

**Bioprospecting of the goat's GIT microbiota and pretreatment strategies
for enhanced sugarcane bagasse degradation and biomethane production**

by

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DEDICATION

To my beloved family:

To my angel, Ziphozenkosi Mabaso, whose name reflects the gift of God's glory. You remind me daily of the strength it takes to carry love beyond words and time. Your memory has been my guiding light through this journey. Your presence in my heart has been a source of enduring strength and purpose. Although your time with us was brief, your impact will be eternal.

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You are my greatest blessings, my anchors, and my eternal motivation.

DECLARATION

I, **Kgodiso Judith Mabaso-Rabapane (10459928)**, declare that this thesis submitted for the Ph.D degree (90040) at the University of South Africa titled “**Bioprospecting of the Goat’s GIT Microbiota and Pretreatment Strategies for Enhanced Sugarcane Bagasse Degradation and Biomethane Production**” is my own work and that all the sources that I have used or quoted have been indicated and acknowledged through complete references.

I further declare that I submitted the thesis to an originality-checking software and that it meets the accepted requirements for originality. I further declare that I have not previously submitted this work, or part of it, for examination at Unisa for another qualification or at any other higher education institution.

Signature:  Date: 13 November 2025

Kgodiso Judith Mabaso-Rabapane (Candidate)

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SUMMARY

The gastrointestinal tract of goats (*Capra hircus*) harbors a diverse array of carbohydrate-active enzyme (CAZyme)-producing microorganisms that play a crucial role in fiber degradation and nutrient cycling. However, the dynamics and functional potential of these microbial communities remain underexplored, limiting their application in sustainable bioenergy production. This study applied functional metagenomics and microbiological profiling to investigate CAZyme-producing microorganisms in South African goats. The ruminal gastrointestinal tract samples were collected to evaluate microbial communities capable of producing cellulases and other carbohydrate-degrading enzymes. Microbial diversity varied across gastrointestinal compartments, with the abomasum exhibiting reduced alpha diversity, likely due to its acidic environment. Proteobacteria, Bacteroidetes, and Firmicutes were the dominant phyla, with Proteobacteria being particularly abundant in the abomasum. Functional profiling revealed that most genes were involved in metabolism, including xenobiotic biodegradation, while carbohydrate-active enzyme (CAZyme) analysis showed hlycoside hydrolases as the predominant class. Higher CAZyme abundance in the reticulum and omasum suggested compartmentalized microbial activity and adaptation to dietary substrates. Genera such as *Proteus* and *Bacillus* were isolated, identified, and screened for enzyme activity, growth rates, and substrate utilization after further processing of the GIT samples. This revealed *Proteus mirabilis* KC94 (KC94) as the most promising strain. KC94 exhibited strong, stable enzyme activity across a broad pH and temperature range, with peak production at 84 hours. Genetic analysis confirmed the presence of key glycoside hydrolase genes (GH39, GH45, GH48). Short-term pretreatment with KC94 significantly improved cellulose hydrolysis, as shown by higher glucose release, and altered the elemental composition of sugarcane bagasse (SCB) by balancing nutrients and reducing sulfur content. Biomethane potential (BMP) assays demonstrated that KC94-pretreated SCB achieved the highest methane yield, with up to 25% improvement over controls and faster, sustained production. The goat rumen microbiota displayed dynamic adaptations to varying dietary inputs, highlighting their potential for bioprospecting in sustainable bioenergy systems. Collectively, the findings demonstrate the applicability of goat-derived microbial communities for cellulase production and lignocellulosic biomass conversion, offering an eco-friendly alternative to chemical pretreatment methods.

Keywords: Goat rumen microbiota, CAZymes, cellulase, biomethane, sugarcane bagasse, sustainable bioenergy, microbial adaptation

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LIST OF ABBREVIATIONS

AD	Anaerobic Digestion
AMPTS	Automatic Methane Potential Test System
BHIB	Brain Heart Infusion Broth
BMP	Biochemical Methane Potential
CAZyme	Carbohydrate-Active enZymes
CHNS	Carbon, Hydrogen, Nitrogen, Sulfur
CMC	Carboxymethyl Cellulose
CZ	Colony Diameter
CZD	Clear Zone Diameter
DNA	Deoxyribonucleic acid
GIT	Gastrointestinal Tract
LB	Lauria Berthani

OTUs	Operational Taxonomic Units
PCR	Polymerase Chain Reaction
REA	Relative Enzymatic Activity
SCB	Sugarcane Bagasse
SEM	Scanning Electron Microscope
TS	Total Solids
VFAs	Volatile Fatty Acids
VS	Volatile Solids

CHAPTER 1: INTRODUCTION

1.1 Background

The energy demand has escalated due to the increasing global population and industrialization (Mudakkar *et al.*, 2013, Østergaard *et al.*, 2022). Many nations heavily rely on conventional fossil fuels, contributing significantly to environmental deterioration, including greenhouse gas emissions and global warming (Wang *et al.*, 2022, Mudakkar *et al.*, 2013). Consequently, there has been a notable worldwide progression towards renewable and sustainable energy sources. One of the promising alternatives is bioenergy derived from biomass due to its potential to minimize environmental impact while utilizing agricultural residues as feedstocks (Moustakas *et al.*, 2020, Østergaard *et al.*, 2022, Hassan *et al.*, 2024). As a byproduct of agricultural activities, agro-waste presents a dual benefit: reducing environmental waste and providing a sustainable resource for bioenergy production (Singh *et al.*, 2024b, Nair *et al.*, 2022).

One of the most abundant agricultural residues with a high potential for utilization in bioenergy is SCB, a fibrous byproduct generated in significant quantities by the sugar and bioethanol industries (Kumar *et al.*, 2021, Ajala *et al.*, 2021). Approximately 250-280 kg of bagasse is produced for every ton of sugarcane processed (Kabeyi and Olanrewaju, 2023, Spyropoulos, 2024). SCB is rich in lignocellulose and comprises 47%–52% cellulose, 25–28% hemicellulose, and 20-21% lignin, as well as ashes (5%) and extractives (2%) (Ajala *et al.*, 2021), making it a valuable substrate for producing biofuels such as biogas, ethanol, and other value-added chemicals (Kumar *et al.*, 2021, Ajala *et al.*, 2021). However, its dense lignocellulosic structure and high lignin content present drawbacks to microbial accessibility and enzymatic degradation (Kumar *et al.*, 2021, Yadav *et al.*, 2024). Hence, efficient SCB utilization requires innovative approaches combining microbial and other pretreatment technologies.

Although enzymes can be produced by other biological sources, such as plants and animals, microorganisms are preferred due to their rapid growth, large biomass capacity, and higher product titers (Çorbacı *et al.*, 2017). Microorganisms, particularly those residing in the GIT of ruminants, have evolved under selective pressure to digest fibrous plant material, making them a rich source of lignocellulolytic enzymes (Rabapane and Matambo, 2024). For example, goats

(*Capra hircus*) consume diets rich in plant biomass, and their gastrointestinal tracts harbor diverse microbial populations, with cumulative densities comprising approximately (10^{11} cells/mL), methanogens (10^6 cells), fungi (10^3 – 10^6 zoospores/mL), protozoa (10^4 – 10^6 cells/mL), and bacteriophages (10^9 particles/mL) (Singh *et al.*, 2024a, Mahayri, 2022, Ribeiro *et al.*, 2016, Han *et al.*, 2015). These microorganisms work in synergy to break down plant cell walls, producing carbohydrate-active enzymes (CAZymes), including cellulases, hemicellulases, and lignin-degrading enzymes.

Several studies to date have investigated varied ecosystems, with the intention of identifying potential sources of these enzymes (Legin *et al.*, 1997, Aly *et al.*, 2012, Roy *et al.*, 2018, Priyanka *et al.*, 2018, Salgado *et al.*, 2019). The nearly limitless potential to identify new strains of evolving microorganisms capable of overproducing and expressing varied enzymes implies that bio-prospecting remains an active research area for identifying new microorganisms to produce known enzymes, as well as isozymes and novel enzymes. The gastrointestinal tract of goats is particularly promising for isolating microorganisms with lignocellulolytic capabilities. The natural adaptation of these microorganisms to degrade complex lignocellulosic substrates, coupled with their resilience to environmental stressors like low pH and anaerobic conditions, makes them a valuable and robust resource for industrial applications (Rabapane *et al.*, 2022, Ribeiro *et al.*, 2016). Thus, these microorganisms hold significant potential for enhancing SCB degradation, paving the way for improved bioconversion processes.

Regardless of its potential, SCB's recalcitrant nature, attributed to its lignin content, poses a challenge to efficient microbial and enzymatic degradation (Kumar *et al.*, 2021, Yadav *et al.*, 2024). Lignin creates a protective matrix around cellulose and hemicellulose, inhibiting enzyme interaction (Ali *et al.*, 2024). Therefore, pretreatment is essential in breaking down the lignin structure and enhancing the availability of fermentable sugars to overcome this challenge. Many pretreatment methods have been developed, including chemical (acid and alkali), biological, and physical techniques. However, these methods differ in efficiency, environmental impact, and cost-effectiveness, necessitating the exploration of optimized approaches tailored to specific feedstocks, such as SCB.

Therefore, integrating microbial pretreatment technologies may provide a pathway to optimize SCB degradation and biomethane production. This research contributes to developing sustainable bioenergy solutions and aligns with global efforts to reduce environmental waste and reliance on fossil fuels. The outcomes of this study can inform scalable bioconversion processes, benefiting the environment and the energy sector while promoting a circular economy.

1.2 Research Problem Statement

Climate change and the urgent global demand for sustainable energy solutions have highlighted the importance of efficient valorization of agricultural waste, which requires advanced approaches to biomass conversion (Moustakas *et al.*, 2020, Østergaard *et al.*, 2022, Hassan *et al.*, 2024). Biomethane production from agricultural waste, such as SCB, is a promising approach since it holds significant potential as a feedstock for bioenergy production due to its abundance and high cellulose content. However, inadequate pretreatment methods often hamper its efficiency due to its complex lignocellulosic structure (Kumar *et al.*, 2021, Yadav *et al.*, 2024). Profiling and isolating microbial communities in the gastrointestinal tracts of ruminants provides potential enzymatic and metabolic capabilities that could enhance SCB degradation and subsequent biogas production. Regardless of this potential, the functional capacity, diversity, and optimization of microbial-driven bioconversion techniques for SCB have yet to be thoroughly investigated. Additionally, the relative efficiency of various pretreatment strategies in enhancing these processes warrants further exploration.

1.3 Research Aim, Objectives, and Hypothesis

1.3.1 Aim

This proposed research study is based on the knowledge that microorganisms from unusual sources adapt with time to secrete enzymes with unique attributes that can be improved for potential contribution to industrial value. Hence, the study aims to harness microbial communities and bacterial isolates for the efficient bioconversion of SCB into biomethane through functional genomics and, ultimately, to promote a circular economy.

1.3.2 Objectives

- Profile microbial communities in the various compartments of the GIT of 10 goats using a culture-independent method.
- Isolate and evaluate bacterial isolates for their CAZyme-producing potential, focusing on surfactants, oxidising agent, and solvent tolerance, as well as growth rate, particularly for glucose.
- Assess the efficiency of biological pretreatment using the selected bacterial isolate.
 - Characterize the biomass using the CHNS analyzer and evaluate the morphological differences using SEM before and after pretreatment.
 - Evaluate the BMP of pretreated SCB using an Automatic Methane Potential Test System (AMPTS II) in order to enhance yield and shorten digestion time.

1.3.3 Hypothesis

Microbial communities from the goat GIT harbour CAZymes-producing bacteria with unique tolerance traits that can be harnessed for biological pretreatment of lignocellulosic biomass, thereby enhancing biomethane yield and reducing digestion time.

1.4 Thesis Structure

Chapter One:

This section outlines the foundational aspects of the research, its core objectives, and the broader implications for the fields of biotechnology and renewable energy.

Chapter Two:

This chapter discusses the use of microorganisms for the pretreatment of agricultural wastes, specifically SCB, with a focus on the mode of action and the role of CAZymes. It further explores how to enhance conditions that facilitate the high yield of CAZyme-producing strains and examines the associated metabolic pathways.

Chapter Three

In this chapter, independent culture techniques were utilized to profile the dynamic adaptations of CAZyme-producing microorganisms within the gastrointestinal tract of South African goats. It explored the diversity and functionality of these microorganisms, highlighting their crucial role in the digestion of complex carbohydrates.

Chapter Four

The bacterial communities identified as dominant (Chapter 3) were isolated in Chapter 4 and evaluated for their cellulolytic degradation capabilities. Growth rates, tolerance to oxidative stress, solvents and surfactants, and glucose production were among the evaluation criteria.

Chapter Five

This chapter describes the pretreatment of SCB using the biological method with the isolated strain selected in Chapter 4. The BMP was further compared between the untreated and pretreated bagasse using the Automated Methane Potential Test System (AMPTS II). To assess the effectiveness of each method, the structural and compositional changes of SCB before and after pretreatment were investigated using Scanning Electron Microscopy (SEM) and a CHNS analyzer, respectively. The study investigated methane production, degradation rates, and enzymatic activity to compare the efficiency of untreated and pretreated strategies in enhancing biogenic methane yield.

Chapter Six

This chapter provides a comprehensive general discussion that integrates the findings from the preceding chapters. It synthesizes the principal results, situates them within the broader scientific context, and draws overarching conclusions from the study. In addition, the chapter outlines actionable recommendations for future research and practical applications, thereby ensuring coherence between the individual studies and the thesis's overall aim.

1.5 References

- AJALA, E., IGHALO, J., AJALA, M., ADENIYI, A. & AYANSHOLA, A. 2021. Sugarcane bagasse: a biomass sufficiently applied for improving global energy, environment and economic sustainability. *Bioresources and Bioprocessing*, 8, 1-25.
- ALI, S., RANI, A., DAR, M. A., QAISRANI, M. M., NOMAN, M., YOGANATHAN, K., ASAD, M., BERHANU, A., BARWANT, M. & ZHU, D. 2024. Recent Advances in Characterization and Valorization of Lignin and Its Value-Added Products: Challenges and Future Perspectives. *Biomass*, 4, 947-977.
- ALY, M. M., TORK, S., AL-GARNI, S. M. & NAWAR, L. 2012. Production of lipase from genetically improved *Streptomyces exfoliates* LP10 isolated from oil-contaminated soil. *Afr J Microbiol Res*, 5, 1125-1137.
- ÇORBACI, C., UYAR, E. & YALÇIN, H. T. 2017. Determination of Enzyme Profiles and Molecular Characterization of Yeast Species Isolated from Butter Samples. *Celal Bayar Üniversitesi Fen Bilimleri Dergisi*, 13, 833-837.
- HAN, X., YANG, Y., YAN, H., WANG, X., QU, L. & CHEN, Y. 2015. Rumen Bacterial Diversity of 80 to 110-Day Old Goats Using 16SrRNA Sequencing. *PLoS One*, 10, 1-12.
- HASSAN, Q., VIKTOR, P., AL-MUSAWI, T. J., ALI, B. M., ALGBURI, S., ALZOUBI, H. M., AL-JIBOORY, A. K., SAMEEN, A. Z., SALMAN, H. M. & JASZCZUR, M. 2024. The renewable energy role in the global energy Transformations. *Renewable Energy Focus*, 48, 100545.
- KABEYI, M. J. B. & OLANREWaju, O. A. 2023. Bagasse electricity potential of conventional sugarcane factories. *Journal of Energy*, 2023, 5749122.
- KUMAR, A., KUMAR, V. & SINGH, B. 2021. Cellulosic and hemicellulosic fractions of sugarcane bagasse: Potential, challenges and future perspective. *International Journal of Biological Macromolecules*, 169, 564-582.
- LEGIN, E., LADRAT, C., GODFROY, A., BARBIER, G. & DUCHIRON, F. 1997. Thermostable amylolytic enzymes of thermophilic microorganisms from deep-sea hydrothermal vents. *Comptes Rendus de l'Académie des Sciences-Series III-Sciences de la Vie*, 320, 893-898.
- MAHAYRI, T. M. 2022. Diversity and composition of the bacterial community in the feces of cattle and goats.
- MOUSTAKAS, K., LOIZIDOU, M., REHAN, M. & NIZAMI, A. 2020. A review of recent developments in renewable and sustainable energy systems: Key challenges and future perspective. Elsevier.
- MUDAKKAR, S. R., ZAMAN, K., KHAN, M. M. & AHMAD, M. 2013. Energy for economic growth, industrialization, environment and natural resources: living with just enough. *Renewable and Sustainable Energy Reviews*, 25, 580-595.
- NAIR, L. G., AGRAWAL, K. & VERMA, P. 2022. An overview of sustainable approaches for bioenergy production from agro-industrial wastes. *Energy Nexus*, 6, 100086.
- ØSTERGAARD, P. A., DUIC, N., NOOROLLAHI, Y. & KALOGIROU, S. 2022. Renewable energy for sustainable development. *Renewable energy*, 199, 1145-1152.

- PRIYANKA, S., JAYASHREE, M., SHIVANI, R., ANWESHA, S. & RAO, B. K. 2018. A Study on Marine Lipolytic Yeasts: Optimization and Characterization of Lipase Enzyme. *Research Journal of Pharmacy and Technology*, 11, 593-598.
- RABAPANE, K. J., IJOMA, G. N. & MATAMBO, T. S. 2022. Insufficiency in functional genomics studies, data, and applications: A case study of bio-prospecting research in ruminant microbiome. *Frontiers in Genetics*, 13.
- RABAPANE, K. J. & MATAMBO, T. S. 2024. Profiling the dynamic adaptations of CAZyme-Producing microorganisms in the gastrointestinal tract of South African goats. *Heliyon*, 10.
- RIBEIRO, G., GRUNINGER, R., BADHAN, A. & MCALLISTER, T. 2016. Mining the rumen for fibrolytic feed enzymes. *Animal Frontiers*, 6, 20-26.
- ROY, M., KUMAR, R., RAMTEKE, A. & SIT, N. 2018. Identification of lipase producing fungus isolated from dairy waste contaminated soil and optimization of culture conditions for lipase production by the isolated fungus. *Journal of Microbiology, Biotechnology & Food Sciences*, 8.
- SALGADO, V., FONSECA, C., DA SILVA, T. L., ROSEIRO, J. C. & EUSÉBIO, A. 2019. Isolation and Identification of *Magnusiomyces capitatus* as a Lipase-Producing Yeast from Olive Mill Wastewater. *Waste and Biomass Valorization*, 1-15.
- SINGH, B., MAL, G., KALRA, R. S. & MAROTTA, F. 2024a. Microbial-Rich Niches in Herbivores. *Probiotics as Live Biotherapeutics for Veterinary and Human Health, Volume 1: Functional Feed and Industrial Applications*. Springer.
- SINGH, N., GAUTAM, S. B., SAWOOD, G. M., YADAV, V. L., MISHRA, G., DIXIT, S. & GUPTA, S. 2024b. Bioenergy from Agro-waste: A Sustainable Solution for Energy Needs. *Sustainable Clean Energy Production Using Waste Biomass: Sustainable Energy Production and Utilization*. Springer.
- SPYROPOULOS, G. 2024. *Bagasse Utilization and Management Strategies in São Paulo Sugarcane Mills*.
- WANG, J., GHOSH, S., OLAYINKA, O. A., DOĞAN, B., SHAH, M. I. & ZHONG, K. 2022. Achieving energy security amidst the world uncertainty in newly industrialized economies: The role of technological advancement. *Energy*, 261, 125265.
- YADAV, P., ANU, KUMAR TIWARI, S., KUMAR, V., SINGH, D., KUMAR, S., MANISHA, MALIK, V. & SINGH, B. 2024. Sugarcane bagasse: An important lignocellulosic substrate for production of enzymes and biofuels. *Biomass Conversion and Biorefinery*, 14, 6111-6142.

CHAPTER TWO: LITERATURE REVIEW

2.1 Biomethane and Its Potential as a Sustainable Source of Renewable Energy

Biomethane is a renewable energy source derived from the purification of biogas, which is produced through the anaerobic digestion (AD) of organic matter by microorganisms under anoxic conditions. The raw biogas is a heterogeneous gas mixture, typically consisting primarily of 60-70% methane (CH_4) and 30-40% carbon dioxide (CO_2), as well as trace gases such as hydrogen sulfide (H_2S), ammonia (NH_3), and water vapour (H_2O), depending on the organic composition of the substrate (Kabeyi and Olanrewaju, 2022, Ekwebelem *et al.*, 2020, Mignogna *et al.*, 2023). The process of biogas formation occurs in several stages (Figure 2.1), including hydrolysis, which involves the breaking down of complex organic molecules, such as carbohydrates, proteins, and lipids, into fermentable molecules, including sugars, amino acids, and fatty acids (Sivamani *et al.*, 2021, Singh, 2025). In the second stage, acidogenesis, the simple molecules produced are further degraded by acidogenic bacteria to produce volatile fatty acids (VFAs), alcohols, hydrogen (H_2), and CO_2 . Acetogenic bacteria in the third stage (acetogenesis) then convert the VFAs and alcohols into acetic acid, H_2 , and CO_2 . Lastly, the methanogenic archaea then convert the acetic acid, H_2 , and CO_2 into methane and water during the methanogenesis stage (Kabeyi and Olanrewaju, 2022, Harirchi *et al.*, 2022).

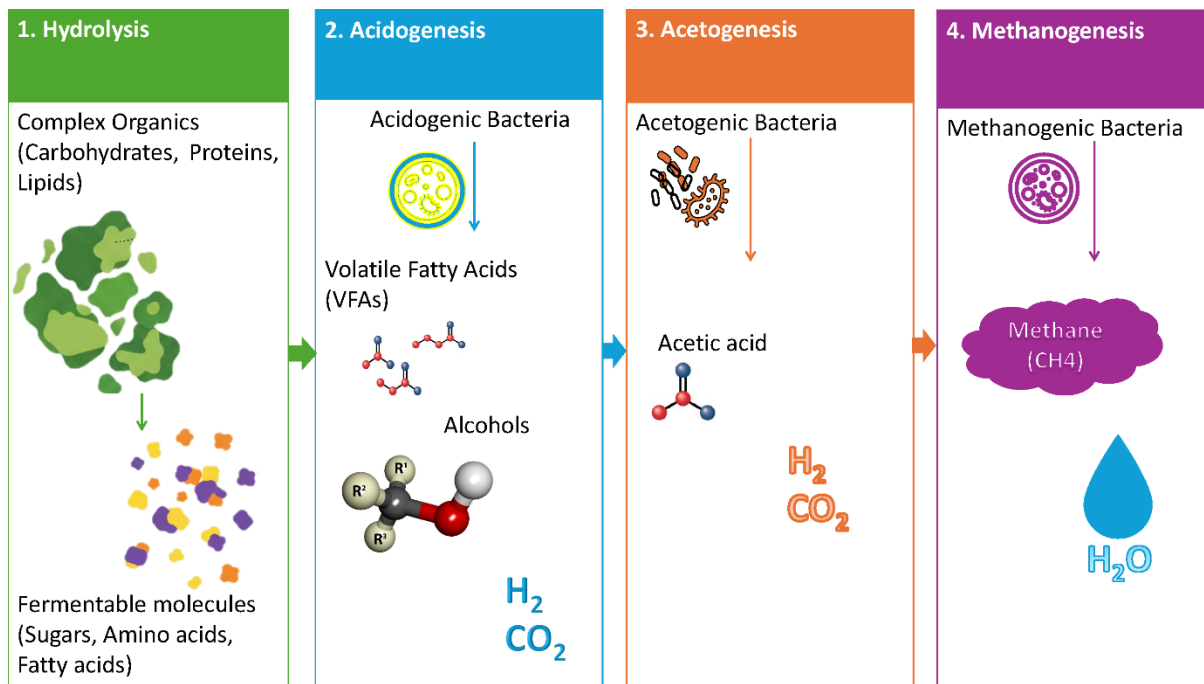


Figure 2.1: Sequential stages of biogas production, illustrating the microbial processes and key intermediates from hydrolysis to methanogenesis (Generated by author).

Although biogas comprises a mixture of methane, carbon dioxide, and other trace gases, its energy value is primarily determined by the methane fraction. As such, biogas yield is often interpreted relative to methane production, with higher methane content indicating more efficient substrate conversion and greater bioenergy potential (Kabeyi and Olanrewaju, 2022). Raw biogas can be used directly for heat and electricity generation (Rafiee *et al.*, 2021; Malode *et al.*, 2021; Abanades *et al.*, 2022). Furthermore, the AD process also produces digestate, a byproduct rich in nutrients with the potential to be used as a biofertilizer, enhancing soil fertility and structure (Kabeyi and Olanrewaju, 2022). This approach of biogas production promotes a biological loop process that facilitates the 'recovery and reuse' of bio-based raw materials (Figure 2.2), thereby contributing to the establishment of a circular economy (Vanhamäki *et al.*, 2020, Spyridonidis *et al.*, 2020).

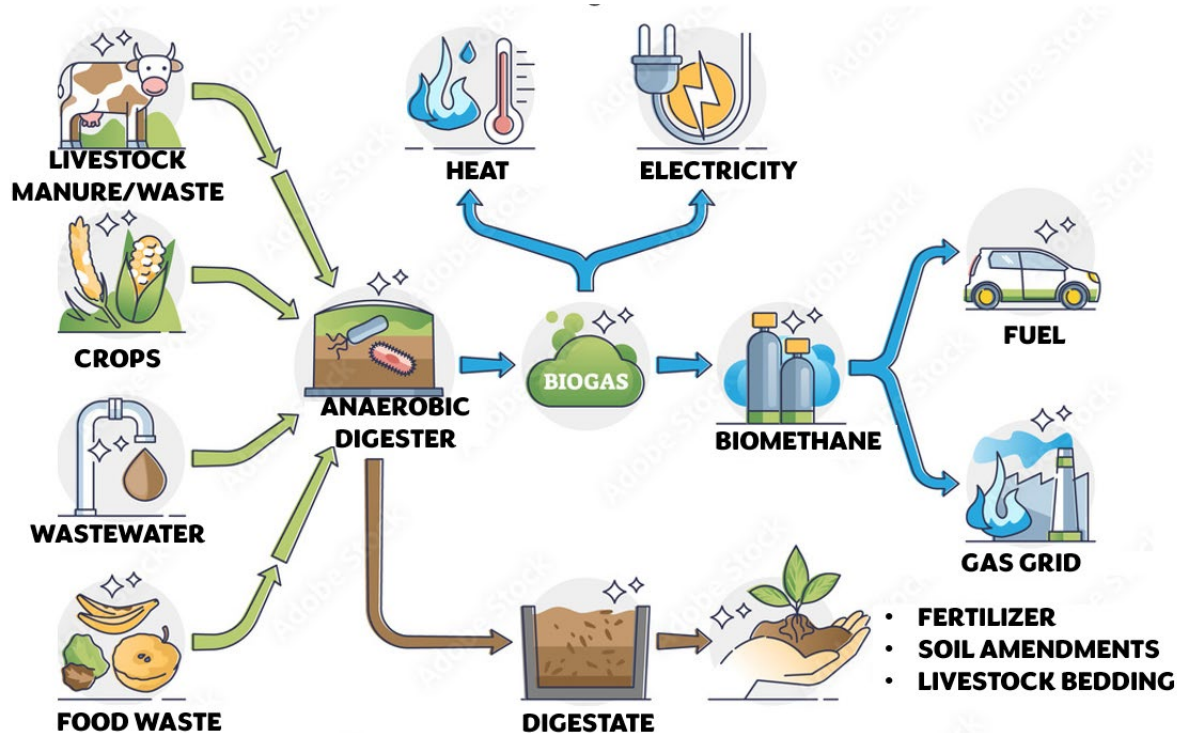


Figure 2.2: Biogas and biomethane processes from the introduction of feedstock to their bioproducts (VectorMine/Getty images)

In addition to the various applications mentioned above, biogas production offers numerous environmental, economic, and social benefits. Firstly, by capturing methane that would otherwise be released from decomposing organic waste in landfills, biogas production mitigates the impact of climate change by reducing greenhouse gas emissions. Additionally, utilizing biogas in this manner reduces reliance on fossil fuels, resulting in decreased carbon dioxide emissions. Biogas production further creates a sustainable solution for waste management, including agricultural residues, food waste, and municipal solid waste, diverting it from landfills and reducing environmental pollution (Srivastava *et al.*, 2020, Rama *et al.*, 2023, Blair and Mataraarachchi, 2021, Francisco López *et al.*, 2024). When utilized to produce biogas, readily available and local organic feedstocks can reduce dependence on imported fossil fuels and enhance energy security (Rocha-Meneses *et al.*, 2023). Research has also shown that the biogas sector can stimulate economic activity and job creation in rural and urban areas. Farmers and waste management companies can generate additional income from biogas production and the sale of digestate as fertilizer (Sørensen and Jørgensen, 2022, Verner *et al.*, 2021). South Africa's biogas industry is still in its early stages of development, but pilot studies

and initiatives suggest that it has the potential to drive rural economic growth, such as those documented by the Department of Fisheries, Forestry and the Environmental ([biogasguidebook_smme.pdf](#)) and industry reports from Biogas SA and the Southern African Biogas Industry Association (SABIA) suggest that it has the potential to drive rural economic growth. According to the South African National Energy Development Institute (SANEDI), small-scale biogas digesters in rural areas can contribute to job creation, improved waste management, and increased energy access (Rasimphi *et al.*, 2024). Projects in the Eastern Cape, KwaZulu-Natal, and Limpopo have demonstrated how farmers and communities can use organic waste to generate biogas and digestate, creating income streams through fertilizer sales and plant maintenance. However, the sector is hindered by limited policy incentives and financial support (Rasimphi *et al.*, 2024). Beyond these economic and energy benefits, effective management of organic waste through biogas production also improves sanitation and helps mitigate diseases related to waste and water contamination (Werkneh and Gebru, 2023, Ashokkumar *et al.*, 2022).

2.2 Biogas Upgrading Technologies: Pathways to Biomethane Purification

While raw biogas offers multiple environmental and economic benefits, its direct use is limited by the presence of impurities, including carbon dioxide (CO₂), hydrogen sulfide (H₂S), ammonia (NH₃), and water vapor (Werkneh, 2022, Kabeyi and Olanrewaju, 2023). These components reduce the energy value of biogas, posing challenges related to combustion efficiency, equipment corrosion, and compliance with energy standards (Kabeyi and Olanrewaju, 2023). Thus, to unlock the full energy potential of biogas and enable its use in high-value applications, it must be upgraded into biomethane, a purified methane stream with a content of at least 90% CH₄ (Kazimierowicz *et al.*, 2025).

Several biogas upgrading technologies are available, each with distinct mechanisms, efficiencies, and suitability depending on the scale of operation, feedstock composition, and intended end use (Ahmed *et al.*, 2021). Pressure swing adsorption (PSA) utilizes adsorbent materials, such as activated carbon or zeolites, to selectively capture CO₂ under high pressure. When the pressure is reduced, the adsorbed gases are released, leaving behind a high-purity methane stream. PSA is widely adopted due to its modularity and dry operation (Molatudi *et*

al., 2025, Francisco López *et al.*, 2024). Membrane separation, on the other hand, utilizes semi-permeable membranes that allow smaller molecules, such as CO₂ and H₂S, to pass through while retaining methane (Francisco López *et al.*, 2024, Soto Guzmán, 2021). Multi-stage membrane systems can achieve high methane purity with relatively low energy demand, making them compact and scalable, which makes them ideal for decentralized biogas plants (Avezova *et al.*, 2024, Villarim, 2023).

Water scrubbing is another common method, where CO₂ and H₂S are absorbed into water under pressure. This physical absorption process is simple and cost-effective, particularly for agricultural and small-scale digesters, although it requires large volumes of water and subsequent treatment of the scrubbing liquid. Chemical scrubbing, such as amine absorption using monoethanolamine (MEA), involves reversible chemical reactions that bind CO₂ with high selectivity and efficiency (Joseph and Sawant, 2021). However, this method requires chemical regeneration and raises environmental concerns related to solvent handling (Francisco López *et al.*, 2024, Joseph and Sawant, 2021). Cryogenic separation, which cools biogas to extremely low temperatures to condense and separate gases based on their boiling points, yields very high methane purity but is energy-intensive and best suited for large-scale operations. Emerging biological upgrading technologies, which use microalgae or methanotrophic bacteria to biologically remove CO₂, are still under development but show promise for low-energy, sustainable purification (Francisco López *et al.*, 2024).

The choice of upgrading technology depends on several factors, including the methane concentration in raw biogas, flow rate, operational costs, and regulatory requirements (Ahmed *et al.*, 2021). In regions like South Africa, where small-scale digesters are more common, biogas is often used directly without upgrading due to cost constraints. Nevertheless, when upgrading is required for grid injection or vehicle fuel, membrane separation and water scrubbing may offer practical and adaptable solutions because of their relative simplicity and lower infrastructure demands.

At a laboratory scale, BMP is commonly quantified using systems such as the Automatic Methane Potential Test System (AMPTS), which enables precise measurement of methane production under controlled anaerobic conditions. In this study, the BMP was quantified using the Automatic Methane Potential Test System (AMPTS) (Figure 2.3).

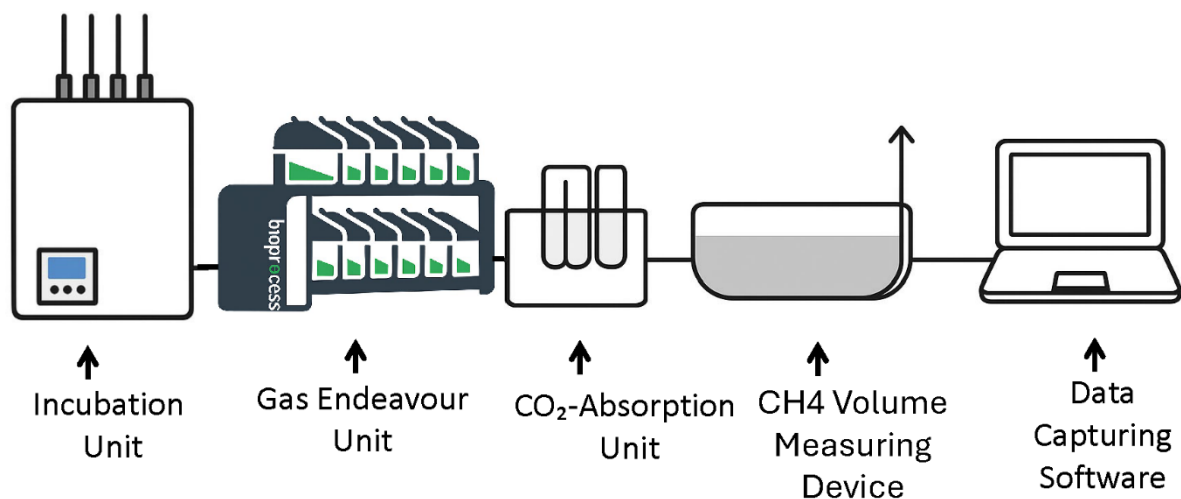


Figure 2.3: Schematic representation of the AMPTS II system (Bioprocess Control, Sweden) showing the main components involved in BMP analysis, including the incubation unit, Gas endeavour CO₂-absorption unit, CH₄ volume measuring device, and data acquisition software (Generated by author).

The AMPTS System does not perform biogas upgrading in the industrial sense; however, it isolates methane for measurement purposes. The system operates by passing the produced biogas through a carbon dioxide absorption unit, typically containing a sodium hydroxide (NaOH) solution, which chemically removes CO₂ from the gas stream. The remaining methane is then measured using a high-precision volumetric sensor (BPC Instruments, 2022). This setup ensures that only methane is quantified, allowing for accurate determination of the methane yield from each substrate. By focusing on BMP rather than total biogas volume, this study aligns with the broader technological and policy shift toward the use of purified methane.

2.3 Current Drawbacks in Biogas Production Processes

Despite its advantages, biogas production faces several challenges that must be addressed to maximize its potential. For instance, lignocellulosic biomass, typically used as a feedstock such as agricultural residues, comprises complex polymers that must be effectively broken down into fermentable sugars (Figure 2.4). Due to their availability, abundance, and sustainability, lignocellulosic biomass is preferred for biogas production (Gao *et al.*, 2022, Kainthola *et al.*, 2019). These renewable and often cost-effective materials, including agricultural residues such as corn stover and wheat straw, sugarcane bagasse, forestry by-products, and organic waste —

represent attractive feedstocks for bioenergy production. They contain elevated levels of organic matter, primarily cellulose, hemicellulose, and lignin, which microbes can break down to produce biogas under suitable conditions (Ghatak, 2011, Priya *et al.*, 2025, Guo *et al.*, 2024). An effective pretreatment method releases sugars from cellulose and hemicellulose, thereby enhancing methane production and yielding higher biogas yields compared to non-pretreated feedstocks. Furthermore, using lignocellulosic biomass mitigates environmental issues such as waste accumulation and pollution from open burning, supporting waste-to-energy initiatives and a circular economy (Guo *et al.*, 2024, Olatunji *et al.*, 2021, Hernández-Beltrán *et al.*, 2019, Mirmohamadsadeghi *et al.*, 2021).

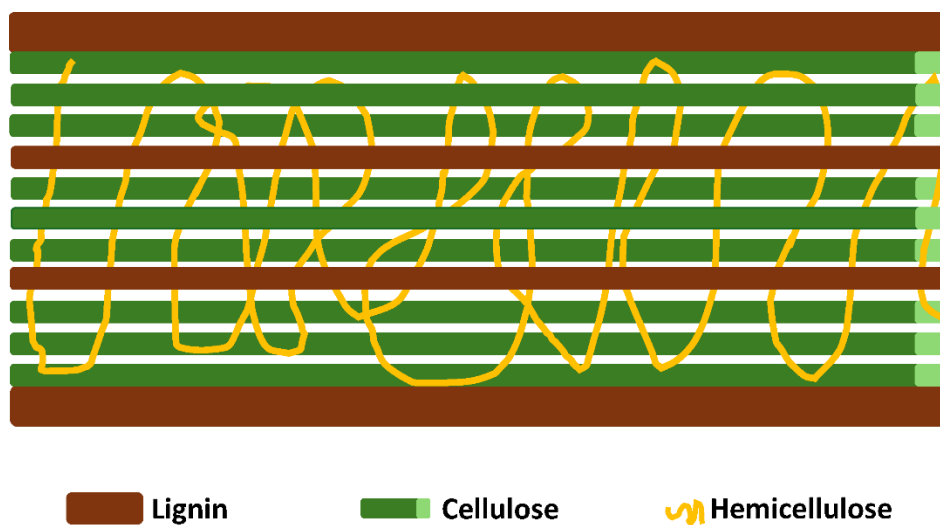


Figure 2.4: Schematic representation of lignocellulose structure, highlighting cellulose microfibrils (green), hemicellulose matrix (yellow), and lignin network (brown) that collectively reinforce plant cell wall integrity and resistance to degradation (Generated by author).

These materials can also be co-digested with other wastes, such as manure or food scraps, thereby improving the carbon-to-nitrogen (C/N) ratio for more efficient digestion. For instance, co-digesting materials such as crop residues or food waste (which are carbon-rich) with nitrogen-rich substrates like animal manure has proven effective in balancing the C/N ratio. This synergy not only stabilizes the digestion process but also increases biogas yield and methane content (Zhang *et al.*, 2014). Field and lab-scale studies confirm that blending these diverse organic wastes improves digestion kinetics, reduces lag phases, and enhances overall energy recovery (Li *et al.*, 2011). In a recent study, the anaerobic co-digestion of cooked food waste collected from a cafeteria and groundnut shells was explored, and it was found that the process increased the C/N ratio, balanced nutrients, mitigated toxicity, and improved

biomethane production, demonstrating the benefits of combining carbon-rich and nitrogen-rich substrates (Olatunji *et al.*, 2025). Another study evaluated biogas production from the co-digestion of food waste and cow dung under mesophilic conditions using a batch digester. The study assessed key physicochemical parameters, including total solids, volatile solids, moisture content, C/N ratio, and pH, before and after digestion, highlighting the impact of substrate composition on biogas yield and methane composition (Bidiko *et al.*, 2025). They found that under optimized conditions (Cafeteria Food Waste: Cow Dung ratio of 3, pH 7.0, 37.5 °C), co-digestion achieved a maximum biogas yield of 197 mL with 62.5% methane, demonstrating the strong influence of substrate composition on biogas yield and quality. The co-digestion of dairy manure and cucumber vines also significantly improved the bioconversion efficiency of livestock manure and crop straw, suggesting that high-carbon crop residues, when combined with nitrogen-rich manure, can enhance nutrient balance and process stability in AD. This synergy not only stabilizes the digestion process but also increases biogas yield and methane content (Wang *et al.*, 2025). As such, integrating diverse organic inputs into anaerobic systems is a vital consideration in the design and operation of efficient biogas facilities. Thus, improving the C/N ratio through co-digestion is widely recognized as a critical strategy for enhancing AD efficiency. The C/N ratio significantly influences microbial activity during digestion, with an optimal range generally falling between 20:1 and 30:1 (Yen and Brune, 2007). Feedstocks with an imbalanced C/N ratio can hinder the process, for instance excess nitrogen may cause ammonia inhibition, while excess carbon can lead to nutrient deficiency and reduced microbial growth. For example, a recent study reported that when the total nitrogen (TN) concentration reached 3000 mg/L, methane yield dropped by more than 55.7% compared to a TN concentration of 2000 mg/L, indicating that ammonia inhibition becomes significant above 2000 mg/L during co-digestion of food waste and livestock manure (Lee *et al.*, 2024).

In addition to balanced feedstock requirements, some drawbacks associated with utilizing lignocellulosic biomass include the need for pretreatment to address lignin's recalcitrance to microbial degradation, which can render the process expensive and complex. Despite these challenges, ongoing research continues to optimize processes, making lignocellulosic biomass increasingly viable for sustainable biogas production (Woźniak *et al.*, 2025, Mirmohamadsadeghi *et al.*, 2021). These drawbacks highlight the importance of precise substrate evaluation and accurate quantification of methane yield, which can inform feedstock

selection, process optimization, and the selection of the most suitable technology. In this context, the use of AMPTS to determine BMP becomes a critical tool for addressing inefficiencies and unlocking the full value of biogas systems.

2.4 The Crucial Role of Pretreatment in Enhancing Biogas and Biomethane Yield: A Potential for Optimization

Pretreatment is a crucial stage in the biogas production process, especially for lignocellulosic biomass, which comprises complex structures that can be resistant to microbial disintegration. Effective pretreatment methods can significantly enhance the biogas yield by improving biomass accessibility to anaerobic microorganisms (Karimi and Taherzadeh, 2016). One of the primary purposes of pretreatment is increased substrate accessibility (Bidiko *et al.*, 2025). Methods such as chemical, mechanical, and biological processes (Figure 2.5) work by disrupting the lignocellulosic structure, which then exposes cellulose and hemicellulose to enzymatic hydrolysis, ultimately increasing the availability of fermentable sugars for biogas production (Olatunji *et al.*, 2021, Poddar *et al.*, 2022). Another factor is the reduction of inhibitory compounds found in some substrates containing compounds such as lignin, phenolic compounds, and tannins, which inhibit microbial activity (Cai *et al.*, 2021, Balasundaram *et al.*, 2022, Bidiko *et al.*, 2025). An effective pretreatment method could help remove or modify these inhibitory compounds, thereby promoting microbial degradation and biogas production. Digestion rates can also be accelerated by breaking complex organic materials into simpler molecules, leading to shorter retention times and increased biogas production rates (Uddin and Wright, 2023, Wang *et al.*, 2023, Abubakar *et al.*, 2022).

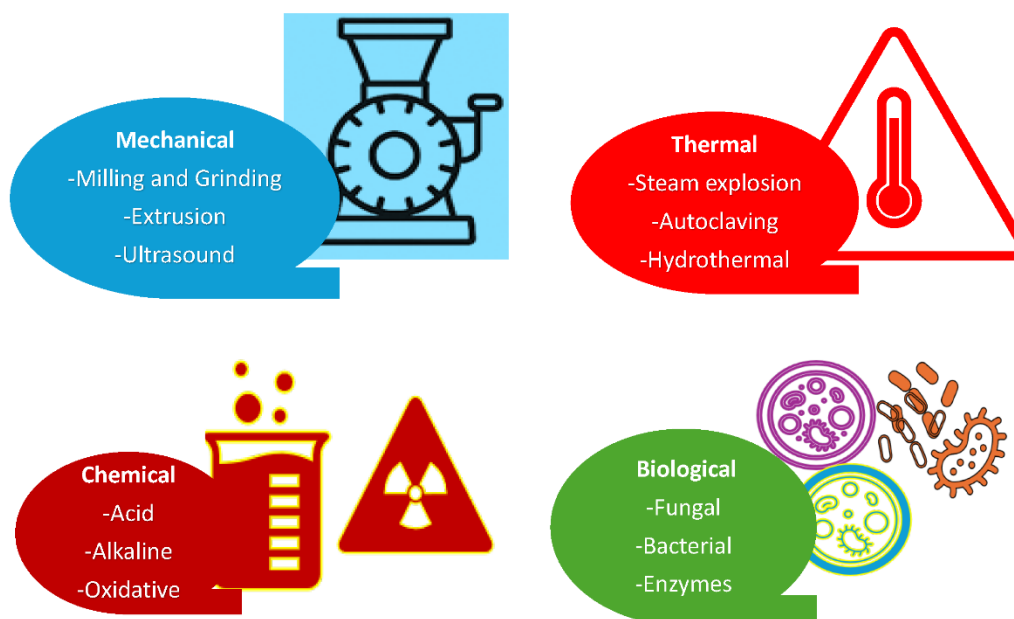


Figure 2.5: The main pretreatment strategies for lignocellulosic biomass: mechanical, chemical, thermal, and biological (Generated by author)

To promote the digestibility and biodegradability of biomass, numerous pretreatment methods have been implemented and tested, each with varying effectiveness, cost implications, and environmental impact. These methods are categorized into mechanical, chemical, thermal, biological, and combined approaches (Olatunji *et al.*, 2021).

2.4.1 Mechanical Pretreatment

Mechanical pretreatment involves physical processes that aim to reduce the particle size of biomass, thereby increasing its surface area and enhancing its digestibility (Arce and Kratky, 2022). Milling and grinding are common mechanical methods used to break down biomass into smaller particles. While effective, these processes can be energy-intensive (Poddar *et al.*, 2022, Olatunji *et al.*, 2021). The extrusion method forces biomass through a small orifice under high pressure, causing shearing of biomass and structural breakdown, as well as increasing the solubility of carbohydrates, facilitating their breakdown (Pennells *et al.*, 2023, John *et al.*, 2024, Jicsinszky *et al.*, 2023). In the ultrasound method, ultrasonic waves are used to create

cavitation bubbles that implode, generating localized elevated temperatures and pressures that disrupt the cell walls of biomass (Liu *et al.*, 2022). This promotes the accessibility of cellulose and hemicellulose for microbial degradation (Liu *et al.*, 2022).

2.4.2 Chemical Pretreatment

Chemical pretreatment methods use acids, alkalis, or other chemical agents to solubilize lignin and hemicellulose, making cellulose more accessible. Key chemical techniques include acid pretreatment, which uses dilute and concentrated acids, such as sulfuric and hydrochloric acid, to hydrolyze hemicellulose and break down the lignocellulosic structure. While effective, this method can produce inhibitory byproducts and requires neutralization before AD (Liu *et al.*, 2022, Baksi *et al.*, 2023, Nguyen *et al.*, 2021, Varjani *et al.*, 2022, Wolfaardt *et al.*, 2021). In alkaline pretreatment, alkaline agents such as sodium hydroxide, potassium hydroxide, and ammonia are used to saponify lignin and hemicellulose, thereby enhancing cellulose accessibility. Alkaline pretreatment is less likely to produce inhibitory byproducts than acid pretreatment (Alino *et al.*, 2022, Kim and Karthikeyan, 2021). Oxidative pretreatment utilizes oxidizing agents such as hydrogen peroxide or ozone to degrade lignin and hemicellulose. While highly effective, this method may require careful handling of chemicals and controlled reaction conditions (Zhou *et al.*, 2023, Di Fraia *et al.*, 2024).

2.4.3 Thermal Pretreatment

Thermal pretreatment uses heat to disrupt the lignocellulosic structure of biomass, increasing its digestibility. Common thermal methods include steam explosion, where biomass is treated with high-pressure steam and then rapidly depressurized, causing the material to explode and break down (Yu *et al.*, 2022). This process effectively disrupts the lignocellulosic matrix, promoting enzymatic hydrolysis. In autoclaving, the biomass is subjected to high temperatures and pressures in an autoclave, which denatures lignin and hemicellulose, making cellulose more accessible. Although effective, autoclaving can be energy-intensive (Das *et al.*, 2021, Bansod *et al.*, 2024). In contrast, hydrothermal treatment treats biomass with hot water or steam

at moderate temperatures and pressures. This method effectively solubilizes hemicellulose and partially breaks down lignin (Sun *et al.*, 2022, Scapini *et al.*, 2021).

2.4.4 Biological Pretreatment

Biological pretreatment utilizes microorganisms or enzymes to break down lignocellulosic biomass. These methods are environmentally friendly and effective at improving digestibility. Common biological techniques include fungal, bacterial, and enzymatic pretreatment (Li *et al.*, 2022, Mustafa *et al.*, 2022, Chhetri *et al.*, 2022, Wu *et al.*, 2022). White-rot and brown-rot fungi are employed to degrade lignin and hemicellulose, making cellulose more accessible. Although slow, fungal pretreatment is highly effective and environmentally friendly. Certain bacteria, including cellulolytic and hemicellulolytic strains, degrade lignocellulosic biomass. Bacterial pretreatment is effective but may require specific environmental conditions. Enzymes such as cellulases, hemicellulases, and ligninases hydrolyze lignocellulosic biomass. This method is highly specific and efficient, but can be costly due to enzyme production (Wu *et al.*, 2022, Mustafa *et al.*, 2022)

2.4.5 Combined Pretreatment Approaches

Recent research has focused on integrating mechanical, chemical, and biological methods to enhance the efficiency of lignocellulosic degradation (Woźniak *et al.*, 2025, Kumar *et al.*, 2019, Dutta *et al.*, 2022). For instance, an alkali pretreatment can be followed by biological treatment to remove remaining lignin and enhance cellulose breakdown (Chen *et al.*, 2010, Kim *et al.*, 2016). In a comparative study, SCB treated with NaOH at 5% and 10% (w/v) concentrations yielded methane increases of 21.2% and 34.1%, respectively, compared to the untreated control. Although these results affirm the efficacy of alkaline pretreatment, the study also noted the generation of toxic effluents and higher operational costs associated with chemical use. By contrast, a more sustainable combined pretreatment. Thus, pre-storing SCB with fruit and vegetable waste (FVW) enhanced anaerobic digestion by introducing readily degradable substrates and additional microbial consortia. This co-storage not only improved substrate accessibility but also balanced the C:N ratio, resulting in the highest methane yield (520 ± 7

NL CH₄/kg VS), a 27.6% increase over the control, and shortened the degradation time from 13 to 7 days. While alkaline pretreatment remains a powerful method, these findings highlight the growing relevance of biological or hybrid pretreatments for enhancing AD while minimizing environmental impact and chemical dependency (Alino *et al.*, 2022). Furthermore, this combination approach improves digestibility and reduces reliance on harsh chemicals, making the process more cost-effective. Biological pretreatment can mitigate inhibitors produced during chemical treatments, such as furfural and Hydroxymethylfurfural (HMF), thus improving microbial activity during AD. This approach further aligns with sustainability goals by optimizing energy use and minimizing the environmental footprint. It is also adaptable and scalable, allowing for the efficient processing of various feedstocks. It also reduces the overall process complexity by leveraging milder chemical conditions and more efficient biological processes. This balanced approach addresses energy consumption, cost, and scalability challenges while improving biogas yield.

2.5 Environmental Benefits and Sustainability Challenges of SCB Utilization

SCB, a highly abundant lignocellulosic agricultural residue generated as a byproduct during sugar extraction, holds significant potential for biogas production (Kumar *et al.*, 2021, Negrão *et al.*, 2021). Comprising approximately 30–35% of the total sugarcane weight, bagasse is rich in cellulose and hemicellulose but contains a significant amount of lignin, making it resistant to degradation (Kumar *et al.*, 2021). This lignocellulosic structure poses a challenge for AD, necessitating effective pretreatment methods to enhance its digestibility. SCB's relatively high carbon content offers substantial potential for biogas production; however, its high lignin content limits microbial access to fermentable sugars (Mubarak Alqahtani, 2024, Konde *et al.*, 2021). To overcome this, a combination of alkali-acid and biological pretreatments can break down the complex structure, improving the availability of sugars and, thus, the efficiency and yield of biogas (Poddar *et al.*, 2022, Konde *et al.*, 2021, Ajala *et al.*, 2021).

SCB is produced in areas with substantial sugarcane farming, particularly in tropical and subtropical regions like Brazil, India, China, Thailand, and several parts of Africa, including South Africa, Eswatini, Egypt, Kenya, and Mauritius (Tiema *et al.*, Omuse *et al.*, 2024).

Despite being globally abundant and having great potential, SCB is often underutilized in many areas, with its use primarily restricted to bioenergy production within sugar mills (Bezerra and Ragauskas, 2016, Mubarak *et al.*, 2024, Konde *et al.*, 2021). The considerable environmental and economic challenges associated with the underutilization of SCB highlight the need to explore its potential, particularly for sustainable biogas production. Furthermore, bagasse is a low-cost waste product from the sugar industry, making it an economically viable substrate for large-scale, cost-effective, and sustainable biogas generation (Hiranobe *et al.*, 2024).

2.6 The Abundance and Environmental Impact of Underutilized SCB

Globally, sugarcane production exceeds 1.9 billion tons annually, with about one-quarter to one-third of this amount converted into bagasse (Kabeyi and Olanrewaju, 2023, Negrão *et al.*, 2021). Bagasse typically represents 25–30% of the processed cane weight, while sugar accounts for around 10–15%, with the remainder consisting of water and other residues. (Mankar *et al.*, 2021, Nhleko and Sekoai, 2025). In South Africa, sugarcane production ranges from 19 to 22 million tons each year, resulting in approximately 6 to 8 million tons of bagasse (Mnisi and Dlamini, 2012, Pryor *et al.*, 2017, Nhleko and Sekoai, 2025). This agricultural residue is primarily used for cogenerating electricity in sugar mills, but its broader applications have been limited. However, the plentiful supply of bagasse presents a significant opportunity to develop bio-based products, such as biofuels, bioplastics, and biofertilizers. In many African countries, particularly South Africa, this abundant raw material could support local industries, reduce dependence on imported materials, and create new economic opportunities (Hugo, 2010, Seebaluck *et al.*, 2008, Smithers, 2014, Kohler, 2016).

Despite its abundance, countries like South Africa have not fully utilized SCB due to several challenges, such as resource constraints, limited technological capabilities, and inadequate investments in research and development, which are significant barriers. Additionally, the economic feasibility of large-scale bagasse utilization remains a concern, as there is often a lack of infrastructure for bioprocessing and advanced biorefineries (Thibane *et al.*, 2023, Mohlala *et al.*, 2016, Meghana and Shastri, 2020). These obstacles highlight the need for targeted strategies to unlock the potential of SCB in South Africa and other regions.

When SCB is not utilized properly, it creates significant environmental challenges (Ajala *et al.*, 2021). In many areas, bagasse is either discarded in landfills or burned openly, leading to air pollution and increased greenhouse gas emissions. Open burning releases particulate matter, carbon dioxide, and other harmful pollutants, which worsen climate change and pose public health risks (Kumar *et al.*, 2021, Sarker *et al.*, 2017). Meanwhile, landfilling bagasse generates methane, a potent greenhouse gas, while also occupying valuable landfill space that could be utilized for other purposes.

In South Africa, waste management infrastructure often faces significant challenges, and the improper disposal of bagasse contributes to the environmental burden (Mbazima *et al.*, 2022). Reports indicate that unutilized agricultural residues, such as bagasse, are major contributors to waste-related emissions in the country (Mandree *et al.*, 2023). Agricultural residues represent a largely untapped resource in South Africa, yet their mismanagement contributes directly to the country's waste burden and greenhouse gas emissions. SCB, which makes up nearly 270–280 kg per ton of cane harvested, is often left underutilized beyond limited energy recovery in mills (Nhleko and Sekoai, 2025). When residues such as bagasse are discarded or burned inappropriately, they add to South Africa's emissions profile. Although crop residues contribute only about 0.1% of total waste (Bega, 2025), their mismanagement accounts for approximately 13% of field crop-related greenhouse gas emissions. This equated to 5.2 million tonnes of CO₂-eq in 2012, while nitrous oxide emissions from residues and fertilizer use together reached 7.3 million tonnes CO₂-eq in 2018 (Tongwane *et al.*, 2016, Bega, 2025, Tongwane *et al.*, 2020). These figures underscore the environmental cost of failing to integrate agricultural residues into value-added, circular economy processes.

This challenge cannot be separated from South Africa's wider waste crisis. The country produces an estimated 50–60 million tonnes of waste annually, of which only about 10% is recycled, leaving the vast majority destined for landfills or illegal dumping (Nqcongo, 2025). Alarmingly, 60% of the country's landfill sites are reported as non-compliant, raising serious concerns about public health and environmental safety (Dawood, 2025). Municipalities such as eThekweni spend hundreds of millions of rands (R180 million) annually on waste removal, with the costs of illegal dumping often surpassing routine collection budgets (Nqcongo, 2025).

Communities living near informal dumping grounds frequently report respiratory illnesses, water contamination, pest infestations, and degraded living conditions (Khan, 2025).

The case of Kya Sands, located in the north of Johannesburg, has become one of the most visible examples of this escalating crisis. A landfill discontinued in 2010 has since been overtaken by criminal networks that manage up to 14 informal dumping sites, which receive hundreds of truckloads of various waste types daily, including garden refuse, building rubble, household garbage, and even toxic and medical waste (Masuku, 2024, Khuboni, 2025). A significant portion of this waste is burned in the open, producing persistent toxic fumes and releasing hazardous pollutants, including dioxins, which pose a threat to community health. Nearby residents have long complained about the deteriorating air quality, and as a result, in 2024, the Johannesburg High Court ordered the municipality to conduct air quality monitoring in the area (Masuku, 2024, Khuboni, 2025). Consequently, the City of Johannesburg allocated R140 million for site rehabilitation, including security measures, waste removal, and monitoring systems (Khuboni, 2025). The Kya Sands example, along with similar dumping crises in Durban (Khan, 2025), Nelson Mandela Bay (Chirume, 2024), Tshwane (Moatshe, 2025), and other regions, highlight how inadequate waste management exacerbates environmental injustice and disproportionately affects vulnerable communities.

