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Research Article

Effect of Different Inoculum Combination on Biohydrogen Production from Melon Fruit Waste

Yumechris Amekan[a], Dyah Sekar Ayu Pudak Wangi[a], Muhammad Nur Cahyanto[b], Sarto[c,*] and Jaka Widada[d]

[a]*Department of Biotechnology, Gadjah Mada University, Yogyakarta, Indonesia, 55281*

[b]*Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Gadjah Mada University, Yogyakarta, Indonesia, 55281*

[*c]*Department of Chemical Engineering, Gadjah Mada University, Yogyakarta, Indonesia, 55281*

[d]*Department Agriculture Microbiology, Gadjah Mada University, Yogyakarta, Indonesia, 55281*

ABSTRACT. The natural microbial consortium from many sources widely used for hydrogen production. Type of substrate and operating conditions applied on the biodigesters of the natural consortium used as inoculum impact the variation of species and number of microbes that induce biogas formation, so this study examined the effect of different inoculum source and its combination of biohydrogen production performance. The hydrogen producing bacteria from fruit waste digester (FW), cow dung digester (CD), and tofu waste digester (TW) enriched under strictly anaerobic conditions at 37°C. Inoculums from 3 different digesters (FW, CD, and TW) and its combination (FW-CD, CD-TW, FW-TW, and FW-CD-TW) were used to test the hydrogen production from melon waste with volatile solids (VS) concentration of 9.65 g/L, 37°C and initial pH 7.05 ± 0.05. The results showed that individual and combined inoculum produced the gas comprising hydrogen and carbon dioxide without any detectable methane. The highest cumulative hydrogen production of 743 mL (yield 207.56 mL/gVS) and 1,132 mL (yield 231.02 mL/gVS) was shown by FW and FW-CD-TW, respectively. Butyric, acetate, formic and propionic were the primary soluble metabolites produced by all the cultures, and the result proves that higher production of propionic acid can decrease hydrogen yield. *Clostridium perfringens* and *Clostridium baratii* prominently seen in all single and combination inoculum. Experimental evidence suggests that the inoculum from different biodigesters able to adapt well to the environmental conditions and the new substrate after a combination process as a result of metabolic flexibility derived from the microbial diversity in the community to produce hydrogen. Therefore, inoculum combination could be used as a strategy to improve systems for on-farm energy recovery from animal and plant waste to processing of food and municipal waste.

Keywords: Inoculum; Biohydrogen; Melon fruit waste; Dark Fermentation; Denaturing Gradient Gel Electrophoresis

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1. Introduction

Currently, the high rate of economic and population growth led to an increase in fuel demand as the main energy source for industry, transportation, and even electricity generation. Concern about rapid depletion of fossil fuels and environmental pollution are stimulating studies on alternative energy sources to reduce dependence on fossil fuels (Das and Veziroglu, 2001). Hydrogen (H_2) is a potential energy carrier because it is renewable (Karthic and Joseph, 2012), clean (Sivagurunathan et al., 2016) and has a very high energy yield (122 kJ/g). It is 2.75 times greater than fossil fuels (Hand and Shin, 2004; Kapdan and Kargi, 2006; Choi and Ahn, 2015). Hydrogen can be produced biologically and chemically (Sivagurunathan et al., 2016; Hand and Shin, 2004; Kumar et al., 2015). Biological hydrogen production through photosynthesis and fermentation are more environmentally friendly with lower energy requirements than chemical production processes (Kumar et al., 2015; Pachapur et al., 2015). Anaerobic fermentation is simpler because the process does not require light (Sivagurunathan et al., 2016; Hand and Shin, 2004) and it can apply to wide range of feedstocks (Nath and Das, 2004). Current research has studied various types of substrates for hydrogen production, such as glucose (Li et al., 2008), sucrose (Choi and Ahn, 2015), galactose (Sivagurunathan et al., 2016), cassava starch (Tien et al, 2016), the starch residue of sweet potato (Yokoi et al., 2001), sugarcane bagasse (Pattra et al., 2008) and extracts of pineapple wastes (Ruknongsaeng et al., 2005). The

* Corresponding author: sarto@chemeng.ugm.ac.id

availability, cost, carbohydrate content and biodegradability are the important criteria for the substrate selection (Kapdan and Kargi, 2006; Cai, et al., 2009). In Indonesia, melon production reached 129,706 tons/year with the potential for waste generated ±12,970 tons/year. Melon waste containing 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%). Cahyari et al. (2011) using melon fruit waste as a substrate for hydrogen production in batch at thermophilic conditions (55°C) yield 5.96 mmole H_2/gVS, and the potential H_2 production reached 185,808,197 m3 STP.

