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Studies on Enrichment of Hydrogenotrophic Methanogens for Biomethane Production

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ABSTRACT: The presence of CO_2 in biogas affects its applicability and hence methanation of CO_2 in biogas by exogenous addition of H_2 is one of the promising technologies for biogas upgradation. Accordingly this study was aimed to convert CO_2 directly to methane. Biomethanation of H_2 and CO_2 was studied in lab-scale reactors using hydrogenotrophic methanogens. They are obligate anaerobes and hence the samples were collected from anoxic environments *viz.*, paddy field, anaerobic digestor, rumen fluid, and rumen digesta of cattle. The isolates were screened for their efficiency to produce methane and developed into a hydrogenotrophic consortium. This consortium was used for biomethanation studies. A lab-scale anaerobe reactor with slaughterhouse wastewater was seeded with the hydrogenotrophic consortium and H_2:CO_2 (4:1, *v/v*) was given as substrate. A temporal microbial shift in substrate utilization from dissolved organic nutrients to H_2 and CO_2 is influenced by the addition of H_2 and CO_2 which enhances hydrogenotrophic methanogenic activity. This resulted in a 29.8% increase in biomethane production in test reactors compared to control. The developed hydrogenotrophic consortium can be utilized for scrubbing CO_2 directly from the atmosphere thereby reducing greenhouse gas emissions.

Keywords: Biogas, Biomethane, Biomethanation, Hydrogenotrophic methanogens, and methane upgradation.

INTRODUCTION

Humans generate over 105 tonnes of organic wastes globally each year but only 2% of them are properly treated and disposed of. There are copious methods available for treating wastes but anaerobic digestion (AD) proves to be the effective and economic way of converting wastes into valuable byproducts (Primmer, 2021). AD is a series of biological process that is gaining popularity in the era of a growing need for sustainable energy sources and concerns about global warming caused by greenhouse gas emissions. It is estimated that 50 million micro anaerobic digesters are currently in use around the world for the production of biogas, which is then employed for cooking, heating, and lighting (Jain, 2019). AD breaks down organic substances into renewable products like biogas and biofuels which can be utilized as a substitute for non-renewable energy sources (Li *et al.,* 2015; Patel *et al.*, 2017). It is an intricate process that relies on the synergistic effort of various bacterial communities performing distinct metabolic reactions. The vital steps in anaerobic digestion include (i) *hydrolysis* of complex organic biopolymers to simple monomers (ii) *acidogenesis* - fermentation of products of hydrolysis to short chain fatty acids, ammonia, organic acids, and alcohols by a fermentative group of bacteria (iii) *acetogenesis* - anaerobic oxidation of volatile fatty acids and alcohols to acetate, CO_2 and hydrogen by acetogens (iv) *methanogenesis* - methane production by methanogens using methanol, methylamines, dimethyl sulfide, methanethiol, acetate, CO_2 and hydrogen (Nishio & Nakashimada 2013). Anaerobic digestion is a sensitive process that requires an equal rate of degradation of all intermediates and each microbial community in the digestion is interlinked, and its population dynamics and metabolic activity have a significant impact on the stability and rate of decomposition (Achinas *et al.*, 2020; Gerardi, 2003; Schink, 1997).

The major product of AD is – biogas primarily composed of methane (CH_4) in the range of 55 – 60 % and carbon dioxide (CO_2) in the range of 35 – 45 % (Balat & Balat 2009). The presence of CO_2 in biogas reduces its calorific value which limits its wide application. Various techniques and technologies have been developed and thoroughly scrutinized for biogas upgradation. The addition of hydrogen to the anaerobic reactor which boosts the hydrogenotrophic methanogenic community to convert CO_2 to CH_4 is known as chemoautotrophic upgradation (Muñoz *et al.,*

2015). The principle of chemoautotrophic biogas upgradation using hydrogenotrophic methanogens is based on the Sabateir reaction (1) (Leonzio, 2016),

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad \Delta G^\circ = -130.7 kJ/mol \quad (1)$$

