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The Effect of Aeration, Agitation and Light on Biohydrogen Production by *Rhodobacter sphaeroides* NCIMB 8253

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Abstract: Photo fermentation is a biological process that can be applied for hydrogen production. The process is environmental friendly which is operated under mild conditions using renewable resources. In order to increase yield of H_2 produced by *Rhodobacter sphaeroides*, some experimental factors that may enhance H_2 production were studied. The effect of operating parameters including agitation, aeration and light on hydrogen production using *R. sphaeroides* NCIMB 8253 was investigated. *Rhodobacter sphaeroides* NCIMB 8253 was grown in 100 mL serum bottle containing growth medium with maliec acid as the sole organic carbon source. The cultures were incubated anaerobically at 30°C with tungsten lamp (100 W) as the light source (3.8 klux) and argon gas was purged for maintaining anaerobic condition. The results show that maximum hydrogen produced was higher (54.37 mL) in static culture with 69.98% of H_2 in the total gas compared with shake culture (11.57 mL) with 57.86% of H_2. By using static culture, H_2 produced was five times higher compared with non-static in both aerobic and anaerobic condition. It was found that growth and H_2 production with fluorescent lamp showed better results than growth and H_2 production with tungsten light.

Key words: *Rhodobacter sphaeroides*, photo fermentation, hydrogen production, anaerobic fermentation

INTRODUCTION

Biological hydrogen production can be carried out through photo- and dark-fermentation processes. Hydrogen production by dark fermentation is relatively well known technology and carried out under anaerobic conditions by certain bacterial species, i.e., Clostridium and Enterobacter. A wide range of substrates from sugar to complex carbohydrates or biomass to waste materials, i.e., domestic or agricultural residues and wastewater can be used for H_2 production using dark fermentation. However, H_2 evolution by dark-fermentation has been treated with little attention, while hydrogen evolution by photosynthetic microorganisms has been extensively studied. Photo-fermentation is performed by anaerobic, photoheterothrophic bacteria like Rhodobacter or Rhodopseudomonas in the presence of light by using organic acids, i.e., acetic and butyric acids, as substrate for hydrogen production (Kapdan *et al*., 2009).

Photosynthetic bacteria are favorable for biological hydrogen production due to their high conversion efficiency and versatility in the substrates they can utilize (Koku *et al*., 2002). This is mainly because of their high theoretical conversion yields, lack of O_2-evolving activity which causes problems of O_2 inactivation of different biological systems, ability to use wide spectral light energy and, ability to consume organic substrates from wastes (Fascetti *et al*., 1998). Photosynthetic bacteria can produce hydrogen at the expense of solar energy and small-chain organic acids as electron donors. The conversion efficiency of light energy to hydrogen, with the supply of an appropriate carbon source, is the key factor for hydrogen production by biological systems. Photosynthetic bacteria produce hydrogen from organic compounds by an anaerobic light-dependent electron transfer process (Barbosa *et al*., 2001).

Two enzymes namely nitrogenase and hydrogenase play an important role for biohydrogen production in these bacteria. Photo fermentation by Purple Non-Sulfur (PNS) bacteria is a major field of research through which the overall yield for biological hydrogen production can be improved significantly by optimization of growth conditions and immobilization of active cells (Basak and Das, 2007). *Rhodobacter sphaeroides* NCIMB 8253 used in this study is a PNS bacteria which is capable of producing molecular hydrogen under anaerobic

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conditions by photofermentation of organic acids. PNS bacteria evolve molecular H_2 catalyzed by nitrogenase under nitrogen-deficient conditions using light energy and reduced compounds (organic acids). Basak and Das (2007) reported that the overall biochemical pathways for the photo fermentation process can be expressed as follows (Das and Iu, 2001). Ferredoxin (Fd) acts as electron carrier in presence of nitrogenase in the cell membrane:

$$(CH_2O)_n \rightarrow \underset{\uparrow}{\text{Ferredoxin}} \rightarrow \underset{\uparrow}{\text{Nitrogenase}} \rightarrow H_2 \quad \begin{matrix} \uparrow & \uparrow \\ \text{ATP} & \text{ATP} \end{matrix} \qquad (1)$$

The nitrogenase enzyme also catalyses the evolution of H_2, particularly in the absence of N_2. Akkerman *et al.* (2002) showed that the overall reaction for conversion of the organic substrate (acetate at the example below) into hydrogen demands energy and this is obtained from light.

