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16S rRNA BASED T-RFLP ANALYSIS OF METHANOGENS IN BIOGAS PLANT WITH *P. HYSTEROPHORUS* L. AS SUBSTRATE

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ABSTRACT

Background: Methanogenic microbiome plays an important role in contributing to global renewable energy resource. Globally, biogas production totally estimates to 1/4th of total consumption of fossil fuel. The bioconversion of organic material by anaerobic digestion process to a valuable energy source has a promising outlook. Though there is lot of research contribution to the biogas technology, the knowledge about the microbiome involved in the bioconversion of plant biomass is limited.

Objective: In this study, batch fermenter with *P. hysterophorus* as mono-substrate and along with co-additives of bovine animal faeces at different ratios is presented in the context of molecular data on the microbial composition.

Material and methods: A slightly modified protocol of the kit RKN15 to extract the metagenomic DNA from the microbial community participating in the bioconversion of the mono-substrate *P.hysterophorus*, *P. hysterophorus* with cow dung and *P. hysterophorus* with goat dropping was performed. Thus extracted and purified DNA from the microbial consortium was subjected for 16S rRNA analysis by terminal restriction fragment length polymorphism (T-RFLP).

Result: The minimum estimate number of bacterial community present among the samples with high methane yield revealed the presence of 18 and 14 different types of microbes correspondingly.

Key Words: Bioconversion, Co-additive, Methanogenic, Microbiome, *P. hysterophorus*

INTRODUCTION

The diversity and functional analysis of the microbial community involved in the biogas technology is the principle aspect of upgrading the production efficiency in terms of methane yield. Hence a better understanding regarding the composition and abundance of the microorganisms involved in these systems becomes the need of the hour. Though there are reports of cultivable methanogens from diverse habitats (Garcia, 1990), cultivation of indigenous methanogens were unsuccessful (Williams & Crawford 1985; Goodwin & Zeikus, 1987). For the past few decades, researchers depend on culture independent methods of microbial community analysis using molecular techniques to isolate, identify and compare gene sequences (Atlas et. al., 1992; Madson, 1998; Schneegurt & Kulpa, 1998). Anaerobic digestion, an attractive option for the weed management at ambient environmental conditions offers technical and economical benefits. The potential limitations previously associated with weed biomass batch reactor operation have been largely overcome by optimizing various pretreatments and co-additive options. However, much scope still exists via application of the ambient microbial consortium which will allow in improvising the efficiency in terms of methane yield. In the present study, batch reactor fermenter operations were investigated by monitoring the effect of pretreatments and co-additives on reactor performance over hydraulic retention time of 35 days. DNA extracted from the whole microbial consortium was subjected to PCR amplification and 16S rRNA analysis by T-RFLP was performed.

MATERIALS AND METHODS

Batch Reactor Set-up

Biochemical Methane Potential (BMP) vials of 100ml were used as reactor bottles with *P. Hysterophorus* as

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substrate, which was pretreated (Ramya R and Shree M. P., 2014a). Inoculum substrate (I/S) ratio of 0.5%, 1.0%, 2.0% were done in triplicates. The inoculum was collected from a plant-litter based biogas plant, which was filtered with muslin cloth and stored at refrigeration temperature until use. Substrate along with co-additives (C/S ratio), cow dung and goat dropping at ratio of 1:0.25, 1:0.5 and 1:1 were performed in triplicates for a hydraulic retention time of 35 days in batch fermentation process at ambient temperature.

Biogas analysis

The composition of the biogas collected in the head space of the digester was analyzed using a Nano HP-1 Model BG 1000 Gas Chromatograph equipped with a Flame Ionization Detector (FID) and Thermal Conductivity Detector (TCD). Argon (Ar) was used as carrier gas with flow rate of 8ml/min. Oven temperature was set up to 60°C. Methanator temperature was set at 360°C. All the digesters were operated in triplicate. Biogas was sampled by directly inserting the intra venous syringe into the Anaerobic Digestion (AD) vial and volumetric composition of biogas was analyzed by using gas chromatography. Gas chromatograph (Nano HP-1) equipped with FID was used to analyze gas composition (Ramya R and Shree M. P., 2014b).

