ISOLATION AND CHARACTERIZATION OF ANTIBIOTIC AND EXOENZYMES PRODUCING ACTINOBACTERIA FROM GUTS OF FUNGUS-CULTIVATING TERMITES (*Macrotermes michaelseni)*

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Isolation and Characterization of Antibiotic and Exoenzymes Producing Actinobacteria from Guts of Fungus-Cultivating Termites (*Macrotermes Michaelseni)*

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DECLARATION

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

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DEDICATION

This work is dedicated to my family and all who made this course a success.

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LIST OF ABBREVIATIONS

Bp	Base pairs
DNA	Deoxyribonucleic Acid
EDTA	Ethylene diamine tetra-acetic acid
G+C	Guanine and Cytosine
ISP	International Streptomyces protocol
KMM1	Kenya Minimal Medium 1
LB	Luria Bertani
Nm	nanometers
BSS	Basic saline solution
PCR	Polymerase chain reaction

ABSTRACT

Termites are an important group of insects that harbor a complex community of gut microbes which contribute to digestion and termite nutrition. Fungus-cultivating termites harbour dense populations of bacteria and archaea in the gut. Actinobacteria are known to produce a wide variety of secondary metabolites including many commercially important enzymes and antibiotics. The aim of this study was to isolate Actinobacteria from guts of fungus-cultivating termites *Macrotermes michaelseni* using solid KMM1 medium and screen for their ability to produce industrially useful enzymes and antibiotics. The isolates were characterized using morphological, physiochemical, biochemical and molecular methods. A total of ten isolates were obtained. The isolates produced amylases, lipases, proteases and esterases. All the isolates, apart from KMM1, KMGC6, KMGC8 and KMGT9 produced gelatinases, xylanases and cellulases. All the isolates showed inhibition against *Escherichia coli* and *Staphylococcus aureus.* The isolates grew well at pH 6 and temperature of 40^{o}C. They preferentially utilized glucose and did not require sodium chloride for growth. Analysis of partial sequences of 16S rRNA genes confirmed isolate KMM2, KMM3, KMM4, KMM5, KMGC7, KMGC8 and KMGT10 belonged to the genus *Streptomyces* while KMM1 and KMGC6 were close relatives of Bacillus. These results confirmed that guts of fungus-cultivating termites harbor Actinobacteria that can produce enzymes and antibiotics.

CHAPTER 1.0

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Termites are terrestrial social ants that belong to the order Isoptera and are among the most abundant and important soil micro-invertebrates, playing a role in the decomposition of wood or plant litter (Nutting, 1990).

Phylogenetically, termites can be classified into two subgroups constituting of lower termites and higher termites. Lower termites include Mastotermitidae, Hodotermitidae, Kalotermitidae, Rhinotermitidae and Serritermitidae, characterised by having symbiotic intestinal protozoa on which they depend for digestion of complex polysaccharides (Wood and Johnson, 1986). Higher termites are termites from Termitidae (Edwards and Mill, 1986) that constitute approximately 75% of all termite species (Breznak, 1983). Higher termites harbour dense populations of gut bacteria (Wood and Johnson, 1986). Their digestive processes remain obscure, although they rely partly on their own digestive enzymes including cellulases and symbiotic gut microorganisms (Breznak, 1983).

Termites are found in a wide geographical area spanning from tropical to temperate zones in great abundance, and are capable of degrading various derivatives of plant materials such as wood, leaves and humus and play an important role in the carbon cycle. The xylophagy of termites depends on digestive symbionts established in there

hindgut, which consists of both eukaryotes and prokaryotes. These microbial symbionts in termite gut play an important role in lignocellulose digestion and termite nutrition (Breznak and Brune, 1994).

The African continent is climatically and geographically diverse and contains the world's largest deserts and one of the greatest mountain peaks. Termite diversity also reflects this topological and climatological diversity. More than 1,000 species of the over 2,600 species of termites recognised occur in Africa (UNEP, 2000).

The termites present in a colony consist of several castes, which are morphologically and functionally distinct (Noirot, 1992). The castes may be divided into two broad groups; reproductive and sterile. The most important of the sterile castes are the soldiers and the workers. The workers are the most numerous. They are responsible for building the nest and for all foraging activity. The workers care for the eggs, feed the larvae, the soldiers and the queen, all of which are incapable of feeding themselves. Most biochemical and microbiological studies of termites have been carried out on the worker caste because of there importance in feeding other castes (O'Brien and Slaytor, 1982).

In this study, we propose to isolate Actinobacteria from guts of fungus-cultivating termites *Macrotermes michaelseni* using KMM1 medium, screen for production of enzymes and antibiotics and characterize using physiological, biochemical and molecular techniques.

1.2 Literature Review

Although frequently thought of as feeding on wood, majority of termites feed on other resources (Wood and Johnson, 1986). Half of the genera feed on soil (Noirot, 1992), while others cultivate gardens of fungi, which they ingest along with different components of plant litter, such as roots, leaves, and dead wood among others (Breznak, 1983).

The diet of termites is diverse but is rich in cellulose, hemicellulose and lignin or derivatives of lignin. These termites colonize various biotypes due to their different dietary demands. Most of these thrive on relatively refractory nitrogen poor resources. These include soil feeding, wood feeding and fungus-cultivating termites (Noirot, 1992).

1.2.1 Soil feeding termites

Soil feeding termites ingest large amounts of soil (Wood, 1978; Okwakol, 1980). Due to their high biomass densities, their feeding activity is important for the biomass turnover in the tropical and sub-tropical ecosystems (Wood and Johnson, 1986; Wood, 1978; Martius, 1994; Bignell *et al.,* 1994). The food ingested by soil-feeding termites is quite heterogeneous. The gut predominantly contains soil minerals and humus, but also plant tissue fragments, plant roots, fungal mycelia and macerated organic material. The hindguts of soil feeding termites are more elongated and compartmentalised than those of any other feeding guild and characterised by steep pH changes along the axis (Bignell and Eggleton, 1995). Microscale pH measurements have shown that pH increases

sharply in the anterior hindgut, culminating in the paunch region with the most alkaline values encountered in the biological systems (pH >12), and decrease towards the rectum (Brune, 1998).

1.2.2 Wood feeding termites

These are termites that feed on wood and woody litter, including dead branches still attached on trees. Lower termites are wood feeders and there are wood feeding species in all the families of the Termitidae except the Apicotermitinae (Bignell and Eggleton, 1995). The main part of the digestion takes place in the hindgut especially in the paunch, under the action of symbiotic micro-organisms. It is well established that lower termites digest cellulose using synergetic actions of the cellulolytic enzymes that originate from the termite guts and symbiotic protozoa in the hindgut. The protozoan fauna accounts for the most of the cellulolytic activities in the hindgut (Breznak, 1982).

1.2.3 Fungus cultivating termites

Termites of the subfamily Macrotermitinae, so called fungus-cultivating termites, have a sophisticated and highly efficient symbiotic relationship with fungi (Wood and Sands, 1978). Fungus-cultivating termites are abundant in Asian and African tropics and have a great impact on the decomposition of dead plant material in those ecosystems (Abe and Matsumoto, 1979). The symbiosis between termites and the fungi of the genus *Termitomyces* is crucial to the digestive process. Despite the symbiosis with the fungi

the fungus-cultivating termites are also reported to harbour dense populations of bacteria and *Archaea* (Anklin-Muhlemann *et al*., 2001). The symbiotic fungi grow on a special culture within the nest maintained by the termites called fungus comb (Hyodo *et al*., 2000). The fungus comb is made from partly digested foraged plant litter, which passes rapidly through the termite‘s guts. The resulting faecal pellets are pressed together to make a comb-like matrix. As the comb matures, mycelium develops and produces conidial nodules, which together with older, senescent comb are consumed by workers. Many studies have been conducted on the termite-fungus association because of their unique symbiotic relationship (Darlington, 1994). Several roles have been suggested for the fungal symbionts for example, the provision of heat and moisture (Lusher, 1951), the provision of concentrated nitrogen source as conidia (Matsumoto, 1976) and the enrichment of nitrogen in foraged foodstuffs by the virtue of the fungal metabolism (Collins, 1983). It has been noted that by associating with the lignin decomposers, the fungus-cultivating termites make it possible to utilize lignocellulose nearly completely as reflected in the small volume of there final faeces (Darlington, 1994) and therefore play a dominant role in the decomposition processes in many parts of the tropics (Abe, 1980; Buxton, 1981).

The relationship between termites and their gut micro-organisms is a well known example of symbiosis. Culture independent molecular studies on the gut microbial community reveal that a great majority of the gut symbionts are difficult to cultivate, rendering our knowledge of this symbiotic system limited (Ohkuma, 2003; Hongoh *et*

al., 2005). Most studies have focussed on wood feeders. Analogous studies on other feeding guilds, especially fungus-cultivating termites remain sparse owing to their habitat, delicate nature and the difficulty of establishing permanent laboratory cultures (Bignell *et al*., 1980; Rouland *et al*., 1993).

1.3 Intestinal tracts of higher termites

In Macrotermitinae, the structure of the digestive tube is very uniform and very close to that of the lower termites, notably the Kalotermitidae and Rhinotermitidae due to the relatively unextended nature of the hindgut (Noirot and Noirot-Timothee, 1969; Bignell *et al*., 1979). The crop posses well developed typical armature (Figure 1). The midgut is relatively long and there are four malpighian tubules which are inserted in a symmetrical manner at the junction of the mesenteron-proctodeum. In the mixed segment, the intestinal lumen is limited on one side by a cuticulated hindgut wall and on the other side by an extension of the midgut epithelium. The proctodeal begins with a very short, narrow segment and terminates in a muscular enteric valve, which commands the entrance to a voluminous paunch (Grasse and Noirot, 1954).

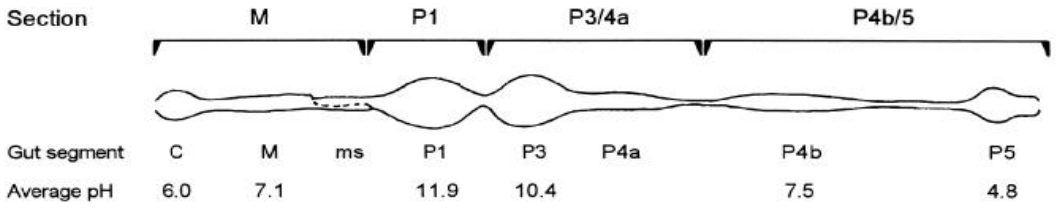

Figure 1: Schematic diagram of gut morphology representative of higher termites. The gut was drawn in its unraveled state to illustrate the various segments: C, crop; M, midgut; ms, mixed segment; P1 to 5, proctodeal segments 1 to 5 respectively (Schmitt-Wagner and Brune, 1999).

Bignell *et al* (1979) using electron microscope, observed Actinomycete-like bacteria in the guts of termites. Actinomycetes were later isolated from termite guts by Pasti and Belli (1985), and were found to have cellulolytic activity. The Actinomycetes were then observed to have lignin-solubilizing activity (Pasti and Belli, 1985; Pasti *et al.,* 1990).

1.4 Actinobacteria

Actinobacteria are filamentous bacteria that are widely distributed in a variety of natural and man-made environments. They constitute a significant component of the microbial population in most soils (Watve *et al*., 2001). Actinobacteria are about 1µm in diameter. Their colonies look like a mass of unicellular mycelium, with branching filament extensions of the original cell or cells, in addition to spores and degradation of products. They reproduce by fission or by spores and this is why they were originally classified as fungi (Waksman, 1968).

Actinobacteria are gram-positive with a high G+C content of their DNA (60-70 mol %) belonging to the order *Actinomycetales*. The mycelial spore forming habit of Actinobacteria is of both Phylogenetic as well as taxonomic importance (Madigan *et al*., 2005). They produce extensive substrate and aerial mycelium. The substrate hyphae are approximately 0.5-1.0µm in diameter and often lack cross-walls during vegetative phase. Growth occurs at the hyphal apices producing a complex tightly woven matrix of hyphae during vegetative growth. As the colony ages, aerial mycelia (sporophores) are produced which develop into chains of spores by the formation of cross walls in the

multinucleate aerial filaments. This is followed by separation of individual cells into spores (Wildermuth and Hopwood, 1970).

