ROLE OF INITIAL SUCROSE AND PH LEVELS ON NATURAL, HYDROGEN-PRODUCING, ANAEROBE GERMINATION

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Abstract

Anaerobic batch cultures were established to assess natural anaerobic sporulation, germination, and hydrogen production. Heat-shocked soil inocula obtained from a potato field was cultured using sucrose as the substrate. Eleven batch experimental results suggested that baking was an excellent heat-shock treatment to select for spore forming hydrogen-producing bacteria i.e. clostridia from the soil. Sucrose could induce clostridial spore germination and subsequent hydrogen production. Hydrogen production potential and rate were selected for monitoring clostridial spore germination and vegetative cell activity, and could be systematically estimated using a modified Gompertz equation. Multivariate analysis (i.e., polynomial regression with stepwise) and response surface plots performed with a central composite design (sucrose: 15 – 45 g COD/L; pH: 4.5 – 6.5) demonstrated that the constant hydrogen production rate increased and

the potential decreased with a decrease in the sucrose concentration from 45 to 15 g COD/L. The results indicate that substrate deprivation was more important than initial pH on hydrogen-production/clostridial germination. Based on the results of this study, at a substrate concentration of 15 g COD/L, the pH value of 5.5 might be a trigger for clostridial germination and a pH of 5.5 or greater may shift the dynamic metabolic balance from solventogensis to hydrogenesis.

Introduction

Hydrogen is an excellent alternative energy candidate for the future. It has a high calorific value and water is the only by-product resulting from the combustion of hydrogen. It can also easily stored as a metal hydride (Billings, 1991). While hydrogen can be easily produced by water electrolysis, thermochemical, and radiolytic processes, these processes are not very economical owing to intensive energy consumption and are viable only in areas where cheap electricity is readily available (Rajeshwar *et al*., 1994). From the perspective of global environmental impacts, such as the greenhouse effect and resource recovery, microbial hydrogen production from renewable biomass reduces dependence on fossil fuel, decreases carbon dioxide emission, and recovers bioenergy (Gray and Gest, 1965; Borkris, 1973).

The microbial conversion of agricultural and industrial wastes and residues into hydrogen is attracting increasing interest. Apart from surplus agricultural products, suitable source materials are now readily available in the form of wastes from the sugar producing and processing industry and fruit processing factories (Afschar and Schaller, 1991). Hydrogen is a major intermediate in organic matter degradation in the digested sludge ecosystem. It is produced by fibrolytic and fermentative microorganisms and could be potentially re-utilized by the hydrogentrophic bacteria (Giallo *et al.*, 1985). Interspecies hydrogen transfer between hydrogen-producing and hydrogen-utilizing microorganisms allows growth and activity of fermentative and hydrolytic microorganisms. Several studies have been carried out using pure cultures of anaerobic bacteria, such as *Clostridium* sp., to study the conversion of carbohydrates (e.g., glucose) to hydrogen gas (Zeikus, 1980; Miyake *et al*., 1984; Taguchi *et al*., 1992). Recently, some investigators have used anaerobic microorganisms, taken from sludge compost, to generate hydrogen from sugary wastewater in a chemostat culture (Ueno *et al*., 1996). However, only traces of hydrogen are usually evolved with continuous flow digesters due to the ubiquitous nature of hydrogen consumers (Kibdy and Nedwell, 1991). If the activity of hydrogentrophic bacteria contained in anaerobic cultures is inhibited, the culture possesses significant capacity for the transformation of sugary processing wastes into hydrogen gas. The review by Zajic and coworkers (Zajic *et al*., 1978) indicates that anaerobic spore-forming bacteria form an important part of the acidogenic bacterial population performing acetate/butyrate fermentation and the main anaerobic hydrogen-producing sporeformers are *Clostridium* sp. The clostridial bacteria could be enriched from soil, cracked cereals and comparable sources using a heat-shock treatment to inactivate non-spore-formers (Biebl, 1999). Preliminary experiments have successfully used a heat-shock digested sludge to convert the organic fraction of municipal solid wastes into hydrogen gas and demonstrated that the sludge possessed clostridial characteristics and no methanogenesis occurred (Lay *et al*., 1999).

