Molecular characterization of culturable thermophilic prokaryotes from Chinyunyu hot spring in central Zambia

M. P. Kalumbilo, E. Kaimoyo,* J. Phiri
University of Zambia, School of Natural Sciences, Department of Biological Sciences,
P. O. Box 32379, Lusaka, Zambia

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ABSTRACT

Hot springs are among some of the naturally-occurring extreme environments that have generated considerable interest in researchers worldwide. Thermophilic prokaryotes present in hot spring habitats are considered valuable sources for biotechnological products including thermally-stable enzymes applied in many research and manufacturing process. Despite the numerous hot springs in Zambia, there is limited information on the diversity of thermophilic prokaryotes in these places. In this study, characterization of thermophilic prokaryotes isolated from Chinyunyu hot spring in Lusaka province, Zambia was conducted using phenotypic and molecular-methods. The recorded temperature of the hot spring at the time of sampling was $60°C$ and the pH was 9.0 indicating alkaline environment. A total of 13 phenotypically distinct isolates were identified on nutrient agar medium at $55°C$ and pH 7.0. All isolates were Gram-positive, rod-shaped cells. Their genomic DNA was PCR-amplified using 16S rRNA primers and sequenced using the Big Dye Terminator v3.1 cycle sequencing kit on the ABI Prism 3130xl Genetic Analyzer (Life Technologies Corp). Amplicon sequences were analyzed using Basic Local Alignment Search Tool (BLAST) and revealed that all isolates belonged to the bacterial phylum Firmicutes. Seven isolates were affiliated to the genus Anoxybacillus and six were affiliated to the genus Bacillus. The study revealed that Chinyunyu hot spring harbours genetically diverse thermophilic prokaryotes which could be amenable to further studies to exploit the hot spring microbiome for its biotechnological potential.

1 Introduction

Temperature is an essential parameter for growth of microorganisms. Based on their optimum growth temperature, microorganisms are categorized as psychrophiles, mesophiles and thermophiles (Baltaci et al., 2017; Mohammed et al., 2017). Ever since Thomas Brock and Hudson Freeze reported Thermus aquaticus in hot springs of Yellowstone National Park in the United States of America, a lot of interest has been directed towards the isolation of thermophiles from similar habitats (Brock and Freeze, 1969). These are a group of extremophilic microorganisms whose optimum growth temperature lies between $45°C$ and $80°C$ (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 (Daupan and Rivera, 2015). Growth at elevated temperatures enables them to exhibit high metabolic rates which result in greater end

*Corresponding author: *E-mail address:* ekaimoyo@gmail.com

products as compared to mesophiles. Thermophilic prokaryotes express thermostable enzymes that are used as biocatalysts in pharmaceutical, food processing and several other industries (Baltaci et al., 2017; Mohammed et al., 2017). In addition to thermal stability, the enzymes are also stable against detergents, organic solvents, high acidity and alkalinity, hence their use in various industries (Bhalla et al., 2013; Dettmer et al., 2013).

Despite difficulties in isolation and maintenance as pure cultures, thermophilic prokaryotes have been isolated from various geothermally heated regions such as hot springs, deep sea hydrothermal vents and volcanic craters (Mohammed et al., 2017). They have been isolated from hot springs in Rwanda (Nshimiyimana et al., 2018), Turkey (Mohammed et al., 2017), India (Lele and Deshmukh, 2016), Italy (Maugeri et al., 2001), Bulgaria (Derekova et al., 2008), Greece (Sievert et al., 2000), Japan (Kawasaki et al., 2012), Jordan (Malkawi and Al-Omari, 2010) and Morocco (Aanniz et al., 2015).

