Isolation of Alkalophilic Bacteria from Lonar Crater and its Insecticidal Protein Producing Ability

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Abstract - Alkalophilic bacteria were isolated from water and sediment samples collected in rainy season,June 2010 from alkaline Lonar lake.India having pH -10.5 .The total viable count (TVC) were found to be in the range of (10^3 to 10^4 cfu ml^{-1}) and (10^3 to 10^5 cfu g^{-1}) in water and sediment sample respectively. Five different bacterial strain were isolated using different enrichment media. These five isolated bacterial strain was furture studied for Morphological characterization on the basis of the different characters such as colony colour, colony shape, colony surface, colony elevation and colony edge.These five isolates were furthur subjected to biochemical characterization and 16SRNA sequencing. Biochemical characterization of these isolates showed that out of five isolates four isolates were gram positive and one was gram negative when observed under light microscopy. The taxonomic identification of the all bacterial isolates by 16S rRNA sequencing showed that four isolates were from Bacillus species and one isolate from Halomonas species. The BLAST results of these isolates are Bacillus thurengeinsis serovar finitimus, Bacillus licheniformis, Bacillus cereus, Halomonas campisalis, Bacillus pseudofirmus .These five alkalophilic bacteria were furthur studied for isolation and characterization of enzyme/protein having insecticidal potential.

Keywords - TVC, alkalophilic bacteria.

I. INTRODUCTION

Alkalophiles, bacteria living in alkaline environment, have been have been flourishing everywhere on our planet. However, only a few scientists, have shown interests in this microbial domain and its application in various agricultural fields. They can thrive in neutral as well as alkaline environments because they can change their surrounding from neutral to alkaline pH value by producing basic compounds or by symbiosis. However, alkalophiles were thought to require only higher pH values for their growth. The term "Alkalophiles" is applied only to microorganism that grow optimally or very well at pH values above 9.0 ,but cannot grow or grow slowly at neutral pH values of 6.5 -7.0 (Horikoshi,1992).

Alkalophilic microorganism are widely distributed throughout the world. There are three largest lake in the world where alkalophiles are found. Among them, Lonar crater in Maharashtra is the third largest and meteriotic crater having alkaline pH. (Siddiqui2008).

Takami et al (1997) isolated thousands of microbes from mud samples collected from the Mariana Trench. The microbial flora found at a depth of 10,897m was composed of actinomycetes,fungi, non exteromophilic bacteria and various exteromophilic bacteria such as alkaliphiles thermophiles and psychrophilies.

Lonar lake ecosystem has reported to contain rich bacterial diversity. The microorganism, alkalophilic bacteria, in this environment would therefore be unique.

Lonar crater is a classic beautiful bowl shaped depression in the basaltic flows of the Deccan traps in Southern India believed to be formed as a result of high volecity impact of huge meteror of extra terrestrial origin. Rightly rated as the third largest and oldest meterotic crater is about 52000 year old crater size 1800-2000m in diameter, height is 20-30m,depth 150m and placid water spread areas 77.69 ha. The water of this lake are characterised by very high alkaline pH of 8 to 10.5.(Gopalkrishna, 2000)

Lonar Lake is rich in microbial diversity. Microbial ecology of Lonar Lake have been earlier studied by various scientific groups. Lonar Lake represent most stable naturally occurring alkaline environment on earth. This environments typically contain high concentration of sodium carbonate or complexes of salt. (Jhingram and Rao,1912).

Joshi et al (2006) have found that, microorganisms like Arthorospira, Algae, Spirulina, Clostridium, Chlorella and various type of bacteria are abundant in water of this lake. Spirulina sp. growing in high alkaline area has been studied for various microbiological interaction and categories as alkalophiles. Despite the temperature optima for these bacteria is in mesophilic range but some isolate show thermotolent character. Nitrogen fixing microorganism have also found in this lake which are all halophilic in nature and grow at pH 11.

Attempts were made to study the biodiversity of cultivable bacteria of lonar lake Aerobically alkalophilic bacteria was isolated and characterized from water and sediment sample from lonar lake. Number of microorganism in sediment sample and water sample was found to be at $10^2 – 10^6$ cfu g^{-1} and 10^2-10^4 cfu ml^{-1} respectively. One hundred and ninety six microbial strain were isolated using different enrichment media. Sixty four isolates was subjected to phenotypic biochemical analysis. Phylognetic analysis indicated that most of Lonar lake isolates was related to phylum firmicutes containing low G + C having Gram positive bacteria with different genera Bacillus, Alkalibacillus, Exiguobacterium. Seven strains constituted a Gram negative bacterial growth with different genera *Halomonas, Strenotrophomonas* (Joshi *et al* 2008) .