$$C_2H_4O_2liq + 2H_2Oliq + \text{"light energy"} \rightarrow 2CO_2gas + 4H_2gas \ (\Delta G^0 = 75.2 \text{ kJ}) \qquad (2)$$

The nitrogenase enzyme is also highly sensitive to oxygen, inhibited by ammonium ions or inhibited at high N/C ratio. Therefore, the process requires ammonium limited and oxygen free conditions (Kapdan and Kargi, 2006). This explains why bioreactors must usually operate under anaerobic conditions free of N_2, with illumination and limiting concentrations of nitrogen sources. Matsunaga *et al.* (2000) reported that the theoretical value of H_2 production was calculated from:

$$C_4H_6O_5 + 3H_2O + 5NAD^+ + FAD^+ \rightarrow 4CO2 + 5NADH + FADH_2 + 5H^+ \qquad (3)$$

and 6 mol of molecular hydrogen can be produced per mole of L-malate under anaerobic conditions. Therefore, maximum hydrogen production from 15 mmol of L-malate is 90 mmol. Small amounts of L-malate are utilized by respiration. One mole of L-malate is necessary for the uptake of 3 mol O_2 to produce 36 mol of ATP:

$$C_4H_6O_5 + 3O_2 + 36ADP + Pi \ \ 4CO_2 + 3H_2O + 36ATP \qquad (4)$$

These bacteria use enzyme nitrogenase to catalyze nitrogen fixation for reduction of molecular nitrogen to ammonia (Akkerman *et al.*, 2002):

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i \qquad (5)$$

Nitrogenase has interesting property that it can evolve hydrogen simultaneously with nitrogen reduction and it is the key enzyme that catalyzes hydrogen gas production by photosynthetic bacteria (Kapdan and Kargi, 2006). Stressful concentrations of nitrogen are therefore required for H_2 evolution (Gadhamshetty *et al.*, 2008).

Unlike nitrogenase, hydrogenase enzyme in photo-fermentative bacteria is an uptake hydrogenase which utilizes H_2 gas and therefore is antagonistic to nitrogenase activity (Kapdan and Kargi, 2006). This enzyme was able to take up H_2 at low partial pressures, reducing a relatively high potential electron acceptor (at the level of the NAD/NADH couple or even FAD/FADH), but producing little or no measurable hydrogen (Das and Iu, 2001). Uptake hydrogenase activity should be limited for enhanced H_2 gas production (Kapdan and Kargi, 2006). These photo heterotrophic 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 species of photosynthetic bacterium, *R. sphaeroides* (formerly known as *Rhodopseudomonas sphaeroides*) has been studied most widely for hydrogen production (Fang *et al.*, 2006). In this study we are reporting the effect of operational parameters that will affect the growth and hydrogen production by *R. sphaeroides* NCIMB 8253 including aeration, agitation and light.

MATERIALS AND METHODS

Experimental site: This research project was conducted at the Biotechnology Pilot Plant Lab., Department of Chemical and Process Engineering, Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia and was carried from February 2007 until May 2009.

Microorganism: *Rhodopseudomonas sphaeroides* NCIMB 8253 was obtained from NCIMB Limited, Scotland in freeze-dried culture form. The bacteria were activated by rehydration of dried cultures with a sterile liquid rich medium (medium 27) and transferred at least 3 times until they were activated. Activated cultures were transferred continuously into new growth medium which was modified Biebl and Pfennig medium (Eroglu *et al.*, 1999; Koku *et al.*, 2003) aerobically to keep them active. For long term reserved the active cultures were keep in glycerol stock (-29°C) and for short term they were keep in agar plate (4°C). For H_2 production experiment, the bacteria were activated anaerobically. After 72 h incubation at 30°C (anaerobic), the culture was transferred

into modified Biebl and Pfennig growth medium and incubated for 48 h at 30°C. Then culture was used to inoculate the hydrogen production medium. The amount of 10% v/v inoculum was used throughout this project except otherwise stated.

Culture condition: Growth of *R. sphaeroides* NCIMB 8253 was carried out in modified medium of Biebl and Pfennig. The modified medium of Biebl and Pfennig contains Maliec acid (7.5 mM) as the organic carbon source and sodium glutamate (10 mM) as the nitrogen source (Koku *et al*., 2003). Solid agar medium was prepared by adding 20 g agar Bacteriological No. 1 into 1 L modified medium of Biebl and Pfennig. The pH of the medium was adjusted to 6.82 with 1 M sodium hydroxide solution. The liquid culture medium used for the hydrogen production was similar to the growth medium except that the concentrations of malate and glutamate were 15 and 2 mM, respectively (Koku *et al*., 2003). Both medium were sterilized at 121°C for 15 min in an autoclave before being used.

