Available online at www.ijpab.com

ISSN: 2320 – 7051
Int. J. Pure App. Biosci. **3 (4):** 202-207 (2015)

INTERNATIONAL JOURNAL OF PURE & APPLIED BIOSCIENCE

Research Article

OPEN ACCESS

Activity Screening of Mangrove Microbes through a Metagenomic Approach

Ayona Jayadev*, Rajika Lekshman V. and Parvathy V.S.

Dept. of Environmental Science, All Saints' College, Thiruvananthapuram, Kerala, India

*Corresponding Author E-mail: sureshayona@gmail.com

ABSTRACT

Mangrove ecosystems constitute about 60 – 70% of costline of earth. There are many factors which make mangroves hotspots of microbial diversity. And these communities play an important role in maintenance of this ecosystem. The countless roles of these microbes in this ecosystem is appreciated but the inability to culture microbes in any environmental sample completely is also realized. Hence, this study was undertaken for accomplishing the potentials of environmental DNA of a mangrove ecosystem by constructing a metagenomic library using the isolated DNA. Metagenomic DNA (good quality, 225 µg/mL) was isolated as per standard procedure and subjected to restriction digestion using three restriction enzymes, Bam HI, Eco RI and Hind III. Plasmid with high purity and good concentration (470µg/ml) was also isolated using standard protocols. The digested fragments were ligated to a vector, pGEX 6p-1 digested with the same enzymes. The recombinant vector was used to transform the host bacterium, E.coli DH5α and a small insert metagenomic library was constructed. Transformed colonies were selected on the basis of blue-white selection. The metagenomic library was screened for amylase activity. Fifty four colonies were found to be transformed. Out of the 54 clones obtained 14 were found to show amylase activity.

Key words: *Metagenomics, Environmental genomics, Metagenomic DNA, Metagenomic Library, Vector, Host, Restriction Enzymes, Restriction digestion, Clones*

INTRODUCTION

Mangroves are one of the unique ecosystems on earth. The global microbial diversity comes to an enormous, largely untapped genetic and biological pool[12] that could be exploited for the recovery of novel genes, biomolecules for metabolic pathways and various valuable products[3]. In spite of the obvious importance of microbes, very little is known of their diversity, for example, how many species are present in the environment, and what each individual species does, its ecological function[11]. Until recently, the traditional methods of culturing microorganisms limit analysis to those that grow under laboratory conditions[5,7]. However, it is widely accepted that up to 99% of the microbes in the environment cannot be readily cultivated [4,6,9]. This 99% carries genetic potentials that is unavailable in the culturable subset.

Diversity of microbial communities inhabiting mangroves, which are unique swampy, saline, partially anaerobic environment, is useful as it provides clue of the microorganism and their adaptability in such

habitats[8]. Many industrially important extracellular enzymes are reported to be produced by microbes. As microbes inhabiting this unique environment needs many potential metabolic activities[1], this piece of work was undertaken with the view of identifying the potent activities by looking for the production of extracellular enzymes of unculturable lot of microbial community.

MATERIALS AND METHODS

Sampling site

Water samples were collected from a mangrove system, Ottaplammodu estuarine region located in Kollam district. Collection was done according to standard procedures.

Bacterial strain and plasmid

The host bacterium used for the study was *E coli* DH5α (Bangalore Genei) and the plasmid vector was pGEX 6p-1(Department of Biotechnology, University of Kerala). This is a 4.9kb vector consisting of ORI (Origin of Replication), Ampicillin resistance gene, Lac Z gene with multiple cloning sites (MCS) consisting sites for different restriction enzymes such as Bam H1, Eco R1, Sma1, Not1 and PstI. The Host cells were cultured in Luria-Bertani medium at 30°C, culture with vector in LB broth substituted with (50µg/mL) ampicillin. Screening for transformed cells was done in LB agar plate substituted with antibiotic ampicillin (50µg/mL), X-gal (20 mg/mL) and of IPTG (200 mg/mL).

