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

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Abstract

In this work, the scale-up from 0.01 to 10 L process for the co-production of hydrogen (H_2) and ethanol (EtOH) by a genetically engineered *Escherichia coli* that utilizes hemicellulosic hydrolysates from wheat straw as substrate is presented. Co-production of biofuels was performed through the redirection of carbon-flux to ethanol by deleting *ldhA* (D-lactate dehydrogenase) and *frdD* (fumarate reductase) genes in an H_2-overproducer strain (*E. coli* WDH). Resulting strain, *E. coli* WDH-LF (Δ*hycA* Δ*frdD* Δ*ldhA*), increased up to 70% and 167% the H_2 and EtOH production compared with the parenteral strain. The yields of H_2 and EtOH remained constant at all the evaluated scales. In 10 L bioreactors, the production parameters such as maximum production, production rate and yield were 5603.0 ± 233.5 mL H_2/L, 41.4 ± 4.0 mL H_2/L·h, 342.7 ± 14.3 mL H_2/g TRS, 7.90 ± 0.28 g EtOH/L and 0.48 ± 0.01 g EtOH/g TRS, respectively. The results demonstrate the potential of the co-production of H_2 and EtOH at different production scales by the engineered *E. coli* strain using lignocellulosic biomass as feedstock, such as wheat straw hydrolysates.

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

1 Introduction

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

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

simultaneously hydrogen (H_2) and ethanol (EtOH), because the anaerobic pyruvate breaking produces formate and acetyl-CoA. Then, H_2 is produced by formate hydrogenlyase system through the decarboxylation of formate [11], while EtOH formation is carried out by the action of alcohol dehydrogenase, which catalyzes the reaction from acetyl-CoA to EtOH [12]. However under anaerobic conditions, other end-products such as lactate, acetate, and succinate are synthetized to maintain the $NADH/NAD^+$ balance and intracellular pH [13]. Since these metabolites are reduced molecules, their synthesis reduce both H_2 and EtOH production [14]. Therefore, the removal of the synthesis of one or more of these end-products will increase the carbon flux towards H_2 and EtOH synthesis.

Although metabolic engineering in *E. coli* have been widely studied and employed to improve biofuels performance [14–16], to our knowledge, studies on the scale-up have not been reported. Therefore, the scale-up of the co-production of H_2 and EtOH by an engineered *E. coli* and using wheat straw hemicellulosic hydrolysates as substrate is presented.

2 Material and methods

2.1 Lignocellulosic biomass

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

2.2 Hydrolysis of hemicellulosic hydrolysate

The hydrolysate was obtained by auto-hydrolysis followed by a 0.25% H_2SO_4 pre-treatment [18]. Auto-hydrolysis was carried out in a semi-pilot scale pre-treatment continuous tubular reactor (PCTR) at 150 psi (about 180°C) with a mean residence time of 18 min [18]. The biomass from auto-hydrolysis was further hydrolyzed with 0.25% (v/v) H_2SO_4 in a 1:2 (w/v) solids loading ratio, in an autoclave at 121°C for 60 min. The liquid fraction from the pre-treatment was separated by centrifugation and collected for further analysis, and it was identified as wheat straw pretreated (WSP); which contained 1.8 g/L glucose, 39.8 g/L xylose, 7.9 g/L arabinose, 2.6 g/L formate, 7.8 g/L acetate and 1.2 g/L furfural. Further dilutions were made to obtain a hydrolysate with a final concentration of 16.1 ± 0.2 g/L of total reducing sugars (TRS) to perform the scale-up experiments.

2.3 Construction of mutant strains

Strains, plasmids, and primers used for the construction of the mutant strains are shown in Table 1. W3110 Δ*frdD* and W3110 Δ*ldhA* strains from Keio collection [19] were used as donor. The deletion of *frdD* and *ldhA* genes was achieved using P1 transduction method [20], with some modification as follows: To prepare liquid P1 lysate, an overnight culture of *E. coli* donor strain (W3110 Δ*frdD* or W3110 Δ*ldhA*) in LB broth with 30 mg/L kanamycin was washed and suspended in MC medium (10 mM $MgSO_4$ and 5 mM $CaCl_2$). Phage P1 was added with multiplicity of infection between 0.1 and 1.0, and the culture was incubated for 30 min at room temperature. After incubation, the culture was added into soft agar and finally plated in LB agar plates and incubated overnight at 37°C. After lysis, the culture was treated with chloroform and the debris was removed by centrifugation. The phage was stored at 4°C until its application. For transduction, an overnight culture of *E.*

