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Biomethanation and anaerobic co-digestion via microbial communities of microalgal *Hydrodictyon reticulatum* biomass residues with sewage sludge

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ABSTRACT

The performance of co-digestion via the combination of microalgae residues and wasted activated sludge (WAS) was evaluated in batch and semi-continuous type anaerobic digestion (AD) reactors. Simultaneously, AD with WAS alone (R_1) and with the combination of raw microalgae and WAS (R_2) were conducted, respectively. In batch tests, compared with R_1 (344 mL-CH_4/g-VS), co-digestion of WAS with microalgae residues (R_3) achieved 40% higher methane yield (498 mL-CH_4/g-VS), while R_2 exhibited the lowest value of 148 mL-CH_4/g-VS. The semi-continuous type R_3 digester reduced higher volatile solids (VS; 39%) at an organic loading rate of 1.0 kg-VS/m^3d, producing the methane yield of 292 mL/g-VS d. The R_3 semi-continuous type digester had the highest concentrations of both total bacteria and archaea, showing a ratio of 1:1 among hydrogenotrophic and acetoclastic methanogens. The bacterial community was characterized as existence of the *Lactobacillus* genus as well as fermentative bacteria belonging to the *Clostridia* class syntrophically associated with hydrogenotrophic methanogens.

Keywords: Anaerobic co-digestion; Microalgae residues; Wasted activated sludge; Biogas; Microbial community

1. Introduction

Microalgae are increasingly viewed as a promising source of biomass for biofuel production including bioethanol, biodiesel and biomethane due to their potential for high biomass yields as well as the capability of carbon dioxide fixation and lipid accumulation [1]. However, biofuel production from microalgae entails high inputs of energy and costs for harvesting, lipid extraction and conversion processes [2]. During the steps in producing biofuel, microalgal biomass residues are generated from ethanol fermentation and lipid extraction. These residues, which still have remaining carbohydrates and lipids, allow further utilization as organic wastes [3].

Anaerobic digestion (AD) represents one potential option for producing bioenergy from microalgae residues. AD is a series of biochemical reactions producing methane and hydrogen from organic compounds [4,5]. Biogas as an end product of AD generally comprises ~60% methane, a potential source of heat or energy [6]. Energy production via AD of microalgae residues can partially offset the cost of biofuel conversion processes using microalgae. Moreover, wet microalgae residues can be directly utilized as a substrate in the AD process [1]. Microalgae residues pretreated for biofuel conversion are more fermentable in AD than raw microalgal biomass, which have high resistance to biodegradation [7]. Previous studies have reported the feasibility of AD using algal residues, showing higher methane potentials than using raw microalgae samples [3,8]. This can lead to

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more sustainable and efficient AD requiring no further steps nor incurring additional costs.

To encourage biogas production in AD, co-digestion has been applied with combinations of different organic wastes [9,10]. In wastewater treatment plants (WWTPs), microalgae have been used to provide benefits both in biofuel production and nutrient removal [1]. For efficient processing in co-digestion, microalgae residues generated from biofuel conversion offer an attractive option in combination with wasted activated sludge (WAS) in the existing waste stream of a WWTP, reducing costs but increasing energy yields. Thus, integrated biofuel production from microalgae could present a feasible platform in WWTPs as a useful energy recovery system.

Although anaerobic co-digestion of raw algal biomass with sewage sludge has been investigated [11,12], previous work has paid little attention to the events and microbial ecology in the co-digestion of microalgae residues with sewage sludge. Given the AD process relies on a balance between functioning groups of microbes, information about the microbial community would be helpful to better understand the interactions of those microbes involved in anaerobic co-digestion systems. In this study, batch and semi-continuous anaerobic reactors were operated under mesophilic conditions with a combination of microalgae residues and WAS. To evaluate the performance of co-digestion, batch and semi-continuous type AD reactors were operated simultaneously with WAS alone and with the combination of raw microalgae and WAS, respectively. To characterize bacterial and archaeal communities in the anaerobic digesters, a combination of different molecular techniques was applied using denaturing gradient gel electrophoresis (DGGE) for qualitative assay and quantitative polymerase chain reaction (qPCR) for quantitative assay.

