Int J Pharm Bio Sci 2015 Oct ; 6(4): (B) 279 - 290

Research Article **Life sciences**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

PHYLOGENETIC ANALYSIS OF BACILLI FROM HALOALKALINE LONAR SODA CRATER

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ABSTRACT

In the last decade, special attention has been given to the investigation of the microbial communities in soda lakes using traditional isolation methods and molecular biology techniques. Aerobic, haloalkaliphilic bacteria were isolated and characterized from sediment and water samples collected from Lonar Soda Lake. The uniqueness of the Lonar Lake water is its salinity and alkalinity. Culture dependent phenotypic characterization and 16S rRNA based phylogenetic analysis were applied. One hundred and fourteen bacterial strains were isolated using different enrichment media. Out of One hundred and fourteen, twenty nine bacilli strain were selected for 16S rRNA sequencing. The phylogenetic position indicated the Lonar lake bacterial strains were related to phylum Firmicutes and belongs to three genera Bacillus, Lysinibacillus, Oceanobacillus. Most of the isolates produced biotechnologically important amylase, lipase and protease enzymes at alkaline pH. *Bacillus cereus, Bacillus pseudofirmus* and *Bacillus flexus, Bacillus sp.* were found multienzyme producer. Lonar Lake harbors a wealth of diverse microorganisms with useful commercial properties. Thus, the culture-dependent approach used in the present study contributes to our understanding of Lonar lake bacilli diversity and provides useful information on many fascinating cultures in this extreme environment.

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INTRODUCTION

Soda lakes are a specific type of salt lake with high to extremely high carbonate alkalinity, a pH from 9 to 11, and a moderate to extremely high salinity. They are spread all over the world, but located, as most inland salt lakes, in arid and semi-arid areas where the evaporative climate favors accumulation of salts in local depressions. These equally extreme conditions make soda lakes a unique ecosystem. In the last decade, special attention has been given to the investigation of the microbial communities in soda lakes using traditional isolation methods and molecular biology techniques[1-6.] Alkaline saline habitats are constant inhabited by a wealth of microbial communities suitable to these ecosystems. Among the microorganisms, the bacteria play a major role as important and dominant inhabitants of alkaline saline and hypersaline environments. Moderately halophilic bacteria are defined as prokaryotes that grow optimally in media containing 3–15% NaCl. Studies have been carried out in variable distinctive feature of moderately halophilic alkaliphilic bacteria, such as their physiology and phylogenetic association[7]. There are obtain fact to allow that the extreme haloalkaliphilic bacteria have strong expedient for promising relevance as a sources of compatible solutes, fermented foods, enzymes, polymers and degradation of toxic compounds[8-11]. Alkaliphilic microorganisms, in particular *Bacillus* species, have attracted much curiosity because of their ability to synthesize extracellular enzymes and metabolite that are operative and established at high pH and NaCl[12-19.] The well studied soda lakes are those of the East African rift valley[20]. With to a greater range of investigation into alkaline halobiotic environments more, archaeal extremophiles were isolated and identified. Foti et al.[21] few species identified from hypersaline habitats via culture-independent approach and described species were defined as haloalkalitolerant. Very few reports were documenting the presence of haloalkaliphilic bacterial diversity from the Indian soda lake. We have applied this strategy to characterized diversity of biotechnological potential bacterial strains isolated from Lonar Lake. The aim of the present study was to get insight into the species composition of microbial communities in Lonar crater by cultivation dependent approach. In order to isolation and identify the organism, by using conventional bacterial classification methods based on the cultural, morphological, physiological, biochemical character, 16S rDNA phylogenetic diversity of bacterial strains from Lonar lake.

MATERIALS AND METHODS

Sampling site description

The alkaline Lonar Lake in Buldhana district of Maharashtra is unique ecosystem and wonder in the India (latitude 19º58', Longitude 76º36'). Lonar lake has a periphery of 1.7 km and is situated in hallow, 0.14 km below the ground level, with an amphitheater of practically vertical cliffs. Based on geological studies, it is postulated that the lake was created as a meteoritic impact crater near about 50-60 thousand years ago. It is the third largest crater in the world and the only known crater formed by meteoritic impact in basaltic rock. Lonar Lake is originated due to the meteor impact on basaltic rock is unique. The uniqueness of Lonar lake water is its alkalinity and salinity. The water is alkaline (pH 10–10.5); this high alkalinity is due to the high concentration of sodium carbonate. This high alkalinity due to Water enters the lake through rain, ground water seepage and springs situated in the cliffs at the edge of the Lake no any outlet were present the Lonar lake. Lonar lake water and sediment sample were collected in sterile bottles and polythene bags respectively, from defined sampling site[22-23].

