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Hydrogen fermentation of food waste by alkali-shock pretreatment: Microbial community analysis and limitation of continuous operation

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HIGHLIGHTS

- Increased H_2 production from food waste by alkali-pretreatment.
- High H_2 production and dominance of *Clostridium* sp. at pH 11.0 and 12.0.
- Limited performance in continuous operation due to H_2-consuming reactions.
- The repeated batch performance: 1.6 ± 0.1 mol H_2/mol hexose$_{added}$ and 4.4 ± 0.3 L H_2/L/d.

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ABSTRACT

In the study, at first, batch tests were performed to investigate the effect of alkali-shock on H_2 production from food waste (FW). After alkali-pretreatment of FW at pH 9.0–13.0, the FW was cultivated under mesophilic condition at pH 6.0 for 30 h without external inoculum addition. The amount of H_2 production from FW pretreated at pH 11.0 and 12.0 was higher than that achieved in other pretreatment pH. The main metabolite was butyrate, and *Clostridium* were dominant at pH 11.0 and 12.0. Meanwhile, lactate was the main metabolite with *Enterococcus* and *Streptococcus* being the dominant genus at other pretreatment pH. When the batch process was switched to a continuous mode, H_2 production was significantly dropped due to the increased activity of H_2-consumers. The reliability of alkali-pretreatment at pH 11.0 was proven by repeating the scale-up batch process, recording 1.57 ± 0.11 mol H_2/mol hexose$_{added}$ (17 ± 2 L H_2/kg FW) and 4.39 ± 0.32 L H_2/L/d.

1. Introduction

Food waste (FW), accounting for 15–63% of total municipal solid wastes (MSW) in the world, may cause odor and other environmental problems due to its high organic and nutrient levels, and water content, unless properly treated (AIT, 2010; Van Dyk et al., 2013). Since 2005, when landfilling of FW was prohibited in Korea, most FW has been transported to recycling facilities, where it is converted to compost and animal feed (MOE, 2014). However, the demand for these recycling products is very low due to the low quality; moreover, a large amount of secondary wastewater (>60% of FW, v/v) is generated during the recycling process (Kim et al., 2013) Therefore, a proper way of handling FW is a critical issue today, and energy, product or chemicals generation via biological methods are considered the best option owing to its economic and environmentally friendly aspect (Kim et al., 2010). In the US and EU, the landfilling portion of FW is decreasing while biological treatment methods are gaining a lot of attention (EPA, 2012; EEA, 2013).

Bioconversion to chemicals and biofuels such as ethanol, biodiesel, and methane as well as H_2 from FW is possible (Kiran et al., 2014). However, among them, H_2 production is the best option since H_2 has high energy content and produces only water when combusted (Nathao et al., 2013). Currently, H_2 is exclusively made by physico-chemical methods such as electrolysis, methane

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steam reforming, oil/naptha reforming (Obradović et al., 2013), and coal gasification owing to their cost advantage. However, there is an environmental contradiction that a clean fuel is generated from polluting and limited sources. On the other hand, biological H_2 production processes are more environmentally friendly and less energy consumptive than physico-chemical ones. In particular, H_2 fermentation by anaerobic digestion is considered the most practically applicable method since it does not require external energy and its H_2 production rate is much faster than that of other processes (Kim et al., 2011).

Anaerobic digestion (AD) is a traditional biological waste treatment process in which microorganisms convert organic substances to methane (CH_4). The produced CH_4 can be used as a heat source, to produce electricity, or as vehicle fuels (Frac and Zieminski, 2012). The AD process consists of various microbial reactions, largely acidogenesis and methanogenesis. During the acidogenesis step, organic substances are converted to various acids such as acetate, lactate, and butyrate, and to the gaseous product, hydrogen (H_2) (Luo et al., 2011). Since the 1990s, extensive research has been conducted on manipulating the acidogenesis reaction step to obtain more H_2 (Kiran et al., 2014). In the beginning, pure carbohydrates such as glucose and sucrose were used as the main feedstock, but actual organic wastes including FW are currently used for economic reasons since dark fermentation of FW is attractive to cheaply produce hydrogen as well as to efficiently treat the problematic wastes in biorefinery concept. Since FW has high carbohydrate content compared to other organic wastes such as sewage sludge and animal manure, it generally shows higher H_2 yield (Kim et al., 2008b; Kim et al., 2013).

In using FW as a feedstock, the suppression of indigenous non-H_2-producers (NHP) has often been raised as a key strategy for successful H_2 production (Kim et al., 2009). The mostly well-known NHP are lactic acid bacteria (LAB), whose main reaction is to convert carbohydrate to lactate without H_2 production (glucose → 2lactate). Due to their unique metabolic characteristics and antibiotic function, LAB are enriched in many fermentation processes of milk, meat, cereals, and vegetables (Stiles and Holzapfel, 1997). Most LAB are not capable of forming spores; thus, heat-, acid-, and alkali-shock of FW can lead to enhanced H_2 fermentation performance by killing indigenous LAB (Kim et al., 2009). In particular, alkali-shock has often applied in continuous H_2 production from FW, in which FW was pretreated at a very high pH level, 12.0–13.0, by pouring a large amount of alkaline solution (Kim and Shin, 2008; Kim et al., 2009). Alkali-shock has also been used to increase the biodegradable portion of sludge and algae for enhanced anaerobic digestion (Yan et al., 2010). However, alkali-shock pretreatment for H_2 production has never been optimized, which is important to minimize the consumption of alkaline solution. In addition, in spite of alkali-pretreatment, the obtained H_2 yields in the above studies were limited, ranging from 0.6–0.9 mol H_2/mol $hexose_{added}$, which was less than 50% of the theoretical value. The theoretical H_2 yield is known to be 2.0 mol H_2/mol hexose in treating organic solid wastes, with production of butyrate as the main soluble by-product (Kim and Kim, 2011). This low yield can possibly be attributed to the revival of NHP during continuous operation, or to some other reasons.

