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Research Article

Effect of Hydrogen Peroxide on Biohydrogen Production from Melon Fruit (*Cucumis melo* L.) Waste by Anaerobic Digestion Microbial Community

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Abstract. Biohydrogen (H_2) production has the potential to provide clean, environmentally friendly, and cost-effective energy sources. The effect of increasing oxidative stress on biohydrogen production by acid-treated anaerobic digestion microbial communities was studied. The use of varying amounts of hydrogen peroxide (H_2O_2; 0.1, 0.2, and 0.4 mM) for enhancing hydrogen production from melon fruit waste was investigated. It was found that H_2O_2 amendment to the H_2-producing mixed culture increased hydrogen production. Treatment with 0.4 mM H_2O_2 increased cumulative H_2 output by 7.7% (954.6 mL/L), whereas treatment with 0.1 mM H_2O_2 enhanced H_2 yield by 23.8% (228.2 mL/gVS) compared to the untreated control. All treatments showed a high H_2 production rate when the pH was 4.5 – 7.0. H_2O_2-treated samples exhibited greater resilience to pH reduction and maintained their H_2 production rate as the system became more acidic during H_2 fermentation. The application of H_2O_2 affected the volatile fatty acid (VFA) profile during biohydrogen fermentation, with an increase in acetic and propionic acid and a reduction in formic acid concentration. The H_2O_2 treatment positively affects H_2 production and is proposed as an alternative way of improving H_2 fermentation..

Keywords: Biohydrogen, dark fermentation, fruit wastes, mixed culture, oxidative pressure

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1. Introduction

The development of bioenergy from waste is vital to minimize dependence on fossil fuels as the global primary energy source and contribute to the security of sustainable and environmentally friendly energy supply (Amekan 2020; Hao and Shao 2021; Martins *et al.* 2019). In Indonesia, the government has supported the development of renewable energy sources via the issuance of Presidential Regulation No. 5 of 2006 (Amekan and Guntoro 2017). Biohydrogen is one of the feasible alternative energy sources as it has a higher energy density than fossil fuels (Choi and Ahn 2014) and its utilization seems cleaner and carbon-free (Kim *et al.* 2021).

Hydrogen (H_2) can be produced biologically via photosynthesis, fermentation, and combination of photo/dark fermentation (Ding *et al.* 2016; Eroglu and Melis 2011; Hassan *et al.* 2020). Biological hydrogen production via fermentation is simpler as it does not require light and can be applied to a wide range of biomass wastes or residues (Amekan *et al.* 2018; Kumar *et al.* 2015; Shaojie *et al.* 2020; Sivagurunathan *et al.* 2016). Current research has studied various types of agricultural wastes as feedstock for biohydrogen production, such as extracts of pineapple wastes (Reungsang and Sreela-or 2013), cassava starch (Tien *et al.*, 2016), sweet potato (Chu *et al.* 2012), sugarcane bagasse (Reddy *et al.* 2017) and melon waste (Amekan *et al.* 2018; Cahyari et al. 2019). Melon waste meets the criteria as a substrate for biohydrogen production because it has high carbohydrate content, such as lignin (8.26%), hemicellulose (22.71%), cellulose (19.01%), soluble starch (17.22%), total sugar (30.42%), lipid (6.91%), total N (0.89%), total solids (7.67%) and volatile solids (6.45%) (Amekan *et al.* 2018)

In a fermentation-based system, hydrogen-producing bacteria (anaerobes and facultative anaerobes) break down organic matter to produce hydrogen via hydrogenase enzymes that catalyze the reversible oxidation of H_2 (Choi and Ahn 2014; Das *et al.* 2006). *Clostridia* are potential hydrogen producers that is widely found in sewage sludge, cow dung, and pig manure (Sivagurunathan *et al.* 2014a). However, *Clostridia* are obligate anaerobe (oxygen-sensitive bacteria) that can lose viability when oxygen

