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Isolation and Identification of Two Aerobic Thermophilic Bacilli from Jowshan (Iran) Hot Spring

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Keywords: *thermophiles, bacilli, hot springs, 16S rRNA genes, phylogenetic analysis*

Hot springs are a type of extreme environments widely distributed all over the world and represent a challenge for searching of new thermophilic microbes [1, 2]. Recently molecular phylogenetic studies based on 16S rDNA conducted culture-dependent methods, have been used to examine the microbial diversity of various hot springs [3]. A number of aerobic thermophiles have been isolated from a variety of geothermal environments such as hot springs, solfataric fields and hydrothermal vents throughout the world [4-6]. Members of the genus *Bacillus*and related genera are probably the most frequently isolated thermophilic aerobes from terrestrial and marine hot water environments [7].

Numerous geothermal springs represented a challenge for searching of new biotechnological resources was found on the territory of Iran [8]. In this study isolation and identification based on henol- and phylotypic characterization of two aerobic thermotolerant bacilli from hot springs of Jowshan (Iran) hot spring was reported.

Materials and methods. **Study sites and sampling.**The samples were collected from Jowshan hot spring located about 70 kilometers south-eastern part of Kerman, at the center of Iran, on the eastern side of Sirch Mountain (Fig.1). Jowshan geothermal system comprises of 6 thermal springs with outlet temperatures ranging from 39.3 to 46.6°C. The pH value of these springs is from slightly acidic to neutral (6.8-7) and the conductivities about 1500 μS/Cm. The use of different chemical geothermometers suggests temperature of about 110°C in the depth of Jowshan geothermal system [8]. Adjacent sediment

samples were collected using sterile glass flasks and were maintained on ice until processed.

Enrichment and isolation. Samples were incubated in the medium contained the following per liter: sodium chloride, 5g; glucose 20 g; yeast extract, 5 g; peptone 10 g; $CaCO_3$ 6 g; agar-agar 20 g; pH 7.2. One gram of each sediments sample was suspended in 9 ml sterile distilled water and by means of serial dilutions concentrations of 10^{-1}-10^{-6}were prepared and 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 [9].

Fig. 1. Location of Jowshan hot spring on map [8].

Phenotypic characteristics. Morphological feature of strains were investigated using a Nikon light microscope and TEM Zeiss EM10electron microscope. Characterization of each bacterial isolate was performed morphologically according to colony color, size, elevation, margin and Gram staining [9].

Physiological and biochemical characteristics of the isolates were studied by standard methods [10]. 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 (0-15%) the same liquid medium mentioned above was applied. Growth was tested by measuring the optical density (OD) of cell suspension at 600nm with a spectrophotometer (Model 722G UV-Visible).

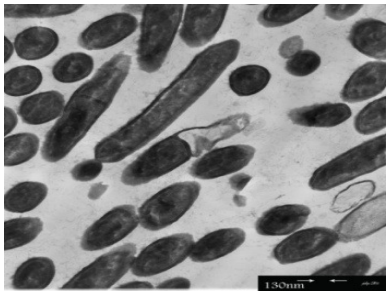
The utilization of various compounds (glucose, maltose, sucrose, galactose, lactose D-manitol, D-sorbitol and salicin) as carbon and energy sources by the bacteria was tested on a mineral medium. The substrates were sterilized separately and added to the medium at a concentration of 1%. Fermentation of various sugars and gas production was examined by change in the colour of indicator. Catalase, urease, oxidase, methyl red and Voges-Proskauer activity was determined by standard methods described in [9, 10]. Citrate utilization was determined by the appearance of change in colour of citrate agar medium. Caseinolitic activity was determined by growing the bacterial strains on milk agar and observing presence or absence of clearing around the colony. Lipolitic activity was determined by growing the bacterial strains on tween-80 agar (1%) and observing the absence or presence of zone around the colony. Starch

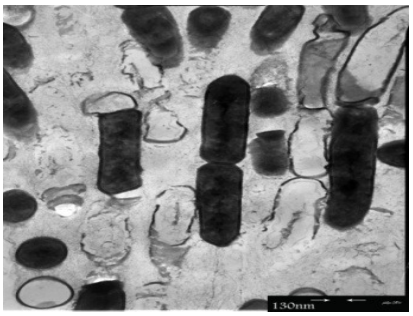
hydrolysis was tested by flooding iodine solution (0.3% I2 in 3% KJ) on the colonies of the strains grown on the starch agar containing 2% starch and observing presence or absence of clearing around the colony [9, 10]. Each experiment was carried out in triplicate; the averaged values were used.