This study, at first, aimed to investigate the effect of alkali-shock on H_2 production from FW by varying the pretreatment pH range, 9.0–13.0. After the alkali-shock pretreatment for 6 h, the pH was readjusted to 8.0 ± 0.1, and FW was fermented under mesophilic condition without external inoculum addition. This batch-type fermentation method using indigenous H_2-producers was proven to be effective in a previous work (Kim et al., 2009). The produced gas and organic acids were periodically monitored. For detailed analysis, the microbial community was analyzed using a next generation sequencing (NGS) tool, which enabled us to determine the communities with high sequencing depth (Sanapareddy et al., 2009). In addition, the batch process was switched to a continuous mode to determine the performance stability during the continuous operation.

2. Methods

2.1. Feedstock

FW collected from the cafeteria in Korea Institute of Energy Research was used as a feedstock for H_2 production. Before fermentation, the feedstock was shredded using a grinder, to pieces, smaller than 2 mm in diameter. As the FW was not collected at the same time, the characteristics of the FW used in all experiments were different, as shown in Table 1. The acidic condition of the FW indicates the presence of indigenous acidogens.

2.2. Batch test

A batch test was performed to investigate the effect of pretreatment pH on H_2 fermentation of FW. As an alkali-shock, the pH of FW was increased to pH 9.0, 10.0, 11.0, 12.0, and 13.0 in five fermenters using 6 N KOH in five fermenters in parallel, respectively. During the pretreatment process, the FW was agitated at 60 rpm using a mechanical stirrer for 6 h. The expected pH levels were achieved instantaneously, but dropped slightly with time. Therefore, the injection of alkaline solution was continued for 6 h.

The predetermined amount of pretreated FW corresponding to 30 g carbo. COD/L (g/L as carbohydrate chemical oxygen demand (COD)) was then added to the batch fermenter, which was equipped with a pH sensor (working volume 300 mL); the remainder of the effective volume was filled with tap water. Neither external inoculum nor basal medium was added. The initial pH was readjusted at 8.0 ± 0.1 by the addition of 6 N HCl solution. After purging with argon gas for 10 min to provide anaerobic conditions, the fermentation started. During the fermentation, the pH was allowed to drop from 8.0 to 6.0, after which point the pH was automatically controlled at 6.0 ± 0.1 by adding 3 N KOH solution. The fermenters were agitated at 150 rpm by a mechanical mixer

Table 1
Characteristics of feedstock (food waste) used (A: a batch test was performed to investigate the effect of alkali-pretreatment pH on H_2 fermentation; B: a repeated batch fermentation was performed to show the reliability of alkali-shock; C: a continuous fermentation was performed to determine the performance stability).

Item	Unit	A	B	C
Total COD	g COD/L	$(1.5 \pm 0.1) \times 10^2$	$(1.6 \pm 0.1) \times 10^2$	$(1.6 \pm 0.2) \times 10^2$
Soluble COD	g COD/L	$(3.7 \pm 0.3) \times 10^1$	$(4.5 \pm 0.6) \times 10^1$	$(4.4 \pm 0.7) \times 10^1$
Total solids (TS)	g/L	$(1.3 \pm 0.1) \times 10^2$	$(1.4 \pm 0.1) \times 10^2$	$(1.5 \pm 0.2) \times 10^2$
Volatile solids (VS)	g/L	$(1.2 \pm 0.1) \times 10^2$	$(1.3 \pm 0.1) \times 10^2$	$(1.4 \pm 0.2) \times 10^2$
Carbohydrate	g COD/L	$(9.6 \pm 0.3) \times 10^1$	$(9.5 \pm 0.8) \times 10^1$	$(1.0 \pm 0.1) \times 10^2$
Total nitrogen (TN)	mg N/L	$(2.5 \pm 0.1) \times 10^3$	$(2.5 \pm 0.2) \times 10^3$	$(2.6 \pm 0.2) \times 10^3$
Ammonia	mg NH_4-N/L	$(1.9 \pm 0.1) \times 10^2$	$(1.6 \pm 0.3) \times 10^2$	$(1.6 \pm 0.3) \times 10^2$
pH		4.9 ± 0.1	5.2 ± 0.3	5.0 ± 0.3

Table 2
The procedure of batch, scale-up-repeated batch and continuous operations.

