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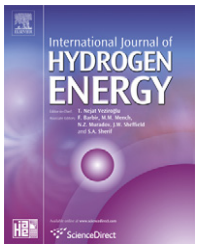

Fermentative hydrogen production in batch experiments using lactose, cheese whey and glucose: Influence of initial substrate concentration and pH

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ABSTRACT

Biologically produced hydrogen using biomass and mixed bacterial cultures is one approach to generate renewable H_2. Response surface methodology (RSM) was used to study the effect of initial pH (3.88–8.12) and initial substrate concentration (0.86–29.14 g/L) on both hydrogen molar yield (HMY) and volumetric H_2 production rate (VHPR). Lactose, cheese whey powder (CWP) and glucose were used as substrates and heat-treated anaerobic granular sludge as inoculum. For lactose, 3.6 mol H_2/mol lactose and 5.6 mmol H_2/L/h were found at pH 7.5 and 5 g lactose/L. CWP yielded 3.1 mol H_2/mol lactose at pH 6 and 15 g CWP/L while 8.1 mmol H_2/L/h were attained at pH 7.5 and 25 g CWP/L. Glucose yielded 1.46 mol H_2/mol substrate (pH 7.5, 5 g glucose/L), with a VHPR of 8.9 mmol H_2/L/h, at pH 8.12 and 15 g glucose/L. Acetic and butyric acids were the main organic metabolites detected. HMY and VHPR obtained in this study were found at initial pH above the reported optimum pH value for hydrogen production. These findings could be of significance when alkaline pretreatments are performed on organic feedstock by eliminating the need to lower the pH to acidic levels before fermentation start-up.

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1. Introduction

Hydrogen gas (H_2) is considered a valuable energy carrier, and an alternative to fossil fuels, since its combustion or utilization in fuel cells to produce electricity only yields water and heat as by-products [1]. Under anaerobic conditions, a wide variety of microorganisms evolve H_2 (biohydrogen) from organic matter [2]. Among the known biochemical routes to produce H_2, fermentative hydrogen production is a promising one [3].

Mixed anaerobic microbial populations from different sources (soil, sediment, compost, aerobic and anaerobic sludges) have been studied as inocula for H_2 production [4]. In these processes, most of the microbial populations were treated, before inoculation, with heat or acid to select for biohydrogen-producing communities. According to a review

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by Kraemer and Bagley [5], heat treatment has been a common method for killing methanogens (hydrogen-consuming microorganisms), leaving behind sporogenic bacteria such as *Clostridium*, *Bacillus* and *Thermoanaerobacterium*. However, in some cases heat treatment was not effective in selecting only H_2-producing microorganisms because few hydrogen-consuming bacteria, such as lactic or propionic acid producers and acetogens, could survive [5].

There are reports in which pure cultures or microbial populations have been used for biohydrogen production using sugars or complex substrates such as organic wastes [6]. Among the sugars used extensively are glucose, sucrose and to a lesser extent lactose. Due to thermodynamic constraints, a maximum of 4 mol of H_2 can be produced from 1 mol of glucose when acetic acid is the main organic product. This yield is lower ($\leq$2 mol H_2/mol glucose) when more reduced metabolites, such as butyric acid, are also produced [7,8].

Glucose and sucrose are of interest as model substrates due to their easy biodegradability. On the other hand lactose is also an interesting model substrate because it is present in wastes or by-products from the dairy industry. One lactose-containing by-product is cheese whey, that represents around 85–90% of the total volume of processed milk and it is a potential substrate for fermentative processes [9]. Dry cheese whey powder (CWP) is obtained from cheese whey by spray or drum drying with a cost of around 0.30 USD/kg CWP [10]. Therefore, CWP represents a cheap concentrated source of lactose (>61% w/w).

One of the approaches used to study the effect of parameters such as temperature, pH, substrate concentration and others, as independent variables, is by using an *a priori* statistical experimental design along with the analysis of the results using response surface methodology (RSM). There are few reports in the literature in which this approach had been used to find optimal conditions for Bio-H_2 production using starch [11,12], sucrose [13–15] and food waste [16].

Thus, the aim of this work was to study the kinetics of hydrogen production using an enriched mixed population and glucose, lactose or CWP as carbon substrates in batch experiments. The effect of different levels of initial substrate concentration ($[S_0]$) and initial pH on both, the volumetric hydrogen production rate (VHPR) and hydrogen molar yield (HMY), was evaluated using a central composite experimental design and RSM. The concentration of fermentation end products was also measured.

2. Materials and methods

2.1. Inoculum and substrate

Anaerobic granular sludge from a full-scale up-flow anaerobic sludge blanket (UASB) reactor was used as inoculum for biohydrogen production. The UASB reactor treats wastewater from a candy factory in San Luis Potosí, México. The granular sludge was washed with three volumes of tap water and then boiled for 40 min to inactivate methanogenic microflora and stored at 4 °C before use. Glucose and lactose were obtained from Sigma–Aldrich (Minnesota, USA), and CWP was purchased from Land O'Lakes Inc. (Minnesota, USA). The lactose content of CWP was 77% with 11% protein (w/w). All chemicals were purchased as reagent grade.

