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Research Paper

MODIFIED ALKALINE LYSIS METHOD FOR TOTAL DNA EXTRATION FROM MARSLEY SOIL OF BARACK VALLEY: IN SEARCH OF METHANOGENIC GENOME

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Abstract

More than 99% of the microorganisms are unculturable with the standard techniques available now-a-days which limits ours understanding of genetic diversity, microbial physiology and ecological roles of the most important microbial species. Metagenomic study has overcome the problem of analyzing the unculturable bacterial groups. Methanogen are the most diverse group of microorganism living in anoxic environment with the ability to produce methane as a byproduct of their metabolism which contribute half of the greenhouse gases. Keeping this in mind, this work was intended to isolate methanogen bacteria from soil sample, particularly in the marshy land of Barak Valley.As, most of them are unculturable and extraction of genomic DNA is difficult due the presence complex cell wall structure. Furthermore the direct extraction of total DNA from soil results in co-extraction of humic acid substances which negatively interferes the effect of taq-polymerase during polymerase chain reaction. Various extraction methodologies has been used but the quality of yield was tram in every single method including a manufacturer kit so we have further modified the standard alkaline lysis method to get the better yield and was proved much efficient in comparison to the standard ones. The extracted DNA was amplified using methanogen specific primer, archeal 16S rRNA gene and universal 16S rRNA for validation.

Key words: Methanogen, Barack Valley, Metagenomic, Total DNA, Alkaline lysis method.

INTRODUCTION

Climate change is considered to be the vilest environmental problem of recent century with gradual increase in earth temperature as an accumulation of green-house gases causing catastrophic inhabitable situations. Along with the concentration of Carbon Dioxide (CO_2) the concentration of Methane gas which is mostly overlooked as a greenhouse gas is increasing at a rate of 3% per year [1]. It has been predicted that the concentration will rise upto 3.0 to 4.0 ppm within the next hundred years from current 1.783 ppm [2]. Major contributors towards the increase of environmental methane are the archeal methanogens which releases on an average 600 million tons of methane gas per year [3]. They use carbon dioxide and acetic acid as a

terminal electron acceptor to produce methane gas or the marshy gas in process of methanogenesis [4]. Methanogens are mainly found in anoxic environment of soils, wetlands, bogs, swamps and especially in the rumen of cows and human. The distribution of methanogens in natural environments is variable with various temperature, pH and salinity [5].Most of these species are unculturable for their strict anaerobic nature and additionally complexity in cellular envelope often restricts the isolation of good quality DNA for molecular analysis and so cause their study is still exudious or in preliminary level. The cell wall lacks the peptidoglycan layer and instead has a pseudomurinlayer with an array of paracrystalline protein and glycerol ether lipids that fit together like a jigsaw puzzle [6]. Keeping the facts in view, in this paper we will describe the methodology for extraction of good quality total DNA from swampy soil samples with the modification to remove the excess humic acid like substances which often denatures the biological molecules viz. restriction enzyme and taq-polymerase by binding to amides or oxidatively forms quinone which covalently binds to DNA which repress the downstream PCR reaction [7].

METHODS AND MATERIALS

Sample collection

The collection of appropriate soil samples for probable occurrence of methanogen is very important because methanogens are the residence of anoxic environment in marshy soils. For this reason, we have selected some of the swamp soils around Barak Valley region and collected samples at varying depth ranging from 10 cm to 100 cm of soils (**Table. 1**)

Total DNA extraction from soil

Total soil DNA was extracted by alkaline lysis method as described by Zaporozhenko *et. al.* [8] with few modifications and compared with native alkaline method and with a manufacturer kit (MoBio, USA) method for better results. The collected soil samples were properly homogenized with glass beads and centrifuged at 5000 rpm for 5 minutes at room temperature. The top interface layer was taken and again centrifuged at 12000 rpm for 10 minutes at 4^0C and supernatant was decanted with addition of 100 µlTris-EDTA (TE) buffers to the soil pallets. Then 300 µl of cell lysis buffer (10%SDS + 2mM Tris-Acetate + 1mM polyvinylpolypyrrolydone + 0.5mM lysozyme) was added and mixed well with vortexing. Separation of cell lysate was carried out with Phenol: Chloroform: Isoamyl alcohol and then precipitation was done with double volume of 2-Isopropanol. The precipitated DNA was first gradient centrifuged with cesium chloride (CsCl) to dilute the humic acid and then aggregated with Bovine Serum Albumin (BSA) at concentration of 0.1 – 0.5 µg/µl to minimize the effects of co-extracted humic acid. The amount of the DNA was quantified with nanodrop reading at an absorbance of 260/280nm.

