Effects of Different Initial pH, Argon Gas and Nitrogen Gas on Cell Growth and Hydrogen Production using *Rhodobacter sphaeroides*

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ABSTRACT

Hydrogen is considered as a promising alternative fuel and energy carrier by virtue of the fact that it does not evolve the CO₂ in combustion. Some non-sulfur photosynthetic bacteria are potent hydrogen producers, utilizing organic acids or alcohols under specific environmental conditions. Effects of different parameters including different initial pH, use of argon and nitrogen gas on hydrogen production were investigated in detail using *R. sphaeroides* NCIMB 8253 in batch culture. The cultures were grown aerobically and anaerobically and hydrogen production was investigated at different initial pH ranging from 6-11. It was found that initial pH 7 can be designated as optimum pH for maximum cumulative H₂ production of 45.03 mL/g yield per gram of substrate (225.17 mL·g⁻¹) and rate of H₂ production (3.13 mL/L·h) compared to other initial pH within the range of 6-11. Argon gas was found better than nitrogen gas to create more suitable anaerobic conditions for maximum H₂ production using *R. sphaeroides* NCIMB 8253. Culture sparged with argon gas at the beginning produced 45.03 mL H₂ after 144 h incubation; whereas culture sparged with N₂ gas only produced 23.50 mL H₂ after 120 h incubation. The findings reveal that the use of argon gas to create anaerobic environment and the initial pH 7 could favor the enhanced hydrogen production using *Rhodobacter sphaeroides* NCIMB 8253.

Key words: *Rhodobacter sphaeroides*, photofermentation, biohydrogen, biofuel, nitrogen gas, argon gas

INTRODUCTION

Depletion of fossil fuel reserves and emerging environmental problems (Asif and Muneer, 2007) have stimulated the research in the field of new sustainable energy sources that could be environment friendly and a substitute to fossil fuels.

Hydrogen is considered as a promising alternative fuel and energy carrier by virtue of the fact that it does not evolve the CO₂ in combustion. Hydrogen gas is clean fuel with no green house gas emissions and can easily be used in fuel cells for generation of electricity. Hydrogen has a high energy yield of 122 kJ g⁻¹ which is 2.75 times greater than hydrocarbon fuels. Due to increasing need for hydrogen energy, development of cost-effective and efficient hydrogen production technologies has gained significant attention in recent years. Photosynthetic bacteria undergo anoxicogenic photosynthesis with organic compounds or reduced sulfur compounds as electron donors (Truper and Fischer, 1982). Some non-sulfur photosynthetic bacteria are potent hydrogen...
producers, utilizing organic acids or alcohols as electron donors. Since light energy is not required for water oxidation, the efficiency of light energy conversion to hydrogen gas by photosynthetic bacteria is in principle much higher than that by Cyanobacteria. Clostridia such as *C. butyricum* (Yokoi et al., 2001), *C. thermolaeticum* (Collect et al., 2004) and some other anaerobic Clostridia produce hydrogen gas during the exponential growth phase.

Conventional hydrogen production involves non-catalytic partial oxidation of fossil fuels and autothermal reforming which requires high temperature. However, some cost-effective techniques have also been introduced including membrane processes, selective oxidation of methane and oxidative dehydrogenation (Armor, 1999). Biological hydrogen from photosynthetic bacteria has advantages because of the ability of the bacteria to utilize a variety of substrates including waste material to produce H$_2$ with high yield and their ability to act photosynthetically in a wide spectrum of light energy (Fascetti et al., 1998; Koku et al., 2002).

Hydrogen gas production is more environmentally friendly by using biological methods as compared to the chemical methods because the chemical methods may cause some unusual environmental and industrial problems, e.g., production of hydrogen gas from the gasification of biomass comes with many problems such as the existence of unacceptable level of tars and low efficiency of the catalysts because of coke deposition (Alalayah et al., 2010; Misi et al., 2011). Hydrogen can also be generated using *Clostridium saccharoperbutyliceticum* N1-4 from the different raw material including cellulosic biomass or glucose (Alalayah et al., 2009). Hydrogen production by *R. sphaeroides* and some Purple Non Sulfur (PNS) bacteria can be achieved under illumination in the presence of an inert, anaerobic atmosphere, from the breakdown of organic substrates. The culture medium should be under a nitrogen limitation (i.e., a high C/N ratio) which forces the bacteria to 'dump' the excess energy and reducing power by producing H$_2$. Several individual components constitute the overall production system and these may include the enzyme systems, the carbon flow specifically the TCA cycle and the photosynthetic membrane apparatus. These components are interconnected within the H$_2$ production scheme by means of the exchange of electrons, protons and ATP. It can be inferred from the preceding description that for the PNS bacteria, H$_2$ production of any significance occurs under a photoheterotrophic growth mode which is a preferred growth mode for these microorganisms. Yet, PNS bacteria are capable of several alternative metabolic modes such as aerobic or anaerobic respiration, fermentation and photoautotrophy (Koku et al., 2002). The studies revealed that PNS bacteria have been investigated for their potential to convert light energy into H$_2$ using waste organic compounds as substrate (Levin et al., 2004). Among all of the photosynthetic bacteria, *R. sphaeroides* has been studied widely for H$_2$ production (Fang et al., 2006). Fermentation is a process which is highly influenced by different environmental parameters (Emily et al., 2009) and each strain need to be studied for the optimum values required establishing a set of standard conditions for enhanced hydrogen production. Effects of operating parameters including agitation, aeration and light on hydrogen production using *R. sphaeroides* NCIMB 8253 has already been investigated (Jaapar et al., 2009). Effects of age of inoculum, size of inoculum and headspace on hydrogen production using *Rhodobacter sphaeroides* have also been reported by Jaapar et al. (2011).

