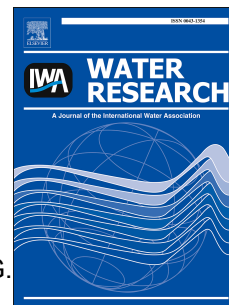


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Simultaneous biogas upgrading and biochemicals production using anaerobic bacterial mixed cultures

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19 Abstract

20 A novel biological process to upgrade biogas was developed and optimised during the current
21 study. In this process, CO₂ in the biogas and externally provided H₂ were fermented under
22 mesophilic conditions to volatile fatty acids (VFAs), which are building blocks of higher-value
23 biofuels. Meanwhile, the biogas was upgraded to biomethane (CH₄ >95%), which can be used as a
24 vehicle fuel or injected into the natural gas grid. To establish an efficient fermentative microbial
25 platform, a thermal (at two different temperatures of 70°C and 90°C) and a chemical pretreatment
26 method using 2-bromoethanesulfonate were investigated initially to inhibit methanogenesis and
27 enrich the acetogenic bacterial inoculum. Subsequently, the effect of different H₂:CO₂ ratios on the
28 efficiency of biogas upgrading and production of VFAs were further explored. The composition of
29 the microbial community under different treatment methods and gas ratios has also been unravelled
30 using 16S rRNA analysis. The chemical treatment of the inoculum had successfully blocked the
31 activity of methanogens and enhanced the VFAs production, especially acetate. The chemical
32 treatment led to a significantly better acetate production (291 mg HAc/L) compared to the thermal
33 treatment. Based upon 16S rRNA gene sequencing, it was found that H₂-utilizing methanogens
34 were the dominant species in the thermally treated inoculum, while a significantly lower abundance
35 of methanogens was observed in the chemically treated inoculum. The highest biogas content (96%
36 (v/v)) and acetate production were achieved for 2H₂:1CO₂ ratio (v/v), with *Acetoanaerobium*
37 *noterae*, as the dominant homoacetogenic hydrogen scavenger. Results from the present study can
38 pave the way towards more development with respect to microorganisms and conditions for high
39 efficient VFAs production and biogas upgrading.

40 **Keywords:** Biogas upgrading; Acetogens; Biomethane; CO₂ mitigation; Gas fermentation; 16S
41 rRNA genes

1 Introduction

Anaerobic digestion of organic wastes has gained considerable attention as a promising technology for bioenergy production and environmental pollution control. Biogas produced from the anaerobic digestion process consists mainly of CH₄ (40-75%, v/v) and CO₂ (25-60%, v/v) with several trace compounds depending on the organic source (Rotunno et al., 2017). The low CH₄ content of biogas limits its application to heating and electricity production (Leonzio, 2016). In order to increase the application possibilities, biogas can be upgraded to more than 95% (v/v) CH₄ (biomethane) by removing CO₂ and increasing the methane content (Micale, 2015). Biogas upgrading increases the heating value and relative density of biogas. The upgraded biogas can be used as vehicle fuel or injected to the natural gas grid.

To date, biogas upgrading is performed in approximately 280 biogas plants around the globe, using several different technologies (Kougias et al., 2017). The most common established technologies are based on: adsorption (pressure swing adsorption), absorption (pressurized water wash, physical or chemical absorption), membranes (high pressure, low pressure), and cryogenic separation (Sun et al., 2015). The costs of these technologies are relatively high as they require either high pressure, or addition of chemicals, or membranes (Luo and Angelidaki, 2013; Yan et al., 2016). Moreover, CH₄ loss during separation of CO₂ from biogas by these technologies is another significant limitation (Luo and Angelidaki, 2012). Therefore, there is room for the development of new technologies that will improve the overall biogas upgrade efficiency and, at the same time, will reduce the investment, operation and maintenance costs.

Biological biogas upgrading is a promising technology with high efficiency and low investment and operational costs (Kao et al., 2012; Xu et al., 2014; Bassani et al., 2016). Different biological systems have been used for biogas upgrading, such as microalgae to capture CO₂ from biogas (Sun et al., 2015), or hydrogenotrophic methanogenic archaea to convert *in-situ* or *ex-situ* CO₂ and H₂ to

66 CH₄ (Rittmann, 2015). However, the selection of appropriate microorganisms for efficient and
67 economical biogas upgrading has still not been explored.

68 Recently, acetogenic bacteria have attracted great interest in the bioenergy technology due to
69 their ability to efficiently fix C1 compounds (including CO₂) using H₂ as an electron donor via the
70 acetyl-CoA pathway and produce valuable chemicals (e.g. organic acids) that can be used in the
71 chemical industry (Fernández-naveira et al., 2017). These bacteria with 23 different genera and over
72 100 species identified up to date, are strictly anaerobic and produce billions of tons of acetate
73 globally each year as their major fermentation product (Groher and Weuster-Botz, 2016).
74 Acetogens can inhabit very diverse ecosystems ranging from different soils to termite hindgut, with
75 wide variations in temperature, pH, and salinity (Vandecasteele, 2016). Several acetogenic pure
76 cultures especially within the *Clostridium* spp. have been used for biochemical production
77 (Fernández-naveira et al., 2017). Recently, mixed culture fermentation has gained more attention
78 due to several advantages compared to the pure culture; such as process robustness during
79 continuous processes and no need for sterile conditions (Redl et al., 2017). Taking all together, CO₂
80 from biogas could be converted to building blocks of higher value biofuels (e.g., VFAs) instead of
81 CH₄, by using mixed culture acetogenic consortia. However, the ability of the acetogenic bacteria to
82 simultaneously upgrade biogas and produce biochemicals has never been assessed.

83 The necessary H₂ for the CO₂ fixation by the acetogens can potentially be produced from large
84 point sources, including coal gasification, petroleum refinery, petrochemical plants, and soda
85 manufacture (Luo and Angelidaki, 2012). However, to make the whole process economically and
86 environmentally sustainable, hydrogen should be produced from renewable resources. One of the
87 most promising renewable sources of H₂ is the electrolysis of water using surplus electricity
88 generated by wind or solar energy (Kougiass et al., 2017). However, H₂ is not yet a competitive
89 transportation fuel due to high transportation cost, development of country scale infrastructure and

low volumetric energy content (Ramachandriya et al., 2013). On the contrary, utilization of H_2 produced from renewable resources for biogas upgrading has several advantages, such as usage of the existing infrastructure of biogas plants and using the energy of hydrogen for chemicals production (Luo et al., 2012).

