Biotechnology Reports 4 (2014) 134–138

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Evaluation of five in situ lysis protocols for PCR amenable metagenomic DNA from mangrove soils

Harisree P. Nair, Helvin Vincent, Sarita G. Bhat *

Department of Biotechnology, Cochin University of Science and Technology, Cochin, Kerala 682022, India

ARTICLE INFO

Article history:
Received 15 July 2014
Received in revised form 25 September 2014
Accepted 26 September 2014
Available online 30 September 2014

Keywords:
Metagenome
In situ lysis
DNA extraction

ABSTRACT

Microbes in nature are rarely amenable to growth by standard microbiological methods, with the majority being unculturable. Metagenomic methods help to bypass and overcome the limitations of traditional culturing method; wherein total community DNA is isolated, cloned into suitable vector and host systems. However, isolation of total community DNA itself remains a challenge. In this study five methods of total community DNA isolation from three different mangrove soils were evaluated to test its PCR amenability. The yield and purity of the isolated DNA was also analysed. The quantity of DNA by all 5 methods although reasonably high, contained residual humic contaminants. Of the five, the method employing liquid nitrogen yielded readily amplifiable DNA, while that by all others required further downstream processing to achieve purity and PCR amenability.

© 2014 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

1. Introduction

Soil being a complex habitat for diverse microorganisms, is a rich source of novel enzymes and bioactive molecules for application in health and other industries. Estimations reveal that less than 1% of the total microbial communities from the environment are readily cultivable by standard microbiological methods [1]. The unculturable microbes remain uncharacterised, the deficiency of information about their culturing parameters, allowing their continuation as unexplored reservoir of metabolic and genetic diversity.

Mangrove ecosystems present at the intertidal zones of estuaries, lagoons or marshes of tropical and subtropical latitudes, are unique ecological niches, habitat to multiple microbes playing significant roles in nutrient recycling and various ecological processes; thereby necessitating a thorough exploration of these microflora. Mangrove soils are commonly nutrient rich and hence exceedingly diverse in their microbial content. By the same rationale, community DNA isolation is a challenging process owing to co-extraction of humic substances.

DNA extraction methods are classified as direct (in situ) and indirect (ex situ) methods. In direct methods, cells are lysed within the soil sample, followed by consequent separation of DNA from cell debris and soil matrix [2]; and indirect method employs cell separation followed by cell lysis and DNA recovery [3]. These approaches have advantages as well as disadvantages concerning DNA yields, purity for molecular analysis and unbiased representation of the entire microbiome.

However as soil compositions vary greatly with regard to the organic and inorganic content, standardisation of total DNA isolation protocols become a prerequisite to any analysis. The objective of this study was to investigate the effectiveness of different direct lysis methods on yield and purity of DNA from mangrove soils to enable PCR amplification and further metagenomic analysis.

2. Materials and methods

2.1. Sample collection

Mangrove soils were collected from 3 different islands located in Kochi, Kerala, India, by removing surface leaf litter and collecting the top soil. Samples were transferred with sterilised spatula in sterile containers and were stored at −20 °C until further analysis. Sampling location details are given in Table 1.

2.2. DNA extraction

The five direct lysis methods tested for isolation and purification of DNA from the three mangrove soils include the methods of Zhou et al. (1996), slightly modified method of Volossiouk et al.

* Corresponding author. Tel.: +91 484 2576267.
E-mail address: saritagbhat@gmail.com (S.G. Bhat).

http://dx.doi.org/10.1016/j.btre.2014.09.008
2215-017X/© 2014 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

Table 1
Coordinates of sampling location.

Soil	Sampling station	Latitude	Longitude
1	Vypin	10° 4' 7.3272"N	76° 12' 47.3292"E
2	Bolgatty	10° 0' 16.2864"N	76° 15' 42.0120"E
3	Ponnarimangalam	10° 0' 16.2864"N	76° 15′ 42.0120"E

(1995), Dong et al. (1996), Tsai and Olson, (1991) and that of Siddhapura et al. (2010).