Starting a bioprocess with a spore-soil mixture to generate hydrogen, the spores germinate to vegetative cells in a triggering stage. Proper pH control and substrate concentration are key factors improving the germination of the clostridia, as well as in operating a hydrogen-producing bioprocess (Craven, 1998). This is due to the fact that bacterial germination/sporulation involves several metabolic pathways associated with morphological, physiological, and chemical changes and can affect end products associated with energy metabolism (Labbé and Shih, 1997). Specific carbohydrate composition of the growth media also affects the ability of the culture to germinate. Rapidly metabolizable carbohydrates such as glucose are generally avoided in germination media because they are vigorously fermented by certain saccharolytic species of *Clostridium*, resulting in considerable acid production (Labbé and Rey, 1979).

The objective of this study was to investigate the role of the environmental factors of initial pH and carbohydrate concentration on clostridia-rich, natural anaerobe germination for hydrogen production. For these purposes, the experiments in this paper were performed with heat-shock treatment on small batch reactors using sucrose and an undefined bacterial consortium derived from potato soil as inocula. A fractional factorial central composite design was employed in planning experiments for learning the effects of these environmental factors on clostridial germination using hydrogen production potential and rate as indicators summarized with a predictive polynomial quadratic equation incorporating stepwise regression and response surface methodology (Box *et al.*, 1978).

Materials and Methods

Seed Microorganisms

In most natural environments, clostridia have significant ability to transform organics to acids, alcohols, and hydrogen. They are easily obtained from soil, roots (especially of leguminous plants), cracked cereals, and comparable sources using starchy mashes or media containing sugar (Biebl, 1999). In this study, the natural anaerobes were taken from the Iowa State University Student Farms in Ames, Iowa and respectively brought to a two-hour bake and a fifteen-minute boil to inhibit the bioactivity of hydrogen consumers and to harvest hydrogen-producing spore-forming anaerobes (i.e., clostridia). This soil was obtained in February and potatoes were grown the previous year.

Experimental Design

To assess the soil having the capability of biohydrogen production, two sets of experiments were conducted with 250-mL bottles with two grams of sucrose added, while the pH varied from 4.0 to 8.0 in increments of 0.5. The boiling and the baking heat-shock treatments were used for the first and the second experiments, respectively. Additionally, experiments were run with and without sucrose added in the 250-mL bottles containing the same quantity of soil and nutrients in order to observe no hydrogen production resulting from degradation of organic matter contained in the soil.

To evaluate the characteristics of clostridial germination for hydrogen production, a fractional factorial central composite experimental design of key variables of initial pH and sucrose concentration was performed by measuring their hydrogen-producing rate and potential. This design (Table 1) used a central value consisting of four grams of sucrose and an initial pH of 5.5. This central value was replicated three times. The variables, sucrose and pH, were coded by the following equation in order to be used in a regression equation.

$$x_i = (X_i - X_i^*)/\Delta X_i \quad (1)$$

where x_i is the coded value of the ith test variable, X_i is an uncoded value of the ith test variable, X_i^* is an uncoded value of the ith test variable at the center point and ΔX_i is the step change value. Response Surface Methodology (Box *et al.*, 1978) is a statistical technique involving the solution of multivariate polynomial regression equation (refer to Eq. 3) simultaneously. The visual evaluation of these graphs can give insight to the main effects and the mutual interactions of the variables used in the design.

Table 1. Full factorial central composite design variables in coded and natural units.

Sucrose (g/L)	pH Range and Levels				
	4.5 (-2)	5.0 (-1)	5.5 (0)	6.0 (1)	6.5 (2)
2 (-2)			+		
3 (-1)		+		+	
4 (0)	+		+		+
5 (1)		+		+	
6 (2)			+		

* Numbers in parentheses are x_i).

* Central value (5.5, 4) was replicated three times.

Experimental Apparatus and Procedures

The experiment was conducted at random in 250 mL batch serum bottles filled to 150 mL with 30 grams of the soil, varying amounts of initial sucrose and initial pH values (Table 1), and 0.5 mL of nutrient stock solution. Each liter of nutrient stock solution contained 200 g of NH_4HCO_3, 100g of KH_2PO_4, 10g $MgSO_4 \cdot 7H_2O$, 1.0 g of NaCl, 1.0 g of $Na_2MoO_4 \cdot 2H_2O$, 1.0 g of $CaCl_2 \cdot 2H_2O$, 1.5g of $MnSO_4 \cdot 7H_2O$ and 0.278g of $FeCl_2$. After addition of substrate and nutrients, the bottles were flushed for 15 seconds with nitrogen gas. The optimal temperature for vegetative clostridia is between 35 and 40°C although a few strains of this species can also sporulate at the same temperature range (Garcia-Alvarado *et al*., 1992). The bottles were then incubated at 37°C in an orbital shaker at a rotational speed of 150 rpm. The volumes of the biogas collected were measured using 2 to 50 mL syringes as described by Owen *et al*. (1979).

