CELLULOLYTIC ACTIVITY AND DIVERSITY OF BACTERIA ISOLATED FROM THE WHOLE BODY OF (*Macrotermes michaelseni)* FROM NANDI AND VIHIGA COUNTY

BY

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A THESIS SUBMITTED TO THE SCHOOL OF SCIENCE IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DEGREE OF MASTER OF SCIENCE IN MICROBIOLOGY,

UNIVERSITY OF ELDORET, KENYA

MARCH, 2022

DECLARATION

Declaration by the Candidate

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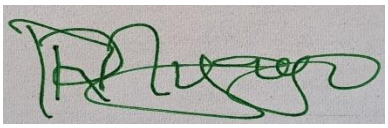

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DEDICATION

This thesis is dedicated to my loving and caring parents, Lameck Miyayo and Yunia Miyayo, my dear beloved twin, Samwel Miyayo and my lovely husband, Nicanor Sidho for their patience and understanding over the period of this study.

ABSTRACT

The accumulation of plant waste in the environment has increased recently since plants are not easily degraded. Consequently, there is a rising demand for efficient cellulolytic bacteria that can break done cellulose efficiently thus increasing the rate of enzymatic hydrolysis, fermentation, and product recovery. Termites are known have their ability of degrading plants with the help of cellulolytic bacteria in them. This project analyses the diversity of cellulolytic bacteria in termites (*Macrotermes michaelseni)* collected from different locations. Therefore, three *Macrotermes spp* termites were collected from Vihiga County and compared with three distinct termite hills in Nandi County. The cellulolytic bacteria were distinguished from non-cellulolytic bacteria using carboxymethyl cellulose media. Isolates containing cellulolytic bacteria were identified by amplifying the 16S rRNA gene and sequencing. The results showed that 17 isolates possessed cellulolytic activity based on formation of clear zone around their colony. The cellulolytic index values ranged from 1.50 to 5.80. The cellulolytic bacteria were identified as *Staphylococcus saprophyticus, Arthrobacter defluvii*, *Klebsiella oxytoca*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Bacillus thuringiensis*, *Serratia marcescens*, *Paenibacillus polymyxa*, *Bacillus cereus*, *Dietzia natronolimnae,* and *Exiguobacterium aurantiacum* strain VMG12. Among all isolated strains, *Paenibacillus polymyxa* showed the highest cellulolytic activity of 5.8. Therefore, bacteria from this study's findings, could be potential candidates for the degradation of cellulose, and hence could be employed to convert cellulose into valuable bio-products.

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LIST OF ABBREVIATIONS

%	Percentage
°C	Centigrade
ANOVA	Analysis of variance
BLAST	Basic local alignment search tool
CI	Cellulolytic index
CMC	Carboxymethyl cellulose
DNA	Deoxyribonucleic acid
LSD	Least significant difference
MAFFT	Multiple Alignment using Fast Fourier Transform
Mm	Millimeter
MUSCLE	Multiple sequence comparison by Log-expectation
NCBI	National Center of Biotechnology Information
NRF	National research fund
PCR	Polymerase chain reaction
rDNA	Ribosomal Deoxyribonucleic acid
RNA	Ribonucleic acid
SPSS	Statistical Package for the social sciences
TAE	Tris -Acetate-EDTA
UV	Ultraviolet radiation

ACKNOWLEDGMENTS

Most importantly, I thank God for enabling me to go through my education successfully, for the strength and wisdom, and good health He granted upon me during my years of study and accomplishment of this thesis.

My deepest appreciation goes to my supervisors late Dr. Lexa G. Matasyoh University of Eldoret and Dr. George Obiero from the University of Nairobi, for their constant encouragement and supervision of expert opinions over the period of this research study. I thank Dr. George On'gamo (The University of Nairobi) for his contribution in the identity of termites used in this research.

My sincere appreciation goes to the National Research Fund (NRF) for providing the resources to carry out this project. Additionally, I thank the Center for Biotechnology and Bioinformatics at the University of Nairobi for permitting me to carry out this project in their laboratory.

I wish to extend my gratefulness and special thanks to my friends and entire laboratory fellows in the Center for Biotechnology and Bioinformatics laboratory for their assistance, excellent cooperation, scientific knowledge, and skills that made my research a success. I'm greatly indebted to you all.

Lastly, I thank my parents, brother, and husband for forever encouraging, supporting and their endless prayers, God bless you all.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Sub-Saharan Africa is experiencing increasing pollution and garbage disposal issues as a result of increased population and poor solid waste management by the government, which has led in waste generation (Njenga et al., 2010). This is a major problem in Kenya, where the city of Nairobi alone creates 760,000 metric tons of waste each year from kitchen and garden waste, as well as agricultural and industrial waste. These wastes are typically dumped in landfills, allowed to burn, or scattered in drainage systems or disposed of in water, resulting in environmental pollution (Scheinberg et al., 2011). Moreover, the rising energy consumption and reduction of fossil fuels have shifted the focus of energy production using fossil fuel towards biofuel use (Dashtban et al., 2009; Sreena et al., 2016). Therefore, utilization of these natural resources will meet the energy demand in return leading to sustainable development.

The hindrance to the effective utilization of these plants is the complex structure of the plant cell wall that slows degradation (Sharma et al., 2015). Middle lamella, primary, and secondary cell walls are the three layers that make up a plant's cell walls (Kameshwar and Qin, 2016). The secondary cell wall is further grouped into complex structures known as cellulose, hemicellulose pectin, and lignin (Kameshwar and Qin, 2016; Sakolvaree and Deevong, 2016). Cellulose is the most important plant component, accounting for 20-50% of the dry weight (Patagundi et al., 2014). In contrast, hemicellulose and lignin

constitutes of (15–35%), and (18–35%) respectively (Ni and Tokuda, 2013). Each of these polymers has inherent complexity, and when combined, they make a resistance substance to bacterial attack (Auer et al., 2017).

For effective hydrolysis of cellulose to occur, pretreatment methods are used to eliminate lignin and hemicelluloses or break their connections with cellulose (Mtui and Nakamura 2009; Li et al., 2014) enabling the susceptibility of cellulose to enzyme action (Mtui and Nakamura 2009). Physical, physicochemical, chemical, and biological pretreatment are examples of these approaches. Although the chemical pretreatment method is commonly used, this method is expensive due to the cost of chemicals used (Saini et al., 2014), and it involves high temperatures and acid concentration that pose harsh conditions to the environment (Musatto and Teixeira, 2010).

Recently, researchers have shown an increased interest in biological pretreatment because microbes are highly efficient cellulose degraders that can degrade a broad variety of plant biomass (Liao et al., 2016; Wong et al., 2014). This process involves enzymatic hydrolysis with a highly specific process that can be performed under lower reacting conditions with lower energy consumption thus posing little threat to the environment (Liao et al., 2016). Therefore, this makes the use of the biological pretreatment method to be more effective and efficient compared with the other methods.

Cellulase enzyme facilitates the degradation of cellulose by hydrolyzing β-1–4 bonds in the polymer (Phuong et al., 2016). Microorganisms produce cellulases during their growth on a cellulosic material (Phuong et al., 2016; Patagundi et al., 2014). Bacteria, fungi, and actinomycetes harboring different environments have extensively been

researched for cellulose degradation. Although most commercial cellulase producers originate from fungi, current findings have adjusted their focus on bacteria (Auer et al., 2017; Sreena et al., 2016) because bacteria have a faster rate of growth than fungi, resulting in increased enzyme production (Patagundi et al., 2014; Pourramezan et al., 2012; Sreena et al., 2016). Additionally, bacterial enzymes are more effective catalysts that allow product recovery than for fungi (Pourramezan et al., 2012; Sreena et al., 2016). Furthermore, they can easily adapt to numerous specific genetic manipulations (Patagundi et al., 2014; Saini et al., 2017). Lastly, is their ability to inhabit diverse habitations that enables their resistance under extreme conditions (Pourramezan et al., 2012).

The degradation of cellulose in plants has long been a subject of great interest in scientific and industrial processes. Extensive studies have shown the ability of bacteria in soils, animals, and termites to degrade complex cellulose (Saini et al., 2014; Kameshwar and Qin, 2016 Sreeremya et al, 2016). However, the bacteria in termites are the most efficient cellulose degraders (Pourramezana et al., 2012; Kudo, 2016; Mikaelyan et al., 2015; Upadhyaya et al., 2012) as demonstrated through their ability to consume wood which is difficult to decompose in nature (Auer et al., 2017; Sakolvaree and Deevong, 2016; Batool et al., 2018). Termites effectively digest wood through the help of microbes in their guts that are involved in the carbon substrate, thus supplying termites with nutrients (Igwo-Ezikpe, et al., 2013; Muwawa et al., 2016; Sreena et al., 2014).

On the other hand, bacteria efficiently degrade cellulose because of the cellulase enzyme released during their growth on cellulosic material that acts as a carbon source. This

enzyme consequently has an extensive variety of applications in the productions of biofuel, animal feed, ethanol, food, paper, and textile industry (Phuong et al., 2016).

Several studies have been conducted to identify cellulolytic bacteria in termites around the world. For instance, a study by Igwo-Ezikpe et al., (2013) isolated six cellulolytic bacteria obtained from the guts of wood-eating worker termites (*Amitermes evuncifer*) collected in two locations in Lagos. Fem-Ola and Oyebamiji (2019), who isolated four cellulolytic bacteria of the phyla Pseudomonas, Bacillus, Achromobacter, and Lysinibacillus, report similar findings.

Another study by Sharma et al. (2015) identified three isolates of the genus *Bacillus spp, Staphylococcus spp* and *Paenibacillus spp* in a wood-feeding termite from India. Out of the three species, *Paenibacillus* demonstrated the highest cellulolytic activity. Similarly, Sreena et al. (2015) identified three cellulose-degrading bacteria bacillus, *Staphylococcus,* and *Enterobacter spp* from *Ondototermes* and *heterotrimers spp*.

Sakolvareea and Deevong 2016 isolated cellulose producing strains of bacillus in a higher termite *Termes propinquus* in Thailand 2 cellulolytic bacterial such as *Bacillus megaterium* and *Paracoccus yeei*, were isolated from the gut of *Macrotermes gilvus* in Indonesia (Ferbiyanto et al., 2016). Pourramezan et al. (2012) identified *Acinetobacter*, *Pseudomonas*, and *Staphylococcus* from Iran termites. *Bacillus* and *Acinetobacter* destroyed cellulose rapidly than the other isolates.

These and other studies indicate the importance of this study in cellulose degradation as well as the diversity and the role bacteria play in termites. In Kenya, Muwawa et al. (2016) isolated and characterized gut symbionts of a fungus cultivating termites,

Macrotermes and *Odontotermes* spp associated in nitrogen metabolism. A similar study by Ntabo et al. (2010) identified *Escherichia coli, Bacillus subtilis, Candida albicans* from *Cubitermes spp*, and soils. The isolates were potential antibiotic manufacturers with variable degradability of gelatin, casein, and cellulose. A different study determined the bacteria diversity in *Microtermes* and *Ondotermes spp*. According to their findings, *Ondototermes* had greater bacterial diversity than *Microtermes*. The goal of this investigation was to observe the diversity of cellulolytic bacteria in *Macrotermes mishaelseni*.

1.2 Statement of the Problem

The accumulation of plant waste in the environment from agriculture, kitchen and industrial wastes especially in urban areas that require degradation have led to pollution because of poor disposal thus causing health problems to the people (Njenga et al., 2010). Furthermore, reliance on fossil fuels for the production of oil, power, and other products has resulted in increased pollution, greenhouse gas emissions in the atmosphere, global warming, and increased global energy demand to support the growing human population (Sarkar et al., 2012; Awasthi et al., 2015).

Termites are recognized for their immense destruction of wood in the environment Auer et al., 2017; Sakolvaree and Deevong, 2016; Batool et al., 2018. They harbor diverse and unique microbes that assist in their digestion of plant material (Muwawa et al., 2016; Sreena et al., 2015). Several studies have sought to examine termite gut symbiotic bacteria with the potential to breakdown cellulose. However, because microorganisms are native to specific geographic regions, different habitats in Kenya may house diverse

bacteria due to differences in soil composition and particle size (Ntabo et al 2010). The detected bacterial strains, on the other hand, exhibit limited cellulolytic activity. Therefore, studies on the investigations of unique bacteria that will utilize cellulose are still a significant task.

1.3 Justification of the study

The ability of bacteria in soils, animals and insects including termites to degrade complex cellulose has been recognized in past decades and is still an ongoing research study in the degradation of the recalcitrant cellulose. These studies confirm the presence of bacteria, their cellulose degrading abilities, and the roles they play in those environments. However, the bacteria found in termites have been found to be most significant cellulose degraders (Pourramezana, et al., 2012). The ability of bacteria to efficiently degrade is credited by the cellulase enzyme released during their growth on cellulosic material that acts as a carbon source. This enzyme in return has a wide range of application in industries and biotechnology.

The accumulation of plant waste produced through forestry, agricultural, and paper pulp industrial operations has caused contamination in the environment especially in urban areas. Consequently, much plant waste is frequently burned (Njenga et al., 2010). To reduce environmental contamination, it is vital to understand the processes and microorganisms involved in plant degradation. While cellulolytic bacteria in termites are a well-established phenomenon, what remains in black box is the effectiveness of these strains in cellulose degradation. Termites, discovered in microbial biotechnology, are utilized to explore biocatalysts originating from symbiotic eukaryotes and prokaryotes

(Korsa et al 2022). Therefore, in order to understand how bacteria aid in cellulose digestion in termites, we must isolate from different sites and different termites' species.

Cellulolytic bacteria can be collected and used to catalyze industrial processes and in biofuel production. This study will contribute to solid waste management, a healthy environment, and sustainable energy production since most of the plant waste will be recycled and processed to yield useful products. Therefore, the purpose of this investigation was to isolate and detect bacteria with cellulolytic activities that would catalyze plant degradation.

1.4 Objectives

1.4.1 General objective

The main goal of this investigation was to determine the cellulolytic activity and variety of bacteria in *Macrotermes michaelseni* for their use in the degradation of plant waste.

1.4.2 Specific objectives

1. To compare the cellulolytic activity of bacteria between the termites in Vihiga and Nandi County using carboxymethyl cellulose media.
2. To determine the diversity of cellulolytic bacteria in *Macrotermes michaelseni*.
3. To evaluate the evolutionary association among the cellulolytic isolates with other celluloltic bacteria in the DNA databases.

1.5 Hypotheses

1. There is no statistical difference in cellulolytic activity between the termites in Vihiga and Nandi County.

2. There is no significant difference in the diversity of cellulolytic bacteria present in *Macrotermes michaelseni.*
3. There is no evolutionary relationship among the characterized isolates with other cellulolytic bacteria in the DNA databases.

CHAPTER TWO

LITERATURE REVIEW

2.1 Cellulose

Plant biomass, comprises of wood residues, agricultural residues, domestic wastes, food industry residues and municipal solid wastes (Mtui, 2009). It is the world's most abundant and renewable carbon source. Plant biomass is made up of numerous inorganic elements as well as cellulose, hemicellulose, and lignin extractives (Jaiswal and Ravindran, 2015). Agricultural wastes are inexpensive, renewable, and plentiful (Sarkar, 2012). Examples of lignocellulose-rich agricultural waste include wheat and rice straw, bagasse, cotton stalk, and wheat bran. Sabiti Saini et al., 2015. Hemicellulose and lignin act as a matrix and encrusting components, and cellulose forms a skeleton that is surrounded by hemicellulose and lignin (Saini et al., 2015).

