**New method for enhancement of bioenergy production from
municipal organic wastes via regulation of anaerobic fermentation
process**

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Abstract: Although biological recovery of energy (such as hydrogen and methane) from municipal organic wastes (MOW) is a hot topic, the bio-energy production yield is usually unsatisfied due to the low efficiency of anaerobic fermentation process regulation. In this paper, a new method for increasing the production yields of hydrogen and methane from MOW via efficiently regulating the anaerobic fermentation process was reported. It was found that by firstly fermenting the MOW under alkaline conditions to produce hydrogen and volatile fatty acids (VFA)-enriched fermentation liquid (stage I), and then anaerobically treating the fermentation liquid at neutral pH value for methane production (stage II), the yields of both hydrogen and methane were significantly increased. The highest yields of H₂ and VFAs were obtained at C/N of 25.2 and pH of 8 in stage I, and the corresponding fermentation liquid generated the highest CH₄ yield in stage II. The overall energy gain of this method was estimated as 4.54 kW·h/kg-COD. The mechanism study showed that the enzymatic activities with MOW hydrolysis (protease and amylase), acidification (phosphotransacetylase, phosphotransbutyrylase, acetate kinase, butyrate kinase, oxaloacetate transcarboxylase, and CoA transferase) and hydrogen generation (pyruvate-ferredoxin oxidoreductase) exhibited the greatest at alkaline pH, which resulted in the increase of hydrogen production together with remarkably high VFA (especially acetic acid) accumulation. When this VFA-enriched fermentation liquid was used to produce methane, the enhancement of methane yield was therefore observed.

Keywords: Municipal organic wastes, hydrogen, methane, fermentation, regulation

1. Introduction

China has a population of nearly 1.3 billion, and the municipal waste has reached more than 150 million tons each year. With the rapid economic development, the level of urbanization and the improvement of people's living standards, the amount of municipal solid waste in China is increasing

greatly with a rate of around 8%. It is an urgent task faced by China's environmental protection to develop an effective management of municipal organic wastes (MOW) with low environmental pollution. As there are large numbers of organic compounds, such as protein and polysaccharide, in MOW, it is attractive to recover valuable bioproducts from municipal organic wastes [1].

Anaerobic treatment of organic wastes is a highly sustainable process because it can reduce the environmental pollution of wastes and simultaneously generate energy (such as hydrogen and methane) [2-4]. During anaerobic treatment the organic compounds usually undergo the stages of hydrolysis (i.e., carbohydrates and proteins in wastes are hydrolyzed to their soluble monomers), acidification (the hydrolyzed soluble monomers are bio-converted to volatile fatty acids (VFA), especially acetic acid, and carbon dioxide and hydrogen), and methanogenesis (methane is produced from acetic acid and hydrogen) [5]. As hydrolysis is generally the rate-limited step during anaerobic treatment of MOW, in order to increase the energy recovery efficiency physical or chemical pretreatment methods have been applied to accelerate the hydrolysis process [6-9]. Nevertheless, very few publications reported the simultaneous increase of hydrogen and methane production from municipal organic wastes by improving the hydrolysis, acidification and methanogenesis steps.

It is well known that acetic acid is a preferred substrate for methane production. It has been reported that acetic acid and hydrogen are produced in the acidification step, and higher acetic acid generation is usually accompanied with greater hydrogen production [10]. However, few studies considered that the simultaneous improvement of both hydrogen and methane yields could be achieved during wastes anaerobic treatment by firstly increasing the acetic acid accumulation, and then using the acetic acid as the substrate to produce methane. The purpose of this paper was therefore to report a new strategy for increasing both hydrogen and methane production from municipal organic wastes by

regulating the anaerobic fermentation process, especially the hydrolysis and acidification steps. Unlike the traditional method, in this study, municipal organic waste was firstly fermented under alkaline conditions (i.e. stage-I) to produce hydrogen and VFA-enriched fermentation liquid, and then the fermentation liquid was treated at neutral pH value for methane productions (stage-II). As the yields of both hydrogen and methane were significantly increased by the use of this new method, the fundamental microbial mechanisms were then investigated in detail.

