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SHORT COMMUNICATION

Bacterial Diversity of Lonar Soda Lake of India

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Abstract Total seventy four bacteria were isolated from Lonar soda lake of Maharashtra state, India. Eleven isolates were identified using morphological, biochemical and molecular analysis. The bacteria isolated belonged to phylum firmicutes and proteobacteria. Majorities (eight) were firmicutes and three were proteobacteria. For the first time we are reporting *Alcanivorax* spp. which is a genus well known for its oil degradation capacity, indicate the probable existence of oil reservoir in vicinity of Lonar lake. In addition all the eleven bacteria are potential producers of industrially important enzymes, pigments, antibiotics as well.

Keywords Lonar lake · Firmicutes · Proteobacteria and *Alcanivorax* spp

Introduction

Soda lakes are naturally occurring alkaline environments. The best studied soda lakes are those of the East African Rift Valley, where detailed limnological and microbiological investigations have been carried out over many years [1, 2]. In India the Lonar crater lake, popularly called as the Lonar soda lake is situated in the Buldhana district of the Maharashtra state. It is one of the three largest craters in the world and is the only crater which is formed due to high velocity meteoritic impact on basaltic rock, more than 50,000 years old [3]. Studies on microbial diversity of alkaline/saline environments are important for two reasons. First, some of the earliest microbial life on earth might have been haloalkaliphiles, thus research on microbial community in soda lakes may gives clues into the evolution on life earth [4]. Secondly, because of the presence of hypersaline conditions on Mars, terrestrial saline environments may act as good models for studies on life on Mars [5].

The ecology and diversity of an East African soda lake is studied for its biotechnological potential and it was found to contain prokaryotic groups of considerable phylogenetic diversity, including haloalkaliphilic archea [2]. Diversity of the Kenyan soda lake was assessed by using molecular techniques and lipase producing and starch degrading microorganisms are reported [6–8]. Microbial diversity assessment of Lonar and isolation of amylase, protease, chitinase and antibiotic producers are reported by various workers [9–11]. In the present study we attempted to isolate and characterize industrially important and novel haloalkaliphiles from Lonar soda lake.

Materials and Methods

Lonar lake (19°58′N and 76°31′E) is in the formerly volcanic Deccan trap geological region. Sediment (SD) and surface (SU) water samples were collected in the pre-monsoon season. Water samples were treated and analyzed for chemical and physical properties. Metals were analyzed using a Flame Photometer (CL-361 ELICO, India) [12]. The samples were inoculated into three different media such as, nutrient broth at pH 10.5 [**A**]; nutrient broth at pH 10.5 with 30 g/l sodium chloride [**B**] and Tindal's medium [**C**] and incubated at 30°C for 8 days on a shaking incubator at 200 rpm speed. After enrichment, bacteria were isolated on respective agar media and pure cultures were maintained.

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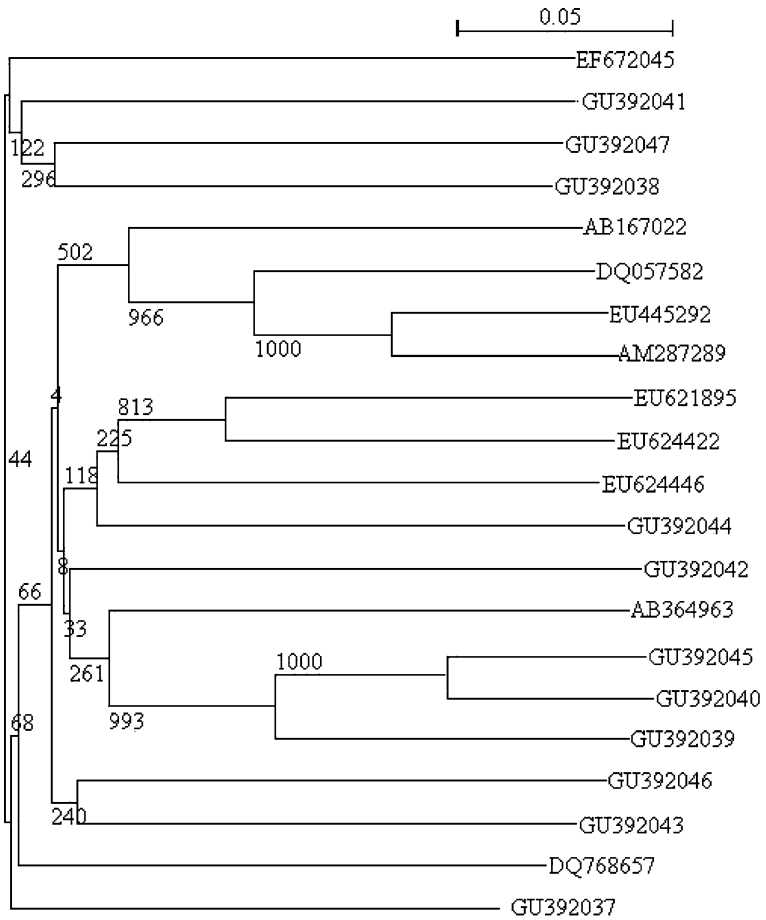

