions by 45% in 2030, and to be climate-neutral and create an economy with net-zero GHG emissions by 2050 (Tol, 2021).

Low-carbon energy sources can generate multiple forms of natural, clean and usable energy. They include nuclear energy and renewable energy (i.e bioenergy, geothermal resources, solar, hydropower and wind). Renewable energy contributes 11.3% to the global primary energy mix (Ritchie et al., 2022). Disaggregating this information by source is shown in Figure 2.1. It is evident that around 0.7% of the renewable energy comes from biofuels. The use of biofuels as an energy source is still underestimated.

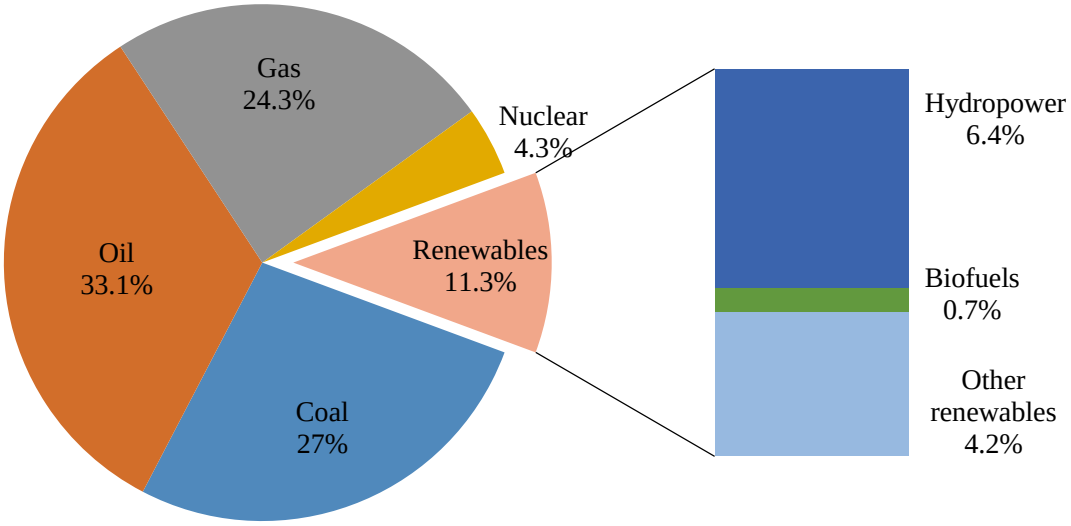

Figure 2.1 Structure of the energy supply mix in the world (Ritchie et al., 2022)

Biofuels such as biogas, biohydrogen, bioethanol, bioelectricity, biodiesel and biobutanol are contemporary sources of energy that can replace petroleum for transportation, heat and power generation. They are derived from biomass, mainly microorganisms, plants, organic wastes and animals (Datta et al., 2019; Mahapatra et al., 2021). As compared to petroleum fuels, biofuels are ecofriendly, sulfur-free, biodegradable and renewable. However, biofuels are labor intensive; require large amounts of storage space, land and water; interfere with life cycles; and their viscosity can influence engine performance (Malode et al., 2021).

There are four generations of biofuels depending on the type of feed stocks: first, second, third and fourth generation biofuels (Rodionova et al., 2017; Mahapatra et al., 2021). First generation biofuels come from edible oils, sugars and starches that lead to food-energy dispute. Second generation biofuels are acquired from organic wastes. The third and fourth generation biofuels come from microalgae and engineered cyanobacteria, respectively (Mahapatra et al., 2021; Malode et al., 2021).

2.3 Types of Organic Feedstock

Organic wastes are non-food feedstocks, primarily of agricultural and forestry in origin (Mahapatra et al., 2021; Malode et al., 2021). Examples of second generation feedstocks include wood, forest waste, food crop waste, vegetable oil waste, industrial waste and ecological biomass crops (Kumar et al., 2022). Second-generation materials are useful for two reasons. Firstly, the crops are harvested to provide food or feed. Secondly, the wastes from harvesting and processing of crops are a potential source of feedstock for energy production (Patinvoh et al., 2016).

However, the economic viability of second generation biofuels is a key concern due to high cost of feedstock pretreatment. For instance, pretreatment was estimated to consume around 16 - 19% of the total cost of equipment in a lignocellulosic biorefinery (Sambusiti, 2013).

2.4 Types of Biofuels Produced from Organic Feedstocks

Different types of second generation biofuels can be generated from crop residues. The types of biofuels produced from crop residues are shown in Figure 2.2. Biofuels fall into three broad categories: solid, liquid and gaseous fuels (Zhang & Zhang, 2019). Solid biofuels are “primary fuels” including fire wood, wood chips and pellets that are used in their unprocessed form whilst liquid and gaseous biofuels are “secondary fuels” obtained through biomass conversion. Liquid biofuels have more advantages over solid and gaseous biofuels. This is due to their high density, ease of transportation and storage, and ability to power modern engines, turbines and boilers (Sindhu et al., 2019). Yields of biofuels may differ due to variations in lignocellulosic composition and reactor conditions (Sindhu et al., 2019).

Biodiesel, bioethanol and biogas are the most commercially available biofuels and they constitute 90% of the global biofuel market (Arshad et al., 2018). Amongst them, bioethanol is

the most extensively utilized biofuel in transportation (Inyinbor et al., 2017). Sustainability is a major concern in establishment of full-scale biorefineries for second generation biofuels. This needs special attention with regards to food security issues and resource consumption during biomass production. Losses of biodiversity and land use change are new concerns (Patinvoh et al., 2016).

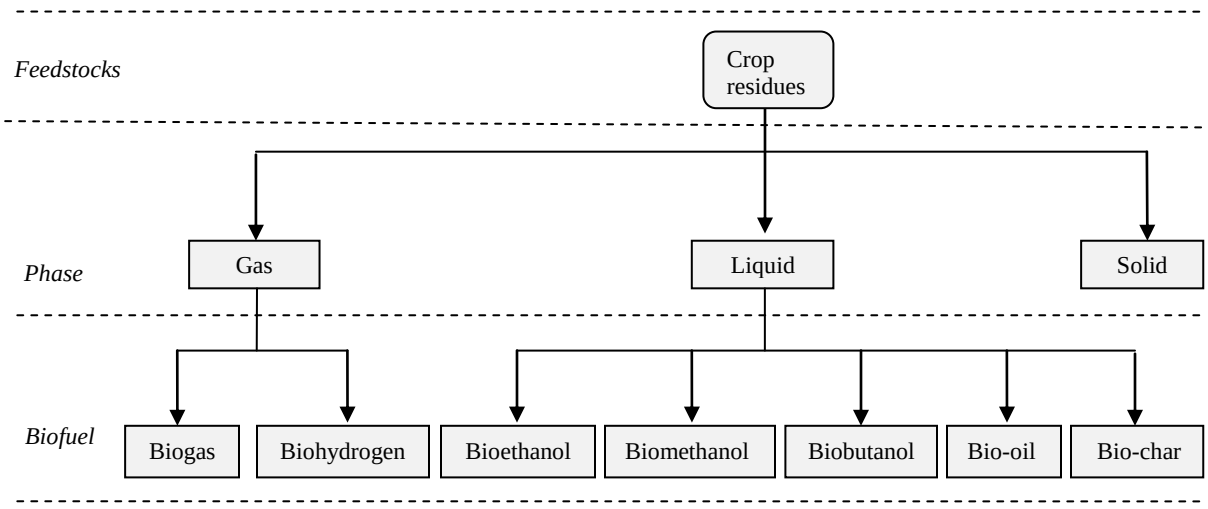

Figure 2.2 Types of biofuels produced from crop residues

2.5 Crop Residues as a Source of Biofuels

2.5.1 World Crop Residues Production

Crop residues are one of the most plenteous, cheap and renewable resources available on earth and have multifarious benefits (Santana-Meridas et al., 2012). They are non-edible phytomass that arise from crop production or processing systems (Santana-Meridas et al., 2012; Inyinbor et al., 2017; Mohammed et al., 2018; Shinde et al., 2022). Over 5 billion tonnes of crop residues are produced annually all over the world (Shinde et al., 2022). Information on crop residues production in several continents is shown in Figure 2.3. Asia is the world's largest producer of

crop residues and accounts for 47% of the total global annual crop residues production. Approximately 7% of crop residues are produced in Africa.

Crop residues have multiple uses. Traditionally, they are utilized as livestock feed or cooking fuel, buried into the soil to improve fertility, and burned to prepare fields for farming and to eradicate pests and diseases (Jingura & Matengaifa, 2008; Berazneva, 2013). These crop residues management practices present several techno-economic challenges. For example, burying of crop residues requires new and expensive technologies (Bhuvaneshwari et al., 2013). Burning of crop residues contributes to global warming and climate change, and has several adverse effects to man's health (Santana-Meridas et al., 2012).

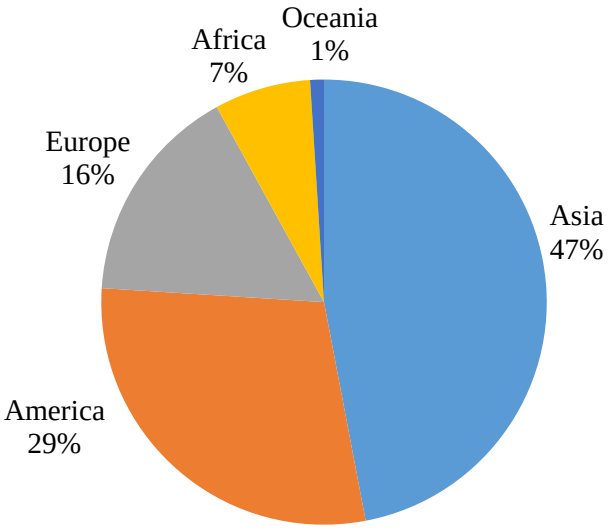

Figure 2.3 Structure of crop residues production in the world (Shinde et al., 2022)

The common types of crop residues include maize stover, rice straw and husks, wheat straw and sugarcane bagasse (Batidzirai et al., 2016; Mohammed et al., 2018). Crop residues are generated mainly from crops such as rice, wheat, maize, barley, soybean and sugar cane. These crops account for nearly 85% of the world crop residues production (Shinde et al., 2022).

About 74% of the global annual crop residues come from cereals (Maqsood et al., 2022). Cereal straws from maize, wheat and rice contribute approximately 27.2%, 21.9% and 26.7%, respectively, to the global agricultural by-product yield (Wang et al., 2017). According to Lal (2005), crop residues production is calculated based on cultivated area and productivity of various crops, and available information in literature on straw to grain ratio (Equation 2.1).

Crop residues production = grain production x straw/grain ratio (Equation 2.1)

The generation of residues from different crops in the world is shown in Table 2.1. The global annual production of cereal crop residues was estimated at more than 3 000 Mt in 2020. Cereal crops yield residues ranging from 22.53 - 1 162.35 Mt per year (Shinde et al., 2022). Cereal straw is the most abundant crop residues in the world. Other producers of crop residues are legumes, sugar crops, oil crops and fibers (Cherubin et al., 2018).

Table 2.1 Crop residues production in the world

Category	Crops	Yield (Mt)
Cereals	Barley, maize, millet, oats, rice, rye, sorghum, wheat	3855.87
Legumes	Beans, broad beans, groundnut, chickpeas, lentils, peas, pulses, soybeans	481.02
Oil crops	Linseed, rapeseed, safflower, seed cotton, sesame, sunflower	301.09
Sugar crops	Sugar beet, sugarcane	530.67
Tuber crops	Potato, sweet potato	112.14
Grand total		5280.80

Source: Shinde et al. (2022)

2.5.2 Structural Composition of Crop Residues

Cell walls provide the bulk of crop residues. Lignocellulosic compounds are the major constituents of cell walls (Sambusiti, 2013; Sun, 2015; Achinas et al., 2017). It is important to know the structural composition of lignocellulose so as to fully utilize crop residues. The main lignocellulosic compounds found in crop residues are cellulose, hemicellulose and lignin (Figure 2.4). The composition of crop residues may vary depending on their sources (Table 2.2). Typically, crop residues consist of 30 - 44%, 30 - 50% and 8 - 21% of cellulose, hemicellulose and lignin, respectively. The composition of crop residues strongly affects the biodegradability and efficiency of use, and determines the most appropriate strategy for their valorization (Sukhesh & Rao, 2018).

Cellulose and hemicellulose are sugar-producing polysaccharides while lignin is a structural non-carbohydrate compound that serves as a protective coat surrounding cellulose and hemicellulose. The biodegradation of cellulose and hemicellulose produces glucose and xylose, respectively. However, the lignocellulosic matrix is extremely resistant to microbial and enzymatic degradation. An efficient pretreatment is required to destroy this matrix, thus making cellulose and hemicellulose amenable for further bioprocessing (Moodley & Trois, 2021). Other compounds that support plant cell walls include non-structural carbohydrates (e.g. glucose, fructose, sucrose and fructans), proteins and pectins (Bajpai, 2016; Hughes et al., 2017).

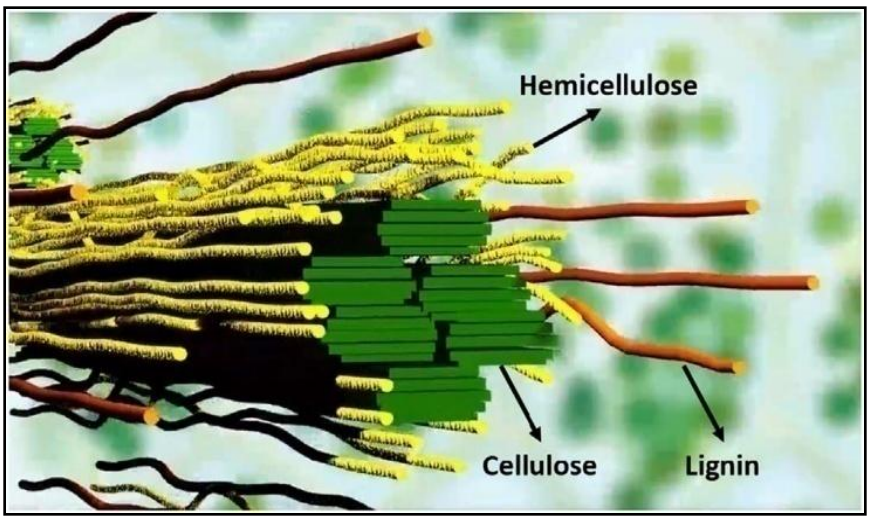

Figure 2.4 Structural composition of crop residues (Moodley & Trois, 2021)

Table 2.2 Chemical composition of selected crop residues

Crop residue	Cellulose (wt %)	Hemicelluloses (wt %)	Lignin (wt %)
Wheat straw	27 - 42	11 - 27	14 - 21
Maize stems	36 - 38	10 - 30	4 - 11
Rice straw	27 - 44	14 - 34	13 - 26
Sunflower stalk	34 - 42	19 - 21	12 - 30
Barley straw	36	12 - 29	8 - 15
Corn stover	37 - 39	23 - 31	14 - 18
Sweet sorghum bagasse	27 - 38	15 - 20	10 - 20

Source: Sambusiti (2013)

2.5.2.1 Cellulose

Cellulose is the most abundant, renewable and sustainable bioresource available on planet and it is the main polymeric compound in plant cells (Chen, 2014; George & Sabapathi, 2015). Global production of cellulose is estimated at around 1.5 trillion tonnes every year (Baghaei & Skrifars,

2020). All crop residues have a similar cellulose structure with a common formula $(C_6H_{12}O_5)_n$ (Moodley & Trois, 2021). Cellulose is mainly composed of 44.44% carbon, 6.17% hydrogen and 49.39% oxygen (Hu et al., 2020). The degree of polymerization and the length of the main chain of cellulose differ across plant biomass (Moodley & Trois, 2021). The degree of polymerization of cellulose varies with source from 10 000 to 15 000 glucose units (Hu et al., 2020). The primary structure of cellulose is shown in Figure 2.5.

Cellulose is a linear homopolymer of cellobiose units that are linked by β-1,4-glycosidic bonds to form parallel cellulose chains of 20 to 300 glucose units. These parallel units are held by hydrogen bonds and van der Waals forces to form microfibrils that are packed in a matrix of hemicelluloses and lignin (Sun, 2015; Bajpai, 2016). Cellulose is a tough, fibrous and water insoluble compound (Chen, 2014; George & Sabapathi, 2015). However, cellulose is biodegradable and biocompatible making it the most important source of fermentable sugars in lignocellulosic biomass. Fermentable sugars can be transformed into useful multiple products in pulp and paper, textile, and fibrous chemical industries (Sambusiti, 2013; Singhania, 2009). Cellulose is also a potential source of biofuels that can provide clean energy and substitute fossil fuels (Chen, 2014; George & Sabapathi, 2015).

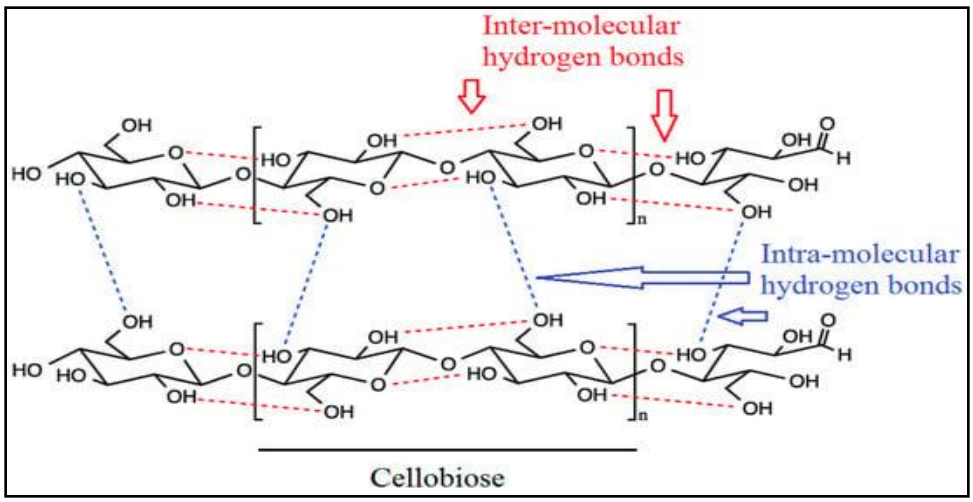

Figure 2.5 Molecular configuration of cellulose (Baghaei & Skrifars, 2020)

2.5.2.2 Hemicellulose

Hemicellulose is the world's second most abundant polysaccharide in plant cell walls. It is naturally found in primary and secondary walls of wood and annual plants, where it is strongly bound to cellulose and lignin (Hu et al., 2020). The structure and composition of hemicellulose vary depending on the type of plant. However, the well-studied chemical structure of hemicellulose is the backbone chain and side chains of glucans (Chen et al., 2014). Figure 2.6 illustrates the typical chemical structure of hemicellulose.

Hemicellulose is a complex heteropolymer of various compounds including pentoses (xylose and arabinose), hexoses (mannose, glucose and galactose) and many organic acids (Chen et al., 2014; Florez-Pardo et al., 2019; Hu et al., 2020; Huang et al., 2021; Moodley & Trois, 2021). The backbone of hemicellulose is either a homopolymer (xylan, glycan and galactan) or heteropolymer (glucomannan) chain with short armophous side chains that are not readily soluble in water (Hu et al., 2020; Moodley & Trois, 2021). Each backbone consists of monomer

sugar units that are joined by β-1,4-glycosidic bonds or β-1,3-glycosidic bonds (Moodley & Trois, 2021).

Hemicellulose has a wide range of industrial applications. For example, biomaterials such as biofilms, hydrogels, wound dressing, drug delivery vessels, etc can be derived from hemicellulose (Hu et al., 2020).

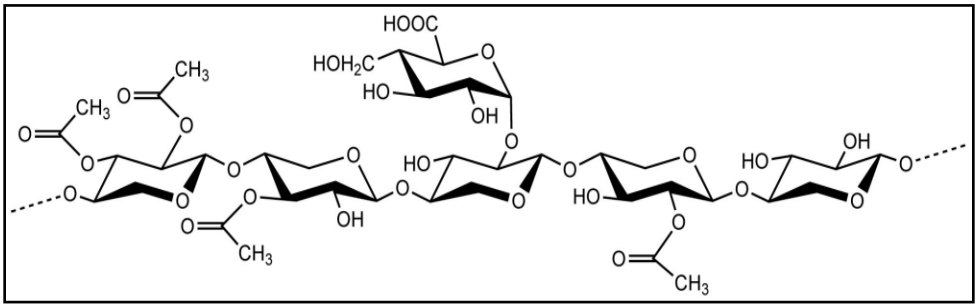

Figure 2.6 Chemical configuration of hemicellulose (Hu et al., 2020)

2.5.2.3 Lignin

Lignin is the most widely available non-polysaccharide component of crop residues. Plant biomass generates about 150 billion tons of lignin each year (Moodley & Trois, 2021). The word "lignin" originates from Latin noun *lignum*, meaning wood. Many functional units such as aliphatics, phenolic hydroxyl and carbonyl groups are found in lignin (Moodley & Trois, 2021; Perez-Pimienta et al., 2021). Lignin is a methoxylated polyphenolic polymer of syringyl (S), guaiacyl (G) and *p*-hydroxyphenyl (H) that are cross-linked by ester bonds of monolignols sinapyl, coniferyl and *p*-coumaryl alcohols to form three-dimensional structures (Lu & Ralph, 2010; Moodley & Trois, 2021; Perez-Pimienta et al., 2021). Apart from *p*-coumaryl alcohols, the general structure of lignin is a phenyl-propanol backbone with methyl ether groups (Perez-Pimienta et al., 2021). The general structure of lignin and its elemental monomers is shown in Figure 2.7.

Lignin plays a significant role in wood and other terrestrial plants. It acts as the main supporting compound and prevents water loss in plants (Holtzapple, 2003). Lignin is amorphous in nature, and highly resistant to chemical and microbial degradation (Holtzapple, 2003; Bajpai, 2016). It serves as a defence mechanism against arthropods and pathogenic microbes amongst plants (Holtzapple, 2003; Perez-Pimienta et al., 2021). Syngas and several biochemicals can be obtained from extraction and processing of lignin (Moodley & Trois, 2021; Perez-Pimienta et al., 2021).

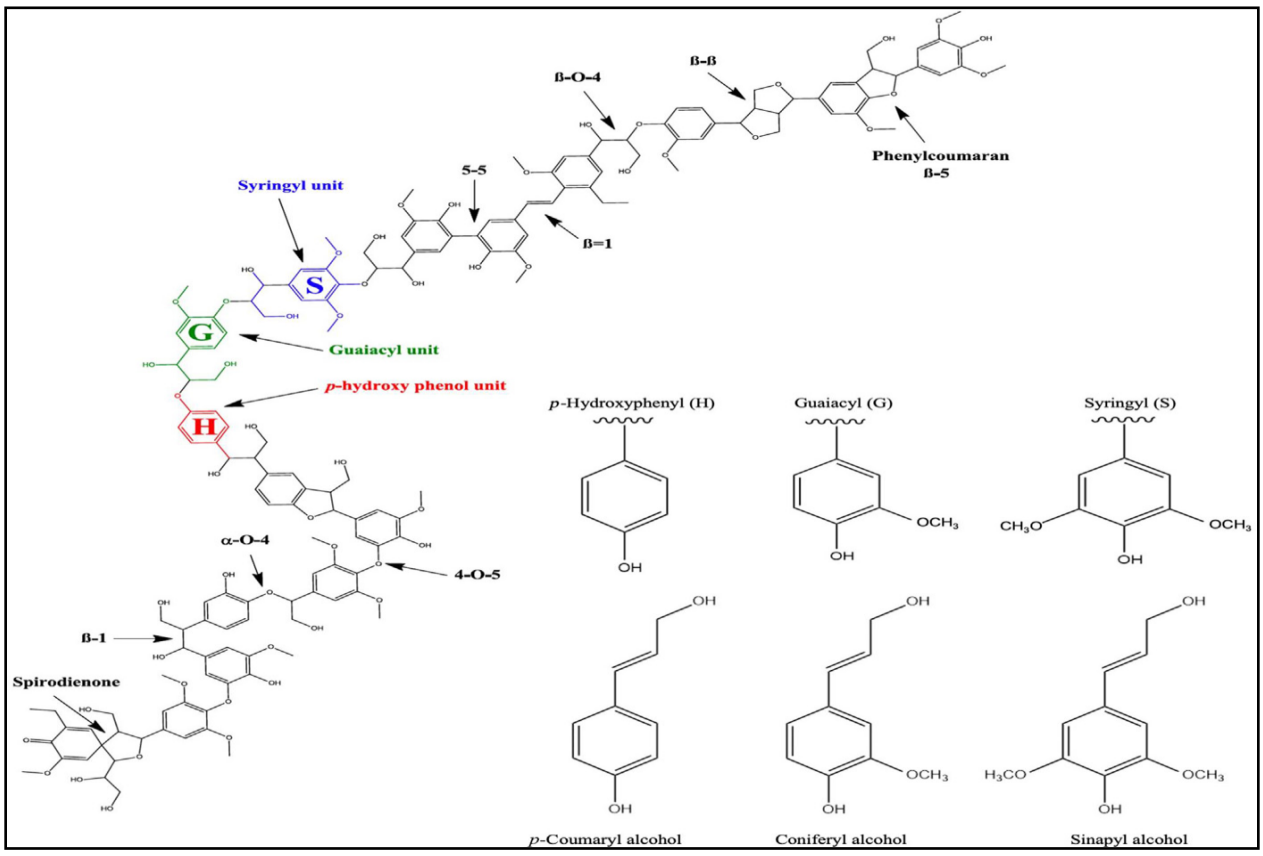

Figure 2.7 Chemical configuration of lignin, and its monomeric units and monolignols (Perez-Pimienta et al., 2021)

2.5.3 Biomethane Production from Crop Residues

Crop residues are suitable substrates for biogas production via the AD process (Liguori et al., 2013; Patinvoh et al., 2016). The AD process is an efficient and established option that merges biofuel production with sustainable waste management (Achinas et al., 2017). The technology produces biogas and a rich digestate waste that can be used to condition soil and improve its water holding capacity (Sukhesh & Rao, 2018; Sindhu et al., 2019). The biodigestate is also useful as a substrate for white oyster mushroom (*Pleurotus florida*) production.

Crop residues contain high amounts of cellulose that is amenable to AD. In fact, up to 80% cellulose in crop residues can be degraded via the AD process to generate high net energy in the form of biomethane (Sukhesh & Rao, 2018). Biomethane is generated in the final stages of AD in which methanogens utilize hydrogen. It is the main constituent (50 - 85%) and a source of energy in biogas (Sindhu et al., 2019). Biomethane can be used in boilers for heat and power generation, and as a fuel for transportation (Weiland, 2010; Sindhu et al., 2019).

Several studies have been conducted to evaluate the potential of crop residues to produce biogas (Table 2.3). Biomethane yields ranging from 124 to 307 mL g^{-1} VS were reported from these studies. The methane yield of crop residues is limited due to high lignin content and imbalanced nutrient composition (Sukhesh & Rao, 2018). This can be surpassed by use of suitable pretreatment strategies and codigestion with nitrogen-enriched substrates, respectively (Sukhesh & Rao, 2018; Kamusoko et al., 2019). Lignin is extremely resistant to degradation. Various studies have showed an inverse relationship between methane yield and lignin composition (Sukhesh & Rao, 2018). Further exploration is still needed to reduce operational costs and

optimize biogas production from crop residues although many biogas plants have been established and commissioned in the world.

Table 2.3 Biomethane yields of various selected crop residues fractions

Crop residues	Test method	Biomethane (mL g^{-1} VS)	Reference
Maize stalks	Batch trials by VDI 4360 (2006)	234	Menardo et al. (2015)
	Batch tests by VDI 4360 (2006)	246	Menardo et al. (2012)
Maize leaves	Batch trials by VDI 4360 (2006)	245	Menardo et al. (2015)
Maize stalk	Batch by water displacement	217	Chen et al. (2010)
Maize husks	Batch trials by VDI 4360 (2006)	307	Menardo et al. (2015)
Maize cobs	Batch trials by VDI 4360 (2006)	207	Menardo et al. (2015)
Sunflower stalk	Batch tests by gas chromatograph	124	Hesami et al. (2015)
	BMP test by displacement method	128	Zhurka et al. (2020)
Sunflower heads	BMP test by displacement method	211	Zhurka et al. (2020)
Wheat straw	Automatic Methane Potential Test System II	293	Peng et al. (2016)
	BMP assay	270	Jackowiak et al. (2011)
	Batch anaerobic digestion by liquid displacement	274	Mancini et al. (2018)
	Automatic Methane Potential Test System II	237	Victorin et al. (2020)
Maize stover	BMP test	261	Li et al. (2013)
Rice straw	Batch tests by VDI 4360 (2006)	197	Menardo et al. (2012)

2.6 The Anaerobic Digestion Process

Anaerobic digestion is a biochemical process in which anaerobic bacteria degrade organic matter and convert it into biogas (Zheng et al., 2014; Ge et al., 2016; Majd et al., 2017). The composition of biogas may differ from source to source. However, biogas from agricultural wastes consists of 50 - 60% methane, 30 - 40% CO_2 and trace amounts of intermediary byproducts, such as hydrogen sulfide (H_2S), ammonia (NH_3) and water vapor (Ge et al., 2016; Scholes, 2020). The stages of the AD process are shown in Figure 2.8. The AD is a multi-stage complex process which involves hydrolysis, acidogenesis, acetogenesis and methanogenesis. Each stage is facilitated by a different group of a consortium of bacteria. In general, the end products of one microbial group are a source of energy for the next consortium (Wang, 2016). Figure 2.8 presents a process-flow map which helps to track the microbiological activities of the AD process and how they are affected by process parameters.

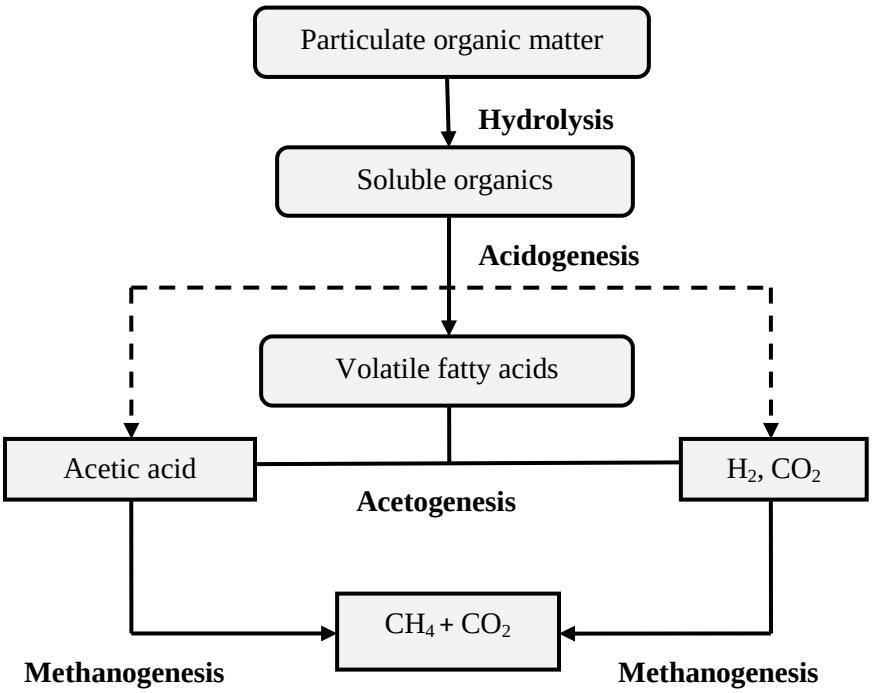

Figure 2.8 Schematic presentation of the anaerobic digestion process (Majd et al., 2017)

Hydrolysis is the first step and often rate-limiting in AD of lignocelluloses. This can be attributed to generation of toxic complex heterocyclic compounds and detrimental volatile fatty acids (VFAs) (Rehman et al., 2019). Hydrolysis utilizes hydrolytic bacteria which secrete extracellular enzymes (cellulases, lipases, proteases and amylases) to degrade complex organic polymers, such as lipids, carbohydrates and proteins into long chain fatty acids, simple sugars and amino acids, respectively (Sambusiti, 2013; Jain et al. 2015). The genera that have been isolated from AD systems include *Cellulomonas*, *Clostridium*, *Bacillus*, *Thermonospora*, *Ruminococcus*, *Bacteroides*, *Erwinia*, *Acetovibrio*, *Microbispora*, *Streptomyces* (Manyi-Loh et al., 2013; Jain et al., 2015). The overall reaction for the hydrolysis step is shown in Equation 2.2 (Anukam et al., 2019). Cellulose ($C_6H_{10}O_5$) is hydrolyzed by water (H_2O) to give off glucose ($C_6H_{12}O_6$) as the main product along hydrogen (H_2) as a byproduct.

$$(C_6H_{10}O_5)n + nH_2O \rightarrow nC_6H_{12}O_6 + nH_2 \qquad \text{(Equation 2.2)}$$

Acidogenesis is a fermentation process where products of hydrolysis are degraded by acidogenic bacteria to produce alcohols, aldehydes, VFAs, acetate, H_2 and CO_2 (Sambusiti, 2013). Degradation of amino and acids also liberates NH_3 gas. Acidogenic bacteria can be either facultative anaerobes or strict anaerobes. Those belonging to the family *Enterobacteriaceae* have been identified as active fermenters (Manyi-Loh et al., 2013). Species found in anaerobic digesters include *Lactobacillus*, *Escherichia*, *Staphylococcus*, *Pseudomonas*, *Desulfovibrio*, *Selenomonas*, *Sarcina*, *Streptococcus*, *Desulfobacter* and *Desulforomonas*. Bacteria convert amino acids to fatty acids, acetate and NH_3 (Stronach et al., 1986). Other known species, such as *Clostridium*, *Eubacterium limosum* and *Streptococcus* transform sugars into intermediary fermentation products (Stronach et al., 1986). The acidogenic stage is represented by Equations 2.3 - 2.5 (Anukam et al., 2019).

$$C_6H_{12}O_6 \leftrightarrow 2CH_3CH_2OH + 2CO_2 \quad \text{(Equation 2.3)}$$

$$C_6H_{12}O_6 + 2H_2 \leftrightarrow 2CH_3CH_2COOH + 2H_2O \quad \text{(Equation 2.4)}$$

$$C_6H_{12}O_6 \leftrightarrow 3CH_3COOH \quad \text{(Equation 2.5)}$$

Methanogenic bacteria cannot directly utilize the products of acidogenesis. This is because methane-forming bacteria do not consume H_2 gas. The products of acidogenesis are further converted via acetogenesis or dehydrogenation, before final transformation into biogas. Acetogenesis converts the products of acidification and the residues of hydrolysis to acetic acid, CO_2 and H_2 (Giard, 2011). Acetogens can be distinguished from acidogens by their ability to reduce CO_2 to acetate using the Wood-Ljungdahl pathway (Jain et al., 2015). Genera comprising *Acetobacterium* and *Sporomusa* are completely isolated acetogens (Jain et al. (2015). Equations 2.6 - 2.8 provide a sequence of reactions that occur during acetogenesis (Anukam et al., 2019).

$$CH_3CH_2COO^- + 3H_2O \leftrightarrow 2CH_3COO^- + H^+HCO_3^- + 3H_2 \quad \text{(Equation 2.6)}$$

$$C_6H_{12}O_6 + 2H_2O \leftrightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad \text{(Equation 2.7)}$$

$$CH_3CH_2OH + H_2O \leftrightarrow CH_3COO^- + 3H_2 + H^+ \quad \text{(Equation 2.8)}$$

Methanogenesis is the final phase of the AD process. Methanogens convert acetate, CO_2 and H_2 into biogas (Sambusiti, 2013). Methanogens occupy diverse anaerobic conditions including freshwater and marine habitats, sewage, digestive systems of herbivores and mammals, wood and humus feeding insects (Manyi-Loh et al., 2013). Species that have been identified as methanogens include *Methanobacterium formicicum*, *M. bryantic* and *M. thermoautotrophicum*; *Methanobrevibacter ruminantium*, *M. arboriphilus* and *M. smithii*; *Methanococcus vannielli* and *M. voltae*; *Methanomicrobium mobile*; *Methanogenium cariaci* and *M. marinsnigri*; *Methanospirilum hungatei*; and *Methanosarcina barkei* (Bajpai, 2017).

