Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 13 [7] June 2024: 94-102

Online ISSN 2277-1808
Journal's URL: http://www.bepls.com
CODEN: BEPLAD

ORIGINAL ARTICLE **OPEN ACCESS**

Methanogenic Population Dynamics of Anaerobic Night Soil Biodegradation

Brijendra Kumar Kashyap[1]*, Jose Mathew[1]
Department of Biotechnology, Bundelkhand University, Jhansi-284128, Uttar Pradesh, India

ABSTRACT
Human excreta comprises faeces (night soil), and its disposal to the environment requires an energy-efficient, self-sustainable anaerobic approach. Based on night soil (NS) feeding, the anaerobic NS biodegradation can be operated optimally, underfeed, or overfeed. The feeding is governed by certain groups of microorganisms, specially rate-limiting methanogenic bacteria. The problem of delayed startup of underfeed and biodigester failure of overfeed NS can be turned to optimal operating conditions by inoculating methanogens into the biodigester. Therefore, understanding the methanogenic dynamics of the digester using a molecular approach is essential, which necessitates the rapid isolation of DNA for metagenomics approach by using a commercially available DNA isolation kit. In this study, four different commercially available DNA isolation kits were evaluated for NS biodegradation. Among the four kits, Zymo Research Soil Microbe DNA Kit was found to be suitable for methanogenic population dynamics and was superior to other kits in quality, quantity, and purity of the DNA. For the dynamic study, 50% anaerobic microbial inoculum was filled in five 2L anaerobic biodigesters (2D,5D,8D,10D, and 15D) and fed in fed-batch mode with a fixed volatile solid of NS with varying hydraulic retention time of 2,5,8,10, and 15 days, respectively. The VS reduction was maximum (50-70%) for 15D HRT and methane was found to be maximum of 55-70%. Among the methanogens, only methanosaetacae group of methanogens (2.75×10^3 16S rDNA/25ng) were detected in the inoculum. After the first HRT of 15D biodigester, the number of Methanosaetacae methanogens increased to 100 fold.
Keywords: *Biodigester, HRT, Methanosaetacae, Methanogen, Night soil, DNA Kit*

Received 09.04.2024 Revised 29.04.2024 Accepted 06.06.2024

INTRODUCTION

Human excreta commonly refer to the by-product of digestion and comprises faeces and urine. Human faeces is also called night soil (NS)[1], and it comprises undigested food, bacterial mass (mainly proteins), foul-smelling compounds such as fatty acids (i.e., acetic acid, propionic acid and butyric acid), and a small amount of indole and skatole [2],[3]. The proper treatment and disposal of NS is important which require an energy-efficient, eco-friendly anaerobic biodegradation. The proper treatment of NS in the absence of molecular oxygen may take place in a closed vessel called a digester (figure 1). This can be installed and operated either at a small capacity family level or large capacity community level, including airways, roadways, railways etc. The efficiency of NS biodegradation depends upon the various ways of NS feeding including, total solid (TS), volatile solid (VS), hydraulic retention time (HRT), chemical oxygen demand (COD), biological oxygen demand (BOD), and organic loading rate (OLR) etc. In the presented study, varying HRT (2, 5, 8, 10 and 15 days) at fixed concentrations of TS and VS of NS feeding was used for the optimization of NS biodegradation. Anaerobic digestion of NS involves a series of metabolic interactions among four major groups of microorganisms, namely hydrolytic-fermentative bacteria, proton-reducing acetogenic bacteria, and methanogens (acetoclastic and hydrogenotrophic methanogens)[4],[6]. These microorganisms depolymerise the waste materials into simpler molecules such as carbon dioxide, methane, and water through a series of reactions[7]. Among all these microorganisms, methanogens are the key indicator for energy-efficient organic waste biodegradation[8]. There are mainly seven orders of methanogens *Methanosarcinales, Methanobacteriales, Methanomicrobiales, Methanococcales, Methanopyrales, Methanocellales*, and *Methanomassiliicoccales* responsible for methanogenesis [9]. Among these *Methanopyrales* are growing at high temperatures, *Methanomassiliicoccales* are present in gastrointestinal tracts (GIT) of animals while *Methanocellales* are isolated from rice paddy soil[10],[12]. Therefore, only four methanogens group is expected to be present in NS biodegrading biodigester. Approximately 70% of methane is produced from acetoclastic methanogen while the rest 30% of methane is produced from hydrogenotrophic methanogen[13]. The major methane production occurs through