According to Reith et al. (2003), the formation of biogas (anaerobic digestion) induced by various types of microbes, usually obtained through a natural enrichment of each substrate used. The composition and amount of bacteria involved in this process will vary depending on the type of substrate and fermentation conditions were applied, causing the variation of yield and biogas production rate (Sivagurunathan et al., 2016; Pachapur et al., 2015; Insam et al., 2010). Sivagurunathan et al. (2014) conducted a study on the use of a combination of three different mixed cultures (cow dung, pig slurry, and sewage sludge) to increase hydrogen production using glucose as a substrate. The results showed an increase in hydrogen yield and production rate by mixed cultures of pig slurry+sewage sludge (2.34mole H_2/mole glucose and 6.76 L/day) compared to the single culture of pig slurry (1.59 mole H_2/mole glucose and 4.43 L/day). An understanding of the effect of an increase in the interaction between microbial diversity and the production of hydrogen is essential to form a stable hydrogen production process. Therefore, in the present study, we investigated the effect of inoculum from different biogas digesters (tofu waste, fruit waste and cow dung anaerobic digester) and their combinations to the biohydrogen production performance and soluble metabolites production using melon fruit waste as the substrate in the batch test.

2. Materials and Methods

2.1 Inoculum and substrate preparation

The seed sludge collected from fruit waste, cow dung and tofu waste biodigester in Yogyakarta (Indonesia), designated as FW, CD, and TW, respectively, for enriching the mixed cultures. All microbial sources were acidified to pH 3 through the addition of 2 M HCl and maintaining for 24 hours, then adjusting back to pH 6.8 with the addition of 2 M NaOH (Ren et al., 2008) to deactivate the hydrogenotrophic methanogens before use in the enrichment of hydrogen-producing bacteria using glucose as the sole carbon source. The melon fruit waste used in this study collected from Gemah Ripah fruit market located in Yogyakarta. The characteristics of the melon waste were: water (92.30%), total solids (7.71%), ash (1.25%) and volatile solid (6.48%). The melon slurry was stored at 4°C until used as the hydrogen fermentation substrate (Ruggeri and Tommasi, 2012).

2.2 Enrichment of hydrogen-producing bacteria

Enrichment of H_2 producing bacteria was carried out in 100 mL serum vials with 50 mL working volume at 37°C, following a method described elsewhere (Sivagurunathan et al., 2014). The sterile pre-reduced peptone-yeast extract-glucose (PYG) medium contains peptone 10 g/L, yeast extract 10 g/L, resazurin 0.001 g/L, L-cysteine-HCl0.5 g/L, glucose 10 g/L. The pH adjusted to 7.0 using either 1 N HCl or NaOH before the autoclave. Three successive transfers in PYG medium with 0.05% cysteine-HCl were done to obtain the mixed-cultures FW, CD, and TW from fruit waste, cow dung, and tofu waste digester, respectively. Freshly grown (24 h) enriched mixed cultures used as the inoculum for all the fermentation experiments.

2.3 Batch hydrogen fermentation

A series of batch hydrogen fermentation tests were carried out to check the hydrogen production performance of individual and combined inoculum. Batch experiments were carried out in serum vials (100 mL) with 50 mL working volume under strict anaerobic condition. Five mL of enriched culture was added as the inoculum to the fermentation medium, following the method described by Sivagurunathan et al. (2014). The batch hydrogen fermentation of melon waste was carried out by the individual, and combined inoculum in basal medium contains volatile solid 9.65 g/L, Na_2HPO_4 5 g/L, KH_2PO_4 2 g/L, $MgSO_4 \cdot 7H_2O$ 0.50 g/L. The single inoculum was mixed in equal volumes to prepare the combined inoculum. All batch serum vials incubated in an incubator with temperature controlled at 37°C. The initial cultivation pH was adjusted to 7.0 using either 1 N HCl or NaOH before the autoclave. The volatile fatty acids (VFA) analysed for samples collected at the end of the fermentation. Fermentation was carried out until the cessation of gas production. All experiments were carried out in duplicates, and the mean ± SD (standard deviation) reported.

2.4 Analytical methods

The volume of biogas was measured using an airtight glass syringe. The biogas composition (H_2, CH_4, and CO_2) analysed with a gas chromatograph having a thermal conductivity detector (GC-14B, Shimadzu, Japan). The analytical procedures of standard methods (APHA, 1998) were used to determine the pH, and VS. Volatile fatty acids (VFA) were analysed using gas chromatograph having a flame ionisation detector (Hewlett Packard 5890 Series II).

