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Effects of Lipase Addition, Hydrothermal Processing, Their Combination, and Co-Digestion with Crude Glycerol on Food Waste Anaerobic Digestion

Xiaojue Li [1] and Naoto Shimizu [2,*]

[1] Laboratory of Agricultural Bio-System Engineering, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Hokkaido, Japan; jue@bpe.agr.hokudai.ac.jp
[2] Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Hokkaido, Japan
* Correspondence: shimizu@bpe.agr.hokudai.ac.jp

Abstract: To enhance anaerobic fermentation during food waste (FW) digestion, pretreatments can be applied or the FW can be co-digested with other waste. In this study, lipase addition (LA), hydrothermal pretreatment (HTP), and a combination of both methods (HL) were applied to hydrolyze organic matter in FW. Furthermore, the effects of crude glycerol (CG), which provided 5%, 10%, and 15% of the volatile solids (VS) as co-substrate (denoted as CG5, CG10, and CG15, respectively), on the anaerobic digestion of FW were assessed. With an increasing proportion of CG in the co-digestion experiment, CG10 showed higher methane production, while CG15 negatively affected the anaerobic digestion (AD) performance owing to propionic acid accumulation acidifying the reactors and inhibiting methanogen growth. As the pretreatments partially decomposed hard-to-degrade substances in advance, pretreated FW showed a stronger methane production ability compared with raw FW, especially using the HL method, which was significantly better than co-digestion. HL pretreatment was shown to be a promising option for enhancing the methane potential value (1.773 NL CH_4/g VS) according to the modified Gompertz model.

Keywords: food waste; lipase addition; hydrothermal pretreatment; anaerobic digestion; biogas

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1. Introduction

According to FAO estimates, approximately 1.3 billion tons of food waste (FW) are generated annually across the entire food supply chain [1]. Between 2010 and 2016, global food waste accounted for 8–10% of all man-made greenhouse gas emissions, leading to an annual loss of approximately USD 1 trillion [2]. Current traditional practices, such as incineration and landfill, help release some stress from garbage siege; however, a series of problems require urgent attention, including the further cost of waste disposal, the lack of land space, groundwater pollution by leachate, and the emission of greenhouse gases that need further treatment [3]. As a major component of municipal solid waste [4], FW also promotes the growth of various pathogens, risking harm to human health [5]. Therefore, developing appropriate countermeasures to tackle FW is emerging as a key issue associated with sustainable development and the bioeconomy concept [6]. Anaerobic digestion (AD) is now widely accepted as the most effective technology for energy production and adds value to agronomic organic waste [7], while simultaneously reducing secondary environmental pollution during the digestion process [8,9].

Regarding the characteristics of FW, the crude lipid and crude protein contents are in the ranges of 22.8–31.45% and 14.71–28.64%, respectively, and the main component is carbohydrate [10]. The reported hydrolysis rates for municipal organic waste are in the order of lipids < proteins < carbohydrates [11]. This implies that lipid hydrolysis is the rate-limiting step of the whole anaerobic process for FW. The high lipid content can also result in the accumulation of lipid dross in the digester, which adversely affects organic

matter usage by methanogens and equipment cleanliness. Accordingly, a technology that is highly efficient and requires only mild reaction conditions, with no secondary pollution pretreatment, is urgently needed. In the present study, lipase is investigated as an efficient catalyst for hydrolyzing FW lipids into free long-chain fatty acids (LCFAs), which are further converted to hydrogen and acetate by acetogenic bacteria (β-oxidation process), and finally to methane by methanogenic archaea [12]. Lipase addition (LA) is harmless to anaerobic treatment processes, and its contribution to biochemical oxygen demand in the waste stream is negligible [13]. The biomethane production (1704 mL) of the hydrolysis products of crude lipid in food waste by enzymatic pretreatment was enhanced by 26.9–157.7% [14]. The amount of biogas produced has been proven to be larger when biomass is subjected to thermal preprocessing [15]. Hydrothermal pretreatment (HTP) is a thermal treatment that requires no extra drying [16], and it promotes the dissolution of recalcitrant organic compounds by decomposing cell membranes efficiently at an appropriate temperature and residence time [17]. The application of HTP has focused on lignocellulosic substrates [18], sewage sludge [19], lipid-rich wastewater [20], and babassu oil processing [21]. Wang et al. reported that use of thermal pretreated food waste halved the time needed to produce the same quantity of methane in comparison with fresh food waste [22]. Therefore, this study proposed the HTP method as a technique for effectively shortening the FW hydrolysis time by changing FW properties, resulting in improved efficiency of the subsequent AD process. Subjecting FW to HTP benefitted the two-stage fermentative hydrogen and methane co-production, which exhibited an increase of 31.9% compared with untreated FW (387.9 mL/gVS). However, new methods are still required to process rich lipids that remain stable in hydrothermally pretreated FW [23]. Therefore, the subsequent lipase addition after HTP might also lead to further biomass decomposition.

