CO_2 sequestration by methanogens in activated sludge for methane production

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HIGHLIGHTS

- Methane (CH_4) produced stems from CO_2 sequestration by methanogens.
- Enriched methanogens from WAS was dominated by an active archaeal community.
- CO_2 which has a single carbon atom is a profitable resource for energy production.

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ABSTRACT

Carbon dioxide (CO_2) is the main greenhouse gas; hence, processes are needed to remove it from the environment. Here, CO_2 was used as the substrate to generate methane (CH_4) by using enriched methanogens after anaerobic enrichment of waste activated sludge (WAS); therefore, we demonstrate that methanogens from WAS have significant potential for converting the greenhouse gas CO_2 into the fuel methane. Methane production was found to increase 70 fold by active methanogens in the enriched methanogens culture after 3 days in the presence of H_2 and CO_2. Throughout the process, CO_2 was completely consumed after 4 days of incubation in the vials after sparging with a mixture of H_2 and CO_2, resulting in significant biological CO_2 sequestration by methanogens. Using a mixture of H_2 and $^{13}CO_2$, we also demonstrated that the methane produced is due to the utilization of CO_2. Microbial community studies via by quantitative real time PCR (qRT-PCR) indicate the dominance of archaea in the enriched methanogens culture of WAS. Archaeal community studies of the enriched methanogens via high-throughput 16S rRNA sequencing also showed that the archaea consist mainly of hydrogenotrophic and aceticlastic methanogens such as *Methanobacteriaceae*, *Methanospirillaceae* and *Methanosarcinaceae* spp. which are actively grown in H_2 and CO_2. We envision that CO_2 gas from power plants can be directed to enriched methanogens of WAS to prevent release of this greenhouse gas while generating a useful biofuel (methane) or other valuable products using this single carbon atom.

1. Introduction

The atmospheric concentration of carbon dioxide (CO_2) has increased significantly from year to year and has become an issue for mitigation and control as outlined in the United Nation Framework Convention on Climate Change in 1992 and the Kyoto protocol in 1997 [1,2]. Unfortunately, excess atmospheric CO_2 does not provide significant balance in the carbon cycle. In addition, CO_2 is abundant, inflammable, and nontoxic and can be used as a carbon source in producing valuable products [3]. Recent studies have been carried out to investigate CO_2 utilization by plants through photosynthesis [4,5], supercritical fluid extraction by CO_2 [6], and CO_2 oxidation by catalysts to form a wide variety of chemicals such as methanol, formic acid, urea, carboxylic acid, lactones, carbonates, and other petrochemicals [1,7–11]. However, CO_2 pressurized in coal bed systems, and gasification treatment for methane

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production by carbon capture and storage (CCS) systems require fossil fired power plants, large facilities, and high energy inputs [10,12–14].

An alternative method for sustainable use of CO_2 is to convert this gas to an energy source such as methane. Thereby, this strategy can be one of the options to trap CO_2 and exploit it for other CO_2 applications to reduce the effect of global warming. In addition, this process is economical due to the low energy requirements [3,15,16]. Methane produced from indigenous anaerobic microorganisms is a safe gas which can be used as a source of electricity and energy for internal combustion engines for power generation and automobiles [17,18]. In addition, methane also can be used as a starting material for methanol, biodiesel and other hydrocarbons [19,20]. Biological methane production from CO_2 is also an exciting approach for the implementation of CO_2 capture and storage (CCS) at power plants to utilize CO_2 for bioenergy [21]. Thus, application of this system might be more beneficial by using indigenous microorganisms readily available in the environment. In this study, we use waste activated sludge (WAS) as the inoculum for CO_2 sequestration for methane production.

WAS is the most abundant waste produced by activated sludge systems in wastewater treatment plants [22]. Reported by Maeda et al. [23], WAS has been produced from the Hiagari wastewater treatment plant in Kitakyushu, Japan where about $7530 m^3$ of excess sludge is generated daily through the treatment of domestic sewage. To date, several attempts have been made to reduce excess sludge for biogas production [24] and other treatments such as thermal or pH treatment [25] in order to reduce the volume of WAS. Many studies have been carried out for methane production from high-molecular-weight compounds present in municipal solid waste, food waste and biomass feedstock by using WAS as a bacterial source [24,26].

Biological methane production through anaerobic digestion of WAS is divided into the four phases, hydrolysis, acidogenesis, acetogenesis and methanogenesis, and these processes are conducted by different microorganisms [18]. In these processes, organic components in WAS are the main substrate for methane production, which are degraded and converted into smaller molecules through hydrolysis, then converted to organic acids (mainly acetate) through acidogenesis and acetogenesis, and finally methane is formed through methanogenesis. Therefore, methane production from WAS itself is indeed complicated. WAS can also be a rich source of microorganisms including archaea and bacteria, which are able to produce useful products such as methane.

Theoretically, methane evolution comes from three different sources which are (i) hydrogen and carbon dioxide, (ii) acetate and (iii) formate [27]. Reported by Demirel and Scherer [27], hydrogenotrophic and aceticlastic methanogens are two of the important methanogens in methane production. Thus, WAS has potential as a microbial source to utilize carbon dioxide; however, it was difficult for us to obtain effective microbial communities which have the ability to clearly utilize carbon dioxide into methane because methane gas can be produced from WAS itself rather than from carbon dioxide sequestration by microbial activity in WAS. Hence, in order to evaluate only the microbial activity of WAS, we created conditions in which methane is not produced from WAS itself. In fact, although there exists an article reporting methane production from CO_2 as a source of carbon by using H_2 as a reductant [16], there was no evidence that the methane was derived from CO_2 as opposed to coming from the WAS itself.

In this study, instead of using high-molecular-weight organics in WAS for methane production, we used enriched methanogens in WAS to see the real potential for assimilating CO_2 as the sole source of carbon. This work demonstrates that a biofuel (methane) may be derived from CO_2 as the sole carbon source by using enriched methanogens prepared from WAS.