The bacterium was grown in liquid medium anaerobically at 30°C with 100 W tungsten lamp (3.0 -3.8 kLux) or 30 W fluorescent lamp (4 lamps) with light intensity of 3.8-4.5 klux. Argon or nitrogen gas was used to create anaerobic conditions (Kalil *et al*., 2003; Dogrusoz *et al*., 2004). The bacterium that was grown in modified of Biebl and Pfennig medium agar was incubated in anaerobic jar with light intensity of 3.0-3.8 klux at 30°C with tungsten lamp (100 W) as the light source.

Hydrogen fermentation: Hydrogen gas production experiments were done in batch culture systems using 100 mL serum bottle containing 100 mL medium with 10% v/v inoculum. The temperature was maintained at 30°C under the illumination of a tungsten lamp (100 W) with light intensity of 3.8 klux. For all hydrogen production experiments, the reactor was flushed with pure argon in order to create an anaerobic atmosphere. After flushing with argon, 10% v/v inoculum of the pre-activated bacteria (in minimal medium of Biebl and Pfennig) was transferred into the hydrogen production medium. During the experiments, the evolved gas was collected and measured volumetrically in a measuring cylinder (Fig. 1) using method modified from Pang (2005) which was classical quantitative method (Standard Method-American Public Health Association, American Water Works Association and Water Environment Federation, 1989).

Analysis: Growth of the culture in liquid medium was monitored by measuring the Optical Density (OD) at 660 nm using a Thermo Spectronic UV-visible spectrophotometer (Model: Genesys 10 UV). A relationship of cells dry weight and OD was obtained by plotting a graph of OD versus cell dry weight. It was found that an optical density of 1.0 at 660 nm corresponded to a cell density of 0.341 g dry weight per liter culture.

The volume fraction of H_2 in the gas produced was analyzed using a SRI 8610C GC, USA series Gas Chromatograph (GC) equipped with a packed column (length 2 m, internal diameter 2.1 mm, mol sieve 13X, 60-80 mesh) and a thermal conductivity detector (TCD). Helium was used as the carrier gas at a flow rate of 25 mL min^{-1}. Oven and detector temperatures were 50 and 150°C, respectively. A volume of 1 mL gas samples were taken using 1 mL gas tight syringe and the samples were injected into the GC immediately. The actual volume of

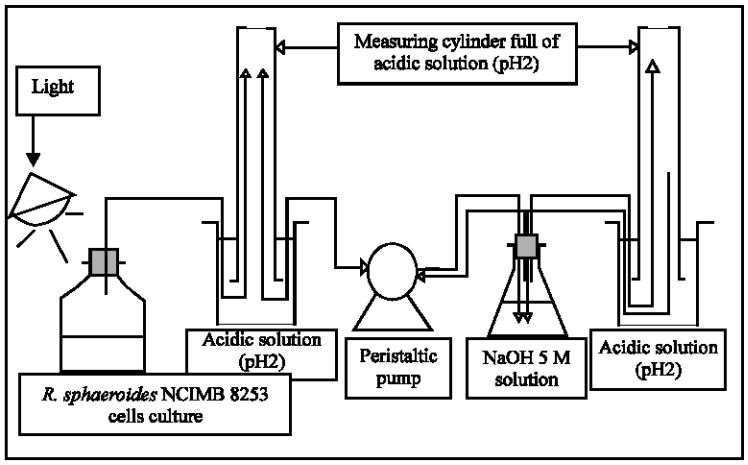

Fig. 1: A schematic diagram of hydrogen gas production by *R. sphaeroides*

hydrogen gas produced was obtained by multiplying the percentage of H_2 gas measured by the GC with the total volume of gas collected.

RESULTS AND DISCUSSION

Effect of agitation: The effect of agitation on H_2 production was studied in two sets of culture, incubated at two different ways, one with shaking and the other without shaking. Results in Table 1 show the percentage of H_2, volume of H_2 and rate of H_2 production in both static and shake culture. More hydrogen is produced in the static culture which is 5 times higher than the amount produced in the shake culture. The maximum amount of hydrogen produced in shake culture was only 11.57 mL while in the static culture was 54.37 mL. However, the time taken for H_2 produced to reach its maximum value was longer than the shake culture. The yield of H_2 produced also higher in the static culture and almost five times higher than shake culture.

