Biohydrogen production from wastewater by *Clostridium beijerinckii*: effect of pH and substrate concentration

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Abstract

An investigation of biological hydrogen production from glucose by *Clostridium beijerinckii* was conducted in a synthetic wastewater solution. A study examining the effect of initial pH (range 5.7 to 6.5) and substrate loading (range 1 to 3 g COD/L) on the specific conversion and hydrogen production rate has shown interaction behaviour between the two independent variables. Highest conversion of 10.3 mL H_2/(g COD/L) was achieved at pH of 6.1 and glucose concentration of 3 g COD/L, whereas the highest production rate of 71 mL H_2/(h*L) was measured at pH 6.3 and substrate loading of 2.5 g COD/L. In general, there appears to be a strong trend of increasing hydrogen production rate with an increase in both substrate concentration and pH. Butyrate (14% to 63%), formate (10% to 45%) and ethanol (16% to 40%) were the main soluble products with other volatile fatty acids and alcohols present in smaller quantities.

Keywords: Biohydrogen, Wastewater, Clostridium

1 Introduction

The rising costs in conventional energy supplies and the established link between climate change and the burning of fossil fuels [1] have revitalized the search for alternative fuels and modes of energy production. Hydrogen has been identified as a possible alternative to fossil-based fuels and a worldwide investigation of hydrogen as a future energy carrier is now underway. Currently, commercial hydrogen is produced mainly through the reforming of fossil fuels a process that is energy and environmentally intensive due to the large amount of greenhouse gases (GHGs) that it generates. Biological hydrogen production has two main advantages over the conventional

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method; it generates less GHGs and couples the metabolic activity of hydrogen-emitting micro-organisms with the simultaneous disposal of human-derived wastes rich in organics, such as domestic and food industry wastewaters.

A number of studies have examined the potential of using mixed communities of fermentative bacteria, mainly from the *Clostridium* genus, obtained from anaerobic sludge digesters [2-4] and compost piles [5] in order to degrade both simple sugars such as glucose and sucrose and more complex substrates such as industrial effluents from food manufacturers [6-9] and chemical wastewaters containing pharmaceuticals, drugs and pesticides [10]. Fewer studies have dealt with pure cultures of known species of hydrogen-producing bacterium [11-13], yet by studying pure organisms of anaerobic bacteria much can be learned about operating conditions, such as pH and substrate concentration, which are favourable to high hydrogen yield and production rate. Experiments have demonstrated that the optimal pH for cell growth does not appear to be the same as that for obtaining high hydrogen potential [14]. For the degradation of simple substrates, the optimum initial pH for Clostridia has been reported in the range of 4.5 – 7.0 [4,5,14-16], though high yields have been reported at a pH value as large as 9.0 [16].

In general, it has been shown in both batch and continuous experiments that the initial pH has a significant effect on both the yield and rate of hydrogen production; however, neither the optimal value of pH nor the trends observed during initial pH manipulation are uniform from one author to the other [17]. While some works report an increase in both H_2 yield and production rate with an increasing pH [5,16] for a mixed culture inoculum, other authors report a positive effect in only one of the two variables of interest [15], while others still observe a reverse trend for both H_2 yield and production rate [4]. All these results strongly suggest that optimum pH is system specific and as such should be assessed for the specific application in question.

Only handful of studies have considered the effect of substrate concentration on the biohydrogen production rate and yield [4,5,17] and even fewer using low substrate concentrations such as those sometimes found in industrial wastewater streams [10]. This paper aims to investigate the effect of initial pH and low substrate concentration (glucose) on the specific H_2 production potential and maximum production rate on the batch scale using a pure culture bacterium of *Clostridium beijerinckii* as a preliminary stage of assessing the suitability of using industrial wastewater as a substrate for continuous biological hydrogen production.

2 Materials and Methods

2.1 Bacterial Cultivation

Pure culture of *Clostridium beijerinckii* was purchased from ATCC (#8260) and used for the duration of the study. The bacterium was pre-cultured in the recommended nutrient broth (Difco ™ Reinforced Clostridial Medium) at 30°C in serum bottles inside a dark incubator shaker. The bacteria were transferred 2-3 times and cultivated for 12 hours (till stationary phase) between each transfer prior to use in the experiments in order to ensure a healthy and active culture population.