Zambia has several hot springs located in different regions of the country. Chinyunyu hot spring located in Rufunsa district, Lusaka province, is one of the most famous hot springs in Zambia. Previous studies conducted at Chinyunyu 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 thermal energy, either for power generation or for other purposes (Legg, 1974). However, no studies describe the compositions of thermophilic prokaryotes in Chinyunyu hot spring especially at the molecular level. The purpose of the current study was to isolate and characterize thermophilic prokaryotes from Chinyunyu hot spring in Zambia and assess their phylogenetic relationships

2 Materials and Methods

2.1 Study area

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 located in Lusaka province of Zambia is situated at $15°15'40.9"S$ latitude and $29°01'24.6"E$ longitude at an elevation of about $990m$ above sea level (Figure 1). 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 1: A Google map showing the location of Chinyunyu hot spring

2.2 Collection of Samples

Water samples were collected from Chinyunyu hot spring on 21st June, 2018. Fifteen water samples from a depth of 10 cm were randomly collected in 25 ml sterile screw cap bottles. Hot spring water samples were enclosed in a zip-lock bag and placed in a cooler box to maintain the temperature of the samples. Samples were transported to the laboratory at the University of Zambia within 2 hours of collection. Temperature and pH of the hot spring water at the sampling sites were measured at the time of sampling using a portable thermometer (Fisher Scientific, Mumbai, India) and a portable pH meter (Thermo Fisher Scientific, Singapore) respectively.

2.3 Isolation of thermophilic prokaryotes

The modified method of Adhikari et al. (2015) was adopted for the isolation of thermophilic prokaryotes from the water samples collected. Attempts were made to plate $100\,\mu l$ volumes from each sample after serial dilutions up to the dilution factor of 103 but very few colonies grew on the nutrient agar medium. To improve colony numbers, $100\,\mu l$ volumes were thus drawn directly from each sample without dilution and spread-plated on nutrient agar (NA) medium (peptone $[5.0\,g]$, NaCl $[5.0\,g]$, HM peptone B $[1.5\,g]$, yeast extract $[1.5\,g]$ and agar $[15.0\,g]$ per litre) (Himedia, Mumbai, India). The inoculated plates were covered with aluminium foil to prevent the NA from drying and incubated at $50°C$, $55°C$ and $60°C$ respectively for 24h. Different colonies were selected based on morphology and purified using streak plate method (Sanders, 2012). The purified isolates were stored in a refrigerator at $4°C$ for further study.

2.4 Morphological and biochemical characterization of isolates

Purified colonies were characterized based on morphology and selected biochemical characteristics according to the standard methods described in Bergey's manual of Systematic Bacteriology (Vos et al., 2009). Tests conducted included examination of colony characteristics, Gram staining, catalase test, Kovac's oxidase test and motility test.

2.5 Screening of isolates for temperature tolerance

The effect of incubation temperature on the growth of isolates was studied. Isolates were spread plated on NA plates and incubated at $37°C$, $45°C$, $50°C$, $55°C$, $60°C$ and $65°C$. Growth as measured by colony size was examined after 48 hours of incubation. Colony growth rate was scored using four levels of positive sign, whereby one positive $(+)$ indicated minimal growth, two positives $(++)$ indicated average growth, and three positives $(+++)$ indicated satisfactory growth while four positives $(++++)$ indicated excellent growth. Negative $(-)$ sign was used to denote no growth.

2.6 Molecular characterization of isolates

To determine the diversity of the isolates, colonies showing distinct phenotypes were selected for molecular characterization. Isolates were grown on NA at $55°C$ and genomic DNA was isolated and used in PCR-amplification reactions.

2.6.1 Extraction of genomic DNA

Genomic DNA extraction was performed according to Dashti et al. (2009) with slight modifications using isolates grown on NA for 24 h at $55°C$. A loopful of two colonies of each isolate was transferred and suspended in eppendorf tubes containing $100\,\mu l$ of sterile distilled water and boiled for 10 minutes on a heating block (Thomas Scientific Brand) to lyse the cells. Boiled samples were centrifuged at $25°C$ for five minutes at 6000 rpm in a

Hemle Z233 MK microcentrifuge (Hermle Labortechnik, Wehingen, Germany) and $5\,\mu l$ of the supernatant from each isolate was used for the PCR.

