ISOLATION AND CHARACTERIZATION OF ANTIBIOTIC PRODUCING THERMOPHILIC \textit{Bacillus} IN SELECTED HOT-SPRINGS ALONG LAKE BOGORIA, KENYA

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2015
DECLARATION

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

Signature……………………… Date…………………………

Tom Kintet Torome

I56/CTY/PT/24751/2011

We confirm that the work reported in this thesis was carried out by the student under our supervision.

Signature……………………… Date…………………………

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Kenyatta University

Signature……………………… Date…………………………

Dr. Francis Gakuya

Kenya Wildlife Service
DEDICATION

This work is dedicated to my family, my dear wife and my parents for their support and encouragement throughout the course of this study.
ACKNOWLEDGEMENT

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<tr>
<td>BECA</td>
<td>Biosciences Eastern and Central Africa</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>bp</td>
<td>Base Pairs</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic Acid</td>
</tr>
<tr>
<td>ILRI</td>
<td>International Livestock Research Institute</td>
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<tr>
<td>KOH</td>
<td>Potassium Hydroxide</td>
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<td>KWS</td>
<td>Kenya Wildlife Service</td>
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<tr>
<td>MUSCLE</td>
<td>Multiple Sequence Comparison by Log Expectation</td>
</tr>
<tr>
<td>NBA</td>
<td>Nutrient Broth Agar</td>
</tr>
<tr>
<td>NCLS</td>
<td>National Committee for Clinical Laboratory Standards</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>pH</td>
<td>Hydrogen Potential</td>
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<tr>
<td>rDNA</td>
<td>Ribosomal Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>RDP</td>
<td>Ribosomal Database Project</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal Ribonucleic Acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SIM</td>
<td>Sulphur Indole Motility</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
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Extreme habitats are increasingly being recognized as sources of secondary metabolites which provide an encouraging source for development of novel natural pharmaceuticals. Saline and hot water lakes are now becoming appreciated as rich and untapped reservoirs of extremophilic microorganisms with previously uncharacterized functions. Bacilli, a large homogeneous group of bacteria that survive in a wide range of environmental conditions, is one such microorganism. Formation of resistant spores allows it to survive in high temperatures zones where other organisms cannot. The objective of this study was to isolate, identify, and characterize Bacillus organisms with the potential to produce secondary metabolites with antibacterial properties from the hot springs of Lake Bogoria. Eighty samples were collected from surface and sediment waters of selected hot springs and inoculated directly into nutrient broth in universal bottles. Samples were initially incubated at 45°C for 48 hours and growth was examined by checking for turbidity. Out of the 80 bottles inoculated with sample, thirty three exhibited growth. Subsequent streaking on nutrient agar and Gram staining showed that nine of the isolated organisms were Gram-positive rods, twenty were Gram-negative rods, and four were Gram-positive cocci. Only Gram-positive rods were processed further. Biochemical characterization revealed that all nine processed isolates were catalase-positive and non produced gas upon sugar fermentation. They however had varying results for indole, motility, lactose fermentation, glucose fermentation and \( \text{H}_2\text{S} \) production. Antibacterial profiling was performed using ATCC organisms: Staphylococcus aureus ATCC 29213, Escherichia coli ATCC 25922, Escherichia coli 35218, and Pseudomonas aeruginosa ATCC 27853. Isolates D1, D5, D8, D22 and S15 inhibited growth of Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 which was shown by clear zones of inhibition around the discs. No inhibition was noted against S. aureus ATCC 29213 and E. coli 35218. Phylogenetic analysis of amplified 16S rDNA gene showed that D1, D5, D8, D22 and S15 formed close phylogenetic clusters with known members of Bacillus organisms with a (88-99\%) sequence identity. The current study shows the presence of thermophilic Bacillus species, which are producers of biomolecules with antibacterial properties within the hot springs of Lake Bogoria. Characterization of the antibacterial compounds produced will be useful for the discovery of novel antimicrobial substances that are effective against wide range of pathogens.
CHAPTER ONE

INTRODUCTION

1.1 Background information

The number of multi-resistant bacterial strains as etiological agents of infectious diseases has increased at an alarming rate, challenging physicians to find effective therapy that ensures effective result (Bansidhar et al., 2013). For example, in 2013, there were about 480 000 new cases of multidrug-resistant tuberculosis (MDR-TB). Extensively drug-resistant tuberculosis (XDR-TB) has been identified in 100 countries. MDR-TB requires treatment courses that are much longer and less effective than those for non-resistant TB. Treatment failures due to resistance to treatments of last resort for gonorrhoea (third-generation cephalosporins) have been reported from 10 countries. Gonorrhoea may soon become untreatable as no vaccines or new drugs are in development. There are high proportions of antibiotic resistance in bacteria that cause common infections (e.g. urinary tract infections, pneumonia, bloodstream infections) in all regions of the world. A high percentage of hospital-acquired infections are caused by highly resistant bacteria such as methicillin-resistant Staphylococcus aureus (MRSA) or multidrug-resistant Gram-negative bacteria (WHO fact sheet, 2015).

Beside the search for new antibiotics that microorganisms have not developed resistance against, the other major impetus driving extremophile research is the biotechnological potential associated with extremophiles. Indeed, the application of extremophiles in industrial processes has opened a new era in biotechnology. Until recently, majority of
antimicrobial compounds were isolated from terrestrial microorganisms. In the last two decades spanning between the 1980s and 1990s, the rate of discovery of novel compounds from this source has significantly declined, as exemplified by the fact that extracts from soil-derived actinomycetes have yielded high numbers of clinically unacceptable metabolites (Mincer et al., 2002). Saline and hot water lakes are now becoming increasingly appreciated as rich and untapped reservoirs of useful novel natural products. These environments contain taxonomically diverse bacterial groups which exhibit unique physiological and structural characteristics that enable them to survive in extreme environmental conditions, with the potential production of novel secondary metabolites not observed in terrestrial microorganisms. Discovery and identification of new sources of natural products, therefore, plays an important role in the uncovering of novel drug candidates and drug development process (Jorgensen et al., 2003).

Generally antibiotics are widely distributed in nature where they play an important role in regulating the microbial population of soil, water, sewage, and compost (Brock, 1986). Of the several hundred naturally produced antibiotics that have been purified, only a few have been sufficiently non-toxic to be of use in medical practice. Those that are currently of greatest use have been derived from a relatively small group of microorganisms belonging to the genera *Penicillium, Streptomyces, Cephalosporium, Micomonospora* and *Bacillus*; a few of which occupy some extreme environments (Brock, 1986).

It is commonly understood that only a fraction of microbial life (less than 10 %) is known and explored. The microbes from extreme habitats constitute an important component of
the hugely unexplored biotechnological potential. The limited studies on the diversity of the extreme organisms from varied habitats have indicated highly diversified population dynamics (Romano et al., 2005). Therefore, exploration of extreme habitats for organisms with novel properties would be of great significance in bringing into focus their significance as sources of bioactive molecules.

1.2 Statement of the problem

Bacilli have been known for a long time to be sources of antimicrobial compounds which form the backbone for treatment of infectious diseases. However, there is little information available on the presence of antibiotic producing bacilli in Lake Bogoria. This necessitates carrying out studies that will generate information on the availability of bacilli in the hot springs of the lake, and also determine their ability to produce secondary metabolites with antibacterial properties by carrying out antibacterial assays. This study is an attempt to identify Bacillus organisms able to produce secondary metabolites with antibacterial properties from selected geysers in Lake Bogoria.

1.3 Justification of the study

Bacillus have been known and used for decades in the production of antibiotics that have been and are still being used commercially. New knowledge is also coming up about thermophilic Bacillus organisms that have the potential to produce secondary metabolites with microbicidal and microbiostatic properties.
Virgin unexplored salty hot waters of Lake Bogoria offer a widely unexplored environment that could offer enormous potential from microorganisms if enough studies are carried out on them. There is therefore need to generate information on the group bacilli in what is regarded as extreme environments in order to determine their potential as ideal candidates for commercial antibiotic production especially in this era of development of antibiotic resistance by most pathogens.

1.4 Hypothesis

Lake Bogoria hot springs contain *Bacillus* organisms that have the potential to produce secondary metabolites with antibacterial properties.

1.5 Objectives of the study

1.5.1 General objective

To isolate, identify and characterize thermophilic *Bacillus* organisms with the ability to produce antibiotics from the hot water springs of Lake Bogoria.

1.5.2 Specific objectives

- To isolate and identify thermophilic *Bacillus* organisms from Lake Bogoria hot-springs using morphological and biochemical methods.
- To screen for antibacterial activity of secreted compounds from isolated thermophilic *Bacillus* species against selected bacteria.
- To characterize thermophilic *Bacillus* organisms that secret compounds with antibacterial properties from Lake Bogoria hot-springs using 16S rDNA genotyping.
CHAPTER TWO

LITERATURE REVIEW

2.1 Description of the genus Bacillus

*Bacillus* is a genus of Gram-positive, rod-shaped (*Bacillus*), bacteria and a member of the phylum Firmicutes. *Bacillus* species can be obligate aerobes or facultative anaerobes. They will test positive for the enzyme catalase when there has been oxygen used or present (Tumbull, 1996). Ubiquitous in nature, *Bacillus* includes both free-living (non-parasitic) and parasitic pathogenic species. Under stressful environmental conditions, the bacteria can produce oval endospores that are not true spores but which the bacteria can reduce themselves to and remain in a dormant state for very long periods. These characteristics originally defined the genus, but not all such species are closely related, and many have been moved to other genera of Firmicutes (Madigan, 2005). Bacilli exhibit a wide range of physiological capabilities that allow them to flourish in almost every environment. They compete favourably with other organisms mainly due to the ability to form spores that are heat stable, resistant to cold, radiation, and desiccation. Bacilli are also able to produce secondary metabolites that exhibit antagonistic effect on other microorganisms (Urdaci, 2004).

2.2 Bacteria identification

Several tools have over time been used in identification of bacteria. Classical microbiology uses both gross and microscopic morphology to identify microbes. Gross morphology includes colony shape, size, and surface features. One of the most intriguing
aspects of this approach is the observation of similar colony patterns in different systems and the existence of distinct patterns when culturing a sole strain in analogous conditions. The large number of reckonable patterns turns the identification of colony morphologies a real challenge for microbiologists and technicians (Ana et al., 2013).

The first credible approaches to the systematic classification of bacteria began in the latter part of the 19th century. These early studies separated groups of bacteria primarily on the basis of morphology, size, and motility. A pioneer investigator during this period was Ferdinand Cohn, who supported the concept of diversity of microorganisms and argued that, within species; varieties emerged and transmitted their characteristics to the next generation (Rosello et al., 2001). Over the first half of the 20th century, a number of approaches to the identification and classification of bacteria were identified. Among these were the use of physiologic tests to assess the diversity of bacteria and the first codification of biochemical test results (Rosello et al., 2001).

Thermophilic microorganisms have the adaptability to survive in high environmental conditions. Many researchers believe that such capability may be due to their molecular modifications at molecular and sub-molecular level. Many biochemical identification methods are based on the physiology of organisms and have been used reliably for many decades. The Gram stain is one of the most useful and commonly used microscopic tools to differentiate bacteria. This staining procedure named for its inventor, Hans Christian Gram, supplies both the biochemical information about the composition of bacteria and special information about the distribution of chemicals within the cell. Other tests that
determine the biological nature of organisms have been used (Rosello et al., 2001). Catalase test is a test that detects the activity of enzyme catalase, present in most cytochrome-containing aerobic bacteria. These microbes produce hydrogen peroxide during the aerobic breakdown of sugars. Catalase decomposes hydrogen peroxide to water and oxygen (Chelikani et al., 2004). It is a very important enzyme protecting the cell from oxidative damage by reactive oxygen species (ROS).