Enrichment and isolation of microorganisms

Enrichment of water samples and sediment samples were carried out in various enrichment media. All flasks were incubated at 37°C on a rotary shaker (100 rpm) for 48h. After enrichment, the organisms were isolated on respective media agar plates and incubated at 37°C for 24h. Well isolated and morphologically distinct colonies from these plates were transferred on the respective medium slants and maintained as stocks.

Media Composition

Glucose, NaCl, Na_2CO_3 and trace element solutions were autoclaved separately and added before pouring the agar media

		The different media used in this study for enrichment and isolation of bacteria
Medium		Medium composition per litre [20,24]
A	:	glucose 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,
B	:	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, agar 20.0.
C	:	peptic digest of animal tissue 5.0, yeast extract 1.5, beef extract 1.5, sodium chloride 5.0, agar 20.0. pH adjusted to 10.0 with 1N NaOH solution.
D	:	peptic digest of animal tissue 5.0, yeast extract 1.5, beef extract 1.5, sodium chloride 35.0, agar 20.0. pH adjusted to 10.0 with 1N NaOH solution.
E	:	KH_2PO_4 7.0, K_2HPO_4 3.0, $MgSO_4.7H_2O$ 0.1, peptone 1.0, trace element solution ($FeSO_4.7H_2O$ 0.5, $ZnSO_4.7H_2O$ 0.5, $MnSO_4.3H_2O$ 0.5, H_2SO_4 0.1N 10 ml, pH adjusted to 10.0 with 1N NaOH solution.
F	:	Potassium chloride 1.0, sodium glutamate 1.0, NH_4Cl 1.0, KH_2PO_4 1, 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, agar 20.0.
G	:	Casein 10, yeast extract 4, Peptone 2, Agar 15
H	:	Casein 2, yeast extract 0.8, Peptone 0.1, Agar 15
I	:	Peptone 5.0, yeast extract 5.0, KH_2PO_4 1, $MgSO_4.7H_2O$ 0.2, Glucose 1, Agar 15
J	:	Peptone 5.0, yeast extract 5.0, KH_2PO_4 1, $MgSO_4.7H_2O$ 0.2, Xylose 1, Agar 15

Identification of the bacterial culture

Bacterial cultures were examined for their cultural, morphological character, and standard biochemical test were performed according to Bergey's Manual of systematic bacteriology.

Screening for enzymes

Utilization of various substrates which is an indication of the enzymes produced by an organism was assayed on a Nutrient agar containing 1% casein, starch, egg yolk reaction for protease and amylase, lipase respectively[11].

Statistical analysis

Statistical analysis of cultural, morphological and biochemical characteristic data were analyzed by the Statistical package for Social Sciences (SPSS) and MATLAB.

16S rDNA sequences and Phylogenetic analysis

DNA was extracted from bacilli culture using standard phenol chloroform protocol[25]. The partial sequence of the 16S rRNA gene was amplified by using polymerase chain reaction and universal primer Eubacteria specific primers, 16F 27 (5' CCAGAATTGATCMTGGCTCAG- 3') and 16R 1525 (5' TTCTGCAGTCTAGAAGGAGGTGWTCCAGC C – 3'). The PCR condition used were an initial denaturation at 94° C for two minutes, followed by 35 cycles of denaturation at 95° C for one minutes and extension at 72° C for one minutes and final extension at 72° C for 10 minutes. The amplified 16S rRNA gene PCR products from these isolates were directly sequenced after purification by precipitation with polyethylene glycol and NaCl procedure[26] and directly sequenced on the Applied Bio systems Model 3730 DNA sequence (Foster, California USA). The 16S rRNA sequence were analysed using BLAST program Multiple Sequence Alignment of approximately 900 bp sequences were performed using CLUSTAL W, version 1.8. A phylogenetic tree was constructed from evolutionary distances using the neighbor-joining method of MEGA 4 program package[27].

