MOLECULAR CHARACTERIZATION OF THERMOPHILIC PROKARYOTES FROM CHINYUNYU HOT SPRING IN ZAMBIA

By

Kalumbilo Pascal Mubonda

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DECLARATION

I, **Kalumbilo Pascal Mubonda**, hereby declare that this dissertation represents my own work and that it has not previously been submitted for a degree, diploma or other qualification at this or another University.

Signature : .....

Date : .....

APPROVAL

This dissertation of **Kalumbilo Pascal Mubonda** is approved as fulfilling part of the requirements for the award of the degree of Master of Science in Molecular Biology by the University of Zambia.

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Examiner II	**Signature**	**Date**
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Supervisor	**Signature**	**Date**

ABSTRACT

Hot springs are among some of the naturally-occurring extreme environments that have generated considerable interest in microbial ecologists worldwide even though there is very little information on hotspring ecosystems in Zambia. Thermophilic prokaryotes present in such extreme habitats are considered valuable sources of biotechnological products including thermally-stable enzymes applied in many research and manufacturing processes. Fifteen water samples were aseptically collected from three different sampling points at three different temperatures of 50°C, 55°C and 60°C at Chinyunyu hot spring in Zambia. The recorded pH of the water at the sampling time was 9.0. One hundred microlitres of each sample was inoculated on nutrient agar medium and incubated at 50°C, 55°C and 60°C. A total of 13 phenotypically distinct isolates were identified from all the three incubation temperatures. The isolated colonies were subsequently cultured at 55°C and pH of 7.0. Their genomic DNA was PCR-amplified using 16S rRNA primers and sequenced by the Sanger dideoxy chain termination method. Amplicon sequences were analyzed using Basic Local Alignment Search Tool (BLAST) algorithm. All the thirteen isolates were Gram-positive, catalase positive, rod-shaped cells. All isolates belonged to the bacterial phylum Firmicutes and seven were affiliated to the genus *Anoxybacillus* while six were affiliated to the genus *Bacillus*. The study revealed that Chinyunyu hot spring harbors genetically diverse thermophilic prokaryotes which could be amenable to further studies to exploit the hot spring microbiome for its biotechnological potential.

Key words: Chinyunyu hot spring, Thermophilic prokaryotes, 16S rRNA and thermally-stable enzymes

DEDICATION

I dedicate this dissertation to my family. Thank you for the support that you have continued to give me and may God bless you abundantly.

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ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
bp	Basepair
Corp	Corporation
cDNA	Complementary deoxyribonucleic acid
cm	Centimetre
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
e.g.	For example
et al.	And others
etc.	And so on
Fig	Figure
g	Gram
h	Hour
i.e.	That is
M	Molar
MEGA	Molecular Evolutionary Genetics Analysis
mg	Milligram
min	Minute
mL	Millilitre
mM	Millimolar
NA	Nutrient Agar
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
NJ	Neighbor Joining
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
pH	Potential of Hydrogen
pmol	Picomoles
RNA	Ribonucleic acid

rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
sp.	Species
v	version
µg	Microgram
µL	Microlitre
µM	Micromolar
°C	Degree Celsius
%	Per cent

CHAPTER ONE - INTRODUCTION

1.1 Overview

This chapter establishes the background to the study and a statement of the problem. The aim of the study is stated as well as the specific objectives and hypothesis. The significance of the study is also elaborated.

1.2 Background

Microorganisms comprise a major part of living organisms on earth and represent the largest source of unexplored biodiversity (Sogin *et al*., 2006; Vitorino and Bessa, 2018). They perform numerous functions essential to the biosphere such as decomposition of organic matter and recycling of nutrients. They are also used for the commercial and industrial production of biotechnological products such as antibiotics, organic acids and enzymes (Hardwaj and Garg, 2012; Poli *et al*., 2017). The numerous functions of microorganisms essential to the biosphere and to human economics provide a strong rationale for understanding their diversity, conservation and exploitation (Verma *et al*., 2014; Naeem *et al*., 2016).

Temperature is a vital parameter for microbial growth. Based on their optimum growth temperature, microorganisms are categorized as psychrophiles, mesophiles and thermophiles (Baltaci *et al*., 2017). Psychrophiles have an optimum growth temperature that lies between -5°C and 15°C. They inhabit terrestrial and aquatic habitats that are permanently or seasonally subjected to cold temperatures (Cavicchioli, 2015; Dalmaso *et al*., 2015). Mesophi1es grow optimally between 25°Cand 45°C. They represent the majority of microorganisms and inhabit almost all terrestrial environments and water bodies such as rivers, lakes, wells, springs and marine environments (Bauman, 2004; Willey *et al*., 2008). Thermophiles are a group of extremophilic microorganisms whose optimum growth temperature lies between 45°C and 80°C (Rothschild and Mancinelli, 2001; Bendia *et al*., 2018). Another group of thermophiles has recently been classified as hyperthermophiles. These grow optimally at temperatures above 80°C (Adhikari *et al*., 2015; Nshimiyimana *et al*., 2018). Most thermophiles and hyperthermophiles are prokaryotes, that is, bacteria and archaea. They are able to grow at elevated

temperatures because they possess unique thermal stable macromolecules (Stetter *et al*., 1993; Haki and Rakshit, 2003). Growth at elevated temperatures enables them to exhibit high metabolic rates which result in greater end products as compared to mesophiles. Thermophilic prokaryotes express enzymes that are stable at high temperatures, which make them useful for pharmaceutical, food processing and chemical industries (Baltaci *et al*., 2017). In addition to thermostability, enzymes extracted from thermophiles are also stable against detergents, organic solvents, high acidity and alkalinity, hence their wide application in various industries (Bhalla *et al*., 2013; Dettmer *et al*., 2013).

Natural habitats for thermophiles include various geothermally heated regions such as hot springs, deep sea hydrothermal vents and volcanic craters (Mohammad *et al*., 2017). They have been discovered in thermal lands, deserts and in engineered systems operated at high temperatures (Aanniz *et al*., 2015; Yanmis *et al*., 2015). Thermophiles have also been isolated from fermenting material that can produce heat such as compost heaps (Saha and Santra, 2014) and garbage landfills (Bosma *et al*., 2015). Among the many extreme environments for thermophiles, hot springs have received a lot of interest from researchers. They are a good source of thermophilic prokaryotes that have become valuable sources for biotechnological products (Adhikari *et al*., 2015).

Hot springs are produced by the emergence of geothermally heated groundwater that rises from the earth's crust (Arya *et al*., 2015). The temperature of hot spring water ranges from warm to that of boiling water and it contains a range of dissolved minerals and traces of gases whose composition is determined by the properties of the parent rock (Homma and Tsukahara, 2008). The diversity of thermophiles in hot springs is highly dependent on parameters such as temperature, pH and mineral composition of the hot spring water (Narayan, 2008; Lau *et al*., 2009).

Zambia is home to several hot springs located in different regions of the country. According to Legg (1974), most of these are related to young faults affecting Karroo rocks, and some of them are related to the rift valley system of East Africa. Chinyunyu hot spring located in Rufunsa district, Lusaka province, is one of the most famous hot springs in Zambia. Previous studies conducted at the hot spring were aimed at

determining the potential of the hot spring for production on a commercial scale of dissolved salts and also the potential for exploitation of thermal energy, either for power generation or for other purposes (Legg, 1974). However, no studies describe thoroughly the diversity of thermophilic prokaryotes in Chinyunyu hot spring especially at the molecular level.

Characterization of thermophilic prokaryotes from the hot springs requires the use of conventional methods (morphological, physiological and biochemical methods). However, conventional methods alone are not sufficient and reliable for the differentiation of microorganisms at species and sub-species level (Donelli *et al*., 2013). The limitation of conventional methods lies in part in their reliance on qualitative characteristics such as colony morphology as well as their dependency on culturing techniques. The latter point is of importance since not all microorganisms may be easily culturable. Molecular based methods such as 16S ribosomal RNA gene sequencing which involve analysis of genomic DNA extracted from the prokaryotic isolates are more reliable in terms of microbial identification. In addition, molecular methods are able to reveal the phylogenetic relationships between isolated thermophilic strains (Farber, 1996; Emerson *et al*., 2008). The aim of this study was to characterize thermophilic prokaryotes from Chinyunyu hot spring in Zambia and to assess their phylogenetic relationships.

1.3 Statement of the Problem

Hot springs are a good source of thermophilic prokaryotes which express thermostable enzymes that are useful in pharmaceutical, food processing and other industries (Mehta *et al*., 2016; Mohammad *et al*., 2017). Chinyunyu hot spring in Zambia provides a unique location that could be a potential source of thermophilic prokaryotes with potential to develop industrial products. However, there are currently no records of microbial diversity in this and other hot springs in the country. This could be because there have not been any studies so far conducted in the hot spring with a view to understanding the microbial diversity of the environment. The biotechnological potential of the hot spring microbiome remains unexploited.

1.4 Significance of the Study

This study was undertaken to generate baseline data on the diversity of thermophilic prokaryotes in Chinyunyu hot spring. These findings could be useful in understanding the diversity of the prokaryotic microorganisms in the hot spring and the generated results could serve as a springboard for comprehensive analysis of the microbial ecology within Zambian hot spring ecosystems as well as the exploitation of the biotechnological products such as thermally-stable enzymes from identified microorganisms of the hot spring microbiome.

1.5 Aim of the Study

The aim of the study was to isolate and characterize thermophilic prokaryotes from Chinyunyu hot spring in Zambia and to examine their phylogenetic relationships.

1.6 Specific Objectives

The specific objectives of the study were to:

i. Isolate thermophilic prokaryotes from Chinyunyu hot spring.
ii. Characterize and identify thermophilic prokaryotes using morphological, biochemical and molecular approaches.
iii. Examine the phylogenetic relationships of thermophilic prokaryotic isolates.

1.7 Study Hypothesis

Chinyunyu hot spring harbors genetically diverse thermophilic prokaryotes.

