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Construction and Preliminary Screening of Metagenomic Library from Lonar Crater Lake, India

Ms. Priya Borekar

Department of Biotechnology Santaji Mahavidhyalaya, Nagpur.
Email: priyaborekar1@gmail.com

Abstract*—Lonar Lake is a eutrophic, saline soda lake with permanently anoxic deep water. Saline water lake microbes are a large and diverse group, which are exposed to a wide variety of pressure, temperature, salinity, nutrient availability and other environmental conditions. Soil metagenomics, which comprises isolation of soil DNA and the production and screening of clone libraries, can provide a cultivation-independent assessment of the largely untapped genetic reservoir of soil microbial communities. Metagenomics as a new field of research has been developed over the past decade to analyzed collective genomes of an assemblage of organisms, of the non-cultured microbes with the goal to better understand global microbial ecology on the one side and on the other side has been successfully applied to isolate novel biocatalysts from the uncultured microbiota in the environment. Recent progress in molecular microbial ecology has been applied to diverse problems in microbiology and has yielded insight into the physiology of uncultured organisms to access the potentially useful enzymes and secondary metabolites they produce. They provide a huge potential source of novel enzymes with unique properties that may be useful in biotechnological industry and further development of high throughput technologies for accelerating the pace of discovery in microbial ecology.*

***Keywords*—**Metagenomics, Lonar Lake soil, Library, Amylase.

I. INTRODUCTION

In India, the Lonar crater, popularly called as the Lonar soda lake. Lonar (19°58′N, 76°31′E) is situated in the Buldhana district (Maharashtra, India) in the formerly volcanic Deccan-Trap geological region. It is almost circular, occupied by saline water. Its longest and shortest diameters being 1875 m and 1787 m, respectively, with a raised rim of about 30 m and a depth of 135 m (Touche *et.al* 1912). Based on geological studies, it is postulated that the lake originated as a meteorite impact crater around 50,000 years ago. It is the third largest crater in the world and the only known crater formed by meteoritic impact in basaltic rock (Fredriksson *et.al* 1973). Soil is probably the most challenging of all natural environments for microbiologists, with respect to the microbial community size and the diversity of species present. One gram of forest soil contains an estimated 4 x 10^7 prokaryotic cells (Richter, D. D. and Markewitz, D.,1995) whereas one gram of cultivated soils and grasslands contains an estimated 2 x 10^9 prokaryotic cells (Paul, E. A. and Clark, F. E. 1989). Only about 0.1-1.o% of bacteria present in the environmental sample can be cultured by existing techniques (Kellenberger 2001). Metagenomics can be used to address the challenges of studying prokaryotes in the environment that are, as yet to be culturable and which represent more than 98% of the organism in some micro environment.

Cultured microorganisms represent only a minor component in the existing diversity of soda lakes because of the difficulty in culturing most of the microbial assemblage (Jones *et.al* 1998). Microorganisms are ubiquitous, live in most in most inhospitable sites across the various ecosystem and are considered by some as "Master of the Biosphere" Their total number on earth is estimated to be (4-6) × 10^{30}, but not more than 99% of microorganism in the environment cannot be cultured in the laboratory so called as "hidden" or "un-culturable world." The phenomenon that limits, the understanding of microbial physiology, genetics and community ecology, and the one way around this (problem) "Metagenomics"- "The genomic analysis of a population of microorganism".

Soda lakes and deserts represent the most stable naturally occurring alkaline environments on Earth. These environments typically contain high concentrations of sodium carbonate or complexes of this salt formed by evaporation (Grant *et.al* 1990) which generate pH values greater than 11.5. The microbial community structure of only a few soda lakes has been studied throughout the

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world (Rees *et.al* 2003). This ecosystem is unique in nature and having niche for salt resistant alkalophiles. Therefore this soil was used for the isolation, identification and screening of various bioactive compounds which are present in microflora of Lonar creater i.e. for metagenomic studies. The development of new tools exploiting expression capabilities of multiple host, libraries prepared from metagenomic DNA from various habitats, in combination with high throughout screening is expected to yield novel and potent bioactive compounds in near future for that taken as Lonar soil sample.