Item	Unit	Batch tests					Repeated batch operation	Continuous operation	
Working volume	L	0.3	0.3	0.3	0.3	0.3	3.0	0.7	0.7
Fermentation time/HRT	d	1.2	1.2	1.2	1.2	1.2	1.0	0.7	1.0
Pretreatment pH		9.0 ± 0.1	10.0 ± 0.1	11.0 ± 0.1	12.0 ± 0.1	13.0 ± 0.1	11.0 ± 0.1	11.0 ± 0.1	11.0 ± 0.1
Pretreatment time	h	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Fermentation pH		6.0 ± 0.1	6.0 ± 0.1	6.0 ± 0.1	6.0 ± 0.1	6.0 ± 0.1	6.0 ± 0.1	6.0 ± 0.1	6.0 ± 0.1
Substrate concentration	g carbo. COD/L	30 ± 1	30 ± 1	30 ± 1	30 ± 1	30 ± 1	30 ± 1	30 ± 1	30 ± 1
Temperature	°C	37 ± 1	37 ± 1	37 ± 1	37 ± 1	37 ± 1	37 ± 1	37 ± 1	37 ± 1

and placed in a temperature controlled room at 37 ± 1 °C. The produced gas and its constituents were monitored at 1–4 h intervals. Batch tests were carried out in triplicate and the results were averaged. The detailed operation conditions are reported in the Table 2.

2.3. Repeated batch operation

In the repeated batch operation, a scale-up fermenter of 5 L (3 L working volume) was used. FW was pretreated at pH 11.0 and the batch operation was repeated 20 times. The produced gas and its constituents were analyzed at the end of fermentation. The remaining experimental procedure was the same as that described in Section 2.2. The detailed operation conditions are reported in the Table 2.

2.4. Continuous operation

The continuous operation was conducted using a 1 L fermenter (effective volume 700 mL) equipped with a pH sensor. Having found the optimum pretreatment pH to be 11.0, the predetermined amount of alkali-pretreated FW at pH 11.0 was injected into the fermenter, followed by adding tap water to fix the substrate concentration at 30 g carbo. COD/L. Before fermentation, the initial pH was readjusted to 8.0 ± 0.1 by 6 N KOH addition, and the broth was purged with nitrogen gas for 10 min to provide anaerobic conditions. The pH was allowed to drop from 8.0 to 6.0, after which point pH was automatically controlled at 6.0 ± 0.1 by adding 3 N KOH solution. When the H_2 yield reached 0.7 ± 0.05 mol H_2/mol $hexose_{added}$, a certain amount of the fermented broth corresponding to the hydraulic retention time (HRT) of 1.0 d and 0.7 d (0.50 L and 0.71 L for HRT 1.0 d and 0.7 d, respectively) was decanted and alkali-pretreated FW at pH 11.0 was newly injected. This decanting/feeding cycle was conducted twice a day. The produced gas immediately moved to gas collector in all operation period.

The fermenters were mechanically agitated at 150 rpm and installed in the temperature controlled room at 37 ± 1 °C. The experiments were operated in duplicate and the results were averaged. The detailed operation conditions are reported in the Table 2.

2.5. Analytical methods

The concentrations of total solids (TS), volatile solids (VS), COD, total nitrogen (TN), and ammonia were measured according to Standard Methods (Clescerl et al., 1998). Carbohydrate concentration was determined by the colorimetric method (Dubois et al., 1956). The measured biogas ($H_2 + CO_2$) was adjusted to the standard conditions of temperature (0 °C) and pressure (1.01 bar) (STP). H_2 content in biogas was measured by gas chromatography (GC, Gow Mac series 580) using a thermal conductivity detector and a 2.0 m × 3.2 mm stainless-steel column packed with molecular sieve 5A with N_2 as a carrier gas. The content of CH_4 was determined using a GC of the same model noted previously with a 1.8 m × 3.2 mm Porapak Q (80/100 mesh) column using N_2 as a carrier gas. The temperatures of injector, detector, and column were kept at 80, 90, and 50 °C, respectively, in both GCs. Organic acids were analyzed by a high performance liquid chromatograph (HPLC) (Finnigan Spectra SYSTEM LC, Thermo Electron Co.) with an ultraviolet (210 nm) detector (UV1000, Thermo Electron) and an 100 mm × 7.8 mm Fast Acid Analysis column (Bio-Rad Lab.) using 0.005 M H_2SO_4 as a mobile phase. The liquid samples were pretreated with a 0.22 μm membrane filter before injection into HPLC.

2.6. Microbial community analysis

2.6.1. Sampling, DNA extraction, and PCR

The procedures of sampling, DNA extraction, and PCR were conducted according to the method in paper of Moon et al. (2015).

2.6.2. High throughput pyrosequencing and sequence analysis

The PCR products were purified by using AMPure beads (Beckman coulter). After the products were purified and quantified, sequencing was performed using a 454 pyrosequencing Genome Sequencer FLX Titanium (Life Sciences, CT, USA), according to manufacturer instructions, by a commercial sequencing facility (Macrogen, Seoul, Korea). The overall procedures of pyrosequencing and sequence analysis were performed according to the method in paper of Moon et al. (2015).

