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Characterization and Identification of Moderately Thermophilic Bacteria Isolated from Jazan Hot Springs in Saudi Arabia

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ABSTRACT

Three new moderately thermophilic bacteria were isolated from three (Al-Khawbah, Bani Malik, and Al-Bozah) hot springs located in Jazan district, south-western of Saudi Arabia. The isolates were identified by the sequence analysis of the 16S rRNA gene. The 16S rRNA gene sequence alignment with GenBank resulted in a percent identity (% ID) with closest GenBank match of 98% suggesting that the isolates are new strains within the branching of well-defined genus Bacillus. The phylogenetic analysis of these strains using their 16S rDNA sequence data showed that strains SA and SS30 had highest homology (98%) with *B. licheniformis,* whereas strain SA09 showed 98% similarity with *B. subtilis*. The morphological, biochemical, and physiological characteristics of the isolates were also studied. They were aerobic, gram-positive, rod-shaped, and moderately thermophilic bacteria (with an optimum growth temperature of 45–50˚C, and pH of 6).

INTRODUCTION

Microorganisms, specifically bacteria, continue to be found living under extreme environmental conditions previously thought to be unable to contain life. These organisms are called extremophiles which are generally divided into five groups; thermophiles, acidophiles, alkophiles, halophiles, and psychrophiles. Temperature is one of the most important factors that controls microbial species leading to the selection of specific flora capable of tolerating and surviving heat stress (Eugene *et al.,* 1999). The thermophiles are defined as organisms, which have optimal growth temperatures of 45 and 70°C (Madigan and Martino, 2006).

Currently, the scientists have a great interest to study thermophilic microorganisms due to their biotechnological importance (Turner *et al.,* 2007).

The majorities of thermophilic microorganisms surviving above 60˚C belongs to Archaea, which always lacks cell wall and cannot survive in some industrial processes such as pulp density (Wei-min *et al.,* 2009). Alternatively, moderately thermophilic bacteria can adapt to survive at an elevated temperature in different habitats such as hot springs. Although the optimal growing temperature values for moderately thermophilic bacteria are 45-60˚C, they can produce unique biocatalysts that function under extreme conditions comparable to those prevailing in various industrial processes (Abou-Shanab, 2007).

A variety of thermophilic bacteria have been isolated for many geothermal areas in different regions in the World, including Turkey (Gul-Guven *et al.,* 2008), Italy (Maugeri *et al.,* 2001), Bulgaria (Derekova *et al.,* 2008), Greece (Sievert *et al.,* 2000), China (Lau *et al.,* 2009), India (Sharma *et al.,* 2008), and Iceland (Takacs *et al.,* 2001). Jazan hot springs, located in the south-western of Saudi Arabia, are the most extensive and active hot springs in Saudi Arabia. However, not much is known about the thermophilic bacteria present in these hot springs. The only recorded study is that conducted by Mohammed (2008) who investigated the presence of toxic cyanobacteria and cyanotoxins in these hot springs. This makes them interesting environment for study, as they may have some thermophilic bacteria. In addition, the thermophilic bacteria that are present in these hot springs could have unique evolutionary lineages that are not found in other parts of the world.

Bacterial communities are difficult to study because of their complexity and potential problems in culturability of many of the members (Bae *et al.,* 2005). However, advances in molecular biology techniques such as fatty acid methyl ester and rep-PCR profiling, and 16S rRNA sequencing to have provided an excellent opportunity for identification and characterization of a microorganism at species and subspecies levels (Zaliha *et al.,* 2007). Although there is not a perfect and simple identification method, PCR-based approaches for direct recovery and analysis of 16S rRNA gene sequences from the environment provide a relatively rapid and effective means of detection; enhance identification beyond that available by conventional technique (Dawson and Pace, 2002; DeLong and Pace, 2001). The aim of this study was to isolate and identify moderately thermophilic bacteria isolated from Jazan hot springs located in the south-western of Saudi Arabia, by using conventional as well as 16S RNA genes.

