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1 Scale-up of hydrogen and ethanol co-production by an engineered *Escherichia coli*

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19

20 **Abstract**

21 In this work, the scale-up from 0.01 to 10 L process for the co-production of hydrogen (H₂)
22 and ethanol (EtOH) by a genetically engineered *Escherichia coli* that utilizes
23 hemicellulosic hydrolysates from wheat straw as substrate is presented. Co-production of
24 biofuels was performed through the redirection of carbon-flux to ethanol by deleting *ldhA*
25 (D-lactate dehydrogenase) and *frdD* (fumarate reductase) genes in an H₂-overproducer
26 strain (*E. coli* WDH). Resulting strain, *E. coli* WDH-LF ($\Delta hycA \Delta frdD \Delta ldhA$), increased
27 up to 70% and 167% the H₂ and EtOH production compared with the parenteral strain. The
28 yields of H₂ and EtOH remained constant at all the evaluated scales. In 10 L bioreactors,
29 the production parameters such as maximum production, production rate and yield were
30 5603.0 ± 233.5 mL H₂/L, 41.4 ± 4.0 mL H₂/L·h, 342.7 ± 14.3 mL H₂/g TRS, 7.90 ± 0.28 g
31 EtOH/L and 0.48 ± 0.01 g EtOH/g TRS, respectively. The results demonstrate the potential
32 of the co-production of H₂ and EtOH at different production scales by the engineered *E.*
33 *coli* strain using lignocellulosic biomass as feedstock, such as wheat straw hydrolysates.

34

35 **Keywords:** Hemicellulosic hydrolysates, dark fermentation, metabolic engineering,
36 biofuels, phage P1 transduction

37

38 **1 Introduction**

39 The main criteria for the selection of feedstock for biofuels production are their availability,
40 cost, carbohydrate content and biodegradability. Lignocellulosic biomass (LCB) meets with
41 them and it is the most abundant renewable resource on earth [1,2]. From them, wheat
42 straw is one of the most abundant LCB worldwide, with a production of 529 million tons
43 [3]. The greater production of agricultural residues is obtained from cereals (*e.g.*, wheat
44 among others) and is directly associated with the production of grains. For every kilogram
45 of grain produced, approximately one kg of lignocellulosic residue (*i.e.* straw) is obtained
46 [4]. At the end of 2019, Agricultural and Fisheries Information Service (SIAP by its
47 acronym in Spanish) reported a production of 3 million tons of wheat-grain [5]. Their
48 residues are mainly used as fodder in livestock, as well soils improvers, construction
49 materials, and composting [4]. Notwithstanding, with the search for alternative and
50 renewable energy sources, the use of agricultural residues as feedstock for biofuels
51 production is seen as a prominent option.

52 For biorefining purposes, the complex LCB lignocellulosic matrix must be debilitated and
53 deconstructed to simpler chemical forms by pre-treatment methods before further
54 processing [6,7]. After pre-treatment, the simple carbohydrates from LCB can be converted
55 into biofuels through fermentation. Microbial fermentations offer an attractive alternative to
56 produce sustainable energy because metabolic diversity of microorganisms enables the use
57 of different substrates as the starting point for biofuels (*e.g.*, ethanol, hydrogen, biodiesel,
58 among others) production. However, high yields and productivities in every step during
59 biofuel production by microorganisms are required for profitable industrial-scale
60 production. Genetically modified microorganisms with redesigned metabolic pathways can
61 improve both yield and productivity [8–10]. *Escherichia coli* has the capability to generate

62 simultaneously hydrogen (H₂) and ethanol (EtOH), because the anaerobic pyruvate
63 breaking produces formate and acetyl-CoA. Then, H₂ is produced by formate
64 hydrogenlyase system through the decarboxylation of formate [11], while EtOH formation
65 is carried out by the action of alcohol dehydrogenase, which catalyzes the reaction from
66 acetyl-CoA to EtOH [12]. However under anaerobic conditions, other end-products such as
67 lactate, acetate, and succinate are synthesized to maintain the NADH/NAD⁺ balance and
68 intracellular pH [13]. Since these metabolites are reduced molecules, their synthesis reduce
69 both H₂ and EtOH production [14]. Therefore, the removal of the synthesis of one or more
70 of these end-products will increase the carbon flux towards H₂ and EtOH synthesis.

71 Although metabolic engineering in *E. coli* have been widely studied and employed to
72 improve biofuels performance [14–16], to our knowledge, studies on the scale-up have not
73 been reported. Therefore, the scale-up of the co-production of H₂ and EtOH by an
74 engineered *E. coli* and using wheat straw hemicellulosic hydrolysates as substrate is
75 presented.

76

77 **2 Material and methods**

78 **2.1 Lignocellulosic biomass**

79 Wheat straw (WS) used in this work was harvested in the spring of 2017 in La Barca
80 (Jalisco, Mexico). The feedstock was milled with a hammer mill (Azteca 301012) using a
81 1.27 cm screen. LCB composition was determined according to NREL laboratory analytical
82 procedures [17]. Glucan, xylan and lignin content in the LCB (dry basis) were 41.39, 21.0
83 and 16% for WS, respectively.

84

85 2.2 Hydrolysis of hemicellulosic hydrolysate

86 The hydrolysate was obtained by auto-hydrolysis followed by a 0.25% H₂SO₄ pre-treatment
87 [18]. Auto-hydrolysis was carried out in a semi-pilot scale pre-treatment continuous tubular
88 reactor (PCTR) at 150 psi (about 180°C) with a mean residence time of 18 min [18]. The
89 biomass from auto-hydrolysis was further hydrolyzed with 0.25% (v/v) H₂SO₄ in a 1:2
90 (w/v) solids loading ratio, in an autoclave at 121°C for 60 min. The liquid fraction from the
91 pre-treatment was separated by centrifugation and collected for further analysis, and it was
92 identified as wheat straw pretreated (WSP); which contained 1.8 g/L glucose, 39.8 g/L
93 xylose, 7.9 g/L arabinose, 2.6 g/L formate, 7.8 g/L acetate and 1.2 g/L furfural. Further
94 dilutions were made to obtain a hydrolysate with a final concentration of 16.1 ± 0.2 g/L of
95 total reducing sugars (TRS) to perform the scale-up experiments.