2.6.2 PCR amplification of 16S rRNA gene

Amplification of the 16S rRNA gene was performed using universal primer pair combination of forward primer 8F (5'-AGAGTTTGATCCTGGCTAG-3') and reverse primer 1492R (5'-CGGCTACCTTGTTACGACTT-3') (Baker et al., 2003). Amplification was performed using a Veriti 96 well Thermal Cycler (Applied Biosystem, USA). Amplification was carried out in $40\,\mu l$ reaction mixtures containing $5\,\mu l$ of template DNA, $4\,\mu l$ of PCR buffer ($\times 10$), $3\,\mu l$ dNTP's (2.5 mM), $1\,\mu l$ (5 pmol) of 8F forward primer, $1\,\mu l$ (5pmol) of 1492R reverse primer, $0.3\,\mu l$ Taq polymerase and $25.7\,\mu l$ of water. The control contained all the reagents except 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 $96^{\circ}C$ for five minutes, cycle denaturation at $95^{\circ}C$ for 30 seconds, primer annealing at $53^{\circ}C$ for 30 seconds, chain extension at $72^{\circ}C$ for 1.0 minute and a final extension at $72^{\circ}C$ for 10 minutes (Roux, 2018). Amplification products ($5\,\mu l$) were separated on 1% agarose gel stained with $10\,\mu g/ml$ ethidium bromide in $1\times$ TBE buffer and visualized under ultraviolet light transilluminator using standard procedures (Sambrook et al., 1989).

2.6.3 Purification of PCR products

To obtain DNA for sequencing, PCR products were extracted from the agarose gel and purified using the QIAquick PCR purification Kit protocol (Qiagen GmbH, Germany) according to manufacturer's instructions. Briefly, 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.

2.6.4 Sequencing and BLAST analyses of PCR amplified products

PCR products were sequenced at the University of Zambia, School of Veterinary Medicine using the Big Dye Terminator v3.1 cycle sequencing kit on the ABI Prism 3130xl Genetic Analyzer (Life Technologies Corp., Carlsbad, CA, USA). Partial sequences were generated using the universal primers (8F and 1492R). The 16S rDNA gene sequences of the 13 isolates were compared to 16S rRNA database sequences using the Basic Local Alignment Search Tool (BLAST) algorithm at the National Center for Biotechnology Information website (`http://www.ncbi.nlm.gov`).

2.6.5 Phylogenetic analysis of sequences

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 (Tamura et al., 2013).

3 Results

3.1 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 1. At the

time of sampling the ambient temperature at the sampling site was $20°C$. While the pH of the water was similar at the three sites, water temperatures varied from $50°C$ to $60°C$.

Table 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

3.2 Thermophilic prokaryotes isolated at different temperatures varied in growth characteristics

To isolate thermophilic prokaryotes, inoculated NA plates were incubated at $50°C$, $55°C$ and $60°C$ and observations were recorded after 24 h. Different colony morphologies were observed on all NA agar plates incubated at different temperatures as illustrated in Figure 2. Isolates grown on NA plates incubated at $55°C$, Figure 2B showed the highest diversity in colony morphology and characteristics. The $55°C$ temperature also supported growth of the largest number of bacterial or archeal cells. Nutrient agar plates incubated at $50°C$, Figure 2A had intermediate number and diversity of colonies. Cells that grew at this temperature also formed special colony features including biofilm formation. The lowest diversity of isolated colonies was observed on NA plates incubated at $60°C$. Subsequent culturing of isolates was conducted on NA plates at $55°C$ and pH 7.0.

