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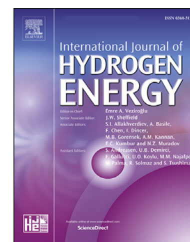

# Hydrogen production from acidogenic food waste fermentation using untreated inoculum: Effect of substrate concentrations

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## HIGHLIGHTS

- Biohydrogen fermentation was established using the untreated inoculum.
- High substrate concentration is adverse for hydrogen fermentation.
- Heat-treated FW was preferable for hydrogen production than fresh FW.
- Lactate-type biohydrogen fermentation was observed using the fresh FW.

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## ABSTRACT

The effect of substrate concentrations (0, 7.5, 15, 22.5, 30, and 37.5 g-VS/L) on hydrogen production from heat-treated and fresh food waste (FW) using untreated inoculums was investigated in this work. The highest hydrogen yield (75.3 mL/g-VS) was obtained with heat-treated FW at 15 g-VS/L. Lower substrate content could not provide enough organic matter for hydrogen fermentation, while higher substrate concentrations shifted the metabolic pathways from hydrogen fermentation to lactic acid fermentation by enriching the lactic acid bacteria (LAB), which lowered the slurry pH and decreased enzyme activity, resulting in a lower chemical oxygen demand (COD), volatile solid (VS), carbohydrate removal rate, and hydrogen yield. Compared with fresh FW, heat-treated FW is preferred for biohydrogen process with acetate as the main organic product. Additionally, at the optimal concentration (15 g-VS/L) using

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Substrate concentration
Untreated inoculum

fresh FW, lactic acid is first accumulated and then degraded to produce hydrogen with butyrate as the main metabolite.

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## Introduction

With rapid urbanization, food waste (FW) has become a key issue in cities and adversely affects the environment and social economy. It was reported that over $1.3 \times 10^9$ t of FW are generated and disposed globally every year [1]. Issues related to FW such as odors, greenhouse gas (GHG) emissions, and contamination of soil and groundwater by the leachate produced from food waste decomposition are major threats to the sustainable development of cities and the environment. Moreover, the growing environmental consciousness of government and citizens has led to the rapid development of FW treatment capacity, and the circular economy has been proposed to reduce, reuse, and recycle the FW [2]. Therefore, timely and effective management is required in order to conserve the energy and minimize the environmental impacts associated with FW.

Based on the properties of high content of easily biodegradable organics and high moisture content, FW has been widely treated through anaerobic digestion (AD) to recover energy [1]. This treatment consists of four steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. However, acid accumulation caused by rapid acidification seriously inhibits the methanogenesis processes and results in poor system stability [2]. Thus, researchers have attempted to use FW to produce organic acids and hydrogen through acidogenesis fermentation [3–6]. Hydrogen ($H_2$) has been widely recognized as a clean energy source with zero pollutant emissions and high specific energy content (142 kJ/g), and could be an ideal alternative to fossil fuels [1,6,7]. Moreover, the biological hydrogen production process has several excellent properties such as lower operation cost, ease of control, and simultaneous waste remediation associated with clean bioenergy generation in a sustainable approach. FW contains a significant amount of easily hydrolyzable and biodegradable organic matter, and has high hydrogen production potential [6,8]. Thus, using FW as a substrate for dark fermentative hydrogen production could not only solve food waste problems but also recover clean energy.

The efficacy of acidogenic $H_2$ production is influenced by many biotic and abiotic factors. Inoculum is generally considered as one of the crucial factors that affect the $H_2$ yield [9–11]. Owing to the extremely rigorous conditions when using pure hydrogen-producing bacterial (HPB) as inoculum, mixed cultures from anaerobic sludge have been widely used in lab-scale tests or full-scale fermenters because of their merits such as a broader choice of feedstock, low operating costs, and ease of control [11]. However, this kind of inoculum often introduces hydrogen consumers (e.g. hydrogenotrophic methanogens, homoacetogens, LAB, propionate-producing bacteria, and sulfate reducers) that consume the generated $H_2$ [6,11,12]. Various pretreatment methods such as heat shock, alkaline/acidic treatment, ionizing irradiation, and pretreatment using waste frying oil (WFO) have been attempted to alleviate the effect of non-hydrogen-producing bacteria and increase the hydrogen yield and productivity [10,13,14].

However, Wang et al. recently reviewed hydrogen production enhancement using different inoculums pretreatment methods and found no agreement as to which method is the most effective for enriching hydrogen producers owing to differences in inoculum sources and microbial community structures [11]. Moreover, these pretreatment methods are likely to increase the overall AD operating cost, which is less attractive for large-scale applications [6]. In addition, the effects of pretreatment on $H_2$ production might not continue during the long-term operation owing to changes in the microbial communities [15,16]. It was documented that hydrogen fermentation using untreated seed sludge could achieve a high product yield for two reasons: the lowered fermentative pH could inhibit the methanogenic activity and promote biohydrogen fermentation [17], and although pretreatment methods could selectively enrich spore-forming HPB, such extreme pretreatment conditions might inhibit non-spore-forming $H_2$ producers such as *Bacillus* sp. and *Enterobacter* sp. [11,18]. This leads to a decline in total hydrogen production capacity compared with mixed inoculum without any pretreatment. Thus, if unpretreated seed sludge can be successfully utilized as inoculum for hydrogen fermentation, then the processes can be simplified. The energy for inoculum pretreatment will be saved, and this could realize a net energy production for biohydrogen processes. But seldom study focused on the effect of untreated seed sludge on biohydrogen process.

Microflora in substrates also affect hydrogen fermentation. It was found that large amounts of microorganisms exist in fresh FW [3], and different types of microorganisms in the system can synergistically degrade the substrate and promote fermentation processes [19,20]. However, the LAB and other acidogens in the FW or seed sludge adversely affect the hydrogen production processes [5]. Under certain conditions, these indigenous microorganisms can selectively accumulate in the fermenters and compete with HPBs for substrates, resulting in a lower hydrogen yield [21,22]. More significantly, some metabolic products of these bacteria (e.g., organic acids) have adverse effects on the HPB or disturb the bio$H_2$ fermentation, which further lowers the hydrogen yield and productivity or even stops the fermentation process [23]. Additionally, the input of microorganisms from substrates may change the bacterial community dynamics and further affect the hydrogen yield and productivity [24].