Indole is produced by bacteria as a degradation product of the amino acid tryptophan. The test to detect indole identifies isolates with the ability to produce the enzymes tryptophanase that removes the amino group from tryptophan to form Indole, pyruvic acid and ammonia; and cysteine desulfurase that produces pyruvate and ammonia. Ammonia produced in the breakdown of tryptophan raises the pH of the medium and changes the color from purple to yellow (Cappuccino and Sherman, 2002). Another identification media, Triple Sugar Iron Agar (TSI), differentiates microorganisms on the basis of dextrose, lactose, and sucrose fermentation and hydrogen sulphide production. Triple Sugar Iron Agar formula was originally described by Sulkin and Willett (1940). Enzymatic digest of casein, enzymatic digest of animal tissue, and yeast enriched peptone provide the nitrogen, carbon, and vitamins required for organism growth. Triple sugar iron agar contains three carbohydrates, dextrose, lactose and sucrose. When the carbohydrates are fermented, acid production is detected by the Phenol Red pH indicator. Sodium thiosulfate is reduced to hydrogen sulfide, and hydrogen sulfide reacts with an iron salt yielding the typical black iron sulphide (Cappuccino and Sherman, 2002).
Conventionally, Bacilli have been identified in the laboratory through biochemical tests and fatty acid methyl ester profiling (Bobbie and White, 1980; Vaerewijck et al., 2001). These are technically complex and labour intensive procedures and the scarcity of reproducible and distinguishable phenotypic characteristics for several bacterial species often makes precise identifications difficult (Khamis et al., 2003). To date, the development of gene amplification and sequencing, especially that of the 16S rDNA gene, has simplified the identification and the detection of specific bacteria (Woese, 1987; Yamada et al., 1997; Kolbert and Persing, 1999; Shaver et al., 2002; Wang et al., 2003; Wu et al., 2006), especially those lacking distinguishable phenotypic characteristics. The 16S rDNA gene is the most conserved DNA in all cells. Portions of the 16S rDNA sequence from distantly related organisms are remarkably similar. This means that sequences from distantly related organisms can be precisely aligned, making the true difference easy to measure. Comparison of 16S rDNA sequences thus shows evolutionary relatedness among microorganisms.

The 16S rDNA gene sequence is about 1,500 bp long and is composed of both variable and conserved regions. The gene is large enough, with sufficient interspecific polymorphisms of 16S rDNA gene, to provide distinguishing and statistically valid measurements. Universal primers are usually chosen as complementary to the conserved regions at the beginning of the gene and at either the 540-bp region or at the end of the whole sequence (about the 1,500-bp region), and the sequence of the variable region in between is used for the comparative taxonomy (Chen et al., 1989). Although 500 and
1,500 bp are common lengths to sequence and compare, sequences in databases can be of various lengths.

_Bacillus_ has for a long time been regarded as a phylogenetic heterogenous group (Ash _et al._, 1991). Since 1990, 16S rDNA has been successfully applied in determining phylogenetic relationships of the aerobic, endospore forming bacteria which played an important role in the creation of several families and genera Bacillales (Garrity _et al._, 2007). To date 16S rDNA forms a vital standard of taxonomy not only for _Bacilli_ organisms but bacteria in general. Goto _et al._ (2000) used partial 16S rDNA sequence for rapid identification of _Bacillus_ organisms. Then Xu and Cote (2003) used 3’ end 16S rDNA and 5’ end 16S-23S ITS nucleotide sequences to infer phylogenetic relationships among _Bacillus_ species and related genera.

### 2.3 Resistance to antibiotics

Antibiotics have been recognized as the most significant means of effective microbial growth control (Bertrand, 2008) after the discovery of penicillin and other antimicrobial agents by Alexander Fleming in 1928 (Fleming, 1929). Since that time up to now, there has been ongoing search for more effective antibiotics that can withstand the emergence of drug resistance among microorganisms (Song, 2008). In Sub Saharan Africa for example, resistance to most available antibiotics has resulted in morbidity and mortality from treatment failures and the ever increasing costs of antibiotics (Bertrand, 2008). The increase in antibiotics resistance has been mainly due to inappropriate use and substandard medicines from rogue manufacturers (Blomberg, 2004), which leads to the
steady decline of effective antibiotics (Byarugaba, 2004). This situation has become a serious challenge to drug manufacturers and public health practitioners worldwide.

On the other hand, antibiotics and antibiotic biosynthetic pathways are believed to have evolved over millions of years suggesting that antibiotic resistance is an equally ancient phenomenon (Hall et al., 2007; Balz, 2008; Knapp et al., 2010). Indeed, it has recently been shown that antibiotic resistance elements were abundant and diverse in ancient DNA dating from the Pleistocene (30,000 years ago) (D’Costa 2011). Which raises the question, whether the extensive presence of resistance elements in microbes is primarily the result of human activity (Kirandeep et al., 2012). The development of antibiotic resistance tends to be related to the degree of simplicity of the DNA present in the microorganism becoming resistant and to the ease with which it can acquire DNA from other microorganisms. For antibiotic resistance to develop, it is necessary that two key elements combine: the presence of an antibiotic capable of inhibiting the majority of bacteria present in a colony and a heterogeneous colony of bacteria where at least one of these bacterium carries the genetic determinant capable of expressing resistance to the antibiotic (Levi and Marshall, 2004).

Once this happens, susceptible bacteria in the colony will die whereas the resistant strains will survive. These surviving bacteria possess the genetic determinants that codify the type and intensity of resistance to be expressed by the bacterial cell. Selection of these bacteria results in the selection of these genes that can now spread and propagate to other bacteria (Levi and Marshall, 2004). Resistance to antibiotics can be natural (intrinsic) or
acquired and can be transmitted horizontally or vertically. Whereas the natural form of antibiotic resistance is caused by a spontaneous gene mutation in the lack of selective pressure due to the presence of antibiotics and is far much less common than the acquired one, it can also play a role in the development of resistance.

For the most part, however, the micro-ecological pressure exerted by the presence of an antibiotic is a potent stimulus to elicit a bacterial adaptation response and is the most common cause of bacterial resistance to antibiotics (Sefton, 2002). Susceptible bacteria can acquire resistance to antimicrobial agents by either genetic mutation or by accepting antimicrobial resistance genes from other bacteria. The genes that codify this resistance (the “resistant genes”) are normally located in specialized fragments of DNA known as transposons (sections of DNA containing “sticky endings”), which allow the resistance genes to easily move from one plasmid to another (Sefton, 2002).

2.4 Screening for new antibiotics

Antibiotics, in one form or another, have been in use for many years. The vast majority of novel antibiotics have been detected by screening of wild isolates obtained from natural habitats. Many of these wild habitats vary widely and range from extremely cold to extremely hot, highly alkaline or acidic and some which are extremely salty. As mentioned previously the vast majority of antibiotics have come from non-extreme terrestrial microorganisms. However, while these microorganisms continue to be studied extensively, the rate of discovery of novel metabolites from them is decreasing (Bentley et al., 2002).
Genomic studies indicate that the genetic potential for producing secondary metabolites is not uniformly distributed within the bacterial world. In fact, most bacterial genomes lack any detectable gene cluster for secondary metabolism (Bently et al., 2002). It is interesting to point out that genomic data parallel the observed discovery events and indicate that streptomycetes, pseudomonads and bacilli have yielded large numbers of metabolites since they can be easily isolated and have a relatively large genetic potential for producing secondary metabolites. It is thus likely that most microbial strains that could be isolated in large numbers have actually been screened for bioactivities (Bull et al., 2000).

The main approach in which new antibiotics have been discovered has been by screening groups of microorganisms such as *Bacillus*, *Penicillium*, *Streptomyces* and other microorganisms (Demain et al., 2000; Oluoch et al., 2010). The classical method for testing for potential antibiotic producing microbial isolates is the cross-streak method used by Flemming in his studies on penicillin. Currently, standard reference strains such as the America Type Culture Collection (ATCC) organisms are used to standardize screening tests. Isolates that demonstrate evidence of antibiotic production are then subjected to further studies to determine if the antibiotic they produce is new. When an organism producing a new antibiotic is discovered, it is produced in large quantities, purified and tested for cytotoxicity and therapeutic activity in infected animals (Tolaro, 2006). Most of new antibiotics will fail the *in vivo* testing, but a few of the new antibiotics that prove medically useful are then produced commercially (Yarbohrough et al., 2009). Since antibiotic producing microorganisms from nature rarely produce the
desired antibiotic at sufficient quantities and concentrations, it is necessary to isolate new high yielding strains. A lot of research is carried out on these newly identified antibiotic producers to come up with the best fermentation protocol that will maximize antibiotic production in an economical way (Demain et al., 2000).

To date, marketed antibiotics such as streptomycin have been derived from bacteria that grow on artificial solid or liquid media. However, most species of bacteria will not grow on artificial media (Diaz-Torres et al., 2006). Marketed antibiotics have not been isolated from non-culturable bacteria, since growth on solid media has been an essential step to the development antibiotics. Now, it is possible to clone large fragments of non-culturable bacterial genomes and to express them using recombinant DNA technology. DNA is extracted from a mixture of bacteria from, for example, soil, and is inserted into a vector, such as a bacterial artificial chromosome (Rondon et al., 2000) that can accept large DNA fragments. Open-reading frames within the fragment are then expressed in a culturable cell such as Streptomyces spp and are screened for antibiotic activity. Possible problems with this approach are that the productive DNA fragments occur too infrequently to be detected by cloning. Also, DNA fragments may not contain all the genes that are required for the production of the antibiotic, and the host organism may not express the genes in the DNA fragment correctly. Culture of previously uncultivated microbes is notoriously difficult, and though success may be limited, the prospects are promising. No compounds that have been produced in this way are yet in clinical trials (Coates et al., 2009).
2.5 Antibiotic production by *Bacillus*

Production of antimicrobial compounds is a general phenomenon for most bacteria. *Bacillus* species are among the most common organisms used in the commercial production of antibiotics. The prevalence of antimicrobial resistance among key microbial pathogens is increasing at an alarming rate worldwide. Current solutions involve development of a more rational approach to antibiotic use and discovering of new antimicrobials. Thermophilic *Bacillus* species produce a large number of biological compounds active against bacteria, fungi, protozoa and viruses. Most of the peptide antibiotics produced by *Bacilli* are active against gram-positive bacteria; however, compounds such as Polymyxin, Colistin, and Circulin exhibit activity almost exclusively upon gram-negative bacteria, whereas as Bacillomycin, Mycobacillin, and Fungistatin are effective against molds and yeasts (Katz and Demain, 1997).

In spite of the great attention to microorganisms living under extreme environmental conditions, including thermophiles and numerous studies of their physiology, genetics, and biochemistry (Sonnleittner et al., 2005), the secondary metabolism of these microorganisms is poorly understood. It has been proposed that secondary metabolism will have evolved so that traits that optimize the production and retention of chemical diversity at minimum cost will have been selected (Firn and Jones 2000). Antimicrobial secondary metabolites occur in some well-studied species of Thermophilic *Actinomycetes*, but little is known of thermophilic *Bacillus* species producing antibiotic substances, especially in the hot Lakes of the East African Rift Valley (Roy et al., 2002). The very potent antimicrobial activities of *Bacillus* lantibiotics against pathogenic
microorganisms such as *B. cereus*, *Listeria monocytogenes* and *Staphylococcus* spp. make them good candidates for application in the food and medical industry.