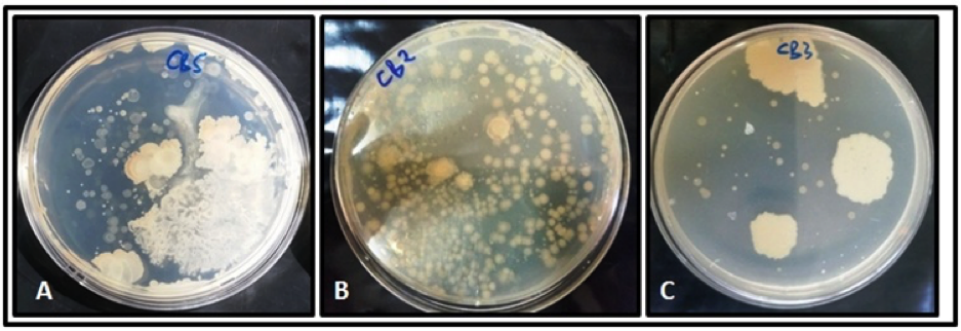

Figure 2: Diversity in colony morphology of microbial isolates on NA plates incubated at $50°C$, A, $55°C$, B and $60°C$. One hundred microliters($100\,\mu l$) from each sampling point were incubated at the respective temperatures and the highest colony numbers were observed in samples on plates incubated at $55°C$ while the least growth was at $60°C$. Growth at $50°C$ also appeared to promote biofilm formation in colonies. Experiments were conducted in triplicates with similar results.

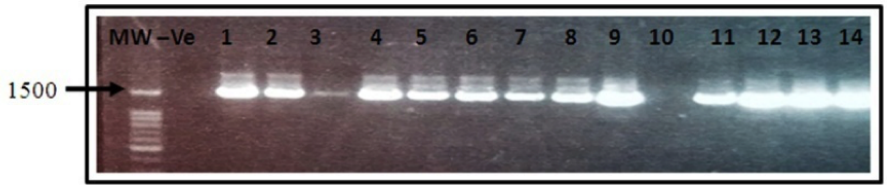

Figure 3: PCR amplification of 16S rRNA gene region of the thermophilic isolates. Genomic DNA was amplified using universal primers 8F and 1492R targeting a 1522 bp region. Thirteen of the 14 samples were positive for a 1522 bp fragment.

3.3 Isolates showed diversity in colony colour, shape and oxidase phenotype

To determine the diversity of the isolates, colonies were examined and those that showed distinct colony morphology were selected and purified by sub-culturing on NA medium and incubation at $55°C$. A total of 13 phenotypically different isolates were selected and subjected to morphological and selected biochemical tests, and the results are shown in Table 2. In terms of colony colour, six isolates were yellow, four were white, two were cream white in colour and one 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. To determine the biochemical properties of the isolates, they were tested for catalase and oxidase presence. As shown in Table 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 were motile and four non-motile.

Table 2: Morphological and biochemical characterization of isolates.

ISOLATE CODE	CULTURE CHARACTERISTICS		GRAM STAINS	CELL SHAPE	CATALASE	OXIDASE	MOTILITY
	COLOR	SHAPE					
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	+	+	+

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.

3.4 Isolates exhibited varied tolerance to different temperatures

Isolates were grown on NA plates and incubated for 24-48h at $37°C$, $45°C$, $50°C$, $55°C$, $60°C$ and $65°C$. All the isolates were able to grow at temperatures between $37 - 55°C$ (Table 3). Excellent growth for most of the isolates was observed at $55°C$. Isolate C10 showed good growth at $37°C$ and isolates C05 and C07 were the only isolates that showed growth at $65°C$. The rest of the isolates were unable to grow at the temperature of $65°C$.

3.5 Molecular Identification of Isolates

3.5.1 Most of the samples were positive for the expected 1500bp fragment for 16SrRNA gene

To determine the diversity of isolates at molecular level, genomic DNA was isolated from all the 13 phenotypically distinct isolates. DNA was amplified by the PCR using 16S rRNA gene universal primer pair (8F and 1492R) and yielded an amplification product of approximately 1500 bp, as shown in Figure 3.

3.5.2 Isolated nucleotide sequences show relationships to known thermophilic species

To determine the diversity of the isolate at molecular level, the amplified DNA was extracted from the agarose gel and purified. Determined near full-length 16S rDNA nucleotide sequences for the 13 thermophilic isolates were compared with those available in GenBank using BLAST algorithm on NCBI (Table 3).

