Rapid and efficient method to extract metagenomic DNA from estuarine sediments

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Abstract Metagenomic DNA from sediments of selective estuaries of Goa, India was extracted using a simple, fast, efficient and environment friendly method. The recovery of pure metagenomic DNA from our method was significantly high as compared to other well-known methods since the concentration of recovered metagenomic DNA ranged from 1185.1 to 4579.7 µg/g of sediment. The purity of metagenomic DNA was also considerably high as the ratio of absorbance at 260 and 280 nm ranged from 1.88 to 1.94. Therefore, the recovered metagenomic DNA was directly used to perform various molecular biology experiments viz. restriction digestion, PCR amplification, cloning and metagenomic library construction. This clearly proved that our protocol for metagenomic DNA extraction using silica gel efficiently removed the contaminants and prevented shearing of the metagenomic DNA. Thus, this modified method can be used to recover pure metagenomic DNA from various estuarine sediments in a rapid, efficient and eco-friendly manner.

Keywords Metagenomics · PCR amplification · Restriction digestion · Spectrophotometry · Metagenomic library · Gene cloning

Introduction

The marine microbial diversity is largely untapped with reference to its biotechnological potential which can be exploited for the discovery of novel genes, entire metabolic pathways and bioactive compounds including protein pharmaceuticals (Sharma et al. 2014). The advancement in field of biotechnology with new and improved technologies in molecular biology, genetic engineering, protein engineering, fermentation technology as well as bioinformatics has immensely facilitated the process of discovering novel biomolecules especially from microorganisms. The diverse and extreme eco-niches viz. estuary, mangrove, salt pan and coastal sediments harbor several microorganisms that are potential sources of various industrially important novel enzymes and other bioactive molecules (Nair et al. 2014; Solomon et al. 2016). However, all the microorganisms cannot be cultivated using standard culture media conditions and >99% of these microorganisms remain uncultured (Hugenholtz and Pace 1996; Lorenz and Schleper 2002; Daniel 2005).

To overcome the limitations of culture dependent method, metagenomic approach has been adopted to explore the microbial community present in a specific eco-niche. Metagenomic DNA recovered from an environmental sample represents DNA of the entire community of a particular eco-niche (Handelsman et al. 1998; Rondon et al. 1999; Nordgard et al. 2005). Metagenomics coupled with PCR and bioinformatics has proved to be a powerful tool to identify novel genes encoding industrially important enzymes, biomolecules apart from determining the phylogeny and taxonomy of specific microbial communities (Theron and Cloete 2000; Torsvik and Ovreas 2002). Cloning of metagenomic DNA in an appropriate vector and then transforming the resulting recombinant DNA

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molecules into a suitable host enables us to access the gene pool of unculturable microorganisms (Rondon et al. 2000).

Recovery of metagenomic DNA suitable for PCR and metagenomic library construction remains a challenging task since humic and fulvic acids, being the major contaminants predominantly present in soil as well as sediment interfere with downstream DNA purification processes (Tsai and Olson 1992; Tebbe and Vahjen 1993; Alm et al. 2000). Recovery of high molecular weight metagenomic DNA is important for metagenomic library construction to improve the possibility of retaining the full length gene cluster involved in any biosynthetic pathway. Moreover, optimization of the lysis methods also play a crucial role in obtaining a good quantity of metagenomic DNA representing all taxonomic groups (Bag et al. 2016). It is also necessary to recover pure metagenomic DNA in large quantities to ensure representation of all the genomes of a particular community (Martin-Laurent et al. 2001; Lorenz and Schleper 2002; Bertrand et al. 2005).

Therefore, an efficient, cost effective and rapid method is needed to recover highly purified metagenomic DNA from the environmental samples viz. estuarine, mangrove and salt pan. Although molecular microbial ecologists have developed several diverse methods for recovery of metagenomic DNA from soil and sediment, but majority of these methods are time consuming and expensive. Besides various hazardous chemicals are involved and shearing results in an extremely poor yield of metagenomic DNA making it unsuitable to construct metagenomic library (More et al. 1994; Zhou et al. 1996; Clegg et al. 1997; Yeates et al. 1998; Miller et al. 1999; Niemi et al. 2001; Roose-Amsaleg et al. 2001; Santosa 2001; Lakay et al. 2006; Luna et al. 2006; Sagar et al. 2014; Solomon et al. 2016).