2.2. Biohydrogen production experiments

Batch experiments were conducted in 120 mL serum vials with a working volume of 80 mL. Calculated masses of substrate, 4.5 g volatile suspended solids (VSS)/L of inoculum and 1 mL of mineral medium modified from van Ginkel et al. [15], were added to each vial. One liter of this medium contained: 200 g NH_4HCO_3, 100 g KH_2PO_4, 10 g $MgSO_4{\cdot}7H_2O$, 1.0 g NaCl, 1.0 g $Na_2MoO_4{\cdot}2H_2O$, 1.0 g $CaCl_2{\cdot}2H_2O$, 1.5 g $MnSO_4{\cdot}7H_2O$, 0.278 g $FeCl_2$, 0.24 g $CoCl_2{\cdot}8H_2O$, 0.12 g $NiCl_2{\cdot}6H_2O$ and 0.06 g $ZnCl_2$. Vials were filled to the working volume with deionized water and pH was adjusted using HCl 10 N or NaOH 2 M. After sealing the vials with Wheaton rubber septum stoppers and aluminum rings, the headspace was purged with nitrogen gas for 15 s. Finally, for glucose and lactose experiments the vials were incubated under static conditions and hand-shaken before the headspace gas composition was measured. For CWP, the bottles were placed in a horizontal shaker at 150 rpm in an incubation room. All experiments were carried out at 37 °C. Gas production and composition in the headspace were measured periodically as described in analytical methods.

2.3. Analytical methods

Gas production was measured using a liquid-replacement device filled with water (pH = 2). Hydrogen cumulative production was calculated for each vial considering the headspace composition and the volume of gas released at each time interval, using a mass balance equation [17,18]. All gas volumes are reported at 1 atm and 25 °C.

H_2, CO_2 and CH_4 were measured with a 1.0 mL Pressure-Lok® syringe (Valco Instruments, Houston, Texas, USA) by comparing a 300 μl sample with high purity standards (Alltech, Deerfield, Illinois, USA) using a gas chromatograph (GC, 6890N Network GC System, Agilent Technologies, Waldbronn, Germany) equipped with a thermal conductivity detector. The column used was a Hayesep D (Alltech, Deerfield, Illinois, USA) with the following dimensions: $10' \times 1/8'' \times 0.085''$. Temperatures of the injection port, oven and the detector were 250, 60 and 250 °C, respectively. Nitrogen was used as carrier gas with a flow-rate of 12 mL/min.

At the end of each experiment, 3 mL of liquid samples were taken and 60 μL of $HgCl_2$ (16 g/L) were added before centrifugation at 6610 *g* for 15 min to minimize microorganisms activity [19]. The supernatant was diluted and filtered through a 0.22 μm membrane (Millipore, Bedford, Massachusetts, USA). Remaining substrate and fermentation end products, such as formic, acetic, propionic and butyric acids (VFA) were analyzed in the filtrate by capillary electrophoresis in the same run [20]. Analytes were quantified by comparison with high purity standards. For this purpose a capillary electrophoresis system (Agilent 1600A, Waldbronn, Germany) was used with a basic anion buffer (Agilent, pH = 12.1) and a fused silica capillary column (Agilent, id = 50 μm, L = 80.5 cm, effective length = 72 cm). Temperature and voltage were 20 °C and −30 kV, respectively. The samples were injected with a pressure of 300 mbar for 6 s. Detection was carried out with

indirect UV using a diode-array detector. The signal wavelength was set at 350 nm with a reference at 230 nm. A buffer flush for 4 min at 1 bar was performed prior to each run. Solvents such as acetone, ethanol, propanol and butanol were analyzed by injecting a 1 μL sample in a gas chromatograph 6890N equipped with an auto-sampler 7863 (Agilent, Wilmington, USA) and a capillary column HP-Innowax (30 m × 0.25 mm i.d. × 0.25 m film thickness; Agilent, Wilmington, USA). Helium was used as carrier gas at a flow-rate of 1.5 mL/min. Temperatures for the injector and flame ionization detector (FID) were 220 and 250 °C, respectively. The solvents' analyses were performed with a split ratio of 1:0.1 and a temperature program of 35 °C for 2 min, increased to 80 °C (10 °C/min), and was maintained at this temperature to a final time of 15 min. VSS were analyzed according to the Standard Methods [21].

2.4. Experimental design and data analysis

Once cumulative hydrogen production was calculated from experimental data, a modified Gompertz equation was used to fit the kinetics of biohydrogen production using KaleidaGraph 4.0 (Synergy software). This equation has been widely used to model gas production data [22–25]:

$$H(t) = H_{max}\exp\left\{-\exp\left[\frac{2.71828R_{max}}{H_{max}}(\lambda - t) + 1\right]\right\} \quad (1)$$

where $H(t)$ (mL) is the total amount of hydrogen produced at culture time t (h); H_{max} (mL) is the maximal amount of hydrogen produced. R_{max} (mL/h) is the maximum hydrogen production rate; λ (h) is the lag time before exponential hydrogen production. HMY and VHPR were defined as response variables. HMY was calculated from H_{max} and defined as mol H_2/mol consumed substrate. VHPR was obtained from R_{max} standardized to the working volume (mmol H_2/L/h). As one aim of this work was to evaluate the effect of initial substrate concentration [S_0] and initial pH on both HMY and VHPR, experiments were conducted following a central composite experimental design (Table 1). As can be seen from Table 1, [S_0] varied from 5 to 25 g/L with a central value of 15 g/L and axial points at 0.86 and 29.14 g/L, while pH varied from 4.5 to 7.5 with a central point at 6.0 and axial points at 3.88 and 8.12. The central point was a triplicate run and the experimental design was run in duplicate for data analysis.