Fig. 1 Phylogenetic trees showing the relationship among 16S rDNA gene sequences from Lonar soda lake obtained in this study. The trees were constructed using the neighbour-joining tree. The values indicate the percentage of occurence in 1000 bootstrapped trees and the scale bar represents 0.05 nucleotide substitution

For DNA extraction isolates were suspended in an extraction buffer (10 mM Tris HCL, pH 8.0; 1 mM EDTA, pH 8.0). Proteinase K solution was added to a final concentration of 100 ug/ml and incubated at 55°C for 2 h with continuous shaking. 0.5 M NaCl was added and incubated at 72°C for 30 min. DNA was extracted by phenol–chloroform extraction. DNA was washed with 70% ethanol and dissolved in Tris–EDTA buffer (pH 8.0). Extracted DNA was analyzed by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining [13]. The amplification of 16S rDNA fragments were performed by using an Applied Biosystem thermocycler, model 9700 (Foster, California, USA) with 27f (5′ → CAGAGTTTGATCGT GGCTCAG ← 3′) and 1488R (5′CGGTTACCTTGT-TACGACTTCACC 3′) primer pair. The PCR reaction mixture contained 1.5 mM $MgCl_2$, 200 uM dNTP mixture and 0.3 μM of each primer and 1 U of Taq DNA polymerase with a reaction mixture supplied by the manufacturer in a total volume of 100 μl. Reaction mixture was first denatured at 94°C for 3 min, followed denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 1 min. Amplification was completed by a final extension step at 72°C for 7 min reaction was carried out for 30 cycles. PCR products were run on a 1% agarose gel. PCR

Table 1 Physico-chemical analysis of Lonar water samples

Components	Lake water (mg/lit)
Temperature	32
pH	10.5
Total solids	3950
Carbonate as CaCO3	2680
Alkalinity	4350
Total hardness as CaCO3	140
Chloride	3953
Calcium	12
Magnesium	27
Sulphate	192
Nitrate	<0.1
Phosphorus	5.3
Iron	0.05
Sodium	4120
Potassium	20
Sulphide	0.2
Chromium	0.036
DO	5
BOD	9
COD	16

products were purified by the PEG/NaCl method [14] and directly sequenced using Applied Biosystem model 3730 DNA analyzer (Foster, California, USA). The 16S rDNA sequences were initially analyzed using BLAST program (www.ncbi.nlm.nih.gov/blast/blast.cgi). Multiple sequence alignments of approximately 900 base pair sequences were performed using CLUSTALW program version 1.8 [15]. Phylogenetic tree was constructed using the neighbor joining method [16]. Tree files were generated by PHYLIP and viewed by TREE VIEW program. Bootstrap analysis (1000 replications) was also carried out. The 16S rDNA sequences from GenBank used in the phylogenetic analysis are shown in Fig 1. The 16S rDNA sequences determined in this study are deposited in the GenBank databases, under accession numbers, GU392037 to GU392047.

