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Biological hydrogen production: effects of pH and intermediate products

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Abstract

A series of batch tests were conducted to investigate the effects of pH and intermediate products on biological hydrogen production. The tests were run in serum bottles to determine the optimal operating conditions to maximize hydrogen production using sucrose and starch as organic substrates. Apart from hydrogen, variations in pH, volatile fatty acids, and solvent concentrations were also monitored. Initial pH was found to have a profound effect on both hydrogen production potential and hydrogen production rate. A mixed microbial culture was involved in the fermentation process with H_2, propionate, acetate, butyrate, and CO_2 as major products. The lowest initial pH of 4.5 gave the highest specific hydrogen production potentials of 214.0 ml H_2/g chemical oxygen demand (COD) and 125.0 ml H_2/g COD for sucrose and starch respectively, but with the lowest specific hydrogen production rate. Although hydrogen production started earlier with the high production rate at a higher initial pH, the duration of the production was shorter. The rapid pH depletion could have caused a metabolic alteration of the microorganisms involved in hydrogen production, thereby resulting in the shift of intermediates production pathway [variation of the acetate/butyrate (HAc/HBu) ratio] and a consequent decrease in hydrogen production. The specific hydrogen production rate was highest for the pH range of 5.5–5.7. For the optimum pH range, the HAc/HBu ratio was in the range of 3–4 for both sucrose and starch. The findings of this study can be applied in the design of a high rate hydrogen bioreactor.

Keywords: pH; Hydrogen fermentation; Biological hydrogen production; *Clostridium*; Acetate/butyrate ratio; Mixed culture

1. Introduction

The environmental consequences of extensive use of fossil fuels have already begun to surface. The excessive use of fossil fuels is one of the primary causes of global warming and acid rain, which have started to affect the earth's climate, weather condition, vegetation and aquatic ecosystems [1]. Considering the energy security and the global environment, there is a pressing need to develop non-polluting and renewable energy source. Hydrogen is a clean energy source, producing water as its only by-product when it burns. Hydrogen can be produced from renewable raw materials such as organic wastes. Therefore, hydrogen is a potential clean energy substitute for fossil fuels. Additionally, there is also a need to dispose of human-derived wastes in an environmentally friendly manner. Some of these wastes are by-products/residuals of food processing plants and agricultural residues and are rich in carbohydrate content. Microbial hydrogen production through anaerobic fermentation could be a bioenergy process that couples the need for waste reduction/treatment and byproduct recovery.

Acidogens of genus clostridia have been studied for their ability to form spores and for their potential to generate hydrogen gas [2–4]. Clostridia are classified as proteolytic or saccharolytic depending of types of organics they ferment.

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Proteolytic acidogens degrade proteins or amino acids. Saccharolytic acidogens ferment carbohydrate and are widely studied because of their ability to produce higher level of hydrogen. One of the widely studied saccharolytic clostridia is *Clostridium butyricum* which produces butyric acid as the major fermentation product together with CO_2, acetate and H_2 [5]. The hydrogen fermentation reactions for sucrose as organic substrate are shown by Eqs. (1) and (2):

$$\underset{\text{(sucrose)}}{C_{12}H_{22}O_{11}} + 5H_2O \rightarrow \underset{\text{(acetic acid)}}{4CH_3COOH} + 4CO_2 + 8H_2 \uparrow, \quad (1)$$

$$\underset{\text{(sucrose)}}{C_{12}H_{22}O_{11}} + H_2O \rightarrow \underset{\text{(butyric acid)}}{2CH_3CH_2CH_2COOH} + 4CO_2 + 4H_2 \uparrow . \quad (2)$$

This pathway is found in approximately 50% of all clostridia that have been isolated to date. Other fermentation pathways found in sacchrolytic clostridia are those leading to the production of propionate by *Clostridium arcticum* [6], succinate by *Clostridium coccoides* [7], and lactate by *Clostridium barkeri* [8]. The clostridial genus is an obligate anaerobic heterotroph that does not contain a cytochrome system [9]. This genus produces hydrogen using the activities of pyruvate-ferredoxin-oxidoreductase and hydrogenase enzymes. The activity of hydrogenase, an iron-containing enzyme is inhibited by low pH, which was reported to be one of the most important factors in the overall hydrogen fermentation [10–13]. In certain cases, the hydrogen evolution activity of hydrogenase may be suppressed in the presence of high levels of hydrogen [14]. A decrease in hydrogen concentration will favor hydrogen formation and permit bacteria to metabolize acetyl-CoA through the energy-efficient path leading to acetate and ATP production.

In a typical anaerobic process, hydrogen is produced during the exponential growth phase of clostridia [5]. When the population reaches the stationary growth phase, the reactions shift from a hydrogen/acid production phase to a solvent production phase. This shift occurs when the pH drops to 4.5 or below [15]. Apparently the build up of volatile fatty acids and hydrogen during the exponential growth phase induces this shift. While other researchers found that the shift occurred at pH levels above 5.7, due to enzyme synthesis or enzyme activation, which is necessary for solvent production [16]. Thus, it is important to remove excess hydrogen from the system and control the pH at an optimal range to maintain hydrogen production. If hydrogen builds up, higher molecular weight acids such as butyric and propionic acids are accumulated in the system [17]. On the other hand, if the pH is not maintained in the desired range, it could inhibit hydrogen production or cause a microbial population shift resulting in cessation of hydrogen production.

