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## Removal of Headspace CO<sub>2</sub> Increases Biological Hydrogen Production by *C. acetobutylicum*

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**Abstract:** The effect of removal of resultant gas resulted in enhancement of the H<sub>2</sub> yield. The technique of CO<sub>2</sub> scavenging resulted in H<sub>2</sub> yield being improved from 408 mL g<sup>-1</sup> to reach the maximum of 422 mL g<sup>-1</sup>. The highest hydrogen productivity of 87.9 mL L<sup>-1</sup> h<sup>-1</sup> was obtained by CO<sub>2</sub> scavenging. Biomass concentration was enhanced to 1.47 g L<sup>-1</sup>, Y<sub>PK</sub> of 287 mL g<sup>-1</sup> L<sup>-1</sup>, Y<sub>X/S</sub> of 0.294 and Y<sub>H<sub>2</sub>/S</sub> of 0.0377 by the use of CO<sub>2</sub> scavenging. The results suggested that the presence of the gaseous products in fermentation medium and headspace adversely effect biomass growth and hydrogen production.

**Key words:** Glucose, batch fermentation, CO<sub>2</sub> scavenging, KOH

### INTRODUCTION

Hydrogen has been suggested as a promising alternative to fossil fuels as an energy carrier for transportation needs. Hydrogen gas has a high-energy capacity per unit mass (118.2 kJ g<sup>-1</sup>) and its reaction with oxygen does not produce greenhouse gases such as CO<sub>2</sub>. In contrast, methane has a relatively lower energy capacity (36.3 kJ g<sup>-1</sup>) and CO<sub>2</sub> is released during its combustion in conventional engines. However, most hydrogen is usually produced from stored methane reserves and other nonrenewable materials, resulting in net increases of CO<sub>2</sub> in the environment. Shifting a fossil fuel economy to a hydrogen economy offers few environmental advantages if both are based on the net consumption of fossil fuels (Wooshin *et al.*, 2005). Thus, it is essential for reducing CO<sub>2</sub> emissions that hydrogen production not release a net amount of CO<sub>2</sub> into the atmosphere and that the technologies and materials used to produce hydrogen are sustainable.

Major advantages of fermentative H<sub>2</sub> production processes are higher evolution rate of H<sub>2</sub> and a wide gamut of substrate utilization. However, the inherent disadvantage of these processes is lower yield of H<sub>2</sub>. This is one of the major deterrents of the fermentative H<sub>2</sub> production process. At most a maximum of 4 mol H<sub>2</sub> could be obtained per mol glucose during acetate fermentation. To address this problem, efforts are needed to improve the operating conditions to overcome thermodynamic limitations (Fabiano and Perego, 2002) of the acetate fermentation reaction represented below.



Decrease of H<sub>2</sub> partial pressure could be considered as an approach towards improvement of H<sub>2</sub> productivity (Levin *et al.*, 2004). Equilibrium constant of the above reaction is according to Le Chatelier's principle; the equilibrium of the above reaction will shift to the right if one or both of the gaseous products of the reaction is removed. Decrease in partial pressure of H<sub>2</sub> by reducing total pressure of the system allows the reaction equilibrium to shift towards right side and thereby enhance the H<sub>2</sub> production.

Reducing the partial pressure of hydrogen in reactor vessels can increase hydrogen production. In batch tests, continuous versus intermittent release of gas pressure. Logan *et al.* (2002) found that continuous release increased hydrogen production by 43%. Reducing the hydrogen partial pressure using a vacuum or by sparging the reactor vessel with nitrogen gas has also increased hydrogen yields (Logan *et al.*, 2002; Tanisho *et al.*, 1998). It was assumed in these tests that this increase in hydrogen production was related to reduction in the hydrogen partial pressure. However, hydrogen can also be consumed via acetogenesis, according to Oh *et al.* (2003) they mentioned that hydrogen yields were lowered by acetogenesis in the absence of glucose, a heat-treated inoculum produced acetic acid from hydrogen and CO<sub>2</sub>. The fact that both CO<sub>2</sub> and H<sub>2</sub> are

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required for acetogenesis suggested to us that limiting CO<sub>2</sub> concentrations in the reactor might provide a viable strategy for reducing acetogenesis. Several studies have shown that produced gases during biohydrogen production can affect hydrogen production. While this increase in production has typically been attributed to a reduced percent of hydrogen in the gas phase, Wooshin *et al.* (2005) demonstrated that changes in CO<sub>2</sub> concentration can also be a factor. They found that overall hydrogen production increased by 43% with chemical scavenging of the CO<sub>2</sub> despite an increase in the hydrogen concentration in the headspace. Previous studies by Logan *et al.* (2002) have shown that an increase in gas pressure in bottles during these tests can reduce hydrogen production. The present study shows that reduction the CO<sub>2</sub>% of produced gas substantially improves H<sub>2</sub> production in an anaerobic fermentation process.