acetoclastic mode involving *Methanosarcinales* (*Methanosarcinacae* (MSc) and *Methanosaetacae* (MSt)). At higher substrate loadings, that is, during the periods of startup and overload, hydrogenotrophic methanogen fails to consume a surplus amount of hydrogen produced during fermentation and acetogenesis, leading to the accumulation of propionate, butyrate, lactate, and ethanol (more reduced metabolites). The lipid-containing food waste produces volatile fatty acids (VFAs) after the hydrolysis of triglycerides. The accumulation of VFAs, which causes even more imbalance, may decrease the pH (<7.0) and inhibit the growth of methanogen, thereby inhibiting methane production[14]. In anaerobic digestion, methanogens, sulfate-reducing bacteria, and acetogens are responsible for the removal of hydrogen[15],[16]. For effective anaerobic NS biodegradation, the presence of methanogens is desirable for methane generation and energy production[7], [17], [18], [19]. Therefore, the problems of delayed startup and failure of the biodigester, along with maximum energy requirement, can be addressed by ensuring a large number of methanogenic communities during startup and periods of overload [20],[21]. Methanogen culturing is a labour-intensive process that requires expertise, and it becomes more difficult when it is, sometimes, associated with syntrophic with other microbes. Hence, the metagenomic approach is preferable for determining the structure and population dynamics of methanogens during anaerobic NS biodegradation[22], [26]. This approach can also resolve the problems of delayed startup of anaerobic digestion and failure of anaerobic NS digestion [28],[29]. Therefore, in the present study, metagenome/ genomic DNA was isolated using four commercially available DNA isolation kits; the quality and quantity of the DNA isolated from the four kits were compared to determine the most suitable kit for methanogenic population dynamics studies. The methanogenic population dynamics were detected at the level of order and family level for NS biodegradation through quantitative real-time PCR.

MATERIAL AND METHODS

Biodigester setup

Five biodigesters (2D, 5D, 8D, 10D, and 15D) were set up (figure 2). Each biodigester has a cork and glass rod fitted biodigester flask (2L capacity; filled with 50% inoculum [30] interconnected through a lazem tube with a biogas flask (2L capacity; filled with tap water).

NS feeding

Night soil was collected from the local region of Jhansi, Uttar Pradesh. It was weighed and an equal volume of tap water was added to it to make a 1:1 dilution (weight/volume) of NS. An Aliquot of this was made in a polythene bag and stored at -80 °C in the fridge. Before feeding the biodigester NS was allowed to bring at room temperature [19].

Biodigester Operation

The biodigester operating volume was 2 L. The five biodigesters (2D, 5D, 8D, 10D, and 15D) were set up and filled with 50% AMI inoculum while the biogas flask was filled with tap water. The five biodigesters were operated at varying hydraulic retention times (HRT) of 2, 5, 8, 10 and 15 days with NS feeding (1:1 diluted), at fixed TS (61.40 g/L) and VS (53.338 g/L), with volumes of 1000 mL, 400 mL, 250 mL, 200 mL, and 134 mL, respectively in fed-batch mode[19]. Initially, biodigester NS feeding was done up to 2 L capacity without spending out the digested slurry and later on in semicontinuous mode i.e., an equal volume of the digested slurry was spent out and the same amount was fed with NS. Before and after every feeding, biodigester was mixed for 30 sec. The digestion experiment was allowed to operate at least for 5 HRT and the digested slurry sample (for TS, VS, and biogas) was collected at each HRT and stored at -80 °C in the fridge. All the digestion experiment was executed in a controlled environment of a BOD incubator at 35± 2°C.

Total Solid (TS) and Volatile Solid (VS)

A silica crucible was dried at 105 °C in a hot air oven for 1 hour and allowed to cool in an air-tight desiccator. The crucible was weighed (w_1) to the constant weight. The inoculum sample of 25 mL was poured into a crucible and was allowed to dry at 105 °C for 4 hr and cooled in a desiccator for weighing till the constant weight (w_2). The TS (gm/ L) was calculated with the difference in weight (w_2-w_1) between the dried sample along with the crucible (w_2) and the empty crucible (w_1)[19] [31]

The crucible was further put at 550 °C in a muffle furnace for 1 hr and was allowed to cool in a desiccator followed by weighing till constant weight (w_3). The VS (gm/ L) was calculated with the difference in weight (w_3-w_1)[31]. The total dissolved solid was measured through a portable TDS meter. All the experiment of TS and VS was done in triplicate.

