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*Full Length Article*

# Phylogenetic Analysis of Methanogenic Archaea by *mcrA* Gene in Anaerobic Digester

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## Abstract

Methanogenesis is catalyzed by syntrophic cooperation between anaerobes, acetogenic bacteria and methanogenic archaea in any anaerobic biogas digester. The objective of the current study was to explore the archaea community, predominantly the methanogenic bacteria in the inoculum of a full-scale anaerobic digester. A sludge sample of the biogas reactor was tested for batch fermentation process. On the optimum methane production and fermentation time, the sludge sample was analysed by *mcrA* gene (a functional molecular marker of methanogenic archaea) to discover the active and functional methanogenic archaea community of the working digester. It was found that some *mcrA* genes phylotypes were found to be different from known methanogens and belonged to an unidentified group called "unculturable unclassified archaea", which are might be derived from a novel methanogen group in the mesophilic biogas digester. These unclassified archaeal group may represent a putative new taxon within the methanogenic archaeal group. It was also found that some species belonging to genera *Methanoculleus*, *Methanosarcina*, and *Methanobacterium* might contribute to methane production in anaerobic digesters. These studies provide insight towards prediction of the ecosystem present in mesophilic biogas digester through non-culture based approach. © 2019 Friends Science Publishers

**Keywords:** Methanogens; Archaea; Biogas digester; *mcrA* gene; Phylogeny

## Introduction

Methanogens are members of the domain Archaea and fall within the kingdom Euryarchaeota. These are obligate anaerobes and can be differentiated from other microorganisms based on the production of methane gas as a major catabolic end product (Garcia *et al*., 2000). Methanogenic archaea (methanogens) convert $H_2/CO_2$, formate, methanol, methylamines and/or acetate to methane (Zhu *et al*., 2011). Methanogens are a diverse group of organisms found in different environments such as blanket bog peat (Hales *et al*., 1996), rice paddies (Fey *et al*., 2001), marine environments (Hinrichs *et al*., 1999), the wet wood of trees and rumen. Approximately 50% of total worldwide methane emission by methanogenic archeal communities is found in freshwater sediments like wetlands, rice paddy fields and lakes (Rulik *et al*., 2013). Rumen sample contains only seven species of recognizable methanogens (Sirohi *et al*., 2010) and associated with genera *Methanosarcina*, *Methanobrevibacter*, *Methanomicrobium*, *Methanobacterium* and *Methanoculleus* (Sirohi *et al*., 2012). Methanogens are key players in biogas production. The anaerobic biogas reactor is a closed and relatively established environment. In a process of anaerobic fermentation, a better understanding of microbial community structure is mandatory for maximum methane production (Acharya and Mukundan, 2014) to further optimize technical solutions for anaerobic digestion (Krause *et al*., 2008).

During methane biosynthesis, methyl coenzyme M reductase (MCR) catalyzes reduction of a methyl group bound to coenzyme-M with simultaneous production of methane gas. This enzyme complex is an appropriate tool for the development of activity-based detection of methanogens (Thauer, 1998). MCR operon occurs in two forms, *MCRI* and *MCRII*. All methanogens have *MCRI* form, whereas all the members of order *Methanobacteriales* and *Methanococcales* have only *MCRII* form. Studies have highlighted that *mcrA* gene was used as a selectable marker in the PCR-based detection of methanogens because *mcrA* gene encoded one peptide of the MCR complex (Springer *et al*., 1995). Methanogens are characterized by a *mcrA* gene, which expresses the methyl coenzyme M reductase (MCR) and it plays a key role during anaerobic fermentation of biomass (Ferry, 1999). 16S rRNA gene is considered to be a gold standard for studying the microbial diversity in environmental sample (Nocker *et al*., 2007) However, *mcrA* gene can also be used for biodiversity studies of methanogens because of its exclusive occurrence and functional significance from different environments (Sirohi

*et al*., 2013). The samples of rumen fluid (RF), rectal dung (RD) and barn floor manure (BFM) of Korean Hanwoo cattle were analysed using *mcrA* gene sequence to understand diversity of methanogenic archaea in these samples. The major species found in the rumen fluid and bran floor manure was *Methanobrevibacter ruminantiumin* and *Methanocorpusculum labreanumin* in rectal dung (Daquiado *et al*., 2014). However, molecular diversity studies in freshwater sediments revealed the dominance of order *Methanomicrobiales* and *Methanosarcinales* of methanogens (Rulik *et al*., 2013). Culture dependent traditional techniques for identification of methanogens are very laborious, involve direct culturing, microscopy and require a high degree of accuracy for accurate identification of methanogens (Chaudhary *et al*., 2013). Huge differences were observed using culture-dependent and culture-independent (Laskar *et al*., 2018). Thus, in any natural environment, various factors including high sensitivity, competency and acquiescence make culture independent techniques as primary choice for microbial diversity study.