2. Materials and Methods

2.1 Municipal organic wastes and anaerobic granular sludge

Polysaccharide (or carbohydrate) and protein were the main organic components of MOW, and the variations of their ratio were observed to affect both hydrogen and methane yields. In this study, the food waste and municipal waste sludge were mixed, and then used as the MOW. The food waste came from a dining hall in Shanghai, China, which was milled to slurry state and diluted with tap water to final total COD (TCOD) of 142.3 ± 15.1 g/L before experiments. The data showed it had total carbohydrate of 84.1 ± 5.3 g COD/L, total protein of 21.4 ± 3.2 g COD/L, total solids (TS) of 80.6 ± 4.2 g/L and volatile solids (VS) of 76.8 ± 3.8 g/L, respectively. The municipal waste sludge was withdrawn from a municipal wastewater treatment plant. After concentrated by settling at 4°C for 24 h, its main characteristics were TCOD 17.5 ± 1.1 g/L, total carbohydrate 1.7 ± 0.2 g COD/L, total protein 9.5 ± 1.1 g COD/L, TS 17.4 ± 2.6 g/L, and VS 12.2 ± 1.3 g/L. The anaerobic granular sludge (AGS), obtained from an upward-flow anaerobic sludge blanket (UASB) reactor of a food wastewater treatment plant in Wuxi (Jiangsu province, China), was used as the inoculums for methane production from the VFA-enriched fermentation liquid of MOW. In order to eliminate the possible influences of methanogens, which might appear in different content in the fermentation substrate (food waste and

municipal waste sludge), on the experiments, the MOW was pretreated at 102 °C for 30 min to kill methanogens [11], and then cooled down to room temperature for the tests.

2.2 Tests of bio-hydrogen production and VFA generation from municipal organic wastes

The experiments were conducted in serum bottles with working volume 600 mL each. The initial TCOD of MOW in all tests maintained almost identical (5143 ± 204 mg/L), and the changes of carbon to nitrogen ratio of MOW were achieved by mixing different fraction of food waste and sludge. At each carbon to nitrogen ratio six pH values (5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) were studied. Every two hours the fermentation pH was adjusted by 4 M hydrochloric acid or 4 M sodium hydroxide to their initial one. The serum bottles were flushed with nitrogen gas, capped with rubber stoppers, and then placed in an air-bath shaker (120 rpm, 35 °C). Samples were obtained at different fermentation time for chemical analyses, such as VFA and hydrogen. For the assays of enzymatic activity, samples were withdrawn from the continuous fermentation serum bottles. The serum bottles were operated almost the same as described above with minor modifications. The fermentation mixture was wasted every day to maintain the hydrolytic retention time (HRT) to be approximately 8 d in each bottle, and the same amount of fresh food waste and waste sludge were supplemented. It took 86 days' culture before the generations of H₂ and VFA in all reactors reached relatively stable, and then the activities of key enzymes were determined.

2.3 Experiments of producing methane from the fermentation liquid of MOW

Our previous study indicated that the expanded granular sludge bed (EGSB) showed a much higher methane production yield from the fermentation liquid of waste activated sludge than the up-flow anaerobic sludge bed (UASB) and anaerobic sequencing batch reactor (ASBR) [12]. Therefore, in this study, the methane production from three fermentation liquids (i.e., the fermentation liquid of carbon to

nitrogen ratio=7.2 and pH=10, the fermentation liquid of carbon to nitrogen ratio=25.2 and pH=8, and the fermentation liquid of carbon to nitrogen ratio=40.1 and pH=5) was studied and compared in three identical EGSB reactors. Each reactor had a working volume of 4.5 L with the internal diameter of 60 mm and height of 1400 mm, and was maintained at a constant temperature room ($35 \pm 2^{\circ}\text{C}$). The operation of EGSB and the culture of anaerobic granular sludge were the same as that described by Zhang et al [12]. The pH values of all fermentation liquids were adjusted to pH 7 before experiments. The final organic loading rate (OLR) was controlled at 40 kg-COD/m³/d. After the methane production in all reactors did not change significantly with time, the data were reported.

2.4 Analytical methods.

The content of ATP and the activities of protease, mylase, pyruvate-ferredoxin oxidoreductase (POR) and H₂-uptake hydrogenase were measured according to the references [13-16]. The gas components, such as methane and hydrogen, were determined via a gas chromatograph (6890N, Agilent, Santa Clara, CA, USA) equipped with a thermal conductivity detector using nitrogen as a carrier gas [10]. The energy gain of anaerobic treatment was calculated according the literature [29]. Other enzymes (including phosphotransacetylase (PTA), phosphotransbutyrylase (PTB), acetate kinase (AK), butyrate kinase (BK), oxaloacetate transcarboxylase (OAATC), CoA transferase, pyruvate-ferredoxin oxidoreductase (POR), formate dehydrogenase (FDH) and formyltetrahydrofolate synthetase (FTHFS)), VFA, protein and carbohydrate were assayed as described in our previous publications [17-19]. In this study the sum of measured acetic, propionic, n-butyric, iso-butyric, n-valeric, and iso-valeric acids was recorded as the concentration of total VFA with proper COD conversion (i.e. 1 g acetic acid=1.07 g-COD, 1 g propionic acid=1.51 g-COD, 1 g butyric acid=1.82 g-COD, and 1 g valeric acid=2.04 g-COD [20]).