Methane estimation from biogas

For the collection of produced biogases, a 10 mL serum vial (filled with water) was sealed with butyl rubber and an aluminium crimp. The biogas was collected in the serum vial through water displacement and kept in an inverted position for methane analysis.

The determination of the methane content within the biogas was performed by GC analysis. For this, 30 µL of collected biogas from a serum vial was injected into a Thermo GC Ultra gas chromatograph with a gas-tight syringe (Hamilton). The GC was equipped with a flame ionisation detector (FID) using Free Fatty Acid Phase-Fused Silica Capillary Column (0.25mm X 0.25 µm X 30 m). The temperature of the column was kept at 50 °C in the first 50 sec followed by a linear increase of 4 °C sec^{-1} to 120 °C and the final temperature was kept at 200 °C with a retention time of 3 min. Nitrogen was used as the carrier gas with a flow rate of 30 mL/ min. The calibration of the GC was performed with standard methane gas (Sigma Gases and Services, New Delhi, India).

Methanogenic population dynamics

Isolation of DNA

Four commercially available DNA isolation kits, namely Zymo Research Soil Microbe DNA Isolation Kit (ZR), Power Soil DNA Isolation Kit (PS), Ultra Clean Fecal DNA Kit (UCF), and Ultra Clean Soil DNA Isolation Kit (UCS), were compared for genomic DNA isolation. The sample was collected from inoculum (Anaerobic microbial inoculum) maintained at a mesophilic temperature of 35 °C. For comparison of the DNA isolation kits and their evaluation, 5.0 mL of inoculum sample was centrifuged at 8000 g for 10 min in a 15-mL centrifuge tube, followed by washing with 10 mL TE buffer twice. The washed sample was used for genomic DNA isolation as per the recommendation of the manufacturers of the four commercially available DNA isolation kits i.e., ZR, PS, UCF, and UCS. All the experiment was done in replicate. The final extraction of DNA was performed in an elution volume of 50 µL. The eluted DNA was quantified spectrophotometrically at 260 nm, 280 nm, and 230 nm (nanodrop) (Table 1). Further, 5.0 µL of the DNA sample was loaded along with 6X DNA loading dye (Fermentas) in the wells of 0.8% agarose gel and allowed for electrophoresis (BioRad) in 1X TAE buffer at 50 volts for 45 min. The gel was visualised using the gel documentation system (BioRad) under ultraviolet light, and the images were captured.

Reference strain

The 16s rDNA of various members of the methanogenic group, namely methanobacteriale (MBT), methanococcale (MCC), methanosarcinaceae (MSc), and methanosaetaceae (MSt), were retrieved from the National Centre for Biotechnology Information (NCBI) gene data bank and were allowed to be aligned. The strains of all four methanogenic groups, namely MBT, MCC, MSc, and MSt were selected (data not shown). A strain of methanosaetaceae group, namely *Methanothrix soehngenii*, Opfikon (DSM 2139) of 164 bp, was selected. This 16S rDNA was having methanosaetaceae specific region of forward primer MSt F (5'TAATCCTYGARGGACCACCA3'), reverse primer MSt R (5'CCTACGGCACCRACMAC), and TaqMan probe (FAM-ACGGCAAGGGACGAAAGCTAGG-BHQ1) [32]. The corresponding 16S rDNA gene of each methanogenic group was oligosynthesized (as culturing of anaerobic methanogen is tedious and cumbersome) from Biotek Desk Pvt. Ltd., India and inserted into the pUC57 vector. This oligo-synthesised 16S rDNA gene was used as a methanogens reference DNA for real-time PCR analysis of inoculum and collected sample at each HRT.

