INFLUENCE OF CULTURE MEDIA AND INCUBATION CONDITIONS ON THE POPULATION AND DIVERSITY OF MICRORGANISMS CULTIVATED FROM SOILS AND TERMITE GUTS

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Influence of Culture Media and Incubation Conditions on the Population and Diversity of Microorganisms Cultivated from Soils and Termite Guts

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Declaration

This thesis is my original work and has not been presented for a degree in any other university.

Signature……..Date……..

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This thesis has been submitted for examination with our approval as university supervisors

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Dedication

This work is dedicated to my Father Mr. Thomas Ntabo who laid the foundation, supported and encouraged me to attain even greater heights in the academic ladder.

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Abbreviations

DNBA	Dilute nutrient broth plus Agar
LB	Luria Bertani
DNA	Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
G+C	Guanine and Cytosine
EDTA	Ethylene diamine tetra-acetic acid
Bp	Base pairs
Cfu	Colony forming units
Nacl	Sodium Chloride
rDNA	ribosomal Deoxyribonucleic Acid
JKUAT	Jomo Kenyatta University of Agriculture and Technology
IBR	Institute for biotechnology research
BSS	Basal salt solution
Rpm	Revolutions per minute
K_2HPO_4	Dipottasium hydrogen phosphate
$MgSO_4$	Magnesium sulphate
$FeSO_4$.	Iron sulphate
H_2O	Water molecule
$CuSO_4$	Copper sulphate
$ZnSO_4$	Zinc sulphate
$MnSO_4$	Manganese sulphate
KH_2PO_4	Potassium dihydrophosphate
KCL	Potassium chloride
rRNA	ribosomal ribonucleic acids

Abstract

In the last several years information on the gut ecosystem of termites has continued to be gathered. Most studies on termite gut microbial communities have been focused on wood feeding termites but studies on soil feeders remain sparse owing to their typically remote habitat, delicate nature and the difficulty of establishing permanent laboratory cultures. Studies have shown soil feeding termites' house important antibiotic producing bacteria in their gut, nest and surrounding soil. An alternative approach was used in this study to isolate such bacteria from the soil, gut and mound of soil feeding termites from Kakamega Forest. The samples were collected from two sites Kalunya Glade and Lirhanda Hill. The study was also extended to the soils found in Juja. The cultivation procedure included: use of media with minimal nutrients, incubation at three different temperatures (25 ^{o}C, 30 ^{o}C and 37 ^{o}C) and observing the trend and counting of colony forming units (cfu) for an extended cultivation period. Dilution and heat shock method was also used during cultivation to target isolation of Actinobacteria. Through statistical analysis, Kakamega Forest soil samples had higher counts of cultivable microorganisms' (cfu/g) with a mean of 1.65×10^{8} cfu/g than Juja soils samples that had a mean of 8.5×10^{7} cfu/g. However clay soils from Juja and kakamega Forest had higher cfu/g counts than all the other soils samples with means of 1×10^{8}cfu/g and 2.02×10^{8} cfu/g respectively. The termite gut had high cfu/g counts with a mean of 3.10×10^{9}cfu/g with an optimum cultivation temperature of 37 ^{o}C while the mound samples had the least number of cfu/g counts with a mean of 6.25×10^{7} cfu/g at optimum cultivation temperatures of 25 ^{o}C and 30 ^{o}C. The results showed that the effect of incubation temperature on a number of cultivable microorganisms was significant when the dilution and heat shock method was used where the optimum cultivation temperature for termite guts, soil and termite mound samples was 30^{o}C. Extending the incubation period was significant as cfu/g count increased with time on most samples. Hundred and thirty seven (137) isolates were

screened for their antagonistic effects on various test organisms. Fifty one percent of the isolates were antagonist to *Escherichia coli*. Fifty seven percent of the isolates were antagonists to *Bacillus subtilis* while 55% of the isolates were antagonist to *Candida albicans*. Enzymatic activities of the isolates showed that 65% of the total isolates were starch degraders, 54% were casein degraders and 68% of the isolates were able to liquefy gelatin. Lastly, 11% of the isolates were cellulose degraders the majority of which were obtained from termite gut and mound. Isolates from Juja soil had the highest number of non degraders as compared to Kakamega Forest soils. The isolates were characterized using morphological, biochemical and molecular methods. Phylogenetic analysis of amplified 16S rRNA gene sequence revealed eight isolates from gut, mound and soil were closely related to *Bacillus thuringiensis.* An isolate from surrounding soil was closely related to *Bacillus pumilus* while two isolates from the mound were closely related to *Bacillus subtilis*. An isolate from surrounding soil of termites was closely related to *Brachybacterium paraconglomeratum* and showed very strong *in vitro* antagonistic effects. Two Gram negative bacterial isolates obtained from surrounding soil were closely related to *Pseudomonas aeruginosa* and *Serratia marcescens*. In conclusion, the study was able to cultivate microorganisms in low nutrient media from the gut, mound and surrounding soil of *Cubitermes severus.* These isolates were antibiotic producers and had the ability to degrade gelatin, casein, starch and cellulose an indication of the role they play in their habitat.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Soil microorganisms

Soil is a highly heterogeneous environment (Alexander, 1977). It contains a high diversity of microorganisms (Liesack *et al*., 1997) which can be found as single cells or micro colonies developing around the soil particles (Torsvik, 2002). The numbers and kinds of microorganisms present in the soil are depended on many environmental factors such as soil type, nutrient availability, degree of aeration, pH, temperature and soil moisture (Prescott *et al.*, 1999). Soil is therefore regarded heterogeneous with respect to conditions for microbial growth and for the distribution of microorganisms and matrix substances. As a result there is a wide variety of microbial niches and a high diversity of soil microorganisms (Rolf, 2004). Soil microorganisms influence above ground ecosystems by contributing to plant nutrition, plant health, soil structure and soil fertility (O'Dounell *et al*., 2001). They are responsible for many of the key processes in the biogeochemical cycling on earth such as nitrogen, sulphur and carbon cycle (Lengeler *et al*., 1999). In addition they are a valuable sources of natural products providing important antibiotics for pharmaceuticals, important enzymes and bioactive compounds for industries (Strohl, 2000).

In recent years the emergence of multidrug resistance pathogens has rekindled the need to discover new antimicrobials from the environment. The tremendous diversity of microorganisms could represent one of the richest sources of new antimicrobials (Keller and Zengler, 2004). However, numerous attempts made using traditional cultivation methods have failed to discover new antimicrobial products (Strohl, 2000). This is because isolate culture extracts often produced numerous previously described metabolites (Silva *et al*., 2001) and the rate of rediscovery of known antimicrobial has approached 99.9% (Zahner and Fiedler, 1995). It's reported that only

1% of the greater than a million cells in each gram of soil seems able to form colonies on laboratory media implying that majority have eluded cultivation hence their biotechnology potential cannot be explored (Jensen, 1968).

1.2 Termites

Termites are a ubiquitous feature of tropical and sub-tropical soils, where their number exceeds 6000 m^{-2} and their biomass densities (> 50 g m^{-2}) often surpass those of grazing mammalian herbivores (0.01–17.5 g $m^{-2)}$ (Collins and Wood, 1984). They belong to the order Isoptera (Nutting, 1990). This order contains five families of lower termites and one family of higher termites (Noirot, 1992). Termites are recognized as one of the major ecosystems engineers in tropical soils (Holt and Lepage, 2000). The impact of termites on soils is caused by their feeding habits where they play a vital role as mediators of decomposition (Wood and Johnson, 1986), humification, soil conditioning, and fragmentation of organic detritus, nitrogen-fixation, aggregate-binding and formation of clay-mineral complexes (Lee and Wood, 1971; Collins, 1983; Tayasu *et al*., 1994). They also construct mounds from mineral matrix mixed with faeces or saliva depending on termite species (Grassie, 1984). These mounds have increased the microbial density as result of high organic matter (Brauman *et al*., 2000).

Diverse microorganisms "inhabit" the intestinal tracts of all termite feeding groups (Brune, 1998; Brauman *et al*., 2001). Most studies on termite gut microbial communities have focused on wood feeding termites; analogous studies of other feeding guilds, especially soil feeders are lagging behind, owing to their typically remote habitat, delicate nature, and the difficulty of establishing

permanent laboratory cultures (Bignell *et al*., 1980; Rouland *et al*., 1993). As a result their biotechnological potential cannot be exploited.

1.3 Cultivation independent molecular approaches

Culture-independent studies mostly based on PCR amplification, cloning and 16s rRNA gene sequence analysis have revealed unexplored bacterial diversity that have previously not been cultured in soil (Liesack *et al.,* 1997) and termites (Mackenzie *et al.*, 2007). Molecular analysis have also revealed drastic difference between the genetic structures of bacterial communities in soil feeding termite mound, gut and surrounding soil (Fall *et al.,* 2005). However these methods do not provide further information on the microbial physiology, abundance and ecological significance hence the need for cultivation dependent approaches (Embley and Stackebrandt, 1996).

1.4 Cultivation dependent approaches

The diversity of soil microorganisms has been exploited for many years based on the cultivation and isolation of microbial species (Rolf, 2004). Various unconventional culture media such as low nutrient media have been used in the recent past to isolate rarely isolated groups of bacteria in soil. Such media prevent growth of fast growing bacteria and substrate accelerated death of bacteria from low nutrients habitats (Hunter and Postgate, 1964), a situation that is common with high nutrient standard cultivation media (Zengler *et al*., 2002). Janssen *et al*., (2002) cultivated novel bacteria using DNB plus agar/gellan media and extended incubation period that allowed cultivation of slow growing bacteria at an incubation temperature of 25^{o}C. This media has also

been used to cultivate uncultured groups of bacteria from wood feeding termites (Stevenson *et al.,* 2004).

Temperature is an important factor in regulating microbial activity and shaping the soil microbial community (Pietikainen *et al*., 2005). However little is known on how temperature affects microbes found in termite gut, mound and soil. This study intended to isolate antibiotic and enzyme producing bacteria from soil, termite gut and mound obtained from a rain forest and soils from dry grassland using low nutrient media with a view of understanding cultivable bacteria population dynamics in the sample units under varied incubation temperatures and extended incubation periods.

1.5 Statement of the problem

Bacteria are known producers of antimicrobials and enzymes used in pharmaceuticals and industries respectively. However the emergence of multidrug resistance pathogens has rekindled the need to discover new antimicrobials from the environment. Low discovery rate of these antimicrobials is attributed to standard cultivation methods that use nutrient rich media (Strohl, 2000). Low nutrient media has therefore been used in the recent past to cultivate uncultured groups of bacteria from soil and termites. However little is known on how temperature affects bacteria cultured from termite gut, mound and soil while using low nutrient media to isolate antibiotic and enzyme producing bacteria from these samples. The findings of this study will therefore shed light on optimum cultivation temperatures as it seeks to isolate antibiotic and enzyme producing bacteria from the termite gut, mound and soils.

1.6 Justification

There still exits large discrepancy between the numbers of bacterial colonies that form on solid media and actual total number of bacterial cells present in that same soil (Rappé and Giovannoni, 2003). This discrepancy has limited our understanding of species diversity of soil bacterial communities and it has been associated with the inadequacy of standard cultivation methods (Joseph *et al.*, 2004). These unrecovered microorganisms represent an unexplored reservoir of novel strains that may produce novel natural products (Rolf, 2004). Further more, investigations have always focused on old and known producers of natural products. It is time that this search is diversified to include the little or previously uninvestigated species or strains of organisms.

The problem of resistance to drugs currently available in the market by pathogens coupled with toxicity of most of them has called for urgent development of newer and more effective substances that could improve the fight against infectious diseases (Keller and Zengler, 2004). Most pathogens have evolved mechanisms that always keep them one step ahead of us, and therefore the need to try and also stay one or two steps ahead of them. This can only be achieved by discovering and developing new substances capable of interfering with certain key processes inherent in them (Leeb, 2004). It is a belief that such compounds could be derived from natural products and more so from untapped sources (Keller and Zengler, 2004). This can only be achieved by modifying cultivation conditions that can accommodate some bacteria if not all. Though cultivation of soil bacteria has been done elsewhere in the world, the heterogeneity of the soil in terms of composition and size of particles (Torsvik, 2002) is different in various ecosystems in the world. Microbes are therefore endemic to certain geographic regions hence various habitats in Kenya may harbor unique microbes for biotechnology use This could lead to

discovery of new potent substances that could find use in the pharmaceutical industry and help in the fight to combat infectious diseases afflicting mankind.

1.7 Main objective

To isolate and characterize microorganisms which can produce antibiotics and/or extra cellular enzymes from the soil, mound and guts of soil-feeding termites.

1.8 Specific objectives

1. To determine the impact of temperature on cultivation efficiency of microorganisms from different soil types, termite gut and termite mound
2. Improve the cultivability of bacteria in soil and termite gut through modification of cultivation conditions
3. To isolate, characterize and identify bacteria resident in the guts, mound and parent soil of soil feeding termites *Cubitermes* species.
4. Screen isolates for antibiotic and enzyme production.

1.9 Hypotheses

- There is no difference in the population of viable bacteria found in the different types of soil, mound and termite gut under different incubation conditions.
- The soil feeding termite gut, mound and soil are a potential source of antibiotic and enzyme producing bacteria.

1.10 Expected outputs

It was expected that at the end of this project the following would be achieved:

- Obtain bacteria isolates that produce Antibiotics and Enzymes
- Determine cultivation temperature of termite gut, mound and soil bacteria.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Soil feeding termites

Soil-feeding termites make up 62% of the genera of higher termites and nearly 50% of all termite species (Brauman *et al*., 2000). In Africa more energy flows *via* soil-feeding termites than through wood-feeding termites (Eggleton *et al*., 1995; 1997). However, little is known about the exact nature of the dietary components exploited by these ecologically important soil macro invertebrates (Brauman *et al*., 2000). Besides fragments of plant tissue, fungal hyphae, and numerous microorganisms, their diet consists largely of undefined humic material intimately associated with the mineral soil matrix (Donovan *et al*., 2001). While it was initially assumed that the principal substrate of soil-feeding termites is the aromatic component of humus (Noirot, 1992; Bignell, 1994), feeding trials with soil-feeding *Cubitermes* species have shown that peptidic soil components – free or polymerized into humic model compounds – are preferentially digested and mineralized (Ji *et al*., 2000; Ji and Brune, 2001). The anterior hindgut of soil-feeding termites is extremely alkaline, which favours the extraction of organic matter from the soil (Brune, 1998; Kappler and Brune, 1999). Microbial biomass and its structural components are assimilated more efficiently than cellulose, which supports the hypothesis that soil microorganisms and the nitrogenous components of humus are an important dietary resource for humivorous soil macro invertebrates (Ji *et al*., 2000, Ji and Brune, 2001).

In tropical ecosystems the termite mound soils constitute an important soil compartment covering 10% of African soils (Holt and Lepage, 2000). Soil feeding termite mound have very specific properties arising from the combination of materials of two distinct origins, feces and

soil (Brauman, 2000). This increases richness in clay, minerals (Ndiaye *et al*., 2004) and organic matter in form of carbon and nitrogen (Fall *et al*., 2001) with respect to surrounding soil (Fall *et al*., 2007). The construction of these mounds cause changes in water holding capacity, bulk density, structural ability and chemical changes in the surrounding environment for microbes (Brauman, 2000). Consequently the mound has bacterial communities that are different from those in surrounding soil (Fall *et al.*, 2004).

