PHENETIC DIVERSITY OF ALKALINE PROTEASE PRODUCING BACTERIA FROM ALKALIPHILIC ENVIRONMENT

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ABSTRACT

Culture dependent phenotypic characterization analyses were applied to study the protease producing bacteria from Indian Soda (Lonar Crater). The uniqueness of the Lonar Lake water is its salinity and alkalinity. One hundred and fourteen bacterial cultures were isolated from the water and sediment samples collected from the hyperalkaline saline environment of Lonar crater using eleven different enrichment media. Out of one hundred and fourteen, thirty two bacterial strains were selected. Proteases producing bacterial strains were selected on the basis of their proteolytic activity. All these bacterial strains showed optimum growth and protease production at pH 10. Majority of the alkaliphiles were identified as different species of the genus *Bacillus* on the basis of morphological and biochemical characterizations. The study indicated that the isolated and traditional base identified thirty two proteolytic microorganisms have ability to hydrolyze casein by producing protease enzyme which can be commercially exploited for detergent and leather industry. The present study is screenings of diversity belong to Bacillus species and their enzyme producing potential from Lonar crater as well as revealed a high taxonomic diversity among these isolated bacterial species. Isolation of bacterial strains from Lonar Lake would also provide extensive scope to assess their biotechnological potential

Key Words: Bacillus, Diversity, Enzyme, Protease, Application

Extremozymes are now-a-day replacing chemical catalyst in manufacturing of chemicals, textiles, pharmaceuticals, paper, food, leather processing and agricultural chemicals since the enzymes prepared with suitable properties with the advent of new knowledge in biotechnology (Kanekar et al., 1997; Yang et al., 2000; Alagarsamy et al., 2005). The Lime and sodium sulphide were used for the removal of hair from leather but presence of these chemicals in tannery waste is responsible for remarkable pollution, causing health hazards to the tannery workers and Lime produces a poisonous sludge while sodium sulphide is highly toxic and has obnoxious odor (Malathi and Chakraborty 1991; Alessandro et al., 2003; Thanikaivelan et al., 2003). Although the use of enzyme for dehairing process reduces the pollution burden to some extent, a technology based on enzyme alone, without the use of sulphide and other chemical inputs, has yet to be explored (Purushotham et al., 1996). Alkaline protease producing bacteria are of also great importance in detergent and textile industry due to their high thermostability and pH stability and most important industrial enzymes, accounting for about 60% of total enzyme market. As there is large demand of protease, isolation and production of extremozyme is most important to fulfill this demand (Zambare et al., 2007). But such types of extremozyme are produced from microorganisms which survive in extremophilic environment such as Soda Lake and alkaline hot springs. As far as Indian alkaline saline lakes are concerned, a culture-dependent approach has still not been used to analyze bacterial diversity. Haloalkaliphiles is focused on microbiological classification and genetic characterization, with limited work to discover their industrial application. We have applied this strategy to explore the proteolytic diversity of aerobic bacteria from Lonar Lake.

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MATERIALS AND METHODS

Enrichment and isolation: Enrichment and isolation of microorganisms Lonar lake water and sediment sample will be collected in sterile bottles and polythene bags respectively, from defined sampling site. Enrichment of water samples and sediment samples were carried out in eleven enrichment media. Well-isolated and differentiated colonies will be transferred to respective medium agar slants (Joshi et al., 2007; Tambekar and Dhundale 2012).

Screening for protease production

Screening of bacterial alkaliphiles Individual bacterial colonies will be screened for proteolytic activities on casein agar medium (Casein 1.0, Peptone 5.0, Yeast Extract 1.5, Beef extract 1.5, Sodium Chloride 5.0, Agar 20.0, pH 10). The inoculated plates will be incubated at 37°C for 48 h. The halo zone will be observed for proteolytic activity of the isolates. The isolate will be selected for further characterization and detailed studies.

Identification of the bacterial culture

Bacteria identify on the basis of physiological and standard biochemical test according to Bergey's Manual of systematic bacteriology.

Statistical Analysis

Statistical analysis of cultural, morphological and biochemical characteristic data were analyzed. 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 METLAB program.

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 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 for there biotechnological potential. In the present study, Total one hundred and fourteen isolates obtained in the isolation exercise, cultural, morphological characteristics of all the strains were studied their tolerance of pH, salt and temperature.