Previous studies mostly define cellulose to be a polysaccharide linear chain of glucose with glycosidic linkages (Taherzadeh and Karimi, 2008; Kameshwar and Qin, 2016; Ni and Tokuda 2013; Gupta et al., 2019). It is usually found in cellular walls, stems and woody parts of the plant (Kameshwar and Qin, 2016) where it accounts for 50% dry weight of a plant material thus making it the most abundant biopolymer in the environment (Gupta et al., 2012). Its abundance is related to higher plants' continuous photosynthetic cycles, which can synthesize roughly 1011 ± 1012 tons of cellulose in a relatively pure form (Gupta et al., 2019). Cellulose functions are, to give mechanical strength and chemical stability to plants by serving as a structural component of cell walls (Harmsen et al., 2010). Finally, cellulose is the best polymer because it is a non-toxic, biodegradable polymer with great tensile and compressive strength (Gupta et al., 2012).

2.1.1 Chemical Structure of cellulose

Cellulose is a high-molecular-weight linear polysaccharide polymer of D-glucose composed up of cellobiose units. Hydrogen and vander Walls bonds connect the cellulose chains, causing the cellulose to compact into microfibrils. The chains are organized in a crystalline structure and are parallel. This makes cellulose structure to become resistant to biological and chemical treatments (Taherzadeh and Karimi, 2008; Mussatto and Teixeira, 2010; Ravindran and Jaiswal,, 2015).

Cellulose's structural features are due to its ability to maintain a semi-crystalline state of aggregation in aqueous environments, which is remarkable for a polysaccharide (Gupta et al., 2019).

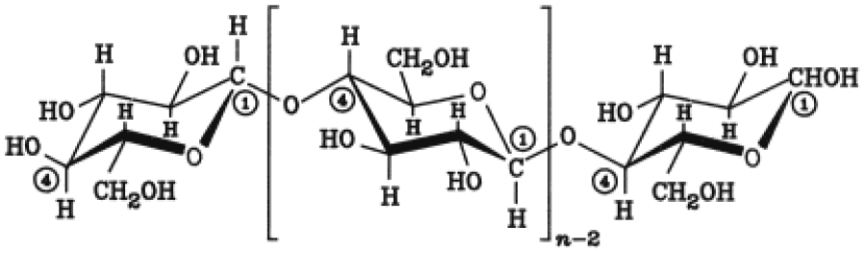

Figure 1 chemical structure of cellullose

2.1.2 Properties of cellulose

Cellulose is crystalline and connected to lignin and hemicelluloses, making it inaccessible to microbial cellulase synthesis (Li et al., 2014). The crystalline structure of cellulose is made up of numerous cellulose fiber chains connected by hydrogen bonds between

adjacent molecules' hydroxyl groups. Together, these hydrogen bonds and Vander Wall forces produce cellulose crystals that are strong and stable (Nandy et al., 2021).

Cellulose is a D-glucose homopolysaccharide that is linear and unbranched. The number of D-glucose units might be anything between 10,000 and 15,000 units. A 1-4 glycosidic bond (Nandy et al., 2021) links glucose residues in cellulose. According to (Zoghlami and Paes 2019) the degree of polymerisation is the number of glucose molecules formed in a polymer. This ranges from 100 to 14,000 residues by cellulose source (Lakhundi et al., 2015). The gylcosidic bonds between the molecules make the glucose molecules to be arranged in a straight chain (Harmsen et al., 2010).

Under normal air-conditions (20 °C, 60% relative humidity), cellulose is a relatively hygroscopic substance that absorbs 8-14 % water. However, it is insoluble in water, where it swells. At low temperatures, cellulose is also insoluble in dilute acid solutions. The degree of hydrolysis performed has a strong relationship with polymer solubility. As a result, factors affecting cellulose hydrolysis rate also affect its solubility, which occurs with the molecule in a different form than the native one. It becomes soluble at higher temperatures because the energy provided is sufficient to dissolve the hydrogen bonds that hold the crystalline structure of the molecule together.

Cellulose is also soluble in concentrated acids, but hydrolysis causes severe destruction of the polymer. In alkaline solutions, cellulose is extensively swollen, and the low molecular weight portions of the dissolve cellulose (Harmsen et al., 2010).

2.1.3 Mechanism of cellulose degradation by microorganisms

It is renowned that the digestion of plant waste in termite gut involves a complex action between the host and gut microbes (Li et al., 2014; Wong et al., 2014). This implies that the termite's digestives system works together with the microbial flora inside termite's gut to break down the plant material. These microbes inside termite gut are highly specific that each work individually in transforming different plant substrates (Wong et al., 2014).

Cellulose is broken down by the enzyme cellulase, which is produced by bacteria as they grow on cellulose (Sreena et al., 2015). Cellulases hydrolyze b-1-4 glycosidic linkages in the cellulose polymer to break it down (Behera et al., 2017). At least three enzymes (Endo-glucanases, exo-glucanases, and -glucosidases) must work together to break down cellulose into glucose (Sharma et al., 2015; Sreena et al., 2015; Shinde et al., 2017), as shown in figure 2.2.

The endoglucanses randomly initiates an attack at the numerous internal locations of the amphorous region of celluose. This makes sites more accessible for future attacks by exoglucanase. After that, mono and dimers are removed at the ends of the glucose chain, releasing the cellobiose and oligosaccharides. Finally, the β-glucosidase cleaves the cellobiose to yield monomers of glucose. Glucose is transferred across the membrane to participate in metabolic activities that generate energy (Malherbe and Cloete 2002; Howard et al., 2003).

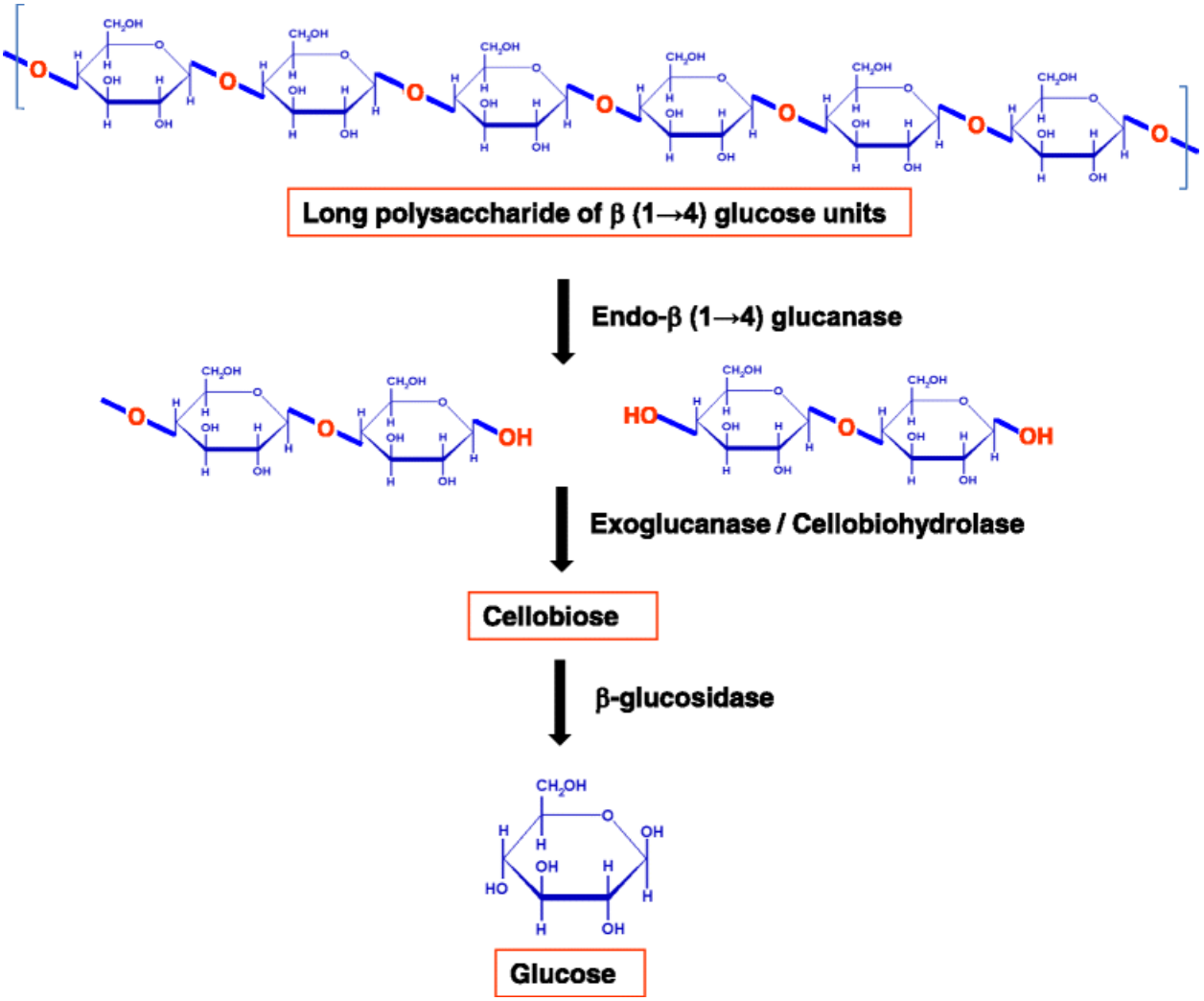

Figure 2 Diagram of a mechanism of cellulose hydrolysis

2.2 Termites

Termites are communal pests that occur in the world's hot, semitropical, and moderate areas (Ni and Tokuda, 2013; Sakolvaree and Deevong, 2016). Approximately there are 2800 termite species identified (Ali et al., 2019). They are commonly referred to as white ants because of their tiny to medium sizes, dull-white to light brown bodies, and colonial activities (Tochukwu Frank and Osita Gabriel, 2018). The size, shape, and venation of

termite wings are largely the same, however the length and span of wings varies per species. Other distinctive features of this group include social insects with caste difference, moniliform antennae, four-segmented tarsi, and mouthparts designed for biting. Termites develop colonies ranging in size from a few hundred to seven million individuals (Gomati et al., 2011).

Termites are categorized into lower termites and greater termites (Eutick et al., 1978; Tsegaye et al., 2018). There are six families within this order whereby five belong (*Mastotermitidae, Kalotermitidae, Hodotermitidae, Rhinotermitidae* and *Serritermitidae*) belong to lower termites while higher termites consist of only one family known as Termitidae. In their guts, lower termites have prokaryotes and protists, whereas higher termites have a broad array of prokaryotes Wong et al., 2014; Sreena et al., 2015; Upadhyaya S et al., 2015. Microenvironmental variables including as pH, accessible substrates, and oxygen and hydrogen gradients influence the prokaryotic gut bacteria of higher termites (Korsa et al., 2022). Higher termites, on the other hand, lack the protists that distinguish them from lower termites. Furthermore, lesser termites eat wood contaminated by the fungus mycelia, which makes it easier for termites to consume. Higher termites, on the other hand, consume a wide range of lignocelluloses, including leaves, roots, grass, feces, and soil. Higher termites have a more complex external and internal anatomy, as well as a social structure, than lower termites (Wong et al., 2014; Sreena et al., 2015; Upadhyaya S et al., 2015).

Termites are unique among communal insects in that they undergo partial metamorphosis and exhibit caste polyphenism. In a termite colony, worker, soldier, reproductive, and

undifferentiated immature forms work together in an integrated way (Berlanga et al., 2011). Termites live in colonies that consist of reproductive (queen) termites are in charge of breeding and guarding eggs, and non-reproductive (soldier) termites are in charge of protecting the colony from intruders, while worker termites build and maintain the galleries, repair, and transport food to the queen termites (Berlanga et al., 2011; Oktiarni et al 2022).

Termites feed on cellulose, present in the form of living or dead wood, plant woody tissues, or manure. Some even eat soil, while others have developed the fascinating habit of maintaining fungal gardens as a source of nutrients (Gomati et al., 2011). The digestion of cellulose in termites begins with the flowing of food through the digestive tract, which takes around 24 hours. The digestive process begins with the reduction of organic waste to minute particles in the mandible. Salivary gland enzymes aid in the mechanical breakup of food. The particles are carried over the digestive system, which is divided into three compartments: the foregut, midgut, and hindgut, each of which secretes various cellulolytic enzymes. The food is pulverized and the lignin is processed in the foregut of wood-feeding termites before it enters the midgut, which is where lignin-hemicellulose dissociation, esterase secretion, and endogenous cellulose digestion take place. Finally, it passes through the hindgut, where symbiotic bacteria and archaea can be found. The bacterial symbionts also create many distinct hemicellulolytic enzymes in this compartment, which is where cellulose hydrolysis takes place (Ben Gurrero et al., 2015).

2.2.1 Fungus growing termites

The fungus-growing termites belong to subfamily *Macrotermitinae*, (Zhu et al., 2012; Vesala et al., 2017). Macrotermitinae are among the most abundant and influential insects in Asia's hot and semitropical environments, as well as the African tropical rain forest (Nobre, 2010; Zhu et al., 2012). In many areas of savanna Africa, they are the major decomposing organisms of plant material (Vesala et al., 2017). *Macrotermitinae* are recognized by their large mounds constructions (Sileshi et al., 2009). Termite mounds maintain humidity, vapor exchange, and temperature condition (Vesala et al., 2017). Additionally, (Nobre, 2010; Femi-Ola and Oyebamiji, 2019) noted the distinctive symbiotic relationship of the macro terminal with fungus termitomyces. The fungus emerges on a fungus comb within the mound that is made and sustained by the termites. The relationship between termite and fungus results in the degradation of cellulose (Dangerfield et al., 1998)

Numerous authors from different part of the world have investigated on the fungus-cultivating termites and documented the presence and cellulolytic capacity of symbiotic bacteria. For instance, (Ferbiyanto et al., 2016) identified *Bacillus megaterium* and *Paracoccus yeei*, with a cellulolytic catalog of 0.81 and 2.5 respectively in *Macrotermes gilvus*. Others studies by (Kakkar et al., 2015; Kavitha et al., 2014; Sreena et al., 2015) demonstrated cellulose-degrading bacteria in *Odontotermes spp*.

In Kenya (Makonde et al., 2013) characterized and compared bacterial diversity of *Microtermes* and *Odontotermes* species. Their findings revealed that *Odontotermes* species had more diverse bacteria than *Microtermes*. Another study as explained above

characterized gut bacteria with nitrogen metabolism potential from *Macrotermes* and *Odontotermes* spp (Muwawa et al., 2016). Ayitso and Onyango (2016) identified antibiotic-producing bacteria from the gut of *Macrotermes michaelseni*. Research on the bacteria that break down cellulose in termites has not kept up with the effectiveness of these investigations in revealing the diversity and role of bacteria in termites.

2.2.2 Soil feeding termites

Soil feeding termites are soil macroinvertebrates abundantly distributed in the tropical ecosystem (Köhler et al., 2008). (Harry et al., 2001) suggests that these termites spread from rainforest to savannah. Unlike the *macrotermitinae* subfamily that feeds on plant biomass or wood, soil-feeding termites thrive highly on decayed wood or organic soil rich in nitrogen (Ngugi and Brune, 2011) in return increase the fertility of soils.

In many African Savanna habitats, including Kenya, *M. michaelseni* is a main decomposer of plant waste (Vesala et al., 2017). The majority of widely consumed edible insects in Africa are *Macrotermes spp*., which are often eaten as a delicacy and dietary supplement. Due to its big size and high protein and fat content, it is the most popular and well-known edible termite (Egan et al., 2021). *M. michaelseni* is Kenya's most abundant species, that is widely distributed in regions such as Kiambu County, Muwawa et al. (2016), Thika County Makonde et al. (2015), Taita –taveta County Vesala et al. (2017), and Kisumu County Ayisto and Onyango (2016).Since termite are abundant distributed in Kenya, and the abundance of plant waste accumulated in the environment, therefore the isolation of cellulose-degrading bacteria in termites remains a significant task.