3. Results and discussion

3.1. Effect of pretreatment pH on batch H_2 fermentation

Fig. 1 shows the time courses of cumulative H_2 production from the alkali-pretreated FW at various pHs. The production curves were well fitted by the modified Gompertz Eq. ($R^2 > 0.99$) (Chen

Table 3
Average H_2 fermentation performance of alkali-pretreated food waste at various pHs.

Pretreatment pH	H_2 yield			Carbohydrate removal (%)	VS reduction (%)	H_2 production $rate_{maximum}$ (L H_2/L/d)
	H_2/mol $hexose_{added}$	mL H_2/g VS_{added}	mL H_2/g COD_{added}			
pH 9.0	0.67	63	52	91 ± 1	52 ± 4	7.25
pH 10.0	0.87	82	67	92 ± 1	52 ± 5	9.94
pH 11.0	1.65	156	128	92 ± 2	53 ± 0	20.27
pH 12.0	1.71	162	133	90 ± 3	51 ± 4	19.29
pH 13.0	0.92	88	72	72 ± 4	33 ± 2	9.85

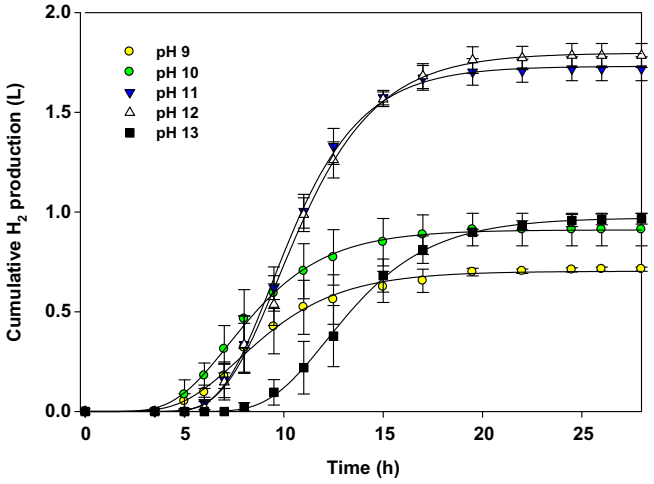

Fig. 1. Cumulative H_2 production from alkali-pretreated food waste at various pHs.

et al., 2006), and CH_4 was not detected during the entire fermentation period.

It was observed that H_2 production from FW pretreated at pH 11.0 and 12.0 was higher than that at pH 9.0, 10.0, and 13.0. At pH 11.0 and 12.0, 1.7–1.8 L of H_2 was produced from each batch fermenter, which were comparable to those in previous work using organic solid wastes as feedstock (Kiran et al., 2014). It indicates that the activity of H_2-producers was more enhanced by pretreating at pH 11.0 and 12.0, compared to other pretreatment pH. At pH 9.0 and 10.0, it seemed that the alkaline strength was not enough to suppress the activity of NHP, resulting in low H_2 yield. Substrate degradation in terms of carbohydrate degradation and VS reduction was in a similar range at pH 9.0–12.0. However, there was a drop of substrate degradation at pH 13.0, with the longest lag period being observed and low H_2 yield of 0.92 mol H_2/mol $hexose_{added}$ (Table 3). It seemed that pH 13.0 was a severe condition for even H_2-producers to survive. The lag period generally represents the inactivation status of microorganism. The increase of lag period by increasing of pH level from pH 10.0 to 11.0 and 12.0 to 13.0 indicates that as alkali-shock intensity increased, the more time is required to recover from inactive state. The main organic acids were butyrate and lactate and their compositions were varied depending on pH levels (Fig. 2). While the production of H_2 is accompanied by the production of acetate and butyrate, the production of lactate is not related with H_2 production (Kim et al., 2013).

At pH 11.0 and 12.0, the main metabolite was butyrate, accounting for around 80% of total organic acids produced, while lactate occupied less than 1% of the total organic acids produced. On the other hand, the main metabolite was lactate, accounting for 57–62% of total organic acids produced at pH 9.0 and 10.0. The highest lactate concentration of 15.4 g COD/L was observed at pH 9.0, under which condition the lowest H_2 yield was achieved. Meanwhile, at pH 13.0, lactate and butyrate occupied 30% and 50% of the total organic acids produced, respectively. The total production of organic acids was 23–25 g COD/L at pH 9.0–12.0, while only 16 g COD/L was produced at pH 13.0 due to low substrate degradation. The reason for this difference of patterns in organic acids might be the change of the microbial community at various levels of pretreatment pH, which will be discussed in the next section.

3.2. Repeated batch performance

Although, similar fermentation performance were achieved at pH 11.0 and 12.0, pH 11.0 was chosen at further experiment, owing to the more than 70% less requirement of alkaline solution compared to that needed at pH 12.0. The required amount of alkaline to adjust pH at 9.0, 10.0, 11.0, 12.0, and 13.0 were 0.9, 1.2, 1.9, 3.2, 7.3 meq OH^-/g VS, respectively.

As the characteristics of FW, such as pH, nutrient content, biodegradability, and diversity of indigenous bacteria vary in each collection, obtaining the same performance is unrealistic. However, it is important to show the reliability of the invented process by repeating it.