MATERIALS AND METHODS

Water Sampling and Bacterial Isolation

Water samples were collected in sterile 200 ml glass screw cap bottles from three (Al-Khawbah, Bani Malik, and Al-Bozah) hot springs located in Jazan district, south-western of Saudi Arabia. Temperature was measured *in situ* with a mercury bulb thermometer, and collected at the depth of 40 cm away from the border to obtain representative samples. The bottles were fully filled following by tightly closing to prevent the loss of dissolved gases.

The water samples were transported to the laboratory and analyzed within 24 h. Bacteria were isolated using the streaking method on Nutrient agar containing **(**Downes and Ito, 2001) the pH of the medium was adjusted to 7.2 before autoclaving plates were incubated at 50°C for 24 h, and purities of the colonies were checked microscopically and enumerated using the standard plate method.

Characterization and Identification of the Isolates:

Morphological Studies

Morphological properties were investigated by using 36 h old bacterial cultures growing on Nutrient agar plates. These included the wet mount preparations using light microscope, Gram staining to confirm Gram reaction, Motility, and spore position according to Gudni *et al.* (1988).

Biochemical Tests

A range of biochemical tests were carried out to characterize the isolates. These tests included endospore formation, Gram reaction, catalase**,** and oxidase activities, hydrolysis of protein, starch, and acid production from sugar (Bae *et al.,* 2005).

Determination of the Optimum pH

The optimum pH for growth was determined by using phosphate buffer, universal buffer, and Tris-HCI buffer to obtain different pH values in the range of 4.0 to 11.0 Bae *et al*. (2005). pH was confirmed using pH meter. The isolates were cultured aerobically in 50 ml tubes containing 10 ml of Nutrient broth medium and incubated at 55°C in an orbital shaker running at 200 rpm. All cultivations were done in duplicate. After incubation for 24 h, the optical density (OD600) was measured using a spectrometer.

Determination of the Optimum Temperature

To evaluate the optimal growth temperature of the thermophilic bacteria, the pH of the isolation medium was adjusted to getting a final value of 6.0 before autoclaving; the isolates were cultured as described above and incubated at a range of temperatures from 45-55°C (Khalil 2002). All cultivations were done in duplicate. After incubation for 24 h, the optical density (OD_{600}) was measured using a spectrometer.

Hydrolysis of skimmed milk (casein)

The ability of bacterial isolate to hydrolyze casein was tested by streaking on skimmed milk agar plates containing (1.0 % skimmed milk, 0.2 % yeast extract, 0.01 % KH2PO4, 0.03 % K2HPO4, 0.5 % NaCl, and 2 % agar) (pH 6.5) and incubation at 50°C for 24 h (Fujio and Kume, 1991). The production of a clearing halo around the colonies indicated casein hydrolysis.

Hydrolysis of Starch

Starch hydrolysis was tested on Nutrient agar plates containing 1% starch (pH 6.5) (Fisher *et al.,* 1995). Plates were incubated at 50°C for 24 hour. Appearance of clear halos around the colonies upon the addition of Lugol's iodine indicated the presence of amylase activity.

DNA Extraction, RAPD and 16S Ribosomal RNA -PCR Analysis

Bacterial DNA isolates was extracted from 5 ml bacterial cultures grown overnight using QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA) (L. J. *et al.,* 2006). Briefly, the pellet was suspended in 180 µl of (20 mg/ml lysozyme in20 mM Tris·HCl, pH 8.0; 2 mM EDTA; 1.2% Triton), followed by incubation for 30 min at 37°C. Twenty µl of Proteinase K was added then mixed by vortexing and incubated at 56°C for 30 min. DNA amplification reactions were conducted in a Bio Rad My Cycler thermocycler. RAPD-PCR amplification was performed using the primers: A01, 5'-CGGCTGGAG-3'; and A02, 5'-CCCCCAGAT-3'. The PCR protocol was a 35-cycle PCR (initial denaturation, 94°C for 5 min; subsequent denaturation, 94°C for 1 min; annealing temperature, 30°C for 2 min; extension temperature, 72°C for 1 min; and final extension, 72°C for 10 min). The genes encoding the small-subunit rRNA (16S rDNA) were amplified through the application of primers targeted to universally conserved regions. The oligonucleotide primers have the following sequences: 1F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1R (5'-GGTTACCTTGTTACGACTT-3') used to amplify bacterial 16S rRNA. The PCR was initiated by incubating the reaction mixture at 95°C for 3 min, followed by 30 cycles of 50 sec at 95°C; 1 min at 50°C; and 2 min at 72°C. The reaction was terminated with an extension step consisting of 10 min incubation at 72°C. The PCR products were analyzed on 1.0% agarose gel containing 0.5 µg/ml ethidium bromide and visualized by Bio Rad Gel Documentation System 2000.

