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PRELIMINARY ISOLATION AND CHARACTERIZATION OF HALOTOLERANT AND HALOPHILIC BACTERIA FROM SALT MINES OF KARAK, PAKISTAN

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Abstract

Halophiles are extremophile organisms that thrive in environments with very high concentrations of salt. The salt mines of Karak region, is an extremely saline environment and its microbial communities have not yet been explored. In the present study, twenty one halotolerant and halophilic bacterial strains were isolated from salt mines of Karak region. These strains can grow in media with 5-40% NaCl concentrations. Morphological, physiological and biochemical characteristics of these strains were studied by optimizing their growth conditions such as pH, NaCl and temperature. A high microbial density was observed at low NaCl concentration. Halophilic bacterial strains were divided into three groups on the basis of NaCl concentration; first was slightly halotolerant / halophilic; second, moderately halophilic and third, extreme halophilic bacteria. The phylogenetic analyses inferred from 16S rRNA gene sequence of these strains demonstrated that these are closely related to species belonging to different genera: *Thalassobacillus, Halomonas, Brevibacterium, Oceanobacillus, Terribacillus, Pseudomonas, Bacillus, Enterobacter, Halobacillus, Staphylococcus* and *Virgibacillus*. This preliminary study showed that the salt mine of Karak are rich in halotolerant / halophilic bacterial population with diverse bacterial communities, which may be utilized in various industrial applications.

Introduction

Biodiversity is an attribute of an area and specifically refers to the species/varieties within and among living organisms, assemblage of the living organisms, biotic communities and biotic processes, whether naturally occurring or modified by humans (DeLong, 1996). Microbial organisms occupy a peculiar place in the world. A handful of soil contains billions of microbial organisms, so many different types that accurate numbers remain unknown (Satyanarayana *et al*., 2005). The ability of microorganisms to survive under harsh conditions has recently prompted researchers to study these organisms to better understand their characteristics and eventually utilize them in various applications. Exploration of microbial biodiversity in extreme environments has opened a new era for microbiologists already blessed by the establishment of *Archaea* as a separate domain and the successive introduction of molecular biology and genomics as basic and powerful tools (Woese & Fox, 1977).

Most extremophiles are found in microbial world. The range of environmental extremes tolerated by microbes is much broader than other life forms. Extremophiles may be divided into five categories; thermophiles, acidophiles, alkophiles, halophiles, and psychrophiles. This clearly indicates the nature of habitats used by these microorganisms (Austain, I988). Many extremophilic microorganisms possess properties suitable for biotechnological and commercial uses (Tango & Islam, 2002).

Halophiles are extremophile organisms that thrive in environments with very high concentrations of salt. Halophiles are categorized as slight, moderate or extreme, based on the extent of their halotolerance. Halophiles can be found in areas where the concentration of salt is five times greater than that salt concentration of the ocean, such as the Great Salt Lake in Utah, Owens Lake in California, the Dead Sea, and in evaporation ponds. Miteva *et al*., (2004) successfully isolated nearly 800 bacterial strains and classified them into different groups based on morphology, amplified rDNA restriction analysis (ARDRA) patterns followed by phylogenetic and physiological characterization. Halophilic microorganisms have several biotechnological applications like β-carotene production of fermented foods. In recent years, uses of halophilic microorganisms have significantly increased. Many enzymes, stabilizers and valuable compounds from halophiles may present advantages for the development of biotechnological production processes.

Pakistan is renowned for its unique biodiversity. It is especially endowed with a richness of extremophilic environments. A number of thermal springs, many glacial areas and salt mines exist in remote areas of Pakistan. Studies of these areas are far from complete. Many strains of microorganisms have been isolated from various sources but stll these are not yet validated from Pakistani ecology. The purpose of this research was to explore any novel extremely halotolerant / halophilic aerobic or facultative anaerobic bacteria, and to examine their phenotypic, physiological and biochemical characteristics. It was also aimed to assess the bacterial biodiversity of halophilic bacteria in Pakistan using biochemical and molecular techniques.

