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ISOLATION AND IDENTIFICATION AEROBIC THERMOPHILIC BACTERIA FROM HOT SPRING RAMSAR

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ABSTRACT

Members of the genus *Bacillus* are considered the most frequently isolated thermophilic aerobes from terrestrial and marine hot water environments. Thermophilic microorganisms are found to be potential and good alternative source of thermostable enzymes. Terrestrial hot springs support thermophilic prokaryotic communities. In this study, the samples were enriched and cultivated in the modified medium named mBTM. SO, phenotyping and genotyping characteristics of isolated bacteria have been shown by morphological, biochemical and molecular tests. According to the results of this study, isolated bacteria are belong to the thermophilic genera of *Bacillu*s. according to a study of diversity is wide aerobic bacilli. 16S rDNA sequence analysis showed that have higher similarity with *B. licheniformis.* Existence of *B. licheniformis* in thermal area was also reported in many other studies. However, this is study that demonstrated that *B. licheniformis* population is common bacterial species present in hot spring Ramsar, Ramsar, IRAN.

Keywords: Thermophiles, mBTM, Hot Spring, 16S rDNA Sequence

INTRODUCTION

Thermophilic bacteria show optimal growth at temperatures ranging from 45 to 70 °C and can be isolated from both thermophilic and mesophilic environments **[1]**.Members of the genus *Bacillus* are considered the most frequently isolated thermophilic aerobes from terrestrial and marine hot-water environments **[2, 3]**. Aerobic spores forming thermophilic bacteria growing at 70 °C were characterized for the fi rst time by Miquel in 1888 **[4]**. Then

a number of strains of the spore forming thermophilic bacteria particularly those belonging to the genera *Bacillus* and *Clostridium* have been studied **[5]**. Numerical classification based on a series of phonetic characters was used for classification of 368 *Bacillus* strains into 79 clusters **[6]**. After 1990, 16S rDNA has been successfully applied in determining phylogenetic relationships of the aerobic, endospore-forming bacteria, which played an important role in the creation of several families and genera of Bacillales **[7]**. Thermophilic microorganisms are found to be potential and good alternative source of thermostable enzymes **[8]**.

They are source of products for industrial use, such exopolysaccharides or compatible solutes and thermostable enzymes, named thermozymes **[9, 10, 11]**. Thermal stability enables thermozymes to be active in the presence of chemical denaturants and to resist harsh process conditions **[12]**. Terrestrial hot springs support thermophilic prokaryotic communities and significant research attention has centered upon the lithic laminated microbial mats that occur in thermal waters from 50–75_C **[13]**. There are 308 hot spring areas in Iran **[14]** and most of them are located in the north and northwest of the country. The waters of the hottest spring (Geynarjeh), located in the NWSabalan geothermal field, have a temperature of 86ºC. **[15]**. Ramsar hot springs are located in north of IRAN the Latitude 36° 54′ 1.08″N, and Longitude 50° 40′ 57″ E.

MATERIALS AND METHODS

Sample Collection and Sampling

Water and suspended sediment samples from hot springs at 30 to 50 cm in summer 1390-91 of hot spring Ramsar have collected located in north of Iran in the Ramsar area **(Figure 1)**. Samples was transferred immediately to the microbiology laboratory. Water temperature at the time of sampling and 48-50ºC and pH of water 6.5- 7 respectively.

Medium and Growth Conditions

Samples were cultured in mediummBTM (moditied Basal Medium Thermophilc) include ($10^{-1}{}_g$): 10_g Peptone of meat , 0.1_{ml} (%10) $FeSO_4.7H_2O$, 0.2_g $MgCL_2.6H_2o$, 0.9_g NH_4CL. 1.5_g k_2HPO_4, 3_g yeast extract, 5_g Glucose, 5_{ml} wolf's vitamin, 10_{ml} Trace element Sulation, Trace 5, 1_g Tryptone, 1_g, NaCL. pH:7.2±0.2. The cultures for 24 to 48 hours incubation at rpm 200/ 50 degrees and use the same medium by adding 1.5% agar to isolate single colony. Bacteria isolated and purified in mBTM containing 1% DMSO in -70°C to be stored for subsequent studies.

