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Polymerase chain reaction and real-time PCR parameters for amplification of hydrolytic microorganisms and hydrogenotrophic methanogens in anaerobic bioreactor

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The objective of the present research is to envisage microbial assorted variety in the reactor through systems of general polymerase chain reaction (PCR) and real-time PCR, which can be further applied for production of methane using anaerobic reactor by employing vegetable waste as a feed. The outcomes proposed the names of species identified with the hydrolytic microorganisms, for example, *Bacillus subtilis* and hydrogenotrophic methanogenic archaea like i.e. *Methano culleus* and i.e. *Methano corpusculum* of the methanogenic microbes. Results demonstrated that, presence of methanogenic archaea in the reactor was confirmed using general PCR.

Keywords: Methanogenic archaea, hydrolytic microorganisms, anaerobic baffled reactor, PCR, RT-PCR

Introduction

Urbanization and the subsequent expansive scale relocation of individuals from provincial to urban territories in creating nations prompts age of immense heaps of metropolitan solid waste (MSW)[1-2]. The issue anticipated that would amplify in future because of absence of appropriate transport of the waste, reasonable treatment and transfer offices. This comprises of the biodegradable waste such as sustenance, kitchen waste, green waste and paper wastes. The biodegradable waste involves natural tissue that can be disintegrated in two or three weeks. The high substance of starch, cellulose, potassium in nourishment squander, particularly vegetable and organic product sustenance squander makes it perfect for change into biofuels, as biogas[3-6].

The change of natural tissue into biogas is completed by microorganisms utilizing the procedure of anaerobic digestion. Anaerobic digestion (AD) is a progression of organic procedures in which microorganisms separate biodegradable material without oxygen[7]. The digestion process comprises of four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis. Different microorganisms are involved in each step. Hydrolysis basically implies the cleavage of synthetic bonds by the expansion of water atoms. Cases of hydrolytic microorganisms are *Bacillus, Cellulomonas, Eubacteria* and so forth. In the second acidogenesis progression, the hydrolytically created monomers are changed over into unpredictable unsaturated fats, ketones, alcohols, hydrogen and carbon dioxide. Cases of acidogenic bacteria are *Clostridium, Pseudomonas, Bacillus, Micrococcus, Flavobacteria* and so on. Acitogenesis is trailed by oxidation of the aging items to acetic acid derivation, hydrogen and carbon dioxide. This includes two noteworthy gatherings of acetogens – syntrophic acetogens (or the commit hydrogen creating acetogens) and homoacetogens. The last advance, methanogenesis is in charge of development of methane. Here, the hydrogen and acetic acid are changed over to methane and carbon dioxide. It is the most significant advance in biogas generation, and it relies upon pH, temperature, stacking rate, natural and inorganic mixes. Methanogens are grouped into two sorts-acetotrophic, which use acidic corrosive and hydrogenotrophic, which utilize hydrogen as their substrate[8-11]. Biogas generation is a complex process and requires various organisms and each progression of anaerobic processing includes an alternate microbial group[12]. The life forms engaged with biogas creation by anaerobic assimilation can be entirely

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grouped into four classes to be specific the hydrolytic fermentative microorganisms, the proton-diminishing acetogenic microorganisms, hydrogenotrophic methanogens, and aceticlastic methanogens[13-16]. The methanogenic life forms included are different archaea that can change over the acetic acid derivation into methane and carbon dioxide, water and so forth[17-20]. Anaerobic digestion can be executed as a batch process or a continuous process.

Research has exhibited that the polymerase chain reaction (PCR) method is stable and suitable for the quantitative measurement of source-specific genetic markers of microorganisms. The PCR technique involves simultaneous amplification, detection and quantification of a specific nucleic acid target in a biological sample. Real-time PCR takes measurements at the exponential phase of the PCR and is a more sensitive and reproducible method[21-23]. Therefore, the amplification of the hydrolytic microorganisms and hydrogenotrophic methanogens used for biogas production were quantified by PCR techniques.