It was hypothesized that different communities of microbial consortia may be involved in the process of biogas production from biogas digester fed with agricultural waste by amplification of *mcrA* gene. From the sequence information, *mcrA* gene can provide us the methanogens flora inside reactor. The objective was to explore the archaea community, predominantly the methanogenic bacteria in the inoculum of a full-scale anaerobic digester.

## Materials and Methods

Anaerobic digestion samples in triplicates were collected from a pilot plant biogas reactor fed with agricultural waste. These samples were properly mixed in one liter of serum flask. The anaerobic sludge was filtered by stainless steel wire having 40 mesh size and stored at -20°C. An agriculture waste substrate of 20 gVS/L was added into 500 mL anaerobic reactor along with 300 mL sludge sample. The reaction mixture was added with 50 mL of macronutrients ($NaHCO_3$ 2 g/L, $KH_2PO_4$ 2.4 g/L, and NaCl 2.59 g/L), 10 mL of micronutrients ($MnCl_2$ $4H_2O$ 0.03 g/L, $CoCl_2$ $6H_2O$ 0.01 g/L, $CuCl_2$ $2H_2O$ 0.03 g/L and $NiCl_2$ 6 $H_2O$ 0.02 g/L), The total volume of the reactor was adjusted at 400 mL by adding distilled water. A second reactor fed with 300 mL sludge sample without substrate and reaction mixture of 50 mL of macronutrients, 10 mL of micronutrients having the same total volume of 400 mL was kept as control reactor. The initial pH of reactor bottles was adjusted 7.5 and bottles were closed tightly using rubber cork with aluminum crimp. The reactors were flushed with nitrogen for 10 min. Both the rectors were kept for 35 days of batch incubation on 37°C and the volume of biogas was measured regularly by the water displacement method. The biogas samples were analyzed using GFM series gas analyzer for $CH_4$ and $CO_2$ content gas composition.

The DNA from the sample reactor was extracted on the time point of maximum activity of the reactor inoculum when the composition of the biogas reached up to 60% $CH_4$ content in gas composition. Power Soil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) was used for metagenomic DNA extraction according to the protocol provided by the Kit. DNA quantity was assessed using a Nano Drop Spectrophotometer (Thermo Scientific, USA).

### Analysis of Sludge Composition

The standard laboratory analytical procedure (LAP) was used to measure the Total solid (TS), volatile solid (VS), ash contents of the sludge sample. The National Renewable Energy Laboratory (NREL)'s method was used for lignin content, cellulose and hemicellulose.

### Amplification of *mcrA* Gene Sequence and Phylogenetic Analysis

The *mcrA* gene was amplified using specific primers: mcrAF 5′GGTGGTGTMGGATTCACACARTAYGCWACAGC3′ and mcrAR 5′ TTCATTGCRTAGTTWGGRTAGTT3′ (Vaksmaa *et al*., 2017). AccuPrime™ SuperMix II (Invitrogen, USA) was used for Polymerase chain reaction (PCR). The PCR mixtures consisted of 25 $\mu$L of components:40 m*M* Tris-HCl (pH 8.4); 3 m*M* $MgCl_2$; 100 m*M* KCl; AccuPrime™ Taq DNA Polymerase; 400 $\mu$M each dTTP, dGTP, dCTP, dATP, stabilizers; thermostable AccuPrime™ protein; 10 $\mu$M of both primers and 50 ng of metagenomic DNA. The PCR profile was run on 95°C denaturation for 3 min, followed by 30 cycles of 95°C for 1 min, 60°C annealing for 45 sec and 72°C for 1 min and a final extension at 72°C for 10 min. Zyppy™ PCR purification kit (Zymo Research Corp, USA) was used for PCR products purification, purified PCR product was ligated into pTZ57R/T vector of TA cloning kit (Invitrogen, USA) and transformed into *Escherichia coli* TOP10 cells by heat shock method (Cohen *et al*., 1972). LB ampicillin X-gal-IPTG agar plates were used for the selection of transformants. The development of white colonies showed the transformed vectors. The plasmid DNA was extracted and purified using QIA prep spin mini prep kit (QIAGEN Inc CA, USA) and sequenced with M13 primer pair (forward primer 5′-CAGGAAACAGCTATGAC-3′ and reverse primer 5′-GTTTTCCCAGTCACGAC-3′. The sequence reads were trimmed, assembled and analyzed in Chromas pro 2.6.5 software. The *mcrA* gene sequences were blast in NCBI database to search out the most similar sequences using BLASTn algorithm (http://www.ncbi.nlm.nih.gov/BLAST) and retrieved. Sequence demarcation tool (SDT v 1.2) was used for pairwise sequence identity and multiple sequence alignment was carried out by Muscle algorithm in SDT following default setting. MEGA 7 software was used for phylogenetic study. The sequences of the isolates were

