Thermophilic two-phase anaerobic digestion of source-sorted organic fraction of municipal solid waste for bio-hythane production: effect of recirculation sludge on process stability and microbiology over a long-term pilot-scale experience

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ABSTRACT

A two-stage thermophilic anaerobic digestion process for the concurrent production of hydrogen and methane through the treatment of the source-sorted organic fraction of municipal solid waste was carried out over a long-term pilot scale experience. Two continuously stirred tank reactors were operated for about 1 year. The results showed that stable production of bio-hythane without inoculum treatment could be obtained. The pH of the dark fermentation reactor was maintained in the optimal range for hydrogen-producing bacteria activity through sludge recirculation from a methanogenic reactor. An average specific bio-hythane production of 0.65 m^3 per kg of volatile solids fed was achieved when the recirculation flow was controlled through an evaporation unit in order to avoid inhibition problems for both microbial communities. Microbial analysis indicated that dominant bacterial species in the dark fermentation reactor are related to the *Lactobacillus* family, while the population of the methanogenic reactor was mainly composed of *Defluviitoga tunisiensis*. The archaeal community of the methanogenic reactor shifted, moving from *Methanothermobacter*-like to *Methanobacteriales* and *Methanosarcinales*, the latter found also in the dark fermentation reactor when a considerable methane production was detected.

Key words | bio-hythane, dark fermentation, food waste, free ammonia inhibition, microbial community

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INTRODUCTION

The two-stage anaerobic digestion (AD) system is a promising technology since it allows for efficient stabilization of organic biodegradable waste, providing, at the same time, a valuable energy carrier, bio-hythane, characterized by a hydrogen/methane (H_2/CH_4) ratio suitable for improving both combustion engine performance and environmental impact (Liu *et al.* 2006; Porpatham *et al.* 2007). In the two-stage AD system, H_2 production occurs in a first reactor through the dark fermentation process that involves strict or facultative anaerobic bacteria (Guo *et al.* 2010), while CH_4 production takes place in a second reactor fed with the acidic effluent that comes from the first reactor.

Liu *et al.* (2013) reviewed the feasibility of bio-hythane production through the two-stage AD system and optimal design parameters such as pH, hydraulic retention time (HRT) and temperature, related to different biomass and process configurations. One of the main bottlenecks, which still makes the scale-up of the two-stage process expensive, is the alkali addition in the dark fermentation reactor in order to maintain the optimal pH range for efficient H_2 production (Valdez-Vazquez & Poggi-Varaldo 2009). A cost-effective way to avoid external pH control in the dark fermentation reactor is to recirculate the effluent of the methanogenic reactor, since it is characterized by a high buffer capacity (Cavinato *et al.* 2011; Chinellato *et al.* 2013). However, in long-term trials this choice may affect the reliability of the process because of the increase in ammonia concentration due to the high hydrolysis rate of food waste

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causing inhibition in both reactors (Cavinato *et al.* 2012). Among the different microorganisms of the anaerobic trophic chain, the methanogens are the least tolerant, and free ammonia (NH_3) rather than total ammonia nitrogen (TAN) has been suggested to be the main cause of inhibition because of its ability to penetrate the cell membrane, causing proton imbalance and potassium deficiency (Sung & Liu 2003; Chen *et al.* 2008). However, there is conflicting information in literature about the sensitivity of both acetoclastic and hydrogenotrophic methanogens, since the ammonia inhibition level depends on different factors such as inocula, environmental conditions and acclimation level (Chen *et al.* 2008). Another important aspect that may affect the reliability of the process in long-term operation is the development of H_2-consuming microorganisms in the recirculation flow, which can contaminate the dark fermentation reactor, negatively affecting the syntrophic association with H_2-producing microorganisms for suitable H_2 production (Wang & Wan 2009). Several strategies have been adopted to avoid the involvement of H_2-consuming microorganisms in the first dark fermentation reactor, such as thermal treatment or chemical treatment of the recirculated digested sludge (Guo *et al.* 2010), but they significantly increase operation costs. In order to ensure the stability of the microbial communities and to design a strategy for enhanced bio-hythane production, it is important to understand microflora development during the experimental test, which depends on the operating conditions adopted (Wang & Wan 2009).

The aim of the present study is to provide information about the natural selection of the microbial communities structure involved in a two-stage thermophilic AD approach for H_2 and CH_4 co-production, treating the source-sorted organic fraction of municipal solid waste (SS-OFMSW) and applying the recirculation of the methanogenic reactor liquid effluent as buffer supplier in the dark fermentation reactor. Ammonia accumulation in the system was controlled by an evaporation unit and the microbial community structure was investigated in a long-term experimental test.

MATERIALS AND METHODS

Substrates, inoculum and operating conditions

A schematic diagram of the proposed process in this study is given in Figure 1.