Extraction of metagenomic DNA from digested slurry

In this study, extraction of DNA of two samples PT4cCDI and PT4cGDIII were performed using the bacterial genomic DNA isolation kit RKN15 with slight modification. 1.5 ml of bacterial culture was taken in 2 ml vial after spinning the BMP vial at 70rpm for 10 mins. The supernatant thus obtained was spun at 10,000 rpm for 2 min. Supernatant was completely discarded. To the pellet 750 μl of 1X suspension buffer was added and subjected for vortexing. 5 μl of RNase was then added and incubated at 65°C for 10 min with intermittent mixing. To this, 1 ml of lysis Buffer was added and mixed by inverting the tube 10-12 times. The tubes were incubated at 65°C for 15 min and spun at 10,000 rpm for 5min. The clear lysate was loaded (unlysed Cells will settle as pellet leaving clear supernatant) onto spin column. The mini-spin column was placed into 2ml collection tube and 600μl of the lysate was loaded on the spin column each time, spun at 10,000 rpm for 1min at room temperature. The content of the collection tube was discarded and the spin column was placed back in the collection tube. 500 μl of 1X wash buffer was added to the column, spun at 10,000 rpm for 1 min at room temperature. The contents were discarded of the collection tube. The spin column was placed back in the same collection tube and the procedure was repeated. The empty column was spun at 10,000 rpm for 3 min at room temperature. 50 μl of warm elution buffer was added to the spin column placed in a fresh 1.5ml vial, at 65°C for 1 min and spun at 10,000 rpm for 1 min at room temperature. The elution was collected in the same vial. Thus obtained DNA concentration was determined by quantitative analysis on 1% (w/v) agarose gel with 0.5xTBE as electrode buffer as described elsewhere (Sun, et al., 2009).

T-RFLP analysis

The ~1.5 Kb bacterial 16s rRNA fragment was amplified from the metagenomic DNA extracts by using high–fidelity Polymerase Chain Reaction (PCR) polymerase as described by Sun, et. al., 2009, with the labelled primers marked with 6-carboxyfluorescein (FAM). The PCR products were subjected to restriction digestion with a 4-base cutter (HpaII). The fluorescent labelled t-RFs (terminal restriction fragments) were size separated on an ABI 3130 automated sequencer (Applied Biosystems) using an internal size standard (LIZ-500). t-RFLP (terminal restriction fragment length polymorphism) electropherograms were analyzed with GeneScan 3.7 software (Applied Biosystems) at chromous biotech Pvt Ltd, Bangalore (Wayan Suardana, 2014). The numbers of peaks obtained in t-RFs represents the minimum number of bacteria present in the sample.

RESULT

The biogas compositions of the samples subjected for AD are presented in Table 1 & 2. It is very evident from the GC result that the PT4 at the I/S ratio of 2%, C/S ratio of 1:1 with CD and C/S ratio of 1:0.25 with GD showed the maximum methane yield of 149.24% and 120.81%. Thus these two samples where further subjected for extraction of metagenomic DNA (Fig 1 & 2) and analysis by T-RFLP (Fig 3 & 4).

DISCUSSION

In our study, the DNA extracted from the BMP vials using slightly modified protocol of RKN15 kit gave us a good quality DNA which was amplified by PCR. T-RFLP is a technique widely used for study of microbial diversity in environmental samples (Salvador Embarcadero-Jiménex, et al., 2014). With the metagenomic DNAs obtained in this study, 35-500 T-RFs were obtained from the 16S rRNA amplicons digested by HpaII restriction endonuclease. Fig 3 & 4 indicated that the bacterial communities in the anaerobically digested *P.hysterophorus* present in the 100ml BMP vials were very diverse and composed of many species.

CONCLUSION

The effect of pretreatment and co-additive on the biomethanation efficiency of *P.hysterophorus* was studied by performing batch anaerobic digestion process experiment using BMP vials. The experimental results in this study demonstrated that the suitable pretreatment for biomethanation of *P.hysterophorus* for a HRT time of 35 days is PT4 with the maximum methane yield of 149.24% with CD as co-additive and 120.81% with GD as co-additive. The most important finding of this research is the microbial consortium involved in the biomethanation process. Therefore, the evaluation process with respect to the microbial consortium should be considered carefully. In the present study, extraction of high quality metagenomic DNA from anaerobically digested BMP vials was performed. Thus obtained DNA samples were subjected for PCR amplification and T-RFLP analysis, which showed the presence of diverse bacterial communities. Studies related to the diversity are still under progress.

