ANALYSIS OF POME MICROBIAL COMMUNITY FOR HYDROGEN PRODUCTION: COMPARISON OF FEEDSTOCK FROM SOUTHERN AND EASTERN THAILAND

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Received: July 25, 2019; Revised: December 13, 2019; Accepted: December 14, 2019

Abstract

The palm oil industry generates a large volume of waste known as palm oil mill effluent (POME), which must to be treated appropriately before the final discharge. The most powerful refining treatment method is anaerobic digestion to produce hydrogen which is a promising alternative energy. This study aims to investigate and compare the hydrogen production efficiency, metabolites, and microbial community from POME obtained from two different sites, Southern (TSPOME) and the Eastern (SBPOME) parts of Thailand. Our finding suggests that TSPOME is a better hydrogen-producing inoculum than SBPOME. The TSPOME produced a hydrogen content of 34.35% and a maximum hydrogen yield of 0.74±0.10 mol-H_2/mol-glucose, which is 24.63% and 0.63 mol-H_2/mol-glucose higher than those of SBPOME (9.72% and 0.11±0.01 mol-H_2/mol-glucose). Furthermore, the metabolite analysis in TSPOME showed the increase in butyric acid concentration had led to higher hydrogen production. In addition, the total relative abundance of potential hydrogen producer in TSPOME was 2.42% while that from SBPOME was only 0.24%. The hydrogen-producing bacteria found in TSPOME were *Ruminococcus* sp. (2.40 %), *Bacillus* sp. (0.01%), and *Clostridium* sp. (0.01%). Our results allow a better understanding of H_2 production in different regions of Thailand and how the microbial community involved can affect H_2 yield. Hence, this provides the key information for efficient selection of inoculum for future applications.

Keywords: Hydrogen, microbial community, POME, anaerobic digestion, renewable energy

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Introduction

Hydrogen gas is regarded as a source of clean energy since the combustion of hydrogen yields only water as a product while releasing a high amount of energy (Nielsen *et al*., 2001). It has a high heating value, which can be used for massive applications relating to heat and power generation, as well as transportation (Raheem *et al*., 2015). The hydrogen produced from non-valuable materials or waste can alleviate problems such as greenhouse gas emission, wastewater discharging, and waste management. Currently, hydrogen is widely generated from electrochemical, thermochemical, and biological processes. Hydrogen production from the biological process is can be operated at mild conditions with low energy consumption (Norfadilah *et al*., 2016). During the process of anaerobic digestion, the organic substances such as proteins, lipids and carbohydrates are initially hydrolyzed to their monomers that include amino acids, fatty acids and sugars, respectively, which are then converted to acetate and hydrogen by further fermentation (Wang and Yin, 2017). Generally, acetate and hydrogen continue to be converted to methane by methanogens. Therefore, the methanogens need to be inhibited in order to enhance the yield of hydrogen production (Valdez-Vazquez and Poggi-Varaldo, 2009). Hydrogen can be produced from a wide variety of microbial inoculum sources, which are likely to be pre-treated to eliminate the activity of H_2-consuming species and/or enrich the microbial community with H_2-producing bacteria. Mixed cultures coming from natural and engineered ecosystems have also been used as inoculums, with the advantage of providing better adaptation capacity in response to environmental stresses. The higher robustness of mixed cultures has been attributed to the diversity of the microbial community, enabling positive or negative interspecies interactions (*Ziesack et al*., 2019). Therefore, the origin of the inoculum is one of the most important factors to ensure H_2-producer enrichment in order to achieve high and stable H_2-production performance.

