Nitrogen sources impact hydrogen production by *Escherichia coli* using cheese whey as substrate

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Abstract

The impact of nitrogen source on hydrogen production by *Escherichia coli* WDHL (*ΔhycAΔlacI*) strain using cheese whey as a substrate was evaluated. To improve the assimilation of complex proteins such as lactalbumin, we assessed treatment with a protease. Also, five external nitrogen sources were tested: NH_4Cl, $(NH_4)_2SO_4$, urea, yeast extract, and tryptone. The treatments in 110 mL serological bottles with pancreatin 1,000 mg/L produced 1.75-fold more hydrogen than the cultures without pancreatin. In the bottle cultures supplemented with yeast extract or tryptone 5 g/L, hydrogen production increased up to 3.2- and 3.5-fold, respectively, whereas inorganic salts and urea had no statistical difference with respect to the control cultures. In bioreactors, the use of tryptone improved 2.1-fold hydrogen production. Tryptone or yeast extract enable the total consumption of lactose in 40 h, whereas in the control assay the lactose was not completely consumed. Our results demonstrate that it is necessary to select an adequate nitrogen source, which allows both carbon source consumption and high hydrogen production.

Keywords: Biofuels, Hydrogen, Cheese whey, Nitrogen sources.

Highlights:

- Type of nitrogen source affects hydrogen production by *E. coli.*
- Ammonium salts have no effect on hydrogen production
- Yeast extract and tryptone positively affect hydrogen production

Introduction

CO_2 emissions and their consequent adverse effects on the environment such as global warming have made it necessary to find alternative sustainable energy sources [1]. Among them, hydrogen is an attractive option because is a renewable energy source, its combustion generates only water and heat, and it has a high-energy yield (122 kJ/g) [2].

There are a variety of methods and substrates to produce hydrogen. Electrolysis, steam reforming of coal and natural gas, thermochemical and photochemical decomposition of the water are the most non-biological methods used to produce hydrogen [1]. However, they require high-energy inputs and fossil fuels as raw materials. Therefore, biological processes, mainly dark fermentation, have been explored because many of them use agro-industrial waste, household wastewater, and municipal waste as raw material, while also attacking the problem of environmental pollution [3-7]. Dark fermentation seems to be the best promise process due to its low cost, a relatively high production efficiency, and stable hydrogen-evolving enzymes [8].

One of the main factors that influences bacterial productivity and hydrogen yield is the type and concentration of the nitrogen source, because microorganisms need a nitrogen supplement for their metabolism during fermentation. For instance, Ferchichi *et al.* [9] reported that inorganic nitrogen sources dramatically inhibited the hydrogen production by *Clostridium saccharoperbutylacetonicum*, whereas 0.1% yeast extract increased it 1.12-fold ~~[9]~~. Wang *et al.* [10, 11] found that hydrogen production with digested sludge as inoculum increased 1.94-fold using 0.1 g/L NH_4Cl, whereas 10 g/L NH_4Cl reduced it 4.8-fold ~~[10, 11]~~.

In previous works, we described hydrogen production by genetically modified *Escherichia coli* WDHL using cheese whey as substrate [2]. However, *E. coli* cannot assimilate protein complex such as those present in cheese whey; thus, we decided to evaluate the effect of adding a protease or supplementing the culture medium with external nitrogen sources. To our knowledge, this is the first report on the effect of nitrogen source on hydrogen production by *E. coli.*

Materials and methods

Strain and culture media

In this study, a hydrogen overproducing *E. coli* Δ*hycA* Δ*lacI* strain (WDHL) was used [2]. The strain was grown routinely in LB plates (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl). Hydrogen production experiments were performed in HP medium, which contains per liter: 0.8 g NaCl, 0.2 g KCl, 1.43 g Na_2HPO_4, 0.2 g KH_2PO_4, 1 mL of trace elements solution (0.015 g/L $FeCl_3.4H_2O$, 0.00036 g/L $Na_2MoO_4.2H_2O$, 0.00024 g/L $NiCl_2.6H_2O$, 0.0007 g/L $CoCl_2.6H_2O$, 0.0002 g/L $CuCl_2.2H_2O$, 0.0002 g/L Na_2SeO_3, 0.01 g/L $MgSO_4$), and cheese whey (CW) powder (Land O'Lakes, Arden Hills, Minnesota) were added as carbon source. The pH was adjusted to 7.5 and the medium was pasteurized for 25 min at 65°C and chilled 20 min on ice. For all experiments, pre-inocula were grown overnight in 25 mL of LB medium at 37°C, shaken at 200 rpm, and used to inoculate 900 mL of fresh LB medium in closed twist-cover bottles incubated at 37°C for 48 h. Cells were harvested, washed once with HP medium and inoculated into the 120 mL serological bottles or 1-L bioreactor. Experiments were started with an optical density (OD_{600nm}) of 1.5.

