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Effect of Some Environmental Parameters on Hydrogen Production Usimg *C. acetobutylicum*

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Abstract: The aim of this study was to investigate the influence of some environmental factors on bacterial metabolism. Fermentative hydrogen production by *C. acetobutylicum*, using glucose as the substrate. The effect of initial pH (4-8), inoculum size (1-20% (v/v)) and glucose concentration (1-30 g L^{-1}) on hydrogen production were studied. The optimum cultivation temperature for hydrogen production was at 30°C. The results show that substrate concentration and inoculum size resulted in hydrogen yield ($Y_{P/S}$) of 391 mL g^{-1} glucose utilized with maximum hydrogen productivity of 77.5 mL/L/h. Higher substrate concentration or inoculum size adversely affects hydrogen production, which decreases hydrogen yield by 15% to 334 mL g^{-1} glucose utilized when 30% (v/v) inoculum size was used. The use of 30 g L^{-1} substrate concentration resulted in a 75% decrease to 97 mL g^{-1} glucose supplied. Concluded that proper Xo/So enhanced the hydrogen production.

Key words: Biohydrogen, inoculum size, pH, glucose

INTRODUCTION

The trend of global environmental goals of lower emissions and carbon dioxide reduction and depletion of known crude oil reserves have intensified the development of non-polluting or clean energy carriers from renewable resources. Among recognized alternatives to fossil fuels, hydrogen is regarded as a clean energy carrier because water is the only product of its combustion. Moreover, its energy value of 231 BTU mol^{-1} (244 kJ mol^{-1}) is higher than that of any hydrocarbon. Although it can be generated from both renewable and non-renewable resources, more than 96% of the H_2 produced worldwide depends on fossilized energy in one form or another (Wunschiers and Lindblad, 2002). By the year 2002, producers were generating about 45 million tonnes of H_2 from fossil fuel (Gorman, 2002).

In contrast, microbial production of H_2, also referred to as biohydrogen production, is a much less capital and energy-intensive process. In particular, H_2 production by anaerobic fermentation is technically much simpler and can generate H_2 from a large a number of carbohydrate materials obtained in refuse and waste products (Nandi, and Sengupta, 1998). It is superior in terms of the rate of production to photosynthetic H_2 production and it can also make use of the existing infrastructure for biogas and fermentative acetone-butanol production without elaborate adjustments (Das and Veziroglu, 2001).

Clostridia have the capacity to utilize a wide variety of sugars, thus broadening the spectrum of potential carbon sources for H_2 production. Optimizing fermentation conditions that favour clostridial growth should therefore, essentially enhance H_2 production. However, what controls the mechanisms of clostridial H_2 production has not been determined in detail, since the culture conditions that affect H_2 production are only beginning to be ascertained (Logan *et al*., 2002). To date, studies have shown that iron availability, H_2 partial pressure, substrate concentration, temperature (Zhang *et al*., 2003) and the carbon-to-nitrogen ratio (Lin and Lay, 2004) all in some way affect the H_2-producing potential of a microorganism. Nonetheless, most clostridia produce both organic solvents (alcohols and ketones) and acids. Since alcohol production involves the consumption of H_2 in the form reducing equivalents such as NADH, it is inevitable that fermentation conditions that favour the metabolism of sugar to alcohols reduce H_2 production.

Clostridium acetobutylicum NCIMB13357 is a known alcohol-producing microorganism. Only one study was conducted using this microorganism for fermentative acetone, butanol and ethanol (ABE) production

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(Kalil *et al*., 2003). Its hydrogen-producing potential, however, has not been reported. Hence this study was conducted to investigate the influence of culture parameters on H_2 production by the microorganism. Hence, this study was conducted to investigate the influence of some environmental factors have been reported to affect on bacterial metabolism like initial culture pH, substrate concentration and inoculum size on hydrogen production by this microorganism.

MATERIALS AND METHODS

Microorganism and culture conditions: *C. aceto-butylicum* NCIMB 13357 was purchased from a British culture collection, NCIMB Ltd. Scotland, UK. This study was conducted in Chemical Lab, Department of Chemical and Bioprocess Engineering: Universiti Kebangsaan Malaysia at the period between 2006 to 2008. The bacterium was cultivated in anaerobic condition in Reinforced Clostridial 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 study.

Cultivation medium: New medium was formulated in the lab to be used for hydrogen production by bacterium species we used in this study have the following composition in (g L^{-1}): glucose (5), yeast extract (5), L-Cystine. HCl (1.0) and bacteriological agar (0.5).

The initial anaerobic condition in the reactor after inoculation inside the anaerobic glove box was established by replacing the gaseous phase with nitrogen at start of cultivation. Then incubated at 30°C in temperature controlled water bath without shaking. The evolved gas was monitored and collected in a gas collection 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 (inverted), 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^{-1}; 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_{550} nm). Individual batch experiments were observed until the hydrogen production from each bottle stopped. Final medium pH was measured by pH meter (Mettler Teldo) and final biomass was measured by Spectrophotometer (UV 1601IPC, Shimadzu Corporation-Japan) at optical density (OD_{600} nm). All of these data were the average (mean) of three trials.