CHAPTER TWO - LITERATURE REVIEW

2.1 Overview

This chapter reviews literature pertinent to this study. The discussion is intended to highlight efforts by several researchers on the isolation and characterization of thermophilic prokaryotes from hot springs. The review was also conducted to identify the current themes and methods used to study extreme microbial ecosystems, as well as recent results and gaps in results generated from related studies. The review is presented under the following subheadings; (1) Thermophilic microorganisms (2) Thermophilic environments (3) Diversity of thermophilic prokaryotes in hot springs (4) Identification methods for thermophilic prokaryotes (5) Biotechnological and industrial applications of thermophilic prokaryotes.

2.2 Thermophilic microorganisms

Microorganisms living under extreme environmental conditions continue to be discovered. Such microorganisms, known as extremophiles thrive in environments that previously were thought to be uninhabitable by any form of life. They thrive in extreme environments characterized by, for example, physical conditions such as extreme temperatures, pressures and chemical conditions such as high salinity and high acidity or alkalinity (Bauman, 2004; Rampelotto, 2013). Extremophiles are classified on the basis of the characteristics of the environments in which they live. Thus, for example, thermophiles and halophiles are found in environments characterized by high temperature and salinity, respectively (Satyanarayana, 2005). Thermophiles are one of the most studied groups of extremophiles. They are a group of extremophilic microorganisms that thrive in high temperature environments (Mohammad *et al*., 2017; Nshimiyimana *et al*., 2018). Their ability to survive in conditions that would kill other microbes is attributed to the highly thermostable macromolecules they posses (Stetter *et al*., 1993; Haki and Rakshit, 2003). Thermophilic microorganisms express enzymes that are not only stable at high temperatures but also stable against denaturing agents such as detergents, organic solvents, high acidity and high alkalinity (Bhalla *et al*., 2013; Dettmer *et al*., 2013). The thermostable enzymes expressed by thermophiles have a wide range of applications, particularly in pharmaceutical, food processing, detergent

manufacturing, agriculture, paper manufacturing, water purification, bioremediation, mining and petroleum recovery industries (Demirjian, 2001; Nigam, 2013; Lele and Deshmukh, 2016; Dumorné *et al*., 2017).

2.2.1 Classification of thermophiles

Temperature is a vital parameter for growth of microorganisms and has always been used in microbial systematics as a basis for classifying microbes. Based on optimum growth temperatures, microorganisms are categorized as psychrophiles, mesophiles and thermophiles (Baltaci *et al*., 2017). Psychrophiles have an optimum growth temperature that is below 20°C (Abe and Horikoshi, 2001; Siddiqui *et al.*, 2013; Cavicchioli, 2015; Dalmaso *et al*., 2015). Mesophiles grow optimally between 20°C to 40°C whilst thermophiles thrive in temperatures above 45 °C (Rothschild and Mancinelli, 2001; Willey *et al*., 2008).

Thermophiles are placed in different categories according to their temperature requirement. They are classified as moderate thermophiles, extreme thermophiles and hyperthermophiles. According to Sharma *et al*. (2013), moderate thermophiles have an optimum growth temperature that lies between 50°C and 60°C whereas extreme thermophiles grow optimally between 60°C and 80°C. Hyperthermophiles, however, require very hot environmental conditions for survival. Their optimum growth temperature lies between 80°C and 110°C (Stetter, 2006). Thermophiles can also be classified as obligate and facultative thermophiles. Obligate thermophiles, also known as true thermophiles, grow optimally at 60-70°C but show little or no growth at temperatures below 45°C. Facultative thermophilic microorganisms show optimal growth between 50°C and 55°C. However, they exhibit little growth even at 25°C (Baker *et al*., 2001; Demirjian *et al*., 2001; Reed *et al*., 2013).

2.2.2 Thermophilic prokaryotes

According to Woese *et al*. (1990), all living organisms belong to one of three domains of life, namely, Bacteria, Archaea, and Eukarya. Bacteria and archaea are prokaryotic domains, that is, all living organisms belonging to these domains lack a true nucleus and other membrane bound organelles. The eukarya domain includes all other living

organisms that have a true nucleus and other membrane bound organelles (Shively, 2006; Murat *et al*., 2010). The classification of all organisms into the three domains by Woese *et al*. (1990) was based on comparisons of nucleotide sequences from the small subunits of the ribosomal RNA genes of living organisms. Thermophilic microorganisms from all the three domains of life have been discovered. Studies have showed that most thermophilic microorganisms isolated from high-temperature environments are thermophilic prokaryotes (Madigan *et al.*, 1997). Thermophilic fungi are the only thermophilic eukaryotic microorganisms that have been discovered (Maheshwari, 2000).

Thermophilic prokaryotes thrive in environments characterized by temperatures exceeding45°C. They have been isolated from different geothermally heated regions such as hot springs, deserts, thermal lands, compost heaps, deep sea hydrothermal vents and volcanic craters (Yanmis *et al*., 2015; Mohammed *et al*., 2017). Based on their optimum growth temperatures, several thermophilic bacteria and archaea have been classified as described in Table 2.1.

Table 0.1: Examples of members of the various classes of thermophilic microorganisms

Microbial class	**Examples**	**References**
Moderate thermophiles	*Bacillus caldolyticus*, *Geobacillus stearothermophilus*, *Thermoactinomyces vulgaris*, *Clostridium thermohydrosulfuricum*, *Thermoanaerobacter ethanolicus*, *Thermoplasma acidophilum.*	Stetter *et al*., 1998; Rampelotto, 2013
Extreme thermophiles	*Thermus aquaticus*, *Thermodesulfobacterium commune*, *Sulfolobus acidocaldarius*, *Thermomicrobium roseum Dictyoglomus thermophilum*, *Methanococcus vulcanicus*, *Sulfurococcus mirabilis*, *Thermotoga maritima.*	Brock and Freeze, 1969; Bertoldo *et al.*, 2002; Ghosh *et al*., 2003
Hyperthermophiles	*Methanoccus jannaschii*, *Acidianus infernos*, *Archaeoglobus profundus*, *Methanopyrus kandleri*, *Pyrobaculum islandicum*, *Pyrococcus furiosus*.	Rampelotto,2013; Dennett and Blamey,2016

Most hyperthermophiles belong to the archeal domain. *Thermotoga* and *Aquifex* are among few bacterial species belonging to the hyperthermophile group and are able to survive temperatures above 80°C (Huber and Stetter, 1998; Bocchetta *et al*., 2000).

2.3 Thermophilic environments

Microbial thermophiles have been discovered in diverse extreme environments including hot water springs, deep sea hydrothermal vents and volcanic craters (Aanniz

et al., 2015; Yanmis *et al*., 2015; Mohammed *et al*., 2017). They also have been isolated from engineered systems operated at high temperatures such as laundry heaters (Clive, 1990; Roberto *et al*., 2018), compost heaps (Saha and Santra, 2014), garbage landfills (Bosma *et al*., 2015), ultra-deep mines and deep basalt aquifers (Brett *et al*., 2003).

2.3.1 Hot springs

Hot springs, also called thermal springs are a good source of thermophilic prokaryotes (Hussein *et al*., 2017). They are hot water pools created by the emergence of geothermally heated underground water. According to Power *et al*. (2018), a hot spring can either be volcanic or non-volcanic. In areas near active volcanic zones, underground water becomes heated when it comes in contact with magma. The heated water finds its way to the surface through cracks and faults in the earth's crust which results in formation of either a hot spring or a geyser. A hot spring is formed when the heated water rises slowly whilst a geyser is formed when the water rises quickly from the ground (Satyanarayana *et al*., 2005; Power *et al*., 2018). A non-volcanic spring on the other hand is formed when water becomes heated as it comes into contact with hot rocks within the earth's crust (Zaher *et al*., 2012). The heated water then rises up to create hot springs.

The diversity of thermophiles in a hot spring is to a large extent dependent on parameters such as temperature and pH of the hot spring water (Narayan, 2008; Lau *et al*. 2009). While the temperature of water in one hot spring tends to remain constant over time, the temperature among different hot springs greatly varies and can range from being slightly warm to being too hot to touch (Huber and Stetter, 1998). The pH among different hot springs can also vary significantly (Rajapaksha *et al*., 2014). Most terrestrial hot springs are alkaline whilst hot springs found in areas with active volcanoes or shallow magma pools are usually acidic. According to Huber and Stetter (1998), the difference in pH is reflected in the biodiversity of the hot spring. Acidophilic thermophiles such as *Sulfulobus* thrive in acidic hot springs whereas in neutral or moderate hot springs, a diverse number of thermophiles such as *Thermoproteus, Pyrobaculum, Methanothermus, Desulforococcus and Thermofilum* are present (Huber and Stetter 1998). Hot springs also contain minerals and traces of gases

(Clive, 1990; Lengeler *et al*., 1999). The chemical composition of hot springs varies from one hot spring to another and is dependent on the properties of the parent rock (Homma and Tsukahara, 2008).

Hot springs are widely distributed and have been studied by various groups around the world. Examples of places where they have been discovered include Rwanda (Nshimiyimana *et al*., 2018), South Africa (Tekere *et al*., 2011), Turkey (Mohammed *et al*., 2017), Italy (Maugeri, 2001), India (Sharma *et al*., 2012), Morocco (Aanniz *et al*., 2014), China (Lau *et al*., 2009), Greece (Sievert *et al*., 2000), and Iceland (Takacs *et al*., 2001) among many other places.

2.3.2 Hot springs in Zambia

Although there is no evidence of recent volcanic activity in Zambia, there are numerous hot springs that are located in different regions of the country. According to Legg (1974), most of the hot springs are related to young faults affecting Karroo rocks, and some of them related to the rift system of East Africa. Chinyunyu hot spring is one of the most famous hot springs in Zambia and is located in Rufunsa district about 80 kilometers from the capital, Lusaka. According to Legg (1974), the spring belongs to a heterogeneous group of isolated hot springs known as the South-eastern group. The group stretches from the northern part of Lukasashi Valley, through the Luano Valley, to the Zambezi River at Lake Kariba. The spring occurs in an area underlain by basement rocks on an extension of the young faults which define the margins of the Mwapula re-entrant on the southern side of the Luano Valley (Legg, 1974). Previous studies conducted on the hot spring were aimed at determining the potential for production on a commercial scale of dissolved salts and also the potential for thermal energy, either for power generation or for other purposes.