Table 3: Growth of isolates from Chinyunyu hot spring at different temperatures.

ISOLATE	$\mathbf{37^{\circ}C}$	$\mathbf{45^{\circ}C}$	$\mathbf{50^{\circ}C}$	$\mathbf{55^{\circ}C}$	$\mathbf{60^{\circ}C}$	$\mathbf{65^{\circ}C}$
C01	+	++	+++	++++	++	-
C02	+	++	+++	++++	++	-
C03	+	++	+++	++++	++	-
C04	+	++	+++	++++	++	-
C05	+	+	+	++	+++	+++
C06	+	++	+++	++++	++	-
C07	+	+	++	++	+++	+++
C08	++	+++	++++	+++	+	-
C09	++	++	++++	+++	+	-
C10	+++	+++	++	+	+	-
C11	++	+++	++++	+++	+	-
C12	++	++	++++	+++	+	-
C13	++	++	++++	+++	+	-

All isolates were able to grow at temperatures between $37 - 55^{\circ}C$. Excellent growth for most of the isolates was observed at $55^{\circ}C$. Isolate C10 showed significant growth at $37^{\circ}C$ and isolates C05 and C07 were the only isolates able to grow at $65^{\circ}C$. The rest of the isolates were unable to grow at the temperature of $65^{\circ}C$.

Four isolates (C01, C02, C04 and C06) were determined to be strains belonging to *Anoxybacillus suryakundensis* at a similarity rate of 99%. Further, two of the isolates (C05 and C07) were related to *Anoxybacillus thermarum* (with 99% similarity ratio), one isolate (C03) to *Anoxybacillus aydrerensis* with 99% ratio, three isolates (C098, 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 rate of 95% and one isolate (C13) was affiliated to *Bacillus subtilis* (99% ratio of similarity).

3.5.3 Phylogenetic analysis reveal that all isolates belonged to the genus Bacillus or Anoxybacillus

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.

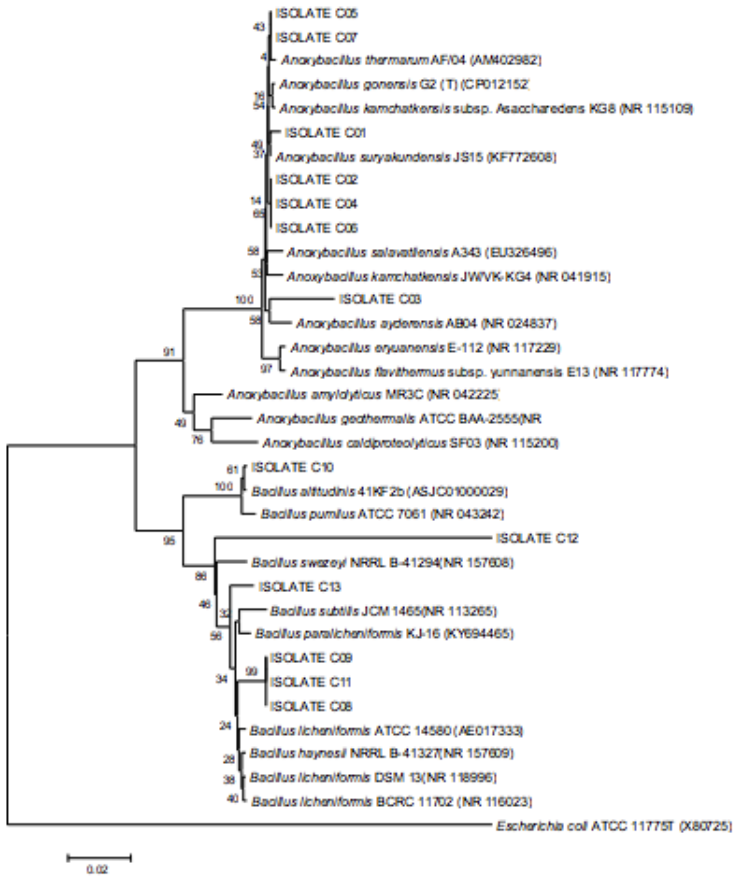

Figure 4: Phylogenetic analysis of isolates by the Neighbour- joining phylogenetic analysis method.