Antibiotics, as secondary metabolites, are generally produced by multi-step biosynthetic pathways starting from intermediates of primary metabolism to specific moieties with different functional groups. Biosynthetic steps are catalyzed, by specific enzymes for each antibiotic (Demain *et al.*, 2000). Selective toxicity is possible because of occasional differences in the biochemistry of the host and target microbial cell. For example, bacitracin interferes with the dephosphorylation of the C55-isoprenyl pyrophosphate, a molecule that carries the building-blocks of the peptidoglycan bacterial cell wall outside of the inner membrane. Antimicrobial agents may have a low or high selective toxicity (Belkeva *et al.*, 2007).

### 2.6 Secondary metabolites produced by *Bacillus* organisms

Secondary metabolites are produced by bacteria and serve survival functions for the organism producing them. They act as competitive weapons against other members of the community, as metal transporting agents, as agents of symbiosis between microbes and plants, nematodes, insects and higher animals and also as regulators of cellular differentiation (Demain *et al.*, 2000). Also their production appears to be a response to various ecological pressures within the environment. The search for active secondary metabolites produced by environmental isolates, using poorly explored microorganisms, could provide a new source of discovering novel bioactive compounds.
More than 500 antimicrobial compounds have been described so far. *Bacillus* genus is known to produce more than 45 antimicrobial molecules some of which are of clinical significance (Stein, 2005). These metabolites can be grouped into three categories according to the synthetic pathway:

- ribosomally synthesized peptides also known as bacteriocin;
- small microbial peptides synthesized enzymatically by non-ribosomal pathways.
- non-peptide based antibiotics.

### 2.7.1 Bacteriocins and bacteriocin-like inhibitory substances

Bacteriocins usually show a high degree of specificity against target related bacteria, although some have a wide spectrum of activity (Nissen-Meyer *et al.*, 2007). Based on Klaenhammer’s classification (Klaenhammer, 1993), several antimicrobial substances produced by *Bacillus* organisms are grouped into bacteriocin Class I (also known as lantibiotics), within which antibiotics are also classified into sub-groups A and B based on their general structure, molecular weight and biological activity.

Type A lantibiotic exhibits a more linear secondary structure and kills Gram-positive target cells by forming voltage-dependent pores into the cytoplasmic membrane. This group includes subtilin produced by *B. subtilis* ATCC 6633 which inhibits a broad range of Gram-positive bacteria including other species of *Bacillus*. Type B lantibiotics includes globular and uncharged antibiotics (Jung *et al.*, 2001).
Recently, Parisot et al. (2008) reported that subtilin shows a more complex mechanism of action, involving the binding to a specific target or “docking molecules”, the membrane-anchored cell wall precursor lipid II also targeted by vancomycin. The interaction with the lipid II "stabilizes" the formation of pores leading to antimicrobial effects at very low concentrations of bacteriocins.

Type B mersacidin lantibiotic, produced by *Bacillus* organisms exhibits a more globular structure due to the formation of four intermolecular thioether bridges (Chatterjee et al., 2002). Mersacidin exerts its antibacterial activity by the inhibition of cell wall biosynthesis; this compound forms a complex with the peptidoglycan precursor lipid II. Several works showed that this peptide successfully inhibited *in vitro* and *in vivo* the growth of Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* strains (Kruszewska et al., 2004) as well as enterococci expressing the vancomycin resistance phenotype. Mesarcidin like subtilin is produced in stationary phase under the regulation of the sporulation transcription factor SigH (Schmitz et al., 2006).

Class II bacteriocins include small ribosomally synthesised peptides (> 5kDa) that do not display any modification in their amino acids except for, in some cases, the presence of disulfide bridges. Class II bacteriocin can be further subdivided into three subclasses (IIa, IIb, IIc) based on structural properties, activity and mode of action (Nes et al., 1996). Class IIa bacteriocins are characterized by hydrophobic N-termini containing the YGNGV consensus sequence and disulfide bridges. Class IIb feature two-component
non-modified bacteriocin whereas class IIc regroups all the other molecules that do not correspond to class IIa and IIb (Nes et al., 1996).

2.7.2 Non-ribosomal biosynthesized peptides

*Bacillus* species produce several non-ribosomal compounds through a multi-step mechanism including the selection and condensation of amino acid residues such as cyclic lipopeptides (iturin group) and macrolactones (fengycins, surfactins and plipastatins) (Urdaci et al., 2004; Stein, 2005)

Iturin group includes A, C, D and E isoforms (Besson et al., 2008), bacillomycin D, F and L (Peypoux et al., 2004) and mycosubtilin (Peypoux et al., 2004). Bacillomycins and mycosubtilin form channels in bacterial cell membrane (Maget-Dana et al., 2004). Mycosubtilin alters the permeability of the plasma membrane, releasing nucleotides, proteins and lipids (Peypoux et al., 2004).

Fengycin class, including the closest plipastatin, consists of beta hydroxyl fatty acid connected to the N-terminus of a decapeptide (Wang et al., 2004).

2.7.3 Non-peptide-based antibiotics

*Bacillus* organisms also produce a variety of non-peptide antibiotics with different chemical structures (Pinchuk et al., 2001). They include:

- Amicoumacins which are a family of low molecular weight dihydroisocoumarin
derivatives, showing inflammatory, anti-ulcer and gastro-protective effects in addition to antibacterial activities (Itoh et al., 1992).

- Macrolactins and their derivatives of which about 18 from *Bacillus* species have been chemically described (Nagao et al., 2001). They are considered to be potent antiviral and cytotoxic agents that also have antibacterial activity against *S. aureus* (Romero-Tabarez et al., 2006).

- Difficidin and oxydifficidin, isolated from fermentation of *B. subtilis* ATCC 39320, represent a class of antibiotics characterized by highly unsaturated 22-membered macrolide phosphates and exhibit a good antibacterial activity against both aerobic and anaerobic organisms (Wilson et al., 1997).

- *B. subtilis* also produces rhizocticins displaying antifungal activity (Kugler et al., 1990).

Due to the fact that *Bacillus* species produce antibiotics in a soluble protein form which they synthesize and secret directly into the growing medium, these antibiotics have been found to be cheaper and more effective, hence the preference of these organisms in commercial production (Baruzzi et al., 2011).
CHAPTER THREE

MATERIAL AND METHODS

Samples were collected from four hot-springs along Lake Bogoria. Morphological and biochemical methods as outlined in this chapter were used in identification of members of the group bacilli. Secreted compounds of the isolates that were found to be within the group bacilli were subjected to antibacterial testing using the disc diffusion method against selected Gram positive and Gram negative bacteria. 16S rDNA analysis of the isolates that inhibited growth of selected bacteria was carried out to determine phylogenetic relationship with known Bacillus species.

3.1 Study site

The study was conducted in Lake Bogoria. The lake is a saline and alkaline lake that lies in a volcanic region in a basin south of Lake Baringo, and a little north of the equator. It is located in the greater Baringo-Bogoria basin, about 250 km from the city of Nairobi on the floor of the Kenya Rift Valley. It is bound by latitudes 0°00’ and 0°30’N and longitudes 35°45’E and 36°15’E within the rift graben. The lake is a Ramsar site and has been a protected National Reserve since November 29, 1973 (Boon et al., 2000). Lake Bogoria is shallow, about 10 m deep and is about 34 km long by 3.5 km wide, with a drainage basin of 700 km². The lake waters are of sodium hydrogen carbonate composition with a pH of 9.8-10.3 and alkalinity of 35%. The waters of the lake originate from inflow from the Sandai and Emsos rivers, and from about 200 alkaline hot springs that are present at four onshore sites. The western shore is home to the Loburu hot
springs which is made up of some sixty hot springs with six geysers. Further south is the Chemurkei group made up of forty springs, including four geysers. At the south eastern end are the Koibobei springs made up of at least three geysers. At the eastern side are the Losaramat springs with seventeen springs and three geysers (Figure 3.1). The lake itself is stratified with less dense surface waters lying on a denser more saline bottom waters. Although hyper-saline, the lake is highly productive with abundant cyanobacteria that feed the flamingos. However, few other organisms inhabit the lake (David et al., 2003).
Figure 3.1 Map of Lake Bogoria showing positions of the hot springs.

Key: (A) Loburu hot springs (B) Chemurkei hot springs (C) Losaramat hot springs (D) Koibobei hot springs
(Map courtesy of Lake Bogoria Integrated Management Plan 2007-2012)
3.2 Collection of water samples

Samples were collected randomly from each of the four hot springs of Lake Bogoria. Ten sediment and ten surface water samples were collected from the four hot springs of Chemurkei, Loburu, Koiobei and Losaramat to make a total of 80 samples. The samples were obtained within a temperature range of 45-60°C. Samples were randomly collected and inoculated directly into nutrient broth in universal bottles. In every hot spring, each of ten universal bottles containing 15 ml nutrient broth was directly inoculated with 5 ml sediment sample. A sterile scoop was used in collecting samples from near the bottom of the water. From the scooped sample, 5 ml was aspirated using a 10 ml sterile syringe and transferred into 15 ml broth in a universal bottle. Equally, 5 ml of surface water sample was inoculated into ten 15 ml of nutrient broth in universal bottles from each of the four hot springs. A 10 ml sterile syringe was used to aspirate 5ml of the surface water which was aseptically inoculated into 15 ml nutrient broth in universal bottles. The bottles with surface water were labelled as S while those with sediment material were labelled as D. The inoculated bottles were put in insulated boxes and immediately transported to the laboratory.

3.3 Bacteria Isolation and culture

Samples in nutrient broth were incubated without shaking at 45°C for 48 hours. Growth was determined by visually checking for turbidity. All tubes that showed growth were streaked on nutrient agar using the streak plate method recommended by Rath and
Subramanyam (1998) in order to isolate single colonies. The plates were incubated at 40°C overnight.

### 3.3.1 Gram staining of the isolated bacteria

All different colonies in nutrient agar obtained after incubation at 40°C overnight were used to make smears on clean labelled slides. These were allowed to air dry and then fixed by passing the slides; smear facing upwards, twice across a flame. Slides were placed on the staining rack and flooded with crystal violet (Appendix 1). This was allowed to stand for 30 secs. The slide was then rinsed with water for 5 secs then covered with Gram’s iodine (Appendix 1). The slide was allowed to stand for 1 min and rinsed with water for 5 secs. Decolonization was done with 95% ethanol for 15 to 30 secs. This was followed by rinsing with water for 5 secs. Counterstaining was done using Safranin (appendix 1) for about 60 to 80 secs and the slides were rinsed again with water for 5 secs. Blot drying was done with bibulous paper and examined using microscope at x100 in oil immersion (Keast et al., 1984). Gram-positive organisms stained blue to purple; Gram negative organisms stained pink to red.

Gram staining results were confirmed by using 3% KOH string test (Halebian et al., 1981). This test was performed by adding a drop of 3% KOH on a slide. A loopful of the bacteria was introduced and mixed thoroughly. Positive results were observed if the culture pulled along with the wire loop when raised up and negative results were recorded if nothing was pulled along the wire loop. Their Gram reaction was described and the results obtained from each slide recorded. All colonies with Gram positive rods (nine)
were streaked in fresh nutrient agar and incubated for 24 hours at 40°C as pure cultures. Based on the fact that all *Bacillus* are Gram positive rods, all Gram positive cocci and Gram negative rods were discarded. Stock cultures of each selected strain were prepared and preserved in nutrient agar slants at 4°C.

### 3.3.1 Morphological characterization

Colony features obtained after 24 hour growth of the isolates in pure culture were examined and used in the presumptive identification of the organisms in nutrient agar. Colony morphological characteristics including size, shape, elevation and margins were described and recorded.