Over years several studies have been presented to biologically convert CO_2 to CH_4. The process of converting biogas to biomethane can be carried out in one of three ways: either directly injecting H_2 into the anaerobic fermenter ("*in-situ*"), or via an external bioreactor containing an enhanced culture of hydrogenotrophic methanogens ("*ex-situ*") or hybrid method combining both *ex-situ* and *in-situ* methods (Angelidaki *et al.,* 2018; Aryal *et al.*, 2018). *In-situ* biogas upgrading enables more efficient use of AD while minimizing the need for additional infrastructure for post-treatment. In this method, H_2 is directly fed into an anaerobic digester to boost the activity of endogenous hydrogenotrophic methanogens, promoting CO_2 reduction to CH_4 (Fu *et al.*, 2021). The exogenous addition of H_2 was demonstrated by Mulat *et al.* (2017) using a thermophilic pressurized reactor fed with maize leaf as substrate, which resulted in 89% CH_4 content in biogas with a decrease of CO_2 content from 60 to 11%. Addition of H_2 directly into the reactor showed inhibition of anaerobic digestion process due to consumption of bicarbonate (Luo & Angelidaki 2012) and this can be alleviated by co-digestion with acidic waste (Luo & Angelidaki 2013). Another important parameter to consider during *in situ* upgradation is the level of H_2 in the reactor which critically affects other microflora in the anaerobic digestion. High levels of H_2 (> 10 Pa) in the reactor leads to inhibition of anaerobic digestion and accumulation of volatile fatty acids (Liu & Whitman 2008). However long term exposure of H_2 increases the population of hydrogenotrophic community which in turn increases the H_2 utilization rate and reverts the inhibition (Treu *et al.,* 2018). To date, only minimal studies have been conducted to employ hydrogenotrophic consortium for biogas upgradation and biomethane production. Accordingly, this study aims to develop a consortium of hydrogenotrophic methanogens solely for *in situ* biomethane production.

MATERIALS AND METHODS

Source of inoculum for isolation of methanogens. For this study, hydrogenotrophic methanogens were isolated from different environmental niches viz., soil samples from Tamil Nadu Agricultural University's paddy field and Pichavaram mangrove forest, Chidambaram, Tamil Nadu; anaerobic digestor slurry from biogas plant located at Tamil Nadu Agricultural University; rumen fluid and rumen digesta from the rumen of freshly slaughtered cattle from Government Slaughterhouse located at Coimbatore. Before the sample collection, all the sample containers were sterilized and flushed with O_2-free N_2 gas. The samples were brought to the lab and processed immediately or stored at -20º C.

Enrichment of methanogens. The enrichment of methanogens was performed in Wheat on serum vials containing 50 mL of broth. For this purpose, 2.0 mL aliquots of anaerobic soil and digestor samples were inoculated in serum bottles containing Basal Carbonate Yeast Trypticase (BCYT; pH 7.0) broth (Touzel & Albagnac 1983) and 2.0 mL aliquots of rumen fluid and rumen digesta were inoculated in BY broth (Joblin, 2005) with H_2:CO_2 (80:20, *v*/v) as a substrate. The bottles were sealed tightly with butyl rubber stoppers, crimped firmly with aluminum caps, and sealed with parafilm wax. The enrichment was carried out in three sets with two negative controls – one without inoculum with substrate and the other with inoculum without substrate. The enriched bottles were incubated at 37ºC.

Transfer of enrichment and methane analysis. Methane production in the enriched serum vials was periodically assessed during the enrichment phase using gas chromatography. The gas from the headspace was analyzed for methane using a 'Gas Chromatograph' (NUCON, 5765) equipped with Flame Ionization Detector (FID) and a glass column packed with 80/100 Poropak Q. The flow rate of carrier gas (N_2) was 25 mL min^{-1}; injector temperature was 100ºC; detector temperature was 200ºC and the column temperature was set to 75ºC. A Standard methane canister (99.9% purity from Covai Air Products, Tamil Nadu) was used to standardize methane estimation.

Isolation and purification of methanogens. The enriched serum vials with a methane content of more than 30% were subjected to two-stage successive transfer to fresh BY and BCYT broth to eliminate non-methanogenic bacterial contaminants using (i) serial dilution - a high decimal dilution showing the presence of methanogens was inoculated into the fresh broth and incubated at 37ºC and (ii) treatment with antibiotics. The roll technique proposed by Hungate (1969) was adapted for the purification of methanogens from enriched samples. 1 mL of enriched sample and 10 mL of the medium were added to a pre-sterilized roll tube under anoxic conditions and sealed with butyl rubber cork. The roll tube was rolled immediately on ice cold sponge so that the media forms a uniform layer on the roll tube. The roll tubes were then incubated in an upright position in the anaerobic jar (3.5 L) containing Anaerogas Pack (HiMedia®). After the incubation period, individual colonies of different colony morphology were picked using a sterile lumbar needle and inoculated in sterile anaerobic broth under anoxic conditions. The isolates were again serially diluted and re-cultured in a solid medium in roll tubes to ensure purity. Reculturing was repeated until a single colony type was obtained. The absence of non-methanogens in the culture was confirmed with two separate growth media devoid of methanogenic substrates namely, (i) PYG medium and (ii) glucose-rich nutrient broth (Jain *et al.*, 2021).