Prompted by above facts and findings we have developed a simple, fast and yet high yielding protocol for recovery of pure metagenomic DNA avoiding usage of toxic chemicals. The metagenomic DNA recovered by this method is suitable for various downstream processes viz. restriction digestion, PCR amplification, molecular cloning and metagenomic library construction.

Materials and methods

Collection of sediment samples

Sediment samples, i.e., GUMD1 (15°24'09.8"N 73°54'26.0"E), GUMD2 (15°30'03.5"N 73°50'43.2"E) and GUMD3 (15°30'21.3"N 73°49'31.9"E) were collected from three different estuarine sites and were sieved through a 2 mm mesh to remove larger particles. The pH of the samples was measured by 10× dilution of the original

sediment samples. Soil moisture as well as total organic content (by weight) of the samples was also determined after drying the samples in the oven at 100 °C for 2 days and at 500 °C for 3 h, respectively.

Isolation of metagenomic DNA

Sediment samples (400 mg) were suspended in 875 µL of 0.12 M sodium phosphate buffer (pH 8) followed by addition of 125 µL of 20% SDS in 2 mL eppendorf tubes which were mixed by inversion several times. Lysozyme (100 µL) from stock solution of 10 mg/mL was added to each sample, this was followed by vortexing for 30 s. Eppendorf tubes were incubated at 37 °C for 30 min along with inversion after every 5 min. Samples were subjected to centrifugation at 13,000 rpm and 4 °C for 10 min and the supernatant was transferred to a clean eppendorf tube to which 250 µL of ammonium sulphate (2.5 M) was added. The samples were mixed by inversion several times to remove protein contamination. Samples were centrifuged at 13,000 rpm at 4 °C for 10 min and supernatant was transferred to a clean eppendorf tube again. The supernatant was mixed with equal volume of silica gel in presence of 3 M guanidine hydrochloride (pH 7.5) and incubated at 25 °C for 10 min. The mixture of supernatant and silica gel slurry was applied to the spin column (MP Biomedicals, USA) and centrifuged at 13,000 rpm at 4 °C for 10 min. Metagenomic DNA bound to the column was eluted in 1.5 mL clean eppendorf tube using 50 µL sterile Milli-Q water. Eluted metagenomic DNA was further purified by mixing with equal volume of chloroform and isoamyl alcohol (24:1). Aqueous phase was recovered after centrifugation at 13,000 rpm at 4 °C for 10 min and metagenomic DNA was precipitated by incubating aqueous phase with 0.6 volume of isopropanol at 25 °C for 1 h. Subsequently the mixture was centrifuged at 13,000 rpm at 4 °C for 10 min and the pellet containing metagenomic DNA was washed with 70% ethanol, vacuum dried and finally resuspended in 25 µL of TE buffer (pH 8.0).

Comparison of performance of the modified method with commonly used methods

Metagenomic DNA of the environmental sediment samples was also recovered using other commonly used methods to compare the performance of our method in terms of DNA yield, purity of DNA, duration of the protocol and type of chemicals and solvents used (Zhou et al. 1996; Yeates et al. 1997; Burgmann et al. 2001; Amorim et al. 2008). The extracted metagenomic DNA samples (10 µL) were electrophoresed on 0.8% agarose gel.

Determination of yield and purity of metagenomic DNA

The yield and purity of the recovered metagenomic DNA was determined using spectrophotometer (Biospectrometer, Eppendorf, Germany). Absorbance of the DNA sample was recorded at 230, 260 and 280 nm to determine the concentration and purity of the metagenomic DNA (Holben 1994; Tien et al. 1999).

Restriction digestion and PCR amplification of 16S rDNA

Metagenomic DNA was digested with restriction enzyme *Sau3AI* to check the purity of metagenomic DNA in terms of digestion efficiency of the restriction enzyme. Restriction digestion was performed as per the standard molecular biology procedure (Sambrook et al. 1989). After incubating the restriction mixture at 37 °C for 30 min, the restriction digested metagenomic DNA samples (10 µL) were electrophoresed on 0.8% (w/v) agarose gel at 80 V for 30 min.