In light of these circumstances, utilizing SCB through sustainable technologies such as microbial pretreatment gains significance beyond its technical merits. By diverting residues from harmful disposal pathways and converting them into fuels, enzymes, or high-value bioproducts, microbial pretreatment is not only a potential approach to reduce emissions but also addresses systemic waste challenges. More importantly, it aligns with South Africa's circular bioeconomy aspirations while offering socioeconomic benefits through decentralized, community-level biorefineries. In this way, the utilization of bagasse extends beyond the laboratory scale and becomes part of a broader solution that addresses challenges related to emissions, waste management, and environmental justice in South Africa. Properly utilizing bagasse can further mitigate these impacts by diverting waste from landfills and reducing reliance on fossil fuels for energy and materials. Therefore, addressing the environmental consequences of underutilized bagasse is a key driver for sustainable innovation in the sugarcane industry.

2.7 Biotechnological Potential, Challenges, and Sustainable Opportunities

To effectively break down the complex lignocellulosic structure and enable the extraction of fermentable sugars for bioethanol production and other applications, biological pretreatment methods are essential. Recent biotechnological advancements have highlighted the potential of SCB as a substrate for microbial fermentation and enzymatic hydrolysis (Pandey *et al.*, 2000, Pérez-Contreras *et al.*, 2025). These advancements highlight the potential of utilizing cellulose-degrading strains sourced from various sources as a promising approach to enhance enzymatic hydrolysis. These microorganisms, which are naturally adapted to degrading complex plant materials, provide an efficient means of processing bagasse. By utilizing biological pretreatment methods, it is possible to significantly increase the yield of fermentable sugars, thereby facilitating the production of biofuels and bioplastics (Alawad and Ibrahim, 2024).

Countries such as Brazil and India have made significant advancements in utilizing bagasse for bioethanol production, leveraging large-scale biorefineries and government incentives (Chandel *et al.*, 2019, Pattnaik *et al.*, 2024, Bento *et al.*, 2024). In contrast, South Africa has yet to establish comparable infrastructure or policy frameworks to fully harness this potential. This disparity highlights the importance of creating a supportive environment for research, investment, and industrial-scale applications of bagasse.

Despite its abundance, SCB is underutilized in South Africa due to several challenges, including technological limitations, economic barriers, policy gaps, and a lack of awareness and collaboration among stakeholders. Advanced bioprocessing technologies necessary for large-scale bagasse conversion are not widely accessible. Additionally, research institutions and industries often lack the resources and expertise to implement state-of-the-art biorefinery processes (Gbadeyan *et al.*, 2024, Rosa and Martius, 2021). The initial costs of establishing biorefineries and scaling up production can be significantly high. Without sufficient government support or private investment, the economic viability of bagasse-based industries remains uncertain. Moreover, unlike countries such as Brazil, South Africa has limited policy frameworks and incentives to promote the use of agricultural residues for bio-based industries, hindering sector growth (Gbadeyan *et al.*, 2024, Uzoagba, 2024). Limited awareness among

stakeholders regarding the potential of bagasse, as well as insufficient collaboration between academia, industry, and government, further restricts its utilization. Addressing these challenges requires a multifaceted approach, including investment in research and development, capacity building, and the establishment of public-private partnerships to drive innovation in the sugarcane industry.

Utilizing SCB aligns with global efforts to promote sustainable development and circular economy practices (Uzoagba, 2024). By transforming agricultural waste into valuable products, South Africa can achieve multiple objectives, including environmental sustainability through reduced greenhouse gas emissions, minimized waste, and alleviation of environmental degradation. Developing bagasse-based industries can also create jobs, stimulate local economies, and reduce dependence on imported chemicals and fuels. Additionally, bagasse-derived bioenergy offers a renewable alternative to fossil fuels, contributing to energy security and climate resilience. Advancing bagasse utilization fosters innovation in biotechnology, creating opportunities for academic research and industrial applications. By leveraging its abundant agricultural resources, South Africa has the potential to emerge as a leader in sustainable bioprocessing. However, achieving this vision requires collective efforts to overcome existing challenges and capitalize on the opportunities presented by SCB.

2.8 Goat Microbiota as a Potential Source for Pretreatment of SCB for Biogas Production

The goat's gastrointestinal tract reflects evolutionary adaptations that enable efficient processing of fibrous plant materials in resource-scarce environments. As a small ruminant, the goat possesses a four-chambered stomach comprising the rumen, reticulum, omasum, and abomasum (Figure 2.6), each playing a specialized role in the digestion of feed (Hungate, 1947). The rumen, a large fermentation chamber, harbors a diverse and highly specialized microbial community capable of breaking down lignocellulosic biomass. Unlike other ruminants, goats are highly selective browsers, consuming a varied diet of fibrous plants, including shrubs and coarse vegetation (Smith, 2010, Cunha *et al.*, 2011). This dietary flexibility is complemented by the goat's rumen physiology, which maintains conditions that favor the proliferation of microorganisms adept at lignocellulosic degradation (Hungate, 1947, Russell, 2002). The reticulum facilitates particle size reduction and regurgitation, enabling

thorough mechanical and microbial processing, while the omasum absorbs water and nutrients. Like a monogastric stomach, the abomasum secretes gastric juices that further break down proteins for nutrient absorption (Hungate, 1947, Russell, 2002, Moran, 2005). This system's efficiency makes goats particularly effective in fostering microbial diversity, a crucial factor for bioprospecting microorganisms relevant to SCB pretreatment (Smith, 2010, Cunha *et al.*, 2011).

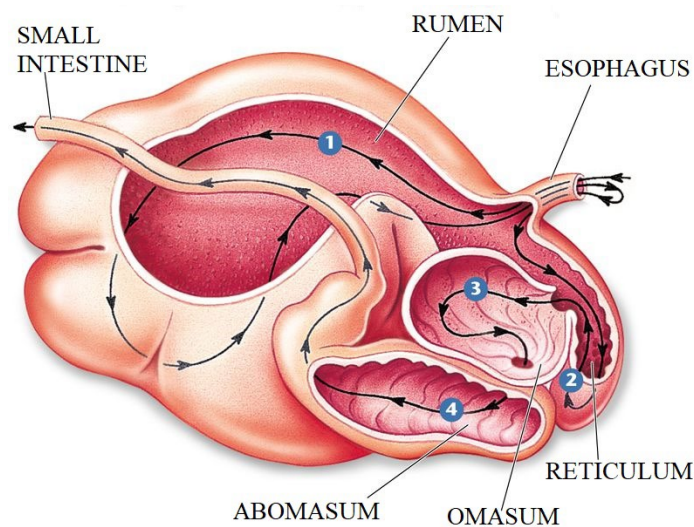


Figure 2.6: Ruminant digestive system showing the flow of feed at the point of ingestion to the esophagus, rumen (1), reticulum (2), omasum (3), and abomasum (4) until it enters the small intestine (Vodopich and Moore, 2002).

Goats exhibit unique characteristics that position their microbiota as superior for the pretreatment of lignocellulosic biomass compared to those of other ruminants. Their microbial ecosystems are highly adaptable and can process a broader range of fibrous materials, including agricultural residues such as SCB. The rumen of goats houses diverse lignocellulose-degrading microbes, including cellulolytic bacteria, lignin-degrading fungi, and protozoa (Smith, 2010, Cunha *et al.*, 2011). These microorganisms live in synergy to break down complex plant polymers, making them ideal candidates for bioprospecting efforts. Furthermore, goat-derived microbial consortia have demonstrated superior enzymatic activity in degrading cellulose and hemicellulose compared to those isolated from cattle or sheep (Thapa *et al.*, 2023). This high enzymatic efficiency, combined with the resilience of goat microbiota to environmental

variations, underscores their suitability for industrial applications targeting lignocellulosic biomass.

Currently, there is a lack of studies specifically examining the pretreatment of SCB by rumen microbiota. Although several research papers discuss the role of the rumen microbiome in lignocellulose degradation (Gharechahi *et al.*, 2022, Hua *et al.*, 2022) and the nutritional value of SCB (Selim *et al.*, 2022), none addresses the specific process of pretreatment by rumen microorganisms. This gap indicates a significant opportunity for future research.

A study conducted by Gharechahi *et al.* (Gharechahi *et al.*, 2022) offers indirect insights into the capabilities of the camel rumen microbiota to degrade various lignocellulosic feeds, including SCB. The research revealed significant variations in the structure and function of rumen microbial communities, influenced by the physicochemical properties of different substrates. Although the study does not directly examine the effects of pretreatment by the rumen microbiota, it suggests the potential for such processes. The observed variations indicate that the rumen microbiome could be manipulated or adapted to enhance the pretreatment of SCB. The authors identified low-abundance taxa, such as members of Verrucomicrobiota, Planctomycetota, and Fibrobacterota, which possessed a disproportionately large number of carbohydrate-active enzymes (CAZymes) per Mb genome. This finding suggests that these microbes may play a significant role in degrading SCB. However, further research is necessary to confirm this potential and to explore the specific mechanisms involved.

Another study conducted by Selim *et al.* (Selim *et al.*, 2022) evaluated the nutrient content and in vitro degradation kinetics of several unconventional feed resources in Bangladesh, including SCB. The researchers used rumen liquor to assess the in vitro degradation of SCB, which provided valuable insights into its digestibility. While this study is crucial for understanding the nutritional aspects of SCB, it does not address the pretreatment process. The use of rumen liquor indicates that the researchers recognized the significance of rumen microbes in the degradation process; however, the study does not investigate the microbial mechanisms underlying the pretreatment of SCB.

Other studies in the literature, while not directly focusing on SCB pretreatment, offer valuable insights into the broader capabilities of rumen microbiota in lignocellulose degradation. These studies provide a contextual framework for understanding the potential of rumen microorganisms for SCB pretreatment. For instance, Hua and colleagues (Hua *et al.*, 2022) reviewed the role of rumen microorganisms in degrading starch and cellulose, emphasizing the use of metagenomics to study the microbial community. This highlights the approach that could be applied to further research on SCB pretreatment. The authors identified various amylases and cellulases, providing a foundation for understanding the enzymatic potential available within the rumen microbiome network. The study by Cui *et al.* (Cui *et al.*, 2023) examined the dynamics of the rumen microbiome in Tibetan sheep under different feeding strategies. This research highlights the rumen microbiome's adaptability and plasticity, suggesting its potential to degrade recalcitrant substrates, such as SCB. The lack of direct studies on SCB pretreatment by rumen microbiota necessitates a critical evaluation of the methods that could be employed in future research. This scarcity has important implications for the sugarcane industry. In countries like South Africa, where SCB is underutilized beyond cogeneration and often accumulates as agricultural waste, the lack of research on microbial pretreatment further represents a missed opportunity to convert a low-value by-product into biofuels, biogas, animal feed, or platform chemicals. Without effective valorization strategies, unutilized SCB contributes to waste-related emissions (CO₂-eq, CH₄, and leachates) and environmental burdens from open dumping or incineration. By contrast, rumen-derived pretreatment strategies, particularly those from goats adapted to fibrous diets, offer a pathway to integrate bagasse into a circular economy, reducing environmental impacts, diversifying end uses, and creating value from agricultural residues. The current research gap thus underscores both the scientific novelty and industrial relevance of investigating goat rumen consortia as a next-generation pretreatment option.

2.9 The Utilization of Metagenomics and Functional Genomics to Explore the Diversity of the GIT Microbiota

Metagenomics, as highlighted by Hua *et al.* (Hua *et al.*, 2022), offers a culture-independent, high-resolution view of the gastrointestinal (GIT) microbial community and the genetic potential for lignocellulose degradation. Shotgun metagenomic sequencing of rumen samples recovers both taxonomic composition and functional gene content, enabling direct detection of

carbohydrate-active enzymes (CAZymes), oxidative enzymes involved in lignin modification, and other accessory proteins implicated in plant cell-wall breakdown (Rabapane and Matambo, 2024). Recent metagenomic studies have revealed extensive repertoires of glycoside hydrolases (GHs), carbohydrate esterases (CEs), polysaccharide lyases (PLs), and auxiliary activity (AA) enzymes in ruminant microbiomes, confirming that the rumen ecosystem is a valuable reservoir of cellulases, xylanases, and lignin-modifying enzymes with potential for industrial bioprocessing (Rabapane and Matambo, 2024).

Functional genomics approaches, which include metatranscriptomics (RNA sequencing of community RNA), metaproteomics, and targeted enzyme activity assays, extend these insights by clarifying which genes are actively expressed and translated under specific conditions. For instance, when SCB is present as a substrate. These methods help identify not only which microbial taxa are present, but which are contributing to fibrolytic activity in situ (Prabha *et al.*, 2025, Tomazetto *et al.*, 2020). Commonly, members of the genera *Ruminococcus*, *Fibrobacter*, and *Prevotella*, as well as cellulolytic fungi, have been implicated in cellulose and hemicellulose degradation, while functional assays also highlight lignin-degrading oxidative enzymes such as laccases and peroxidases. Thus, linking gene presence to gene expression and enzymatic activity is important for selecting candidate microorganisms and enzymes that can be harnessed for biological pretreatment of lignocellulosic biomass (Serrano-Gamboa José *et al.*, 2022, Chirania *et al.*, 2022).

Applied to SCB pretreatment, the combined use of metagenomics and functional genomics has the potential to offer three key benefits. The first being bioprospecting through the discovery of novel cellulases, xylanases, and oxidative enzymes from goat-rumen microbiota for enzymatic pretreatment strategies, followed by mechanistic optimization. Secondly, this will further reveal the microbial interactions and metabolic pathways that correlate with efficient biomass deconstruction, thereby guiding the design of enzyme cocktails or microbial consortia. Thirdly, process monitoring through tracking functional shifts during pretreatment to optimize incubation times, inoculum levels, and process conditions. This integrated approach highlights the value of goat-rumen microbiota as both a source of novel lignocellulolytic enzymes and as an applied pretreatment strategy for enhancing the bioenergy potential of agro-industrial residues such as SCB.

2.10 Catalytic Mechanisms of Lignocellulose-Degrading Enzymes and Microbial Processes

The enzymatic degradation of lignocellulosic biomass by goat-derived microbiota involves a well-coordinated interplay of enzymes and microbial activity (Figure 2.4). Cellulases, hemicellulases, and ligninases are the primary enzymes facilitating the breakdown of SCB (Wu *et al.*, 2022). Cellulases hydrolyze β -1,4-glycosidic bonds in cellulose, converting it into fermentable sugars. This process occurs in three stages. First, endoglucanases cleave internal bonds within cellulose chains, creating free chain ends. Next, exoglucanases act on these chain ends, releasing cellobiose units (Jayasekara and Ratnayake, 2019, Hamid *et al.*, 2015). Finally, β -glucosidases hydrolyze cellobiose into glucose, which fermentative microbes can then metabolize (Ribeiro *et al.*, 2016, Rahman *et al.*, 2018). The precise cleavage of β -1,4-glycosidic bonds ensures the efficient breakdown of cellulose into its monomeric components (Dos Santos *et al.*, 2023), a critical step in biogas production.

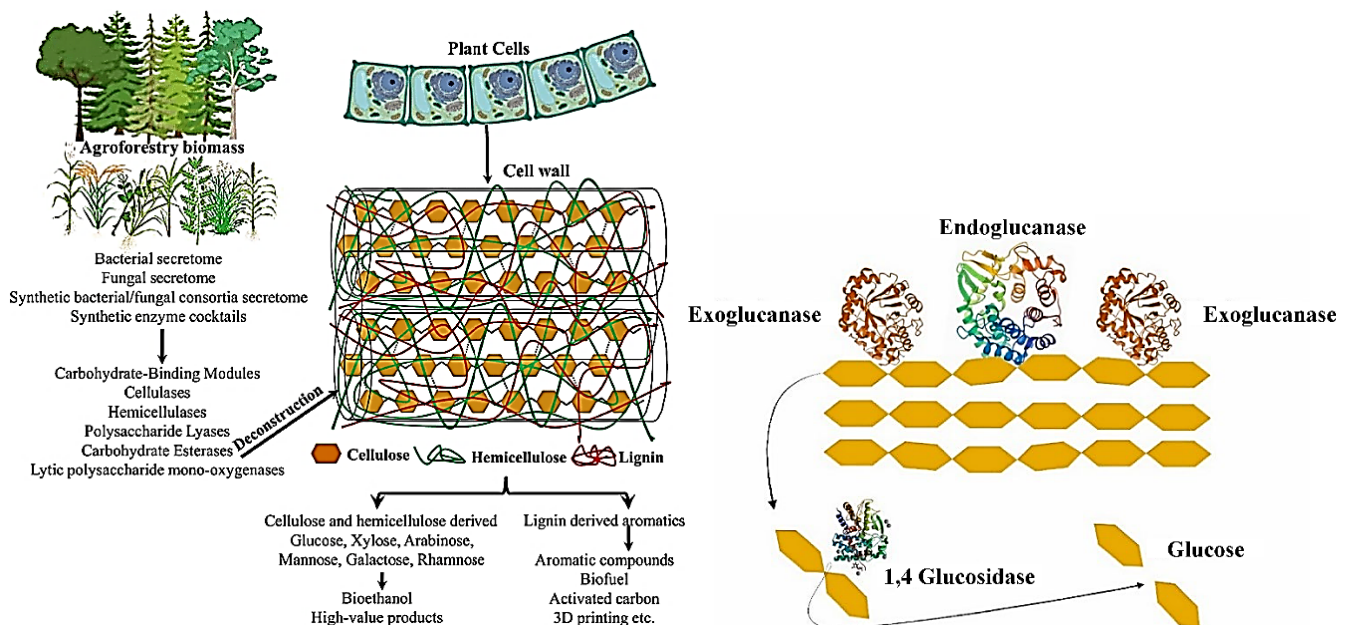


Figure 2.7: Degradation of cellulose polymers by microbial enzymes (Sethupathy, 2021; Datta, 2024).

Hemicellulases target hemicellulose, a heteropolysaccharide composed of various sugar monomers such as xylose, mannose, and arabinose (Rodrigues *et al.*, 2024). Enzymes like xylanases hydrolyze the backbone of xylan, releasing xylooligosaccharides, which are further degraded by β -xylosidases into xylose. Arabinofuranosidases and mannanases also break down

the side chains and backbone of hemicellulose, enhancing substrate accessibility (Kaur *et al.*, 2024, Pisalwar *et al.*, 2023, Monica *et al.*, 2024). Ligninases, including laccases and peroxidases, degrade lignin, a complex aromatic polymer that provides structural integrity to plant cell walls. By cleaving lignin bonds, these enzymes increase the exposure of cellulose and hemicellulose, facilitating enzymatic hydrolysis (Rasool and Irfan, 2024, Chauhan, 2020). The breakdown of lignin is particularly important in SCB pretreatment, as its removal significantly improves biomass digestibility.

2.11 Applications and Limitations of Goat Microbiota to SCB for Biogas Production

A significant research gap exists in the lack of understanding of the specific microbial interactions and synergistic effects that contribute to SCB degradation in the rumen microbiome. Further research is needed to identify the primary species, their functional roles, and the specific enzymes involved in this process. The influence of various environmental factors, such as pH, temperature, and substrate composition, on the efficiency of SCB pretreatment also warrants investigation. Furthermore, concerns regarding the reliance on goat rumen microbiota for biogas production can be alleviated by highlighting their demonstrated benefits, adaptability, replicability, and sustainability (Thapa *et al.*, 2023, Nleya *et al.*, 2025, Rabee *et al.*, 2025). The unique lignocellulolytic capabilities of the goat rumen microbiota offer an effective pretreatment strategy for SCB, enhancing biogas production by improving degradation.

Controlled anaerobic bioreactors that simulate the goat rumen environment facilitate the breakdown of cellulose and hemicellulose into fermentable sugars, thereby increasing methane yields (Ozbayram *et al.*, 2018, Zhang *et al.*, 2013). This biological approach eliminates the need for harsh chemical pretreatments, reducing the formation of inhibitory byproducts and lowering operational costs. Empirical studies have demonstrated improvements in biomethane production and enzymatic hydrolysis efficiency when using microbial consortia derived from goats. For instance, in a wheat straw batch-digestion trial, bioaugmentation with a 2% goat rumen-derived enrichment culture resulted in approximately a 27% higher biomethane yield, and when the enrichment was increased to 6%, the increase reached 36% compared to the standard inoculum (anaerobic sludge from a wastewater treatment plant) (Ozbayram *et al.*,

2018). These findings suggest that goat rumen microbiota are effective at enhancing hydrolysis and fermentation. However, yields depend on the type and concentration of substrate, inoculum loading, and process conditions. In another study, they used cattle rumen fluid to pretreat corn straw, which yielded ~3.6-fold more biomethane than untreated substrate, with the 6% pretreatment concentration showing yields about 20-45% higher than other pretreatment concentrations (3% and 9%) under similar conditions (Yu *et al.*, 2024).

Although rumen-derived inocula and rumen-like enrichment cultures have been widely explored for the hydrolysis of lignocellulosic substrates, explicit reports of goat-rumen microbiota used as a pretreatment specifically for SCB are scarce. Several studies, however, do demonstrate the feasibility and effectiveness of rumen-based approaches on related substrates. Rumen fluid has enhanced hydrolysis and biogas yields from a variety of crop residues (including wheat and corn straw) and has been used to pretreat agave bagasse (Barragán-Trinidad *et al.*, 2017) and other lignocellulosic wastes (Sema *et al.*, 2020, Sumardiono *et al.*, 2023) producing substantial gains in saccharification and biogas production. Taken together, these results indicate strong mechanistic plausibility that rumen communities (including goat-associated microbiota) contain diverse CAZymes and accessory oxidative activities capable of breaking lignocellulosic biomass. However, they also reveal a gap: targeted, well-controlled pretreatment studies that utilize goat-rumen consortia on SCB, with downstream validation such as AMPTS biogas/biomethane assays or saccharification metrics, remain limited or unpublished.

This gap confers clear novelty for studies that aim to isolate or enrich goat-rumen consortia specifically applied to SCB, in order to characterize the functional capacity of CAZyme profiles and expression, as well as enzymatic performance. In addition, validation at the process level encompasses reductions in lignin content or cellulose crystallinity, increases in reducing sugars, and enhanced methane yields in AMPTS or pilot-scale reactors. Demonstrating reproducible enrichment, enzyme activity, and methane yields using goat-derived consortia would therefore both fill an empirical gap and provide a transferable, sustainable pretreatment route for the valorization of SCB.

Notably, the use of live goats for continuous rumen sampling raises significant ethical and logistical concerns, including concerns about animal welfare, invasive procedures, and high

maintenance costs. Recent work has demonstrated that cryopreservable consortia derived from goat feces and selected on lignocellulosic substrates, such as SCB, retain stable degradative capacity, thereby reducing reliance on live animals (Peng *et al.*, 2021). Creating biobanks and sourcing microbial biobanks from such preserved consortia offers a sustainable and ethically sound resource for future bioprospecting and industrial applications.

2.12 References

- ABANADES, S., ABBASPOUR, H., AHMADI, A., DAS, B., EHYAEI, M. A., ESMAEILION, F., EL HAJ ASSAD, M., HAJILOUNEZHAD, T., HMIDA, A. & ROSEN, M. A. 2022. A conceptual review of sustainable electrical power generation from biogas. *Energy Science & Engineering*, 10, 630-655.
- ABUBAKAR, A. M., SILAS, K. & AJI, M. M. 2022. An elaborate breakdown of the essentials of biogas production. *J. Eng. Res. Sci*, 1, 93-118.
- AHMED, S. F., MOFIJUR, M., TARANNUM, K., CHOWDHURY, A. T., RAFA, N., NUZHAT, S., KUMAR, P. S., VO, D.-V. N., LICHTFOUSE, E. & MAHLIA, T. 2021. Biogas upgrading, economy and utilization: a review. *Environmental Chemistry Letters*, 19, 4137-4164.

- AJALA, E., IGHALO, J., AJALA, M., ADENIYI, A. & AYANSHOLA, A. 2021. Sugarcane bagasse: a biomass sufficiently applied for improving global energy, environment and economic sustainability. *Bioresources and Bioprocessing*, 8, 1-25.
- ALAWAD, I. & IBRAHIM, H. 2024. Pretreatment of agricultural lignocellulosic biomass for fermentable sugar: opportunities, challenges, and future trends. *Biomass Conversion and Biorefinery*, 14, 6155-6183.
- ALINO, J. H. L., BASTOS, J. A., REMOR, P. V., FRARE, L. M., ORSSATTO, F., DAMACENO, F. M. & EDWIGES, T. 2022. Alkaline Pretreatment and Pre-Hydrolysis Using Acidic Biowastes to Increase Methane Production from Sugarcane Bagasse. *Methane*, 1, 189-200.
- ARCE, C. & KRATKY, L. 2022. Mechanical pretreatment of lignocellulosic biomass toward enzymatic/fermentative valorization. *IScience*, 25.
- ASHOKKUMAR, V., FLORA, G., VENKATKARTHICK, R., SENTHILKANNAN, K., KUPPAM, C., STEPHY, G. M., KAMYAB, H., CHEN, W.-H., THOMAS, J. & NGAMCHARUSSRIVICHAI, C. 2022. Advanced technologies on the sustainable approaches for conversion of organic waste to valuable bioproducts: Emerging circular bioeconomy perspective. *Fuel*, 324, 124313.
- AVEZOVA, N., TOIROV, O. & USMANOV, A. 2024. Review of Modern Approaches in the Development of Hybrid Biogas Systems. *Applied Solar Energy*, 60, 868-879.
- BAKSI, S., SAHA, D., SAHA, S., SARKAR, U., BASU, D. & KUNIYAL, J. 2023. Pre-treatment of lignocellulosic biomass: review of various physico-chemical and biological methods influencing the extent of biomass depolymerization. *International journal of environmental science and technology*, 20, 13895-13922.
- BALASUNDARAM, G., BANU, R., VARJANI, S., KAZMI, A. & TYAGI, V. K. 2022. Recalcitrant compounds formation, their toxicity, and mitigation: key issues in biomass pretreatment and anaerobic digestion. *Chemosphere*, 291, 132930.
- BANSOD, S. P., MAKWANA, K., SARANGI, P. K. & PARIKH, J. K. 2024. Advanced pretreatment processes for lignocellulosic biomass to biofuels production: Path towards circular bioeconomy. *Sustainable Chemistry and Pharmacy*, 39, 101514.
- BARRAGÁN-TRINIDAD, M., CARRILLO-REYES, J. & BUITRÓN, G. 2017. Hydrolysis of microalgal biomass using ruminal microorganisms as a pretreatment to increase methane recovery. *Bioresource Technology*, 244, 100-107.
- BEGA, S. 2025. Energy sector contributes most of SA's carbon emissions. *Mail & Guardian*, 22 February 2025.
- BENTO, H. B., TESSARO, Í., THEODORO, J. M., DE SOUZA MATIAS REIS, W., POLICARPO, G., REIS, C. E. R. & DE CARVALHO, A. K. F. 2024. Economic, Social, and Organizational Challenges in Biorefineries. *Clean Energy Transition-via-Biomass Resource Utilization: A Way to Mitigate Climate Change*. Springer.
- BEZERRA, T. L. & RAGAUSKAS, A. J. 2016. A review of sugarcane bagasse for second-generation bioethanol and biopower production. *Biofuels, Bioproducts and Biorefining*, 10, 634-647.
- BIDIKO, G. B., SANGIB, E. B. & GNARO, M. A. 2025. Optimization of biogas production through co-digestion of cafeteria food waste and cow dung using the response surface methodology. *Frontiers in Energy Research*, 13, 1568478.
- BLAIR, J. & MATARAARACHCHI, S. 2021. A review of landfills, waste and the nearly forgotten nexus with climate change. *Environments*, 8, 73.
- BPC Instruments. (2022). *AMPTS II & AMPTS II Light: Operation and Maintenance Manual* (Version 3.3.2). Retrieved from https://bpcinstruments.com/wp-content/uploads/2022/02/2022_AMPTS-II-and-AMPTS-II-Light-Manual.pdf
- CAI, Y., ZHENG, Z. & WANG, X. 2021. Obstacles faced by methanogenic archaea originating from substrate-driven toxicants in anaerobic digestion. *Journal of Hazardous Materials*, 403, 123938.
- CHANDEL, A. K., ALBARELLI, J. Q., SANTOS, D. T., CHUNDAWAT, S. P., PURI, M. & MEIRELES, M. A. A. 2019. Comparative analysis of key technologies for cellulosic ethanol production from Brazilian sugarcane bagasse at a commercial scale. *Biofuels, bioproducts and biorefining*, 13, 994-1014.

- CHAUHAN, P. S. 2020. Role of various bacterial enzymes in complete depolymerization of lignin: a review. *Biocatalysis and agricultural biotechnology*, 23, 101498.
- CHEN, S., ZHANG, X., SINGH, D., YU, H. & YANG, X. 2010. Biological pretreatment of lignocellulosics: potential, progress and challenges. *Biofuels*, 1, 177-199.
- CHHETRI, B. R., ACHARYA, D., GAUTAM, A., BAJRACHARYA, N., SHRESTHA, A. & KHADKA, S. 2022. Microbial pre-treatment of lignocellulosic biomass for biofuel production: a review. *International Journal of Applied Sciences and Biotechnology*, 10, 140-148.
- CHIRANIA, P., HOLWERDA, E. K., GIANNONE, R. J., LIANG, X., POUDEL, S., ELLIS, J. C., BOMBLE, Y. J., HETTICH, R. L. & LYND, L. R. 2022. Metaproteomics reveals enzymatic strategies deployed by anaerobic microbiomes to maintain lignocellulose deconstruction at high solids. *Nature Communications*, 13, 3870.
- CHIRUME, J. 2024. Illegal dumping swamps Nelson Mandela Bay. South Africa: Future Media (Pty) Ltd.
- CUI, X., WANG, Z., GUO, P., LI, F., CHANG, S., YAN, T., ZHENG, H. & HOU, F. 2023. Shift of feeding strategies from grazing to different forage feeds reshapes the rumen microbiota to improve the ability of Tibetan sheep (*Ovis aries*) to adapt to the cold season. *Microbiology spectrum*, 11, e02816-22.
- CUNHA, I. S., BARRETO, C. C., COSTA, O. Y. A., BOMFIM, M. A. & CASTRO, A. P. 2011. Bacteria and Archaea community structure in the rumen microbiome of goats (*Capra hircus*) from the semiarid region of Brazil. *Anaerobe*, 17, 118-124.
- DAS, N., JENA, P. K., PADHI, D., KUMAR MOHANTY, M. & SAHOO, G. 2021. A comprehensive review of characterization, pretreatment and its applications on different lignocellulosic biomass for bioethanol production. *Biomass conversion and biorefinery*, 1-25.
- DATTA, R., 2024. Enzymatic degradation of cellulose in soil: A review. *Heliyon*, 10(1).
- DAWOOD, Z. 2025. 60% of SA landfill sites non-compliant as political instability cripples waste management. *iOL*.
- DI FRAIA, A., DI FRAIA, S. & MASSAROTTI, N. 2024. Role of advanced oxidation processes in lignocellulose pretreatment towards biorefinery applications: a review on emerging trends and economic considerations. *Green Chemistry*.
- DOS SANTOS, J. R., DE SOUZA MOREIRA, L. R. & FILHO, E. X. F. 2023. Cellulose-degrading enzymes: key players in biorefinery development. *Biologia*, 78, 1759-1772.
- DUTTA, N., USMAN, M., LUO, G. & ZHANG, S. 2022. An insight into valorization of lignocellulosic biomass by optimization with the combination of hydrothermal (HT) and biological techniques: A review. *Sustainable Chemistry*, 3, 35-55.
- EKWEBELEM, O. C., OFIELU, E. S., DIKE, O. V. N., ALEKE, J. C. & NWACHUKWU, G. A. 2020. Towards Sustainable Energy: The Requisite Role of Microorganisms in the Production of Biogas and Bioethanol. *Journal of Energy Research and Reviews*, 6, 20-32.
- FRANCISCO LÓPEZ, A., LAGO RODRÍGUEZ, T., FARAJI ABDOLMALEKI, S., GALERA MARTÍNEZ, M. & BELLO BUGALLO, P. M. 2024. From Biogas to Biomethane: An In-Depth Review of Upgrading Technologies That Enhance Sustainability and Reduce Greenhouse Gas Emissions. *Applied Sciences*, 14, 2342.
- GAO, Z., ALSHEHRI, K., LI, Y., QIAN, H., SAPSFORD, D., CLEALL, P. & HARBOTTLE, M. 2022. Advances in biological techniques for sustainable lignocellulosic waste utilization in biogas production. *Renewable and Sustainable Energy Reviews*, 170, 112995.
- GBADEYAN, O. J., SIBIYA, L., LINGANISO, L. Z. & DEENADAYALU, N. 2024. Waste-to-energy: the recycling and reuse of sugar industry waste for different value-added products such as bioenergy in selected countries—a critical review. *Biofuels, Bioproducts and Biorefining*.
- GHARECHAH, J., SARIKHAN, S., HAN, J.-L., DING, X.-Z. & SALEKDEH, G. H. 2022. Functional and phylogenetic analyses of camel rumen microbiota associated with different lignocellulosic substrates. *npj Biofilms and Microbiomes*, 8, 46.
- GHATAK, H. R. 2011. Biorefineries from the perspective of sustainability: Feedstocks, products, and processes. *Renewable and Sustainable Energy Reviews*, 15, 4042-4052.
- GUO, J., ZHANG, Y., FANG, J., MA, Z., LI, C., YAN, M., QIAO, N., LIU, Y. & BIAN, M. 2024. Reduction and reuse of forestry and agricultural bio-waste through innovative green utilization approaches: A review. *Forests*, 15, 1372.

- HAMID, S. B. A., ISLAM, M. M. & DAS, R. 2015. Cellulase biocatalysis: key influencing factors and mode of action. *Cellulose*, 22, 2157-2182.
- HARIRCHI, S., WAINAINA, S., SAR, T., NOJOURI, S. A., PARHAM, M., PARHAM, M., VARJANI, S., KHANAL, S. K., WONG, J. & AWASTHI, M. K. 2022. Microbiological insights into anaerobic digestion for biogas, hydrogen or volatile fatty acids (VFAs): a review. *Bioengineered*, 13, 6521-6557.
- HERNÁNDEZ-BELTRÁN, J. U., HERNÁNDEZ-DE LIRA, I. O., CRUZ-SANTOS, M. M., SAUCEDO-LUEVANOS, A., HERNÁNDEZ-TERÁN, F. & BALAGURUSAMY, N. 2019. Insight into pretreatment methods of lignocellulosic biomass to increase biogas yield: current state, challenges, and opportunities. *Applied sciences*, 9, 3721.
- HIRANOBE, C. T., GOMES, A. S., PAIVA, F. F., TOLOSA, G. R., PAIM, L. L., DOGNANI, G., CARDIM, G. P., CARDIM, H. P., DOS SANTOS, R. J. & CABRERA, F. C. 2024. Sugarcane Bagasse: Challenges and Opportunities for Waste Recycling. *Clean Technologies*, 6, 662-699.
- HUA, D., HENDRIKS, W. H., XIONG, B. & PELLIKAAN, W. F. 2022. Starch and cellulose degradation in the rumen and applications of metagenomics on ruminal microorganisms. *Animals*, 12, 3020.
- HUGO, T. J. 2010. *Pyrolysis of sugarcane bagasse*. Stellenbosch: University of Stellenbosch.
- HUNGATE, R. E. 1947. Studies on Cellulose Fermentation: III. The Culture and Isolation for Cellulose-decomposing Bacteria from the Rumen of Cattle. *J Bacteriol*, 53, 631-45.
- JAYASEKARA, S. & RATNAYAKE, R. 2019. Microbial Cellulases: An Overview and Applications. *Cellulose*. IntechOpen.
- JICSINSZKY, L., BUCCIOL, F., CHAJI, S. & CRAVOTTO, G. 2023. Mechanochemical Degradation of Biopolymers. *Molecules*, 28, 8031.
- JOHN, N., FATHIMA, P., HARSHA, V., PAUL, N. M. & NISHA, P. 2024. Physical Conversion of Biomass: Dewatering, Drying, Size Reduction, Densification, and Separation. *Handbook of Biomass*. Springer.
- JOSEPH, D. & SAWANT, R. 2021. Recent Trends in the Use of Amine Solvents for Carbon Dioxide Scrubbing Process. *IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT)*, 15.
- KABEYI, M. J. B. & OLANREWaju, O. A. 2022. Biogas production and applications in the sustainable energy transition. *Journal of Energy*, 2022, 8750221.
- KABEYI, M. J. B. & OLANREWaju, O. A. 2023. Bagasse electricity potential of conventional sugarcane factories. *Journal of Energy*, 2023, 5749122.
- KABEYI, M. J. B. & OLANREWaju, A. 2023 Biogas treatment and upgrading techniques. Fourth Asia Pacific International Conference on Industrial Engineering and Operations Management. Michigan: *IEOM Society International*.
- KAINTHOLA, J., KALAMDHAD, A. S. & GOUD, V. V. 2019. A review on enhanced biogas production from anaerobic digestion of lignocellulosic biomass by different enhancement techniques. *Process Biochemistry*, 84, 81-90.
- KARIMI, K. & TAHERZADEH, M. J. 2016. A critical review on analysis in pretreatment of lignocelluloses: Degree of polymerization, adsorption/desorption, and accessibility. *Bioresource Technology*, 203, 348-356.
- KAUR, G., KAUR, P., KAUR, J., SINGLA, D. & TAGGAR, M. S. 2024. Xylanase, xylooligosaccharide and xylitol production from lignocellulosic biomass: Exploring biovalorization of xylan from a sustainable biorefinery perspective. *Industrial Crops and Products*, 215, 118610.
- KAZIMIEROWICZ, J., DĘBOWSKI, M. & ZIELIŃSKI, M. 2025. Innovative Method for Biomethane Production Based on a Closed Cycle of Biogas Upgrading and Organic Substrate Pretreatment—Technical, Economic, and Technological Fundamentals. *Energies*, 18, 1033.
- KHAN, N. 2025. Pollution and waste management: residents speak. *Post*.
- KHUBONI, M. L. 2025. City of Joburg allocates funds for the Kya Sand landfill site. *The Citizen* June 2025.
- KIM, J. R. & KARTHIKEYAN, K. 2021. Effects of severe pretreatment conditions and lignocellulose-derived furan byproducts on anaerobic digestion of dairy manure. *Bioresource Technology*, 340, 125632.

- KIM, J. S., LEE, Y. & KIM, T. H. 2016. A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass. *Bioresource technology*, 199, 42-48.
- KOHLER, M. 2016. An economic assessment of bioethanol production from sugar cane: The case of South Africa. *Economic Research Southern Africa-ERSA. Working paper*, 630.
- KONDE, K. S., NAGARAJAN, S., KUMAR, V., PATIL, S. V. & RANADE, V. V. 2021. Sugarcane bagasse based biorefineries in India: potential and challenges. *Sustainable Energy & Fuels*, 5, 52-78.
- KUMAR, A., KUMAR, V. & SINGH, B. 2021. Cellulosic and hemicellulosic fractions of sugarcane bagasse: Potential, challenges and future perspective. *International Journal of Biological Macromolecules*, 169, 564-582.
- KUMAR, G., DHARMARAJA, J., ARVINDNARAYAN, S., SHOBAN, S., BAKONYI, P., SARATALE, G. D., NEMESTÓTHY, N., BÉLAFI-BAKÓ, K., YOON, J. J. & KIM, S. H. 2019. A comprehensive review on thermochemical, biological, biochemical and hybrid conversion methods of bio-derived lignocellulosic molecules into renewable fuels. *Fuel*, 251, 352-367.
- LEE, W., KIM, Y., KIM, H. & KIM, M. 2024. Comparison of Anaerobic Co-Digestion of Food Waste and Livestock Manure at Various Mixing Ratios under Mesophilic and Thermophilic Temperatures. *Sustainability*, 16, 7653.
- LI, X., SHI, Y., KONG, W., WEI, J., SONG, W. & WANG, S. 2022. Improving enzymatic hydrolysis of lignocellulosic biomass by bio-coordinated physicochemical pretreatment—A review. *Energy Reports*, 8, 696-709.
- LI, Y., PARK, S. Y. & ZHU, J. 2011. Solid-state anaerobic digestion for methane production from organic waste. *Renewable and Sustainable Energy Reviews*, 15, 821-826.
- LIU, Y., LIU, X., CUI, Y. & YUAN, W. 2022. Ultrasound for microalgal cell disruption and product extraction: A review. *Ultrasonics Sonochemistry*, 87, 106054.
- MALODE, S. J., PRABHU, K. K., MASCARENHAS, R. J., SHETTI, N. P. & AMINABHAVI, T. M. 2021. Recent advances and viability in biofuel production. *Energy Conversion and Management: X*, 10, 100070.
- MANDREE, P., THOPI, G. A. & RAMCHURAN, S. 2023. Potential opportunities to convert waste to bio-based chemicals at an industrial scale in South Africa. *Fermentation*, 9, 908.
- MANKAR, A. R., PANDEY, A., MODAK, A. & PANT, K. K. 2021. Pretreatment of lignocellulosic biomass: A review on recent advances. *Bioresource Technology*, 334, 125235.
- MASUKU, D. 2024. CoJ hauled to court for ignoring Kya Sand's illegal dumping crisis. *The Citizen*, 3 July 2024.
- MBAZIMA, S. J., MASEKAMENI, M. D. & MMEREKI, D. 2022. Waste-to-energy in a developing country: The state of landfill gas to energy in the Republic of South Africa. *Energy Exploration & Exploitation*, 40, 1287-1312.
- MEGHANA, M. & SHASTRI, Y. 2020. Sustainable valorization of sugar industry waste: Status, opportunities, and challenges. *Bioresource technology*, 303, 122929.
- MIGNOGNA, D., CECI, P., CAFARO, C., CORAZZI, G. & AVINO, P. 2023. Production of Biogas and Biomethane as Renewable Energy Sources: A Review. *Applied Sciences*, 13, 10219.
- MIRMOHAMADSADEGHI, S., KARIMI, K., AZARBAIJANI, R., YEGANEH, L. P., ANGELIDAKI, I., NIZAMI, A.-S., BHAT, R., DASHORA, K., VIJAY, V. K. & AGHBASHLO, M. 2021. Pretreatment of lignocelluloses for enhanced biogas production: A review on influencing mechanisms and the importance of microbial diversity. *Renewable and Sustainable Energy Reviews*, 135, 110173.
- MNISI, M. S. & DLAMINI, C. S. 2012. The concept of sustainable sugarcane production: Global, African and South African perceptions. *African Journal of Agricultural Research*, 7, 4337-4343.
- MOATSHE, R. 2025. Illegal dumping sites dangerous to health. *iOL*.
- MOHLALA, L. M., BODUNRIN, M. O., AWOSUSI, A. A., DARAMOLA, M. O., CELE, N. P. & OLUBAMBI, P. A. 2016. Beneficiation of corncob and sugarcane bagasse for energy generation and materials development in Nigeria and South Africa: A short overview. *Alexandria Engineering Journal*, 55, 3025-3036.

- MOLATUDI, L. E., KUNENE, T. J. & MASHIFANA, T. 2025. Strategies for Biomethane Purification: A Critical Review and New Approaches. *GCB Bioenergy*, 17, e70040.
- MONICA, P., RANJAN, R. & KAPOOR, M. 2024. Lignocellulose-degrading chimeras: Emerging perspectives for catalytic aspects, stability, and industrial applications. *Renewable and Sustainable Energy Reviews*, 199, 114425.
- MORAN, J. 2005. *Tropical dairy farming: feeding management for small holder dairy farmers in the humid tropics*, Csiro Publishing.
- MUBARAK, A. A., ILYAS, R., NORDIN, A. H., NGADI, N. & ALKBIR, M. 2024. Recent developments in sugarcane bagasse fibre-based adsorbent and their potential industrial applications: a review. *International Journal of Biological Macromolecules*, 134165.
- MUBARAK ALQAHTANI, A. 2024. Sweet sorghum and bagasse: a comprehensive review of feedstock traits, conversion processes, and economic viability for bioethanol and biogas production. *Biofuels*, 15, 575-585.
- MUSTAFA, A., RASHID, S., RAHIM, M., ROSLAN, R., MUSA, W., SIKDER, B. & SASI, A. 2022. Enzymatic pretreatment of lignocellulosic biomass: an overview. *Journal of Chemical Engineering and Industrial Biotechnology*, 8, 1-7.
- NEGRÃO, D. R., GRANDIS, A., BUCKERIDGE, M. S., ROCHA, G. J., LEAL, M. R. L. & DRIEMEIER, C. 2021. Inorganics in sugarcane bagasse and straw and their impacts for bioenergy and biorefining: A review. *Renewable and Sustainable Energy Reviews*, 148, 111268.
- NGUYEN, V. K., CHAUDHARY, D. K., DAHAL, R. H., TRINH, N. H., KIM, J., CHANG, S. W., HONG, Y., LA, D. D., NGUYEN, X. C. & NGO, H. H. 2021. Review on pretreatment techniques to improve anaerobic digestion of sewage sludge. *Fuel*, 285, 119105.
- NHLEKO, L. & SEKOAI, P. T. 2025. Sugarcane Bagasse: A Sustainable Feedstock for Biorefinery Portfolios in South Africa. *Fermentation*, 11, 489.
- NLEYA, Y., YOUNG, B., NOORAE, E. & BAROUTIAN, S. 2025. Anaerobic digestion of dairy cow and goat manure: Comparative assessment of biodegradability and greenhouse gas mitigation. *Fuel*, 381, 133458.
- NQCONGO, B. 2025. Growing crises of illegal dumping at Ethekwini. Medium.
- OLATUNJI, K. O., AHMED, N. A. & OGUNKUNLE, O. 2021. Optimization of biogas yield from lignocellulosic materials with different pretreatment methods: a review. *Biotechnology for Biofuels*, 14, 1-34.
- OLATUNJI, K. O., MOOTSWI, K. D., OLATUNJI, O. O., ZWANE, M. I., VAN RENSBURG, N. J. & MADYIRA, D. M. 2025. Anaerobic Co-Digestion of Food Waste and Groundnut Shells: Synergistic Impact Assessment and Kinetic Modeling. *Waste and Biomass Valorization*, 16, 3745-3760.
- OMUSE, E. R., TONNANG, H. E., YUSUF, A. A., MACHEKANO, H., EGONYU, J. P., KIMATHI, E., MOHAMED, S. F., KASSIE, M., SUBRAMANIAN, S. & ONDITI, J. 2024. The global atlas of edible insects: analysis of diversity and commonality contributing to food systems and sustainability. *Scientific Reports*, 14, 5045.
- OZBAYRAM, E. G., KLEINSTEUBER, S., NIKOLAUSZ, M., INCE, B. & INCE, O. 2018. Bioaugmentation of anaerobic digesters treating lignocellulosic feedstock by enriched microbial consortia. *Eng Life Sci*, 18, 440-446.
- PANDEY, A., SOCCOL, C. R., NIGAM, P. & SOCCOL, V. T. 2000. Biotechnological potential of agro-industrial residues. I: sugarcane bagasse. *Bioresource technology*, 74, 69-80.
- PATTNAIK, F., PATRA, B. R., NANDA, S., MOHANTY, M. K., DALAI, A. K. & RAWAT, J. 2024. Drivers and barriers in the production and utilization of second-generation bioethanol in India. *Recycling*, 9, 19.
- PENG, X., WILKEN, S. E., LANKIEWICZ, T. S., GILMORE, S. P., BROWN, J. L., HENSKE, J. K., SWIFT, C. L., SALAMOV, A., BARRY, K., GRIGORIEV, I. V., THEODOROU, M. K., VALENTINE, D. L. & O'MALLEY, M. A. 2021. Genomic and functional analyses of fungal and bacterial consortia that enable lignocellulose breakdown in goat gut microbiomes. *Nature Microbiology*, 6, 499-511.
- PENNELLS, J., BLESS, I., JULIANO, P. & YING, D. 2023. Extrusion Processing of Biomass By-Products for Sustainable Food Production. *From Biomass to Biobased Products*. IntechOpen.

- PÉREZ-CONTRERAS, S., HERNÁNDEZ-ROSAS, F., LIZARDI-JIMÉNEZ, M. A., HERRERA-CORREDOR, J. A., BALTAZAR-BERNAL, O., AVALOS-DE LA CRUZ, D. A. & HERNÁNDEZ-MARTÍNEZ, R. 2025. Sugarcane Industry By-Products: A Decade of Research Using Biotechnological Approaches. *Recycling*, 10, 154.
- PISALWAR, P., FERNANDES, A., TRIBHUVAN, D., GITE, S. & AHMED, S. 2023. Arabinofuranosidases. *Glycoside Hydrolases*. Elsevier.
- PODDAR, B., NAKHATE, S., GUPTA, R., CHAVAN, A., SINGH, A., KHARDENAVIS, A. & PUROHIT, H. 2022. A comprehensive review on the pretreatment of lignocellulosic wastes for improved biogas production by anaerobic digestion. *International Journal of Environmental Science and Technology*, 1-28.
- PRABHA, R., KUMARI, R., KUMAR, S., BHATI, J., MISHRA, D. C., RAI, A., SAMOTA, M. K. & SINGH, D. P. 2025. Metatranscriptome analysis to identify the influence of diet over metabolic proficiency of rumen in Mehsani buffalo. *Dairy Science and Management*, 2, 1.
- PRIYA, K., RANI, J. & GWAL, S. 2025. Transforming agricultural residues to value-added products: waste to wealth. *Sustainable Management of Agro-Food Waste*. Elsevier.
- PRYOR, S. W., SMITHERS, J., LYNE, P. & VAN ANTWERPEN, R. 2017. Impact of agricultural practices on energy use and greenhouse gas emissions for South African sugarcane production. *Journal of Cleaner Production*, 141, 137-145.
- RABAPANE, K. J. & MATAMBO, T. S. 2024. Profiling the dynamic adaptations of CAZyme-Producing microorganisms in the gastrointestinal tract of South African goats. *Heliyon*, 10.
- RABEE, A. E., ABOU-SOULIMAN, I., YOUSIF, A. I., LAMARA, M., EL-SHERBIENY, M. A., ELWAKEEL, E. A. & SALLAM, A. M. 2025. Variations in rumen microbiota and host genome impacted feed efficiency in goat breeds. *Front Microbiol*, 16, 1492742.
- RAFIEE, A., KHALILPOUR, K. R., PREST, J. & SKRYABIN, I. 2021. Biogas as an energy vector. *Biomass and Bioenergy*, 144, 105935.
- RAHMAN, M. S., FERNANDO, S., ROSS, B., WU, J. & QIN, W. 2018. Endoglucanase (EG) Activity Assays. *Cellulases*. Springer.
- RAMA, H., AKINDOLIRE, M., OBI, L., BELLO-AKINOSHO, M., NDABA, B., DHLAMINI, M. S., MAAZA, M. & ROOPNARAIN, A. 2023. Anaerobic Digestion: Climate Change Mitigation Through Sustainable Organic Waste Valorization. *Handbook of Nature-Based Solutions to Mitigation and Adaptation to Climate Change*. Springer.
- RASIMPHI, T., KILONZO, B., TJALE, M., TINARWO, D. & NYAMUKONDIWA, P. 2024. Review of implementation of biogas technology in rural communities of South Africa. *Cogent Social Sciences*, 10, 2419536.
- RASOOL, G. & IRFAN, M. 2024. The Role of Microbial Diversity in Lignocellulosic Biomass Degradation: A Biotechnological Perspective. *ChemBioEng Reviews*.
- RIBEIRO, G., GRUNINGER, R., BADHAN, A. & MCALLISTER, T. 2016. Mining the rumen for fibrolytic feed enzymes. *Animal Frontiers*, 6, 20-26.
- ROCHA-MENESES, L., LUNA-DELRISCO, M., GONZÁLEZ, C. A., MONCADA, S. V., MORENO, A., SIERRA-DEL RIO, J. & CASTILLO-MEZA, L. E. 2023. An overview of the socio-economic, technological, and environmental opportunities and challenges for renewable energy generation from residual biomass: a case study of biogas production in Colombia. *Energies*, 16, 5901.
- RODRIGUES, P. D. O., CORRÊA, A. G., BAFFI, M. A. & PASQUINI, D. 2024. Potential applications of hemicellulose. *Handbook of Biomass*. Springer.
- ROSA, S. F. & MARTIUS, C. 2021. Forest-based bioeconomy in sub-Saharan Africa: looking at benefits, barriers and burdens from a social sustainability standpoint.
- RUSSELL, J. 2002. Rumen Microbiology and its Role in Ruminant Nutrition. Ithaca, NY: James B. Russell Publishing. Available online at: <http://www.ars.usda.gov/services>
- SARKER, T. C., AZAM, S. M. G. G., EL-GAWAD, A. M. A., GAGLIONE, S. A. & BONANOMI, G. 2017. Sugarcane bagasse: a potential low-cost biosorbent for the removal of hazardous materials. *Clean Technologies and Environmental Policy*, 19, 2343-2362.
- SCAPINI, T., DOS SANTOS, M. S., BONATTO, C., WANCURA, J. H., MULINARI, J., CAMARGO, A. F., KLANOVICZ, N., ZABOT, G. L., TRES, M. V. & FONGARO, G. 2021. Hydrothermal

- pretreatment of lignocellulosic biomass for hemicellulose recovery. *Bioresource Technology*, 342, 126033.
- SEEBALUCK, V., MOHEE, R., SOBHANBABU, P., ROSILLO-CALLE, F., LEAL, M. & JOHNSON, F. 2008. Bioenergy for sustainable development and global competitiveness: the case of sugar cane in Southern Africa. *Thematic Report*, 2.
- SELIM, A. S. M., HASAN, M. N., RAHMAN, M. A., RAHMAN, M. M., ISLAM, M. R., BOSTAMI, A. R., ISLAM, S. & TEDESCHI, L. O. 2022. Nutrient content and in vitro degradation study of some unconventional feed resources of Bangladesh. *Heliyon*, 8.
- SEMA, Y. A., URGESSA, O. E. & AYELE, A. K. 2020. Evaluating rumen fluid-inoculated lignocellulosic substrates for biogas production. *East African Journal of Sciences*, 14, 99-110.
- SERRANO-GAMBOA JOSÉ, G., FÓCIL-ESPINOSA CARLOS, A., CABELLO-YEVES PEDRO, J., COUTINHO FELIPE, H., ROJAS-HERRERA RAFAEL, A. & SÁNCHEZ-GONZÁLEZ MÓNICA, N. 2022. Metatranscriptome Profiling of a Specialized Microbial Consortium during the Degradation of Nixtamalized Maize Pericarp. *Microbiology Spectrum*, 10, e02318-21.
- SETHUPATHY, S., MORALES, G.M., LI, Y., WANG, Y., JIANG, J., SUN, J. AND ZHU, D., 2021. Harnessing microbial wealth for lignocellulose biomass valorization through secretomics: a review. *Biotechnology for Biofuels*, 14(1), p.154.
- SINGH, M. 2025. Enzymatic reactions and their impact on bioenergy in anaerobic digestion. *Waste-to-Energy*. Elsevier.
- SIVAMANI, S., SAIKAT, B., NAVEEN PRASAD, B., BAALAWY, A. A. S. & AL-MASHALI, S. M. A. 2021. A comprehensive review on microbial technology for biogas production. *Bioenergy Research: Revisiting Latest Development*, 53-78.
- SMITH, C. K. 2010. *Raising goats for dummies*, John Wiley & Sons.
- SMITHERS, J. 2014. Review of sugarcane trash recovery systems for energy cogeneration in South Africa. *Renewable and Sustainable Energy Reviews*, 32, 915-925.
- SOTO GUZMÁN, M. C. 2021. Development of membrane processes for the selective separation of CO₂ from biogas and biohydrogen.
- SØRENSEN, J. F. L. & JØRGENSEN, H. P. 2022. Rural development potential in the bioeconomy in developed countries: The case of biogas production in Denmark. *Sustainability*, 14, 11077.
- SPYRIDONIDIS, A., VASILIADOU, I. A., AKRATOS, C. S. & STAMATELATOU, K. 2020. Performance of a full-scale biogas plant operation in Greece and its impact on the circular economy. *Water*, 12, 3074.
- SRIVASTAVA, R. K., SHETTI, N. P., REDDY, K. R. & AMINABHAVI, T. M. 2020. Sustainable energy from waste organic matters via efficient microbial processes. *science of the total environment*, 722, 137927.
- SUMARDIONO, S., MATIN, H. H. A., SULISTIANINGTIAS, I., NUGROHO, T. Y. & BUDIYONO, B. 2023. Effect of physical and biological pretreatment on sugarcane bagasse waste-based biogas production. *Materials Today: Proceedings*, 87, 41-44.
- SUN, D., LV, Z.-W., RAO, J., TIAN, R., SUN, S.-N. & PENG, F. 2022. Effects of hydrothermal pretreatment on the dissolution and structural evolution of hemicelluloses and lignin: A review. *Carbohydrate Polymers*, 281, 119050.
- THAPA, S., ZHOU, S., O'HAIR, J., AL NASR, K., ROPELEWSKI, A. & LI, H. 2023. Exploring the microbial diversity and characterization of cellulase and hemicellulase genes in goat rumen: a metagenomic approach. *BMC Biotechnology*, 23, 51.
- THIBANE, Z., SONI, S., PHALI, L. & MDODA, L. 2023. RETRACTED: Factors impacting sugarcane production by small-scale farmers in KwaZulu-Natal Province-South Africa. *Heliyon*, 9.
- TIEMA, C., OCHUNG, A. & OMWOMA, S. Sugarcane Production in Kenya.
- TOMAZETTO, G., PIMENTEL AGNES, C., WIBBERG, D., DIXON, N. & SQUINA FABIO, M. 2020. Multi-omic Directed Discovery of Cellulosomes, Polysaccharide Utilization Loci, and Lignocellulases from an Enriched Rumen Anaerobic Consortium. *Applied and Environmental Microbiology*, 86, e00199-20.
- TONGWANE, M., MDLAMBUZI, T., MOELETSI, M., TSUBO, M., MLISWA, V. & GROOTBOOM, L. 2016. Greenhouse gas emissions from different crop production and management practices in South Africa. *Environmental Development*, 19, 23-35.