In practice, during the fermentation process both butyrate and acetate are formed, but the ratio may vary with growth conditions within thermodynamically determined limits [18]. Van Andel et al. [19] found that decreasing the partial pressure of hydrogen results in an increase in the *acetate/butyrate* ratio accompanied by an increased production of hydrogen. Therefore, if we know the actual metabolic pattern, it would be possible to drive the pathway towards a higher *acetate/butyrate* ratio so as to enhance hydrogen production by controlling environmental conditions such as pH, mixing intensity, hydraulic retention time (HRT), organic loading rate and nutrients.

Based on these backgrounds, the objectives of this study were to investigate the effects of environmental factors such as initial pH, partial pressure of hydrogen, and intermediate products on hydrogen production and to determine the optimum operational pH range for hydrogen production based on specific hydrogen production rate.

2. Materials and methods

2.1. Seed microorganisms

Compost material obtained from Iowa State University composting pile was used as a seed source. It was baked in the oven at 105°C for two hours and then cooled to room temperature in a desiccator. The baked compost material was ground and sieved through a No. 30 sieve. The sieved fines were mixed with nano-pure water to make a 2.0 l slurry solution. It was settled for 30 s to remove sands and the final volume was adjusted to 2.0 l again with nano-pure water. 8.0 g K_2HPO_4 and 21.0 g Na_2HPO_4 were added as pH buffer.

2.2. Experimental procedure

The experiment was conducted in a series of 250 ml serum bottles at different initial pH. The seed slurry was mixed completely and an aliquot of the slurry was added into each serum bottle. To each bottle, 1.5 g sucrose or 10.0 ml concentrated starch solution (150.0 g/l) and 0.5 ml of nutrient stock solution were added. Each liter of nutrient stock solution contained 200.0 g of NH_4HCO_3, 100.0 g of KH_2PO_4, 10.0 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of NaCl, 1.0 g of $Na_2MoO_4 \cdot 2H_2O$, 1.0 g of $CaCl_2 \cdot 2H_2O$, 1.5 g of $MgSO_4 \cdot 7H_2O$ and 0.278 g of $FeCl_2$, which was slightly modified from Lay et al. [3]. The bottles were filled to the 150 ml mark using nano-pure water. pH was adjusted by adding few drops of concentrated HCl or NaOH solution to the desired initial levels of 4.5, 5.0, 5.5, 6.0 and 6.5. The bottles were flushed with oxygen free nitrogen gas and capped tightly with butyl rubber before being put on an orbital shaker table running at 180 rpm in a 37 ± 1°C constant temperature room. Each sample was duplicated and two control bottles (blank) were also prepared without addition of substrate. Biogas produced was measured by

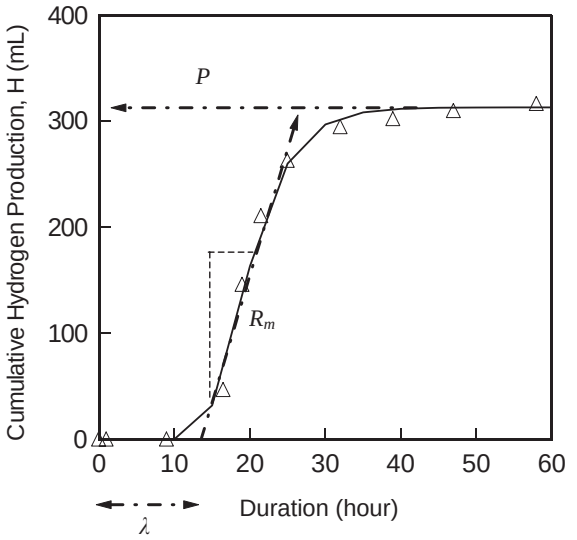

Fig. 1. Typical cumulative hydrogen production curve fitted by the Modified Gompertz equation at initial pH of 5.5 with sucrose as substrate. Open triangles are experimental data. Line is nonlinearly modeled using Eq. (3).

the plunger displacement method with appropriately sized wetted glass syringes [20].

2.3. Analytical methods

The composition of biogas in the headspace was determined using two gas chromatographs (Gow Mac series 350) equipped with thermal conductivity detectors (TCD). Hydrogen was analyzed using one GC-TCD connected with an $8' \times 1''$ stainless column—SS 350A Molesieve 13X (80/100 mesh). Nitrogen was used as the carrier gas at a flow rate of 30 ml/min. The operational temperatures of the injection port, the oven and the detector were 100°C, 50°C, and 100°C, respectively. Methane and carbon dioxide in biogas were measured using another GC-TCD connected with a 3.3′ stainless column packed with Porapak T (60/80 mesh). The operational temperatures of the injection port, the oven and the detector were maintained at 100°C, 50°C, and 100°C, respectively. Helium was used as a carrier gas at a flow rate of 35 ml/min. A modified Gompertz equation [Eq. (3)] was used to fit the cumulative hydrogen production curves for each bottle to obtain the hydrogen production potential P, the hydrogen production rate R and lag phase λ as illustrated in Fig. 1 [21].

$$H = P \exp\left\{-\exp\left[\frac{R_m.e}{P}(\lambda - t) + 1\right]\right\} \quad (3)$$

where, H is the cumulative hydrogen production (ml), λ the lag-phase time (h), P the hydrogen production potential (ml), R_m the maximum hydrogen production rate (ml/h), t the incubation time (h), e the exp(1) = 2.718.

