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SCREENING OF ALKALIPHILIC, HALOALKALIPHILIC BACTERIA AND ALKALITHERMOPHILIC ACTINOMYCETES ISOLATED FROM ALKALINE SODA LAKE OF LONAR, INDIA FOR ANTIMICROBIAL ACTIVITY

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ABSTRACT

Twelve moderately haloalkaliphilic bacteria were isolated from sediment and water sample collected from the alkaline Soda Lake of Lonar, India using Lonar Lake water as enrichment medium. All the isolates were identified based on biochemical characterization, 16S rRNA sequencing and phylogenetic analysis. Isolation of haloalkaliphilic *Marinobacter excellens, Alkalimonas delamerensis, Roseinatronobacter monicus* and *Rhodobaca bogoriensis* from Lonar Lake appears to be the first report. Three alkalithermophilic actinomycetes isolated from sediment sample of Lonar Lake using glucose yeast-extract peptone medium were identified as *Laceyella sacchari* belonging to family *Thermoactinomycetaceae*. All the 12 bacterial isolates along with 66 previously isolated and identified isolates from Lonar Lake (total 78 isolates) and three isolates of alkalithermophilic actinomycetes were screened for their antimicrobial activity. The alkaliphilic bacteria isolated from Lonar Lake exhibited inhibitory effect against clinical isolates of pathogenic bacteria and phytopathogenic fungi which indicates the possibility of using alkaliphilic bacteria as biological control agents.

KEYWORDS: Lonar Lake, Moderately haloalkaliphilic bacteria, Phylogenetic analysis, Alkalithermophilic actinomycete, Antimicrobial activity

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INTRODUCTION

To survive in the environment and compete with other microorganisms for resources, many bacteria produce antimicrobial compounds to inhibit or kill other competing strains, including human and animal pathogens[1]. Search for new antimicrobial compounds is important because use or misuse of antibiotics has resulted in the development of antibiotic resistance by the infecting organisms, similar to the development of pesticide resistance in insects. Increased public concern about the development of resistance among pathogens against conventional pesticides and the accumulation of pesticide residues in the biosphere has led scientists towards the development of alternative strategies for plant disease suppression[2]. Alkaliphiles are the microorganisms that grow optimally at pH values above 9, often between 10 and 12, but cannot grow or grow only slowly at the near neutral pH[3]. Habitats of alkaliphiles are alkaline environments like paper and pulp production, alkaline hot springs and soda lakes and soda deserts. Alkaliphilic members of the genus *Bacillus* were majority in number[4]. Haloalkaliphiles are bacteria that thrive in both saline and alkaline environments such as soda lakes including Lake Magadi, Mono Lake and Soap Lake[5]. Systematic studies of different soda lakes have shown that the microorganisms are adapted to the extreme condition of pH and salt, many of which are alkaliphilic and halophilic or extremely halotolerant and many represent separate alkaliphilic lineages within recognized taxa[6]. *Halomonas campaniensis* sp. nov., a haloalkaliphilic bacterium was isolated from a mineral pool of Campania Region, Italy[7]. *Halomonas campisalis*, sp. nov., a denitrifying moderately haloalkaliphilic bacterium was isolated from the salt plain of Alkali Lake in Washington State[8]. To date, most published research on haloalkaliphiles is focused on microbiological classification and genetic characterization, with limited work to discover their biotechnological potential[9].

Alkalithermophiles are an exciting subset of extremophilic organisms and represent extremophiles that are adapted to two extreme conditions, alkaline and thermo biotic growth conditions. Alkalithermophiles occur in alkaline hot springs, the new alkaline hydrothermal vents of the 'Lost City' or alkaline lakes like Lake Bogoria (Africa) containing hot springs[10]. *Cladialkalibacillus thermarum* gen. nov. sp. nov., a novel alkalithermophilic bacterium was isolated from a hot spring in China[11]. Alkalithermophilic actinomycetes in subtropical area of Argentina were reported by Carrillo et al. (2009)[12]. The soda lake of Lonar is a unique basaltic rock meteorite impact crater which is filled with saline water. The uniqueness of the lake water is its salinity and high alkalinity. Cultivable bacterial diversity of Lonar Lake was studied using phenotypic characterization and 16S rDNA based phylogenetic analyses by Joshi *et al.*[13] wherein six different nutrient rich enrichment media were used. However, isolation of haloalkaliphilic microorganisms using lake water itself as a nutrient medium and isolation of actinomycetes was not attempted. In the present studies, isolation, characterization and identification of moderately haloalkaliphilic bacteria and alkalithermophilic actinomycetes and their screening for antimicrobial activity has been described.

MATERIALS AND METHODS

(i) Sample collection -

Sampling site was located at Lonar Lake in Buldhana district of Maharashtra state, India. The sediment and water samples were collected from Lonar Lake and the pH and temperature was noted immediately at the site.

