Mining the metagenome of activated biomass of an industrial wastewater treatment plant by a novel method

Nandita Sharma · Himgouri Tanksale ·
Atya Kapley · Hemant J. Purohit

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Abstract Metagenomic libraries herald the era of magnifying the microbial world, tapping into the vast metabolic potential of uncultivated microbes, and enhancing the rate of discovery of novel genes and pathways. In this paper, we describe a method that facilitates the extraction of metagenomic DNA from activated sludge of an industrial wastewater treatment plant and its use in mining the metagenome via library construction. The efficiency of this method was demonstrated by the large representation of the bacterial genome in the constructed metagenomic libraries and by the functional clones obtained. The BAC library represented 95.6 times the bacterial genome, while, the pUC library represented 41.7 times the bacterial genome. Twelve clones in the BAC library demonstrated lipolytic activity, while four clones demonstrated dioxygenase activity. Four clones in pUC library tested positive for cellulase activity. This method, using FTA cards, not only can be used for library construction, but can also store the metagenome at room temperature.

Keywords Activated biomass · Cellulase · FTA elute micro card · Lipase · Metagenomic library

Introduction

A Pandora's Box was opened when Jo Handelsman [8] coined the word metagenomics. Since then, metagenomic approach is used extensively to seek out several novel biocatalysts with the help of genetic information contained in the microbes inhabiting the niche. Quite a few innovative methods have been reported to analyze environmental niches and decipher the secrets hidden [3, 4, 22, 27]. The biggest asset of metagenomics is that it retrieves information without any bias to the culturable microorganisms of the microbiota. This ensures a fair representation of the total genomic content of all the microbial communities residing in the environmental sample, thereby allowing access to the gene pool of the yet uncultured microorganisms. There are two techniques by which the metagenome can be explored; one, creating libraries and screening for desired function [1, 29, 31] and second, sequencing the DNA directly (deep sequencing) and analyzing sequence data. Both approaches have the pre-requisite of good quality DNA.

During the last 10 years, many protocols for the extraction of environmental DNA have been reported [6, 7, 33, 34]. The methods vary with respect to removal of contaminants, shearing, purity and quantity of the isolated DNA. The most popular niche, the soil metagenome has been largely explored and is known to be the source of various novel biocatalysts such as enzymes and antibiotics [14]. The activated biomass of industrial wastewater treatment plants, comprising a complex microbial community [23], though less explored in comparison to other environmental niches, has also been a mining site for several enzymes and antibiotics [15, 19]. A wastewater treatment plant is an arrangement to facilitate the degradation of organic matter by the enzymatic action of

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N. Sharma · H. Tanksale · A. Kapley (✉) · H. J. Purohit
Environmental Genomics Division, National Environmental
Engineering Research Institute, CSIR, Nehru Marg,
Nagpur 440020, Maharashtra, India
e-mail: a_kapley@neeri.res.in; atyadrd@hotmail.com

microbes present in the activated sludge. Thus, metagenomic libraries generated from activated sludge as a source, can be beneficial for identifying novel biocatalysts which are of industrial and pharmaceutical importance.

Industrial wastewaters contain a wide variety of chemicals/non-degradable intermediates that are difficult to remove in DNA extraction procedures [26]. These are some of the prime obstacles for isolation of pure microbial DNA from effluent treatment plants. We have previously reported a protocol for extraction of PCR compatible DNA from activated sludge [21]. While we could use this template efficiently in amplification reactions [16, 17], the method did not yield high number of transformants in metagenomic libraries. In this study, we have developed a novel protocol for construction of metagenomic libraries, from the complex matrix of activated sludge of an industrial wastewater treatment plant using FTA Elute Cards (Whatman, USA). The libraries were developed in two vectors, pUC18 and pBeloBAC11, and efficacy was demonstrated by the functional screening of clones and large representation of bacterial genome.

Materials and Methods

Bacterial Strains, Plasmids and Growth Conditions

Plasmids pUC18 (2.686 Kb) and pBeloBAC11 (7.507 Kb) were used to construct metagenomic libraries from activated sludge. *Escherichia coli* DH5 α and *E. coli* DH10B were used as host cells for construction of pUC18 and pBeloBAC11 libraries respectively. The strains were grown at 37 °C on Luria–Bertani (LB) agar or in LB broth and *E. coli* DH5 α growth media was supplemented with 100 μ g/ml ampicillin, while growth media of *E. coli* DH10B was supplemented with 12.5 μ g/ml chloramphenicol.