2.2 Experimental procedure

Batch hydrogen production experiments were carried out in 100 mL serum bottles with a working volume of 50 mL. The media contained a prescribed amount of glucose as well as essential growth nutrients, adapted from pervious work [11] (in mg/L) 850, KH_2PO_4; 750, K_2HPO_4; 3, H_3BO_3; 200, $MgSO_4.7H_2O$; 1, $Na_2MoO_4.2H_2O$; 1, $ZnSO_4.7H_2O$; 2, $MnSO_4.4H_2O$; 0.1, $Cu(NO_3)_2.3H_2O$; 1, $CaCl_2.2H_2O$; 2, EDTA; 12, $FeSO_4.7H_2O$; 4, thiamine; 3, biotin; 5, *p*-aminobenzoic acid; 6.5, nicotinamide; 420, glutamic acid; 1, resazurin. A 0.1 M phosphate buffer was also added, in order to prevent pH decrease due to organic acid accumulation during the bacterial metabolism. The initial pH was adjusted to a desired value using 5N NaOH or 5N HCl. The media was boiled under a condenser set-up for 30 minutes to activate the oxidation indicator and drive off dissolved oxygen from the solution. Empty serum bottles were placed in a water/ice bath and continuously flushed with oxygen-free argon gas. The media was then dispensed into the serum bottles and cooled under the flow of argon for 10 minutes. Once cool, the bottles were capped with a butyl rubber stopper, sealed with an aluminium crimp and sterilized in an autoclave.

In order to remove any residual oxygen in the media, prior to inoculation a reducing agent, $Na_2S.9H_2O$ was added at a 0.025% (w/w) concentration. All bottles were inoculated with 3% (v/v) *C. beijerinckii* in the stationary phase and incubated in an orbital shaker (New Brunswick

Scientific) at 30°C ± 1°C and rotational speed of 180 rpm. Biogas and hydrogen concentration measurements were conducted at regular time intervals (within each batch) throughout the experiment, after an initial acclimatization period of approximately 12 hours. Experiments were deemed complete when no biogas production was observed for at least 24 hours. All trials were done in triplicate to ensure reproducibility.

2.3 Factorial Design

In order to analyze the effect of initial glucose concentration and initial pH as well as any interactions between the two variables, a fractional factorial design was employed [18]. A nine trial design was constructed as shown in Figure 1a to cover the area of interest. The substrate concentration, expressed in term of chemical oxygen demand (COD), varied from 1 to 3 g COD/L with the central value of 2 g COD/L and the pH varied from 5.7 to 6.5 with a central value of 6.1. Both of the ranges were based on values previously observed in a number of local industrial wastewater streams (data not shown). The pH range was modified from the original design of 4.5 - 6.5 after no growth was observed in the lower end of the range.

As shall be shown later, the extrapolated regions turned out to be of particular interest and for this reason four additional experiments, covering the corner points of the matrix, were carried out in order to validate the initial results. The revised fractional factorial design can be seen in Figure 1b.

2.4 Analytical methods

Biogas production was periodically measured using 50, 25 and 10 mL glass syringes, depending on the expected biogas production, fitted with hypodermic needles as described by Owen *et al* [19]. The biogas was sampled from the headspace with a 2 mL gas tight syringe and analyzed for the amount of hydrogen (H_2%) using a gas chromatogram (Hewlett-Packard 5890) equipped with a thermal conductivity detector (TCD). The GC was fitted with a stainless steel molecular sieve column (6' x 1/8") and the injector, oven and detector temperatures were set at 100°C, 80 °C and 100 °C, respectively. Argon was used as the carrier gas with a flow rate of 2 mL/min. Following the completion of the biohydrogen experiments, samples were taken from each serum bottle in order to analyze the organic make up of the effluent. The soluble

metabolites were analyzed using a gas chromatogram equipped with a flame ionization detector (Hewlett- Packard 5890) fitted with a Stabilwax column (30 m, 0.32 mmID, 0.25 μm film thickness) and the injector, oven and detector temperatures were set at 45°C, 55 °C and 180 °C, respectively with the oven ramp rate set at 5.0°C/min. Helium was used as the carrier gas with a flow rate of 30 mL/min. In order to quantify the formic and acetic acids samples were injected into the ion chromatogram (Metrohm 820) using a disposable syringe fitted with a 0.45 μm filter (PTFE, Fisher Scientific) to remove any particulate matter from the solution. The IC was fitted with a Metrosep A Supp column, set at a temperature of 45°C and 3 mM solution of Na_2CO_3 was used as the eluent at a flowrate of 0.8 mL/min. Initial and final glucose concentrations were analyzed using a glucose (HK) assay kit (Sigma-Aldrich).

