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# Physiological properties of facultative and obligate alkalophilic *Bacillus sp.* strains isolated from Saudi Arabia

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**Isolation and identification of new alkalophilic *Bacillus* strains have increasing interest due to their possessing valuable and commercially interesting enzymes. To date, several researchers have studied the identification and characterization of alkalophilic *Bacillus* strains based on the phenotypic characterization as phylogenetic analysis of 16SrRNA. In the present study, six obligate and facultatively alkalophilic isolates were purified from dessert soil around Al-qunfotha city, in Saudi Arabia. All isolates were phenotypically and genotypically characterized. Among these isolates, AS3, AS4, AS5 and AS6 could grow at pH 9, 10, 11 and 12, but could not grow at pH 7 indicating that this isolates are obligate alkalophiles while, isolates AS1 and AS2 grew at pH range from 7 to 10, but could not grow at pH 11 and 12, suggesting that they could be facultative alkalophiles. All isolates could hydrolyze casein and starch, indicating that they possess interesting amylase and protease enzymes. Comparative sequence analysis of 16s rRNA of the six alkalophilic Bacillus strains indicated that these isolates share 99% identity with the previously isolated genes and belong to *Bacillus cohnii* at the full length gene nucleotide sequence level. The nucleotide sequences of 16SrRNA gene for the six isolates were given Gene-bank accession numbers: KP053301, KP053302, KP053303, KP053304, KP053305 and KP053306, respectively.**

**Key words:** *Bacillus cohnii,* 16SrRNA, obligate alkalophiles, facultative alkalophiles.

## INTRODUCTION

Genus *Bacillus* are more phenotypically heterogeneous than most other bacterial genera (Claus and Berkeley, 1986; Osman, 2012). There is a diverse group of *Bacillus* species living in highly alkaline terrestrial and aquatic environments. In the past decade there was a full revision of alkaliphilic *Bacillus* classification according to their phylogenetic and phenotypic characteristics (Takami and Horikoshi, 2000; Osman et al., 2013). The alkaliphilic and nonalkaliphilic species of the genus *Bacillus* are difficult to identify by traditional methods based on phenotypic characteristics (Woese, 1987; EL-Ghareeb et al., 2102). All the morphological and physiological characteristics of the native strains indicated that these isolates were from genus *Bacillus*. Native alkaliphilic *Bacillus* isolates are

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similar according to the biochemical characteristics. Some native isolates did not utilize much carbohydrate for growth, and no glucose. As a result, it was determined that the conventional tests based on phenotypic characteristics were insufficient for the differentiation of native alkaliphilic *Bacillus* isolates. Bacteria are widely distributed in nature. Most of the known microorganisms grow best at around neutral pH. However, there are bacteria that can grow in extreme alkaline environments (Muntyan et al., 2005). They are classified into two groups: alkalophilic bacteria, which are able to grow at pH above 10 and their optimal growth about pH 9 (Xu and Cote, 2003). The other group is called alkalotolerante bacteria, which show optimum growth at pH around 7, but able to grow at pH around 10 (Joung and Cote, 2002). Alkalophilic bacteria can be further divided into obligate and facultative alkaliphilic (Marie et al., 2005). The former group shows optimal growth of pH around 10 and cannot grow at pH around seven; whereas, the latter group grows at pH 7 and around 10 with optimal growth at pH 10 or above (Schallmey et al., 2004).

Interest in alkalophilic bacteria has increased during the last few decays due to their applications in ecological, industrial and biotechnology fields. Alkalophilic microorganisms represent a challenge to the basis of Michell's chemiosmotic theory. It has been reported that the cytoplasmic pH for alkalophiles growth at 10 to 11 is in the range of 8 to 9 (Ashis and Sudhir, 2011). It has been demonstrated that alkalophilic bacteria have a reversed transmembrane proton gradient. Despite that, alkalophilic bacteria have a higher growth rate than those of neutrophils (Felske et al., 2003). Furthermore, facultative alkalophilic show higher growth rate at alkaline pH than at neutral pH. To date, several researchers have identified and characterized alkalophilic *Bacillus* strains using phenotypic characteristics, DNA-DNA relatedness data, and analysis of the 16SrRNA sequence (Assaeedi and Osman, 2012). Although, these methods have been used for the classification of alkalophilic *Bacillus* species, but the characterization of these microorganisms is considered complicated due to their slow growth and their extreme pH which interfere with the results of phenotypic tests. Alkalophilic bacteria that grow well at pH range of 10 to 11 are widely distributed throughout the world and have been isolated from a variety of ecosystems including soil (Guffanti et al., 1986). There are only a few reports on alkalophilic bacteria isolated from Saudi Arabia (Salama et al., 1993).

