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Identification of metabolically active methanogens in anaerobic digester by DNA Stable-Isotope Probing using ^{13}C-acetate

V. Gowdaman, M. Srikanth

Applied and Environmental Biotechnology Laboratory, Department of Biological Sciences,
Birla Institute of Technology and Science - Pilani, K. K .Birla Goa campus, Goa – 403726, India.

Abstract: Anaerobic digestion is gaining enormous attention due to the ability to covert organic wastes into biogas, an alternative sustainable energy. Methanogenic community plays a significant role in biogas production and also for proficient functioning of the anaerobic digester. Therefore, this study was carried out to investigate the methanogen diversity of a food waste anaerobic digester. After endogenous respiration, the digester samples were supplemented with isotopes of acetate to enrich methanogen population, and were analyzed using DNA-SIP (Stable-Isotope Probing). Following separation and fractionation of heavy (^{13}C) and light (^{12}C) DNA, PCR amplification was carried out using archaeal 16S rRNA gene followed by DGGE analysis. Sequencing of the prominent DGGE bands revealed the dominance of *Methanocorpusculum labreanum* species belonging to hydrogenotrophic Methanomicrobiales, which can produce methane in the presence of H_2/CO_2 and requires acetate for its growth. This is the first instance where *Methanocorpusculum labreanum* is being reported as a dominant species in an anaerobic digester operative on food waste.

Keywords: Methanogens, anaerobic digester, food waste, stable-isotope, DNA-SIP, DGGE.

1 Introduction: Anaerobic digestion (AD) is considered to be an appealing source of renewable energy in the form of biogas and also a feasible method to treat various organic wastes [1]. Anaerobic conversion of organic wastes involves different bacterial species, such as hydrolytic, acidogenic, acetogenic, and methanogenic bacteria that produce CO_2 and CH_4 as the main products of the digestion process [2]. The production of biogas from food waste and other organic materials is a feasible strategy in view of both ecology and economy [3]. During biomass conversion to methane, process stability is a key issue in anaerobic digestion, as increasing use of residual and waste materials together with variable substrate quality requires a high flexibility of microbial community. High fluctuation of various bacterial populations can sustain stable performance of hydrolysis, acidogenesis and acetogenesis [4]. However, methanogens are sensitive to process imbalances that are reflected in the bioreactor performance via accumulation of intermediates such as volatile fatty acids (mainly C2 and C3), via pH changes or via reduced efficiency [5] and also by different substrates and operation conditions [6]. So the stable function of the methanogenic archaeal community determines the success or failure of anaerobic digestion systems. Therefore, investigation of methanogens might provide the most sensitive indicators for understanding the parameters influencing the biogas process.

Current research work focuses on the methanogens, which directly reduce CO_2 to CH_4 or use acetate as the main substrate to yield methane [7]. Active microbial population can be studied efficiently by

utilizing Stable isotope probing (SIP) technique, which relies on the incorporation of stable isotopes (^{13}C) into DNA **[8]**, RNA **[9]** or phospholipid fatty acid (PLFA) **[10]**. Successive separation and identification of labeled DNA, RNA or PLFAs offer culture-independent insight into microorganisms involved in the incorporation of particular labeled compounds, therefore relating the phylogeny of microorganisms to their functions **[11]**. DNA-SIP is regarded as the most informative method for taxonomic purposes **[12]**. The objective of this study was to identify the methanogenic archaea involved in methanogenesis of food waste digester by employing DNA-SIP with ^{13}C-acetate as the labeled substrate. Identifying the active methanogenic archaea will help in efficient functioning of food waste anaerobic digesters.

2. Materials and Methods

2.1 Collection and characterization of food waste: Food waste samples were collected from BITS Pilani KK Birla Goa campus institute mess. Effluent from the anaerobic pilot plant treating food waste in the institute campus was used as the inoculum. The effluent was brought in the laboratory in closed container and was monitored for gas production. It was pre-incubated at 32^{o}C to deplete the residual biodegradable organic material present in it and was used for BMP assay when it reached the endogenous respiration stage. TS were determined after drying the sample at 105°C overnight. VS in organic wastes were measured as total solids minus the ash content after ignition at 550°C **[13]**. Based on the VS/TS %, amount of inoculum and substrate to be added for the experiment were calculated.