Screening and characterization of isolates. The isolates obtained from purification were characterized and screened for methane production. The obtained isolates were characterized based on colony morphology, gram reaction, catalase test, and methane production. Morphological characters of the isolates were studied under a bright field microscope and gram staining was done using modified Hucker or Burke's method (Smith & Hussey 2005). The methane

production was analyzed at three days intervals using GC-FID. Among fifteen isolates, seven isolates showed maximum growth and methane production in H-minimal broth and they were further developed as a consortium for biomethanation studies.

Consortium development for biomethanation. The seven hydrogenotrophic isolates were studied together for their ability to grow as a consortium and for methane production. The consortium was investigated for (i) growth at temperature (20-50ºC), pH (5-10), and requirement of sodium chloride for methanogenesis (0-5M) using H_2:CO_2 (4:1, *v*/v) as substrate. The substrate utilization spectrum of the consortium was studied by culturing in a minimal medium containing 20mM of formate, acetate and propanol, and butanol. The utilization of these substrates by the consortium was determined by observing growth and methane levels in comparison with blank control. The utilization of formate and acetate was quantified using 'High-Pressure Liquid Chromatography (HPLC)'. About 1 mL of sample was taken from representative vials centrifuged, acidified with 5N H_2SO_4, and filtered through a 0.2 µm syringe filter before being run on Shimadzu High-pressure liquid chromatography fitted with a C_{18} column. The compounds were eluted with 0.01M NaH_2PO_4 buffer containing 10% methanol as eluent and detected at 210nm using a UV detector (Bush *et al.,* 1979).

Lab scale biomethanation study. Wastewater was collected from slaughterhouse located in Coimbatore, Tamil Nadu, India. Batch tests were conducted using 125 mL Wheaton serum vials as anaerobic reactors with a working volume of 50 mL. The reactors were filled with filtered slaughterhouse wastewater and seeded using 10% of the developed hydrogenotrophic consortium. The gas in the headspace was removed using a vacuum pump and filled with H_2:CO_2 (4:1, *v*/v). The gas was bubbled through the samples using a PVDF membrane filter to remove the contaminants if any and sealed tightly with butyl rubber stoppers, crimped firmly with aluminum caps, and sealed with parafilm wax. A serum bottle with distilled water H_2:CO_2 in head space was used as blank and a serum bottle with wastewater and inoculum with N_2 in the headspace was used as control. The reactors were incubated at 35ºC in water bath and pH was maintained at 7.2 as per results obtained by above studies. The gas concentration in the headspace was analyzed using GC-FID.

RESULTS AND DISCUSSION

Transfer of enrichment and methane analysis. The selection of enrichment culture and the transfer was carried out based on 'the theory of selection and elimination. The enrichment cultures showing methane productivity greater than 30% were further enriched and the rest were eliminated. The methane productivity of fifteen enriched cultures in the presence of H_2:CO_2 (4:1, *v*/v) were 31, 38, 36, 42, 33, 39, 54, 62, 34, 51, 53, 49, 47, 62, and 39% respectively. These fifteen enrichment cultures were subjected to further enrichment. After five enrichments, the cultures were microscopically observed for the presence of methanogens and non-methanogens.

Isolation and purification. The selected enrichment (6^{th}) was serially diluted (10^{-5}) and transferred to BY and BCYT broth which still showed the presence of non-methanogens. Hence the enrichment cultures were grown in respective broths with ampicillin, chloramphenicol cycloheximide, kanamycin, ketoconazole, gentamicin, and streptomycin at the final concentration of 25µL/mL to eliminate non-methanogens (Kumar *et al.,* 2012). Several antibiotics, either in single or in combination has been proven to be effective in eliminating non-methanogens during purification (Whitman *et al.*, 2006). After two successive transfers, the load non-methanogens were significantly reduced which was confirmed using the absence of growth in PYG medium and glucose-rich nutrient broth. This strategy to confirm the absence of non-methanogens was carried out by Jain *et al.* (2021).

Table 1: Characteristics of the anaerobic isolates obtained in the study from various environments.