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concentration exceeds 1% (Kawasaki *et al.* 1998). Nonetheless, it is known that *Clostridia* may tolerate transient oxygen exposure and be able to detoxify it by accepting electrons from NADH-dependent rubredoxin oxidoreductase (NROR) and then reducing them to hydrogen peroxide (H_2O_2) (Hillmann *et al.* 2008). *C. acetobutyricum* defends itself against the lethal effect of oxygen by using the anaerobic pathway of detoxification of reactive oxygen species (ROS). *C. acetobutyricum* express scavenging enzyme, such as superoxide reductases (SOR), peroxidases or oxygen reductases which act as electron carriers from NADPH to O_2^- (superoxide), H_2O_2 or O_2 providing a reductive environment that enables cells to nullify the ROS. NADH, unlike NADPH, is a potent prooxidant that produces the majority of the ROS generated by the cells under oxidative stress. Alternatively, *Clostridia*-type fermentations can generate H_2 via NADH oxidation (Hallenbeck 2009). Hence, increased oxidative stress by excessive ROS exposure could possibly evoke a metabolic adaptation of hydrogen-producing bacteria to limit NADH consumption as a prooxidant and trigger H_2 production via NADH oxidation in the cell.

Therefore, in the present study, the effect of H_2O_2 exposures to induce oxidative stress on mixed culture of hydrogen-producing bacteria during biohydrogen production from melon fruit (*Cucumis melo* L.) waste was investigated. Anaerobic digestion microbial communities were used as the source of hydrogen-producing bacteria as it provides diverse metabolic, high productivity, and economic advantages over pure culture (Shaojie *et al.* 2020). During biohydrogen fermentation, the gas production (H_2 and CO_2) and soluble organic acids profile were monitored. This is the first study to look at the effect of radical agent treatment on hydrogen production from agricultural organic wastes using a mixed cultures inoculum. The findings offer new insight into an alternative approach for increasing biohydrogen production from agricultural waste substrate using a dark fermentation.

2. Materials and Methods

2.1 Hydrogen-producing mixed culture and substrate preparation

The seed sludge was collected from biogas digester treating fruit waste at Gemah Ripah fruit market (Yogyakarta, Indonesia) for enriching the hydrogen-producing bacteria. The seeds were acidified to pH 3 by adding 2 M HCl and then maintaining for 24 hours and then adjusting back to pH 6.8 with the addition of 2 M NaOH (Amekan *et al.* 2018; Damayanti *et al.* 2020) to inactivate the hydrogen-consuming microbes before use in the enrichment of hydrogen-producing bacteria using glucose as the sole carbon source. The melon fruit waste used in this study was collected from Gemah Ripah fruit market located in Yogyakarta. The melon slurry was prepared by chopping melon fruit wastes into small pieces and mashing them with a kitchen blender. The melon slurry, which contained 9.625 g/L of volatile solids (VS), was adjusted to pH 7 before being used as substrates for H_2 fermentation. The melon slurry was stored at 4 °C until used as the hydrogen fermentation substrate (Amekan *et al.*, 2018).

2.2 Enrichment of hydrogen-producing mixed cultures

The mixed cultures were enriched using glucose-based media to increase hydrogen-producing bacteria (Sivagurunathan *et al.* 2014a). Enrichment of hydrogen-producing mixed cultures was carried out in 100 mL serum vials with a 50 mL working volume as described in Amekan *et al.* (2018). The peptone-yeast extract-glucose (PYG) medium containing glucose 10 g/L, yeast extract 10 g/L, peptones 10 g/L, L-cysteine-HCl 0.5 g/L, and resazurin 0.001 g/L was prepared as the enrichment media for hydrogen-producing bacteria. L-cysteine-HCL and resazurin were applied to reduce the oxygen content in the substrate and anaerobic indicator, respectively. The PYG medium was inoculated with 2 mL of enriched hydrogen-producer mixed culture and incubated at 37 °C for 24 h. Freshly grown (24 h) enriched mixed cultures were used as the inoculum for all the fermentation experiments.