(ii) Media composition

Lonar Lake Water Medium (LLWM-A) (g L^{-1}): Lonar Lake water 1L; Agar 20.0; pH 9.8 (adjusted with 10N NaOH). Lonar Lake Water

Medium with peptone and yeast extract (LLWM-B) (g L^{-1}): Lonar Lake water 1L; Peptone 0.5; yeast extract 0.5; agar 20.0; pH 9.8 (adjusted with 10N NaOH).
Glucose Yeast-extract Peptone (GYP) Medium (g L^{-1}): Glucose 10.0; Yeast extract 5.0; Peptone 5.0; NaCl 5.0; $CaCl_2$ 0.2; Agar 15.0; pH 9.2 (adjusted with 10N NaOH).

(iii) Enrichment and Isolation

Enrichment of sediment and water sample was carried out using two enrichment media namely LLWM-A and LLWM-B for bacteria. The flasks were incubated on the orbital shaker (120 rpm) at room temperature (28 ± 2°C) for 7 days. The enrichment of sediment sample was then tenfold diluted with sterile saline and 0.1ml of that and undiluted water sample (0.1ml) were spread on respective media agar plates and incubated at room temperature (28 ± 2°C) for 7 days. Well isolated and differentiated colonies from these enrichment media were transferred on the respective media slants and cultures maintained as glycerol stocks and lyophilized vials.
For alkalithermophiles, enrichment was set up using 50 g of sediment sample in GYP broth (pH 9) prepared in filtered Lonar Lake water and incubated at 55ºC. 0.1 ml of enriched sample was spread on respective medium after 5 days of incubation at 55ºC. Chalky white colonies were observed and maintained on GYP agar plate at 4ºC.

(iv) Identification of isolates

Isolated bacterial strains were studied for their phenotypic and biochemical characterization according to *Bergey's Manual of Systematic Bacteriology* Volume I (1984)[14]. Isolated actinomycetes were studied morphologically and biochemically according to *Bergey's Manual of Systematic Bacteriology* Volume IV (1989)[15]. Identification of the isolates was confirmed by 16S rRNA sequencing. Procedure for DNA isolation and sequencing of all the isolated strains was carried out as per Joshi *et al.*[13]. All the sequences were compared with 16S rRNA sequences available in the Gene Bank databases by BLASTn search[16]. Multiple sequence alignments were performed using CLUSTALW version 1.8[17]. A phylogenetic tree was constructed from evolutionary distances using the neighbor-joining method of MEGA 4 program package[18]. The 16S rRNA sequences of the isolated bacteria and actinomycetes were submitted to NCBI Gene Bank Database and all the cultures were deposited in MCM culture collection with MCM B numbers.

(v) Screening of alkaliphilic bacteria for antimicrobial activity

Twelve haloalkaliphilic isolates obtained under present investigation and sixty six alkaliphilic bacteria previously obtained from water and sediment samples from Lonar Lake[13] were employed for study.

Indicator pathogenic microorganisms used

Five multi-drug resistant (MDR) human pathogenic bacterial strains namely *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi* and *Klebsiella pneumoniae* were procured from K. E. M Hospital, Pune, India and five phytopathogenic fungal cultures namely *Fusarium oxysporum* (NFCCI 745), *Fusarium moniliforme* (NFCCI 1256), *Rhizoctonia solani* (NFCCI 611), *Aspergillus parasiticus* (NFCCI 1325) and *Colletotrichum gloeosporioides* (NFCCI 1280) were obtained from National Fungal Culture Collection of India (NFCCI-WDCM-932), ARI, Pune, India.

Media used

Mueller Hinton medium (MH) was used as growth medium for indicator bacteria (g L^{-1}): casein acid hydrolysate 17.5; beef heart infusion 2.0; starch soluble 1.5; agar 17.0; pH 7.3. Potato dextrose agar (PDA) was used as growth medium for fungi (g L^{-1}): potato infusion 200.0; dextrose 20.0; agar 15.0; pH 5.6.

Primary screening of alkaliphilic bacteria for antimicrobial activity

Following media were used for growing alkaliphilic bacteria for primary screening Horikoshi-I (g L^{-1}): soluble starch 10.0, peptone 5.0, yeast extract 5.0, KH_2PO_4 1.0, $MgSO_4.7H_2O$ 0.2, Na_2CO_3 10.0, pH 10, Agar

