CTAB influenced differential elution of metagenomic DNA from saltpan and marine sediments

Bhavya Kachiprath¹ · G. Jayanath¹ · Solly Solomon¹ · Manomi Sarasan¹

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Abstract

A simple, reliable method for genomic DNA extraction from sediments with minimum contaminants was developed to address the risk of poor quality DNA in metagenomic studies. Nine DNA extraction methods using 20% cetyl-trimethyl-ammonium bromide (CTAB) were performed and compared to develop an extraction protocol that can offer humic acid-free metagenomic DNA from marine and saltpan sediments. Community DNA extraction was executed via., Zhou et al. modified protocol using 20% CTAB treatment at different steps to compare the efficacy of humic acid removal. Out of nine DNA extraction methods, method 6 significantly improved the quality of DNA with efficient removal of humic substances. 16S rRNA gene amplification and spectrophotometric analysis confirmed the efficiency of method 6 to remove DNA inhibitors from marine sediments as well as saltpan samples. Inhibitors extracted along with metagenomic DNA outcome increased DNA yield and PCR inhibition in method 1 and 3. However, repeated 20% CTAB wash in method 6 ensured 16S amplification and least yield and concentration. Current study explains a detailed protocol based on 20% CTAB wash for the extraction of humic acid-free DNA from diverse sediment samples.

Keywords Metagenomic DNA · DNA extraction · PCR inhibitory compounds · CTAB · Humic acid removal · Saltpan · Marine sediment

Introduction

Community DNA extraction methods from soil and sediments have been characterized by inherent biases because of the co-precipitation of contaminants, especially humic acids and phenolic compounds along with nucleic acids, and their interference to other downstream processes. The quality and quantity of template DNA plays a crucial role in a PCR-based experiment since the presence of various inhibitors has adverse effects on accuracy and reproducibility of PCR amplification (Wilson 1997; Huggett et al. 2008). As the physico-chemical properties of soil and sediment samples

vary worldwide, it is difficult to develop a single, ideal total community DNA extraction protocol (Felczykowska et al. 2015). A number of chemical, mechanical, and enzymatic methods and commercial kits have been developed to encounter this problem (Tsai and Olson 1991; Canto-Canche et al. 2013; Tian et al. 2013; Gutierrez-Lucas et al. 2014; Devi et al. 2015; Tanveer et al. 2016). Moreover, several cell lysis methods and purification steps have been recommended for the efficient recovery of high quality metagenomic DNA. These methods aid the lysis of entire microbial community within a sample and successive revival and purification of nucleic acids from lysed cells. As commonly co-precipitated PCR inhibitors like humic acids have physical and chemical properties similar to that of nucleic acids, it is challenging to remove humic acids and phenolic compounds from DNA samples. Techniques established for the removal of inhibitory compounds comprise Cesium chloride density gradient centrifugation (Lee and Hallam 2009), Sephadex G-200 column purification (Miller et al. 1999), gel electrophoresis (Zhou et al. 1996) and methods using cetyl-trimethyl-ammonium bromide (CTAB) (Farkas et al. 2017) and polyvinylpyrrolidone (PVPP) (Lakay et al. 2007).

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✉ Bhavya Kachiprath
bhavyanirmalagiri@gmail.com

¹ Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Cochin, Kerala 682016, India

Many DNA extraction protocols with CTAB facilitate cell breakage and effective deproteinization (Zhou et al. 1996; Porteous et al. 1997; Sei et al. 2000; Minas et al. 2011). Several studies do indicate that an increased CTAB concentration might enhance the formation and precipitation of insoluble complexes with proteins, polysaccharides and cell debris, resulting in a better quality DNA (Edgcomb et al. 1999; Schneegurt et al. 2003; Balestrazzi et al. 2009). Herein, we standardised and evaluated a CTAB-based DNA extraction protocol applicable to variety of sediment samples and recommended it for pure, intact and high-quality DNA with minimum shearing and better yield.