Parameters (P, R_m and λ) were estimated using the solver function in Microsoft Excel version 5.0 (Microsoft, Inc.). This program uses a Newton algorithm. Up to a hundred iterations were used to converge the ratio of sum of square error (SSE) to correlation coefficient (R^2) between the experiment and the estimate to a minimum. Starting parameter values were estimated using a built-in visual procedure based on a limited fit algorithm [22]. The specific hydrogen production potential, P_s (ml/g chemical oxygen demand—COD), was obtained by dividing P by substrate COD applied. The specific hydrogen production rate, R_s (ml/h g VSS), was obtained by dividing R_m by volatile suspended solids (VSS) added. Hydrogen conversion efficiency for different substrates was compared based on P_s and R_s.

Liquid samples were collected 6–7 times during the process that lasted as long as 160 h using a 10 ml plastic syringe. 2 ml sample was taken each time and placed into a 15 ml centrifuge tube. pH was measured using a calibrated pH probe (Cole Parmer, Model 05669-20). The liquid was centrifuged and the supernatant was transferred to a 2 ml centrifuge tube with the addition of few drops of concentrated sulfuric acid. The samples were mixed, capped and kept in a 4°C before being analyzed. Individual volatile fatty acids (VFA) and solvents were analyzed using another gas chromatograph (Gow Mac series 580) equipped with a flame ionization detector (FID). The column used was a $6' \times 8''$ stainless column—SS 580 FID, 10%SP-1200/1% H_3PO_4 (80/100 mesh). The operational temperatures of the injection port, the oven and detector were maintained at 140°C, 100°C, and 140°C, respectively for volatile acids analysis, and 170°C, 70°C, and 170°C, respectively for solvent analysis. Helium was used as the carrier gas at a flow rate of 40 ml/min. COD, suspended solids (SS), VSS were measured according to Standard Methods [23].

3. Results and discussion

3.1. Effects of initial pH

In a previous exploratory study in our laboratory by Van Ginkel et al. [24], it was concluded that initial pH did not have as profound effect on hydrogen production rate as it had on specific hydrogen production potential. To confirm these results, a series of batch tests were conducted using two different substrates—starch and sucrose at the same concentration (on COD basis) but with different initial pHs. The cumulative hydrogen productions from sucrose and starch are shown in Figs. 2a and b.

The plot showed that the initial pH did have an effect on both hydrogen production potential and hydrogen production rate. The higher the initial pH was, the lower the total hydrogen production potential was. Visual observation showed no difference in hydrogen production rates at initial pH above 5.0 for both starch and sucrose, however a paired T-test showed significant difference between

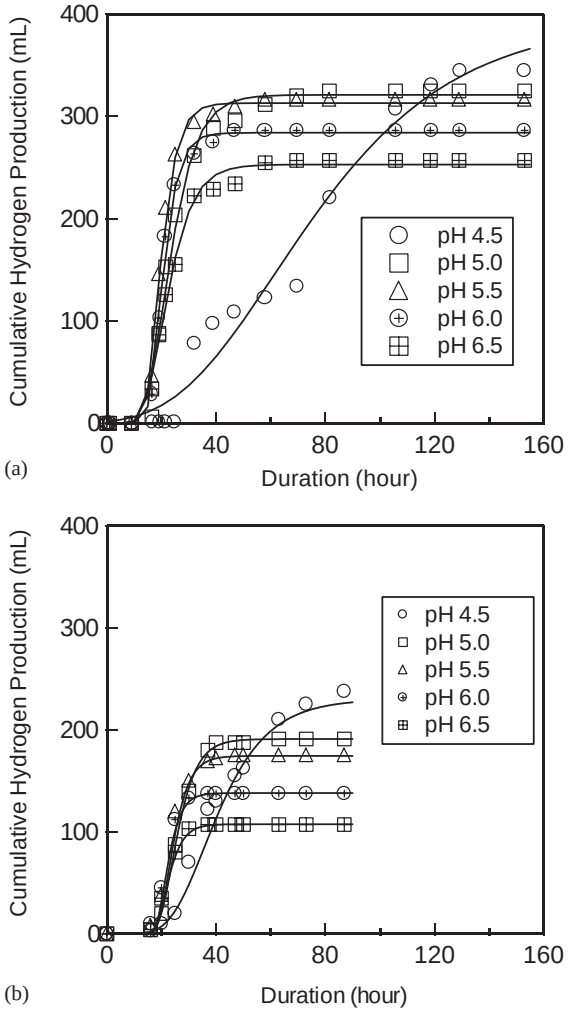

Fig. 2. Cumulative hydrogen production at different initial pH levels: (a) from sucrose and (b) from starch.

