Molecular Identification and Phylogenetic Analysis of Some Rare Actinomycetes Obtained from Al-Lith Hot Spring Area of Saudi Arabia

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ABSTRACT

Isolating microbes from diverse natural ecological units has led to achieve metabolite structural diversity. Hot springs have been less explored ecological sects for discovery of novel microbial bioactive compounds as compared to other terrestrial samples. The capability of thermophilic microorganisms to flourish at high temperatures makes their enzyme systems ideal for various biotechnological applications. In this study, a total of 11 bacterial strains were isolated from Al-Lith hot spring which named Oyun Al-Haar, located about 17 km northeast of Gomika, at about 250 km south of Jeddah, Saudi Arabia. All these isolates were characterized and screened for some enzymes production. They were able to abundantly grow on starch nitrate agar medium and grew optimally at 45⁰ with pH 7.0. According to bioinformatics analysis, the eleven bacterial isolates were encompassing 9 actinomycetes and 2 eubacteria namely, *Streptomyces tendae* (3 isolates) and only one isolate for each of *Streptomyces mutabilis, Streptomyces chitinivorans, Streptomyces barkulensis, Leclercia adecarboxylata, Streptomyces fradiae, Streptomyces azureus, Streptomyces macrosporus* and *Enterobacter cloacae*. Based on the enzymatic activities, all isolates were positive for keratinase, gelatinase, chitinase, and lipase. Nine out of the eleven isolates showed protease production in good levels, and out of these 11 isolates, 6 isolates exhibited remarkable amylase activity. Bacteria, especially actinomycetes isolated from hot springs area have gained commercial significance as source of thermostable enzymes. There are few reports of enzymes production from the microflora of hot springs, which opens a window for exploring this resource as potential cache of novel strains with bioactive compounds. .

KEY WORDS: Isolating Microbes, Enzymes, Microflora, Encompassing.

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INTRODUCTION

Secondary products play important roles in several sectors of our society because they are valuable for industrial, biotechnological and pharmacological uses in addition to treat human diseases, such as cancer and bacterial infections (Harvey, 2008). The natural products are largely produced from primary and secondary metabolism of plants and microorganisms (Jose and Jebakumar, 2013). Microbial natural products have made an incredible contribution to the diverse industrial applications (Salehghamari and Najafi, 2016). There are more than 22,000 known microbial secondary metabolites, 70% of which are produced by actinomycetes, 20% from fungi, 7% from *Bacillus* spp. and 1-2% by other bacteria (Subramani and Aalbersberg, 2013). The industrial enzyme market is one of the fastest growing revenue generating sectors in the world. Biocatalysis offers green and clean solutions to chemical processes and is emerging as a challenging and revered alternative to chemical technology. Only 20 enzymes are currently utilized on the industrial level indicating the need for further research and development of low-cost enzymes and their production (Prakash et al., 2013). Enzymes derived from microbial source are generally regarded as safe and they are functional at wide range of temperature, pH, salinity or other extreme conditions (Mukhtar et al., 2017).

Nowadays, the search for new bioactive secondary metabolites has switched to rare genera of actinomycetes from normal habitats or to discovery of strains/species found in unusual habitats. The logic behind these approaches is that such strains may be producers of novel bioactive compounds (Khanna et al., 2011). The unexplored and underexplored environments are promising sources of rare isolates that are believed to be rich sources of interestingly new compounds (Subramani and Aalbersberg, 2013).The extreme habitats are examples of unexplored environments and characterized by chemical or physical conditions that differ significantly from those found in environments that support more abundant and varied life forms (Zhao et al., 2011). Microorganisms have been detected in a variety of extreme environments (Merino et al., 2019).

In recent years, researchers have shown great interest in thermophilic bacteria because of their economic potential, either in useful biological processes such as biodegradation, or in the production of antibiotics and enzymes (Agarwal and Mathur, 2016). Thermophiles including bacterial and archaeal species are found in various geothermally heated regions of the earth such as hot springs (El-Gayar et al., 2017). Hot springs are defined as springs with water at temperatures substantially higher than the air temperature of the surrounding region (Mahajan and Balachandran, 2017). Consistent exposure to high temperatures, high free water content, high moisture content, and a typical chemical composition facilitates the growth of a typical range of hyperthermophilic microorganisms (Mahajan, 2015).

Many microbial strains of following major groups/genera have been reported from hot springs as sporulating and non-sporulating bacilli, actinomycetes and cyanobacteria (Jiang and Xu, 1993). In Saudi Arabia, there are about ten geothermal springs with varying deep temperatures and different flow rates. They are distributed in Gizan and Al-Lith areas (El-Gayar et al., 2017). The aim of the present investigation is the characterization of some thermophilic bacteria, especially actinomycetes isolated from Al-Lith hot spring area, Saudi Arabia in addition to the determination of their potential production of some important enzymes.

MATERIAL AND METHODS

Chemicals and media: Cultivation media and components were obtained from different companies: BDH Limited Poole (England), Biomatic Corporation (Canada), Difco Laboratories (USA), Fluka Chemie (Switzerland), Himedia (India), Holyland (Saudi Arabia), Merck (Germany), and Techno Pharmchem Haryana (India).