Methanogenesis is affected by various conditions such as temperature, feedstock composition and organic loading rate, therefore the stage is rate-limiting in AD of substrates that are easy to biodegrade. Equations 2.9 - 2.11 show a series of reactions that represents methanogenesis (Anukam et al., 2019).

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad \text{(Equation 2.9)}$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad \text{(Equation 2.10)}$$

$$2CH_3CH_2OH + CO_2 \rightarrow CH_4 + 2CH_3COOH \quad \text{(Equation 2.11)}$$

2.7 Factors that Affect the Anaerobic Digestion of Crop Residues

The AD process is dependent on the rate of microbial growth which is affected by several biotic and abiotic operating factors (Figure 2.9). It is essential to monitor and control the operating factors in order to optimize microbial activity and ultimately the AD efficiency of crop residues. The importance of each operating parameter is discussed in this section. In general, methanogens are the mostly affected consortium by operating factors.

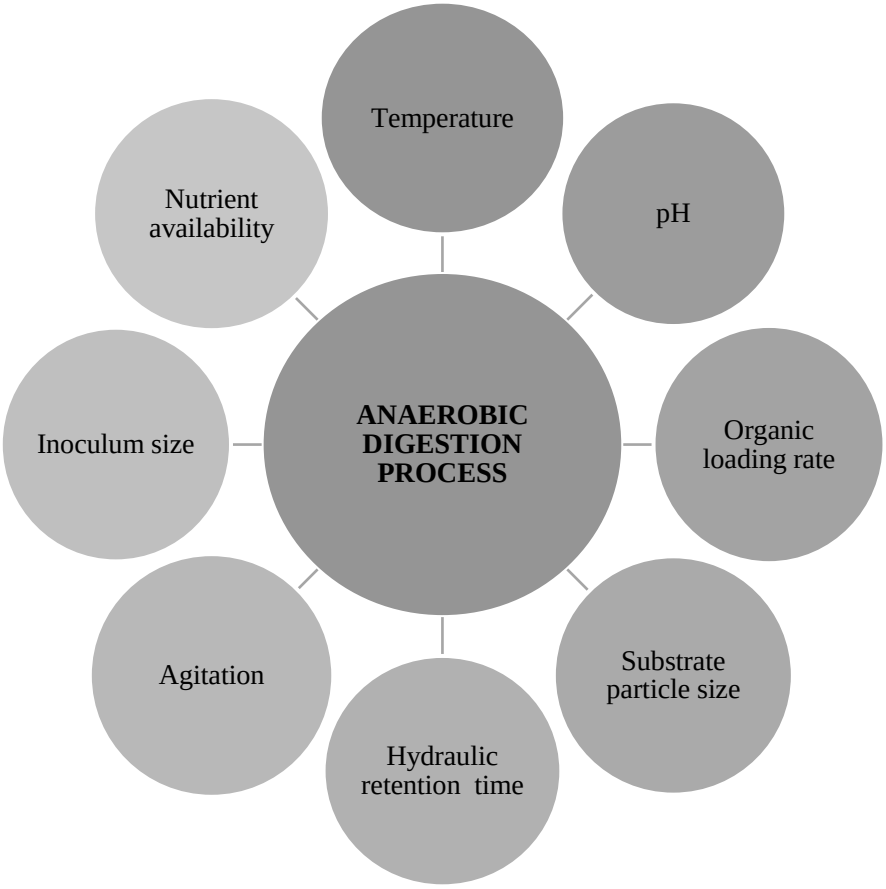

Figure 2.9 Process parameters affecting the anaerobic digestion

2.7.1 The pH Value

Enzymatic activity or digester performance is mostly affected by changes in pH conditions (Wang, 2016). The pH of digester contents varies depending on the stage of digestion. In the early phases of AD, pH conditions are mostly acidic (≤ 6) and tend to increase in the later phases due to degradation of VFAs and the release of methane (Jain et al., 2015). Acidic pH conditions are caused by accumulation of VFAs and evolution of CO_2 (Jain et al., 2015; Wang, 2016).

Various consortia of microorganisms function well at different pH ranges during AD (Wang, 2016). Acid-forming bacteria grow best at pH range of 5 - 6 while methane-forming bacteria thrive at pH range of 6.5 - 7.8 (Majd et al., 2017). However, appropriate pH range must be

maintained so that biogas production is always at steady state. The most favorable pH for optimum AD vary from 6.5 - 7.5 (Jain et al., 2015; Wang, 2016). Extreme pH values are harmful to digester microorganisms. The mostly affected microorganisms are methanogenic bacteria that cannot tolerate acidic pH conditions (Jain et al., 2015; Wang, 2016).

2.7.2 Temperature

Digester microorganisms and enzymes are highly susceptible to variations in temperature (Wang, 2016). Temperatures below 15°C lower microbial growth, substrate utilization and biogas yield. This is due to accumulation of VFAs that inhibits the AD process (Wang, 2016). Lower temperatures also promote liquefaction of CO_2 to give off carbonic acid. Carbonic acid reacts with water and decrease the reactor pH (Siddigue & Ab. Wahid, 2018). Elevated temperatures may lead to a decline in biogas production due to accumulation of free NH_3 inhibitors. Methanogens are not resistant to high temperature conditions (Wang, 2016; Robles et al., 2018). It is vital to maintain temperature at an optimum to ensure satisfactory digester performance.

Digester microorganisms can be classified into three main groups, such as psychrophiles, mesophiles and thermophiles depending on temperature zones (Jain et al., 2015; Majd et al., 2017). The optimum temperatures for psychrophiles, mesophiles and thermophiles are 20ºC, 35ºC and 55ºC, respectively. In addition, there is another group of microorganisms known as hyperthermophiles (> 65ºC) (Wang, 2016). Mesophiles are the commonly utilized group of microorganisms in AD since thermophiles require higher energy to heat the biodigesters (Jain et al., 2015, Wang, 2016). However, mesophilic digestion is normally associated with reduced start-up performance in solid-state AD (SSAD). Thermophiles are now considered to be more stable and effective than the counterpart microorganisms during SSAD (Jain et al., 2015; Ge et

al., 2016). Performing SSAD under thermophilic conditions at 55ºC can improve digester performance and destroy pathogenic microorganisms. Furthermore, high energy demand is compensated by rapid digestion rate and high biogas yield (Jain et al., 2015).

2.7.3 Inoculum Type and Size

Inoculum type and size are critical parameters that affect stability and start of digestion (Ge et al., 2016; Demichelis et al., 2022). A suitable inoculum minimizes the lag phase, stabilizes AD, and accelerates degradation and biomethane production (Demichelis et al., 2022). Animal manure or digested sludge from an operating anaerobic reactor or wastewater treatment plant is mainly utilized as inoculum (Li et al., 2013; Gu et al., 2014). Studies on AD of rice straw indicated that inoculum of digested manure origin is more suitable than sludge in enhancing the hydrolysis of cellulosic material and biogas production (Gu et al., 2014; Demichelis et al., 2022). This can be ascribed to improved adaptability, higher cellulase and xylanase activities, and adequate supply of nutrients from digested manure (Gu et al., 2014; Demichelis et al., 2022).

A mixture of cow dung and water at an optimal ratio of 1:1 provides the most favorable inoculum size for AD (Jain et al., 2015). In addition, substrate to inoculum (S/I) ratio can stabilize microbial composition of the inoculum and improve the hydrolysis rate. The S/I ratio of 1:1 is frequently preferred as it is sufficient to avert accumulation of acids during AD (Demichelis et al., 2022).

Recycling of inoculum from one reactor to another is encouraged to select microorganisms that are able to work on a wide range of feedstocks (Godon et al., 2013). It is suggested to choose an inoculum with mixed microbial population and high affinity towards a feedstock. This helps to counteract the problems of overloading the digester (Saady & Masse, 2013). Overloading of

inoculum may result in release of intermediary products, such as acetic, propionic and butyric acids by acidogens (Jain et al., 2015). The acids can lower digester pH and inhibit the activity of methanogenic bacteria (Amani et al., 2010; Vogeli et al., 2014; Jain et al., 2015).

However, a large inoculum size was proposed to offer rapid and successful start-up for methanogens in SSAD. For instance, the effect of inoculum size was found to be more pronounced in the early stages than the final phases of SSAD (Ge et al., 2016).

2.7.4 Nutrient Availability

Various nutrients are required for optimal growth of microbes in digesters. The main nutrients include carbon (C), H_2, oxygen (O_2), nitrogen (N_2), phosphorous (P) and sulfur (S) (Jain et al., 2015). Amongst these nutrients, C and N are the most vital elements that form the diet of anaerobic bacteria. Carbon and N are needed for energy provision and synthesis of cellular structure, respectively (Jain et al., 2015). A stable AD process needs a balance between C and N content (Khalid et al., 2011). The C/N ratio must be maintained at an optimum for an efficient AD system. The optimum C/N ratio may differ depending of the type of feedstock. However, several authors have suggested a C/N ratio varying from 20 - 30 with an optimum of 25 (Jain et al., 2015).

Inappropriate C/N ratio may lead to accumulation of total ammonical nitrogen and VFAs that can inhibit methanogens and retard the AD process (Jain et al., 2015; Einarsson & Persson, 2017). Codigestion of crop residues with livestock manure has been suggested to provide an adequate supply of nutrients and balance the C/N ratio. Another example is codigestion of livestock manure and straw which produced highest methane yield at C/N ratio in the range 25 - 30, whereas ratios varying from 15 - 20 had adverse effects on methane production (Wang et al.,

2014). An optimum C/N ratio of 27 was proposed for codigestion of livestock and poultry manure with wheat bran (Majd et al., 2017).

2.7.5 Agitation

Agitation plays a significant role in biogas production as it affects mass and heat transfer, and the discharge of gas bubbles entrapped in the bioreactor fluid (Wang, 2016). It enables the homogeneity of nutrients, temperature and other environmental factors in digester tanks. This prevents stratification, development of surface crust, and accumulation of foam and solids that may be problematic to AD (Kaparaju et al., 2008; Mir et al., 2016). In addition, agitation allows heat transfer, particle size reduction and the discharge of gas generated from the digester slurry (Kaparaju et al., 2008).

Jain et al. (2015) reported that slow agitation can enhance AD while violent mixing may restrain the activity of digester microorganisms. For example, minimal mixing improved methane yield by 12.5% compared to continuous and intermittent mixing in a study to optimize mixing conditions for biogas production (Kaparaju et al., 2008). Digester slurry can be mixed by mechanical, hydraulic and pneumatic agitation (Wang, 2016; Majd et al., 2017). However, mechanical mixing is commonly preferred for most digester designs (Wang, 2016). Mixing strategy, mixing intensity, mixing period and site of mixer were cited as the major factors that may influence digester agitation (Kaparaju et al., 2008). Further, the choice of agitation is affected by composition of the substrate and the type of bioreactor (Majd et al., 2017).

2.7.6 Substrate Particle Size

Substrate particle size can influence the performance of AD (Hajji & Rhachi, 2013; Robles et al., 2018; Siddigue & Ab. Wahid, 2018). Hajji & Rhachi (2013) reported a positive relationship

between substrate particle size and biogas yield, and observed maximum biogas yield from smaller sieve pore size (10 mm) for crop residues. Similarly, Kaur (2022) showed maximum biogas yield from smaller particle size (<1 mm) of rice straw and bagasse. Luo et al. (2021) also produced the highest methane yield (176.47 mL g^{-1} VS) from 1 mm particle size of rice straw. Small particle size increases the surface area for microbial and enzymatic attack, thus improving the hydrolysis of crop residues.

On the other hand, large particle size may cause blockages and process instability in digesters (Siddigue & Ab. Wahid, 2018). In a study by Wall et al. (2015), large particle size of greater than 3 cm was observed to cause major operational challenges in AD of silage. Therefore, mechanical pretreatment is a prerequisite to enlarge the surface area of crop residues and improve hydrolysis rate (Kaur, 2022).

2.7.7 Organic Loading Rate

Organic loading rate (OLR) is a critical factor for optimizing the performance of microorganisms in a digester. It is defined as the quantity of dry organic solids loaded in a reactor per unit time and per unit volume (Manyi-Loh et al., 2013; Mir et al., 2016; Siddigue & Ab. Wahid, 2018). Low OLR reduces the efficacy of an AD system. In contrast, high OLR promotes microbial activity, and minimizes energy use, digester size and operational cost (Siddigue & Ab. Wahid, 2018). However, operating further than the specified optimal range of OLR leads to overloading, poor heat transfer and heterogeneity in a digester (Manyi-Loh et al., 2013; Mir et al., 2016; Siddigue & Ab. Wahid, 2018).

Overloading causes accumulation of fatty acids, extension of the acidogenesis stage, low pH, death of methanogens, process inhibition and low biogas yield (Manyi-Loh et al., 2013; Mir et

al., 2016). Operating a circulating pump at a higher OLR than its carrying capacity may also destroy the pump (Siddigue & Ab. Wahid, 2018). Therefore, it is very imperative to operate a digester at its optimal OLR. The optimal OLR may vary depending on the type of feedstock. Jiang et al. (2020) reported that an OLR of 7.50 [gVS] L^{-1} d^{-1} was optimal for AD of organic fraction of municipal solid waste. The maximum methane yield was recorded at an optimum OLR of 1.4 [kgVS] m^{-3} d^{-1} during AD of vegetable waste (Babee & Shayegan, 2011).

2.7.8 Hydraulic Retention Time

Hydraulic retention time (HRT) is an important parameter in design and performance of an AD system. It is referred to as the residence time taken by microbes to utilize and synthesize a particular substrate in a digester (Manyi-Loh et al., 2013; Mir et al., 2016; Siddigue & Ab. Wahid, 2018). The HRT varies depending on the type of substrate, environmental factors and the intended use of the digested material (Manyi-Loh et al., 2013; Mir et al., 2016). However, it is recommended to operate a digester within stipulated optimal HRTs for efficient AD.

The most favorable HRTs for thermophiles and mesophiles are 12 - 24 days and 15 - 30 days, respectively (Manyi-Loh et al., 2013; Mir et al., 2016). Extended HRTs can deprive digester microbes of nutrients leading to process failure. Therefore, short HRT was proposed for commercial use due to reduced reactor size and capital costs, and improved biogas yield (Siddigue & Ab. Wahid, 2018). The HRT can be decreased by mixing the substrate with water. Conversely, if the doubling time of digester microbes exceeds the HRT, the microbes will die and halt the AD pathway (Siddigue & Ab. Wahid, 2018).

2.8 Methods to Enhance Biomethane Production from Crop Residues

There are several practices that can enhance the efficiency and regulation of AD. These include bioaugmentation, microbial community monitoring, pretreatment of substrates and codigestion (Kamusoko et al., 2022). Bioaugmentation involves the addition of cultured microorganisms to a digester (Mohan et al., 2011; Tsapekos et al., 2017; Lebiocka et al., 2018). This alters the endogenous microbial communities so that they perform specialized functions. Bioaugmentation tends to enhance the start-up and process efficiency of a digester, thus safeguarding the endogenous microbial community against adverse conditions, and recompense for organic or hydraulic overloading (Mohan et al., 2011). Bioaugmentation has been reported to enhance biogas production from wheat straw (Ozbayram et al., 2017; Tsapekos et al., 2017), cellulose and stover (Strang et al., 2017) and maize silage (Acs et al., 2015).

Microbial community monitoring ensures a thorough understanding on microbial composition and function, which improves the performance of the AD process (Lim et al., 2018). The method uses multiple techniques to provide in-depth discernment on microbial diversity and dynamics in anaerobic digesters. Microbial community monitoring relies on both culture-enrichment and PCR-based methods (Usman & Ali, 2009; Chaudhary et al., 2019). However, PCR-based methods provide rapid, reliable and economic tools for elucidating the taxonomy and phylogeny of microbial structure (Chaudhary et al., 2019). Pretreatment of feedstock and codigestion are discussed in detail in this section. The effect of these two methods on biomethane production from crop residues was evaluated in this study. Pretreatment and codigestion were performed in an attempt to improve the hydrolysis rate of crop residues and complement nutrients, respectively.

2.8.1 Pretreatment of Crop Residues

Pretreatment enhances the hydrolysis of complex organic substances, thus increasing the availability of sugars and other small molecules to microbes (Siddigue & Ab. Wahid, 2018). It is primarily designed to destroy lignin seal and decrease the crystallinity of cellulosic materials (Teghammar, 2013; Gumirisiza et al., 2017). Ultimately, pretreatment raises chemical oxygen demand (COD) levels and discharges the intracellular components of a substrate. The aim of pretreatment is to: enhance the efficiency of AD; improve biogas and methane yield; utilize new or locally available substrates; and prevent processing problems, such as high electricity demands or the development of floating layers (Montgomery & Bochmann, 2014).

The choice of a pretreatment method is critical in developing a viable system for biogas production. Many pretreatment methods are available to enhance the microbial digestion efficiency of crop residues (Figure 2.10). These can be grouped into five categories including physical, chemical, biological, nanoscale and physicochemical processes (Sahay, 2022). Each pretreatment method has its own merits and drawbacks (Awogbemi & Kallon, 2022). Physical and chemical methods are fast and effective, but however, they have limited value at industrial-scale due to high cost of resources, energy and operation, and formation of toxic compounds.

Biological and nanoscale pretreatments are still at their infant stages of development. Pretreatment with microbial consortia is one of the most promising biological methods to increase methane production (Yuan et al., 2011; Yan et al., 2012). Cellulases, xylanases, pectinases and lignolytic enzymes are also commercially available biological agents from submerged cultivation of fungi or bacteria (Cater et al., 2014).

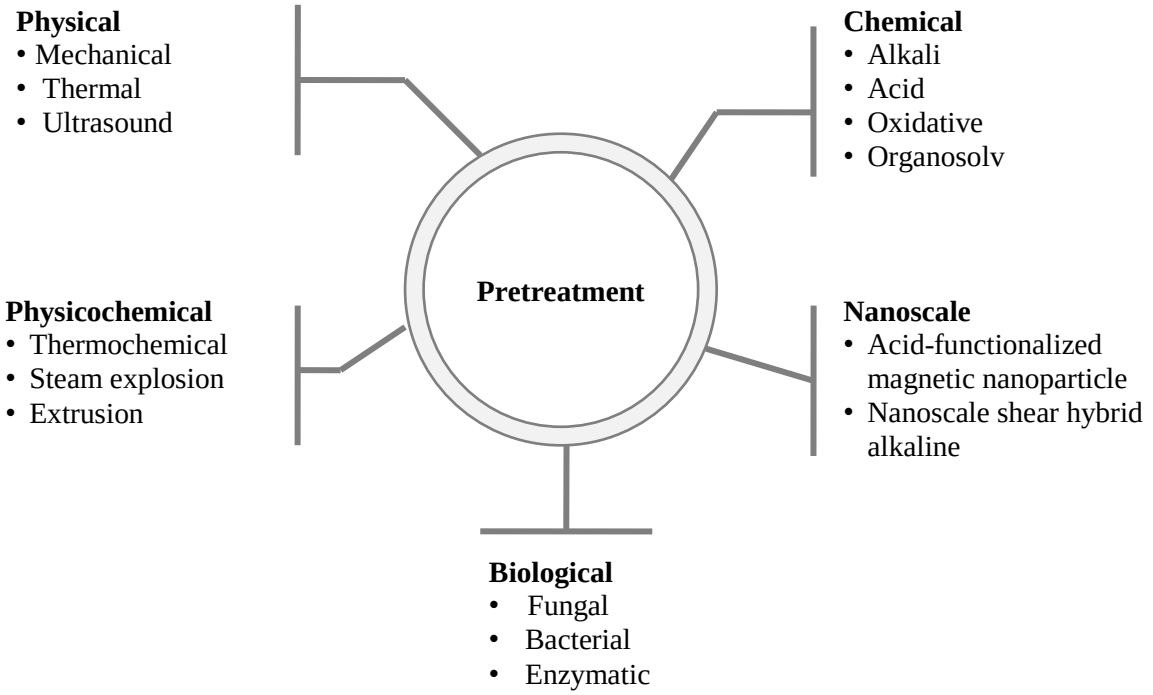

Figure 2.10 A schematic presentation of pretreatment methods for crop residues

2.8.1.1 Physical Pretreatment

Physical pretreatment is designed to trim down the size of particles of lignocelluloses by mechanical comminution or to enhance the surface area of biomass through mechanical refining. The aim is to enhance the rate of hydrolysis and the yield of biogas (Jain et al., 2015; Moodley & Trois, 2021). For example, size reduction of maize stover to 53 - 75 μm was reported to improve hydrolysis efficiency by 5 - 25% (Kumar et al., 2022). All methods that do not utilize water, chemicals and microorganisms are generally regarded as physical pretreatment methods (Zheng et al., 2014; Rodriguez et al., 2016).

In general, physical pretreatment can enhance the efficiency of hydrolysis and methane yield of crop residues (Table 2.4). However, the method has limited commercial application due to high energy demand, high operational cost, generation of inhibitory compounds, such as phenolic

compounds and furfural, and stringent monitoring of equipmment (Sambusiti, 2013; Kamusoko et al., 2019). The most important types of physical pretreatments include mechanical, ultrasound and microwave methods (Rodriguez et al., 2016; Moodley & Trois, 2021).

Mechanical pretreatment includes a wide range of methods, such as grinding, milling, chipping or extrusion. Chipping is considered as the most suitable mechanical technique for treatment of crop residues, such as straw and corn stover, and forestry residues. Mechanical pretreatment reduces the size of particles, decrease the degree of crystallinity of cellulose, and increase the surface area and pore size of the feedstock (Sambusiti, 2013; Moodley & Trois, 2021). Studies performed to evaluate the effect of mechanical pretreatment on biogas production have generated useful information for designing AD systems. For example, around 13% increase in methane production was observed from rice straw pretreated with knife milling (Garuti et al., 2022). Similarly, mechanical pretreatment of six disparate lignocellulosic materials improved methane yield by 22% (Dahunsi, 2019). However, mechanical pretreatment is not economically viable as it requires high energy (Sambusiti, 2013; Moodley & Trois, 2021)

The microwave method utilizes thermal energy produced through stimulation of vibration of molecules by non-ionizing radiation (Moodley & Trois, 2021). The process works by cutting β-1,4-glucan bonds of the recalcitrant biomass, thus enhancing the surface area and decreasing the crystallinity of cellulose (Rodriguez et al., 2016). Microwave method can replace conventional heating in the foreseeable future (Zheng et al., 2014). With microwave pretreatment, high temperatures are attained within a short period of time, thus saving energy. The major drawback of this method is high processing times that may lead to sugar degradation (Moodley & Trois, 2021).

There is limited information available in literature on microwave pretreatment of crop residues to enhance methane production. According to Sapci (2013), there was no significant difference in methane yield between microwave pretreated and non-treated agricultural straws. An increase of 28% in methane production was reported from wheat straw solubilized with microwave irradiation at 150°C (Jackowiak et al., 2011). Another study by Qian et al. (2019) revealed a 25% improvement in biogas production from combined alkaline and microwave pretreated rice straw.

Ultrasound pretreatment uses acoustic energy in form of high frequency waves to induce cell lysis. Microbubbles are generated due to cavitation of cells in liquid solutions by high frequency sonic waves. Physical effects caused by the disintegration of microbubbles lead to rupturing of the plant cell wall. This exposes cellular contents resulting in improved hydrolysis (Zheng et al., 2014; Jain et al., 2015; Rodriguez et al., 2016). Ultrasound pretreatment has advantages of short treatment time and low processing temperature, although it integrates the use of chemicals (Moodley & Trois, 2021). There are few reported studies on ultrasound pretreatment of crop residues for biomethane production. However, an increase in the range of 18.75 - 21.95% was recorded from ultrasound pretreatment of rice straw by Pansripong et al. (2019).

Table 2.4 Physical pretreatment of crop residues for biogas production

Physical pretreatment	Feedstock	Pretreatment conditions	Findings	References
Mechanical	Rice straw	Knife milling, 6 - 2 mm particle size	13% increase in specific methane	Garuti et al. (2022)
	Rice straw	Grinding, 10 - 25 mm, 0 - 100°C	Biogas improved by 17.5%	Zhang & Zhang, 1999
	Wheat straw	Knife mill, 0.3 - 1.2 mm particle size	Methane yield increased by 49.3%	Dell'Omo & La Froscia (2018)
	Wheat straw	Roll mill	21% increase in methane yield	Victorin et al. (2020)
	Rice straw, rice straw	Force mill/centrifugal grinder, 0.3 - 1.5 mm	Methane yield increased by 38.7%	Chandra et al. (2015)

Table 2.4 Physical pretreatment of crop residues for biogas production

Physical pretreatment	Feedstock	Pretreatment conditions	Findings	References
Microwave	Rice straw	130 - 230°C, 2 - 5 min	41.3% increase in methane yield	Kainthola et al. (2019)
	Cauliflower and cabbage leaves	Power of 87.5 - 350 W, 15 min	64.7% increment in methane	Savoo & Mudhoo (2018)
	Wheat straw	Power of 400 - 1600 W, 150°C	28% increase in methane yield	Jackowiak et al., (2011)
Ultrasound	Rice straw	Frequency: of 37 - 102 kHz, 45 days	18.8 - 21.9% increase in methane yield	Pansripong et al. (2019)
	Maize straw	Frequency of 50 kHz, power of 250 W	Improved methane yield by 69.7 %	Zou et al. (2016)

2.8.1.2 Chemical Pretreatment

Chemical pretreatment is the application of chemical substances like acids, alkalis and ionic liquids to degrade the crystal structure of crop residues. It is intended to improve the biodegradability of cellulose and hemicellulose (Kumar et al., 2018; Moodley & Trois, 2021; Awogbemi & Kallon, 2022). Chemical pretreatment is the most widely reported pretreatment method in extant literature. However, more chemical pretreatments were reported in cellulosic bioethanol production as compared to biogas production (Zheng et al., 2014). Table 2.5 shows some studies that were performed on chemical pretreatment of crop residues for biogas production. Chemical pretreatment can be broadly classified into acid, alkaline, oxidative and organic solvent pretreatments (Abraham et al., 2020).

Alkali pretreatment can be deployed to solubilize lignin, hemicellulose and/or cellulose making lignocellulosic materials more amenable to microbial and enzymatic degradation. The method uses bases, such as sodium hydroxide (NaOH), calcium hydroxide ($Ca(OH)_2$), potassium hydroxide (KOH) and ammonium hydroxide ($NH_3.H_2O$) to liquefy and cleave lignin-carbohydrate cross links. Alkali pretreatment increase pore size and surface area, reduce the degree of polymerization and crystallinity, and deconstruct the lignin structure of the feedstock (Zheng et al., 2014; Rodriguez et al., 2016; Kumar et al., 2018; Abraham et al., 2020; Awogbemi & Kallon, 2022).

The effectiveness of alkali pretreatment depends on lignin composition of crop residues. Sodium hydroxide is the most effective and extensively studied alkali pretreatment of crop residues, such as wheat straw, rice straw, maize stover and sunflower stalks during AD (Zheng et al., 2014). For instance, Chandra et al. (2012) reported a 112% increase in methane yield from NaOH pretreated

wheat straw. In addition, biogas yield of rice straw was improved by 50% through NaOH and KOH solubilization (Dong et al., 2009). However, NaOH must be treated with caution as it generates Na^+ ions which can inhibit the AD process (Abraham et al., 2020).

Acid pretreatment is another chemical method considered to be effective against hemicelluloses and lignin (Zheng et al., 2014; Moodley & Trois, 2021). This technique leads to ease access of cellulose hydrolysis by microbial agents (Zheng et al., 2014). Nitric acid (HNO_3), sulfuric acid (H_2SO_4), phosphoric acid (H_3PO_4) and hydrochloric acid (HCl) are common examples of inorganic acids that can be used for pretreatment of crop residues (Kumat et al., 2018; Awogbemi & Kallon, 2022; Kumar et al., 2022). Dilute acid is believed to be more effective than concentrated acid for pretreatment of lignocellulose and can solubilize hemicelluloses up to 100% into its constituent sugar units. It can also destroy lignin to a high degree, although it is considered to be less efficient in dissolving lignin (Zheng et al., 2014; Kumat et al., 2018).

Concentrated acid is very effective in pretreatment of cellulose, but it is highly toxic, corrosive and requires specialized equipment. Sulfuric acid is the most widely studied acid in acid pretreatment. Conversely, there is limited information on pretreatment of cellulosic material using acid for biogas production (Zheng et al., 2014). Song et al. (2014) obtained a highest increase in methane yield of 115% from maize straw pretreated with H_2O_2. Taherdanak et al. (2016) also reported a 16% rise in methane yield from wheat straw pretreated with H_2SO_4.

Oxidative pretreatment is the degradation of lignin and hemicellulose compounds by oxidants, such as H_2O_2 and ozone gas (Zheng et al., 2014; Abraham et al., 2020; Awogbemi & Kallon, 2022). This leads to nucleophilic substitution, destruction of aromatic nuclei, removal of side chains and dislocation of alkyl aryl ether bonds (Paudel et al., 2017; Abraham et al., 2020).

Hydroxyl radicals (-OH) and superoxides (O_2^-) released from H_2O_2 promote the delignification of lignocellulosic materials and release more fermentable sugars (Paudel et al., 2017; Awogbemi & Kallon, 2022). The H_2O_2 is very effective in alkaline solutions of pH 11.5, and does not generate toxic compounds (Paudel et al., 2017). Oxidative pretreatment of sunflower stalks with H_2O_2 increased methane yield by 33% (Monlau et al., 2011). Song et al. (2013) reported an increase by 88% in methane yield from rice straw pretreated with H_2O_2. Furthermore, increase in methane yield varying from 20 - 30% was obtained from oxidative pretreatment of crop residues with fentone, ozone and ozone combined with H_2O_2 and Fe(II) (Almomani et al., 2019).

During organosolv pretreatment, organic solvents are used to destroy internal linkages of lignin and hemicelluloses to ensure pure cellulose is available for AD (Abraham et al., 2020; Awogbemi & Kallon, 2022). Commonly deployed organic solvents include methanol, ethanol, tetrahydrofuranol, acetone and ethylene glycol. Organosolv reaction is catalyzed by acids like H_2SO_4 and HCl or bases, such as NaOH, NH_3 and calcium carbonate ($CaCO_3$) (Awogbemi & Kallon, 2022). Mancini et al. (2018) observed up to 11% enhanced methane yield from pretreatment of wheat straw with 50% ethanol. Likewise, Ostovareh et al. (2015) improved biogas production by 270% through pretreatment of sweet sorghum stalks with 50 - 70% ethanol.

Various ionic liquids have also the potential to liquefy cellulose with absolute recovery at the end of pretreatment. Frequently used ionic liquid for pretreatment of cellulosic materials during biogas production is N-methylmorpholine-N-oxide monohydrate (NMMO) (Abraham et al., 2020). For instance, Mancini et al. (2018) reported 11% methane increase from wheat straw pretreated with NMMO while Akhand & Blancas (2012) recorded a 47% methane increase. Purwandari et al. (2013) also evaluated the effect of NMMO pretreatment on biogas production from oil palm empty fruit bunch. A significant increase in biogas yield of 167% was reported.

Table 2.5 Chemical pretreatment of crop residues for biogas production

Chemical pretreatment	Feedstock	Pretreatment conditions	Results	References
Acid	Wheat straw	1% H_2SO_4, 121°C, 10 - 120 minutes	Increased methane by 16%	Taherdanak et al. (2016)
	Maize stover	Dilute H_2SO_4, HCl, CH_3COOH and H_2O_2 (1%, 2%, 3%, 4%), 25°C, 7 days	H_2O_2 improved methane by 115%	Song et al. (2014)
	Sunflower stalks	4% HCl, 170°C	21% methane increase	Monlau et al. (2012)
Alkaline	Wheat straw	1.6% NaOH, 30°C, 24 hours	15% methane increase	Mancini et al. (2018)
	Maize stover	2% NaOH, 20°C, 72 hours	73.4% increase in methane yield	Zheng et al. (2009)
	Maize straw, grass and sprout stem	7.5% $Ca(OH)_2$, 10°C, 20 hours	37% methane improvement	Khor et al. (2015)
	Wheat straw	NH_3 (2,4,6%), 35°C, 7 days	52% increase in methane yield	Yang et al. (2014)
	Maize stover	6% NaOH, 35°C	48.5% methane improvement	Pang et al. (2008)
	Wheat straw	4% NaOH, 37°C, 5 days	Biogas increased by 87.5%	Chandra et al. (2012)

Table 2.5 Chemical pretreatment of crop residues for biogas production

Chemical pretreatment	Feedstock	Pretreatment conditions	Results	References
Oxidative	Coffee hemicellulose hydrolysate	Ozone (6 - 81 mg0_3/gCH), pH 3 - 11	Methane yield increased by 37%	Santos et al. (2018)
	Sunflower stalks	4% H_2O_2, 55°C, 24 hours	Methane increased by 33%	Monlau et al. (2011)
	Agricultural residues	Fentone, ozone and ozone combined with F(II) and H_2O_2	20 - 30% methane increase	Almomani et al. (2019)
	Rice straw	2.68% H_2O_2, 6 - 18 days	88% methane increment	Song et al. (2013)
	Greenhouse crop residues	1% H_2O_2, 50°C, 6 hours	Methane enhanced by 77%	Perendeci et al. (2018)
Organosolv	Wheat straw	NMMO, 90°C, 7 hours	47% increase in methane	Akhand & Blancas (2012)
	Sweet sorghum stalks	50-70% ethanol	Biogas yield enhanced by 270%	Ostovareh et al (2015)
	Wheat straw	50% ethanol, 180°C, 1 hours	15% methane increase	Mancini et al. (2018)
	Wheat straw	NMMO, 120°C, 3 hours	11% methane improvement	Mancini et al. (2018)

2.8.1.3 Physicochemical Pretreatment

Physicochemical pretreatment amalgamates physical and chemical methods to depolymerize lignin and hemicelluloses so that more fermentable sugars are released for AD (Abraham et al., 2020; Awogbemi & Kallon, 2022). The most suitable temperature for physicochemical pretreatment of crop biomass ranges from 50 - 250°C (Abraham et al., 2020). Heat is applied to disrupt the hydrogen bonds in lignocellulosic biomass, thereby increasing the surface area for microorganisms. It is prudent to recycle heat as a strategy to save energy during physicochemical pretreatment. In contrast, extended pretreatment times must be avoided to prevent accumulation of inhibitory byproducts (Rodriguez et al., 2017; Abraham et al., 2020). The most predominant physicochemical pretreatments include extrusion, steam explosion, ammonia fiber explosion and thermochemical pretreatment methods (Awogbemi & Kallon, 2022).

