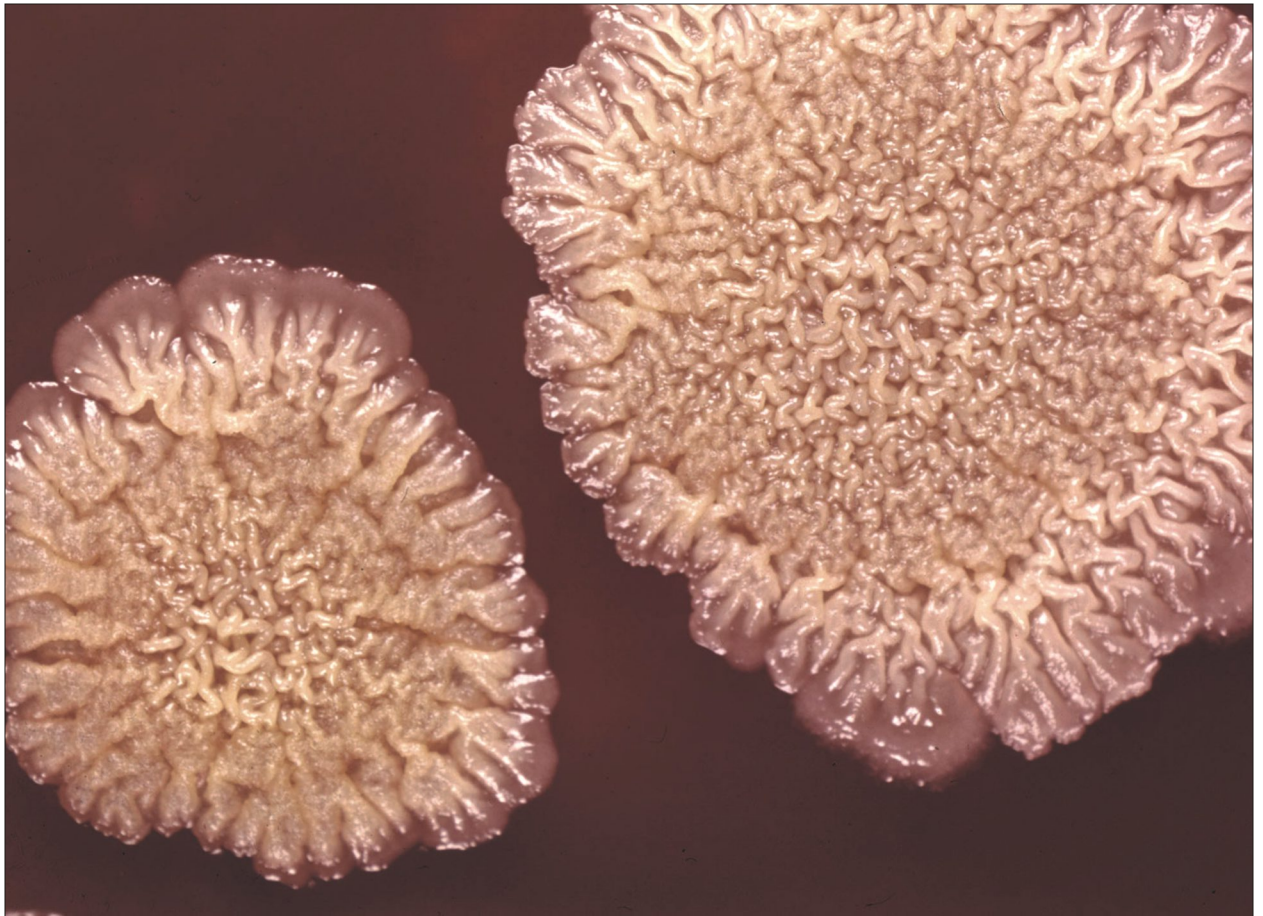




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Effects of Age of Inoculum, Size of Inoculum and Headspace on Hydrogen Production using *Rhodobacter sphaeroides*

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ABSTRACT

Effects of different parameters including age of inoculum, size of inoculum and headspace on hydrogen production were investigated in detail using *Rhodobacter sphaeroides* NCIMB 8253 in batch culture. The effects of aerobic and anaerobic conditions for growth of this bacterium were also studied. The growth of *R. sphaeroides* NCIMB 8253 in aerobic condition reached exponential phase faster as compared to the anaerobic condition. The maximum absorbance at 660nm for the aerobic culture was 20% higher than the cells in anaerobic condition. The lag phase has been shortened when same medium was used for inoculum and H₂ production but the H₂ yield was found 9.26% lower as compared when different media were used for inoculum and H₂ production. It was found that inoculum age 24 h, 10% v/v inoculum size with initial pH 7 can be designated as optimum conditions for maximum H₂ production.