Table 1 – Central composite experimental design

Run	Real values		Coded values	
	X_1	X_2	x_1	x_2
1	5.0	4.5	−1	−1
2	5.0	7.5	−1	1
3	25.0	4.5	1	−1
4	25.0	7.5	1	1
5	15.0	3.88	0	−1.414
6	15.0	8.12	0	1.414
7	0.86	6.0	−1.414	0
8	29.14	6.0	1.414	0
9	15.0	6.0	0	0
10	15.0	6.0	0	0
11	15.0	6.0	0	0

To perform the fitting of the experimental design for both initial pH and [S_0] levels, coded variables were used according to Eq. (2).

$$x_i = \frac{X_i - X_i^*}{\Delta X_i} \quad (2)$$

where x_i is the coded value of the ith test variable, X_i is the uncoded or normal value of the ith test variable, X_i^* is the uncoded value of the ith test variable at the center point, and ΔX_i is the step change value in the normal variables [14,26]. X_1 (g/L) and x_1 correspond to the real and coded values for [S_0], respectively, while X_2 and x_2 correspond to the real and coded values for pH. The step change values for [S_0] and pH were set at 10.0 and 1.5, respectively. Experimental results obtained with this *a priori* design were analyzed using RSM due to its suitability in finding optimal values for the response variables as a function of experimental treatments.

The response variables (HMY and VHPR) were fitted using a polynomial quadratic equation to correlate each response variable to the independent variables ([S_0] and pH). The mathematical form of each quadratic equation is described in Eq. (3):

$$y = \beta_0 + \sum_{i=1}^{k}\beta_i x_i + \sum_{i=1}^{k}\beta_{ii}x_i^2 + \sum_{i=1}^{k}\sum_{j=1}^{k}\beta_{ij}x_i x_j \quad (3)$$

where x_i are the independent variables, which could have an influence on the response variable y; β_0 is the constant of the model, β_i is the ith linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the coefficient for the ijth interaction. RSM analyses were made using three-dimensional response surface plots constructed for each polynomial equation with Statgraphics Plus 5.0 software (Statistical Graphics Corp.).

3. Results and discussion

3.1. Kinetics of hydrogen production with lactose, cheese whey and glucose

As an example, kinetic experimental data obtained from each treatment during 46 h of the experiment using CWP are shown in Fig. 1a. After a lag period that ranged from 8 to 15 h depending on the substrate and treatment, biohydrogen production started at different rates and to a different extent. For the three substrates, the hydrogen content in the headspace peaked at around 50–55%, with 50–45% CO_2. Methane was detected (<10%) when CW was used as substrate in all treatments. The profile of hydrogen content in the vial headspace, at central point conditions for glucose conversion to biohydrogen is shown in Fig. 1b. For all substrates, Eq. (1) adequately described biohydrogen production showing regression coefficients (R^2) above 0.87 (Table 2).

The parameters H_{max} and R_{max} obtained from fitting Eq. (1) to the cumulative Bio-H_2 production data for each substrate are shown in Table 2. These parameters were used to calculate the response variables HMY and VHPR. Response variables were analyzed using RSM. Because R_{max} is not normalized to

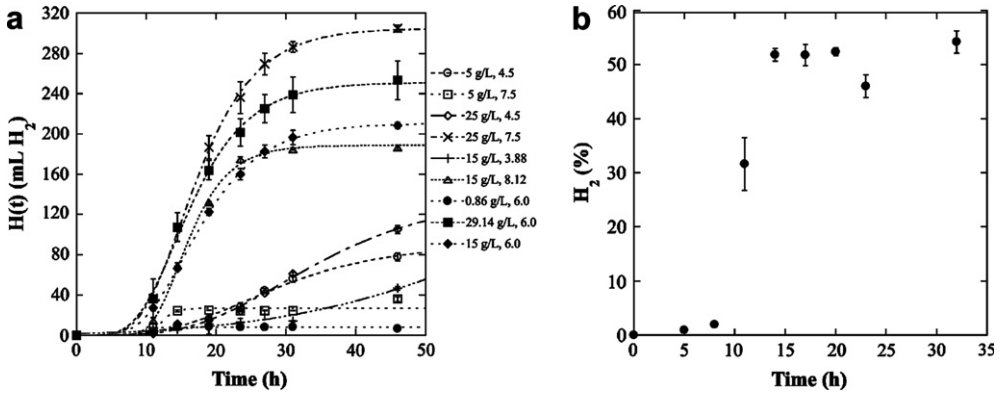

Fig. 1 – (a) Cumulative production of Bio-H_2 from CWP. Modified Gompertz equation fit for each treatment is shown as broken lines. (b) Hydrogen content in the biogas during the batch experiment with glucose at central point conditions (15 g glucose/L, pH 6.0). Standard deviations (SD) are presented as error bars.

the reactor volume, it is not possible to make comparisons between the performances of different reactors. Therefore, VHPR was used as response variable instead of R_{max}. Furthermore, the use of standardized units for hydrogen production rates has been proposed by Levin et al. [27] in order to facilitate the sizing of a bioreactor that would be needed to supply hydrogen to a specific fuel cell for electricity generation.