To alleviate these negative effects, various FW pretreatment methods such as heat shock, microwave pretreatment, alkali shock, and acid pretreatment have been reported to

inactivate the hydrogen-consuming bacteria [5,19,25]. Ortigueira et al. utilized microwave pretreatment to eliminate the microbial counts in FW, and successfully improved the hydrogen yield and productivity [5]. Heat-shock pretreatment was regarded as an effective method to inactivate the LAB and other non-spore-forming microorganisms. Noike et al. found that a pretreatment temperature of 50 °C for 30 min is adequate for preventing the growth of LAB and increasing hydrogen production [23]. Additionally, high-temperature pretreatment processes could be favorable for releasing encapsulated organics, breaking macromolecules, increasing the substrate hydrolysis rate, and finally promoting the hydrogen yield [26]. However, the properties of biological hydrogen fermentation using heat-treated and fresh FW as substrates have not been adequately studied and compared. The substrate transformation processes and mechanisms have not been clarified and should be further investigated.

In addition, the substrate load plays a critical role in the overall process efficiency, which not only influences the fermentation output but also determines the populations and communities of microbiota during fermentation [9,27]. Increasing the substrate concentration benefits $H_2$ production by providing enough organics for microorganisms and promoting bacterial enzyme activity. However, excess organic loading to a certain extent negatively impacts the $H_2$ fermentation process owing to the production of excess volatile fatty acids (VFAs) and a consequent reduction in pH and the system buffer capacity [27]. Moreover, high organics concentrations decrease the efficiency of hydrogen production via changes in the microbial communities and metabolic pathways [24,28]. Thus, hydrogen production and acidogenic fermentation are balanced by controlling the substrate loading. However, to the best knowledge of the authors, few studies have focused on the effect of the substrate concentration on hydrogen production using nonsterilized seed sludge as inoculum for food waste fermentation.

Therefore, determining a more effective, simple, and economical way to improve the $H_2$ yield is still a challenge. In this study, the effect of substrate concentrations on hydrogen fermentation using unsterilized seed sludge (raw anaerobic sludge) was investigated. The variations in hydrogen production from FW were compared under different organic loading rates to analyze the effect of substrate concentrations on hydrogen fermentation processes. Then, the variations of metabolic products and enzyme activities were investigated to further explain the biological hydrogen fermentation process.

## Materials and methods

### Food waste substrate

Food wastes (FW) collected from the student canteen in a university campus in Xi'an was mainly composed of rice, vegetables and meat. The animal bones, plastic bags and other non-biodegradable and inert materials (e.g., egg shells, napkin tissues) in the FW were firstly sorted out, and the residuals were then crushed using an electrical blender [3]. Thereafter, the FW slurry was sieved (1 mm) and stored in the refrigerator (4 °C) until further use. To avoid the effect of indigenous microorganisms in the fresh FW on hydrogen production, a part of raw FW slurry was added in a sealed bottle and heat-treated at 100 °C for 30 min to inactivate the indigenous bacteria. After that, the pretreated FW was cooled down and conserved in refrigerator (4 °C). The characteristics of fresh and pretreated FW slurry were shown in Table 1.

### Inoculum

Anaerobic seed sludge was obtained from a full-scale upflow anaerobic sludge blanket (UASB) reactor of a brewery company in Xi'an, China. The UASB was utilized to produce methane from the wastewater of the beer production process. The raw anaerobic sludge was sampled and screened to remove the large particles and then hermetically stored in the refrigerator (4 °C) until the experiment. Characteristics of the inoculum sludge were shown in Table 1.

### Fermentative hydrogen production

Batch hydrogen production experiments were performed in the 100 mL Erlenmeyer flasks. To investigate the effect of FW pretreatment on hydrogen fermentation, two groups of experiments were simultaneously conducted. 20 mL of the inoculum sludge was firstly added into each flask, then different volume (0, 10, 20, 30, 40, 50 mL) of heat-treated FW (group 1) or fresh FW slurry (group 2) was added in the reactors. After that pure water was added to make up a volume of 80 mL in all reactors. The substrate concentrations (based on VS) were then fixed at approximately 0, 7.5, 15, 22.5, 30 and 37.5 g-VS/L. The other two reactors as control groups were solely added with 20 mL of pretreated and fresh FW, respectively. No other nutrient solution was added in this study. Thereafter, the HCl or NaOH solution was used to adjust the initial pH to approximately 6.5. $O_2$ gas in the reactors was removed by flushing with $N_2$ gas for 3 min. Then, all flasks were sealed and agitated on a shaker with 100 rpm at 37 °C. Three replicates were carried out for each substrate concentration.

### Analytical methods and data analysis

Fermentation slurry were collected from each reactor and centrifuged at 5000 rpm for 10 min at 4 °C. The clarified

**Table 1 – Characteristics of food waste slurry and inoculum sludge.**

| | Anaerobic sludge | Fresh food waste | Heat-treated food waste |
|---|---|---|---|
| pH (–) | 7.5 ± 0.3 | 5.0 ± 0.3 | 5.2 ± 0.2 |
| TS (g/L) | 16.2 ± 3.2 | 66.2 ± 2.6 | 65.5 ± 1.7 |
| VS (g/L) | 10.4 ± 2.7 | 60.4 ± 2.3 | 60.3 ± 1.5 |
| VS/TS (%) | 0.65 ± 0.04 | 0.91 ± 0.02 | 0.92 ± 0.03 |
| COD (g/L) | 23.1 ± 4.2 | 60.4 ± 7.8 | 62.2 ± 6.5 |
| Carbohydrate (g/L) | 2.3 ± 0.5 | 52.7 ± 1.2 | 55.4 ± 1.8 |
| Protein (g/L) | 5.5 ± 1.2 | 1.9 ± 0.3 | 1.8 ± 0.5 |
| Lactic acid (mg/L) | 16.8 ± 1.1 | 1226.7 ± 30.7 | 945.0 ± 20.5 |
| VFAs (mg/L) | 342.6 ± 33.1 | 1250.2 ± 39.7 | 611.0 ± 22.5 |

supernatants were used to measure pH, soluble COD (SCOD) and VFAs. The total solids (TS), VS, pH and COD measurements were performed in accordance with the American Public Health Association (APHA) standard methods [29]. Total and soluble carbohydrate and protein in the fermentation slurry were detected using the methods described in our previous studies [3]. Prior to determining the VFAs and lactic acid, the liquid samples were filtered through the 0.22-μm filter membranes. Then, the filtrate was analyzed by the high performance liquid chromatograph (LC-10AD, Shimadzu, Japan) equipped with an ultraviolet detector (210 nm) using sulfate acid (5 mmol/L) as eluent. The biogas was collected in the airbags, and its volume was measured by the syringe. The content of the biogas was monitored using a gas chromatograph (GC2010, Shimadzu, Japan) equipped with a packed column (TDX-01, China) and thermal conductivity detector analyser (TCD), which require one carrier of argon. All the analyses of individual samples were performed in triplicates. The dehydrogenase is an important enzyme involved in the hydrogen production, and its activity was analyzed by measuring the triphenyl formazan (TPF) generated from the reduction of the triphenyl tetrazolium chloride (TTC) at 37 °C for 24 h in darkness using spectrophotometry at 485 nm according to the process described in the previous studies [30,31]. The population of viable LAB in the slurry during the fermentation was detected using De Man-Rogosa-Sharpe (MRS) agar by counting the colony-forming units (cfu) according to our previous study [4]. The bio$H_2$ production was analyzed by kinetic analysis using the modified Gompertz model [32].