Methods

Sample collection

Sediment samples were collected from different saltpan regions of Ribander, Goa (15°29'57.12"N; 73°50'49.06"E) as well as marine regions of Kakinada, Bay of Bengal (17°09'37.2"N; 83°07'13.2"E) during cruise no. 353 *FORV Sagar Sampada* and Kovalam, Arabian Sea (8°30'43.0"N; 76°52'54.0"E) during cruise no. 360 *FORV Sagar Sampada*. Samples were kept in sealed sterile containers, transported to laboratory and stored at − 80 °C till analysis.

DNA extraction

DNA extraction was accomplished by modifying Zhou et al.'s (1996) protocol. Sediment samples (200 mg) were suspended in 500 µl of TEN buffer [100 mM of Tris–HCl, 100 mM of EDTA, (pH 8.0), 100 mM of sodium phosphate (pH 8.0) and 1.5 M of NaCl] followed by centrifugation at 12,000 rpm for 15 min (Step 1). Residue was treated with 500 µl of TEN buffer, 50 µl of 10% cetyl–trimethyl–ammonium bromide (CTAB), 50 µl of 20% sodium dodecyl sulphate (SDS) and 10 µl of Proteinase K (20 mg/ml) (Step 2) and the suspension was incubated at 55 °C for 2 h. Genomic DNA in resulting lysate was centrifuged at 10,000 rpm for

10 min and supernatant was purified by extraction with equal volume of chloroform: iso-amyl alcohol (24:1, vol/vol). The aqueous phase was precipitated with 600 µl of iso-propanol and kept at 4 °C overnight. Crude nucleic acid from aqueous phase was pelletized by centrifugation at 12,000 rpm for 30 min. DNA pellet washed with 500 µl of ice-cold 70% (vol/vol) ethanol followed by absolute ethanol was air-dried and re-suspended in TE buffer. We modified the above-mentioned extraction protocol by incorporating 20% CTAB instead of usual 10% in step 2. Besides, an additional 20% CTAB wash was included in step 1 as well as prior to ethanol wash to check its efficiency in removing inhibitory compounds. Genomic DNA extraction from all three stations was performed using nine different methods (Table 1). Experiments were repeated to get triplicate values.

Quantitative and qualitative assessment of DNA

Yield and purity of DNA was estimated using a Varioskan™ LUX µDrop Plate (Thermo Fisher Scientific, Finland). Protein and humic acid contaminants were assessed by calculating the absorbance ratio 260/280 (DNA/protein) and 260/230 (DNA/humic acid), respectively. Usually, 260/280 ratio smaller than 1.8 indicates the occurrence of proteins, whereas a value higher than 2.0 specifies probable contamination such as phenols. On the other hand, ideal 260/230 ratio is 2 and a value less than 2 denotes humic acid contamination. DNA integrity was evaluated by ethidium bromide-stained agarose gel electrophoresis.

PCR amplification of 16S rRNA gene

16S rRNA regions were amplified employing universal primers (27F: 5'-AGAGTTTGATCTGGCTCAG-3'; 1492R: 5'-TACGGYTACCTTGTTACGACTT-3'; Lane 1991). Thermal cycling program was as follows: 5 min at 95 °C; 35 cycles of 45 s at 94 °C, 45 s at 58 °C and 1 min at 72 °C; and 10 min at 72 °C. All PCR reactions were performed in 25 µl volume containing 1× standard Taq buffer (10 mM of Tris–HCl, 50 mM of KCl, pH 8.3), 2.5 mM of MgCl₂,

Table 1 Description of metagenomic DNA extraction methods employed in this study

Method	Modifications in Zhou et al. (1996) protocol
1	Normal Zhou et al. protocol using 10% CTAB
2	Normal Zhou et al. protocol using 20% CTAB
3	20% CTAB wash in step 1 and step 2
4	20% CTAB wash in first 2 steps + single 20% CTAB wash just before 70% ethanol wash
5	20% CTAB wash in first 2 steps + 2 times 20% CTAB wash just before 70% ethanol wash
6	20% CTAB wash in first 2 steps + 3 times 20% CTAB wash just before 70% ethanol wash
7	20% CTAB wash in step 2 + single 20% CTAB wash just before 70% ethanol wash
8	20% CTAB wash in step 2 + 2 times 20% CTAB wash just before 70% ethanol wash
9	20% CTAB wash in step 2 + 3 times 20% CTAB wash just before 70% ethanol wash