3.2. *Response surface analysis of HMY and VHPR*

To examine the behavior of both HMY and VHPR, surface response plots were built. The following quadratic equations were used to draw these plots for HMY and VHPR:

Lactose

$$HMY = 2.609 - 0.568x_1 + 0.415x_2 + 0.036x_1^2 + 0.108x_1x_2 - 0.166x_2^2 \quad (4)$$

$$VHPR = 2.691 - 0.074x_1 + 1.606x_2 - 0.183x_1^2 - 0.180x_1x_2 + 0.429x_2^2 \quad (5)$$

CWP

$$HMY = 2.764 - 0.285x_1 + 0.064x_2 + 0.090x_1^2 + 0.508x_1x_2 - 0.822x_2^2 \quad (6)$$

$$VHPR = 5.318 + 1.183x_1 + 2.305x_2 - 0.323x_1^2 + 1.076x_1x_2 - 0.945x_2^2 \quad (7)$$

Glucose

$$HMY = 1.319 - 0.086x_1 + 0.179x_2 - 0.181x_1^2 + 0.098x_1x_2 - 0.057x_2^2 \quad (8)$$

$$VHPR = 4.609 - 0.533x_1 + 3.141x_2 + 0.453x_1^2 + 0.118x_1x_2 + 0.075x_2^2 \quad (9)$$

In Eqs. (4)–(9), x_1 and x_2 are the coded variables for $[S_0]$ and pH respectively. Since the p values for each quadratic model equation (Eqs. (4)–(9)) were below 0.005, thus these equations adequately described the behavior of experimental data. The regression coefficients (R^2) for HMY were 0.78, 0.63 and 0.63 for lactose, CWP, and glucose, respectively, while for VHPR, R^2 were 0.83, 0.94 and 0.91 for lactose, CWP and glucose, respectively.

Table 2 – Adjusted H_{max} and R_{max} for lactose, CWP and glucose

Initial conditions: $[S_0]$, pH	Lactose			CWP			Glucose		
	H_{max} (mL H_2)	R_{max} (mL H_2/h)	R^2	H_{max} (mL H_2)	R_{max} (mL H_2/h)	R^2	H_{max} (mL H_2)	R_{max} (mL H_2/h)	R^2
5 g/L, 4.5	62.4 ± 4.6	4.3 ± 1.7	0.99	91.5 ± 14	3.2 ± 0.8	0.99	54.8 ± 8.8	2.9 ± 0.1	0.99
5 g/L, 7.5	105.3 ± 1.2	13.6 ± 1.2	0.99	26.6 ± 0.2	8.3 ± 0.9	0.87	96.5 ± 4.3	19.6 ± 1.9	0.99
25 g/L, 4.5	32.6 ± 1.2	3.4 ± 1.6	0.97	140 ± 12.2	4.0 ± 0.1	0.99	48.8 ± 2.7	3.4 ± 0.6	0.98
25 g/L, 7.5	171.8 ± 1.4	10.9 ± 1.9	0.99	305.2 ± 3.1	19.6 ± 3.7	0.99	173.2 ± 4.5	21.2 ± 1.0	0.99
15 g/L, 3.88	42.8 ± 0.6	2.6 ± 0.3	0.99	29.8 ± 6.7	0.9 ± 0.1	0.91	34.9 ± 7.0	2.9 ± 0.6	0.98
15 g/L, 8.12	165.1 ± 7.9	12.8 ± 3.4	0.99	188.7 ± 0.5	17.9 ± 0.7	0.99	176.8 ± 15.2	21.6 ± 2.1	0.99
0.86 g/L, 6.0	20 ± 0.1	4.0 ± 2.1	0.90	7.0 ± 0.3	8.5 ± 0.1	0.93	9.0 ± 0.2	18.5 ± 1.7	0.98
29.14 g/L, 6.0	112.5 ± 11.6	5.5 ± 0.2	0.99	251.2 ± 19	16.3 ± 1.4	0.99	95.9 ± 1.4	9.7 ± 1.1	0.99
15 g/L, 6.0	108.7 ± 3.1	6.5 ± 0.1	0.99	210 ± 2.1	12.6 ± 1.5	0.99	101.1 ± 1.3	10.2 ± 0.5	0.99
15 g/L, 6.0	124.4 ± 0.4	6.3 ± 0.2	0.99	211 ± 2.7	13.2 ± 1.9	0.99	104.1 ± 3.9	11.9 ± 2.5	0.99
15 g/L, 6.0	116.1 ± 12.2	6.8 ± 1.1	0.99	197 ± 3.9	13.1 ± 0.1	0.99	115.7 ± 8.0	11.3 ± 0.6	0.99

Note: Data are given as mean values ± SD, $n = 2$.

Table 3 summarizes the highest HMY and VHPR predicted by the response surface analysis. Also, the parameters with the most significant effects on HMY and VHPR are indicated in Table 3.

3.2.1. *Lactose and CWP*

The analysis of variance (ANOVA) for HMY using lactose as substrate showed that both initial lactose concentration and initial pH had a significant effect on HMY (Table 3). The highest experimental HMY was 3.6 mol H_2/mol lactose and was achieved under the same conditions as glucose. This yield represents 45% of the theoretical maximum (8 mol H_2/mol lactose consumed) [28]. According to Fig. 2a, predicted by the quadratic model (Eq. (4)), there is a tendency for HMY to increase as both initial lactose concentration decreases and initial pH increases. For VHPR, the effect of initial pH was greater than the initial lactose concentration. It was reflected in a low p value for initial pH and a higher figure for [S_0] ($p = 0.65$). The highest experimental VHPR obtained was 5.6 mmol H_2/L/h under the same initial conditions as HMY (Table 3).