2.5 DNA extraction and PCR amplification

Total DNA extracted from bacterial cell pellet of H2 fermentation using Wizard® Genomic DNA extraction kit. PCR was performed using 12.5µL Gotaq Green Master Mix, one µl of each primer (10 µmole/L), one µL (10 ng/µL) of template DNA, and 9.5 µL of nuclease-free water to give a final volume of 25 µL. The first primer was amplified with 16S rDNA using 27f (5'-GAGAGTTTGACTCTGGCTCAG-3') and 1495r (5'-CTACGGCTACCTTGTTACGA-3'). Those primers are

universal primer set (Liu et al., 2009). Bacterial 16S rDNA were amplified at a temperature of 95^{O}C for 3 min, followed by 30 cycles at 95℃-30 sec, 55℃-30 sec, 72℃-1 min and the final extension at 72℃-5 min. The second primers used for the PCR were EuB 984f with GC-clamps (5'CGCCCGGGGCGCGCCCCCGGCGGGGCGGGGGCACGGGGGGAACGCGAACGCGAAGAACCTTAC-3') and EuB1398r (5'CGGTGTGTCCAAGGCCCGGGAACG-3') (Eurofins MWG Operon, Japan). The second primer target was 500 bp reflecting a sequence of V6-8 region in 16S rDNA. V6-8 regions were amplified using the following program; 95℃ for 3 min, followed by 34 cycles at 95℃ for 15 sec, 55℃ for 30 sec, 72℃ for 30 sec and the final extension at 72℃ for 5 min. The size of PCR product was confirmed by electrophoresis in 1.3 % agarose gel in ethidium bromide solution.

2.6 Analysis of Denaturing Gradient Gel Electrophoresis (DGGE) bands

Digitized Denaturing Gradient Gel Electrophoresis (DGGE) images were also analysed using gel compare II software, version 6.6 (Applied Maths, Kortjnk, Belgium). This software carries out relative intensity value of each band to the total intensity of bands in all cultures. Shannon index was calculated using (Eq.1.) and (Eq.2.) as described below

$$H = -\sum_{i=1}^{S} pi \ln pi \quad (1)$$

$$EH = \frac{H}{Hmax} = H/\ln S \quad (2)$$

Where H is the value of the Shannon index, pi is the ratio of the specific band intensity to the total intensity of all bands. The Richness (S) of the bacterial community determined from the number of bands in each lane (Sun et al., 2015; Diez et al., 2001). Two matrices constructed; the first took into account intensity of individual bands related to intensities of the FW-CW7 band (intensity matrix), whereas the second matrix (distance matrix) measure the distance between individual band to FW-CW 7 bands. The intensity matrix was used to calculate bacterial diversity. Both matrices were then used to construct a non-metric multidimensional scaling (NMDS) diagram. The diagram places each sample at a point in a plane (with dimensions of no special significance). A similar bacterial diversity index in inoculum combinations will plot together.

2.7 Bacterial species identification

Predominant Denaturing Gradient Gel Electrophoresis (DGGE) bands (1-16) were excised and eluted in 50 µL of Mili-Q water at 4^{O}C overnight. They were then were centrifuged at 11,000xg for 2 min using MX-105 refrigerated microcentrifuge (Tomy Seiko, Japan). The DNA concentration of the supernatant used as the template for the next PCR using the second primer without GC-clamps. PCR products were purified using the FastGene Gel/PCR extraction kit (Nippon genetics, Japan) and sequenced in DNA sequencer of Eurofins Company, Japan. Basic local alignment search tool (BLAST) used to evaluate the similarity sequence data of DGGE bands with 16S rDNA reference sequences at GenBank (http://www.ncbi.nlm.nih.gov). MEGA 6.0 software used for phylogenetic tree construction.

3. Results and Discussion

3.1 Influence of origin of inoculum on hydrogen production from melon waste

The anaerobic fermentation process includes four biochemical functions, namely hydrolysis, acidogenesis, acetogenesis, and methanogenesis. H_2 products can obtain during the acidogenesis and acetogenesis stages, whereas methanogenesis used H_2 as an electron donor and converted to methane (CH_4) (Mohan, 2009; Saady, 2013). Therefore, hydrogenotrophic methanogens should be inhibited or eliminated in the utilisation of anaerobic microflora for H_2 fermentation (Hawkes et al., 2007). The inhibition did by acid treatment (pH 3; ±24 h) against the seed sludge (Ren et al., 2008; Chang et al., 2011). The results prove that H_2-producing inoculum from fruit waste (FW), cow dung (CD) and tofu waste (TW) digester are capable of producing gas consisting of hydrogen (46 – 65%) and carbon dioxide (35 – 54%). Methane was undetectable for seven days of fermentation (Table 1).

Table 1.
Hydrogen production from melon waste in batch fermenter by Single inoculum from fruit waste (FW), cow dung (CD), and tofu waste (TW) digester (37^{o}C, start pH 7, volatile solid 9.65 g/L).