RESULTS

Effect of initial pH: pH is one of the important controlling factors of anaerobic fermentation process. The results shown in Fig. 1a demonstrate the effect of initial pH on bacterial conversion of glucose to hydrogen gas. At acidic initial pH, low hydrogen was produced, whereas the quantity of produced hydrogen increased as the initial pH near to neutral pH. As shown in Fig. 1a, increasing the pH at intervals of 0.5 unit's affected H_2 production. The full utilization of 1 g of glucose at initial pH of 4.0 was 357 mL g^{-1} glucose utilized but only 50 mL g^{-1} glucose supplied was obtained, suggested that at acidic pH, the bacterial productivity of hydrogen by this species was inhibited.

As described earlier that optimal pH gave the highest hydrogen yield and that also shown in Fig. 1b which indicated that percentage of glucose consumption was increased with culture pH and the maximum of 87% of glucose was consumed within 22 h of batch process at pH of 7.0. Increased or decreased initial pH reduces the percentage of glucose consumed and finally affect adversely on total evolved hydrogen. Furthermore, the results shown in Fig. 1d revealed that neutral pH enhanced the biomass concentration and the maximum of 1.34 g L^{-1} was obtained at pH of 7.0. Above results showed that between pH 4.5 and 7.0, H_2 production was enhanced by every 1 unit increase in pH. While the highest yield of 391 mL g^{-1} glucose utilized (340 mL g^{-1} glucose supplied), was obtained at pH of 7.0. Further increased in culture pH reduce the hydrogen yield to 360 mL g^{-1} glucose utilized. Increasing the biomass shown to have positive effect on the bacterial productivity of hydrogen as shown in Fig. 1c. The results shown in Fig. 1c indicated that the bacterial productivity of hydrogen was increased from 11.5 mL/L/h at pH of 4.0 to 77.5 mL/L/h at pH of 7.0 then decreased to 50 mL/L/h at pH of 8.0 indicating that H_2 production by *C. acetobutylicum* NCIMB13357 was favored by a neutral initial pH.

The results shown in Table 1 shown that the hydrogen yield from glucose found in this study is higher than the 125 mL g^{-1} glucose supplied for the pure culture of *Enterobacter aerogenes* (Tanisho *et al*., 1989) and of

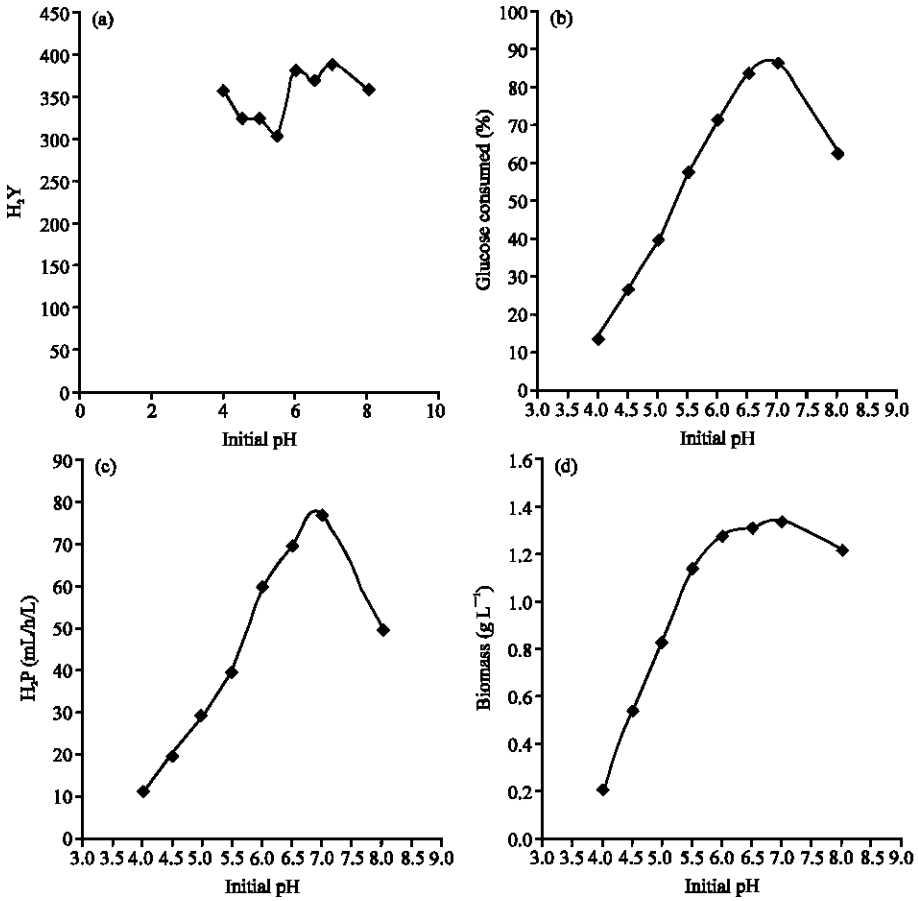

Fig. 1: Results of initial pH effect on (a) H_2 Y (mL g^{-1} utilized), (b) Glucose consumed (%), (c) H_2 P (Productivity (mL/L/h) = (H_2 volume/L culture volume. Duration time and (d) [Biomass] (g L^{-1}): [Glucose]: 5 g L^{-1}, inoculum size 10% (v/v), Temp. 30°C