Nucleotide sequence accession numbers

The 16S rRNA sequences of the representative isolated strains have been deposited in the GenBank under accession numbers JQ319523- JQ319545, JX076852-JX076856, JX134050

RESULTS AND DISCUSSION

The alkaline Lonar Lake is a unique basaltic rock meteorite impact crater. Lonar Lake is a one such soda Lake in which the indigenous microflora is present, and such microbial flora has ability thrives in alkaline condition. The data on the physicochemical analysis of the water samples (Table 1) indicated that the Lonar lake water samples were alkaline with the pH 10 and alkalinity 2756 mg /l. The salinity of water samples 8017 mg /L (table 1).

Table 1
Physiochemical analysis of the water samples from Lonar crater

pH	10.0-10.5
TDS	7.0- 11.5 g/L
DO	2.0-3.2
Conductivity	12.4-15.8
Chlorides	3585-4366 mg/L
Salinity	6539-8017 mg/L
Alkalinity	1220-2756mg/L
Total Hardness	220-520mg/L
Ca Hardness	105-130mg/L
Mg Hardness	305mg/L
Phosphate	4
Nitrate	6.6mg
sulphate	20.2mg/L

These findings revealed that the Lonar lake water was alkaline (pH 10.3) and characterized by high concentration of salts, chloride (3492 mg/L), total hardness (520 mg/L), calcium hardness (130mg/L), magnesium hardness (305 mg/L), sulphate (20.2 mg/L), phosphate (4 mg/L), nitrate (6.6 mg/L) and dissolved oxygen (3.2 mg/L). The Lonar Lake is unique in the world for its alkalinity and salinity of the water but it is seen that chlorides and salinity of the lake water is decreasing day by day[28]. Hence, it should be protected and preserved for its uniqueness and a scientific phenomenon. On the basis physicochemical analysis, the various enrichment media were selected for the maximum isolation of the bacterial strains. Total one hundred and fourteen isolates obtained in the isolation exercise, cultural, morphological characteristics of all the strains were studied and 55 isolates were selected on the basis of their pH salt and temperature tolerance. The isolates were screened on the basis of biochemical characteristics as described earlier and further confirmed by 16S rDNA sequencing and twenty nine bacterial isolates were selected on the basis of temperature tolerance and enzyme profile. This was done to avoid sequencing several identical isolates from the total isolates. Similar study was performed by Joshi et al.[20] and Mwirichia et al.[24] All the twenty nine strains were revealed to be Bacillus by their positive Gram reaction, spore bearing, motile, and rods in cell morphology. Then a various conventional phenotypic tests were used to identify them tentatively. Out of these twenty nine, six bacillus stains isolated from sediment sample and twenty bacilli strains isolated from water sample. In present investigation was to determine biotechnological potential diversity of Lonar Lake using different enrichment media with various substrates as peptone, yeast extract, glucose, and starch, Casein and egg yolk. The media composition seemed to have an effect on the recovery of different species of the *Bacilli.* In the present study media A and B suitable for the Oceanobacillus, and Bacillus species, C and D medium were suitable for the Bacillus but Lysinibacillus was supported by medium C. By morphologically including gram reaction, spore forming, position and shape of spore, swollen sporangia, capsulated, motility, the bacilli strains were found twelve groups of diversified group of bacilli. Physiologically all these selected bacilli showed three group of bacilli which were all strains grow in 7-12 pH and 0 - 7% NaCl except bacterial strain AS1(1) having pH 8 for the growth and DW2(3) and DW2(5) having pH 8 and 3% NaCl require for the growth. All bacterial isolates were catalase positive, most of this oxidase positive except three bacilli AS1(1), OCW3(1), and AW3(2). All these isolates were Indole and urease negative (Fig 2). The microbial diversity of saline lakes has been studied primarily by focusing on the isolation and characterization of individual organisms with potential industrial application[20]. In present study, out of twenty nine, fifteen bacterial strain were found starch hydrolyzing, fourteen bacterial strain were found protease producer and eleven bacterial strains were found Lipase producing microorganisms. Out of this OCW3(1), AW3(2), DW1(1), DW4(1), BS1(1), CS4(1), AW4(3) and OBW3(2) has been found to have produced all the three enzymes Amylase, Lipase, Protease at alkaline