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

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

2.4 Effect of *frdD* and *ldhA* genes deletion on co-production of H_2 and EtOH

The effect of *frdD* and *ldhA* genes deletion on co-production of H_2 and EtOH by *Escherichia coli* strains (Table 1) was determined by using glucose as substrate (20 g/L). The experiments were done in anaerobic serological bottles containing 0.01 L of B buffer [21], 1 mL/L trace elements solution [22], 0.01 g/L $MgSO_4$ and 1 g/L yeast extract (Difco). The cultures were started with an optical density of 0.2 measured at a wavelength of 600 nm (OD_{600}), pH 7.5, and they were incubated at 31°C and 175 rpm, until no longer production of H_2 was observed. All the experiments were carried out in triplicate. The production of H_2, EtOH, and other metabolites was measured as indicated in section 2.6.

2.5 Scale-up of H_2 and EtOH co-production

E. coli WDH–LF strain was selected to perform the scale-up of H_2 and EtOH co-production using WSP as substrate (16 g/L TRS). The working volumes used were 0.01, 0.1, 1 and 10 L. In 0.1, 1 and 10 L experiment, a volume of the headspace relative to that of the liquid approximately of 40% was used and for 0.01 L reactors was 20%. The experiments were done under anaerobic conditions using the B buffer [21] plus 1 mL/L trace elements solution [22], 0.01 g/L $MgSO_4$ and 1 g/L yeast extract (Difco). The cultures were started with an OD_{600} of 0.2 and pH 8.2, incubating them at 31°C and shaking at 200 rpm, until no longer production of H_2 was observed. 10 L bioreactors were stirred at 250 rpm during first 29 h and then it was increased at 400 rpm. Batch cultures with working volumes 1 and 10 L were performed in 1.5-L and 13.5-L bioreactors equipped with two six-blade Rushton turbines flat, with a H/D ration of 2.1 and 1.5, respectively. The pH was monitored using an autocleavable electrode (Applikon® Biotechnology) and connected to Bioconsole ADI 1035 (Applikon® Biotechnology) controlled by the ADI 1030 Biocontroller (Applikon® Biotechnology). BioXpert 1.3 software (Applikon® Biotechnology) was used for data acquisition. The experiments in serological bottles and bioreactors were carried out in quadruplicate and duplicates, respectively. The production of H_2 and EtOH was measured as indicated in Section 2.6.

2.6 Analytical Methods

TRS determination was performed by the dinitrosalicylic acid (DNS) method [23], with some modifications, as follow: 250 µL of diluted sample with 750 µL of DNS reagent (10 g/L NaOH, 200 g/L $KNaC_4H_4O_6 \cdot 4H_2O$, 0.5 g/L $Na_2S_2O_5$, 2 g/L C_6H_6O, 10 g/L 3,5-Dinitrosalicylic acid) were heated for 5 minutes at 100ºC and then cooled down to room

156 temperature. Then, 400 µL 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 H_2SO_4 0.0025 M as a mobile phase
162 (0.550 cm^3/min). Furfural was analyzed by Gas Chromatography (Agilent Technologies
163 6890N Network GC Systems) using a capillary column HP-Innowax (30 m × 0.25 mm i.d.
164 × 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 ≤ 2) displacement in an inverted
169 burette connected to serological bottles/bioreactor with rubber tubing and a needle. H_2
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 µm 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. H_2 volume was corrected to standard conditions of
176 temperature and pressure (298.15K and 10^5 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 H_2 and EtOH co-production by *frdD* and *ldhA* genes deletion in
185 H_2-overproducer *Escherichia coli* strains