Crude glycerol (CG) is the main byproduct of the biodiesel industry from the transesterification of vegetable oil, animal fat, or used kitchen oil with alcohol, accounting for about 10% of the initial feedstock weight. Due to their low cost, sodium and potassium hydroxide are principally implicated in the alkali-based transesterification and introduce heavy metals into CG [24]. As a complex mixture, CG contains glycerol, ethanol, water, salt, heavy metals, free fatty acids, unreacted monoglycerides, diglycerides, triglycerides, and methyl esters [25]. There is an oversupply of CG containing a large amount of impurities, and its purification is difficult and expensive. Therefore, CG is treated as waste in many areas of industry, resulting in a waste of resources. The concept of mixing FW with CG has been proposed because the high water content of FW could act as a solvent for CG. Nuchdang [26] reported that the AD of acid-treated glycerol in a synthetic medium had a maximum methane yield of 0.32 L g^{-1} at standard temperature and pressure, with chemical oxygen demand (COD) removal achieved at an organic loading rate (OLR) of 1.6 g COD L^{-1} d^{-1}. Astals et al. [27] reported an increase of about 400% in biogas production under mesophilic conditions when pig manure was co-digested with 4% glycerol, on a wet basis, compared with mono-digestion. Nartker et al. [28] showed that biogas and consequent energy production were significantly increased by a 25% glycerol loading within an anaerobic co-digestion process using primary sewage sludge.

Developing a system that is sustainable, and capable of handling the large amounts of organic waste currently produced in urban and rural areas with high efficiency, is a major current challenge. Therefore, this study aimed to improve the anaerobic digestion of FW by conducting LA, HTP, and their combination (HL) as pretreatments and co-digestion with different ratios of CG, and to evaluate the fermentation quality of these pretreatments and co-digestion methods.

2. Materials and Methods

2.1. Materials and Sample Preparation Procedures

Anaerobic sludge used as the inoculum was obtained from Hokkaido No.1 farm and stored at 52 °C, with its characteristics shown in Table 1. The raw materials used as

substrates were FW collected from the central restaurant of Hokkaido University and CG derived from the transesterification process during biodiesel production provided by Revo International Co., Ltd. (Kyoto, Japan). The FW was minced, homogenized using a blender, and then stored at −4 °C before use, with its characteristics shown in Table 1.

Table 1. Characteristics of FW and CG used as substrates, and the sludge used as inoculum (TS, total solid content; VS, volatile solid content; VFA, volatile fatty acids; TAN, total ammonia nitrogen; FAN, free ammonia nitrogen; C, carbon content; N, nitrogen content).

	TS (%w.b.) [1]	VS (%w.b.)	C (%d.b.) [2]	N (%d.b.)	C/N	VFA (mg/L)	TAN (mg/L)	FAN (mg/L)
FW	19.7 ± 0.17	17.5 ± 0.16	30	3	10	nd [3]	nd	nd
CG	84.51 ± 0.12	79.82 ± 0.14	41.7	0.24	173.75	nd	nd	nd
Inoculum	2.17 ± 0.37	1.40 ± 0.26	nd	nd	nd	829.012 ± 20.226	1507.724 ± 16.034	1108.301 ± 11.786

[1] % Wet basis. [2] % Dry basis. [3] Not determined.

The lipase used in the present study was sourced from *Pseudomonas fluorescens* and purchased from Amano Enzyme Inc. (Nagoya, Japan). The optimal growth conditions were pH 7–8.5 and a temperature of 50–60 °C, and the enzyme activity was 20,000 U/g.

2.2. Pretreatment Methods

2.2.1. Lipase Addition for Food Waste (LA)

Lipase (25 mg) was accurately weighed, dissolved in sodium chloride solution (10 g/L) to a final volume of 1 L, and cooled to below 10 °C. To obtain appropriate conditions for lipase application, the FW pH was adjusted to 8.0 using sodium carbonate solution (4 g/L). A 50% (*w*/*w*) lipase solution was then added to the FW, followed by incubation at 52 °C for 24 h, with no further pH adjustment during the subsequent process.

2.2.2. Hydrothermal Pretreatment (HTP)

HTP of FW was conducted in a 50 mL autoclave (PPY-CTRL, Tokyo Rikakikai Co., Ltd., Tokyo, Japan). FW (30 g) and water (10 mL) were placed in the autoclave. The reactor was operated at 120 °C and 0.3 MPa, held for 60 min from when the autoclave reached the set temperature. The reactor was then cooled to ambient temperature. To generate little of furfural and hydroxymethylfurfural (HMF) during the hydrothermal process, the temperature at a relatively low severity (120 °C) was chosen [29,30]. As FW, which was stored in a refrigerator, had a certain viscosity that can act as a protective shield around the microbes, hydrothermal reaction conditions of 120 °C for at least 40 min were necessary to obtain sufficient sterilization [31].

2.2.3. Combination of Hydrothermal Pretreatment and Lipase Addition (HL)

After HTP treatment of the FW (see Section 2.2.2), 50% (*w*/*w*) lipase solution was added. The mixture was stirred evenly and then left to stand at 52 °C for 24 h.

2.3. Anaerobic Biodegradability Tests

Lipase addition and hydrothermal treatment were both expected to somewhat decrease the contents of total solids (TS) and volatile solids (VS). The VS removal rate was calculated using Equation (1) [32].

$$VS_{removed} = 1 - VS_{output}\left(1 - VS_{input}\right)/VS_{input}\left(1 - VS_{output}\right) \quad (1)$$

These changes are shown in Table 2 and were used to recalculate the feed amounts using Equation (2) to ensure that the OLR was equal to 1 g VS/kg inoculum/day. Experiment design regarding the proportions of the feed mixture is shown in Table 3.

$$\text{Organic loading rate} = \frac{\text{Volatile solid input [g VS]}}{(\text{Inoculum [kg sludge]} \times \text{Fermentation time day})} \quad (2)$$

Table 2. Contents of TS and VS in FW used for different pretreatments.

	Raw FW	LA	HTP	HL
TS (%)	18.15 ± 0.68	7.93 ± 0.34	7.60 ± 0.80	7.07 ± 0.64
VS (%)	17.51 ± 0.09	7.23 ± 0.90	6.75 ± 0.64	5.80 ± 0.20

Table 3. Fermentation substrates used as feed for different experimental groups.