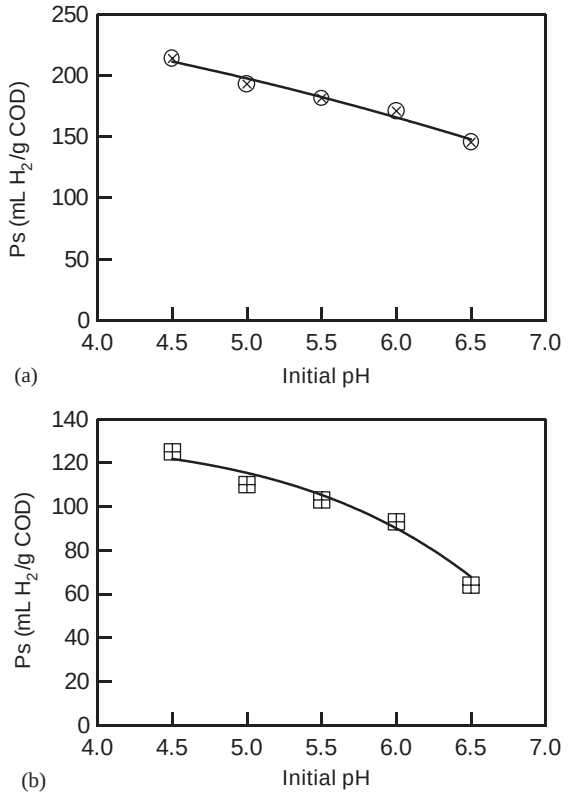

Fig. 3. Specific hydrogen production potential at different initial pH levels: (a) sucrose and (b) starch.

samples with a p-value of 0.05. The initial pH effect was more pronounced for starch than that for sucrose. This might be because of easy degradability of sucrose compared to starch, which needs to be hydrolyzed before being readily available.

To better understand pH effect on hydrogen production, the specific hydrogen production potential (P_s) and lag phase (λ) obtained from the Gompertz model were plotted against the corresponding initial pH values as shown in Figs. 3 and 4, respectively. Sucrose and starch batches showed similar trends for specific hydrogen production potential (Fig. 3). The specific hydrogen production potential showed a declining trend with an increasing pH for both sucrose and starch (Fig. 4).

As to the lag time, a similar trend was also observed for both sucrose and starch as shown in Fig. 4. However, the bottles with starch had 3–4 h longer lag phase than that with sucrose at all pH levels. For both starch and sucrose, the bottles with initial pH of 4.5 had the longest lag phase, while the others had similar lag phase with 14 ± 1 and 18 ± 1 h for sucrose and starch batch, respectively. The longer lag phase for starch was primarily attributed to the complex nature of substrate (α-linkage) compared to sucrose.

To further elucidate the observed results, the pH profile during the course of hydrogen production was plotted for each bottle as shown in Figs. 5a and b. From the figures, it was apparent that at a higher initial pH, there was a greater drop in pH with a shorter duration of hydrogen production. It seems to suggest that the rapid hydrogen production was accompanied with rapid acid production to the inhibitory levels which simultaneously depleted the buffering capacity (Figs. 6a and b). The hydrogen producers could not adapt to the fast change in environment and may have been inhibited. On the other hand, at a lower initial pH level (especially pH of 4.5), the starting environment might not be suitable for hydrogen producers. However, with their adaptation and limited self-adjustment of environmental conditions such as pH [16], they started to produce hydrogen gradually at a moderate rate. The level of acids produced was insufficient to cause a dramatic depletion of pH. The active hydrogen

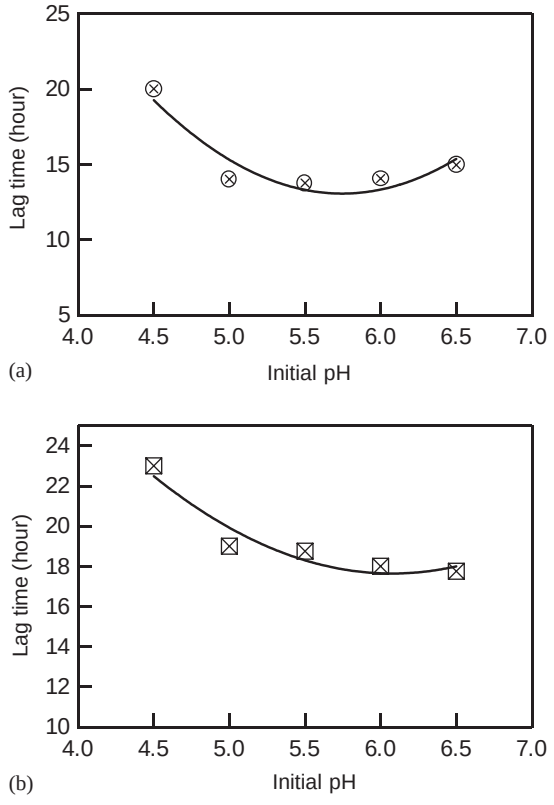

Fig. 4. Lag phase at different initial pH levels for starch: (a) sucrose and (b) starch.

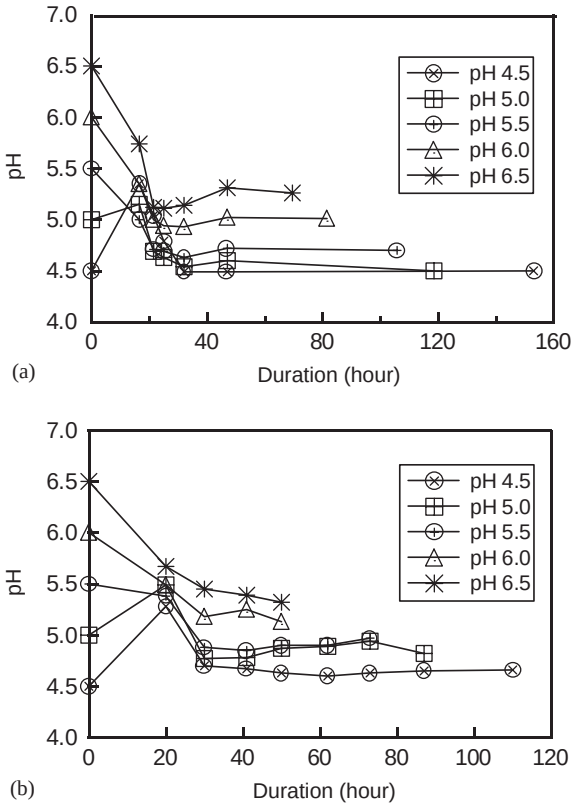

Fig. 5. pH profile during hydrogen production at different initial pH levels: (a) for sucrose and (b) for starch.

producers could exist longer in a relatively consistent environment.