Sample collection: Samples from different six locations were obtained from Al-Lith hot spring, Saudi Arabia. The hot spring of Al-Lith which named Oyun Al-Haar (Figures 1) was located about 17 km northeast of Gomika, at about 250 km south of Jeddah at 20˚27'39.4776''N, 40˚28'14.7252''E. The samples (mainly from the area not tampered by human activities) were obtained from: a) soil sediments from surface and at a depth of 1-2 cm, b) water from hot spring, and c) scraping of the inner wall of small channels. The samples were collected aseptically using sterile bottles along with a sterile spatula, transported at 4ºC, and processed directly.

Figure 1.(A): A mapshowing the sampling site atAl-Lith,and (B):The studied hot spring area, Gomika, Saudi Arabia.

Isolation and purification of bacteria: The soil samples were air dried at room temperature for about 7 days prior to inoculation onto isolation plates. Then, soil suspension of each sample was made separately by mixing 1g with 10 ml distilled water and vortexed for 2-5 min, then the mixtures could settle (Istianto et al., 2012). For each soil dilution, about 0.5 ml was taken (Al-Dhabi et al., 2016) and 0.1 ml was taking from hot spring water (Chaudhary and Prabhu, 2016). All these samples were separately spread evenly with a sterile glass rod onto starch nitrate agar medium (Shirling and Gottileb, 1966). Samples from the inner walls of hot spring were directly inoculated on starch nitrate agar medium by cotton swap. The inoculated plates were then incubated at 45°C for a week with presence of humidity (Chaudhary and Prabhu, 2016). After the incubation period, morphologically

distinct colonies were chosen and sub-cultured to obtain pure isolates. The pure cultures were maintained on starch nitrate agar slants at 4⁰C; for long-term storage, cultures were maintained in glycerol broth (50%, v/v) at -20⁰C (Uzel et al., 2011). Taxonomic characterization and producing some important enzymes were studied for all isolates.

Taxonomical studies of the isolates: Cultural characteristics of the isolates: Growth capability (heavy, moderate, scanty, or no growth), color of substrate and aerial mycelia, and presence of soluble pigment were examined using various media. These media were International *Streptomyces* Project (ISP) including yeast extract-malt extract agar (ISP-2) (Pridham et al., 1956, 1957), inorganic salts-starch agar (ISP-4) (Küster, 1959), tyrosine agar (ISP-7) (Shinobu, 1958), and starch nitrate agar (Shirling and Gottileb, 1966). The production of melanin pigment was examined using ISP-7 and dark brown to black color pigment means positive result (Srinivasan et al., 2016). In all previous media, the plates were incubated at 45⁰ for a week with presence of humidity. The color of substrate mycelia was described by the colors of RAL code (Vishwanatha et al., 2017).

Micromorphological characteristics of the isolates: Light microscope was used to observe the morphology of the isolates. Chitin agar plate was separately inoculated by the isolates and incubated at 45⁰ for 7 days to examine by direct microscopic observation at 400X magnification (Caviedes et al., 2000).

Physiological characteristics of the isolates: To determine the optimal temperature and pH of all isolates, the growth capability was observed using incubated the isolates at different temperatures (45, 50 and 55°C) and at various pH (5, 6, 7, 8 and 9). The pH of the media was adjusted by adding 1N NaOH or 1N HCl solution (Al-Dhabi et al., 2016).

Biochemical characteristics of the isolates: Gram staining was carried out by using standard Gram reaction. Antibiotic susceptibility was examined on Muller-Hinton agar (Himedia, India) plates. The isolates were separately spread over the agar plates with disks containing different antibiotics such as amikacin (AK), ceftazidime (CAZ), aztreonam (ATM), piperacillin (PRL), imipenem (IMI), and ciprofloxacin (CIP). After incubation at 45⁰ for 24-72 hr., the plates were examined for the presence of inhibition zones around the disc of antibiotic (Aly et al., 2012; Karp et al., 2017).

Molecular identification of the isolates: DNA extraction and PCR amplification: Genomic DNA extraction was performed at King Fahd Medical Research Centre, KAU according to Kumar et al., (2010) with some modifications. The purified isolates were grown for approximately 5 days on starch nitrate agar plates. In a clean tube, bacterial colonies of each isolate were separately mixed with 500 µl of TE buffer. This was followed by 3 cycles of heating in a water bath at 99⁰C

and then treating by liquid nitrogen (3 min in each per cycle). The crushed cells were treated with lysozyme (20 mg/ml) and incubated in a water bath at 37⁰ for 30 min. After cell lysis with 10% SDS (w/v) and proteinase K at 55⁰C for 30 min, the lysate was cooled, extracted for 5 min with a mixture of phenol: chloroform (1:1 v/v) at 10,000 rpm. In the aqueous phase, DNA was precipitated by adding 70-90% ethanol at -20⁰C for 30 min. After centrifugation, the formed pellet was washed twice with 90% ethanol and dissolved in TE buffer. To obtain RNA free DNA, 20 µl of RNAase solution (20 µg/ml) was added and then incubated at 37⁰C for 1 hr. The sample was once again extracted with equal volume of phenol: chloroform and precipitated as above. The purity and concentration of DNA was checked using NanoDrop 2000 spectrophotometer (Thermo Scientific).

