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THERMOPHILIC BACILLI ISOLATED FROM GEOTHERMAL SOILS OF GANDOM BERYAN IN LUT DESERT, IRAN AND THEIR IDENTIFICATION BASED ON PHENOTYPIC AND PHYLOGENETIC APPROACHES

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Two thermophilic bacilli strains isolated from geothermal soil samples collected from Gandom-Beryan in Lut desert, Iran, were identified based on phenotypic and phylogenetic approaches and designed as *Bacillus* sp. Iranian S1 and *Bacillus* sp. Iranian S2. They both grew aerobically at 15–80 °C (optimum 56°C) and at pH 3-10 (optimum pH 7) and could tolerate up to 20% NaCl (optimum 2.5%). The 16S rRNA gene sequences of strains *Bacillus* sp. Iranian S1 and *Bacillus* sp. Iranian S2 have been deposited in the GenBank under accession numbers HQ823666 and HQ823667, respectively.

Thermophiles – geothermal soil bacilli –16S rRNA genes – phylogenetic analysis

Լութ անապատի (Իրան) Գանդոմ-Բերյան երկրաջերմային տեղամասի հողից մեկուսացվել են և ֆենոտիպական ու ֆիլոգենետիկական բնութագրերի հիման վրա նույնականացվել են թերմոֆիլ աերոբ էնդոսպոր առաջացնող բակտերիաների երկու շտամներ՝ *Bacillus* sp. Iranian S1 և *Bacillus.* sp. Iranian S2: Մեկուսացված շտամներըն աճումեն 15-80՛C ջերմաստիճանային միջակայքում (օպտիմումը՝ 50՛C), pH-ի 3-10 միջակայքում (օպտիմումը 7), ընդունակ են դիմակայելու NaCl-ի մինչև 20 % կոնցենտրացիաներին (օպտիմումը՝ 2.5%): *Bacillus* sp. Iranian S1 և *Bacillus* sp. Iranian S2 շտամների16S ռՌՆԹ գեների նուկլեոտիդային հաջորդականություններն ավանդադրվել են GenBank տվյալների բազայում համապատասխանաբար HQ823666 և HQ823667 համարներով:

Թերմոֆիլներ – երկրաջերմային հողերի բակտերիաներ – 16S ռՌՆԹ գեներ – ֆիլոգենետիկական վերլուծություն

Из почв участка Гандом-Берян пустыни Лут, Иран, изолированы и по фенотипическим и филогенетическим характеристикам идентифицированы два штамма аэробных термофильных эндоспорообразуюших бактерий, обозначенных как *Bacillus* sp. Iranian S1 и *Bacillus* sp. Iranian S2. Изоляты растут при температуре 15–80°С (оптимальная температура 56°С), pH 3-10 (оптимальный pH 7), толерантны к 20% - ному NaCl (оптимальный при росте 2.5% NaCl). Последовательности гена 16Sp РНК штаммов *Bacillus* sp. Iranian S1 и *Bacillus* sp. Iranian S2 депонированы в базе данных GenBank под инвентарными номерами HQ823666 и HQ823667.

Термофилы –бактерии геотермальных почв – ген 16S рРНК – филогенетический анализ

A number of aerobic thermophiles have been isolated from variety of geothermal environments such as terrestrial hot springs and hydrothermal vents, sulfataric fields, volcanic area [5, 8]. Hot habitats other than geothermal are solar-heated soils and deserts.

The deserts represent extreme environments for microorganisms [9]. Although the conditions of deserts vary strongly in the different regions of the world, all of them are characterized by a combination of extreme temperatures and desiccation, high soil salinity, low nutrient levels, high summer UV radiation levels, and physical instability caused by strong winds. Several investigations based on culture-dependant and molecular methods showed a unique and extraordinary microbial diversity in desert soils [3, 6, 12, 13]. The great majority of the bacteria isolated from desert soils was proved to be aerobic endospore-forming bacteria belonging to the genus *Bacillus* and related genera [9].

At the present study two bacilli strains from geothermalsoil of Gandom-Beryan in Lut desert, Iran were isolated and identified based on phenotypic and phylogenetic approaches.