The batch fermentation is a popular strategy to produce value added products using microorganisms. A lot of enzymes and other value added products can be produced by using bacterial fermentation (Fonseca and Antonio, 2007; Ray, 2011).

The proposed study is a continuity of our previous studies regarding important parameters which may contribute well for hydrogen production from *R. sphaeroides* NCIMB 8253 on being
optimized (Jaapar et al., 2011). Influence of different initial pH, argon gas and nitrogen gas on growth and hydrogen production using *R. sphaeroides* NCIMB 8253 was investigated in detail in batch culture. This study may help to establish a set of parameters to enhance the hydrogen production from *R. sphaeroides* NCIMB 8253 in batch culture.

**MATERIALS AND METHODS**

This study was conducted starting from April 2009 to March 2010 at the Laboratory of Synthetic Biology, Department of Chemical and Process Engineering, University Kebangsaan Malaysia.

**Bacterial strain:** *R. sphaeroides* NCIMB 8253 was obtained from NCIMB Limited Scotland, in freeze-dried form and the activation of the bacterial strains were performed as previously reported, inoculum of 10% *v/v* was used throughout this study except unless stated clearly. The culture conditions, strategic use of bacterial medium, growth conditions, analysis and gas collection procedure were also kept same as reported and discussed in our previous study and discussed in this study to relate the parameters under investigation (Jaapar et al., 2011).

**Culture conditions:** *R. sphaeroides* NCIMB 8253 was grown in modified medium of Biebl and Pfennig. The modified medium of Biebl and Pfennig contains Malic acid (7.5 mM) as the organic carbon source and sodium glutamate (10 mM) as the nitrogen source. Solid agar medium was prepared by using 2% agar Bacteriological No. 1 into 1 L of the modified medium of Biebl and Pfennig. The pH of the medium was adjusted to 6.82 with 1 M sodium hydroxide. The liquid culture medium used for the H₂ production was similar to the growth medium except that the concentrations of malate and glutamate were 15 and 2 mM, respectively. Both medium were sterilized at 121°C for 15 min in the autoclave.

The bacterium was grown anaerobically at 30°C in 100 mL serum bottle containing 100 mL liquid medium under 100 W tungsten lamp (3.0-3.8 klux) or 30 W fluorescent lamp having light intensity of 3.8-4.5 Klux. Argon gas was used to create anaerobic conditions by purging it into the medium. The agar cultures were incubated in anaerobic jar at 30°C with tungsten lamp (100 W) as the light source.

**Hydrogen production:** H₂ gas production was performed in batch culture systems using 100 mL serum bottle containing 100 mL medium using 10% (*v/v*) inoculum. The temperature was adjusted to 30°C under the light of a tungsten lamp (100 W) with light intensity of 3.8 klux. For all H₂ production experiments, the reactor was flushed with pure argon to provide an anaerobic atmosphere to the cultures. After flushing with argon gas, 10% *v/v* inoculum of the pre-activated bacteria (in minimal medium of Biebl and Pfennig) was transferred into the H₂ production medium. During the experiments, the produced gas was collected and measured volumetrically in syringes directly connected to the reaction vessel without any water trap.

**Analysis:** Growth of the culture was monitored by measuring the Optical Density (OD) at 660 nm using a Thermo Spectronic UV-visible spectrophotometer (Model: Genesys 10 UV). Fresh medium was used as reference solution. Cell’s dry weight was obtained by centrifuging 10 mL of cells suspension at 13,000 rpm for 10 min and then the pellet was washed twice with deionized water and dried in an oven at 105°C until constant weight was gained. A relationship of cells dry weight
and OD was obtained by plotting a graph of OD versus dry weight of cells. For Gas Chromatograph (GC) analysis, 1 mL gas sample was taken from collected gas by H₂ fermentation. The H₂ gas produced was analyzed by GC equipped with a Thermal Conductivity Detector (TCD) and packed column (SRI 8610C GC, USA). Helium was used as the carrier gas. Oven and detector temperatures were 50 and 150°C, respectively. Percentage of H₂ in total gas by GC analysis was used to determine the total of H₂ gas collected from each experiment. The pH of the culture medium was measured with Eutech Instruments pH meter (Model: pH 510; pH/mVFC; Cyberscan). Light intensities were measured by a luxmeter (Model: LX-103; Digital Instruments; Lutron).