Fig. 2c shows that the maximum HMY using CWP as substrate was found at pH 6.0. At that pH, HMY is barely sensitive to changes in [S_0]. That is to say, HMY has slightly higher values at low [S_0] than at higher [S_0]. This is because the HMY response surface resembles a saddle [29], with a zone at pH 6.0 in which HMY values decrease with increasing [S_0] and then increase again but at a lower value than the initial one. The significant effects were [S_0] and pH^2 (Table 3). Using CWP the experimental values for HMY at pH 6.0 decrease from 5.9 to 2.8 mol H_2/mol lactose as [S_0] increases from 0.86 to 29.14 g CWP/L. Although 5.9 mol H_2/mol lactose is a very high yield, the low H_{max} (7 mL H_2) found with this treatment (Table 2, CWP: 0.86 g/L, pH 6.0) makes it impractical and therefore this condition is not reported as maximum in Table 3.

For VHPR, the effects of [S_0], pH, the interaction ([S_0] pH) and pH^2 were all significant (Table 3). A clear trend is observed in Fig. 2d with higher VHPR values for both, higher pH and [S_0]. Therefore, it may be possible to find an optimal VHPR value by performing experiments at [S_0] higher than 30 g CWP/L and pH above 8.12. The highest VHPR found using CWP was 8.1 mmol H_2/L/h achieved at near neutral pH and under higher concentration than glucose and lactose (Table 3).

Ferchichi et al. [30] used diluted crude CW (ca. 41.1 g lactose/L) as carbon substrate and a pure *Clostridium* strain, and studied the influence of different initial pH values (5–10) on the hydrogen production rate and yield in batch experiments. The authors found that HMY peaked at pH 6.0 with a value of 2.7 mol H_2/mol lactose and the VHPR was 9.4 mmol H_2/L/h. In another study with a *Clostridium* strain, Collet et al. [28] used lactose (10 g/L) as carbon substrate in continuous culture obtaining HMY from 2.1 to 3 mol H_2/mol lactose and VHPR around 2.5 mmol H_2/L/h depending on the dilution rate at pH 7. Recently, Yang et al. [31] performed both batch and continuous experiments using cheese whey permeate powder as substrate. In batch experiments with initial pH that ranged from 7.28 to 7.33 the authors obtained yields between 8 and 10 mM/g chemical oxygen demand (COD) fed, achieved with anaerobic sludge as inoculum and uncontrolled pH. However, the hydrogen production rate was not reported for batch experiments. Best results were found by the authors in the continuous system (hydraulic retention time (HRT) = 24 h, organic loading rate = 12 g COD/L/d), at controlled pH (4–5) attaining yields between 1.8 and 2.3 mM/g COD and volumetric production rates up to 18.75 mL H_2/L/h.

In the present study, biohydrogen production from the lactose and protein present in cheese whey powder solution resulted in comparable HMY and VHPR values as reported in the previous works mentioned above [28,30]. However, in this study the highest VHPR was obtained under more alkaline initial conditions.

3.2.2. *Glucose*

The analysis of variance showed a stronger effect of initial pH than initial glucose concentration ([S_0], $p = 0.1148$) on HMY. However, there was a significant effect of the quadratic term $[S_0]^2$ (Table 3). From Fig. 2e it is clear that a simultaneous increase in [S_0] and decrease in pH lowers HMY.

Some authors had reported HMY and VHPR from batch experiments using glucose as substrate and mixed microbial populations. Among these, Kawagoshi et al. [32] obtained an HMY of 1.4 mol H_2/mol glucose working at an initial glucose concentration of 20 g/L; they found two pH values as suitable initial conditions for biohydrogen production: 6.5 and 7.0. Salerno et al. [33] found the highest HMY (1.17 mol H_2/mol glucose) using low glucose concentration (3.76 g/L) at pH 6.2. Park et al. [19] obtained 2 mol H_2/mol glucose also at pH 6.12. Furthermore, Zheng and Yu [34] attained an HMY of 1.75 mol H_2/mol glucose at an initial glucose concentration of 10 g/L and pH 6.0. The highest experimental HMY found in this work was 1.46 mol H_2/mol glucose at initial pH above the values reported by Kawagoshi et al. [32] and [S_0] above 3.76 g/L used by Salerno et al. [33] (Table 3).

Table 3 – Summary of significant effects from the ANOVA analysis and experimental conditions for best HMY and VHPR

Substrate	Significant effects (p value < 0.10)		Highest HMY (mol H_2/mol substrate) and VHPR (mmol H_2/L/h) obtained, and conditions at which they were found	
	HMY	VHPR	HMY	VHPR
Lactose	pH (0.0005), [S_0] (0.0001)	pH (0.0001)	3.6 ± 0.03, pH = 7.5, [S_0] = 5 g/L	5.6 ± 0.48, pH = 7.5, [S_0] = 5 g/L
CWP	pH^2 (0.0032), [S_0] (0.0394)	pH (0.0001), [S_0] (0.0001), pH [S_0] (0.0004), pH^2 (0.0002)	3.1 ± 0.04 (mol H_2/mol lactose), pH = 6.0, [S_0] = 15 g/L	8.1 ± 1.5, pH = 7.5, [S_0] = 25 g/L
Glucose	pH (0.0018), $[S_0]^2$ (0.0108)	pH (0.0001), [S_0] (0.0557)	1.46 ± 0.07, pH = 7.5, [S_0] = 5 g/L	8.9 ± 0.87, pH = 8.12, [S_0] = 15 g/L