Analytical Methods

The hydrogen gas percentage was calculated by comparing the sample biogas with a standard of pure hydrogen using a GOW-MAC Series 350 GC equipped with a thermal conductivity detector. The column was an 8' by 1" SS 350A Molesieve 13X 80/100. The operational temperatures of the injection port, the oven and the detector were 100, 50, and 100°C, respectively. Nitrogen was used as the carrier gas with a flow rate of 40 mL/min. The concentrations of total solids (TS) and volatile solids (VS) were determined by placing a 10 mL sample in 105 and 550°C ovens, respectively, according to the procedures described in APHA *et al*. (1995).

Data Analysis

A modified Gompertz equation was used to fit the experimental data to estimate the hydrogen production potential, *P* (mL) and the maximum hydrogen production rate, *R* (mL/h).

$$H = P \cdot \exp\left\{-\exp\left[\frac{R \cdot e}{P}(\lambda - t) + 1\right]\right\} \qquad (2)$$

where λ is lag phase time (h) and *e* is 2.718. The specific hydrogen production potential (mL/g sucrose), *Ps* was calculated by dividing *P* by the appropriate grams of sucrose. The specific hydrogen production rate, *Rs* was calculated by dividing *R* by the Δ VS (the difference of final and initial VS in each individual bottle) because VS contained biomass and organics in the soil. The parameters of Eq. (2) were estimated using the function of "Solver" in Microsoft Excel version 5.0 (Microsoft, Inc., 1985 - 1995). This program uses a Newton algorithm. Up to 100 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 (Lay *et al.*, 1998).

The influences of pH and sucrose concentration on clostridial germination corresponding to *Ps* and *Rs* were obtained by solving the second order polynomial regression equation according to the least squares approach.

$$y = a_0 + a_1 x_1 + a_2 x_2 + a_{11} x_1^2 + a_{22} x_2^2 + a_{12} x_1 x_2 \quad (3)$$

where y represents *Ps* and *Rs*, x_1 is pH, x_2 is sucrose (g), and a_{xx} are the coefficients of the equation. All coefficients were diagnosed by: sum of the square errors (SSE), correlation coefficient (R^2), standard errors (SE), 95% confidence limits, and F-test. Here, Statistica V.5.5 was employed for polynomial regression and response surface contour plots.

Results and Discussions

Feasibility of Natural Anaerobes Converting Sucrose to Hydrogen

Table 2 lists the biohydrogen potential of heat-shocked anaerobes consuming sucrose under a pH range from 4.0 to 8.0. In Table 2, the range of biohydrogen potential of the anaerobes consuming sucrose was from 62 to 185 mL, while the potential increased and decreased with increasing pH for the baking and boiling methods, respectively. It is obvious that the cultures contained a significant population of hydrogen-producing clostridia although a high variation in potential occurred. This variation resulted mainly from poor mixing and poor soil sample homogeneity among the batch bottles. Experience learned from soil inoculum preparation indicated that the baked soil grinded into meal easily obtained homogeneous soil samples. Additionally, experiments were run with and without added sucrose to confirm that no hydrogen production resulted from degradation of soil organic matter. As shown in curve "D" of Figure 1, no significant hydrogen production occurred from the bottle without added sucrose, indicating that soil organic matter was not converted to hydrogen.

Table 2. Biohydrogen potential of natural anaerobes using sucrose with various pH and pretreatment approaches.

Pretreatment Approach	pH Value								
	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0
Baking	97 (14%)	82 (11%)	80 (8%)	120 (50%)	78 (6%)	165 (7%)	116 (36%)	134 (63%)	185 (6%)
Boiling	138 (19%)	118 (4%)	110 (30%)	157 (60%)	86 (41%)	164 (20%)	118 (53%)	62 (45%)	89 (7%)

* Numbers in parentheses are relative standard errors.
* Unit of potential is mL
* Data are the mean of two replicate cultures.