1.4.1 Actinobacteria as source of secondary metabolites

Streptomycetes produce a wide variety of secondary metabolites, many of which have potent biological activities. These metabolites vary enormously in structural complexity and biological activity (Demain and Davies, 1999). They are used to produce more than half of the known biologically active microbial products including many commercially important antibiotics, immunosuppressive compounds, animal health products, and agrochemicals (Demain and Fang, 2000). They are used to obtain various enzymes that are commercially and academically valuable. This vast reservoir of diverse products makes the *Streptomycetes* one of the most important industrial microbial genera (Kieser *et al*., 2000).

The metabolites include antibiotics, enzymes, and bioactive products (Bull *et al.*, 1992). The first identified antibiotic was streptomycin, detected about 60 years ago. Since then, thousands of low molecular weight, chemically different compounds with antibacterial, antifungal, antiparasitic, agroactive, herbicidal, cytostatic, and other activities have been found within many *Streptomyces* species and some other Actinomycetes (Demain and Davies, 1999). Because of their ability to synthesize numerous compounds that exhibit extreme chemical diversity, *Streptomyces* strains are major part of industrial strain collections used in screening for new bioactive molecules. Most of these compounds are

synthesized as secondary metabolites. Genes encoding these pharmacologically active substances have been found clustered within DNA stretches of 20 kb to more than 100 kb (Demain and Davies, 1999).

To discover useful metabolites, it is critical not only to design suitable and sensitive assays for screening microbial extracts but also to test extracts that contain most or all of the metabolites from culture broths with a minimum interference (Demain and Davies, 1999). In addition to sensitive assays, novel and diverse microorganisms are critical to the success of any natural product program, hence biological diversity may lead to chemical diversity (Porter and Fox, 1993).

1.4.2 Actinobacteria in degradation of waste

Actinobacteria are active in the decomposition of organic materials including lignin, starch, cellulose and other recalcitrant polymers, and can degrade agricultural and urban wastes (Crawford, 1988). Mycolic acid-containing Actinomycetes are involved in filamentous foaming in activated sludge systems (Reyes *et al.*, 1997). The capability of Actinomycetes to degrade a variety of biopolymers, to modify xenobiotics, and to detoxify harmful compounds is of great biotechnological and ecological relevance (Demain and Davies, 1999). In addition to enzymes degrading macromolecules, *Streptomycetes* produce a large repertoire of enzymes, including those for the

modification of pharmacologically relevant compounds and xenobiotics (Goodfellow *et a*l., 1990).

1.4.3 Actinobacteria in farming

Actinomycetes have also been used in biological control of plant pathogens (Liu *et al.*, 1996), and a few are known to be plant pathogenic (Takeuchi *et al.*, 1996). Preliminary descriptive research shows that Actinomycetes are a promising group of the fungus-antagonistic root colonizing microbes (Crawford *et al*., 1993). *Streptomyces* species and a few other Actinomycetes have been shown to protect several different plants to various degrees from soil-borne fungal pathogens (Reddi and Rao, 1971). Actinomycetes have also been shown to produce herbicidal and insecticidal compounds. Members of the Genus *Frankia* fix nitrogen in nodules of non-leguminous plants (Liu *et al.*, 1996).

1.4.4 Characterization of Actinobacteria

Actinobacteria can be separated into different genera on the basis of morphological, physical chemical and molecular criteria (Goodfellow, 1989). Actinobcteria grow as vegetative, long, branching hyphae that rarely contain septae and are thus multinucleoid (Kieser *et al*., 2000). Cells of *Actinomyces* species are variable in shape showing various degrees of true branching and a more or less wavy appearance (Slack and Gerencser, 1975). All of the *Actinomyces* species are Gram positive and they are nonmotile. The cellular morphology vary from strain to strain within one species and may be influenced

additionally by the composition of the growth medium, its pH, cultural conditions, or the age of cultures (Schaal and Goldmann, 1986).

Actinomyces colonies mature between seven and seventeen days of incubation at 36-40^{o}C and tend to grow into the agar below its surface, which reduces their optical refractivity. In broth, *Actinomyces* species may grow as discrete cotton pad-like colonies or compact masses of variable size adhering to the inner glass surface of the tube or forming granular or pellicular sediment so that the broth remains clear. They may produce diffuse turbidity with varying amounts of granular, flaky, pellicular, sediments (Slack and Gerencser, 1975). The minimal nutritional requirements of *Actinomyces* species are not well understood. However, good growth has only been obtained in or on complex media containing either rich biological substrates such as brain heart infusion, meat extract, yeast extract, or serum or a defined and complex mixture of a large variety of organic and inorganic compounds. These organisms apparently require organic nitrogen like peptides and amino acids, a fermentable carbohydrate and possibly also vitamins and other growth factors for optimum growth (Schaal and Goldmann, 1986).

Molecular biology techniques have become increasingly popular methods for discovering bacterial diversity. Actinobacteria have been characterized by their 16S rRNA gene sequences (Muyzer and Ramsing, 1996; Andrew, 2000). A whole array of taxonomic tools has been used to define genera and suprageneric groups of Actinomycetes (Goodfellow, 1989), but partial sequence analysis of 16S rRNA is the

most significant. The characterization of bacteria has a great potential to assist in further investigation and exploitation of the organisms (Teske *et al.*, 1996). One of these molecular tools is the PCR amplification of variable region of genes encoding 16S rRNA by use of primers homologous to the conserved regions of the gene. Subsequent electrophoretic separation of the PCR products in a polyacrylamide matrix, give comparable fingerprints of microbial communities (Muyzer *et al.*, 1993).

1.5 Justification

There is substantive data on wood-feeding and soil-feeding termites in which intestinal microbiota has been characterized (Schultz and Breznak 1978; Bignell *et al.*, 1980; Brune and Friedrich, 2000; Schmitt-Wagner *et al*., 2003), but little has been done to describe the microbiota of the gut of fungus-cultivators. Fungus-cultivating termites are able to utilize lignocellulose (Darlington, 1994), and therefore play a dominant role in decomposition processes in many parts of the tropics (Buxton, 1981).

Termites digest high molecular weight food substances from their diet such as cellulose and hemicellulose (Brune, 1998) using enzymatic action present in the termite and from the symbiotic microbiota that include bacteria, protozoa and fungi. Processes in the termite gut attributed to the microbiota include digestion and termite nutrition derived from lignocellulose digestion (Breznak and Brune, 1994). The efficiency in degrading cellulosic compounds and the complex diversity of microbes resident in the termite gut

may provide strains of microorganisms with equally new metabolic activities like hydrolyzing cellulose to produce bio-fuel (Veiver *et al*., 1982).

Despite the isolation and cultivation of several bacteria and protists from within the termite guts (Odelson and Breznak, 1985) the biology and the physiology of intestinal microbiota remains limited as many of the predominant species within the community, have not yet been cultured and characterized This is due to there habitat, delicate nature and difficulty in establishing permanent laboratory cultures (Ohkuma and Kudo, 1996).

The frequency of discovering structurally new natural compounds is decreasing. This trend seems to imply that the easily accessible microorganisms in nature have been exhausted and there is need to seek unutilized microorganisms from unexplored sources (Fenical, 1993). Since the role of natural products in the drug discovery is still large, new approaches such as the utilization of cDNA, combinatorial biosynthesis and screening of microorganisms from extreme environment are investigated to discover novel natural chemical structures (Hutchinson, 2002).

It has been suggested by Shiburaj (2003) that screening and isolation of promising strains of Actinomycetes with potential antibiotics is an important area of research. The exploration of materials from new areas and habitats has a pivotal role to play in the search for new microbes and novel metabolites and is urgent to counter the threats posed by the fast emerging phenomenon of antibiotic resistance. It is likely that the diversity of

secondary metabolites relies more or less on the isolation source, namely, the habitat of the producers.

Studies have shown that the termite symbiotic system includes many species yet uncultured in the laboratory (Ohkuma and Kudo, 1998). A large discrepancy between the high diversity of microbial phenotypes and the few numerically and metabolically relevant species isolated underscores that new concepts for cultivation are necessary.

1.6 General objectives

To isolate antibiotic and exoenzymes producing Actinobacteria from fungus-cultivating termites *Macrotermes michaelseni*.

1.7 Specific objectives

1. To isolate and morphologically characterize Actinobacterial isolates from fungus cultivating termites *Macrotermes michaelseni*
2. To screen for production of exo-enzymes and antibiotics from the Actinobacterial isolates.
3. Physiochemical, biochemical and molecular characterization of exo-enzymes and antibiotic producing Actinobacterial isolates from termite gut.

1.8 Hypothesis

Guts of fungus-cultivating termites *Macrotermes michaelseni* harbor Actinobacteria that can produce antibiotics and enzymes.

CHAPTER 2.0

MATERIALS AND METHODS

2.1 Termite collection

The study was conducted on fungus-cultivating termites *Macrotermes michaelseni* which belong to the higher termites; Family *Termitidae,* Subfamily *Macrotermitinae* (Ruelle *et al.,* 1975).

A small number of termites, workers major caste of fungus-cultivating termites *Macrotermes michaelseni,* were freshly collected once from one mound in the compound of Jomo Kenyatta University of Agriculture and Technology in Juja, Thika District, Kenya.

The sampling site Juja is located in central Province of Kenya (Latitude 10 05^{o}S, Longitude 370 00^{o}E) at an altitude of 1525m above sea level. It is located in the transitional zone between the high rainfall area to the North and Northwest and the low rainfall area to the East. The mean annual rainfall ranges from 782-933 mm. The vegetation is grassland with scattered bushes, dominated by *Digitaria scalarum* (Schweinf.) Chiov, *Eragrostis atroviren* (Desf.) Steud and herb *Ocinum* species. *Acacia seyal* is the dominant bush. The soils are generally poorly drained and the texture of the topsoil is clay (Muchena *et al*., 1978).

The mound was dug and termites together with nest fragments were collected in sterile containers and transported to the laboratory. The termites were collected from chimney-like mounds (Figure 2a). Adult workers major caste termites were used (Figure 2b). The presence of fungus-combs (Figure 2c) confirmed that the species was fungus-cultivating termites.

Figure 2a: A termite mound of fungus-cultivating termites.

Figure 2b: Workers major caste of fungus-cultivating termites

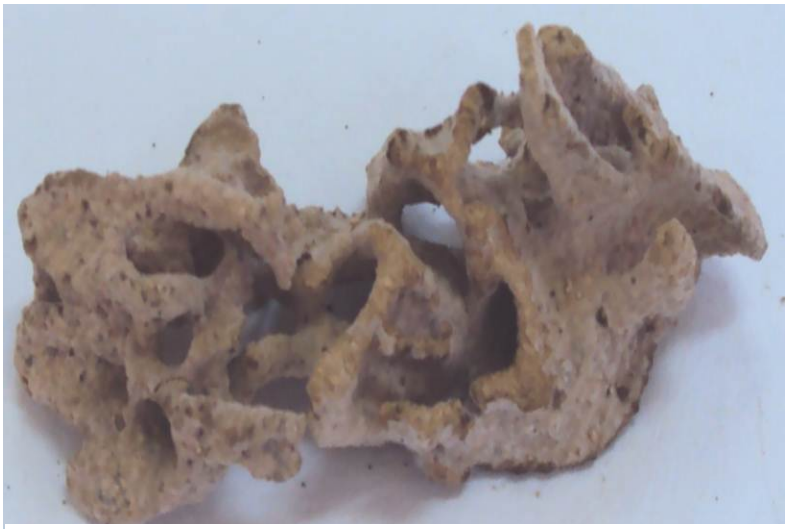

Figure 2c: A sample of fungus comb on which fungi is grown by the fungus-cultivating termites**.**

Under a hood, individual termites were degutted in aseptic conditions. Ten termites were degutted using sterile fine tipped forceps as previously described by Schmitt-Wagner *et al*., 2003. The whole gut of each termite was extracted by pulling the rare tip with one set of the forceps while securing the head with the other. Ten guts (73mg) were homogenized in sterile glass homogenizer in 1ml of sterile buffered salt solution (Breznak and Switzer, 1986).

