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Research Article

Design of Substrate Specific Consortium for Improved Biogas Production

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Abstract

The current study was aimed at design of substrate specific synthetic consortium for improved biogas production. Pure cultures specific to their roles were isolated from biogas digesters and used to design the synthetic consortium. The designed consortium was compared with natural consortium for biogas production efficiency in batch mode fermentation. The biogas yields obtained with designed consortium and natural consortium were 66% and 60% of theoretical maximum, respectively. The complete conversion of substrate to biogas was observed in 7 days with designed consortium in comparison to 35 days in case of natural consortium. The performance of designed consortium with respect to enhanced rate of digestion was attributed to presence of substrate specific microbes and their initial abundance. The higher abundance of bacteria and archaebacteria in the designed consortium as estimated using qPCR quantification was evident of the fact that initial abundance of specific microbes increases the rate of biogas production. This report is first of its kind in constructing substrate specific synthetic consortium and demonstrating its effectiveness in improvement of anaerobic digestion process. Overall, the presented work shall pave ways to implement substrate specific consortium design strategy for development of economically sustainable biogas production from diverse substrates.

Keywords: Anaerobic Digestion; Consortium Design; Isolation and Screening; Batch Mode Fermentation; QPCR Quantification

Abbreviations

AD: Anaerobic Digestion; HPLC: High Performance Liquid Chromatography; GC: Gas Chromatography; QPCR: Quantitative Polymerase Chain Reaction.

Introduction

AD (Anaerobic digestion) has gained widespread importance as it serves dual purpose of waste management as well as energy generation in the form of methane for versatile applications [1-4]. It has potential to treat diverse waste like food waste, agricultural waste, municipal solid and liquid wastes, and industrial effluents thereby reducing the environmental hazard due to these wastes. Regardless of its advantages and versatility, commercial sustenance of AD is uncertain owing to low process efficiency in terms of incomplete substrate utilization and partial recovery of energy linked to the process. The overall process efficiency is determined interdependently by both physicochemical and microbiological factors that include substrate composition, operational conditions, reactor design, inoculum source and syntrophic interactions amongst the microbial communities [5]. However, any alteration in the substrate and physicochemical parameters leads to drastic changes in the microbial community structure and abundance, thereby contributing to major process variations. This infers that the process performance is primarily coupled to its microbial community structure, abundance and syntrophic interactions amongst the community members [6].

AD process for long has been worked at the level of reactor design and maintenance of physicochemical parameters for optimum performance [7]. The microbial consortium, however, was not much explored and manipulations at the level of microbial communities involved in different steps of AD for optimum process efficiency were not considered till the development of advanced molecular biology tools. AD being a multistep process is governed by different microbial communities. Microbes from each of these stages if targeted for manipulation of natural consortia in terms of their substrate specificity, abundance and diversity, can possibly address the bottlenecks associated with slow digestion rates, incomplete substrate utilization and overall process efficiency. With recent advancements in molecular biology tools several studies have been performed on natural consortia in order to characterize the microbial communities with respect to diversity and abundance of different species and their influence on process efficacy. Jo and co-workers studied the effect of different inoculums on biomethane potential of different wastes. The results indicated that presence and absence of specific organisms and their initial abundance is the crucial factor for high substrate conversion efficiencies and respective biogas production [8]. The insights from several

such studies have been used to improve the natural consortia by bioaugmentation of specific microbes (pure isolates) into natural consortium to increase their initial abundance [9-13]. Though bioaugmentation results in improved process performance, maintaining the functional activity and monitoring of augmented microbes still remains a challenge due to presence of multiple species in natural consortium. Besides bioaugmentation, researchers have also characterized the microbial communities present in the biogas digesters in order to understand their role in driving the overall process. Characterization of microbial consortium of a biogas digester was attempted by Wirth., *et al.* 2012 [1] to understand the community structure and abundance. The results clearly indicated that an optimum consortium can be designed from the insights gained and this would promote greater efficacy in large-scale practical systems. Several researchers have performed studies on characterization of microbial consortium of biogas digester [14-17] but none of them have actually implemented the knowledge gained for design of an optimal consortium.

The usual practice for seeding the biogas digesters is random selection of nonspecific natural consortia which are composed of different species of hydrolyzing bacteria, fermenting bacteria (acidogens), syntrophs (acetogens) and methanogens. These natural consortia often exhibit poor substrate specificity thus leading to longer adaptation times or start up period. This further results in slow digestion rates and incomplete substrate utilization. Moreover, monitoring of such undefined and multispecies culture during the process remains a challenge. A substrate specific consortium comprised of microbes with defined functional roles and optimum abundance can really be a promising approach to address the challenges associated with natural consortia. The substrate specificity and optimum abundance will help reduce the start-up period as well as enhance both substrate utilization and rate of digestion. Since designed consortium is comprised of known microbes with defined functional roles, monitoring of the process with respect to changes in microbial community at different conditions becomes more feasible. AD systems with designed substrate specific consortia would serve as prototypes to understand performance and stability of microbial communities under different operating conditions. The knowledge gained from such systems can be used to develop early diagnostic tools (microbial) to monitor commercial biogas digesters [2]. Owing to importance of microbes, their specificity and abundance in driving the AD process performance, there is need to design synthetic microbial communities to overcome the bottlenecks of natural consortia. Synthetic microbial communities are abstractions of natural consortia and are often inspired by microbial interactions present in them [18].