The pilot trial was carried out in two continuous stirred tank reactors fed with the food waste collected daily at the integrated waste and wastewater treatment plant of Treviso municipality (North Italy). The incoming organic waste was mechanically pretreated in order to remove the inert fraction (plastics, metals, glass, etc.) (Bolzonella *et al.* 2006) and showed an average total solids (TS) content of about 270 g kg^{-1} with an inert fraction of less than 5% of total wet weight. The dark fermentation reactor (volume = 0.2 m^3) was inoculated with a mixture of minced organic waste and tap water resulting in TS content of about 8%, while the methanogenic reactor ($V = 0.76$ m^3) was inoculated with an active mesophilic biomass collected from a full-scale anaerobic digester which co-treats both SS-OFMSW and waste activated sludge.

The design parameters of the thermophilic (55 °C) two-stage process are reported in Table 1.

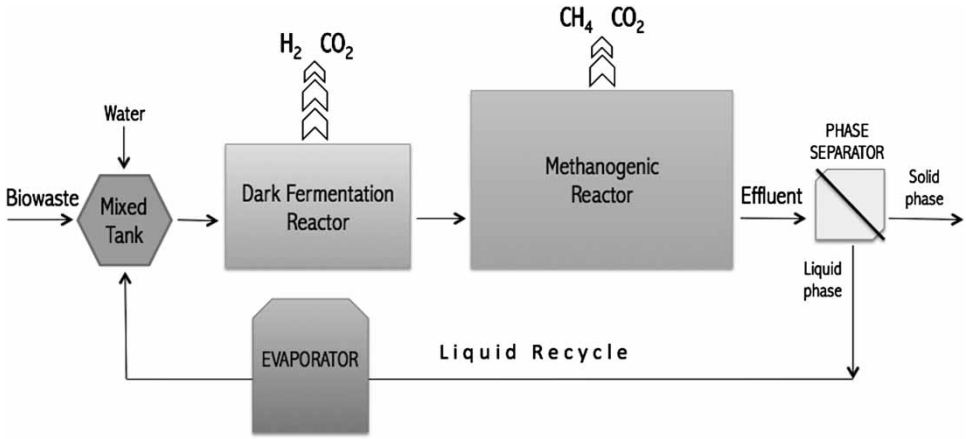

Figure 1 | Flow scheme of two-stage technology adopted for bio-hythane production.

Table 1 | Operating conditions of two-stage AD process

	RUN 1 (0–90 days)	RUN 2 (91–345)
Evaporator	No	Yes
Dark fermentation reactor		
HRT (days)	3.3	3.3
OLR (kg TVS $m^3 d^{-1}$)	16–18	16–18
T (°C)	55	55
V (m^3)	0.2	0.2
Methanogenic reactor		
HRT (days)	12.6	12.6
OLR (kg TVS $m^3 d^{-1}$)	4–5	4–5
T (°C)	55	55
V (m^3)	0.76	0.76

Maintenance periods: 180–200 days; 220–240 days.

The authors' previous studies (Cavinato *et al.* 2011) have demonstrated that the best performance in terms of bio-hythane production was obtained with an organic loading rate (OLR) of about 16 kg TVS $m^{-3} d^{-1}$ and HRT of 3 days in the first fermentative reactor resulting in 4.2 kg TVS (total volatile solids) $m^{-3} d^{-1}$ applied in the second methanogenic reactor, with a retention time of 12 days. In accordance with these results, a long-term operation was designed in order to asses process stability (Cavinato *et al.* 2012). Recirculation of anaerobic digested sludge was applied from the methanogenic reactor to the dark fermentation reactor after a mild solids separation, in order to support the fermentative step with alkalinity and to keep the pH in the optimal range (5–6) for thermophilic hydrogen-producing microorganisms (Guo *et al.* 2010). The process was continuously operated for 345 days with the exception of two maintenance periods (from 180 to 200 and from 220 to 240 days). The long-term experience was divided into two runs based on different recirculation strategies: during RUN1, the recirculation flow was fixed as half of the total flow rate fed (data published, Cavinato *et al.* 2012) while during RUN2 the recirculation flow was treated by an evaporation unit (R150v3, Veolia Water S&T, Italy). The operation time unit was set daily in order to avoid ammonia accumulation. Ammonia removal was dynamically controlled depending on daily stability behavior observed through pH, volatile fatty acids (VFAs) and alkalinity values.

Chemical analysis

The substrates and the effluents of both reactors were monitored weekly according to *Standard Methods* (APHA 1998) in terms of TS, TVS, chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN) and phosphorus (P), whereas the process stability parameters, namely pH, VFAs, alkalinity and TAN, were checked at least three times per week. VFA concentration (acetate, propionate, i-butyrate, butyrate, i-valerate, valerate, i-caproate, caproate and heptanoate) was monitored using a gas chromatograph (Carlo Erba instruments) according to the procedure given in Cavinato *et al.* (2011). NH_3 concentration was calculated according to the formula given in Angelidaki & Ahring (1994).