3. Results and Discussion

3.1 Bio-hydrogen production and VFA generation from municipal organic wastes

It was observed that the hydrogen yield during anaerobic fermentation of MOW was affected by time, and the time required for achieving maximal hydrogen yield was observed to be influenced by the carbon to nitrogen ratio and pH value. As seen in Figure 1A, the required fermentation time increased with the carbon/nitrogen ratio at a certain pH value, which might be attributed to be more carbohydrate, a preferred substrate for bio-hydrogen production [21-23], present in the fermentation system at higher carbon to nitrogen ratio. For example, at pH 5 the fermentation time was 55 h at carbon to nitrogen ratio of 7.2, but it was increased to 103 h at carbon to nitrogen ratio of 15.3, which was further increased to 175 h at carbon to nitrogen ratio of 40.1. Figure 1A also showed that at a certain carbon to nitrogen ratio the time necessary for achieving the maximal hydrogen was not significantly affected by the fermentation pH in the range of pH 5-8, but a longer time was needed at pH 9 and pH 10 due to the adaptation time being required by the microbes under alkaline fermentation conditions. Taking the carbon to nitrogen ratio of 25.2 as an example, the fermentation time was 127 h in the range of pH 5-8, but it became 168 h and 192 h at pH 9 and pH 10, respectively. It was noted that the fermentation time should be controlled at 192 h under the pH 10 condition no matter what the carbon to nitrogen ratio was, which suggested that the adaptation time was a more important element affecting bio-hydrogen production than the carbon to nitrogen ratio if the anaerobic fermentation occurred at a stronger alkaline pH (such as pH 10).

Figure 1. Effect of pH and carbon to nitrogen ratio on the time required for achieving the maximal hydrogen production (A), the maximal hydrogen yield (B), and the VFA generation at the time of maximal hydrogen production. The carbon to nitrogen ratio of sole waste activated sludge and food waste was respectively 7.2 and 40.1 g/g. The data reported are the averages and their standard deviations.

As shown in Figure 1B, at carbon to nitrogen ratio of 7.2 the maximal hydrogen production was 21 mL/g-TCOD, which occurred at pH 10. When the ratio was increased to 10.1, the maximal hydrogen yield (36.6 mL/g-TCOD) was observed at pH 9. Further increasing the ratio to 15.3, 20.4, and 25.2, respectively, the corresponding maximal hydrogen yields were increased to 44.8, 84.9 and 100.9 mL/g-TCOD, which all occurred at pH 8. However, when the carbon to nitrogen ratio increased to 30.4 and 40.1, respectively, the maximal bio-hydrogen yields (77.8 and 64.8 mL/g-TCOD) appeared at pH 6 and pH 5. Apparently, the highest hydrogen production (100.9 mL/g-TCOD) occurred at carbon to nitrogen ratio of 25.2 and pH of 8, which was almost 5 folds of sole sludge (carbon to nitrogen ratio of 7.2 and pH 10) and 1.6 folds of sole food waste (ratio 40.1 and pH 5).

The influences of carbon to nitrogen ratio and pH on VFA generation were the same as that on bio-hydrogen production. As seen in Figure 1C, in the sole waste sludge fermentation reactor (carbon to nitrogen ratio =7.2), the generated VFA was increased with pH value, and the maximal VFA (117 mg COD/g TCOD) was observed at pH 10, which was in correspondence with our previous publication [24]. After food waste (i.e., the carbohydrate enriched organic matter) was mixed with sludge at a carbon to nitrogen ratio of 10.1, however, the fermented maximal VFA was 172 mg COD/g TCOD, which appeared at pH 9. With the increase of carbon to nitrogen ratio from 15.3 to 25.2, the VFA generation was increased significantly from 209 to 311 mg COD/g TCOD, and the optimal pH changed to pH 8. At the ratio of 30.4 both the maximal VFA production (254 mg COD/g TCOD) and the optimal pH value (pH 6), compared with those at carbon to nitrogen ratio of 25.2, were declined. Further increasing the ratio to 40.1 (i.e., the sole food waste) caused a much lower maximal VFA production (215 mg COD/g TCOD) and the optimal pH value (pH 5). Obviously, the greatest VFA production (311 mg COD/g TCOD) was observed under conditions of pH 8, carbon to nitrogen ratio of 25.2, and fermentation time

of 127 h, which was respectively 2.66 and 1.45-fold of the maximal VFA produced with the sole sludge fermentation at pH 10 and the sole food waste fermentation at pH 5.