Figure 1: Hot spring Ramsar

Morphology, Physiology and Biochemical Characteristics of Isolated

The isolates were Gram stained and observed for cell morphology, spores production and motility. Oxidase and catalase activity was also tested. Temperature and pH range for growth was determined following incubation of the strains for 48 h days at 37 to 70°C and pH 5 to 12 in BTM medium. Biochemical characteristics were screened by the miniaturized systems API 20E, API 50CHB (bioMerieux) according to Maugeri *et al.* **[5]**. Strips were incubated at 55°C for 24 to 48 h.

DNA Extraction and PCR Amplification

Genomic DNA from isolates was extracted according to Ausubel *et al.*, **[16]**. The 16S rRNA genes were amplified by PCR using universal bacterial primers 27F (5' GAGTTTGATCCTGGCTCAG- 3'; position 9-27 in *E. coli* numbering) and 1492R (5' GGTTACCTTGTTACGACT 3') **[17]**. PCR reactions were performed with a HotStar Master mix (Takara, Japan) and The reaction mixtures contained (per 25 µl) 12 µl PCR Master mix, each primer at a concentration of 1 µM, and 5 µl of extracted DNA as templates and 6 µl Distilled Deionized Water. The temperature profile for the PCR was as follows: initial denaturation at 95°C for 5 min, followed by denaturation at 95°C for 1 min, annealing at 59°C for 1.15 min, and primer extension at 72 °C for 1 min. After the 35th cycle, the extension step was prolonged for 10 min to complete synthesis of all strands, and then the samples were kept at 4 °C until analysis. PCR products were detected by gel electrophoresis. Samples (5 µl) of final PCR products were loaded onto 1.5% agarose gels and subjected to electrophoresis in 1X TAE buffer for 45 min at 100 V. The gels were stained with ethidium bromide and photographed with UV light transillumination.

16S rDNA Sequence Determination and Analysis

Isolates selected for 16S rDNA sequencing. The PCR products were purified using the Wizard Genomic DNA Purification kit and sequenced by Macrogen (Korea). BLAST software (http://www.ncbi.nlm.nih.gov/blast/)

RESULTS AND DISCUSSION

RESULTS

Phenotypic Characteristics

Ten strains of thermophilic bacteria spore forming, Gram-positive, catalase and oxidase positive were isolated from Hot spring Ramsar. All strains were able to grow at 45 °C and most from 40 °C until 62 °C. Most of field isolates was able to grow at pH 10. All the environmental isolates were able to hydrolyse gelatine. Majority of the isolates here studied showed lipolytic and amylolytic activities **(Table 1)**.

16srDNA Sequence Analysis and Phylogenic Tree

BLAST analysis of the most complete sequencing results revealed the similarity percentages among field isolates and reference strains **(Table 2)**. The nucleotide sequences of isolated have been deposited in the GenBank database **(Table 2)**. Based on phylogenetic comparisons of sequenced 16S rDNA genes, RA19 and RA30 represented two groups of novel species **(Figure 2)**.

Table 1: Phenotypic and chemo-taxonomic properties of isolated bacteria of Hot spring

isolated of hot spring										
	RA_{23}	**RA_{12}**	**RA_3**	**RA_6**	**RA_1**	**RA**	**R5**	**RA19**	**RA30**	**R4**
Morphology	**Bacilli**	**Bacilli**	**Bacilli**	**Bacilli**	**Bacilli**	**Bacilli**	**Bacilli**	**Bacilli**	**Bacilli**	**Bacilli**
Gram Reaction	+	+	+	+	+	+	+	+	+	+
Range temperature for growth	**45-60**	**45-55**	**45-62**	**45-62**	**45-55**	**45-62**	**45-55**	**45-55**	**45-55**	**50-55**
Range pH for growth	**6.5-10**	**6-9**	**5-7**	**5-8.5**	**6.5-9**	**6-10**	**6-10**	**5-7**	**6-8.5**	**5-9**
Catalase	+	+	+	+	+	+	+	+	+	+
Cellulase	-	-	-	+	-	-	-	-	-	-
Oxidase	+	+	+	-	+	-	+	+	+	+
Lipase	+	+	**weak**	**weak**	+	**weak**	+	+	+	+
Amylase	+	+	+	+	+	+	+	+	+	+
Protease	-	+	-	**weak**	+	+	+	+	+	+
Carbone source:										
Glycerol	-	-	-	-	-	+	+	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-
L-Arabinose	+	+	-	+	+	-	-	+	+	-
D-ribose	+	+	-	+	+	+	+	+	+	-
D-xylose	+	-	-	-	+	-	+	+	+	-
L-xylose	-	-	-	-	-	-	-	-	-	-
D-Adonitol	-	-	-	-	-	-	-	-	-	-
Methyl-B-D-xylopyranoaside	-	-	-	-	-	-	-	-	-	-
D-Galactose	-	-	-	-	-	-	-	-	-	-
D-Glucose	+	+	+	+	+	+	-	+	+	-
D-Fructose	+	+	+	+	+	+	-	+	+	-
D-Mannose	+	+	+	+	+	+	+	+	+	-
L-Sorbose	+	-	-	-	-	-	-	-	-	-

