Hydrogen production by *Escherichia coli* Δ*hycA* Δ*lacI* using cheese whey as substrate

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Abstract

This study reports a fermentative hydrogen production by *Escherichia coli* using cheese whey as substrate. To improve the biohydrogen production, an *E. coli* Δ*hycA* Δ*lacI* strain (WDHL) was constructed. The absence of *hycA* and *lacI* genes had a positive effect on the biohydrogen production. The strain produced 22% more biohydrogen in a shorter time than the wild-type (WT) strain. A Box-Behnken experimental design was used to optimize pH, temperature and substrate concentration. The optimal initial conditions for biohydrogen production by WDHL strain were pH 7.5, 37°C and 20 g/L of cheese whey. The specific production rate was improved from 3.29 mL H_2/optical density at 600nm ($OD_{600\ nm}$) unit-h produced by WDHL under non-optimal conditions to 5.88 mL $H_2/OD_{600\ nm}$ unit-h under optimal conditions. Using optimal initial conditions, galactose can be metabolized by WDHL strain. The maximum yield obtained was 2.74 mol H_2/mol lactose consumed, which is comparable with the yield reached in other hydrogen production processes with *Clostridium* sp. or mixed cultures.

Keywords: biohydrogen, bioenergy, biofuels, dark fermentation, galactose, lactose.

1. Introduction

Hydrogen has been considered a viable alternative energy carrier. It has a high-energy yield of 122 kJ/g, which is 2.75 fold greater than hydrocarbon fuels [1]. The main advantage of hydrogen is the absence of polluting emissions, since its utilization either via combustion or fuel cells, results in pure water [2]. Although the hydrogen storage is still a challenge, efficient adsorption-desorption systems are being developed [3-6]. The biological hydrogen production or biohydrogen is an attractive method because it is carried out at ambient temperature and pressure. Despite photosynthetic and fermentative processes can produce biohydrogen, the fermentative hydrogen production utilizes a wide range of carbon sources, does not need light and generally yields higher rates than the photosynthetic processes [1, 7-9]. In addition, it can be coupled to the use of organic industrial wastes [9-14].

Among the fermentative microorganisms, *Escherichia coli* has been the main microorganism genetically modified to improve the biohydrogen production. This is because its metabolic pathways and genomic sequence are known [10]. Glucose is the main substrate used for biohydrogen production by *E. coli* genetically modified strains [15-22] and few works have reported biohydrogen production from formate [23, 24]. There are few reports on biohydrogen production using mutant *E. coli* strains and industrial wastes as a raw material [25, 26].

Cheese whey (CW) is the by-product from cheese production and represents an 85-90% of the total volume of processed milk. Only a minor proportion is used in the food industry and for animal feeding. The rest is a matter of concern because of the risk of being a pollutant if its not disposed in a proper way. However, CW is an inexpensive potential raw material for biohydrogen production by fermentative processes considering its high content

of lactose [27]. The aim of this work was to produce hydrogen from CW by *E. coli*. To improve the hydrogen production an *E. coli* W3110 Δ*hycA*, Δ*lacI* strain was constructed. Hydrogen is produced from formate in *E. coli* and the required enzymes are encoded in the formate regulon [10]. The *hycA* gene codes for the negative regulator of the formate regulon and strains with defective *hycA* gene are hydrogen overproducers' strains [24, 25]. In *E. coli*, the genes necessary to metabolize lactose are coded by the *lac* operon. The *lacI* gene was deleted to express constitutively the *lac* operon and increase the lactose consumption rate. This is the first work showing the biohydrogen production by a genetically engineered *E. coli* strain using cheese whey as substrate.