2.1.1 Microbial diversity in soil feeding termites gut

The digestive tract of soil-feeding termites provides a favourable environment for micro organisms and harbours a dense and varied micro biota (Brune, 1998; Brauman *et al.*, 2000), which is thought to be involved in the transformation processes inside the termite gut (Bignell *et al*., 1980). However the exact composition and role played by the micro biota in the digestive tract of soil-feeding termites is still poorly understood. Early studies on the hindgut micro biota of soil feeders revealed that the hindgut is packed with morphologically diverse bacteria (Noirot and Noirot-Timothee, 1969; Honigberg, 1970; Bignell *et al*., 1980; Noirot, 1992) whose metabolic products are thought to contribute to the nutrition of the termite (Noirot, 1992; Breznak and Brune, 1994). Electron microscopy studies on the soil-feeding termites *Procubitermes aburiensis* and *Cubitermes severus* revealed an abundant colonization of the hindgut by bacteria and actinomycete-like organisms (Bignell *et al*., 1979;1980).

In a number of studies combining feeding experiments and microinjection with radiolabel tracers into the gut, transformation and mineralization of a number of possible substrates has been

followed (Ji *et al*., 2000, Ji and Brune, 2001; Tholen and Brune, 1999). Using feeding experiments, researchers have been able to show that soil-feeding termites transform peptides, protein, cellulose, peptidoglycan, and microbial biomass (Ji and Brune, 2001), but not the aromatic components, in the soil (Ji *et al*., 2000, Ji and Brune, 2001). Microinjection studies have been used to localize homoacetogenic and methanogenic activities in the hindgut of soil-feeding termites (Tholen and Brune, 1999). In addition, hindguts of soil-feeding termites were observed to have very high levels of ammonia (Ji and Brune, 2006).

Studies on the microbiota of soil-feeding termites, especially their functional role in digestion, are still lagging behind (Brune, 2006)). Enumeration of microorganisms using the DAPI-staining technique confirmed abundant colonization of the guts of *Cubitermes umbratus* and *Cubitermes ugandensis* with bacteria and numerous filamentous organisms (Schmitt-Wagner *et al*., 2003a). Studies on the metabolic diversity of microganisms present in the guts of soil-feeders have revealed the presence of diverse metabolic groups of bacteria, including homoacetogenic (Boga *et al*., 2003) and propionigenic bacteria (Boga *et al*., 2007), as well as methanogenic archaea (Tholen and Brune, 1999). Lactic acid bacteria (Bauer *et al*., 2000) and aerobic organisms capable of degrading glucose, gelatin, casein and short-chain fatty acids are also present (Miambi, *et al*., unpublished results).

Apart from lactic acid bacteria (Bauer *et al*., 2000), isolates of other microorganisms that could be playing a significant role in the gut of soil-feeding termites have not been obtained. Boga *et al*., (2003) isolated and described a new homoacetogenic bacterium, *Sporomusa aerivorans*, which was found to have the unique ability to consume large amounts of oxygen (Boga and

Brune, 2003). However the isolate probably represents only a small population and it is not likely to play a significant role in the gut. The same reservations apply to a novel propionigenic bacterium, which was tentatively assigned to a new genus (Boga *et al*., 2007). Cultivation attempts have generally been very dissapointing and have yielded only 0.1% of the gut microorganisms enumerated by direct counts (Miambi, *et al.*,, unpublished results). As a result, still very little is known about the physiology of the larger proportion of microorganisms that have escaped cultivation.

Following the observations of filamentous organisms made in electron microscopy studies of hindguts of soil feeders (Bignell *et al.*, 1980), attempts were made to cultivate these organisms. Numerous *Streptomyces* species from the gut, parent soil and mound materials of *Procubitermes aburiensis* and *Cubitermes severus* were isolated (Bignell *et al.*, 1991). Some cellulolytic and lignin-solubilizing actinomycetes were also isolated from several termites (Pasti and Belli, 1985; Kuhnigk and König, 1997). Recently, Watanabe *et al.*, (2003) isolated and characterized actinomycetes from termites guts and established that the actinomycetes isolated depended largely on the geographical origin of the termite. Preliminary studies in the laboratory have also led to the isolation of a number of actinomycetes from the intestinal tracts of fungus-cultivating *Macrotermes michaelseni* and *Odontotermes* species (Mackenzie *et al.*, 2007*).* However, most of these studies employed classical techniques used for enrichment of actinomycetes from soil and might have captured transient populations of actinomycetes or their spores.

2.2 Microbial diversity in soil

Soils contain phylogenetic groups of bacteria that are globally distributed and abundant in terms of the contributions of individuals of those groups to total soil bacterial communities (Buckley and Schmidt, 2002). These groups include the phyla *Acidobacteria, Bacteroidetes-Cytophaga-Flexibacter, Firmicutes* and *Proteobacteria* (Durbar *et al.,* 1999). The *Planctomycetes, Verrucomicrobia and Gemmatimonadetes* phyla are also found in the soil but they have very few known members of their respective division (Joseph *et al.,* 2004). Natural ecosystems such as forests are characterized by high biological diversity. The continuous plant cover results in increased soil organic matter leading to improved soil structure, nutrient storage capacity, water holding capacity and microbial density (Gulick *et al.,* 1994). It is estimated that a forest sample contained about 4,000-6,000 genome that could represent between 12,000 and 18,000 different species (Torsvik *et al.,* 1996).

Microbial diversity in soil is dependent upon many environmental factors for instance microbial survival and abundance is dependent upon soil moisture content linked with oxygen status of the soil (Entry *et al*., 2002). Slight anaerobic conditions during high moisture content favor persistence of microbes in addition to microbial die off rate increases with a decrease in soil moisture (Mubiru *et al.,* 2000). However under conditions of excessive moisture, a considerable dilution of usable organic carbon may occur resulting in unfavorable conditions for microbial survival (Klein and Casida, 1967).

The role of nutrients in modulating microorganisms' survival rate cannot be underrated since multiplication rate of bacteria cells is determined by the level of utilizable nutrients available

(Lechevallier *et al*., 1991). Temperature also regulates microbial activity while shaping the soil microbial community (Pietikainen *et al.,* 2005). Most soil bacteria remain viable for lengthy periods provided soil temperatures are relatively low. Soil pH also affects the soil bacterial community and it is suggested that a soil pH of between 6 and 7 offers optimal conditions for the majority of bacterial survival (Reddy *et al*., 1981).

Soil type dictates the soil texture hence influencing the general microorganism's survival (England *et al*., 1993). For instance, soil texture determines the water content holding capacities. Consequently soils that are poor at retaining water will accommodate great numbers of discontinuities in water films, thus restricting the movements of grazing protozoa and improving the survival potential of prey cells (Heijnen and Vanven, 1991). Soil texture through the amalgamation of soil aggregates and organic matter also acts as a provider of microhabitats that may affect the survival of microorganisms in the soil. Hence the presence of clay enhances retention of microorganisms and increases the provision of protective niches (England *et al.*, 1993). Soil type also dictates the soil structure and physical make up of the soil pore network. The habitable pore space that arises through the given soil structure means that organisms of different diameters may only inhabit pores to which they can gain physical access. Cells that inhabit smaller pores become less susceptible to predation by larger microorganisms which cannot access the narrow pore networks (Young and Ritz, 2000).

2.3 Detection of Microbial diversity in Soils and termite guts using Molecular tools

Molecular techniques such as the PCR denaturing gradient gel electrophoresis (DGGE) analysis, cloning and sequencing of PCR amplified 16S rRNA gene fragments have been used to show

bacterial genetic structure of the mounds, gut and surrounding soil of soil feeding termites *Cubitermes niokoloensis* (Fall *et al.,* 2007). The mound is dominated by phyla *Actinobacteria* whereas *Firmicutes* and *Proteobacteria* phyla dominated the gut sections of termites and surrounding soil respectively. The surrounding soil was also characterized by the members of the family *Dermabacteriaceae* (Fall *et al.,* 2007). Restriction fragment length polymorphism analysis of PCR product from clones obtained from a fungus cultivating termite gut have been used to show presence of high G+C content bacteria in the gut that have previously escaped cultivation (Mackenzie *et al*., 2007). Molecular techniques such as the Terminal Restriction Fragment Length Polymorphism have revealed presence of diverse phylogenetic groups of bacteria that do not have cultured representatives (Brunk *et al*., 1996). In recent studies, environmental clone libraries of soil metagenome have been generated using shotgun, Fosmid and bacterial artificial chromosomes (BAC) cloning (Santosa, 2001). Sequencing DNA of these clone libraries have enabled access to potential new gene products for example enzymes and secondary metabolites from uncultivated microorganisms (Robertson *et al*., 1996) circumventing the need to culture them in the laboratory (Sharma *et al*., 2005).

However these molecular methods do not provide information on their physiology, abundance and ecological significance (Embley and Stackebrandt, 1996). Therefore pure culture isolates greatly simplifies investigations of the physiology and roles of bacteria (Sait *et al*., 2002). However parallel study of laboratory cultures and molecular ecological investigations would together strongly compliment and enhance research into the roles of soil bacteria and their biotechnology potential (Joseph *et al*., 2004).

2.4 Strategies for Increasing Cultivation of Microorganisms

It is estimated that 99% of microorganism observed in nature typically are not cultivated by using standard techniques (Amann *et al*., 1995) hence there exists a large discrepancy between the number of bacterial colonies that form on solid media and the total number of bacteria in that same soil (Rappé and Giovannoni, 2003). The rate of discovery of novel natural products derived from isolated microorganisms has significantly decreased (Strohl, 2000). This is due to standard cultivation methods that often produced numerous previously described metabolites (Silva *et al*., 2001) and as a result the rate of rediscovery of known antimicrobials has approached 99% (Zahner and Fiedler, 1995). It has often been suggested that only the easily cultured fraction of the soil micro flora has been isolated and that the more numerous, difficult to isolate bacteria have been more or less ignored (Torsvik *et al.*, 1990). Consequently more or less sophisticated techniques have been developed for the isolation of novel bacteria from complex microbial habitat.

Studies have shown that the transfer of bacteria from low nutrient soil environment to high nutrient laboratory media results in substrate accelerated death (Postgate and Hunter, 1964). Low nutrient level media compares to those present in the natural environments (Button *et al.*, 1993). They prevent growth of fast growing bacteria, a situation that is common with high nutrient level in standard cultivation media (Zengler *et al.*, 2002). Cultivation in dilute nutrient broth media (DNB) solidified with Gellan gum/Agar at 25^{o}C incubation temperature and incubation period of ten weeks has been used successfully to cultivate uncultured groups of bacteria belonging to phyla *Verrucomicrobia, Gemmatimonadetes and Planctomycetes* (Janssen *et al.*, 2002). Davis *et al.*, (2004) recommended DNB plus agar medium because it allows least growth of fungal

contaminants, permits growth of as large a range as possible of bacteria present and prevents growth of spreading colonies. Dilution and heat shock method on media that contains agar with no nutrients added has been used successfully to isolate actinomycetes found in marine ecosystems (Watanabe, 2003).

VL55 medium has also been used to cultivate less cultured groups of bacteria. The medium is formulated to mimic the low concentration of inorganic ions in soils with increased concentrations of ammonium and phosphate ions to allow sufficient biomass formation to produce visible colonies of rarely isolated groups of bacteria (Sait *et al.,* 2002). Various polymers are used since the substrates eliminate sudden exposure of soil bacteria to high substrate concentrations, as polymers must be hydrolyzed before microorganisms can utilize them (Liesack *et al.*, 1997).

2.5 Microorganisms as sources of natural products

2.5.1 Enzyme production

Microorganisms are used in numerous applications such as the synthesis of specific enzymes and commodity chemicals (Keller and Zengler, 2004). Microorganisms have become important as producers of industrial enzymes due to their biochemical diversity and ease with which enzyme concentration may be increased by genetic and environmental manipulation (Pandey *et al.*, 2000). Proteolytic enzymes for instance are used as industrial process aids contributing to more than 50% of the worlds commercially produced enzymes (Godfrey and Reichet, 1983). Microbial proteases derived from a large number of *Bacillus* species and *Streptomyces* species have been used in dehairing of hides and skins (Taylor *et al.*, 1987). Microbial source of amylases is

preferred to other sources like synthetic, plant and animal sources because of its plasticity and vast availability (Pandey *et al*., 2000). Starch degrading amylolytic enzymes are most important in the biotechnology industries with huge application in food, fermentation, textile and paper industries (Pandey *et al*., 2000). Biomass such as starch and cellulose that are used in the production of chemicals, biodegradable plastics, pesticides, new fibres and biofuels also require enzymes.

2.5.2 Antibiotic production

Antibiotic production is a feature of several kinds of soil bacteria (Talaro and Talaro, 1996). The order Actinomycetales became renowned as a source of antibiotics (Waksmans *et al.*, 1942). By 1980's members of Actinomycetales accounted for almost 70% of the worlds occurring antibiotics used in humans (Lazzarini *et al*., 2001) while non actinomycetes bacteria accounts for 12% of known secondary metabolites (Agrawal, 2003). The phyla *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes* and *Proteobacteria* are known to have species that produce bioactive compounds (Keller, 2004).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Kakamega Forest

3.1.1 Study site

Kakamega Forest is located in western part of Kenya and lies in the Lake Victoria basin 150 kilometers west of the great African Rift Valley (Government of Kenya, 1994). It measures approximately 19,649 hectares. Of this dense indigenous forest covers 11,345 hectares. Semi dense indigenous forest covers 2,705 hectares while the area under degraded indigenous cover is negligible. It lies within an altitude that ranges between 1,500 m and 1,600 m above sea level and up to 2,060 m. There are a few scattered forested hills such as Lirhanda where termites were collected. The forest also has a marshy clay swampy region that lies between 800 m-1000 m above sea level which is the Kalunya Glade where soil feeding termites were collected (Government of Kenya, 1994).

3.1.2 Climate

The Forest receives 2, 080 mm rainfall annually with long rains in the months of April and May and short rains in September to November. The driest months are January and February. The temperatures range between 11 ^{o}C and 26 ^{o}C (Government of Kenya, 1994).

3.1.3 Soils

Soils are generally Acrisols of low fertility, which are heavily leached, medium to heavy textured clay loams and clay (FAO, 1989). The soils are acidic with pH below 5.5. The bedrock substrate on which the forest lies consists of basalt, phenolites and ancient gneisses. These rock formations are overlaid by a layer of clay-loam soils (FAO, 1989).