- TONGWANE, M. I., MOELETSI, M. E. & TSUBO, M. 2020. Trends of carbon emissions from applications of nitrogen fertiliser and crop residues to agricultural soils in South Africa. *J Environ Manage*, 272, 111056.
- UDDIN, M. M. & WRIGHT, M. M. 2023. Anaerobic digestion fundamentals, challenges, and technological advances. *Physical Sciences Reviews*, 8, 2819-2837.
- UZOAGBA, C. E. 2024. *Valorization of Underutilized Lignocellulosic Biomass Wastes for Biofuel Production*. AUST.
- VANHAMÄKI, S., VIRTANEN, M., LUSTE, S. & MANSKINEN, K. 2020. Transition towards a circular economy at a regional level: A case study on closing biological loops. *Resources, Conservation and Recycling*, 156, 104716.
- VARJANI, S., SIVASHANMUGAM, P., TYAGI, V. K. & GUNASEKARAN, M. 2022. Breakthrough in hydrolysis of waste biomass by physico-chemical pretreatment processes for efficient anaerobic digestion. *Chemosphere*, 294, 133617.
- VERNER, V., MAZANCOVÁ, J., JELÍNEK, M., PHUNG, L. D., VAN DUNG, D., BANOUT, J. & ROUBÍK, H. 2021. Economics and perception of small-scale biogas plant benefits installed among peri-urban and rural areas in central Vietnam. *Biomass Conversion and Biorefinery*, 1-13.
- VILLARIM, P. C. New compacts systems for biogas upgrading. 2023. Université du Littoral Côte d'Opale.
- VODOPICH, D. S. & MOORE, R. 2002. *Biology laboratory manual*.
- WANG, Y., LI, Y., QI, Y., FU, L., LI, G., LIU, Z., BO, L. & JING, Y. 2025. Anaerobic Co-Digestion of Dairy Manure and Cucumber Residues: Methane Production Efficiency and Microbial Community Characteristics. *Agronomy*, 15, 1610.
- WANG, Z., HU, Y., WANG, S., WU, G. & ZHAN, X. 2023. A critical review on dry anaerobic digestion of organic waste: Characteristics, operational conditions, and improvement strategies. *Renewable and Sustainable Energy Reviews*, 176, 113208.
- WERKNEH, A. A. 2022. Biogas impurities: environmental and health implications, removal technologies and future perspectives. *Heliyon*, 8.
- WERKNEH, A. A. & GEBRU, S. B. 2023. Development of ecological sanitation approaches for integrated recovery of biogas, nutrients and clean water from domestic wastewater. *Resources, Environment and Sustainability*, 11, 100095.
- WOLFAARDT, F. J., FERNANDES, L. G. L., OLIVEIRA, S. K. C., DURET, X., GÖRGENS, J. F. & LAVOIE, J.-M. 2021. Recovery approaches for sulfuric acid from the concentrated acid hydrolysis of lignocellulosic feedstocks: A mini-review. *Energy Conversion and Management: X*, 10, 100074.
- WOŹNIAK, A., KULIGOWSKI, K., ŚWIERCZEK, L. & CENIAN, A. 2025. Review of Lignocellulosic Biomass Pretreatment Using Physical, Thermal and Chemical Methods for Higher Yields in Bioethanol Production. *Sustainability*, 17, 287.
- WU, D., WEI, Z., MOHAMED, T. A., ZHENG, G., QU, F., WANG, F., ZHAO, Y. & SONG, C. 2022. Lignocellulose biomass bioconversion during composting: Mechanism of action of lignocellulase, pretreatment methods and future perspectives. *Chemosphere*, 286, 131635.
- YEN, H.-W. & BRUNE, D. E. 2007. Anaerobic co-digestion of algal sludge and waste paper to produce methane. *Bioresource Technology*, 98, 130-134.
- YU, Q., SUN, C., CAO, W., LIU, R., ABD-ALLA, M. H. & RASMEY, A.-H. M. 2024. Rumen fluid pretreatment promotes anaerobic methane production: revealing microbial dynamics driving increased acid yield from different concentrations of corn straw. *Environmental Science and Pollution Research*.
- YU, Y., WU, J., REN, X., LAU, A., REZAEI, H., TAKADA, M., BI, X. & SOKHANSANJ, S. 2022. Steam explosion of lignocellulosic biomass for multiple advanced bioenergy processes: A review. *Renewable and Sustainable Energy Reviews*, 154, 111871.
- ZHANG, C., SU, H., BAEYENS, J. & TAN, T. 2014. Reviewing the anaerobic digestion of food waste for biogas production. *Renewable and Sustainable Energy Reviews*, 38, 383-392.
- ZHANG, T., LIU, L., SONG, Z., REN, G., FENG, Y., HAN, X. & YANG, G. 2013. Biogas production by co-digestion of goat manure with three crop residues. *PLoS One*, 8, e66845.

ZHOU, Z., OUYANG, D., LIU, D. & ZHAO, X. 2023. Oxidative pretreatment of lignocellulosic biomass for enzymatic hydrolysis: Progress and challenges. *Bioresource Technology*, 367, 128208.

CHAPTER THREE: MICROBIAL PROFILING OF TEN GOATS' GASTROINTESTINAL TRACT

Objective

The purpose of this chapter was to profile the dynamic adaptations of CAZyme-producing microorganisms in the gastrointestinal tract of South African goats. It aimed to understand how these microorganisms adapted to different dietary and environmental conditions and how these adaptations contributed to digestion in the GIT. Additionally, the chapter aimed to investigate

the diversity of microbial species in goats' GIT and how this diversity affects the efficiency of carbohydrate breakdown. By examining the specific CAZymes produced and their activity levels, the research aimed to uncover the mechanisms behind the goats' ability to thrive on a varied diet. This comprehensive profiling provided insights into optimizing nutrient absorption and maintaining gut health, ultimately leading to enhanced livestock management practices.

Publication

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Abstract

The gastrointestinal tract of goats serves as a habitat for anaerobic microbial populations that work together to break down complex plant material, including lignocellulose. This study explored the microbial diversity and metabolic profiles across different gastrointestinal tract compartments. Significant diversity differences among the compartments were observed (ANOSIM $p < 0.006$), with the abomasum showing a distinct species composition and a decreased alpha diversity (Mann-Whitney/Kruskal-Wallis test $p = 0.00096$), possibly due to its acidic environment. Dominant microbial phyla included Proteobacteria, Bacteroidetes, and Firmicutes, with Proteobacteria being the most prevalent in the abomasum (50.06%). Genera like *Proteus* and *Bacteroides* were particularly prominent in the rumen and reticulum, highlighting their significant role in feed degradation and fermentation processes. Over 65% of Kyoto Encyclopedia of Genes and Genomes (KEGG) level 1 genes were associated with metabolism, with significant xenobiotic biodegradation detected in the abomasum. The dbCAN2 search identified Glycoside Hydrolases as the most prevalent CAZyme class (79%), followed by Glycosyltransferases, Polysaccharide Lyases, and Carbohydrate Esterases, with Carbohydrate-Binding Modules and Auxiliary Activities accounting for 1% of the hits. Higher CAZyme abundance was observed in the reticulum and omasum compartments, possibly due to MAGs diversity. In conclusion, the gastrointestinal tract of South African goats harbors diverse CAZyme classes, with Glycoside Hydrolases predominating. Interestingly, higher CAZyme abundance in specific compartments suggested compartmentalized microbial activity, reflecting adaptation to dietary substrates.

Keywords: Goat, Gastrointestinal, Xenobiotic, lignocellulose, CAZymes

3.1 Introduction

Ruminants, including goats (*Capra aegagrus hircus*), possess a complex GIT comprising the rumen, reticulum, omasum, and abomasum, which house a diverse and dynamic microbial ecosystem (Gruninger *et al.*, 2019, Russell and Rychlik, 2001, Cholewińska *et al.*, 2020). This intricate mutual beneficial relationship between the host and the ruminal microbiome is crucial for the efficient fermentation of complex plant material ruminants consume, including lignocellulose (Huws *et al.*, 2018, Zhang *et al.*, 2019). Central to this process is the secretion of specialized enzymes known as CAZymes by the ruminal microbiome, facilitating the breakdown of complex plant cell walls into digestible nutrients (Hungate, 1947, Han *et al.*, 2015, Ribeiro *et al.*, 2016). While the importance of CAZymes in lignocellulosic biomass degradation is well-established, there remains a gap in understanding the specific genomic makeup and functional potential of the microbial communities within the goat GIT, particularly in the context of South African goats.

This knowledge gap presents an opportunity to elucidate the genetic basis of CAZyme production and its implications for lignocellulosic biomass utilization in this unique population (Röder and Welfle, 2019, Reddy, 2015, Adewuyi, 2022, Pahla *et al.*, 2017). This knowledge gap presents an opportunity to elucidate the genetic basis of CAZyme production and its impact on lignocellulosic biomass utilization in this unique population (Kaza *et al.*, 2018, Gasparatos *et al.*, 2015, Tauqeer *et al.*, 2022, Abatenh *et al.*, 2018). One key challenge in harnessing the potential of lignocellulosic biomass lies in its recalcitrant nature, characterized by cellulose's complex structure, lignin's presence, and hemicellulose composition (Röder and Welfle, 2019, Hu *et al.*, 2023, Shi *et al.*, 2023). Overcoming these challenges requires a deeper understanding of the microbial enzymes involved in biomass degradation and the genetic mechanisms underlying their production (Zoghalmi and Paës, 2019, Rojas *et al.*, 2022).

To address these gaps in knowledge, our study aims to characterize the metagenomic profiles of microbial communities within each compartment of the South African goats' GIT, focusing

on the genomic abundance of CAZymes and their potential for lignocellulosic biomass degradation; investigate the functional roles of specific microbial taxa and their associated CAZymes in biomass conversion, with a particular emphasis on overcoming the recalcitrant nature of lignocellulosic substrates; and then assess the impact of geographic location on the composition and functional potential of the goat GIT microbiome, hypothesizing that this factor influence CAZyme gene expression and microbial community dynamics. By rigorously testing these hypotheses, we aim to advance our understanding of the genetic basis of lignocellulosic biomass degradation in South African goats and pave the way for the development of sustainable biotechnological solutions for biomass conversion and potential bioenergy production.

3.2 Methods and Materials

3.2.1 Ethical statement

All necessary research permits and relevant ethical committee approvals were obtained. Ethical guidelines from the College of Agricultural and Environmental Sciences Animal Research Ethics Committee (CAESAREC) of the University of South Africa approved this study and assigned the reference number 2021/CAES_AREC/097 under Dr. A Wilson. The Unisa SOE Ethics Review committee also approved the study and allocated the reference number 2020/CSET/SOE/028 under Prof. E Onyari-Benecha. Ensuring humane treatment of animals during slaughter and compliance with regulations, the Chief State Veterinarian of South African Agriculture and Rural Development, Dr. S. Kamudyariwa, also approved this study and assigned it the reference number UNISA-01-2021-KJR.

3.2.2 Sample information and collection

A total of 10 Saanen Boer domestic goats, aged 2.5-3 years, were selected based on weight and dental development to ensure they were at a similar physiological stage. This age range was chosen based on the hypothesis that older goats harbor a microbiome better adapted to efficiently breaking down plant material. The goats were sourced from two provinces in South Africa: nine goats were procured from three free-range farmers, and one goat (G10) was designated as the control. As a result, the goats were kept under free-range conditions, allowing them to exhibit natural grazing behavior and select their diet. Specifically, three goats were

sourced from Springfontein (30°15'14"S 25°42'14"E), Free State Province, Verkeerdevlei (28°50'S 26°47'E), Free State Province, and Moremela (24.6663°S 30.801°E), Mpumalanga Province (Supplementary table S1. The 10th goat, G10 from Volkrust (27°22'S 29°53'E), Mpumalanga province, was included as a control, with dietary conditions carefully controlled and nutritional information provided in supplementary table S2. To our knowledge, no antibiotics or other drugs were administered to the animals before procurement.

The goats were taken to a registered abattoir and slaughtered by a designated butcher, in accordance with the guidelines set by the National Council of Societies for the Prevention of Cruelty to Animals (NSPCA). Each compartment (rumen, reticulum, omasum, and abomasum) from the stomach was sampled aseptically using a spatula and labelled accordingly. The samples were transported to the University of South Africa laboratory on ice and stored at 4°C for no more than 24 hours after metagenomic DNA extraction.

3.2.3 Metagenomic DNA extraction and sequencing

A total of 40 samples (4 compartments per goat) were weighed between 146-150 mg and were extracted using the Quick-DNA fecal/soil microbe miniprep kit (Zymo Research, Irvine, CA) following manufacturer's instructions. The quantity of extracted DNA was evaluated using the Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). The metagenomic library was prepared using the MGIEasy Universal DNA Library Set (MGI Tech Co., Ltd., Shenzhen, China) according to the manufacturer's instructions, and the library was sequenced on the MGI DNBSEQ-400 sequencer with 2x150 bp reads.

3.2.4 Diversity and Taxonomy of the microbial community in various compartments

Raw metagenomic paired-end read sets were imported into the Nephele platform (version 2.32.1) (Weber et al., 2018) for analysis. Additional trim and filters were run on default settings, and the system outputs were specified for TEDread fastq files, gene-based taxonomic profiling, and taxonomic annotations of scaffolds. At the same time, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used for metabolic pathways (Weber *et al.*, 2018). The merged taxa tables were then uploaded onto the MicrobiomeAnalyst database. The total read

counts amounted to 316,315,689, with an average of 7,892 per sample and a maximum count of 150,696,18, and a minimum of 48,739.77 per sample. To filter the data, the low-count filter minimum was set to 4, and the prevalence in samples was 20% with a mean abundance value, while the low-variance removal filter was set to 10% based on the interquartile range (IQR). A total of 2139 low-abundance features were removed based on prevalence, and approximately 668 low-variance features were removed based on IQR—the number of features that remained after the data filtering step was 6003. The data were further normalized using the cumulative sum scaling (CSS) option, and an abundance-stacked bar was generated for taxonomic profiling of compartments at the phylum and genus levels, merging small taxa with a count <10 based on the total read count (n=10). Furthermore, alpha and diversity plots were generated (Dhariwal *et al.*, 2017).

3.2.5 Functional profiles of whole metagenomes of each compartment group

Raw metagenomics reads were imported to Nephele (version 2.32.1) to analyze the diversity and relationship of the functional annotations (Weber *et al.*, 2018) using KEGG, and the resulting gene abundance table was then imported to Statistical Analysis of Metagenomic Profiles (STAMP) (Parks *et al.*, 2014) in order to determine the abundance and significant gene families in each compartment.

3.2.6 Classification of Metagenome-Assembled Genomes and CAZymes Identification

To facilitate this classification, the DOE Systems Biology Knowledgebase (KBase), an open-source software and data platform that promotes the sharing, integration, and analysis of data on microbes, plants, and their communities (Arkin *et al.*, 2018), was employed. Quality control of the sequence reads was performed on all 40 metagenomic read libraries using FastQC v.0.11.9, and the resulting reads were then trimmed using Trimmomatic v0.36. This was further run on FastQC to assess the quality of trimmed reads. The trimmed sequences were assembled into contiguous fragments (contigs) to create scaffolds for whole-genome assembly using metaSPAdes v3.13, MEGAHIT v1.29, and IDBA-UD v1.1.3 (Gharechahi *et al.*, 2022). Assemblies from all three methods were merged using KB v1.0.1 and compared for assembled contig distribution and quality using the Quality Assessment Tool (QUAST) report. The

assembly platform that yielded the highest-quality contigs was selected for each library. The assembled contigs were clustered into bins, each corresponding to a putative population genome, using MetaBAT2 v1.7, CONCOCT v1.1, and MaxBin2 v2.2.4. DAS Tool improved binning quality by using consensus assignments from the binned contigs outputs of MetaBAT2, CONCOCT, and MaxBin2. We explored the abundance and diversity of CAZymes within the Metagenome-Assembled Genomes (MAGs) from the various compartments of the goat's GIT, focusing on their potential role in lignocellulosic biomass degradation. The assembled contigs clustered into bins, each corresponding to a putative population genome, resulting in 180 Metagenomic Assembled Genomes (MAGs). CheckM v1.0.18 analysis revealed high completeness (averaging 98.40%) and low contamination (averaging 0.57%) of MAGs. A 95% or higher completeness score and contamination less than 5% was generally considered good (Eisenhofer *et al.*, 2023, Parks *et al.*, 2015). Hence, this confirmed that the assembly process was successful and that the assembled genomes were relatively pure, not containing significant genetic material from other organisms or sources (Eisenhofer *et al.*, 2023). The quality and completeness of all the MAGs are represented in Table S2. The MAGs filtered by quality were extracted to individual genome assembly objects with BinUtil v1.0.2. To obtain KBase (Arkin *et al.*, 2018) genomes, genes were annotated with RAST and classified with the Genome Taxonomy Database (GTDB-Tk). To construct a phylogenetic distribution analysis of the predicted CAZymes and draw a correlation between the GIT microbiome, the MAGs were taxonomically classified based on a concatenated protein-based phylogeny representing more than 250 000 prokaryote genomes using the GTDB-Tk. The phylogenetic tree was generated using iTOL (Letunic and Bork, 2007). We then examined all 180 MAGs for CAZyme diversity related to lignocellulosic biomass degradation, including cellulase, lignin, hemicellulase, and pectinase/esterase, using the dbCAN2 database with an E-value of 1e-15 (Gharechahi *et al.*, 2022). Parameters were set to profile all groups of CAZymes, including cellulosome Fams, Glycoside Hydrolases (GH), Glycosyl Transferases (GT), Carbohydrate-Binding Modules (CBM), Carbohydrate Esterases (CE), Polysaccharide Lyases (PL), and Auxiliary Activities (AA) with an E-Value of 1e-15 (Chivian *et al.*, 2023, Chivian *et al.*, 2021, Gharechahi *et al.*, 2022).

3.2.7 Statistical Analysis

Alpha diversity metrics, including Chao1 and Shannon Observed_species, were utilized to measure species richness and evenness across different compartments. Statistical significance was determined using nonparametric tests: the Mann-Whitney U test for two independent groups and the Kruskal-Wallis test for three or more independent groups. The post hoc analysis was also conducted using the Tukey-Kramer test to identify specific group differences when significant differences were observed. All these tests were done on the web-based MicrobiomeAnalyst using the Marker Data Profiling feature (Dhariwal *et al.*, 2017). In the multivariate analysis, Principal Coordinate Analysis (PCoA) was conducted using the Bray-Curtis Index as a distance metric in MicrobiomeAnalyst. The number of dimensions (principal coordinates) used was determined based on Eigenvalues and scree plots, which indicate the variance each principal coordinate explains. To perform PCoA in MicrobiomeAnalyst, the software uses internal algorithms and computations. The significance of differences in microbial community composition among groups was assessed using the Analysis of Similarities (ANOSIM) statistical method, with a conventional threshold of $p < 0.05$ considered significant (Guerra *et al.*, 2022, Guo *et al.*, 2020). Kruskal-Wallis H-test statistical test and the Turkey-Kramer Post-hoc test to the value of 0.95 was done on the KEGG functional annotation heatmap that was generated on STAMP (Parks *et al.*, 2014). The PCA was done using the SRplot (Tang *et al.*, 2023) with an eclipse of 68% confidence interval.

3.3 Results and Discussion

3.3.1 The richness and diversity of the microbial community in various compartments

Comparative analysis of diversity among all compartments (beta-diversity), visualized by Principal Coordinate Analysis (PCoA) using the Bray–Curtis dissimilarity index, revealed clustering of microbial communities from adjacent GIT compartments, such as the reticulo-rumen, reticulum, and omasum, indicating related taxonomy composition (Figure 3.1).

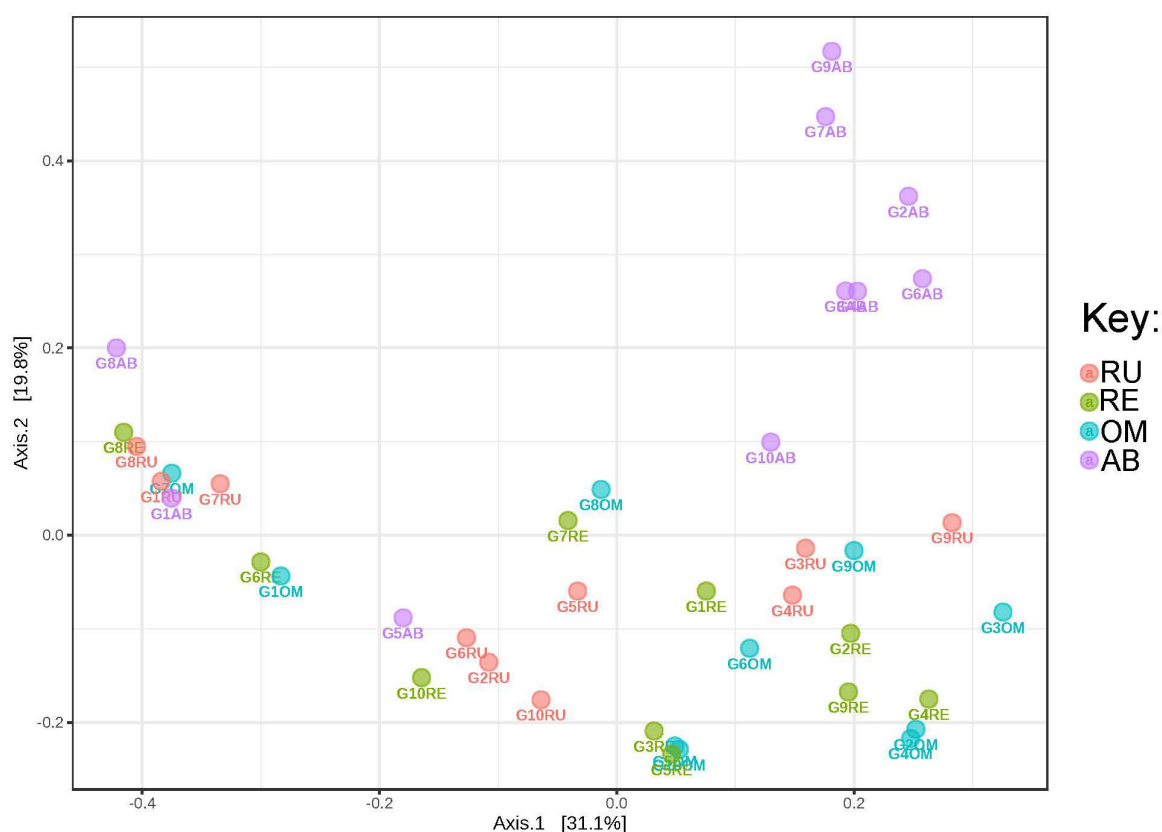
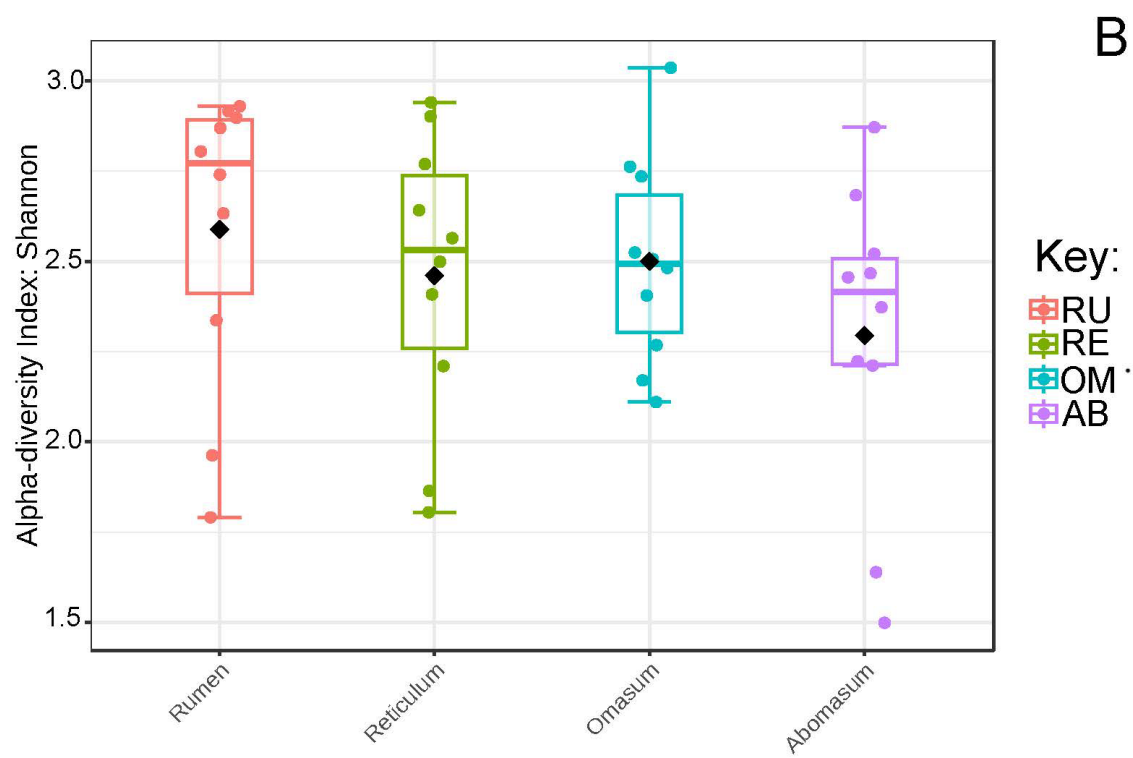
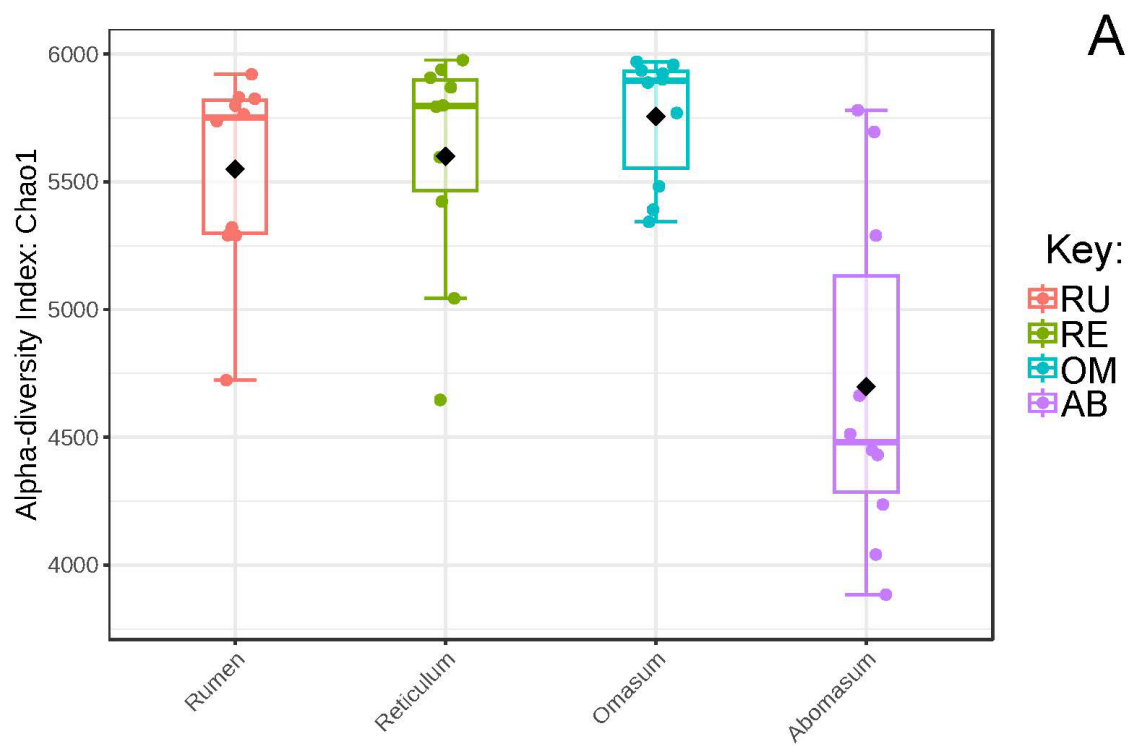


Figure 3.1: Principal Coordinate Analysis (PCoA) plot with the compartment as the primary factor using the Bray-Curtis Dissimilarity index (ANOSIM $p < 0.006$). In the legend, RU represent the rumen, RE the reticulum, OM the omasum, and AB the abomasum.

Conversely, OTUs from the abomasum were mainly separated, suggesting a distinct species composition. ANOSIM analysis confirmed significant compartment differences ($p < 0.006$; $R = 0.12261$). Consistent with prior research (Guerra *et al.*, 2022), the abomasum exhibited notably different microbial communities, suggesting physiological and environmental distinctions.

Alpha diversity analyses, including Chao1 (richness), Shannon index (diversity), and Observed_species (estimated OTUs), further elucidated microbial diversity profiles. The omasum displayed the highest Chao1 diversity (5756.4), followed by the reticulum (5599.6), rumen (5550.2), and abomasum (4698.3), indicating lower species richness in the latter (Figure 2A).



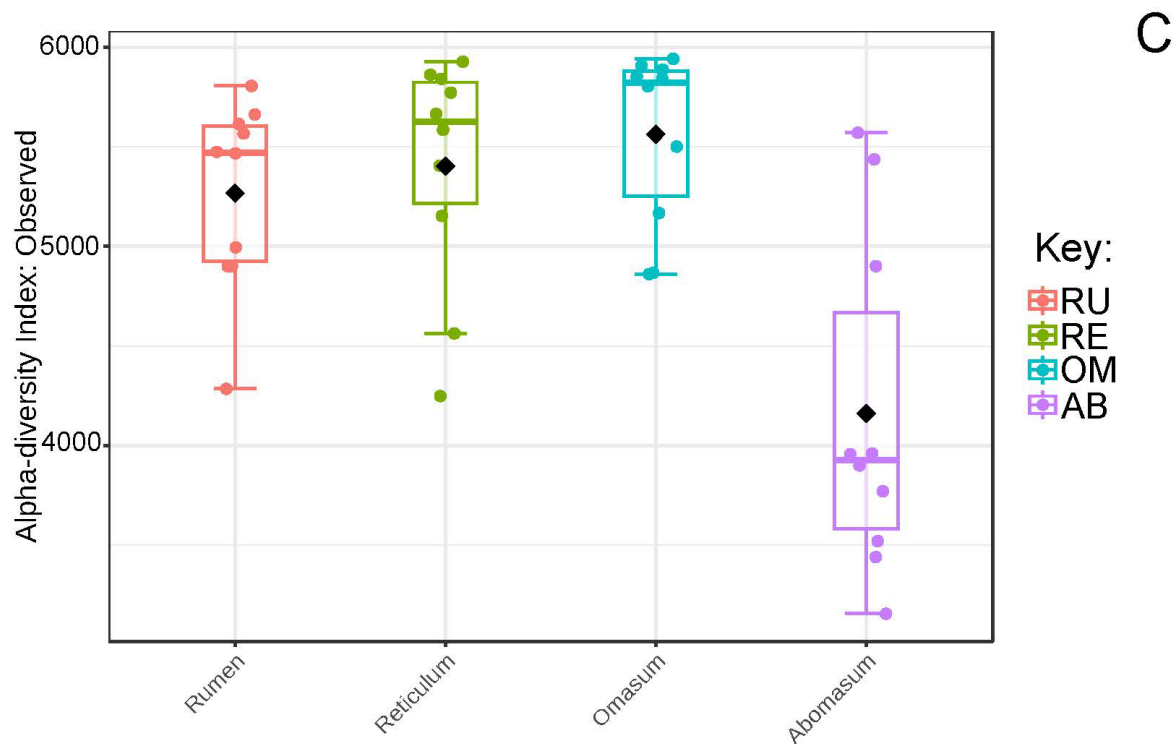


Figure 3.2: A) Alpha diversity indices Chao1, B) Shannon, and C) Observed species derived from the rumen (RU), reticulum (RE), omasum (OM), and abomasum (AB).

The Shannon diversity (Figure 3.2) values varied slightly among compartments, with the rumen exhibiting the highest diversity (2.59), followed by the omasum (2.50), reticulum (2.49), and abomasum (2.29). Observed diversity (Figure 3.2C) followed a similar trend, with the omasum having the highest values (5562.4), followed by reticulum (5401.6), rumen (5266.7), and abomasum (4161.6). Statistical analysis using Mann-Whitney/Kruskal-Wallis tests revealed significant differences in Chao1 diversity ($p = 0.00096$), indicating non-random variations among compartments.

However, Shannon diversity differences were not statistically significant ($p = 0.34399$), suggesting similar evenness across compartments. Meanwhile, like the Chao1 diversity, Observed diversity differences were statistically significant ($p = 0.00157$), indicating actual differences in species richness. These findings suggest decreased richness and evenness in the abomasum, attributed to its acidic environment resembling that of monogastric animals

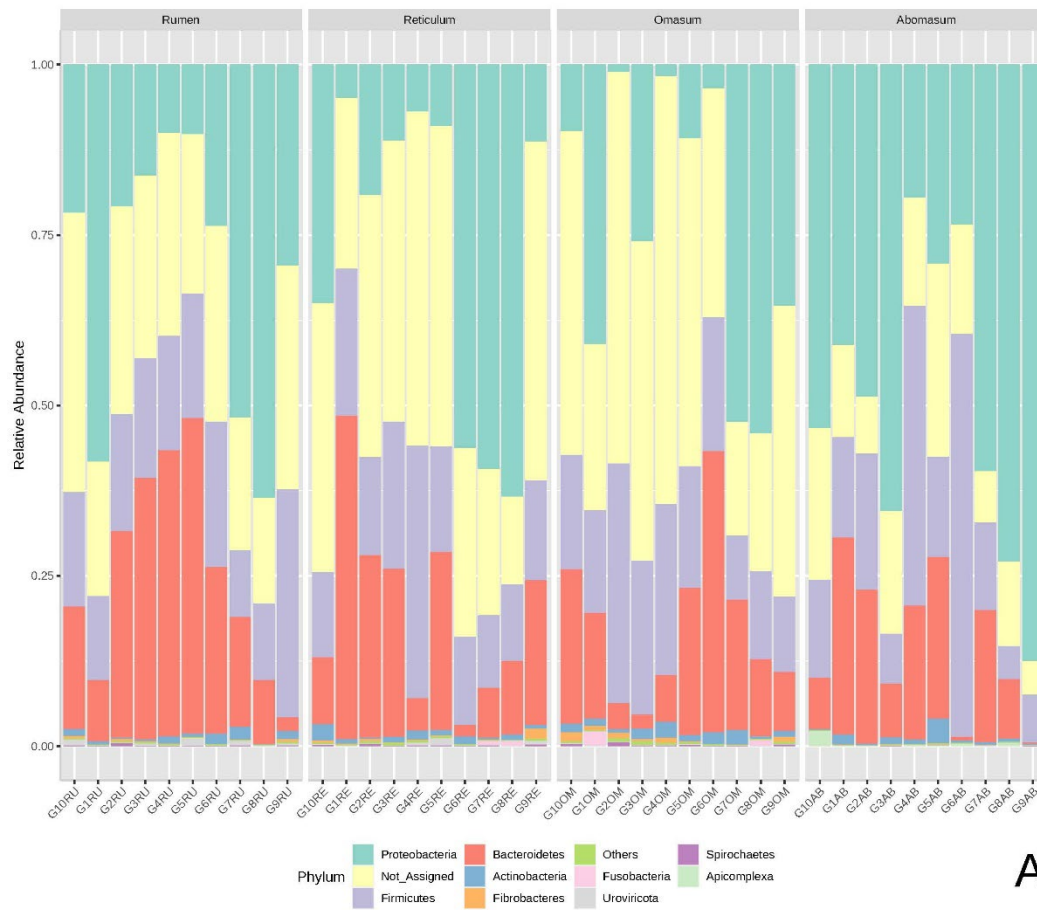
(Firrman *et al.*, 2022, Guerra *et al.*, 2022). Acidic conditions can inhibit microbial growth and favor acid-tolerant species, as observed in prior goat studies (Guerra *et al.*, 2022).

3.3.2 Taxonomic classification of microbial communities in various compartments

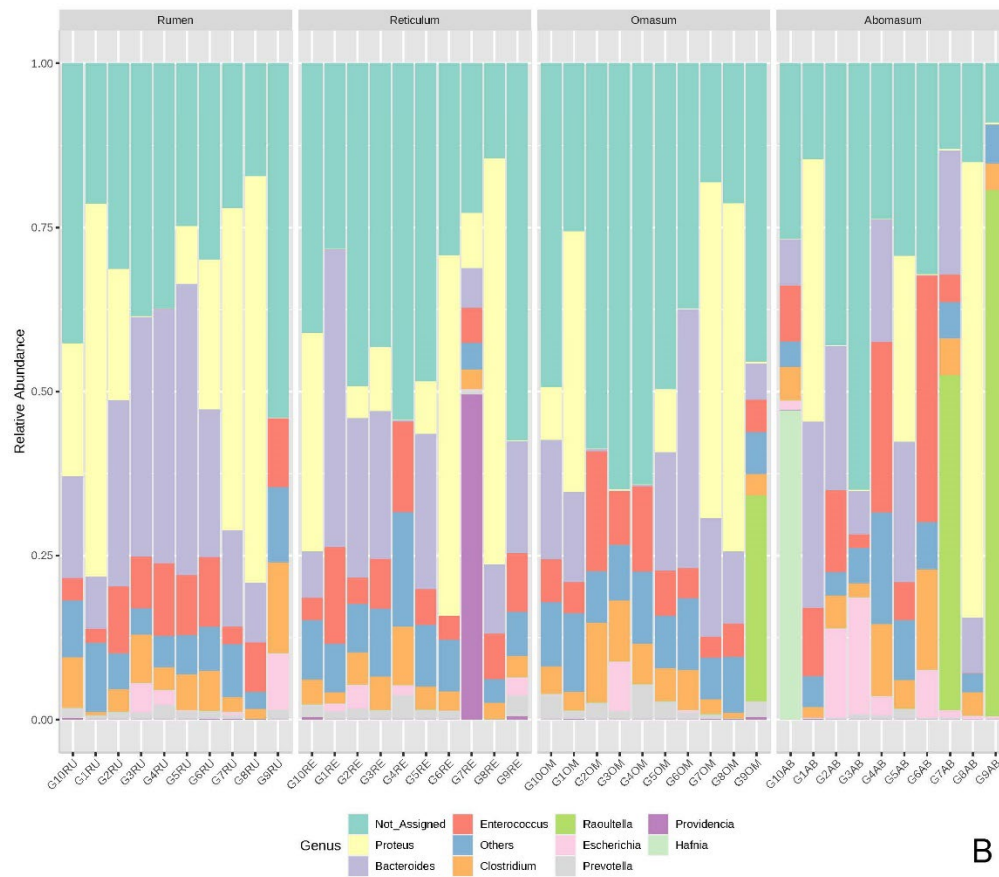
The taxonomic analysis revealed that the compartments were dominated by Proteobacteria, Bacteroidetes, and Firmicutes, as well as by a notably higher abundance of unclassified phyla (n=10). However, the distribution in all the compartments varied (Figure 3.3A). The abomasum had the highest prevalence of Proteobacteria (50.06%), followed by the rumen (30.52%), reticulum (27.58%), and the omasum (23.52%). A notable decrease in Bacteroidetes was observed from the rumen to the abomasum. The rumen consisted of 23.58%, the reticulum had 18.05%, the omasum had 15.29%, and the abomasum had the least abundance at 13.9%.

On the other hand, Firmicutes were relatively more abundant in the abomasum and least prevalent in the other compartments, particularly the reticulum and rumen. The Firmicutes accounted for 19.9% of the abomasum, 18.55% of the omasum, 17.49% of the rumen, and 17.22% of the reticulum. Actinobacteria were present in amounts of less than 1% in the rumen, reticulum, and abomasum and approximately 1.39% in the omasum. The remaining phyla were found in relatively lower levels in all compartments, with no phylum accounting for more than 0.6% of the total biomass. The unclassified phyla were also present in relatively higher abundance in the omasum, reticulum, and rumen, accounting for 40.03%, 35.2%, and 26.77% of total biomass, respectively, with the abomasum having the least abundance (14.71%) among the other compartments. These findings suggest that the goats' digestive tract microbial communities are diverse and are attributed to specific physiological aspects of each compartment. For example, a notable population of Proteobacteria has been reported to thrive in acidic environments, while Bacteroidetes thrives well in alkaline environments (Firrman *et al.*, 2022). As the true stomach of ruminants, the abomasum is characterized by acidic and proteolytic conditions essential for the digestion of protein-rich feed (Guerra *et al.*, 2022, Moran, 2005). The higher prevalence of Proteobacteria might be associated with their ability to thrive in low pH environments (Firrman *et al.*, 2022). In other studies, Bacteroidetes, Firmicutes, and Proteobacteria were also found to be the primary dominant phyla (Wang *et al.*, 2016, Pitta *et al.*, 2016, Wang *et al.*, 2019). In similar studies, Bacteroidetes was also found in

higher abundance in the rumen compartment (Guerra *et al.*, 2022, Wang *et al.*, 2019, Mao *et al.*, 2015). Anaerobic bacteria from the two major phyla, Bacteroidetes and Firmicutes, comprise most of the microbiome in a healthy GIT (Zhang *et al.*, 2021). Bacteroidetes play a significant role in the breakdown of complex carbohydrates due to the number of genes encoding CAZymes (Patel *et al.*, 2014a). Firmicutes are primarily responsible for degrading complex polymers, including cellulose, hemicellulose, and other fibrous materials (Patel *et al.*, 2014a). Therefore, these two phyla and their functions in stimulating the breakdown of dietary fibers and other complex compounds are indicators of a healthy GIT.



A



B

Figure 3.3: A) The relative abundance of the ten most prevalent Phyla, **B)** Genus across all goats' compartments; Rumen, Reticulum, Omasum, Abomasum.

The microbial composition analysis across different compartments of the goats' GIT also revealed variations in the abundance of genera (Figure 3.3B). Among the analyzed genera, *Proteus*, *Bacteroides*, *Enterococcus*, and *Clostridium* were prominent across all compartments. Among the identified taxa, *the Proteus and Bacteroides genera were the most prevalent in the rumen, accounting for approximately 21.88% and 23.92%, respectively*. The rumen exhibited the highest diversity of both genera, and this observation could be attributed to the unique physiological conditions of the rumen, which serves as the primary fermentation chamber in the goat's digestive system (Moran, 2005). The rumen provides an anaerobic environment rich in fermentable substrates from plant materials, such as cellulose and hemicellulose, facilitating the proliferation of cellulolytic and fibrolytic microorganisms (Hungate, 1947).

The reticulum was the second compartment in which both these genera were prevalent, *Proteus* accounting for 18.08% and *Bacteroides* present at 15.74%. The efficient degradation of feed in the rumen would not be possible without the cooperative role of the reticulum, which is located adjacent to the rumen and acts as a mixing chamber by facilitating the regurgitation and rumination of partially fermented feed, forming the cud (Hungate, 1947, Moran, 2005). This rumination process is crucial in breaking down large feed particles into smaller ones, significantly increasing the surface area available for microbial colonization in the rumen. As a result, the microbial population can efficiently ferment the feed, leading to enhanced nutrient availability for the host (Moran, 2005, Rabapane *et al.*, 2022). Furthermore, the reticulum's contractions play a pivotal role in particle size reduction, fiber processing, and the uniform distribution of ingested feed among the microbial population. Its honeycomb-like structure helps trap large fibrous particles, providing extended retention time for microbial activity. This dynamic process allows the microorganisms to break down complex plant material further, ensuring optimal nutrient utilization from the feed (Moran, 2005, Puniya *et al.*, 2015). Therefore, these results emphasize the intricate interplay between the rumen and reticulum in the partial degradation of feed.

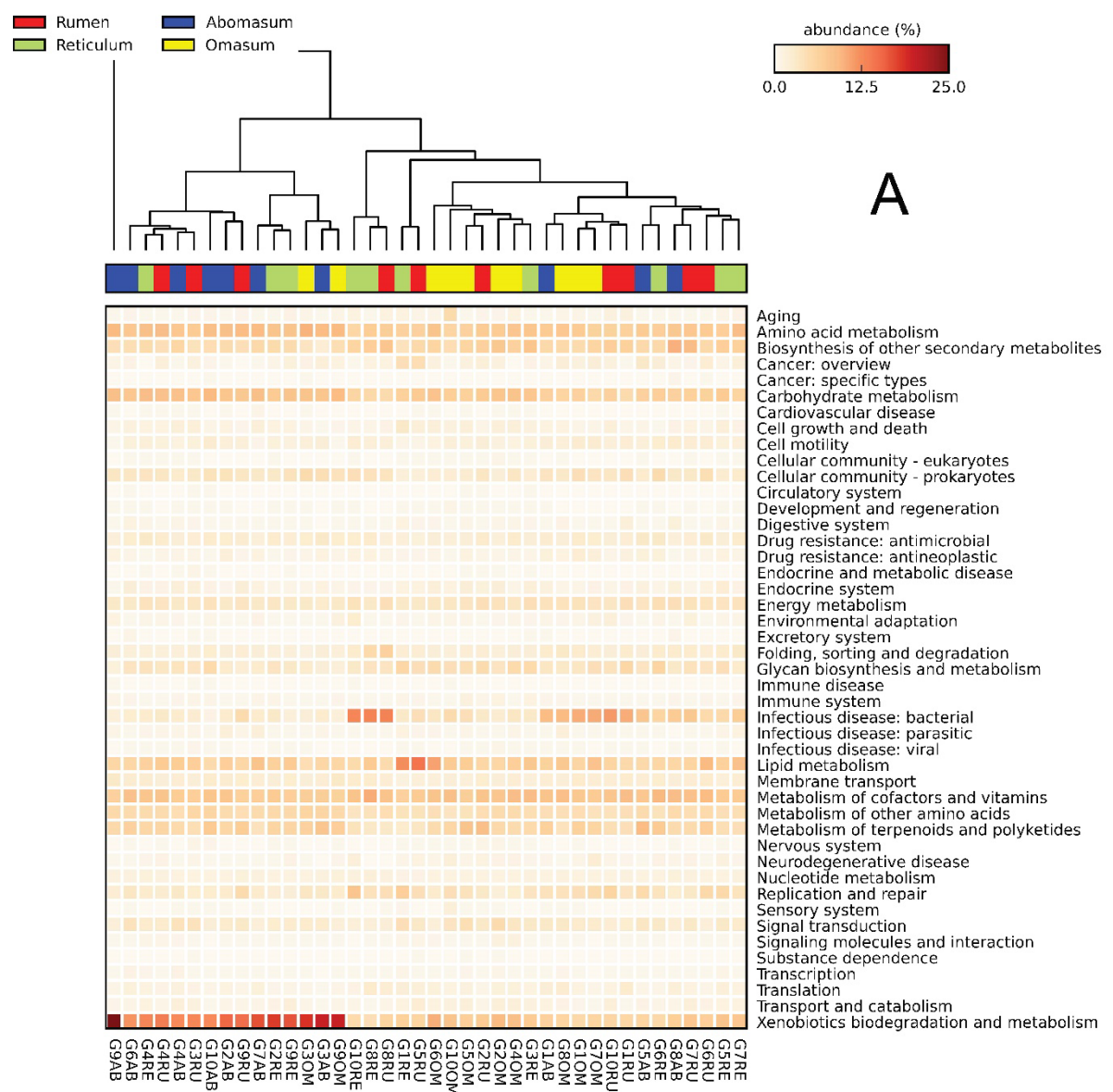
In contrast, the omasum exhibited a relatively lower prevalence of genera than the other three compartments but exhibited a notably higher abundance of unclassified genera (43.51%) and *Prevotella* genus (2.12%). The omasum is a filtration chamber where water and fine particles are absorbed before the ingesta progresses to the abomasum. The reduced abundance of genera in the omasum could be attributed to its role in fine particle retention and water absorption, leading to a less favorable environment for diverse microbial growth (Moran, 2005). Meanwhile, the abomasum displayed a distinct microbial profile compared to the other compartments, with *Raoultella*, *Hafnia*, and *Klebsiella* among the top ten in this compartment. Even though *Proteus* and *Bacteroides* were dominant, a significant decrease was observed, with lower quantities at 13.81% and 13.22%, respectively. A relatively higher abundance of *Enterococcus* (10.7%), *Clostridium* (5.8%), and *Escherichia* (4.54%) was observed in the abomasum, whereas these genera were less prevalent in the other three compartments. This observation could be due to their ability to cope under lower pH conditions, like the ones reported in the abomasum (Wang *et al.*, 2019). While the other observed genera are similar to previous research (Guerra *et al.*, 2022, Mao *et al.*, 2015, Wang *et al.*, 2019), identifying the *Proteus* genus as a prevalent genus in this study presents a unique and potentially significant finding. Sequences below 0.5% were derived from archaeal communities, which were present in lower quantities than bacterial communities. A similar study also reported low observed quantities of archaeal communities, which suggested their low levels of participation in the degradation of polysaccharides in the GIT (Guerra *et al.*, 2022).

The following sections explore the putative functional roles of these microbial communities in each compartment, focusing on the pathways involved and the production of CAZymes.

3.3.3 Functional profiles of whole metagenomes of each compartment

Building on the preceding discussions on diversity and taxonomy, the functional profiles of the entire metagenomes within each compartment, in the context of significant gene families, will be discussed. The functional profiles of the microbial population represented in each compartment were predicted using the KEGG database. The heatmap (Figure 4A) depicts all the gene families predicted for each class, including the metabolic pathways and their abundance in various compartments. At level 1 KEGG, more than 65% of the predicted genes were found to be involved in metabolism. The most prevalent gene families at level 2 of KEGG,

including xenobiotic biodegradation and metabolism (12%), metabolism of cofactors and vitamins (9%), amino acid metabolism (9%), carbohydrate metabolism (7%), and lipid metabolism (7%), were identified. According to the findings, the quantities of these functional classes varied greatly across compartments, with some demonstrating greater dominance than others (Figure S2).



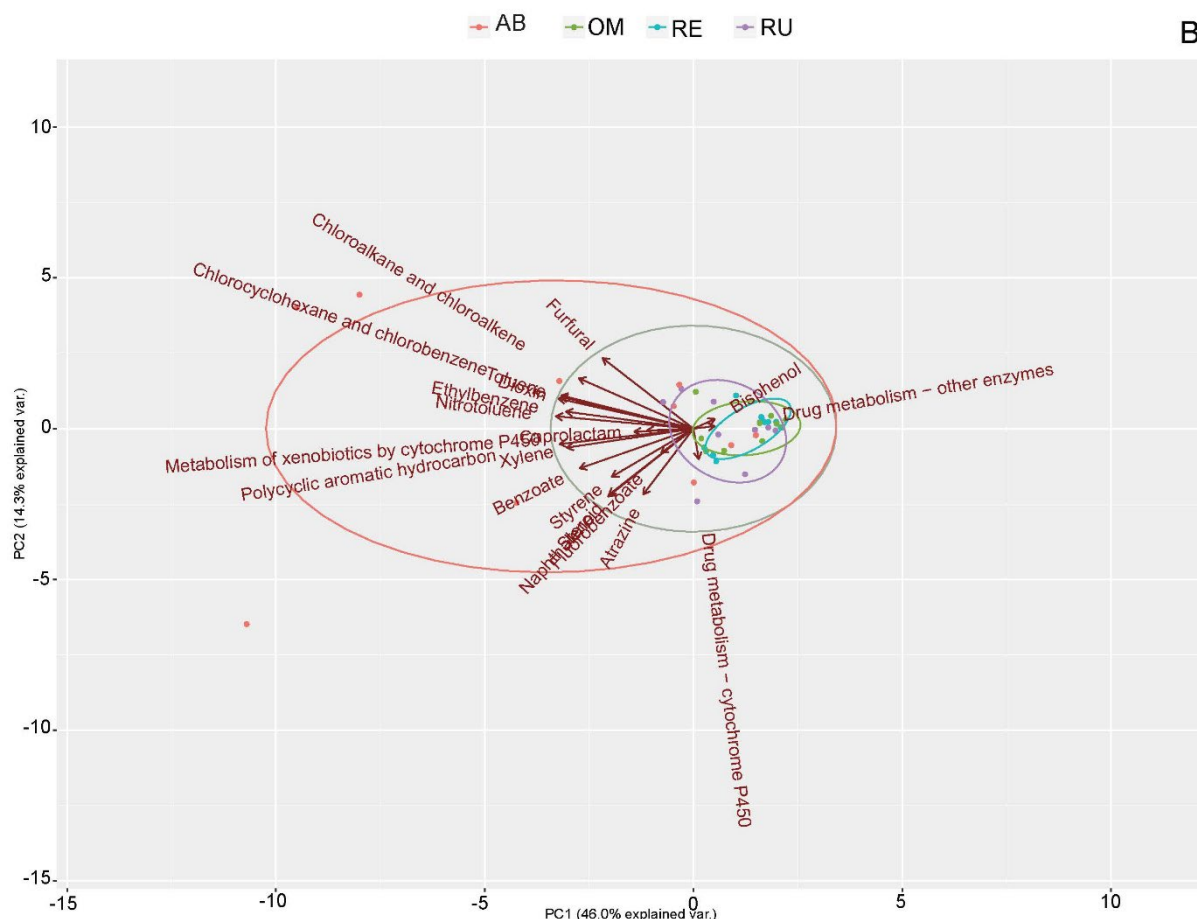


Figure 3.4: **A)** Abundance of functional profiles of gene families in all the compartments based on the KEGG database. **B)** Principal Component Analysis (PCA) plot showing the distribution of various xenobiotics degradation pathways across the four compartments, Rumen (RU), Reticulum (RE), Omasum (OM), Abomasum (AB).

Compared to other pathways, the number of genes associated with xenobiotic biodegradation and metabolism was relatively high in the abomasum compartment. The xenobiotics biodegradation and metabolism pathway in the KEGG database provides information on the enzymes and metabolic pathways involved in the biodegradation and metabolism of xenobiotic compounds, including environmental pollutants, drugs, and other foreign compounds, which further highlights the adaptability and complexity of the microbiota in these compartments. Xenobiotics are foreign substances not naturally produced or expected to be present in an organism's body (Patel and Sen, 2013).

Certain compounds can be regarded as xenobiotics in plant cell walls, as they may be recalcitrant or difficult to metabolize without the assistance of specific microbial communities (Röder and Welfle, 2019, Hu *et al.*, 2023). Lignin, a complex polymer that provides rigidity to the cell walls and its derivatives, can be considered as such a compound due to its recalcitrant complex structure, and requires lignolytic enzymes produced by certain microbial taxa for it to be broken down (Röder and Welfle, 2019, Hu *et al.*, 2023, Shi *et al.*, 2023). Furthermore, phyla Firmicutes, Bacillota, and Proteobacteria have been reported to produce lignolytic enzymes, including lignin peroxidases, laccase, and manganese peroxidase (Silva *et al.*, 2021). Thus, the prevalence of Firmicutes and Proteobacteria, particularly in the omasum and abomasum compartments, might be attributed to their involvement in lignin degradation, which assists the host in accessing nutrients encapsulated within plant cell walls. The xenobiotic pathways in this study related to lignin degradation were toluene, ethylbenzene, xylene, styrene, and polycyclic aromatic hydrocarbons, among others (Lonchay *et al.*, 2022).

Hemicellulose is another plant cell wall compound that can be considered a xenobiotic, and the furfural degradation pathway was detected in this study during its degradation (Figure 4B). Pathways related to the degradation of environmental pollutants and industrial chemicals, such as atrazine, chloroalkanes, and chloroalkenes, show significant activity across all compartments but are particularly abundant in the abomasum (Figure 4B). This suggests that the abomasum plays a crucial role in detoxifying and breaking down ingested toxic compounds into less harmful metabolites. Furthermore, pathways associated with the metabolism of pharmaceuticals and drugs, including cytochrome P450-mediated metabolism, exhibit varying levels of activity across compartments, with some showing prominent abundance in the abomasum. This indicates that the abomasum is actively involved in processing and detoxifying ingested medications and pharmaceutical compounds, converting them into less toxic forms. Moreover, the presence of pathways for the degradation of industrial chemicals such as caprolactam, styrene, and xylene in the abomasum highlights the potential for ruminant exposure to these compounds through their diet. The significant activity of these pathways highlights the importance of the abomasum in handling and metabolizing xenobiotic compounds, ultimately converting them into less harmful substances.

Interestingly, the high abundance of xenobiotic degradation and metabolism across all compartments in this study is inconsistent with other studies conducted on goats (Zhang *et al.*, 2021, Lonchay *et al.*, 2022), and ruminants, including dairy cows (Mao *et al.*, 2015). These observations offer a unique and intriguing perspective within the context of existing research, providing an opportunity to explore new dimensions of xenobiotic interactions. The involvement of the microbial community in the abomasum compartment in xenobiotic degradation and metabolism, as observed in our study, offers thought-provoking insights into established findings in this field. While existing research may not align with our results, it is crucial to consider the potential influences of various factors that might contribute to this disparity. Environmental factors, such as diet and exposure to different browse species (shrubs, trees, and forage plants), may play a pivotal role in shaping the metabolic responses observed in our study (Wang *et al.*, 2019).

Likely, specific microbial populations residing within the goat's GIT could also contribute to xenobiotic metabolism, potentially introducing a previously unexplored dimension to this intricate process. Unlike in previous research studies, which were dominated by Firmicutes and Bacteroidota (Guerra *et al.*, 2022, Wang *et al.*, 2019), the GIT samples in this study were dominated by the presence of the *Proteus* genus of the Proteobacteria phylum, which primarily consists of bacterial strains suspected of their involvement in pollutant degradation and metabolism (Drzewiecka, 2016, Apines-Amar *et al.*, 2022). They have been reported to possess mechanisms that enable them to break down and utilize complex organic compounds, including hydrocarbons (Survery *et al.*, 2004). These properties make them essential contributors to bioremediation and environmental clean-up processes (Drzewiecka, 2016, Apines-Amar *et al.*, 2022). While our findings may differ from prior research, they open new avenues of inquiry and highlight the need for a more comprehensive understanding of species-specific and contextual influences on xenobiotic degradation and metabolism. Therefore, elucidating the mechanisms underlying these findings should be warranted to establish a holistic perspective on the complex interplay between the *Proteus* genus and xenobiotic degradation and metabolism.

The carbohydrate metabolic pathway observed was also relatively abundant across all samples. This pathway is significant to the goat's GIT microbiome because it ferments complex

carbohydrates into simple sugars that microbial communities can consume for energy and replenishment in the GIT. The number of carbohydrate-metabolism pathways detected across all compartments indicated that the microbial community was well adapted to plant cell wall degradation and absorption. Furthermore, all compartments had abundant amino acid and lipid metabolism. This was also a crucial observation because amino acids are required for protein synthesis and play a role in numerous physiological processes. These findings were similar to studies conducted on other goats (Guerra *et al.*, 2022, Wang *et al.*, 2019).

3.3.4 Classification of Metagenome-Assembled Genomes and CAZymes Identification

The reticulum and omasum exhibited higher MAGs representation of 32% and 29%, respectively, with the abomasum having the lowest (18%) (See table S2 and figure 3.5). At the genus level, MAGs from *Bacteroides* were consistently present across all compartments, suggesting their importance in GIT degradation and metabolism. *Proteus* MAGs were less abundant in the abomasum. Meanwhile, *Enterococcus_B* and *Escherichia* MAGs exhibited compartment-specific variations, with higher counts in the abomasum. Furthermore, *Enterococcus_C* and *Peptostreptococcus* were only present in the first three compartments, and none were observed in the abomasum. *Bifidobacterium*, *Fibrobacter*, and *Butyrivibrio* MAGs appeared to be relatively more abundant in the omasum, potentially indicating their involvement in later digestion and nutrient absorption stages.