Van Ginkel et al. [24] reported that maximum hydrogen production rate occurred before any inhibitory effects were observed. On the basis of this observation, they might have undermined the effect of initial pH value on hydrogen production rate. Without proper monitoring of pH profile during the course of the reaction, it would be difficult to determine the role of pH in hydrogen fermentation. Moreover, by varying both the initial pH and substrate concentration in their tests, it would not be possible to study the sole effect of two variables at the same time. Nevertheless, it is interesting to note that with the lag phase, which was about 14 h for sucrose and 18 h for starch, the optimal pH for hydrogen production was found to be in the range of 5.5–6.0 (Fig. 4). This is in close agreement with the earlier works [3,24,25] where pH of 5.5 was deemed the optimum initial pH for hydrogen production.

3.2. Optimum operational pH

With continuous monitoring of pH and hydrogen production during hydrogen fermentation in the serum bottle test, it is possible to determine the actual pH values that yield maximum hydrogen. This pH data could be employed in developing continuous flow reactor system for the maximum hydrogen yield. In a continuous flow system, it is possible to maintain a constant high rate of microbial growth by controlling the operational parameters such as HRT/organic loading rate, solids retention time (SRT), among others [26,27]. However, pH being the most pivotal parameter for *Clostridium*-rich bioprocess [28], the operation of hydrogen fermentation in the continuous reactor system is driven by its operating pH. With this in mind, a combined plot was developed by superimposing the cumulative hydrogen production curve fitted with Gompertz model and pH profile plot for the individual bottles. The pH values corresponding to maximum hydrogen production rate were determined from the combined plot. Specific hydrogen production rates were then obtained by dividing the maximum hydrogen production rates by the corresponding biomass (VSS) in each bottle. The results are presented in Figs. 7a and b. The results showed that for both sucrose and starch, the maximum specific hydrogen production rate occurred at the same pH range of 5.5–5.7. In field application, the required optimum pH needs to be assessed for each specific waste stream.

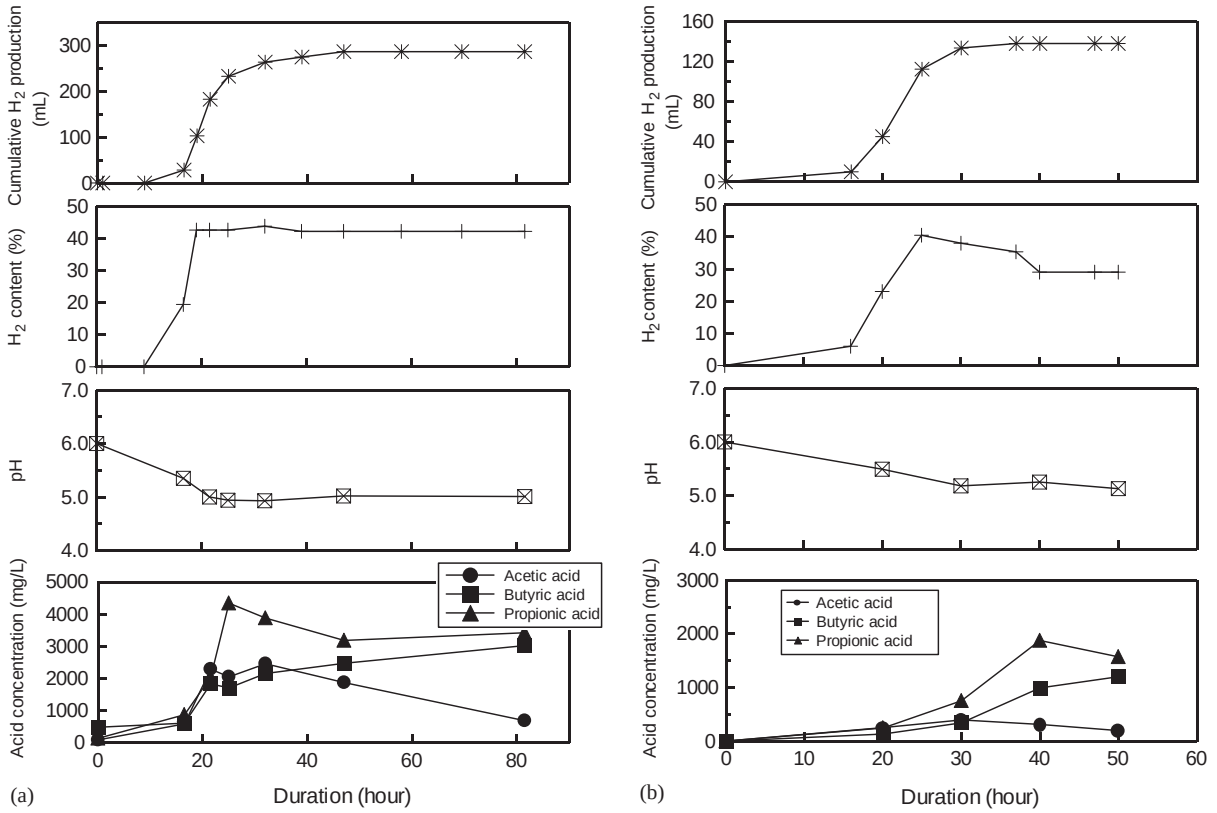

Fig. 6. Hydrogen production, hydrogen content, pH and volatile acids profiles: (a) for sucrose (at initial pH = 6.0) and (b) for starch (at initial pH = 6.0).