Sr. No.	Isolates	Habitat	Microscopic observation	Colony Morphology	Gram reaction	Catalase activity	Methane production (%)
1.	ADS 1	Anaerobic digestor	Cocci	White pin headed colony	Cells stain Gram positive	Negative	42
2.	ADS 3	Anaerobic digestor	Cocci	Creamy colony with entire margin	Cells stain Gram positive	Negative	62
3.	PFW 2	Paddy Field	Elongated rods	Creamy colony with entire margin	Cells stain Gram positive	Negative	56
4.	PFW 3	Paddy Field	Curved rods	Translucent colony	Cells stain Gram negative	Negative	48
5.	RFC 1	Rumen fluid	Slightly curved rods	Light brown colonies with entire margin	Cells stain Gram negative	Negative	59
6.	RFC 2	Rumen fluid	Cocci	Brown colonies with regular margin	Cells stain Gram positive	Negative	65
7.	RDC 3	Rumen Digesta	Curved Rods	Colorless colonies with entire margin	Cells stain Gram negative	Negative	48

Screening and characterization of the isolates. The morphological characters of the isolates are presented in the table. Screening of efficient hydrogenotrophic methanogens for biomethanation study was assessed based on the methane production by the isolates in H-minimal broth with H_2:CO_2 (4:1, *v*/v) as substrate. The isolates that were able to grow and generate methane in H-minimal broth confirms its hydrgenotrophic nature (Morii *et al.,* 1983). All the isolates were able to utilize H_2:CO_2 (4:1, *v*/v) and generate methane, this confirms that the isolates are hydorgenotrphic in nature and can be employed for biomethanation. The methane produced by individual isolates is presented in the table. The isolate from rumen fluid (RFC 2) showed maximum methane production (65%) followed by an anaerobic digestor isolate ADS 3 (62%). The least methane production was observed by ADS 1 (42%), an anaerobic digestor isolate.

Consortium development for biomethanation. The seven hydrogenotrophic isolates obtained in this study were cultured together as a consortium for biomethanation studies. They were grown in H-minimal broth with H_2:CO_2 (4:1, *v*/v) as substrate. The consortium exhibited growth and the maximum methane production (85%) was observed at the end of the 20th day with 98% CO_2 conversion (Fig. 1). This proves their ability to utilize H_2:CO_2 for biomethanation and confirms the hydrogenotrophic nature. In the study conducted by Burkhardt and Busch (2013) using immobilized hydrogenotrophs in a trickle bed reactor investigating the conversion of H_2:CO_2 at 37ºC and ambient pressure resulted in a hydrogen conversion up to gH_2 = 99% and the maximum methane concentration of C_{CH4} = 97.9 vol% which supports this study. A similar study was carried by Alitalo *et al.* (2015) to investigate the conversion of H_2 and CO_2 using a fixed bed reactor. By recirculation of gas mixtures, maximal methane productivity of 6.35 $l/l_{reactor}d$ was obtained, while the hydrogen conversion rate was 100%.

The optimum conditions for biomethanation were assessed by growing the consortium at different ranges of i) pH (2-9), ii) temperature (20-50ºC), and iii) NaCl (0-3 M). i) pH: The maximum methane production of the consortium was at pH 7.2 and no methane production was observed below pH 6 and above pH 8.5 as shown in Fig. 1. Chen *et al.* (2021) studied the effects of pH on hydrogenotrophic methanation, which showed that stable and higher methane production was observed at pH 7.0-7.5 and 8.5-9.0 which supports this study. ii) Temperature: The optimal temperature for methane production was observed at 32-35ºC with 35ºC and 28º being the maximum and minimum temperature requirements for biomethanation (Fig. 1). Palù *et al.* (2022) reported that higher and durable methane production by hydrogenotrophic methanogens in CSTR co-digesting manure and cheese whey under *in situ* biomethanation was 37ºC. (iii) NaCl: The consortium was able to tolerate 0.1 – 0.8 M NaCl concentrations above which the growth and methane production abruptly stopped as shown in Fig. 1. Zhang *et al.* (2017) investigated the effects of salinity in the anaerobic digestion of marine macroalgae by acclimating inoculums at varying salinity. Methanogenesis was considerably delayed at salinity over 55 g L^{-1}. Hydrogenotrophic methanogens such as *Methanobacterium* were able to tolerate salinity up to 85 g L^{-1}, whereas acetoclastic methanogens, *Methanosaeta* and *Methanosarcina* were severely inhibited at salinity is greater than 65 g L^{-1}.

