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Maximizing hydrogen production and substrate consumption by *Escherichia coli* WDHL cheese whey fermentation

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E. coli, fermentative metabolism, lactose, biohydrogen, biofuels.

Abbreviations:

CW	Cheese Whey
LB	Lysogeny Broth
MSHPR	Maximum specific hydrogen production rate
WT	Wild Type

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Practical Application.

The suitable treatment and disposal of CW is a big problem for the dairy industry. On the other hand, the production of biofuels from wastes or by-products has been of increasing interest in recent years, and this by-product can be used as cheap substrate for biohydrogen production, due to its high lactose content.

In order to maximize hydrogen production and substrate consumption, one has to control pH, which is one of the most important factors in *E. coli* Δ*hycA,* Δ*lacI* (WDHL) CW fermentation. The results obtained shown that pH controlled at 6.5 resulted in highest cumulative hydrogen production, yield and carbohydrates consumption. This information is of the outmost practical application in the use of CW as substrate in batch, continuous and semi continuous hydrogen fermentations and very useful for the scale-up of energy production processes.

Abstract.

In order to maximize hydrogen production and substrate consumption in *E. coli* Δ*hycA,* Δ*lacI* (WDHL) cheese whey fermentation, the influence of pH control at values of 5.5, 6, and 6.5 was studied in batch stirred tank bioreactors. From the conditions evaluated, pH 6.5 was the best condition, at which highest cumulative hydrogen production and yield were obtained. Moreover, all carbohydrates from the cheese whey were consumed, and a mix of ethanol and organic acids, mainly lactate, were produced from glucose, whereas galactose yielded acetate, ethanol and succinate. Operating the reactor at pH 5.5 resulted in the highest MSHPR but smaller hydrogen yield because only glucose was metabolized. At pH 6, not all cheese whey carbohydrates were consumed, and it was not favorable for hydrogen production. Lactose consumption and growth kinetics were not affected by the pH. The results show the importance of controlling pH to maximize hydrogen production and substrate consumption using cheese whey as substrate.

1 Introduction.

Hydrogen has been recognized as a clean substitute of fossil fuels because of its higher energy yield of 122 kJ/g, which is 2.75-fold greater than hydrocarbon fuels [1]. Moreover, its use is environmentally benign because its combustion or use in fuel cells only produces water [2]. Biological hydrogen production by fermentation is an attractive method because it is carried out at ambient temperature and pressure. In addition, a wide range of substrate types can be used [3-7].

To be economically competitive, fermentative hydrogen production must use carbohydrate-rich wastes or by-products. Cheese whey (CW) is a green-yellowish liquid resulting from the precipitation and removal of casein in cheese production [8, 9]. This by-product represents 85-90% of the total volume of processed milk. Disposal of CW is a major problem for the dairy industry [10]. Most of this by-product is discharged into the environment [11] and only a minor proportion is used in the food industry and for animal feeding. Therefore, disposal of untreated CW is considered a source of environmental pollution due to its bulk quantities and high organic content [12].

The main components of CW are lactose (70-72% dried extract), proteins (8-10%), mineral salts (12-15% dried extract) mainly calcium salts, phosphate, and chloride [13, 14]. Fat content fluctuates between 0.99 and 10.8 g/L [9]. Considering its components, CW is an inexpensive potential raw material for fermentative processes [15, 16].

There are two kinds of whey: the by-product of the production of hard, semi-hard and soft cheese is known as sweet whey; the manufacture of mineral-acid

precipitated casein yields acidic whey [17]. The pHs of sweet whey and acidic whey are 5.9-6.6 and 4.3-4.6 respectively.

Fermentative hydrogen production and the proportions of end products are strongly affected by the culture medium's pH [18]. The initial pH is considered one of the most important parameters that influences fermentative hydrogen production with axenic [19-21] and non axenic cultures [22]. The fermentative pathway in *E. coli* is linked to the production of organic acids such as acetic, formic, lactic, and succinic. These products accumulate in the medium and can affect both hydrogen production and substrate consumption if pH is not controlled in an optimum range. Although the effect of initial pH on hydrogen production has been widely described using a variety of inocula [19-22], few works described the influence of online control of pH in non-axenic cultures [23, 24]. To our knowledge, the effect of pH control on hydrogen production and substrate consumption by *Escherichia coli* has been poorly studied. In this work, the influence of online control of pH on hydrogen production by *Escherichia coli* WDHL [19] using sweet cheese whey as substrate was studied.