Materials and methods. *Study site and sampling*. Gandom-Beryan (meaning "Scorched Wheat" in Persian) is a 480 km² large plateau covered with dark volcanic lava that made extreme atmosphere of climate in this area (fig.1). Temperatures can often reach even 71°C and pH 6.2 [14]. Soil samples were collected using sterile glass flasks and mentioned it at 4°C until processed at laboratory.

Fig. 1. Location of Gandom Beryan in Lut Desert on map [11].

Enrichment and isolation. Samples were incubated in the medium contained (gram per liter) sodium chloride, 5; glucose, 20; yeast extract, 5; peptone, 10; $CaCO_3$, 6; agar-agar, 20; pH 7.2. One gram of soil sample was suspended in 9 ml sterile distilled water and by means of serial dilutions concentrations of 10^{-1}-10^{-6} were prepared. Then 1ml of each aliquot was spread on medium and incubated at 56°C for 24h. The pure cultures were obtained by plating the enrichment culture onto nutrient agar with subsequent subculturing [1].

Phenotypic characteristics. Phenotypic characteristics of the isolates were studied by standard methods described in [1, 2]. Morphological features of strains were investigated using a Nikon light microscope and TEM Zeiss EM10 electron microscope. Characterization of each bacterial isolate was performed morphologically according to colony color, size, elevation, margin and Gram staining [1]. To determine the ability of the strains to grow at different temperature (10-70°C) and pH (3-10) values and with different NaCl concentrations (0-22%) the same liquid medium mentioned above was applied. Growth was tested by measuring the optical density (OD) of cell suspension at 600 nm with a spectrophotometer (Model 722G UV-Visible). Hydrolitic activities were determined using as substrates milk, tween-80 and soluble starch [1, 2].

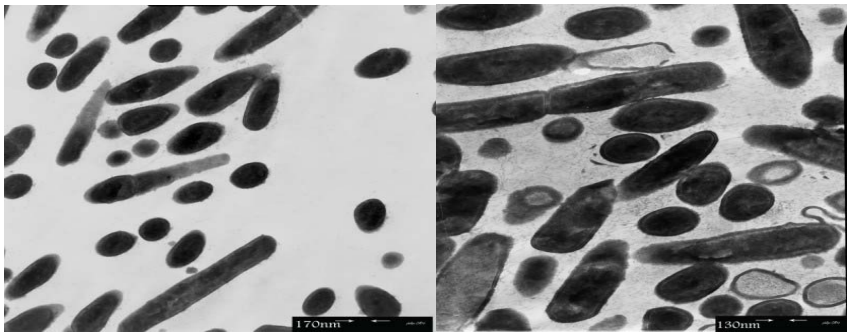
Phylogenetic analysis. Total DNA from pure culture was done by the CTAB/NaCl method described in [10]. The 16S rRNA gene fragments were PCR-amplified applying bacterial specificprimers PIB16F (5'-AGAGTTTGATCCTGGCTCAG-3') and MIB16R (5'-GGCTGCTGGCACGTAGTTAG-3').

The PCR conditions used were an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 95°C for one minute, annealing at 55°C for one minute then a final extension was given at 72°C for ten minute. The identity of the isolates was determined through a BLAST search [4]. Nucleotide sequences of the PCR products were sent to SinaGene Company of Iranian order to determine DNA sequencing (www.cinnaGen.com).

Phylogenetic analyses were conducted using MEGA version 5 software package and GeneBank database (http://www.ncbi.nlm.nih.gov) as a source for DNA sequences of closely related species. The phylogenetic tree was constructed by the neighbour-joining method using the distance matrix from the alignment [7]. The 16S rRNA gene sequences reported in this study have been deposited in the GenBank database under accession numbers HQ823666 and HQ823667.

Results and Discussion. Two isolates of aerobic endospore-forming Gram-positive, rod shaped bacilli designated as Iranian S1 andIranian S2, respectively, were isolated and further characterized from the soil samples of Gandom-Beryanarea in Lut Desert of Iran. Colonies of isolate Iranian S1 were flat, very small, transparent, and without color. Colonies of isolate Iranian S2 were flat, with a denticulate edge, irregular shape and whitish.

Electronic microscopic studies of strains revealed that all endospores formation stages and envelopes during the stages were typical to spore-forming bacteria. The cell morphology of isolate Iranian S1 was differed from that of Iranian S2 grown under similar conditions. The cells of isolate Iranian S1 were short cylindrical, whereas cells of isolate Iranian S2 were short filamentous (fig. 2). In the cells of studied strains, elliptic endospores were located centrally and terminally (Iranian S1) or subterminally (Iranian S1) along the cell axis. Sporangium wasn't swollen for both strains.