Isolation of Metagenomic and Plasmid DNA

The method adopted for the isolation of metagenomic DNA was by modified Zhou et. al.[13] . The cells were harvested from the water sample by continuous centrifugation at 5000 rpm, till visible pellet is observed. The pellet was mixed with 200µl of resuspension solution (TE Buffer), 50µl Lysozyme and 20µl Proteinase K and treated with 200µl of Extraction buffer. This was extracted with equal amount of phenol: chloroform: isoamyl alcohol and centrifuged at 12,000 rpm for 5 minutes. 20µl RNase was added and incubated at 65ºC for 10 minutes. The solution was centrifuged at 12,000 rpm and DNA was precipitated from aqueous phase adding 0.1 volume of 3M sodium acetate and 0.7 volume of isopropanol. The DNA containing pellet was washed, dried, resuspended in TE buffer and stored in -20ºC.

Plasmid DNA was isolated according to modified alkaline lysis method[8]. The selected colonies of plasmid carrying culture were used to inoculate 10 ml of Luria Bertani broth supplemented with antibiotic ampicillin (50mg/ml). 1ml of this overnight culture was inoculated into 100ml LB broth containing ampicillin. Cells were harvested from culture by centrifugation (5000 rpm for 15 minutes at 4ºC), suspended in 200µl lysis solution (Tris pH8 and 0.5M EDTA). 100µl of lysozyme was added followed by 400µl lytic mixture (1N NaOH and 10% SDS) and 300µl of 3M sodium acetate, was added and centrifuged. The aqueous phase was treated with 0.6 volume of isopropanol for the precipitation of DNA. DNA was pelleted by centrifugation at 10, 000 rpm for 15 minutes at 4ºC. The pellet was dried, suspended in TE buffer and stored at -20ºC.

The genomic and plasmid DNA were digested with two different restriction enzymes *viz.*, Bam H1 and Eco R1 to obtain DNA fragments for cloning. The method included mixing 20µl of genomic DNA with 2µl buffer, 7.5µl of sterile water and 0.5µl of restriction enzyme. The restricted fragments were detected in 0.8% of agarose gel electrophoresis at 100V and analyzed. The fragments of digestion were ligated by mixing 15µl of restricted genomic and plasmid DNA with 1.5µl sterile water, 3µl ligation buffer and 0.5µl T4 DNA ligase.

Competent cells were prepared according to Sambrook and Russell[8]. The culture of *E.coli* DH5α was centrifuged at 5000 rpm for 10 minutes. The pellet was suspended in 7.5 ml of pre-chilled 100 mM

calcium chloride, mixed and incubated in ice for 30 minutes. After incubation, centrifugation was done at 5000 rpm for 10 minutes at 4ºC. 1ml of calcium chloride was added to the pellet and incubated in ice for 1 hour. The competent cells thus prepared were stored at 4ºC for transformation experiments.

The *E coli* DH 5α cells were transformed with recombinant vector according to standard procedures[8]. The transformation of *E.coli* using pGEX 6p-1 was done by mixing 100µl of competent cells and 15µl of ligation products. The mixture was exposed to cold and heat shock. LB agar plates were prepared with and without antibiotic ampicillin as control. To the LB agar plate containing antibiotic ampicillin 40µl X-gal (20 mg/mL) and 4µlof IPTG (200 mg/mL) was added and uniformly spreaded using an L rod and incubate at 37ºC for 1 hour. The LB plates without ampicillin (control) were plated with both competent *E.coli* cells and with the transformed mixture. All the plates were incubated at 37ºC overnight. The transformants containing recombinant plasmid will appear as white and non-transformants as blue.

The metagenomic library was constructed using the recombinant white colonies. The colonies were transferred to fresh ampicillin containing LB plates spread with X-gal and IPTG. The plates were incubated at 37ºC overnight. The clones were stored for activity screening.

Functional Screening of metagenomic Library

The metagenomic library was screened for the production of extracellular enzyme, amylase. Starch agar medium was used to check amylase activity. All the plates were incubated at 37ºC overnight. After incubation the starch agar plate was flooded with Gram's iodine solution and observed[2]

Plasmid DNA was isolated from the positive amylase producing clones using modified alkaline lysis method[8].