20.0. Nutrient broth of pH 10 (NA-10) (g L^{-1}): Peptic digest of animal tissue 5.0, yeast extract 1.5, beef extract 1.5, sodium chloride 5.0, Agar 20.0 (pH adjusted with 10N NaOH). Nutrient broth of pH 10 with 30 g L^{-1} sodium chloride (NA-NaCl), Agar 20.0 (pH adjusted with 10N NaOH). Davis Mingioli's synthetic medium with pH 10 and 5 g L^{-1} peptone (DMP) (g L^{-1}): K_2HPO_4 7.0, KH_2PO_4 3.0, $MgSO_4.7H_2O$ 0.1, peptone 1.0, trace element solution 5 ml, Agar 20.0 (pH adjusted with 10 N NaOH). Tindall's medium (g L^{-1}): Potassium chloride 1.0, sodium glutamate 1.0, NH_4Cl 1.0, KH_2PO_4 1.0, yeast extract 5.0, casein hydrolysate 5.0, $FeCl_2.4H_2O$ 0.036, $MnCl_2.4H_2O$ 0.36, NaCl 20.0, Na_2CO_3 5.0, PH 10.0, Agar 20.0. Lonar Lake Water Medium without peptone and yeast extract (LLWM-A) (g L^{-1}): Lonar Lake water 1.0 L, pH 9.8, Agar 20.0. Lonar Lake Water Medium with 0.5 g L^{-1} peptone and yeast extract (LLWM-B) (g L^{-1}): Lonar Lake water 1.0 L, peptone 0.5, yeast extract 0.5, pH 9.8, Agar 20.0.

Alkaliphilic isolates were spot inoculated on petriplates containing 20 ml growth medium agar and incubated for 48 h at 37°C. The spot grown cultures were overlaid with a 10 ml of Mueller Hinton agar medium containing 1.7% agar and 10^6 CFU/ml of pathogenic bacteria test cultures. Plates were incubated for two days at 37°C. Antibacterial activity was assessed by observing the zone of clearance around the growth of alkaliphilic bacteria[19]. Antibacterial antibiotics, Streptomycin (10 mcg) and Tetracycline (30 mcg) (Hi-Media, India) were used as positive controls.

To study the spectrum of antifungal activity, agar plugs (5 mm diameter) were picked up from actively growing regions of phytopathogenic fungal cultures and placed at the centre of PDA plates. A loopful cell suspension of alkaliphilic isolates was streaked 2 cm away from the fungal disc and the plates were incubated at 30°C for 7 days. The plates were observed for the zone of inhibition around the fungal colony. Antifungal antibiotic, Nystatin (100 Units) (Hi-Media, India) was used as a positive control.

Antimicrobial activity of culture supernatants in secondary screening

Preparation of the fungal spore's suspension

The indicator phytopathogenic fungi were grown in PDA until growth reached the edge of the Petri dishes. 0.85% sterile saline solution was poured onto fungal growth and spores were loosened using a sterile swab by gently scraping the colony surface. A micropipette was used to remove the spore suspension, density of which was adjusted to 10^6 – 10^7 spores/ml concentration[20].Twenty five alkaliphilic bacteria were selected and screened for their antimicrobial activity using different production media namely Horikoshi-I, Nutrient broth of pH 10 (NA-10), Nutrient broth of pH 10 with 30 g L^{-1} sodium chloride (NA-NaCl), Davis Mingioli's synthetic medium with pH 10 and 5 g L^{-1} peptone (DMP), Tindall's medium, Lonar Lake Water Medium without peptone and yeast extract (LLWM-A) and Lonar Lake Water Medium with 0.5 g L^{-1} peptone and yeast extract (LLWM-B) as described above omitting agar from the medium. After growing these cultures under shaking conditions (150 rev min^{-1}) for different periods (24, 48, 72 and 96 h) at 37°C, culture broths were centrifuged at 16,770 x g for 30 min followed by membrane filter (0.22 µm pore size) sterilization. The filtrate was then concentrated tenfold by lyophilization. About 50 µl of concentrated filtrate was added to wells (6 mm diameter) in Mueller Hinton agar plates and PDA plates containing 10^6 bacterial cells ml^{-1} and 10^6 spores ml^{-1}, respectively and all plates were incubated at an appropriate temperature. The inhibition of growth is expressed as the diameter of the zone of inhibition around the well. The uninoculated medium was used as a negative control. All tests were carried out in triplicate. Diameters of inhibition zones were scored for test organisms as follows: (-) no inhibition, (+) weak inhibition of the tested strain around the spot or well (clear zones of inhibition <7 mm), (++) moderate inhibition (clear zones between 7 and 15 mm) and (+++) strong inhibition of the tested strain around the spot or well (large and clear zones >15 mm). The bacteria showing zone of inhibition >15 mm against both bacterial and fungal pathogens were selected.

Solvent extraction of antimicrobial compounds

Different solvents (diethyl ether, acetone, toluene, ethanol, methanol, acetonitrile, *n*-propanol, *n*-butanol, ethyl acetate and chloroform) were used for extraction of antimicrobial compounds. Antimicrobial activity of the solvent extracts was determined by agar well diffusion assay as described earlier.

RESULTS

(i) Isolation of moderately haloalkaliphilic bacteria

Identification of the isolates and their optimum pH and salt tolerance are described in Table 1 and phylogenetic relatedness in Fig. 1. In this study, nine moderately haloalkaliphilic bacteria and three haloalkalitolerant bacteria were isolated. For confirmation of microorganisms belonging to moderately halophilic bacteria, the growth of bacteria at different salt concentrations was determined. The results showed that most of the strains could be cultured at the salt concentration ranging from 0-15% and the optimal growth condition was 5% salinity in terms of NaCl for all the strains. All the isolated strains were grown optimally at the pH ranging between 9 to 11. On the basis of these results the isolated bacteria could be classified as moderately haloalkaliphilic and haloalkalitolerant.