2. Materials and methods

2.1. Sludge source and preparation

WAS was from the Hiagari wastewater treatment plant in Kitakyushu, Japan. The sludge was washed using distilled water by centrifugation as described previously [22]. The total solids content in the washed sludge was adjusted to 5% (wet sludge pellet, w/v) with distilled water prior to the preparation of inoculum (enriched methanogen). The characteristics of the raw sludge are presented in Table 1.

2.2. Inoculum preparation (inoculum enriched methanogen)

The total volume of 30 mL 5% (w/v) waste activated sludge was filled in the tightly crimped 66 mL serum vials. The serum vials were sparged with N_2 for 5 min to provide anaerobic conditions prior to the fermentation at 37 °C with shaking at 120 rpm. The methane that was produced was monitored daily. To enrich the microbial culture for methanogens in the samples, the inoculum was prepared over 40 days by periodically sparging with N_2 every 4 days. This step eliminates the trace methane from the WAS itself and provides anaerobic conditions for the indigenous microorganisms that are responsible for carbon utilization in the sludge. Then, N_2 was replaced by H_2 and the same procedure was conducted until no further methane was detected in the vials. In order to remove any residual CO_2 present in the headspace and liquid of the vials, which can contribute to methane production, the vials were further sparged with H_2 every 4 days for about 10 days until no methane was detected. Therefore, the enriched methanogen procedure takes about 50 days (Fig. 1) indicating that the activated sludge in the vials contained methanogens without substrates for further methane production from WAS. The prepared sludge was used as the inoculum of enriched methanogen for the subsequent experiments. The characteristics of the sludge after inoculum preparation (enriched methanogen) are presented in Table 1.

Table 1
Characteristics and solid removal efficiencies for the raw sludge and the inoculum enriched in methanogens after 50 days.

	Unit	Raw sludge	Inoculum enriched in methanogens
pH	–	6.3	9.2
Total carbohydrates	mg/l	555 ± 3	238 ± 3
Total protein	mg/l	537 ± 8	238 ± 8
Total COD	mg/l	808 ± 8	163 ± 5
Removal efficiencies			
Total solid	%	–	36.1 ± 0.2
Total volatile solid	%	–	39.52 ± 0.02
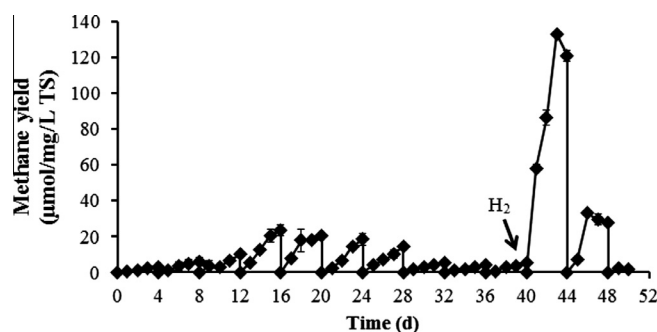
Fig. 1. Time-course of the methane yield while enriching methanogens in the waste activated sludge. N_2 sparging occurred for the first 36 days with 4 day intervals while H_2 sparging started on day 40 with 4 day intervals for 50 days.

2.3. Methane (CH_4) assay

Different sets of 66 mL serum vials with 30 mL of enriched methanogens were sparged with N_2 (control), H_2, CO_2, and a mixture of H_2 and CO_2 (4:1) for 5 min. All gases were purchased from Kifune Shoji Co., Ltd., Japan. Each experiment was conducted in triplicate. Methane production was assayed in an incubator shaker at 37 °C, 120 rpm. Biogas was monitored daily by gas chromatograph as mentioned below.

2.4. Confirmation of $^{13}CH_4$ from $^{13}CO_2$

Confirmation that $^{13}CH_4$ produced from $^{13}CO_2$ was determined by sparging the samples of enriched methanogens (30 mL in 66 mL serum vials) with a mixture of H_2 and $^{13}CO_2$ (4:1) for 2 min. The control vial was filled with autoclaved enriched methanogens and sparged with the mixture of H_2 and $^{13}CO_2$. All vials were incubated at 37 °C at 120 rpm as stated in the methane assay. $^{13}CH_4$ production was analyzed by determination of the $^{13}C/^{12}C$ ratio of the headspace of the vials by Stable Isotope Ratio Mass Spectrometer (SIRMS) as described by Wang et al. [28].

2.5. Analytical methods

50 μl of gas from the headspace of the fermentation vials were analyzed for methane and carbon dioxide by gas chromatography using a GC-3200 gas chromatograph (GL Sciences, Japan) equipped with a thermal conductivity detector. Helium was used as a carrier gas at flow rate of 40 mL/min. Current was set at 100 mA. Methane was analyzed by a Molecular Sieve 13X 60/80 mesh column, SUS 2 m × 3 mm I.D (GL Science, Japan). The oven temperature was set at 40 °C, while the injector and detector temperature are set at 50 °C and 65 °C, respectively. Carbon dioxide was analyzed by WG-100 SUS 1.8 m × ∅¼″ O.D column (GL Science, Japan). The oven temperature was set at 50 °C, while the injector and detector temperature are set at 50 °C and 50 °C, respectively. Hydrogen was measured by a 6890-N gas chromatograph (Agilent Technologies, Glastonbury, CT) as described previously [22]. The measurement of organic acids was conducted by high performance liquid chromatography (Shimadzu LC-10AD) [29]. pH was measured by a AS ONE compact pH meter, AS-211 (Horiba Ltd, Kyoto, Japan). Total COD was measured using COD measuring unit (COD-60A, TOA-DKK, Japan) according to the manufacturer protocol. The protein measurement was according to the Lowry method [30]. Total solids and volatile solids were measured according to the Standard Method for Water and Wastewater, APHA [31].