## MATERIALS AND METHODS

**Microorganism and culture conditions:** *Clostridium acetobutylicum* NCIMB 13357 was purchased from a British culture collection, NCIMB Ltd. Scotland, UK. This study was conducted from 2006 to 2007 in Department of Chemical Engineering, Universiti Kebangsaan Malaysia. The bacterium was cultivated in anaerobic condition in Reinforced Clostridia. Medium (RCM) for 24 h at 30°C. Liquid medium of RCM was used for inoculum preparation. The growth of culture in RCM was monitored by measuring an optical density at 600 nm using a spectrophotometer. Only inoculum with Optical Density (OD) values greater than 0.4-0.6 after 18 h cultivation was used as inoculum. An inoculum of 10% v/v was used throughout this work. Batch fermentation was carried out at a working volume of 100 in 500 mL reactor bottles at 30°C, each medium was seeded with a 10% inoculum inside anaerobic cabinet and sparged with nitrogen gas (99.9%). All the bottles were tightly closed. The culture pH was not controlled during fermentation and the initial pH was fixed to 7.0 before sterilization process. To quantify H<sub>2</sub>, gas produced during fermentation was recorded at the end of its production. The evolved gas was collected in a gas collection inverted cylinder and the volume of evolved gas was measured at room temperature by the water displacement method (Morimoto *et al.*, 2004) in a graduated cylinder that had been filled with water of pH 3 or less in order to prevent dissolution of the gas components.

**Analytical methods:** The gas composition was determined by gas chromatography (Shimadzu Co., Kyoto, GC-8A) under the following conditions: column: Porapack-Q, carrier gas: Nitrogen, flow rate: 33 mL min<sup>-1</sup>; column

temperature: 50°C, injection temperature: 100°C, detector temperature: 50°C, detector: Thermal Conductivity Detector (TCD). The soluble glucose concentration was measured at the end of each batch experiment for the calculation of the amount of glucose consumed by DNS method modified by Miller (1959) using spectrophotometer (UV 1601IPC, Shimadzu Corporation-Japan) optical density (OD<sub>550nm</sub>). Individual batch experiments were observed until the hydrogen production from each bottle stopped. All bottles (500 mL Scott bottle) with working volume of 100 mL (90% culture medium and 10% inoculum), were initially flushed with nitrogen gas for 5 min and then sealed. CO<sub>2</sub> was removed from the headspace using 6 mL of KOH (30% (w/v) (Wooshin *et al.*, 2005) placed in a small tube open only to the headspace. Biotic controls were prepared in the same manner using distilled water instead of KOH.

Gas production was measured using water displacement method (Morimoto *et al.*, 2004) with biohydrogen production calculated from headspace gas measurements and the total volume of biogas produced at the end of gas production. The difference in gas production between the treatment and control was used to calculate total CO<sub>2</sub> gas reduced by (30% (w/v)) KOH. Hydrogen gas quantity was measured manually from the quantity of the collected gas in inverted cylinder after CO<sub>2</sub> absorption by 30% (w/v) KOH. Control was used to calculate the percentage of CO<sub>2</sub> absorbed by KOH scavenging method (Wooshin *et al.*, 2005). All of these data were the average (mean) of three trials.

V<sub>1</sub> = Total gas volume (Control)

V<sub>2</sub> = Total gas (Scavenging)

V<sub>3</sub> = Collected gas after absorbed by (30% (w/v) KOH) (Control)

V<sub>4</sub> = Collected gas after absorbed by (30% (w/v) KOH) (Scavenging)

CO<sub>2</sub> (mL) (Scavenging) = V<sub>1</sub>-V<sub>2</sub>

H<sub>2</sub> (mL) (Scavenging) = V<sub>3</sub>-V<sub>4</sub>

To confirm the results gas was collected using plastic bag and analyzed after absorption by 30% (w/v) KOH, by GC-TCD.

