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Thermophilic Dark Fermentation for Simultaneous Mixed Volatile Fatty Acids and Biohydrogen Production from Food Waste

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Abstract: Food waste is categorized as organic solid waste, which has a negative impact on environmental sustainability. Food waste was simultaneously used for the feasible generation of mixed volatile fatty acids (VFAs) and bio-hydrogen by deploying dark fermentation. Original anaerobic digested sludge was prepared via the shock technique with 50 g/L glucose under thermophilic temperature (55 °C). The pretreated inoculum was found capable of converting 10 g VS/L food waste to hydrogen with a rather high yield of 135.2 ± 7 mL H_2/VS_{added}. The effect of various concentrations of food waste, including 10.2, 16.3, 20.3, and 26.4 g VS/L, on mixed VFAs production was subsequently carried out in batch dark fermentation. The highest butyric acid concentration (5.26 ± 0.22 g/L) in soluble metabolites was obtained from batch dark fermentation with 26.4 g VS/L of food waste. The dominant *Clostridium thermobutyricum*, *Clostridium sporogenes*, and *Octadecobacter* sp. found in the batch of dark fermentation of food waste could confirm the effectiveness of the load shock pretreatment method for inoculum preparation. The continuous stirred tank reactor (CSTR) inoculated with mixed cultures, also prepared via the load shock pretreatment method and without the addition of external nutrients, was operated by feeding 26.4 g VS/L food waste at the kinetically designed HRT for 4 days, corresponding to an organic loading rate (OLR) of 7.6 g VS/L·d. Under steady state conditions, promising butyric acid (5.65 ± 0.51 g/L)-rich mixed VFAs were achieved along with the hydrogen yield of 104.9 ± 11.0 mL-H_2/g VS_{added}, which is similar to the upper side of the previously reported yields (8.8 ± 0.6–103.6 ± 0.6 mL-H_2/g VS).

Keywords: organic waste; mixed culture fermentation; volatile fatty acids; bio-hydrogen

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1. Introduction

Food waste presently collected in Thailand amounts to more than 18 million tons/year, accounting for approximately 46% of municipal solid wastes. It represents one of the critical problems related to environmental concerns and accounts for more than 8% of global greenhouse gas emissions [1]. Landfilling, incineration, and composting, defined as first-generation processing technologies, are deployed for the final destination of food waste in Asian countries (India, Thailand, Singapore, Malaysia, Indonesia, etc.). Food

waste is rich in carbohydrates, proteins, and lipids [2]. Nevertheless, food waste has attracted attention in recent years as the potential substrate for valuable bio-chemicals production using microbial processes [3]. Furthermore, producing new chemicals and material from carbon-rich waste streams is a highly attractive motivation to overcome worldwide pollution levels and climate change primarily caused by the overuse of fossil fuels. Volatile fatty acids (VFAs) such as butyric acid, acetic acid, and propionic acid are important building block chemicals with an increasing market demand in the industrial sector for bioplastics, foods, composites, synthetic fibers, and coating [4]. The bio-chemicals industry, using either biomass or organic waste as feedstock, is one of Thailand's ten S-curve industries being targeted for investment, according to the 20-year national strategic plan (2018–2037), with which Thailand aims to escape from the middle-income country trap [5].

Mixed culture fermentation for the potential production of mixed VFAs, lactic acid, and alcohols, which are the building block chemicals for other environmentally friendly industries, is potentially feasible, according to kinetic and thermodynamic modeling [6]. When the product of interest generated from the mixed culture fermentation of carbohydrate-rich substrate is hydrogen, which is thermodynamically directed to only butyric acid and acetic acid, the fermentation process is typically called dark fermentation [2]. Additionally, the conversion of complex mixtures of carbohydrates to VFAs by applying the mixed culture process is more suitable for industrial application than applying the pure culture process because of the lack of requirements for sterilization, a better adaptation capacity due to its high microbial diversity, the possibility of mixed substrate co-fermentation, and the possibility of a continuous mode of operation [7,8]. The key parameters including the type of waste stream, reaction time/hydraulic retention time (HRT), pH, and temperature could affect the production of VFAs [9]. Fermentative microorganisms cultivated at pH either above 8.0 or below 6.0 could provide a rather high specific growth rate and, thus, could enhance VFA production [10]. Additionally, the concentration and composition of produced VFAs are usually dependent on both pH and temperature. Using thermophilic mixed cultures, the fermentation of glucose at 55 °C generated acetate, butyrate, and hydrogen at pH 4. When adjusted to pH 7, the main products were shifted to acetate, ethanol, and propionate [11].

Thermophilic conditions (55–60 °C) usually provide several advantages over mesophilic conditions due to the increasing thermodynamic favorability of microorganisms leading to the acceleration of biological and chemical conversion. Consequently, the higher production yield and smaller reactors required due to shorter HRTs; the enhanced liquid solubilization, leading to a lesser need for energy in the mixing process; and a reduction in the retention time required in pathogenic destruction are accomplished [12].

The corresponding microbial population containing the mixed cultures capable of providing required metabolic capacities for the typical dark fermentation process could potentially be accomplished by manipulating the operation of the fermentation process initiated with various natural inoculum sources [13]. One of the feasibly practical and economical methods to suppress methanogenic microorganisms to avoid generated VFAs consumption is the load shock pretreatment method, which was previously applied by adding 50 g of sugar/L of desugared molasses (DM) to originally digested manure under a thermophilic temperature (55 °C). Later on, the pretreated inoculum was successfully established in the UASB reactor at 55 °C for hydrogen production from DM. A satisfactory yield of 269.5 mL-H_2/g of sugar was achieved by feeding 16.7 g of sugar/L of DM with 1-day HRT at 55 °C. The soluble end products were dominated mainly by butyrate, followed by acetate [14].