2.6. Total RNA extraction, cDNA synthesis and quantitative RT-PCR

Prior to the total RNA extraction, a sludge pellet was collected from the raw sludge as well as from the inoculum enriched in methanogens. A 10 mL of sludge sample was added to 2 mL of RNA later buffer (Ambion, Cat#AM7020) in RNAse free falcon tubes before centrifugation at 13,000 rpm for 2 min at 4 °C. The supernatant was discarded and the pellet was dissolved in 1 mL of RNA later buffer. The mixture was immediately transferred in 2 mL screw capped tubes before centrifuging at 13,000 rpm for 1 min. The pellet was quick frozen in dry ice with ethanol for 30 s before storage at −70 °C prior to the RNA extraction. Total RNA was extracted by an RNeasy kit (Qiagen Inc, Valencia, CA) as described by Mohd Yusoff et al. [29]. cDNA was synthesized using PrimeScript RT reagent kit Perfect Real Time (TAKARA Bio Inc, Cat#RR037A) according to the manufacturer's protocol. Five μg of total RNA was mixed with 2 μL of 5× Prime Script buffer, 0.5 μL reverse transcriptase enzyme, 0.5 μL oligo dT primer and 2 μL random primers in a 10 μL reaction mixture. The mixture

was incubated at 25 °C for 10 min and 37 °C for 30 min followed by enzyme deactivation at 85 °C for 5 s. The cDNA was stored in −20 °C prior to being used for quantitative reverse transcription real-time PCR (qRT-PCR). qRT-PCR was performed using the StepOne Real Time PCR System (Applied Biosystem) with primers and probes as listed in Table 2. The real time PCR mixture of 20 μL was prepared by mixing of 100 ng of cDNA in 10 μL Taqman Fast Advance master mix (Life Technologies, Cat#444455), 0.34 μL Taqman probe (11.8 μM), and 0.72 μL of each primer (25 μM). Each template cDNA was analyzed in triplicate. The PCR amplification included UNG incubation at 50 °C for 2 min followed by polymerase activation at 95 °C for 20 min. The 40 cycles of annealing and extension were performed at 95 °C for 1 s and 60 °C for 20 s, respectively. The standard curve for the universal bacteria was constructed using Escherichia coli BW25113 from the Yale Coli Genetic Stock Center (USA). The standard curve for archaea was constructed with a mixture of Methanosarcina barkeri (JCM 10043) and Methanobacterium formicicum (JCM 10132) from the Japan Collection of Microorganisms. Genomic DNA was extracted from each pure culture using the Ultra Clean Microbial DNA Isolation kit (MOBIO, Cat#12224) prior to amplification with real time PCR using the primer sets shown in Table 2. The slope and intercept values for the universal bacterial primers and archaea primers were calculated by plotting C_T values against the logarithm of the template DNA copy number [32]. The slope and intercept values are indicated in Table 3. The calculation of copy number based on DNA concentration was according to Lee et al. [33].

2.7. High-throughput 16S rRNA sequencing

DNA extraction was performed using PowerSoil DNA Isolation kit (MO BIO Laboratory Inc, Cat#12800-50). The V6 region of archaeal 16S rRNA genes was amplified using the primer pair Illumina adaptor-linker (AG)-barcode-958F (5′-AAT TGG ANT CAA CGC CGG-3′) and an equal mixture of 1048R major (5′-CGR CGG CCA TGC ACC WC-3′) and 1048R minor (5′-CGR CRG CCA TGY ACC WC-3′) (http://vamps.mbl.edu/resources/primers.php). Each sample was tagged with a unique 8 bp barcode. The PCR reaction mixture (25 μL) contained 20–50 ng DNA template, 1 × TransStart® FastPfu buffer (TransGen, Beijing, China), 0.25 mM dNTPs, 2 U of TransStart® FastPfu DNA Polymerase (TransGen) and 0.3 μM of each primer. PCR was performed under the following conditions: 94 °C for 5 min, followed by 35 cycles of 94 °C for 20 s, 57 °C for 30 s, and 72 °C for 30 s, and a cycle of 72 °C for 5 min. Each sample was amplified in triplicate, pooled and purified using the MinElute Gel Extraction Kit (Qiagen, Valencia, CA, USA). The concentration of amplicon was measured in duplicate using a Quant-iT dsDNA HS assay kit (Molecular Probes, Sunnyvale, CA, USA). All amplicons were mixed in equimolar concentration and sent to the Beijing Genomics Institute (Shenzhen, China). Paired-end sequencing (2 × 91 bp) was performed on a HiSeq 2000 instrument (Illumina Inc., San Diego, CA, USA).

The raw paired-end reads were assembled following the barcoded Illumina PE sequencing (BIPES) pipeline to improve the sequencing accuracy of the reads [34], and then the clean data were analyzed using Mothur v1.31 [35] and QIIME v1.7.0 [36]. The reads shorter than 50 bp or longer than 90 bp were discarded, and any read containing ambiguous bases (N) or incorrect barcode or primer sequences was also excluded from further analysis. The potential chimeras were removed using UCHIME. The remainder of the reads were clustered into operational taxonomic units (OTUs) using an open-reference OTU picking pipeline, where reads were clustered against the Greengenes database v13_8 [37], and any read that failed to match a reference sequence was subsequently clustered de novo (http://qiime.org/tutorials/otu_picking.html) (an identity cutoff value of 97% was used). Singletons were

Table 2
Primer and probe sets used in qRT-PCR.

Target group	Primer or probes	Sequences (5′–3′)	*E. coli* numbering	Product size (bp)	Reference
Universal bacteria	Forward	TCCTACGGGAGGCAGCAGT	331-349	466	[32]
	Reverse	GGACTACCAGGGTATCTAATCCTGTT	772-797		
	Probe	CGTATTACCGCGGCTGCTGGCAC	506-528		
Universal Archaea	Forward	ATTAGATACCCSBGTAGTCC	787-806	273	[33]
	Reverse	GCCATGCACCWCCTCT	1044-1059		
	Probe	AGGAATTGGCGGGGGAGCAC	9915-934		

Table 3
Real-time PCR standard curve using universal bacteria and archaea set.