3.3. Intermediate products and its effects

Hydrogen production is usually accompanied by acid production coupled with solvent production. The production of these intermediates reflects changes in the metabolic pathway of the microorganisms involved and a better knowledge of such changes could improve our understanding of the conditions favorable for hydrogen production. Thus, during the course of hydrogen production, liquid samples were collected and analyzed for individual VFAs and solvents.

The major VFAs detected in the process were propionate, acetate and butyrate. The typical VFAs production profile is shown in Figs. 6a and b for sucrose and starch, respectively. These results were obtained from serum bottles with an initial pH of 6.0. A gradual production of acids depleted the buffering capacity that resulted in a concomitant decline in pH to about 5.5 before hydrogen production began. This was followed by a rapid production of hydrogen with an increase in acids production until the stationary phase of hydrogen production was achieved. The hydrogen content in the biogas reached a maximum value of about 40%, before the maximum hydrogen gas production was attained. In the stationary phase, the production of acetate decreased slightly but an increase in butyrate production was noticed. Nevertheless, acetate and butyrate levels were independent of pH in the range studied. This finding was in close agreement with the results obtained by Van Andel et al. [19] based on a study with pure culture of *Clostridium butyricum*.

To have a better understanding of the change in metabolic pathway, the ratio of acetate (HAc) and butyrate (HBu) was plotted together with the cumulative hydrogen production for different bottles in the two batches as shown in Figs. 8a and b. The HAc/HBu ratio during hydrogen production showed a similar pattern for all the bottles—the maximum ratio was observed during the exponential growth phase and this was followed by the stationary phase of hydrogen production at which the ratio showed a declining trend. Although acetate and butyrate levels were independent of the initial pH, the maximum ratio was higher at lower initial pH. This coincided with the trend of total hydrogen production for all bottles. For optimum pH range of 5.5–5.7, the HAc/HBu ratio was found in the same range of 3–4 for both sucrose and starch.

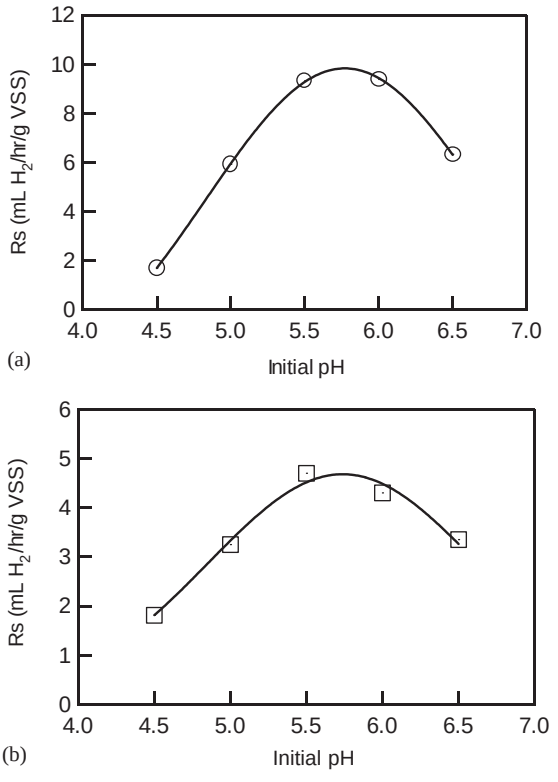

Fig. 7. Maximum specific hydrogen production rate (R_s) at different initial pH levels: (a) for sucrose and (b) for starch.

According to the major metabolic pathway involved in hydrogen production as shown by Eqs. (1) and (2), it might be interpreted that a lower initial pH could have driven the reaction predominantly towards acetate production resulting in higher production of hydrogen. The changes in acetate/butyrate ratio implied a metabolic alteration due to environmental changes such as pH, partial pressure of hydrogen (hydrogen content in the biogas) and the accumulation of intermediate products in the bottles.

It is interesting to note that the levels of propionate detected in the present study were consistently higher than acetate and butyrate. This was different from the results obtained by other researchers [25,29,30]. In most of these experiments, butyrate was the predominant byproduct. This suggests the presence of not only *C. butyricum* but also other species of propionate producing saccharolytic clostridia e.g. *C. arcticum* [31], *C. novyi* [32] and *C. propionicum* [33] in the serum bottles. This fact strengthened the view that a mixed culture was involved in the hydrogen production. This adds significant meaning to the engineering prospect since a mixed culture is more viable than a pure one in hydrogen fermentation from organic wastes.

During hydrogen production, the concentration of solvents produced in all serum bottles was less than 500 mg/l. This implied that microbial shift to solventogenesis did not occur in the pH range of 4.5–6.5. In his earlier work, Lay [25] stated that microbial shift from hydrogen/acid production to solventogenesis would occur at a pH level as low as 4.1. Above this level, metabolic alternation but not hydrogenase activity is the most likely cause for decrease in hydrogen production rate.