Group	Effects of Pretreatments on FW Anaerobic Digestion	Group	Effects of Co-Digestion with CG on FW Anaerobic Digestion
	Pretreatments for FW		Substrates ratio (FW:CG)
Raw	-	Raw	100:0
LA	Lipase addition	CG5	95:5
HTP	Hydrothermal processing	CG10	90:10
HL	Hydrothermal processing + Lipase addition	CG15	85:15

Laboratory batch anaerobic tests were conducted in Schott Duran bottles as reactors, each with a working volume of 1.0 L and fed with 0.2 kg of sludge as the inoculum. All the reactors were fed as the ration of 1.54 g $VS_{substrate}/VS_{inoculum}$. The feedstock for the group labeled "Raw" was raw FW for mono-digestion, while the feedstocks for the other three groups were FW pretreated by lipase addition (LA group), hydrothermal processing (HTP group), and a combination of these two methods (HL group). The co-digestion group contained CG added in proportions of 5%, 10%, and 15% (denoted as CG5, CG10, and CG15). After flushing with nitrogen for 3 min to remove oxygen, all reactors were capped, sealed, and kept in an incubator (MIR-153, SANYO Electric Co., Ltd., Osaka, Japan) at 52 °C (thermophilic condition) for a hydraulic retention time (HRT) of 21 days.

2.4. Analytical Methods

Element analysis (CE440, Exeter Analytical, Inc., Coventry, UK) was performed to determine the carbon and nitrogen content. Generated gas was collected in gas bags, and its volume was measured using a wet gas meter (W-NK, Shinagawa Corp., Tokyo, Japan). The CH_4 contents of the gas samples were further characterized using a gas chromatograph (GC-4000, GL Science, Tokyo, Japan) equipped with a flame ionization detector. In this study, evaluation of methane production was based on corrected methane yields according to standard temperature and pressure. The daily methane volume was normalized (T = 0 °C, P = 1 bar (1 bar = 105 Pa)) according to Equation (3)

$$V_N = \frac{V \times 273 \times (760 - P_w)}{(273 + T) \times 760} \quad (3)$$

where V_N is the volume of the gas under standard conditions (NL), V is the volume of the biogas (NL), P_w is the water vapor pressure as a function of ambient temperature (mmHg, 1 mmHg ≈ 133.322 Pa), and T is the ambient temperature (°C).

Alkalinity was determined according to standard methods [33]. The concentrations of volatile fatty acids (VFA) and total ammonia nitrogen (TAN) in the digestate were assessed with a titration method using a BUCHI Distillation Unit Type B-323 (BUCHI Corp., Tokyo, Japan). The free ammonia nitrogen (FAN) concentration was calculated using Equation (4) [34]:

$$[NH_3] = \frac{[TAN]}{1 + \frac{[H^+]}{K_b}} \quad (4)$$

where $[NH_3]$ is the free ammonia concentration and K_b is the dissociation constant (34.4×10^{-10} at 52 °C).

TS and VS were determined by drying wet samples at 105 °C for 24 h, followed by incineration at 600 °C for 3 h. The pH of each sample was determined using a pH meter. Each measurement was performed in triplicate, and the mean result was calculated. The

composition of the liquid phases of FW following pretreatments was analyzed using high-performance liquid chromatography (HPLC, Agilent 1260 Infinity, Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a Shodex SUGAR SH 1821 column (Showa Denko, K.K, Tokyo, Japan) and an Optilabrex 1260 GPC differential refractive index detector. VFA components in the digestate were analyzed by HPLC equipped with a Shodex RSpak KC811 column (Showa Denko KK, Tokyo, Japan). The mobile phase was 0.1% H_3PO_4 at a flow rate of 0.9 mL/min, with a reflective index detector and a column temperature of 40 °C used.

2.5. Modified Gompertz Model

The modified Gompertz model [35], shown in Equation (5), was used for curve fitting of the biogas and methane production values. The kinetic constants for anaerobic digestion (AD) under different treatment conditions were determined, the dynamic process was simulated, and the biogas and methane production potential of all groups was quantitatively analyzed. The fitting of this model was achieved using Origin 2020b software (OriginLab Corporation, Northampton, MA, USA). Data obtained from all experimental groups were checked for goodness of fit with the model and evaluated using Pearson correlation coefficients (SPSS Statistics, IBM, Armonk, NJ, USA).

$$H = P * \exp\left\{-\exp\left[\frac{R_m e}{P}(\lambda - t)\right] + 1\right\} \quad (5)$$

where H is the cumulative methane production (NL/g VS) recorded at time t (d), P is the methane potential (NL/g VS), R_m is the maximum methane production rate (NL/g VS d), e is exp (1) = 2.718, and λ is the lag-phase period (d). The fitness of this model was evaluated using analysis of variance (ANOVA), and the significance (*p*-value) was considered according to a 95% confidence level.