2.2 Isolation and culturing of Actinobacterial cultures

Ten microlitres of the gut homogenates prepared from different termites (Tholen and Brune, 1999) were inoculated on Kenya Minimal Medium for aerobes (KMM1), which was based on MM5 medium (Boga *et al*., 2003), except that Napthoquinone, 4-hydroxyacetic acid, 3-indolyl acetic acid, aromatic fatty acids (AFAs) and Menadion were excluded. Media with additional substrate glucose and gelatin, each 0.1% w/v was

prepared and was solidified with agar (1.5% w/v). Cultures were incubated at 30^{o}C in the dark. All solutions, cultures and media were prepared and maintained under aerobic conditions and at pH 7.0.

Individual colonies which grew on the plates were re-inoculated on freshly prepared LB-Kanamycin agar (Appendix 1). Colonies were selected based on morphology and purified using the streak plating technique. The isolated pure Actinobacterial cultures were streaked on LB agar. After incubation, the cultures were maintained at 4^{o}C and sub-cultured after every three months (Demain and Davies, 1999).

2.3 Characterization of the isolates

Preliminary characterization was performed using morphological and cultural characteristics. Morphological identification of the isolates was done under the dissecting (×16) and phase contrast microscope to observe colony and growth characteristics. Further characterization was done through physiochemical, biochemical and molecular studies to support the finding of the morphological characterization.

2.3.1 Morphological characterization

Morphological characterization of the isolates was determined under a dissecting microscope (×16) and phase contrast microscope supplemented by the classical gram staining method (Cappuccino and Sherman, 2002).

2.3.2 Enzymatic and anti-microbial activity

2.3.2.1 Starch hydrolysis

Starch agar containing 1.5% agar and 0.2% soluble starch served as the polysaccharide substrate. The detection of hydrolytic activity following the growth period of seven days at 30^{o}C was made by examining for clearing zones around the colonies and by carrying out starch hydrolysis test. This was done by flooding the surface of agar with iodine. The presence of blue black color around the colonies indicated a negative test hence no starch hydrolyzed and absence of blue black color around the colonies and presence of a zone of clearing indicated a positive test (Cappuccino and Sherman, 2002).

2.3.2.2 Gelatin hydrolysis

The hydrolysis of gelatin was determined using the media of Frazier's gelatin agar, containing nutrient agar (pH 7.0) and bacteriological gelatin (4.0gl^{-1}). After incubation at 30^{o}C for 72 hours, the plates were covered with Frazier's revealers (Appendix 8). (Smibert and Krieg, 1981). The presence of a clear halo around the bacterial growth was observed and recorded.

2.3.2.3 Cellulose hydrolysis

Well developed colonies of the isolate were spot inoculated on agar supplemented with 1% powdered cellulose and incubation of cultures was done for 15 days at 30^{o}C (Smibert and Krieg, 1981). After incubation, the plates were flooded with Congo red for 2 minutes. The stain was poured off and the plates rinsed with 1M NaOH. Colonies were then examined using dissecting microscope for the presence of zones of clearing (Rheins *et al*., 1998).

2.3.2.4 Xylan hydrolysis

The ability of the isolates to degrade xylan was tested by inoculating agar containing 1% xylan as the only carbon source. After incubation for 14 days at 30^{o}C, the plates were flooded with Congo red for 2 minutes. The stain was poured off and the plates rinsed with 1M NaOH. Clearing zones around the growth was checked for (Rheins *et al*., 1998).

2.3.2.5 Skim milk proteolysis

Isolates ability to degrade casein was done using agar supplemented with 1% skim milk as sole carbon source. After incubation for three days at 30^{o}C, Organisms secreting proteases exhibited a zone of proteolysis demonstrated by clear zone surrounding the

Actinobacterial growth. In absence of protease activity the medium surrounding the growth of the organism remained opaque which was a negative reaction (Cappuccino and Sherman 2002).

2.3.2.6 Esterasic activity

The media used was as described by Sierra (1957), containing (g l^{-1}): peptone 10.0, NaCl 5.0, $CaCl_2.2H_2O$ 0.1, agar 18.0, pH 7.4. To the sterilized culture media, previously sterilized Tween 80 was added in a final concentration of 1% (v/v). This medium was inoculated with the isolates and incubated at 30^oC. The presence of halos was observed after seven days of incubation.

2.3.2.7 Lipolytic activity

The previously described methodology was used to determine the lipolytic activity (Sierra, 1957). In this determination, Tween 80 was substituted by Tween 20. The presence of clear halos around the colonies was observed after seven days of incubation at 30^oC.

2.3.2.8 Antibiotic screening assays

To test the ability of individual Actinobacterial isolates to inhibit the growth of test organisms [*Escherichia coli* (NCTC 10418) and *Staphylococcus aureus* (NCTC 10788)] an invitro plate assay was adapted. The plates were covered with test organisms. Actinobacterial cultures were spot inoculated on the surface using paper discs and incubated for 18 hours at 25^{o}C after which the bioassay results were recorded. Inhibition activity was evaluated visually under a dissecting microscope by scoring for inhibition of growth of test bacteria on the plates. Samples on plates where the growth of bacteria was observed were recorded as negative. The samples that inhibited bacterial growth were scored as positive (Fatope *et al.*, 2000).Positive control consisted of commercial Kanamycin antibiotic (10mg/ml) and negative consisted of un-inoculated plate.

2.3.3 Physiochemical and biochemical characterization

Physiochemical and biochemical characterization unless otherwise indicated were done according to Cappuccino and Sherman (2002).

2.3.3.1 pH requirements

LB broth was prepared in five batches whose pH was adjusted to 3, 6, 8, 10 and 12 using 1M HCl and 1M NaOH. The media was then dispensed in tubes in duplicates and autoclaved. Each batch was inoculated with the isolates. An uninoculated tube with

complete medium for each served as negative control. Observation was made after seven days, incubated at 30^{o}C. Growth was determined by optical densities using spectrophotometer at 660nm in cuvettes with a 1centimeter light path.

2.3.3.2 Growth at various temperatures

LB broth at pH 7.0 was prepared in five batches. The medium was then dispensed in tubes in duplicates and autoclaved. Each batch was inoculated with the isolates and incubated at temperatures 20, 30, 40, 50 and 60^{o}C. An uninoculated tube with medium for each served as negative control. Observation was made after seven days. Growth was determined by optical densities using spectrophotometer at 660nm in cuvettes with a 1centimeter light path.

2.3.3.3 Sodium Chloride tolerance

LB broth without NaCl was prepared in five batches that were supplemented with NaCl: 0, 20, 40, 70 and 100g/l. The medium was dispensed into tubes. Tubes were divided to five sections in duplicates, each being inoculated with isolates. An uninoculated tube with medium for each served as negative control. Observations were made after seven days. Growth was determined by optical densities using spectrophotometer at 660nm in cuvettes with a 1centimeter light path.

2.3.3.4 Utilization of sugars and similar compounds

The following compounds were used as sole carbon source; glucose, sucrose, xylose, mannitol, lactose, rhamnose, mannitose, galactose, mannose and maltose. 1% of these compounds supplemented to ISP medium 2 (Appendix 3). The medium was dispensed in tubes in duplicates, autoclaved and inoculated with the isolates. Growth was recorded after seven days in terms of optical densities. An uninoculated tube with complete medium for each sugar served as negative control (Williams *et al.*, 1989). Growth was determined by optical densities using spectrophotometer at 660nm in cuvettes with a 1centimeter light.

2.3.3.5 Citrate utilization

Simmons' Citrate agar slants were used to determine the capability of the isolates to use citrate as a carbon source for their energy. Bromothymol blue indicator incorporated in the media turned from green to prussian blue indicating positive tests.

2.3.3.6 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. Following inoculation and incubation, addition of sulfanilic acid and alpha-naphthylamine produced a cherry red coloration, which was indicative of positive results.

2.3.3.7 Methyl Red-Voges Proskauer (MR-VP) test

MR-VP test was used to determine the ability of the isolates to oxidize glucose with production and stabilization of high concentrations of acid end products. MR-VP broth was inoculated with each of the isolates and incubated at 27°C for 48 hours. Methyl red indicator or Barrit's reagent was added to aliquots of each culture. For positive culture, methyl red appeared red and in VP positive culture gave a rose coloration.

2.3.3.8 Indole production and Hydrogen sulfide production

Sulfur-Indole Mortility 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. Presence of indole was detected by addition of Kovac's reagent to 48-hour cultures of each isolates. 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.

2.3.3.9 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. A positive reaction was indicated by development of deep pink color.

2.3.4 Molecular characterization

2.3.4.1 DNA extraction

Pure cultures of the isolates were inoculated in 20ml of freshly prepared LB broth in universal bottles and incubated for six days in a shaker incubator at 35^{o}C and 200rpm. 1.5ml of the culture was added to eppendorf tube and centrifuged at 4000rpm for five minutes, the supernatant was discarded. 1ml of TE buffer was added and centrifuged again at 4000rpm for five minutes and the supernatant discarded.

The mycelial pellet was re-suspended in 200µl of solution 1 (50mM Tris pH 8.5, 50mM EDTA pH 8.0 and 25% sucrose solution). 5µl of lysozyme (20mg/ml) was added and contents gently mixed. 10µl of RNAse A (20mg/ml) was added and gently mixed then incubated at 37^{o}C for one hour. Following incubation, 600µl of solution 2 (10mM Tris pH 8.5, 5mM EDTA pH 8.0 and 1% SDS) was added and contents were mixed by inverting the eppendorf several times. 10µl of proteinase K (20mg/ml) was added and mixed gently followed by incubation at 50^{o}C for 30minutes. DNA was extracted by adding equal volumes of phenol: chloroform and centrifuged for five minutes at 13000rpm and carefully pipetted out the aqueous phase which contained the crude DNA. Proteins and lipids were removed by addition of 0.3 volumes of Diethyl ether and phases were mixed by inversion and centrifuged at 12000rpm for five minutes. The aqueous phase was transferred to a new tube and DNA was stabilized by addition of 0.1 volumes of 3M NaCl and precipitated with an equal volume of absolute ethanol. After the genomic DNA was centrifuged, the pellet was rinsed with 70% ethanol to

remove traces of salt. The DNA was air-dried for one hour to completely remove the ethanol then re-dissolved in 200µl of TE and stored at –20^{o}C (Magarvey *et al*., 2004). The DNA was semi quantified on a 1% agarose and visualized under ultraviolet by staining with ethidium bromide (Sambrook *et al*., 1989).

2.3.4.2 PCR amplification of 16S rRNA

Total DNA from each isolate was used as a template for amplification of the 16S rRNA genes. Nearly full-length 16S rRNA gene sequences were PCR-amplified using bacterial primer pair 8F forward 5'-AG (A/G) GTTTGATCCTGGCT-3') and 1492R reverse, 5'-CGGCTACCTTGTTACGACTT-3' (Sigma) according to the position in relation to *Escherichia coli* gene sequence (Embley and Stackebrandt, 1994; Lane, 1991). Amplification was performed using Peqlab primus 96 pcr machine. Amplification was carried out in a 41µl mixture containing 4µl of pcr buffer (×10), 3µl dNTP's (2mM), 0.6µl (5 pmol) of 8F forward primer, 0.6µl (5pmol) of 1492R reverse primer, 0.3µl taq polymerase, 1µl of template DNA and 30.6µl of water. The control contained all the above except the DNA template. Reaction mixtures were subjected to the following temperature cycling profiles repeated for 35 cycles: Initial activation of the enzyme at 94^{o}C for five minutes, denaturation at 94^{o}C for 45 seconds, primer annealing at 55^{o}C for 50 seconds, chain extension at 72^{o}C for 1.5 minutes and a final extension at 72^{o}C for eight minutes (Roux, 1995). Amplification products (5µl) were separated on a 1%

agarose gel in 1× TAE buffer (Appendix 4) and visualized under ultraviolet by staining with ethidium bromide (Sambrook *et al.*, 1989).