During extrusion pretreatment, thermal and mechanical methods are combined in a single unit to modify the physical and chemical properties of plant biomass (Awogbemi & Kallon, 2022). Biomass is subjected to distressful conditions like heating and mixing with rapid fall in pressure (Abrahham et al., 2020). As the biomass is discharged from the extruder, cellulose dissociates from complex polymers by breaking the β-*O*-4 linkage in lignin and the plant cell wall structure is destroyed (Awogbemi & Kallon, 2022). Extrusion results in deconstruction of cellulose, hemicelluloses, lignin and protein (Hjorth et al., 2011). The most favorable operating conditions for extrusion include a temperature range of 160 - 250°C and pressure varying from 0.5 - 5.0 MPa (Awogbemi & Kallon, 2022).

Hjorth et al. (2011) evaluated the effect of twin-screw extrusion on 13 different types of biomass. The highest increase in methane content of 70% and 11% were obtained from barley straw after

28 days and 90 days, respectively. In a related study, Chen et al. (2022) reported improved methane and biogas yields by 27.01% and 22.55%, respectively, from extruded wheat straw. Kozłowski et al. (2019) observed extruded maize straw to enhance biogas and methane yields by 7.50% and 8.51%, respectively. Extrusion pretreatment of maize straw was found to influence methane and biogas production efficiency by 51.63% (Kupryaniuk et al. 2020).

Steam explosion is one of the most promising ecofriendly techniques for pretreatment of plant biomass during AD (Ziegler-Devin et al., 2021). During steam explosion, complex plant polymers are exposed to high pressure (5 - 50 bar) and saturated steam at 160 - 250°C for short residence times (Abraham et al., 2020; Ziegler-Devin et al., 2021). Pressure is then rapidly discharged leading to depolymerization of plant biomass (Abraham et al., 2020). The conversion of crop residues into biofuels and other multiple products via steam explosion pretreatment has gained interest in the 21st century. For example, the steam explosion pretreatment of rice straw was studied by Steinbach et al (2019), who reported a 32% increase in methane content. Comparably, Zhou et al. (2016) pretreated rice straw through steam explosion at a temperature range of 200 - 220°C and at time intervals of 1 - 4 minutes. The highest rise of 51% in biogas production at 200°C and residence time of 2 minutes was reported from this study.

Thermochemical pretreatment uses an acid or alkali at high temperature (>100°C) or low temperature (<100°C) to delignify the crystal structure of biomass and improve the accessible surface area of cellulose (Hajji & Rhachi, 2022). This process is mainly affected by the structure and composition of biomass (Hajji & Rhachi, 2022). Alkali-thermal pretreatment shows more potential for pretreatment of lignocellulosic biomass in AD technology. However, the method is associated with high processing cost due to elevated temperatures. Commonly used alkalis include NaOH, sodium carbonate ($NaCO_3$) and alkaline peroxide.

Akalis are preheated to a temperature range of 75 - 125°C for saccharification of hemicelluloses and lignin (Awogbemi & Kallon, 2022). For instance, saccharification efficiency of lignin and hemicelluloses varying from 54.09 - 67.67% with increase in cellulose yield of 52.65% was observed from NaOH-heat pretreated maize straw (Lopez et al., 2019; Zhang et al., 2020). Few studies are reported in literature on application of thermochemical pretreatment of crop residues for AD. An increase of 11% in methane content was reported from thermochemically pretreated sweet potato root waste using 2.9 g L^{-1} of NaOH at 82°C for 102 minutes (Catherine & Twizerimana, 2022).

Ammonia fiber explosion pretreatment was developed as an alternative solution to alkali-heat pretreatment. In this process, liquid NH_3 is subjected to high pressure, and liberates NH_3 and oxyhydrogen ions leading to a rapid rise in temperature. Elevated temperatures will destroy ester and ether linkages in lignin and hemicelluloses, thus increasing cellulose content (Awogbemi & Kallon, 2022). Ammonia fiber explosion was found to be successfully in deconstruction of lignin and increasing the availability of glucose in barley straw and maize stover (Awogbemi & Kallon, 2022).

2.8.1.4 Biological Pretreatment

Biological pretreatment entails the use of microbial metabolism or byproducts to transform recalcitrant biomass into valuable products (Abraham et al., 2020). Frequently used biological agents include fungi, bacteria and enzymes (Awogbemi & Kallon, 2022). As shown on Figure 2.11, a sole or a consortium of microbes is generally applied to solubilize a feedstock. The main effect of biological pretreatment is delignification, which provides more cellulose and hemicellulose for fermentation (Abraham et al., 2020).

Biological pretreatment is more favorable for AD than the other pretreatment methods. It is an eco-friendly technology that does not pollute the environment, generates little or no toxic byproducts and has lower energy demands (Zheng et al., 2014; Abraham et al., 2020; Awogbemi & Kallon, 2022). Contrarily, the method is considered to be slow, often with prolonged incubation times (Awogbemi & Kallon, 2022). Studies on application of biological pretreatment to improve biogas production from crop residues are shown on Table 2.6.

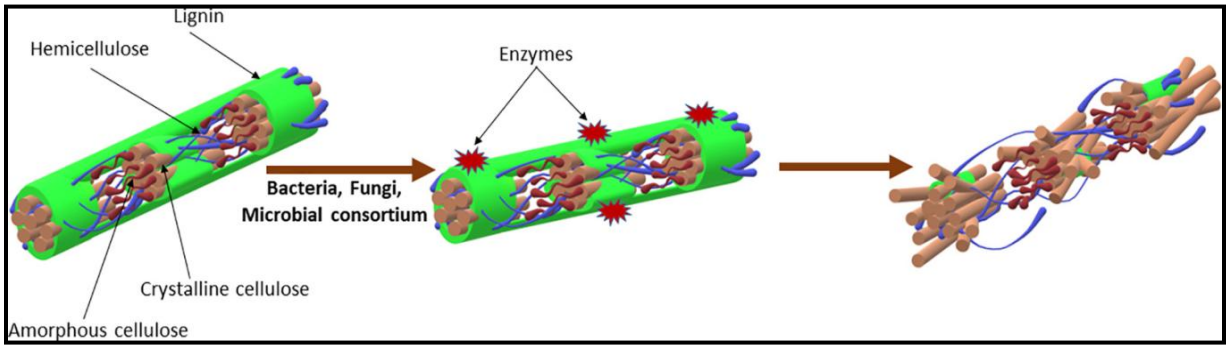

Figure 2.11 Microbial pretreatment of polymeric plant biomass

Fungal pretreatment is a method that utilizes white-rot fungi, brown-rot fungi, soft-rot fungi or other fungi to remove lignin in plant biomass (Zheng et al., 2014; Abraham et al., 2020; Awogbemi & Kallon, 2022). The removal of lignin and hemicelluloses by fungi improves the availability of cellulose for AD. Cellulose is highly resistant to fungal degradation than counterpart fractions. White-rot fungi are the most effective fungal pretreatment agents (Zheng et al., 2014). Basidiomycetes like *Phanerochaete chrysosporium* are the commonly utilized white-rot fungi for mineralization of lignin (Awogbemi & Kallon, 2022). Besides, white-rot fungi consume fermentable sugars such as cellulose and hemicelluloses during pretreatment, and have prolonged retention times (Kainthola et al., 2021).

Fungal pretreatment is affected by several parameters such as moisture content, particle size of the substrate, temperature, pH, oxygen concentration, incubation time and nutrient availability (Abraham et al., 2020; Awogbemi & Kallon, 2022). These factors must be optimized for efficient degradation of lignin. In a study by Rouches et al. (2018), the pretreatment of wheat straw using *Polyporus brumalis* promoted methane production by 52%. Wyman et al. (2018) reported a 19% increase in biogas yield from maize stover pretreated with *Pleurotus eryngii* for 40 days. In addition, *P. ostreatus, Garnodema lucidum* and *P. chrysosporium* were evaluated for their ability to treat rice straw for enhanced methane production. The reported methane yields of 269.99 mL g^{-1} VS, 295.91 mL g^{-1} VS and 339.31 mL g^{-1} VS, respectively, were greater by 1.64 - 2.22 times than the control (Kainthola et al., 2019).

Bacteria pretreatment involves the use of enzyme-secreting bacteria to combat the polymerization of agricultural wastes (Awogbemi & Kallon, 2022). Many anaerobic bacteria have high hydrolysis capacity towards plant biomass (Abraham et al., 2020). Delignification using bacteria possess several distinct traits compared to fungal pretreatment. Notable unique features in bacteria include the potential to induce C_α-oxidation and cleave of C_β-C_β linkages in lignin (Abraham et al., 2020). Bacteria have rapid growth with continuous conversion of substrates into usable products. They also have shorter incubation periods and are more cost-effective than fungal pretreatment. In addition, the genome of bacteria can be more easily modified than fungal genome. Therefore, bacteria are more preferred biological pretreatment agents compared to fungi and enzymes (Barati et al., 2021).

Bacteria, such as *Clostridium, Bacillus and Pseudomonas* are capable of degrading plant cell wall through secretion of cellulases, xylanases and other hydrolytic enzymes. These bacteria occupy diverse extreme conditions including decomposing forestry matter, compost material,

agricultural waste, organic matter and soil, and hot springs (Barati et al., 2021). *Bacillus* is one of the most promising genera for decomposing organic matter due to its strong cellulose-degrading capacity. Furthermore, the bacteria can tolerate high temperatures and a wide range of pH conditions (Barati et al., 2021).

A coculture of *Bacillus* sp. was investigated for its ability to pretreat rice straw using single-stage batch AD for 50 days. Lignin content was greatly decreased and biogas yield was higher by 76% compared to untreated biomass (Shah et al., 2019). In another study, pretreatment of maize straw in a single-stage batch mode using *B. subtilis* produced 17.35% higher methane content than control (Xu et al., 2018). *Citrobacter werkmanii* VKVVG4 isolated from the gut of silver fish was also investigated for its potential to solubilize water hyacinth and a three-fold increase in biogas yield was noted (Barua et al., 2018).

Construction of microbial consortium was proposed as a panacea to limited utility of biological pretreament at pilot-scale due to prolonged residence time (Kumar et al., 2018). The method is believed to be more effective than a single microorganism in enhancing the degradation of agricultural wastes. A microbial consortium is a group of species with varying delignification efficiencies and can perform in diverse ecological conditions. The microbes can deploy distinct delignification mechanisms with improved potential to exploit a substrate compared to indigenous microorganisms (Abraham et al., 2020).

Unlike fungi, which mostly act on lignin, microbial consortium has high affinity for holocellulose (cellulose and hemicellulose) (Zheng et al., 2014; Awogbemi & Kallon, 2022). The advantage of using microbial consortium for pretreatment over fungi is that sterilization may not be required in microbial consortium pretreatment. In general, the microbes are isolated from

natural conditions where decomposing lignocellulosic waste is the main substrate (Zheng et al., 2014).

A thermophilic consortium containing microbes isolated from landfills, decaying straws and wastewater was reported to enhance methane production by 96% following pretreatment of cassava residues (Zhang et al., 2011). Pretreatment of wheat straw using a microbial consortium improved methane production by 80.34% than control (Zhong et al., 2016). Bai et al. (2010) constructed an MEG microbial consortium in an attempt to pretreat cotton stalks and enhanced biogas production by 25%. A complex microbial consortium prepared from a mixed culture of pure strains of yeast and cellulolytic bacteria in freeze-dried powder was exploited in pretreatment of maize straw for biogas production. Methane yield was improved by more than 75% while a decrease in digestion time of 34.6% was reported (Zhong et al., 2011).

The exogenous incorporation of hydrolytic or oxidative enzymes prior to AD can promote the degradation of lignocellulosic materials (Abraham et al., 2020; Awogbemi & Kallon, 2022). Various classes of ligninolytic enzymes that can be applied for pretreatment of agricultural wastes are shown in Figure 2.12. The most widely studied classes of enzymes for pretreatment of agricultural wastes are cellulases and hemicellulases (Abraham et al., 2020; Awogbemi & Kallon, 2022). Enzymes have short reaction periods and can reduce the loss of holocellulose during hydrolysis. Furthermore, enzymes have ease of access to a substrate with increased mass transfer rate (Abraham et al., 2020). However, the use of enzymatic pretreatment is limited due to high cost of commercial enzymes (Zheng et al., 2014). Enzymatic pretreatment is affected by the activity and specificity of the enzyme, enzyme concentration, inhibitors, pretreatment time, digester design, temperature and pH (Abraham et al., 2020).

Combining different enzymes is a strategy to improve the efficiency of pretreatment. Screening enzymes with high specific activity and cross specificity can lower the quantity of enzyme required and reduce the pretreatment cost (Abraham et al., 2020). Zieminiski & Kowalska (2015) evaluated the effect of enzyme pretreatment using a mixture of endoglucanase, xylanase and pectinase on codigestion of sugar beet pulp silage and vinasse, and 27.9% higher biogas yield than control was achieved. Wang et al. (2018) pretreated maize stover using cellulases at an enzyme loading of 30 FPU g^{-1} substrate. The biogas yield obtained was 36.9% higher than untreated conditions. Rollini et al. (2014) reported 15% increase in biogas from ensiled sorghum forage pretreated with a mixture of commercial enzymes.

SUBSTRATE	**Cellulose**	**Hemicelluloses**	**Lignin**	**Pectin**
ENZYME	• cellobiohydrolase • endoglucanase • β-glucosidase	• endoxylanase • acety xylan esterase • β-xylosidase • endomannanase • β-mannosidase • α-L-arabinofuranosidae • α-glucuronidase • ferulic acid esterase • α-galactosidase • ρ-coumaric acid esterase	• laccase • Mn peroxidase • lignin peroxidse	• pectin methyl esterase • pectate lyase • polygalacturonase • rhamnogalacturonan lyase

Figure 2.12 Classification of cellulose-degrading enzymes (Kamusoko et al., 2022b)

Termites also belong to a class of microorganisms that can degrade plant biomass. Various species of termites, such as *Microcerotermes parvus, Termes hospes* and *Nasutitermes ephratae* were shown to produce hydrolytic enzymes that can solubilize wheat straw during AD (Awogbemi & Kallon, 2022).

Ensiling is traditional practice which boosts the storage life of plant biomass by restraining microbial growth. The process creates an acidic environment through lactate and acetate fermentation in order to preserve the feedstock (Abraham et al., 2020). Ensiling was also examined for potential pretreatment of feedstocks in biogas production. However, most authors reported little or no significant improvement in biogas production (Zheng et al., 2014; Abraham et al., 2020). Pakarinen et al. (2011) showed a 50% rise in methane from ensiled hemp. Ensiling is affected by loss of organic material as bacteria proliferate in the substrate (Abraham et al., 2020).

Table 2.6 Biological pretreatment of crop residues for biogas production

Biological pretreatment	Microbes and enzymes	Feed stock	Pretreatment conditions	Results	References
Fungal pretreatment	*Penicillium aurantiogriseum, Trichoderma reesei, Gilbertella persicaria, Rhizomucor miehei*	Wheat straw, maize stover, willow chips	Batch, 37ºC, 10 days	Highest methane yield (281 mL_N/g oTS) from *P. aurantiogriseum* pretreated maize stover	Kovacs et al. (2022)
	P. brumalis	Wheat straw	Batch, 36ºC, 57 days	52% methane increase	Rouches et al. (2018)
	P. eryngii	Maize stover	Batch, mesophilic, 50 days	19% increase in biogas yield	Wyman et al. (2018)
	P. ostreatus, P. chrysosporium, G. lucidum	Rice straw	Batch, 36ºC, 5 weeks	Maximum methane yield (339.31 mL g^{-1} VS) from *P. chrysosporium*	Kainthola et al. (2019)
	T. reesei	Rice straw	Batch, 37ºC, 45 days	78.3% methane increase	Mustafa et al. (2016)
	Auricularia auricula-judae	Sweet chest nut leaves	Batch, 37ºC, 4-5 weeks	15% higher biogas yield	Mackulak et al. (2012)

Table 2.6 Biological pretreatment of crop residues for biogas production

Biological pretreatment	Microbes and enzymes	Feed stock	Pretreatment conditions	Results	References
Bacteria pretreatment	*B. subtilis*	Maize straw	Batch, 37ºC, 50 days	17.35% higher methane content	Xu et al. (2018)
	Bacillus sp.	Rice straw	Batch, 37ºC, 50 days	76% more biogas	Shah et al. (2019)
Microbial consortium	Microbial consortium	Cassava waste	Batch, 55ºC, 12 hours	96% higher methane yield	Zhang et al. (2011)
	Microbial consortium TC-5	Wheat straw	Batch, 45ºC, 35 days	36.6% increase in methane	Kong et al. (2018)
	Microbial (BSAM) consortium	Barley straw and hay	Batch, 33-36 ºC, 45 days	58% higher methane	Raut et al. (2021)
	Rumen fluid	Rice straw	Batch, 35ºC, 30 days	82.6% increase in methane	Zhang et al. (2016)
	Microbial consortium	Wheat straw	Batch, 37ºC, 20 days	80.34% increase in methane	Zhong et al. (2016)
	MEG microbial system	Cotton stalks	Batch, 35±2ºC, 7 days	25% higher biogas yield	Bai et al. (2010)

Table 2.6 Biological pretreatment of crop residues for biogas production

Biological pretreatment	Microbes and enzymes	Feed stock	Pretreatment conditions	Results	References
Enzymatic pre-treatment	Cellulase	Maize stover	Batch, 37ºC, 18 days	36.9% methane increase	Wang et al. (2018)
	Endoglucanase, xylanase and pectinase	Sugar beet pulp silage and vinasse	Batch, 37ºC, 30 days	27.9% methane increase	Zieminiski & Kowalska (2015)
	Endoglucanase, exoglucanase and xylanase	Ensiled sorghum forage	Batch, 35ºC, 30 days	Maximum 15% increase in biogas yield	Rollini et al. (2014)
	Laccase	Maize stover	Batch, 37ºC, 30 days	25% methane increase	Schroyen et al. (2014)
	Mn peroxidase and versatile peroxidase	Maize stover	Batch, 37ºC, 30 days	17% methane increase	Schroyen et al. (2014)

2.8.2 Anaerobic Codigestion of Crop Residues with Animal Manure

A nutrient balance between C and N content is required for an efficient AD system. The AcoD of a mixture of biosolids is one of the most effective strategies to ensure this balance. A flow chart of the AcoD process is shown in Figure 2.13. Codigestion is the simultaneous digestion of multiple feedstocks and cosubstrates. Generally, an AD system is designed to degrade a sole feedstock. The principal aim of codigestion is to enhance biogas yield by supplementing substrates that have higher methane potential than the base substrate (Siddigue & Ab. Wahid, 2018).

The commonly used base feedstocks are animal manure and MSW. Codigestion creates synergism in the digestion medium, and reduces inhibition, operational costs and GHG emissions. Furthermore, it improves buffering action and process stability during AD (Shu et al., 2015; Siddigue & Ab. Wahid, 2018). However, AcoD has some drawbacks, such as high levels of COD in effluent, and increased mixing, energy and pretreatment requirements. Mathematical modeling can be used as a tool to predict these challenges (Siddigue & Ab. Wahid, 2018).

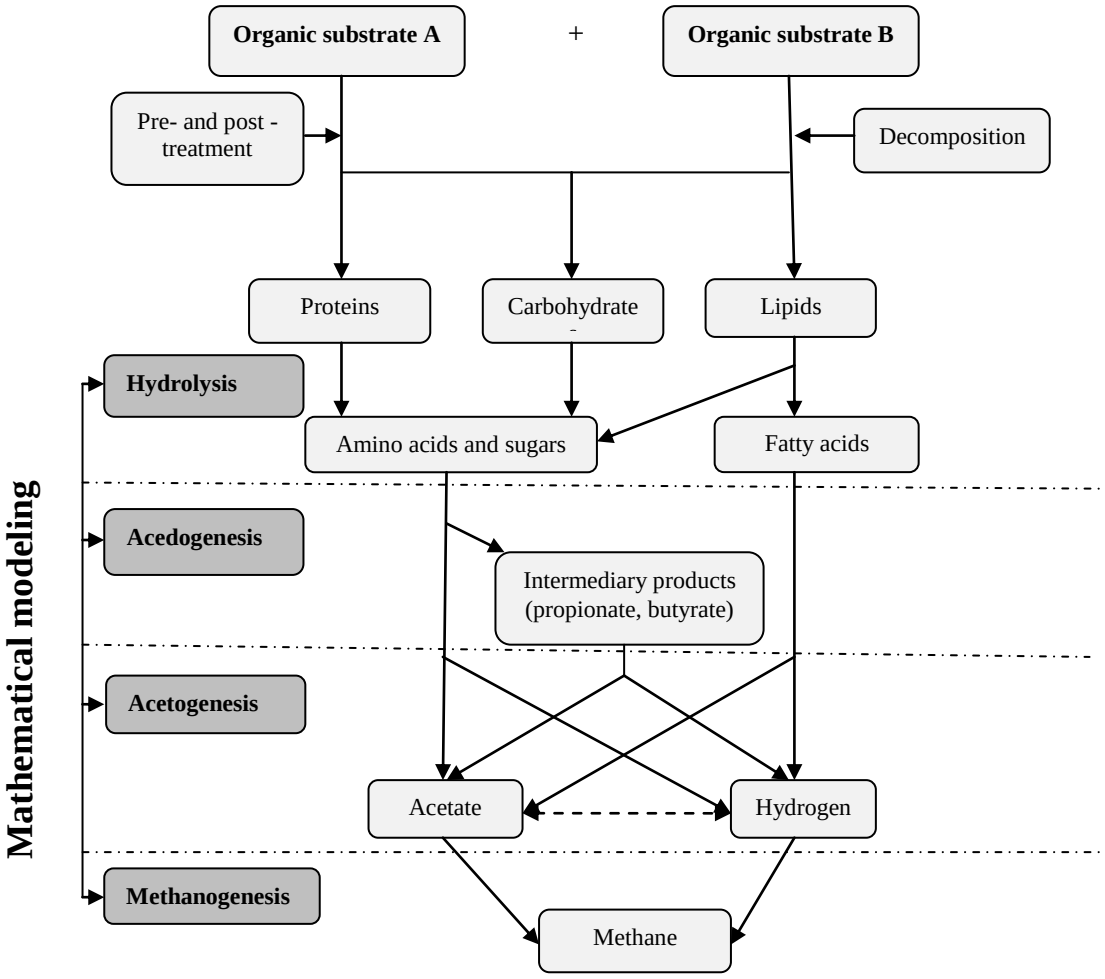

Figure 2.13 An overview of codigestion process and mathematical modeling application (Siddigue & Ab. Wahid, 2018)

Codigestion utilizes a variety of substrates to enhance methane production. Several authors have reported a wide array of methane yields from different codigestion experimental set-ups (Table 2.7). The instantaneous digestion of tomato residues, maize stover and dairy manure produced the highest methane (415 mL g^{-1} VS) (Li et al., 2016) while codigestion of tomato residues and cow manure generated the lowest methane (130 mL g^{-1} VS) under mesophilic conditions (Akman et al., 2015).

Table 2.7 Codigestion of different organic feedstocks and their biomethane potential

Feedstocks	Mixing ratio	Experimental set-up	Methane (mL g^{-1} VS)	Reference
Maize stover: chicken manure	3 : 1	1 L wet anaerobic digestion, mesophilic (37°C)	219	Li et al. (2013)
Maize stover: chicken manure	1.4 : 1	I L batch digestion, mesophilic (37°C)	281	Li et al. (2014)
Maize stover: chicken manure	1.4 : 1	1 L continuous stirrer tank reactors, mesophilic (37°C)	223	Li et al. (2014)
Tomato residues: maize stover: dairy manure	1 : 3 : 4	1 L solid-state anaerobic digestion, mesophilic (35°C)	415	Li et al. (2016)
Aloe peel waste: dairy manure	3 : 1	500 mL batch reactions, mesophilic (36°C)	195	Huang et al. (2016)
Tomato residues: cow manure	1 : 1.2	500 mL batch tests, mesophilic (36°C)	130	Akman et al. (2015)
Maize straw: swine manure	3:7	1 L batch digesters, mesophilic (35°C)	220	Mao et al. (2017)
Banana waste: cow manure	3:2	1 L semi-continuous stirrer tank, mesophilic (37°C)	229	Joute et al. (2016)

2.9 Cellulolytic Bacteria in Biogas Production

2.9.1 Bacteria as a Source of Cellulolytic Enzymes

Naturally occurring organisms such as bacteria, fungi, protozoans and animals are able to produce cellulases that can decompose cellulose (Hussain et al., 2017; Verma et al., 2021). Extensive research was performed on cellulolytic potential of fungi, including *Trichoderma, Aspergillus* and *Penicillium*. However, limited studies are reported on bacterial cellulases (Verma et al., 2021). Further exploration is essential to acquire more novel bacteria strains with hypercellulolytic activity. Table 2.8 shows the taxonomic diversity of bacteria with capability to degrade cellulose. Cellulolytic bacteria can be isolated from multifarious habitats such as sewage, agricultural wastes, soil, hot springs, and guts of ruminants and insects (Martin-Ryals, 2012; Pinheiro et al., 2015). Genera include *Bacillus*, *Clostridium*, *Cellulomonas*, *Micrococcus*, *Alteromonas*, *Acetivibrio*, *Pseudomonas* and *Bacteriodes* (Seo et al., 2013; Hussain et al., 2017).

Table 2.8 Bacteria involved in cellulolytic activities

Genus	Species
Acidothermus	*A. cellulolyticus*
Bacillus	*Bacillus* sp., *B. megaterium, B. amyloliquefaciens, B. subtilis* and *Anoxybacillus flavithermus*
Clostridium	*C. thermocellum, C. acitobutylicum* and *C. cellulovorans*
Pseudomonas	*P. cellulose*
Rhodothermus	*R. marinus*
Cellulomonas	*C. fim* and *C. uda*

Source: Singhania (2009) and Hussain et al. (2017)

2.9.2 Classification and Mode of Action of Cellulolytic Enzymes

Cellulolytic enzymes produced from thermotolerant bacteria have contributed considerably to developments in biogas industry. Generally, cellulase is produced during growth of microorganisms on lignocellulosic material. It acts as a biocatalyst for transformation of cellulosic material into fermentable sugars (Akhtar et al., 2014; deb Dutta et al., 2018). Cellulase is a complex mixture of three different hydrolytic enzymes: exoglucanase (cellobiohydrolases), endoglucanase and β-glucosidase. These multienzyme systems interact in a synergistic way to degrade lignocellulosic biomass into constituent glucose, cellobiose and other oligosaccharides units (Singhania, 2009; Dashtban et al., 2010; Verma et al., 2021).

The classification of cellulases is based on the degradation phase of the target substrate (Bhardwaj et al., 2021). Cellulase functions in a synchronized mechanism to hydrolyze β-1,4-glycosidic bonds found in cellulose (Figure 2.14). Firstly, endoglucanase attacks amorphous regions in cellulose fibers, allowing exoglucanase to enter the crystalline regions of the fibers. Oligomers varying in level of polymerization are the end products of this reaction. Exoglucanase destroys non-reducing ends of the microcrystalline structure of the cellulose polymer releasing cellobiose or glucose. Lastly, β-glucosidase converts cellobiose and cellodextrin into glucose, thus further blocking the accumulation of cellobiose which is inhibitory to cellobiohydrolase (Verma et al., 2021). Glucose is an intermediary substrate that can be converted via AD or fermentation into value-added products like biogas and bioethanol (deb Dutta et al., 2018).

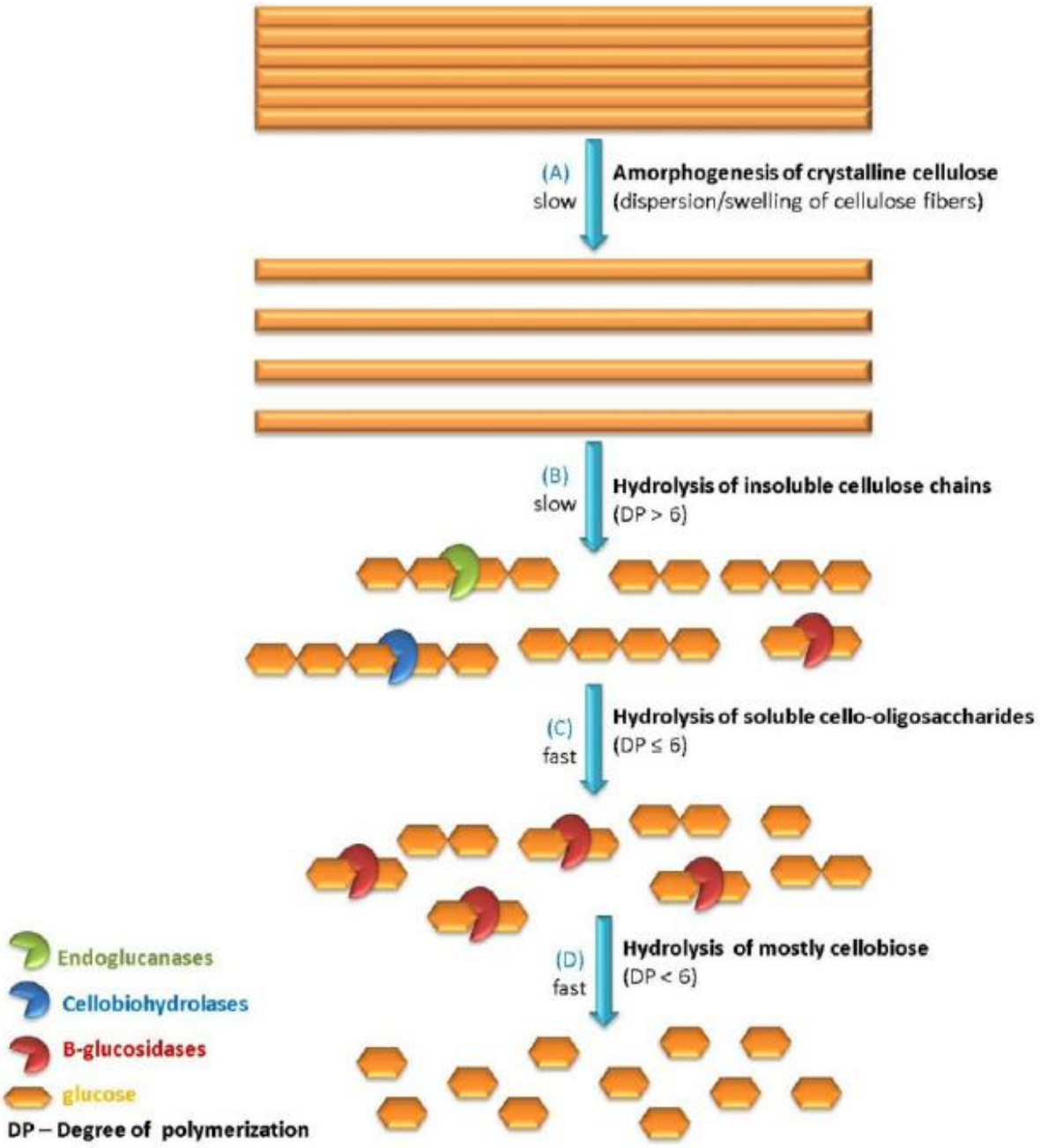

Figure 2.14 Schematic presentation of the mechanism of action of cellulases on cellulose substrate (Arantes & Saddler, 2010)

2.9.3 Methods to Detect Cellulolytic Bacteria

Traditional phenotypic techniques are commonly used for primary identification of novel cellulolytic bacteria from diverse ecological sources. They are based on phenotypic

characteristics, including morphological, physiological and biochemical features (Bisen et al., 2012; Li et al., 2015). The majority of phenotypic variables are not sensitive enough for strain differentiation. Moreover, phenotypic analysis is a very tedious exercise in identification of bacteria (Bisen et al., 2012). Traditional methods involve enrichment of bacteria in media. However, not all bacteria are able to grow in media. Current focus has been placed on searching for fast and unambiguous culture-independent tools to detect cellulolytic bacteria.

Traditional methods have been accelerated, simplified and automated due to the advancement in technology (Bisen et al., 2012; Li et al., 2015). Advances in molecular biology have led to the advent of new approaches for identification and characterization of bacteria taxonomy. These new frontiers of technology rely mostly on 16S rRNA gene sequencing, polymerase chain reaction (PCR) and other PCR-related techniques (Bisen et al., 2012; Buszewski et al., 2017). The popularity of these methods has been derived from their high sensitivity and reproducibility. The 16S rRNA sequencing is touted the most accurate method and declared the "gold standard" for detection of bacteria up to species level (Buszewski et al., 2017). The method is the most sensitive, very rapid, highly specific, reliable, reproducible and allows the detection of bacterial strains that cannot be easily cultured under laboratory conditions (Buszewski et al., 2017; Franco-Duarte et al., 2019).