Inoculum pretreatments, process optimization to achieve high and stable yields, the development of efficient techniques for selective recovery, and the cost-effectiveness of the whole process are the main challenges for the attainment of the full-scale implementation of food waste dark fermentation. Indeed, the dark fermentation of food waste to efficiently recover mixed VFAs, which are currently the products of interest for further application

in sustainable chemical industries, is highly dependent on sludge pretreatment for deactivated methanogens and physicochemical and operational parameters involved in the fermentative system. Applying thermal, acid, and alkali pretreatments was reported as an efficient method to select high-activity microorganisms for dark fermentation [15,16]. Nevertheless, these pretreatment methods are still in doubt for the purpose of economic viability. Therefore, in this investigation, original anaerobic digested sludge was pretreated by load shock means, which is considered to be a practical and economic pretreatment for subsequently establishing the mixed culture dark fermentation of food waste under a thermophilic temperature (55 °C). A batch fermentation experiment was later conducted to estimate the first-order kinetic constant (k_h), which was further used to simply design a suitable HRT for a CSTR operation of mixed culture anaerobic fermentation of food waste in order to evaluate long-term performance under a continuous operation mode.

2. Materials and Methods

2.1. Food Waste and Inoculum Preparation

Food waste was collected from the main canteen of Prince of Songkla University, Pattani campus, Thailand. The bones in the food waste were picked out. Free bone food waste was then crushed with an electric blender to homogenize the food waste. Crushed food waste was later stored at −4 °C until later use. It was then characterized for total solids (TS), volatile solid (VS), ash, pH, chemical oxygen demand (COD), proteins, lipids, carbohydrates, and carbon-to-nitrogen (C/N) ratio.

Anaerobic digested sludge was collected from commercial biogas digester for palm oil mill effluents located in Pattani, Thailand. It was later pretreated to suppress methane-producing microorganisms and to select mixed-VFA-producing bacteria simultaneously using load shock pretreatment [14] with 50 g/L of glucose below 55 °C.

2.2. Batch Dark Fermentation Test

2.2.1. Performance Test for Hydrogen Production Using Pretreated Sludge

Pretreated sludge was further used as the inoculum for dark fermentation of food waste. The inoculum performance for hydrogen production from food waste and glucose was tested in 125 mL serum bottles with working volume of 60 mL, containing 10 g TS/L of either food waste or glucose without any additional nutrient. Tap water was replaced as a substrate for a set of blank controls. All experiments were performed in triplicates. Each bottle was subsequently sealed with butyl stopper and aluminum crimp cap. Closed bottle was purged with N_2 for 3–5 min to create anaerobic conditions by inserting a pair of syringe needles through the butyl stopper. Then, the bottle was subsequently incubated in a 55 °C incubator for 7 days. Generated gas volume and composition was measured daily.

2.2.2. Batch VFAs Production at Various Initial Food Waste Concentrations

Batch fermentation was carried out in a 5 L jacketed fermenter with 3.5 L working volume to investigate kinetics of VFAs formation and carbohydrate consumption at various food waste concentrations of 5, 8, 10, and 13 (% wet weight of food waste per working volume, % w/v), corresponding to VS loading of 10.2, 16.3, 20.3, and 26.4 g VS/L and COD loading of 13.2, 21.1, 26.4, and 34.4 g COD/L, respectively. The thermophilic inoculum around 30% of the working volume was added to a fermenter. Tap water was subsequently added to the fermenter until reaching 3.5 L working volume. For each initial food waste concentration, batch fermentation was restarted with the same original inoculum obtained from the pretreatment method described in the previous section. The fermenter temperature was controlled at 55 °C by circulating hot water via the water jacket of the fermenter. Liquid sample was taken periodically for metabolite (acetic acid, propionic acid, butyric acid, lactic acid, and ethanol) analysis. Sludge samples were collected at the end of stationary phase for further analysis of microbial communities by using the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) method.

The cumulative results of butyric acid production obtained from batch food waste dark fermentation at different initial concentrations were used to estimate the first-order kinetic constant (k_h, h^{-1}) according to Equation (1) [17]:

$$\boldsymbol{B}=\boldsymbol{B}_{\infty}[1-\mathbf{exp}(-\boldsymbol{k_h t})] \quad (1)$$

B_{∞}: maximum butyric acid concentration (g/L); B: cumulative butyric acid concentration (g/L) at a given time; t: incubating time (h).

2.3. CSTR Start-Up and Operation

The sludge obtained from a previous batch assay (food waste 13% w/v) providing the highest butyric concentration was then used as inoculum to start up the continuous dark fermentation by using 10 L CSTR, with an active volume of 7 L. The CSTR was first added with inoculum of mixed cultures around 2100 mL, accounting for 30% of the active volume. Subsequently, the rest of the reactor active volume was filled up with food waste and tap water to a concentration of 26.4 g VS/L (13% w/v). The CSTR was maintained at thermophilic temperature (55 °C) by circulating hot water through the water jacket of the CSTR. The reactor was kept in batch mode operation for 4 days. After that, food waste with 26.4 g VS/L concentration was continuously fed to the CSTR at 4-day *HRT*, which was derived accordingly from Equation (2) [18]:

$$HRT = \frac{1}{k_h}\left(\frac{y}{y_m - y}\right) \quad (2)$$

where y_m is an ultimate/maximum hydrogen yield obtained from the stationary phase of the cumulative hydrogen yield of batch food waste dark fermentation as shown in Figure 1, and y is an expected yield, which is around 80% of the y_m. Kongjan et al. [19] reported that the HRT calculated by using the expected yield (y) at around 80% of the ultimate yield (y_m) is practically feasible for operating the CSTR for dark fermentation at the maximum organic loading rate (OLR) of 11.3 g VS/L·d. The OLR of 7.6 g VS/L·d was achieved for food waste fermentation by using calculated HRT of 4 days and initial food waste concentration of 26.4 g VS/L. It should be noted that hydrogen generation from dark fermentation is normally linked to the butyric acid and acetic acid pathway and catalyzed by pyruvate, ferredoxin oxidoreductase (PFOR) [20], whereby the product generation time data for HRT estimation is better adopted from hydrogen production than the VFAs.