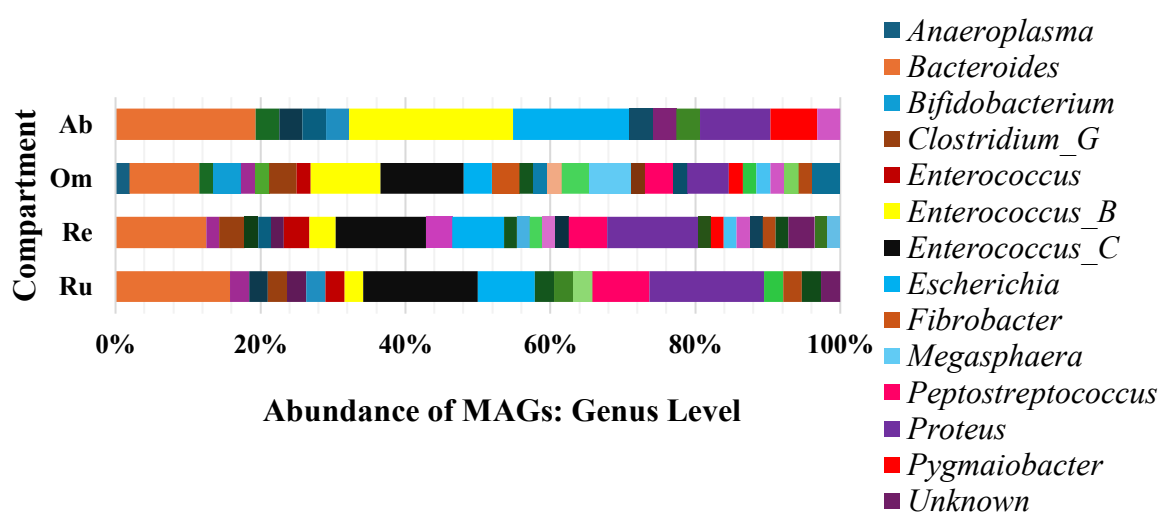


Figure 3.5: The abundance (%) of MAGs represented in each compartment at the genus level.

The dbCAN2 prediction search revealed various CAZyme classes, including glycoside hydrolases (GHs), glycosyl transferases (GTs), carbohydrate esterases (CEs), carbohydrate-binding modules (CBMs), polysaccharide lyases (PLs), and auxiliary activities (AAs). All the hits are profiled in supplementary table S4. The search resulted in a total of 32 455 hits, of which 79% encoded for GHs (25 551 hits), which are a large family of enzymes that are responsible for the hydrolysis of glycosidic bonds between carbohydrates or between a carbohydrate and a non-carbohydrate moiety. These enzymes are involved in the degradation of various carbohydrates, including cellulose, hemicellulose, and pectin, which are the major components of plant cell walls (Jakeer *et al.*, 2020, Park *et al.*, 2018). GTs (3 144 hits), PLs (1896 hits), and CEs (1 242 hits) accounted for 10%, 6%, and 4% of the hits, respectively (Figure 6A). GTs transfer sugar moieties from activated donor molecules to acceptor molecules, forming glycosidic bonds (Park *et al.*, 2018), while PLs facilitate the depolymerization of anionic polysaccharide chains via a β -elimination mechanism (MacDonald and Berger, 2014). CEs play an essential role in the breakdown of lignocellulosic biomass by cleaving ester bonds between lignin and hemicellulose or pectin, making the cellulose and hemicellulose more accessible to other enzymes (Jakeer *et al.*, 2020). CBMs and AAs domains accounted for only 1% of the hits. CBMs domains bind specifically to carbohydrate substrates and allow CAZymes to target and degrade or modify carbohydrates, while AAs are involved in the breakdown and modification of complex carbohydrate structures such as lignocellulose, chitin, and hemicellulose (Park *et al.*, 2018).

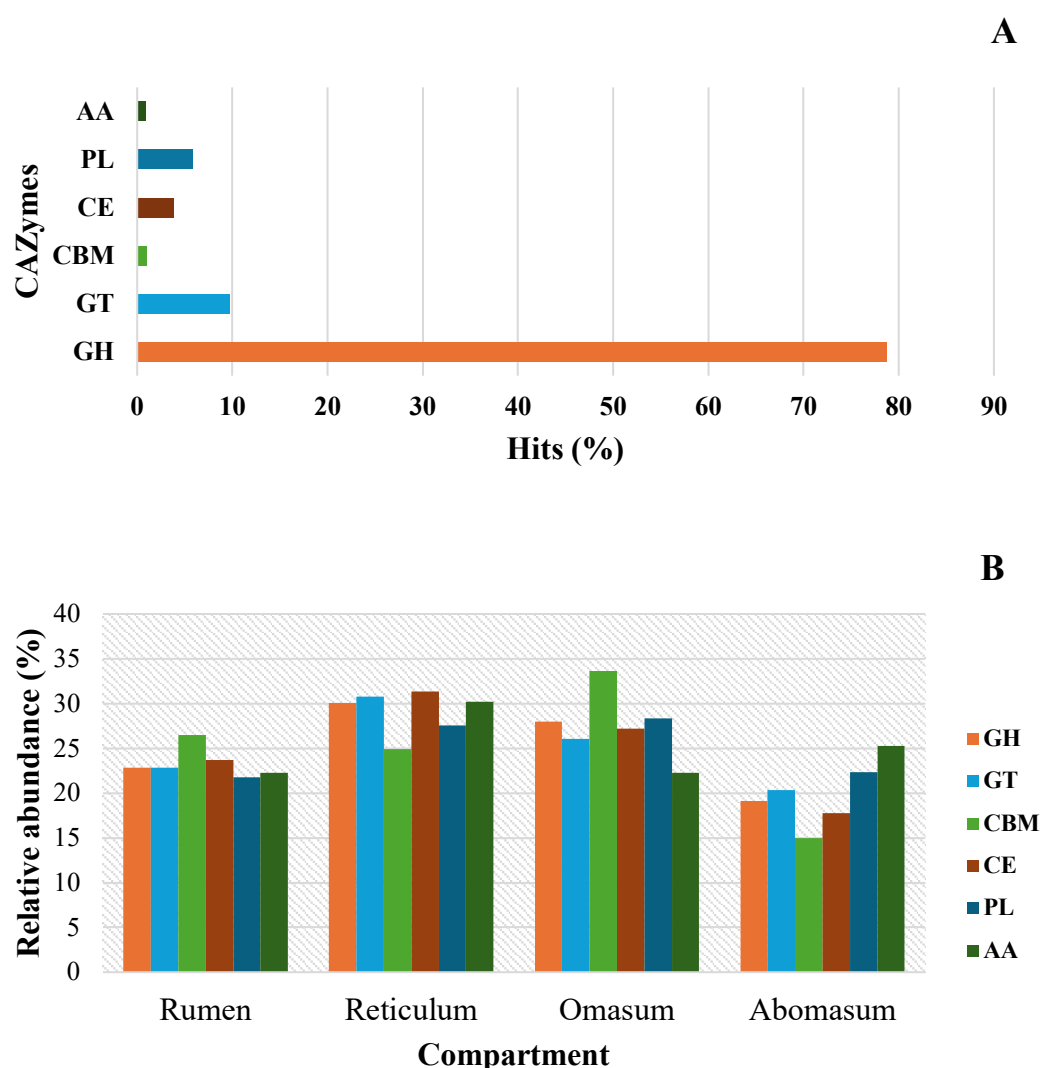


Figure 3.6: **A)** The abundance (%) and prevalence of CAZymes across from all four compartments: glycoside hydrolases (GHs), glycosyl transferases (GT), carbohydrate esterases (CEs), carbohydrate-binding modules (CBMs), polysaccharide lyases (PLs), and auxiliary activities (AAs) **B)** The distribution of CAZymes hits in all compartments.

Previous studies have also investigated the ruminant microbiome for the presence of CAZymes responsible for the efficient degradation of lignocellulosic biomass. An analysis of the camel rumen microbiome found that among the detected CAZymes, GHs were the most abundant, accounting for approximately 49.5%, followed by GTs at 30.8%, and the remaining CAZymes accounted for 19.61% (Gharechahi *et al.*, 2022). Similarly, another study of the buffalo rumen metagenome identified GHs as the most abundant, followed by GTs (Patel *et al.*, 2014a). The

CAZymes were relatively higher in the reticulum and omasum compartments (Figure 6B), possibly due to the MAGs diversity observed.

Table S2 shows the number of hits for various GHs, with the GH13 and GH43 families having the highest hits at 7300 and 6590, respectively. The reticulum had higher hits in both GH13 and GH43 families, accounting for 2278 and 2056 hits, respectively. The abomasum had the least amount of hits in both. GH13 includes α -amylases, branching enzymes, and α -glucosidases, which hydrolyze α -1,4-glycosidic bonds in starch and related polysaccharides. GH43 enzymes include xylanases, arabinases, β -1,3-xylosidase, and β -xylosidase, which hydrolyze xylan, a major hemicellulose component. The high number of hits for these two families can provide a snapshot of the goats' diet. Other GHs with a high number of hits include GH2 (885 hits), GH3 (883 hits), GH5 (804 hits), and GH30 (522 hits). The GH2 family has a range of possible functions, including β -galactosidase activity, which breaks down lactose and related complex carbohydrates. GH3 enzymes are β -glucosidases that break down cellobiose and related carbohydrates; meanwhile, GH5 enzymes are endo- β -1,4-glucanases involved in cellulose degradation. GH30 enzymes are also involved in cellulose degradation, specifically cleaving β -1,4-glycosidic bonds in the backbone of cellulose (Jakeer *et al.*, 2020).

The presence of various GTs in the goat gut microbiome also indicated the diversity of microorganisms in these goats' digestive systems. GTs are responsible for transferring sugar moieties between molecules, forming glycosidic bonds (Jakeer *et al.*, 2020). However, the ability of GTs to act on lignocellulosic biomass is still under research. While some studies suggest that GTs may not play a significant role in the degradation of lignocellulosic biomass (Jakeer *et al.*, 2020), it has been reported that GTs may be involved in modifying hemicelluloses (Scheller and Ulvskov, 2010, Phitsuwan *et al.*, 2013). Therefore, depending on the microbial source, GTs can modify hemicellulose by attaching a sugar molecule or modifying an existing sugar molecule, thereby altering its physical and chemical properties and potentially making it more accessible to CAZymes involved in the degradation of lignocellulosic biomass. Accounting for 362 hits, PL11 was the most abundant family among the known PLs, followed by PL1 and PL8 with 314 and 277 hits, respectively. PL4, PL27, and PL31, on the other hand, had the fewest hits. Based on the number of matches for each CE, CE1, CE9, and CE12, they appear to be the most common in the sample. CE8 and CE4 also

alter glycan structures, critical for cell signaling and recognition. CE7, which is engaged in glycan structure synthesis, had fewer hits.

Few hits for CE11, CE6, CE19, CE2, CE15, CE17, CE5, and CE14 indicated that they were less common in the samples. CBM67 was the most common family, with 105 hits, followed by CBM34 (45), CBM6 (40), and CBM37 (32) in the CBMs search results. CBMs are non-catalytic domains found in CAZymes that identify and bind carbohydrates in lignocellulosic biomass. CBMs are divided into families based on sequence similarity and carbohydrate-binding specificity. In this analysis, CBM67 was the most common domain. CBM67 is found in enzymes that degrade crystalline cellulose and has a high affinity for L-rhamnose, a sugar present in the cell walls of various plants, particularly lignocellulosic biomass. A substantial number of CBM67 domains in the goat microbiome indicates that goats have a high potential for effective cellulose degradation (Bredon *et al.*, 2018).

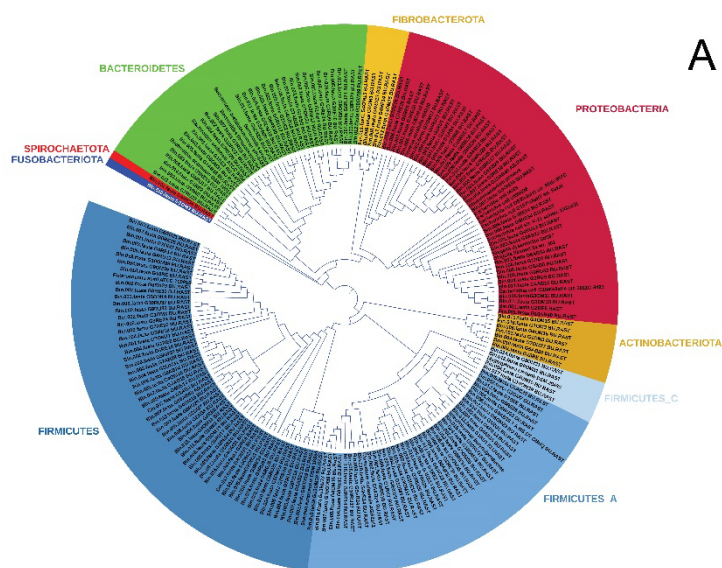
AAs are proteins that do not belong to the CAZymes families but are required for the complete degradation of complex carbohydrates and organic compounds such as lignin. These lignin-modifying enzymes (LMEs) include oxidoreductases, such as laccases and peroxidases, carbohydrate esterases, and other accessory enzymes. Lytic polysaccharide monooxygenases (LPMOs), by contrast, act on recalcitrant cellulose and hemicelluloses rather than lignin. The hits for AAs were relatively low compared to the different families of enzymes discussed earlier because AAs are not directly involved in the depolymerization of complex carbohydrates but instead assist in the degradation process by breaking down the non-carbohydrate components of these substrates or modifying the carbohydrate polymers to make them more accessible to other enzymes. AA3 was the most prevalent family, with 150 hits. The AA3 family comprises oxidoreductases (e.g., glucose oxidases, aryl-alcohol oxidases, and cellobiose dehydrogenases) that can enhance cellulase and hemicellulase activity by generating hydrogen peroxide or electron donors (Jakeer *et al.*, 2020, Kameshwar and Qin, 2017). Although most AA hits are from the reticulum, the abomasum had a relatively higher number of AA hits than the other two compartments.

The Firmicutes phylum was the most dominant group of microorganisms in the goats' GIT responsible for lignocellulosic biomass, with approximately 32.2% coverage. This phylum contains many species that can efficiently degrade lignocellulose, including genera *Butyrivibrio*, *Ruminococcus*, and *Clostridium* (Patel *et al.*, 2014a). These microorganisms produce a range of lignocellulolytic enzymes such as cellulases, hemicellulases, and ligninases, which break down the complex lignocellulosic biomass into simpler sugars that can be further utilized by the microorganisms as a source of energy (Gharechahi *et al.*, 2022). Most of the hits in the GIT microbiome belonged to the genera *Enterococcus*, specifically *Enterococcus_B* and *Enterococcus_C*, and were primarily associated with the GH family of CAZymes.

Proteobacteria were the second-most abundant phylum responsible for producing CAZymes, with approximately 20% coverage, and are among several members shown to play a role in lignocellulose degradation. Members of the genus *Succinivibrio* can degrade lignocellulose to varying degrees through the secretion of various hydrolytic enzymes (Takizawa *et al.*, 2021, Gharechahi *et al.*, 2020). Similarly, Bacteroidetes accounted for approximately 17.2% of the MAGs. They include several members reported to be associated with butyrate production in the rumen and are involved in the degradation of lignocellulosic biomass (Naas *et al.*, 2014). Members of the genera *Bacteroides* and *Prevotella* are known to produce a range of lignocellulolytic enzymes that help break down complex plant material (Patel *et al.*, 2014a). Actinobacteriota and Fibrobacterota phyla accounted for 3.89% and 2.78% of the MAGs, respectively. Although they were less abundant than Firmicutes, Proteobacteria, and Bacteroidetes phyla, they are also known to play a vital role in lignocellulosic biomass degradation (Patel *et al.*, 2014a).

Firmicutes_C, Spirochaetota, and Fusobacteriota phyla, on the other hand, were identified in significantly lower percentages, at 2.2%, 0.56%, and 0.56%, respectively. Although they were less prevalent, members of these phyla are also capable of lignocellulose degradation, producing a variety of enzymes that aid in the breakdown of complex plant material. In a previous study investigating the CAZymes from a buffalo metagenome fed with green and dry roughage, Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria, in descending order, were found to be the most abundant phyla involved in the production of various CAZymes (Patel *et al.*, 2014a).

Out of 180 MAGs, about 14 amounted to 3938 genomes that could not be accurately identified as any known species (Table S2). Considering the high quality of these MAGs, this suggests that there were likely more unidentified and uncultured microbial species in goat GIT, which are not represented in existing reference databases. These observations are essential for understanding the complex microbial communities in the GIT and their role in lignocellulosic biomass degradation. The unassigned MAGs at the species level belonged to the genera *Anaeroplasma* (1), *Butyrivibrio* (1), *Enterococcus_C* (1), *Fusobacterium_A* (1), *JAAME01* (1), *Massilibacterium* (3), *Ruminococcus_E* (2), *Treponema_D* (1), and Unassigned genera (3). Furthermore, these findings suggested that there is still much to be discovered about the microbial population within the GIT of free-range goats and their ability to break down lignocellulosic biomass. A phylogenetic tree showing the relatedness of the taxa responsible for CAZyme production at the phylum level is shown in Figure 7A.



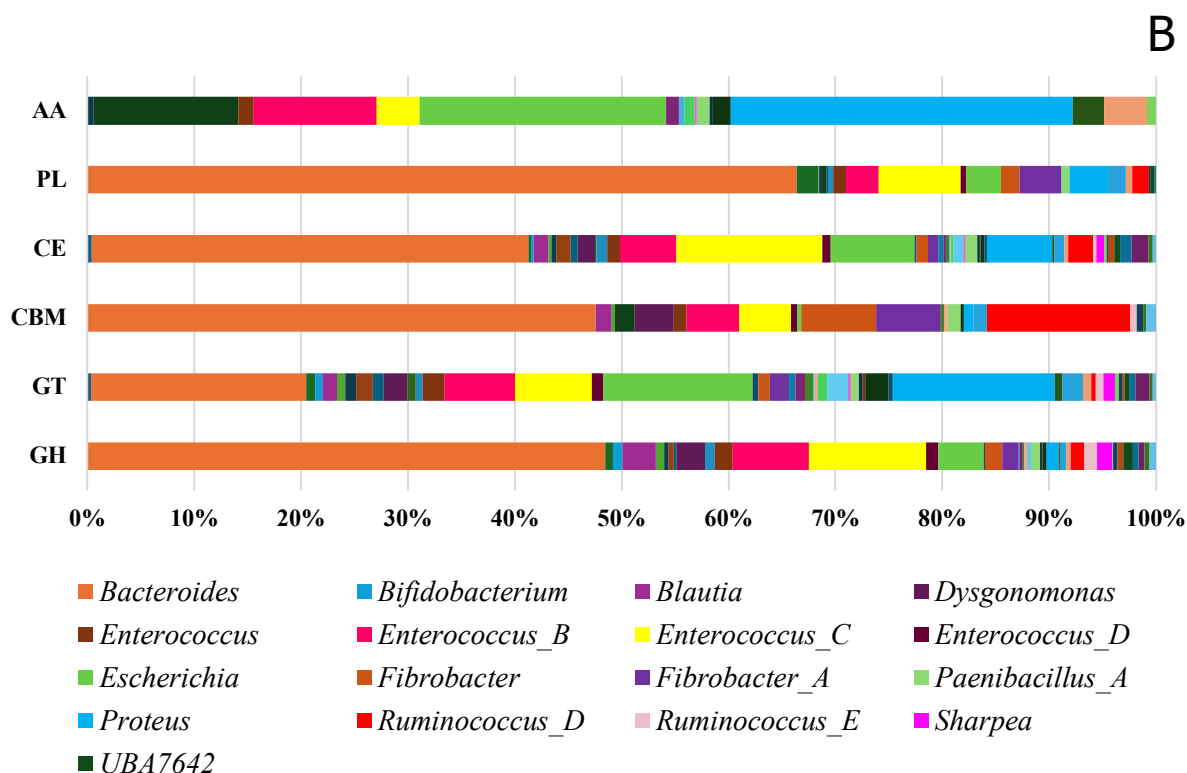


Figure 3.7: A) Phylogenetic tree showing the relationship of the taxa responsible for secreting CAZymes (Letunic and Bork, 2007). **B)** The bar chart shows the abundance of different CAZyme families produced by each genus. GH, GT, CBM, CE, PL, and AA represent glycoside hydrolases, glycosyltransferases, carbohydrate-binding modules, carbohydrate esterases, polysaccharide lyases, and auxiliary activities, respectively.

Enterococcus_B, *Enterococcus_C*, *Escherichia*, and *Proteus* were found in all CAZyme families (Figure 7B). The *Bacteroides* genus was found in all families. However, no hits for AAs were observed. *Proteus* is a genus commonly found in the GIT of goats and other ruminants, such as cattle and sheep (Drzewiecka, 2016). Although *Proteus* is considered an opportunistic pathogen, its presence is often associated with a symbiotic relationship between different groups of bacteria and the host animal, including ruminants (Drzewiecka, 2016). Few studies have directly examined the role of the genus *Proteus* in the GIT, but it is suspected that *Proteus* bacteria in the GIT play an essential role in the breakdown of complex carbohydrates, such as cellulose and hemicellulose. This is due to *Proteus*'s ability to secrete a consortia of enzymes, including cellulases and xylanases, which are involved in the breakdown of complex carbohydrates into fermentable sugar molecules that can then be absorbed by the goat's

digestive system and also produce ammonia, which is a crucial source of nitrogen for other microorganisms in the GIT (Liu *et al.*, 2021b). Thus, the presence of the genus *Proteus* may be essential in ruminants' GIT due to its role in degrading plant material. In addition, the likelihood of successfully isolating and characterizing various *Proteus* strains to advance scientific knowledge of lignocellulosic biomass degradation using these goats' microbiomes is significantly higher.

Bacteroides and *Enterococcus* are also common commensal bacteria found in the GIT. While the dominance of specific genera suggests their significant presence within the goat's GIT, it is important to note that other bacterial groups, though less abundant, may still play a critical role in the overall function of the GIT. *Bacteroides* and other groups, such as *Clostridia*, *Ruminococcus*, and *Clostridium*, have been reported to produce fermentative hydrogen in the rumen (Gharechahi *et al.*, 2022). Meanwhile, the *Enterococcus* genus has been reported as one of the primary lactic acid producers and is utilized as a probiotic. In addition, *Enterococcus* strains are known to be resistant to bile salts and gastric juices (Eisenhofer *et al.*, 2023) and can secrete crucial enzymes, including lipase, proteases, peptidases, and esterases (Sarantinopoulos *et al.*, 2001), and can restrict the growth of harmful microorganisms (Mansour *et al.*, 2018). Mansour *et al.* (2018) used three *Enterococcus* strains to inhibit *Clostridium difficile* in Mice to control *C. difficile* infection. *Enterococcus faecium* supports the function of lactate-fermenting microorganisms and promotes the development of rumen microorganisms. Accordingly, this may increase the supply of glucogenic propionate energy for the host (Pang *et al.*, 2014). Li *et al.* (2018) reported two *Enterococcus* strains, *E. faecalis* JF85, and *E. faecium* Y83, from the Tibetan Yak rumen, with the potential to degrade cellulose (Li *et al.*, 2018). These findings suggest that *Enterococcus* may play various roles in the GIT, including enhancing lignocellulosic biomass degradation by producing VFAs, which eventually replenish the nutritional balance in the overall GIT feed flow. These findings suggest a diverse range in the microbial population within the goat's GIT capable of breaking down lignocellulose, further highlighting the complex and efficient nature of microbial degradation of lignocellulosic biomass in the goat's GIT.

Acknowledging the limitations of our study is crucial for providing a comprehensive understanding of our research findings and guiding future investigations. One limitation is that our focus was solely on the metagenomics of the ruminal samples without concurrent

physicochemical, elemental, or nutrient analyses. While metagenomic data provide valuable insights into microbial composition and potential capabilities, integrating additional analyses could offer a more holistic understanding of the ruminal environment and its metabolic activity. Future studies should consider incorporating these analyses to complement microbial profiling data and provide a more comprehensive characterization of the GIT ecosystem. Furthermore, exploring correlations between microbial composition and environmental factors, such as pH, temperature, moisture content, and nutrient availability, through physicochemical and nutrient analyses would deepen our understanding of microbial dynamics and adaptations.

3.4 Conclusion

In conclusion, this study has illuminated the complex microbial diversity and functional profiles within a goat's forestomach, particularly in the production of CAZymes. The distinct variations in species composition between compartments, coupled with the high richness and diversity observed in the omasum, may emphasize the importance of each compartment in the GIT. Notably, the higher prevalence of the *Proteus* genus observed in this study adds to scientific knowledge by addressing gaps in the understanding of how this genus interacts with other microorganisms in a natural environment. The highlighted gene families associated with xenobiotic degradation and metabolism, as well as amino acid, lipid, and carbohydrate metabolism, hold great potential for advancing bioremediation strategies in environmental biotechnology.

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3.7 Data Availability

Data is in the Appendix section

3.8 References

- ABATENH, E., GIZAW, B., TSEGAYE, Z. & TEFERA, G. 2018. Microbial function on climate change-a review. *Environment Pollution and Climate Change*, 2, 1-6.
- ADEWUYI, A. 2022. Underutilized lignocellulosic waste as sources of feedstock for biofuel production in developing countries. *Frontiers in Energy Research*, 10, 741570.
- AJALA, E., IGHALO, J., AJALA, M., ADENIYI, A. & AYANSHOLA, A. 2021. Sugarcane bagasse: a biomass sufficiently applied for improving global energy, environment and economic sustainability. *Bioresources and Bioprocessing*, 8, 1-25.
- ALAMRI, S. A., MOSTAFA, Y. S. & ALRUMMAN, S. A. 2016. Isolation, Screening and Optimization of *Geobacillus stearothermophilus* Cellulase Production using Date Palm Cellulosic Wastes. *Journal of Pure & Applied Microbiology*, 10.
- ALI, S., RANI, A., DAR, M. A., QAISRANI, M. M., NOMAN, M., YOGANATHAN, K., ASAD, M., BERHANU, A., BARWANT, M. & ZHU, D. 2024. Recent Advances in Characterization and Valorization of Lignin and Its Value-Added Products: Challenges and Future Perspectives. *Biomass*, 4, 947-977.
- ALY, M. M., TORK, S., AL-GARNI, S. M. & NAWAR, L. 2012. Production of lipase from genetically improved *Streptomyces exfoliates* LP10 isolated from oil-contaminated soil. *Afr J Microbiol Res*, 5, 1125-1137.
- APINES-AMAR, M. J. S., CAIPANG, C. M. A., LOPEZ, J. D. M., MURILLO, M. N. A., AMAR, E. C., PIÑOSA, L. A. G. & PEDROSO, F. L. 2022. *Proteus mirabilis* (MJA 2. 6S) from saline-tolerant tilapia exhibits potent antagonistic activity against *Vibrio* spp., enhances immunity, controls NH₃ levels and improves growth and survival in juvenile giant tiger shrimp, *Penaeus monodon*. *Aquaculture Research*, 53, 5510-5520.
- ARAKI, Y., MUKAISHO, K., SUGIHARA, H., FUJIYAMA, Y. & HATTORI, T. 2010. *Proteus mirabilis* sp. intestinal microflora grow in a dextran sulfate sodium-rich environment. *Int J Mol Med*, 25, 203-8.
- ARKIN, A. P., COTTINGHAM, R. W., HENRY, C. S., HARRIS, N. L., STEVENS, R. L., MASLOV, S., DEHAL, P., WARE, D., PEREZ, F. & CANON, S. 2018. KBase: the United States department of energy systems biology knowledgebase. *Nature biotechnology*, 36, 566-569.
- ASHA, B. M. & SAKTHIVEL, N. 2014. Production, purification and characterization of a new cellulase from *Bacillus subtilis* that exhibit halophilic, alkalophilic and solvent-tolerant properties. *Annals of Microbiology*, 64, 1839-1848.
- BARMAN, D. & DKHAR, M. S. 2022. Characterization and purification of esterase from *Cellulomonas fimi* DB19 isolated from *Zanthoxylum armatum* with its possible role in diesel biodegradation. *Archives of Microbiology*, 204, 580.
- BARRAGÁN-TRINIDAD, M., CARRILLO-REYES, J. & BUITRÓN, G. 2017. Hydrolysis of microalgal biomass using ruminal microorganisms as a pretreatment to increase methane recovery. *Bioresource Technology*, 244, 100-107.
- BERNFELD, P. 1955. [17] Amylases, α and β . *Methods in Enzymology*. Academic Press.
- BHATT, B., BHATT, K., LAL, S. & BHATT, V. 2024. Production of a novel cellulase by *Bacillus amyloliquefaciens* OKB3 isolated from soil: Purification and characterization. *International Journal of Biological Macromolecules*, 282, 137454.
- BOHRA, V., DAFALE, N. A., HATHI, Z. & PUROHIT, H. J. 2019. Genomic annotation and validation of bacterial consortium NDMC-1 for enhanced degradation of sugarcane bagasse. *Annals of Microbiology*, 69, 695-711.
- BREDON, M., DITTMER, J., NOËL, C., MOUMEN, B. & BOUCHON, D. 2018. Lignocellulose degradation at the holobiont level: teamwork in a keystone soil invertebrate. *Microbiome*, 6, 1-19.
- CASTRO-COSTA, A., SALAMA, A. A., MOLL, X., AGUILÓ, J. & CAJA, G. 2015. Using wireless rumen sensors for evaluating the effects of diet and ambient temperature in nonlactating dairy goats. *J Dairy Sci*, 98, 4646-58.

- CELI, P. 2011. Oxidative Stress in Ruminants. *In*: MANDELKER, L. & VAJDOVICH, P. (eds.) *Studies on Veterinary Medicine*. Totowa, NJ: Humana Press.
- CELI, P., CHAUHAN, S., COTTRELL, J., DUNSHEA, F., LEAN, I., LEURY, B. & LIU, F. 2014. Oxidative stress in ruminants: enhancing productivity through antioxidant supplementation. *Feedipedia Broadening Horizons*, 13.
- CHAKKOUR, M., HAMMOUD, Z., FARHAT, S., EL ROZ, A., EZZEDDINE, Z. & GHSSEIN, G. 2024. Overview of *Proteus mirabilis* pathogenicity and virulence. Insights into the role of metals. *Frontiers in Microbiology*, 15, 1383618.
- CHENG, B., WU, S., LIU, S., RODRIGUEZ-ALIAGA, P., YU, J. & CUI, S. 2015. Protein denaturation at a single-molecule level: the effect of nonpolar environments and its implications on the unfolding mechanism by proteases. *Nanoscale*, 7, 2970-2977.
- CHENG, J., HUANG, S., JIANG, H., ZHANG, Y., LI, L., WANG, J. & FAN, C. 2016. Isolation and characterization of a non-specific endoglucanase from a metagenomic library of goat rumen. *World Journal of Microbiology and Biotechnology*, 32, 1-8.
- CHIVIAN, D., JUNGBLUTH, S. P., DEHAL, P. S., CANON, R. S., ALLEN, B. H., CLARK, M. M., GU, T., LAND, M. L., PRICE, G. A. & RIEHL, W. J. 2021. Genome extraction from shotgun metagenome sequence data. US Department of Energy Systems Biology Knowledgebase.
- CHIVIAN, D., JUNGBLUTH, S. P., DEHAL, P. S., WOOD-CHARLSON, E. M., CANON, R. S., ALLEN, B. H., CLARK, M. M., GU, T., LAND, M. L. & PRICE, G. A. 2023. Metagenome-assembled genome extraction and analysis from microbiomes using KBase. *Nature Protocols*, 18, 208-238.
- CHOLEWIŃSKA, P., CZYŻ, K., NOWAKOWSKI, P. & WYROSTEK, A. 2020. The microbiome of the digestive system of ruminants—a review. *Animal health research reviews*, 21, 3-14.
- ÇORBACI, C., UYAR, E. & YALÇIN, H. T. 2017. Determination of Enzyme Profiles and Molecular Characterization of Yeast Species Isolated from Butter Samples. *Celal Bayar Üniversitesi Fen Bilimleri Dergisi*, 13, 833-837.
- DEMISSIE, M. S., LEGESSE, N. H. & TESEMA, A. A. 2024. Isolation and characterization of cellulase producing bacteria from forest, cow dung, Dashen brewery and agro-industrial waste. *PLOS ONE*, 19, e0301607.
- DHARIWAL, A., CHONG, J., HABIB, S., KING, I. L., AGELLON, L. B. & XIA, J. 2017. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic acids research*, 45, W180-W188.
- DRZEWIECKA, D. 2016. Significance and roles of *Proteus* spp. bacteria in natural environments. *Microbial ecology*, 72, 741-758.
- EISENHOFER, R., ODRIOZOLA, I. & ALBERDI, A. 2023. Impact of microbial genome completeness on metagenomic functional inference. *ISME communications*, 3, 12.
- EL-KHAMISI, E. F., SOLIMAN, E. A., EL-SAYED, G. M., NOUR, S. A., ABDEL-MONEM, M. O. & HASSAN, M. G. 2024. Optimization, gene cloning, expression, and molecular docking insights for enhanced cellulase enzyme production by *Bacillus amyloliquefaciens* strain elh1. *Microbial Cell Factories*, 23, 191.
- EL ASRI, O. 2023. Anaerobic biodegradation: the anaerobic digestion process. *Handbook of Biodegradable Materials*. Springer.
- FARGHALI, M., OSMAN, A. I., UMETSU, K. & ROONEY, D. W. 2022. Integration of biogas systems into a carbon zero and hydrogen economy: a review. *Environmental Chemistry Letters*, 20, 2853-2927.
- FIRRINCIELI, A., MINUTI, A., CAPPELLETTI, M., FERILLI, M., AJMONE-MARSAN, P., BANI, P., PETRUCCIOLI, M. & HARFOUCHE, A. L. 2024. Structural and functional analysis of the active cow rumen's microbial community provides a catalogue of genes and microbes participating in the deconstruction of cardoon biomass. *Biotechnology for Biofuels and Bioproducts*, 17, 53.
- FIRMAN, J., LIU, L., MAHALAK, K., TANES, C., BITTINGER, K., TU, V., BOBOKALONOV, J., MATTEI, L., ZHANG, H. & VAN DEN ABBEELE, P. 2022. The impact of environmental pH on the gut microbiota community structure and short chain fatty acid production. *FEMS Microbiology Ecology*, 98.

- GASPARATOS, A., VON MALTITZ, G. P., JOHNSON, F. X., LEE, L., MATHAI, M., DE OLIVEIRA, J. P. & WILLIS, K. J. 2015. Biofuels in sub-Saharan Africa: Drivers, impacts and priority policy areas. *Renewable and Sustainable Energy Reviews*, 45, 879-901.
- GAUR, R. & TIWARI, S. 2015. Isolation, production, purification and characterization of an organic-solvent-thermostable alkalophilic cellulase from *Bacillus vallismortis* RG-07. *BMC biotechnology*, 15, 1-12.
- GEBBIE, L., DAM, T. T., AINSCOUGH, R., PALFREYMAN, R., CAO, L., HARRISON, M., O'HARA, I. & SPEIGHT, R. 2020. A snapshot of microbial diversity and function in an undisturbed sugarcane bagasse pile. *BMC biotechnology*, 20, 1-16.
- GHARECHAH, J., SARIKHAN, S., HAN, J.-L., DING, X.-Z. & SALEKDEH, G. H. 2022. Functional and phylogenetic analyses of camel rumen microbiota associated with different lignocellulosic substrates. *npj Biofilms and Microbiomes*, 8, 46.
- GHARECHAH, J., VAHIDI, M. F., DING, X.-Z., HAN, J.-L. & SALEKDEH, G. H. 2020. Temporal changes in microbial communities attached to forages with different lignocellulosic compositions in cattle rumen. *FEMS Microbiology Ecology*, 96, fiae069.
- GRUNINGER, R., RIBEIRO, G., CAMERON, A. & MCALLISTER, T. 2019. Invited review: Application of meta-omics to understand the dynamic nature of the rumen microbiome and how it responds to diet in ruminants. *Animal*, 13, 1843-1854.
- GUERRA, V., TIAGO, I., AIRES, A., COELHO, C., NUNES, J., MARTINS, L. O. & VERISSIMO, A. 2022. The gastrointestinal microbiome of browsing goats (*Capra hircus*). *Plos one*, 17, e0276262.
- GÜNEL, G., İNCE, O., UZUN, Ö., ERDEM, E. I. & İNCE, B. 2025. Enhancing biomethane production from cattle manure by integrating rumen bacteria: a microbial analyses with next-generation sequencing and quantitative PCR. *Biomass Conversion and Biorefinery*.
- GUO, W., WANG, W., BI, S., LONG, R., ULLAH, F., SHAFIQ, M., ZHOU, M. & ZHANG, Y. 2020. Characterization of anaerobic rumen fungal community composition in yak, Tibetan sheep and small tail han sheep grazing on the Qinghai-Tibetan Plateau. *Animals*, 10, 144.
- HALL, T. A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic acids symposium series, 1999. [London]: Information Retrieval Ltd., c1979-c2000., 95-98.
- HAN, X., YANG, Y., YAN, H., WANG, X., QU, L. & CHEN, Y. 2015. Rumen Bacterial Diversity of 80 to 110-Day Old Goats Using 16SrRNA Sequencing. *PLoS One*, 10, 1-12.
- HARUN, N., OTHMAN, N., ZAKI, N., RASUL, N. M., SAMAH, R. & HASHIM, H. 2019. Simulation of anaerobic digestion for biogas production from food waste using SuperPro designer. *Materials Today: Proceedings*, 19, 1315-1320.
- HASSAN, Q., VIKTOR, P., AL-MUSAWI, T. J., ALI, B. M., ALGBURI, S., ALZOUBI, H. M., AL-JIBOORY, A. K., SAMEEN, A. Z., SALMAN, H. M. & JASZCZUR, M. 2024. The renewable energy role in the global energy Transformations. *Renewable Energy Focus*, 48, 100545.
- HERNÁNDEZ-BELTRÁN, J. U., HERNÁNDEZ-DE LIRA, I. O., CRUZ-SANTOS, M. M., SAUCEDO-LUEVANOS, A., HERNÁNDEZ-TERÁN, F. & BALAGURUSAMY, N. 2019. Insight into Pretreatment Methods of Lignocellulosic Biomass to Increase Biogas Yield: Current State, Challenges, and Opportunities. *Applied Sciences*, 9, 3721.
- HOSSAIN, M. 2020. Sub-acute ruminal acidosis in dairy cows: Its causes, consequences and preventive measures. *Online J. Anim. Feed Res*, 10, 302-312.
- HSIAO, N.-W., CHEN, Y., KUANG, Y.-C., LEE, Y.-C., LEE, S.-K., CHAN, H.-H. & KAO, C.-H. 2014. Purification and characterization of an aspartic protease from the *Rhizopus oryzae* protease extract, Peptidase R. *Electronic Journal of Biotechnology*, 17, 89-94.
- HU, Y., PRIYA, A., CHEN, C., LIANG, C., WANG, W., WANG, Q., LIN, C. S. K. & QI, W. 2023. Recent advances in substrate-enzyme interactions facilitating efficient biodegradation of lignocellulosic biomass: A review. *International Biodeterioration & Biodegradation*, 180, 105594.
- HUA, M., YU, S., MA, Y., CHEN, S. & LI, F. 2018. Genetic diversity detection and gene discovery of novel glycoside hydrolase family 48 from soil environmental genomic DNA. *Annals of Microbiology*, 68, 163-174.

- HUNGATE, R. E. 1947. Studies on Cellulose Fermentation: III. The Culture and Isolation for Cellulose-decomposing Bacteria from the Rumen of Cattle. *J Bacteriol*, 53, 631-45.
- HUSSAIN, A. A., ABDEL-SALAM, M. S., ABO-GHALIA, H. H., HEGAZY, W. K. & HAFEZ, S. S. 2017. Optimization and molecular identification of novel cellulose degrading bacteria isolated from Egyptian environment. *Journal of Genetic Engineering and Biotechnology*, 15, 77-85.
- HUWS, S. A., CREEVEY, C. J., OYAMA, L. B., MIZRAHI, I., DENMAN, S. E., POPOVA, M., MUÑOZ-TAMAYO, R., FORANO, E., WATERS, S. M. & HESS, M. 2018. Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present, and future. *Frontiers in microbiology*, 9, 2161.
- HYDER, I., RAVI KANTH REDDY, P., RAJU, J., MANJARI, P., SRINIVASA PRASAD, C., ASWANI KUMAR, K. & SEJIAN, V. 2017. Alteration in rumen functions and diet digestibility during heat stress in sheep. *Sheep production adapting to climate change*. Springer.
- IRFAN, M., MUSHTAQ, Q., TABSSUM, F., SHAKIR, H. A. & QAZI, J. I. 2017a. Carboxymethyl cellulase production optimization from newly isolated thermophilic *Bacillus subtilis* K-18 for saccharification using response surface methodology. *AMB Express*, 7, 29.
- IRFAN, M., TAYYAB, A., HASAN, F., KHAN, S., BADSHAH, M. & SHAH, A. A. 2017b. Production and characterization of organic solvent-tolerant cellulase from *Bacillus amyloliquefaciens* AK9 isolated from hot spring. *Applied Biochemistry and Biotechnology*, 182, 1390-1402.
- ISLAM, M., SARKAR, P. K., MOHIUDDIN, A. & SUZAUDDULA, M. 2019. Optimization of fermentation condition for cellulase enzyme production from *Bacillus* sp. *Malaysian Journal of Halal Research*, 2, 19-24.
- JAKEER, S., VARMA, M., SHARMA, J., MATTOO, F., GUPTA, D., SINGH, J., KUMAR, M. & GAUR, N. A. 2020. Metagenomic analysis of the fecal microbiome of an adult elephant reveals the diversity of CAZymes related to lignocellulosic biomass degradation. *Symbiosis*, 81, 209-222.
- JAYASEKARA, S. & RATNAYAKE, R. 2019. Microbial Cellulases: An Overview and Applications. *Cellulose*. IntechOpen.
- JIANG, X., ZHAI, R., LI, H., LI, C., YUAN, Y., DENG, Q., XU, Z., SHA, Y. & JIN, M. 2024. Unveiling the inhibition of enzymatic hydrolysis of cellulose by lignin-derived phenolics: interfacial kinetics and molecular simulations. *ACS Sustainable Chemistry & Engineering*, 12, 9957-9968.
- KABEYI, M. J. B. & OLANREWaju, O. A. 2023. Bagasse electricity potential of conventional sugarcane factories. *Journal of Energy*, 2023, 5749122.
- KAMESHWAR, A. K. S. & QIN, W. 2017. Metadata Analysis of *Phanerochaete chrysosporium* gene expression data identified common CAZymes encoding gene expression profiles involved in cellulose and hemicellulose degradation. *International journal of biological sciences*, 13, 85.
- KAMRA, D. N. 2005. Rumen microbial ecosystem. *Current science*, 124-135.
- KAZA, S., YAO, L., BHADA-TATA, P. & VAN WOERDEN, F. 2018. *What a waste 2.0: a global snapshot of solid waste management to 2050*, World Bank Publications.
- KIM, Y.-K., LEE, S.-C., CHO, Y.-Y., OH, H.-J. & KO, Y. H. 2012. Isolation of cellulolytic *Bacillus subtilis* strains from agricultural environments. *International Scholarly Research Notices*, 2012, 650563.
- KOIKE, S. & KOBAYASHI, Y. 2009. Fibrolytic Rumen Bacteria: Their Ecology and Functions. *Asian-Australasian Journal of Animal Sciences*, 22.
- KONDE, K. S., NAGARAJAN, S., KUMAR, V., PATIL, S. V. & RANADE, V. V. 2021. Sugarcane bagasse based biorefineries in India: potential and challenges. *Sustainable Energy & Fuels*, 5, 52-78.
- KUCHARSKA, K., RYBARCZYK, P., HOŁOWACZ, I., ŁUKAJTIS, R., GLINKA, M. & KAMIŃSKI, M. 2018. Pretreatment of lignocellulosic materials as substrates for fermentation processes. *Molecules*, 23, 2937.
- KULICHKOVA, G. I., IVANOVA, T. S., KÖTTNER, M., VOLODKO, O. I., SPIVAK, S. I., TSYGANKOV, S. P. & BLUME, Y. B. 2020. Plant feedstocks and their biogas production potentials. *The Open Agriculture Journal*, 14.

- KUMAR, A., KUMAR, V. & SINGH, B. 2021. Cellulosic and hemicellulosic fractions of sugarcane bagasse: Potential, challenges and future perspective. *International Journal of Biological Macromolecules*, 169, 564-582.
- KUMARI, S., SHARMA, U., KRISHNA, R., SINHA, K. & KUMAR, S. 2019. Screening and molecular characterization of cellulase producing actinobacteria from Litchi Orchard. *Current Chemical Biology*, 13, 90-101.
- LEE, K.-T., TOUSHIK, S. H., BAEK, J.-Y., KIM, J.-E., LEE, J.-S. & KIM, K.-S. 2018. Metagenomic mining and functional characterization of a novel KG51 bifunctional cellulase/hemicellulase from black goat rumen. *Journal of agricultural and food chemistry*, 66, 9034-9041.
- LEGIN, E., LADRAT, C., GODFROY, A., BARBIER, G. & DUCHIRON, F. 1997. Thermostable amylolytic enzymes of thermophilic microorganisms from deep-sea hydrothermal vents. *Comptes Rendus de l'Académie des Sciences-Series III-Sciences de la Vie*, 320, 893-898.
- LETUNIC, I. & BORK, P. 2007. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics*, 23, 127-128.
- LI, J., YUAN, X., DESTA, S. T., DONG, Z., MUGABE, W. & SHAO, T. 2018. Characterization of *Enterococcus faecalis* JF85 and *Enterococcus faecium* Y83 isolated from Tibetan yak (*Bos grunniens*) for ensiling *Pennisetum sinense*. *Bioresource Technology*, 257, 76-83.
- LIAN, H., ZHANG, C., LIU, Y., LI, W., FU, T., GAO, T. & ZHANG, L. 2022. In Vitro Gene Expression Responses of Bovine Rumen Epithelial Cells to Different pH Stresses. *Animals*, 12, 2621.
- LIANG, B., LIANG, Y., CHEN, J., ZHU, Y., FU, F., GAN, T., HUANG, Z., HU, H. & ZHANG, Y. 2024. Stepwise processing strategy for efficient and comprehensive utilization of sugarcane bagasse via enzymatic hydrolysis and catalytic conversion to produce 5-hydroxymethylfurfural. *Industrial Crops and Products*, 214, 118592.
- LIAO, Y., WU, S., ZHOU, G., MEI, S., YANG, Z., LI, S., JIN, Z., DENG, Y., WEN, M. & YANG, Y. 2024. Cellulolytic *Bacillus cereus* produces a variety of short-chain fatty acids and has potential as a probiotic. *Microbiology Spectrum*, 12, e03267-23.
- LIU, K., ZHANG, Y., YU, Z., XU, Q., ZHENG, N., ZHAO, S., HUANG, G. & WANG, J. 2021a. Ruminant microbiota-host interaction and its effect on nutrient metabolism. *Anim Nutr*, 7, 49-55.
- LIU, Y., TANG, Y., GAO, H., ZHANG, W., JIANG, Y., XIN, F. & JIANG, M. 2021b. Challenges and future perspectives of promising biotechnologies for lignocellulosic biorefinery. *Molecules*, 26, 5411.
- LONCHAY, W., BAGNATO, G. & SANNA, A. 2022. Highly selective hydrolysis of lignin waste to benzene, toluene and xylene in presence of zirconia supported iron catalyst. *Bioresource Technology*, 361, 127727.
- MACDONALD, L. C. & BERGER, B. W. 2014. A polysaccharide lyase from *Stenotrophomonas maltophilia* with a unique, pH-regulated substrate specificity. *Journal of Biological Chemistry*, 289, 312-325.
- MAHAPATRA, S., VICKRAM, A., SRIDHARAN, T., PARAMESWARI, R. & PATHY, M. R. 2016. Screening, production, optimization and characterization of β -glucosidase using microbes from shellfish waste. *3 Biotech*, 6, 1-10.
- MAHAYRI, T. M. 2022. Diversity and composition of the bacterial community in the feces of cattle and goats.
- MALIK, W. A., KHAN, H. M. & JAVED, S. 2021. Bioprocess optimization for enhanced production of bacterial cellulase and hydrolysis of sugarcane bagasse. *BioEnergy Research*, 1-14.
- MANIKANDAN, S., KRISHNAN, R. Y., VICKRAM, S., SUBBAIYA, R., KIM, W., GOVARTHANAN, M. & KARMEGAM, N. 2023a. Emerging nanotechnology in renewable biogas production from biowastes: Impact and optimization strategies—A review. *Renewable and Sustainable Energy Reviews*, 181, 113345.
- MANIKANDAN, S., VICKRAM, S., SIROHI, R., SUBBAIYA, R., KRISHNAN, R. Y., KARMEGAM, N., SUMATHIJONES, C., RAJAGOPAL, R., CHANG, S. W. & RAVINDRAN, B. 2023b. Critical review of biochemical pathways to transformation of waste and biomass into bioenergy. *Bioresource Technology*, 128679.

- MANSOUR, N. M., ELKHATIB, W. F., ABOSHANAB, K. M. & BAHR, M. M. 2018. Inhibition of *Clostridium difficile* in mice using a mixture of potential probiotic strains *Enterococcus faecalis* NM815, *E. faecalis* NM915, and *E. faecium* NM1015: novel candidates to control *C. difficile* infection (CDI). *Probiotics and antimicrobial proteins*, 10, 511-522.
- MAO, S., ZHANG, M., LIU, J. & ZHU, W. 2015. Characterising the bacterial microbiota across the gastrointestinal tracts of dairy cattle: membership and potential function. *Scientific reports*, 5, 16116.
- MCDUGALL, E. I. 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. *Biochem J*, 43, 99-109.
- MOBLEY, H. L. T. 2019. *Proteus mirabilis* Overview. In: PEARSON, M. M. (ed.) *Proteus mirabilis: Methods and Protocols*. New York, NY: Springer New York.
- MOKALE KOGNOU, A. L., CHIO, C., KHATIWADA, J. R., SHRESTHA, S., CHEN, X., HAN, S., LI, H., JIANG, Z.-H., XU, C. C. & QIN, W. 2022. Characterization of cellulose-degrading bacteria isolated from soil and the optimization of their culture conditions for cellulase production. *Applied Biochemistry and Biotechnology*, 194, 5060-5082.
- MORAN, J. 2005. *Tropical dairy farming: feeding management for small holder dairy farmers in the humid tropics*, Csiro Publishing.
- MOUSTAKAS, K., LOIZIDOU, M., REHAN, M. & NIZAMI, A. 2020. A review of recent developments in renewable and sustainable energy systems: Key challenges and future perspective. Elsevier.
- MUDAKKAR, S. R., ZAMAN, K., KHAN, M. M. & AHMAD, M. 2013. Energy for economic growth, industrialization, environment and natural resources: living with just enough. *Renewable and Sustainable Energy Reviews*, 25, 580-595.
- MUSHTAQ, Q., ISHTIAQ, U., JOLY, N., QAZI, J. I. & MARTIN, P. 2024. Amylase and cellulase production from newly isolated *Bacillus subtilis* using acid treated potato peel waste. *Microorganisms*, 12, 1106.
- MUSTAFA, A. M., POULSEN, T. G. & SHENG, K. 2016. Fungal pretreatment of rice straw with *Pleurotus ostreatus* and *Trichoderma reesei* to enhance methane production under solid-state anaerobic digestion. *Applied Energy*, 180, 661-671.
- NAAS, A. E., MACKENZIE, A., MRAVEC, J., SCHÜCKEL, J., WILLATS, W., EIJSINK, V. & POPE, P. 2014. Do rumen Bacteroidetes utilize an alternative mechanism for cellulose degradation? *MBio*, 5, e01401-14.
- NAIR, L. G., AGRAWAL, K. & VERMA, P. 2022. An overview of sustainable approaches for bioenergy production from agro-industrial wastes. *Energy Nexus*, 6, 100086.
- NEGRÃO, D. R., GRANDIS, A., BUCKERIDGE, M. S., ROCHA, G. J., LEAL, M. R. L. & DRIEMEIER, C. 2021. Inorganics in sugarcane bagasse and straw and their impacts for bioenergy and biorefining: A review. *Renewable and Sustainable Energy Reviews*, 148, 111268.
- NKUNA, R. & MATAMBO, T. S. 2024. Determining the metabolic processes of metal-tolerant fungi isolated from mine tailings for bioleaching. *Minerals*, 14, 235.
- ØSTERGAARD, P. A., DUIC, N., NOOROLLAHI, Y. & KALOGIROU, S. 2022. Renewable energy for sustainable development. *Renewable energy*, 199, 1145-1152.
- OZTAT, K., YAVUZ, A. A. & IŞÇEN, C. F. 2024. Optimization studies on laccase activity of *Proteus mirabilis* isolated from treatment sludge of textile industry factories. *Brazilian Journal of Microbiology*, 55, 1231-1241.
- PAHLA, G., MAMVURA, T. A., NTULI, F. & MUZENDA, E. 2017. Energy densification of animal waste lignocellulose biomass and raw biomass. *south african journal of chemical engineering*, 24, 168-175.
- PAN, S., ZABED, H. M., WEI, Y. & QI, X. 2022. Technoeconomic and environmental perspectives of biofuel production from sugarcane bagasse: Current status, challenges and future outlook. *Industrial Crops and Products*, 188, 115684.
- PANĂ, A.-M., ORDODI, V., GHERMAN, V., SFÎRLOAGĂ, P. & DUMITREL, G.-A. 2023. Study on the Biodegradation Process of D-Mannose Glycopolymers in Liquid Media and Soil. *Polymers*, 15, 3194.

- PANG, D., YANG, H., CAO, B., WU, T. & WANG, J. 2014. The beneficial effect of *Enterococcus faecium* on the in vitro ruminal fermentation rate and extent of three typical total mixed rations in northern China. *Livestock Science*, 167, 154-160.
- PARK, Y.-J., JEONG, Y.-U. & KONG, W.-S. 2018. Genome sequencing and carbohydrate-active enzyme (CAZyme) repertoire of the white rot fungus *Flammulina elastica*. *International journal of molecular sciences*, 19, 2379.
- PARKS, D. H., IMELFORT, M., SKENNERTON, C. T., HUGENHOLTZ, P. & TYSON, G. W. 2015. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome research*, 25, 1043-1055.
- PARKS, D. H., TYSON, G. W., HUGENHOLTZ, P. & BEIKO, R. G. 2014. STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics*, 30, 3123-3124.
- PATAKOVA, P., KOLEK, J., SEDLAR, K., KOSCOVA, P., BRANSKA, B., KUPKOVA, K., PAULOVA, L. & PROVAZNIK, I. 2018. Comparative analysis of high butanol tolerance and production in clostridia. *Biotechnology advances*, 36, 721-738.
- PATEL, D. & SEN, D. J. 2013. Xenobiotics: An essential precursor for living system. *Am J Adv Drug Deliv*, 1, 262-270.
- PATEL, D. D., PATEL, A. K., PARMAR, N. R., SHAH, T. M., PATEL, J. B., PANDYA, P. R. & JOSHI, C. G. 2014a. Microbial and Carbohydrate Active Enzyme profile of buffalo rumen metagenome and their alteration in response to variation in the diet. *Gene* 545, 88-94.
- PATEL, V., PATEL, A. K., PARMAR, N. R., PATEL, A. B., REDDY, B. & JOSHI, C. G. 2014b. Characterization of the rumen microbiome of Indian Kankrej cattle (*Bos indicus*) adapted to different forage diet. *Applied microbiology and biotechnology*, 98, 9749-9761.
- PHITSUWAN, P., SAKKA, K. & RATANAKHANOKCHAI, K. 2013. Improvement of lignocellulosic biomass in planta: a review of feedstocks, biomass recalcitrance, and strategic manipulation of ideal plants designed for ethanol production and processability. *Biomass and Bioenergy*, 58, 390-405.
- PITTA, D. W., INDUGU, N., KUMAR, S., VECCHIARELLI, B., SINHA, R., BAKER, L. D., BHUKYA, B. & FERGUSON, J. D. 2016. Metagenomic assessment of the functional potential of the rumen microbiome in Holstein dairy cows. *Anaerobe*, 38, 50-60.
- POTPROMMANEE, L., WANG, X.-Q., HAN, Y.-J., NYOBE, D., PENG, Y.-P., HUANG, Q., LIU, J.-Y., LIAO, Y.-L. & CHANG, K.-L. 2017. Characterization of a thermophilic cellulase from *Geobacillus* sp. HTA426, an efficient cellulase-producer on alkali pretreated of lignocellulosic biomass. *PLoS one*, 12, e0175004.
- PRASAD, R. K., SHARMA, A., MAZUMDER, P. B. & DHUSSA, A. 2024. A comprehensive pre-treatment strategy evaluation of ligno-hemicellulosic biomass to enhance biogas potential in the anaerobic digestion process. *RSC Sustainability*, 2, 2444-2467.
- PRIYANKA, S., JAYASHREE, M., SHIVANI, R., ANWESHA, S. & RAO, B. K. 2018. A Study on Marine Lipolytic Yeasts: Optimization and Characterization of Lipase Enzyme. *Research Journal of Pharmacy and Technology*, 11, 593-598.
- PUNIYA, A. K., SINGH, R. & KAMRA, D. N. 2015. Rumen microbiology: from evolution to revolution.
- RABAPANE, K. J., IJOMA, G. N. & MATAMBO, T. S. 2022. Insufficiency in functional genomics studies, data, and applications: A case study of bio-prospecting research in ruminant microbiome. *Frontiers in Genetics*, 13.
- RABAPANE, K. J. & MATAMBO, T. S. 2024. Profiling the dynamic adaptations of CAZyme-Producing microorganisms in the gastrointestinal tract of South African goats. *Heliyon*, 10.
- RAHBAN, M., ZOLGHADRI, S., SALEHI, N., AHMAD, F., HAERTLÉ, T., REZAEI-GHALEH, N., SAWYER, L. & SABOURY, A. A. 2022. Thermal stability enhancement: Fundamental concepts of protein engineering strategies to manipulate the flexible structure. *International journal of biological macromolecules*, 214, 642-654.
- RASHAMUSE, K., VISSER, D. F., HENNESSY, F., KEMP, J., ROUX-VAN DER MERWE, M., BADENHORST, J., RONNEBURG, T., FRANCIS-POPE, R. & BRADY, D. 2013. Characterisation of two bifunctional cellulase-xylanase enzymes isolated from a bovine rumen metagenome library. *Current microbiology*, 66, 145-151.