## Results and discussions

### *Hydrogen production properties*

Accumulative hydrogen with different substrate concentrations during fermentation is shown in Fig. 1. Clearly, the hydrogen production process in all reactors exhibited a rapid-slow pattern, which is consistent with data from other researchers [31,33]. In the reactors with heat-treated FW (Fig. 1a), hydrogen production was very fast during the initial 24 h and slowed down thereafter. This was possibly owing to the fact that easily biodegradable organics in the substrates were consumed in this period. In control tests with FW only, although a sufficient amount of substrates was supplied, very low content of hydrogen was detected. This might be because few HPBs existed in the reactor, showing the importance of inoculation to initiate the hydrogen fermentation [6]. According to previous studies, $H_2$-producing microflora can be enriched from FW through acid or heat pretreatment [19,20], and hydrogen production can be established without additional inoculums. However, no hydrogen accumulation was observed in this study with sole pretreated FW, possibly because of the diverse sources of FW and different microbial communities in the reactors. In the reactor without FW added (only seed sludge), no hydrogen was produced, which was mainly owing to the insufficiency of easily biodegradable organics for hydrogen production. Except for the reactor with a VS content of 15 g/L, the production rate of hydrogen in the

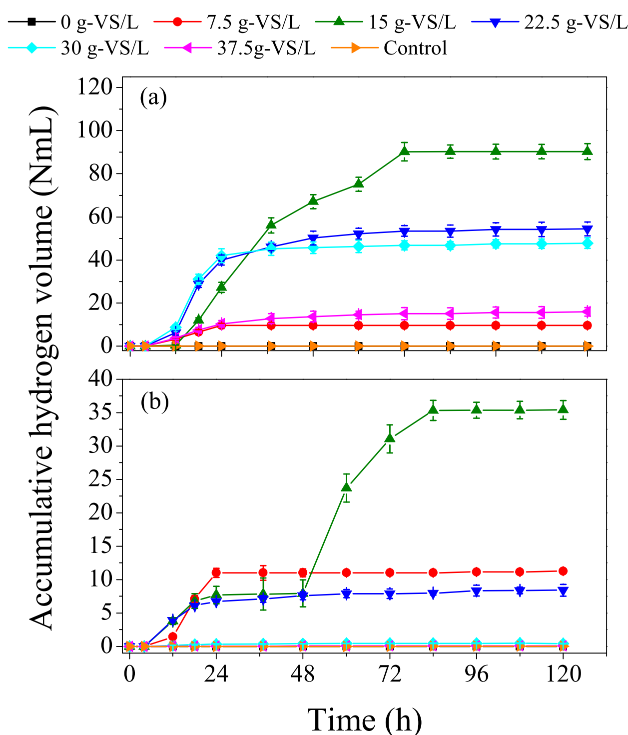


**Fig. 1 – Cumulative hydrogen production at various substrate concentrations. (a) heat-treated FW, (b) fresh FW.**

reactors became almost constant after 48 h, which may have two causes. First, the easily biodegradable organic substrates in the reactors were largely utilized by the microorganisms, and the residual organics were not suitable for hydrogen production, which retarded the hydrogen generation processes. Second, the accumulation of metabolic intermediates and the decrease in the pH value in the reactors during fermentation restricted the activity of HPBs and further reduced the hydrogen production rate [18].

However, the hydrogen continuously produced in the reactor with a substrate concentration of 15 g-VS/L and reached a maximal value of 90.3 mL at 72 h, which might have resulted from the suitable conditions under proper substrate concentrations. In this reactor, the initial carbohydrate content was around 13.7 g/L (Fig. 3), which did not restrict the hydrogen fermentation by a substrate shortage. In addition, the ratio of substrate to inoculum in this reactor might have provided a proper nutrient structure for organics degradation and alleviated the restriction of acids with a lower final total organic acid content of 3.0 g/L (Fig. 4). It was reported that the proper substrate content can alleviate the inhibition of substrates and metabolites, enhance bacterial activity, and improve the hydrogen yield and productivity [31]. Moreover, the proper organics concentration can balance the substrate availability and metabolic rates, which ensures the best substrate assimilation and consequently an increase in hydrogen production [24]. Further increasing the substrate concentration inhibited the hydrogen production as less $H_2$ was collected, and is similar to the results reported in previous studies [27]. This might occur because of the restriction by the accumulation of organic acids and low pH value. After 120 h, the hydrogen production in all reactors stopped, and the

ultimate hydrogen production was 0.01, 9.6, 90.3, 54.4, 47.8, and 16.0 mL at concentrations of 0, 7.5, 15, 22.5, 30, and 37.5 g-VS/L, respectively. This indicated that the substrate concentrations significantly influenced the hydrogen yield [6,31].

In the reactors with fresh FW as feedstock, much lower hydrogen production was observed (Fig. 1b). Only the reactors with substrate concentrations of 7.5, 15, and 22.5 g-VS/L showed an obvious hydrogen accumulation during fermentation. Similar to the reactors with pretreated FW, the hydrogen rapidly accumulated during the initial 24 h and exhibited a rapid-slow pattern. In the reactors with 7.5 and 22.5 g-VS/L, the accumulative hydrogen reached 11.3 and 8.4 mL, respectively, and then remained stable until the end of the experiment. The highest hydrogen yield was obtained in the reactor with a substrate concentration of 15 g-VS/L, but different from that in the reactor with heat-treated FW, the hydrogen in this reactor increased to 7.7 mL during the initial 24 h and after a short-term stagnation further increased to 35.4 mL from 48 h to 120 h. This exhibited a two-stage hydrogen-producing pattern. It was deduced that the substrates were first transformed into organic acids (lactic acid and acetate) and then into hydrogen gas owing to the shift of microbial communities during fermentation [34]. Similar to the previous study, after the glucose was rapidly depleted in the first stage, $H_2$ could be continuously generated using the lactate with the production of butyrate in the second stage [32]. The lower hydrogen production using fresh FW than that of pretreated FW might be owing to competition between HPB and other bacteria such as LAB for substrates. In our previous studies, LAB was largely detected in fresh food waste and could be easily enriched in the fermentation slurry and rapidly transform the carbohydrates into lactic acid [3]. LAB, acetogenic bacteria, and solventogenic bacteria consume large amounts of carbon sources without hydrogen production, and their metabolites can inhibit or even kill the HPB, thus decreasing the hydrogen production efficiency [21]. Additionally, LAB and acetogenic bacteria lead to acid accumulation during fermentation and directly reduce the pH of the fermentation slurry, which further restricts the enzyme activity. Furthermore, the presence of LAB could hamper bio$H_2$ production through substrate competition, the acidification of cultivation broth, and the excretion of bacteriocins [7,35].