Key words: *Rhodobacter sphaeroides*, photofermentation, biohydrogen, biofuel, anaerobic fermentation, head space

INTRODUCTION

Photosynthetic bacteria are favorable for biological H₂ production due to several reasons. The bacteria can convert a wide range of substrates including waste materials into H₂ with high yield and able to use wide spectrum of light energy (Fascetti *et al.*, 1998; Koku *et al.*, 2002). Hydrogen gas can be produced using biological or chemical methods but the chemical methods may present 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 due to coke deposition (Alalayah *et al.*, 2010; Misi *et al.*, 2011). Hydrogen can also be produced using *Clostridium saccharoperbutylacetonicum* N1-4 from the different feedstock including cellulosic biomass or glucose (Alalayah *et al.*, 2009).

Hydrogen production by *R. sphaeroides* and other Purple Non Sulfur (PNS) bacteria occurs under illumination in the presence of an inert, anaerobic atmosphere (such as argon), from the breakdown of organic substrates such as malate and lactate. 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 through the production of H₂. Several individual components make up the overall production system and these may conveniently be grouped as: (i) the enzyme systems, (ii) the carbon flow, specifically the TCA cycle and (iii) the photosynthetic membrane apparatus.

These groups are interconnected within the H₂ 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, H₂ production of any significance occurs under a photoheterotrophic growth mode which is also the 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).

These PNS bacteria have been investigated for their potential to convert light energy into H₂ 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 widely for H₂ production (Fang *et al.*, 2006). It has been reported that pH 6.8-7.0 and temperature 30-35°C are the optimum conditions for growth of *R. sphaeroides*, the optimum conditions for H₂ production can be achieved at pH 7.0, temperature at 30-40°C and photoproduction of H₂ saturated around 5000 lux (Arik *et al.*, 1996). The efficiency of biological hydrogen production is influenced by different environmental parameters and each strain need to be standardized for the optimum values required for enhanced hydrogen production. A detail of the effect of operating parameters including agitation, aeration and light on hydrogen production using *R. sphaeroides* NCIMB 8253 has been investigated (Jaapar *et al.*, 2009).

Fermentation is a process which is usually influenced by different environmental parameters (Emily *et al.*, 2009). Temperature and pH are usually considered as most important parameters for microbial growth and production of value added products. The proposed study was designed to concentrate on some unusual parameters which may contribute well for hydrogen production from *Rhodobacter sphaeroides* NCIMB 8253 on being optimized. Influence of the operational parameters including age and size of inoculum and headspace on hydrogen production was investigated in detail using *Rhodobacter sphaeroides* NCIMB 8253 in batch culture. This study may help to establish a set of parameters to enhance the hydrogen production from *Rhodobacter sphaeroides* NCIMB 8253 in batch culture.

MATERIALS AND METHODS

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

Microorganism: *R. sphaeroides* NCIMB 8253 was obtained from NCIMB Limited Scotland, in freeze-dried form. The bacteria were activated by rehydration of dried cultures with a sterile fresh rich medium named Basal Carbonate Yeast Extract Trypticase (BCYT) medium and transferred continuously into new fresh medium (BCYT) at least 3 times until they were activated. This procedure was done aerobically and incubates under tungsten light at 30°C. To start H₂ production experiment, activated cultures were transferred continuously into new growth medium (modified Biebl and Pfennig) anaerobically. After 72 h anaerobic incubation at 30°C, the culture was transferred into modified Biebl and Pfennig growth medium and incubated for 24 h at 30°C. Then culture was used to inoculate the H₂ production medium. The amount of 10% v/v inoculum was used throughout this project except otherwise stated.

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 adding 20 g 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 solution. 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 an 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 with 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.

H₂ production: H₂ gas production was performed 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 H₂ 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 H₂ production medium. During the experiments, the evolved 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 blank solution. Cell's dry weight was estimated 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 attained. A relationship of cells dry weight and OD was obtained by plotting a graph of OD versus cell dry weight. 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 collected from each experiment. The pH of the culture medium was measured with Eutech Instruments pH meter (Model: pH 510; pH/ mV/ °C; Cyberscan). Light intensities were measured by a luxmeter (Model: LX-103; Digital Instruments; Lutron).

