Dark fermentative hydrogen production from cheese whey using hydrogen-producing bacteria isolated from Mount Pancar hot spring, West Java

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Abstract. One of the most promising ways to produce high hydrogen yield is through dark fermentation by using dark fermentative bacteria due to the capability of these microbial agents to convert various organic compounds, particularly sugar, into hydrogen gas. In this study, three Gram-positive hydrogen-producing bacteria with a different character of colony on agar, namely as RP 009, RP 010, and RP 011, had been successfully isolated from Mount Pancar hot spring, West Java. All these isolates were able to produce hydrogen gas in all cheese whey concentration, consisting of cheese whey 30%, 60%, and 100%. RP 011 was the most favorable hydrogen producers in this study due to its high hydrogen productivity (4,400.625 ml biogas/L medium) as well as its ability to adapt and consecutively produce hydrogen even in the very high concentration of the organic compound. The best cheese whey concentration for hydrogen production in this study was 60%, considering the efficiency and effectiveness of the organic compound conversion into hydrogen gas. Ultimately, this study presented the potential of high hydrogen productivity of indigenous hot spring bacteria isolated from Mount Pancar hot spring in which had major potential for environmentally friendly bioenergy and biomass refineries.

1. Introduction
The world is seeking a renewable and environmentally friendly source of energy to overcome the problem of energy demand and environmental pollution [1]. In that case, Hydrogen (H₂) is considered as one of the most promising alternative energy, acting as a strong energy carrier that produces high energy yield [2]. Hydrogen has energy yield 122 kJ/g in which is 2.75 times higher than that of fossil fuel [3]. Furthermore, hydrogen is highly clean energy which only produces water vapor in the combustion process [4]. Thus, it represents a highly suitable future energy with a high content of energy and without any environmental pollution to be questioned [5].

Hydrogen produced by biological methods, called biohydrogen, has outstandingly attracted more attention rather than physical or chemical methods due to its potential as inexhaustible bioenergy, low-cost production, renewable feedstock, as well as solid waste and wastewater recycling [6]. Furthermore, the dark-fermentation process using anaerobic bacteria seemed to be the best choice of all biological methods for hydrogen production because it exhibited a high rate of hydrogen production and had a wide range of organic compound usage [7]. The latter demonstrated that this process showed high versatility in employing various types of solid waste as well as wastewater, including cheese whey [8,9].

Acting as the by-product from cheese or casein manufacturing, cheese whey contained high organic load with a biological oxygen demand and chemical oxygen demand in the interval 27 – 60 g/L and 50 – 102 g/L, respectively [10]. It was considered as the most important pollutant in dairy wastewaters which mainly harboring lactose and fat [11]. High biodegradable nutrients in cheese whey made it as one of the best feedstock for bioprocessing, including for energy conversion [12]. Ultimately, the utilization of cheese whey for hydrogen production through the dark-fermentation process showed
notable advantages, particularly from the environment and economic perspectives due to the conversion of waste into high-value product hydrogen gas [10,13].

Due to the biomass-containing waste from the industrial process was seemingly released in high temperature, the hydrogen production from thermal-resistant bacteria, such as hyperthermophilic, thermophilic, and thermo-tolerant bacteria showed great potential as the better agents for biological hydrogen production, in addition to its capability of high rate hydrogen production [9]. Furthermore, biohydrogen production from thermo-tolerant bacteria seemed to be more attractive because of its potential for enhancing the production by co-culturing with mesophilic photosynthetic bacteria [14,15]. The efficiency level for bio-waste usage was highly increased by a co-culture system of the dark- and photo-fermentation [16]. Thus, the utilization of thermo-tolerant bacteria, which could be isolated from hot spring, was principal for biohydrogen production from biological waste.

In this present study, we have isolated three potential bacteria from Mount Pancar hot spring for hydrogen production through dark fermentation using cheese whey. This study was aimed to investigate the hydrogen production capability of the three isolates using different concentration of cheese whey. This is the first report of the bioproduction of hydrogen gas using Indonesian indigenous hot spring bacterial isolates with major potential for biomass refinery.