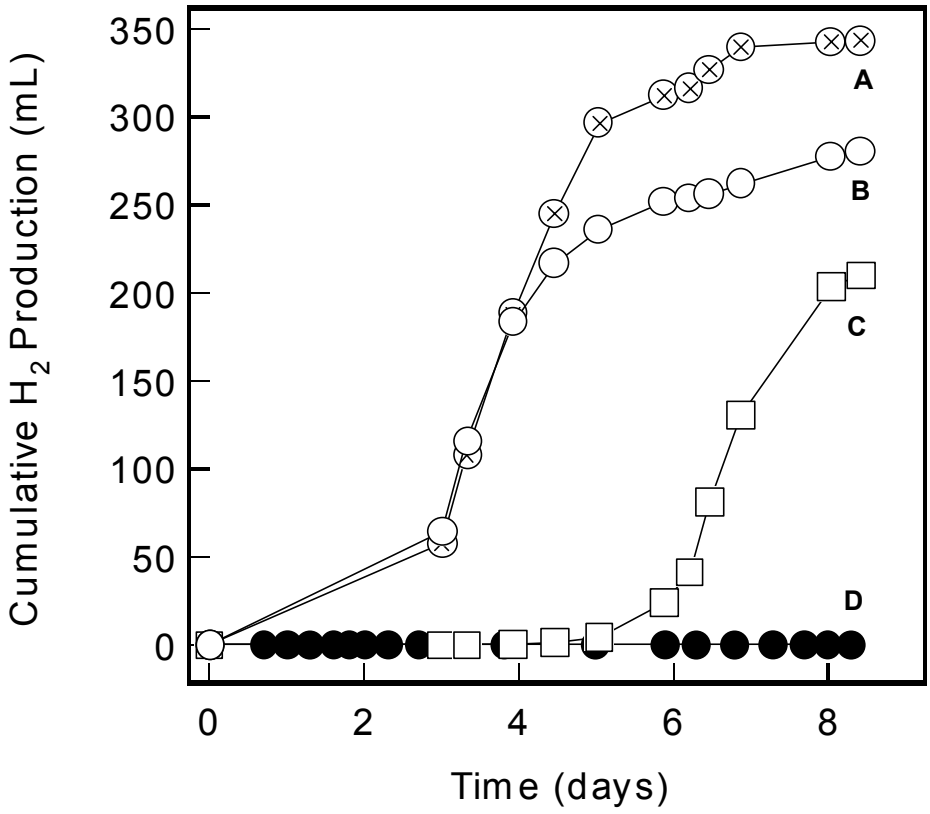

Figure 1. Feasibility of the natural anaerobes converting sucrose into hydrogen. "A" and "B" are 250-mL bottle adding with 2 and 4 grams of sucrose, respectively. "C" is 250-mL bottle adding with 2 grams of sucrose but delay 2 days. "D" is without adding sucrose.

To ensure hydrogen-producing sporeformers (i.e. clostridia) in the soil inocula consumed sucrose, two bottles containing the same soil and nutrients as described previously were individually charged with 2 and 4 grams of sucrose, which are respectively shown in curves "A" and "B" of Figure 1. Moreover, two grams of sucrose was anaerobically injected into a bottle after a 2-day incubation period ("C" curve of Figure 1). Graphically, in Figure 1, the hydrogen production shows a phase in which the production rate started at a value of zero and accelerated to a maximal value after a certain period of time (lag phase, λ). In addition, the curves contain a final phase in which the rate decreased and finally reached zero, (i.e. an asymptote or potential, P, was reached). In Figure 1, the lag-periods of curves "A" and "B" (approximately 3 days) were significantly less than that of curve "C" (approximately 5 days), indicating that the sucrose addition significantly affected the hydrogen-producing anaerobes.