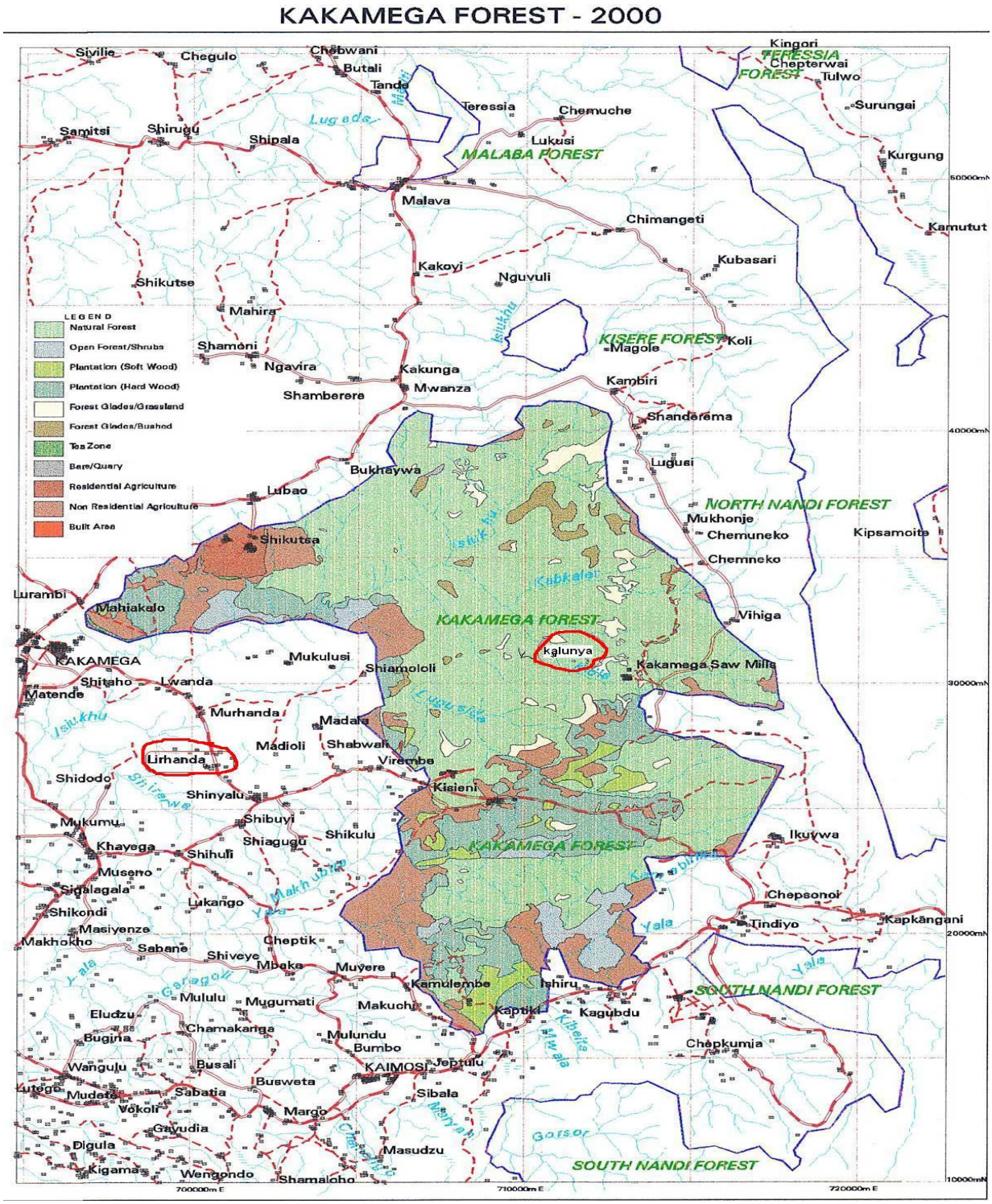
3.1.4 Vegetation

Forest vegetation covers 32 hectares, scattered trees and glades cover 1,557 hectares and cleared or cultivated area covers 2,002 hectares (Government of Kenya, 1994). Throughout the forest are a series of grassy glades such as the Kalunya Glade ranging in size from about 1 to 50 hectares. The glades vary a great deal in structure, some open grass and others having a considerable number of trees or shrubs. A number of streams and small creeks run through the glades of open grassland forming small marshy patches. The marshy patches are dominated by sedges and the grass *Echinocloa pyramidalis*. The glades have small trees such as the *Combretum molle, Psidium guajava, Maesa lancelata, Hamungana madagascariensis* and *Chaetacmearistatas* species. Conspicuous flowering plants such as the *Gladiolus* species are also found (Mutangah *et al*., 1992).

3.1.5 Kakamega Soil and Termite sampling

Soil termites, mound and soil were sampled from Kakamega forest in western Kenya from two sampling sites; Lirhanda Hill and Kalunya Glade as shown in Kakamega forest map (figure 1).

Figure 1: Illustrates a map of Kakamega forest showing Kalunya Glade and Lirhanda hill sampling points (ICIPE, 2000)



The soil feeding termites *Cubitermes severus* species (Figure 2a) were collected in their respective mound (figure 2b). They have a characteristic small white head and dark abdomen because of their soil diet. The surrounding soil was collected one meter apart in three directions from the termite mound using a clean metal soil core with dimensions: 25 mm diameter and 100 mm depth. A total of four mounds with their respective termites and surrounding soil were collected and transported to Jomo Kenyatta University of agriculture and technology laboratory. The termite mounds were carried in paper boxes to the laboratory. The mounds were cut to fit in plastic containers that measured 15 cm by 40 cm by 20 cm. The termites survived for six months periodically changing moist tissue paper and parent soil.

Figure 2(a): Soil feeding termites-*Cubitermes severus*. **Figure 2(b):** Termite mound at Kalunya Glade

Figure 2(a)

Figure 2(b)

3.2 Juja

3.2.1 Study site

Juja is located in lowland areas in the eastern parts of the Thika District. The District is located in the southern part of Central Province in the Kenya. It is situated 32 kms from Nairobi and nine kilometers from Thika town. It lies between latitudes 3° 35" and 1°45" south of the Equator and Longitudes 36º 35" and 37º 25" East (Government of Kenya, 1997).

3.2.2 Climate

The area is generally semi arid and receives low rainfall (Government of Kenya, 1997). The average amount of rainfall is 856 mm: Its distribution in the year is bimodal with a primary peak in April and a secondary one in November. There is a dry period of about 4 months from June to October and a relatively shorter one extending from December to February (Muchena *et al*., 1978). The area lies within an attitude of 1,060nm above sea level. The mean annual temperature is 20 ºC and the mean maximum temperature is

30 ºC. Relative humidity ranges from 57% in February to 74% in July. Evaporation Rate ranges from 2.6 mm in July to 6.3 mm in February (Muchena *et al.*, 1978).

3.2.3 Soils

Juja area falls under the same physiographic unit (plains) with slopes of less than 2%. The soils develop on pyroclastic rocks-trachytic tuff. The soils in the area can be divided into three types:

3.2.3.1 Shallow clay soils (Murram).

These soils are generally well drained with a gravely sandy clay-to-clay texture and friable moist consistence. They are all underlain by petroplinthite i.e. indurated murram

or loose murram at depths ranging from 10 cm to 60 cm. The soil structure is in general weak and the fertility low. They consist of mapping units' pp1m, pp2m and pp3m as shown in figure 1(Muchena *et al*., 1978).

3.2.3.2 Deep clay soils (Vertisols).

These soils are in general poorly drained to very poorly drain and are in many places water logged. They are mottled and when dry have cracks reaching the surface. The permeability of these soils is low. The soil structure is in general moderate while the fertility is generally low. They consist of mapping units' pp1d, pp2d and pp3d as shown in figure 1 (Muchena *et al*., 1978).

3.2.3.3 Soil associations and complexes

This unit is an association of well drained, moderately deep to deep, dark brown, friable, gravely clay soils and well drained, very shallow to shallow, dark brown to dark yellowish brown, gravely clay soils. The two components of the association are all over petroplinthite (indurated murram). They consist of mapping units' pp1c, pp2c and pp3c as shown in figure 1 **(**Muchena *et al*., 1978).

3.2.4 Vegetation

The deep clay soil is characterized by vegetation that can tolerate flooding for a considerable period of time. Important species are *Fimbristylis complanata, Echnochlo*a species, *Cyperus* species and *Eragrostis atrovirens steud* and sorghum species that form a grassland type. *Acacia – Digitari*a – *Eragrostis* group are found on slightly drier habitats than the preceding group. They consist of grassland with scattered bushes. *Acacia seyal* is the dominant bush here, while grasses are dominated by *Digitaria scalarum* **(**Muchena et al., 1978). *Acacia- Themeda group* represents a bushed grassland aspect and is fully developed on the chromic vertisols and chromic gleysols.

Vegetation of well drained shallow clay is mainly grassland with small areas of wooded bushed grasslands. The common grasses are *Themeda triandra, Bothriochoa insculpta. Accacia camus and Hyparrhenia rufa* (Muchena *et al.,* 1978).

3.2.5 Juja soil sampling

Samples of soil were randomly collected from the three types of soil commonly found in the area as indicated in the Juja map (Figure 3). Soil cores were collected from the soil types using a clean metal soil core with the following dimensions: 25 mm diameter and 100 mm depth. The soil cores were transported to the laboratory in sealed polyethylene bag at room temperature. The upper 30 mm of each core was discarded and large roots and stones removed from the remainder were then sieved through a sterile brass sieve of 2 mm aperture size.

Figure 3: Illustrates a map of Juja soil classification system showing sampling points (Muchena et al., 1978).

Key

Ppc1 – cultivated land (no sampling took place here).

Ppc2- This unit is an association of well drained, moderately deep to deep, dark brown, friable, gravely clay soils and well drained, very shallow to shallow, dark brown to dark yellowish brown, gravely clay soils.

Ppd3- These soils are in general poorly drained to very poorly drain and are in many places water logged. They are mottled and when dry have cracks reaching the surface. The permeability of these soils is low.

Pp3m-These soils are generally well drained with a gravely sandy clay-to-clay texture and friable moist consistence. They are all underlain by petroplinthite (indurated murram) or loose murram at depths ranging from 10 cm to 60 cm.

3.3 Dry weight determination

Fresh samples of ten termites and five grams of soil and mound were dried at 105 ºC to constant weight. The samples were then measured to determine the dry weight so as to calculate the conversion factor from fresh weight to dry weight. This factor was multiplied by the number of colony forming units per ml and the results expressed per gram of dry weight (Janssen *et al*., 2002).

3.4 Cultivation and Isolation of Microorganisms

3.4.1 Termite gut

Ten worker caste termites weighing 0.18 g fresh weight were degutted using sterile fine-tipped forceps. The gut sections were pooled in 1ml basal salt solution (BSS) that consisted of the following compounds; K_2HPO_4 (2.0 g), KH_2PO_4 (1.0 g) KCl (1.5 g), NaCl (1.5 g) and one liter of distilled water (Leadbetter and Breznak**,** 1996). Using a sterile glass homogenizer the guts were crushed and the solution was then agitated using a vortex mixer at approximately 150 rpm for five minutes. Serial dilutions were carried out up to 10^{-6} from the gut solution. Spread plates of DNBA that constituted 0.08 g of nutrient broth solidified with 15 g agar and having a pH of 6.23 were then prepared from 0.1 ml of dilutions 10^{-4} and 10^{-5}. In the case of heat shock method where the dilutions were first preheated in a hot water bath (50 ^{o}C) for six minutes before spreading 0.1 ml of dilutions 10^{-4} and 10^{-5} on to DNBA plates. Cultivation was done in triplicate and incubated in the dark at temperatures 25 ^{o}C, 30 ^{o}C and 37 ^{o}C for two weeks. The plates were observed after two, three, seven and fourteen days during which the colony forming units were counted (Janssen *et al*., 2002).

3.4.2 Mounds and soil

One gram of freshly sieved soil/ mound was accurately weighed and added to 9 ml aliquots of sterile distilled water in universal bottles. The solution was then agitated using a vortex mixer at approximately 150 rpm for five minutes so as to dislodge bacteria from soil particles. Serial dilutions were carried out up to 10^{-6} from the soil/mound suspension. Spread plates of DNBA (Janssen *et al.,* 2002) at pH of 6.23 were then prepared from 0.1 ml of dilutions of 10^{-4} and 10^{-5}. In the case of heat shock method, Serial dilution was carried out up to 10^{-6} from the soil/mound suspension that was first preheated in a water bath (50 ^{o}C) for six minutes (Mincer *et al.,* 2002). Spread plates of DNBA with pH of 6.23 were then prepared from 0.1 ml of dilutions 10^{-4} and 10^{-5}. Cultivation was done in triplicate and incubated at temperatures; 25 ^{o}C, 30 ^{o}C and 37 ^{o}C in the dark for fourteen days. The plates were observed after two, three, seven and fourteen days during which the colony forming units were counted (Janssen *et al.,* 2002).

3.5 Statistical analysis

Numbers of cultivable microorganisms were examined through counting the Colony forming units on primary culture plates after two, three, seven and fourteen days for each sample unit at their respective incubation temperatures. Colony forming units per gram of dry weight was then calculated and analyzed with the Genstat program version 2.0 (VSN international oxford UK). The association of colony forming units per gram of dry weight (cfu/g) of soil, mound and termite gut samples under different incubation temperatures was analyzed to determine the significance of the differences. The influence of incubation period on cfu/g was also determined

using two way analysis of variance. Comparisons having significant F values (p <0.01) were further compared using Least Significant Differences test. Mean values and standard errors for counts were generated for each sample and its respective incubation temperature and time. The software used for computation of statistics was Genstat program version 2.0 (VSN international oxford, UK).

3.6 Isolation of pure cultures

Isolates were selected and purified in nutrient agar and M1 media (Mincer *et al*., 2002) that contained starch (1%), yeast extract (0.5%), peptone water (0.2%) and agar (1.5%). Selection of isolates was done based on morphology and those that formed zones of inhibition in the primary culture. Isolates that formed spores were purified in starch casein agar that contained: starch (1%), casein (0.03%), KNO_3 (0.2%), K_2HPO_4 (0.2%), NaCl (0.2%), $MgSO_4$ (0.05%), $CaCO_3$ (0.002%) and $FeSO_4.7H_2O$ (0.001%). Isolates were labeled based on the sample they were obtained from and temperature at which they were cultivated in the primary culture.

3.7 Stocking of the pure culture

The pure cultures were stored in several media such as, M1 that contained 1% of starch, 0.5% yeast extract and 0.2% peptone water. Starch casein broth that contained of starch (1%), casein (0.03%), KNO_3 (0.2%), K_2HPO_4 (0.2%), NaCl (0.2%), $MgSO_4$ (0.05%), $CaCO_3$ (0.002%), $FeSO_4.7H_2O$ (0.001%). Nutrient broth was also used in stocking isolates. Glycerol was added into the broth culture and deep-frozen at -80^{o}C until the time of use.

3.8 Screening for antibiotic producing isolates

Isolates that formed zones of inhibition in the primary culture were selected and purified in nutrient agar. The screening method consisted of primary and secondary screening stages. In primary screening the antimicrobial activity of pure isolates was determined by perpendicular streak method on nutrient agar. The test organisms used were *Bacillus subtilis* (NCIB 3610*)*, *Escherichia coli* (NCTC 10418) and *Candida albicans* (CACBS 562). 0.1 ml of the test organism was spread on the surface of nutrient agar and a colony of the bacterial isolate picked using a sterile loop and inoculated by perpendicular streak method before incubating at the temperature in which that particular isolate grew at initially for 24 hours. After incubation isolates that had clear zones of inhibition (primary screening) were inoculated in Tripticase soy broth and incubated for seven days to allow release of secondary metabolites. After incubation, the broth cultures were centrifuged at 12000 rpm for 5 minutes. Secondary screening was performed by disc diffusion test (Cappuccino and Sherman, 2002) against the standard test organisms; *Bacillus subtilis* (NCIB3610*), Escherichia coli* (NCTC 10418) and *Candida albicans* (CACBS 562). A control disc was included in the experimental set up using sterile distilled water for each test organism. After 24 hours of incubation, zones of inhibition around the discs were measured for each isolate and recorded in a table.

3.9 Screening for enzyme activity in isolates

3.9.1 Starch hydrolysis

Starch agar contained 0.2% soluble starch which served as the polysaccharide substrate and 1.5% agar. Freshly prepared isolates were inoculated by streak method using a sterile loop. The plates were then incubated for 24 hours to allow bacterial growth. The detection of the hydrolytic activity of the isolate was determined by addition of iodine. A clear zone around the colonies indicated positive test (Cappuccino and Sherman, 2002; Harold, 2002).

3.9.2 Gelatin liquefaction

Nutrient Broth supplemented with 12% gelatin was used to demonstrate the hydrolytic activity of gelatinases (Harold, 2002). Freshly prepared isolates were inoculated by stab method in to gelatin tubes using a sterile straight loop. The tubes were incubated at 24 ^{o}C for 48 hours. Grown culture tubes were placed in the refrigerator at 4 ^{o}C for thirty minutes. Cultures that remained liquefied were considered positive for gelatin hydrolysis (Cappuccino and Sherman, 2002).

3.9.3 Casein hydrolysis

Milk agar containing 10% skimmed milk powder which served as served as the polysaccharide substrate and 1.5% agar was used to test for casein hydrolysis. Freshly prepared isolates were inoculated by streak method using a sterile loop on the surface of the milk agar. The plates were then incubated for 24 hours at incubation temperature of the respective isolates to allow bacterial growth. The detection of the hydrolytic activity was determined by a clear zone around the colonies indicating positive test (Cappuccino and Sherman, 2002; Harold, 2002).