Antibiotic resistance of the strains was tested by using the commercial discs contained (µg): kanamycin (30), lincomycin (2), cephalexin (30), oxacilin (1), ofloxacin (5), stereptomycin (25), oxytetracyclin (30), cefazolin (30), gentamycin (10), nalidixic acid (30), amoxicillin (10), ampicillin (25), penicillin (10), chloramphenicol (30), methicillin (5), erythromycin (10).

Phylogenetic analysis. Total DNA from pure culture was done by the CTAB/NaCl method described in [11]. The 16S rRNA gene fragments were PCR-amplified applying eubacterial specific primers PIB16F (5'-AGAGTT TGATCCTGGCTCAG-3') and MIB16R (5'-GGCTGCTGG CACGTAGTTAG-3'). The PCR conditions used were an initial denaturation at 94°C for 2 minutes, 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 [12]. Nucleotide sequences of the PCR products were sent to cinnaGene Company of Iranin order to determine DNA sequencing (www.cinnaGen.co). 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 [13]. The 16S rRNA gene sequences reported in this study has been deposited in the GenBank database under accession number HQ702748 and HQ734816.

Result and discussion. Two strains of aerobic endospore-forming Gram-positive, rod shaped bacilli designated as B1 and B2, respectively, were isolated and further characterized from the sediment samples of Jowshan hot spring (Kerman, Iran). Colonies of strain B1 was compact, small and yellowish and strainB2 was sprawling, flat, irregular shape and whitish. Electronic microscopic examination of the studied strains revealed that all endospores formation stages and envelopes during the stages were typical to spore-forming bacteria. The cell morphology of isolate B1 differed from that of B2 grown under similar conditions.The cells of isolate B1 were short filamentous, whereas cells of isolate B2 were slightly shorter and cylindrical (Fig. 2). In the cells of studied strains, elliptic endospores were located centrally (B1) or subterminally (B2) along the cell axis. Sporangium wasn't enlarged for both strains.





B1 B2

Fig.2.Electron microscope images of isolates (×21000).

The growth rates of isolates B1 and B2 were determined at the temperature range of 10-70 °C. For isolate B1 the highest maximum growth rate was at 60°C, while isolate B2 had the highest maximum growth rate at 65 °C. The optimum growth temperature was 50°C for both isolates. Isolate B1 was unable to grow at 65°C or below 20°C, and isolate B2 was unable to grow above 70°C or below 20°C (Table 1). Regarding the pH growth limitation, both B1 and B2 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 isolates B1 and B2, respectively (Table 1). Both strains grew with 0-10 % NaCl. The isolate B2 was grown at a NaCl concentration up to 12.5%, and isolate B1was able to grow at 10% NaCl (Table 1).Thus, our isolates grew within the temperature and the pH ranges characteristic to their habitat.

Table 1.The temperature, pH and NaCl concentration range for growth of isolates

Strains	Growth range											
	Temperature (^{0}C)			pH			NaCl concentration (%)					
	Min	Opt	Max	Min	Opt	Max	0	2.5	5.5	7.5	10	12.5
B1	20	50	60	4	7	9	--	+	+	+	+	--
B2	20	50	65	3	7	9.5	--	+	+`	+	+	+

Results of the biochemical characters of isolates are presented in Table 2. Both strains were catalase- and oxidase-positive, showed a positive reaction in the methyl red experiment, able to utilize carbon sources such as glucose, maltose, sucrose, galactose, lactose D-manitol, D-sorbitol and salicin and unable to produce indole from tryptophan. Isolate B2 was unable to utilize citrate, but was urease positive.

Among the tested antibiotics both strains were resistant to penicillin, oxacilin, ampicillin, methicillin and oxytetracyclin. They were highly sensitive to stereptomycin, kanamycin, gentamycin, chloramphenicol, erythromycin, ofloxacin, nalidixic acid and lincomycin. The strain B1 was sensitive also to amoxicillin and strain B2 was sensitive to cephalexin and cefazolin. Antibiotic sensitivity of thermophilic isolates is shown in Table 3.

Strains were showed positive results for starch, milk and tween-80 hydrolysis (Figs. 3, 4, 5).

Table 2. Biochemical characteristic of thermophilic isolates

Biochemical characters	Strains	
	B1	B2
Oxidase	+	+
Urease	--	+
Catalase	+	+
Methyl Red test	+	+
VogesProskauer test	w	+
Production of :		
Indole	--	--
Hydrolysis of :		
Casein	+	+
Gelatin	+	+
Starch	+	+
Citrate utilization	+	--
Gas production from glucose	w	+
Acid formation from:		
Glucose	+	+
Galactose	w	+
Lactose	w	w
Maltose	w	w
Sucrose	+	w
D-manitol	w	+
D-sorbitol	w	w
Salicin	w	w