L-Rhamnose	+	-	-	-	-	-	-	-	-	-
Dulicitol	-	-	-	-	-	-	-	-	-	-
Inositol	+	+	-	+	+	-	+	-	-	-
D-Manitol	+	+	+	+	+	+	+	+	+	-
D-sorbitol	+	+	-	-	-	-	+	-	+	-
Methyl-D-mannopyranoside	-	-	-	-	-	-	-	-	-	-
D-Clucopyranoside-Methyl	+	+	+	+	+	+	+	+	+	-
N-Acetylglucosamine	+	+	+	+	+	-	-	+	+	-
Amygdaline	+	+	+	+	+	-	+	+	+	-
ARButine	-	-	-	-	-	-	-	-	-	-
Esculine	+	+	+	+	+	+	+	+	+	-
Salicine	+	+	+	+	+	-	+	+	+	-
D-Celiobiose	+	+	+	+	+	-	-	+	+	-
D-Maltose	+	+	+	+	+	+	+	+	+	-
D-lactose	-	-	-	-	-	-	-	-	-	-
D-Melibiose	-	-	-	-	-	+	-	-	-	-
D-sacaharose	+	+	+	+	+	+	+	+	+	-
D-Trehalose	+	+	-	+	+	+	+	+	+	-
Inuline	-	-	-	-	-	-	-	-	-	-
D-melezitose	-	-	-	-	-	+	-	-	-	-
D-RAF finose	-	-	-	-	-	+	-	-	-	-
Amidon	+	-	+	-	-	+	-	-	+	-
Glycogene	+	-	+	-	-	+	-	-	+	-
Xylitol	-	-	-	-	-	-	-	-	-	-
Gentiobiose	-	-	-	-	+	-	+	-	-	-
D-Turanose	-	-	-	-	-	-	-	-	-	-
D-lyxose	-	-	-	-	-	-	-	-	-	-
D-Tagatose	+	+	+	+	+	-	-	+	+	-
D-Fucose	-	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-	-
L-Arabital	-	-	-	-	-	-	-	-	-	-
Potassium gluconate	-	-	-	-	-	-	-	-	-	-
Potossium2-ceto gluconate	-	-	-	-	-	-	-	-	-	-
Potossium 5-ceto gluconate	-	-	-	-	-	-	-	-	-	-

Table 2: Similarity percentage (%) based on the most complete 16S rDNA gene sequences of isolates to their closest bacteria present in the NCBI database and environmental origin of reference strains

Isolate	Sequence similarity (%)	Affiliation	Reference strain	Genbank Accession number
RA_{23}	**99**	***Bacillus licheniformis***	***GYPB30***	**JF346897.1**
RA_{12}	**99**	***Bacillus licheniformis***	***D13AO6***	**AY030337.1**
RA_3	**99**	***Bacillus amyloliquefaciens***	***Zy44***	**JN035230.1**
RA_6	**100**	***Anoxybacillus flavithermus***	**8POT11**	**KJ722464.1**
RA_1	**99**	***Bacillus subtilis***	***GYPB04***	**JF346887.1**
RA	**100**	***Bacillus sp.***	**Z.H tonekaboniansis**	**KC121318.1**
RA5	**99**	**Uncultured Bacillus sp.**	**clone DB-F15**	**JQ966227.1**

