Utilization of Acid-Hydrolysed Microalgal Biomass Collected from Eutrophication-Affected Freshwater Pond as a Substrate for Biogas (Biohydrogen) Production by means of Dark- and Photo-Fermentation

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Abstract. Eutrophication-affected waters could be a potential source in providing abundant microalgal biomass which is rich in carbohydrates and can be utilized as a promising substrate for biogas (biohydrogen) production. In the present study, microalgal biomass were collected from the eutrophication-affected freshwater pond and then were treated by acid thermal process. Three dilute acid solution (2.25%) were used as hydrolytic agent, namely sulphuric-, hydrochloric- and nitric acid. Alternate biogas production first by anaerobic bacteria and second by Rhodobium marinum were conducted to convert microalgal biomass into clean energy in the form of biogas (biohydrogen). At first stage, dark-fermentation was carried out by anaerobic bacteria to decompose macromolecular organic matter contained in the microalgal slurry or hydrolysate into organic acids. At second stage, photo-fermentative bacteria, Rhodobium marinum, will utilize organic acids and monosaccharides in the fermented liquid from the first stage to produce (hydrogen) gas. The highest value of biogas evolution (426,88±26,88 mL/L) and biogas yield (839,93±49,41 mL/g COD) was achieved when sulphuric-acid hydrolysate was used as substrate.

1. Introduction

Human activities in many fields could bring serious impacts to the environment surroundings. Environmental issue related to greenhouse gas or carbon dioxide (CO₂) emission could cause some serious global problems in many aspects [1], [2]. On the other hand, agricultural and also husbandry wastes spill out directly to the environment could cause the increasing of nutrients in many waters, deteriorating quality and limiting use for drinking, recreation and food production. Higher nutrient concentrations (eutrophication) have increased algal biomass in lakes by approximately 60% and could cause algal blooming incident [3].

The high accumulation of algal biomass in waters could cause several serious effects, including change the colour, smell and tastes of drinking water [4]. Other effects of algal blooming include reduced species diversity, food chain disturbances and also habitat loss, as well as the decline in the aesthetic and scenic values of an ecosystem [5]. Algal blooms have become global epidemic and the outbreaks has changed considerably over the last several decades [6], [7]. Recently, countries including the USA, Canada, Greece, Norway, Spain, Portugal, Ireland, China, Japan, and Korea have invested considerable funds and effort to manage (prevent, control and mitigate) algal blooming [8].
In contrast, algal biomass provides huge potential as a potent bioresource at which diverting the algal blooming biomass to other value added applications is very promising. Algal biomass contains primary metabolites, such as carbohydrate which is potential to be utilized as a feedstock for various applications, including bioenergy production [9]. One of energy form which is regarded as a future energy economy is hydrogen or biohydrogen [10]. Biohydrogen is the only carbon-free fuel produced biologically, with water as its final combustion product. Therefore, the production of biohydrogen will significantly contribute to the reduction of energy-related environmental problems, such as greenhouse gas emission and algal blooming [11]. This research was aiming at utilizing microalgal biomass which was acquired from eutrophication-affected freshwater ponds as a substrate or feedstock to produce biogas (biohydrogen) by means of dark- (anaerobic bacteria) and photo-(photosynthetic bacteria) fermentation.

2. Methods

2.1. Sampling Site
The microalgal biomass was obtained from freshwater pond which is affected by eutrophication occurrence. Freshwater pond was located at the sub-district of Cimandala, Bogor, West Java. The GPS coordinate of the sampling site is 6°31'23.988" S; 106°49'28.344" E. In situ measurements of pH and temperature (°C) from a depth of 0.5 m were made at the time of water sampling using universal indicator and thermometer, respectively.

2.2. Microalgal Biomass Collection
Water samples were collected from a depth of 0.5 m at onetime sampling moment. Water samples were directly brought to the laboratory and the microalgal biomass were concentrated by centrifugation at 6000 rpm for 5 minutes [12]. Wet microalgal biomass was obtained to be subsequently treated by thermal-acidic hydrolysis.