2.4. Analytical Methods

TS, VS, ash, pH, and COD were determined following the procedures described in the Standard Methods (APHA, 2005) [21]. The protein was determined by the micro-Kjeldahl method using a general factor of n = 6.25 (AOAC, 2000; method 976.05). The lipid content was determined by the Soxhlet method (AOAC, 2000; method 920.39). The carbohydrates content was estimated by subtracting the protein lipid and total phenolic compounds from the volatile solids [22]. C/N ratio was determined by using a CHNSO analyzer (Thermo Quest Flash EA 1112, Rochester, NY, USA).

Gas chromatography (Hewlett Packard, G1530A, East Lyme, CT, USA) equipped with a flame ionization detector (FID) and HP-INNO Wax column (19091N-113, 30 m × 0.32 mm × 0.25 µm), which contains a polyethylene glycol (PEG) stationary phase was deployed for VFAs and ethanol determination. The oven was heated up with ramping rate of 25 °C/min to increase temperature from 70 °C to 230 °C. By using helium carrier gas at a flow rate of 1 mL/min, the injector and detector temperature was then set to 240 °C. High-performance liquid chromatography (HP1100, Hewlett-Packard GMGH, Waldbronn, Germany) equipped with Pinnacle® II C18 Columns, ultraviolet (UV) detector at 210 nm under oven temperature of 45 °C was deployed for lactic acid (LA) analysis by using 2.5 mM of H_2SO_4 as a mobile phase with a flow rate of 0.8 mL/min. Collected liquid

samples were first centrifuged at 10,000 rpm for 10 min and subsequently filtered through 0.45 µm nylon membrane.

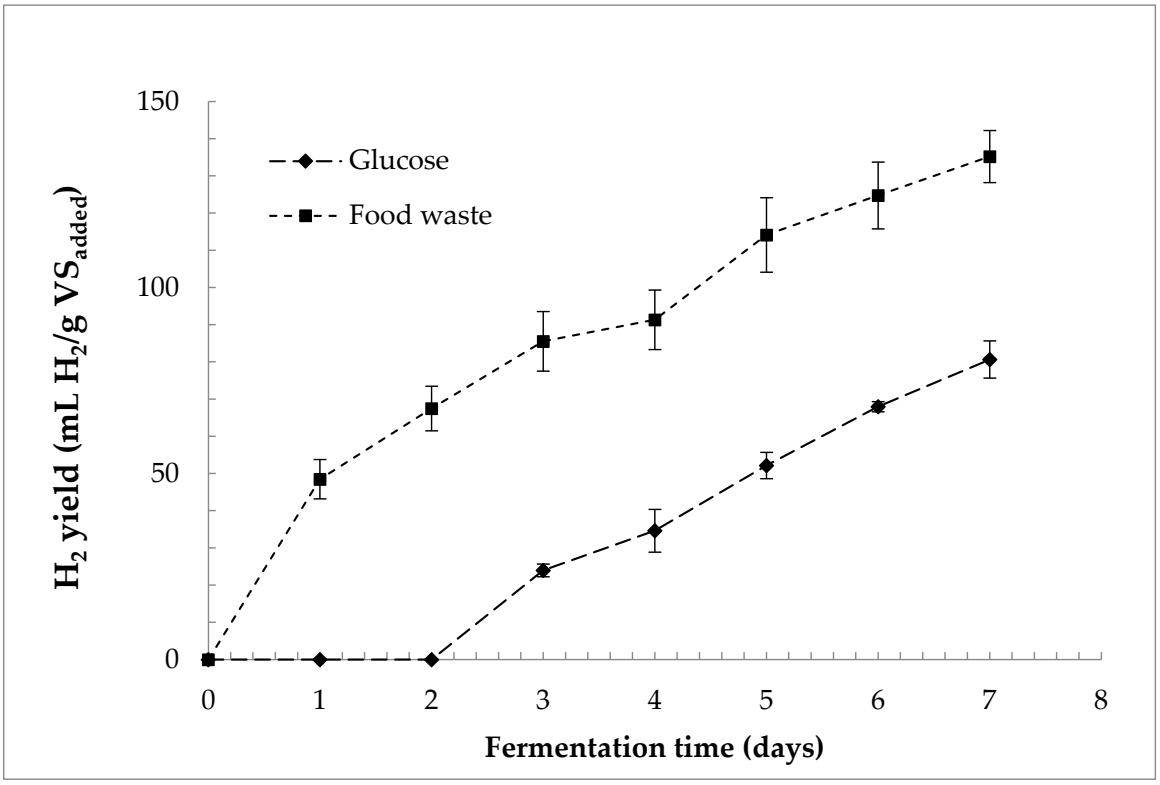

Figure 1. Hydrogen production yield of inocula without the addition of a medium.

Meanwhile, hydrogen content in the produced gas was measured by using thermal conductivity detector (TCD) gas chromatography (Shimadzu, GC-14A, Kyoto, Japan) connected with a stainless column (1.5 m) packed with molecular sieve 58 (80/100 mesh). Argon, as a carrier gas was controlled at the flow rate of 15 mL/min. The temperatures of 100 °C, 50 °C, and 100 °C were, respectively, applied for injector, oven, and detector.