2.2 Biomethanation Potential (BMP) of food waste: BMP of food waste was studied **[14]** using 125 ml serum bottles. The concentration of the substrate and inoculum used were in the ratio of 1:2, followed by the addition of 1 ml of micronutrients, 1 ml of macronutrients and 5 ml of 5% $NaHCO_3$ to each bottles. The final volume was made to 100 ml with distilled water. A control without substrate was setup to account for the endogenous biogas produced from the inoculums. The bottles were flushed with nitrogen gas followed by immediate sealing. All the bottles were incubated at 32^{o}C. The experiments were carried out in duplicate. Biogas produced from each bottle was measured everyday using water displacement method till endogenous respiration stage.

2.3 DNA Stable-Isotope Probing (DNA-SIP): Once endogenous respiration is attained, the samples were supplemented with stable isotopes of ^{13}C and ^{12}C Sodium acetate **[15]** at a concentration of 50 µM, as a substrate on every third day for a period of 9 days. One set of bottles with no substrates added served as a control.

2.4 DNA Extraction and Ultracentrifugation: Following the incubation of sample with labeled substrate, DNA was extracted from each vial using Qiamp DNA stool mini kit (Qiagen) using manufacturers protocol. Extracted DNA was quantified using a nanodrop and alternatively visualized using agarose gel electrophoresis. Separation of ^{13}C and ^{12}C DNA was carried out using Cesium chloride gradient ultracentrifugation **[16].** The required amount of DNA (5-10 µg) was mixed with 2.167 ml of Gradient Buffer, 2.167 g of CsCl and 10.4 ml of 7.163 M CsCl to a total volume of ~13 ml in a sterile disposable 50 ml tube. Using a pasteur pipette the 13-ml polyallomer ultracentrifuge tubes were carefully filled with gradient solutions. Seal the tubes using a torque wrench according to the manufacturer‘s instructions. Insert the tubes into Beckman-Coulter MLA 55 rotor and place the rotor onto Optima MAX-HP Ultracentrifuge. Set the rotation speed to 46,000 rpm at 20°C, and time for 48 hours, select vacuum, maximum acceleration, and deceleration at coast.

2.5 Gradient fractionation and DNA precipitation: After ultracentrifugation the tubes were removed and using a syringe each fraction of 500 µl was collected from top of the tube. DNA were precipitated from all 26 fractions by adding 2 µl of Glycogen, followed by two volumes of PEG-NaCl solution and mixed by inversion **[16]**. Tubes were kept at room temperature for 2 hours to overnight to allow DNA

precipitation followed by centrifugation at 13,000 rpm for 30 minutes. Supernatant was discarded and the pellet was washed with 500 µl of 70% ethanol, dry the pellet at room temperature for 15 min. Suspend each pellet in 50 µl of TE buffer. Check the presence of DNA in the each fraction by running 5 µl of the dissolved DNA by agarose gel electrophoresis.

2.6 Polymerase chain reaction: Archaeal specific primers 340F (5'-CCCTAYGGGGYGCASCAG-3') and 1000R (5'-GAGARGWRGTGCATGGCC-3') were used to amplify the V3-V6 region of the 16S rRNA gene sequence **[17]**. Each PCR amplification was performed with a total volume of 20 µl, which contained 2 µl of 10X buffer, 2 µl 25 mM $MgCl_2$, 5 pmol of each primer (forward and reverse), 1 µl of 100 nM dNTP mixture, 0.5 U of Taq DNA polymerase (Sigma Aldrich), and 2.0 µl of template. PCR conditions were 95^{o}C for 5 min followed by 30 cycles of 95^{o}C for 1 min, 55^{o}C for 1 min, and 72^{o}C for 1 min and final extension at 72^{o}C for 10 min. PCR fragment of 660 bp in length were amplified. For DGGE analysis of archaeal 16S rRNA gene a GC clamp of 40 bases at 5' end **[18]** of 340F primer were added. Same PCR conditions used for archaeal 16S rRNA gene was retained with final extension of 30 min. at 72^{o}C.

2.7 Denaturing gradient gel electrophoresis (DGGE): The fractions that showed amplification for archaeal 16S rRNA gene were analysed using DGGE **[18]**. The polyacrylamide gels used were having 7 % denaturant at a gradient of 30-70% with a temperature of 60^{o}C. DNA concentration of 3 µg was loaded onto each lane of the polyacrylamide gel and run for 14 hours at 70 V. Electrophoresis was performed using the DGGE system (C.B.S. Scientific Inc., California, USA) according to the manufactures directions. The gels were stained using silver nitrate. Bands of interest were excised and eluted in 40 µl of sterile distilled water. Two microliters of the eluted sample were re-amplified with the same primers as for DGGE but without GC-clamp.