2 Material and Methods.

2.1 Strain and culture media.

Escherichia coli WDHL strain [19] was used in this work. Inocula were pre-grown overnight in 25 mL of LB medium at 37°C and shaken at 200 rpm, then added to 900 mL of fresh LB medium in twist cover bottles closed and incubated at 37°C for

48 h. Cells were harvested, washed, and inoculated into the bioreactor at an average initial $OD_{600\ nm}$ of 2.18 ± 0.4. Bioreactor cultures were done using HP medium (a complete description of the medium was reported elsewhere [19]) with 20 g/L of cheese whey powder (Land O´Lakes, Arden Hills, Minnesota).

2.2 Cultures on bioreactor.

Batch cultures were performed in a 1-L bioreactor (Applikon, Foster City, CA.). The pH was monitored on-line using an autoclavable electrode (Applikon) connected to the ADI 1035 Bioconsole (Applikon). The initial pH was 7.5 in all the experiments and was allowed to decrease to the desired value (6.5, 6 or 5.5). Once the pH reached the value indicated in each experiment, was automatically controlled at the set point indicated and using 2.5 N NaOH and HCl solutions. The control parameters were a dead zone of 0.1 and hysteresis of 1. BioXpert 1.3 software (Applikon) was used for data acquisition. The cultures were maintained at 37°C and stirred at 175 rpm with two six-blade Rushton turbines. The fermentations at pH of 5.5 and 6 were done in triplicate.

2.3 Analytical methods.

Cell growth was monitored at $OD_{600\ nm}$ using a spectrophotometer Cary BIO-50 (Varian, Palo Alto, CA). Culture samples were periodically taken from the bioreactor, centrifuged, and the supernatant was filtered through a 0.22 µm filter (Millipore). The gas produced was measured by water displacement in an inverted burette connected to the bioreactor with rubber tubing and a needle. The hydrogen

content in the gas phase, sugars, and organic acids were determined as described elsewhere [22]. Ethanol was determined by gas chromatography as described elsewhere [25].

3 Results and discussion.

3.1 Online pH control influence on hydrogen production.

The pH is one of the most important factors in hydrogen production by *Escherichia coli* [19, 26, 27]. In a previous work, it was observed that the hydrogen production by WDHL (Δ*hycA,* Δ*lacI*) strain was better with respect to the wild type (WT) strain, and the initial pH is an important factor for the hydrogen production using cheese whey as substrate [19]. In order to study the effect of pH control on hydrogen production a set of experiments was conducted at values of 5.5, 6, and 6.5.

Figure 1 shows cumulative hydrogen production and cell growth at pH values of 5.5, 6 and, 6.5. The growth kinetics showed a similar behavior in the 3 cases. A slight increment in biomass concentration was observed during the first 12 h, and then the biomass decreased slowly reaching initial values. The highest increase in biomass was observed at a pH of 6.5. In this work, no additional nitrogen source was added to the culture media to decouple hydrogen production from growth [28] as used in other works [29, 30].

The control of pH at 5.5 resulted in a maximum hydrogen volume of 868 mL (Figure 1A), and it was the lowest hydrogen production of the conditions tested. Hydrogen was only produced in the first 20 hours, and then hydrogen production

stopped. At a pH 6 (Figure 1B), two phases of hydrogen production were observed; 1157 mL were produced in the first 56 h, and 689 mL were produced in 164 h to yield a maximum cumulative volume of 1846 mL. As shown in Figure 1C, the highest cumulative hydrogen production of 2402 mL was attained at 6.5.

3.2 Substrate consumption.

Lactose is the main component of CW, which is hydrolyzed by the β−galactosidase enzyme, producing glucose plus galactose. The concentration of these carbohydrates was analyzed in the fermentation samples to study the effect of pH on the up-take of these sugars. Figure 2 shows the lactose and galactose consumption at pH values studied. Lactose was quickly consumed during the first hours of fermentation at pHs of 5.5, and 6.5; at pH 6, the lactose was completely consumed after 45 h (Figure 2A). This could be due to the lowest initial OD used in this experiment. As lactose concentration decreased galactose accumulated in the three conditions (Figure 2B), whereas glucose was not detected. Galactose was completely consumed when the pH value was controlled at 6.5 after 170 h, and partially consumed at 6. Interestingly, when the pH was 5.5, the galactose was not metabolized even after 150 h of fermentation.

The response of *E. coli* to the pH of the culture media is important to survive. The increase or decrease of the expression of specific genes to adapt to high or low pH has been studied before in cultures of *E. coli* [31, 32]. For instance, Yohannes *et al.* [33] found high pH induction of glycolitic enzymes under anaerobic conditions, and it was suggested that an increment of the fermentation rate and acids

129 production helps to neutralize the high alkalinity. The accumulation of galactose
130 observed at pH of 5.5 in the present work, could be explained by a low expression
131 of the genes related to the galactose catabolism caused by the harsh pH.