Comparison of Boiling and Baking Heat Shock Treatments on Hydrogen-producing Spore Germination

Preliminary experimental experience (Lay, 2000a) indicates that if a culture of clostridia were regularly transferred as vegetative cells, the ability to form hydrogen may be permanently lost. This unusual property is named degeneration and has been circumvented by inoculating with only dry spores which were heat-shocked before incubation to eliminate the "weak" spore. In order to avoid degeneration of the culture, the spores can be activated by heat shock (Biebl, 1999). This phenomena is due to a set of so-called heat shock proteins that are clearly induced when the clostridial cells are suddenly exposed to a higher temperature (Bahl *et al*., 1995). These proteins may play a role in the complex cycle of metabolic and morphological differentiation of clostridial bacteria. Especially, the baked soil could be ground to provide a homogeneous spore-soil mixture for initiating hydrogen-producing batch experiments. Hydrogen production potential was, therefore, introduced to monitor the effect of heat-shock treatment on clostridial germination and the transformation of sucrose into hydrogen. An eleven-repetition average indicates that the hydrogen production potential and rate of the boiling experiments (137 mL and 2.9 mL/hr, respectively) was less than that of the baking experiments (189 mL and 4.1 mL/hr, respectively), implying that the latter was superior to the former on clostridial germination. In addition, an eighteen repetition average of the batch experiments conducted with 2 grams of sucrose with a pH range of 4 to 8 indicates that the hydrogen production potential and rate of the boiling experiments (165 mL and 3.9 mL/hr, respectively) was less than that of the baking experiments (210 mL and 5.1 mL/hr, respectively), implying that the latter was superior to the former on clostridial germination. Interestingly, the environments triggering the initiation of clostridial germination still play key roles in affecting their metabolic characteristics, including hydrogen-producing activity and ability. As a result, the baking heat shock treatment might be a good candidate to induce sporulation of hydrogen-producing clostridial spores subsequently providing stable hydrogen production in addition to inhibiting hydrogentrophic bacterial activity contained in the natural anaerobic culture.

Indicators of Hydrogen-producing Spore Germination

The cultures of hydrogen-producing anaerobes (i.e. clostridia) obtained from natural inocula typically displayed a lag-period (Figure 1). During this period, the spore suspensions transform into vegetative cells. Biosystems created with these cells initially transform sucrose into hydrogen with an exponential phase (R) and a final saturation phase (P) obtained throughout the whole culture history. Dividing the R and the P by the Δ VS and the amount of sucrose could correspond to the hydrogen-producing activity (Rs) and the specific potential (Ps) of the sucrose converted into hydrogen, respectively (Lay *et al*., 1999).

The Ps and the Rs were, therefore, selected as indicators for monitoring spore germination. A set of batch experiments were performed by changing the initial sucrose concentrations from 13 to 40 g/L (corresponding to 15 to 45 gCOD/L) and initial pH from 4.5 to 6.5 in order to examine the effects of these environmental factors on spore germination for generating hydrogen. Equation (2) was then used to fit the experimental data of each cumulative hydrogen production curve. Typical results are plotted in Figure 2. Table 2 summaries the design matrix of the

variables in both coded and natural units along with the best values of the parameters. The diagnosis results presented that all of the correlation coefficients, R^2, and the statistics test, $F_{.05}$, were larger than 0.90 and the tabulated value, respectively. The F is defined as MSR/MSE, where MSR is the mean square of regression, obtained by dividing the sum of squares of regression by the degrees of freedom. MSE is the mean squares of error from the analysis of variance. If the calculated value of F exceeds that in the F table at a specified probability level (i.e., $F(P-1, \nu, 1-\alpha)$), then a "statistically significant" regression model is obtained, where ν is the degree of freedom of error and P is the number of parameters. $F(P-1, \nu, 1-\alpha)$ is the F value at the α probability level. This means that a perfect fit to the experimental data and the evaluated parameters were taken to be statistically significant at a confidence interval of 95% (Box *et al.*, 1978). Moreover, both the curve fitting and statistical analysis demonstrated that Equation (2) was suitable for estimating the hydrogen production lag-period, rate, and potential of the clostridia in converting sucrose to hydrogen.