Microbial analysis

Molecular analysis of microbial communities was performed on the triplicate samples at two different times, when the process showed good performance in terms of bio-hythane production and composition: during RUN1 at the end of start-up phase (day 35) and at the steady-state conditions (SSC) of RUN2 (day 300). Bacterial 16S rRNA genes were selectively amplified using F8/R11 primers (Weisburg *et al.* 1991) with the following thermocycling program: initial denaturation at 94 °C for 2 min; 30 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 30 s and extension at 72 °C for 2.5 min; final extension at 72 °C for 5 min. Afterwards a nested polymerase chain reaction (PCR) was performed on the hyper-variable V3 region of the 16S rRNA gene using primers P3 (with a GC clamp) and P2 (Muyzer *et al.* 1993); conditions were as above, except for number of cycles, 35, the annealing temperature, 57 °C, and extension time, 35 s. For *Archaea*, primers A109-f and A934b-r (Grosskopf *et al.* 1998) were used for nearly complete 16S rRNA gene amplification. Afterwards a nested PCR was performed on the hypervariable V2–V3 region using primers A109(T)-f and 515-GC-r (Roest *et al.* 2005), with a GC clamp. The first archaeal PCR reaction was performed with the following thermocycle program: initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for 30 s, extension at 72 °C for 1 min; and final extension at 72 °C for 5 min. The nested PCR was as above but with 35 cycles. The PCR products were quantified using Low DNA Mass Ladder (Celbio, Italy) in a 2.0% agarose gel. Denaturing gradient gel electrophoresis (DGGE) analyses were performed in duplicate on amplicons obtained both for bacterial V3 and archaeal V2–V3 regions. Gels (8% acrylamide/bisacrylamide 19:1, BioRad) were cast using a denaturing gradient of 30–60%, with 100% denaturant defined as 7 mol L^{-1} urea and 20% (v/v) formamide. Electrophoresis was performed at 45 V for 18 h at 65 °C with the Dcode® Universal Detection

System (Biorad) and gels were stained with ethidium bromide (1 mg L^{-1}). Representative DGGE bands were excised and incubated for 4 h in 50 mL of sterile water. DGGE bands containing DNA to be sequenced were re-amplified. PCR amplification was carried out as described before, except for the use of non-GC-clamped primers. PCR products were transformed in *Escherichia coli* DH5α using the pGEM-T vector system according to the manufacturer's instructions (Promega, Italy), sequenced on both strands, and finally searched for homology using the BLASTN database (Altschul *et al.* 1997). Similarity of the sequences with Type Strains was checked using EzTaxon server 2.1 (Chun *et al.* 2007). The sequences were initially aligned using the multiple alignment program CLUSTAL_X 1.83 (Thompson *et al.* 1997). A phylogenetic tree was constructed using the neighbour-joining method with the MEGA version 5.1 software package (Tamura *et al.* 2011). Bootstrap analysis was performed from 1,000 bootstrap replications. Chimeras were checked using the DECIPHER database (Wright *et al.* 2012).

Sequenced bands have been submitted to the GenBank database with accession numbers from KJ209714 to KJ209727.

Fluorescence *in-situ* hybridization (FISH) analysis was performed to investigate the presence of *Archaea* population in the dark fermentation reactor at 300 days, when significant CH_4 production was detected. The samples were centrifuged in order to separate the biomass from undigested food waste materials; supernatant was removed and replaced with phosphate buffer saline solution. The separated microbial biomass was then fixed with 4% of paraformaldehyde solution and used for FISH analysis (Daims *et al.* 2005). The oligonucleotide probes as detailed in Table 2 and the hybridization stringency were chosen based on Banks *et al.* (2012). The hybridized samples were observed using Leica DMRX epifluorescence microscopy. To quantify the archaeal population, Daime 1.3.1 software was used (Daims *et al.* 2006).

RESULTS AND DISCUSSION

Process stability

The stability of the process was evaluated considering the temporal profile of the pH, VFAs, alkalinity, biogas production and composition. The data obtained during RUN1 concerning process stability have already been exhaustively described in the authors' previous publication, where, after 30 days, the gas mixture produced by the reactors met the typical composition of bio-hythane (Cavinato *et al.* 2012).

Throughout the overall experimental trials, the behavior of the dark fermentation reactor was stable, showing an average specific gas production (SGP) of 0.175 m^3 kg TVS^{-1} with H_2, CH_4 and CO_2 content of 31, 11 and 58% (on average) respectively (Figures 2(a) and 2(b)).On the other hand the SGP of the methanogenic reactor fe significantly at 85–109 days reaching values of less than 0.1 m^3 kg TVS^{-1}.