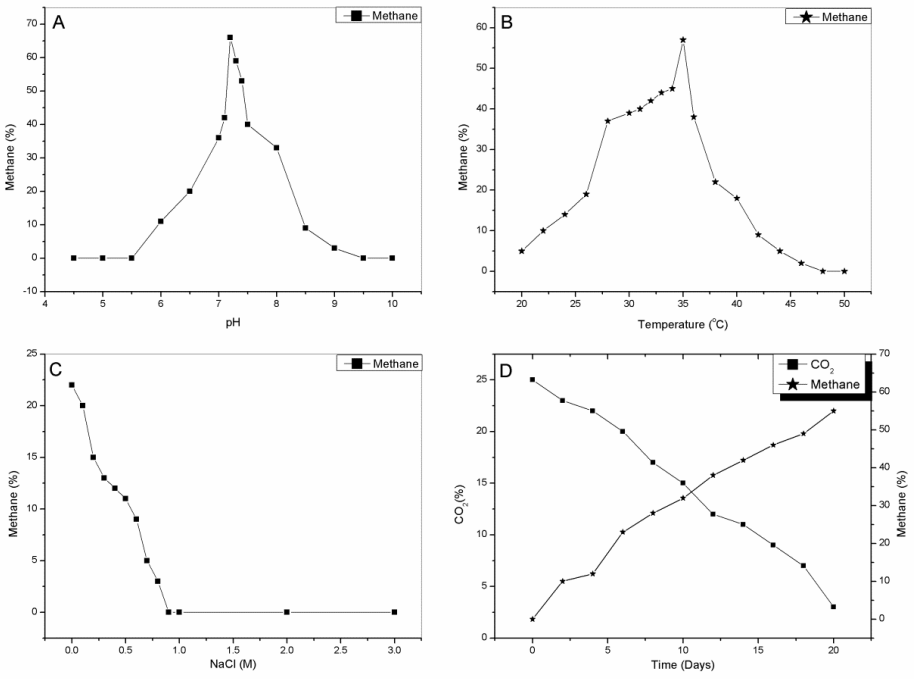

Fig. 1. (A) Effect of pH on methane production (B) Effect of temperature on methane production (C) Effect of salt concentration on methane production (D) CO_2 utilization and methane production by hydrogenotrophic consortium in minimal medium.

The substrate utilization spectrum of the consortium was studied by culturing them in a minimal medium containing 20 mM of formate, acetate, propanol, and butanol. The consortium was able to utilize all four substrates but the growth and methane production was slower compared to the medium containing H_2:CO_2 as substrate (Fig. 2). The primary electron source for hydrogenotrophic methanogens to carry out methanogenesis is H_2, but they can also utilize formate, ethanol, carbon monoxide, or some secondary alcohols as electron donors for methane production (Kurth *et al.*, 2020). For the conversion of for mate, many hydrogenotrophic methanogens utilize the enzyme formate dehydrogenase, which catalyzes the formation of reduced coenzyme F_{420} which then supplies electrons for methane production (Jones & Stadtman 1980). Hydrogenotrophic methanogens use acetate as the carbon source for their cell assimilation but do not compete with acetoclastic methanogens in the natural environment (Jetten *et al.*, 1990). Many hydrogenotrophic methanogens contain alcohol dehydrogenases which convert secondary alcohols to their respective ketones and serves as an electron donor for methanation (Kurth *et al.*, 2020). The results obtained from this experiment shows that the hydrogenotrophic consortium has a wide substrate range.