2.5. Bacteria Community Analysis

Sludge samples were taken from batch dark fermentation at 168 h with different initial concentrations of food waste. Total genomic DNA was extracted from 0.5 g sludge samples using TIAN amp Soil DNA Kit (TIANGEN Biotech (Beijing, China) Co., Ltd.). Extracted DNA was checked for quality by electrophoresis on a 1.0% agarose in 1X TAE buffer. 16S rRNA gene of bacteria was PCR-amplified using universal primer 1525r (5′ AAGGAGGTGWTCCARCC 3′) and 27f (5′ GAGTTTGATCCTTGGCTCAG 3′). The polymerase chain reaction (PCR) product was used as templates for amplification of the V3 region of 16S rRNA gene using primer 518r (5′ GTATTACCGCGGCTGCTGG 3′) and 357f (5′ CTCCTACGGGAGGCAGCAG 3′) with CG clamp (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGG GG). The PCR products were stored at 4 °C and analyzed on 1.5% agarose before sequencing with denaturing gradient gel electrophoresis (DGGE). By using 8% polyacrylamide gel, the DGGE was constructed with linear urea-formamide gradients of denaturant ranging from 40% to 70% in 0.5 × TAE buffer at a constant temperature of 60 °C (DGGE unit, V20-HCDC, Scie-Plas Limited, Cambridge, England). DGGE gel was performed by electrophoresis at 20 V for 20 min, followed by 70 V for 16 h, Sybr Gold-stained, and visualized on a UV transilluminator. Most of the DGGE bands were excised from the polyacrylamide gel and sequenced by Macrogen Inc. (Seoul, Republic of Korea). The closest matches for partial 16S rRNA gene sequences were identified by a database search in Gene Bank using BLAST [23].

3. Results and Discussion:

3.1. Performance of Digested Sludge Pretreated by Load Shock Method

The main characteristics of food waste and original inoculum used in this investigation are demonstrated in Table 1. The 24 C/N ratio of food waste collected from the university canteen indicates that amounts of carbon and nitrogen contained in food waste are suitable to be used as a major substrate for dark fermentation. Such a high C/N ratio for food waste indicates that the used food waste must be composed mainly of carbohydrates (rice, vegetables, and fruits), which could be considerably degraded by fermentative bacteria of VFAs alongside mixed hydrogen–carbon dioxide gas [24]. Food waste richer in meat and cooked food are also richer in proteins, giving rise to N-content and, consequently, decreasing the C/N ratio. An optimal C/N ratio range between 15 and 30 has been reported for short-chain fatty acid production via dark fermentation [25].

Table 1. Characteristics of food waste and original inoculum.

Characteristic	Unit	Food Waste	Original Inoculum
pH	-	-	7.1 ± 0.03
Total solid (TS)	% (*w*/*w*)	20.93 ± 1.83	5.64 ± 0.78
Volatile solid (VS)	% (*w*/*w*)	20.33 ± 1.68	4.14 ± 0.27
Ash	% (*w*/*w*)	0.60 ± 0.05	1.50 ± 0.13
Carbohydrates	% TS	81.5 ± 2.08	-
Proteins	% TS	11.0 ± 0.65	-
Lipids	% TS	4.75 ± 0.21	-
VS/TS ratio	%	97.13	73.40
COD/VS ratio	-	1.30	-
C/N ratio	-	24.10	8.0

Mixed cultures obtained by the load shock pretreatment of anaerobic digested sludge were used as inoculum without the addition of nutrients and buffer in batch fermentation. Hydrogen yields of 135.2 ± 8.6 mL H_2/VS_{added} and 80.6 ± 5.3 mL H_2/VS_{added} were consequently obtained in the stationary phase of batch fermentation of food waste and glucose, respectively, at initial concentrations of 10 g VS/L (Figure 1). The hydrogen yield obtained from food waste is around 40% higher than that obtained from glucose. Hydrogen production rate and yield from food waste were higher than those from glucose during dark fermentation, indicating that food waste has an appropriate ratio of carbon and nitrogen, sufficient nutrients and buffer, which are favorable for microorganism growth and enhancing hydrogen production via dark fermentation, which is thermodynamically linked to butyric acid and acetic acid production as demonstrated in Equation (3), providing a theoretical yield of 373.57 mL H_2/g VS:

$$2C_6H_{12}O_6 + 2H_2O \rightarrow C_4H_8O_2 + 2C_2H_4O_2 + 6H_2 + 4CO_2,\ \Delta G^0 = -239.88\ \text{kJ/mol} \quad (3)$$

However, other metabolic products such as propionic acid, lactic acid, and ethanol are usually generated without hydrogen formation during anaerobic mixed microbial culture fermentation [16]. Consequently, the hydrogen yield obtained from mixed culture fermentation is usually lower than the stated theoretical yield.

3.2. Batch Dark Fermentation of Food Waste at Various Initial Loadings

The enriched inoculum capable of producing hydrogen by using food waste without adding external nutrients was subsequently used to investigate the effect of initial food waste concentration on VFA production. Figure 2 shows profiles of the metabolites, butyric acid, acetic acid, propionic acid, lactic acid, and ethanol during batch fermentation at various food waste concentrations. The metabolites were produced exponentially in the first 72 h and, later on, approached a stationary stat. Butyric acid presented as the main metabolite, followed by acetic acid, lactic acid, ethanol, and propionic acid for all food waste