The increase, over time, of TAN (Figure 2(c)), which was formed during the degradation of proteinous organic materials, was observed. It is well known that NH_3 may restrain the growth rate of anaerobic microflora. Since the fraction of NH_3 increases with both temperature and pH, its inhibition effect on thermophilic methanogens may become significant under specific conditions. Moreover, the authors

Table 2 | Oligonucleotide probes used with target groups

Probe name	Probe sequence (5′–3′)	Stringency (%)	Target group	Fluoro-Chrome
EUB338	GCTGCCTCCCGTAGGAG	0–50	Bacteria domain	FITC
Arch915	GTGCTCCCCCGCCAATTCCT	35	Archaea	CY3
MX825	TCGCACCGTGGCCGACACCTAGC	50	*Methanosaetaceae*	CY3
MS1414	CTCACCCATACCTCACTCGGG	50	*Methanosarcinaceae*	CY3
hMS1395	GGTTTGACGGGCGGTGTG	–	MS1414-helper	–
hMS1480	CGACTTAACCCCCCTTGC	–	MS1414-helper	–
MB1174	ACCGTCGTCCACTCCTTCCTC	45	*Methanobacteriaceae*	FITC
MG1200	CGGATAATTCGGGGCATGCTG	30	*Methanomicrobiaceae* & *Methanospirillaceae*	FITC
MC1109	GCAACATAGGGCACGGGTCT	45	*Methanococcales*	CY3
MX1361	ACGTATTCACCGCGTTCTGT	25	*Methanosaetaceae*	CY3
MC1109	GCAACATAGGGCACGGGTCT	45	*Methanococcales*	CY3

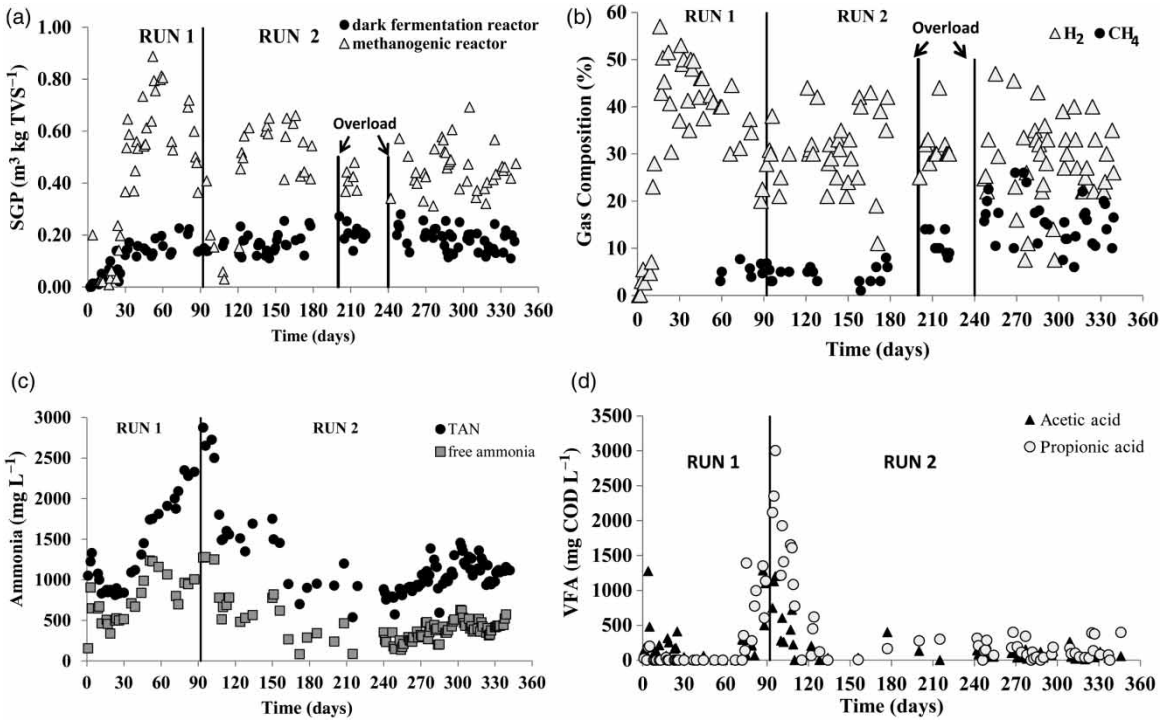

Figure 2 | (a) SGP of both reactors; (b) gas composition of the dark fermentation reactor; (c) total ammonia nitrogen (TAN) and free ammonia of methanogenic reactor; (d) acetate and propionate of dark fermentation reactor.