Materials and Methods

Sampling: The strains were isolated from water, sediments and rocks of the Karak Salt mines of Pakistan. The samples were collected from different sites (Fig. 1). All samples were collected into sterile bottles and stored at 4ºC in the laboratory until isolation of the strains.

Isolation and enrichment of bacteria: Enrichment cultures and isolation procedures were performed to recover moderately to extremely halotolerant / halophilic bacteria by dilution plate technique on Tryptic Soya Agar medium. An appropriate volume of diluted sample was streaked on agar medium containing various concentrations (5-20%) of NaCl and incubated at 28ºC. The isolated strains were sub-cultured several times under same conditions to obtain pure cultures of morphologically different bacteria. The purified strains were further characterized and also stored at – 80ºC.

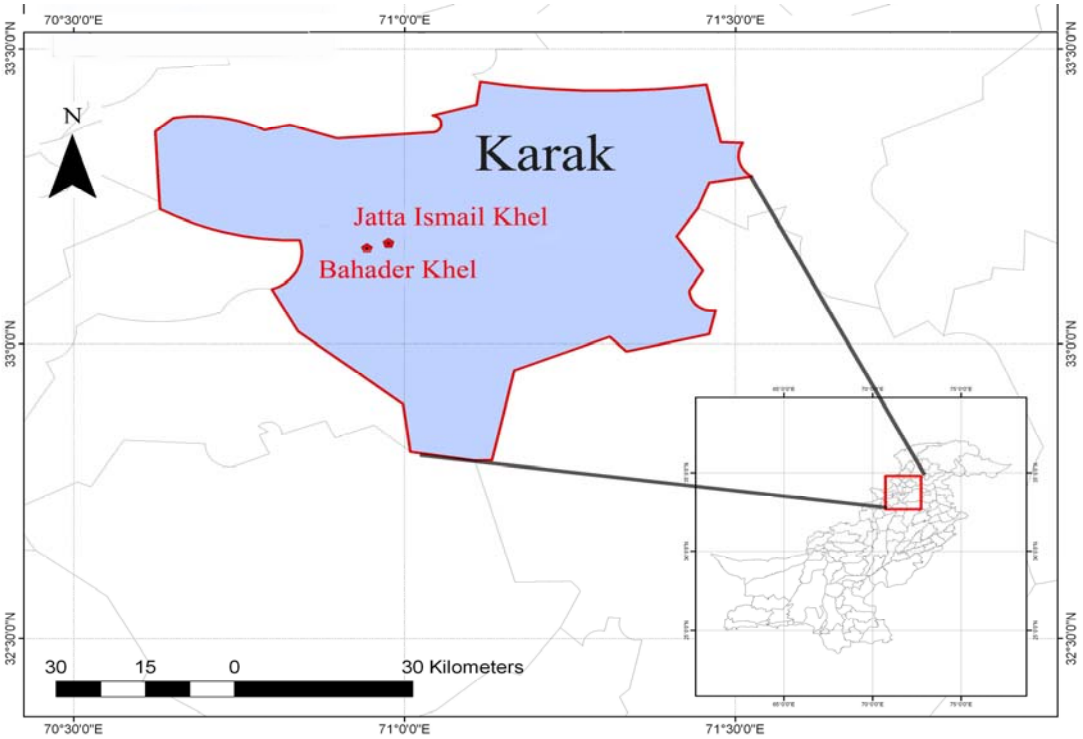

Fig. 1. Location of sample collection sites in Karak Salt Mines, Khyber Pakhtunkhwa Province, Pakistan.

Morphological studies of isolated bacteria: Pure bacterial colonies were characterized for color, form, elevation, margin etc. Bacterial isolates were also characterized on the basis of Gram's staining, cell morphology and motility using microscope.

Optimization of growth conditions: Bacterial isolates were evaluated at various pH (4-11), sodium chloride concentrations (1-40%) and temperature (4, 10, 20, 28, 35, 37, 40, 45 and 50ºC) to find out the optimum growth conditions. The optical density at 600 nm wavelength was measured for evaluating bacterial growth in broth culture.