Real-time PCR analysis

A serial dilution of the oligo-synthesised reference DNA was made from 10^0 to 10^5 copy numbers of the gene in duplicate. Quantitative real-time PCR (Cepheid Smart Cycler, U.S.A.) was executed with the serially diluted reference DNA as a positive control (and milliQ sterile water as negative control). It was performed in a 25 µL reaction mixture containing 1000 nM each of forward and reverse primers, 200 nM probe (Table 1), and 25 ng/ µL template with Takara Master mix. Amplification was performed in a two-step thermal cycling procedure: predenaturation of 10 min at 94 °C followed by 40 cycles of 15 sec at 94 °C and 30 sec at 56 °C. The fluorescence and threshold cycle (Ct) values were observed and a standard curve was plotted between the log concentration of gene copy number and Ct value. The minimum limit of detection (LOD) of the gene was also observed for each group. This graph was further used to determine the number of methanogenic 16S rDNA gene copy numbers of genomic DNA of inoculum, night soil, and sample collected at each HRT along with positive (10^6 gene copy number of 16S rDNA of reference strain) and negative (sterile Milli-Q water) controls. The genomic DNA was isolated by using the commercially available Zymo Research DNA kit.

RESULT AND DISCUSSION

The five biodigester (2D, 5D, 8D, 10D, and 15D) was allowed to operate with varying HRT of 2, 5, 8, 10, and 15 days in a controlled environment of BOD incubator at mesophilic temperature (35±2 °C). Initially, the biodigesters were filled with 50% inoculum and were fed with fixed VS of NS with volumes of 1000 mL, 400 mL, 250 mL, 200 mL, and 134 mL, respectively till the capacity of 2L without spending out slurry waste. After NS feeding till the 2 L of biodigester corresponding digested slurry volume was spent out every day from each biodigester.

The performance of biodigester was observed in terms of VS reduction (%) and methane content in biogas. Initially, during the first HRT % VS reduction increased rapidly due to the low VS content of inoculum (18.815 g/L) and the high VS content of NS feed (53.338 g/L) while no spent out of slurry was done from the biodigester till the volume of biodigester reach 2 L (biodigester content only 1 L inoculum) with NS feeding. After the first HRT, biodigesters get stabilised in terms of TS, VS, % VS reduction, and methane content of biogas and methanogens. The methane content of biodigester 2D and 5D was <20 % after the first HRT indicating overfeeding and failure of the digester. In 8D and 10D HRT biodigester, after 2nd HRT the % VS reduction was 45-60% and methane content in biogas was 45-55% and 50-65%, respectively. While the maximum VS reduction was 50-70% for 15D HRT and methane was found to be a maximum of 55-70 % for 15D HRT (figure 3). This showed that 15D HRT biodigester works more optimally and has sufficient numbers of methanogens to produce 60% methane. The optimum biodegradation of NS organic waste depends upon a higher % VS reduction to methane indicating the more conversion of organic waste to methane from methanogens. Thus, 15D HRT shows a 70% VS reduction along with more methane content ~70 % of biogas from a greater number of methanogens. The quality and quantity of the DNA isolated using the four commercially available DNA isolation kits, Zymo Research Soil Microbe DNA Isolation Kit (ZR**)**, Power Soil DNA Isolation Kit (PS), Ultra Clean Fecal DNA Kit (UCF), and Ultra Clean Soil DNA Isolation Kit (UCS), were compared. Table 1 shows that the time consumption/incubation time for DNA isolation is minimum (15 min) for the ZR DNA isolation kit among all four DNA isolation kits (28, 21, and 22 min for PS, UCF, and UCS, respectively). The quality of the genomic DNA isolated using all four kits was assessed spectrophotometrically (nanodrop). DNA concentration (OD_{260}) was highest for the UCS DNA isolation kit (81.0 ng/µl) and lowest for the ZR isolation kit (50.6 ng/µl). However, the ratio of optical density at wavelengths 260 and 280 nm ($OD_{260/280}$) was optimal (i.e., 1.8) for genomic DNA isolated using ZR (1.84), whereas for those isolated using PS, UCF, and UCS, the ratios were 1.24, 1.25, and 1.39, respectively, all less than 1.8, which indicated the presence of protein/ inhibitor component. Additionally, the ratio of optical density at wavelengths 260 and 230 nm ($OD_{260/230}$) for ZR was 1.55, approaching toward purity (i.e., 2.0–2.2), which was higher than those for the other kits (<0.8)[33]. Further, the quality of DNA was assessed through agarose gel electrophoresis by loading an equal quantity (5 µL) of all four genomic DNA samples. The intensity and thickness of the DNA band for the genomic DNA isolated using the ZR kit were found more intense than those of the DNA isolated using the PS, UCS, and UCF DNA kits. Further, the purity of ZR genomic DNA (presence of PCR inhibitors) was assessed through quantitative PCR (TaqMan based). The amplification of a methanogenic (methanosaetaceae group) specific probe and primer set was used for plotting the standard graph. This graph was further used to determine the number of genes of 16S rDNA in the inoculum, NS and the sample collected at each HRT of the 15D digester along with positive and negative (sterile miliQ water) controls (figure 4). Among all the methanogens, only methanosaetaceae group of methanogens above the LOD (2.75×10^3 16S rDNA copy number) was detected in the inoculum genomic DNA isolated using the ZR DNA isolation kit. The amplification results confirmed that the genomic DNA was pure and did not contain any inhibitor. Thus, based on time consumption/incubation required during DNA isolation, intensity and thickness of the genomic DNA band on agarose gel, $OD_{260/280}$, $OD_{260/230}$, and purity assessed through quantitative real-time PCR, we infer that the ZR DNA isolation kit is superior to the other three kits. The AMI inoculum used for methanogenic population dynamics of NS biodegradation contains dominating MSt group of methanogens which is classified in acetoclastic methanogens responsible for 70% of methane production[6],[13],[29],[33]. Since the inoculum contains only the methanosaetaceae group of methanogens, therefore, major methane production takes place by MSt group only[35]. The genomic DNA of the collected biodigested slurry at each HRT was isolated from a ZR DNA isolation kit. Equal genomic DNA concentration (i.e., 25 ng) of each HRT sample of 15D HRT biodigester was used for MSt group of methanogens. Initially, the inoculum methanogens number in 15D biodigester was 2.75×10^3 16S rDNA copy number which increased rapidly after NS feeding during the first HRT to 6.0×10^5 16S rDNA copy number of MSt /25ng of genomic DNA (figure 5). This increment was proportionate to more % VS reduction and methane content. This initial increment can be explained as the spending out of biodigester slurry (containing methanogens) starting after 7 days. After the first HRT, the number of methanogens (Methanosaetacae) gets stabilized which ranges between 8.0×10^4 to 2.0×10^5 16S rDNA/ 25 ng.