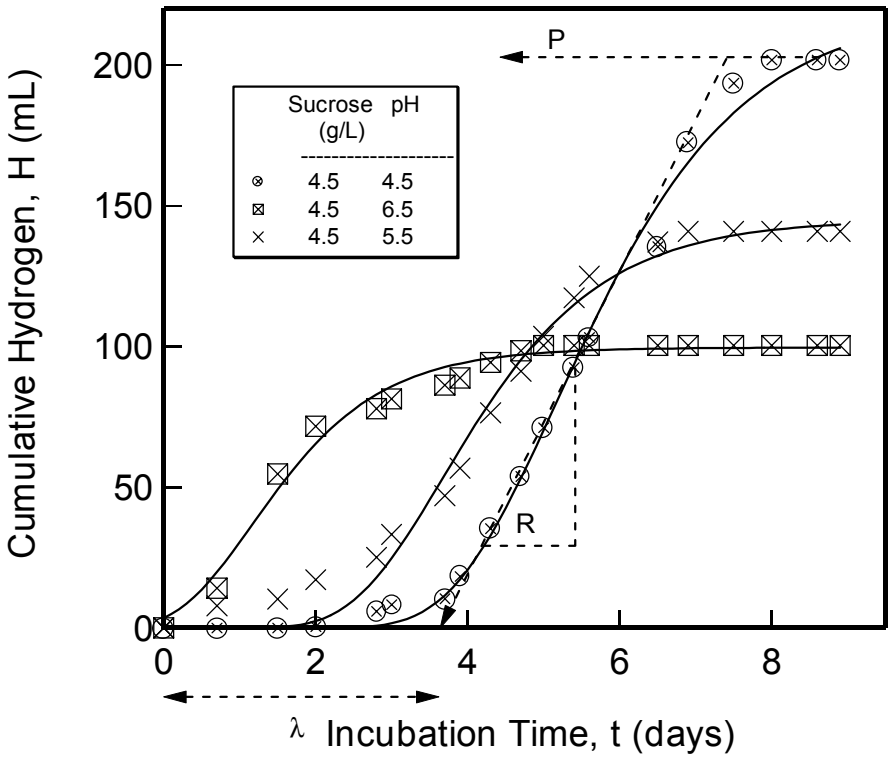

Figure 2. A typical cumulative hydrogen production curve in batch experiments (markers are experimental data; lines are nonlinearly estimated using Eq. 2; and the variables R, λ, and P are defined as in Eq. 2).

Specific Hydrogen Production Potential and Rate

To depict the relationships between pH (x_1) and sucrose concentration (x_2) on specific hydrogen production potential (Ps) and rate (Rs), the design matrix with corresponding results listed in Table 3 was subjected to multivariate analysis; that is, multiple regression with stepwise method, depending on the quadratic polynomial model used (refer to Equation (3)). The following equations could arrange the rank of linear, squared, and interaction effects between pH and sucrose concentration on spore germination for hydrogen production.

$$Ps = 13.57 - 0.45767x_2 + 0.00552x_2^2 + 0.00727x_1x_2 - 0.46523x_1 \quad (4)$$
$$(F = 43.9,\ df = 4, 6,\ R^2 = 0.9670)$$

$$Rs = 3.16 - 0.19165x_2 + 0.002268x_2^2 + 0.332233x_1 - 0.002538x_1x_2 \quad (5)$$
$$(F = 20.6,\ df = 4, 6,\ R^2 = 0.9320)$$

The significance of Equations (4) and (5) was estimated using the same statistical approach that was used for Equation (2) and the values of R^2 and F were larger than 0.90 and the tabulated

Table 3. Full factorial central composite design matrix of two variables in coded and natural units along with the observed responses.

x_1	x_2	pH	Sucrose (g)	Sucrose (gCOD/L)	Baking Experiments	
					Ps (mL/gCOD$_{sucrose}$)	*Rs* (mL/TVS/hr)
-1	-1	5.0	3.0	22.5	3.9	1.0
1	-1	6.0	3.0	22.5	4.8	1.5
-1	1	5.0	5.0	37.5	2.4	0.6
1	1	6.0	5.0	37.5	2.3	0.5
0	0	5.5	4.0	30.0	3.3	0.9
-2	0	4.5	4.0	30.0	3.3	0.8
2	0	6.5	4.0	30.0	3.5	0.9
0	-2	5.5	2.0	15.0	6.7	2.3
0	2	5.5	6.0	45.0	2.2	0.5
0	0	5.5	4.0	30.0	3.2	1.0
0	0	5.5	4.0	30.0	3.4	0.8

*Data are the mean of two replicate cultures.