Table 1
Characteristics of Lonar Lake isolates and their phylogenetic affiliations

Isolate No.	MCM Numbers	Enrichment Medium	pH Tolerance	Optimum pH for growth	Salt Tolerance	Optimum Salt for growth	Nearest Phylogenetic neighbor and (%) similarity to nearest neighbor	Gene Bank Accession Number
ARI 401	MCM B-1046	LLMW (A)	7-10	8	5-15 %	5 %	*Alkalimonas delamerensis* (98%)	EF423727
ARI 402	-	LLMW (A)	7-11	8	0-10 %	5 %	*Bacillus* sp. Y (99%)	EF428124
ARI 403	MCM B-1047	LLMW (A)	7-12	11	0-5 %	5 %	*Alkalimonas delamerensis* (98%)	EF428125
ARI 404	MCM B-1048	LLMW (A)	7-12	9	0-15 %	NR	*Halomonas* sp. IB-559 (99%)	EF503591
ARI 406	MCM B-1049	LLMW (B)	7-12	9	5-15 %	5 %	*Marinobacter excellens* (98%)	EF428127
ARI 407	MCM B-1050	LLMW (B)	7-12	9	5-10 %	5 %	*Marinobacter alkaliphilus* (99%)	EF503593
ARI 408	MCM B-1051	LLMW (B)	7-11	9	5-15 %	5 %	*Roseinatronobacter monicus* (96%)	EF503594
ARI 409	MCM B-1052	LLMW (B)	7-11	9	5 %	5 %	*Rhodobacteraceae* bacterium D3-7bb (97%)	EF503595
ARI 410	MCM B-1053	LLMW (B)	7-12	10	5 %	5 %	*Rhodobaca bogoriensis* (97%)	EF428126
ARI 412	-	LLMW (B)	7-9	8	5-10 %	5 %	*Bacillus* sp. Y (99%)	EF423726
ARI 413	MCM B-1054	LLMW (B)	7-12	10	5-10 %	5 %	*Paracoccus koreensis* (99%)	EF503592
ARI 414	-	LLMW (B)	7-9	8	5-10 %	5 %	*Paracoccus* sp. DSG 13 (100%)	EF544994