Sample Collection

Activated biomass was collected from an industrial effluent treatment plant (ETP), treating wastewater generated at a pharmaceutical industry. One liter activated sludge sample was collected from nine different random sampling points, mixed to represent homogeneity of sample, kept on ice and transported to the laboratory within 6 h. The sample was stored overnight at 4 °C and used for the preparation of metagenome.

Metagenomic DNA Isolation

Hundred milligram (X5) of the sample was harvested by centrifugation at 10,000 $\times$ g for 5 min and washed with 1 ml sterile phosphate buffer. The biomass pellet was

resuspended in 50 μ l of sterile phosphate buffer and samples were directly spotted onto FTA Elute Micro card (5 repeats) (FTA cards were purchased from Whatman, UK). The card was allowed to dry at room temperature and placed at the bottom of a 2 ml centrifuge tube. 500 μ l of FTA purification reagent was added and incubated for 1 min. The solution was decanted after centrifugation at 6,000 $\times$ g for 30 s. After three washes with FTA purification reagent, the cards were incubated with 50 μ g Proteinase K (stock solution; 10 mg/ml) in 495 μ l of FTA purification reagent and incubated at 60 °C for 18 h at 100 rpm in a rocking chamber. The samples were centrifuged at 6,000 $\times$ g for 30 s and supernatant discarded. The cards were then suspended in 500 μ l of TE buffer and incubated for 1 min at room temperature. Supernatant was discarded after centrifugation and after three such washes the cards were washed with 1 ml sterile phosphate buffer to remove traces of TE. DNA immobilized on the cards was digested at 37 °C for 1½ h using *HindIII* enzyme in a total reaction volume of 100 μ l. Digested DNA, released from the paper matrix, was recovered in the supernatant after centrifugation at 12,000 $\times$ g for 2 min.

The quality of DNA was analysed by Nanodrop (ND-1000, Thermo scientific) UV–Vis Spectrophotometer and monitored for the values of OD260/OD280 and tested by 0.8 % gel electrophoresis.

Metagenomic Library Generation

The DNA recovered in the supernatant was used for library construction. The library was constructed by ligation of the insert DNA to *HindIII* digested and phosphatased pUC18 and pBeloBAC11 vectors. Ligated DNA was transformed into *E. coli* DH5 α and *E. coli* DH10B respectively by electroporation. Transformants were selected on LB agar plates supplemented with ampicillin (100 μ g/ml) for pUC18 recombinants and chloramphenicol (12.5 μ g/ml) for pBeloBAC11 recombinants. Blue-white screening of the recombinant clones was carried out after incubation of plates overnight at 37 °C. Clones were scored and selected for functional screening.

Functional Screening of Metagenomic Library

Spirit blue agar medium supplemented with 1 % tributyrin was used to screen for lipolytic activity [5]. Two strategies were used to screen the clones. One, wherein the transformants from LB-agar plates were lifted using sterile nitrocellulose membranes and transferred to target plates, and the other, where overnight grown cultures of the transformed clones were spotted onto spirit blue agar with 1 % tributyrin and incubated for 2 days at 37 °C. Formation of a clear halo around the colony as a result of

biocatalytic conversion of the indicator substrate, demonstrated lipolytic activity of the clone.

Cellulase- positive clones were screened by spotting them onto Minimal agar medium with 1 % carboxy methyl cellulose [32]. Cellulase expressing clones were detected by the formation of a yellow halo against a red background after staining with congo red and subsequent destaining with sodium chloride.

To screen for the catabolic capacities, overnight grown clones were spotted onto $0.1 \times$ LB plates containing 1 mM phenol or catechol, as reported earlier [30]. To further confirm that the observed phenotype was attributed to the metagenomic DNA insert, recombinant plasmid DNA was isolated and the presence of an insert was confirmed by restriction analysis for all clones isolated.