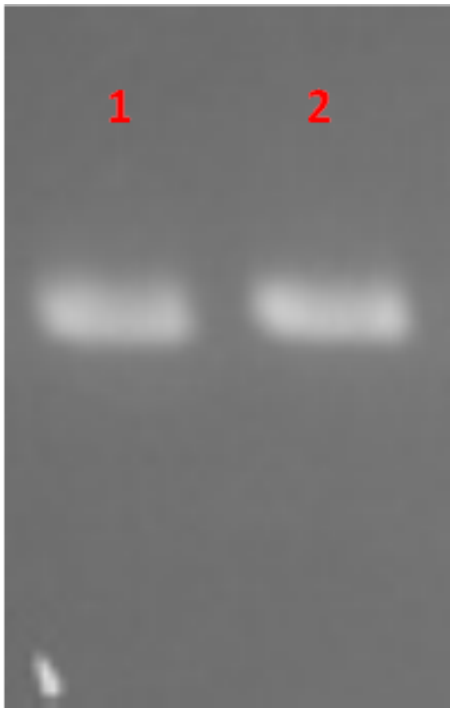

Figure 1: Extraction of Genomic DNA from Bacterial sample using the Bacterial Genomic DNA Isolation Kit (RKN15).

Lane description:

1. SAMPLE PT4cCDI
2. SAMPLE PT4cGDIII

PCR Cycle Condition:

94°C	**94°C**	**55°C**	**72°C**	**72°C**
5 min	30 sec	30 sec	1.30 min	7 min
	35 cycles			

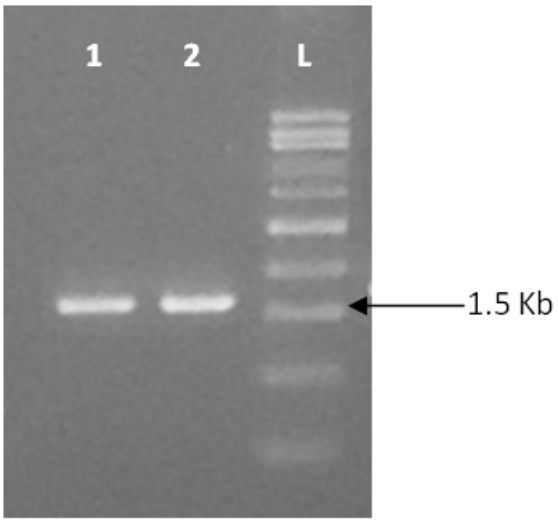

Figure 2: PCR Amplicon loaded on 1% Agarose Gel

Lane Description:

1 – PCR amplicon of PT4cCDI
2 - PCR amplicon of PT4cGDIII
L – 500bp DNA Ladder

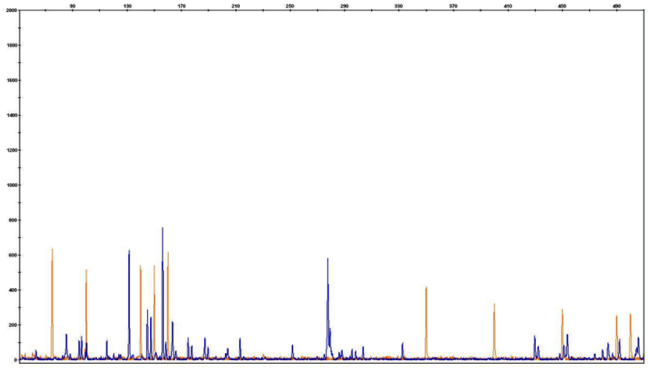

Figure 3: t-RFLP of PT4cCDI

Minimum numbers of Bacteria present in the given sample are 18, (Refer the t-RF's in Fig 5)