2.4 Diversity of thermophilic prokaryotes in hot springs

Hot springs are a source of novel thermophilic prokaryotes that are capable of providing biotechnologically useful products (Mehta *et al*., 2016). The discovery of *Thermus aquaticus* in hot springs of Yellow Stone National Park in the United States of America, from which thermotolerant DNA polymerase used in Polymerase Chain Reaction was

isolated, sparked interest in the study of hot springs and the biodiversity within these extreme environments (Garibyan and Avashia, 2013). Since then, diverse thermophilic prokaryotes have been and continue to be isolated from hot springs around the world. The diversity of thermophilic prokaryotes in hot springs varies greatly due to differences in physical and chemical conditions, biogeography and geological history (Narayan, 2008; Lau *et al*., 2009).

Nshimiyimana *et al*. (2018) isolated and characterized thermophiles from Nyamyumba hot spring in Rwanda. Morphological and biochemical characterizations revealed that *Bacillus* species and *Thermus aquaticus* were present in the hot spring. The researchers also established that the isolates were capable of producing thermostable enzymes.

Kambura *et al.* (2016) determined the diversity and community structure of bacteria and archaea within three hot springs of Lake Magadi and Little Magadi in Kenya. The study combined illumina sequencing and analysis of amplicons of both total community rDNA and 16S rRNA cDNA. Three thousand four hundred twenty-six and one thousand nine hundred thirteen OTUs were recovered from 16S rDNA and 16S rRNA cDNA respectively. Uncultured diversity accounted for 89.4 % 16S rDNA and 87.6 % 16S rRNA cDNA reads. The most abundant phyla in both the 16S rDNA and 16S rRNA cDNA datasets included Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Euryarchaeota in all samples.

Baltaci *et al*. (2017) isolated, identified and characterized thermophilic bacteria from different hot springs in Turkey using conventional (morphological, physiological and biochemical tests) and molecular methods (fatty acid methyl esters, GTG5-PCR and 16S rRNA sequencing). The isolates were then tested for their ability to produce enzymes such as lipase, protease, amylase and cellulase. All the isolates were capable of producing industrially valuable enzymes based on screening. Most of the isolates could produce at least two or more enzymes.

Tekere *et al*. (2011) investigated bacterial diversity of Siloam hot water spring, Limpopo, South Africa using 454 pyrosequencing of two 16S rRNA variable regions (V1-3) and (V4-7). Analysis of the community DNA revealed that the phyla

Proteobacteria, Cyanobacteria, Bacteriodetes, Planctomycetes, Firmicutes, Chloroflexi and Verrucomicrobia were the most abundant. The bacterial diversity was greater when the V4-7 variable region was used compared to the V1-3 region. The most abundant bacteria detected with region V1-3 were *Stenotrophomonas, Aquaspirillum*, *Zavarzinella*, *Haliscomenobacteria*, *Rheinheimera* and *Tepidomonas*.

The microbial diversity of five major hot springs in Jordan was studied by Malkawi and Al-Omari (2010) using both phenotypic and molecular approaches. Out of 132 isolates, 125 were Gram positive rods, while the other seven were Gram positive coccobacilli. Majority of isolates were affiliated to the genus *Bacillus*.

Culture-dependent and culture-independent approaches were used to study the diversity of *Crenarchaeota* in terrestrial hot springs of the Kamchatka Peninsula, Lake Baikal region (Russia) and Iceland by Perevalova *et al.* (2008). They reported a large and diverse group of uncultured *Crenarchaeota* inhabiting these hot springs which were moderately thermophilic in nature (55 to 70° C). One of the lineages of this group was identified as '*Fervidococcus fontis'*, which was moderately thermophilic, neutrophilic (optimum pH of 6.0-7.5) and anaerobic organotroph.

As is clear from the foregoing, hot springs and other extreme environments harbor a large diversity of microorganisms, most of which have not yet been isolated and characterized (Saxena *et al*., 2017). Chinyunyu hot spring in Zambia provides a unique location to explore novel thermophilic prokaryotes and evaluate their biotechnological potential.

2.5 Identification methods for thermophilic prokaryotes

Classification methods for thermophilic prokaryotes can essentially be divided into two separate groups, namely phenotypic and genotypic characterization methods.

2.5.1 Phenotypic characterization methods

Phenotypic methods comprise all characterization methods that are not based on DNA or RNA (Willey *et al*., 2008). They include methods to characterize the morphological, physiological and biochemical features of the microorganism. Morphological

characterization involves studying features such as shape, colony color, dimension, form, endospore formation, flagella, inclusion bodies and Gram staining properties of the microorganism among others (Willey *et al*., 2008). Physiological characterization involves examination of features such as growth at different temperatures, pH, salt concentrations, atmospheric conditions, ability to metabolize different carbon sources, resistance to antibiotics, and production of different metabolic products and enzymes (Sokolova *et al*., 2001; Seyfried *et al*., 2002; Zavarzina *et al*., 2002). Biochemical characterization is composed of a varied set of methodologies. These include analysis of cell wall composition, polar lipids and fatty acid composition, whole protein analysis, antigenic analysis and isoenzyme analysis (Kim *et al*., 1999; Chung *et al*., 2000).

Phenotypic characterization is useful in preliminary description of prokaryotes from species up to genus and family. However, some phenotypic characteristics are affected by certain environmental or culture conditions, hence phenotypic methods alone may not be adequate and reliable for the differentiation of microorganisms (Farber, 1996). Results from phenotypic characterization of microorganisms must therefore be evaluated together with genotypic results in order to identify an unknown organism.

2.5.2 Genotypic characterization methods

Genotypic characterization methods are based on the analysis of DNA or RNA from an organism (Farber, 1996). As compared to phenotypic characterization, genotypic characterization is not affected by the environment or physiological state an organism (Donelli *et al*., 2013). In addition, genotypic methods have many advantages over phenotypic methods in terms of factors such as reproducibility (the ability to repeatedly obtain the same typing profile), discriminatory power (ability to produce results that can distinguish two closely related strains) and ease of interpretation (Farber, 1996; Donelli *et al*., 2013).

2.5.2.1 16S ribosomal RNA gene (16S rDNA) sequencing

One of the most common genotypic methods for identification of thermophilic prokaryotes is 16S rRNA gene sequence analysis (Janda and Abbott, 2007). According to Willey *et al*. (2008), ribosomes are complex structures that occur in all living cells whose function is to synthesize proteins. They consist of two subunits, each of which is composed of protein and a type of RNA, known as ribosomal RNA (rRNA). Prokaryotic ribosomes consist of 30S subunit (small sub unit) and 50S subunit (large sub unit) which together make up the complete 70S ribosome. The 16S ribosomal RNA is a component of the 30S small subunit and the gene encoding for its rRNA is called 16S rDNA (Madigan *et al*., 1997). The approximate length of the 16S rRNA gene is 1522 bp and it consists of conserved and hyper variable regions. The hyper variable regions present in the 16S rRNA gene provide a species-specific signature sequence which is useful for prokaryotic identification (Rosselli *et al*., 2016). Some regions of the gene are universally conserved and suitable for phylogenetic studies of distantly related organisms (Willey *et al*., 2008). The 16S rRNA gene sequencing method has been used to characterize thermophilic prokaryotes from hot springs by many research groups.

Thermophilic bacteria from hot spring of Bhurung, Nepal were isolated and characterized by Adhikari *et al*. (2015). Bacteria isolates that could grow at high temperatures and tolerate extreme temperature were characterized by morphology, biochemistry and 16S rRNA gene sequence. The isolates were also screened for production of extracellular enzymes like proteases, amylases, lipases, cellulases, caseinases, pectinases and xylanases. Fifteen isolates with ability to tolerate high temperatures were identified as *Bacillus* sp. BLAST search analysis of the isolated nucleotide sequence showed maximum identity (99% similarity) with *Bacillus licheniformis, Bacillus subtilis and Bacillus pumilus*. The study confirmed the isolated *Bacillus* sp. to be true thermophiles that could be a source of various thermostable exozymes.

Kumar *et al.* (2014) analyzed and characterized the diversity of culturable thermotolerant bacteria in Manikaran hot springs (India) by employing culture methods and 16S rRNA gene sequencing. Sequencing of the 16S rRNA gene of 42 representative isolates revealed that the majority of isolates belonged to Firmicutes, followed by equal representation of Actinobacteria and Proteobacteria.

2.6 Biotechnological and industrial applications of thermophilic prokaryotes

Thermophilic prokaryotes express thermostable enzymes that are of great importance in various industries where they are used as biocatalyst (Adhikari *et al*., 2015; Daupan and Rivera, 2015). Thermostable enzymes catalyze biochemical reactions at higher temperatures resulting in greater end products than enzymes derived from mesophiles (Demirjian *et al*., 2001). In addition, the thermostable enzymes are also stable against denaturing agents such as detergents, organic solvents, high acidity and high alkalinity (Bhalla *et al*., 2013; Dettmer *et al.,* 2013). These attributes enable them to resist harsh industrial processing conditions which make them more attractive than enzymes from mesophiles. In addition, biotechnological reactions catalyzed by thermostable enzymes are less prone to contamination because of the increased temperature (Turner *et al.,* 2007). The increase in temperature of thermostable enzyme catalyzed biotechnological processes enhances bioavailability and solubility of organic compounds (Nigam, 2013). Thermostable enzymes are useful in fermentation processes. Unlike heat sensitive enzymes that require cooling to prevent them from being denatured during fermentation, thermostable enzymes do not require cooling. The use of thermostable enzymes as opposed to heat sensitive enzymes is cost effective as the process of cooling contributes about ten percent of the total energy cost in fermentation processes (Niehaus *et al.,* 1999). The main advantages of thermostable enzymes are shown in Table 2.2.