The 16S rRNA was amplified using a thermal cycler (Applied Biosystems Veriti™ 96-Well Thermal Cycler). Amplification was performed using GoTaq® Green Master Mix, 2X (Promega) and two selected primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 519R (5'- GWATTACCGCGGCKGCTG -3') (Lane, 1991). PCR conditions consisted of an initial denaturation at 95⁰C for 5 min; followed by 35 cycles of denaturation (94⁰C for 40 sec), annealing (58⁰C for 40 sec), and extension (72⁰C for 1 min), and a final extension at 72⁰C for 10 min. The amplification products were examined by 1% agarose gel electrophoresis and the gel was visualized by Ultra-Violet Product (UVP BioSpectrum ® Imaging System).

Bioinformatics analysis: The sequences of 11 isolates were subjected to NCBI BLAST search tool to detect non-chance sequence similarity. BLAST search was restricted to 16S ribosomal RNA sequences, where models (XM/XP) as well as uncultured/environmental samples were also filtered out, such that more reliable results would be attained. Each individual sequence was solely blasted, where the BLAST hit with the lowest expect-value (which indicates the number of non-chance alignments) was picked. In order to ensure that BLAST outputs were governed by expected-value (aka e-value), BLAST algorithm parameter was decreased such the expected threshold was set to more stringent value of 1e−6. Alignment of the 11 sequences was carried out using version 2 of Clustalx (Larkin et al., 2007).

Exploratory data and phylogenetic analyses were carried out under R Project for Statistical Computing (R Core Team, 2019) where exploratory data analysis was done using SeqinR package (Charif and Lobry, 2007). Phylogenetic analysis was carried out by ape package (Paradis et al., 2004). Reconstruction of the phylogenetic tree was done using neighbor-joining method (Nei, 1987). DNA Sequence Polymorphism (DnaSP) (Librado and Rozas, 2009) software was used to analyze the haplotype diversity (Hd), average number of nucleotide differences (Tajima, 1983), the nucleotide diversity (π), polymorphic sites (S), singleton variable sites (SP), parsimony-informative sites (PIP) for each gene, and

the average number of nucleotide substitutions per site between species (Dxy) (Lynch and Crease, 1990).

Screening of the isolates for their extracellular enzymes production: Production of extracellular enzymes by potential isolates was studied by inoculated individually on different agar media at 45^0 for 7-14 days. Bacterial isolates were subjected to screen for amylase, protease, keratinase, gelatinase, chitinase, and lipase activity. For screening amylolytic activity, all isolates were individually grown on starch agar medium (Mohseni et al., 2013). After growth, the plates were flooded with iodine solution. Amylase production was detected as a zone of clearance around the colony on bluish black background due to starch digestion. Iodine forms a bluish black complex with starch but not with hydrolyzed starch (Singh et al., 2019). Indicator of hydrolysis of casein was assessed by growing the isolates individually on skim milk agar medium (Mohseni et al., 2013). Protease production was observed by appearing a clear zone around the colony in the medium (Das et al., 2010).

Regarding the screening for keratinase, feather meal agar medium (Agrahari and Wadhwa, 2010) was individually inoculated with the bacterial isolates. A clearing zone around the growth indicates that this isolate has keratinase. Gelatinase production by the isolates was tested using nutrient gelatin medium in test tubes (Ekpenyong et al., 2016). Partial or total gelatin liquefaction of the inoculated tubes even after exposure to cold temperature of refrigerator (4°C) was considered that these isolates produced gelatinase (Kole et al., 1998).

The bacterial cultures were further examined for their chitinase activity on plates containing chitin agar medium (Kim et al., 2003). The isolates that produced chitinase can grow on the medium and their growth depends, at least partially, on their ability to solubilize chitin. Also, appearance of a clear zone surrounding the colony on a creamish background was regarded that this isolate positive for chitinase activity (Sukalkar et al., 2017; Lacombe-Harvey et al., 2018). To determine lipase activity, all isolated bacteria were individually streaked on solid agar medium with Tween 80 as substrate (Niyonzima and More, 2013). The lipolysis was detected due to occurrence of a zone of clearance around the growth and subsequent formation of white precipitate of calcium monolaurate (Sierra, 1957; Cardenas et al., 2001) because of the combination of released fatty acids and Ca^{+2} ions (Bernal et al., 2015).

Table 1. Cultural characteristics of hot spring isolates grown on starch nitrate agar medium at 45°C for 7 days.

Isolate no.	Growth	*Source of isolation	Color of aerial mycelium	Color of substratemycelium	Soluble pigment
HL1	Heavy	Sediment 1	Pale grey	Terra brown	-
HL2	Heavy	Sediment 1	Grey	Sepia brown	-
HL3	Heavy	Sediment 1	Grey	Terra brown	-
HL4	Moderate	Sediment 2	White	Signal yellow	-
HL5	Heavy	Sediment 2	Pale grey	Golden yellow	-
HL6	Heavy	Sediment 3	Grey	ND	-
HL7	Heavy	Sediment 3	Brown	Brown beige	-
HL8	Heavy	Wall 1	Pale brown	Brown beige	-
HL9	Heavy	Wall 2	Dark green	Olive brown	-
HL10	Heavy	Wall 1	Dark grey	ND	-
HL11	Heavy	Water	Grey	Terra brown	-

*Sediment 1 and sediment 3: at a depth of 1-2 cm; Sediment 2: from surface; -: Soluble pigment absent; ND: Not detected.