RESULTS AND DISCUSSION

Growth and pH profile under aerobic and anaerobic conditions: The growth of *R. sphaeroides* NCIMB 8253 in aerobic and anaerobic culture conditions were monitored by measuring the absorbance at 660 nm at certain time intervals. As can be observed in Fig. 1, the growth of the cells in aerobic culture reached exponential phase earlier than the cells grown in the anaerobic condition. The maximum OD_{660nm} of 1.243 achieved at 160 h incubation in the aerobic culture and decline when the culture was incubated furthers. The decline of the OD of the culture might be due to cell lysis. In case of the anaerobic culture, the maximum OD_{660nm} 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 (Kars *et al.*, 2006), higher cell masses were reported for the non H₂ producing cells, on the other hand lower cell masses were related to enhanced H₂ production.

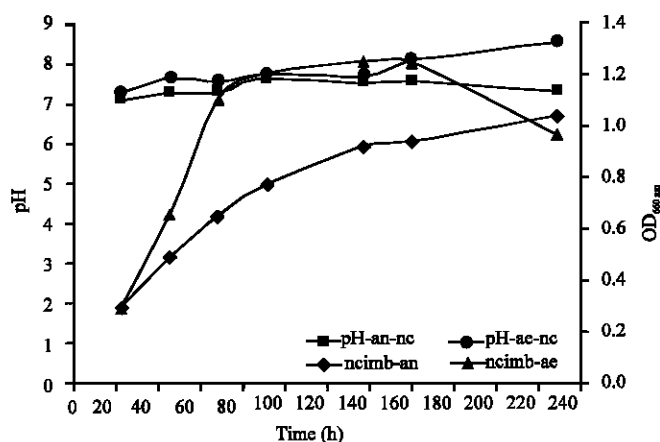


Fig. 1: Changes of absorbance and pH during the growth of *R. sphaeroides* NCIMB 8253 in aerobic and anaerobic condition. (pH-an-nc) pH for *R. sphaeroides* NCIMB 8253 in anaerobic condition, (pH-ac-nc) pH for *R. sphaeroides* NCIMB 8253 in aerobic condition, (ncimb-an) growth of *R. sphaeroides* NCIMB 8253 in anaerobic condition (OD_{660nm}), (ncimb-ac) growth of *R. sphaeroides* NCIMB 8253 in aerobic condition (OD_{660nm})

Therefore, the results are in consistent with the previous study, as the H₂ production was targeted in anaerobic conditions but 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 materials. 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 also in agreement with the previous research reported that during H₂ production by *R. sphaeroides* in anaerobic fermentation, a slight decrease in the pH of the medium is observed during the cell growth period, but the pH increased with the H₂ production (Eroglu *et al.*, 1999).

The bacterial cell concentration was determined 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 correspond 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. It has been have reported that 1OD_{660nm} is equivalent to 0.6 g L⁻¹ of cell dry weight (Uyar *et al.*, 2007). They have used the same bacterial strain but dried the cells 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). Bratbak and Dundas (1984) have also used 105°C to get the constant dry cell mass (Bratbak and Dundas, 1984).

Effect of medium: The growth of *R. sphaeroides* NCIMB 8253 in aerobic and anaerobic culture conditions were monitored by measuring the absorbance at 660 nm at certain time intervals. H₂ production was studied using two different media, growth medium and H₂ production medium of modified Biebl and Pfennig. Growth Medium (GrM) was used for starter culture or inoculum

Table 1: Effect of age of inoculum and nature of medium on hydrogen gas production

Inoculum medium	Production medium	Age of inoculum		Total gas		Total of H ₂ (mL)	Y H ₂ /S (mL g ⁻¹)	Rate of H ₂ production (mL/L/h)
		(h)	Total hours	(mL)	H ₂ %			
H ₂ M ^a	H ₂ M	24	144	67.20	18.64	12.53	125.26	0.87
GrM ^b	H ₂ M	24	144	66.50	20.76	13.81	138.05	0.96
H ₂ M	H ₂ M	48	144	1.00	0.00	0.00	0.00	0.00
GrM	H ₂ M	48	144	60.50	14.73	8.91	89.10	0.62