The present study was envisioned to design a substrate specific consortium for biogas production and compare its performance with the natural consortium. The insights gained from prior studies performed at our laboratory to understand changes in microbial community dynamics in terms of their predominance and abundance (data not discussed in current report) formed the basis for the present work. The study focusses on isolation and screening of potential microbes involved in different stages of AD process and further using the screened candidates for design of substrate specific consortium. The biogas production efficiency of the designed synthetic consortium in terms of digestion rate and biogas yields was validated in batch mode fermentation and compared with natural consortium. The overall aim of the study was to explore the possibility of developing a substrate specific synthetic consortium and demonstrate its role in improved process efficiency.

Materials and Methods

Medium composition and preparation

The Wolfe medium was used throughout the work for carrying out isolations, screening as well as final reactor studies with natural and designed consortium. The Wolfe medium was prepared as per methods described by Wolfe., *et al* [19].

Wolfe medium composition (g/L)

NH_4Cl, 1; NaCl, 0.6; $NaHCO_3$, 5; KH_2PO_4, 0.3; K_2HPO_4, 0.3; $MgCl_2$.6H/2O, 0.16; $CaCl_2.2H_2O$, 0.009; resazurin 0.1% solution, 1 ml/L; cysteine-HCl 0.2M solution; 15 ml /L, Na2S.9H_2O 0.2M solution 8 ml /L and vitamin and trace element stock 10 ml /L each.

Vitamin stock composition (mg/L)

10 mg/L of each of p-aminobenzoic acid, nicotinic acid, calcium pantothenate, pyridoxine, riboflavin, thiamine, and 5 mg/L each of biotin, folic acid, a-lipoic acid, and B12.

Trace minerals stock composition (g/L)

Tri-sodium nitrilotriacetic acid, 1.5; $Fe(NH_4)_2(SO_4)_2$, 0.8; $NaSeO_3$, 0.2; $CoCl_2.6H_2O$, 0.1; $MnSO_4.H_2O$, 0.1; $Na_2MoO_4.2H_2O$, 0.1; $NaWO_4.2H_2O$, 0.1; $ZnSO_4.7H_2O$, 0.l; $NiCl_2.6H_2O$, 0.1; H_3BO_3, 0.01; $CuSO_4.5H_2O$, 0.01.

All mentioned chemicals were of AR grade and were procured from Hi-Media Laboratories Pvt. Ltd., Mumbai.

Media preparation

To prepare anoxic medium, the salt components along with resazurin were dissolved in a serum bottle containing distilled water and boiled till the solution turned pink. Further, nitrogen gas was purged through the boiled solution till it cooled down and bottles were sealed with resealable rubber septum and crimped with aluminum caps. These bottles were then autoclaved at 121˚C for 20 min. The stock solutions of vitamins, trace element, reducing agents and carbon sources were prepared separately and cold sterilized

using a 0.2µm syringe filters. The cold sterilized components were then added to the autoclaved salt solution just before inoculation. Resazurin is the commonly used anaerobicity indicator which on addition of reducing agents turns colourless indicating complete absence of oxygen with redox potential below -100mV.

Analytical methods

HPLC (High Performance Liquid Chromatography) analysis was performed using HPLC system (Agilent 1100 series, Agilent Technologies, New Castle DE) equipped with an Aminex 87H column (Bio Rad, Richmond, CA) at 65˚C and RI detector (G1362A) at 35˚C was used. Solution of 5mM H_2SO_4 was used as a mobile phase at the flow rate of 0.6 ml/min. Chem Station/ EZ chrome software was used for data processing. Concentrations of the compounds were assessed using HPLC grade standards procured from Sigma Aldrich, USA. For sample preparation, samples were filtered through 0.2µm pore sized nylon filters (Axiva) to obtain a cell free filtrate.

Qualitative analysis of methane was carried out using GC (Gas Chromatography) (Agilent series # CN10911028, Agilent Technologies, New Castle DE) equipped with Flame Ionization Detector (FID) and HPPLOTQ column (Bio-Rad). High purity nitrogen gas was used as a carrier gas with flow rate of 1ml/min. The detector was set at 250^0C and oven at 50^0C. The injector temperature was 50^0C and split ratio was 50:1. The gas sample was injected using an airtight syringe (1002RN, 2.5 ml, Hamilton). 70% methane was used as standard for quantification and Chem Station/ EZ chrome software was used for data processing.

Sample collection and enrichment

The aim was to isolate substrate specific microbes and hence digesters running on different substartes were targeted for sample collection. Samples were collected from digesters of Ashoka Biogreen Pvt. Ltd., Nashik, Khandoba Distilleries Pvt. Ltd., Baramati and Kirloskar Integrated Technologies Limited, Pune, running on cow dung, spent wash and kitchen waste, respectively. All three samples were mixed together to obtain a composite consortium with diverse microbes and further used for isolation of acidogens, acetogens and methanogens.

Enrichment was performed in 125 ml serum bottles using Wolfe medium with different carbon sources (glucose and xylose for isolation of acidogens, mixture of H_2-CO_2, butyrate and propionate for isolation of acetogens, sodium acetate, mixture of H_2-CO_2 and methanol for isolation of methanogens). The medium was inoculated with 10% of composite sample using syringe and the bottles were then incubated in a shaker incubator (Innova) at 37^0C and 100 rpm for 15days. The substrate consumption and respective product formation was monitored in all enriched bottles using HPLC (High Performance Liquid Chromatography) and GC (Gas Chromatography) methods described in analytical methods section. Further samples from enriched bottles were used for isolation of pure cultures.

