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THE EFFECT OF *Rhodobacter sphaeroides* CULTIVATION TECHNIQUES FOR HYDROGEN GAS PRODUCTION

Mohd Sahaid Kalil and Ahmad Razi Othman*

Department of Chemical Engineering and Process, Faculty Engineering and Built Environment, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan, Malaysia

*Corresponding Author: ahmadrazi@ukm.edu.my

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Abstract

Rhodobacter sphaeroides NCIMB 8523 is a facultative anaerobic bacterium that can generate hydrogen (H_2) under aerobic and anaerobic conditions. To compare hydrogen production by *R. sphaeroides*, three parameters were chosen in this report. First, hydrogen gas production was compared under aerobic and anaerobic conditions. Second, under the different culture chamber (headspace) condition and finally, the effect of different culture volume towards hydrogen production were evaluated. Gas chromatography with thermal conductivity detector (GC-TCD) analysis was used to measure total H_2 production, approximately 9.09 mL and 3.57 mL of H_2 for anaerobic and aerobic respectively. Different headspace was tested under anaerobic condition and H_2 production was measured. Hydrogen gas production increased proportionally with the increment of the headspace area percentage. Hydrogen production for this test was at 4.18 mL, 6.51 mL, 7.65 mL and 10.74 mL for 50%, 30%, 20% and 0% of headspace area respectively. Different culture volume (250 mL and 500mL) was chosen in H_2 production under anaerobic condition. Results showed that higher H_2 production achieved with 250 mL culture volume with H_2 volume at 10.44 mL and 7.58 mL for 250 mL and 500 mL respectively. Obtained results showed that *R. sphaeroides* NCIMB 8523 produce higher H_2 gas under anaerobic conditions at 0% of headspace with optimum culture volume at 250 mL.

INTRODUCTION

Fossil fuel is a current fuel that used to generate energy worldwide with more than 30% comes from oil itself [1]. Fossil fuel source is predicted to be depleted with an increase in usage as this source considered as non-renewable source [2]. Fossil fuel reservoir is not the only major issue that concerned worldwide. Environmental issues also contributed to fossil fuel concern. Combustion of fossil fuel contributes to the greenhouse gas (CO_2) emission and this amount increasing yearly [3]. Carbon dioxide (CO_2) emission to the atmosphere leads to global warming thus affecting worldwide climate [4]. Due to this effect, a new clean and renewable energy source is needed to replace fossil fuel in the future. Hydrogen is considered a new energy source that have both clean and renewable characteristic [5].

Hydrogen becoming the main attraction due to its high energy yield from its combustion [6]. Hydrogen can be produced via two techniques, through conventional technique and biological technique. The conventional method uses physical chemistry approach to synthesis hydrogen. For example, water electrolysis is considered as the simplest method in hydrogen production [7]. Using metal electrodes, water undergoes an electrochemical reaction that separates oxygen and hydrogen [8]. Steaming is another technique applied in hydrogen production. Almost 50% of world hydrogen demand is produced through the steaming technique [9]. However, this process also contributes to the greenhouse gas emission to the environment due to the combustion of natural gas [10]. A report claims that CO_2 produced from the steaming process is almost the same as the direct combustion of fossil fuel [10]. Besides that, hydrogen production through physico-chemical approach consumes high electricity and require high temperature [11]. Due to these factors, an alternative approach using living microorganism is promoted to produce hydrogen.

Hydrogen produced from microorganisms or known as biohydrogen is first discovered in the 1930s by Gaffron and his coworker using green algae as the source of microorganism [12]. Technology in biohydrogen production grows year by year. Currently, researches on biohydrogen is not only focusing on the type of feeds but also focusing on the type of microorganisms used in this process [13]. One of the areas in biohydrogen study is focusing on the utilization of bacteria in hydrogen production. Several bacteria strain used such as *Escherichia coli*, *Clostridium*

acetobutylicum , *Enterobacter cloacae, Kluyveromyces marxianus and Rhodobacter sp.*[14–16].

Thus, this analysis aims to evaluate the hydrogen production by *Rhodobacter sphaeroides* NCIMB 8523 in aerobic and anaerobic conditions with different reactor headspace and culture volume.