RESULT AND DISCUSSION

After many attempts of standardizations, metagenomic DNA was obtained (Plate 1). It was purified using phenol: chloroform extraction method. Spectrophotometric analysis to determine the purity of metagenomic DNA gave a value 0.9. From this the concentration of DNA was calculated to be 225µg/ml. The DNA isolated by this method was used for further studies. As phenol: chloroform extraction and Proteinase K treatment was done, the obtained DNA was almost pure.

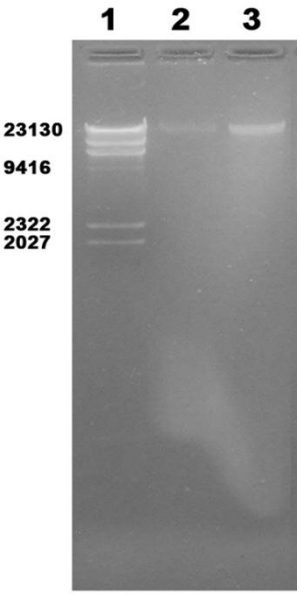
Isolated and purified plasmid DNA was observed as three distinct bands. The spectrophotometric ratio was found to be 1.83 indicating a pure DNA sample. The concentration was calculated to be 470µg/ml.

Metagenomic DNA may have contaminating humic acids which are hard to remove as their molecular weight, charge etc. are comparable to metagenomic DNA. Unless density gradient centrifugation is carried out by which pure DNA can be isolated the purity of DNA needs compromising. Even then the impurities in the isolated DNA in this study was not to that limit which would limit downstream applications like restriction digestion, ligation etc.

Normally to construct a large insert metagenomic Library, metagenomic DNA would be subjected to partial digestion only. But since in this study a small insert library was constructed, in order for the plasmid to take up fragments of DNA, complete digestion of metagenomic DNA was done (Plate 2).

The transformation was done with the competent cells and the ligated mixture of metagenomic and plasmid DNA restriction digest (Plate 3). Transformed recombinant colonies were observed as white colonies, while transformed non-recombinant colonies were observed as blue colonies (Plate 4). The restriction product of Bam H1 ligated with plasmid vector was transformed and showed good recombination i.e., more number of white colonies than blue colonies whereas no other restriction enzyme digest provide distinct bands.

The metagenomic library was constructed using the recombinant white colonies (Plate 5). Fifty four recombinant clones were successfully obtained. Of these 14 clones showed to have amylase activity.