Isolation and screening of acidogens, acetogens and methanogens

Isolation

The isolation experiments were performed in Don Whitley A85 Anaerobic Workstation manufactured by Don Whitley Scientific Limited, UK. The chamber was maintained at 37^0C with 50% relative humidity. The anoxic conditions inside the chamber were maintained by anaerobic gas mix of N_2:H_2:CO_2 in the ratio of 80:10:10. To maintain sterility standard aseptic procedures were followed.

Acidogens

Serial dilutions (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) of enriched samples were spread on solid Wolfe agar medium plates containing 0.5% of C5 and C6 sugars as carbon source. The plates were incubated in Don Whitley anaerobic chamber for 5 days.

Acetogens and methanogens

The Hungate Roll Tube technique [20] was used for isolations of acetogens and methanogens. To obtain the segregated colonies of acetogens and methanogens, enriched samples were serial diluted up to 10^{-6} and poured in different hungate tubes containing solid Wolfe agar medium and different carbon sources (H_2:CO_2 80:20, 1bar pressure; butyrate, 0.5% v/v; propionate, 0.5% v/v for acetogens and sodium acetate, 0.5% w/v; H_2:CO_2 80:20, 1bar pressure; methanol, 0.5% v/v for methanogens). Bromoethane sulfonic acid (BrES) was added to the medium while isolating acidogens and acetogens to inhibit growth of methanogens. The inoculated Hungate tubes were then incubated in anaerobic workstation for 30 days.

Screening

Acidogens

The isolated acidogens obtained after repetitive sub culturing were screened on the basis of zone of clearance on Wolfe medium agar plate containing $CaCO_3$ [9]. These screened pure colonies were then studied for acid production in 125 ml serum bottles containing Wolfe medium broth with 0.3% sugars as carbon source. The organic acid production profile was studied using HPLC analysis.

Acetogens

Isolated pure colonies of acetogens were screened on the basis of type of substrate utilized and acetate produced. The isolated colonies were inoculated in 125 ml serum bottles containing Wolfe medium broth with different carbon sources as mentioned above. The substrate utilization and acetate production in the inoculated serum bottles were measured by HPLC analysis.

Methanogens

The isolated colonies of methanogens were grown in 125 ml serum bottles containing Wolfe medium broth with different car-

bon sources as mentioned above and the isolates were further screened on the basis of different characteristics like fluorescence, methane produced, substrate utilization and presence of *mcrA* gene (specific to methanogens). Inverted phase contrast microscopy was performed to check fluorescence in methanogens using Olympus IX 51 microscope equipped with filters of specific wavelengths {Excitation Filter Wavelengths: 330 - 380 nm (BP, 355 CWL), Dichromatic Mirror Cut-on Wavelength: 400 nm (LP) and Barrier Filter Wavelengths: 420 nm cut-on (LP)}. The methane produced was withdrawn from head space of incubated serum bottles and analyzed using GC. The isolated methanogens were further screened for type of substrate utilized using acetate or H_2:CO_2 as carbon source. The colony PCR was performed to screen the isolated methanogens for presence of *mcrA* gene using standard PCR protocol [21] with degenerate primers specific for *mcr A* (Fwd_*mcr A*: 3'-GCACATAGGGTGGTGTMGGWTTCRC-5'; Rev_*mcr A*: 3'-CAGCCGAGTCYTGMARRTCRTABCCG-5').

Identification of selected microbes

Morphological identification

The screened acidogens, acetogens and methanogens were examined for morphological characteristics under phase contrast microscope (BX 51, Olympus). The acidogens and acetogens were gram stained, whereas the methanogens were as such observed without staining.

Molecular identification

DNA extraction

Total genomic DNAs were extracted by NucleoSpin® Soil DNA extraction kit manufactured by Machery-Nagel (MN) using 5 ml sample. Quality of g DNA was checked on 0.8% agarose gel (loaded 5 µl) for single intact band. The gel was run at 110 V for 30 mins. 1 µl of each sample was loaded in Nanodrop 8000 for determining A260/280 ratio.

Sequencing

Identification of screened acidogens and methanogens was carried out based on 16S *rRNA* gene and *mcrA* sequencing, respectively. The purified DNA samples of acidogens after QC analysis were outsourced to Xcelris Labs Ltd., Ahmedabad, India for 16S *rRNA* sequencing. However, in case of methanogens the amplified *mcrA* gene product was sent for *mcrA* sequencing. The phylogenetic analysis of sequences was carried out at laboratory using blastn algorithm from NCBI (National Centre for Biological Information).

Batch mode reactor studies of selected acidogen and methanogen

The selected acidogen and methanogen based on their performance were further studied individually in batch mode reactors (1.2 L heat pad jacketed glass reactor, Eppendorf) equipped with temperature and pH probe. The reactors were sterilized by autoclaving at 121˚C for 20 min and after autoclaving sterilized nitrogen gas was purged in headspace of reactor to ensure anaerobic conditions. Sterility of nitrogen gas was maintained using a 0.2µm pore sized PTFE filters. pH was maintained at 7.0 by automated addition of alkali (2N NaOH). Fermentation runs were carried out under controlled anaerobic conditions at 37^{0}C and agitation of 50 rpm with specific substrate (0.1% sugars for acidogens and 0.1% sodium acetate for methanogen). The working volume for the fermentation runs was maintained at 1L. The reactors were monitored for substrate utilization, intermediate acids production and methane produced using HPLC and GC methods as described above. The volume of biogas produced was measured by in-house designed gas traps based on water displacement method.