The batch fermentation was repeated 20 times at pH 11.0 using a scale-up fermenter (3 L working volume). The pH was controlled in range of 6.0 ± 0.1 and agitation was sufficient. The average H_2 yield was 1.57 mol H_2/mol $hexose_{added}$ (17 L H_2/kg FW) with a margin of 7% error. Average H_2 production rate was 4.39 ± 0.32 L/L/d, which was obtained by dividing the volumetric amount of H_2 produced by the total fermentation time, 30 h (pretreatment time of 6 h and fermentation time of 24 h). This result indicates that repeatability regardless of the fermenter size and the characteristics food waste was demonstrated, and this performance was comparable to the previous ones in the continuous H_2 fermentation of FW, ranging 0.9–2.4 mol H_2/mol $hexose_{added}$ (65–283 mL H_2/g VS) and 1.47–6.18 L/L/d (Kiran et al., 2014). VS reduction of approximately 50% and carbohydrate degradation of 91% of were also achieved, indicating a indicative of strong waste treatment during H_2 fermentation. Butyrate was found to be the predominant acid (72% of total organic acids produced), while lactate production was suppressed.

3.3. Microbial community analysis

Table 4 shows the total reads number, OTUs, and SHANNON INDEX. The 454 GS-FLX pyrosequencing of bacterial 16S rRNA gene amplicons from five samples resulted in a total of 84,939 reads and 155 OTUs. The low values in OTUs and SHANNON INDEX at pH 13.0 indicate not only low species richness but also evenness among all the species in the community, which could be linked to the low substrate degradation because of H_2-producers inhibition as well as NHP at extreme alkaline condition above pH 13. The sequences of bacteria were assigned to genus and species levels to see the relative abundance in each sample.

As can be seen in Fig. 3, a huge change in microbial community was observed depending on the pretreatment pH. At pH 9.0 and 10.0, the dominant genus were *Enterococcus* and *Streptococcus*, which occupied 45–55% and 35–45% of the total number of sequences, respectively. They are all belonged to LAB, and this could be the clear reason for the low H_2 yield with large amount of lactate production at pH 9.0 and 10.0. At pH 11.0 and 12.0, the genus *Clostridium* became dominant, comprising 50% of the total number of sequences. *Clostridium* are known to have the unique feature of forming endospores under alkali-conditions, and have often been observed in H_2 fermentation of organic wastes (Yun et al., 2014). Interestingly, though *Enterobacter* do not have the ability to form endospores (Ohad et al., 2014), they were detected at levels of 12.9% and 15.5% under strong alkaline conditions of pH 11.0 and 12.0. At pH 13.0, the relative abundance of *Enterococcus*, non-endospore forming bacteria, was 93.2%, while *Clostridium* only accounted for 6.5% of the total number of sequences, which is the same level that it had at pH 9.0 and 10.0 (6% of total number of sequences). It is speculated that even the endospores were broken by the extreme alkali-shock at pH 13.0. It has been reported that spores of *Clostridium perfringens* showed a 4-log reduction when treated with 100 g lime/kg biosolids (Abu-Orf et al., 2004).

In terms of species level, fifteen representative OTUs (sequences > 3%) with similarity 97–100% were chosen from all the representative sequences, as shown in Table 4. In all samples, there were seven representative OTUs that belonged to *Clostridium*

