Effects of different pre-treatment methods on anaerobic mixed microflora for hydrogen production and COD reduction from domestic effluent

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Abstract. Effects of different pretreatment methods on sludge inoculum were evaluated concerning hydrogen (H₂) production enhancement and COD (chemical oxygen demand) reduction, using domestic effluent in a batch system. The sludge was taken from a recycled line of the activated sludge reactor. Two types of pretreatment were investigated, heat treatment and chloroform treatment. The experiment was conducted at pH 4 - 6 and inoculum sizes of microbes were 10%, 20%, and 30% respectively; and experiment without sludge pretreatment was also conducted as control. The result showed that 30% COD reduction was achieved for chloroform pretreatment at pH 3 and 10% inoculum size. For heat treatment, a maximum COD removal of 60% was achieved in the experiment at pH 6 and 10% inoculum size. In chloroform pretreatment, a maximum volume of gas evolved was 3.6 mL, at pH 3 and 20% inoculum size. For heat pretreatment, maximum biogas evolved was 2.1 mL, at pH 3 and 10% inoculum size. The experimental results showed that the pretreatment methods (heat treatment and chloroform treatment) at 35 °C and initial pH 5.5 had a positive influence on H₂ production yield and COD removal efficiency during the fermentative H₂ production as compared to the control experiments (without pretreatment). Heat treatment method was shown to be a simple and useful method for enhancing both H₂ producing and COD removal processes from domestic effluent with highest H₂ yield and COD removal efficiency at 0.314 mmol H₂/g COD and 86%, respectively.

1. Introduction
Wastewater can be a suitable substrate for economical production of biological hydrogen. Converting waste to a product would reduce waste treatment cost. Domestic wastewater can be biologically treated to produce hydrogen and reduce its pollutant load simultaneously [1]. Amount of domestic wastewater that goes to sewage increases from year to year with the increasing population. Therefore, treatment of sewage wastewater to produce hydrogen seems to be a beneficial spin-off. Sewage wastewater contains a moderate concentration of COD.

Many applications of the treatment are directed towards the removal of organic pollution in wastewater, slurries, and sludge. Anaerobic microorganisms convert organic pollutant to a gas containing methane and carbon dioxide, known as biogas. Anaerobic fermentation and oxidation
processes are used for the treatment of waste sludge and high-strength organic wastes. Anaerobic fermentation processes are advantageous because of the lower biomass yields and source of energy being produced, in the form of methane, or hydrogen. The removal of pollutant and gas production is a result of the biological conversion of organic substrates [2]. Three basic steps are involved in the overall anaerobic oxidation of waste; the first is hydrolysis. Second, fermentation and the third is methanogenesis [3].

Anaerobic mixed fermentation can also be used to produce hydrogen as long as methanogenesis is blocked. The major hydrogen producing microorganisms in anaerobic digestion is Clostridia, Gram-positive, spore-forming, rod-shaped bacteria [4]. Clostridia can form protective spores when they are in a restrictive environment such as high temperature, extreme acidity, and alkalinity, but methanogens have no such capability. Methanogens are also obligative anaerobic archaeobacteria; when they are exposed to an aerobic environment, the oxygen lowers their adenylate charge and causes them to die. Conversely, Clostridia are facultative bacteria that can also grow in the presence of oxygen [5]. Many ways such as acid treatment, heat treatment, alkaline treatment have been demonstrated for turning methane production system into the hydrogen production system because these treatments eliminated most of the methanogens [6].

Chloroform treatment was compared against acid and heat treatments of sewage sludge and methanogenic granules regarding effectiveness to eliminate methanogenic activity [7]. The results showed that chloroform treatment was the most effective among the three methods tested. Acid and heat treatments that were effective for sewage sludge treatment were observed not highly useful for application to granules because of the protection from the granular structure. Acid pretreatment and heat pretreatment have already been proved effective for obtaining hydrogen producing sludge from sewage sludge. Chloroform addition showed the same effectiveness for treating sewage sludge if the concentration was appropriately controlled. The chloroform treatment is also a simple step that can be easily implemented [2].