The present study aimed to develop a novel bioprocess that combines renewable electricity utilization and CO_2 fixation by acetogenic bacteria, to efficiently upgrade biogas and produce biochemicals. Therefore, a series of experiments were performed to identify the best pre-treatment method that inactivates the methanogenic archaea in the inoculum and enriches the acetogenic bacteria. Subsequently, the optimum H_2 : CO_2 gas ratio for maximum biogas upgrading efficiency and production of chemicals was assessed. In addition, 16S rRNA analysis of the microbial community was conducted to better understand the process from the microbiological point of view.

2 Materials and methods

2.1 Inoculum

Mesophilic digested sewage sludge (wastewater treatment plant, Lundtofte, Denmark) was used as inoculum in this study. Inoculum was sieved through a 2 mm net after collection to remove large particles and then was kept three days in the incubator at $37 \pm 1^\circ C$ before starting the batch experiments. The basic characteristics of the inoculum are presented in Table 1.

2.2 Experimental setup

The mixed culture was maintained under strictly anaerobic conditions in a basic anaerobic medium (BA-medium) (Angelidaki et al., 1990), with the exception that all the carbon source (e.g. $NaHCO_3$) were omitted to minimize the interference of carbon compounds during the fermentation process. All the experiments were performed in 540 mL serum bottles with 100 mL working volume. The bottles were sealed with butyl rubber stoppers and aluminium crimps and then

autoclaved at 121°C and 15 psi for 20 min. The filter sterilized vitamin solution using micro syringe filter (0.2 µm) was added to the bottles after cooling at room temperature. Before inoculation, sodium sulphide was added to the serum bottles (final concentration of 0.025% w/v) to ensure anaerobic conditions. The mixed culture was used as a starting inoculum at the rate of 10% of the working volume. The initial pH of the medium was adjusted at 6.0 ± 0.1 with 2 N KOH and 2 N HCl solutions. All bottles were incubated horizontally (to maximize gas-liquid transfer) at $37 \pm 1^\circ\text{C}$ with a constant agitation of 150 rpm in an orbital shaker (IKA® KS 4000i control).

2.2.1 Pre-treatment methods for inactivation of methanogens

The potential methanogenesis inactivation methods were a crucial step of the current study. Therefore, batch experiments were performed to elucidate the efficiency of different methanogens inactivation methods (thermal and chemical treatments) and promotion of acetate production under mesophilic conditions. For the heating pre-treatment, two different temperatures (70°C and 90°C) were tested. In both temperatures, the inoculum was heat shocked for 30 min in an aluminium coated pan, followed by cooling to room temperature (Yun et al., 2015; Zhou et al., 2013). For the chemical treatment, 50 mM of 2-bromoethanesulfonate (BES, $\geq 95\%$; Sigma-Aldrich) was used to inhibit the methanogens (Lins et al., 2015). All, the inoculated bottles were filled with a synthetic gas (H_2 : CO_2 : CH_4 2:1:1.5 v/v) to a final pressure of 1.5 bar. The characteristics of the treated inoculum are shown in Table 1.

2.2.2 Optimization experiment: effect of the gas compositions

The inoculum with the most efficient pre-treatment methods (in terms of chemical production and biogas upgrading) from the previous experiment was used in a new batch experiment under four different gas compositions (Table 2). The synthetic gas used in this experiment was a mixture of biogas (40% CO_2 :60% CH_4) and pure (100%) H_2 . The difference between the four gas ratios (GR_{1-4x}) was based on different ratios between H_2 and CO_2 (Table 2). For controls, the mixed cultures

were kept under 100% N₂ in the headspace. Triplicates were used for each ratio and analysis of variance was performed.

Table 1 and Table 2 is here

2.3 Microbial communities analysis

Genomic DNA was extracted from sludge samples with RNA PowerSoil® DNA Elution Accessory Kit (MO BIO Laboratories, Carlsbad, CA). Samples were filtered through a 100 µm nylon cell strainer filter to remove the animal fibers residues. The samples were centrifuged at 10000 g for 10 min at 4°C and the supernatant was discarded recovering ~2 g of pellet. Quality and quantity of the DNA extracted were determined using NanoDrop (ThermoFisher Scientific, Waltham, MA) and Qbit fluorimeter (Life Technologies, Carlsbad, CA). Samples were sent to the Ramaciotti Centre for Genomics (UNSW, Sydney) for sequencing amplified hypervariable V4 region of bacterial and archaeal 16S rRNA genes using universal primers 515F/806R and MiSeq platform (Illumina). Bioinformatics' analyses on raw data were conducted using CLC Workbench software (V.8.0.2) with microbial genomics module plug in (QIAGEN) as previously described by Kougias et al. (2017). Raw reads were deposited in Sequence Read Archive (SRA) database (<http://www.ncbi.nlm.nih.gov/sra>) under the project number PRJNA388850. The samples' details are given in Table 3 and Table S1 (Supplementary materials).

Table 3 is here

2.4 Analytical methods

Total solid (TS), volatile solid (VS), total chemical oxygen demand (TCOD), total Kjeldhal nitrogen (TKN) and total ammonia (NH₄⁺-N) were measured according to APHA standard methods for the examination of water and wastewater (APHA, 2005). The pH was measured immediately after the collection of samples to avoid the CO₂ removal from the liquid phase by a digital PHM210

pH meter connected to the Gel pH electrode (pHC3105–8; Radiometer analytical). VFAs were determine using a gas chromatograph (GC) (Shimadzu GC-2010, Kyoto, Japan), which was equipped with a flame ionization detector (FID) and a FFAP fused-silica capillary column (30 m × 0.53 mm I.D., film thickness 1.0 μm) using nitrogen as a carrier gas.