2. Materials and methods

2.1. Biochemical methane potential test

To determine the anaerobic digestibility of organic waste mixed with WAS and microalgae residues, biochemical methane potential (BMP) tests were performed for 50 d in 160 mL serum bottles with a working volume of 100 mL. Methane production from the batch tests was compared by digestion of WAS alone to co-digestion of WAS with raw algal biomass and algal residues from bioethanol fermentation, respectively. In the anaerobic co-digestion, the ratio of mixtures with WAS and algal biomass was determined as 50:50 based on the concentration of VS. The WAS was collected from the Chunnang municipal WWTP in Seoul, Korea. The filamentous algae, *Hydrodictyon reticulatum* (*H. reticulatum*), and its residues saccharified for bioethanol production were used as algal biomass in the AD [8]. The bottles were inoculated with anaerobic sludge from an anaerobic digester in the same municipal WWTP. The inoculum and substrate were mixed at a ratio of 0.5:1.0 based on the concentration of VS. All BMP tests at 35°C ± 1°C and 120 rpm were performed in duplicate.

2.2. Semi-continuous anaerobic digestion test

Three lab-scale semi-continuous AD digesters were operated in 2 L glass bottles with a working volume of 1.5 L under mesophilic conditions (R_1, R_2 and R_3). The digesters were inoculated with the same anaerobic sludge used in the BMP tests. The control, the R_1 digester, was fed with 100% WAS alone as a substrate. To compare the performance of co-digestion, the R_2 digester was fed with a mixture of 50% WAS and 50% raw *H. reticulatum* biomass. The R_3 digester was supplied with a substrate, a combination of 50% WAS and 50% *H. reticulatum* biomass residues. The mixture ratio in co-digestion was based on the concentration of VS. During the operation of 85 d, feeding the substrate and the wasting digested sludge were manually conducted at rates reflecting variations in hydraulic retention time (HRT) (Table 1). Operating conditions of the digesters were divided into two phases according to the HRT. The digesters were operated for 32 d at an HRT of 13 d, corresponding to an organic loading rate (OLR) of 0.75 kg-VS/m^{3}d (Run 1). After confirmation of stable production of methane from the digesters, OLR was increased to 1.0 kg-VS/m^{3}d, corresponding to an HRT of 10 d (Run 2). The second phase was performed for 52 d (Table 1).

2.3. Chemical analysis

Total solids (TS), VS and chemical oxygen demand (COD) were analyzed according to standard methods [13]. The contents of C, H, O, N and S in the microalgae and algal residues were determined using an elemental analyzer (2400 Series

Table 1
Operational conditions of semi-continuous digesters

Run	Digester[a]	Time (d)	HRT (d)	OLR (kg-VS/m^{3}d)	Mixture ratio (%)		
					WAS	*H. reticulatum*	Algae residues
1	R_1	0–32	13	0.75	100	0	0
	R_2				50	50	0
	R_3				50	0	50
2	R_1	33–85	10	1	100	0	0
	R_2				50	50	0
	R_3				50	0	50

[a]Substrates mixtures of each digester, R_1: wasted activated sludge (WAS); R_2: raw *H. reticulatum* + WAS; R_3: *H. reticulatum* residues + WAS.

II CHNS/O system, PerkinElmer Instrument, USA). Biogas production was measured by gas chromatography (HP 5890, PA, USA) equipped with a thermal conductivity detector and helium as a carrier gas. The injector was operated in splitless mode (column flow: 19 mL/min). The temperatures of the oven, injector and detector were 35°C, 150°C and 180°C, respectively [14]. All the chemical measurements were carried out in duplicate.

2.4. DNA extraction

Total DNA from the sludge was extracted and purified using a Nucleo Spin® Soil kit (MACHEREY–NAGEL, Germany) according to the manufacturer's protocol. Purified DNA was eluted with 100 μL of Tris–HCl buffer (pH 8.0) and stored at –20°C for further analyses.

2.5. DGGE and sequencing analysis

Bacterial communities were analyzed via polymerase chain reaction (PCR)–DGGE using a primer set BAC338F/805R with a GC-clamp as previously described (Table 2) [15]. The PCR protocol was performed as follows: (1) initial denaturation at 95°C for 10 min; (2) 30 cycles of 95°C for 5 min, 55°C for 30 s and 72°C for 30 s; and (3) a final extension at 72°C for 10 min. The PCR product was loaded onto each well of an 8% (w/v) acrylamide gel (acrylamide:bisacrylamide solution, 37.5:1) containing a 30%–60% denaturant gradient. Electrophoresis was performed in 0.5x Tris-acetate-EDTA buffer for 720 min at 100 V and 60°C.