The objectives of this study were to isolate, characterize and identify alkalophilic bacteria from Al-qunfotha region at Kingdom of Saudi Arabia. Strains were characterized using phenotypic characteristics, 16SrRNA gene sequencing.

## MATERIALS AND METHODS

### Collection of soil samples

A total 50 soil samples were collected from the top few centimeters of different locations surrounding Al-qunfotha city located in the western region of Saudi Arabia. Samples were collected from 2 to 5 cm below the surface with a shovel (Horikoshi and Akiba, 1982). Samples were stored on ice until were transported to laboratory where they were stored at +4°C.

### Growth medium

The medium used in this study consists of 1% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% $K_2HPO_4$, 0.02% $MgSO_4$ $7H_2O$, 2% Agar, pH 10.5 (Horikoshi and Akiba, 1982). The solution of $Na_2CO_3$ was autoclaved separately and added to the medium.

### Isolation and screening of alkalophilic isolates

For isolation of alkalophilic strains, 1 g soil samples were suspended in 10 ml of sterilized $H_2O$ and 1 ml of soil suspension were then plated on M1 agar medium. Plates were incubated at 30°C for 48 h. Single colonies showing different morphologies were picked and re-streaked for 2 to 3 times on agar medium until single uniform colonies were obtained. Isolates were then stored in 20% glycerol at -80°C. The recipe for liquid media was the same as the composition of M1 medium but without addition of agar. Single colonies were inoculated on MI medium at pH10 and re-streaked several times for purity check. Six isolates were designated as AS1, AS2, AS3, AS4, AS5 and AS6. Growth on M1 broth medium with and without peptone was measured by determination of the optical density at 660 nm using a Hitach specterophotometer (type 124).

### Morphological and phenotypic characterization

Cells actively growing on nutrient agar plates (pH 7.0 and 9.0) were used for cell and colony morphology. The formation of spores was tested by using nutrient broth cultures of 18 to 24 h supplemented with 5 mg/L of $MnSO_4.4H_2O$ and observed under a phase contrast microscope. Temperature (20 to 60°C), pH (6 to 12), and salinity (2 to 10% NaCl) ranges for growth were tested in nutrient broth, and after 24 h of incubation at 37°C the optic density of the cells at 600 nm was measured. Physiological characterization tests including Gram staining; anaerobic growth; catalase and amylase activities; casein, citrate, starch, tyrosine, gelatin, and urea utilization; reduction of nitrate to nitrite; acid production from sugars; the methyl red test; the Voges Proskauer test; indole and $H_2S$ production; and susceptibility to lysozyme were carried out according to the methods of Murray et al. (1994).

### PCR of 16S rRNA gene sequencing

16S rRNA were performed according to El-Menofy et al. (2014) using Forward primer GF: 5'-AGTTTGACTCTGGCTCAG-'3 and reverse Primer GR: 5'-TACGGCTACCTTGTTACGACTT-'3. These primers were also used for PCR amplification of the 16S rRNA gene. Genomic DNA was isolated as described by Abulreesh et al. (2012). Cells from 5 ml overnight culture for each isolate were harvested. Cell pellets were rinsed with 200 µl of NET buffer (0.1 M NaCl, 50 mM EDTA, 10 mMTris-Cl, pH 8.0) and re-suspended in 200 µl of GET buffer (50 mM glucose,10 mM EDTA, 25 mMTris-Cl, pH 8.0). 0.001 ug of lysozyme was added and the mixture was incubated at 37°C for 3 h. Twenty microliter of 10 mg/ml proteinase K was then added and the mixture was incubated at 37°C for 1 h. One hundred microliter 10% SDS was then added, and the mixture was incubated at 37°C for 1 h. The mixture was extracted several times with phenol: chloroform: isoamyl alcohol (24 : 24 : 1, v/v) until the interface was clear. DNA was precipitated by adding 1/25 volume

