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ISOLATION OF METAGENOMIC DNA FROM MANGROOVE SOIL AND CONSTRUCTION OF COSMID LIBRARY

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ABSTRACT

Microorganisms present in the marine environment, can act as a good source of large number of biologically active molecules like antibiotics, anti cancer compounds, a wide range of enzymes etc. Screening for such organisms based on currently available culture based techniques is yielding only limited results because only around 2 percent of microorganisms present in marine environment are culturable. Metagenomics is a promising technique for the screening and isolation of biologically active molecules even from unculturables. Environmental mega-DNA(eDNA) was isolated from marine sediments. It represents DNA from both culturables and unculturables. Isolated mega-DNA was ligated with pWEB-TNC vector from Epicentre. It was cloned into the EPI100-T1^R *E.coli*, to construct cosmid library of the mega-DNA. Library was screened for antibiotic producing clones and enzyme producing clones. Positive clones were separated from the library and studied in detail. Out of the positive clones clone ML3 was known to produce maximum amount of lipase

KEYWORDS

Metagenomics, eDNA, Marine sediment, Cosmid library, Lipase

INTRODUCTION

Microorganisms are considered to be main sources of metabolites like antibiotics, enzymes and growth factors. A large number of metabolite producer are isolated from soil. Now marine environment is attracting much attention. Mangroves are hotspots of biodiversity. Isolation of novel metabolite from microorganisms based on culture based techniques are yielding limited amount of success when considering the number of microbes available there. Screening for metabolite producer based on culture independent methods is gaining importance.

Metagenomics is now gaining popularity. (Meta=environment). Metagenomics is the study of metagenomes, genetic material recovered directly from environmental samples. Currently two different approaches are followed in metagenomics. In first approach, the meta DNA or eDNA is subjected to massive sequencing and genes present in the sequence are annotated and identified. In second approach eDNA is isolated and metagenomic library is constructed in a host organism using suitable vectors. Cosmids, Fosmids and Bacterial Artificial Chromosome (BAC) are some of the suitable vectors available.

MATERIALS AND METHODS

(i) Collection of Mangrove soil

Soil sample for this study was collected from Pichavaram in Cuddalore district, Tamilnadu, South India. Organic rich soil from mangrove sediment was scooped into sterile polythene bag and stored at 4°C 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.

(ii) Isolation of eDNA

125 g of soil was placed in 500 mL nalgene bottle. Another bottle was kept with the same quantity of soil. 150 mL of preheated (70°C) lysis buffer (100mM Tris-HCl, 100mM NaEDTA, 1.5 M NaCl, 1%(w/v) CTAB, 2% (w/v) SDS, pH 8.0) was added to each bottle and mixed well. The bottles were incubated in a 70°C water bath for 2 hours. The bottles were inverted every 30 minutes. After 2 hours the bottles were removed from water bath and allowed to cool. Cooled crude soil lysate was transferred to 1L centrifuge bottle and centrifuged at 3,500g for 10 minutes at 4°C. Supernatant was centrifuged again at 3,500g for 10 minutes at 4°C. Supernatant was transferred to a clean tube and 0.7 volumes of isopropanol was added and gently mixed. Tubes were incubated at room temperature for 30 minutes. eDNA was precipitated as pellet by centrifugation at 3,500g for 30 minutes at 4°C. Pellet was washed with 100mL of 70% ethanol and centrifuged at 3,500g for 10 minutes at 4°C. Pellet was air dried.

(iii)Purification of eDNA

Using 0.5X TBE buffer 20cm X 10cm EtBr free 1% agarose gel was prepared. eDNA was pre-warmed to 50°C and loaded to the well. Gel was run at 100V for 1 hour and 20V overnight. Buffer was replaced after 5 hours. On the next day gel was cut at the border along with the part of the well and stained with EtBr. The place of the band was marked on stained gel. Stained and unstained gel pieces were kept together and position of the DNA band in unstained gel was removed.

(iv) Electroelution and concentration of eDNA

Gel segment with eDNA was placed in a piece of dialysis tubing (MWCO 10,000) and clamped. Dialysis tube was kept in electo-eluter and electricity was applied at 100V for 2-3 hours. Buffer in the dialysis bag with eDNA was collected and concentrated to less than 1 mL with Amicon centrifugal concentrator.

(v) Cloning of purified eDNA

Cloning was done with pWEB-TNC cosmid cloning kit from EPICENTRE Biotechnologies, Madison, Wisconsin. Blunt ended eDNA was endrepaired using the kit. The composition of the reaction mixture as follows,

42 μl sterile water (the final reaction volume will be 80 μl)
8 μl of 10X end-repair buffer
8 μl of 2.5 mM dNTP mix
8 μl of 10 mM ATP
10 μl of HMW eDNA (2.5 mg)
4 μl of end-repair enzyme mix
80 μl final reaction volume

The mixture was incubated for 45 minutes at room temperature. Later enzyme was heat inactivated by heating at 70°C for 10 minutes. Endrepaired eDNA was precipitated by adding 120 μl of sterile nuclease free water, 20 μl of 3M sodium acetate(pH 5.0) and 140 μl isopropanol. Mixture was incubated for 30 minutes at room temperature and centrifuged at 14,000g for 30 minutes at 4°C. Supernatant was removed and 500 μl of 70% ethanol was added to the pellet. Tube was centrifuged at 14,000g for 5 minutes at room temperature, supernatant was discarded and tube was centrifuged again. Pellet was air dried and dissolved in 25 μl of TE buffer. 1 μl of eDNA was run in 1% agarose gel with Ethidium Bromide (EtBr) to find out the concentration.