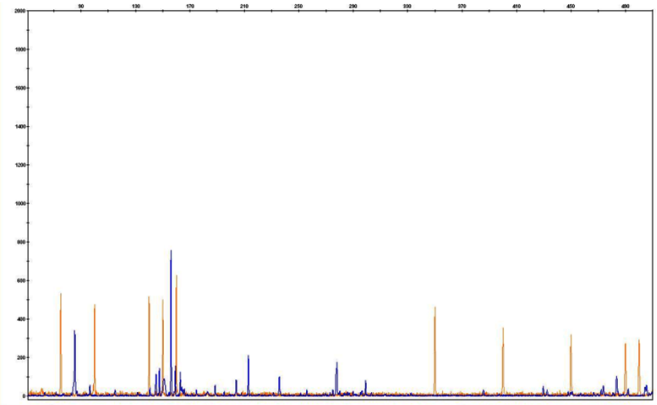

Figure 4: t-RFLP of PT4cGDIII

Minimum numbers of Bacteria present in the given sample are 14, (Refer the t-RF's Fig 5)

6-carboxyfluorescein (FAM)-labeled primers gives blue color in Genescan analysis. (Blue colored peaks which corresponds to tRF's are sized by internal size standard)

Orange peaks are internal size standard (LIZ 500, Chromous)

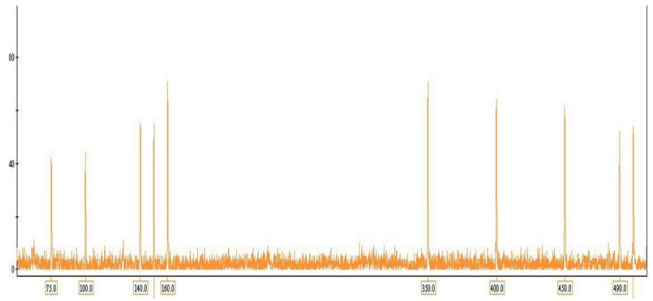

Figure 5: Standard t-RFs

The GeneScan™ 500 LIZ® Size Standard is a fifth dye-labelled size standard for the reproducible sizing of fragment analysis data. This size standard is used for fragments between 35 and 500 bp. The standard contains 16 LIZ® dye-labelled, single-stranded DNA fragments. Since the standard is labeled with the fifth dye, can genotype a greater number of markers in a given lane, compared to the four-dye system.

Size Fragments in the 35-500 Nucleotides Range:

GeneScan™ 500 LIZ® Size Standard is designed for sizing DNA fragments in the 35-500 nucleotides range and provides 16 single-stranded labeled fragment of: 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490 and 500 nucleotides. The sizing curve generated from these fragments make the GeneScan™ 500 LIZ® Size Standard ideal for a variety of fragment analysis applications such as Microsatellites, Fragment Length Polymorphisms and Relative Fluorescent Quantitation. Each of the DNA fragments is labelled with the LIZ® fluorophore which results in a single peak when run under denaturing conditions. With the 5th dye LIZ® your marker fragments can be labelled with the dyes FAM™, VIC™, NED™ or PET®.

Table 1: Methane yield of *P. hysterophorus* substrate at various I/S ratios, C/S ratios and pretreatments.

Sample	Methane yield in %*	Sample	Methane yield in %*	Sample	Methane yield in %*
UT a	11.59	PT1 a	42.42	PT2 a	65.02
UT b	11.00	PT1 b	86.87	PT2 b	47.99
UT c	9.04	PT1 c	71.87	PT2 c	89.95

Table 1: (Continued)