200 μM of dNTPs, 0.4 μM of each primer and 1U Taq DNA polymerase (New England Biolabs, USA) and 1 μl of genomic DNA using Veriti™ 96-well thermal cycler (Applied Biosystems, USA). After PCR, the amplicons were separated in 1% agarose-TBE gel (40 mM of Tris-HCl, 20 mM of Boric acid, 1 mM of EDTA, pH 8.0), comprising ethidium bromide for nucleic acid staining and visualization. DNA fragment size was calculated using a 100 bp ladder (New England Biolabs/NEB) as DNA marker. Electrophoresis was carried out in 1× TBE (Tris/Borate/EDTA) buffer for 60 min at 70 V and photographed using a gel documentation system (BioRad, USA).

Results and discussion

We followed conventional DNA extraction protocol developed by Zhou et al. in 1996 as the basic method for total community DNA isolation. Several studies had optimised Zhou et al. protocol (1996) by adapting a number of modifications in different reagents, their concentration, incubation time, etc. enabling the efficient removal of inhibitory compounds from different environment samples (Kauffmann et al. 2004; Sharma et al. 2007; Singh et al. 2014; Natarajan et al. 2016).

Generally, 2% CTAB is used for the removal of inhibitory compounds like humic acids. Studies comparing various procedures revealed that increasing CTAB concentration results in better quality DNA and efficient humic acid removal. This is consistent with those testified by several authors viz., DNA isolation from arctic sediments (10% CTAB; Solomon et al. 2016), filamentous fungi (3.5% CTAB; Gontia-Mishra et al. 2014), rhizospheric soil (10% CTAB; Fatima et al. 2014), coastal sediments (5% CTAB; Tatti et al. 2016), food borne fungal pathogens (10% CTAB; Umesha et al. 2016), tissues like leaves, seeds, etc. (8% CTAB; Rani and Sharma 2016) and diversity study of human respiratory microbiome (10% CTAB; Willner et al. 2012). Herein, we included 20% CTAB wash in different steps in Zhou et al. protocol and compared each method for the effective removal of inhibitory compounds. Metagenomic DNA was hence mined from marine and saltpan sediment samples by different isolation methods and DNA bands were appeared in electrophoretogram for Saltpan, Bay of Bengal and Arabian Sea samples (Fig. 1). Presence of PCR inhibitors were assessed by comparing DNA samples based on their absorbance ratio. While comparing the quality of nucleic acid from all three stations, highest $A_{260/280}$ ratio (least protein contamination among nine methods) was reported from method 7 (1.38) for saltpan and from method 3 (1.16 and 1.26) for Bay of Bengal as well as Arabian Sea (Table 2). Out of nine extraction protocols, DNA obtained by method 6 expressed values 1.18, 0.96 and 1.15 as highest

Fig. 1 Lane 1 to 9—metagenomic DNA isolated from saltpan, Goa (a), from Bay of Bengal (b) and from Arabian Sea (c) by nine different DNA extraction methods

Table 2 Absorbance $A_{260/280}$ of metagenomic DNA extracted from saltpan and marine sediment samples to determine protein contamination

Methods	Saltpan, Goa	Bay of Bengal	Arabian Sea
Method 1	1.2493 $\pm$ 0.0006	1.1220 $\pm$ 0.0000	1.2527 $\pm$ 0.0021
Method 2	1.3320 $\pm$ 0.0120	1.1457 $\pm$ 0.0040	1.2273 $\pm$ 0.0015
Method 3	1.2530 $\pm$ 0.0030	1.1667 $\pm$ 0.0015	1.2670 $\pm$ 0.0010
Method 4	1.2290 $\pm$ 0.0040	1.1120 $\pm$ 0.0062	1.1537 $\pm$ 0.0015
Method 5	1.2180 $\pm$ 0.0506	1.1137 $\pm$ 0.0021	1.1700 $\pm$ 0.0010
Method 6	1.3630 $\pm$ 0.0957	1.1310 $\pm$ 0.0017	1.1163 $\pm$ 0.0109
Method 7	1.3860 $\pm$ 0.0234	1.1237 $\pm$ 0.0006	1.1763 $\pm$ 0.0025
Method 8	1.3743 $\pm$ 0.0441	1.1203 $\pm$ 0.0012	1.1353 $\pm$ 0.0136
Method 9	1.3077 $\pm$ 0.0163	1.1317 $\pm$ 0.0021	1.2137 $\pm$ 0.0136