(+) Positive, (-) Negative, (NR) Not required

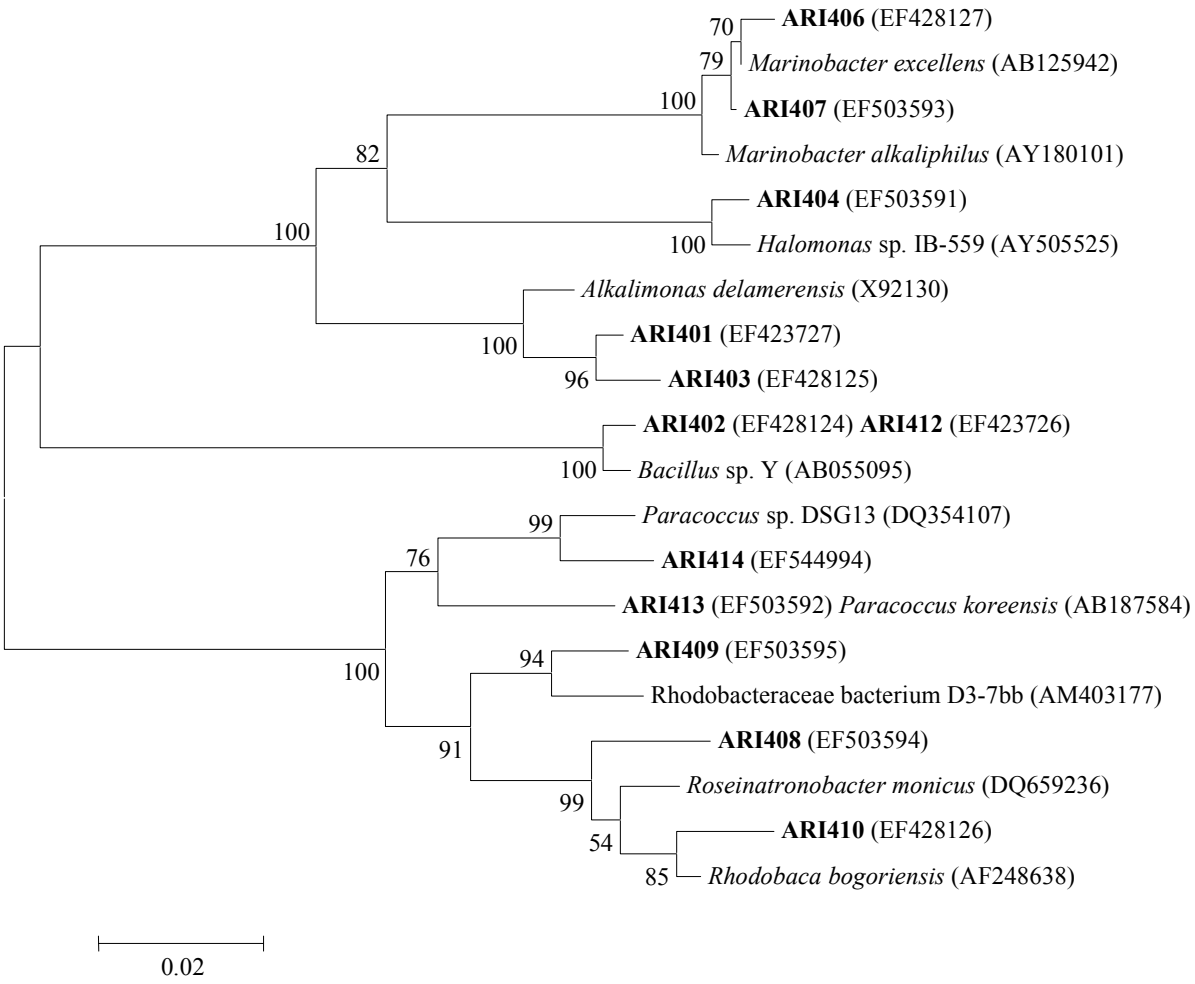


Figure 1

Unrooted Phylogenetic tree based on a comparison of the 16S ribosomal DNA sequences of Lonar Lake isolates and some closest phylogenetic relatives. The tree was created by the neighbor-joining method. The numbers on the tree indicates the percentages of bootstrap sampling derived from 1000 replications. Isolates characterized in the present study are indicated in bold. Bar inferred nucleotide substitutions per nucleotides

(ii) Isolation of alkalithermophilic actinomycetes

Morphological characteristics of all the actinomycetes strains were studied and the isolates were identified on the basis of biochemical characteristics as described earlier and further confirmed by 16S rRNA sequencing (Table 2). All the isolated strains grew optimally at pH 9 and 55°C temperature. This data suggested that the isolated actinomycetes could be classified as alkalithermophilic actinomycetes. Sessile spores (Fig. 2) and growth on 25 µm ml^{-1} Novobiocin were also the special characteristics of these cultures.

Table 2
Characteristics of alkalithermophilic actinomycetes and their phylogenetic affiliations

Biochemical Tests		Alkalithermophilic actinomycetes		
		MCM B-381	MCM B-382	MCM B-383
Soluble pigment (Pink-red)		+	+	+
Growth at 30°C		-	-	-
55°C		+	+	+
Spores sessile		+	+	+
Growth on 25 µm ml^{-1} Novobiocin		+	+	+
Growth on 1% NaCl (W/V)		+	+	+
Melanin production on CYC agar with 0.5% L-Tyrosine (W/V)		+	+	+
Growth in presence of Lysozyme		+	+	+
Degradation of	Casein	+	+	+
	Chitin	-	-	-
	Esculin	-	-	-
	Gelatin	+	-	+
	Starch	+	+	-
	Tyrosine	-	-	-
	Cellulose	-	-	-
	Xanthine	-	-	-
	Hypoxanthine	-	-	-
Utilization as 'C' source	Fructose	+	+	+
	Mannitol	+	+	+
	Sucrose	+	+	+
	Trehalose	+	+	+
	Glucose	+	+	+
	Arabinose	+	+	+
	Lactose	+	+	+
	Xylose	+	+	+
	Maltose	+	+	+
	Galactose	-	+	+
	Glycerol	-	+	+
Sensitivity to	Ampicillin	+	+	+
	Chloramphenicol	+	+	+
	Streptomycin	+	+	-
	Gentamycin	+	-	-
	Kanamycin	+	-	+
	Neomycin	-	-	-
	Nalidixic acid	+	+	-
Nearest phylogenetic neighbor and (%) similarity to nearest neighbor		*Laceyella sachhari* (100%)	*Laceyella sachhari* (100%)	*Laceyella sachhari* (100%)

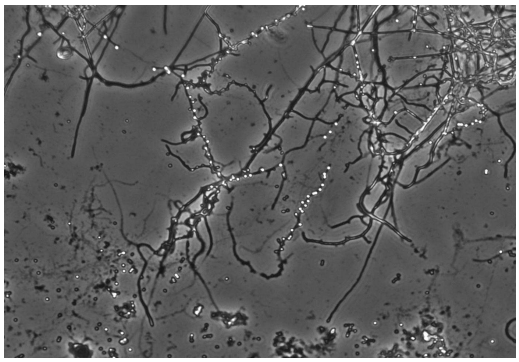


Figure 2
Inclined cover slip preparation of growth of Laceyella sacchari (isolate No. 381) GYP agar, incubation 55°C, showing single sessile spores under phase contrast microscope, total magnification x 400

All the three strains of alkalithermophilic actinomycetes exhibited 100% similarity with *Laceyella sacchari* which is previously described as *Thermoactinomyces sacchari* (Fig. 3).