MATERIALS AND METHODS

Materials

All chemicals used in this experiment such as L-Malic acid, Sodium glutamate, KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$ were purchased from Merck (M) Sdn Bhd.

Methods

Source of Bacteria
Rhodobacter sphaeroides NCIMB 8253 was purchased from National Collection of Industrial, Marine and Food Bacteria (NCIMB) Limited, Scotland. Bacteria culture was in lyophilized form and kept in -5ºC chiller. Bacteria stock was prepared fresh upon analysis.

Culture Media
Media used for bacteria growth in this analysis was known as Beibl-Pfennig Modified Medium (BPMM). BPMM composition as listed in gL^{-1}: L-Malic Acid (1.0), Sodium Glutamate (1.8), NaCl (0.4), KH_2PO_4 (0.5), $MgSO_4.7H_2O$ (0.2), $CaCl_2.2H_2O$ (0.05). BPMM media was fortified with 1 mL of trace element solution (**Table 1**). For hydrogen production under aerobic and anaerobic conditions, BPMM media (100ml) was used with slight modification. All compositions for hydrogen production media were the same as growth media except for L-Malic acid and Sodium Glutamate concentration at 2.0 gL^{-1} and 0.36 gL^{-1} respectively.

Table 1. Media component in Beible-Pfenning Modified Medium (BRMM)

Component	Quantity in Liter
HCl (25% v/v)	1 ml
$ZnCl_2$	70 mg
$MnCl_2.4H_2O$	100 mg
H_3BO_3	60 mg
$CoCl_2.6H_2O$	200 mg
$CuCl_2.2H_2O$	20 mg
$NiCl_2.6H_2O$	20 mg
Thiamine	500 mg
Niacin	500 mg
Biotin	100 mg

Hydrogen Gas Percentage Analysis by Gas Chromatography
Gas chromatography equipped with a thermal conductivity detector (TCD) was used in this analysis. Helium was used as a carrier gas with a flow rate of 25 $mLmin^{-1}$. Capillary column with dimension at 2 m length, 2.1 mm for inner diameter, 13X for mol sieve and mesh at 60-80 was used. Temperature for the detector and oven was set at 150ºC and 50ºC respectively. About 1 mL of biogas was injected into Gas chromatography and analyzed under the condition stated above. The percentage value of hydrogen in the biogas sample was recorded.

Effect of Headspace on Hydrogen Production Under Anaerobic Condition
This analysis was meant to assay hydrogen production under different size of reactor headspace. A hundred milliliters (100 mL) serum bottles were used in this analysis. Culture volume at 50 mL (50%), 70 mL (30%), 80 mL (20%) and 100 mL (0%) was chosen. *R. sphaeroides* NCIMB 8523 was cultured in hydrogen production media with respected volume stated above for 168 hours under anaerobic condition. Hydrogen production was measured using gas chromatography analysis.

Effect of Media Volume on Hydrogen Production Under Anaerobic Condition
Blue cap bottle with different volume was chosen for this analysis. Bottle volume at 100 mL, 250 mL and 500 mL was used to measure the effect of media volume on the hydrogen production. *R. sphaeroides* NCIMB 8523 was cultured in hydrogen production media with respected volume stated above for 168 hours under anaerobic condition. Hydrogen production was measured using gas chromatography analysis.

RESULTS AND DISCUSSION

R. sphaeroides are grouped under facultative anaerobic bacteria. Analysis of the hydrogen production under both anaerobic and aerobic conditions was conducted to compare the best condition for *R. sphaeroides* in producing hydrogen. **Table 2** shows a summary of hydrogen production and hydrogen production rate under anaerobic and aerobic conditions by *R. sphaeroides*.