### 3.4 Biochemical tests

Pure colonies were subjected to biochemical tests for partial identification. The tests used included glucose and lactose fermentation, gas production, catalase production, hydrogen sulfide production, indole production and motility tests according to the methods described by Sneath (1984).

#### 3.4.1 Catalase test

Catalase test was done by scooping a colony of a 24-hour culture, placing it on a glass slide and adding a drop of 3% hydrogen peroxide solution. A positive reaction was indicated by the formation of bubbles, while the absence of air bubbles indicated a negative catalase test (Cappuccino and Sherman, 2002).
3.4.2 Indole production, Motility tests

The isolates were inoculated in Indole Motility (IM) agar (Appendix 1) by stabbing the media in duplicate for replication. The inoculated medium was then incubated at 37°C for 48 hours. Two un-inoculated tubes were used as controls. Lack of motility was detected by the confinement of the bacteria along the line of inoculation. Motility was detected by the spreading of growth, visualized as turbidity, away from the stab line. The media is semi-solid and hence allows movement of motile bacteria (Cappuccino and Sherman, 2002).

3.4.3 Triple Sugar Iron (TSI) test

Slants were inoculated in TSI (Appendix 1) duplicate using a straight wire and the surface streaked with the same wire. The tubes were incubated at 37°C and the results read the following day. An alkaline slant acid butt (red/yellow) indicated fermentation of glucose only. An acid slant-acid butt (yellow/yellow) indicated fermentation of glucose, lactose and/or sucrose. An alkaline slant-alkaline butt (red/red) indicated glucose or lactose were not fermented (non-fermenter). Cracks, splits, or bubbles in medium indicated gas production. A black precipitate in butt indicated hydrogen sulfide production (Cappuccino and Sherman, 2002).
3.5 Antimicrobial activity testing

3.5.1 Preparation of secretion containing supernatant

Nine duplicated 50 ml conical flasks with nutrient broth enriched with 1% glucose were inoculated with a 24 hour Mac Farland 0.5 turbidity standard (appendix 1) from each of the nine organisms and appropriately labelled. The flasks were incubated in an orbital shaker incubator (100 rpm) for 4 days at 40°C. 5 ml of the broth from each of the tubes was taken and put in labelled sterile tubes and centrifuged at 10,000 rpm for 15 minutes. The cell free supernatant was sterilized using 0.2 µl filter paper and then put into sterile eppendorf tubes. Six mm sterile filter papers were dipped in each of the sterile supernatant in eppendorf tubes and dried in a vacuum drier for 10 minutes.

3.5.2 Antimicrobial assay

Four standard ATCC organisms were used to test for production of antibacterial compounds:

*Staphylococcus aureus* ATCC 29213-(Gram positive cocci)

*Escherichia coli* ATCC 25922-(Gram negative rods)

*Escherichia coli* ATCC 35218-(Gram negative rods)

*Pseudomonas aeruginosa* ATCC 27853-(Gram negative rods)

Antibiotic assay was performed on 9 cm Petri-plates containing 20 ml of Mueller Hinton medium (Appendix1). Agar disk diffusion assay was used to check the production of antimicrobial compounds. Care was taken to ensure each plate got between 18 and 20 ml (according to guidelines given by the Clinical and Laboratory Standards Institute, NCLS,
on agar diffusion sensitivity testing) of medium as any antimicrobial substance in the discs would diffuse in all directions and depth would affect the size of the zones of inhibition. This was achieved by pouring the medium from one side of the plate slowly until it converged on the opposite side and letting the medium set on a completely flat surface. Test cultures of *S. aureus* (ATCC 29213), from overnight growth on blood agar, *E. coli* (ATCC 25922), *E. coli* (ATCC 35218) and *P. aeruginosa* (ATCC 27853) from overnight growth cultures on nutrient agar were emulsified in 3 ml of sterile normal saline to achieve the correct inoculum turbidity that matched that of the 0.5 Mac Farland turbidity standard in test tubes. Sterile, non-toxic swabs were dipped into the inoculum suspension and excess fluid removed by pressing the swab against inside wall of the test tube. The swabs were used to streak the entire agar surface of each of the labeled 90 mm plate, rotating the plate approximately 90º each time to ensure an even distribution of the inoculum.

The plates were then allowed to dry for ten minutes. With the aid of sterile forceps, the discs impregnated with the supernatant were placed on Mueller Hinton sensitivity medium. Each plate received 9 discs which were placed at equidistant to each other with the help of a template that was placed underneath the plate. The broad spectrum antibiotic Trimethoprim/sulfamethoxazole which consists of one part trimethoprim to five parts sulfamethoxazole was used as a control. These petri-dishes were incubated overnight at optimum temperature of 37ºC for 24 hours. Zones of inhibition seen as clear zones around the paper discs were observed and scored semi-quantitatively depending on the size for the activity of antimicrobial compounds. The organisms that showed no
inhibitory properties against the selected standard organisms were not processed any further.

3.6 Molecular characterization

3.6.1 DNA extraction

ZR Fungal/Bacterial DNA Miniprep™ (Zymo Research USA) was used to extract DNA from the bacterial cells according to the manufacturer’s protocol. Approximately five colonies of the bacterial cells were suspended in 200 µl of water and added to ZR bashingbead™ Lysis Tubes. A 750µl volume lysis solution was then added to the tube. The tubes were vortexed for 5 min at maximum speed after which they were centrifuged in a microcentrifuge at 10,000 rpm for 1 min. A 400 µl volume of the supernatant was transferred to a Zymo-spin™ IV spin filter in a collection tube and centrifuged at 7,000 rpm for 1 min. A 1,200 µl volume of the Fungal/Bacterial DNA binding buffer was added to the filtrate in the collection tube. An 800 µl volume of the mixture was transferred to a Zymo-spin™ IIC column in a collection tube and centrifuged at 10,000 rpm for 1 min.

The flow through from the collection tube was discarded and the step repeated. A 200 µl aliquote DNA pre-wash buffer was added to the Zymo-spin™ IIC column in a new collection tube and centrifuged at 10,000 rpm for 1 min. 500 µl of Fungal/Bacterial DNA wash buffer was added to the Zymo-spin™ IIC column and centrifuged at 10,000 rpm for 1 min. The Zymo-spin™ IIC column was then transferred to a clean 1.5 ml
microcentrifuge tube and 50 µl DNA elution buffer added directly to the column matrix. It was then centrifuged at 10,000 rpm for 30 seconds to elute the DNA.

The DNA product was quantified using agarose gel electrophoresis (FisherBiotech Electrophoresis system) in 1% agarose gel at 80 volts for one hour and visualised by UV (Uvitec Cambridge). The eluted DNA was stored at -20°C pending further analysis.

3.6.2 PCR amplification of 16S rDNA

Target 16S rDNA sequences were amplified using universal bacterial primers 27f AGAGTTTGATCCTGGCTCAG and 1525r AAGGAGGTGATCCAGCCGCA (Bioneer, USA) in relation to *Escherichia coli* gene sequence (Lane, 1991; Embley and Stackebrandt, 1994). Reagent content and volume per PCR tube for DNA amplification is shown in Table 3.1. Amplification was performed using an advanced Eppendorf 96 AG, model 22331 thermal cycler (Hamburg, Germany).
Table 3.1: Reagent preparation for PCR DNA amplification.

<table>
<thead>
<tr>
<th>REAGENTS</th>
<th>X1'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Master mix*</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Primer-27f (10pmol/µl)</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Primer-1525r (10pmol/µl)</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>6.5 µl</td>
</tr>
<tr>
<td>Template DNA (1500 bp)</td>
<td>4.0 µl</td>
</tr>
<tr>
<td><strong>TOTAL VOLUME/PCR TUBE</strong></td>
<td><strong>25.0 µl</strong></td>
</tr>
</tbody>
</table>

Key: X1'-Reagent composition per PCR tube for each reaction.

Master mix* (appendix 3)

The control contained all the above except the DNA template (Sambrook et al., 1989). Initial heating was done at 94°C for 3 mins. The cycles included heating at 94°C for 30 secs followed by 60°C for 30 secs followed by 68°C for 1 min and finally 68°C for 5 mins. A total of 35 cycles were run. The holding temperature was 4°C. The PCR products were separated on agarose gel electrophoresis (FisherBiotech Electrophoresis system Australia) at 80 volts for one hour and visualized by ethidium bromide staining (Sambrook et al., 1989).

3.6.3 Purification of amplified DNA

The amplified PCR product was purified using a GeneJET PCR Purification Kit (Thermo scientific) according to the manufacture’s protocol. For each sample, 25 µl of the PCR mixture was added to 25 µl of Binding Buffer and mixed forming a yellow color. The
above mixture was transferred to the GeneJET purification column, centrifuged for 60 secs and the flow through discarded. A 700 µl volume of Wash Buffer was added to the Gene JET purification column and centrifuged for 60 secs. The flow through was discarded and the purification column re-centrifuged for an additional 1 min. The GeneJET purification column was then transferred to a clean 1.5 ml microcentrifuge tube onto which 50 µl of elution buffer was added and centrifuged for 1 min. The GeneJET purification column was discarded and the purified DNA stored at -20°C awaiting sequencing.

3.6.4 Data processing of the isolates’ 16S rDNA

Data entry management and preliminary summaries such as averages, percentages were done in Microsoft Excel Spreadsheet. Observations were made on the cultures to define the nature of each of the qualitative tests as either positive or negative. Sequencing of purified PCR product was done in both directions using a commercial service provider (BECA, ILRI). Sequencing reactions were carried out with ABI PRISM Dye Terminator Cycle Sequence Ready reaction Kit (Applied Biosystems Inc. USA) on an ABI 310 DNA sequencer according to manufacturer’s specifications using forward bacterial universal primer 27f AGAGTTTGATCCTGGCTCAG and reverse primer 1525r AAGGAGGTGATCCAGCCGCA (Bioneer, USA). Sequence data was analyzed with CLC Main Workbench version 7.0.3 software package (http://www.clcbio.com). Alignments were checked and corrected manually where necessary.
The obtained consensus sequences were exported and saved in FASTA format. The 16S rDNA gene sequences obtained were compared to sequences in the public database using basic local alignment search tool (BLAST) on the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nih.gov) in order to determine similarity with sequences in the GeneBank database (Altschul et al., 1990, Shyne et al., 2003). 16S rDNA sequences of the isolates that exhibited antibacterial activity were deposited in the NCBI gene bank to be assigned accession numbers. 16S rDNA sequence identity was confirmed using the RDP Naive Bayesian rRNA Classifier Version 2.6, Sep 2013 (Wang et al., 2007) Michigan State University, (https://rdp.cme.msu.edu). The 16S rDNA gene sequences with high similarities to those determined in the study were retrieved and aligned using Molecular Evolutionary Genetics Analysis version six (MEGA6) (Tamura et al., 2013) software and the aligned sequences used to construct a maximum likelihood phylogenetic tree.
CHAPTER FOUR

RESULTS

A total of 80 samples were collected and inoculated in universal bottles containing nutrient broth from four hot-springs sites along Lake Bogoria. Growth was obtained in 33 of them. Preliminary identification was carried out using morphological and biochemical methods the results of which are outlined in this chapter. From the 33 isolates, 9 were identified as Gram positive rods within the group bacilli. Secreted compounds from the nine isolates were tested for antibacterial activity against selected bacteria and five were found to inhibit growth of the bacteria. The five isolates were subjected to molecular characterization using 16S rDNA to infer phylogenetic relationship with known Bacillus organisms and were found to have sequence identities of between 88-99% with members of the genus Bacillus.