Thus, the hydrogen production in this group was much lower than that with the pretreated FW using the same substrate concentrations. Interestingly, reactors with heat-treated FW at a content of 7.5 g-VS/L exhibited hydrogen production similar to that with fresh FW, which might occur because the LAB in the fresh FW had a slight influence on the hydrogen production process owing to their lower population under this conditions, or because the HPB coexisted with LAB in the reactors [24], and further demonstrated the importance of the substrate concentration on hydrogen fermentation. The sharp increase of hydrogen in reactors with 15 g-VS/L might be owing to the fact that the microorganisms in the reactor were cultured to utilize lactic acid as a substrate for hydrogen production, which is consistent with the findings of other researchers [34,36,37], and will be further explained by the variations of organic acids in Variations of metabolites.

The hydrogen yield and productivity were calculated based on the variations in hydrogen production (Table 2). No methane was detected in all reactors, probably owing to the serious restriction of methanogens by the low-pH condition. Hydrogen yield increased from 16.1 mL/g-VS to 75.3 mL/g-VS when the heat-treated FW content increased from 7.5 to 15 g-VS/L, but gradually decreased as the substrate concentration increased. This indicated that proper organic loading is crucial for hydrogen fermentation, and further proved that a higher organic loading rate restricts hydrogen production, as documented previously [38,39]. The hydrogen productivity in the reactors showed tendencies similar to those in the variations of hydrogen yield, demonstrating that the concentrations of organic matter in the reactors not only affect the hydrogen yield but also the productivity. This might be related to the effects of organic content on bacterial metabolism processes. Additionally, the concentrations of substrate influence the types and concentrations of intermediate products in the reactors, which also impacts the hydrogen fermentation. A two-stage hydrogen productivity was observed in the reactor with fresh food waste at 15 g-VS/L, and a longer lag time phase was found when it was compared with that using heat-treated FW, probably owing to the cultivation of microbial communities in the reactor [32].

The hydrogen yields from FW fermentation using different inoculum pretreatment methods are compared (Table 3). As it can be seen, various pretreatment methods have been attempted to enhance the hydrogen production. However, the maximum $H_2$ yield achieved in most studies was still lower as compared to the present study, showing the feasibility and superiority of no inoculum pre-treatment for biohydrogen fermentation.

From the above analysis, it was concluded that the substrate concentration is an important factor that influences the hydrogen yield. The most suitable substrate concentration for hydrogen production in this study was 15 g-VS/L. Although hydrogen yield with heat-treated FW was higher than that using fresh FW, the cost-benefit analysis of hydrogen fermentation using fresh FW or heat-treated FW should be further compared in the future because more energy and higher operation cost are required for FW heat pretreatment.

### *Variations of TCOD and VS removal rates*

Variations in the total COD (TCOD) removal rate during fermentation are shown in Fig. 2. With the addition of heat-treated FW (Fig. 2a), the concentrations of TCOD in the substrate increased from 16.7 g/L to 59.1 g/L. After 120 h, the TCOD content in all reactors decreased, which was mainly attributed to the production of biogas or other bacterial metabolisms during fermentation. The TCOD removal efficiencies in the reactors increased from 4.8% to 36.3% with an increase in the substrate concentrations from 0 to 15 g-VS/L but showed a decreasing tendency when the FW amount was further increased (Fig. 2a). This indicates that the proper addition of FW promotes bacterial activity and substrate degradation, but higher substrate concentrations restrict the substrate digestion processes. It has been documented that high substrate concentrations impair the mass transfer, which results in an imbalance of cellular osmosis and disturbs the substrate assimilation and degradation processes [41]. The lower TCOD removal rate in the reactors with higher substrate content

**Table 2 – Hydrogen yield and productivity at various substrate concentrations.**

| | | 0 g-VS/L | 7.5 g-VS/L | 15 g-VS/L | 22.5 g-VS/L | 30 g-VS/L | 37.5 g-VS/L | Control |
|---|---|---|---|---|---|---|---|---|
| Heat-treated FW | Maximal hydrogen volume (mL) | 0.01 | 9.6 | 90.3 | 54.4 | 47.8 | 16.0 | 0.01 |
| | Hydrogen content in biogas (%) | 0.2 | 16.3 | 40.1 | 23.2 | 22.9 | 7.4 | 0.5 |
| | Hydrogen yield (mL/g-VS) | – | 16.1 | **75.3** | 30.2 | 19.9 | 5.3 | – |
| | Hydrogen productivity (mL/g-VS.h) | – | 1.26 | 1.89 | 1.58 | 1.60 | 0.16 | – |
| | Lag phase time (h) | – | 7.7 | 13.1 | 8.9 | 9.7 | 3.9 | – |
| Fresh FW | Maximal hydrogen volume (mL) | 0.003 | 11.3 | 35.4 | 8.4 | 0.41 | 0.07 | 0.04 |
| | Hydrogen content in biogas (%) | 0.6 | 13.2 | 26.8 | 9.1 | 0.7 | 0.1 | 0.2 |
| | Hydrogen yield (mL/g-VS) | – | 18.8 | **29.5** | 4.7 | 0.17 | 0.02 | – |
| | Hydrogen productivity (mL/g-VS.h) | – | 1.90 | 0.63 (stage 1)<br>0.53 (stage 1) | 0.24 | 0.01 | – | – |
| | Lag phase time (h) | – | 11.2 | 7.0 (stage 1)<br>39.9 (stage 2) | 3.6 | 0.9 | – | – |

- represents unavailable date.