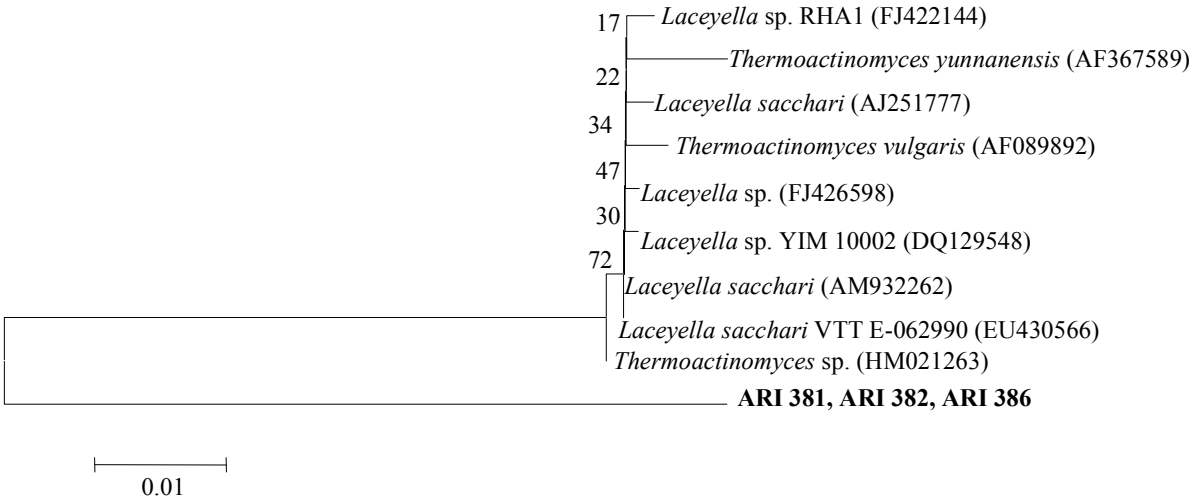
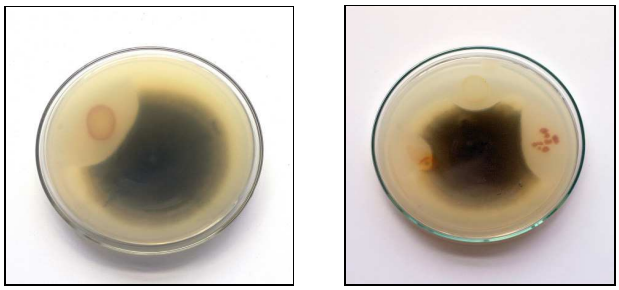


Figure 3
Unrooted phylogenetic tree based on a comparison of the 16S ribosomal DNA sequences of alkalithermophilic actinomycetes isolated from Lonar Lake and some closest phylogenetic relatives. The tree was created by the neighbor-joining method. The numbers on the tree indicates the percentages of bootstrap sampling derived from 1000 replications. Isolates characterized in the present study are indicated in bold. Bar inferred nucleotide substitutions per nucleotides.

(iii) Antimicrobial activity of alkaliphilic isolates

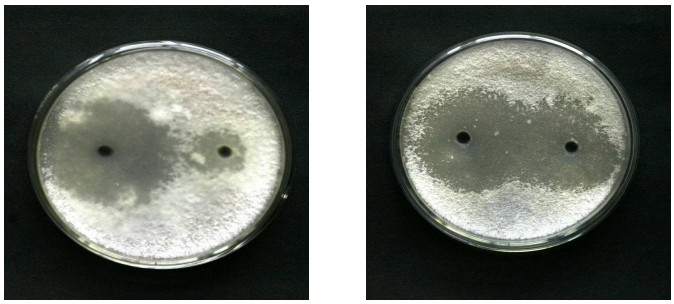
In the primary screening, out of 78 isolates, 25 exhibited antibiosis, inhibiting at least one of the indicator bacteria (Table 3) (Fig. 4). In secondary screening, three isolates namely *Paenibacillus* sp. L55 (MCM B-1034), *Halomonas campisalis* (MCM B-1027) and *Planococcus maritimus* (MCM B-1024) (Fig. 5) stood out inhibitors of at least nine of the ten indicator strains and hence selected for further studies. The growth of *Fusarium oxysporum*

and *Fusarium moniliforme* was strongly inhibited by 8 alkaliphilic bacteria, showing the average inhibition zones >19 mm. The zone of inhibition of standard antifungal compound Nystatin was 15 mm thus indicating higher antifungal activity of the alkaliphilic bacteria.