4.1 Isolation and identification of bacilli colonies.

Samples were collected from four geyser sites along the shores of Lake Bogoria namely Loburu, losaramat, Koibobei, and Chemurkei (Table 4.1). Isolation and identification of bacilli was crucial in the initial stages to limit the scope of the study to only Bacillus organisms. For this reason, colony morphology description and Gram staining were the first criteria used in determining the identity of the isolated organisms.
Table 4.1: Summary of the geysers where samples were obtained and the number of universal bottles that growth was obtained. Each universal bottle containing 15 ml of broth and was inoculated with 5 ml of sample.

<table>
<thead>
<tr>
<th>Location</th>
<th>15ml Nutrient Broth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth</td>
</tr>
<tr>
<td>Loburu</td>
<td>8</td>
</tr>
<tr>
<td>Losaramat</td>
<td>8</td>
</tr>
<tr>
<td>Koibobei</td>
<td>9</td>
</tr>
<tr>
<td>Chemurkei</td>
<td>8</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>33</strong></td>
</tr>
</tbody>
</table>

4.1 Morphologic and physiologic characterization

Growth from the 33 universal bottles were streaked onto nutrient agar and incubated at 40°C overnight. Colonies obtained were stained to determine their Gram reaction. From the Gram reactions, four (12.1%) were Gram positive cocci, twenty (60.6%) were Gram negative rods and nine (27.3%) were Gram positive rods. Only Gram positive rods were considered for further screening for the basic reason that Bacillus are Gram positive rods. On subculture all the Gram positive rods grew well on nutrient agar incubated at 40°C within a period of 24 hours producing varying colonial morphology types (Figure 4.1) that are described in Table 4.2. However they tended to lose the ability to form spores on subculture and the spores were less pronounced on subsequent Gram stains. D22 had densely staining rods, whereas others had branched network of rods (Figure 4.2).
Figure 4.1: Colonies streaked as pure cultures from Gram positive rods exhibiting antibacterial activity as seen after 24 hours incubation at 40°C. (D1) white, raised, undulate (S15) cream colored, flat, spreading, (D5) white, flat undulate, (D8), white, flat, undulate (D22) large, white, undulate

Table 4.2: Morphological characterization of Gram positive rods isolated from Lake Bogoria.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Colony Characterization</th>
<th>Cell characterization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colour</td>
<td>Form</td>
</tr>
<tr>
<td>S7</td>
<td>White</td>
<td>Irregular</td>
</tr>
<tr>
<td>D8</td>
<td>white</td>
<td>Irregular</td>
</tr>
<tr>
<td>S17</td>
<td>White</td>
<td>Irregular</td>
</tr>
<tr>
<td>D22</td>
<td>White</td>
<td>Irregular</td>
</tr>
<tr>
<td>D5</td>
<td>White</td>
<td>Irregular</td>
</tr>
<tr>
<td>D18</td>
<td>Cream</td>
<td>Irregular</td>
</tr>
<tr>
<td>D10</td>
<td>Cream</td>
<td>Spreading</td>
</tr>
<tr>
<td>S15</td>
<td>Cream</td>
<td>Spreading</td>
</tr>
<tr>
<td>D1</td>
<td>White</td>
<td>Irregular</td>
</tr>
</tbody>
</table>
**Figure 4.2:** Gram stain images of isolates exhibiting antibacterial action against selected Gram positive and Gram negative bacteria. (D1) Gram positive filamentous rods, (D5) Gram positive filamentous rods. (D8) Small Gram positive rods, (S15) Small Gram positive rods, (D22) Large Gram positive rods.
Physiological studies (Table 4.3) showed that all the isolates were catalase positive. All but S17, D5, D18, and D1 were motile, fermented lactose/sucrose with acid production and also broke down tryptophan to produce indole. S15, D10 and D8 produced hydrogen sulfide whereas none of the isolates produced gas from carbohydrate fermentation.

**Table 4.3:** Biochemical results for all nine Gram positive isolates.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Biochemical test</th>
<th>Catalase</th>
<th>motility</th>
<th>indole</th>
<th>lactose fermentation</th>
<th>glucose fermentation</th>
<th>H$_2$S production</th>
<th>Gas production</th>
</tr>
</thead>
<tbody>
<tr>
<td>S13</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>D10</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>S7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D22</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S17</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D5</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D18</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Key:** (+) Positive, (-) Negative
4.2 Susceptibility testing

Disc diffusion assay was used to test for antimicrobial activity of the nine isolates. All the nine (9) isolates were subjected to screening for antimicrobial activity against selected standard Gram positive and Gram negative bacteria. Five (5) out of the nine (9) organisms that were assayed showed antibacterial activity against one or more of the standard organisms (Table 4.4) used. The other three that showed no activity were not investigated further. No activity was noted against *S. aureus* ATCC 29213 and *E. coli* 35218. The broad spectrum antibiotic, trimethoprim/sulfamethoxazole, was used as a control. The control did not inhibit growth of *S. aureus* ATCC 29213 and *E. coli* 35218. However, it inhibited *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 which was seen as clear zones around the discs. Zones of inhibition were determined semi-quantitatively with the smallest zone being given a score of + and the largest inhibition zone being given a score of ++++. The control showed a similar reaction pattern on the four test organisms with *S. aureus* ATCC 29213 and *E. coli* ATCC 35218 being non-responsive to it. *P. aeruginosa* ATCC 27853 was the most responsive of the four test organisms with the biggest inhibition zone being recorded against extracts from isolate D22.
Table 4.4: Semi-quantitative antimicrobial assay of extracts obtained from the Gram positive isolates against *P. aeruginosa*, *S. aureus* and two strains of *E. coli* using disc diffusion method.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Zones of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P. aeruginosa</em> ATCC 27853</td>
</tr>
<tr>
<td>D10</td>
<td>X</td>
</tr>
<tr>
<td>S17</td>
<td>X</td>
</tr>
<tr>
<td>D18</td>
<td>X</td>
</tr>
<tr>
<td>S7</td>
<td>X</td>
</tr>
<tr>
<td>D22</td>
<td>++</td>
</tr>
<tr>
<td>D1</td>
<td>+</td>
</tr>
<tr>
<td>D8</td>
<td>+</td>
</tr>
<tr>
<td>S13</td>
<td>+</td>
</tr>
<tr>
<td>D5</td>
<td>++</td>
</tr>
<tr>
<td>T</td>
<td>+++</td>
</tr>
</tbody>
</table>

**KEY:** Inhibition was regarded as clear zones around the discs; (X) no inhibition, (+) weak inhibition, (++) strong inhibition, (+++) very strong inhibition. The row highlighted in red is the control. (T) Trimethoprim/sulfamethoxazole.

4.3 Extraction and amplification of DNA

Genomic DNA was extracted from the five isolates that showed antibacterial activity using the phenol/chloroform method (appendix 2). Amplification of 16S rDNA gene using universal primers 27F and 1525R yielded an amplification product of approximately 1500bp (Figure 4.3). The amplicons were visualized in ethidium bromide under UV light on 1% agarose gel.
Figure 4.3: Image of PCR purified DNA product

Key: (N) Negative control (M) Molecular marker 1500 bp Molecular size. (D1*), (D22*), (D5*), (S15*), (D8*), (D10*).
* represents isolate numbers

4.4 Isolate sequences

The 16S rDNA nucleotide sequences from the five isolates that inhibited growth of the tested bacteria were submitted to the NCBI database and were assigned accession numbers from KT277950-KT277954 for isolates D1, D5, D8, D22 and S15 respectively. They were annotated as Seq1 for isolate D1, Seq2 for isolate D5, Seq3 for isolate D8, Seq4 for isolate D22 and Seq5 for isolate S15. The sequences of the five isolates are given below.

>Seq1. Accession number KT277950
TGACGTCGAGCGGACCGAAGGGAGCTTTGCTTCTTGAGGTTAGCGGCAACGGCTTGATACACGCGGCTACCTTCAAGCTGGGATGAGATCGGCCACACTGGGACTGAGAC
CGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAAGAACAAGTGCCGTTCGAAAGGGCGGCACCTTGACGGTACCTAACGAGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAAATTATTGGGCGTAAAGCGCGCGCAGGGGTCTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTACAGAAGAGGAGAGTGGATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAGGGGTTTCGATGCCTCTTA

1200 bp

700 bp

4200 bp

700 bp

1500 bp

1200 bp

700 bp
Accession number KT277951

>Seq2.

>Accession number KT277952

>Accession number KT277953

>Accession number KT277954
4.5 Phylogenetic analysis of the sequences

Sequence data was analyzed with CLC Main Workbench version 7.0.3 software package (http://www.clcbio.com). Alignments were checked and corrected manually where necessary. BLAST analysis of partial 16S rDNA gene sequences showed that all the five isolates with antibacterial activity were closely affiliated with members of the genus *Bacillus*. Evolutionary analyses were conducted in MEGA6 software. A phylogenetic tree was constructed using nearest neighbour obtained from the GeneBank (Table 4.5) to show the phylogenetic position of each of the isolates studied (Figure 4.4). The isolates shared sequence identities of between 88-99% with known *Bacillus* species. D1 and D5 clustered together with a sequence identity of 96%. These isolates also clustered very closely with *B. halodurans* (Acc. NR_025446.1), with isolate D5 having a sequence identity of 99% while isolate D1 had a sequence identity of 95% with the organism. Isolate D8 clustered with isolate S15 with a sequence identity of 88%. They also clustered with *P. thiaminolyticus* (Acc. NR_113266 and NR_040887) and *P. dendritiformis* (Acc. NR_042861) with sequence identities of 89%, 89% and 90% respectively for D8 and 88%, 92% and 94% for S8 respectively. D22 clustered with *B. cereus* group with sequence identities of 93%. 
Table 4.5: Blast analysis results of isolates from Lake Bogoria nearest neighbours in the data bank and their percentage relatedness. *Mycobacterium smegmatis* was used as the outgroup.