The 16S rRNA sequencing is based on PCR amplification and sequencing of amplified DNA. Polymerase chain reaction requires sufficient yield of high purity genomic DNA (Zhang et al., 2014). Extracted DNA should be free of proteins, RNA and PCR inhibitors (Abbas & Al Musawi, 2016). DNA extraction from cellulolytic bacteria can be done using various cell lysis methods, such as chemical, mechanical, enzymatic and heat (Vesty et al., 2017). The methods

that have been commonly used to extract genomic DNA from cellulolytic bacteria include commercial kits, phenol-chloroform and the boiling methods (Zhang et al., 2014).

Table 2.9 provides a comparison of disparate methods that can be used to purify DNA from cellulolytic bacteria. It is important to select the most appropriate method to identify cellulolytic bacteria. An ideal DNA extraction protocol should be sensitive, rapid, easy-to-use, minimize use of specialized equipment, pose minimum risk to users, avoid cross-contamination of samples and elute DNA that is highly pure (Chacon-Cortes & Griffiths, 2014). The 16S RNA sequencing was previously used to identify cellulase-producing bacteria from soil and ward poultry (Hussain et al., 2017), hot springs (Adiguzel et al., 2009; Abdollahi et al., 2021), termite guts of worker *Macrotermes gilvus* (Ferbiyanto et al., 2015) and pig's intestine (Yang et al., 2014). The isolated cellulolytic bacteria strains can be potentially incorporated in biogas production from polymeric biomass compounds.

Table 2.9 Comparison of different methods to isolate genomic DNA from cellulolytic bacteria

Method	Parameter						
	Cost	Yield of DNA	Purity of DNA	Time efficiency	Technical requirements	Sensitivity	Toxic compounds
Commercial kits	High	Very high	Very high	Rapid	Simple	High	None
Phenol-chloroform	Moderate	Low	Low	Slow	Simple-complex	Low	Phenol, chloroform
Boiling	Low	High	Low	Rapid	Very simple	Low	None

Source: Abbas & Al Musawi (2016), Ahmed et al. (2017) and Wright et al. (2017)

2.10 Summary of Findings

This Chapter reviewed extant literature on biofuel production from crop residues, seeking to identify the most studied pretreatment methods and cosubstrates, and research gaps arising in this context. Biofuels can play an important role in sustainable development with respect to combating climate change, and improving energy security and the livelihoods of people. However, the contribution of biofuels to the global energy sector is insignificant compared to the growing energy demands. The main challenge on implementing biofuel policies is that most biofuels are derived from starches, sugars and edible vegetable oils, thus risking food security. Even though, organic wastes emerged as alternative feedstocks, further research is required to diversify the feedstock reserves for biofuel production.

The agricultural sector generates large amounts of crop residues in form of straws, husks, cobs, stovers and hulls throughout the world. Disposal of crop residues still needs to be addressed as current management practices, such as burying and burning present adverse effects to the society and environment. The AD is a well-established and environmentally friendly technology to control the quantity of crop residues. Crop residues are endowed with large amounts of organic matter, which can be converted into energy via AD. In spite of this fact, valorization of crop residues into biogas is undervalued and poorly understood as they are naturally recalcitrant. High cost of feedstock pretreatment remains a critical issue in AD of crop residues. Biogas production from crop residues needs to be enhanced and promoted through innovation.

The AD of crop residues needs to be complemented with suitable cosubstrates in a codigestion system to balance nutrient availability and minimize toxicity. Thus, authors suggested further research to optimize process parameters such as C/N ratio, pH, temperature, OLR, HRT, etc.

Animal manure is highly abundant and rich in nitrogen; therefore it could be an ideal cosubstrate to accelerate the AD of crop residues.

The most widely reported pretreatment strategies fall into three broad categories: chemical, physical and biological methods. However, chemical and physical methods have pitfalls, such as infrastructural and technological limitations. Further, they release inhibitory compounds that may interfere with microbial activities during AD. It is important to acknowledge that biological pretreatment of crop residues is still naive; hence there is limited data towards its valorization into biogas. Literature abounds with information that fungi are the most reported biological pretreatment agents, but they often consume fermentable sugars during pretreatment, and have long retention times.

Several authors recommend that research should focus on pretreatment with microbial consortium systems, which are more effective than a single microorganism in improving the hydrolysis of crop residues. However, there is lack of research on how pretreatment of disparate crop residues with consortia of bacteria influence biogas production. This research gap limits our understanding of how biogas production from crop residues can be optimized and commercialized through biological pretreatment. In addition, isolation of novel cellulolytic bacteria strains remains a critical step towards development of sustainable pretreatment systems. Focus area should be expansion of the database of cellulolytic bacteria strains through development of novel technologies.

CHAPTER 3: COMPARISON OF PRETREATMENT METHODS THAT ENHANCE BIOMETHANE PRODUCTION FROM CROP RESIDUES: A SYSTEMATIC REVIEW

3.1 Introduction

Renewable energy platforms can be an alternative way to alleviate problems of energy crisis and global climate change in the 21st century (Wen et al., 2015; Sasaki et al., 2016; Paudel et al., 2017). According to the UN, a total of 77% of the global energy supply will be replaced by renewable energy sources by 2050 (Yao et al., 2018). The AD of organic feedstock streams is a cheap and renewable source of energy, although it only contributes about 11% to the global energy mix (Ritchie et al., 2022). It converts organic wastes into biomethane and a solid digestate fertilizer (Schroyen et al., 2015; Panepinto & Genon, 2016; Khalid et al., 2019). Biomethane can be used for energy purposes in vehicles, and heat and power engines, after upgrading the methane content to around 96% (Sasaki et al., 2016; Zhao et al., 2018).

A wide range of agricultural waste streams can be used for biomethane production (Påledal et al., 2013; Gould, 2015; Achinas et al., 2017). These include animal manure, fruit and vegetable waste, grass, forest waste, various crops and their residues (Paudel et al., 2017). Crop residues are an abundant waste material and hold much promise as a bioresource for biomethane production. According to Shinde et al. (2022), more than 5 billion tonnes of crop residues are produced annually all over the world. Crop residues primarily contain a heterogeneous mixture of cellulose, hemicelluloses and lignin (Schroyen et al., 2015; Horváth et al., 2016). Lignin forms a protective layer surrounding holocellulose (cellulose and hemicelluloses). This protects holocellulose from biodegradation, thus restricting the use of crop residues in biomethanation (Schimpf & Schulz, 2019; Moodley & Trois, 2021). Therefore, a suitable pretreatment method is

required to rupture the lignin seal and accelerate the bioconversion of crop residues into biogas (Lalak et al., 2016; Guan et al., 2018).

As reported in extant literature (Chapter 2), a number of pretreatment methods are available to optimize biomethane production from crop residues (Ge et al., 2016; Lalak et al., 2016; Achinas et al., 2017; Wagner et al., 2018; Liu et al., 2019). Pretreatment methods can be divided into three major categories, namely physical, chemical and biological processes (Wen et al., 2015; Amin et al., 2017; Patinvoh et al., 2017; Kumar et al., 2018; Mustafa et al., 2018; Venturin et al., 2018; Dahunsi, 2019; Karrupiah & Azariah, 2019). The success of chemical and physical pretreatments has been hindered by high energy demands, need for specialized equipment and emission of toxic byproducts (Liu et al., 2019). Biological pretreatments can combat challenges of inhibitor formation and metabolite suppression, but operation at industrial scale has not been viable (Wen et al., 2015).

In this study, a comparative analysis of pretreatment methods for crop residues was conducted through gathering of extant literature. The purpose was to provide an informed decision on the choice of pretreatment method to be used for crop residues prior to biomethane production. Such information is useful for planning and enhancing of AD plants that utilize crop residues. Based on this systematic review, bacteria strains with high cellulolytic activities were isolated and identified for biological pretreatment of crop residues (Chapter 4).

3.2 Specific Objectives

The objectives of this study were to:

(a) Conduct an aggregated comparative analysis of the efficacy of pretreatment approaches for crop residues by reviewing extant literature.

(b) Establish a suitable pretreatment method for improving the AD of crop residues.

3.3 Methodology

3.3.1 Procedure

The standard procedure on performing a systematic literature review was used (Kitchenham & Charters, 2007; Okoli & Schabram, 2010; Mittal et al., 2018). The search period was January 2014 to November 2018. Most recent work on enhancing biogas production was published during this period (Prasad et al., 2017; Kougias & Angelidaki, 2018). Three databases comprising Science Direct, EBSCOhost and PubMed were chosen on the basis of their availability in the University library and being among the top ten online research databases. The search was delineated to online full-text journal articles. Gray literature covering government reports, conference proceedings, graduate dissertations and unpublished papers was excluded. The challenges of searching gray literature were pointed out by Paez (2017) and Mahood et al. (2014).

The search protocol for identification and selection of articles for inclusion in this study is shown in Figure 3.1. The protocol was in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Moher et al., 2009; Forbes et al., 2018). Key search terms used for extracting relevant articles are shown in Table 3.1. These were selected using the model of a concept map (Asiksoy, 2019). ‘Biogas’ and ‘crop residues’ were the major search terms. The major search terms were held as constant in order to eliminate articles not concerned with the use of crop residues for biogas production.

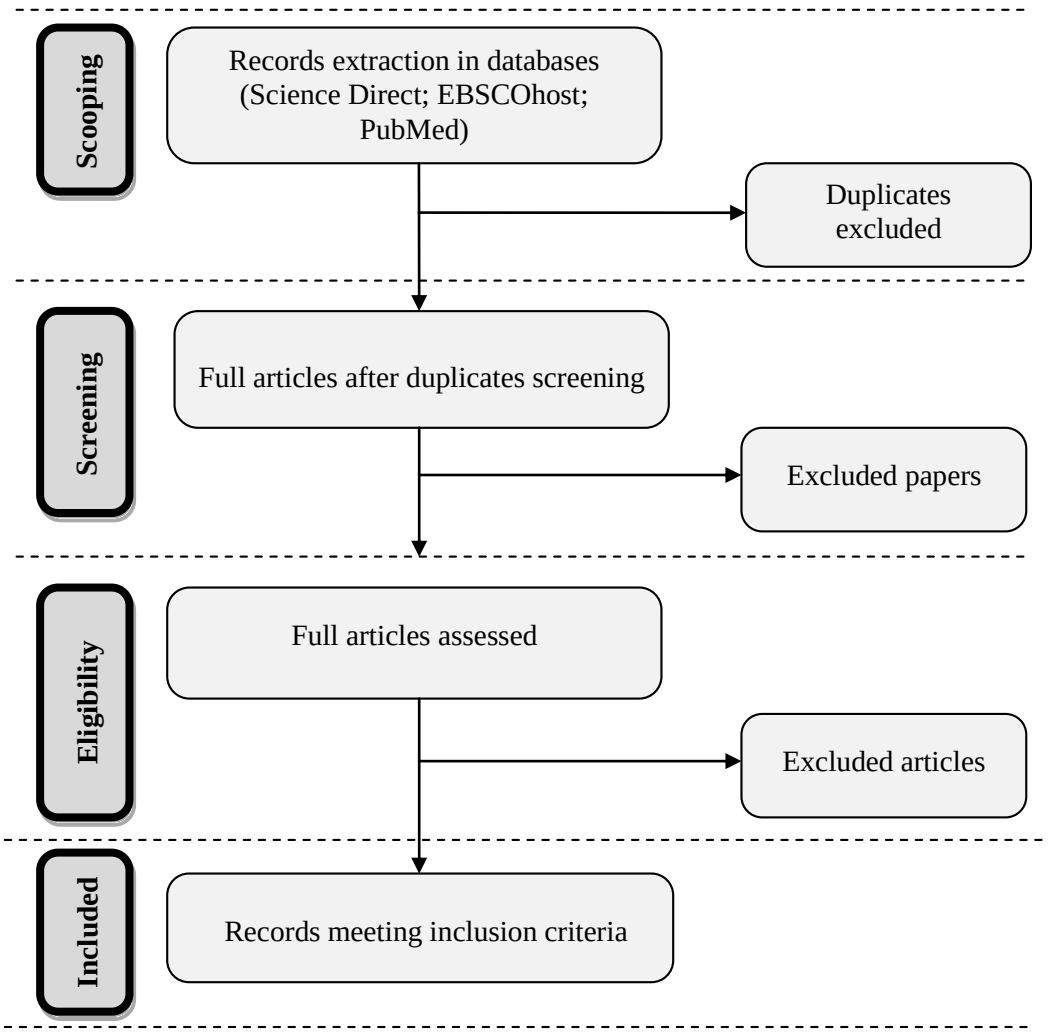

Figure 3.1 Flow diagram of systematic literature review according to PRISMA (Mittal et al., 2018)

3.3.2 Comparison Criteria

The main variables of interest were advantages and disadvantages of each pretreatment method in terms of techno-economic aspects. The techno-economic aspects used were: rate of hydrolysis, energy use, effectiveness, cost input and formation of toxic compounds (Wagner et al., 2018; Venturin et al., 2018).

3.4 Results and Discussion

3.4.1 Identification and Selection of Articles for Inclusion

A total of 3 167 full text articles were screened for relevance to the study (Figure 3.2). Disaggregation of the number of scooped articles by search terms is shown in Table 3.1. The majority of the publications (n = 1017) were extracted using the search terms, "chemical pretreatment" and "crop residues" and "biogas".

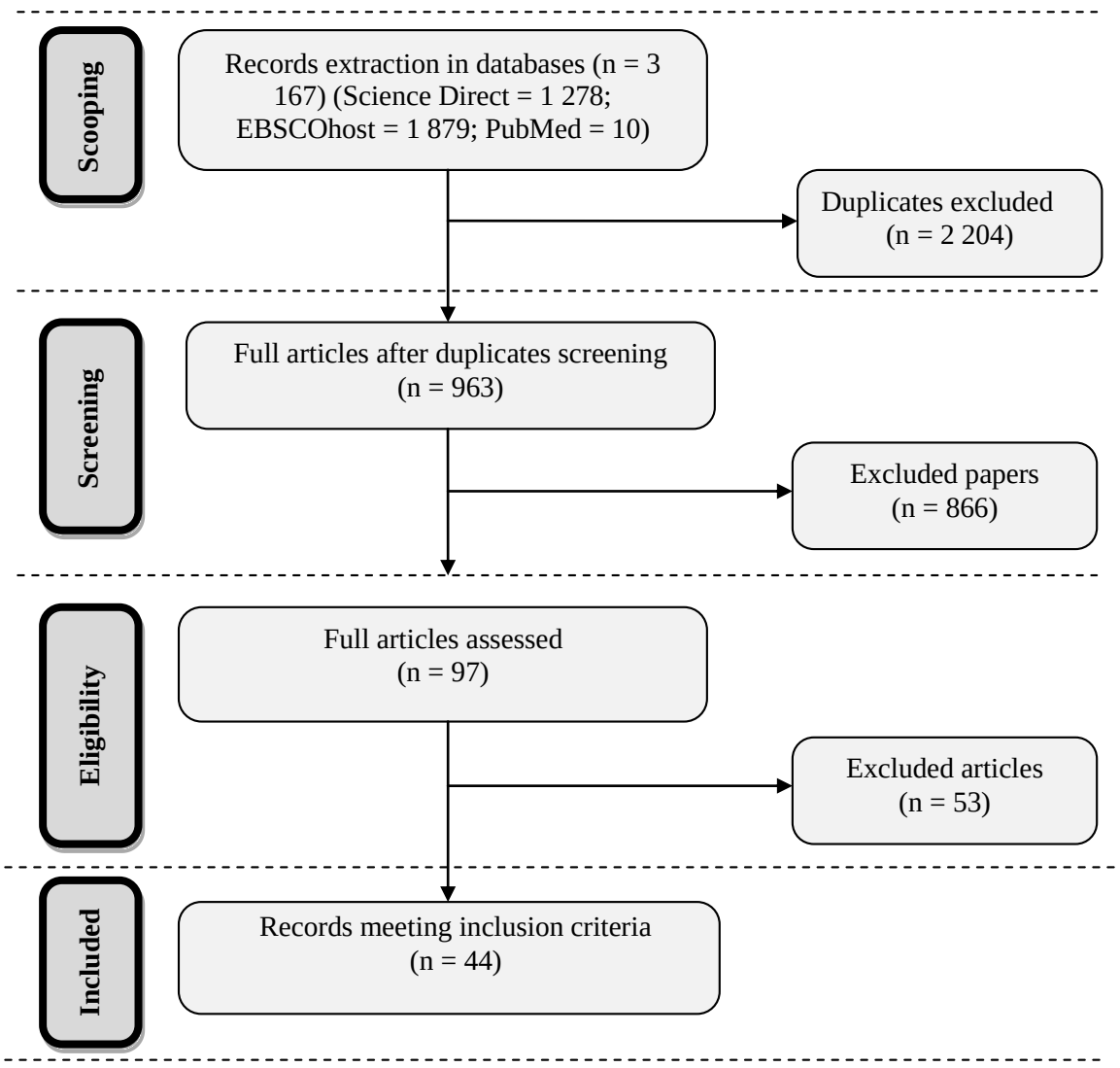

Figure 3.2 Summary of identification and selection of articles for the study inclusion process

Table 3.1 Number of articles extracted from databases using different search terms

Search terms	No. of articles scooped			
	Science Direct	EBSCOhost	PubMed	Total
"Pretreatment" and "crop residues" and "biogas"	384	214	6	604
"Chemical pretreatment" and "crop residues" and "biogas"	365	651	1	1 017
"Biological pretreatment" and "crop residues" and " biogas"	309	521	2	832
"Physical pretreatment" and "crop residues" and " biogas"	220	493	1	714

Forty-four (44) full text articles met the inclusion criteria. Thirty-six (36) articles were research papers and eight (8) articles were narrative reviews. The trend in the number of publications on pretreatment of crop residues for biogas production from 2014 to 2018 is shown in Figure 3.3. The number of publications on pretreatment of crop residues for biogas production rose between 2014 and 2017. This trend points to sustained research efforts on the subject. In recent years, pretreatment of feedstocks is a focal technology in biogas production (Kigozi et al., 2014; Achinas et al., 2017; Horváth et al., 2016).

Comparably, Kougias & Angelidaki (2018) reported a surge in the number of articles on biogas production from 1977 to 2017 by retrieving the Scopus and Web of Science databases. Carrere et al. (2016) reported a similar phenomenon from 2009 to 2014 based on the Web of Science database. Ge et al. (2016) reported an increase in the number of articles in which the phrase

"solid state anaerobic digestion" appeared in the Google Scholar database from 2005 to 2015. They noted a predominant and steady increase in the period between 2010 and 2015. This indicates the increasing focus on pretreatment technology.

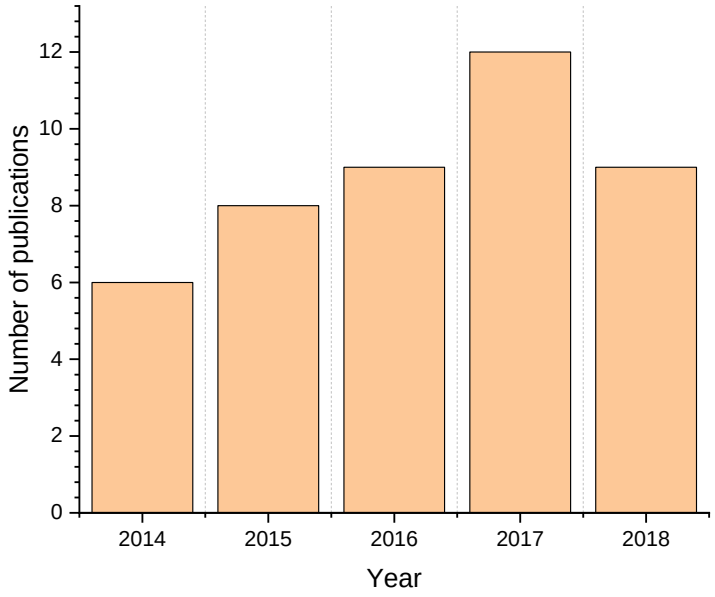

Figure 3.3 Number of publications in which "pretreatment of crop residues for biogas production" appeared, based on search results by Science Direct, EBSCOhost and PubMed

3.4.2 Characteristics of Selected Articles

The number of selected articles and their authors are shown in Table 3.2. The articles are organized according to four main pretreatment methods. Various pretreatment techniques are used to depolymerize crop residues into simple units. As shown in Figure 3.4, the key pretreatment methods are chemical, physical, biological and a combination of processes (Montgomery & Bochmann, 2014; Yuan et al., 2014; Kumar et al., 2018).

Most selected articles (21) focused on chemical pretreatment, with the alkali technique being dominant. The dominance of the alkali technique arises from its efficacy (Kumar et al., 2018).

Alkali pretreatment has been reported to have high efficiency and ability to improve the degradation of complex compounds (Amin et al., 2017). Physical and biological methods follow with 19 and 15 articles, respectively. However, fewer articles (11) focused on combined pretreatment methods. The main limitation of combined processes is their complexity (Montgomery & Bochmann, 2014).

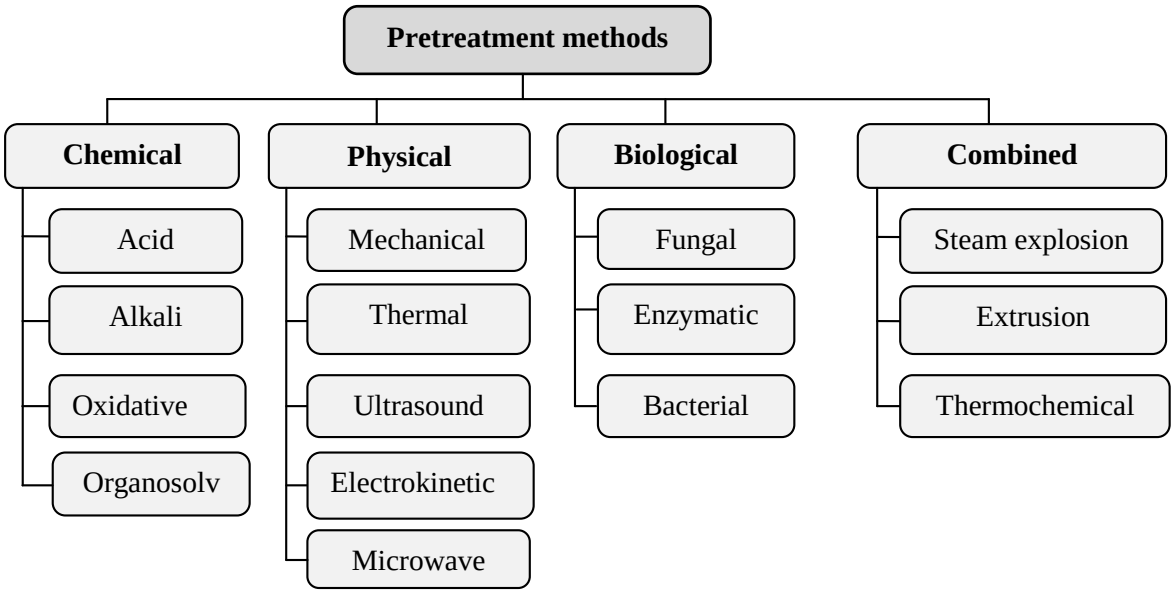

Figure 3.4 Summary of pretreatment methods (Montgomery & Bochmann, 2014)

Table 3.2 Characteristics of articles meeting inclusion criteria for comparison of pretreatment methods for crop residues

Pretreatment method	No. of articles	Authors
Chemical	21	Perendeci et al. (2018), Den et al. (2018), Nair et al. (2018), Zhang et al. (2018), Thomas et al. (2018), Chen et al. (2017), Ismail et al. (2017), Gumisiriza et al. (2017), Talha et al. (2016), Strauber et al. (2015), Li et al. (2015), Pei et al. (2014), Sahito & Mahar (2014), Song et al. (2014), Yuan et al. (2014), Amin et al. (2017), Kumar & Sharma (2017), Ge et al. (2016), Yao & Chen (2016), Constant et al. (2016), Wikandari et al. (2014)
Biological	15	Rouches et al. (2017), Thomsen et al. (2016), Mulakhudair et al. (2016), Singh et al. (2014), Wagner et al. (2018), Byrne et al. (2018), Gumisiriza et al. (2017), Amin et al. (2017), Kumar & Sharma (2017), Speda et al. (2017), Oszust et al. (2017), Ge et al. (2016), Zieminski & Kowalska-Wentel (2017), Li et al. (2016), Kudanga & Le Roes-Hill (2014)

Table 3.2 Characteristics of articles meeting inclusion criteria for comparison of pretreatment methods for crop residues

Pretreatment method	No. of articles	Authors
Physical	19	Paul et al. (2018), Zieminski & Kowalska-Wentel (2017), Li et al. (2016), Dumas et al. (2015), Chandra et al. (2015), Luo et al. (2015), Sahito & Mahar (2014), Gumisiriza et al. (2017), Amin et al. (2017), Kumar & Sharma (2017), Xi et al. (2015), Wu et al. (2015), Kostas et al. (2017), Mulakhudair et al. (2016), Eskicioglu et al. (2017), Gaworski et al. (2017), Baeta et al. (2016), Ge et al. (2016), Sadhukhan et al. (2018)
Combined processes	11	Zhang et al. (2018), Byrne et al. (2018), Gaworski et al. (2017), Zieminski & Kowalska-Wentel (2017), Siddhu et al. (2016), Gumisiriza et al. (2017), Amin et al. (2017), Kumar & Sharma (2017), Ge et al. (2016), Kim et al. (2018), Paul et al. (2018)

3.4.3 Comparison of Pretreatment Methods

3.4.3.1 Rate of Hydrolysis

Table 3.3 provides information on pretreatment methods with reference to rate of hydrolysis. According to information in Table 3.3, physical methods appeared to be the fastest amongst the pretreatment methods. This is more so for microwave pretreatment (Wu et al., 2015; Kumar & Sharma, 2017). This is in agreement with Yuan et al. (2014) who posited that short duration time is an advantage of mechanical pretreatment. Conversely, Gumisiriza et al. (2017) reported that irradiation processes are slow.

Two studies (Li et al., 2015; Yuan et al., 2014) reported that chemical pretreatment is a fast process, and three studies reported that it is a slow process (Gumisiriza et al., 2017; Amin et al., 2017; Kumar & Sharma, 2017). This can be explained in terms of variation in treatment conditions. For example, retention time of chemical pretreatment is affected by temperature (Theuretzbacher et al., 2015; Sambusiti, 2013). Data on combined methods is rather limited. However, combined processes were reported to be fast methods (Kim et al., 2018; Kumar & Sharma, 2017).

As shown in Table 3.3, studies considered in this review cited the major drawback of biological pretreatment as being a slow process (Kudanga & Le Roes-Hill, 2014; Singh et al., 2014; Mulakhudair et al., 2016; Amin et al., 2017; Gumisiriza et al., 2017; Kumar & Sharma, 2017; Den et al., 2018; Wagner et al., 2018). Generally, the required residence time is 10 - 14 days (Amin et al., 2017). The hydrolysis rate can be enhanced by optimizing parameters, such as nature of biomass, microbial composition, temperature, pH, incubation time, inoculum size, moisture content and aeration rate (Sindhu et al., 2016).

Table 3.3 Comparison of pretreatment methods in terms of rate of hydrolysis

Pretreatment method	Observations	References
Chemical	• Fast	Li et al. (2015), Yuan et al. (2014)
	• Slow	Amin et al. (2017) Gumisiriza et al. (2017), Kumar & Sharma (2017)
Biological	• Slow	Kudanga & Le Roes-Hill (2014), Singh et al. (2014), Mulakhudair et al. (2016), Amin et al. (2017), Gumisiriza et al. (2017), Kumar & Sharma (2017), Den et al. (2018) Wagner et al. (2018)
Physical	• Fast	Yuan et al. (2014), Wu et al. (2015), Kumar & Sharma (2017)
	• Slow	Gumisiriza et al. (2017)
Combined processes	• Fast	Kumar & Sharma (2017), Kim et al. (2018)

3.4.3.2 Energy Use

Seven studies indicated the main advantage of biological pretreatment as low energy consumption (Table 3.4). Biological pretreatment saves chemicals and energy (Gumisiriza et al., 2017; Kumar & Sharma, 2017; Zieminski & Kowalska-Wentel, 2017; Den et al., 2018; Wagner et al., 2018). Singh et al. (2014) posited that fungal pretreatment offers many advantages compared to abiotic pretreatments, including low energy needs and mild reaction conditions. Amin et al. (2017) reported that microaerobic pretreatment can be considered to be a pretreatment option for AD of maize straw due to low energy requirements and limited supply of oxygen. As an example, Hua et al. (2016) reported increased biogas yield from biomass pretreated by microbial consortium and ascribed this to minimal energy needs.

Physical pretreatment was reported as a high energy consumption process (Table 3.4). It is very energy intensive given high temperatures and pressures involved (Mulakhudair et al., 2016; Gumisiriza et al., 2017; Speda et al., 2017; Wagner et al., 2018). For example, milling, extrusion and crushing used for particle size reduction of straw have high energy demands (Luo et al., 2015). Kostas et al. (2017) reported microwave pretreatment to have a challenge of high energy demand. It was reported in three studies (Yuan et al., 2014; Chandra et al., 2015; Kumar & Sharma, 2017) that size reduction by mechanical comminution requires large amounts of energy.

For instance, materials, such as maize stover and switch grass consume energy worth of 11.0 kWh Mt^{-1} and 27.6 kWh Mt^{-1}, respectively (Baruah et al., 2018). This is equivalent to a third of total electricity required for the whole biogas production process (Zheng et al., 2014). For this reason, physical pretreatment is considered to be economically unviable for large scale operation (Zheng et al., 2014). Conversely, Wu et al. (2015), Kostas et al. (2017) and Kumar & Sharma (2017) reported that physical pretreatment with respect to microwave pretreatment is energy efficient.

Generally, chemical methods are considered as high energy-consuming processes. This was reported in five studies shown in Table 2.4 (Wikandari et al., 2015; Speda et al., 2017; Rouches at al., 2017; Gumisiriza et al., 2017; Wagner et al., 2018). Three studies showed that combined processes are high energy-use methods (Perendeci et al., 2018; Speda et al., 2017; Gumisiriza et al., 2017). High temperatures and limited heat recovery during steam explosion pretreatment may lead to high energy use and reduced methane yield (Gumisiriza et al., 2017). Extrusion pretreatment consumes high energy of about 10 - 15 kW of power to pretreat a tonne of substrate (Montgomery & Bochmann, 2014).

In contrast, three studies reported combined processes to be low energy-consuming. This observation was sustained by Kumar & Sharma (2017), Kim et al. (2018) and Zhang et al. (2018). It can be postulated that this variation in results is due to the complexity of combined methods and further research is needed.

Table 3.4 Comparison of pretreatment methods in terms of energy use

Pretreatment method	Observations	References
Chemical	• Low energy needs	Sahito & Mahar (2014), Ismail et al. (2017)
	• Energy intensive	Wikandari et al. (2015), Speda et al. (2017), Rouches at al. (2017), Gumisiriza et al. (2017), Wagner et al. (2018)
Biological	• Low energy demand	Singh et al. (2014), Gumisiriza et al. (2017), Kumar & Sharma (2017), Amin et al. (2017), Zieminski & Kowalska-Wentel (2017), Den et al. (2018), Wagner et al. (2018)
Physical	• Saves energy	Wu et al. (2015), Kostas et al. (2017), Kumar & Sharma (2017)
	• Very energy intensive	Yuan et al. (2014), Chandra et al. (2015), Luo et al. (2015), Mulakhudair et al. (2016), Gumisiriza et al. (2017), Kostas et al. (2017), Kumar & Sharma (2017), Speda et al. (2017), Wagner et al. (2018)
Combined processes	• Require low energy	Kumar & Sharma (2017), Kim et al. (2018), Zhang et al. (2018)
	• Consume high energy	Speda et al. (2017), Gumisiriza et al. (2017), Perendeci et al. (2018)

3.4.3.3 Effectiveness

Information in Table 3.5 suggests that chemical pretreatment is the most effective method. Song et al. (2014), Strauber et al. (2015), Jiang et al. (2016), Amin et al. (2017), Ismail et al. (2017), Kumar & Sharma (2017) and Rouches at al. (2017) reported that chemical pretreatments are effective for enhancing the degradation of complex compounds, such as crop residues and herbaceous crops. For example, Perendeci et al. (2018) observed enhanced methane by 78% from alkaline H_2O_2 pretreated greenhouse crop waste. Ammonia pretreated wheat straw effectively increased total methane yield by 17.5% (Li et al., 2015). Thomas et al. (2018) obtained 32% more methane from lime pretreated *Mischanthus x giganteus*. Den et al. (2018) reported that acid and alkali pretreated oil palm empty fruit bunches increased methane yield by 40% and 100%, respectively.

Data in Table 3.5 show that combined processes are effective methods (Amin et al., 2017; Kumar & Sharma, 2017; Kim et al., 2018; Perendeci et al., 2018; Zhang et al., 2018). Steam explosion was found to be effective in pretreatment of crop residues (Amin et al., 2017; Kumar & Sharma, 2017). As an example, Zhang et al. (2018) reported improved cumulative methane yield of 226.6% and 216.4% at 1.2 MPa for 15 minutes and 1.5 MPa for 5 minutes, respectively, from steam-exploded crop straw.

As shown in Table 3.5, biological pretreatment is not as effective as the other methods. None of biological methods are efficient as standalone pretreatment methods (Thomsen et al., 2016). For instance, no significant difference in methane yields between enzymatic pretreated banana stems and non-treated stems was observed by Li et al. (2016). Paul et al. (2018) reported that fungal pretreatment of agricultural biomass do not improve methane production. However, pretreatment

of rice straw with fungal strains such as *P. ostreatus* and *T. reesei* increased methane yield by 120% (Wagner et al., 2018). This variation in results is expected as biological pretreatment is still under development.

Physical pretreatment appears to be generally effective as shown in Table 3.5. For example, hot water pretreatment increased methane yield of rice straw by 222% (Ge et al., 2016) and the microwave method increased methane yield by 28% (Wu et al., 2015). Baeta et al. (2016) reported that autohydrolysis is a highly effective process. Den et al. (2018) showed that microwave pretreatment can not increase biogas production.