(A) **(B)**

Figure 4
Zone of inhibition of Rhizoctonia solani by (A) Bacillus subtilis and (B) Paenibacillus sp.L55



(A) **(B)**

Figure 5
Zone of inhibition of Fusarium oxysporum around the well produced by culture filtrate of (A) Halomonas campisalis and (B) Planococcus maritimus after 24 and 48 h

Antimicrobial compounds produced by *Paenibacillus* sp. L55, *Bacillus flexus, Alkalibacillus haloalkaliphilus* and *Planococcus maritimus* were extracted in *n*-butanol while *Halomonas campisalis* and *Alkalimonas delamerensis* were extracted using diethyl ether. These solvent extracts also exhibited antimicrobial activity against indicator pathogenic strains (Table 4).

Table 4
Antimicrobial activity of solvent extracts of selected alkaliphilic isolates towards test organisms

Alkaliphilic isolates	Antimicrobial activity of cell free culture extracted with solvents					
	Diethyl ether	Toluene	*n*-Propanol	*n*-Butanol	Ethyl acetate	Chloroform
Paenibacillus sp. *L55* (MCM B-1034)	+	-	+	+	-	-
Bacillus flexus (MCM B-1036)	-	-	-	+	+	-
Alkalibacillus haloalkaliphilus (MCM B-1041)	-	-	-	-	-	+
Halomonas campisalis (MCM B-1027)	+	-	-	-	-	-
Alkalimonas delamerensis (MCM B-1047)	-	-	-	-	-	-
Planococcus maritimus (MCM B-1015)	-	+	-	-	-	-
Planococcus maritimus (MCM B-1029)	-	+	-	+	-	-

(+) Positive antimicrobial activity (-) No antimicrobial activity

Table 3
Screening of alkaliphilic bacteria for antimicrobial activity

Alkaliphilic Isolates	MCM B-No.	Growth medium	Clinical Pathogens (K.E. M. Hospital, Pune)					Phytopathogenic Fungi (NFCCI-ARI, Pune)				
			A	B	C	D	E	*a*	*B*	*c*	*d*	*e*
Water Isolates												
Bacillus licheniformis	1010	DMP	+	+	+	+	-	+	++	+	-	+
Bacillus subtilis	1012	NA-NaCl	+	+	-	+	+	++	+++	+++	+	-
Bacillus fusiformis	1020	NA-10	+	-	+	+	-	-	++	+ +	-	-
Bacillus flexus	1036	Horikoshi-I	+	+	-	+	+	++	+++	++	++	-
Halomonas sp. M3-10C	1013	NA-NaCl	-	+	+	+	+	+	-	+	-	-
Exiguobacterium aurantiacum	1021	NA-10	-	-	-	-	-	-	-	-	-	+
Alkalimonas delamerensis	1047	LLWM-A	-	-	+	+	-	++	+++	++	-	+
Halomonas sp. IB559	1048	LLWM-A	-	+	-	-	+	-	-	-	-	-
Bacillus cereus	1033	NA-10	-	+	+	+	+	-	+	+	-	-
Uncultured bacterium clone	1028	NA-10	+	-	+	+	-	-	+++	+++	-	+
Sediment Isolates												
Bacillus licheniformis	ARI 30	NA-10	-	+	+	+	+	-	+	++	-	-
Paenibacillus sp. L55	1034	Horikoshi-I	+	+	++	++	+	++	+++ +	+++	-	+
Alkalibacillus haloalkaliphilus	1041	Tindall's	+	-	+	+	+	-	-	-	-	-
Bacillus sp.	1042	Tindall's	+	+	+	+	-	++	++	+	-	+
Alcaligenes sp. A72	1010	DMP	+	-	+	+	+	-	+	-	-	-
Arthrobacter sp. CW1	1006	NA-10	+	+	-	+	+	+	+	-	-	-
Alcaligenes faecalis	1011	NA-10	+	-	+	-	+	-	+	++	-	-
Planococcus maritimus	1015	NA-10	+	-	+	+	+	++	++	++	-	-
Cellulosimicrobium cellulans	1043	DMP	+	-	+	+	+	-	-	-	-	+

Halomonas campisalis	1027	NA-NaCl		+	+				+++				
Planococcus maritimus	1024	NA-NaCl	++	+	+	+	+	+++	+++	++	-	+++	
Marinobacter excellens	1049	LLWM-B	-	-	-	+	+	-	-	-	-	-	
Roseinatronobacter monicus	1051	LLWM-B	+	-	+	+	-	-	+	+	-	-	
Rhodobacteraceae bacterium	1052	LLWM-B	-	-	+	+	-	+	+++	+++	-	-	
Rhodobaca bogoriensis	1053	LLWM-B	-	+	+	-	+	-	+	-	-	+	

A- ***Staphylococcus aureus, B- Pseudomonas aeruginosa, C- Salmonella typhi, D- Escherichia coli, E- Klebsiella pneumoniae***
a- Rhizoctonia solani, b- Fusarium oxysporum, c- Fusarium moniliforme, d- Colletotrichum gloeosporioides, e- Aspergillus parasiticus
(-) no inhibition; (+) inhibition zones from 7 to11 mm; (++) from 12 to 18 mm; (+++) >19 mm
Zone of inhibition of standard antibiotics: Streptomycin 12 mm, Nystatin 15 mm.