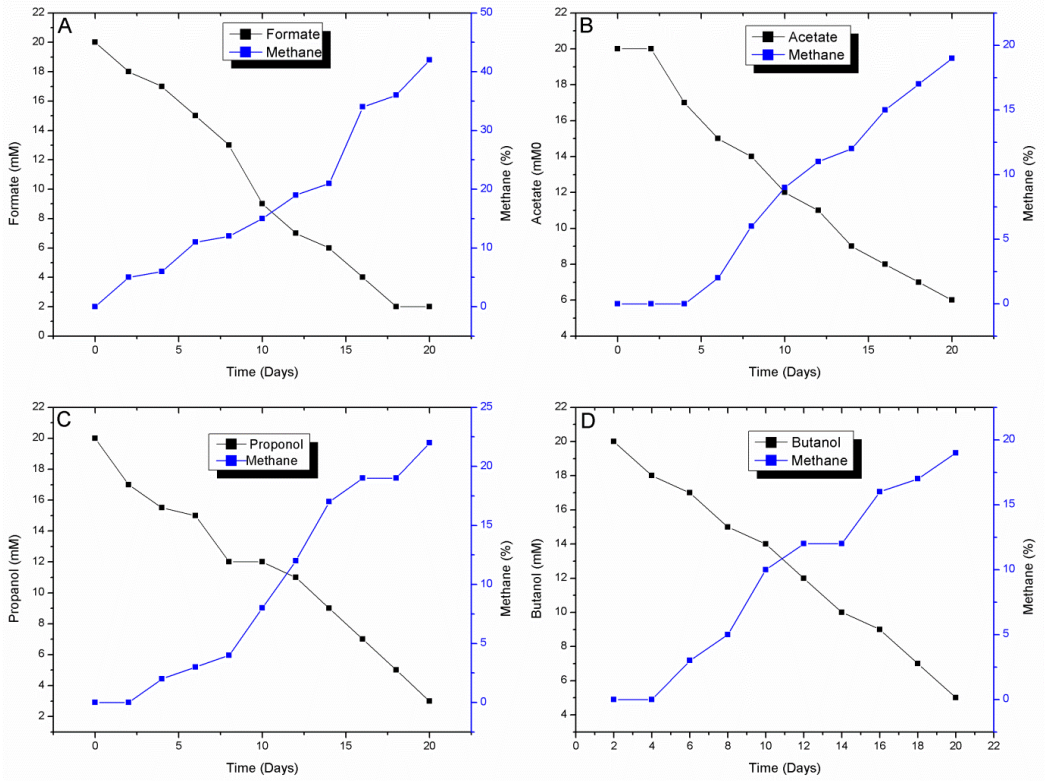

Fig. 2. Substrate utilization spectrum of developed hydrogenotrophic consortium (A) Formate utilization and methane production (B) Acetate utilization and methane production (C) Propanol utilization and methane production (D) Butanol utilization and methane production by hydrogenotrophic methanogens in minimal medium.

Biomethanation study. The developed hydrogenotrophic consortium was cultured in anaerobic reactors with H_2:CO_2 (4:1, *v*/v) as substrate. The addition of H_2 and CO_2 to the reactor causes a temporal shift in microbial substrate utilization of H_2 and CO_2 from dissolved organic nutrients (Kougias *et al.,* 2017). This boosts the hydrogenotrophic methanogenic community to utilize H_2 and CO_2. The volumetric change in CO_2 and CH_4 in the headspace of the reactors in test and control reactors was compared for H_2:CO_2 utilization. The methane production was higher in the reactors supplemented with H_2:CO_2 compared to the control. The final biogas from the test reactor was composed of 82.6% CH_4 and 17.4% CO_2, while the biogas from the control reactor was composed of 52.8% CH_4 and 47.2% CO_2 respectively, resulting in a 29.8% biomethane increase. A study conducted by Alitalo *et al*. (2015); Okoro-Shekwaga *et al.* (2019) with a similar experimental study utilizing hydrogenotrophs also showed increased methane productivity. This study proves that methane content in biogas can be alleviated using hydrogenotrophic methanogenic community.

CONCLUSION

In the present study, we demonstrated that biogas with a methane content of 82.6% can be produced with aid of a developed hydrogenotrophic consortium with support biomethanation in a simple reactor without continuous stirring or recirculation. The results obtained are promising and this mechanism can be up-scaled to produce biomethane of high purity. Further study should be carried out to optimize the reactor variables

while up-scaling. The optimizing process becomes more complex during up-scaling since the addition of H_2 increases the partial pressure which affects other microbial processes in methanation *viz*., fermentation and acetogenesis. As a result, oxidation of volatile fatty acids in the anaerobic digestion ceases which leads to process failure. Hence future studies should be carried out to maintain the H_2 partial pressure as low as possible while employing *in situ* H_2 addition for biomethane production.

FUTURE SCOPE

The hydrogenotrophic consortium developed in this study can be utilized as a biological alternative to the Sabateir reaction to produce biomethane. The biomethanation process requires H_2, which can be harnessed from a renewable source and carbon dioxide can be provided by capturing CO_2 from fossil fuel utilizing industries thereby reducing the emission of CO_2 into the atmosphere. This opens a path for eco-friendly production of biomethane which has wide applications.

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Conflict of Interest. None.

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