2.5 Data Analysis

The cumulative hydrogen gas production curves were constructed as previously described [20] by measuring the gas composition in the headspace of the bottle and the total volume of the biogas produced at each time interval, and applying the mass balance equation (1),

$$V_{H,i} = V_{H,i-1} + C_{H,i}\left(V_{G,i} - V_{G,i-1}\right) + V_H\left(C_{H,i} - C_{H,i-1}\right) \qquad (1)$$

where $V_{H,i}$ and $V_{H,i-1}$ are cumulative hydrogen gas volumes at the current *(i)* and previous *(i-1)* time interval, $V_{G,i}$ and $V_{G,i-1}$ are the total biogas volumes at the current and previous time interval, $C_{H,i}$ and $C_{H,i-1}$ are the fractions of hydrogen gas in the headspace of the bottle as determined by gas chromatography in the current and previous interval, and V_H is the total volume of headspace in the bottle.

Each of the cumulative hydrogen production curves was modeled using the modified Gompertz equation (eqn 2),

$$H(t) = H_{\max} * \exp\left\{-\exp\left[\frac{R_m * e}{H_{\max}}(\lambda - 1) + 1\right]\right\} \qquad (2)$$

where $H(t)$ is the cumulative hydrogen production (mL) during the course of the incubation time, t (hours), H_{max} is the hydrogen production potential (mL), R_m is the maximum production rate (mL H_2/h) and λ is the duration of the lag phase (h). This model has been commonly used to describe biological production of various gases such as methane, hydrogen and biogas in a batch set up [15,21]. The cumulative production curves were fit using Matlab 6.5 by minimizing the sum of square error (SSE). Initial estimates for the parameters (H_{max}, R_m and λ) were selected based on visual inspection. The hydrogen production potential was normalized with respect to substrate concentration to give the conversion efficiency, P_s (mL H_2/g COD/L); the hydrogen production rate, R was normalized with respect to working volume and defined as R_m/V_{media} (mL H_2/h-L).

In order to assess the effect of substrate concentration and initial pH on the conversion efficiency and the specific production rate of hydrogen, the data obtained from the Gompertz modelling was graphed in two three-dimensional plots. A second-order polynomial regression was conducted in order to interpolate/extrapolate results to cover the entire region of interest as outlined in Figure 1b. The main objective of the regression was to enhance visual understanding of the types of trends that exist within the matrix.

3 Results and Discussion

3.1 Cumulative Hydrogen Production

The cumulative hydrogen production curves were generated for each trial by fitting each of the replicates to the Gompertz model and the parameters of interest (R_m, H_{max}, λ) were calculated by averaging the individual results from the triplicates. The cumulative hydrogen production for the replicates of trial nine (pH: 6.1, [S]: 3 g COD/L) is shown in Figure 2. It can be seen from the figure that all data fit the model well ($R^2 > 0.996$ for all curves) and the variation between the replicates was small. In general, all the experiments showed good reproducibility and the average values for the parameters of interest are shown in Table 1.

3.2 Specific Hydrogen Production Potential

The specific hydrogen production potential, or the conversion efficiency, fitted based on the original design, is plotted in Figure 3a. It is clear from the data that COD loading and initial pH play a role on the yield. The trends indicate that higher conversion is achieved at higher glucose concentration and a mid-range pH in the vicinity of 6.0; the highest P_s of 10.3 mL H_2/g (COD/L) was measured at substrate concentration of 3 g COD/L and pH of 6.1. Both high and low-end pH appears to be unfavourable, but this effect is secondary to that of substrate concentration where the difference between the highest and lowest observed value is almost double. When the extra four trials (corner points) were added to the matrix, some changes were observed in the regression of the response variable, P_s (see Figure 3b). In general, though, the trends discussed above remain valid and the highest P_s is still found in the same region of the surface as before.

The trends observed in this study are similar to those reported by Chen *et al* [4] and Van Ginkel *et al* [5] who, using a different substrate, saw a rise in the specific hydrogen production potential with an increase in sucrose concentration until a certain maximum. The authors hypothesized that high substrate concentrations become inhibitory to the microorganisms as a result of pH depletion and/or hydrogen partial pressure increase. Conversely, at low substrate concentrations bacteria are thought to utilize the carbon source mainly for biomass growth and not biogas production. It appears that similar trends are true when glucose is used as a carbon source.