Primer used for PCR amplification

16S rRNA sequence analysis has been used to clarify the taxonomic affinities of a wide range of taxa and as powerful tool for assessing the genetic diversity of environmental samples. We have observed that, for methanogenthere are a lot of 16S rRNA primers which are being used till date but none of them could able to amplify entire community of methanogen. For that reason we have analyzed almost all the methanogenic primer reported so far and selected three primers *viz.*methanogen 16S rRNA, universal 16S rRNA, archeal 16S rRNA gene (**Table. 2**) for proper validation of methanogenic genome with high fidelity.

PCR amplification

A volume of 20µL PCR reaction of isolated genomic DNA from the soil sample was carried out using a thermocycler system(Applied Biosystem, UK). Reaction mixture was prepared as follows: 20 pmol of each primer, 3 µL of template DNA, 1.25 µL of $MgCl_2$, 0.5 µL of tag polymerase, 2.0 µL of dNTP and sterile water for volume makeup.Thermal profile for this PCR reaction is carried out as, **i**nitial Denaturation- 95º C for 2min, denaturation- 94ºC for 30sec.Annealing temperature for each primer was kept different for different primer depending on the sequence variation and Tm profile –for universal 16S primer (55ºC for 1min); Archeal 16S primer(58ºC for 1min); Methanogen 16S primer (58ºC for 1min).Primary extension-72ºC for 1: 30 min and final Extension-72ºC for 7min.

RESULTS AND DISCUSSION

Total DNA from soil

The isolated gDNA from soil was subjectedto nanodrop reading for quantitative density (**Table. 3**) and 1% agarose gel electrophoresis for confirmation, distinct fluorescence band with different intensities was recorded in gel documentation system for each methods. The modified alkaline method was found comparatively better than the other two methods in having high quality non-biased gDNA from soil.

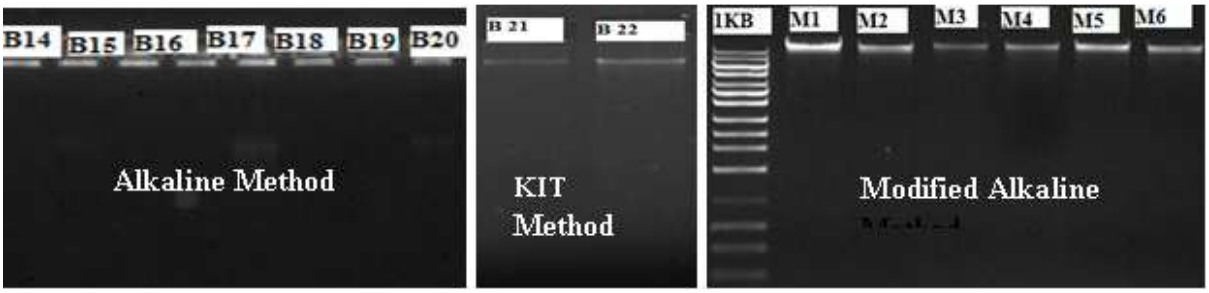

Fig. 1: Gel Electrophoresis of Total gDNA isolated from Soil for different Soil ID.