Table 2. Summary of total hydrogen and hydrogen production rate by *R. sphaeroides*

Culture Condition	Hydrogen (mL)	Hydrogen production rate ($H_2mL^{-1}\ H^{-1}$)
Anaerobic	9.09	$5.41x10^{-4}$
Aerobic	3.57	$2.13x10^{-4}$

Figure 1 shows hydrogen production for both conditions increases through time. Incubation under anaerobic conditions showed higher production compared to aerobic conditions. The highest hydrogen volume for anaerobic is 1.82 mL whereas for aerobic condition is 0.91 mL. Total hydrogen produced under both conditions is 9.09 mL and 3.57 mL for anaerobic and aerobic respectively. A comparison between several types of facultative anaerobic showed that the hydrogen production by *R. sphaeroides* is low. *Bacterium.E*, Gram-negative facultative anaerobic bacteria gave more than 10 times higher in hydrogen production when compared to *R. sphaeroides*. This is most probably due to the glucose presence as a carbon source in the fermentation media [17]. The same results also obtained with *Enterobacter aerogens*, where the hydrogen production in this bacterium is 10 times greater when compared with current strain [18]. A comparison between aerobic and anaerobic fermentation result in hydrogen production is higher under the anaerobic condition. This is because, hydrogen production requires Nitrogenase enzyme that utilized nitrogen to ammonia and hydrogen [19]. Nitrogenase activity is proved to be inhibited by the presence of oxygen [20]. Previous report claims that using the same bacteria strain, the production of total H_2 is higher. Approximately 54.37 mL of total hydrogen was recorded

under anaerobic fermentation [21]. The difference is mainly caused by the presence of nitrogen gas used in this experiment. High nitrogen and ammonia (NH^{4+}) concentration is claimed to be effecting hydrogen production in bacteria [22].

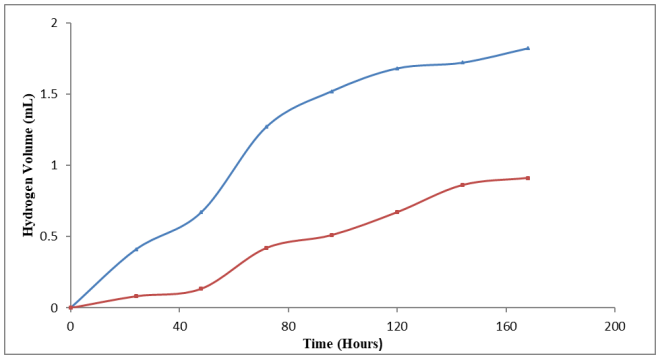

Figure 1. Hydrogen production (mL) by *R. sphaeroides* under anaerobic and aerobic conditions. Marker (▲) represents for anaerobic condition and marker (■) represents anaerobic condition.

Analysis of hydrogen production under different headspace is continued under anaerobic condition. Using a 100 mL serum bottle, the percentage of headspace was determined at 0%, 20%, 30% and 50% respectively. In this analysis, the amount of hydrogen production is compared with the total biogas amount produce. **Figure 2** shows an ascending pattern for biogas production and descending patterns for hydrogen production from 0% to 50% headspace. The lowest biogas produce is at 0%, followed by 20%, 30% and 50% headspace with volume at 20.8 mL, 24.8 mL, 28.9 mL and 39.7 mL respectively. The hydrogen amount showed a reduction from 0% to 50% with the value at 10.74 mL, 7.65 mL, 6.51 mL and 4.18 mL respectively.

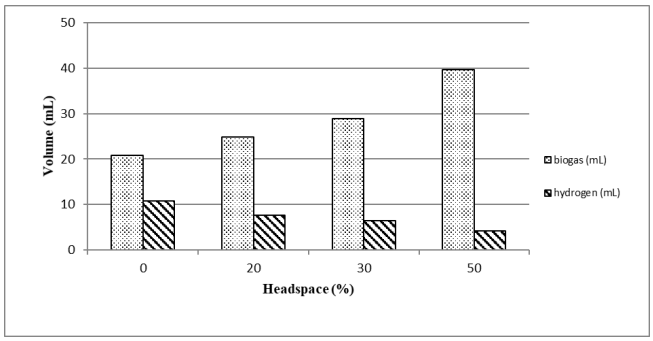

Figure 2. Total biogas production and hydrogen production in different percentage headspace.