loading. The highest concentration of butyrate 5.26 g/L was obtained at 13% w/v, followed by 4.87 g/L at 10% w/v, 3.78 g/L at 8% w/v, and 2.1 g/L at 5% w/v. As demonstrated in Figure 2, butyric acid, acetic acid, lactic acid, propionic acid, and ethanol were generated differently according to the different food waste concentrations used. The k_h, representing butyric acid formation, was increased from 0.030 h^{-1} to 0.044 h^{-1}, when increasing the food waste concentration from 5% w/v to 10% w/v. It was then slightly constant after further increasing the food waste concentration to 13% w/v (Table 2). Presumably, after further increasing the food waste concentration higher than 13% w/v, mixed microbial cultures in the fermentation system could have declined hydrolysis activity. For batch dark fermentation of food waste, Yahya et al. [26] previously reported an inoculum obtained by applying an autoclave heat pretreatment (100 °C and 20 min) of anaerobic digestate sludge, providing a k_h in a range between 0.021 h^{-1} and 0.041 h^{-1}, which is a similar range of k_h obtained from batch food waste dark fermentation in the current investigation. This could be evidence that load shock pretreatment might be one of the effective methods for inoculum preparation for dark fermentation. During the load shock operation, mixed organic acids are rapidly generated in a rather high concentration; consequently, the pH in the fermentative system was rapidly decreased to an acidic range between pH 5 and pH 6 by self-adjustment via a high concentration of generated organic-acid-based acidogenesis. Methanogens in the original anaerobic sludge usually prefer a pH range between pH 7 and pH 8 for their growth [27], which could eventually be deactivated under acidic conditions. The butyrate and acetate as the major metabolites in the mixed-acid metabolic pathway were previously generated during dark fermentation [28]. The production of the mixed acids can be affected by various factors such as the composition of the microbial consortium [15], concentration, characteristics of the substrates, pH, redox potential, etc. [29].

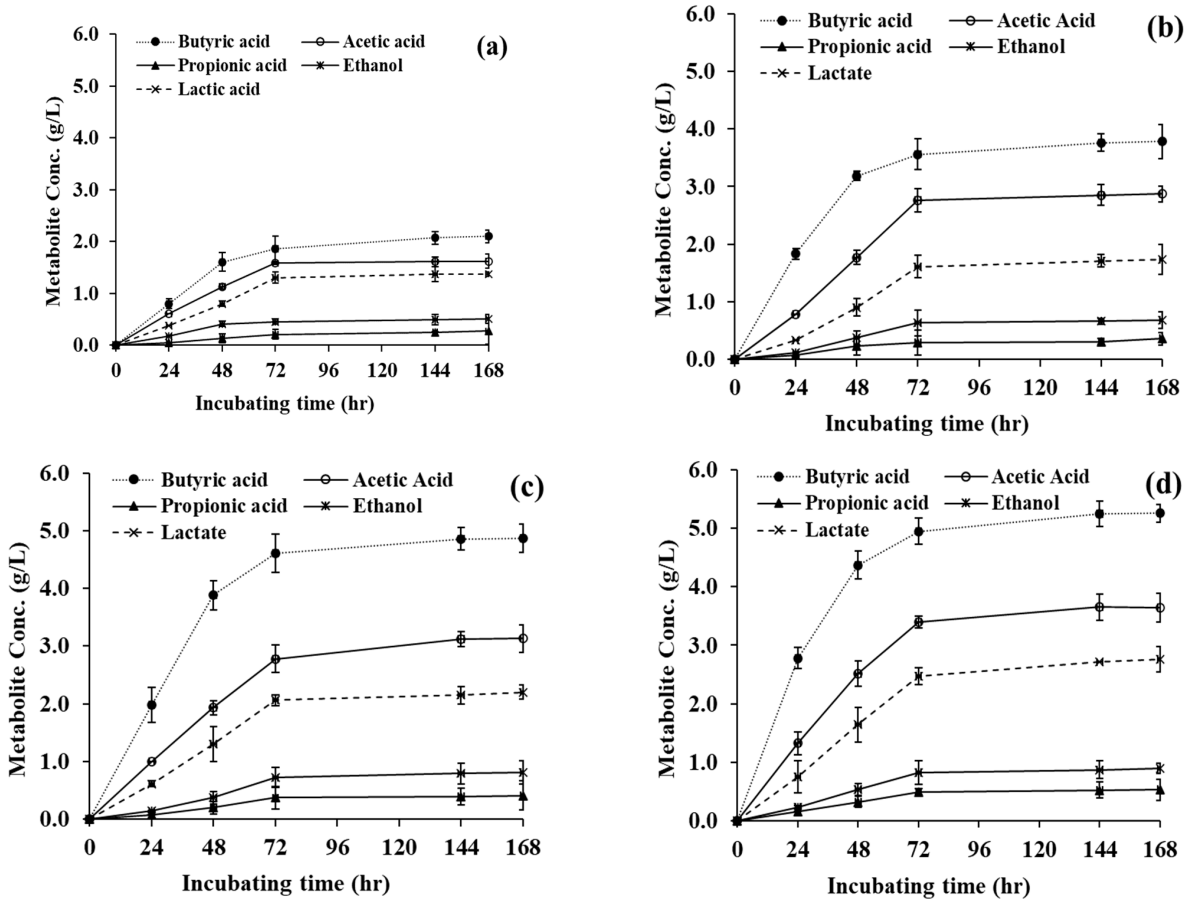

Figure 2. Metabolites from batch dark fermentation of food waste at various initial food waste concentrations (% w/v) of 5 (**a**), 8 (**b**), 10 (**c**), and 13 (**d**).

Table 2. Mass balance and the first-order kinetic constant (k_h) of batch fermentation at various food waste concentrations.

Food Waste Conc.			Metabolite Conc. (g/L)					Metabolite Conc. (g COD/L)						COD_{VFASS}/COD_{FW} (%)	k_h (h^{-1})	R^2
%*w*/*v*	g VS/L	g COD/L	BA	AA	PA	ETOH	LA	BA	AA	PA	ETOH	LA	Total			
5	10.2	13.2	2.1	1.62	0.27	0.51	1.37	3.82	1.73	0.58	1.06	1.47	7.19	65.54	0.030	0.996
8	16.3	21.1	3.78	2.87	0.36	0.68	1.73	6.87	3.06	0.78	1.42	1.85	12.13	66.13	0.037	0.993
10	20.3	26.4	4.87	3.13	0.41	0.81	2.20	8.85	3.34	0.89	1.69	2.35	14.77	64.19	0.044	0.991
13	26.4	34.4	5.26	3.64	0.53	0.89	2.76	9.56	3.88	1.15	1.86	2.95	16.45	56.47	0.044	0.996

BA = butyric acid, AA = acetic acid, PA = propionic acid, LA = lactic acid, ETOH = ethanol.