Abbreviations used

ZR	Zymo Research Soil Microbe DNA Kit
PS	Power Soil DNA Isolation Kit
UCF	Ultra Clean Faecal DNA Kit
UCS	Ultra Clean Soil DNA Isolation Kit
IN	Inoculum
NS	night Soil
LOD	limit of detection
VFA	volatile fatty acid
MSt	Methanosaetaceae
µL	Microlitre
ng	Nanogram
°C	degree Celsius
OD	optical density
DNA	deoxyribonucleic acid
qPCR	quantitative polymerase chain reaction
mL	Mililitre
TE	Tris EDTA
nM	Nanomolar
TAE	Tris-acetate-EDTA
Ct	threshold cycle

Table1: Comparison of commercially available DNA isolation kits for anaerobic night soil biodegradation

DNA Isolation Kit	**ZR Soil Microbe DNA Kit**	**Power Soil DNA Isolation Kit**	**Ultra Clean Faecal DNA Kit**	**Ultra Clean Soil DNA Isolation Kit**
Abbreviation	ZR	PS	UCF	UCS
Make	United States	MO Bio Laboratories, CA, USA	MO Bio Laboratories, CA, USA	MO Bio Laboratories, CA, USA
Lysis of the cells	Bead beating	Bead beating	Bead beating	Bead beating
Time Incubation/ Consumption	~15 min	~28 min	~21 min	~22min
DNA Conc. (ng/µl)	50.6 (1.3)	55.1 (0.9)	69.1 (1.9)	81.0 (2.1)
OD 260/280	1.84 (0.2)	1.24 (0.18)	1.25 (0.15)	1.39 (0.23)
OD260/230	1.55 (0.11)	0.76 (0.03)	0.8 (0.08)	0.65 (0.04)
The values in parentheses indicate the standard deviation				