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

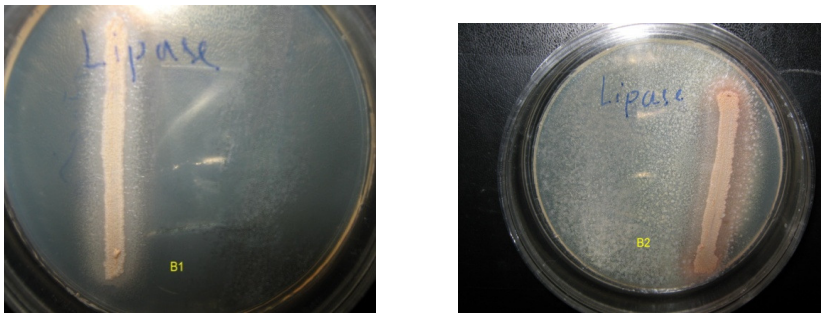


Fig. 3. Lipolitic activity of isolates on tween-80 agar.

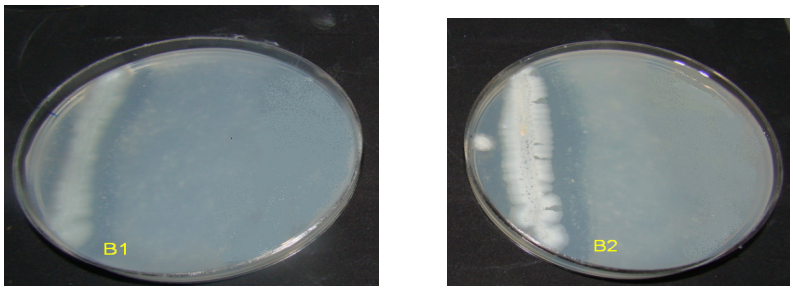


Fig. 4. Caseinolitic activity of isolates on milk agar

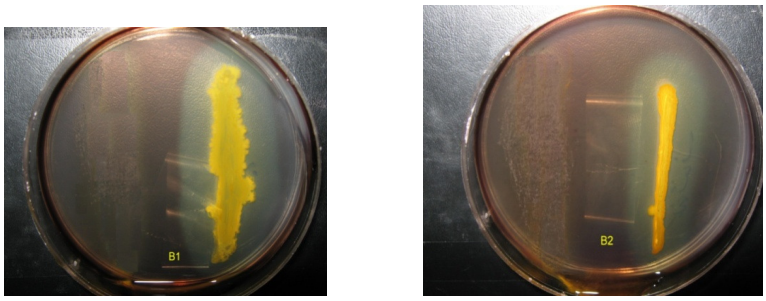


Fig .5. Amylolitic activity on starch agar.

Table 3. Antibiotic sensitivity of thermophilic isolates

Strains	Antibiotics/zones of cell lysis or growth suppression (mm)															
	β- lactames							Aminoglycosides					Macro lides	Others		
	Penicillin	Oxacillin	Ampicillin	Meticillin	Amoxicillin	Cefazollin	Carbenicillin	Streptomycin	Kanamycin	Gentamycin	Chloramphenicol	Oxytetracyclin	Erythromycin	Ofloxacin	Nalidixic acid	Lincomycin
B1	0	0	0	0	9	0	0	10	30	35	22	0	15	5	7	5
B2	0	0	0	0	0	7	5	12	32	30	5	0	10	5	28	32

Based on their morphology, physiological and biochemical properties isolates B1 and B2 were tentatively identified as *Bacillus* species. Comparative analysis of 16S rRNA gene nucleotide sequences of isolates B1 and B2 confirmed their close homology to the members of the genus *Bacillus.* The strain B1 was identified as *Bacillus spp*. (99%) and strain B2 was identified as *B. licheniformis* (99%) (Table 4). A phylogenetic tree was constructed by the neighbor-jointing method identified that both isolates were part of the cluster of genus *Bacillus*. The 16S rRNA gene sequences have been deposited in the GenBank database under accession number HQ702748 (B1) and HQ734816 (B2).

Table 4. Closest sequences and % similarity of studied isolates

Strains	The lengths of the DNA fragments, (bp.)	Closest Sequence	% Similarity	Accession no
B1	526	***Bacillus Sp*** St. TGS 437	99	HQ702748
B2	527	***B. licheniformis***st.B8	99	HQ734816