S1 **S2**

Fig. 2. Electron microscope images of isolates (x21000).

The growth rates of isolates Iranian S1 and Iranian S2 were determined at the temperature range of 20-80°C. For Iranian S1 the highest maximum growth rate was at 75°C, while isolate Iranian S2 had the highest maximum growth rate at 65°C. The optimum growth temperature was 56°C for both isolates. Iranian S1 was unable to grow at 70°C or below 25°C, and Iranian S2 was unable to grow above 75°C or below 25°C (fig. 3). Regarding the pH growth limitation, both isolates were grown in medium that had a range of pH values between 3 and 10. A pH range around neutrality favored optimal growth of the both isolates. The isolates did not grow at pH lower than pH 3. The maximum growth at pH 9 and 9.5 was noted for Iranian S1 and S2, respectively (fig.4). Both isolates grew with 0-22 % NaCl. The Iranian S1 was grown at a NaCl concentration up to 22%, and Iranian S2 was able to grow at 20% NaCl (fig.5). Thus, our isolates grew within the temperature and the pH ranges characteristic to their habitat [8].

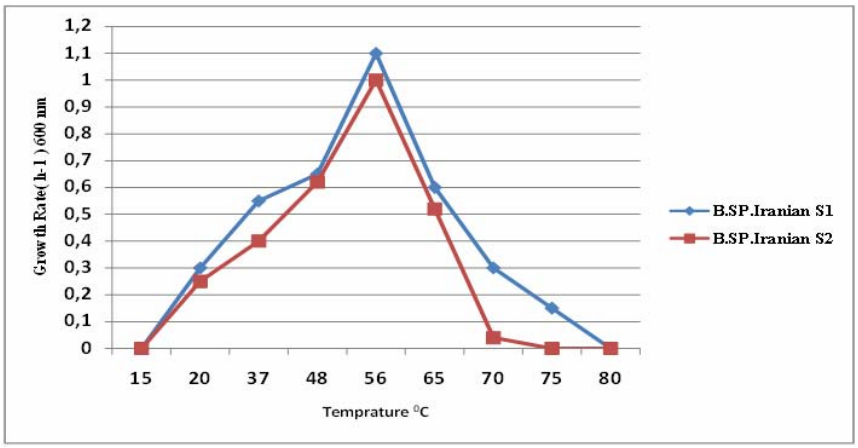


Fig. 3. Growth of strains at different temperature.

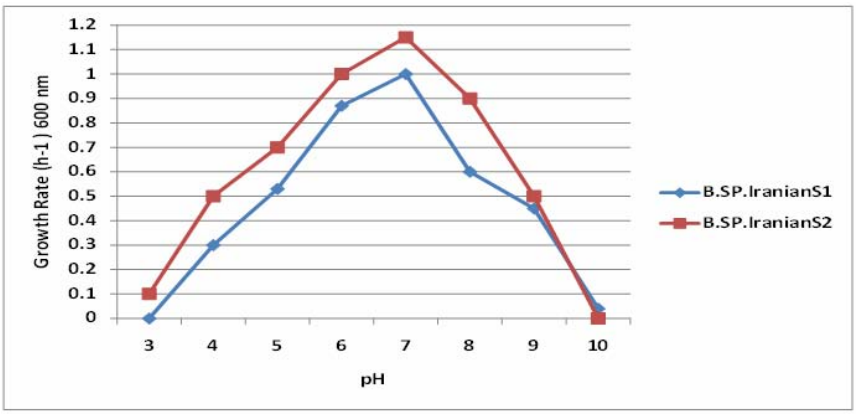


Fig. 4. pH range of isolates.