16S rRNA Sequence Analysis

The 16S rRNA gene PCR product amplified from genomic DNA isolated from pure bacterial colonies was sequenced by Mubarak City for Science and Technology in Egypt. Homology of the 16S rRNA gene sequence of the isolates with reference 16S

rRNA sequences was analyzed using the BLAST algorithm in Gen Bank (available in NCBI in the internet). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura *et al.,* 2007; Tamura *et al.,* 2007). Only the highest-scored BLAST result was considered for phylotype identification, with 98% minimum similarity.

Statistical Analysis

Analysis of variance (one-way ANOVA) was used for data analysis. If the data and residues were not normally distributed or did not have equal variance, even after transformation, then the Kruskal-Wallis test was used. All analyses were performed at $P \leq 0.05$ using MINITAB, version 13.1. Graphs were plotted using Sigma Plot 2001.

RESULTS

Physical and Chemical Characteristics of Jazan Hot Springs

Three different locations of hot springs in Jazan district were sampled. The results of physical and chemical characteristics of the three hot springs are summarized in Table 1. The results showed that Jazan hot springs had temperature gradients ranging from 48 to 70˚C. These springs had slight alkaline waters (pH = 7.7–8.2), and high concentrations of nitrate, ammonium, phosphate, silicate, and sulfate. The statistical analysis revealed that the water temperature differed significantly among all hot springs studied. The difference in pH asset value was not significant among different hot springs. Conductivity showed a significant variation among all hot springs, except between Al Khawbah and Beni Mlaik hot springs. The remaining measured parameters, including nitrate, ammonium, phosphate, silicate, and sulfate varied significantly between Al Khawbah hot spring and the other two hot springs, but not significantly between Beni malik and Al Bozah hot springs.

Table 1: Physiochemical characteristics of Jazan hot spring waters.

Characteristics	Hot springs location		
	Al Khawbah	Bani Malik	Al Bozah
Temperature (˚C)	70	48	52
pH	7.7	8.1	8.2
Conductivity ($\mu moscm^{-2}\ s^{-1}$)	3300	3200	3000
Alkalinity ($mg\ l^{-1}$)	267±5.4	258±4.3	255±3.9
NO_3^- ($mg\ l^{-1}$)	7.46±0.7	6.76±0.6	7.223±0.3
NH_4^+ ($mg\ l^{-1}$)	47.1±1.1	45.2±1.8	46.4±1.6
PO_4^{3-} ($mg\ l^{-1}$)	8.4±1.1	7.6±1.6	8.1±1.4
SiO_2 ($mg\ l^{-1}$)	36±2.2	41±1.8	38±2.4
SO_4^{2-} ($mg\ l^{-1}$)	2300±23.6	986±11.3	1040±16.2

under a light microscope.

Morphological, Biochemical, and Physiological Characterization

Three moderately thermophilic bacilli isolated from hot springs located in Jazan distract, south-western of Saudi Arabia, were identified and characterized by conventional and molecular techniques. The investigation of these strains in terms of morphologic, biochemical, and physiological properties (Table 2) showed that the isolated strains are Gram positive, catalase, and oxidase positive as well as moderately thermophiles. In addition, they are rod-shaped and amylase positive. Motility (except for the strain 3) and sporulation (except for the strain 2 and 3) were clearly detected.

Table 2: Morphological, biochemical, and physiological characteristics of the thermophilic strains.