In this context some earlier research work on Lonar Lake, India have been undertaken by (Tambekar D.H. *et al*. 2013) on isolation of biosurfactant , (Rebecca S. *et.al* 2013) Produced Cyclodextrin Glycosyl Transferase from Alkaliphilic *Paenibacillus sp* L55 MCM B -1034, biodegradation of phenol (Tambekar D. H. *et al*. 2012). The microbial ecosystem of this lake has been studied by (Aijaz Ahmad *et. al* 2006), methanogenic archaea of genus *Methanosarcina* have been isolated by (Thakker C.D.2002).

II. MATERIALS AND METHODS

Sampling (Lake Soil Collection)

The 20 cm deep soil sample was scooped into sterile polythene bags from the alkaline Lonar Lake. Stored at 4oC and transported to laboratory for analysis. The soil was passed through coarse mesh screen and then a fine mesh screen to remove rocks, insects and large plant material. The soil sample was air dried and grind to fine powder with the help of morter and pestle. Homogenization and sonication is done by using sonicator at 5 min at pulser 30 having power set at 30 V/T (ultrasonic homogenizer model 150/VT) to get better yield of nucleic acid. Genomic DNA from soil is isolated using modified protocol of (Henne et al.2000) describes the genomic DNA isolation based on direct lysis of the microorganisms from soil sample. DNA was isolated from the soil microflora using two different methods.

i) Enzymatic mechanical method and ii) RNA-based method.

Isolation of Metagenomic DNA By using RNA based DNA extraction method: 1 ml of TENP buffer (50 mM Tris HCl), 20 mM EDTA, 100 mM NaCl, 1% (w/v) polyvinyl polypyrrolidone + sodium dodecyl (0.3%) PH-9) (Qualigen fine chemicals, Mumbai) is added to 1 g of soil, 500 ul of lysozyme (10 mg/ml) (MBI Fermentas) and 50 mg/g of RNA was added to soil sample. Then suspention was vortexed for 10 min and centrifuged at 4,000 xg for 5 min. Supernatant was extrated with phenol: Chloroform (24:1) (sigma chemie, Germany) extraction. Aquous layer was precipitated by using chilled icecold ethanol. Pellet was washed with 70% ethanol. The pellet was resuspended in 1 X Tris – EDTA buffer (1.0 m Tris HCl and 0.1 M-EDTA) (Sigma chemie, Germany) dissolved nucleic acid was stored at 200C for long term storage.

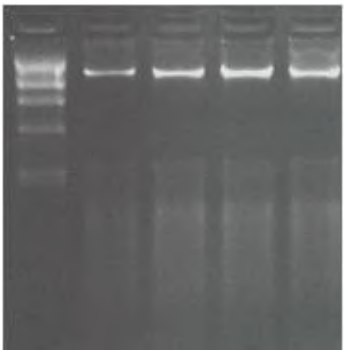

Fig-1:Photograph (Alpha Imager 2000) showing genomic DNA. Isolated from Lonar soil. M, Lambda Hind III digest (MBI Fermentas). Samples were loaded on 0.7 % TAE agarose gel with 0.5 µg/mL ethidium bromide (Sigma Aldrich).

DNA Quantification: In case, purity of DNA was determined or DNA was quantified by Spectrophotometric method. In case of purification the ratio of absorbance of 260/280 was obtained using U.V. spectrophotometer (Backman DU 64 OB). As the ratio of absorbance 260/280 was recorded 1.3 i.e. from its 100 time dilution with TE buffer and considering O.D. of DNA the approximately 16µg. of DNA per gram of soil was obtained.

Evaluation of purity of DNA:

Sample	260/280 ratio
	1.87

As the ratio of 260/280 was recorded on spectrophotometer not more than 1.9 that means protein contamination should be suspected.