Biogas production from natural and designed consortium

Inocula

Natural consortium

Culture from full scale mesophilic digestion plant running on cow dung at Ashoka Biogreen Pvt. Ltd., Nashik was used as a natural consortium for batch reactor study. Preceding to batch set up, the culture was sieved and preincubated at 37^{0}C for 7 days to ensure gas production using residual substrate. The substrate to inoculum ratio for batch fermentation was kept 1:1 and the inoculum was seeded on dry cell weight basis.

Synthetic consortium

The substrate specific consortium was designed using isolated pure cultures of *Clostridium beijerinckii* (acidogen), isolate A1 (unidentified acetogen), *Methanosarcina mazei* (acetoclastic methanogen), *Methanoculleus* species (hydrogenotrophic methanogen) and acquired cultures of propionate and butyrate degrading syntrophic bacteria (unidentified) from Agharkar Research Institute, Pune. The pure cultures were separately grown in 125 ml serum bottles and were then mixed on dry cell weight basis in the ratio of 1:1:4:4 of acidogen, acetogen, syntrophs and methanogen, respectively. The substrate to inoculum ratio for batch fermentation was kept 1:1.

Batch fermentation

Both consortia (natural and designed) were evaluated for biogas production in batch mode reactor operations. Two separate fermenters (1.2 L heat pad jacketed glass reactor, Eppendorf) equipped with temperature and pH probe for continuous monitoring of temperature and pH were used for fermentation studies. The reactors were sterilized by autoclaving at 121˚C for 20 min and after autoclaving sterilized nitrogen gas was purged in headspace of reactor to ensure anaerobic conditions. Sterility of nitrogen gas was maintained using a 0.2µm pore sized PTFE filters. pH was maintained at 7.0 by automated addition of alkali (2N NaOH). Fermentation runs were carried out under controlled anaerobic conditions at 370C and agitation of 50 rpm with glucose (1g/L) as substrate.

The working volume for the fermentation runs was maintained at 1L. The reactors were monitored till the gas production ceased. In-house designed gas traps were connected to the fermenters to measure the volume of biogas produced. The substrate utilization, intermediate acids production and methane analysis were estimated at different time intervals using HPLC and GC methods as described above. To determine the abundance total bacteria (acidogens and acetogens) and total methanogens in the natural and designed consortium, samples were withdrawn at initial and final day of fermenter runs and analysed with quantitative polymerase chain reaction (qPCR) technique. The abundance of total bacteria and total methanogens was projected based on abundance of 16S *rRNA* (conserved gene) and mcrA (marker gene) respectively. The quantification (qPCR) of 16S *rRNA* and *mcrA* gene was performed as per prior optimised protocols for 16S *rRNA* and *mcrA* gene quantification at our laboratory (optimisation study not mentioned here). The gene copies of samples were extrapolated by comparing with the standard curves.

Results and Discussion

Isolation and screening of acidogens, acetogens and methanogens

AD is a complex process, which is divided into four phases: hydrolysis, acidogenesis, acetogenesis and methanogenesis [22]. Each of these phases involve different microbial communities which partly syntrophiycally interrelated and play different functions in the environment [23]. The microorganisms involved in hydrolysis step are the hydrolytic bacteria which hydrolyze the polymeric materials to monomers such as glucose and amino acids through extracellular hydrolytic enzymes (cellulase, xylanase, amylase, protease, lipase) they excrete. The second step involves acidogenic bacteria that convert the monomeric components into volatile fatty acids (lactate, succinate, formate, acetate, propionate, butyrate, isovalerate, etc.), alcohols (methanol, ethanol), aldehydes, carbon dioxide, hydrogen, ammonia and hydrogen sulfide. These volatile fatty acids are acted upon by acetogenic bacteria for conversion of the acid phase products to acetate, carbon dioxide and hydrogen. The terminal phase of AD process is carried out by methanogens which use the intermediate products of the preceding stages and convert them into methane, carbon dioxide, and water. The efficiency and stability of the process is primarily dependent upon the concerted and syntrophic activity of these microorganisms belonging to different phases [24,25]. Isolation of pure cultures from anaerobic digesters has built up a milestone for improvement of AD process as pure cultures allow researchers to have a deeper understanding about their morphological, physiological, biochemical, and genetic characteristics and their application in manipulation of natural consortium [26]. With recent advancements in molecular biology and microbiology for understanding complex microbial communities and role of specific microbes in AD process, several researchers have investigated bioaugmentation of pure cultures into natural consortia for improved AD performance [9-13]. However, targeted isolation of substrate specific microbes involved at different stages of AD process and their use for design of a substrate specific composite consortium has not yet been attempted. The current work was focused on design of substrate specific consortium and examine its potential to improve AD process efficiency. The idea was to select pure isolates from each step of AD process based on the substrate specificity to make a composite consortium and validate its performance using a simple and easy to monitor substrate like glucose. With this prospect isolation and screening of potential microbes involved in each step of biogas production were carried.

A total of 36 colonies of acidogens were observed on Wolfe agar medium plate and out of these 20 were selected (HLN 1 - 20) on the basis of zone of clearance observed on $CaCO_3$ containing Wolfe agar plates. $CaCO_3$ dissolves upon release of extracellular acids by acidogens resulting in a clear zone around the colony on agar plate and hence is used as an indicator for screening of acid producers [27,28]. The selected acidogens were further screened for organic acids production and the average yield of mixture of organic acids obtained was 0.6g/g sugars with complete utilization in 24 h (Figure 1). The yield and profile of acids produced in all the 20 isolates was found to be almost similar, however, HLN 5 showed slightly higher acetate production of 0.19 g/g of sugars as compared to other cultures which showed average acetate yield of 0.15 g/g sugars. Based on this observation, HLN 5 was considered as an acidogen candidate for consortium design. Microscopic examination of HLN 5 showed gram positive rod-shaped cells with oval, sub- terminal spores (Figure 2). The 16S rRNA sequence of HLN 5 when analyzed by BLAST, showed high degree similarity with many *Clostridium beijerinckii* sequences, including many partial sequences and uncultured clostridium species clone. Only those sequences which showed 98% identity were compared and phylogenetic tree was drawn as shown in figure 3. The HLN 5 isolate was identified as *Clostridium beijerinckii* based on 98% sequence similarity with *Clostridium beijerinckii* (Accession number LN908213.1). The performance of HLN 5, identified as *Clostridium beijerinckii* was evaluated for acid production in batch mode reactor studies which resulted in total acid yield of 0.68g/g sugars. The profile of acids produced is as shown in figure 4.