4. Summary and conclusions

pH and intermediate products especially volatile fatty acids drive the hydrogenase reaction during hydrogen fermentation. Besides, pH control is also important to suppress hydrogen consumers and to obtain an enriched culture of hydrogen producing clostridia. A series of serum bottle tests were conducted to examine the effect of pH and intermediate products on hydrogen production using sucrose and starch as organic substrates. By detailed monitoring of pH changes and intermediates formation during hydrogen fermentation, it was found that initial pH did have an effect on both hydrogen production potential and hydrogen production rate. At lower initial pH, higher total hydrogen production potential, but lower hydrogen production rate was observed. This effect was more pronounced for hydrogen production from starch than from sucrose. The maximum specific hydrogen production potentials were 214 ml H_2/g COD and 125 ml H_2/g COD at initial pH of 4.5 for sucrose and starch respectively. An initial pH of 4.5 delayed the hydrogen production, but it lasted longer in comparison to a higher initial pH. For the samples with higher initial pH, the changes in environmental conditions caused by the rapid depletion of pH might have resulted in a metabolic alteration, and subsequent inhibition of hydrogen production. The changes in the intermediates production pattern, especially the concentration ratio of HAc/HBu support this hypothesis. The stationary phase of hydrogen production for all serum bottles occurred after the HAc/HBu ratio decreased. For pH range of 5.5–5.7, the HAc/HBu ratio was found in the same range of 3–4. Based on the evaluation of maximum hydrogen production rate, the optimum operational pH range was about 5.5–5.7. This result could be applied in future continuous flow processes to maintain a high rate of hydrogen production.

Acknowledgements

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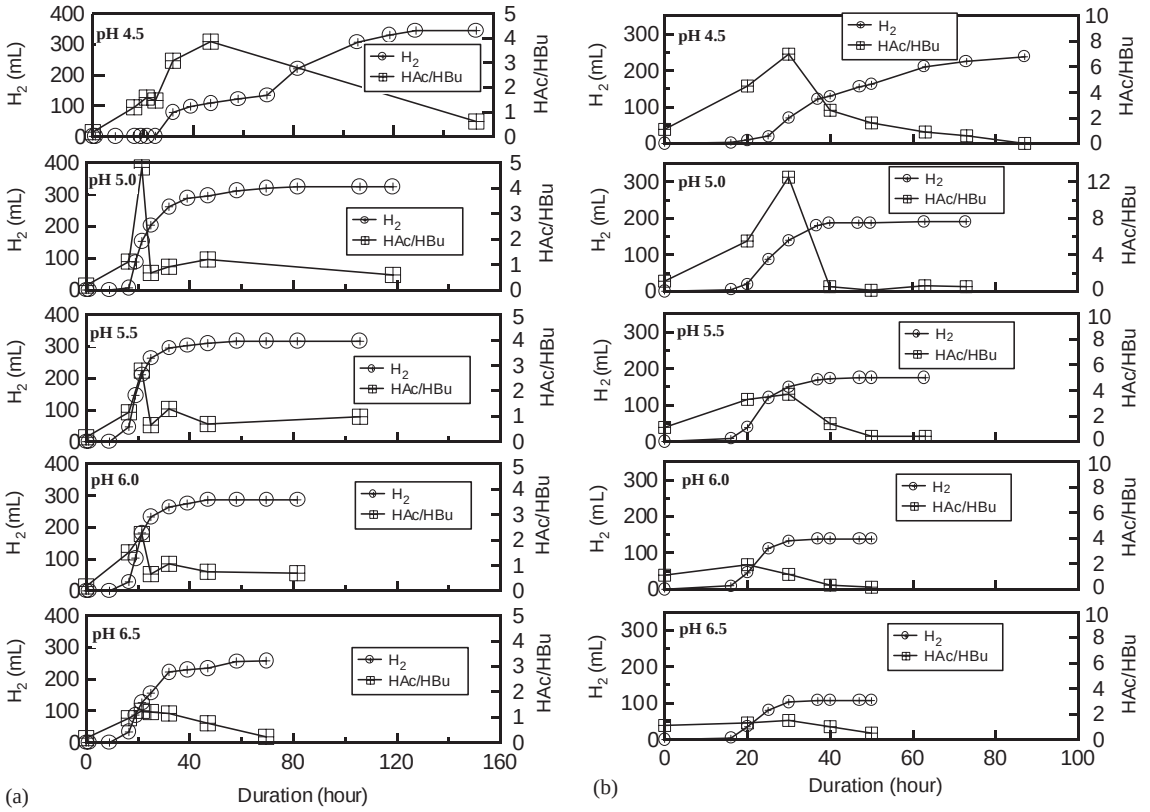

Fig. 8. The profile of HAc/HBu ratio and cumulative hydrogen production at different initial pH levels: (a) for sucrose and (b) for starch.

References

[1] Hansen J, Johnson D, Lacis A, Lebedeff S, Lee P, Rind D, Russell G. Climate impact of increasing atmospheric carbon dioxide. Science 1981;213(4511):957–66.

[2] Brosseau JD, Zajic JE. Hydrogen gas production with *Citrobacter intermedius* and *Clostridium pasteurianum*. J Chem Technol Biotechnol 1982;32:496.

[3] Lay JJ, Lee YJ, Noike T. Feasibility of biological hydrogen production from organic fraction of municipal solid waste. Water Res 1999;33:2576–86.