Table 1: Comparison of hydrogen yield using glucose as substrate

Microorganism	pH	Hydrogen yield	References
	7.0	340	Present study
E. œrogenes	5.5-6.0	125	Tanisho *et al.* (1989)
E. œrogenes	5.0-6.0	274	Kumar and Das (2000)
C. butyricum	6.7	175-288	Kataoka *et al.* (1997)
Mixed culture	5.7	213	Lin and Chang (1999)

Hydrogen yield: (mL g^{-1} glucose supplied)

Table 2: Comparison of Hydrogen yield using different substrate

Feedstock	pH	Hydrogen yield	References
Rice	4.5	346	Fang *et al.* (2006)
Starch	6.0	92	Zhang *et al.* (2003)
Cellulose	7.0	72	Lay (2000)
Cellulose	7.0	193	Ueno *et al.* (1995)
Sucrose b	5.5	280	Fang and Liu (2002)
Glucose b	5.5	261	Fang and Liu (2002)

Hydrogen yield: (mL g^{-1} carbohydrate supplied), b: Continuous experiments

275 mL g^{-1} glucose supplied, using *E. cloacae* by Kumur and Das (2000) and also than 175-288 mL g^{-1} glucose supplied, using *C. butyricum* reported by Kataoka *et al.* (1997). Whereas by using mixed culture, the results of this study was higher than that of 213 mL g^{-1} glucose supplied reported by Lin and Chang (1999). Results of this study and those reported for pure culture seem to suggest that the maximum hydrogen yield by glucose fermentation is varied, depend on the bacterial species used.

Compared with reported hydrogen yield per carbohydrate supplied, but with varied pH and carbon source using mixed culture, Table 2 shows that the results of this study was lower than the yield reported by Fang *et al.* (2006) of 346 mL g^{-1} carbohydrate supplied but higher than other reported values, suggested that medium pH play an important role in pure culture but the lower yield in mixed culture it might because that not all hydrogen producing organisms would be active in selected pH.

The results shown in Table 4 were used to calculate different yields like $Y_{P/X}$ (mL/g/L): (H_2 mL g^{-1} Biomass L^{-1}), $Y_{X/S}$: (Biomass production per g glucose supplied) and $Y_{H2/s}$ (conversion of H_2 (mL) to H_2 (g) per g glucose

Table 3: Results of initial pH on glucose consumption and H_2P (mL/L/h) by *C. acetobutylicum* NCIMB13357

Initial pH	Final pH	Glucose consumed (%)	H_2P
4.0	4.07	14	11.5
4.5	4.39	27	20.0
5.0	4.44	40	30.0
5.5	4.51	58	40.0
6.0	4.48	72	60.0
6.5	4.45	84	70.0
7.00	4.46	87	77.5
8.0	4.56	63	50.0

[Glucose]: 5 g L^{-1}, inoculum size 10% (v/v) I pH 7.0, Temp. 30°C

Table 4: Results of initial pH on H_2 production by *C. acetobutylicum* NCIMB13357

Initial pH	$Y^1_{P/S}$	$Y^2_{P/S}$	[Biomass]	$Y_{P/X}$	$Y_{X/S}$	$Y_{H2/s}$
4.0	50	357	0.213	1677	0.04	0.032
4.5	88	325	0.543	599	0.10	0.030
5.0	130	325	0.830	392	0.17	0.030
5.5	176	303	1.140	268	0.23	0.028
6.0	267	383	1.280	299	0.26	0.034
6.5	310	369	1.310	282	0.26	0.032
7.0	340	391	1.340	292	0.27	0.036
8.0	227	360	1.220	295	0.24	0.032

$Y^1_{P/S}$: (H_2 mL g^{-1} glucose supplied) (mL g^{-1}), $Y^2_{P/S}$ (mL g^{-1}) (Utilized): (H_2 mL g^{-1} glucose utilized), [Biomass] (g L^{-1}): Biomass production g L^{-1} culture, $Y_{P/X}$ (mL/g/L): (H_2 mL g^{-1} Biomass L^{-1}), $Y_{X/S}$ (Biomass production per g glucose supplied), $Y_{H2/S}$: (conversion of H_2 (mL) to H_2 (g) per g glucose utilized) [Glucose], 5 g L^{-1}, inoculum size 10% (v/v), I pH 7.0, Temp. 30°C

Table 5: Results of inoculum size on glucose consumption and H_2P (mL/L/h) by *C. acetobutylicum* NCIMB13357

Inoculum size (%(v/v))	pH	Glucose consumed (%)	H_2P
1	4.41	60	19
3	4.46	72	22
5	4.49	72	34
7	4.55	77	57
10	4.46	87	77.5
20	4.63	94	82
30	4.66	97	88