Table 0.2: Main advantages of high temperature and thermostable enzymes

Property	**Advantage in process**
Thermostability	Tolerate high temperatures
High optimum temperature	Little activity at low temperatures, long shelf life
Resistance to denaturing agents	Tolerate organic solvents, high and low pH
Solubility	High concentrations of poorly soluble compounds
Viscosity	Decreases, mixing and pumping can be accelerated, mass transfer rate increases
Microbial contamination	Growth of pathogens and undesired microbial contaminants is prevented
Reaction rates	Diffusion and chemical reaction rates are accelerated

Source: (Niehaus *et al*., 1999; Yavuz *et al*., 2004).

Thermophilic prokaryotes produce several industrially important thermostable enzymes. These are broadly grouped into three major categories, namely: protein-degrading enzymes (proteases), lipid-degrading enzymes (lipases and phopholipases) and carbohydrate-degrading enzymes such amylases, cellulases, xylanases and chitinases (Haki and Rakshit, 2003).

2.6.1 Protein degrading enzymes

Proteases (peptidases) are enzymes that catalyze the digestion of proteins into short peptides or free amino acids by cleavage of peptide bonds (Sookkheo *et al*., 2000; Haki and Rakshit, 2003). Based on their site of action, proteases are classified as either exopeptidases or endopeptidases. According to Rao *et al*. (1998), exopeptidases cleave the peptide bond close to the amino or carboxy termini of the substrate, whereas endopeptidases cleave the peptide bonds distant from the termini of the substrate. They can also be further divided into four groups based on the functional group present at the active site. These are serine proteases, aspartic proteases, cysteine proteases and metalloproteases (Rao *et al*., 1998).

Proteases are widely used in various industries and account for about 60 % of the world enzyme market (Rao *et al.*, 1998; Rigoldi *et al.*, 2018). They are produced by various organisms including animals, plants, fungi and bacteria. Thermophilic prokaryotes, however, are a preferred source of proteases because they produce thermostable proteases. Some thermostable proteases are used in leather treatment and bioremediation processes (Rao *et al.*, 1998; Rigoldi *et al.*, 2018). They are also used in detergent, pharmaceutical, animal feed, leather and food industries (Gupta *et al.*, 2002; Tambekar *et al.*, 2009; Dabananda and Kshetri, 2010; Rigoldi *et al.*, 2018; Roberto *et al.*, 2018). The main uses of proteases in several industries are shown in Table 2.3.

Table 0.3: Industrial applications of thermostable proteases

Industry	**Applications of Proteases**
Photography	Bioprocessing of used X-ray or photographic films for silver recovery.
Detergent	To remove protein-based stains from clothes.
Food Bread / confectionery Cheese production Meat Beverage	 To modify gluten elasticity. Casein coagulation and cheese ripening. Meat tenderization. Solubilisation of grain proteins and stabilization of beer.
Leather Industry	To dehair hides and soften leather
Waste management	Lowering the biological oxygen demand of aquatic systems. Management of waste feathers from poultry slaughterhouses
Silk	Degumming of silk.
Chemical	Sucrose-polyester synthesis (biodegradable plastic)

Source: (Rigoldi et al., 2018; Roberto et al., 2018)

2.6.2 Lipid degrading enzymes

Lipases are a group of enzymes that catalyze the hydrolysis and synthesis of esters formed from glycerol and long-chain fatty acids (Haki and Rakshit, 2003). They are produced by plants, animals and microorganisms. Lipases produced by thermophilic

prokaryotes catalyze a wide range of reactions because of their thermostability and resilience to harsh industrial processing conditions (Salihu and Alam, 2015). Lipases are used in industries to catalyze various reactions that include hydrolysis, alcoholysis, esterification, inter-esterification, acidolysis and aminolysis (Joseph *et al*., 2008; Salihu and Alam, 2015). In the manufacturing of detergents, lipases are added for the removal of fat and lipid based strain from clothes. Lipases are also used to produce esters that are useful in the food industry as flavour and aroma constituents (Joseph *et al*., 2008). Other applications include the removal of the pitch from pulp produced in the paper industry, hydrolysis of milk fat in the dairy industry, removal of non-cellulosic impurities from raw cotton before further processing into dyed and finished products, drug formulations in the pharmaceutical industry and in the removal of subcutaneous fat in the leather industry (Gupta *et al,* 2004; Park *et al*., 2005; Joseph *et al*., 2008).

2.6.3 Carbohydrate degrading enzymes

Carbohydrate degrading enzymes are enzymes that hydrolyze glycosidic bonds between two or more carbohydrates (such as starch, cellulose, xylan, chitin and pectin). They can also degrade glycosidic bonds between a carbohydrate and non-carbohydrate (Eichler, 2001). Carbohydrate-degrading thermostable enzymes of industrial importance include enzymes such as amylases, cellulases, xylanases, pectinases and pullulanases. Amylases and pullulanases are widely used in starch industry to hydrolyze and modify starch to produce glucose and various other products (Haki and Rakshit, 2003; Rigoldi *et al*., 2018). Several cellulases, xylanases and pectinases have been applied in biotechnological processes such as biobleaching of paper pulp, production of animal feed, production of fermentable sugars for obtaining biofuel from cellulosic wastes, fruit juice extraction and clarification, refinement of vegetable fibers, degumming of natural fibers, curing of coffee, cocoa and tobacco and also for waste-water treatment (Beg *et al*., 2001; Haki and Rakshit, 2003; Rigoldi *et al*., 2018).

CHAPTER THREE-MATERIALS AND METHODS

3.1 Overview

This chapter describes the methods, approaches and strategies used to execute the study. The methods include a description of how water samples were collected, the isolation of thermophilic prokaryotes, biochemical and non-biochemical characterization of the isolates and analysis of the phylogenetic relationships between the isolates and isolates from related studies.

3.2 Sampling site

Chinyunyu hot spring is located in Rufunsa district, Lusaka province, Zambia (Figure 3.1). Zambia is situated in south-central Africa and is surrounded by Zimbabwe and Botswana to the south, Mozambique to the southeast, Malawi to the east, Tanzania to the northeast, Democratic Republic of Congo to the north, Namibia to the southwest and Angola to the west. Chinyunyu hot spring is situated at15°15'40.9"S Latitude and 29°01'24.6"E Longitude at an elevation of about 990 m above sea level. The spring occurs on the eastern slope of the Chinyunyu Valley, which is about 100m north of the Great East Road. According to Legg (1974), the spring occurs in an area underlain by basement rocks on an extension of the young faults which define the margins of the Mwapula re-entrant on the southern side of the Luano Valley.

Figure 3.1: A Google map showing the location of Chinyunyu hot spring (Google Maps, 2019).

3.3 Collection of samples

Water samples from which prokaryotes used in this study were isolated were collected from Chinyunyu hot spring. The location of the hot spring was determined to be at latitude 15°15'40.9"S and longitude 29°01'24.6"E at the point of sampling using a hand-held Garmin eTrex® GPS system (Southampton, United Kingdom). The ambient temperature at the time of sampling was recorded to be 20°C using a portable thermometer (Fisher Scientific, Mumbai, India) while the pH of the hot spring water measured using a portable pH meter (Thermo Fisher Scientific, Singapore) was pH 9.0.

Water samples were collected in 25ml sterile screw cap bottles from the spring on 21st June, 2018 at three points at which the water was at 60°C, 55°C and 50°C respectively (Figure 3.2). Five water samples were collected at random from each of the three sampling points giving a total of fifteen samples and samples were coded as shown in Table 3.1. Water samples in the screw-cap bottles were enclosed in a zip-lock bag and transported in cooler box to the laboratory at the University of Zambia within 2 hours of collection.

Figure 3.2: Points at which water samples from which prokaryotes used in the study were collected. Five samples of twenty-five milliliters each were collected from the three sampling points with water temperature at 60°C, A; 55°C, B and 50°C, C.

Table 3.1: Water samples collected from Chinyunyu hot spring. Samples collected from sampling point A at 60°C were coded CA1 to CA5, those from point B at 55°C were coded CB1 to CB5 and those from point C were coded CC1 to CC5.

NUMBER	SAMPLING SITE	SAMPLE CODE
1	A	CA1
2	A	CA2
3	A	CA3
4	A	CA4
5	A	CA5
6	B	CB1
7	B	CB2
8	B	CB3
9	B	CB4
10	B	CB5
11	C	CC1
12	C	CC2
13	C	CC3
14	C	CC4
15	C	CC5

3.4 Isolation of thermophilic prokaryotes

The modified method of Adhikari *et al*., (2015) was adopted for the isolation of thermophilic prokaryotes from all the water samples collected from Chinyunyu hot spring. For each of the fifteen water samples (Table 3.1), 100 µl was directly spread plated on nutrient agar (NA) medium (Appendix A), with three replicates every time. The inoculated plates were covered with aluminium foil to prevent the NA from drying and each replicate was incubated at 50°C, 55°C and 60°C respectively, for 24h.

After the incubation period, prokaryotic colony growth was visually inspected to determine the growth characteristics of the isolates from the water samples. Many of the colonies were observed to be morphologically similar on Petri dishes incubated at the three different temperatures but thirteen were observed to be distinct. The distinct colonies were observed on each of the replicate plates incubated at the three different temperatures. Visual inspection of the culture plates after the incubation period also showed that in all the experimental replicates the highest number of colony growth was

at 55°C. This was selected as the temperature choice for subsequent sub-culturing of isolates. The thirteen colonies selected based on distinct colony morphology were purified by sub-culturing on NA medium at 55°C using the streak plate method (Sanders, 2012). These isolates were used in all the subsequent analyses and constituted the study population in the methods described below. The purified colonies were kept in a refrigerator at 4°C for further study.

3.5 Morphological, physiological and biochemical characterization of isolates

The thirteen selected, purified and sub-cultured isolates were coded (C01-C13) and further characterized based on morphology, physiological and selected biochemical characteristics according to the standard methods described in Bergey's manual of Systematic Bacteriology (Vos *et al*., 2009).