96

97 2.3 Construction of mutant strains

98 Strains, plasmids, and primers used for the construction of the mutant strains are shown in
99 Table 1. W3110 Δ *frdD* and W3110 Δ *ldhA* strains from Keio collection [19] were used as
100 donor. The deletion of *frdD* and *ldhA* genes was achieved using P1 transduction method
101 [20], with some modification as follows: To prepare liquid P1 lysate, an overnight culture
102 of *E. coli* donor strain (W3110 Δ *frdD* or W3110 Δ *ldhA*) in LB broth with 30 mg/L
103 kanamycin was washed and suspended in MC medium (10 mM MgSO₄ and 5 mM CaCl₂).
104 Phage P1 was added with multiplicity of infection between 0.1 and 1.0, and the culture was
105 incubated for 30 min at room temperature. After incubation, the culture was added into soft
106 agar and finally plated in LB agar plates and incubated overnight at 37°C. After lysis, the
107 culture was treated with chloroform and the debris was removed by centrifugation. The
108 phage was stored at 4°C until its application. For transduction, an overnight culture of *E.*

109 *coli* recipient strain (WDH or WDHL) in LB medium was washed and suspended in MC
110 medium. Phage P1 lysate with the donor strain (W3110 $\Delta frdD$ or W3110 $\Delta ldhA$) was added
111 with multiplicity of infection between 0.1 and 1.0. The phage was allowed to absorb for 30
112 min at room temperature, and then 1 M sodium citrate was added. Finally, all the mixture
113 was plated on selective LB plates with 30 mg/L kanamycin and incubated at 37°C until
114 colonies appeared.

115 The deletion of *frdD* and *ldhA* genes was verified by colony PCR with OGF-F, OGF-R,
116 OG-L-F and OG-L-R primers (Table 1). The $\Delta frdD$, $\Delta ldhA$, or $\Delta ldhA \Delta frdD$ strains were
117 transformed by electroporation with the pCP20 plasmid [22], and selected by ampicillin
118 resistance in LB + 30 mg/L ampicillin plates at 28°C. Kanamycin resistance loss was
119 verified by subcultivation on LB plates plus 30 mg/L kanamycin. Selected colonies were
120 tested by PCR to confirm the loss of kanamycin resistance.

121

122 **2.4 Effect of *frdD* and *ldhA* genes deletion on co-production of H₂ and EtOH**

123 The effect of *frdD* and *ldhA* genes deletion on co-production of H₂ and EtOH by
124 *Escherichia coli* strains (Table 1) was determined by using glucose as substrate (20 g/L).

125 The experiments were done in anaerobic serological bottles containing 0.01 L of B buffer
126 [21], 1 mL/L trace elements solution [22], 0.01 g/L MgSO₄ and 1 g/L yeast extract (Difco).

127 The cultures were started with an optical density of 0.2 measured at a wavelength of 600
128 nm (OD₆₀₀), pH 7.5, and they were incubated at 31°C and 175 rpm, until no longer
129 production of H₂ was observed. All the experiments were carried out in triplicate. The
130 production of H₂, EtOH, and other metabolites was measured as indicated in section 2.6.

131

132 **2.5 Scale-up of H₂ and EtOH co-production**

133 *E. coli* WDH-LF strain was selected to perform the scale-up of H₂ and EtOH co-production
134 using WSP as substrate (16 g/L TRS). The working volumes used were 0.01, 0.1, 1 and 10
135 L. In 0.1, 1 and 10 L experiment, a volume of the headspace relative to that of the liquid
136 approximately of 40% was used and for 0.01 L reactors was 20%. The experiments were
137 done under anaerobic conditions using the B buffer [21] plus 1 mL/L trace elements
138 solution [22], 0.01 g/L MgSO₄ and 1 g/L yeast extract (Difco). The cultures were started
139 with an OD₆₀₀ of 0.2 and pH 8.2, incubating them at 31°C and shaking at 200 rpm, until no
140 longer production of H₂ was observed. 10 L bioreactors were stirred at 250 rpm during first
141 29 h and then it was increased at 400 rpm. Batch cultures with working volumes 1 and 10 L
142 were performed in 1.5-L and 13.5-L bioreactors equipped with two six-blade Rushton
143 turbines flat, with a H/D ration of 2.1 and 1.5, respectively. The pH was monitored using an
144 autocleavable electrode (Applikon® Biotechnology) and connected to Bioconsole ADI 1035
145 (Applikon® Biotechnology) controlled by the ADI 1030 Biocontroller (Applikon®
146 Biotechnology). BioXpert 1.3 software (Applikon® Biotechnology) was used for data
147 acquisition. The experiments in serological bottles and bioreactors were carried out in
148 quadruplicate and duplicates, respectively. The production of H₂ and EtOH was measured
149 as indicated in Section 2.6.

150

151 **2.6 Analytical Methods**

152 TRS determination was performed by the dinitrosalicylic acid (DNS) method [23], with
153 some modifications, as follow: 250 µL of diluted sample with 750 µL of DNS reagent (10
154 g/L NaOH, 200 g/L KNaC₄H₄O₆·4H₂O, 0.5 g/L Na₂S₂O₅, 2 g/L C₆H₆O, 10 g/L 3,5-
155 Dinitrosalicylic acid) were heated for 5 minutes at 100°C and then cooled down to room

156 temperature. Then, 400 μ L of distilled water were added. Xylose (0.1 to 1.0 g/L, Sigma)
157 was used as the reference standard. The absorbance was measured at 595 nm (iMark™
158 Microplate Absorbance Reader).

159 Simple carbohydrates and metabolites were quantified by an Agilent HPLC equipped with
160 a refractive index (Agilent Technologies 1220 Infinity LC), using a Rezex™ ROA-Organic
161 Acid H+ (Phenomenex) column, operated at 60°C with H₂SO₄ 0.0025 M as a mobile phase
162 (0.550 cm³/min). Furfural was analyzed by Gas Chromatography (Agilent Technologies
163 6890N Network GC Systems) using a capillary column HP-Innowax (30 m \times 0.25 mm i.d.
164 \times 0.25 m film thickness; Agilent Technologies). Injector and flame ionization detector
165 (FID) temperatures were 220 and 250°C, respectively. Helium was used as carrier gas at 25
166 mL/min. Analyses were performed with a split ratio of 10:1 and a temperature program of
167 35 °C for 2 min, then 10°C/min to 210°C for 1 min.

168 Gas production was measured by acidified water (pH \leq 2) displacement in an inverted
169 burette connected to serological bottles/bioreactor with rubber tubing and a needle. H₂
170 concentration (% , v/v) in the gas was determined by with a Gas Chromatography (Agilent
171 Technologies 6890N Network GC Systems) coupled to a thermal conductivity detector
172 (Agilent Technologies 6890N Network GC Systems) and using Agilent J&W HP-PLOT
173 Molesieve column (0.32 mm ID, 30 m length, 12 μ m film) under the following conditions:
174 200°C, injector temperature; 280°C, detector temperature; 300°C, oven temperature.
175 Helium was used as carrier gas. H₂ volume was corrected to standard conditions of
176 temperature and pressure (298.15K and 10⁵ Pa).

177

178 **2.7 Statistical analysis**

179 For comparisons between samples, the data was analyzed by analysis of variance
180 (ANOVA) and Tukey's HSD (honestly significant difference) test (Origin® 9). A
181 probability of 5% was accepted as statistically significant.