2.3 Hydrogen peroxide treatments in hydrogen production

Batch experiments were carried out under strictly anaerobic conditions in 100 mL serum vials with a 50 mL working volume (5 ml inoculum, 15 ml substrate, and 30 ml nutrition). Melon fruit waste was used as substrate with the addition of micronutrients (peptone 5 g, yeast extract 0.5 g, KH_2PO_4 1.2 g, $Na2HPO_4$ 5.1 g, $MgSO_4.7H_2O$ 0.5 g, and L-sistein-HCl 0.5 g). The pH was adjusted to pH 7 using either 1 M HCl or 1 M NaOH before sterilization. 2 M H_2O_2 stock solution was made by dissolved H_2O_2 in sterilized water and then injected aseptically into the sterile media prior to the addition of inoculum to obtain 0.1, 0.2, and 0.4 mM H_2O_2 final concentrations. Non-treated (NT) sample was included as a control. Each treatment was conducted in duplicate. The vials were capped with rubber bungs, sealed with aluminum rings, and flushed with nitrogen gas for 3 minutes to provide anaerobic conditions. All batch 100 mL vials were incubated at 37 °C and agitated continuously at 120 rpm. Gas samples for H_2, CO_2, CH_4, and volatile solids (VS) were collected four times during seven days of fermentation, while volatile fatty acids (VFAs) samples were collected at the end of the fermentation process.

2.4 Analysis of fermentation products

The volume of biogas was measured using an airtight glass syringe. The analysis of biogas (H_2, CH_4, and CO_2) was performed by gas chromatography equipped with a thermal conductivity detector (GC-14B, Shimadzu, Japan) and a molecular column sieve 5A (MS-5A). Nitrogen was applied as the carrier gas at 100 kPa. Volatile fatty acids (VFA) were determined by gas chromatography equipped with a flame ionization detector (Hewlett Packard 5890 Series II). Standard of VFA analysis was obtained from Sigma-Aldric (Standard Volatile Free Acid No. Cat: 46975-U Supelco). The analytical procedures of standard methods (APHA, 1998) were used to determine the pH and VS.

3. Results and Discussion

3.1 H_2O_2 effect on biohydrogen production from melon fruit waste

No methane was detected in all samples during biohydrogen production via anaerobic degradation of melon waste organic content. It suggests that acid treatment (pH 3, 24 h) has successfully eliminated hydrogen-consuming bacteria and archaea from anaerobic microflora used in this study. All H_2O_2 treatments are capable of producing gas consisting of H_2 (48 – 52%) and CO_2 (48 – 52%) (Table 1).

Cumulative hydrogen production of all H_2O_2 treated samples were higher than untreated sample. 0.4 mM H_2O_2 treatment produced the highest hydrogen (954.6 mL/L; Table 1) among the H_2O_2 treatments. This is 7.7% higher than the untreated samples (NT; 880.6 mL/L; Table 1; Fig. 1a), while 0.1 and 0.2 mM H_2O_2 increased H_2 production by 1.2% and 1.6%, respectively. Our results showed higher H_2 production by 22 – 88% compared to the reported hydrogen production from melon waste by Amekan *et al.* (2018) using inoculum from fruit waste (743 mL/L), cow

Table 1
Hydrogen production from melon fruit waste by acid treated anaerobic digestion microbial community under H_2O_2 stress (initial pH 7, 37°C, VS 9.625 g/L).

Treatment	Final pH	Total gas (mL/L)	Total CO_2 (mL/L)	Total H_2 (mL/L)	*Yield* H_2 (mL/gVS)
NT	4.04 ± 0.01	1,828 ± 33	947 ± 10	880.6 ± 33	175.5 ± 9
0.1 mM H_2O_2	3.85 ± 0.02	1,847 ± 24	955 ± 13	891.6 ± 24	228.2 ± 47
0.2 mM H_2O_2	3.82 ± 0.01	1,798 ± 58	902 ± 25	895.1 ± 58	161.7 ± 44
0.4 mM H_2O_2	3.74 ± 0.04	1,821 ± 16	866 ± 17	954.6 ± 16	190.2 ± 8

Notes: Total CO_2 and H_2 represented the cumulative gas production and yield H_2 showed the total yield H_2 during 7 days fermentation periods. NT: Control with no H_2O_2.