2.6. qPCR analysis

To quantify total bacterial 16S rRNA gene copy numbers, qPCR amplification and fluorescence detection were conducted using an Applied Biosystems 7300 qPCR system (Applied Biosystems, Forster City, USA) with six primer and probe sets targeting 16S rRNA genes of different microbial groups, the domains bacteria and archaea, and the methanogenic orders *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, and *Methanosarcinales* (Table 2) [15]. The qPCR was conducted in duplicate on a thermal cycler using the following protocol: (1) 95°C for 10 s and (2) 40 cycles of 95°C for 5 s, 56°C for 10 s and 72°C for 27 s (fluorescence detection step).

2.7. Statistical analysis

To predict methane yields of the combinations of substrates with WAS and microalgae in the AD, the values of cumulative methane production were analyzed by the modified Gompertz model using the following equation:

$$M = P \times \exp\left\{-\exp\left[\frac{R_m \cdot e}{P}(\lambda - t) + 1\right]\right\} \quad (1)$$

where M is the cumulative methane production; P is the methane production potential; R_m is the methane production rate and λ is the lag phase time.

The significance levels of 1% ($p < 0.01$) and 0.01% ($p < 0.0001$) were considered for estimated parameter and regression model, respectively. Sigmaplot 8.0 (Systat Software Inc., San Jose, USA) software was used for the statistical analysis.

3. Results and discussion

3.1. Digestibility of microalgae residues in batch and semi-continuous anaerobic digesters

Fig. 1 represents cumulative methane production in AD with mixtures of different wastes (WAS + raw microalgae and WAS + microalgae residues) and WAS alone. Compared with AD with WAS alone (344 mL-CH_4/g-VS), co-digestion of WAS with microalgae residues achieved 40% higher methane yield (498 mL-CH_4/g-VS), while co-digestion with WAS with raw microalgae exhibited a 60% decrease (148 mL-CH_4/g-VS).

Table 2
Detailed information of the primers used in the study of Shin et al. [15]

Target group	Primers	Sequence	Annealing temperature (°C)
Bacteria	F: BAC338F	ACTCCTACGGGAGGCAG	55.0
	R: BAC805R	GACTACCAGGGTATCTAATCC	
Archaea	F: ARC787F	ATTAGATACCCSBGTAGTCC	
	R: ARC1059R	GCCATGCACCWCCTCT	
Methanobacteriales	F: MBT857F	CGWAGGGAAGCTGTTAAGT	60.0
	R: MBT1196R	TACCGTCGTCCACTCCTT	
Methanococcales	F: MCC495F	TAAGGGCTGGGCAAGT	
	R: MCC832R	CACCTAGTYCGCARAGTTTA	
Methanosarcinales	F: MSL812F	GTAAACGATRYTCGC	
	R: MSL1159R	GGTCCCCACAGWGTACC	
Methanomicrobiales	F: MMB282F	ATCGRTACGGGTTGTGGG	63.0
	R: MMB832R	CACCTAACGCRCATHGTTTAC	

These results indicate that anaerobic co-digestion of WAS with algae residues could be viewed as a potential energy source. Generally, raw algae are known as biomass possessing poor digestibility due to high resistance of the algal cell wall's cellulose or hemicellulose structure [16], leading to the lowest biomethanation of mixtures with raw algae and WAS (Fig. 1). On the other hand, microalgae residues offer more readily accessible substrates for microbes, due to higher amounts of soluble organic matter released from disintegrated microalgae [8]. The microalgae *H. reticulatum* residues used in this study had already been hydrolyzed with a combination of enzyme and acid during bioethanol fermentation. Algae biomass pretreated using ultrasound, alkaline and thermal methods has shown further increases in the rate of methane production in AD systems [7,17].

To predict methane yields of the combinations of substrates with WAS and microalgae in the AD, the values of cumulative methane production were analyzed by the modified Gompertz model. Mixed substrates of WAS and microalgae residues displayed both the highest potentials (P) and rates (R_m) of methane production (Table 3), leading to an increase in anaerobic digestibility of sewage sludge when combined with microalgae residues. Co-digestion of sewage sludge could be enhanced through a balance of nutrients by adding readily biodegradable carbon such as microalgae residues [10]. Results from the batch digesters show that integrated management of microalgae residues with sewage sludge might provides a viable platform in WWTPs as an energy recovery system by improving methane production in AD, along with simultaneous management of sewage sludge and microalgae residues. As can be seen in Table 3, AD of WAS with microalgae residues produced approximately 3 times greater R_m than that of WAS alone, while AD of a mixture of WAS and raw microalgae brought a 20% decrease in R_m. To optimize biogas production by facilitating substrate utilization, the ideal mixing ratio of WAS and microalgae residues needs to be determined in future research.