PCR amplification and sequencing of 16S rRNA gene: Genomic DNA extraction for amplification of 16S rRNA gene was performed as described previously (Ahmed *et al*., 2007) by suspending few well isolated colonies in TE buffer in a micro-centrifuge tube. These cells were heated for 10 minutes at 95ºC and were centrifuged at 6,000 rpm for 5 minutes. The supernatant was used as template DNA for the amplification of 16S rRNA gene.

The 16S rRNA gene of all the isolated strains were amplified by polymerase chain reaction using primers 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1510R (5'-GGCTACCTTGTTACGA-3') using PreMix ExTaq (Takara, Japan) as described previously (Ahmed *et al.,* 2007). The polymerase chain reaction was carried out in ABI Veriti PCR Machine (Applied Biosystems, USA) using optimized PCR Program: initial denaturation at 94ºC for 2 min; 30 cycles of denaturation at 94ºC for 1 min, annealing at 50ºC for 1 min and extension at 72ºC for 1:30 min. The final extension was performed at 72ºC for 5 min. The amplified PCR products of 16S rRNA gene of bacterial strains were purified and sequenced using the primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-ACCTTGTTACGACTT-3') using commercial service of Macrogen Inc. Korea (www.dna.macrogen.com).

Phylogenetic analysis of bacterial isolates: BioEdit software (Hall, 1999) was used to assemble the fragment sequences of 16S rRNA gene. 16S rRNA gene sequences were submitted to DDBJ (http://www.ddbj.nig.ac.jp/) and accession numbers are mentioned in Table 2. Using 16S rRNA gene sequences, the strains were identified by BLAST search on EzTaxon Server (http://147.47.212.35:8080). The sequences of closely related type strains were retrieved for constructing the phylogenetic trees. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 5 (Tamura *et al*., 2011). A phylogenetic tree was constructed from unambiguously aligned nucleotides using the neighbour-joining algorithm (Saitou & Nei, 1987). The stability of the relationship was assessed by bootstrap analysis by performing 1000 resamplings for the tree topology of the neighbour-joining data.

Results

Colony and cell morphology: Twenty one strains were isolated during this preliminary study of halotolerant / halophilic bacteria from Karak salt mines. The strains were purified on the basis of morphology of bacteria. Colonial pigmentation of the isolates included pale yellow, creamy and white (Table 1). These colonies were irregular and circular in form. Colony elevations were raised, flat, umbonate and pulvinate. Most of the colonies had entire margin, while some had undulate and wavy filamentous margins. Few of them also had lobate margins. Different isolates displayed different cell sizes and morphologies when viewed under the microscope. Some rod shaped isolates in small or long chains were also observed.

Table 1. Phenotypic characteristics of isolated halotolerant / halophilic strains.