3. Results and Discussion

3.1. Effect of Pretreatments on the Properties of FW

After LA, HTP, and HL pretreatments, the VS ratio dropped to 7.23%, 6.75%, and 5.80%, respectively (Table 2), indicating that pretreatments promoted the solubilization of solids in FW. The main hydrolysates of lipids from FW were VFAs and LCFAs, which should be converted to acetate through β-oxidation and finally to biomethane [12]. When HTP was conducted, high-molecular-weight carbohydrate polymers (such as starch, cellulose, and hemicellulose) were hydrolyzed into low-molecular-weight oligosaccharides and monosaccharides (such as glucose and xylose) [36]. In terms of solubilization, HL was the most efficient method. HTP first alters the structure of the insoluble fraction to make it more amenable to biodegradability, resulting in shortened hydraulic retention times for solubilization [23], and then LA further promotes the hydrolysis of residual lipids [18]. Therefore, the combined HL method achieved the highest decomposition rate of TS and VS contents. This promoted hydrolysis and acidogenesis steps of the anaerobic fermentation substrate, and provided essential nutrients for the growth and activity of anaerobic bacteria. The properties of the filtered liquid phase of FW following pretreatments were detected by HPLC. The concentrations of glucose, fructose, acetic acid, and ethanol in the filtered liquid phase following pretreatments are summarized in Table 4.

Table 4. Compositions of the filtered liquid phase of FW after (a) LA, (b) HTP, and (c) HL pretreatments.

	Glucose (g/L)	Fructose (g/L)	Acetic Acid (g/L)	Ethanol (g/L)
LA	0.0108	0.000870	2.20	1.48
HTP	0.0168	0.00259	5.47	2.16
HL	0.0102	0.0023	6.02	1.97

The carbohydrates in FW include starch, cellulose, and hemicellulose, which can be easily hydrolyzed under HTP conditions, with glucose and xylose generated as the main intermediates [37]. Continued heating of water further decomposes the intermediate components formed (oligomers and glucose) into organic acids, such as acetic acid. After pretreatments, the generated acetic acid and ethanol in the liquid phase of FW could be used by methanogens, serving as a precursor for two-thirds of methane generation [38]. After HTP, more glucose, fructose, and ethanol accumulated in the liquid phase. As lipids from plant oils and animal fat are difficult to dissolve in water unless under supercritical conditions, the lipase was used to hydrolyze lipids into fatty acids [39]. After adding lipase solution, the acetic acid concentration increased correspondingly.

3.2. Anaerobic Biodegradation Assays

3.2.1. Effects of Pretreatments on FW Anaerobic Reactors

Under the pretreated FW digestion conditions, the daily biogas and methane generated from raw FW were much lower than those obtained after pretreatments. Figure 1 shows the biogas and methane accumulated over 21 d through acidification and methanogenesis processes occurring in the batch reactors. For the LA group, peak methane production occurred on the third, fifth, and sixth day after feeding. During the subsequent digestion time, the methane production rate gradually slowed down, and tended to stop from the sixth day because the hydrolysate was consumed and hydrolysis rate decelerated with increasing fermentation time. The methane production peak for the HTP group occurred on the second and fifth day, possibly owing to high-molecular-weight carbohydrate polymers, such as starch, cellulose, and hemicellulose, hydrolyzing into smaller oligosaccharide and monosaccharides molecules [40], and started to weaken on the seventh day. The possible reason is that these reducing sugars react with amino acids and proteins in FW to undergo a Maillard reaction under heating conditions, which produces ketones, aldehydes, and heterocyclic compounds, and finally generate melanoidin. This is the reason for FW turning brown after hydrothermal treatment. The production of these Maillard reactants consumed part of the carbon source and, owing to the difficulty of their degradation, inhibited the AD process to some extent. Daily methane production on the first day in the HL group, which combined the two LA and HTP pretreatments, was not much different to that in the HL pretreated groups, but a higher daily methane production rate was maintained from the second day. This was possibly due to HTP pretreatment breaking down the recalcitrant structure of the biomass and accelerating hydrolysis, with the release of water-soluble sugars and added lipase further decomposing the insoluble lipids. Therefore, this step accelerated the accumulation of VFAs in the substrate, a proportion of which was directly used by methanogens to produce biomethane. The HL method led to the rapid release of organic matter in FW, resulting in a balance between hydrolysis and methane generation [20], and maintained a high biogas production rate until the sixth day. The HL group exhibited the highest increase in VS removal rate of 63.5%, which was in stark contrast to that of the Raw group owing to complete decomposition of the organic components.

Ultimately, the proposed pretreatments had a significant effect on improving FW AD. The digestion of FW treated by LA, HTP, and HL afforded cumulative methane yields of 1.263, 1.384, and 1.686 NL CH_4/g VS, respectively, and VS removal rate increases of 51.2%, 54.5%, and 63.5%, respectively, compared with raw FW AD and co-digestion with CG (Table 5). Overall, biogas and methane production from the HL group increased at a faster rate during the digestion process. After the sixth day, relatively stable methane production was maintained, probably owing to increased solubilization of the organic solids making the substrates more available to the anaerobic microorganisms [41].