The purified metagenomic DNA recovered from sediment samples was also used directly as a template for the PCR experiment to amplify 16S rDNA. PCR mix consisted of 4 µL metagenomic DNA (50 ng/µL), 10 µL 10× reaction buffer, 4 µL dNTP mix (10 mM), 1 µL forward and reverse primers (20 µM) each, 2 µL *Taq* polymerase (2.5 U/µL) and 33 µL sterile Milli-Q water to make the final reaction volume of 50 µL. Thermal cycler (Mastercycler Nexus Gradient, Eppendorf, Germany) was used for PCR amplification. PCR conditions were as follows: 94 °C for 5 min (hot start) followed by 35 cycles of 94 °C for 1 min (denaturation), 57 °C for 1 min (annealing), 72 °C for 1 min (extension), final extension at 72 °C for 10 min and hold at 4 °C for infinity. PCR amplicon (10 µL) was analyzed by gel electrophoresis on (1%) agarose gel to determine the size of the amplicon.

Following primers (Fredriksson et al. 2013) were used for amplification of 16S rDNA:

Forward primer: 27F 5'-AGAGTTTGATCMTGGCT-CAG-3'.

Reverse primer: 1492R 5'-TACGGYTACCTTGTTAC GACTT-3'.

Construction of metagenomic DNA library

The recovered metagenomic DNA was also used directly to develop metagenomic library following standard molecular biology techniques (Sambrook et al. 1989). Metagenomic DNA was subjected to partial digestion with *Sau3AI* followed by resolution on 1% agarose gel. Metagenomic DNA fragments in the range of 1 to

10 Kbps were gel purified using gel extraction kit (Qiagen, India). Ligation of metagenomic fragments with *Bam*HI cut and dephosphorylated (alkaline phosphatase) pUC 18 was done by incubating the ligation mix overnight at 16 °C. The ligation mix was used for transformation using *E. coli* HB 101 as a host using heat shock method (Hanahan 1983). The transformation mix (100 µL) was plated on LB agar plate containing ampicillin (50 µg/mL) along with X-gal and IPTG followed by overnight incubation at 37 °C. The positive transformants (metagenomic clones) were screened based on blue white screening method.

Results and discussion

Isolation of metagenomic DNA

The moisture content and pH of the estuarine sediment samples (GUMD1, GUMD2 and GUMD3) ranged from 59 to 65% and pH 7.9 to 8.2 whereas total organic carbon ranged from 9 to 15%, respectively (Table 1). Although several metagenomic DNA extraction protocols and commercial kits are in use for isolation of metagenomic DNA from environmental samples but we have achieved substantially high yield of pure metagenomic DNA using our modified method. The commonly used cell lysis reagents include lysozyme, SDS, Tween-80 and DMSO (Zhou et al. 1996; Kresk and Wellington 1999; Rondon et al. 2000). Alternatively mechanical disruption of cells is also used to lyse the cells which include bead beating, freeze thawing and grinding of environmental samples, but the environmental DNA gets significantly sheared which thereby may not remain suitable for any molecular biology experiments (Amorim et al. 2008).

It is interesting to note that the quantity of metagenomic DNA recovered using our method was significantly high since the amount of metagenomic DNA ranged from 1185.1 to 4579.7 µg/g for various sediment samples (Fig. 1A, B; Table 2). The recovered metagenomic DNA was considerably pure as the A_{260}/A_{280} ranged from 1.88 to 1.94 and A_{260}/A_{230} varied from 2.21 to 2.30 confirming that our methodology ensured successful elimination of humic acid as well as fulvic acid contamination (Table 2). In contrast, earlier methods resulted in a comparatively poor metagenomic yield ranging from 0.6 to 748.6 µg/g for soil samples with a purity ratio (A_{260}/A_{280}) varying from 0.8 to 1.9 (Nair et al. 2014; Sagar et al. 2014; Sharma et al. 2014; Devi et al. 2015; Bag et al. 2016; Solomon et al. 2016). Therefore, our method is highly efficient for extraction of metagenomic DNA from sediment samples.

