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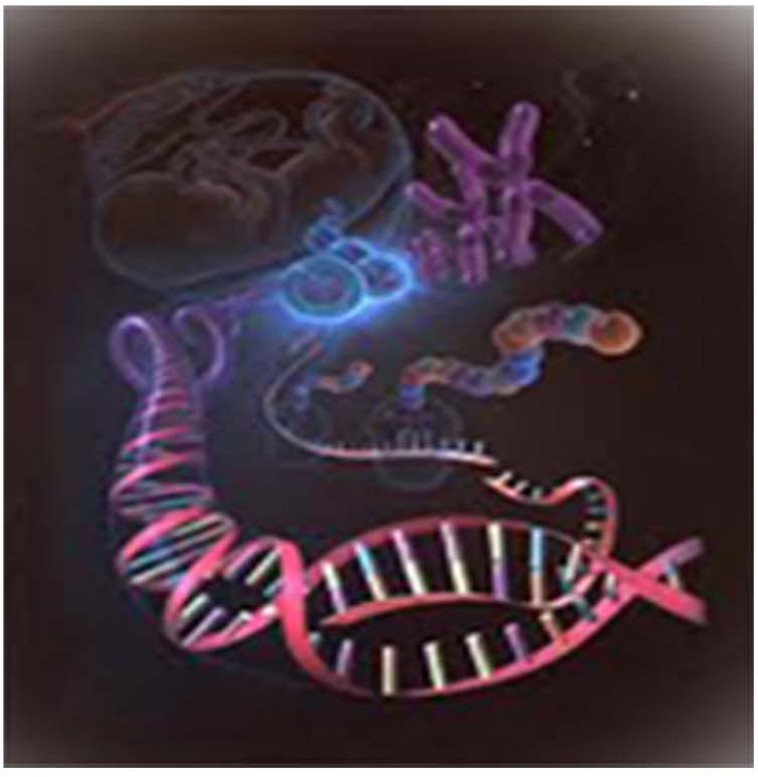

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Research Paper

PHYLOGENETIC ANALYSIS OF AMYLASE PRODUCING BACILLI FROM EXTRIMOPHILLIC ENVIRONMENT

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Culture dependent phenotypic characterization analyses were applied to study the amylase producing bacteria from the Lonar crater. The uniqueness of the Lonar Lake water is its salinity and alkalinity. A total of 55 bacterial strains were isolated from the water and sediment samples collected from the hyperalkalinesaline environment of Lonar crater. These haloalkalitolerant isolates were analyzed by numerical taxonomy techniques by using the average linkage; clustering was achieved using the between groups with averages. Amylase producing fifteen bacterial strains was selected on the basis of their starch hydrolysis activities and studies physiological taxonomy as well as molecular phylogeny. The phenotypic characterization and 16S rRNA indicated that bacterial cultures belongs to genera *Bacillus*; species were *B. flexus, B. pseudofirmus, B. lehensis*, *B. circulans, B. agaradhaerens, B. alkalogaya, B. krulwichiae, B. cereus, Bacillus sp* AW4(3) and *Bacillus sp* OBW 3(2). All these bacterial strains were cultured at pH range 7-12 and 37-50 °C temperature, most of them showed optimum growth at pH 10 and 45 °C. These bacterial strains may have biotechnological potential by being useful for the production of amylase.

Keywords: Amylase, *Bacillus,* Lonar crater, Numerical taxonomy

INTRODUCTION

Microbial amylases could be usable resources in the pharmaceutical since the enzymes prepared with suitable properties. With the advent of new knowledge in biotechnology, the spectrum of amylase application has widened in many other fields, such as clinical, medicinal, and analytical chemistries, as well as their widespread relevance in starch saccharification (Shanmugha-priya, 2009). The stipulations prevailing in the industrial relevance in which enzymes are used are rather extreme, particularly with regards to temperature and pH. Therefore, there is a continuing attention to become better the stability of enzymes and to encounter the fulfillment set by particular applications. In this respect, thermostable enzymes have been proposed to be industrially relevant (Prakash and Jaiswal, 2010). Members of diverse genera have been reported to produce different types of amylase enzymes. So far not many amylases with optimum activity under alkaline conditions have