<table>
<thead>
<tr>
<th>DESCRIPTION</th>
<th>QUERY COVER</th>
<th>E VALUE</th>
<th>IDENTITY</th>
<th>ACCESION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D5</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus halodurans C-125 16S ribosomal RNA, complete sequence</td>
<td>100%</td>
<td>0</td>
<td>99%</td>
<td>KT277951</td>
</tr>
<tr>
<td>Bacillus halodurans strain DSM 497 16S ribosomal RNA gene, partial sequence</td>
<td>100%</td>
<td>0</td>
<td>99%</td>
<td>NR_025446.1</td>
</tr>
<tr>
<td>Bacillus halodurans strain ATCC 27557 16S ribosomal RNA gene, complete sequence</td>
<td>100%</td>
<td>0</td>
<td>99%</td>
<td>NR_112056.1</td>
</tr>
<tr>
<td>Bacillus okuhidensis strain GTC 854 16S ribosomal RNA gene, partial sequence</td>
<td>99%</td>
<td>0</td>
<td>99%</td>
<td>NR_024766.1</td>
</tr>
<tr>
<td><strong>D8</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paenibacillus popilliae strain ATCC 14706 16S ribosomal RNA gene, partial sequence</td>
<td>99%</td>
<td>0</td>
<td>97%</td>
<td>KT277952</td>
</tr>
<tr>
<td>Paenibacillus thiaminolyticus strain NBRC 15656 16S ribosomal RNA gene, partial sequence</td>
<td>99%</td>
<td>0</td>
<td>97%</td>
<td>NR_113795.1</td>
</tr>
<tr>
<td>Paenibacillus thiaminolyticus strain IFO 15656 16S ribosomal RNA gene, partial sequence</td>
<td>99%</td>
<td>0</td>
<td>97%</td>
<td>NR_040887.1</td>
</tr>
<tr>
<td><strong>D22</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA) gene, complete sequence</td>
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<td>0</td>
<td>99%</td>
<td>KT277953</td>
</tr>
<tr>
<td>Bacillus cereus strain JCM 2152 16S ribosomal RNA gene, partial sequence</td>
<td>99%</td>
<td>0</td>
<td>99%</td>
<td>NR_113266.1</td>
</tr>
<tr>
<td>Bacillus cereus strain CCM 2010 16S ribosomal RNA gene, complete sequence</td>
<td>100%</td>
<td>0</td>
<td>99%</td>
<td>NR_115714.1</td>
</tr>
<tr>
<td>Bacillus cereus strain NBRC 15305 16S ribosomal RNA gene, partial sequence</td>
<td>100%</td>
<td>0</td>
<td>99%</td>
<td>NR_112630.1</td>
</tr>
<tr>
<td><strong>S15</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paenibacillus dendritiformis strain T168 16S ribosomal RNA gene, partial sequence</td>
<td>99%</td>
<td>0</td>
<td>99%</td>
<td>KT277954</td>
</tr>
<tr>
<td>Paenibacillus thiaminolyticus strain IFO 15656 16S ribosomal RNA gene, partial sequence</td>
<td>97%</td>
<td>0</td>
<td>99%</td>
<td>NR_040887.1</td>
</tr>
<tr>
<td>Paenibacillus thiaminolyticus strain NBRC 15656 16S ribosomal RNA gene, partial sequence</td>
<td>97%</td>
<td>0</td>
<td>99%</td>
<td>NR_113795.1</td>
</tr>
<tr>
<td>Paenibacillus popilliae strain ATCC 14706 16S ribosomal RNA gene, partial sequence</td>
<td>97%</td>
<td>0</td>
<td>99%</td>
<td>NR_040888.1</td>
</tr>
<tr>
<td><strong>D1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus hemicellulosilyticus strain C-11 16S ribosomal RNA gene, complete sequence</td>
<td>100%</td>
<td>0</td>
<td>97%</td>
<td>KT277950</td>
</tr>
<tr>
<td>Bacillus alcalophilus strain NBRC 15653 16S ribosomal RNA gene, partial sequence</td>
<td>99%</td>
<td>0</td>
<td>96%</td>
<td>NR_112722.1</td>
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<tr>
<td>Bacillus alcalophilus strain 1 16S ribosomal RNA gene, partial sequence</td>
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<td>0</td>
<td>96%</td>
<td>NR_036889.1</td>
</tr>
<tr>
<td>Bacillus pseudofirmus OF4 strain OF4 16S ribosomal RNA, complete sequence</td>
<td>100%</td>
<td>0</td>
<td>96%</td>
<td>NR_102774.1</td>
</tr>
</tbody>
</table>

*Mycobacterium smegmatis* ATCC 607 16S rRNA gene. 0% 0.34 100% AM905265.1
Figure 4.4: A maximum likelihood tree based on partial 16S rDNA gene sequences showing the phylogenetic relationships between the isolates with antibacterial activity against standard ATCC bacteria and related to Bacillus species. The tree is drawn to scale, with the branch lengths measured in the number of substitutions per site. The 16S rDNA sequence of *Mycobacterium smegmatis* was used as an outgroup.
CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Bacilli have been used widely in industrial applications. The main goal in this research was to bioprospect for thermophilic bacilli from selected hot springs in Lake Bogoria and to characterize those with antibacterial activity using morphological, biochemical and molecular methods. The results obtained showed the presence of different bacteria in these environments predominantly Gram-negative rods. However our main interest was focused on Gram-positive rods as they represent the group bacilli. Sampling was done on both surface (denoted as S) and sediment (denoted as D) samples. We found that more growth was obtained from samples obtained from below the surface. This region was warmer than the surface. However we could only speculate that bacilli preferred living in warmer waters below the surface, since extensive literature search did not reveal any theory to support this.

Colonial morphology description (Table 4.2, Figure 4.1) and Gram stain reaction (Figure 4.2) of the isolates were the initial identification criteria used in the identification process. Bacteria have a short generation time and will grow to produce different looking colonies. The shape, size and color of colonies produced by bacteria can be used to give a general indication on the type of bacteria. Indeed, the recognition of typical colony morphologies is crucial, among other considerations for bacterial
identification. Scientific laboratories frequently use the colony morphology displayed by bacteria on agar media as an auxiliary means to identify bacteria because of their different and specific growth patterns (Ana et al., 2013). As stated earlier, members of the group Bacilli are Gram positive rods, meaning they take up the primary stain during Gram staining and resist de-colorization using acetone or alcohol. Of the thirty three organisms that were able to grow on nutrient media 27.3% were Gram positive rods.

Biochemical identification of bacteria has been in practice since the late 19th and early 20th centuries. However because of the limit of biochemical and phenotypic tests available, characterization of proposed species has been imprecise and inadequate. This problem has often led to discovery and rediscovery of the same bacterial species by different investigators who gave the same taxa new names based upon slightly different morphological, cultural and phenotypic criteria (Micheal and Sharon, 2002). All the 9 isolates were subjected to baseline biochemical tests as suggested by Brock (1986) and all were found to be catalase positive (Table 4.3). Catalase, an enzyme that was discovered in 1818 by Louis Jacques, is present in almost all aerobic microorganisms including some anaerobic ones (Micheal and Sharon, 2002). Catalase is frequently used by cells to rapidly catalyze decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules (Gili et al., 2006). Because hydrogen peroxide is mainly formed in aerobes or facultative anaerobes which in one way or the other use oxygen for their cellular processes, catalase was found in all the isolates due to its protective role against reactive hydrogen peroxide.
Indole test was done to determine the ability of the organisms to produce the enzyme tryptophanase. Bacteria are able to sense environmental cues, regulate their overall gene expression, and control their phenotypes. As a well-known metabolite, indole has recently shown diverse biological roles as an intercellular signal in microbial communities (Lee and Lee, 2010). Indole production is significantly increased at high temperatures and probably plays a role in drug resistance (Hirakawa et al., 2005) and plasmid stability (Chant and Summers, 2007). All the isolates apart from isolate D22, D5, D18 and D1 were indole positive (Table 4.3) indicating that they were able to produce the enzyme tryptophanase and convert tryptophan into indole.

Most the organisms utilized glucose as an energy source whereas none produced gas (Table 4.3). Heterotrophic bacteria often use carbohydrates as energy sources. Each bacterium has its own collection of enzymes that enable it to use diverse carbohydrates; and this is often exploited in the identification of bacterial species. One can determine if a given bacterial species can utilize a given carbohydrate by checking for the presence of by-products that are produced by the oxidation or fermentation of these carbohydrates. To this end, pH indicators may be added to the media to detect metabolic acids that have been produced by bacteria after the oxidation and fermentation of sugars. TSI medium was used to determine sugar fermentation. Phenol red is incorporated in the media as a pH indicator which turns yellow in acid pH of below 6.8. S15 and D10 were lactose and/or sucrose fermenters as indicated by the butt and slant turning yellow. D22, S7 and D8 were only able to ferment glucose and therefore produced only enough acid to turn the butt yellow with the slant remaining
red. D5, S17 and D18 did not change the color of the medium indicating that they are non-fermenters and therefore by inference, are obligate aerobes.

Hydrogen sulfide production was tested to determine anaerobic fermentation of sulfate. Sulfate-reducing bacteria are those bacteria that can obtain energy by oxidizing organic compounds or molecular hydrogen while reducing sulfate to hydrogen sulfide. Sulfate occurs widely in seawater, sediment, or water rich in decaying organic material. Sulfate-reducing bacteria are common in anaerobic environments where they aid in the degradation of organic materials (Dexter, 2003). Isolates S15, D10 and D8 (Table 4.3) were able to produce hydrogen sulfide which was shown by blackening of the butt of the slant medium.

Different scientists have reported inhibition of various organisms by microbial products produced by *Bacillus* organisms. Marahiel *et al.* (1997) isolated a strain of *Bacillus subtilis* C126 from sugar cane fermentation, which produced a polypeptide antibiotic, bacitracin, which inhibited the growth of *Micrococcus flavus*. A *Bacillus licheniformis* strain, 189, isolated from a hot spring environment in the Azores, Portugal, was found to strongly inhibit growth of Gram-positive bacteria by producing peptide antibiotic (Mendo *et al.*, 2004), further confirming the significance of extreme environments as sources of antimicrobials. Initial screening in the present study which was carried out at 37°C against four (4) ATCC organisms showed maximum inhibition achieved against *P. aeruginosa* ATCC 27853 by secreted metabolites from isolate D22 (Table 4.4). No inhibitory activity was noted against *S. aureus* ATCC 29213 and *E. coli* ATCC 35218. Testing was done using the disk diffusion method. The diameter of the zones of growth
inhibition around each of the disks was approximated, and was taken to be related to the susceptibility of the isolate to the extracts, and to the diffusion rate of the antimicrobial compound through the agar medium (Wayne, 2009).

The secreted metabolites antimicrobial profile we obtained against the standard ATCC organisms agrees with the findings of another study by researchers Maria et al. (2006) who also found *E. coli* ATCC 35218 to be resistant and *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 to be susceptible to phenolic extracts obtained from apple fruits. *S. aureus* was resistant to all extracts. In a different study, the effect of a “non-antibiotic” trimebutine was investigated against reference ATCC organisms (Kountouras et al., 2012). It was noted that the minimum inhibitory concentration and minimum bacteriocidal concentration were similar for *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 whereas *S. aureus* ATCC 29213 and *E. coli* ATCC 35218 the minimum inhibitory concentration was higher. This comparison is supported by studies carried out by researchers Dimitriu et al. (2006) who found that there was significant correlation between minimum inhibition values and inhibition zone diameters in the disc diffusion method. It was further confirmed by the use of a standard commercial broad spectrum antibiotic trimethoprim/sulfamethoxazole which displayed the same pattern of reaction. Five of the nine isolates showed some activity against the standard organisms used (Table 4.4) indicating that the remaining isolates were not able to produce antimicrobial substances effective against any of the tested organisms.

It is important to note however that on secondary culture most bacteria lose their ability to produce bioactive compounds. A critical element for the establishment of an efficient production of secondary metabolites is the condition in which the producer
microorganism is grown (Higgs et al., 2001). It has been reported that over 40% of microorganisms produce active secondary metabolites when they are freshly isolated from nature but this ability is lost in many of them during storage (Demain et al., 2000). Isolates of the *Bacillus* genus continue to be promising sources of biologically active secondary metabolites and the experience accumulated with the *Bacillus* strains highlight once more the fact that the production of bioactive secondary metabolites is strain and not species related.

The taxonomic classification of the isolates performed using morphological characteristics, biochemical tests and 16S ribosomal DNA sequences of their genomic DNA placed the isolates in the genera *Bacillus*. The genus *Bacillus* contains more than two hundred and twenty two (222) recognized species distributed widely across both terrestrial and aquatic habitats (Koo et al., 2013).

Molecular taxonomy was revolutionized in the mid-1980s with the advent of full sequence analysis of molecular chronometers such as rDNA (Rosello et al., 2001). By mid-1990s, sequencing of the small subunit 16S rDNA genes had become commonplace, considered a standard tool of microbial taxonomists not only for elucidating phylogenetic relatedness, but also as a means of bacterial identification (Kolbert et al., 1999; Rosello et al., 2000). The current classification of species within the genus *Bacillus* and related genera is well established and is based on a combination of numerous experimental approaches (Xu and Côté, 2003). In addition, many phylogenetic studies of the *Bacillus* (Goto et al., 2000) have been done, most of which are biased towards terrestrial isolates (e.g. *B. subtilis*), particularly because of clinical
concerns about certain pathogens such as *B. cereus*, *B. thuringiensis*, and *B. Anthracis* (Sneath *et al.*, 1986). However, relatively few taxonomic works on aquatic *Bacillus* have been attempted so far. In the present study 16S rDNA was amplified by polymerase chain reaction, sequenced and then used for phylogenetic comparisons (Figure 4.4).