RA_{19}	**100**	***Bacillus sp.***	**clone Jing-G-41**	**HM135037.1**
RA30	**99**	***Bacillus licheniformis***	***KL-185***	**FJ655803.1**
RA4	**99**	***Bacillus licheniformis***	**PS-6**	**KJ933861.1**

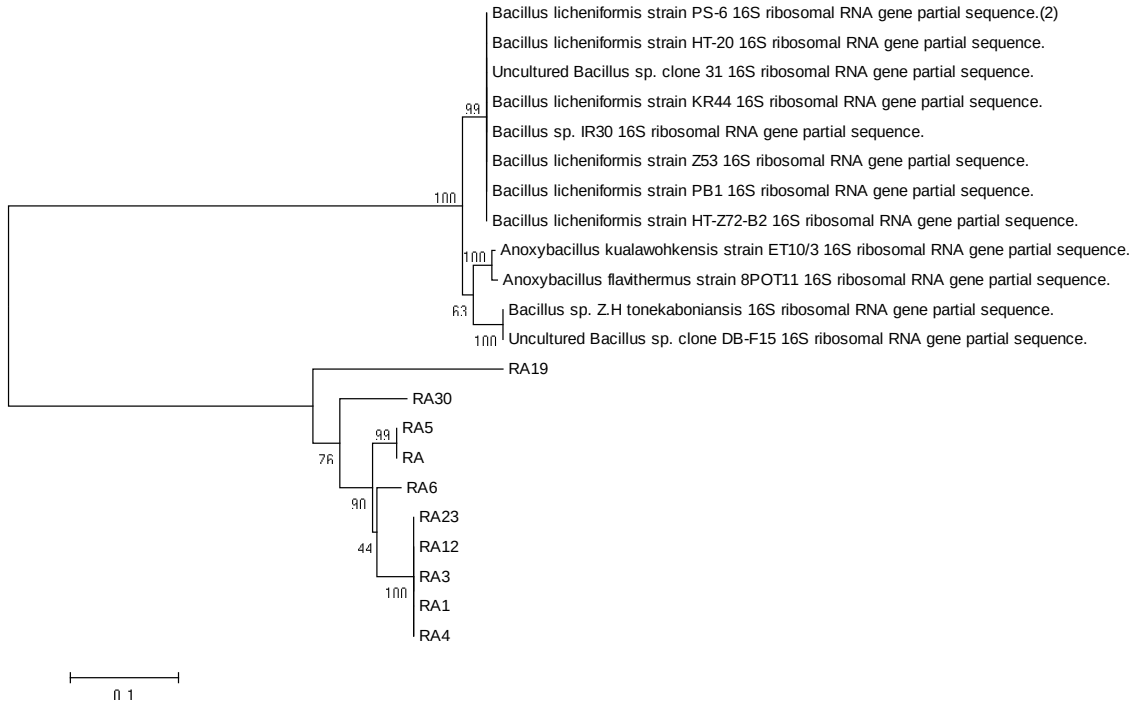

Figure 2: Phylogenetic relationship of isolated from Hot spring Ramsar. The tree is constructed using 16S rRNAgene sequence using the Neighbour-joining method. Bar, 0.1 nucleotide substitutions per site

DISCUSSION

The new isolates from hot spring Ramsar have been identified as strains of *Anoxybacillus* or *Bacillus* genera. Most of was similar at high level (≥ 99%) to different reference strains of the species *Bacillus licheniformis*. According to a study of diversity is wide aerobic bacilli and many of bacilli have the ability to produced very high the metabolites. Our results revealed presence of different thermophilic bacilli in hot spring Ramsar, some of which are phylogenetically novel ones. Investigation on their carbohydrate degrading activity demonstrated presence of biotechnologically valuable enzyme producers. Since most microbes from nature (about 99%) are difficult to cultivate **[18]**. According to a study, showed that *Bacillus* are strong degraders.

16S rDNA sequence analysis showed that the strains in the three groups. Although the 16S rDNA gene is used as a framework for modern bacterial classifi cation, it has oft en been seen that its usage shows limited

variation for the discrimination of closely related taxa and strains **[19, 20]**. Existence of *B. licheniformis* in thermal area was also reported in many other studies **[21]**. However, this is study that demonstrated that *B. licheniformis* population are common bacterial species present in hot spring Ramsar, Ramsar, IRAN.

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