Chloroform inhibited the production of CH$_4$ from both $H_2$ or CO$_2$ and acetate. CHCl$_3$ also inhibits acetate consumption by sulfate reducers. Furthermore, it was shown that the synthesis of acetate from CO$_2$ by cells of Clostridium thermoaceticum fermenting glucose was inhibited by CHCl$_3$. Chloroform was added into the culture medium at the first batch culture for the chloroform pretreatments [8]. Chloroform showed strong inhibitory effects when added to both granules and sewage sludge culture systems. More than 2.5% of chloroform addition severally inhibited both hydrogen and methane production for both the granule culture and sewage sludge culture. However, the toxicity of chloroform showed a difference between hydrogen-producing bacteria and methanogens at a low level of chloroform addition into the culture medium. Methanogens were so sensitive to the chloroform that only 0.05% addition into the culture medium almost completely inhibited methane production even for granulated methanogens.

Heat treatment has been reported as being more effective than pH control for enhancing biological hydrogen production; however, substantial hydrogen lost occurs during a long time of batch culture due to homoacetogenic activity that consent acetogenesis, especially in most cases where some homoacetogenic bacteria are also spore-forming bacteria. For the continuous fermentation, short hydraulic retention time (HRT) is also a method used to wash out the methanogens because Clostridium grow faster than methanogens. In the heat pretreatment, the sludge or granules was heated in a boiling water bath for a short period (10 to 30 minutes) first, and then cooled down [6].

Methanogenic activity was almost eliminated from sewage sludge culture after 10 minutes heat treatment of sewage sludge. This method has already been proved to be effective and the results of this study confirm that. For granules, on the other hand, methanogen was still very active with massive methane production after the granules were treated by heat for 10 minutes. Longer treatment time was needed to eliminate all methanogenic activity. After 30 minutes heat treatment, granule culture could be able to produce hydrogen instead of methane. Similar reason to acid treatment, the granular structure protected methanogens against the harsh environment, leading to longer time required for heat treatment to eliminate methanogens [6].
Meanwhile, with the decrease of chloroform addition from 2.5% to 0.05%, hydrogen production increased dramatically for both granule and sewage sludge culture. When only 0.1% chloroform added into the culture mediums, hydrogen production was even higher than the sewage sludge control culture. The inhibition of chloroform to hydrogen production also showed a strong correlation to the mass of chloroform added to the culture system. Hydrogen production was inhibited with the chloroform at a treatment level of above 2.5%. Meanwhile, the system was transferred to methane production with no chloroform addition[8]. In addition to eliminating the methanogenic activity for the hydrogen production system, chloroform can also inhibit the activity of hydrogen-producing bacteria, mostly \textit{Clostridia}, but if the concentration was controlled at a low level, chloroform selectively inhibited methanogenic activity while did not affect the hydrogen production activity. It was also reported that chloroform can inhibit the activity of other hydrogen-consuming bacteria such as homoacetogens and acetate-consuming sulfate-reducing bacteria. It might be part of the reason why hydrogen production at 0.1% chloroform addition for sewage sludge culture was even higher than any other sewage sludge from other treatment methods [6].

Chloroform treatment might cause some chloroform pollution, but the concentration required is low (0.05%) without a need for repeated treatment. Chloroform treatment showed many advantages over acid and heat treatments when dealing with granules because granular structure forms a protective environment. Chloroform resulted in more completely methanogens elimination. Furthermore, chloroform also postponed the activity of homoacetogens, another hydrogen-consuming bacteria, while no reports about acid treatment and heat treatment mentioning this possibility. Some homoacetogens are also spore-forming bacteria, which are hardly eliminated by the heat treatment method [9].

This research aimed to investigate the effect of pre-treated sludge, with chloroform treatment and heat treatment, to the bio-hydrogen and total biogas production, and COD removal. Sewage wastewater was treated by anaerobic microflora under a dark condition in a batch reactor[10]. Wastewater was taken from sewage treatment, and sludge was taken from a recycled line of clarifier of the treatment plant. To enhance hydrogen production and to increase pollutant removal, two types of sludge pre-treatment were investigated and compared with the process without pre-treatment (control experiment).

2. Material and Method

Raw wastewater was taken from the tank inlet after screening. pH value of the raw domestic wastewater was 6.4 and COD was 750 mg/l. COD value were measured using the Open Reflux Method [11].

2.1 Inoculum

Sludge was taken from the recycled stream of the activated sludge reactor (clarifiers). pH of the sludge was adjusted to 5, and 1 mg/L of glucose was added to provide an easily metabolized source of carbon. Inoculum production was carried out under dark conditions at 180 rpm.