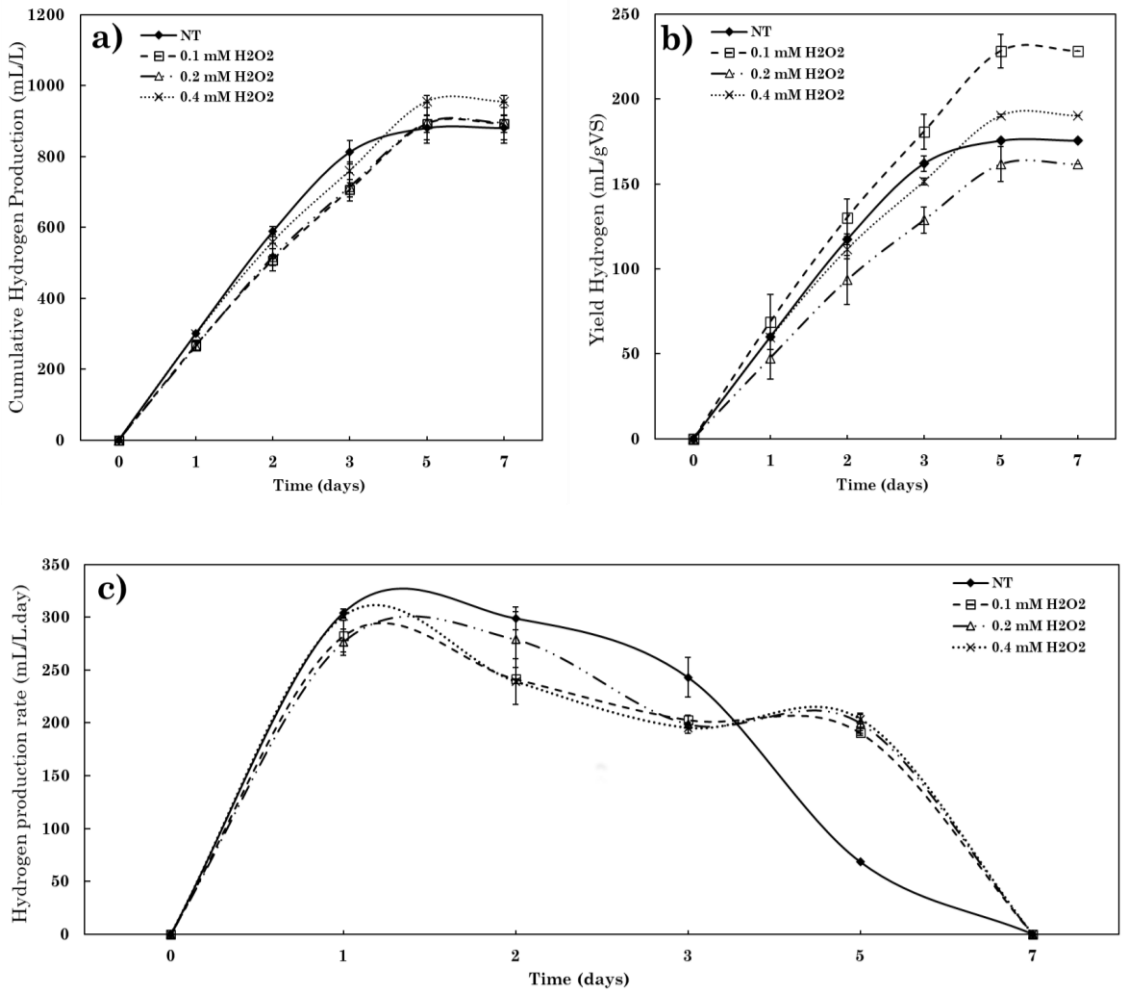

Fig. 1 Cumulative H_2 production **(a)**, yield H_2 **(b)**, and H_2 production rate **(c)** during H_2 fermentation with melon wastes as substrates and inoculum from fruit waste digester (37°C, start pH 7). Error bar represented standard deviation of experimental data.

dung (426 mL/L), and tofu waste digester (323 mL/L) (Table 1; Fig. 1a). The results indicate that increased H_2O_2 exposure induced H_2-producing bacteria to produce more H_2 seemingly via NADH oxidation (Hallenbeck 2009). Interestingly, 0.1 mM H_2O_2 showed higher yield with 228.2 mL/gVS relative to 0.4 mM (190.2 mL/gVS; Table 1; Fig 1b). Overall, the increase in H_2 production was likely caused by changing metabolism pathways after H_2O_2 treatment. Here, the application of H_2O_2 seemingly affected the metabolism pathways of anaerobic microbial H_2 production, resulting in the detoxification system activation by NADH oxidation. This pathway was used by the H_2-producing bacteria to overcome ROS and possibly affect hydrogen production (in surplus NADH condition) by oxidizing NADH-ferredoxin oxidoreductase and hydrogenase. Tanisho and Ishiwata (1995) reported that the ratio of NADH in the cell would adjust to overcome the effect of free radicals, such as H_2O_2. These results suggest that 0.1 mM H_2O_2 is an efficient alternative method for increasing H_2 yield by inducing NADH formation. Future investigation is required to ensure the role of NADH in fermentative H_2 production under ROS conditions.