3.9.4 Cellulose hydrolysis

Cellulose agar contained 1% cellulose and 2.8% nutrient agar was prepared for cellulose hydrolysis. Freshly prepared isolates were inoculated by streak method using a sterile loop onto the surface of the milk agar. The plates were then incubated for 24 hours at incubation temperature of the respective isolates to allow bacterial growth. The detection of the hydrolytic activity following bacterial growth was determined by a clear zone around the colonies indicating positive test (Cappuccino and Sherman, 2002; Harold, 2002).

3.10 Characterization of isolates

Isolates that showed antagonistic activity against the test organisms and enzymatic activities to various substrates were selected for characterization. Preliminary characterization was performed using morphological and cultural characteristics. Morphological identification of the isolate was done under the dissecting microscope to observe colony and growth characteristics. Further characterization was done through biochemical and molecular studies to support the findings of the morphological characterization.

3.10.1 Morphological characterization

In order to study the morphology of the isolates, Gram stain was first performed. Fresh cultures were heat fixed on slides and stained using crystal violet for one minute and then washed off. The smear was then stained with iodine for one minute and then washed off. The smears was then decolorized using 95% alcohol before washing off and counter staining with 0.25% safranin

for 30 seconds. The smear was then blot dried and observed under inverted microscope at ×100 oil immersion (Cappuccino and Sherman, 2002).

3.10.2 Biochemical characterization

3.10.2.1 Indole production and Hydrogen sulfide production

Sulfur-Indole Motility (SIM) agar media was used to test the production of tryptophanase enzyme and the ability to produce hydrogen sulfide from substrates such as sulfur containing amino acids and organic sulfur. The SIM agar tubes were inoculated with the isolate and incubated at their respective incubation temperatures. Presence of indole was detected by addition of Kovac's reagent to 48hour cultures of each isolate (Harold, 2002). Positive results were indicated by production of a cherry red layer. Absence of black coloration in the media following incubation indicated absence of hydrogen sulfide (Cappuccino and Sherman, 2002).

3.10.2.2 Nitrate reduction test

The ability of the isolates to reduce nitrates to nitrites or beyond was carried out using nitrate broth medium containing 1% potassium nitrate according to the methods of Harold (2002) and that of Cappuccino and Sherman (2002). Following inoculation with the isolate and incubation for 48 hour, addition of sulfanilic acid and alpha-naphthylamine produced a cherry red coloration, which was indicative of positive results (Cappuccino and Sherman, 2002).

3.10.2.3 Urease test

The ability of the isolates to attack nitrogen and carbon bonds in amide compounds was determined using urea broth media containing phenol red indicator according to the methods of Harold (2002). The urea broth tubes were inoculated with the isolate and incubated at their respective incubation temperatures for 48 hours. A positive reaction was indicated by development of deep pink color (Cappuccino and Sherman, 2002).

3.10.2.4 Catalase test

Catalase production was determined by addition of 3% hydrogen peroxide to Trypticase Soy Agar (TSA) cultures of each isolate based on the methods outlined by Cappuccino and Sherman (2002). A positive reaction was indicated by formation of bubbles, which indicated production of oxygen gas.

3.10.2.5 Ability of isolates to grow in 7% sodium chloride

The ability of isolates to grow in saline was performed using nutrient agar with additional 7% sodium chloride added to it. The plates were inoculated with the isolate and incubated at their respective incubation temperatures. Organisms that were able to grow in this media were a positive result (Cappuccino and Sherman, 2002).

3.10.2.6 Substrate utilization of isolates

The ability of the isolates to utilize the following substrates: arabinose, maltose, fructose, sucrose, lactose, mannitol, xylose and xylan was determined using 2% of the substrate added to a

solution that consisted of Nacl (0.1%), K_2HPO_4 (0.1%), $MgSO_4$ (0.05%), $FeSO_4.7H_2O$ (0.001%), $CuSO_4.7H_2O$ (0.0001%), $ZnSO_4.7H_2O$ (0.0001%g), $MnSO_4.7H_2O$ (0.0001%). Substrate utilization was determined after inoculation of the tubes and incubation at their respective incubation temperatures for 24 hours. Turbidity was used to indicate growth and was measured using a Spectrophotometer at 560 nm. Uninoculated control for each substrate was used as a standard when measuring turbidity using a Spectrophotometer at 560 nm (Cappuccino and Sherman, 2002).

3.10.3 Molecular characterization

3.10.3.1 DNA isolation protocol

Pure subcultures of the isolates were inoculated in freshly prepared LB broth and incubated for six days in a shaker incubator at 28 ^oC and 200 rpm. Two milliters of the broth was put into a sterile Eppendorf® micro tube and centrifuged at 13000 rpm for five minutes. The supernatant was then discarded. Five hundred microliters of solution A (50 mM Tris pH 8.5, 50 mM EDTA pH 8.0 and 25 % Sucrose solution) that was previously prepared was added and mixed by inverting several times and centrifuge at 13000 rpm for one minute and supernatant discarded. 200 µl of solution A (50 mM Tris pH 8.5, 50 mM EDTA pH 8.0 and 25 % Sucrose solution) and 5 µl of Lysozyme (20 mg/ml) were added into the Eppendorf® micro tube before gently mixing the solution. 5 µl of RNAse A (20 mg/ml) was then added and mixed gently. The solution was then incubated at 37^oC for one hour after which 600 µl of solution B (10 mM Tris pH 8.5, 5 mM EDTA pH 8.0 and 1% SDS) was added and the resultant solution was mixed several times. 10 µl of Proteinase K (20 mg/ml) was added and mixed gently. The solution was then incubated at 50 ^oC for 30 minutes. The solution was divided into two halves before proceeding to the next step.

One half was stored at -80 ^{o}C while the other half proceeded to be used in DNA extraction procedure.

DNA was extracted by adding 200 µl of phenol and 200 µl of chloroform and the solution centrifuged for 15 minutes at 13000 rpm. The aqueous phase which contains the crude DNA was pipette out and put in sterile Eppendorf® micro tube. 200 µl of phenol and 200 µl of chloroform were again added and the mixture centrifuged for 15 minutes at 13000 rpm. The aqueous phase which contains crude DNA was pipette out and put in the Eppendorf® micro tube. An equal volume of Chloroform: Isoamylalcohol (24:1) was added to the crude DNA solution and centrifuged at 13000rpm for 15 minutes to remove the phenol. The aqueous phase was then pipetted to a clean Eppendorf® micro tube. Equal volumes of Chloroform: Isoamylalcohol (24:1) were added to the DNA solution in the Eppendorf® micro tube and centrifuged at 13000 rpm for 15 minutes to remove phenol from the DNA. The aqueous phase was then pipetted to a clean Eppendorf® micro tube. An equal volume of Isopropanol and 1 microliter of 3 M Nacl were added to the mixture. The DNA was left at -80 ^{o}C overnight.

The DNA samples were then thawed and later centrifuge at 4 ^{o}C for 30 minutes to pellet the DNA. The pellet was then washed with 70% ethanol and centrifuged at 13000 rpm for five minutes at 4 ^{o}C. The ethanol was pipette out leaving the pellet in the micro tube. The pellet was air dried on the bench at room temperature for 20 minutes. The DNA pellet was then dissolved in 100 µl of TE buffer pre-warmed at 55 ^{o}C. The DNA was stored at
-20 ^{o}C. The DNA was semi quantified on a 1% agarose gel in 1xTAE buffer and visualized under UV by staining with ethidium bromide (Sambrook *et al.*, 1989).

3.10.3.2 DNA purification Quick Clean 5M Gel Extraction Kit

A clean, sharp razor blade or scalpel was used to excise the DNA band from the agarose gel and excess agarose removed. The gel slice was placed in a colorless, pre-weighed tube to weigh the gel slice. Three volumes of binding solution II was added to one volume of gel slice (100 mg ≈ 100 µl). The tube was incubated at 50 °C for ten minutes with occasional vortexing until the gel slice has completely dissolved. The color of the mixture was yellow. Ten µl of 3 M sodium acetate (pH 5.0) was added to turn the violet color to yellow and mix. One volume of Isopropanol (with respect to the original gel volume) was added and mixed. The mixture was transferred to 600 µl Quick Clean column (Gel extraction kit) to remove impurities form the DNA. The column was centrifuged at 12,000 rpm for 30 seconds. Five hundred microliters of wash solution was added to the column and centrifuge at 12,000 rpm for one minute. The column was centrifuged at 12,000 rpm for an additional 60 seconds to remove residual wash solution. The column was then transferred to a clean 1.5 ml micro centrifuge tube and 50 µl of elution buffer added to the center of the column membrane and later incubated at room temperature for one minute. The tube was centrifuged at 12,000 rpm for one minute to elute and collect the DNA.

3.10.3.3 PCR amplification of bacteria 16S rRNA gene

Purified total DNA from each isolate was used as a template for amplification of the 16S ribosomal RNA genes. This was done using the QIAquick PCR purification kit (Qiagen, Germany) according to the manufacturer's instructions. Nearly full-length 16S rRNA gene sequences were PCR-amplified from DNA extracts using bacterial primer pair 27F forward 5'-AGA GTT TGA TCC TGG CTC AG-3' in relation to *Escherichia coli* positions 8 to 27

(Edwards *et al*., 1989) and 1492R reverse, 5'-TAC GGY TAC CTT GTT ACG ACT T-3' *Escherichia coli* positions 1492 to 1512 (Wesburg *et al*., 1991). Amplification was performed using thermal block Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). Each 50 µl PCR mixture stored at 4 °C contained a 0.5 µM concentration of each primer, a 100 µM concentration of each dNTPs (Amersham Life Science, Braunschweig, Germany), 1 µl of template DNA, PCR buffer (20 mM Tris-HCl [pH 8.3], 50 mM KCl) and 1.0 U of *Taq* DNA polymerase ((Perkin-Elmer Applied Biosystems, Weiterstadt, Germany). Amplifications were started by placing cooled (4 °C) PCR tubes immediately into the preheated (94 °C) thermal block Mastercycler Gradient thermocycler. The thermal cycle profile consisted of initial denaturation for 3 minutes at 94 °C, followed by 30 cycles consisting of 30 seconds at 94 °C, 30 seconds at the annealing temperature of 55 °C, 45 seconds at 72 °C elongation and a single final extension step consisting of 72 °C for 5 minutes. Five µl of each DNA sample was loaded on an ethidium bromide containing agarose gel (1%) in 1X TAE buffer and run at 80V for 1 hour (Sambrook *et al*., 1989). Gel documentation was done using the Gel Logic 200 Imaging System.

3.10.3.4 Purification of PCR products

The PCR products were purified using the QIAquick PCR purification Kit protocol (Qiagen, Germany) according to manufacturer's instructions. Five volumes of buffer PB (Qiagen, Germany) was added to one volume of the PCR sample and thoroughly mixed. The QIAquick spin column was placed in a 2 ml collection tube, the sample applied to the QIAquick column to bind the DNA, and then centrifuged for 30-60 seconds at 13000 rpm. The flow-through was discarded, and the QIAquick column placed back into the same tubes. To wash the DNA, 0.75 ml buffer PE was added to the QIAquick column and centrifuged for one minute. The flow-

through was discarded and the column centrifuged again for an additional one minute at 13000 rpm to remove residual ethanol from buffer PE. The Qiaquick column was placed in a 1.5 ml micro centrifuge and 30 µl of buffer EB (10 mM Tris-Cl, pH 8.5) added to elute DNA. The tubes were then centrifuged for 1 minute, the spin column removed and DNA stored at –20^{o}C for application (Sambrook *et al.,* 1989).

3.10.3.5 Phylogenetic data analysis

Sequencing of purified PCR products was done without cloning, using a commercial service provider. The CHECK-CHIMERA program (http://rdp.cme.msu.edu/html/) of the Ribosomal Database Project (Maidak *et al*., 2001) was used to check for the presence of possible chimeric artifacts (Janssen *et al*., 2002). Sequence data was analyzed with ARB software package [version 2.5b; O.Strunk and Ludwig, Technishe Universitat Munchem (http://www.arb-home.de)]. The new sequences were added to the ARB database and aligned with the Fast Aligner Tool (version 1.03). Alignments were checked and corrected manually where necessary, based on conserved regions. The 16S rRNA gene sequences were compared to sequences in the public database using Basic Local Alignment Search Tool (BLAST) on the National Center for biotechnology Information (NCBI) website (http://www.ncbi.nih.gov) in order to determine similarity to sequences in the Genebank database (Altschul *et al*., 1990; Shayne *et al*., 2003). The 16S rRNA gene sequences with high similarities to those determined in the study were retrieved and added to the alignment based on BLAST results. Phylogenetic trees were constructed by Maximum likelihood method. Bootstrap analysis-using PHYLIP for 100 replicates was performed to attach confidence estimates for the tree topologies (Felsentein, 1989; Saitou and Nei, 1987).

CHAPTER FOUR

4.0 RESULTS

4.1 Enumeration of microorganisms from sample units under different conditions

4.1.1 Impact of incubation temperature on number of cultivable microorganisms in soil, gut and mound samples

The effect of incubation temperatures on number of cultivable microorganisms was not significant (F=0.143; n=4; P=0.05). However, a number of important observations were made that concern optimal cultivation temperatures (Table 1). The termite gut had a high number of cultivable microorganisms when incubated at 37^{o}C and least number of cultivable microorganisms when incubated at 25^{o}C. The termite mound from Kalunya Glade had high numbers of cultivable microorganisms when incubated at 30^{o}C while Lirhanda Hill termite mounds had high number of cultivable microorganisms at an incubation temperature of 25^{o}C followed by incubation temperature of 30^{o}C. The least number of cultivable microorganisms in mounds grew at the incubation temperature of 37^{o}C in samples from both sites. In Juja soils, high numbers of cultivable microorganisms were observed in clay soils and soil associations at 25^{o}C whereas in Murrum soil high numbers of cultivable microorganisms were observed at 30^{o}C. All soils in Juja had the least number of cultivable microorganisms' (counts on plate) at 37^{o}C as shown in Table 1.