^aHydrogen production medium, ^bGrowth medium

purposes. H₂ production Medium (H₂M) was used for bulk H₂ production. The purpose of this experiment was to investigate the effects of nature of the starter culture/inoculum on the growth and potential of the bacteria to adopt the H₂ production medium for optimum functioning. The results show that the shortened lag phase and enhanced H₂ production can be achieved when the H₂ production medium (H₂M) was used for inoculum and bulk production, this combination of media is abbreviated as H₂M-H₂M 24 h. The age of inoculum was also contributing to the bacterial efficiency as discussed in next paragraph. Table 1 shows the comparison of the percentage of H₂ produced, total H₂ produced, Yield of H₂ produced per substrate consumed (YH₂/S) and the rate of H₂ produced by different cultures. The shortened lag phase and enhanced hydrogen production while using same media for inoculums and fermentation reveal that microbes were in cooperative mode to utilize the medium components and start producing H₂ from the beginning. On the other hand, the change of medium from starter culture to production medium might have slowed down the bacterial efficiency being engaged in acclimatization and adaptation to the new environment.

Effect of age of inoculum: The potential of growth of bacteria in the starter culture with respect to the time duration was investigated by using the inoculum grown for 18, 24, 40 and 48 h. It was designed to monitor the effects of age of inoculum on reproducing ability and H₂ production efficiency of *R. sphaeroides* after inoculating into production medium (Table 1). 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 production. It was found that the culture that was inoculated with 24 h inoculum age produced the highest percentage of H₂ (20.76%), highest yields of H₂ per gram of the substrate consumed i.e. YH₂/S (138.05 mLg⁻¹) and the highest rate of H₂ (9.6×10⁻⁴ L/L/h) compared to the culture grown for 48 h in this experiment. Koku *et al.* (2003) have reported that large retention times within such media might be causing the bacterial metabolism to drive in the production of other metabolites rather than H₂ production such as the production of Poly-Beta-Hydroxybutyrate (PHB) along with the H₂ production (Koku *et al.*, 2003).

Jee *et al.* (1987) has reported the loss of H₂ production activity with the passage of time for batch cultures of *R. sphaeroides* S associated with the declined activity of the electron carrier ferredoxin (Jee *et al.*, 1987). Continuous chemostat culturing of *R. sphaeroides* RV causes the bacteria to change their metabolism and start producing reserve products such as PHB rather than H₂ (Fascetti *et al.*, 1998). For PNS bacteria an inoculum from the exponential phase of growth is most suitable for a higher yield of H₂ production (Basak and Das, 2007). All of the above mentioned references reporting the effect of age of inoculum on different strains with a common conclusion of declined hydrogen production with increased time span and the proposed study also illustrate that H₂ production by *R. sphaeroides* NCIMB 8253 can be attained maximum at the inoculum age of 24 h and reduced if age of inoculum is increased.

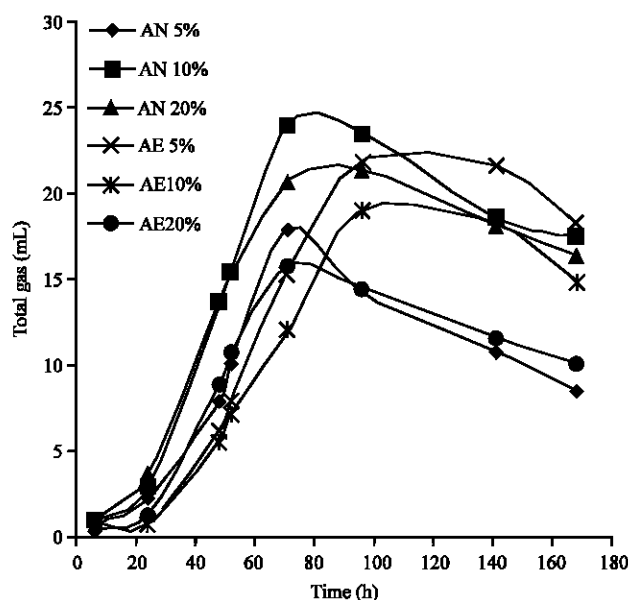


Fig. 2: Total gas produced by cultures with different inoculum size. AN: Anaerobic, Ae: Aerobic