Four chambered semi continuous baffled anaerobic reactor was used for biogas production in the laboratory using vegetable waste. In this study we are reporting PCR and real-time PCR parameters for amplification of hydrolytic microorganisms and hydrogenotrophic methanogens in the reactor. The presented work in this paper is focused on microbial assorted variety in the reactor through systems of general PCR and real-time PCR.

Materials and Methods

Isolation of DNA from Soil Sample

Five gram of soil sample was mixed with 13.5 mL of DNA extraction buffer and 50 µL of proteinase K (20 µg/mL). The mixture was subjected to temperature controlled shaking at 120 rpm for 30 min at 30ºC. 1.5 mL of 20% sodium dodecyl sulphate (SDS) was added and the tubes were again incubated for 2 h at 65ºC. The supernatant was collected after the samples were transferred to centrifuge tubes and centrifuged at 6,000 rpm for 10 min. The soil pellet was again re-extracted by adding 4.5 mL of extraction buffer and 0.5 mL of 20% SDS. Tubes were incubated for 10 min at 65ºC and centrifuged as above. Equal volume of chloroform-isoamyl alcohol was added in the ratio of 24:1 in the centrifuge tubes and the upper aqueous layer was collected. This mixture was incubated at 30ºC for overnight and the pellet was recovered by centrifugation at 12,000 rpm for 30 min followed by washing with 70% ethanol. Finally, the obtained pellet was suspended in 500 µL of 10 mM Tris buffer (pH 8.0).

NaOH/Tris DNA Preparation

The extraction of DNA from bacterial cell was performed by using PCR analysis. The harvested cells were washed twice with double distilled water re-suspended in 25 µL of 0.5 N NaOH solutions. The suspension was incubated for 30 min, followed by addition of 25 µL of 1 M Tris pH 7.5 for neutralization and mixed in vortex mixer and the debris was discarded. The extracted DNA was stored at –20ºC.

Agarose Gel Electrophoresis

Electrophoresis Conditions and Accessories

The flat gel electrophoresis framework of standard measurements from Genei (Bangalore, India) was utilized as a part of the investigations. The power supply was given from the electrophoresis power pack (Biotech Analytical Model), and electrophoresis completed at 100 - 120 V for around 1 - 2 h as per the convergence of agarose utilized. An aliquot (5 µl) of each amplification sample was analyzed on 2% w/v agarose gel cast and run in TAE buffer (pH 8.3). Gels were stained with ethidium bromide and photographed. A 100 base pair marker (Pharmacia, LKB) was added on every gel.

Polymerase Chain Reaction

DNA amplification by PCR was carried out in Peltier thermal cycler and M J research thermal cycler, with slight deviations for optimal temperatures. The thermo cycling programmed with PCR conditions for 35 cycles consisting 94ºC for 1 min, 60 sec at the annealing temperature of 60ºC and 72ºC for 1 min. The steps were examined with various concentrations of $MgCl_2$, and various annealing temperatures and total reaction volume of 50 µL with additional 2 drops of mineral oil to avoid evaporation. The constituents (as per kits of Perkin & Invitrogen) for PCR and primers used were listed in Table 1 and 2. The PCR product was then eluted with

Table 1 — The constituents for PCR

S. No	Components*	Volume*
1	Autoclaved distilled water	29 µL
2	10 X PCR buffer	5 µL
3	$MgCl_2$ (25 mM)	6 µL
4	dNTPs (200 µM)	2.5 µL
5	Primer (25 pM)	2 µL
6	*Taq* polymerase (3 units/µL)	0.5 µL
7	Template DNA	5 µL

*as per kits of Perkin-Elmer and Invitrogen

Table 2 — Primers used for PCR

S. No.	Primer	Target group
1	1 AF/1100 AR	Archaea
2	MET	Methanogens
3	MCR	Methyl coenzyme reductase gene

the silica-based QIA quick gel extraction kit from Qiagen, according to the kit instruction.

Screening and Selection of Clones

Blue-white shading screening is entrenched as methods for distinguishing a ligation item or showing the nearness of a DNA insert. The technique depends on the capacity of ß-galactosidase to hydrolyze X-gal to form 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside, bringing about the trademark blue staining of a phage plaque. In this way, when the *Escherichia coli* host exchange the carboxy terminal that divides ß-galactosidase, a plasmid vector do not bear any change suggesting a phage without a DNA insert.