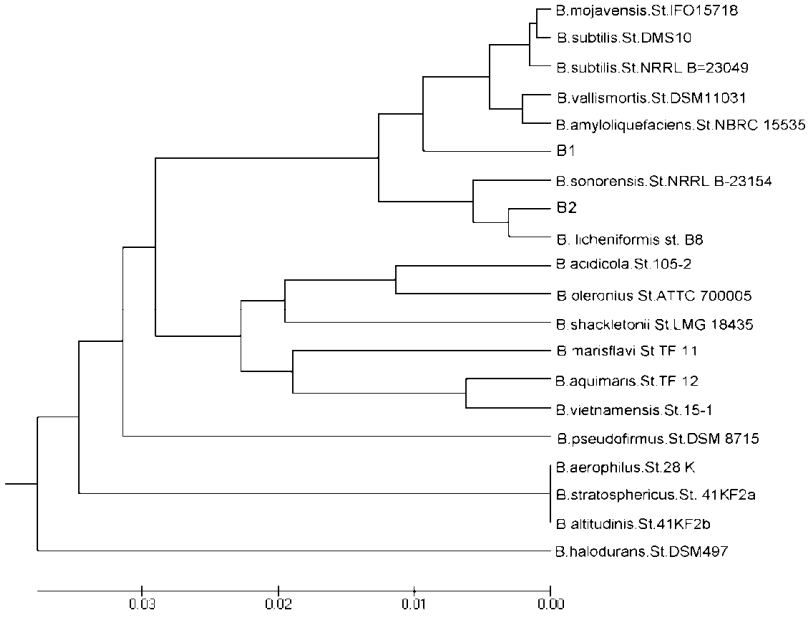


Fig. 6. Phylogenetic tree was constructed by the neighbour-joining method using the distance matrix from the alignment.

In the present study, for characterization and identification of two isolates, both phenotypic and genotypic characters are given emphases. Biochemical and physiological characters are important as they provide clues for selection of

efficient strains for further investigations mainly for applied value. Although studied strains based on 16S rRNA genes sequences showed 99% homology values to *B. licheniformis* 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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Isolation and Identification of Two Aerobic Thermophilic Bacilli from Jowshan (Iran) Hot Spring

Two strains of aerobic thermophilic endospore-forming bacteria designed as B1 and B2 were isolated from Jowshan hot spring (Iran) have been identified using phenotypic and phylogenetic approaches. The isolates were grown optimally at temperature of 50°C, at pH 7 and could tolerate 10-12 % (w/v) NaCl. They use a wide range of carbohydrates as carbon and energy sources. Comparative analysis of 16S rRNA gene nucleotide sequences of isolates B1 and B2 confirmed their close homology to the members of the genus *Bacillus*.The 16S rRNA gene sequences have been deposited in the GenBank database under accession number HQ702748 and HQ734816.

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Изолирование и идентифицирование двух аэробных термофильных бацилл из горячего источника Джовшана (Иран)

Из горячего источника Джовшана (Иран) изолированы и по фенотипическим и филогенетическим характеристикам идентифицированы два штамма аэробных термофильных эндоспорообразующих бактерий, обозначенных как B1 и B2. Оптимальный рост изолятов наблюдается при температуре 50° C, pH 7, толерантны к 10-12% NaCl. Изоляты используют широкий спектр углеводов в качестве источников углерода и энергии. Сравнительный анализ нуклеотидных последовательностей гена 16S рРНК штаммов B1 и B2 показали их близкую гомологию с представителями рода *Bacillus*. Последовательности гена 16S рРНК депонированы в базе данных GenBank под инвентарными номерами HQ702748 и HQ734816.

Ս. Ֆ. Շարիֆի Ալղաբփուր, Հ. Հ. Փանոսյան, Յու. Գ. Պոպով,
ՀՀ ԳԱԱ թղթակից անդամ Ա. Հ. Թռչունյան

Ջովշանի (Իրան) երկրաջերմային աղբյուրից բացիլների աերոբ թերմոֆիլ երկու շտամների մեկուսացումը և նույնականացումը

Ջովշանի (Իրան) երկրաջերմային աղբյուրից մեկուսացվել են և ֆենոտիպական ու ֆիլոգենետիկական հատկանիշների հիման վրա նույնականացվել են թերմոֆիլ աերոբ էնդոսպոր առաջացնող բակտերիաների երկու շտամներ՝ B1 և B2: Մեկուսացված շտամների աճի ջերմաստիճանային օպտիմումը 50°C է, pH-ը՝ 7, ընդունակ են դիմակայելու NaCl-ի 10-12% կոնցենտրացիաներին, որպես ածխածնի և էներգիայի աղբյուր օգտագործում են տարբեր ածխաջրեր: Շտամների 16S ռՌՆԹ գեների նուկլեոտիդային հաջորդականությունների համեմատական վերլուծությունը հաստատել է դրանց պատկանելիությունը *Bacillus* ցեղին: 16S ռՌՆԹ գեների նուկլեոտիդային հաջորդականություններն ավանդադրվել են GenBank տվյալների բազայում HQ702748և HQ734816 համարներով:

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