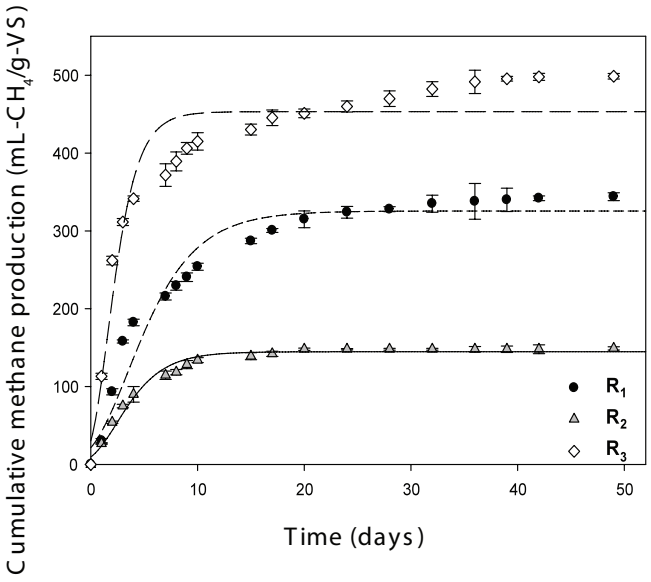

Fig. 1. Cumulative methane production in batch type anaerobic digestion with mixtures of different wastes: R_1: wasted activated sludge (WAS); R_2: raw *H. reticulatum* + WAS; and R_3: *H. reticulatum* residues + WAS.

Studies of semi-continuous anaerobic digesters were conducted to evaluate the long-term performance of digesters (Table 4 and Fig. 2). R_1 was operated as a control with feeding WAS alone, while R_2 and R_3 were conducted as co-digestion of WAS with raw algae and algal residues from bioethanol fermentation, respectively (Tables 1 and 4). During Run 1, each digester was initially operated at an OLR of 0.75 kg-VS/m^{3}d, which was then increased to 1.0 kg-VS/m^{3}d for operation of Run 2. All digesters (R_1, R_2 and R_3) at an OLR in the range of 0.75–1.0 kg-VS/m^{3}d demonstrated stable performance, maintaining consistent values of pH, alkalinity, VS and volatile fatty acids in the effluent from the digesters.

Concentrations of VS in influent and effluent showed similar patterns among the digesters as shown in Table 4. Average concentrations of VS in the influent of all digesters were around 9,600 ± 622 mg/L; concentrations of discharging VS in the effluent, in the range of 5,576–6,275 mg/L. These results corresponded to a VS reduction (VSR) of 36%–44%. A higher VSR was obtained in the R_3 feeding mixtures with WAS and microalgae residues, while the R_2 digester showed a lower VSR (Table 4). The substrate of R_3 contained around 2.5 times higher levels of soluble COD than those of the other digesters, but the R_3 digester produced removal efficiency of 95%, leading to higher methane production (Table 4 and Fig. 2). Concentrations of total N and total P in the effluent of all digesters surpassed those in the influent (data not shown). Ammonium ion concentrations were also detected to be higher in the effluents (in the range of 278–392 mg-N/L) than in the influents. Concentrations of acetic acid in the effluent of all digesters were low in the range of 33–39 mg/L, indicating stable digester performance and synergism of hydrolysis and methanogenic microorganisms. Operation of the three digesters until attaining an OLR of 1.0 kg-VS/m^{3}d did not cause any accumulation of volatile fatty acids, showing consistent pH values of around 7.0 ± 0.2.

Table 3
Parameters of Gompertz model obtained from fitting methane production curve

Substrates	P (mL/g-VS)	R_m (mL/g-VS d)	λ (d)	R^2	p Value
WAS	345.9	27.5	<0.01	0.95	<0.0001
WAS + raw microalgae[a]	147.6	21.8	<0.01	0.98	<0.0001
WAS + microalgae residues[b]	465.5	77.6	<0.01	0.92	<0.0001

[a]*H. reticulatum*.
[b]*H. reticulatum* residues from bioethanol fermentation.
Note: P: methane production potential; R_m: methane production rate; and λ: lag phase time.