3.5.1 Colony morphology

Colony morphologies of the purified isolates were described with special emphasis on color and form.

3.5.2 Gram stain

The Gram-stain was performed according to standard procedure (Cappuccino and Sherman, 2002). A loopful of the overnight culture was spread onto a drop of sterile water on microscope slides using an inoculation loop to form a thin smear. Air dried colony smears were heat-fixed by passing the slide through an open flame for 1-3 seconds. The heat-fixed smears were first stained with crystal violet for 1 min. After rinsing under gentle running tap water, the slides were placed in iodine solution for 1 min followed by washing in 95% laboratory grade ethanol for 6 seconds. They were then counter-stained with safranin for 30 seconds and after staining, the slides were dried on paper towels. The cells were examined under oil immersion using a light microscope at ×100 and observations were recorded.

3.5.3 Biochemical and motility tests

Two biochemical (catalase and oxidase) and motility tests were performed according to standard procedure (Aygan, 2007; Narayan, 2008) to assist in the identification and characterization of the isolates.

3.5.3.1 Catalase test

Isolates were grown on nutrient agar at 55°C for 24 h. 2ml of 3% hydrogen peroxide was poured onto the colonies and observations were recorded.

3.5.3.2 Oxidase test

Isolates were grown on nutrient agar at 55°C for 24 h. A filter paper was placed into a Petri dish and was wetted with 2-3 drops oxidase reagent (1% tetramethyl-*p*-phenylenediamine). One large colony was taken with a sterile glass rod and placed onto the wet filter paper and observations were recorded.

3.5.3.3 Motility test

The Craigie tube method was used to detect the motility of the isolates (Aygan, 2007). Isolates were grown on nutrient agar at 55°C for 24 hours. A hollow tube was placed in semi-solid NA inside a bottle. A loop full of culture was inoculated into the medium in the hollow tube and the setup was incubated at 55°C for 24 h and observations were recorded.

3.5.4 Screening of isolates for temperature tolerance

The effect of incubation temperature on the growth of members of the selected population of the isolates was studied. The isolates were spread-plated on NA media and incubated at 37°C, 45°C, 50°C, 55°C, 60°C and 65°C. Growth as measured by colony size was visually inspected after 48 hours of incubation at the different temperatures. The level of growth was scored using four levels of positive sign, whereby one positive (+) indicated minimal growth, two positives (+ +) indicated moderate growth, and three positives (+ + +) indicated satisfactory growth while four

positives (+ + + +) indicated excellent growth. No growth was indicated by a negative (-) sign.

3.6 Molecular characterization of isolates

To determine the diversity of the isolates, colonies showing distinct colony phenotypes were selected for molecular characterization. Molecular characterization of the isolates was performed at the School of Veterinary Medicine, Disease Control Department. Isolates were grown on NA at 55°C and DNA was isolated and used in PCR-amplification reactions as described below.

3.6.1 Extraction of genomic DNA

The modified method of Dashti *et al*. (2009) was adopted for extraction of genomic DNA from isolates which were grown on NA at 55°C for 24 h. A loopful of two colonies of each isolate was transferred and suspended in eppendorf tubes containing 100 µl of sterile distilled water and boiled for 10 minutes on a heating block (Thomas Scientific Brand). Boiled samples were centrifuged at 25°C for five minutes at 6000 ×g in a Hemle Z233 MK microcentrifuge (HermleLabortechnik, Wehingen, Germany) and 5 µl of the supernatant from each isolate was used for the PCR.

3.6.2 PCR amplification of 16S rRNA gene

Amplification of the 16S rRNA gene was conducted using universal primer pair combination of forward primer 8F (5'AGAGTTTGATCCTGGCTAG3') and reverse primer 1492R (5'CGGCTACCTTGTTACGACTT3') (Baker *et al*., 2003). The amplification was performed using a Veriti 96 well Thermal Cycler (Applied Biosystem, USA). Amplification was carried out in 40µl reaction mixtures containing 5 µl of template DNA, 5 µl of PCR buffer (×10), 3 µl dNTP's (2.5mM), 1 µl (5 pmol) of 8F forward primer, 1 µl (5pmol) of 1492R reverse primer, 0.3 µl *Taq* polymerase and 24.7 µl of water. The control contained all the above except the DNA template. Reaction mixtures were subjected to the following temperature cycling profiles repeated for 35 cycles: Initial DNA denaturation step and activation of the enzyme at 96 °C for five minutes, cycle denaturation at 95 °C for 30 seconds, primer annealing at 53 °C for

30 seconds, chain extension at 72 °C for 1.0 minute and a final extension at 72 °C for 10 minutes (Roux, 1995). Amplification products (5 µl) were separated on 1 % agarose gel stained with 10 µg/ml ethidium bromide in 1× TBE buffer and visualized under ultraviolet light (Sambrook *et al*., 1989).

3.6.3 Purification of PCR products

To obtain DNA for sequencing, PCR products were isolated from the agarose gel and purified using the QIAquick PCR purification Kit protocol (Qiagen, Germany) according to manufacturer's instructions.Agarose gel with DNA fragments was cut and transferred to eppendorf tubes. The gel was then dissolved in Buffer QG and loaded to spin column and isolated using a simple bind/wash/elute procedure. The purified DNA was then eluted into elution buffer and sequenced.

3.6.4 DNA Sequencing

PCR products were sequenced using the Big Dye Terminator v3.1 cycle sequencing kit on the ABI Prism 3130*xl*Genetic Analyzer (Life Technologies Corp). Partial sequences were generated using the universal primers (8F and 1492R).

3.6.5 BLAST and phylogenetic analyses of nucleotide sequences of the isolates

The 16S rDNA gene sequences of the 13 isolates were compared to the 16S rRNA databases using Basic Local Alignment Search Tool (BLAST) algorithm at the National Center for Biotechnology Information website (http://www.ncbi.nlm.gov). The 16S rDNA gene sequences on NCBI with high similarity to the isolated sequences were retrieved and added to the sequences from this study. Sequence alignment was done using MUSCLE in MEGA 6 (Tamura *et al*., 2013). A phylogenetic tree was constructed from the aligned dataset using neighbor-joining (NJ) (Saitou and Nei, 1987) with the MEGA 6 package.

CHAPTER FOUR- RESULTS

4.1 Overview

This chapter presents the findings on the isolation, characterization and identification of thermophilic prokaryotes from Chinyunyu hot spring in Zambia.

4.2 Physical characteristics of the sampling site

The population structure and diversity of microorganisms in a natural ecosystem depends in part on the prevailing pH and temperature of the ecosystem being considered. The temperature and pH at each of the three sampling sites were recorded at the time of sampling and the results were tabulated as shown in Table 4.1. While the pH of the water was similar at the three sites, temperatures varied from 50°C to 60°C.

Table 4.1: Temperature and pH of water samples at the time of collection. While the pH of the water was similar at the three sites, temperatures varied from 50°C to 60°C.

Sampling site	**pH**	**Temperature (°C)**
A	9.0	60
B	9.0	55
C	9.0	50

[The GPS Position of sampling was Latitude: 15°15'40.9"S and Longitude: 29°01'24.6"E.]

4.3 Isolated thermophilic prokaryotes

Different colony morphologies were observed on all NA agar plates incubated at different temperatures. Isolates were selected based on differences in colony morphologies and purified (Figure 4.1). Some colonies had spreading and irregular edges (for example Figure 4.1A and Figure 4.1C) while others such as those recorded in Figure 4.1 B, E and F had colonies with distinct and regular edges. Other colonies also secreted significant amounts of exopolysaccharides. The purified isolates were clearly different from each other.

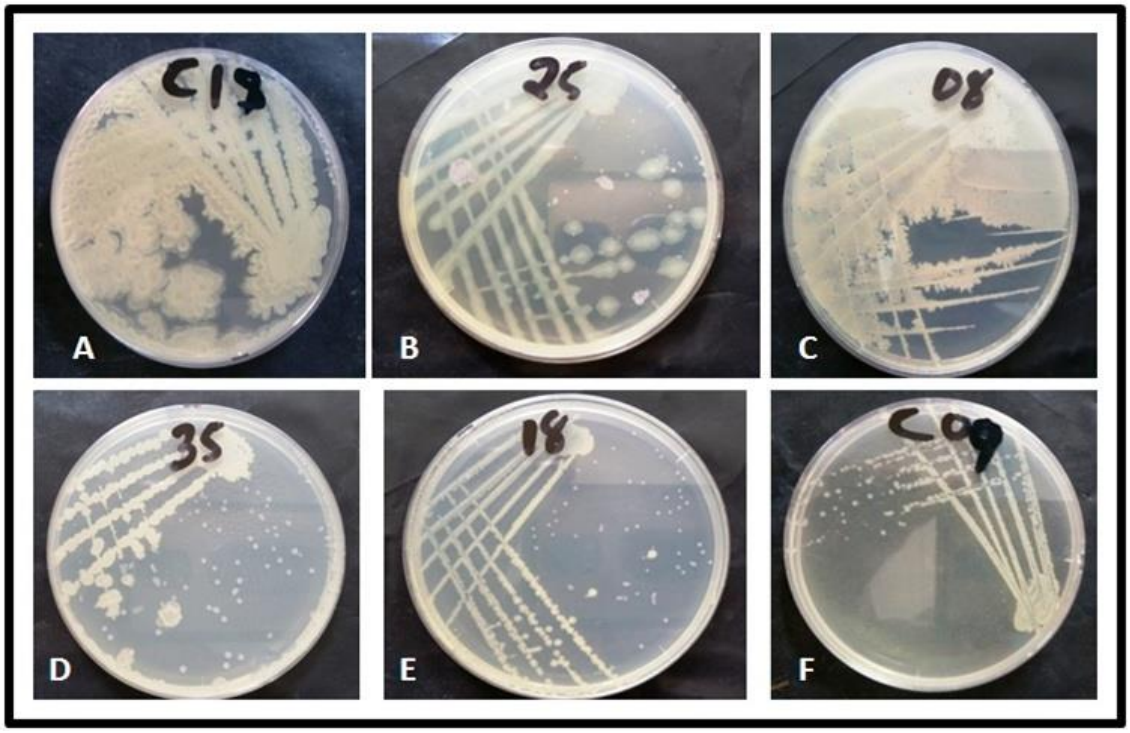

Figure 4.1: Selected purified colony morphotypes obtained from samples from Chinyunyu hot spring.