Construction of a Metagenomic Library :

Genomic libraries were constructed by cloning segments of DNA generated by partial digestion of high molecular weight genomic DNA with restriction enzyme (Maniatis *et al.,* 1978) every DNA sequence in the target genome should be proportionally represented in a library of recombinant clones. The probability that any given DNA sequence will be represented in a genomic library can be calculated from the equation.

$$N = \frac{\ln(1-P)}{\ln(1-f)}$$ (Seed, 1982 and Seed *et al*., 1982)

Where 'P' is the desired probability, is the fractional proportion of the genome in a single recombinant and 'N'

is necessary no of clones in the library (Clarke and Carbon, 1976)

Generating genomic DNA libraries (Maniaties *et al.*, 1978) on the other hand, partial digestion with restriction enzymes that recognize frequently occuring tetranucleotide sequences within eukaryotic DNA yields a population of fragments that is close enough to random for many purpose and yet can be used without further manipulation to generate genomic libraries. The best enzyme for this purposes Mbo I and Sal III AI, although Taq I or Eco RI activity can also be used.

pUC: pBR 322 is replaced by a revolutionary series of pUC vectors in which the number of restriction enzymes cleavage sites was expanded and distribution within the vector was rationalized (Messing *et al.*, 1983 ; Norrander *et al.*, 1983 ; Vaniskh –Perron *et al.*, 1985 ; Vieira and Messing, 1987). The pUC vectors were the first plasmids to contain a closely arranged series of synthetic cloning sites, termed polylinkers multiple cloning sites, or polycloning sites that consist of banks of sequences recognized by restriction enzymes. Polycloning site from the vector pUC19 consist of a tandom array of unique cleavage sites for 13 restriction enzymes viz. Hind III, Pst I, Sal III ,Acc I, Hind II, Xba I, Bam HI, Sma I, Xma I, Kma I,Sac l, and EcoRI,.

1) Genomic library have been Constructed using Partial digestion of Genomic DNA:

I) **Pilot reaction**: (Sambrook and Rossel, 2001)

II**) Large scale reaction**: (Sambrook and Rossel, 2001)

2) Linearization of plasmid: (Sambrook and Rossel, 2001): 15 ul (2.5 ug) of plasmid DNA that is pUC^{18} (MBI Fermentation) was mixed with 10 ul of restriction enzyme buffer (MBI Fermentus) and 10 ul of (1M) NaCl. 8 ul of restriction enzyme EcoRI (MBI Fermentas) was added to the tube and volume of reaction mixture was make up by adding nuclease free distilled water and the sample wee incubated at 37^0C for one hour. After incubation for the deactivation of enzyme the sample were incubated at 37^0C for 15-20 minutes. Then reaction mixture was extracted one with standard phenol:Chloroform extraction followed by standard ethanol precipitation subsequent washing with 70% ethanol. Digested plasmid was then dissolved in 1x Tris-EDTA buffer (MBI Fermentus) 5 ul of digested plasmid is mixed with 3 ul of digested plasmid is mixed with 3 ul of 6x gel loading dye and sample was electrophorised at on 1% W/V M.B. grade agarose gel at 50 volt in 1x TAE buffer. (MBI Fermentas). Gene ruler 1 kb is used as standard molecular size standard. The gel were stain with ethidium bromide (sigma) 0.5 ug/ml and visualized under Ultra violet light and recorded with an Alpha Imager (2000) (Alpha Innotech, San Leandro CA).

3)Ligation of plasmid to metagenomic DNA: (Sambrook and Rossel, 2001): Restriction digested metagenomic DNA was dissolved in 10 ul of 1x ligation buffer (MBI Fermentus) 10 ul of linearised plasmid and 2 weiss unit bacteriophase T4 DNA ligases were added and reaction mixture were incubated at 37^0 for 1 hour and after this the reaction mixture was stored at 4^0C until the tranformation was done.