2.3. Acidic Hydrolysis
Three solution of acid (H₂SO₄, HCl and HNO₃) in the concentration of 2.25% were used as hydrolysis catalytic agent. Each of acidic solutions (10 mL) were added to the microalgal biomass (250 mg in dried weight) placed in the 50 mL of glass tube, then it was autoclaved at 121°C for 15 minutes [13]. The acidic hydrolysate was centrifuged to separate the biomass residue and the supernatant in which soluble-carbohydrate fraction was contained. Furthermore, the supernatant of acidic hydrolysate would be analysed for its total sugar content and Chemical Oxygen Demand (COD) parameter.

2.4. Fermentation Conditions and Biogas (Biohydrogen) Production
The biogas production was conducted by means of two-stage fermentations, dark- and photo-fermentation. The both cultures were grown anaerobically by transferring the microbial culture broth from growth medium into the production medium which contains acidic hydrolysates. In dark fermentation process (1st step), anaerobic bacteria of RP010 isolated from Mount Pancar Hot Spring was employed as biological agent to produce biogas (biohydrogen). In photo-fermentation process (2nd step), Rhodobium marinum was used to utilize the fermentate produced from dark fermentation process to produce biogas (biohydrogen) [14].

The anaerobic bacteria of RP010 were grown anaerobically by transferring the single colony from ASY agar media into GY medium. Rhodobium marinum were grown anaerobically in the ASY broth medium. ASY Medium was prepared by weighing its components which was comprised of ammonium sulfate (1.35 g/L), disodium succinate (2.75 g/L), yeast extract (1 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 autoclaving. GY medium was contained of glucose (10 g/L), yeast extract (1 g/L), and 10 ml modified basal medium.

The grown cultures were eventually re-transferred into each growth medium (GY medium for anaerobic bacteria and ASY medium for \textit{R. marinum}) to ensure the cultures were in the best condition for biogas production. The batch fermentation process was performed by inoculating the 24-hour cultures from growth medium into serum bottle 125 ml containing acidic-treated hydrolysates with total working volume of 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 rubber stopper and capped with aluminum seal. The batch fermentation was run for 7 consecutive days and the produced gas was collected every 24 hours. All batch fermentation procedures were performed at room temperature (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:

\[ \text{Biogas Total in L Medium} = \frac{\text{Biogas Total in the actual experiment}}{\text{ml Medium in the actual experiment}} \times 1000 \]

2.5. Analytical Measurements

The evolved gas was periodically measured by using a glass tight gas syringe. The produced hydrogen was analyzed qualitatively by injecting the gas into the PEM fuel cell (Horizon Fuel Cell Technologies). Total sugar and organic compound (COD) were determined using phenol-sulphuric acid methods [15] and dichromate spectrophotometric methods [16], respectively. The measurements of total sugar and COD were conducted before and after the fermentation process occurred. Glucose with concentration 10, 20, 30, 40, 50, 60, 70, and 80 ppm was used to build standard curve for total sugar examination. Potassium hydrogen phthalate with concentration 0, 200, 400, 600, 800, and 1000 ppm was used to create standard curve for total organic compound (COD) examination. Dry cell weight of anaerobic bacteria and of \textit{Rhodobium marinum} were analyzed gravimetrically.

2.6. Statistical Analysis

The effects of acidic hydrolysate as a substrate for biogas production was analyzed statistically by using ANOVA (One-way and Two-way). The data were presented in mean ± standard deviation. The software of Microsoft Excel was employed for operating statistical analysis.

3. Results and Discussions

3.1. Sampling Site and Microalgal Biomass

The site of freshwater pond was located in the sub-district of Cimandala, Bogor, West Java. Visually, the pond looked very green, meaning that it dense with microalgal cells. The dense of green color could be caused by the high concentration of nutrients (eutrophication) come from fish feed. The species of microalgae occupied the pond water was suspected to be originated from Chlorophyte familia. Most species of Chlorophytes known to be mainly composed of carbohydrates, besides protein and lipid [17][18]. Carbohydrates in microalgae are present as both cell wall components (generally cellulose and soluble hemicellulose) and plastids (mainly in the form of starch) [17]. Glucose was found as the predominant monosaccharides in the microalgal biomass, which accounts for more than 70% of total sugars [19]. Therefore, collected microalgal biomass were expected to be suitable to be utilized as substrate in anaerobical fermentation, as well as in photofermentative process, in order to produce biogas (biohydrogen).

