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Isolation and screening of multiple enzyme producing Alkaliphilic *Bacillus species* from Lonar Lake

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Abstract
Lonar Lake is a unique ecosystem in India and was formed by meteorite impact on basaltic rock. It is situated in a village Lonar in the Buldhana district of Maharashtra State, India. Lonar Lake has been reported for the presence of diverse bacterial genera which are able to produce a number of different enzymes. These enzymes particularly lipases, proteases and amylases show their activity at high pH. The objective of this study was to isolate multienzyme producing *Bacillus* species from Lonar Lake source. In the present study, a total of 20 water samples were collected from Lonar Lake in sterilized bottle. 28 *Bacillus* species were isolated and identified on the basis of morphological and biochemical characteristics as well as screened for amylase, protease and lipase enzyme production. Among the 28 isolates, three isolates were found multienzyme producers namely AB03, AB10 and AB21. These isolates were confirmed by 16srRNA. Among these three isolates AB10 isolate was found most prominent species for multienzyme production. The promising single strain which can produce multienzyme has potential to be used in various economic industrial processes as it can withstand high pH conditions.

Keywords: amylase, protease, lipase, 16s rRNA, Lonar lake

Introduction

Lonar Lake of Maharashtra is a unique ecosystem with an alkaline environment including alkaliphilic and halophilic bacteria such as *Bacillus sp.*, *Halomonas campisalis*, *Alkalibacillus haloalkaliphilus* etc and was created due to a meteorite impact (Deshmukh *et al*., 2011; Kanekar *et al*., 2007) [3, 6]. Lonar crater is the third natural salt water lake in world and has pH 10.5 of lake water. It is an almost circular depression (Borul, 2012) [1]. Lonar Lake is most suitable for alkaliphilic bacteria. The term alkaliphile is used for microorganisms that grow optimally or very well at pH values above 9, often between 10 and 12, but cannot grow or grow only slowly near neutral pH value of 6.5 (Horikoshi, 1999) [5]. Members of diverse bacterial genera which are present in Lonar Lake have been reported to produce different types of enzymes (Tambekar and Tambekar, 2012) [13, 14].

Microbial enzymes are usable resources with the advent of new knowledge in the pharmaceutical, biotechnology, clinical, medicinal fields, as well as its widespread use in starch saccharification. Alkaline enzymes have a dominant position at worldwide market in pharmaceutical, leather, detergent and textile industry (Shanmughapriya *et al*., 2009; Sarethy *et al.,* 2011) [11, 10]. Lipase can be used to accelerate the degradation of fatty waste, polyurethane, removal of oil stains from fabrics, development of flavoring agents in milk, cheese, and butter, fat removal, (Masse *et al*., 2001; Takamoto *et al*., 2001) [8, 12]. Proteases are also one of the most important of industrial enzymes and account for almost 60% of the total enzyme sale (Brown and Yada, 1991 and Escobar and Barnett, 1993) [2, 4]. The major uses of free proteases significantly occur in dry cleaning, detergents, meat processing, cheese making etc (Nout and Rombouts, 1990) [9]. α-amylases are some of the most versatile enzymes in the industrial enzyme sector Many applications of these enzymes have been reported in various literatures. The aim of our screening was to obtain these enzymes from a single alkaliphle that can work at high P^H. The attempt of this study was to isolate the multienzyme producing *Bacillus* species from Lonar Lake source.

Materials and Methods

Collection of samples

Twenty water **s**amples were collected from Lonar Lake by wearing hand gloves. All the samples were collected by keeping a 5 feet distance between two samples. These samples were kept in icebox and immediately transported to laboratory and stored in refrigerator.P^H of the samples was determined by using P^H meter (McLean, 1982) at the site and in the laboratory as well. For the isolation of only *Bacillus* species, 10ml water sample was taken in sterilized glass tube and boiled at 80^0C in water bath to kill the vegetative cells and cooled the samples and inoculated on Nutrient agar figure containing pH 10 and incubated for 24 to 48h at 37^0C.

Isolation of Multiple Enzyme Producing Microorganisms

The isolation of the multiple enzyme producing microorganisms was carried out on different media. For that purpose Skimmed milk agar (SMA) was used for protease producing activity, Tributyrin agar for Lipase producing activity and Starch agar was used for amylase producing activity**.** A loopful from each sample out of 20 samples were streaked on to Skimmed milk agar, Tributyrin agar and Starch agar. All the figure were then incubated for 24 hours at the 37^0C. After incubation is over the figure were observed for the growth of the microorganisms. Some microorganisms producing the respective enzyme were identified.