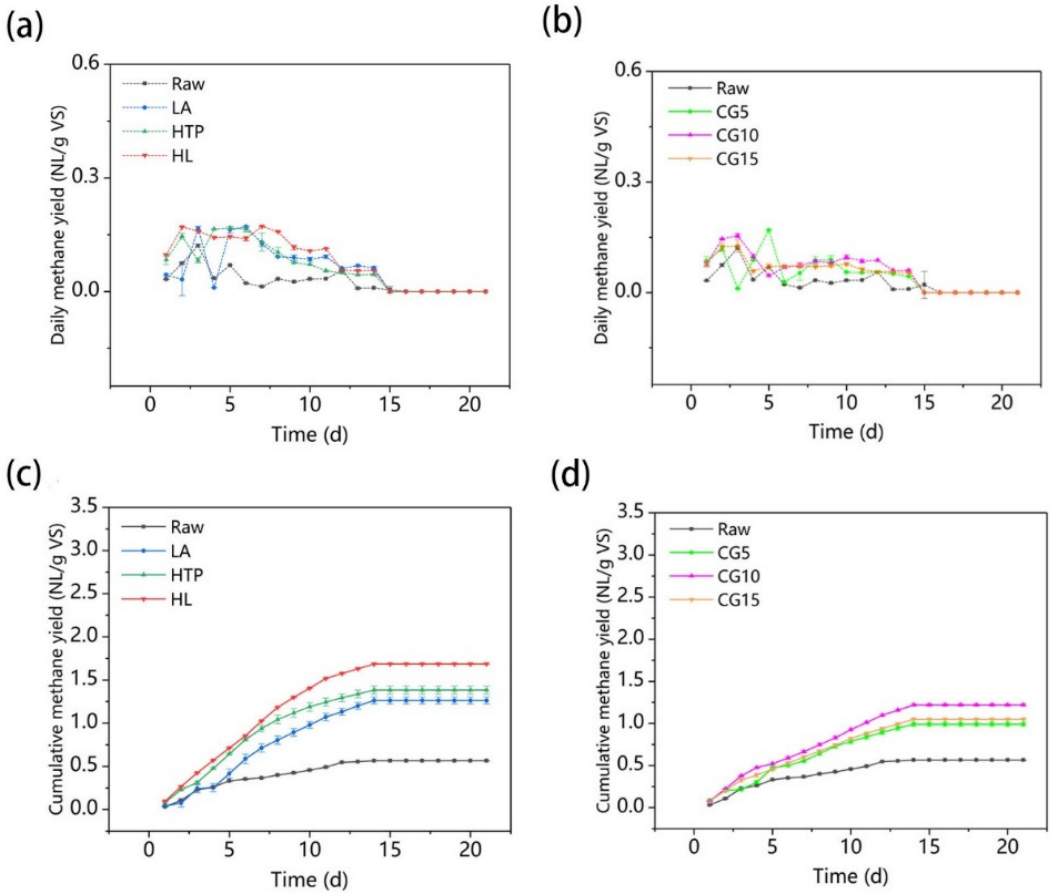

Figure 1. Daily methane yield: (**a**) LA, HTP, and HL pretreated FW groups; (**b**) FW co-digestion with 5, 10, and 15% CG) and cumulative methane yield: (**c**) LA, HTP, and HL pretreated FW groups; (**d**) FW co-digestion with 5, 10, and 15% CG).

Table 5. VS_{input}, VS_{output}, and $VS_{removed}$ values of all experimental groups.

	VS_{input} (%)	VS_{output} (%)	$VS_{removed}$ (%)
Raw	2.45	1.90	22.9
LA	2.43	1.20	51.2
HTP	2.45	1.13	54.5
HL	2.48	0.92	63.5
CG5	2.47	1.93	22.3
CG10	2.48	1.86	25.5
CG15	2.48	1.91	23.4

3.2.2. FW Anaerobic Co-Digestion with 5%, 10%, and 15% (of VS Provided) CG

Co-digestion of FW with CG at a FW/CG ratio (% of VS provided) of 90:10 (CG10 group) produced better results regarding enhanced methane production and the rapid peak appearance on the second day after feeding. Adding CG had an important effect on the parameters related to matter content (C/N ratio). Under these feeding conditions, adding CG (5%, 10%, 15% of VS replaced) increased the C/N ratio from 10 to 18.2, 26.4, and 34.1, respectively, while methane accumulation nearly doubled compared with raw FW mono-digestion. The C/N ratio is an indicator of nutrient availability for AD process. For a well-operated AD reactor [42], C/N can be between 20 and 30, with 25 being optimal [43]. The C/N ratio of CG10 was in the optimal range. It indicated that methane generation was substantially affected by the availability and characteristics of the substrate, which would contribute to meeting the nutritional requirements of microorganisms and achieving optimal microbial activities.

Combined analysis of Figure 1b,d and Table 5 showed that the daily and cumulative methane production performance and VS removal rate of the CG15 group, containing a higher proportion of CG, were decreased, possibly owing to overloading with CG and its various impurities. The disadvantages of CG overloading have also been reported previously, with the considerable dissolved inorganic salts content in CG causing inhibition of the biomass activity [44]. Rétfalvi et al. [45] studied the effects of overloading (an OLR increase of 10% per day) on a laboratory scale using CG as the only substrate. The study showed that the concentration of acetic acid and propionic acid increased significantly, possibly due to the suppression of methanogenic bacteria or CG containing other ingredients or impurities (methanol, potassium and sodium salts, heavy metals, and soap) [46] that led to poor anaerobic digestibility or toxicity to anaerobic microorganisms. Therefore, adding 15% CG can reasonably be inferred to have overloaded the FW anaerobic reactors. Fountoulakis [47] reported that, after 20 d, biogas production decreased significantly when 3% CG was added to the sewage sludge digestion tank compared with the group containing 1% CG, because overloading the reactor with CG had increased the propionate concentration. Furthermore, lipid hydrolysis is a reversible reaction [48–50], with the added CG potentially pushing the reaction in the negative direction and inhibiting lipid hydrolysis to some extent.