of 5 M NaCl and 2.5 volumes of 95% chilled ethanol. The precipitated DNA was rinsed with 1 ml of ice cold 70% ethanol, air dried, and re-suspended in 30 µl of sterilized distilled water. Selection of primers (Invitrogen, Paisley, UK) for PCR was according to Marchesi et al. (1998). The PCR reaction mixture (50 µl total volume) contained 200 µM of each dNTP, 0.5 for each µM primer, 10 mM trisHCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mMKCl, 2.5 U Taq polymerase (ABgene, Surry, UK) and 100 ng of template DNA. DNA amplification using primer was performed at the following temperature cycle: denaturation at 94°C for 2 min, 30 cycles at 94°C for 60 s, 50°C for 60 s, and 72°C for 90 s, final extension at 72°C for 7 min, respectively. A total of 10 µl of PCR products were analyzed by 1% agarose gel (Bioline, London, UK) electrophoresis and made visible by ethidium bromide (0.5 mg/ml) staining and ultraviolet (UV) transillumination. Sequencing of PCR products was performed by the research team of the biotechnology lab company, Cairo, Egypt; following the procedure described by (Sanger et al., 1977; Assaeedi et al., 2011). The deduced sequence was subjected to blast search tool from the national center of biotechnology, Bethesda, MD, USA (http://www.ncbi.nlm.nih.gov) the full length 16SrRNA sequence was aligned with reference homologues DNA sequence from NBCI database using multiple sequence alignment program of MEGA4. Phylogenetic trees were constructed by distance matrix based cluster algorithms viz weighted pair group with average (UPGMAs) (Saitou, 1987). *Bacillus cohnii* APT5 been used as a reference group and *Escherichia coli* as out-group.

## RESULTS AND DISCUSSION

### Isolation and screening of alkalophilic microorganisms

A total of six alkalophilic bacterial isolates were isolated from soil samples collected from Al-qunfotha area, Saudi Arabia. All strains were purified as a single colony and microscopically investigated. For routine work, all strain was grown in nutrient agar plate and kept at 4°C as well as they were grown in nutrient broth and stored in 20% glycerol under -80°C.

### Phenotypic characterization

Colonies of all six isolates are creamy white when grown on alkaline peptone medium. All six isolates were gram positive, motile rods, sub-terminal to terminal ellipsoidal spore in swollen sporangia. As presented in Table 1, isolates AS3, AS 4, AS 5 and AS 6 could grow at pH 9, 10, 11 and 12 with 9, but could not grow at pH7, indicating that this isolates are obligate alkalophiles while isolates AS 1 and AS 2 grew at pH ranged from 7 to 10, but could not grow at pH 11 and 12, suggesting that they could be facultative alkalophiles. In this context, it was reported that alkalophilic microorganisms constitute a diverse group that thrives in highly alkaline environments. They have been further categorized into two broad groups, namely, alkalophiles and alkalotolerants. The term alkalophiles is used for those organisms that were capable of growth above pH 10, with an optimal growth around pH 9, and are unable to grow at pH 7 or less. On the other hand, alkalotolerant organisms are capable of growing at pH values in excess of 10, but have an optimal growth rate nearer to neutrality (Guffanti et al., 1986). The extreme alkalophiles have been further subdivided into two groups, namely, facultative and obligate alkalophiles. Facultative alkalophiles have optimal growth at pH 10 or above but can grow well at neutrality, while obligate alkalophiles fail to grow at neutrality (Guffanti et al., 1986). All isolates could grow at 2% NaCl but could not grow at 5 and 10%. Only strain AS3 appeared as halotolerant, whereas, it could grow at 5% NaCl. All isolates grew at 45°C, but no growth was observed at 50°C. Negative reactions for all strains were recorded for lysis by KOH. The data in Table 1 shows that most of these isolates utilize a wide range of carbon sources including maltose, D-fructose, D-glucose, Sucrose and D-mannitol, but was not able to ferment lactose, D-xylose, raffinose, D-galactose, D-sorbitol or L-arabinose. Casein, gelatin, starch, citrate utilizations and amylase and catalase activities were all positive, but urea and tyrosine could not be utilized. Also, they were able to reduce nitrate to nitrite, but gas production was not observed from nitrate. The methyl red and Voges-Proskauer tests were negative for all isolates and indole and $H_2S$ were not produced. Isolates AS1 and AS2 could not utilize maltose and mannitol. Since the soils in Al-qunfotha region of western of Saudi Arabia contain high concentrations of arsenic and sodium chloride and are highly alkaline (pH ranging from 8.9 to 9.9), we expected that the extreme environment of the Al-qunfotha would be a good location for the discovery of previously unidentified alkalitolerant, halotolerant, endospore-forming organisms that maybe of ecological and/or commercial interest. One of the most important and noteworthy features of many alkalophiles is their ability to modulate their environment. They can alkalinize neutral medium or acidify high alkaline medium to optimize external pH for growth. However, their internal pH is between pH 7 and 9, always lower than the external medium. Thus, alkalophilicity is maintained by these organisms through bioenergetic membrane properties and transport mechanisms, and does not necessarily rely on alkali-resistant intracellular enzymes (Guffanti et al., 1986).