2.2.3 Role of bacteria in termite's gut

Termites have evolved to be highly efficient at degrading cellulose, based on their effectiveness and the quantity of wood devoured per year. This is attributed to the dual cellulolytic system existing in termite's gut that combines its own mechanical and enzymatic machinery together with the gut endo-symbiotic cellulolytic microbe (Ben Gurrero et al., 2015).

Microbes in the termite gut help break down lignocellulose into simple carbohydrates for easy metabolization into pyruvate, which is then converted into carbon dioxide, acetate, methane, and ethanol (Wong et al., 2014; Brune and Dietrich, 2015). In addition, microbes also play a role of nitrogen metabolism in termites through fixation and recycling of nitrogen to their host (Ohkuma, 2003; Wong et al., 2014). For instance, a study by (Muwawa et al., 2016) established role of nitrogen fixing bacteria in two fungus-growing termites. Consequently, seventeen isolates demonstrated the ability to target nitrogen by reducing nitrates and nitrites. Some bacteria reutilize nitrogen from uric acid while others produce amino acid needed by their host (Radek, 1999).

Facultative and strictly aerobic bacteria play a significant role of providing oxygen in termite guts that are so small, the influx of oxygen across the hindgut wall is enormous (Brune and Dietrich, 2015). Other roles of microbes are protection of the termite gut from pathogenic bacteria, removal of hydrogen and carbon dioxide through hydrogenosomes of protozoans (Radek, 1999) and fermenting bacteria that support the respiratory requirement of termites (Ohkuma, 2003).

2.2.4 Isolation of bacteria in termites

Research studies show several approaches carried out to isolate bacteria in termites. For instance, Studies by (Shinde et al., 2017) successfully isolated cellulolytic bacteria in termites through inoculation of their samples on a nutrient agar media and incubated for 48 hours. Other studies reported using a medium containing Whatman filter paper (Pourramezan et al., 2012; Tochukwu Frank and Osita Gabriel 2018). This technique, however, seemed to take a longer period of incubation of up to 30days. All the methods yielded valid results as noted by each study but the nutrient agar media appeared to be more efficient less time, and easier to use.

Bacterial isolates from termite stomachs have Gram-negative or Gram-positive morphological traits, with varying forms and colors.

2.2.5 Screening of Cellulose degrading bacteria

The biodegradation of plant biomass is not the only dependant on environmental factors but also the degradation capacity of bacteria (Patagundi, 2015). Most screenings of cellulose-degrading bacteria in termites preferably use carboxymethylcellulose due to its high solubility in water. Carboxymethyl cellulose is an artificial substrate used for determining the cellulolytic potential of an organism. To achieve this, pure isolates obtained are grown in circular batches on CMC (Carboxymethyl cellulose) media (Kakkar et al., 2015). To detect the cellulase activity of the isolates each agar plate is flooded with a Congo red stain for ten to fifteen minutes. After that, it is rinsed with NaCl solution for another fifteen minutes (Sakolvaree and Deevong, 2016). The presence of a colony and a clear zone of surrounding the colony after staining indicate the cellulase activity of bacteria (Sharma et al., 2015). Although Congo red staining is the most

commonly used method, Gohel et al. (2014), discovered Congo red to be less efficient in their study comparing several staining methods for evaluating extracellular cellulase activity. They utilized several stains in their experiment, including congo red gram's iodine, coomassie brilliant blue, and safranin. As an outcome, Gram’s iodine produced the most visible and obvious clear zones within two minutes. Kakkar et al. (2016), found a considerable clean zone after employing gram's iodine stain to determine cellulase activity of gut bacteria in Odontotermes obesus. Gram's iodine is thus the best stain to employ for assessing bacterial extracellular activity.

2.3 Diversity of cellulolytic bacteria in termites

The termite gut community is extremely diverse, although the function of each group of symbionts is poorly understood (Ramin, et al., 2008). Bacteria, Archaea, and Eukarya are all represented in the termite gut microbiota. The majority of flagellates may be identified based on their visual characteristics; however, characterization of bacterial and archaeal communities necessitates the use of genetic methods (Brune and Dietrich 2015).

The action of these intestinal bacteria, which include numerous uncultured species, is credited with the termite's capacity to digest plant biomass (Mackenzie et al., 2007). The termites require these bacteria in order to survive. Spirochetes, which are highly phylogenetically diversified and comprise distinct monophylotic groupings of termite lineages, are examples of bacteria with various functions recovered from termite stomach. They can be found as free-swimming cells or as flagellates on the surface. They are highly found in wood feeding termites (Brune and Dietrich 2015).

Some of the bacteria cultured are difficult to recognize using phenotypic characteristics since bacteria consist of a diverse group of microorganisms (Khayalethu, 2013). However, the introduction of 16S ribosomal RNA (rRNA) sequence technique has enabled the identification isolates into the genus and species of isolates hence providing accurate information of the isolates (Drancourt et al., 2000; Janda and Abbott, 2007). Additionally, highly related species can be distinguished (Aisha, 2017). Adequate variations and presence of 16S rRNA gene among bacteria enables differentiation between taxa. This means several bacterial species can be targeted with one PCR primer pair (Khayalethu, 2013). Lastly, it is possible to detect PCR on DNA derived from crude sample bypassing bacterial isolation and thus identifying uncultivable bacteria (Khayalethu, 2013). Therefore, 16S ribosomal DNA sequencing serves as the best alternative for the identification of isolates over phenotypic method.

Various bacteria species have efficaciously been cultured and identified in termite's gut that can break down cellulose (Table 2.1).

Table 1 Examples of cellulolytic bacteria in termites

Author	Country	Termite	Isolated cellulolytic bacteria
Kavitha et al., (2014)	India	*Ondontermes spp*	*Citrobacter freundii, Bacillus, Pseudomonas aeruginosa Salmonella entrica , Enterococcus casseliflavus Staphylococcus gallinarum and Serratia marcescens*
Igwo-Ezikpe et al., (2013)	Nigeria	*Amitermes evuncifer*	*Bacillus subtilis, Bacillus cereus, Enterobacter cloacae Acinetobacter spp and Chryseobacterium sp*
Femi-Ola and Oyebamiji, 2019	Nigeria	*Amitermes evuncifer*	*Pseudomonas, Bacillus, Achromobacter Lysinibacillus.*
Ferbiyanto et al., (2016)	Indonesia	*Macrotermes gilvus*	*Bacillus megaterium Paracoccus yeei*
Sakolvaree and Deevong (2016	Thailand	*Termes propinquus*	*Bacillus amyloliquefaciens Bacillus methylotropicus*
Tochukwu and Osita (2018)	Nigeria	*Coptotermes formosanus*	*Chryseobacterium luteola Pseudomonas mendocina Burkholderia pseudomallei Klebsiella oxytoca Klebsiella terrigena*
Pourramezan et al., (2012)	Iran	*Microcerotermes diversus*	*Acinetobacter Pseudomonas Staphylococcus*
Muwawaet al.,	Kenya	*Macrotermes &*	*Pseudomonas, Citrobacter,*

(2016)		*Odontotermes spp*	*Enterobacter, Proteus, Klebssiella, Bacillus, Staphylococcus, Rhodococcus and Micrococcus*
Oktiarni et al., 2022	Indonesia	*Macrotermes gilvus*	*Enterobacter cloacae Klebsiella pneumoniae, Klebsiella quasipneumoniae, Klebsiella varicolla Enterobacter roggenkampii, Enterobacter asburiae*
Ntabo et al., 2010	Kenya	*Cubitermes*	*Bacillus spp Brachybacterium spp*
Ben Guerrero et al., 2015			

Cellulolytic bacteria are common in nature, however due to their ability to digest cellulose; unique cellulolytic bacteria in termites have been isolated and characterized in recent years. For instance Kavitha et al., (2014) isolated *Citrobacter freundii, Bacillus, Pseudomonas aeruginosa Salmonella entrica , Enterococcus casseliflavus Staphylococcus gallinarum and Serratia marcescens* from *Ondontermes spp* in India. Igwo-Ezikpe et al., (2013) and Femi-Ola and Oyebamiji, (2019), have isolated *B. subtilis, B. cereus, Enterobacter cloacae Acinetobacter spp Chryseobacterium sp Pseudomonas, Achromobacter* and *Lysinibacillus* from *Amitermes evuncifer* in Nigeria while Tochukwu and Osita (2018) isolated *Chryseobacterium luteola Pseudomonas mendocina ,Burkholderia pseudomallei, Klebsiella oxytoca* from *Coptotermes formosanus.*

Several authors have identified cellulolytic activity in various termites for their cellulolytic activity have identified these and many other species.

Based on 16s rRNA gene sequencing of bacteria often isolated from termites are dominated by members of the phyla Actinobacteria, γ-Proteobacteria and Firmicutes (Dantur et al 2015; Muwawa et al., 2016). Firmicutes are the most prevalent bacteria in termites (Long et al., 2010; Xie et al., 2017). The firmicutes are either Gram-positive rod or cocci producing endospores. The most commonly isolated firmicutes with cellulose degrading abilities in termites from this phylum is *Bacillus sp, Staphylococcus* and *Paenibacillus* genus.

The genus *Bacillus* is the most commonly reported genus of bacteria in termite gut with cellulolytic activities (Wenzel et al, 2002 Pourramezan et al., 2012; Kavitha et al., 2014; Sreena, et al., 2015; Ferbiyanto et al., 2016 ; Sakolvaree and Deevong 2016). *Bacillus* sp are widely distributed and are the most dominant group of bacteria commonly isolated (Bashir et al., 2013; Xie et al., 2013) observed the occurrence and dominance of bacilli among group of firmicutes involved in cellulose degradation in eastern subterranean termite. Amongst all *Bacillus* species *B. cereus* have been documented with the highest cellulolytic activity (Patagundi et al., 2014; D. Sharma et al., 2015)**.** Some of the *Bacillus* species have been observed to thrive on media containing xlyan thus suggesting their role in hemi-cellulose degradation (Butera et al., 2015). Other *Bacillus spp* recognized are *B. amyloliquefaciens, B. methylotropicus* Sakolvaree and Deevong (2016), *B. megaterium* Ferbiyanto et al. (2016), and *B. subtilis* Igwo-Ezikpe et al., (2013).

Staphylococcus spp are Gram-positive cocci and coagulase-negative bacteria. Although cellulose degrading activity is not a common property of *Staphylococcus* spp (Pourramezan et al., 2012), they have been isolated in termites such as *Microcerotermes diversus Silvestri* (Pourramezan et al., 2012), *Ondontotermes formosanus* (Kavitha et al., 2014) and *Neotermes spp* (Sharma et al., 2015) showing significant cellulolytic potential.

The genus *Paenibacillus* as classified by Priest (2015), are rod-shaped Gram-positive cells. They are either strictly aerobic or partially anaerobic. The majority of the species are catalase- positive with smooth and transparent colonies observed in different colors such as light brown, white, or light pink. They generally grow best at 28–40°C and pH 7.0. *Paenibacillus* is derived from the Latin adverb paene, to mean almost a *Bacillus* (Grady et al., 2016). The most well-known species for cellulolytic and xylanolytic activity is *Paenibacillus polymyxa.* Pasari et al. (2019), isolated *P. polymyxa* isolated form a termite gut and found it to be the maximum producer of cellulase amongst all the isolates. Ben Guerrero et al. (2015) also obtained *P. polymyxa* as their highest cellulolytic bacteria from two Argentinian termites. This clearly shows that *Paenibacillus spp* should be exploited for their cellulolytic potential. Furthermore, Paenibacillus species have the ability to fix nitrogen in addition to degrading cellulose (Grady et al. (2016), thus improving soil which leads to high crop production.

Proteobacteria are the second dominant phyla of gut bacteria in termites. Proteobacteria are mostly constitutes Gram-negative pathogenic bacteria. Proteobacteria isolated are cellulolytic and are also involved in nitrogen fixation thus providing nitrogen to their host (Muwawa et al., 2016). The most commonly isolated cellulose degrading bacteria include, *Klesbiella, Enterobacter*, *Pseudomonas, Citrobacter Salmonella* and *Serratia*

(Pourramezan et al., 2012; Igwo-Ezikpe et al., 2013; Kavitha et al., 2014 ;Muwawaet al., 2016; Oktiarni et al., 2022).

Pseudomonas aeruginosa is an example of a highly bacteria that degrade cellulose in termites (Kavitha et al., 2014; Muwawa et al., 2016; Femi-Ola and Oyembamji, 2019) but other species known, such as *P. putida* Pourramezan et al.(2012) and *P. mendocina* Tochukwu and Osita (2018). *Pseudomonas* species are recognized for their extraordinary diversity of metabolic activities in different ecological unit. Several *Pseudomonas* species have been investigated for their ability to breakdown several polymers like cellulose and lignin (Kameshwar and Qin 2016). Other habitats such as soils (Gunavathy and Boominathan 2015) and stained painted walls (Obidi et al., 2015) have also been discovered with members of this genus with cellulolytic activities. Cellulolytic *Pseudomonas* has also demonstrated other enzyme activities for instance xylanases and amylases Muwawa et al., 2016 and Femi-Ola and Oyembamji, (2019) respectively. Furthermore Muwawa et al., 2016 demonstrated their role in Nitrogen metabolism in termites. Therefore, this indicates the significance role of *Pseudomonas* not only in breaking down cellulose but also in other activities in termites.

In termites, the species Enterobacter is also the most common cellulolytic bacteria (Pourramezan et al., 2012; Igwo-Ezikpe et al., 2013; Sreena et al., 2015). The genus *Enterobacter* is the most dominant group in termites with cellulose degrading activities (Shinde et al., 2017). They are gram-negative, facultative anaerobic rods that do not generate spores. They are common in nature and highly predominant in intestines of animals resulting in wide distribution in soils water and sewage (Octavia and Lan 2014).

Enterobacter cloacae are commonly isolated species in termites (Igwo-Ezikpe et al., (2013); Oktiarni et al., 2022). Other species include *Enterobacter roggenkampii and Enterobacter asburiae* (Oktiarni et al., 2022). Apart from cellulolytic properties, this species fixes nitrogen Muwawa et al. (2016), promotes plant development Taghavi et al. (2010), produce of electricity in a microbial fuel cell. Thus, suggesting the diverse application of *Enterobacter* species. Furthermore, according to Waghmare et al. (2018), *Enterobacter sp*. SUK-Bio isolated from a plant litter soil was found to utilize various agricultural waste. Sorghum husk is used more than other materials, resulting in high levels of cellulolytic and hemicellulolytic enzymes.

Citrobacter members are Gram-negative, facultative anaerobic, motile bacilli that grow on Simmons citrate media (Arens et al., 1997). The most prevalent isolated *Citrobacter* in termites is *Citrobacter freundii* (Kavitha et al., 2014). Aside from cellulolytic capabilities, this genus is also known for nitrogen fixation and aromatic chemical breakdown (Horazono et al., 2003; Muwawa et al., 2016).