Table 1 Physico-chemical characteristics of the estuarine sediment samples

S. no.	Sample	pH	Moisture (%)	Organic content (%)
1	GUMD1	8.20 $\pm$ 0.05	59	9.80
2	GUMD2	7.98 $\pm$ 0.05	65	15.25
3	GUMD3	8.12 $\pm$ 0.05	62	12.10

Fig. 1 (A) Agarose gel analysis of metagenomic DNA isolated from estuarine samples. Lane 1–2 DNA isolated using present method from sample GUMD1. Lane 3–4 DNA isolated using present method from sample GUMD2. Lane 5–6 DNA isolated following the method of Amorim et al. (2008) from sample GUMD1 and GUMD2. Lane 7–8 DNA isolated following the method of Bürgmann et al. (2001) from sample GUMD1 and GUMD2. Lane 9–10 DNA isolated following the method of Yeates et al. (1997) from sample GUMD1 and GUMD2. Lane 11–12 DNA isolated following the method of Zhou

et al. (1996) from sample GUMD1 and GUMD2. Lane 13 DNA isolated from cultural bacteria as a control. (B) Lane 1 DNA isolated following the method of Amorim et al. (2008) from sample GUMD3. Lane 2 DNA isolated following the method of Bürgmann et al. (2001) from sample GUMD3. Lane 3 DNA isolated following the method of Yeates et al. (1997) from sample GUMD3. Lane 4 DNA isolated following the method of Zhou et al. (1996) from sample GUMD3. Lane 5 DNA isolated following the present method from sample GUMD 3

Table 2 Yield and purity of the metagenomic DNA from different estuarine sediment samples

S. no.	Samples	DNA yield ($\mu\text{g/g}$)	DNA purity (A_{260}/A_{280})	DNA purity A_{260}/A_{230}
1	GUMD1	2255.7 ± 2.1	1.89 ± 0.03	2.21 ± 0.02
2	GUMD2	1185.1 ± 2.2	1.94 ± 0.02	2.30 ± 0.02
3	GUMD3	4579.7 ± 2.5	1.88 ± 0.03	2.25 ± 0.03

Ratio of $A_{260}/A_{230} > 2$ indicates pure DNA and < 2 indicates contamination of humic acids

Ratio of $A_{260}/A_{280} > 1.7$ indicates pure DNA

Comparative performance of the modified method with commonly used methods

Our extraction procedure is comparatively simple, fast, cost effective and works well without using any toxic reagents (Table 3). We were able to recover metagenomic DNA from the estuarine sediments using liquid nitrogen and phenol in the range of $310\text{--}1000 \pm 2.4 \mu\text{g/g}$ using the protocol of Amorim et al. (2008). Likewise the protocol of Burgmann et al. (2001) yielded metagenomic DNA in the range of $136\text{--}176 \pm 2.3 \mu\text{g/g}$. We have recovered metagenomic DNA in the range of $15\text{--}23.5 \pm 2.1$ and $10\text{--}17 \pm 1.8 \mu\text{g/g}$ by following the protocol of Yeates et al. (1997) and Zhou et al. (1996), respectively. Keeping in view the comparative performance of these commonly used protocols for metagenomic DNA extraction, it is evident that our modified method is far more superior as the metagenomic DNA yield is significantly high, i.e., $1185.1\text{--}4579.9 \pm 2.2 \mu\text{g/g}$ of sediment.

The ratio of absorbance at 260 and 280 nm of the recovered metagenomic DNA using our method was in the range of 1.88–1.94 which clearly demonstrated that the metagenomic DNA was highly pure in nature. However, this ratio ranged from 1.68 to 1.76 for the protocol of Zhou et al. (1996), 1.75 to 1.82 for Yeates et al. (1997), 1.68 to 1.83 for Burgmann et al. (2001) and 1.71 to 1.79 for Amorim et al. (2008).

Our modified protocol required only lysozyme, SDS and sodium phosphate for metagenomic DNA extraction whereas other protocols include the use of chemicals viz.