132 The effects of pH on cumulative hydrogen production, yield, and maximum specific
133 hydrogen production rate (MSHPR) are shown on Table 1. When the pH was 5.5,
134 the MSHPR was the highest but smaller cumulative hydrogen production and yield
135 per mol of lactose were obtained. This is because at pH 5.5, only glucose was
136 metabolized and all the galactose produced was accumulated, driving the yield and
137 hydrogen production to low values. The pH 6.5 resulted in the best condition for
138 cumulative hydrogen production and yield, but the MSHPR was the lowest. At a pH
139 6 the cumulative hydrogen production and yield were slightly lower than 6.5, but
140 were twice as that obtained at 5.5. The MSHPR also showed an intermediate
141 value.

142

143 **3.3 Production of metabolites.**

144 The hydrogen production pathway in *E. coli* involves the conversion of sugars to
145 pyruvate that is broken into formate and acetyl-coenzyme A. Formate is
146 metabolized to hydrogen and CO_2, whereas acetyl-coenzyme A is converted to
147 acetate or ethanol [34]. However, lactate can be produced from pyruvate and
148 succinate from phosphoenolpyruvate and CO_2 [35]. Therefore, formate, acetate,
149 and ethanol are desirable metabolic by-products in the hydrogen fermentations,
150 whereas lactate and succinate must be avoided.

151 The production of organic acids is related to the pH. Table 2 shows the acids
152 produced in the fermentative pathway and their pKa values. The pKa is an
153 important parameter because it determines the amount of dissociated and
154 undissociated acid present at a specific pH. The undissociated form of the acids is
155 able to cross the membrane, and it can affect hydrogen production [36]. The
156 fermentative metabolites were analyzed to evaluate the effect of pH on the
157 metabolite ratio.

158 The profiles of the pH and metabolites produced at pH 5.5 are shown in Figure 3.
159 The accumulation of organic acids was very strong during the first 20 h (Figure
160 3A). Although the pH was controlled, during the first 20 h it oscillated between 5.4
161 and 5.5 (Figure 3B). Then acids production stopped and the pH remained at 5.5.

162 The main product was lactate, which reached a concentration of 6.5 g/L, followed
163 by succinate, which reached a maximum concentration of 2.5 g/L. Acetate and
164 ethanol were produced at a final concentration of 1.4 and 0.6 g/L. Only a slight
165 amount of formate was detected, with a maximum concentration of 0.2 g/L at 7.5 h.
166 The low pH and the high amount of lactate mean a high concentration of the
167 undissociated form of lactic acid which could affect hydrogen production and the
168 inhibition of metabolic functions of the cell [37] such as sugar metabolism.

169 Figure 4 shows the profiles of the pH and metabolites produced at pH 6
170 fermentation. Similar to the previous case, a variation in the pH between 5.9 and 6
171 can be observed in the first 20 h (Figure 4A) due to the organic acids production
172 (Figure 4B), mainly lactate. This acid reached a concentration of 4 g/L at 33 h and

then remained constant until the fermentation was sttoped. However, the concentration of lactate was lower than that observed at pH 5.5. Lactate production seems to be the principal factor that is affected by the pH. Among the acids produced in *E. coli* fermentations, the pKa of this acid is the lowest (Table 2). Succinate is the other product that must be avoided in hydrogen fermentations; in this case, it was produced and reached a maximum value of 1.7 g/L at 142 h, and then a slightly decrease was observed. This concentration was also lower than in the case of pH 5.5. Besides the differences in the substrate consumption caused by the pH, the production of metabolites not involved in hydrogen production is different. At pH 6, the production of lactate and succinate was diminished. Acetate and ethanol were produced at a maximum concentration of 1.2 and 1.9 g/L, respectively. Interestingly, in this case an accumulation of formate was observed, its concentration reached 0.8 g/L at 58 h and then decreased to a final concentration of 0.4 g/L at 215 h.

The profiles of pH and metabolites produced at pH 6.5 are shown in Figure 5. As in the previous cases, pH oscillated between 6.4 and 6.5 when the metabolite production was very active. In this case, the main metabolites were lactate and ethanol with a final concentration of 3.3 g/L for both (Figure 5A). It can be noted that lactate production was the lowest of the conditions tested, and it was produced only during the first 27 h, reaching a maximum concentration of 3.6 g/L and then remained constant. Production of the other metabolites was constant during the fermentation. Acetate reached a maximum concentration of 2.5 g/L, whereas highest concentration of succinate was 1.9 g/L. Propionate was detected also and

196 reached a concentration of 2.7 g/L. This observation is consistent with previously
197 reported propionate production in *E. coli* fermentations [34, 38-40]. As observed in
198 Figure 5A, the concentration of final products from alternative pathways that do not
199 involve hydrogen production was low and therefore, the highest cumulative
200 hydrogen production and yield were obtained at this pH.