2.3.4.3 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 1 volume of the PCR sample and thoroughly mixed. The QIAquick spin column was placed in a 2ml collection tube; the sample was applied to the QIAquick column to bind the DNA, and then centrifuged for 60 seconds at 13000 rpm. The flow-through was discarded, and the QIAquick column placed back into the same tubes. To wash the DNA, 0.75ml 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 13000rpm to remove residual ethanol from buffer PE. The Qiaquick column was placed in a 1.5ml microcentrifuge tube and 30µl of buffer EB (10mM Tris-Cl, pH 8.5) added to elute DNA. The tubes were then centrifuged for one minute, the spin column removed and DNA stored at -20^{o}C for application (Sambrook *et al.*, 1989).

2.3.4.4 Phylogenetic data analysis

Sequencing of purified PCR products was done without cloning, using a commercial service provider. The CHROMAS-LITE program was used to check for the presence of

possible chimeric artifacts. 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 in order to determine similarity to sequences in the Gene bank 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 tree was constructed by Neighbour-Joining method (Saitou and Nei, 1987). Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

2.3.5 Data analysis

Data on pH, temperature, Sodium Chloride requirements and utilization of sugars was computed and analyzed using Statistical Package for Social Sciences (SPSS v.11).

CHAPTER 3.0

RESULTS

3.1 Isolation of Actinobacterial isolates

The inoculated plates incubated at 30^{o}C for two weeks results are shown in Figure 3a-c. KMM1 media (Figure 3a) yielded more diversity and large numbers of colonies compared to glucose based (Figure 3b) and gelatin based media (Figure 3c).

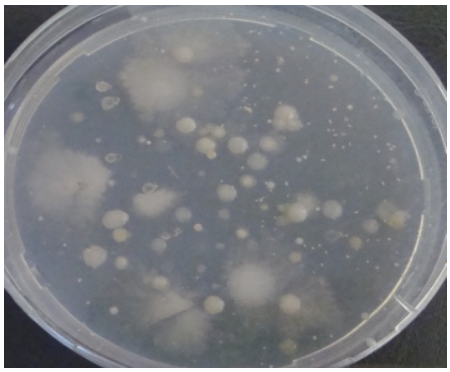

Figure 3a: A photograph showing a KMM1 medium culture plate with different colonies before isolation of individual colonies.

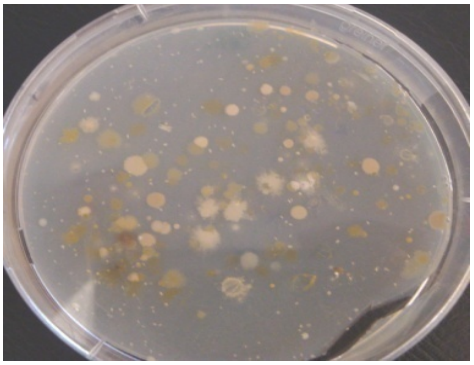

Figure 3b: A photograph showing a KMM+glucose medium culture plate with different colonies before isolation of individual colonies.

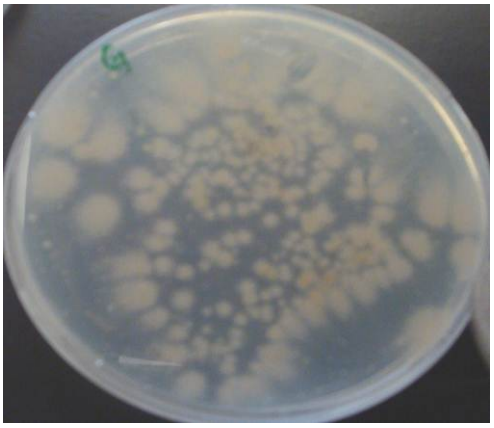

Figure 3c: A photograph showing a KMM+gelatin medium culture plate with different colonies before isolation of individual colonies**.**

Despite the many colonies on the initial plates, the isolates reduced due to the inability to grow after sub-culturing on LB-Kanamycin agar that contained antibiotic. Ten isolates were obtained from gut homogenates of *Macrotermes michaelseni* (Table 1).

Table 1: Summary of isolates obtained from intestinal tracts of M. michaelseni on different media

Medium	Code	Number of isolates
KMM1	KMM	5
KMM1+GLUCOSE	KMGC	3
KMM1+GELATIN	KMGT	2
Total		10

3.2 Morphological characterization of the isolates

All the isolates were gram positive. They grew well in both LB and ISP2 broth (Figure 4). They form granular or pellicular sediment so that the broth remained clear.

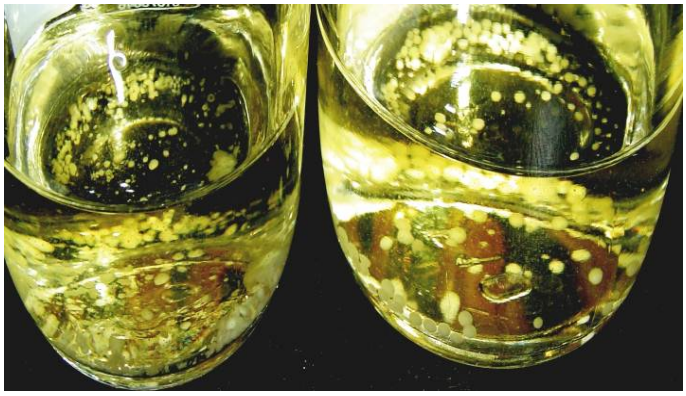

Figure 4: Growth of Actinobacteria in broth LB and ISP2 respectively**.** Formed granular sediments so that the broth remained clear. Isolates KMM3 and KMM4 were used.

Pure isolates grew well on both LB and ISP2 agar forming well-isolated colonies (Figures 5-14). The isolates formed tough, leathery colonies that were hard to pick from the culture media. They exhibited moderate to abundant growth. The colonies not only had characteristic morphologies but some were arranged in concentric rings. The isolates displayed a branching network of mycelia under phase contrast microscope that is characteristic of Actinobacteria (Table 2).

Morphology of cells under dissecting and phase contrast microscope

Table 2: Morphological characteristics of isolates obtained from termite gut, as observed under dissecting microscope (x16) and phase contrast microscope.

Isolate	Characteristics			
	Colony color	Growth form under dissecting microscope (×16)	Growth form under phase contrast microscope	Gram reaction
KMM1	Pale-brown	Irregular, tough colonies with smooth margins	Filamentous	Positive
KMM2	White	Abundant, tough, round colonies with smooth margins.	Long branching mycelia	Positive
KMM3	White	Tough, leathery, smooth round margins with wrinkled surface.	Branching network of mycelia	Positive
KMM4	White	Tough, leathery and smooth margins raised at the centre	Long branching mycelia	Positive
KMM5	White	Round, tough, wrinkled with raised surface	Long branching mycelia	Positive
KMGC6	White	Big, irregular colonies with smooth margins	Branched network of mycelia	Positive
KMGC7	Grey	Abundant, tough irregular colonies with fibrous margins	Filamentous	Positive
KMGC8	White	Big, tough leathery and concentric with smooth margins	Branching network of mycelia	Positive
KMGT9	Brown	Abundant, small, tough, leathery with scattered colonies	Branching network of mycelia	Positive
KMGT10	Dark-grey	Tough, leathery, round raised colonies with smooth margins.	Long branching mycelia	Positive

Morphology of cells under dissecting and phase contrast microscope

Figure 5a: KMM1 colonies under a dissecting microscope (×16). Irregular colonies with smooth margins.

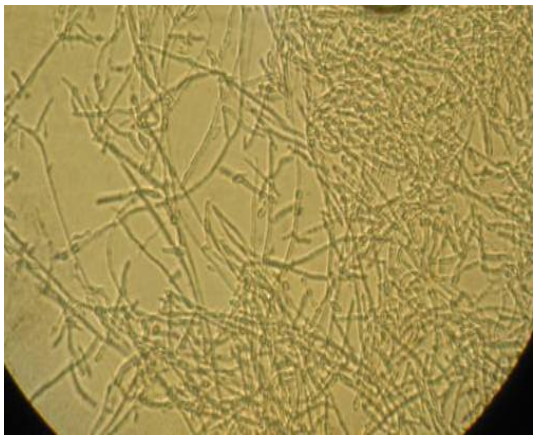

Figure 5b: KMM1 under phase contrast microscope (×20). Has got long branching mycelia.

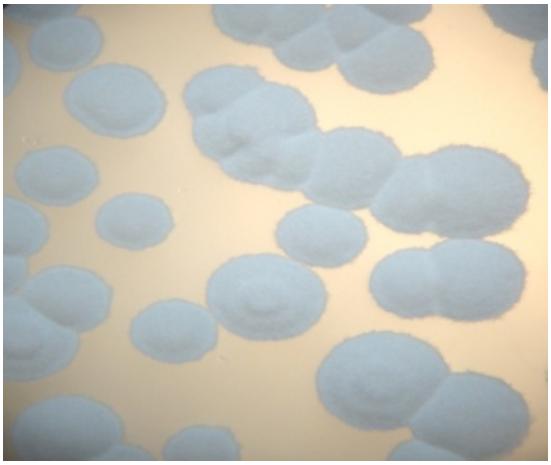

Figure 6a: KMM2 colonies under a dissecting microscope (×16). Abundant, round colonies with smooth margins.

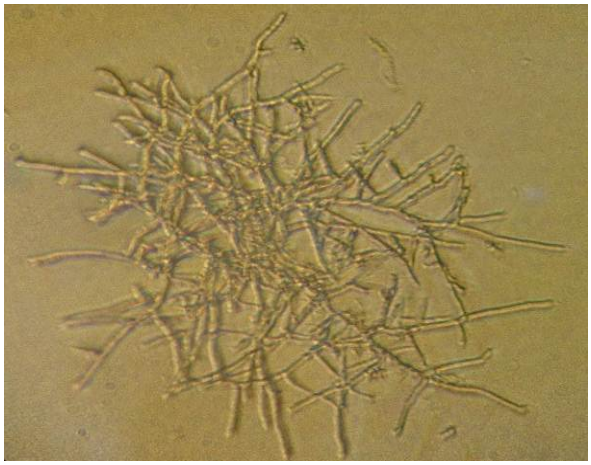

Figure 6b: KMM2 under phase contrast microscope (×40). Has got long branching mycelia.

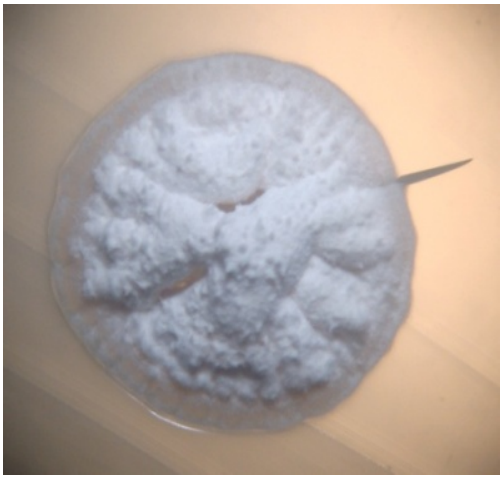

Figure 7a: KMM3 colonies under a dissecting microscope (×16)**.** Has got smooth round margins with wrinkled surface.