Table 4
Chemical parameters of influent and effluent from semi-continuous digesters

Period	Run 1 (days 0–32), OLR 0.75 kg-VS/m^{3}d						Run 2 (days 33–85), OLR 1.0 kg-VS/m^{3}d					
Digesters	R_1		R_2		R_3		R_1		R_2		R_3	
Parameters	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
TS (mg/L)	13,313 ± 367	8,639 ± 636	11,737 ± 313	7,601 ± 398	13,203 ± 127	8,171 ± 611	13,206 ± 598	9,089 ± 280	11,206 ± 858	7,413 ± 178	13,057 ± 113	8,631 ± 262
VS (mg/L)	9,993 ± 380	6,139 ± 373	8,803 ± 384	5,576 ± 419	10,037 ± 98	5,789 ± 432	9,970 ± 55	6,275 ± 280	8,775 ± 544	5,620 ± 177	9,977 ± 100	6,050 ± 263
VSR (%)	N.D.	38.5 ± 4.3	N.D.	35.7 ± 6.9	N.D.	43.8 ± 2.2	N.D.	37.1 ± 3.7	N.D.	35.9 ± 7.7	N.D.	39.3 ± 7.8
tCOD (mg/L)	16,594 ± 60	8,919 ± 458	13,620 ± 60	7,750 ± 155	19,004 ± 68	8,500 ± 526	16,253 ± 151	8,881 ± 495	13,318 ± 151	6,700 ± 240	19,245 ± 144	8,216 ± 465
sCOD (mg/L)	2,279 ± 20	439 ± 107	2,279 ± 202	319 ± 75	5,953 ± 31	351 ± 47	2,339 ± 101	505 ± 69	2,339 ± 101	293 ± 45	6,518 ± 28	328 ± 63
sCOD removal (%)	N.D.	81.7 ± 4.8	N.D.	79.7 ± 3.3	N.D.	94.1 ± 0.8	N.D.	78.3 ± 2.1	N.D.	76 ± 1.6	N.D.	95.2 ± 0.9

Note: R_1: wasted activated sludge (WAS); R_2: raw *H. reticulatum* + WAS; R_3: *H. reticulatum* residues + WAS; and N.D.: Not detectable.

During Run 1, average biogas productions for R_1, R_2 and R_3 were 802 ± 342 mL/d, 723 ± 290 mL/d and 1,080 ± 437 mL/d, respectively (Fig. 2). The ratio of average methane in biogas from R_1 (65%) and R_3 (67%) was similar, irrespective of the amount of biogas production, but R_2 (53%) produced 10% less methane ratio in biogas than the others. Average methane yields of R_3 were 244 ± 100 mL/g-VS d, indicating 1.8 times higher methane yields than for R_2. Feeding WAS alone (R_1) also produced 50% less methane than R_3, indicating the beneficial effects of co-digestion of WAS and algal biomass residues. An increase in OLR (Run 2) led to improved biogas production for all digesters (Fig. 2), showing around 1.7 times higher production than during Run 1. However, variations in OLR did not affect the ratio of average methane in biogas, maintaining the ratio during Run 1. Average methane yields for all digesters were improved approximately 20% by increasing the OLR and achieved around 50% higher yields from R_3 than those from R_1 and R_2 (Fig. 2). These results support co-digestion of WAS with algal residues as an efficient way to recover energy, thereby reducing the costs of microalgae biofuel production.