Strain ID	Gram staining	Colony morphology					Range of pH for growth (Optimum)	Range of NaCl (%) for growth (Optimum)	Range of temperature (°C) for growth (Optimum)
		Color	Form	Surface	Margin	Opacity			
NCCP-58	+	Pale yellow	Circular	Smooth	Entire	Opaque	6-9 (7)	1-15 (10)	4-50 (28)
NCCP-64	+	Pale yellow	Circular	Smooth	Entire	Opaque	6-9 (7)	2-15 (10)	4-50 (28)
NCCP-67	–	White	Irregular	Smooth	Lobate	Opaque	5-9 (7)	1-19 (10)	10-45 (37)
NCCP-68	+	White	Round	Shiny	Entire	Opaque	6-9 (8)	2-17 (10)	4-50 (37)
NCCP-69	+	White	Round	Shiny	Entire	Opaque	5-9 (7)	1-20 (15)	4-50 (35)
NCCP-70	+	Pale yellow	Round	Shiny	Entire	Opaque	6-9 (8)	1-15 (8)	10-45 (28)
NCCP-72	+	Pale yellow	Circular	Smooth	Entire	Opaque	6-9 (7)	2-15 (10)	4-50 (28)
NCCP-76	+	White	Circular	Smooth	Entire	Transparent	6-9 (7)	1-25 (10)	10-45 (28)
NCCP-89	+	Pale yellow	Circular	Smooth	Entire	Opaque	5-10 (8)	1-20 (5)	10-45 (35)
NCCP-90	+	White	Circular	Viscous	Entire	Transparent	6-9 (8)	0-15 (5)	4-45 (28)
NCCP-164	–	White	Round	Mucoid	Entire	Transparent	6-9 (7)	0-20 (5)	4-45 (37)
NCCP-165	+	White	Circular	Smooth	Entire	Transparent	5-9 (8)	2-25 (10)	10-45 (35)
NCCP-167	–	White	Round	Smooth	Entire	Transparent	6-9 (7)	0-25 (15)	4-45 (37)
NCCP-169	+	White	Circular	Mucoid	Entire	Transparent	6-9 (7)	2-25 (15)	4-40 (35)
NCCP-177	+	Creamy	Circular	Smooth	Entire	Opaque	5-8 (7)	1-25 (15)	4-45 (35)
NCCP-178	–	White	Irregular	Smooth	Lobate	Opaque	5-9 (7)	3-30 (20)	10-45 (37)
NCCP-179	–	White	Circular	Smooth	Entire	Opaque	6-9 (8)	3-35 (25)	4-45 (37)
NCCP-180	+	Creamy	Round	Smooth	Entire	Opaque	5-9 (8)	2-30 (25)	10-40 (35)
NCCP-181	–	White	Irregular	Smooth	Lobate	Opaque	5-9 (7)	3-35 (20)	10-45 (37)
NCCP-182	+	White	Circular	Smooth	Filamentous	Opaque	6-9 (7)	2-35 (20)	4-40 (37)
NCCP-183	–	White	Irregular	Smooth	Lobate	Opaque	5-9 (8)	2-40 (30)	10-45 (37)

Table 2. Identification of isolated halotolerant / halophilic strains based on 16S rRNA gene sequence and their accession numbers published in DNA database.

Strain ID	Strain name / Genus	Number of nucleotides of 16S rRNA gene	Accession number of 16S rRNA gene	Closely related validly published taxa	Sequence similarity (%) of 16S rRNA gene
NCCP-58	*Thalassobacillus* sp.	1417	AB541110	*Thalassobacillus devorans* (AJ717299)	99.011
NCCP-64	*Thalassobacillus* sp.	1446	AB698781	*Thalassobacillus devorans* (AJ717299)	98.958
NCCP-67	*Halomonas* sp.	1421	AB698782	*Halomonas elongate* (FN869568)	98.872
NCCP-68	*Brevibacterium* sp.	1382	AB698783	*Brevibacterium linens* (X76567)	98.524
NCCP-69	*Brevibacterium* sp.	1407	AB698784	*Brevibacterium epidermidis* (X76567)	98.927
NCCP-70	*Brevibacterium* sp.	1409	AB698785	*Brevibacterium permense* (X76565)	98.927
NCCP-72	*Thalassobacillus* sp.	1437	AB698786	*Thalassobacillus cyri* (AJ717299)	98.739
NCCP-76	*Oceanobacillus* sp.	1437	AB698787	*Oceanobacillus kapialis* (FJ386518)	99.102
NCCP-89	*Terribacillus* sp.	1437	AB698791	*Terribacillus goriensis*(AB243845)	99.512
NCCP-90	*Terribacillus* sp.	1455	AB698792	*Terribacillus saccharophilus* (DQ519571)	99.650
NCCP-164	*Pseudomonas* sp.	1436	AB698796	*Pseudomonas xanthomarina* (CP002881)	99.646
NCCP-165	*Bacillus* sp.	1436	AB698797	*Bacillus pumilus* (AF234854)	99.789
NCCP-167	*Enterobacter* sp.	1416	AB698799	*Escherichia hermannii* (AJ508303)	99.925
NCCP-169	*Oceanobacillus* sp.	1445	AB698800	*Oceanobacillus manasiensis* (AJ315060)	99.445
NCCP-177	*Halobacillus* sp.	1449	AB698808	*Halobacillus profundi* (X94558)	99.515
NCCP-178	*Halomonas* sp.	1478	AB698809	*Halomonas eurihalina* (FN869568)	99.717
NCCP-179	*Halomonas* sp.	1418	AB698810	*Halomonas halophila* (AJ295145)	99.435
NCCP-180	*Staphylococcus* sp.	1418	AB698811	*Staphylococcus equorum* (AB009939)	99.441
NCCP-181	*Halomonas* sp.	1418	AB698812	*Halomonas eurihalina* (FN869568)	99.717
NCCP-182	*Virgibacillus* sp.	1446	AB698813	*Virgibacillus marismortui* (AB197851)	99.792
NCCP-183	*Halomonas* sp.	1420	AB698814	*Halomonas eurihalina* (FN869568)	99.366