Sequences of 16S rRNA PCR products from Chinyunyu hot spring isolates were aligned by MUSCLE together with 22 DNA sequences from thermophilic bacteria downloaded from the NCBI website (`http://www.ncbi.nlm.gov`) and analyzed using MEGA version 6 (MEGA6). Escherichia coli was used as an out-group. Bootstrap values based on 1000 replications are listed as percentages at branching points. Inferences on tentative identification were based on relatedness to DNA sequences archived at the NCBI database at `http://www.ncbi.nlm.gov`.

The deduced phylogenetic tree revealed two clades supported by high boot strap values. The clades were represented by two major lineages, one belonging to the genus *Anoxybacillus* and the other to the genus Bacillus. Isolates C01 to C07 were closely clustered with bacterial species belonging to the genus *Anoxybacillus*. Among these were; *Anoxybacillus suryakundensis, Anoxybacillus aydrerensis* 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*.

4 Discussion

Hot springs are among many extreme environments that are of considerable interest to researchers worldwide. Studies have revealed that thermophilic prokaryotes present in hot springs are a good source of biotechnologically useful products including novel genes and metabolites (Baltaci et al., 2017; Mohammed et al., 2017). Therefore,

the isolation and identification of thermophilic prokaryotes from hot springs is important.

This study is the first to describe the thermophilic prokaryotic compositions from Chinyunyu hot spring in Zambia. A total of 13 isolates were obtained on nutrient agar at $55°C$ and pH 7.0. The isolates were recognized as thermophiles, since they were able to grow at temperature of $55°C$. The purified isolates were encoded in the form of C01-C13 and then subjected to phenotypic and molecular tests. Morphological analysis revealed that all isolates were Gram positive and their cells were rod-shaped (Table 2). 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 colours and shapes suggesting a great diversity at the species levels among the isolates.

The isolates 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 can 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. 16S rDNA for all 13 isolates were analyzed by BLAST. Based on MEGA alignment of the isolates to GenBank sequences, a phylogenetic tree was constructed (Figure 4). The results 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*. Among these were bacterial species *Anoxybacillus aydrerensis, Anoxybacillus thermarum* and *Anoxybacillus suryakundensis* (Table 4). 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). 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). *Thermophilic Bacillus* species 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 et al., 2001), Bulgaria (Derekova et al., 2008), Greece (Sievert et al., 2000), Japan (Kawasaki et al., 2012), Jordan (Malkawi and Al-Omari, 2010) and Morocco (Aanniz et al., 2015). *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).

Table 4: Comparison of 16S rRNA gene sequences of isolates under study with those in GenBank.

ISOLATE	ACCESSION	SEQUENCE LENGTH (bp)	CLOSEST PHYLOGENETIC MATCH	% IDENTITY
C01	MK473915	696	*Anoxybacillus suryakundensis*	99
C02	MK473916	1455	*Anoxybacillus suryakundensis*	99
C03	MK473917	1419	*Anoxybacillus aydrerensis*	99
C04	MK473918	1425	*Anoxybacillus suryakundensis*	99
C05	MK473919	1445	*Anoxybacillus thermarum*	99
C06	MK473920	1443	*Anoxybacillus suryakundensis*	99
C07	MK473921	1446	*Anoxybacillus thermarum*	99
C08	MK473922	1436	*Bacillus licheniformis*	99
C09	MK473923	1436	*Bacillus licheniformis*	99
C10	MK473924	1452	*Bacillus altitudinis*	99
C11	MK473925	1446	*Bacillus licheniformis*	99
C12	MK473926	1399	*Bacillus swezeyi*	99
C13	MK473927	1444	*Bacillus subtilis*	99

Four isolates (C01, C02, C04 and C06) were determined to be strains belonging to Anoxybacillus suryakundensis. Further, two of the isolates (C05 and C07) were related to *Anoxybacillus thermarum*, one isolate (C03) to *Anoxybacillus aydrerensis*, three (C08, C09 and C11) to *Bacillus licheniformis*, one (C10) to *Bacillus altitudinis*, one isolate (C12) to *Bacillus swezeyi* and one isolate (C13) to *Bacillus subtilis*.