Parameter	Universal bacteria set	Universal archaea set
Linear range (copy μL^{-1})	2.1×10^6–2.1×10^{11}	2.5×10^6–2.5×10^{11}
Slope	−3.71	−3.55
R^2 of slope	0.994	0.998
Intercept	54.731	53.082
Source strains	*Escherichia coli* BW25113	*Methanobacterium formicicum* and *Methanosarcina barkeri*

excluded for the further analysis since many of them tended to have higher sequencing error rates [38]. The remaining OTUs were assigned to taxonomic rank using RDP Classifier with a 50% bootstrap confidence threshold [39]. The raw sequence data were deposited into the NCBI short reads archive database under accession number: SRP010829.

3. Results and discussion

3.1. Methanogen enrichment

Fig. 1 shows how the culture of 5% (w/v) WAS was enriched for methannogens. The process of periodically sparging with N_2 for the first 36 days and H_2 starting from day 40 until day 50 was carried out to ensure that the enriched methanogens culture was ready for methane production by using CO_2 as the sole source of carbon. Low methane production after 28 days (Fig. 1) demonstrated the low available substrate content in the broth. Also, analysis of the inoculum after 50 days showed that the amount of carbohydrates, protein, and COD were reduced significantly compared to the initial raw sludge (Table 1). The reduced carbon content in the enriched methanogen sample confirmed that the enriched methanogens use the supplied gas such as CO_2 as the main source of carbon. The reduction of fermentable sludge components in the enriched methanogen sample compared to raw sludge (Table 1) shows that the microbial community in the sludge consumed the available carbon and nitrogen sources through the 50 days of anaerobic degradation for methane production [27]. Thus, due to the limitation of carbon content in the WAS, the active methanogens utilized gaseous CO_2 for methane generation.

3.2. Methane production assay

To assay the amount of methane that could be generated from CO_2 by the enriched methanogens in the WAS, different gases (N_2 as a negative control, H_2, CO_2 and a mixture of H_2 and CO_2 at a 4:1 ratio) were added to the enriched methanogens, and the methane yield was determined (Fig. 2a). The vials sparged with both H_2 and CO_2 gave the highest methane yield which was as high as 70 fold greater than the other treatments (after 3 day). During the 14-day anaerobic incubation, the methane yield from the vials sparged with H_2 and CO_2 was stable and increased up to
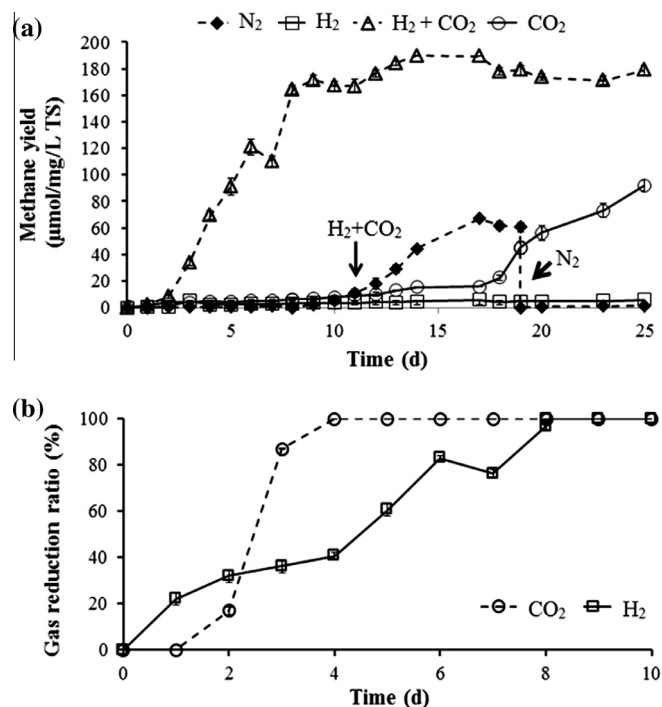

Fig. 2. (a) Time course of the methane yield with different supplemented gases such as N_2 (negative control), H_2, CO_2 and the mixture of H_2 and CO_2. The headspace of the control vials (first sparged with N_2) was replaced with the mixture of H_2 and CO_2 after 10 days of incubation. The headspace was returned to N_2 during 19 days of incubation. (b) The reduction ratio of H_2 and CO_2 in the enriched methanogens supplemented with a mixture of H_2 and CO_2.

189 ± 2 μmol mg^{-1} L^{-1} TS from 34 ± 7 μmol mg^{-1} L^{-1} TS after 3 days of incubation.

The increase in methane yield was corroborated by the reduction of CO_2 and H_2 in the vials after sparging with H_2 and CO_2 (Fig. 2b). The vials sparged with H_2 and CO_2 showed complete reduction of CO_2 and H_2 after 4 days and 8 days, respectively. However, no reduction of H_2 was seen in the vials sparged with only H_2 (data not shown). Hence, the methane generated was due to the activity of the hydrogenotrophic methanogens which require CO_2 as the source of carbon and H_2 as a reducing agent for methane production [16]. Although the reaction of $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ is known as the methane generation pathway from power sources [40], Kim and colleagues found that the ratio of H_2–CO_2 of 2–1 also have the potential for producing methane [16]. Thus, H_2 can act as a source of reducing agent in this reaction with minimal spiking of H_2 gas capable of stimulating CO_2 sequestration by methanogens.

Mayumi and colleagues indicate that CO_2 usage has the potential to reduce 20% of future global CO_2 emissions [41]. In line with our study, we aim mostly to recover the greenhouse gas CO_2 for CH_4 production at a mesophilic temperature. Our results also show that no methane is produced when CO_2 was not supplemented in
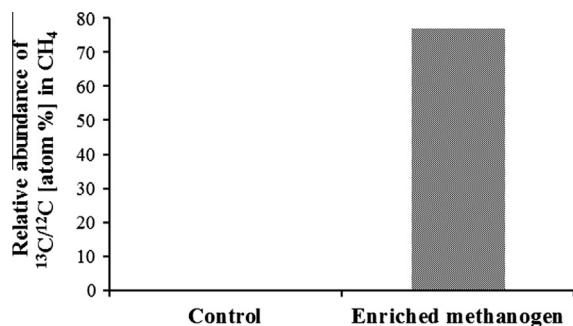
Fig. 3. Relative abundance of $^{13}C/^{12}C$ in CH_4 from the control and inoculum enriched in methanogens sparged with the mixture of H_2 and $^{13}CO_2$. Results were obtained by a Stable Ratio Mass Spectrophotometer (SIRMS).

the vials. Hence, active methanogens cannot produce methane in the absence of a carbon source (Fig. 2a). Thus, CO_2 is the main source of carbon in influencing methane production in this study.