Density of halotolerant / halophilic bacteria: Halotolerant / halophilic bacteria isolated from salt mines were slightly halotolerant (1-5% NaCl), moderately halophilic (5-15% NaCl) and extreme halophilic (15-40% NaCl).

Optimization of growth conditions: The Growth conditions of all the strains were optimized for pH, NaCl tolerance and temperature (Table 1). The purpose of optimization of the strains was to find their optimum growth on different pH. From the results it was concluded that the halophilic bacterial strains grow best on 7-9 pH. Similarly, NaCl tolerance was checked for these strains, most strains grow best in the range of 5-20% at the temperature range of 28-37^{o}C.

Phylogenetic analysis: Twenty one bacterial strains were identified taxonomically using robust method of 16S rRNA gene sequence (Table 2). To determine their phylogenetic position, the 16S rRNA gene sequence of each strain was analyzed, and phylogenetic trees were constructed (Fig. 2a, 2b and 2c). Phylogenetic analysis indicated that the majority of isolated strains belonged to genera: *Thallossobacillus, Halobacillus, Halomonas, Brevibacterium, Terribacillus* and *Bacillus* (Table 2). All strains shared more than 97% identity with their closest phylogenetic relatives. However, the phylogenetic analyses showed that few strains can further be studied taxonomically to delineate as novel species. These strains belonged to genera: *Brevibacterium, Thallossobacillus, Halobacillus, Halomonas* and *Terribacillus.* The Phylogenetic analysis reflected the evolutionary relationships among halotolerant / halophilic bacteria.