As the nature of he Lonar lake is alkaline most of the strains were alkali tolerant and only two strains were obligate alkalophilic bacteria These bacteria were found to produce biotechnologically important enzymes at alkaline pH. However production and characterization of insecticidal enzyme/protein have not be reported so far. These insecticidal enzymes/protein thought to act effectively in insect gut (alkaline condition) because of their alkaline stability. Considering this present investigation is planned with the objective to isolate alkalophilic bacteria and to explore their insecticidal protein producing ability.

II. MATERIAL AND METHODS

Agar powder, Leuria broth, crystal violet, Gram iodine, Saffranin Glucose peptone broth Kovac's reagent α-napthol 40%KOH Simmons Citrate agar

SAMPLING SITE AND SAMPLE COLLECTIONS

Different samples comprising of sediment sample and water sample were collected from different sites of Lonar lake. as shown in table 1 and fig 1The sediment sample were collected with the help of scooper in sterile polyethylene bags or sterile bottle. Water samples were collected directly into sterile bottle. They werel labelled, and store at 4°C until further analysis. The pH, temperature and depth of sampling were noted, immediately at site of sample collection. The pH of the water and sediment sample was 10.5 at the time of collection of sample.

III. ENEMERATION OF BACTERIA FROM LONAR LAKE

Determination of total viable cell count (TVC) present in the water and sediment of Lonar Lake were done by serial dilution method. Serial dilution were carried out in sterilized saline water and diluted samples were poured on the Leuria broth of various pH viz, pH 10,11,12 by Pour plate technique. The plate were incubated at room temperature (28$\pm$2°C) for 48 h.

IV. CHARACTERIZATION OF ISOLATED ALKALOPHILES

Bacterial strain were examined for their colony and cell morphology, Gram staining and standard biochemical tests IMVic test.

V. MORPHOLOGICAL CHARATERIZATION

For the purpose of the morphological characterization of the alkalophilic isolate. All the isolates were grown on LB agar. Isolated bacterial strain was observed for colony morphology under stereoscopic zoom microscope under low magnification. Colonies were observed for colony colour, shape ,margin ,surface, edge and elevation.

VI. CHARATERIZATION BASED ON STAINING

GRAM STAINING

Twenty four hrs old bacterial cultures were used to observe the morphology of vegetative cells and differential characterization of alkalophilic isolates by using gram staining. procedure given by Krieg and Holt1984.

VII. BOCHEMICAL CHARATERIZATION

Morphologically and microscopically characterized probable Bacterial isolates and its characterization based on staining were further confirmed by the biochemical characterization which comprises of IMViC test (Indole production, methyl red, Voges-Proskauer, citrate utilization test).

VIII. IDENTIFICATION OF ALKALOPHILES BY 16S rRNA SEQUENCE ANALYSIS

The taxonomic identification of the all bacterial isolates was confirmed by 16S rRNA sequencing. 16S rRNA molecules are almost ideal for studies of evolution and relatedness in bacteria since they are found in all bacteria and conserved in the nature. The 16S rRNA sequencing was carried out at NCCS Pune.

IX. PRODUCTION OF THE DIFFERENT INSECTICIDAL ENZYMES/PROTEINS FROM THE ALKALOPHILES BY USING SPECIFIC PRODUCTION MEDIA

Five different enzymes/proteins such as Amylase inhibitor, Protease inhibitor, Alkaline protease, Cholesterol oxidase, Chitinase were produce from the alkalophiles obtained from Lonar crater by using specific production media.

1.Amylase inhibitor

Alkalophiles were subjected for production of Amylase inhibitor by using the media given by Murao et al. 1976. and the inhibitory activity assay were performed according to the protocol given by Moreno et.al 1989.

2.Protease inhibitor

Alkalophiles were subjected for production of Protease inhibitors by using media given by Dash and Rao 2001 and the protocol given by Ceelia et al. 2002 was followed for decipiting the Protease inhibitory assay to its insecticidal activity

3.Alkaline protease production

Alkalophiles were subjected for production Alkaline protease by using media given by Jackeline et al. (2006). And the protocol developed by Kalpana Devi et al in 2006 was used to calculate alkaline protease activity .

4.Cholesterol oxidase production

Alkalophiles were subjected for production of Cholesterol Oxidase by using media given by Yazdi et.al 2001. and the protocol developed by Richmond, in 1973 was used to calculate the Cholesterol oxidase activity.