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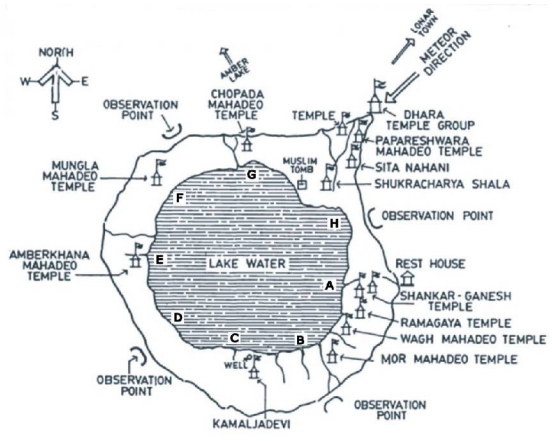
been studied from the various soda lakes (Martins *et al*., 2001; and Hashim *et al.,* 2004). The alkaline Lonar crater is a unique basaltic rock meteorite impact crater, ranking third in the world. Lonar crater is filled with saline water (Latitude 19° 582", Longitude 76° 362"). The lake water is alkaline 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. 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). The metalloprotease from alkaline *Streptomyces* isolated from Lonar lake silt sample have studied (Chaphalakar and Dey, 1994). Methanol degrading microorganism also isolated and identified from water and sediment samples of Lonar Lake (Tambekar *et al.,* 2011). A preliminary data of bacterial diversity of the Lonar Lake has been reported (Joshi *et al.,* 2005; and Tambekar and Dhundale 2012) which includes some of the biochemically identified isolates. Alkaliphilic microorganisms have attracted much interest because of their ability to produce extracellular enzymes that are active and stable at high pH values (Tambekar and Tambekar, 2011). The unusual properties of these enzymes offer a potential opportunity for their utilization in processes demanding such extreme conditions. 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 (Takami *et al.,* 1999). The object of the study was to numerical taxonomy studies of haloalkaliphilic amylase producing bacteria from Lonar Lake, using traditional methods such as morphological, cultural, physiological studies.

MATERIAL AND METHODS

Collection of samples: Thirty two water samples were collected in the sterile sampling bottles and sixteen sediment sample collected into the sterile zip lock polyethylene bags. Total eight sites (A, B, C, D, E, F, G and H) of Lonar Lake were selected for the collection of samples. From each spot two water samples were collected one from periphery and another from inner side of about 10-20 m from periphery (Figure 1).

Figure 1: Sites of Sample Collection in Lonar Crater



Source: Taiwade (1995)

Enrichment and Isolation of Microorganisms: Enrichment strategy was established by four different liquid media (Table 1). Enrichment of water and sediment samples was carried out in Horikoshi I (A), Horikoshi II (B), Peptone 5.0, yeast

Table 1: Composition of Enrichment and Isolation Medium

Component	A	B	C	D
Glucose	10 g	–	–	–
Starch	–	10 g	–	–
Peptone	5 g	5 g	5 g	5 g
Yeast extract	5 g	5 g	1.5 g	1.5 g
Beef extract	–	–	1.5 g	1.5 g
KH_2PO_4	1 g	1 g	–	–
$MgSO_4.7H_2O$	0.2 g	0.2 g	–	–
Na_2CO_3	10 g	10 g	–	–
NaCl	–	–	5 g	35 g
Agar	20 g	20 g	20 g	20 g
pH	10	10	10	10

Source: Joshi et al. (2007)

extract 1.5, beef extract 1.5, sodium chloride 5.0, agar 20.0, pH 10 (C). Peptone 5.0, yeast extract 1.5, beef extract 1.5, sodium chloride 35.0, agar 20.0 pH 10 (D). pH of the medium was adjusted using 1 N NaOH solution.

One gram sediment sample from each site was dissolved in sterile distilled water and poured the water and sediment samples in enrichment medium. All the flasks were incubated at room temperature on a rotary shaker (100 rpm) for 7 days. After enrichment, the organisms were isolated on respective medium agar plates and incubated at 37 °C. Well isolated and differentiated colonies from these enrichment media were transferred on the respective media slants and same were maintained as stocks for further study (Joshi *et al*., 2007).

Screening of amylase activity: Bacterial cultures were inoculated on starch medium. After 48 h incubation, floods the iodine solution into the plate. The halo zone was observed for amylase activity of isolates.

Identification of the bacterial culture: Bacterial cultures were examined for their morphological, cultural and standard biochemical test according to Bergey's Manual of systematic bacteriology (Sneath 1986).