Palm oil is one of the important agricultural products and, therefore, an economic driver of Thailand and Southeast Asian countries (Nutongkaew *et al*., 2019). As the third biggest palm oil producer in the world, Thailand produces about 2.4 million metric tons of palm oil annually (U.S. Department of Agriculture, 2016; Nutongkaew *et al*., 2019). Oil palm is distributed throughout the country; however, the southern region is the dominant area of oil palm farms which produces palm oil of approximately 2 kg/sqm. The eastern region is another large palm oil producer by Producing palm oil of around 1 kg/kg/sqm (Office of Agricultural Economics, 2017). Palm oil industries process oil palm to produce crude palm oil which can be used as a starting material for several products such as cooking oil, sweets, biodiesel, cosmetics and soap (Nutongkaew *et al*., 2019). Along the processes of palm oil production, the industry also produces a huge volume of wastewater called palm oil mill effluent (POME). Most of the palm oil industries employ anaerobic digestion systems to treat wastewater before discharging. Such anaerobic digestion yields biogas, an alternative energy source. The variation of microbial community leads to different biogas production, typically consisting of 40-75% CH_4, 15-60% CO_2, 0-5% N_2, <1% H_2, <1% H_2S, <2% O_2, 1-5% moisture, and <2% trace gases. The microbial community depends on various plantation parameters of oil palm which include soil quality, weather, palm species, sunlight, temperature, moisture, etc. (CPI Agrotech, 2017). The contour mapping of several parameters related to palm oil plantation in Thailand has recently been reported (Nutongkaew *et al*., 2019). The results showed that the annual mean rainfall in the southern part of Thailand is in moderate to high range, while that of the east is in moderate to low range. Regarding the annual mean temperature, it is in moderate range in the south whereas it is in high range in the east. Moreover, the sunshine hours of the south are in low range, but in moderate range for the east. Hence, these reports showed that the southern and eastern regions have significantly different plantation parameters, which might affect the microbial community inside the anaerobic digestion system for POME treatment and, therefore, affect the hydrogen production efficiency. Morimoto *et al*. (2004) and had reported that microbes from the anaerobic digestion system of palm oil mill wastewater treatment plant can be used as an inoculum for hydrogen production (Morimoto *et al*., 2004; Seengenyoung, 2013). Therefore, the objective of this study is to compare inoculums obtained from different anaerobic digestion systems of palm oil mills from the southern and eastern regions of Thailand in terms of hydrogen and metabolite production, and to investigate the microbial community in order to use POME as a hydrogen-producing inoculum and reduce waste being discharged into the environment along with promotion for the use of alternative energy.

Materials and Methods

Inoculums

The inoculum samples were collected from anaerobic digestion systems in summer season of two palm oil factories which are Suksomboon Vegetable Oil Co., Ltd. (SBPOME; 13°09'26.1"N 101°20'03.1"E) located in Chon Buri from the Eastern region and Southern Palm (1978) Co., Ltd. (TSPOME; 9.1145° N, 99.2657° E) located in Surat Thani from the South. The moisture content, total solid (TS), volatile solid (VS), volatile suspended solid (VSS) and ash content of inoculum from TSPOME and SBPOME were reported in Table 1. TS, VS and VSS (centrifuged at 12,000 rpm for 5 min) tests were performed at 105°C for overnight using oven and 550°C for 3 h using furnace, respectively (Eaton *et al*., 2005). The amount of moisture and ash were estimated based on the mass loss after the heating samples at 105°C and 550°C, respectively. In order to study the hydrogen production, the inoculums were heated at 100°C for 60 min to inactivate hydrogen-consuming bacteria such as methanogens, sulfate reducing bacteria and homoacetogens (Valdez-Vazquez and Poggi-Varaldo, 2009). The VSS concentration of the inoculums was 2.5 g/L.