Effect of inoculum size: Inoculum size also plays an important role in H_2 gas production. Three different inoculum sizes (5% v/v, 10% v/v, 20% v/v) in two different culture conditions (aerobic and anaerobic) were studied. This experiment was carried out using 100 mL culture in triplicates. The results regarding bacterial behaviour in aerobic and anaerobic conditions are presented in Fig. 2. In case of anaerobic culture the most productive percentage of inoculum was 10 % to produce 23.95 mL of H_2 but 20 and 5% inoculum size were able to produce 21.4 and 17.9 mL of H_2 , respectively. On the other hand, aerobic culture was able to produce maximum H_2 of 21.8 mL when 5% (v/v) inoculum was used. Other inoculum sizes were producing less H_2 as compared to 5%. The results show that 10% inoculum size in anaerobic conditions can give highest H_2 production rate which was $2.6986 \text{ mmol h}^{-1}$ and the same size of inoculum in aerobic conditions could generate rate of hydrogen production $1.5833 \text{ mmol h}^{-1}$. Ferchichi *et al.* (2005) reported that the H_2 production rate increased from 2 mmol h^{-1} at 1% inoculum size to 2.36 mmol h^{-1} with 10% inoculum size but the highest yield can be obtained with 1% inoculums (Ferchichi *et al.*, 2005). This shows that for the same concentration of substrate, an initially high substrate to cell ratio would prolong the growth phase and facilitate a longer duration of high rates of H_2 production by the growing cells and that would result in high H_2 production. This however, disagrees with the findings of Lay (2001), they have reported that a low substrate (cellulose) to cell density facilitated high H_2 generation by a mixed H_2 producing culture (Lay, 2001). The reduction in total gas or H_2 at the end of the reaction can be attributed to the consumption of gas by bacteria because the collected gas was directly in contact with the culture without any water trap system.

Effect of headspace: Effect of headspace was studied using 50, 70, 80 and 100 mL H_2 fermentation culture medium in 100 mL serum bottle. The results showed that 100 mL H_2 fermentation culture medium in 100 mL serum bottle produced the highest volume of H_2 as shown in Fig. 3. Wooshin *et al.* (2006) have used a chemical scavenger (KOH) to reduce H_2 losses via acetogenesis, CO_2 concentrations in the headspace can be reduced during H_2 production using KOH (Wooshin *et al.*, 2006). In their studies, CO_2 in the headspace was decreased from 24.5% (control)

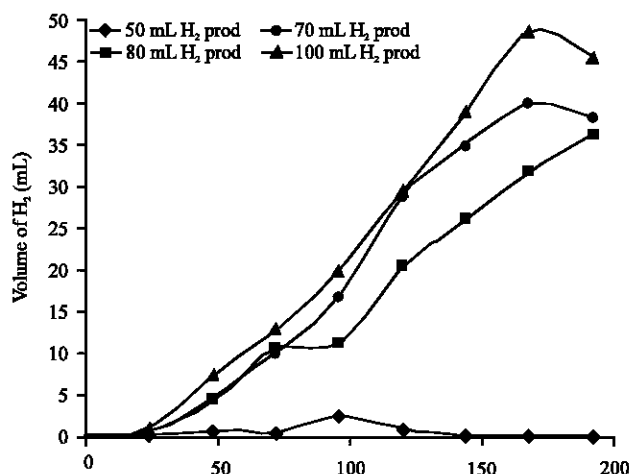


Fig. 3: Effect of headspace to produce hydrogen gas during batch fermentation

to a minimum 5.2% during the highest gas production phase, resulting in a H₂ partial pressure of 87.4%. This reduction in CO₂ increased the H₂ yield by 43% (from 1.4 to 2.0 mol of H₂ mol⁻¹ of glucose). These results show that H₂ production can be increased by removing CO₂ in the reactor vessel, likely as a result of suppression of acetogenesis. These results are enough convincing to allow minimum head space over the reaction contents in the bottle/reactor for the maximum H₂ production.

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 type of medium being used for inoculum and H₂ production, age of inoculum, inoculum size and effect of headspace. The highest gas production phase was observed by *R. sphaeroides* NCIMB 8253 in, 24 h inoculum and 10% v/v inoculum size. *R. sphaeroides* NCIMB 8253 may grow well in Growth Medium (GrM) for inoculum and H₂ production can be achieved maximum using H₂ medium, highest H₂ volume produced was also contributed by minimum headspace of H₂ fermentation vessel. This study may lead to establish the optimized conditions at laboratory scale and industrial scale for the production of maximum H₂ using *R. sphaeroides* NCIMB 8253.

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