In case of acetogens, total of 8 morphologically different colonies were selected from isolation batches and these colonies were further studied for acetate production. Out of these 8 colonies only one (A1) was found to assimilate H_2-CO_2 mixture and produce acetate. This isolate was further profiled for different substrate (glucose, propionate and butyrate) uptake and it did not show preference for propionate and butyrate. On the other hand, it showed complete utilization of glucose and gave mixed organic acid yield of 0.64 g/g glucose. The profile of different acids produced with

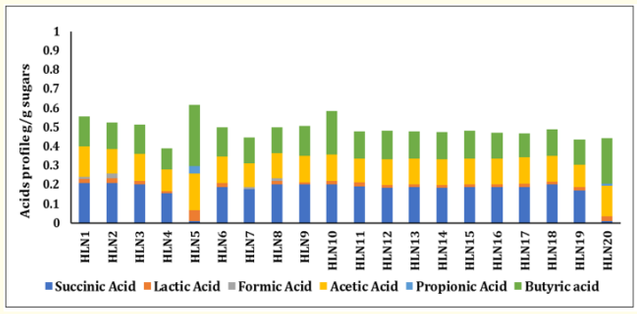

Figure 1: Screening of isolated acidogens for acid production.

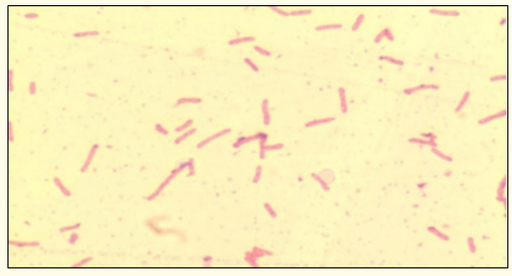

Figure 2: Gram staining of HLN 5.

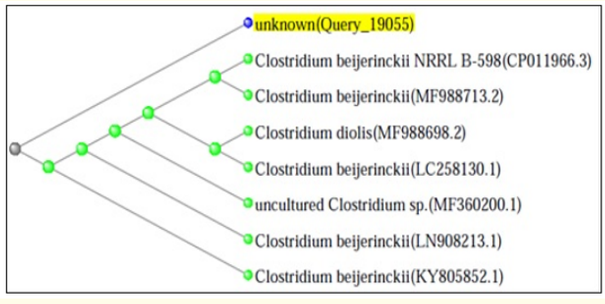

Figure 3: Phylogenetic tree of isolate HLN 5 based on 16S rRNA gene sequence.

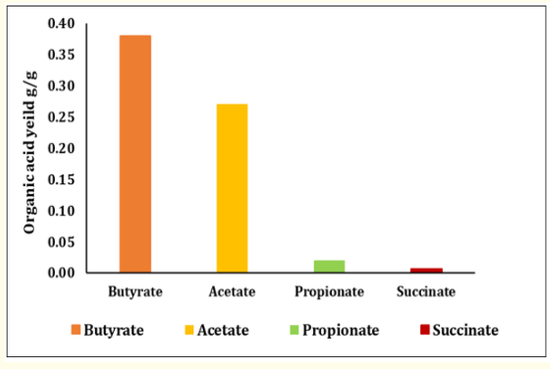

Figure 4: Total mixed acid yield/g sugars of reactor batch study of Clostridium beijerinckii.

their respective yields are as shown in figure 5. The isolate A1 was chosen as an acetogen candidate for consortium design.

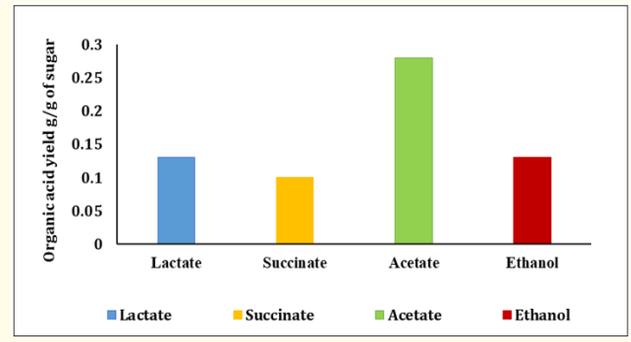

Figure 5: Total mixed acid yield g/g glucose of isolate A1.

A total of 40 colonies of methanogens were isolated and 20 (M1 - M20) were randomly selected based on phenotypic characteristics. These 20 isolates were screened on the basis of fluorescence, methane produced, substrate utilized and presence of *mcrA* gene (specific to methanogens). These characteristics are exclusively present in methanogens and widely used for screening the methanogens [29,30]. All the isolates were found positive for the aforementioned characteristics. Out of 20 screened isolates, 11 were found to be acetoclastic methanogens and 9 hydrogenotrophic methanogens (Table 1). As all the isolates were found to have positive characteristics, one isolate each from acetoclastic and hydrogenotrophic methanogens was randomly selected for further identification.