Table 1. Characterization of inoculums

Characteristics	TSPOME	SBPOME
Moisture, %	97.29±0.01	97.52±0.05
Total solid (TS), %	2.71±0.01	2.48±0.05
Volatile sold (VS), %	1.44±0.06	1.21±0.03
VS/TS, %	53.03±1.94	48.89±0.11
Ash, %	1.27±0.05	1.27±0.02

Hydrogen Fermentation

After the pretreatment, the inoculums were acclimated and degassed by using 100 mM glucose until no pressure was produced. Hydrogen fermentation was performed by using 100 mM glucose as a substrate in 20 mL airtight sealed glass bottles, each with 5 mL working volume under anaerobic atmosphere at 37°C, 220 rpm, and the initial pH was adjusted to 5.5. The hydrogen fermentation was performed for 84 h. Before sampling biogas, the pressure inside the sealed glass bottle was equilibrated to the ambient pressure using 60 mL syringe. The hydrogen yield was calculated from the hydrogen content in the headspace and the total gas volume using a mass balance equation as previously described (Sen and Suttar, 2012). By taking the control experiment (using deionized water instead glucose) into account, the hydrogen yields reported had already been subtracted with the value from the control experiment.

Hydrogen and Metabolite Analysis

The hydrogen content was measured as previously described (Wang and Wan, 2008) with a slight modification. Headspace gas was collected to measure the hydrogen content via gas chromatography thermal conductivity detector (GC-TCD) equipped with CP-Molsieve 5A column (10 m × 30 µm × 0.32 mm). Nitrogen was used as a carrier gas with a flow rate of 4 mL/min. The temperatures of injection port and detector were 40 and 250°C, respectively. The oven temperature was programed at 40°C and to hold for 1 min. The hydrogen content was measured in triplicate.

For metabolite analysis, volatile fatty acids (VFAs) were extracted and measured as described (Pruksatrakul *et al*., 2017) with slight modifications. Fermentation sample was mixed with tert-butyl methyl ether (TBME) to extract the VFAs and centrifuged at 6,000 rpm for 30 min for phase separation. The VFAs (acetate, propionate, butyrate, iso-butyrate, valerate, and iso-valerate) were analyzed by gas chromatography flame ionization detector (GC-FID) equipped with HP-INNOWAX polyethylene glycol column (30 m × 0.25 µm × 0.25 mm). Helium was used as a carrier gas with a flow rate of 2 mL/min. The temperature of injection port and detector were 240°C. The oven temperature was programed as follows: the initial oven temperature, 95°C; increase to 140°C at 10°C/min; increase to 200°C at 40°C/min; hold for 5 min. The data were determined in triplicate.

Microbial Community Analysis

The Fecal/Soil Microbe Miniprep Kit (Zymo research, USA) was used to extract genomic DNA, following the manufacturer's protocol. The genomic DNA was amplified with 16S rRNA primers using V3-V4 region. The 16S rRNA amplification primer was 16S rRNA forward primer 5' AGAGTTTG ATCCTGGCTCAG 3'; and 16S rRNA reverse primer 5' ACGGTTACCTTGTTACGACTT 3'. Then, PCR products were used for library preparation and subsequently sequenced using Ilumina Miseq (Macrogen, Korea). Raw data were pre-processed to filter short reads and trim extra-long tails. The filtered reads were clustered using a greedy algorithm into OTUs at specific OTU cutoff. In the final step, the representative sequences from each OTU were used to assign the resulted taxonomy.

Kinetic Analysis

The cumulative hydrogen production profile was fitted to a modified Gompertz equation using Solver function in Microsoft Excel 365 (Zwietering *et al*., 1990):

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

Where H is the cumulative hydrogen production (mol-H_2/mol-glucose), P is the hydrogen production potential (mol-H_2/mol-glucose), R_m is the maximum hydrogen production rate (mol-H_2/mol-glucose/h), λ is the lag-phase time (h), and e is 2.718. The Solver function was used to identify parameters (P, R_m, λ) for the generation of H value which provided the best fit with an experimental hydrogen production value.