However, *Bacillus* species are difficult to identify by traditional methods based on phenotypic characteristics (Woese, 1987). In past decades, there was a full revision of alkalophilic *Bacillus* classification according to their phenotypic characteristics (Fritze et al., 1990). Sequence analysis of a 16s rRNA hyper-variant region has been a widely accepted technique (Saitou, 1987), and was reported to be a useful tool in the discrimination between the species in the *Bacillus* group (Jill, 2004). So far, genetic methods used in the characterization of alkaliphilic *Bacillus* have included 16s rRNA sequence data analysis (Nielsen et al., 1995).

Generally, when discriminating between closely related

**Table 1.** Phenotypic and biochemical characterization of alkalophilic isolates isolated from soil samples collected from Al-qunfotha area, KSA.

| Character | Alkalophilic Strains | | | | | |
|---|---|---|---|---|---|---|
| | AS1 | AS2 | AS3 | AS4 | AS5 | AS6 |
| Colony morphology | White , creamy | | | | | |
| Cell morphology | Rod | | | | | |
| Gram stain | + | + | + | + | + | + |
| Sporulation | + | + | + | + | + | + |
| Oxidase | + | + | + | + | + | + |
| Catalase | + | + | + | + | + | + |
| **Carbon source utilization** | | | | | | |
| D-Glucose | + | + | + | + | + | + |
| D-fructose | + | + | + | + | + | + |
| D-mannitol | - | - | + | + | + | + |
| Sucrose | + | + | + | + | + | + |
| Maltose | - | - | + | + | + | + |
| L-arabinose | - | - | - | - | - | - |
| D-glactose | - | - | - | - | - | - |
| D-sorbitol | - | - | - | - | - | - |
| D-xylose | - | - | - | - | - | - |
| D-raffinose | - | - | - | - | - | - |
| Tween 20 | + | + | + | + | + | + |
| Tween 40 | + | + | + | + | + | + |
| Tween 80 | + | + | + | + | + | + |
| D-lactose | - | - | - | - | - | - |
| **Hydrolysis of** | | | | | | |
| Gelatin | + | + | + | + | + | + |
| Casein | + | + | + | + | + | + |
| Starch | + | + | + | + | + | + |
| Urea | - | - | - | - | - | - |
| **Other biochemical tests** | | | | | | |
| Reduction of Nitrate to nitrite | + | + | + | + | + | + |
| Voges and Proskaur reaction | - | - | - | - | - | - |
| Growth at pH 7 | + | + | - | - | - | - |
| 8 | + | + | + | + | + | + |
| 9 | + | + | + | + | + | + |
| 10 | - | - | + | + | + | + |
| 12 | - | - | + | + | + | + |
| Growth at NaCl 2% | + | + | + | + | + | + |
| 5% | - | - | | - | - | - |
| 10% | - | - | - | - | - | - |
| Growth at (°C) :30 | + | + | + | + | + | + |
| 40 | + | + | + | + | + | + |
| 50 | - | - | - | - | - | - |

species of the same genus, DNA-DNA hybridization, as well as housekeeping genes sequences should be the methods of choice, in accordance with the proposed molecular definition of species (Berkum et al., 1996). Since, all isolates that were identified in this study are closely related to *B. cohnii,* thus, our results indicate that

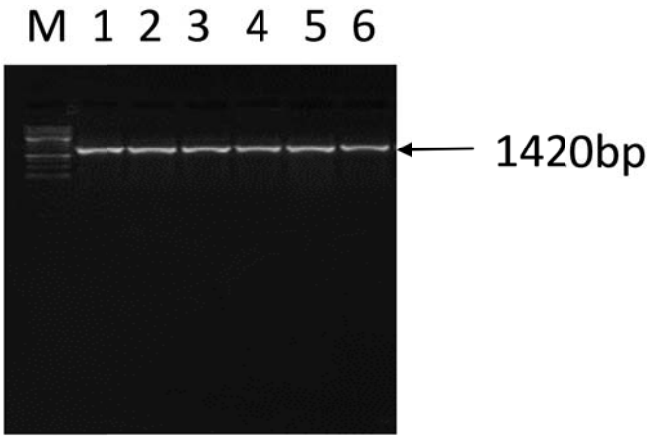


**Figure 1.** Agarose gel electrophoresis of PCR products of the 16S rRNA fragments for isolates number 1 to 6 (AS1, AS2, AS3, AS4, AS5 and AS6 respectively). M, 1 Kb DNA ladder marker.