Table 3.5 Comparison of pretreatment methods in terms of effectiveness

Pretreatment method	Observations	References
Chemical	• Effective	Song et al. (2014), Li et al. (2015), Strauber et al. (2015), Jiang et al. (2016), Talha et al. (2016), Amin et al. (2017), Kumar & Sharma (2017), Ismail et al. (2017), Rouches at al. (2017), Den et al. (2018), Perendeci et al. (2018), Thomas et al. (2018)
Biological	• Effective	Wagner et al. (2018)
	• Not effective	Li et al. (2016), Thomsen et al. (2016), Paul et al. (2018)
Physical	• Effective	Baeta et al. (2016), Ge et al. (2016), Wu et al. (2015)
	• Not effective	Den et al. (2018)
Combined processes	• Effective	Amin et al. (2017), Kumar & Sharma (2017), Kim et al. (2018), Perendeci et al. (2018), Zhang et al. (2018)

3.4.3.4 Cost

Table 3.6 compares different pretreatment methods in terms of cost of operation. Despite being less effective, biological pretreatment is variable in cost-effectiveness. Biological pretreatment was reported to be inexpensive (Mulakhudair et al., 2016; Gumisiriza et al., 2017; Den et al., 2018; Wagner et al., 2018). Rouches et al. (2017) stated that fungal pretreatment is cost-effective. Fungi reduce the number of pretreatment steps and cost by avoiding enzyme recovery steps (Carrerre et al., 2016). Kudanga & Le Roes-Hill (2014) reported that enzyme pretreatment has low utility cost due to use of mild conditions. Microaerobic pretreatment is more economic and ecofriendly than other pretreatments (Amin et al., 2017). Contrarily, biological pretreatment was reported to be costly with respect to use of commercial enzymes (Kudanga & Le Roes-Hill, 2014; Sahito & Mahar, 2014; Li et al., 2016; Mulakhudair et al., 2016).

Despite variations in Table 3.6, chemical and physical methods appear to be expensive and not economically viable for biogas production. Chemical pretreatment was reported to be expensive due to high cost of disposing the digestate waste (Wagner et al., 2018), use of extraneous agent which incurs high cost of chemicals and downstream processing (Strauber et al., 2015; Amin et al., 2017; Kumar & Sharma, 2017; Den et al., 2018; Sadhukhan et al., 2018), the need for expensive auxiliary equipment (Wikaandari et al., 2015; Speda et al., 2017) and high operational and maintenance cost (Rouches at al., 2017). Other studies reported chemical methods with respect to alkali pretreatment to be inexpensive (Song et al., 2014; Amin et al., 2017; Gumisiriza et al., 2017; Ismail et al., 2017; Kumar & Sharma, 2017).

Kostas et al. (2017) reported that microwave heating offers opportunities to reduce processing cost. However, most studies reported that physical pretreatment is expensive due to high energy

and capital cost (Sadhukhan et al., 2018). Milling, grinding, ultrasonic and irradiation processes have high energy and maintenance costs (Sahito & Mahar, 2014; Yuan et al., 2014; Xi et al., 2015; Kumar & Sharma, 2017; Perendeci et al., 2018; Sadhukhan et al., 2018). Combined methods are affected by costs associated with the other pretreatment methods. Steam explosion pretreatment requires expensive auxiliary equipment (Speda et al., 2017), although it is regarded as inexpensive (Gumisiriza et al., 2017; Kumar & Sharma, 2017; Zhang et al., 2018).

Table 3.6 Comparison of pretreatment methods in terms of cost

Pretreatment method	Observations	References
Chemical	• Relatively inexpensive	Song et al. (2014), Amin et al. (2017), Gumisiriza et al. (2017), Ismail et al. (2017), Kumar & Sharma (2017)
	• Expensive	Strauber et al. (2015), Wikaandari et al. (2015), Amin et al. (2017), Speda et al. (2017), Kumar & Sharma (2017), Den et al. (2018), Rouches at al. (2017), Sadhukhan et al. (2018), Wagner et al. (2018)
Biological	• Cost-effective	Kudanga & Le Roes-Hill (2014), Mulakhudair et al. (2016), Amin et al. (2017), Gumisiriza et al. (2017), Rouches et al. (2017), Den et al. (2018), Wagner et al. (2018)
	• Costly	Kudanga & Roes-Hill (2014), Sahito & Mahar (2014), Li et al. (2016), Mulakhudair et al. (2016)

Table 3.6 Comparison of pretreatment methods in terms of cost

Pretreatment method	Observations	References
Physical	• Inexpensive	Kostas et al. (2017)
	• Expensive	Sahito & Mahar (2014), Song et al. (2014), Yuan et al. (2014), Xi et al. (2015), Amin et al. (2017), Gumisiriza et al. (2017), Kumar & Sharma (2017), Speda et al. (2017), Perendeci et al. (2018), Sadhukhan et al. (2018)
Combined processes	• Cost-effective	Gumisiriza et al. (2017), Kumar & Sharma (2017), Zhang et al. (2018)
	• Expensive	Speda et al. (2017)

3.4.3.5 Formation of Toxic Compounds

Table 3.7 provides information regarding formation of toxic inhibitory compounds during pretreatment. Biological pretreatment avoids formation of inhibitors during biogas production (Singh et al., 2014; Amin et al., 2017; Wagner et al., 2018). This is a positive attribute of biological pretreatment. While some chemical pretreatment methods do not produce inhibitory compounds (Sahito & Mahar, 2014; Den et al., 2018; Gumisiriza et al., 2017; Paul et al., 2018), eleven studies reported that chemical pretreatment causes formation of toxic compounds. As such, formation of inhibitory compounds is one of the disadvantages of chemical pretreatment.

As shown in Table 3.7, physical processes lead to formation of inhibitory compounds. For instance, thermal pretreatment at temperatures above 160°C may lead to degradation of polysaccharides and lignin to release phenolic and heterocyclic compounds (Zieminski &

Kowalska-Wentel, 2017). However, some of the studies (Baeta et al., 2016; Kumar & Sharma, 2017) showed that autohydrolysis and mill pretreatment do not generate toxic compounds.

Seven studies in Table 3.7 showed that combined processes generate inhibitory compounds, especially steam explosion pretreatment. Zheng et al. (2014) reported that the efficacy of steam explosion and extrusion can be affected by production of inhibitory substances, such as furfural and hydromethylfurfural. This can explain the methane yield loss during high temperature steam explosion pretreatment of late harvested hay (Bauer et al., 2014).

Table 3.7 Comparison of pretreatment methods in terms of formation of toxic compounds

Pretreatment method	Observations	References
Chemical	• Avoids formation of toxic inhibitory compounds	Sahito & Mahar (2014), Gumisiriza et al. (2017), Paul et al. (2018), Den et al. (2018)
	• Produces toxic inhibitory compounds	Kudanga & Roes-Hill (2014), Pei et al. (2014), Amin et al. (2017), Eskicioglu et al. (2017), Gumisiriza et al. (2017), Kumar & Sharma (2017), Speda et al. (2017), Rouches at al. (2017), Den et al. (2018), Nair et al. (2018), Paul et al. (2018)
Biological	• Reduces formation of inhibitory substances	Singh et al. (2014), Amin et al. (2017), Wagner et al. (2018)

Table 3.7 Comparison of pretreatment methods in terms of formation of toxic compounds

Pretreatment method	Observations	References
Physical	• Avoids formation of toxic inhibitory compounds	Baeta et al. (2016), Kumar & Sharma (2017)
	• Produces toxic inhibitory compounds	Wu et al. (2015), Speda et al. (2017), Zieminski & Kowalska-Wentel (2017), Den et al. (2018), Perendeci et al. (2018)
Combined processes	• Generates toxic inhibitory compounds	Gumisiriza et al. (2017), Speda et al. (2017), Zhang et al. (2018), Eskicioglu et al. (2017), Kumar & Sharma (2017), Amin et al. (2017)

3.4.3.6 Aggregated Results

Table 3.8 provides aggregated results of the five parameters for all the pretreatment methods. Put together, biological methods have more techno-economic advantages across the five parameters compared to other methods. The advantages of biological pretreatments are tagged with low energy use, low cost and ability to avoid formation of byproducts that are toxic to methanogens. However, there is need to improve on the efficacy of biological pretreatment. Focus area should be enhancement of the rate of hydrolysis. Despite high effectiveness, the main limitations of chemical and physical methods are high energy use and cost.

Table 3.8 Aggregated comparison of the pretreatment methods

Pretreatment method	Rate of hydrolysis	Energy use	Effectiveness	Cost	Generation of toxic compounds
Chemical	Fast	High	Very effective	Very expensive	Yes
Biological	Slow	Very low	Less effective	Cost-effective	No
Physical	Very fast	Very high	Moderately effective	Very expensive	Yes
Combined processes	Fast	Moderate	Effective	Cost-effective	Yes

3.5 Conclusion

It is evident from this study that pretreatment methods used for crop residues are variable in their effect. As such, a multi-factor evaluation conducted in this study provides information that can assist selection of methods to use. Rate of hydrolysis, energy use, effectiveness, cost and formation of toxic compounds are critical parameters that inform selection of a pretreatment method. Physical and chemical pretreatment methods have been utilized to some extent for delignification of crop residues to enhance biomethane production. However, these methods are energy intensive, expensive, not environmentally safe and generate toxic compounds, including carboxylic acids, furans and phenolic compounds which may be inhibit methanogenic activity.

In comparison to other methods, biological pretreatment offers more techno-economic advantages. It is regarded as inexpensive and low energy need process that can minimize formation of inhibitory compounds. Thus, biological pretreatment is one of the most promising technologies for enhancing biomethane production of crop residues. Moreover, rigorous research

is still needed to develop novel microorganisms and more efficient pretreatment options for crop residues to yielding potential results.

CHAPTER 4: ISOLATION AND CHARACTERIZATION OF CELLULOLYTIC BACTERIA FROM LUBIMBI HOT SPRINGS IN BINGA, ZIMBABWE

4.1 Introduction

Lignocellulose is the most abundant and cheap renewable resource on earth (Ge et al., 2018). Plant cell wall consists of 85 - 90% complex polymers of lignocellulose (Jung et al., 2015), which are made up of 40 - 50% cellulose, 15 - 25% hemicelluloses and 20 - 25% lignin (Zing et al., 2017). Cellulose is the major constituent of plant cell walls. It has a wide range of applications in agriculture, fuel and paper industry (Yang et al., 2014). In terms of bioenergy production, about 80% of cellulose can be converted into glucose (Sukhesh & Rao, 2018). However, cellulose has a water-insoluble crystalline structure that is covered by a heterogeneous layer of hemicelluloses and lignin. This limits its conversion into fermentable sugars.

Pretreatment is a promising strategy to improve the biodegradability of lignocellulose (Yang et al., 2014; Kasinath et al., 2021; Naik et al., 2021). Several attempts have shown the potential of chemical and physical methods to pretreat lignocellulose. The limited utility of these methods has been due to high energy demand, the need for complex equipment and release of inhibitory products (Yang et al., 2014; Kamusoko et al., 2019).

Biological pretreatment is considered to be a better option than chemical and physical methods (Chapter 3). It is an inexpensive and low energy method that does not produce secondary pollution (Zheng et al., 2014; Kamusoko et al., 2019). Pretreatment of cellulosic material using fungi, microbial consortium and enzymes is extensively reported in literature (Zheng et al.,

2014). Cellulases and hemicellulases are the most widely used enzymes for pretreatment of lignocellulosic substrates (Zheng et al., 2014).

Cellulases are a complex structure of hydrolytic enzymes, including cellobiohydrolase or exoglucanase (EC 3.2.1.91), endo β-glucanase (EC 3.2.1.4) and β-glucanase (3.2.1.21) that interact in a synergistic manner to convert cellulose into fermentable sugars. The enzymes are mainly produced by fungi, bacteria and actinomycetes (Yang et al., 2014; Kamusoko et al., 2021a).

Cellulases of fungal origin are mainly exploited in food, feed, textiles, fuel and chemical industries. Limited growth of fungi coupled with high cost of cellulase production is the main challenge of using cellulolytic fungi. Comparatively, cellulolytic bacteria have simple cultivation, rapid growth, short generation time and good potential applicability (Yang et al., 2014). Cellulolytic bacteria occupy multiple habitats including sewage, agricultural wastes, soil, hot springs, and guts of ruminants and insects. Genera consist of *Bacillus*, *Clostridium*, *Cellulomonas*, *Micrococcus*, *Alteromonas*, *Acetivibrio*, *Pseudomonas* and *Bacteriodes* (Kamusoko et al., 2021).

A large research gap still exists in the diversity of novel bacteria strains with high cellulolytic capability. Hence, it was necessary to isolate bacteria from the local environment for biological pretreatment. This Chapter focused on isolation, identification and characterization of cellulolytic bacteria from hot springs. The main conceptual framework was premised on the fact that cellulolytic activities of microorganisms are specific to microbial species and its environment.

Lubimbi hot springs located in Binga, Matabeleland North of Zimbabwe are characterized by extremely high temperatures with an average of about 73ºC (Chikwama et al., 2022). Expectations are very high that these hot springs are perfect sources of novel bacteria strains with multifarious applications. However, the microbial diversity of Lubimbi hot springs is not yet fully studied.

The aim of the study was to isolate and identify cellulolytic bacteria from local hot springs for potential use in pretreatment of crop residues for biogas production. The ability of cellulolytic bacteria to degrade lignocellulose is linked to biomass structure and composition. Therefore, the proximate composition of crop residues was analyzed towards biological pretreatment with novel bacteria strains (Chapter 5).

4.2 Specific Objectives

The specific objectives were to:

(a) Isolate and identify cellulolytic bacteria from hot springs.

(b) Optimize fermentations conditions for cellulase production from the isolated bacteria.

4.3 Materials and Methods

4.3.1 Experimental Design

The statistical design was a completely randomized design (CRD) with three replications. Submerged fermentation (SmF) was optimized using the one-variable-at-a-time (OVAT) approach. The main variables were temperature, incubation time, pH, and nitrogen and carbon source.

4.3.2 Study Site and Sample Collection

Cellulolytic bacteria were isolated from water samples collected from Lubimbi hot springs (18.4761° S, 27.3061° E). The hot springs are located in Binga District, Matabeleland North Province, Zimbabwe. Samples of water were collected at a depth of 20 - 50 cm in sterile 500 mL thermos flasks at four different sites. Temperature, pH, electrical conductivity and total dissolved solids were measured in-situ using a digital meter (PH-686, Juanjuan, China). These physicochemical parameters varied from 71 - 82°C, 7.1 - 7.8, 1098 - 2058 μs cm^{-1} and 1023 - 1029 mg L^{-1}, respectively. Samples were carried to the Biotechnology Laboratory, Department of Biotechnology, Chinhoyi University of Technology, Zimbabwe and preserved at 4°C.

4.3.3 Isolation and Screening of Cellulolytic Bacteria

Cellulolytic bacteria were isolated from hot spring water samples according to Kunasundari et al. (2016). One milliliter of a sample was serially diluted up to a dilution factor of 10^{-10}. A volume of 0.1 mL of the dilution (10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) was inoculated on carboxymethyl cellulose (CMC) agar: 1.36 g KH_2PO_4, 2 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 1 g $(NH_4)_2SO_4$, 0.01 g $FeSO_4 \cdot 7H_2O$, 3 g CMC, 1 g yeast extract and 15 g agar in 1 000 mL of distilled water. The inoculated plates were incubated at 37ºC for 5 days. After incubation, the plates were flooded with 0.1% Congo red for 20 minutes and counter-stained with 1 M NaCl for 15 minutes (Samira et al., 2011). Formation of a clear zone of hydrolysis around a colony indicated a positive test for cellulose degradation. Strains with the largest zone of clearance were isolated for repeated screening to obtain pure isolates as suggested by Naresh et al. (2019). Cellulolytic index was calculated using the following equation:

$$\text{Cellulolytic index} = \frac{\text{Diameter of clearance zone} - \text{Diameter of bacteria colony}}{\text{Diameter of bacteria colony}}$$

4.3.4 Morphological and Biochemical Identification of Cellulolytic Bacteria

Colony morphology of pure bacteria strains was checked using Gram staining. Biochemical characteristics, such as motility, citrate utilization, indole, urease, catalase and starch hydrolysis tests were conducted to further identify the isolates. The tests were performed according to the standard protocols in Bergey's Manual of Systematic Bacteriology (Garrity et al., 2004).

4.3.5 16S rRNA Sequencing of Cellulolytic Bacteria

4.3.5.1 Genomic DNA Extraction

Total genomic DNA was extracted from cellulolytic bacteria using a DNA extraction kit (Zymo Research, USA). Pure strains of bacteria were cultured overnight in LB broth at 37ºC. Thereafter, about 1.5 mL of each culture was transferred to a microcentrifuge tube and centrifuged at 13 000 rpm for 2 minutes. The supernatant was decanted and drained on tissue paper. The remaining pellet was resuspended in 200 µL of sterile water and 750 µL of lysis buffer was added. The contents were then mixed by repeated pipetting before transferring to ZR bashing bead lysis tubes. The tubes were vortexed at a maximum speed for 5 minutes, followed by centrifugation at 10 000 rpm for 1 minute. About 400 µL of the supernatant was transferred to a Zymo-spin IV spin filter in a collection tube and centrifuged at 7 000 rpm for 1 minute. The DNA binding buffer (1 200 µL) was added to the filtrate in a collection tube.

A total volume of 800 µL of the resultant mixture from each collection tube was transferred to a Zymo-spin IIC spin column in a collection tube and centrifuged at 10 000 rpm for 1 minute. The flow through was discarded from the collection tube and the previous step was repeated. Addition of 200 µL DNA pre-wash buffer to the Zymo-spin IIC spin column in each new collection tube preceded centrifugation at 10 000 rpm for 1 minute. After spinning, 500 µL of

DNA wash buffer was added to each Zymo-spin IIC spin column and centrifuged at 10 000 rpm for I minute. Each spin column was transferred to a clean 1.5 mL microcentrifuge tube and 100 µL of DNA elution buffer was added to the column matrix, followed by centrifugation at 10 000 rpm for 30 seconds to elute DNA. Quantification of DNA was done by resolving the DNA on 0.8% agarose gel alongside O'GeneRuler 1 kb DNA ladder (Thermo Scientific, USA). Ultra-pure DNA was stored at -20°C until further use.

4.3.5.2 PCR Amplification and Sequencing

The protocol for 16S rRNA sequencing was previously described by Kamusoko et al. (2021a). The 16S rRNA gene was amplified by PCR in a Peltier Thermal Cycler (PTC-200, Bio-Rad/MJ Research, USA) using universal primers: 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (Inqaba Biotech, Pretoria, South Africa). The reaction mixture of 25 µL for each strain consisted of 14.5 µL Maxima Hot Taq^{TM} PCR Master Mix (2X) (Fementas Life Sciences, Lithuania), I µL bacteria genomic DNA, 0.5 µL each of the two 25 µM primers and 8.5 µL nuclease free water. Amplification conditions were as follows: 5 minutes for 95°C for initial denaturation of DNA, followed by 35 cycles of denaturation at 95°C for 30 seconds, primer annealing at 60 °C for 30 seconds, strand extension at 72°C for 1 minute 30 seconds, final extension at 72 °C for 15 minutes and termination at 4°C.

After amplification, the PCR products were electrophoretically seperated on 1.5% agarose gel in 1 x TBE buffer (pH 8.0) at 100 volts for 1 hour 30 minutes. The DNA was stained and visualized using GR Green Nucleic Acid stain, and photographed using Vilber Lourmat Gel Documentation System (Infinity ST5, Fisher Biotech, Australia). The banding patterns of the 16S rRNA gene fragments were compared with O'GeneRuler 1 kb DNA ladder (Thermo Scientific, USA) to

obtain the size of the fragments. The amplified fragments were excised from the agarose gel into 1.5 mL microcentrifuge tubes and stored at -20°C. The PCR amplicons were sequenced at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa).

4.3.6 Preparation of Crude Enzyme Extract from Cellulolytic Bacteria

Bacteria strains were grown in 1% CMC broth for production of crude enzymes (Yang et al., 2014). A bacterial isolate was inoculated into a conical flask containing 150 mL of Luria Bertani (LB) broth + 1% CMC sterile medium and SmF was performed by incubating in a shaker at 37°C for 48 hours at an agitation speed of 160 rpm. After incubation, the broth was centrifuged at 10 000 rpm for 10 minutes. The supernatant was collected and preserved at 4°C as the crude enzyme extract.

4.3.7 Quantification of Crude Enzyme Activity

Enzyme activity was estimated by the carboxymethyl cellulase (CMCase) activity assay (Yang et al., 2014). About I mL of the culture supernatant was mixed with 1 mL of 1% CMC in 0.05 M sodium acetate buffer (pH 5.5) and incubated in a water bath at 65ºC for 5 minutes. The reaction was terminated by adding 1.5 mL of dinitrosalicylic acid (DNS), subsequently boiled at 98ºC in a water bath for 15 minutes and cooled on ice. Reducing sugars were determined by measuring absorbance at 540 nm using a spectrophotometer (UV-1900i, Shimadzu, Germany). One unit (U) of cellulase activity was measured as the amount of enzyme required to release 1 mole of glucose per minute under standard assay conditions and it was expressed as units per milliliter (U mL^{-1}).

4.3.8 Optimization of Fermentation Conditions for Cellulase Production

Cellulase production of the bacteria strains was optimized for carbon source, nitrogen source, incubation time, temperature and pH (Hussain et al., 2017). Incubation time was determined by

inoculating 5 mL of overnight grown bacteria in 100 mL of 1% CMC, followed by incubation at 37ºC for 24 hours and 48 hours at 120 rpm in a shaker. The effect of a carbon source on cellulase activity was evaluated by inoculating bacteria in 1% CMC medium (pH 7) at four different concentrations (1%, 2%, 3% and 4%). Bacteria strains were incubated in 1% CMC media containing three different nitrogen sources (1% peptone, 1% yeast extract and 1% urea) to investigate the nitrogen requirements for cellulase production.

Cellulase activity was optimized for pH by growing bacteria at four different pH conditions (3.0, 5.0, 7.0 and 9.0). The pH was adjusted using 1 M HCl and 1M NaOH. Production media were then incubated at 37ºC for 24 hours. Temperature was studied by incubating bacteria culture in 1% CMC broth at various temperatures (20ºC, 25ºC, 30ºC, 35ºC, 40ºC, 45ºC and 50ºC). After incubation, the wort was centrifuged at 10 000 rpm for 10 minutes. The cellulase activity was measured by adding 0.1 mL of the cell free supernatant to 0.1 mL of 1% CMC in 10 Mm sodium phosphate buffer at 37ºC for 60 minutes. About 1.5 mL of DNS reagent was added to the mixture, boiled for 15 minutes and cooled in ice to terminate the reaction. The optical density was measured at 540 nm using a spectrophotometer (UV-1900i, Shimadzu, Germany).

4.3.9 Data Analysis

All experiments were conducted in triplicate and statistical data were presented as mean ± standard deviation using Microsoft Excel. The 16S rRNA sequences were quality edited using BioEdit version 7.2 Software (Hall, 1999). Sequence relationships were determined by comparing with known representative sequences in the GenBank database by multiple sequence alignment using Basic Alignment Search Tool (BLAST) algorithm of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov).

4.4 Results and Discussion

4.4.1 Isolation and Screening of Cellulolytic Bacteria

Sustained efforts have been made to screen novel cellulolytic strains, such as *Anoxybacillus flavithermus*, *B. subtilis* and *B. amyloliquefaciens* for potential use in biofuel production (Hussain et al., 2017). Hot springs are a potential source of bacteria with high cellulolytic activity due to their high temperatures and alkaline conditions. Growing bacteria on CMC medium, followed by Congo red staining is believed to be a rapid and simple method for isolation and screening of cellulolytic bacteria (Yang et al., 2014). Out of the total isolates grown on CMC medium, only 11 were found to produce large clear zones around colonies (Table 4.1). The ability of bacteria to produce clear zones indicates their potential to degrade cellulose. Cellulolytic index of the studied bacteria isolates varied from 0.1 - 2.0. Isolate LB-6 showed the highest cellulolytic index whereas LB-9 had the lowest index.

Among the 11 isolates, only three (LB-4, LB-6 and LB-8) reported high cellulolytic activity, with cellulolytic indices of 1.8, 2.0 and 1.5, respectively. Hence, LB-4, LB-6 and LB-8 were regarded as active cellulose degraders. The strains were subjected to morphological and biochemical identification, 16S rRNA sequencing, and evaluated for cellulase production. The maximum index (1.32) of cellulolytic bacteria screened from termites' guts (*Cryptotermes* sp.) was lower than the indices of LB-4, LB-6 and LB-8 (Peristiwati et al., 2018). The isolates showed lower cellulolytic indices than those isolated from tropical mangrove soil (Naresh et al., 2019). Ferbiyanto et al. (2015) reported higher cellulolytic indices in the range of 0.75 - 2.5 from termite gut of worker *Macrotermes gilvus*.

Table 4.1 Cellulolytic activity of bacterial isolates from hot springs

Isolate code	Clear zone diameter (mm)	Colony diameter (mm)	Cellulolytic index
LB-1	4.0	2.0	1.0
LB-2	2.0	1.0	1.0
LB-3	3.0	2.0	0.5
LB-4	2.8	1.0	1.8
LB-5	2.0	1.0	1.0
LB-6	3.0	1.0	2.0
LB-7	4.5	3.0	0.5
LB-8	5.0	2.0	1.5
LB-9	1.0	0.5	1.0
LB-10	4.5	2.0	1.3
LB-11	1.0	0.5	1.0

4.4.2 Morphological and Biochemical Identification of Bacteria Isolates

Figure 4.1 shows the morphological characteristics of the bacterial colonies. Using Gram's reaction, microscopic observation showed all the strains (LB-4, LB-6 and LB-8) to stain blue. This revealed the strains to be Gram positive. Microscopically, the strains were rod-shaped and found to belong to a group of motile bacillus.

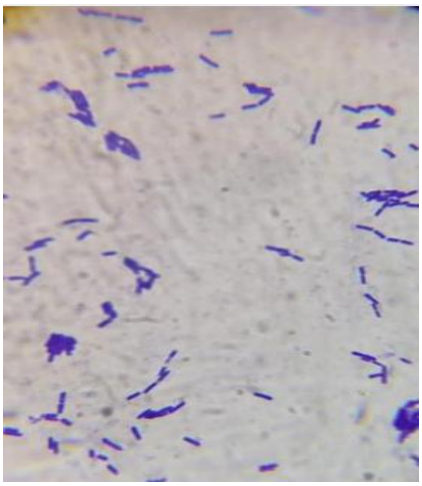

Figure 4.1 Gram reaction of the bacterial strains

Gram staining and biochemical characteristics of the three bacteria strains are shown in Table 4.2. According to morphological and biochemical characteristics, the strains belong to the genus *Bacillus. Bacillus* spp., with high cellulolytic potential have been previously isolated from hot springs and other harsh ecological habitats (Adiguzel et al., 2009; Yang et al., 2014; Hussain et al., 2017; Naresh et al., 2019).

Table 4.2 Gram staining and biochemical characteristics of bacteria isolates

Isolate	Gram's reaction	Motility	Catalase	Indole	Urease	Citrate	Starch hydrolysis	Triple sugar
LB-4	+	+	+	-	+	+	+	+
LB-6	+	+	+	-	+	+	+	-
LB-8	+	+	+	-	+	+	+	-

+: positive test -: negative test

4.4.3 16 rRNA Sequencing of Cellulolytic Bacteria

The 16S rRNA gene is a highly conserved region that encodes the 30S ribosomal subunit of bacteria and archaea. Analysis of 16S rRNA is a precise and suitable way for molecular taxonomy of prokaryotes (Ferbiyanto et al., 2015; Kamusoko et al., 2021a). Amplification of 16S rRNA sequences of cellulolytic bacteria with universal primers 27F/1492R confirmed DNA amplicons of 1.5 kb in size (Figure 4.2). The findings are supported by previous work of Yang et al. (2014) and Ferbiyanto et al. (2015).

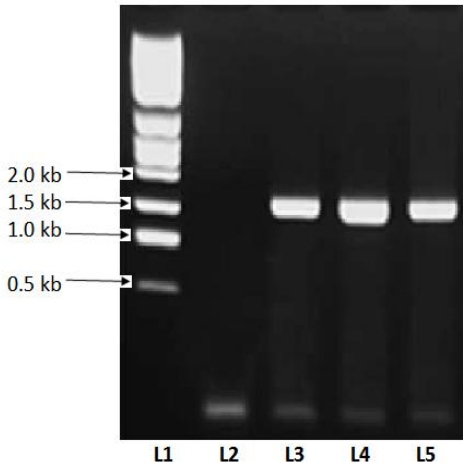

Figure 4.2 16S rRNA amplicons from cellulolytic bacteria isolated from hot springs. Lane 1, 1 kb molecular weight marker; lane 2, negative control; lane 3, strain LB-4; lane 4, strain LB-6; lane 5, strain LB-8

Homology analysis of the strains reported a strong degree of similarity (>98%) between isolates LB-4, LB-6 and LB-8, and strains in the NCBI GenBank of *Bacillus subtilis*, *Bacillus* sp., and *Bacillus licheniformis,* respectively (Table 4.3). The BLAST algorithm with a similarity threshold of 98.65% is used to compare the16S rRNA sequences with potential phylogenetic neighbors (Kim and Chun, 2014). Previous studies have demonstrated that strains belonging to

the genus *Bacillus* are capable of producing cellulolytic enzymes (Adiguzel et al., 2009; Yang et al., 2014; Hussain et al., 2017).

Table 4.3 Affiliations of 16S rRNA gene sequences of cellulolytic bacteria isolated from hot springs

Isolate	Query cover	*e* Value	Maximum identity	Identity in BLAST	Accession number
LB-4	100%	0	99.13%	*Bacillus subtilis*	CP032865.1
LB-6	100%	0	98.26%	*Bacillus* sp.	MF355364.1
LB-8	100%	0	98.91%	*Bacillus licheniformis*	MT642943.1

4.4.4 Optimization of Fermentation Conditions for Cellulase Production

4.4.4.1 Effect of Incubation Time on Cellulase Activity

Figure 4.3 compares the cellulolytic activity of LB-4, LB-6 and LB-8 strains after 24 hours and 48 hours of incubation. The study indicates that LB-4 and LB-6 attained an optimum cellulase productivity of 0.14 U mL^{-1} and 0.20 U mL^{-1}, respectively, after 24 hours. However, strain LB-8 maintained a steady state of CMCase activity (0.13 U mL^{-1}) between 24 hours and 48 hours.

Similarly, Hussain et al. (2017) reported maximum cellulase activity from *B. subtilis* BTN7A, *B. amyloliquefaciens* SA5 and *B.megaterium* BMS4 after 24 hours, whereas cellulase productivity of *B. flavithermus* BTN7B did not change after 24 hours and 48 hours. Contrary to our findings, Pramanik et al. (2021) reported maximum cellulase activity after 72 hours from *B. pseudomycoides*. A decline in cellulase productivity with increasing incubation time may be attributed to exhaustion of nutrients and accumulation of toxins (Hussain et al., 2017).

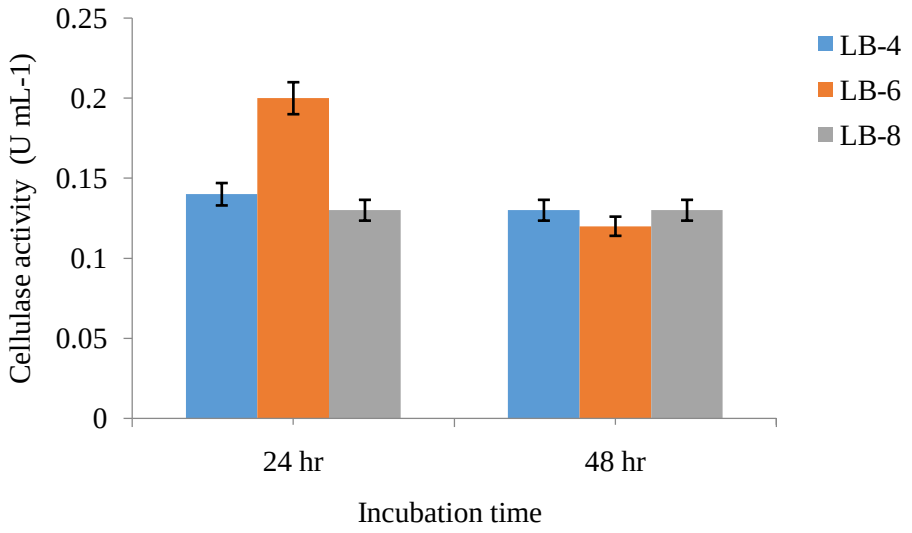

Figure 4.3 Effect of incubation time on cellulase activity. Error bars represent standard deviations (n = 3)

4.4.4.2 Effect of pH on Cellulase Activity

Cellulolytic bacteria occupy habitats with diverse pH conditions. The pH determines how specific substrates are metabolized and affects the cellulolytic potential of bacteria. This study examined how cellulolytic bacteria performed under varying pH from 3.0 to 9.0. All test strains (LB-4, LB-6 and LB-8) reported the lowest cellulase activity (0.15 - 0.16 U mL^{-1}) at pH 3, whereas the highest cellulase activity (0.17 - 0.19 U mL^{-1}) was found at pH 7 (Figure 4.4). These findings corroborate with cellulase activity of *B. Pseudomycoides* (Pramanik et al., 2021), *B. subtilis* BTN7A, *B. amyloliquefaciens* SA5, *B.megaterium* BSM4, *B. flavithermus* BTN7B (Hussain et al., 2017) and *B. coagulans* (Romsaiyud et al., 2009).

However, Acharya and Chaudhary (2011) reported an optimum cellulase activity at pH 9 from *B. subtilis* isolated from hot springs, while Yang et al. (2014) observed maximum cellulase activity at pH 5.5 from *B. subtilis* BY-2 isolated from pig's intestine. Varying pH of the fermentation medium alters ionic properties of the enzyme's active site causing damage to the three-dimensional structure and reducing its affinity towards the substrate (Naresh et al., 2019).