- RASOOL, K., HUSSAIN, S., SHAHZAD, A., MIRAN, W., MAHMOUD, K. A., ALI, N. & ALMOMANI, F. 2023. Comprehensive insights into sustainable conversion of agricultural and food waste into microbial protein for animal feed production. *Reviews in Environmental Science and Bio/Technology*, 22, 527-562.
- RAVIKUMAR, R., RANGANATHAN, B. V., CHATHOTH, K. N. & GOBIKRISHNAN, S. 2013. Innovative and intensified technology for the biological pretreatment of agro waste for ethanol production. *Korean Journal of Chemical Engineering*, 30, 1051-1057.
- REDDY, P. 2015. A critical review of ionic liquids for the pretreatment of lignocellulosic biomass. *South African Journal of Science*, 111, 1-9.
- RIBEIRO, G., GRUNINGER, R., BADHAN, A. & MCALLISTER, T. 2016. Mining the rumen for fibrolytic feed enzymes. *Animal Frontiers*, 6, 20-26.
- RÖDER, M. & WELFLE, A. 2019. Bioenergy. *Managing Global Warming*. Elsevier.
- ROJAS, L. F., ZAPATA, P. & RUIZ-TIRADO, L. 2022. Agro-industrial waste enzymes: Perspectives in circular economy. *Current Opinion in Green and Sustainable Chemistry*, 34, 100585.
- ROUT, P. G. & BEHERA, S. K. 2024. Evaluation of organosulfur compound degradation property of a noble bacterial strain of *Proteus mirabilis*. *Environmental Chemistry and Ecotoxicology*, 6, 223-228.
- ROY, D., GUNRI, S. K. & PAL, K. K. 2024. Isolation, screening and characterization of efficient cellulose-degrading fungal and bacterial strains and preparation of their consortium under in vitro studies. *3 Biotech*, 14, 131.
- ROY, M., KUMAR, R., RAMTEKE, A. & SIT, N. 2018. Identification of lipase producing fungus isolated from dairy waste contaminated soil and optimization of culture conditions for lipase production by the isolated fungus. *Journal of Microbiology, Biotechnology & Food Sciences*, 8.
- RUSSELL, J. B. & RYCHLIK, J. L. 2001. Factors that alter rumen microbial ecology. *Science*, 292, 1119-1122.
- RUSSELL, J. B. & WILSON, D. B. 1996. Why Are Ruminant Cellulolytic Bacteria Unable to Digest Cellulose at Low pH? *Journal of Dairy Science*, 79, 1503-1509.
- RUSSMAYER, H., MARX, H. & SAUER, M. 2019. Microbial 2-butanol production with *Lactobacillus diolivorans*. *Biotechnology for biofuels*, 12, 1-11.
- SAINI, A., AGGARWAL, N. K. & YADAV, A. 2017. Isolation and screening of cellulose hydrolyzing bacteria from different ecological niches. *Bioengineering and Bioscience*, 5, 7-13.
- SALGADO, V., FONSECA, C., DA SILVA, T. L., ROSEIRO, J. C. & EUSÉBIO, A. 2019. Isolation and Identification of *Magnusiomyces capitatus* as a Lipase-Producing Yeast from Olive Mill Wastewater. *Waste and Biomass Valorization*, 1-15.
- SARANTINOPOULOS, P., ANDRIGHETTO, C., GEORGALAKI, M. D., REA, M. C., LOMBARDI, A., COGAN, T. M., KALANTZOPOULOS, G. & TSAKALIDOU, E. 2001. Biochemical properties of enterococci relevant to their technological performance. *International Dairy Journal*, 11, 621-647.
- SARKER, T. C., AZAM, S. M. G. G. & BONANOMI, G. 2017. Recent advances in sugarcane industry solid by-products valorization. *Waste and Biomass Valorization*, 8, 241-266.
- SARSAN, S. & MERUGU, R. 2019. Role of bioprocess parameters to improve cellulase production: Part II. *New and future developments in microbial biotechnology and bioengineering*. Elsevier.
- SCHELLER, H. V. & ULVSKOV, P. 2010. Hemicelluloses. *Annual review of plant biology*, 61, 263-289.
- SHAH, T. A., LEE, C. C., ORTS, W. J. & TABASSUM, R. 2019. Biological pretreatment of rice straw by ligninolytic *Bacillus* sp. strains for enhancing biogas production. *Environmental Progress & Sustainable Energy*, 38, e13036.
- SHAREEF, I., GOPINATH, S., SATHEESH, M. & CHRISTOPHER, S. X. 2015. Isolation and identification of cellulose degrading microbes.
- SHENG, P., LI, Y., MARSHALL, S. D. & ZHANG, H. 2015. High genetic diversity of microbial cellulase and hemicellulase genes in the hindgut of *Holotrichia parallela* larvae. *International Journal of Molecular Sciences*, 16, 16545-16559.
- SHI, Q., ABDEL-HAMID, A. M., SUN, Z., CHENG, Y., TU, T., CANN, I., YAO, B. & ZHU, W. 2023. Carbohydrate-binding modules facilitate the enzymatic hydrolysis of lignocellulosic

- biomass: Releasing reducing sugars and dissociative lignin available for producing biofuels and chemicals. *Biotechnology Advances*, 108126.
- SILVA, É. B. R. D., SILVA, J. A. R. D., SILVA, W. C. D., BELO, T. S., SOUSA, C. E. L., SANTOS, M. R. P. D., NEVES, K. A. L., RODRIGUES, T. C. G. D. C., CAMARGO-JÚNIOR, R. N. C. & LOURENÇO-JÚNIOR, J. D. B. 2024. A Review of the Rumen Microbiota and the Different Molecular Techniques Used to Identify Microorganisms Found in the Rumen Fluid of Ruminants. *Animals*, 14, 1448.
- SILVA, J. P., TICONA, A. R., HAMANN, P. R., QUIRINO, B. F. & NORONHA, E. F. 2021. Deconstruction of lignin: from enzymes to microorganisms. *Molecules*, 26, 2299.
- SINGH, B., MAL, G., KALRA, R. S. & MAROTTA, F. 2024a. Microbial-Rich Niches in Herbivores. *Probiotics as Live Biotherapeutics for Veterinary and Human Health, Volume 1: Functional Feed and Industrial Applications*. Springer.
- SINGH, N., GAUTAM, S. B., SAWOOD, G. M., YADAV, V. L., MISHRA, G., DIXIT, S. & GUPTA, S. 2024b. Bioenergy from Agro-waste: A Sustainable Solution for Energy Needs. *Sustainable Clean Energy Production Using Waste Biomass: Sustainable Energy Production and Utilization*. Springer.
- SINGH, S., JAISWAL, D. K., SIVAKUMAR, N. & VERMA, J. P. 2019. Developing efficient thermophilic cellulose degrading consortium for glucose production from different agro-residues. *Frontiers in Energy Research*, 7, 61.
- SIVAMANI, S., SAIKAT, B., NAVEEN PRASAD, B., BAALAWY, A. A. S. & AL-MASHALI, S. M. A. 2021. A comprehensive review on microbial technology for biogas production. *Bioenergy Research: Revisiting Latest Development*, 53-78.
- SKOVGAARD, P. A. & JØRGENSEN, H. 2013. Influence of high temperature and ethanol on thermostable lignocellulolytic enzymes. *Journal of Industrial Microbiology and Biotechnology*, 40, 447-456.
- SPYROPOULOS, G. 2024. *Bagasse Utilization and Management Strategies in São Paulo Sugarcane Mills*.
- SUMARDIONO, S., MATIN, H. H. A., SULISTIANINGTIAS, I., NUGROHO, T. Y. & BUDIYONO, B. 2023. Effect of physical and biological pretreatment on sugarcane bagasse waste-based biogas production. *Materials Today: Proceedings*, 87, 41-44.
- SURVERY, S., AHMAD, S., SUBHAN, S. A., AJAZ, M. & RASOOL, S. A. 2004. Hydrocarbon degrading bacteria from Pakistani soil: isolation, identification, screening and genetical studies. *Pak J Biol Sci*, 7, 1518-1522.
- TAKIZAWA, S., ASANO, R., FUKUDA, Y., BABA, Y., TADA, C. & NAKAI, Y. 2021. Characteristics of various fibrolytic isozyme activities in the rumen microbial communities of Japanese Black and Holstein Friesian cattle under different conditions. *Animal Science Journal*, 92, e13653.
- TANG, D., CHEN, M., HUANG, X., ZHANG, G., ZENG, L., ZHANG, G., WU, S. & WANG, Y. 2023. SRplot: A free online platform for data visualization and graphing. *PLoS One*, 18, e0294236.
- TAUQEER, H. M., TURAN, V., FARHAD, M. & IQBAL, M. 2022. Sustainable agriculture and plant production by virtue of biochar in the era of climate change. *Managing plant production under changing environment*. Springer.
- THAPA, S., MISHRA, J., ARORA, N., MISHRA, P., LI, H., O' HAIR, J., BHATTI, S. & ZHOU, S. 2020. Microbial cellulolytic enzymes: diversity and biotechnology with reference to lignocellulosic biomass degradation. *Reviews in Environmental Science and Bio/Technology*, 19, 621-648.
- THEKKILA-VEEDU, S., MOHANAN, D. P., BANERJEE, S., RAVICHANDIRAN, V. & NATESAN, S. 2023. Natural biopolymers in ophthalmology. *Natural Biopolymers in Drug Delivery and Tissue Engineering*. Elsevier.
- THOMAS, L., RAM, H., KUMAR, A. & SINGH, V. P. 2016. Production, Optimization, and Characterization of Organic Solvent Tolerant Cellulases from a Lignocellulosic Waste-Degrading Actinobacterium, *Promicromonospora* sp. VP111. *Applied Biochemistry and Biotechnology*, 179, 863-879.

- USMAN, A., MOHAMMED, S. & MAMO, J. 2021. Production, Optimization, and Characterization of an Acid Protease from a Filamentous Fungus by Solid-State Fermentation. *International journal of microbiology*, 2021, 6685963.
- VALDEZ-VAZQUEZ, I., ALATRISTE-MONDRAGÓN, F., ARREOLA-VARGAS, J., BUITRÓN, G., CARRILLO-REYES, J., LEÓN-BECERRIL, E., MENDEZ-ACOSTA, H. O., ORTÍZ, I. & WEBER, B. 2020. A comparison of biological, enzymatic, chemical and hydrothermal pretreatments for producing biomethane from Agave bagasse. *Industrial Crops and Products*, 145, 112160.
- WAHEEB, M. S., ELKHATIB, W. F., YASSIEN, M. A. & HASSOUNA, N. A. 2024. Optimized production and characterization of a thermostable cellulase from *Streptomyces thermodiastaticus* strain. *AMB Express*, 14, 129.
- WANG, J., GHOSH, S., OLAYINKA, O. A., DOĞAN, B., SHAH, M. I. & ZHONG, K. 2022. Achieving energy security amidst the world uncertainty in newly industrialized economies: The role of technological advancement. *Energy*, 261, 125265.
- WANG, J., WANG, X., BIAN, C., LIU, J. & XIAO, B. 2025. Effect of aeration pretreatment on anaerobic digestion of swine manure. *Environ Technol*, 46, 2035-2051.
- WANG, L., JIN, L., XUE, B., WANG, Z. & PENG, Q. 2019. Characterizing the bacterial community across the gastrointestinal tract of goats: Composition and potential function. *Microbiologyopen*, 8, e00820.
- WANG, L., XU, Q., KONG, F., YANG, Y., WU, D., MISHRA, S. & LI, Y. 2016. Exploring the goat rumen microbiome from seven days to two years. *PLoS One*, 11, e0154354.
- WASFI, R., HAMED, S. M., AMER, M. A. & FAHMY, L. I. 2020. *Proteus mirabilis* biofilm: development and therapeutic strategies. *Frontiers in cellular and infection microbiology*, 10, 414.
- WEBER, N., LIOU, D., DOMMER, J., MACMENAMIN, P., QUIÑONES, M., MISNER, I., OLER, A. J., WAN, J., KIM, L. & COAKLEY MCCARTHY, M. 2018. Nephele: a cloud platform for simplified, standardized and reproducible microbiome data analysis. *Bioinformatics*, 34, 1411-1413.
- WEIMER, P. J. 2022. Degradation of Cellulose and Hemicellulose by Ruminant Microorganisms. *Microorganisms*, 10.
- WEISBURG, W. G., BARNS, S. M., PELLETIER, D. A. & LANE, D. J. 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of bacteriology*, 173, 697-703.
- WILSON, K. H., BLITCHINGTON, R. B. & GREENE, R. C. 1990. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. *Journal of clinical microbiology*, 28, 1942-1946.
- WONGFAED, N., O-THONG, S., SITTIJUNDA, S. & REUNGSANG, A. 2023. Taxonomic and enzymatic basis of the cellulolytic microbial consortium KKU-MC1 and its application in enhancing biomethane production. *Scientific Reports*, 13, 2968.
- WU, H., CHEN, Q., ZHANG, W. & MU, W. 2023. Overview of strategies for developing high thermostability industrial enzymes: Discovery, mechanism, modification and challenges. *Critical Reviews in Food Science and Nutrition*, 63, 2057-2073.
- WYMAN, V., HENRÍQUEZ, J., PALMA, C. & CARVAJAL, A. 2018. Lignocellulosic waste valorisation strategy through enzyme and biogas production. *Bioresource Technology*, 247, 402-411.
- XU, Z., CEN, Y.-K., ZOU, S.-P., XUE, Y.-P. & ZHENG, Y.-G. 2020. Recent advances in the improvement of enzyme thermostability by structure modification. *Critical reviews in biotechnology*, 40, 83-98.
- YADAV, P., ANU, KUMAR TIWARI, S., KUMAR, V., SINGH, D., KUMAR, S., MANISHA, MALIK, V. & SINGH, B. 2024. Sugarcane bagasse: An important lignocellulosic substrate for production of enzymes and biofuels. *Biomass Conversion and Biorefinery*, 14, 6111-6142.
- YANG, W. & BEAUCHEMIN, K. 2006. Physically effective fiber: method of determination and effects on chewing, ruminal acidosis, and digestion by dairy cows. *Journal of dairy science*, 89, 2618-2633.

- YE, Y., CAI, Y., WANG, F., HE, Y., YANG, Y., GUO, Z., LIU, M., REN, H., WANG, S., LIU, D., XU, J. & WANG, Z. 2025. Industrial Microbial Technologies for Feed Protein Production from Non-Protein Nitrogen. *Microorganisms*, 13, 742.
- YIN, L.-J., LIN, H.-H. & XIAO, Z.-R. 2010. Purification and characterization of a cellulase from *Bacillus subtilis* YJ1. *Journal of Marine Science and Technology*, 18, 19.
- ZAKS, A. & KLIBANOV, A. M. 1988. Enzymatic catalysis in nonaqueous solvents. *Journal of Biological Chemistry*, 263, 3194-3201.
- ZHANG, K., LI, B., GUO, M., LIU, G., YANG, Y., WANG, X., CHEN, Y. & ZHANG, E. 2019. Maturation of the goat rumen microbiota involves three stages of microbial colonization. *Animals*, 9, 1028.
- ZHANG, Q., JIAN, L., YAO, D., RAO, B., XIA, Y., HU, K., LI, S., SHEN, Y., CAO, M., QIN, A., ZHAO, J. & CAO, Y. 2023. The structural basis of the pH-homeostasis mediated by the Cl⁻/HCO₃⁻ exchanger, AE2. *Nature Communications*, 14, 1812.
- ZHANG, X.-Z. & ZHANG, Y.-H. P. 2013. Cellulases: characteristics, sources, production, and applications. *Bioprocessing technologies in biorefinery for sustainable production of fuels, chemicals, and polymers*, 1, 131-146.
- ZHANG, Y., ZHANG, X., LI, F., LI, C., LI, G., ZHANG, D., SONG, Q., LI, X., ZHAO, Y. & WANG, W. 2021. Characterization of the rumen microbiota and its relationship with residual feed intake in sheep. *Animal*, 15, 100161.
- ZHOU, Z., WANG, Y., WANG, M. & ZHOU, Z. 2021. Co-metabolic effect of glucose on methane production and phenanthrene removal in an enriched phenanthrene-degrading consortium under methanogenesis. *Frontiers in Microbiology*, 12, 749967.
- ZOGHLAMI, A. & PAËS, G. 2019. Lignocellulosic biomass: understanding recalcitrance and predicting hydrolysis. *Frontiers in chemistry*, 7, 874.

CHAPTER FOUR: ISOLATION, IDENTIFICATION, AND OPTIMIZATION OF BACTERIAL ISOLATES

Objective

In the previous chapter, culture-independent techniques were used to profile the diversity and functionality of CAZyme-producing microorganisms in the gastrointestinal tract of South African goats. These findings highlighted their crucial role in the digestion of complex carbohydrates. The purpose of this chapter was to investigate the cellulolytic capacity of microorganisms isolated from the gastrointestinal tract of goats. It aimed to characterize and identify cellulase-producing strains, and to optimize key environmental factors, such as pH, temperature, and incubation time, that influence their enzyme production and activity. The chapter further sought to evaluate the effects of surfactants, solvents, and oxidative stress on cellulase performance, and to check for the presence of lignocellulose-degrading genes in strain KC94 that underpin its potential for biomass conversion. By integrating physiological, biochemical, and genomic approaches, this study provided new insights into the cellulolytic potential of *Proteus mirabilis* as an underexplored candidate for microbial pretreatment. Ultimately, the findings advance strategies for sustainable lignocellulosic biomass utilization and expand the application of cellulolytic bacteria in bioconversion processes.

Publication

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Abstract

This study reports the isolation and optimization of cellulase-producing bacteria from the gastrointestinal tract of South African goats for the pretreatment of lignocellulosic biomass in bioenergy applications. Among the isolates, three strains, *Bacillus* KC50, *Bacillus* KC70, and KC94, were identified by 16S rDNA sequencing. To our knowledge, this is the first report of cellulolytic optimization in *P. mirabilis* derived from goat rumen. Enzyme production was optimized under varying pH, temperature, and incubation conditions. KC94 exhibited robust enzyme activity at pH 7 and 35 °C, with greater stability than the *Bacillus* strains. Peak activity occurred at 84 h of incubation, reflecting strain-specific metabolic adaptation. The presence of organic solvents and surfactants inhibited enzyme activity, whereas mild oxidative stress induced by H₂O₂ stimulated cellulase production. Amplification of GH39, GH45, and GH48

genes revealed KC94's strong genetic potential for efficient lignocellulose degradation. These findings highlight the biotechnological potential of rumen-derived cellulolytic bacteria, particularly KC94, for advancing sustainable bioenergy systems.

Keywords: pretreatment; lignocellulosic biomass; bioenergy; cellulase

4.1 Introduction

Lignocellulosic biomass presents an abundant and renewable resource for biofuel and biochemical production (Ajala *et al.*, 2021, Pan *et al.*, 2022). Effective enzymatic hydrolysis of cellulose and hemicellulose is essential to unlock the fermentable sugars within these complex substrates (Kumar *et al.*, 2021, Negrão *et al.*, 2021, Konde *et al.*, 2021, Sarker *et al.*, 2017). Therefore, utilizing cellulase-producing bacteria offers an efficient and environmentally friendly approach to lignocellulosic degradation, paving the way for enhanced biofuel and value-added chemical production (Liang *et al.*, 2024).

Among cellulase-producing bacteria, members of the *Bacillus* genus, such as *Bacillus subtilis* (Kim *et al.*, 2012, Yin *et al.*, 2010, Mushtaq *et al.*, 2024), *Bacillus amyloliquefaciens* (Bhatt *et al.*, 2024), and *Bacillus cereus* (Liao *et al.*, 2024), have demonstrated significant cellulolytic activity due to their robust enzyme production and adaptability to various environmental conditions, as well as rapid growth rates. In contrast, *P. mirabilis* is rarely associated with cellulose-degrading or cellulolytic activity. While limited studies have described weak to moderate degradation of polysaccharide substrates such as cellulose, dextran sulfate, and mannose-based glycopolymers (Pană *et al.*, 2023, Araki *et al.*, 2010), only one study has demonstrated extracellular β -glucosidase production in *P. mirabilis* VIT117, isolated from shellfish waste. In that study, enzyme activity was maximized at pH 7.0 and ~72 h of incubation, with stable activity under mesophilic conditions (35–37 °C), highlighting its adaptability to physiological environments (Mahapatra *et al.*, 2016). However, its presence in the gastrointestinal tracts of ruminants suggests a possible role in fiber degradation, a role that remains poorly understood and warrants further investigation. Exploring such unconventional cellulolytic candidates may reveal overlooked microbial contributions and expand the diversity of biocatalysts for biomass utilization. The goat gastrointestinal tract represents a particularly underexplored microbial niche. Ruminant microbiota is well known for its role in breaking

down complex plant materials, and isolating novel cellulolytic strains from this environment could offer new opportunities for optimizing lignocellulosic pretreatment (Patel *et al.*, 2014a, Patel *et al.*, 2014b, Rabapane and Matambo, 2024). Despite global interest in microbial cellulases, limited knowledge of cellulolytic diversity within African goat populations leaves a gap in both microbial ecology and applied biotechnology.

Some of the key factors influencing microbial cellulase production include pH, temperature, incubation time, inoculum size, and the presence of surfactants and organic solvents (Sarsan and Merugu, 2019). Therefore, it is crucial to optimize these parameters in order to achieve maximum enzyme activity and efficient substrate degradation. In this study, cellulase-producing bacteria were isolated from the gastrointestinal tract of South African goats, and three promising strains were identified: *Bacillus* KC50, *Bacillus* KC70, and *Proteus mirabilis* KC94. To our knowledge, this is the first report of cellulolytic optimization of *P. mirabilis* in the goat rumen. The specific objectives were to characterize and identify cellulase-producing strains from goat gastrointestinal samples, to optimize key environmental factors (pH, temperature, and incubation time) influencing their cellulase production and activity, and to assess the effects of surfactants, solvents, and oxidative stress on enzyme performance, as well as to characterize lignocellulose-degrading genes in KC94 that underpin its potential for biomass conversion. By addressing these objectives, the study contributes to expanding the potential of cellulolytic bacteria and advancing microbial pretreatment strategies for sustainable lignocellulosic biomass utilization. This study was informed by our previous profiling of CAZyme-producing microorganisms (Rabapane and Matambo, 2024), which revealed the presence of *P. mirabilis* harboring genes associated with cellulose-oligosaccharide metabolism, suggesting its potential as a previously underexplored cellulolytic candidate.

4.2 Materials and Methods

4.2.1 Ethical Statement

This study was conducted in the College of Science, Engineering, and Technology of the University of South Africa. Ethics approval was granted by the School of Engineering Ethics Review committee, chaired by Dr. Walied A. Elsaigh, and assigned reference number 2020/CSET/SOE/028. Because of the nature of the study involving isolation of microorganisms from goat GIT it was submitted for further approval within the College of Agricultural and Environmental Sciences Animal Research Ethics Committee (CAESAREC)

of the University of South Africa, chaired Dr. A Wilson., where it was also granted and assigned the following reference number: 2021/CAES_AREC/097, to ensure humane treatment of the animals during slaughtering and compliance with regulations. The Chief State Veterinarian of South African Agriculture and Rural Development, Dr. S. Kamudyariwa, also approved this study and allocated it the reference number UNISA-01-2021-KJR.

4.2.2 Bacterial Screening, Isolation, and Qualitative Assay

Forty gut samples from different compartments (rumen, reticulum, omasum, and abomasum) of ten goats were grown in Brain Heart Infusion Broth (BHIB) (Lasec, SA, (Pty) Ltd) to promote the growth of fastidious microorganisms under anaerobic conditions. The gastrointestinal samples were acquired as outlined in our previous study (Rabapane and Matambo, 2024). About 1% of the samples were inoculated into 250 mL BHIB at pH 7.01 and incubated at 41 °C in a water bath for 5 days with regular shaking to mimic the goat's gastrointestinal (GIT) environment (Rabapane *et al.*, 2022). Anaerobic conditions were maintained using 500 mL specialized reactors equipped with a hook for controlled gas release and a separate sampling port, thereby preventing oxygen ingress. Gas release was quantified using the water-displacement method, and a schematic of the reactor design is shown in Appendix A4.5. After cultivation, the cultures were stored at –86 °C in 20% glycerol for long-term preservation, creating a biobank. This biobank enables future research without requiring additional goat slaughter, thereby promoting food security. Approximately 250 µL from the enrichment was used to inoculate 50 mL of the cellulase production medium, as listed in Supplementary Material Table S1. The pH was maintained at 7.0, and the sample was incubated at 37 °C for 48 h after serial dilution in 0.9% NaCl. A volume of 100 µL from each 10⁻⁸ dilution was plated and spread on 0.5% (w/v) Carboxymethyl Cellulose (CMC) (St. Louis, MO, USA) for cellulolytic activity. Plates were incubated at 37 °C for 48 h in an inverted position. Isolated cultures were purified by repeatedly picking and streaking on agar plates to obtain pure isolates. The plates were then flooded with 0.1% Congo red (w/v) solution for 30 min and then washed with 1 M NaCl for 15 min (Kumari *et al.*, 2019, Gaur and Tiwari, 2015, Hussain *et al.*, 2017, Irfan *et al.*, 2017a). A light yellow color around the isolate confirmed the hydrolysis of cellulose. The zone of hydrolysis was measured using a digital caliper (mm). A negative control (a non-cellulolytic strain) was used to rule out false positives. The relative enzyme activity was calculated using Equation (1) (Hsiao *et al.*, 2014, Usman *et al.*, 2021):

$$REA = \frac{CZD}{CD} \quad (1)$$

where REA: relative enzyme activity, CZD: clear zone diameter, and CD: colony diameter. Positive isolates were stored in 20% glycerol stocks at -86°C .

4.2.3 Identification of the Cellulase-Producing Bacteria by 16S rDNA Sequencing

For identification, primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') (Weisburg *et al.*, 1991, Wilson *et al.*, 1990) were used to amplify the 16S rDNA gene of the pure isolates using extracted genomic DNA (*Quick-DNA Fungal/Bacterial extraction kit*, Zymo Research, Irvine, CA, USA) as template in a polymerase chain reaction (PCR). Amplified genes were visualized using 1% agarose gel electrophoresis. The PCR products of the amplified 16S rDNA genes were sequenced at Inqaba Biotech (Pty) Ltd. (Pretoria, South Africa) using the ABI Big dye V3.1 kit following the manufacturer's protocol and sequenced using the ABI 3730xl Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) (Irfan *et al.*, 2017b). Sequences for KC40, 50, 70, and 94 were aligned using ClustalW multiple alignment on BioEdit v7.2 software (Hall, 1999) and MAFFT—an online version of a multiple sequence alignment program. The obtained 16S rRNA gene sequence was submitted to NCBI GenBank, and a phylogenetic tree was constructed using the neighbor-joining method (using MEGA 11.0) (Waheeb *et al.*, 2024). Bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence of the tree topologies (Potprommanee *et al.*, 2017). Operational taxonomic units (OTUs) were generated in MOTHUR (v1.48.0) using a 98% similarity threshold to group similar species. The representative OTU sequences were then compared to sequences in the GenBank database for identification (Nkuna and Matambo, 2024).

4.2.4 Effects of pH, Temperature, and Incubation Period

The selected isolates were grown at 37°C for 24 h. After which, they were used as a 1% inoculum to inoculate CMC production media as a carbon source at various pHs ranging from 4.0 to 9.0 (pH 4.0 and 5.0 with Acetate Buffer, pH 6.0 and 7.0 with Phosphate Buffer, pH 8.0

and 9.0 with Tris-HCl) at temperatures 35–65 °C. Cellulase activity was measured using the DNS method (Bernfeld, 1955). The pH and temperature with the highest activity were selected and analyzed further for the effect of the incubation period at 12 h intervals. To determine the cellulase activity, the cultures were centrifuged at 4 °C (4400 rpm) for 15 min, and the cell-free extracts (CFEs) were assayed for cellulase activity (Alamri *et al.*, 2016). Two hundred and fifty microliters (250 µL) of the buffered CMC (0.5%) at pH 7.0 was equilibrated for 5 min at 35 °C in a water bath. After 5 min of equilibration, 250 µL of the CFE was transferred to a test tube to start the reaction and stopped after 30 min (Demissie *et al.*, 2024). Then, 500 µL of DNS (3,5-dinitrosalicylic acid) reagent (Glenthams Life Sciences Ltd, Corsham, UK) was added to each test tube to stop the reaction. The test tubes were placed in boiling water for 5 min (Gebbie *et al.*, 2020), then cooled to room temperature under running water. Thereafter, 2 mL of distilled water was added to the test tubes, and the absorbance was measured at 540 nm. Then, the absorbance was converted to glucose concentration (mg/mL) using the calibration curve (Appendix A.4.4). The substrate and DNS reagent were mixed first, and the CFE was added afterward for the blank reaction (Gebbie *et al.*, 2020). This experiment was performed in triplicate, and the means ± standard deviation were used to plot the figures. One unit of cellulase activity was defined as the amount of enzyme that liberated 1 µmol of glucose equivalents under the assay conditions. The formula below (Equation (2)) was used to calculate the enzyme activity (Shareef *et al.*, 2015, Singh *et al.*, 2019, Demissie *et al.*, 2024).

$$\frac{U}{ml} = \frac{\left[\text{glucose} \frac{mg}{ml} \right]}{\text{Time (min)} \times \text{VS (ml)} \times \text{MW glucose} \frac{mg}{mmol}} \times 1000 \quad (2)$$

where VS = Volume of Substrate and MW = Molecular Weight.

4.2.5 Effects of Organic Solvents and Surfactants on Cellulase Production

To investigate the effects of organic solvents and surfactants on the isolate's ability to produce cellulase, the production medium was supplemented with a low concentration (0.5%) and a high concentration (2%) of various organic solvents and surfactants under optimum conditions. We tested two solvent/surfactant/oxidizing levels, 0.5% (low, process-relevant) and 2% (high, stress-level), deliberately to capture both typical operational exposure and an upper bound for enzyme tolerance. While many previous studies use 0.1–0.2% for low concentrations and ~1% for higher levels (Barman and Dkhar, 2022, Gaur and Tiwari, 2015), these are often specific to

conventional pretreatment of industrial waste. In contrast, we included 0.5% as a more realistic low-level condition relevant to some detergent-assisted pretreatment streams, and 2% as an extreme stress condition to explore enzyme stability at the upper range of reported or conceivable industrial surfactant exposure. This approach allows us to evaluate both practical applicability and enzyme robustness under more challenging conditions.

4.2.6 PCR Amplification of Possible Cellulase and Hemi-Cellulase Gene Fragments on KC94

The Touchdown (TD) PCR settings on Supplementary Table S3 were used to amplify the targeted fragments using the T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The DNA template concentration was 9.04 ng/μL as determined by the Qubit 3® (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) high-sensitivity assay. OneTaq 2X Master Mix (New England Biolabs, Ipswich, MA, USA) was used for amplification of GH45 and GH48, while Q5 High-Fidelity 2X Master Mix (NEB) was used for GH39. The targeted genes and their corresponding enzymes were as follows: GH39 (PF01229), β-xylosidase; GH45 (PF02015), β-1,4-endoglucanase; and GH48 (PF02011), cellobiohydrolase. The list of degenerate primers used in this study is also included in the Supplementary Materials Table S3, as adopted from (Sheng *et al.*, 2015). KC94 was selected for PCR-based gene amplification and enzyme-specific validation. This decision was based on preliminary optimization experiments, which consistently identified KC94 as the most robust cellulolytic strain. Therefore, KC94 was considered the most representative candidate for further molecular characterization.

4.2.7 Statistical Analysis

Each experiment was performed in triplicate ($n = 3$) to accurately capture variability within the dataset, and the standard deviation for each experimental result was calculated and populated using Microsoft® Excel® for Microsoft 365 MSO (Version 2508 Build 16.0.19127.20192). Statistical analysis was performed using two-way ANOVA with Tukey's post hoc test ($p < 0.05$) using IBM SPSS Statistics (version 30.0.0.0).

4.3 Results

4.3.1 Qualitative Assay of Cellulase-Producing Bacteria

Twelve (12) bacterial isolates were obtained from ruminal fluid samples, of which 4 (33%) showed clear zone hydrolysis on CMC agar during primary screening. Following performance screening, the isolates KC40 (reticulum), KC50 (rumen), KC70 (abomasum), and KC94 (abomasum) were advanced for further study, as they demonstrated the highest cellulolytic activity, confirmed by sub-culturing on 1% CMC for 72 h at 37 °C, flooding the plates with 0.1% Congo red, and washing off with 1 M NaCl. The bacterial isolates exhibited a significant clear zone diameter and relative enzyme activity (REA) ranging from 16.21 mm to 78.62 mm and between 1.26 and 4.15, respectively (Table 1). A univariate analysis of variance (GLM) was conducted to assess differences in REA among isolates. The analysis revealed a significant effect of isolate on REA ($F_{3,8} = 5480.94$, $p < 0.001$, partial $\eta^2 = 1.000$), indicating that REA differed substantially between isolates. Levene's test confirmed homogeneity of variance ($p = 0.143$), satisfying the assumption for ANOVA. Post hoc comparisons using Tukey's HSD indicated that all pairwise differences between isolates were statistically significant at the 0.05 level ($p < 0.001$ for all comparisons). Specifically, KC94, which exhibited the highest REA, was significantly greater than KC70, KC40, and KC50, while KC50 had the lowest REA.

Table 4.1. Comparison of cellulolytic potential among isolates based on clear zone diameter. Error bars represent $\pm$ SD; $n = 3$. Strain KC94 was significantly higher than KC50 and KC70 across all the plates (Tukey's HSD, $p < 0.001$).

Isolate	Colony Diameter (mm)	Clear Zone Diameter (mm)	Relative Enzyme Activity
KC40	7.33 $\pm$ 0.12	16.21 $\pm$ 0.12	2.21 $\pm$ 0.03
KC50	62.16 $\pm$ 0.12	78.62 $\pm$ 0.11	1.26 $\pm$ 0.01
KC70	8.81 $\pm$ 0.10	23.41 $\pm$ 0.10	2.66 $\pm$ 0.02
KC94	5.99 $\pm$ 0.10	24.86 $\pm$ 0.15	4.15 $\pm$ 0.05

Correlation analysis revealed strong relationships among the measured variables. Colony diameter was highly positively correlated with clear zone diameter ($r = 0.990$, $p < 0.001$), and both colony diameter. Of the 4 isolates subjected to secondary screening, 3 showed significant

cellulase activity (KC50, KC70, and KC94), producing relatively high amounts of glucose when assayed under physiological conditions (37 °C and pH 7) (Figure 4.2).

4.3.2 Bacterial Identification

Identification using the 16S rDNA sequence revealed that KC40 was closely related to *Bacillus licheniformis*, KC50 to *Bacillus subtilis*, and KC70 to *P. mirabilis*.

Pseudomonas sp. IRG-1 was used as an outlier to root the tree, representing a reference point for comparison. Based on the phylogenetic findings, isolate KC40 was identified as *Bacillus licheniformis*. Meanwhile, KC50 and KC70 were classified within the *Bacillus* genus but may represent different subspecies or closely related species, warranting further investigation. KC94 was identified as *P. mirabilis* (Figure 4.1). Bootstrap analysis provided additional confidence, with high values (100) observed in the clusters involving *P. mirabilis* and among the *Bacillus* sp., supporting the robustness of these relationships. The inclusion of *Pseudomonas* sp. IRG-1 as an outgroup confirmed that the isolates are more closely related to each other than the outlier. This separation validated the tree's internal structure and highlighted the distinct evolutionary pathways of the *Bacillus* and *Proteus* sp.

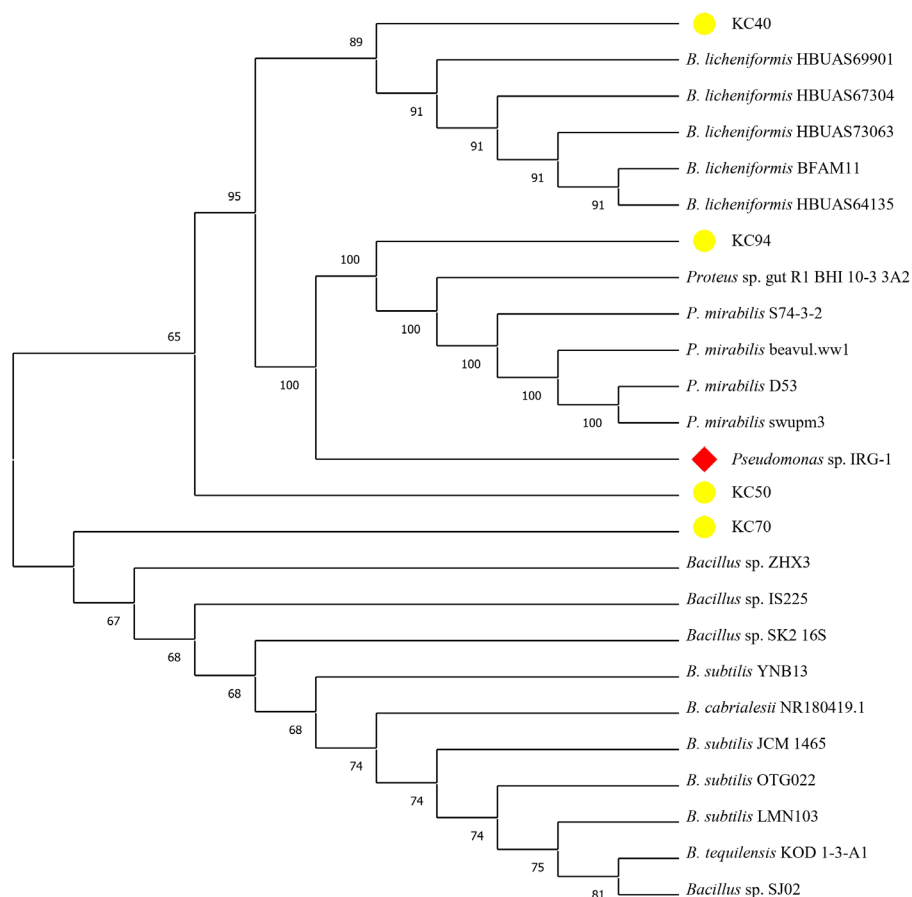


Figure 4.1. Phylogenetic tree showing the relationships among isolates KC40, KC50, KC70, and KC94 (highlighted in yellow). The phylogenetic analysis used the neighbor-joining method, and the significance of junctions was established using the bootstrap method (1000 replicates). *Pseudomonas* sp. *IRG-1*, highlighted in red, was used as an outlier.

4.3.3 Optimization Studies

4.3.3.1 The Effect of pH

The effects of pH on bacterial cell growth and cellulase production were assessed using CMC after growing at pH levels ranging from 4 to 9 for 24 h. As shown in Figure 2, KC50, KC70, and KC94 have optimum pHs of 5, 6, and 7, respectively. KC50 strain exhibited a significant increase in cellulase activity at pH 5 and maintained a relatively stable activity level from pH 5 to pH 7, with a slight decrease at pH 8. However, under extreme acidic (pH 4) and alkaline (pH 9) conditions, KC50 exhibited negligible cellulase activity, indicating a narrow optimal pH range.

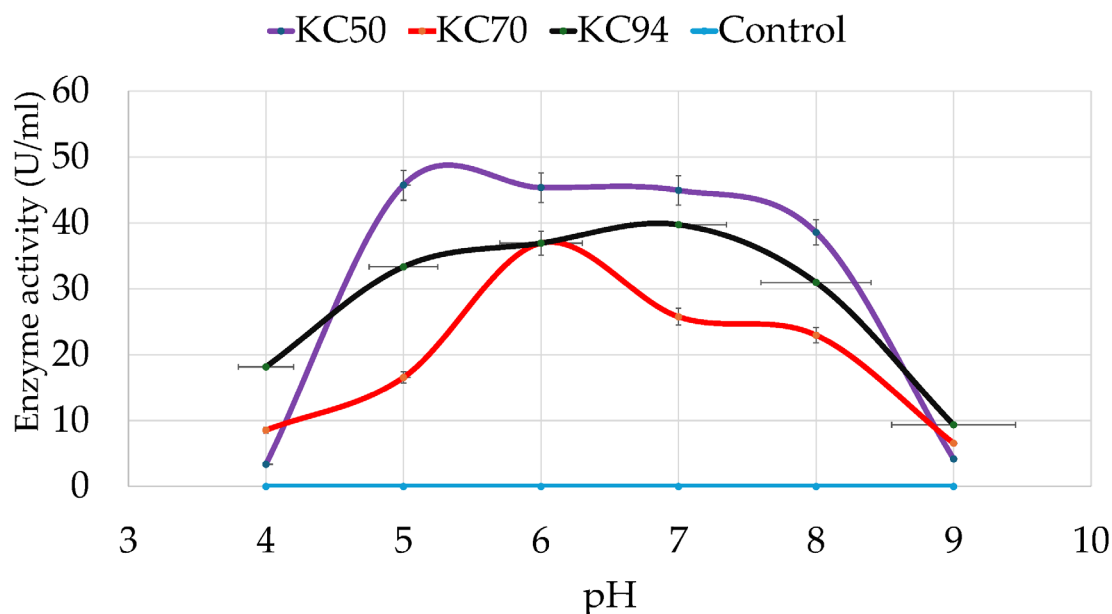


Figure 4.2. pH-dependent growth and enzyme activity profiles of the isolated strains. Error bars represent mean $\pm$ SD of three independent replicates ($n = 3$). Mean standard deviation for all the values is $<\pm 5.0\%$. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test ($p < 0.05$). All pairwise differences among pH levels and strains were statistically significant (Tukey's HSD, $p < 0.05$).

KC70, on the other hand, showed a more consistent but lower cellulase activity across the pH spectrum. At pH 5 and 6, KC70 demonstrated relatively good activity, but it dropped significantly as the pH approached both extremes, with a notable decrease at pH 8 and minimal activity at pH 9. KC94 exhibited significant growth over a wide pH range, reaching its optimum at pH 7 while retaining considerable activity between pH 5 and 8. A similar optimum pH strain was also observed (Demissie *et al.*, 2024), and a significant drop was observed at pH 8. At pH 9, KC94 also showed higher average enzyme activity than the other isolates, although the variability between replicates was larger, as indicated by the error bar.

4.3.3.2 The Effects of Temperature

The effect of temperature on cellulase production was also determined for all the strains, revealing distinct thermal tolerance patterns. Accordingly, KC50 exhibited optimal cellulase activity at 35 °C, indicating its mesophilic nature. At 25 °C, KC50 retained significant activity,

but enzyme efficiency declined sharply at 45 °C and 55 °C (Figure 3). On the other hand, KC70 demonstrated lower enzyme activity than KC50 across all temperatures, with peak cellulase production at 35 °C. A steady decline was observed as the temperature increased, and no detectable activity was recorded at 55 °C.

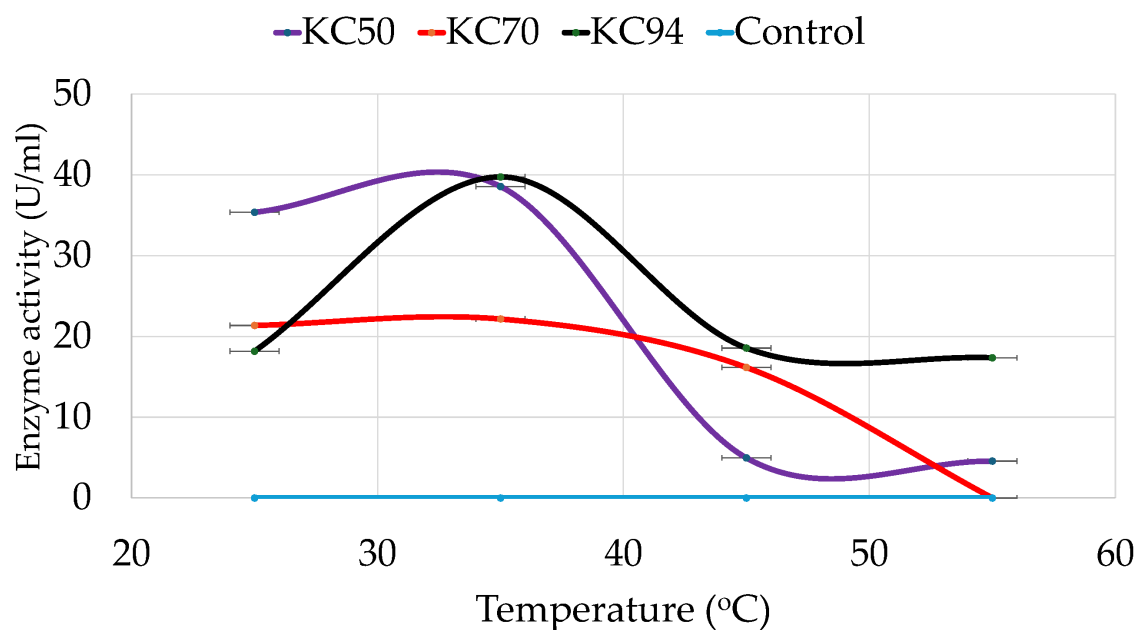


Figure 4.3. Effect of temperature on growth and cellulase production of KC50, KC70, and KC94. Error bars represent mean $\pm$ SD of three independent replicates ($n = 3$). Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test ($p < 0.05$). All pairwise differences among temperature levels and strains were statistically significant (Tukey's HSD, $p < 0.05$).

Meanwhile, KC94 exhibited the highest temperature tolerance among the tested strains, maintaining substantial activity from 25 °C to 55 °C. Like KC50 and KC70, KC94 displayed peak activity at 35 °C but retained significant cellulase function at both 45 °C and 55 °C. Interestingly, although KC70's activity was less throughout the thermal levels, it retained noticeable activity at 45 °C compared to KC50.

4.3.3.3 The Effects of the Incubation Period

Interest has been placed on cellulase-producing bacteria due to their rapid growth. Various studies have reported high enzyme production at 24 (Islam *et al.*, 2019), and 48 h (Demissie *et*

al., 2024). Meanwhile, others have reported 72 h (Shareef *et al.*, 2015, Mahapatra *et al.*, 2016, Demissie *et al.*, 2024, El-Khamisi *et al.*, 2024), and as long as 5–7 days (Waheeb *et al.*, 2024). In a study by Gaur and Tiwari, enzyme production was observed at 12 h, with maximum production observed at 48 h when investigated on various agro-wastes (Gaur and Tiwari, 2015). In this study, cellulase production was monitored over time up to 84 h. As a result, KC50 displays consistent performance and sustained productivity over time, marked by an impressive early growth phase characterized by a sharp increase at the beginning. It quickly rises to about 50 units after 36 h, indicating that KC50 has a fast initial growth rate due to either rapid cellulase production or expansion. However, its growth reaches a peak of around 50 units and then gradually declines (Malik *et al.*, 2021). Meanwhile, KC70 shows a slower but steady increase, reaching around 30 units. Its growth rate is less steep than that of KC50, suggesting a prolonged but less intense period of activity.

Interestingly, KC94 demonstrates the best performance, peaking at approximately 90 units at 84 h and consistently maintaining a high level (Figure 4.4). This suggests that KC94 is highly effective throughout the entire duration, likely indicating strong cellulase activity or steady biomass production (Mobley, 2019). A two-way ANOVA revealed significant main effects of incubation time and strain on activity, as well as a significant interaction between incubation time and strain, indicating that strain effects varied across time points. Post hoc tests showed that KC94 consistently yielded the highest values, followed by KC50 and KC70, with significant differences across all incubation time points.

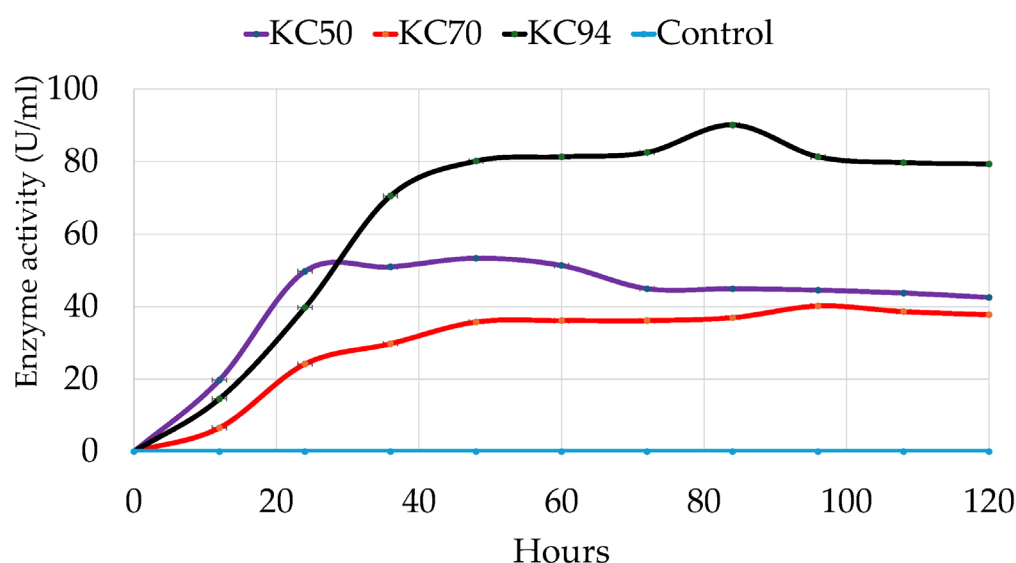
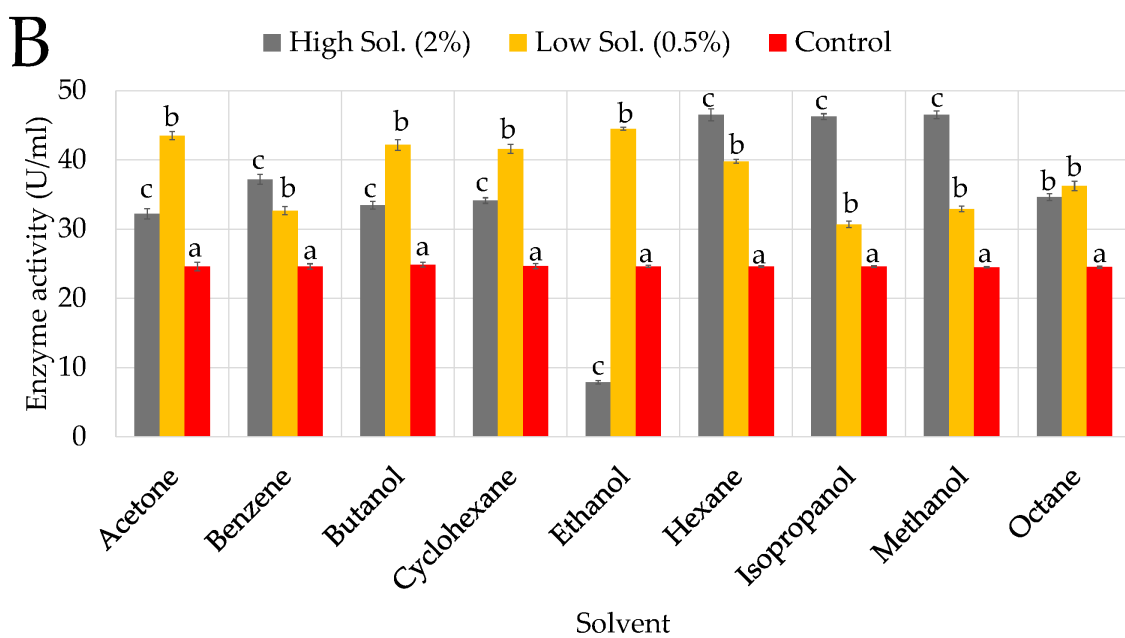
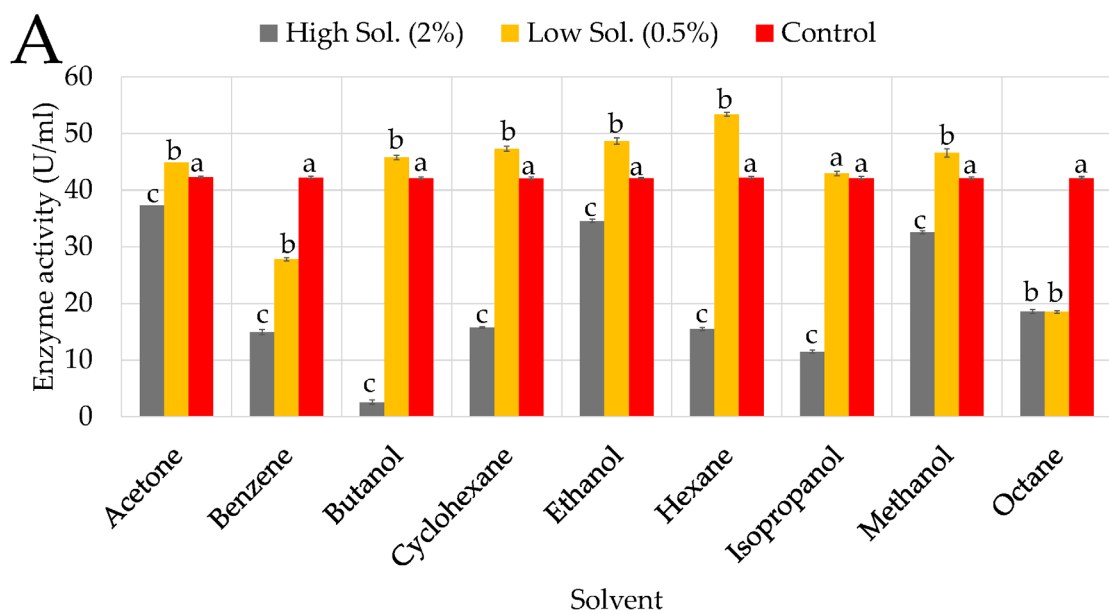


Figure 4.4. Determination of optimal incubation time for maximal cellulase activity in all tested isolates. Error bars represent mean $\pm$ SD of three independent replicates ($n = 3$). Statistical analysis was performed using two-way ANOVA with Tukey's post hoc test ($p < 0.05$). All pairwise differences among time points and trains were statistically significant (Tukey's HSD, $p < 0.05$).

4.3.4 The Effects of Organic Solvents, Surfactants, and Oxidizing Agent on Cellulase Production

4.3.4.1 Solvents

KC50's ability to produce cellulase was significantly inhibited in the presence of butanol, isopropanol, and benzene, with residual activities (RA) of 2.55%, 11.36%, and 14.95%, respectively (Figure 4.5A). Cyclohexane, hexane, and octane exhibited moderate inhibition, with RAs of 15.75%, 15.35%, and 18.56%, respectively, indicating that KC50 is less tolerant to non-polar solvents. Moderate tolerance was observed in the presence of ethanol (34.56%) and acetone (37.36%), suggesting that KC50 retains some activity in the presence of these less toxic solvents. KC70 generally exhibited moderate tolerance to organic solvents but was particularly sensitive to ethanol at higher concentrations (Figure 4.5B), which strongly inhibited cellulase production. Non-polar solvents such as hexane and octane caused moderate inhibition but, in some cases, even outperformed the control, suggesting KC70 maintains enzyme structure stability in hydrophobic solvents.



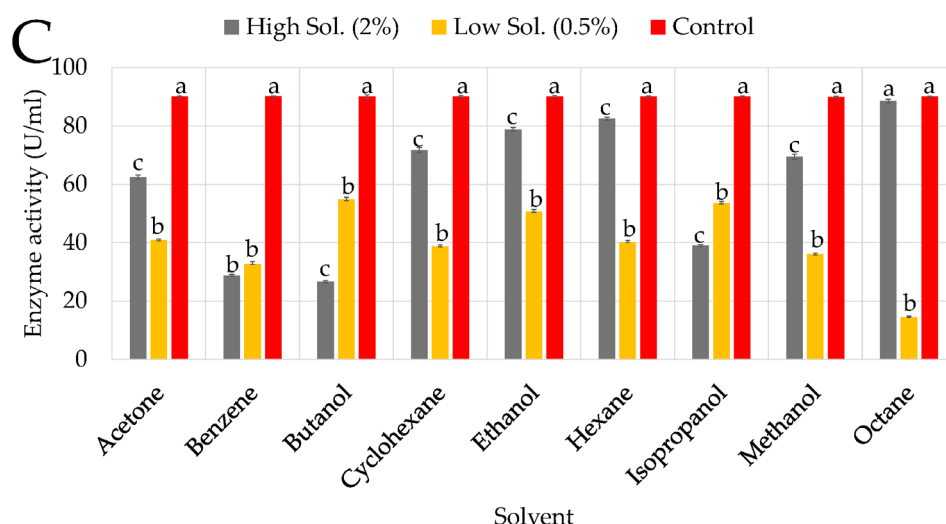
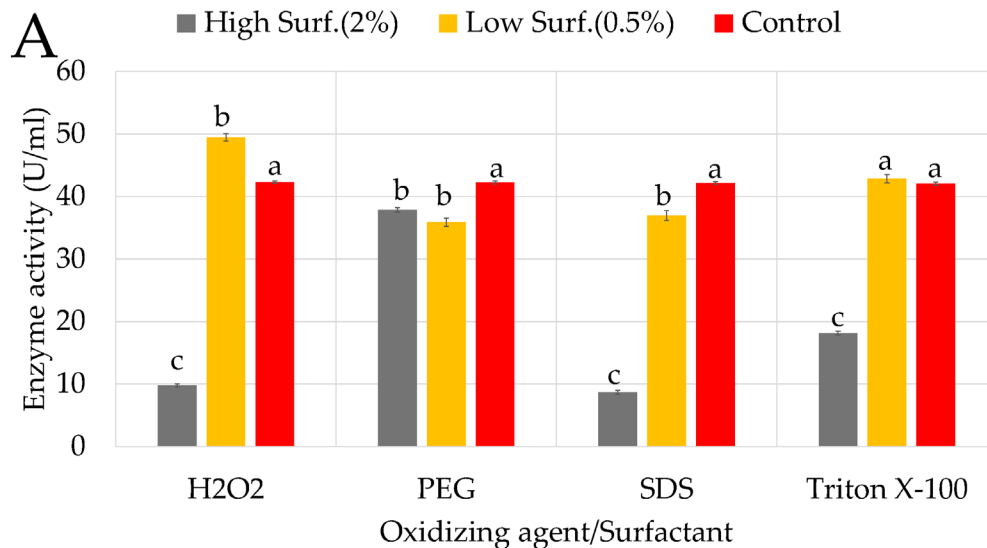


Figure 4.5. Influence of organic solvents on growth and enzyme activity of the three isolates: KC50 (A), KC70 (B), KC94 (C). Error bars represent mean $\pm$ SD of three independent replicates ($n = 3$). Statistical analysis was performed using two-way ANOVA with Tukey’s post hoc test ($p < 0.05$). Letters above bars indicate statistical differences within each solvent: bars labeled “a” are the control (baseline), “b” = significantly different from control, “c” = significantly different from both control and low (0.5%) concentration, and bars sharing the same letter are not significantly different from each other (Tukey’s HSD post hoc test, $p < 0.05$).