Characteristics	***B. licheniformis* SA**	***B. licheniformis* SS30**	***B. Subtilis* SA09**
***Colony* Configuration**	Round with radiating margin	Rhizoid	Round
***Colony* Margins**	Branching	Branching	Irregular
***Colony* Elevations**	Drop like	Hilly	Hilly
Cell Shape	Long Bacilli	Streptobacilli	Bacilli
Gram reaction	+ ve	+ ve	+ ve
Spore formation	+	-	-
Motility	+	+	-
Catalase	+	+	+
Oxidase	+	+	+
Acid from glucose	-	-	-
Acid from Mannitol	-	-	-
Amylase production	-	-	+
Protease production	+	+	+
Optimal temperature	50°C	45°C	45°C
Optimal pH	6	6	6

Characterization of Optimal Temperature and pH Value for Strains Growth

Optimal temperature and pH were assessed in order to identify optimal growth conditions for the isolates. The results shown in Figure 1 suggest that the optimal temperature for the strains SA, SS30, and SA09 are 50°C, 45°C, and 45°C, respectively. Temperatures greater than the optimal temperature are lethal to the growth of strains. The strains were also grown at different pH values ranging from 4 to 11 in order to identify optimal pH for growth. The strains achieved an optimal growth at pH 6 ± 0.4 (Figure 2). The growth of isolates was highly inhibited at pH values higher or lower than the optimal pH indicating that the strains cannot grow in high acid or alkaline conditions.

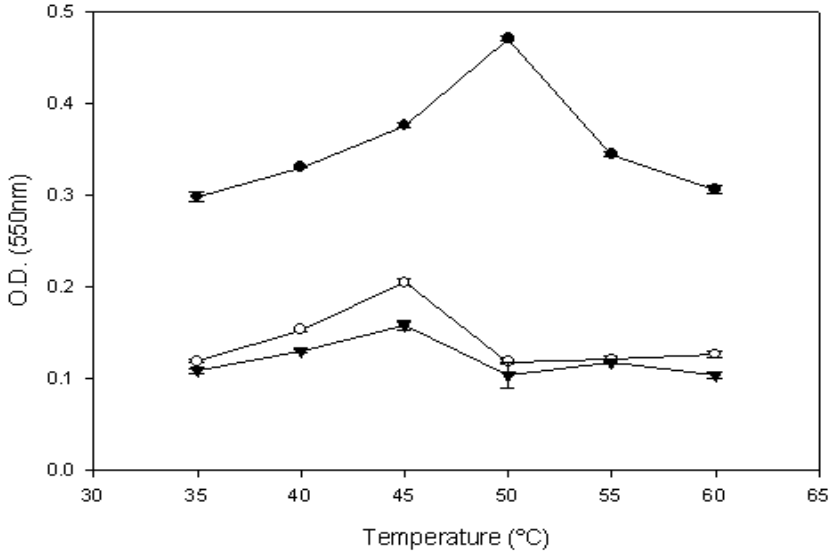

Fig. 1: Assessment of an optimal temperature for growth of the isolated thermophili bacteria, where (●) is *B. licheniformis* SA, (○) is *B. licheniformis* SS30 and (▼) is *B. Subtilis* SA09. Bars indicate standard errors of the mean (n = 3).

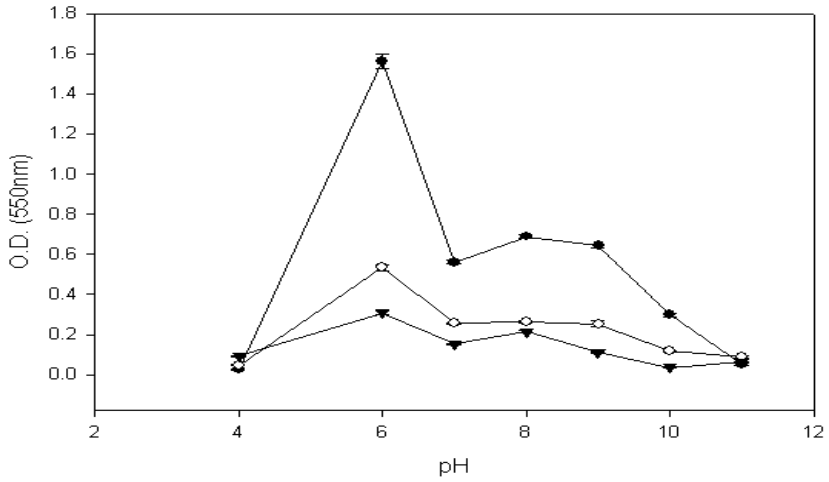

Fig. 2: Assessment of an optimal pH for growth of the isolated thermophili bacteria, where (●) is *B. licheniformis* SA, (○) is *B. licheniformis* SS30 and (▼) is *B. Subtilis* SA09. Bars indicate standard errors of the mean (n = 3).

