Study on the effect of headspace on biohydrogen production using palm oil mill effluent (POME) via immobilized and suspended growth

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Abstract. The world has been using fossil fuels to generate energy for centuries and has had adverse effects on the environment; hence renewable energy needs to be discovered and developed. Biohydrogen production is renewable energy since it emits no greenhouse gases and may provide clean energy. Therefore, this study aimed to investigate the optimum headspace ratio and biohydrogen production for suspended and immobilized cells using Palm Oil Mill Effluent (POME) as the fermentation substrate, while its anaerobic sludge acted as the inoculum. Five different ratios were investigated, which are 0.2, 0.3, 0.4, 0.5, and 0.6. These are equivalent to working volume (WV) of 80 mL, 70 mL, 60 mL, 50 mL, and 40 mL, respectively. The solution contained 10 % of inoculum and 90 % (v/v) of the feedstock. For immobilized cells, additional of glass beads as carrier material was added into the solution, using the ratio of 1:1 for anaerobic sludge (mL) to support carrier (g). The kinetic study was investigated using a modified Gompertz equation whereby for suspended cells, the best ratio was 0.3, with the highest biohydrogen concentration of 357.6 ppm. Meanwhile, the optimum ratio for the immobilized cell was 0.2, with the highest biohydrogen concentration of 479.3 ppm. Based on the kinetic studies, the kinetic parameters for suspended cells were: \( H_m = 89.8 \) mL, \( R_m = 6.8 \) mL/h, and \( \lambda = 0.1 \) hr. Meanwhile for immobilized cell, the kinetic parameters were: \( H_m = 73.6 \) mL, \( R_m = 6.9 \) mL/h and \( \lambda = 0 \) hr. In conclusion, selecting the suitable headspace ratio could affect the biohydrogen quality and improve the effectiveness of the production rate.

1. Introduction
Human has depended on the use of fossil fuel to generate energy for centuries. New solutions are needed to discover and replace it as fossil fuel caused humungous emission of greenhouse gases. Biohydrogen is a clean source of energy that can be produced by using agricultural waste. Anaerobic sludge from wastewater treatment plants has a high potential to be used as inoculum to produce biohydrogen due to the wide range of hydrogen-producing microorganisms present in it [1].

Headspace is the unoccupied space at the top of a serum bottle. The ratio of headspace is very much related to its working volume. During fermentation, biogas produced will accumulate at the top of the bottle that is capped with a rubber stopper and an aluminum seal. It plays a crucial role in fermentation because this could contribute to the partial pressure inside the bottle. Using a lower
headspace ratio or higher working volume will cause higher partial pressure because the unoccupied space left available for the biogas to accumulate is decreased and limited. The partial pressure will lead to suppression and diverted to the pathway that does not favor biohydrogen production. Its insufficient headspace will favor the ethanol pathway as the end product instead of biohydrogen. Therefore, it is crucial to conduct the experiment with suitable headspace that could produce the optimum yield of biohydrogen.

Several studies have shown that biohydrogen production using the immobilized cell system will have a higher biohydrogen yield than suspended cells [2][3]. The suspended cell does not involve any carrier material during the fermentation process, and the production of biohydrogen will be observed only for a batch. On the other hand, immobilized cells continuously involve several batches of fermentation by keeping the same inoculum as the microbes to attach to the carrier as immobilized cells.

The main objective of this research is to determine the effect of headspace on biohydrogen production using Palm Oil Mill Effluent (POME) anaerobic sludge. Several other objectives studied along with the course of research are (i) to investigate the optimum headspace ratio and the effect on biohydrogen production, (ii) to investigate the relationship between suspended and immobilized cells in biohydrogen production, and (iii) to evaluate the kinetic study of the biohydrogen production using batch profiling based on the headspace ratio that produces the highest biohydrogen concentration.

2. Materials and methodology

2.1. Feedstock and seed sludge

Raw POME was used as the feedstock, while the POME anaerobic sludge was used as the seed sludge for the study. The samples were collected from Hiltop Sdn. Bhd., Bagan Serai, Perak, Malaysia and were preserved and refrigerated at 4°C prior to use in the study to decrease the biological degradation and acidification.

2.2. Head space ratio

In the study, five different ratios were investigated, which are 0.2, 0.3, 0.4, 0.5, and 0.6. These are equivalent to working volume (WV) of 80 mL, 70 mL, 60 mL, 50 mL, and 40 mL, respectively. The solution contained 10% of inoculum and 90% (v/v) of the feedstock. For immobilized cells, additional carrier material was added into the solution, using the ratio of 1:1 for anaerobic sludge (mL) to support carrier (g). The support carrier used in this research was glass beads with uniform size of 3mm.

