STUDIES ON THE PHYSIOLOGICAL AND CULTURAL DIVERSITY OF *BACILLI* CHARACTERIZED FROM LONAR LAKE (MS) INDIA

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ABSTRACT

Culture dependent phenotypic characterization analysis was applied to study the bacilli diversity of the Lonar Lake. The uniqueness of the Lonar Lake water is its salinity and alkalinity. Aerobic, haloalkaliphilic bacteria were isolated and characterized from sediment and water samples collected from Lonar Lake. Fifty five bacterial strains were isolated using different enrichment media. Out of them twenty nine spore bearing bacteria were selected for further analysis. The phenotypic characterization indicated that bacterial strains were related to genera Bacillus and species were *Bacillus flexus, Bacillus cellulosilyticus, Bacillus pseudofirmus, Bacillus clausii, Bacillus krulwichiae, Bacillus pumilus, Bacillus lehensis, Bacillus halodurans, Bacillus circulans, Bacillus cereus, Bacillus agaradhaerens, Bacillus sphaericus, Bacillus fusiformis,* and *Bacillus asahii*, *Bacillus pseudalcalophilus, Bacillus okuhidensis and Bacillus gibsonii.* The abilities of many of the strains to produce extracellular protease, amylase and lipase suggest that they might be of potential value for biotechnological exploitation.

Key Word: Bacillus, Diversity, Lonar Lake, Enzyme.

INTRODUCTION

The alkaline Lonar Lake (Latitude 19° 58', Longitude 76° 36') is a unique basaltic rock meteorite impact crater, ranking third in the world. Lonar crater is filled with saline water. The uniqueness of the lake water is its salinity and high alkalinity. The lake water is alkaline having an average pH of 9.5- 10. Lonar Lake is a closed one without any outlet and unique due to its salinity, alkalinity and biodiversity. Due to the uniqueness, the lake has evoked much scientific value among researchers (Tambekar *et al.,* 2010). Several studies revealed that its salinity was 40.78, 31.52 and 30.87 in 1910, 1958 and 1960, respectively (Malu *et al.,* 2000). The presence of brackish water inside the crater having pH 10 is distinctive feature of the ecosystem along with the concentration of chlorides, calcium carbonate and water over a long period of time (Tambekar *et al.,* 2010). Depending on the water chemistry of the individual lakes they are likely to be green, pink, red, orange, due to massive permanent or seasonal blooms of microorganism. This is reflected in the extreme high primary productivity associated with some of these lakes (Grant *et al.,* 1990; Duckworth *et al.,* 1996; Foti *et al.,* 2007). Alkaliphilic microorganisms, in particular *Bacillus* species, have attracted much interest because of their ability to produce extracellular enzymes that are active and stable at high pH values. The unusual properties of these enzymes offer a potential opportunity for their utilization in processes demanding such extreme conditions. (Guffanti *et al.,* 1980; Horikoshi and Akiba, 1982; krulwich and Guffanti 1983; Sharp and Munster, 1986; Horikoshi, 1996; Aguilar *et al.,* 1998; Takami *et al.,* 1999). Chaphalkar and Dey (1994), have studied the metalloprotease from alkaline Streptomyces isolated from Lonar lake silt sample. Methanol degrading microorganism also isolated and identified from Lonar Lake water and sediment samples was good finding (Tambekar *et al.,* 2011; Surakashi *et al.,* 2010). The main object of the study was, the haloalkaliphilic bacilli species isolate from Lonar Lake, using traditional methods such as cultural, morphological and physiological studies. The isolation and characterization of these bacterial strains could provide knowledge on the diversity of the *Bacillus* genus in this unexplored haloalkaliphilic Lonar lake environment.

MATERIALS AND METHODS

Enrichment and isolation of microorganisms Lonar lake water and sediment sample were collected in sterile bottles and polythene bags respectively, from defined sampling site. Enrichment of water samples and sediment samples were carried out in Horikoshi I, Horikoshi II, and nutrient agar at pH 10, nutrient agar at pH 10.0 with 30 g l−1 sodium chloride (Table 1). 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

Table 1. Enrichment medium and their composition

	Horikoshi I g/L	Horikoshi II g/L	Nutrient agar g/L	Nutrient agar g/L
Glucose	10	-	-	-
Soluble starch	-	10	-	-
Peptone	5	5	5	5
Yeast extract	5	5	1.5	1.5
Beef extract	-	-	1.5	1.5
KH_2PO_4	1	1	-	-
$MgSO_4.7H_2O$	0.2	0.2	-	-
Na_2CO_3	10	10	-	-
Sodium Chloride	-	-	5	35
Agar	20	20	20	20

Identification of the bacterial culture

Bacterial culture were examined for their colony, morphological character, motility, capsule staining, spore staining and standard biochemical test such as catalase, oxidase, nitrate reduction, methyl red reaction, voges prauskar test, citrate utilisation by Simmons, urease activity, Indol production and production of acid from glucose, arabinose, mannitol, xylose, lactose, trehalose, sucrose, cellobiose, galactose, maltose, fructose, salicin, sorbitol, raffinose 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.