Restriction Enzyme Digestion

The colonies obtained on spreading the transformation resultant were inoculated in 1X Luria Bertani (LB) media with the antibiotic respective of the plasmid. After overnight development, restriction digestion of this detached plasmid was done to check the presence of insert. The reaction mixture was incubated at 37ºC inside a water shower for 2 hours. The resultant was then stacked on to 0.8% agarose gel to check for the nearness of embed by affirming a band at the position of the insert size.

Real Time PCR

Real time PCR was performed to consider the intensification design quantitatively and additionally subjectively. Beside the new preliminaries, an arrangement of responses were likewise included utilizing a pre-considered groundwork as a standard. A positive control and a negative control were likewise kept for examination. For the positive control, the reaction mix is set up except for the preliminary and including proportional volume of autoclaved distilled water to influence the response volume to up to 25 µL. For the negative control, the template was supplanted by water.

Results and Discussion

Metagenomic DNA Isolation

Metagenomic approaches have been connected to consider a scope of soil conditions, and examinations with development systems ought to incorporate inclinations in the strategies used to isolate DNA from soil. In the present research a quick modest technique has been used for efficient extraction of high quality metagenomic DNA from limited environmental sample with adequate microbial community information that were extensively reasonable for metagenomic applications. Since, cell lysis and purification are the key strides in metagenomic DNA extraction; this examination incorporates a specific focus on these two elements. Cell lysis is expert by homogenizing with glass powder that is acquired from research center waste dish sets. Silica, the real segment of ground glass powder, has been broadly utilized for DNA extraction from different sources including soils and residue[24-25]. The metagenomic DNA isolated from the reactor sludge samples is run against the 1 kb plus ladder. Figure 1(a) shows the gel image of DNA isolated from sludge.

Evaluation of Polymerase Chain Reaction Parameters

BSA as Enhancer

Our outcomes predict that among natural solvents, bovine serum albumin (BSA) act as an effective co-enhancer of PCR amplification of these DNA layouts. Addition of BSA to PCR responses in nearness of natural solvents likewise permits high PCR yields of GC-rich DNA of different sizes to be acquired. Figure 1(b) shows the gel image of PCR products to compare the effect of BSA as an enhancer. This explains the role of BSA as an enhancer to help the primer bind with the template.

Study of Annealing Temperature

Archaeal Primer

Metagenome sample was used to optimize annealing temperature for the universal archaeal primer 1AF/1100AR. Bands are obtained at the expected band size of approximately 1100 bp and show increase in intensity with temperature with maximum intensity at 60ºC. Figure 1(c) confirms that presence of archaeal community in the reactor and also shows the PCR products annealed at varying temperatures for the archaeal primer 1AF/1100AR.

MCR Primer

Metagenome sample was used to optimize annealing temperature for the functional marker or MCR (methyl coenzyme M reductase) primer. Bands were obtained at the expected band size of approximately 450 bp and show increase in band intensity with temperature and maximum band intensity was observed at 60ºC. The gene codes for