Klebsiella are Gram negative, bacillus and facultative anaerobic bacteria (Muwawa et al., 2016). *K. pneumona* and *K. oxytoca* are most commonly isolated species with cellulolse degrading abilities (Muwawa et al., 2016; Tochukwu and Osita 2018; Oktiarni et al., 2022) however, *K oxytoca* is has been identified as with the highest cellulolytic activity (Dantur et al., 2015). Other *Klebsiella* species such as *K. pneumonia*, *K. oxytoca* and *K. planticola* are capable of fixing nitrogen (Muwawa et al., 2016).

Actinobacteria make up a minor percentage of termites' total gut microbiome (Arango et al., 2016). Members of this phylum include Streptomycetaceae, Nocardiopsaceae, and

Micromonosporaceae, with Streptomycetaceae being the most numerous and usually isolated in termites (Malavika et al., 2022). Streptomyces is a Gram-positive bacteria genus that grows in a variety of habitats characterized as a filamentous fungus-like form. Streptomyces forms a layer of hyphae that can differentiate into a chain of spores during morphological differentiation (de Lima Procópio et al., 2012). Cellulolytic activity of *Streptomyces spp*. are extensively studied (Watanabe et al., 2003; Book et al., 2014). Additionally, member of this genus has been extensively isolated from termites for it used in the production of antibiotics (Arango et al., 2016; Zang et al., 2020; Zhou et al., 2021). Apart from their cellulolytic abilities, actinobacteria also have probiotic properties in herbivores and antagonistic properties against infections, making them important in therapeutic development (Malavika et al., 2022). Benndrouf et al. (2018) have discovered that Actinobacteria obtained from the fungus-eating termite immediately defends termites against invasive fungus, therefore defending termites from foreign invaders.

2.4 Applications of bacterial cellulase

2.4.1 Biofuel

Bacterial cellulases have numerous applications in industries, medicine and agriculture. One of the most significant applications of bacterial cellulase is in biofuel production (Menendez et al., 2015). Since the rising demands of fuel in the world and the depletion of crude oil production, many efforts to provide a sustainable fuel production have been conducted (Shweta 2012). Although countries such as Brazil and USA have successfully produced ethanol from corn and sugar cane, focus has been shifted towards plant waste to reduce on using food crops and lower production cost (Howard et al., 2003). While

fungal strains such as *Trichoderma reseei* are utilized to breakdown plant waste to monosaccharide however, recent studies are directed to bacterial cellulases that have the ability of hydrolysis of plant biomass and fermentation to bioethanol (Menendez et al., 2015).

2.4.2 Agriculture

Cellulose degrading bacteria are not only involved in breaking down of cellulose but also used in various processes of enhancing crop growth and plant protection from pathogens and diseases (Kuhad et al., 2011; Jayasekara and Ratnayake 2018). Cellulase enzyme is employed in the environment for decomposing plant waste remaining in the agricultural field after harvesting (Shinde et al., 2017). Cellulases and -glucanases can breakdown the cell wall and prevent phytopathogen spores from germinating. Different hydrolytic enzyme mixtures make it easier to digest desirable plant or fungal cell walls and yield protoplast, which can be utilized to create hybrid strains with desired traits for research (Nandy et al., 2021).

2.4.3 Animal feed

Cellulases in animal feeds increases nutritional content and give extra intestinal enzymes namely proteases, amylase, and glucanase (Kuhad et al., 2011), hence improving animal feed digestibility (Gunavathy and Boomainathan 2015).

Dietary fiber in animal feed is made up of non-starch polysaccharides like cellulose and arabinoxylans as well as other plant-based substances such waxes, chitins, pectins, b-glucan, and oligosaccharides. These substances can operate as anti-nutritional factors for a variety of animals. By removing these antinutritional elements from raw materials and adding additional digestive enzymes including proteases, amylases, and glucanases,

cellulase increases the nutritional value of feed. This enhances grain quality while also ensuring a high milk and meat yield (Behera at el 2017; Nandy et al., 2021).

Additionally, cellulases can improve feed's nutritional value by breaking down particular grain components. For example, b-glucanases hydrolyze cereal b-glucans in monogastric animal feed, reducing intestinal thickness and discharging nutrients from grains, resulting in significantly improved feed digestion and absorption, along with weight increase in broiler chickens (Behera at el 2017).

By increasing the production of propionic acid, a bacteriostatic agent that helps to inhibit the colonization of dangerous bacteria, cellulases enhance the processes involved in caecal fermentation. Cellulases have also been utilized to help ruminants improve feed utilization, milk output, and body weight gain (Behera at el 2017).

2.4.4 Industrial applications

Several industries use cellulase enzyme for various functions. For instance, textile industries use cellulases for biopolishing and producing a stonewashed-look in denims (Menendez et al., 2015). This cellulase break off fiber ends on cotton fabric slackening the dye to be easily removed by washing thus there is reduced fiber damage (Kuhad et al., 2011).

On the other hand, detergent industry uses cellulase to enhance the softness, brightness color, and dirt removal of cotton fabrics (Kuhad et al., 2011; Menendez et al., 2015). Additionally, cellulase, xylanases, and pectinases are employed in the food processing industry for extracting and clarifying fruit and vegetable juices designed to increase juice

yield, stability, and texture, as well as lower the viscosity of nectars and purees from tropical fruits Kuhad et al. (2011), resulting in reduced processing time (Behera et al., 2017). Macerating enzymes employed to liquefy fruit pastes to the largest extent feasible, together with the increase the cloud stability and texture of nectars and purees, besides swiftly reduce their viscosity and extract olive oil (Behera et al., 2017).

Cellulases are utilized in the manufacturing of food coloring agents. Carotenoid is the most common group of natural colorants that are responsible for various plant colors ranging from red to yellow. Cellulase and pectinase enzymes are employed to destroy the cell walls of sweet potatoes, carrots, and orange peels, releasing the carotenoids. Cellulases enzyme also helps to increase the nutritional content of fermented foods, uniform water absorption by cereals and dried vegetables, and the production of low-calorie food ingredients such as oligosaccharides (Behera et al., 2017).

In order to make beer, barley must first be malted in a malt house, and then the wort must be prepared and fermented in the brewery. The primary process of malting is seed germination, which starts the production and activation of amylases, carboxypeptidases, and cellulases. Under ideal circumstances, these enzymes work in harmony to produce high-quality malt (Behera et al., 2017).

In brewery, use of unmalted or poor-quality barley during malting and fermentation, poor filtration of the wort, slow run-off times, low extract yields and development of haze in the final product lead to gel or precipitate formation and low extract yield of beers. To overcome this problem endoglucanases are used. Glucanases are added either during mashing or primary fermentation to hydrolyse glucan, reduce the viscosity of wort, and

improve the filterability. Therefore, the addition of cellulases is known to improve not only the beer qualities but also their overall production efficiency (Behera et al., 2017).

In the process of making wine, cellulases along with other enzymes aid in the hydrolysis of polysaccharides found in plant cell walls, enhancing skin maceration, grape color extraction, quality, stability, clarity, and aroma. By altering glycosylated precursors, b-glucosidases can enhance the scent of wines. Macerating enzymes help boost the pressing, settling, and juice outputs of grapes utilized during wine fermentation (Behera et al., 2017).

The utilizaion of cellulase in the bio-stoning of denim and jeans products has proven to be a huge success. The use of cellulase in bio polishing cotton fabric has another benefit: the enzyme may easily remove surface fibers and fluff, giving cotton clothes a glossier, smoother, and brighter appearance. After several washes, cotton clothing normally turn fluffy and drab. The addition of cellulase enzyme to household detergents aids in the removal of fluffy fibrils from cotton, improving the clothing' look and brightness (Nandy et al., 2021).Lastly, cellulase are best in recycling of paper waste such as books and newspapers by deinking them and reusing the fiber for manufacturing ethanol, production of soft paper towel and sanitary papers (Behera et al., 2017). These industries demand for proper cellulase producers with highly stable and actively extreme pH and temperature at low-cost production (Menendez et al., 2015).

2.4.5 Waste management

Unused cellulose in trash from woods, agricultural areas, and agro-industries pollutes the environment. These wastes are now being strategically used to make valuable goods such

as enzymes, sugars, biofuels, chemicals, cheap energy sources for fermentation, enhanced animal feeds, and human nutrients via enzymes such as cellulase (Behera et al., 2017; Nandy et al., 2021).

2.4.6 Biotechnology

To acquire these qualities and increase enzyme output, various genetic strategies are being employed to improve microbial strains. Several industrially important fungal and bacterial strains, including *A. niger, T. reesei, Saccharomyces cerevisae, Pichia pastoris*, and *Escherichia coli* and *Bacillus subtilis*, have been genetically modified to produce a recombinant enzyme with significant industrial potential (Nandy et al., 2021).

CHAPTER THREE

METHODOLOGY

3.1 Study Area and Sample Collection

3.1.1 Study site

Three distinct termite hills in Vihiga and three additional termite hills in Kapsabet, Nandi County, were the sites of sample collection. At an elevation of 1500–1600 meters above the surface of the earth, Vihiga is located in the eastern portion of Kakamega Forest, approximately 0.0768N latitude and 34.7078E longitude. The woodland receives 2080mm of rain per year and temperatures range from 11°C to 26°C (Mwangi, 2019). The soils are usually Aerosols of poor fertility medium to heavy grained clay loams and clay with pH less than 5.5. (Ntabo et al., 2010). The termites were collected from the mounds found in Vihiga village at a distance of one meter apart.

On the route to Chavakali, 40 kilometers southwest of Eldoret is the settlement of Kapsabet in Nandi County. It is located between latitude 0.034N and longitude 340.45E. With an area of 2,884.4 km^3 and an elevation of 2,020 m above the ground, it receives between 1200 and 2000 mm of rain on average annually (Nandi County development plan, 2018). The termite mound near the Kimondi River is where the termite samples were taken.

3.2 Materials

Materials applied in this investigation were a hoe for digging termite mounds, boxes, polythene bags, 70% alcohol, Parafilm, Petri dishes termites from Vihiga and Nandi County, used for this study.

3.3 Sampling

Six locations established with termite mounds in Vihiga and Kapsabet were selected for sampling to find the cellulolytic activity and diversity of bacteria in *M. mishaelseni.* The samples were randomly collected using a hoe to excavate the mounds and picked a piece of mound containing termites. Six pieces of mound were placed in different boxes, and transported to the laboratory for further studies (Peristiwati et al., 2018). In the laboratory, the boxes were stored in cabinet at room temperate.

The soldier's major factors were used to identify termites. Each of the collected items was placed in its own vial containing 70% ethanol. Soldiers were observed for their structural characteristics using a phototube camera approach beneath a microscope stereomicroscope STEMI 2000 (Arif et al., 2019). Heads, antennal segments, fontanelle, mandibles, postmentum, and pronotum is the main morphological traits or body components utilized for identification. (2019, Hidayat)

3.3.1 Culturing of bacteria

Six termites were chosen at random from the six mound pieces and sterilized with 70% ethanol for 10 minutes before being rinsed in sterile distilled water (Femi-Ola and Oyebamiji, 2019). Each termite was crushed with a glass rod in the Eppendorf tube with 1.5 microliters of distilled water to make a paste that was used for isolation of bacteria.

The tubes were placed on a shaker, for 37°C at a speed of 150rpm for 24 hours (Kavitha et al., 2014).

The homogenate solutions above were used for the culturing of the bacteria. The Nutrient media used for culturing bacteria was prepared and sterilized by autoclaving at 121ºC for 15 minutes. Streak plating was applied for cultivating bacteria. Each plate was sealed to prevent contamination using parafilm and nurtured at 37°C upside down for 72 hours. To obtain pure isolates, distinguished single colonies were selected on the plate using a sterile inoculating loop and streaked on a fresh Nutrient medium plate for a full day (Kavitha et al., 2014).

3.3.2 Identification of bacteria

The identification of bacteria was done using physical characterization and molecular. Morphological properties of the pure isolates' colonies, such as shape (circular or filamentous), surface(smooth, glistening, roungh wrinkled or dull), margin the edge of a colony, and color pigmentation, observation on an agar plate as stated by (Sharma et al., 2015).

Gram staining was used to observe whether the isolates were Gram-negative or Gram-positive as described by (Ayitso and Onyango 2016). This process was performed by the collection of a portion of the colony using an inoculating loop under aseptic conditions, which was transferred to a water-containing slide to form a thin coating. The slide was then passed through the Bunsen burner to fix the bacteria. The slide cooled in the air and then poured crystal purple stain for a minute, followed by a wash through flowing tap water. Following that, the slides were drenched with Gram iodine for a minute before

being rinsed with water. To prevent the cells from bleaching, the decolorizer was poured for a few seconds and promptly washed with water. Finally, the saffron stain was put on the slide to stain the Gram-negative bacteria for two minutes, rinsed with water, and dried. The slides were viewed under a microscope by applying oil immersion and analyzed under 100x objectives lens to determine whether or not the bacteria were Gram-positive (purple or blue) or Gram-negative (red) and cocci or bacillus.

3.4 Screening for cellulolytic activity of bacteria in termites

The isolates obtained were cultured on the agar media with complemented by 1 % level CMC previously prepared and sterilized by autoclaving at 121ºC for 15 minutes. Isolates from pure cultures were used. Single colonies were picked using a sterile inoculating loop and placed in circular patches on the agar containing CMC. . Each plate was sealed with a parafilm and incubated at 30°C upside down for 72 hours. After incubation, Gram-iodine solution was poured on the plates for 15 minutes. Following that, the solution was drained out and observed for a presence of a clear zone around the colonies. Isolates with a clear zone around their colonies were measured to determine their cellulolytic potential. The cellulolytic potential of the positive isolates was assessed using the cellulolytic index (CI), which is defined as the ratio of the diameter of the zone of hydrolysis to the diameter of the colony specified by (Saini et al., 2017) as shown in formula below.

Celluloytic index = (Diameter of clear zone-Diameter of colony)/ Diameter of colony.

3.5 Molecular characterization of the bacterial isolates

The cellulolytic isolates (isolates that formed a clear zone around their colonies) were further used for the identification by 16S rRNA gene.

3.5.1 DNA extraction

The isolates with cellulolytic activity used for this extraction were cultured in a 20ml prepared previously nutrient broth and incubated at 37˚C on a shaker at a speed of 150 rpm for 72 hours. The culture was centrifuged at 10,000 rpm for 10 minutes in a laboratory centrifuge machine. The supernatant was discarded and the pellet was used for the DNA extraction as detailed on (Ramin et al., 2009). PureLink™ Genomic DNA extraction Kit (Invitrogen, Carlsbad, California, United States)was used to extract DNA according to the manufacturer's instructions. The recovered DNA was passed on a 1% agarose gel in 1xTAE buffer and stained with ethidium bromide for visualization under UV light (Sakolvaree and Deevong, 2016).

3.5.2 PCR amplification

Each purified DNA of the seventeen isolates was utilized as template for 16S rDNA gene amplification. The nearly full length of the gene was amplified using universal 16S rRNA gene primers ACGGTTACCTTGTTACGACTT -16Reverse and AGAGTTTGATCCTGGCTCAG -16 forward. The total composition of the PCR reaction was 12.5 µL of Taq polymerase, 9.5 µL of distilled water 1µL of primers and the DNA as a template. The thermocycling condition were: the first reaction started at 94 for 5 min, followed by 35 cycles of 94 ˚C for 4 min, 94 ˚C, for 40 sec, 55 ˚C for 1 min after that 72 ˚C for 10 min as prescribed by (Aisha et al., 2017). The PCR products were analyzed on

1.5 kb agarose gel (0.375 g agarose + 25 mL 1× TBE) electrophoresis to determine its size. The PCR products were then gel purified using QIAquick purification kit (Qiagen, USA) as per manufacturer's protocol and sent to Macrogen for sequencing (Ben Guerrero et al., 2015).