At a higher concentration (2%), butanol significantly inhibited KC94’s cellulase production (Figure 4.5C), resulting in approximately 26.55% RA, likely due to its toxic effects, causing membrane disruption and enzyme inactivation (Patakova *et al.*, 2018, Russmayer *et al.*, 2019). Isopropanol exhibited similar inhibition (38.96% RA), and benzene also had a notable inhibitory effect (28.96% RA). Moderate inhibition was observed with acetone (62.56% RA), reflecting its intermediate polarity (Zaks and Klibanov, 1988). Despite these effects, KC94 showed notable tolerance to ethanol (78.96% RA), likely due to ethanol’s stabilizing effects on the enzyme’s hydrophobic core (Skovgaard and Jørgensen, 2013). Likewise, KC94 maintained significant cellulase production in cyclohexane (71.76% RA), hexane (82.56% RA), and octane (88.57% RA).

4.3.4.2 Surfactants and Oxidizing Agent

The effects of surfactants at high concentrations showed strong inhibition by SDS and Triton X-100 on KC50 (Figure 4.6A), with only 8.56% and 18.16% RA, respectively. Triton X-100, a non-ionic surfactant, likely disrupted the enzyme's hydrophobic regions, leading to destabilization and reduced activity. PEG exhibited moderate inhibition with 37.75% RA, likely due to its interference with enzyme stability via modification of the hydration layer. Hydrogen peroxide (H_2O_2) as an oxidizing agent appeared particularly unfavorable for KC50 (9.75% RA), though cellulase activity was slightly higher at lower concentrations (0.5%). Notably, H_2O_2 stimulated cellulase production (49.36% RA), suggesting that mild oxidative stress might activate protective pathways.



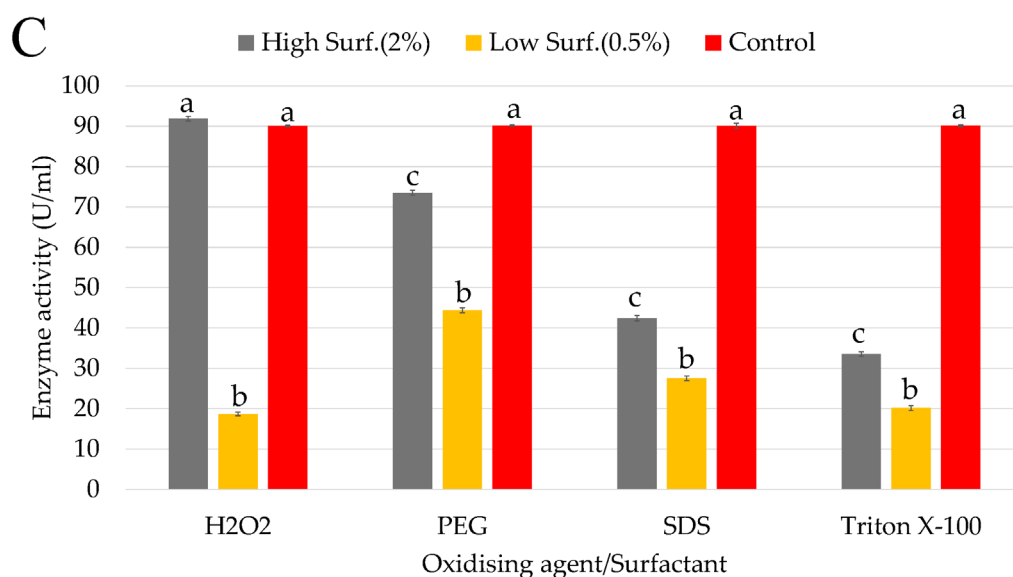
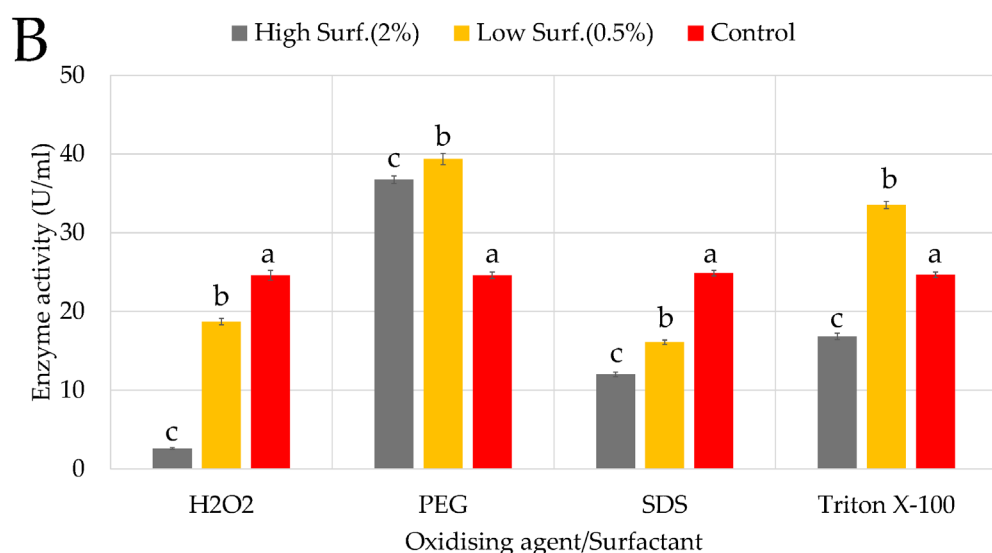


Figure 4.6. Impact of selected surfactants and oxidizing agents on enzyme activity profiles of the three isolates: KC50 (A), KC70 (B), and KC94 (C). Error bars represent mean $\pm$ SD of three independent replicates ($n = 3$). Statistical analysis was performed using two-way ANOVA with Tukey's post hoc test ($p < 0.05$). Letters above bars indicate statistical differences within each surfactant/oxidizing agent: bars labeled "a" are the control (baseline), "b" = significantly different from control, "c" = significantly different from both control and low (0.5%) concentration, and bars sharing the same letter are not significantly different from each other (Tukey's HSD post hoc test, $p < 0.05$).

KC70 exhibited varied responses to different surfactants, with enzyme production significantly influenced by both type and concentration (Figure 4.6B). High concentrations (2%) of SDS

and H₂O₂ strongly inhibited cellulase production, while lower concentrations (0.5%) of PEG and Triton X-100 stimulated enzyme activity. Regarding surfactants, SDS and Triton X-100 strongly inhibited cellulase activity on KC94, with RAs of 19.56% and 22.76%, respectively (Figure 4.6C). These surfactants likely disrupted enzyme conformation by interfering with hydrophobic regions, reducing functionality. PEG exhibited moderate inhibition (47.65% RA), while H₂O₂ had a relatively mild effect (54.36% RA), suggesting that KC94 possesses mechanisms to tolerate oxidative stress. At lower concentrations (0.5%), PEG, SDS, and Triton X-100 had less inhibitory effects, while H₂O₂ slightly stimulated cellulase production (64.36% RA), possibly due to the activation of oxidative stress response pathways.

4.3.5 Diversity of Possible Cellulase and Hemi-Cellulase Gene Fragments in KC94

The GH45 family gene coding for β -1,4-endoglucanase was successfully amplified at the expected size range of 377–412 bp (Figure 7). Similarly, amplification of the GH39 family, which codes for β -xylosidase, produced clear products at the expected size of 223–230 bp. Interestingly, additional bands were observed in the GH39 amplification, ranging in size from 321 to 665 bp. For GH48, two distinct amplicons were obtained, with sizes between ~480–702 bp.

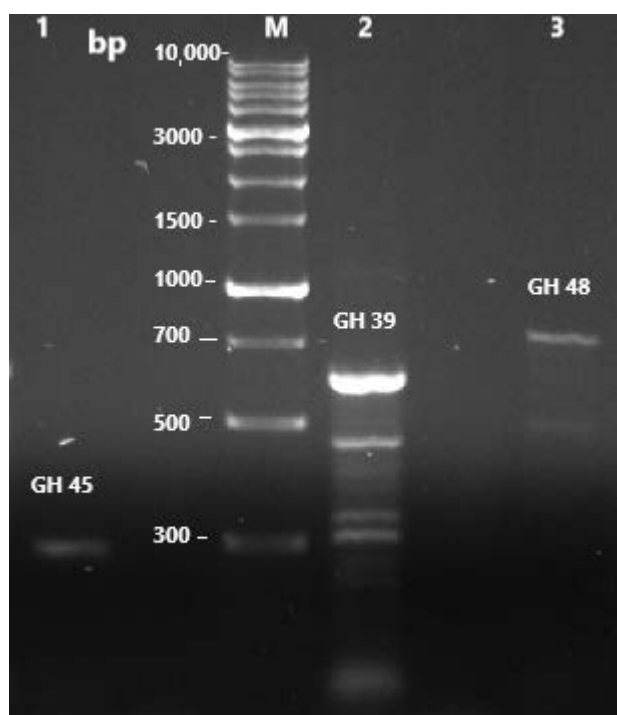


Figure 4.7. 1% agarose gel electrophoresis of the Touchdown PCRs. M: 1 kb DNA marker; 1: GH45 coding for β -1,4-endoglucanase (377–412 bp), 2: GH39 coding for β -xylosidase (223–

230 bp), 3: GH48 coding for cellobio-hydrolase (420 bp). The empty lane between lanes 2 and 3 served as the control, containing all PCR reagents except the DNA fragment.

4.4 Discussion

4.4.1 Cellulase Activity Identified by Qualitative Assay

The qualitative CMC plate assay confirmed that the hydrolysis zone size correlates with cellulase production capacity, consistent with earlier reports (Saini *et al.*, 2017). Moderate cellulase activity is exhibited by KC40, as indicated by the CZD, which is over twice the size of the colony diameter. The REA of 2.21 suggests it is a decent cellulase producer but less efficient than strains such as KC94 and KC70. Thus, the moderate CZD indicates that KC40 can degrade cellulose but not as aggressively as the other strains. Interestingly, the largest CD was observed in KC50, suggesting rapid growth, as its CZD is slightly larger than its CD, resulting in a lower REA of 1.26. Therefore, although KC50 grows rapidly, its ability to produce cellulase may not be correlated with its growth rate, suggesting it may not produce significantly higher levels of cellulase than the other strains. Meanwhile, KC70 exhibits a CZD that is almost three times its CD, indicating strong cellulase activity relative to its size. The REA of 2.66 is substantially higher and indicative of a potent cellulase producer (Hsiao *et al.*, 2014). Although KC70 is more efficient at cellulase production than KC40 and KC50, it still falls short of KC94, which has the highest REA (4.15) and thus the highest production per colony. The CZD of KC94 is approximately 4 times the CD, suggesting its ability to efficiently break down cellulose. Although its colony size is smaller, it produces the highest degradation activity, making it the most efficient cellulase producer among all the strains. This pattern highlights the importance of considering relative activity measures rather than absolute colony or zone size when comparing isolates (Saini *et al.*, 2017). The variation in CZD and REA could be attributed to differences in pH preference. The media for the primary screening were at pH 7, favoring the KC94 strain as determined by the pH assay conducted in subsequent sections, and may have affected the growth of isolates KC50 and KC70, which thrived at pH 5 and 6, respectively.

4.4.2 The Effects of pH, Temperature, and Incubation Period

4.4.2.1 pH

The differences in pH response among isolates highlight their potential ecological roles and industrial relevance. KC50 showed a narrow pH optimum centered at 5–7, which aligns with earlier reports of *Bacillus subtilis* cellulases exhibiting optimal activity at slightly acidic to neutral conditions (Asha and Sakthivel, 2014). Given that the rumen environment is generally maintained within a regulated pH range of 6.2–7.0 (Rabapane *et al.*, 2022). KC50's stable activity within this range indicates that the rumen environment is conducive to its production of cellulase. Its efficiency at near-neutral pH suggests that KC50 could contribute substantially to cellulose degradation under typical rumen conditions, particularly compared with strains that lose activity at neutral pH. This supports the potential relevance of KC50 as an effective cellulase producer within the rumen ecosystem, where maintaining fiber digestion efficiency is crucial for host nutrition.

Meanwhile, KC70 exhibited moderate, more evenly distributed activity, which is somewhat more resilient to pH changes than KC50 but has a lower overall cellulase production capacity. The moderate performance of KC70 could be attributed to its ability to maintain some enzyme functionality under diverse conditions, though it still experiences a decrease at higher pH. Although KC70 produces less cellulase than KC50 under optimal conditions, it could still contribute to cellulose degradation in the rumen environment (6.2–7.0) because its activity is sustained within the physiological pH of the rumen. This observation supports the notion that *Bacillus* species, including KC70, can retain some cellulase activity across a broader pH range. However, efficiency decreases as the pH moves away from its optimal conditions (Asha and Sakthivel, 2014). This is a common observation in microorganisms found in neutral or slightly acidic environments (Malik *et al.*, 2021, Demissie *et al.*, 2024).

KC94 demonstrated both high activity and broad pH tolerance, with optimum cellulase activity at pH 7 and retention across pH 5–8. This broad pH tolerance suggests that KC94 may possess efficient pH-regulation mechanisms, such as effective ion-exchange systems that protect cellular components from pH-induced stress (Zhang *et al.*, 2023). These properties make KC94 an ideal option for applications such as lignocellulosic biomass pretreatment, where pH levels often fluctuate as the substrate is gradually broken down. This finding aligns with previous studies reporting that *P. mirabilis* VIT117 can tolerate a broad pH range, suggesting the presence of mechanisms that help maintain cellular stability under varying pH conditions

(Mahapatra *et al.*, 2016). Meanwhile, urease activity has been identified as a common pH-regulating mechanism in *P. mirabilis* (Wasfi *et al.*, 2020, Chakkour *et al.*, 2024). The current study did not evaluate urease production; therefore, while urease may have contributed to KC94's pH resilience, other regulatory pathways, such as ion exchange systems or membrane adaptations, could also play a role in enabling efficient function across different pH levels.

Unlike KC50, which performs best in a narrow pH range, and KC70, which shows lower overall activity, KC94 combines both high activity and tolerance across a broader pH spectrum. A two-way ANOVA revealed that pH significantly affected activity ($p < 0.001$) and that activity also differed among strains. There was a significant pH and strain interaction ($p < 0.001$), indicating that strain activity depended on pH. Post hoc comparisons showed that KC94 and KC50 generally exhibited the highest activity, followed by KC70. Activity varied significantly across pH levels, with maximal values observed around pH 6–7 and minimal activity at extreme pH values.

In the rumen, pH fluctuates with diet, often ranging from 5.5 to 5.8 (Yang and Beauchemin, 2006, Hossain, 2020, Lian *et al.*, 2022) when fed a high-grain diet, and to approximately 7.0 when fed forage-rich diets. Many fibrolytic bacteria, such as *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*, are highly sensitive to pH drops, losing cellulase activity below 6.0 (Russell and Wilson, 1996). KC94's acid tolerance contrasts with these typical rumen cellulolytics, suggesting it could persist and remain active in suboptimal ruminal pH conditions, such as during sub-acute ruminal acidosis (SARA). This trait could provide ecological resilience and industrial utilization in acidic pretreatment environments.

4.4.2.2 Temperature

The three strains exhibited distinct thermal tolerance patterns. KC50, peaking at 35 °C, demonstrates a mesophilic profile similar to *Bacillus subtilis* cellulases, which also lose stability at elevated temperatures (Asha and Sakthivel, 2014). Although its peak activity is slightly below the rumen temperature (38–42 °C) (Hyder *et al.*, 2017, Castro-Costa *et al.*, 2015). KC50 still retains significant activity at moderate temperatures. This suggests that in the rumen environment, KC50 could maintain functional cellulase activity, but may not perform at maximum efficiency due to its reduced stability at higher temperatures. This further

suggests that KC50 is well adapted to moderate temperatures but lacks the thermostability required for high-temperature industrial processes. KC70 showed reduced overall activity and a sharp loss at 55 °C, indicating weak thermal adaptation. This suggests that KC70 possesses limited thermal adaptation, making it less suitable for applications requiring high-temperature tolerance. The strain's limited thermal tolerance makes it less adaptable to the rumen's constant temperature range of 38–42 °C, potentially limiting its contribution to in situ fiber degradation efficiency. In contrast, KC94 combined high activity with broad tolerance, maintaining substantial cellulase function even at 55 °C. These findings were also supported by the two-way ANOVA, which revealed significant main effects of temperature and a significant interaction between temperature and strain, indicating that strain's effect depended on temperature. Post hoc tests showed that KC94 and KC50 generally yielded the highest values, followed by KC70 and Control. Pairwise comparisons between temperatures revealed significant differences across all strains. This suggests that KC94 may possess mechanisms that enhance enzyme stability at elevated temperatures, such as increased hydrogen bonding or enhanced protein folding (Xu *et al.*, 2020, Rahban *et al.*, 2022, Wu *et al.*, 2023). Most ruminal species display temperature optima close to 38 °C and decline sharply from 43 °C (Günel *et al.*, 2025). By contrast, KC94 maintains activity above 45 °C, which is unusual for rumen isolates, suggesting an advantage for consistent cellulose degradation in the rumen while also making it suitable for industrial processes that require higher operational temperatures. To our knowledge, no prior study has reported thermostable cellulases from rumen *P. mirabilis*. These findings align with previous research on *P. mirabilis* isolated from textile industry treatment sludge, which reported laccase enzyme stability over a broader temperature range (17–47 °C) due to adaptive stress responses (Oztat *et al.*, 2024). The ability of KC94 to produce cellulases at higher temperatures suggests potential applications in biofuel production and industrial biomass degradation, where thermostability is a desirable trait.

4.4.2.3 Incubation Period

The three isolates differed in their growth and enzyme production dynamics. KC94's sustained high activity indicates efficient and prolonged cellulase production, making it the most reliable strain for continuous cellulose degradation (Mobley, 2019). KC50 displayed rapid early growth, which may be attributed to heightened metabolic activity in the early stages. The

leveling off and subsequent decline could imply constraints such as a lack of nutrients, excess substrate, or the accumulation of inhibitory substances, a limitation on enzyme production, or a stress response following the initial rapid expansion (Waheeb *et al.*, 2024). Visible colonies that expand rapidly on plates can be observed (Supplementary Materials, Figure S1), confirming the fast growth noted on the plates. Hence, the high initial activity may also lead to rapid substrate exhaustion, restricting further growth or cellulase production. KC70 produced cellulase consistently, although at lower efficiency than KC50 and KC94. Rumen studies frequently report that fibrolytic bacteria exhibit distinct growth kinetics: *Fibrobacter succinogenes* rapidly colonizes fibers but declines as soluble sugars accumulate, while *Ruminococcus albus* maintains prolonged activity (Koike and Kobayashi, 2009). Therefore, KC50 is similar to *Fibrobacter*, while KC94 resembles *Ruminococcus*-like persistence. By demonstrating prolonged stability in cellulase production, KC94 may represent an untapped “slow but steady” cellulolytic niche within the rumen microbiome, complementing faster-growing degraders like KC50. Such kinetics may be advantageous for maintaining baseline cellulase activity over extended periods, though with lower efficiency than KC94, which demonstrated the most favorable profile, combining high and sustained enzyme production, underscoring its potential suitability for industrial processes and rumen biomass degradation.

4.4.3 Response of Cellulase Production to Organic Solvents, Surfactants, and Oxidizing Agent

4.4.3.1 Organic Solvents

A two-way ANOVA was performed for each strain to assess the effects of solvent and concentration on activity. In all cases, there were significant main effects of solvent and concentration, as well as significant solvent and concentration interactions (all $p < 0.001$), demonstrating that solvent effects were strongly dependent on concentration. Post hoc Tukey HSD tests further revealed that the ranking of solvents varied somewhat among strains. For instance, KC94 exhibited superior stability, particularly in non-polar solvents. Similar observations have been reported in *Bacillus amyloliquefaciens* AK9, where cellulase showed remarkable solvent stability, with some solvents even enhancing activity (Irfan *et al.*, 2017b). A study on *Bacillus vallismortis* RG-07 demonstrated similar solvent tolerance, with cellulase activity remaining stable even at high concentrations of organic solvents (Gaur and Tiwari, 2015). It is reported in literature that non-polar solvents likely interact favorably with

hydrophobic protein regions, preventing aggregation and denaturation (Cheng *et al.*, 2015, Gaur and Tiwari, 2015, Zaks and Klibanov, 1988). In a similar study, the cellulases from *Promicromonospora* sp. *VP111* exhibited significant tolerance to organic solvents, particularly non-polar solvents. CMCase showed high stability and even apparent stimulation in the presence of solvents like *n*-hexane and isooctane, while FPase and β -glucosidase retained 77–95% and 79–89% residual activities, respectively. However, cellulase activity was significantly reduced in polar organic solvents, likely due to enzyme destabilization caused by solvent interactions with the enzyme's hydration layer. Also, like the previous studies mentioned above, it suggests that non-polar solvents might enhance enzyme conformation, preventing denaturation, whereas polar solvents can wash away essential water molecules needed for enzymatic activity (Thomas *et al.*, 2016). It is important to note that the rumen microbiome operates in a strictly anaerobic, plant fiber-rich environment and is not naturally exposed to industrial solvents used in pretreatment, such as butanol or benzene; however, microbial fermentation generates short-chain alcohols. These environments are dominated by fermentative bacteria, archaea, protozoa, and fungi specialized for cellulose breakdown, not solvent tolerance (Liu *et al.*, 2021a, Silva *et al.*, 2024). Therefore, the solvent resilience observed in KC94 indicates an extraordinary capacity that may be valuable for industrial applications where such solvents are present. Reports on rumen cellulases rarely test solvent effects, making this study one of the first to evaluate such tolerance in *Proteus mirabilis* from rumen origin. Furthermore, KC94's high tolerance to ethanol (78.9% RA) is remarkable and indicates potential for bioethanol-integrated lignocellulose degradation systems, where solvent accumulation often inhibits enzyme function.

4.4.3.2 Surfactants and Oxidizing Agent

A two-way ANOVA was also conducted to assess the effects of surfactants (PEG, SDS, Triton X-100), an oxidizing agent (H_2O_2), and concentration on activity. Across the three strains, there were significant main effects of both factors and their interaction (all $p < 0.001$, $\eta^2 > 0.99$), confirming that the impact of concentration depended strongly on the surfactant and oxidizing type. Post hoc comparisons revealed consistent strain-specific trends. For instance, KC50 and KC70 showed similar trends, with high concentrations of SDS and Triton X-100 strongly inhibiting cellulase production. However, lower concentrations of PEG and oxidizing agent

H₂O₂ resulted in mild effects or slight stimulation in both strains. KC94 showed the strongest overall tolerance to surfactants, with mild inhibition by PEG and a slight increase in activity upon H₂O₂ exposure. Ionic surfactants (SDS, Triton X-100) disrupted enzyme hydrophobic interactions, leading to denaturation, whereas non-ionic surfactants (PEG, Tween) occasionally stabilized enzymes and enhanced activity, consistent with prior reports (Russmayer *et al.*, 2019). Studies have also indicated that surfactants can protect cellulases by reducing non-specific binding and enhancing enzyme stability during hydrolysis. In a study by Thomas and colleagues, Tween-80 slightly enhanced CMCase production, whereas Triton X-100, sodium lauryl sulfate (SLS), and sodium *N*-lauryl sarcosine completely inhibited cellulase activity (Thomas *et al.*, 2016). A similar study on *Bacillus subtilis* SU40, isolated from agricultural soil, also examined the effects of surfactants on enzyme activity and found relatively stable enzyme production across treatments. Tween 20 showed the highest activity, while phenylmethylsulfonyl fluoride (PMSF) showed the greatest inhibition at a lower concentration (0.5%). Unlike KC70, *B. subtilis* SU40 maintained high activity even in the presence of SDS and Triton X-100, indicating a more robust mechanism for enzyme stabilization (Asha and Sakthivel, 2014). The differences observed between KC70 and *B. subtilis* SU40 suggest variations in enzyme folding, membrane composition, or stress response mechanisms that influence surfactant tolerance. These findings reinforce the importance of strain-specific optimization when selecting microbial candidates for industrial enzyme production. This suggests that ionic surfactants disrupt the enzyme's structural integrity, likely by interfering with hydrophobic interactions that are crucial to maintaining enzyme stability. The positive effect of Tween 80, a non-ionic surfactant, is consistent with previous findings indicating that non-ionic surfactants can help prevent enzyme aggregation and enhance solubility (Thomas *et al.*, 2016). In the rumen environment, oxidative stress can be triggered by rapid microbial fermentation, dietary shifts, and metabolic overload, leading to reactive oxygen species (ROS) generation that challenges microbial survival (Celi, 2011, Celi *et al.*, 2014). Therefore, KC94's demonstrated tolerance to oxidative stress may offer a colonization advantage in this highly competitive ecosystem. Few rumen studies have directly linked cellulase activity to oxidative/surfactant tolerance. KC94's performance fills this gap and suggests a unique adaptation that could be exploited in industrial enzyme cocktails that require stability under pretreated biomass conditions.

4.4.3.3 Diversity of Possible Cellulase and Hemicellulase Gene Fragments in KC94

The successful amplification of GH39, GH45, and GH48 genes indicates that KC94 possesses a strong genetic basis for lignocellulose degradation. While the expected fragments were confirmed in agreement with published data (Sheng *et al.*, 2015), the presence of additional PCR products suggests that the KC94 strain may contain multiple isoforms or gene variants of β -xylosidase and cellobiohydrolase. This observation is consistent with the genetic diversity often reported in cellulolytic microbial systems (Hua *et al.*, 2018) and may indicate that KC94 possesses a broader enzymatic diversity for lignocellulose degradation than initially anticipated. One possible explanation for these additional products is the presence of paralogous genes, reflecting evolutionary adaptation of KC94 to efficiently hydrolyze diverse plant polysaccharides. Another explanation could be primer slippage due to primer degeneracy, as previously noted by Sheng *et al.* (2015). Nevertheless, the detection of multiple amplicons is encouraging, suggesting functional redundancy or specialization within KC94 and potentially enhancing its lignocellulolytic efficiency, a characteristic of the rumen environment. Future sequencing of these amplicons will clarify whether they represent novel gene variants or amplification artifacts, but the current findings already highlight KC94 as a promising source of diverse cellulolytic enzymes.

4.5 Conclusions

The cellulase enzymes produced by *Bacillus* spp. (KC50 and KC70) and KC94 exhibit robust activity, pH and temperature stability, and tolerance to mild oxidative stress, making them suitable candidates for industrial applications in lignocellulosic biomass processing. The molecular identification of KC94 as a cellulase producer adds to the diversity of microorganisms that can be utilized for lignocellulose degradation. This strain may complement traditional cellulolytic bacteria by providing enzymes that function under diverse environmental and process conditions. Incorporating a broader diversity of bacterial strains could improve pretreatment efficiency, enzyme stability, and overall bioconversion performance in biofuel or biochemical production systems.

The cellulases produced by KC94 have several features that could support integration into industrial bioenergy systems. Its enzyme activity at neutral pH and 35 °C, combined with tolerance to mild oxidative stress, makes KC94 a promising candidate for pretreatment of lignocellulosic biomass under moderate conditions. However, as KC94 exhibits sensitivity to surfactants such as SDS and Triton X-100, its application may require formulation strategies or co-culture systems to enhance enzyme stability and performance in industrial settings. The amplification of GH39, GH45, and GH48 genes confirms that KC94 harbors key cellulolytic genes and potentially multiple isoforms, reflecting genetic diversity that may enhance lignocellulose degradation. Such diversity is valuable for the industry, where enzyme robustness and redundancy can improve efficiency in biomass conversion. Moreover, these findings complement lignin-degrading enzyme systems, supporting the design of co-culture strategies that integrate cellulose and lignin degraders for more effective bioprocessing. Although further amplicon sequencing is still needed to validate the variants, KC94 already stands out as a promising candidate for biotechnological applications. Integrating KC94 cellulases with more robust *Bacillus* spp. could provide complementary enzymatic activities, potentially improving overall hydrolysis efficiency. Future work will focus on evaluating enzyme yield at scale, co-culture synergy, tolerance to industrial stressors, and pretreatment of various lignocellulosic biomass, as well as evaluating apparent K_m and V_{max} for the most promising isolates (KC50, KC70, KC94) under their identified optimal conditions, thereby determining the feasibility of deploying them in sustainable bioenergy production workflows.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/microorganisms13092170/s1>. Table S1 lists the media used for cellulase production. Figure S1 illustrates the qualitative screening assay, demonstrating cellulase activity on 0.5% CMC agar plates after flooding with Congo Red dye. Table S2 includes the nucleotide accession numbers submitted to GenBank. Table S3: Relative abundance (number of isolates) of bacteria identified in this study. Table S4 outlines the touchdown PCR cycling parameters for amplifying GH39, GH45, and GH48 gene fragments, as well as the degenerate primers used to amplify cellulase and hemicellulase gene fragments. Statistical analysis for all the statistics in the manuscript is presented separately for each factor and strain in the Excel sheets. References (Singh *et al.*, 2014; Wei *et al.*, 2011; Sheng *et al.*, 2015) are cited in the Supplementary Materials.

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original draft, K.J.R.; Visualization, K.J.R.; Supervision, T.S.M.; Project administration, T.S.M. All authors have read and agreed to the published version of the manuscript.

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4.6 References

- AJALA, E., IGHALO, J., AJALA, M., ADENIYI, A. & AYANSHOLA, A. 2021. Sugarcane bagasse: a biomass sufficiently applied for improving global energy, environment and economic sustainability. *Bioresources and Bioprocessing*, 8, 1-25.
- ALAMRI, S. A., MOSTAFA, Y. S. & ALRUMMAN, S. A. 2016. Isolation, Screening and Optimization of *Geobacillus stearothermophilus* Cellulase Production using Date Palm Cellulosic Wastes. *Journal of Pure & Applied Microbiology*, 10.
- ARAKI, Y., MUKAISHO, K., SUGIHARA, H., FUJIYAMA, Y. & HATTORI, T. 2010. *Proteus mirabilis* sp. intestinal microflora grow in a dextran sulfate sodium-rich environment. *Int J Mol Med*, 25, 203-8.
- ASHA, B. M. & SAKTHIVEL, N. 2014. Production, purification and characterization of a new cellulase from *Bacillus subtilis* that exhibit halophilic, alkalophilic and solvent-tolerant properties. *Annals of Microbiology*, 64, 1839-1848.
- BERNFELD, P. 1955. [17] Amylases, α and β . *Methods in Enzymology*. Academic Press.
- BHATT, B., BHATT, K., LAL, S. & BHATT, V. 2024. Production of a novel cellulase by *Bacillus amyloliquefaciens* OKB3 isolated from soil: Purification and characterization. *International Journal of Biological Macromolecules*, 282, 137454.
- CASTRO-COSTA, A., SALAMA, A. A., MOLL, X., AGUILÓ, J. & CAJA, G. 2015. Using wireless rumen sensors for evaluating the effects of diet and ambient temperature in nonlactating dairy goats. *J Dairy Sci*, 98, 4646-58.
- CHAKKOUR, M., HAMMOUD, Z., FARHAT, S., EL ROZ, A., EZZEDDINE, Z. & GHSSEIN, G. 2024. Overview of *Proteus mirabilis* pathogenicity and virulence. Insights into the role of metals. *Frontiers in Microbiology*, 15, 1383618.
- CHENG, B., WU, S., LIU, S., RODRIGUEZ-ALIAGA, P., YU, J. & CUI, S. 2015. Protein denaturation at a single-molecule level: the effect of nonpolar environments and its implications on the unfolding mechanism by proteases. *Nanoscale*, 7, 2970-2977.
- DEMISSIE, M. S., LEGESSE, N. H. & TESEMA, A. A. 2024. Isolation and characterization of cellulase producing bacteria from forest, cow dung, Dashen brewery and agro-industrial waste. *PLOS ONE*, 19, e0301607.
- EL-KHAMISI, E. F., SOLIMAN, E. A., EL-SAYED, G. M., NOUR, S. A., ABDEL-MONEM, M. O. & HASSAN, M. G. 2024. Optimization, gene cloning, expression, and molecular docking insights for

enhanced cellulase enzyme production by *Bacillus amyloliquefaciens* strain elh1. *Microbial Cell Factories*, 23, 191.

GAUR, R. & TIWARI, S. 2015. Isolation, production, purification and characterization of an organic-solvent-thermostable alkalophilic cellulase from *Bacillus vallismortis* RG-07. *BMC biotechnology*, 15, 1-12.

GEbbie, L., DAM, T. T., AINSCOUGH, R., PALFREYMAN, R., CAO, L., HARRISON, M., O'HARA, I. & SPEIGHT, R. 2020. A snapshot of microbial diversity and function in an undisturbed sugarcane bagasse pile. *BMC biotechnology*, 20, 1-16.

HALL, T. A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic acids symposium series, 1999. [London]: Information Retrieval Ltd., c1979-c2000., 95-98.

HSIAO, N.-W., CHEN, Y., KUAN, Y.-C., LEE, Y.-C., LEE, S.-K., CHAN, H.-H. & KAO, C.-H. 2014. Purification and characterization of an aspartic protease from the *Rhizopus oryzae* protease extract, Peptidase R. *Electronic Journal of Biotechnology*, 17, 89-94.

HUSSAIN, A. A., ABDEL-SALAM, M. S., ABO-GHALIA, H. H., HEGAZY, W. K. & HAFEZ, S. S. 2017. Optimization and molecular identification of novel cellulose degrading bacteria isolated from Egyptian environment. *Journal of Genetic Engineering and Biotechnology*, 15, 77-85.

HYDER, I., RAVI KANTH REDDY, P., RAJU, J., MANJARI, P., SRINIVASA PRASAD, C., ASWANI KUMAR, K. & SEJIAN, V. 2017. Alteration in rumen functions and diet digestibility during heat stress in sheep. *Sheep production adapting to climate change*. Springer.

IRFAN, M., TAYYAB, A., HASAN, F., KHAN, S., BADSHAH, M. & SHAH, A. A. 2017a. Production and characterization of organic solvent-tolerant cellulase from *Bacillus amyloliquefaciens* AK9 isolated from hot spring. *Applied Biochemistry and Biotechnology*, 182, 1390-1402.

IRFAN, M., TAYYAB, A., HASAN, F., KHAN, S., BADSHAH, M. & SHAH, A. A. 2017b. Production and characterization of organic solvent-tolerant cellulase from *Bacillus amyloliquefaciens* AK9 isolated from hot spring. *Applied Biochemistry and Biotechnology*, 182, 1390-1402.

ISLAM, M., SARKAR, P. K., MOHIUDDIN, A. & SUZAUDDULA, M. 2019. Optimization of fermentation condition for cellulase enzyme production from *Bacillus* sp. *Malaysian Journal of Halal Research*, 2, 19-24.

KIM, Y.-K., LEE, S.-C., CHO, Y.-Y., OH, H.-J. & KO, Y. H. 2012. Isolation of cellulolytic *Bacillus subtilis* strains from agricultural environments. *International Scholarly Research Notices*, 2012, 650563.

- KONDE, K. S., NAGARAJAN, S., KUMAR, V., PATIL, S. V. & RANADE, V. V. 2021. Sugarcane bagasse based biorefineries in India: potential and challenges. *Sustainable Energy & Fuels*, 5, 52-78.
- KUMAR, A., KUMAR, V. & SINGH, B. 2021. Cellulosic and hemicellulosic fractions of sugarcane bagasse: Potential, challenges and future perspective. *International Journal of Biological Macromolecules*, 169, 564-582.
- KUMARI, S., SHARMA, U., KRISHNA, R., SINHA, K. & KUMAR, S. 2019. Screening and molecular characterization of cellulase producing actinobacteria from Litchi Orchard. *Current Chemical Biology*, 13, 90-101.
- LIANG, B., LIANG, Y., CHEN, J., ZHU, Y., FU, F., GAN, T., HUANG, Z., HU, H. & ZHANG, Y. 2024. Stepwise processing strategy for efficient and comprehensive utilization of sugarcane bagasse via enzymatic hydrolysis and catalytic conversion to produce 5-hydroxymethylfurfural. *Industrial Crops and Products*, 214, 118592.
- LIAO, Y., WU, S., ZHOU, G., MEI, S., YANG, Z., LI, S., JIN, Z., DENG, Y., WEN, M. & YANG, Y. 2024. Cellulolytic *Bacillus cereus* produces a variety of short-chain fatty acids and has potential as a probiotic. *Microbiology Spectrum*, 12, e03267-23.
- MAHAPATRA, S., VICKRAM, A., SRIDHARAN, T., PARAMESWARI, R. & PATHY, M. R. 2016. Screening, production, optimization and characterization of β -glucosidase using microbes from shellfish waste. *3 Biotech*, 6, 1-10.
- MALIK, W. A., KHAN, H. M. & JAVED, S. 2021. Bioprocess optimization for enhanced production of bacterial cellulase and hydrolysis of sugarcane bagasse. *BioEnergy Research*, 1-14.
- MOBLEY, H. L. T. 2019. *Proteus mirabilis* Overview. In: PEARSON, M. M. (ed.) *Proteus mirabilis: Methods and Protocols*. New York, NY: Springer New York.
- MUSHTAQ, Q., ISHTIAQ, U., JOLY, N., QAZI, J. I. & MARTIN, P. 2024. Amylase and cellulase production from newly isolated *Bacillus subtilis* using acid treated potato peel waste. *Microorganisms*, 12, 1106.
- NEGRÃO, D. R., GRANDIS, A., BUCKERIDGE, M. S., ROCHA, G. J., LEAL, M. R. L. & DRIEMEIER, C. 2021. Inorganics in sugarcane bagasse and straw and their impacts for bioenergy and biorefining: A review. *Renewable and Sustainable Energy Reviews*, 148, 111268.
- NKUNA, R. & MATAMBO, T. S. 2024. Determining the metabolic processes of metal-tolerant fungi isolated from mine tailings for bioleaching. *Minerals*, 14, 235.

- OZTAT, K., YAVUZ, A. A. & IŞÇEN, C. F. 2024. Optimization studies on laccase activity of *Proteus mirabilis* isolated from treatment sludge of textile industry factories. *Brazilian Journal of Microbiology*, 55, 1231-1241.
- PAN, S., ZABED, H. M., WEI, Y. & QI, X. 2022. Technoeconomic and environmental perspectives of biofuel production from sugarcane bagasse: Current status, challenges and future outlook. *Industrial Crops and Products*, 188, 115684.
- PANĂ, A.-M., ORDODI, V., GHERMAN, V., SFÎRLOAGĂ, P. & DUMITREL, G.-A. 2023. Study on the Biodegradation Process of D-Mannose Glycopolymers in Liquid Media and Soil. *Polymers*, 15, 3194.
- PATAKOVA, P., KOLEK, J., SEDLAR, K., KOSCOVA, P., BRANSKA, B., KUPKOVA, K., PAULOVA, L. & PROVAZNIK, I. 2018. Comparative analysis of high butanol tolerance and production in clostridia. *Biotechnology advances*, 36, 721-738.
- PATEL, D. D., PATEL, A. K., PARMAR, N. R., SHAH, T. M., PATEL, J. B., PANDYA, P. R. & JOSHI, C. G. 2014a. Microbial and Carbohydrate Active Enzyme profile of buffalo rumen metagenome and their alteration in response to variation in the diet. *Gene* 545, 88-94.
- PATEL, V., PATEL, A. K., PARMAR, N. R., PATEL, A. B., REDDY, B. & JOSHI, C. G. 2014b. Characterization of the rumen microbiome of Indian Kankrej cattle (*Bos indicus*) adapted to different forage diet. *Applied microbiology and biotechnology*, 98, 9749-9761.
- POTPROMMANEE, L., WANG, X.-Q., HAN, Y.-J., NYOBE, D., PENG, Y.-P., HUANG, Q., LIU, J.-Y., LIAO, Y.-L. & CHANG, K.-L. 2017. Characterization of a thermophilic cellulase from *Geobacillus* sp. HTA426, an efficient cellulase-producer on alkali pretreated of lignocellulosic biomass. *PLoS one*, 12, e0175004.
- RABAPANE, K. J., IJOMA, G. N. & MATAMBO, T. S. 2022. Insufficiency in functional genomics studies, data, and applications: A case study of bio-prospecting research in ruminant microbiome. *Frontiers in Genetics*, 13.
- RABAPANE, K. J. & MATAMBO, T. S. 2024. Profiling the dynamic adaptations of CAZyme-Producing microorganisms in the gastrointestinal tract of South African goats. *Heliyon*, 10.
- RAHBAN, M., ZOLGHADRI, S., SALEHI, N., AHMAD, F., HAERTLÉ, T., REZAEI-GHALEH, N., SAWYER, L. & SABOURY, A. A. 2022. Thermal stability enhancement: Fundamental concepts of protein engineering strategies to manipulate the flexible structure. *International journal of biological macromolecules*, 214, 642-654.

- RUSSMAYER, H., MARX, H. & SAUER, M. 2019. Microbial 2-butanol production with *Lactobacillus diolivorans*. *Biotechnology for biofuels*, 12, 1-11.
- SAINI, A., AGGARWAL, N. K. & YADAV, A. 2017. Isolation and screening of cellulose hydrolyzing bacteria from different ecological niches. *Bioengineering and Bioscience*, 5, 7-13.
- SARKER, T. C., AZAM, S. M. G. G. & BONANOMI, G. 2017. Recent advances in sugarcane industry solid by-products valorization. *Waste and Biomass Valorization*, 8, 241-266.
- SARSAN, S. & MERUGU, R. 2019. Role of bioprocess parameters to improve cellulase production: Part II. *New and future developments in microbial biotechnology and bioengineering*. Elsevier.
- SHAREEF, I., GOPINATH, S., SATHEESH, M. & CHRISTOPHER, S. X. 2015. Isolation and identification of cellulose degrading microbes.
- SINGH, S., JAISWAL, D. K., SIVAKUMAR, N. & VERMA, J. P. 2019. Developing efficient thermophilic cellulose degrading consortium for glucose production from different agro-residues. *Frontiers in Energy Research*, 7, 61.
- SKOVGAARD, P. A. & JØRGENSEN, H. 2013. Influence of high temperature and ethanol on thermostable lignocellulolytic enzymes. *Journal of Industrial Microbiology and Biotechnology*, 40, 447-456.
- THOMAS, L., RAM, H., KUMAR, A. & SINGH, V. P. 2016. Production, Optimization, and Characterization of Organic Solvent Tolerant Cellulases from a Lignocellulosic Waste-Degrading Actinobacterium, *Promicromonospora* sp. VP111. *Applied Biochemistry and Biotechnology*, 179, 863-879.
- USMAN, A., MOHAMMED, S. & MAMO, J. 2021. Production, Optimization, and Characterization of an Acid Protease from a Filamentous Fungus by Solid-State Fermentation. *International journal of microbiology*, 2021, 6685963.
- WAHEEB, M. S., ELKHATIB, W. F., YASSIEN, M. A. & HASSOUNA, N. A. 2024. Optimized production and characterization of a thermostable cellulase from *Streptomyces thermodiastaticus* strain. *AMB Express*, 14, 129.
- WASFI, R., HAMED, S. M., AMER, M. A. & FAHMY, L. I. 2020. *Proteus mirabilis* biofilm: development and therapeutic strategies. *Frontiers in cellular and infection microbiology*, 10, 414.
- WEISBURG, W. G., BARNS, S. M., PELLETIER, D. A. & LANE, D. J. 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of bacteriology*, 173, 697-703.
- WILSON, K. H., BLITCHINGTON, R. B. & GREENE, R. C. 1990. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. *Journal of clinical microbiology*, 28, 1942-1946.

- WU, H., CHEN, Q., ZHANG, W. & MU, W. 2023. Overview of strategies for developing high thermostability industrial enzymes: Discovery, mechanism, modification and challenges. *Critical Reviews in Food Science and Nutrition*, 63, 2057-2073.
- XU, Z., CEN, Y.-K., ZOU, S.-P., XUE, Y.-P. & ZHENG, Y.-G. 2020. Recent advances in the improvement of enzyme thermostability by structure modification. *Critical reviews in biotechnology*, 40, 83-98.
- YIN, L.-J., LIN, H.-H. & XIAO, Z.-R. 2010. Purification and characterization of a cellulase from *Bacillus subtilis* YJ1. *Journal of Marine Science and Technology*, 18, 19.
- ZAKS, A. & KLIBANOV, A. M. 1988. Enzymatic catalysis in nonaqueous solvents. *Journal of Biological Chemistry*, 263, 3194-3201.
- ZHANG, Q., JIAN, L., YAO, D., RAO, B., XIA, Y., HU, K., LI, S., SHEN, Y., CAO, M., QIN, A., ZHAO, J. & CAO, Y. 2023. The structural basis of the pH-homeostasis mediated by the Cl⁻/HCO₃⁻ exchanger, AE2. *Nature Communications*, 14, 1812.

**CHAPTER 5: PRE-TREATMENT STUDIES BY *PROTEUS MIRABILIS_KC94*:
BIOGAS PRODUCTION**

Objective:

In the previous chapter, cultivable bacterial isolates were obtained and assessed for their cellulolytic degradation capabilities. Evaluations included growth rates, tolerance to oxidative stress, responses to solvents and surfactants, and glucose production. The purpose of this chapter was to evaluate the effect of a short-term biological pretreatment with the cellulolytic bacterium KC94 on SCB to enhance its bioconversion potential. Specifically, it aimed to compare untreated, sterilized, and KC94-pretreated bagasse with respect to hydrolysis efficiency, glucose release, and elemental composition. The chapter further sought to assess morphological changes and their implications for substrate accessibility, and to determine the BMP of pretreated SCB via AD assays. By integrating biochemical, structural, and bioenergetic analyses, this study demonstrated how a 5-day microbial pretreatment could improve cellulose degradation, nutrient stabilization, and methane yield. Ultimately, the findings contribute to the development of eco-friendly strategies for utilizing sugarcane bagasse, thereby supporting renewable energy generation and advancing circular bioeconomy practices.

Rabapane, K.J., Rashama, C. and Matambo, T.S., 2025. Assessing Sugarcane Bagasse Biomethanation After a Pretreatment with Proteus mirabilis KC94. Bioresources and Bioproducts, 2(1), p.1.

Abstract

SCB is a lignocellulosic byproduct with low biodegradability, limiting its potential for biological processes such as biogas production. The objective of this study was to evaluate whether a short-term biological pretreatment with the cellulolytic bacterium KC94 could enhance SCB hydrolysis, improve nutrient balance, and increase BMP. Three treatments were compared: untreated bagasse (UB), sterilized bagasse (SB), and KC94-pretreated bagasse (PB). Glucose release was highest in PB (61.83 ± 0.8 mg/mL), indicating enhanced cellulose degradation in PB relative to UB (53.19 ± 0.9 mg/mL) and SB (44.00 ± 0.5 mg/mL). Elemental analysis revealed a more balanced nutrient profile in PB, characterized by optimal carbon and nitrogen levels and reduced sulfur content, indicating microbial assimilation and potential biological desulfurization. Scanning electron microscopy revealed pronounced structural disruption, increased porosity, and fiber delamination in PB, confirming the efficacy of KC94-mediated lignocellulosic pretreatment. BMP assays conducted over a 31-day incubation period revealed that PB produced the highest cumulative methane yield (99 ± 0.7 mL CH₄/g VS), representing 19% and 25% increases over UB and SB, respectively. PB biomethanation was

also faster compared to the other two substrates. These findings demonstrate the novelty of a 5-day bacterial pretreatment strategy, which significantly improves lignocellulosic hydrolysis and methane yield. Specifically, KC94 pretreatment increased glucose release by 16–40% and cumulative methane yield by 19–25% compared to untreated and sterilized controls. This cost-effective, environmentally friendly approach highlights KC94's potential to valorize SCB, advancing sustainable energy recovery and circular bioeconomy practices.

Keywords: biomethane; biogas; pretreatment; desulfurization; cellulolytic bacterium; biodegradation

5.1 Introduction

SCB, a lignocellulosic byproduct of the sugar industry, can pose environmental challenges if not managed sustainably. Alternatively, SCB can serve as a low-cost feedstock for biogas production via AD due to its high abundance and organic content (Konde *et al.*, 2021, Agarwal

et al., 2022). However, its high lignin content (>19%) is higher than that of other common biomasses used for biogas production, such as water hyacinth (with a lignin content of 0.6%) (Teofilo *et al.*, 2025, Simbayi *et al.*, 2023). Additionally, the crystalline cellulose structure of SCB poses significant barriers to effective microbial hydrolysis, as demonstrated by Xu *et al.*, 2019. The aforementioned factors result in low reducing sugar assays from SCB hydrolysis, which subsequently lower methane yields and extend retention times when SCB is subjected to AD (Kumar *et al.*, 2021, do Espirito Santo *et al.*, 2022). To overcome SCB's limitations in biomethanation, biological pretreatment can be employed to enhance cellulose breakdown. Biological pretreatment using enzymes and/or microorganisms has emerged as a sustainable alternative to chemical and thermal methods due to the process's environmental friendliness (Woźniak *et al.*, 2025). Several cellulose-degrading microorganisms exist that produce a variety of cellulolytic enzymes, including cellulases and hemicellulases. These enzymes synergistically act on cellulose, releasing monosaccharides and disaccharides that are required for subsequent steps in biogas production, which are acidogenesis, acetogenesis, and methanogenesis (Weimer, 2022, Harun *et al.*, 2019, Sivamani *et al.*, 2021). Thus, understanding the potential, mechanisms, and efficiency of different cellulose-degrading microorganisms, as well as those capable of degrading lignin, is essential for developing strategies to optimize biogas production from lignocellulosic biomass. This study aims to evaluate the hydrolytic capacity of KC94 to enhance biomethanation of SCB.

It is crucial to expand the sources of these beneficial microorganisms through bioprospecting and testing readily accessible strains, thereby facilitating the rapid commercialization of scientific discoveries. *Bacillus pumilus* SL8, isolated from oriental leafworm, *Spodoptera litura*, has demonstrated cellulolytic breakdown capability on different lignocellulosic materials, although its performance was lower in SCB at 52.6% compared to other biomass, such as corn cob powder, which was 59.2%, and sawdust, which was 54.2% (Dar *et al.*, 2022). Another potentially useful microorganism applicable in lignocellulosic hydrolysis is *Bacillus tequilensis* G9, previously isolated from *Achatina fulica* (African Giant snails), as cellulase extracted from it has been successfully tested for lignocellulosic biomass hydrolysis (Dar *et al.*, 2019). A continued search for other microbial strains and cellulases with improved SCB hydrolytic capacity, sourced from sustainable hosts, is a justified research effort if SCB is to be used sustainably as an AD feedstock. The present study investigates the use of KC94, a cellulolytic bacterium isolated from the goat gastrointestinal tract, as a biological pretreatment for SCB. Goats are widely distributed in South Africa to reliably supply this bacterial strain (Mohlatlole *et al.*, 2015). While *P. mirabilis* is traditionally known for its metabolic versatility

and sulfur-transforming capabilities (Drzewiecka, 2016), its application in lignocellulosic biomass pretreatment remains underexplored. Emerging research has demonstrated its enzymatic versatility, particularly in degrading complex polysaccharides. Recent studies have shown that *P. mirabilis* can actively participate in cellulose and xylan degradation in the intestine of the Indian flying fox and the millipede gut, suggesting the presence of robust cellulolytic systems in this strain (Anand and Sripathi, 2004, Alagesan, 2003). These observations lead us to hypothesize that the *KC94* strain, previously adapted to the goat gastrointestinal tract, may possess improved lignocellulosic hydrolytic capacity, as ruminants primarily graze on lignocellulosic material (Aquino *et al.*, 2019). Our previous metagenomic study of goat rumen microbiota identified *P. mirabilis* as a dominant CAZyme-producing species, suggesting its potential role in lignocellulose degradation (Rabapane and Matambo, 2024). Its demonstrated ability to degrade cellulose further strengthens its candidacy as a biological pretreatment agent for lignocellulosic substrates, such as SCB.

This study represents one of the first to evaluate *P. mirabilis* as a biological pretreatment agent for SCB, aiming to enhance methane generation during BMP testing. By integrating microbial pretreatment, glucose release assays, and automated BMP testing, this work presents a comprehensive approach to valorising SCB and enhancing biogas production through improved cellulose accessibility and potential sulfur mitigation.

5.2 Materials and Methods

5.2.1 Ethical Compliance, Substrate Preparation, and Analytical Methods

Due to the nature of the study, which involved the isolation of microorganisms from goats' GIT, ethical clearance was obtained from the College of Agricultural and Environmental Sciences Animal Research Ethics Committee (CAESAREC) of the University of South Africa (Ref: 2021/CAES_AREC/097). All laboratory work with *KC94* was conducted under Biosafety Level 2 (BSL-2) conditions, following strict protocols for sterilization, containment, and waste disposal. The *KC94* strain was previously isolated from goat GIT (Rabapane and Matambo, 2024) and screened for cellulase activity on carboxymethyl cellulose (CMC) agar (Bacteriological agar (5 g/L), CMC (10 g/L), supplemented with 0.1% Congo red (Mokale Kognou *et al.*, 2022). Hydrolysis was confirmed by halo formation after flooding with 1 M NaCl for 30 min (Roy *et al.*, 2024). Enzyme activities were determined by the 3,5-dinitrosalicylic acid (DNSA) method (Miller, 1959) following previously optimized protocols

(Rabapane and Matambo, 2024, Dar *et al.*, 2018). For biomass preparation, KC94 was cultured in bulk (1×8 L flask) overnight in Luria–Bertani broth, harvested by centrifugation ($4400 \times g$ for 15 min), washed, and resuspended in McDougall’s buffer (comprising the following components (in g/L): $(\text{NH}_4)_2\text{SO}_4$ (1.3), K_2HPO_4 (2.04), NaHCO_3 (0.4), NaCl (0.08), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.0192), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.0011), CaCl_2 (0.008), and KH_2PO_4 (0.04) (McDougall, 1948). The pH was adjusted to 7.0 using 1 M HCl. McDougall’s buffer was used to provide balanced nutrients, buffering capacity, and trace minerals to sustain enzymatic activity and mimic rumen fluid conditions during pretreatment (McDougall, 1948, Valdez-Vazquez *et al.*, 2020). All chemicals used in the experiments were sourced from Sigma-Aldrich (Pty) Ltd (Kempton Park, South Africa) and were of analytical grade.

SCB samples were collected from a plantation in KwaZulu-Natal, South Africa. The fibrous residues were air-dried for 40 days (Figure 5.1), ground to a particle size of 0.6–1 mm (Armaha *et al.*, 2019) using the DNATM Health Super blender (Midrand, South Africa), and used as a substrate. Anaerobic digestate sludge from a commercial biogas plant (cow dung with brewers’ spent grain co-substrate) served as inoculum for BMP assays. The inoculum was transported on the same day of sampling in 5 L sealed high-density polythene containers to the University of South Africa Biotechnology laboratories, where it was screened the following day to remove solids exceeding a 3 mm mesh size. Immediately after screening, the screened portion was pre-incubated for 4 days at 37 °C to exhaust residual volatile solids, as recommended by Holliger *et al.* (Holliger *et al.*, 2016). Therefore, the inoculum was used within five days of sampling from the digesters. These digesters operate in a semi-continuous batch mode with intermittent feeding at a rate of 3 g COD/L per day, average organic loading rates, and a hydraulic retention time (HRT) of 25 days. A mesophilic temperature of 38 °C is maintained in these CSTR digesters.

Feedstocks and digestates were characterized for total solids (TSs), volatile solids (VSs), and ash using standard biofuel protocols I.S. EN 14774-3:2009 (EN14774-3:2009, 2015), I.S. EN 15148:2009 (15148:2009, 2015), and I.S. EN 14775:2009 (14775:2009, 2015). Elemental composition (C, H, N, S) was determined using a CHNS analyzer (Elementar Vario EL Cube, Separations, Johannesburg, South Africa). Samples were dried, powdered, and combusted under a flow of helium and oxygen. Morphological changes in SCB before and after pretreatment were examined using scanning electron microscopy (SEM; Crossbeam 340, Carl Zeiss Carl Zeiss Microscopy Ltd., Oberkochen, Germany) after gold sputter coating (Quorum Q150R ES, ALS, Laughton, East Sussex), and observed using an acceleration voltage range of

1–3 kV and a working distance of approximately 2–4 mm. Multiple SEM images were obtained from different sample areas to ensure reproducibility of the results.



Figure 5.1: Air-dried SCB collected after juice extraction.

5.2.2 Experimental Activities

5.2.3 Experimental Design

The study included three distinct bagasse treatments designed to isolate the effects of sterilization and biological pretreatment, as well as a blank test involving only the inoculum and no SCB. Untreated bagasse (UB) was air-dried for approximately forty days and used directly without sterilization or microbial inoculation, serving as the baseline control to assess the native lignocellulosic structure (Valdez-Vazquez *et al.*, 2020). Sterilized bagasse (SB) was prepared in the same manner but subsequently ground to a particle size of 0.6–1 mm, autoclaved at 121 °C for 15 min to eliminate autochthonous microbial populations. This group enabled the evaluation of the impact of both mechanical and sterilization processes on substrate characteristics and biomethane production. The third treatment, KC94-pretreated bagasse (PB), was processed identically to SB but inoculated with KC94 resuspended in McDougall's buffer. Pretreatment was carried out for five days at 37 °C and pH 7.0, providing balanced nutrients and buffering capacity to sustain enzymatic activity. This group served as the main experimental treatment, designed to test whether KC94 could accelerate hydrolysis, improve nutrient balance, and shorten the lag phase before biomethane production compared with the other two groups. All samples were incubated aerobically at 35 °C with agitation at 105 rpm for 5 days in McDougall's buffer. All treatments were evaluated for BMP using the Automatic Methane Potential Testing System (AMPTS II; Bioprocess Control Instruments AB, Lund,

Sweden). For clarity, SB after incubation is not a distinct treatment but rather SB subjected to the incubation process, and this incubated form was subsequently used in the AD assays. After 5 days of incubation, glucose levels in each sample were measured, and morphological changes were assessed by scanning electron microscopy (SEM). The solid portion of digestate in each treatment was tested for residual TS and VS content.