5.Chitinase production :

Alkalophiles were subjected for production of Cholesterol Oxidase by using media given by Bansode and Bajekal 2006.and The assay system of Monreal and Reese (1969) estimating reducing sugars released by enzyme action, was adapted to study its protein producing ability.

X. DETERMINATION OF OPTIMUM pH AND TEMPERATURE OF INSECTICIDAL ENZYMES OBTAINED FROM ALKALOPHILES

The optimum pH and optimum temperature were determined by incubating the insecticidal enzymes at different temperature range viz 30°C 40 °C, 50°C, 60°C. during enzyme assay . The assay was carried out as decribed above only the incubation temperature was changed from 30°C to 60°C. Similar way the optimum pH was examined only by changing the pH of the Tris –HCl buffer used during assay was changed. Different pH range viz.8, 9, 10, 11, 12 was used during assay.

XI. DETERMINATION OF OPTIMUM pH AND TEMPERATURE STABILITY OF INSECTICIDAL ENZYMES OBTAINED FROM ALKALOPHILES

The measure the pH stability of the insecticidal enzymes isolated from alkalophiles a solution of enymes (50 µl/ml) was diluted with an equal volume of buffer with different pH range(pH 8-12).After 1 hr incubation in each buffer at 37°C, the residual inhibitory activity of all enzymes was measured as described above.(Yoshizaki et al 2007).

To measure the heat stability of the insecticidal enzyme isolated from alkalophiles was determined by incubation of the insecticidal enzyme (50 µl enzyme extract and 60µl 0.1M Tris-HCl pH 9.0) at various temperature (30°C-60°C) for 1h. After treatment the aliquots were cooled on ice and inhibitory assay was carried out for determination of residual activity of insecticidal enzymes from alkalophiles.

XII. NSECT BIOASSY AGAINST Plutella xylostella TO STUDY THE INSECTICIDAL POTENTIAL

The larvae and pupae of *P. xylostella* were collected from cabbage and Cauliflower field from outskirts of Akola. They were reared in the laboratory on the mustard seedlings up to F_4 generations for establishing homologous laboratory population. The rearing procedure described by Lu and Sun (1984) was followed to maintain the test culture of *P. xylostella.*

XIII. BIOASSAY OF SELECTED NATIVE ISOLATES AGAINST *Plutella xylostella*

The bioassay was carried out by cabbage leaf disc dip method in triplicate as described by Tabashnik *et al*. (1987). Mortality in *Plutella xylostella* larvae was recorded and cumulative mortality after 72 hrs. was calculate

XIV. RESULT AND DISCUSSION

For isolation Alkalophiles and their characterization. The water and sediment samples were collected from five different locations of Lonar crater. Table 1.The alkalophilic bacteria were enumerated by counting total viable cells from water and sediment samples of Lonar lake. Enumeration was carried out by two ways i.e with enrichment (by incubating for seven days on rotary shaker) and without enrichment. The leuria agar plates with different pH range (pH 7 to 10) were used for plating the serially diluted samples.

The total number of the aerobic bacteria obtained in Lonar lake water samples was in the range of (10^3 to 10^4)which was comparatively less than that of TVC of sediment sample ($10^3 – 10^5$ cfu g^{-1}). It is evident from Table 3 that, maximum TVC of bacteria was present in the alkaline pH (9-10) than neutral pH.

On the basis of their distinct morphological characteristics only five isolates [designated as A,B,C,D and E] were selected for further study. They were further screened for its detailed morphological, biochemical and microscopic characterization. After characterization they were exploited for the production of different insecticidal enzymes.

Joshi *et al.* (2006) isolated and characterized alkalophilic bacteria from water and sediment sample collected from Lonar lake having pH 10.5. They had isolated one hundred and ninty six strains using different enrichment media while studying the bacterial diversity of Lonar lake. They found that the TVC of the aerobic bacteria from sediment samples was in the range of(10^2 to 10^5 cfu g^{-1}) which was

comparatively more than that of the TVC of the water sample(10^2–10^4 cfu ml^{-1}).

Result of Morphological characteristics were studied under low magnification by using stereoscopic zoom microscope. Table 3 gives the glimpes of the respevtive data. The results of Gram staining were helpful in determining the shape of bacteria and its Gram reaction. as shown in Table 4.

Five isolates obtained were further screened on the basis of biochemical tests. IMViC test i.e. Indole test, Methy Red, Vogus Proskeus and Citrate utilization was carried out to see the difference in the reaction. All five alkalophiles showed positive test for indole, methyl red and citrate utilization tests, while negative for vogas proskaus test. Details are given in Table 5.