The values are averages $\pm$ standard errors based on three replications

$A_{260/230}$ ratio (least humic acid contamination among nine methods) for sediments from saltpan, Bay of Bengal and Arabian Sea, respectively (Table 3).

16S rRNA gene amplification was carried out as quality control for DNA extracts from saltpan and marine sediments using universal primers (Fig. 2). DNA obtained from method 6 exhibited amplicon with a product size of 1500 bp for all three stations. Method 7 and 8 also demonstrated positive results in the case of saltpan sediments.

Comparative analysis of DNA quantification from all extraction procedures showed highest yield and concentration from both method 3 for saltpan samples and method 1 for Bay of Bengal and Arabian Sea samples (Figs. 3, 4), whereas method 6 provided lowest DNA concentration and yield. Figures 3 and 4 indicated successive decrease in both concentration and yield according to increase in number of CTAB wash along with TEN buffer and 70% ethanol. Although, repeated washing resulted a decrease in

Table 3 260/230 ratio of metagenomic DNA extracted from saltpan and marine sediment samples as an indication of humic acid contamination

Methods	Saltpan, Goa	Bay of Bengal	Arabian Sea
Method 1	0.6527 ± 0.0049	0.8646 ± 0.0034	0.8236 ± 0.0121
Method 2	0.9504 ± 0.0103	0.8995 ± 0.0019	0.7217 ± 0.0142
Method 3	0.8102 ± 0.0025	0.7504 ± 0.0044	0.8209 ± 0.0128
Method 4	0.8363 ± 0.0091	0.9141 ± 0.0069	0.8652 ± 0.0713
Method 5	0.9493 ± 0.0059	0.9367 ± 0.0100	0.9333 ± 0.0193
Method 6	1.1860 ± 0.0647	0.9656 ± 0.0140	1.1480 ± 0.2103
Method 7	1.1177 ± 0.0085	0.8974 ± 0.0065	0.8864 ± 0.0143
Method 8	1.1620 ± 0.0344	0.9097 ± 0.0050	1.1833 ± 0.0352
Method 9	1.1303 ± 0.0199	0.9035 ± 0.0068	1.8737 ± 0.2829

The values are averages ± standard errors based on three replications

Fig. 2 16S amplified products of isolated DNA from saltpan sediment samples (a), from Bay of Bengal (b) and from Arabian Sea (c) by different isolation methods. Lane 1—Method 1, Lane 2—Method 2, Lane 3—Method 3, Lane 4—Method 4, Lane 5—Method 5, Lane 6—Method 6, Lane 7—Method 7, Lane 8—Method 8, Lane 9—Method 9, Lane 10—100 bp ladder (M)

concentration and yield, an increase in quality of DNA was observed as 16S rRNA amplification could be obtained by method 6 even in those samples which previously showed no amplification. The discrepancy in the yield of DNA and 16S rRNA gene amplification clearly shows the influence of co-eluted PCR inhibitors that can lead to a negative result by its interference in the normal quantification procedure.