Luo et al. [16] also reported that the highest butyric acid concentration over other metabolites (acetic acid, propionic acid, lactic acid, and formic acid) was generated from food waste fermentation using mixed microbial cultures previously pretreated by acid, alkali, and thermal pretreatments. These pretreatment techniques seem to be expensive for implementation on the industrial scale because chemicals and energy are required for inoculum pretreatments. Therefore, inoculum pretreatments could affect the economic viability for the mixed culture dark fermentation process and require careful consideration.

The obtained soluble metabolites (Figure 2 and Table 2) are obviously affected by the initial food waste concentration. Mixed microbial cultures in the fermentative system could be directed in different pathways, when having different microorganisms, substrates, and environmental conditions [30]. According to the COD balance shown in Table 3, the detected soluble metabolites account for more than 56.47% of the COD input. The remaining COD should probably be attributed to microbial cell mass, hydrogen, and other soluble metabolites (formic acid valeric acid, acetone, and butanol) [9]. Microbial cells generally uptake around 15% of the COD input [27]. H_2 also practically contributed around 10% to 15% of the COD input. Farouk et al. [31] report that the highest VFAs yield of 823.55 mg/g SCOD (about 82% VFAs/SCOD) and the highest H_2 yield of 5.19 mL/g SCOD were obtained from the dark fermentation of food waste when the chemical pretreatment of mixed culture and external nutrients was applied.

Table 3. Product concentrations and COD balance of CSTR under steady state conditions (days 29–51).

	Concentration (g VS/L)	COD (g COD/L)	% COD Distribution
Influent (food waste)	26.40	34.32	100
Output products		27.71	82.94
BA	5.74	10.33	30.10
AA	3.85	4.12	12.00
PA	0.56	1.21	3.52
ETOH	0.83	1.73	5.05
LA	2.96	3.17	11.43
H_2	0.25	2	5.83
* Cell mass		5.15	15
Balance		−6.61	−17.06

* An average cell mass production around 15% was previously used by Kotsopoulos et al. [27].

Different metabolites obtained from dark fermentation at different concentrations of food waste could be explained by different dominant microorganisms analyzed using the PCR-DGGE assay. With this analytical method, structures and quantities of microorganisms contained in mixed microbial cultures could be provided according to the DNA band intensities [32]. As demonstrated in Figure 3, the dominant microbial communities in the batch fermentation of food waste at initial concentrations of 5% *w*/*v*, 8% *w*/*v*, and 10% *w*/*v* were *Clostridium thermobutyricum*, a butyric acid producer [33], and genus Lactobacillus (*Lactobacillus fermentum*, *Lactobacillus delbrueckii*, and *Lactobacillus crispatus*), a lactic-acid-

producing bacteria [34]. The existence of various *Lactobacillus* sp. could be strong evidence for having lactic acid as one of the major metabolites generated from the dark fermentation of food waste in this investigation. Laothanachareon et al. [35] reported the co-production of butyric acid and lactic acid from the dark fermentation of food waste using mixed microbial cultures. The formation of a considerably high concentration of lactic acid in the dark fermentation process could be an indicator for having a lower hydrogen yield and unstable or overloading conditions [23].

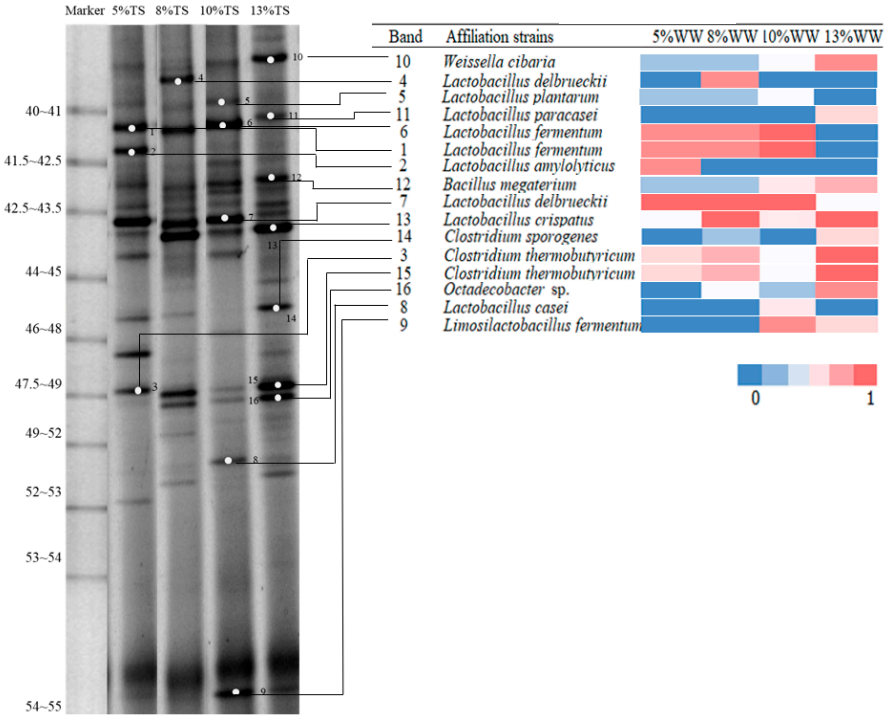

Figure 3. Micro-community identified by PCR-DGGE of sludge of different initial concentrations of food waste after 168 h of batch dark fermentation.