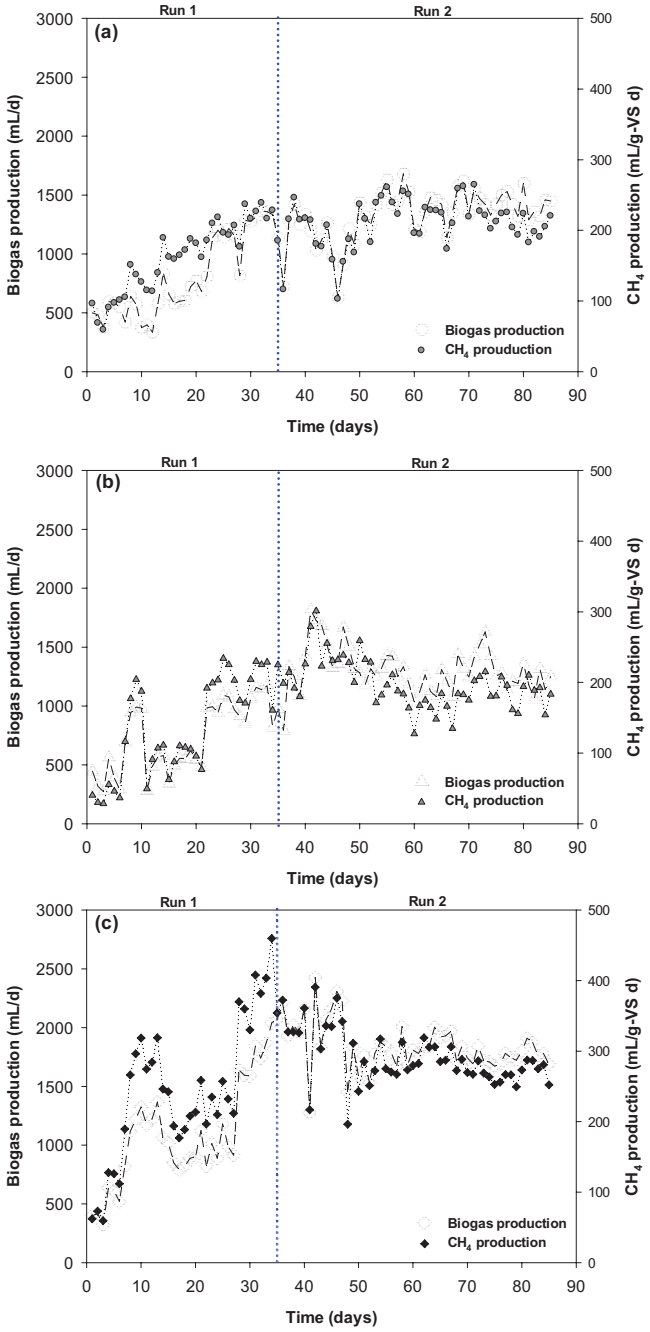

Fig. 2. Biogas production from semi-continuous digesters at different ORLs: (a) R_1: wasted activated sludge (WAS); (b) R_2: raw *H. reticulatum* + WAS; and (c) R_3: *H. reticulatum* residues + WAS.

3.2. Microbial community

Bacterial communities in the semi-continuous anaerobic digesters were analyzed according to the DGGE results (Fig. 3 and Table 5). The DGGE profiles were retrieved at the end point of Run 2 stage for each digester. The pattern of bacterial DGGE bands did not indicate big differences among the three digesters, illustrating less dynamic variations in the bacterial community structure originating from WAS used as an inoculum (Fig. 3). However, a lower number of bacterial DGGE bands were observed in the R_2 digester (10 bands), indicating a less diverse community compared with R_1 and R_3 (each having 13 bands). This might be associated with both lower VSR and methane production from R_2.

The 14 bacterial sequences mostly comprised the phyla *Bacteroidetes* (bands A and N), *Firmicutes* (bands B, G, I, L and M) and *Actinobacteria* (bands D, J and K) (Table 5). The predominant bacterial group, *Firmicutes*, was known to metabolize a variety of substrates including protein, lipids, cellulose, sugars and amino acids, all common constituents of organic wastes [18]. This *Fimicutes* group was observed less in the R_2 digester (not shown band G and M), reflecting a less enhanced fermentation step in R_2. Among the various bands, B and I detected in all samples (Fig. 3) were affiliated with the order *Clostridiale*. These members are frequently found in AD processes [19] associated with diverse AD pathways especially relating with hydrolysis and H_2 production [20]. *Clostridium ultunense* sp. (band I) are known as a mesophilic bacterium oxidizing acetate in syntrophic association with hydrogenotrophic methanogens [21]. *Syntrophomonas wolfei* sp. corresponding to band B, which appeared with high intensity, have also been widely reported as an anaerobic, syntrophic, fatty acid oxidizing bacteria in partnership with hydrogenotrophic *Methanoculleus* and *Methabacterium* methanogens in anaerobic digesters treating sewage sludge and swine manure [22,23]. Members belonging to the *Lactobacillus* genus affiliated with band L are known to have metabolic pathways producing lactic acid or other acids as a primary or end product of fermentation [15]. These results support that existence of the *Lactobacillus* genus as well as fermentative bacteria belong to the *Clostridia* class may be syntrophically associated with hydrogenotrophic methanogens in semi-continuous anaerobic digesters.