182

183 **3 Results and discussion**

184 **3.1 Improvement of H₂ and EtOH co-production by *frdD* and *ldhA* genes deletion in**
185 **H₂-overproducer *Escherichia coli* strains**

186

187 To enhance the co-production of H₂ and EtOH, the effect of deletion of *ldhA* and *frdD*
188 genes encoding lactate dehydrogenase and fumarate reductase was determined. The
189 modified pathway is shown in Fig. 1A, and the genotypes of the constructed strains are
190 shown in Table 1. The first step it was performed the deletion of *ldhA* gen in two H₂-
191 overproducer strains, *E. coli* $\Delta hycA$ (WDH) and *E. coli* $\Delta hycA \Delta lacI$ (WDHL) [22],
192 through P1 transduction method (Fig. 1B). Then, $\Delta hycA \Delta ldhA$ (WDH-L) and $\Delta hycA \Delta lacI$
193 $\Delta ldhA$ (WDHL-L) *E. coli* strains were generated. The effect of *ldhA* gen deletion on co-
194 production of H₂ and EtOH was determined in batch cultures (0.1 L) using glucose (20 g/L)
195 as substrate at 37°C and initial pH of 7.5. After 160 h (Fig. 2A), the WDH-L and WDHL-L
196 strains produced 1639.7 ± 231.3 and 1870.5 ± 286.0 mL H₂/L, respectively. According to
197 the analysis of variance, not statistically significant difference (Table A1, $p < 0.05$) was
198 found in the H₂ production achieved by each strain. The H₂ production rate (r_{H_2}) obtained
199 by WDHL-L strain was 1.7-fold higher than the achieved by WDH-L strain (Fig. 2A).
200 However, comparing it with their parental (WDH and WDHL) strains [22,24], r_{H_2} seems to
201 not have been affected by *ldhA* gen deletion, which agrees with previously reported for

202 *ldhA E. coli* mutants strains [25,26]. Regarding to metabolites distribution at the end of
203 dark fermentation (Fig. 2B), the WDH-L strain produced 1.20 ± 0.10 g/L succinate, $0.90 \pm$
204 0.45 g/L lactate, 3.18 ± 0.33 g/L acetate and 5.90 ± 0.75 g/L ethanol. Whereas, the WDHL-
205 L strain achieved 1.58 ± 0.02 g/L succinate, 3.60 ± 0.34 g/L lactate, 3.82 ± 0.13 g/L acetate
206 and 3.60 ± 0.30 g/L ethanol. The end pH was ranging between 4.75 and 4.93. According to
207 the results, the WDH-L strain produced 1.6-fold more ethanol than the WDHL-L strain.
208 There was statistically significant difference in the final concentration of the metabolites in
209 the cultures after 160 h of fermentation (Table A2). Both strains (WDH-L and WDHL-L)
210 achieved approximately the same cumulative H₂ production, but the WDH-L strain
211 produced the higher concentration of EtOH. This finding helps us to select WDH strain for
212 the following experiments.

213 The deletion of *frdD* gen was performed in WDH and WDH-L strains to obtain the WDH-F
214 (*E. coli* $\Delta hycA \Delta frdD$) and WDH-LF (*E. coli* $\Delta hycA \Delta ldhA \Delta frdD$) strains. To determine
215 the effect of *frdD* gen deletion on co-production of H₂ and EtOH by *E. coli*, the
216 fermentation of 20 g/L of glucose in a batch culture during 180 h at 31°C and initial pH of
217 7.5 was conducted (Fig. 3). The WDH-F strain obtained 1.3- and 1.4-fold more H₂ than the
218 wild-type (*E. coli* W3110, WT) strain and its parental (WDH) strain, respectively.
219 Regarding to *r*H₂, the WT, WDH, and WDH-F obtained almost the same results –ranging
220 between 53.9 and 59.4 mL H₂/L/h without statistical difference, as shows Table A3.
221 However, WDH-F achieved only 50% and 70% of the EtOH obtained by WT and WDH
222 strains, respectively, since flux of glucose was utilized mainly to produce lactate instead
223 EtOH or another metabolite. Comparing both double mutant strains, WDH-F strain
224 produced 1.5-fold more H₂ and achieved 2.4-fold higher *r*H₂ than WDH-L. But the latter

225 produced 4.5-fold more EtOH than WDH-F due to the fact flux of glucose seems to be
226 partially redirected to EtOH by the *ldhA* gene deletion.

227 The co-production of H₂ and EtOH was significantly improved by both *ldhA* and *frdD*
228 genes deletion. The WDH-LF strain produced $2,950.3 \pm 261.8$ mL H₂/L, 1.7-fold and 1.5-
229 fold more hydrogen than the WT and WDH strains, respectively (Fig. 3A). Also, WDH-LF
230 strain produced more H₂ than single mutant strains –WDH-L and WDH-F–, which
231 achieved $1,650.83 \pm 234.20$ and $2,523.03 \pm 93.54$ ml H₂/L, respectively. Although, H₂
232 production was increased by *ldhA* and *frdD* genes deletion, *r*H₂ seems to be decreased in
233 WDH-LF strain. WDH-LF strain achieved 40.35 ± 3.89 mL H₂/L/h, whereas WT and
234 WDH obtained $1.3-53.91 \pm 3.15$ and 55.69 ± 3.23 mL H₂/L/h (Fig. 3B), respectively.

235 Succinate and lactate production were dramatically reduced by the deletion of *frdD* and
236 *ldhA* genes, whereas the EtOH production was improved compared to WT strain (Fig. 3C).

237 The WDH-LF strain produced 7.20 ± 0.26 g EtOH/L, which is 2.7-, 3.8-, 1.2, and 5.5-fold
238 more EtOH than obtained by WT, WDH, WDH-L, and WDH-F strains (Table A4),
239 respectively. These results are consistent with the previously reported [26,27], and
240 confirmed that the absence of *frdD* and *ldhA* genes improves the co-production of H₂ and
241 EtOH by *E. coli* using glucose as substrate.

242 Mutagenesis of competing metabolic pathways has been widely employed to improve H₂
243 and/or EtOH production performance [28]. To improve H₂ production, the most utilized
244 strategies are related to disruption of genes –such as *ldhA*, *frdAB*, *hycA*, *hya*, and *hyb*–,
245 inactivation of formate-hydrogen lyase (FHL) repressor (encoded by *hycA*), and/or
246 overexpression of FHL (encoded by *fhIA*). Regarding to the EtOH production, EtOH-
247 producer strains [*e.g.* *E. coli* SE2378 ($\Delta ldh \Delta pfl$) and *E. coli* SZ420 ($\Delta frdBC \Delta ldh \Delta ackA$
248 $\Delta folA-pfl \Delta pdhR::pflBp6-aceEF-lpd$)] have been constructed by deleting the competing

249 fermentation pathways, and implementing another molecular strategies such gene
250 overexpression. However, the low H₂ yields still a limitation for large-scale H₂ production
251 *via* dark fermentation and it is not sustainable economically. To overcome this issue, co-
252 production of H₂ and EtOH has been proposed [29]. In this regard, using *E. coli* $\Delta hycA$
253 \DeltahyaAB \DeltahybBC \DeltaldhA \DeltafrdAB as parental strain, mutant strains devoid *pta-ackA*
254 (encoding to phosphate acetyltransferase and acetate kinase, respectively) or *pfkA*
255 (encoding to phosphofructokinase 1) genes were constructed to increase the H₂ and EtOH
256 co-production [30]. The authors concluded that $\Delta ack-pta$ strain does not improve the
257 biofuels co-production. However, the $\Delta pfkA$ strain resulted in an increase of approximately
258 50% and 9.5% of H₂ and EtOH, respectively. In the present study, we used the *E. coli* Keio
259 collection to introduce two mutations into a single strain by the repetition of resistance-
260 gene elimination and P1 transduction; this method may be used to engineer *E. coli* for many
261 applications where multiple chromosomal genes must be eliminated. The deletion of the
262 *lhdA* and *frdD* genes in WDH strain, produce an improvement of 70 and 167% on H₂ and
263 EtOH production, respectively.