5.2.4 Pretreatment Details

For biological pretreatment of bagasse, only the PB group was inoculated with the KC94 cellulolytic strain. KC94 was cultivated overnight (16 h) in bulk (1 × 8 L flask) using Luria–Bertani broth to an optical density at 600 nm (OD₆₀₀) of approximately 0.6, corresponding to the late exponential phase, to ensure metabolic activity at the time of inoculation. Then, it was centrifuged, washed, and resuspended in McDougal buffer as previously described (Section 5.2). The objective was to enhance the digestibility of SCB prior to AD. Pretreatment was conducted in triplicate using 250 mL Erlenmeyer flasks with a working volume of 66.6 mL of McDougall’s buffer. Each flask contained 2.3 g of sterilized SCB (dry weight) and was inoculated with the KC94 strain at a substrate-to-inoculum (S/I) ratio of 3 g VS/g VS as described by Baba *et al.* (2017). All other treatment groups (UB and SB) were incubated in McDougall’s buffer in triplicate in 250 mL Erlenmeyer flasks with a working volume of 66.6 mL. Flasks were incubated at 35 °C in a shaking incubator (105 rpm) until the optimum incubation period of 5 days was reached. After incubation, a 3 mL aliquot was taken from each of the three replicate groups for glucose analysis. The aliquots were then centrifuged at 4000 rpm for 30 min to obtain the cell-free extract (CFE). Glucose release was quantified from the CFE prior to AD using the DNS method (Bernfeld, 1955, Miller, 1959). This was performed before and after the 5-day pretreatment to establish baseline hydrolysis levels and evaluate whether *KC94* pretreatment can shorten the lag phase before biomethane production.

All treatments were tested for their biomethane production potential (BMP) using the Automatic Methane Potential Testing System (AMPTS II; Bioprocess Control Instruments AB, Lund, Sweden). BMP was evaluated using the Automatic Methane Potential Test System (AMPTS), a laboratory-scale analytical platform that enables real-time, high-resolution measurement of methane production under controlled anaerobic conditions. The solid portion of digestate from each treatment, after completion of the BMP test, was characterized for

residual TS and VS content. Following the manufacturer's recommendation and based on the system calibrations, bottles with a working volume of 400 mL were loaded with a mixture of 2.8 g-VS of inoculum, 4 g-VS of each hydrolysate, and topped to the mark with McDougall's buffer. This mixture's initial pH was adjusted to 7.00 (Angelidaki and Sanders, 2004). After purging with nitrogen for 30 s, the mixtures were incubated at 37 °C and agitated automatically on the AMPTS II until the daily biogas production reached less than 1% of the cumulative biogas collected over the entire incubation period. Assays for each hydrolysate were performed in triplicate, and average values were used in the BMP calculation for each sample according to the AMPTS II equipment manufacturer's guidelines (Figure 5.2).

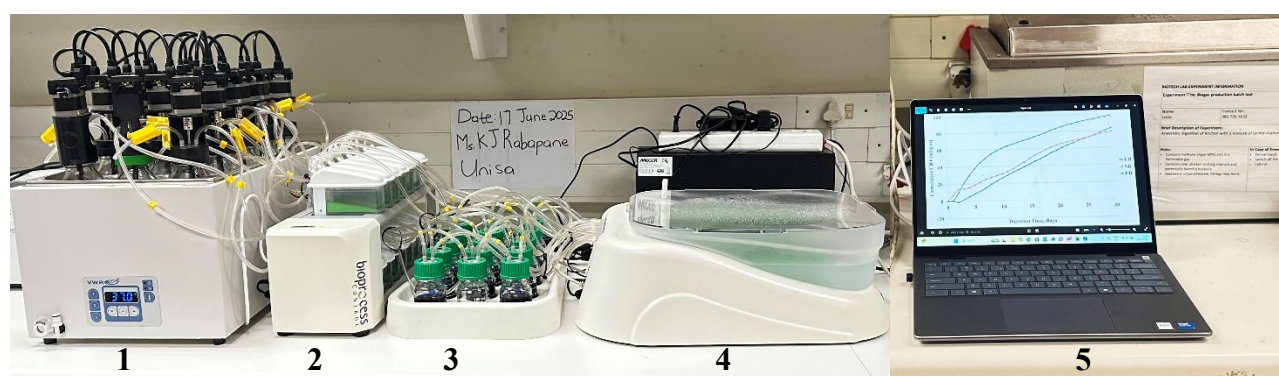


Figure 5.2: Experimental setup of the AMPTS II. The numbers identify each component within the setup. 1: Incubation unit, 2: Gas endeavor, 3: CO₂-absorption unit, 4: CH₄ volume measuring device, 5: Data Acquisition Software.

5.2.5 Statistical Analysis

The experiments were conducted in triplicate ($n = 3$) to accurately capture variability within the dataset. Statistical analyses were performed in IBM SPSS Statistics (version 31.0.1.0). One-way analysis of variance (ANOVA) was used to assess differences among the three treatments (UB, SB, PB) for BMP daily values, as well as for glucose concentration and enzyme activity at each time point, with treatment as the fixed factor. Pearson's correlation examined the relationship between treatment duration and BMP daily values. Where ANOVA indicated significant effects, Tukey's HSD test identified pairwise differences and generated homogeneous subsets used for graph annotations. Statistical significance was set at $p < 0.05$, and results are presented as mean $\pm$ standard deviation or mean difference with 95% confidence intervals.

5.3 Results and Discussion

5.3.1 Effect of Pretreatment on Glucose Yield from SCB

The glucose content analysis of the hydrolysates before and after treatment highlights the impact of each condition on biomass saccharification (Figure 5.3). In the UB group, glucose concentration increased from 19.13 ± 0.6 mg/mL to 53.19 ± 0.9 mg/mL post-hydrolysis. Authors speculate that this moderate increase may be due to spontaneous hydrolysis resulting from native enzymatic activity or pre-existing microbial consortia within the unsterilized biomass, as also postulated by Gebbie *et al.* (2020) and Valdez-Vazquez *et al.* (2020). This moderate increase in glucose in the UB group is consistent with comparative pretreatment studies, which report that native enzymatic activity and resident microbial consortia in unsterilized lignocellulosic biomass can produce measurable spontaneous hydrolysis, particularly where hydrothermal or mild biological pretreatments expose carbohydrates to enzymatic degradation (Valdez-Vazquez *et al.*, 2020). In contrast, the SB group began with a substantially lower glucose concentration (3.99 ± 0.1 mg/mL), confirming the sterilization's effectiveness in eliminating native microbes and preventing premature saccharification. Another possible contributor to the initially low glucose level in SB before hydrolysis could be the formation of inhibitors, such as formic acid, under the harsh conditions of autoclaving, as observed by Ladeira-Ázar *et al.* (2019).

After incubation, the glucose concentration rose to 44.00 ± 0.05 mg/mL (10-fold increase), indicating that mechanical and thermal pretreatment at 35 °C can enhance hydrolysis efficiency by disrupting the lignocellulosic structure and improving enzyme accessibility, even in the absence of microbial contribution. Milling SCB prior to enzymatic evaluations was reported to produce similar effects in a separate study, where glucose yields exceeded 77% after these interventions (Da Silva *et al.*, 2010). Meanwhile, the PB group showed the most substantial glucose release, with levels increasing from 3.99 ± 0.2 mg/mL to 61.83 ± 0.8 mg/mL. One-way ANOVA showed significant treatment effects across all measured variables (Figure 3). For glucose concentration before incubation, UB differed significantly from both SB and PB ($p < 0.001$), whereas SB and PB did not differ, forming two homogeneous subsets (UB = a; SB = b; PB = b). Meanwhile, enzymatic activity before incubation followed the same pattern, with UB significantly lower than both SB and PB ($p < 0.001$), and no difference between SB and PB (UB = a; SB = b; PB = b). In contrast, for glucose concentration after incubation, all

treatments differed significantly from one another ($p < 0.001$), producing three distinct subsets (UB = aa; SB = ab; PB = bb). Additionally, enzymatic activity after incubation mirrored this trend, with UB, SB, and PB each forming distinct groups (UB = aa, SB = ab, PB = bb). Accordingly, these findings indicate that although SB and PB begin at comparable baseline levels, PB produces the greatest post-treatment increases in both glucose concentration and enzymatic activity.

This finding illustrates the pronounced biological breakdown effect of the KC94 strain on lignocellulose degradation. In our previous goat rumen bioprospecting study, *KC94* was identified as a potent cellulolytic candidate, and genomic analysis confirmed the presence of key glycosyl hydrolase genes, including β -1,4-endoglucanase (GH45), cellobiohydrolase (GH48), and β -xylosidase (GH39) (Rabapane and Matambo, 2024). These genes encode enzymes that collectively target the depolymerization of lignocellulose. GH45 initiates hydrolysis by cleaving internal glycosidic bonds within amorphous cellulose regions (Saharan *et al.*, 2025, Dar *et al.*, 2018), GH48 progressively releases cellobiose units from chain termini (You *et al.*, 2023), and GH39 contributes to hemicellulose degradation by converting xylo-oligosaccharides into xylose (Teramoto *et al.*, 2021). In the present study, this genomic potential was functionally validated, as KC94 pretreatment of SCB (PB group) resulted in the highest glucose release (61.83 ± 0.8 mg/mL) and enzyme activity (45.66 ± 0.4 U/mL), surpassing both untreated and sterilized controls. These findings demonstrate that KC94's enzymatic machinery directly dismantles the polysaccharide backbone into fermentable monomers, reducing cellulose crystallinity and enhancing substrate accessibility. Together, the genomic evidence from our earlier work and the functional outcomes reported here reinforce KC94's suitability for bioenergy applications through microbial saccharification of lignocellulosic biomass.

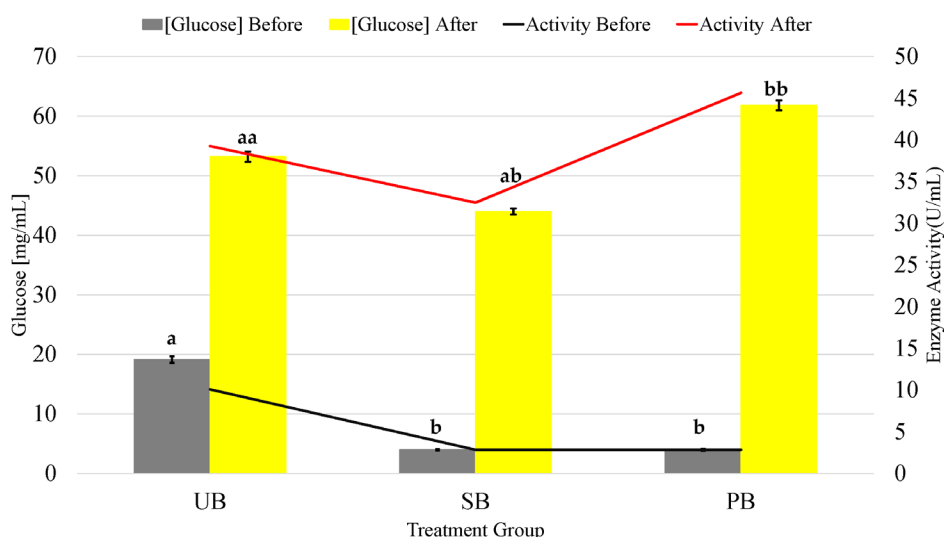


Figure 5.3. Glucose concentration (mg/mL) and enzyme activity (U/mL) before and after hydrolysis across treatment groups (UB—Untreated Bagasse; SB—Sterile Bagasse; PB—Pretreated Bagasse). Error bars represent mean $\pm$ SD of three independent replicates ($n = 3$). Different letters above bars indicate statistically significant differences between treatments based on post hoc comparisons ($p < 0.05$).

These findings further underscore the synergistic benefit of combining mechanical pretreatment with targeted microbial inoculation for efficient biomass hydrolysis. The enhanced glucose release in the PB group suggests a higher potential for downstream biogas production, as glucose is a readily fermentable substrate for methanogenic consortia (Zhou *et al.*, 2021). The results support the inclusion of mechanical and biological pretreatment steps in biogas process optimization strategies, particularly for recalcitrant lignocellulosic feedstocks like bagasse.

5.3.2 Elemental Shifts in SCB After Biological Pretreatment

The elemental composition of SCB varied across treatments, indicating distinct effects of sterilization, incubation, and microbial inoculation. A significant reduction in carbon content was observed between sterile bagasse before incubation ($43.70 \pm 0.18\%$) and the SB after incubation in McDougall's buffer ($36.48 \pm 0.34\%$), suggesting substantial carbon loss during incubation. This likely reflects the leaching of soluble organic compounds or mild thermal degradation at 35°C over 5 days. Interestingly, the pretreated bagasse inoculated with KC94 (PB) showed a higher carbon content ($40.55 \pm 0.26\%$) than SB after incubation, indicating microbial stabilization or re-incorporation of solubilized carbon into bagasse. The carbon

content differences between SB before and after incubation, as well as between PB and SB, were significant, highlighting the biochemical impact of both incubation and microbial metabolism.

Sulfur was detected in the incubated SB ($1.25 \pm 0.03\%$) only, but none in the SB before incubation or the pretreated PB samples. Given that all samples received identical concentrations of McDougall's buffer, which contains about 0.0192 g/L of MgSO_4 . The presence of sulfur in SB after incubation suggests that it may have become bound to the substrate or concentrated during incubation and was present in sufficient quantities to be detected during analysis (Macena *et al.*, 2025). This may also suggest that sulfur retention is not merely a function of buffer exposure, but rather a result of incubation-driven physicochemical interactions (Rahman and Reza, 2025). During the 5-day incubation at 35 °C, SB (after incubation) likely underwent mild thermal and moisture loss, which concentrated solutes, including sulfate ions, within the biomass matrix. In contrast, the absence of sulfur in SB before incubation and PB (inoculated with *KC94*) can be attributed to two distinct mechanisms. In SB (before incubation), the lack of incubation time and thermal exposure likely prevented any significant sulfur binding or concentration, and the concentration was too low to be detected during analysis. Meanwhile, in PB, the disappearance of sulfur may be due to microbial or chemical transformations during incubation. Although *P. mirabilis* is known to reduce sulfate to hydrogen sulfide (H_2S) under anaerobic conditions (Rout and Behera, 2024), the pretreatment in this study was not conducted under anaerobic conditions, and therefore the observed sulfur changes cannot be attributed to anaerobic sulfate reduction. Rout and Bhehera (2024) further highlight that microbial pretreatment can lead to volatilization of sulfur compounds, especially in facultative anaerobes capable of sulfur respiration. This finding aligns with previous research demonstrating that *P. mirabilis* can desulfurize organosulfur compounds, such as dibenzothiophene, via the 4S pathway, releasing volatile sulfur species, including H_2S (Rout and Behera, 2024). This interpretation is supported by the literature, which shows that hydrogen sulfide is a common byproduct of sulfur reduction in facultative anaerobes, including *P. mirabilis*. The sulfur reduction pathway, particularly the 4S pathway, has been implicated in the degradation of organosulfur compounds, with H_2S as the terminal product. Therefore, the presence of sulfur in SB after incubation indicates abiotic retention, whereas its absence in PB indicates biological transformation and volatilization. This distinction highlights the dual role of microbial pretreatment: not only in modifying biomass

but also in pre-digestive desulfurization, which can reduce the sulfur burden on anaerobic digesters and enhance biogas quality (Ye *et al.*, 2025, Rasool *et al.*, 2023).

Meanwhile, nitrogen was detected only in SB (after incubation), ($1.12 \pm 0.02\%$), and PB ($1.24 \pm 0.03\%$), but not in SB (before incubation), indicating nitrogen incorporation during incubation. The slightly elevated levels of PB likely reflect active microbial protein synthesis. These findings further support the role of *P. mirabilis* in modifying the elemental composition through sulfur assimilation and biomass accumulation, which then enriches the substrate with microbial protein, thereby improving the C:N:S balance of SCB (Ye *et al.*, 2025). This nutrient enrichment has the potential to promote microbial activity and stability during digestion, particularly given the carbon-rich, nitrogen-poor nature of lignocellulosic feedstocks (Rasool *et al.*, 2023). While some assimilated sulfur may eventually be mineralized to H_2S during digestion, its incorporation into biomass likely moderates the rate of release, reducing the risk of acute methanogen inhibition. Taken together, these results suggest that microbial pretreatment with *KC94* not only contributes to biomass valorisation but also offers a strategy for preconditioning substrates in ways that can both mitigate sulfur-related challenges and improve digester performance. To the best of our knowledge, this is one of the first studies to assess elemental shifts, particularly the disappearance of sulfur, during the microbial pretreatment of SCB using *P. mirabilis*.

5.3.3 Scanning Electron Microscopy (SEM) Analysis of Bagasse Morphology

The scanning electron micrographs showed distinct morphological differences across the compared treatments (Figure 5.4). The untreated bagasse (UB) displayed intact and compact fibrillar structures with smooth surfaces, indicating the natural integrity of the lignocellulosic matrix (Singh *et al.*, 2023). The fibers appeared tightly packed, with minimal visible disruption and pore formation, characteristic of native cellulose–hemicellulose–lignin complexes. In contrast, the sterile bagasse (SB) exhibited slight surface disruption, attributable to mechanical grinding and autoclaving. However, the general fibrous structure remained largely intact, suggesting that grinding and sterilization alone did not significantly alter the lignocellulosic structure. Notably, the biologically pretreated bagasse (PB) exhibited substantial morphological alterations. The SEM images revealed fragmented fibers, visible fissures, and increased surface roughness compared to UB and SB. The formation of pores, erosion of the

cell wall surface, and partial delamination of fiber bundles indicated that the KC94 strain degraded lignin and hemicellulose components. Supplementary Figure S1 shows the morphology of the pretreatment groups at magnification 1 k $\times$, 5 k $\times$, and 100 $\times$. These microstructural changes are consistent with microbial depolymerization processes, reflecting the effective biological pretreatment observed after bacterial fermentation and white-rot fungal pretreatment (Abbas *et al.*, 2023). Overall, the SEM observations confirmed that microbial pretreatment with KC94 markedly disrupted the structural integrity of bagasse, thereby enhancing fiber porosity and surface accessibility for potential downstream enzymatic hydrolysis.

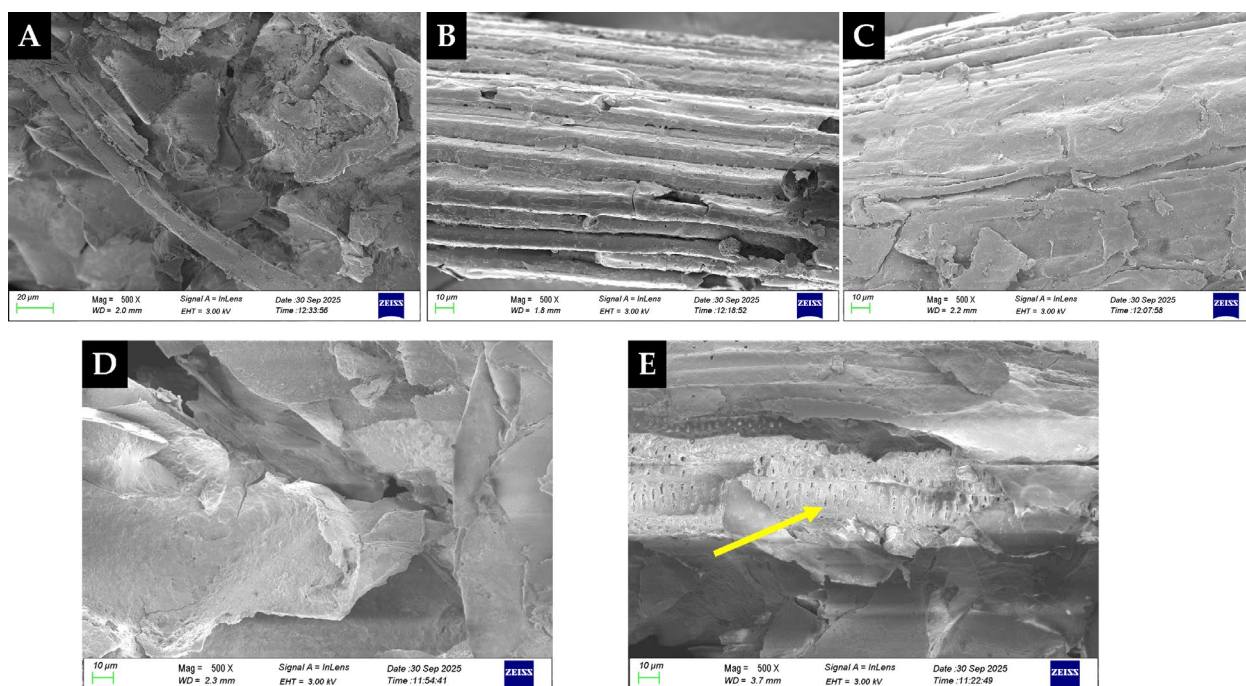


Figure 5.4: Scanning electron micrographs of untreated bagasse (UB) ((A)—before incubation and (B)—after incubation), sterile bagasse (SB) ((C)—before incubation and (D)—after incubation), and biologically pretreated bagasse (PB) (E) with KC94 after 5 days of incubation at 500 $\times$ magnification. The arrow indicates areas of fiber disruption and surface erosion in PB, compared with the intact structures of UB and SB.

5.3.4 Biochemical Methane Potential (BMP) of Pretreated SCB

The BMP assay was conducted over 31 days to assess the effect of biological pretreatment with the KC94 cellulolytic strain on the anaerobic digestibility of SCB. The cumulative methane

yields (expressed in mL CH₄/g VS) for untreated bagasse inoculum (UB), sterilized bagasse inoculum (SB), and pretreated bagasse (PB) are presented in Figures 5.5 and 5.6.

5.3.5 Methane Production Trends

As expected, the methane production was negligible in all samples on Day 0. The methane yield increased progressively over the incubation period. At the end of the experiment (Day 31), the cumulative BMP values were 83 ± 1.6 mL/g VS for UB, 79 ± 0.7 mL/g VS for SB, and 99 ± 0.7 mL/g VS for PB. The PB group exhibited the highest methane yield, indicating a clear enhancement in the biodegradability of SCB following KC94 biological pretreatment. Compared with the untreated control (UB), pretreated bagasse (PB) showed a 19.3% increase in cumulative methane production. Similarly, the improvement over PB (sterilized control) was approximately 25%, suggesting a clear link between the biological action of the cellulolytic strain and the enhanced methane output.

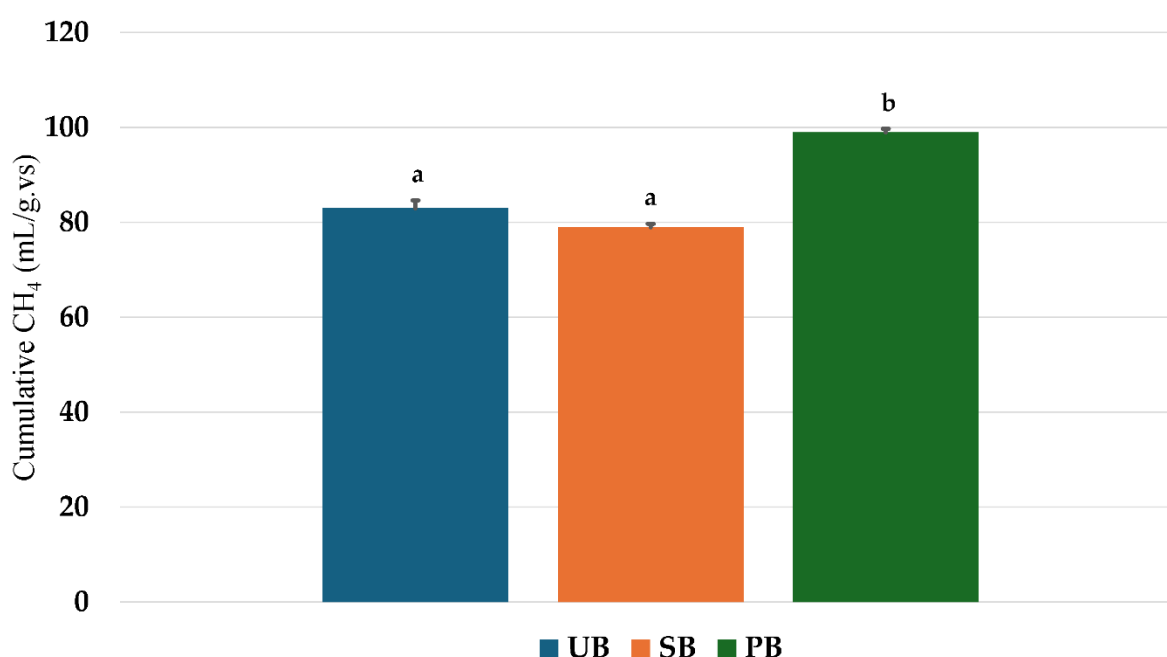


Figure 5.5: Methane production over 31 days for all the treatment groups (UB—Untreated Bagasse; SB—sterile Bagasse; PB—Pretreated Bagasse). Error bars represent mean $\pm$ SD of three independent replicates ($n = 3$). Different letters above bars indicate statistically significant differences between treatments based on post hoc comparisons ($p < 0.05$).

5.3.6 Kinetics of Methane Production

Post hoc comparisons using Tukey's HSD test revealed no significant differences among the three treatments (UB, SB, and PB) on a daily indicating that treatment had no effect on this variable. In contrast, clear differences emerged for BMP Daily (mL/g VS). PB produced significantly higher BMP values than both UB (mean difference = 24.26 mL/g VS, $p < 0.001$) and SB (mean difference = 19.47 mL/g VS, $p < 0.001$), with confidence intervals that did not include zero. However, UB and SB did not differ significantly from each other ($p = 0.429$). These results demonstrate that while UB and SB perform similarly, PB consistently yields the highest daily methane production, highlighting its superior effectiveness in enhancing BMP. Homogeneity of variances was confirmed for BMP Daily using Levene's test ($p = 0.398$), supporting the use of one-way ANOVA and Tukey's HSD for multiple comparisons. These results indicate that treatment type exerted a measurable effect on the outcome variable, with PB consistently outperforming the other groups. The approximately 24-unit difference between PB and UB is not only statistically significant but also potentially functionally relevant in the context of glucose release. In the PB group, a more rapid and sustained increase in daily BMP was observed from day 1 to day 15, reaching 70 ± 0.7 mL/g VS by day 15, compared to 39 ± 4.9 mL/g VS in the UB and 44 ± 0.4 mL/g VS in the SB. The faster onset and progression of methane generation suggest that the 5-day pretreatment reduced the structural complexity of lignocellulosic biomass, likely by hydrolysing cellulose into fermentable sugars, thereby making the substrate more readily available for anaerobic microorganisms. This finding provides robust evidence of a time-dependent effect, whereby outcomes improved progressively with treatment duration.

Interestingly, the PB group showed a slower start in the first few days compared to the SB group. The initial lag in methane production observed in the PB group could be attributed to residual metabolic byproducts (Kulichkova *et al.*, 2020) or microbial competition from the aerobic pretreatment phase (Wang *et al.*, 2025), temporarily inhibiting anaerobic microbial activity. Despite this early delay, the PB ultimately surpassed both SB and UB in cumulative methane yield, highlighting the overall effectiveness of the biological pretreatment in enhancing substrate biodegradability. The curve for UB showed slower, more linear methane accumulation, characteristic of untreated lignocellulosic biomass, where hydrolysis is typically the rate-limiting step due to lignin's recalcitrant structure (Jiang *et al.*, 2024). The SB curve closely followed the UB curve, indicating that sterilization alone did not significantly alter the substrate's biodegradability. Tukey's HSD test grouped UB and SB into the same

homogeneous subset ($p = 0.429$), indicating no significant difference in BMP Daily between these treatments. In contrast, PB formed a separate subset with a substantially higher mean BMP value (65.90 mL/g VS), confirming that PB produced significantly greater methane yields than both UB and SB. Hence, the observed improvement in BMP in the PB group can be attributed to the KC94 cellulolytic strain, which presumably enhanced cellulose degradation during the 5-day aerobic pretreatment phase. This is supported by prior glucose monitoring in Section 5.3.1, which showed a notable increase post-pretreatment, indicating effective hydrolysis of complex carbohydrates.

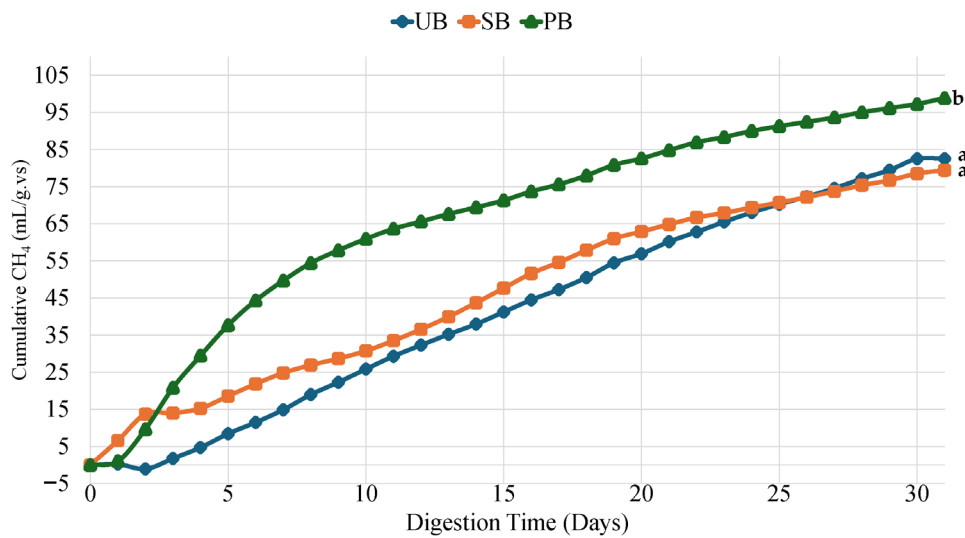


Figure 5.6: Cumulative BMP (BMP, expressed as mL CH₄/g VS) from the three different treatment groups, UB (untreated bagasse), SB (sterile bagasse), and PB (pretreated bagasse), monitored over a 31-day digestion period. Different letters above bars indicate statistically significant differences between treatments based on post hoc comparisons ($p < 0.05$).

These findings align with previous studies demonstrating the efficacy of microbial pretreatment in improving anaerobic digestibility. By disrupting the lignocellulosic matrix and converting cellulose into fermentable sugars, pretreatment significantly reduced the energy and time barriers to AD. In a similar study, they used ruminal consortia for pretreatment to hydrolyze microalgal biomass, aiming to promote methane production during AD (Barragán-Trinidad *et al.*, 2017). Although microalgal biomass differs structurally from lignocellulosic materials like SCB, their approach leveraged the natural enzymatic capabilities of rumen microorganisms to break down complex algal cell walls, which are typically resistant to degradation. The pretreatment effectively increased hydrolysis rates and methane recovery, demonstrating the potential of ruminal consortia to improve biogas yields from microalgal substrates.

The enhancement in BMP observed with the 5-day KC94 pretreatment, which shows a 19.3% increase over UB and 25% over SB, is both significant and competitive compared with existing microbial pretreatment strategies (Prasad *et al.*, 2024). This improvement falls well within the range reported for other biological pretreatment approaches and, in some instances, even surpasses the benchmarks of single-strain approaches. For example, fungal pretreatments such as *Pleurotus eryngii* applied to corn stover have yielded approximately 19% higher biogas production (Wyman *et al.*, 2018), while *Trichoderma reesei* pretreatment of rice straw achieved a methane yield of 214 L per kg VS, albeit typically requiring extended incubation periods of 45 days or more (Mustafa *et al.*, 2016). Microbial consortia methods, such as WSD-5 on Napier grass, have reported improvements in methane yield of 49% (Mustafa *et al.*, 2016). Bacterial treatments, such as those employing *Bacillus* species on rice straw, also show substantial rises of up to 76%, often necessitating prolonged pretreatment durations (Shah *et al.*, 2019).

In contrast, the KC94 pretreatment in this study combines a relatively short aerobic pretreatment phase (only 5 days) with considerable methane yield gains, highlighting its efficiency and operational simplicity. Many of the higher-yielding studies referenced used pretreatment times that were 4 to 16 times longer, suggesting that further extending the KC94 incubation period could improve lignocellulose hydrolysis and further boost BMP. This presents a promising avenue for future research optimization. Moreover, the simplicity of the KC94 method, which relies on a single microbial strain and a short pretreatment duration, contrasts favorably with more complex microbial consortia or lengthy fungal pretreatments, which often pose operational challenges. Taken together, these findings demonstrate that KC94 pretreatment offers a competitive, practical approach to enhancing methane production from lignocellulosic biomass.

5.3.7 Organics Reduction During BMP Testing

Biodegradabilities for the tested substrates during BMPs ranged from 43 to 46% as measured by substrate VS reductions (Table 5.1). These values are on the lower side when compared to biodegradabilities reported in other studies using different substrates and conditions. Neves *et al.* (2009) reported biodegradabilities ranging from 49.1 to 55.6% for AD tests involving cow manure and food waste digestion with pulses of fat added to reactors. Studies involving highly degradable food waste achieved a VS reduction of 79% when digested under mesophilic conditions, with a 35-day hydraulic retention time (HRT) (Pramanik *et al.*, 2019). These results suggest that there is potential to further enhance the biodegradability of SCB. The absence of

co-substrates, such as easily degradable food waste or fat pulses, may have further constrained the extent of VS reduction. These factors highlight SCB's inherent resistance to anaerobic degradation, suggesting that pretreatment or co-digestion strategies could enhance its biodegradability.

Table 5.1: Characterization of the SCB (before and after BMPs), KC94, and inoculum. $\pm$ represent mean $\pm$ SD of three independent replicates ($n = 3$).

Material	Before BMP Tests		Elemental Analysis				After BMP Tests		
	TS (%)	VS (%)	C (%)	H (%)	N (%)	S (%)	TS (%)	VS (%)	VS Reduction (%)
KC94	0.07 $\pm$ 0.01	0.04 $\pm$ 0.01	-	-	-	-			
Sludge	96 $\pm$ 1.0	89 $\pm$ 1.0	-	-	-	-			
Untreated Bagasse (UB)	88 $\pm$ 1.0	87 $\pm$ 1.0	-	-	-	-	78 $\pm$ 0.02	76 $\pm$ 0.01	46 $\pm$ 0.04
Sterile Bagasse (SB) before incubation			43.70 $\pm$ 0.18	6.17 $\pm$ 0.01	-	-			
Sterile Bagasse (SB) After Incubation	86 $\pm$ 0.05	82 $\pm$ 0.04	36.48 $\pm$ 0.34	5.21 $\pm$ 0.06	1.12 $\pm$ 0.02	1.25 $\pm$ 0.03	82 $\pm$ 0.03	71 $\pm$ 0.02	43 $\pm$ 0.06
Pretreated Bagasse (PB)	85 $\pm$ 0.06	82 $\pm$ 0.02	40.55 $\pm$ 0.26	5.62 $\pm$ 0.07	1.24 $\pm$ 0.03	-	70 $\pm$ 0.04	79 $\pm$ 0.02	46 $\pm$ 0.03

Note: All VS and elemental composition values in Table 1 are expressed on the material's dry basis. SB (before incubation) also represents PB under the same conditions. The CHNS analyzer used was only suitable for ground samples; hence, UB is not reported.

5.3.8 Study Strengths and Weaknesses

The current study's main strength lies in successfully identifying a matching local microbial source (goat gastrointestinal tract) and a local problem (bagasse waste-to-energy conversion) that this microorganism can address. The local availability of both the microorganism and the waste offers scale and cost advantages for fast-tracking commercialization decisions or, if needed, for further trials to advance this research. However, the study has limitations, as it focuses solely on SCB as the target lignocellulosic biomass. South Africa, like many other countries, has numerous lignocellulosic biomass wastes, such as forestry residues, corn cobs, and sawdust, which could also benefit from this research but would need to be tested first

before commercialization efforts can be pursued. The study can also be strengthened by comparing different pretreatment methods or different microorganisms under similar conditions (biomass, end use of hydrolysate, and laboratory personnel) to demonstrate superior or inferior performance. This study could also have included quantification of inhibitors in the hydrolysate and microbial dynamics during AD trials to better understand the observed results.

5.4 Conclusion

The mechanical and biological pretreatment strategy applied in the PB group led to significant lignocellulosic degradation and microbial enrichment, both of which are desirable outcomes for enhancing BMP. The observed elemental changes confirm that KC94 actively influences carbon and sulfur dynamics during SCB pretreatment, likely through microbial assimilation and sulfur metabolism. These findings highlight the potential of targeted microbial strains to enhance biomass transformation and uncover novel biochemical interactions during incubation. This study provides new evidence of *P. mirabilis*' ability to alter the elemental profile of lignocellulosic biomass, offering a novel approach to microbial pretreatment strategies. The detection of sulfur, often overlooked in lignocellulosic analysis, may warrant further investigation as a potential indicator of advanced microbial processing or sulfur-cycling activity relevant to downstream AD. Biological pretreatment of SCB using the KC94 cellulolytic strain resulted in a substantial improvement in both cumulative methane yield and production kinetics, although there is scope to further enhance substrate biodegradability through inoculum acclimatization and other strategies. These results support the application of microbial pretreatment as an environmentally friendly strategy to enhance the energy recovery potential of lignocellulosic waste in biogas systems. Furthermore, relatively few studies in the literature focus specifically on biomethane production from SCB compared to other lignocellulosic wastes, such as rice straw or corn stover. This scarcity highlights an underexploited potential for utilizing sugarcane waste, which is produced in large quantities globally. Developing efficient pretreatment and digestion strategies for SCB could significantly contribute to sustainable waste management and renewable energy generation in sugarcane-producing regions. To address biosafety concerns associated with opportunistic pathogens such as *Proteus mirabilis*, future work should prioritize transferring the genetic determinants of lignocellulosic biomass degradation into non-pathogenic, well-characterized hosts. In particular, cloning and expressing these genes in laboratory strains such as *Escherichia coli* BL21(DE3) or other GRAS (Generally Recognized as Safe) organisms would provide a safer, more scalable platform. This approach reduces biosafety risks during industrial

applications, leverages the extensive genetic tools available for these hosts, and facilitates regulatory compliance. It is recommended that future studies focus on evaluating the effects of extending pretreatment duration, increasing I/S ratios during pretreatment/AD tests, and inoculum acclimatization. The detection/suppression of potential inhibitors during bagasse hydrolysis pretreatment also deserves attention to improve bagasse biodegradability for biomethane production. Furthermore, a comparison study between biological, physical, and chemical pretreatments should also be explored. Future AD studies linked to these hydrolytic experiments should incorporate metagenomics and transcriptomics elements to fully understand how yield enhancements and the role of microorganisms in this process can be utilized to inform future optimization strategies.

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5.5 References

- 14775:2009, E. 2015. Solid Biofuels—Determination of Ash Content. Brussels, Belgium.
- 15148:2009, E. 2015. Solid Biofuels—Determination of the Content of Volatile Matter. Brussels, Belgium.
- ABBAS, M., EJAZ, U., SHAFIQUE, M., NAZ, S. A. & SOHAIL, M. 2023. Biological pretreatment of sugarcane bagasse for the production of fungal laccase and bacterial cellulase. *J Basic Microbiol*, 63, 722-733.
- AGARWAL, N. K., KUMAR, M., GHOSH, P., KUMAR, S. S., SINGH, L., VIJAY, V. K. & KUMAR, V. 2022. Anaerobic digestion of sugarcane bagasse for biogas production and digestate valorization. *Chemosphere*, 295, 133893.
- ALAGESAN, P. 2003. Isolation and characterization of gut bacteria of millipede, *Xenobolus carnifex* (Fabricius). *Indian J. Microbiol.*, 43, 111-113.
- ANAND, A. A. P. & SRIPATHI, K. 2004. Digestion of cellulose and xylan by symbiotic bacteria in the intestine of the Indian flying fox (*Pteropus giganteus*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 139, 65-69.
- ANGELIDAKI, I. & SANDERS, W. 2004. Assessment of the anaerobic biodegradability of macropollutants. *Re/Views in Environmental Science & Bio/Technology*, 3, 117-129.
- AQUINO, D., DEL BARRIO, A., TRACH, N. X., HAI, N. T., KHANG, D. N., TOAN, N. T. & VAN HUNG, N. 2019. Rice straw-based fodder for ruminants. *Sustainable rice straw management*. Springer International Publishing Cham.
- ARMAHA, E. K., CHETTYA, M. & DEENADAYALUB, N. 2019. Effect of particle size on biogas generation from sugarcane bagasse and corn silage. *CHEMICAL ENGINEERING*, 76.
- BABA, Y., MATSUKI, Y., MORI, Y., SUYAMA, Y., TADA, C., FUKUDA, Y., SAITO, M. & NAKAI, Y. 2017. Pretreatment of lignocellulosic biomass by cattle rumen fluid for methane production: bacterial flora and enzyme activity analysis. *Journal of bioscience and bioengineering*, 123, 489-496.
- BARRAGÁN-TRINIDAD, M., CARRILLO-REYES, J. & BUITRÓN, G. 2017. Hydrolysis of microalgal biomass using ruminal microorganisms as a pretreatment to increase methane recovery. *Bioresource Technology*, 244, 100-107.
- BERNFELD, P. 1955. [17] Amylases, α and β . *Methods in Enzymology*. Academic Press.
- DA SILVA, A. S. A., INOUE, H., ENDO, T., YANO, S. & BON, E. P. 2010. Milling pretreatment of sugarcane bagasse and straw for enzymatic hydrolysis and ethanol fermentation. *Bioresource technology*, 101, 7402-7409.
- DAR, M. A., PAWAR, K. D., RAJPUT, B. P., RAHI, P. & PANDIT, R. S. 2019. Purification of a cellulase from cellulolytic gut bacterium, *Bacillus tequilensis* G9 and its evaluation for

- valorization of agro-wastes into added value byproducts. *Biocatalysis and Agricultural Biotechnology*, 20, 101219.
- DAR, M. A., SHAIKH, A. A., PAWAR, K. D. & PANDIT, R. S. 2018. Exploring the gut of *Helicoverpa armigera* for cellulose degrading bacteria and evaluation of a potential strain for lignocellulosic biomass deconstruction. *Process Biochemistry*, 73, 142-153.
- DAR, M. A., SYED, R., PAWAR, K. D., DHOLE, N. P., XIE, R., PANDIT, R. S. & SUN, J. 2022. Evaluation and characterization of the cellulolytic bacterium, *Bacillus pumilus* SL8 isolated from the gut of oriental leafworm *Spodoptera litura*: An assessment of its potential value for lignocellulose bioconversion. *Environmental Technology & Innovation*, 27, 102459.
- DO ESPIRITO SANTO, M. C., THEMA, F. T., PELLEGRINI, V. D. O. A., KANE, A. O., GUIMARAES, F. E. G., FILGUEIRAS, J. G., NOVOTNY, E. H., DEAZEVEDO, E. R. & POLIKARPOV, I. 2022. When the order matters: Impacts of lignin removal and xylan conformation on the physical structure and enzymatic hydrolysis of sugarcane bagasse. *Industrial Crops and Products*, 180, 114708.
- DRZEWIECKA, D. 2016. Significance and roles of *Proteus* spp. bacteria in natural environments. *Microbial ecology*, 72, 741-758.
- EN14774-3:2009 2015. Solid Biofuels—Determination of Moisture Content—Oven Dry Method—Part 3: Moisture in General Analysis Sample. Brussels, Belgium.
- GEBBIE, L., DAM, T. T., AINSCOUGH, R., PALFREYMAN, R., CAO, L., HARRISON, M., O'HARA, I. & SPEIGHT, R. 2020. A snapshot of microbial diversity and function in an undisturbed sugarcane bagasse pile. *BMC biotechnology*, 20, 12.
- HARUN, N., OTHMAN, N., ZAKI, N., RASUL, N. M., SAMAH, R. & HASHIM, H. 2019. Simulation of anaerobic digestion for biogas production from food waste using SuperPro designer. *Materials Today: Proceedings*, 19, 1315-1320.
- HOLLIGER, C., ALVES, M., ANDRADE, D., ANGELIDAKI, I., ASTALS, S., BAIER, U., BOUGRIER, C., BUFFIÈRE, P., CARBALLA, M. & DE WILDE, V. 2016. Towards a standardization of biomethane potential tests. *Water Science and Technology*, 74, 2515-2522.
- JIANG, X., ZHAI, R., LI, H., LI, C., YUAN, Y., DENG, Q., XU, Z., SHA, Y. & JIN, M. 2024. Unveiling the inhibition of enzymatic hydrolysis of cellulose by lignin-derived phenolics: interfacial kinetics and molecular simulations. *ACS Sustainable Chemistry & Engineering*, 12, 9957-9968.
- KONDE, K. S., NAGARAJAN, S., KUMAR, V., PATIL, S. V. & RANADE, V. V. 2021. Sugarcane bagasse based biorefineries in India: potential and challenges. *Sustainable Energy & Fuels*, 5, 52-78.
- KULICHKOVA, G. I., IVANOVA, T. S., KÖTTNER, M., VOLODKO, O. I., SPIVAK, S. I., TSYGANKOV, S. P. & BLUME, Y. B. 2020. Plant feedstocks and their biogas production potentials. *The Open Agriculture Journal*, 14.

- KUMAR, A., KUMAR, V. & SINGH, B. 2021. Cellulosic and hemicellulosic fractions of sugarcane bagasse: Potential, challenges and future perspective. *International Journal of Biological Macromolecules*, 169, 564-582.
- LADEIRA-ÁZAR, R. I., MORGAN, T., MAITAN-ALFENAS, G. P. & GUIMARÃES, V. M. 2019. Inhibitors compounds on sugarcane bagasse saccharification: effects of pretreatment methods and alternatives to decrease inhibition. *Applied biochemistry and biotechnology*, 188, 29-42.
- MACENA, M., PEREIRA, H., CRUZ-LOPES, L., GROSCHE, L. & ESTEVES, B. 2025. Competitive Adsorption of Metal Ions by Lignocellulosic Materials: A Review of Applications, Mechanisms and Influencing Factors. *Separations*, 12, 70.
- MCDUGALL, E. I. 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. *Biochem J*, 43, 99-109.
- MILLER, G. 1959. Modified DNS method for reducing sugars. *Anal. Chem*, 31, 426-428.
- MOHLATLOLE, R. P., DZOMBA, E. F. & MUCHADEYI, F. C. 2015. Addressing production challenges in goat production systems of South Africa: The genomics approach. *Small Ruminant Research*, 131, 43-49.
- MOKALE KOGNOU, A. L., CHIO, C., KHATIWADA, J. R., SHRESTHA, S., CHEN, X., HAN, S., LI, H., JIANG, Z.-H., XU, C. C. & QIN, W. 2022. Characterization of cellulose-degrading bacteria isolated from soil and the optimization of their culture conditions for cellulase production. *Applied Biochemistry and Biotechnology*, 194, 5060-5082.
- MUSTAFA, A. M., POULSEN, T. G. & SHENG, K. 2016. Fungal pretreatment of rice straw with *Pleurotus ostreatus* and *Trichoderma reesei* to enhance methane production under solid-state anaerobic digestion. *Applied Energy*, 180, 661-671.
- NEVES, L., OLIVEIRA, R. & ALVES, M. 2009. Co-digestion of cow manure, food waste and intermittent input of fat. *Bioresource technology*, 100, 1957-1962.
- PRAMANIK, S. K., SUJA, F. B., PORHEMMAT, M. & PRAMANIK, B. K. 2019. Performance and kinetic model of a single-stage anaerobic digestion system operated at different successive operating stages for the treatment of food waste. *Processes*, 7, 600.
- PRASAD, R. K., SHARMA, A., MAZUMDER, P. B. & DHUSSA, A. 2024. A comprehensive pre-treatment strategy evaluation of ligno-hemicellulosic biomass to enhance biogas potential in the anaerobic digestion process. *RSC Sustainability*, 2, 2444-2467.
- RABAPANE, K. J. & MATAMBO, T. S. 2024. Profiling the dynamic adaptations of CAZyme-Producing microorganisms in the gastrointestinal tract of South African goats. *Heliyon*, 10.
- RAHMAN, M. M. & REZA, T. 2025. Hydrothermal Liquefaction of Structurally Diverse Lignins: Insights into Biocrude Yield, Fuel Properties, and Reaction Mechanisms. *Energies*, 18, 4773.
- RASOOL, K., HUSSAIN, S., SHAHZAD, A., MIRAN, W., MAHMOUD, K. A., ALI, N. & ALMOMANI, F. 2023. Comprehensive insights into sustainable conversion of agricultural and

- food waste into microbial protein for animal feed production. *Reviews in Environmental Science and Bio/Technology*, 22, 527-562.
- ROUT, P. G. & BEHERA, S. K. 2024. Evaluation of organosulfur compound degradation property of a noble bacterial strain of *Proteus mirabilis*. *Environmental Chemistry and Ecotoxicology*, 6, 223-228.
- ROY, D., GUNRI, S. K. & PAL, K. K. 2024. Isolation, screening and characterization of efficient cellulose-degrading fungal and bacterial strains and preparation of their consortium under in vitro studies. *3 Biotech*, 14, 131.
- SAHARAN, V., TUSHIR, S., YADAV, M., DHAKA, N. & KAPOOR, R. K. 2025. Hydrolysis: Challenges and Future. *Lignocellulosic Biomass and Enzymes: Fundamentals, Emerging Technologies and Applications*, 127.
- SHAH, T. A., LEE, C. C., ORTS, W. J. & TABASSUM, R. 2019. Biological pretreatment of rice straw by ligninolytic *Bacillus* sp. strains for enhancing biogas production. *Environmental Progress & Sustainable Energy*, 38, e13036.
- SIMBAYI, T. M., RASHAMA, C., AWOSUSI, A. A., NKUNA, R., CHRISTIAN, R. & MATAMBO, T. S. 2023. Investigating the Anaerobic Digestion of Water Hyacinth (*Eichhornia crassipes*) Sourced from Hartbeespoort Dam in South Africa. *Fermentation*, 9, 685.
- SINGH, S. S., LIM, L.-T. & MANICKAVASAGAN, A. 2023. Imaging and spectroscopic techniques for microstructural and compositional analysis of lignocellulosic materials: a review. *Biomass Conversion and Biorefinery*, 13, 499-517.
- SIVAMANI, S., SAIKAT, B., NAVEEN PRASAD, B., BAALAWY, A. A. S. & AL-MASHALI, S. M. A. 2021. A comprehensive review on microbial technology for biogas production. *Bioenergy Research: Revisiting Latest Development*, 53-78.
- TEOFILO, R. F., CHAVES, D. M., GONCALVES, I. M., RUFINO, A. B., ANTUNES, T. R., QUINTAO, A. J., DA SILVA, R. C., SUKARNO, O., DA SILVA, G. A. & PETERNELLI, L. A. 2025. A Comprehensive Study of Cellulignin Production from Sugarcane Bagasse, Sugarcane Straw, and Energy Cane for Advanced Biofuels: Liquor Composition and Cellulignin Characterizations. *Waste and Biomass Valorization*, 16, 2195-2208.
- TERAMOTO, K., TSUTSUI, S., SATO, T., FUJIMOTO, Z. & KANEKO, S. 2021. Substrate Specificities of GH8, GH39, and GH52 β -xylosidases from *Bacillus halodurans* C-125 Toward Substituted Xylooligosaccharides. *Applied Biochemistry and Biotechnology*, 193, 1042-1055.
- VALDEZ-VAZQUEZ, I., ALATRISTE-MONDRAGÓN, F., ARREOLA-VARGAS, J., BUITRÓN, G., CARRILLO-REYES, J., LEÓN-BECERRIL, E., MENDEZ-ACOSTA, H. O., ORTÍZ, I. & WEBER, B. 2020. A comparison of biological, enzymatic, chemical and hydrothermal pretreatments for producing biomethane from Agave bagasse. *Industrial Crops and Products*, 145, 112160.

- WANG, J., WANG, X., BIAN, C., LIU, J. & XIAO, B. 2025. Effect of aeration pretreatment on anaerobic digestion of swine manure. *Environ Technol*, 46, 2035-2051.
- WEIMER, P. J. 2022. Degradation of Cellulose and Hemicellulose by Ruminal Microorganisms. *Microorganisms*, 10.
- WOŹNIAK, A., KULIGOWSKI, K., ŚWIERCZEK, L. & CENIAN, A. 2025. Review of lignocellulosic biomass pretreatment using physical, thermal and chemical methods for higher yields in bioethanol production. *Sustainability*, 17, 287.
- WYMAN, V., HENRÍQUEZ, J., PALMA, C. & CARVAJAL, A. 2018. Lignocellulosic waste valorisation strategy through enzyme and biogas production. *Bioresource Technology*, 247, 402-411.
- XU, H., CHE, X., DING, Y., KONG, Y., LI, B. & TIAN, W. 2019. Effect of crystallinity on pretreatment and enzymatic hydrolysis of lignocellulosic biomass based on multivariate analysis. *Bioresource technology*, 279, 271-280.
- YE, Y., CAI, Y., WANG, F., HE, Y., YANG, Y., GUO, Z., LIU, M., REN, H., WANG, S., LIU, D., XU, J. & WANG, Z. 2025. Industrial Microbial Technologies for Feed Protein Production from Non-Protein Nitrogen. *Microorganisms*, 13, 742.
- YOU, C., LIU, Y.-J., CUI, Q. & FENG, Y. 2023. Glycoside hydrolase family 48 cellulase: A key player in cellulolytic bacteria for lignocellulose biorefinery. *Fermentation*, 9, 204.
- ZHOU, Z., WANG, Y., WANG, M. & ZHOU, Z. 2021. Co-metabolic effect of glucose on methane production and phenanthrene removal in an enriched phenanthrene-degrading consortium under methanogenesis. *Frontiers in Microbiology*, 12, 749967.

CHAPTER 6: GENERAL DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

6.1 General Discussion

The growing demand for sustainable energy sources and environmentally responsible waste management strategies has intensified interest in using lignocellulosic agricultural residues for bioenergy production. SCB, one of the most abundant agro-industrial residues worldwide, is a promising substrate for renewable biomethane production. However, its complex lignocellulosic structure, composed primarily of cellulose, hemicellulose, and lignin, limits microbial accessibility and reduces biodegradability during AD. Consequently, the development of effective, environmentally friendly pretreatment strategies remains a critical challenge. This study addressed this challenge by investigating the GIT microbiome of South African goats as a source of cellulolytic microorganisms and CAZymes capable of enhancing lignocellulose degradation and biomethane production.

The findings presented in Chapter 3 demonstrated that the goat GIT harbors a highly specialized and compartmentalized microbial ecosystem with significant taxonomic and functional diversity. Distinct microbial communities were observed across the different GIT compartments, reflecting the varying physicochemical conditions and nutritional niches present throughout the digestive tract. The reduced microbial diversity observed in the abomasum was likely attributable to its acidic environment, which acts as a selective barrier against many microorganisms. In contrast, the rumen, reticulum, and omasum exhibited greater microbial diversity and functional potential, supporting their established roles as primary sites of plant biomass degradation.

The predominance of Proteobacteria, Bacteroidetes, and Firmicutes across the GIT aligns with previous reports describing these phyla as major contributors to lignocellulose degradation in herbivorous animals. Importantly, the high abundance of GHs identified through CAZyme profiling highlighted the considerable hydrolytic potential of the goat microbiome. Glycoside hydrolases are key enzymes that depolymerize cellulose and hemicellulose, making them fundamental to lignocellulosic biomass conversion. The elevated abundance of CAZymes in the reticulum and omasum further suggests that these compartments serve as important reservoirs of lignocellulose-degrading microorganisms. Collectively, these findings support the hypothesis that the goat GIT is a valuable, largely underexplored source of microbial resources for biotechnological applications, particularly in biomass conversion and renewable energy production.

Building on these findings, Chapter 4 focused on translating microbial diversity into practical biotechnological applications by isolating and characterizing cellulolytic bacteria. Three cellulase-producing strains were successfully isolated and identified, with KC94 emerging as the most promising candidate. The successful isolation of a highly cellulolytic *P. mirabilis* strain is noteworthy, particularly because reports describing cellulolytic optimization in goat-derived *P. mirabilis* remain limited. The strain exhibited strong cellulase production under moderate cultivation conditions, demonstrating optimal activity at pH 7 and 35°C. These conditions are advantageous for industrial implementation because they minimize energy requirements and reduce operational costs compared with thermophilic systems.

The physiological characterization of KC94 revealed an organism capable of maintaining cellulolytic activity across a relatively broad range of environmental conditions. Such adaptability is desirable for industrial processes, where fluctuations in operating conditions are often unavoidable. Interestingly, mild oxidative stress stimulated cellulase production, suggesting that stress-response mechanisms may influence enzyme synthesis and secretion. Similar responses have been reported in other cellulolytic microorganisms, where reactive oxygen species act as signaling molecules that trigger adaptive metabolic pathways. In contrast, the inhibitory effects of organic solvents and surfactants indicate that membrane integrity and enzyme stability remain important considerations for process optimization.

The molecular characterization of KC94 further reinforced its lignocellulose-degrading potential. The successful amplification of the GH39, GH45, and GH48 genes confirmed the presence of key glycoside hydrolase families involved in cellulose and hemicellulose degradation. These findings provide a direct link between the functional potential predicted from the metagenomic analyses in Chapter 3 and the experimentally verified cellulolytic capabilities observed in Chapter 4. Thus, the study effectively progressed from microbial community-level observations to the identification of a specific bacterial strain with demonstrated biotechnological value.

The application of KC94 for SCB pretreatment in Chapter 5 represented the culmination of the study and provided practical evidence supporting the use of goat-derived cellulolytic bacteria in bioenergy production. Biological pretreatment with KC94 significantly enhanced cellulose hydrolysis, as demonstrated by increased glucose release compared with untreated and sterilized controls. Enhanced glucose availability indicates improved cellulose accessibility and serves as an important precursor to subsequent AD processes. The increased hydrolysis

efficiency observed following only five days of pretreatment highlights the effectiveness of KC94 in rapidly modifying lignocellulosic substrates.

Structural analyses provided additional evidence supporting the effectiveness of biological pretreatment. Scanning electron microscopy revealed substantial disruption of the compact lignocellulosic matrix, including increased porosity, fiber separation, and surface erosion. These structural modifications likely improved microbial access to cellulose and hemicellulose during AD, thereby facilitating enhanced biodegradation. The observed changes are consistent with the action of cellulolytic enzymes identified in Chapter 4 and illustrate how microbial pretreatment can alter biomass recalcitrance without the need for harsh chemical or energy-intensive physical treatments.

Elemental analysis further demonstrated that KC94 pretreatment influenced substrate composition by improving nutrient balance and reducing sulfur content. The reduction in sulfur is particularly significant because sulfur-containing compounds can contribute to hydrogen sulfide formation during AD, negatively affecting biogas quality and causing corrosion of processing equipment. Therefore, the apparent biological desulfurization observed in this study may provide additional operational benefits beyond improved methane production.

The ultimate objective of biomass pretreatment is to improve bioenergy recovery, and the BMP results confirmed the effectiveness of the KC94 pretreatment strategy. Pretreated SCB produced significantly higher methane yields than both untreated and sterilized controls, while also exhibiting faster biomethanation kinetics. These findings demonstrate that enhanced hydrolysis directly translates into improved methane production, supporting the widely accepted relationship between substrate accessibility and AD performance. Although the observed methane yield improvements were moderate compared with some intensive chemical pretreatments reported in the literature, the biological approach offers distinct advantages, including lower energy requirements, reduced chemical consumption, and improved environmental sustainability.