pH 10. Thus these alkaline enzymes may be useful wide industrial and biotechnological interest due to the fact their enzyme are better suited for harsh industrial process. Martin *et al.*[29] reported the amylase producing *Bacillus pseudofirmus*, *Bacillus cohnii*, *Bacillus vedderi* and *Bacillus agaradhaerens* from Ethiopian soda lakes and Hashim *et al.*[30] isolate the starch hydrolyzing *Bacillus halodurans* from a Kenyan soda lake. In the present studies the phylogenetic analysis of isolates indicated that all the bacterial isolates affiliated to Firmicutes, which included family of bacilliace, with the three genera such as *Bacillus, Oceanobacillus, Lysinibacillus* and species were *Bacillus flexus, Bacillus cellulosilyticus, Bacillus pseudofirmus, Bacillus alkalogaya, Bacillus pumilus, , Bacillus halodurans, Bacillus circulans, Bacillus cereus, Bacillus agaradhaerens, Bacillus sp. Lysinibacillus sphaericus, Lysinibacillus fusiformis,* and *Oceanobacillus iheyensis,* which were reported from various soda lakes as the highly alkaline lake, Van in Turkey, Inner Mongolian Baer Soda Lake, and a also from a Kenyan Soda Lake[31-34]. A novel group of bacteria were isolated on the various enrichment media from lake Elementia, Kenya by Mwirchia *et al.*[24]. They revealed that gammaproteobacteria Halomonas and Firmicutes to the genus bacillus. Joshi *et al*[20], studied on isolation of various group of bacteria for the phylogenetic diversity from Lonar Lake. They revealed that, phylum Firmicutes, α-Proteobacteria, β-Proteobacteria and γ-Proteobacteria. In the present investigation various bacterial strains were firstly reported from the Lonar crater such as *Bacillus lehensis, Bacillus cellulosilyticus, Bacillus alkalogaya, Bacillus halodurans, Bacillus circulans, Bacillus agaradhaerens, Lysinibacillus sphaericus,* and *Lysinibacillus fusiformis.* These organisms previously reported from the various soda lake and alkaliphilic environment over the world wide it suggests that the Lonar Lake is the treasurer of alkaliphilic and alkalitolerant microorganism[35]. In present study, BW4(4) were found *Bacillus gibsonii.* Nielsen *et al.*[36] was described the phenetic diversity of alkaliphilic *Bacillus* strains proposal for nine new species including *Bacillus gibsonii,* while 16S rRNA gene sequence similarity showed that strain BW4(3) and CS1(1) were member of the Bacillaceae. The highest similarity value was observed with the obligate alkaliphiles and the phylogenetic analysis based on 16S rRNA gene sequences indicated that the taxonomic position of strain BW4(3) and CS1(1) belong to the *Bacillus krulwichiae* 97%[37]. Naturally occurring alkaline environments, such as soda lakes, deserts and arid soils, harbour a wide range of alkaliphilic and alkalitolerant strains not only such type of the environment is treasurer of alkaliphilic microorganism but also alkaliphilic microorganism also isolated from pristine soil samples[38]. The phylogenetic analysis based on 16S rRNA gene sequences indicated that the taxonomic position of strain BW3(2) related to *Bacillus lehensis.* Previously Blanco *et al.*[39] was isolated *Bacillus lehensis* from cassava starch wastewater and studied optimization of parameters for cyclodextrin glycosyltransferase production.The 16S rDNA of bacterial strains DW3(1), CW3(3), DW2(1) and ODW3(2) which were 99% similarity showed with three types of bacterial species such as *Bacillus safensis* isolated from Longshan potassium mines, Nanjing, China[40].*Bacillus pumilus* AY167883 and *Bacillus* sp. In the present study the physiological and molecular methods were used for the identification of bacterial strains but the biochemical results suggest that, the strains DW3(1), CW3(3), DW2(1) and ODW3(2) were belonging to *Bacillus pumilus*. However, the conventional bacterial classification methods based on the morphology, physiology and biochemical test were time consuming. But at the same time it is necessary for the exact identification of the bacterial strain in combine with molecular techniques. In our research, we revealed that the each method has its own advantages and disadvantages. Moreover, both of them are necessary to identify a strain accurately.