In all the lanes of the bacterial DGGE gel (Fig. 3), bands G, H and M were detected only in R_1 and R_3 digesters. Band G was closely related to *Acetoanaerobium* sp. with 99% sequence similarity (Table 5). These acetogenic

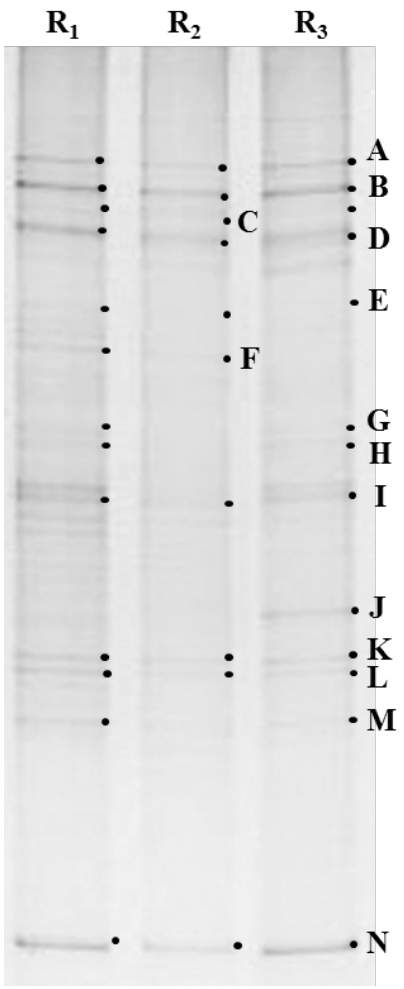

Fig. 3. Bacterial DGGE profiles analyzed from semi-continuous anaerobic digesters. Fourteen bacterial DGGE bands were excised for sequencing analysis. R_1: wasted activated sludge (WAS); R_2: raw *H. reticulatum* + WAS; and R_3: *H. reticulatum* residues + WAS. (For more detailed microbial information for each labels (A to N), please refer to the Table 5.)

bacteria are strictly anaerobic microorganisms, which play a vital role in catalyzing the formation of acetate from hydrogen and carbon dioxide [24]. The acetogens, *Acetoanaerobium* sp., are syntrophs participating in an interspecies hydrogen transfer process maintaining low hydrogen concentrations [25]. Moreover, acetate produced by *Acetoanaerobium* sp. might support growth of the acetoclastic methanogens, *Methanosarcinales*, detected in this study (Fig. 4). *Syntrophus* affiliated with band H has been described as syntrophic benzoate oxidizing bacteria coupled with *Methanoculleus* as H_2 and *Methanosaeta* as an acetate utilizer in AD [23]. Through partnering with both methanogens, *Syntrophus* species might syntrophically contribute to both the acetoclastic and hydrogenotrophic pathways by providing available substrates. *Fusibacter* sp., corresponding to band M, has represented an enriched anaerobic fermentation community utilizing carbohydrates and then producing acetate and butyrate [26]. These diverse bacterial communities (*Acetoanaerobium*, *Syntrophus* and *Fusibacter*) in the reactors of R_1 and R_3 might be synergistically involved in the additional degradation of complex organic matter or higher methane production.

Concentrations of the 16S rRNA gene within the target microbial groups in the semi-continuous digesters are shown in Fig. 4. Based on the 16S rRNA gene concentrations, co-digestion (R_3) of the WAS and algal biomass residues indicated both the highest bacterial (9.9×10^8 copies/mL) and archaeal (9.7×10^7 copies/mL) populations. The lowest bacterial and archaeal amounts were detected in the co-digestion (R_2) of WAS and raw microalgae (Fig. 4), reflecting the reactors' digestibility depending on having access to their substrates. In all the digesters, methanogens belonging to orders *Methanosarcinales*, *Methanobacteriales* and *Methanomicrobiales* constituted more than 97% of the concentrations of total archaeal 16S rRNA genes. *Methanococcales* was not detected in any digesters.

Concentrations of the acetoclastic *Methanosarcinales* 16S rRNA gene accounted for ~50% of total methanogen in the digester of WAS and algal biomass residues (R_3). The remaining 50% methanogens consisted of hydrogenotrophic *Methanomicrobiales* and *Methanobacteriales* (Fig. 4). This demonstrates that methane formation in the co-digestion of WAS and algal biomass residues in this study may be dependent equally on the acetoclastic and hydrogenotrophic pathways. In the digesters of R_1 and R_2, hydrogenotrophic methanogens were dominant, indicating concentrations of two hyrogenotrophic orders *Methanomicrobiales* and *Methanobacteriales* being 3–4 times higher than those of acetoclastic *Methanosarcinales*. Thus, one may conclude that the main mechanism of methane production in the digesters of R_1 and R_2 might be largely dependent on hydrogenotrophic pathways associated with *Clostridia* populations detected in this study. The ratio of two hydrogenotrophic methanogens (*Methanomicrobiales* and *Methanobacteriales*) was nearly 1:1 in the semi-continuous digester of R_1 feeding only WAS, but in the semi-continuous digester of R_2 feeding WAS and raw microalgae, *Methanomicrobiales* was 3 times more prevalent. Based on the qPCR results in Fig. 4, differences in bacterial and archaeal populations closely reflected the reactors' performance such as digestibility as well as methane production. Depending on the digestibility of substrates, characterized archaeal communities revealed different methane producing pathways.