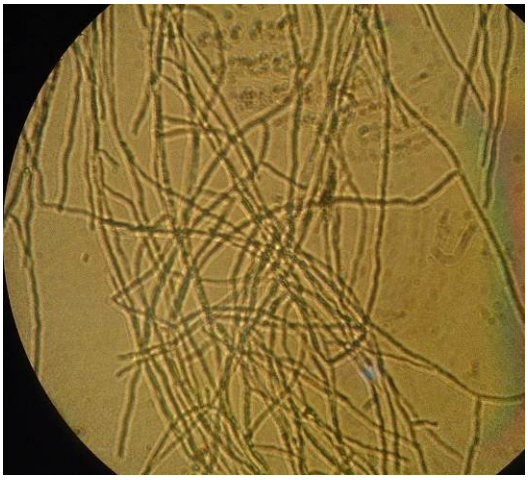

Figure 7b: KMM3 under face contrast microscope (×20). Has got long branching mycelia

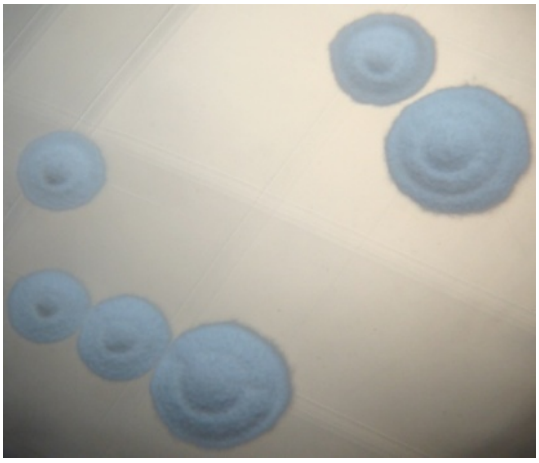

Figure 8a: KMM4 colonies under a dissecting microscope (×16)**.** Has smooth round margins and raised at the centre.

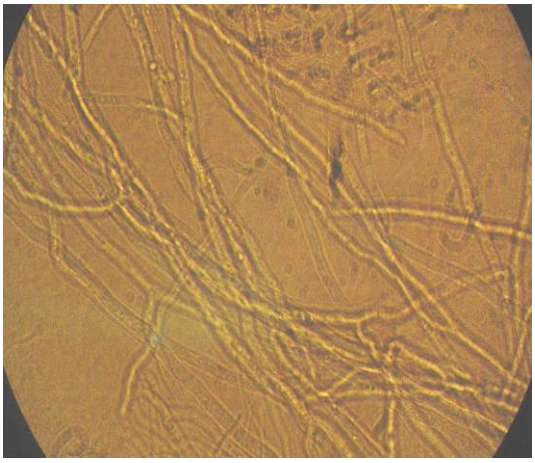

Figure 8b: KMM4 under phase contrast microscope (×20)**.** Has got long branching mycelia.

Figure 9a: KMM5 colonies under a dissecting microscope (×16)**.** Has smooth margins and wrinkled raised surface.

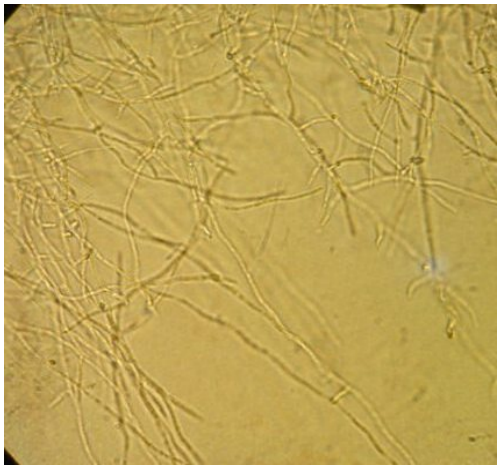

Figure 9b: KMM5 under phase contrast microscope (×20). Has got long branching mycelia.

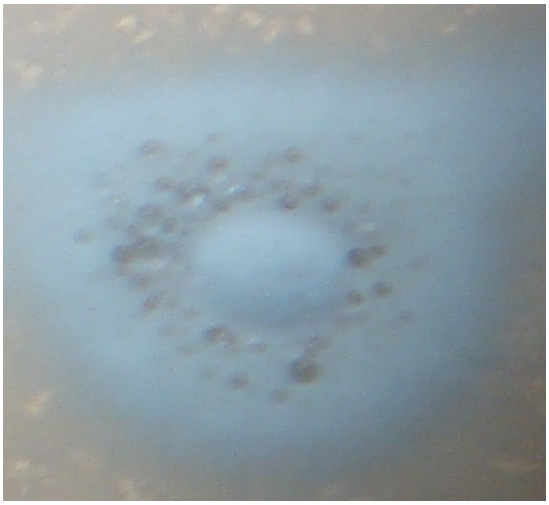

Figure 10a: KMGC6 colonies under dissecting microscope (×16). Exhibited irregular colonies.

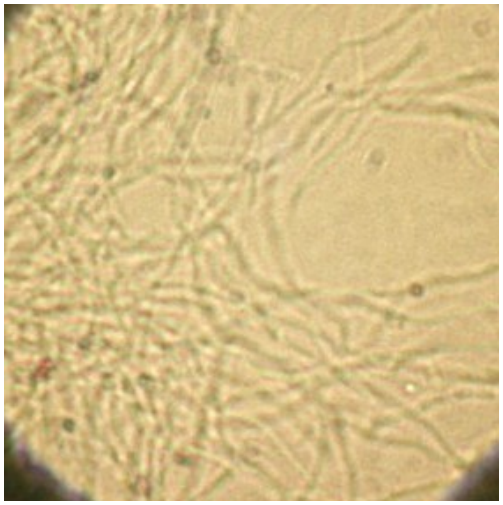

Figure10b: KMGC6 under phase contrast microscope (×20)**.** The cells are filamentous.

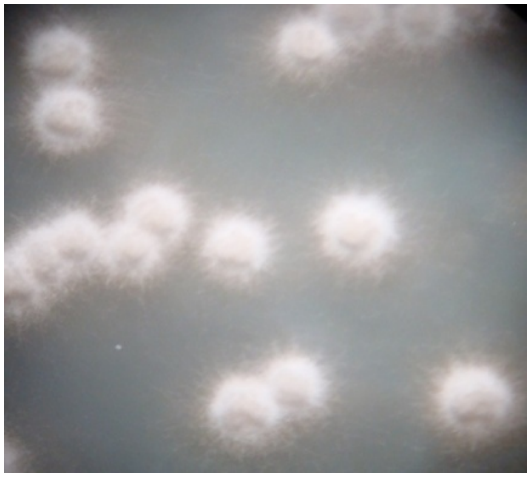

Figure 11a: KMGC7 colonies under a dissecting microscope (×16). Irregular colonies with fibrous margins.

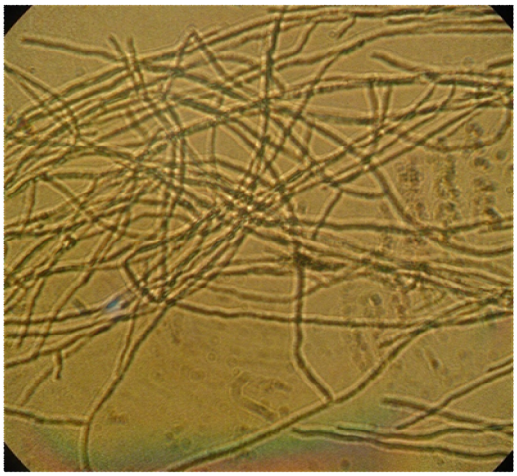

Figure 11b: KMGC7 under phase contrast microscope (×20)**.** Has got long branching mycelia.

Figure 12a: KMGC8 colony under a dissecting microscope (×16). Round, leathery with smooth margins.

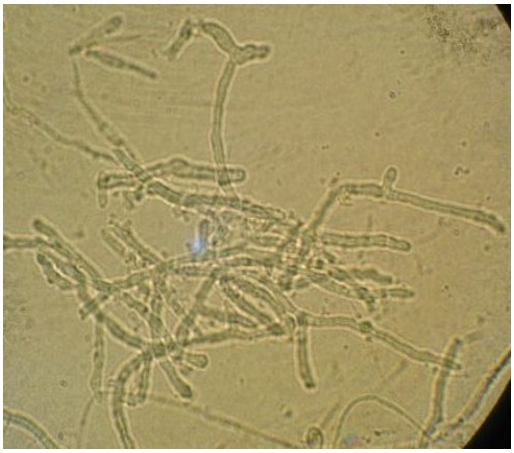

Figure 12b: KMGC8 under phase contrast microscope (×40)**.** Has got branched network of mycelia.

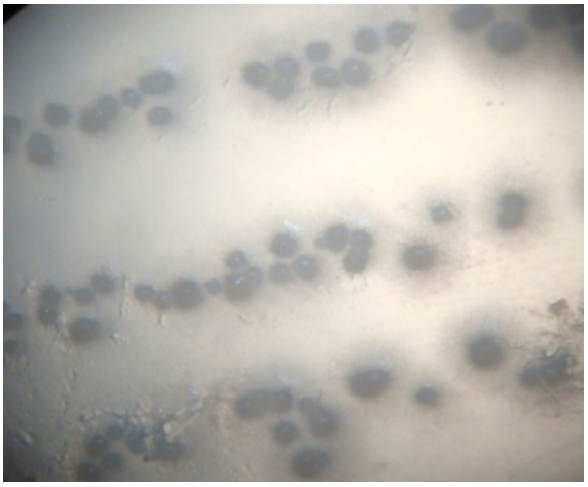

Figure 13a: KMGT9 colonies under a dissecting microscope (×16). Irregular scattered colonies

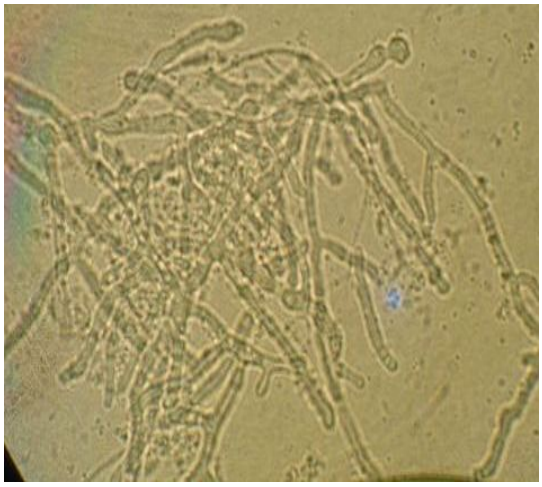

Figure 13b: KMGT9 under phase contrast microscope (×40)**.** Has got branching network of mycelia.

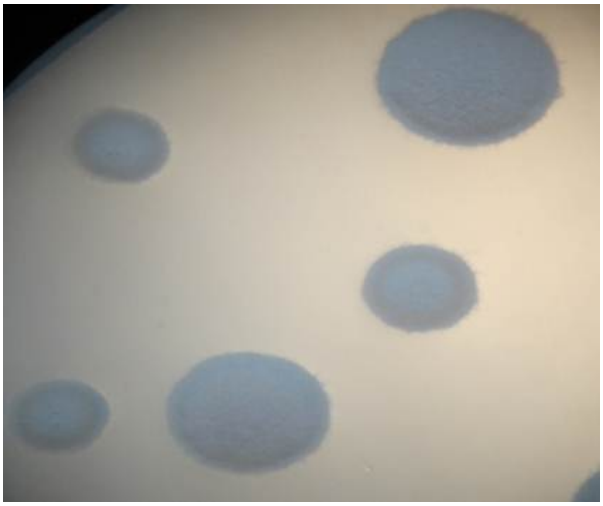

Figure 14a: KMGT10 colonies under a dissecting microscope (×16)**.** Round raised colonies with smooth margins.

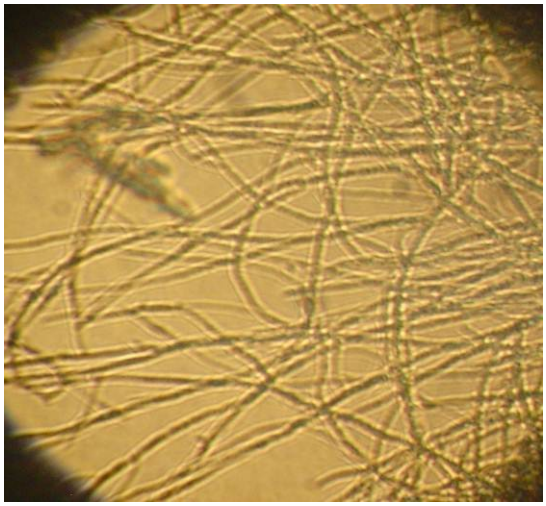

Figure 14b: KMGT10 under phase contrast microscope (×20)**.** Has got long branching mycelia.