264

265 **3.2 Scale-up of H₂ and EtOH co-production from wheat straw hemicellulosic**

266 **hydrolysate**

267 The scaling-up is a vital tool to development bioprocesses since it can reduce errors in the
268 industrial-scale designs, as well as lack of information. For this reason, the effect of the
269 reactor size on co-production of H₂ and EtOH by *E. coli* WDH-LF was determined. Since
270 lignocellulose is a sustainable and worldwide available biomass, it was selected as
271 feedstock to perform the scaling-up of dark fermentation. Therefore, wheat straw
272 hemicellulosic hydrolysate (WSP) obtained by autohydrolysis followed by a 0.25% diluted

273 sulphuric acid pre-treatment was used as substrate for H₂ and EtOH co-production by *E.*
274 *coli* WDH-LF. The reactor sizes used to scale-up the process were 0.01, 0.1, 1 and 10 L.
275 The average of the maximum H₂ concentration (considering all working volumes) was 50.9
276 ± 7.4 %. The H₂ production obtained in 0.01, 0.1, 1 and 10 L reactors was $5,259.9 \pm 540.2$,
277 $4,076.1 \pm 468.4$, $5,574.8 \pm 156.6$, and $5,603.0 \pm 233.5$ mL H₂/L, respectively, as shows in
278 Fig. 4A. H₂ production was similar in all working volumes except in 0.1 L (Table A5),
279 which was approximately 25% lower than the other sizes. Regarding to the H₂ yield, the
280 results obtained were 328.7 ± 33.8 , 253.2 ± 29.1 , 350.6 ± 9.9 , and 342.7 ± 14.3 mL H/g
281 TRS for 0.01, 0.1, 1 and 10 L reactors, respectively. H₂ yield seems to be lower in 0.1 L
282 reactors compare with the other reactor sizes (Fig. 4B). These behaviors respond to the fact
283 that the volume of the headspace relative to that of the liquid (working volume) may affect
284 dark fermentation [38,39], because both H₂ and CO₂ accumulate to high pressures in the
285 headspace and inhibits H₂ production [40]. As described in Section 2.5, 0.1 L reactors
286 employed lower volume of the headspace relative to that of the working volume than the
287 other reactor sizes. The maximum r_{H_2} achieved by WDH-LF strain were 40.7 ± 2.3 , $39.2 \pm$
288 5.8 , 36.9 ± 1.4 and 41.5 ± 4.0 mL H₂/L·h for 0.01, 0.1, 1 and 10 L reactors, respectively
289 (Fig. 4B). Kinetic of H₂ production are shown in Fig. 5. As noted, 10 L reactor attained the
290 longest lag phase compare to the other reactor sizes. This can be explained by the agitation
291 rate utilized in 10 L reactors, which was maintained at 250 rpm during first 29 h and then it
292 was increased at 400 rpm, as described Section 2.5. Mixing may promote dark fermentation
293 performance by enhancing liquid-gas mass transfer, heat transfer, as well as
294 homogenization. However, inappropriate stirring can be harmful to H₂ producing bacteria,
295 and thus H₂ production may be inhibited [41,42]. The EtOH productions obtained by
296 WDH-LF strain were 8.9 ± 1.3 , 7.8 ± 1.7 , 7.9 ± 0.6 , and 7.9 ± 0.3 g EtOH/L in 0.01, 0.1, 1,

297 and 10 L reactors, respectively. And the EtOH yields achieved during dark fermentation by
298 WDH-LF strain were 0.56 ± 0.08 , 0.48 ± 0.11 , 0.50 ± 0.04 , and 0.48 ± 0.01 in 0.01, 0.1, 1,
299 and 10 L reactors, respectively. The production and yield of EtOH seems not to be affected
300 by the change of reactor size, as shown in Fig. 4C.

301 The pH profile of H₂ and EtOH co-production for 1 and 10 L appear in Fig. 6. In 1 L
302 reactors, H₂ and EtOH production started in the first 15 h of fermentation, while for 10 L
303 reactors, the first evidence of H₂ and EtOH production was after 36 h of fermentation. The
304 pH decreased only two units due to the low organic acids production, which is a
305 consequence of the *frdD* and *ldhA* genes absence, involved in the succinate and lactate
306 production, respectively. The optimal conditions of temperature and initial pH used in this
307 work were established previously using the WDHL strain [43]. By comparing the results
308 obtained in this work by WDH-LF strain with those previously reported for WDHL strain,
309 the following is noticed: (a) the WDH-LF strain increased 38% the EtOH yield; (b) the H₂
310 production rate by *E. coli* WDH-LF was approximately 4-fold than the achieved by *E. coli*
311 WDHL; (c) the H₂ yield increased 2-fold by the deletion of *ldhA* and *frd* genes.

312 H₂ yields reported for microbial consortia and pure cultures from lignocellulosic sources,
313 under mesophilic or thermophilic conditions, are in the range of 200 to 500 mL H₂/g
314 consumed sugar [44]. Also, theoretical yields between 70 and 99% has been reported for
315 lignocellulosic ethanol achieved by yeast and bacteria [45]. Nonetheless, mainly these
316 biofuels are obtained in separate processes or coupled to the production of other
317 components (Table 2). In the present work, it was obtained in one step hydrogen and
318 ethanol with yields up to 342.7 mL H₂/g TRS and 97% (0.48 ± 0.01 g EtOH/g TRS) of the
319 maximum theoretical of ethanol; this strategy could be used in the conceptual design of 2G
320 biorefineries [29]. Dark fermentation has been shown as the most realistic opportunity to

321 leave laboratory scale production behind, which is attributed to the relatively low energy
322 requirements, high biofuels production rates and the chance of using a broad spectrum of
323 organic matter as substrate [46]. However, they still have certain limitations that could be
324 crucial for scaling-up, as the stability of the process in self [47,48]. As an answer to the
325 limitations of the dark fermentation, several researchers have proposed the use of
326 genetically modified microorganisms with advanced selected properties [49]. In the present
327 work, we demonstrate that: (1) the increase of biofuels production yields by deletion of the
328 *ldhA* and *frd* genes in a H₂-overproducer *E. coli* strain; (2) *E. coli* WDH-LF can produce
329 efficiently and simultaneously H₂ and EtOH from hemicellulosic hydrolysates *via* dark
330 fermentation; (3) the yields achieved by WDH-LF strain are stable despite the change in the
331 reactor size.