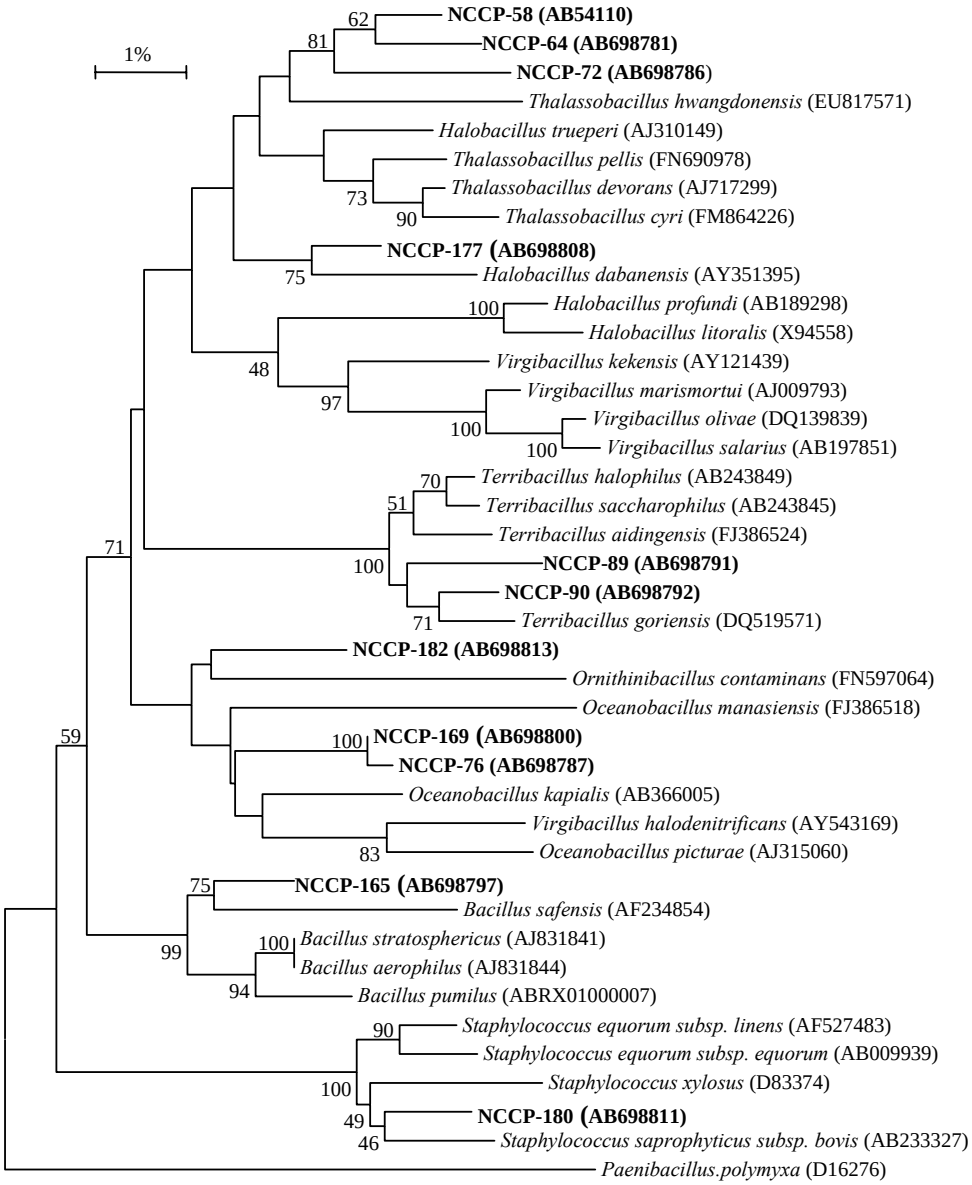

Fig. 2a. Phylogenetic tree showing the interrelationships of isolated halotolerant / halophilic strains belonging to *Firmicutes* and their close relatives inferred from 16SrRNA gene sequence. Data with gaps were removed after alignment by CLUXTAL X. The rooted tree was constructed using the neighbour-joining method contained in the MEGA5 software (Tamura *et al*., 2011). Bootstrap values, expressed as percentages of 1000 replications, are given at branching points. The sequence of *Paenibacillus polymyxa* (D16276) was used as the outgroup. Bar, 1% sequence divergence.

Discussion

Bacteria isolated from samples collected from the various sites of salt mines were found highly diverse group of halotolerant and halophilic bacteria with different phenotypic characteristics (Table 1). However, the phenotypic characteristics alone were not enough to differentiate the bacterial isolates and could lead to identification problems. The main reason for this is the standardization of a conventional method, when it was applied to halophilic bacteria because their growth characteristic highly dependent on many factors such as NaCl concentrations, temperature, pH and medium composition. Our results of phenotypic characteristics are in accordance with Fritze (2002) who recommended that phenotypic characterization results cannot be directly compared without full background knowledge of the precise conditions used for a particular test.

Several studies have been conducted on the ecology, taxonomy, and phylogeny of halophilic bacteria as well as their biotechnological applications (Lichfield and Gillevet, 2002). Halophilic bacteria were categorized on the basis of tolerance to different NaCl concentrations into slightly-, moderately-, and extremely-halophilic bacteria (Table 1). However, this approach is practically ineffective for this purpose as the optimum and range of halophilic bacteria for tolerating NaCl is critical. Therefore, in order to classify the halotolerant / halophilic bacterial isolates in response to NaCl. Previously, the

halophilic bacteria were grouped by using the results of salt tolerant test proposed by Kushner (1993). Our results showed that the slightly- and moderately halophilic bacteria were more abundant than the extremely halophilic bacteria (Table 1). These results are in agreement with those reported by Quesada *et al*., (1982), and Rodriguez-Valera (1988), who reported higher frequencies of moderately halotolerant and halophilic bacteria compared to extremely halophilic bacteria in saline environments.