2.2.1. Method 1 [4]

Mixed 5 g soil with 13.5 mL DNA extraction buffer (in an Oakridge tube) (100 mM Tris–HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 100 mM sodium phosphate (pH 8.0), 1.5 M NaCl, 1% CTAB) and 100 mL of proteinase K (10 mg/mL) (Fermentas, USA) and the sample was incubated by horizontal shaking at 225 rpm for 30 min at 37 °C (Orbitek, Scigenics India). This was followed by addition of 1.5 mL of 20% SDS and incubated in a 65 °C water bath for 2 h (Remi, India) with gentle end-over-end inversions every 15–20 min. The supernatant was transferred to new tubes after centrifugation at 6000 × *g* for 10 min (Sigma, 2–16 K, Germany) at room temperature. The soil pellets were further extracted twice using the same protocol. Supernatants from the three extractions were pooled, mixed with equal volume of chloroform: isoamyl alcohol (24:1, v/v), followed by recovery of the aqueous phase by centrifugation and finally precipitation with 0.6 volume of isopropanol at room temperature for 1 h. The nucleic acids obtained were pelleted by centrifugation at 16,000 × *g* for 20 min and washed with cold 70% ethanol, air dried and resuspended in sterile deionised water to a final volume of 500 μL.

2.2.2. Method 2 [5]

After adding liquid nitrogen the 0.25 g soil sample was ground to fine powder using sterile mortar and pestle, suspended in 0.5 mL of skim milk powder solution (0.1 g skim milk in 25 mL of water), vortexed well and centrifuged for 10 min at 12,000 × *g* at 4 °C. To the supernatant 2 mL of SDS extraction buffer (0.3% SDS in 0.14 M NaCl, 50 mM sodium acetate (pH 5.1) was added and vortexed to mix. An equal volume of Tris-saturated phenol solution was added and vortexed for 2 min at room temperature. Aqueous phase was collected by centrifugation at 12,000 × *g* for 10 min and the nucleic acid was precipitated with 1 volume of ice cold isopropanol at −20 °C for 1 h, followed by centrifugation at 12,000 × *g* for 10 min to pellet the DNA. The pellet was washed twice with cold 70% ethanol, with centrifugation between each rinse, air dried, dissolved in 150 μL of sterile deionised water and stored at −20 °C until further analyses.

2.2.3. Method 3 [6]

In this method 0.30 g of soil sample was mixed with 0.35 g of glass beads (diameter 2.0 mm) and 300 μL of phosphate buffer (0.1 M NaH_2PO_4–$NaHPO_4$ (pH 8.0)) in a microcentrifuge tube, vortexed well, followed by addition of 250 μL of SDS lysis buffer (100 mM NaCl, 500 mM Tris (pH 8.0), 10% SDS). This was vortexed horizontally for 10 min at 225 rpm. The supernatant was transferred to new tube after centrifugation at 10,000 × *g* for 30 s. 250 μL of chloroform: isoamyl alcohol (24:1) was added and incubated at 4 °C for 5 min, followed by centrifugation at 10,000 × *g* for 1 min. Nucleic acids were precipitated by addition of 0.5 volume of 7.5 M ammonium acetate and 1volume of isopropanol, and incubated at −20 °C for 15 min. DNA was pelleted at 12,000 x *g* for 10 min, was washed thrice with 70% ethanol and air-dried. Pellets were dissolved in 100 μL of 10 mM Tris (pH 8.1), 100 μL of 10 mM Tris [pH 7.4], 100 μL of 10 mM Tris (pH 6.7) and 100 μL of 10 mM Tris (pH 6.0) and flocculated with 10 mM aluminium sulfate. Precipitate of humic substances was removed by centrifuging at 10,000 × *g* for 5 min.

2.2.4. Method 4 [7]

One gram soil was washed twice with 2 mL of 120 mM sodium phosphate buffer (pH 8.0), suspended in 2 mL of lysis solution (0.15 M NaCl, 0.1 M Na_2EDTA [pH 8.0]) containing lysozyme [15 mg/mL], incubated for 2 h in a 37 °C water bath with mixing at 20–30 min intervals, followed by addition of 2 mL of 0.1 M NaCl, 0.5 M Tris–HCl [pH 8.0], 10% SDS. Cells were lysed by three cycles of alternating freeze-thaw at −80 °C and 65 °C respectively. After phenol–choloroform extraction, the nucleic acid was precipitated with ice cold isopropanol, dried and resuspended in 100 μL of TE buffer (20 mM Tris–HCl, 1 mM EDTA (pH 8.0)).