[Glucose]: 5 g L^{-1}, initial pH 7.0, Temp. 30°C

Table 6: Results of inoculum size (%) (v/v), on H_2 production by *C. acetobutylicum* NCIMB13357

Inoculum size (%(v/v))	$Y^1_{P/S}$	$Y^2_{P/S}$	[Biomass]	$Y_{P/X}$	$Y_{X/S}$	$Y_{H2/s}$
1	170	283	1.09	260	0.02	0.025
3	202	281	1.13	249	0.23	0.025
5	189	263	1.26	208	0.25	0.0234
7	214	278	1.31	212	0.26	0.0248
10	340	391	1.34	292	0.27	0.0348
20	308	328	1.53	214	0.31	0.0292
30	321	331	1.67	198	0.33	0.0296

$Y^1_{P/S}$: (H_2 mL g^{-1} glucose supplied) (mL g^{-1}), $Y^2_{P/S}$ (mL g^{-1}) (Utilized): (H_2 mL/g glucose utilized), [Biomass] (g L^{-1}): Biomass production g L^{-1} culture, $Y_{P/X}$ (mL/g/L): (H_2 mL g^{-1} Biomass L^{-1}), $Y_{X/S}$: (Biomass production per g glucose supplied), $Y_{H2/S}$: (conversion of H_2 (mL) to H_2 (g) per g glucose utilized) [Glucose], 5 g L^{-1}, initial pH 7.0, Temp. 30°C

utilized). These results showed that optimum pH was necessary for bacterial growth which reversed to enhance both bacterial growth as well as hydrogen production.

Finally the result in Table 3 and 4 was in agreement also with results which showed that H_2 production by Clostridia is completely inhibited below a pH range of 4 to 5 (Bahl *et al*., 1986; Dabrock *et al*., 1992; Wu and Lin, 2004).

According to Bahl *et al*. (1982) results, the drop in culture pH was the critical factor for major shifting from acids production to solvent production. They reported that at neutral pH and glucose or nitrogen limitation, the main product was acids, CO_2 and H_2 by using *C. acetobutylicum* species, but when pH drop down because of acid production and reach to certain concentration the bacteria start forming the solvent and stop first metabolites production. They reported also that solvent production was not observed when *C. acetobutylicum* was grown in continuous culture with glucose or nitrogen limitation at pH values of 6.5 or 5.7. They stated that this bacterium is governed by physiological parameter. The optimal pH for their study for solvent production was found to be 4.3. From above finding of Bahl *et al*. (1982), the result of present study was strongly agreed with Bahl *et al*. (1982) regarding the initial pH and its effect on both metabolites and the final growth. It concluded that the pH for optimal H_2 production was dependent on the hydrogen-producing microorganism(s). The highest productivity and hydrogen yield were obtained at the optimum pH of 7.0; all data were shown in Table 3 and 4.

Effect of inoculum size: The results shown in Table 5 and 6 shown that hydrogen production was affected by the inoculum size. Obtained data of tested range shows how the importance to choose the proper inoculum size to influent substrate. The results of using 5 g L^{-1} of glucose in formulated medium and 10% inoculum size gave the highest hydrogen yield of 391 mL g^{-1} glucose utilized. Decreased or increased of inoculum size lead to decrease of hydrogen yield. The results shown in Fig. 2a indicated that inoculum size from 1 to 7% (v/v), the hydrogen yield was same with maximum of 283 mL g^{-1} glucose utilized using inoculum size of 7% (v/v) but further increased to 10% (v/v), the yield was enhanced resulted of 391 mL g^{-1} glucose utilized then started to decreased to 331 mL g^{-1} glucose utilized using 30% (v/v). The results shown in Table 6 shown that the inoculum size should be compatible with available substrate for maximum bacterial productivity due to substrate limitation would affect adversely on bacterial growth and finally on its metabolites. The reason for that mainly attributed to both glucose concentration and inoculum size in fermentation medium and as inoculum size increased the batch process finished early due to exhausted of available substrate and that was clear from Fig. 2c regarding the bacterial productivity which the maximum of 88 mL/L/h was