Cultures with a protease or an external nitrogen source

Since the WDHL strain does not assimilate lactalbumin of CW, 1 to 1,000 mg/L of pancreatin (Lab. Quimica Son's, Puebla, Mex) were added to the cultures for an enzymatic hydrolysis into 120 mL anaerobic serological bottles (Prisma, DF, Mex) containing 110 mL of HP medium plus 10 g/L CW.

To evaluate the effect of external nitrogen sources, experiments were performed in serological bottles with 110 mL of HP plus 20 g/L CW and each of the following nitrogen sources: NH_4Cl (Sigma), $(NH_4)_2SO_4$ (Fermont), urea (Sigma), tryptone (Difco) or yeast extract (Difco) at three concentrations (0.5, 2.75, and 5 g/L). The experiments were conducted in triplicate.

Cultures in bioreactor

Batch fermentations were performed using 1-L of HP medium plus 20 g/L CW and 2.75 g/L tryptone in a 1-L bioreactor (Applikon, Foster City, CA) equipped with two six-blade Rushton turbines. pH was monitored using an autocleavable electrode (Applikon) and controlled at 6 by a Bioconsole ADI 1035/Biocontroller 1030 (Applikon). BioXpert 1.3 software (Applikon) was used for data acquisition. The experiments were performed at 37°C and stirred at 175 rpm. Culture samples of 3 mL were taken every 4 h from the bioreactor and centrifuged at 6,000 rpm. The supernatant was filtered through a 0.22 µm filter (Millipore, Bedford, MA, USA) before analysis of fermentation products.

Analytical methods

The gas was measured by water displacement in an inverted burette connected to the bioreactor or to serological bottles with rubber tubing and a needle. The hydrogen content in the gas phase was measured by Gas Chromatograph model 6890N (Agilent Technologies, Wilmington, DE) as described elsewhere [12]. Sugars and organic acids were determined by liquid chromatography in a Waters 600 Controller as described by Rosales-Colunga *et al.* [13]. Ethanol was measured by GC-FID as described in De León-Rodríguez *et al.* [14]. Cell growth was monitored at OD_{600nm} using a Cary BIO-50 spectrophotometer (Varian, Palo Alto, CA). Protein concentration was determined by the Lowry method using bovine serum albumin (BioRad, Hercules, CA, USA) as the standard. Proteins were separated by 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a Miniprotean III System (BioRad). Proteins were stained with Coomassie Blue R-250 (BioRad).

Statistics

The statistical analysis of the treatments was determined by analysis of variance (ANOVA) and unpaired Student's *t*-test. Treatments with $p<0.0001$ were statistically significant. The statistical analysis was performed using Microsoft Excel v 14.0.

Results and discussion

Hydrogen production by WDHL strain using cheese whey as substrate

We previously reported the construction of an overproducing hydrogen *E. coli* WDHL strain, through the deletion of the *lacI* and *hycA* regulatory genes for the lactose operon and formate regulon, respectively [2]. A 1-L typical batch culture using cheese whey as carbon source in bioreactor is shown in Fig. 1. For this culture, the biomass increased from 1.5 to 1.7 $OD_{600\ nm}$ and the maximum cumulative hydrogen was 1,300 mL at 140 h (Fig. 1A). Lactose was consumed fast during the first 10 h of the culture and, galactose and glucose were accumulated up to 5.8 and 4 g/L, respectively, and were consumed. Residual lactose of 4 g/L was observed at the end of the culture (Fig. 1B). The by-products at the end of fermentation were 4.9 g/L succinate, 2 g/L lactate, 1.4 g/L acetate, 1 g/L formate and 3.3 g/L ethanol (Fig. 1C). Despite the genetic improvement of the WDHL strain, the lactose was not completely consumed and the hydrogen production rate was low, because there was insufficient nitrogen availability in the culture medium. Since *E. coli* cannot assimilate the lactalbumin, two strategies were evaluated to resolve this issue: the proteolytic hydrolysis of the lactalbumin, and the addition of an external nitrogen source.