Identification of enzyme producers

Protease producer was identified on the skimmed milk agar by observing the zone of hydrolysis around the colony or growth. In similar way, Lipase producer was identified on the Tributyrin agar by observing the zone of hydrolysis around the colony or growth. The amylase producer was identified by spreading iodine solution on the starch agar figure containing growth and then it was observed for the white zone around growth with media getting blue colour. (Note: Starch agar figure was streaked in duplicate) After identification of enzyme producer each colony was then isolated by streaking on the slants of nutrient agar. Then these slants were incubated at 37^0C for 24-48 hrs.

Screening of multienzyme producing microorganism

After 24-48 hrs. of incubation, slants containing different enzyme producers were observed for growth. Each colony irrespective of its source was spot inoculated on the all three media figure and labeled with the source of media on which it was isolated. (Note: Starch agar figure were streaked in duplicate) Then all the figure were incubated at 37^0C for 24 to 72 hrs. After incubation (may be different for different microorganism) figure were observed for growth and enzyme production. Single organism giving zone on all the three agar figure viz; Starch agar, Skimmed milk agar and Tributyrin agar was selected as a multiple enzyme producer. The diameter of the zone on each figure was measured and noted down.

Isolated multienzyme producer then identified and sequenced

The morphological and biochemical characteristics of the isolates were studied. The biochemical reactions included glucose fermentation, catalase and oxidase production. The 16S rDNA sequences analysis was carried out at the National Centre for Cell Science, Pune (MS), India, and sequences were submitted to NCBI GenBank Database for the accession Numbers.

Sequence matching by RDP

The isolates which have maximum multienzyme producing activity were selected for confirming identification. These isolates were initially identified by Ribosomal Database Project (RDP), from the RDP website (http://rdp.cme.msu.edu).

Sequences obtained through RDP were subjected to Basic Local Alignment Search Tool (BLAST) to obtain significant relationships from chance similarities.

Results and Discussion

In the present study, a total of 20 water samples were collected from Lonar Lake in sterilized bottle. From the 20 water samples, 28 *Bacillus* species were isolated and identified on the basis of Morphological and Biochemical characteristics as well as screened for Amylase, Protease and Lipase enzyme production. Out of 28, three bacterial strains produced all the three enzymes screened for while 10 bacterial strains were found to produce two enzymes and 11 bacterial strains produced one enzyme. (Fig. 1) Few moderate alkaliphilic strains especially representatives of the genus *Bacillus*, are able to produce extracellular amylase, lipase and proteases that are even active at alkaline pH (Martins *et al.,* 2001; Vargas *et al*., 2004; Tambekar and Tambekar, 2012 [13, 14].

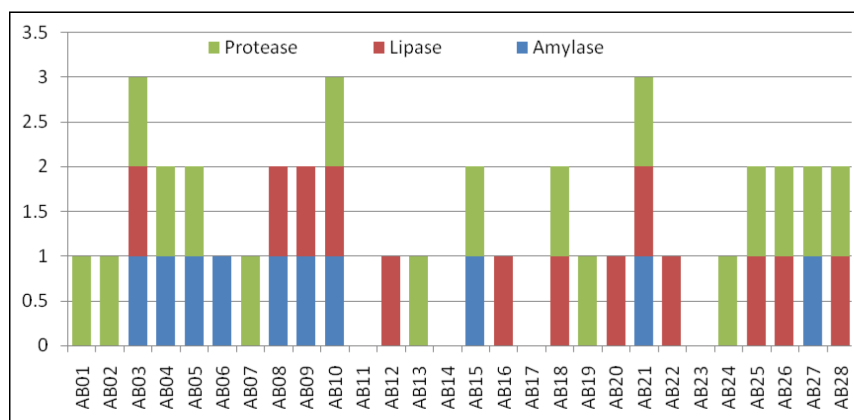

Fig 1: Production of Multienzymes from Alkalophilic Lonar lake *Bacillus* sp.