Result and Discussion

The pH of the surface and sediment lake water was 10.5 and the temperature was 32°C. The physical and chemical properties of lake water are given in Table 1. Total dissolved solids (TDS) recorded in present investigation was higher as compared to the very well studied African soda lake and Kenyan soda lake [2, 6]. Green cyanobacterial mass was observed indicating eutrophication. High phosphate content could be another reason for eutrophication.

Luxuriant growth was observed at pH 10.5. Total viable bacterial count was 2.2×10^3 cfu/ml for surface water and 2.3×10^4 cfu/ml for sediment water samples at optimum pH. Total viable count (TVC) of Lonar lake water is less. Medium **A**, **B** and **C** were used for the cultivation of isolates. Luxuriant growth of alkaliphilic bacteria was observed on medium **A**, while, medium **B** and **C** supported growth of haloalkaliphiles. The selected media were suitable for growth of firmicutes and proteobacteria. 74 isolated bacteria were selected based on morphological characteristics and pH tolerance and eleven were studied detail. Out of 11, seven were Gram positive rods, one Gram positive cocci and three Gram negative rods. Morphological and physiological analysis based identification of Lonar lake isolates is given in Table 2.

Approximately 900 base pairs of 16S rDNA fragments were amplified from 5′ terminus. Phylogenetic analysis of

Table 2 Morphological and physiological analysis based identification of Lonar lake isolates

Characters	KBDL1	KBDL2	KBDL3	KBDL4	KBDL5	KBDL6	KBDL7	KBDL8	KBDL9	KBDL10	KBDL11
Morphology	Cocci	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Gram nature	+	+	+	+	−	+	+	−	−	+	+
Motility	−	+	+	+	−	+	+	−	−	+	+
Oxidase	−	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+
pH range	8–10.5	8.5–10.5	8.0–10.0	8.0–10.0	9.0–11.0	9.0–11.0	8.5–10.5	9.0–11.0	9.0–11.0	8.5–10.5	
Salt range	3.0–15	0–5	0–8	0–8	3.0–10	5.0–15	0–5	3.0–10	3.0–7	0–5	3.0–15
Temperature range	20–45	20–50	20–50	20–60	15–40	15–40	20–50	15–40	15–40	20–50	20–50
Urease	−	−	−	−	−	+	−	−	−	−	−
Nitrate reduction	−	+	−	+	+	+	+	+	+	+	+
H2S production	ND	−	−	−	+	−	−	+	ND	−	−
Lipase	−	−	−	+	+	−	−	+	+	−	−
Protease	−	−	+	+	−	+	−	−	−	−	+
Cellulase	−	−	+	−	−	+	+	−	−	+	−
Hydrolysis of:											
Casein	−	−	+	+	−	−	+	−	−	+	+
Gelatin	+	−	+	+	−	+	+	−	−	+	+
Starch	−	+	+	+	−	+	+	−	−	+	+
Utilization of:											
Arabinose	−	+	−	−	−	−	+	−	−	−	+
Fructose	+	+	−	+	−	+	−	−	−	+	+
D-Glucose	+	+	+	+	−	+	+	−	−	+	+
D-Galactose	−	−	+	−	−	+	+	−	−	+	−
D-Mannose	−	ND	+	+	−	+	+	−	−	+	+
Lactose	+	+	−	+	−	+	+	−	−	+	−