The use of 16S rDNA gene sequences for determining phylogeny was initially based on the assumption that sequence diversity was purely due to evolutionary change and that the 16S rRNA gene was not influenced by horizontal gene transfer (Jain *et al.*, 1999). However, as early as 1993, Sneath provided evidence for horizontal exchange of 16S rDNA gene fragments in *Aeromonas* species (Sneath, 1993). These findings were extended by Wang and Zhang (2000), who examined actinomycetes. They found evidence consistent with lateral gene transfer of short gene segments corresponding to hairpins in the 16S rDNA gene. They suggested that this mode of lateral gene transfer would create mosaic rRNA genes and gradually destroy the evolutionary history recorded in the sequence. Thus, it is not in the least surprising that bacilli 16S rDNA gene sequences have been modified by horizontal exchange and their phylogenetic signal degraded. It is however important to note that it is still possible, in the presence of genetic exchange to construct an organismal phylogeny. What is required for reliable phylogenetic analysis is inclusion of a sufficiently large number of informative bases to recover the true phylogenetic signal from the noise created by horizontal transfer of genetic information. Roughly 1500 bp obtained from Sanger dye terminator sequencing revealed that the five isolates D1, D5, D8, D22 and S15 lay within the group bacilli based on 16S rDNA gene sequences as confirmed using the RDP Naïve Bayesian
classifier for Rapid Assignment of rRNA Sequences into new bacterial taxonomy (Wang et al., 2007).

Isolate D1 and D5 were closely related with 96% sequence identity and grouped with *B. halodurans* (Acc. NR_025446.1) showing that the two organisms could be different strains of the same organism. However, the two could morphologically be separated from each other by D1 having raised colonies whereas D5 had flat colonies. *B. halodurans* has been known to produce a two-peptide lantibiotics, haloduracin, with activity against a wide range of Gram-positive bacteria (Lawton et al., 2007). Trent and Wilfred (2009) reported that though Haloduracin was active against Gram-positive bacteria, it had no potency against Gram-negative bacteria. Secreted secondary metabolites from isolate D1 and D5 were active against Gram-negative bacteria showing the two strains were different from *B. halodurans*.

Isolate D22 clustered with a group of different strains of *Bacillus cereus* with a sequence identity of 93% to the closest neighbor. Typical *B. cereus* colonies are large, raised, and opaque with undulate margins and lack the ability to split indole from the amino acid tryptophan (Wong et al., 1988). The colonies of isolate D22 were flat and the organism degraded tryptophan showing it to be different from typical colonies of *B. cereus*. Strains of *Bacillus cereus* have been known to produce a few antibiotics such as zwittermicin A (Laura et al., 1994) that has a wide spectrum of activity (Laura et al., 1998) including against diverse Gram-negative bacteria and certain Gram-positive bacteria. *B. cereus* has also been found to produce an emetic toxin, cerulide, like its structural homologue, valinomycin, produced by soil dwelling *Streptomyces* species that
dissipates the membrane potential of Gram positive bacteria at a wide pH range (Mercele et al., 2011).

The inhibitory effect of D22 was highly pronounced against *P. aeruginosa* ATCC 27853. In the same line of thinking, it is important to note that *P. aeruginosa* produces a biosurfactant which is a rhamnolipid that possesses antimicrobial properties. Rhamnolipids have been shown to have activity against a range of bacteria including *Seratia marcescens*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Bacillus subtilis*. The mode of killing has been shown to result from intercalation of rhamnolipids into the cell membrane causing pores to form which result in cell lysis, at least in the case of *Bacillus subtilis* (Sotirova et al., 2008).

Isolate S15 was the only isolate obtained from surface water that had any inhibitory effect against the standard organisms. The isolate clustered with D8 with a sequence identity of 88% showing that their relation is significant. Isolate D8 clustered with *P. thiaminolyticus* (Acc. NR_113795.1 and NR_040887.1) and *P. dentritiformis* (Acc. NR_042861.1) with sequence identities of 89%, 89% and 90% respectively, whereas S15 clustered with the same organisms with sequence identities of 88%, 92% and 94% respectively. Both D8 and S15 produced cream colored colonies with undulate margins and the Gram appearance presented small Gram-positive rods. D8 had irregular colonies whereas S15 had spreading colonies. D8 and S15 were able to produce the enzyme catalase, hydrogen sulfide and indole indicating that they could be different trains of the same organism. *Paenibacillus*, a genus initially included in the genus *Bacillus* was reclassified as a separate genus in 1993 (Ash et al., 1993). Like D8 and S15, *Paenibacillus* are Gram-positive endospore forming bacteria (Ash et al., 1993) that
develop complex colonies with intricate architectures (Ingham and Jacob 2003). Various *Paenibacillus* species also produce antimicrobial substances that affect a wide spectrum of microorganisms (Piuri *et al.*, 1998; Von Der Weid *et al.*, 2003) such as fungi, soil bacteria, plant pathogenic bacteria and even important anaerobic pathogens as *Clostridium botulinum*.

Lastly, it is important to note that although 16S rDNA gene sequencing is highly useful in bacterial identification and classification, it has low phylogenetic power at the species level and poor discriminatory power for some genera (Bosshard *et al.*, 2006; Mignard *et al.*, 2006), and DNA relatedness studies are necessary to provide absolute resolution to this taxonomic problems. For example the genus *Bacillus globisporus* and *Bacillus psychrophilus* share 99.5% sequence similarity with regard to their 16S rDNA genes, and yet at the DNA level exhibit only 23 to 50% relatedness in reciprocal hybridization reactions (Fox *et al.*, 1992). In some instances, the difference between the closest and next closest match to the unknown strain is <0.5% divergence (>99.5% similarity). In these circumstances, such small differences cannot justify choosing the closest match as a definitive identification, although in some studies this is exactly what was done resulting in wrong taxonomic classification (Fontana *et al.*, 2005).
5.2 Conclusions

The antimicrobial potential of isolates from selected hot-springs along Lake Bogoria that are antagonistic to selected bacteria was demonstrated in this work based on results from antibacterial assays. The isolates showed antimicrobial activity and are likely to be potential candidates for discovery of novel secondary metabolites for application as antibiotics. Importantly, this study shows that extreme habitats may harbor Bacillus organisms which could be useful as sources of antimicrobial compounds especially in this era of increasing antimicrobial resistance of microbes to the existing antibiotics. Although the in vitro evaluation of the isolates for antibacterial activity may not give any correlation with in vivo assays, they may aid in screening studies to provide positive antagonists for further testing by in vivo assays. Furthermore, the five isolates that were active on all the test organisms could potentially be developed as producers of novel metabolites.

5.3 Recommendations

Throughout history, secondary metabolites have been and continue being an unlimited source of natural products with different biological activities, one of them being their use as antibiotics. Extracts from five (5) isolates were found to possess inhibitory activity against other organisms. It was however found necessary to:

i. Formulate culture conditions that best mimic the natural habitat of the organisms so as to better isolate and preserve the abilities in terms of production of secondary metabolites of the organisms as much as possible.
ii. Purify the crude secretions using chromatographic or other appropriate methods and determine the biological activity of the purified product against a wide range of susceptible and resistant bacteria; then go a step further to elucidate their chemical structure so as to determine whether they are new chemical structures or known structures belonging to known compounds.

iii. Complement 16S rDNA phylogenetic analysis with other molecular tools such as DNA-DNA hybridization for proposed new species and for the definitive assignment of a strain with ambiguous properties to the correct taxonomic unit.
REFERENCES


APPENDICES

APPENDIX 1: Media and reagent preparation

Dilute nutrient agar

**Formula:** 8 g of difco Nutrient broth, 15 g of bacteriological agar, 0.1M Hcl.

**Preparation:** Suspend the components in 1 litre of distilled water. Stir well to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 45-50°C and pour in 15–20 ml amounts into Petri dishes.

**Use:** General purpose and Minimal media isolates non-fastidious bacteria.

Nutrient broth

**Formula:** 1.3% nutrient broth, 3% sodium chloride, 1% sodium carbonate

**Preparation:** Suspend the components in 1 litre of distilled water. Stir well to dissolve the medium completely. Dispense in 3-10mls in tubes then Sterilize by autoclaving at 121°C for 15 minutes.

**Use:** General purpose broth media.

Triple sugar iron (TSI)

TSI slant is a tube that contains agar, a pH sensitive dye (phenol red), 1% lactose, 1% sucrose, 0.1% glucose, sodium thiosulfate, ferous sulfate or ferrous ammonium sulfate.
Recipe

Pancreatic digest of casein USP 10.0 g
(see Note)

Peptic digest of animal tissue 10.0 g
USP (see Note)

Glucose 1.0 g

Lactose 10.0 g

Sucrose 10.0 g

Ferrous sulfate or ferrous ammonium sulfate 0.2 g

NaCl 5.0 g

Sodium thiosulfate 0.3 g

Phenol red 0.024 g

Agar 13.0 g

Distilled water 1,000 mL

Note: The following combination of ingredients can substitute for the first two components listed: beef extract, 3.0 g; yeast extract, 3.0 g; and peptone, 20.0 g.

Combine ingredients, and adjust the pH to 7.3. Boil to dissolve the agar, and dispense into tubes. Sterilize by autoclaving at 121°C for 15 min. Cool in a slanted position to give a 2.5-cm butt and a 3.8-cm slant.

TSI agar is also available commercially.
Indole motility medium

SIM Medium is used for the differentiation of microorganisms on the basis of indole production and motility. It is a semisolid media that have been used extensively in the determination of bacterial motility. Indole formation, and motility are useful diagnostic tests in the identification of bacteria.

Principles of the Procedure

The nitrogen, carbon, and amino acids sources in SIM Medium are provided by Enzymatic Digest of Casein and Enzymatic Digest of Animal Tissue. SIM Medium is semi-solid, due to the low concentration of agar. The semi-solid nature of this medium allows for easy visual determination of motility which appears as growth extending outward from the original line of inoculation.

Enzymatic Digest of Casein contains tryptophane, which is converted to indole. Indole is detected after incubation by the addition of Kovac’s Reagent.

Formula / Liter

Enzymatic Digest of Casein .................................................... 20 g

Enzymatic Digest of Animal Tissue........................................ 6.1 g

Agar ....................................................................................... 3.5 g

Final pH: 7.3 ± 0.2 at 25°C

Formula may be adjusted and/or supplemented as required to meet performance specifications.

Procedure

1. Dissolve 30 g of the medium in one liter of purified water.
2. Heat with frequent agitation to completely dissolve the medium.

3. Autoclave at 121°C for 15 minutes.

**Mueller Hinton Agar**

Mueller Hinton agar is used in the tests for organism's susceptibility to antimicrobial agents by the disk diffusion method.

**Preparation**

Suspend 38 g of the medium in one liter of distilled or deionized water. Mix well and heat with frequent agitation. Boil for one minute and sterilize at 121°C (15 lbs. of pressure) for 15 minutes.

Cool to 40-45°C and pour into Petri dishes.