Despite consistency in the trends observed, the maximum P_s seen in this work is significantly lower than that reported elsewhere. Khanal *et al* [15] and Sung *et al* [22] reported production potential values of 28 and 89 mL H_2/(g COD/L) respectively, in sucrose degradation with mixed anaerobic inoculum. In both studies, however, the nature of the substrate was different and its concentration was much higher (>11 g COD/L) than in the work done here. Thus, it is possible that at higher substrate levels P_s obtained in these experiments could be in the same vicinity as that seen elsewhere. In fact, a linear extrapolation of the observed P_s trend along a constant pH of 6.1 gives a value of approximately 25 mL H_2/ g COD/L, thus making the

comparison to Khanal's value reasonable. In order to test this hypothesis, though, experiments with higher glucose concentrations would need to be carried out.

3.3 Specific Hydrogen Production Rate

The hydrogen production rate obtained from the nine-trial data is shown in Figure 4a. The data indicates that both initial pH and glucose concentration have noticeable effects on the rate of hydrogen production (R), and also that there is interaction between these two parameters. At low substrate loadings, the largest R appears to be located in the middle of the pH range, in the vicinity of 6.1. As the glucose concentration increases, however, the high-end pH values seem to favour greater hydrogen production rates. Low pH gives poor R across the entire substrate concentration span. This is somewhat expected, as similar trends have been reported in other works [15,16]; as low pH tends to have an initial inhibitory effect on the bacteria causing a longer lag phase and lower rate of production.

Based on Figure 4a, the largest R appears to be at high initial pH and substrate concentration, a region of the plot that is mostly extrapolated. As a result, conducting experiments at the corners of the matrix became crucial prior to drawing final conclusions regarding the location of highest hydrogen production rate. The new surface plot can be seen in Figure 4b. In general the shape, as well as the scale of the graph, remained unchanged, with slight alterations to the extrapolated areas. The highest R of 71 mL H_2/(h*L) was measured at pH 6.3 and substrate loading of 2.5 g COD/L, and similarly high rates were observed in the region around these parameter values. This value is in agreement with what was found in literature; maximum volumetric rates of hydrogen production from pure substrates in batch systems have been reported in the range of 30 – 140 mL H_2/(L*h) [4,11,21,23].

In practice, any treatment/conversion process strives for high yield and fast rate but often, such as in this case, the most promising operating conditions are different for each variable of interest. When this occurs, a compromise must be made. In this system, out of the two response variables, P_s and R, the rate is of more interest since continuous biogas systems operate more optimally at low to mid-range hydraulic retention times [24,25] meaning that maximum conversion is hardly ever achieved. For this reason, more effort should be devoted to maximizing the rate of hydrogen production with partial sacrifice to the conversion efficiency.

3.4 Soluble products

The composition of the liquid media, following the completion of biogas production, was of significant interest and the distribution of the soluble products resulting from the fermentation of glucose for all thirteen experiments is shown in Table 2. In all cases, the most prominent product was in the form of either butyrate (14% to 63%), formate (10% to 45%) or ethanol (16% to 40%) with propionate, acetate and propanol present in smaller quantities. No glucose was detected in the media at the completion of the experiment. To test for correlation between the soluble products and the two variables of interest, plots of R and P_s as a function of each metabolite concentration were generated (data not shown); in all cases, no correlation was observed.

Presence of formate, butyrate, ethanol, propanol, butanol and propionate during anaerobic fermentation by clostridia has been widely reported [13,14,26] and is in accordance with fermentative metabolism, however the concentrations of ethanol and formate are much higher in this study than that reported elsewhere [11,13,26]. From a hydrogen production perspective volatile fatty acids (VFAs), acetate and butyrate, are the desirable soluble products since hydrogen generation occurs via those reactions. Presence of ethanol is particularly undesirable due to its added toxic effect on bacteria. High concentrations of formate and ethanol are in agreement with the low P_s values that were observed in all experiments (see Table 1) since these metabolites represent hydrogen that has not been released as gas. Clearly, the metabolism witnessed in this work is far from ideal for obtaining optimal biohydrogen production parameters. In order to maximize hydrogen yield, substrate metabolism should be steered towards VFAs and away from alcohol (solventogenesis) or reduced acid production (ex. formate); more work is needed in order to understand how *C. beijerinckii* can be directed towards the desired metabolic pathways.