4.4 Morphological, biochemical and motility tests

Isolates showed diversity in colony colour, shape and oxidase phenotype (Table 4.2). In terms of colony color, six isolates were yellow, four were white, two were cream white and one isolate was light-brown. The colony shapes of the isolates ranged from circular to spherical whilst others were irregularly shaped. With reference to the Gram-stain results all isolates were identified to be Gram-positive and the cells were all rod-shaped as Figure 4.2 shows. The biochemical properties and motility of the isolates were also tested and the results are summarized in Table 4.2. As shown in Table 4.2, all 13 isolates tested positive for the catalase test, seven were oxidase positive and six were oxidase negative. Motility varied among the isolates, of which nine isolates were found to be motile and four non-motile.

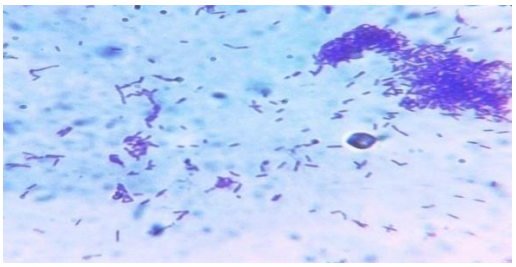

Figure 4.2: Gram-stain reaction of the isolates. All isolates were Gram-positive and the cells were all rod-shaped.

Table 4.2: Morphological and biochemical characterization of isolates.All isolates were Gram-positive rods and colony shapes varied from circular (for instance C01 and C03) and irregular (for instance C08 and C09). Variations were also observed in the oxidase and motility tests as seen in the relevant columns.

Isolate code	**Culture characteristic**		**Test**				
	Colour	**Shape**	**Gram stain**	**Cell shape**	**Catalase**	**Oxidase**	**Motility**
C01	yellow	Circular	+	Rods	+	+	-
C02	yellow	Circular	+	Rods	+	+	-
C03	cream	Circular	+	Rods	+	+	+
C04	yellow	Circular	+	Rods	+	+	-
C05	yellow	Spherical	+	Rods	+	-	+
C06	Yellow	Circular	+	Rods	+	+	-
C07	Yellow	Spherical	+	Rods	+	-	+
C08	white	Irregular	+	Rods	+	-	+
C09	white	Irregular	+	Rods	+	-	+
C10	white	Circular	+	Rods	+	+	+
C11	white	Irregular	+	Rods	+	-	+
C12	cream	Circular	+	Rods	+	-	+
C13	Light-brown	Circular	+	Rods	+	+	+

4.5 Isolates exhibited varied tolerance to different temperatures

Isolates exhibited varied growth at different temperatures, as shown in Table 4.3. All the isolates were able to grow at temperatures between 37-55°C. Excellent growth for most of the isolates was observed at 55 °C. Isolate C10 showed satisfactory growth at

37°C. Isolates C05 and C07 were the only isolates that showed growth at 65°C while the rest of the isolates were unable to grow at that temperature.

Table 4.3: Growth rates of the thirteen isolates from Chinyunyu hot spring at different temperatures. While all the isolates grew satisfactorily at temperatures between 37-55°C, isolates C05 and C07 were the only isolates that showed growth at 65°C.

Isolate	37°C	45°C	50°C	55°C	60°C	65°C
C01	+	++	+++	++++	++	-
C02	+	++	+++	++++	++	-
C03	+	++	+++	++++	++	-
C04	+	++	+++	++++	++	-
C05	+	+	+	++	+++	+++
C06	+	++	+++	++++	++	-
C07	+	+	++	++	+++	+++
C08	++	+++	++++	+++	+	-
C09	++	++	++++	+++	+	-
C10	+++	+++	++	+	+	-
C11	++	+++	++++	+++	+	-
C12	++	++	++++	+++	+	-
C13	++	++	++++	+++	+	-

4.6 Molecular characterization of isolates

4.6.1 Isolates were positive for the expected 1500bp fragment for 16SrRNA gene

The genomic DNA extracted from all 13 phenotypically distinct isolates and amplified by the PCR using 16S rRNA gene universal primer pair (8F and 1492R), yielded an amplification product of approximately 1500 bp, as shown in Figure 4.3.

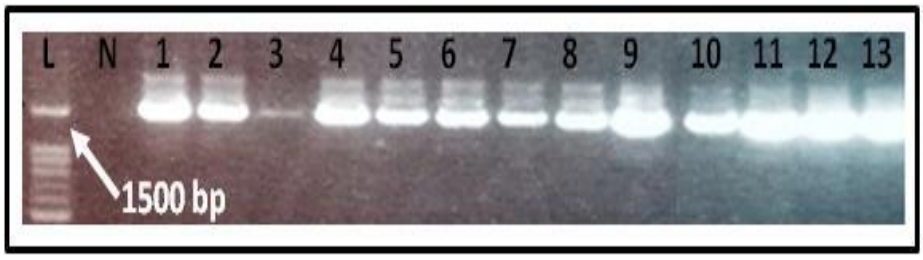

Figure 4.3: Agarose gel electrophoresis analysis of PCR amplification products of genomic DNA isolated from the thirteen prokaryotic cell isolates. Primers targeting the

16S rRNA gene region of the thermophilic isolates were used in the PCR. Lane L, 100 bp DNA ladder; white arrow shows the 1500 bp fragment of the ladder.

4.6.2 BLAST and phylogenetic analyses of nucleotide sequences of the isolates

The determined 16S rRNA nucleotide sequences for the 13 thermophilic isolates showed relationships to known thermophilic species in GenBank on NCBI (http://www.ncbi.nlm.gov) as shown in Table 4.4. Four isolates (C01, C02, C04 and C06) were identified to be strains belonging to *Anoxybacillus suryakundensis* at a similarity ratio of 99%. Further, two of the isolates (C05 and C07) were related to *Anoxybacillus thermarum* (with 99% similarity ratio), one isolate (C03) to *Anoxybacillus ayderensis* with 99% ratio, three isolates (C08, C09 and C11) to *Bacillus licheniformis* (with 99% similarity ratio), one isolate (C10) to *Bacillus altitudinis* (99% ratio of similarity), one isolate (C12) to *Bacillus swezeyi* with a similarity ratio of 95% and one isolate (C13) was affiliated to *Bacillus subtilis* (99% ratio of similarity).

Table 4.4: Comparison of 16S rRNA gene sequences of isolates under study with those in GenBank. Seven isolates (C01-C07) were determined to be strains belonging to the genus *Anoxybacillus* and six isolates (C08-C13) were related to strains belonging to the genus *Bacillus*.

Isolate	**Sequence Length (bp)**	**Closest phylogenetic match and accession**	**% Similarity**
C01	696	*Anoxybacillus suryakundensis* [KF772608]	99
C02	1455	*Anoxybacillus suryakundensis* [KF772608]	99
C03	1419	*Anoxybacillus ayderensis* [NR024837]	99
C04	1425	*Anoxybacillus suryakundensis* [KF772608]	99
C05	1445	*Anoxybacillus thermarum* [AM402982]	99
C06	1443	*Anoxybacillus suryakundensis* [KF772608]	99
C07	1446	*Anoxybacillus thermarum* [AM402982]	99
C08	1436	*Bacillus licheniformis* [NR074923]	99
C09	1436	*Bacillus licheniformis* [NR074923]	99
C10	1452	*Bacillus altitudinis* [ASJCO100029]	99
C11	1446	*Bacillus licheniformis* [NR074923]	99
C12	1399	*Bacillus swezeyi*[NR157608]	95
C13	1444	*Bacillus subtilis* [NR115063]	99

Phylogenetic analysis of the isolates and closely-related species was performed using the neighbor-joining method, and the deduced tree is presented in Figure 4.4. The deduced phylogenetic tree revealed two clades supported by high boot strap values. The clades are represented by the two major lineages, one being the genus *Anoxybacillus* and the other being the genus *Bacillus.* Isolates C01 to C07 were closely clustered with bacterial species belonging to the genus *Anoxybacillus*. Among these were; *Anoxybacillus suryakundensis, Anoxybacillus ayderensis* and *Anoxybacillus thermarum.* Isolates C08-C13 were clustered with bacterial species belonging to the genus *Bacillus* and among these were *Bacillus licheniformis, Bacillus altitudinis, Bacillus swezeyi* and *Bacillus subtilis.*