### 3.3. Confirmation of CH_4 from CO_2

In order to confirm that the enriched methanogens use H_2 and CO_2 supplemented to the vials to produce methane, the head space of some of the control vials that were initially sparged with N_2 was replaced by a mixture of H_2 and CO_2 after 10 days of incubation (Fig. 2a). The methane yield increased simultaneously after adding H_2 and CO_2, while no methane was detected with N_2 sparging during this timeframe. Methane production increased to 67 $\mu mol\ mg^{-1}\ L^{-1}$ TS after 7 days of sparging with the mixture with H_2 and CO_2. After 9 days of sparging with the mixture of H_2 and CO_2, the headspace of the control vials was then replaced with N_2. Without CO_2 in the vials, no methane was produced when the headspace of the vials was replaced with N_2 (Fig. 2a). Therefore, these results demonstrate that the methanogens require additional carbon and hydrogen sources for methane production [16].

Another set of experiments was carried out to prove that the methane production was from the supplemented CO_2. The enriched methanogens and autoclaved enriched methanogens (control) in different vials were sparged with a mixture of H_2 and $^{13}CO_2$ and analyzed for the production of $^{13}CH_4$ from $^{13}CO_2$. Fig. 3 shows that the percentage of $^{13}C/^{12}C$ of the headspace of methane obtained from the enriched methanogens vials was 77 (atom%) $^{13}C/^{12}C$. Hence, the $^{13}CH_4$ that was produced by the methanogens was from the microbial conversion of $^{13}CO_2$.

3.4. Soluble metabolites and pH

Fig. 4 shows the organic acids and pH profile for 0 (Fig. 4a), 11 (Fig. 4b) and 25 days (Fig. 4c) of methane production after sparging with different gases. No changes in acetate concentrations were found in the vials sparged with both H_2 and CO_2 which shows that the methane production was from the H_2 and CO_2 in the headspace (Fig. 2b). Also, the slight formate consumption did not contribute much to the evolved methane in the vials sparged with the mixture of H_2 and CO_2. Furthermore, the vials sparged with only N_2 and H_2 showed no increment of acetate and no significant reduction of formate which is in agreement with the lack of methane evolution.

The vials sparged with CO_2 produced more acetate from 6.1 ± 0.4 mM to 15 ± 2 mM over the 11 days of incubation (Fig. 4b). Fig. 2a shows that the vials sparged with CO_2 started to produce methane after 18 day of anaerobic incubation. The result indicates that the vials sparged with CO_2 that tended to produce acetate after 11 days due to the reducing power left in the sludge. Acetate production in the enriched methanogens sparged by CO_2
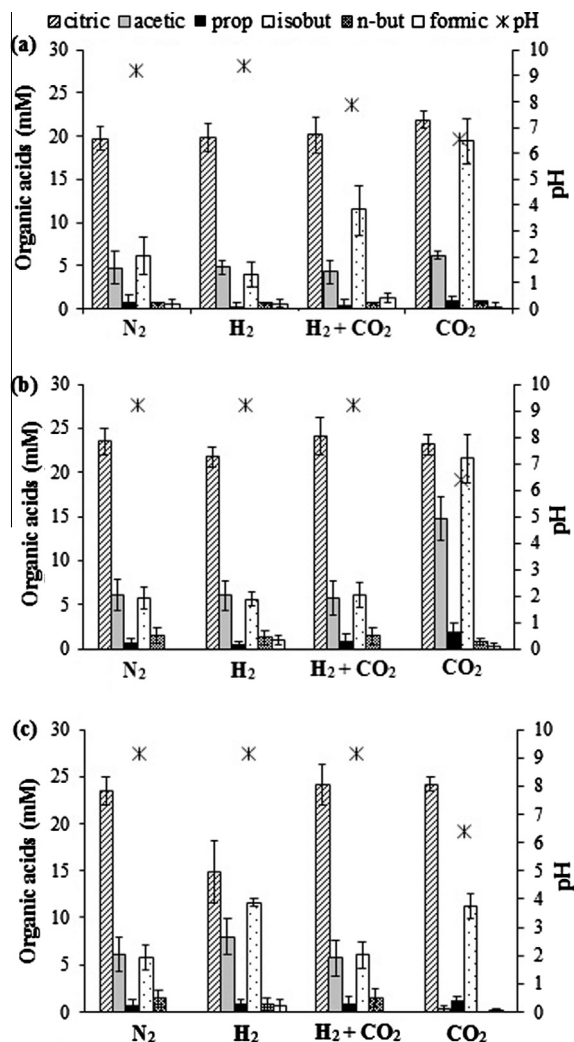
Fig. 4. Organic acids and pH profiles after (a) 0 day, (b) 11 day and (c) 25 days of sparging with different gases.

might be due to Wood/Ljungdhal pathway (also known as CO dehydrogenase/acetyl-CoA synthase pathway) directed by acetogens and several anaerobic microbes that are very diverse in sludge and employ the complex degradation pathway [42,43]. Then, the acetate was used by the acetoclastic methanogens for methane production [33]. Methane production starting from day 18 until day 25 of incubation in the vials sparged with CO_2 (Fig. 2a) was responsible for reducing the acetate concentration from day 11 to day 25 of the incubation (Fig. 4b and c).