DISCUSSION

The present study revealed that most of the bacteria isolated from Lonar Lake are alkaliphilic as the pH of the lake water is alkaline but the presence of moderately halophilic bacterial isolates is very interesting as the salinity of lake water is now lowered down to 0.5-1% NaCl. It could be hypothesized that these physiological groups of microorganisms might be present in lake from years ago, which got adapted to the extreme environment. The alkaliphilic nature of Lonar Lake isolates with their broad pH range for growth may be an important physiological feature for survival under fluctuating pH conditions in the soda lake environment. In this study, we have used selective media to isolate haloalkaliphilic bacteria and a total of 12 moderately haloalkaliphilic bacterial strains were isolated from alkaline Lonar Lake. In our earlier report on cultivable bacterial diversity of Lonar Lake[13], the nutrient rich media supported the abundant growth of Firmicutes belonging to the low G+C group. Use of Lonar Lake water as medium has supported growth of haloalkaliphilic bacteria. It is interesting to note that the genera *Alkalimonas, Marinobacter, Roseinatronobacter*, Rhodobacteraceae bacterium and *Rhodobaca* could be obtained only when the Lonar lake water was used as the nutrient medium. There is no report on the presence of this physiological group of alkaliphilic and moderately halophilic bacteria from Lonar Lake. Thus, occurrence of *Marinobacter excellens, Alkalimonas delamerensis* and *Roseinatronobacter monicus* in the Lonar Lake seems to be the first report. Isolation of *Rhodobaca bogoriensis* from Lonar Lake is a new finding. Alkalithermophiles represent an exciting group of extremophilic microorganisms which contains representatives of both bacteria and archaea. The review by Bowers *et al.*[21] focuses on the correlation between the extent of alkaline pH and elevated temperature optima and the extent of salt tolerance of extremely halophilic eubacteria. In the present study, we isolated three alkalithermophilic actinomycetes (ARI 381, ARI 382 and ARI 386) which require pH 9 and 55°C temperature for their optimum growth and tolerate 1% NaCl. This is the first report of halotolerant, alkaliphilic as well as thermophilic actinomycetes isolated from Lonar Lake. Extremophiles are being looked upon as a source of active biomolecules and secondary metabolites. Total 78 alkaliphilic bacterial isolates obtained from sediment and water samples of Lonar Lake were studied for their antagonistic activity. An antimicrobial and cytotoxic activity of moderately halophilic bacteria isolated from the Weihai Solar Saltern (China) was investigated[22]. Microbial resources from East China Sea were studied for antibacterial, cytotoxic and antioxidation activities[23]. Some studies have shown that alkaliphilic bacteria produce substances with antibacterial and antifungal activities[3, 24] and the alkaliphilic actinomycetes produced some potent antibiotics[25]. Alkaliphilic *Streptomyces* and cyanobacteria from Lonar Lake were reported for their antimicrobial activity[26, 27]. Actinomycetes isolated from rhizosphere soil of medicinal plants have been demonstrated to produce antimicrobial compounds[28].

Lonar Lake harbors a wealth of diverse microorganisms with useful commercial properties[13]. In our study, alkaliphilic isolates from Lonar Lake belonging to genera *Bacillus, Paenibacillus, Alkalibacillus*, *Halomonas, Alkalimonas, Planococcus, Alcaligenes, Arthrobacter, Exiguobacterium, Cellulosimicrobium, Marinobacter, Roseinatronobacter* and *Rhodobaca* exhibited antibiosis against indicator strains and showed biologically active metabolites, confirming microbial strains as a prolific source for active compounds. Moreover, the great taxonomical variety of the producer microorganisms demonstrates that the production of active secondary metabolites is not restricted to particular genera or species[2]. Antimicrobial activity of 11 seaweed species from the coast of Urla, Turkey was studied by extracting the compounds in solvents like methanol,

acetone, diethyl ether and ethanol. The solvent diethyl ether was found to extract the antimicrobial compounds from most of the species followed by ethanol[29]. In the present investigation also, diethyl ether was found to be suitable for extraction of antimicrobial compounds followed by n-butanol. In conclusion, it is interesting to note that the rare bacteria like *Marinobacter, Alkalimonas, Rhodobaca,* and *Roseinatronobacter* could be isolated from Lonar Lake using Lonar Lake Water Medium as enrichment medium. Halotolerant alkalithermophilic actinomycetes showed polyextremophilic nature of the organism[21] which is reported for the first time from Lonar Lake. Our study also provides primary evidence of the antimicrobial activity of alkaliphilic bacteria isolated from Lonar Lake. Secondary metabolites produced by these species showed promising antifungal activity compared to standard antifungal antibiotics against phytopathogenic fungal cultures suggesting that their metabolites could be used as an alternative or supplementary method to chemical crop protection.

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