332

333 **4 Conclusions**

334 Deletion of *frdD* and *ldhA* genes positively impact the co-production of H₂ and EtOH by *E.*
335 *coli*, since production, production rate and yield of both biofuels were improved. The
336 engineered WDH-LF strain may utilize hemicellulosic hydrolysates as substrates to
337 produce simultaneously and efficiently H₂ and EtOH, under optimal temperature and pH
338 conditions. The change in the reactor size have not impact on the co-production of H₂ and
339 EtOH by *E. coli* WDH-LF using wheat straw as feedstock, which is inferred because the H₂
340 and EtOH yields were similar despite the working volumes employed. Still, further genetic
341 modifications, as well as engineering techniques must be employed to improve H₂ and
342 EtOH production rates and makes them competitive in the actual biofuels framework.

343

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539

540 **Figure captions**

541 **Fig. 1. Carbon flux in *E. coli* during anaerobic metabolism and the strategy used to**
542 **improve the co-production of H₂ and EtOH.** (A) Improving the co-production of H₂ and
543 EtOH by deletion of *ldhA* and *frdD* genes. The *dashed orange lines* are the pathways
544 inactivated, and the *bold yellow lines* are the pathways enhanced. Chemical structures are
545 shown for dark fermentation products and pyruvic acid. (B) *Escherichia coli* genome
546 manipulation by phage P1 transduction. Homologous regions are indicated by red and
547 green boxes; dtg, deletion target gen (*ldhA* and *frdD* genes for this study); res, antibiotic
548 resistance gen (kanamycin for this study). Flp recognition sites are indicated.

549

550 **Fig. 2. Effect of *ldhA* gene deletion on the H₂ and metabolites production by WDH**
551 **and WDHL strains.** Kinetic of H₂ production (A) and final concentration of metabolites
552 (B). Data are presented as mean ± standard deviation. Batch culture were done in 0.1 L
553 anaerobic serological bottles using glucose (20 g/L) as substrate at 37°C and initial pH of
554 7.5.

555

556 **Fig. 3. Improvement of H₂ and EtOH co-production by metabolic engineering in *E.***
557 ***coli* strains.** Kinetic of H₂ production (D), cumulative H₂ production (E) and final
558 concentration of metabolites (F). Data are presented as mean ± standard deviation. *
559 indicates statistically significant differences compared with the wild type (WT) cultures (*p*
560 < 0.05). Batch culture were done in 0.1 L anaerobic serological bottles using glucose (20
561 g/L) as substrate at 37°C and initial pH of 7.5.

562

563 **Fig. 4. Effect of the increase of reactor size on co-production of H₂ and EtOH by *E.***
564 ***coli* WDH-LF using WSP as substrate.** Batch cultures were done in 0.01, 0.1, 1 and 10 L
565 at 31°C and initial pH of 8.2. Production (A), production rate and yield (B) of H₂.
566 Production and yield of EtOH (C). Data are presented as mean ± standard deviation.

567

568 **Fig. 5. Kinetics of H₂ production by *E. coli* WDH-LF using WSP hydrolysate as**
569 **substrate.** Batch cultures were done in 0.01, 0.1, 1 and 10 L at 31°C and initial pH of 8.2.

570

571 **Fig. 6. pH profile during co-production of H₂ and EtOH by *E. coli* WDH-LF using**
572 **WSP hydrolysate as substrate.** Batch cultures were done in 1 and 10 L at 31°C.

573

Table 1

574

Strains, plasmid, and primers used in this work.

Strains	Relevant genotype	Source
WT	<i>Escherichia coli</i> W3110 (lac ⁺ , gal ⁺ , F ⁻ IN (rrnD-rrnE)1, rph-1)	Laboratory stock
W3110 Δ <i>frdD</i>	WT Δ <i>frdD</i>	Laboratory stock
W3110 Δ <i>ldhA</i>	WT Δ <i>ldhA</i>	Laboratory stock
WDH	WT Δ <i>hycA</i>	[22]
WDHL	WT Δ <i>hycA</i> Δ <i>lacI</i>	[22]
WDHL-L	WT Δ <i>hycA</i> Δ <i>lacI</i> Δ <i>ldhA</i>	This work
WDH-L	WT Δ <i>hycA</i> Δ <i>ldhA</i>	This work
WDH-F	WT Δ <i>hycA</i> Δ <i>frdD</i>	This work
WDH-LF	WT Δ <i>hycA</i> Δ <i>ldhA</i> Δ <i>frdD</i>	This work
Plasmid		
pCP20	FLP recombinase expression plasmid (<i>bla</i> , <i>cat</i>) <i>p_R</i> FLP ⁺ , cI857 ⁺ , pSC101 ori TS	[50]
Primers		
OGF-F	GAGGGGCAGCAAATGTGGAG	This work
OGF-R	TGAACTGGCACCGAAAGCGG	This work
OG-L-F	CGCGGCTACTTTCTTCATTG	This work
OG-L-R	GGTTGCGCCTACACTAAGCAT	This work

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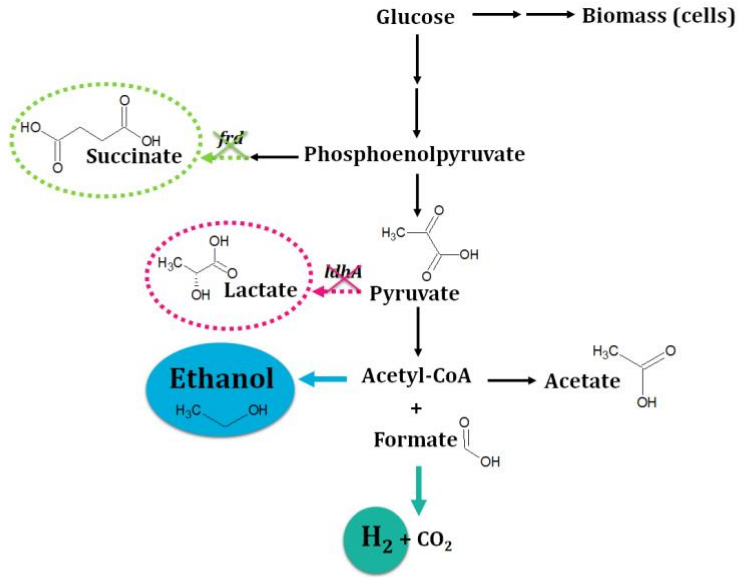
577 **Table 2**
 578 Comparison of the production and yield of hydrogen and ethanol from wheat straw biomass.