Results and Discussion

Performance Indicator During Hygrogen Production

Figure 1 shows the comparison of hydrogen content and cumulative hydrogen production of TSPOME and SBPOME, which shows that TSPOME produced hydrogen content and maximum hydrogen yield of 34.35% and 0.74±0.10 mol-H_2/mol-glucose which is 24.63% and 0.63 mol-H_2/mol-glucose higher than that of SBPOME (9.72% and 0.11±0.01 mol-H_2/mol-glucose). In general, the hydrogen content is within the range that has been reported in the literature (10-58%) (Ghimire *et al.*, 2015). These results indicate that TSPOME is a better hydrogen-producing inoculum than SBPOME. It should be noted that the hydrogen content from both POME sources significantly decreased because the fermentation did not produce any gas volume in the longer fermentation period (48-84 h).

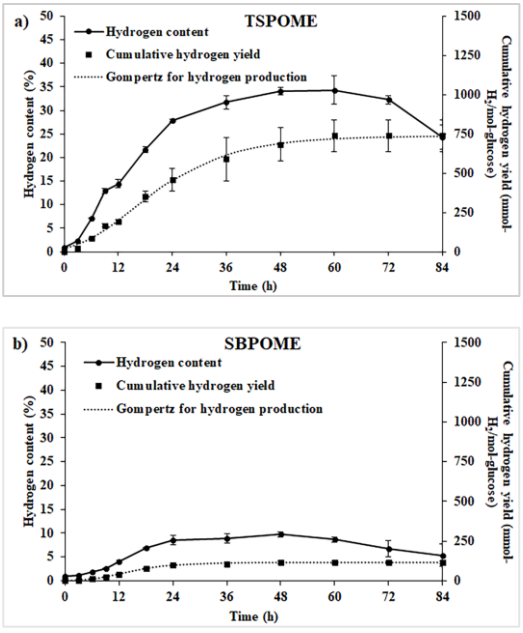

Figure 1. Comparison of the hydrogen production from (a) TSPOME and (b) SBPOME digested with 100 mM glucose. The result shows in triplicates

Furthermore, the hydrogen production profile was evaluated by Equation 1. resulting in the optimum kinetic parameters as summarized in Table 2 which contains the information on hydrogen production potential, maximum hydrogen production rate, and lag-phase time. Here, the hydrogen production potential of TSPOME (740.22 mol-H_2/mol-glucose) presented 6.5-fold higher than that of SBPOME (113.39 mol-H_2/mol-glucose). Similarly, the maximum hydrogen production rate of TSPOME (22.45 mol-H_2/mol-glucose/h) is 3.5-fold higher than that of SBPOME (6.34 mol-H_2/mol-glucose/h). This would also solidly confirm our reported findings that TSPOME can establish hydrogen better than SBPOME. In addition, the lag-phase time of TSPOME (3.10 h) showed 2.41 h faster than that of SBPOME (5.51 h) which indicates that the TSPOME inoculum has faster adaptation ability with feedstock than that of SBPOME. The trends observed for high hydrogen production in POME from the south (TSPOME) correlated well with the previous studies where most POME inoculums for high energy generation are located in southern region of Thailand (Pattanapongchai and Limmeechokchai, 2011). Consecutively, in this study, the correlation of hydrogen efficiency, metabolite production, and microbial community between TSPOME and SBPOME will be discussed in more details in the following sections.

Table 2. Kinetic parameters for the hydrogen production from different sources of POME

Samples	Hydrogen production potential, P (mmol-H_2/ mol-glucose)	Maximum hydrogen production rate, R_m (mmol-H_2/ mol-glucose/h)	Lag-phase time, λ (h)
TSPOME	740.22	22.45	3.10
SBPOME	113.39	6.34	5.51

Metabolic Patterns During H2 Production

Since hydrogen is one of the main metabolites which is a byproduct from VFAs production, the relationship between hydrogen and VFAs were analyzed in both sources. The VFAs were measured at the starting of fermentation (0 h) and at the maximum hydrogen production (48 h). The dominant VFAs in both POME samples are butyric acid, acetic acid, isobutyric acid, and propionic acid, which showed in Figure 2.