Out of one hundred and fourteen, thirty two bacterial culture which were found proteolytic activity. All the bacterial strains were found both alkaliphilic (7-12) and the optimum pH was revealed 10 for all the bacterial culture. Some protease producing bacterial strains were also studied from some moderately halophilic environment (Joshi et al., 2007). Gessesse and Gashe (1997) found both alkalitolerant and obligate alkaliphiles were found and identified by phylogenetic analysis as microbial species found in soda lake microbial population and known for being good protease producers. All the bacterial cultures were found gram positive and spore forming bacilli (Table 1). All the bacterial strains were found stable upto 45°C and several were found thermotolerent upto 50°C. Majority of the alkaliphiles were identified as different species of the genus *Bacillus* on the basis of morphological and biochemical characterizations, which are known to produce a wide variety of enzymes with tolerance to thermal and alkaline conditions (Nthangeni et al., 2001). These alkaliphilic *Bacillus* strains are of considerable industrial interest, especially for the production of proteases for laundry detergents. A wide diversity of physiological abilities is exhibited, ranging from psychrophilic to thermophilic and alkaliphilic some strains were salt tolerant and some are halophilic. Seven carbohydrate substrates were catabolized by essentially all strains tested. These were glucose, mannitol, sucrose, fructose, maltose, xylose and arabinose. Similar study also performed by NieIsen *et al.* (1995).

Dendrogram was based on comparison of the morphological, cultural and biochemical characteristics of thirty bacterial species found in six main cluster. In first cluster seven bacterial species were found. These bacteria make two subclusters. In this CW2, CW3, CW1 and EW1 are highly similar. In second cluster sixteen bacterial species were found. These bacteria make seven subclusters. AW4(3) and BW4(1) were made third cluster. The high similarity on the basis of their physiological characters between *Bacillus* sp. HS3 and OCW3(1) were found and separately made fourth and fifth

cluster respectively, on the basis of their physiological characters. In sixth cluster, three bacterial species were found. The high similarity on the basis of their physiological characters between DS3, DS7 and HS4 (Fig 2).

OCW3(1) shows highest proteolytic activity with 40 mm zone. BW4(4), DW4(1), BS1(1), CW3(3) and ODW3(2) also show high proteolytic activity by producing zone within range of 21-25 mm. From BW1(1) to DS7 bacteria shows moderate proteolytic activity by producing zone within the range of 16-20 mm. The minimum proteolytic activity was seen from IW3 to HS3. Our current work is interested with casein 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 (Fig 3).

Finding of this study provides a opportunity for protease producing bacterial diversity from Lonar Lake. Thus these alkaline enzymes have industrially usable due to their tolerance to industrial process (Horikoshi. 1999). This study indicated the alkaliphilic indigenous bacteria as wealth origination of enzyme but it needs to explore for the enzyme production and purification. Now a day the high demand of enzyme has great regard to the future for improvement in properties for biotechnological applications and producing novel enzymes for broad range to industrial level because of molecular enzymology. The developing novel techniques in genetic engineering with great knowledge of structure and function are allowing industrial needs and the exploration of novel applications (Gupta et al., 2004). For understanding of role and structures of microbial communities for multipurpose approach is necessary, cultivability of the microorganisms important for the study of their physiology and metabolism (Borsodi et al., 2005; Joshi et al., 2007).

CONCLUSION

The present study was carried out with the purpose of defining proteolytic enzyme from haloalkaliphilic bacterial strain, which were adapted to live at extreme salt and alkaline environments. In our study, the culturable dependent approach was applied to study the protease producing bacteria from Lonar crater. These extreme haloalkaliphiles in general were specialist since them able to survive under alkaliphilic conditions and may be useful for industrial application. The cultivation based methods have a great importance in research, providing the possibility in investigations of biotechnologically significant bacterial isolates under laboratorial stipulation and this work revealed a data for supporting further studies of enzyme producing bacteria from these evidently alkaliphilic habitats. In the present study the physiological and biochemical were used for the identification of bacterial culture but the biochemical results suggest that, the bacterial culture were belonging to *Bacillus.* 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 screening of the bacterial culture to avoid the replicating culture for sequencing and other genetic method for identification of bacteria in future. However, It would be more instructive if proteolytic enzymes are purified from the isolates and then characterized, which is the further planning of the currentinvestigation

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Fig 1: Casein hydrolysis activity shown by *bacilli* strain isolated from Lonar crater