Treatment of the cheese whey with pancreatin

The effect of pancreatin on hydrogen production and protein assimilation by the WDHL strain in serological bottles of 110 mL is shown in Fig. 2. A clear increase in hydrogen production was only observed for the experiments using 1,000 mg/L pancreatin, which produced 1.75-fold more hydrogen than the cultures without pancreatin (Fig. 2A). The protein concentration in the control culture without pancreatin as expected remained constant at 2 g/L, since no proteolytic activity was present, whereas in the cultures plus

143 pancreatin a decrease of 50% of protein concentration was observed at 24 h of culture (Fig.
144 2B). The cell concentration for the culture with 1,000 mg/L pancreatin increased from 1.5
145 to 2.2 OD_{600nm} at 2 h of culture in the beginning and then remained constant. For the other
146 cultures, biomass increased slowly at 2 OD_{600nm} and was maintained after 18 h of culture
147 (Fig. 2C). Fig. 3 shows a SDS-PAGE of culture samples taken at 24 h. In all cases a protein
148 digestion pattern was observed indicating the proteolytic hydrolysis of the lactalbumin,
149 whereas in the lane of the control culture, a band of 14.2 kDa corresponding to alpha-
150 lactalbumin was observed.

151

152 **Effect of external nitrogen sources on the hydrogen production**

153 The effect of adding external nitrogen sources on hydrogen production by *Clostridium sp.*
154 and mixture cultures has been documented [10, 15-17]; however, to our knowledge, there is
155 no report for hydrogen production processes using *E. coli*. For this reason, we decided to
156 evaluate the effect of five nitrogen sources: tryptone, yeast extract, urea, NH_4Cl, and
157 $(NH_4)_2SO_4$, in cultures with 110 mL of production medium, and the results are shown in
158 Fig. 4. Both inorganic sources and the urea had no statistical difference with respect to the
159 control cultures, which attained 33±1.2 mL of hydrogen. Yeast extract and tryptone had a
160 positive effect on hydrogen production at the three concentrations tested. Using 0.5, 2.75,
161 or 5 g/L of yeast extract in cultures, 84±1.5, 86±2.5, and 106±2.5 mL of hydrogen were
162 obtained, respectively, whereas using tryptone, hydrogen production was 87±2, 102±2, and
163 114±1.4 mL, respectively. Hydrogen yield based on tryptone was 37.1 and 22.8 mL/g for
164 2.75 and 5 g/L tryptone, respectively. These results led us to use production medium plus

2.75 g/L tryptone in subsequent experiments in bioreactor (Fig. 5). In this case the biomass increased from 1.55 to 1.81 OD_{600nm}, and remained constant until 100 h of culture, and then decreased to 1.7 (Fig. 5A). Hydrogen production reached a maximum of 2,733 mL at 80 h of culture, which is 2.1-fold higher than that attained in the culture without an external nitrogen source. This can be explained by the total sugar consumption. In this culture, lactose was undetectable after 4 h of culture, and a maximum of 5 g/L glucose was detected only in the first hours of culture (Fig. 5B). Galactose was accumulated and a maximum of 9.6 g/L was detected at 12 h, and then it was consumed and was undetectable after 28 h of culture (Fig. 5B). The by-products of fermentation were 3 g/L succinate, 2.3 g/L acetate, 2.6 g/L ethanol, formate was not detected and lactate was accumulated to 2.2 g/L at the first 4 h and then decreased until 0.8 g/L (Fig. 5C).

Table 1 shows the effect of different types of nitrogen sources on hydrogen production for various microorganisms. For *Clostridium butyricum* both organic and inorganic nitrogen sources increased hydrogen production [10]. While in *C. saccharoperbutylacetonicum* cultures only yeast extract had a slight positive effect, and inorganic salts and urea inhibited hydrogen production drastically [9]. In mixed cultures reported by Sivaramakrishna *et al.* [17], urea had a negative effect, ammonium sulfate had no effect, whereas ammonium chloride, tryptone, and yeast extract had a positive effect. Wang *et al.* [11] evaluated the use of ammonium chloride as nitrogen source with a digested sludge, and reported that concentrations between 0.01 to 2 g/L increased hydrogen production, but above 5 g/L it decreased. Salerno *et al.* [18] reported that NH_4Cl directly affected the hydrogen production in heat-treated agricultural soil cultures in a continuous flow reactor. In our study for *E. coli*, urea and both inorganic salts had a negative effect, whereas tryptone and yeast extract