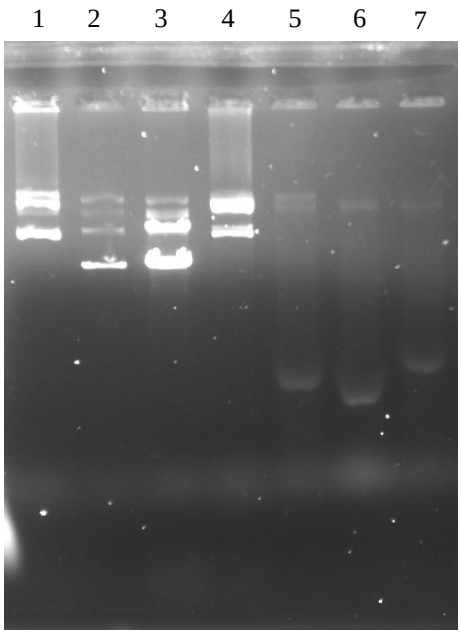
Lane 1

Molecular Weight Marker

Lane 2 &3

Metagenomic DNA

Plate 1 Metagenomic DNA



Lane 1- Plsmid Control

Lane 2- Bam HI digest of Plasmid

Lane 3- Bam HI digest of Plasmid

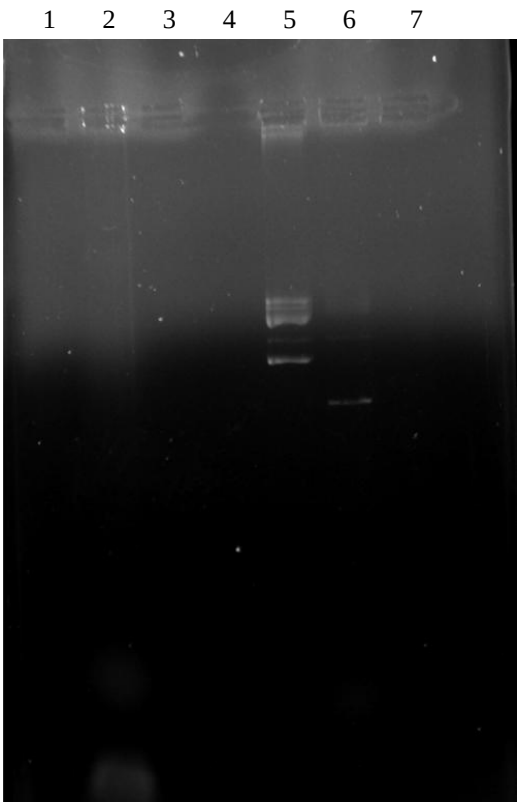
Lane 4- EcoRI digest of Plasmid

Lane 5- Metagenomic DNA control

Lane 6- Bam HI digest of Metagenomic DNA

Lane 7- EcoRI digest of Metagenomic DNA

Plate 2 Restriction Digestion (Metagenomic and Plasmid DNA)



Ligation product (Bam HI digest of Metagenomic and Plasmid DNA)
Lane 2- Bam HI digest of Metagenomic DNA
Lane 4- Bam HI digest of Plasmid DNA
Lane 5- Ligation product

Plate 3 Digestion and Ligation products

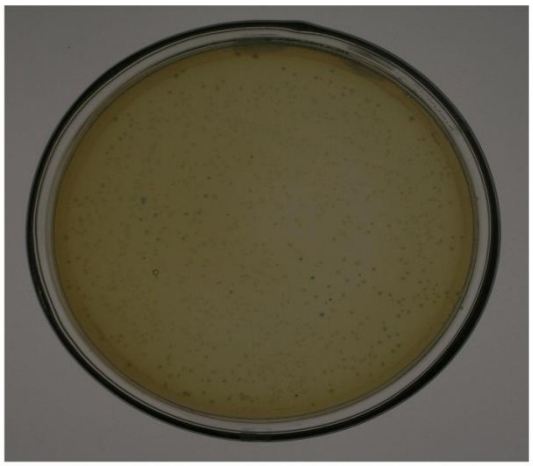


Plate 4 Blue White Selection

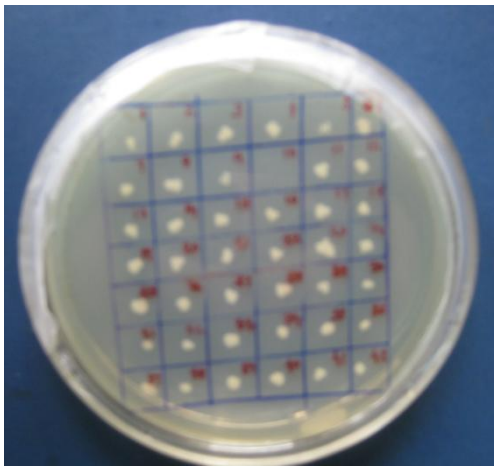


Plate 5 Metagenomic Library

CONCLUSION

Small insert metagenomic library was constructed in *E coli* DH 5α using pGEX-6p-1 from restriction digestion of metagenomic DNA using Bam HI. Activity screening of the clones for amylase, protease and lipase gave positive reaction for lipase activity only. The failure for not giving protease and lipase activity

may be due to the absence of expressible genes in the library. In the study, 14 out of 54 clones were found to be positive for amylase activity. Construction of large-insert metagenomic libraries will harbor large stretches of gene sequences which will give still more putative activities of microbial metagenome of the selected mangrove ecosystem.

Acknowledgement

I acknowledge the financial support received from the 'Kerala Biotechnology Commission', Kerala State Council for Science, Technology and Environment, in the form of Post Doctoral Fellowship and the space and materials provided by the Department of Biotechnology, University of Kerala for doing this piece of work.