value, respectively, indicating that the models were suitable for accurately representing the experimental results. Examining the terms listed in Equations (4) and (5), sucrose concentration played a more important role than the pH value on specific hydrogen production rate and potential. For the examination of the dependence of the potential and rate on both factors, the contour plots (Figure 3) were constructed using Equations (4) and (5). Consider the fitted equations (Equations (4) and (5)) graphed in Figure 3. The constant potential and rate curves have the shape commonly referred to as a "ridge" system. The constant rate and potential increased with a decrease in the sucrose concentration from 45 to 15 gCOD/L. This phenomena appears to be correlated with the anaerobic biohydrogenization of microcrystalline cellulose (Lay, 2000b). The low initial ratio of substrate concentration (S_o) to sludge density (X_o) corresponding to low S_o and constant X_o accompanied high-level hydrogen production. It was also tempting to consider a high concentration of organic acids produced by the hydrogen-producing clostridia consuming high levels of sucrose to cause the internal pH to decrease to below critical values, resulting in the cessation of growth and the loss of viability (Gottwald and Gottschalk, 1985).

Compared to sucrose concentration, there was very little increase in hydrogen production potential and rate in the pH range of 4.5 to 6.5 (Figure 3). To assess the role of pH on hydrogen-producing spore germination, consider the hydrogen production potential and rate graphed in Figure 3 using Equations (4) and (5), respectively, while the sucrose concentration was 15 g COD/L. As shown in Figure 4, the relative hydrogen production potential decreased and the rate

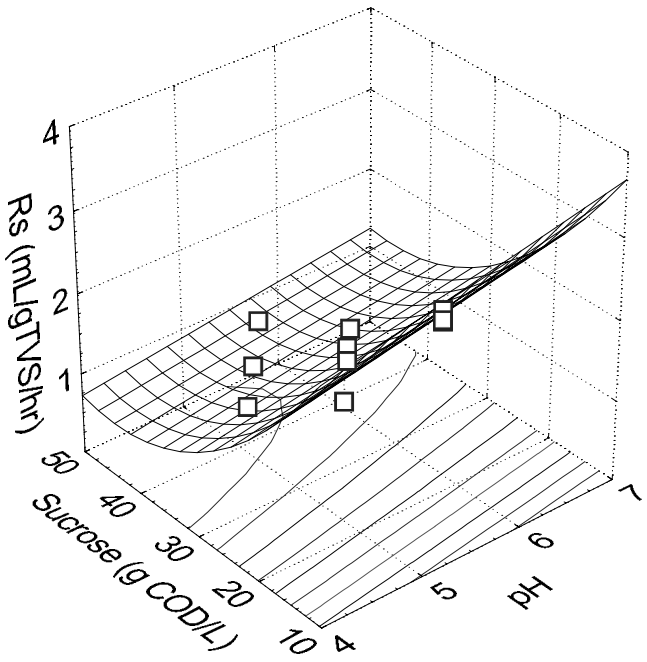

Figure 3. Constant specific hydrogen-producing potential (Ps, mL H_2/$gCOD_{sucrose}$) and rate (Rs, mL H_2/ Δ gVS/hr) contour lines of baking soil against initial pH and sucrose concentration.

increased with increase in initial pH. Although no significant evidence could explain this phenomena, on the examination of the two lines plotted in Figure 4, the rate increased 50% and the potential decreased 50% at a pH of 5.5. Furthermore, the pH value of 5.2 was the optimum pH for controlling a chemostat reactor using heat-shocked anaerobic digested sludge to transform starch into hydrogen (Lay, 2000a). Similarly, the experimental results of Bahl and coworkers demonstrated that an acidic pH below 5.0 was found optimal for butanol-producing clostridia (Bahl et al., 1982). However, what is the optimal pH for hydrogen-producing clostridial spore germination or hydrogen formation is still an open question. According to the description in Figure 4, the pH of 5.5 might be a trigger for hydrogen-producing clostridial germination and for the dynamic metabolic balance between hydrogenesis and solventogenesis. Nevertheless, according to multivariate analysis (refer to Eq. (4) and (5)) and contour plots (Figure 3), substrate deprivation was more important than initial pH value on spore germination for generating hydrogen although very little has been done to clarify the mechanisms of hydrogen-producing spore germination associated with metabolites.