Interestingly, *Lactobacilli*, such as *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus casei*, and *Lactobacillus delbrueckii*, were not dominant for dark fermentation using an initial food waste concentration of 13% *w*/*v*. This could imply that even less lactic acid could be produced using a food waste concentration of 10% *w*/*v*. However, *Clostridium thermobutyricum* was still dominant, corresponding to the highest butyric acid concentration obtained from fermentation using 13% *w*/*v* food waste concentration. *Clostridium sporogenes* and *Octadecobacter* sp. were other dominant species during batch fermentation with a food waste concentration of 13% *w*/*v*. *C. sporogenes* is capable of fermenting amino acid to possibly produce both butyrate and acetate, as reported by Batstone et al. [36]. Meanwhile, *Octadecobacter* sp., psychrophilic microorganisms could produce polyunsaturated fatty acids (PUFA), as reported by Nichols et al. [37]. Their dominance during the thermophilic fermentation of food waste at 13% *w*/*v* is strong evidence of being thermotolerant species. According to Chen et al. [38], VFAs and hydrogen-producing bacteria (*Thermoanaerobacterium,* and *Clostridium sensu stricto* 1) were found as domain bacteria more often at the thermophilic temperature than the mesophilic temperature in the dark fermentation process due to these bacteria's ability to tolerate high temperatures and an acid–base range 5–6.

3.3. Dark Fermentation of Food Waste in the CSTR

During the first 4-day start-up period, hydrogen composition in the fermented gas of 5, 11, 25, and 36 (% *v*/*v*) was detected on day 1, day 2, day 3, and 4, respectively. Food waste with 26.4 g VS/L was fed to the CSTR at a designed HRT of 4 days, corresponding to

an organic loading rate (OLR) of 7.6 g VS/L d under a 55 °C thermophilic temperature. The appropriate HRT between 2 days and 6 days is usually selected to evaluate the optimum performance of the continuous dark fermentation of an organic waste in the CSTR [39]. Since they have a slow growth rate, methanogens normally require an HRT longer than 10 days for the traditional anaerobic digestion of organic wastes under a 55 °C thermophilic temperature in the CSTR [18]. Methane was not detected during the continuous operation for 51 days, indicating that methanogenic archaea were completely deactivated by operating with an HRT below 6 days and using an initial inoculum obtained by the load shock pretreatment of anaerobic sludge, leading to a high VFA concentration [14]. As demonstrated in Figure 4, catabolic metabolites including butyric acid, acetic acid, lactic acid, ethanol, propionic acid, and hydrogen were generated from food waste fermentation. The formation of such soluble metabolites must be the major cause for pH reduction in the fermentation system.

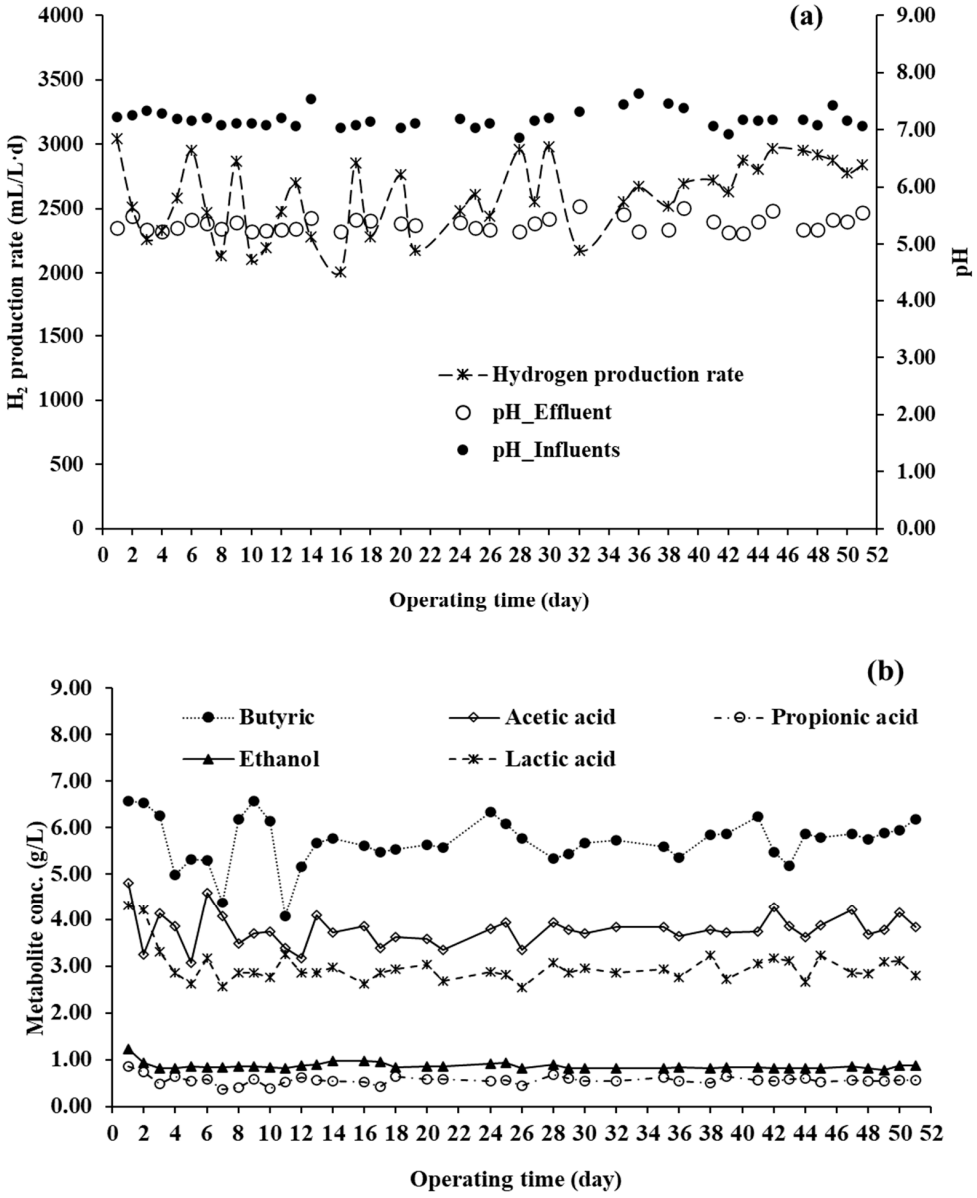

Figure 4. Hydrogen production rate and pH (**a**) and metabolite profiles (**b**) during continuous dark fermentation at 55 °C in the CSTR fed with 13% *w*/*v*-food waste during a 4-day HRT anaerobic digestion of food waste using volatile fatty acids and hydrogen at high organic loading rates in immersed membrane bioreactors.