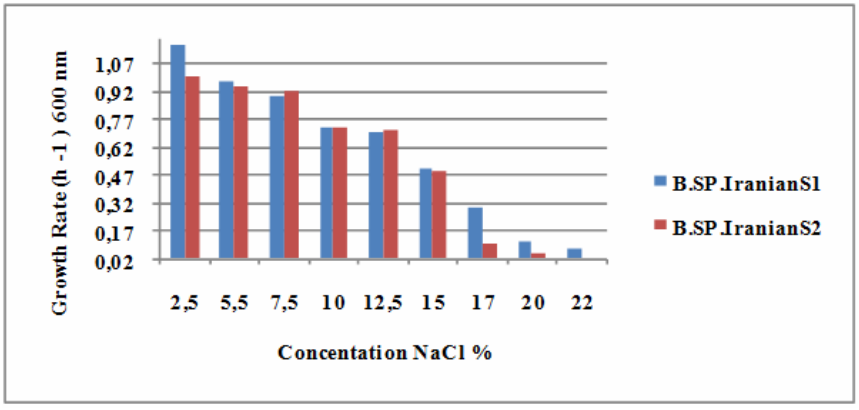


Fig. 5. Growth of strains in the presence of NaCl

Results of the biochemical properties of isolates are presented in Table 1 below.

Table 1. Biochemical characteristic of thermophilic isolates

Biochemical characteristic	Strains	
	S1	S2
Oxidase	+	+
Urease	--	--
Catalase	+	+
Methyl Red test	+	--
VogesProskauer test	w	w
Production of :		
Indole	--	--
Hydrolysis of :		
Tween-80	+	+
Gelatin	+	+
Starch	+	+
Citrate utilization	--	--
Gas production from glucose	+	+
Acid formation from:		
Glucose	+	+
Galactose	+	+
Lactose	+	+
Maltose	+	+
Sucrose	+	+
D-manitol	+	--
D-sorbitol	+	--
Salicin	+	+

Designation: (+), (-) *and w for positive, negative and slight positive reaction*

A phylogenetic tree was constructed by the neighbor-joining method identified that both isolates were part of the cluster of genus *Bacillus* (fig.6).

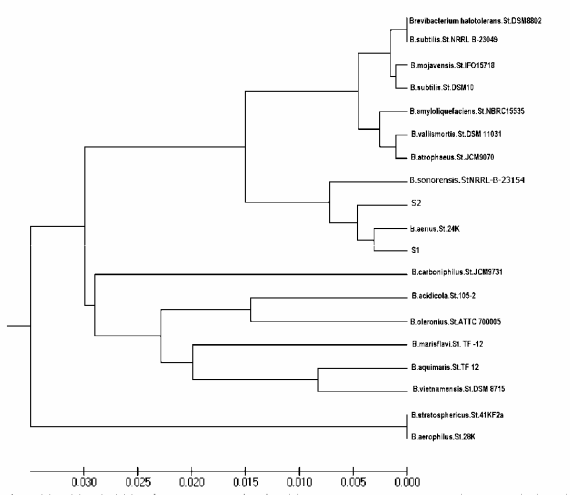


Fig.6. Phylogenetic tree was constructed by the neighbour-joining method using the distance matrix from the alignment.

Both strains were catalase- and oxidase-positive, able to utilize carbon sources such as glucose, maltose, sucrose, galactose, lactose and salicin, unable to produce indole from tryptophan. Only Iranian S1 showed a positive reaction in the methyl red experiment. Iranian S2 strain unable utilized D-manitol, D-sorbitol. Isolates were unable to utilize citrate, and were urease negative. Strains were showed positive results for starch, tween-80 and casein (milk) hydrolysis.

Based on their morphological, physiological and biochemical properties, isolates Iranian S1 and S2 were tentatively identified as *Bacillus* species. Comparative analysis of 16S rRNA gene nucleotide sequences of isolates Iranian S1 and S2 confirmed their close homology to the members of the genus *Bacillus*. The strain S1 was identified as *Bacillus* sp. LS04 (99%) and strain S2was identified as *Bacillus* sp.DY17 (98%) (tab. 2).

Table 2. Closest sequences and % similarity of studied isolates

Strain no	The lengths of the DNA fragments, (bp.)	Closest Sequence	Similarity %	Accession no
Iranian S1	527	*Bacillus sp. LS04*	99	GU972598.1
Iranian S2	527	*Bacillussp. DY17*	98	DQ821489.1

Although studied strains based on 16S rRNA genes sequences showed 98% homology values to *Bacillus* spp. and related species, this need to be further confirmed by fatty acid analysis, DNA-DNA hybridization, etc. While these results are important for further taxonomic work, positive results on lipolitic, caseinolitic and amylolitic activities are indicative of potential application of these bacterial cultures.

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