have shown that under high ammonia concentrations, hydrogenotrophic methanogenesis is the principal route to CH_4 formation (Banks *et al.* 2012) and NH_3 plays a role as a strong inhibitor of this metabolic pathway (Wiegant & Zeeman 1986). Gallert & Winter (1997) reported that NH_3 of about 560 mg L^{-1} causes significant inhibition of methanogens at pH 7.6 In the present study, when biogas production falls (days 60–108), the NH_3 content ranged from 698 to 1,280 mg L^{-1} (Figure 2(c)) at pH range 8.0–8.71. Moreover, propionate was the first VFA to increase (Figure 2(d)) and it was the predominant metabolic by-product (31% of total VFAs) when the total VFAs reached the maximum level (9,346 mg COD L^{-1} at day 96), while the rest was represented by caproate (20%), butyrate (20%), acetate (13%) i-valerate (11%) and valerate (5%). Angelidaki & Ahring (1994) indicated that at thermophilic temperature NH_3 above 700 mg L^{-1} registered poor treatment performance, affecting the propionate breakdown. Therefore, since propionate was the main by-product during the development of digestion instability, the poor process performance observed in the methanogenic reactor may be explained by the toxic effect caused by passive diffusion of the hydrophobic NH_3 in thermophilic methanogens.

In the light of these observations, an evaporation unit was adopted during RUN2 in order to control the ammonia content in the recirculation flow as well as to avoid inhibition problems in the reactors. As reported in Figure 2(c) after 20 operation days of the evaporation unit the TAN concentration was reduced in the methanogenic reactor, reaching a value of around, 1,500 mg L^{-1} (500 mg NH_3 L^{-1}). Consequently, a rapid increase of SGP was found after 10 days. The balance of the anaerobic trophic chain was confirmed by the low VFA concentration detected (less than 1 g COD L^{-1}) until the end of experimental trials. On the other hand, since ammonium nitrogen is one of the most important parameters that affect the mechanism of buffering in the AD process, the partial removal of ammonia from the recirculation flow led to a significant decrease in total alkalinity (data not shown). Consequently, the decrease in alkalinity in the recirculation sludge also affected the pH of the dark fermentation reactor (Figure 3(a)) with a negative impact on hydrogenase activity. In fact, the fraction of H_2 in the gas decreased at days 166–173 reaching a value less than 20% (v/v) (Figure 2(b)).

Moreover, a continuous decrease of C4/C2 ratio (butyrate/acetate) was observed (Figure 3(b)), probably due to the shift from the H_2/butyrate fermentation pathway to the solvent/lactate fermentation pathway (Valdez-Vazquez & Poggi-Varaldo 2009). In fact, previous studies have shown that the H_2 yield in thermophilic conditions is improved

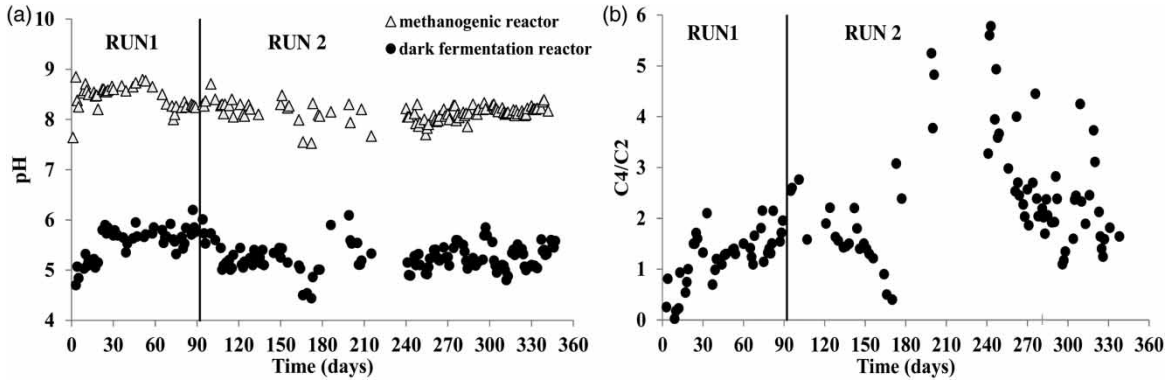

Figure 3 | (a) pH in the dark fermentation reactor; (b) butyrate and acetate ratio (C4/C2) in the dark fermentation reactor.

by maintaining the pH values within the range 5–6.5 (with an optimum value of 5.5 for food waste), since it optimizes the microbial hydrogenase activity. Moreover, pH not only affects the hydrogenase activity in mixed cultures, but can also modify the by-product spectrum as well as the structure of the microbial communities (Guo *et al.* 2010). Therefore, since pH reached values less than 5 at day 166–178 (Figure 3(a)), the recirculation flow treated through the evaporation unit was changed daily in order to maintain NH_3 concentration below the critical value observed (600–700 mg L^{-1}) in the methanogenic reactor, meanwhile ensuring a sufficient alkalinity supply to the fermentative reactor so as to buffer the high VFA content and to optimize H_2 production. This strategy allows all the main stability parameters such us pH, VFAs, biogas composition and alkalinity to be maintained within a suitable range, thus avoiding the phenomena of imbalance of the anaerobic trophic chain (Figures 2 and 3). Steady-state operation (days 270–345) during RUN2 was considered when continuous bio-hythane production in terms of gas composition was detected. The overall performance of the reactors is summarized in Table 3.