Under steady state conditions from day 11 onward, defined by having a standard deviation of generated main metabolites (hydrogen, butyric, and acetic acid) less than 10%, an influent pH around 7.18 ± 0.18 was reduced to an effluent pH around 5.36 ± 0.13. Consequently, the butyric acid concentration (5.65 ± 0.51 g/L) at 1.5 times higher than the acetic acid concentration (3.76 ± 0.33 g/L) was achieved along with a hydrogen production rate of 2623.4 ± 274.7 mL H_2/L·d, corresponding to a hydrogen yield of 104.9± 11.0 mL H_2/g VS_{added}. Villanueva-Galindo et al. [15] reviewed that hydrogen yields obtained from the continuous dark fermentation of various types of food waste are in the range of 8.8 ± 0.6 mL H_2/g VS to 103.6 ± 0.6 mL H_2/g VS. Total soluble metabolites of 13.94 g/L (0.53 g/g VS) contained butyric acid at 41.2%, acetic acid at 27.6%, lactic acid at 21.1%, ethanol at 5.9%, and propionic acid at 4.0% of the total composition. Astoy et al. [40] reported that butyric (37 ± 4%) and acetic acids (35 ± 8%) were dominantly co-produced during the mixed culture fermentation of glucose at acidic pH 5. Additionally, the distribution of generated metabolites can be significantly different following as small a change as half a pH unit [41]. Additionally, cyclic hydrogen production (Figure 4a) with around 10% standard deviation is observed under steady state conditions. This cyclic fluctuation could possibly be due to pH always changing in the continuous fermentation system (without pH control). Hydrogen is generated from proton (H^+) reduction by hydrogenase, which is highly inhibited by pH. Protons are poor electron acceptors. Therefore, electrons are easily shifted to other reduced products of lactic acid and propionic acid [42].

Furthermore, glucose fermentation using extremely thermophilic (70 °C) mixed culture fermentation at controlled pH 4 could prevent sugar uptake due to the toxicity of undissociated acid to microorganisms, which is known as a so-called acid crash [42]. This toxic phenomenon occurs when undissociated acids at a low pH can permeate through a cell [43].

Table 3 demonstrates COD distribution for the generation of metabolic products and cell mass from food waste with a COD/VS ratio of 1.30 under steady state conditions. The satisfied COD recovery with an error of 17.06% was achieved with 15% COD distribution for cell mass [14], indicating a good establishment of operating conditions to enhance the growth of mixed cultures for converting organic matters in food waste to metabolite products of organic acids and ethanol. During the mixed culture dark fermentation of food waste in this investigation, butyric acid could majorly produce butyric acid with around 30% COD distribution. Dark fermentation is thermodynamically controlled by hydrogen partial pressure. Butyric acid is preferably favorable to be generated when hydrogen partial pressure in the system is higher than 60 Pa [20].

A supernatant of dark fermentation of rice hydrolysate with 6.87 g/L butyric acid, for a total VFA content of 9.52 g/L (60%) mixed with rice hydrolysate (40%), could be further converted to butanol with a high concentration of 13.8 g/L by using *Clostridium beijerinckii* NCIMB 8052 ABE fermentation [44].

Meanwhile, hydrogen-rich bio-syngas after being used as sparging gas to recover butanol from ABE fermentation broth, as previously stated, could be possibly converted to ethanol by novel gas fermentation. Monir et al. [45] reported that hydrogen-rich gas containing 13.05% H_2, 22.92% CO, 7.9% CO_2, and 1.13% CH_4 could be converted to ethanol by using *Saccharomyces cerevisiae* yeast. Additionally, solventogenic *Clostridium aceticum* is capable of converting mixed syngas with a $CO:CO_2:H_2:N_2$ ratio of 30:5:15:50 to ethanol with a concentration of 5.6 g/L, as reported by Arslan et al. [46]. Therefore, clean bio-ethanol fuel is produced, and greenhouse gases (CO and CO_2) are sequestrated by applying syngas fermentation using suitable microorganisms. Furthermore, bio-based VFAs from organic wastes as a carbon source could be potentially utilized for biological nutrient removal in the denitrification process and for microbial lipids for further producing bio-diesel [4]. Interestingly, Vu et al. [47] reported that a PHA content of 1.3 g/L was achieved successfully after 128 h of cultivation in the immersed membrane bioreactor (IMBR) fed with potato liquor and apple-pomace-based VFAs with a total concentration of 8.8 g/L.

4. Conclusions

Without the addition of nutrients and buffers, inoculum pretreated with the load shock technique, which was detected dominantly with *Clostridium thermobutyricum*, *Clostridium sporogenes*, and *Octadecobacter* sp., could produce satisfactory mixed VFAs and hydrogen during batch and continuous food waste dark fermentation. The appropriate HRT for the CSTR operation can be simply designed by using kinetic data obtained from batch food waste dark fermentation. The thermophilic dark fermentation of food waste using mixed cultures to simultaneously produce butyric-acid-rich VFAs and hydrogen-rich biogas was eventually established for continuous operation in the CSTR successfully. Therefore, the experimental results obtained in this study can be extrapolated to pilot scale implementation. To be more economically feasible, further investigation to replace glucose with food waste for inoculum load shock pretreatment should be studied further.

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