The H_2 yield was decreased as the H_2O_2 concentration increase, suggesting that 0.4 mM H_2O_2 seemingly induced too much oxidative stress that slowed down the activity of H_2-producing bacteria. The highest hydrogen production rate was achieved on day-1 of fermentation, and it slowed down gradually as fermentation progressed up to day-7. The highest production rate was achieved when the pH was around 7 and then slowed down as pH decreases caused by the accumulation of VFAs in the media. Hydrogen production stopped when pH ~4 (Table 1; Fig. 1c). Interestingly, all H_2O_2 treatment still show higher H_2 production rate (0.1 mM – 190.45 mL/L.day; 0.2 mM – 199.72 mL/L.day; 0.4 mM – 203.43 mL/L.day) compared to the NT (68.34 mL/L.day) at the day-5 of H_2 fermentation (Fig. 1c). It suggests that H_2O_2 treated samples have higher resistance with decreases in pH and maintained their H_2 production rate where the systems became more acidic at day-5 of H_2 fermentation. The pH at day-5 fermentation of 0.1, 0.2, 0.4 mM H_2O_2, and control (NT) were 3.91, 3.88, 3.83 and 4.13, respectively (Fig. 2a).

3.2 The change of initial pH and total volatile solids degradation after H_2O_2 treatment

In the H_2 fermentation process, pH is crucial factor in controlling enzyme activity and metabolic transporters of H_2-producing bacteria (Cappai *et al.* 2014). The result shows that pH decreased gradually from pH 7 on day-1 to pH 3.7 – 3.8 at the end fermentation periods in all treatments and control samples (Fig. 2a). The H_2 production showed the highest rate at 24 h fermentation, in line with optimum pH for H_2 generation in the pH range 4.5 to 7.0 (Hawkes *et al.* 2002; Wei *et al.* 2010). As the pH decreased, the H_2 production slowed down gradually. The H_2 production was ceased when the pH reached 3.7 – 3.8 at day-7 fermentation. The decrease in pH was caused by the accumulation of organic acids produced as end-products during the acidogenesis (Fig. 2a). Moreover, it affects hydrogen-producing microbial growth and their metabolic activity, resulting in the inhibition of metabolic pathways and then stopping hydrogen production (Melis and Melnicki 2006). The trend of initial pH after H_2O_2 treatment was noticeably similar to NT, indicating that the change of pH was not affected by H_2O_2 treatment during H_2 fermentation.

Degradation of organic material in melon waste for H_2 production was monitored by determining VS every 24 h of fermentation. The hydrogen yields were calculated by using Eq. 1 (Chen *et al.* 2006; Dong *et al.* 2009):

$$\text{Yield} = \frac{\text{volume of hydrogen produced (mL)}}{\text{volatile solids consumed (gVS)}} \qquad (1)$$

The VS concentration was gradually decreased during H_2 fermentation as the H_2-producing bacteria consumed it. The higher consumption coincided with the optimum pH in the system at day-1 fermentation and getting slow as the system became more acidic (lower pH). The highest consumption of VS was detected by 0.1 mM H_2O_2 treatment with 61% VS consumption, while other H_2O_2 treatments showed similar or lower than control (NT) during fermentation with an average of 51.9% VS consumption (Fig. 2b). Generally, microbes used organic materials as an energy source for growth and gas production or other products (Table 2). The treatment of 0.1 mM H_2O_2 seemingly not limiting consumption of VS by the H_2-producing bacteria during the fermentation process to produce a fermentation product. An important question for future studies is to provide better information regarding the relationship between VS consumption and ROS condition in fermentative hydrogen production.