3.2.3. Kinetic Parameters from Modified Gompertz Model

The Gompertz model can be used to obtain kinetic parameters, providing a preliminary basic design for development into an industrial-scale process. Table 6 shows the kinetic parameters for P (NL/g VS), R_m (NL/g VS d), and λ (d) in all experimental groups. As the rate of methane production in a batch digester has been assumed to correspond to the specific growth rate during the methanogenic bacteria growth phase, kinetic models are usually used to simulate the anaerobic biodegradation process [51]. In the present study, biogas and methane production were successfully fitted using the modified Gompertz equation, as shown in Equation (5). Lag phase (λ) is an important parameter to determine the substrate biodegradability and utilization rate. The negative λ (d) value fitted from the experimental data occurred when bioproducts were generated almost immediately without a lag period [52]. The shortest λ in pretreated groups was for the HL group (0.881 d), and in co-digestion groups, it was for the CG10 group (−0.122 d), while the longest was in raw FW mono-digestion (1.794 d). The results for FW co-digestion were in agreement with the description above, with maximum methane production rates (R_m) of 0.109 NL/g VS d recorded at a substrate ratio of 90:10 (FW/CG, %VS, Raw10 group) and methane production potential (P) of 1.272 NL/g VS, respectively. When 15% CG was added (according to the VS proportion provided), the P and R_m values both gradually declined, possibly owing to the complexity of the CG substrate. For the LA, HTP, and HL pretreatments, the maximum *p*-value of methane increased to 1.312, 1.420, and 1.773 NL/g VS, respectively. The predicted P and R_m values were consistent with the experimental results and verified that biomethane production was enhanced by the pretreatments, particularly in the HL group, which exhibited the highest methane potential. This meant that under the same VS input from substrate, pretreatments can better promote methane production performance than co-digestion can. The high coefficients of determination (R^2) of 0.988–0.999 showed that the models using the kinetic parameters predicted by the Gompertz model fitted the experimental data well. The actual methane production was lower than the predicted value owing to the complexity of the anaerobic systems consisting of substrate, inoculum, acidification products, ammonia dissolution, and changes in microbial flora.

Table 6. Gompertz kinetics for methane production under different experimental conditions.

	Gompertz Kinetics for Methane Production			
	P (NL/g VS)	**R_m (NL/g VS d)**	**λ(d)**	**R^2**
Raw	0.571 ± 0.007	0.070 ± 0.004	1.794 ± 0.048	0.997
LA	1.312 ± 0.013	0.136 ± 0.002	1.024 ± 0.127	0.999
HTP	1.420 ± 0.018	0.157 ± 0.004	0.881 ± 0.076	0.997
HL	1.773 ± 0.028	0.172 ± 0.004	0.830 ± 0.135	0.996
CG5	1.047 ± 0.022	0.090 ± 0.004	0.437 ± 0.225	0.992
CG10	1.272 ± 0.036	0.109 ± 0.008	−0.122 ± 0.269	0.988
CG15	1.091 ± 0.014	0.100 ± 0.005	0.163 ± 0.197	0.995

3.3. Characteristics of Digesters and System Stability during the Anaerobic Digestion Process

Figure 2 shows the contents of VFA, alkalinity, TAN, and FAN, and the pH values during the AD processes. After pretreatments, more proteins, carbohydrates, and lipids were dissolved from FW into liquid phase and VFAs production was significantly improved. Regarding pH, the LA and HTP pretreatments helped volatile acids to accumulate, and significantly lowered the pH of the four experimental groups below the initial pH value in the first two weeks.

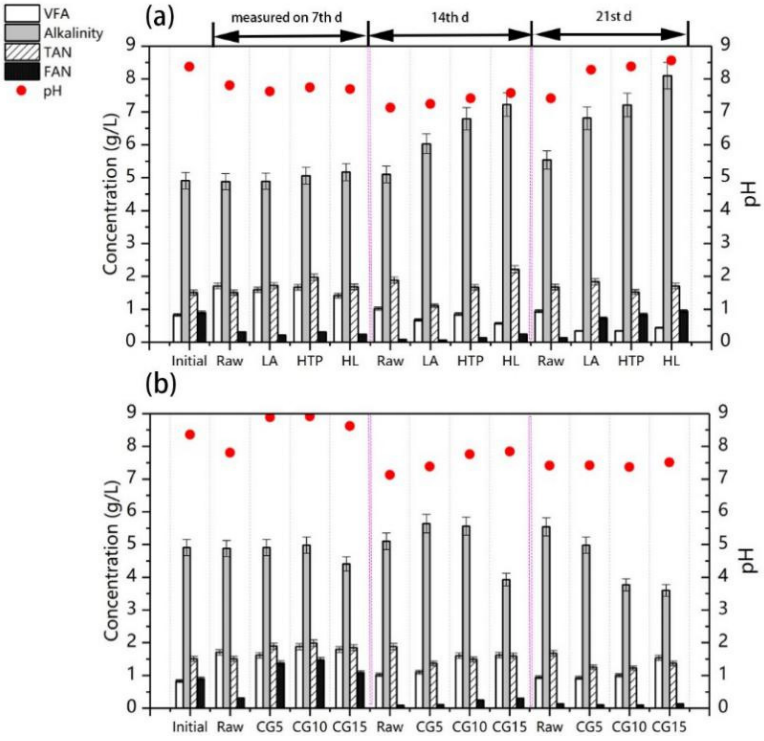

Figure 2. VFA, alkalinity, TAN, and FAN contents, and pH of digesters (measured every 7 d) during the whole AD process: (**a**) raw and pretreated FW mono-digestion; (**b**) FW co-digestion with CG (5%, 10%, and 15% of VS).

Because the pretreatment promotes the dissolution of organic matter, it produces VFAs faster, which can be quickly used by microorganisms to produce methane. The raw food waste needs to go through a long hydrolysis stage. If the generated volatile acid cannot be consumed in time, the VFAs will accumulate and the reactor will gradually acidify. The relatively stable pH of co-pretreated HL groups probably resulted from the rapid consumption of VFAs and increase in the buffering capacity during ammonia release, which effectively neutralized a proportion of the generated VFAs. After two weeks of acid production and adaptation, the pH values increased in the third week. According to conditions in the bioreactor on the seventh day, co-digestion can also maintain higher pH

and ammonia levels, but the ammonia concentration gradually decreased and the system gradually acidified with continued fermentation, especially in the CG15 group, reflecting that no biogas production occurred in the third week. Alkalinity is a source of the system stability and is recommended to be 2 to 4 g $CaCO_3$ L^{-1} in a laboratory-scale anaerobic digestor [53].