Inoculum	Substrate treatment	Operation mode	Working volume (L)	Hydrogen production (mL H ₂ /L)	Hydrogen yield (mL H ₂ /g consumed substrate)	Ethanol production (g EtOH/L)	Ethanol yield (g EtOH/g consumed sugars)	Reference
<i>Caldicellulosiruptor saccharolyticus</i> and <i>C. owensensis</i>	Steam explosion (190 °C, 10 min), enzymatic hydrolysis	Continuous	1.5	3350 ^a (134 mmol H ₂ /L)	-	-	-	[51]
A three-species culture of epiphytic <i>Enterococcus Clostridium beijerinckii</i> and <i>C. cellulovorans</i>	Hydration of fibres	Batch	0.1	-	386	-	-	[52]
	Biologically treated WS enriched in cellulose	Batch	0.1	-	-	3.7	-	
<i>Pichia stipites</i>	5% H ₂ O ₂ (pH 11.5, 50°C, 120 rpm, 60 min)	Batch	0.025	-	-	17.37	0.44	[53]
<i>Caldicellulosiruptor saccharolyticus</i>	Acid pretreatment (H ₃ PO ₄ , 190°C, 5 min), enzymatic hydrolysis	Continuous	1	-	413 ^a (3.04 mol H ₂ /mol sugars)	-	0.05 ^a (0.19 mol EtOH/mol sugars)	[54]
<i>Escherichia coli</i> WDH-LF	Autohydrolysis (180°C, 150 psi, retention time: 18 min) followed by acid hydrolysis (0.25% H ₂ SO ₄ v/v, 121°C, 21 psi, 1h)	Batch	0.01	5259.9 ± 540.2	328.7 ± 33.8	8.9 ± 1.3	0.56 ± 0.08	This work
		Batch	0.1	4076.1 ± 468.4	253.2 ± 29.1	7.8 ± 1.7	0.48 ± 0.11	
		Batch	1	5574.8 ± 156.6	350.6 ± 9.8	7.9 ± 0.6	0.50 ± 0.04	
		Batch	10	5063.0 ± 233.5	342.7 ± 14.3	7.9 ± 0.3	0.48 ± 0.01	

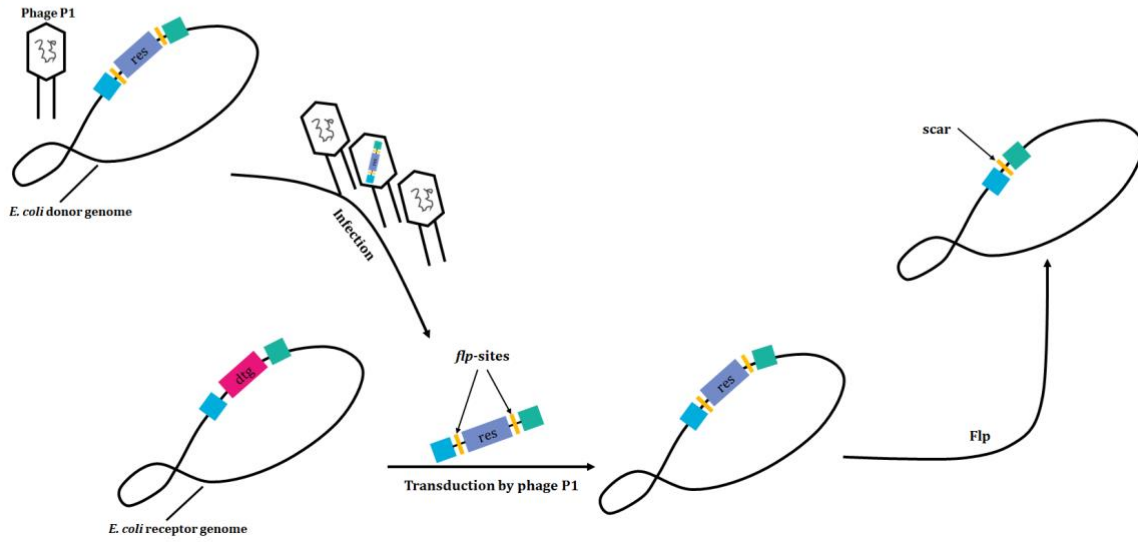
^a Converted unit from the original data

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580

A

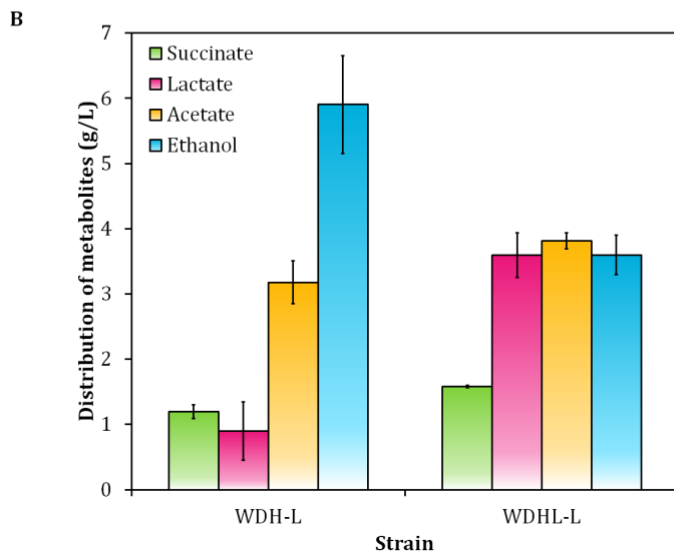
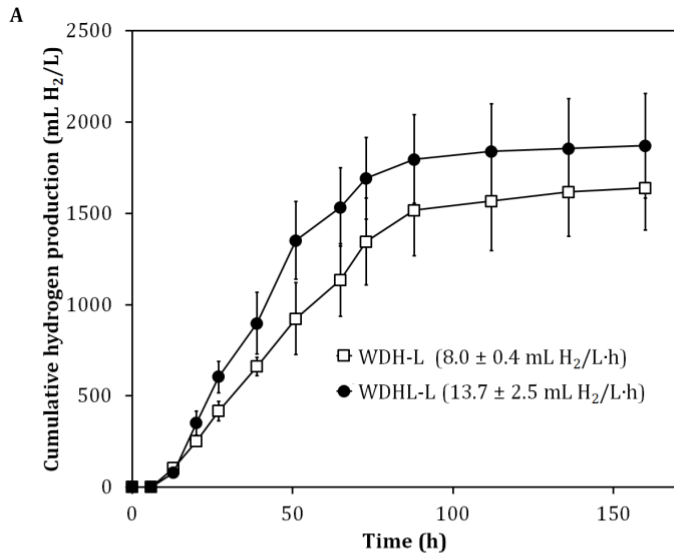