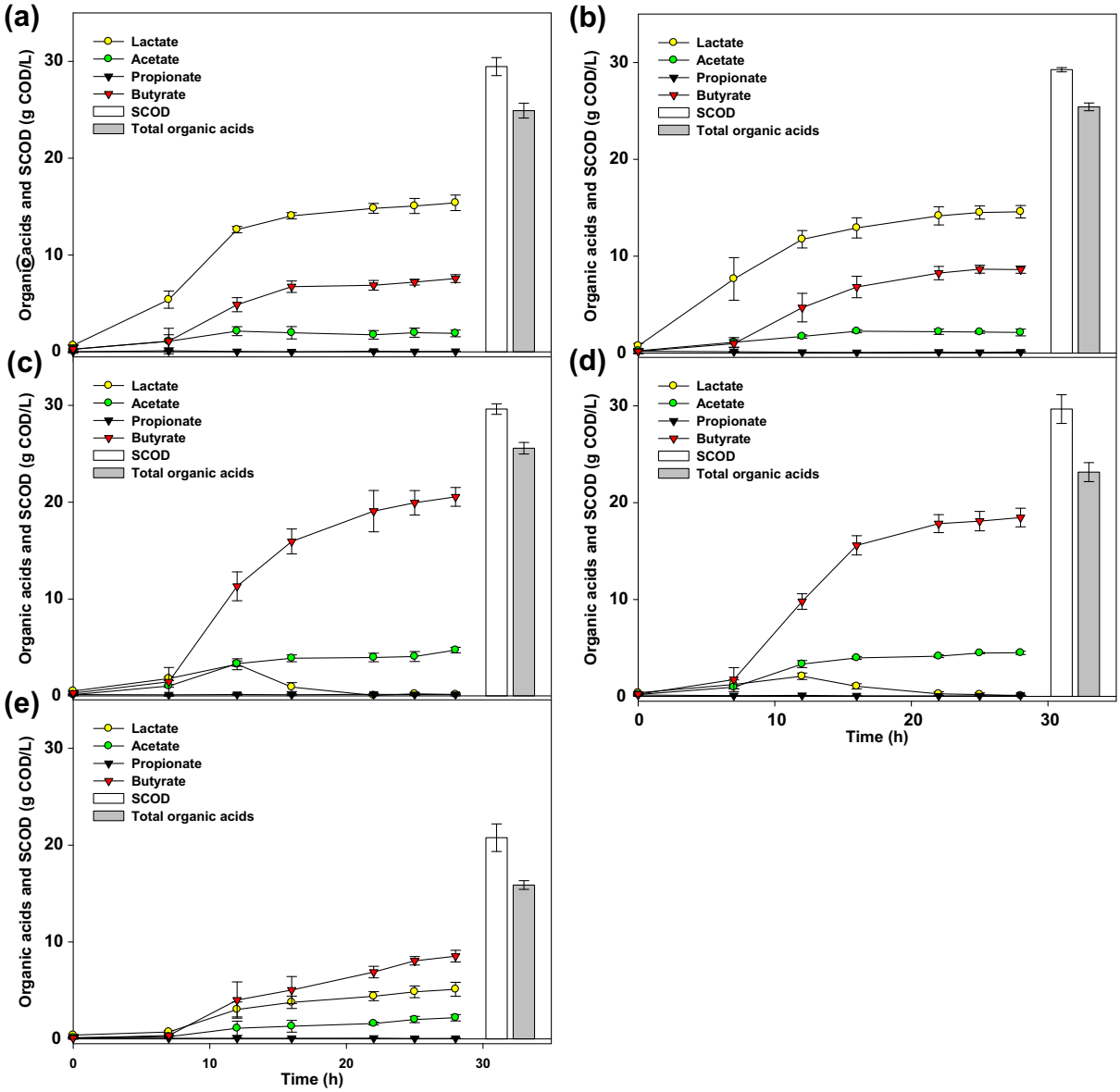

Fig. 2. Organic acids production profile of alkali-pretreated food waste at various pHs, and the amount of soluble chemical oxygen demand and total organic acids produced at the end of fermentation ((a) pH 9.0, (b) pH 10.0, (c) pH 11.0, (d) pH 12.0, and (e) pH 13.0).

sp., three that belonged to *Enterococcus* sp., two that belonged to *Streptococcus* sp. and *Enterobacter* sp., and one that belonged to *Lactococcus* sp. *Enterococcus faecalis* V583 was observed in all conditions (43.3% at pH 9.0, 40.7% at pH 10.0, 24.7% at pH 11.0, 19.8% at pH 12.0, and 89.7% at pH 13.0, respectively). The survival mechanism of *E. faecalis* under alkaline condition has been reported, and has been correlated with an increase in Na^+ and K^+-ATPase activity and cell-surface hydrophobicity, in addition to the up-regulation of genes involved in the stress response and biofilm formation (Ran et al., 2013). Because of this alkali-tolerant characteristic, it seemed that *E. faecalis* were dominant in all alkali-pretreatment conditions, in particular at pH 13.0. On the other hand, *Streptococcus* sp. were not tolerant to high pH. The abundance of *Streptococcus lutetiensis* and *Streptococcus infantarius* sub sp. *infantarius* CJ18 decreased with pH increase. Meanwhile, the distribution of *Clostridium* sp., the main H_2-producers, was found to be different depending on the pH level. *Clostridium acetobutylicum* ATCC 824 was observed as the most dominant species at pH 11.0, accounting for 27.1% of the total number of sequences. On the other hand, the abundance of *Clostridium butyricum*, *Clostridium saccharoperbutylacetonicum* N1-4(HMT), and *Clostridium sardiniense* were enhanced at pH 12.0. However, at pH 13.0, the dominance of *Clostridium butyricum* decreased to 5.0%, while there was a drastic increase of *E. faecalis*.

3.4. Limitation of continuous operation

The main purpose of a continuous biological process in treating organic wastes is to retain active biomass for fast reaction without lag period (Kim et al., 2009), but also to increase productivity by eliminating batch process bottlenecks, such as reactor emptying, filling, cleaning and so on. Thus, the HRT applied in the continuous operation was set at 1.0 d and 0.7 d, considering that the batch fermentation was completed within 1 d. The continuous operation lasted for 12 d.

As can be easily seen in Fig. 4, H_2 production performance fluctuated during the continuous operation, showing a negligible yield after 3 d. There was little difference in the fermentation performance between the two HRTs, and the whole operation period

Table 4
Analytical results of the microbial gene libraries obtained from next generation sequencing, and species level identification of the dominant OTUs from each sample (>3% of total sequences).