It is difficult to know whether the production of ethanol was happening simultaneously with hydrogen generation or if there was a shift in the metabolism at some point during the experiment. Lay *et al* [27] has indicated that a shift from H_2/VFA production to solventogenesis occurs around pH 5.6, but no significant pH decrease was observed in any of the experiments. Other authors suggest that alcohol production occurs once the bacteria enter the stationary growth phase [14,28], while still other works attribute the shift to increasing hydrogen partial

pressure [5,9]. In order to gain a better understanding of the metabolic activity occurring in the system additional experiments are required; periodically monitoring the composition of the liquid media throughout the duration of the experiment is suggested. This information could help discover not only the cause of the metabolic shift and hence possible methods for steering the systems away from solventogenesis, but more importantly identify a performance variable (ex. formate concentration) that could be used in operating a continuous biohydrogen production process.

4 Concluding remarks

This work has demonstrated that substrate concentration (glucose) and initial pH have an effect on both the hydrogen production potential and rate of hydrogen production for a pure anaerobic bacterium of *C. beijerinckii*. Specifically, following conclusions can be drawn:

1) **Greatest conversion efficiency is achieved at high glucose concentration and mid-range pH.** Increasing substrate concentration gives rise to increasing conversion efficiency. In the range studied, highest P_s of 10.3 mL H_2/g (COD/L) was observed at substrate concentration and pH of 3 g COD/L and 6.1, respectively. Trends indicate that both high and low-end pH is unfavourable.
2) **Highest rate of hydrogen production occurs at high pH and high glucose concentration.** Increasing both the COD and pH gives results in increasing rate of hydrogen production. In the range studied, highest R of 71 mL H_2/(h*L) was achieved at pH 6.3 and substrate loading of 2.5 g COD/L. Low pH gives poor R across the entire substrate concentration range.
3) **Volatile fatty acids and alcohols are the main soluble products resulting from the fermentation process.** In all experiments, butyrate (14% to 63%) formate (10% to 45%) and ethanol (16% to 40%) were the main soluble metabolites with pronanoic acid (<20%), propanol (<8%) and acetate (<5%) present in smaller quantities. No correlation between the final concentration of each soluble product and neither R nor P_s was observed.

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6 References

1. Climate Change 2007: The Physical Science Basis – Summary for Policymakers. Paris: Intergovernmental Panel on Climate Change; 2007 February 5th.
2. Atif AAY, Fakhru'l-Razi A, Ngan MA, Morimoto M, Iyuke SE, Veziroglu NT. Fed batch production of hydrogen from palm oil mill effluent using anaerobic microflora. Int J Hydrogen Energy 2005;30(13-14):1393-1397.
3. Benitez JF, Acero JL, Gonzalez T, Garcia J. Organic matter removal from wastewaters of the black olive industry by chemical and biological procedures. Process Biochem 2001;37(3):257-265.
4. Chen WM, Tseng ZJ, Lee KS, Chang JS. Fermentative hydrogen production with Clostridium butyricum CGS5 isolated from anaerobic sewage sludge. Int J Hydrogen Energy 2005;30(10):1063-1070.
5. Van Ginkel S, Sung S, Lay JJ. Biohydrogen production as a function of pH and substrate concentration. Environ Sci Technol 2001;35(24):4726-4730.
6. Mohan SV, Babu VL, Sarma PN. Anaerobic biohydrogen production from dairy wastewater treatment in sequencing batch reactor (AnSBR): Effect of organic loading rate. Enzyme Microb Tech 2007;41(4):506-515.
7. Oh SE, Logan BE. Hydrogen and electricity production from a food processing wastewater using fermentation and microbial fuel cell technologies. Water Res 2005;39(19):4673-4682.
8. Van Ginkel SW, Oh SE, Logan BE. Biohydrogen gas production from food processing and domestic wastewaters. Int J Hydrogen Energy 2005;30(15):1535-1542.
9. Yu H, Zhu Z, Hu W, Zhang H. Hydrogen production from rice winery wastewater in an upflow anaerobic reactor by using mixed anaerobic cultures. Int J Hydrogen Energy 2002;27(11-12):1359-1365.
10. Mohan SV, Bhaskar YV, Krishna PM, Rao NC, Babu VL, Sarma PN. Biohydrogen production from chemical wastewater as substrate by selectively enriched anaerobic mixed consortia: Influence of fermentation pH and substrate composition. Int J Hydrogen Energy 2007;32(13):2286-2295.
11. Fang HHP, Zhu H, Zhang T. Phototrophic hydrogen production from glucose by pure and co-cultures of Clostridium butyricum and Rhodobacter sphaeroides. Int J Hydrogen Energy 2006;31(15):2223-2230.
12. Lin PY, Whang LM, Wu YR, Ren WJ, Hsiao CJ, Li SL, Chang JS. Biological hydrogen production of the genus Clostridium: Metabolic study and mathematical model simulation. Int J Hydrogen Energy 2007;32(12):1728-1735.
13. Kim MS, Baek JS, Yun YS, Sim SJ, Park S, Sunghun, Kim SC. Hydrogen production from Chlamydomonas reinhardtii biomass using a two-step conversion process: Anaerobic conversion and photosynthetic fermentation. Int J Hydrogen Energy 2006;31(6):812-816.