2.8 Phylogenetic analysis: The resulting PCR products were sequenced and identified to their closest neighbor with the help of EzTaxon server (http://www.ezbiocloud.net/eztaxon) **[19]** on the basis of 16S rRNA sequence data. The DNA sequences of closest neighbor and sample were taken form EzTaxon and their alignment and phylogenetic analysis were performed using MEGA 5 **[20]**. The nucleotide sequences were submitted in DDBJ with accession numbers LC014947-49.

3 Results and Discussion

3.1 Food waste characteristics: The total organic and inorganic matter of food waste digestate represented by total solids was indicated in Table (1). The high proportion of volatile solid (VS) to total solid (TS) (82.8%) shows that a large fraction of food waste was biodegradable and could serve as an important feedstock for biogas production **[21]**.

Table (1): Characterization of Food waste

Parameters	**Food waste**	
	a	b
Total solids (TS) %	5.14	5
Volatile solids (VS) %	4.21	4.14
VS/TS %	81.9	82.8

We studied BMP for food waste sample for a period of 80 days under mesophilic conditions, as there are previous studies where BMP was determined for fruits and vegetable wastes at 35^{o}C with digestion time of 100 days **[22]**. Biogas production from food waste showed gradual increase from day 10, and reached maximum biogas production on 28th day producing 38 ml/day, endogenous respiration attained on 72nd day [Figure (1)].

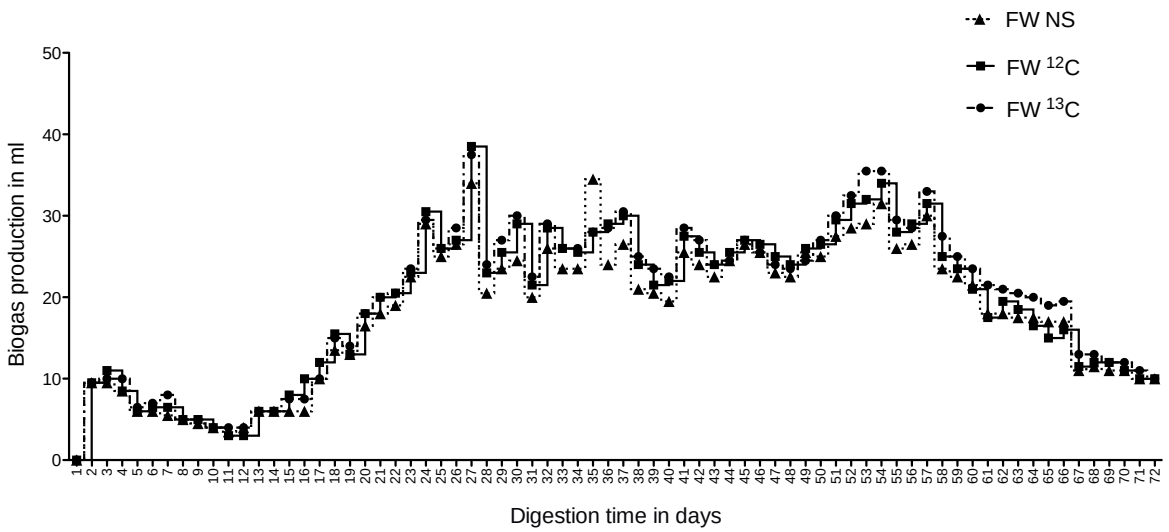

Figure (1): Biogas production using Food waste in anaerobic digestion process. FW-NS - No substrate; FW-^{12}C – unlabeled sodium acetate; FW-^{13}C – labeled sodium acetate.

Following endogenous respiration, samples were enriched with stable isotopes of acetate ^{13}C and ^{12}C **[15]** and their biogas production was monitored every 24 h, which showed negligible amount of biogas production [Figure (2)]. Labeled ^{13}C-acetate was used to detect the active methanogenic populations in the food waste digesters, there are previous reports were ^{13}C-acetate has been used to detect active methanogenic populations from swine manure storage tanks **[15]** however not studied for food waste samples.