**Table 3 – Hydrogen yield from FW fermentation using various inoculum pre-treatment methods.**

| Inoculum | Fermentation conditions | Hydrogen yield (mL-$H_2$/g-VS) | References |
|---|---|---|---|
| FW, Acidic pretreatment (pH 2) | Batch 35 °C, pH 6 | 158.0 | [19] |
| FW, Acidic pretreatment (pH 4) | | 27 | |
| Granular sludge, alkaline pretreatment (pH 12, 24 h) | Batch 35 °C, uncontrolled pH | 42.8 | [10] |
| Granular sludge, heat shock (90 °C, 30 min) | | 53.8 | |
| Anaerobic sludge, heat shock (100 °C, 30 min) | Batch 35 °C, uncontrolled pH | 43.0 | [33] |
| Anaerobic sludge, heat shock (100 °C, 15 min) | CSTR, 35 °C, pH 5.5, HRT 2 days | 64.7 | [40] |
| Anaerobic sludge, untreated | Batch 37 °C, uncontrolled pH | 75.3 | This study |

Note: CSTR represents continuous stirred-tank reactor, HRT represents hydraulic retention time.

further explains the unsatisfactory hydrogen yield in Fig. 1. In the reactor containing solely seed sludge (0 g-VS/L), the TCOD removal rate was 4.8%, which mainly resulted from the low content of biodegradable carbon sources in the seed sludge and further verified the low hydrogen yield in this reactor. Interestingly, the reactor with only heat-treated FW still exhibited a slight COD removal rate (approximately 1.4%), which indicates that the heat treatment eliminated most of the indigenous microorganisms. The reduction of COD was probably caused by the spore-forming bacteria that were revived during fermentation and utilized the organics in the substrate to proliferate. It has been documented that many $H_2$-consuming groups of bacteria can form spores and

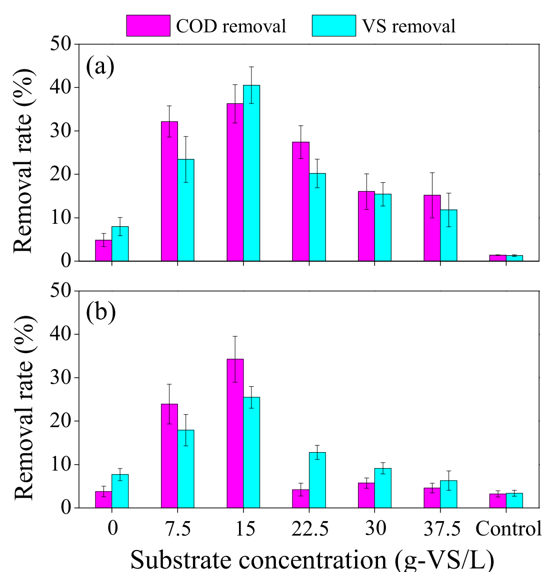


**Fig. 2 – COD and VS removal efficiencies at various substrate concentrations. (a) heat-treated FW, (b) fresh FW.**

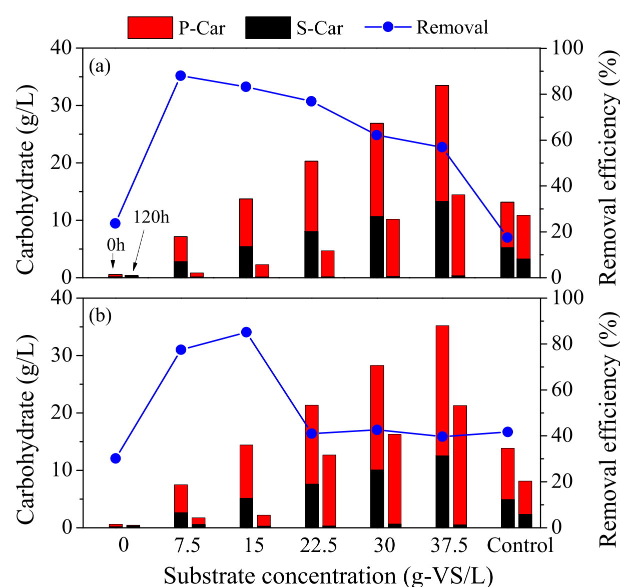


**Fig. 3 – Variations of carbohydrates concentrations and removal rate during the hydrogen fermentation at various substrate concentrations. (a) heat-treated FW, (b) fresh FW.**

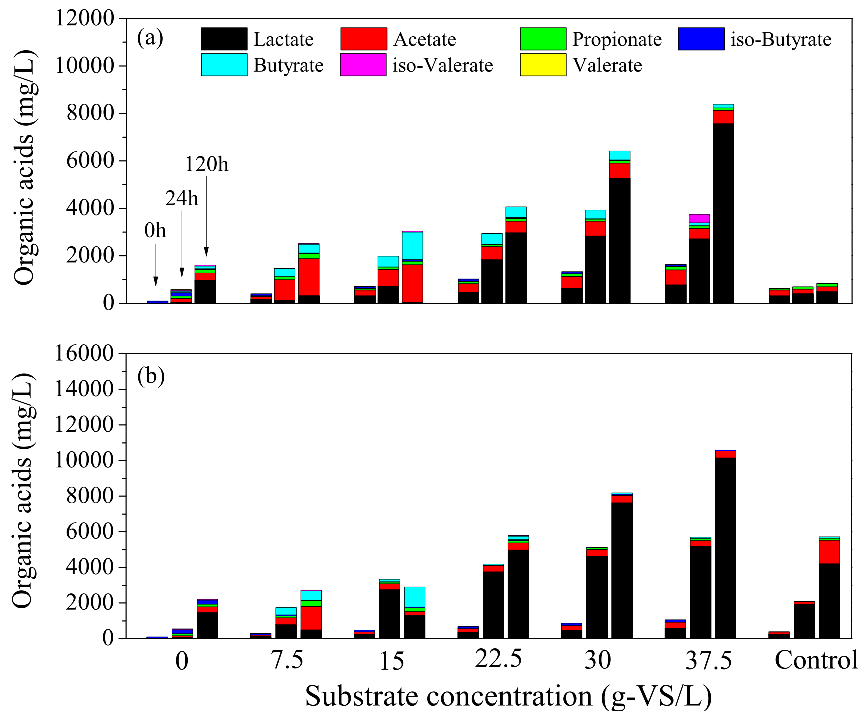


**Fig. 4 – Variations of metabolites during the hydrogen fermentation at various substrate concentrations. (a) heat-treated FW, (b) fresh FW.**

therefore survive heat treatment, including acetogens (*Acetobacterium*, some *Clostridium* sp., and *Sporomusa*), certain propionate and lactate producers (*Propionibacterium* and *Sporolactobacillus*), and a sulphate reducer (*Desulfotomaculum*) [18]. Moreover, the slight decrease in carbohydrates (Fig. 3), increase in organic acids (Fig. 4), and the detection of $CO_2$ in this reactor (data not shown) further demonstrate the existence of alive microorganisms in the fermentation slurry.