Items	pH 9.0	pH 10.0	pH 11.0	pH 12.0	pH 13.0
Reads	16,954	17,266	17,704	14,601	18,414
OTUs	35	24	39	36	21
SHANNON	1.5193 ± 0.0328	1.6815 ± 0.0304	2.1616 ± 0.0313	2.6019 ± 0.0377	0.5465 ± 0.0272

OTU	Species	pH 9.0	pH 10.0	pH 11.0	pH 12.0	pH 13.0	Accession (#)	Similarity (%)
1	*Enterococcus faecalis V583*	43.3	40.7	24.7	19.8	89.7	NR_036922.1	100
2	*Streptococcus lutetiensis*	36.1	29.1	4.9	0.1	0.0	NR_037096.1	99
3	*Clostridium acetobutylicum ATCC 824*	0.5	0.0	27.1	0.2	0.0	NR_074511.1	100
4	*Clostridium butyricum*	1.1	1.3	11.6	16.0	5.0	NR_042144.1	97
5	*Streptococcus infantarius sub sp. infantarius CJ18*	8.6	8.9	1.3	0.0	0.0	NR_102799.1	99
6	*Clostridium saccharoperbutylacetonicum N1-4(HMT)*	0.8	1.1	3.6	8.1	0.2	NR_102516.1	100
7	*Enterobacter asburiae*	0.1	0.0	12.5	0.0	0.0	NR_024640.1	99
8	*Lactococcus lactis sub sp. lactis Il1403*	0.3	0.2	5.4	5.3	0.1	NR_103918.1	98
9	*Enterobacter sakazakii*	0.3	0.0	0.4	15.5	0.0	NR_102794.1	99
11	*Clostridium perfringens*	1.6	1.7	0.2	3.8	0.0	NR_074482.1	100
12	*Enterococcus thailandicus*	0.0	9.3	0.1	0.1	0.1	NR_044160.1	100
13	*Clostridium sardiniense*	0.2	0.0	0.5	7.3	0.0	NR_041006.1	99
14	*Enterococcus casseliflavus EC20*	1.7	3.2	0.2	2.6	1.8	NR_102793.1	97
18	*Clostridium roseum*	0.5	0.6	0.3	3.2	0.5	NR_029354.1	99
19	*Clostridium tertium*	0.0	0.0	0.0	3.1	0.0	NR_037086.1	99

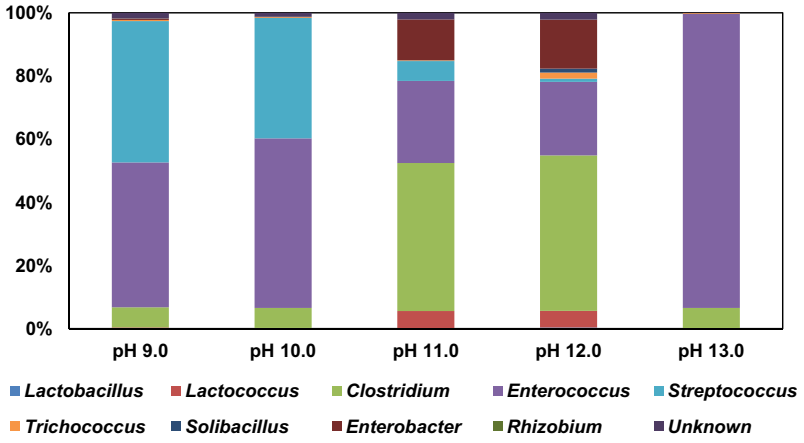

Fig. 3. Next generation sequencing results of fermentation broth in genus level. (The fermentation was conducted using alkali-pretreated food waste at pH 9.0–13.0).

could be divided into three phases depending on the production of H_2 and organic acids. Carbohydrate degradation did not change during the continuous operation, reaching around 90%.

In Phase I, some amount of H_2 production was detected, with butyrate being the main metabolite. However, as operation went on, acetate production increased while butyrate production decreased. Also, H_2 yield decreased to less than 1.0 mol H_2/mol $hexose_{added}$. At Phase II, there was a significant drop of H_2 production (<0.2 mol H_2/mol $hexose_{added}$), during which phase not only acetate but also propionate production became dominant. Meanwhile, lactate was not detected. At Phase III, H_2 production was negligible, and the production profile of organic acids seemed stable. The concentrations of acetate, propionate, and butyrate ranged 5–8 g COD/L, respectively.

In early study of H_2 fermentation, acetate production was often correlated with H_2 production, However, some authors have argued that acetate production is irrelevant to H_2 production: it is rather related with the H_2-consuming reaction, which consumes four moles of H_2 with one mole of CO_2 (called as 'homoacetogenic reaction'). The existence of this homoacetogenic reaction was first reported by Oh et al. (2003), in which a decreased H_2 content was observed at the final stage of the batch process with acetate production. Also, Kim et al. (2006) found a large difference between the actual and theoretical amount of H_2 production. The theoretical amount was calculated by analyzing the production of liquid metabolites, assuming that acetate was produced. Lalman et al. (2013), by constructing a flux balance model, estimated the activity of homoacetogenic reaction, which was found to be active. To date, nearly 100 homoacetogenic species from 20 different genera have been isolated, and some *Clostridium* sp., e.g. *Clostridium aceticum*, *Clostridium scatologens*, *Clostridium coccoides*, and *Clostridium magnum*, are included (Drake et al., 2002). All these *Clostridium* sp. are spore-forming bacteria, and they might have survived from alkali-shock. Meanwhile, two enzymes, Fe–Fe hydrogenases and NADH-Fd oxidoreductase, could catalyze the reversible biohydrogen process, and their activities are influenced by environmental conditions such as pH, cell growth rate, and H_2 partial pressure (Kim and Kim, 2011). Therefore, a plausible explanation for the decrease of H_2 production with an increase of acetate production would be the increased activity of homoacetogens or/and the inverse reactions of enzymes as operation went on. It seemed that the H_2 content in the headspace over 40% at Phase I triggered this reaction.