2.2.5. Method 5 [8]

In this method 1 g soil was mixed with 10 mL extraction buffer (100 mM Tris–HCl (pH 8.2); 100 mM EDTA (pH 8); 1.5 M NaCl), incubated at 37 °C for 10 h with shaking at 150 rpm and supernatant was collected by centrifugation at 5000 rpm for 10 min. Samples were re-extracted with 1 mL of extraction buffer. To the supernatant 4 mL of lysis buffer (20%, w/v) SDS, lysozyme (20 mg/mL), Proteinase K (10 mg/mL), *N*-lauryl sarcosine (10 mg/mL), 1% (w/v) CTAB (cetyltrimethylammonium bromide) was added and incubated at 65 °C for 2 h with intermittent shaking every 15 min. Centrifuged at 10,000 rpm for 10 min at 4 °C to collect the supernatant. The preparation after phenol–chloroform extraction was treated with 1/10 volume of 7.5 M potassium acetate and precipitated by 2 volumes of chilled absolute alcohol. DNA was pelleted by centrifugation at 10,000 rpm for 10 min, air dried and suspended in 50 μL sterile deionised water.

2.3. Determination of yield and purity of DNA

The yield and purity of DNA obtained by all the five methods was quantified using spectroscopic methods, by calculating A_{260}/A_{280} and A_{260}/A_{230} ratios for protein and humic acid contaminants in the preparation. A_{260}/A_{280} ratio less than 1.8 indicates protein contamination and A_{260}/A_{230} ratio less than 2 indicates the presence of humic acid substances.

2.4. Gel electrophoresis

The extracted DNA were analysed by agarose gel electrophoresis in 0.8% gel containing 10 mg/mL ethidium bromide solution under UV light. Gel pictures were captured using gel documentation system (Syngene, USA)

2.5. Purity of DNA by PCR

To determine whether PCR inhibitors were present, DNA preparations isolated by all protocols were used as template to amplify the region encoding 16S rRNA gene in a thermal cycler (Biorad, USA) using universal primers [9]. 50 ng template DNA was used in a 20 μL reaction with an initial denaturation for 2 min at 94 °C, 34 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 2 min with a final extension for 10 min at 72 °C. The amplicons were separated electrophoretically in 1% agarose gel and visualised using ethidium bromide under ultraviolet illumination and gel pictures are captured using gel documentation system (Syngene, USA)

2.6. Statistical analysis

All experiments repeated thrice and statistical analysis was done by Microsoft Excel 2007 calculating mean and standard error.

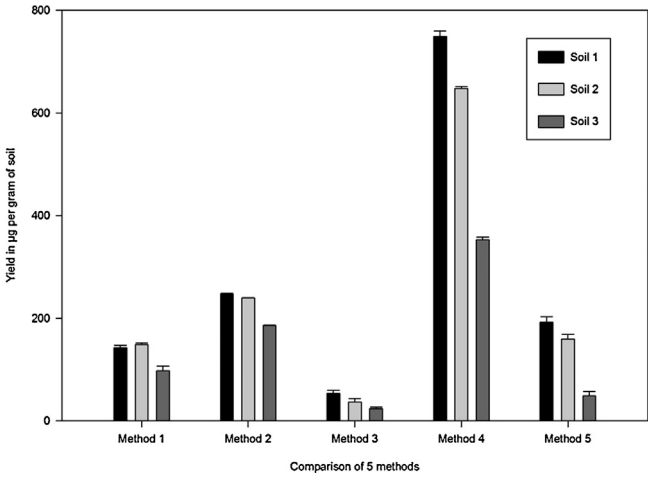

Fig. 1. DNA yield from different Mangrove soils by five methods.

3. Results

3.1. Metagenomic DNA extraction

Five different methods of metagenomic DNA isolation using three different soil samples from mangroves were compared with respect to DNA yield, purity, humic acid content, and suitability for PCR. Highest yield was obtained by method 4, giving 748.6, 647.3 and 353 µg DNA/gram of soil with soil 1, 2 and 3 respectively; while the lowest yield was obtained by method 3 which is calculated as follows: Yield in µg per gram of soil = concentration of DNA (µg/µL)/weight of soil (g) × volume in which DNA suspended (µL). The results are indicated in Fig. 1.

