JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2015. Vol. **9**(2), p. 1257-1261

Prospecting for Industrial Enzymes in a Hot Spring Metagenome

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(Received: 10 February 2015; accepted: 06 March 2015)

In the present study metagenomic library was created from soil sample collected from a hot water spring located in the district Chamoli, Uttarakhand, India. The genomic DNA was partially digested with *Sau*3AI and the restriction fragments were eluted from the agarose gel. The purified fragments were ligated with plasmid vector and transformed into *E.coli* cells. A metagenomic library of insert size approximately 1.6 kb was constructed. A total number of 60,000 clones were screened for secretion of industrial enzymes of which two were detected positive for amylase activity.

Key words: Metagenomics, Hot spring, Amylase.

The soil is a potential source of diverse group of microorganisms that can be explored for several industrial, environmental and agriculture applications. Microorganisms isolated from soil samples can continue to play a major role in the development of new biocatalysts and drugs. However, it is now known that from most of the ecosystems just 1% or even fewer can actually be grown by the traditional culture dependent approach (Torsvik *et al*., 1990). This limitation is largely overcome by the developments in the area of metagenomics which involves the study of metagenome, i.e genetic material recovered directly from the environmental sample (Handelsman *et al*., 1998). This culture independent approach is therefore, highly useful in understanding and accessing microbial genomes and their functions (Abbai *et al*., 2011). In recent years a number of important metabolites, enzymes, therapeutic molecules etc. have been isolated through this technique. The recent advances in the area of metagenomics have opened up an entire new way of looking into the plant-microbe interaction by providing the ability to collect and store the genetic information from the unculturable microbes.

Hot springs are manifestations of geological activity and represents extreme environments that emerge in Himalayan region. They give a wide range of diversity and new genes, producing useful biocatalysts or industrially important enzymes. So there is a need to explore the genes present in the extreme environments as hot springs by using metagenomic approach. In the present study metagenomic library was created from a hot spring soil located in the district Chamoli, Uttarakhand and the metagemoic library construction was done for screening of several industrially important enzymes.

MATERIALS AND METHODS

Soil Sample collection

In the present study, soil sample was collected from the hot spring located at Ringi (latitude 39° 29’ 25’’N, longitude 79° 39’ 29’’; altitude 1900m amsl), Chamoli district, Uttarakhand, India. The soil sample was collected from the hot spring at a depth of 10 to 15cm with temperature of 95°C. The samples were pooled and immediately sealed in sterile capped containers and transported to the laboratory in a thermos flask containing hot water to maintain the habitat temperature.

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Metagenomic DNA isolation

The soil sample was lyophilized and the DNA isolation was done by using the method as developed by Zhou *et al*., 1996 with slight modifications. Sieved fine soil (0.5 g) was extracted with 1.3 ml of extraction buffer (100 mM Tris Cl, pH 8.0, 100 mM EDTA, pH 8.0, 1.5 M NaCl, 100 mM sodium phosphate, pH 8.0, 1% CTAB). After proper mixing, 10 µl of proteinase K (20 mg/ml) was added. The eppendorf tubes containing the reaction mixture was incubated horizontally at 37°C with shaking at 225 rpm for 45 min. After that 150 µl of 20% SDS was added and vortexed for 30 sec with further incubation at 65°C for 2 hours. The sample in each eppendorf was mixed thoroughly after every 15 min interval. The samples were centrifuged at 7000 rpm for 10 min at 4°C. The supernatant was transferred into new eppendorf tubes. The remaining soil pellets were treated three times with 400 µl of extraction buffer, 60 µl of SDS (20%) and kept at 65°C for 15 min with intermittent shaking after every 5 min. The supernatants collected from all four extractions were mixed with equal quantity of chloroform and isoamyl alcohol (24:1) and extracted three to four times. Aqueous layer was separated and precipitated with 0.6 volume of isopropanol at -10°C for 1 h. After centrifugation at 14,000×g for 15-20 min at 4°C, the pellet was washed with ice cold 70% ethanol. The same was dried and resuspended in TE (10 mM Tris Cl, 1 mM EDTA, pH 8.0)