The total VFA concentrations of TSPOME at 0 and 48 h were 74.91±8.54 and 115.22±0.89 mM, whereas, the total VFA concentrations of SBPOME at 0 and 48 h were 54.64±2.59 and 61.47±2.76 mM.

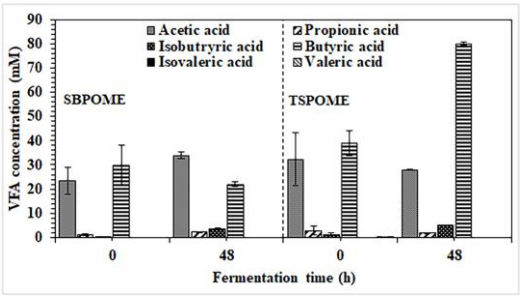

Figure 2. Volatile fatty acid concentrations from SBPOME and TSPOME at 0 and 48 h of the fermentation time. The results show in triplicates

Higher total VFA production was found in TSPOME (190.16 mM) compared to that of SBPOME (116.11 mM) after subtracting baseline at 0 h. This is probably a key factor for the high hydrogen production efficiency in TSPOME. Butyrate was a main metabolite found in all experiments, detected between 22.02±1.03 and 29.83±8.17 mM in SBPOME, and 38.82±5.21 and 80.11±0.64 mM in TSPOME. These results indicate that hydrogen production in TSPOME belongs to the conversion of sugar to butyrate. Similarly, acetate production was present with the concentration between 23.36±5.68 and 33.81±1.41 mM in SBPOME, and 27.89±0.25 and 32.27±10.86 mM in TSPOME. The propionic acid, isobutyric acid, isovaleric acid and valeric acid were also detected in all experiments but at low concentrations (<7.0 mM).

The glucose substrate can produce 2 and 4 mol of hydrogen with butyric acid and acetic acid as an end product, respectively, which indicates that the main metabolites of hydrogen fermentation are acetic acid and butyric acid (Ren *et al*., 2006). In the case of SBPOME, acetic acid and butyric acid were marginally changed throughout the process, which correspond to very low hydrogen production, while TSPOME showed the increase in butyric acid concentration leading to high hydrogen production. This might be due to the conversion of sugar into butyric acid, carbon dioxide, and hydrogen in acidogenesis pathway as showed in Equation 2. (Reungsang, 2019). The decrease in acetic acid concentration in TSPOME might be due to the reaction between acetic acid and ethanol to produce ethyl acetate as showed in Equation 3. (Wagner *et al*., 1980). The ethanol was produced in the acidogenesis pathway as showed in Equation 4-5., (Reungsang, 2019).

$$C_6H_{12}O_6 + H_2O \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (2)$$

$$C_2H_5OH + CH_3COOH \rightarrow CH_3COOC_2H_5 + H_2O \quad (3)$$

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \quad (4)$$

$$C_6H_{12}O_6 + H_2O \rightarrow 2CH_3COOH + C_2H_5OH + 2CO_2 + 2H_2 \quad (5)$$

Microbial Communities in Inoculums

Based on the results of hydrogen production and volatile fatty acid dynamics, it can be seen that TSPOME is a better hydrogen-producing inoculum than SBPOME. To confirm that the microbial community in TSPOME is indeed correlating well with the hydrogen production efficiency and metabolic pattern, NGS data from TSPOME were further compared with SBPOME. The total relative abundance of potential hydrogen producers in TSPOME was 2.42% while only 0.24% was present in SBPOME. In TSPOME, three genera of hydrogen-producing bacteria were found, which are *Ruminococcus* sp., *Bacillus* sp. and *Clostridium* sp. as shown in Figure 3. The *Ruminococcus* sp., *Ruminococcus callidus* (1.08%), *Ruminococcus gauvreauii* (0.61%), *Ruminococcus champanellensis* (0.58%), and *Ruminococcus albus* (0.13%), are found with high relative abundance of potential hydrogen producer (2.40% of total relative abundance). The *Bacillus* sp., *Bacillus wudalianchiensis* is found at a relative abundance of 0.01%. Moreover, *Clostridium*