Inc.	Final pH	Total gas* (mL/L)	Total CO_2* (mL/L)	Total H_2* (mL/L)	Yield H_2 (mL/g VS)	Yield CO_2 (mL/g VS)
FW	4.67±0.01	1,254±28^{a}	511±4	743±32^{d}	207.56±14.61^{f}	142.74±2.75
CD	4.66±0.04	796±65^{b}	369±29	428±36^{e}	129.74±9.82^{g}	111.83±7.83
TW	4.64±0.01	641±25^{c}	319±15	323±40^{e}	92.39±12.41^{g}	91.17±3.39

Legend: *= gas volume at the standard conditions (1 atm. 25^{o}C); different letter in the same column indicate significant difference statistically at the 0.05 level

Inoculum from fruit waste digester produces the highest hydrogen (743 mL/L; yield 207.56 mL/gVS) among the single inoculums. Sivagurunathan et al. [20] showed similar results using three different inoculums, i.e., cow dung (2,139 mL H_2), sewage sludge (2,330 mL H_2) and pig slurry (1.633 mL H_2). The result is supporting the notion that the difference in the performance of hydrogen production caused by the variation of species and number of microbes that induce anaerobic digestion which is affected by the type of substrate and operating conditions applied on the biodigesters of natural consortium used as inoculum (Akutsu et al., 2008).

Inoculum from fruit waste digester showed the highest hydrogen production activity on day 1 of fermentation (Fig. 1 A and B) which the hydrogen production rate was reaching 559 mL/L.day. However, the production rate slows to 127 mL/L.day in the next time span and reaches the lowest point of 4 mL/L.day on the 5th day of fermentation, before stopping on the 7th day. The same trend observed for inoculum from cow dung and tofu waste digester. This phenomenon is influenced by the degree of acidity (pH) of the fermentation medium (Fig. 1B). High hydrogen production rate indicates the optimal pH range, i.e. FW has an optimal pH of 5.59 – 7.10, CD 5.63 – 7.04 (283 mL/L.day) and TW 5.71 – 7.10 (224 mL/L.day). The pH values of all treatments correspond to the optimal pH range of hydrogen production, i.e., 5.5 to 7 (Van Ginkel et al., 2001; Fang and Liu, 2002; Khanal et al., 2004; Kawagoshi et al., 2005).

The hydrogen production rate is high along with a significant increase in the consumption of organic matter over the same time span of 0.74 to 1.09 g/L (7 – 11%). Rotten fruits (such as melon waste used in this study) contain simpler and more easily degraded compounds (e.g., glucose, sucrose, fructose, and maltose). When pH conditions are optimal, acidogenic bacteria that produce hydrogen will transform simple sugars into other products to meet the needs of metabolism and cell growth. Such as acetate together with the formation of ATP as an energy source of cells and alcohol compounds are accompanied by regeneration of nicotinamide adenine dinucleotide (NAD^+) of the reduced form NADH (an essential molecule for continuing glycolysis) (Hallenbeck et al., 2012).

The hydrogen production rate is slower due to the decrease in acidity (pH) of the fermentation media (Fig. 1B). Fermentative hydrogen production by acidogenic bacteria in conjunction with the formation of short chain fatty acids (volatile fatty acid/VFA) such as acetate, butyrate, and propionate (Mohanakrishna et al., 2010; Mohan et al., 2009). Inhibition of acetogenic bacteria and methanogenic (acetoclastic and hydrogenotrophic) activity led to the accumulation of VFA. Which resulted in a decrease in pH and the cessation of production of H_2 as indicated by the slowing of hydrogen production rate (Lee et al., 2008; Zhang et al., 2012; Srikanth and Mohan, 2014). According to Van Ginkel et al. (2001) and Das et al. (2014), the inhibition process occurs when a non-polar undissociated acid penetrates into the cell and releasing protons that would destabilise the intracellular pH and cause denaturation of intracellular enzymes, including hydrogenases (essential hydrogen-producing enzyme). The pH of the media at the end of the fermentation process decreased to 4.63 – 4.78 and hydrogen were not produced under this condition (Fig. 1B).

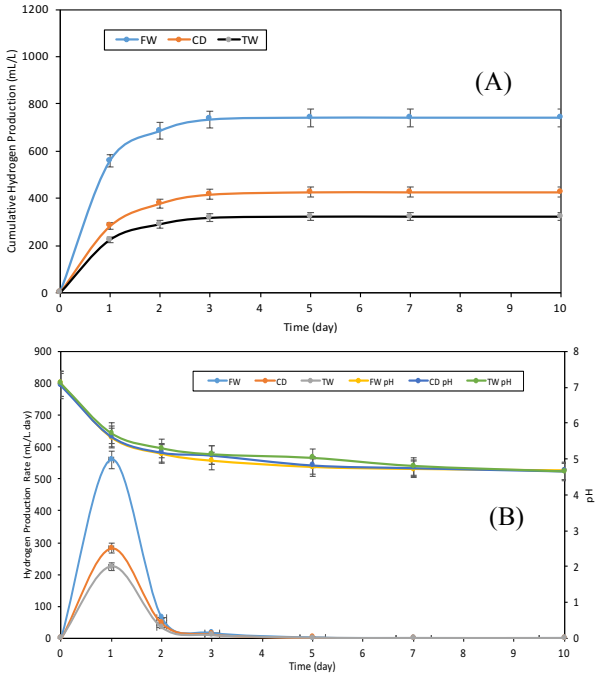

Figure 1. Cumulative production* (**A**), hydrogen production rate and pH profile (**B**) during dark fermentation of melon waste by inoculum from fruit waste digester (FW; ;), cow dung digester (CD; ;) and tofu waste digester (TW; ;) (37°C, start pH 7). *= gas volume at the standard conditions (1 atm, 25°C); *Error bar* represented deviation standard of experimental data.