## RESULTS

It was noted that investigators have reported H<sub>2</sub> yield as mol H<sub>2</sub> per mol substrate, mol H<sub>2</sub> per gram substrate or H<sub>2</sub> produced (mL) per gram substrate; hence, for ease of comparison with values reported, the H<sub>2</sub> yields were all converted to H<sub>2</sub> produced (mL) per gram substrate utilized. The results shown in Table 2 indicated that hydrogen was enhanced 3% by reducing the CO<sub>2</sub> concentration in the headspace. Overall, hydrogen yields

Table 1: Results of CO<sub>2</sub> scavenging by 30% (KOH) on glucose consumption and hydrogen productivity by *C. acetobutylicum* NCIMB13357. H<sub>2</sub> productivity

	pH	Gas volume (mL)	CO <sub>2</sub> (mL)	H <sub>2</sub> (mL)	Glucose consumed (%)	H <sub>2</sub> P
Control	4.45	550	185	365	89	81.5
Scavenging	4.53	460	80	380	90	87.9

[Glucose]: 5 g L<sup>-1</sup>, inoculum size 10% (v/v), initial pH 7.0. Temp. 30°C

Table 2: Results of CO<sub>2</sub> scavenging by 30% (KOH) on Hydrogen production by *C. acetobutylicum* NCIMB13357

	Y <sub>PS</sub> <sup>1</sup>	Y <sub>PS</sub> <sup>2</sup>	[Biomass]	Y <sub>PK</sub>	Y <sub>XS</sub>	Y <sub>H<sub>2</sub>/2</sub>
Control	365	408	1.38	296	0.276	0.036
Scavenging	380	422	1.47	287	0.294	0.038

Y<sub>PS</sub><sup>1</sup> (H<sub>2</sub> mL g<sup>-1</sup> glucose supplied) (mL g<sup>-1</sup>), Y<sub>PS</sub><sup>2</sup> (mL g<sup>-1</sup>) (Utilized): (H<sub>2</sub> mL g<sup>-1</sup> glucose utilized (mL g<sup>-1</sup>), [Biomass] (g L<sup>-1</sup>), Biomass production/L culture, Y<sub>PK</sub> (mL g<sup>-1</sup>): (H<sub>2</sub> mL g<sup>-1</sup> Biomass), Y<sub>XS</sub>: (Biomass production/g glucose supplied), Y<sub>H<sub>2</sub>/2</sub> (conversion of H<sub>2</sub> (mL) to H<sub>2</sub> (g) per g glucose utilized) [Glucose]: 5 g L<sup>-1</sup>, inoculum size 10% (v/v), initial pH 7.0. Temp 30°C

increased from 408 mL g<sup>-1</sup> glucose utilized to 422 mL g<sup>-1</sup> glucose utilized with CO<sub>2</sub> scavenging. CO<sub>2</sub> concentrations in the headspace could not be completely depleted, with CO<sub>2</sub> concentrations reduced from 185 mL g<sup>-1</sup> (control) to 80 mL g<sup>-1</sup> glucose supplied by using the 30% (v/v) KOH trap. As shown in Table 1, trapping the CO<sub>2</sub> reduced the measured gas production (compared to that of the control), but increased overall hydrogen production.

In the absence of CO<sub>2</sub> removal, the pH decrease from pH 7.0 to 4.45 was larger than that of the scavenging route final pH 4.53 due to the higher concentration of CO<sub>2</sub> and volatile acids. This slight difference in final pH should not have substantially affected hydrogen production as maximum hydrogen yields in batch tests have been reported to be in the pH range of 5.5-6.0 (Van Ginkel *et al.*, 2001). The results shown in Table 1 indicated that the rate of glucose consumed was increased slightly when CO<sub>2</sub> was removed. With CO<sub>2</sub> scavenging the bacterial productivity of hydrogen as shown in Table 2 was increased from 81.6 to 87.9 mL L<sup>-1</sup> h<sup>-1</sup> and that was attributed to the enhancement of biomass concentration due to reduction of CO<sub>2</sub>% from the produced gas. Reduction of CO<sub>2</sub>% from the produced gas reduced the pressure of produced gas inside the reactor and finally gave the chance for bacteria for more utilization of substrate to produce gas and to grow and that was clear from the difference between the bacterial growth in both experiments. The results shown in Table 1 demonstrate that bacterial growth was enhanced from 1.38 to 1.47 g L<sup>-1</sup> and that was not connected with consumed glucose due to the percentage of glucose consumed mainly same.