Headspace gas samples were collected using lock gas tight syringes inserted through the rubber stoppers of the bottle reactors. The CH₄ and CO₂ content in biogas were measured with a gas-chromatograph (Thermoscientific GC-8A, Japan) equipped with an Agilent column (30 m, 20μm OD, 0.32 mm ID) packed with Porapak Q 80/100 mesh (Supelco, Bellefonte, PA, USA) and with a TCD detector. The H₂ gas was determined with a gas-chromatograph (Shimadzu GC-11A, Tokyo-Japan) equipped with a glass column (2 m, 5 mm OD, 2.6 mm ID) packed with Porapak Q 80/100 mesh (Supelco, Bellefonte, PA, USA) and with a flame ionization detector (FID).

2.5 Calculations and statistical analyses

The obtained data were statistically analysed using analysis of variance (ANOVA) with F test. The treatment means were compared using Duncan's multiple range Test at $p < 0.05$, using the MSTAT-C statistical computer package (Michigan State University, East Lansing, MI, USA). The figures were made using the OriginLab program (OriginLab Corporation, Northampton, Massachusetts). Mass balance was based on COD balance. Finally, the utilisations of the CO₂ and H₂ (%) were calculated using Eq. (1):

$$\% \text{ CO}_2 \text{ (or H}_2\text{) utilized} = \frac{\text{moles of CO}_2 \text{ (or H}_2\text{) consumed}}{\text{moles of CO}_2 \text{ (or H}_2\text{) supplied}} \times 100 \quad (1)$$

3 Results and Discussion

3.1 The effect of different pre-treatment methods on gas fermentation

The chemical (50mM BES) treatment of the inoculum had clearly the most significant effect on the production efficiency of VFAs compared to all other treatments (Fig. 1). Specifically, BES completely blocked the activity of methanogenic archaea and enhanced the VFAs production, especially acetate, which accounted for up to 93% of the total VFAs production (Fig. 1, Table 4). The maximum concentration of acetate (291 mg/L) was achieved by the BES treated inoculum while almost no VFAs were produced by the heat-treated inocula after 4 days incubation (Fig. 1). BES has the ability to limit the Co-enzyme M (Co-M) availability to methanogens that subsequently suppresses the activity of methyl-CoM reductase (MCR) enzyme in methanogens, which in turn reduces the methanogenesis rate (Vogels et al., 1988). In accordance with the current study findings, Lins et al. (2015) has reported that 50 mM of BES inhibited completely the VFAs degradation and methanogenesis of a thermophilic sludge-derived inoculum. (Yujiao et al., 2016) also reported that BES addition in a bio-electrochemical system selectively suppressed the growth of methanogens and results in a shift of the dominant activity to acetogenesis.

Fig.1 and Table 4 are here

Interestingly, thermal treatment did not inhibit methanogenesis; this is in contrast to previous studies reported that heating treatment of sludge-derived inoculum at 70 or 90°C for 30 min efficiently inhibited methanogenesis (Table 4) (Zhou et al., 2013; Yun et al., 2015). This could be explained by the presence of heat resistant methanogens (i.e. extreme- and/or hyper-thermophiles) in the sludge-derived inoculum, as reported before (Oh et al., 2003), which were resistant to the applied temperature. The maximum CO₂ consumption (95.4%) was observed with the chemical treatment (BES), which was 45 and 43% higher than in 70 and 90°C treated sludge-derived inoculum, respectively (Table 4); while the consumption of H₂ did not differ significantly among all

treatment methods. Finally, both thermal treatment fermentations had higher final pH compared to the BES treatment fermentation due to the lack of organic acids (VFAs) production (Table 4).

3.2 Evaluation of optimal H₂/CO₂ ratio

3.2.1 Biogas upgrading

The maximum biogas upgrading efficiency of 96% (v/v) was achieved by GR_(2x) after 10 days of incubation, which was 19.7, 43.2 and 74.6% higher than GR_(1x), GR_(3x) and GR_(4x), respectively (Fig. 2). This was associated with the high uptake of both CO₂ and H₂ by acetogenic bacteria for this gas ratio with only 2.7% CO₂ and 1.6% H₂ left. Thus, based to the gas quality standard regarding to biogas utilization (Sun et al., 2015), the upgraded biogas can be used as a vehicle fuel or injected into the natural gas grid. Comparing to other biological biogas upgrading methods, the current study is very promising. Beside the significant role in CO₂ mitigation, wastes management and biogas upgrading, valuable chemicals (e.g. acetate) that can be used in the chemical and biofuel industry were also produced. Yan and Zheng (2014) found that the concentration of CH₄ in upgraded biogas by the microalgae *Chlorella* sp. could reach 93.7%, while Luo and Angelidaki (2013) and Bassani et al. (2015) reported an average biogas upgrading of 89-96.1%. Thus the upgrading method of the current study is approximately efficient compared to the previously reported methods and it has the pivotal advantage of high acetate production.

Fig.2 is here

3.2.2 Volatile fatty acids production and pH

Acetate was produced as the main metabolite under all tested gas ratios (Fig. 3). The acetate concentration increased rapidly through the first two days of inoculation for all gas ratios, owing to the rapid consumption of gaseous substrates. The highest acetate concentration (358 HAc mg/L) was achieved in GR_(4x) with 62% H₂ consumption after 4 days of incubation (Fig. 3 and 4). The acetate produced by GR_(4x) was significantly higher (22%) than that produced by GR_(2x) ($p < 0.05$),

while there is no significant ($p > 0.05$) difference was observed between GR_(4x) and GR_(3x). The lowest acetate production of 141 mg/L was observed in GR_(1x), which was 61% lower than the acetate produced in GR_(4x). The same behaviour was observed by Groher and Weuster-Botz (2016), who made a comparative study between eight different acetogenic bacteria in stirred-tank reactors with continuous supply of gaseous substrates (20% CO₂ and 80% H₂). In that study, acetate was the main product and the maximum volumetric acetate formation rate was associated with the maximum gas uptake rates. The COD balance showed that acetate accounted for approx. 89 to 93% of total VFA products for all fermentations (Table 5). Acetate is an important industrial feedstock, which used as a starting material for vinyl acetate and acetic anhydride synthesis (Jang et al., 2012). Moreover, acetate can be converted to valuable metabolites, such as butyrate and ethanol by acetogenic bacteria (Chen et al., 2016). Biological production of acetate by acetogenic bacteria could replace the traditional methods of acetate production, which depends on petrochemicals through methanol carbonylation or acetaldehyde oxidation (Daniell et al., 2012).