14. Hawkes FR, Dinsdale R, Hawkes DL, Hussy I. Sustainable fermentative hydrogen production: challenges for process optimisation. Int J Hydrogen Energy 2002;27(11-12):1339-1347.

15. Khanal SK, Chen WH, Li L, Sung S. Biological hydrogen production: effects of pH and intermediate products. Int J Hydrogen Energy 2004;29(11):1123-1131.

16. Zhao QB, Yu HQ. Fermentative H_2 production in an upflow anaerobic sludge blanket reactor at various pH values. Bioresource Technol 2008;99(5):1353-1358.

17. Wang J, Wan W. Factors influencing fermentative hydrogen production: A review. Int J Hydrogen Energy;In Press, Corrected Proof.

18. Box GEPH, Hunter WG, Hunter JS. Statistics for experimenters:an introduction to design, data analysis, and model building. New York, NY: Wiley, 1978.

19. Owen WF, Stuckey DC, Healy JB, Young LY, McCarty PL. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. Water Res 1979;13(6):485-492.

20. Oh SE, VanGinkel S, Logan BE. The Relative Effectiveness of pH Control and Heat Treatment for Enhancing Biohydrogen Gas Production. Environ Sci Technol 2003;37(22):5186-5190.

21. Jeong TY, Cha GC, Yeom SH, Choi SS. Comparison of hydrogen production by four representative hydrogen-producing bacteria. J Ind Eng Chem 2008;14(3):333-337.

22. Sung S, Raskin L, Duangmanee T, Padmasiri S, Simmons JJ. Hydrogen production by anaerobic microbial communities exposed to repeated heat treatments. 2002; US DOE Hydrogen Program Review.

23. Ogino H, Miura T, Ishimi K, Seki M, Yoshida H. Hydrogen Production from Glucose by Anaerobes. Biotechnol Progr 2005;21(6):1786-1788.

24. Chang FY, Lin CY. Biohydrogen production using an up-flow anaerobic sludge blanket reactor. Int JHydrogen Energy 2004;29(1):33-39.

25. Yang H, Shao P, Lu T, Shen J, Wang D, Xu Z, Yuan X. Continuous bio-hydrogen production from citric acid wastewater via facultative anaerobic bacteria. Int J Hydrogen Energy 2006;31(10):1306-1313.

26. Fang HHP, Liu H. Effect of pH on hydrogen production from glucose by a mixed culture. Bioresource Technol 2002;82(1):87-93.

27. Lay J, Lee Y, Noike T. Feasibility of biological hydrogen production from organic fraction of municipal solid waste. Water Res 1999;33(11):2579-2586.