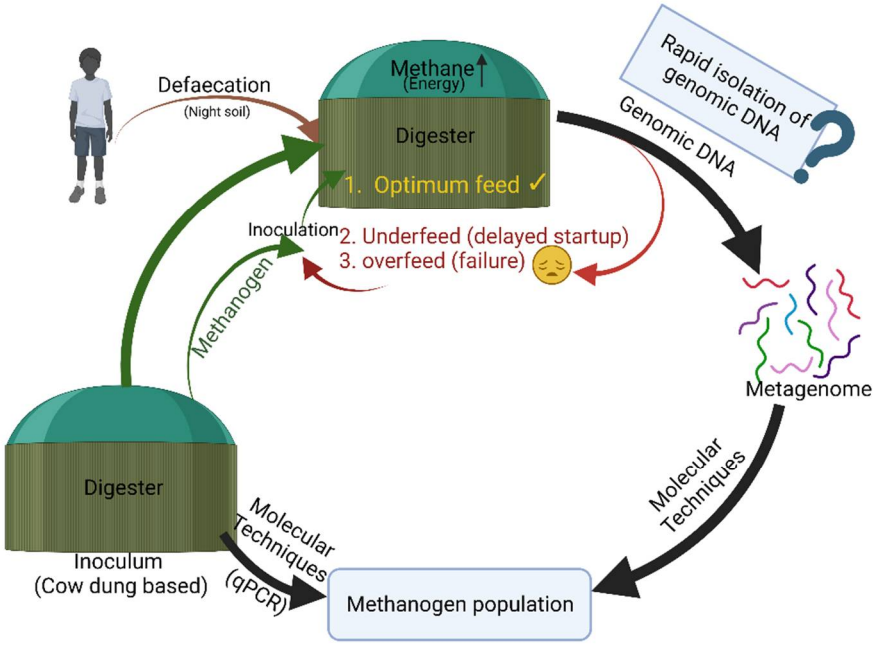

Figure 1: Schematic representation and methanogen involvement in night soil biodegradation

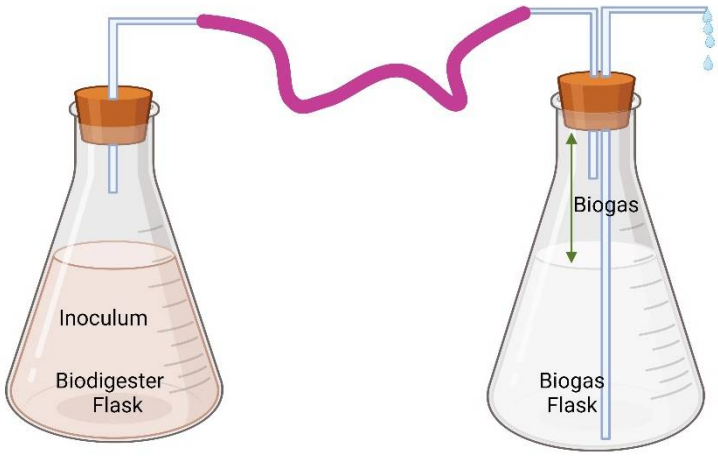

Figure 2: Night soil biodigester assembly

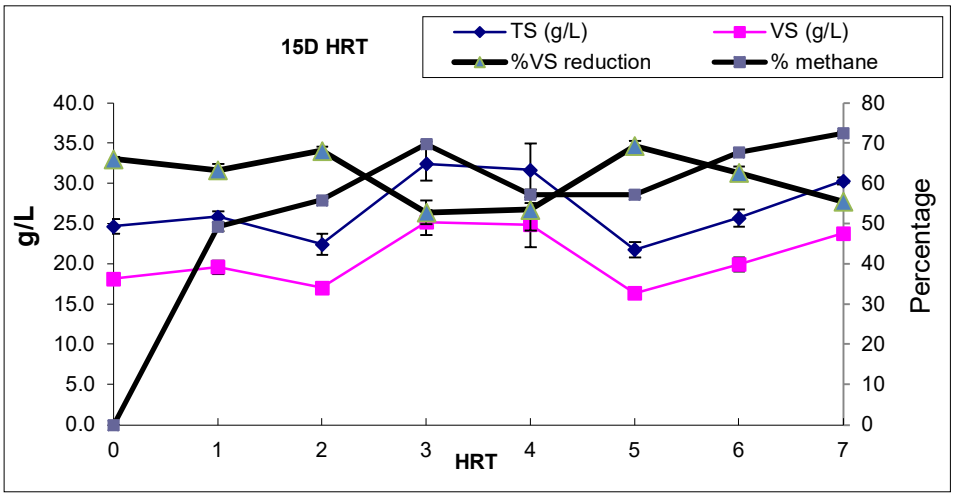

Figure3: Anaerobic night soil biodegradation of 15 D HRT (HRT-hydraulic retention time; TS-total solid; VS-volatile solid)