Figure 2: Colonies morphology and color of some hot spring isolates grown on starch nitrate agar medium at 45°C for 7 days.
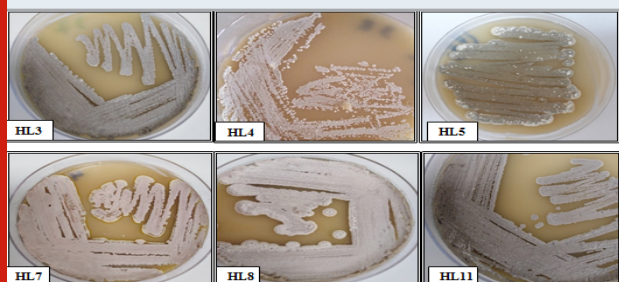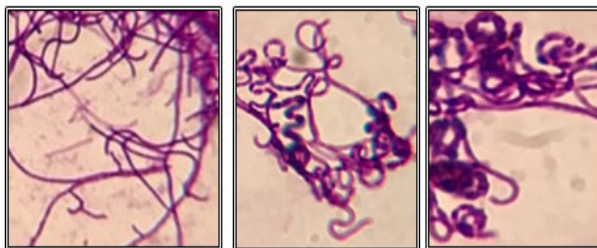

Figure 3: Gram staining of some hot spring actinomycetes under a light microscope at 1000X magnification.

RESULTS

Isolation of actinomycetes: A total of 11 morphologically distinct colonies were obtained from Oyun Al-Haar hot spring using starch nitrate agar medium. The temperature of water in the spring ranged from 46°C to 75°C with pH 5.0-6.0. A total of 7 isolates, named from HL1 to HL7 were obtained from air-dried sediments, while isolates named HL8, HL9, and HL10 were obtained from inner wall of channels, and only one isolate HL11 was obtained from spring water.

Table 2. Some cultural and physiological characters of hot spring bacterial isolates.

Tested character	Isolate no.	Result
Melanin pigment on IPS-7	Isolate HL6	+ ve
	Isolates HL1, HL2, HL3, HL4, HL5, HL7, HL8, HL9, HL10, and HL11	-ve
Growth temperature range	Isolates HL1, HL2, HL3, HL6, and HL11	45-50°C
	Isolates HL4, HL5, HL7, HL8, HL9, and HL10	45-55°C
pH range	Isolate HL5	6.0-9.0
	Isolates HL1, HL2, HL3, HL4, HL6, HL7, HL8, HL9, HL10, and HL11	5.0-9.0
+ve: Positive result; -ve: Negative result.		

Table 3. Antibiotic susceptibility of hot spring bacterial isolates to some antibiotics.

Isolate no.	*Resistance to antibiotics					
	AK	CAZ	ATM	PRL	IMI	CIP
HL1	S	R	R	R	S	S
HL2	S	R	R	R	S	S
HL3	S	R	R	R	S	S
HL4	S	R	R	S	S	S
HL5	S	R	R	R	S	S
HL6	S	R	R	R	S	S
HL7	S	R	R	R	S	S
HL8	S	R	R	R	S	S
HL9	S	R	R	S	S	S
HL10	S	R	R	R	S	S
HL11	S	R	R	R	S	S

*(AK): Amikacin; (CAZ): Ceftazidime; (ATM): Aztreonam; (PRL): Piperacillin; (IMI): Imipenem; (CIP): Ciprofloxacin. R: Resistant (16 mm or less); S: Sensitive (20 mm or more).

Cultural characterization of the isolates: From the result, it was indicated that cultural characteristics of the isolates on starch nitrate agar medium showed various colonies appearance and no soluble pigments were observed in the medium. Also, it was noticed that most isolates (7 isolates) had grey color in different degrees, 2 isolates were brown, one isolate was white and one isolate was green (Table 1 and Figure 2). Colonies characteristics of the isolates on isolation medium can be transformed into different appearance when the organism is sub-cultured on another growth medium. All isolates showed good growth (heavy or moderate) on starch nitrate agar; however, the growth on ISP-2, ISP-4, and ISP-7 plates was varied.

On ISP-2 medium, one isolate HL8 had heavy growth, one isolate HL6 showed moderate growth, 7 isolates HL1, HL2, HL3, HL7, HL9, HL10, and HL11 had poor growth, and isolates HL4 and HL5 showed no growth. On ISP-4 medium, the isolates were divided into two groups; the isolates HL2, HL3, HL6, HL7, HL8, HL9, HL10, and HL11 showed heavy growth and the isolates HL1, HL4, and HL5 showed moderate growth. The growth and melanin pigment production were recorded on IPS-7 (tyrosine agar). On the previous medium, the growth was heavy for isolate HL6, moderate for isolates HL1, HL7, HL8, and HL9, and scanty for these isolates HL2, HL3, HL4, HL5, HL10, and HL11, while melanin pigment was noticed only for isolate HL6.

Figure 4: Antibiotic resistance patterns of the two isolates HL1 and HL9, AK: Amikacin; CAZ: Ceftazidime; ATM: Aztreonam; PRL: Piperacillin; IMI: Imipenem; CIP: Ciprofloxacin.

Micromorphological characterization of the isolates: The morphology of the isolates was examined under a light microscope. All isolates, except isolates HL6 and HL10, were Gram-positive with filamentous hyphae (Figure 3). Isolates HL6 and HL10 were Gram-negative with rod-shaped.