A arrays of biochemical tests are needed for the classification of bacteria, therefore, these IMViC results help us in i) initial chacterization of the alkalophiles ii) to support nucleotide the sequencing and BLAST result.

The taxonomic identification of the all bacterial isolates was confirmed by 16S rRNA sequencing. and furthur confirmed by 16S rRNA sequencing 16S rRNA molecules are almost ideal for studies of evolution and relatedness in bacteria since they are found in all bacteria and conserved in the nature. The 16S rRNA sequencing was carried out at NCCS, Pune. The16S rRNA sequences in a FASTA format were used for performing the nucleotide- BLAST available at NCBI web portal. BLAST was carried out by searcing the non redundant (nr) nucleotide collection with default parameters. The results of the sequencing and BLAST are detailed in Figure 1 to Figure 5. The BLAST results confirm the taxonomic classification of alkalophiles is as below.

Isolate A :*Bacillus thuriengiensis* serovar finitimus

Isolate B :*Bacillus licheniformis*

Isolate C :*Bacillus cereus*

Isolate D : *Halomonas campisalis*

Isolate E : *Bacillus pseudofirmus*

First isolate was confirmed as *Bacillus thuriengiensis* (*Bt*). (*Bt*) is a facultative anerobic Gram positive bacterium, which forms characteristics protein inclusion having insecticidal activity. Apart from its insecticidal activity *Bt* found to produce lots of important enzymes e.g. alkaline protease (Alvarez et al in 2009).

Second isolate was confirmed as *Bacillus licheniformis* It is a bacterium that is commonly found in soil and bird feathers. *Bacillus licheniformis* is a rod-shaped, Gram-positive bacterium. It tends to form spores in soil which makes it desirable to be used for the industrial purposes such as the production of enzymes, antibiotics, and small metabolite.It produces a variety of extracellular enzymes including protease with having high optimal growth temperature (50°C) and high pH (pH 9-12) levels (Edward et al 2010).

Third isolate was confirmed as *Bacillus cereus* after 16S rRNA sequencing. It is an endemic, spore-forming ,soil -dwelling, Gram-positive, rod-shaped and beta hemolyticbacterium *Bacillus cereus* is a bacterium that can be frequently isolated from soil and some food .*B. cereus* bacteria are aerobes, and like other members of the genus *Bacillus* can produce protective endospores. Some strains are harmful to humans and cause foodborne illness, while other strains can be beneficial as probiotics for animals. *B. cereus* spores are more resistant to heat and chemical treatments (Kramer and Gilbert 1989).

Fourth isolate was found to belong in *Halomonas genus* (*Halomonas campisalis)* *Halomonas* bacteria genus is halophiles, requiring high NaCl for growth. They are highly versatile in terms of their ability to successfully grow in a variety of temperature and pH conditions. This versatility may eventually lead to *Halomonas* species being used as a substitute for the utilization of starch-derived raw materials. *Halomonas* are Gram-negative rod-shaped cells that are usually unpigmented or yellow-tinted in color. Because *Halomonas* species are typically halophiles, they are usually found in water sources with high salinity levels, such as the Dead Sea and even within the frigid waters of Antarctica. *Halomonas*can also inhabit deep-sea sediment, deep-sea waters affected by hydrothermal plumes, and hydrothermal vent fluids (Mormile et al 1999).

Lastly, fifth isolate was confirmed as *Bacillus pseudofirmus.* It is a gram positive, spore forming, rod shaped bacterium and it is the best studied species of the alkalopihilic *Bacillus.* This facultatively alkaliphilic *Bacillus* grow at high pH environment 7.5 to 10.5 by maintianing high cytoplasmic pH than most other bacteria on malate containing medium. This bacterium shows tolerence to high NaCl concentration upto 18% (3.1M) due to which it appears more close to the recently identified extremely alkalophilic and halotolerent *Oceanobacillus iheynesis isolated* from deep sea sediment (Muntyan et al 2005).

The result confirms the abundance of *Bacillus* genus in the Lonar lake. The Joshi *et al*, 2008 showed the same kind of results, they also confirmed the abundance of phylum Firmuicutes, which include low G+C Gram positive bacteria related with different families of Bacillaceae

which include *Bacillus* as one of genera. They also found that Gram positive bacteria are predominant in Lonar lake. The presense of *Bacillus* in other soda lakes are already been reported by various scientists.