PCR Amplification

The presences of the respective methanogenic genome were confirmed with the successful amplification of the methanogenic primer and Archeal primer from the isolated DNA samples of the marshy soil. The amplification profile of Universal primer was used for further fidelity. The Archeal primer gives a product of size 900kb in length, Methanogenic Primer 1100kb and Universal Primer with a product size of 1500 kb in length respectively. Eight out of the fifteen samples were found to be total positive for the methanogenic genome as well as for the both archeal and universal 16S rRNA primer, which validate the presence of methanogenic genome to extracted bulk microbial DNA from soil (**Table. 4**)

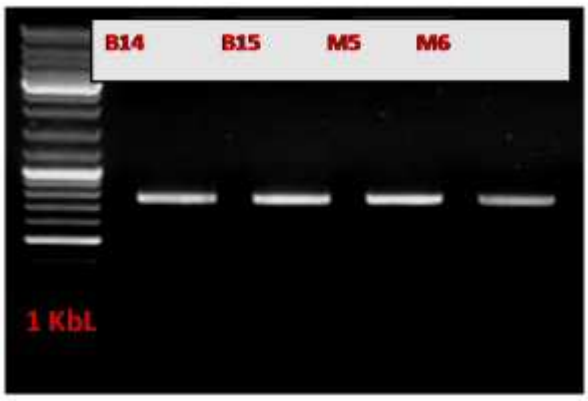

Fig. 2: Gel electrophoresis of PCR Product generated by methanogenic primer (MP).

CONCLUSION

Methanogenic bacteria are considered to be one of the few major factor responsible for continuous rising of methane gas in the environment but they are hardly explored at the molecular level due to lack in proper *in-vitro* cultural method and as well as due to hindrance in isolation the DNA from the natural samples. So, in the contrary we have developed the standard alkaline lysis method with few steps to get the quality DNA with less PCR inhibiting factors like humic acid, which is mainly responsible for taq-polymerase inactivation and results in null response. In comparison with the native method and one kit method; we have found averagely our method is much more promising for taking out the further molecular studies.

Table 1. Sample profiles collected from different swamp dales of Barack valley.

Season (S)	Name of Place	Month	Types of soil	PH of soil	Water Depth (cm)
S1	Ramnagar	June-August	Paddy Field	5.7 ±2	40- 60
	Shadinbazar		Marshy	6.2 ±2	
	Sunabarighat		Marshy	6.1 ±2	
S2	Tarapur	October-November	Paddy Field	6.7 ±2	40 – 60
	Shilkudi		Paddy Field	5.9 ±2	
S3	Dakbanglow	December - January	Paddy Field	6.4 ±2	40 - 60
	Duarband		Paddy Field	5.8 ±2	
	Barjalenga		Marshy	6.1 ±2	
S4	Chutojalenga	January-March	marshy	6.3 ±2	40 - 60
S5	Assam University Campus	March -June	marshy	5.7 ±2	50 - 100
	Hailakandi		marshy	5.7 ±2	

Table 2. Primer Sequence used for the methanogen genomic amplification.

Strand	Archeal16S rRNA primer (AP)	Universal 16S rRNA primer (UP)	Methanogenic 16S rRNA primer (MP)
Forward	5'-CCTAGGGGRBGCAGCAGG-3'	5'-AGAGTTTGATCCTGGCTCAG-3'	5'-GCTCAGTAACACGTGG-3'
Reverse	5'-GCGGTGTGTGCAAGGAGC-3'	5'-GGTTACCTTGTTACGACTT-3'	5'GTGCTCCCCCGCCAATTCCT-3'

Table 3. Nanodrop reading for gDNA (µg) extracted for the respective soil sample.

Sample ID.	Kit Method	Alkaline Method	Modified Alkaline Method
B14	323	320	469
B15	343	355	380
B16	385	367	370
B17	372	363	389
B18	364	334	377
B19	335	339	320
B20	361	385	377
B21	396	400	493
B22	389	354	420
M1	335	337	397
M2	326	310	473
M3	339	342	398
M4	367	340	411
M5	329	334	387
M6	397	385	404

Table 4: PCR Results of the isolated DNA Samples.

Sl. No.	Sample ID.	Archeal Primer	Methanogenic Primer	Universal Primer
1.	B14	+	+	+
2.	B15	+	+	+
3.	B16	-	-	+
4.	B17	-	-	-
5.	B18	+	+	+
6.	B19	+	+	+
7.	B20	-	-	+
8.	B21	-	+	+
9.	B22	+	+	+
10.	M1	-	-	-
11.	M2	-	-	+
12.	M3	-	-	+
13.	M4	+	-	+
14.	M5	+	+	+
15.	M6	+	+	+

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