Microscopic observations of isolate M9 (acetoclastic) showed irregular spheroid shaped aggregates with large cyst like structure (Figure 6 and 7). On the other hand, microscopic observations of M13 (hydrogenotrophic) showed irregular short rods as shown in figure 8 and 9. The selected methanogens (M9 and M13) were identified using *mcrA* gene sequencing. The *mcrA* gene is exclusively present in methanogens and has been widely used as a marker for identification of methanogenic population. Luton and co-workers demonstrated use of *mcrA* gene as an alternative to 16S *rRNA* based sequences for identification of methanogens [31]. The *mcrA* sequence of M9 when analyzed by BLAST showed high degree similarity to *Methanosarcina mazei* sequences, including many partial sequences and uncultured *Methanosarcinales species* clones. Only sequences which showed more than 80% identity were compared and phylogenetic tree was drawn as shown in figure 10. The sequence of M9 *mcrA* gene showed 86% similarity with *Methanosarcina mazei* (Accession number EF452663.1) and this isolate was identified as *Methanosarcina mazei*. The sequence of M13 showed high degree similarity to sequences of *Methanoculleus* species, including many partial sequences of Methanoculleus chikugoensis and *Methanoculleus marisnigri* as shown in figure 11. M13 *mcrA*

Isolate Name	Methane Production	Presence Of *McrA* Gene	Colony Fluorescence	Type Of Substrate Utilized
M1	+ve	+ve	+ve	Acetate
M2	+ve	+ve	+ve	Acetate
M3	+ve	+ve	+ve	Acetate
M4	+ve	+ve	+ve	Acetate
M5	+ve	+ve	+ve	Acetate
M6	+ve	+ve	+ve	Acetate
M7	+ve	+ve	+ve	Acetate
M8	+ve	+ve	+ve	Acetate
M9	+ve	+ve	+ve	Acetate
M10	+ve	+ve	+ve	Acetate
M11	+ve	+ve	+ve	Acetate
M12	+ve	+ve	+ve	H_2, Formate
M13	+ve	+ve	+ve	H_2, Formate
M14	+ve	+ve	+ve	H_2, Formate
M15	+ve	+ve	+ve	H_2, Formate
M16	+ve	+ve	+ve	H_2, Formate
M17	+ve	+ve	+ve	H_2, Formate
M18	+ve	+ve	+ve	H_2, Formate
M19	+ve	+ve	+ve	H_2, Formate
M20	+ve	+ve	+ve	H_2, Formate

Table 1: Screening of isolated methanogens based on different characteristics.

gene sequence showed 82% similarity with *Methanoculleus chikugoensis* (Accession number EFAB288270.1) and was identified as *Methanoculleus chikugoensis*.

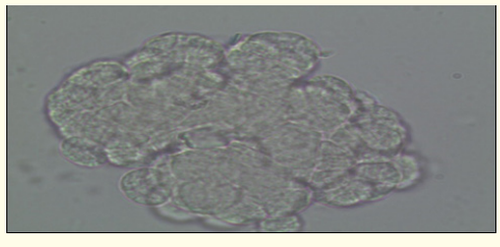

Figure 6: Microscopic image of M9.

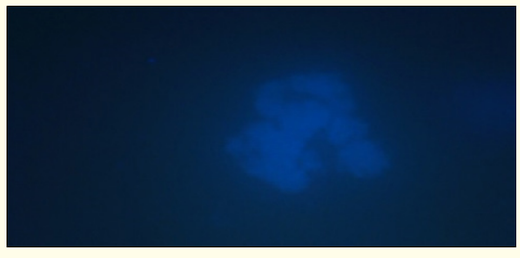

Figure 7: Fluorescence microscopic image of M9.

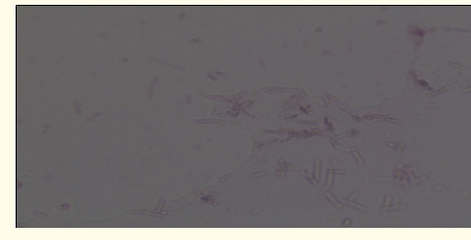

Figure 8: Microscopic image of M13.

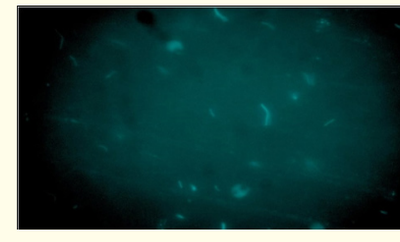

Figure 9: Fluorescence microscopic image of M13.

Amongst both the identified methanogens, Methanosarcina mazei is known to utilize multiple substrates such as acetate, methanol and H_2:CO_2 [32]. Due to its versatile behavior and capability to utilize multiple substrates it was used as a potential methano-

gen candidate for consortium design and was evaluated for biogas production in batch mode fermentation. The batch fermentation with sodium acetate as substrate resulted in methane yield of 43% of theoretical maximum as shown in figure 12. The theoretical amount of methane produced per gram of substrate was calculated using the Buswell formula [33,34]. It took 10days for complete utilization of acetate.

Based on the results of isolation and screening experiments HLN 5, A1, M9 and M13 were found to be promising candidates with respect to their functional roles in different stages of AD process for biogas production. However, isolation of syntrophic acetogens was not successful, despite of taking multiple efforts. It might be because of requirement of stringent nutritional and environmental conditions which were difficult to mimic at lab scale. Keeping in mind the acid profile of acidogens, syntrophs for utilization of propionate and butyrate were required in order to make a substrate specific composite consortium. The syntrophic acetogens procured from ARI, Pune were used for the consortium design along with our lab isolates.