Physiological and biochemical characterization of the isolates: As shown in Table 2, the obtained data from physiological and biochemical examination showed that the optimal growth temperature for all isolates was 45°C. Out of 11 isolates, 6 isolates HL4, HL5, HL7, HL8, HL9, and HL10 grew approximately up to 55°C and the isolates HL1, HL2, HL3, HL6, and HL11 could grow up to 50°C only. It was noticed that all isolates, except isolate HL5, grew well at pH between 5.0 and 9.0 and the pH growth range for isolate HL5 was 6.0-9.0. The optimum pH for all isolates was 7.0. Using antibiotic susceptibility pattern, the growth ability of the isolates was evaluated in the presence of six different antibiotics. All isolates except isolates HL4 and HL9 recorded a clear sensitivity against amikacin, imipenem, and ciprofloxacin with different degrees. Meanwhile, isolates HL4 and HL9 demonstrated resistance against ceftazidime and aztreonam. Table 3 clarified antibiotic susceptibility profiles of all isolates. Figure 4 illustrated antibiotic susceptibility of the isolates HL1 and HL9.

Table 4. Results of NCBI BLAST query for the 11 sequences isolated from hot spring.

Isolate no.	Species	Query coverage (%)	Identity (%)
HL1	*Streptomyces tendae*1	40	99
HL2	*Streptomyces tendae*2	30	99
HL3	*Streptomyces mutabilis*	36	100
HL4	*Streptomyces chitinivorans*	34	97
HL5	*Streptomyces barkulensis*	34	98
HL6	*Leclercia adecarboxylata*	34	97
HL7	*Streptomyces fradiae*	27	100
HL8	*Streptomyces azureus*	90	96
HL9	*Streptomyces macrosporus*	85	100
HL10	*Enterobacter cloacae*	96	95
HL11	*Streptomyces tendae*3	30	99

Bioinformatics analysis of the data: NCBI BLAST query: Table 4 showed results of NCBI BLAST query for the 11 sequenced isolates. The criteria used for query sequence aimed to narrow down the search space (database), such that a smaller the database has more likely to contain the sequence of interest. Therefore, search query was restricted to 16S ribosomal RNA sequences. For all the 11 queries, zero e-values were attained indicated that all alignments were non-chance alignments. The percentages of query coverage ranged from 27 to 96%, where identities % were also high which ranged from 95 to 100%. The results of BLAST query indicated that 11 strains of bacteria encompassing 9 actinomycetes

and 2 eubacteria namely, *Streptomyces tendae* (3 isolates) and only one isolate for each of *Streptomyces mutabilis*, *Streptomyces chitinivorans*, *Streptomyces barkulensis*, *Leclercia adecarboxylata*, *Streptomyces fradiae*, *Streptomyces azureus*, *Streptomyces macrosporus* and *Enterobacter cloacae*.

Exploratory data analysis: The base frequencies of the 11 isolates were presented in Figure 5. Base frequencies varied greatly among the 11 isolates, this may be result of great fluctuation in sequence lengths (Figure 6). Sequence length ranged from 499 for *S. azureus* to 1587 base pair (bp) for *S. fradiae* (Table 5). Similarly, GC percentage (%) also varied greatly, where GC% ranged from 49% for *S. fradiae* to 60% for *S. tendae* 2.

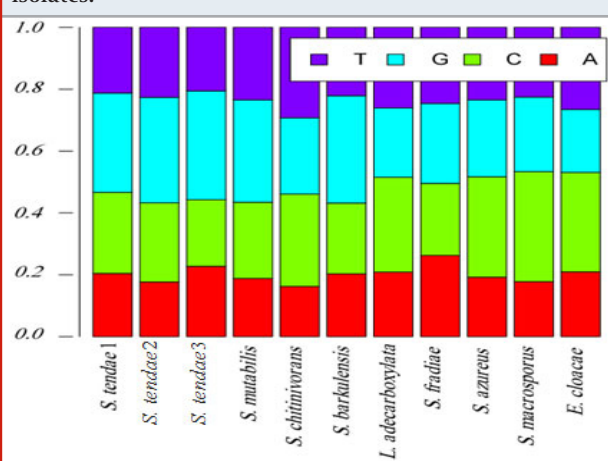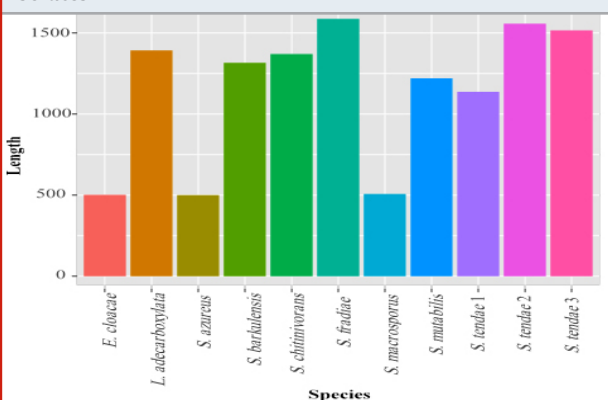Figure 5: Base frequencies of hot spring bacterial isolates.

Figure 6: Sequence lengths of hot spring bacterial isolates

Cluster and phylogenetic analysis: Cluster analysis was carried out as pre-processing step to glean an insight into the data distribution. Results of cluster analysis were shown graphically in Figure 7 and Table 6. The resulted dendrogram comprised of 2 large clusters, where *Leclercia* and *Enterobacter* species clustered in one cluster and *Streptomyces* species clustered in the second cluster. The evolutionary history of the 11 isolates was inferred based on neighbor-joining method (Figure 8). The optimal tree with the sum of branch length = 2.5 is

shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and were expressed as the number of base substitutions per site. This analysis involved 11 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1650 positions in the final dataset.