3.2 Effect of the combination of inoculum from different anaerobic digester on hydrogen production from melon waste

Inoculum from fruit waste (FW), cow dung (CD), and tofu waste (TW) digester are combined to see the effect of increased microbial diversity on hydrogen production performance. The low pH treatment (pH 3; ± 24 hours) was able to limit the activity of the hydrogen users' bacteria, especially methanogens, which were active in a narrow pH range of 6.3 – 7.8. It proved that no methane (0%) was found to 7 days of hydrogen fermentation of melon waste, although the batch test performed at an initial pH of 7.05 ± 0.05. The test results showed that four combined treatments were capable of producing gases comprising H_2 (744 – 1,132 mL/L) and CO_2 (586 – 792 mL/L) (Table 2).

Table 2.
Hydrogen production from melon waste in batch fermenter by combined inoculum from fruit waste digester (FW), cow dung digester (CD), tofu waste digester (TW) (37°C, start pH 7, volatile solid 9.65 g/L)

Inc.	Final pH	Total gas* (mL/L)	Total CO_2* (mL/L)	Total H_2* (mL/L)	Yield H_2 (mL/gVS)	Yield CO_2 (mL/gVS)
FW-CD	4.71±0.01	1,549±98^{a}	546±57	1,003±41^{d}	288.30±0.66^{g}	156.75±10.21
CD-TW	4.70±0.09	1,387±69^{b}	643±30	744±39^{e}	173.48±0.70^{h}	150.06±1.52
FW-TW	4.65±0.06	1,804±75^{c}	728±8	1,077±67^{d,f}	195.15±7.13^{i}	131.97±2.01
FW-CD-TW	4.68±0.03	1,902±70^{c}	770±31	1,132±39^{f}	231.02±1.62^{j}	157.20±2.04

Legend: Inoculum combination of fruit waste and cow dung digester (FW-CD). Inoculum combination of cow dung and tofu waste digester (CD-TW). Inoculum combination of tofu waste and fruit waste digester (FW-TW). Inoculum combination of fruit waste, cow dung, and tofu waste digester (FW-CD-TW). *= gas volume at the standard conditions (1 atm, 25°C); different letter in the same column indicate significant difference statistically at the 0.05 level.

Increased in diversity and interaction between microbial effects the hydrogen production performance. The combination of inoculum can increase the total hydrogen production (Table 2; Fig. 2) 1.74 to 3.50 times compared to the single inoculum. The combination of FW-CD-TW produces the highest hydrogen compared to the combination of FW-CD, FW-TW, CD-TW, and its sole inoculum. These results indicate that the inoculum from different bio digesters able to adapt well to the environmental conditions and the new substrate after a combination process as a result of metabolic flexibility derived from the microbial diversity in the community.