In the reactors with fresh FW, similar tendencies of TCOD removal were observed (Fig. 2b). This further explains the variations of hydrogen production in Fig. 1 and indicates the importance of substrate content in hydrogen fermentation. However, compared with the reactors using heat-treated FW, the COD removal efficiencies were relatively lower. Higher COD removal rates of 23.9% and 34.3% were obtained in the reactors with 7.5 and 15 g-VS/L, respectively. The rate sharply decreased to 4.2% when the substrate concentration increased to 22.5 g-VS/L and remained below 5% in reactors with more FW, which further explains the lower $H_2$ yield in these reactors, and might be owing to the restriction by the rapid acidification and lower pH during the fermentation process. Given the existence of indigenous microorganisms such as LAB and acetogens in the fresh FW, most of the substrates were transformed into organic acids and led to a decrease in pH, which in turn restricted the substrate degradation [5]. Additionally, in the reactors with 15 g-VS/L, the COD removal rate using fresh FW was very close to that using heat-treated FW as feedstock, but a much lower amount of hydrogen was observed. This could be explained by two reasons. First, owing to the existence of indigenous microbiota in the fresh FW, part of the substrate was transformed into organic acids, and less organic matter could be utilized by the HPB. Second, the hydrogen was produced in different metabolic pathways, and more substrates were required to produce the same volume of hydrogen when fresh FW was used as feedstock.

In the reactor with fresh food waste, the organics were first transformed into lactic acid by the indigenous LAB, and then into hydrogen via the lactate-type pathway [34], which can be explained by the variations of organic acids during the tests in Fig. 4. Theoretically, 1-mol LA can produce 1 mol of $H_2$ and 0.5 mol of butyrate (Eq. (A.6)) or 0.5 mol of $H_2$ and 0.75 mol of butyrate (Eq. (A.4)). This means that 1 mol of glucose can produce a maximum of 2 mol of $H_2$ through lactate-type pathway (Eq. (A.1–6), Supporting Information), which is lower than that by the acetate-type or butyrate-type pathway (1 mol glucose produced 2–4 mol of $H_2$) mainly exhibiting in the reactor with the pretreated FW (Eq. (A.7–10)). Based on the above analysis, it can be concluded that the pretreatment processes were beneficial for promoting the substrate utilization rate during the hydrogen fermentation processes.

The variations in the VS removal rate were similar to those of the TCOD, and the highest value was obtained for a substrate content of 15 g-VS/L in reactors with both heat-treated (40.5%) and fresh FW (25.5%). This further explains the high hydrogen production in these reactors. The lower VS removal rate under higher substrate concentrations is probably attributable to the inhibition of hydrolysis by the rapid acid accumulation [42]. Due to the large populations of indigenous acidogenic bacteria in the fresh FW, the easily biodegradable substrates were rapidly transformed into acids and restricted the hydrolysis processes, which resulted in a lower VS removal rate than that of heat-treated FW. Owing to the inactivation of indigenous bacteria during heat treatment, the VS removal in the control group with pretreated FW was only

1.3%, which was lower than that of fresh FW (3.4%) and consistent with the variations in TCOD removal. In addition, the tendencies of the VS removal rate were not identical with those of the COD reduction. In some groups, the COD removal rate was higher than that of VS, while in other groups they were opposite. This might be caused by the different proportions of volatile organics (e.g., VFAs and ethanol) in the fermentation slurry. In addition, perhaps the organic components in the fermentation products were distinct, or the metabolism and assimilation processes in the reactors were diverse. This will be studied in the future.

### *Carbohydrate degradation properties*

Carbohydrates are the major components of FW and the main substrates in hydrogen fermentation. To further explain the hydrogen production and bacterial metabolism processes, Fig. 3 illustrates the carbohydrate degradation during fermentation at various substrate concentrations. Clearly, with heat-treated FW as feedstock (Fig. 3a), the total carbohydrate content decreased after 120 h of fermentation, and almost all of the soluble carbohydrates were consumed during the fermentation. The high residual particulate carbohydrate content was owing to the restriction of hydrolysis by low-pH conditions. The carbohydrate degradation efficiency achieved its maximal value of 88.1% at the substrate concentration of 7.5 g-VS/L but exhibited a decreasing trend when the substrate concentration was further increased. This indicates that a proper substrate concentration facilitates the degradation of carbohydrates for hydrogen production [9].

In the reactor with the highest hydrogen yield (VS content of 15 g/L), the carbohydrate removal rate was 83.2%, exhibiting a high efficacy of substrate transformation. In the reactor with only pretreated FW (control group), the total carbohydrate content decreased slightly from 13.2 g/L to 10.9 g/L, showing a removal rate of 17.5%. However, no hydrogen gas was detected in this reactor. It was deduced that the carbohydrates were transformed into organic acids or other intermediates and will be discussed in Variations of metabolites. In the reactor with only seed sludge, the lowest carbohydrate removal rate (23.6%) was found, which was mainly attributed to the harsh degradation properties of anaerobic sludge as reported by Yang et al. [31].

In the reactors using fresh FW as a substrate (Fig. 3b), the carbohydrates were degraded by microorganisms from the seed sludge and the food waste. In the reactors with a substrate content lower than 15 g-VS/L, the carbohydrate removal rate increased from 30.2% to 85.1%. However, the removal rate decreased when more FW was added, which might have resulted from the inhibition of high organic loading. In the reactor with fresh FW only, the carbohydrates decreased from 13.9 g/L to 8.1 g/L, exhibiting a removal rate of 41.7%. This was higher than that using heat-treated FW, which was the function of indigenous microorganisms in the fresh FW. Moreover, a higher content of particulate carbohydrate retained in the reactor, which can be explained by the fact that the hydrolysis was restricted by the rapid accumulation of organic acids in the reactor [3].

### *Variations of metabolites*

In the hydrogen fermentation process, soluble metabolites such as ethanol, acetate, propionate, butyrate, and lactate are simultaneously formed with the production of hydrogen. Based on previous discussions, carbohydrates in the reactors were largely degraded, but low hydrogen gas was produced during the fermentation (Fig. 1). Thus, it was deduced that the substrates were transformed into intermediate metabolic products. Metabolites are useful indicators for characterizing the performance of the biohydrogen fermentation process, but they can also inhibit hydrogen production [31]. Fig. 4 illustrates the soluble metabolite distributions at 0 h (the initial value), 24 h (maximal biogas production), and 120 h (end of fermentation), which further explains the bio$H_2$ production processes under various substrate concentrations.

It was found that the total organic acid content in the substrates (0 h) increased with the addition of FW, and lactate and acetate accounting for 70.8%–90.1% of the total organic acids were the dominant components in all of the reactors. This is related to the properties of FW and seed sludge as shown in Table 1. During fermentation, organic acids gradually accumulated in the reactors, indicating that the production of hydrogen was accompanied by organic acid generation. Moreover, the concentration of organic acids increased with the addition of FW, which can be explained by the fact that the acidification was accelerated at a higher content of substrates.