In the literature several researchers reported the bio-hydrogen and VFA production by fermentation of sole sludge and sole carbohydrate-enriched wastes. For example, the studies of Zhao et al. and Yuan et al. indicated that the maximal hydrogen and VFA production occurred at pH 10 when sole waste activated sludge was fermented to produce hydrogen [10, 24]. This was the same as that discussed in the above text at the carbon to nitrogen ratio of 7.2. Ginkel et al. reported that when sucrose-enriched synthetic wastewater was treated anaerobically, the optimal pH for the production of hydrogen was pH 5.5 [25], which was similar to that observed in this study at the ratio of 40.1. It was firstly reported in this study that the maximal hydrogen and VFA production could be achieved simultaneously, and their yields were increased by the co-fermentation of sludge and food waste. It can also be seen from that the optimal fermentation pH and time changed with the carbon to nitrogen ratio during the co-fermentation of sludge and food waste, and the highest hydrogen and VFA production could be achieved at carbon to nitrogen ratio of 25.2, pH of 8, and time of 127 h.

Usually, acetic, propionic, butyric, and valeric acids are generated in the fermentation system of organic wastes, such as waste activated sludge [18, 19]. As seen in Figure 2, all these VFA were detectable in the reactors of different carbon to nitrogen ratios under conditions of optimal pH values for maximal VFA production, and acetic, propionic, and butyric acids were the three most prevalent VFAs. When the carbon to nitrogen ratio was in the range of 7.2-25.2 g/g acetic content was the greatest one, and propionic acid ran the second. For example, at carbon to nitrogen ratio of 10.1, pH 9 and time 103 h, the acetic, propionic, butyric and valeric acids were respectively 89, 44, 25, and 14 mg COD/L. Nevertheless, at carbon to nitrogen ratio of greater than 25.2, the concentration of propionic acid was

greater than that of acetic acid. It seems that the increase of carbon to nitrogen ratio resulted in the improvement of propionic acid fraction in the generated VFA during organic waste anaerobic fermentation. Macromolecular organic compounds (such as polysaccharide) are firstly hydrolyzed to monomers such as glucose, which is then fermented by acidogenic bacteria to mainly form VFA. It can be seen in Figures 1 and 2 that the effect of carbon to nitrogen ratio on the sum of acetic and butyric acids was in correspondence with that on hydrogen, because hydrogen is generated during acetic acid and butyric acid fermentations, but no hydrogen is generated in propionic acid type fermentation (reactions 1-3) [26, 27].

Figure 2. Effect of carbon to nitrogen ratio on the main composition of VFA and the sum of acetic and butyric acids under the conditions of optimal fermentation pH and time. The data reported are the averages and their standard deviations.

3.2 Effects of pH and carbon to nitrogen ratio on hydrolysis of fermentative substrates

Hydrolysis, acidification and methanogenesis are usually involved in organic matter anaerobic fermentation, and hydrogen and VFA are produced in the acidification stage. As the fermentation substrate was pre-treated at 102°C for 30 min to kill the methane-forming microorganisms and there was no methane detectable during hydrogen fermentation, the hydrogen and VFA yields were affected mainly by the hydrolysis and acidification stages. In this study, protein and polysaccharide are the main compositions of sludge and food waste, and they are macromolecular polymers which can not be utilized directly by fermentative microbes until being hydrolyzed. The efficiencies of protein and polysaccharide hydrolysis can be expressed by their degradations [19].

As seen from Figures 3A and 3B, at the carbon to nitrogen ratios of 7.2, 10.1, 15.3, 20.4, 25.2, 30.4 and 40.1 g/g, the maximal protein and carbohydrate hydrolysis appeared respectively at pH 10, pH 9, pH 8, pH 8, pH 6 and pH 5 with the highest one at carbon to nitrogen ratio of 25.2 and pH of 8. The bio-hydrolysis of organic substrates is carried out by hydrolytic enzymes, such as protease and amylase. The data in Figures 3C and 3D further showed that the activities of both protease and amylase exhibited the greatest ones at ratio of 25.2 and pH of 8. All these observations consisted with the influence of pH on the hydrogen and VFA yield (Figure 1).

Figure 3. The hydrolysis of protein (A) and carbohydrate (B) at different pH and carbon to nitrogen ratios, and the activities of protease (C) and amylase (D) under the conditions of maximal hydrogen and VFA production. The data reported are the averages and their standard deviations.