2.2 Preliminary

Preliminary experiments were conducted to determine the reaction time in the batch reactor based on the volume of total biogas production. Three inoculum sizes were studied, i.e., 10%, 20%, and 30%. The wastewater was put in the 22 mL headspace vials and the working volume was 10 mL. The working pH was pH 5. Nitrogen gas was spurge to create an anaerobic condition. The experiment was run for 7 days. COD values, the volume of biogas and pH were recorded daily.

2.3 Pre-treatment of sludge

Pretreatment with heat (heat pre-treatment), the sludge was heated in a boiling water bath (101°C) for 30 minutes before cooling [12]. While in chloroform pre-treatment, 2.5% chloroform (v/v) was added to the sludge and homogenized.[8]
2.4 Main Experiment

The raw wastewater was put into the vial reactor. The nitrogen sparged to the wastewater, and different sizes of inoculum (sludge) were applied, i.e. 10%, 20%, and 30%. The working pH was pH 4, 5 and 6 and the process was conducted under dark condition. The samples were put in the shaker at 200rpm for 60 hours. The experiment without sludge pretreatment was also conducted as control.[13].

2.5 Analytical Method

The volume of biogas is withdrawn and measured using a syringe. Measurement of COD follows the procedure of Open reflux Method [11]. Hydrogen concentration was measured using a gas chromatograph.

3. Result and Discussion

Volumes of biogas produced during the preliminary experiment are shown in figure 1. It showed that the highest volume of biogas was produced by 10 % inoculums size at day 1 (1.3 mL). For 10 % inoculums of size, it reached the highest volume in the first day, and it decreased continuously until the sixth day, and the volume decreased again on the seventh day. For 20 % inoculums size, the volume of biogas produced increased until the second day before the volume decreased and the volume increased again until reaching the highest volume at day 4 (1.0 mL). Then the biogas volume decreased again on the fifth day, and for the next day, the volume increased until it stops produce biogas at day 7. For 30 % inoculums size, the volume of biogas produced increase until the first day before it cannot produce any biogas on day 2. After that, the biogas volume increased continuously and reaches the peak volume at day 6 (0.4 mL) before it stops produced biogas at day 7. 10 % and 20 % inoculums size efficiently produced biogas than 30 % inoculums size. It caused by low conversion of domestic wastewater to biogas. The volume of total biogas was the highest between the first and second day.

Figure 1 showed the COD value slightly decreased at day one until day three, but it increased at day four and was the highest value at that day. However, for 30 % inoculums size, it showed a different phenomenon at day 6, the COD value increased and decreased thereafter. All the COD value on each day was almost the same for all inoculums size except at day 5 and 6. At day 5, 30 % inoculums size showed a lowest value (208 mg/L) than 10 % and 20 % inoculums size (360 mg/L and 352 mg/L). At day 6, 20 % inoculums size resulted a lowest value of COD (114.24 mg/L) than the others (344 mg/L and 297.92 mg/L).
For 10 % inoculums size, the lowest COD value was at day 3 (80 mg/L) and the highest COD value at day 5 (456 mg/L) where the COD value more than the value of raw wastewater (400 mg/L). For 20 % inoculums size, the lowest COD value was at day 3 (112 mg/L) and the highest COD value at day 4 (408 mg/L). For 30 % inoculums size, the lowest COD value is at day 1 (96 mg/L) and the highest COD value at day 4 (432 mg/L). It showed that at day 4, the COD value for all inoculums sizes higher than COD value of raw wastewater. Based on COD value, the removal was highest at the second and the third day.

COD removal in heat-treatment was higher (8-66%) than without treatment and chloroform pretreatment. Figure 2 showed the COD removal for all samples, with and without pretreatment. At pH 4, 10 % inoculums size without pretreatment showed the highest removal of COD (78 %). 10 % inoculums size with chloroform and heat pretreatment showed the same ability to reduce raw wastewater COD value at pH 3 (58%). Only 20 % and 30 % inoculums size with chloroform pretreatment had a low percentage of COD removal at pH 3. For pH 4, 10 % inoculums size without pretreatment and heat pretreatment showed similar COD removal (60 %). At pH 4, 20 % and 30 % inoculums size with chloroform pretreatment and 30 % inoculums size without pretreatment resulted high COD value.
At pH 5, 10 % inoculums size, heat pretreatment resulted in the highest percentage of COD removal (61%), followed by 10 % inoculums size without pretreatment (55%) and 20 % inoculums size with heat pretreatment (49%). At pH 6, 10 % inoculums size, heat pretreatment showed the highest COD removal (66%) followed by 10 % inoculums size without pretreatment (48%) and 20 % inoculums size heat pretreatment (47%). It was similar to the treatment at pH 5. At pH 5, 30 % inoculums size without pretreatment could not reduce the COD value of raw wastewater.