Figure 3a shows the VFA/alkalinity ratios in the experimental groups. The low C/N ratio 10 of raw FW (Table 2) might have caused the high concentrations of ammonia [54]. Callaghan et al. [55] found that the VFA/alkalinity (both g/L) ratio can be used to judge system stability. At a VFA/alkalinity ratio of <0.4, the digester should be stable; between 0.4 and 0.8, some instability will occur, and at >0.8, the digester will be significantly unstable.

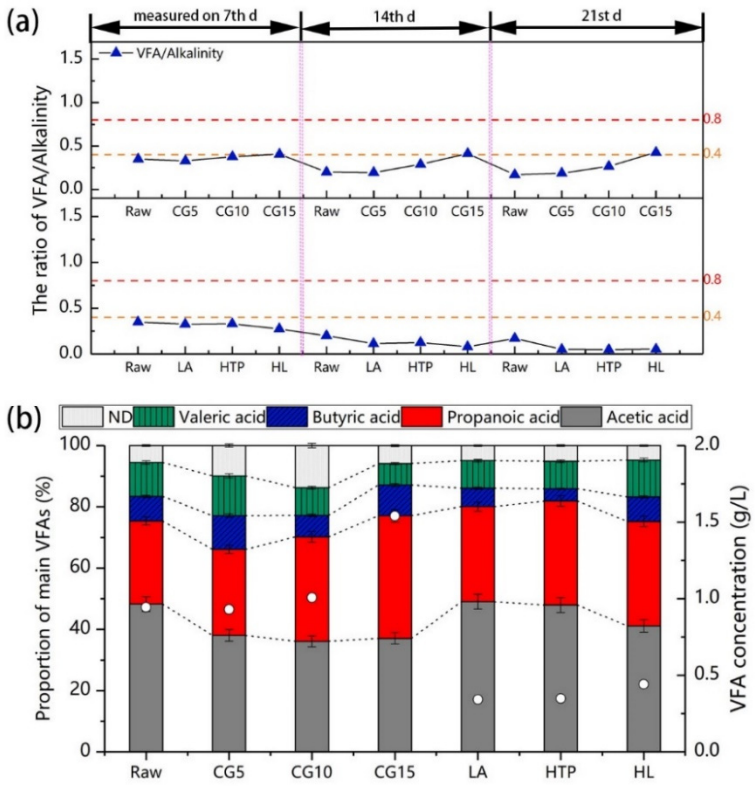

Figure 3. (**a**) VFA/alkalinity ratio; (**b**) main VFA compositions and proportions measured on the 21st day.

The VFA/alkalinity ratio values of CG5, CG10, and all pretreated groups were always found to be lower than 0.40, thus indicating that the process operated favorably without the risk of acidification. The VFA/alkalinity ratio was closest to 0.4 as the proportion of CG increased to 15% VS provided, showing that the AD system tended to become unstable at higher CG contents. Therefore, the gradual accumulation of VFA might have caused the anaerobic fermentation capacity to decrease during the later stages of the experiment. Overall, the combined HL pretreatment method helped to improve the system stability, because the pH of FW after HTP needed to be adjusted to 8.0 before adding lipase to maintain lipase activation, giving the substrate a certain buffering capacity. Adding a biological enzyme preparation to FW helped to improve the buffering acidity by releasing NH_4^+ ions, diluting the concentrations of toxic chemicals, and adjusting the nutrient availability.

The composition of VFAs produced is an important factor that can provide useful information regarding the degree of hydrolysis and fermentation processes. The compositions of the main VFAs in digesters from different experimental groups after three weeks are shown in Figure 3b. The most prevalent products in all reactors were acetic acid (HAc) and propionic acid (HPa). The high non-fiber carbohydrate and fat contents in FW might have increased the rate of HPa production to a level that exceeded its consumption

rate [56]. However, as the amount of CG increased, HPa gradually replaced HAc as the most prevalent product. A previous study [57] showed that the conversion rates of VFAs to methane were in the order of HAc > butyric acid (HBu) > HPa. Owing to differences in their order of utilization, microbial populations did not appear to use accumulated HPa efficiently while rapidly consuming HAc [58]. This might be due to the conversion of HPa to HAc (related to standard conditions) being an endothermic reaction that requires additional energy [59].

Syntrophic bacteria must absorb energy to convert HPa to HAc because the Gibbs free energy is positive. In contrast, methane generation from substrates such as HAc and hydrogen involves exothermic reactions that can proceed spontaneously. This extra energy demand makes transforming HPa into HAc, which can be directly used by methanogens, very difficult. When the rate of VFA consumption is less than its accumulation rate, the reactors will gradually acidify.