3.3 Effects of pH and carbon to nitrogen ratio on the activity of key enzymes involved in acidification step

Hydrogen and VFA are produced in the acidification step (see equations (1-3) and Figure 4). As large numbers of enzymes took part in the production of different VFA [28], in this study only some key enzymes relevant to the generations of acetic, propionic and butyric acids, which were the top three VFAs, were determined. In the metabolic pathway, acetyl-CoA and butyryl-CoA are first converted to acetyl and butyryl phosphate by PTA and PTB, and then converted to acetic acid and butyric acid by AK

	PTA	AK	PTB	BK	OAATC	CoAT
C/N=7.2, pH=10	0.09±0.01	1.01±0.12	0.0013±0.0002	0.0021±0.0002	0.57±0.04	0.93±0.07
C/N=10.1, pH=9	0.19±0.02	2.03±0.18	0.0022±0.0002	0.0051±0.0004	1.32±0.11	1.36±0.12
C/N=15.3, pH=8	0.23±0.02	3.42±0.23	0.0032±0.0003	0.0068±0.0007	1.72±0.18	1.66±0.17
C/N=20.4, pH=8	0.27±0.03	4.01±0.37	0.0041±0.0004	0.0079±0.0006	1.84±0.16	1.73±0.14
C/N=25.2, pH=8	0.36±0.03	4.13±0.41	0.0047±0.0004	0.0087±0.0005	1.98±0.18	1.86±0.15
C/N=30.4, pH=6	0.29±0.03	3.78±0.34	0.0037±0.0003	0.0069±0.0006	1.79±0.18	1.63±0.16
C/N=40.1, pH=5	0.18±0.02	2.68±0.27	0.0029±0.0002	0.0054±0.0004	1.62±0.14	1.48±0.13

* The unit of enzyme activity is U/mg-protein. The data reported are the averages and their standard deviations.

3.4 Effects of pH and carbon to nitrogen ratio on the activity of hydrogen generation and consumption related enzymes

POR is an important enzyme for hydrogen generation. Also, it has been reported that the produced hydrogen can be consumed by hydrogen consumers, such as methanogens and acetobacteria. In this study, the activity of methanogens was inhibited due to the fermentation substrates being pretreated at 102°C for 30 min, which had been reported to be an efficient method to kill methanogens [11]. Nevertheless, under anaerobic conditions, acetobacteria can use hydrogen and carbon dioxide to form acetic acid catalyzed by PTA and FTHFS (Figure 4). Thus, both hydrogen generation and consumption by acetobacteria should be considered, when the bio-hydrogen production is studied.

In this study it was observed that the influences of carbon to nitrogen ratio and pH on the activity of POR were the same as those on hydrogen. The POR was 1.16, 1.41, 1.55, 2.39, 3.38, 1.78, and 1.64 U/mg-protein under the optimal conditions for hydrogen production at carbon to nitrogen ratio of 7.2, 10.1, 15.3, 20.4, 25.2, 30.4, and 40.1, respectively (Figure 5A). The activity of POR reached the greatest one at ratio of 25.2, pH 8 and time 127 h, which was respectively 2.91-fold of that at ratio of 7.2 (sole sludge) with pH 10 and time 192 h, and 2.01-fold of that at ratio 40.1 (sole food waste) with pH 5 and time 175 h. The data in Figures 5B and 5C revealed that the activities of both FTHFS and FDH

were measurable at any carbon to nitrogen ratio and pH value, suggesting that H₂-uptake appeared. Although the activities of FTHFS and FDH reached the greatest at carbon to nitrogen ratio of 25.2, pH 8 and time 127 h, they were only 1.64 and 1.67-fold of the sole sludge (carbon to nitrogen ratio 7.2) and 1.36 and 1.43-fold of the sole food waste (carbon to nitrogen ratio 40.1). Apparently, controlling MOW fermentation under the condition of carbon to nitrogen ratio of 25.2, pH 8 and time 127 h could enhance the enzyme activities of both hydrogen generation and consumption, but the increase of the former was much greater than the latter. Thus, a significantly high hydrogen yield was observed.

Figure 5. The activity of POR (A), FTHFS (B), and FDH (C) under conditions of different carbon to nitrogen ratios and optimal pH and time. Error bars represent standard deviations of triplicate tests.

3.5 Methane production from the VFA-enriched fermentation liquid of MOW

At different carbon to nitrogen ratios and pH values, the produced fermentation liquid was then used as the substrate for methane production. The overall energy gain was estimated according to the

literature [29], and the data were recorded in Table 2. It was found that at C/N of 25.2 the energy gain at pH 8 was 4.54 kW h/kg-COD, which was higher than that of the control (pH 7).

Table 2. Energy gain at different pH values

(C/N=25.2)	H ₂ yield (m ³ /kg-COD)	CH ₄ yield (m ³ /kg-COD)	Energy (kW h/kg-COD)
pH 7 (control)	0.090	0.38	2.08
pH 8	0.101	0.16	4.54

At different C/N ratio and corresponding optimal pH, the methane yield with the fermentation liquid of sole sludge (carbon to nitrogen ratio of 7.2), sole food waste (carbon to nitrogen ratio of 40.1), and their mixture (carbon to nitrogen ratio of 25.2) is shown in Figure 6. During the 90 d operational time the use of fermentation liquid of sole sludge as the substrate resulted in the average methane yield of 0.29 m³/kg-COD. The production of methane was 0.33 m³/kg-COD with the use of sole food waste fermentation liquid. The methane yield could reach 0.38 m³/kg-COD in the reactor of fermentation liquid of the mixture of sludge and food waste, which was the highest one in this study. It was shown in table 3 that the overall energy gains upon different conditions was 3.27, 3.86 and 4.54 kW·h/kg-COD, respectively, which could be utilized by wastewater treatment plant itself.