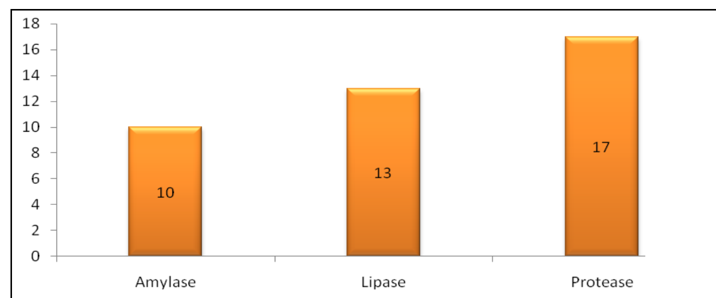

Fig 2: Enzyme producer isolates from Lonar Lake

In the present study, *Bacillus* species were screened for Amylase, Lipase and Protease production and result showed that out of 28 *Bacillus* strains 17 isolates (60%) were protease producer followed by 13 isolates (46%) Lipase producer while 10 isolates (35%) were Amylase producer (Fig. 2 and 3).

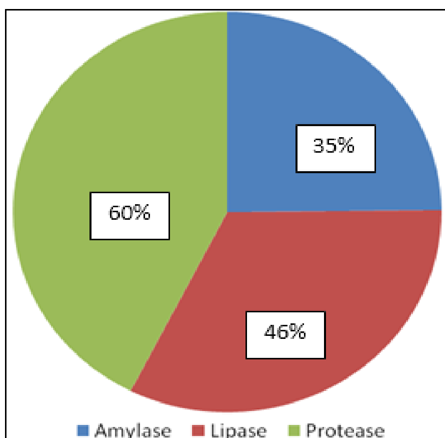

Fig 3: Percentage of enzymes production from isolated bacteria from lonar lake

Among the 28 isolates, 3 isolates of *Bacillus* sp. were found multienzyme producers such as AB03, AB10 and AB21. Multienzyme production activities of these isolates was checked at intervals of 24, 48 and 72h. These isolates were confirmed by 16srRNA as *Bacillus subtilis and Bacillus pumilus*. Among these three isolates AB10 isolate was found most prominent species for multienzyme producers.

Table 2: Screening for amylase activity on starch agar

Isolates	Zone of hydrolysis (mm)		
	24hrs	48hrs	72hrs
AB 03	12	19	24
AB 10	15	28	34
AB 21	14	15	18

Table3: Screening for lipase activity on Tributyrin agar

Isolates	Zone of clearance (mm)		
	24hrs	48hrs	72hrs
AB 03	19	22	27
AB 10	14	12	17
AB 21	13	15	16

Table 4: Screening for Protease activity on Skimmed milk agar egg yolk agar

Isolates	Zone of solubilization (mm)		
	24hrs	48hrs	72hrs
AB 03	15	30	35
AB 10	18	22	26
AB 21	9	14	18

Fig 4: 24 hour incubation

Fig 5: 48 hour incubation

Fig 6: 72 hour incubation

Fig 4-6: Protease production activity of isolates after 24, 48 and 72 h. respectively