compared and analyzed using alignment tool Clustral W (1.6) by downloading the closely related sequences from NCBI data base (Tamura *et al*., 2013). The evolutionary history was inferred by using neighbor-joining algorithm with the bootstrap test based on 1000 replicates to represent well-supported nodes and evolutionary history of the taxa analyzed (Hillis and Bull, 1993).

### Nucleotide Sequence Accession Numbers

The nucleotide sequences obtained in this study were deposited in the GenBank database as sequence sets under accession numbers (MH004446 –MH004454).

## Results

### Composition of the Sludge

During the sampling, the pH of the sludge was 7.4. The inoculum sample was comprised of 19.6, 16.4 and 1.6% of total solid, volatile solid and ash content. As the sludge was fed with agriculture biomass, it was also composed of 24.2, 9.5 and 19.4% cellulose, lignin and hemicellulose respectively as showed in (Table 1).

### Anaerobic Digestion for Biogas Production

The sludge sample from the full-scale anaerobic digester was tested on the same substrate composition fed to the digester in lab scale one-liter serum bottle. The anaerobic digestion experiment was conducted to assess the biogas potential of the inoculum and to identify the active methanogenic culture community. During anaerobic digestion experiment, the sampling point 20$^{th}$ day was selected based on the high methane content production on the particular time period. Initially, a 7 days of lag phase was observed in both tested and control reactor, however a cumulative biogas of 920.3 mL/gVS was obtained from test reactor bottles. Whereas a lower cumulative biogas of 143.2 mL/gVS was obtained from the control sludge bottles. The biogas production continued for 30 days and stopped in the tested reactor, whereas, in case of control sludge sample the biogas stopped after 25 days of fermentation time (Fig. 1).

The $CH_4$ and $CO_2$ composition was detected on the 9$^{th}$ day of fermentation time. The $CH_4$ percentage gradually increased and reached to a maximum of 65% on the 20$^{th}$ day of fermentation time in batch anaerobic digestion experiment. The $CH_4$ percentage started to decreased after 24$^{th}$ day of the batch anaerobic digestion experiment.

### Morphological Study

The sample of the anaerobic digester inoculum on the 20$^{th}$ day of fermentation was the optimum time period for the methanogens producer of the respective sludge sample. The sample containing microbial consortia were picked from the reactor bottles using sterile needle and was spotted on slide to visualize the existing microbioata. Their morphologies were observed by light microscopy showed the diversity of coccai, rods and spiral shaped microflora from an anaerobic digester (Fig. 2). Previous studies also observed the different morphologies of Methanogens present in the anaerobic digesters (Scherer and Neumann, 2013). It was also reported that *Methanomicrobiales* occurred in coccoids forms in agricultural biogas plants (Wirth *et al*., 2012). Whereas *Methanoculleus* and *Methanomicrobiales* (MMB) were reported as a coccoid-type while *Methanobacteriales* (MBT) as rod-type methanogens from biogas reactor (Kim

**Table 1:** Composition of anaerobic digester sludge

| Parameters | Sludge |
|---|---|
| Total solid (%) | 19.6 |
| Volatile l solid (%) | 16.4 |
| Ash (%) | 1.6 |
| pH | 7.4 |
| cellulose | 24.2 |
| Lignin | 9.5 |
| Hemicellulose | 19.4 |