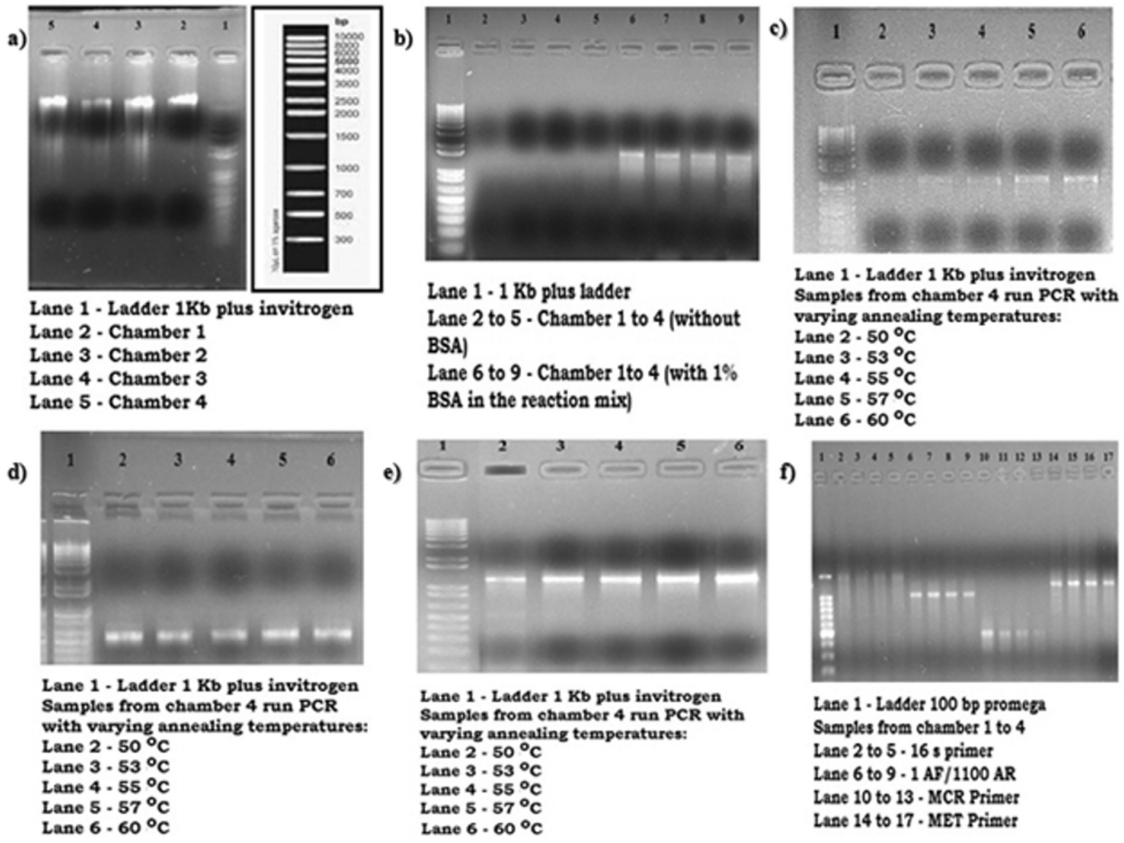


Fig. 1— (a) Gel image of DNA isolated from sludge, (b) Gel image of PCR products to compare the effect of BSA as an enhancer, (c) Gel image of PCR products annealed at varying temperatures for the archaeal primer 1AF/1100AR, (d) Gel image of PCR products annealed at varying temperatures for the MCR primer, (e) Gel image of PCR products annealed at varying temperatures for the MET primer, and (f) Gel image of PCR products of DNA samples from all chambers, with various primers.

the alpha subunit of the enzyme methyl coenzyme reductase which plays a key role in the final step of methanogenesis i.e. the cleavage of methyl coenzyme M to produce methane. Figure 1(d) shows the gel image of PCR products annealed at varying temperatures for the MCR primer.

MET Primer

Metagenome sample was used to optimize annealing temperature for the universal methanogenic primer MET. Bands are obtained at the expected band size of approximately 1200 bp and show increase in intensity with temperature with maximum intensity at 60ºC.This gene is known as a phylogenetic marker for methanogenic archaea and amplification of corresponding amplicon conveys presence of methanogenic community. Figure 1(e) gel image of PCR products annealed at varying temperatures for the MET primer.

Different Chambers Using Different Primers

The isolated DNA from different chambers was amplified using different primers at their appropriate band length. When PCR has been carried out using universal 16S primer, no amplification took place. This is because of presence of a large archaeal community which responds to amplification using the archaeal homologous primer 1AF/1100AR. Figure 1(f) shows that gel image of PCR products of DNA samples from all chamber, with various primers.