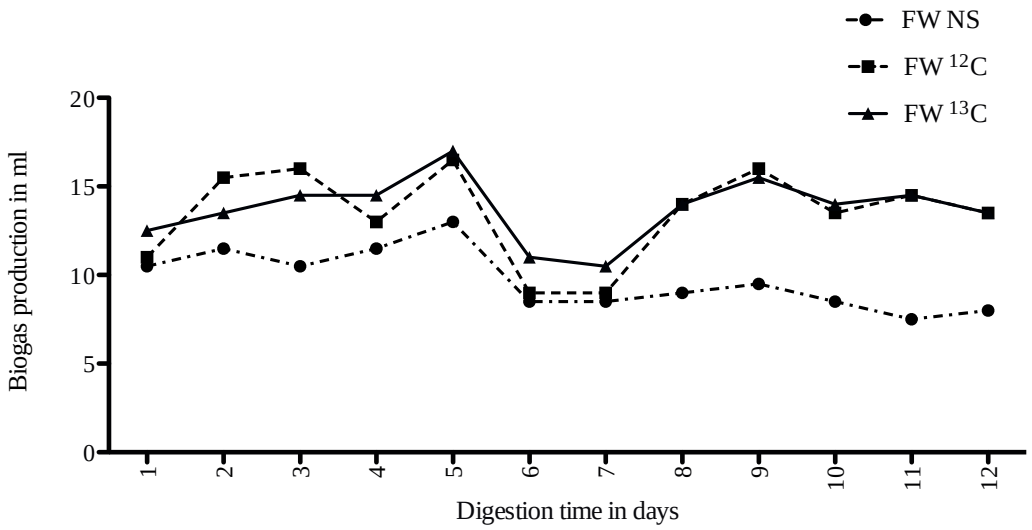

Figure (2): Biogas production after addition of Stable isotope Sodium acetate (^{13}C and ^{12}C) in Food waste samples. FW-NS - No substrate; FW-^{12}C – unlabeled sodium acetate; FW-^{13}C – labeled sodium acetate.

3.2 Metagenomic DNA extractions and separation of ^{13}C and ^{12}C DNA: The incubation period of DNA-SIP was for 9 days, as relatively long incubation periods (~40 days) might result in detection of organisms that do not directly incorporate the substrate but instead use ^{13}C-labeled intermediates **[23]**. After the incubation period, metagenomic DNA was extracted which carries both heavy (^{13}C) and light (^{12}C) DNA, and were separated using cesium chloride (CsCl) density-gradient centrifugation **[16]**.

Samples enriched with labeled acetate will facilitate the incorporation of ^{13}C isotope into their DNA, which greatly enhances the density of labeled DNA when compared with unlabeled DNA **[23]**. Around 26 fractions were collected (each 500 µl) from 13 ml tube, fractions 1 to 8 showed positive for the presence of DNA in agarose gel electrophoresis.

3.3 Archaeal 16S rRNA gene based DGGE community profile: The positive fractions (1 to 8) were amplified using archaeal primers 340F and 1000R **[17]** that amplified the specific 660 bp product [Figure (2)].

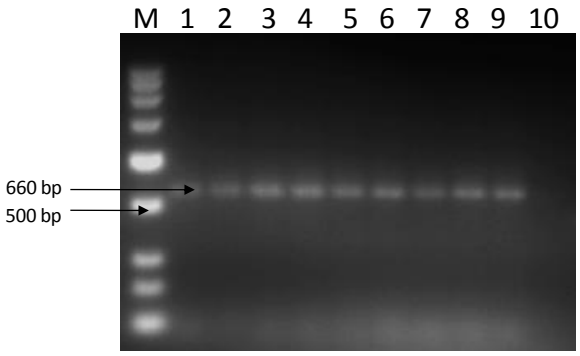

Figure (3): PCR amplification of food waste fractions using archaeal primers. Lane M - Marker; Lane 1-8 - Fractions, Lane 9 - No substrate, Lane 10 - Negative control

Archaeal community of food waste digester was analyzed by DGGE [Figure (3)], which is highly recommended fingerprinting method for downstream analysis after DNA-SIP **[24]**. There was no variation of the banding patterns among the fractions. Lanes 2-5 showed high intensity of the band BGFW1 than the other lanes [Figure (3)]. Three prominent archaeal bands of interest (BGFW1, BGFW2, BGFW3) were retrieved from DGGE gel and were sequenced. The sequences were identified to the nearest neighbor using the EzTaxon server **[19]**.