RESULTS AND DISCUSSION

Growth and pH profile in aerobic and anaerobic photofermentation: Absorbance at 660 nm at specific time intervals was monitored to evaluate the growth of R. sphaeroides NCIMB 8253 in aerobic and anaerobic conditions. It is evident from Fig. 1, the growth of the cells in aerobic culture reached exponential phase faster as compared to the culture in the anaerobic condition. The maximum OD of 1.245 was achieved after 160 h incubation in the aerobic culture and declined on further incubation. The decline of the OD of the culture might be attributed to the cell lysis. In case of the anaerobic culture, the maximum OD of 1.033 was achieved after 240 h incubation with a quite low level growth as compared to aerobic culture. According to the previous study of Can et al. (2006) and Kars et al. (2006), higher cell masses were reported for the non H₂ producing bacterial cells, on the other hand lower cell masses were related to the enhanced H₂ gas production. Therefore, the H₂ production was targeted in anaerobic conditions, as aerobic conditions were unable to produce enough H₂. This phenomenon could be attributed to the total amount of energy and electron spent either to H₂ production or cell components. Comparison of the percentage of H₂, total H₂ produced, yield of H₂ produced per gram of the substrate consumed (YH₂/S) and the rate of H₂ produced from inoculums grown in 24 and 48 h are shown in Table 1. The results demonstrate that the age of inoculum has a strong relation with the healthy growth of the culture and H₂.

![Fig. 1: pH and growth profile of R. sphaeroides NCIMB 8253 in aerobic and anaerobic conditions, (pH-an) pH in anaerobic condition, (pH-a) pH aerobic condition, (ncimb-an) growth in anaerobic condition, (ncimb-a) growth in aerobic condition](image-url)
production. It was found that the culture that was inoculated with 24 h inoculum age produced the highest percentage of $H_2$ (20.76%), highest yields of $H_2$ per gram of the substrate consumed i.e., $Y_{H_2}$s (138.05 mL g${}^{-1}$) and the highest rate of $H_2$ ($9.6 \times 10^{-4}$ L/Lh) compared to the culture grown for 48 h in this experiment. The pH changes during the growth of aerobic and anaerobic conditions of cells were monitored and illustrated in Fig. 1. The initial pH was buffered to 7.0 and was not controlled during the growth in either aerobic or anaerobic experiments. There was no considerable difference in the pH values in aerobic and anaerobic conditions of culture and the pH values varied between 7.0 and 8.54 during the cultivation. These results are in agreement with the previous research reported that during $H_2$ production by $R$. sphaeroides in an aerobic fermentation, a slight decrease in the pH of the medium is observed during the cell growth period but the pH increased with the $H_2$ production (Eroglu et al., 1999).

The bacterial cell concentration was analyzed spectrophotometrically at absorbance 660 nm. Dry cell weight versus OD 660 nm curve was obtained from the samples corresponding to the various points of growth curve. It was established that an optical density of 1.0 at 660 nm corresponds to a cell density of 0.3441 g dry weight per liter of culture or 1 OD$_{660nm}$ is equivalent to 0.3441 g L${}^{-1}$ of cell biomass (Fig. 2). It has been reported that 1 OD$_{660nm}$ is equivalent to 0.6 g L${}^{-1}$ of cell dry weight (Uyar et al., 2007), as they have used the same bacterial strain but cells were dried at 55°C. In present study, the drying was continued until the constant dry weight was achieved at 105°C. Some reports are available to perform drying of cells at 105°C to get the constant dry weight (Chang et al., 2005). Some other scientists have also used 105°C to get the constant dry cell mass (Bratbak and Dundas, 1984).
Table 2: Effect of pH on hydrogen gas production