Table 3 shows the hydrogen production rate. Data obtained showed that the rate for hydrogen produce increase from 50% to 0% headspace. Rate of H_2 production is recorded as $4.5x10^{-4}$, $5.54x10^{-4}$, $5.7x10^{-4}$ and $6.4x10^{-4}$ for 50%, 30%, 20% and 0% respectively. Even though the hydrogen production rate is highest at 0% headspace, the value is still low when compared with other reports. Report claim that at 0% headspace, the average rate of hydrogen production for several facultative anaerobic was recorded at 212.2 mL/(L.H) [17]. Other claims that the hydrogen production rate by *E.coli* strain ATCC 8739 was at 56.84 mL (L.H) [23]. This difference is due to the low headspace capacity will produce high CO_2 content, thus introduce high pressure to the reactor environment. High pressure was reported previously to inhibit hydrogen production when the pressure set above 1 bar [24]. The hydrogen production rate can be increased by removing CO_2 from the reactor. This technique is reported to increase in hydrogen production by up to 25.48% [25].

Table 3. Rate of hydrogen production by *R. sphaeroides* under different headspace

Percentage Headspace (%)	Total Biogas (mL)	Hydrogen (mL)	H_2 production rate (mL (L.H))
50	39.70	4.18	4.50×10^{-4}
30	28.90	6.51	5.54×10^{-4}
20	24.80	7.65	5.70×10^{-4}
0	20.80	10.74	6.40×10^{-4}

Analysis of hydrogen production under anaerobic conditions is continued with different media volume effect. Three different volumes (100 mL, 250 mL and 500 mL) are chosen for this particular analysis. Total hydrogen produced is compared with total biogas produced under this condition. **Figure 3** shows that the highest biogas production is at 250 mL culture media, followed by 500 mL culture media and 100 mL culture media with the biogas volume at 33.6 mL, 24.8 mL and 20.8 mL respectively. However, a different pattern is observed for the hydrogen production. The highest hydrogen volume produced is given by the 100 mL culture media followed by 250 mL and 500 mL culture media with the value at 10.74 mL, 10.44 mL and 7.58 mL respectively.

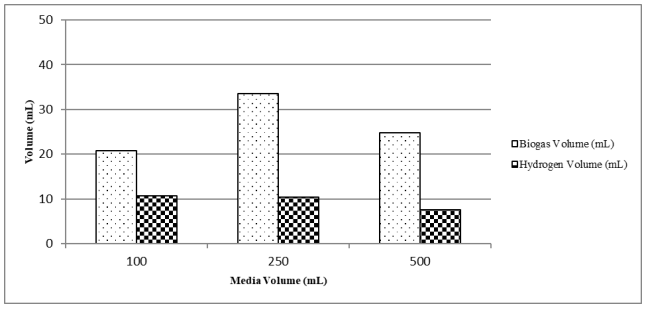

Figure 3. Total biogas production and hydrogen production in different media volume

Table 4 shows the hydrogen production rate under different culture volume. Data obtained showed that the highest hydrogen production rate is at 100 mL culture media, followed by 250 mL ad 500 mL with the rate value at 6.40×10^{-4} mL (L.H), 2.49×10^{-4} mL (L.H) and 9.02×10^{-5} mL (L.H) respectively. The total volume of culture media also can affect the hydrogen production by *R. sphaeroides*. This is proven by the highest hydrogen production rate is at 100 mL culture media when compared to others. The same pattern was observed for *Clostridium butyricum* KBH1where lower culture volume has a high hydrogen production rate compared to higher culture volume [26]. This situation is due to the nature of the biohydrogen process itself, where products from the metabolic

reaction can inhibit hydrogen production [27]. In this situation, the high production of hydrogen leads to the production of acid [28]. In the presence of an acid, pH media will decrease thus effecting the hydrogen synthesis process [28].

Table 4. Data represent the rate of hydrogen production by *R. sphaeroides* under different media volume

Media Volume (mL)	Total Biogas (mL)	Hydrogen (mL)	H_2 production rate (mL (L.H))
100	20.80	10.74	6.40×10^{-4}
250	33.6	10.44	2.49×10^{-4}
500	24.8	7.58	9.02×10^{-5}

CONCLUSION

R. sphaeroides NCIMB 8523 is proved to produce hydrogen better under anaerobic conditions compared to aerobic conditions. Analysis of different headspace conclude that at 0% headspace, *R. sphaeroides* produce the highest hydrogen volume and the highest hydrogen production rate. Comparison with different culture volume indicates that hydrogen production is highest at 100 mL culture volume compared to higher culture volume. This factor is contributed by the feedback inhibition mechanism of the metabolism product.

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