Zhang et al, (2001) studied the diversity of alkalophiles in Hailaer Soda lake inner Mongolia an autonomous region of china. They also suggested the abundance of *Bacillus* genus in the soda lake, which can tolerate high alkaline pH (pH- 12) and high salt concentration (2.5M).

Lopez-Garicea et al. in 2005 studied the bacterial diversity and carbonate precipitation in the giant microbilites from the highly alkaline lake Van, Turkey. Their results also showed the higher relative abundance of *Bacillus* Species

Primary goal of the present investigation was to isolate alkalophilic bacteria from Lonar lake using different culture media. Phylogenetic analysis of the representative isolates indicated that most of the isolates were related to phylum Firmicutes, which included Low G+C Gram posotive bacteria related to different families of *Bacillus* Species.

XIV. DIAGRAMS AND FIGURES

Table 1) Different Sites of Collection

Collection Point	Location
Collection Point 1	Guest House
Collection Point 2	Ramgaya temple
Collection Point 3	Kamlja Devi temple
Collection Point 4	Banana Farm
Collection Point 5	Shiv temple

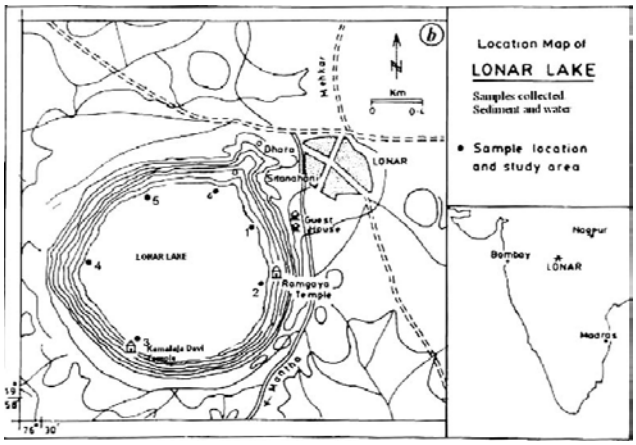

Fig 1 Location map of Lonar Lake

Table 2. Total viable count of bacteria (TVC) from Lonar alkalophiles

Location	Sample type	Total Viable Count (TVC) (cfu ml^{-1} for water) and (cfu $g^{-1}$for sediment)			
		pH range			
		7	8	9	10
GuestHouse	Water Sample	1×10^3	1×10^4	3×10^4	5×10^3
Ramgaya temple	Water Sample	2×10^3	1×10^5	4×10^4	5×10^4
KamljaDevi temple	Water Sample	3×10^3	3×10^3	3×10^4	4×10^4
Banana Farm	Sediment Sample	1×10^3	2×10^3	2×10^5	2×10^5
Shiv temple	Sediment Sample	1×10^3	1×10^1	2×10^5	2×10^5

Table 3 .Morphological characterization of bacterial isolates obtained from Lonar alkalophiles

Sr. No.	Isolates name	Colony morphological characters				
		Colony colour	*Colony shape*	*Colony surface*	*Colony elevation*	Colony edge
1	A	CR	P	S	RA	E
2	B	CR	C	S	F	E
3	C	WH	I	WR	P1	UN
4	D	WH	I	R	P1	UN
5	E	CR	O	SW	P1	E

Where

CR- Creamish	P – Punctiform	WR – wrinkled
C- Circular	O – Oval	SW- Smoothwrinkled
I - Irregular	S – Smooth	RA – Raised
WH- Whitish	R – Rough	Pl - Pulvinate
F- Flat	E – Entire	UN – Undulated

Table 4 Characterization based on Gram staining of bacterial isolates Lonar alkalophiles

Sr. No.	Isolate name	Gram staining	
		Shape	Reaction
1	A	Rod shaped	Gram positive
2.	B	Rod shaped	Gram positive
3.	C	Rod shaped	Gram positive
4.	D	Short rod shaped	Gram postitive
5.	E	Short rod shaped	Gram positive

Table 5 .Biochemical characterization of alkalophiles obtained from Lonar lake.

Isolate name	Indole	MRVP test		Citrate utilization
		MR	VP	
A	+	+	-	+
B	+	+	-	+
C	+	+	-	+
D	+	+	-	+
E	+	+	-	+

(+) - Positive test ,(-) - Negative test

ACKNOWLEDGEMENT

Present investigation entitled “Isolation of alkalophilic bacteria from Lonar crater for insecticidal proteins’’ was carried out during 2010-2011 in Plant Protection Biotechnology Laboratory, Biotechnology Centre, Department of Agricultural Botany, Post Graduate Institute, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola

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