REFERENCES

1. Fernando Dini Andreote mail, Diego Javier Jiménez, Diego Chaves, Armando Cavalcante Franco Dias, Danice Mazzer Luvizotto, Francisco Dini-Andreote, Cristiane Cipola Fasanella,Maryeimy Varon Lopez, Sandra Baena, Rodrigo Gouvêa Taketani and Itamar Soares de Melo, The Microbiome of Brazilian Mangrove Sediments as Revealed by Metagenomics, *PLOS ONE,* **7(6)** (2012).
2. Cho, H.Y., Kim, Y.W. Kim, T.J., Lee, H.S., Kim, D.Y., Kim, J.W., Lee, Y.W., Lee, S.B. and Park, K.H., Molecular characterization of a dimeric intracellular maltogenic amylase of *Bacillus subtilis* SUH4-2, *Biochem. Biophys. Acta,* **(1478):** 333–340 (2000).
3. Cowan, D.A.., Microbial genomes-the untapped resource, *Trends Biotechnol,* **(18)**: 14 (2000).
4. Hanada, S., Filamentous anoxygenic phototrophs in hot springs, *Microbes.Environ,* **(18)**: 51-61 (2003).
5. Hugenholtz, P.B., Goebel, M. and Pace, N.R., Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity, *J. Bacteriol*., **(180)**: 4765-4774 (1998).
6. Rappe, M.S. and Giovannoni, S.J., The uncultured microbial majority, *Annu. Rev. Microbiol*. **(57)**: 369-394 (2003).
7. Rondon, M.R., August, P.R., Bettermann, A.D., Brady, S.F., Grossman, T.H., Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms,. *Appl. Environ. Microbiol.*, **(66)**: 2541–4797 (2000).
8. Sambrook,, J., and Russell, D.W., *Molecular cloning: a laboratory manual* 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (2001).
9. Sekiguchi, Y., Yet-to-be cultural microorganisms relevant to methane fermentation processes, *Microbes. Environ,* **(21)**: 1-15 (2006).
10. Semenov, A.M., Van Bruggen, A.H. and Zelenev, V.V., Moving waves of bacterial populations and total organic carbon along roots of wheat, *Microbial Ecol.,* **(37)**: 116-128 (1999).
11. Singh,. L., Gautam, S.K., Verma, V., Kumar, M. and Singh, B., Metagenomics in animal gastrointestinal ecosystem: Potential biotechnological prospects, *Anaerobe*., **(14)**: 138 (2008).
12. Whitman, W.B., Coleman, D.C. and Wiebe, W.J., Prokayotes: the unseen majority. Proc. Natl. Acad. Sci. USA **95**: 6578-6583 (1998).
13. Zhou, J.M., Bruns, A. and Tiedje, J.M., DNA recovery from soils of diverse composition, *Appl. Environ. Microbiol*, **(62)**: 316–322 (1996).
14. Panmei, K. and Soram, J.S., Evaluation of Antioxidant Properties of Certain Traditional Medicinal Plants Used to Treat Diabetes mellitus in Manipur, *Int. J. Pure App. Biosci.* **2(2):** 24-27 (2014).
15. Yousef, N.M.H. and Nafady, N.A., Combining Biological Silver Nanoparticles with Antiseptic Agent and their Antimicrobial Activity, *Int. J. Pure App. Biosci.* **2(2):** 39-47 (2014).
16. Zulfiquar, M.B. and Battalwar, R., Nutritional Assessment and Health Status of Patients Undergoing Dialysis, *Int. J. Pure App. Biosci.* **3 (3):** 45-51 (2015).
17. Zuraida, A.R., Erny Sabrina, M.N., Mohd Shukri, M.A., Razali, M., Norma, H., Wan Zaliha, W.S. and Ayu Nazreena, O., *In vitro* Micropropagation of a Valuable Medicinal Plant, Piper crocatum. *Int. J. Pure App. Biosci.* **3 (3):** 10-16 (2015).