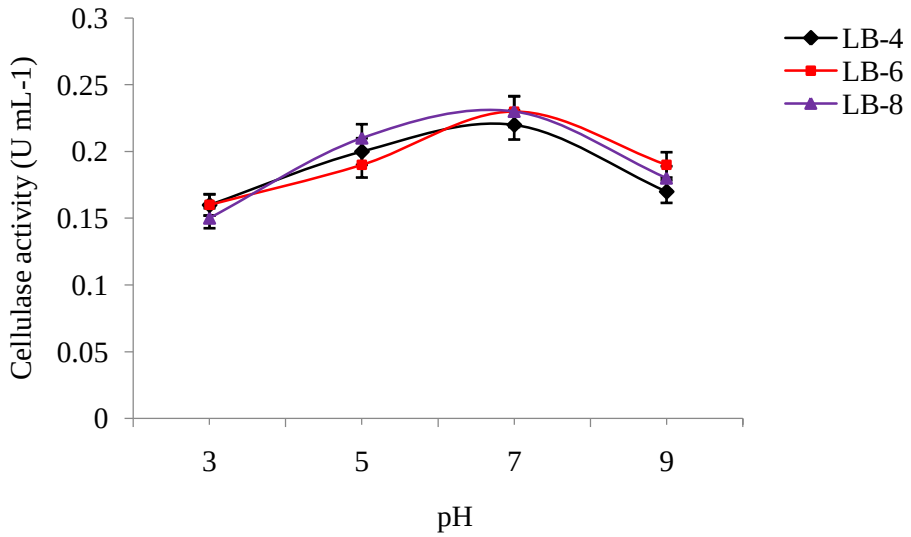

Figure 4.4 Effect of pH on cellulase activity. Error bars represent standard deviations (n = 3)

4.4.4.3 Effect of Carbon Source on Cellulase Activity

In the present study, the effect of different concentrations of the carbon source on cellulase fermentation was investigated. Results indicated that carbon utilization efficiency of the strains decreased with rising CMC concentration (Figure 4.5). The maximum cellulase activity (0.11 U mL^{-1}) was reported from isolate LB-6 at 1% CMC. This shows that strain LB-6 could efficiently utilize CMC as a carbon source compared to other strains. Isolate LB-8 exhibited the lowest cellulase activity of 0.02 U mL^{-1} at 4% CMC. Overall, the cellulase activity of all the strains (LB-4, LB-6 and LB-8) was found to be optimum at 1% CMC concentration.

This research is supported by several authors (Sakthivel et al., 2010; Behera et al., 2016; Islam and Roy, 2018). According to Yang et al. (2014), cellulases are inducible enzymes that require cellulose-containing substrate as a carbon source for fermentation. The most favorable carbon source for cellulase production is CMC medium (Das et al., 2010).

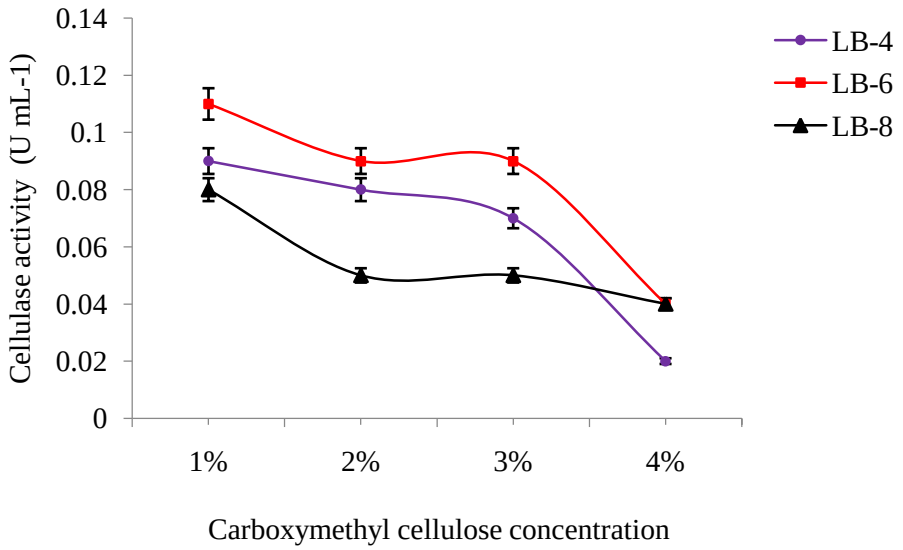

Figure 4.5 Effect of carbon source on cellulase activity. Error bars represent standard deviations (n = 3)

4.4.4.4 Effect of Nitrogen Source on Cellulase Activity

Nitrogen plays a significant role as the main building block for proteins and nucleic acids in microbial cells. Effect of various nitrogen sources on SmF of isolated bacteria strains was evaluated (Figure 4.6). All the strains optimally produced cellulases whilst utilizing 1% yeast extract as the sole nitrogen source. Strain LB-8 reported the highest cellulase activity (0.19 U mL^{-1}) suggesting that it could efficiently exploit yeast extract as a nitrogen source. Yeast extract is enriched with nitrogen, vitamin B complex and trace mineral nutrients that could have promoted cellulase production (Jach et al., 2022). The results are in agreement with the works of Sakthivel et al. (2010) and Pramanik et al. (2021) who obtained maximum cellulase activity from yeast extract.

Peptone at a concentration of 1% had better cellulase activity than 1% urea. Peptone was found to be an effective source of nitrogen for cellulase production by Lugani et al. (2015). However, all the strains (LB-4, LB-6 and LB-8) recorded the lowest cellulase activity when 1% urea was

used as a nitrogen source, with LB-4 having the lowest cellulase activity of 0.01 U mL^{-1}. Degradation of urea might have lowered the pH of the medium, thus affecting cellulase production (Yang et al., 2014). Contrarily, ammonium sulfate which is an inorganic salt was found to be the best source of nitrogen for cellulase production. This could be due to direct incorporation of ammonium sulfate in protein synthesis (Sethi et al., 2013).

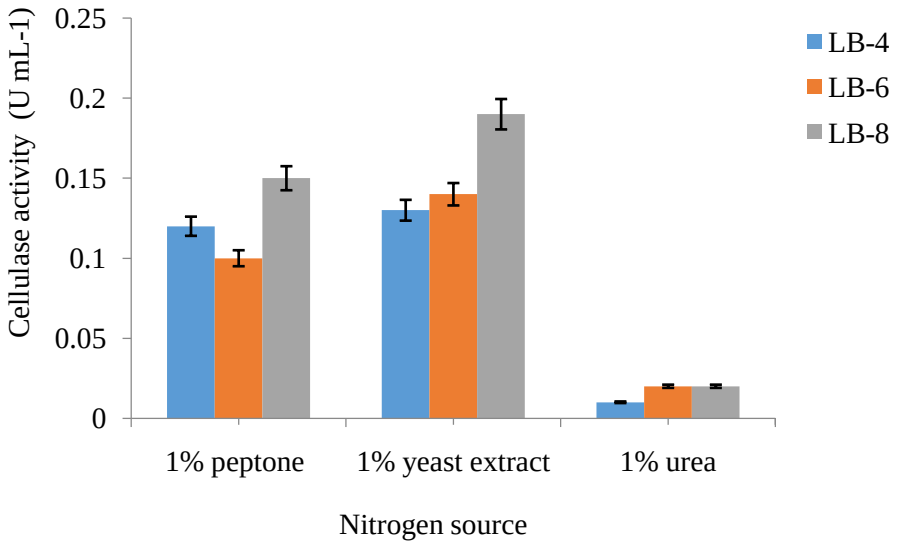

Figure 4.6 Effect of different nitrogen source on cellulase activity. Error bars represent standard deviations (n = 3)

4.4.4.5 Effect of Temperature on Cellulase Activity

Enzyme activity of the isolates was studied at various temperatures ranging from 20 to 50 ºC. All the isolates showed effective cellulase production from 35 to 45°C with the highest activity at 40°C (Figure 4.7). Temperature might have affected exoenzyme secretion by altering the physical properties of the microbial cell membrane. Isolate LB-6 reported the highest cellulase activity of 0.32 U mL^{-1}, whereas the lowest activity of 0.01 U mL^{-1} was observed from isolates LB-4 and LB-8. The optimum temperature of 40°C was also reported from cellulase fermentation of several bacteria species (Sethi et al., 2013; Gozanet al., 2018). Pramanik et al.

(2021) observed the highest cellulase activity (2.24 U mL^{-1}) from fermentation of *B. pseudomycoides* at 40°C. Generally, the most frequently used temperature for bacteria fermentation is 37ºC (Hussain et al., 2017; Gozan et al., 2018).

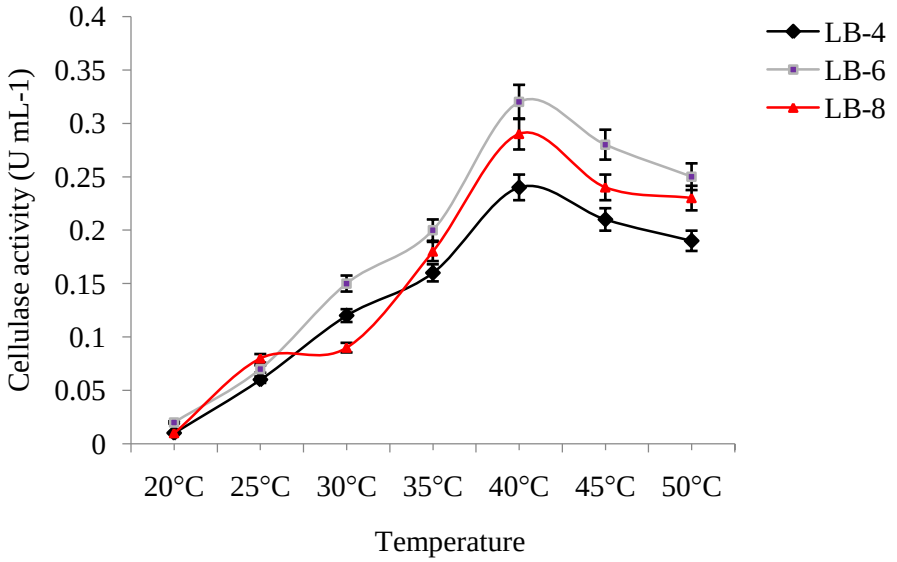

Figure 4.7 Effect of temperature on cellulase activity. Error bars indicate standard deviations (n = 3)

4.5 Conclusion

Hot springs are an ideal source of many cellulolytic bacteria with a wide range of applications. In this study, three bacteria strains (LB-4, LB-6 and LB-8) with high cellulolytic activity were isolated on culture-enrichment medium. All the strains were found to belong to a genus of motile *Bacillus* on the basis of morphological and biochemical characteristics. Using 16S rRNA homology analysis, strains LB-4, LB-6 and LB-8 were identified as *B. subtilis*, *Bacillus* sp., and *B. licheniformis*, with similarities of 99.13%, 98.26% and 98.91% in the NCBI GenBank, respectively.

The optimum fermentation conditions of the strains for cellulase production were at pH 7, 1% yeast extract, 1% CMC and 40ºC after 24 hours. The three bacteria isolates can be translated to

commercial production under these optimal fermentations conditions. However, more research still needs to be done to further optimize the strains for inoculum size, medium additives, aeration rate, etc and evaluate their ability to degrade crop residues in biogas production.

CHAPTER 5: CHARACTERIZATION OF LIGNOCELLULOSIC CROP RESIDUES FOR POTENTIAL BIOGAS PRODUCTION IN ZIMBABWE

5.1 Introduction

Valorization of lignocellulose through generation of biofuels is a rising opportunity to reduce energy crisis and global warming (Soni et al., 2019; Malode et al., 2021). A worldwide deficiency of petroleum is expected by 2070 - 2080, while GHG emissions from petroleum are estimated to rise to 43 billion metric tonnes by 2040 (Malode et al., 2021). Biogas production is a promising technology that can replace petroleum fuels. Biogas is a multipurpose fuel for transportation, heat and power generation. It is mainly produced by AD of organic matter.

The AD can supply clean, readily available and renewable energy to rural communities in the developing world (Khanal et al., 2021). It is an efficient and established technology which couples biofuel production with sustainable waste management (Khanal et al., 2021; Malode et al., 2021). A rich-digestate produced during AD can be used as fertilizer or substrate for mushroom production (Dar et al., 2021; Khanal et al., 2021). The AD performance is affected by biochemical composition of the feedstock (Bohutskyi et al., 2014; Kamusoko et al., 2019). Therefore, it is a standard practice to characterize feedstocks for total solids (TS), volatile matter (VM), COD, N, P, lignin, hemicellulose and cellulose (Angelidaki et al., 2009).

Lignocellulose is the most abundant and economical natural resource on earth (Qian, 2014; Ge et al., 2018). A huge portion of this biomass comes from harvesting and processing of agricultural crops (Soni et al., 2019). The biomass is known as crop residues. The world annual production of crop residues is around 200 billion tonnes. Zimbabwe contributes about 7.8 Mt to the global

annual crop residues production (Kamusoko et al., 2021b). Crop residues are mostly produced by cereal crops (e.g. rice, wheat, maize, millet) and sugar cane. They include straws, husks, bran, bagasse, molasses, etc (Bhuvaneshwari et al., 2019). Legumes, sugar crops, oil crops and fibers also generate substantial amounts crop residues (Cherubini et al., 2018).

Crop residues are second generation feedstocks and are considered to be cheaper than crude petroleum (Ge et al., 2018). They are rich in cellulose which can serve as an energy source (Sukhesh & Rao, 2018). Crop residues are non-edible with limited impact on food production (Malode et al., 2021). For these reasons, crop residues hold much promise as a feedstock for biogas production.

Crop residues are made up of 85 - 90% complex polymers of cellulose, hemicelluloses and lignin (Pasangulapati et al., 2012; Jung et al., 2015). They are composed of 40 - 50% cellulose, 15 - 25% hemicelluloses and 20 - 25% lignin (Zing et al., 2017). The actual composition and nature of crop residues differ widely depending on type, harvesting and storage techniques of thhe biomass (Pasangulapati et al., 2012; Soni et al., 2019). Lignin is a complex heteropolymer of phenolic monomer units (Jung et al., 2015). It is the portion that is resistant to microbial degradation in lignocellulose (Bajpai, 2016). Hemicelluloses are branched heteropolysaccharides of different pentose and hexose sugars (Bajpai, 2016).

Cellulose is a linear homopolymer of D-glucose units linked by β-1,4-glycosidic bonds. The elemental fibrils of cellulose are joined together by hydrogen bonds and van dar Waals forces to create microfibrils that are covered by hemicelluloses and lignin (Sun, 2015). The heterogeneity and complexity of lignocellulose is the main impediment in processing crop residues into viable

products. Pretreatment is an efficient option that can improve the hydrolysis of crop residues (Kasinath et al., 2021; Naik et al., 2021).

In order to assess the potential of cellulolytic bacteria isolated in the preceeding Chapter for pretreatment of plant biomass, it was relevant to understand the actual composition of the locally available crop residues (Chapter 5). The actual composition of crop residues differs widely according to biomass type and geographical location. This may affect the biodegradability and BMP of crop residues. Although literature widely reports on characterization of biomass, the composition of locally abundant crop residues is not yet fully understood. Hence, the potential of these feedstocks for biogas production remains untapped. The effect of biological pretreatment on BMP of crop residues was investigated in the next Chapter.

5.2 Specific Objectives

The specific objectives were to:

(a) Determine the physicochemical composition of crop residues i.e pH, total alkalinity (TA), VM, total suspended solids (TSS) and total nitrogen (TN).

(b) Assess the lignocellulosic content of crop residues (cellulose, hemicelluloses and lignin).

5.3 Materials and Methods

5.3.1 Experimental Design

A CRD with three replications was used to design experiments to determine the proximate composition of feedstocks. Each feedstock was assigned to eight treatment factors. The treatment factors were pH, TA, VM, TSS, TN, cellulose, hemicelluloses and lignin.

5.3.2 Sample Collection

Three types of crop residues, namely maize stover, wheat straw and soybean straw were chosen for this study. These are the most abundant based on calculated theoretical availability in Zimbabwe (Jingura & Matengaifa, 2008). Dry crop residues were collected after harvesting crops from local farms in Chinhoyi, Zimbabwe. Soybean straw was obtained from a soybean field near Alaska Mine. Maize stover and wheat straw were collected from Chinhoyi University of Technology farm. Samples were shredded into small pieces using a hammer mill, sieved into 1 mm meshes using a standard testing sieve and kept in air tight containers.

5.3.3 Determination of Physicochemical Characteristics of Crop Residues

The biochemical composition of crop residues was determined using standard methods (American Public Health Association (APHA), 2005). Pieces of equipment were calibrated and equilibrated using standard procedures from manufacturers. The pH was determined in 0.5 M $CaCl_2$ using a digital ATC pH meter. Total alkalinity (TA) was measured using the titration method and VM content was determined by gravimetric analysis. Total nitrogen (TN) content was measured at a wavelength of 240 nm using UV-Vis spectroscopy (721-100, Huanghua, China). Total suspended solids (TSS) were determined using a standard electrode (711, Victoria, Australia).

5.3.4 Determination of Lignocellulosic Composition of Crop Residues

5.3.4.1 Preparation of Extractive-free Plant Biomass

Extractive-free samples were obtained using Soxhlet apparatus (Jung et al., 2015). Approximately 3 g of plant biomass was put in a thimble filter of the Soxhlet system, followed by incubation in ethanol-toluene (1:2) solvent for 4 hours. The plant material was filtered

through No. 1 Whatman filter paper. Toluene was removed by rinsing the thimble filter and the sample with 80% ethanol (1:10 w/w). Soxhlet extraction was repeated until ethanol in the siphon became colorless. The sample was extracted again using distilled water, filtered and washed with boiling water to remove ethanol. The sample was oven-dried at 100°C and stored in a polythene bag.

5.3.4.2 Determination of Lignin Composition

Lignin content of extractive-free samples was determined by adapting the standard method (Moreira et al., 2020). About 300 mg of the sample was weighed and hydrolyzed in 3 mL of 72% H_2SO_4 in an Erlenmeyer flask. The flask was incubated in water bath at 30°C for 1 hour with intermittent stirring. The hydrolyzate was diluted with 84 mL of distilled water, autoclaved at 121°C for 45 minutes and left to cool at room temperature. The sample was then filtered using No. 1 Whatman filter paper. The residue remaining in the filter paper was rinsed with distilled water and dried in an oven at 105°C until a steady weight was obtained.

Thereafter, the residue was used for gravimetric determination of acid-insoluble lignin. Approximately 2 mL of H_2SO_4 was mixed with 2 mL of the primary filtrate and the mixture was filtered. The filtrate was used to estimate acid-soluble lignin by measuring the absorbance of the solution at 205 nm using a UV-Vis spectrophotometer (721-100, Huanghua, china).

5.3.4.3 Extraction of Holocellulose

Delignification using sodium chlorite was done to extract holocellulose from extractive-free materials (Jung et al., 2015). Approximately 500 mg of the extractive-free sample was weighed into a conical flask and 200 mg of sodium chlorite was added. About 30 mL of water and 0.04 mL of 10% peracetic acid (pH 3.5) was added. The mixture was incubated in water bath at 85 ºC

for 30 minutes and the step was repeated 7 - 8 times. The sample was filtered through No. 1 Whatman filter paper and rinsed with hot water. The residue remaining on filter paper was dried in an oven at 100ºC and used as holocellulose.

5.3.4.4 Determination of Hemicelluloses and Cellulose Composition

Cellulose in holocellulose was estimated using a standard protocol (Jung et al., 2015). About 300 mg of holocellulose was weighed into a beaker and 3 mL of 17.5% NaOH was added. The sample was left for 3.5 minutes and pulverized with a glass rod for 5 minutes. After flattening, the sample was incubated at 20ºC in a water bath for 20 minutes. The sample was diluted with 3 mL of distilled water, stirred for 1 minute and allowed to settle for 5 minutes. The sample was then filtered and washed with 54 mL of distilled water. The rotary evaporator (QYRE-2A-2L, Shanghai, China) was switched off for 4 minutes and 2.4 mL of acetic acid was added to the sample followed by rinsing with hot distilled water. The sample was then heated in an oven at 100ºC until a constant weight of cellulose was reported. The weight of hemicelluloses was estimated as the difference between the weight of holocellulose and cellulose.

5.3.5 Statistical Analysis

All analyses were conducted in triplicate and the results were expressed as mean values and standard deviations. The data were subjected to one-way analysis of variance (ANOVA) at $\alpha = 0.05$ to test for significant differences among means using Microsoft Excel.

5.4 Results and Discussion

5.4.1 Physicochemical Characteristics of Crop Residues

Characterization of feedstocks is an essential step when converting biomass into biogas. Feedstock composition affects digester stability and methane yield (Angelidaki et al., 2009; Kamusoko et al., 2019). The physicochemical characteristics of crop residues are shown in Table 5.1. The values for VM and TN were expressed in terms of percent dry weight. Wheat straw, maize stover and soybean straw were variable in physicochemical composition. This variability may be due to different agronomic, handling and storage conditions from which the crop residues were collected (Danish et al., 2015).

The pH is a critical factor that affects metabolic growth of microorganisms. In this study, all the crop residues were acidic in nature, with pH varying from 5.3 - 5.5. Wheat straw reported the lowest pH while soybean straw recorded the highest pH. The pH values fall within the range 5.3 - 6.8 (Sahito et al., 2013). Contrarily, Almomani & Bhosale (2020) reported a close to neutral pH of 6.95 for wheat straw, while a pH of 7.5 was recorded from maize stover (Lizasoain et al., 2017). The ideal pH range for AD is 6.6 - 7.4. Neutral pH is favorable as it supports metabolic activity and prevents accumulation of VFAs (Jain et al., 2015). Animal manure can provide buffering capacity if codigested with crop residues (Sahito et al., 2013).

Total alkalinity (TA) ranged from 232.7 - 448.3 mg L^{-1}, of which the highest and lowest values were reported from maize stover and wheat straw, respectively. Typically, TA for crop residues ranges from 100 - 1 350 mg L^{-1} (Sahito et al., 2013). Higher TA of 1 050 mg L^{-1} was reported from wheat straw (Almomani & Bhosale, 2020). High alkalinity improves the buffering capacity and maintains pH at optimum (Almomani & Bhosale, 2020).

The VM is an important parameter for evaluating AD and it is directly related to biogas production (Jingura & Kamusoko, 2017; Pan et al., 2021). The VM was reported in the range 74.1 - 78.1%, of which soybean straw showed the highest content. The values are close to those reported by Danish et al. (2015) and Tan et al. (2019). Lower VM of 63.2% was observed from wheat straw (Sahito et al., 2013). According to Kumar et al. (2018), VM of wheat straw can range from 87.8 - 92.7%. Volatile matter content reported from maize stover was significantly higher than our findings (Lizasoain et al., 2017). The VM content of soybean straw was comparably lower than the values from literature (Kovacic et al., 2017; Xiong et al., 2020).

Total nitrogen (TN) content varying from 3.1 - 8.2% was typically high. This may result in release of excess NH_3 that can inhibit AD and lower biogas yield. Wheat straw reported the highest TN content, whereas maize stover reported the lowest content. Our TN content exceeded most values from established literature (Xiong et al., 2020). Optimal amount of nitrogen promotes microbial growth and activity (Zhang et al., 2013). Total suspended solids (TSS) content ranged from 610.0 - 2 039.5 mg L^{-1}. The highest level of TSS was recorded from maize stover while the lowest level was reported from soybean straw.

Table 5.1 Physicochemical characteristics of crop residues (mean ± standard deviation)

Parameter	Wheat straw	Maize stover	Soybean straw
pH (pH units)	5.3 ± 0.00	5.4 ± 0.00	5.5 ± 0.00
Total alkalinity (mg L^{-1})	232.7 ± 2.20	448.3 ± 2.20	256.7 ± 2.20
Volatile matter (%)*	74.1 ± 0.02	74.8 ± 0.02	78.1 ± 0.02
Total nitrogen (%)*	8.2 ± 0.02	3.1 ± 0.03	5.1 ± 0.02
Total suspended solids (mg L^{-1})	1 943.1 ± 7.10	2 039.5 ± 2.80	699.95 ± 0.60

*Based on dry weight basis

5.4.2 Lignocellulosic Composition of Crop Residues

Cellulose, hemicelluloses and lignin are important structural components of plant cells. The structural compositions of crop residues are shown in Figure 5.1. The values are expressed in terms of percent weight on a dry matter basis. Crop residues varied widely regarding cellulose, hemicelluloses, lignin and extractives composition. This disparity may be due to differing plant types, varieties, maturities and sources (Passoth & Sandgren, 2019; Soni et al., 2019). Cellulose, hemicelluloses, lignin and extractives varied from 34.6 - 37.8%, 19.7 - 28.2%, 16.2 - 23.5% and 12.0 - 23.7%, respectively. Wheat straw showed the highest cellulose and hemicelluloses content of 37.8% and 28.2%, respectively. This indicates that wheat straw can be a more promising feedstock for biogas than maize stover and soybean straw.

Despite, all the crop residues showed their potential for biogas production by containing more than 30% cellulose. Cellulose content in crop residues is directly related to biogas yield. Approximately 80% of this cellulose can undergo AD to produce energy (Kamusoko et al., 2021b). Currently, biomass with high cellulose and hemicelluloses, and low lignin are believed to be more attractive for biofuel production (Jung et al., 2015). Cellulose content of crop residues was found within the range of 33 - 46% (Ho et al., 2014). However, cellulose content was lower than 40 - 50% reported for most agricultural residues (Zing et al., 2017; Soni et al., 2019). Hemicelluloses content was comparable to the range of 18 - 30% (Ho et al., 2014).

Soybean straw reported the lowest cellulose and hemicelluloses content of 34.6% and 19.7%, respectively. However, lignin content (23.5%) of soybean straw was found to be higher than the counterpart residues. High lignin content restrains AD because lignin is highly resistant to degradation (Bajpai, 2016). This lowers the hydrolysis rate and biogas production. For instance,

methane yield from lignocellulose was observed to decrease with more than 10% lignin content (Triolo et al., 2012). Despite, soybean straw remains a valuable resource for biogas production that still needs further investigation to surpass the barriers associated with high lignin content (Kovacic et al., 2017). Our lignin values are comparable to other authors (Ho et al., 2014; Sahito et al., 2013).

Extractives content ranging from 12 - 23.7% was considerably high. Maize stover had the highest extractives content while wheat straw reported the lowest extractives content. This could be the reason for high amounts of hemicelluloses and cellulose in wheat straw. Extractives are non-cellular components of plants, which include resin acids, fatty acids and sterols. High extractives content implies that there could be some hindrances in the extraction process (Nadiha & Jamilah, 2020). This calls for the removal of extractives before extraction of lignocelluloses.

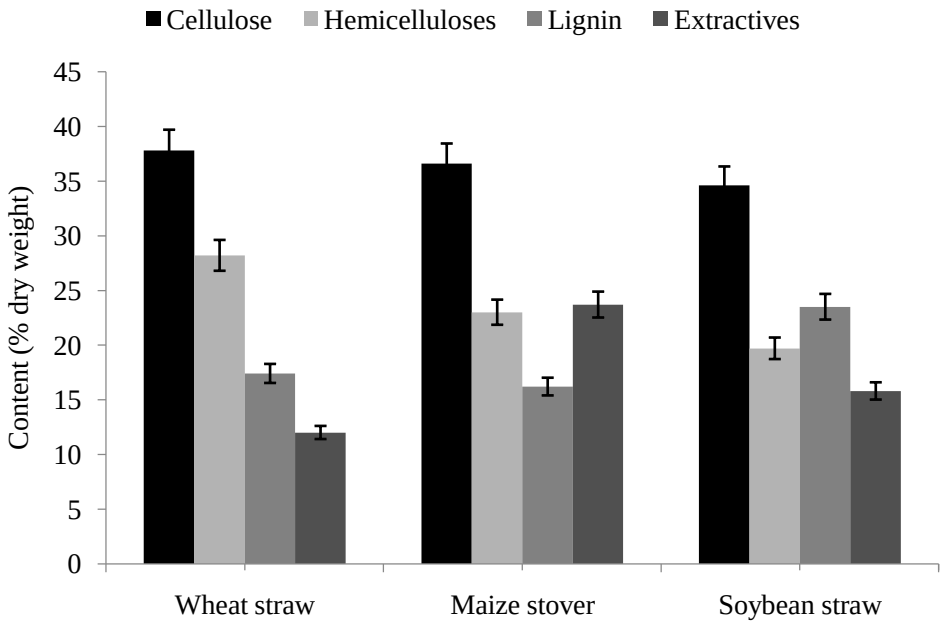

Figure 5.1 Lignocellulosic composition of crop residues based on percent dry weight. Error bars show the standard deviations of the three replicates

5.5 Conclusion

Proximate analyses provide useful information for modeling and designing the AD of crop residues. In this study, crop residues showed significant variation ($\alpha = 0.05$) amongst different proximate characteristics. Crop residues were acidic in nature with pH varying from 5.3 - 5.5 and have high TN content (3.1 - 8.2%). All crop residues have more than 30% cellulose indicating their potential use for biogas production. Cellulose, hemicelluloses and lignin content ranged from 34.6 - 37.8%, 19.7 - 28.2% and 16.2 - 23.5%, respectively.

Wheat straw had higher cellulose (37.8%) and hemicelluloses (28.2%) than maize stover and soybean straw. Soybean straw exhibited the highest lignin content of 23.5%. This study suggests that wheat straw could be a more valuable resource for biogas production as compared to maize stover and soybean straw. However, there are still many obstacles that must be surpassed for efficient AD of crop residues. Further research should be done to destruct the structural complexity of crop residues and develop optimization strategies for an AD system utilizing crop residues as feedstocks.

CHAPTER 6: EFFECT OF BIOLOGICAL PRETREATMENT USING CELLULOLYTIC BACTERIA CONSORTIUM ON BIOMETHANE PRODUCTION FROM CROP RESIDUES

7.1 Introduction

Rapid exhaustion of fossil fuels accompanied with continuous population growth has led to ever-increasing energy crisis in the world (Ali et al., 2017; Kong et al., 2018). The utilization of fossil fuels is estimated to increase by 105-fold by 2050, outweighing the supply from natural fossil reserves. At this rate, the natural oil reserves are likely to be depleted in less than 30 years, putting the global energy security at risk (Ali et al., 2017). In addition, concerns about global climate change have motivated to search for affordable, clean and sustainable sources of energy (Kamusoko et al., 2022b).

Crop residues, such as straws, husks, stover, stalks, baggase, hulls, cobs, etc, are potential sources of renewable energy that can replace fossil fuels (Jingura & Matengaifa, 2008; Zhong et al., 2011). Vast amounts of crop residues are generated each year in the world, but their valorization in a biorefinery is still undervalued. The annual crop residues production exceeds 5 billion tonnes the world over, with Asia, America, Europe, Africa and Oceania accounting for 47%, 29%, 16%, 7% and 1%, respectively (Shinde et al., 2022).

Zimbabwe is an agro-based economy, which produces large quantities of crop residues (7.8 Mt per year) from harvesting and processing of crops (Kamusoko et al., 2021b). Cereals are the major producers of crop residues, while stover and straw dominate crop residues production systems (Jingura & Matengaifa, 2008). Crop residues are mostly exploited via traditional

practices, such as use as animal feed, burying into the soil and burning (Zhong et al., 2011; Kamusoko et al., 2021b). Traditional management practices present some techno-economic problems and have widespread environmental impacts. For instance, burning of crop residues release GHGs, contributing to global warming and climate change, whilst burying of crop residues into the soil decreases crop yields through resurgence of diseases and degraded soil conditions (Zhong et al., 2011). In this context, development of cost-effective strategies to manage and recycle crop residues is needed.

The AD of crop residues is considered a well-established and efficient technology that amalgamates waste management and biofuel production (Achinas et al., 2017). In addition to biogas, a rich digestate substance that can be used to improve soil fertility and as a substrate for edible mushroom cultivation is produced (Sukhesh & Rao, 2018; Sindhu et al., 2019; Dar et al., 2021). However, crop residues are highly lignocellulosic due to the structural complexity and rigidity of the cellulose, hemicellulose and lignin matrix which is resistant to microbial degradation (Ali et al., 2017).

Holocellulose (cellulose and hemicellulose) is the most fermentable substrate in crop residues and about 80% of the cellulose is converted into biogas (Sukhesh & Rao, 2018). Lignin is the hard-to-digest material which is resistant to hydrolysis during AD (Zhong et al., 2011). The recalcitrant nature of lignin limits the use of crop residues for biogas production. The hydrolysis step is often rate-limiting in AD of crop residues (Yuan et al., 2012). This restriction may be overcome by deploying suitable pretreatment technologies to enhance methane production.

Several pretreatment options, including chemical, physical and biological are widely reported to improve biogas production (Abraham et al., 2020; Awogbemi & Kallon, 2022; Catherine &

Twizerimana, 2022). Biological pretreatment is more advantageous over the other pretreatment methods. It is considered to be a simple, low capital and energy venture, with minimum pollution to the natural environment. Furthermore, it does not produce toxic byproducts that may inhibit the AD process (Abraham et al., 2020; Awogbemi & Kallon, 2022). Literature extensively report on use of single strain systems for pretreatment of lignocellulose. However, this strategy is not in compliance with the natural degradation of cellulosic biomass, in which consortia of microbes work synergistically to deconstruct the substrate (Zhang et al., 2011).

Utilization of microbial cocultures or complex microbial systems has been posited to be a very competent strategy for pretreatment of crop residues. It can eliminate drawbacks of feedback regulation and metabolic suppression associated with single strain pretreatment (Zhang et al., 2011). Therefore, several authors have designed mixed microbial consortia yielding promising results in pretreatment of AD feedstocks. The application of crop residues pretreated using microbial consortia in AD significantly enhanced methane yield compared to untreated conditions (Bai et al., 2010; Zhang et al., 2011; Zhong et al., 2016; Kong et al., 2018).

Despite, most researchers reporting on use of single strain as a pretreatment strategy, microbial consortium system has been proposed to be a more effective method for cellulose degradation. However, there is limited information on the feasibility of microbial consortium for pretreatment of crop residues to improve biogas production. In this Chapter, a microbial consortium with high cellulolytic activity was constructed using thermotolerant bacteria isolated from local hot springs. The bacteria strains were identified using morphological, biochemical and molecular methods (Chapter 4). The microbial consortium was investigated for its ability to improve the hydrolysis of crop residues that were initially characterized in Chapter 5. Lastly, the BMP of the pretreated agricultural wastes was evaluated in this study.

6.2 Specific Objectives

The specific objectives were to:

(a) Develop a microbial consortium from cellulolytic bacteria isolated from hot springs.

(b) Pretreat crop residues using a consortium of bacteria.

(c) Determine the chemical characteristics of pretreated crop residues.

(d) Produce biomethane from pretreated crop residues.

6.3 Materials and Methods

6.3.1 Experimental Design

A CRD layout was used in this study. Two treatments were assigned to each of the three crop residues during biological pretreatment: (a) treatment with a bacteria consortium; and (b) control (without inoculation). Three batch setups were designed for the AD of crop residues. Two treatments were allotted to each experimental set up: (a) pretreated with a bacteria consortium; and (b) non-treated crop residues. A batch experimental set up containing inoculum only was included. Each experimental setup was replicated thrice.

6.3.2 Raw Materials

Maize stover, wheat straw and soybean straw were collected in clean polythene bags from crop fields located in Chinhoyi, Zimbabwe. Crop residues were air-dried at room temperature and utilized as substrates for pretreatment using microbial consortium. The residues were ground into small pieces using a hammer mill. The final powder was obtained by passing through a 1 mm pore size standard testing sieve. The powder samples were then kept in air tight containers until further use. The initial characterization of untreated crop residues was performed in Chapter 5. Rumen waste of cattle obtained from a local abattoir was used as inoculum for this study. The

inoculum was in a semi-solid form. The ash, volatile solids (VS) and total solids (TS) content of the inoculum were 3.49%, 6.18% and 7.19%, respectively.