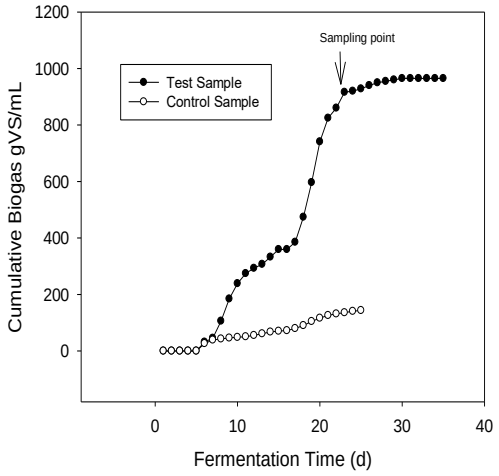


**Fig. 1:** Cumulative Biogas Production

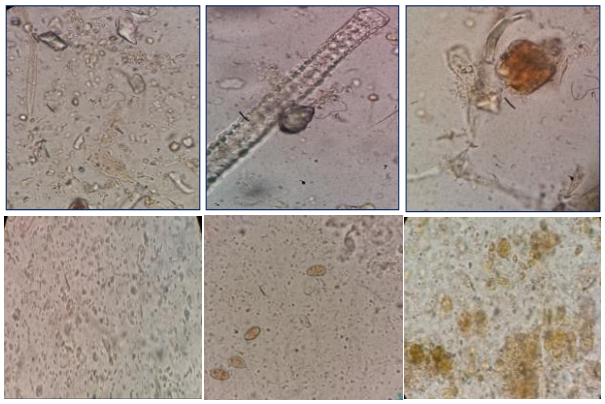

**Fig. 2:** Microscopic view of coccai, rods and spiral shaped microflora from an anaerobic mesophilic biogas digester

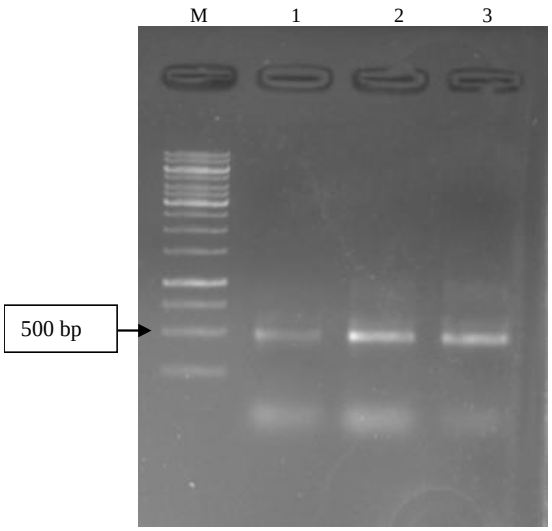


**Fig. 3:** Lane M 1kb DNA ladder (Fermentas SM#0313), Lane 1 represents the positive control from Kallar grass, Lane 2 and 3 represents PCR Amplification of *mcrA* gene (500 bp) from NIBGE biogas digester

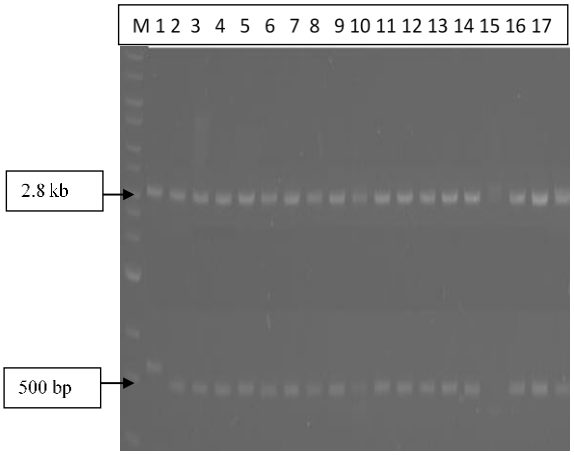


**Fig. 4:** Restriction digestion of pTZ57R/T-mcrA with *Eco*RI and *Hind III* to excise the cloned fragment. 1 kb Fermentas ladder; other Lanes, represents double restriction of *mcrA* gene 500 bp and pTZ57R/T vector 2.8 kb

*et al*., 2014).