As a substrate with high degradability, glycerol shifts the metabolic pathway toward HPa production to make cells maintain the redox balance [60]. This occurs because 1 mol of glycerol converted to pyruvate generates 2 mol of NADH (the reduced form of nicotinamide adenine dinucleotide (NAD)). The propionic acid synthesis pathway must then completely regenerate NAD^+ (an oxidized form of NAD) to maintain the redox balance (Figure 4). Pyruvate, which can be transformed into various acid products, is key in the network of acid production metabolic pathways. Conversely, 1 mol of NADH will be produced by the conversion of pyruvate to acetate. In contrast, 1 mol of pyruvate used in cell biomass production consumes only 1.44 moles of NADH, resulting in an imbalance of the $NADH/NAD^+$ ratio, meaning that cell growth on glycerol can be inhibited. Wang [61] concluded that no significant inhibition of the activity of methanogenic bacteria occurred when the highest concentrations of ethanol, HAc, and butyric acid were 2400, 2400, and 1800 mg/L, respectively. However, significant inhibition occurred when the HPa concentration was increased to 900 mg/L, with the bacterial concentration decreased from 6×10^7 to 0.6–1 $\times$ 10^7 CFU/mL, and this activity could not be recovered. This also provided relevant information to prove CG overloading in pretreated groups. Barbirato et al. [62] suggested that, instead of using conventional carbon sources for propionic acid biosynthesis, glycerol was a promising substrate for producing propionic acid, in terms of both conversion yield and productivity. This phenomenon might explain why the increasing CG ratio inhibited digestibility. In this study, methanogens were able to tolerate CG ratios of up to 10%, but a significant inhibitory effect was observed for FW co-digestion with 15% CG. Furthermore, the methane production capacity and system stability of the co-digestion groups were obviously lower than those of the pretreatment groups, especially for the HL method.

$$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2 \Delta G = +48.1 kJ \quad (6)$$

$$CH_3CH_2COO^- + 6H_2O \rightarrow 2CH_3COO^- + 2HCO_3{}^- + 2H^+ + 6H_2 \Delta G = +152.2\ kJ \quad (7)$$

$$2CH_3COO^- + 2H_2O \rightarrow 2CH_4 + 2HCO_3{}^- \Delta G = -62.0\ kJ \quad (8)$$

$$4H_2 + HCO_3{}^- + H^+ \rightarrow CH_4 + 3H_2O\ \Delta G = -135.6 kJ \quad (9)$$

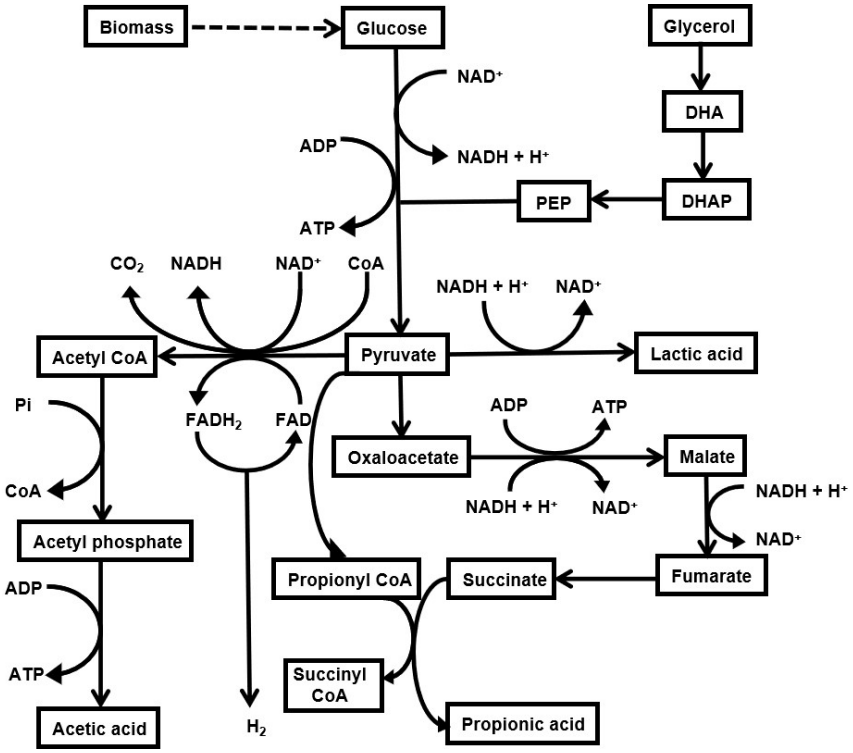

Figure 4. Schematic diagram of the fermentation pathway for biomass from FW under the action of microorganisms and the dicarboxylic acid pathway for propionic acid fermentation of glycerol. (DHA, docosahexaenoic acid; DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvic acid; ADP, adenosine diphosphate; ATP, adenosine triphosphate; FAD, flavin adenine dinucleotide; $FADH_2$, reduced flavin adenine dinucleotide; Pi, phosphate group; CoA, coenzyme A).

4. Conclusions

Co-digestion with 10% (VS replaced) CG improved the biodegradability of raw FW most effectively owing to the decomposability and sufficient supplementation of the carbon source. However, using CG to replace 15% of VS provided by FW led to suppression of the digestion activity owing to significant accumulation of propionic acid and decreasing alkalinity. The pretreatments used further benefited the digestion process by significantly increasing the total volume of methane and accelerating the methane production peak. Compared with other pretreatments and co-digestion, the highest cumulative methane yield was observed for HL-treated FW, which also led to a higher solids solubilization and VS removal rate of 63.5%, and higher methane potential and maximum methane production rate from Modified Gompertz model.

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Nomenclature

Abbreviation	Meaning
FW	food waste
CG	crude glycerol
LA	lipase addition
HTP	hydrothermal pretreatment
HL	a combination of hydrothermal pretreatment and lipase addition
AD	anaerobic digestion
VS	volatile solids
VFA	volatile fatty acids
TAN	total ammonia nitrogen
FAN	free ammonia nitrogen
HAc	acetic acid
HPa	propionic acid
HBu	butyric acid

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