5 Conclusion

This is the first report on the isolation of culturable thermophilic prokaryotes from Chinyunyu hot spring in central Zambia. Thermophilic prokaryotes were identified and characterized using phenotypic methods and sequencing of their 16S rRNA genes. The results revealed the presence of bacterial species belonging to the two genera, *Anoxybacillus* and *Bacillus*. This work paves the way for further studies to exploit the hot spring microbiome for its biotechnological potential.

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References

Aanniz T., Ouadghiri M., Melloul M., Swings J., Elfahime E., Ibijbijen J., Ismaili M., Amar M. Thermophilic bacteria in Moroccan hot springs, salt marshes and desert soils. *Brazilian Journal of Microbiology*, 46(2), 443–53 (2015).

Adhikari H., Ghimire S., Khatri B., Yuvraj K.C. Enzymatic Screening and Molecular Characterization of Thermophilic Bacterial Strains Isolated from Hot spring of Tatopani, Bhurung, Nepal. *International Journal of Applied Sciences and Biotechnology*, 3(3), 392–397 (2015).

Amnin A. L., Waraganegara F. M., Akhmaloka A. P., 2008. Culture independent and culture dependent approaches on microbial community analysis at Gedongsongo hot spring. *International Journal of Integrative Biology*, 2(3):245–252 (2008).

Baker G, Gaffar S, Cowan DA and Suharto AR. Bacterial Community Analysis of Indonesian Hot springs. *FEMS Microbiology Letters* 200:103–109 (2001).

Baker G., Smith J. J., Cowan D. A. Review and reanalysis of domain specific 16S primers. *J. Microbiol. Methods* 55:541–555 (2003).

Baltaci M. O., Genc B., Arslan S., Adiguzel G., Adiguzel A. Isolation and Characterization of Thermophilic Bacteria from Geothermal Areas in Turkey and Preliminary Research on Biotechnologically Important Enzyme Production. *Geomicrobiology Journal*, 34:1, 53–62 (2017).

Bendia A., Araujo G., Pulschen A. A., Contro B., Duarte R., Rodrigues F., Galante D., Pellizari V. Surviving in hot and cold: psychrophiles and thermophiles from Deception Island volcano, Antarctica. *Extremophiles*, 22 (2018).

Bhalla A., Bansal N., Kumar S., Bischoff K. M., Sani R. K. Improved lignocellulose conversion to biofuels with thermophilic bacteria and thermostable enzymes. *Bioresource Technology*, vol. 128, pp. 751–759 (2013).

Brock T., Freeze H. Thermus aquaticus gen. n. and sp. n., a non-sporulating extreme thermophile. *J Bacteriol*; 98(1):289–297 (1969).

Dashti A., Mehrez J., Abdulsamad M. A., Dashti H. Heat Treatment of Bacteria: A Simple Method of DNA Extraction for Molecular Techniques. *Kuwait Medical Journal*, 41 (2009).

Daupan M.S., Rivera W.L. Isolation and characterization of culturable thermophilic bacteria from hot springs in Benguet, Philippines. *International Journal of Philippine Science and Technology*. 8:14–18 (2015).

Derekova A., Mandeva R., Kambourova M. Phylogenetic diversity of thermophilic carbohydrate degrading Bacilli from Bulgarian hotsprings. *World J. Microbiology and Biotechnology*. 24(9):1697–1702 (2008).

Dettmer A., Dos Anjos P. S., Gutterres M. Special review paper: Enzymes in the leather industry. *Journal of the American Leather Chemists Association*, vol. 108, no. 4, pp. 146–158 (2013).