2. Experimental procedures

2.1 Construction of mutant strains

Strains, plasmids and primers used for the construction of the mutant strains are shown in Table 1. *E. coli* W3110 (WT) [28] strain was used because it grows well using CW as carbon source [27]. Mutant strains were constructed according to the method of Datsenko and Wanner [29] as follows: to generate the *E. coli* W3110 Δ*hycA*, WT strain was transformed with pKD46 plasmid and grown at 30°C to a 0.6 $OD_{600\ nm}$ in SOB medium (Invitrogen) plus 1 mM L-arabinose (Sigma, St Louis, MO) and ampicillin 200 µg/mL. These cells were transformed by electroporation with the PCR product obtained from the plasmid pKD3 used as template with HYCF and HYCR primers. Afterward 900 µL of SOC medium (Invitrogen) were added to shocked cells and incubated 3-4 h at 30°C. 200 µL of this culture were plated on LB agar with 25 µg/ml of chloramphenicol, and incubated again

at 30°C. The deletion of *hycA* was verified by colony PCR with OGHF and OGHR primers, which bind upstream and downstream of *hycA* gene. The mutants were incubated at 42°C to induce the loss of pKD46 plasmid and then tested for ampicillin sensitivity. The Δ*hycA* strain was transformed by electroporation with the pCP20 plasmid, and selected by both chloramphenicol and ampicillin resistant at 30°C. Transforming cells were incubated overnight in LB medium without antibiotic at 42°C, and then they were tested for sensitivity for both antibiotics. Sensible colonies were tested by PCR to confirm the lost of *hycA* or *cat* genes with OGHF and OGHR primers. The resultant strain was named as WDH. This strain was transformed with pKD46 again and *lacI* gene was deleted as described above but using the LACF and LACR primers to obtain the PCR product. OGLF and OGLR primers were used to verify the deletion. The Δ*hycA* Δ*lacI* resultant strain was named as WDHL.

2.2 Culture media.

Strains were maintained in LB plates. Biohydrogen production experiments were done 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 $FeCl_2.4H_2O$, 0.00036 g/L $Na_2MoO_4.2H_2O$, 0.00024 g/L $NiCl_3.6H_2O$, 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 $MgSO_4$, 0.05 g/L rezasurine as redox indicator) and the concentration of CW powder (Land O´Lakes, Arden Hills, Minnesota) specified in each experiment. The pH was adjusted to 6.8 for general purpose or according to the Box-Behnken design described below. The HP medium was pasteurized during 25 min at 65 °C and chilled 20 min on ice.

2.3 Comparative growth kinetics of mutant strains using glucose or lactose

WDH and WDHL strains were aerobically cultured (37 °C; 175 rpm) in HP medium plus 1 g/L NH_4Cl, 40 µg/L thiamine (Sigma), but using 5 g/L glucose or 5 g/L lactose instead CW. Samples were taken and the optical density at 600nm ($OD_{600\ nm}$) was measured as described in the section 2.7. Preinocula of each strain were grown overnight using the HP medium with glucose plus 5 g/L yeast extract (BD, Le Pont-de-Claix, France).

2.4 Comparative hydrogen production ability by the WDH and WDHL mutant strains

To evaluate the hydrogen production by the WT, WDH and WDHL strains, they were cultured in 120 mL anaerobic serological bottles containing 110 mL of HP medium with 16.5 g/L CW. The cultures were started with 1.5 $OD_{600\ nm}$, pH of 6.8 and were incubated at 37°C and 175 rpm. Preinocula were grown 48 h in LB medium in anaerobic conditions. Cells were harvested, centrifuged, washed and inoculated into the serological bottles. Nitrogen gas was sparged into the bottles to ensure the anaerobic condition.

2.5 Experimental design.

A Box-Behnken experimental design (Table 2) was used to find the optimal conditions for the hydrogen production using CW as substrate. The independent variables were pH, temperature and CW concentration. Three levels for each variable were included. The response variables were volumetric hydrogen production (VHP) and hydrogen production

112 rate (HPR). The experiments were done in 120 mL anaerobic serological bottles; the
113 cultures were adjusted to initial $OD_{600\ nm}$ of 1.5 and were shaken at 175 rpm. Data were
114 analyzed according to response surface methodology (RSM). Analysis of variance
115 (ANOVA), RSM and the optimal conditions were performed using Statgraphics Plus *v* 5.0
116 software (Statistical Graphics Co). *F*-test from ANOVA was used to evaluate the adjusted
117 models. The significance of each coefficient was determined with the *t* test with a *P*-value
118 smaller than 0.05.