Table 2

Characteristics of representative strains of Lonar lake and their phylogenetic related strains (Sale 2004)

Isolation code	Pigment	Colony Shape	Colony Elevation	Colony Edge	Internal structure of Colony	Growth at 50º C	Starch hydrolysis	lipid Hydrolysis	casein Hydrolysis	Bacteria
AS1(1)	Colourless	Circular	Effuse	Entire	Transperent	+	-	-	-	*Bacillus cellulosilyticus*
AS1(2)	White	Circular	Effuse	Entire	Wavy Interlaced	+	-	-	+	*Oceanobacillus iheyensis*
BW2(1)	White	Circular	Convex	Entire	Wavy Interlaced	-	+	-	-	*Bacillus alkalogaya*
BS1(2)	White	Circular	Effuse	Entire	Wavy Interlaced	+	-	-	+	*Oceanobacillus iheyensis*
BW1(1)	White	Circular	Convex	Entire	Transperent	+	-	-	+	*Oceanobacillus iheyensis*
BW4(3)	White	Circular	Umbonate	Entire	Wavy Interlaced	+	+	-	-	*Bacillus krulwichiae*
BW4(4)	Colourless	Circular	Effuse	Entire	Transperent	-	-	-	+	*Bacillus gibsonii*
CS1(1)	White	Circular	Umbonate	Entire	Wavy Interlaced	+	+	-	-	*Bacillus krulwichiae*
CW1(3)	White	Circular	Convex	Entire	Wavy Interlaced	-	-	-	-	*Lysinibacillus fusiformis*
CW4(1)	White	Circular	Convex	Entire	Wavy Interlaced	-	-	-	-	*Lysinibacillus fusiformis*
CW4(2)	White	Circular	Umbonate	Entire	Wavy Interlaced	-	-	-	+	*Lysinibacillus sphaericus*
CW4(3)	White	Circular	Convex	Entire	Wavy Interlaced	-	-	-	-	*Lysinibacillus fusiformis*
DW3(1)	White	Circular	Effuse	Entire	Wavy Interlaced	+	-	+	+	*Bacillus pumilus*
DW4(1)	White	Circular	Umbonate	Entire	Transperent	-	+	+	+	*Bacillus pseudofirmus*
AW3(2)	White	Circular	Effuse	Conves papilate	Wavy Interlaced	-	+	+	+	*Bacillus flexus*
AW4(3)	White	Irregular	Effuse	cerenate	Wavy Interlaced	+	+	+	+	*Bacillus* sp.
BS1(1)	Colourless	Circular	Umbonate	Entire	Transperent	-	+	+	+	*Bacillus pseudofirmus*
BW3(2)	White	Circular	Raised with concave	Entire	Transperent	+	+	-	-	*Bacillus lehensis*
BW4(1)1	Colourless	Curled	Effuse	cerenate	Wavy Interlaced	+	-	+	+	*Bacillus halodurans*
CW2(2)	White	Toruloied	Effuse	Undulate	Arboresent	-	+	-	-	*Bacillus circulans*
CW3(3)	White	Circular	Effuse	Entire	Wavy Interlaced	+	-	+	+	*Bacillus pumilus*
CS4(1)	White	Circular	Umbonate	Entire	Transperent	-	+	+	+	*Bacillus pseudofirmus*
OCW3(1)	White	Irregular	Umbonate	Undulate	Wavy Interlaced	+	+	+	+	*Bacillus cereus*
DW1(1)	White	Circular	Umbonate	Entire	Transperent	-	+	+	+	*Bacillus pseudofirmus*
DW2(1)	White	Circular	Effuse	Entire	Wavy Interlaced	+	-	+	+	*Bacillus pumilus*
DW2(3)	White	Circular	Raised with concave	Entire	Transperent	+	+	-	-	*Bacillus agaradhaerens*
DW2(5)	White	Circular	Raised with concave	Entire	Transperent	+	+	-	-	*Bacillus agaradhaerens*
ODW3(2)	White	Circular	Effuse	Entire	Wavy Interlaced	+	-	+	+	*Bacillus pumilus*
OBW3(2)	Colourless	Circular	Raised with concave	Entire	Transperent	-	+	+	+	*Bacillus* sp.