186

187 To enhance the co-production of H_2 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 H_2-
191 overproducer strains, *E. coli* Δ*hycA* (WDH) and *E. coli* Δ*hycA* Δ*lacI* (WDHL) [22],
192 through P1 transduction method (Fig. 1B). Then, Δ*hycA* Δ*ldhA* (WDH-L) and Δ*hycA* Δ*lacI*
193 Δ*ldhA* (WDHL-L) *E. coli* strains were generated. The effect of *ldhA* gen deletion on co-
194 production of H_2 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 H_2/L, respectively. According to
197 the analysis of variance, not statistically significant difference (Table A1, $p < 0.05$) was
198 found in the H_2 production achieved by each strain. The H_2 production rate (rH_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], rH_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 ±
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 H_2 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* Δ*hycA* Δ*frdD*) and WDH-LF (*E. coli* Δ*hycA* Δ*ldhA* Δ*frdD*) strains. To determine
215 the effect of *frdD* gen deletion on co-production of H_2 and EtOH by *E. coli*, the
216 fermentation of 20 g/L of glucose in a batch culture during 180 h at 31ºC and initial pH of
217 7.5 was conducted (Fig. 3). The WDH-F strain obtained 1.3- and 1.4-fold more H_2 than the
218 wild-type (*E. coli* W3110, WT) strain and its parental (WDH) strain, respectively.
219 Regarding to rH_2, the WT, WDH, and WDH-F obtained almost the same results –ranging
220 between 53.9 and 59.4 mL H_2/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 H_2 and achieved 2.4-fold higher rH_2 than WDH-L. But the latter

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

The co-production of H_2 and EtOH was significantly improved by both *ldhA* and *frdD* genes deletion. The WDH-LF strain produced 2,950.3 ± 261.8 mL H_2/L, 1.7-fold and 1.5-fold more hydrogen than the WT and WDH strains, respectively (Fig. 3A). Also, WDH-LF strain produced more H_2 than single mutant strains –WDH-L and WDH-F–, which achieved 1,650.83 ± 234.20 and 2,523.03 ± 93.54 ml H_2/L, respectively. Although, H_2 production was increased by *ldhA* and *frdD* genes deletion, *r*H_2 seems to be decreased in WDH-LF strain. WDH-LF strain achieved 40.35 ± 3.89 mL H_2/L/h, whereas WT and WDH obtained 1.3-53.91 ± 3.15 and 55.69 ± 3.23 mL H_2/L/h (Fig. 3B), respectively. Succinate and lactate production were dramatically reduced by the deletion of *frdD* and *ldhA* genes, whereas the EtOH production was improved compared to WT strain (Fig. 3C). The WDH-LF strain produced 7.20 ± 0.26 g EtOH/L, which is 2.7-, 3.8-, 1.2, and 5.5-fold more EtOH than obtained by WT, WDH, WDH-L, and WDH-F strains (Table A4), respectively. These results are consistent with the previously reported [26,27], and confirmed that the absence of *frdD* and *ldhA* genes improves the co-production of H_2 and EtOH by *E. coli* using glucose as substrate.

Mutagenesis of competing metabolic pathways has been widely employed to improve H_2 and/or EtOH production performance [28]. To improve H_2 production, the most utilized strategies are related to disruption of genes –such as *ldhA*, *frdAB*, *hycA*, *hya*, and *hyb*–, inactivation of formate-hydrogen lyase (FHL) repressor (encoded by *hycA*), and/or overexpression of FHL (encoded by *fhlA*). Regarding to the EtOH production, EtOH-producer strains [*e.g. E. coli* SE2378 (Δ*ldh* Δ*pfl*) and *E. coli* SZ420 (Δ*frdBC* Δ*ldh* Δ*ackA* Δ*folA-pfl* Δ*pdhR*::*pflBp6-aceEF-lpd*)] have been constructed by deleting the competing

fermentation pathways, and implementing another molecular strategies such gene overexpression. However, the low H_2 yields still a limitation for large-scale H_2 production *via* dark fermentation and it is not sustainable economically. To overcome this issue, co-production of H_2 and EtOH has been proposed [29]. In this regard, using *E. coli* Δ*hycA* Δ*hyaAB* Δ*hybBC* Δ*ldhA* Δ*frdAB* as parental strain, mutant strains devoid *pta-ackA* (encoding to phosphate acetyltransferase and acetate kinase, respectively) or *pfkA* (encoding to phosphofructokinase 1) genes were constructed to increase the H_2 and EtOH co-production [30]. The authors concluded that Δ*ack-pta* strain does not improve the biofuels co-production. However, the Δ*pfkA* strain resulted in an increase of approximately 50% and 9.5% of H_2 and EtOH, respectively. In the present study, we used the *E. coli* Keio collection to introduce two mutations into a single strain by the repetition of resistance-gene elimination and P1 transduction; this method may be used to engineer *E. coli* for many applications where multiple chromosomal genes must be eliminated. The deletion of the *lhdA* and *frdD* genes in WDH strain, produce an improvement of 70 and 167% on H_2 and EtOH production, respectively.