119

120 **2.6 Batch cultures on bioreactor.**

121 Batch cultures were performed using HP medium plus 20 g/L of CW in a 1 L bioreactor
122 (Applikon, Foster City, CA) equipped with two six-blade Rushton turbines. Redox
123 potential, pH and dissolved oxygen were monitored using autocleavable electrodes
124 (Applikon) and connected to the Bioconsole ADI 1035 (Applikon) controlled by the ADI
125 1030 Biocontroller (Applikon). The redox electrode was calibrated at 215 mV using the
126 reference solution HI7020 (Hanna Instruments, Armazem, Portugal) and was corrected
127 using the pH modified Nernst equation [30]. BioXpert 1.3 software (Applikon) for data
128 acquisition was used. The cultures were maintained at 37°C and stirred at 175 rpm. Culture
129 samples were periodically taken from the bioreactor and centrifuged. The supernatant was
130 filtered through a 0.22 µm filter (Millipore) before analysis of fermentation products.
131 Preinocula were grown overnight in 25 mL of LB medium at 37°C, shaken at 200 rpm and
132 used to inoculate 900 mL of fresh LB medium in closed twist cover bottles incubated at
133 37°C for 48 h. Cells were harvested, washed and inoculated into the bioreactor.

2.7 Analytical methods.

The gas produced was measured by water displacement in an inverted burette connected either to the bioreactor or to serological bottles with rubber tubing and a needle. The hydrogen content in the gas phase, sugars and organic acids were determined as described elsewhere [31]. Ethanol was determined by De Leon Rodriguez *et al.* [32]. Cell growth was monitored at $OD_{600\ nm}$ using a spectrophotometer Cary BIO-50 (Varian, Palo Alto, CA).

3. Results and discussion

3.1 Hydrogen production using CW as substrate by WDH and WDHL mutant strains

E. coli W3110 Δ*hycA* strain (WDH) was constructed to improve the hydrogen production. In the first experiment, WDH strain was grown on lactose or glucose in aerobic condition to measure the lag phase duration on these substrates (Fig. 1). It can be observed that the lag time using lactose was 1.5 h larger that the culture using glucose. Since this behavior can be more dramatic under anaerobic conditions a WDHL strain was constructed deleting the *lacI* gene in the WDH strain. As expected, the resultant WDHL strain showed the same lag-time using glucose or lactose as substrate. No effect of the *lacI* deletion was observed on the overall biomass yield (Fig. 1).

The hydrogen production by the WT, WDH and WDHL strains was evaluated using CW as substrate. These experiments were conducted in serological bottles and the gas volume was measured periodically by water displacement, which allowed an increase in the partial hydrogen pressure. Therefore, hydrogen production in our experiments could be affected by

157 the high partial pressure as reported for other microorganisms [7]. Fig. 2 shows that the WT
158 strain produced 94.7 mL of hydrogen. The deletion of the *hycA* gene had no effect on the
159 final hydrogen production, whereas the WDHL strain produced 22% more hydrogen than
160 the WT, leading to a final hydrogen production of 115.5 mL. In WDHL strain the *lac*
161 operon transcription becomes constitutive and the induction of the formate regulon is
162 constant. Moreover WDHL produces almost 110 mL of hydrogen in 170 h, which is 95%
163 of the final production, whereas the WT and WDH strains only produced 72% and 76% of
164 the final production. Therefore the WDHL strain was selected for subsequent experiments.

165

166 **3.2 Hydrogen production by the WDHL strain in bioreactor.**

167 Kinetic behavior of *E. coli* WDHL batch culture conducted in bioreactor is shown in Fig. 3.
168 Only a slight biomass concentration increment from 1.3 to 2.35 $OD_{600\ nm}$ was attained (Fig.
169 3A). Cell growth was observed on the first 6 h and between 50 and 66 h. The lactose from
170 the CW was consumed quickly and galactose accumulated to a maximum concentration of
171 22 mM. For this culture, lactose decreased from 42 to 15 mM (Fig 3A). A rapid production
172 of hydrogen was observed from the beginning of the culture and it attained a maximum
173 production of 983.8 mL for this culture (Fig 3B). Besides the hydrogen production *E. coli*
174 WDHL produced succinate, acetate, lactate and ethanol. Only a slight amount of formate
175 was detected (less than 5 mM); hence this was used immediately to produce hydrogen
176 instead of the formate exportation to the medium (Fig 3C). A notorious decrease of pH was
177 observed due to the accumulation of these organic acids (Fig 3D). This behavior continued
178 until 40 h of fermentation, but the rate of hydrogen and acids production decreased and the
179 pH dropped slowly. This strain began to metabolize acetate and lactate due to the low pH.