Cloning

Elution from Gel

The widely used method involves packing the gel piece into an open-finished tube that has a dialysis film sack connected to one end. This elution of sack is submerged in the anode cushion tank, and DNA is constrained out of the gel and against the dividers of the sack by electrophoresis. This strategy works sufficiently, however it retained as an opportunity to set up, and desert the dialysis layer or, on the other hand the connection of the film can bring about the misfortune of a valuable specimen. The opening is made quickly in front of the DNA band to be eluted. The space is loaded with gel cushion (90 mM Tris-

borate, 3 mM EDTA, pH 8.3), and the portability of the ethidium bromide-recolored band of DNA are taken after with a hand-held UV light. The DNA is expelled from the space before it re-enters the gel on the opposite side. The elution is for the most part performed at 3 V/cm, and the gel support in a 5 broad space is gathered and supplanted with new cushion at 5 to 10 min interims. The DNA can be cleaned from colored agarose, followed by ethidium bromide by butanol or isopropanol extraction by ethanol precipitation. Figure 2 (a) and (b) shows that gel image of the eluted PCR amplified product with 1AF/ 1100AR and gel image of the eluted PCR amplified product with MCR primer, respectively.

Transformation

Transformation of a vector with insert DNA brings about a white or clear state plaque. Color determination was acquainted with segregating a ligation insert, or to recognize vectors with or without cloned DNA fragment amid library development and screening. With the appearance of progressively effective cloning frameworks, this procedure has turned out to be less basic for routine cloning. Be that as it may, the development of the genomic sequence for different species and the broad utilization of libraries for various purposes, including drug development, and reference pathways related with improvement and illness, keep on depending widely on screening determination as a crucial segment of the high-throughput process. In addition, plaque investigations require a convenient insight of recombinants from non-recombinants, and recognizable positive flag against the foundation of the medium.

In spite of its utility, the utilization of X-gal has numerous downsides. Blue and white colonies are regularly hard to recognize, especially amid early settlement development. Moderate shading advancement and uncertain re-coloring regularly make it important to chill plates at 4ºC before states or plaques can be picked, including an extra holding up period before clones can be additionally investigated. X-gal is light delicate, requiring that stock arrangements and put away plates be shielded from light.

Real Time PCR

Figure 3 shows that melt curve of real time PCR products with various primers. The graph shows the amplification pattern of the samples in the RT-PCR. The pink color peaks represent the sample which has amplified the most. This is correlated with the following plot of the samples against their respective $1/C_t$ values. The values of $1/C_t$ are directly proportional to the extent to which the sample has

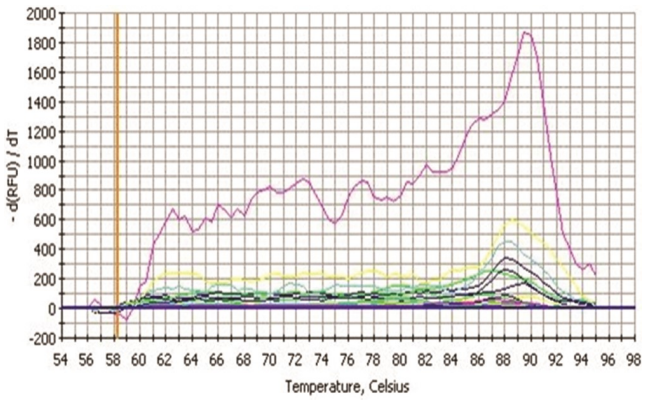
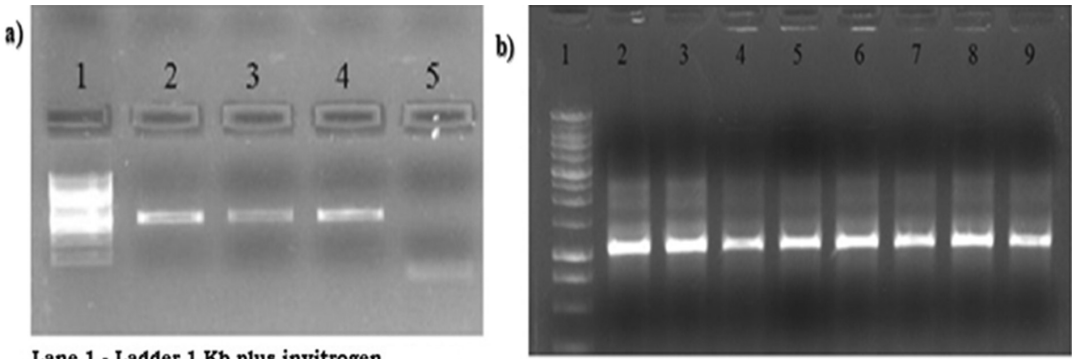


Fig. 3 — Melt curve of real time PCR products with various primers.