Fig 1: Morphological characteristics of bacterial isolates from Lonar lake

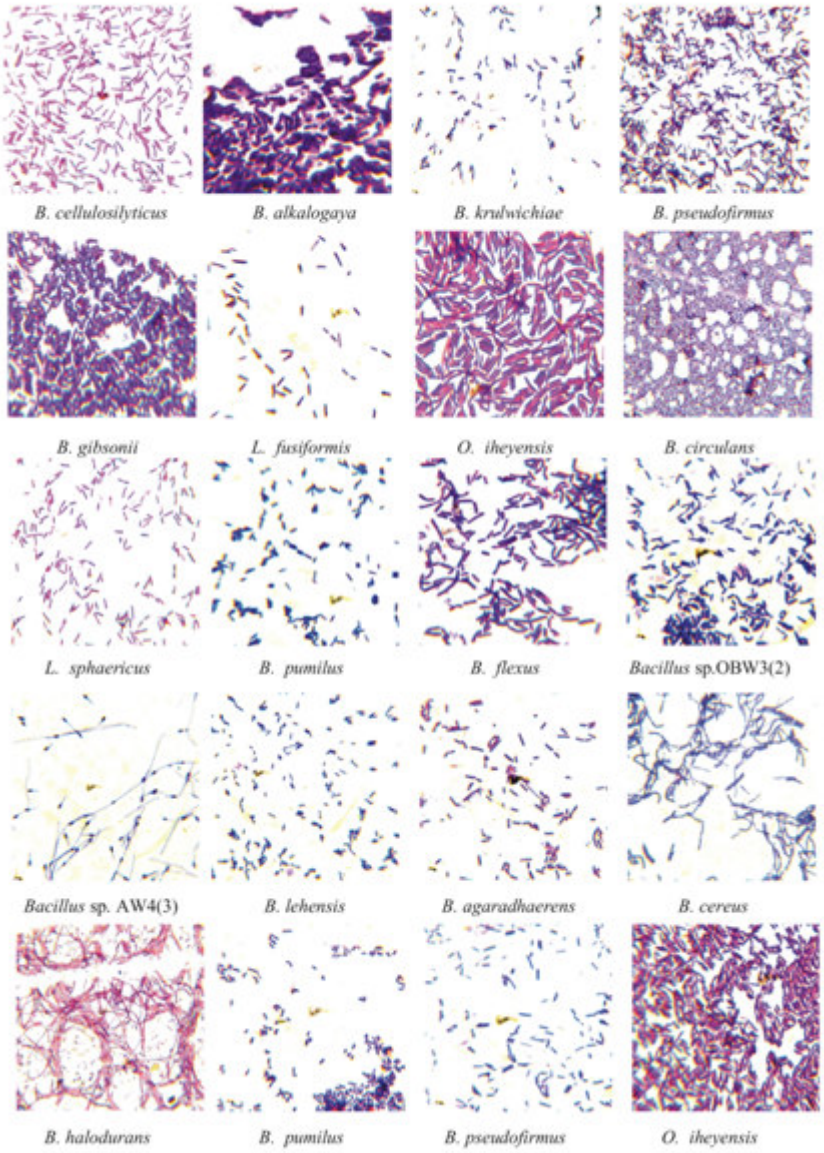
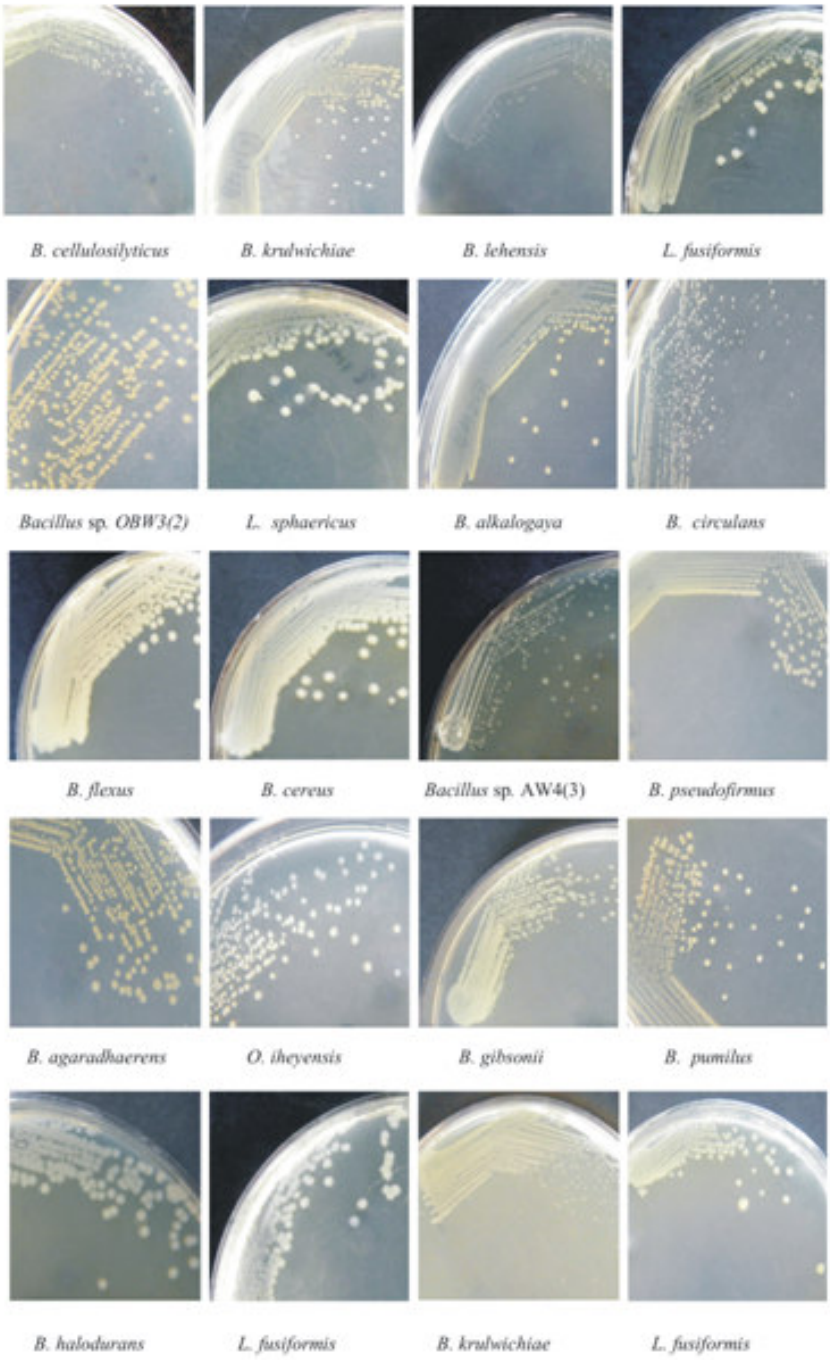