The amount of acetate was 12.6 mM at 42 h and dropped to 3.7 mM at 117 h and this amount was constant until the end of fermentation. The lactate accumulated was 13.4 mM at 42 h and dropped to 1.7 mM at 100 h and became undetectable at 163 h of fermentation. The acetate and lactate consumption increased the pH from 4.65 to 4.9 at 65 h, and then began to decrease slowly to 4.75 at the end of fermentation (Fig. 3D). Succinate was also produced during the culture and it attained a maximum concentration of 30 mM and then it remained constant until the end of fermentation. Ethanol was produced at the beginning of fermentation and it remained constant at 13 mM. The redox potential decreased from -104 to -450 mV as a result of the metabolic activity. The decrease was related to the cell growth, since the redox potential dropped dramatically when the cell concentration increased. The specific production rate was 3.29 mL $H_2/OD_{600\,nm}$ unit-h respect to the initial $OD_{600\,nm}$ and the yield was 1.21 mol H_2/mol lactose consumed or 0.97 mol H_2/ mol hexose consumed.

3.3 Optimization of the culture conditions to improve the hydrogen production.

In order to find the best conditions for the hydrogen production by the WDHL strain using CW as substrate, an experimental Box-Behnken design was done. The effect of the substrate concentration, pH and temperature on the hydrogen production was evaluated. The experimental design used and results obtained from these 15 experiments are shown in Table 2. The maximum hydrogen production and hydrogen production rate were reached by experiment 2 (pH 7.5, 37°C, 20 g/L of CW). The mathematical model representing the hydrogen production as a function of the evaluated variables in the experimental region is expressed by the following equation:

Hydrogen production (mL) = 1272.17 – 496.992*A + 40.3002*B - 33.5917*C + 30.85*A^2 + 0.916667*A*B + 3.7*A*C - 0.659105*B^2 + 0.0866667*B*C + 0.2675*C^2 (1)

Where A is the pH, B is the temperature in °C and C is the CW concentration. The standard error was 5.47 and the R^2 value was 99.07%. These values indicate a good fit between the model and the experimental data indicating that the treatment was highly significant. The analysis of variance (ANOVA) for the adjusted model showed the hydrogen production was significantly affected by A, B, C, AA, AC and BB (Table 3).

The response surface plots for hydrogen production are shown in Fig. 4A to 4C. CW concentration had a positive effect on the hydrogen production, *i.e.* high CW concentration produced more hydrogen. A similar effect but less intense was observed with the pH, whereas the temperature presents a maximum value of hydrogen production around 37°C.

The effect of the temperature, pH and CW concentration on the hydrogen production rate was also evaluated. The mathematical model representing the hydrogen production rate is represented by the following equation:

Hydrogen production rate (mL/h)= 47.0488 - 16.5747*A + 1.04193*B -1.06736*C + 1.07667*A^2 + 0.00388889*A*B + 0.114*A*C - 0.0144856*B^2 - 0.00138889* B *C + 0.0117667*C^2 (2)

In this case the R^2 was 95.8% and the standard error was 0.2513. Table 4 shows that the hydrogen production rate was significantly affected only by BB. The response surface plots of Figs. 4D to 4F were obtained based on this equation. The Figs. 4D and 4F show that the amount of CW also affects hydrogen production rate; at high concentrations the production rate also increased. The best parameters for the hydrogen production rate were 20 g/L of cheese whey, pH 7.5 and 36°C. Whereas, the best conditions for improving both the hydrogen production and hydrogen production rate were 20 g/L of cheese whey, pH of 7.5 and 37°C, since similar hydrogen production rate was observed at 36 and 37°C according to response surface plot. Similar to the results obtained here, Li *et al.* [28] found a direct relationship between the initial pH in the range 5 to 7 with the hydrogen production rate and the hydrogen yield in batch cultures using natural sludge as inoculum and glucose as substrate. A possible reason for it is that the higher initial pH could help to buffer the acid production associated to hydrogen production. This could also explain that the higher pH tested in our Box-Behnken design was the better condition. Ghosh and Hallenbeck [33] reported that the maximum hydrogen production was attained at an initial pH of 6.5 by a metabolically engineered *E. coli* using glucose as substrate. Yoshida *et al.* [24] established the maximum hydrogen production rate at 42°C and pH around 6.5, with *E. coli* W3110, using sodium formate as substrate. Ferchichi *et al.* [34] reported that the hydrogen production rate from cheese whey peaked at an initial pH 6 with *Clostridium saccharoperbutylacetonicum*. Davila-Vazquez *et al* [31] found the highest hydrogen molar yield at pH of 7.5 and 6.5 using lactose and CW, respectively in mixed cultures. Therefore, the pH is one of the most important parameters that affect the hydrogen production on different microorganisms.