The microbial biomass formation according to the COD balance accounted 8.6-14.7% of the H₂. The rapid increase in the consumption of the gaseous substrate and the accumulation of acetate significantly reflected on the pH profiles (Fig. 5). In the experiments with high consumption of gases (GR_(2x)-GR_(4x)) the pH decreased rapidly between days 2 and 5, and maintained these low levels (pH<5.5) till the end of the experiment. While in GR_(1x), no significant ($p > 0.05$) changes on the pH values was observed throughout the experiment.

Table 5 and Fig.3 , Fig.4 and Fig.5 are here

3.3 Microbial Community Composition

Alpha diversity based on the number of OTUs (Supplementary Fig. S1) showed that the sequencing depth was adequate to cover the microbial species richness. In addition, it was observed that the microbial community complexity varied depending on the different treatment methods and

the different gas compositions. The lowest microbial complexity was observed in the untreated sludge inoculum (BA01), while the highest was observed with samples exposed to gases: BA04, BA06, and BA09. Phylogenetic tree (Fig. 6) and a heat map were used for representing all the microbial communities on the samples and the abundance of the identified OTUs (Fig. 7). Principal coordinate analyses (PCoA) as an assessment tool of the similarities between the microbial communities among samples are depicted in Fig. S2 (Supplementary Information). The samples were well defined into three clusters corresponding to the treatment methods. PCoA also illustrated the significant difference in similarity between the untreated sample BA01 and the samples with different treatment methods, demonstrating the effect of these treatments on the microbial community compositions.

Fig.6 and Fig.7 are here

3.3.1 The effect of pre-treatment methods of inoculum on microbial community compositions

The different treatment methods of the inoculum had a significant effect on the composition of microbial community. There was a shift in the microbial community after exposing the thermally or chemically treated inoculum to fermentation gases ($2\text{H}_2 : 1\text{CO}_2$) compared to untreated inoculum. *Methanobacterium beijingense* 1 was the most dominant methanogen with a high relative abundance of 37.9% after exposing the 90°C to gases, while *Methanobacterium formicicum* 4 was the most dominant (40.4%) after exposing the 70°C treated inoculum to fermentation gases. Therefore, the high productivity of methane during the first batch experiment (Table 5) was due to the high abundance of these H_2 -utilizing methanogens, which have the ability to use H_2/CO_2 for their growth and methane production (Ma et al., 2005; Battumur et al., 2016). Sulphate reducing bacteria (SRB) were also present in the thermal treated inoculum after fermentation of the gases. SRB can compete with methanogens or grow syntrophically with them depending on the

276 availability of sulphate (Muyzer and Stams, 2008). Under sulfate limited conditions, SRB especially
 277 *Desulfovibrio* species can produce acetate, H₂ and CO₂ in co-occurrence with a hydrogenotrophic
 278 methanogens (Bassani et al., 2015), *Desulfovibrio oxamicus* was one of the primary species that
 279 observed in 70°C treated inoculum BA05 with a relatively high abundance of 4.02%, which
 280 indicates the existence of a potential syntrophic interaction between this species and
 281 *Methanobacterium formicicum* 4. Similarly, *Desulfovibrio vulgaris* has been observed in 90°C
 282 treated inoculum BA04 with 1.87% relative abundance, which also indicates the existence of a
 283 potential syntrophic interaction between this species and *Methanobacterium beijingense* 1.
 284 *Wolinella succinogenes* 36 was the second dominant bacterium in the both thermal treated inocula
 285 with high relative abundance of 15.74% and 12%. *W. succinogenes* is one of the most thoroughly
 286 studied fumarate reducing organisms which uses hydrogen and formate as both electron donors and
 287 energy sources, while fumarate and NO₃ serve as electron acceptors (Stackebrandt et al., 1987).
 288 While, a significant lower abundance of methanogens was observed in the chemically treated
 289 inoculum that was exposed to fermentation gases compared to the other inoculum treated at 70°C
 290 and 90°C. The most dominant bacteria in the chemically treated inoculum were taxonomically
 291 assigned as *Cloacamonaceae* sp. 18 (23.08%) which is a member of the WWE1 (Waste Water of
 292 Evry 1) phylum which was frequently reported in anaerobic environments such as AD (Ozbayram
 293 et al., 2017). WWE1 appear to be responsible for the early phase of cellulose degradation and/or the
 294 fermentation of substrates resulting from cellulose hydrolysis (Limam et al., 2014). The presence of
 295 genes coding for several hydrogenases, together with five different ferredoxin oxidoreductase in
 296 WWE1 have suggested its involvement in amino acid fermentation (Pelletier et al., 2008).
 297 *Acetoanaerobium noterae* 10 was the second dominant bacteria with a relative abundance of 6.37%.
 298 *Acetoanaerobium* spp acts as homo-acetogenic hydrogen scavenger that has the ability of acetate

production from H₂ and CO₂ (Lesnik and Liu, 2014), which explained the higher acetate productivity compared to the thermally treated inocula (Table 4).

3.3.2 The effect of different gas ratios on the microbial community composition

The fermentation gas-composition had a significant effect on the microbial community through the second batch compared to the samples without gases (BA10). The most dominant bacterium in the control was related to *Thermovirgaceae* sp. 8 (10.96%) followed by *Lysinibacillus contaminans* 74 (7.95%), *Anaerolinaceae* sp. 12 (6.27%), BA021 sp.11 (6.22%), *SHA-1* sp. 9 (5.51%), *Cloacamonaceae* sp. 18 (5.10%), *SB-1* sp. 14 (4.94%), and *Bacteroidales* sp. 16 (2.72%). It should be noted that the taxonomic assignment of the most highly abundant bacteria was only possible at high taxonomic levels, even by performing a further analysis (i.e. except from the one conducted using CLC Workbench software) using BLASTn search against the NCBI (16S rRNA sequence database). This is particularly interesting as it highlights the novelty of the microbiome residing in gas fermentation systems demonstrating that still remains largely unexplored. For example, *Cloacamonaceae* sp. 18 was the most dominant bacteria in all the samples with different gas ratio (BA06 - BA09) with an abundance that ranged from approximately 13% up to 27% of the total microbial community in samples BA06 and BA09, respectively. Unfortunately, the low 16S rRNA gene sequence similarity against the genomes deposited in Greengenes and NCBI database do not allow a comparison with other findings in the literature. However, the significant proliferation of this OTU upon BES addition (i.e. almost absent in all the other treatments) indicates a potential key-role during gas fermentation for acetate production. Moreover, it was found that the variation of the samples based on the gas ratio led to a significant difference in microbial structure of the community. *Thermovirgaceae* sp. 8 (phylum Synergistetes) was the second dominant species in the samples with lower gas ratio BA06 followed by *SHA-1* sp. 9 and BA021 sp. 11. Members of phylum Synergistetes were previously reported in various anaerobic environments, i.e. anaerobic digesters,