Lane 1 - Ladder 1 Kb plus invitrogen
Eluted samples
Lane 2 - Chamber 4
Lane 3 - Chamber 3
Lane 4 - Chamber 2
Lane 5 - Chamber 1

Lane 1 - Ladder 1 Kb plus invitrogen
Eluted samples from
Lane 2, 3 - Chamber 1
Lane 4, 5 - Chamber 2
Lane 6, 7 - Chamber 3
Lane 8, 9 - Chamber 4

Fig. 2 — (a) Gel image of the eluted PCR amplified product with 1AF/ 1100AR and (b) Gel image of the eluted PCR amplified product with MCR primer.

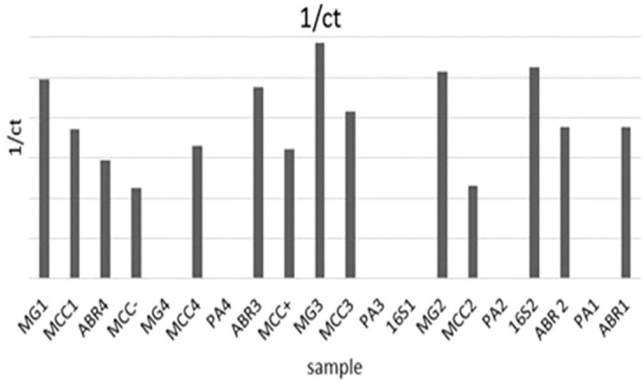


Fig. 4 — Sample vs. $1/c_t$ graph for various RT-PCR products.

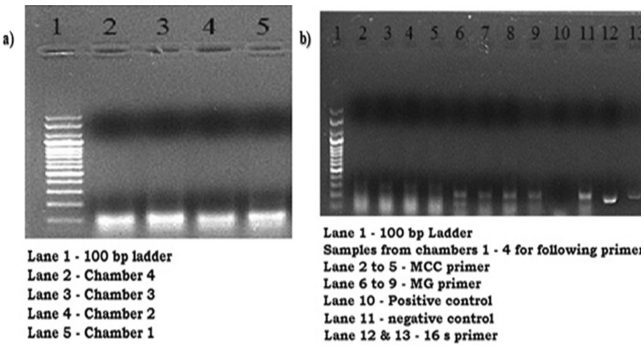


Fig. 5 — (a) Gel image of RT-PCR product amplified with ABRB07f/ABRB07r and (b) Gel image of RT-PCR product amplified with 934f/MG1200b and MCCf/MCCr.

been amplified (or C_t values are inverse of the copy number) and the results were shown in Figure 4. Figure 5 (a) shows that gel image of RT-PCR product amplified with ABRB07f/ABRB07r. Likewise, metagenome sample from all chambers are amplified using the primer ABRB07f/ABRB07r. The obtained bands are at the expected band size of approximately 110 bp and at 60ºC and primer target the ARBR07 strain of *Bacillus subtilis.* Figure 5(b) shows that gel image of RT-PCR product of the metagenome sample amplified with 934f/MG1200b and MCCf/ MCCr primers. The former targets the genus *Methano culleus* while the latter targets on the genus *Methano corpusculum*. Bands were obtained at the expected band size of approximately 265 bp and 300 bp respectively at 60ºC. Therefore, this amplification confirms presence of methanogenic archaea in the reactor.

Conclusion

Through the evaluation of PCR process conditions for all the primers used (by trial and error method) the optimum annealing temperature was observed to be 60ºC, at which all primers produced good results. Using the universal archaeal primer and methanogenic phylogenetic and functional markers, the presence of methanogenic archaea in the reactor was confirmed using general PCR. Further, using various pre-reported real-time primers, presence of hydrolytic bacteria like the ABRB07 strain of *B. subtilis* and hydrogenotrophic methanogens like species of *M. culleus* and *M. corpusculum* of the methano microbials family were also found. Biogas (methane) production using vegetable wastes in anaerobic reactor will be envisaged using the results obtained from this study.

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