<table>
<thead>
<tr>
<th>pH</th>
<th>h</th>
<th>Volume of gas (mL)</th>
<th>%H₂</th>
<th>Volume of H₂ (mL)</th>
<th>YieldH₂ (mL g⁻¹)</th>
<th>Rate of H₂ production (mL/L/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>144</td>
<td>45</td>
<td>74.52</td>
<td>33.53</td>
<td>167.67</td>
<td>2.33</td>
</tr>
<tr>
<td>7</td>
<td>144</td>
<td>66</td>
<td>68.23</td>
<td>45.03</td>
<td>225.17</td>
<td>3.13</td>
</tr>
<tr>
<td>8</td>
<td>24</td>
<td>2</td>
<td>94.71</td>
<td>0.69</td>
<td>3.47</td>
<td>0.29</td>
</tr>
<tr>
<td>9</td>
<td>72</td>
<td>30</td>
<td>90.98</td>
<td>11.88</td>
<td>59.38</td>
<td>1.65</td>
</tr>
<tr>
<td>10</td>
<td>168</td>
<td>0</td>
<td>59.68</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>192</td>
<td>0</td>
<td>12.84</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3: Effect of nature of gas to create anaerobic environment on hydrogen production

<table>
<thead>
<tr>
<th>Gas</th>
<th>Total gas (mL)</th>
<th>Total H₂ (mL)</th>
<th>% H₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argon (y)</td>
<td>66.0</td>
<td>45.03</td>
<td>68.22</td>
</tr>
<tr>
<td>Nitrogen (x)</td>
<td>36</td>
<td>23.50</td>
<td>66.28</td>
</tr>
</tbody>
</table>

Equation y = 1.83x y = 1.92x

Effect of initial pH: The effect of initial medium pH on the photoproduction of H₂ from modified Bibel and Pfennig medium was studied at varying pH conditions ranging from 6 to 11 at intervals of 1.0 unit. The experiments were conducted without controlling the pH of medium. The results shown in Table 2 indicate that pH 7 gave the highest cumulative H₂ production of 45.03 mL, yield per gram of substrate (225.17 mL g⁻¹) and rate of H₂ production (3.13 mL/L/h) compared to others initial pH. It shows that initial medium pH 7 was the most suitable pH for the highest cumulative of H₂ volume by *R. sphaeroides* NCIMB 8253, followed by the cultures with initial medium pH 6 and 9 and no gas was produced in the cultures with initial medium pH 10 and 11. The studies regarding pH in batch fermentation to produce hydrogen from food waste also present that maximum hydrogen was produced at pH 7 (Nazlina et al., 2009). Some scientists have reported maximum H₂ production by *R. sphaeroides* O.U.001 at pH 7 using spent media (Nath and Das, 2009). Kim et al. (1982) have reported maximum biomass growth of *R. sphaeroides* KD181 at pH 6.8-7.2 using Sistrom’s media. Present experimental findings are in agreement with the results reported before regarding a slight decrease in pH occurred during the biomass growth and the pH increased during the H₂ production by *R. sphaeroides* O.U.001 in modified Bibel and Pfennig medium with initial pH 7.5. Eroglu et al. (1999) have also described that H₂ production occurred with medium having pH below than 7.5 (Eroglu et al., 1999).

Effect of using argon and nitrogen gas to create anaerobic atmosphere: The anaerobic condition for growth and H₂ production by *R. sphaeroides* can be achieved by sparging the culture with argon or N₂ gas before inoculation. This study investigates the effect of argon and nitrogen gas on H₂ production by *R. sphaeroides* NCIMB 8253. Culture sparged with argon gas at the beginning produced 45.03 mL H₂ after 144 h incubation; whereas culture sparged with N₂ gas only produced 23.50 mL H₂ after 120 h incubation (Table 3). The use of argon gas could double the H₂ production by *R. sphaeroides* as compared to the use of N₂ gas. Koku et al. (2003) have reported that high nitrogen contents in the medium can inhibit H₂ production (Koku et al., 2003). It was observed that the percentage of H₂ in the total gas produced using argon gas was about 5% higher than the culture with nitrogen. H₂ production by anoxicogenic phototrophic bacteria also can be inhibited by NH₄⁺ because it represses the synthesis of key enzyme nitrogenase (Zhu et al., 2001). The presence of NH₄⁺ ion as nitrogen source may reduce the rate of H₂ production and cumulative H₂ production, since the activity of nitrogenase enzyme is hampered (Basak and Das, 2007).
CONCLUSION

The extract of this study is an established set of information regarding the influence of some common and influencing parameters for H₂ production using *R. sphaeroides* NCIMB 8253. Effects of the culture conditions on the H₂ producing efficiency of the *R. sphaeroides* NCIMB 8253 were investigated to establish the optimized values for a maximum level of H₂ production. The parameters studied were including the effect of pH in aerobic and anaerobic photo fermentation. The highest gas production phase was observed by *R. sphaeroides* NCIMB 8253 at initial pH 7 and by using argon gas instead of nitrogen to create the anaerobic atmosphere. *R. sphaeroides* NCIMB 8253 may grow optimally in growth medium as stated in results and discussion. This report may lead to establish the optimized conditions at laboratory scale and industrial scale for the production of maximum H₂ using *R. sphaeroides* NCIMB 8253.

REFERENCES