Table 5. Sequence lengths and GC contents of hot spring bacterial isolates.

Species	Sequence length	*GC content
S. tendae1	1138	0.58
S. tendae2	1556	0.60
S. tendae3	1515	0.57
S. mutabilis	1219	0.58
S. chitinivorans	1370	0.54
S. barkulensis	1315	0.58
L. adecarboxylata	1392	0.53
S. fradiae	1587	0.49
S. azureus	499	0.57
S. macrosporus	506	0.60
E. cloacae	501	0.52

*GC content: Guanine + Cytosine content

Polymorphism and genetic diversity among species: General information about the polymorphisms on the 11 isolates was found in Table 7. The number of sites was 1650, the number of monomorphic informative sites was 296 sites, and the number of polymorphic sites was 175. Of polymorphic sites, 151 sites were parsimony-informative sites (i.e. sites that have a minimum of two nucleotides that are present at least twice) and 24 sites were singletons. The 11 sequences were also analyzed to characterize the sequence diversity. The results of the analysis were presented in Table 8, the haplotype diversity was 1±0.04 and nucleotide diversity was only 0.15, where the average number of nucleotide differences was 68.6. Only one conserved region was found among the 11 isolates in the region between 18 to 552. Measurements of conservation (C)=0.59, homozygosity =0.82 and P-value <0.001. Conservation (C) was calculated as the proportion of conserved site in the alignment region, where homozygosity was measured as 1- heterozygosity.

Screening for enzymes production: From the results (Table 9), it was noticed that all isolates produced keratinase, gelatinase, chitinase, and lipase. Out of 11 isolates, 6 isolates HL1, HL6, HL8, HL9, HL10, and HL11 produced amylase, while isolates HL2, HL3, HL4, HL5, and HL7 showed weak amylase production. On skim milk agar medium, all isolates showed protease production, but the isolates HL1 and HL2 exhibited protease activity

in very low levels. Some results for enzymes production were depicted in Figure 9.

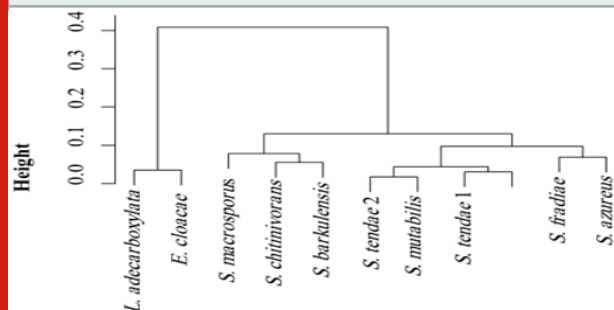Figure 7. Cluster analysis of hot spring bacterial isolates.

Table 6. Distribution of hot spring bacterial isolates in 3 clusters

Species	Clusters		
	1	2	3
E. cloacae	1	0	0
L. adecarboxylata	1	0	0
S. azureus	0	0	1
S. barkulensis	0	1	0
S. chitinivorans	0	1	0
S. fradiae	0	0	1
S. macrosporus	0	1	0
S. mutabilis	0	0	1
S. tendae	0	0	3

Figure 8: Neighbor-joining phylogenetic tree of hot spring bacterial isolates.
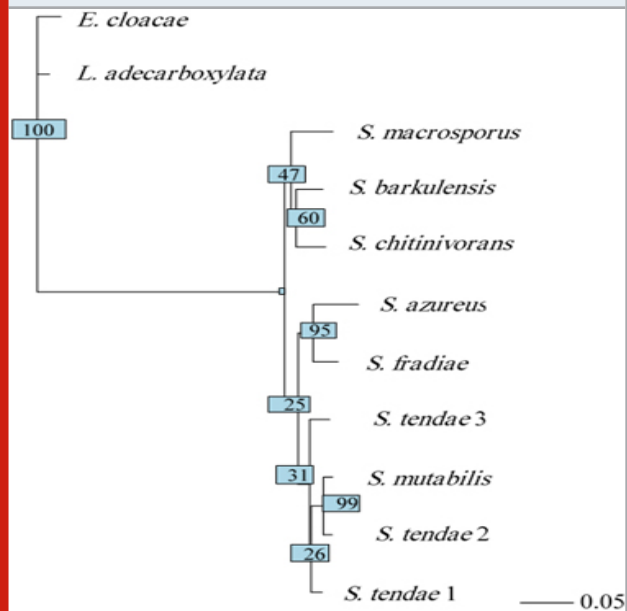
Table 7. Estimated parameters of the polymorphic sites of hot spring bacterial isolates.

| No. of sites	No. of monomorphic informative sites	No. of polymorphic sites	Parsimony-informative sites	Singleton variable sites
1650	296	175	151	24

Table 8. Estimated parameters of DNA polymorphism of hot spring bacterial isolates.

No. of Haplotypes	Haplotype diversity±sd	Nucleotide diversityπ	Average number of nucleotide differences
11	1±0.04	0.15	68.6

Table 9. Enzymes production by bacterialisolates.