3.5.3 Analysis of 16S rDNA gene and phylogenetic tree construction

Sequence data were edited and aligned using Bioedit (Biological sequence alignment editor) upon quality trimming with Codon Code aligner. The 16S rRNA gene sequences were compared to the sequences in the public databases using Basic Local Alignment Search Tool (BLAST) in the National Centre For Biotechnology Information (NCBI) website (htt://www.ncbi.nih.gov) to determine the similarity to sequences in the gene bank database (Zhu et al., 2012).

The sequence alignment was done using MAFFT (Katoh & Standley, 2013), viewed and manually edited using Aliview (Larsson, 2014). Phylogenetic trees were then constructed based on the nucleotide sequences with the neighbor-joining tree with 80% bootstraps value. The trees were then visualized using fig tree software obtained at http://tree.bio.ed.ac.uk/.

3.6 Data analysis

The experimental data was analyzed using one way Analysis of variance (ANOVA) using Statistical Package for Social Sciences (SPSS, 2007) version 16.0.

CHAPTER FOUR

RESULTS

4.1 Cellulolytic activity of bacteria in *Macrotermes michaelseni*

24 isolates were taken from termites in Vihiga and Nandi County after the isolates had been satisfactorily decontaminated (Appendix I). The isolates from Vihiga County (KG) and Nandi County (EG) were identified.

The bacteria found in M. michaelseni from Vihiga and Nandi County were distinguished from one another using microscopic characteristics of the isolates along with single, independent colonies. The colony features of the 24 isolates of M. michaelseni that were taken from the termite hills are displayed in Table 4.1. Six isolates from the species found in Nandi County and eighteen isolates from termites in Vihiga County were produced as a consequence of the use of nutrient agar in the isolation process.

Table 2 Morphological characteristics of the bacterial isolates

No	Isolates	Termite	Shape	Surface	Colour	Shape of bacteria	Gram stain
1	KG11	Macrotermes	irregular	raised	cream	bacillus	negative
2	KG12	Macrotermes	spherical	raised	cream	coccus	positive
3	KG13	Macrotermes	spherical	raised	orange	coccus	negative
4	KG14	Macrotermes	irregular	raised	brownish	coccus	negative
5	KG15	Macrotermes	irregular	raised	cream	bacillus	positive
6	KG16	Macrotermes	spherical	raised	red	bacillus	negative
7	KG21	Macrotermes	spherical	raised	yellow	coccus	positive

8	KG22	Macrotermes	spherical	raised	cream	coccus	negative
9	KG23	Macrotermes	spherical	raised	cream	coccus	negative
10	KG24	Macrotermes	spherical	raised	greyish	coccus	negative
11	KG25	Macrotermes	spherical	raised	greyish	bacillus	positive
12	KG26	Macrotermes	spherical	raised	red	bacillus	negative
13	KG31	Macrotermes	spherical	raised	yellow	bacillus	positive
14	KG32	Macrotermes	spherical	raised	orange	bacillus	positive
15	KG33	Macrotermes	spherical	raised	white	coccus	positive
16	KG34	Macrotermes	spherical	raised	cream	coccus	positive
17	KG35	Macrotermes	irregular	raised	yellow	coccus	negative
18	KG36	Macrotermes	irregular	raised	cream	coccus	negative
19	EG11	Macrotermes	spherical	raised	cream	bacillus	positive
20	EG12	Macrotermes	spherical	raised	cream	bacillus	positive
21	EG13	Macrotermes	spherical	raised	orange	coccus	positive
22	EG21	Macrotermes	oval	flat	greyish	bacillus	positive
23	EG31	Macrotermes	filamentous	flat	orange	coccus	positive
24	EG32	Macrotermes	spherical	raised	orange	coccus	positive

The features of each isolate's colony, which vary in terms of form, elevation margin, and color, are displayed in Table 4.1. Of the twenty-four isolates, seventeen had elevated surfaces and circular shapes, five had irregular shapes (KG11, KG14, KG15, KG35, and KG36), and the other two (EG21 and EG31) had egg-shaped and threadlike shapes, respectively.

Gram stain analysis revealed that eight isolates (KG11, KG13, KG14, KG16, KG25, KG26, KG35, and KG36) stained gram negative, while the bulk of isolates (KG12,

KG15, KG21, KG22, KG23, KG24, KG31, KG32, KG33, KG34, EG11, EG12, EG13, EG21, EG31, and EG32) stained gram positive (Table 4.1). Besides, 10 isolates were rod-shaped and 14 isolates were spherical, according to their morphological appearance under a microscope.

4.2 Cellulolytic activity between the termites in Vihiga and Nandi County using carboxymethyl cellulose media

The ability of 24 isolates to break down cell walls was tested. Consequently, a clear zone was observed surrounding the colonies of 17 of the 24 isolates that were examined, confirming the existence of cellulolytic activity (Appendix II). The isolates' cellulolytic activity is displayed in Table 4.3 following gram's iodine staining. Fourteen isolates from termites in Vihiga County and three from Nandi County were among the seventeen that displayed a distinct zone. Table 3 Carboxymethyl cellulose activity of the isolates

Table 3 Carboxymethyl cellulose activity of the isolates

Isolate	Diameter of clear zone	Diameter of colony	Cellulolytic index
KG12	7	5	0.15
KG13	8	6.9	0.40
KG14	29	16	0.81
KG15	29	23	0.26
KG16	79	45	0.76
KG21	18	5	2.60
KG23	15	3	4.00
KG24	24	18	0.33
KG25	32	15	1.13
KG26	67	41	0.63
KG31	20	10	1.00
KG32	10	5	1.00
KG33	17	7	1.43
KG35	16	5	2.20
EG11	31	6	4.17
EG12	41	6	5.83
EG21	22	17	0.29

While isolates from Nandi termites ranged in diameter from 22 to 31 mm, those from Vihiga termites ranged in diameter from 7 to 79 mm. Cellulolytic index measurements revealed that isolate KG23 had the greatest value at 4.00, while KG12 had the lowest index at 0.15. Conversely, isolate EG 12 from Nandi termites exhibited the most cellulolytic index (5.83), whereas isolate EG21 displayed the lowest, at 0.29.

The statistical findings of diameter of hydrolysis of *M. michaelseni* showed no significant difference among the termite mounds collected at Vihiga County (F= 0.97, P- value = 0.4029).

Table 4 ANOVA table for diameter of hydrolysis by differences within termite mound in Vihiga County

Source	Sum of squares	Df	Mean Square	F-Ratio	P-Value
Between groups	921.444	2	460.722	0.97	0.4029
Within groups	7150.83	15	476.722		
Total	8072.28	17			

Table 5 : Table of means for diameter of hydrolysis area by difference within termite mound with 95.0% LSD interval

Level	Count	Mean	(Pooled s)	Lower limit	Upper limit
1	6	25.3333	8.91368	11.8989	38.7677
2	6	26	8.91368	12.5656	39.4344
3	6	10.5	8.91368	-2.93439	23.9344
Total	18	20.6111			

The diameter of hydrolysis for the termite mound collected in Nandi County did not exhibit any significant variation (F= 1.35, P= 0.3102)

Table 6 ANOVA Table for Diameter of hydrolysis area by Differences within termite mound in Nandi County

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	416.667	1	416.667	1.35	0.3102
Within groups	1236.67	4	309.167		
Total (Corr.)	1653.33	5			

Table 7 Means for Hydrolysis area by Differences within antihills with 95.0 percent LSD intervals

Level	Count	Mean	Stnd. Error (pooled s)	Lower limit	Upper limit
1	3	24	10.1516	4.06982	43.9302
2	3	7.33333	10.1516	-12.5968	27.2635
Total	6	15.6667			

There was no significant difference in the diameter of hydrolysis in comparison between the termite mounds collected from the two sites (F= 1.3, P=0.2667).

Comparison of the two locations hydrolysis area

Table 8 Means for Hydrolysis area by Differences within termite mounds of Vihiga and Nandi County

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	183.361	1	183.361	1.3	0.2667
Within groups	3106.1	22	141.186		
Total (Corr.)	3289.46	23			

Table 9: Means for Diameter by Differences within mounds with 95.0 percent LSD intervals

Level	Count	Mean	Stnd. Error (pooled s)	Lower limit	Upper limit
1	18	11.65	2.80066	7.54297	15.757
2	6	5.26667	4.85088	-1.84692	12.3803
Total	24	10.0542			

4.3 Diversity of cellulolytic bacteria in *Macrotermes michaelseni*

The seventeen cellulolytic positive isolates' nearly full-length 16S rDNA gene sequences were PCR amplified using the universal bacterial primer pair 27F (forward) primer and 1492R (reverse) prime by using gel electrophoresis. PCR successfully amplified all isolates. The 16S rRNA amplification yielded a 1500bp amplicon for all of the isolates (Figure 4.4) as seen by the thick single band on the gel.

Figure 3 Amplification of the 16 rDNA gene for the seventeen isolates

Photograph of the DNA ; (1) KG21, (2) KG12, (3) KG13, (4) KG10, (5) KG15, (6) KG23, (7) KG24, (8) KG24, (9) KG25, (10) KG26, (11) KG16, (12) EG11, (13) EG12, (14) EG21, and (15) KG31 (16) KG32 and (17) KG33.

Furthermore, the 16S rRNA gene fragment analyzed for its nucleotide base sequence to determine the species of each bacterial isolate. BLAST-N of 16S rRNA genes of isolates. Sequencing was done on the 17 isolates that revealed a distinct zone surrounding their colony. Out of the 17 isolates, only12 sequences were identified using the BLASTI-N search. The other five sequences had poor quality reads with multiple peaks hence could not be analyzed. The query cover, e value, and maximum identity percentage formed the basis of assigning the isolates with their corresponding species with 16S rRNA sequences deposited in GenBank (Table 4.4).

Each sequence was edited before being uploaded to GenBank, where similarity searches versus publicly available bacterial sequences were carried out using the BLAST and FASTA algorithms to compare with similar sequences in the GenBank. The results of each sequence search that provided the closest match to the sample were utilized to identify the species of bacterial isolates. When Blastn analysis was used to compare the newly recovered 16S rRNA gene sequences with the sequences already present in the Genbank database, it was found that there were more than 95% of sequence similarities with known sequences (Table 4.4).

Table 10: NCBI BLAST search for each amplified 16S rDNA gene of the cellulolytic bacteria

Isolate name	Accession number	Closest database match	Query cover	e Value	Maximum identity %
KG21	**MN173448.1**	***Staphylococcus saprophyticus***	**99**	**0.0**	**99**
KG12	**KT072093.1**	***Arthrobacter defluvii***	**99**	**0.0**	**99**
KG13	**MK282890.1**	***Klebsiella oxytoca***	**100**	**0.0**	**100**
KG23	**MT356128.1**	***Citrobacter freundii***	**100**	**0.0**	**100**
KG24	**MN175140.1**	***Klebsiella pneumoniae***	**100**	**0.0**	**98**
KG25	**MN911366.1**	***Bacillus thuringiensis***	**100**	**0.0**	**100**
KG26	**MN252011.1**	***Serratia marcescens***	**100**	**0.0**	**100**
EG52	**JX945883.1**	***Paenibacillus polymyxa***	**100**	**0.0**	**99**
KG31	**MT337533.1**	***Bacillus cereus***	**100**	**0.0**	**100**
KG32	**MF029649.2**	***Dietzia natronolimnae***	**99**	**0.0**	**98**
KG33	**LN614643.1**	***Arthrobacter sp***	**99**	**0.0**	**99**
KG36	**MN636436.1**	***Exiguobacterium aurantiacum strain VMG12***	**100**	**0.0**	**100**

Results of the BLAST-N 16S r RNA gene (Table4.4) showed that 6 isolates namely; KG13, KG23, KG 25, KG26, 31, and KG36 had 100% similar identity K. Oxytoca, C. freundii, B. thuringiensis, Serratia, B. cereus and E. aurantiacum respectively. On the

other hand, 4 isolates KG21, KG12, EG52 and KG33 had 99% identity similarity with *S. saprophyticus, A. defluvii, P. polymyxa and Arthrobacter sp* respectively. Lastly, the remaining two isolates KG24 and KG32 identified with *K. pneumoniae* and D. *natronolimnae* respectively at 98%.

4.4 Evolutionary association of cellulolytic bacteria with other cellulolytic bacteria in DNA database

The PCR amplified products from the 17 isolates were sequenced and analyzed. Only eleven sequences (5 forward and 6 reverse strands) produced quality sequences and thus used to build a Phylogenetic tree. The rest of the sequences had poorly resolved sequences hence were excluded from the analysis of the phylogenetic tree. The Neighbor-Joining approach was used to reconstruct the evolutionary history of the detected isolates. The evolutionary distances were calculated and expressed in percentages using the Maximum Composite Likelihood method. The phylogenetic trees displayed each isolate's evolutionary position (Figure 4.4 A and B). In the reconstructed phylogenetic tree, the isolates from this investigation (prefixed as RM) were phylogenetically varied and linked with known individuals from distinct genera in the DNA database, as supported by a bootstrap value of > 95%.

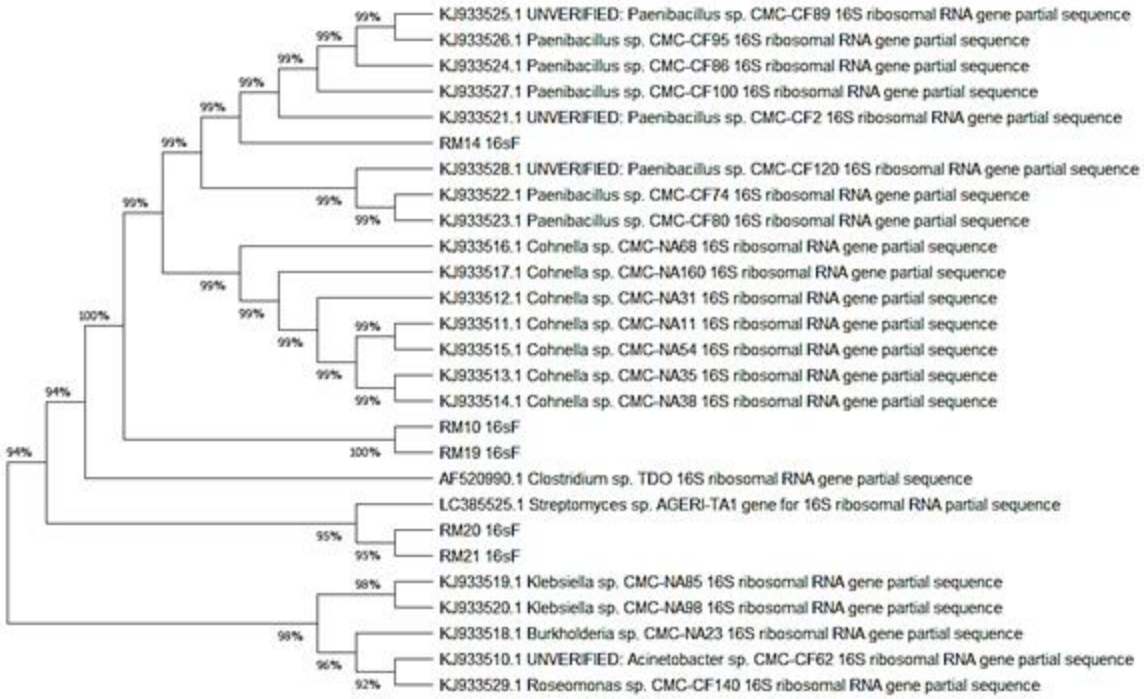

Figure 4 Phylogenetic tree based on 16S rDNA forward sequences

The samples from this tree are entitled as RM14 16sF as isolate EM12, RM10 16sF as isolate KG25, RM19 16sF as KG31, RM20 16sF as KG32, and RM21 16sF as KG33.