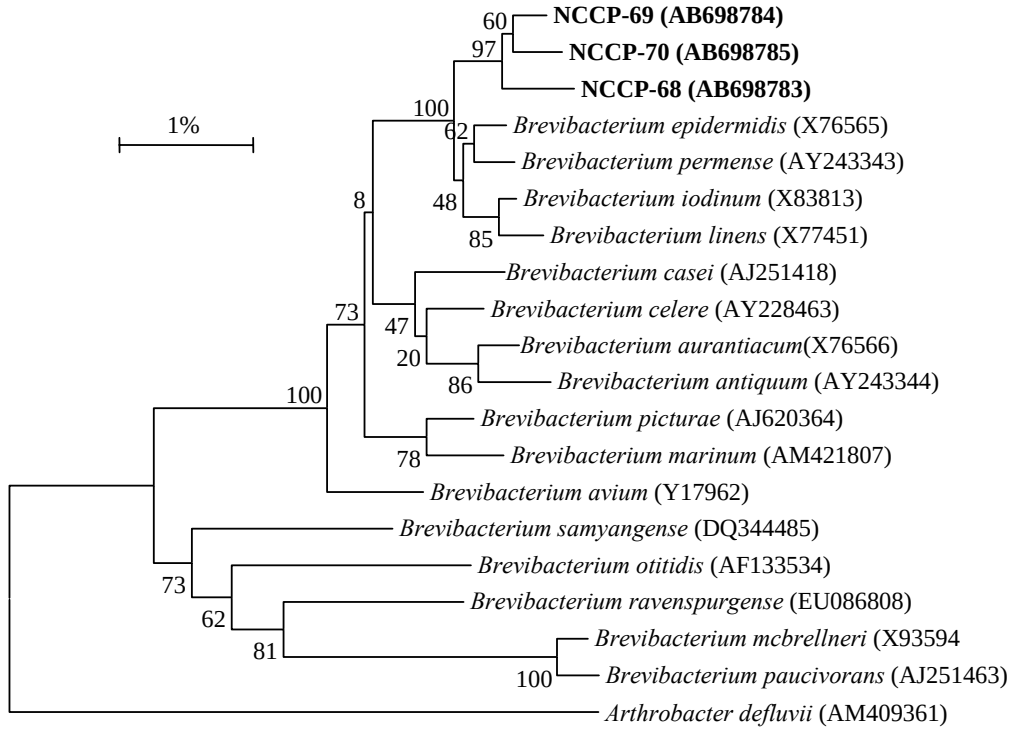

Fig. 2b. Phylogenetic tree showing the interrelationships of isolated halotolerant / halophilic strains belonging to genus *Brevibacterium* and the close relatives inferred from 16SrRNA gene sequence.