Table 3. Energy gain at different conditions

Conditions	C/N=7.2, pH=10	C/N=40.1, pH=5	C/N=25.2, pH=8
H ₂ yield(m ³ /kg-COD)	0.021	0.0648	0.1009
CH ₄ yield (m ³ /kg-COD)	0.29	0.33	0.38
Energy gain (kW·h/kg-COD)	3.27	3.86	4.54

Although these data were obtained from the lab-scale experiments, this study has confirmed the feasibility of simultaneous improvement of both hydrogen and methane production from municipal organic wastes. Compared with other pre-treatment methods (Table 4), for example, ultrasonic

pretreatment, this study has two advantages. Technically, it is easily operated and needs no large equipment except the lye tank, and economically, it needs less energy input than ultrasonic pre-treatment. If this method was applied on a full-scale project, the produced biogas might be further purified for production of vehicle grade fuel in the future.

Table 4. Comparison between different pre-treatment methods

Pretreatment	Technically	Economically	Ref.
Ultrasonic	Need ultrasonic equipment; Total CH ₄ production: 186 mL/g TS;	Energy input: 1090 kJ/kg TS; Energy gain: 6040 kJ/kg TS; Energy efficiency: 2880 kJ/kg TS.	[30]
Ultrasonic	Need ultrasonic device with probe; 24% increase in biogas yield;	Energy input: 5000-10000 kJ/kg TS; Energy gain: 24% increase in biogas yield.	[31]
Ultrasonic	Need ultrasonic equipment; Biogas production with pretreatment was 1.7 folds of control;	Energy input: 284 kJ/kg TS; Energy gain: 240 mL biogas/kg TS.	[32]
Alkaline (pH8, C/N=25.2)	Need no large equipment; Easy operation; High H ₂ and CH ₄ yields;	Low additional energy cost; Reagent input: 80 g NaOH/kg-COD; Energy gain: 4.54 kW h/kg-COD Energy gain > Energy input;	This study

It is well-known that methane production is increased with the concentration of VFA. The data in Figure 1C showed that the fermentation liquid of the mixture of sludge and food waste with carbon to nitrogen ratio of 25.2 had the highest VFA concentration, which might be one reason for its greatest methane production. It has also been reported that the composition of VFA significantly affects methane yield, and acetic acid is the most preferred substrate for methane generation [33, 34]. As seen in Figure 2, the fermentation liquid of the mixture of sludge and food waste (carbon to nitrogen ratio 25.2) had the highest acetic acid concentration, which was another reason for its highest methane yield.

Figure 6. Comparison of the long-term performance of methane production (A) and ATP concentration (B) in three EGSB feeding with different fermentation liquid. Error bars represent standard deviations of triplicate samples.

The production of methane is also affected by the activity of microbes. In literatures the measurement of ATP has been used to assess the general physiological activity of anaerobic cells [35, 36], and the increase of methane production during anaerobic digestion was observed to be in correspondence with that of ATP [37]. In the current study the ATP contents in three EGSB reactors are shown in Figure 6B. The average ATP in the reactor of fermentation liquid of sole sludge, mixture of

sludge and food waste, and sole food waste was 13.2, 21.3, and 15.9 $\mu\text{g/L}$, respectively. The general physiological activity of anaerobic cells in the sludge and food waste fermentation liquid was the highest one. Obviously, the variations of ATP trends in three EGSB consisted with the production of methane.

4. Conclusion

When municipal organic wastes were anaerobically treated for bioenergy recovery, the optimal fermentation pH and time changed with the carbon to nitrogen ratio, and the yield of both bio-hydrogen and methane could be remarkably enhanced by firstly fermenting the MOW under alkaline conditions, and then using the fermentation liquid as the substrate for methane production at pH 7. It was found that under the optimal fermentation conditions ($\text{C/N}=25.2$ and $\text{pH}=8$) the highest VFA and hydrogen production were 311 g-COD/kg-TCOD and 0.1 $\text{m}^3/\text{kg-TCOD}$, respectively, and the subsequent methane production was 0.38 $\text{m}^3/\text{kg-TCOD}$. The overall energy gain was estimated as 4.54 $\text{kW}\cdot\text{h/kg-COD}$. Further investigation showed that both the hydrolysis and acidification steps involved in MOW anaerobic treatment were significantly improved, leading to the increase of hydrogen production. Also, under the optimal fermentation conditions the enzymatic activity of hydrogen generation was much greater than that of hydrogen consumption. As there were more total VFA amount and acetic acid content in VFA generated in the fermentation liquid of MOW, and the microbes showed the greatest activity, the methane production from the fermentation liquid could be effectively enhanced. This study provided an efficient strategy for bio-recovering hydrogen, VFA, and methane from municipal organic wastes. In the future, the biogas produced from anaerobic treatment could be used to refine methane, and then used as vehicle grade fuel.