In the reactor with a substrate concentration of 7.5 g-VS/L, the total organic acids were only 2.52 g/L (Fig. 4a), which mainly resulted from the lower organic substrate content and the limited hydrolysis and acidification. However, with more heat-treated FW added to the reactors, the content of organic acids obviously increased and achieved a maximal value of 8.39 g/L at 37.5 g-VS/L after 120 h. This indicates that with the addition of FW, more easily biodegradable substrates were supplied, which resulted in the accumulation of organic acids in the reactors. In the reactor without FW addition, owing to the insufficiency of easily biodegradable organic matter and the low hydrolysis rate of the substrates, the organic acid concentration was only 1.6 g/L. However, in the reactor with only heat-treated FW, the organic acid increased from 0.62 to 0.83 g/L with an increase in lactic acid from 0.31 to 0.49 g/L. This indicates that the LAB in FW were almost eliminated by the heat treatment, and the production of lactic acid in other reactors was mainly attributed to the LAB in the seed sludge [4]. It was reported that acetate, propionate, butyrate, and ethanol had similar inhibitive impacts on hydrogen fermentation in the concentration range of 0–100 mmol/L, and that the inhibitory effect was enhanced with an increase in these metabolite concentrations [43]. These results indicate that the accumulation of metabolites might restrain the hydrogen yield in reactors with high substrate concentrations, which is possibly due to the fact that undissociated soluble metabolites can penetrate the cell membrane of HPB and disrupt the intracellular physiological balance [44].

Organic acid components also varied with the addition of FW (Fig. 4a). In the reactors with less FW added (VS less than 15 g/L), acetic acid in proportions of 57% and 55% was the main

organic acid. However, with a VS content higher than 15 g/L, lactic acid dominated the organic acids and achieved a proportion of 92%, indicating that the hydrogen fermentation was replaced with lactic acid fermentation, which might be relevant to the inhibition of HPB by higher organic loading [24]. This result indicates that LAB competed with hydrogen producers for available substrate and produced lactate at the expense of hydrogen, which might be a crucial factor that results in the decrease of hydrogen yield at higher substrate concentrations.

In the reactors with a VS of 7.5 and 15 g/L, the concentration of organic acids also changed over time. With a substrate concentration of 7.5 g-VS/L, bacteria preferred to produce acetate rather than lactate during the entire fermentation period. The acetate increased from 0.13 g/L to 0.87 g/L at 24 h and 1.55 g/L at 120 h, while the lactate decreased from 0.16 g/L to 0.13 g/L at 24 h and then increased to 0.32 g/L at 120 h. Meanwhile, the butyrate increased from 0 g/L to 0.32 g/L at 24 h and remained stable thereafter. However, in the reactor with a substrate concentration of 15 g-VS/L, lactic acid was first accumulated from 0.31 g/L to 0.73 g/L in the initial 24 h, but decreased to 0.02 g/L after 120 h. The acetate first increased from 0.25 g/L to 0.69 g/L and further climbed to 1.6 g/L, accompanied by an increase in butyrate from 0.46 g/L to 1.15 g/L, from which it can be deduced that the lactic acid was degraded by the microorganisms during fermentation. It was reported that the lactate could be preferentially utilized as a carbon source for hydrogen production, and that *Clostridium butyricum* was a performant candidate for hydrogen production from lactate [8,24]. Thus, acetate and butyrate were the main intermediates during hydrogen fermentation with heat-treated FW at the substrate concentration of 7.5 and 15 g-VS/L, from which it was deduced that the biohydrogen might be achieved by a combination of acetate-type (Eq. (A.7–8)), butyrate-type (Eq. (A.9–10)) and lactate-acetate (Eq. (A.4)) type metabolic pathways.

In other reactors with higher substrate content, owing to the accumulation of acids and a decrease in pH, HPB was seriously restricted, but LAB was largely enriched in the reactors and transformed the substrates into lactate. However, the FW has been pretreated by high temperature, and the LAB should be inactivated during the pretreatment process. Thus, the LAB in these reactors were mainly from the seed sludge. It was well known that LAB can coexist in methanogenic sludge.

In the reactors with fresh FW (Fig. 4b), more organic acids were produced. The final total organic acid (TOA) content increased from 2.21 g/L (0 g-VS/L) to 10.59 g/L (37.5 g-VS/L), which was mainly owing to the existence of indigenous microbiota in the FW and inoculum. Similarly, the lactate was the dominant component and increased with the addition of FW. The generated lactate may further inhibit hydrogen production in the reactors with high substrate concentrations, which explains the low hydrogen production under a high FW loading rate as discussed previously. In the reactor with a substrate concentration of 15 g-VS/L, lactate was only 0.24 g/L at 0 h, while it sharply increased to 2.76 g/L after 24 h, and gradually decreased to 1.31 g/L, accompanied with an increase in butyrate from 0 g/L to 1.34 g/L. It was deduced that the hydrogen production in this reactor was realized through lactate-type fermentation, in which lactate was utilized by the bacteria to produce hydrogen and butyrate, as it was reported by Kim et al. [45].

The accumulation of organic acids usually leads to a decrease in pH during biological hydrogen fermentation and in turn affects the hydrogen fermentation. Most studies found that the optimum pH range for biohydrogen production is 5–6, which facilitates the transport of nutrients between the cells and surrounding fluids by maintaining the surface charge on the microbial cell membrane [46]. A lower and unstable pH not only affects the bacterial activities and growth rates but also changes the metabolic pathways [46–49]. It was found that the final pH decreased with increasing substrate concentrations (Table A1, Supporting Information), which was consistent with the results of organic acid formation. In the reactors with lower VS content (less than 15 g-VS/L), the final pH was between 5 and 6, which was suitable for hydrogen production. However, in the reactor with more FW, the pH value decreased to 3.8–5, which seriously restricted the activity of HPB and further explains the low hydrogen yield in these reactors. It was reported that hydrogen production by *Clostridium* sp. stopped as the pH decreased from 7.0 to 5.0, which was possibly caused by restrictions in hydrogenase activity due to the low pH [6]. In reactors added with fresh food waste, a much lower pH (3.3–4.5) was detected in reactors with the same substrate concentrations. This may have resulted from the rapid production of LA owing to the existence of indigenous LAB in the FW. It was documented that an increase of pH deviation from 0.1 to 0.9 would change the microbial communities and reduce the hydrogen yield [48]. Therefore, the significant decline in pH may also be an important inhibitory factor for hydrogen production at high substrate concentrations during fermentation. Thus, it can be expected that if the pH in reactors were controlled, the restriction of low pH and high organic acids content will be relieved, stable microbiota can be maintained, higher hydrogen yield and substrate transformation rate might be obtained under higher substrate concentrations [48,49].