Sparging of either only CO_2 or the mixture of H_2 and CO_2 gas both resulted in reduced pH and higher isobutyrate formation as indicated in Fig. 4a. The supplementation of the mixture of H_2 and CO_2 in the vials resulted in a pH decrease to 7.9 (Fig. 4a). However, the pH was increased to 8.8 during 25 days of incubation due to the complete CO_2 utilization in the vials prior to that time (Fig. 2b); the CO_2 sparging influenced the low pH conditions due to the carbonic acid formation as reported by Peterson and Daugulis [44]. Similarly, CO_2 is used in industry to neutralize pH in alkaline wastewater [45]. Furthermore, CO_2 dissolving in water produces hydrogen ions through the reaction: CO_2 (g) + H_2O (aq) $\leftrightarrow H_2CO_3$ (aq) $\leftrightarrow H^+ + HCO_3^-$ (aq) $\leftrightarrow 2H^+ + CO_3^{2-}$ (aq) [46]. Hence, the hydrogen ions produced can be the electron donor for methane evolution in the vials supplemented with only CO_2 gas (Fig. 2a).

Fig. 4c shows that CO_2 supplementation resulted in significant acetate and isobutyrate formation in which both metabolites are utilized by aceticlastic methanogens such as *Methanosarcinaceae* [47,48]. This process resulted in CH_4 evolution in the vials sparged only with CO_2 (Fig. 2a). The addition of CO_2 to the vials favors the growth of acidogenic microorganisms since they can grow at low pH (5.2–6.5) for acetate and isobutyrate formation [49].

The *Methanosarcinaceae* which comprise of 30% out of the total archaea population in the inoculum enriched methanogens (Fig. 6), probably utilized acetate in the CO_2 vials for methane production [27]. The exergonic reaction $CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$ ($\Delta G° = -31$ kJ/mol) indicates the aceticlastic methanogens can produce CH_4 from acetate [42].

3.5. Microbial community analysis

Different sets of universal primers and probes targeting bacteria and Archaea were used in order to evaluate active bacteria and archaeal species in the raw sludge and in the inoculum with the enriched methanogens as indicated in Table 2. Fig. 5 shows the quantitative changes of the bacterial and archaeal population in the raw sludge and in the inoculum enriched in methanogens. A significant difference was found for the bacterial and archeal populations between raw sludge and inoculum enriched methanogens. The bacterial population dominated the Archaea in raw sludge (4.02×10^{10} rRNA gene copies mL^{-1} vs. 6.28×10^8 rRNA gene copies mL^{-1}). In contrast, the archaeal population was drastically increased in the inoculum enriched in methanogens to 4.64×10^{13} rRNA gene copies mL^{-1} while the bacterial population dropped to 1.08×10^9 rRNA gene copies mL^{-1}. The increase in the archaeal population after enrichment shows that the culture was dominated with methanogens which were ready to use the supplemented gases as a substrate for CH_4 production. Thus, CH_4 evolution from the supplemented CO_2 was from the assimilation of CO_2 by the enrichment of WAS under anaerobic conditions.

In order to determine more details about the composition of the microbial community in the enriched inoculum, a microbial community analysis was performed by Illumina high-throughput 16S rRNA sequencing. The raw sludge was compared to the enriched methanogen inoculum after 1 day of sparging with N_2, H_2 and the mixture of H_2 and CO_2. Overall, a total of 221,349 high-quality reads were obtained with an average of $74,000 \pm 23,000$ reads per sample. Fig. 6 shows the relative abundance of the archaeal taxa from the high-throughput sequencing. The results demonstrate that the raw sludge is comprised of different types of archaeal phylotypes. However, after 1 day of sparging with N_2, H_2 and the mixture of H_2 and CO_2, the archaeal population was dominated by members of the *Methanobacteriaceae*, *Methanospirillaceae* and *Methanosarcinaceae* family. The *Methanosarcinaceae* family increased after 1 day of sparging in the vials sparged with
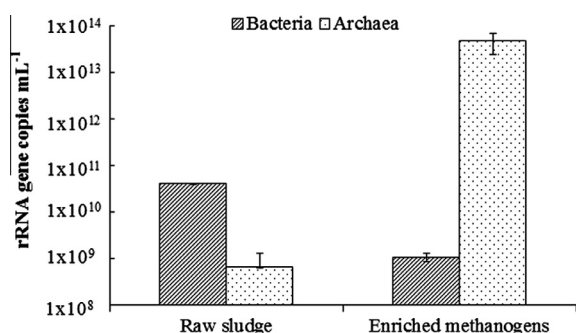
N_2 (31%), H_2 (54%) and the mixture of H_2 and CO_2 (36%) when compared to the raw sludge (13%). The increment of *Methanobacteriaceae* out of the total archaeal population increased from 9.3% in raw sludge to 22.4%, 43.3% and 32.5% in the H_2, N_2 and the vial with the mixture of H_2 and CO_2, respectively. *Methanospirillaceae* was also abundant in all vials (especially in the vial sparged with H_2 and CO_2) which indicates that hydrogenotrophic methanogens were actively growing in the H_2 and CO_2 environment; i.e., methanogens that grow on H_2/CO_2 and formate [27,50]. The other microbial taxa in the raw sludge and sludge after 1 day of sparging with different gases were also represented by members of other hydrogenotrophic methanogens such as *Methanobacteriaceae*, *Methanospirillaceae*, *Methanoregulaceae*, *Methanocorpusculaceae* and *Methanobacteriales*. Other hydrogenotrophic methanogens such as *Methanoregulaceae*, *Methanocorpusculaceae* and *Methanobacteriales* were presented in the raw sludge but their population decreased after sparging with N_2, H_2 and the mixture of H_2 and CO_2. In the overall microbial community analysis from Fig. 6, the raw sludge contained a mixture of different archaeal phylotypes but the community shifted to a dominant archaeal family comprised of *Methanobacteriaceae*, *Methanospirillaceae* and *Methanosarcinaceae* in the vials after 1 d of sparging with N_2, H_2 and the mixture of H_2 and CO_2. The hydrogenotrophic methanogens in all samples was about 64–87% which indicates the dominant role of methanogens that grew in the H_2/CO_2 environment.

The likely reaction of the hydrogenotrophic methanogens is $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ ($\Delta G° = -130.7$ kJ/reaction) due to the higher CO_2 pressure [41]. Thus, the use of biological reactor such as CSTR could be used for our process by supplementation of high CO_2 pressure with intermittent spiking of H_2 for methane production.