2. Methods

2.1. Bacterial Isolation, Screening, and Characterization

The bacteria were isolated from the sediment samples in the Red Crater of Mount Pancar hot spring, West Java. The temperatures of the hot spring are ranging from 60 – 70°C. The sediment samples were collected in the bottom layer of the hot spring for ensuring the availability of anaerobic bacteria. The isolation procedure was conducted using ASY Agar Medium, containing ammonium sulfate (1.35 g/L), disodium succinate (2.75 g/L), yeast extract (1 g/L), Agar (30 g/L) and 10 ml modified basal medium stock 100x (Basal medium stock 100x (g/L): K2HPO4 94, KH2PO4 63, EDTA.2Na 0.2, H2BO3 0.28, NaMoO4.2H2O 0.075, ZnSO4.7H2O 0.024, MnCl2 0.21, Cu(NO3)2.3H2O 0.004, FeSO4.7H2O 1, CaCl2.2H2O 0.075, MgSO4.7H2O 20). The pH was adjusted to 7.0 using NaOH 4 M before adding Agar and autoclaving. The serial dilution was performed before transferring the sediment samples into ASY Agar media by doing spread plate methods. The cultures were incubated in the anaerobic jar for 24 hours at 28°C. The grown colonies were selected by the different character of the colony. The selected colonies were eventually re-streaked into freshly made sterile ASY Agar media. This step was repeated 2 to 3 times to ensure the purity of the isolates.

The pure isolates were grown in GY media to determine the capability of the isolates for gas production through the fermentation process. This media contained glucose (10 g/L), yeast extract (1 g/L), and 10 ml modified basal medium as described in the latter paragraph. The bacteria isolates were transferred into 125 ml serum bottle containing 50 ml GY media. The cultures were incubated in 120 rpm orbital shaker for 24 hours at 28°C. The gas production was determined by collecting the gas (if produced) by injecting a gas-tight syringe through the rubber cap.

The colony and cell characteristics of the selected pure isolates were determined. The colony properties of the isolates in the Agar media were identified by observing the form, margin, opacity, and pigmentation of the colony. The cell characteristics were determined by observing the shapes and Gram types of the cell at 1000x magnification under light microscopy (Leica DM 750), after performing the Gram staining procedure.

2.2. Collection and Preparation of Feedstock

Fresh cheese whey was collected from Cheese Industry, Research Center for Biotechnology, Indonesian Institute of Sciences. The pH of fresh whey wastewater was 5.17 which will eventually be adjusted to 7.0 using NaOH 10 M. Furthermore, three concentrations of cheese whey consisting of 100%, 60%, and 30% of the cheese whey were made by dilution with distilled water. Ultimately, the pre-treated whey wastewaters were autoclaved at 121°C for 15 min. The concentration of sugar and
organic compound (COD) in pre-treated cheese whey (cheese whey 100%) was 36016.69 ppm and 77819.6 ppm respectively.

2.3. *Culture Conditions and Biogas Production*

The cultures were grown anaerobically by transferring the single colony from ASY Agar media into GY medium. The grown cultures were eventually re-transferred into freshly made GY medium to ensure the cultures were in the best condition for biogas production. The batch fermentation process was performed by inoculating the 24 hours cultures from GY medium into serum bottle 125 ml containing treated whey wastewaters with total working volume 80 ml. The Optical Density of the initial culture was adjusted to 0.1 at wavelength A600 nm using UV/Vis Spectrophotometer (UV-1700; Shimadzu Scientific Instruments, Japan). Each bottle was sealed by a rubber stopper and capped with aluminum seal. The batch fermentation was run for 7 consecutive days and the gas was harvested every 24 hours. All batch fermentation procedures were performed at 28°C in an orbital shaker with rotation speed at 120 rpm. All the batch experiments were carried out independently in duplicate. The biogas data were presented in 1 L medium with the following equation:

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\text{Biogas Production in 1 L Medium} = \frac{\text{Observed Biogas Production (ml)}}{\text{Total Working Volume (ml)}} \times 1000
\]

2.4. *Analytical Methods*

The evolved gas was periodically measured by using a glass tight gas syringe. The data of gas showed in this study represented the biogas defining the total mixture of gas produced by the bacterial isolates. The hydrogen appearance was determined qualitatively by injecting the gas into the Polymer Electrolyte Membrane (PEM) fuel cell. Total sugar and organic compound (COD) were determined using phenol-sulfuric acid methods and dichromate spectrophotometric methods [17], respectively. Glucose with concentrations of 10, 20, 30, 40, 50, 60, 70, and 80 ppm was used to build a standard curve for total sugar examination. Potassium hydrogen phthalate with concentrations of 0, 200, 400, 600, 800, and 1000 ppm was used to create a standard curve for total organic compound (COD) examination. The regression equation with \( R^2 \) value > 0.99 was used to convert the Optical Density value into the concentration unit (ppm).