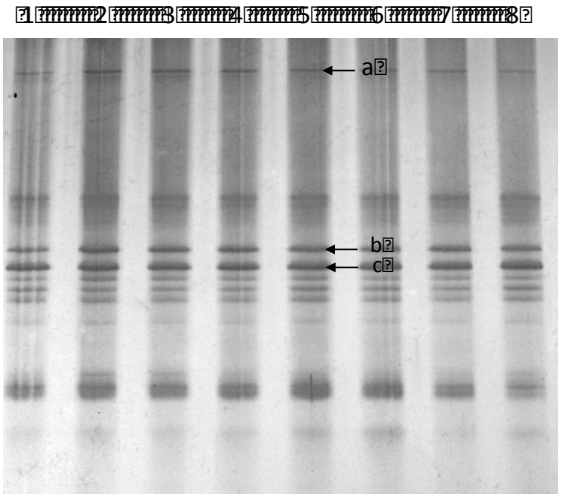

Figure (4): DGGE profile of food waste samples. Lane 1- No substrate; Lane 2-8 food waste fractions. Arrow mark indicates the band positions that were sequenced.
a - BGFW1, b - BGFW2, c - BGFW3

3.4 Phylogenetic analysis: All three bands showed taxonomic affiliation towards hydrogenotrophic methanogens belonging to the order of Methanomicrobiales. Hydrogenotrophic methanogenesis is one of the major methanogenic pathways in anaerobic digesters **[25]**. There are earlier reports suggesting the

dominance of hydrogenotrophic methanogens like in sewage sludge [26], secondary sludge [27] municipal wastewater sludge [28] and in biogas reactor utilizing swine feces [29]. Under the order Methanomicrobiales, the samples belonged to the genera *Methanocorpusculum*, exhibiting close relatedness to *M. labreanum*, *M. sinense* and *M. parvum* with similarities ranging from 98.21 to 99.20 % as shown in Table (2).

Table (2): Samples were identified to their phylogenetic neighbors using EzTaxon server

Sample	Accession no	Phylogenetic affiliation	% similarity
BGFW1	LC014947	***Methanocorpusculum labreanum* Z(T)**	**99.20**
		Methanocorpusculum sinense DSM 4274(T)	99.04
		Methanocorpusculum parvum DSM 3825(T)	99.02
BGFW2	LC014948	***Methanocorpusculum labreanum* Z(T)**	**98.41**
		Methanocorpusculum sinense DSM 4274(T)	98.25
		Methanocorpusculum parvum DSM 3825(T)	98.21
BGFW3	LC014949	***Methanocorpusculum labreanum* Z(T)**	**99.02**
		Methanocorpusculum sinense DSM 4274(T)	98.86
		Methanocorpusculum parvum DSM 3825(T)	98.83