4)Transformation and plating recombinants: Was done by preparing competent cells, Transformation and Plating recombinant (Sambrook and Rossel, 2001)

5) Screening of metagenomic library for starch degrading enzymatic Activity: Each clone represents a different insert and they may contain *amylase* gene in it. So for screening of metagenomic library, Starch agar (sigma chemie, Germany) was used. Starch agar plates containing ampicilin 40ug/ml (MBI fermentas were prepared and grid of 0.8cm were paired on the plates. Replica plating was done with the help of sterile wooden tooth peak on laminar flow hood. Plates were incubated at 37^0C for 24 hours and development of clear zone and growth was observed by addition of Lugot's Iodine on plate after incubation.

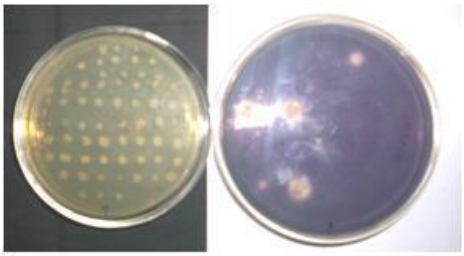

FIG-2: CONFIRMATORY SCREENING OF AMYLASE POSITIVE CLONES FROM M-1/R-1 SET ON STARCH AGAR. COLONIES SHOWING CLEAR ZONE AFTER ADDITION OF IODINE SOLUTION.

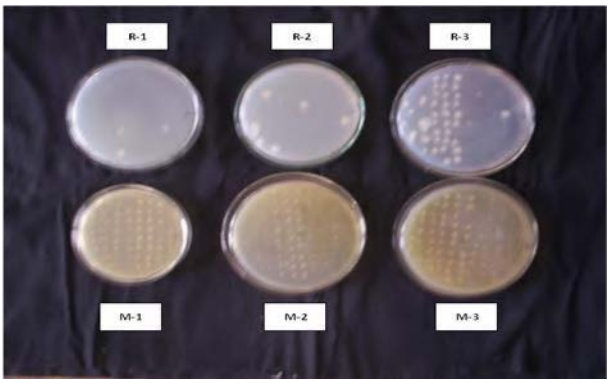

Fig-3: Replica plating for the selection of amylase positive recombinant E. Coli clones. M-1, M-2 and M-3 represents master plates (L.B.+ ampicillin) and R-1 ,R-2 and R-3 represents replicas on Starch agar ampicillin plates respectively

It is confirmed by iodine, getting zone of clearance the resulting clones can also be used for biochemical analysis

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of amylases again. Many enzymes of industrial important have been discovered using the functional based screening. For screening of metagenomic library a functional based analysis was performed because for most of the enzyme assay a functional analysis is important. Amylases E.C. (3.2.1.-) are enzymes that hydrolyze starch, and some of them performed trans-glycosylation or condensation as well as hydrolysis. Amylases and related enzymes have been among the most important enzymes in many industrial fields, especially in the food industry. The application of any amylase in industrial reactions depends on its unique characteristics, such as its action pattern, substrate specificity major reaction products, optimal temperature, and optimal pH.

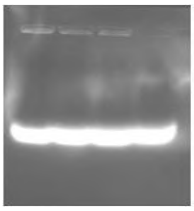

Fig-4: Photograph (Alpha Imager 2000) showing RNA isolated from Bakers Yeast. Samples were loaded on a 2.0% agarose gel with 0.5 µg/mL ethidium bromide (Sigma Aldrich, Germany)

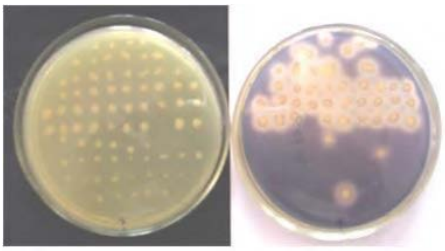

Fig-5: Confirmatory screening of amylase positive clones from M-3/R-3 set on starch agar. Colonies showing clear zone after addition of iodine solution.