16S rDNA Sequence analysis: The partial sequence of the 16S rDNA gene was amplified by polymerase chain reaction using universal primer Eubacteria specific primers, 16F 27 (5' CCAGAATTGATCMTGGCTCAG-3') and 16R 1525 (5' TTCTGCAGTCTAGAAGGAGGT GWTC CAGCC-3'). The amplified 16S rDNA gene PCR products from these isolates, after purification by precipitation using polyethylene glycol and NaCl procedure (Ausubel *et al.,* 2002) were directly sequenced on the Applied Biosystems Model 3730 DNA sequence (Foster, California USA).

Phylogenetic Analysis: The 16S rDNA sequences were analyzed using BLAST program. In addition, sequences were analyzed via RDPII using SEQUENCE-MATCH (Version 2.7) to identify the most closely related database sequences. Multiple Sequence Alignment of approximately 900 bp sequence was done by using Clustal X version 1.8. The phylogenetic tree was constructed from evolutionary distances using the neighbor-joining method of Mega 4 program package (Kumar *et al.,* 2004). The 16S rDNA sequences analysis was carried out at the National Centre for Cell Science, Pune, and sequences were submitted to NCBI GenBank Database for the accession numbers.

Statistical Analysis: Statistical analysis of cultural, morphological and biochemical characteristic data were analyzed. The sixty differential features obtained were used for a numerical analysis. Positive and negative results were coded as 1 and 0, respectively. Strain similarities were estimated separately for each physiological group with both simple matching coefficients, and clustering was achieved by average linkage. Cophenetic correlation was also obtained in each method. These computations were performed by the Statistical Package for Social Sciences (SPSS) and METLAB program.

RESULTS AND DISCUSSION

In the present study, a total fifty five isolates were obtained by culture dependent methods. The morphological, cultural and pH, NaCl tolerance of isolated bacterial species were studied. Out of fifty five, twenty nine bacterial cultures were gram positive bacilli and five bacteria were cocci and twenty one were gram negative bacteria. All the bacterial species were screened for the starch hydrolytic activity. Out of them fifteen gram positive bacilli showed their starch hydrolyzing activity. All the bacterial strains were found gram positive and spore forming bacilli. All the bacterial strains were found stable upto 45 °C and seven were found thermotolerent upto 50 °C. Majority of the alkaliphiles were identified as different species of the genus *Bacillus*, which are known to produce a wide variety of enzymes with tolerance to thermal and alkaline conditions (Nthangeni *et al.,* 2001). All the bacterial strains were found both alkaliphilic (7-12) and halophilic (0.5-7% NaCl), the optimum pH were 10 for all the bacterial strains. Some amylase producing bacterial strains also studied from the some soda lake. Martin *et al.* (2001) were found both alkalitolerant and obligate alkaliphiles were found and identified by phylogenetic analysis as the microbial species found in Ethiopian soda lake microbial population and known for being good amylase producers.

The phenotypic and 16S rRNA characterization indicated that the bacterial cultures belongs to genera *Bacillus* and species were *B. flexus* (1), *B. pseudofirmus* (4)*, B. cereus* (1), *B. lehensis* (1)*, B. circulans* (1), *B. agaradhaerens* (2), *B. alkalogaya* (1)*, B. krulwichiae* (2) and *Bacillus sp.* (2). This study indicated the presence of *bacillus* species which were also reported from the various soda lakes, Van in Turkey, inner Mongolian Bear soda lake (Ma *et al.,* 2004, Joshi *et al.*, 2007). Martin *et al*. (2001) reported the amylase producing *B. pseudofirmus*, *B. cohnii*, *B. vedderi* and *B. agaradhaerens* from Ethiopian soda lakes and Hashim *et al*. (2004) isolate the Starch hydrolysing *Bacillus halodurans* isolates from a Kenyan soda lake. In present study, *B. cereus* was showed zone of starch hydrolysis (30 mm), *B. lehensis* and *B. circulans* (23 mm), *B. flexus* (22 mm) while *B. pseudofirmus* (15-18 mm),

Bacillus sp. (17-22 mm), *B. agaradhaerens* (13-15 mm), *B. alkalogaya* and *B. krulwichiae* (10 mm) (Figure 7) at alkaline pH (10) and 45 °C. Lonar Lake bacterial strains a promising source of the extracellular amylase and produced at alkaline condition with having thermostability. However, it would be more informative if crude amylase is purified from the isolates and characterized, in future research.