B



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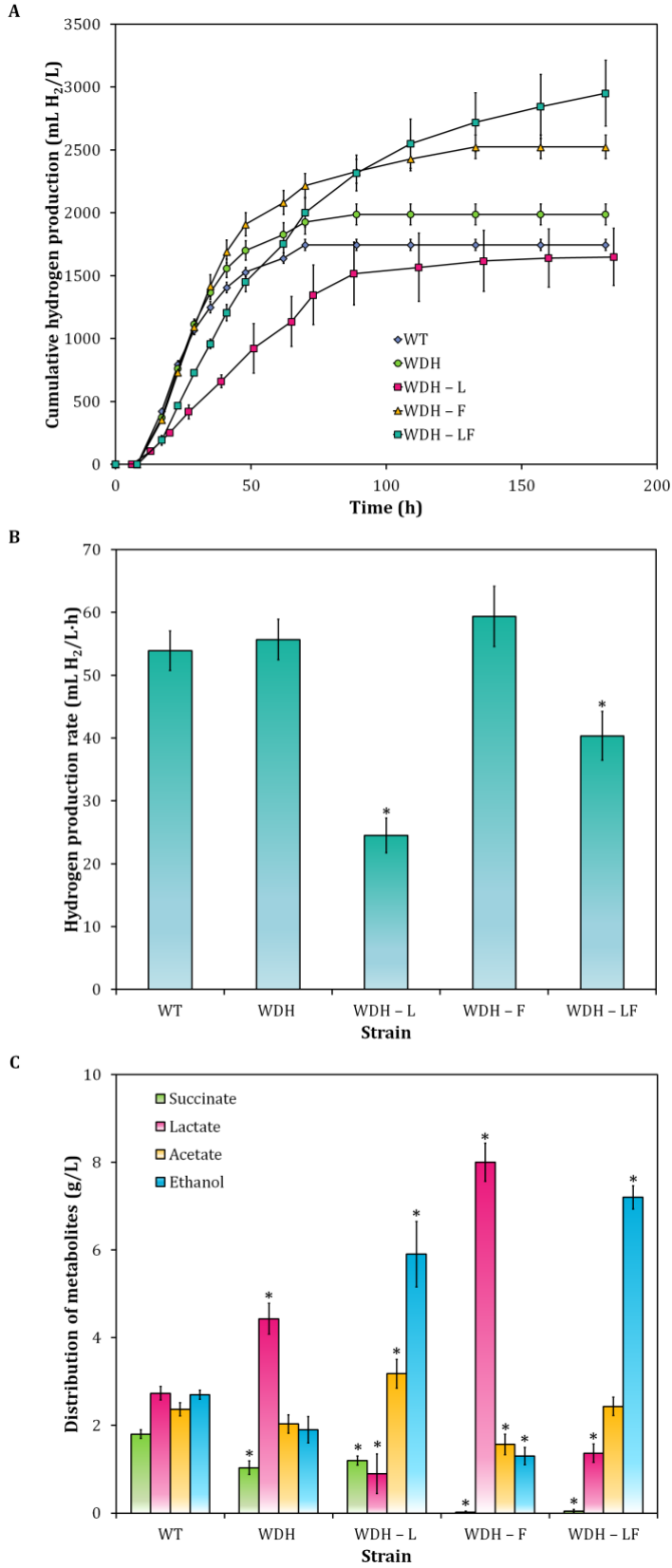
582 **Fig. 7.**



583

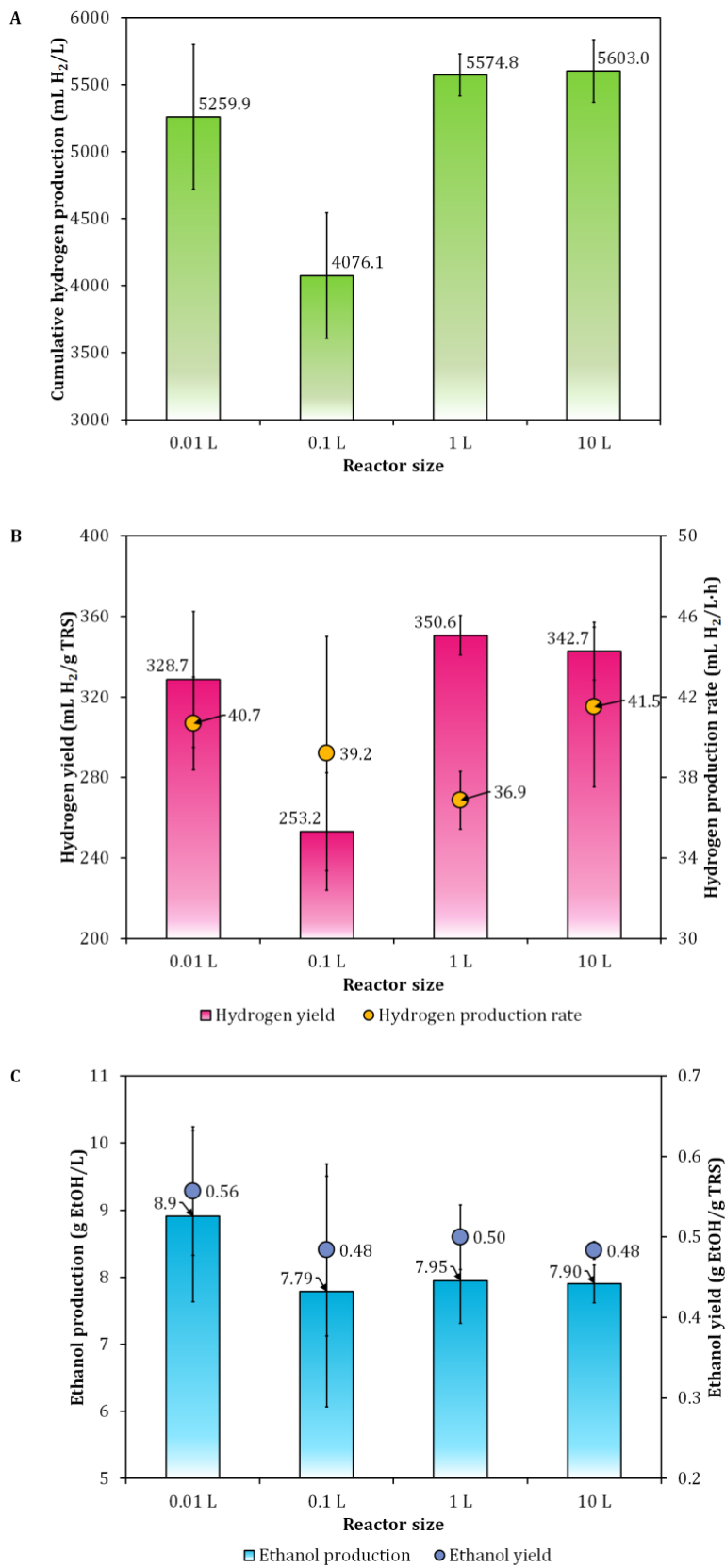
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Fig. 8.

