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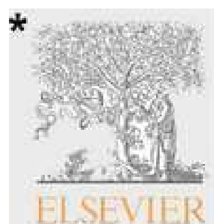

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RESEARCH ARTICLE MICROBIOLOGY

ISOLATION OF GENOMIC DNA AND CONSTRUCTION OF METAGENOMIC LIBRARY FROM MARINE SOIL SEDIMENTS

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ABSTRACT

Microorganisms constitute two third of the earth's biological diversity. As many as 99% of the microorganisms present in certain environments cannot be cultured by standard techniques. Culture-independent methods are required to understand the genetic diversity of microbial population, structure and ecological role of the majority of organisms. Metagenomics is the genomic analysis of microorganisms by direct extraction and cloning of DNA from their natural environment. To develop methods to investigate the full extent of microbial diversity, we have used a direct lysis method for the isolation of genomic DNA from soil sediments and also pWEB –TNC cosmid cloning kit have been used as a vector for construction of environmental metagenomic library for the detection of novel biocatalyst. To date, we have constructed a library , which contain 10,000 clones, further it will be submitted for functional based screening to detect the novel biocatalyst for various purpose.

KEY WORDS

Marine soil, genomic DNA, cosmid vector, metagenomics.

INTRODUCTION

Soil considered as a complex environment which appears to be the major reservoir of microbial genetic diversity[1.] Most of the soil microorganisms seem to be extremely well adapted to their environment, because they cannot be cultured under the usual laboratory conditions. The recent surge of research in molecular microbial ecology provides compelling evidence for the existence of many novel types of microorganisms in the environment in numbers and varieties that dwarf those of the comparatively few microorganisms amenable to laboratory cultivation [2,3,4].

Modern biotechnology has a steadily increasing demand for novel biocatalyst, which has prompted the development of novel experimental approaches to find and identify novel biocatalyst encoding genes. Recent studies have reported to investigate the metagenome [5], which represents the genomes of uncultured microbes as a rich source for isolation of many novel genes. One gram of soil may contain upto 4,000 different species [6]. However, current estimates indicate that less than 1% of these organisms are readily culturable with known cultivation techniques[7]. This problem can be solved by direct isolation and cloning of metagenomic DNA, thereby circumventing cultivation, which may result in the loss of major proteins of the microbial communities because the different growth requirements of the different microbes[8].

The microbial populations are vital to life on the earth and are of enormous practical significance in medicine, engineering and agriculture[9]. Microbes have many of the properties similar to complex organisms like humans. They also exhibit unique properties such as ability to degrade waste products. As a result, the genetic and biological diversity of the microorganisms is an important area of scientific research[10].

The current metagenomic studies have revealed that only 0.001%-0.1% of the total microbes in sea water, 0.25% in sediments and 0.3% soil microorganisms only could be cultivable *in vitro*[7].The current metagenomic studies have largely progressed due to the construction of efficient gene cloning vectors like Bacterial Artificial Chromosomes (BACs) or cosmid vectors etc[11.12], which allow cloning and expression of larger and complex DNA segments genes and the development of methods for generation and analysis of the data.

Metagenomics analyses are usually initiated by the isolation of environmental DNAs. A major difficulty associated with the metagenomeics approach is related to the contamination of purified DNA with polyphenolic compounds, which are co-purified with the DNA. Although it is expected that laboratory enrichment cultures having only a limited biodiversity this technique has proven to be highly efficient for the rapid isolation of large DNA of fragments and for cloning of operons and genes with high biotechnological value[13,14,15,16]. DNA isolation and purification is followed by the construction of DNA libraries in suitable cloning vectors and host strains. The classical approach includes the construction of small insert libraries (<10) in a standard sequencing vector and in *Escherichia coli* as a host strain [17].

However, small insert libraries do not allow detection of large gene clusters or operons. to circumvent this limitation researchers have been employing large insert libraries, such as cosmid DNA libraries (mostly in the pWEB15 vector of sratagene) with insert sizes ranging from 25-35 kb [15] and/or bacterial artificial chromosome (BAC) libraries with insert sizes up

to almost 200 kb [18,19]. *E.coli* is still the preferred host for the cloning and expression of any metagenome- derived genes. Recently have other host such as *streptomyces lividans* been employed to identify genes involved in the biosynthesis of novel antibiotics[20]. Also to our concerned, metagenomic libraries are currently developed in other Gram-negative hosts by several laboratories working in the field, which will became available soon. There are many published methods for extracting DNA from soil sediment. Commercial kits were also available for extraction and purification of soil DNA, which are easy to use with considerable reproducibility.