Fig 2: Cultural characteristics of bacterial isolates from Lonar lake



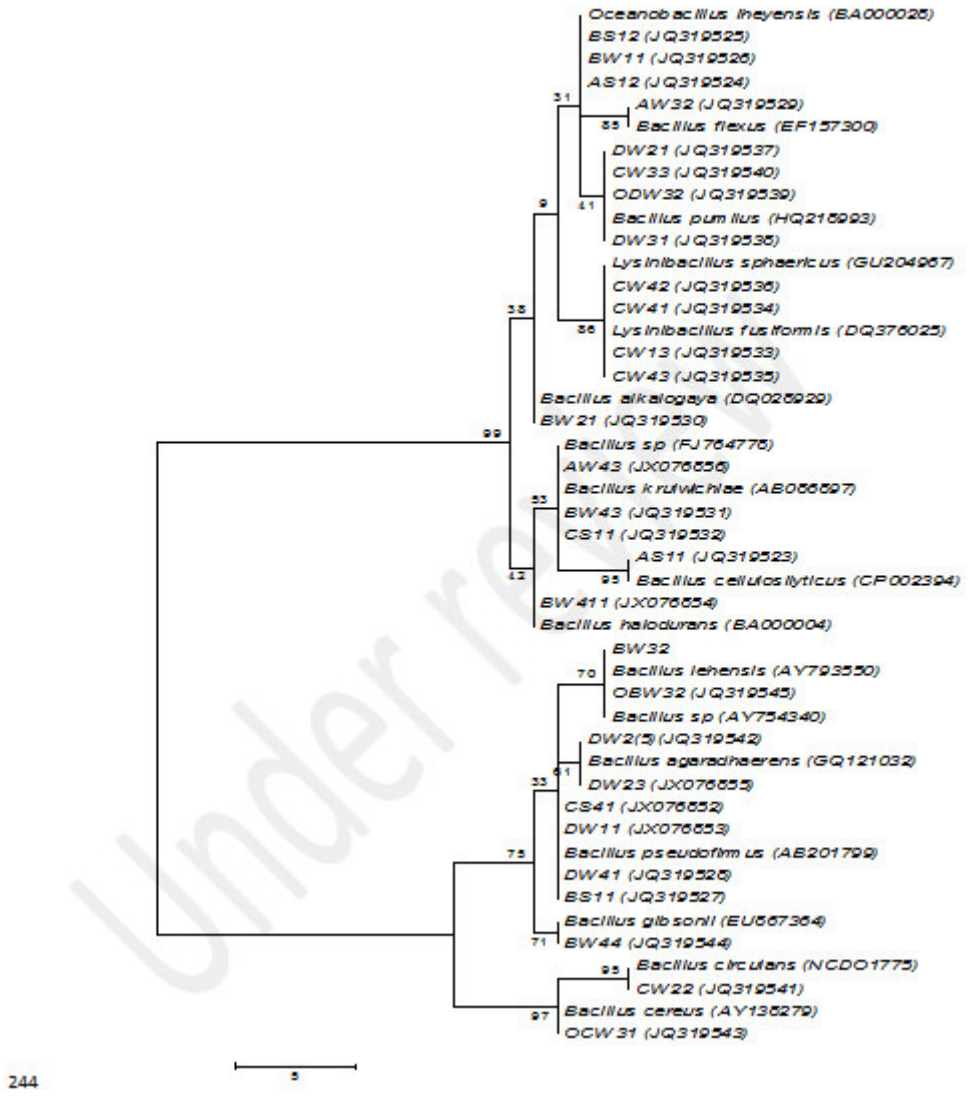


Figure 3

Phylogenetic tree based on a comparison of the 16S ribosomal DNA sequences of Lonar lake isolates and some of their closest phylogenetic relatives. The numbers on the tree indicates the percentages of bootstrap sampling derived from 1,000 replications.

Bacterial classification methods based on morphology, physiology and biochemical tests are time-consuming and not fully and reliable. On the basis of 16S rDNA, various bacterial strains were revealed less than 97% similarity with reported sequence in database. These bacterial strains which need to be further confirmed by recommended scientific method for novality of the organism[35].

CONCLUSION

In conclusion this work elucidates the existence of complex and unique bacterial diversity associated with alkaline saline habitat of Lonar Lake. The structure and diversity of a microbial community adapted to a particular environment reflect conditions of the habitat, including those alkaliphilic and halophilic environment. On the base of the phylogenetic affiliation of the identified *Bacillus, Lysinibacillus, Oceanobacillus* it is possible to predict some physiological characteristics. The results of salt and pH tolerance tests revealed principally alkaliphilic and alkalitolerant as well as moderately halophilic *Bacilli* from the Lonar lake sample, referring to the adaptation of these bacteria to the studied environments. Biotechnological potential diversity assessment should contribute to the understanding of ecology in alkaline saline and to the exploring of industrially important

strains associated with unique environment. Certain differentiating phenotypic characters and the ability of amylase, lipase and protease production of these isolates were studied and biotechnologically valuable haloalkaliphilic enzyme producing isolates were determined in order to use in further studies. Cultivation-independent methods that are based solely responsible on phylogenetic sequence information are not adequate physiological and metabolic study of strains in a given environment. On the other hand culture dependent based strategy is neither reliable for the description of bacterial diversity in the case of such a complex community in this extremophilic environment. However, our findings in this study, traditional cultivation based methods have a great importance in research providing the opportunity in investigations of potentially bacterial isolates under laboratorial conditions and this work provides a framework for further studies of these evidently important habitats and other soda lake.

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