201 In the case of formate, it accumulated reaching 1.3 g/L at 84 h; afterwards its
202 concentration decreased, becoming undetectable at the end of the experiment.
203 This metabolite is initially exported out from the cells to avoid the acidification of
204 cytoplasm by the protein FocA [41, 42]. The import of formate depends on the pH
205 of culture media, and at a pH below 6.8, formate is re-imported [42]. A possible
206 explanation of the accumulation of formate observed in the present work is a
207 balance between formate import and export. The formate metabolism and
208 subsequent hydrogen production is affected by alkaline pH in *E. coli*, and for this
209 reason, pH values higher than 6.5 were not tested. Bagramyan *et al.* [43] observed
210 that the inclusion of 30 mM formate in the growth medium did not increase
211 hydrogen production rates at pH 6.5 or 7.5.
212 The optimal pH for hydrogen production depends on the inocula and substrate. A
213 pH of 5 and 5.3 were reported as optimal for hydrogen production using xylose or
214 lactose respectively with mixed cultures at 55°C [24]. Li *et al.* [23] reported an
215 optimal constant pH of 6 using 7.5 g/L of glucose with a natural sludge as
216 inoculum. Using *Clostridium butyricum* CWBI1009, Masset *et al.* [44] reported the
217 maximum yield for glucose when the pH was maintained at 5.2 and a maximum
218 yield and production using starch at pH 5.6. Liu *et al*. [45] evaluated the effect of

pH on hydrogen production by three *Clostridium* species using glucose. The maximum hydrogen yield for *Clostridium butyricum* CGS2 was achieved at pH 6, whereas a high hydrogen production with *Clostridium beijerinckii* L9 and *Clostridium tyrobutyricum* FYa102 could be achieved under uncontrolled pH conditions. In the present work, using cheese whey and *E. coli* WDHL, the optimal pH was 6.5 to maximize the cumulative hydrogen production and yield.

4 Concluding Remarks

The pH has an important effect on the fermentative metabolism of *Escherichia coli,* including hydrogen production because it influences the formate metabolism. In this work, the effect of operating the reactor at controlled pH values of 5.5, 6, and 6.5 on the hydrogen production was evaluated.

Controlling pH at 6.5 resulted in the best condition since both higher cumulative hydrogen production and yield were obtained, and all the sugars of the cheese whey were metabolized. At this pH a mix of ethanol and acids, mainly lactate, was produced from glucose; the metabolism of galactose yielded other acids than lactate and ethanol. On the other hand, operating at pH of 5.5 resulted in the highest MSHPR but both smaller cumulative hydrogen production and yield because only glucose was metabolized. At pH 6 not all the carbohydrates of cheese whey were consumed, and this was not favorable for hydrogen production.

The results show the importance of controlling the pH to improve substrate consumption, kind of metabolites produced and finally, maximize hydrogen

production. It could be interesting to determine how pH affects galactose catabolism in this system.

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Table 1. Comparison of hydrogen production at different pHs.

pH	Hydrogen (mL)	Yield (mol H_2/mol lactose)	MSHPR[a] (mL/L h $O.D_{600}$)
5.5[b]	835.5 (63.6)	0.66 (0.05)	17.07 (0.74)
6.0[b]	1788.6 (53.4)	1.38 (0.04)	15.36 (2.71)
6.5	2402.0	1.78	11.9

[a]MSHPR- Maximum Specific Hydrogen Production Rate. It was calculated by dividing the maximum slope of hydrogen production kinetics by the $O.D_{600}$.

[b] Experiments were done by triplicate, average values are showed and standard deviations are in ().

Table 2. Organic acids involved in *E. coli* fermentative pathway.

Acid	pKa
Lactic	3.5
Formic	3.74
Succinic	4.2, 5.6
Acetic	4.76

Figure captions:

Figure 1 Biomass (●) and hydrogen production (■) of the cultures at pH of 5.5 (**A**), 6 (**B**) and 6.5 (**C**).

Figure 2 Lactose (**A**) or Galactose (**B**) consumption at pH of 5.5 (●), 6 (□) and 6.5 (▲).

Figure 3 A Production of fermentative metabolites: formate (▲), succinate (□), acetate (■), lactate (○) and ethanol (●) and **B** pH (--), controlling the pH at 5.5.

Figure 4 A Production of fermentative metabolites: formate (▲), succinate (□), acetate (■), lactate (○) and ethanol (●) and **B** pH (--), controlling the pH at 6.

Figure 5 A Production of fermentative metabolites: formate (▲), succinate (□), acetate (■), lactate (○) propionate (△) and ethanol (●) and **B** pH (--), controlling the pH at 6.5.