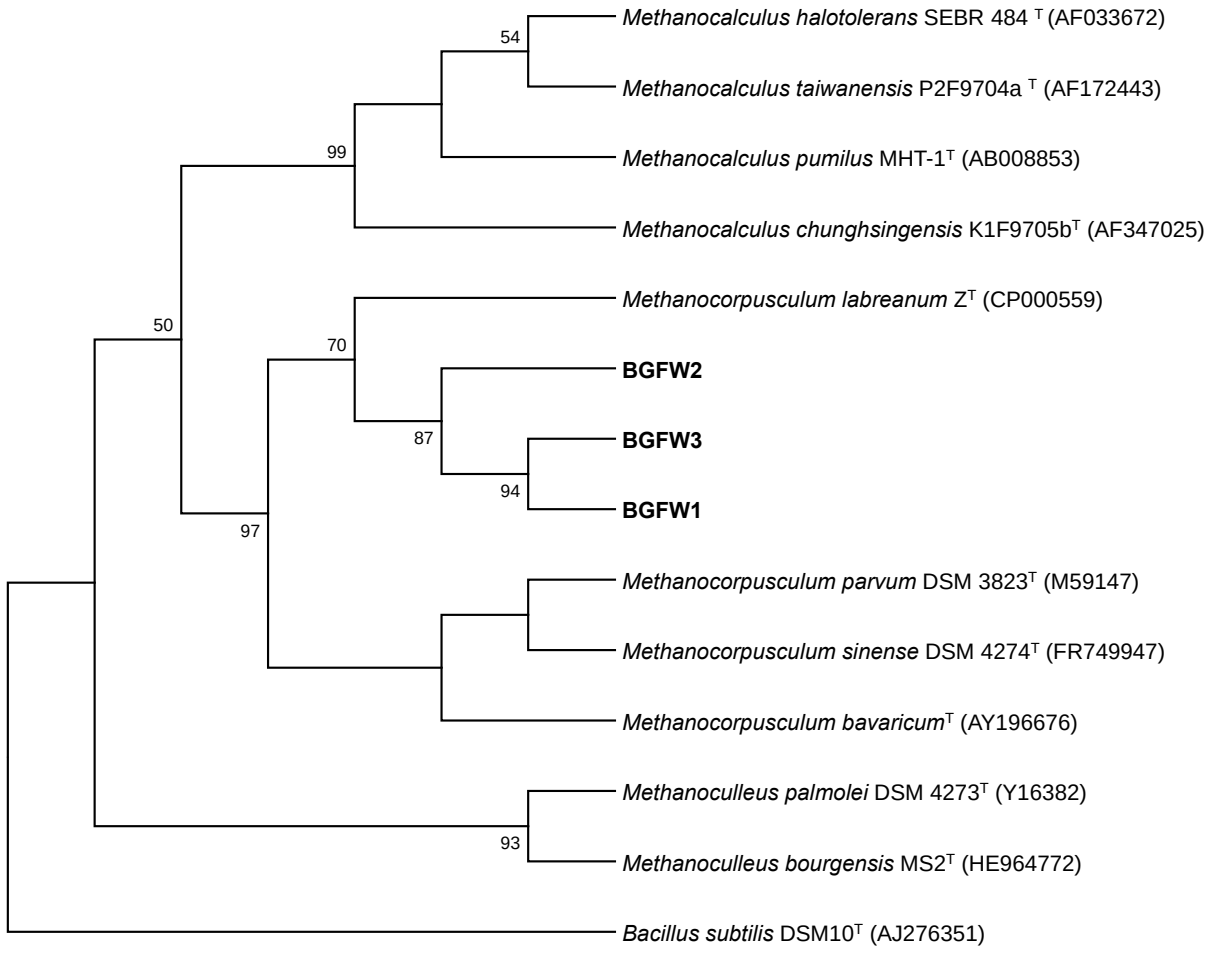

Figure (5): Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship of DGGE excised bands of food waste samples among the members of *Methanocorpusculum* species. Bootstrap values (expressed as percentage of 1000 replications) greater than 50% are given at nodes.

Neigbhour joining tree [Figure (5)] illustrates the phylogenetic affiliation of BGFW1, BGFW2 and BGFW3 towards *Methanocorpusculum labreanum* Z^T **[30]**. The genus *Methanocorpuscullum* belongs to a group of organisms that utilizes H_2/CO_2 and formate for methanogenesis and some species can use 2-propanol + CO_2. They cannot produce methane by consuming acetate, but they require acetate as an additional carbon source for biomass synthesis **[31]**. There are five species in this genus with growth temperature range of 25-40^oC with optimum at 37^oC and having G+C content of 48-52 mol% **[32]**. *Methanocorpusculum* have been previously isolated from anaerobic digesters and sewage sludge **[33]**.

Methanogens are more sensitive to low temperatures and is one of the most common factors affecting methanogenic processes **[34]**. Strains of the genus *Methanocorpusculum* are psychrotolerant and can be grown at temperatures as low as 1-5^oC but optimally between 25 and 35^oC. Psychrotolerant methanogens are considered to play an important role in methane production in habitats where seasonal variations in temperature or in permanent cold areas reported by Rastogi et al. **[35]**, the author also found the increase in percentage of *Methanocorpusculum* related sequences in winter samples of cattle manure. O'Reilly et al. **[36]** also reported the dominance of *Methanocorpusculum* in lab-scale digesters operated at 15°C using qPCR. These results imply that the genus *Methanocorpusculum* has high tolerance for cold conditions. This is one of the first reports mentioning about the presence of genus *Methanocorpusculum* in a food waste digester.

4 Conclusions: Using stable isotope probing and DGGE approaches we investigated methanogenic community in anaerobic digesters treating food waste of institute mess. All 3 archaeal DGGE band sequences were affiliated to hydrogenotrophic methanogens, particularly the order Methanomicrobiales. The bands were closely related to the species *Methanocorpusculum labreanum*, this is one of the first reports of this species being identified in food waste digesters. Strains of *Methanocorpusculum* were considered to be psychrotolerant, suggesting it can be a potential candidate in consortia for anaerobic digesters running at very low temperatures during winter season facilitating in continuous running of the digester without process failure. Further understanding of the entire microbial community structure and dynamics in anaerobic digester will help to optimize efficient functioning of anaerobic digestion process for renewable energy.

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