A hydrogen concentration of 100% could not be achieved due to incomplete CO<sub>2</sub> removal. On the basis of earlier results of Thauer *et al.* (1977), we would expect that increased hydrogen partial pressures would have inhibited bio-hydrogen production and not resulted in an

increase of hydrogen production as observed here. Therefore, the increase in hydrogen production must result from an aspect related to the reduced CO<sub>2</sub> concentration in the system. The most likely explanation is that a reduction in CO<sub>2</sub> concentrations reduced hydrogen losses via acetogenesis, which could not be completely inhibited. While there was an overall increase in hydrogen production from 408 mL g<sup>-1</sup> glucose utilized to 422 mL g<sup>-1</sup> glucose utilized with CO<sub>2</sub> removal. CO<sub>2</sub> could not be completely removed from the headspace. CO<sub>2</sub> concentrations may have been disproportionately higher in the liquid phase (relative to equilibrium) where, the CO<sub>2</sub> was generated. Thus, it was not possible to completely inhibit acetogenesis due to the remaining CO<sub>2</sub> concentration in the liquid phase.

The results shown in Table 1 and 2 suggested that removal the CO<sub>2</sub> by chemical scavenging using 30% (KOH) resulted in enhanced the hydrogen yield by 3% and that mainly due to the increased in biomass concentration which was enhanced by reduction of CO<sub>2</sub>% in produced gas. Different yields were obtained show that the reduction of CO<sub>2</sub> from the produced gas reduce the hydrogen production by biomass suggested that the biomass concentration affect inversely on hydrogen production due to byproducts inhibition whereas the biomass per substrate was increased suggested that reduction the gaseous products from the reactor enhanced the growth of biomass.

The results shown in Table 1 demonstrate that CO<sub>2</sub> percentage of produced gas was decreased from 34 to 17% (17%) and that was related to the hydrogen percentage in produced gas, which was increased from 66 to 82% (15%). The increase in hydrogen % in produced gas attributed to reduction in CO<sub>2</sub>% in produced gas and that would reduce the effect of total gas and its pressure inside the reactor. These results were agreed with Wooshin *et al.* (2005) they reported that by reducing the CO<sub>2</sub> concentration in the headspace from 24.5 to 5.2%, hydrogen yield was increased by 43%. They reasoned that to reduction of CO<sub>2</sub>% in produced gas and its pressure.

The results shown in Table 2 were used to calculate different yields like Y<sub>PK</sub> (mL g<sup>-1</sup> L<sup>-1</sup>): (H<sub>2</sub> mL per g Biomass per L), Y<sub>XS</sub>: (Biomass production per g glucose supplied) and Y<sub>H<sub>2</sub>/2</sub> (conversion of H<sub>2</sub> (mL) to H<sub>2</sub> (g) per g glucose utilized). These results showed that reducing the produced CO<sub>2</sub> from headspace that would reduce the percentage of dissolved CO<sub>2</sub> in culture medium which showed to enhanced the bacterial growth and finally enhanced the hydrogen productivity as well as hydrogen yield suggested that minimize or removal of produced CO<sub>2</sub> from the reactor headspace would enhance the hydrogen production.

## DISCUSSION

In general, biological  $H_2$  production from organic substrates is limited by the thermodynamics of the hydrogenase reaction, involving the enzyme-catalyzed transfer of electrons from an intracellular electron carrier molecule to protons. On the contrary, protons are poor electron acceptors, so the electron donor must be a strong electron reducing agent. Ferredoxin is a low-potential iron sulfur containing protein that is capable of reducing proton to  $H_2$ . Another important intracellular electron carrier, NADH, has a higher redox potential. Under actual conditions the ability of reduced ferredoxin and NADH to reduce protons is determined by the redox potential of the overall reaction.

