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Research Article

## Phenotypic and Genotypic Characterization of facultative and obligate Alkalophilic *Bacillus* sp. isolated from Saudi Arabia alkaline soils

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**Abstract:** Isolation and identification of new alkalophilic *Bacillus* strains have been increasing interest due to their possessing valuable and commercially interesting enzymes. In the present study, a total six obligate and facultative alkalophilic isolates were isolated from desert soil around the Al-qunfotha city, 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, indicates that these isolates are obligate alkalophiles. While, isolates AS1 and AS2 grew at pH ranged 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. Based on 16S rDNA data, the phylogenetic analysis of these strains showed that all six alkalophilic *Bacillus* belonged to *Bacillus cohnii* with 99% similarity. The nucleotide sequences of 16S rRNA gene for the six isolates were deposited in a Gene-bank under accession numbers; KP053301, KP053302, KP053303, KP053304, KP053305 and KP053306, respectively.

**Keywords:** *Bacillus cohnii*, 16S rRNA, obligate alkalophiles, facultative alkalophiles, Al-qunfotha city, Saudi Arabia.

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### 1. Introduction

Extremophiles are organisms evolved to live in a variety of extreme environments like deep-sea hydrothermal vents, hot springs and hypersaline environments. They can be classified as thermophiles, psychrophiles, acidophiles, alkalophiles, halophiles and others (Atomi, 2005). All microorganisms follow a normal distribution pattern based on the pH dependence for their optimal growth, and the majority of these microorganisms is known to proliferate well at near-neutral pH values. As the pH moves away from this neutral range, the number of microorganisms decreases. The number of alkalophilic bacteria found in the soil is about 1/10 to 1/100 of that of neutrophilic bacteria (Krulwich and Guffanti, 1986; Krulwich and Guffanti, 1990). Alkalophilic microorganisms are widely distributed in nature and can be found in almost all environments without the restriction of alkalinity. However, a few of the naturally-occurring alkaline environments, namely soda soils, lakes, and deserts, harbor a wide range of these types (Kumar *et al.*, 1997). Their ecological and chemical aspects have been

studied in detail (Grant, 1986; Grant *et al.*, 1990). 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 called alkalotolerant 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 alkalophilic (Guffanti *et al.*, 1986; Marie *et al.*, 2005). The former group shows optimal growth of pH around 10 and cannot grow at pH around 7. Whereas, the latter group grows at pH 7 and around 10 with optimal growth at pH 10 or above (Schallmeyer *et al.*, 2004). Interest in alkalophilic bacteria has increased during the last few decades due to their applications in ecological, industrial and biotechnology fields. Alkaliphilic *Bacillus* strains constitute an important source of extracellular enzymes that are useful for numerous industrial processes (Ito *et al.*, 1998; Horikoshi, 1999). This group includes facultative and

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obligate alkaliphilic species that grow well at pH values higher than 9.0.

Highly saline, alkaline environments are relatively rare in the world compared with high saline, neutral environments. However, there is a possibility that such environments harbor a unique microbial population (Grant, 1988; Tindall *et al.*, 1980). In Saudi Arabia, there are only a few reports on alkalophilic bacteria (Salama *et al.*, 1993; Assaedi and Osman, 2012). The objectives of this study were to isolate, characterize and identify alkalophilic bacteria from the Al-qunfotha region in the Kingdom of Saudi Arabia.

## 2. Materials and Methods

### 2.1 Soil samples and the medium used in this study

A total 50 Soil samples were collected from different locations surrounding Al-qunfotha city located in the western region of Saudi Arabia. Samples were collected from 2-5cm below the surface with a shovel. Samples were stored on ice until were transported to the laboratory where they were stored at +4°C.

The medium used in this study consists of 1% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K<sub>2</sub>HPO<sub>4</sub>, 0.02% MgSO<sub>4</sub>.7H<sub>2</sub>O, 2% Agar, pH 10.5 (Horikoshi and Akiba, 1982). The solution of Na<sub>2</sub>CO<sub>3</sub> was autoclaved separately and added to the medium.

### 2.2 Isolation and screening of alkalophilic isolates

For isolation of alkalophilic strains, 1gm soil samples were suspended in 10ml of sterilized H<sub>2</sub>O and 1ml of soil suspension were then plated on M1 agar medium. Plates were incubated at 30°C for 48 hr. 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 M1 medium at pH 10 and restreaked 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 660nm using a Hitachi spectrophotometer (type 124).