Fig. 3 Concentration of DNA isolated from saltpan and marine sediment samples by different extraction methods **Fig. 4** Yield of DNA isolated from saltpan and marine sediment samples by different isolation protocols

Conclusion

Among the main factors responsible for the success of metagenomic studies, DNA extraction is the most challenging and vital step since humic acids are extremely inhibitory for PCR amplifications. It is worth considering that no single method of cell lysis or purification will be applicable for all samples. Since purity is of the greatest concern, several modifications have been made by researchers to allow the extraction of better quality DNA from complex environmental settings such as marine sediments and saltpan. In the present study, repeated washing with higher concentration of CTAB at the second step and before 70% ethanol wash in the DNA-extraction protocol (method 6) performed better for the effective removal of humic acids and extraction of good quality DNA. The protocol presented here is ideal for most sediments and it also offers valuable guidelines that can be applied to developing extraction methods for other kind of samples.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Balestrazzi A, Bonadei M, Calvio C, Galizzi A, Carbonera D (2009) DNA extraction from soil: comparison of different methods using spore-forming bacteria and the *swrAA* gene as indicators. *Ann Microbiol* 59(4):827–832
- Canto-Canche B, Tzec-Sima M, Vázquez-Loria JI, Espadas-Alvarez H, Chi-Manzanero BH, Rojas-Herrera R, Valdez-Ojeda R, Alzate-Gaviria L (2013) Simple and inexpensive DNA extraction protocol for studying the bacterial composition of sludges used in microbial fuel cells. *Genet Mol Res* 12(1):282–292
- Devi SG, Fathima AA, Radha S, Arunraj R, Curtis WR, Ramya M (2015) A rapid and economical method for efficient DNA extraction from diverse soils suitable for metagenomic applications. *PLoS One* 10(7):e0132441. <https://doi.org/10.1371/journal.pone.0132441>
- Edgcomb VP, McDonald JH, Devereux R, Smith DW (1999) Estimation of bacterial cell numbers in humic acid-rich salt marsh sediments with probes directed to 16S ribosomal DNA. *Appl Environ Microbiol* 65:1516–1523
- Farkas K, Hassard F, McDonald JE, Malham SK, Jones DL (2017) Evaluation of molecular methods for the detection and quantification of pathogen-derived nucleic acids in sediment. *Front Microbiol* 8:53. <https://doi.org/10.3389/fmicb.2017.00053>
- Fatima F, Pathak N, Verma SR (2014) An improved method for soil DNA extraction to study the microbial assortment within rhizospheric region. *Mol Biol Int*. <https://doi.org/10.1155/2014/518960>
- Felczykowska A, Krajewska A, Zielińska S, Los JM (2015) Sampling, metadata and DNA extraction—important steps in metagenomic studies. *Acta Biochim Pol* 62(1):151–160
- Gontia-Mishra I, Tripathi N, Tiwari S (2014) A simple and rapid DNA extraction protocol for filamentous fungi efficient for molecular studies. *Indian J Biotechnol* 13:536–539
- Gutierrez-Lucas LR, Montor-Antonio JJ, Cortes-Lopez NG, del Moral S (2014) Strategies for the extraction, purification and amplification of metagenomic DNA from soil growing sugarcane. *Adv Biol Chem* 4:281–289. <https://doi.org/10.4236/abc.2014.44034>
- Lee S, Hallam, SJ (2009) extraction of high molecular weight genomic DNA from soils and sediments. *J Vis Exp* 33. <http://www.jove.com/details.php?id=1569>. <https://doi.org/10.3791/1569>
- Huggett JF, Novak T, Garson JA, Green C, Morris-Jones SD, Miller RF, Zumla A (2008) Differential susceptibility of PCR reactions to inhibitors: an important and unrecognised phenomenon. *BMC Res Notes* 1:70–79
- Kauffmann IM, Schmitt J, Schmid RD (2004) DNA Isolation from soil samples for cloning in different hosts. *Appl Microbiol Biotechnol* 64:665–670. <https://doi.org/10.1007/S00253-003-1528-8>