4. Conclusions

Co-digestion of WAS with algal residues resulted in the highest methane yields as well as VSR. These results supported both the highest bacterial and archaeal populations. A more diverse bacterial community was observed such as *Acetoanaerobium Syntrophus* and *Fusibacter* sp. The ratio of hydrogenotrophic methanogens (*Methanomicrobiales* and *Methanobacteriales*) and acetoclastic methanogen (*Methanosarcinales*) was nearly 1:1, demonstrating equal dependence on both acetoclastic and hydrogenotrophic pathways in the co-digestion of WAS with algal residues. These results show that integrated management of microalgae residues with sewage sludge may offer a viable platform in WWTPs as an energy recovery system.

Table 5
Phylogenetic affiliation of the 16S rRNA gene sequences from DGGE bands

Bands	Closest sequence	Accession number	% Similarity	Class/Phylum
A	*Prolixibacter bellariivorans* strain JCM 13498	NR_113041	99	Unclassified/*Bacteroidetes*
B	*Syntrophomonas wolfei* subsp. saponavida strain DSM4212	NR_115849	99	*Clostridia/Firmicutes*
C	Uncultured bacterium clone LBAC134	KJ601176	99	Unclassified bacteria
D	*Bifidobacterium aerophilum*	AY174104	100	*Actinobacteria/Actinobacteria*
E	Uncultured bacterium gene clone UA01	AB456223	100	Unclassified bacteria
F	Uncultured *Betaproteobacteria* bacterium clone QEDN10DF12	CU926928	99	*Betaproteobacteria/Proteobacteria*
G	*Acetoanaerobium* sp. WJDL-Y2	KF176997	99	*Clostridia/Firmicutes*
H	*Syntrophus* sp. Clone B3	AJ133796	100	*Deltaproteobacteria/Proteobacteria*
I	*Clostridium ultunense* strain Esp	GQ487664	99	*Clostridia/Firmicutes*
J	*Streptomyces* sp. 13 (2014)	KJ573803	99	*Actinobacteria/Actinobacteria*
K	*Nocardioides* sp. DN36	AB508351	99	*Actinobacteria/Actinobacteria*
L	*Lactobacillus panis* strain FQ084	KF418828	100	*Bacilli/Firmicutes*
M	*Fusibacter* sp. enrichment culture clone 22-7A	EU517558	99	*Clostridia/Firmicutes*
N	Uncultured *Bacteroidetes* bacterium clone PG-5-1-3-L	EU626571	99	Unclassified/*Bacteroidetes*

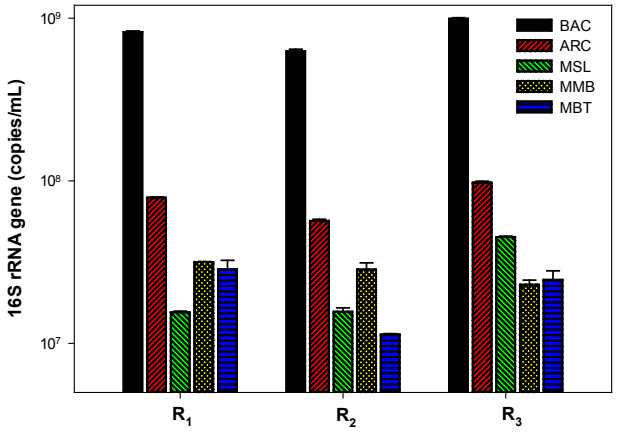

Fig. 4. Bacterial, archaeal and methanogenic 16S rRNA gene concentrations in semi-continuous digesters: BAC: Bacteria; ARC: Archaea; MSL: *Methanosarcinales*; MMB: *Methanomicrobiales*; MBT: *Methanobacteriales*; R_1: wasted activated sludge (WAS); R_2: raw *H. reticulatum* + WAS; and R_3: *H. reticulatum* residues + WAS.

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