28. Kapdan IK, Kargi F. Bio-hydrogen production from waste materials. Enzyme Microb Tech 2006;38(5):569-582.

Figure 1

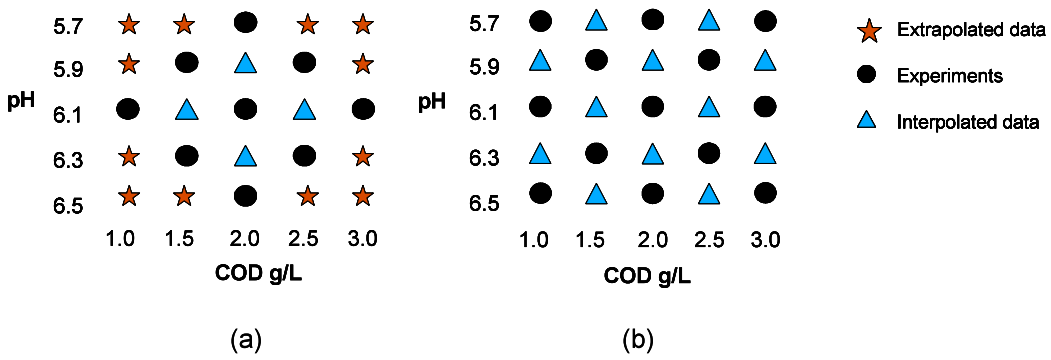


Figure 1: Fractional factorial designs.

Figure 2

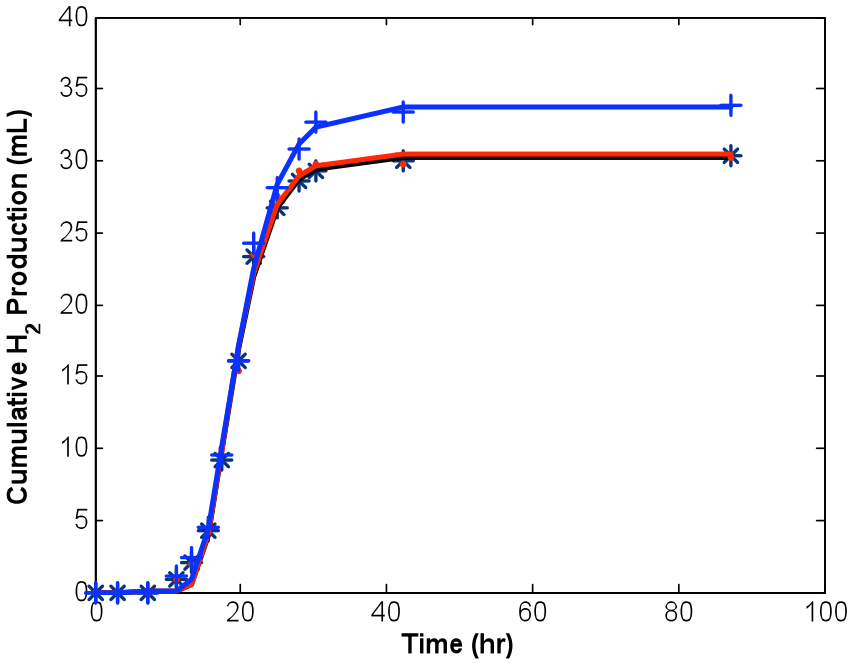


Figure 2: Cumulative hydrogen production curves for trial nine (pH: 6.1, [S]: 3 g COD/L) reported at 25°C and 1 atm. Each curve represents one of the triplicates and in all three cases, R^2 > 0.996.