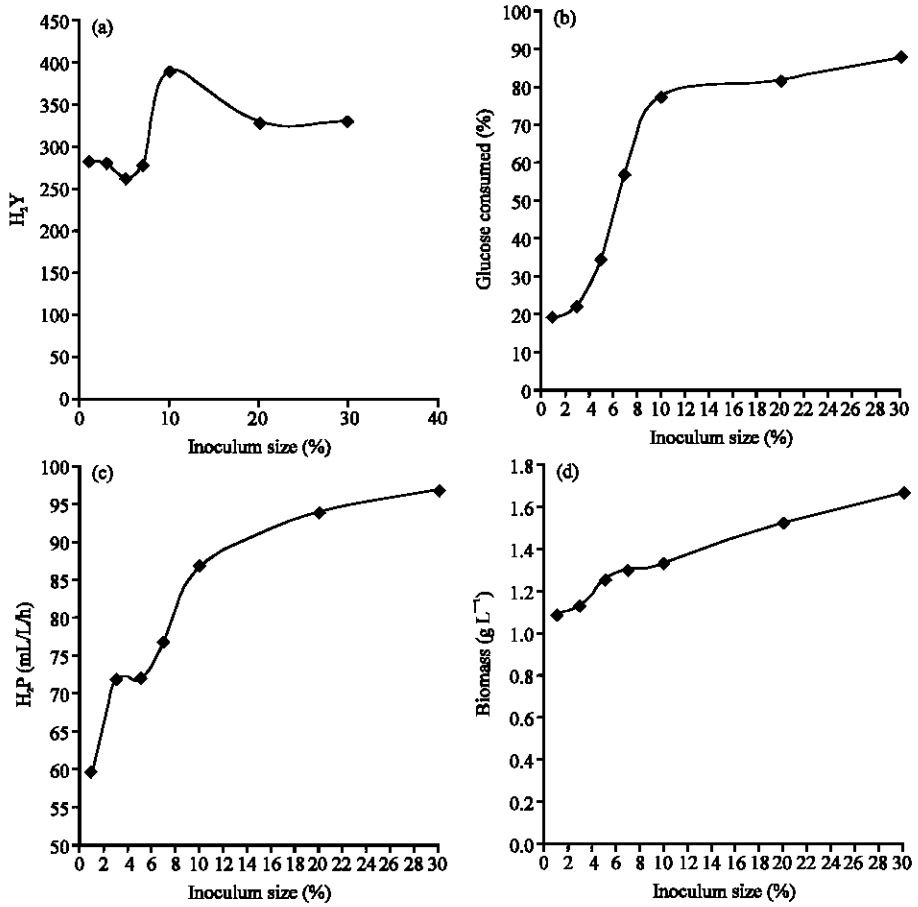

Fig. 2: Results of Inoculum size (%) (v/v): effect on, (a) H_2 Y (mL g^{-1} utilized), (b) Glucose consumed (%), (c) H_2 P (Productivity (mL/L/h) = (H_2 volume/L culture volume, duration time and (d) [Biomass] (g L^{-1}): [Glucose]: 5 g L^{-1}, initial pH 7.0, Temp. 30°C

obtained by using inoculum size of 30% (v/v). These results of bacterial productivity of hydrogen indicated that as the inoculum size increased, the bacterial metabolites would increased which are acids and that would affected adversely due to dropping in culture pH due to higher acids production that would made the culture more favorable for solvent production (second growth phase) and stop the first metabolites production (acids and gases). The shift from acid production to solvent phase was clear from the final pH of culture medium and that connected with the hydrogen yield which was maximized at inoculum size of 10%. The results shown in Fig. 2b indicated that the percentage of consumed glucose at 30% inoculum size was higher than others and that due to higher biomass concentration which resulted to increase the final biomass as shown in Fig. 2d. The results shown in Fig. 2d shown that final biomass concentration was increased as the inoculum size increased but that increase not enhanced the hydrogen yield suggested that inoculum size should be used in proper percentage to culture volume to maximize the bacterial productivity of hydrogen and not to enforce the bacterial shift from acid phase to solvent phase.

The results shown in Table 6 were used to calculate different yields like $Y_{P/X}$ (mL/g/L): (H_2 mL g^{-1} Biomass L^{-1}), $Y_{X/S}$: (Biomass production per g glucose supplied) and $Y_{H2}/_s$ (conversion of H_2 (mL) to H_2 (g) per g glucose utilized). These results showed that proper inoculum size to influent substrate (glucose) enhanced the bacterial productivity of hydrogen. Further increased of inoculum size was shown to affect on negative effect on bacterial productivity of hydrogen suggested that available substrate restrict the metabolism of initial inoculum size (acid or solvent: for *Clostidium*). Concluded that proper inoculum size should be used for maximum bacterial production of any products (here hydrogen).

Hydrogen evolution by *Clostridium* is primarily a growth-associated process and therefore it is expected