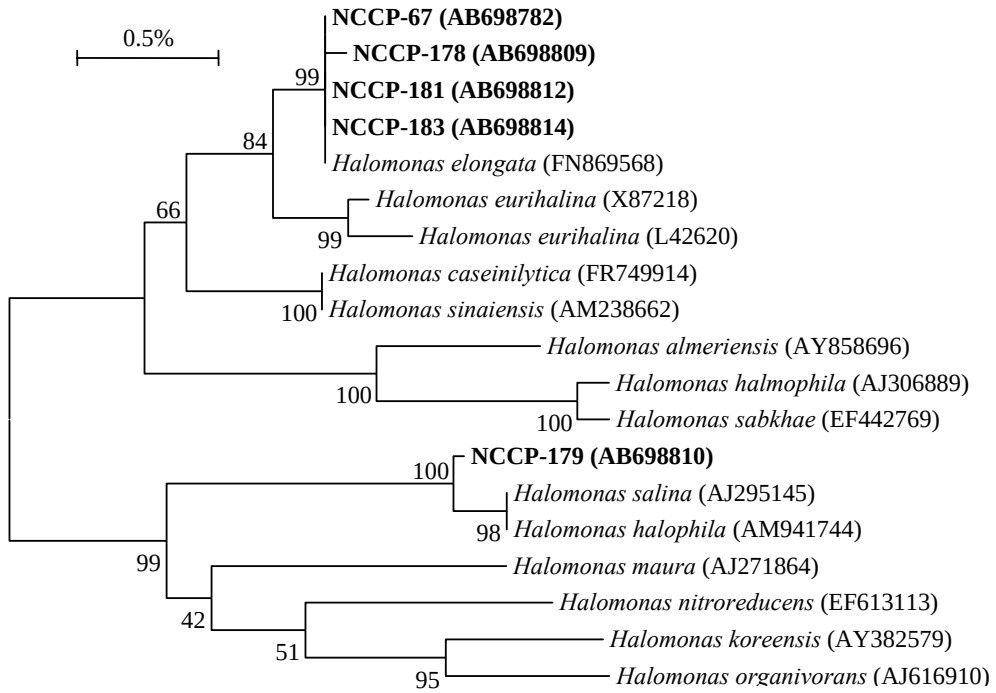

Fig. 2c. Phylogenetic tree showing the interrelationships of isolated halotolerant / halophilic strains belonging to genus *Halomonas* and the close relatives inferred from 16SrRNA gene sequence.

The growth of extremely halophilic bacteria requires relatively high NaCl and the majority of them require magnesium ion (Mg^{2+}) for their growth whereas slightly- and moderately-halophilic bacteria do not require magnesium ion for growth (Grant *et al*., 2001). Halophilic bacteria grew better at the temperature of 28–37ºC and at pH 7.0–8.0 on medium supplemented with 5–20% NaCl concentration. Our results agreed with the study of Hongyu, *et al*., (2009) who isolated the halophilic microorganisms from salt ponds of China and observed the growth of these microorganisms at the temperature of 35–40ºC and at pH 7.0–8.0 with 20% (w/v) NaCl.

Fritze (2002) recommended that phenotypic characterization results should not be directly compared without full background knowledge of the precise conditions used for a particular test. This can be particularly true for the group of Gram-positive endospore-forming bacteria that were formerly classified as the genus *Bacillus* but have now been reclassified based upon phylogenetic diversity into 6 RNA groups and separate lineages (Stackebrandt & Swiderski, 2002). Therefore, we also used 16S rDNA sequence analysis to ensure the accurate taxonomic position of the halotolerant / halophilic strains reported in this study. On the basis of the phenotypic characteristics and the comparison of partial 16S rRNA gene sequences, the isolates were identified as members of the genera: *Thallosobacillus, Halobacillus, Brevibacterium, Bacillus* and *Terribacillus* (Table 2). Molecular phylogeny increasingly supports our understanding to the organism relationships and provides the basis for the conventional identification techniques (Singh *et al.,* 2007).Comparative sequence analysis of 16S rRNA is currently the most widely used approach for the reconstruction of microbial phylogeny (Dewhirst *et al.*, 2005). Results of this study are in accordance with those of Rohban *et al*., (2009), who isolated bacterial strains belonging to the *genera Salicola, Halovibrio, Halomonas, Bacillus, Oceanobacillus, Thalasobacillus, Virgibacillus, Gracilibacillus, Halobacillus, Piscibacillus* and *Salinicoccus* from Saltan lake of Iran.

This is the first study on investigating the bacterial diversity of the Karak salt mines of Pakistan. Culturing microbes and molecular analysis give us an edge to have more cultured microorganisms with their taxonomy from the extreme environments. The microbial diversity can prove to be a valuable future resource in various industrial and biotechnological processes. Such microbes can also be used as a source of gene(s) that can increase salt tolerance in different crop species through genetic transformation.

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