### The *mcrA* Gene Sequence and Phylogenetic Analysis

Metagenomic DNA was successfully extracted from an agricultural waste biogas digester. A single PCR product of *mcrA* gene (expected size, 500 bp) was amplified using the template metagenomic DNA from all communities (Fig. 3). The purified plasmid was digested with *Eco*RI and *Hind*III restriction enzymes. The two fragments of 2886 bp and 500 bp appeared on the gel, showing that the insert was cloned in right orientation (Fig. 4). An analysis of *mcrA* clone library sequences was carried out and results revealed affiliation of clones with the genera *Methanoculleus, Methanosarcina, Methanobacterium* and other unclassified archaea (Fig. 5). Low affiliation was found with known species for most of the clones and possibly corresponded to genes of novel methanogenic archaeal genera and species. The global function of these species in the biogas production could be revealed by the *mcrA* clone's composition and phylogenetic analysis.

The phylogenetic analysis also showed that these clones were classified into 5 clades which are unknown *Archaea*, *Methanoculleus*, *Methanosarcina*, uncultured *Methanogenic Archaea* and *Methanobacterium* in mesophilic biogas digester (Fig. 5). Most of the clones named as *mcrA AR22* (MH004453), *mcrA AR3* (MH004447), *mcrA AR6* (MH004449) and *mcrA AR9* (MH004450) were affiliated with unclassified archaea and having an own branch in the tree representing novel archaeal species (Fig. 5). Therefore, clone *mcrA AR22* (MH004453) showed 99.5% sequence similarity with an uncultured archaeon clone *ATBEN* 5763M095 (FG226694) and *mcrA AR3* (MH004447) was 94% identical with uncultured archaeon clone *P3A 32* (KT314404). Similarly, *mcrA AR6* (MH004449) and *mcrA AR9* (MH004450) showed sequence similarity 97.7 and 99.1% with uncultured archaeon clone *P3A 32* (KT314404) (Table 2).

*Methanomicrobia* included three genera *Methanospirillium, Methanoculleus* and *Methanosarcina*. Clones *mcrA AR12* (MH004451) were clustered in genus *Methanoculleus* and *mcrA AR18* (MH004454) in genus *Methanosarcina* (Fig. 5). In addition, *mcrA AR12* (MH004451) had the greatest sequence identity (99.5%) to uncultured *Methanoculleus* sp. clone *HB93* (KF836871) and *mcrA AR18* (MH004454) showed 99.3% sequence similarity with *Methanosarcina* clone *CM2a* (KU555351) (Table 2). Clones *mcrA AR1* (MH004446) and *mcrA AR5* (MH004448) could not be clustered in a certain genus as the similarity of these clones were with the uncultured methanogenic archaeon. Therefore, clones were nominated as ''uncultured methanogenic archaeon'' (Fig. 5). These methanogenic archaeon *mcrA AR1* (MH004446) and *mcrA AR5* (MH004448) sequences were 99.5% similar to uncultured archeon clone-*AQmcrA3-26* (LC002158) (Table 2*). Methanobacterium* included *mcrA AR15* (MH004452) which showed 99.8% sequence similarity with uncultured archaeon clone *B8C20L621* (DQ260955) (Table 2).

## Discussion

Identification of non-culturable anaerobes by PCR based technology that helps to identify the novel sequences of new species, provide absolute description of methanogenic community. Gene cloning is the technology that has been improving understanding of genetic systems by analysing the structures and functional relationship. In this study, the *mcrA* gene from the mesophilic biogas digester was cloned and characterized. The TA cloning method is used, which is

**Table 2:** Relationship of archaeal mcrA nucleotide sequences from biogas reactor compared with GenBank database

| Clone Name | Accession Number | Most Closely Related Organism (accession number) | Sequence Similarity (%) | Functional Group |
|---|---|---|---|---|
| *mcrA AR1* | MH004446 | Uncultured archeaon clone-*AQmcrA3-26*(LC002158) | 99.5 | Unknown |
| *mcrA AR3* | MH004447 | Uncultured archeaon clone *P3A 32* (KT314404) | 94 | Unknown |
| *mcrA AR5* | MH004448 | Uncultured archeon clone-*AQmcrA3-26*(LC002158) | 99.5 | Unknown |
| *mcrA AR6* | MH004449 | Uncultured archeaon clone *P3A 32* (KT314404) | 97.7 | Unknown |
| *mcrA AR9* | MH004450 | Uncultured archeaon clone *P3A 32* (KT314404) | 99.1 | Unknown |
| *mcrA AR12* | MH004451 | Uncultured Methanoculleus sp. Clone *HB93* (KF836871) | 99.5 | Hydrogenotrophic |
| *mcrA AR15* | MH004452 | Uncultured archeaon clone *B8C20L621* (DQ260955) | 99.8 | Hydrogenotrophic |
| *mcrA AR22* | MH004453 | Uncultured archeaon clone *ATBEN 5763M095* (FG226694) | 99.5 | Unknown |
| *mcrA AR18* | MH004454 | Methanosarcina clone *CM2a* (KU555351) | 99.3 | Hydrogenotrophic/aceticlastic |