human oral cavity and goat rumen (Hamdi et al., 2015) with a vital role in amino acid degradation (Milton et al., 2015). *A. noterae* 10, which is an anaerobic bacterium that produces acetate from H_2 and CO_2 , was observed in all treated samples exposed to different gas ratios (BA06-BA09). This, which is consistent with the higher acetate production in the samples with treated inocula compared to the control (Fig. 3) (Sleat et al., 1985).

3.4 Technology potentials for biogas upgrading and biochemicals production

The present study is proposing a new method for biogas upgrading and VFAs production through the mixed culture fermentation of CO_2 and H_2 . However, this research area is still at its infancy and further improvements are necessary to optimise this novel process. These improvements should focus on:

- The type of biocatalyst. Using effluents from gas fermentation processes that are already rich in acetogenic bacteria, could lead to higher process productivity.
- Omitting the pretreatment step which would reduce the operational costs.
- Optimising the gas composition. CO , which is considered as a gaseous pollutant from various industrial activities (Miller et al., 2008), can be used as carbon and energy source instead of H_2 through the fermentation process.
- Optimising substrate composition. Some *Clostridia* species have been reported for their ability to reduce volatile fatty acids (VFAs) to their corresponding alcohols (e.g., n-propanol, n-butanol, n-pentanol, n-hexanol, and isobutanol) by using syngas as electron donor (Liu et al., 2014). Therefore, the addition of VFAs to the fermentation media through the gas fermentation processes or the use of VFAs rich waste could lead to the production of higher alcohols.
- Improving reactor configuration which is affecting gas liquid mass transfer. As the solubility of CO and H_2 is low (Bredwell et al., 1999), use of gas dispersion systems (e.g. hollow fibre

membranes), instead of gas addition in the headspace of batch reactors, could increase the liquid gas transfer and thus improve the productivity of the overall process.

Therefore, fermentation of CO and CO₂ (in biogas) using fermentative effluent and VFAs rich wastes can significantly contribute in the production of various types of biofuels (biomethane and alcohols).

5 Conclusions

A novel fermentation process that converts CO₂ from biogas and H₂ from renewable electricity to valuable chemicals and biomethane was developed. In order to achieve that, chemical treatment with BES was applied to a methanogenic inoculum that completely blocked the activity of methanogens and promoted homoacetogenic acetate production. The homoacetogenic pathway in the chemically treated inoculum was mainly mediated by *A. noterae* 10 (a hydrogen scavenger) that had the highest relative abundance when a gas ratio of 2H₂:1CO₂ was applied. These results offer insight into future bio-economy concepts based on integrating existing technologies, such as biogas upgrading and biosynthesis of value-added biofuels.

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ACCEPTED MANUSCRIPT

513 Table and Figure Captions

514 **Table 1** Characteristics of digested sludge-derived inoculum before and after thermal treatment.

515 **Table 2** Composition of different gas ratios used in the batch experiment.

516 **Table 3** The samples' information for the 16S rRNA analysis.

517 **Table 4** Effect of different treatment methods on gas fermentation.

518 **Table 5** COD balance during batch fermentations under different gas ratio.

519 **Fig. 1.** VFA production profile observed under different treatment methods.

520 **Fig. 2.** The relative concentration of the CH₄, CO₂ and H₂ through the upgrading process under
521 different gas ratios: A) GR_(1x) (H₂:CO₂), B) GR_(2x) (2H₂:CO₂), C) GR_(3x) (3H₂:CO₂), and D) GR_(4x)
522 (4H₂:CO₂), respectively.

523 **Fig. 3.** The acetate production under different gas ratios.

524 **Fig. 4.** H₂ consumption profile under different gas ratio.

525 **Fig. 5.** The pH fluctuation during the incubation under different gas ratio.

526 **Fig.6.** Phylogenetic tree of the complete microbial community under different gas ratios and
527 treatment methods.

528 **Fig.7.** Heat map representing the OTUs with the higher abundance in the samples. The gradient
529 scale above the heat map illustrates the correspondence between the colours and relative abundance.

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Table 1

Characteristics	Unit	NT [*] / BES	90°C	70°C
pH	-	7.6	7.6	7.8
TS	g/kg	47±0.31	56±0.06	45±0.52
VS	g/kg	29.3±0.02	31.7±0.06	30.5±0.06
NH ₄ ⁺ -N	g/kg	1.6±0.02	1.3±0.05	1.4±0.03
TKN	g/kg	2.7±0.03	2.5±0.03	2.4±0.06
Protein	g/kg	16.8±0.03	15.6±0.03	15.1±0.06
TCOD	g/L	32.1±0.36	43.0±1.27	35.0±0.37
Acetate	g/L	0.77±0.03	1.57±0.08	1.08±0.11
Propionate	g/L	0.15±0.006	0.32±0.01	0.19±0.02
Iso-butyrate	g/L	0.07±0.002	0.16±0.002	0.10±0.004
Butyrate	g/L	0.06±0.002	0.22±0.004	0.11±0.008
Iso-valerate	g/L	0.11±0.003	0.23±0.001	0.14±0.006
Valerate	g/L	0.015±0.003	0.07±0.001	0.04±0.006

^{*} Untreated inoculum

Table 2

Gas ratios	(GR _{1x})	(GR _{2x})	(GR _{3x})	(GR _{4x})
H ₂ :CO ₂ :CH ₄	1:1:1.5	2:1:1.5	3:1:1.5	4:1:1.5