Dendrogram based on comparison of the morphological characteristics of fifteen bacterial strains were found in three phenon (Figure 2) and on the basis of cultural characters these bacterial strains were found in four phenon (Figure 3) while on the basis of biochemical characters these bacterial strains were found in five phenon (Figure 4 and Table 2) but while on the basis of biochemical and physiological characters of these bacterial strains were found in four phenon (Figure 5). The Dendrogram showed the physiological similarity and dissimilarity in the genus and it was used to elucidate taxonomical position based on their physiological characters.

Figure 2: Dendrogram Based on Percent Similarity of the Physiological Characteristics of Bacterial Isolates From Lonar Crater, The Dendrogram was Created by the Using Average Linkage (Between Groups)

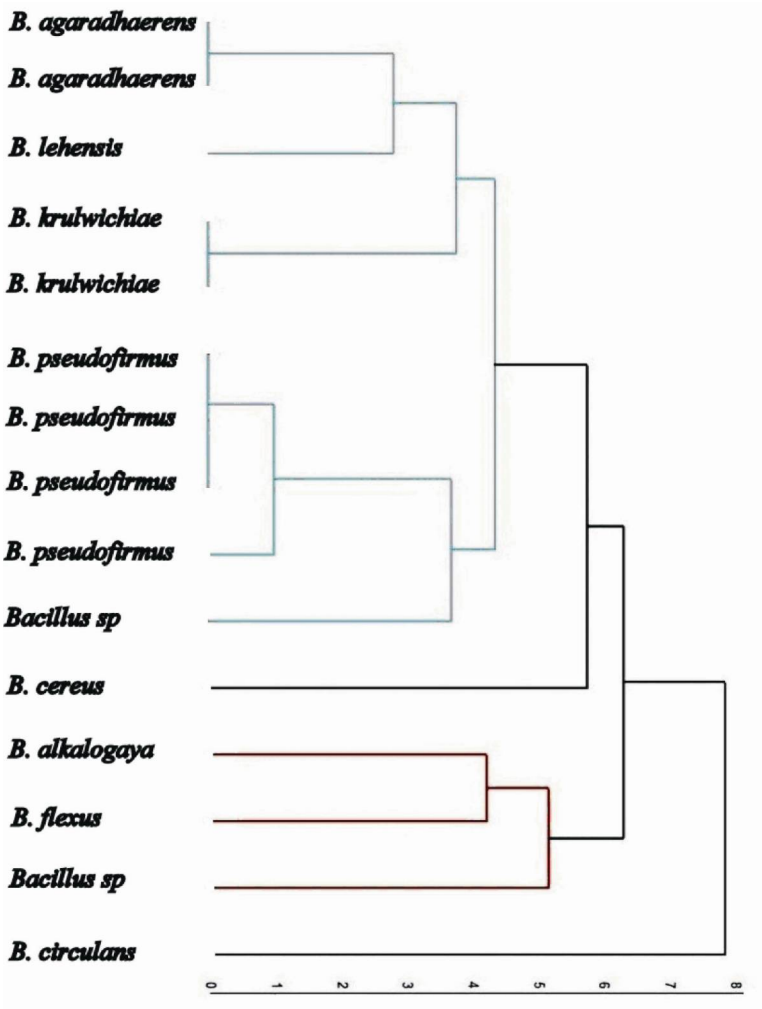
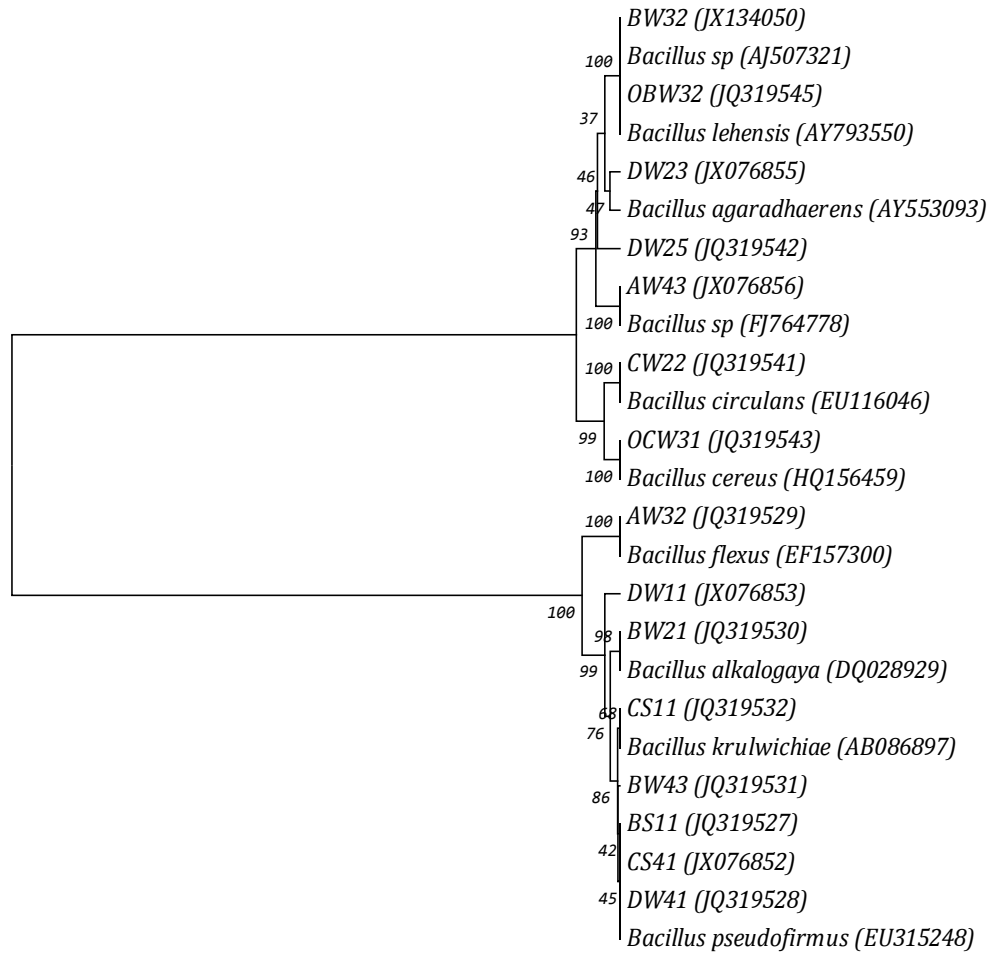