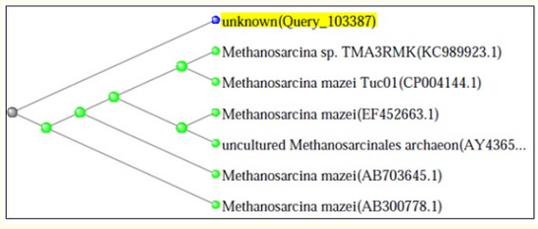

Figure 10: Phylogenetic tree of isolate M9 based on mcrA gene sequence.

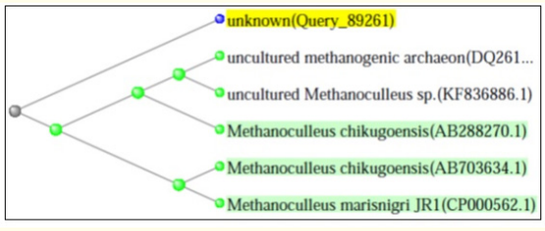

Figure 11: Phylogenetic tree of isolate M13 based on mcrA gene sequence.

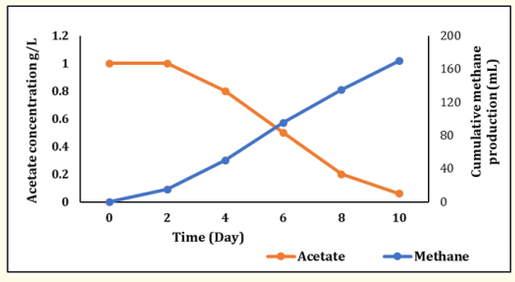

Figure 12: Acetate utilization and methane production by Methanosarcina mazei.

Substrate specific synthetic consortium design

The performance of AD process is closely coupled to its microbial communities and their syntrophic interactions. The biogas digesters are usually seeded with randomly selected natural consortia which are composed of multiple species with often unknown functions and poor substrate specificities. As a result, the consortium requires longer adaptation periods thus resulting in slow digestion rates and incomplete substrate utilization. This consequently affects the viability and economy of the overall process. To address the challenges associated with natural consortia, the novel approach of designing a substrate specific consortium was attempted. Insights and understanding gained from prior studies performed on microbial community dynamics with different substrate and different inocula formed the basis for synthetic consortium design. The results from microbial community dynamics study very well indicated that the initial abundance of substrate specific microbes significantly affects the rate of digestion (data not presented). Besides this, the ratio in which the bacterial (acidogens and syntrophic acetogens) and archaebacterial communities are present also plays a substantial role in AD process efficacy. Keeping in mind these observations, the initial abundance of methanogens and syntrophs in the designed consortium was kept twofold the initial abundance of acidogen.

The biogas production performance of this designed substrate specific consortium was compared to that of natural consortium in batch mode fermentation using glucose as substrate. The biogas yields obtained in batch fermentation were found to be 66% and 60% of theoretical maximum with the designed and natural consortium, respectively (Figure 13 and Figure 14). Significant differences were also observed in substrate and intermediate products (acetate and butyrate) utilization patterns. The glucose fed was utilized within 36 h in case of designed consortium, whereas the natural consortium took almost 72 h for the same. The utilization of intermediate products i.e. volatile fatty acids (lactate, propionate, butyrate and acetate) formed during the digestion process was also monitored. The HPLC results clearly indicated that their uptake was initiated much earlier with designed consortium (on 4th day) in comparison to that of natural consortium (15th day) as shown in figure 15. Though the difference in biogas yield obtained was not very significant, but there was considerable improvement in the rate of digestion with designed consortium as it took 7 days in comparison to 35 days with natural consortium for complete conversion of substrate to biogas (Figure 16). The performance of designed consortium with respect to substrate utilization and enhanced rate of digestion was attributed to the presence of substrate specific microbes and their initial abundance.

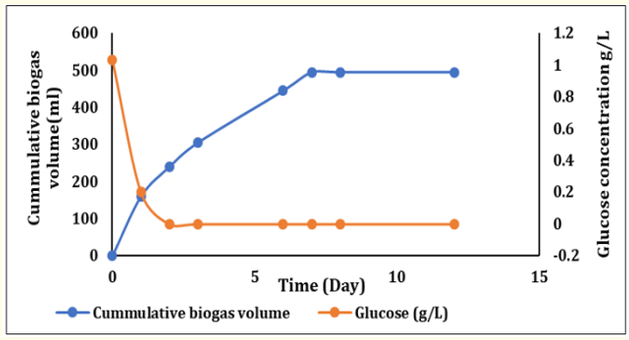

Figure 13: Substrate utilization and biogas production by designed consortium.

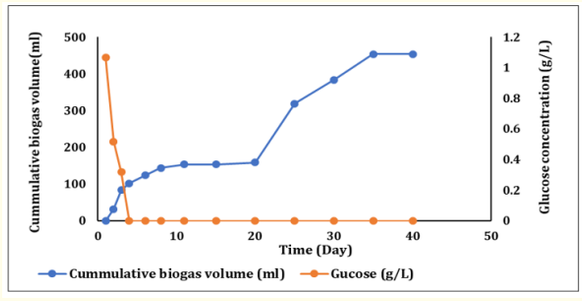

Figure 14: Substrate utilization and biogas production by natural consortium.