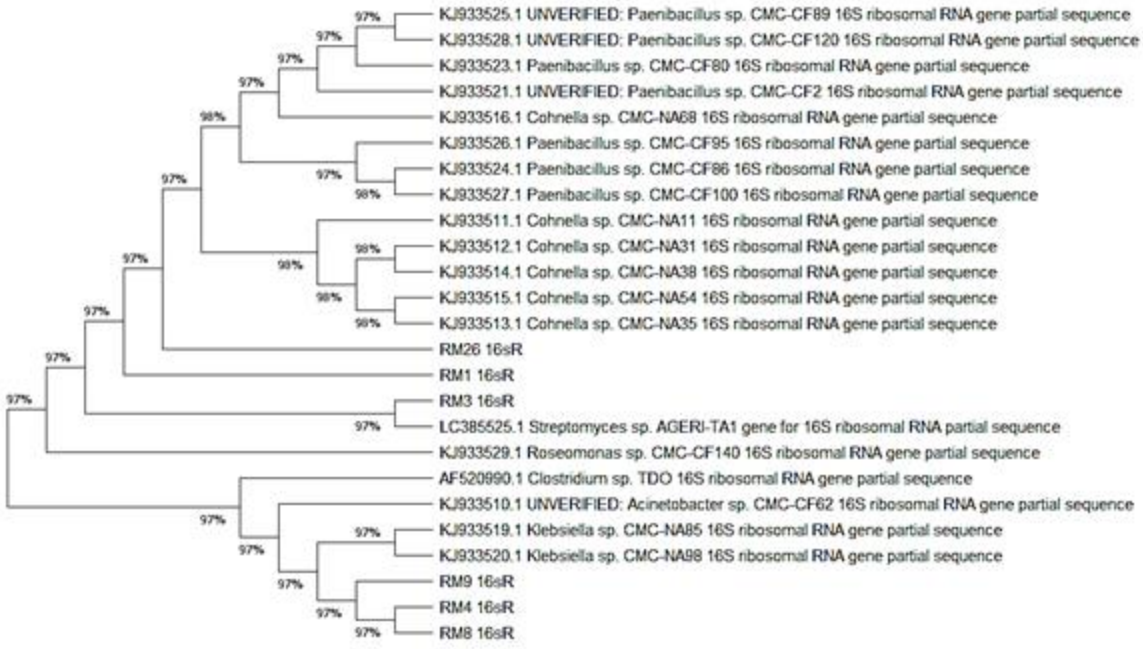

Figure 5 Phylogenetic tree based on 16S rDNA Reverse sequences

Phylogenetic tree based on 16S rDNA sequences. The samples from this study are entitled as RM 26 16sR as KG36, RM1 16sR as KG21, RM3 16sR as KG12, RM9 16sR as KG24, RM4 16sR as KG23, and RM8 16sR as KG26

CHAPTER FIVE

DISCUSSION

5.1 Cellulolytic activity of bacteria in *Macrotermes michaelseni*

Cellulose degrading bacteria in the termites are naturally correlated with plant degradation due to the capacity of termite to digest wood fully. Not only are cellulolytic bacteria present in termites, but they can also be acquired in various environments where plants are either eaten or plant degradation takes place. This research successfully isolated and established cellulolytic bacteria in termites as potential producers of cellulase for plant degradation.

Termites for this investigation were collected from two diverse sites in Vihiga and Nandi County. In both sites, termites were found in mounds that were stronger than normal soil. In Vihiga County, the termites mound contained fungus combs in them with and the mound was built in with plant stems. While Nandi County, the termites were found in mound composed of various networks connected to each other.

Determining the variety of cellulolytic bacteria present in *M. michaelseni* was the aim of this investigation. After cultivating on nutrient agar, 24 isolates were collected. The bacterial population was higher in Vihiga termites compared to Nandi termites. This study supports evidence from previous observations indicating the prevalence of bacteria in termite (e.g. Pourramezana et al., 2012; Igwo-Ezikpe et al., 2013; Ferbiyanto et al., 2015). Although it is known that one termite can harbors abundant microbial species in its gut, during this study, only a handful were culturable. The possible explanation of this might be that the problem of mimicking natural conditions in the gut atmospheres (Bodor et al., 2020). However, the emergences of molecular based methods have disclosed the

variety and role of gut microbes in termites providing information of the exclusive and important microbes useful for plant degradation.

The colony characteristics obtained in this study were diverse and different between the two locations. These findings corroborate the idea of Goyari et al., (2014), who suggested that a sample with more different and unique isolates is more diverse than another sample. Therefore, termites from Vihiga County of this imvestigation had high diversity of culturable bacteria as compared with termites from Nandi County since it had many diverse isolates. These findings are in accord with (Ntabo et al., 2010; Kavitha et al., 2014) who isolated diverse bacteria in soil feeding termites and surrounding soil from Juja and Kakamega forest and fungus growing termites in Chennai respectively.

Few isolates were able to maintain the primary stain, while the majority of isolates were able to retain violet stain, based on the findings of Gram staining. These outcomes align with the information of (Ntabo et al., 2010; Sreena et al., 2015) who also identified more Gram positive and few Gram-negative isolates in termite gut. However, comparing the two locations in this study, Kakamega termites had both Gram-positive and few Gram-negatives bacteria unlike Kapsabet termites that consisted of only Gram-positive bacteria. These findings relates with the investigation of Femi-Ola and Oyebamiji (2019) who observed termite species from similar ecological site with similar gut bacteria. The possible explanation to this difference is that microorganisms are endemic to certain geographical regions due to different in soil composition, food type, amount of rainfall received and agricultural activity carried out in the area that may influence the type of microbes in termites (Ayisto and Onyango 2016). Since the majority of the isolates

detected in this investigation were rod-shaped, it is likely that they are members of the *Bacilli* genus. These findings concur with earlier research that frequently observed bacillus to be the dominant isolates (Sreena, et al., 2015; Ferbiyanto et al., 2016; Sakolvaree and Deevong 2016 Ayitso and Onyango 2016).

The cellulolytic activity between the termites in Vihiga and Nandi County was ascertained using carboxymethyl cellulose media. To accomplish this, all isolates acquired were tested on Carboxymethyl cellulose media to determine the isolates' cellulolytic ability. The measures utilized to evaluate an isolate's ability were its growth and the presence of a distinct hydrolysis zone around the colony. The clear zone around the colony indicates the hydrolytic capacity that is used to determine the cellullase capacity of bacteria (Sharma et al., 2015). Although 24 isolates grew well on CMC media, only seventeen isolates displayed clear zones around their colonies. Because of the cellulolytic enzymes produced by the bacteria, the cellulose in CMC media was hydrolyzed. The hydrolysis process led to the production of clear zones around the colony for the reason that of the binding between gram's iodine with polysaccharide forming a clear zone (Gohel, et al., 2014).

The cellulolytic index of the 17 isolates varied from one organism to another. Florencio.et al., 2012; Vimal, et al., 2016, considered isolates with a ratio ≥of 1.5 to be an efficient producer of cellulase, so in this analysis, five isolates are more promising bacteria for cellulose degradation. The larger the clear zone of hydrolysis on CMC media plates, the stronger the enzyme activity of the bacterial strain (Sharma et al., 2015). For this cause, Isolate EG12 showed a significant high cellular activity with a cellulolytic

index of 5.8. These findings are consistent with those obtained by (Kakkar et al., 2015), who examined cellulolytic activity in the guts of *O. parvidens*. As a result, out of 19 culturable bacterial isolates, 15 isolates showed a presence of a clear zone surrounding their colonies. However, all the 15 isolates had a cellulolytic index greater than 1.5. While most of the isolates in their study had a ratio greater than 1.5, their overall cellulolytic index (3.50) was underneath the highest cellulolytic index in the current investigation. The explanation of these variations is that termites explored are different organisms from different locations.

5.2 Diversity of cellulolytic bacteria in *Macrotermes michaelseni*

Termites have a symbiotic gut microbial population that allows them to survive on tough plant debris (Zhu et al., 2012). These microorganisms are common in termites and have distinct metabolic abilities, allowing them to play a variety of roles in termites. Cellulolytic bacteria use enzymes that produce hydrogen as a byproduct to break down complex plant material (Tochukwu Frank and Osita Gabriel 2018).The microbial community consists of a wide range of microorganisms, the most of which are currently uncultivable, and the precise symbiotic mechanism is unknown (Muwawa et al., 2016; Zhu et al., 2012). Identifying cellulose-degrading microorganisms is critical not only for understanding nutrient cycling in the ecosystem, especially in soil, but also for understanding food digestion in animals, such as termites. Identification of cellulolytic bacteria will also aid in the development of next-generation bioethanol technology based on the immensely abundant cellulosic plant resources (Xie et al 2017). The intention of this objective was to explore a diversity of cellulolytic bacteria in *M. michaelseni*

gathered from two distinct places used to produce cellulase enzyme for plant biomass utilization.

Amplification of the 16S rRNA gene with universal primers indicated 1500bp DNA amplicons. This result is similar with those of (Ferbiyanto et al., 2016; Muwawa et al., 2016) who used universal primer to amplify their cellulose degrading bacteria. Although CMC plate assay allowed the identification of 17 cellulolytic gut microbes, only 12 could be identified with great confidence (>98% similarity). This strongly suggests that the *M. michaelseni* contains uncharacterized microbes with uncharacterized cellulolytic systems. Furthermore, the isolates from this investigation grouped with sequences resulting from another termites and varied environments, such as soil, water, and plants, pharmaceutical sludge, and milk, with a similarity of > 98 percent to known 16S rRNA sequences.

The isolates from this study linked to the genera *Citrobacter, Paenibacillus Klebsiella, Bacillus, Staphylococcus, Arthrobacter, Dietzia,* and *Serratia*. The genera to which these isolates belonged have been identified previously in multiple studies of the termite gut. For instance a study by Kavitha et al., (2014), identified nine bacterial isolates on the gut content of a termite (*O. formosanus*) involved in cellulolytic activities. They identified microorganisms from the genera *Bacillus, Citrobacter, Pseudomonas Salmonella, Enterococcus, Staphylococcus*, and *Serratia*. Other studies such as, Pourramezan et al. (2012), also reported *Acinetobacter*, *Pseudomonas* and *Staphylococcus* isolated from Iran termites. Sharma et al. (2015), identified three isolates of the genus *Bacillus* sp, *Staphylococcus* sp and *Paenibacillu*s sp in a wood-feeding termite from India while Ferbiyanto et al. (2016), identified two isolates from the gut of *M. gilvus* and many

others. Therefore, as predicted, the majority of the detected genera of the isolates from this study have cellulolytic activities.

The genus *Bacillus* has been commonly isolated and identified for its cellulolytic activities. It is among the most dominant groups of bacteria in termites that are widely distributed (Bashir et al., 2013). For instance, (Xie et al., 2017) observed the occurrence and dominance of bacilli among groups of firmicutes involved in cellulose degradation in an eastern subterranean termite. The two isolates recognized from this inestigation were established to have cellulolytic activities. *B. cereus* is a pathogenic bacteria causing food poisoning often isolated in soil and growing plants (Granum and Lindbäck, 2013; Ivanova et al., 2003). Furthermore, while *B. cereus* demonstrated the highest cellulolytic activity among all *Bacillus* species (Patagundi et al., 2014; Sharma et al., 2015), the *B. cereus* obtained in this investigation was among the lowest, with a cellulolytic index of 1.00. Similar results were found by Sreena et al., (2015), who reported B. cereus with a low cellulolytic activity of 1.8 isolated from *Odontotermes spp*.

Staphylococcus saprophyticus has been widely reported for their tremendous ability of degrading numerous materials (Ogawa et al., 2009; Chebbi et al., 2018; Flimban et al., 2019). One of the isolates obtained from this study had a cellulolytic activity. This was comparable to the findings of *Staphylococcus* spp in the gut of fungus-growing termites by (Sreena et al., 2015; Kavitha et al., 2014). In addition to their cellulolytic ability, they can survive severe conditions and drug resistance (Humphreys, 2012; Varadaraj, 2010). Despite the fact that most *Staphylococcus spp* are pathogenic strains, they can also be investigated for their role in degradation processes.

The remaining two isolates from this investigation were from the Arthrobacter genus. *Arthrobacter* species are frequently found in soil. (Gobbetti and Rizzello 2014; Jones and Keddie 2017). *Arthrobacter spp*. is a highly proteolytic obligate aerobic bacteria with a bacillus-coccus growth cycle that do not create endospores (Gobbetti and Rizzello 2014). Apart from its cellulolytic activities, *Arthrobacter spp* has been observed to reduce high amounts of chromium in contaminated soil by Xiao et al., 2017, and also degradation of feathers and leather debris from poultry (Braman et al., 2017) suggesting their role in bioremediation and biodegradation.

In this analysis, the cellulolytic index measured for the two *Klebsiella spp* was lower than the ones obtained in previous studies (Ben Guerrero et al., 2015 Tochukwu Frank and Osita Gabriel, 2018). Members from this genus are characterized as Gram-negative, rod-shaped and facultative anaerobic bacteria (Muwawa et al., 2016). Korsa et al. (2022) established the importance of cellulolytic *Klebsiella oxytoca* and *Klebsiella sp*. Isolated from termites in textile wastewater decolorization. As a result, these isolates decolorized 58 to 94 percent of dyeing wastewater with less energy, released fewer chemicals into textile effluents, increased fabric quality, and partially purified the cellulase enzymes. Another significant role of *Klebsiella spp* in termites is their ability to degrade nitrogen into ammonia and other nitrogenous chemicals that termites can utilise (Muwawa et al., 2016; Doolittl et al., 2008). On the other hand Lin et al. (2010), used the cellulolytic capabilities of *Klebsiella oxytoca* THLC0409 isolated from a lignocellulose-degrading microflora to transform Napier grass to ethanol.

Citrobacter, on the other hand, produces cellulases and nitrogenase enzymes, which break down cellulose and nitrogen. Citrobacter members are bacilli and Gram-negative bacteria, as previously stated. *Citrobacter spp* not only possess cellulolytic activities but are nitrogen fixer and aromatic chemical breakdown Kavitha et al., 2014; Muwawa et al., 2016). For instance, Kavitha et al. (2014) reported a cellulolytic *C. freundii* isolated from the gut of *O. formosanus* with nitrogen fixation capabilities with Muwawa et al (2016) reported a cellulolytic *Citrobacter* strain with a role of nitrogen fixer in *Macrotermes* and *Odontotermes spp.*

Various strains of *Paenibacillus* have also been isolated and identified as cellulolytic bacteria. This study isolated *P. polymyxa* that exhibited the highest cellulolytic activity amongst all the isolates obtained. Wenzel, (2002) who also reported the highest cellulolytic activity of *Paenibacillus sp* isolated from *Zootermopsis angusticollis* observes similar findings. This genus may therefore be among the most cellulolytic bacterial genera found in termite reconstructions. Apart from their ability to break down cell walls, Paenibacillus also has the ability to fix nitrogen, solubilize phosphate, produce the phytohormone indole-3-acetic acid (IAA), and release siderophores that take up iron (Grady et al., 2016).