+ positive, − negative, *ND* not detected

Table 3 Closest relatives of bacterial isolates from 16S rDNA library

Isolate	Water sample	Medium	Nearest neighbour	% similarity	Accession No.	Phylum or subphylum
KBDL1	SD	A	*Planococcus maritimus*	99	EU624446	Firmicute
KBDL2	SU	A	*Bacillus cohnii*	100	AM287289	Firmicute
KBDL3	SU	A	*Bacillus subtilis*	99	AB364963	Firmicute
KBDL4	SD	A	*Bacillus licheniformis*	99	EU445292	Firmicute
KBDL5	SD	A	*Alcanivorax sp.*	97	EU621895	γ-proteobacteria
KBDL6	SD	B	*Oceanobacillus iheyensis*	99	EU624422	Firmicute
KBDL7	SD	B	*Bacillus cohnii*	98	EF672045	Firmicute
KBDL8	SD	B	*Alcanivorax sp.*	96	DQ768657	γ-proteobacteria
KBDL9	SD	C	*Alcanivorax sp.*	95	AB167022	γ-proteobacteria
KBDL10	SU	C	*Bacillus subtilis*	98	DQ057582	Firmicute
KBDL11	SU	C	*Haloalkaliphilic bacterium*	98	EU604320	Firmicute

SD sediment water samples, *SU* surface water samples

these sequences revealed a range of identities to several groups of bacteria (Table 3). The clones fell into two major lineages of domains of bacteria; the firmicutes and Gamma proteobacteria. Eight sequences were placed into the firmicutes. Out of eight, five were closely related to the genus *Bacillus*, with 98–100% identity the isolates KBDL7 and KBDL2 showed 98 and 100% identity with *Bacillus cohnii*, respectively. KBDL 3 and KBDL 10 showed 99 and 98% identity to *Bacillus subtilis*, respectively. KBDL4 showed 99% identity with *Bacillus licheniformis*. All members of *Bacillus sp*. showed effective production of protease, amylase, lipase and cellulase enzymes. Being an inhabitant an alkaline environment, all enzymes secreted by these isolates have remarkable potential in various industries [17] (Table 3). KBDL1 and KBDL6 showed 99% identity to *Planococcus maritimus* and *Oceanobacillus ineyensis*, respectively. *Planococcus maritimus* is reported earlier from Lonar Lake [10]. Starch degrading *Planococcus* is reported from Yellow Sea in Korea [18]. This bacterium is reported to synthesize a red pigment with potent antioxidant activity [19]. *Oceanobacillus sp.* is reported to produce at least 29 proteolytic enzymes and antibiotics effective against *Staphylococcus aureus* [20]. KBDL11 showed 98% identity to the haloalkaliphilic bacteria, it was earlier reported from crude sea salt sample near Qingdao in Eastern China [21]. The isolates KBDL5, 8 and 9 were placed in the phylum gamma proteobacteria. Phylogenetic analysis of these three isolates affiliated it with the genus, *Alcanivorax* (95-97%). This is the first report of *Alcanivorax* from Lonar soda lake. *Alcanivorax borkumensis* is a dominant microorganism in oil polluted marine environments capable of hydrocarbon degradation [22]. *Alcanivorax sp*. is usually halophilic and use aliphatic hydrocarbons as carbon source. It is also found in association with marine dinoflagellates. *Alcanivorax sp.* is reported to produce a biosurfactant and biodegradation of n-alkylcycloalkanes and n-alkylbenzenes [23]. Remarkable number of *Alcanivorax spp* in surface and sediment samples and negligible oil contamination of lake encourage us to predict that there may be oil reservoirs in the vicinity of the lake. Further studies may reveal the significance of *Alcanivorax* in Lonar crater lake.

We have identified two different bacterial lineages. Most of our clones from Lonar crater lake were related to alkaliphilic or haloalkaliphilic bacteria from soda lakes e.g. Kenyan soda lake, East African Rift valley lakes, Inner Mongolian Baer soda lake and Mono lake. Our study describes not only the existence of bacterial diversity in Lonar soda lake, but also indicates industrial potential of cultures like *Planococcus* sp. (KBDL1), *Oceanobacillus* sp.(KBDL6) and *Alcanivorax* (KBDL5, 8 and 9) which could be used as efficient red pigment, antibiotic producers and hydrocarbon degraders. Industrially important alkaline enzyme protease, amylase, lipase etc., production was observed with *Bacillus* sp (KBDL2-4, 7 and 10). The data presented in this report therefore significantly advance the understanding of the microbial ecology of Lonar crater.

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