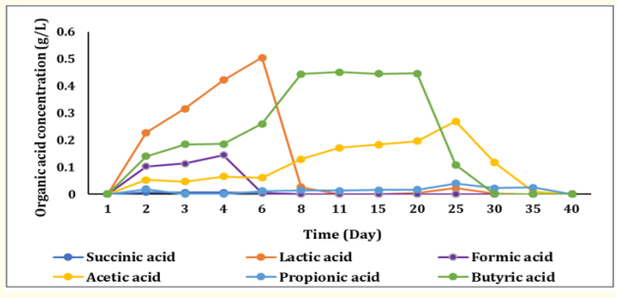

Figure 15: Organic acids profile of batch mode reactor with natural consortium.

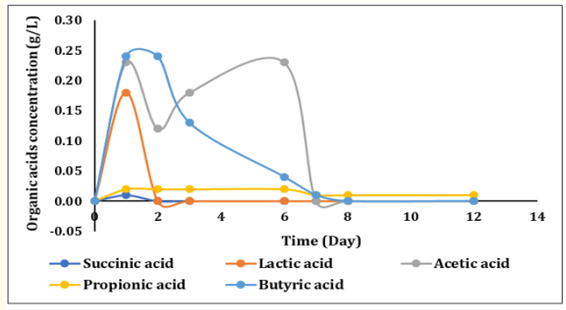

Figure 16: Organic acids profile of batch mode reactor with designed consortium.

The syntrophs and methanogens have slower growth rates as compared to those of acidogens and take longer time to adapt as well as attain optimum abundance [35]. As a result, it becomes difficult to balance them with the fast growing acidogens for stable process performance in case of natural consortium [13]. The two fold concentration of slow growing syntrophs and methanogens in our designed consortium helped in reducing the adaptation period. It is clearly evident from the results witnessed that balancing these populations in an optimum ratio improves the rate of digestion and overall efficiency of biogas production. The difference in abundance of bacterial and archaebacterial populations in both consortia (designed and natural) was determined by qPCR quantification and the standard curves as illustrated in figures 17 and 18 were used to extrapolate the gene copy numbers. The gene copy numbers for bacteria and archaebacteria were found to be 2.8×10^7/ml and 1.01×10^5/ml, respectively, in natural consortium. On the other hand, designed consortium showed 6.7×10^7/ml and 1.02×10^6/ml of the bacterial and archaebacterial gene copy numbers, respectively. The results of qPCR quantification are evident of the fact that initial abundance of specific microbes improves the rate of digestion.

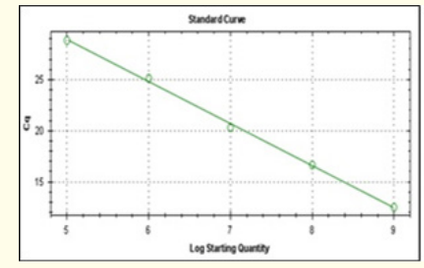

Figure 17: Standard curve for bacterial count.

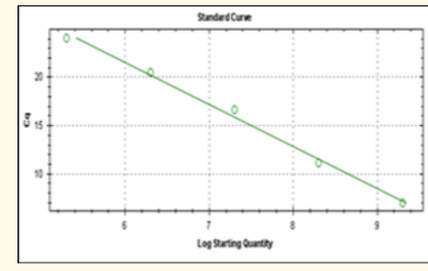

Figure 18: Standard curve for Archaeal count.

Manipulations of natural consortia for improved AD performance have gained wide interest in recent years [36]. Bioaugmentation of standard reference strains into natural consortia has been the most common strategy to alter the natural consortia for improved process efficiency. Routine bioaugmentation for improved rate of digestion rate of cellulolytic waste was performed and it was observed that bioaugmentation of cellulolytic bacteria in the acido-

genesis phase was effective in improvement of the digestion process. However, they concluded that one time bioaugmentation is not as effective as routine bioaugmentation for sustained increase in digestion efficiency [9]. Carter and co-workers studied the effect of bioaugmentation of hydrolytic anaerobic bacteria on hydrolysis of lignocellulosic component present in brewery spent grain. Though the bioaugmentation resulted in hydrolysis of lignocellulosic components, persistence of the bioaugmented strain was not found to be stable. Moreover, bioaugmentation affected the archaeal community significantly [10]. Disappearance of bioaugmented strain during the AD process was also reported by Kovacs., et al. 2012, who studied the effect of bioaugmentation to maintain the hydrogen partial pressure [13]. It is very clear from several such reports that though bioaugmentation helps to improve the biogas production, it is not a long term and sustainable solution. It is difficult to maintain the functional activity and persistence of bioaugmented strains amongst the multiple diverse species present in natural consortium. Also, sometimes the bioaugmented strain adversely affects the other microbial communities present in the digester. A substrate specific synthetic consortium designed using potential species from every step of AD process would serve a better and more promising approach instead of bioaugmentation.

The present study is unique in strategizing and implementing use of isolated pure cultures for design of substrate specific synthetic consortium for improved biogas production efficiency. The results clearly validate that the designed consortium with specific microbes is capable of addressing the issues related to longer start up period and slow digestion rates in spite of having minimal species diversity.

Conclusion

The present study demonstrated the novel approach of substrate specific synthetic consortium design using pure cultures isolates. The enhanced performance of designed consortium with respect to rate of digestion was mainly attributed to the initial abundance of specific microbes present. The understandings and observations from current work will create possibilities for design of synthetic consortia specific to actual and diverse substrates used in AD process.

Acknowledgements

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Conflict of Interest

The authors declare that they have no competing interests.

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