Restriction Digestion of Functional Clones

Clones demonstrating lipase or cellulase activity were differentiated by restriction digestion analysis. Plasmid preparation was carried out using alkaline lysis protocol and plasmid DNA was digested for 3 h with *AluI* enzyme in a 30 μ l reaction using 10 units enzyme. Banding pattern was analyzed on a 2 % agarose gel.

Results and Discussion

The unexplored microbial world is an important source of microbial products. Efforts reported to date are probably only the tip of the iceberg. Activated sludge from an industrial wastewater treatment plant is suspended in various xenobiotics. Extraction of DNA from such niches is always a challenge. As technology develops, new methods appear that overcome problems being faced in this new age of metagenomics. This protocol uses a simple strategy of spotting the sludge onto a commercially available card that is impregnated with a patented chemical formula that lyses cell membranes and denatures proteins upon contact. Nucleic acids are physically entrapped, immobilized, and stabilized for storage at room temperature. We modified the protocol of the manufacturers by incorporating treatment with Proteinase K that could digest any nucleases in the sample and hence, protect from DNA degradation. Addition of this step was found to increase the yield of DNA in our studies by approximately seven fold. Without Proteinase K treatment, metagenome yield was only 20 μ g per gram of wet-weight biomass, but efficiency improved to 122 μ g per gram of wet-weight with the incorporation of Proteinase K treatment. The FTA method, also ensures removal of chemical contaminants lingering in DNA extracts as well as cell moieties that co-precipitate with DNA. The method is

also simple, time saving and generates DNA which is compatible to downstream processes like ligation and cloning. Moreover the metagenomic DNA extraction procedure surpasses the use various chemicals and organic solvents which might otherwise add to the cost and complexity. A direct isolation method generally relies on extensive purification steps post extraction of DNA such as use of Sepharose, Sephadex, PVPP etc. [20, 28] which are not required in this method.

Metagenomic DNA was extracted from activated sludge with a final yield of 122 μ g per gram of wet-weight activated sludge. The 260/280 absorbance ratio of the metagenomic DNA was high (1.78), indicating the purity of DNA with lesser degree of protein contamination. Additionally, a high value (1.83) of the 260/230 absorbance ratio indicated low contamination by humic acid substances. Furthermore, the DNA was competent for downstream applications such as ligation, and cloning, confirming negligible interference by contaminants in enzymatic reactions like restriction digestion and ligation. Two metagenomic libraries were generated, one in pUC18 and the other in pBeloBAC11 cloning vector. Library characteristics are described in Table 1. The average insert size was calculated to be 11.0 kb for pBeloBAC11 library and 6.0 kb for pUC18 library. Multiplying this with the number of clones in each library, and based on the assumption that the *E.coli* genome is 4.6 Mb, the BAC library constructed covers 95.6 times the genome length of bacterial species, while the pUC library covers 41.7 times the size of prokaryotic genome.

Functional screening of metagenomic clones on indicator plates demonstrated diverse enzymatic activities. Screening the 40,000-member BAC library yielded twelve clones with lipolytic activity. Clear halos on tributyrin plates (Fig. 1), indicated positive activity. *E.coli* DH5 α containing pUC18 and *E.coli* DH10B containing pBeloBAC11 vectors were used as controls. The figure demonstrates that there is no lipolytic activity in the controls. The library constructed in pUC vector did not show any clones with lipolytic activity, but, four clones out of 32,000, tested positive for cellulase activity by forming yellow halos on carboxy methyl cellulose plates (Fig. 2). Four clones from the catechol plate showed change in color, demonstrating dioxygenase or monooxygenase activity. Two clones changed the indicator dye from yellow to green and two changed it to red, indicating biotransformation or degradation (Fig. 3).