In this study, we demonstrated that active archaea accumulate in the inoculum by using a qRT-PCR analysis. Furthermore, complete CO_2 utilization shows that hydrogenotrophic methanogens grew actively in H_2/CO_2 environment to produce methane. Thus, this study introduces a promising strategy to sequester CO_2 from the environment. In a similar manner, CO_2 and CH_4 may also be utilized for other hydrocarbon fuels and chemicals production.

3.6. Economic value and potential

Due to increasing interest in CO_2 sequestration, many attempts have been made to utilize CO_2 for methane or electricity production. CO_2 sequestration for energy production has a positive impact on the environment and has favorable economics. Table 4 shows the overall process (inputs and outputs) for methane production in this study using sludge enriched in methanogens and after sparging with different gases (CO_2 sequestration). Initial methane gas generated during the enrichment process for the methanogens was 0.3 L CH_4/L from sequential sparging with N_2 or H_2. During this process, N_2 gas sparged for the first 10 injection with 0.05–0.06 L N_2 per each injection, cost about $ 0.06/L [51] followed by three H_2 injections that cost about $ 3–6/L [52] (Fig. 1). The sparging of both N_2 and H_2 generated methane worth $ 4.16–17.13/L by different categories of the US EIA data in 2014 [53]. Although H_2 might be an issue for methane production, it can be produced by eco-friendly and renewable way such as by wind turbines, solar panels, water electrolysis, and photovoltaic cells [54].

In contrast, after sparging with different gases to investigate CO_2 sequestration, 0.5 g/L of WAS generates 0.3 L CH_4/L of enriched methanogens in 10 days of incubation after sparging with the mixture of H_2 and CO_2. Meanwhile, sparging with only CO_2 generated 0.05 L CH_4/L of enriched methanogens in 25 days of incubation. Throughout the process, 1 mol of CO_2 generated 1 mol of CH_4 in the headspace of the vials. With a proper handling and control, the enriched methanogens after CO_2 sequestration can be used in

Fig. 5. Quantitative changes of active bacterial and archaeal population in the raw sludge and in the inoculum enriched methanogens by qRT-PCR.
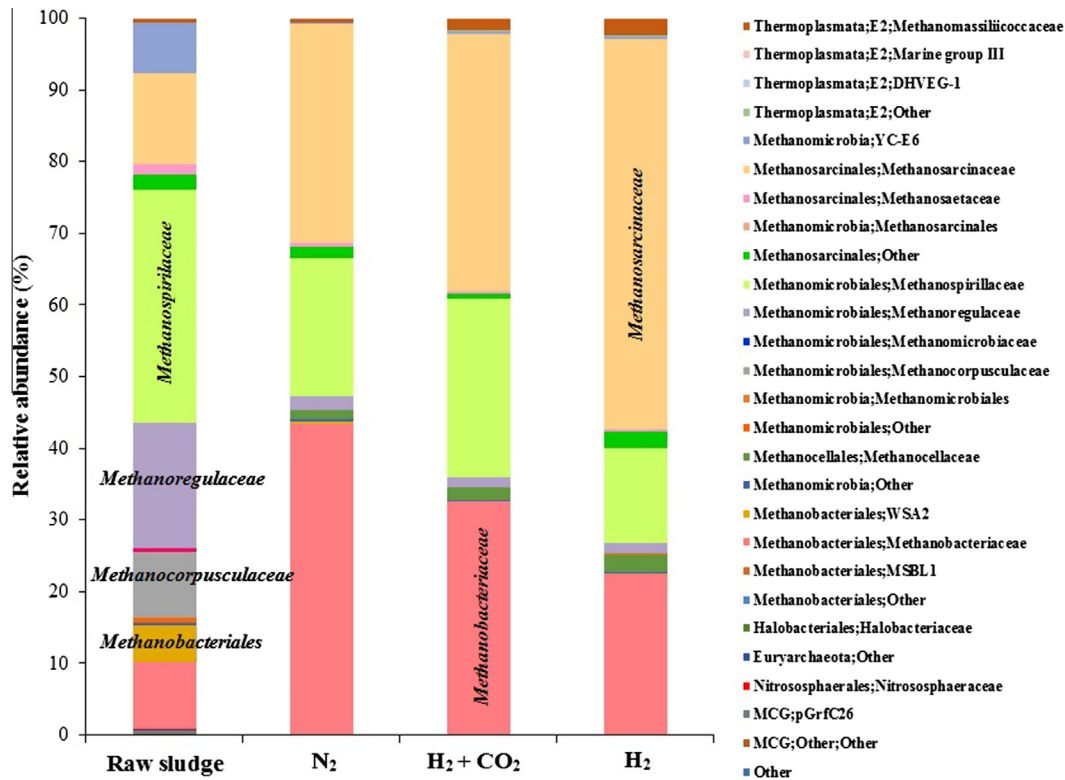
Fig. 6. Relative abundance of the dominant archaeal taxa in the raw sludge and in the inoculum of enriched methanogens after sparging with N_2, H_2 and the mixture of H_2 and CO_2. Results derived from high-throughput 16S rRNA sequencing. Minor phyla accounting for <0.5% of the total sequences are summarized in group marked 'other'.

Table 4
Summary of methane production in the enriched methanogen preparation and after different gases was injected into the enriched methanogens (CO_2 sequestration).

	Enriched methanogen preparation		CO_2 sequestration	
	Input	Output	Input	Output
WAS	50 g/L (no cost)	Enriched methanogens as catalyst for CO_2 sequestration	na	
N_2	~0.05–0.06 L per injection (cost: $ 0.06/L) [51]	Methane gas (0.3 L/L enriched methanogens) (CH_4 price: $ 4.16–17.13 /L) [53]	~0.05–0.06 L per injection	Organic acids
H_2	~0.05–0.06 L per injection (cost: $ 3–6/L) [52]	Methane gas (0.3 L/L enriched methanogens)	~0.05–0.06 L per injection	Organic acids
H_2:CO_2 (4:1)	–	–	~0.05–0.06 L per injection	Methane gas (0.3 L/L enriched methanogens) and organic acids
CO_2	–	–	~0.05–0.06 L per injection (cost: $ 1/L) [10]	Methane gas (0.05 L/L enriched methanogens) and organic acids (mainly isobutyric)

na: not available.

a repeated batch operation or in a continuous system for methane production due to the consistent ability of the enriched methanogens to utilize CO_2 as well as to produce methane. Thus, no waste from the WAS is generated from this system. Also, fermentable components in the WAS were reduced during preparation of the enriched methanogen inoculum. Hence, the overall process yields a significant credit in terms of reducing WAS volume, as well as of course credits in term of CO_2 sequestration and energy production.