The effects of cheese whey concentrations as well as bacterial isolates were analyzed statistically by ANOVA (One-way and Two-way). When ANOVA analysis showed significant results, further analyses were performed by Tukey HSD’s tests. \( p \) values below 0.05 determined that the differences were statistically significant. The data were presented in mean ± standard deviation. The statistical software Statistical Package for the Social Sciences (SPSS) Statistics 25 was used for statistical analysis.

3. Results and Discussions

3.1. *Colony and Cellular Morphology*

Three isolates capable of producing hydrogen were screened and purified from Mount Pancar hot spring. The isolates were able to grow in the presence as well as the absence of the oxygen (facultative anaerobic bacteria). They exhibited Gram-positive staining (Figure 1.) and showed different character for the colony on agar as well as the bacterial morphology under microscope observation (Table 1.). RP 009 showed the fastest growth of all and gets the highest cell density under standard GY media, followed by RP 010 and RP 011 respectively (data not shown). Gram-positive bacteria were reported as one of the strongest hydrogen producer microbes, with a high hydrogen production rate as well as high hydrogen yield per used substrate [18]. These three isolates were then subjected to hydrogen production experiments using cheese whey as the fermentation substrate.
Figure 1. Microscopic view (1000x Magnification) of three hydrogen-producing bacteria from Mount Pancar hot spring. (A) RP 011; (B) RP 010; (C) RP 009.

3.2. Hydrogen Production with Various Cheese Whey Concentration

The concentration of cheese whey is fundamental for the hydrogen production from all the three isolates of the Mount Pancar hot spring. Figure 2 showed the biogas productivity of Cheese whey concentrations as well as bacterial isolates showed significant effects for the biogas production (Two-way ANOVA test). Cheese whey 30% had the lowest biogas productivity in almost all isolates (except RP 009) and statistically showed significant difference with the other concentrations (p-value < 0.05, Two-way ANOVA test). Thus, it was agreed that cheese whey 30% was not the best condition for biohydrogen production. On the other hand, cheese whey 100% and 60% showed high hydrogen productivity in almost all isolates (except RP 009), and cheese whey 100% represented the highest biogas production (RP 011). However, there were no significant differences statistically between cheese whey 100% and 60% (p-value > 0.05, Two-way ANOVA test; Tukey HSD’s test). With no significant differences between cheese whey 100% and 60%, it was confirmed that dilution of cheese whey into the concentration of 60% was the most favorable for biohydrogen production considering the effectiveness and efficiency of substrate conversion into biogas.

Table 1. Characteristics of the hydrogen-producing bacteria from Mount Pancar hot spring.

| Sample   | Gram Test | Bacterial Morphology | Colony on Agar |
|----------|-----------|----------------------|----------------|
|          |           |                      | Form           | Margin  | Opacity  | Pigmentation  |
| RP 009   | +         | Rod                  | Irregular      | Filamentous | Opaque | White         |
| RP 010   | +         | Long Rod             | Irregular      | Undulate  | Opaque | White         |
| RP 011   | +         | Short Rod            | Circular       | Entire    | Transparent | Shiny White |

RP 011 showed the highest biogas production (in cheese whey 100%) with biogas production 4400.625 ml biogas/L medium, as shown in Figure 2. However, it was not statistically different with RP 010 (p-value > 0.05, Two-way ANOVA test; Tukey HSD’s test), but had a significant difference with RP 009 (p-value < 0.05, Two-way ANOVA test). As a result, RP 011 and RP 010 were categorized as the better hydrogen producers in this experiment with higher hydrogen productivity, compare with RP 009. Nevertheless, the best isolate as the hydrogen producer between RP 010 or RP 011 in this study was able to be determined by the statistical analyses (One-way ANOVA) considering particular parameters, such as biogas rate, biogas yield, and organic compound (COD) reduction, in the most suitable cheese whey concentration (Cheese whey 60%).
3.3. Dark Fermentation Characteristics

All bacteria showed a particular pattern of biogas production in different cheese whey concentrations as shown in Figure 3. All three isolates showed a similar pattern of biogas production in cheese whey 30% and so as the 60%. However, as can be seen in Figure 3A, each isolate showed a different pattern in cheese whey 100%. RP 009 was the lowest biogas producers in which the biogas production increased until the third day, and then started to decrease afterward. Likewise, RP 010 was the highest biogas producers from day 1 to day 4 but starts to significantly decrease thereafter. On the other side, RP 011 had a similar pattern with other isolates showing the increase of biogas production until day 3 and starts to decrease until day 5. However, after the fifth day, RP 011 started to show a significant increase in biogas production where no other isolates had ever experienced. We assumed that day 5 was the critical time for all the bacteria to adapt to environmental tension of high concentrations of the organic compound in cheese whey. This study showed that RP 011 was able to adapt after the fifth day of fermentation and continue to produce biogas. This represented the great potential of this isolate for biohydrogen production using waste containing high concentrations of the organic compound.