2.3. Experimental setup and procedures

Dark fermentation was conducted in 100 mL serum bottles with 5 different headspace ratios 0.2, 0.3, 0.4, 0.5 and 0.6 of empty space. In the bottle, 10% of the working volume consist of the inoculum, and 90% of the working volume was the feedstock. The solution was mixed accordingly and then adjusted to pH 6 for each bottle. After adjusting the pH, the bottles were all purged with nitrogen gas for 3-5 minutes to create an anaerobic condition. The bottle was then capped with a rubber stopper and aluminum seal before being placed into the incubator at a mesophilic temperature, 37°C for 24 hours. The immobilized cell had a similar fermentation process as the suspended cell except with the addition of carrier material into the bottles in a ratio of 1:1 of anaerobic sludge volume (mL) to glass beads weight (g) [3]. The fermentation was conducted for three days continuously by keeping the same inoculum where the feedstock was fed with new ones after every 24 hours. After 24 hours fermentation, biogas produced was collected as well as the liquid samples for further analysis. The biogas obtained will then be analyzed using a gas analyzer (GA 5000).

2.4. Analysis of biohydrogen production by using Modified Gompertz Equation
The modified Gompertz equation using Sigma Plot 10.0 (Systat Software Inc., USA) was used to determine the cumulative hydrogen production in this study. Theoretically, the modified Gompertz equation is as follow [4]:

$$H = \frac{H_{\text{max}} \cdot \exp \left( - \exp \left[ R_{\text{max}} \cdot e \cdot H_{\text{max}} (\lambda - t) + 1 \right] \right)}{\rho}$$

Where $H$ is the cumulative hydrogen production (ml), $H_{\text{max}}$ is the maximum hydrogen production (mL), $R_{\text{max}}$ is the maximum hydrogen production rate (mL.h$^{-1}$), $e$ is the Euler's number ($e = 2.73$), $\lambda$ is the lag phase time (h) and $t$ is incubation time (h).

3. Results and discussion

3.1. Biogas and biohydrogen production

Figure 1 shows that headspace 0.3 (WV = 70 mL) produced the highest biogas among the five ratios for suspended cells. The biogas production increased with the increment of working volume, where it started with headspace 0.6 (WV = 40 mL) produced only 80.5 mL of biogas, then increased to its peak, which was 111.50 mL for headspace 0.3 (WV = 70 mL). Then it slowly decreased to 107.3 mL for headspace 0.2 (WV = 80 mL). Nevertheless, the difference between 0.3 and 0.2 was not too noticeable. The high biogas production was most likely due to the greater working volume, which increased the number of bacteria and allowed them to generate more biogas. However, there was a slight reduction for headspace 0.2 since the empty space was restricted. As a result, the biogas was excessively concentrated in the bottle, resulting in decreased biogas production, which may be attributed to hydrogen partial pressure [5].

![Figure 1. Biogas production against headspace ratio for suspended cell](image)

Based on Figure 2, the highest concentration of biohydrogen produced for suspended cells was using working volume 70 mL, equivalent to headspace 0.3, at 357.6 ppm. The second highest was 329.3 ppm, using headspace 0.2 (WV = 80 mL). The lowest concentration of biohydrogen produced was 89.3 ppm with a headspace of 0.6 (WV = 40 mL). The result shows that the concentration of biohydrogen production was parallel to the volume of biogas produced. Thus, the lower the biogas produced, the lower the concentration of biohydrogen contained in the biogas.
On the other hand, for immobilized cells, on the first day, the highest amount of biogas produced was 109.6 mL with headspace 0.2 (WV = 80 mL), followed by headspace 0.3 (WV = 70 mL) that produced 100.6mL of biogas. The least was headspace 0.6 (WV = 40 mL), which only produced 84.1 mL of biogas, as shown in Figure 3. The same trend as for the following days, the amount of biogas for headspace 0.2 still produced the highest volume similar as the day before, while 0.3 increase 8 mL of biogas. On the last batch of the fermentation, the volume of biogas produced has decreased where headspace 0.2 only produced 100 mL of biogas. On the other hand, headspace 0.3 produced a slightly higher amount of biogas at 102 mL. Nevertheless, headspace 0.6 only produced 78 mL of biogas.