6.3.3 Construction of Microbial Consortium

Pure bacteria strains belonging to *B. subtilis, Bacillus* sp., and *B. licheniformis* were used to construct a hot spring cellulolytic microbial consortium called HSCMC consortium. The cellulolytic bacteria strains were isolated from Lubimbi hot springs in Binga, Zimbabwe. The strains were identified using morphological and biochemical identification, and 16S rRNA sequence analysis (Chapter 4). The HSCMC consortium was produced by mixing equal portions (50 mL) of overnight grown cultures of the three bacteria strains on LB broth + 1% CMC medium. The coculture was subcultured numerous times to acquire a steady microbial community that can efficiently degrade lignocellulosic biomass. The final mixed microbial culture was stored in 60% sterile glycerol at -20ºC.

6.3.4 Biological Pretreatment of Crop Residues by Microbial Consortium

The crop residues were pretreated using the HSCMC consortium to improve the hydrolysis rate prior to AD. A mineral salt medium was prepared by dissolving 0.5 g NH_4Cl, 0.5 g NaCl, 0.5 g K_2HPO_4, 0.4 g KH_2PO_4, 0.1 g $MgCl_2.6H_2O$, 1.5 g yeast extract and 1 g peptone in 1 000 mL of distilled water. The pH of the medium was adjusted to 7.0 using I M NaOH or I M HCl solution. Each ground powder of maize stover, wheat straw and soybean straw, with TS concentration of 20% dry weight was mixed with 73 mL of mineral salt medium in 250 mL conical flasks. Distilled water was added to make a total working volume of 100 mL. The flasks were autoclaved at 121ºC for 20 minutes and inoculated with 5 mL of overnight culture (5% v/v) of the HSCMC consortium.

A negative control without inoculation of the HSCMC consortium was prepared for each experimental setup. The flasks were sealed with cotton plugs and foil paper, and incubated in a water bath at 37 ºC and 160 rpm for 7 days. All the experiments were performed in triplicate. The digested powder-containing medium was designated as the hydrolysate of crop residues. The hydrolysates were preserved at -20ºC prior to AD.

6.3.5 Characterization of Pretreated Crop Residues

The pretreated crop residues were characterized for total reducing sugar (TRS), ash, TS and VS content. The DNS assay was used to determine the TRSs present in pretreated crop residues. The reaction mixture consisted of 2 mL sample and 2 mL DNS reagent heated in a water bath at 90ºC for 15 minutes and cooled on ice. Absorbances were measured at 540 nm using a spectrophotomer (UV-1900i, Shimadzu, Germany) and the sugar concentration was estimated by extrapolating from the standard calibration curve of glucose. Ash, VS and TS content were analyzed using APHA standard methods (APHA, 1995). Each experiment was conducted in triplicate.

6.3.6 Anaerobic Digestion of Crop Residues

Bench-scale AD in batch fermentation mode was performed to determine the effect of pretreatment with HSCMC consortium on biogas production from crop residues. The batch reactions were conducted in 500 mL plastic digesters with a working volume of 350 mL. A total of 50 g of start inoculum was seeded into a digester containing the substrate. Nitrogen gas (N_2) was flushed into the digesters for 5 minutes to eliminate aerobic conditions. Lastly, $NaHCO_3$ was added to maintain digester pH at 7.0. The digesters were linked via silicon tubing to plastic

bottles filled with 2% NaOH solution to dissolve CO_2 and H_2S. The digesters were manually shaken once per day to ensure adequate mixing.

Daily methane production was measured using the liquid displacement method, in which the amount of NaOH solution discharged was equal to methane. Control conditions containing only inoculum were run in parallel under similar conditions to evaluate the yield of biogas in the background of the trial digesters. The BMP reactions were performed at ambient temperature for 30 days.

6.3.7 Data Analysis

Statistical analysis was computed using OriginPro Version 8.5 software package. All the experiments were replicated three times. Therefore, each analytical result was presented as the mean of the three measurements ± standard deviation. The standard deviations and statistical differences were analyzed using one-way ANOVA at $p \leq 0.05$. Comparison among means was performed using Tukey's Honest Significant Difference (HSD) test.

6.4 Results and Discussion

6.4.1 Effect of Biological Pretreatment on Chemical Characteristics of Crop Residues

The objective of biological pretreatment was to promote the degradation of crop residues and improve accessibility of holocellulose. Holocellulose is a fermentable substrate that acts as a major carbon source in AD of crop residues (Zhong et al., 2011). The characteristics of pretreated crop residues utilized in this study are shown in Table 6.1. Findings demonstrate a significant variability ($p \leq 0.05$) in different characteristics of pretreated crop residues compared to corresponding control conditions. Ash, VS and TS reductions, and TRS increments in crop

residues were computed and presented in Figure 6.1. The reported reduction in ash content was probably due to wash outs in the hydrolysate that were removed from the biomass to liquid phase. The maximum decline in VS and TS contents of 69.2% and 83.9% were reported from maize stover and wheat straw, respectively. This dry weight loss is directly linked to robust utilization of biomass by complex bacteria population in a synergistic way. According to Zhong et al. (2011), biomass transformation may be shown by a decrease in the quantity of dry matter (TS and VS) of the feedstock. Results obtained in this study are consistent with those found in scientific reports by Wen et al. (2012), Yuan et al. (2012), Zhang et al. (2016) and Ali et al. (2017).

Compared to control experiments, the TRS concentrations of the pretreated hydrolysates of maize stover, wheat straw and soybean straw were significantly increased by 60.9%, 96.3% and 84.7%, respectively. This confirms the efficient degradation of complex carbohydrates to simple sugars by HSCMC consortium. Similarly, Ali et al. (2017) recorded increased dissolved carbohydrate content (70.6%) after pretreatment of saw dust with a novel microbial consortium. In yet another study, Wen et al. (2012) reported the highest reducing sugar content of 3 254 mg L^{-1} after 48 hours of wheat straw saccharification by microbial consortium WSD-5. However, the rate of accumulation of reducing sugars in this study was higher compared to single microbial strain pretreatment (Xu et al., 2018; Shah et al., 2019).

This study implies that an artificial microbial consortium system is more effective than a single bacterial system in solubilizing complex sugars in crop residues to more readily fermentable intermediates. Microbial consortium is associated with cross-feeding which facilitates removal of byproducts, thereby suppressing feedback inhibition during pretreatment. Moreover, it has better

adaptability and stability to harsh conditions over single microorganisms (Cao et al., 2022; Liu et al., 2023).

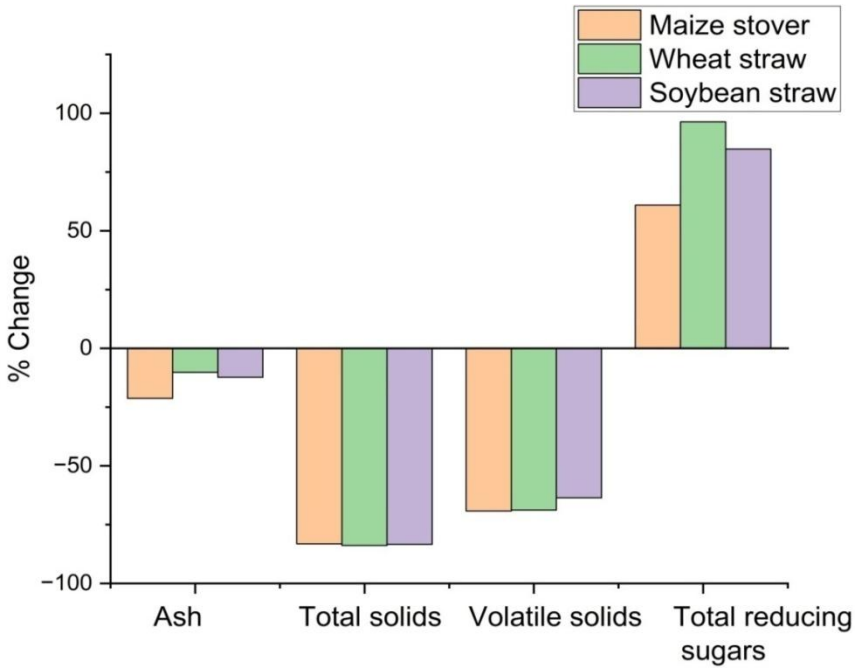

Figure 6.1 Comparison of ash, volatile solids and total solids reductions, and total reducing sugar increments in unpretreated and pretreated crop residues

Table 6.1 Characteristics of crop residues used in this study after microbial consortium pretreatment (% dry basis, apart from reducing sugars)

Parameter	Maize stover		Wheat straw		Soybean straw	
	Control	Treated	Control	Treated	Control	Treated
Ash	5.34 ± 0.49	4.20 ± 0.25*	6.67 ± 0.58	5.99 ± 0.28**	4.55 ± 0.70	3.99 ± 0.21**
Volatile solids	85.53 ± 3.31	26.33 ± 0.51*	89.60 ± 0.40	28.00 ± 0.87*	87.26 ± 2.09	31.73 ± 0.98*
Total solids	94.63 ± 0.78	15.90 ± 0.76*	92.70 ± 0.80	14.93 ± 0.24*	91.23 ± 2.04	15.17 ± 0.44*
Total reducing sugars (mg L^{-1})	65.13 ± 0.71	126.03 ± 2.47*	52.60 ± 1.31	103.27 ± 2.58*	48.80 ± 1.71	90.17 ± 2.74*

Values are means of the three replicates ± standard deviation

*Showed significant difference in comparison to corresponding control conditions using Tukeys test ($p \leq 0.05$)

**Showed no significant difference compared to corresponding control conditions using Tukeys test ($p \leq 0.05$)

6.4.2 Effect of Microbial Pretreatment on Biomethane Production

Pretreatment of crop residues with thermotolerant HSCMC consortium significantly improved methane yield compared to control conditions. The disparity could be due to positive synergistic effect of HSCMC consortium that promoted efficient solubilization of cellulosic compounds in crop residues to make more fermentable sugars available to AD. All experimental treatments in this study showed an analogous trend in terms of daily methane production. Several peaks were observed with maximum methane production appearing from day 13 to day 16 of digestion depending on the nature of pretreated feedstock. The highest methane production for unpretreated crop residues appeared within day 16 to day 18 of AD. Further increase in digestion time caused a gradual decline in methane production conforming loss of organic material and carbon for microbial growth (Zhang et al., 2011).

The profiles for daily methane production and cumulative methane yields of untreated and pretreated wheat straw are shown in Figure 6.2. The maximum methane production of 89 mL per day for non-treated wheat straw was achieved after 18 days of AD. However, pretreatment of wheat straw increased daily methane production to a maximum volume of 124 mL after 13 days of digestion. The maximum cumulative methane yield of pretreated wheat straw was increased by 50.6% over the corresponding control. Besides increased biogas production, the reported enhanced methane yield after pretreatment was also due to increased methane ratio in accumulated biogas (Ali et al., 2017; Kong et al., 2018). The findings are higher than the values of 22.2% and 36.6% obtained after pretreatment of wheat straw using microbial consortium TC-5 under mesophilic and thermophilic conditions, respectively. In contrast, Zhong et al. (2016) remarkably increased the total methane yield of wheat straw by 80.3% through biological pretreatment using a functional microbial consortium comprised of bacteria and fungi.

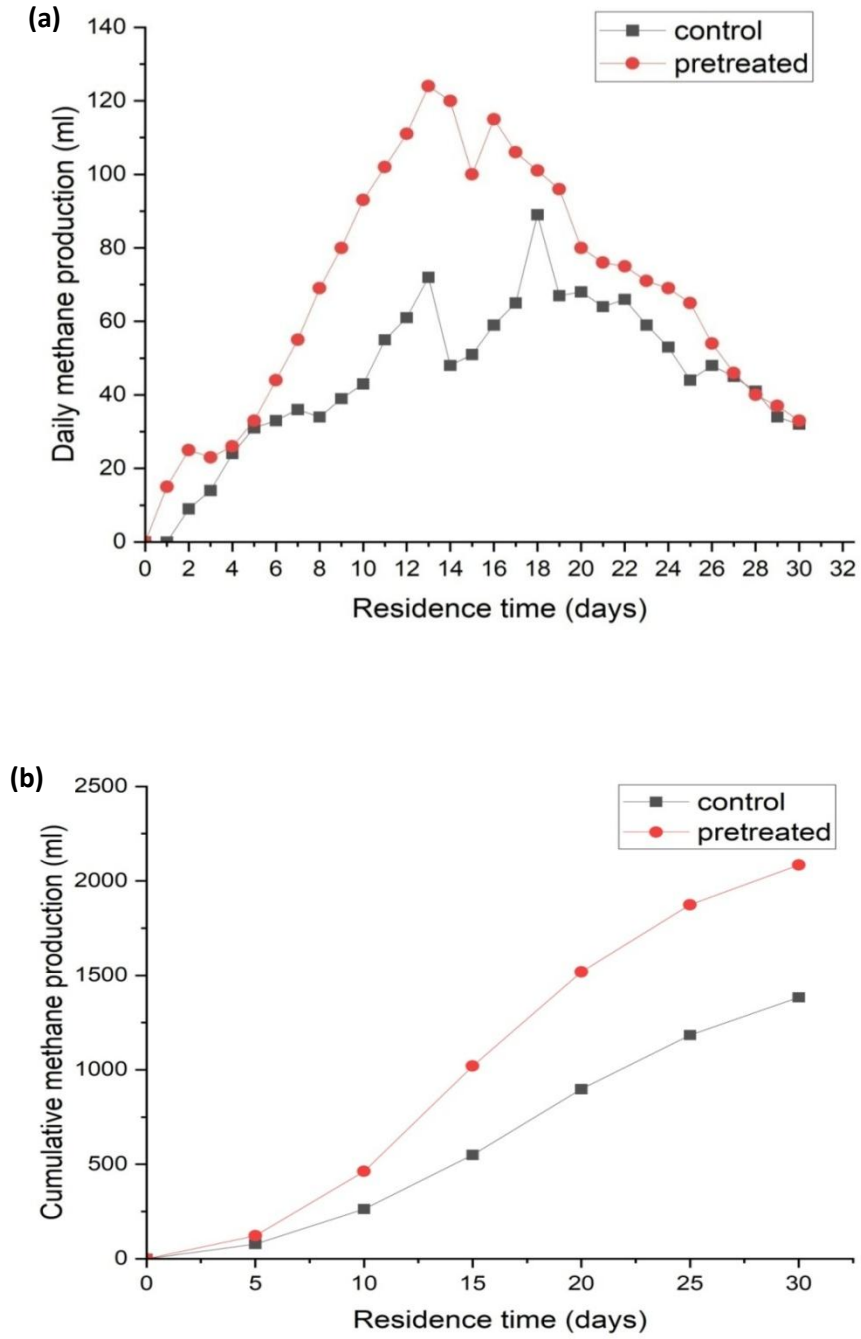

Figure 6.2 Comparison of daily methane production (a) and cumulative methane yield (b) for pretreated and untreated wheat straw

Maize stover consists of leaves, cobs, stalks and husks that are left in maize production fields. This agricultural waste is highly rich in carbohydrates and thus makes it an ideal feedstock for biogas production. The use of maize stover for biogas production is restricted by large amounts lignin of about 16% (Kamusoko et al., 2022b). Microbial pretreatment can improve the methane potential of maize stover.

Figure 6.3 is a comparison of daily methane production and cumulative methane yields of untreated and pretreated maize stover. Variability in terms of daily methane production and cumulative methane yield between pretreated maize stover and control conditions was reported. The total daily methane yield of 96 mL for untreated maize stover was observed after 16 days of bioreactor operation. This value was lower than the maximum methane yield of 129 mL per day obtained after pretreatment of maize stover using HSCMC consortium.

The total cumulative methane yield of pretreated maize stover was found to increase by 50.2% compared to untreated samples. A rise in total methane yield of 6.9% obtained from pretreated maize straw using aerobic microbial consortium was lower than the findings of this study (Zhu et al., 2019). However, Zhong et al. (2011) reported maximum methane yield from microbially pretreated maize straw, which was higher by 75.6% than control.

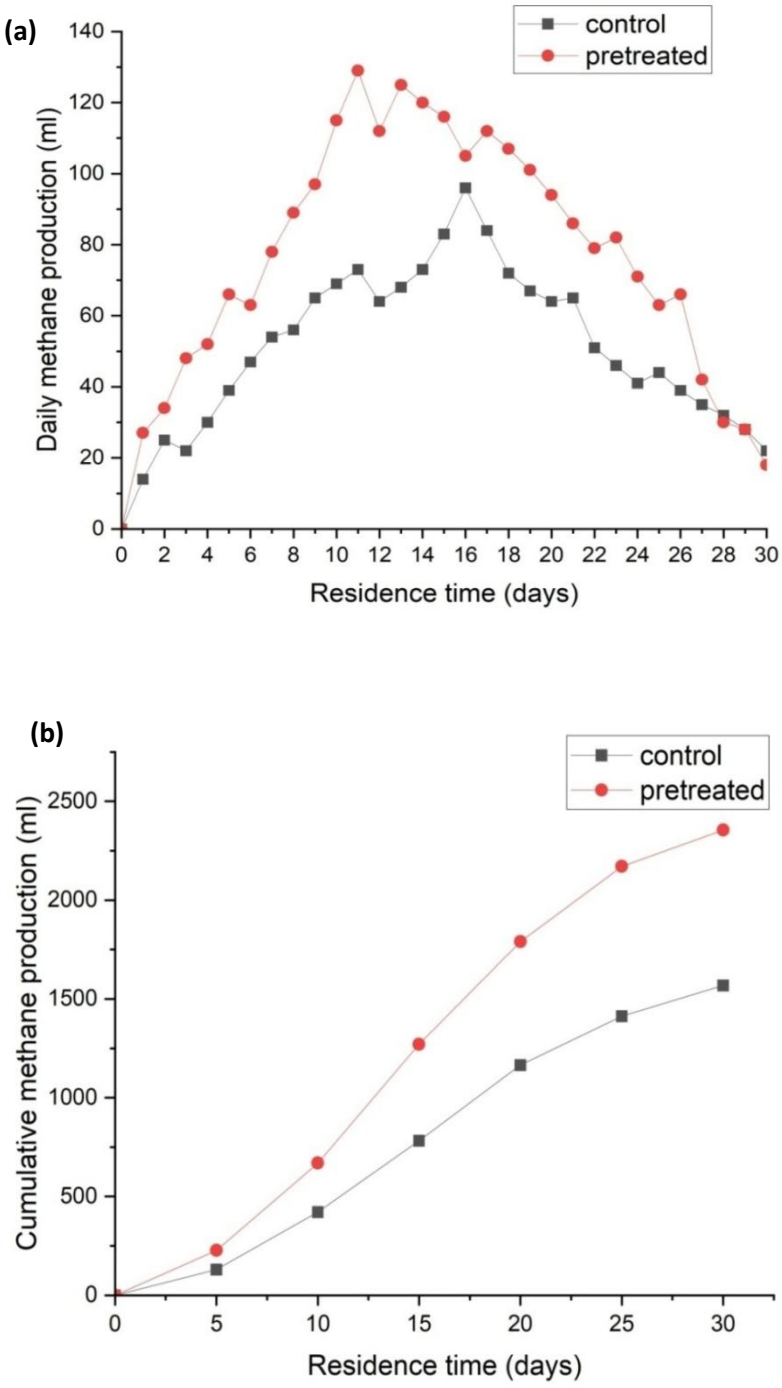

Figure 6.3 Comparison of daily methane production rate (a) and cumulative methane yield (b) for pretreated and untreated maize stover

High lignin concentration (23.5%) in soybean straw also restrains its use in biogas production, hence an effective pretreatment strategy is a prerequisite (Kamusoko et al., 2022b). Daily methane production and cumulative methane yield of untreated and pretreated soybean straw are depicted on Figure 6.4. The maximum daily methane production of 84 mL and 121 mL were observed for unpretreated and pretreated soybean straw after 18 days and 15 days of digestion, respectively.

The highest methane yield of soybean straw was enhanced by 56.6% as a result biological pretreatment using microbial consortium HSCMC. There is limited published data on utility of microbial consortium for pretreatment of soybean straw for enhancement of biogas production. Nonetheless, the HSCMC consortium showed more profound effect on pretreatment of soybean straw for methane production than the MCI consortium. The MC1 consortium increased methane yield of pretreated sterilized and unsterilized soybean straw by 36.9% and 34.3%, respectively (Yan-zhuan et al., 2020).

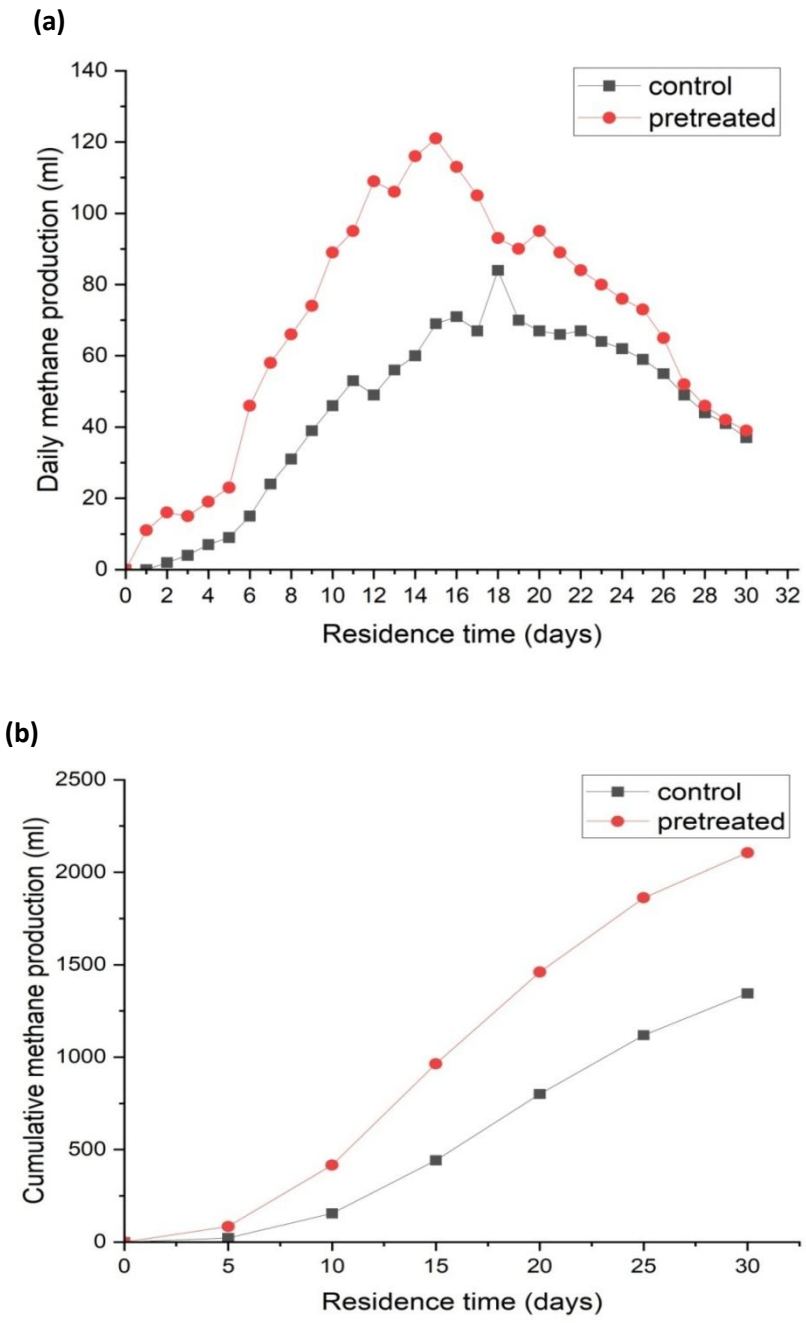

Figure 6.4 Comparison of daily methane production rate (a) and cumulative methane yield (b) for pretreated and untreated soybean straw

6.5 Conclusion

Development of a microbial consortium is an innovative platform for improving the hydrolysis of crop residues. In this study, the HSCMC consortium was enriched from local hot springs and utilized for pretreatment of wheat straw, maize stover and soybean straw. The HSCMC consortium remarkably reduced the ash, VS and TS content of crop residues. It also increased the concentration of TRSs of pretreated hydrolysates of maize stover, wheat straw and soybean straw by 60.9%, 96.3% and 84.7%, respectively. Biological pretreatment using HSCMC consortium enhanced the cumulative methane yield of wheat straw, maize stover and soybean straw by 50.6%, 50.2% and 56.6%, respectively. Microbial consortium pretreatment could be a suitable futuristic option for improving the bioconversion of crop residues into biomethane at large-scale operation.

CHAPTER 7: ANAEROBIC CODIGESTION OF BIOLOGICALLY PRETREATED CROP RESIDUES AND CATTLE MANURE

7.1 Introduction

Anaerobic codigestion is an attractive route for valorization of agricultural waste into biogas (Rowan et al., 2022; Rangseeurichayi et al., 2023). The process has been shown to enhance monodigestion experiments through nutrient balance, toxicity reduction and improved buffering capacity (Karki et al., 2021; Rowan et al., 2022). Generally, the AcoD improves biodegradation and biogas yield due to synergistic interactions in the digester medium (Ameen et al., 2021; Rangseeurichayi et al., 2023).

Crop residues are highly rich in cellulose and their nutritional content is appropriate for microbial growth and biogas production (Sukhesh & Rao, 2018; Kamusoko et al., 2022a). However, the use of crop residues for AD is deterred by high C/N ratio and slow degradation. An ideal C/N ratio for optimum performance of an AD system falls within the range of 20 - 30 (Sukhesh & Rao, 2018). Simultaneous AD of crop residues with low carbon substrates, such as animal manure is a potent strategy to maintain the C/N ratio at an optimum level (Rowan et al., 2022; Rangseeurichayi et al., 2023). Despite high nitrogen content, animal manure is enriched with rumen flora that assists to complete the AD process expeditiously (Ameen et al., 2021).

Many studies have paid much attention on AcoD of unpretreated crop residues with animal manure (Sukhesh & Rao, 2018; Valenti et al., 2020). However, there is little interest on pretreatment of crop residues to enhance biogas production from AcoD with animal manure. This study focused on AcoD of pretreated crop residues with cattle manure for biogas

production. Monodigestion experiments on biogas production from pretreated crop residues are presented in Chapter 6.

7.2 Specific Objectives

The specific objective of this experiment was to:

(a) Determine the effect of codigestion of pretreated crop residues and cattle manure on biomethane production.

7.3 Materials and Methods

7.3.1 Statistical Design

A CRD design with duplicate experiments was used in this study. Three batch setups were designed for AcoD studies. Two treatment combinations were assigned to each experimental set up: (a) mixture of cattle manure and pretreated crop residue; (b) mixture of cattle manure and untreated crop residue. The untreated groups were wheat straw and cattle manure (WS:CM), maize stover and cattle manure (MS:CM), soybean straw and cattle manure (SS:CM), while the pretreated groups were wheat straw and cattle manure (pWS:CM), maize stover and cattle manure (pMS:CM), and soybean straw and cattle manure (pSS:CM).

7.3.2 Feedstocks and Inoculum

The hydrolysates of crop residues pretreated with HSCMC consortium mentioned in Chapter 6 were used as base feedstocks for AcoD experiments. Cattle manure sourced from a livestock farm located in Chinhoyi, Zimbabwe was prepared as a cosubstrate for AcoD. The manure was ground to <1 mm, dried at room temperature and sieved through a 1 mm sieve to remove coarse materials. Triplicate samples of cattle manure were characterized for pH, ash, TS and VS using

APHA standard methods (APHA, 1995). Rumen solid waste of cattle acquired from a local abattoir was used as inoculum for this study. The chemical composition of pretreated crop residues and inoculum were determined as mentioned in Chapter 6.

7.3.3 Anaerobic Codigestion Experiments

Crop residues (untreated and pretreated) were mixed with cattle manure in a 1:1 ratio. The use of 1:1 ratio for AcoD experiments was based on previous research work, in which it was reported to be very effective (Ran et al., 2021). Slurry was prepared by adding distilled water in a 1:10 ratio. The AcoD of crop residues and cattle manure was carried out in duplicate in a batch set up at ambient temperature for 30 days. Plastic bottles (500 mL) with a working volume of 350 mL were used as biodigesters for this study. The biodigesters were purged with N_2 gas for 5 minutes to remove oxygen. The pH was maintained at 7.0 using $NaHCO_3$. The digesters were linked via silicon tubing to plastic bottles filled with 2% NaOH solution.

A control experiment containing rumen waste mixed with distilled water (1:10) was designed to assess the BMP of inoculum. The digesters were manually shaken once per day to ensure adequate mixing. Liquid displacement method was used to quantify the daily methane production.

7.3.4 Data Analysis

The experimental data were presented as means and standard deviations for the two measurements of the variables analyzed. Significant differences between variables were tested at $p \leq 0.05$ by one way ANOVA using OriginPro Version 8.5 software package. Graphs were constructed using Microsoft Excel.

7.4 Results and Discussion

7.4.1 Characteristics of Cattle Manure

The pH, ash, VS and TS content of cattle manure were 7.12, 15.10%, 47.20% and 51.34%, respectively (Table 7.1). This study demonstrates that cattle manure is an appropriate cosubstrate for AcoD with crop residues. Cattle manure could possibly reduce the inhibitory effect of free ammonia nitrogen during AcoD. The pH of cattle manure falls in the range of 6.8 - 7.2 that was suggested to be suitable for an efficient AD (Wang et al., 2023). High TS content of cattle manure can promote degradation of VS leading to enhanced biogas production (Wang et al., 2023). A model cosubstrate for AcoD with crop residues must supply essential nutrients required for higher methane yield and a steady bioreactor operation (Rahmani et al., 2022).

Table 7.1 Characteristics of the cattle manure

Parameter	Value*
pH	7.12 ± 0.14
Ash (%)	15.09 ± 0.82
Volatile solids (%)	47.20 ± 1.14
Total solids (%)	51.34 ± 0.52

*Values are mean ± standard deviation (n = 3)

7.4.2 Effect of Codigesting Crop Residues and Cattle Manure on Bioethane Production

This study infers that synergistic effect of codigesting pretreated crop residues and cattle manure is capable of improving methane production. Overall, the AcoD results indicated higher methane yield compared to monodigestion of untreated and pretreated crop residues discussed in Chapter 6. It was found that pretreated groups (pWS:CM, pMS:CM and pSS:CM) had significantly ($p \leq$

0.05) enhanced methane production compared to untreated groups (WS:CM, MS:CM and SS:CM). This may have been due to pretreatment which conferred accessibility of more holocellulose to microbial attack during AD (Siddigue & Ab. Wahid, 2018). Animal manure has been reported to increase buffering capacity and supplement nutrients, whilst plant biomass stabilizes the C/N ratio and suppress ammonia inhibition during codigestion (Lehtomaki et al., 2007; Shah et al., 2015).

Profiles for daily methane production and cumulative methane yields obtained after AcoD of untreated and pretreated maize stover with cattle manure are shown on Figure 7.1. The highest daily methane production (152 mL) was achieved after 10 days of codigesting pretreated maize stover and cattle manure (pMS:CM). Thus, pretreated maize stover supplemented with cattle manure can release more fermentable sugars within a shorter residence period of AD compared to unpretreated groups. The cumulative methane yield of pretreated groups of maize stover (pMS:CM) was 13.3% higher than non-treated groups (pMS:CM).

There is insufficient information in literature regarding the AcoD of biologically pretreated maize stover and animal manure. However, positive results were observed from AcoD of NaOH pretreated maize stover and cattle manure (Li et al., 2009). You et al. (2019) observed higher methane yields from AcoD of pig manure and maize stover pretreated with NaOH and calcium oxide (CaO). In another study, increased methane yields were reported from concurrent digestion of unpretreated combinations of maize stover and animal manure (Li et al., 2018). Results from this study are lower than those of Zhong et al. (2021) who obtained 45% more methane yield after treating a mixture of rice straw and pig manure with cellulolytic flora.

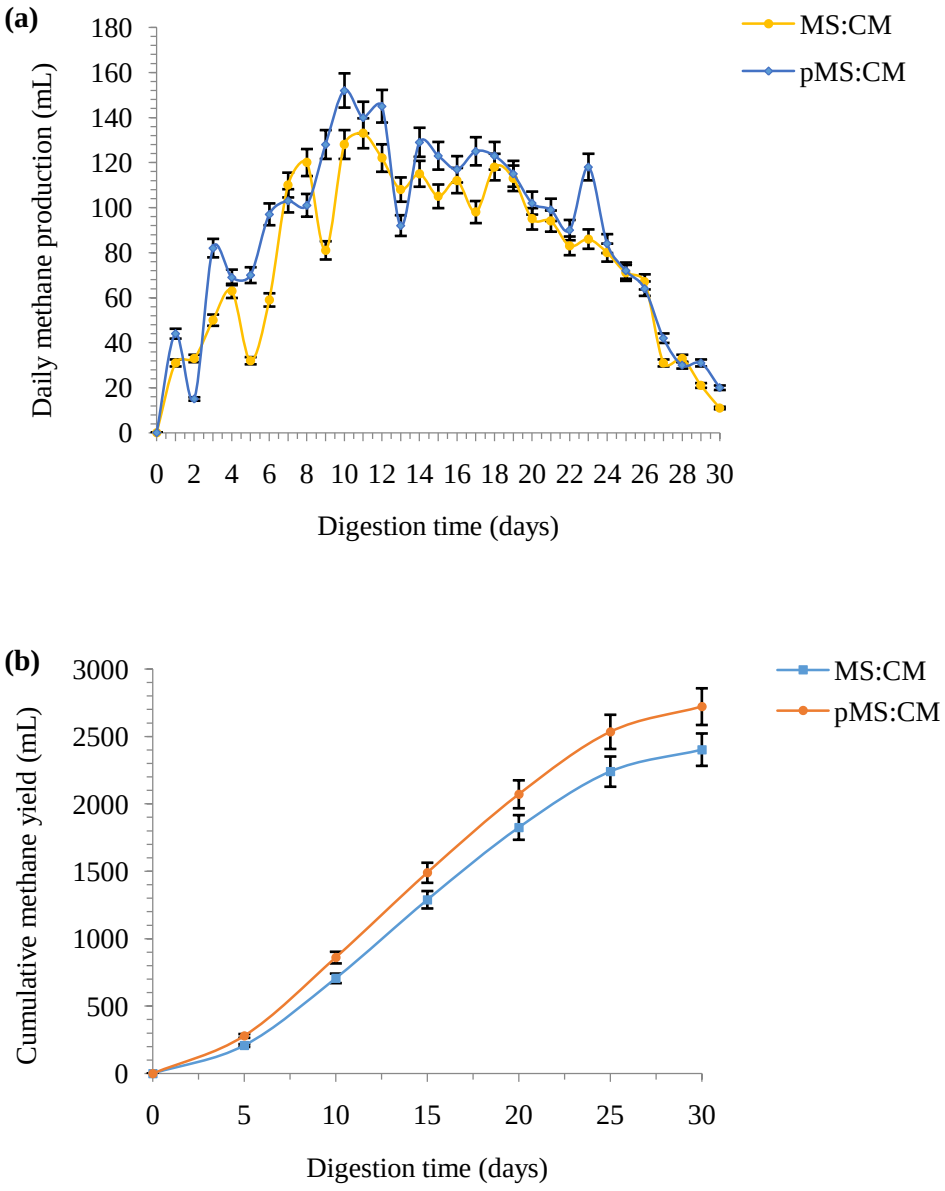

Figure 7.1 Daily methane production (a) and cumulative methane yield (b) from codigestion of pretreated and untreated maize stover with cattle manure

Figure 7.2 shows daily methane production and cumulative methane yields obtained after AcoD of untreated and pretreated wheat straw with cattle manure. Pretreated wheat straw attained the highest daily methane production of 149 mL after 10 days of AcoD with cattle manure. This value was 16% higher than unpretreated combination. Cumulative methane yield from AcoD of cattle manure with pretreated wheat straw (pWS:CM) was 2 916 mL, which was 13.3% higher than untreated group (WS:CM).