Assuming the intracellular concentrations of the oxidized and reduced form of ferredoxin and NADH are equal,  $H_2$  production becomes thermodynamically unfavorable at high  $H_2$  partial pressure. This indicates that for ferredoxin,  $H_2$  production can continue as long as the partial pressure of  $H_2$  is less than  $\approx 0.3$  atm and for NADH, the partial pressure of  $H_2$  must be less than 60 Pa. This implies that at a very low partial pressure ( $< 60$  Pa), NADH could also be used for  $H_2$  production. The first attempt to reduce the partial pressure was to use KOH to remove  $CO_2$  in the headspace, which decreased the partial pressure of  $H_2$  by 87% (Wooshin *et al.*, 2005). This enhanced the yield of  $H_2$  from 1.4 to 2 mol  $H_2$ /mol glucose. Gas sparging is another useful technique to decrease the  $H_2$  partial pressure to enhance its yield. Mizuno *et al.* (2000) observed that the specific  $H_2$  production rate increased from 1.5 mL  $H_2$ /min g biomass to 3.1 mL  $H_2$ /min g biomass under  $N_2$  sparging conditions.

In this study, overall hydrogen production increased here (3%), from 408 to 422 mL  $g^{-1}$  glucose utilized (2.92 to 3.04 mol  $H_2$ /mol glucose supplied: 3.26 to 3.38 mol  $H_2$ /mol glucose utilized), with chemical scavenging of the  $CO_2$  despite an increase in the hydrogen concentration in the headspace. Previous studies have shown that an increase in gas pressure in reactor vessel can reduce hydrogen production. Present results agreed with reports of Logan *et al.* (2002) who stated that reducing the pressures of the evolved gases, either by applying vacuum or by gas sparging, could increase hydrogen production. They stated that continuous release of gases increased  $H_2$  production by 43% versus tests where there an intermittent release of gas and with Kataoka *et al.* (1997) who reported that when a vacuum was applied to a reactor operated in chemostat mode, hydrogen production increased by 10%, resulting in an overall yield of 2.2 mol of  $H_2$ /mol of glucose. For how  $CO_2$  affect the

bacterial metabolism, only one study was conducted by Ballongue *et al.* (1987) they reported that the action of  $CO_2$  has been studied in yeast in which  $CO$  acts on enzyme activities level.

An alternate approach to decrease the dissolved hydrogen concentration in a reactor. Tamisho *et al.* (1998) reported that sparging the reactor with argon gas resulted in an increase in hydrogen yield from 0.52 to 1.58 mol of  $H_2$ /mol of glucose. In both study, they reasoned the increase of hydrogen production to a reduction in the dissolved hydrogen concentration, the concentration of dissolved  $CO_2$  would also have been reduced, contributing to increases in hydrogen production. While applying a vacuum, gas sparging, or  $CO_2$  scavenging may all be effective methods of increasing hydrogen production,  $CO_2$  scavenging may provide the most direct and cost-effective method of reducing  $CO_2$ . Gas sparging may also have the undesirable potential to produce a loss of volatile fatty acids in the reactor off gas. It's known that as the gas pressure increased, the gas would dissolve more in fermentation medium. Finally, there is little information on inhibition by  $CO_2$ . Certainly most organism appear to be unaffected by  $CO_2$  partial pressure up to 0.2 atm.  $CO_2$  at high partial pressure will, however, exert an effect through depression of pH or chemical reaction with other compounds in the culture media (Moo-Young, 1985), whereas Doremus *et al.* (1985) reported that there is no evident that the level of dissolved carbon dioxide affected solvent production by clostridia. Stated that in pure water at 37°C, Henry's Low constant, that  $CO_2$  would be dissolved 34 times than  $H_2$ , on molar basis. According to Doremus *et al.* (1985), the quantity of dissolved  $CO_2$  in fermentation medium would affect on bacterial growth and on its metabolism and that was clear in present study and as the produced  $CO_2$  reduced by KOH scavenging, the bacterial growth and  $H_2$  yield were better than control.

## CONCLUSION

The current study, it was only examined the effect of  $CO_2$  scavenging on hydrogen production by *C. acetobutylicum* NCIMB 13357 in batch tests. Even in these tests, it was not possible to completely scavenge  $CO_2$  from the headspace during high rates of hydrogen production. If hydrogen is produced in continuous culture, it is not known how effectively it could scavenge  $CO_2$  under these conditions. Thus, additional research is needed to extend the batch test results from this test to continuous culture tests, particularly under conditions that affect the hydrogen production rate, such as under different organic loadings and reactor detention times.

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