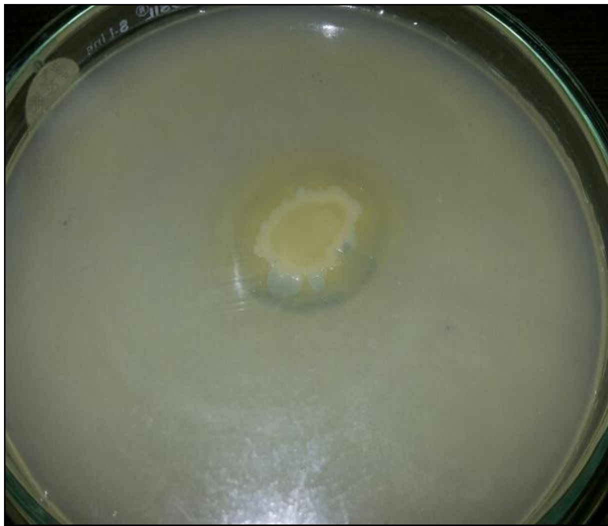

Fig 7: 24 hour incubation

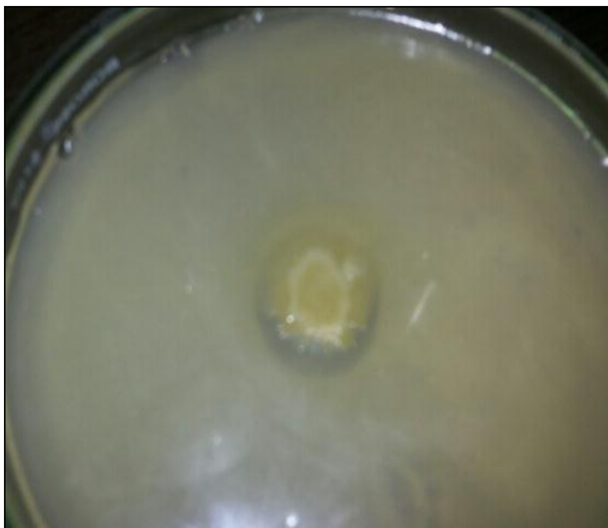

Fig 8: 48 hour incubation

Fig 9: 72 hour incubation

Fig 7-9: Lipase production activity of isolates after 24, 48 and 72 h. respectively

Fig 10: 24 hour incubation

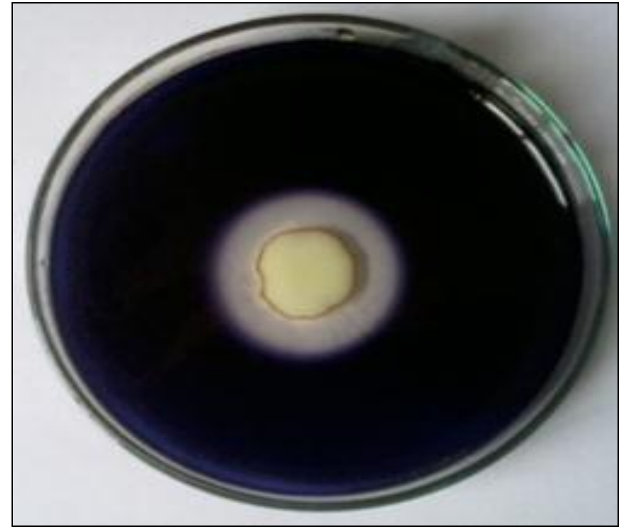

Fig 11: 48 hour incubation

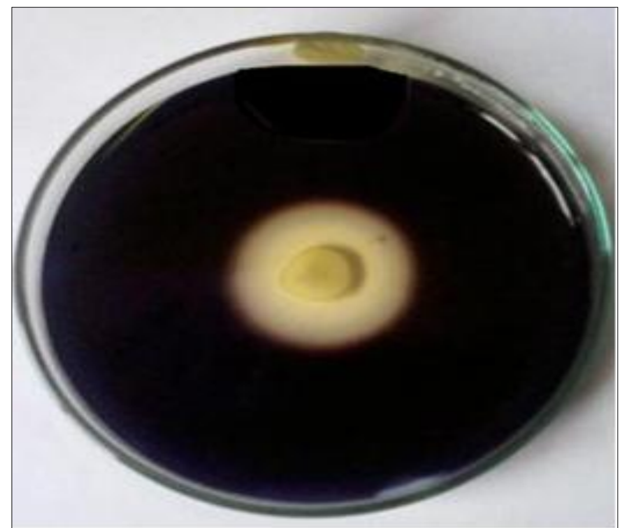

Fig 12: 72 hour incubation

Fig 10-12: Amylase production activity of AB10 isolate after 24, 48 and 72 hours respectively

From the findings, *Bacillus* species isolated from Lonar Lake were promising source of the extracellular enzymes and exhibiting alkaline thermo stability that making it a valuable tool in industrial enzyme production. Thus these alkaline enzymes are of industrial and biotechnological interest due to the fact that they may be used for harsh industrial process.

This study stated that alkalophilic indigenous *Bacilli* can be rich sources of enzyme but it needs to investigate the partial production and purification. These bacteria have potential to produce biotechnologically important enzyme. However, it would be more informative if crude enzymes such as Amylase, Protease and Lipase is purified from the isolate and characterized, in future research.

Conclusion

This study reports multienzyme producer isolate (AB10) from alkaline Lonar Lake. This isolate was found most prominent species for multienzyme production. Hence such promising single strain which can produce multienzyme like Lipase, Protease and Amylase may be used in various economic industrial applications and also for study of their physiology, and understand their importance in such unique environments.

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