[4] Ueno Y, Kawai T, Sato S, Otsuka S, Morimoto M. Biological production of hydrogen from cellulose by nature anaerobic microflora. J Ferment Bioeng 1995;79:395–7.

[5] Minton NP, Clarke DJ. Biotechnology handbooks, vol. 3: *Clostridia*. New York: Plenum Press; 1989.

[6] Jones DT, Woods DR. Solvent production. Biotechnology handbooks, vol. 3: *Clostridia*. New York and London: Plenum Press; 1989.

[7] Kaneuchi C, Benno Y, Mitsuoka T. *Clostridium coccoides*, a new species from feces of mice. Int J Syst Bacteriol 1976;26:482–6.

[8] Stadtman ER, Stadtman TC, Pastan I, Smith LDS. *Clostridium barkeri* sp. J Bacteriol 1972;110:758–60.

[9] Nandi R, Sengupta S. Microbial production of hydrogen: an overview. Crit Rev Microbiol 1998;24:61–84.

[10] Afschar AS, Schaller K, Schurgerl K. Continuous production of acetone and butanol with shear-activated *Clostridium acetobutylicum*. Appl Microbiol Biotechnol 1986;23: 315–22.

[11] Dabrock B, Bahl H, Gottschalk G. Parameters affecting solvent production by *Clostridium pasteurianum*. Appl Environ Microbiol 1992;58:1233–9.

[12] Holt RA, Cairns AJ, Morris GJ. Production of butanol by *Clostridium puniceum* in batch and chemostat culture. Appl Microbiol Biotechnol 1988;27:319–24.

[13] Ueno Y, Otsuka S, Morimoto M. Hydrogen production from industrial wastewater by anaerobic microflora in chemostat culture. J Ferment Bioeng 1996;82:194–7.

[14] Kondratieva EN. Production of molecular hydrogen in microorganisms. Adv Biochem Eng 1983;28:139–91.

[15] Byung HK, Zeikus JG. Importance of hydrogen metabolism in regulation of solventogenesis by *Clostridium acetobutylicum* continuous culture system of hydrogen-producing anaerobic bacteria. Proceedings of the Eighth International Conference on Anaerobic Digestion, vol. 2, 1985. p. 383–90.

[16] Gottwald M, Gottschalk G. The internal pH of *Clostridium acetobutylicum* and its effect on the shift from the acid to solvent formation. Arch Microbiol 1985;143: 42–6.

[17] Grady Jr. CPL, Daigger GT, Lim HC. Biological wastewater treatment, 2nd ed., 1999 [Chapter 13.9].

[18] Thauer RK, Jungermann K, Decker K. Energy conservation in chemotrophic anaerobic bacteria. Bacterial Rev 1977;1: 100–80.
[19] Van Andel JG, Zoutberg GR, Crabbendam PM, Breure AM. Glucose fermentation by *Clostridium butyricum* grown under a self generated gas atmosphere in chemostat culture solvent formation. Appl Microbiol Biotechnol 1985;23: 21–6.
[20] Owen WF, Stuckey DC, Healy Jr. JB, Young LY, McCarty PL. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. Water Res 1979;13:485–93.
[21] Zwietering MH, Jongenburger I, Rombouts FM, Van's Riet K. Modeling of the bacterial growth curve. Appl Environ Microbiol 1990;56:1875–81.
[22] Lay JJ, Li YY, Noike T. Mathematical model for methane production from landfill bioreactor. J Environ Eng ASCE 1998;124:730–6.
[23] APHA. Standard methods for the examination of water and wastewater, 19th ed. Washington, DC, USA: American Public Health Association; 1995.
[24] Van Ginkel S, Sung S, Lay JJ. Biohydrogen production as a function of pH and substrate concentration. Environ Sci Technol 2001;35(24):4726–30.
[25] Lay JJ. Modeling and optimization of anaerobic digested sludge converting starch to hydrogen. Biotechnol Bioeng 2000;68(3):269–78.
[26] Brosseau JD, Zajic JE. Continuous microbial production of hydrogen gas. Int J Hydrogen Energy 1982;7:623–8.
[27] Wang DIC, Cooney CL, Demain AL, Dunnill D, Humphrey AE, Lilly MD. Fermentation and enzyme technology. Toronto: Wiley; 1979.
[28] Lay JJ, Fan KS, Chang JI, Ku CH. Influence of chemical nature of organic wastes on their conversion to hydrogen by heat-shock digested sludge. Int J Hydrogen Energy, 2003;28:1361–7.
[29] Akashah M, Yoshida M, Watanabe M, Nakamura M, Mastsumoto J. Hydrogen gas production from glucose and its microbial kinetics in anaerobic systems. Water Sci Technol 1997;36(6–7):279–86.
[30] Noike T, Mizuno O. Hydrogen fermentation of organic municipal wastes. Water Sci Technol 2000;42(12): 155–62.
[31] Jordan DC, McNicoll PJ. A new nitrogen-fixing *Clostridium* species from a high arctic ecosystem. Can J Microbiol 1979;25:947–8.
[32] Holdemann LV, Cato EP, Moore WEC. Anaerobe laboratory manual, 4th ed. Blacksburg: Anaerobe Laboratory, Virginia Polytechnic Institute and State University; 1977. p. 1–156.
[33] Cardon BP, Barker HA. Amino acid fermentation by *Clostridium propionicum* and *Diplococcus glycinophilus*. Arch Biochem 1947;12:165–80.