Enzyme activities	Isolate no.	Enzyme production
Amylase	Isolates HL1, HL6, HL8, HL9, HL10, and HL11	+ve
	Isolates HL2, HL3, HL4, HL5, and HL7	-ve
Protease	Isolates HL3, HL4, HL5, HL6, HL7, HL8, HL9, HL10, and HL11	+ve
	Isolates HL1and HL2	-ve
Keratinase Gelatinase Chitinase Lipase	Isolates HL1, HL2, HL3, HL4, HL5, HL6, HL7, HL8, HL9, HL10, and HL11	+ve

Figure 9: Enzymes production by hot spring isolates on different agar media, (A): Starch hydrolysis produced by isolate HL9, (B): A clear zone of skim milk hydrolysis by isolate HL4, (C):Keratinase degradation by isolate HL8, (D):Gelatin liquefaction by isolate HL3, (E): Chitinase production by isolate HL8 and (F):Lipolytic activity of isolate HL2.
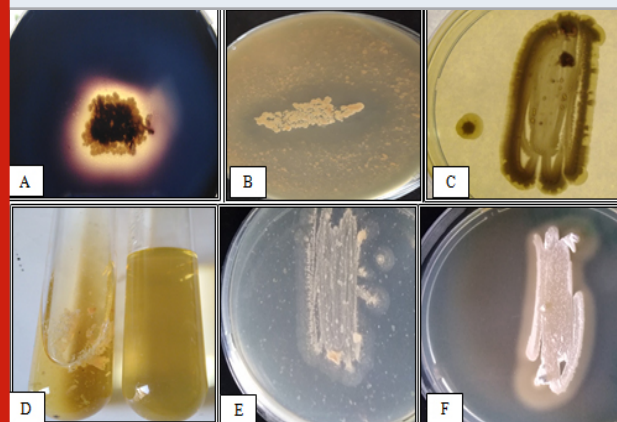
DISCUSSION

Factors such as pH, temperature, pressure, salt and nutrient concentrations, water availability, radiation, harmful heavy metals, and toxic compounds are used in defining extreme habitats (Satyanarayana et al., 2005; Procopio et al., 2016). Microorganisms, including actinomycetes, from extreme environments have attracted a great deal of attention, due to their special mechanisms of adapting to the extreme conditions and because they can produce unusual compounds (Meklat et al., 2011). It is postulated that extremotolerants may have larger genetic and metabolic plasticity (Mohammadipanah and Wink, 2016).

Studies on the microbial potential of extreme environments can be utilized to produce novel enzymes that can become future harbingers of green biotechnology (Mukhtar et al., 2017). It is possible to consider thermophiles as sources of industrially relevant thermostable enzymes. Many industrial processes require enzymes such as amylase, cellulase, xylanase, pectinase, protease, and lipase that are operationally stable at high temperatures (Haki and Rakshit, 2003; Bouzaset al., 2006). Thermophilic bacteria and their hydrolytic enzymes have become a chief research subject due to their valuable biotechnological applications (El-Gayaret al., 2017). Thermophilic actinomycetes play an important role in habitats where decomposition of organic matter takes place at elevated temperatures and under aerobic conditions. Using these isolates for direct hydrolysis agro-industrial waste and in biofuel generation also seems a promising strategy (Chaudhary and Prabhu, 2016).

This research aimed to find novel bacteria, especially actinomycetes that produced some useful enzymes from poorly studied extreme habitat, hot spring ecosystem in Saudi Arabia. In this study, a total of 11 bacterial strains were successfully isolated from sediments, walls, and water samples collected from Al-Lith hot spring named Oyun Al-Haar. This spring is located about 17 km northeast of Gomika, at about 250 km south of Jeddah, Saudi Arabia. From the results, it was noticed that numbers of bacteria obtained from hot spring

walls or water were lower than sediment samples. Some previous studies reported similar findings. Khiyami et al. (2012) and El-Gayar et al. (2017) recorded that the total count of bacteria in hot spring water was low. Also, Chaudhary and Prabhu (2016) reported that the sediment sample from hot spring showed a good diversity of actinomycete colonies, while the water sample had only two types. Akmar et al. (2011) found that isolation rate of actinomycetes from hot spring was higher from sediment and biomat than from water sample.

In the current study, taxonomic characterization of the isolates by morphological, physiological, and biochemical characters were studied. Sequencing of the ribosomal RNA gene of bacteria followed by bioinformatics studies were used to identify the bacteria as reported in previous studies (Caverly et al., 2015; Alsanie et al., 2018). In this study, bioinformatics analysis was performed for 11 hot spring isolates to determine the evolutionary and phylogenetic relationships among the isolates. The following strains were identified from the isolates: *Streptomyces tendae* (3 isolates) and only one isolate for each of *Streptomyces mutabilis, Streptomyces chitinivorans, Streptomyces barkulensis, Leclercia adecarboxylata, Streptomyces fradiae, Streptomyces azureus, Streptomyces macrosporus*, and *Enterobacter cloacae.*

The genus *Streptomyces* is classified in the family Streptomycetaceae and has received attention for three main reasons. First, streptomycetes are abundant and important in the soil, where they play major roles in the cycling of carbon trapped in insoluble organic debris, particularly from plants and fungi. This action is enabled by the production of diverse hydrolytic exoenzymes (Barka et al., 2016). Second, the genus exhibits a wide phylogenetic spread (Aderem, 2005). Thirdly, streptomycetes produce a stunning diversity of bioactive secondary metabolites; consequently, they are of great interest in medicine and industry (Hopwood, 2007).