From the result, 30 % inoculums size with chloroform pretreatment could not reduce COD value for raw wastewater at all pH conditions. 30 % inoculums size without pretreatment also cannot reduce COD value for raw wastewater at pH 4, 5 and 6. It also followed by 20 % inoculums size with chloroform pretreatment at pH 4 and 6. 10 % inoculums size with heat pretreatment showed consistent COD removal and the COD removal ranged from 58 % to 66 %.

Batch experiments performed with the pretreated and selectively enriched anaerobic mixed microflora with domestic sewage wastewater as co-substrates showed after 48 hours fermentation [12], COD value of fermentation with initial pH 5 was about 2600 mg/L. While the same experiment, with initial pH 6 and 7, show the COD value is 1300 mg/L and 1100 mg/L. Domestic sewage wastewater which used as co-substrate had a pH of 7.2, COD of 430 mg/l. The result showed that after fermentation, the COD value of the substrate is high.

Figure 3 showed the volume of the biogas produced by the sample with and without pretreatment. At pH 3, 20 % inoculums size with chloroform produced the highest volume of biogas than the other samples. This sample not only produced the highest volume at pH 3 but also in the entire pH. At pH 3, treatments were done without pretreatment did not produce any biogas in all inoculums size. At pH 4, 10 % inoculums size with heat pretreatment produced the highest volume of biogas followed by 20 % inoculums size with heat pretreatment and 30 % inoculums size with heat pretreatment. The samples with chloroform pretreatment dominated the lowest volumes of biogas.

![Volume of biogas after pretreatment](image)

**Figure 3.** The volume of biogas after pretreatment

Heat pretreatment with 20% inoculums produced the highest volume of biogas at pH five followed by 10 % inoculums with heat pretreatment and 30 % inoculums with heat pretreatment. The lowest volumes of biogas were dominated by the samples with chloroform pretreatment [14]. The entire sample at this pH produces biogas. For pH 6, the highest volume of biogas was produced by 20 % inoculums with heat pretreatment followed by 10 % inoculums with heat pretreatment and 30 %
inoculums with heat pretreatment. 20 % inoculums with heat pretreatment always produced a high volume of biogas at all pH. The samples with chloroform pretreatment dominated the lowest volumes of biogas. Almost all the sample using heat pretreatment produced a high volume of biogas at all pH except at pH 3. 20 % inoculums with chloroform pretreatment produce a less volume of biogas at pH 4 — similar case for 30 % inoculums with chloroform pretreatment. Heat pretreatment produced the highest volume of biogas (2.4 to 3 mL), followed by the process without pretreatment (1.1-2.1 mL), and chloroform pretreatment (0 to 0.3 mL).

Figure 4 showed a concentration of hydrogen for the whole process. In general, the process without pretreatment resulted in a higher concentration of hydrogen compared to the process with pretreatment. The process at pH 6 produced a considerable high hydrogen concentration. Process without pre-treatment at pH gave the best result for the hydrogen concentration. Comparison of the two pretreatments, heat treatment, produces a high concentration of hydrogen [15].

Figure 4. The hydrogen concentration in the produced gas

The similar phenomena were found in the total volume of hydrogen production. At pH 6 [16], hydrogen volume for the process was found to be the highest for the process without pretreatment [13]. The yield of hydrogen was found to be higher in heat treatment. [17], as shown in Table 1.

| pH | Yield (mL gas)/(mg/L removed COD) |
|----|----------------------------------|
| 4  | 0.003 0.000 -                    |
| 5  | 0.027 0.053 -                    |
| 6  | 0.014 - -                         |

| pH | Yield (mL gas)/(mg/L removed COD) |
|----|----------------------------------|
| 4  | 0.045 0.057 0.089                |
| 5  | 0.044 0.062 0.101                |
| 6  | 0.039 0.061 0.314                |

Table 1. Yield of hydrogen
4. Conclusion
Based on COD removal and the total volume of biogas produced, heat pre-treatment showed a better result compared to chloroform pre-treatment and without pre-treatment. The process without pre-treatment produced a higher concentration of hydrogen. The yield of hydrogen production was higher in heat treatment. Yield was range from 0 to 0.313 mL gas/(mg/L) COD.

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