Partial digestion of Metagenomic DNA

About 5 µg of metagenomic DNA was partially digested by using 10 units of *Sau*3AI enzyme (10Units/µl) and the digested product was analyzed on 0.8% agarose gel. A reaction volume of 30 µl was prepared by mixing metagenomic DNA 5µg, *Sau*3AI 0.5µl (10U/µl), Restriction digestion buffer D (Genei) 3µl, 10X BSA 3µl and Sterile water 18.5µl. The reaction mixture was incubated at 37°C for 3 minutes. It was then immediately transferred to 65°C for 15 minutes in order to inactivate the enzyme. The contents were analyzed on 0.8% Agarose gel.

A small part of the agarose gel containing DNA fragments ranging from 1kb to 10kb was carefully excised by a pre-sterilized, sharp razor. The DNA fragments trapped within it were gel eluted using Qiagen gel elution kit by using manufacturer's instructions.

Ligation of DNA fragments into pZEro-2 vector

A zero background vector (3.3 kb) was procured from *Invitrogen*. The vector was linearized with *Bam*HI in a reaction mixture containing pZEro-2 vector (1µl), restriction enzyme *Bam*H1 (0.5µl;10U/µl), BSA 10X (2µl), restriction digestion buffer E (2µl) and DNase free sterile water 14.5µl. The reaction mixture was incubated at 37°C for 3 hours and digested vector was analyzed on 0.8% agarose gel. It was then eluted from the gel using the Qiagen gel elution kit.

The digested vector and metagenomic DNA fragments were ligated in the molar ratio of 1:3. The reaction mixture for ligation contained linearized pZEro-2 vector (1µl), metagenomic DNA fragments (3µl), T4 DNA ligase (1µl), ligation buffer (1µl) and DNase free sterile water (4µl). The contents were incubated at 16°C overnight.

Transformation of ligated products into competent *E.coli* Top10 cells

The ligated products were transformed into chemically competent *E.coli* Top10 cells (Invitrogen) by heat shock according to the protocol developed by Sambrook *et al*.,1989. The transformed cells were then plated on the Kanamycin supplemented LB plates aseptically. The plates were incubated at 37°C overnight. The clones obtained were patched on a separate Kan-LB agar plate and incubated for 12-16 hours at 37ºC.

Plasmid isolation and restriction digestion analysis

Six of the random clones were selected and plasmids were isolated from each of them by using the Qiagen kit for plasmid isolation as per the manufacturer's manual. The plasmids were subjected to double digestion with *Eco*RI and *Hind*III restriction enzymes for analysis of insert size. The reaction mixture contained- Plasmid 3µl, *Eco*RI 0.2µl (10U/µl), *Hind*III 0.2µl (10U/µl), restriction buffer 2µl, BSA(10X) 2µl, and sterile water 12.6µl. The reaction mixture was incubated at 37°C overnight. The digestion was checked for insert fall-out by agarose gel electrophoresis.

Screening for enzyme activity

The metagenomic library thus created was screened for the presence of clones harboring

genes for the enzymes amylase, protease, DNase, Xylanase and Cellulase. For this the standard plate assay methods was used by making starch agar, milk agar, DNA agar, xylan agar and CMC agar plates respectively, and streaking individual clones on each medium. The visual change, if any, was monitored after growth.