3.2. Spectrophotometric analysis for yield and purity of isolated DNA

The isolated DNA was assessed for yield and purity by obtaining OD ratios at 260 nm/280 nm (DNA/Protein) and 260 nm/230 nm (DNA/humic acid). Comparative analysis revealed the considerable variations in yield and purity of DNA obtained by the different methods. As depicted in Figs. 2 and 3 , method 1 gave DNA with A_{260}/A_{280} ratios close to optimum, while A_{260}/A_{230} ratios indicating comparatively reduced humic content was obtained by method 2.

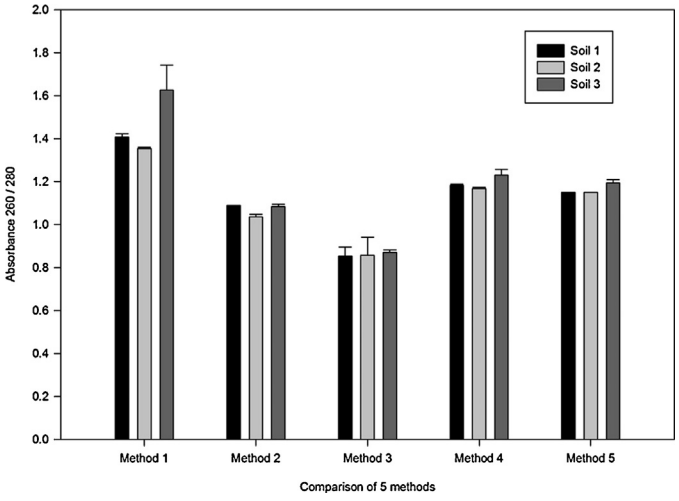

Fig. 2. Purity of DNA (A_{260}/A_{280}) from different Mangrove soils by five methods.

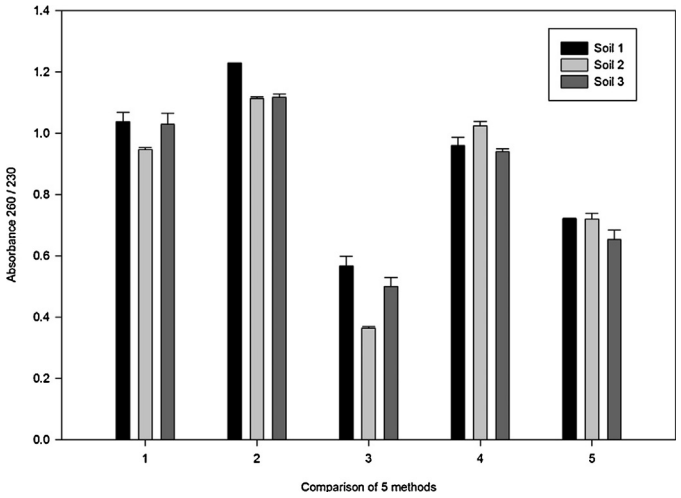

Fig. 3. Purity of DNA (A_{260}/A_{230}) from different Mangrove soils by five methods.

3.3. Visualization of community DNA on agarose gel

Although the quantity of total DNA isolated by the different methods varied considerably, the extracted DNA were of high molecular weight, which was also a DNA quality indicator. The spectophotometric data were supported by the agarose gel analysis. (Fig. 4). Lower DNA concentration obtained by method 2 was clearly visible in the gel picture.

3.4. 16S rRNA gene amplification

PCR amplification of 16S rRNA gene was successful only with DNA obtained by method 2 (Fig. 5), which had comparatively reduced humic acid contaminants.

4. Discussion

To isolate high molecular weight, contaminant free and PCR amplifiable DNA, five different methods of total DNA isolation were utilised. Various environmental DNA isolation protocols have been previously studied [10,11]. Extracting pure DNA from environmental samples is practically as important as yield, however it is also one of the most complex problems associated with the application of molecular techniques on environmental samples. Heterogeneous nature of the environmental samples requires each extraction procedure to be precise and optimised for every soil sample. Most DNA extraction procedures co-extract humic acids, pigments, heavy metals, and other contaminants. Humic