3.2. Acidic Hydrolysis

Acidic hydrolysis was performed in autoclave using temperature 121°C for 15 minutes by using three dilute acidic solutions (H$_2$SO$_4$, HCl, HNO$_3$) at the concentration of 2.25\%. According to Silva and Bertucco [20], the temperature of 120°C is considered as the best temperature for acidic hydrolysis of
microalgal biomass. Our previous study (unpublished research) showed that temperature was the most significant parameter which determined the success of acidic hydrolysis processes. On the other hand, our previous study also showed that the best concentration for acidic solution to be used in hydrolysis process was 2.25%. Therefore, in this study, we used 2.25% as acid solution concentration when performing microalgal biomass hydrolysis. In Table 1, HNO$_3$ showed best performance in hydrolysing microalgal biomass, so the hydrolysed products, namely monosaccharides, could be obtained and be subsequently analysed by using phenol-sulphuric acid method [15]. Higher concentration of sugar solubilized in the hydrolysed-product solution was expected to be able in providing substrate for later fermentation using anaerobic bacteria to produce biogas (biohydrogen). The result in this study recommends that nitric acid is the best acid among others to be used as hydrolytic agent to obtain sugar from microalgal biomass. However, Wang et al. [21] used sulphuric acid in their research to hydrolyse microalgal biomass to obtain sugar for butanol production. Sulphuric acid was also employed by Silva et al. [19] to hydrolyse consortium microalgal biomass to obtain sugar for bioethanol production. The difference between the result of this study and the others in term of the used of acid solution type may be caused by the difference of microalgal biomass characteristics that were used as the substrate.

**Table 1. Initial total sugar and COD concentration of three acid-hydrolysates**

| Acid Treatments          | Total Sugar Concentration (ppm) | COD(mg COD/L) |
|--------------------------|---------------------------------|---------------|
| Sulphuric Acid (H$_2$SO$_4$) | 1472.79 ± 74.26                | 2589.60 ± 230.00 |
| Chloric Acid (HCl)       | 1394.82 ± 3.71                  | 3371.60 ± 92.00 |
| Nitric Acid (HNO$_3$)    | 1804.72 ± 36.39                 | 2579.60 ± 20.00 |

3.3. Dark Fermentation for Biogas Production

Following the hydrolysis process, dark fermentation was conducted by employing anaerobic bacteria in such a way that the closed fermentation system was applied so the gas produced were trapped and collected by using syringe for further volume measurement. Dark-fermentative gas (hydrogen) production by strictly anaerobic or facultative anaerobic bacteria under anaerobic conditions is considered as the practical approach for gas (hydrogen) production [22], [23]. Cumulative gas (hydrogen) production (CGP) during dark fermentation is shown in Table 2. The highest gas production by means of dark fermentation was achieved when using sulphuric acid microalgal-hydrolysate as a substrate. Compared to two other acid hydrolysates, the sulphuric acid hydrolysate was the moderate one in containing sugar, but seemingly it could provide better nutrient, than the nitric acid one, for RP-010 anaerobic bacteria so that the bacteria could produce biogas at the highest volume for 7 days of fermentation time. Prolonging the fermentation time did not result in any further

**Table 2. Biogas Evolution and Yield, Hydrogen Qualitative Detection Test and Dry Weight of Microorganism Biomass after Fermentation**

| Stage          | Biological Agent | Acid Hydrolysis | Biogas Evolution (mL/L) | Biogas Yield (mL. gas/g COD) | H$_2$ Qualitative Detection Test | Biomass Dry Weight (mg) |
|----------------|------------------|-----------------|-------------------------|-----------------------------|-------------------------------|-------------------------|
| Dark-fermentation | Anaerobic bacteria | H$_2$SO$_4$ | 426.88±26.88 | 839.93±49.41 | Positive | 20.60 ± 1.10 |
|                | RP010            | HCl            | 351.25 ± 17.50 | 384.17±10.50 | Positive | 14.95 ± 0.95 |
|                |                  | HNO$_3$        | 311.88 ± 28.13 | 503.02±7.86 | Positive | 9.80 ± 1.10 |
| Photo-fermentation | *Rhodobium* marinum | H$_2$SO$_4$ | 0 | - | - | 93.40 ± 5.70 |
|                |                  | HCl            | 0 | - | - | 73.90 ± 3.30 |
|                |                  | HNO$_3$        | 95±17.50 | 101.12±10.64 | Positive | 41.40 ± 1.00 |
gas production. Figure 1 showed that the biogas production by the bacteria was accompanied by the reduction of sugar content and also COD due to the bacteria’s growth and the digestion of nutrients by anaerobic bacteria into simpler compounds.