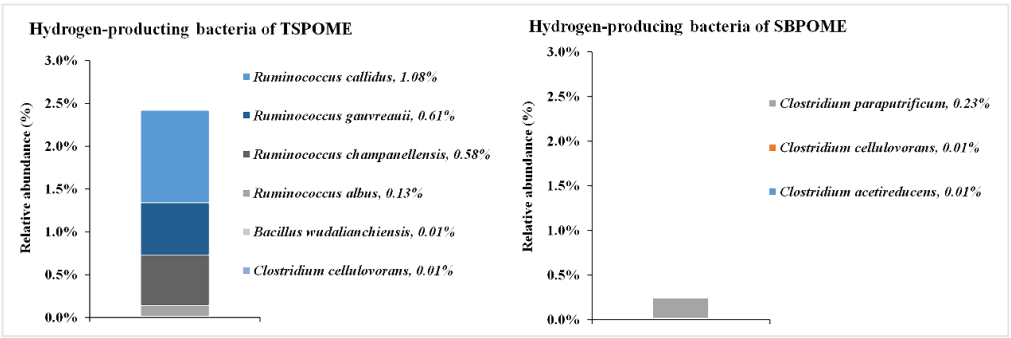

Figure 3. The hydrogen-producing bacteria of inoculums from TSPOME and SBPOME

sp., *Clostridium cellulovorans*, is identified at a relative abundance of 0.01%. *Ruminococcus gauvreauii* can produce acetic acid from sugars, so it should be related to hydrogen production (Domingo *et al*., 2008). *Ruminococcus champanellensis* metabolizes cellulose and cellobiose to acetic acid and succinic acid; therefore, it is also related to hydrogen production (Chassard *et al*., 2012). *Ruminococcus albus* has been reported as anaerobic rumen bacterium, which can hydrolyze cellulose to acetate, ethanol, formate, H_2 and CO_2 (Nandi and Sengupta, 1998). Nevertheless, *Ruminococcus callidus* had not been reported as hydrogen-producing bacteria, while the *Clostridium* sp. and *Bacillus* sp. are well known hydrogen producers (Elsharnouby *et al*., 2013; Dhanasekar and Jonesh, 2018). *Clostridium cellulovorans* can generate H_2, CO_2, acetate, butyrate through the fermentation of sugars and lignocellulosic materials (Tamaru *et al*., 2010). However, *Bacillus wudalianchiensis* had not been reported as hydrogen-producing bacteria. Only the *Clostridium* sp. was found in SBPOME as the potential hydrogen producer as shown in Figure 3. *Clostridium paraputrificum* (0.23% of relative abundance) and *Clostridium acetireducens* (0.01% of relative abundance) had not been reported as hydrogen-producing bacteria. *Clostridium cellulovorans* (0.01% of relative abundance) was only a hydrogen producer in SBPOME. Consequently, the details described above illustrate the hydrogen production profile between two inoculums.

Conclusions

Our findings have shown that the inoculum from the southern region (TSPOME) has a higher efficiency in terms of potential and performance of hydrogen production and adaptation with feeding than that of the inoculum from the eastern region (SBPOME). Acetic acid and butyric acid were mainly found in hydrogen fermentation leading to high hydrogen production in TSPOME. The TSPOME is a favorable hydrogen-producing inoculum which contains hydrogen producers like *Ruminococcus* sp., *Bacillus* sp. and *Clostridium* sp.

Acknowledgments

This research was supported by the Thailand Science Research and Innovation (TSRI) contract no. MRG6080234, Vidyasirimedhi Institute of Science and Technology (VISTEC) and Kasikornbank (KBank). We also thank Mr. Tanarak Pongpetra from the Southern Palm (1987) Co., LTD. and Suksomboon Vegetable Oil Co., Ltd. for kindly providing the samples for this research.

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