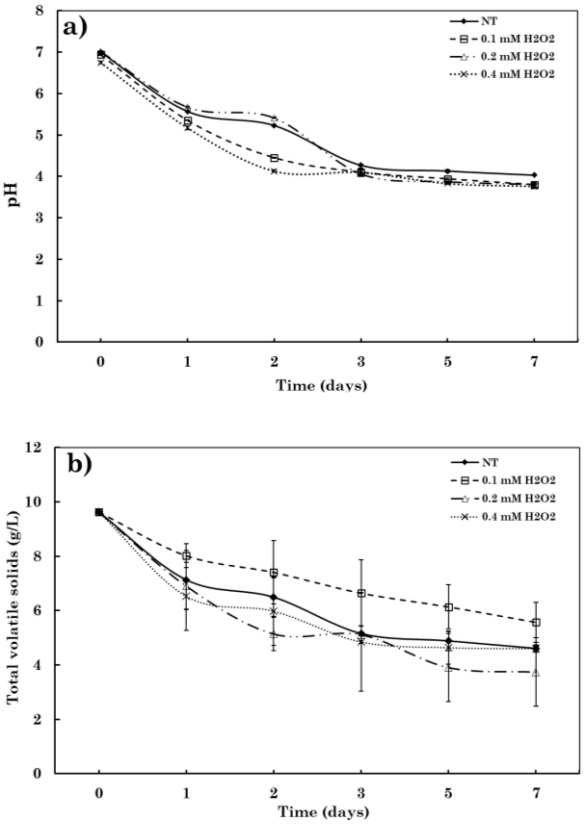

Fig. 2 The profile of pH (**a**) and total volatile solids consumption (**b**) during dark fermentation of melon waste by inoculum from fruit waste digester (37°C, start pH 7). Error bar represented the standard deviation of experimental data.

3.3 Organic acid production during hydrogen fermentation after H_2O_2 treatment

The profile of organic acids production was collected from each fermentor at the end of H_2 fermentation process (7 days). Various organic acids production describes the hydrogen producer's metabolism pathway and affects fermentation (Cai *et al*. 2011). Our result shows that six organic acids were detected at the end fermentation periods: acetic, propionic, formic, butyric, iso-butyric, caproic, and valeric acids. The acetic dan butyric fraction range in total VFAs was 14.2 –41% and 16.1 – 2.22%, respectively (Fig. 3). Acetic production increased in all H_2O_2 treatments (0.1 mM – 517.7 mg/L; 0.2 mM – 607,3 mg/L; 0.3 mM – 550.0 mg/L) compared to NT (327,9 mg/L). Butyric production was detected equal to or lower than NT as it is less profitable in the ROS detoxification process (Hillmann *et al.* 2008). The increase of acetate is theoretically correlated with increased hydrogen yield (Lee *et al.* 2008), which was converted to 4 mol hydrogen yield. For this reason, acetic acid is considered as a critical factor to obtain higher hydrogen production (Khanal *et al*. 2004; Li *et al*. 2009). The results showed an increased acetic acid production under H_2O_2 treatment during H_2 fermentation, positively correlating with the high H_2 production (Table 1). Acetic acid is generated from pyruvate, where the regeneration of NADH and ATP production also occurred (Saady 2013). Acetic acid pathways appear to be utilized by H_2-producing bacteria under H_2O_2 treatment, suggesting that H_2O_2 treatment in all concentrations potentially triggers more acetic acid synthesis that enhances H_2 production.

Formic acid was detected in low concentrations in all H_2O_2 treatments (<40%) than in the NT (51.3%) (Fig. 3). Formic acid is considered a positive metabolic synthesis pathway because of its potential as a source of H_2 production (Wang *et al*. 2018). However, the low formic acid production under H_2O_2 treatment indirectly affected the H_2 yield. Another possibility for this phenomenon is that most formic acid produced may be metabolized by microbes to generate ATP and yield H_2 as a byproduct, resulting in a low formic acid concentration at the end fermentation process. In some studies, the synthesis of several metabolic end-products (such as propionic acid and ethanol) can decrease H_2 production (Bundhoo 2019; Lee *et al.* 2008).