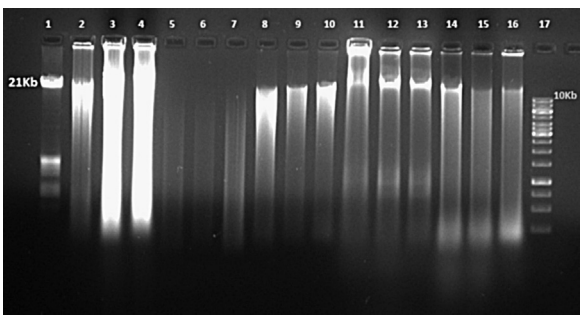

Fig. 4. Agarose gel electrophoresis of metagenomic DNA from three Mangrove soils by five methods. Lane 1–21 Kb ladder (Thermo Scientific, MA, USA) lane 17–1 Kb ladder, lane 2–4: DNA isolated by method 1, lane 5–7: DNA isolated by method 2, lane 8–10: DNA isolated by method 3, lane 11–13: DNA isolated by method 4, lane 14–16: DNA isolated by method 5.

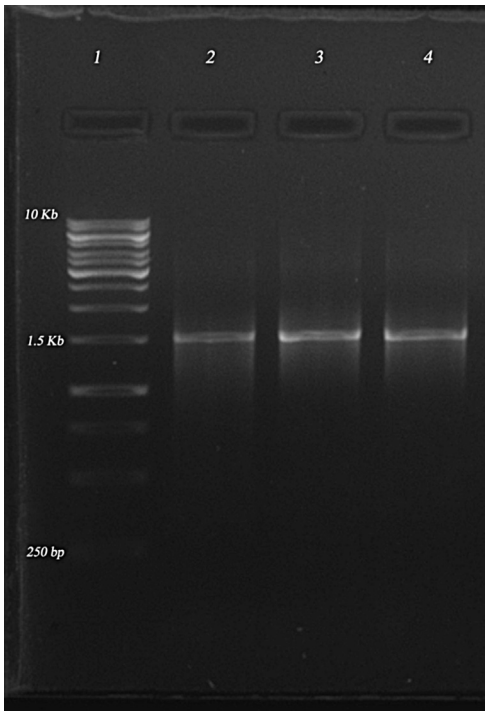

Fig. 5. 16S rDNA amplification profile of DNA by method 2. Lane 1 Kb ladder (Thermo Scientific, MA, USA), lane 2–4 – 16S rDNA amplicon.

contaminants due to their three dimensional structure and functional reactive groups bind with organic compounds [12] and are therefore one of the major problems associated with any soil community DNA isolation. Depending on soil types, crude DNA extracts can be contaminated by approximately 0.7–3.3 μg/μL of humic acid [13]. In addition, due to similar physicochemical properties with nucleic acid they easily co-precipitate with nucleic acid. These contaminants may not only hinder PCR reactions acting as inhibitor, but also can degrade the DNA during storage. Humic acid may through specific binding to DNA inhibit amplification in PCR reactions by limiting the amount of available template [14]. Purification of DNA employing polyvinylpolypyrrolidone, embedding DNA in agarose blocks followed by successive washing steps or by using sephadex columns can help improve quality of soil DNA and subsequent PCR amplification [15–17]. The aim of any extraction protocol is to succeed in obtaining genomic DNA which is a representative of the microbial diversity present within a soil. However different extraction procedures targets only specified group of microbiota present within a soil which results in biased estimates of DNA quantity, evidently due to differences in individual component steps, sorption of DNA to soil particles, DNA degradation or co-extraction of inhibitors [18–20] suggesting that additional measures should be considered when divergent soil types are compared or when comprehensive community analysis is required. SDS based cell lysis is the most widely used DNA extraction method, whereby DNA yield is more compared to freeze thawing and use of other detergents [21]. Physical treatments such as grinding, sonication and bead beating homogenises soil particles and can access individual microbial cells within a sample but with greater possibility of DNA shearing. Previous studies revealed that a combination of chemical and mechanical lysis can yield twice the amount of DNA than by any single method alone [20]. In the present study mechanical disruption of cell wall by grinding with liquid nitrogen and bead beating (method 2 and 3) resulted in increased DNA shearing, when compared to the gentle freeze-thawing method 4. Although the liquid nitrogen method yielded the shortest DNA fragments, it also has reduced amounts of contaminants. Consequently a combination of chemical lysis along with mild physical methods can greatly influence the total DNA content in terms of quantity and quality. Despite the shearing of DNA in all 3 soil samples employing liquid nitrogen extraction technique, they yielded 16S rRNA gene amplification using a single set of primer without the addition of any PCR enhancers or additives, thereby suggesting the suitability of the method in diverse soils and also in diversity studies. Commercial DNA extraction kits are now commonly used for extraction of high molecular weight DNA from complex habitats. Studies evaluating various commercial kits to other methods have shown that DNA yield and purity vary based on methodology and soil type. The mechanism of purification of these kits is based on the adsorption and desorption of the nucleic acids in presence of chaotropic salts [22] which results in contaminants free DNA but the quantity of DNA obtained will be less compared to classical method of DNA extraction. Previous studies recommended that slight modification of protocols employing commercial kits or a combination of classical isolation methods followed by purification of DNA using commercial kits can greatly affect the quantity and quality of the isolated DNA [23,24,25]. In the present study maximum DNA yield was obtained in lysozyme-freeze-thawing protocol (method 4), although the presence of residual amounts of humic and protein contaminants hindered PCR reaction.