Table 1: Number of cultivable microorganisms (cfu/g) in different termite, soil and mound samples (mean ± SD ×10^7)

Site	Soil type	Temp	Days				
			0	**2**	**3**	**7**	**14**
Juja soil	Clay	25 °C	0	5.8±1.4	13.2±1.8	15.8±3.5	18.0±3.4
		30 °C	0	2.8±1.2	3.5±1.3	6.13±2.3	7.9±1.2
		37 °C	0	4.33±1.2	4.3±1.3	6.8±2.4	11±3.8
	Murrum	25 °C	0	4.3±0.6	4.8±1.0	6.9±1.9	6.4±0.9
		30 °C	0	6.4±2.6	7.4±3.7	8.1±2.3	8.8±1.6
		37 °C	0	5.7±1.5	6.5±1.7	7.4±1.7	7.4±1.1
	Soil Association	25 °C	0	7.7±2.1	9.0±1.4	10.0±2.4	15.0±2.5
		30 °C	0	4.0±1.5	5.5±1.6	6.1±1.8	6.1±2.4
		37 °C	0	3.1±2.3	2.6±1.0	2.8±1.1	3.2±1.3
Lirhanda Hill	Parent soil	25 °C	0	11.6±3.2	12.1±3.3	14.1±4.2	14.2±3.6
		30 °C	0	4.7±1.9	10.0±4.8	16.1±3.7	16.1±3.8
		37 °C	0	3.6±1.5	2.9±0.8	6.1±1.0	8.6±0.2
	Termite Mound	25 °C	0	1.5±0.8	7.2±2.4	11.1±4.7	12.4±4.2
		30 °C	0	1.6±0.4	2.7±0.9	3.3±0.8	4.1±1.5
		37 °C	0	0.9±0.2	2.9±0.5	3.2±0.6	3.2±0.4
	Termite gut	25 °C	0	151±12.1	208±13.4	221±6.1	248±31
		30 °C	0	177±16	223±23	303±40	315±34.5
		37 °C	0	203±19.6	235±21	318±24	432±35
Kalunya Glade	Parent soil	25 °C	0	3.98±1.7	6.1±1.5	6.24±1.4	22.0±7.9
		30 °C	0	10±3.5	10.1±2.3	13.9±3.1	10.6±4.5
		37 °C	0	4.7±1.0	15.8±3.9	19.5±7.5	28±6.7
	Termite Mound	25 °C	0	0.6±0.2	2.49±0.2	4.2±0.64	8.0±3.2
		30 °C	0	14.2±0.2	21.8±5.5	21±3.2	22±1.4
		37 °C	0	2.6±0.8	4.4±0.2	4.2±1.3	5.0±1.0
	Termite gut	25 °C	0	81.4±3.8	159.0±12.0	170.0±21.1	200.0±28.1
		30 °C	0	143.0±23.2	147±2.3	148±1.3	246±.53
		37 °C	0	145±6.5	230±8.0	306±18.0	417±16.2

A comparison of cfu/g from each sample unit showed a significant difference in number of cultivable microorganisms (F=0.001; n=4; P=0.05). Results in Table1 showed the termite gut had high number of cultivable microorganisms than all the other samples. Results in Table 1 also

showed Kakamega Forest soils generally had a higher number of cultivable microorganisms with a mean of between 8.6±0.2cfu/g and 28±6.7cfu/g and in the Juja soil samples the number of cultivable microorganism was between mean of 3.2±1.3cfu/g and 18.0±3.4cfu/g at the three cultivation temperatures. Kalunya Glade which is a marshy glade with clay soils had higher number of cultivable microorganisms in a gram of soil sample at the three incubation temperatures used than Lirhanda Hill which is hilly and rocky with loamy soils. In addition deep clay soil in Juja had higher numbers of cultivable microorganisms in a gram of soil sample than the other two soils namely soil associations and murram soil at the three incubation temperatures.

4.1.2 Impact of dilution and heat shock method on number of cultivable microorganisms

The heat shock method used generally reduced the number of colony forming units that grew on plates of all sample units (Table 2**).** Results showed a significant difference (F=0.001; n=4; P=0.05) on number of cultivable microorganisms in the termite gut, mound and soil samples. With least significant difference analysis, the number of cultivable microorganisms in surrounding soil compared with Mound and Termite gut showed significant differences(L.S.D, α=0.05). The number of cultivable microorganisms in the mound compared with those of the gut also showed significant differences (L.S.D, α=0.05).

There was a significant effect of temperature on the number of cultivable microorganisms in colony forming units per gram of termite mound, gut and soil samples (F=0.05; n=4; P=0.05). Statistical analysis further showed the effect at 30^{o}C on the number of cultivable microorganisms was significant when compared with effect at 25^{o}C and 37^{o}C. However, a comparison of the number of cultivable microorganisms at 25^{o}C and 37^{o}C was not significant (L.S.D α=0.05).

Overall results showed 30^{o}C to be the optimum temperature for cultivation of bacteria in termite gut, mound and soil samples as illustrated in Table 2.

Table 2: **Number of cultivable microorganisms (cfu/g) in different termite, soil and nest samples (mean ± SD ×10^{7}) using heat shock method**

Site	Soil type	Temp	Days			
			0	**2**	**3**	**7**	**14**
Lirhanda Hill	Parent soil	25^{o}C	0	25.1±4.9	25.3±6.6	30.1±10.0	30.0±10.1
		30^{o}C	0	11.3±3.5	22.1±4.3	24.2±2.7	32.0±6.2
		37^{o}C	0	12.7±7.0	12.7±3.1	18.2±7.3	19.2±8.5
	Termite Mound	25^{o}C	0	1.12±	1.02±0.4	1.29±.0.15	1.63±0.16
		30^{o}C	0	0.62±0.05	1.2±0.1	1.8±0.2	2.4±0.4
		37^{o}C	0	0.6±0.05	1.2±0.04	1.2±0.2	1.4±0.13
	Termite gut	25^{o}C	0	5.71±1.2	20.3±8.2	32.4±7.5	54.3±1.8
		30^{o}C	0	17.1±11	35.6±16.5	65.3±4.9	81.3±11.6
		37^{o}C	0	15.9±1.3	16.4±1.5	19.8±1.2	44.7±2.1
Kalunya Glade	Parent soil	25^{o}C	0	26.4±1.2	12.5±1.8	30.1±1.4	30.2±1.3
		30^{o}C	0	5.2±1.5	18.2±0.2	31.7±1.0	37.3±6.1
		37^{o}C	0	2.89±1.6	3.0±1.2	12.2±3.2	15.6±4.7
	Termite Mound	25^{o}C	0	74.6±7.3	78.1±7.4	79.2±7.3	82.1±7.0
		30^{o}C	0	42.1±2.4	44.1±2.1	54.5±2.9	61.1±2.1
		37^{o}C	0	2.7.1±0.1	4.1±0.9	8.8±2.6	11.1±0.6
	Termite gut	25^{o}C	0	140±19.5	172.1±20	190. ±4.2	240±24
		30^{o}C	0	166±10.1	169±12.1	298±19..1	316±10.2
		37^{o}C	0	56±16.2	101.3±14.2	209±11.5	225±13.8

4.1.3 Impact of length of incubation period on number of cultivable microorganisms

The number of cultivable microorganisms on most samples showed a general increase with the extended incubation period up to fourteen days. However the rate depended on the incubation temperature and sample unit (Table 1**)**. Statistical analysis showed a significant effect of

extending incubation period on a number of cultivable microorganisms (F=0.014; n=4; P=0.05). The heat shock method showed no significant effect of extended incubation period on number of cultivable microorganisms (F=0.089; n=4; P=0.05). However, some samples were able to show an increase in a number of cultivable microorganisms (cfu/g count) as the incubation period progressed as shown in Table 2.

4.2 Isolation

A total of one hundred and thirty seven (137) pure isolates were obtained from 15 samples that included soil, termite mound and gut (Table 3). Isolates were selected based on colony morphology and those exhibiting zones of inhibition on the primary culture. These samples were later screened for antibiotic and enzyme producing bacteria. It was noted that plates cultivated using the shallow clay soils had high numbers of colonies with zones of inhibition and that DNBA media had minimal fungal growth. In addition plates inoculated with samples from using the heat shock method had high numbers of colonies with zones of inhibition and pigment producing bacteria.

Table 3: A summary of sample unit collection sites and number of isolates screened for enzyme and antibiotic producing bacteria

Location	Code	Isolates (non heat shock)	Isolates (Heat shock method)
Shallow clay soils	PPM	22	0
Soil association	PPC	25	0
Deep clay soils	PPD	15	0
Kalunya parent soil A	SKA	3	3
Kalunya parent soil B	SKB	6	3
Kalunya gut A	KAG	5	2

Kalunya gut B	KBG	3	2
Kalunya nest A	KAN	5	2
Kalunya nest B	KBN	5	1
Lirhanda parent soil A	SLA	5	1
Lirhanda parent soil B	SLB	5	1
Lirhanda gut A	LAG	1	2
Lirhanda gut B	LBG	5	2
Lirhanda nest A	LAN	5	2
Lirhanda nest B	LBN	4	2
Total		114	23

4.3 Screening for antibiotic producing bacteria

Eighty seven isolates from the original culture that had zones of inhibition in the primary culture were considered antagonists hence had the potential of producing antibiotics (Plate 1). These were selected, purified and stored as stock cultures in the refrigerator. The isolates were screened for ability to produce antibiotics against the following test organisms *Bacillus subtilis* (NCIB3610), *Escherichia coli* (NCTC 10418) and *Candida albicans* (CACBS 562). Isolates that had high inhibition zones in the primary screening underwent secondary screening using the disc diffusion (Plate 2) method against the test organisms. Thirty five isolates did not have antagonistic activity while the rest were lost due to contamination or inability to grow after subculture.

Plate 1: Primary culture of Lirhanda Hill A gut at 30ºC (LAG 30^{o}C). Antagonistic activity is seen by a zone of inhibition around the red colonies**.**

Plate 2: Secondary screening of isolates indicating showing antagonistic activity to *Candida albicans.* The antagonistic activity is seen by a zone of inhibition around the paper disc.

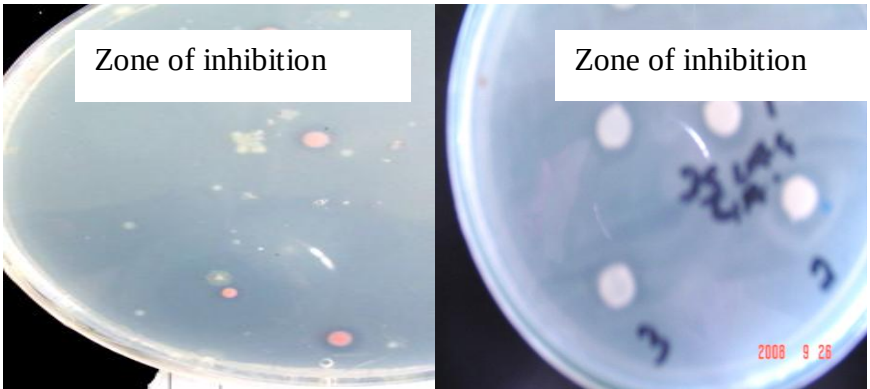
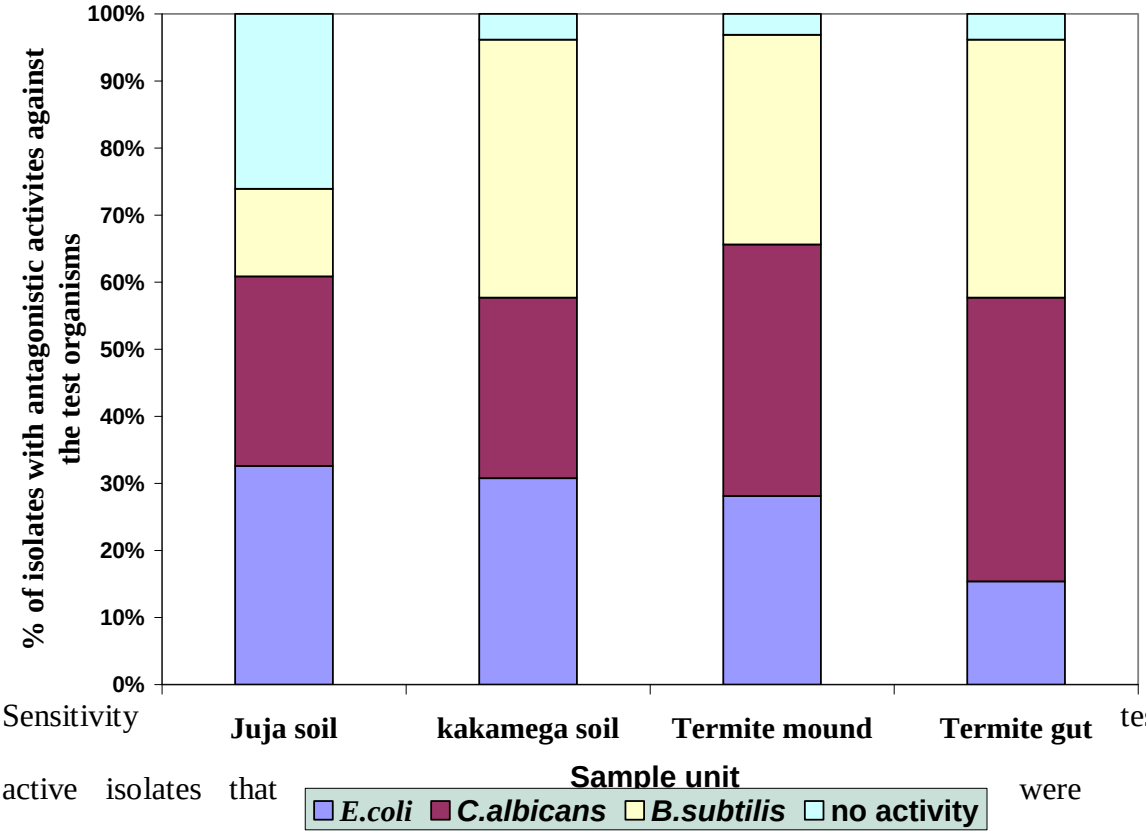


Plate 1 **Plate 2**

The distribution of all antimicrobial producing bacteria from the different sites was observed in the termite gut, mound and soil samples as shown in Figure 4. The results showed the termite mound had a high percentage of antimicrobial producing bacteria. Majority of termite gut isolates were antagonistic to *Bacillus subtilis* and *Candida albicans* than those antagonists of *Escherichia coli* as shown in Figure 4. Overall results indicated that 51% of the isolates were antagonists against *Escherichia coli* (NCTC 10418), 57% were antagonists against *Bacillus subtilis* (NCIB3610), and 55% of the isolates were antagonists against *Candida albicans* (CACBS 562). Isolates in Juja soils had the highest number of non antagonist's bacteria as compared to isolates obtained from Kakamega Forest soils that had the least number of isolates showing no antagonistic activity.

Figure 4: The percentage distribution of antimicrobial producing bacteria against test organisms from various sample units



Sensitivity test for active isolates that were later identified through molecular and biochemical tests was done as shown in Table 4.

Table 4: Sensitivity test of the active isolates on test organisms; *E. coli, B. subtilis* and *C. albicans*, using disc diffusion in cm.