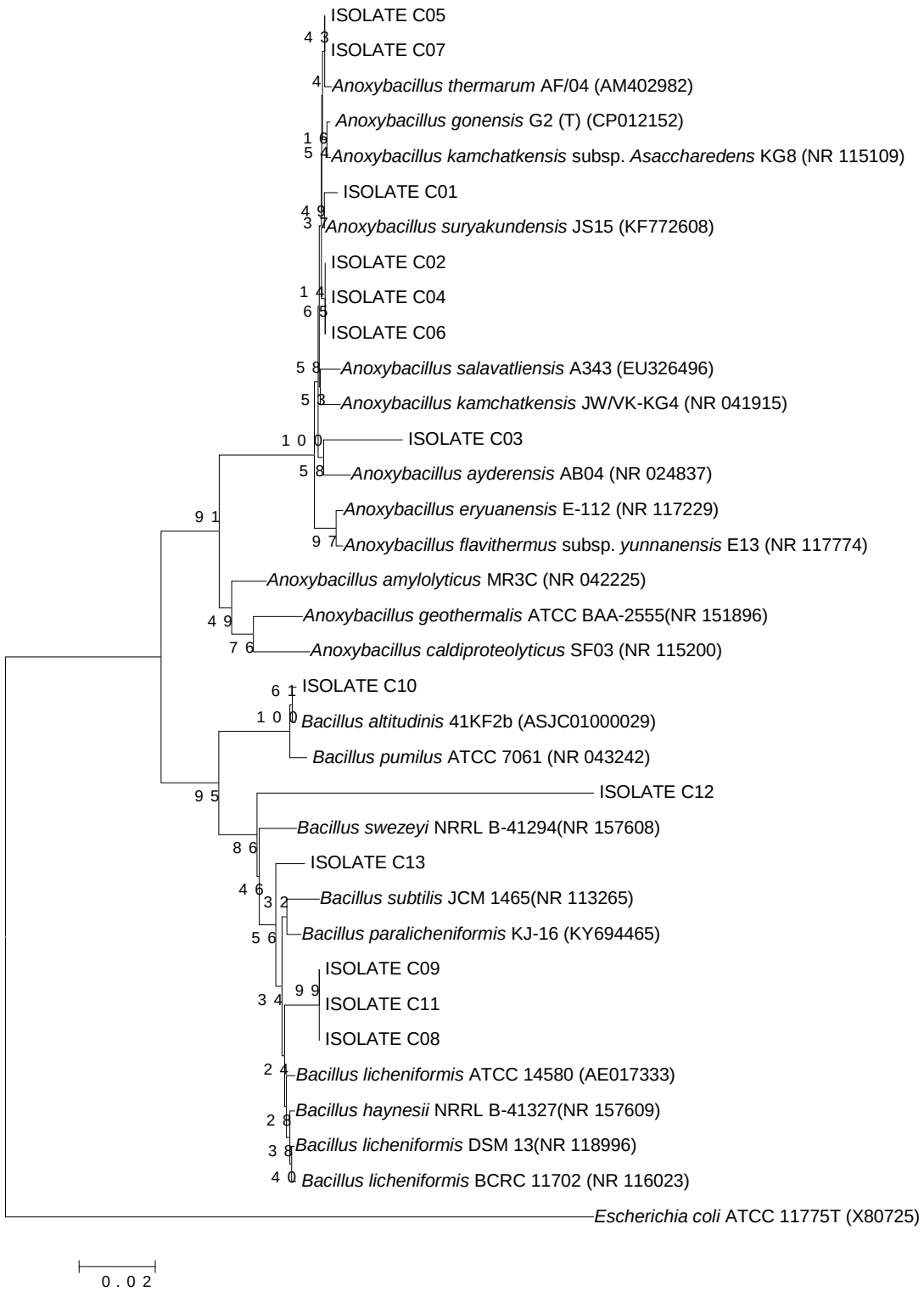

Figure 4.4: Neighbor joining phylogenetic tree based on 16S rRNA gene sequence data of the thermophilic bacterial isolates from Chinyunyu hot spring. *Escherichia coli* was

used as an out-group. Bootstrap values based on 1000 replications are listed as percentages at branching points. Scale bar indicates two nucleotide changes for every 1,000 nucleotides.

CHAPTER FIVE-DISCUSSION

5.1 Overview

This chapter discusses the findings of this study in relation to the objectives and existing knowledge on the isolation, characterization and identification of thermophilic prokaryotes from hot spring environments.

5.2 Discussion

Hot springs are among many extreme environments that are of considerable interest to researchers worldwide. Ever since Thomas Brock and Hudson Freeze reported *Thermus aquaticus* in hot springs of Yellowstone National Park, United States of America, a lot of interest has been directed towards the isolation of thermophiles from similar habitats (Brock and Freeze, 1969; Mohammed *et al*., 2017; Nshimiyimana *et al*., 2018). Studies have revealed that thermophilic prokaryotes present in hot springs are a good source of novel genes and metabolites (Daupan and Rivera, 2015; Baltaci *et al*., 2017). Thermostable enzymes such as proteases, lipases, amylases, xylanases and cellulases that are produced by thermophilic prokaryotes are very important for industrial applications due to their resistance under different operation conditions. These enzymes have been reported to be more stable against denaturing agents such as detergents, organic solvents, high acidity and high alkalinity (Bhalla *et al*., 2013; Dettmer *et al.*, 2013). Because of these properties, the isolation and identification of thermophilic prokaryotes from hot springs is an important task in modern science.

This study is the first to describe the thermophilic prokaryotic compositions in Chinyunyu hot spring in Zambia using conventional (morphological, physiological and biochemical tests) and molecular methods. A total of 13 isolates were obtained from the spring based on colony morphology. The isolates were recognized as thermophiles, since they were able to grow at temperatures above 50°C. The highest number of colony growths was at 55°C and this observation was compatible with studies on thermophilic microorganisms elsewhere, which showed that the optimum growth temperature of most thermophiles lies between 50°C and 60°C (Adhikari *et al*., 2015;Baltaci *et al*., 2017; Mohammed *et al.*, 2017).

Morphological analysis of the isolates revealed that all of them were Gram positive and their cells were rod-shaped. Various other workers have also reported the dominance of Gram positive bacteria in hot springs like Bhurung hot spring in Nepal (Adhikari *et al*., 2015) and Savusavu hot spring in Fiji (Narayan *et al*., 2008). The isolates exhibited diverse colony colors and shapes suggesting a great diversity at the species levels among the isolates.

The isolates from the current study were screened for temperature tolerance and the results revealed that all the isolates were able to grow at temperatures ranging from 37°C to 60°C. According to Stetter (1998), all the isolates could therefore be classified as facultative thermophiles. Facultative thermophiles also called moderate thermophiles have the ability to adapt to low temperature as opposed to obligate thermophiles that require only high temperature for survival. The high adaptability of facultative thermophiles to varying temperature could possibly explain their abundance in extreme habitats such as hot springs. Baker *et al*. (2001) reported the dominance of facultative thermophiles in Indonesian hot springs.

In addition to morphological, physiological and biochemical analyses, the isolates were subjected to molecular analyses. BLAST search and phylogenetic analysis revealed that all isolates belonged to the bacterial phylum Firmicutes. The dominance of Firmicutes has been reported in hot springs elsewhere. Kumar *et al*. (2014) reported the dominance of Firmicutes in Bakreshwar (86%) and Balrampur (93%) hot springs in India. Within the bacterial phylum, seven isolates (C01-C07) were affiliated to the genus *Anoxybacillus.* Members of this genus have been reported to be alkali-tolerant thermophiles that are suitable for many industrial applications including environmental waste treatment, enzyme technology, and bioenergy production (Goh *et al*., 2013). Among the seven isolates affiliated to the genus *Anoxybacillus*, four isolates (C01, C02, C04 and C06) were detected to be *Anoxybacillus suryakundensis* strains. Deep *et al*. (2013) reported the isolation and 16S rDNA sequence based identification of *A. suryakundensis* strains from sediment samples from a hot spring in Suryakund, Jharkhand, India. They reported that the strains were Gram-positive, non-motile and facultative anaerobes, which is in agreement with morphological and physiological

characteristics of the four strains (C01, C02, C04 and C06) isolated from Chinyunyu hot spring. Deep *et al.* (2013) further described *A. suryakundensis* as alkalitolerant and moderately thermophilic with heterotrophic growth occurring at 40 to 60°C and pH 5.5 to 11.5.

Two of the isolates from Chinyunyu hot spring (C05 and C07), were related to *Anoxybacillus thermarum*. Poli *et al.* (2015) revealed the isolation of *A. thermarum*, a novel aerobe thermophilic endospore-forming bacterium designated strain AF/04(T) from thermal mud located in Euganean hot springs, Abano Terme, Padova, Italy. They reported that strain AF/04(T) was Gram-positive, motile, rod-shaped, occurring in pairs, or filamentous. They further reported that the isolate grew between 55°C and 67°C and at pH 6.0-7.5. In contrast, Isolate C05 and C07 exhibited growth even at a low temperature of 37°C (Table 4.3) which shows the possibility of the presence of subspecies among *A. thermarum*.

Isolate C03 was closely matched to *Anoxybacillus ayderensis* with 99% similarity ratio. *A. ayderensis* was first isolated from mud and water samples from the Ayder hot spring located in the province of Rize in Turkey (Belduz *et al*., 2015). The researchers reported that the bacterium is Gram-positive, rod-shaped bacteria and moderately thermophilic (with an optimum temperature for growth of 50-55°C). According to Belduz *et al*. (2015), *A. ayderensis* is a facultative anaerobe that can grow on a wide range of carbon sources including D-glucose, D-raffinose, D-sucrose, D-xylose, D-fructose, L-arabinose, maltose, D-mannose and D-mannitol.

Six isolates (C08-C13) within the bacterial phylum Firmicutes were affiliated to bacterial species belonging to the genus *Bacillus.* Among these were *Bacillus licheniformis*, *Bacillus altitudinis*, *Bacillus swezeyi* and *Bacillus subtilis* (Table 4.4). Thermophilic *Bacillus* sp are among the most studied thermophilic microorganisms and have been isolated from hot springs including Rwanda (Nshimiyimana *et al*., 2018), Turkey (Mohammed *et al*., 2017), India (Lele and Deshmukh, 2016), Italy (Maugeri, 2001), Bulgaria (Derekova *et al.*,2008), Greece (Sievert *et al*., 2000), Japan (Kawasaki *et al*., 2012), Jordan (Malwaki and Al-Omari, 2010) and Morocco (Aanniz *et al*., 2014).

Thermophilic *Bacillus* sp have been reported to produce a wide range of thermostable enzymes, of which amylases, proteases, cellulases and lipases are of significant industrial importance (Lele and Deshmukh, 2016; Amnin *et al.*, 2008).

Among the six isolates affiliated to the genus Bacillus, isolates C08, C09 and C11 were identified as *Bacillus licheniformis* (with 99% similarity ratio). *B. licheniformis* is a Gram-positive, endospore forming, rod-shaped bacterium that occurs in hot springs, plants and soil (Lee *et al*., 2017). The bacterium is a facultative anaerobe that optimally grows at a temperature slightly above 50°C and pH 7, but can also grow at higher or lower pH. *B. licheniformis* expresses enzymes used for the industrial purposes such as the production of enzymes, antibiotics and small metabolites (Aanniz *et al*., 2014).