### 2.3 Morphological and phenotypic characterization

Cells are 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 18-24 hr supplemented with 5mg/L of MnSO<sub>4</sub>.4H<sub>2</sub>O and observed under a phase contrast microscope. Temperature (20-60°C), pH (6-12), and salinity (2%-10% NaCl) range for growth were tested in nutrient broth, and after 24 hr of incubation at 37°C the optical density of the cells at 600nm 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<sub>2</sub>S production; and susceptibility to lysozyme were carried out according to the methods of Claus and Berkeley (1986); Murray *et al.*, (1994); Smibert and Krieg (1994).

### 2.4 PCR of 16S rRNA gene sequencing

PCR amplification of 16S rRNA genes was performed according to (Marchesi *et al.*, 1998) using forward primer GF: 5'-AGTTTGACTCTGGCTCAG-3' and reverse Primer GR: 5'-TACGGCTACCTTGTTACGACTT-3'. These primers also used for sequencing of the 16S rRNA genes. Genomic DNA was isolated as described by (Wang *et al.*, 2001). Cells from 5ml overnight culture for each isolate were harvested. Cell pellets were rinsed with 200µl of NET buffer (0.1M NaCl, 50mM EDTA, 10mM Tris-Cl, pH 8.0) and re-suspended in 200µl of GET buffer (50mM glucose, 10mM EDTA, 25mM Tris-Cl, pH 8.0). 0.001µg of lysozyme was added and the mixture was incubated at 37°C for 3 hr. Twenty microliter of 10mg/ml proteinase K was then added and the mixture was incubated at 37°C for 1 hr. One hundred microliter 10% SDS was then added, and the mixture was incubated at 37°C for 1 hr. 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 5M NaCl and 2.5 volumes of 95% chilled ethanol. The precipitated DNA was rinsed with 1ml of ice cold 70% ethanol, air dried, and re-suspended in 30µl of sterilized distilled water. Selection of primers (Invitrogen, Paisley, UK) for PCR was, according to (Marchesi *et al.*, 1998). The PCR reaction mixture (50µl total volume) contained 200µM of each dNTP, 0.5 for each µM primer, 10mM Tris-HCl (pH 8.3), 1.5mM MgCl<sub>2</sub>, 50mM KCl, 2.5 U Taq polymerase (ABgene, Surrey, UK) and 100ng 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 Sec, 50°C for 60 Sec, and 72°C for 90 Sec, final extension at 72°C for 7 min., respectively. A total of 10µl of PCR products was analyzed by 1% agarose gel (Bioline, London, UK) electrophoresis and made visible by ethidium bromide (0.5mg/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). 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 16S rRNA sequence was aligned with the reference homologous DNA sequence from NCBI database using

multiple sequence alignment program of MEGA 4. Phylogenetic trees were constructed by distance matrix based cluster algorithms pair group with an average (UPGMAs) (Saitou *et al.*, 1987). *Bacillus cohnii* APT5 has been used as a reference group and *Escherichia coli* strain 12 as out-group.

### 3. Results and Discussion

#### 3.1 Isolation and screening of alkalophilic microorganisms

A total of six alkalophilic bacterial isolates were isolated from soil samples collected from the Al-qunfotha area, Saudi Arabia. All strains were purified as a single colony and microscopically investigated. For routine work, all strains were 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.

#### 3.2 Phenotypic characterization

Colonies of all six isolates are creamy white when grown in 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, AS4, AS5 and AS6 could grow at pH 9, 10, 11 and 12 with 9, but could not grow at pH 7, indicating that these isolates are obligate alkalophiles. While, isolates AS1 and AS2 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 are 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 (Krulwich, 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 (Krulwich and Guffanti, 1989). 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) showed 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 utilization 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<sub>2</sub>S was not produced. Isolates AS1 and AS2 could not utilize maltose and mannitol. Since the soils in the 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 may be 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 (Krulwich and Guffanti, 1983).