In conclusion all methods yielded an acceptable amount of DNA, but were not suitable for further downstream processing, except that obtained by method 2. Previous studies suggested that addition of carriers and polyvalent polymers helps to reduce DNA loss due to adsorption and degradation [26]. Similarly in method 2, addition of skim milk prior to addition of extraction buffer may have helped to retain high quality DNA. Our results suggested that addition of skim milk helped to extract DNA amenable to PCR with the three soil samples tested which is in agreement with previous reports [5,27–29] as skim milk by acting as a carrier can reduce the adsorption and degradation of nucleic acids. On the other hand precipitating DNA with isopropanol improved DNA yield compared to the original study which used absolute alcohol instead [5]. Observations from the present study suggest that starting with a low gram weight of soil for DNA isolation as seen in method 2 and addition of skim milk during extraction can possibly help to reduce the humic contaminants, which would otherwise interfere with all other downstream processing of DNA, like amplification and cloning to name a few.

Acknowledgment

This work is supported by grants from University Grants Commission (major project) vide F.No. 41/527/2012 (SR). A portion of the research was also supported by Cochin University of Science and Technology.

Reference

[1] J. Handelsman, Metagenomics: application of genomics to uncultured microorganisms, Microbiol. Mol. Biol. Rev. 68 (2004) 669–685.
[2] A. Ogram, G.S. Sayler, T. Barkay, The extraction and purification of microbial DNA from sediments, J. Microbiol. Methods 7 (1987) 57–66.
[3] W.E. Holben, J.K. Jansson, B.K. Chelm, J.M. Tiedje, DNA probe method for the detection of specific microorganisms in the soil bacterial community, Appl. Environ. Microbiol. 54 (1988) 703–711.
[4] J. Zhou, M.A. Bruns, J.M. Tiedje, DNA recovery from soils of diverse composition, Appl. Environ. Microbiol. 62 (1996) 316–322.
[5] T. Volossiouk, E.J. Robb, R.N. Nazar, Direct DNA extraction for PCR-mediated assays of soil organisms, Appl. Environ. Microbiol. 61 (1995) 3972–3976.
[6] D. Dong, A. Yan, H. Liu, X. Zhang, Y. Xu, Removal of humic substances from soil DNA using aluminium sulfate, J. Microbiol. Methods 66 (2006) 217–222.
[7] Y.L. Tsai, B.H. Olson, Rapid method for direct extraction of DNA from soil and sediments, Appl. Environ. Microbiol. 57 (1991) 1070–1074.