Figure 3: Phylogenetic Tree Based on a Comparison of the 16s Ribosomal DNA Sequences of Amylase Producing Bacterial Isolates From Lonar Lake and Some of Their Closest Phylogenetic Relatives, The Tree Was Created By The Neighbor-joining Method



B. flexus, B. pseudofirmus, B. cereus, B. lehensis, *B. circulans, B. agaradhaerens, B. alkalogaya, B. krulwichiae* and *Bacillus sp.* All these *Bacillus* species are known to be alkaliphilic (Nielsen *et al.*, 1995; Maria *et al.,* 2002; Yumoto *et al.,* 2003; and Ghosh *et al.,* 2007). These and related species have also been isolated from the soda lakes in the Kenyan region of the Rift Valley and Ethiopian soda lakes (Martin *et al.,* 2001; and Hashim *et al.*, 2004). Even though several isolates in this study have been clustered with a specific *Bacillus* species, they appear to be unique isolates according to their physiological differences as well as variance in their enzyme production levels. Some of the alkaliphilic strains, especially those related to *B. cereus, B. flexus, B. circulans, B. lehensis, B. agaradhaerens, B. alkalogaya, B. krulwichiae* and their enzymes, which thus far are less studied, could have applicable for industrial resources. Our current work is interested with starch hydrolyzing enzymes from some of the strains isolated during this study. All these strains and the enzymes are also being evaluated as catalysts in biotechnological applications involving hydrolytic reactions.