Figure 2a and b show the profile of total gas and H_2 produced in both static and shake cultures. In the static culture gas produced increase linearly until 168 h incubation. In the shake culture the rate of gas production reaches stationary phase after 48 of incubation. These results was opposite with what Kim *et al.* (1982) found in his experiment. He reported that the cells had the tendency to flocculate. The flocculation would retard hydrogen production because of the decrease in the efficiency of light absorption. This usually happen in a large scale hydrogen production. Our experiments were carried out in small scale cultures with 100 mL medium and there was no flocculation occurred.

Other explanation on low H_2 produced in the shake culture may be due to stirring will increase the tendency of H_2 to be used in second stage of metabolism to produce other products. Kemavongse and Prasertsan (2008) reported the optimum agitation speed for growth of *Rhodobacter sphaeroides* U7 was 300 rpm while it was 200 rpm for poly-β-hydroxyalkanoate (PHA) production under aerobic condition. Hoekema *et al.* (2002) reported that it is clear that pneumatic agitation at 6.66 l/L/min with nitrogen or argon inhibits bacterial growth and at any flow rate ranging from 0.33 to 6.66 l/L/min, respectively. The extremely low flow rate of 0.33 l/L/min already inhibited growth completely, which made shear stress as an explanation for the absence of growth improbable. Moreover, all bacteria from the Rhodospirillaceae family are contained by a cell wall (Zhu *et al.*, 2001) that offers protection against shear stress. Results show that this operational parameter such as agitation will affect the H_2 production by

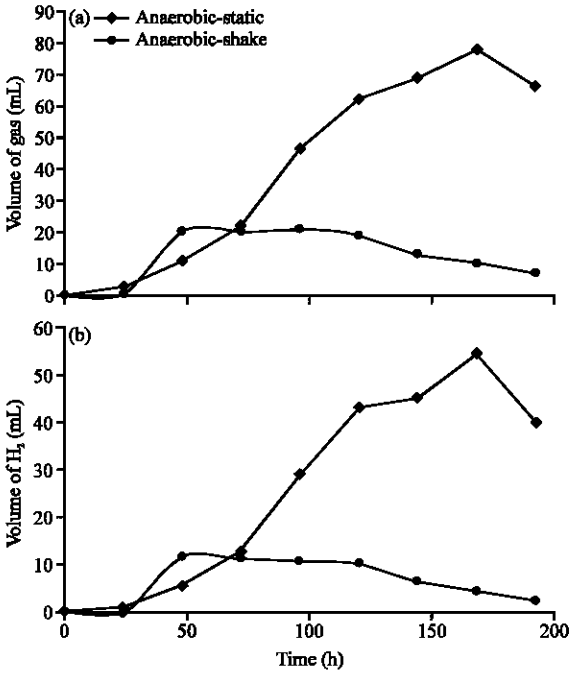

Fig. 2: Hydrogen production by *R. sphaeroides* in static and shake cultures; (a) volume of total gas produced and (b) volume of H_2 produced

Table 1: H_2 production from different culture conditions

	Effect of agitation									
	Anaerobic-static					Anaerobic-shake				
Time (h)	Volume gas (mL)	H_2 (%)	Volume of H_2 (mL)	$Y_{H2/S}$ (mL g^{-1})	Rate of H_2 Production (L/L/h)	Volume gas (mL)	H_2 (%)	Volume of H_2 (mL)	$Y_{H2/S}$ (mL g^{-1})	Rate of H_2 production (L/L/h)
0	0.0	0.00	0.00	0.00	0.00	0.0	0.00	0.00	0.00	0.00
24	3.0	33.59	1.01	5.04	0.4199	0.5	26.15	0.13	0.65	0.0545
48	11.0	52.80	5.81	29.04	1.2101	20.0	57.86	11.57	57.86	2.4109
72	22.0	58.37	12.84	64.20	1.7834	20.0	57.12	11.42	57.12	1.5866
96	46.5	62.23	28.94	144.68	3.0142	21.0	51.54	10.82	54.11	1.1274
120	62.0	69.43	43.04	215.22	3.5870	19.0	53.44	10.15	50.77	0.8461
144	69.0	65.43	45.15	225.74	3.1352	13.0	50.82	6.61	33.04	0.4588
168	77.7	69.98	54.37	271.87	3.2366	10.0	47.26	4.73	23.63	0.2813
192	66.0	60.38	39.85	199.24	2.0754	7.0	36.07	2.52	12.62	0.1315