3.3 Enzymatic activity of the isolates

All the isolates hydrolysed starch, gelatin, skim milk, esters and lipids. All the isolates apart from KMM1, KMGC6, KMGC8 and KMGT10 were able to degrade cellulose and xylan suggesting their role in the degradation of organic matter in the environment (Table 3 and Figure 15).

Table 3: Enzyme activity test results for ten isolates obtained from termite guts, on different substrates

	Enzymatic activity						
ISOLATE	Starch (amylase)	Gelatin (gelatinase)	Xylan (xylanase)	Cellulose (Cellulase)	Lipid (lipase)	Ester (esterase)	Skim milk (protease)
KMM1	+	+	-	-	+	+	+
KMM2	+	+	+	+	+	+	+
KMM3	+	+	+	+	+	+	+
KMM4	+	+	+	+	+	+	+
KMM5	+	+	+	+	+	+	+
KMGC6	+	-	-	-	+	+	+
KMGC7	+	+	+	+	+	+	+
KMGC8	+	-	-	-	+	+	+
KMGT9	+	+	+	+	+	+	+
KMGT10	+	+	-	-	+	+	+

(+) for a positive result for the reaction and (–) for a negative result for the reaction

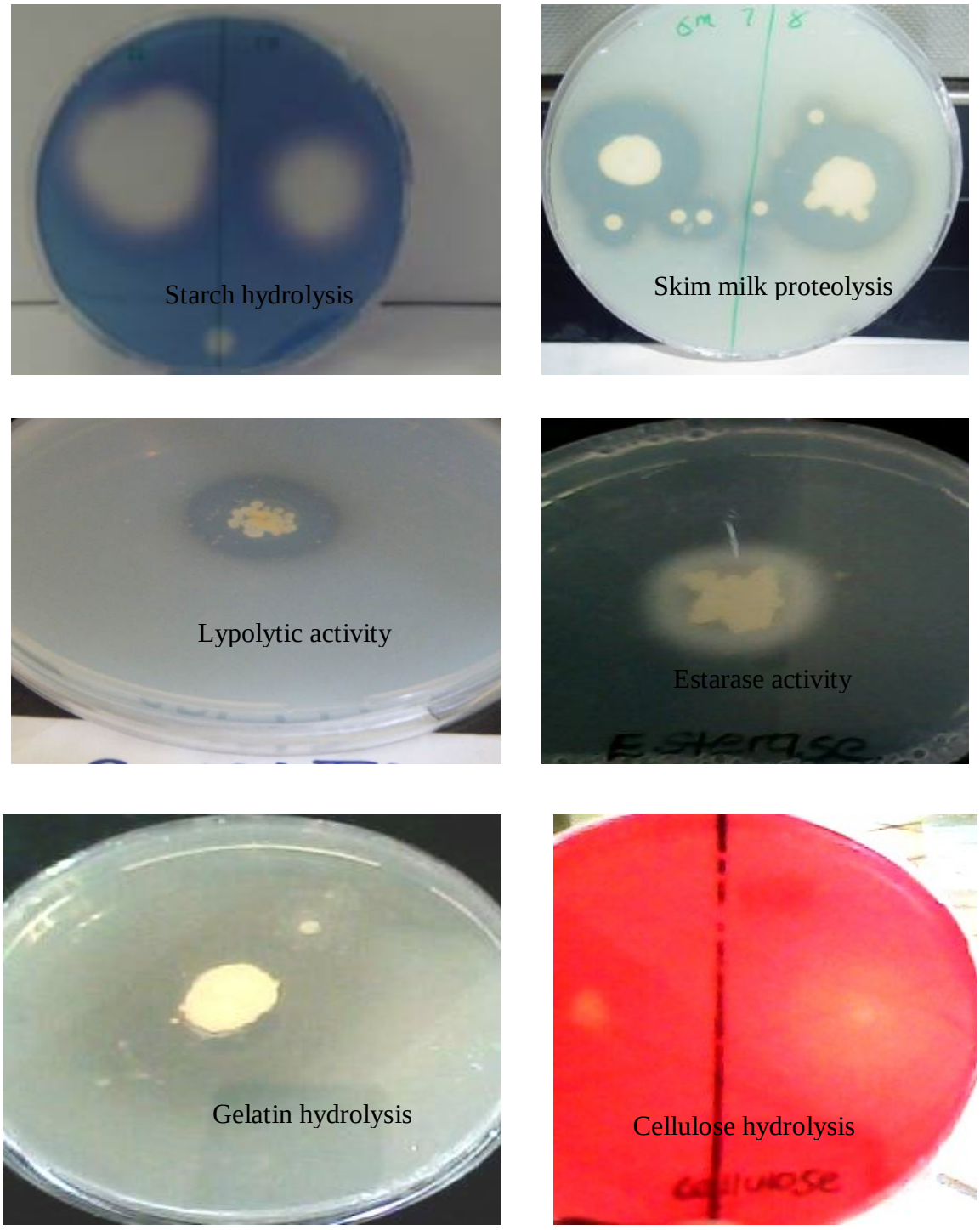

Figure 15: Enzyme activity test results as observed on plates

3.4 Antibiotic production by the isolates obtained from the termite guts

The isolates were tested for inhibition of growth on test organisms *E. coli* and *S. aureus* (Table 4). Isolates were found to suppress the growth of the two test bacteria. Isolate KMM5 and KMGT10 showed stronger inhibition on *S. aureus* while isolate KMM2 and KMGC8 showed stronger inhibition on *E. coli*. Isolates KMM2, KMM5, KMGC8 and KMGT10 showed strong inhibition on both test organisms hence broad-spectrum activity. This showed the potential of the isolates to produce antibiotics. The positive control consisted of commercial Kanamycin antibiotic (1mg/ml) while negative control consisted of un-inoculated plate.

Figure 16: Pictures showing inhibition activity on *E. coli*. positive control, negative control and sample KMM3 respectively. The antagonistic activity can be seen by the clear zone of inhibition around the paper disc.

Table 4: Inhibition of isolates obtained from termite guts, on Gram positive (*S. aureus*) and Gram negative (*E.coli*)**.** Zones of inhibition were measured in millimeters (mm).

Isolate	Disc assay (diameter in mm)			
	S. aureus (NCTC 10788)	*E. coli* (NCTC 10418)	*S. aureus* (NCTC 10788)	*E. coli* (NCTC 10418)
KMM1	+	+	7	7
KMM2	+	+	9	12
KMM3	+	+	7	8
KMM4	+	+	6	7
KMM5	+	+	11	9
KMGC6	+	+	7	7
KMGC7	+	+	9	7
KMGC8	+	+	10	11
KMGT9	+	+	8	9
KMGT10	+	+	11	9
+ve control	+	+	14	16
-ve control	-	-	0	0

(+) for a positive result for the reaction and (–) for a negative result for the reaction. Commercial Kanamycin antibiotic (1mg/ml) was used as positive control and un-inoculated plate as a negative control.

3.5 Physiochemical and biochemical characterization of the isolates

3.5.1 Growth at various pH

The isolates grew well at pH 6 (Table 5). There was generally very poor growth in highly acidic media (pH 3) and highly alkaline medium (pH 12).

Table 5: Growth of isolates obtained from termite guts, at different pH values. Readings were taken after incubation for seven days

	Optical Density (O.D) MEAN±SE				
Isolates	**pH 3**	**pH6**	**pH8**	**pH10**	**pH12**
KMM1	0.005±0.001	0.147±0.001	0.146±0.001	0.133±0.001	0.022±0.001
KMM2	0.003±0.001	0.051±0.001	0.052±0.001	0.031±0.001	0.027±0.002
KMM3	0.011±0.001	0.096±0.001	0.076±0.001	0.032±0.001	0.022±0.001
KMM4	0.001±0.002	0.145±0.001	0.0725±0.001	0.025±0.101	0.026±0.001
KMM5	0.022±0.001	0.087±0.001	0.052±0.001	0.033±0.001	0.025±0.001
KMGC6	0.024±0.003	0.108±0.001	0.097±0.001	0.017±0.003	0.004±0.001
KMGC7	0.002±0.001	0.064±0.004	0.115±0.002	0.034±0.001	0.014±0.011
KMGC8	0.014±0.003	0.009±0.002	0.072±0.001	0.010±0.001	0.038±0.004
KMGT9	0.003±0.001	0.153±0.005	0.139±0.001	0.077±0.001	0.008±0.004
KMGT10	0.005±0.002	0.008±0.001	0.013±0.002	0.017±0.001	0.005±0.001

3.6.2 Growth at various temperatures

The optimum growth temperature for the isolates was from 30 to 40^{o}C. There was poor or no growth at 20^{o}C and at 60^{o}C (Table 6).

Table 6: Growth of isolates obtained from termite guts, at different temperatures. Readings were taken after incubation for seven days

	Optical Density (O.D) MEAN±SE				
Isolates	**20º C**	**30ºC**	**40ºC**	**50ºC**	**60ºC**
KMM1	0.372±0.001	0.433±0.003	1.114±0.014	1.114±0.014	0.066±0.001
KMM2	0.276±0.002	0.394±0.066	0.849±0.028	0.849±0.028	0.013±0.001
KMM3	0.471±0.003	0.549±0.450	0.809±0.008	0.809±0.008	0.011±0.005
KMM4	0.034±0.004	0.392±0.008	0.432±0.002	0.432±0.002	0.030±0.001
KMM5	0.064±0.004	0.480±0.002	0.671±0.001	0.671±0.001	0.011±0.001
KMGC6	0.301±0.018	0.432±0.040	0.756±0.002	0.756±0.002	0.013±0.001
KMGC7	0.351±0.005	0.473±0.003	1.166±0.006	1.166±0.006	0.062±0.002
KMGC8	0.039±0.008	0.133±0.003	0.946±0.046	0.946±0.046	0.008±0.001
KMGT9	0.241±0.001	0.340±0.001	0.803±0.001	0.803±0.001	0.064±0.001
KMGT10	0.335±0.007	0.403±0.003	1.194±0.004	1.194±0.004	0.051±0.001

3.6.3 Sodium Chloride tolerance

The isolates grew well in medium without salt. Growth reduced with increasing Sodium Chloride concentration (Table 7).

Table 7: Growth of isolates obtained from termite guts, at different salt concentrations. Readings were taken after incubation for seven days

	Growth (O.D) MEAN±SE				
Isolate	**0g/l**	**20g/l**	**40g/l**	**70g/l**	**130g/l**
KMM1	0.211±0.001	0.158±0.002	0.042±0.001	0.037±0.001	0.027±0.002
KMM2	0.118±0.001	0.080±0.001	0.047±0.001	0.030±0.001	0.017±0.001
KMM3	0.219±0.001	0.125±0.001	0.069±0.002	0.013±0.001	0.002±0.001
KMM4	0.165±0.006	0.151±0.004	0.074±0.001	0.063±0.002	0.012±0.001
KMM5	0.101±0.004	0.089±0.001	0.036±0.002	0.025±0.001	0.013±0.006
KMGC6	0.086±0.007	0.087±0.002	0.075±0.002	0.073±0.001	0.027±0.001
KMGC7	0.010±0.001	0.074±0.003	0.010±0.002	0.038±0.001	0.032±0.001
KMGC8	0.156±0.001	0.127±0.001	0.106±0.001	0.025±0.001	0.002±0.009
KMGT9	0.166±0.003	0.157±0.001	0.143±0.001	0.078±0.002	0.033±0.001
KMGT10	0.145±0.001	0.033±0.002	0.023±0.001	0.003±0.001	0.002±0.001

3.6.4 Utilization of sugars and similar compounds by the isolates

Glucose, mannose and lactose were generally preferred by most isolates with glucose being highly utilized by all isolates. The least utilized sugars were mannitose, mannitol and galactose (Table 8). Readings were taken after incubation for seven days.

Table 8: Growth of isolates obtained from termite guts, in media with different sugars.