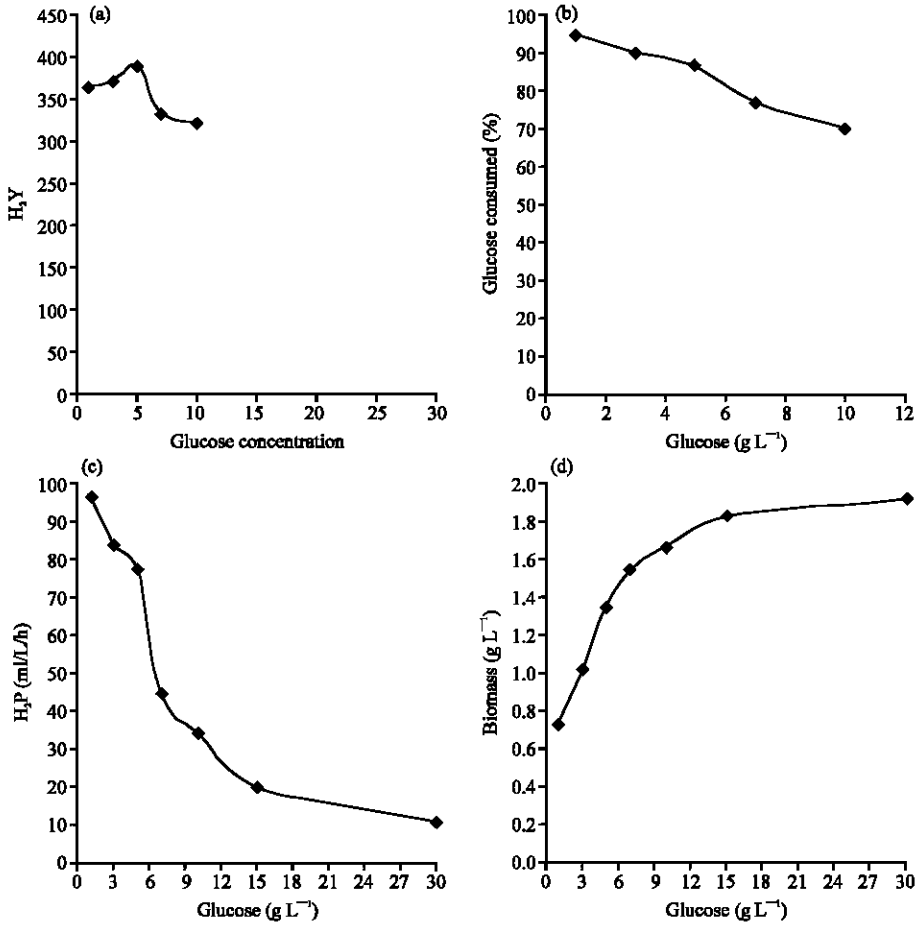

Fig. 3: Results of substrate concentration on (a) H_2 Y (mL g^{-1} utilized), (b) Glucose consumed (%), (c) H_2 P (Productivity (mL/L/h) = (H_2 volume/L culture volume, duration time and (d) [Biomass] (g L^{-1}): Inoculum size 10% (v/v), initial pH 7.0, Temp. 30°C

that the highest rates of evolution be attained during the growth phase. Optimum inoculum size with substrate concentration in fermentation medium gave a better chance for bacteria to meet the available substrate and maximize its production. This implied 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 but not the hydrogen yield. This however disagrees with the findings of Lay (2001) which showed that a low substrate (cellulose) to cell density facilitated high H_2 generation by a mixed hydrogen producing culture.

Effect of substrate concentration: Production of hydrogen in anaerobic fermentation process is accompanied by the breakdown of an organic substrate like glucose in the present study. So, the initial glucose concentration plays an important role in the volume and the rate of hydrogen production during the course of fermentation. The results of earlier study were obtained using 5 g L^{-1} glucose and inoculum size of 10%. The results shown in Table 7 and 8 shown that H_2 production decreased with increasing glucose concentration. Although the highest H_2 production volume was obtained in 30 g L^{-1} glucose supplied, but the H_2 yield reduced by 73% from 347 mL g^{-1} in 1 g L^{-1} glucose supplied to 97 mL g^{-1} in 30 g L^{-1} glucose supplied. Whereas hydrogen yield per glucose utilized decreased with increasing of influent glucose from 365 mL g^{-1} in 1 g L^{-1} to 324 mL g^{-1} in 10 g L^{-1} with maximum yield of 391 mL g^{-1} glucose utilized using 5 g L^{-1}. Furthermore the percentage of consumed glucose of more than 10 g L^{-1} cannot be detected as shown in Fig. 3b. The sharp decreased in hydrogen production for further increased in influent glucose was useful for bacterial growth with maximum biomass concentration of 1.91 g L^{-1} at 30 g L^{-1} of supplied glucose as shown in Fig. 3d. Table 7 and 8 shows all results obtained for the effect of substrate (glucose) concentration on hydrogen production by *C. acetobutylicum* NCIMB13357.

Table 7: Results of substrate concentration on glucose consumption and H_2P (mL/L/h) by *C. acetobutylicum* NCIMB13357

Glucose ($g\ L^{-1}$)	pH	Glucose consumed (%)	H_2P
1	4.55	95	97.0
3	5.45	90	84.0
5	4.44	87	77.5
7	4.49	77	45.0
10	4.51	70	34.5
15	4.59	-	20.0
30	4.66	-	11.0

Inoculum size 10% (v/v) I pH 7.0, Temp. 30°C

Table 8: Results of substrate concentration on H_2 production by *C. acetobutylicum* NCIMB13357

Glucose ($g\ L^{-1}$)	$Y^1_{P/S}$	$Y^2_{P/S}$	[Biomass]	$Y_{P/X}$	$Y_{X/S}$	$Y_{H2/s}$
1	347	365	0.73	500	0.73	0.032
3	336	373	1.02	366	0.34	0.034
5	340	391	1.34	292	0.27	0.036
7	257	334	1.55	215	0.22	0.030
10	227	324	1.67	194	0.17	0.028
15	160	-	1.83	-	0.12	-
30	97	-	1.91	-	0.06	-