RESULTS AND DISCUSSION

Metagenomic library may serve as an unlimited resource for discovery of novel enzymes, biocatalysts, antibiotics etc from uncultured microorganisms of the soil. The technical feasibility of this approach is proved by a large number of reports on isolation of several industrially important enzymes and metabolites like amylase, lipase, protease etc (Lorenz *et al*., 2002; Prabavathi *et al*., 2012). Extraction of a good quality metagenomic DNA from soil is always challenging as the composition of soil affects the DNA yield (Sagar *et al*., 2014). In the present investigation, a good quality metagenomic DNA was extracted from hot spring soil as sharp bands of DNA were seen after agarose gel electrophoresis (Fig. 1). It was also observed that prior lyophilization of hot spring soil improves the quality of metagenomic DNA. The metagenomic DNA was partially digested by *Sau*3AI as uniform DNA smear was observed on agarose gel (figure not shown). Since *Bam*HI and *Sau*3AI are isocaudomers, they will generate compatible ends for ligation of vector and restriction fragments generated from Metagenomic DNA. The pZEro-2 vector was linearized with *Bam*HI (Fig.2) and successfully ligated to the fragments of partially digested metagenomc DNA. The ligated products were successfully transformed in *E. coli* Top10 cells to obtain a genomic library. A very high number of trasformants were calculated to be present on kanamycin LB medium plate (Fig. 3). Over 63,000 clones were estimated to be present in the library thus created. Plasmids were isolated from some of the random transformants. These plasmids were then subjected to double digestion for analysis of insert size. In this analysis, 1-2kb insert fall-out was observed after agarose gel electrophoresis (Fig. 4; results with only 3 transformants are shown). Thus the creation of metagenomic library of average size of 1.6 kb in pZero vector in *E.coli* Top 10 cells is confirmed.

The functional screening of clones in a library depends on the faithful expression of the cloned gene in a heterologous host. However, a very high number of clones need to be screened for identifying the positive clone by plate assay methods. Previous workers have successfully reported a few extracellular enzyme producing clones in metagenomic library of many thousand clones (Yu *et al*., 2011, Neveu *et al*., 2011 and Verma *et. al*., 2013). In the present investigation also the metagenomic library was screened for various industrially important enzymes. Out of

Fig. 1. Metagenomic DNA (lane 1 & 2) run on the agarose gel

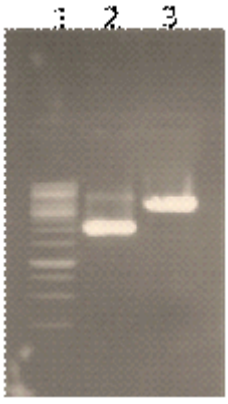
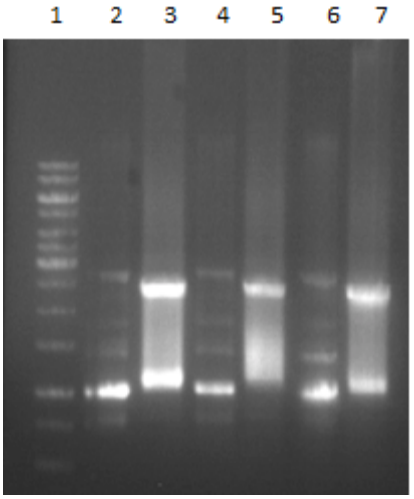


Fig. 2. Vector linearization (Lane1-1 kb DNA ladder, Lane-2 Undigested pZEro-2, Lane-3 pZEro-2 digested with *Bam*HI)

Fig. 3. Transformants observed on kanamycin LB medium



Lane 1: Ladder (I kb)
Lane2: Undigested plasmid from random clone no.1, Lane 3: *Eco*RI and *Hind*III digested plasmid from clone 1
Lane 4: Undigested plasmid from random clone no. 2, Lane 5: *Eco*RI and *Hind*III digested plasmid from clone 2
Lane 6: Undigested plasmid from random clone no, 3, Lane 7: *Eco*RI and *Hind*III digested plasmid from clone 3

Fig. 4. Digestion check for fall-out from clones

Fig. 5. Amylase positive clones on starch agar plate

60,000 clones, only two were found positive for amylase production on starch agar plate (Fig. 5). No positive clone was detected for any of the other enzyme activity tested. This may be due to the improper expression or the small insert size library. The amylase positive clones identified in the present investigation need to be studied further for characterization of associated enzyme gene(s) by DNA sequencing, site-directed mutagenesis, subcloning and expression to realize their industrial applications. Since the site selected in the present study is largely unexplored for any such investigation, it is imperative that the metagenomic approach may be tried by using different vectors with better expression and screening system for identification of many more important genes and associated products.

ACKNOWLEDGEMENTS

The financial support of SERB, Department of Science and Technology, Govt. of India, vide their grant no. SR/FT/LS-078/2009 is duly acknowledged.

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