- Lakay FM, Botha A, Prior BA (2007) Comparative analysis of environmental DNA extraction and purification methods from different humic acid-rich soils. *J Appl Microbiol* 102(1):265–273
- Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) *Nucleic acid techniques in bacterial systematics*. Wiley, New York, pp 115–175
- Miller DN, Bryant JE, Madsen EL, Ghiorse WC (1999) Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. *Appl Environ Microbiol* 65(11):4715–4724
- Minas K, McEwan NR, Newbold CJ, Scott KP (2011) Optimization of a high-throughput CTAB-based protocol for the extraction of qPCR-grade DNA from rumen fluid, plant and bacterial pure cultures. *FEMS Microbiol Lett* 325:162–169. <https://doi.org/10.1111/j.1574-6968.2011.02424.x>
- Natarajan VP, Zhang X, Morono Y, Inagaki F, Wang F (2016) A modified SDS-based DNA extraction method for high quality environmental DNA from seafloor environments. *Front Microbiol* 7:986. <https://doi.org/10.3389/fmicb.2016.00986>
- Porteous LA, Seidler RJ, Watrud LS (1997) An improved method for purifying DNA from soil for polymerase chain reaction amplification and molecular ecology applications. *Mol Ecol* 6:787–791
- Rani B, Sharma VK (2016) A modified CTAB Method for quick extraction of genomic DNA from rice seed/grain/leaves for PCR analysis. *Int J Sci Res Methodol* 4(4):254–260
- Schneegurt MA, Dore SY, Kulpa CF Jr (2003) direct extraction of DNA from soils for studies in microbial ecology. *Curr Issues Mol Biol* 5(1):1–8
- Sei K, Asano K, Tateishi N, Mori K, Ike M, Kohno T, Fujita M (2000) Development of simple methods of DNA extraction from environmental samples for monitoring microbial community based on PCR. *Jpn J Water Treat Biol* 36(4):193–204
- Sharma PK, Capalash N, Kaur J (2007) An improved method for single step purification of metagenomic DNA. *Mol Biotechnol*. <https://doi.org/10.1007/s12033-007-0015-3>
- Singh R, Devi T, Verma V, Rasool S (2014) comparative studies on the extraction of metagenomic DNA from various soil and sediment samples of Jammu and Kashmir region in prospect for novel biocatalysts. *IOSR J Environ Sci Toxicol Food Technol* 8(4):46–56
- Solomon S, Kachiprath B, Jayanath G, Sajeevan TP, Bright Singh IS, Philip R (2016) High-quality metagenomic DNA from marine sediment samples for genomic studies through a preprocessing approach. *3 Biotech* 6:160. <https://doi.org/10.1007/s13205-016-0482-y>
- Tanveer A, Yadav S, Yadav D (2016) Comparative assessment of methods for metagenomic DNA isolation from soils of different crop growing fields. *3 Biotech* 6:220. <https://doi.org/10.1007/s13205-016-0543-2>
- Tatti E, McKew BA, Whitby C, Smith CJ (2016) Simultaneous DNA-RNA extraction from coastal sediments and quantification of 16S rRNA genes and transcripts by real-time PCR. *J Vis Exp* 112:e54067. <https://doi.org/10.3791/54067>
- Tian W, Zhang Z, Liu D, Zhou T, Shen Q, Shen B (2013) An optimized DNA extraction and purification method from dairy manure compost for genetic diversity analysis. *World J Microbiol Biotechnol* 29:815–823
- Tsai YL, Olson BH (1991) Rapid method for direct extraction of DNA from soil and sediments. *Appl Environ Microbiol* 57:1070–1074
- Umesha S, Manukumar HM, Raghava Sri (2016) A rapid method for isolation of genomic DNA from food-borne fungal pathogens. *3 Biotech* 6:123. <https://doi.org/10.1007/s13205-016-0436-4>
- Willner D, Daly J, Whiley D, Grimwood K, Wainwright CE, Hugenholtz P (2012) Comparison of DNA extraction methods for microbial community profiling with an application to pediatric bronchoalveolar lavage samples. *PLoS One* 7(4):e34605. <https://doi.org/10.1371/journal.pone.0034605>
- Wilson IG (1997) Inhibition and facilitation of nucleic acid amplification. *Appl Environ Microbiol* 63(10):3741–3751
- Zhou J, Bruns MA, Tiedje JM (1996) DNA recovery from soils of diverse composition. *Appl Environ Microbiol* 62(2):316–322