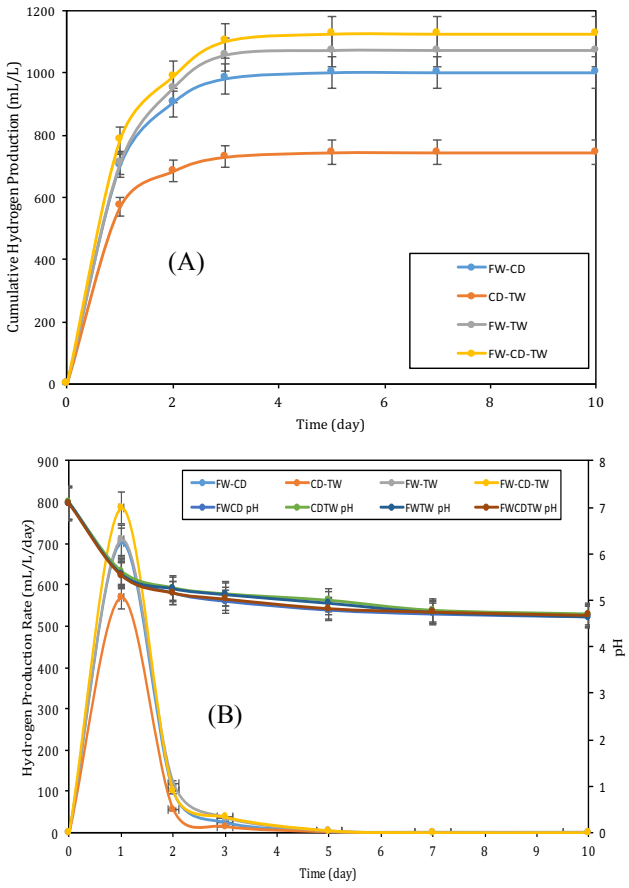

Figure 2. Cumulative production (**A**), hydrogen production rate and pH profile (**B**) during dark fermentation of melon waste by combined inoculum from fruit waste digester (FW), cow dung digester (CD) and tofu waste digester (TW) (37^{o}C, start pH 7). *= gas volume at the standard conditions (1 atm, 25^{o}C); *Error bar* represented deviation standard of experimental data.

Microbial metabolic and physiological diversity means that there are a variety of pathways are utilised by different microbes to produce hydrogen (Hallenbeck and Benemann, 2012). Batch test results prove that the inoculum combination increases the hydrogen production capability. FW inoculum (129.74 mL/g; HPR 85.50 mL/L.d) and TW inoculum (92.39 mL/g; HPR 64.50 mL/L.d) has higher yield and hydrogen production rate after combination FW-TW (173.48 mL/g; 148.70 mL/L.d).

Four inoculum combination tests had the highest rate of hydrogen production (Fig. 2B) on day 1 of fermentation, i.e., 570 – 786 mL/L.day. However, the production rate falls to 110 – 215 mL/L.day in the next time span and reaches a low of 7 – 11 mL/L.day on the 5th day of fermentation, before stopping on the day-7. This phenomenon is similar to the batch test of single inoculum. Hydrogen production associated with VFA formation. The production and accumulation of acid metabolites gradually reduce the medium buffer capacity, so the pH of the medium decreases during the fermentation process (see Fig. 2B). The pH of inoculum and combination batch test media during fermentation showed a similar pattern. Hydrogen not produced by all tests in the pH range of 4.7 – 4.8.

The batch test showed an increase in the yield and hydrogen production rate by four treatment combinations of inoculum up to 1.35 – 3.51 times of individual inoculums. Similarly, Sivagurunathan et al. (2014) using cow dung, sewage sludge and pig slurry show increase of 1.07 to 1.52 times. These results prove that increasing species diversity and microbial interactions due to combinations have a positive effect on improved hydrogen production performance.

3.3 Metabolic end-products of hydrogen fermentation by individual and combined inoculum

Fermentative hydrogen production by all treatments associated with the formation of volatile fatty acid (VFA) under anaerobic/acidogenic conditions. The pattern of VFA in mixed culture fermentation gives a general indication of the concerted metabolism of the community and affects the process performance directly (Ni et al., 2014) as a result of the collective metabolism of mixed culture directly influences the hydrogen yield. The maximum theoretical yield of 4 mole and 2 mole of hydrogen could produce when acetic acid and butyric acid. Respectively are the sole end-product of fermentation. However, the yield significantly drops in case of mixed culture showing mixed-type fermentation due to the accumulation of other end-products like propionic, valeric, and caproic (Lee et al., 2008). As shown in Fig. 3, the VFA composition of single and combined inoculum's fermentation media constituted mainly butyrate (HBu) and acetate (HAc). The range of HAc fraction in VFA was at 20 – 60%. On the other hand, the scope of HBu fraction was at 6.39 – 18.74%.