Isolate	Sugars									
	Sucrose	Xylose	Glucose	Galactose	Mannitol	Lactose	Fructose	Mannose	Mannitose	Maltose
KMM1	0.218±0.001	0.267±0.004	0.651±0.001	0.133±0.001	0.212±0.001	0.312±0.001	0.381±0.202	0.025±0.003	0.072±0.001	0.454±0.001
KMM2	0.131±0.001	0.175±0.007	0.625±0.001	0.091±0.001	0.024±0.001	0.315±0.001	0.008±0.001	0.148±0.001	0.107±0.002	0.093±0.001
KMM3	1.491±0.001	0.408±0.001	1.515±0.001	0.055±0.003	0.529±0.001	1.585±0.001	0.497±0.002	1.217±0.001	0.244±0.004	0.657±0.003
KMM4	0.083±0.001	0.012±0.001	0.132±0.002	0.062±0.001	0.115±0.004	0.044±0.004	0.034±0.001	0.094±0.005	0.036±0.002	0.103±0.001
KMM5	0.152±0.001	0.015±0.001	0.162±0.003	0.148±0.001	0.141±0.005	0.010±0.002	0.053±0.001	0.232±0.002	0.084±0.001	0.074±0.002
KMGC6	1.108±0.001	0.585±0.002	1.517±0.001	0.142±0.001	0.123±0.001	0.875±0.001	1.435±0.001	1.443±0.005	0.085±0.001	1.383±0.001
KMGC7	0.465±0.003	0.765±0.002	1.077±0.046	0.168±0.001	0.026±0.002	0.515±0.001	0.985±0.003	0.274±0.005	0.106±0.001	0.578±0.002
KMGC8	1.666±0.015	0.150±0.003	1.842±0.002	0.141±0.001	0.134±0.001	0.726±0.003	0.010±0.001	0.813±0.001	0.212±0.005	0.071±0.001
KMGT9	1.157±0.003	0.726±0.005	2.275±0.001	0.435±0.001	0.484±0.001	0.592±0.002	2.210±0.009	1.626±0.001	0.772±0.002	2.244±0.006
KMGT10	0.632±0.001	0.831±0.002	2.393±0.003	0.005±0.003	0.002±0.001	0.444±0.006	1.762±0.001	0.897±0.002	0.013±0.001	0.673±0.002

3.6.5 Biochemical tests

All the isolates apart from KMGC6 were positive for the citrate test which indicated there ability to use citrate as the sole carbon source for their energy. The isolates also showed a positive reaction for the urease test indicating there ability to attack nitrogen and carbon bonds in amide compounds. All the isolates were VP test negative indicating there inability to oxidize glucose with production and stabilization of high concentrations of acid end products (Table 9).

Table 9: Biochemical test results for the ten isolates obtained from termite guts

	Biochemical tests						
Isolate	**Citrate**	**Indole**	**Urease**	**MR**	**VP**	**Nitrate**	**H_2S**
KMM1	+	-	+	+	-	-	-
KMM2	+	-	+	+	-	+	-
KMM3	+	-	+	+	-	+	-
KMM4	+	-	+	-	-	+	-
KMM5	+	-	+	-	-	+	-
KMGC6	-	-	+	+	-	-	-
KMGC7	+	+	+	-	-	-	+
KMGC8	+	+	+	+	-	+	+
KMGT9	+	+	+	+	-	+	+
KMGT10	+	+	+	-	-	-	+

Biochemical test results for the 10 isolates defined as (+) a positive result for the reaction and (-) a negative test for the reaction.

3.7 PCR amplification of 16S rRNA genes from the isolates

Genomic DNA was successfully extracted from all the ten isolates. 16S rRNA gene amplification with bacterial based primers specific for this region of DNA yielded an amplification product of approximately 600 bp from all the ten samples (Figure 17).

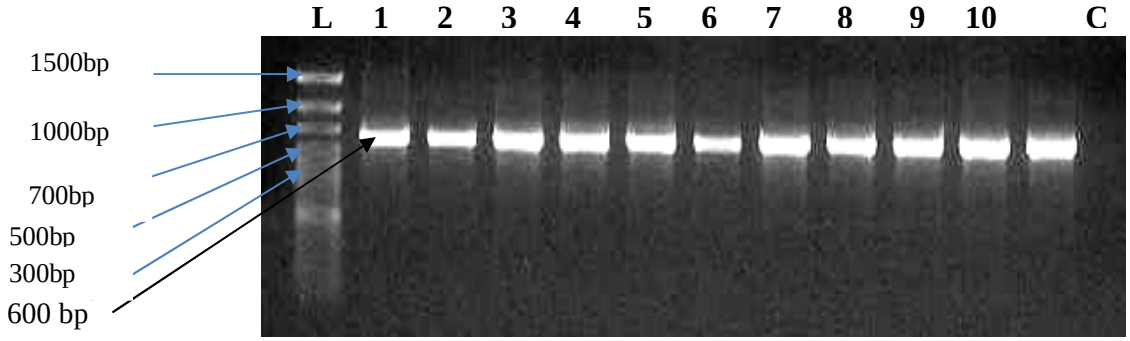

Figure 17: A 1% agarose gel photograph showing PCR amplification of 16S rRNA of the isolates visualized after ethidium bromide staining; L (DNA Ladder; 1.5Kb), 1 (KMM1), 2 (KMM2), 3 (KMM3), 4 (KMM4), 5 (KMM5), 6 (KMGC6), 7 (KMGC7), 8 (KMGC8), 9 (KMGT9), 10 (KMGT10) and C (Control).

The 16S rRNA amplified products from the ten isolates were sequenced. Similarity searching using BLAST showed that they belong to the domain Bacteria. The phylogenetic tree showed three main clusters; the first cluster comprised of genus *Streptomyces* and eight isolates; KMM2, KMM3, KMM4, KMM5, KMGC7, KMGC8, KMGT9 and KMGT10 clustered with this genus. Each isolate shared sequence identity of between 92-97% with known S*treptomyces* species (Table 10). On the phylogenetic tree, the eight isolates formed a cluster of their own with a high bootstrap value of 100% showing that the isolates are very closely related. They did not however cluster with any

known isolate in the database. The eight clustered separately with *Streptomyces* species with a bootstrap value of 98%. The other two clusters comprised of genus *Bacillus.* Two isolates KMM1 and KMGC6 clustered with this genus. Isolate KMM1 clustered with *Bacillus amyloliquefaciens* with a bootstrap value of 97% and a sequence identity of 97%, indicating that it is a close relative of this organism. Isolate KMGC6 clustered with *Bacillus pumilus* and this was supported with a bootstrap value of 96% and a sequence identity of 96% which showed the two are closely related.

Table 10: BLAST results for the ten isolates obtained from termite guts, showing the closest relative and the percentage identity.

Isolate	Closest relative	% identity
KMM1	*Bacillus amyloliquefaciens*	97
KMM2	*Streptomyces coelicolor*	96
KMM3	*Nakumurella multipartita*	92
KMM4	*Streptomyces albus*	96
KMM5	*Streptomyces ramulosus*	96
KMGC6	*Bacillus pumilus*	96
KMGC7	*Nocardia farcinica*	92
KMGC8	*Actinosynnema mirum*	92
KMGT9	*Streptomyces luteoverticillatus*	94
KMGT10	*Streptomyces mutabilis*	94

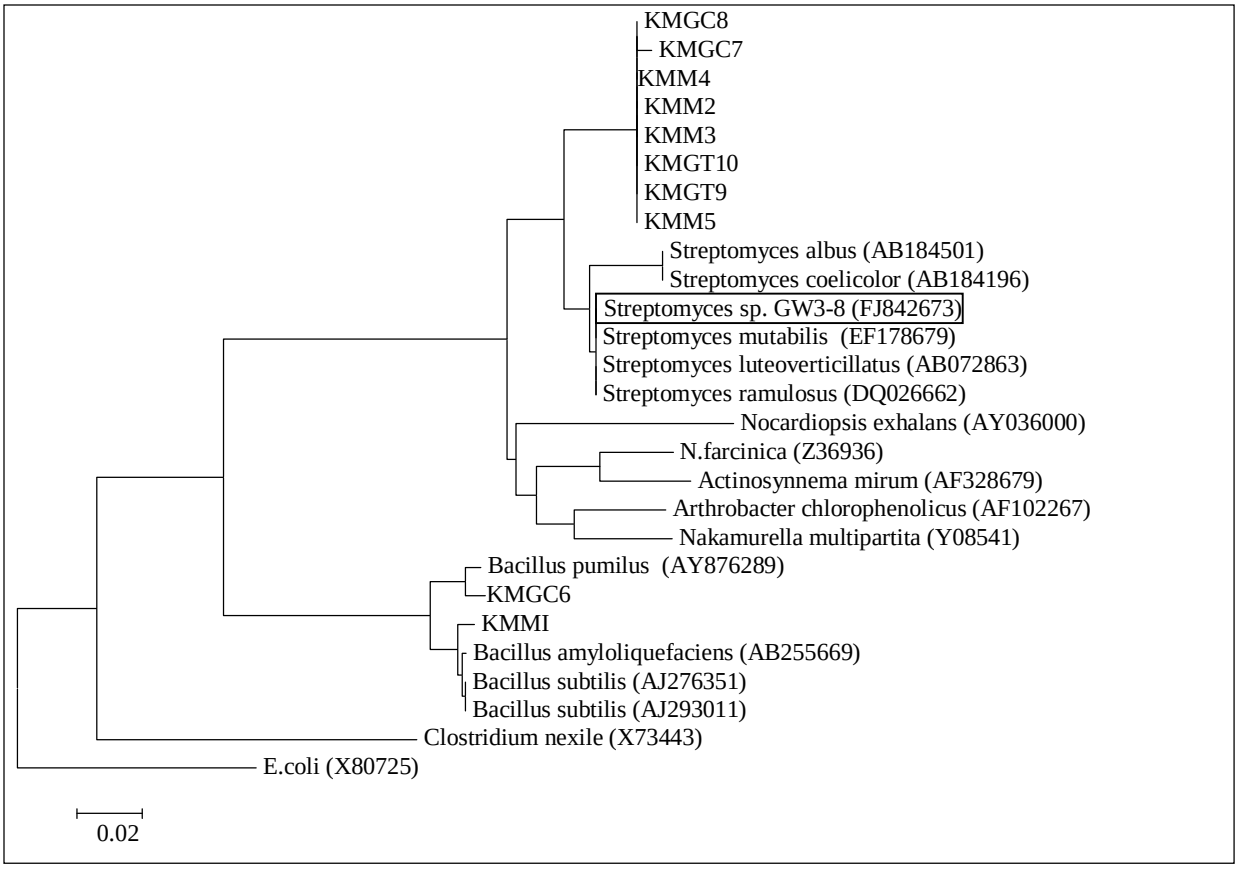

Figure 18: Phylogenetic positions of isolates obtained from termite guts.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

CHAPTER 4.0

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

4.1 Discussion

KMM1 medium was used to isolate Actinobacteria from termite guts. The medium had a variety of organic and inorganic compounds, organic nitrogen (casamino acids), a fermentable carbohydrate (glucose) and vitamins (7-vitamin solution) with other growth factors. This is in line with earlier studies by Schaal and Goldmann (1986) that showed good growth of Actinobacteria were obtained in or on a complex media containing either rich biological substrates or a defined and complex mixture of a large variety of organic and inorganic compounds. The organisms required organic nitrogen like peptides and amino acids and a fermentable carbohydrate and vitamins.

The termites were used within an hour of collection to avoid any physiological changes in the intestinal tract. Ten isolates were obtained. Kanamycin antibiotic was added in the culturing LB agar to inhibit selectively the accompanying flora of the natural habitat (Korn-Wendisch and Kutzner, 1992). This reduced the number of isolates that were obtained. Some did not grow after sub-culturing onto fresh media. The presence of Actinobacteria confirmed previous studies by Breznak and Brune (1994) that termites have got symbionts established in their hindgut, which consists of both eukaryotes (protozoa and fungi) and prokaryotes (bacteria and archea).