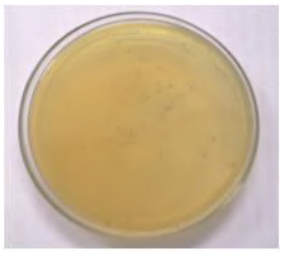

Fig-6: Recombinant selection of E. coli (JM101 Strain) by Blue White Screening. Blue and white (Recombinant) colonies on Luria agar (HiMedia Laboratories) plate with X-gal (2%), IPTG (20%) and Ampicillin (40µg/mL).

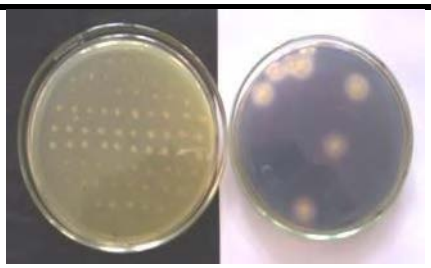

Fig-7: Confirmatory screening of amylase positive clones from M-2/R-2 set on starch agar. Colonies showing clear zone after addition of iodine solution.

III. RESULT AND DISCUSSION

As the amylases are among the most important eanzymes used in industry, particularly in process involving starch hydrolysis. Though amylase originates from different source (plant animals and micro-organism) they are used in industrial application, it is economically important enzyme. Therefore an attempts have been for construct of matagenomic library for the isolation of gene having amylase enzyme. The soil was obtained from lonar creator which was dried grined and sonicated to get better yield of nucleic acid.

Isolation of genomic DNA from soil :- The fundamental investigation on nucleic acid in environmental sample and the development of new methods and application was central to environmental microbiology and molecular ecology (Jacodson & Resmussen 1992, Tsai & Olson 1991). This was demonstrated by direct extraction of DNA from soil and detection of specific DNA sequences diagnostic for presence of specific microbial species or gene. (Trevors and Elsas 1995). DNA was obtained or isolated from the soil microflora using two different methods.

Enzymatic mechanical method and RNA-based method.

In the mechanical-enzymatic yield of DNA was negligible & since there was no use of phenol chloroform the enhances of contamination were supposed to be more using this method therefore DNA was not stored for long time. pH of extraction buffer in the enzymatic-mechanical method was found of affect the recovery of DNA from soil and the recovery of DNA might be affected due to adsorption of DNA to clay particles & therefore DNA was not isolated easily with high yield & it was not free from contamination also. The lysis of cells plays very important role in the isolation of nucleic acids from soil but all the drawbacks were over come in the advanced new one method i.e. RNA based extraction. In this method, to extract the high yield of DNA the different methods were used up such as grinding (to increase extra cellular DNA yield, sonication & thermal shock for lysis of cells etc.) Secondly pH of extraction plays quite

important role for recovers of DNA from soil in the RNA based extraction method. In case of pH 10 more amount & humic acids were released than in case of pH 9. i.e. there is less of amount of humic acid was released at pH 9 and therefore pH 9 was chosen in the extraction buffer of RNA based method for better yield.

In RNA based method, the phenol-chloroform extraction step is added as in enzymatic mechanical method the DNA was degraded, more contamination in recovered DNA. Therefore phenol-chloroform was used to minimize the chances of contamination and for long term storage of DNA. As recovery of DNA might be affected due to adsorption of DNA to clay particles the DNA was not isolated easily with high yield so that, RNA can be used as it saturated the adsorption sites in way particles & adsorption of DNA to clay particles decreases. Hence for the isolation DNA from soil of Lonar Lake, enzymatic mechanical method and RNA based extraction method was adopted. The advantage of RNA-based extraction method over mechanical enzymatic method alone as it gives stability and High yield of DNA.