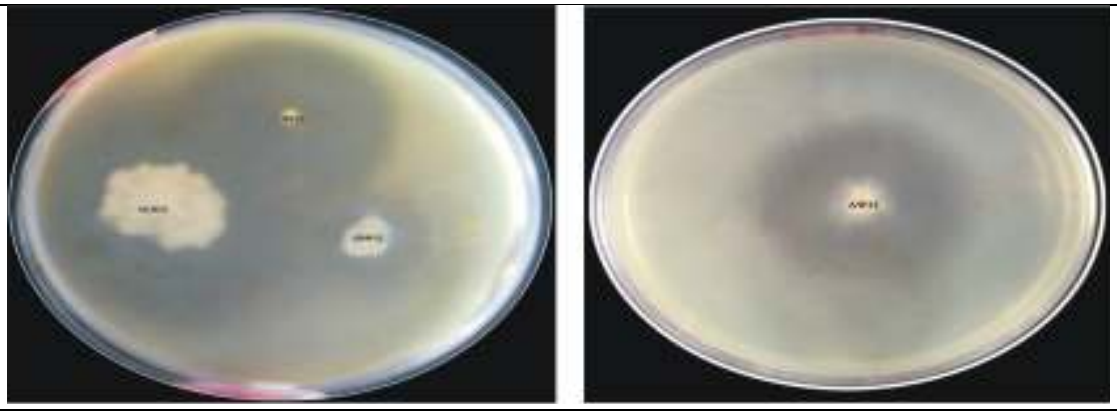

Fig 2: Dendrogram based on percent similarity of the biochemical characteristics of bacterial isolates from Lonar crater. The dendrogram was created by the using average linkage (Between Groups)

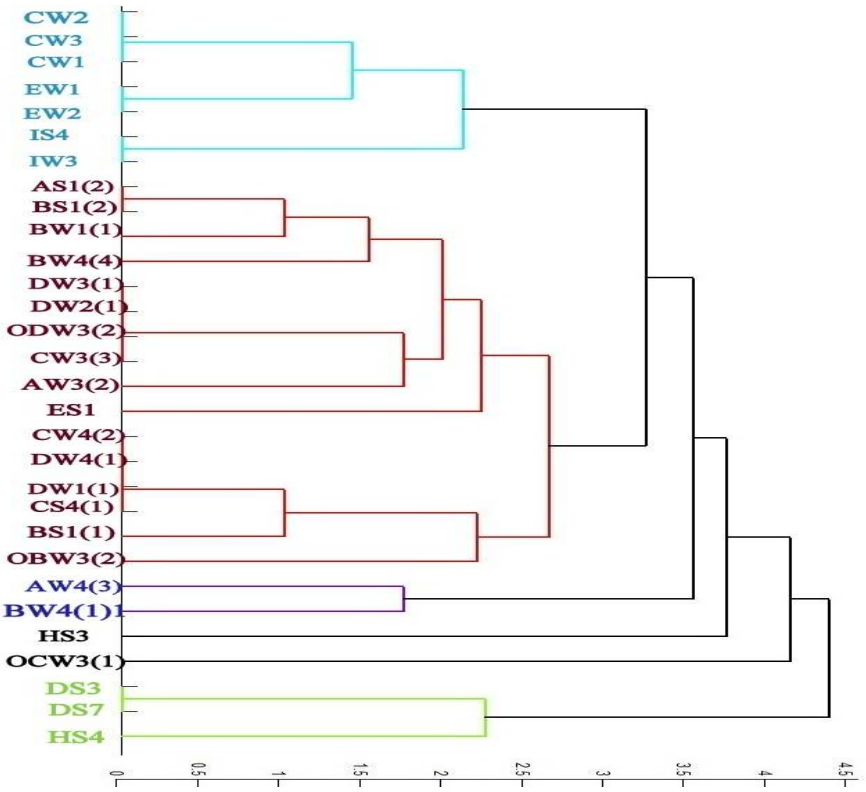

Fig 3: Starch hydrolysis activity shown by bacilli strain isolated from Lonar crater