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Reference

- [1] Cesaro A, Belgiorno V. Pretreatment methods to improve anaerobic biodegradability of organic municipal solid waste fractions. *Chem Eng J*, 2014, 240: 24-37.
- [2] McCarty P L, Smith D P. Anaerobic wastewater treatment. *Environ Sci Technol*, 1986, 20: 1200-1206.
- [3] Lin R, Cheng J, Yang Z, Ding L, Zhang J, Zhou J, Cen K. Enhanced energy recovery from cassava ethanol wastewater through sequential dark hydrogen, photo hydrogen and methane fermentation combined with ammonium removal. *Bioresour Technol*, 2016, 214: 686-691.
- [4] Xia A, Cheng J, Ding L, Lin R, Song W, Zhou J, Cen K. Enhancement of energy production efficiency from mixed biomass of *Chlorella pyrenoidosa* and cassava starch through combined hydrogen fermentation and methanogenesis. *Appl Energy*, 2014, 120: 23-30.
- [5] Kaspar H F, Wuhrmann K. Kinetic parameters and relative turnovers of some important catabolic reactions in digesting sludge. *Appl Environ Microbiol*, 1978, 36, 1-7.
- [6] Ariunbaatar J, Panico A, Esposito G, Pirozzi F, Lens PNL. Pretreatment methods to enhance anaerobic digestion of organic solid waste. *Appl Energy*, 2014, 123(3):143-156.
- [7] Guo J, Wang W, Liu X, Lian S, Zheng L. Effects of thermal pre-treatment on anaerobic co-digestion of municipal biowastes at high organic loading rate. *Chemosphere*, 2014, 101: 66-70.
- [8] Dasgupta BV, Mondal MK. Bioenergy conversion of organic fraction of Varanasi's municipal solid waste. *Energy Procedia*, 2012, 14: 1931-1938.
- [9] Shahriari H, Warith M, Hamoda M, Kennedy KJ. Anaerobic digestion of organic fraction of municipal solid waste combining two pretreatment modalities, high temperature microwave and hydrogen peroxide. *Waste Manag*, 2012, 32(1): 41-52.
- [10] Zhao Y, Chen Y, Zhang D, Zhu X. Waste activated sludge fermentation for hydrogen production enhanced by anaerobic process improvement and acetobacteria inhibition: The role of fermentation pH. *Environ Sci Technol*, 2010, 44: 3317-3323.

-
- 367 [11] Oh S E, Van Ginkel S, Logan BE. The relative effectiveness of pH control and heat treatment for
368 enhancing biohydrogen gas production. *Environ Sci Technol*, 2003, 37(22): 5186-5190.
- 369 [12] Zhang D, Chen Y, Zhao Y, Ye Z. A new process for efficiently producing methane from waste
370 activated sludge: alkaline pretreatment of sludge followed by treatment of fermentation liquid in an
371 EGSB reactor. *Environ Sci Technol*, 2011, 45: 803-808.
- 372 [13] Chung YC, Neetheling JB. ATP as a measure of anaerobic sludge digester activity. *Water Pollut.*
373 *Control Fed*, 1988, 60: 107-112.
- 374 [14] Goel R, Mino T, Satoh H, Matsuo T. Enzyme activities under anaerobic and aerobic conditions in
375 activated sludge sequencing batch reactor. *Water Res*, 1998, 32: 2081-2088.
- 376 [15] Goyal N, Gupta JK, Soni SK. A novel raw starch digesting thermostable α -amylase from *Bacillus* sp.
377 I-3 and its use in the direct hydrolysis of raw potato starch. *Enzyme Microb Technol*, 2005, 37:
378 723-734.
- 379 [16] Yakunin AF, Hallenbeck P C. Purification and characterization of pyruvate oxidoreductase from the
380 photosynthetic bacterium *Rhodobacter capsulatus*. *Biochim Biophys. Acta*, 1998, 1409(1): 39-49.
- 381 [17] Feng L, Chen Y, Zheng X. Enhancement of waste activated sludge protein conversion and volatile
382 fatty acids accumulation during waste activated sludge anaerobic fermentation by carbohydrate
383 substrate addition: The effect of pH. *Environ Sci Technol*, 2009, 43: 4373-4380.
- 384 [18] Liu K, Chen Y, Xiao N, Zheng X, Li M. Effect of humic acids with different characteristics on
385 fermentative short-chain fatty acids production from waste activated sludge. *Environ Sci Technol*,
386 2015, 49(8): 4929-4936.
- 387 [19] Luo J, Chen Y, Feng L. Polycyclic aromatic hydrocarbon affects acetic acid production during
388 anaerobic fermentation of waste activated sludge by altering activity and viability of acetogen.
389 *Environ Sci Technol*, 2016 50(13): 6921-6929.
- 390 [20] Grady CPL, Daigger GT, Lim HC. *Biological Wastewater Treatment*; Marcel Dekker Inc.: New
391 York, 1999.
- 392 [21] Kapdan IK, Kargi F. Bio-hydrogen production from waste materials. *Enzym Microb Technol*, 2006, 38:
393 569-582.