in the range 0.5 to 5 g/L, hydrogen production increased from 2.6 to 3.5 and 2.5 to 3.2 fold, respectively (Fig. 4). A finding in this study was adding tryptone or yeast extract to the culture medium allowed the total consumption of lactose in 40 h. Meanwhile, in the control culture (without tryptone), there was 15.6% lactose remaining at the end of fermentation. There are studies that evaluated sugar consumption by adding a nitrogen source, *i.e*. Kalil *et al.* [16] reported glucose consumption of 80% by adding 13 g/L yeast extract ~~13 g/L~~ and using *C. acetobutylicum* as inoculum. Ferchichi *et al.* [9, 19] reported that glucose consumption was 99.9% by adding 5 g/L yeast extract ~~5 g/L~~ as nitrogen source in *Clostridium saccharoperbutylacetonicum* cultures. Morimoto *et al.* [20] reported that the addition of 2 g/L ~~of~~ yeast extract to the compost enhanced the glucose consumption from 38.2 to 84% using sludge as inoculum. Salerno *et al.* [18] reported that glucose consumption increased from 86 to 92% when NH_4Cl was increased from 0.8 to 7.8 g/L; however, the hydrogen yield decreased. It is not clear how ammonia inhibits hydrogen production, but it could be related to both the intracellular pH changes and inhibition of enzymes related to hydrogen production [11]. Therefore, it is necessary to select an adequate nitrogen source, which allows both carbon source consumption and high hydrogen production.

Conclusions

Cheese whey is an inexpensive potential raw material for hydrogen production by fermentative processes considering its high content of lactose and lactalbumin; however, this complex protein is not accessible for *E. coli*, and thus additional approaches are needed. We evaluated the use of pancreatin and the supplementation of external nitrogen

sources. Pancreatin improves hydrogen production, as well as the use of external organic sources such as tryptone and yeast extract, whereas ammonia salts and urea have no statistical effect. This work demonstrates that the type of nitrogen source directly affects hydrogen production by *E. coli*.

Acknowledgments

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References

[1] Kapdan IK, Kargi F. Bio-hydrogen production from waste materials. Enzyme Microb Techn 2006;38:569-582.

[2] Rosales-Colunga LM, Razo-Flores E, Ordonez LG, Alatriste-Mondragon F, De Leon-Rodriguez A. Hydrogen production by *Escherichia coli* Delta hycA Delta lacI using cheese whey as substrate. Int J Hydrogen Energ 2010;35:491-499.

[3] Claassen PAM, van Lier JB, Contreras AML, van Niel EWJ, Sijtsma L, Stams AJM, de Vries SS, Weusthuis RA. Utilisation of biomass for the supply of energy carriers. Appl Microbiol Biot 1999;52:741-755.

[4] Chong ML, Sabaratnam V, Shirai Y, Hassan MA. Biohydrogen production from biomass and industrial wastes by dark fermentation. Int J Hydrogen Energ 2009;34:3277-3287.

234 [5] Junyapoon S, Buala W, Phunpruch S. Hydrogen Production with Escherichia Coli
235 Isolated from Municipal Sewage Sludge. Thammasat Int J Sc Tech, 2011;16:9-15.
236 [6] Maru BT, Constanti M, Stchigel AM, Medina F, Sueiras JE. Biohydrogen production
237 by dark fermentation of glycerol using *Enterobacter* and *Citrobacter* Sp. Biotechnol
238 progr 2013;29:31-38.
239 [7] Mathews J, Wang GY. Metabolic pathway engineering for enhanced biohydrogen
240 production. Int J Hydrogen Energ 2009;34:7404-7416.
241 [8] Davila-Vazquez G, Arriaga S, Alatriste-Mondragón F, De León Rodríguez A, Rosales
242 Colunga L, Razo-Flores E. Fermentative biohydrogen production: Trends and
243 perspectives. Rev Environ Sci Bio/Technol 2008;7:27-45.
244 [9] Ferchichi M, Crabbe E, Hintz W, Gil GH, Almadidy A. Influence of culture parameters
245 on biological hydrogen production by *Clostridium saccharoperbutylacetonicum* ATCC
246 27021. World J Microb Biot 2005;21:855-862.
247 [10] Wang XY, Jin B, Mulcahy D. Impact of carbon and nitrogen sources on hydrogen
248 production by a newly isolated *Clostridium butyricum* W5. Int J Hydrogen Energ
249 2008;33:4998-5005.
250 [11] Wang B, Wan W, Wang J. Effect of ammonia concentration on fermentative hydrogen
251 production by mixed cultures. Bioresource Technol 2009;100:1211-1213.
252 [12] Davila-Vazquez G, Alatriste-Mondragon F, De Leon-Rodriguez A, Razo-Flores E.
253 Fermentative hydrogen production in batch experiments using lactose, cheese whey
254 and glucose: influence of initial substrate concentration and pH. Int J Hydrogen Energ
255 2008;33:4989-4997.