Taken together, the findings from Chapters 3 to 5 establish a clear link between microbial ecology, enzyme functionality, and bioenergy production. The study demonstrates that the goat gastrointestinal microbiome is a valuable source of cellulolytic microorganisms capable of overcoming key barriers to lignocellulosic biomass conversion. Through a systematic progression from microbial community characterization to strain isolation and practical

application, this research provides evidence that biologically derived pretreatment strategies can effectively enhance SCB biodegradability and biomethane production.

From a broader perspective, the study contributes to the growing body of research supporting the integration of microbial biotechnology into circular bioeconomy frameworks. By converting an abundant agricultural residue into a valuable renewable energy resource using naturally occurring microorganisms, the approach aligns with global efforts to reduce dependence on fossil fuels, minimize agricultural waste accumulation, and promote sustainable energy generation. Furthermore, the identification of KC94 as a robust cellulolytic bacterium underscores the potential to develop tailored microbial inoculants and enzyme systems for lignocellulosic biorefineries.

6.2 Conclusion

This study investigated the potential of the gastrointestinal microbiome of South African goats as a source of cellulolytic microorganisms and CAZymes for biological pretreatment of SCB and for enhancing biomethane production. Metagenomic analysis revealed that the goat GIT harbors a diverse, functionally specialized microbial community rich in CAZymes, particularly glycoside hydrolases involved in lignocellulose degradation. The distribution of these enzymes across different gastrointestinal compartments demonstrated the adaptive nature of microbial communities in response to substrate availability and environmental conditions.

Building upon these findings, cellulolytic bacteria were successfully isolated and characterized. Among the isolates, KC94 exhibited superior cellulase production, environmental adaptability, and possession of key glycoside hydrolase genes associated with lignocellulose degradation. These characteristics identified KC94 as a promising candidate for biomass pretreatment applications. Application of KC94 as a biological pretreatment agent significantly improved the hydrolysis of SCB, resulting in increased glucose release, substantial structural modification of the lignocellulosic matrix, improved nutrient composition, and reduced sulfur content. These changes enhanced substrate accessibility during AD, resulting in higher methane yields and faster biomethanation rates than in untreated controls.

Collectively, the findings demonstrate that microorganisms originating from the goat GIT represent valuable biological resources for sustainable bioenergy production. The study establishes a clear link between microbial diversity, enzymatic functionality, biomass pretreatment, and renewable energy generation. Furthermore, it highlights the potential of environmentally friendly biological pretreatment strategies as alternatives to conventional chemical and physical methods.

By integrating microbial ecology, biotechnology, and AD, this research advances circular bioeconomy principles by valorizing agricultural residues into renewable energy. The successful application of KC94 provides a foundation for future developments in microbial pretreatment technologies and supports the broader goal of achieving sustainable and efficient biomass utilization.

6.3 Recommendations and Future Research

This study makes important progress toward understanding the functional potential of goat GIT microbiota for biomass degradation and bioenergy production. The isolation of KC94 and its demonstrated cellulolytic capacity provide a strong platform for future biotechnological applications. To advance this work, future studies should focus on scaling up KC94-based pretreatment in pilot- and industrial-scale bioreactors to assess technical feasibility, operational stability, and economic viability. Prior to upscaling, however, the pathogenicity of KC94 should be thoroughly verified to ensure biosafety and sustainable application. Integrating microbial pretreatment with mild chemical or enzymatic methods may further enhance substrate digestibility while reducing reliance on energy-intensive or environmentally harmful processes.

Further research should also expand the investigation of microbial mechanisms underlying lignocellulose degradation. While this study focused on individual isolates, microbial consortia are likely to provide synergistic enzyme systems that outperform those of single strains. Omics-based approaches, such as metagenomics, transcriptomics, and proteomics, could reveal additional carbohydrate-active enzymes and pathways of industrial value. Moreover, the cellulase system of KC94 warrants further molecular investigation. Whole-genome sequencing could provide detailed insights into the genetic architecture associated with cellulase production, stress tolerance, substrate utilization, and metabolic regulation. Such

information could facilitate future strain improvement strategies and the development of tailored enzyme formulations.

Future research should also evaluate the performance of KC94 using a wider range of agricultural residues, including maize stover, wheat straw, rice straw, sorghum residues, and forestry wastes. Comparative studies would help determine the strain's versatility and commercial applicability across different biomass resources. Lastly, given the observed reduction in sulfur content following pretreatment, future studies should investigate the mechanisms underlying KC94-mediated biological desulfurization. Understanding these pathways may help develop integrated pretreatment systems that simultaneously improve methane yield and biogas quality.

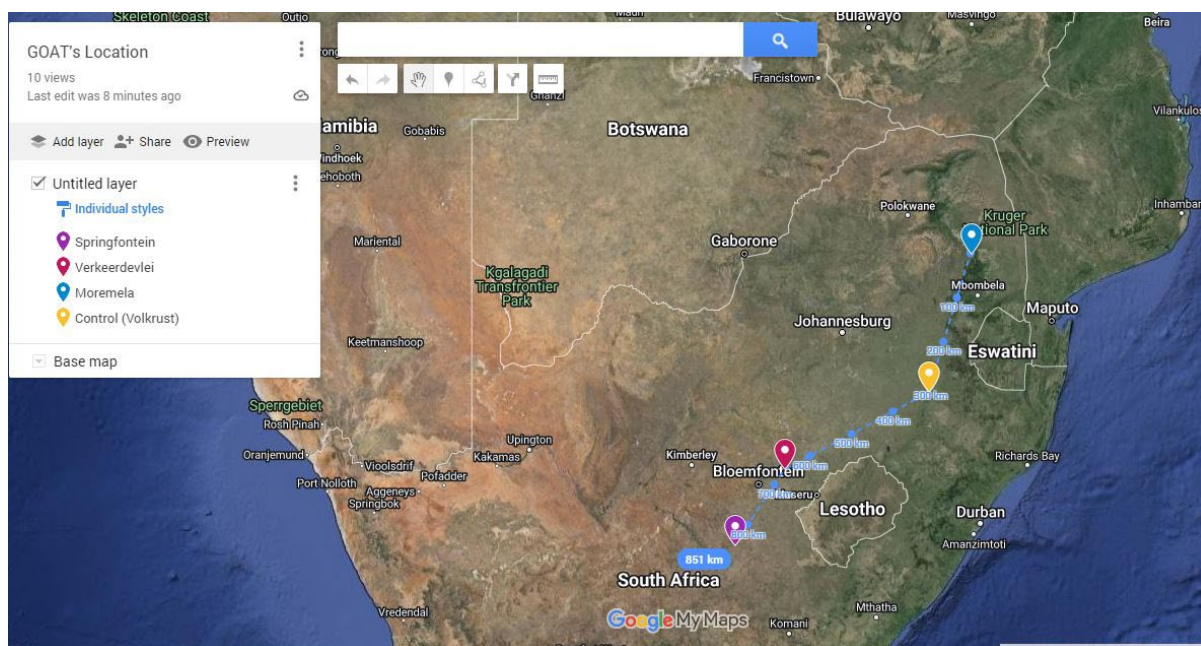
ADDENDUMS

Appendix A3.1: Biological samples information

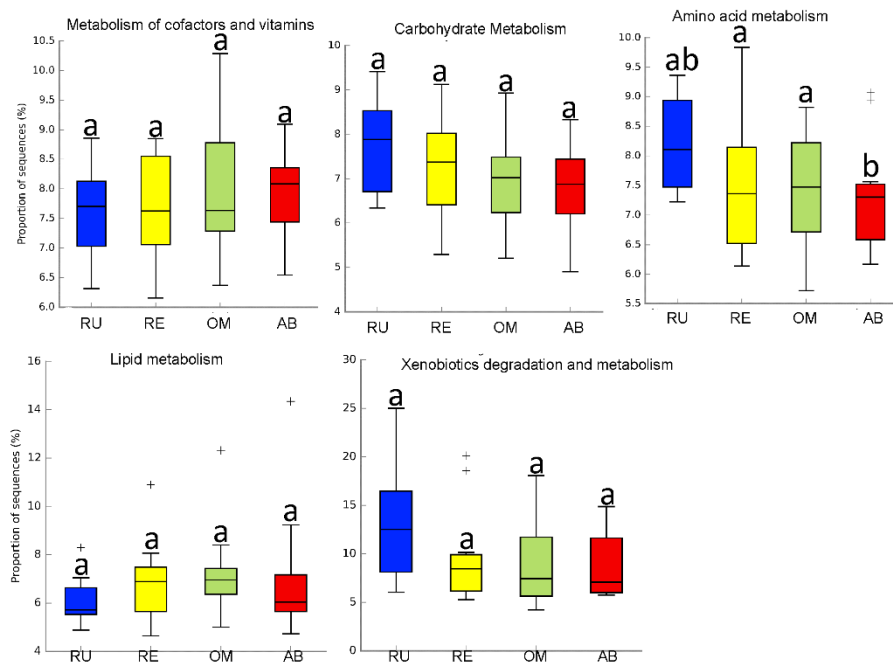
Goat #	Weight (kg)	# of Teeth	Location	C1	C2	C3	C4
1	27,8	7	Springfontein (30°15'14"S 25°42'14"E)	G1-RU	G1-RE	G1-OM	G1-AB
2	27,2	8		G2-RU	G2-RE	G2-OM	G2-AB
3	40,2	8		G3-RU	G3-RE	G3-OM	G3-AB
4	32,7	8	Verkeedevlei (28°50'S 26°47'E)	G4-RU	G4-RE	G4-OM	G4-AB
5	30,9	8		G5-RU	G5-RE	G5-OM	G5-AB
6	31,8	8		G6-RU	G6-RE	G6-OM	G6-AB
7	28,6	8	Moremela (24.6663°50'S 30.801°E)	G7-RU	G7-RE	G7-OM	G7-AB
8	33,2	8		G8-RU	G8-RE	G8-OM	G8-AB
9	29,1	8		G9-RU	G9-RE	G9-OM	G9-AB
10	33,8	8	Volkrust (27°22'S 29°53'E)	G10-RU	G10-RE	G10-OM	G10-AB
Key- ^{C1} :Rumen ^{C2} : Reticulum ^{C3} :Omasum ^{C4} : Abomasum							

Appendix A3.2: Nutritional Information for GOAT 10

Composition			
	Min	Max	Unit
Crude Protein	160	-	g/kg
Prot ex NPN	-	18	%
Urea	-	3	g/kg
Moisture	-	120	g/kg
Crude fat	25	70	g/kg
Crude fibre	120	150	g/kg
Calcium	10	15	g/kg
Phosphorus	3	-	g/kg



Appendix A3.3: Location of the goats on the map



Appendix A3.4: Comparison of the five most prevalent gene families among all the compartments. The boxplots with different superscripts were significantly different based on the Kruskal-Wallis H-test and the Tukey-Kramer post hoc tests

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Research article

Profiling the dynamic adaptations of CAZyme-Producing microorganisms in the gastrointestinal tract of South African goats

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Keywords:
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ABSTRACT

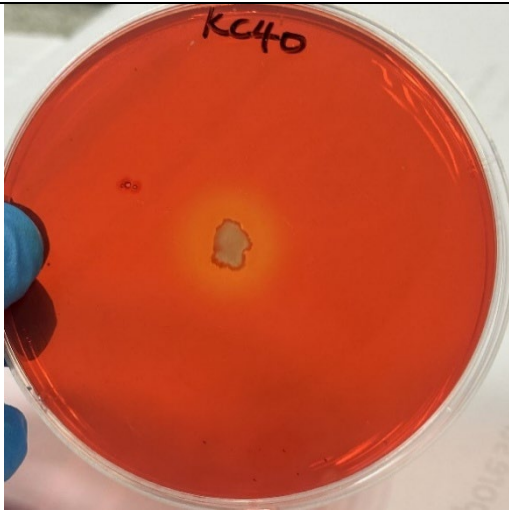
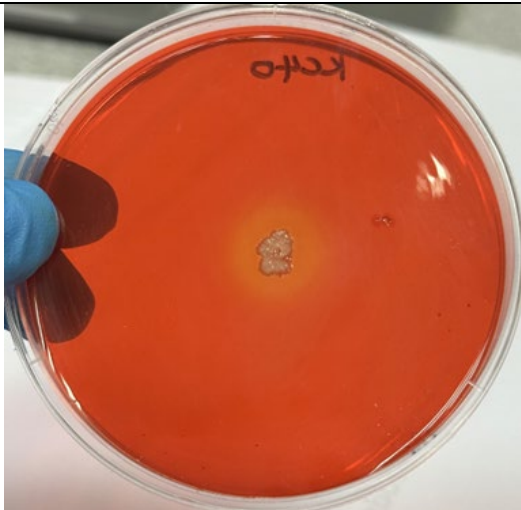
The gastrointestinal tract of goats serves as a habitat for anaerobic microbial populations that work together to break down complex plant material, including lignocellulose. This study explored the microbial diversity and metabolic profiles across different gastrointestinal tract compartments. Significant diversity differences among the compartments were observed (ANO-SIM $p < 0.006$), with the abomasum showing a distinct species composition and a decreased alpha diversity (Mann-Whitney/Kruskal-Wallis test $p = 0.00096$), possibly due to its acidic environment. Dominant microbial phyla included Proteobacteria, Bacteroidetes, and Firmicutes, with Proteobacteria being the most prevalent in the abomasum (50.06 %). Genera like *Proteus* and *Bacteroides* were particularly prominent in the rumen and reticulum, highlighting their significant role in feed degradation and fermentation processes. Over 65 % of genes at Kyoto Encyclopedia of Genes and Genomes level 1 were involved in metabolism with significant xenobiotic biodegradation in the abomasum. The dbCAN2 search identified Glycoside Hydrolases as the most prevalent CAZyme class (79 %), followed by Glycosyltransferases, Polysaccharide Lyases, and Carbohydrate Esterases, with Carbohydrate-Binding Modules and Auxiliary Activities accounting for 1 % of the hits. Higher CAZyme abundance was observed in the reticulum and omasum compartments, possibly due to MAGs diversity. In conclusion, the gastrointestinal tract of South African goats harbors diverse CAZyme classes, with Glycoside Hydrolases predominating. Interestingly, higher CAZyme abundance in specific compartments suggested compartmentalized microbial activity, reflecting adaptation to dietary substrates.

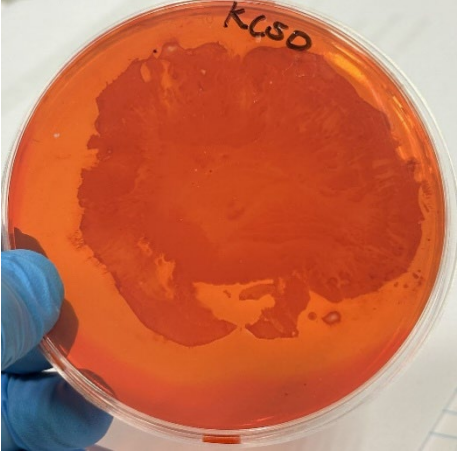
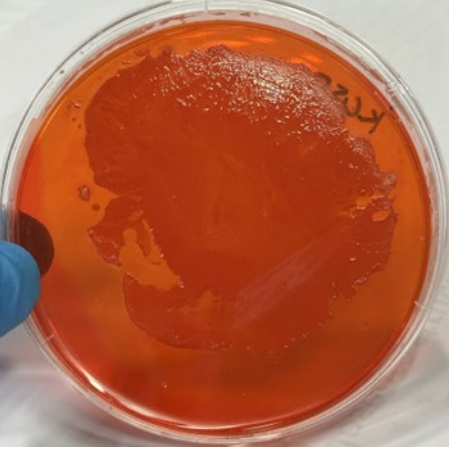
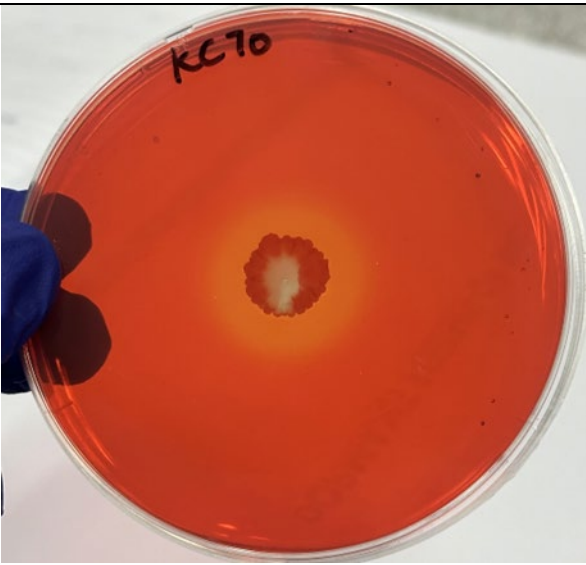
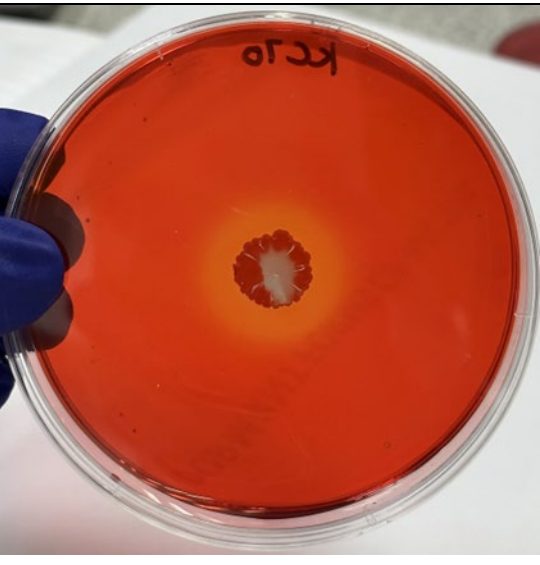
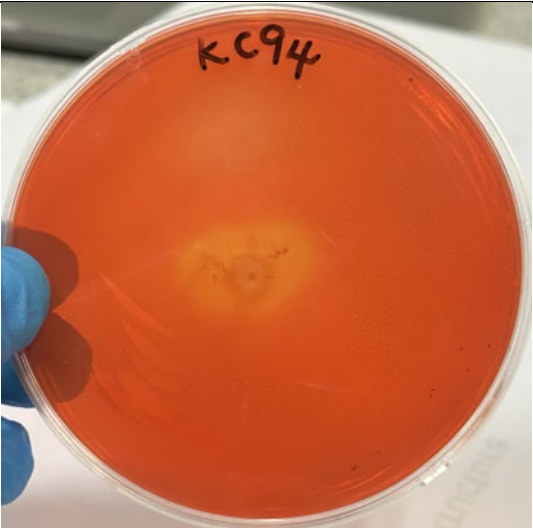
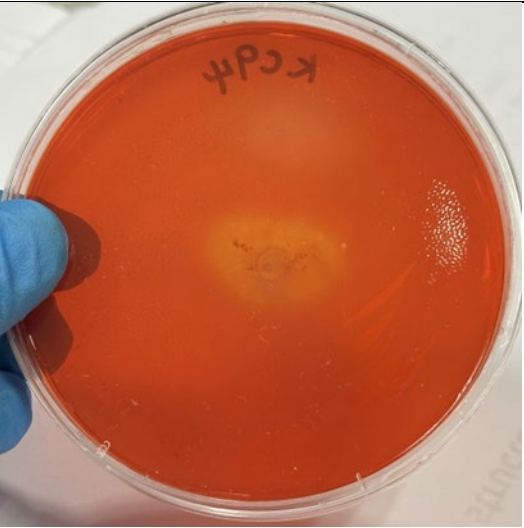
Appendix A3.5: Screenshot that shows the manuscript is published in the Heliyon Journal.

Appendix A4.1: List of media Composition

Name	Composition	Ref
Cellulase production media	(g/L) Carboxymethyl Cellulose (CMC) (10), peptone (10), NaCl (1), CaCl ₂ (0.005), MgSO ₄ (0.82), K ₂ HPO ₄ (1.25), KH ₂ PO ₄ (3), FeSO ₄ (0.01), ZnSO ₄ (0.005), MnCl ₂ (0.0001), and NH ₄ Cl (1)	(Singh <i>et al.</i> , 2014, Wei <i>et al.</i> , 2011)
CMC Agar	Bacteriological agar (5 g/L), CMC (5 g/L)	

Appendix A4.2: Qualitative Assay showing cellulase activity on 0.5% CMC agar

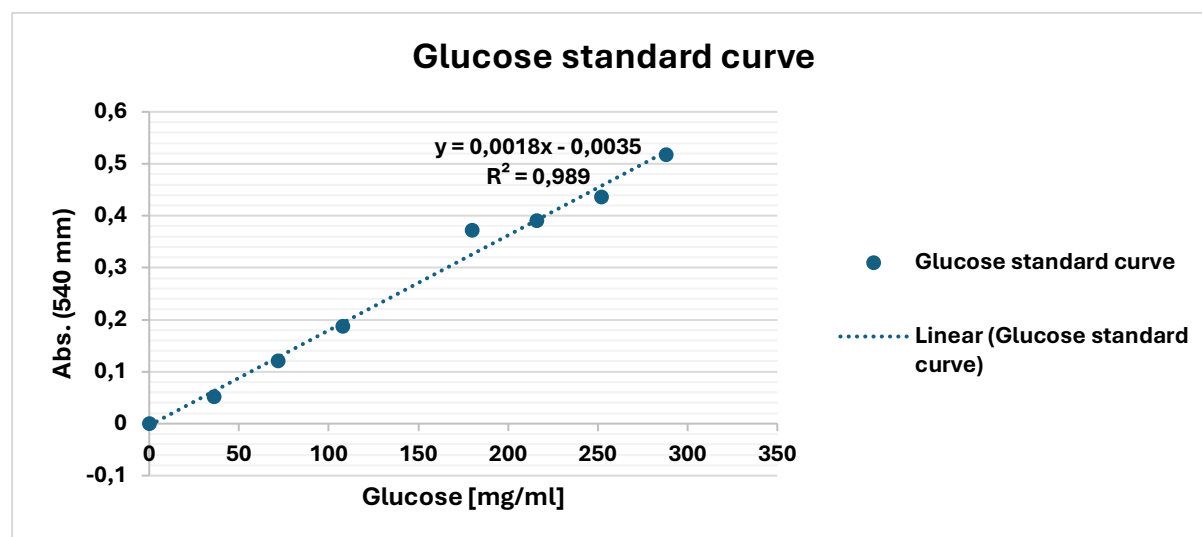
Strain	Back	Front
KC40		

KC50		
KC70		
KC94		

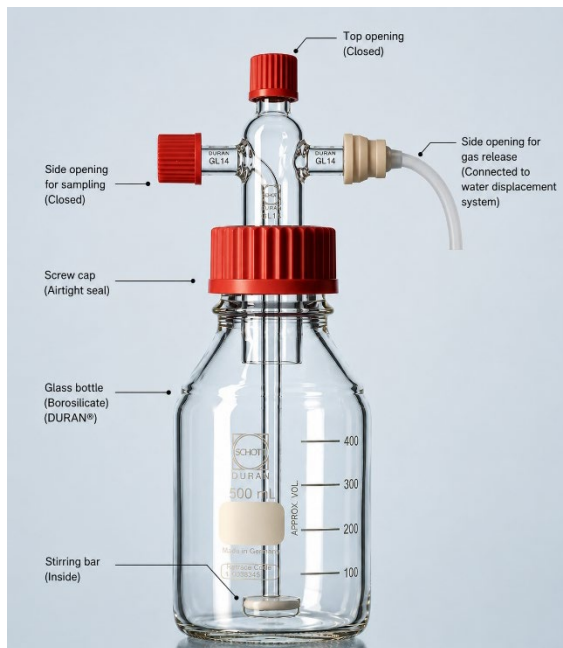
Appendix A4.3: Operational taxonomic units of bacterial isolates clustered at 98% similarity. The identities of the representative isolates are presented at the genus level. The accession numbers for each isolate are also listed.

Representative (OTU)	OTUs (Accession Number and Sample ID)	Frequency	Genera
NR180419.1	SJ02; LMN103; JCM; LMN103; KOD; KC70; OTG022; NR180419.1; ZHX3; SK2; YNB13; KC50; IS225; HBUAS64135; KC40; BFAM11; HBUAS69901; HBUAS73063; HBUAS67304;	18	<i>Bacillus</i>
D53	swupm3; D53; beavul.ww1; S74-3-2; KC94; gut_R1_BHI_10-3_3A2;	6	<i>Proteus</i>
IRG-1	IRG-1	1	<i>Psuedomonas</i>

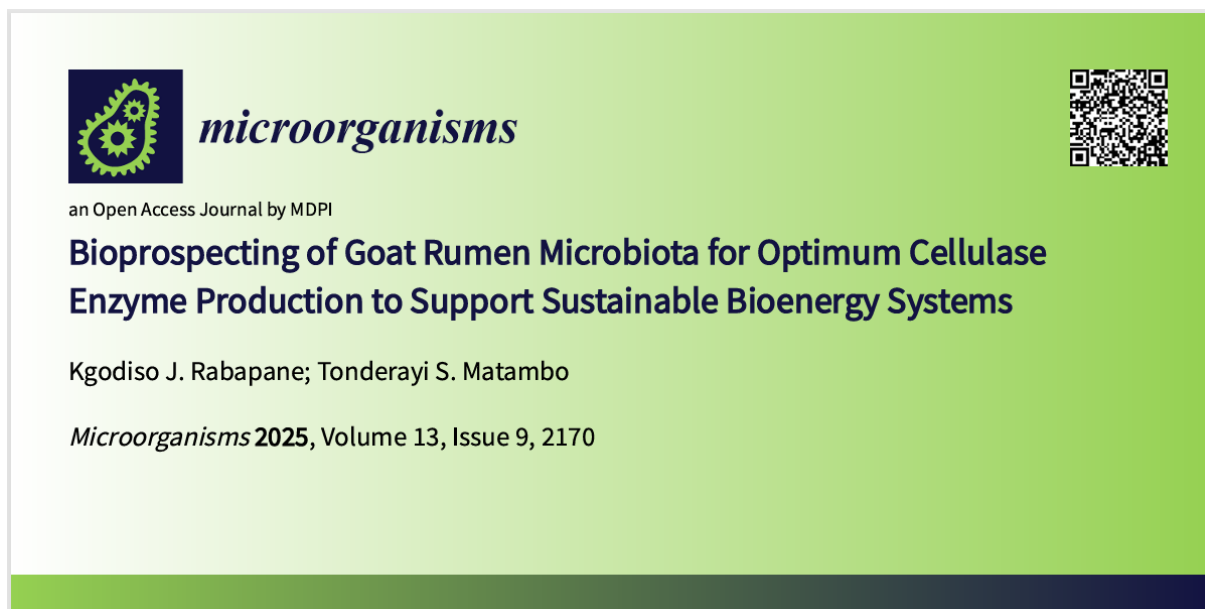
Appendix A4.4: The glucose standard curve used for the glucose quantification.



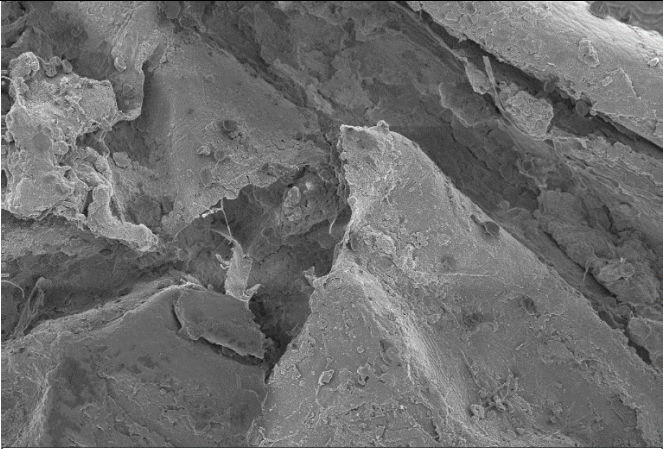
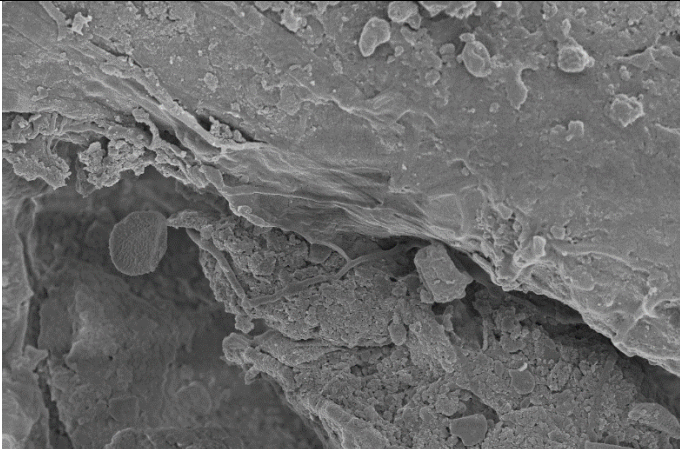
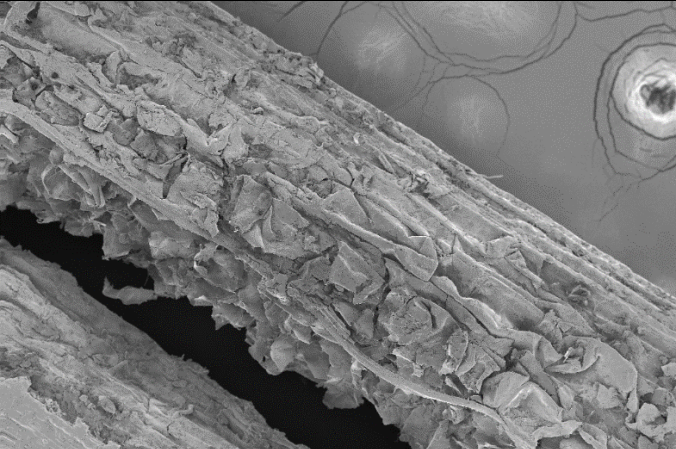
Appendix A4.4: The schematic of the reactors used to ensure anaerobic conditions during enrichment.



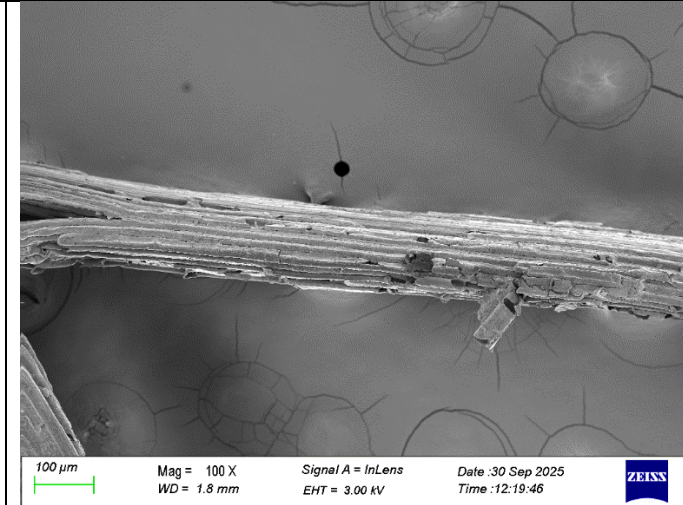
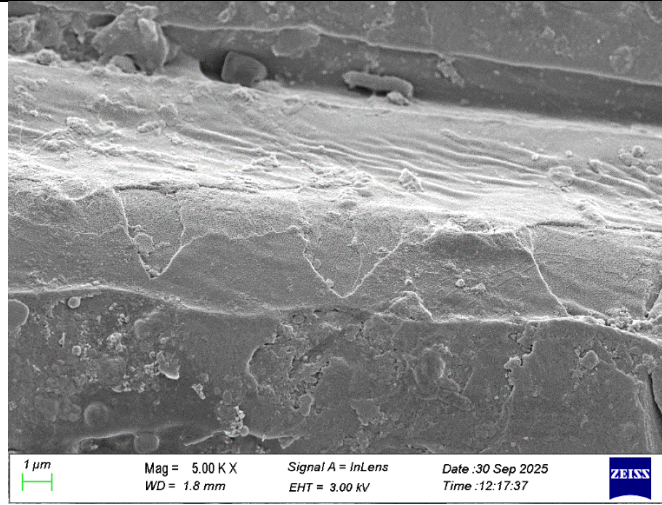
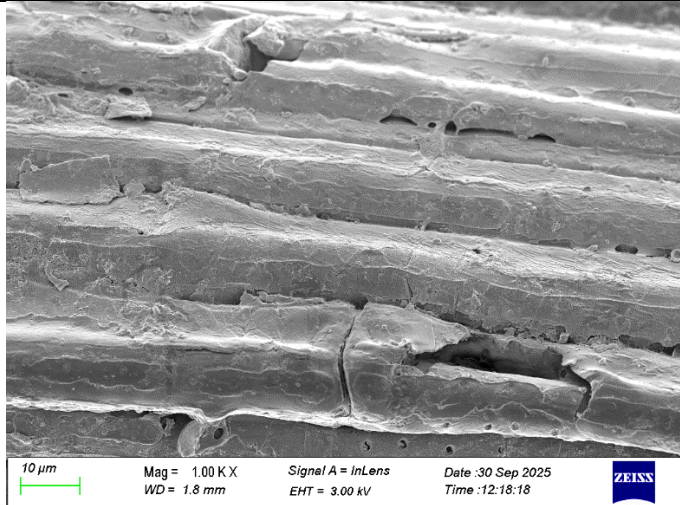
Appendix A4.6: The article banner that shows the manuscript is published in the MDPI-Microorganisms Journal



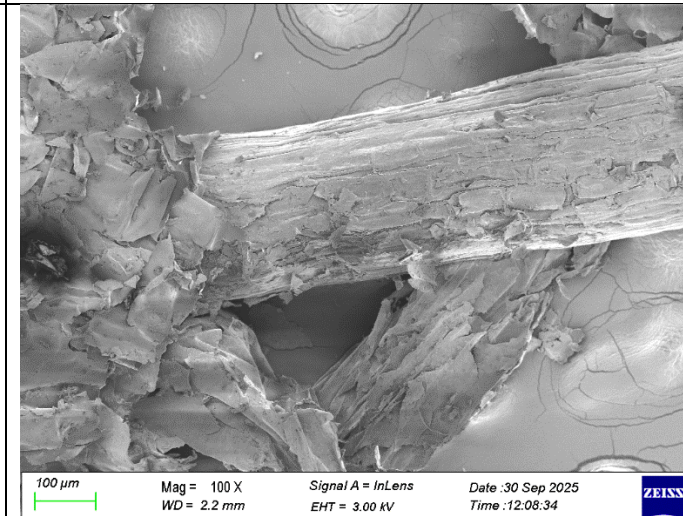
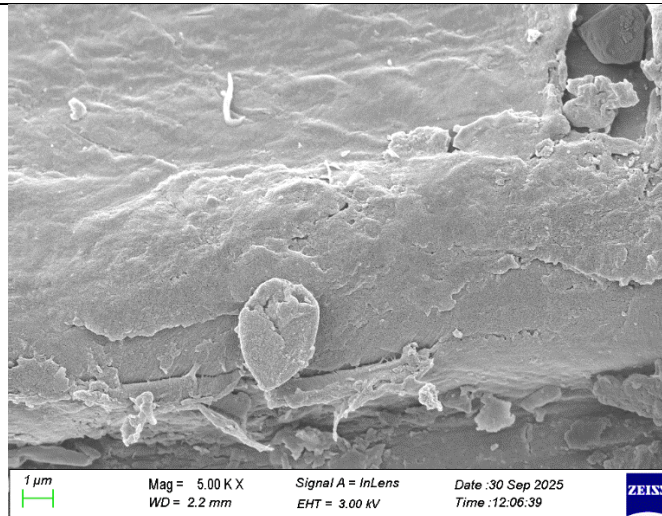
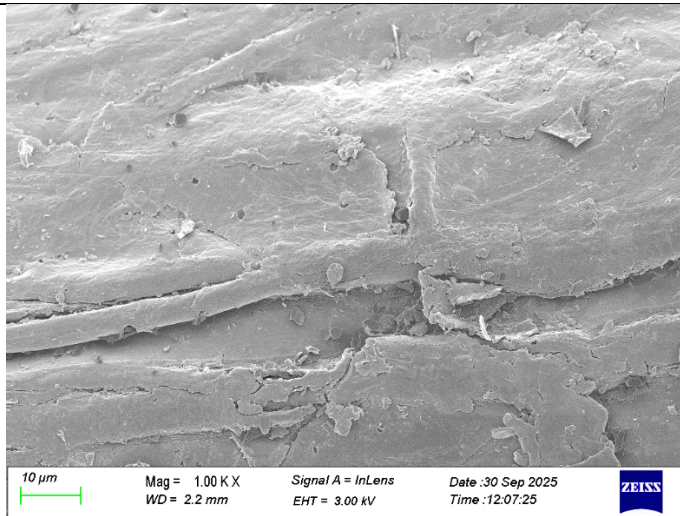
Appendix A5.1. Scanning electron micrographs of untreated bagasse (UB), sterile bagasse (SB), and biologically pretreated bagasse (PB) at magnifications of 1 kX, 5.00 kX, and 100 X. The images illustrate progressive structural disruption, surface roughening, and fiber delamination in PB compared to the intact morphology of UB and SB.

ID	MAGNIFICATION		
	1 kX	5.00 Kx	100X
UBB	 10 µm Mag = 1.00 K X Signal A = InLens Date :30 Sep 2025 WD = 2.0 mm EHT = 3.00 kV Time :12:31:43 ZEISS	 1 µm Mag = 5.00 K X Signal A = InLens Date :30 Sep 2025 WD = 2.0 mm EHT = 3.00 kV Time :12:30:59 ZEISS	 100 µm Mag = 100 X Signal A = InLens Date :30 Sep 2025 WD = 2.0 mm EHT = 3.00 kV Time :12:32:49 ZEISS

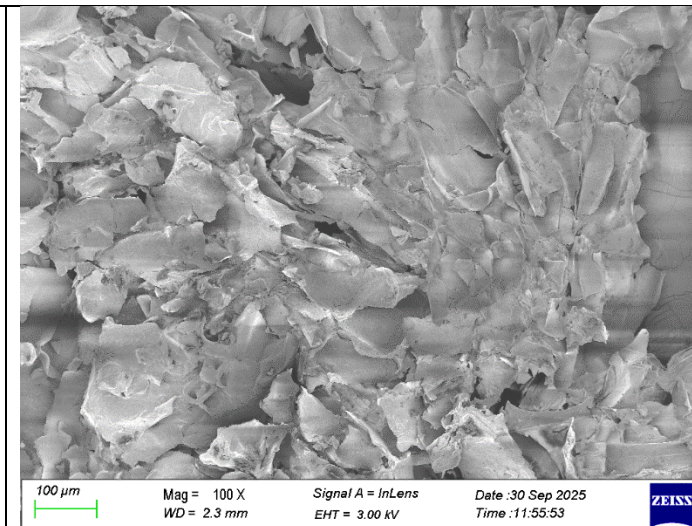
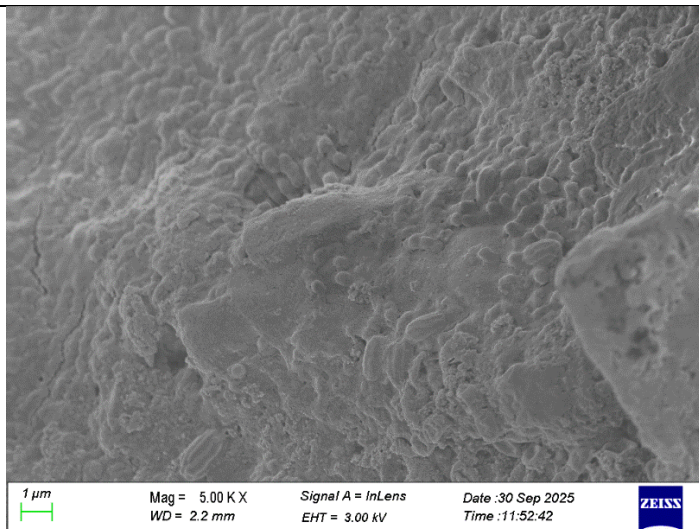
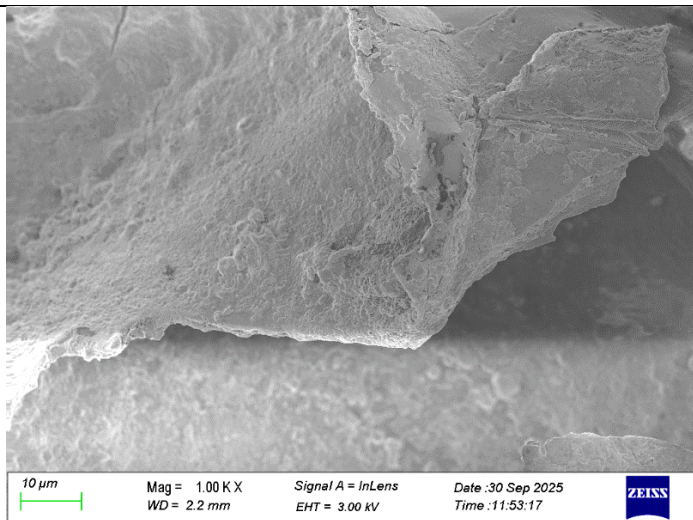
UBA



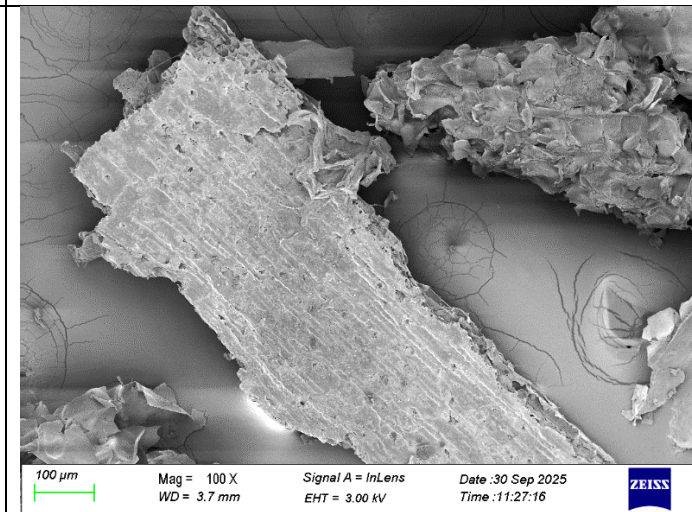
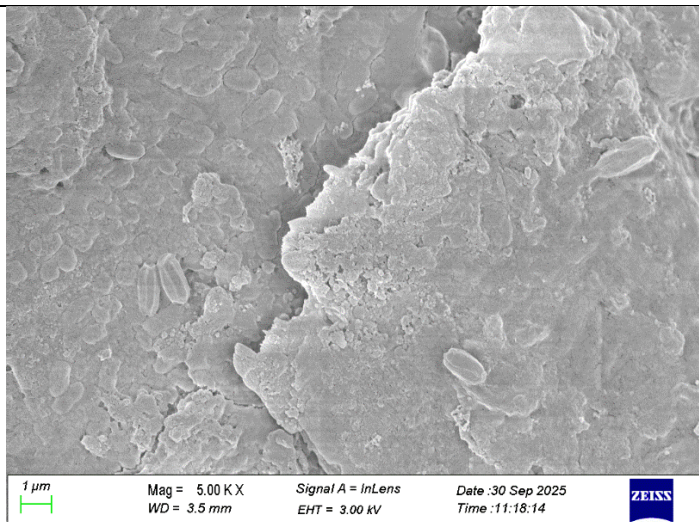
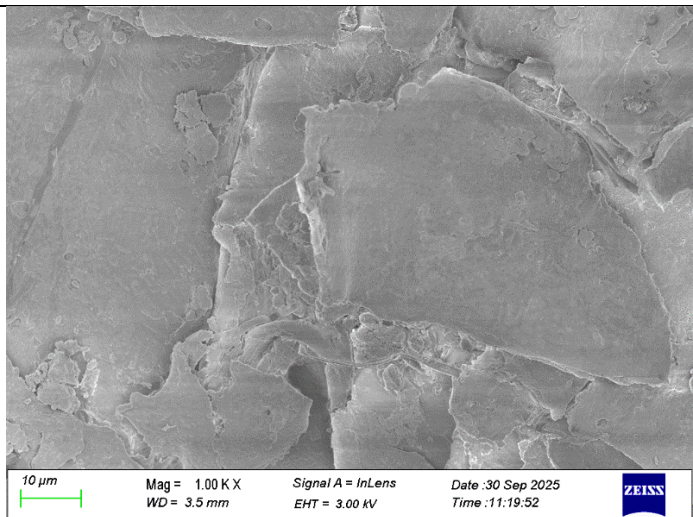
SBB



SBA



PB



Appendix A5.2: CHNS analysis run in triplicate using the CHNS analyser.

Sterile Bagasse Before				
Run	C	H	N	S
1	43,5	6,169	0	0
2	43,76	6,162	0	0
3	43,85	6,174	0	0
Sterile Bagasse After (SBA)				
Run	C	H	N	S
1	36,53	5,209	1,11	1,22
2	36,12	5,152	1,14	1,268
3	36,79	5,278	1,1	1,255
Pretreated Bagasse (PB)				
Run	C	H	N	S
1	40,26	5,598	1,24	0
2	40,76	5,691	1,22	0
3	40,64	5,564	1,27	0

Appendix A5.3: Glucose assay of the hydrolysate done in triplicate using the DNS assay

Sample	Activity Before Pretreatment			Activity After Pretreatment		
	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
UB	19	19,25	19,14	53	53,4	53,17
SB	4	3,95	4,02	44,1	43,9	44
PB	4	3,95	4,02	61,7	61,9	61,89

Appendix A5.4:Automatic Methane Potential Testing System (AMPTS®II) Bio-Methane raw data on a daily basis

Normal Daily Production (Bio-Methane)															
Day 1	UB Volume [Nml]			SB Volume [Nml]			Glucose Volume [Nml]			PB Volume [Nml]			Inoculum Volume [Nml]		
Day	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	61.4	60.9	61.7	86.1	88.6	88.3	258	251.9	256.2	62.63	64.57	66.54	57.8	62.7	60.4
2	142.3	155.2	149.1	208.5	211.6	210.8	1122.4	1092.6	1108.8	190.56	193.47	196.37	140.4	165.4	154.1
3	199.9	218.5	211.3	259.1	261.6	261.2	1636.2	1589.8	1608.1	286.06	288.97	291.87	188.4	215.1	204.3
4	246.9	269.1	255.2	299.4	301.9	301.3	1852	1831.1	1842.6	356.9	359.83	362.73	221.7	251.2	239.6
5	289.5	318	304.4	344.5	347.6	346	1932	1908.3	1924	422.39	425.3	428.21	251.9	286.2	268.7
6	327.4	360.7	346.3	386.3	389.3	387.8	1999.8	1973.9	1986.7	478.12	481.03	483.94	274.1	319.2	298.1
7	360.2	395.4	377.6	417.3	420.4	418.9	2050.9	2025.5	2038.2	519.19	522.1	525.01	291.6	339.8	316.4
8	392	429.6	410.8	442.3	445.4	443.9	2095.5	2067.7	2084.3	554.99	557.9	560.81	308.4	354.6	333.2
9	421.1	460.8	438.4	465.1	468.2	466.7	2137.3	2105.3	2122	584.72	587.63	590.54	323.8	368.4	350.7
10	442.8	484.2	463.1	482.2	485.3	483.7	2170.4	2135.2	2153.9	605.97	608.93	611.9	332.1	377.8	358.4
11	466.6	509.6	487.4	503.7	506.8	505.2	2204	2166.1	2184.2	627.3	630.27	633.26	342.8	387.5	368.3
12	489.6	533.3	511	527.5	530.5	529	2237	2197	2219.6	646.56	649.53	652.52	353.2	398	380.6
13	513.6	555.8	532.3	552	555.1	553.5	2267	2225.8	2246.7	665.52	668.5	671.48	363.4	407.9	392.1
14	534.4	576.4	553.1	576.9	580	578.5	2293.8	2250	2274.5	682.22	685.17	688.11	372.5	416.4	402.3
15	556.3	597.1	576.8	601.9	605	603.5	2317.4	2273.2	2294.9	698.62	701.57	704.51	380.1	425.5	411.5
16	580.7	619.6	599.3	627.9	631	629.4	2342	2296.6	2320.4	718.12	721.07	724.02	389.4	435.7	420.6
17	598.6	636.6	618.4	646.6	649.7	648.1	2357.9	2311.9	2332.6	732.39	735.33	738.34	395.7	441.8	427.4
18	618.3	654.5	635.6	665.1	668.2	666.5	2373.4	2326.9	2351.7	747.16	750.1	753.04	399.7	447.3	432.5
19	639.2	673.5	659.1	682.8	685.9	684.4	2390.3	2341.7	2365	763.9	766.83	769.73	403.4	453.3	437.1
20	655.4	688.4	673	695.5	698.6	697	2400.4	2353	2379.2	775.83	778.77	781.69	407	458.3	442.8
21	673.7	705.4	691.1	708	711.1	709.5	2413.3	2363.6	2387.1	789.47	792.4	795.33	410.6	462.8	447.9
22	691.3	721.7	706.2	721.1	724.2	722.5	2426.7	2375.6	2400.5	803.68	806.63	809.63	414.6	469.4	453.5
23	708.4	737.9	726.5	732.7	735.8	734.2	2437.2	2388.1	2413.9	816.07	819.07	822.06	420.4	476.5	460.3

24	723.3	753.8	742.7	743.9	747	745.4	2448.8	2397.6	2423.5	828.03	831.03	834.07	423.6	482.9	466.2
25	736.8	766.6	755.4	753.3	756.4	754.8	2457.8	2408.6	2432.4	837.2	840.2	843.2	426.9	486.8	470.4
26	750	778.4	765.8	762.6	765.7	764.1	2466.7	2416.5	2443.6	845.35	848.33	851.32	429.5	490.6	474
27	764.1	791.2	777.9	772.9	776	774.4	2476.3	2425.2	2454.1	853.93	856.93	859.93	431.9	495	478.3
28	777.8	804	790.6	781.9	785	783.4	2485.6	2436	2462.8	861.96	864.97	867.97	434	496.9	481.1
29	790.9	814.2	802.3	789.5	792.6	791	2495.5	2443.9	2472.3	868.8	871.83	874.83	436	498.5	483.7
30	808.9	825.1	814.6	799.9	798.9	799.9	2503	2450.2	2480.8	874.27	877.3	880.3	435	501.3	484.9
31	808.9	822.5	815	800.72	801	805.93	2557	2455	2486	880.36	883.33	886.3	435	501.3	484.9

Key -*AS – Activated Sludge

Flow [Nml/day]															
Day	UB			SB			Glucose			PB			Inoculum (*AS)		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	61.4	61.7	60.9	85.6	88.9	87.3	258	251.9	255.7	62.9	64.8	66.6	57.8	60.4	62.7
2	80.9	87.1	94.3	122.7	123.4	122.1	864.5	840.6	853.3	128.1	129.4	130.2	82.6	92.4	102.6
3	57.6	60.2	63.3	51.2	49.8	50.9	513.8	497.2	505.6	95.3	94.7	96.1	48	48.8	49.7
4	46.9	48.7	50.6	40.1	41.4	39.4	215.8	241.3	228.4	70.7	71.2	70.8	33.3	34.6	36.1
5	42.6	45.3	48.9	45.5	44.8	45	79.9	77.2	78.8	65.7	65.3	65.6	30.2	32.5	34.9
6	38	40.1	42.7	41.9	42.3	41.5	67.9	65.6	66.9	55.9	56.1	55.4	22.2	27.0	33
7	32.8	33.9	34.7	38.9	37.1	36.3	51	51.6	51.4	47.1	37.1	39.2	17.5	18.9	20.6
8	31.8	33.2	34.1	33.5	34.1	32.3	44.6	42.3	43.7	39.9	38.3	36.2	16.8	15.9	14.8
9	29.2	30.0	31.2	28.8	26.8	27.7	41.8	37.5	39.8	33.1	27.5	28.5	15.4	14.6	13.8
10	21.6	22.5	23.4	20.5	18.8	19.5	33.2	29.9	31.8	25.1	19.3	19.5	8.3	8.8	9.4
11	23.8	24.6	25.4	22.2	21	21.4	33.6	30.9	32.4	23.7	19.7	20.7	10.8	10.3	9.7
12	23.1	23.5	23.7	23.1	25.7	22.3	33	30.9	31.8	21.3	18.3	18.2	10.3	10.4	10.5

13	24	23.3	22.5	24.5	26.4	22.9	30	28.9	29.4	20.3	18.5	18	10.2	10.1	9.9
14	20.8	20.7	20.5	25.1	24.2	25.5	26.8	24.2	25.5	19.5	15.7	14.8	9.1	8.8	8.5
15	21.9	21.2	20.7	26.9	22.2	26	23.7	23.2	23.5	17.8	16.6	14.8	7.6	8.3	9.2
16	24.4	23.6	22.5	29.1	21.2	27.5	24.5	23.3	24.0	21	19.1	18.3	9.3	9.8	10.2
17	17.9	17.6	17	21.8	13.4	21	15.9	15.3	15.6	16.2	14.6	12	6.2	6.2	6.1
18	19.7	18.9	17.9	21.1	13.6	20.7	15.6	15	15.3	16.4	15.1	12.8	4.1	4.8	5.4
19	21	20.0	19	20.9	17.2	19.1	16.9	14.8	15.8	19.4	15.6	15.3	3.7	4.9	6.1
20	16.2	15.6	14.9	15.2	12.9	14	10.1	11.3	10.7	14.5	10.8	10.4	3.6	4.3	5
21	18.3	17.7	17	14.8	15.4	13.3	13	10.6	11.9	16.1	12	12.9	3.6	4.1	4.5
22	17.6	17.0	16.3	14.3	12.4	13.6	13.4	12	12.6	16.8	13	12.8	4	5.2	6.7
23	17.1	16.8	16.1	13.5	12.6	12.8	10.5	12.5	11.5	15.8	10.8	10.8	5.8	6.5	7.1
24	14.9	15.4	15.9	13.1	10.6	9.9	11.5	9.6	10.6	13.9	10.3	11.6	3.3	4.8	6.4
25	13.6	13.3	12.8	9.5	8.9	9.8	9	10.9	9.8	11.7	7.8	8	3.3	3.5	3.8
26	13.2	12.7	11.8	11.8	8	8.1	8.9	7.9	8.5	9.6	7.3	7.6	2.5	3.1	3.8
27	14	13.5	12.8	13.2	8.1	9.7	9.6	8.7	9.2	10.4	7.5	7.9	2.5	3.3	4.4
28	13.8	13.4	12.8	8.3	8.7	11.9	9.3	10.8	9.9	9.9	7.5	7.2	2.5	2.9	3.5
29	13.1	11.9	10.2	7	6.1	9.6	9.9	8	8.9	7.8	6.2	6.1	2.5	2.7	2.8
30	12.5	11.8	10.9	6.1	6.1	7.3	8.1	7.1	7.7	7.9	5.5	5.4	2.5	2.7	2.8
31	12.5	11.7	10.5	5.5	6.5	5.0	8.0	7.3	7.0	7.3	6.6	7.0	2.5	2.7	2.8

Key -*AS – Activated Sludge

Appendix A5.5: Characterization of the SCB (before and after BMPs), KC94, and inoculum.

Properties	Added to reactors for BMPs		
	TS (%)	VS (%)	VS/TS
KC94	0,07	0,04	57
Sludge	96	89	93
UB	88	87	99
SB	86	82	95
PB	85	82	96

	Added to reactors for BMPs		Total VS (in reactor)
	TS (g)	VS (g)	
KC94	nd	nd	
Sludge	4,3	4	
UB	2,8	2,8	6,8
SB	2,9	2,8	6,8
PB	2,9	2,8	6,8

Remaining TS and VS in reactors after BMPs

TS (g)	TS (%)	VS Measured in Digestate (g)	VS %	Inoculum based (g)	Substrate based (g)	VS Reduction (%)
2,5	78	1,9	76	0,28	1,62	42
2,8	82	2	71	0,28	1,72	39
2,4	70	1,9	79	0,28	1,62	42

In each treatment group(UB, SB, and PB), a total of 6.8g VS was added to each reactor



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CERTIFICATE OF PUBLICATION

The certificate of publication for the article titled:
Assessing Sugarcane Bagasse Biomethanation After a Pretreatment with *Proteus mirabilis* KC94

Authored by:
Kgodiso J. Rabapane; Charles Rashama; Tonderayi S. Matambo

Published in:
Bioresour. Bioprod. **2026**, Volume 2, Issue 1, 1



Basel, February 2026

S. Tochev

Stefan Tochev
Chief Executive Officer

Appendix A5.4: The screenshot showing the certificate of manuscript publication by the Bioresource and Bioproducts Journal of MDPI.

UNISA-CAES ANIMAL RESEARCH ETHICS COMMITTEE

Date: 13/10/2025

Dear Ms Rabapane

NHREC Registration # : AREC-100818-024
REC Reference # : 2021/CAES_AREC/097
Name : Ms KJ Rabapane
Student # : 10459928

**Decision: Ethics Approval Renewal
after Fourth Review from
10/10/2025 to 30/09/2026**

UNISA SOE ETHICS REVIEW COMMITTEE

Date: 14/08/2020

Dear Ms Kgodiso Judith Rabapane

**Decision: Ethics Approval from
14/08/2020 to 14/08/2025**

ERC Reference # :
2020/CSET/SOE/028
Name : Ms Kgodiso Judith
Rabapane
Student # : 10459928
Staff # : N/A



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THE DIRECTOR: ANIMAL HEALTH
DEPARTMENT OF AGRICULTURE, LAND REFORM & RURAL DEVELOPMENT

RE: SECTION 20 REQUEST TO COLLECT SAMPLES AT PRETORIA ABATTOIRS

A request to collect samples was received from Ms. Kgodiso Judith Rabapane for the purposes of conducting research entitled *"Bioprospecting of the goat's GIT microorganisms for the overexpression of enzymes that are of high industrial value"* in partial fulfilment towards PhD studies at the University of South Africa (UNISA) supervised by Diane Hildebrandt.

The slaughtering and collection of the samples will take place at Klipeland Abattoir in Tshwane. Satisfactory methods of disposal as well as methods to prevent contamination have been provided to the State Veterinary office.

The State Veterinarian office has been made aware of the intention to collect samples and there is no objection to the study and sample collection being done within Gauteng as the necessary assurances to prevent disease as well as to ensure proper disposal of the samples are in place.

Regards




Dr S Kamudiyariwa (+27 60 960 5457)
Chief State Veterinarian
Veterinary Public Health And Export Facilitation
Pretoria

Appendix A6.1: CAES, CSET and Chief State Veterinarian proof of ethical clearance