CTAB, proteinase K, polyethylene glycol, phenol and DTT (Zhou et al. 1996; Yeates et al. 1997; Burgmann et al. 2001; Amorim et al. 2008). The extraction of metagenomic DNA was completed in 12 h following the protocol of Amorin et al. (2008) whereas it took 2 h following the protocol of Burgmann et al. (2001), 5 h using the protocol of Yeates et al. (1997) and 3.5 h using the protocol of Zhou et al. (1996). We could successfully obtain pure metagenomic DNA from sediment samples within a considerably less duration of 1.5 h using our method (Fig. 1A, B; Table 3). Therefore, it is evident that our metagenomic DNA extraction method is not only environment friendly and cost effective, but also is less time consuming with greater purity and yield.

Restriction digestion and PCR amplification of 16S rDNA

High molecular weight metagenomic DNA after digestion with restriction enzyme *Sau3AI* when analyzed by agarose gel electrophoresis confirmed that restriction digestion could be performed within 30 min at 37 °C which clearly demonstrated the purity of recovered metagenomic DNA since it was easily cleaved by the restriction endonuclease (Fig. 2). Similarly successful PCR amplification of 16S rDNA amplicon (1.5 Kbps) using recovered metagenomic DNA as a template also confirmed its purity, indicating the complete elimination of humic acid which would have otherwise inhibited the PCR by inactivating *Taq* DNA polymerase (Fig. 3).

Table 3 Comparison of various protocols used for metagenomic DNA extraction

S. no.	Method	Chemicals and reagents used	Time taken in recovery of metagenomic DNA (h)	DNA yield $\mu\text{g/g}$ of sediment	DNA purity A_{260}/A_{280}
1	Amorim et al. (2008)	Liquid nitrogen, phenol	12	$310\text{--}1000 \pm 2.4$	$1.71\text{--}1.79 \pm 0.04$
2	Burgmann et al. (2001)	CTAB, dithiothreitol, phenol, polyethylene glycol	2	$136\text{--}176 \pm 2.3$	$1.68\text{--}1.83 \pm 0.02$
3	Yeates et al. (1997)	Polyethylene glycol, phenol	5	$15\text{--}23.5 \pm 2.1$	$1.75\text{--}1.82 \pm 0.05$
4	Zhou et al. (1996)	CTAB, proteinase K	3.5	$10\text{--}17 \pm 1.8$	$1.68\text{--}1.76 \pm 0.02$
5	Present protocol	Lysozyme, SDS, sodium phosphate	1.5	$1185.1\text{--}4579.7 \pm 2.2$	$1.88\text{--}1.94 \pm 0.03$

Fig. 2 Agarose gel analysis of *Sau3AI* digested metagenomic DNA. Lane 1–3 *Sau3AI* digested metagenomic DNA from estuarine sediments of GUMD1, GUMD2 and GUMD3, respectively

Construction of metagenomic DNA library

Metagenomic DNA library construction is used to assess the suitability of the method for various downstream manipulations (Devi et al. 2015). We successfully developed our metagenomic DNA library using the recovered metagenomic DNA from various sediment samples which resulted in approximately 700 metagenomic clones by means of blue white screening. This evidently demonstrated that ligation as well as transformation efficiency was significantly high even though the metagenomic DNA has been recovered from estuarine sediments. Generally, T4 DNA ligase is prone to inactivation due to presence of common contaminants viz.

Fig. 3 PCR amplification of 16S rDNA using recovered metagenomic DNA samples. Lane 1 1 Kbps DNA marker; Lane 2–4 16S rDNA amplicon

proteins, lipids, humic acids and fulvic acids resulting from the extremely poor quality of metagenomic DNA obtained from environmental samples. The large number of metagenomic clones was an indication of a considerably good quality metagenomic DNA.

Conclusion

We have designed a new modified method to recover metagenomic DNA from estuarine sediment samples which is simple, fast, efficient and cost effective. Additionally, this method is environment friendly since it doesn't involve the use of many toxic reagents as well as feasible because no sophisticated equipments are needed as in case of other commonly used methods. Use of silica significantly reduced the shearing of metagenomic DNA and enhanced its purity as well as yield since proteins and humic acid contaminants commonly present in the sediments got eliminated. It is interesting to mention that the recovered metagenomic DNA was directly suitable for restriction digestion, PCR amplification and construction of metagenomic library without any additional purification steps. Therefore, this modified method may successfully be used to recover pure metagenomic DNA from various environmental samples viz. soil, sediment and sewage sludge.

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Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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