A number of lipolytic enzymes, esterases and other industrially important enzymes have been discovered from the metagenome of activated sludge [2, 9–13, 18, 24, 25, 35]. Characterization of these clones in future will give a clue on the novelty/properties, of these enzymes discovered using the metagenomic approach, but the aim of this study

Table 1 Characteristics of metagenomic libraries developed from activated biomass of an ETP treating wastewater generated at a pharmaceutical industry

Vector used in library construction	Total no. of clones in library	Average insert size ¹	Representative bacterial metagenome screened ^{a,b}	Activity in clone	No. of clones
pBeloBAC11	40,000	11 kb	95.6 times of bacterial genome	Lipase	12
pUC18	32,000	6 kb	41.7 times of bacterial genome	dioxygenase ^c	4
				Cellulase	4

^a Based on the size of *E. coli* genome (4.6 Mb)

^b Multiplied total number of clones by average insert size, viz., 11.0 kb for pBeloBAC11 library and 6.0 kb for pUC18 library

^c Biotransformation of catechol based on plate assay [30]

¹ Calculations of average insert size in (supplementary Figs. 1 and 2)

Fig. 1 Lipase clones from metagenome of activated biomass. Lipase activity was identified by disappearance of blue colour from tributyrin plates (containing *spirit blue* as an indicator). Panel **a** shows the control

plate where overnight grown *E. coli* DH5 α containing pUC18 (1) and *E. coli* DH10B containing pBeloBAC11 (2) have been spotted. Panel **b** and **c** show independent positive clones. (Color figure online)

Fig. 2 Cellulase clones from metagenome of activated biomass. Cellulase activity was identified by halo formation on carboxy methyl cellulose plates after staining with congo red and subsequent wash with sodium chloride. Panel **a** and **b** demonstrate functional activity of clones, C1, C2, C3 and C4. (Color figure online)

was to develop a quick process to create metagenomic libraries. The novelty of the method lies in the metagenome preparation. To analyze if the functional clones were independent or repeats, restriction analysis using *AluI* enzyme was carried out. Banding pattern demonstrating independent nature of the clone can be viewed in Fig. 4a, b. Digestion of only vector has also been carried out for comparison.

The activated sludge matrix is highly complex and different reports are available on obtaining DNA for construction of library. Zhou et al. [36] reports extraction of crude DNA which needs further gel-plus-column methods for purification. Ranjan et al. [24] report that metagenomic DNA prepared needed further purification before downstream applications could be used. Liaw et al. [19] used a PowerSoil DNA isolation kit (Mo Bio Laboratories, US) and obtained a yield of 30 μ g of DNA per gram of activated sludge (wet weight) which was quite low than the yield reported in our method. Our method is rapid and yields DNA that can be used directly for ligation. The functional clones obtained in primary screening, also

Fig. 3 Clones from metagenome of activated biomass exhibiting catechol transforming activity. Catechol transforming activity was identified by appearance of *blue* and *red* colour on Catechol plates. Panel **a** and **b** show positive clones Cat1 and Cat2 respectively which changed colour to *green* as well as Cat3 and Cat4 which changed colour to *red*. (Color figure online)

Fig. 4 Restriction endonuclease digestion analysis of functional clones using *Alul* enzyme. **a** Banding pattern on 2 % agarose gel demonstrating independent nature of clones with cellulase activity. Lane 1 shows the standard 1 Kb Gene Ruler ladder (Fermentas, Canada). Lanes 2–5 show banding pattern of 4 clones and lane 6,

shows banding pattern of pUC18 vector. **b** Banding pattern on 2 % agarose gel demonstrating independent nature of clones with lipase activity. Lane 1 shows the standard 1 Kb Gene Ruler ladder (Fermentas, Canada). Lanes 2–13 show banding pattern of 12 clones and lane 14, shows banding pattern of pBeloBAC11 vector

demonstrate efficiency of the method. JunGang et al. [13] reported, only one lipolytic clone from an activated sludge metagenomic library of size 2.1 Gb, while Roh et al. [25] also reported one clone in 1,00,000 clones. Ranjan et al. [24] reported 13 lipolytic clones from pond water samples. Our protocol revealed as many as twelve lipolytic clones from a library of 40,000 clones of total capacity of

0.44 Gb, thus validating functional efficacy of the metagenomic library. In future, characterization and sequencing of the clones will have to be carried out before establishing novelty of functional activity; however, this paper focuses on an efficient and rapid method to generate metagenomic libraries, thus helping in unraveling the mystery of the microbial world.

Perhaps an additional benefit of our methodology is that DNA immobilized on the FTA card, can be stored at room temperature, avoiding the use of energy in storage.

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