BLAST results revealed that Isolate C10 was affiliated to *Bacillus altitudinis* sp with 99% ratio of similarity. According to Vettath *et al*. (2017), *B. altitudinis* is a Gram-positive, rod-shaped aerobic bacterium. It was first reported to be isolated from air samples and has since been isolated from diverse habitats including freshwater Lakes, soil and silt (Vijay *et al*., 2011; Mao *et al*., 2013; Shafi *et al.*, 2017). Verma *et al.* (2015) reported the occurrence of thermophilic *B. altitudinis* strain in the hot springs of Manikaran, India. The researchers revealed that the bacterium was capable of producing thermostable cellulase that could be used in the bioconversion of lignocellulosic residue to fermentable sugars preferably at high temperature.

Isolate C12 was closely matched to the bacterium *Bacillus swezeyi* with a similarity ratio of 95%. The bacterium is Gram-positive, facultatively anaerobic, motile, endospore-forming and rod-shaped. It grows at pH 5.5–10 and at temperatures of from 15 to 60 °C. The cells are catalase-positive and oxidase-negative (Dunlap *et al*., 2017). Isolate C13 was affiliated to *Bacillus subtilis* (98% ratio of similarity). Thermophilic *Bacillus subtilis* was also isolated from hot springs in Morocco (Aanniz *et al*., 2014). Aanniz *et al*. (2014) reported that the bacterium is Gram-positive, rod-shaped and facultative aerobic bacterium capable of producing industrially important enzymes.

CHAPTER SIX-CONCLUSION AND RECOMMENDATIONS

6.1 Overview

This chapter contains the overall conclusions of the results obtained in this study as well as possible future work.

6.2 Conclusion

The study has shown that Chinyunyu hot spring harbors genetically diverse thermophilic prokaryotes. A total of 13 phenotypically distinct isolates were characterized and identified on nutrient agar medium at 55°C and pH of 7.0. Molecular characterization revealed that all the isolates belonged to the bacterial phylum, Firmicutes. Phylogenetic analysis based on nearly complete 16S rRNA gene sequences revealed that seven isolates were affiliated to the genus *Anoxybacillus* and six isolates were affiliated to the genus *Bacillus*. This study provides the baseline data on the composition of thermophilic prokaryotes in Chinyunyu hot spring. These findings could be amenable to further studies to exploit the hot spring microbiome for its biotechnological potential.

6.3 Recommendations

Further studies should be conducted to comprehensively analyze and screen the identified microorganisms of Chinyunyu hot spring for biotechnological products such as thermally-stable enzymes. This could pave way for commercial enzyme production.

There is also a need to modify the protocols and develop medium to allow the isolation of more diverse genera of thermophilic prokaryotes from Chinyunyu hot spring.

Similar studies should be carried out on other hot springs of Zambia.

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APPENDICES

Appendix A: Nutrient Agar (Grams/Litre) (HI MEDIA)

Peptone	-	5g
Sodium chloride	-	5g
HM peptone B	-	1.5g
Yeast extract	-	1.5g
Agar	-	15g
Distilled water	-	1000ml
pH (at 25°C)	-	7.5

Preparation of nutrient agar medium

1. Suspend a whole 28.0 grams of all the ingredients in 1000 ml purified distilled water.
2. Heat to boiling to dissolve the medium completely.
3. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.
4. Cool to 45-50°C.

Appendix B: Reagents used for Molecular Characterization

1. PCR Mixture

Template DNA - 5 µl

PCR buffer (×10) - 5 µl

dNTP's (2.5mM) - 3 µl

8F forward primer - 1 µl (5 pmol)

1492R reverse primer - 1 µl (5pmol)

Taq polymerase - 0.3 µl

Water - 24.7 µl

2. **Gel Electrophoresis**

Agarose gel (1 per cent)

2.0 g agarose was added to 200ml of 1x TAE buffer and molten down. The molten gel was cooled down to 40°C and 18 µl of ethidium bromide solution was added and mixed well.

Ethidium bromide 10 mg/ml

10 mg of ethidium bromide was dissolved in sterile water and volume made up to 1 ml. The solution was stored in an amber colored bottle at 4°C.

TBE buffer 10×

Chemical	Volume
Tris	108 g
Boric Acid	55 g
$Na_2EDTA.2H_2O$	9.3 g

Adjust the volume to 1 liter with ddH2O

Appendix C: 16S rRNA nucleotide sequences of selected isolates from Chinyunyu hot spring

>ISOLATE C01

CGGGCCCTATAATGCAGTCGAGCGGACGATTCAAAGCTTGCTTTTGAATCGTTAGCGG
CGGACGGGTGAGTAACACGTGGGCAACCTGCCCTGTAGACGGGGATAACACCGAGAAA
TCGGTGCTAATACCGGATAACACGAAGACCGCATGGTTTTTCGTTGAAAGGCGGCGCA
AGCTGTCGCTACAGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCA
CCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTC
TGACGGAGCAACGCCGCGTGAGCGAAGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTTA
GGGAAGAACAAGTACCGCAGTCACTGGCGGTACCTTGACGGTACCTAACGAGAAAGCC
ACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAA
TTATTGGGCGTAAAGCGCGCGCAGGCGGTTCCTTAAGTCTGATGTGAAAGCCCACGGC
TCAACCGTGGAGGGTCATTGAAACTGGGGGACTTGAGTGCAGAAGAGGAGAGCGGAAT
GCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGCGAAAGCGGCT
C

>ISOLATE C02

TGCAAGTCGAGCGGACGATTCAAAGCTTGCTTTTGAATCGTTAGCGGCGGACGGGTGA
GTAACACGTGGGCAACCTGCCCTGTAGACGGGGATAACACCGAGAAATCGGTGCTAAT
ACCGGATAACACGRAAGACCGCATGGTTTTTCGTTGAAAGGCGGCGCAAGCTGTCGCT
ACAGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGAC
GATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGA
CTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCA
ACGCCGCGTGAGCGAAGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTTAGGGAAGAACA
AGTACCGCAGTCACTGGCGGTACCTTGACGGTACCTAACGAGAAAGCCACGGCTAACT
ACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCG
TAAAGCGCGCGCAGGCGGTTCCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGG
AGGGTCATTGGAAACTGGGGGACTTGAGTGCAGAAGAGGAGAGCGGAATTCCACGTGT
AGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTC
TGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA
GTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTATCCACCCTTTAGTGCTGT
AGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGCTCGCAAGAGTGAAACTCAAAGG
AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTTCGAAGCAACGCGA
AGAATCCTTACCAGGTCTTGACATCCCCTGACAACCCGAGAGATCGGGCGTTCCCCCT
TCGGGGGGACAGGGTGACAGGTGGTGCATGGTTGTCCGTCAGCTCGTGTCGTGAGATG
GTTGAGGTTAAGGTCCCGCAACGAGCGCAACCCTCGACCTTAGTTGCCAGCATTCAGT
TGGGCACTCTAAGGTGACTGCCGGCTAAAAGTCGGAGGAAGGTGGGGATGACGTCAAA
TCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCGGTACAAAGGGT
CGCGAACCCGCGAGGGGGAGCCAATCCCAAAAAGCCGCTCTCAGTTCGGATTGCAGGC
TGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGT
GAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGCAACACC
CGAAGTCGGTGAGGTAACCCTACGGGAGCCAGCCGCCGAAG

>ISOLATE C03

AGGGCTAATACTGCAAGTCGAGCGGACGATTCAAGCTTGCTTTTGATCGTAGCGGCGG
ACGGGTGAGTAACACGTGGGCAACCTGCCCTGTAGACGGGGATAACACCGAAAATCGG
TGCTAATACCGGATAACACGAAGACCGCATGGTTTTTCGTTGAAAGGCGGCGCAAGCT
GTCGCTACAGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAA
GGCGACGATGCGTAGCCGACCTGAAGGGATCGGCCACACTGGGACTGAACACGGCCAG
ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGC
AACGCCGCGTGACGAAGAAGGCTTCGGCGTAAAGCTCTTTGTGTAGGAGAACAGTACC
AGTCCATGGCGGTATCCTGACCGGTAACCTTAACGAGGAAAGCCACGGCTAAACTTAT
CGTGCCAGCAGCCGCGGCTAATTACGTATGTKGCAAGCGTTTGTCCGGAATTATTGGG
GCGTAAAGCGCGCGCAGGCGGTTCCTTAAAGTCTGATTGTGAAAGCCCACGGCTCAAC
CGTGAGGGTCATTGGAAACTGGGGGGACTTGAGTGCAGAAGATGAGAGCGGAATTCCC
ACGTGTAGCGGTGAAAATGCGTAGAGATGTGGAGGAACACCAGTGGGAAGGCGGCTCT
CTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAAGAGGGTATCCGCYCTTTA
GTGCTGTAGCTAACGCATTAAGCACTCCGCTGGGGAGTACGCTCGCAAGAGTGAAACT
CAAAGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAC
GCGAAGAACCTTACCAGGTCtTGACATTCCCTGACAACCCGAGAGATCGGGCGTTCCC
CCTTCGGGGGGACAGGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGACCTTAGTTGCCAGCATTCAGTT
GGGCACTATAAGGTGACTGCCGGCTAAAAGTCGGAGGAAGGTGTGGATGACGTCAAAT
CATCATGCCCCTTATGACCTGGGCTACACACGTGGTACAATGGGCGGTACAAAGGGTC
GCGAACCCGCGAGGGGGAGCCAATCCCAAAAAGCCGCTCTCAGTTCGGATGGCAGGGT
GCAACTCGCCTGCATGAAGCCGGAATCCCTAGTAATCGCGGATCAGCATGCGCGGTGA
ATCGTTCCCGGGCCTGTACACACCCCCGTCACACCCGAGAGTTTGCAACCACCGAAGT
CGGTGAGGTAACCCTACGGACCAGCCG