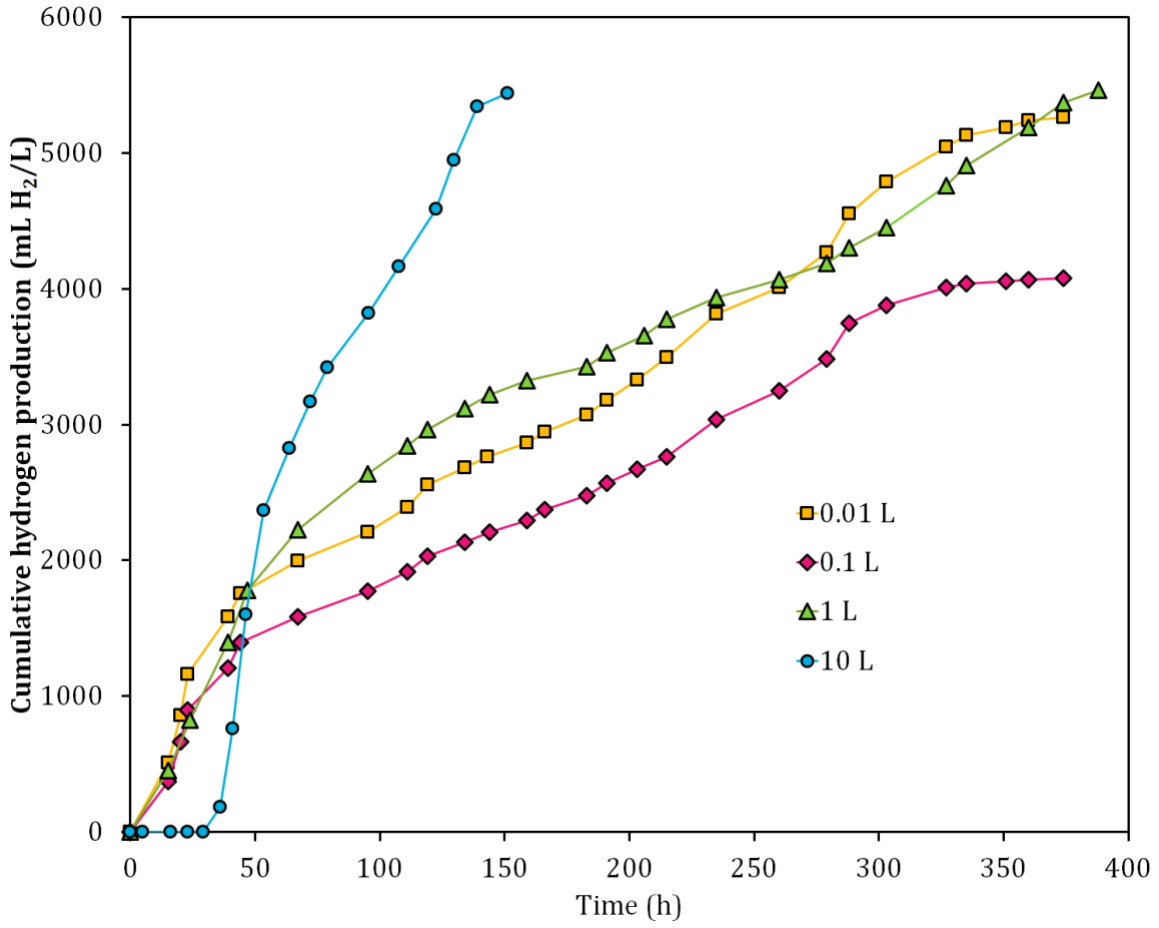


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Fig. 9.



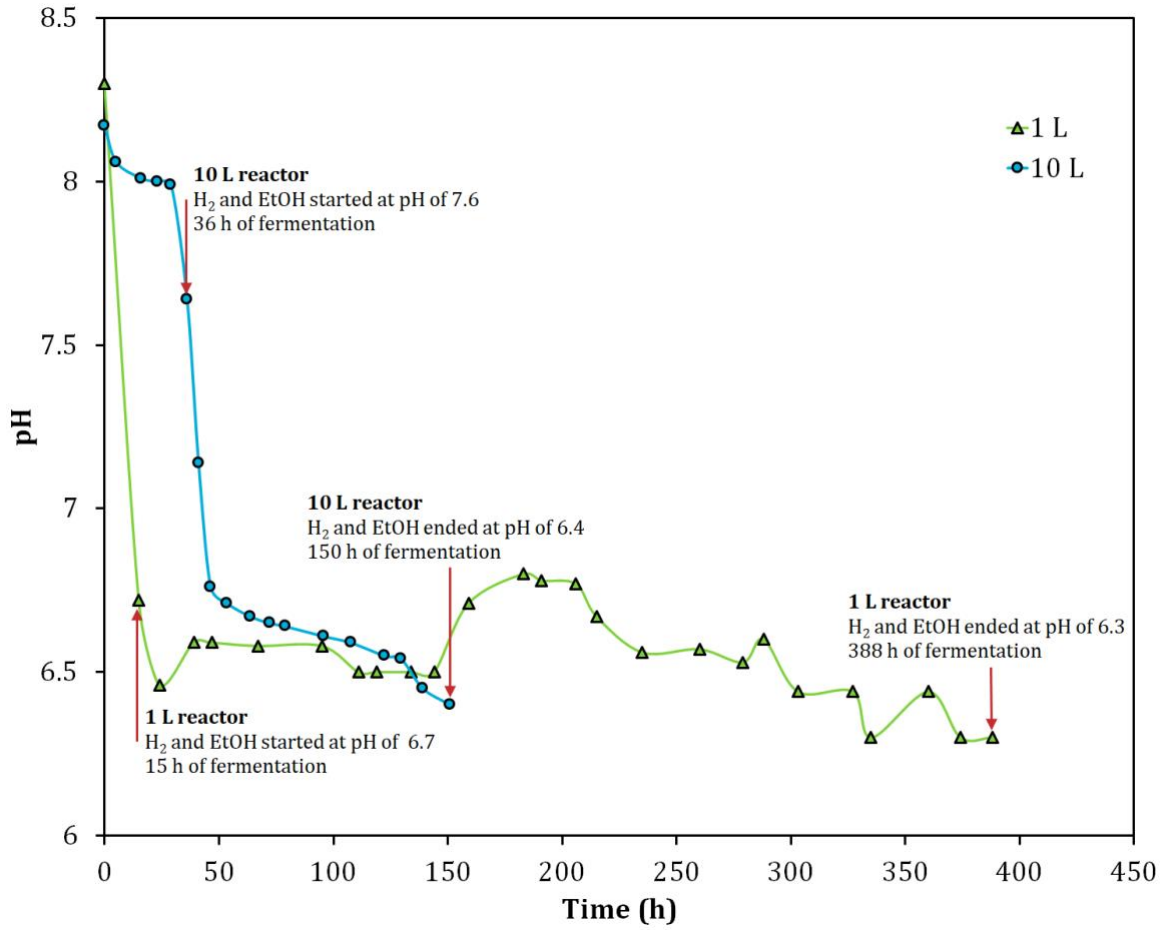
587
588 **Fig. 10.**



589

590 **Fig. 11.**

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592

593 **Fig. 12.**