16S rRNA Gene Sequence

The BLAST program in National Center for Biotechnology Information (NCBI) was used to align the 16S rRNA sequence of the new isolates with previously published sequences in the public database. 16S rRNA sequence analysis showed that there was a strong similarity between the isolates and representative strains of the genus Bacillus in gene bank (Figure 3), indicating that 16S rRNA gene sequence data is helpful for bacterial identification. The phylogenetic analysis of these strains using its 16S rDNA sequence data showed that the strain SA had highest homology (98%) with *B. licheniformis* strain X-14 (EU717842) and SS30 showed 98% similarity with *B. licheniformis* strain YDGW6 (EU334007), whereas SA09 showed 98 % similarities with *B. subtilis* isolate D13AO7 (FJ655806).

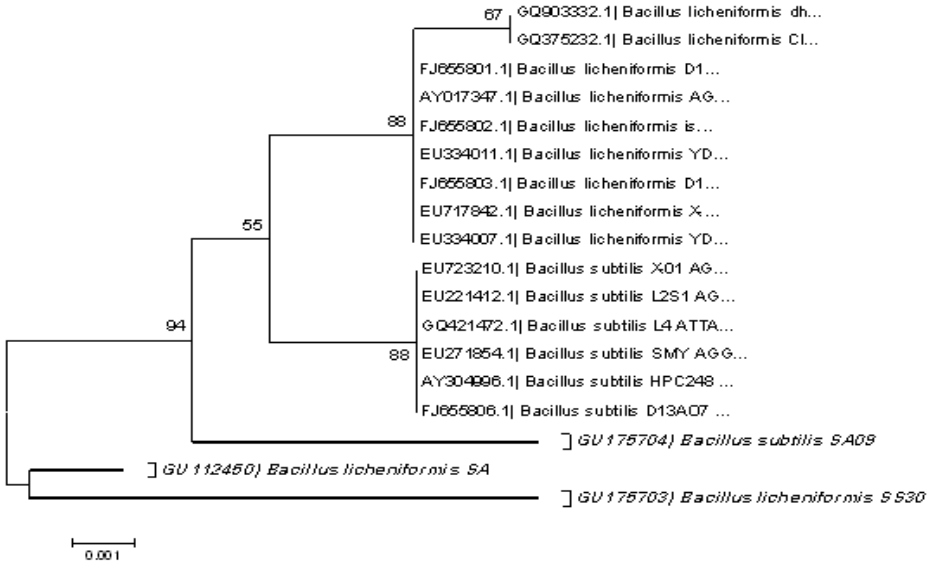

Fig. 3: Phylogenetic tree generated using distance matrix and neighbour-joining methods based on the 16S rRNA gene sequences of the new isolate and related taxa.

Accession

The Gen Bank accession number for the 16S rRNA sequence of *Bacillus licheniformis* SA is (GU112450), *B. licheniformis* SS30 is (GU175703), and *B. subtilis* SA09 is (GU175704).

DISCUSSION

Three wild bacillus thermophilic bacteria isolated from Jazan hot springs were selected from 23 thermophilic isolates. They were chosen for further studies due to their ability to secrete extracellular protease and amylase enzymes during growth on suitable media such as skimmed milk. Enzymes that are suitable to operate at high temperatures in some biological and industrial processes could be only obtained from the thermophilic microbes. Among them, Bacillus species are one of the common microbial enzyme producers at an industrial level, especially hydrolases (Torres *et al.,* 2009).