**Preparation of McFarland Standards**

**Purpose:**

McFarland Standards are turbidity standards that are used to gauge approximately how many bacteria are present in a liquid suspension. The standards are used to visually compare the turbidity of a suspension of bacteria with the turbidity of the appropriate standard. Standards are prepared by adding barium chloride to sulfuric acid to obtain a barium precipitate. The volumes of the two reagents are adjusted to prepare standards of different turbidity that represent different concentrations of bacteria.
Reagents:

- Sulfuric acid, 1%
- Barium Chloride, 1.175%

Procedure for the Preparation of a 0.5 McFarland Standard:

1. Add approximately 85 ml of 1% sulfuric acid (H₂SO₄) to a 100ml volumetric flask.

2. Using a volumetric pipette, add 0.5ml of 1.175% anhydrous barium chloride (BaCl₂) drop-wise to the 1% sulfuric acid (H₂SO₄) while constantly swirling the flask.

3. Bring the volume to 100ml with 1% H₂SO₄.

4. Stir or mix for approximately 3 to 5 minutes while examining visually, until the solution appears homogeneous and free of clumps. A magnetic stirrer can be used for this step if available.

5. Put in tubes and cap the tubes tightly.

6. Store the prepared standards in the dark at room temperature for 3 months or longer.

Use of McFarland Standards:

1. Mix standard and test suspension using a vortex, prior to examination.

2. With good lighting, visually compare the turbidity of the test suspension to the McFarland standard.
3. If the suspension is too dense in comparison to the standard dilute the suspension until it is comparable to the McFarland standard.

4. If the suspension is too dilute in comparison to the standard, inoculate it with additional organism until the concentration matches that of the standard, or prepare a new suspension.

5. All adjustments to the bacterial suspension should be performed using sterile technique.

**Crystal violet**

Crystal violet stain is used in the Gram stain and other staining procedures.

**Crystal Violet Ingredients**

- 2 g crystal violet
- 20 ml 95% ethyl alcohol
- 0.8 g ammonium citrate monohydrate
- 80 ml deionized water

**Preparation of Crystal Violet Stain**

i. Dissolve 2 g crystal violet in 20 ml of 95% ethyl alcohol.

ii. Dissolve 0.8 g ammonium oxalate monohydrate in 80 ml deionized water.
iii. Mix the crystal violet and ammonium oxalate monohydrate solutions to make the crystal violet stain. Filter the stain if necessary.

**Safranin Solution:**

Safranin is used as a secondary stain in the Gram staining procedure.

Dissolve 0.25 g Safranin powder in 95% ethyl alcohol and mix with 100 ml distilled water.

**Gram’s Iodine Solution**

Grams iodine is used in Gram staining procedure for bacteria as a mordant.

Dissolve 6.7 g of potassium iodide in 100 mL of de-ionised water; add 3.3 g of iodine; stir to dissolve, then dilute to 1 L. Store in a dark bottle.
APPENDIX 2: Phenol-Cloroform DNA extraction reagents

Solution 1

50mM Tris pH 8.5

50mM EDTA pH 8.0

25% Sucrose solution

Solution 2

10mM Tris pH 8.5

5mM EDTA pH 8.0

1% SDS

Lysozyme 20 mg/ml

RNaseA 20 mg/ml

Proteinase K 20 mg/ml

Phenol

Chloroform

Absolute ethanol.

3M NaCl

Isopropanol
Electrophoresis buffer Working Concentrated stock

TBE buffer 10 ×

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>108 g</td>
</tr>
<tr>
<td>Boric Acid</td>
<td>55 g</td>
</tr>
<tr>
<td>Na$_2$EDTA.2H$_2$O</td>
<td>9.3 g</td>
</tr>
</tbody>
</table>

Adjust the volume to 1 liter with ddH$_2$O and divide into 500ml bottles

**Running conditions:** use 1× TBE as the running buffer. Pre run the gel at 40W for 30 minutes. Load 2µl of sequencing reactions/well making sure to low out wells with a syringe first then Run the gel at 60W for 1.5-2h interval.

**EDTA 0.5 M pH 8.0**

Dissolve 186.1 g of disodium ethylenediaminetetra-acetate (EDTA .2H$_2$O Sigma ED2SS mw 372.2) in 800 ml of double distilled water. Stir vigorously and adjust the pH to 8.0 with sodium hydroxide pellets (EDTA will not go into solution until the pH is near 8.0, so add some of the pellets before trying to adjust the pH. Bring it to a final volume of 1000 ml. Divide into 100 ml aliquots and autoclave.
**Ethidium Bromide 10X**

Dissolve 1.0 g of EtBr in a final volume of 100 ml double distilled water. Wrap the bottle in aluminum foil and stir several hours to get a true solution. Store at 4°C.

To make the 1X stock used to stain gels take 10 ml of the 10X stock and bring to a final volume of 100 ml using double distilled water. Wrap bottle in aluminum foil and store at room temperature.

**Proteinase K**

To 1 ml of ddH$_2$O add 20 mg of Proteinase K (Promega # 52066). This gives a 20mg /ml stock.

**SDS 10%:** Dissolve 100 g of electrophoresis-grade SDS in 800 ml double distilled water. Heat the solution to dissolve. Bring to a final volume of 1000 ml using double distilled water. Do not autoclave.

**TE pH 7.4 or pH 8.0**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris pH 8.0</td>
<td>2 ml</td>
</tr>
<tr>
<td>0.5 M EDTA pH 8.0</td>
<td>400 µl</td>
</tr>
</tbody>
</table>

Bring it to a final volume of 100ml using double distilled and Autoclave.
Tris 1 M pH 7.4: Dissolve 121.1 g of Tris base in 800 ml of double distilled water and adjust the pH to 7.4 with concentrated hydrochloric acid. Bring the final volume to 1000 ml with double distilled water. Divide into 100 ml bottles and autoclave.
APPENDIX 3: PCR Master Mix.

PCR Master Mix includes Nuclease-Free Water and PCR Master Mix, 2X. PCR Master Mix is a premixed, ready-to-use solution containing *Taq* DNA Polymerase, dNTPs, MgCl$_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR.

<table>
<thead>
<tr>
<th>Component</th>
<th>Per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq polymerase</td>
<td>0.1 µl</td>
</tr>
<tr>
<td>PCR buffer</td>
<td>5 µl</td>
</tr>
<tr>
<td>MgCl$_2$ 1.5 mM</td>
<td>3 µl</td>
</tr>
<tr>
<td>Each NTP 10 mM</td>
<td>1 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>15.4 µl</td>
</tr>
</tbody>
</table>
APPENDIX 4: Agarose gel preparation

Gel electrophoresis is an important molecular biology tool. DNA sequencing, fingerprinting (or “profiling”), and genetic engineering are based upon it. Gel electrophoresis separates DNA fragments by their size or molecular weight. The agarose gel acts like a sieve, separating different sized fragments while the electric current provides electrodes; the voltage determines how fast the DNA will travel through the gel. Larger molecules or DNA fragments become entangled in the gel and travel more slowly, while smaller ones pass through more easily and travel farther down the gel. Similar-sized DNA fragments travel at the same rate and form a tight bunch called a “band”. The DNA in the gel must be stained in order to see the bands.

Preparing the gel

DNA gels are made of agarose, a highly purified agar, which is heated and dissolved in a buffer solution. The agarose molecules form a matrix with pores between them. The more concentrated the agarose, the smaller the pores. 100 ml of 1.2% agarose gels was used (1.2 grams of agarose per 100 ml of TBE buffer).

Procedure:

- Weigh 1.2 grams of agarose powder and place it in a conical flask.
- Add 100 ml of 1X TBE buffer into the flask. Swirl to mix the solution.
- Place the flask in the microwave. Heat on high until the solution is completely clear and no small floating particles are visible (1-2 minutes).
Swirl the flask frequently to mix the solution and prevent the agarose from burning.

- Do not allow the agarose to boil over.
- Use hot mitts when handling the flask because it will be very hot.
- Evaporation during boiling may have caused a reduction in the volume thus increase in concentration. Add water to compensate for the loss and maintain the concentration.
- Cool the solution to 55°C before pouring the gel into the plastic casting tray. Higher temperatures will melt the plastic tray. Ethidium bromide can be added at this point to a final concentration of 0.5 µg/ml; 2 µl of EtBr added per 100 ml of buffer.

Care! Ethidium bromide is an irritant, mutagen, toxic and may cause heritable genetic damage. Always wear gloves when handling the powder, solutions and all gels that contain ethidium bromide. Remember to clean working surface, gloves and equipment using 10% bleach solution.

- Place the plastic comb in the slots on the side of the gel tray. The comb teeth should not touch the bottom of the tray. Push any air bubbles to the side farthest from the wells.
- Allow the agarose gel to cool until solidified. The gel will appear a cloudy white colour and will feel cool to touch (about 20 min). Gels can be stored, wrapped in plastic wrap, in the refrigerators for a few days.
• Once the gel is completely solidified, lift the tray out of the chamber, turn it 90°, and replace it in the chamber with first comb closest to the cathode side of the chamber. The running position exposes the open ends of the agarose to the buffer. The standard agarose should solidify completely in about 30 min.

• Pour the buffer into the unit to fill chamber and completely cover and submerge the gel. A “Fill Line” is located on each unit clearly mark the correct buffer level.

**NB:** Too little buffer may cause the gel to dry out during the run, while excess buffer may slow down DNA migration in the gel.

**Loading and Running an Agarose Gel**

1. Remove the comb from the wells by pulling straight up on the comb. Be careful not to tear the wells as you remove the comb.

2. Place the gel box with the wells closest to the negative (black) electrode.

3. Add 1X TBE buffer to fill the buffer tank and submerge the gel about ¼ inches.

4. Cut a piece of parafilm and place 2 µl of gel loading dye onto the waxy side for each sample to be loaded. Dispense 10 µl of the sample and mix the solution by pipetting the dye up and down into the sample. The gel loading dye contains glycerol that will make your DNA denser so that it will sink into the wells. It also contains dye molecules that are smaller and travel faster through the gel than the DNA molecules. The dye molecules provide a visual tracking method so you know how far the DNA has travelled through the gel.
5. Pipette 5 µl (2 µl loading dye and 3 µl DNA ladder) as reference sample into the first well. Keep the tip of the pipette ABOVE the well. The DNA will sink into the well because it has been mixed with loading dye. If you puncture the bottom of the well your DNA run out through the bottom of the gel into the buffer tank.

- Molecular biologists often use a size standard marker called a 1 kb DNA ladder. The DNA ladder produces several different sized fragments and can be used to estimate the size of an unknown DNA fragment.

6. You are now ready to load the next sample into the next well. Repeat step 4 until all of the samples and controls, both positive and negative (blank without DNA), have been loaded into the gel.

7. Close the top of the box. Plug the leads into the gel box. The black lead is the negative lead and should be plugged in closest to the wells. The red lead is the positive lead and should be plugged-in furthest from the wells.

8. Plug the other end of the leads into the power source and turn it on. Electrophoresis at 80-170 volts until the loading dye has travelled ½ to ¾ of the way down the gel (about 0.5-1 hour).

9. Turn off the power supply. Unplug the leads and power supply before opening the gel box.

10. Visualize the DNA on a long wave UV light box and photograph with Polaroid camera lens aperture set at f/11 and exposure time of 30 milliseconds. Pull out the picture and allow developing for one minute.

11. Photodocument the picture.
Calculations:

Calculate reagent’s concentration according to manufacturer’s specifications.

**Interpretation and reporting of results**

Read the gel picture for the efficient amplification of target sequence. The separated macromolecules in each lane can be seen in a series of bands spread from one end of the gel to the other. Target gene can be read against the standard molecular weight markers (DNA ladder).

The picture can then be documented in a file or record book.