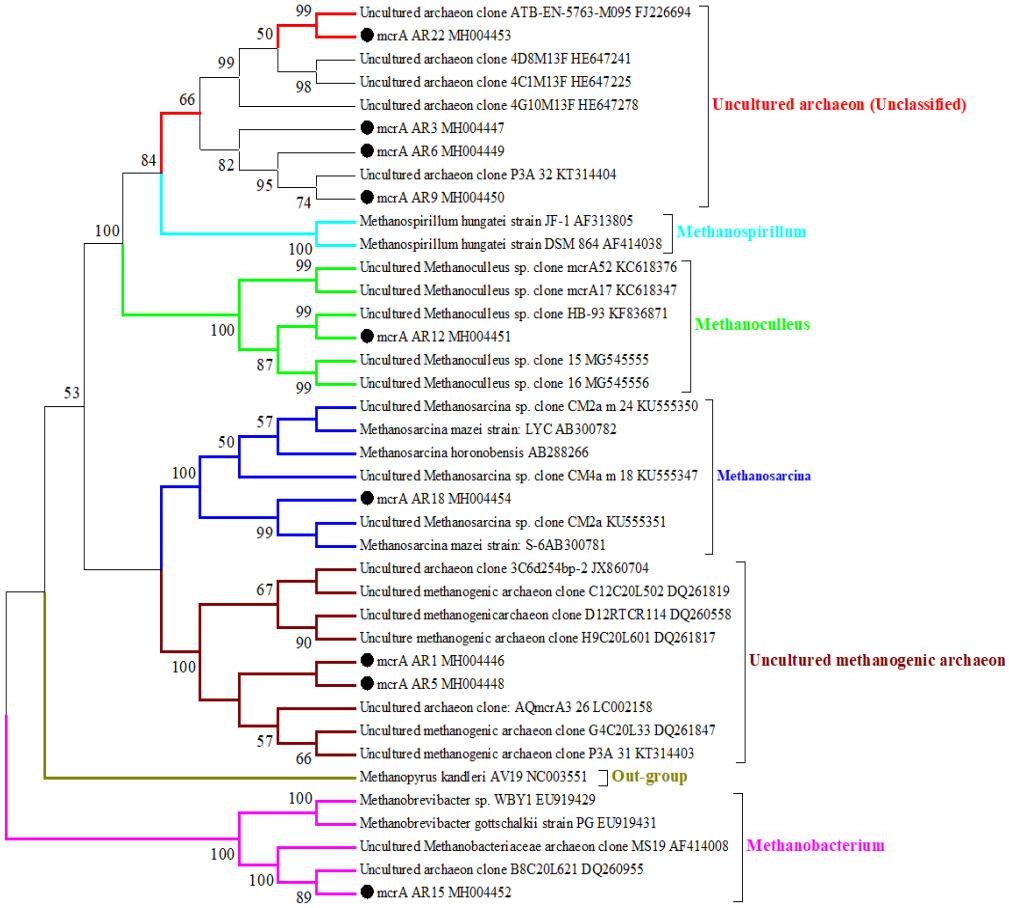


**Fig. 5:** Phylogenetic relationship among the *mcrA* clones with partial *mcrA* nucleotide sequences of known methanogenic archaea based on the neighbour-joining analysis. Reference sequences were retrieved from the GenBank database with their accession numbers. Representative clones name and their accession number are also shown after the clone name. Numbers at nodes represent bootstrap values. Only bootstrap values above 50% are displayed. *Methanopyrus kandleri* strain was used as the outgroup

particularly suitable for cloning of PCR fragments amplified with primers and the use of specifically designed cloning vector; pTZ57R/T ensures high efficiency of this method. The T-overhang prevents recirculization of the vector and the yield of the recombinants is increased as high as 90% (Roslan *et al.*, 2017).