Leclercia adecarboxylata is a Gram-negative bacillus belonging to the family Enterobacteriaceae. It is mainly isolated from environmental or animal specimens but has been recognized as an emerging opportunistic pathogen, with the potential to cause severe infections in immunocompromised patients (Spiegelhauer et al., 2019). *L. adecarboxylata* is a member of the normal gut flora in animals (Hess et al., 2008). *Enterobacter cloacae* is a Gram-negative, facultative anaerobic, rod-shaped, non-spore-forming bacteria belonging to the family Enterobacteriaceae. Species of the *E. cloacae* complex are widely encountered in nature, but they are also pathogens. *E. cloacae* has taken on clinical significance as opportunistic bacterium and has emerged as nosocomial pathogens from intensive care patients pathogenic, especially to those who are on mechanical ventilation (Mezzatesta et al., 2012).

The bioinformatics analysis results in this study revealed that there was a high variability of sequence lengths between and within species, some species contained long sequences, whereas other species contained short sequences. Furthermore, GC contents were highly variable among the target species. Phylogenetic tree construction of isolated hot spring bacterial strains was done using neighbor-joining method (Nei, 1987). Sequence variations and evolutionary distances were calculated using the Maximum Composite Likelihood method (Tamura et al., 2004). Similarly, same phylogenetic analysis methods were applied by Alsanie et al. (2018) for identification of isolated multidrug-resistant (MDR) bacterial strains.

Molecular identification of the isolates demonstrated low diversity of thermophilic bacteria. Out of 11 isolates, 9 strains were belonging to the genus *Streptomyces*, one isolate was identified as *Leclerciaa decarboxylata*, and another isolate was identified as *Enterobacter cloacae.* Similarly, Aanniz et al. (2015) recorded a very low number of thermophilic bacteria in four hot springs in Morocco. In 2012, Khiyami and coauthors have identified about 15 thermo-aerobic bacteria from Jazan and Al-Lith geothermal springs where *Bacillus cereus, Bacillus licheniformis, Bacillus thermoamylovorans, Pseudomonas aeruginosa* and *Enterobacter* sp. were the dominant strains. The low density of the bacterial populations in hot springs could be attributed to the adverse conditions of these environments or due to the application of culture-dependent identification approaches that identify only a small portion of the total microbial communities (Ranjard et al., 2000). In contrast to the results obtained from this study, 73 strains of bacteria encompassing 8 actinomycetes and 65 eubacteria were isolated and purified from the four sampling points of Vajreshwari-Ganeshpurihot springs, India. The isolates reflected the diversity with respect to macroscopic and microscopic characteristics. Twenty-four were Gram-positive and 49 were of Gram-negative bacteria. About 11% of the isolates were actinomycetes (Pednekar et al., 2011).

Valverde et al. (2012) studied actinobacteria in the hot springs in Zambia, China, New Zealand, and Kenya and observed around 28 major operational taxonomic units (OTUs). They opined that the actinobacterial diversity and endemism were very high in hot spring ecosystems. In addition, several strains of actinomycetes have been previously reported from hot springs (Thawai, 2012; Coman et al., 2015; Kambura et al., 2016). Duan et al. (2014) isolated a novel species, *Streptomyces calidiresistens* from a sediment sample collected from Hehua hot spring in China. Regarding enzymes production in this study, all hot spring bacteria showed positive for keratinase, gelatinase, chitinase, and lipase activity. However, out of the total isolates, only 6 isolates exhibited highly amylase activity and 9 isolates showed good protease production. From hot springs in Gazan, Saudi Arabia, two thermophilic bacteria *Brevibacterium linens* and *Bacillus subtilis* were isolated and showed the capability to produce amylolytic and proteolytic enzymes (El-Gayar et al., 2017).

Another study was conducted with Khalil (2011) who isolated 13 thermophilic bacteria from hot springs in Saudi Arabia. Based on the biochemical characterization, all the isolates were lipase positive, 11 isolates showed

amylase activity, while only 3 of them showed cellulase activity. Bacilli like *Thermus aquaticus* and *Thermus brockianus*, from hot springs have gained commercial significance as source of thermostable enzymes (Brock and Freeze, 1969; Breithaupt, 2001). Thermophilic actinobacteria are biotechnologically important producers of several enzymes such as DNA polymerases, pullulanases, amylases, xylanases, lipases, and proteases (Panda et al., 2017). Chaudhary and Prabhu (2016) isolated species of *Streptomyces* from hot spring water, and these strains produced remarkable amount of thermostable amylase and cellulase with temperature optimum at 55. The thermophilic *Streptomyces* Al-Dhabi-2 strain isolated from hot spring produced various enzymes such as amylase, gelatinase, and deoxyribonuclease (DNase) (Al-Dhabi et al., 2019).

CONCLUSION

In conclusion, hot spring environments are underexplored microbiologically and should not be overlooked for the search and discovery of novel bacteria and their chemical diversity of useful compounds. Studies on unique ecological environments could yield enzymes that could be of great commercial importance in near future. The results of this investigation revealed that Al-Lith hot spring named Oyun Al-Haar was potent source of thermophilic bacteria with hydrolytic enzymes production that can catalyze various reactions at high temperatures. Hence, these isolates can be further evaluated and studied in detail for commercial scale production of enzymes to use in various pharmaceutical and industrial applications. Evidently, more studies on optimal cultivation techniques for thermophiles are required, not only for basic research but also for profiling of their unique microbial products.

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