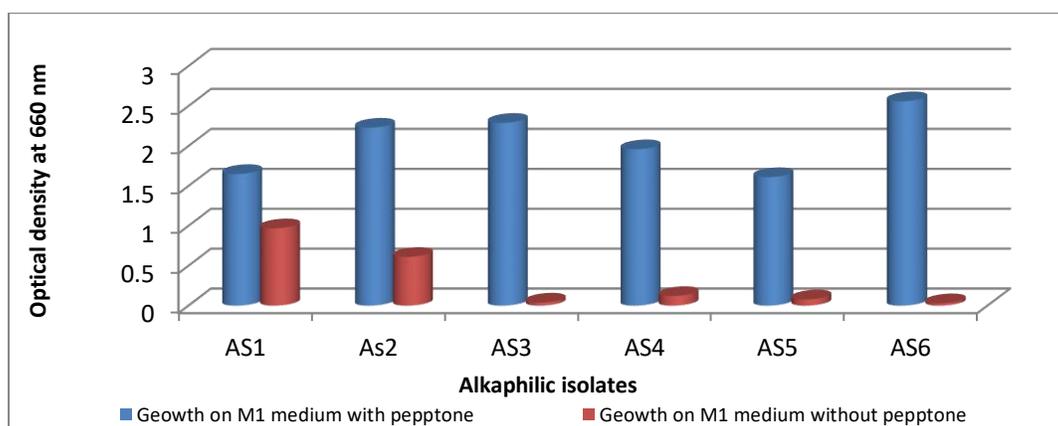


Fig. 1. Growth of alkaliphilic isolates on M1 medium with peptone and without peptone at appropriate pH for each isolate.

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

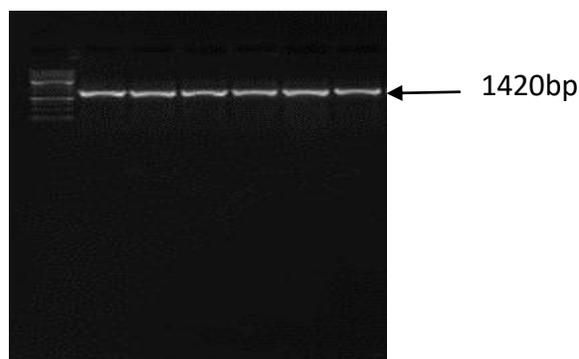
Characters	Alkalophilic Strains					
	AS1	AS2	AS3	AS4	AS5	AS6
Colony morphology	White, creamy					
Cell morphology	Rod					
Gram stain	+	+	+	+	+	+
Sporulation	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
<b>Carbon source utilization</b>						
D-Glucose	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+
D-mannitol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

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; Takami and Horikoshi, 2000; Martins *et al.*, 2001).

### 3.3 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 (Fig. 2).

M 1 2 3 4 5 6

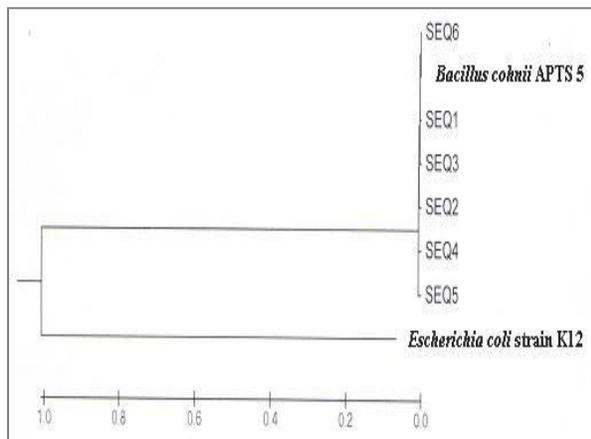


**Fig. 2. Agarose gel electrophoresis of PCR products of the 16S rRNA fragments for isolates AS1, AS2, AS3, AS4, AS5, and AS6, M: 1Kb DNA ladder marker.**

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 program in MEGA 4. Alignment by BLAST showed that the primers only targeted 16S rRNA gene. All sequence data of isolates AS1, AS2, AS3, AS4, AS5 and AS6 were deposited into Gene Bank with accession numbers; KP053301, KP053302, KP053303, KP053304, KP053305 and KP053306, respectively.

Based on 16S rDNA sequence analysis and phenotypic characterization, all six alkaliphilic species were characterized and identified as *B. cohnii* with similarity 99% (Fig. 3). Generally, when discriminating between closely related species of the same genus, DNA-DNA hybridization, as well as housekeeping gene sequences should be the methods of choice, in accordance with the proposed molecular definition of species (Berkum *et al.*, 1996; Elbanna *et al.*, 2009; Elbanna *et al.*, 2014). Since all isolates that were identified in this study are closely related to *Bacillus cohnii*. Thus, our results indicate that *B. cohnii* occurred in the Al-qunfotha area. As reported by (Assaeedi and Osman, 2012), all alkaliphilic 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 alkaliphilic strains use sodium ions to drive the solute uptake. From these results, it could be suggested that AS1 and AS2 isolates were facultative alkaliphilic, 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 isolation and characterization of the interested alkalophilic enzymes from these strains are in progress.



**Fig. 3. Phylogenetic relationship of the six isolates based on 16S rRNA sequence, the tree was generated using the neighbor-joining method and the sequence from *Escherichia coli* strain 12 was considered as outgroup. The sequence of *Bacillus cohnii* APTS 5 was used as reference strain.**

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