Table 2: H_2 production in different culture conditions

Culture	Hours	Total gas (mL)	H_2 (%)	Total of H_2 (mL)	$Y_{H2/S}$ (mL g^{-1})	Rate of H_2 production (L/L/h)
AN-AN	76	62.00	51.71	32.06	160.30	0.00422
AN-AE	76	31.70	34.52	10.94	54.71	0.00144
AE-AN	76	63.60	35.51	22.58	112.92	0.00297
AE-AE	76	21.80	42.40	9.24	46.22	0.00122

*AN-AN = Anaerobic growth and Anaerobic H_2 fermentation, *AN-AE = Anaerobic growth and Aerobic H_2 fermentation, *AE-AN = Aerobic growth and Anaerobic H_2 fermentation, *AE-AE = Aerobic growth and Aerobic H_2 fermentation

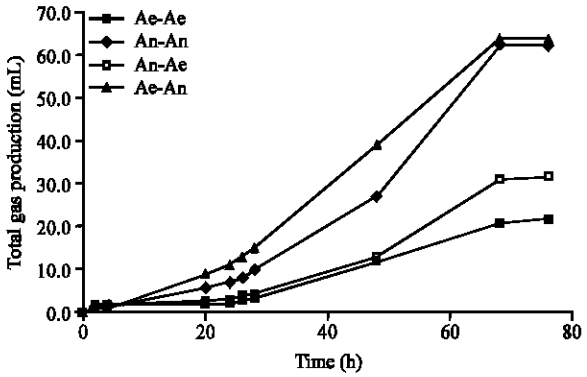

Fig. 3: Total volume of gas mixture produced by *R. sphaeroides* using different culture conditions for H_2 production

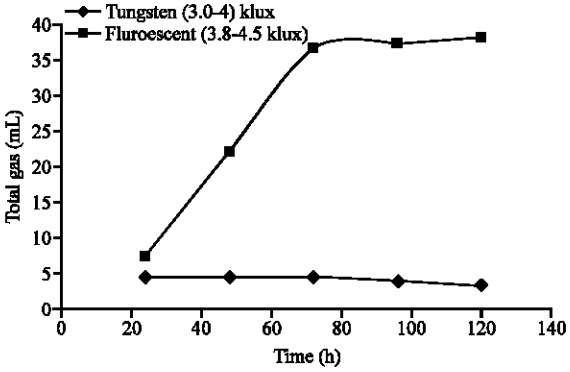

Fig. 4: Total H_2 production by different light sources

R. sphaeroides NCIMB 8253. Therefore, hydrogen production using this bacterium should be carried out in static condition rather than shake condition especially for small scale culture.

Effect of aeration: The effect of the aerobic and anaerobic culture condition was also studied. Figure 3 shows the trends of various conditions used for growth and H_2 production. The results in Table 2 shows that the highest volume of hydrogen produced (32.06 mL H_2) was achieved when both culture for inoculum and H_2 production were incubated in anaerobic condition ($Y_{H2/S}$ = 160.30 mL g^{-1}). However, when the inoculum was prepared in aerobic condition and H_2 fermentation in anaerobic condition the yield of H_2 ($Y_{H2/S}$) decreased to 112.92 mL g^{-1} and the value of $Y_{H2/S}$, 54.71 mL g^{-1} was obtained when the inoculum was grown in anaerobic and H_2 fermentation was in aerobic condition. The lowest yield of H_2 ($Y_{H2/S}$, 46.22 mL g^{-1}) was obtained when both inoculum and H_2 fermentation were prepared in aerobic condition.

Figure 3 shows the trends of total mixture gas produced by *R. sphaeroides*. Results indicated that when the inoculum was grown aerobically and H_2 production was carried out anaerobically the highest volume 63.60 mL of gas mixture was achieved but percentage of H_2 (Table 2) in the mix gas produced was only 35.51% compared to when both culture for inoculum and H_2 production were incubated in anaerobic condition. This result shows that *R. sphaeroides* NCIMB 8253 produced higher yield of H_2 in anaerobic condition and this parameter also give big impact to H_2 production and we found that this is one of the important parameters.

Effect of light sources: Fluorescent and tungsten lamp were used as the light sources for *R. sphaeroides* NCIMB 8253 growth and H_2 production. The effects of both light sources on growth and H_2 production by *R. sphaeroides* NCIMB 8253 were also studied. The light intensities used in this study were around 3.0-4.0 klux by tungsten and 3.8-4.5 klux by fluorescent depends on the distance of the culture to the light source. The 4000 lux was found to be equivalent to 270 W m^{-2} and to 1370 μmol photons m^{-2} sec (photosynthetically active radiation) (Uyar *et al*., 2007).