Thermophilic bacilli, growing optimally over the temperature range 45 to 70˚C, have been isolated from different thermophilic environments. They have been isolated from ocean-bottom mud samples (Bae *et al.,* 2005), deep-sea hydrothermal vents (Marteinsson *et al.,* 1995) and shallow marine hot springs (Maugeri *et al.,* 2002). Currently, thermophilic bacilli are classified into seven genera: *Bacillus, Brevibacillus* (Sneath, 1986), *Alicyclobacillus* (Wisotzkey *et al.,* 1992), *Geobacillus* (Nazina *et al.,* 2005), *Sulfobacillus* (Dufresne *et al.,* 1996), and *Thermobacillus* (Touzel *et al.,* 2000).

Molecular taxonomy shows that most of the thermophilic bacilli described to date belong to either Bacillus rRNA group 1 or Bacillus rRNA group 5 (Ash *et al.,* 1991; Rainey *et al.,* 1994). Bacillus rRNA group 1 contains the thermophilic species *Bacillus smithii* and *Bacillus coagulans* (Ash *et al.,* 1991). While bacillus rRNA group 5 includes the genus: *Geobacillus* and an aerobic, Gram-positive coccus, *Saccharococcus thermophilus* (Ash *et al.,* 1991; Nazina *et al.,* 2005; Rainey *et al.,* 1994).

Although the conventional methods, including morphological, biochemical, physiological characteristics, used in this study characterized the three isolates to the genus of Bacillus, they did not allow further differentiation below the level of the genus. Therefore, the new isolates subjected to 16S rRNA gene sequence studies. The isolate 16S rRNA gene alignment with GenBank resulted in a percent identity (% ID) with closest GenBank match of 98%, indicating that there is no complete match between our isolates, and GenBank deposits sequences. It has been proposed that sequence similarity must be below 95% to qualify as evidence of a novel species (Amann *et al.,* 1995), suggesting that the new isolate may be another strain of *Bacillus* sp. Therefore, the three strains isolated in this study are new species and named *Bacillus licheniformis* SA, *B. licheniformis* SS30, and *B. subtilis SA09.*

In conclusion, the three new species of moderately thermophilic bacteria were isolated from three hot springs, located in Jazan district, south-western of Saudi Arabia. The three isolates were chosen for further studies due to their ability to secrete extracellular enzymes such as protease and amylase during growth on suitable media. According to the investigation of the morphological, biochemical, physiological characteristics and the analysis based on 16S rRNA gene sequence, these new strains are identified to be most closely related to *Bacillus* sp. These strains are named *Bacillus licheniformis* SA, *B. licheniformis* SS30, and *B. subtilis SA09.* Future work will be focused on studying the characterizations of enzymes obtained from these new thermophilic bacteria in order know their potential applications in some biological and industrial processes.

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ARABIC SUMMARY

توصيف وتحديد بكتيريا معزولة الينابيع الساخنة في منطقة جازان في المملكة العربية السعودية

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تم في هذه الدراسة عزل ثلاثة انواع جديدة من البكتيريا من ثلاثة ينابيع ساخنة (الخوبة، بني مالك، البوزة) والتي تقع في منطقة جازان، جنوب غرب المملكة العربية السعودية. وقد تم تحديد العزلات من خلال تحليل تسلسل الجينات 16s rDNA. وقد تبين من خلال مقارنة تتابع النيوكليوتيدات لـ 16s rDNA للعزلات مع مثيلاتها في بنك الجينات ومن خلال شجرة القرابة الوراثية لهذه التتابعات وجود نسبة تقارب حوالي ٩٨٪ مما يدل على أن العزلات هي سلالات جديدة من الجنس Basillus . كما وأظهرت التحاليل باستخدام بيانات تسلسل 16s rDNA أن سلالات SA وSS30 يمثل أعلى التقارب بنسبة (٩٨٪) مع *B.licheniformis* العصوية، في حين أظهرت السلالة SA09 تشابه مع *B. subtilis* بنسبة (٩٨٪). كما أظهرت دراسة الخصائص المورفولوجية، والكيميوحيوية والفسيولوجية للعزلات انها هوائية ، إيجابية لصبغة جرام وعصوية الشكل. كما انها محبة نسبياً لدرجات الحرارة العالية (درجة الحرارة المثلى للنمو ٤٥-٥٠م°) ودرجة الحموضة متعادلة (المثلى حوالي ٦.٠).