Isolate	***B.subtilis* (NCIB3610)**	***E.coli* (NCTC 10418)**	***C.albicans* (CACBS 562)**
Control	0.5	0.5	0.5
84PPD30^{o}C	0.5	0.8	0.8
29 LAN 30^{o}C	0.5	1.2	0.8
57 LBN 30^{o}C	0.5	1.0	1.0
51 LBN 37^{o}C	0.5	1.0	1.1
7 SKB 30^{o}C	0.9	1.0	1.1
14 SLA 30^{o}C	0.8	0.8	0.5
14 KAG 30^{o}C	0.8	0.5	0.5
63KAG 25^{o}C	1.0	0.8	0.5
24 LBN 30^{o}C	1.1	0.9	0.8
1 SKA 37^{o}C	0.8	0.8	0.8
62 LBG 37^{o}C	1.0	0.8	0.5

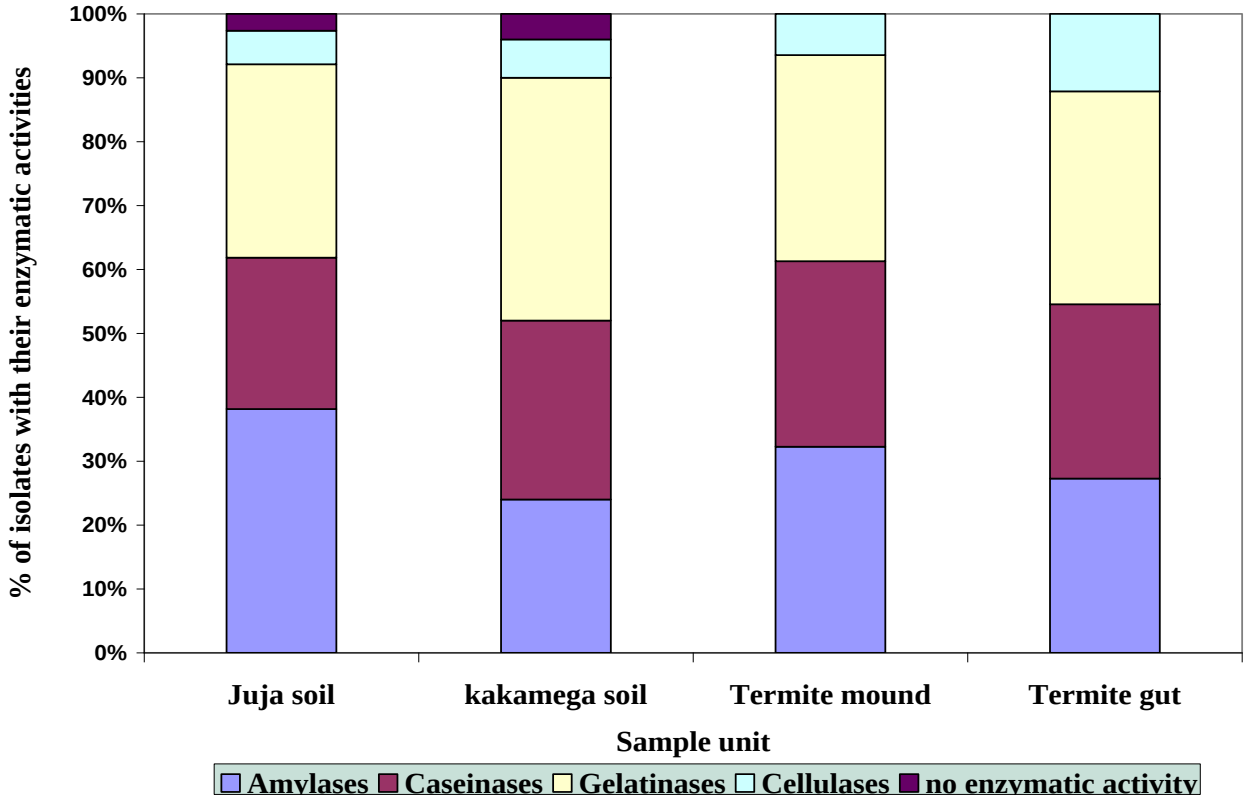
142 M(b) 30^{o}C	1.0	0.7	0.5
58 SLA 25 ^{o}C	1.0	1.0	0.5
11 SKB 30^{o}C	1.0	1.0	0.9
9 SKB 25^{o}C	1.0	0.5	1.0
142 M 30^{o}C	0.5	0.5	0.5
1 1LBG 25^{o}C	0.7	0.5	0.7
LBN37^{o}C	0.8	0.7	0.8
LBG30^{o}C	0.7	0.5	0.7
4KAG30^{o}C	0.7	0.5	0.7

Antimicrobial activity results for the 20 isolates showing measurement of zones of inhibition in cm

4.4 Screening for enzymatic activities of bacteria isolates

Ninety two (92) isolates were also screened for amylases, caseinases, gelatinases and cellulases. The percentage distribution of enzyme producing bacteria in the sample units was studied. The study revealed Sixty five percent (65%) of all isolates were starch degraders while Fifty four percent (54%) of the isolates degraded casein and sixty eight percent (68%) of the isolates degraded gelatin as shown in Figure 5. Cellulose was degraded by 11% of the isolates obtained from termite gut. In addition 7% of isolates obtained from mound were cellulose degraders.

Figure 5: **Percentage of isolates having various enzymatic activities in their respective sample units**



Enzymatic activities of twenty (20) isolates whose antimicrobial activities were previously investigated (Table 4) and that were later identified through biochemical tests and molecular characterization was done as shown in Table 5.

Table 5: Enzymatic activities of isolates

Isolate	Amylases	Caseinases	Gelatinases	Cellulases
84PPD30^{o}C	+	-	+	-
29 LAN 30^{o}C	+	-	+	-
57 LBN 30^{o}C	-	+	+	-
51 LBN 37^{o}C	-	+	+	-

7 SKB 30°C	+	-	+	-
14 SLA 30°C	+	+	+	-
14 KAG 30°C	+	-	+	-
63KAG 25°C	+	-	+	-
24 LBN 30°C	+	-	+	-
1 SKA 37°C	+	-	+	-
62 LBG 37°C	+	-	+	-
142 M(b) 30°C	+	-	+	-
58 SLA 25°C	+	+	+	-
11 SKB 30°C	+	+	-	+
9 SKB 25°C	-	+	+	-
142 M 30 °C	+	+	+	-
1 1LBG 25°C	-	+	+	-
2LBN37°C	+	+	+	+
3LBG30°C	+	+	+	+
4KAG30°C	+	+	+	-

Enzymatic activity results for the 20 isolates (+) a positive result for the reaction and (-) a negative result for the reaction

4.5 Biochemical tests

Isolates were taken through a series of biochemical tests to determine their physiological characteristics. Seventy seven percent (77%) of all isolates were Gram positive while the rest were Gram negative. All isolates were positive for catalase reaction, however none was able to produce hydrogen sulphide apart from the Gram negative bacteria. Results also showed one isolate from termite gut that had the ability to utilize xylan which is a polymer. Isolate 11SKB30 °C and two other isolates from the termite gut and mound were able to grow in 7% sodium chloride.

Table 6: Biochemical test of 20 isolates that were under investigation

Isolate	Nit	Urea	Xylo	Indo	Mann	Arab	Mal	Fruc	Sucr	Lact
24LBN30°C	+	-	Nd	-	nd	+	+	+	+	-
1 SKA37 °C	+	-	Nd	-	nd	+	-	+	+	-
62LBG37°C	+	-	Nd	-	nd	+	-	+	+	-

63KAG37^{o}C	+	-	Nd	-	nd	+	+	+	+	-
58SLA25^{o}C	+	-	Nd	-	nd	+	+	+	+	-
14KAG30 ^{o}C	+	-	Nd	-	nd	+	-	+	+	-
14SLA30 ^{o}C	+	-	Nd	-	nd	+	-	+	+	-
142MB30 ^{o}C	+	-	Nd	-	nd	+	-	+	+	-
142M30 ^{o}C	+	+	Nd	+	nd	-	-	-	-	+
51LBN37 ^{o}C	+	+	Nd	+	nd	-	+	+	-	-
57LAN30 ^{o}C	+	+	Nd	+	nd	-	+	+	-	-
29LAN30 ^{o}C	+	+	Nd	+	nd	-	+	+	-	-
11SKB30 ^{o}C	+	-	+	-	+	-	+	+	+	+
84PPD30 ^{o}C	-	-	Nd	+	nd	-	+	+	+	-
7SKB30 ^{o}C	-	-	Nd	+	nd	+	+	+	+	-
9SKB 25 ^{o}C	+	+	Nd	-	nd	-	-	+	+	+
1LBG25 ^{o}C	-	-	+	-	+	nd	nd	Nd	+	nd
2LBN37 ^{o}C	-	+	-	-	-	nd	nd	Nd	-	nd
3KAG 0 ^{o}C	+	+	+	-	-	nd	nd	Nd	-	nd
4LBG30 ^{o}C	+	+	+	-	+	nd	nd	Nd	+	nd

Biochemical test result for 20 isolates (+) positive reaction,(-) negative reaction and (nd) not determined. Nit-nitrates, Urea-ureas, Xylo-xylose, Indo-indole, Mann-mannitol, Arab-D arabinose, Mal-maltose, Fruc-fructose, Sucr-sucrose and Lact-lactose

4.6 Morphological characterization of isolates

Morphological studies of the isolates were done using the dissecting (×16) and compound microscopes (×100 oil immersion). Majority of the isolates obtained were rod shaped Gram positive bacteria (**Table 7**).

Table 7: Morphological characteristics of isolates

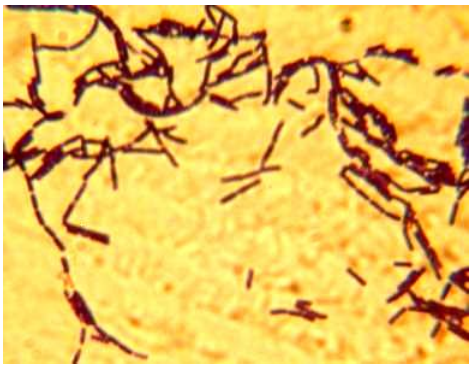
Isolate	Gram	Cell shape	Endospore	Colony morph.	Color	Pigment	Motility
24 LBN30 ^{o}C	+	Rod	+	Circular, slightly raised, entire	Cream white	-	+
1 SKA 37 ^{o}C	+	Rod	+	Circular, slightly raised, entire	Cream white	-	+
62 LBG37 ^{o}C	+	Rod	+	Circular, slightly raised, entire	Cream white	-	+

63KAG 37 ^{o}C	+	Rod	+	Circular, slightly raised, entire	Cream white	-	+
58SLA25 ^{o}C	+	Rod	+	Circular, slightly raised, entire	Cream white	-	+
14KAG30 ^{o}C	+	Rod	+	Circular, slightly raised, entire	Cream white	-	+
14SLA30 ^{o}C	+	Rod	+	Circular, slightly raised, entire	Cream white	-	+
142MB30 ^{o}C	+	Rod	+	Circular, slightly raised, entire	Cream white	-	+
142M30 ^{o}C	-	Rod	-	Circular, serrate flat	Cream white	Green	+
51LBN37 ^{o}C	+	Rod	+	Circular, mucoid, convex,	Cream white	-	+
57LAN30 ^{o}C	+	Rod	+	Circular, mucoid, convex,	Cream white	-	+
29LAN30 ^{o}C	+	Rod	+	Circular, mucoid, convex,	Cream white	-	+
11SKB30 ^{o}C	+	short rod	-	Circular, entire, convex	Cream white	-	-
84PPD30 ^{o}C	+	Rod	+	Circular, mucoid, convex,	cream white	-	+
7SKB30 ^{o}C	+	Rod	+	Circular entire, raised	Cream	-	+
9SKB 25 ^{o}C	-	Coccid rod	-	Circular, entire, slightly raised	Pink	Pink	+

Morphological test for the 16 isolates (+) a positive result or presence and (-) a negative result /no presence. Colony morph.-colony morphology

Observation under compound microscope showed Gram positive rod shaped bacteria of isolate obtained from termite gut as shown in Plate 3.

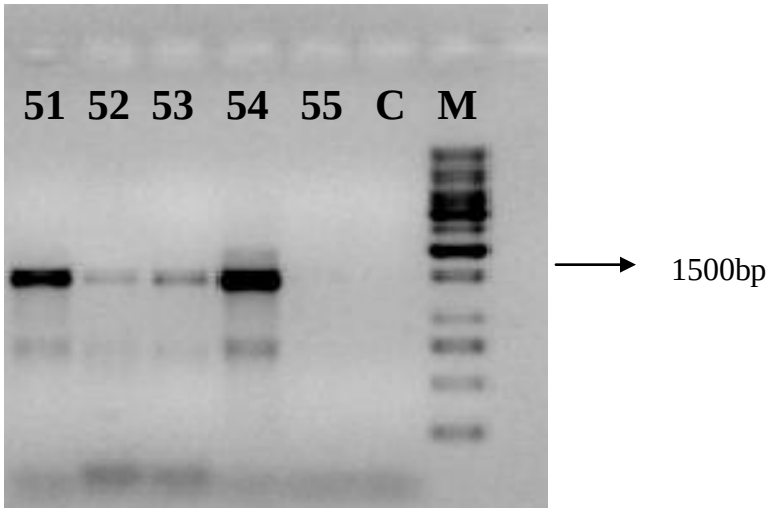
Plate 3: 63KAG 37 ^{o}C isolate under compound microscope x100 under oil immersion



4.7 PCR amplification of 16S rRNA gene from isolates

Bacterial 16S rRNA genes were amplified from DNA extracted from the 16 isolates with bacterial primer 27F 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492R5'-TAC GGY TAC CTT GTT ACG ACT T-3'. PCR amplification of 16S rRNA gene of the isolates was visualized after ethidium bromide staining (Plate 4).

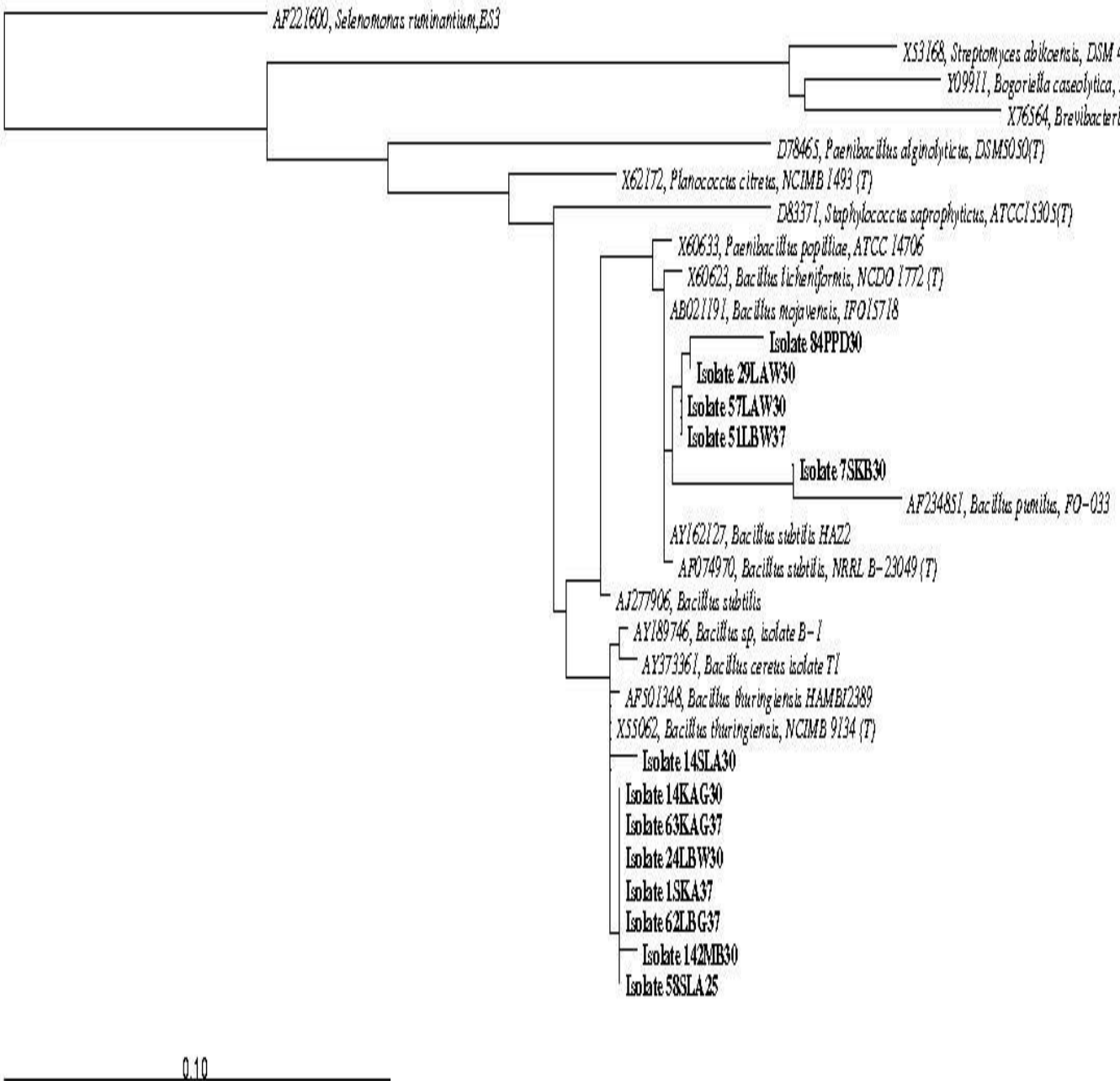
Plate 4: A 1% agarose gel showing PCR amplification of 16S rRNA of the isolates visualized after ethidium bromide staining; (51) LBN37**°C**; (52) PPD 25**°C** (53) SKB 25 **°C**; (54) LBN 37**°C**; (55) SLB 30**°C** (C) Control respectively. M is 100bp plus DNA ladder (Fermentas) used as a molecular marker.