In the present , chromous gel extraction kits have been used to purify the DNA fragments from standard or low melt agarose gels in TAE (tris acetate/EDTA) or TBE (Trisborate/ EDTA) buffer . These kits of rapid protocol were designed to extracts and purify agarose embedded DNA with high quality.In the present investigation, pWEB-TNC™ cosmid cloning kit from Epicenter Biotechnology-USA, has been used as a cloning vector for construction of metagenomic library. *E.coli* has been performed as a host for cloning and expression of any metagenome-derived genes.

MATERIALS AND METHODS

The soil sediments were collected from the study area at coastal regions of Samiyarpettai, Cuddalore District, Tamilnadu, (South India), during the month of April 2010. The sample was taken superficially (0-5 cm depth) during the period of low tide; immediately after sampling, the sediment was stored in ice and subjected to DNA isolation based on direct lysis methods has been modified from nature protocols [21]. One kg of soil has been taken and sieve with mesh screen (1/2 to ¼ inch). Approximately, 125g of soil has been taken into 500 ml conical flask. 150 ml of pre heated lysis buffer has been added with 125g of soil in conical flask, the mixer has been incubated at 70°c for two hours in a water bath. After two hours of cooling the crude soil lysate was transferred to 500 ml centrifuge tube and centrifuged at 3,500g for 20 minutes at 4ºC. After centrifugation, the soil lysate was transferred to a clean centrifuge bottle and 0.7 volume of isopropanol was added, from that 25ml supernatant has been divided into four tubes and 17.5 ml of isopropanol was added to it. The tubes are incubated for 30 minutes at room temperature and gentle mixing is done during incubation. After incubation, the precipitated eDNA was centrifuged at 3500g for 30 minutes at 4ºC DNA pellet was washed by 70% ethanol, air dried and dissolved in 5ml of TE buffer and the pellet was heated to 50ºC for 20-30 minutes. The resuspended pellet was transfer to a falcon tube for long time storage at 20ºC, 20µ l of crude DNA extract was directly added into 1.0% agarose gel and it is run at 100 v until complete separation of DNA. Lambda Hind III was used as molecular weight standard and visualized under U-V trans-illuminator.

ELECTROELUTION

Purification of eDNA procedure was followed by protocol of Chromous Gel Extraction Kits (Chromous biotech, Bangalore). DNA fragment was chopped from the Agarose gel with clean, glass slide. The gel slice was weighed in a 2ml micro centrifuge tube and 3 volumes of gel extraction buffer Ilwas added to 1 volume of gel (100 mg -100µl), and the tube was incubated at 55ºc for 5-10 minutes in dry bath, the gel piece had dissolved completely, to that gel volume one volume of gel isopropanol was added into the tube and mixed well. Total extracted solution along with isopropanol is kept in 2ml spin column and collection tube. The spin column tube is centrifuged at 13000 rpm for 1 minute at room temperature and the contents are removed from the collection tube and again the spin column is kept in the same collection tube. 500µl of wash buffer was added to the column and spun at 13000 rpm for 1 minute at room temperature, and then the contents were removed from the collection tube. The spin column was placed

back in the same collection tube. The above step is repeated for one more time. The empty spin column along with collection tube was spun at 13,000 rpm for 3minutes at room temperature. The spin column was placed in a fresh 1-5 ml micro centrifuge tube, and 20µl of elution buffer was added to the spin column. The vial kept along with the spin column at room temperature for 20minutes then spun at 13, 000 rpm for 1 minute at room temperature. 20 µl of elution buffer was added again to the spin column and vial kept along with the spin column at room temperature for 20 minutes then spun at 13, 000 rpm for 1minute at room temperature. The purified DNA was collected.

CONSTRUCTION OF METAGENOMIC LIBRARY

A metagenomic DNA library was constructed using the pWEB-TNC^{TM} cosmid cloning kit (Epicentre) according to manufacturer instructions. Briefly, the purified HMW DNA was end repaired with end-repair enzyme mix (Epicentre) and ligated to the copy controle pWEB-TNC^{TM} vector .Lamda packaging extracts were added to the ligation mixture and infection of *E.coli* EPI 300-TIR was performed according to the manufacturer protocol. The infected bacteria spreaded on LB-ampicillin selection plates and incubated at 37°C overnight. The number of eDNA clones on each plates were recorded.