Table 3

Sample	Information
BA01	The untreated inoculum
BA02, BA03	The thermally treated inoculum at 90°C and 70°C, respectively
BA04, BA05	The thermally treated inoculum at 90°C and 70°C, respectively, after exposure to the fermentation gas with a constant gas ratio ($GR_{(2x)}$)
BA06, BA07, BA08, BA09	The chemically treated inoculum exposed to different gas ratio $GR_{(1x)}$, $GR_{(2x)}$, $GR_{(3x)}$ and $GR_{(4x)}$, respectively
BA10	The control (without fermentation gas)

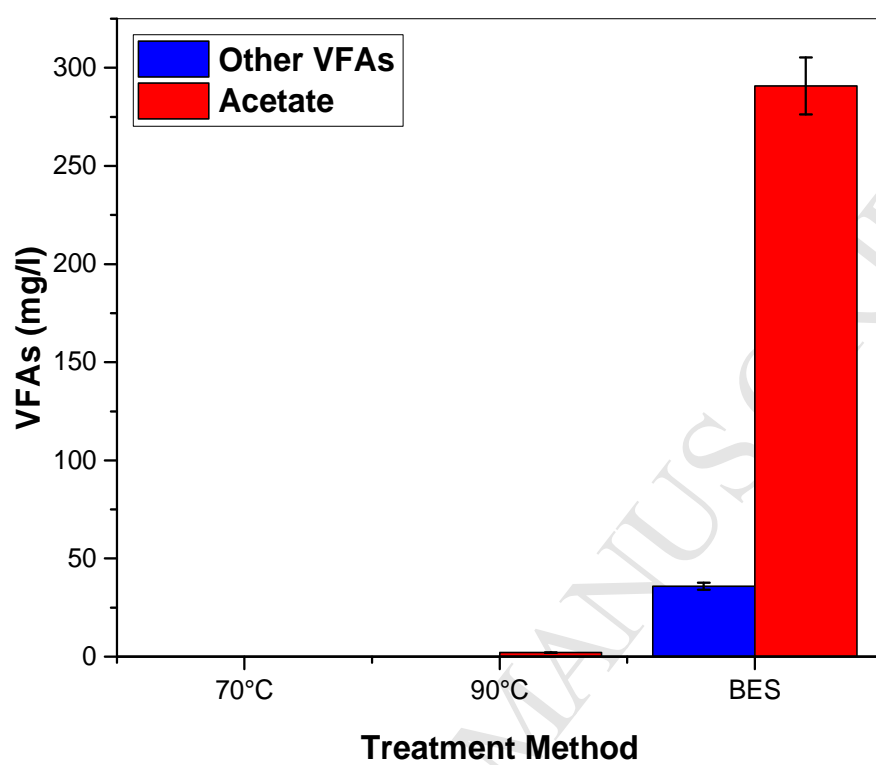
Table 4

Fermentation parameters	Treatment method		
	Chemical	Thermal	
	BES	70°C	90°C
Maximum CO ₂ consumption (%)	95.4	51.5	53
Maximum H ₂ consumption (%)	98.4	100	100
Final pH	5.0	6.7	6.6
CO ₂ -to-acetate conversion (%)	34	0	0
CO ₂ -to-methane conversion (%)	0	100	100

Table 5

	mg-COD_i/L*				mg-COD_f/L*			
	GR _(1x)	GR _(2x)	GR _(3x)	GR _(4x)	GR _(1x)	GR _(2x)	GR _(3x)	GR _(4x)
Ethanol	0.5	0.4	3.1	2.3	0.4	5.7	7.7	8.5
Propanol	4.2	5.3	4.9	3.6	1.4	1.3	1.2	1.3
Butanol	1.5	1.8	1.4	1.2	0.4	0.3	0.4	0.4
Iso-Amyl alcohol	0.2	0.2	-	-	-	-	0.1	0.1
1-Hexanol	0.4	0.4	0.4	-	0.2	0.2	0.2	0.2
Acetate	2.4	2.7	3.0	2.5	168.3	269.2	277.8	298.0
Propionate	1.1	1.0	0.9	0.7	12.0	7.6	8.2	7.6
Butyrate	0.4	0.4	0.4	0.4	6.6	11.2	15.4	12.0
Valerate	0.3	0.5	0.5	0.4	1.0	1.0	1.2	0.9
1-Hexanoate	0.5	0.4	0.4	0.3	0.2	0.3	0.4	0.5
Hydrogen	408.6	788.0	1196.6	1605.2	-	12.6	348.8	607.1
Overall COD balance (%)					93.2	87.1	86.6	85.3