Serratia marccescens is a gram-negative bacillus that produces a red pigment at room temperature, as demonstrated in this study's isolate. Although *Serratia spp* are seldom isolated from termites, they naturally occur in soil and water (Ntabo et al., 2014; Buckle, 2016), and as microbial diversity in termites is regulated by diet and environment, the isolate from this investigation acquired from the nearby soil. Kavitha et al (2014) who

reported a cellulolytic S. marccescens in the guts of O. formosanus have reported a similar study. In addition, Ntabo et al (2014) revealed *S. marccescens* role as nitrogen fixers thus suggesting their role in termites.

Members of the genus *Dietziae* are actinomycetes that do not produce spores are aerobic, Gram-positive, non-acid-alcohol fast, catalase-positive that form cocci that later germinate into short rods or rod-shaped cells that snap and generate V-shaped forms. On the other hand, exhibit morphological traits on agar media, such as colonies with whole edges are created in a round, elevated or convex, sparkling, orange to a red coral pattern, as described by (Koerner et al., 2009; Gharibzahedi et at., 2013). *Dietziae natronolimnaea* enhances plant development in addition to its cellulolytic characteristics. For example, Bharti et al. (2016) looked at the effect of *Dietziae natronolimnaea* in protecting wheat plants from salt stress by altering the transcriptional machinery involved in salinity tolerance.

In 1983, Collins et al. were the first to isolate E. aurantiacum species from a potato-processing effluent. E. aurantiacum are Gram-positive short coryneform bacilli that are aerobic, motile, non-spore-forming, catalase- and DNase-positive, oxidase-negative, and alkaliphilic. On blood agar, they form orange-yellow colored colonies (Gusman et al., 2021). This study exhibited the cellulolytic role of E. aurantiacum in termites and thus explored for the degradation of plants.

Although there are no reports on the cellulolytic activities of *D. natronolimnaea* and *E. aurantiacum*, however, these two had cellulolytic activities in this study. Microbes are found in every environment (Gupta et al., 2016), and some bacterial species in termites

are associated with the microbes present in the termite's hill (Soukup et al., 2020). Since termites feed on plant biomass, soil and humus, *D. natronolimnaea* and *E. aurantiacum* could have been picked from the environments that the termites fed on and thus were able to be isolated. The cellulolytic activity of these isolates are related to the symbiotic relationship prevailing amongst microorganisms and termites, which results in cellulolytic activity, as stated by (Kavitha et al., 2014).

5.3 Evolutionary association of cellulolytic bacteria with other cellulolytic bacteria in DNA database

According to the phylogenetic analysis, several of the typical genes identified in our study cluster with sequences conveyed to have cellulolytic activity from numerous termite guts. Additionally, the isolates displayed up to 98% sequence similarity compared to known sequences in the Gen bank database. However, the results of the phylogenetic tree were inconsistent with those of the BLAST search.

The phylogenetic analysis revealed the affiliation to different organisms namely; *Streptomyces*, *Paenibacillus*, *Cohnella* and *Klebsiella*. This study's findings are congruent with Ben Guerrero et al., 2015. As indicated in the phylogenetic tree, isolates K12, KG32 and KG33 had sequence identities >95% with known members of the genus *Streptomyces* which are corroborated by a bootstrap value of 95%. This implies that *Streptomyces sp*. may be connected to these isolates.

The other isolates (KG31 and KG25) clustered with known *Cohnella sp* members, with a bootstrap value of 100%. Though rarely discovered in termite guts, *Cohnella sp*. has shown itself to be a potent cellulase (Ben Guerrero et al., 2015).

Isolate EG12 linked with 99% bootstrap level with Paenibacillus. As previously observed, Paenibacillus sp. has been shown to be among the microbes capable of breaking down cellulose. The discoveries from this investigation complement those of earlier studies over time (Wenzel, 2002; Ben Guerrero et al., 2015; Pasari et al., 2019) that have shown this strain as the best cellulolytic bacteria in the termite gut.

Two isolates (Isolate KG26 and KG23) were phylogenetically identical that formed a single sub-cluster with isolate KG24. The three isolates affiliated with *Klebsiella spp* are supported by a bootstrap value of 97%. As mentioned earlier, *Klebsiella* have also been recognized as cellulose degrading bacteria in the termite.

Similar bacteria have been found in Macrotermes spp. by other scientists (Muwawa et al.,2016 Ferbiyanto et al., 2016). According to the phylogenetic analysis, members of the same termite species are closely related and share a higher fraction of phylogenetic interconnectivity than microorganisms from other termite species. This relationship pattern shows that co-diversification with their termite host may shape community differences among Macrotermitinae members. This pattern, however, could be the result of termite species with comparable ecologies adopting similar gut bacteria (Femi-ola and Oyebamji 2019).

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The findings from this investigation show seventeen isolates with cellulolytic activity obtained from *Macrotermes mishaelseni* collected from Vihiga and Nandi County. It was also noted that isolates from Nandi County termites had the highest cellulolytic activity as compared to isolates from termites in Vihiga County. However, the cellulolytic activity of the termites from the two sites where they were gathered did not differ statistically significantly.

This study demonstrated a diversity of cellulolytic bacteria in *Macrotermes michaelseni* collected from Vihiga and Nandi County. Statistically there was no significant difference in the diversity of cellulolytic bacteria present in *M. michaelseni* between the two sites were they were collected. Twelve isolates could only be identified with a great confidence (>98% similarity). The isolates from this study linked to several species namely; *Citrobacter freundii, Paenibacillus polymyxa Klebsiella oxytoca, Bacillus thuringiensis, Klebsiella pneumoniae Staphylococcus saprophyticus, Arthrobacter defluvii, Dietzia natronolimnae, Bacillus cereus, Exiguobacterium aurantiacum* and *Serratia marcescens*. *P. polymyxa* exhibited the highest cellulolytic activity on carboxymethyl cellulose media amongst all the isolates obtained.

There was an evolutionary relationship between the characterized isolates in this study with the ones obtained in DNA database with significant bootstrap confidence > 98%.

The phylogenetic tree revealed an affiliation of the bacteria obtained from this study with different organisms previously isolated with cellulose degrading abilities namely; *Streptomyces, Paenibacillus, Cohnella* and *Klebsiella*.

6.2 Recommendations

Termites harbor a wide variety of novel and diverse bacteria that have yet to be cultured and thus investigated due to the use of traditional culturing methods. Future research could be done using a media that simulates the gut environment to explore new and effective bacteria in termite stomachs with cellulose-degrading capacity.

According to this study, *Paenibacillus polymyxa* is among the top bacteria that can break down cellulose after being isolated from termites. Further investigations could be conducted to determine its capacity to produce cellulase for its practical and biotechnological applications.

More research on these isolates are needed to determine the physical conditions (temperature and pH) under which these bacteria thrive in order for them to be used in the biodegradation of plant waste to reduce pollution.

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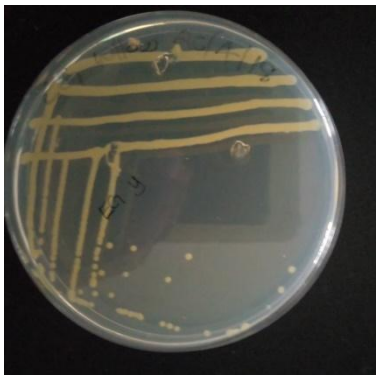
APPENDICES