3.4 Hydrogen production under the best conditions.

The best initial conditions were tested in bioreactor experiments and the results are shown in Fig. 5. Lactose was quickly consumed at the beginning of fermentation and galactose began to accumulate but interestingly in this case, the initial pH of 7.5 allowed galactose consumption. The residual concentration of lactose was 4 mM (Fig 5 A) and glucose was not detected during the whole fermentation. Hydrogen was produced from the beginning of fermentation and its production showed a similar behavior than the non-optimized fermentation, but the hydrogen production was increased. At 200 h of fermentation the hydrogen production was 2488 mL. At this time it seemed that the fermentation process was halted, like in the previous fermentation. However, 630 mL of hydrogen were further produced between 250 and 300 h of fermentation (Fig. 5 B), therefore the cumulative hydrogen production was 3245.4 mL. Organic acids and ethanol production are shown in Fig 5 C. The initial pH of 7.5 allowed a slight increase of the initial organic acid production. Ethanol attained a concentration of 12 mM in the first 19 h and then remained constant until the end of fermentation. The concentration of formate was 5 mM at the beginning of the fermentation and after 9 h of fermentation became less than 0.5 mM. This low amount indicates that it was used to produce hydrogen as soon as it was produced. Succinate, lactate and acetate were produced since the beginning; afterwards cells metabolized these acids, mainly lactate, and to a lesser extent acetate and succinate. It is very likely that the initial conditions used in this experiment caused the succinate consumption. Ren *et al.* [35] reported that redox potential and pH are related with changes on the fermentation type in continuous flow reactor and mixed cultures. Also, Hussy *et al* [36] reported that redox potential is negatively related to the rate of gas production with mixed cultures in a continuous process. As in the case of the previous fermentation under

non optimal conditions, the organic acids production and consumption caused changes on pH, and the drop of redox potential was mainly related with the cell-growth (Fig. 5). According to the data obtained from the optimization experiments, higher CW concentrations should improve the hydrogen production and hydrogen production rate (Fig. 4). For this reason, an additional experiment was carried out using 40 g/L instead of 20 g/L of CW. No improvement of the hydrogen production was observed at the higher CW concentration (data no shown). The specific production rate at optimized conditions was 5.88 mL $H_2/OD_{600\ nm}$ unit-h respect to the initial $OD_{600\ nm}$ and it was 1.8-fold higher than that attained under non-optimal conditions. The hydrogen yield in the optimized fermentation was 2.74 mol H_2/mol lactose consumed or 1.37 mol H_2/mol hexose consumed. The yield obtained in this work using a double mutant strain was similar to the value reported using a multi-gene deleted *E. coli* strain by Maeda *et al* [16]. They obtained a yield of 1.3 mol H_2/mol glucose using an *E. coli* BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*. The *hyaB* and *hybC* genes were deleted to inactivate the hydrogen uptake activity, whereas *fdoG* and *aceE* were deleted to redirect the glucose metabolism to formate. The succinate and lactate synthesis were inactivated by the deletion of *frdC* and *ldhA* genes. Higher yields have been reported, Yoshida *et al.* [20] obtained a maximum yield of 1.82 mol H_2/mol glucose, using an *E. coli* with *hycA*, *frdC* and *ldhA* genes deleted and *fhlA* overexpressed. Bisaillon *et al.* [15] used an *E. coli* JW135 strain carrying deletions on the two uptakes hydrogenases and mutations on *IdhA* and *fhlA* genes, and the hydrogen yield reported approaching 2 mol H_2/mol glucose. In those works glucose was the substrate for the hydrogen production. Although the main way to reduce the cost of hydrogen production is by increasing the yield from glucose another way is to convert inexpensive feedstock into hydrogen [13]. Few works have reported the use of *E. coli* mutant strains consuming