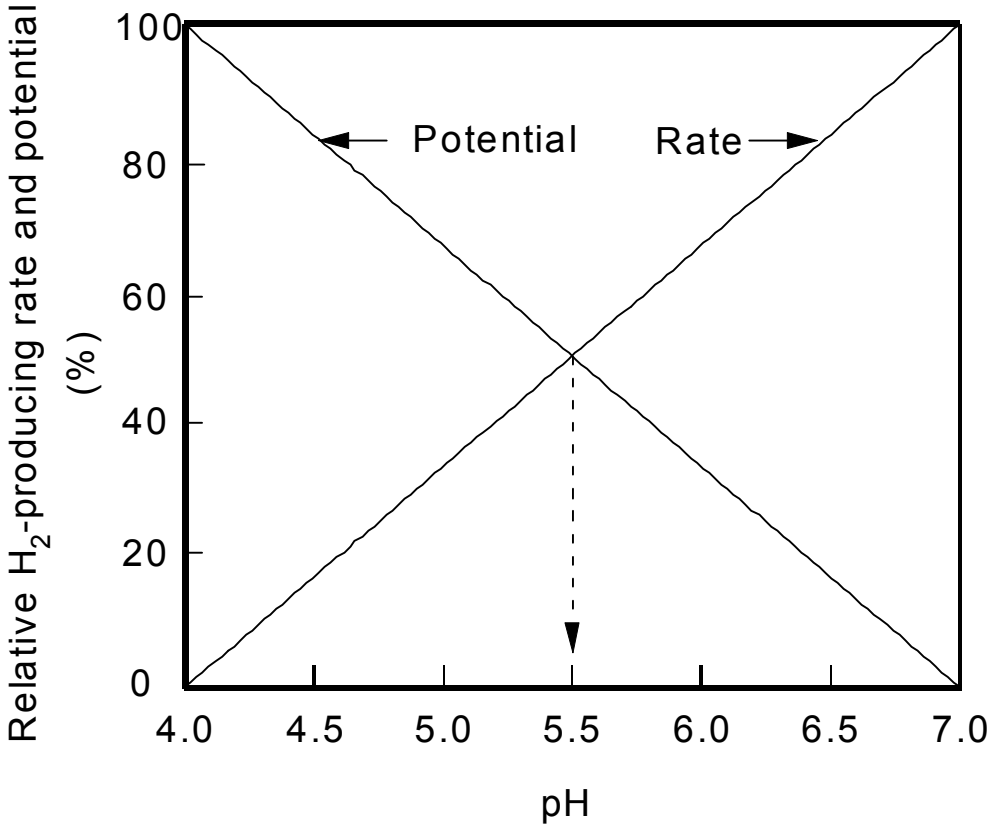

Figure 4. Relationship between initial pH and relative hydrogen-producing potential and rate under sucrose concentration of 15 gCOD/L using Eqs. (4) and (5), respectively.

Conclusions

Naturally obtained, sporeforming, hydrogen producing anaerobic bacteria (i.e. clostridia) have considerable potential in the transformation of carbohydrates into hydrogen. As of yet, there is no selective enrichment procedure for hydrogen-producing spores. Nevertheless, they are easily obtained from soil and the samples can easily be heat-shocked to exclude non-spore-forming, hydrogen-consuming anaerobes. However, obtaining an appropriate approach for hydrogen-producing clostridial germination is complicated by the fact that the genus *Clostridium* and their germination-associated metabolites have yet to be clarified. To overcome this problem, cultures with boiling- and baking-shock treatments were performed to measure the stability of natural anaerobes to form hydrogen, indicating the latter was easier than the former in finding a stable hydrogen-production potential. Experimental observations may indicate that the soil contained a high proportion of hydrogen-producing spore-forming clostridia, however their germination was significantly affected by sucrose addition. Statistical experimental strategies were adopted to systematically collect clostridial hydrogen-producing data sets from batch cultures by varying initial pH and sucrose concentrations from 4.5 to 6.5 and 15 to 45 gCOD/L, respectively. Although what controls the mechanisms of clostridial hydrogen-producing germination has not been determined in detail, substrate deprivation was suggested to be more important than the initial pH value on clostridial germination according to multivariate analysis (i.e., polynomial regression with stepwise) and response surface plots. Correlations between the relative hydrogen production rate and potential plotted against their respective pH value demonstrates clearly that the pH value of 5.5 might be a trigger for clostridial germination and might shift the metabolic balance from hydrogen to solvent formation.

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