$Y^1_{P/S}$: (H_2 mL g^{-1} glucose supplied) (mL g^{-1}), $Y^2_{P/S}$ (mL g^{-1}) (Utilized): (H_2 mL g^{-1} glucose utilized), [Biomass] (g L^{-1}): Biomass production g per L culture, $Y_{P/X}$ (mL/g/L): (H ,mL g^{-1} Biomass L^-),[1] Y $\dot{x}_{/S}$(Biomass production per g glucose supplied), $Y_{H2/s}$: (conversion of H_2 (mL) to H_2 (g) per g glucose utilized) Inoculum size 10% (v/v), initial pH. 7.0 Temp. 30°C

The rate of hydrogen production varied with the variation of glucose content in the feed of the bioreactor. At relatively low initial glucose concentrations of 1-3 g L^{-1}, the rate of fermentation is also low, according to the law of mass action (Fabiano and Perego, 2002). The results shown in Fig. 3a indicated that at initial glucose concentration of 1 to 10 g L^{-1}, the highest hydrogen yield of 391 mg L^{-1} was the maximum by using 5 g L^{-1} compared with further concentration and the batch process was finished earlier than by using higher glucose concentration and that mainly due to the exhausted of available substrate. At 1 to 5 g L^{-1} the results indicated that at these concentrations as shown in Fig. 3c the bacterial productivity with maximum of 97 mL/L/h at 1 g L^{-1} was better than higher substrate concentration and that due to higher inoculum size to glucose concentration, then started too decreased to 77.5 mL/L/h at 5 g L^{-1}. Furthermore, increasing influent glucose gave lower hydrogen productivity and that due to increasing in bacterial production of acids which increased as the glucose substrate increased and that clear from the final pH of the culture medium as shown in Table 7.

The cell concentration was found to increase with increase in glucose concentration in the feed. But the rate of production of hydrogen was maximum by using 0.5% (w/v) glucose concentration in formulated medium, while it decreased with further increase in glucose concentration presumably because of inhibition due to excess substrate. Therefore, Oh *et al.* (2003) attributed that lower H_2 production rate at higher substrate concentration to that the carbon flux at high glucose concentrations is more directed to the production of reduced by-products such as ethanol and organic acids. The trend between the substrate concentration and the H_2 productivity was in an inverse pattern.

The highest productivity of 97 and 11 mL/L/h was obtained in 1 and 30 g L^{-1}, respectively. The reductions in the H_2 yield and bacterial productivity of hydrogen in a concentration more than 5 g L^{-1} in this study were agreed with the suggestion of (Van Ginkel *et al.*, 2001) they attributed that to the shift from the acidogenic to the solventogenic pathways where, H_2 was consumed to reduce the acids to alcohols and with that of a steady decrease in the H_2 yield with increasing substrate concentration observed for starch in starch wastewater (Zhang *et al.*, 2003) and for cellulose (Lay, 2001), whereas disagree with Wu and Lin (2004) they observed a similar trend between the yield and molasses concentration, in which the highest yield was found at a substrate concentration of 40 g COD/L. above finding suggested that hydrogen production by fermentative bacteria depend in both microorganism (s) used and substrate used.

The results shown in Table 8 were used to calculate different yields like $Y_{P/X}$ (mL/g/L): (H_2 mL g^{-1} Biomass L^{-1}), $Y_{X/S}$: (Biomass production per g glucose supplied) and $Y_{H2/s}$ (conversion of H_2 (mL) to H_2 (g) per g glucose utilized). These results showed that as it is worth mentioned in above about the effect of inoculum size, the effect was same and both sections underscore the proper initial substrate concentration to inoculum size would enhanced the bacterial productivity of hydrogen. Further increased of substrate was shown to enforce the bacteria to make shift from acid phase to solvent phase and stop the first metabolites production and that clear from the final pH of different glucose concentration and total hydrogen produced, suggested that lower glucose concentration was necessary for maximum bacterial productivity of hydrogen with the inverse at higher concentration but the yield per glucose was maximum at proper concentration.

It was noted that investigators have reported H_2 yield as mol H_2 per mol substrate, mol H_2 per gram substrate or H_2 produced (mL) per gram substrate; hence, for ease of comparison with values reported, the H_2 yields were all converted to H_2 produced (mL) per gram substrate utilized. On that basis, it was found that the maximum yield of 3.13 molH_2/mol-glucose utilized (391 mL g^{-1}) (2.72 mol/mol glucose supplied) obtained in this study was higher than other reported values even with different substrate. Present results regarding the yield was higher

than the reported values of 132 mL g^{-1} glucose supplied (Oh *et al*., 2003), 92 mL g^{-1} starch supplied in starch wastewater (Zhang *et al*., 2003), 193 mL g^{-1} cellulose supplied (Ueno *et al*., 2001), 134 mL g^{-1} molasses supplied (Logan *et al*., 2002), 126 mL g^{-1} sucrose supplied (Lee *et al*., 2002) and than 314 mL g^{-1} sucrose supplied reported by Lin and Lay (2004). This indicated that *C. acetobutylicum* NCIMB13357 is one of the most efficient hydrogen producers.