industrial wastes as a raw material to produce hydrogen. Penfold *et al.* [25] reported the production hydrogen by *E. coli* HD701 (a hydrogenase upregulated strain) at expense of glucose and fructose, the compounds of sucrose, which is a major constituent of many waste materials. In that work, industrial nougat waste (containing sucrose, fructose and glucose) was used to produce 31.63 mL H_2/h-$OD_{600\ nm}$ unit-$L_{culture}$. *E. coli* HD701 and FTD701 (an isogenic strain of the HD701 that has a deletion of the *tatC* gene) were transformed with the plasmid pUR400 by Penfold and Macaskie [26]. This plasmid carries the genes necessary for sucrose transport and metabolism to produce hydrogen from sucrose. The parental strains did not produce hydrogen, whereas the recombinant strains produced 1.27 and 1.38 mL H_2/mg dry weight-$L_{culture}$. In our study, CW was used as substrate and 2.74 mol H_2/ mol consumed lactose was obtained, this yield is similar to the highest yield of 2.7 mol H_2/ mol lactose reached by Ferchichi *et al* [34] in a pure culture of *Clostridium saccharoperbutylacetonicum* using CW, and it is comparable with 3.1 mol H_2/ mol lactose reported by Davila-Vazquez *et al* [31] with CW powder and mixed cultures.

4. Conclusions

This study showed that the CW can be used to produce hydrogen by *E. coli* W3110 mutant strains. The deletion of *lacI* gene led to a lag-time reduction using lactose as carbon source. Meanwhile, deletion of *lacI* and *hycA* genes on strain WDHL improved hydrogen production by 22% in a shorter time than the WT. The optimal culture conditions were found by RSM. The best initial conditions for hydrogen production were pH 7.5, 37°C and 20 g/L of cheese whey. The specific production rate was improved from 3.29 mL H_2/OD_{600nm} unit-h produced by WDHL under non-optimal conditions to 5.88 mL H_2/OD_{600}

$_{nm}$ unit-h at optimal conditions. The hydrogen yield was improved from 1.21 mol H_2/mol lactose consumed to 2.74 mol H_2/mol lactose consumed under the best conditions. The results showed that the pH is an important variable on the hydrogen production and that the control of pH could improve the hydrogen production. This work enriches the information on hydrogen production using genetically engineered *E. coli* strains and provides the basis for further studies.

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Legends of Figures

Fig. 1. Growth kinetics of the *E. coli* WDH (△) and WDHL (○) using lactose (open symbol) or glucose (filled symbol) as carbon source.

Fig. 2. Hydrogen production kinetics by WT (▲), WDH (□) and WDHL (●) strains using CW as carbon source. The error bars indicate standard deviations.

Fig. 3. Batch culture of the *E. coli* WDHL strain using 20 g/L of cheese whey, initial pH 6.5 and 37°C. (A) Biomass (●), lactose (◆) and galactose conc.(◇). (B) Hydrogen production. (C) Production of fermentative metabolites; succinate (✳), lactate (×), acetate (▲), formate (○), and ethanol (□). (D) pH (—) and redox potential (– –).

Fig. 4. Response surface plot of the hydrogen production (A to C) and hydrogen production rate (D to F) by WDHL strain in HP medium. pH adjusted to 7.5 in A and D, temperature fixed at 37°C in B and E, concentration of CW fixed at 20 g/L in C and F.

Fig. 5. Batch culture of the *E. coli* WDHL strain at optimal initial conditions: 20 g/L of cheese whey, pH 7.5 and 37 °C. (A) Biomass (●), lactose (◆) and galactose conc. (◇). (B) Hydrogen production. (C) Production of organic acids; succinate (✳), lactate (×), acetate (▲), formate (○), and ethanol (□). (D) pH (—) and redox potential (– –).