0.25 μl of endrepaired eDNA was ligated with 0.5 μl of dephosphorylated pWEB-TNC cosmid vector in 20 μl of ligation solution. Ligated eDNA was packed in MAX-PLAX extract according to the manufacturer's instructions. *E.coli* was cultivated a day earlier in LB with 10 mM $MgSO_4$. Packaged eDNA and *E.coli* were mixed in a ratio of 1:10. Mixture was incubated at room temperature for 20 minutes and then at 37°C for 75 minutes with shaking. Absorbed cells were spread on 150 mm LB plates with ampicillin as selection antibiotic. Plates were incubated at 37°C overnight.

(vi) Screening for active clones

The clones were picked using sterile tooth picks and placed on Lipase screening agar with the following composition.

Peptone	-	1 g
Beef extract	-	0.3 g
Calcium chloride	-	0.01g
Tween 80	-	1 ml
Agar	-	2 g
Distilled water	-	100 ml
pH	-	7 $\pm$ 7.2

Clones showing a halo zone after 24 hours of incubation at 37°C after the addition of Rhodamine B were chosen and stored separately.

OBSERVATION

One kilogram of organic rich mangrove sediment was collected. eDNA was isolated from the mangrove soil and made purified using agarose gel electrophoresis. A large band of eDNA was found around 23 kb. eDNA was eluted and concentrated using Amicon centrifugal concentrator. Concentrated eDNA was endrepaired and ligated to dephosphorylated pWEB-TNC vector. Ligated DNA construct was packed in MAX-PLAX and inserted into *E.coli* host. A total number of 8100 clones were obtained. The clones were screened for lipase production activity. A total number of 3 clones were screened to be positive for lipase production.

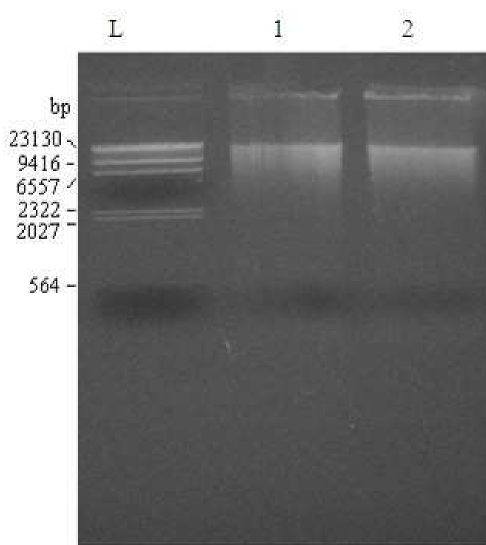

Figure 1
Agarose gel electrophoresis of eDNA(Lane L Lambda DNA HindIII digest; 1,2 eDNA)

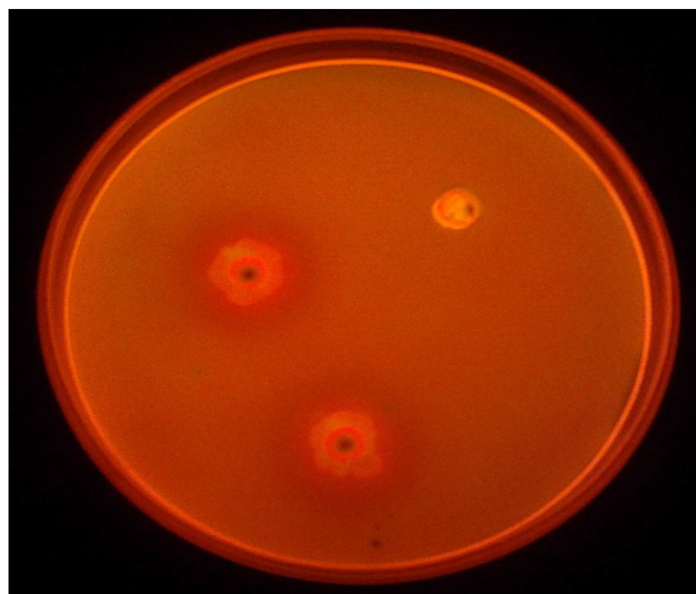

Figure 2
Lipase plate assay

DISCUSSION

Marine microbes are both extremely abundant and diverse. As culture-dependent methods have thus far resulted in the isolation of only a tiny percentage of the marine microbiota the application of metagenomic strategies holds great potential to exploit the enormous microbial biodiversity which is present within these marine environments.(Kennedy2008)In this present study lipase producing clone was isolated from metagenomic library of mangrove sediment. Functional screening of obtained library yielded

0.04% positive clones of lipase activity. Previous studies, using metagenomic libraries for the detection of lipolytic activities, have identified lipases from thermal environments (Rhee *et al*., 2005), saline lake (Rees *et al*., 2003), or forest soil (Lee *et al*., 2004b), or lipases from field soil (Henne *et al*., 2000; Rondon *et al*., 2000) or drinking water (Voget *et al*., 2003).

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