Literature mostly reports data on codigestion of chemically treated wheat straw and cattle manure. For example, Rani et al. (2021) enhanced methane production through simultaneous digestion of cattle manure and wheat straw pretreated with 10% $Ca(OH)_2$. Song & Zhang (2015) reported higher methane yield from AcoD of wheat straw pretreated with 3% H_2O_2 and cattle manure compared to untreated group.

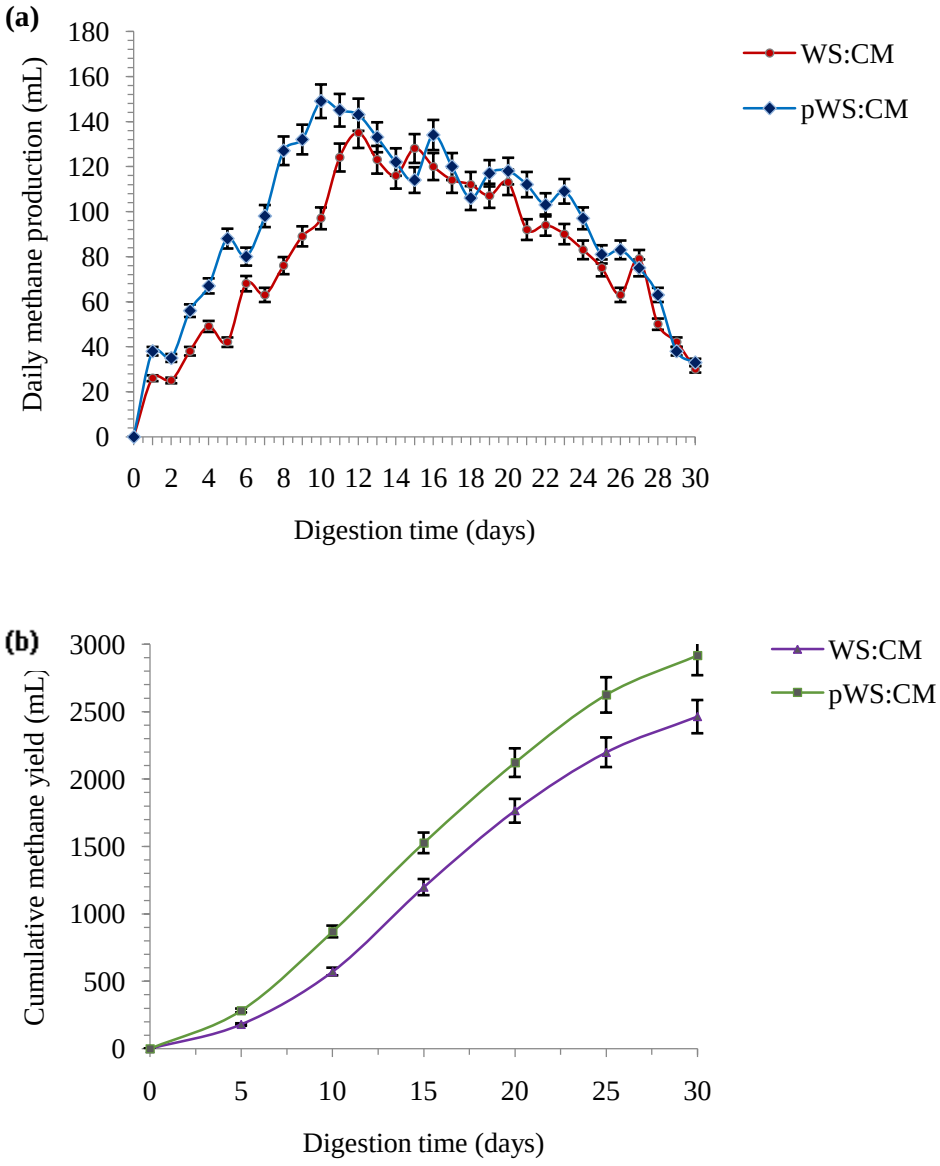

Figure 7.2 Daily methane production (a) and cumulative methane yield (b) from codigestion of pretreated and untreated wheat straw with cattle manure

Daily methane production and cumulative methane yields obtained after AcoD of untreated and pretreated soybean straw with cattle manure is shown in Figure 7.3. Pretreatment of soybean straw before AcoD with cattle manure resulted in maximum daily methane production of 144 mL after 11 days of digestion. Comparatively, the untreated group (SS:CM) yielded relatively lower maximum daily methane (126 mL) after a period of 13 days. Codigestion of pretreated soybean straw with cattle manure resulted in 25.1% increase in cumulative methane production compared to unpretreated combination.

Enhancement of biogas production through AcoD of biologically treated soybean straw and cattle manure is not reported in literature. However, Xiong et al. (2020) found 62% more methane from codigestion of thermochemically treated soybean straw and farm wastewater. Codigestion of untreated soybean straw with activated sludge increased methane production by 2.27 times over monodigestion experiments (Potdukhe et al., 2021).

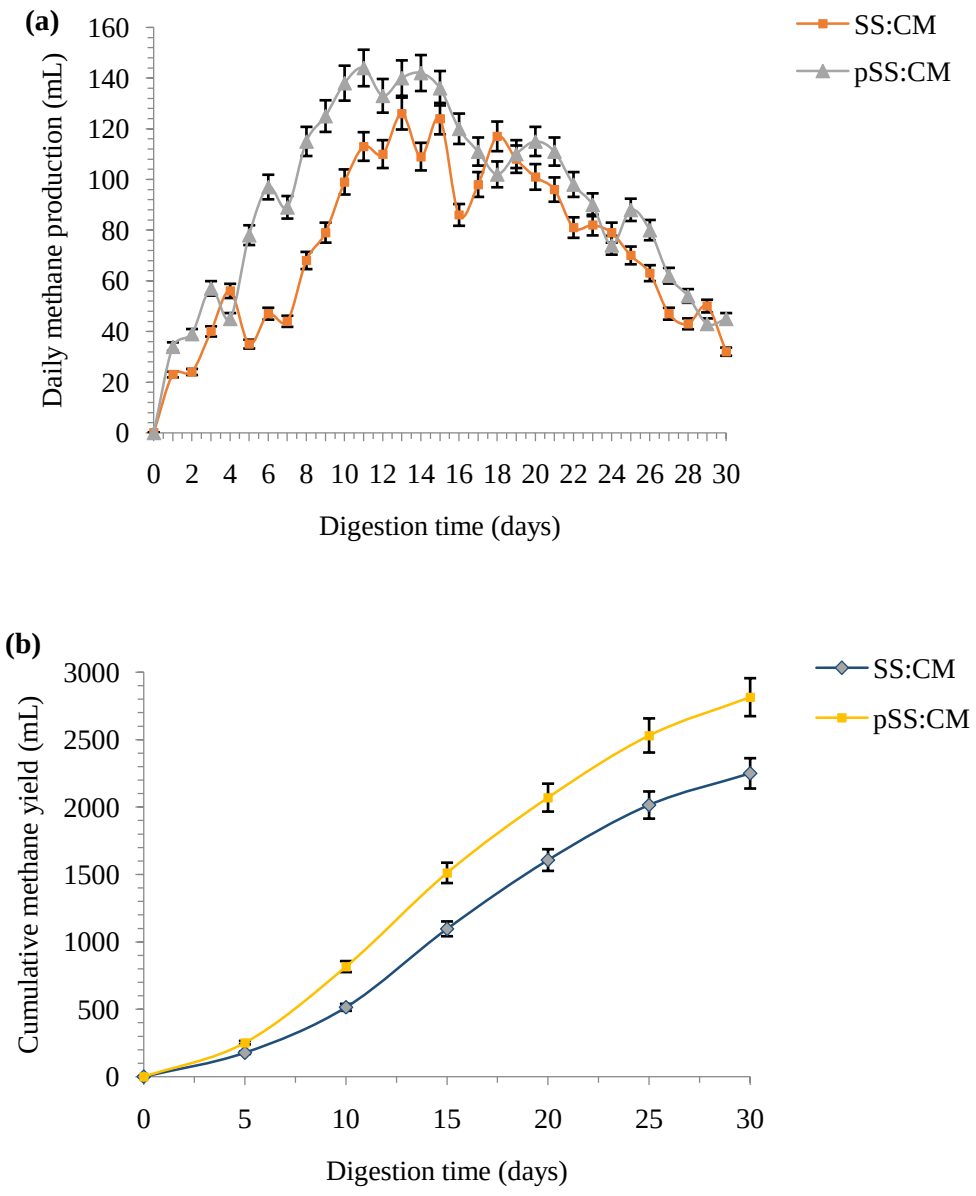

Figure 7.3 Daily methane production (a) and cumulative methane yield (b) from codigestion of pretreated and untreated soybean straw with cattle manure

7.5 Conclusion

Biomethane production could be enhanced by codigestion of biologically pretreated crop residues and cattle manure. The pH, ash, VS and TS content of cattle manure were 7.12, 15.10%, 47.20% and 51.34%, respectively. Higher daily methane production in the range of 144 - 152 mL was recorded from AcoD of crop residues pretreated with a consortium of bacteria and cattle manure compared to unpretreated groups. Cumulative methane yields for pretreated groups of 2 722 mL (pMS:CM), 2 916 mL (pWS:CM) and 2 815 mL (pSS:CM) were 13.3%, 18.4% and 25.1%, respectively, higher than the non-treated experiments. It is shown that AcoD of cattle manure and maize stover pretreated with a consortium of bacteria could be one of the innovative options for efficient biomethane production.

CHAPTER 8: INTEGRATION OF RESEARCH FINDINGS

8.1 Introduction

This study revealed the potential of using crop residues as feedstocks for biogas production through AD. Notable was the high lignocellulosic composition of crop residues that can block the AD pathway and reduce biomethane yield. Biological pretreatment and codigestion were reported to be innovative solutions for improving biogas production from crop residues.

To systematically complete this study, it was imperative to merge the research findings from different components of this study and discuss them against the background of the theory in the context of broader literature. The purpose was to provide an overall synthesis and demonstrate how the findings of this study link to one another in a coherent manner. This Chapter provides an integrated analysis, evaluation, interpretation and explanation of the results obtained in this study.

8.2 Main Findings

8.2.1 Theoretical and Conceptual Framework

The theoretical and conceptual framework of the study presented in Chapter One laid a solid foundation for this study. This work was premised on a sound theoretical and conceptual basis. There is substantial research that has been done which compliments this study. The following were the main issues in extant literature:

a) There is a paradigm shift towards affordable, renewable and sustainable energy sources like biofuels due to depleting fossil fuels and the global climate change (Paudel et al., 2017; Soni et al., 2019). Biofuels are mainly derived from starches, sugars and edible

vegetable oils, putting food security at risk. Organic wastes such as wood, forest waste, food crop waste, vegetable oil waste, industrial waste and energy crops are potential feedstocks for biofuel production.

b) Huge amounts of crop residues are produced from the agricultural sector across the world (Shinde et al., 2022). Crop residues can diversify the feedstock stream for biofuel production. The AD is a versatile biotechnology that converts crop residues into biogas. Anaerobic digestion is affected by several process parameters, such as temperature, pH, OLR, nutrient availability, HRT, inoculum, particle size and mixing (Jain et al., 2015). These factors must be optimized for an efficient AD system. The AD of crop residues is constrained by the recalcitrant nature of crop residues (Moodley & Trois, 2021). This calls for innovative strategies to enhance the hydrolysis of crop residues during biogas production.

c) Pretreatment is an innovative strategy to accelerate the break down of crop residues into fermentable sugars. It can be classified into three broad categories: chemical, physical and biological (Awogbemi & Kallon, 2022; Sahay, 2022). Chemical and physical methods are widely reported in literature, but often associated with several limitations. Although, biological pretreatment appears to have more benefits than physical and chemical methods, the technology is still burgeoning. Pretreatment with microbial consortia is one of the most promising biological methods to increase methane production (Yuan et al., 2011; Yan et al., 2012). Research must focus on microbial consortium pretreatment, which is lacking in literature.

d) Codigestion of crop residues with animal manure is yet another strategy to enhance biogas production from crop residues. The method promotes biogas production by supplementing essential nutrients like nitrogen that are deficient in crop residues (Siddigue & Ab. Wahid, 2018). It is prudent to optimize the C/N ratio of crop residues in order to stabilize the AD system.

Using information in extant literature, it was possible to formulate the research hypotheses that formed the foundation of this study. Subsequently, cellulolytic bacteria were isolated from hot springs and utilized as biological pretreatment agents in an effort to end the ongoing debate on valorization of crop residues into biogas. To validate the hypotheses, crop residues pretreated with cellulolytic bacteria and codigestion were tested for potential to produce biogas. It was realistic to conclude that biological pretreatment and codigestion can enhance biomethane production from crop residues.

8.2.2 Comparison of Pretreatment Methods

A systematic review of literature gathered sufficient evidence to substantiate the claim that biological pretreatment is a better option than physical and chemical methods (Chapter 3). The claims were attributed to low capital investment, reduced energy requirements and inability to produce toxic compounds (Abraham et al., 2020; Awogbemi & Kallon, 2022). Chemical and physical pretreatments were found to be very effective, with limited applications at large-scale due to high energy demand and operational cost, and production of toxic compounds (Liu et al., 2019). It was further observed that the rate of hydrolysis of biological pretreatment still needs to be enhanced through innovation. One plausible approach was to use cellulolytic bacteria as a

pre-processing step to degrade the complex structure of crop residues (Chapter 4). Hence, it was fundamental to screen local hot springs for the presence of cellulose-degrading bacteria.

8.2.3 Screening of Cellulolytic Bacteria from Various Environments

The systematic review of literature in Chapter 3 provided baseline data to support the research hypothesis on enhancing biogas production from crop residues through biological pretreatment. It was possible to isolate cellulolytic bacteria from local hot springs. The purpose was to find novel strains with high cellulolytic activity for pretreatment of crop residues to improve biogas production. Literature abounds with information that cellulolytic bacteria survive in diverse environments, such as insect guts, hot springs, agro-industrial wastes, pig intestines and degraded soil conditions (Ferbiyanto et al., 2015; Hussain et al., 2017; Abdollahi et al., 2021; Pramanik et al., 2021).

The research findings in Chapter 4 established that bacteria strains from Lubimbi hot springs had high cellulolytic activity. It further emphasized that only three bacteria strains identified as *B. subtilis* LB-4, *Bacillus* sp., LB-6 and *B. licheniformis* LB-8 showed high cellulolytic activity with cellulolytic indices of 1.8, 2.0 and 1.5, respectively. High cellulolytic activities indicated the potential of novel isolated strains for pretreatment of crop residues. Accordingly, the strains were further explored to find out the optimum fermentation conditions for cellulase production. It is critical to acknowledge that cellulolytic *Bacillus* ssp., were detected from several hot springs in the world (Adiguzel et al., 2009; Hussin et al., 2011; Panda et al., 2012; Abdollahi et al., 2021).

Cellulolytic bacteria exhibit a myriad of survival mechanisms, such as secondary metabolites that maintain the stability and activity of their cellular proteins in harsh environments (Ree et al., 2013). The prevailing temperatures in the local hot springs in the range of 71 - 82°C were

adequate to support this claim. According to Panda et al. (2012) and Priya et al. (2016), cellulolytic bacteria are more likely to be isolated from hot springs and hydrothermal vents where temperatures vary from 40 - 122ºC.

8.2.4 Optimization of Fermentation Conditions for Cellulase Production

Cellulase production by bacteria is affected by a wide range of environmental factors, such as inoculum size, temperature, pH, inducers, additives, incubation time, aeration time, carbon and nitrogen source, and so on (Abou-Taleb et al., 2009). The ability of isolated bacteria strains to produce crude cellulases was optimized for different fermentation conditions through SmF using the OVAT approach (Chapter 4). This study looked at pH, temperature, carbon source, nitrogen source and incubation time in accordance with the available resources. In addition, the OVAT method used in this study is very cumbersome due to multiple experimental runs (Czitrom, 1999). It is feasible to optimize the other fermentations conditions and purify the crude enzyme extract as a first step towards commercialization of the product.

Incubation time affects cellulase activity of bacteria isolates; hence 24 hour and 48 hour periods were examined for their effect on cellulase production. The highest cellulase yield (0.20 U mL^{-1}) was observed from *Bacillus* sp., after 24 hours of incubation. Prolonged incubation periods results in depletion of nutrients and generations of toxic byproducts that may affect bacteria growth. These findings are similar to the report of Hussain et al. (2017). Optimization of pH conditions from 3.0 - 9.0 confirmed cellulase production in all the media. The highest activity (0.17 - 0.19 U mL^{-1}) was observed at pH 7.0. The pH of the medium is an indicator of how specific substrates are metabolized, and thus it affects the ability of bacteria to produce cellulases. According to Naresh et al. (2019), variations in pH can destroy the three-dimensional

structure of enzymes and lowers its affinity towards the substrate. The findings are in correlation with the works of Romsaiyud et al. (2009), Hussain et al. (2017) and Pramanik et al. (2021).

The CMC is an inducer and carbon source for cellulase fermentation. This study demonstrated that *B. subtilis* LB-4, *Bacillus* sp., LB-6 and *B. licheniformis* LB-8 can efficiently utilize 1% CMC as a carbon source. Strain *Bacillus* sp., LB-6 reported the highest cellulase activity of 0.11 U mL^{-1}. These results are supported by previous authors (Behera et al., 2016; Islam and Roy, 2018). However, the CMC is an expensive substrate that cannot sustain large-scale production of cellulases. It is fundamental to search for cheaper and sustainable substrates for cellulase production. Crop residues hold much promise as a crude carbon source for cellulase production by *Bacillus* spp., (Pramanik et al., 2021).

The effect of three nitrogen sources (yeast extract, peptone and urea) on cellulase production from bacteria isolates was determined. Overall, all the three strains produced maximum yields of cellulases whilst utilizing 1% yeast extract a source of nitrogen. The maximum cellulase production was estimated at 0.19 U mL^{-1}. The vitamin B complex and trace elements present in yeast extract could have promoted cellulase production (Jach et al., 2022). In one study, Sakthivel et al. (2010) and Pramanik et al. (2021) reported maximum cellulase production from bacteria using yeast extract as a sole nitrogen source. The lowest yields of cellulases were attained when bacteria were supplemented with urea as a nitrogen source. Despite, inorganic compounds are more preferred nitrogen sources than organic compounds, such as yeast extract, beef extract and peptone due to their lower cost (Rai et al., 2012).

All the bacteria strains showed effective cellulase production in temperatures varying from 35 - 45ºC. The optimum temperature for cellulase production was observed at 40ºC. Further increase

in temperature led to a decline in cellulase production. Similarly, Gozan et al. (2018) and Pramanik et al. (2021) reported maximum cellulase activity at 40ºC. In contrast, Hussain et al. (2017) obtained highest cellulase production at 37ºC.

8.2.5 Comprehensive Characterization of Crop Residues

Understanding the structure and composition of crop residues was an important phenomenon in this study because of its strong influence on microbial degradation. In Chapter 5, crop residues were consecutively examined for proximate composition to fully exploit cellulolytic bacteria for biological pretreatment. Research findings showed variability with respect to physicochemical characteristics and lignocellulosic composition of crop residues. Crop residues differ in composition depending on agronomic, handling and storage conditions (Danish et al., 2015). In addition, plant type, variety and maturity can also influence the biochemical composition of plant biomass (Soni et al., 2019).

The pH (5.3 - 5.5) and TN content (3.1 - 8.2%) obtained in this study were different from values reported in literature (Xiong et al., 2020). The TN content was rather high while the pH was acidic to some extent. High TN content leads to generation of surplus NH_3 that can obstruct the AD process (Xiong et al., 2020). Most AD systems are operated at neutral pH conditions. Acidic pH conditions promote the release of VFAs which are known to suppress the growth of anaerobic bacteria (Jain et al., 2015). Codigestion with animal manure could be a viable option to stabilize digester medium containing crop residues.

This study revealed that crop residues are potential feedstocks for energy production. All crop residues were observed to contain more than 30% cellulose, which is the most fermentable substrate. However, lignin content ranging from 16.2 - 23.5% was high to some extent. The

reported lignin contents are analogous to values in literature (Ho et al., 2014; Sahito et al., 2013). Lignin is the most recalcitrant material in plant cell walls and could lower the methane yield of the crop residues (Triolo et al., 2012). It is also a source of phenolic compounds which affects the availability of cellulose to AD (Schroyen et al., 2015). Pretreatment is an appropriate strategy that can destroy lignin in crop residues and make holocellulose more accessible to microbial attack. Even so, more research must be done to design an effective pretreatment strategy for lignin degradation.

8.2.6 Biological Pretreatment of Crop Residues

The HSCMC consortium developed from *B. subtilis* LB-4, *Bacillus* sp. LB-6, and *B. licheniformis* LB-8 was utilized for pretreatment of crop residues to enhance biogas production (Chapter 6). The ash, VS, TS and TRS content were estimated to determine the efficacy of the HSCMC consortium in pretreatment of crop residues. The concentration of TRSs was determined using the DNS method, which is simple and rapid to perform. The challenge with the DNS assay is that it cannot estimate individual sugar fractions that vary in color intensities (Kurzyna-Szklarek et al., 2022). Hence, the concentrations of individual sugar units were not examined in this study. Results showed a significant decline in ash, VS and TS, and a rise in TRSs after biological pretreatment. This might be due to efficient transformation of plant biomass into fermentable sugars by the novel HSCMC consortium. Previous studies reported results that are comparable to the trends observed in this study (Wen et al., 2012; Ali et al., 2017; Ali et al., 2021).

The BMP assay is generally carried out to comprehend the biodegradability and methane potential of the organic matter. As a result, crop residues were subjected to BMP assay to further

validate the effect of pretreatment with HSCMC consortium on biogas production. Results indicated that pretreatment using HSCMC consortium can enhance cumulative methane yield of wheat straw, maize stover and soybean straw by 50.6%, 50.2% and 56.6%, respectively. Contrary to this, longer digestion times were taken by pretreated crop residues to achieve their maximum daily methane production. This can be ascribed to fluctuating ambient temperatures used in this study that could have affected metabolic activities of microbial communities. The maximum daily methane production for pretreated hydrolysates ranged from 121 - 129 mL per day.

Bioethane production usually increases with a rise in operating temperature. For this reason, many authors suggest the use of mesophilic and thermophilic conditions for AD as they promote high activity amongst cellulolytic enzymes (Jain et al., 2015, Wang, 2016; Kong et al., 2018). However, an ambient temperature was chosen for this research because it is more linked to realistic conditions than other temperatures. It is fundament to monitor and control the process parameters in order to optimize microbial activity and enhance the biogas yield of crop residues.

Basically, the findings of BMP assays in extant literature may differ and are not easy to compare owing to variability of procedures on the basis of experimental setups, conditions, data analysis, etc (Wang, 2016). However, our results are consistent with the works of Zhong et al. (2011), Zhong et al. (2016) and Yan-zhuan et al. (2020) who improved methane yield of crop residues after microbial consortium pretreatment.

8.2.7 Codigestion of Crop Residues with Cattle Manure

Anaerobic codigestion of crop residues and animal manure is a novel strategy for integrated agricultural waste management. In Chapter 7, pretreated crop residues were codigested with

cattle manure to further enhance biomethane production. The intention was to improve methane production by optimizing the C/N ratio of the digester medium. The findings demonstrated that AcoD of pretreated crop residues with cattle manure can significantly improve methane yield compared to untreated groups. Cumulative methane yields for pretreated groups were found to increase in the range of 13.3% - 25.1%. This infers that the synergistic effect of codigesting pretreated crop residues and cattle manure is capable of improving methane production. Pretreatment of crop residues could have probably conferred accessibility of more cellulose and hemicellulose to microbial attack during AcoD. In addition, animal manure possibly offered buffering capacity and supplemented nutrients, whilst plant biomass stabilized the C/N ratio and suppressed ammonia inhibition during codigestion (Lehtomaki et al., 2007; Shah et al., 2015).

Several authors have reported earlier on codigestion of a combination of animal manure and unpretreated crop residues (Lehtomaki et al., 2007, Babaee et al., 2013; Zhang et al., 2013, Cahyono et al., 2021). There is lack of information in literature regarding AcoD of animal manure with biologically pretreated crop residues. However, it is practical to scale-up biogas production from pretreated crop residues under bench-scale conditions en route to large-scale operation.

8.3 Implications of the Findings

Information produced in this study indicates the benefits of using the isolated bacteria strains to enhance biogas production from crop residues through biological pretreatment and codigestion. Besides, the research may capacitate the local industries through production of cellulase enzymes in an economically viable manner, thus reducing hefty import costs. It is possible to commercialize cellulase production from the bacteria strains under fermentation conditions that

were optimized in this study. It is also practical to scale up, purify and package the crude cellulases extract for future use and as a business. The use of locally available cellulases is capable to power key sectors, such as the textile, paper and pulp, food, feed, detergent, energy and chemical industries that are currently relying on imported enzymes.

Pretreatment of crop residues with a bacteria consortium and AcoD with cattle manure were investigated under bench-scale studies and yielded promising results. The research findings imply that crop residues is a viable feedstock for biogas production via AD. Hence, the AD technology can be fully adopted for handling crop residues in a sustainable and ecofriendly way. It is practical to translate the research findings to pilot- and small-scale towards full-scale biogas operation. Biogas, being a carbon neutral fuel with zero net impact on GHG emissions can help to mitigate climate change on a global perspective. Driven by national policy imperatives, Zimbabwe will boost its energy sector and empower the livelihoods of rural people through bioelectrification. It is viable to create employment and alleviate poverty through installation of large-scale biogas plants that utilize crop residues as feedstocks.

It is also possible to set up small biodigesters at household level that could be directly used for heating or cooking. This could save women and children from tedious tasks of fuel wood collection, thus more time is reserved for productive work. Social risks related to firewood collection, such as injury and violence to women and missed time for education amongst children could be minimized. Fuel wood contributes a huge fraction of GHG emissions in rural areas, thus the use of biogas might assist to alleviate GHG emissions. Firewood consumption is one of the main causes of deforestation; hence environmental benefits through forest conservation could be derived from replacing firewood with biogas.

In rural areas of Zimbabwe, only 31.6% of the population has access to modern electricity (IEA et al., 2023). The majority of rural households rely on traditional fuels, such as firewood and cattle dung as a source of energy. From a health point of view, use of traditional fuels emits smoke and particulate matter that could result in indoor pollution leading to severe respiratory illnesses. Therefore, cooking on biogas stoves could help to reduce indoor air pollution and avert respiratory problems caused by smoke and particulate matter associated with open fire combustion.

Revenue could be generated from multiple end products in a biogas refinery. Biogas can be sold via tariffs or the natural gas grid to consumers for use in power generation. A rich-digestate solid waste that remains after AD can improve food security and the agricultural economy. The digestate contains large quantities of nutrients that are readily available for promoting soil structure and improving crop productivity. The digestate can be utilized or sold as a biofertilizer, minimizing the need of synthetic fertilizers. This saves as a source of revenue and prevents environmental degradation. Additional revenue can be generated from selling the digestate as bedding for farm animals.

CHAPTER 9: CONCLUSION AND RECOMMENDATIONS

9.1 General Conclusion

Crop residues are locally abundant cellulosic materials with potential to diversify the feedstock stream for biogas production. The low nitrogen content, recalcitrant nature and prolonged residence times are the main drawbacks towards full-scale exploitation of crop residues in biorefineries. Innovative strategies, such as pretreatment and codigestion can enhance the BMP of crop residues. It was established that biological pretreatment is more advantageous than physical and chemical methods with respect to capital cost, energy needs and production of inhibitory compounds. As a result, this study focused on biological pretreatment of crop residues and codigestion with cattle manure at bench-scale to improve biomethane production. The findings of this study were in accordance with the proposed hypotheses.

Cellulolytic bacteria were successfully isolated from local hot springs. Of all the isolated bacteria, only three strains (*B. subtilis* LB-4, *Bacillus* sp. LB-6 and *B. licheniformis* LB-8) recorded high cellulolytic activity. These strains were stable in terms of cellulase production under studied SmF fermentation conditions. The bacteria strains might have potential for pretreatment of crop residues to enhance biogas production. However, further research on fermentation conditions is required due to limitations of the present study.

Proximate composition of three crop residues (wheat straw, maize stover and soybean straw) was investigated due to its profound effect on the pretreatment process. Findings demonstrate that crop residues were highly variable with respect to physicochemical and lignocellulosic content. All the crop residues signified to be valuable bioresources for AD by containing more than 30%

cellulose. However, the acidic nature and high TN content of crop residues implies the need for codigestion with other organic substrates to complement nutrients. Further, the high lignin content in crop residues calls for pretreatment before feeding the biodigesters to increase the availability of holocellulose to microbial degradation.

A stable HSCMC consortium was developed from *B. subtilis* LB-4, *Bacillus* sp. LB-6 and *B. licheniformis* LB-8. The bacteria consortium was found to be suitable for pretreatment of crop residues. Ash, VS and TS of crop residues pretreated with a consortium of bacteria was significantly reduced compared control conditions. The converse is true for TRS concentrations of crop residues, which were higher than non-treated samples. Assessing the BMP of crop residues evidenced that pretreatment with HSCMC consortium was able to improve the daily methane production and cumulative methane yields.

The AcoD of pretreated crop residues and cattle manure confirmed to be a promising approach for improving biogas production. Codigestion of pretreated crop residues and cattle manure offered a remarkable increase in methane production. The daily methane production and total methane yields obtained from AcoD of pretreated crop residues and cattle manure were significantly higher than unpretreated groups.

9.2 Recommendations

Biogas production by AD of crop residues is still a burgeoning technology that needs more research in a circular bioeconomy. The following recommendations were suggested for further studies to promote the utility of crop residues for full-scale AD in the foreseeable future:

9.2.1 Pretreatment

It is important to further optimize cellulase production of the isolated bacteria strains for other fermentation conditions, such as inoculum size, medium additives, aeration rate, etc, if full-scale production is to be adopted.

Synergistic effects of *B. subtilis* LB-4, *Bacillus* sp. LB-6 and *B. licheniformis* LB-8 strains on each other's growth and enzyme production should be assessed in future research.

Continuous development of more novel microbial strains that can efficiently degrade crop residues yielding potential results is still necessary.

9.2.2 Codigestion

As highlighted in literature, several process parameters affect the biodegradability and methane potential of crop residues. Experimentation to understand the optimal reactor conditions, including pH, temperature, C/N ratio, substrate particle size, OLR, HRT and inoculum size should be continuously investigated.

Cost-benefit analysis must be carried out to evaluate the feasibility of HSCMC consortium pretreatment and codigestion with animal manure for biogas production from crop residues.

Up-scale the findings of pretreatment and codigestion, and design a full-scale plant or prototype based on data acquired from laboratory-scale studies.

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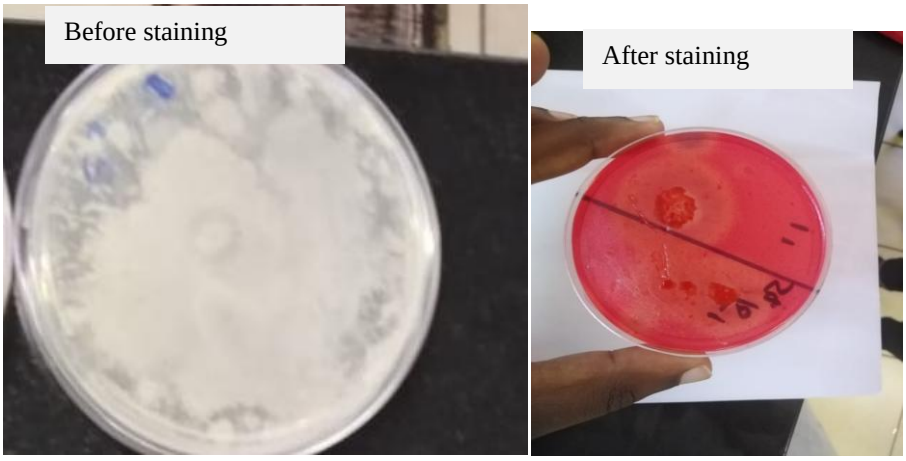
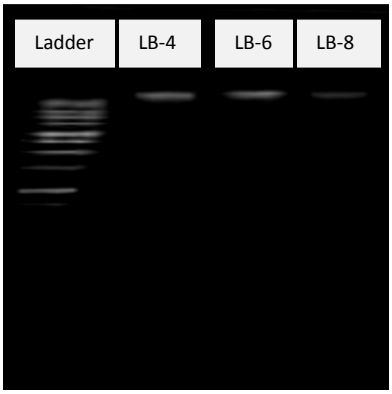
APPENDICES

Appendix 1. Validation of cellulase-producing capacity of the bacterial strains using Congo red staining



Appendix 2. Gel electrophoresis of the isolated bacteria strains



Appendix 3. Crop residues used in this study

soybean straw

wheat straw

maize stover

Appendix 4. Standard calibration curve of glucose for estimating reducing sugar concentration