*COD_f : final COD, COD_i : initial COD

**Fig. 1.**

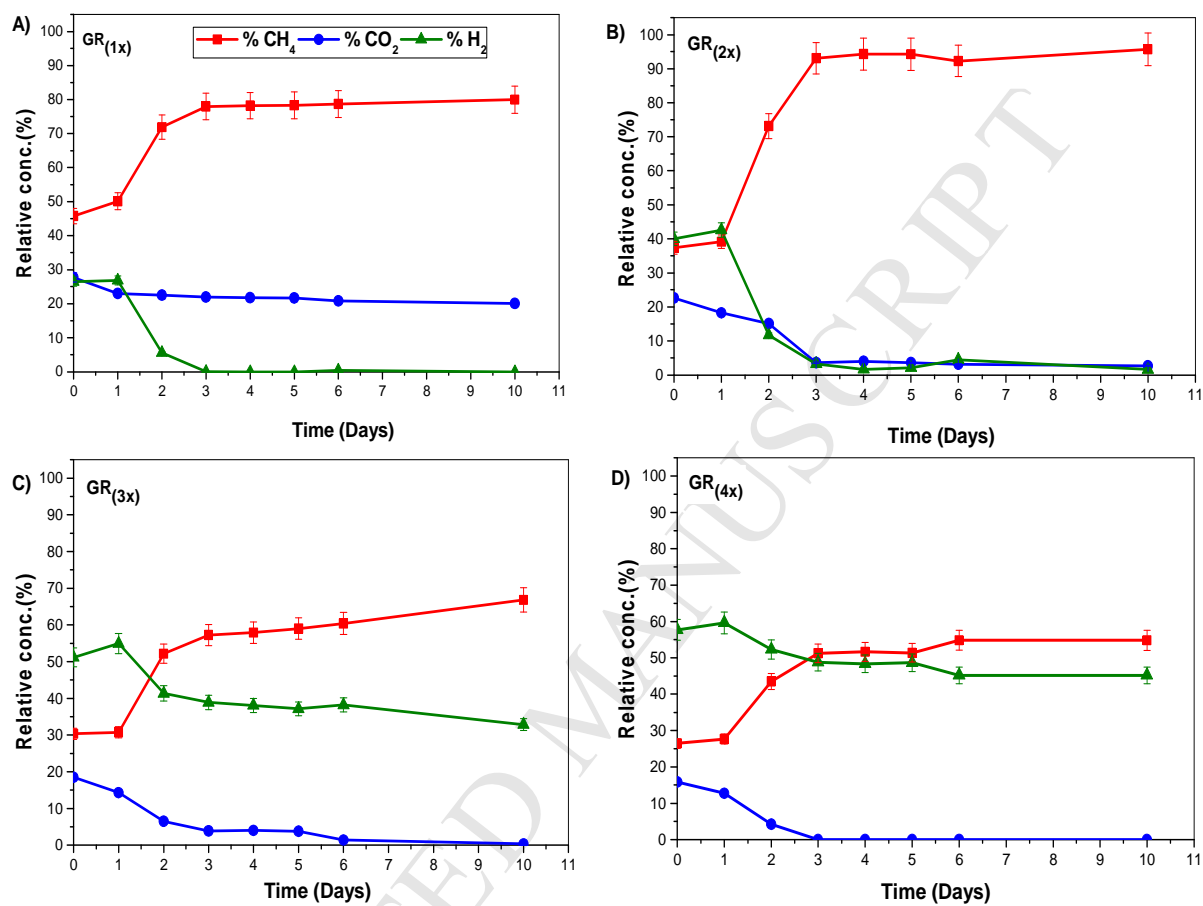
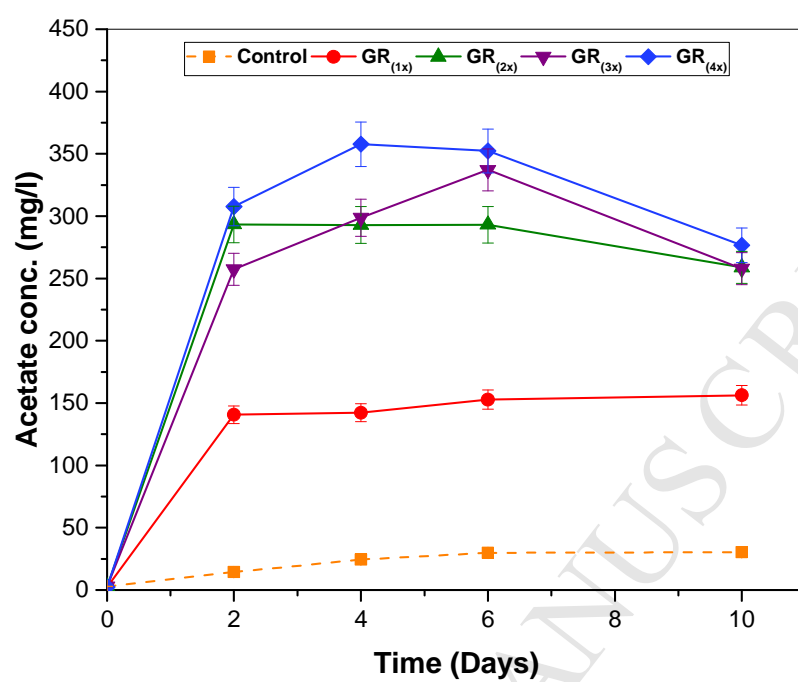
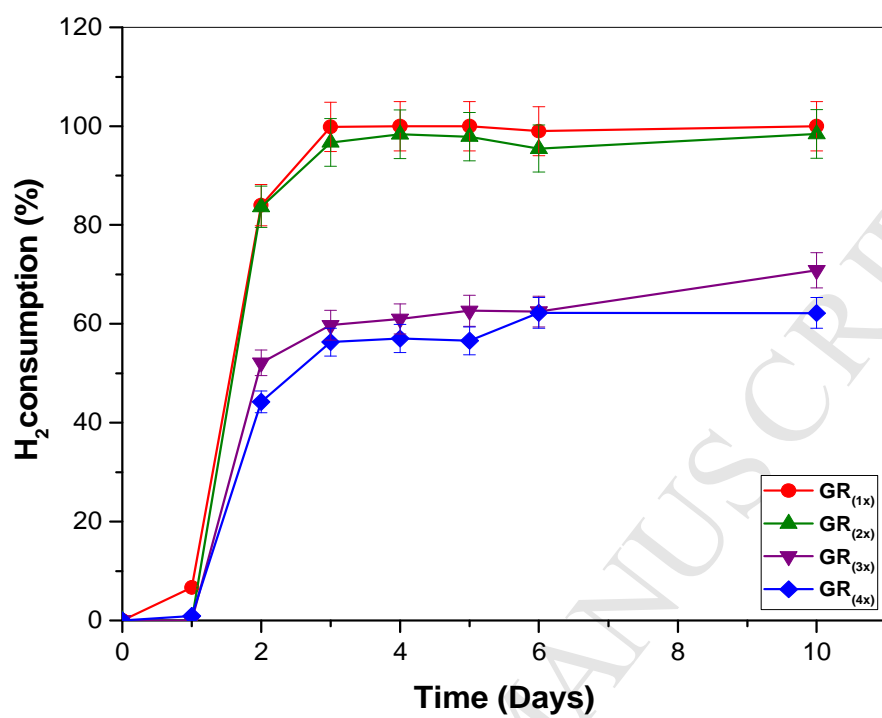


Fig. 2.