Figure 4: Starch Hydrolysis Activity Shown by Bacilli Isolated From Lonar Crater

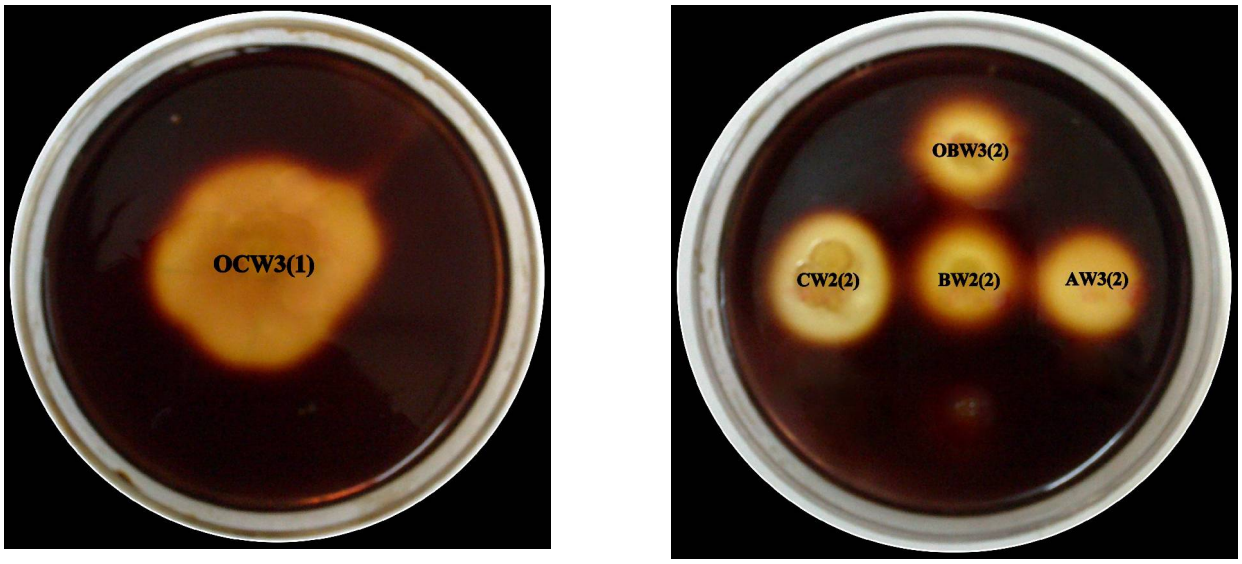
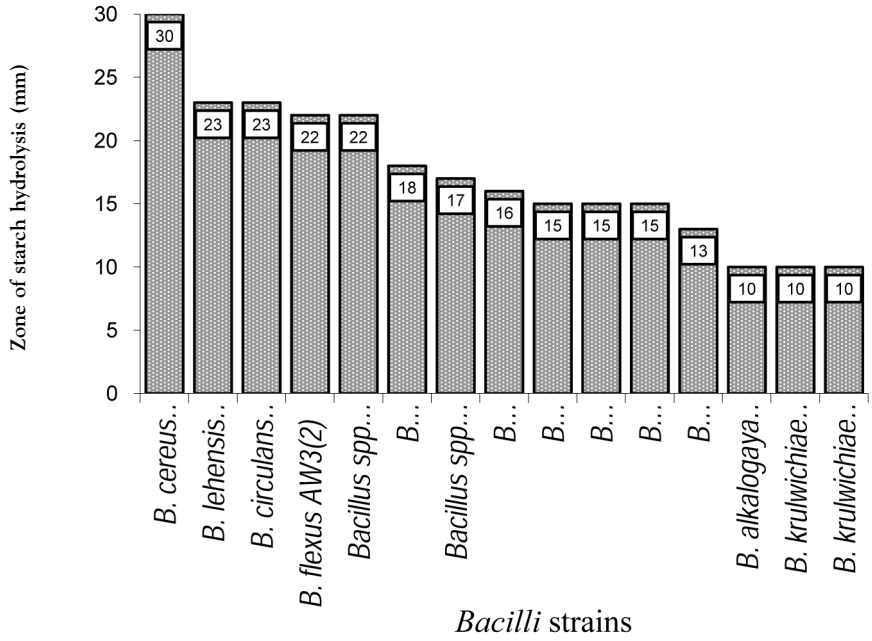


Figure 5: Amylase Producing Bacterial Strains Isolated from Lonar Crater



Finding of this study provides a window for amylase producing *Bacilli* from Lonar Lake. Thus these alkaline enzymes have industrial and biotechnological application due to their tolerance to harsh industrial process (Horikoshi, 1999). In our study, the culturable dependent approach was applied to study the amylase producing bacteria from Lonar crater using physiological and molecular techniques. These extreme halo-alkaliphiles in general were specialist since them able to