Too high concentration of waste placed serious problems for hydrogen production as the process will produce inhibitors in the form of too much VFA accumulation during fermentation. Furthermore, higher concentrations of waste increased the hydrogen pressure in the media as well as the headspace which will significantly affect the growth and the ability of the bacteria on producing the hydrogen gas [19].

Other than that, all isolates showed high productivity in cheese whey 60%. It represented that a proper waste dilution is pivotal to increase the efficiency and effectiveness of biomass conversion into hydrogen gas. Considering those factors, cheese whey 60% was the most proper dilution for hydrogen production in this whole experiment. The comparable study was reported by Wicher et al. (2013) [17] that proper dilution of distillery wastewater could increase the hydrogen production from dark-fermentative bacteria. The proper dilution of bio-waste as the bacterial substrate for hydrogen production was also reported by Anam et al. (2012) [20] in order to optimize the bioproduction of hydrogen gas.

**Figure 2.** Biogas production (ml/L medium) by the hydrogen-producing bacteria (RP 009, RP 010, RP 011) in three concentrations of cheese whey (dotted bar showed cheese whey 100%; filled bar showed cheese whey 60%; blank bar showed cheese whey 30%; error bar showed standard deviation). Biogas evolution was shown as mean ± SD within the duplicate independent experiment.
Figure 3. Average biogas production (dotted line showed biogas per day; plain line showed biogas cumulative) in 7 consecutive days from the hydrogen-producing bacteria (filled diamond showed RP 009; filled rectangle showed RP 010, and filled triangle showed RP 011) in different cheese whey concentrations ((A) cheese whey 100%, (B) cheese whey 60%, and (C) cheese whey 30%).

Each isolate showed similar hydrogen production characteristics at cheese whey 60% (Table 2.). RP 009 showed the highest biogas rate, as well as biogas yield, compared with the other isolates. In fact, it didn’t exhibit any significant difference with the other two isolates (p-value > 0.05; one-way ANOVA test) meaning that RP 009 was not the best hydrogen producers of all. On the other hand, RP 011 showed higher sugar and organic compound (COD) utilization for hydrogen production rather than the other 2 isolates and represented significant difference for organic compound (COD) reduction with p-value < 0.05 (one-way ANOVA test). COD removal was reported as one of the

Table 2. Dark fermentation characteristics of the hydrogen-producing bacterial isolates (cheese whey 60%). The data were presented as mean ± SD from duplicate independent experiments.

| Parameters                  | RP 009       | RP 010       | RP 011       |
|-----------------------------|--------------|--------------|--------------|
| Biogas Rate (ml/L/h)        | 19.29 ± 0.17 | 18.40 ± 0.84 | 17.68 ± 2.55 |
| Biogas Yield (ml/g COD)     | 245.87 ± 31.36 | 212.51 ± 0.32 | 170.28 ± 19.77 |
| H₂ Qualitative Test         | Positive     | Positive     | Positive     |
| Sugar Consumption (%)       | 27.11 ± 1.00 | 28.31 ± 2.09 | 28.79 ± 0.44 |
| Sugar Consumption Rate (g/L/h) | 0.032 ± 0.001 | 0.034 ± 0.003 | 0.034 ± 0.001 |
| COD Reduction (%)           | 31.07 ± 3.69 | 34.03 ± 1.60 | 40.74 ± 1.16 |
| COD Reduction Rate (g/L/h)  | 0.079 ± 0.009 | 0.087 ± 0.004 | 0.104 ± 0.003 |
most important factors in biological waste refinery [10]. Therefore, with high biogas yield and rate as well as high organic compound (COD) reduction, RP 011 was the most favorable isolate as the hydrogen producers, especially on the perspective of bio-waste refinery and its conversion into the biogas.

The hydrogen content from the biogas was not determined in this study. However, qualitatively, all isolates were able to produce hydrogen gas as they showed positive results for hydrogen qualitative test using PEM fuel cell. PEM fuel cell was a technology that is used to generate electricity in the presence of hydrogen gas as well as oxygen gas. Thus, without any hydrogen gas, no electricity will be generated [21]. The dynamo in which was connected with the PEM fuel cell was able to be run after gas injection which literally was indicating the presence of hydrogen gas. Furthermore, according to the literature review, the hydrogen content from the biogas total produced by dark fermentative bacteria was ranging on 30 % [22] to 60% of the biogas total [17]. According to that, our study showed substantial potential results with high hydrogen production in the range of 397.68 ml H2/L medium – 2640.38 ml H2/L medium (data were not shown). It exhibited higher hydrogen production compare with other studies, such as the study of hydrogen production from distillery wastewater using dark-fermentative bacteria presented by Wicher et al. (2013) [17] with the highest hydrogen production was 765 ml H2/L medium. The other study about hydrogen production using Clostridium beijerinckii RZF-1108 described by Zhao et al. (2011) [23] showed comparable results in which the bacteria were able to produce 2209 ml H2/L medium in optimized condition.