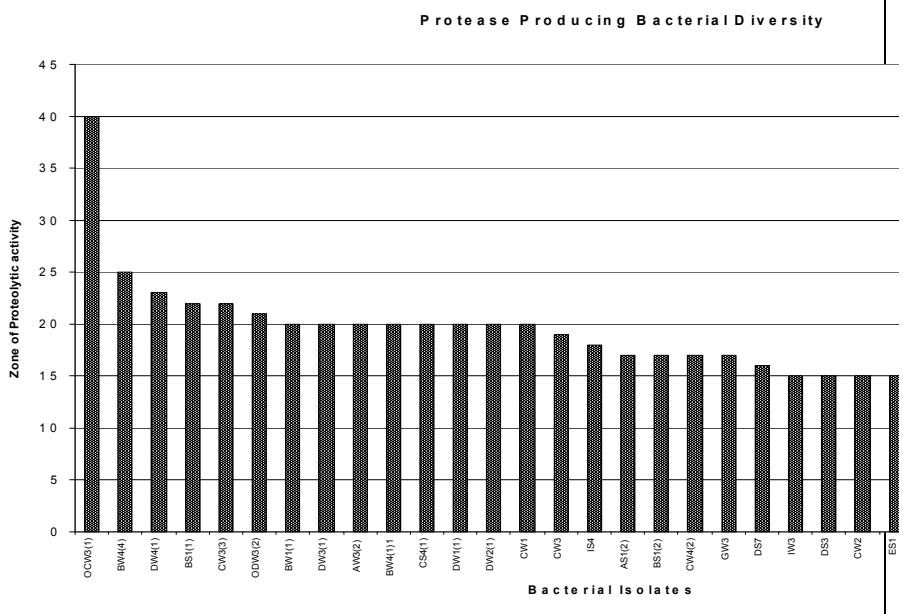

Table 1 : Morphological, cultural and physiological characteristics of bacteria

Isolation Code	Gram Reaction	Shape of Bacteria	Enrichment medium	Size of Colony	Pigment	Shape of Colony	Elevetion	Edge	Catalase	Oxidase	Glucose	Arabinose	Mannitol	Xylose	Sucrose	Maltose	Fructose
AS1(2)	+	Long Rod	A	1	White	Circular	Effuse	Entire	+	+	+	-	-	-	-	-	-
BS1(2)	+	Long Rod	B	1	White	Circular	Effuse	Entire	+	+	+	-	-	-	-	-	-
BW1(1)	+	Long Rod	B	1	White	Circular	Convex	Entire	+	+	+	-	-	-	-	-	-
BW4(4)	+	Long Rod	B	1	Colourless	Circular	Effuse	Entire	+	+	-	-	-	-	-	-	-
CW4(2)	+	Long Rod	C	3	White	Circular	Umbonate	Entire	+	+	-	-	-	-	-	-	-
DW3(1)	+	Long Rod	D	3	White	Circular	Effuse	Entire	+	+	+	+	+	-	+	-	-
DW4(1)	+	Long Rod	D	2	White	Circular	Umbonate	Entire	+	+	-	-	-	-	-	-	-
AW3(2)	+	Long Rod	A	5	White	Circular	Effuse	Conves papilate	+	-	+	-	+	-	+	-	-
AW4(3)	+	Long Rod	A	4	White	Irregular	Effuse	cerenate	+	+	-	-	-	-	-	-	-
BS1(1)	+	Long Rod	B	2	Colourless	Circular	Umbonate	Entire	+	+	-	-	-	-	-	-	-
BW4(1)1	+	Filamentous	B	4	Colourless	Curled	Effuse	cerenate	+	+	-	-	-	-	-	-	-
CW3(3)	+	Long Rod	C	3	White	Circular	Effuse	Entire	+	+	+	+	+	-	+	-	-
CS4(1)	+	Long Rod	C	2	White	Circular	Umbonate	Entire	+	+	-	-	-	-	-	-	-
OCW3(1)	+	Long Rod	C	6	White	Irregular	Umbonate	Undulate	+	-	+	+	+	-	+	-	+
DW1(1)	+	Long Rod	D	2	White	Circular	Umbonate	Entire	+	+	-	-	-	-	-	-	-
DW2(1)	+	Long Rod	D	3	White	Circular	Effuse	Entire	+	+	+	+	+	-	+	-	-
ODW3(2)	+	Long Rod	D	3	White	Circular	Effuse	Entire	+	+	+	+	+	-	+	-	-
OBW3(2)	+	Long Rod	B	1	Colourless	Circular	Raised with concave belaved	Entire	+	-	+	-	+	-	-	-	-
IS4	+	Short Rod	I	1.5	Yellow	Circular	Convex	Entire	+	+	+	+	+	+	+	+	+
IW3	+	Short Rod	I	1	Yellow	Circular	Convex	Entire	+	+	+	+	+	+	+	+	+
EW1	+	Short Rod	E	1	White	Circular	Convex	Entire	+	+	+	+	-	+	-	-	+
EW2	+	Short Rod	E	1	White	Circular	Convex	Entire	+	+	+	+	-	+	-	-	+
DS3	+	Short Rod	D	1.5	Yellow	Oval	Convex	Irregular	+	+	+	+	+	+	+	+	+
DS7	+	Short Rod	D	2	Yellow	Oval	Convex	Irregular	+	+	+	+	+	+	+	+	+
CW1	+	Short Rod	C	1	Colourless	Circular	Convex	Entire	+	+	+	+	-	+	+	-	+

CW2	+	Short Rod	C	1	Colourless	Circular	Convex	Entire	+	+	+	+	-	+	+	-	+
CW3	+	Short Rod	C	1	Colourless	Circular	Convex	Entire	+	+	+	+	-	+	+	-	+
HS3	+	Long Rod	H	2.5	Creamy White	Circular	Convex	Irregular	+	+	+	+	-	+	+	+	+
HS4	+	Long Rod	H	3	Creamy White	Circular	Convex	Irregular	+	+	+	+	-	+	+	+	+
ES1	+	Long Rod	E	1	White	Circular	Convex	Entire	+	+	+	+	-	+	-	-	+
DS2	+	Long Rod	D	1	White	Circular	Convex	Entire	+	+	+	+	-	+	-	-	+
GW3	+	Long Rod	G	1.5	Yellow	Oval	Convex	Irregular	+	+	+	-	+	+	+	+	+

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