Table 2: Biochemical Characteristic of *Bacilli* Isolated from Lonar Lake

Bacteria Isolation code	*B. alkalogaya* BW2(1)	*B. krulwichiae* BW4(3)	*B. krulwichiae* CS1(1)	*B.pseudofirmus* DW4(1)	*B. flexus* AW3(2)	*Bacillus sp* AW4(3)	*B. pseudofirmus* BS1(1)	*B. lehensis* BW3(2)	*B. circulans* CW2(2)	*B. pseudofirmus* CS4(1)	*B. cereus* OCW3(1)	*B. pseudofirmus* DW1(1)	*B. agaradhaerens* DW2(3)	*B. agaradhaerens* DW2(5)	*Bacillus spp.* OBW3(2)
Morphological Character															
Gram character	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Shape of Bacteria	LR	LR	LR	LR	LR	LR	LR	LR	LR	LR	LR	LR	LR	LR	LR
Size of Bacteria (Length(um))	3.5	3	3	4	4	6	4	3.5	2.9	4	5	4	4	4	4
Size of Bacteria (Width (um))	0.4	0.5	0.5	0.7	0.4	0.5	0.7	0.5	0.3	0.7	1	0.7	0.5	0.5	0.6
Arrangement of Cell	S	S	S	S	C	B	S	S	S	S	C	S	S	S	S
Spore bearing	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Position of Spore	C_1	C_1	C_1	C_1	C_1	T	C_1	C_1	C_1	C_1	C_1	C_1	C_1	C_1	C_1
Shape of Spore	C_2	C_2	C_2	C_2	C_2	E	C_2	C_2	E	C_2	C_2	C_2	C_2	C_2	C_2
Swollen Sporangia	+	-	-	+	-	+	+	-	+	+	-	+	-	-	-
Capsule	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at temperature															
37º C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
45º C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
50º C	-	+	+	-	-	+	-	+	-	-	+	-	+	+	-
55º C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at pH															
pH 7	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
pH 8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at NaCl															
1% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
2% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
3% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 2 (Cont.)

Bacteria Isolation code	*B. alkalogaya* BW2(1)	*B. krulwichiae* BW4(3)	*B. krulwichiae* CS1(1)	*B.pseudofirmus* DW4(1)	*B. flexus* AW3(2)	*Bacillus sp* AW4(3)	*B. pseudofirmus* BS1(1)	*B. lehensis* BW3(2)	*B. circulans* CW2(2)	*B. pseudofirmus* CS4(1)	*B. cereus* OCW3(1)	*B. pseudofirmus* DW1(1)	*B. agaradhaerens* DW2(3)	*B. agaradhaerens* DW2(5)	*Bacillus spp.* OBW3(2)
6% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Biochemical characters															
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	-	+	+	+	+	+	-	+	+	+	-
Indol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MR	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-
VP	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-
Citrate Utilization	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Urea Hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	+	+	-	-	-	-	+	-	-	+	-	+	+	-
Utilization															
Glucose	-	+	+	-	+	-	-	-	+	-	+	-	-	-	+
Arabinose	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-
Mannitol	-	+	+	-	+	-	-	+	+	-	+	-	+	+	+
Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Sucrose	-	+	+	-	+	-	-	-	+	-	+	-	-	-	-
Cellobiose	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Galactose	-	+	+	-	+	-	-	-	+	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fructose	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Salicin	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Hydrolysis															
Starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lipid	-	-	-	+	+	+	+	-	-	+	+	+	-	-	+
Casein	-	-	-	+	+	+	+	-	-	+	+	+	-	-	+

Note: LR- Long Rod, S- Single, C- Chain, C_1- Central, T- Terminal, C_2- Cylindrical, E- Ellipsoidal.

thrive under harsh conditions and may be useful for industrial application. The traditional cultivation based methods have a great importance in research, providing the chance in investigations of biotechnologically significant bacterial isolates under in vitro and this work provides a structure for supporting further studies of enzyme producing bacteria from these evidently important habitats.

CONCLUSION

The study indicated that the isolated and identified fifteen amylolytic microorganisms have ability to hydrolyze starch by producing amylase enzymes which can be commercially exploited for detergent and leather industry. The present study is a preliminary screening report of diversity of Bacillus species and their enzyme producing potential from Lonar crater as well as revealed a high taxonomic diversity among these isolated Bacilli. Isolation of bacterial strains from Lonar Lake would also provide extensive scope to assess their biotechnological potential.

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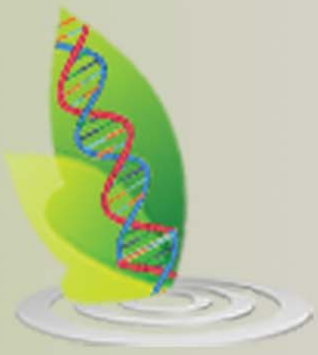

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