The high production of propionate in Phase II and III is also related with the H_2-consuming reaction. Propionate was often detected when the H_2 fermenter performed at low yield (Hussy et al., 2003), and propionic acid bacteria (PAB) such as *Selenomonas* sp. have been detected in H_2-producing anaerobic mixed cultures (Kim et al., 2008a). In addition, Vavilin et al. (1995) demonstrated that propionate production can be accelerated under high H_2 partial pressure condition.

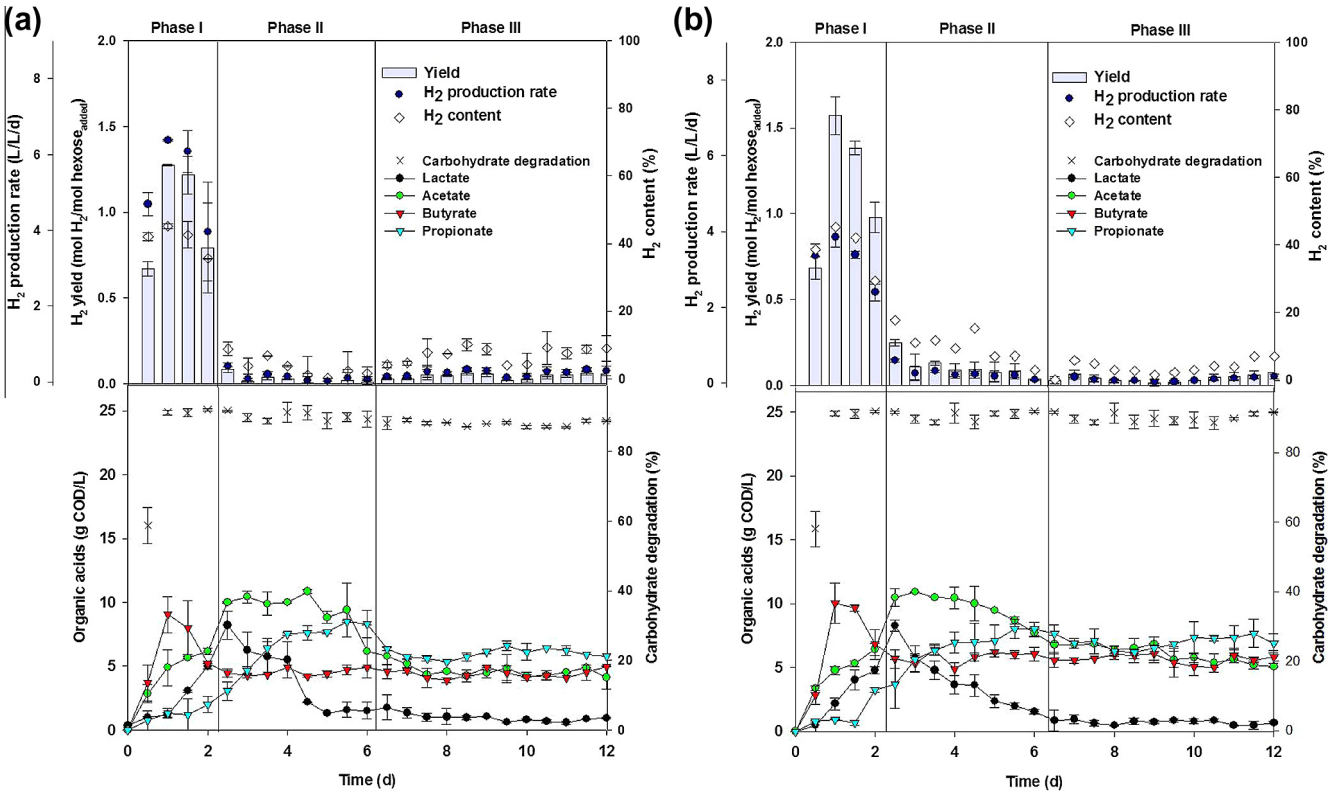

Fig. 4. Continuous H_2 fermentation performance at hydraulic retention time (a) 0.7 d and (b) 1.0 d fed with alkali-pretreated food waste at pH 11.0.

In conclusion, although the activity of indigenous LAB was suppressed by alkali-shock, H_2-consuming acetate and propionate production became dominant as operation went on, resulting in low H_2 yield.

4. Conclusions

It was found that alkali-pretreatment at pH 11.0 and 12.0 was more effective than other pretreatment pHs (9.0, 10.0, and 13.0) for H_2 production. NGS results showed that *Clostridium* were dominant at pH 11.0 and 12.0. When the batch process was switched to a continuous mode, H_2 production decreased with the increase of acetate and propionate production by homoacetogens, and an inverse reaction of hydrogenase and NADH-Fd oxidoreductase under high H_2 partial pressure condition, unlike batch process. The reliability of alkali-pretreatment at pH 11.0 was proven by repeating the scale-up batch process (1.57 ± 0.11 mol H_2/mol hexose$_{added}$ and 4.39 ± 0.32 L H_2/L/d).

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