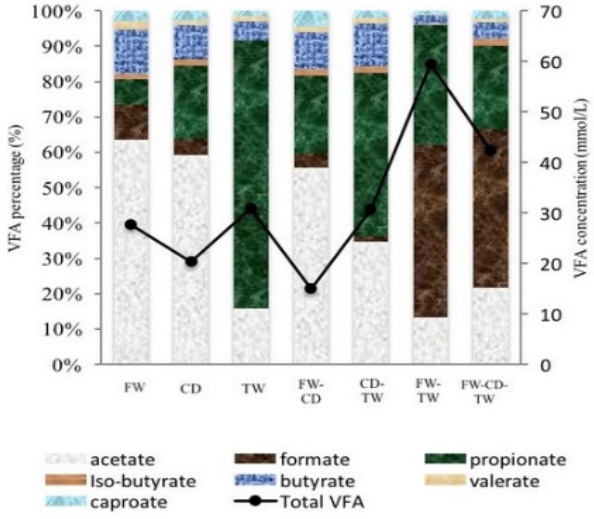

Figure 3. The percent fraction of VFA and total VFA concentration

Hydrogen fermentation by individual inoculum from fruit waste digester produce total organic acids 1821.73 mg/L consisting of acetic 1056.30 mg/L (57.98%), butyric 341.40 mg/L (18.74%), formic 124.25 mg/L (6.8%), propionic 149.38 mg/L (8.20%), valeric 64.30 mg/L (3.53%) and caproic 86.10 mg/L (4.73%). Variation of the final metabolite indicates the diversity of metabolic pathways used by acidogenic microflora in inoculum within seven days of the fermentation process. Pyruvate produced by EMP (Embden-Meyerhoff Pathway) dominantly converted to acetate because in the biochemical reactions of acetate formation occurs NADH regeneration and allow microbes to synthesise ATP (Saady, 2013). Butyrate formed when the hydrogen partial pressure increased (> 60 Pa) to prevent the accumulation of NADH (Saady, 2013). Therefore, the acidogenic bacteria present in the FW has the best hydrogen production capability among CD and TW. Individual inoculum from fruit waste digester (FW) exploit acetate formation pathway for the oxidative decarboxylation of pyruvate and also produce hydrogen through NADH. However, the high production of propionic acid during the batch test of TW (23.38 mmole/L) compared to the FW and CD, each produces 2.02 and 4.21 mmole/L, causing the smaller volume of the hydrogen produced.

The FW-CD combination dominantly oxidises pyruvate to acetate, which is 499.63 mg/L (48.73%), due to the NADH regeneration and allow microbes to synthesise ATP (Saady, 2013). The CD-TW combination exploits propionate formation pathways during fermentation which the concentration of propionic reach 1049.20 mg/L (47.71%). Bio reaction of propionate formation consume H2 as an electron donor in the form of reducing equivalents (NADH2; potential H_2) (Fang and Liu, 2002) causing this treatment has the lowest hydrogen production capability than any other combination. The rank of hydrogen yield of the combination treatment: FW-CD-TW (yield 231.02 mL/g; propionate 28.86%) > FW-TW (yield 195.15 mL/g; propionate 42.22%) > CD-TW (yield 173.48 mL/g; propionate 47.71%). These results prove that higher production of propionic acid decrease hydrogen yield.

The results show that increased in diversity and interactions between microbes effect the yield and hydrogen production rate from the melon waste in batch fermenters. The FW-CD-TW combination shows the best hydrogen production capability compared to CD-TW, FW-CD, CD-TW and single inoculum. Acetate and formic metabolic pathways are used dominantly by the two best combinations during the fermentation process. However, combinations involving the inoculum from the tofu waste digester exhibit a high propionate-forming activity thereby decreasing the hydrogen yield.

3.4 Microbial community analysis

DNA samples collected from all of the treatments at 0, 1, and 7 days of fermentation to see the change in the microbial community during the hydrogen fermentation process. This study found that carbon source replacement using melon waste affect the microbial abundance without any changes in the microbial community that involved in the fermentation. Denaturing Gradient Gel Electrophoresis (DGGE) analysis of the 16s rDNA of single and combined inoculums showed that the microbial community was composed principally of five genera, i.e., Clostridium, *Eubacterium*, *Vagococcus*, *Streptococcus*, and *Lactobacillus* (Fig. 4 and Fig. 5). Interestingly, although FW and TW inoculum have same bacterial species richness and diversity index (lane 2 and 3 on DGGE profiles), FW produces higher hydrogen than TW. The only reason is the high production of propionic acid during fermentation of TW as mentioned before.

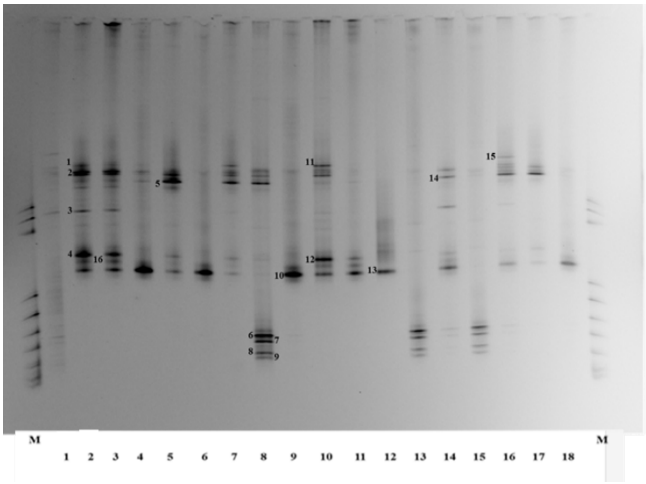

Figure 4. 16S rDNA profiles of H_2 fermenting bacteria from combinations of different inoculum. Lanes 1-3: individual cultures from cow-dung (CD), fruit waste (FW), tofu waste (TW), respectively. Lane 4-18 were combined inoculum took on 0, 1 and 7 day of fermentation. Lanes 4-6: fruit+cow-dung+tofu (FW-CD-TW); 7-9: fruit+tofu (FW-TW); 10-12: fruit+cow-dung (FW-CD); 13-15: fruit (FW); 16-18: cow-dung+tofu (CD-TW). Bands 1-16 were sequenced for identification of bacterial species.