**Fig. 3.**

**Fig. 4.**



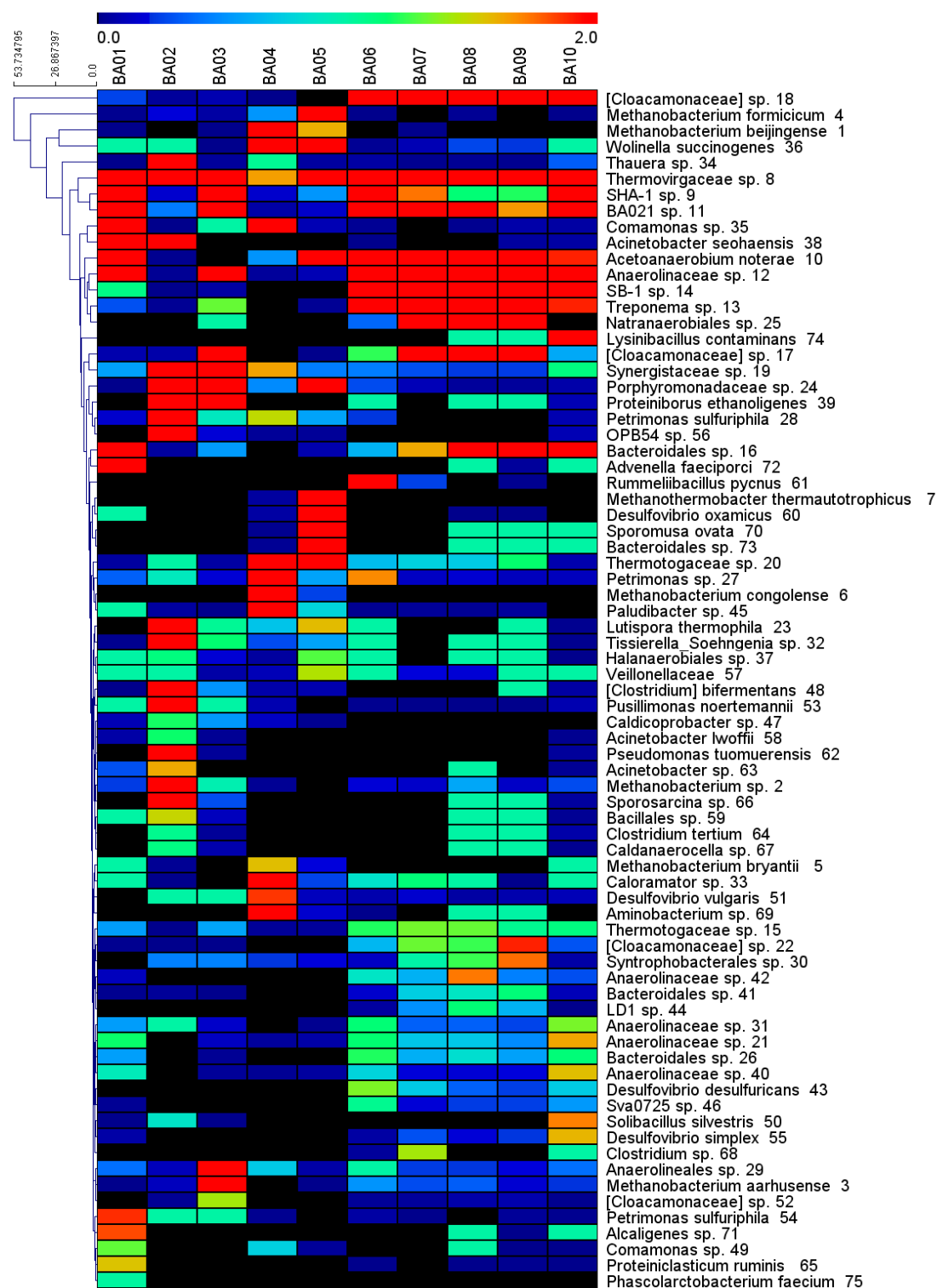


Fig.7.

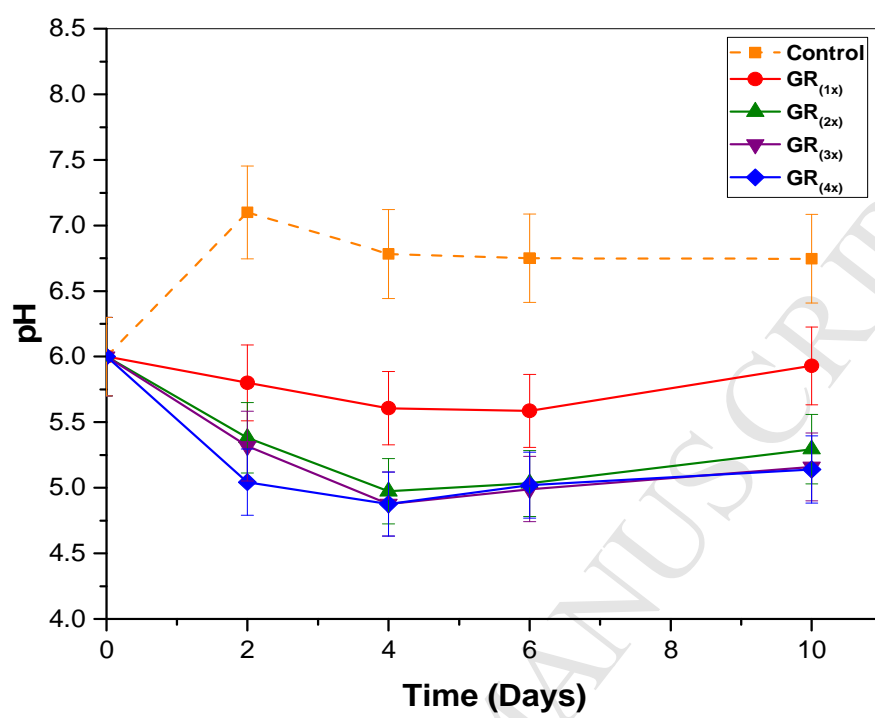


Fig. 5.

Highlights

- A novel technique for biological biogas upgrading and CO₂ mitigation was developed
- Thermal treatment did not suspend the methanogenic activity of the inoculum
- Chemical treatment enhanced biogas upgrading and acetate production
- *Acetoanaerobium noterae* 10 was responsible for the high acetate production
- Biomethane with 96% purity was achieved with 2H₂:1CO₂ ratio