Colonies matured after 14 days of incubation at 30^{o}C. Preliminary characterization through morphological studies indicated that the isolates showed characteristics typical of *Streptomycetes*. Morphological studies under dissecting microscope showed that the isolates formed various growth characteristics on the culture media. They exhibited various texture and color of colonies. The isolates were characterized by their tough and leathery colonies that grew into the agar as earlier described by Mayfield *et al* (1972). The isolates showed profuse sporulation on solid media and had rapid growth in liquid culture. The isolates grew well in LB and ISP2 broth media forming pellicular sediments so that the broth remained clear. The isolates were gram positive which was in line with previous studies by Demain and Davies (1999) that Actinobacteria are gram-positive. Morphological studies under phase contrast microscope showed that the isolates grew as long, branching hypha which is a characteristic of Actinobacteria as earlier recorded by Kieser *et al* (2000). Cells showed various degrees of true branching and a more or less wavy appearance and were non-motile as earlier described by Slack (1974).

According to Williams *et al* (1983), the genus *Streptomyces* of the family *Streptomycetaceae* contains the largest number of species among the genera of *Actinomycetales* and is involved in a number of processes in their habitat. They are found worldwide and are important in soil ecology (Goodfellow *et al.*, 1987). Streptomycetes are metabolically diverse and can "eat" almost anything, including sugars, alcohols, amino acids, organic acids, and aromatic compounds. This is achieved by producing extracellular hydrolytic enzymes (Goodfellow *et al*., 1990).

In this study, the isolates were screened for their ability to hydrolyze various substrates. All the isolates apart from KMM1, KMGC6, KMGC8 and KMGT10 were found to hydrolyze starch, cellulose and xylan, a characteristic that confirm their role in the decomposition of organic matter such as dead plant tissues in their habitats (Kieser *et al*., 2000; Crawford, 1988). This confirms studies by Breznak and Brune (1994) that the microbial symbionts in termite gut play an important role in lignocellulose digestion and termite nutrition. Other enzymes produced by all the isolates included protease, gelatinase, lipase and estarase. These results confirm ealier studies by Kieser *et al* (2000) that Actinobacteria are used to obtain various enzymes that are commercially and academically valuable and that this vast reservoir of diverse products makes the *Streptomycetes* one of the most important industrial microbial genera (Kieser *et al*., 2000). Industrially useful enzymes with novel applications, or which improve upon the activities of ones being currently used, are frequently being sought (Marrs *et al*., 1999). A large number of industrial processes in the areas of environmental and food biotechnology utilize enzymes at some stage or the other and current developments in biotechnology are yielding new applications for enzymes hence there is need to discover new sources so as to meet their demand in industrial uses. Actinobacteria are therefore a probable source of these enzymes as indicated by the enzyme activity test results of this study. This may also be a relatively cheaper source of the enzymes as the Actinobacteria can be made to propagate rapidly and profusely.

Streptomycetes are also of medical and industrial importance because they synthesize antibiotics (Williams *et al*., 1983)**.** The isolates were tested for their *in vitro* activity on type culture collection of gram-positive (*S. aureus*) and gram-negative bacteria (*E. coli*) and were shown to have inhibitory effects. Isolate KMM5 and KMGT10 showed stronger inhibition on *S. aureus* while isolate KMM2 and KMGC8 showed stronger inhibition on *E. coli*. Isolates KMM2, KMM5, KMGC8 and KMGT10 showed strong inhibition on both test organisms hence broad-spectrum activity. This confirms earlier studies that Actinobacteria produce a wide variety of secondary metabolites, many of which have potent biological activities, used to produce more than half of the known biologically active microbial products including many commercially important antibiotics, immunosuppressive compounds, animal health products, and agrochemicals (Demain and Fang, 2000). Actinobacteria can therefore be used to produce antibiotics to counter the threats posed by the fast emerging phenomenon of antibiotic resistance (Shiburaj, 2003).

Physiochemical characterization of the isolates showed that the optimum growth temperature of the isolates was between 30-40^{o}C. Little growth was observed at temperatures 20^{o}C and 60^{o}C. The optimum pH for the growth of the isolates was at pH 6. The isolates grew well in the absence of Sodium Chloride and showed very poor growth in media with high salt concentration. Growth reduced with increasing salt concentration. This shows that the isolates could not tolerate Sodium Chloride. Glucose, mannose and lactose were preferred sugars by most isolates as carbon source. The least

utilized sugars were mannitose, mannitol and galactose. All the isolates showed high growth in medium with glucose, this confirms earlier studies that Actinomycetes multiplication is greatly stimulated by a fermentable carbohydrate (Cato *et al.*, 1984) and that glucose is the only sugar that is universally utilized by nearly all of the species currently included in the genus *Actinomyces* (Wood *et al*., 2003). Biochemical analysis enable to analyse the subsPhysiochemical characteristics are also important especially if the Actinobacteria were to be exploited for industrial and commercial processes.

Biochemical characterization of bacteria helps in analysis of the substances found in living organisms and the chemical reactions underlying life processes. It also has great potential to assist in further investigation and exploitation of the organisms (Teske *et al*, 1996).A series of biochemical test studies were performed on the isolates. All isolates had the ability to reduce nitrates to nitrites as depicted by the nitrate reduction test and also attack nitrogen and carbon bonds in amide compounds as indicated in the urease test. The isolates were also able to oxidize glucose with production and stabilization of high concentrations of acid end products and negative for the production of hydrogen sulfide and indole.

Molecular characterization and phylogenetic position analysis was carried out to help in genus designation of the isolates. Results from molecular characterization of the isolates indicated that all of them belonged to domain Bacteria. Eight of the isolates belonged to the Phylum Actinobacteria, class Actinobacteria, sub-class Actinobacteridae, order

Actinomycetales, sub-order Streptomycineae and family Streptomycetaceae while two were *Bacillus*. The phylogenetic tree showed three main clusters; the first cluster comprised of genus *Streptomyces* and eight isolates; KMM2, KMM3, KMM4, KMM5, KMGC7, KMGC8 and KMGT10 clustered with this genus. Each isolate shared sequence identity of between 92-96% with known S*treptomyces* species as shown by the BLAST results. On the phylogenetic tree, the eight isolates formed a cluster of their own with a high bootstrap value of 100% showing that the isolates are very closely related. They did not however cluster with any known isolate in the database. The eight clustered separately with *Streptomyces* species with a bootstrap value of 98%. The bootstrap value indicates that the eight isolates are very closely related and may belong to the same species. However, morphological studies showed a marked difference among the eight isolates which may indicate that they are different strains. This is supported by Schaal and Goldmann (1986) who recorded that cellular morphology varies from strain to strain within one species of Actinomycetes.16S rRNA sequence analysis is useful in determining evolutionary relationships among organisms at the genus level. It is not however very informative when applied to lower levels of taxonomic treatments such as species delineation (Turner, 2000).

Isolate KMM1 clustered with *Bacillus amyloliquefaciens* (Accession AB255669) with a bootstrap value of 97% and a sequence identity of 97%, indicating that it is a close relative of this organism. *Bacillus amyloliquefaciens* is a species of *Bacillus* that is the source of a natural antibiotic protein barnase, enzyme amylase used in starch hydrolysis,

the protease subtilisin used with detergents and the BamH1 restriction enzyme used in DNA research (Skerman *et al*., 1980). This indicates that isolate KMM1 can be used in the production of enzymes and antibiotics. Isolate KMGC6 clustered with *Bacillus pumilus* (Accession AY876289) and this was supported with a bootstrap value of 96% and a sequence identity of 96% which showed the two are closely related. *Bacillus pumilus is* known for its fungicidal activity by production of an antifungal compound (Bottone and Peluso, 2002) hence isolate KMGC6 may be used to produce fungicide. The study confirms the findings of other researchers that termite gut of *Macrotermes michaelseni* contains Actinobacteria (Bignell *et al.,* 1994; Pasti *et al.,* 1990; Watanabe *et al.,* 2003). The results also show that apart from Actinobacteria, the termite guts harbor other important bacteria.

4.2 Conclusion

The study has demonstrated that guts of fungus-cultivating termites *Macrotermes michaelseni* harbor Actinomycetes species among other bacteria. The study shows that Actinobacteria play role in the nutritional physiology of fungus cultivating termites as indicated by production of enzymes amylases, xylanases, cellulases, proteases, esterases, and lipases.

Physiochemical studies indicate that Actinomycetes grow well at a pH of 6 and temperature range of 30^{o}C to 40^{o}C which is consistent with the requirement of this class.

It also shows that Actinobacteria do not require salt for growth and preferred glucose as a source of carbon. The above conditions are therefore to be adopted if the isolates are to be exploited industrially.

Importantly, industrial potential of the Actinobacterial isolates was demonstrated through production of enzymes and anti-microbial secondary metabolites.

4.3 Recommendations

Culture independent sampling directly from the gut using PCR based approaches is recommended to asses the diversity of Actinobacteria in the intestinal tracts and accommodate microbes that could not be cultivated.

Further, it is necessary to enhance improved culturability of isolatable Actinobacteria as this would help to capture even those isolates that were obtained from the termite gut but were lost through subsequent subcultures. Such isolates may also posses beneficial secondary metabolites.

To get a clear insight into the mechanism of enzyme production, further studies should be done to characterize and identify the proteins or compounds responsible for the enzymes and antibiotic production. That information would be important in the biotechnological processes such as molecular cloning of the genes coding for such proteins.

Further studies should be done to isolates KMM2, KMM3, KMM4, KMM5, KMGC7 and KMGT9 for their commercial potential to produce cellulases that can be used in the production of biofuel.

Further work should also be carried out on isolates KMMI, KMM3, KMM4, KMM5, KMGC7, KMGC8, KMGT9 and KMGT10 to elucidate evolutionary relationship of the 8 isolates that clustered with *Streptomyces* by full sequencing and DNA-DNA hybridization.

Further work should be carried out to establish Actinobacterial diversity in relation to different termite feeding guilds, and biotechnological potential of the diversity of microorganisms isolated from these termites. This is important in understanding the overall diversity of biotechnologically important bacteria from such habitats.

To further expand our knowledge of microbial diversity in the intestinal tracts of termites, it is necessary to carry out further research to investigate the effect of variation of growth parameters such as temperature, pH and culture media composition for isolation of isolates to correspond with those of the natural habitats. Changes could result in differences in the abundance and diversity of isolates obtained as some isolates may grow under some conditions and not others.

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APPENDICES

APPENDIX 1 - LB-Kanamycin Agar (1 Litre)

10g Sodium Chloride

10g Tryptone

5g Yeast extract

15g agar

Add de-ionized water to a final volume of 1Litre

Adjust pH to 7.5 with NaOH and autoclave

Cool to 55^{o}C

Add 10ml of 10mg/ml filter sterilized Kanamycin and pour on plates

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APPENDIX 2 - LB Broth (1 Litre)

10g Sodium Chloride

10g Tryptone

5g Yeast extract

Add de-ionized water to a final volume of 1Litre

Adjust pH to 7.5 with NaOH and autoclave

APPENDIX 3 - International Streptomyces project (ISP) medium 2(1 Litre)

10g Yeast extract

4g Malt extract

4g Glucose

20g Agar

APPENDIX 4 - 1× TAE Buffer (50x)

24g Tris base

57.1ml Glacial acetic acid

100ml 0.5M EDTA (pH 8.0)

Dilute to 1× TAE working solution with dH_2O

APPENDIX 5- 1× TE

10mM Tris

1M EDTA

Adjust pH to 8.0 using HCl

APPENDIX 6 - BSS (1 Litre)

2.0g K_2HPO_4

1.0g KH_2O_4

1.5g KCl

1.5g NaCl

APPENDIX 7 - KMM1 (1 Litre)

1.7g NaCl

6.5g KCl

0.5g $MgCl_2.6H_2O$

0.10g $CaCl_2.2H_2O$

5.6g NH_4Cl

1.0g $NaSO_4$

1.0g KH_2PO_4

Cool the medium and add the following from sterile stock solutions

40ml; pH 7.01M Na-Phosphate buffer

2ml; SL 11

2ml; Se/W solution

2ml; 7-Vitamin solution

2ml; 50mg/L Folic acid

2ml; 50mg/l Riboflavin

2ml; 25mM Branched chain VFAs

2ml; 1mM Lipoic acid

APPENDIX 8 - Frazier's revealers

100ml Distilled H_2O

20ml.HCl

15.0g Mercury dichloride