The concentration of biohydrogen was parallel with the volume of biogas produced since both showed that headspace ratio 0.2 (WV = 80 mL) had the highest volume of biogas and the highest concentration of biohydrogen of 479.3 ppm at the steady-state data of the biohydrogen performance of the last batch fermentation. The same goes for the one which produced the lowest. Headspace ratio 0.6 (WV = 40 mL) produced the least amount of biogas in terms of volume and the lowest concentration of biohydrogen. By referring to Figure 4, the concentration for headspace ratio 0.6 was only 241 ppm on the first day of fermentation; then, it continuously decreased until the last day of batch fermentation with only contained 142.6 ppm of biohydrogen. This study has identified that even though headspace ratio 0.2 was performed with high working volume (80 mL), higher biohydrogen production were achieved with the immobilized mixed cultures. This finding demonstrates that no hydrogen partial pressure inhibits the immobilized cell system. The immobilized cells secrete extra polymeric
substance (EPS) to produce biofilm capable of minimizing mass transfer resistance and stabilizing hydrogen-producing bacteria on biofilm for good biohydrogen performance [6]. As a result, the microorganisms received an adequate quantity of nutrients and food and were able to tolerate the high working volume of the substrate in the serum bottle. In addition, POME anaerobic sludge is very suitable for dark fermentation and can produce a high concentration of biohydrogen [7]. This biomass contained high carbon content to serve as a good nutrient for the growth of microorganisms [8].

![Figure 4](image.png)

**Figure 4.** Concentration of biohydrogen production against days of repeated batch fermentation for immobilized cells

From the studies, we can conclude that WV 70 mL (headspace 0.3) produces the highest amount of biogas for suspended cell culture, while for the immobilized cell was WV 80 mL (headspace 0.2). There was only a slight difference between these two; hence, the optimum headspace ratio for biogas production ranges from 0.2 to 0.3. Besides that, using immobilized cells, the gas produced was higher than using only suspended cell culture. The study proved that immobilized cells were more effective in producing biohydrogen using dark fermentation, which is similar as claimed by Keskin et al. (2012) [9].

3.2. **Kinetic analysis by using Modified Gompertz equation**

The kinetic study of batch fermentation was carried out for both suspended and immobilized cells using the headspace ratio that produces the maximum yield of biohydrogen. For the suspended cell, the best headspace ratio was 0.3, while for the immobilized cell was 0.2. Based on Figure 5, for suspended cells, the highest volume of biohydrogen production was 89.8 mL. Furthermore, from the Gompertz prediction, the following information of the kinetic parameters was obtained, including $H_m = 89.8$ mL, $R_m = 6.8$ mL/h and $\lambda = 0.1$ hr.

Meanwhile for the immobilised cells kinetic study, it was shown that the highest volume of gas was 76.5 mL (Figure 6). Based on the Gompertz prediction, the highest volume is 73.6 mL. The following information of the kinetic parameters was obtained from the Gompertz prediction, including $H_m = 73.6$ mL, $R_m = 6.9$ mL/h and $\lambda = 0$ hr.

Table 2 shows that using Gompertz modified equations, the total volume anticipated for the suspended cell was 16.2 mL greater than the immobilised cell. The lag phase of the immobilised cell, on the other hand, was shorter than that of the suspended cell, which has no lag phase for the production of biohydrogen to initiate [10]. The results of the immobilised cells demonstrated that the
microorganisms quickly adapted to the environment due to the repeated batch fermentation, despite the fact that the maximal hydrogen production rate is only 73.6 mL, which is slightly lower than that of the suspended cells. This is one of the mathematical structure outcomes of the Gompertz model, but it has little influence on its fit.

![Suspended Cell](image1)

**Figure 5.** Gompertz prediction for suspended cell

![Immobilized Cell](image2)

**Figure 6.** Gompertz prediction for immobilized cell

| Table 2. Modified Gompertz equation parameter values for biohydrogen production |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Samples                        | Initial pH | Final pH | Hm (mL) | Rm (mL/h) | λ (h) |
| Suspended Cell                 | 6          | 5.05     | 89.8    | 6.8        | 0.1   |
| Immobilized Cell               | 6          | 5.01     | 73.6    | 6.9        | 0     |
4. Conclusion
The influence of headspace on biohydrogen production was successfully examined in this study. The maximum performance of biohydrogen production for suspended cells was achieved with a headspace ratio of 0.3 (WV = 70 mL) and a biohydrogen concentration of 357.6 ppm. For immobilised cells, the greatest concentration was obtained with a headspace ratio of 0.2 (WV = 80 mL), with 479.3 ppm at the steady-state data of the biohydrogen performance of the last cycle of repeated batch fermentation. According to the Gompertz prediction, the kinetic parameters for suspended cells were $H_m = 89.8$ mL, $R_m = 6.8$ mL/h, and $= 0.1$ hr. The kinetic parameters for an immobilised cell were $H_m = 73.6$ mL, $R_m = 6.9$ mL/h, and $= 0$ hr. For the comparison based on the different headspaces tested, the immobilised cell is more effective since this growth system can survive even at non favourable condition and produce more biohydrogen than the suspended cell.

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