### *Variations of enzyme activity*

Dehydrogenase activity was also detected to explain the variations of the hydrogen yield during fermentation (Fig. 5). The highest dehydrogenase activity (31.7 mg/d·g-VS) was obtained at a VS content of 15 g/L. Further increasing the substrate

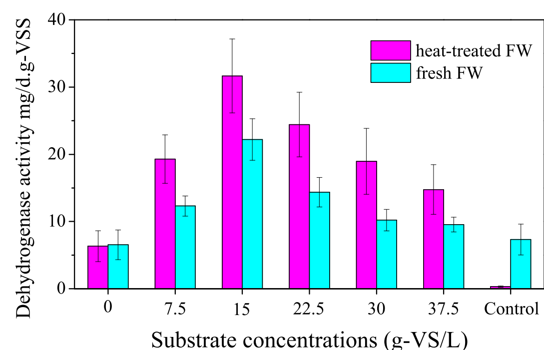


**Fig. 5 – Dehydrogenase activity after the fermentation at various substrate concentrations.**

concentration led to a decreasing tendency of dehydrogenase activity, which confirmed that an appropriate substrate concentration facilitates the metabolic activity of fermentative bacteria. This might be because the proper substrate concentration maintains the dynamic balance of carbon, nitrogen, and phosphorus and provides suitable biodegradation conditions for fermentative bacteria [6]. High microbial activity may contribute to the most efficient process performance. Comparatively, high substrate concentrations inhibited the microbial activity owing to the overloaded accumulation of organic acids and the sharp decrease in pH, which in turn decreased the hydrogen production efficiency. A similar phenomenon was found in hydrogen fermentation fed with food wastewater by Goud et al., who reported that dehydrogenase activity decreased from 3.24 to 1.06 μg/mL of toluene with an increase of the organic load from 30 to 50 g-COD/L owing to the feedback inhibition [50]. In addition, the dehydrogenase activity in reactors with heat-treated FW was higher than that with fresh FW, which further indicates the variations of hydrogen generation in these reactors, and might be related to the pH variations during fermentation.

### *Variations of LAB populations during fermentation*

In Variations of metabolites, it was found that lactic acid was the main component of the metabolites in both groups. It was deduced that the LAB was enriched in the reactor during fermentation. According to Table 4, LAB was seldom detected in the heat-treated FW but was clearly found in the reactors with fresh FW (approximately $1.2 \times 10^3$ cfu/mL). This indicates that a high temperature can indeed inactivate the indigenous LAB in the FW. After 24 h of fermentation, the LAB in reactors with pretreated FW increased slightly to approximately 23 cfu/mL, which fundamentally explains the low lactate content in the reactors. The LAB from seed sludge was the main contributor to lactic acid fermentation. Additionally, a higher population was detected in the reactors with more FW added, which was attributed to the proper conditions for LAB accumulation. The amount of LAB in reactors with higher substrate concentrations (>15 g-VS/L) further increased and reached above $3.6 \times 10^5$ cfu/mL after 120 h, which indicated a rapid increase of lactate content during this period. However, the LAB decreased slightly to $9.5 \times 10^2$ cfu/mL in the reactor with a substrate content of 15 g-VS/L, which might have occurred because the substrate components in this reactor were insufficient for LAB accumulation, further testifying the higher hydrogen yield in this condition. Obviously, the population of LAB in the reactors with fresh FW was much higher than that in the reactor with pretreated FW. This was mainly because the LAB came from both the seed sludge and fresh FW, and further explains the higher concentrations of lactic acid during fermentation. As it was mentioned above, due to the huge population of LAB accumulated in the reactors, the metabolic pathways of the microbiota were transferred from hydrogen fermentation to lactic acid fermentation, which provided an explanation for the lower hydrogen yield in these reactors. Additionally, the LAB increase from $1.3 \times 10^3$ to $2.3 \times 10^4$ cfu/mL in the reactor with fresh FW of 15 g-VS/L during the initial 24 h and drastically decreased to $1.1 \times 10^3$ cfu/mL after 120 h, which is coincident with the variations of lactic acid content

**Table 4 – Variations of LAB populations during the dark fermentation. Unit: cfu/mL.**

| | Pretreated FW (g-VS/L) | | | | | | | Fresh FW (g-VS/L) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 7.5 | 15 | 22.5 | 30 | 37.5 | Control | 0 | 7.5 | 15 | 22.5 | 30 | 37.5 | Control |
| 0 h | 167 | 154 | 136 | 165 | 167 | 171 | – | 179 | $8.9 \times 10^2$ | $1.3 \times 10^3$ | $1.4 \times 10^3$ | $1.5 \times 10^3$ | $1.8 \times 10^3$ | $1.2 \times 10^3$ |
| 24 h | $6.7 \times 10^2$ | $1.2 \times 10^3$ | $1.3 \times 10^3$ | $4.7 \times 10^3$ | $4.8 \times 10^3$ | $5.8 \times 10^3$ | 23 | $7.9 \times 10^2$ | $1.7 \times 10^3$ | $2.3 \times 10^4$ | $3.3 \times 10^4$ | $6.8 \times 10^4$ | $9.4 \times 10^4$ | $5.7 \times 10^4$ |
| 120 h | $4.1 \times 10^2$ | $8.6 \times 10^2$ | $9.5 \times 10^2$ | $3.6 \times 10^5$ | $4.4 \times 10^5$ | $6.5 \times 10^5$ | 35 | $3.2 \times 10^2$ | $4.3 \times 10^2$ | $1.1 \times 10^3$ | $2.3 \times 10^6$ | $3.5 \times 10^6$ | $5.5 \times 10^6$ | $2.9 \times 10^5$ |

Note: represents non detection.

during this period, further verified the lactate-type biohydrogen pathways during the fermentation. Obviously, the hydrogen production was also influenced by the succession of microbial communities during the fermentation processes, which will be studied in the near future.

## Conclusions

Hydrogen production from food waste with different substrate concentrations was investigated using untreated inoculum. It was found that biological hydrogen fermentation could be successfully established and enhanced by providing a proper substrate concentration (15 g-VS/L). Higher substrate concentrations would lower the system pH, shift the metabolic pathways from hydrogen fermentation to lactic acid fermentation by enriching LAB during the fermentation and result in a lower hydrogen yield. Compared with fresh FW, the heat-treated FW exhibited a higher biohydrogen yield (75.3 mL/g-VS) and productivity (1.89 mL/g-VS·h) at 15 g-VS/L. With proper fresh FW concentrations, hydrogen production can be realized through lactate-type fermentation using the lactic acid generated by indigenous lactic acid bacteria. The molecular biological techniques should be utilized in the future to analyze the variations of microbial communities and reveal their biological mechanisms. Additionally, the hydrogen fermentation efficacy in continuous reactors or larger-scale fermenters needs be further investigated to discuss its feasibility for practical use.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijhydene.2019.08.230.

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