Goh K. M., Kahar U. M., Chai Y. Y., Chong C. S., Chai K. P., Ranjani V., Illias R., Chan K.G. Recent discoveries and applications of Anoxybacillus. *Applied Microbiology and Biotechnology*. 97 (4):1474–1488 (2013) .

Kawasaki Y., Aoki M., Makino Y., Sakai H., Tsuboi Y., Ueda J., Sonoda K., Watanabe K., Yamamoto S., Kurosawa N. Characterization of moderately thermophilic bacteria isolated from saline hot spring in Japan. *Microbiology Indonesia*; 5:56–60 (2012).

Kumar M., Nath Yadav A, Tiwari R., Prasanna R., Saxena A.K. Evaluating the Diversity of Culturable Thermotolerant Bacteria from Four Hot Springs of India. *J. Biodivers Biopros Dev*, 1: 127 (2014) .

Legg C. A. A reconnaissance survey of the hot and mineralised springs of Zambia. Lusaka, Geological Survey Department, ER 50 (1974).

Lele O. H., Deshmukh, P. V. Isolation and characterization of thermophilic Bacillus sp. with extracellular enzymatic activities from hot spring of Ganeshpuri, Maharashtra, India. *Int. J. Appl. Res.*, 2:427–430 (2016).

Malkawi H. I., Al-Omari M. N. Culture-dependent and culture-independent approaches to study the bacterial and archaeal diversity from Jordanian hot springs. *African Journal of Microbiology Research*, vol. 4, no. 10, pp. 923–932 (2010).

Maugeri T.L., Gugliandolo C., Caccamo D., Stackebrandt E. A Polyphasic Taxonomic Study of Thermophilic Bacilli from Shallow, Marine Vents. *Systematic and Applied Microbiology*. 24:572–587 (2001).

Mohammed B. T., Daghistani H. I., Jaouani A., Abdel-Latif S., Kennes C. Isolation and Characterization of Thermophilic Bacteria from Jordanian Hot Springs: Bacillus licheniformis and Thermomonas hydrothermalis Isolates as Potential Producers of Thermostable Enzymes. *International Journal of Microbiology*, 1–12.10.1155/6943952 (2017).

Narayan V., Hatha M., Morgan H., Rao D. Isolation and characterization of aerobic thermophilic bacteria from the Savusavu hot springs in Fiji. *Microbes Environ*; 23(4):350–352 (2008).

Nshimiyimana J. B., Khadka S., Mwizerwa M. E., Akimana N., Adhikari S., Nsabimana A. Thermophiles: Isolation, Characterization and Screening for Enzymatic Activity. *Bioscience Discovery*, 9(3):430–437 (2018).

Roux K. H. The neighbor-joining method. A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4:406–425 (1995).

Saitou, N. and Nei, M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*. 4:406–425 (1987).

Sambrook K. J., Fritsch E. F., Maniatis T. Molecular Cloning: a Laboratory Manual. 2nd Edition. *Cold Spring Harbor*, New York (1989).

Sanders E. R. Aseptic laboratory techniques: plating methods. *Journal of visualized experiments: JoVE*, (63), e3064 (2012).

Sievert S.M., Ziebis W., Kuever J., Sahm K. Hydrothermal Microbial systems. Relative abundance of Archaea and Bacteria along a thermal gradient of a shallow water hydrothermal vent quantified by rRNA slot-blot hybridization. *Microbiology*. 146 (6):1287–93 (2000).

Stetter K. Hyperthermophiles: Isolation, Classification, and Properties in Extremophiles: Microbial Life in Extreme Environments. *Wiley-Liss*, Inc. 1–24 (1998).

Tamura, Glen Stecher, Daniel Peterson, Alan Filipski, Sudhir Kumar; MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0, *Molecular Biology and Evolution*, Volume 30, Issue 12, 1: 2725–2729 (2013).

Vos P., Garrity G., Jones D., Krieg N. R., Ludwig W., Rainey F. A., Schleifer K. H., Whitman W. The Firmicutes Volume 3. Bergey's Manual of Systematic Bacteriology. *Williams and Wilkins* (2009).