4.8 Phylogenetic cluster analysis of sequences

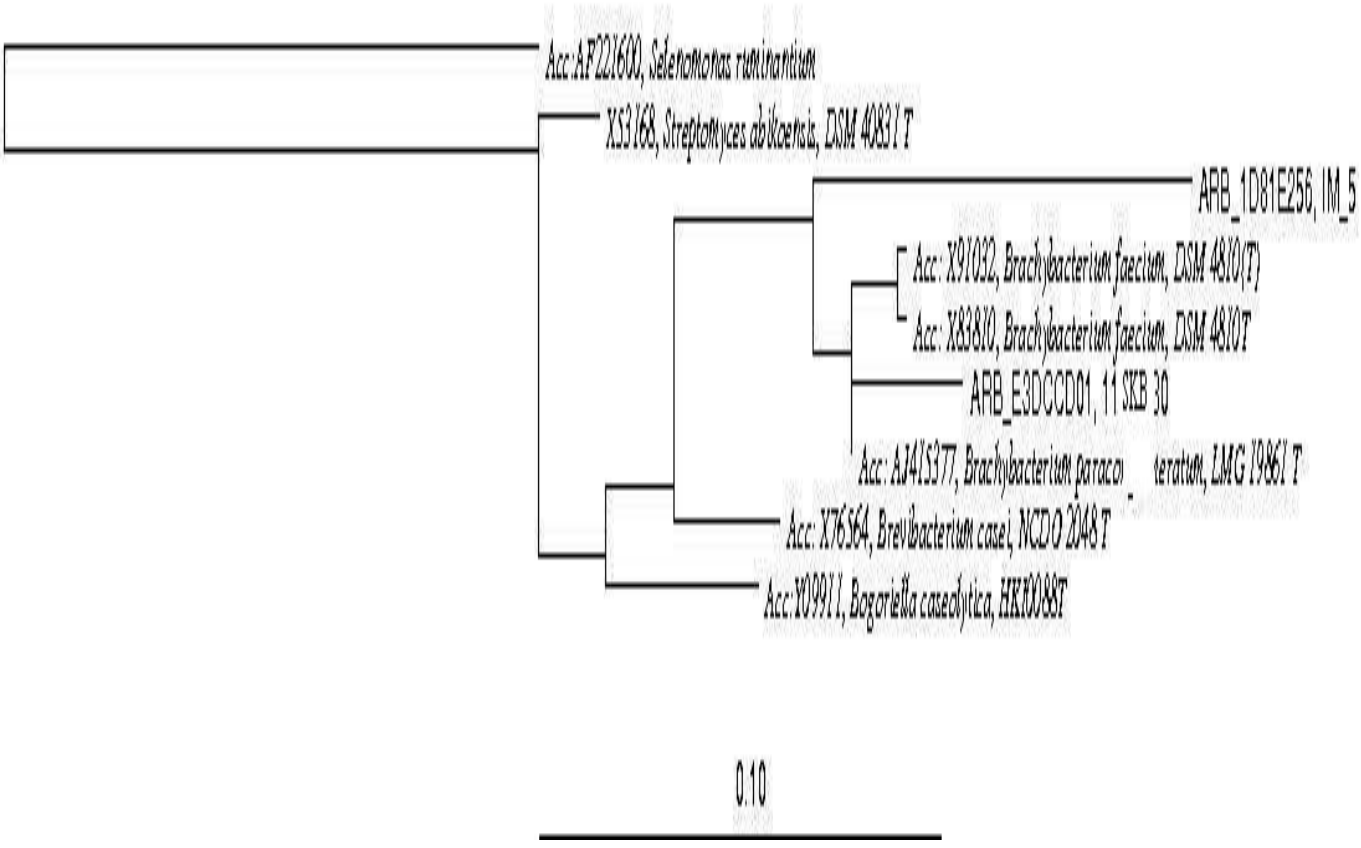
The 16SrDNA amplified products from the sixteen isolates (Table 7) were sequenced. Phylogenic analysis using BLAST software (http://www.ncbi.nih.gov) showed that they belong to the domain Bacteria and phyla *Firmicutes, Proteobacteria* and *Actinobacteria* (Figure 6; Figure 7; Figure 8). Thirteen isolates were clustered with the low G+C content *Firmicutes* in to three clusters (Figure 6**)**. Isolates 1SKA37^{o}C, 58SLA25^{o}C, 142MB30^{o}C, 14SLA30^{o}C 14KAG30^{o}C, 62LBG37^{o}C, 63KAG37^{o}C and 24LBN30^{o}C all clustered with *Bacillus thuringiensis*. It was supported with a sequence similarity of 99.3%-99.7% and bootstrap value of 99%. On the other hand isolates 51LBN37^{o}C and 57LBN37^{o}C from termite mound were clustered with *Bacillus mojavensis* with a sequence similarity of 99.7% and bootstrap value of 99%. Isolate 29LAN 30 ^{o}C and 84PPD30 ^{o}C were clustered with *Bacillus subtilis* with a sequence similarity of 99.5% and bootstrap value of 99%. Lastly, isolate 7SKB 30 ^{o}C from soil was clustered with *Bacillus pumilus* with a sequence similarity of 99.33% and bootstrap value of 99%.

Figure 6: Phylogenetic tree showing positions of isolates closely related to *Firmicutes.* The scale bar indicates approximately 10% sequence difference. Included are sequences from termite gut, mound and soil. 1SKA37^{o}C, 58SLA25^{o}C142MB30^{o}C, 14SLA30^{o}C, 14KAG30^{o}C, 62LBG37^{o}C), 63KAG37^{o}C, 24LBN30^{o}C, 51LBN37^{o}C57LBN37^{o}C, 29LAN30^{o}C, 84PPD30^{o}C, 7SKB30^{o}C



The second phylum that was represented in the phylogenetic analysis was the *Actinobacteria* phylum. Isolate 11SKB 30^{o}C was clustered with *Brachybacterium paraconglomeratum* (Figure 7) with a sequence similarity of 100% and bootstrap value of 99% in the Genbank database **(http://www.ncbi.nih.gov**). This isolate was obtained from Kakamega Forest soil primary culture using the dilution and heat shock method incubated at 30^{o}C.

Figure 7: Phylogenetic tree showing positions of isolates closely related to *Actinobacteria* phyla. The scale bar indicates approximately 10% sequence difference. Included are sequences from soil of isolate 11SKB 30^{o}C.

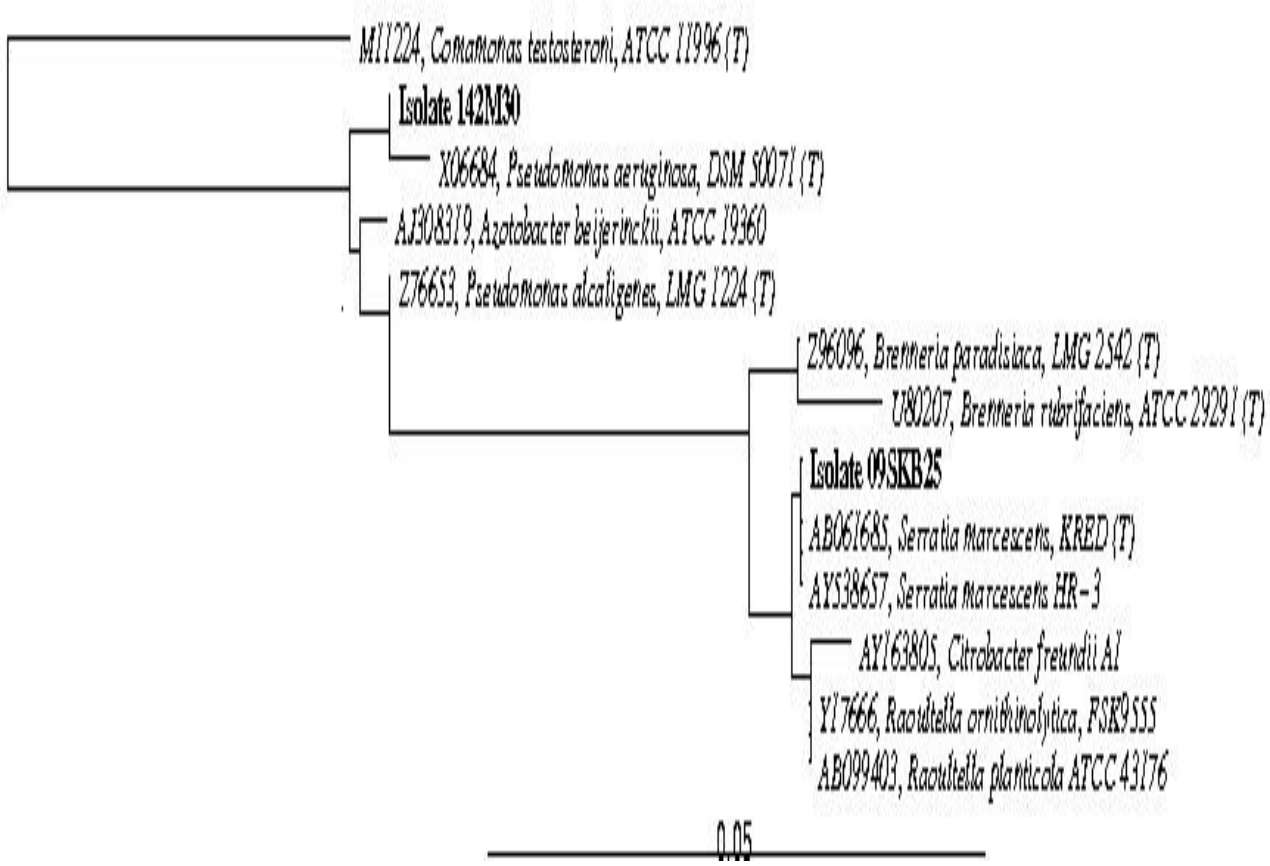


The isolate had the ability to degrade starch in its environment hence grew well in M1 media (Appendix **3**) and has characteristic yellow color (Table 7). As earlier indicated, the isolate is closely related to *Brachybacterium* species which is a high G+C content bacteria and it produces antibiotics against the test organisms (Table 6).

The third phylum represented in the phylogenetic analysis of isolates was the *Proteobacteria* phylum. Isolate 142M30^{o}C was clustered with *Pseudomonas aeruginosa* (Figure 8) with a sequence similarity of 99% and bootstrap value of 98% in the Genbank database (**http://www.ncbi.nih.gov**). The isolate was obtained from murram soil in Juja and belongs to family Pseudomonadaceae. It grew well on minimal media and it hydrolyzes starch.

The phylum *Proteobacteria* was also represented by isolate 9SKB 25 ^{o}C which clustered with *Serratia marcescens* (Figure 8) with a sequence similarity of 99.4% and bootstrap value of 98%. The species *Serratia marcescens* belongs to the family Enterobacteracea. It was also cultivated from the termite gut, mound and soil at Kalunya Glade in Kakamega Forest (results not shown). This isolate grows well at an incubation temperature of 25 ^{o}C and 30 ^{o}C and has a characteristic red colony color.

Figure 8: Phylogenetic tree showing positions of isolates closely related to *Proteobacteria* phyla. The scale bar indicates approximately 50% sequence difference. Included are sequences from soil of isolates 142M30^{o}C and 9SKB 25^{o}C



CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION & RECOMMENDATION

5.1 DISCUSSION

Media with low nutrient was used in this study to cultivate antibiotic and enzyme producing bacteria. This media contained dilute nutrient broth plus agar and was used to study the effect of various incubation temperatures and incubation period in termite gut and mound and soil samples. Dilute nutrient broth plus agar media coupled with lengthy periods of incubation at 25^{o}C have allowed growth of commonly and rarely isolated groups of soil bacteria (Janssen *et al*., 2002). The media was favorable as it allowed the development of a wide range of bacteria present in the sample, prevented growth of spreading colonies and allowed the least possible growth of unwanted non-bacterial colonies such as fungi (Janssen *et al*., 2002).

5.1.1 Comparison of number of cultivable microorganisms in soil, gut and mound samples

Microbial diversity in soil is dependent on environmental factors such as soil type, and nutrient availability (Prescott *et al.,* 1999). Kakamega forest soil samples had higher number of cultivable microorganisms as compared to Juja soil samples. The increased microbial density in the forest is characterized by high biological diversity and continuous plant cover that result in increased soil organic matter leading to improved soil structure, nutrient storage capacity and water holding capacity (Bugg and Van Houn, 1997; Gulick *et al*., 1994). The study also revealed high numbers of cultivable microorganisms in clay soil samples. Consistent with previous

studies (England *et al.,* 1993) that demonstrated presence of clay enhances retention of microorganisms and increases the provision of protective niches. DNBA media was suitable in cultivation of most bacteria since it had a pH of 6.83 (Appendix 2). Previous studies (Reddy *et al.,* 1981) suggested that pH of between 6 and 7 offers optimal conditions for the majority of soil bacterial survival.

In this study the enumeration of cultivable microorganisms showed discrepancies in termite mound, gut and surrounding soil samples. The termite mound samples had a number of cultivable microorganisms of 4.3×10^{7} cfu/g and 9.2×10^{7} cfu/g from Lirhanda and Kalunya Glade sample site which is lower compared to soils that had 1.01×10^{8} and 1.3×10^{8} cfu/g in Lirhanda Hill and Kalunya Glade respectively. Termite gut samples had the highest number of cultivable microorganisms of 2.53×10^{9} cfu/g and 2.0×10^{9} cfu/g in Lirhanda and Kalunya Glade respectively. Previous study (Fall *et al.*, 2007) demonstrated that the bacterial genetic make up of termite mound, gut and surrounding soil are different. This discrepancy in number of cultivable microorganisms could be as a result of different nutrient levels. For instance the termite mound has high organic matter than the surrounding soil (Brauman *et al.*, 2000) however the media used in this study was low in organic matter. This implies majority of bacteria in termite mound may have escaped cultivation due to lack of nutrients.

5.1.2 Impact of cultivation temperature on number of cultivable microorganisms

Temperature is an important factor in regulating microbial activity and shaping the soil microbial community (Pietikainen *et al.,* 2005). Studies by Joseph *et al.,* (2004) where DNBA media was used in cultivation revealed plate counts ranging from 4.82×10^{7} to 3.79×10^{8} cfu/g (dry weight of

soil) at incubation temperature of 25^{o}C. This study is consistent with that study (Joseph *et al.*, 2004), as it revealed plate counts ranging from 6.4×10^{7} to 1.8×10^{8} cfu/g in Juja soil samples and 1.4×10^{8} to 2.2×10^{8} cfu/g in Kakamega Forest soil samples at incubation temperature of 25^{o}C. Cultivation of plates at 30^{o}C and 37^{o}C incubation temperatures revealed most soil samples had higher microbial growth at 25^{o}C and 30^{o}C incubation temperatures and lower values at 37^{o}C. This in line with previous studies of Pietikainen *et al.,* (2005) that showed microbial growth rates had optimum temperatures between 25^{o}C-30^{o}C while lower values were found at higher temperatures.

The termite mound samples had optimal cultivation temperatures at 30^{o}C and 37^{o}C. This may be as a result of the increased microbial activity in the mound that was caused by different bacterial genetic structure compared to the surrounding soils (Fall *et al.,* 2004). This study further revealed the termite gut samples had high numbers of cultivable microorganisms of 4.32×10^{9} cfu/g and 4.17×10^{9} cfu/g in Lirhanda and Kalunya samples at incubation temperature of 37^{o}C. The disparity in optimum cultivation temperatures of termite gut, mound and soil samples could be as a result of different nutrient levels in the microbial habitat. Previous studies have shown the termite mound has increased richness in organic matter in terms of Carbon and Nitrogen arising from the combination of materials of two distinct origins, feces and soils (Brauman, 2000; Fall *et al.*, 2001). Previous studies have further shown that termites raise pH, organic matter and water content in surrounding soils (Schmitt-Wagner and Brune, 1999). Hence the disparity in number of cultivable microorganisms in soils of Juja and Kakamega Forest could be as a result of the effect of termites' mounds.