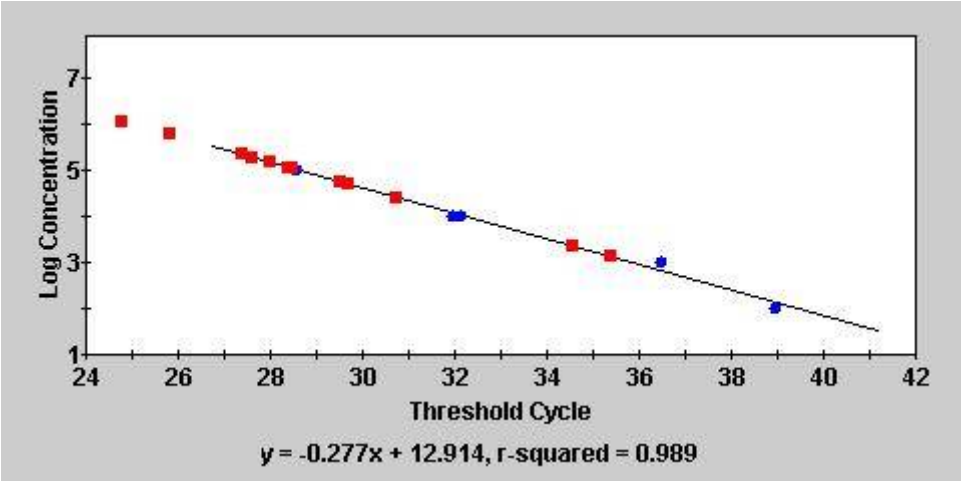

Figure4: Quantitative real time PCR: Log concentration of 16S-rDNA copy and threshold cycle-Standard curve of Methanosaetaceae group of methanogens along with night soil digested sample collected at various hydraulic retention time of 15D biodigester

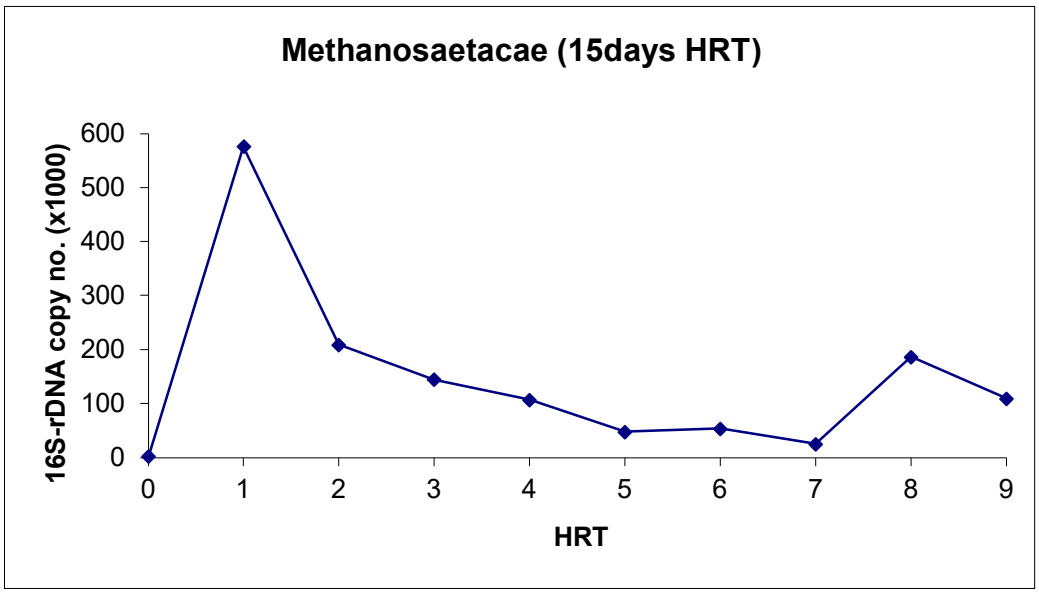

Figure5: Methanogenic (Methanosaetaceae group) population dynamics of night soil biodegradation: 16S-rDNA copy number *vs* hydraulic retention time (collected at various HRT) of 15D biodigester

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CITATION OF THIS ARTICLE

Brijendra Kumar K, Jose M. Methanogenic Population Dynamics of Anaerobic Night Soil Biodegradation. Bull. Env.Pharmacol. Life Sci., Vol 13[7] June 2024: 94-102