RESULTS AND DISCUSSION

For extraction of metagenomic DNA from the marine soil sediment, direct lysis method (Sean F Brady 2007 nature protocol) was used. The distribution of the DNA fragments obtained by this method contained molecules from 35 to 40 kb. The isolated DNA appeared to be contaminated with sediment compounds mainly humic substances as indicated by the brownish color of the isolated DNA solution. Related to humic substances strongly interfere with cloning procedures because those humic substances inhibit the enzymatic reactions such as restriction enzyme digestion, and transformation. To remove that contaminants and also select the HMW DNA fragments suitable for cosmid cloning, the crude eDNA was submitted to electroelution for purification from this procedure resulted in removed of all visible contaminants without further fragmentation of DNA.

In the present study, approximately 6-7 mg of DNA extract has been derived by the direct lysis method. it is designed to release the DNA by breaking the cell wall and membranes of the microorganisms. Although the cell lysis efficiencies have been improved sufficiently by investigators; complete cell lysis has been improved sufficiently by investigators during DNA extraction still exists. It was found that the highest yield was obtained at pH of 9.0-10.0 in buffer lysis. DNA preparation, which is performed to discard the extraction buffer and contaminants, is also a crucial step influencing the quality of metagenomic DNA, preparation of DNA achived by the isopropanol.

However, precipitation with ethanol gave a lower recovery of DNA and more humic substances than the isopropanol. It was reported earlier an efficient modified protocol need for isolation of high amount, un-sheared and good quality of genomic DNA for the construction of metagenomic libraries[22]. It has been reported that the modified troughing method utilizes gel electrophorosis and PEG8000 to effectively remove contaminants, especially humic acids, from genomic DNA obtained from soil; the genomic DNA is larger than 23 kbp and low molecular weight humic acids migrate faster than genomic DNA in agarose gel. The troughing method consistently yields 70% recovery of purified DNA[23]. Factors that have been demonstrated to affect the size of recovered DNA include not only the DNA extraction method used but also the microbial growth status and chemical composition of the soil [24]. In general, DNA extracted from bacterial cells is significantly larger than DNA directly extracted from soil but is also found in lower yields [25,] however, this loss

can be reduced by using wide-bore pipette tips to prevent shearing of DNA, performing multiple rounds of indirect extraction on each soil sample, minimizing the amount of agarose that is retained during size selection, or using electroelution as an alternative to extraction of DNA from the agarose gel[26].

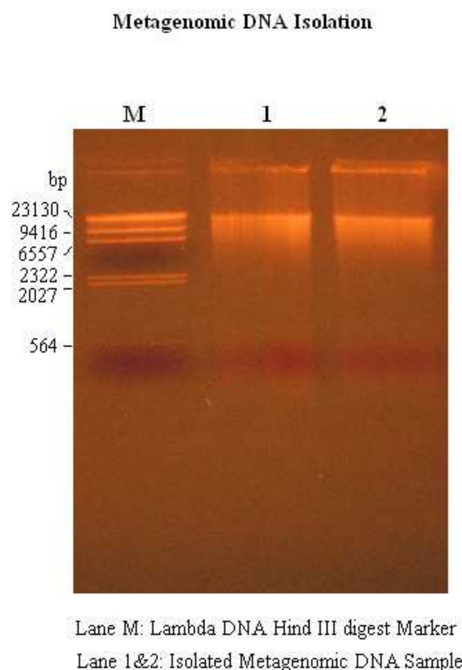

Figure 1
0.8% Agarose gel showing Metagenomic DNA isolated from marine soil sediment

After the migiration of humic compounds out of the agarose block, pure high molecular DNA could be electroeluted. The electroelution could also remove RNA from humic substances. Therefore, electroelution has been considered as an alternate strategy for the soil DNA purification [27]. It was earlier reported that the compared filteration through three different types of spin coloumns ie. Sepharose 4B, sepharose 4B/PVPP (combined coloumn) for purification of soil metagenomic DNA. Following PEG precipitation, sepharose, PVPP and the combined column removed 90.3, 94 and 97.6% of the humic material respectively[28]. Very recently, it has been reported that the manually optimized protocol with either pretreatment of samples with $CaCO_3$ or purification of extraction DNA with $CaCl_2$ was more efficient than commercial kits in yielding PCR-compatible soil metagenomic DNA[29]. The PCR was successful in 95% of the soil DNA sample with $CaCl_2$ and 93% with $CaCO_3$ pretreatment, whereas only 75% of the DNA samples extracted using power soil kit. Similarly, an optimized laboratory method has been reported to yield significantly higher concentrations of DNA than ultra clean and Fast DNA® kits [30].