To understand the economic value and potential of this proposed system, a comparison of this process with other processes for utilization of CO_2 for energy production was made and the results are shown in Table 5. The comparison shows the advantages and disadvantages of each technology. An advantage of the biological approach is that a thermal and electrical energy input are not required. However, other techniques such as enhanced coal-bed methane production with CO_2 injection (CO_2-ECBM) and

integrated gasification combined cycle (IGCC) require high energy inputs. Bajón Fernández et al. [55] reported improved methane production in a digester containing food waste and sewage sludge supplied with saturated CO_2. However Bajón Fernández et al. [55] did not show significant evidence for methane production was come from CO_2 sequestration.

On the other hand, the catalytic synthesis of H_2 and CO_2 to produce energy was also studied and applied for business application. For example, an automobiles company, Audi, has opened a pilot plant which converts an atmospheric CO_2, water and electricity into hydrocarbon fuel [56]. The method for energy conversion was in accordance to the method described by Zuberbuhler et al. [57] with two stages reaction. The first stage is the electrolysis of water to produce H_2, followed by catalytic reaction of H_2 and CO_2. Both chemical reactions require high temperature and pressure. In addition, other studies also reported the catalytic process of H_2O–CO_2 co-electrolysis in solid oxide electrolysis cells for con-

Table 5
Economic comparison between processes for sequestering CO_2.

Treatment	Biological		Others	
	This study	CO_2 injection into biomass	CO_2-ECBM	IGCC
Thermal or electrical energy input	No thermal or electrical energy were used		Energy used for pressurizing CO_2	Thermal energy used for the 360 °C heat exchanger and for the 1300 °C turbine inlet temperature
CH_4/electrical energy production	227 mL CH_4/g VS in vials sparge with H_2 + CO_2) 128 mL CH_4/g VS in vials sparge with CO_2	195 mL CH_4/g VS (in food waste + CO_2)	30 MMcf/month	Electrical energy: 3647 GW h/year)
Fuel consumption	H_2 (in vials sparge with H_2 and CO_2)	No	Coal required	
By-products	Organic acids (isobutyric acid in vials sparged with CO_2)	na	na	Solid sulfur, ash, SOx, NOx
CO_2 reduction efficiency	77%	na	na	85–97%
Time consumption for biogas/electric production	~3–10 days (H_2 + CO_2) and 25 days (CO_2)	~10 days	Monthly injected CO_2 depended on market demand	na
References	This study	[55]	[10]	[13,14]

na: not available.

version of carbon to methane (C (s) + 2H_2 → CH_4) which also requires a high energy input (550–750 °C) [58]. However, Jacquemin and colleague [54] reported the catalytic synthesis of methane from H_2 and CO_2 at low temperature and atmospheric pressure. The process requires three steps, (1) chemisorption of CO_2 on the catalyst, (2) CO_2 dissociation, and (3) H_2 reaction with dissociated product. Compared to our study, our approach used microbial sources from WAS as a catalyst for CO_2 sequestration to produce methane. High temperature and pressure, as well as electricity are not required in our reaction system. Our system requires good enriched methanogens for CO_2 sequestration, with two stages of methane production, (1) methane produced naturally by anaerobic degradation of WAS (Fig. 1), and (2) methane production by CO_2 sequestration (Fig. 2). In addition, our proposed method could be applied in industry after complete anaerobic degradation of organic materials. For example, after a complete anaerobic utilization of organic waste for methane production, CO_2 can be introduced in the microbial community containing enriched methanogens that remain in the sample for methane production. Thus, the microbial community in the sample can be used to generate methane energy from CO_2.

This study shows the great potential of generating methane (227 mL CH_4/g VS in vials sparged with a mixture of H_2 and CO_2, and 128 mL CH_4/g VS in vials sparged with CO_2), when compared to other biomass feedstocks such as food waste supplemented with CO_2 (195 mL CH_4/g VS) [55]. Even though biological methods do not require much capital due to the use of microorganisms to sequester CO_2, this method requires a long incubation period for methane production (from 3 to 25 days) as indicated in Table 5.

The economic value and potential from Table 5 shows that this study can be seen as one of the methods for CO_2 reduction with 77% $^{13}CH_4$ conversion from $^{13}CO_2$. This study can be applicable in industry due to its ability to sequester CO_2 for methane production without the requirement of high cost technology and high energy input. Therefore, methane energy produced by microorganisms has great economic potential with a significant contribution to sustained environment via CO_2 sequestration.

4. Conclusions

Biological methane production from CO_2 sequestration by enriched methanogens from waste activated sludge (WAS) was demonstrated in this study. This approach appears to be reasonable to mitigate global warming. The WAS that was dominated by an active bacterial community was changed to an active

archaeal community after the enrichment for methanogens as demonstrated by quantitative real time PCR (qRT-PCR). The diverse community of Archaea in the raw WAS then dominated by hydrogenotrophic and acetoclastic methanogens such as members of the *Methanobacteriaceae*, *Methanospirillaceae* and *Methanosarcinaceae* family in the enriched methanogen culture as demonstrated by high-throughput sequencing. Methane was not produced without CO_2 supplementation to the enriched methanogens due to the lack of available carbon for the archaeal community, and the carbon in the methane was shown to come from utilization of the CO_2 based on the use of $^{13}CO_2$. Therefore, application of this research can contribute to improving the environment by reducing the greenhouse gas CO_2.

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