256 [13] Rosales-Colunga LM, Garcia RG, De Leon Rodriguez A. Estimation of hydrogen
257 production in genetically modified *E. coli* fermentations using an artificial neural
258 network. Int J Hydrogen Energ 2010;35:13186-13192.
259 [14] De Leon-Rodriguez A, Gonzalez-Hernandez L, Barba De la Rosa AP, Escalante-
260 Minakata P, Lopez MG. Characterization of volatile compounds of mezcal, an ethnic
261 alcoholic beverage obtained from *Agave salmiana*. J Agr Food Chem 2006;54:1337-
262 1341.
263 [15] Collet C, Adler N, Schwitzguebel JP, Peringer P. Hydrogen production by *Clostridium*
264 *thermolacticum* during continuous fermentation of lactose. Int J Hydrogen Energ
265 2004;29:1479-1485.
266 [16] Kalil MS, Alshiyab HS, Yusoff WMW. Effect of nitrogen source and carbon to
267 nitrogen ratio on hydrogen production using *C. acetobutylicum*. Am J Biochem
268 Biotech 2008;4:393-401.
269 [17] Sivaramakrishna D, Sreekanth D, Himabindu V, Lakshmi Narasu M. Thermo-
270 acidophillic biohydrogen production from rice bran de-oiled wastewater by Selectively
271 enriched mixed culture. Int J Energ Environ 2010;1:657-666.
272 [18] Salerno MB, Park W, Zuo Y, Logan BE. Inhibition of biohydrogen production by
273 ammonia. Water Res 2006;40:1167-1172.
274 [19] Mongi Ferchichi EC, William Hintz, Gwang-Hoon Gil and Amer Almadidy. Influence
275 of culture parameters on biological hydrogen production by *Clostridium*
276 *saccharoperbutylacetonicum* ATCC 27021. World J Microb Biot 2005;21:855-862.
277 [20] Morimoto M, Atsuka M, Atif AAY, Ngan MA, Fakhru'l-Razi A, Iyuke SE, Bakir AM.
278 Biological production of hydrogen from glucose by natural anaerobic microflora. Int J
279 Hydrogen Energ 2004;29:709-713.

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Figure caption

Fig. 1. Batch culture of *E. coli* WDHL strain using cheese whey as carbon source in 1-L bioreactor. A) Hydrogen production (●) and Biomass (⬅). B) Carbohydrate consumption: lactose (⁎), glucose (✶) and galactose (□). C) Metabolites: succinate (➲), lactate (⟐), acetate (Δ), formate (+) and ethanol (★).

Fig. 2. Effect of adding different concentrations of pancreatin on hydrogen production and protein assimilation by *E. coli* WDHL strain using 10 g/L CW as carbon source in 110 mL serological bottles. A) Hydrogen production. B) Protein assimilation and C) Biomass. The conditions were: 1 mg/L (●), 10 mg/L (➲), 100 mg/L (□), 1,000 mg/L (Δ) of protease, and control cultures without pancreatin (⬅).

Fig. 3. SDS-PAGE shows protein digestion by different concentrations of pancreatin in cultures of *E. coli* WDHL strain. M: Benchmark molecular weight ladder (Invitrogen), 1: Control culture, 2: 1 mg/L, 3: 10 mg/L, 4: 100 mg/L, 5: 1,000 mg/L.

Fig. 4. Different types of nitrogen sources affect hydrogen production by *E. coli* WDHL strain using 20 g/L CW as substrate. A) NH_4Cl, B) $(NH_4)_2SO_4$, C) Urea, D) Yeast extract, and E) Tryptone. Experiments were done in 110 mL serological bottles. Values are

expressed as mean ± standard deviation (n=3). *** Statistically difference with respect to the control at $p<0.0001$.

Fig. 5. Batch culture of *E. coli* WDHL strain using cheese whey plus 2.75 g/L tryptone ~~2.75 g/L~~ in 1-L bioreactor. A) Hydrogen production (●) and Biomass (⬅). B) Carbohydrate consumption: lactose (⁎), glucose (✶) and galactose (□). C) Metabolites: succinate (➷), lactate (✧), acetate (Δ), formate (+) and ethanol (★).