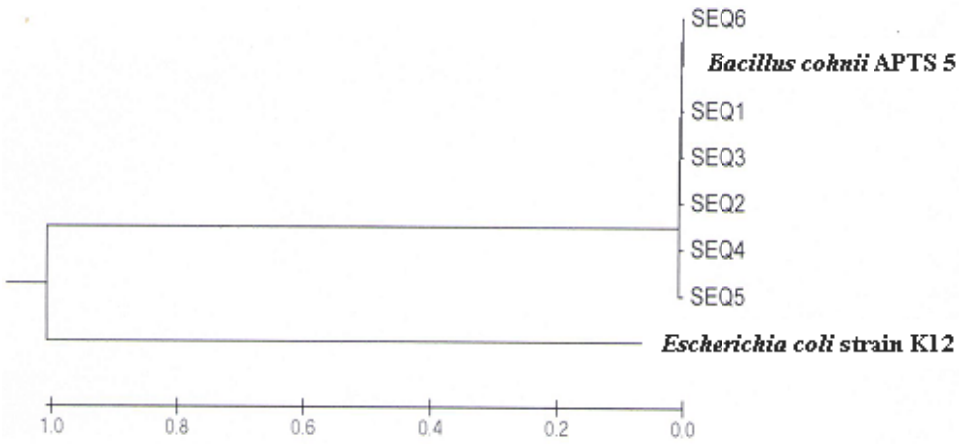


**Figure 2.** Phylogenetic relationship of the six isolates based on 16s rRNA sequence. SEQ1 to 6 represent isolates AS1, AS2, AS3, AS4, AS5 and AS6 respectively.

*B. cohnii* occurred in Al-qunfotha area. As reported by Assaeedi and Osman (2012), all alkalophilic bacteria isolated so far showed no growth in the absence of sodium ions at high pH value. This is due to the presence of acetate inside at pH 10. Therefore as found in the present study alkalkophilic strains use sodium ions to drive the solute uptake. From these results, it could be suggested that AS1 and AS2 isolates were facultative alkalophilic, while isolates AS3, AS4, AS5 and AS6 were obligate alkalophiles. Overall, the results obtained in this study suggest that a variety of alkalophilic and alkalitolerant, endospore- forming bacteria occurred and inhabit the Al-qunfotha area, KSA. However, further work such as isolation and characterization of the interested alkalophilic enzymes from these strains are in progress.

PCR of 16S rRNA gene sequencing and phylogenetic analysis. To identify the taxonomy of alkalophilic isolates, DNA was isolated and PCR amplification of the 16S rRNA was performed using primer. Primer was able to amplify a 1420 bp fragment (Figure 1). Homologs of the deduced sequence were identified using BLAST and Gene Bank from the National Centre of Biotechnology, Bethesda, MD, USA (http://www.ncbi.nlm.nih.gov). The partial 16S rRNA gene sequence was aligned with reference homologous DNA sequences from Gene Bank using the multiple sequence alignment programs in MEGA4. Alignment by BLAST showed that the primers only targeted 16SRNA gene. The results reveal that the six alkalophilic isolates sharing 99% 16SrRNA gene sequence similarity are classified under the same species. The 16SrRNA based phylogenetic analysis demonstrated 99% sequence similarity with *B. chonii*, suggesting that, these six isolates belong to this species (Figure 2). The 16s rRNA sequence data showed that isolates number 1, 2 and 3 had high similarity with *B. cohnii* strain D7023, while isolates number 4, 5 and 6 had

high similarity with *B. cohnii* strain T-46. All sequence data were deposited into Gene Bank with accession numbers: KP053301, KP053302, KP053303, KP053304, KP053305 and KP053306 respectively, (Figure 2). Phylogenetic relationship of the six isolates was based on 16s rRNA sequence; the tree was generated using the neighbor-joining method and the sequence from *E. coli* strain 12 was consider as out group. The sequence of *B. cohnii* APTS 5 was used as reference strain.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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