-
- 394 [22] Hafez H, Nakhla G, El Nagggar H. Biological hydrogen production from corn-syrup waste using a
395 novel system. *Energies*, 2009, 2: 445-455.
- 396 [23] Ntaikou I, Antonopoulou G, Lyberatos G. Biohydrogen production from biomass and wastes via dark
397 fermentation: A review. *Waste Biomass Valor*, 2010, 1: 21-39.
- 398 [24] Yuan H, Chen Y, Zhang H, Jiang S, Zhou Q, Gu G. Improved bioproduction of short-chain fatty acids
399 (SCFAs) from excess sludge under alkaline conditions. *Environ Sci Technol*, 2006, 40: 2025-2029.
- 400 [25] Ginkel SV, Sung SW. Biohydrogen production as a function of pH and substrate concentration.
401 *Environ Sci Technol*, 2001, 35: 4726-4730.
- 402 [26] Himmi EH, Bories A, Boussaid A, Hassani L. Propionic acid fermentation of glycerol and glucose by
403 *Propionibacterium acidipropionci* and *Propionibacterium freudenreichii* ssp. *Shermanii*. *Appl*
404 *Microbiol Biotechnol*, 2000; 3: 435-440.
- 405 [27] Thanakoses P, Black A, Holtzapple M. Fermentation of corn stover to carboxylic acids. *Biotechnol*
406 *Bioeng*, 2003;83: 191-200.
- 407 [28] Zehnder AJB. *Biology of Anaerobic Microorganisms*; John Wiley & Sons: New York, 1988.
- 408 [29] Cano R, Pérez-Elvira SI, Fdz-Polanco F. Energy feasibility study of sludge pretreatments: A review.
409 *Appl Energy*, 2015;149: 176-185
- 410 [30] Zou S, Wang H, Wang X, Zhou S, Li X, Feng Y. Application of experimental design technique in the
411 optimization of the ultrasonic pretreatment time and enhancement of methane production in anaerobic
412 co-digestion. *Appl Energy*, 2016, 179:191-202.
- 413 [31] Rasapoorra M, Ajabshirchia Y, Adlb M, Abdia R, Gharibic A. The effect of ultrasonic pretreatment on
414 biogas generation yield from organic fraction of municipal solid waste under medium solids
415 concentration circumstance. *Energy Convers Manage*, 2016, 119:444-452.
- 416 [32] Zou S, Wang X, Chen Y, Wan H, Feng Y. Enhancement of biogas production in anaerobic co-digestion
417 by ultrasonic pretreatment. *Energy Convers Manage*, 2016, 112:226-235.
- 418 [33] Wang QH.; Kuninobu M, Ogawa HI, Kato Y. Degradation of volatile fatty acids in highly efficient
419 anaerobic digestion. *Biomass Bioenerg*. 1999, 16: 407-416.
- 420 [34] Ozturk M. Conversion of acetate, propionate and butyrate to methane under thermophilic conditions in

-
- 421 batch reactors. *Water Res*, 1991, 25: 1509-1513.
- 422 [35] Fukuzaki S, Nishio N, Shobayashi M, Nagai S. Inhibition of the fermentation of propionate to
423 methane by hydrogen, acetate and propionate. *Appl Environ Microbiol*, 1990, 56: 719-723.
- 424 [36] Salminen EA, Rintala JA. Semi-continuous anaerobic digestion of solid poultry slaughterhouse waste:
425 Effects of hydraulic retention time and loading. *Water Res*, 2002, 36: 3175-3182.
- 426 [37] Perle M, Kimchie S, Shelef G. Some biochemical aspects of the anaerobic degradation of dairy
427 wastewater. *Water Res*, 1995, 29: 1549-1554.