In the RNA based method, the RNA used which is isolated from commercially available Baker's yeast which is shown in following fig. In case, purity of DNA was determined or DNA was quantified by Spectrophotometric method. In case of purification the ratio of absorbance of 260/280 was obtained using U.V. spectrophotometer (Backman DU 64 OB). As the ratio of absorbance 260/280 was recorded 1.3 i.e. from its 100 time dilution with TE buffer and considering O.D. of DNA the approximately 16µg. of DNA per gram of soil was obtained. Evaluation of purity of DNA:

Sample	260/280 ratio
	1.87

As the ratio of 260/280 was recorded on spectrophotometer not more than 1.9 that means protein contamination should be suspected. The quantified DNA was used up for the partial digestion with the restriction enzyme of different concentration of EcoRI. Using the pilot method, optimized partial digestion of genomic DNA and large scale preparation method for digestion of genomic DNA gives the result are as follows:

Photograph of gel under U.V. light shows at adequate amount or units or restriction enzyme used gives the 2-10 kb of fragment of genomic DNA and the intensity of fluorescence in maximum which was used for large scale preparation. The present study pUC 18 plasmid vector was used for construction of metagenomic library. The partially digested genomic DNA was packed for construction of metagenomic library using of vector pUC-18 digested with same restriction enzyme. Mainly the choice of a vector for construction of genomic libraries is depends on the size of the target region and the capacity of the vector. The average size of the structural genes for most enzymes is around 1-2 kb so, for the construction of metagenomic libraries by means of a high copy number plasmid vector has been used up to search for novel enzymes (Tiae *et al* 2004). As the after partial digestion of genomic DNA and pUC-18 plasmid vector the ligation performed with competent cells of E.Coli 101 strain was resulted into step of transformation. As E.Coli (JM 101) is amylase negative and it does not grow on starch agar hence for the present study of transformation E-coli (JM 101) taken as host. The recombinant pUC-18 was transformed into E.Coli JM101 are allowed for blue white screening for selection of recombinant using X-gal and IPTG. Blue color colonies contain non-recombinant plasmid β-galactosidase expressed by the lac-Z gene hydrolyze X-gal, forming blue colour, white colonies were supposed to bear recombinant plasmid in which foreign DNA was inserted at polycloning site in the lac-Z gene. The interrupted lac-Z gene cannot express β-galactosidase activity, therefore the colonies were white. Here approximately 2500 recombinant E.coli strain were isolated. Hence the partially metagenomic library was prepared.

Making the strengthening the step for screening, the step for screening, the specific media was used for the selection of recombinants, as starch as sole source for isolation of amylase gene of which isolation is goal of our work. The starch degrading enzyme is obtained & as it grows on starch agar plate and it is confirmed by iodine, getting zone of clearance the resulting clones can also be used for biochemical analysis of amylases again. The positive colonies of our interest are found to be 26. As it get, from that we argued that there is very less biodiversity is to be seen in the lonar take creater and there is very alkaline nature of water and mainly, there was found very high photosynthetic activity and due to zone a plants are very much large & having leaves decades with high cellulosic material found in it. There are some highly photosynthetic activity organisms such as spirullina is seen. So the numbers of starch degrading enzyme are to be found more.

Functional Driven analysis for screening of amylase gene : In the functional based screening clones expressing desired trait are selected from libraries and aspects of molecular biology & biochemistry of active clones are analyzed. Many enzymes of industrial important have been discovered using the functional based screening. For screening of metagenomic library a functional based analysis is performed because for most of the enzyme assay a functional analysis is important. Amylase E.C.(3.2.1) are enzymes that hydrolyze starch,

and some of them performed transglycolation or condensation as well as hydrolysis. Amylases and related enzymes have been among the most important enzymes in many industrial fields, especially in the food industry. The application of any amylase in industrial reactions depends on its unique characteristics, such as its action pattern, substrate specificity major reaction products, optimal temperature, and optimal pH.

IV. CONCLUSION

Metagenomic study expand and enriches knowledge about unexplored microorganisms as a potential source of the amylases enzyme for releasing short chains of polysaccharides and may provide important data on application of the novel metagenomic biocatalyst for promising biotechnological processes. More specifically, this work points out that screening for microorganisms or biomolecules in a saline environments could provide unconventional and new exciting ways for bioprospecting and its application for sustainable social wellness.

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