In this study, an increase in propionic acid concentration in all H_2O_2 treatments was observed. It was most likely produced by a rise in hydrogen partial pressure in the system, as our inoculum contained no H_2-consuming microorganisms that had been killed by acid treatment prior to use as inoculum. Moreover, microbial valeric and caproic acid synthesis also consumed molecular H_2 (Luo *et al.* 2011). The presence of valeric and caproic acid causes a decrease in H_2 yield during H_2 fermentation process.

3.3 Mass balance in the hydrogen fermentation of melon waste under H_2O_2 treatment

Volatile solids degradation is typically used to evaluate digestion effectiveness. Here, mass balance was calculated based on the conversion of VS to our parameter targets, such as H_2, CO_2, and VFA, during the fermentation periods (Table 2). The VS conversion by the mixed culture of H_2-producing microbes under 0.1, 0.2, and 0.4 mM H_2O_2 treatment mainly resulting in H_2, CO_2, and VFA, although varying in concentration (Table 2). 0.1 mM H_2O_2 treated sample was the highest VS degraders (up to 94.4%) among all treated samples. It suggests that under 0.1 mM H_2O_2, the H_2-producing microbes showed high effectiveness in producing more fermentation products.

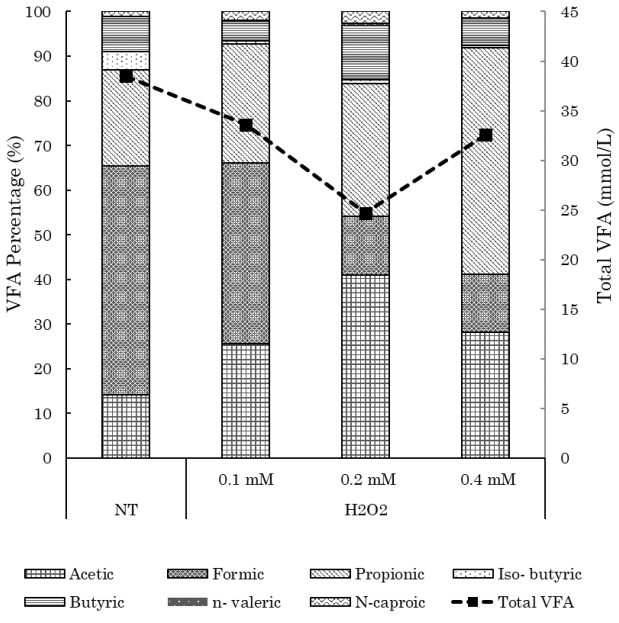

Fig. 3 The percent fraction of VFA and total VFA concentration.

Table 2
Distribution of volatile solids (VS) on the gas (CO_2 and H_2) and volatile fatty acid (VFA) production during seven days fermentation periods.

Treatment	VS availabity (g)	Total production (g)			Total VS consumption		Other product (g)
		VFA	H_2	CO_2	(g)	(%)	
NT	5.020	2.301	0.072	1.704	4.077	81.2	0.943
0.1 mM H_2O_2	4.060	2.040	0.074	1.719	3.833	94.4	0.227
0.2 mM H_2O_2	5.890	1.760	0.074	1.624	3.458	58.7	2.432
0.4 mM H_2O_2	5.030	2.215	0.078	1.558	3.852	76.6	1.178

Note: Total VS consumption was calculated by adding up the total gas production (H_2 and CO_2 [g]) and VFA (g)

4. Conclusion

The findings of this study proposed a promising H_2O_2 treatment approach for increasing hydrogen production from melon fruit waste (*Cucumis melo* L.). The application of 0.4 mM H_2O_2 fairly increased the cumulative hydrogen production up to 7.7 %, while 0.1 mM H_2O_2 enhanced hydrogen yield up to 23.8 %. The primary end products of hydrogen production were acetic, propionic, formic, and butyric acid. The acetic acid was increased by H_2O_2 treatment, leading to enhance H_2 production during fermentation periods. The H_2O_2 treatment appears to positively affect H_2 production and is proposed as an alternative method to enhance H_2 fermentation. This approach is promising and should be explored in the future for providing an in-depth understanding as an alternative method to improve fermentative H_2 production. A further investigation is required to identify NADH profiles under treatment and microbial community analysis to obtain clear information of its mechanism under H_2O_2 treatment as potential alternative methods in H_2 production.

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