DISCUSSION

Unlike other H_2-forming species, such as green algae, production of hydrogen in anaerobic fermentation is accompanied by the breakdown of organic substrate, here glucose. So, the initial glucose concentration plays an important role in the yield and overall production rate of hydrogen during the course of fermentation. It has already been reported that substrate inhibition gets predominant at higher glucose concentration because this modifies the metabolic pathways (Fabiano and Perego, 2002).

pH is one of the important controlling factors of anaerobic fermentation processes. It has a profound effect on evolution rate of hydrogen. A major factor affecting H_2 or solvent production is the pH. Jones and Woods (1986) reported that optimum pH for H_2 production is 5.5, while the optimum pH for solvent production is ~ 4.5. The pH can affect the form of the acids produced during hydrogen production. Mizuno *et al*. (2000) indicated that during fermentation some organic acids were produced as metabolic products. Accumulation of these acids causes a sharp drop of culture pH and subsequent inhibition of bacterial hydrogen production. It was shown from their results that each species have optimum initial pH to initiate and get maximum production. Nath *et al*. (2006) found that the optimum initial pH for hydrogen production was 6.5 using *Enterobacter cloacae* DM11 and Cane Molasses as Feedstock. Mizuno *et al*. (2000) stated that poor hydrogen production rate at low pH; lower than 5.5, could be due to the increased formation of acidic or alcoholic metabolites, which destroys the cell's ability to maintain internal pH. Bowles and Ellefson (1985) suggested that, at low pH it might have resulted in lowering of intracellular level of ATP, thereby inhibiting glucose uptake. Gottschal and Morris (1981) reported that solvent production was also not observed when *C. acetobutylicum* was grown in continuous culture with glucose or nitrogen limitation at pH value of 6.5 or 5.7. A detailed description of the mechanisms of acid inhibition can be found in Jones and Woods (1986). Van Ginkel and Logan (2005b) reported that the concentrations of the undissociated forms of acetic or butryic acid are ten times greater at a pH of 4.5 than at pH 5.5 and thus higher amounts of the undissociated form are present at the lower pH to cause inhibition. Undissociated acid concentrations can also be increased by increasing the substrate concentration from which the acids are produced. However, cultures fed high sugar concentrations are susceptible to both substrate and product inhibition. The substrates most widely used in biohydrogen research are sugars and the products are primarily H_2 and CO_2 gases and acetic and butyric acids. Many researchers have studied the effects of these acids on solvent production in traditional Acetone-Butanol-Ethanol (ABE) fermentation (Monot *et al*., 1984). However, the hydrogen fermentation was found to change into a solvent forming reaction once the undissociated acid concentration reached a critical threshold, but the threshold cannot be well predicted as it varies substantially over the range of ~2-30 mM (undissociated acid concentration) (Grupe and Gottschalk, 1992). It was reported by Bowles and Ellefson (1985), that butanol at higher concentration would inhibit the *C. acetobutylicum* growth, would destroy the ability of the cell to maintain internal pH, lowered the intracellular level of ATP and finally to inhibit the glucose uptake.

Van Ginkel and Logan (2005a) reported that inhibition is caused by nonpolar undissociated acids which being able to cross the cell membrane at a low pH, that then dissociate in the cell at the higher internal pH releasing a proton inside the cell. The uptake of protons in this way uncouples the proton motive force which causes an increase in maintenance energy requirements. The uptake of acid also causes a decrease in the available coenzyme A and phosphate pools which decreases the flux of glucose through glycolysis

Obtained results show that, at higher substrate concentration the quantity of produced gas was higher but the yield per glucose consumed was lower. Produced liquid (acid and solvent mainly butanol) and gas affect inversely on the bacterial metabolism and its growth. It was reported by Ruzicka (1996), that under high gas pressure, the hydrogen gas would dissolved in fermentation medium and that would affect inversely on bacterial metabolism and its growth beside the glucose cleavage. Lee and Zinder (1988) stated that continuous H_2-synthesis requires the partial pressure of H_2 to be <50 kPa at 60°C. At pH_2 <50 kPa, H_2 inhibits NADH reoxidation and electron flow via ferredoxin becomes thermodynamically unfavorable. Thus, high pH_2 may be a principle reason for decrease in H_2 synthesis rates under carbon-excess conditions. Thus the results obtained, also emphasize the need to optimize simultaneously the effect of initial glucose concentration with inoculum size with optimum initial pH for maximizing hydrogen productivity.

CONCLUSION

The results of this study suggested that proper culture pH of 7.0, inoculum size of 10% (v/v) and glucose concentration of 5 g L^{-1} showed to enhance the bacterial production of hydrogen as well as final biomass. Therefore this study demonstrate that hydrogen production can be maximized by optimize both inoculum size and substrate concentration with initial pH.

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