Sample	Methane yield in %*	Sample	Methane yield in %*	Sample	Methane yield in %*
UT aCDI	10.88	PT1 aCDI	72.63	PT2 aCDI	68.37
UT aCDII	11.61	PT1 aCDII	53.71	PT2 aCDII	83.35
UT aCDIII	4.88	PT1 aCDIII	48.15	PT2 aCDIII	71.80
UT bCDI	6.42	PT1 bCDI	45.84	PT2 bCDI	49.97
UT bCDII	14.91	PT1 bCDII	66.96	PT2 bCDII	74.19
UT bCDIII	11.42	PT1 bCDIII	44.24	PT2 bCDIII	86.90
UT cCDI	39.99	PT1 cCDI	35.78	PT2 cCDI	89.11
UT cCDII	21.74	PT1 cCDII	3.26	PT2 cCDII	20.20
UT cCDIII	6.58	PT1 cCDIII	45.25	PT2 cCDIII	69.46
UTaGDI	7.20	PT1 aGDI	40.75	PT2 aGDI	51.71
UT aGDII	15.31	PT1 aGDII	11.12	PT2 aGDII	40.03
UT aGDIII	8.33	PT1 aGDIII	6.88	PT2 aGDIII	38.00
UT bGDI	29.37	PT1 bGDI	50.27	PT2 bGDI	59.13
UT bGDII	44.48	PT1 bGDII	28.06	PT2 bGDII	53.62
UT bGDIII	43.32	PT1 bGDIII	33.89	PT2 bGDIII	59.36
UT cGDI	53.40	PT1 cGDI	85.43	PT2 cGDI	74.43
UT cGDII	68.30	PT1 cGDII	84.15	PT2 cGDII	105.90
UT cGDIII	27.46	PT1 cGDIII	75.33	PT2 cGDIII	112.46

UT: Untreated *P. hysterohorus*
PT1: Alkali treated *P. hysterohorus*
PT2: Acid treated *P. hysterohorus*
a: I/S ratio of 0.5%
b: I/S ratio of 1.0%
c: I/S ratio of 2.0%
I: Co-additive substrate (C/S) ratio of 1:1
II: C/S ratio of 1:0.5
III: C/S ratio of 1:0.25
CD: Cow dung co-additive
GD: Goat dropping co-additive
*Mean value of triplicates

Table 2: Methane yield of P.hysterophorus substrate at various I/S ratios, C/S ratios and pretreatments.

Sample	Methane yield in %*	Sample	Methane yield in %*	Sample	Methane yield in %*
PT3 a	77.41	PT4 a	62.30	PT5 a	49.32
PT3 b	84.27	PT4 b	105.56	PT5 b	30.02
PT3 c	104.78	PT4 c	119.6	PT5 c	64.37
PT3 aCDI	72.73	PT4 aCDI	83.95	PT5 aCDI	54.38
PT3 aCDII	89.19	PT4 aCDII	79.73	PT5 aCDII	29.42
PT3 aCDIII	18.57	PT4 aCDIII	59.61	PT5 aCDIII	23.09
PT3 bCDI	97.25	PT4 bCDI	107.82	PT5 bCDI	59.51
PT3 bCDII	93.47	PT4 bCDII	110.67	PT5 bCDII	47.62
PT3 bCDIII	109.49	PT4 bCDIII	107.54	PT5 bCDIII	57.39
PT3 cCDI	56.17	PT4 cCDI	149.24	PT5 cCDI	95.92
PT3 cCDII	129.6	PT4 cCDII	130.89	PT5 cCDII	48.4
PT3 cCDIII	91.45	PT4 cCDIII	120.91	PT5 cCDIII	70.45
PT3 cGDI	64.7	PT4 aGDI	93.48	PT5 aGDI	58.29
PT3 aGDII	76.66	PT4 aGDII	92.65	PT5 aGDII	46.66
PT3 aGDIII	58.17	PT4 aGDIII	74.83	PT5 aGDIII	28.64
PT3 bGDI	90.73	PT4 bGDI	107.09	PT5 bGDI	71.52
PT3 bGDII	97.35	PT4 bGDII	108.23	PT5 bGDII	84.48
PT3 bGDIII	89.73	PT4 bGDIII	110.65	PT5 bGDIII	76.02
PT3 cGDI	93.62	PT4 cGDI	111.06	PT5 cGDI	90.04
PT3 cGDII	106.10	PT4 cGDII	114.45	PT5 cGDII	94.38
PT3 cGDIII	111.26	PT4 cGDIII	120.81	PT5 cGDIII	54.46

PT3: Water treated P.hysterophorus
PT4: Biologically treated P.hysterohorus
PT5: Whey water treated P.hysterohorus
a: I/S ratio of 0.5%
b: I/S ratio of 1.0%
c: I/S ratio of 2.0%
I: Co-additive substrate (C/S) ratio of 1:1
II: C/S ratio of 1:0.5
III: C/S ratio of 1:0.25
CD: Cow dung co-additive
GD: Goat dropping co-additive
*Mean value of triplicates

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