The isolated DNA from marine soil sediment was used to construct a cosmid library comprising 10,000 clones which were maintained in *E.coli* EPI 300-T1R. In our investigation, from 120 g of soil sediment 6-7 mg of crude DNA has been recovered by direct lysis method. From these crude extractions, one tenth of solutions have been taken for agarose gel electrophoresis submitted to purification process.

Considering the diversity of soil bacteria, an immense number of metagenomic clones

would be needed to completely assay microbial diversity using conventional cloning vectors. Depending on soil type, usually 20,000 to 100,000 clones were obtained in cosmids, using 1 µg of soil DNA with a 35-kb average insert. Our libraries for the present study, consisting of 10,000 clones from marine soil sediments, represent almost 40 kb of genomic DNA, which is equivalent to approximately 1,000 *E. coli* genomes. Cosmids are good vectors for constructing metagenomic libraries because of their high cloning efficiency, improved stability in *E. coli*, and optimum (40 kb) insert size . Given the low chance of finding desirable genes in a metagenomic library of diverse microbial genomes, cloning efficiency is important in constructing a large clone library, which should include most of the microbial DNA in the soil. The genomes of many rare microorganisms were probably not included in our library.

It has been reported that biologically, chemically, and physically heterogeneous nature of soils presents many challenges to the successful characterization of its microbial metagenome. Representative coverage of the soil microbial community requires isolation and cloning of a large amount of DNA from a small sample, and depends on insert size and the number of clones. It has been estimated that the number of plasmid clones (5 kb average insert size) and BAC clones (100 kb average insert size) required for representative coverage of the diverse soil microbial community in 1 g of soil is 107 and 106 respectively[31].

It has been stated in order to achieve substantial representation of the genomes from rare members of the soil community; a 100- to 1000-fold coverage of the metagenome is needed in library construction. Since this translates to about 10,000 Gb of soil DNA, or 1011 BAC clones, it is not reasonable to suppose that bacterial taxa present in lower abundance will be represented within a metagenomic library unless an enrichment method is used[32].

The cosmid, a hybrid plasmid that contains cos sequences from the λ phage genome; was one of the first vectors used for cloning. The packaging capacity of cosmids varies depending on the size of the vector itself but usually lies around 40–45 kb, while typical plasmids can maintain inserts of 1–20 kb, cosmids are capable of containing DNA inserts of about 30 kb up to 40 kb[33]. Large-insert metagenomic libraries are the most challenging to construct, but also can provide significant advantages for some applications since they enable identification and characterization of intact functional pathways encoded on large, contiguous DNA fragments[18,19,20&34]. Cosmids can replicate like plasmids when they contain a suitable origin of replication and they commonly possess selective genes such as antibiotic resistance to facilitate screening of transfected cells. Finally with respect to the construction of large insert libraries by cosmid vectors probably more suitable for the generation of large amounts DNA with functional information for further screening analysis.

CONCLUSION

In conclusion, the direct isolation of DNA method seemed to be an efficient protocol for isolation of high amount, un-sheared and good quality of genomic DNA for the construction of metagenomic libraries. The electroelution method is an effective and straight forward method to purify the genomic DNA, which is usually larger than 10 Kbp, away from humic substances and other contaminants, compared with the various purification kits, the chromous gel extraction kits method consistently resulted in higher rate of DNA recovery. Cloning strategies were dependent strongly on the overall goal of the study. The construction of complex libraries derived from soil sample and cloning of functional genes were dependent on the high quality of the extracted soil DNA, as the enzymatic modifications required during the cloning steps are sensitive to contamination by

various biotic and abiotic components. Metagenomics is a young and exiting technique that has broad application in biology and biotechnology. Collection of metagenomic clones will enhance the potential metagenomics study to provide the address for targeted questions in environmental biotechnology and also this technology is sufficiently powerful to yield bioactive compounds to solve real world problems including biodegrading enzymes antibiotics etc.

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