4. Conclusions
Cheese whey showed considerable potential as the feedstock for biohydrogen production. However, proper dilution was imperative to increase and optimize the production of biohydrogen. This study showed that cheese whey 60% was the most appropriate dilution to be made in which was considered to the efficiency and effectiveness of the conversion of the biological waste into biohydrogen gas. The bacterial isolates from Mount Pancar hot spring showed a particular pattern of biological hydrogen production in the cheese whey. Among the isolates, RP 011 was considered as the most auspicious hydrogen producers in this study because it showed high biogas rate and yield as well as the highest sugar and organic compound (COD) reduction. This study presented the first report of Indonesian indigenous hydrogen-producing bacteria using cheese whey as the primary substrate which showed extremely high biogas production up to 4200.625 ml biogas/L medium.

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6. References
[1] Cardoso V, Romão B B, Silva F T M, Santos J G, Batista F R X and Ferreira J S 2014 Chem. Eng. Trans. 38 481–6
[2] Chandrasekhar K, Lee Y-J and Lee D-W 2015 Int. J. Mol. Sci. 16 8266–93
[3] Preethi, Usman T M M, Rajesh Banu J, Gunasekaran M and Kumar G 2019 Bioresour. Technol. Reports 7 100287
[4] Rajhi H, Conthe M, Puyol D, Diaz E and Sanz J L 2013 Int. Microbiol. 16 53–62
[5] Jaseena K A and Sosamony K J 2016 IRACST – Eng. Sci. Technol. An Int. J. 6 2250–3498
[6] Han W, Yan Y, Shi Y, Gu J, Tang J and Zhao H 2016 Sci. Rep. 6 1–9
[7] Stephen A J, Archer S A, Orozco R L and Macaskie L E 2017 10 1120–7
[8] Show K Y, Lee D J, Tay J H, Lin C Y and Chang J S 2012 Int. J. Hydrogen Energy 37 15616–31
[9] Pawar S S and Van Niel E W J 2013 Appl. Microbiol. Biotechnol. 97 7999–8009
[10] Carvalho F, Prazeres A R and Rivas J 2013 Sci. Total Environ. 445–446 385–96
[11] Romão B B, Batista F R X, Ferreira J S, Costa H C B, Resende M M and Cardoso V L 2014 Appl. Biochem. Biotechnol. 172 3670–85
[12] Montecchio D, Yuan Y and Malpei F 2018 Int. J. Hydrogen Energy 43 17588–601
[13] Valdez-Vazquez I, Castillo-Rubio L G, Pérez-Rangel M, Sepulveda-Gálvez A and Vargas A 2019 Ind. Crops Prod. 137 105–11
[14] Chookaew T, O-Thong S and Prasertsan P 2012 Int. J. Hydrogen Energy 37 13314–22
[15] Chandra R, Nikhil G N and Venkata Mohan S 2015 Int. J. Mol. Sci. 16 9540–56
[16] Patel S K S and Kalia V C 2013 Indian J. Microbiol. 53 3–10
[17] Wicher E, Seifert K, Zagrodnik R, Pietrzyk B and Laniecki M 2013 Int. J. Hydrogen Energy 38 7767–73
[18] Srivastava N, Srivastava M, Kushwaha D, Gupta V K, Manikanta A, Ramteke P W and Mishra P K 2017 Bioresour. Technol. 238 552–8
[19] Pouresmaeil S, Nosrati M and Ebrahimi S 2019 J. Environ. Chem. Eng. 7 103090
[20] Anam K, Habibi M S, Harwati T U and Susilaningsih D 2012 Int. J. Hydrogen Energy 37 15436–42
[21] Koroglu E O, Ozdemir O K, Ozkaya B and Demir A 2019 Int. J. Hydrogen Energy 44 17297–303
[22] Sun L, Huang A, Gu W, Ma Y, Zhu D and Wang G 2015 Int. J. Hydrogen Energy 40 1402–7
[23] Zhao X, Xing D, Fu N, Liu B and Ren N 2011 Bioresour. Technol. 102 8432–6