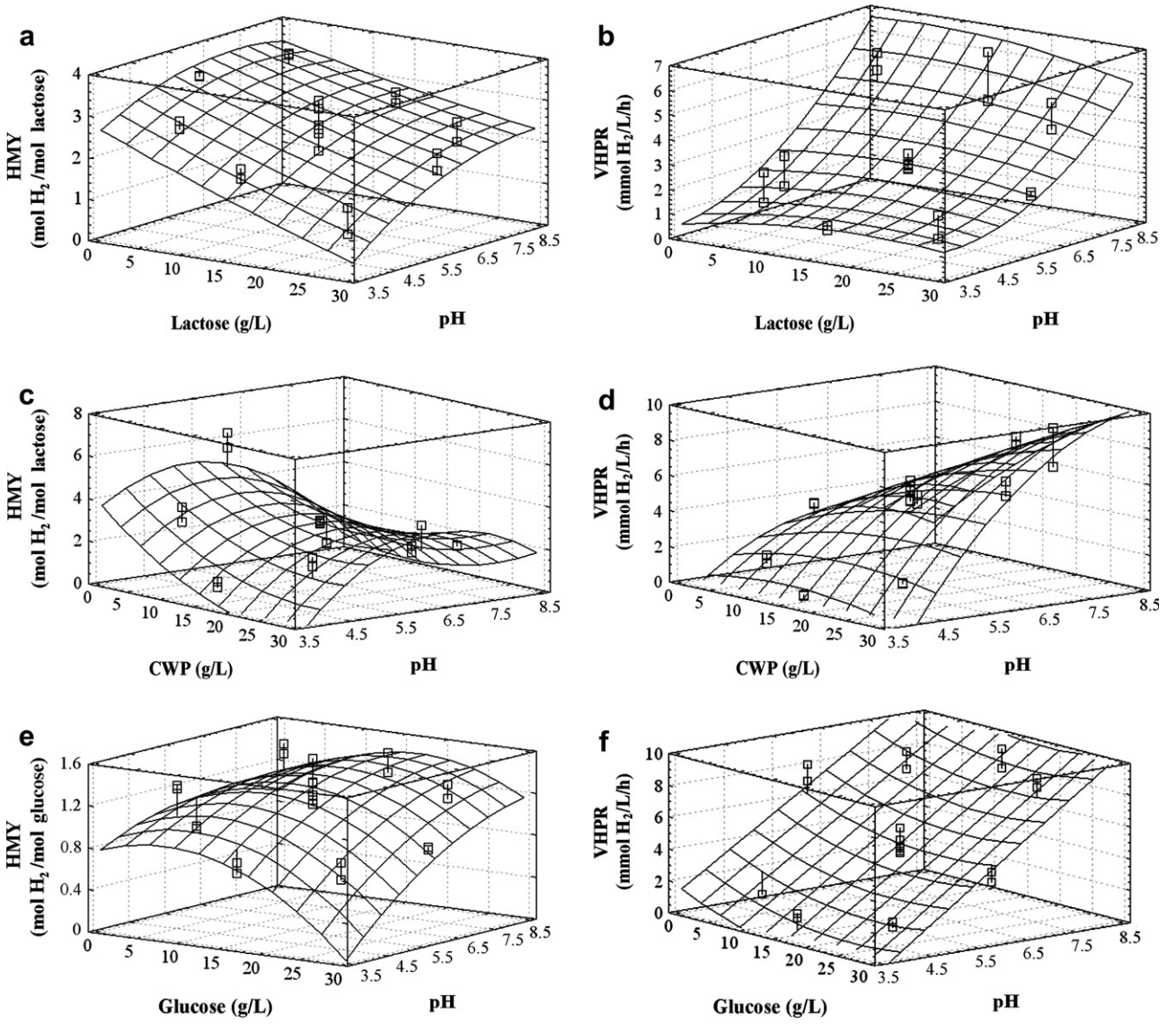

Fig. 2 – Hydrogen molar yield (HMY) and volumetric hydrogen production rate (VHPR) for lactose (a, b), CWP (c, d) and glucose (e, f). Experimental data are shown in squares with the standard error bars.

Regarding the VHPR, the quadratic model (Eq. (9)) adequately described the variance of the experimental data ($R^2 = 0.91$). In this case, the effect of initial pH value was the most significant while [S_0] had a lower effect (Table 3). As can be seen from Fig. 2f there is a clear trend in which an increase in pH, regardless of [S_0], caused an increase in VHPR. The highest experimental rate was 8.9 mmol H_2/L/h (Table 3). Results published by other authors report similar figures for VHPR. Cheong and Hansen obtained 8.6 mmol H_2/L/h at a controlled pH of 5.7 and [S_0] ~ 21.3 g/L [35]. Salerno et al. [33] achieved 9 mmol H_2/L/h at pH 6.2 and [S_0] = 3.76 g/L.