Figure 4 shows that light source from fluorescent gives (about 8 times) higher total volume of gas compared to tungsten. This trend was same to other two graphs which show the % of H_2 (Fig. 5) and the total of H_2 (Fig. 6) gas produced. All these trends proved that light source from fluorescent give better results compared to tungsten. This study only reporting on the effects of two different light sources not on the light intensities, wavelength and illumination protocol as reported by Uyar *et al*. (2007).

Tungsten lamps are a convenient light source but they contain infrared light to a great extent. Infrared light over 1000 nm is not only useless for photo biological hydrogen evolution but it also heats the culture

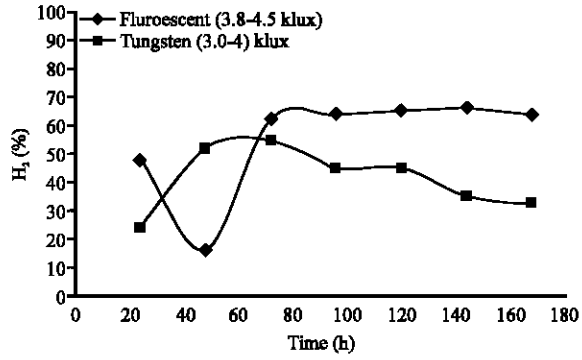

Fig. 5: Total % of H_2 produced by *R. sphaeroides* using different light sources

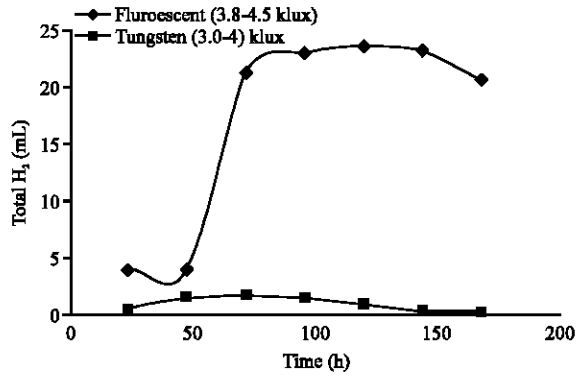

Fig. 6: Total H_2 produced by *R. sphaeroides* using different light sources

suspension (Nakada *et al*., 1999). Uyar *et al*. (2007) have studied the effect of light intensities, wavelength and illumination protocol on the growth and hydrogen production by *R. sphaeroides* O.U. 001 using 150 W tungsten lamp. It was found that lack of infrared light (750-950 nm wavelengths) decreased photo production of hydrogen by 39%. The results showed that the rate of hydrogen production increased up to 33 ml/L/h with increasing light intensity and reached saturation at around 270 W m^{-2} (Uyar *et al*., 2007), compared to our results, the highest rate of H_2 production under fluorescent illumination was 29.26 ml/L/h and under tungsten lamp was 0.32 ml/L/h. These results were lower than that obtained by Uyar *et al*. (2007), which utilized a different *R. sphaeroides* strain (*R. sphaeroides* O.U.001). However, the *R. sphaeroides* NCIMB 8253 also has a great potential to be H_2 producer under suitable parameters.

Another factor evaluated was the effect of different illumination protocols on the growth and hydrogen production. It was observed that illumination after inoculation stimulates hydrogen production, increases substrate conversion efficiency and hydrogen production rate. There was no hydrogen produced during the dark periods. This result shows us that *R. sphaeroides* NCIMB 8253 produced higher yield of H_2 under fluorescent illumination compared to tungsten lamp and this parameter also one of the important parameters that affect H_2 production.

CONCLUSION

This study showed that growth and H_2 production of *R. sphaeroides* NCIMB 8253 was affected by some environmental parameters. H_2 production by *R. sphaeroides* NCIMB 8253 should be carried out in static culture under anaerobic condition with fluorescent lamp as the light source for maximum yield. This work is still in the middle stage and will focus many other factors, i.e., inoculum age and size, media, pH, carbon and nitrogen source, light intensities, effect of NH_4^+ and malate consumption as this bacterium has the bright potential in biological hydrogen production. Once the optimum parameters achieved, this research will be proceed in larger scale using 7L photo bioreactor.

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