5.1.3 Impact of incubation period on number of cultivable microorganisms

Majority of isolates selected after the two week incubation period were Gram positive bacteria. Inline with previous studies that showed skewed abundance of Gram positive isolates as a result of isolates having been selected after three days of incubation (Kasahara and Hattori, 1991).

The effect of incubation period was studied and the results showed that the number of cultivable microorganisms increased as the incubation period extended to two weeks in all samples studied. For instance termite gut samples showed a steady increase in numbers of cultivable microorganisms up to two weeks regardless of the cultivation temperature used. The termite mound on the other hand showed a steady increase of the numbers of cultivable microorganisms to two weeks at 25^{o}C and 30^{o}C temperatures except at 37^{o}C where there was no significant increase after seven days. The effect of extending incubation period to ten weeks using DNBA media showed exponential increase in viable bacteria counts in the first two weeks of cultivating soil samples (Davis *et al*., 2004).

5.1.4 Impact of dilution and heat shock method on number of cultivable Microorganisms

The dilution and heat shock method was used by Mincer *et al*., (2002) on M5 media (low nutrient) that contains 20% agar to isolate Actinomycetes whose members belong to the division Actinobacteria that have unparalleled ability to produce diverse secondary metabolites (Stackebrandt *et al.*, 1997). These Actinomycetes account for almost 70% of the world's naturally occurring antibiotics (Okami and Hotta, 1988) and have been isolated from termite gut

(Watanabe *et al*., 2003). They have also been detected in termite mound and surrounding soils through molecular techniques (Fall *et al.,* 2007).

Dilution and heat shock method used in this study on DNBA media demonstrated no significant increase in the number of cultivable microorganisms in the two week incubation period as opposed to previous studies that showed increase in number of cultivable microorganisms as incubation period was extended (Janssen *et al.,* 2002). This may be attributed to initial heating stage that destroys most bacteria hence the reason for varied results for the same samples. Previous studies by Mincer *et al.,* (2002) showed that incubation period of six weeks allow for further growth however this study did not extend the incubation period further than the two weeks. The effect of temperature on number of cultivable microorganisms was significant when dilution and heat shock method was used. In this study optimal cultivation occurred at 30^{o}C for termite mound, gut and surrounding soil samples when the dilution and heat shock method was used. This may be attributed to the fact that actinomycetes have been isolated in the past at an incubation temperature of 28^{o}C (Mincer *et al*., 2002) and 30^{o}C (Mackenzie *et al.*, 2007).

This study successfully characterized isolate 11SKB 30^{o}C obtained from surrounding soil whose sequences were 100% closely related to *Brachybacterium paraconglomeratum.* This isolate belonging to the division *Actinobacteria* (Takeuchi *et al.,* 1995) was cultivated in low nutrient media (DNBA) at a pH of 6.83 and incubation temperature of 30 ^{o}C in line with previous studies where it was cultivated at a pH of 6-10 and an incubation temperature of 30 ^{o}C (Takeuchi *et al.,* 1995).

5.1.5 Characterization and identification of isolates

The low nutrient media used in the study cultivated mostly Gram positive and a few Gram negative bacteria. Inline with previous phylogenetic diversity of soil samples that revealed presence of Gram positive and *Proteobacteria* phyla (Atlas, 1984). However, phylogenetic analysis in this study revealed few enteric bacteria and this is attributed to the low media used. Enteric bacteria have difficulty competing with natural micro flora for the low concentration of available nutrients in natural ecosystems (Burton *et al.,* 1987). Instead the enteric bacteria grow with much ease in most standard nutrient rich laboratory media preventing growth of the majority micro flora found in natural ecosystems.

Phylogenetic analysis of the isolates did not reveal new or uncultured group of bacteria. However, previous study that used the similar media (DNBA) had twenty two percent of the bacterial isolates cultured belonging to the uncultured group of bacteria (Janssen *et al*., 2002).

Morphological characterisation in this study showed majority of termite gut isolates were Gram positive-rod shaped, endospore forming and motile bacteria. Phylogenetic analysis showed that these isolates were closely related to members in the *Bacillus* group. Prokaryotic microorganisms have been previously shown to inhabit the gut of soil feeding termites (Brune, 1998; Brauman *et al*., 2000). Previous analysis of soil feeding termite gut using PCR- denaturing gradient gel electrophoresis (DGGE) revealed presence of *Firmicutes* phyla in the termite gut library (Fall *et al.,* 2007). Presence of *Bacillus-Clostridium* group that form the low G+C content bacteria in the gut of soil feeding termites was also revealed by sequencing 16Sr RNA gene clones from the

highly alkaline first proctodeal segment of *Cubitermes Orthognathus* (Schmitt-Wagner *et al.*, 2003). Through biochemical characterisation, these isolates showed ability to reduce nitrates to nitrites and nitrogen supporting the hypothesis that nitrogenous components of humus are an important dietary resource for humivorous soil macro invertebrates (Ji *et al.*, 2000; Ji and Brune, 2001). Filamentous micro organisms have been observed in the P3 and P4 gut sections through phase contrast microscopy. However this study failed to obtain these isolates due to homogenization of the gut sections that is known to destroy these bacteria (Schmitt-Wagner *et al.*, 2003a).

Phylogenetic analysis of isolates also revealed members closely related to the *Bacilli* group in the termite mound and surrounding soil. The *Bacilli* species belong to the *Firmicutes* phylum (Claus and Berkeley, 1986). Previous analysis of soil feeding termite mound using PCR- denaturing gradient gel electrophoresis (DGGE) revealed presence of *Firmicutes* phyla in the termite mound and surrounding soil library but to a lesser extent when compared to those found in the gut samples (Fall *et al.*, 2007). Biochemical characterisation showed that these isolates (Bacilli group) were nitrate and nitrites reducers indicating the role these isolates play in the nitrogen cycle of termite mound, gut and soil (Collins, 1983**).**

In this study, termite gut isolates were able to utilize arabinose, maltose, sucrose and fructose an indication of the role played by these isolates in the formation of short chain fatty acids from carbohydrates or synthesis of amino acids (Cummings and Macfarlane, 1997). In line with previous studies by Miambi, Tholen, Boga and Brune (unpublished results) revealed presence of termite gut isolates able to utilize glucose.

Isolates whose sequences were closely related to *Serratia marcescens* and *Pseudomonas aeruginosa* were obtained from surrounding soil and Juja soil samples respectively. Morphological characterisation revealed the isolates were Gram negative, rod-shaped and motile bacteria belonging to *Proteobacteria* phylum (Bergy's manual, 1989). Previous analysis of soil feeding termite surrounding soil using PCR- denaturing gradient gel electrophoresis (DGGE) revealed the presence of *Proteobacteria* phylum in the surrounding soil library (Fall *et al.,* 2007) hence this study was able to cultivate them in low nutrient media. Biochemical characterisation of the isolate closely related to *Serratia marcescens* revealed ability to utilize fructose, sucrose and lactose in line with previous investigations by Thomas and Lewis (1957). The isolate was also able to reduce nitrate indicating the role it could play in the nitrogen cycle (Lengeler *et al*., 1999) in its habitat.

Phylogenetic analysis revealed an isolate closely related to *Brachybacterium paraconglomeratum* obtained in the surrounding soils of termite gut. Previous analysis of soil feeding termite surrounding soil using PCR- denaturing gradient gel electrophoresis (DGGE) revealed presence of members of Dermabacteraceae family in the nearly half of the surrounding soil clone library (Fall *et al*., 2007). This *Brachybacterium* species belongs to the Dermabacteraceae family and *Actinobacteria* phylum (Stackebrandt, 1997). It is a Gram positive, coccid-rod shaped, non spore forming non motile and high G+C content bacteria (Takeuchi *et al.,* 1995). The ability of this isolate to reduce nitrate indicates the role it plays in the nitrogen cycle (Lengeler *et al*., 1999). Substrate utilization tests of the isolate revealed the ability to utilize lactose, xylose, mannose, fructose, galactose, maltose and sucrose (Takeuchi *et al.*, 1995).

5.1.6 Antibiotic production from Bacterial isolates

Antibiotic production from isolates in termite gut and mound samples was clearly shown in this study with 30% of termite mound isolates having ability to secrete antimicrobials against *Candida albicans.* On the other hand, 30% of termites gut isolates had the ability to secret antimicrobials against *Bacillus subtilis*. Isolates closely related to the *Bacillus* species produced antibiotics against gram positive test organism. This is consistent with previous studies that showed antibiotic production is a feature of several kinds of soil bacteria (Talaro and Talaro, 1996). In addition, the *Bacillus* species are capable of producing antibiotic as secondary metabolites (Katz and Demau, 1977). As earlier indicated, isolate 11SKB 30^{o}C obtained from surrounding soil was closely related to *Brachybacterium* species. This isolate was able to produce antimicrobials against the test organisms. *Brachybacterium* species has been used in previous studies to eliminate *Staphylococcus aureus* because of its ability to produce antibiotics (Takeuchi *et al.,* 1995)

5.1.7 Enzyme production

The study showed isolates obtained from termite gut closely related to *Bacillus* species had the ability to degrade starch, an indication of the role the amylases play in extracting organic matter from the soil in the gut, a process that is favoured by alkaline conditions in the gut (Brune, 1998; Kappler and Brune, 1999). Members of genus *Bacillus* are known to secret a wide variety of enzymes such as amylases and proteases that are used to degrade starch and protein (Priest 1977; Mezes and Lampen, 1985).

The study revealed the ability of 33% of the termite gut isolates to degrade gelatin. In line with previous studies that have shown presence of aerobic organisms in the gut capable of degrading gelatin (Miambi, Tholen, Boga and Brune, unpublished results). In addition 33% and 37% of isolates in termite mound and surrounding soil respectively were able to degrade gelatin. Gelatin degrading isolates were closely related to members of Genus *Bacillus* in line with previous investigations by Debabor (1982) that demonstrated ability by *Bacillus* species to degrade gelatin and casein. Isolate 11SKB 30^{o}C that was closely related to *Brachybacterium* species was aslo able to degrade gelatin in line with previous investigations by Takeuchi *et al.,* (1995) that showed the ability to degrade gelatin. This is an indication of the role the isolate plays in transformation of peptides and protein in their habitat (Ji and Brune, 2001).

This study revealed 26% of the isolates in termite gut had the ability to degrade casein. In addition, 38% of isolates in mound had the ability to degrade casein an indication of the role they play in transforming proteins (Ji and Brune, 2001). In this study, 11% of termite gut isolates and 7% of mound isolates were capable of degrading cellulose indicating the role played by these bacterial isolates in degrading cellulotic materials in termite mound. Brune and his co-workers were able to show that soil-feeding termites transform cellulose in the soil an indication of presence of cellulolytic bacteria in the gut (Ji and Brune, 2001). The Ability of the majority of isolates from soil, gut and mound to utilize various sugars is an indication of the role they play in generation of short chain fatty acids from carbohydrates (Cummings and Macfarlane, 1997).

5.2 CONCLUSIONS

- The study has demonstrated using low nutrient media (DNBA) in cultivation that the termite gut, mound and soil is a potential source of antibiotic and enzyme producing bacteria.
- The results showed incubation temperature as a major factor in cultivation as the effect was found to be significant on viable bacterial count through statistical analysis. It further revealed termite gut samples had the highest number of cultivable microorganisms at 37^{o}C, the termite mound had high number of cultivable microorganisms at 30^{o}C and most soils had high numbers of cultivable microorganisms at 25^{o}C and 30^{o}C.
- The study also demonstrated that extending incubation period to fourteen days increased the number of cultivable microorganisms that grew on most sample plates.
- The study modified cultivation condition by using the dilution and heat shock where incubation temperature at 30^{o}C was optimum temperature for termite mound, gut and soil samples. An Actinobacteria species was cultivated through this method.
- The study was also able to cultivate cellulose, casein, starch and gelatin degrading bacteria from the soil feeding termite gut and mound. A xylan degrading bacteria was also cultivated from the termite gut.
- Through molecular techniques, the study was able to identify diverse genera such as *Brachybacterium, Bacillus, Pseudomonas and Serratia* that had diverse antagonist activity against test organisms *E.coli*, *B. subtilis* and *C. albicans.*
- Isolates closely related to *Proteobacteria* and high G+C content bacteria in Dermabacteraceae family were retrieved from soil. Isolates closely related to low G+C

content bacteria in *Firmicutes* phyla were also isolated from termite gut and mound confirming previous molecular studies that showed presence of these bacteria in those habitats.

- Owing to the fact that these isolates were obtained from remote habitat such as termite nest and termite gut, they will add to the pool of already available isolates and may aid in screening studies to provide positive antagonists for further testing by *in vivo and invitro* assays.

5.3 RECOMMENDATIONS

- To get a clear insight into the mechanism of antagonism, further studies should be done to characterize and identify the proteins or compounds with antimicrobial activity. That information would be important in the biotechnological processes such as molecular cloning of the genes coding for such proteins. It will also be necessary to evaluate the bioactivity of such proteins *in vivo* through screen house experiments.
- The most potent antagonistic isolates should be further studied to optimize the conditions of establishment, antimicrobial activity and to investigate dose-response activities.
- Further work needs also to be done to purify the enzymes produced and characterize them. It will also be necessary to evaluate the activity of such enzymes in *vivo* through screen house experiments before scaling them up for large scale production in industries.
- To further expand our knowledge of microbial diversity in various habitats, it may be necessary for further research to investigate the effect of variation of growth parameters such as pH and culture media composition with various substrates for isolation of isolates to correspond to those of the natural habitats of different areas. Changes could result in

differences in the abundance and diversity of isolates obtained from each sample as some isolates may grow under some conditions and not others.

- The incubation period could be extended further to two months to allow growth of slow growers.
- Information of microbes found in soil feeding termite gut has continued to be gathered. The termite gut is alkaline in nature with various pH levels at different sections of the gut. Consequently it is worthwhile to utilize Culture media composition at different alkaline pH levels to accommodate these bacteria.
- Further work needs to be done to establish if there could be any anaerobic bacteria in the gut of soil feeding termites.
- Culture independent sampling directly from the parent soil, termite gut and nest using PCR based approaches has shown presence of Actinomycetes. These filamentous bacteria need to be cultivated as they are known producers of antibiotics production. Further it may be possible to enhance improved cultivability of isolable Actinobacteria as this would help to capture even those isolates that were obtained from the termite gut nest and soil but were lost through subsequent subcultures. Such isolates may also posses' beneficial antagonistic abilities.

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APPENDICES

1. **LB Broth**

   10g Sodium Chloride

   10g Tryptone

   5.0g Yeast extract

   Add deionized water to a final volume of 1 Liter

   Adjust pH to 7.5 with NaOH and autoclave

2. **Dilute nutrient broth (DNBA) medium**

   0.08 Nutrient broths

   18g agar

   1000ml distilled water

3. **M1 media**

   10g of starch

   5g yeast extract

   2g peptone water

   18g Agar

4. **Solution A**

   50mM Tris pH 8.5

   50 mM EDTA pH 8.0

   25 % Sucrose solution

5. **Solution B**

10mM Tris pH 8.5

5mM EDTA pH 8.0

1% SDS

6 **Basal Salt Solution (BSS)**

K_2HPO_4 (2.0g),

KH_2PO_4 (1.0g)

Kcl (1.5g)

Nacl (1.5 g)

1000 ml distilled water.