3.3. *Analysis of fermentation end products and final pH in culture medium*

Analysis of solvents such as acetone, ethanol, propanol and butanol was performed for the treatments with the highest VHPR using CWP as substrate but only ethanol was detected. Ethanol concentrations ranged from 10 to 50 mg/L (0.2–1 mM). As these concentrations were very low compared to those obtained of volatile fatty acids, only VFA were further analyzed as major fermentation end products.

The VFA found in the culture by the end of each experiment are shown in Fig. 3. For the three substrates used, the acetic and butyric acids were the main metabolites (up to 65 mM) while propionic acid was found to a lesser extent (up to 10 mM). As a result of VFA production, pH decreased to acidic conditions for all substrates (Table 4). Final pH values were similar for glucose and lactose, but were higher for CWP. The reason for this is likely due to the anaerobic digestion of protein present in CWP which produces ammonia and, therefore, increases pH [36].

When lactose was used (Fig. 3a), the experimental conditions that yielded the highest H_{max} (Table 2) also resulted in high butyric acid concentration (ca. 35 mM). For these conditions acetic acid content was around 20 mM. At both, central

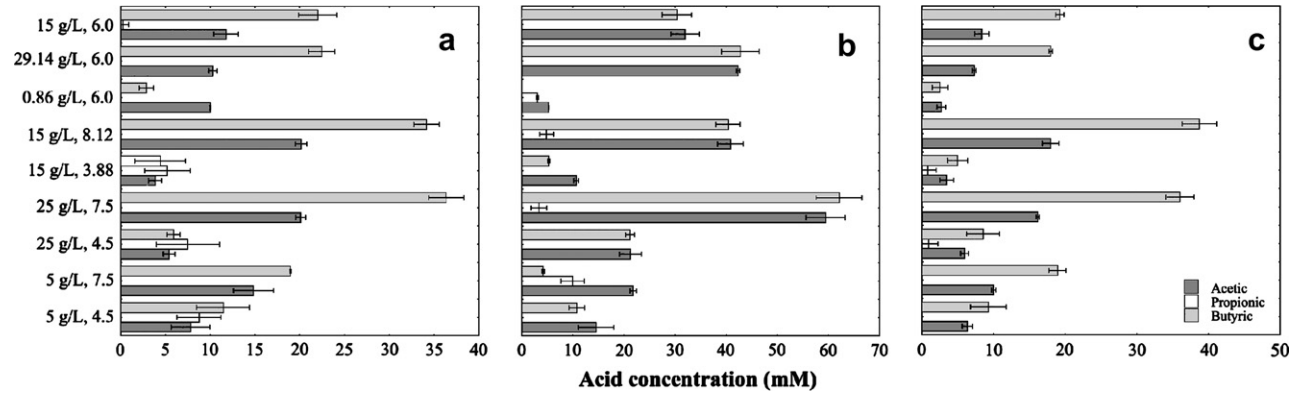

Fig. 3 – Total VFA concentrations (acetic, propionic and butyric acids) at the end of batch experiments for (a) lactose, (b) cheese whey powder (CWP) and (c) glucose.

point (15 g lactose/L, pH 6.0) and axial point (29.14 g/L, pH 6.0) the acids production were similar with acetic acid around 10 mM and butyric acid around 22 mM. Propionic acid was detected (below 10 mM) in treatments with initial pH ≤ 4.5.

In most cases production of acetic and butyric acids, using CWP, was at about the same concentration (1:1, Fig. 3b). The treatment with the high VHPR (25 g CWP/L, pH 7.5) also yielded the highest concentration of both acetic and butyric acids at around 60 mM each. Treatments with [S_0] and pH above 15 g/L and 6.0, respectively, produced between 30 and 40 mM of acetic and butyric acids.

For glucose, the treatments with higher butyric acid concentration (>35 mM) and acetic acid content around 18 mM (Fig. 3c), correlated with the conditions in which both higher volumes of H_2 (H_{max}: Table 2) and higher VHPR were achieved. The treatments with either low pH ($\leq$4.5) or [S_0] = 0.86 g glucose/L resulted in low concentrations (below 10 mM) of both butyric and acetic acids. Except for treatment with [S_0] = 0.86 g glucose/L and pH 6, in which acetic and butyric acids concentration was the same (ca. 3 mM); for the rest of experiments, the butyric content was higher than the acetic acid.

Quantification of fermentation end products such as VFA is important due to their role as regulators in metabolic shifting from acidogenesis (hydrogen production) to solventogenesis (production of acetone, ethanol, propanol or butanol) in clostridia which reduces hydrogen yield [27]. Moreover, some VFA can be toxic or inhibitory to the H_2-producing microbial populations [34]. As discussed by van Ginkel and Logan [37], butyric acid could be more toxic than acetic acid in a hydrogen-saturated system, and although there is no agreement on the threshold value for shifting from acidogenesis to solventogenesis, it is reported that it could be from 2 to 30 mM of undissociated acids. Another important factor in metabolic shifting is pH. The optimum pH reported for solventogenesis is around 4.5 while for acidogenesis, it is 5.5 or higher [15,30,38]. In the present work, high partial pressures were likely to occur (not measured) in the treatments with the highest VHPR, due to high hydrogen accumulation observed in the headspace of the vials. This high hydrogen partial pressure (pH_2) is one of the reasons that lowers HMY because at $pH_2 > 10^{-4}$ atm, metabolic routes deviate from acetate production to other products such as butyrate [7].