Along the process of dark fermentation, several organic acids were produced and contained in the fermentate, namely acetic and butyric acids. The presence of such organic acids in the fermentation broth could contribute to the low of gas production in the dark fermentation process [24]. Such result was caused due to the permeation of organic acids into bacterial cell membrane, interfering with the physiological balance in the cell that will eventually leading to the inhibition of cell growth [25]. The inhibition of anaerobic bacteria growth was seemingly negative-proportionate with the biogas evolution as it could be seen in Table 2. The higher the growth inhibition (less dry biomass produced), the less biogas evolution would be, and vice versa. This result was asserted that the biogas evolution by the bacteria cell was occurred during its growth phase. In that case, the optimization of biogas evolution by anaerobic bacteria of RP-010 could be undertaken along with the optimization of bacterial growth. However, the biogas yield in Table 2 demonstrated different result with the biogas evolution. In Table 2, the biogas yield value demonstrated that anaerobic bacteria of RP-010 converted the substrate more efficient when culturing in the hydrolysate of nitric acid compared to that of hydrochloric acid, but the most efficient one was when the bacteria culturing in the sulphuric acid hydrolysate. Such results hinted that the organic content in each hydrolysate was different, thus, the bacteria would also behave in different way in each hydrolysate regarding its growth and its ability to produce biogas.

**Figure 1.** Comparison of sugar total reduction, COD reduction and biogas evolution (mL) per liter fermentation volume under anaerobic/dark fermentation by RP-010

### 3.4. Photo Fermentation for Biogas Production

The fermentate produced by dark fermentation was subsequently utilized as a substrate in the next process of photo-fermentation using biological agent of purple non-sulfur (PNS) photosynthetic bacteria, *Rhodobium marinum*. PNS photosynthetic bacteria are capable of producing gas (hydrogen) and CO2 using organic acids as substrate in the presence of light under anaerobic conditions [26]. Dark-fermentation was conducted prior to photo-fermentation due to the capacity of anaerobic bacteria in digesting complex compounds extracted from microalgal biomass into organic acids so it can be harnessed by *R. marinum* in later step of photo-fermentation to produce biogas (biohydrogen) [27]. By
means of this way, it was expected that the biogas production would be more optimum rather than using one step only. But, apparently, the biogas production in photo-fermentation step was not optimum as it was expected. In this research, the fermentate of nitric acid was the only one source that could be employed by \textit{R. marinus} to produce biogas, while the other two fermentates tended to more provide nutrients for growing instead of producing biogas. The high reduction of sugar and COD (Figure 2) in the fermentate of sulphuric and hydrochloric acid was seemingly aimed at growing rather than at producing biogas. The growth of \textit{R. marinus} in both fermentates was indicated by its colour transformation visually, the purple-red colour of the culture was getting dense during its culture time.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Comparison of sugar total reduction, COD reduction and biogas evolution (mL) per liter fermentation volume under photo-fermentation by \textit{Rhodobium marinus}}
\end{figure}

In total, the biogas (biohydrogen) produced from acid-hydrolysed-microalgal-biomass by means of integrated dark- and photo-fermentation was highest in volume and yield when the fermentation was carried out by using sulphuric-acid-hydrolysate as substrate. At this rate, dark-fermentation employing anaerobic bacteria was the only one process needed. Photo-fermentation was not necessitated due to its biogas absence, thus, the process of biogas production would be more efficient in one step only. However, fermentation using nitric-acid-hydrolysate was not much different in term of biogas evolution compared to the sulphuric-acid one. Even, based on statistical analysis of ANOVA, both biogas evolution (sulphuric- and nitric-acid) was not significantly different.

4. \textbf{Conclusions}

Integrated of dark- and photo-fermentation in employing acid-hydrolysed-microalgal-biomass collected from eutrophication-affected freshwater pond as substrate to produce biogas (biohydrogen) is a promising strategy to utilize unused biomass and to convert it into valuable product, namely bioenergy in the form of biogas (biohydrogen). Sulphuric-acid hydrolysis is the chosen method to obtain substrate from unused microalgal biomass for biogas (biohydrogen) production.

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