Despite the high VFA concentration measured (13,171 mg COD L^{-1}), the pH of the dark fermentation reactor was kept in the optimal range for hydrogenase activity with an average value of 5.3 ± 0.2. The fraction of CH_4 detected in gas produced by the dark fermentation reactor experienced a marked increase during maintenance periods, probably due to the increase of HRT, which avoids washout of methanogens. Therefore, in order to inhibit the active methanogenic biomass, we overloaded the fermentative reactor by applying an OLR of more than 30 kg TVS m^{-3} d^{-1} for a couple of days when the loading operations were reactivated (days 200 and 240). However, relevant production of CH_4 (18%) was continuously detected (Figure 2(b)), suggesting that acclimation of methanogens could, in time, occur in the dark fermentation reactor (as confirmed by the FISH analysis: see next section) in spite of the low level of pH, high VFA content and low HRT.

Table 3 | Effluent characteristics, gas yields and composition obtained during SSC (270–345 days) of RUN2

Effluent characteristics	Units	Average ± standard deviation	
		H_2-reactor	CH_4-reactor
TS ($n = 14$)	mg L^{-1}	51 ± 11	31 ± 4
TVS ($n = 14$)	mg L^{-1}	42 ± 10	19 ± 8
COD ($n = 14$)	mg L^{-1}	33 ± 3	17 ± 1
TKN ($n = 14$)	mg L^{-1}	1.3 ± 0.3	1.2 ± 0.2
P ($n = 14$)	mg L^{-1}	0.33 ± 0.1	0.31 ± 0.1
pH ($n = 47$)	–	5.3 ± 0.2	8.2 ± 0.1
TAN ($n = 47$)	mg L^{-1}	745 ± 282	1,115 ± 56
VFA ($n = 25$)	mg COD L^{-1}	13,171 ± 5,100	528 ± 393
Acetate ($n = 25$)	mg COD L^{-1}	1,958 ± 1,090	71 ± 54
Propionate ($n = 25$)	mg COD L^{-1}	1,045 ± 766	137 ± 128
Butyrate ($n = 25$)	mg COD L^{-1}	3,994 ± 2,312	116 ± 98
Partial alkalinity ($n = 47$)	mg $CaCO_3$ L^{-1}	–	3,602 ± 392
Total alkalinity ($n = 47$)	mg $CaCO_3$ L^{-1}	4,707 ± 1,070	5,437 ± 471
Total gas composition and bio-hythane yields ($n = 36$)			
H_2	%	8 ± 2	
CH_4	%	53 ± 3	
CO_2	%	38 ± 3	
SGP	m^3 kg TVS^{-1}	0.65 ± 0.10	
GPR	m^3 m^{-3} d^{-1}	2.5 ± 0.32	

n = number of samples.
H_2-reactor = dark fermentation reactor; CH_4-reactor = methanogenic reactor; SGP = specific gas production; GPR = gas production rate.

The methanogenic reactor revealed an average of 5,437 and 3,602 mg $CaCO_3$ L^{-1} for total and partial alkalinity respectively, with a constant difference between these parameters along the CSS of RUN2 (data not shown), indicating good balance of the anaerobic trophic chain. Indeed the methanogenic reactor showed low VFA concentration (528 mg COD L^{-1} on average) and constant pH (8.2 ± 0.1). The resulting TAN concentrations at CSS were 745 and 1,115 mg L^{-1} for the dark fermentation and methanogenic reactor respectively. From day 270 to the end of operations, the specific bio-hythane average was 0.65 m^3 kg TVS^{-1} with an H_2, CH_4 and CO_2 content of 8, 53, and 38% respectively. It can be remarked that TAN concentration was the major factor that influenced system performance during the different operation phases, since all other design parameters remained the same.

Bacterial population diversities

We investigated the microbial community using molecular analysis on two samples taken at day 35 and at day 300, when the process showed good performance in terms of bio-hythane production and composition. After about 30 days, a high H_2 production occurred in the first fermentative reactor, suggesting that the natural selection of H_2-producing bacteria was obtained starting from undefined mixed cultures by feeding the bio-waste directly. Indeed, the analysis of the bacterial community conducted on the sample taken at day 35 from the dark fermentation reactor showed that the two major bands (bands 'b' and 'e') of the dark fermentation reactor (Figure 4(a), R1) included members affiliated with the family of the *Lactobacillaceae* (band 'b') and phylum *Firmicutes* (band 'e'): the first one (band 'b') showed a similarity of 99% to Type Strain *Lactobacillus hamsteri* DSM 5661 (Figure 4(c)), found in thermophilic reactors for H_2 production fed with food waste (Wang *et al.* 2010); band 'e' showed a similarity of 100% to a member of the phylum *Firmicutes*, classified as uncultured cluster I (Tang *et al.* 2004), playing a role in the decomposition of complex and soluble organic matter during the initial step of hydrolysis (Sasaki *et al.* 2011).