Appendix I: Pure Bacterial Isolates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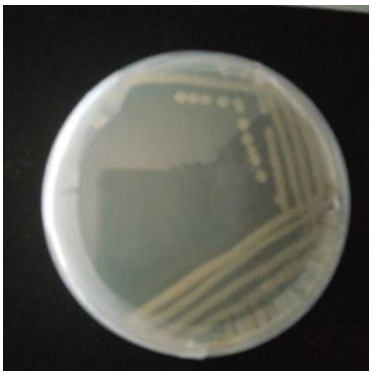
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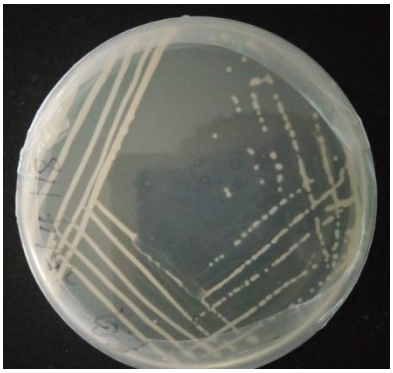
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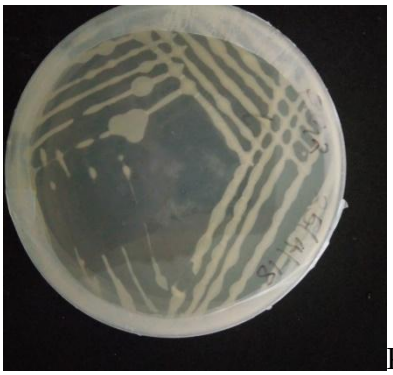
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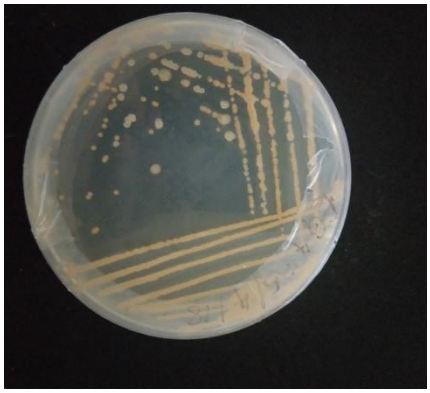
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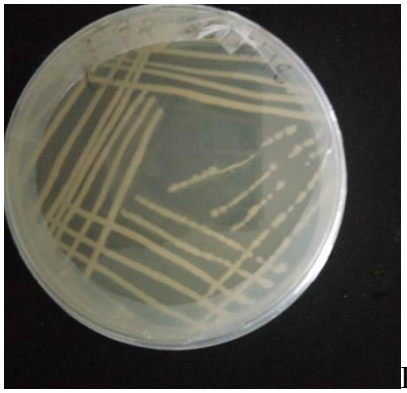
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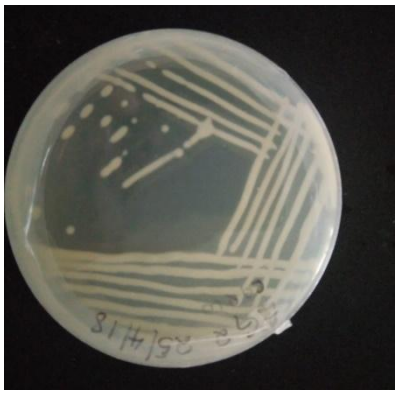
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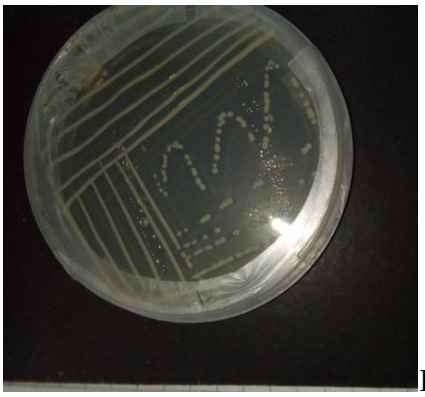
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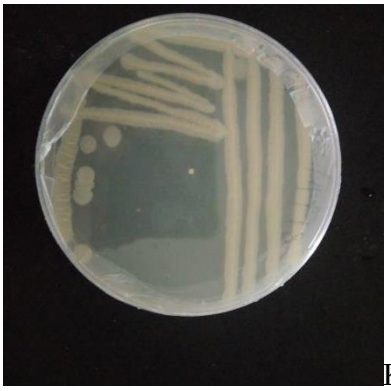
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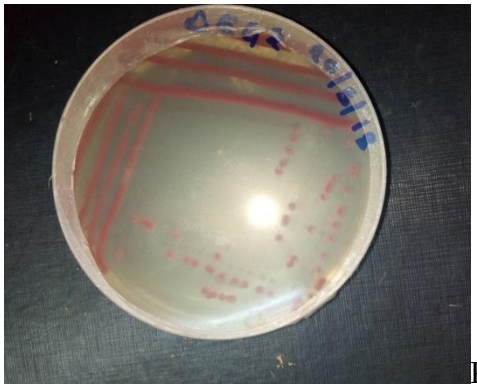
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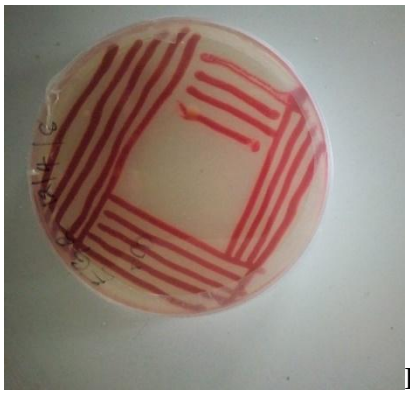
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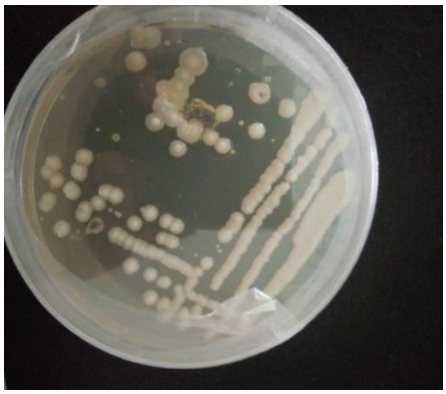
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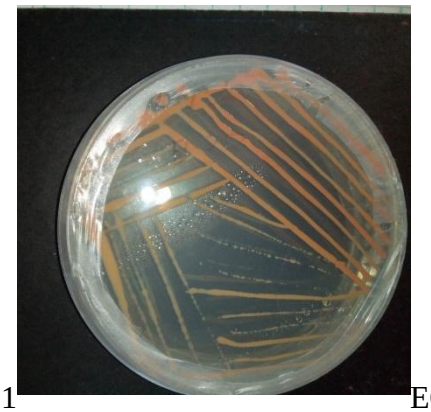
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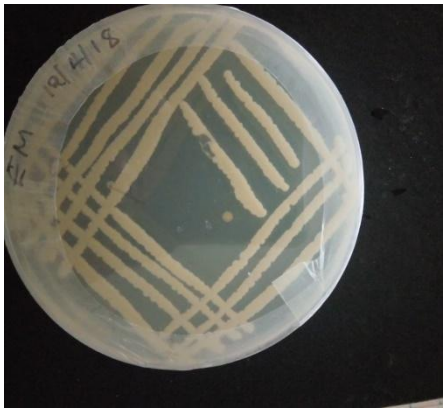
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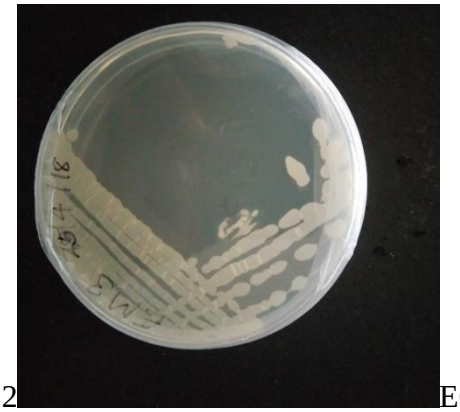
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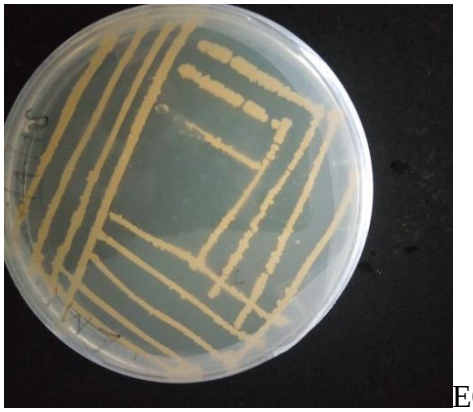
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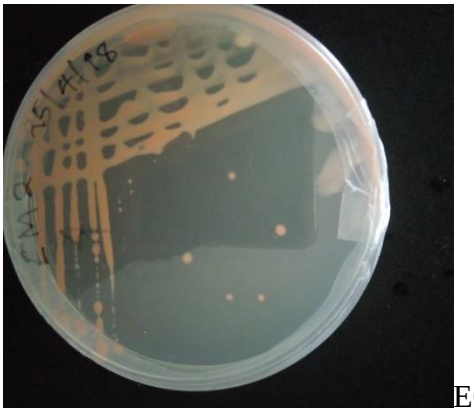
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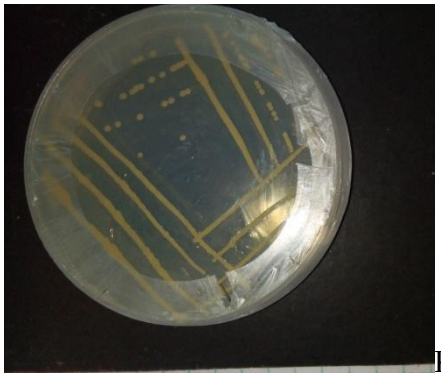
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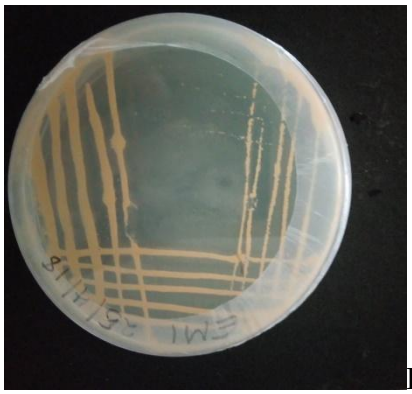
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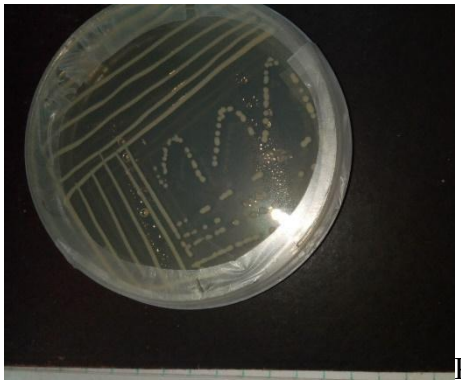
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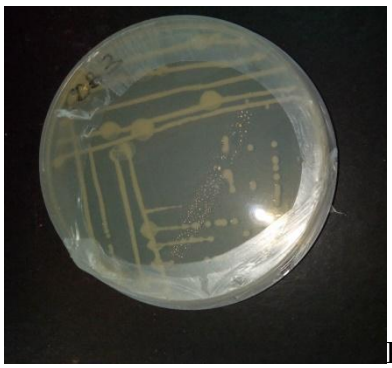
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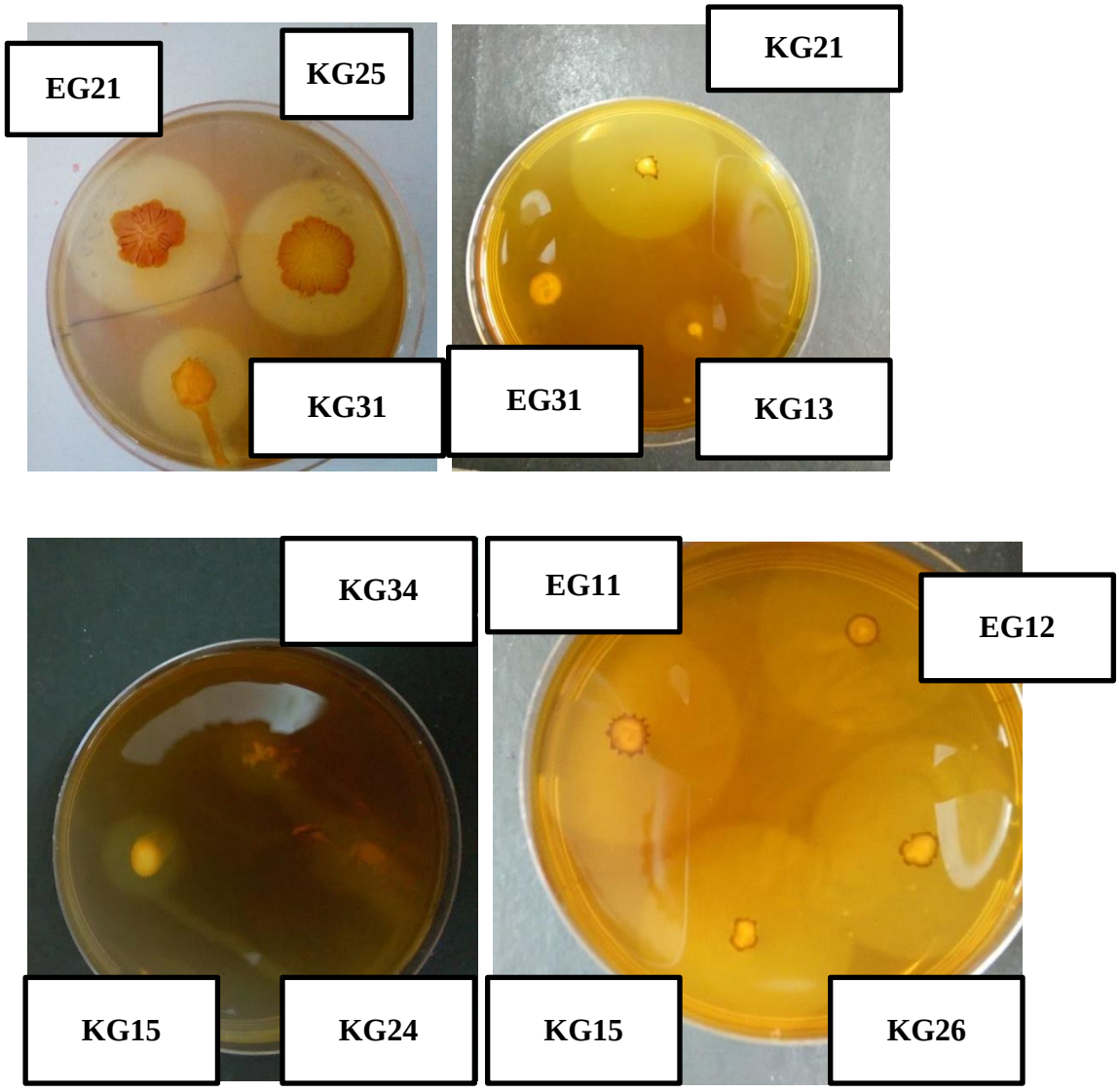
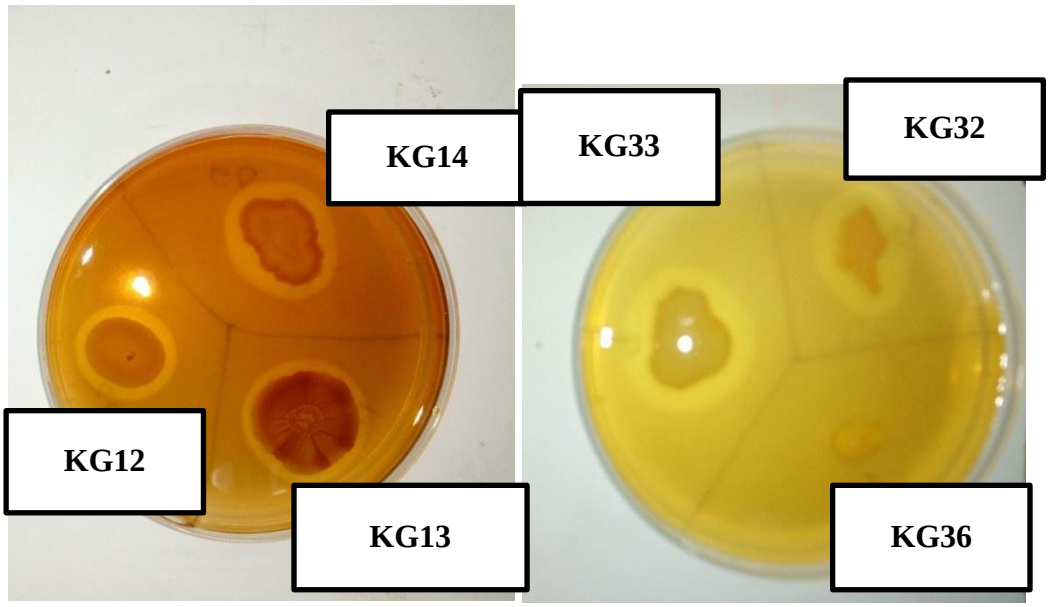
Appendix II: Screening of carboxymethyl cellulose degrading isolates

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KG33
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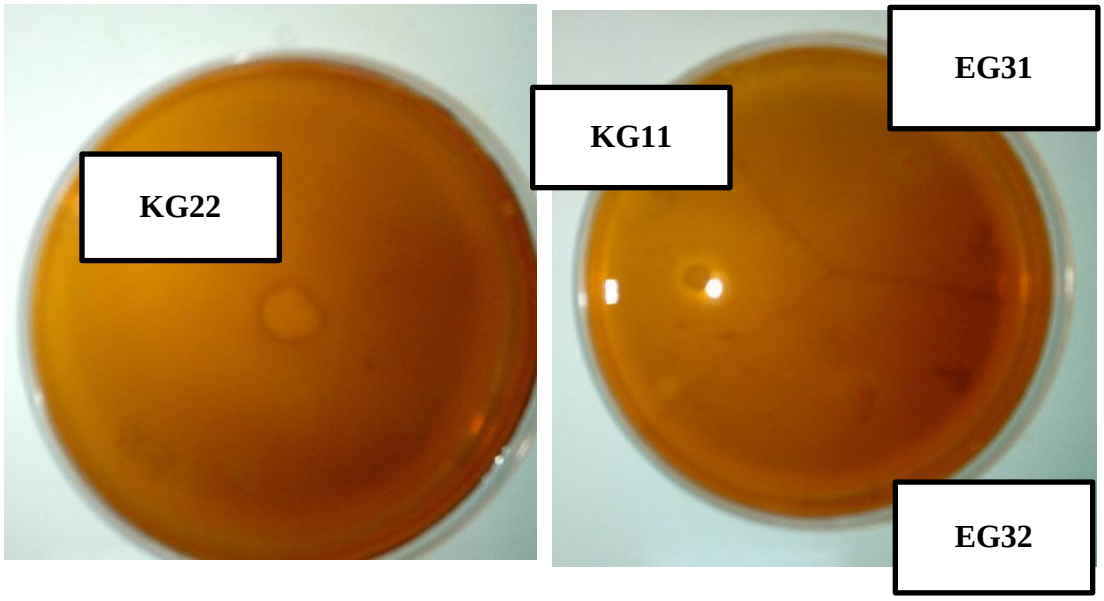
KG12
KG13
KG36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KG11
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EG32

Appendix III: List of sequences

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GGA

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Isolate 26 R

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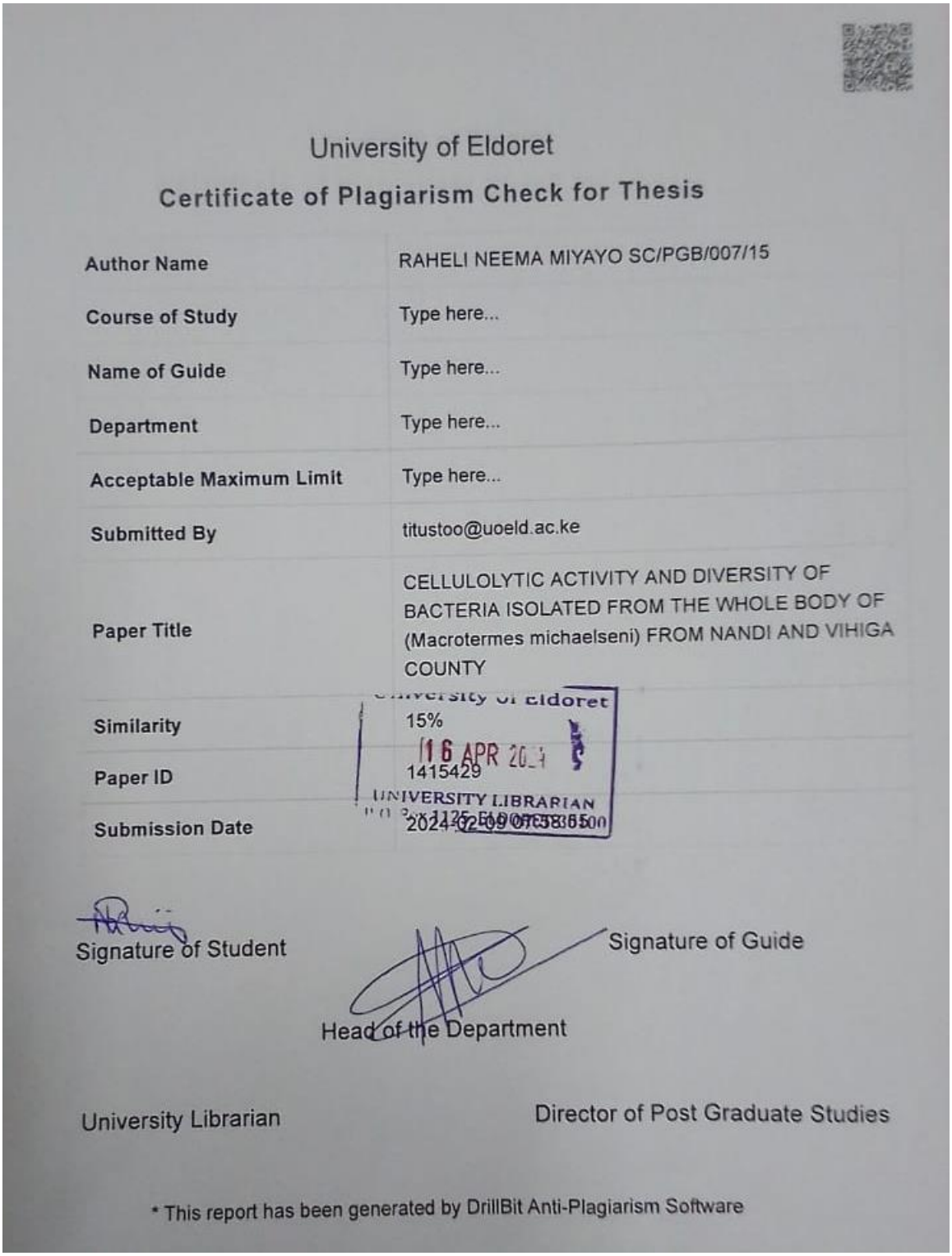
Appendix IV: Similarity Report



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Name of Guide	Type here...
Department	Type here...
Acceptable Maximum Limit	Type here...
Submitted By	titustoo@uoeld.ac.ke
Paper Title	CELLULOLYTIC ACTIVITY AND DIVERSITY OF BACTERIA ISOLATED FROM THE WHOLE BODY OF (Macrotermes michaelseni) FROM NANDI AND VIHIGA COUNTY
Similarity	15%
Paper ID	1415429
Submission Date	2024-02-09 07:38:00

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