RESULTS AND DISCUSSION

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 double extreme conditions (i.e. high pH and high salinity) 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. As far as Indian soda lakes are concerned, a culture-dependent approach has not been yet applied to analyze bacterial diversity. The alkaline Lonar Lake is a unique basaltic rock meteorite impact crater, ranking third in the world. Lonar crater is filled with saline water. 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.

From a total fifty five isolates obtained in the isolation exercise, cultural, morphological characteristics of all the strains were studied and twenty nine bacilli isolates were selected on the basis of gram positive bacillus and spore bearing for further characterization. The isolates were identified on the basis of biochemical characteristics as described earlier in our attempt to isolates different types of bacilli on the basis of colony characteristics and morphological appearance. Out of these twenty nine, six bacillus stains isolated from sediment sample and twenty three bacilli isolated from water sample. All these bacilli were catalase positive, most of this oxidase positive except three bacilli. All these bacilli strains grow in 7-12 pH and also grow in 0-7% NaCl except *Bacillus agaradhaerens* having 3% NaCl require for the growth. In present investigation was to determine *Bacilli* diversity of Lonar Lake using different enrichment media.

In the present studies the phenotypic analysis of representative isolates indicated that all the bacterial isolates were found related to the *Bacillus* genus and species were *Bacillus flexus, Bacillus cellulosilyticus, Bacillus pseudofirmus, Bacillus clausii , Bacillus krulwichiae, Bacillus pumilus, Bacillus lehensis, Bacillus halodurans, Bacillus circulans, Bacillus cereus, Bacillus agaradhaerens, Bacillus sphaericus, Bacillus fusiformis,* and *Bacillus asahii*, *Bacillus pseudalcalophilus, Bacillus okuhidensis and Bacillus gibsonii.*

This study indicated that the presence of bacillus species which were also reported from the various soda lake, Van in Turkey, inner Mongolian Bear soda lake (Ma Y *et al,* 2004). A related work was carried out by Ivanova *et al.,* (1999) from marine environment.

Table 2. Biochemical characteristic of *Bacilli* isolated from Lonar Lake

Biochemical Characters	AS11	AS12	BW21	BS12	BW11	BW43	BW44	CS11	CW13	CW41	CW42	CW43	DW31	DW41	AW32	AW43	BS11	BW32	BW411	CW22	CW33	CS41	OCW31	DW11	DW21	DW23	DW23	ODW32	OBW32
Morphology	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Gram nature	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arrangement	S	S	S	S	S	S	S	B	S	S	C_1	S	S	S	C_1	C_1	S	S	C_1	S	S	S	C_1	S	S	S	S	S	S
Spore	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Position	T	C	C	C	C	C	C	C	T	T	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C	C	C	C
Shape	O	O	O	O	O	O	S_1	O	O	O	E	O	E	O	O	E	O	O	E	E	E	O	O	O	E	O	O	E	O
Swollen	-	-	+	-	-	+	-	+	-	-	-	-	-	+	-	+	+	-	+	+	-	+	-	+	-	-	-	-	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Capsule	-	+	+	+	+	+	-	+	-	-	+	-	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
NaCl Require	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
2%NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5%NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+
7%NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+
pH 7	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH8		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Indol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MR	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+	+	-	-	-	+	-	-	+	-
VP	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Citrate	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	+	-	+	-	-	+	-
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NO_2 reduction	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	+	+	-	-
Glucose	-	+	-	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	+	+	-	+	-	+	-	-	+	-
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-		-	-	-	+	+	-	+	-	+	-	-	+	-
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	-	+	-
Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-
Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	+	-	-	+	-
Galactose	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	-	+	-
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fructose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	+	-	-	+	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	+	-	-	+	-
Amylase	-	-	+	-	-	+	-	-	-	-	-	-	-	-	+	+	+	+	-	+	-	-	+	+	-	+	+	-	+
Lipase	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	-	+	-	+	-	+	+	+	+	-
Protease	-	+	-	+	+	-	+	-	-	-	+	-	+	+	+	+	+	-	+	-	+	-	+	-	+	-	-	+	-

R-Rod, S- Single, B- Bunch, C_1 – Chain, T- Terminal, C- Central, E- Ellipsoidal, O- Oval, S_1 - Spherical