For the methanogenic reactor (Figure 4(b), R2) the dominant band (band 'f') of the bacterial population reactor belongs to the *Thermotogaceae* family (phylum *Thermotogae*) and it was related to *Defluviitoga tunisiensis* SulfLac1 (T) with a 99% similarity. This organism was commonly found in anaerobic reactors (Cardinali-Rezende *et al.* 2009; Riviere *et al.* 2009; Gupta & Bhandari 2011; Kundu *et al.* 2012) since it is able to degrade and utilize complex carbohydrates like xylane and cellulose. The dominance of this organism at 55 °C is most likely due to its broad spectrum of substrate, together with its high optimum temperature for growth (45–80 °C) (Kundu *et al.* 2012). The second dominant microorganism of the methanogenic reactor (band 'g') showed a 97% similarity to the type strain *Clostridium sporosphaeroides* and to other uncultured *Clostridium* spp. (Figure 4(c)), belonging to phylum *Firmicutes*, reported as being effective H_2 producers (Yasin *et al.* 2011). The bacterial community of both reactors showed substantial stability during the operations since no significant differences can be observed between the DGGE profiles obtained at day 35 and day 300 (Figures 4(d) and 4(e)). This stability suggests that it is possible to ensure stable persistence of the natural selected bacteria through control of the recirculation flow. On the other hand, a great difference can be observed among the reactors, showing a speciation to specific bacteria with different characteristics.

Archaea population diversities

PCR-DGGE analysis was also used to investigate the archaeal population at day 35 and day 300 in the methanogenic reactor (Figures 4(d) and 4(e)). The profiles of the two samples taken at different times in the methanogenic reactor (Figure 4(d)) were very different from each other, indicating that the natural selected thermophilic biomass of the second methanogenic reactor was not stable in this long-term experience.

In fact, the phylogenetic tree (Figure 4(e)) showed that the methanogenic archaeal community at day 35 was composed only of species of the genus *Methanothermobacter*, with a similarity of 99% to the *M. thermautotrophicus* strain JW510 (bands 1, 2, 3, 4). In the second sampling time (day 300) the archaeal population shifted, including members of the *Methanobacteriales* families, widely found in thermophilic anaerobic reactors which have very high CH_4 production rates using H_2 and CO_2 as substrate (Kundu *et al.* 2012). Band 7 showed 99% similarity to *M. thermautotrophicus* whereas bands 5 and 6 were respectively related to *M. formicicum* (95% similarity) and *Methanosarcinales* with a similarity of 97% to *M. mazei strain Goe1*. The microbial structure in the second methanogenic reactor showed that the main route for CH_4 formation in thermophilic anaerobic reactors may be the hydrogenotrophic pathway (Banks *et al.* 2012). This result confirms that the imbalance of the anaerobic trophic chain observed during RUN1 was probably due to the free ammonia toxic effect on the hydrogenotrophic metabolism, leading to