[8] P.K. Siddhapura, S. Vanparia, M.K. Purohit, S.P. Singh, Comparative studies on the extraction of metagenomic DNA from the saline habitats of coastal Gujarat and Sambhar Lake, Rajasthan (India) in prospect of molecular diversity and search for novel biocatalysts, Int. J. Biol. Macromol. 47 (2010) 375–379.
[9] S. Shivaji, N.V. Bhanu, R.K. Aggarwal, Identification of Yersinia pestis as the causative organism of plague in India as determined by 16S rDNA sequencing and RAPD-based genomic fingerprinting, FEMS Microbiol. Lett. 189 (2000) 247–252.
[10] T.O. Delmont, P. Robe, I. Clark, P. Simonet, T.M. Vogel, Metagenomic comparison of direct and indirect soil DNA extraction approaches, J. Microbiol. Methods 86 (2011) 397–400.
[11] M.K. Purohit, S.P. Singh, Assessment of various methods for extraction of metagenomic DNA from saline habitats of coastal Gujarat (India) to explore molecular diversity, Lett. Appl. Microbiol. 49 (2009) 338–344.
[12] F.J. Stevenson, Stability constants of Cu^{2+}, Pb_2^{+}, and Cd_2^{+} complexes with humic Acids, Soil Sci. Soc. Am. J. 40 (1976) 665–672.
[13] C.C. Tebbe, W. Vahjen, Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast, Appl. Environ. Microbiol. 59 (1993) 2657–2665.
[14] K.L. Opel, D. Chung, B.R. McCord, A study of PCR inhibition mechanisms using real time PCR, J. Forensic Sci. (2009) .
[15] A. Frostegard, S. Courtois, V. Ramisse, S. Clerc, D. Bernillon, F. LeGall, P. Jeanin, X. Nesme, P. Simonet, Quantification of bias related to the extraction of DNA directly from soils, Appl. Environ. Microbiol. 65 (1999) 5409–5420.
[16] D. Moreira, Efficient removal of PCR inhibitors using agarose-embedded DNA preparations, Nucleic Acids Res. 26 (1998) 3309–3310.
[17] D.N. Miller, J.E. Bryant, E.L. Madsen, W.C. Ghiorse, Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples, Appl. Environ. Microbiol. 65 (1999) 4715–4724.
[18] M.L. Feinstein, W.J. Sul, C.B. Blackwood, Assessment of bias associated with incomplete extraction of microbial DNA from soil, Appl. Environ. Microbiol. 75 (2009) 5428–5433.
[19] E.L. Madsen, A critical analysis of methods for determining the composition and biogeochemical activities of soil microbial communities *in situ*, Soil Biochem. 9 (1996) 287–370.
[20] M.I. More, J.B. Herrick, M.C. Silva, W.C. Ghiorse, E.L. Madsen, Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment, Appl. Environ. Microbiol. 60 (1994) 1572–1580.
[21] J.T. Trevors, H. Lee, S. Cook, Direct extraction of DNA from soil, Microb. Releases 1 (1992) 111–115.
[22] J.P. Gray, R.P. Herwig, Phylogenetic analysis of the bacterial communities in marine sediments, Appl. Environ. Microbiol. 62 (1996) 4049–4059.
[23] L.R. Gutiérrez-Lucas, J.J. Montor-Antonio, N.G. Cortés-López, S. del Moral, Strategies for the extraction: purification and amplification of metagenomic DNA from soil growing sugarcane, Adv. Biol. Chem. 4 (2014) 281–289.
[24] F.M. Lakay, A. Botha, B.A. Prior, Comparative analysis of environmental DNA extraction and purification methods from different humic acid-rich soils, J. Appl. Microbiol. 100 (2006) 1365–1373.
[25] F. Martin-Laurent, L. Philippot, S. Hallet, R. Chaussod, J.C. Germon, G. Soulas, G. Catroux, DNA extraction from soils: old bias for new microbial diversity analysis methods, Appl. Environ. Microbiol. 67 (2001) 2354–2359.
[26] D.T. Denhardt, A membrane-filter technique for the detection of complementary DNA, Biochem. Biophys. Res. Commun. 23 (1966) 641–646.
[27] Y.T. Hoshino, N. Matsumoto, An improved DNA extraction method using skim milk from soils that strongly adsorb DNA, Microbes Environ. 19 (2004) 13–19.
[28] M.D. Garcia-Pedrajas, B.W. Bainbridge, J.B. Heale, E. Perez-Artes, R.M. Jimenez-Diaz, A simple PCR-based method for the detection of the chickpea-wilt pathogen *fusarium oxysporum* f.sp. ciceris in artificial and natural soils, Eur. J. Plant Pathol. 105 (1999) 251–259.
[29] S. Ikeda, H. Tsurumaru, S. Wakai, C. Noritake, K. Fujishiro, M. Akasaka, K. Ando, Evaluation of the effects of different additives in improving the DNA extraction yield and quality from andosol, Microbes Environ. 23 (2008) 159–166.