Table 3. Enzymatic profile of *Bacilli* isolated from Lonar Lake

Amylase	Lipase	Protease	Isolation code	Bacteria	NO. of isolates
Negative	Negative	Negative	AS1(1)	*Bacillus cellulosilyticus*	1
			CW1(3)	*Bacillus fusiformis*	1
			CW4(1)	*Bacillus fusiformis*	1
			CW4(3)	*Bacillus fusiformis*	1
			CS4(1)	*Bacillus pseudofirmus*	1
		Positive	AS1(2)	*Bacillus asahii*	1
			BS1(2)	*Bacillus asahii*	1
			BW1(1)	*Bacillus asahii*	1
			BW4(4)	*Bacillus gibsonii*	1
			CW4(2)	*Bacillus sphaericus*	1
			DW4(1)	*Bacillus pseudofirmus*	1
	Positive	Positive	DW3(1)	*Bacillus pumilus*	1
			BW4(1)1	*Bacillus halodurans*	1
			CW3(3)	*Bacillus pumilus*	1
			DW2(1)	*Bacillus pumilus*	1
			ODW3(2)	*Bacillus pumilus*	1
Positive	Negative	Negative	BW2(1)	*Bacillus clausii*	1
			BW4(3)	*Bacillus krulwichiae*	1
			CS1(1)	*Bacillus krulwichiae*	1
			BW3(2)	*Bacillus lehensis*	1
			CW2(2)	*Bacillus circulans*	1
			DW1(1)	*Bacillus pseudofirmus*	1
			DW2(3)	*Bacillus agaradhaerens*	1
			DW2(5)	*Bacillus agaradhaerens*	1
	Positive	Positive	AW3(2)	*Bacillus flexus*	1
			AW4(3)	*Bacillus okuhidensis*	1
			BS1(1)	*Bacillus pseudofirmus*	1
			OCW3(1)	*Bacillus cereus*	1
			OBW3(2)	*Bacillus pseudalcalophilus*	1

It was evidently found that *B. marinus, B. subtilis, B. pumilus, B. licheniformis, B. cereus* and *B. mycoides* were common inhabitants of the marine habitat. Joshi *et al.,* (2007) also reported the cultivable diversity of Lonar Lake. In present study, the *B. cereus, B. flexus* were found and these *Bacilli* previously reported from the Lonar Lake (Joshi *et al.,* 2007; Kanekar *et al.,* 1999). The isolated *B. cellulosilyticus, B. pseudofirmus, B. krulwichiae, B. pumilus, B. lehensis, B. halodurans, B. circulans, B. agaradhaerens, B. sphaericus* and *B. asahii w*ere new species and not previously recorded *Bacilli* species from Lonar Lake.

The microbial diversity of saline lakes has been studied primarily by focusing on the isolation and characterization of individual organisms with potential industrial application. In present study, out of twenty nine, thirteen *Bacilli* cultures were found starch hydrolyzing, sixteen *Bacilli* cultures were found protease producer and ten *Bacilli* cultures were found Lipase producing *Bacilli* (Table 3).

The ecology and diversity of an East African Soda Lake was studied and extensively reviewed for their biotechnological potential (Grant *et al.,* 1990). Lipase producing *B. agaradhaerens, B. clarkii, B. smithii*, *B. subtilis* from lake Bogoria were isolated and characterized (Vargas *et al.,* 2004) in present studies, *B. flexus, B. cereus, B. okuhidensis, B. pseudofirmus, B. pseudalcalophilus, B. pumilus* (4), *B. halodurans* were found lipase producer. While amylase producer were *B. krulwichiae* (2), *B. lehensis, B. circulans, B. pseudofirmus* (2), *B. agaradhaerens* (2), *B. flexus, B. okuhidensis, B. pseudalcalophilus, B. cereus, B. clausii* reveled Lonar Lake. Similar result also observed from *B. halodurans* were isolated from a Kenyan alkaline soda lake as well as from Ethiopian soda lakes (Hashim *et al.,* 2004; Martins *et al.,* 2001). Haloalkaliphilic bacteria have been relatively less attended, as only few alkaline proteases are reported from these organisms (Studdert *et al.,* 1997; Stan-Lotter *et al.,* 1999; Gimenez *et al.,* 2000; Studdert *et al.,* 2001;

Polosina *et al.,* 2002). In our attempt, *B. asahii* (3), *B. gibsonii, B. sphaericus, B. pseudofirmus* (2), *B. pumilus* (4), *B. halodurans, B. flexus, B. cereus, B. clausii, B. okuhidensis* were found protease producing *Bacilli* while *Arthrobacter ramosus*, MCM B-351 isolated from Lonar Lake by Nilegaonkar *et al.,* (2002) for Production and characterization of extracellular protease. *B. cereus, B. flexus, B. pseudofirmus and B. pseudalcalophilus* and *B. okuhidensis* have been found to have produced all the three enzymes, at alkaline pH 10, making it valuable tool in industrial enzyme production.

Finding of this study provide a window into the diversity of *Bacilli* community members which were enzyme producing from the Lonar lake. 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. These *Bacilli* may be useful for study of their physiology, and understand their importance in such unique environments.

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