As previously mentioned, pH dropped for most of the treatments due to VFA production. For glucose and lactose, the final pH for treatments with high VHPR ranged from 3.8 to 4.7. When CWP was used, the pH of most treatments fell to a range of 4.3–6.1. Considering that the pK_a values for acetic and butyric acids are 4.76 and 4.81, respectively, undissociated butyric acid concentration for the treatments with the highest VHPR would be around 10–30 mM at the end of the experiments. This concentration of protonated butyric acid could have caused inhibition of the metabolism for hydrogen production.

It is known that inhibition of hydrogen production by butyric acid could be reduced by keeping the pH above 4.8, which helps to decrease the concentration of its undissociated acid. As a result, a process under controlled alkaline pH could overcome this inhibition. However, Fig. 3 shows that at alkaline pH (8.12 and 7.5) a larger amount of VFA was formed. These VFA could be fed into a second stage process for additional energy production, i.e. methane or more hydrogen, thus improving the energy/substrate yields [3,4].

3.4. *Overall performance comparison with previous studies*

Although the results obtained in our work were similar to the reports cited above, one novel aspect of this work was the

Table 4 – Final pH measured for lactose, CWP and glucose

Initial conditions: [S_0], pH	Final pH		
	Lactose	CWP	Glucose
5 g/L, 4.5	3.82 ± 0.13	4.36 ± 0.04	3.90 ± 0.04
5 g/L, 7.5	4.7 ± 0.07	6.06 ± 0.05	4.69 ± 0.09
25 g/L, 4.5	3.69 ± 0.13	4.38 ± 0.01	3.69 ± 0.18
25 g/L, 7.5	3.89 ± 0.10	4.86 ± 0.12	3.86 ± 0.12
15 g/L, 3.88	3.79 ± 0.04	4.34 ± 0.09	3.78 ± 0.05
15 g/L, 8.12	4.06 ± 0.02	4.95 ± 0.04	4.05 ± 0.01
0.86 g/L, 6.0	5.56 ± 0.16	6.12 ± 0.03	5.49 ± 0.13
29.14 g/L, 6.0	3.89 ± 0.12	4.55 ± 0.10	3.84 ± 0.09
15 g/L, 6.0	3.86 ± 0.10	4.46 ± 0.01	3.84 ± 0.13
15 g/L, 6.0	3.82 ± 0.10	4.47 ± 0.04	3.80 ± 0.13
15 g/L, 6.0	3.76 ± 0.18	4.48 ± 0.01	3.87 ± 0.01

comparable values of HMY and VHPR at pH above the range (pH 5–6) considered optimum for fermentative biohydrogen production [15]. There are few reports in the literature in which biohydrogen production is achieved using mixed populations at pH above 7. Wang et al. [39] used a mixed population from an acclimated sewage sludge (continuous stirred tank reactor, HRT = 6–12 h), previously acidified. Hydrogen was produced from sucrose up to an initial pH of 8.5, with an optimum initial pH of 7.5. In another work, biohydrogen was produced from starch in a pH range from 5.5 to 8.5, using acclimated sludge (previously heat-treated) [12]. Therefore, it is possible to select hydrogen-producing organisms that can start to grow or germinate at an initial pH above 7. This microbial ability could be useful in processes in which alkaline pretreatments are used for the solubilisation of sugars from lignocellulosic biomass or also when used to enhance hydrogen production from organic matter [40]. This would eliminate the need for reducing the pH to acidic levels before starting hydrogen production experiments after alkaline pretreatments. Due to the wide range between the high starting pH (above 7) and the final acidic pH, the fermentation time would be longer and consequently a larger amount of biohydrogen would evolve, minimizing the need for base addition. This ability may be related to higher values for HMY and VHPR. That is to say, the wider the pH range, the longer the lapse of time before the pH falls to harmful levels for the microbial cells (toxicity by VFA, high hydrogen partial pressure) and/or triggers a switch to hydrogen-consuming metabolic routes (solventogenesis). Regarding the effect of substrate concentration, for glucose and lactose, the higher HMY were found at low substrate concentration, and it was the same case for VHPR with lactose (Table 3). This is in agreement with previous studies in which high initial concentrations caused high initial hydrogen production, increased hydrogen partial pressure and acid toxicity or pH inhibition [15]. Therefore, it seems that low to moderate initial concentrations may be related to better hydrogen yield/production performance. However, we consider that the inhibitory/toxic thresholds are specific to each system (type of substrate and inoculum) and thus RSM is an efficient tool to carry out further research.

4. Conclusions

RSM is a useful tool to model HMY and VHPR with quadratic equations using glucose, lactose and CWP as carbon substrates. The different behavior of the response variables for the tested substrates indicates that RSM is a robust tool to define optimal conditions for biohydrogen production when new substrates or inocula are tested.

Due to the higher trustworthiness of quadratic VHPR models for the three substrates ($R^2 > 0.83$), this variable may be selected as a design parameter. Then, in order to have high VHPR, the best initial conditions for glucose and lactose are: $[S_0] = 5$ g/L and pH 7.5. When CWP is to be used, higher substrate concentrations are recommended ($[S_0] \geq 15$ g/L at pH 7.5). HMY and VHPR obtained in this study were found at an initial pH above the reported optimum pH value for hydrogen production. These findings could also be useful when alkaline pretreatments are performed either for the solubilisation of sugars from lignocellulosic materials or for the conditioning of organic matter from wastes before hydrogen production.

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