Figure 3

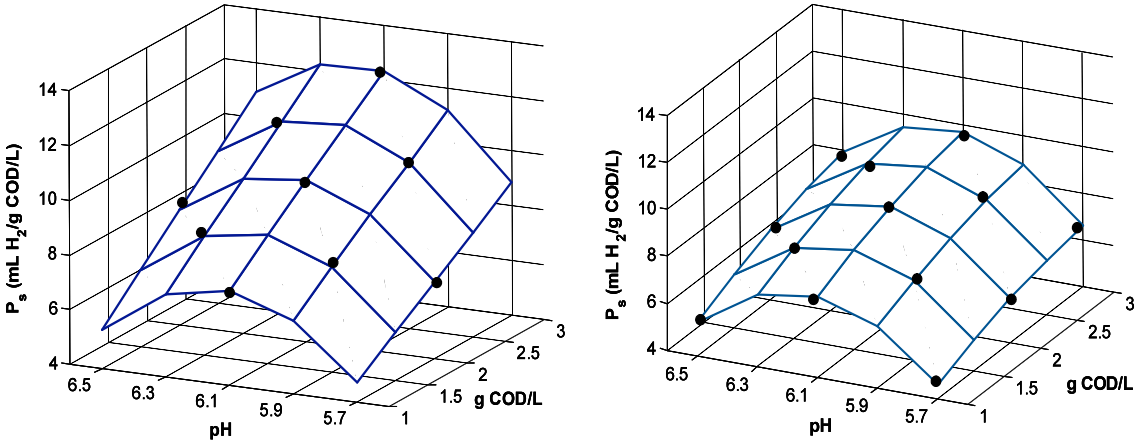
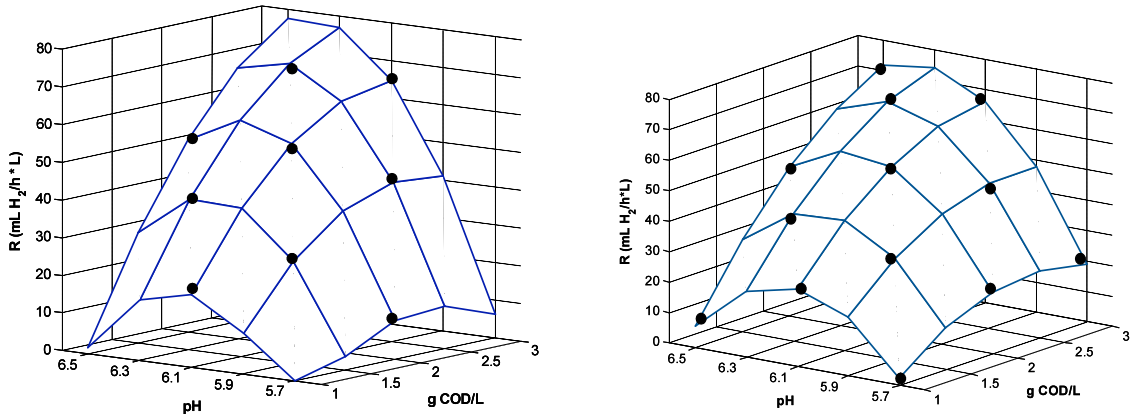


Figure 3: Specific hydrogen production potential 25°C and 1 atm (a) based on the fractional factorial design (9 trials); (b) based on the revisited design (13 trials). The marked points indicate the sum of measured values and the residual of the regression.

Figure 4



(a) (b)

Figure 4: Hydrogen production rate at 25°C and 1 atm (a) based on the initial fractional factorial design (9 trials); (b) based on the revised design (13 trials). The marked points indicate the sum of measured values and the residual of the regression.

Table 1

Table 1: Average values for H_{max}, R_m and λ for the thirteen batch experiments.

COD (g/L)	pH	Trial	H_{max} (mL H_2)		R_m (mL H_2/h)		λ (h)	
			Average	*STDEV*	*Average*	*STDEV*	*Average*	*STDEV*
1	6.1	1	6.9	0.6	1.0	0.2	10.1	0.4
1.5	5.9	2	13	1	2.0	0.5	11.4	1.5
1.5	6.3	3	12.5	0.8	2.1	0.4	16.9	0.9
2	5.7	4	11.6	0.7	0.2	0.0	20	4
2	6.5	5	16	1	2.9	0.6	22.9	0.3
2	6.1	6	19	1	2.4	0.8	23.2	0.4
2.5	5.9	7	21.5	0.6	2.8	0.2	14.4	0.4
2.5	6.3	8	21.5	0.6	3.6	0.2	18.0	0.4
3	6.2	9	31	2	3.3	0.1	14.5	0.1
3	6.5	10	22	1	3.3	0.4	26.3	2.1
3	5.7	11	24	3	1.2	0.4	55	7
1	6.5	12	5.5	0.3	0.2	0.0	5.5	0.4
1	5.7	13	5.0	0.4	0.4	0.2	33	7

Table 2: Soluble product distribution in the effluent.

Trial	COD	pH	Formate	Butyrate	Ethanol	Propionate	Acetate	Propanol
1	1	6.1	35%	17%	22%	17%	3%	6%
2	1.5	5.9	34%	37%	21%	5%	2%	1%
3	1.5	6.3	30%	25%	25%	12%	3%	4%
4	2	5.7	39%	39%	20%	0%	1%	0%
5	2	6.5	16%	35%	30%	13%	4%	3%
6	2	6.1	22%	19%	40%	10%	4%	4%
7	2.5	5.9	13%	52%	30%	0%	4%	0%
8	2.5	6.3	27%	40%	21%	6%	3%	3%
9	3	6.1	10%	63%	20%	4%	3%	0%
10	3	6.5	13%	47%	25%	10%	4%	2%
11	3	5.7	32%	45%	16%	4%	2%	0%
12	1	6.5	21%	14%	36%	16%	4%	8%
13	1	5.7	45%	21%	25%	7%	3%	0%