All bacterial species observed in all treatments usually found in H_2 fermentation that using active sludge from wastewater as inoculum (Choin and Ahn, 2014; Kumar et al., 2015). The role of *Vagococcus salmoninarium* and *Methylobacterium* sp. in hydrogen fermentation was not well known yet. All bacteria in this study belong to Proteobacteria and Firmicutes phyla. Phylum proteobacteria have high ability to adapt and could break down large organic compounds into the simple compound. Phylum Firmicutes often plays a role in hydrogen fermentation (Jang et al., 2015). Inoculum source that used in this study had a source of the potential hydrogen-producing bacteria, i.e. *Clostridium baratii* and *Clostridium perfringens*, which always observed in day 1 of fermentation (peak of hydrogen production) in all treatments.

All single and combination of inoculums were not produce hydrogen 7 days of fermentation. It reflected from the Denaturing Gradient Gel Electrophoresis (DGGE) profiles and bacterial diversity index which showed Lactobacillus paracasei prominently detected during this time point. Noike et al. (2002) reported that Lactobacillus paracasei could inhibit the H2 fermentation by produced bacteriocin in a medium under pH 5.

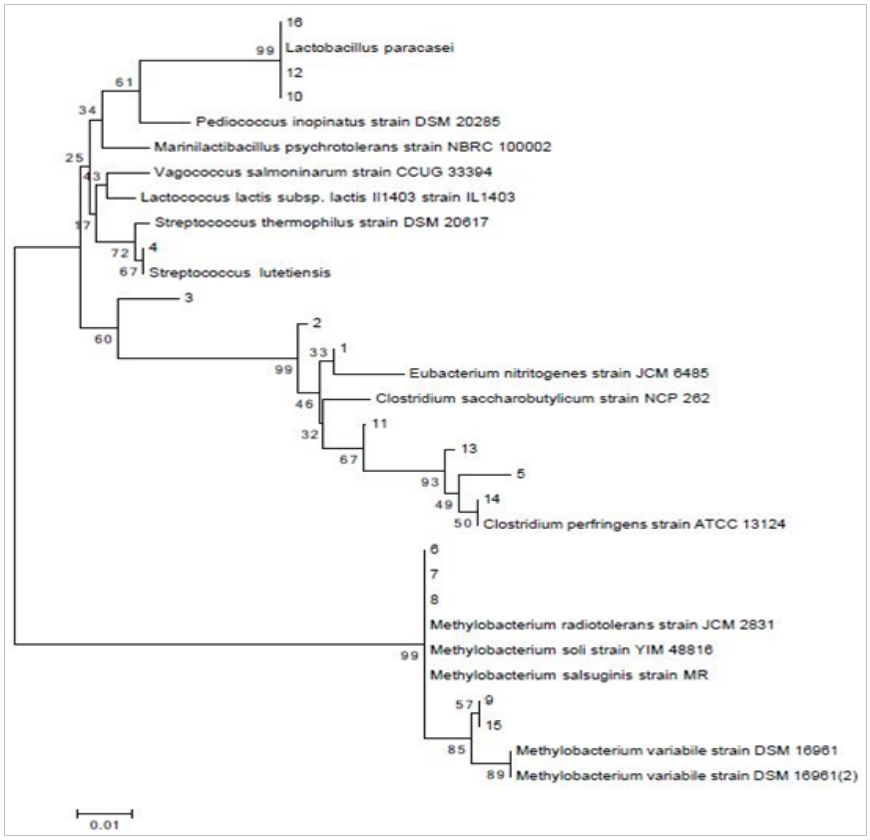

Figure 5. Phylogenetic trees analysis based on Denaturing Gradient Gel Electrophoresis (DGGE) profile. The branching pattern generated by the neighbour-joining method. The topology shown was obtained using 1000 bootstrap replication. The numbers at the nodes indicate the levels of bootstrap support percentage based on 1000 re-samplings.

4. Conclusion

This study demonstrated that increased in diversity and interaction between microbial effects the hydrogen production performance. The combination of inoculum can increase the total hydrogen production. Among the individual and combined inoculums, the highest cumulative hydrogen production of 743 mL/L (yield 207.56 mL/g) and 1,132 mL/L (yield 231.02 mL/g) shown by inoculum from fruit waste digester and the combination of inoculum from fruit waste-cow dung-tofu waste digester. Butyric, acetate, formic and propionic were the primary soluble metabolites produced by all the cultures, and the result proves that higher production of propionic acid can decrease hydrogen yield. Inoculum that used in this study had a source of the potential hydrogen-producing bacteria, i.e. *Clostridium baratii* and *Clostridium perfringens*. Experimental evidence suggests that hydrogen fermentation by inoculum combination could use as a strategy to improve systems for on-farm energy recovery from animal and plant waste to processing of food and municipal solid waste.

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