The analysis of *mcrA* gene from an agricultural waste fed biogas reactor is the first of its kind to show the archaeal diversity in this work. Limited association was shown by most of clones in this study with identified species and possibly characterized genes of novel methanogenic archaeal genera and species. This study showed that novel uncultured unclassified archaea was most abundant in NIBGE biogas digester. All four *mcrA* clones: *mcrA AR22*, *mcrA AR3*, *mcrA AR6* and *mcrA AR9* were clustered in unclassified archaea. The unclassified archaeal clone *mcrA AR22* is comparable to uncultured archaeon clone *ATBEN 5763M095*, which is reported from two-phase biogas plant

of agricultural waste (Nettmann *et al*., 2010). Similarly, *mcrA AR3*, *mcrA AR6* and *mcrA AR9* had maximum resemblance with sequences of uncultured archaeon clone *P3A 32* (KT314404) (Liang *et al*., 2018). These observations revealed that uncultured unclassified archaea are predominant in anaerobic digester fed with agricultural waste.

This study also found *mcrA AR1* and *mcrA AR5* that had maximum similarity to uncultured archeon clone-*AQmcrA3-26*, a methanogen which was obtained from the granulated sludge of USAB digester treating seawater of a fish tank. The clone analysis targeting *mcrA* gene was conducted for anaerobic reactor treating seawater of a fish tank which unveiled taxonomically novel methanogenic archaeal genus/strains. Some *mcrA* gene phylotypes were dissimilar from known methanogens. These lead to unclassified methanogenic archaeal group which was similar to present study (Saito *et al*., 2015).

The kingdom eukaryote consists of two classes; *Methanomicrobia* and *Methanobacteria*. The class *Methanomicrobia* has three genera; *Methanoculleus, Methanosarcina* and *Methanospirillium.* Among *Methanomicrobiales*, *mcrA AR12* was predominant in the present study, which was identical to uncultured *Methanoculleus* sp. Similarly, *mcrA AR18* showed the close association with *Methanosarcinia* sp., which belong to order *Methanosarcinales*. These species can use $CO_2$, formate and hydrogen to yield methane gas (Ferry *et al*., 1974; Karakashev *et al*., 2005). Therefore, these (*mcrA AR12, mcrA AR18*) clones were grouped in hydrogenotrophic (Table 2). The hydrogenotrophic microbial group included *mcrA AR12*, which was closely related to uncultured *Methanoculleus* sp. clone *HB93*. These functional genes were isolated from water samples from oil reservoir (Liu *et al*., 2015). Another member of the hydrogenotrophic and aceticlastic group consisted of *mcrA AR18* which had similarity with *Methanosarcina* clone *CM2*a. These functional genes of *Methanosarcinia* were isolated from methanogenic consortia in suspended sludge and an up-flow anaerobic sludge blanket (UASB) reactors using multiple PCR-based molecular techniques (Chen *et al*., 2017).

The *mcrA AR15* clustered in *Methanobacterium* genus which exhibited the closest association with uncultured archaeon clone *B8C20L621* obtained from methanogens consortia in a biogas digester fed with cattle dung. Most of the *Methanobacterium* sp.were isolated from manure (Rastogi *et al*., 2008).

Based on *mcrA* clone analysis directing *mcrA* gene (a functional molecular marker of methanogenic archaea), which give an insight to discover community composition, taxonomically rare and functional methanogenic archaea were described. Our data suggest the culture independent approach to determine the community structure using *mcrA* gene-based cloning techniques. Most of the clones obtained in this study were originated from unidentified methanogens, showing that the ecosystem is still unexplored environment. Such information will encourage the isolation of best methane culture production from an anaerobic biogas reactor.

## Conclusion

The *mcrA* gene analysis of anaerobic digester fed with agricultural waste showed hydrogenotrophic group comprises of *Methanoculleus*, *Methanosarcinia* and *Methanobacterium* species whereas unclassified archaea were predominant as compared to functional genes. Six novel strains of unclassified archaea from biogas reactor were investigated *mcrA AR1, mcrA AR3, mcrA AR5, mcrA AR6, mcrA AR9 and mcrA AR22*. However, substrate characters and environmental conditions of the mesophilic biogas reactor can be possible reasons, which favours these novel strains to other methanogenic archaea. The study depicts that by understanding the community composition which can possibly enhance the biogas production in an anaerobic biogas reactor.

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