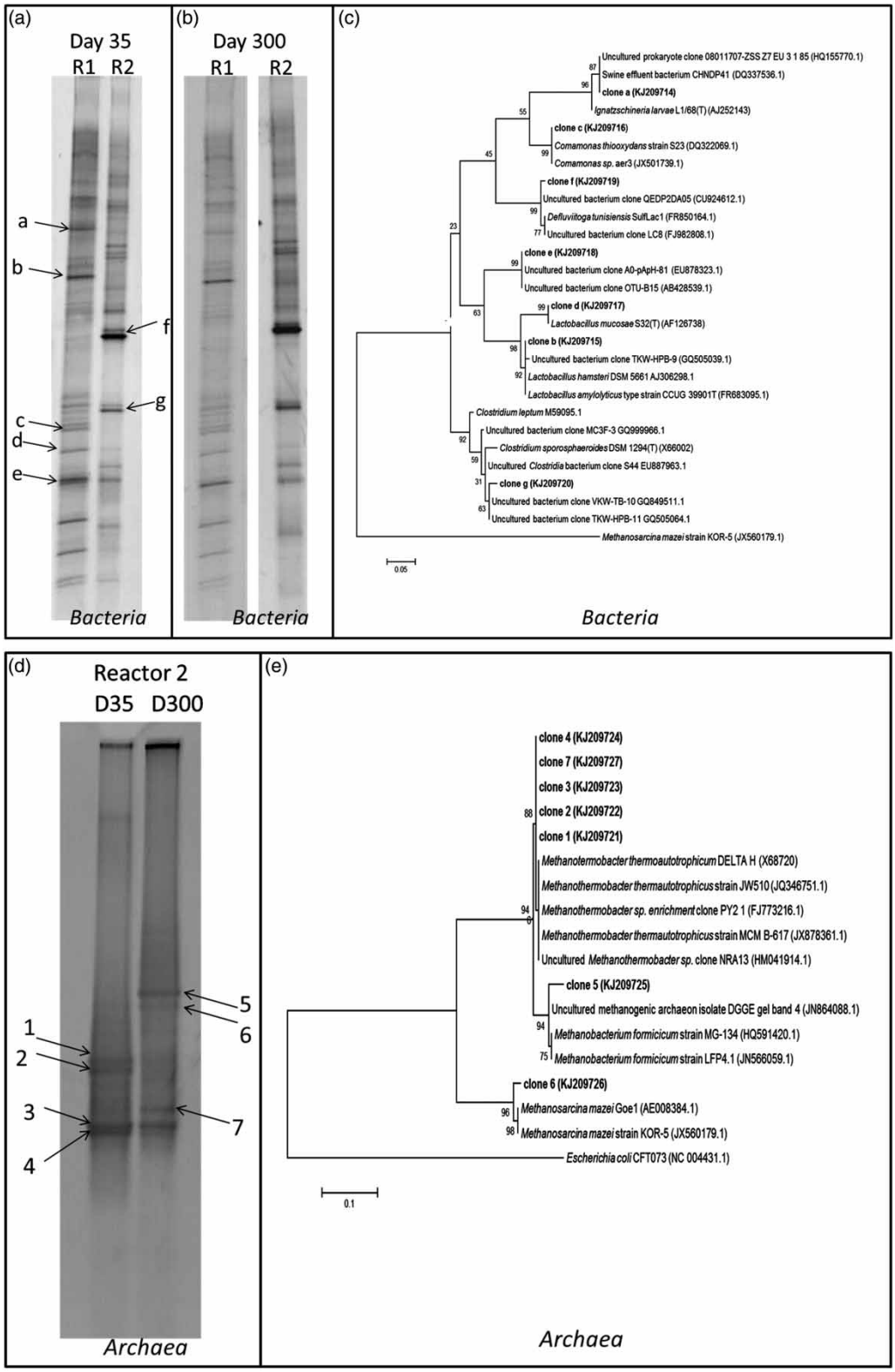

Figure 4 | (a), (b), (c) DGGE profiles using primers for *Bacteria* ((a) and (b)) (R1 = H_2 reactor; R2 = CH_4-reactor) and the related neighbour-joining tree (c) using *Methanosarcina mazei* strain KOR-5 as root. (d), (e) DGGE profiles using primers for *Archaea* (d) (D35 = day 35; D300 = day 300) and the related neighbour-joining tree (e) using *Escherichia coli* CFT073 as root. Sequenced bands highlighted by arrows (reactor 2 = methanogenic reactor).

propionate accumulation in the methanogenic reactor (Wiegant & Zeeman 1986; Angelidaki & Ahring 1994).

Due to the difficulty in amplifying any archaeal fragment from the samples taken from the dark fermentation reactor, mainly caused by the acid composition of the sludge, at day 300 samples from the dark fermentation reactor were taken to investigate the presence of methanogenic population by FISH analysis. The hybridization results (images not shown) showed that the 10–15% microbial biomass was composed of *Archaea* (ARCH915). Among the probes used to detect the archaeal community, the only one which showed positive result was MS1414, thus confirming the active presence of *Methanosarcina* spp., which was able to grow even under acetate concentrations of about 2 g COD L^{-1} (Table 3). These results are in accordance with the study of Díaz *et al*. (2003), who reported that *Methanosarcina* spp. grows at high acetate concentration. Finally, it can be remarked that, in time, the acclimation of the methanogen microorganisms can occur in the dark fermentation reactor, although strict conditions applied.

CONCLUSIONS

Two-stage thermophilic AD of food waste for H_2 and CH_4 co-production was studied at pilot scale. The results of this study demonstrated that a stable H_2 production in the dark fermentation reactor could be achieved when its pH was controlled by means of methanogenic effluent recirculation at the ratio of 50% of the total influent flow rate. The increasing ammonia concentration determined by recycling was controlled, by means of an evaporation unit, below 2 g N L^{-1}, a concentration which determined inhibition on the hydrogenotrophic methanogens since a high level of free ammonia was reached in the methanogenic reactor. Microbial analysis showed that the selection of hydrogen producers was achieved by feeding the biowaste directly in the first reactor without any substrate or inoculum pretreatment and that the bacterial population in the system was stable over the entire experimentation.

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