3.2 Scale-up of H_2 and EtOH co-production from wheat straw hemicellulosic hydrolysate

The scaling-up is a vital tool to development bioprocesses since it can reduce errors in the industrial-scale designs, as well as lack of information. For this reason, the effect of the reactor size on co-production of H_2 and EtOH by *E. coli* WDH-LF was determined. Since lignocellulose is a sustainable and worldwide available biomass, it was selected as feedstock to perform the scaling-up of dark fermentation. Therefore, wheat straw hemicellulosic hydrolysate (WSP) obtained by autohydrolysis followed by a 0.25% diluted

sulphuric acid pre-treatment was used as substrate for H_2 and EtOH co-production by *E. coli* WDH-LF. The reactor sizes used to scale-up the process were 0.01, 0.1, 1 and 10 L. The average of the maximum H_2 concentration (considering all working volumes) was 50.9 ± 7.4 %. The H_2 production obtained in 0.01, 0.1, 1 and 10 L reactors was 5,259.9 ± 540.2, 4,076.1 ± 468.4, 5,574.8 ± 156.6, and 5,603.0 ± 233.5 mL H_2/L, respectively, as shows in Fig. 4A. H_2 production was similar in all working volumes except in 0.1 L (Table A5), which was approximately 25% lower than the other sizes. Regarding to the H_2 yield, the results obtained were 328.7 ± 33.8, 253.2 ± 29.1, 350.6 ± 9.9, and 342.7 ± 14.3 mL H/g TRS for 0.01, 0.1, 1 and 10 L reactors, respectively. H_2 yield seems to be lower in 0.1 L reactors compare with the other reactor sizes (Fig. 4B). These behaviors respond to the fact that the volume of the headspace relative to that of the liquid (working volume) may affect dark fermentation [38,39], because both H_2 and CO_2 accumulate to high pressures in the headspace and inhibits H_2 production [40]. As described in Section 2.5, 0.1 L reactors employed lower volume of the headspace relative to that of the working volume than the other reactor sizes. The maximum rH_2 achieved by WDH-LF strain were 40.7 ± 2.3, 39.2 ± 5.8, 36.9 ± 1.4 and 41.5 ± 4.0 mL H_2/L·h for 0.01, 0.1, 1 and 10 L reactors, respectively (Fig. 4B). Kinetic of H_2 production are shown in Fig. 5. As noted, 10 L reactor attained the longest lag phase compare to the other reactor sizes. This can be explained by the agitation rate utilized in 10 L reactors, which was maintained at 250 rpm during first 29 h and then it was increased at 400 rpm, as described Section 2.5. Mixing may promote dark fermentation performance by enhancing liquid-gas mass transfer, heat transfer, as well as homogenization. However, inappropriate stirring can be harmful to H_2 producing bacteria, and thus H_2 production may be inhibited [41,42]. The EtOH productions obtained by 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 H_2 and EtOH co-production for 1 and 10 L appear in Fig. 6. In 1 L
302 reactors, H_2 and EtOH production started in the first 15 h of fermentation, while for 10 L
303 reactors, the first evidence of H_2 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 H_2
310 production rate by *E. coli* WDH-LF was approximately 4-fold than the achieved by *E. coli*
311 WDHL; (c) the H_2 yield increased 2-fold by the deletion of *ldhA* and *frd* genes.
312 H_2 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 H_2/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 H_2/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

leave laboratory scale production behind, which is attributed to the relatively low energy requirements, high biofuels production rates and the chance of using a broad spectrum of organic matter as substrate [46]. However, they still have certain limitations that could be crucial for scaling-up, as the stability of the process in self [47,48]. As an answer to the limitations of the dark fermentation, several researchers have proposed the use of genetically modified microorganisms with advanced selected properties [49]. In the present work, we demonstrate that: (1) the increase of biofuels production yields by deletion of the *ldhA* and *frd* genes in a H_2-overproducer *E. coli* strain; (2) *E. coli* WDH-LF can produce efficiently and simultaneously H_2 and EtOH from hemicellulosic hydrolysates *via* dark fermentation; (3) the yields achieved by WDH-LF strain are stable despite the change in the reactor size.

4 Conclusions

Deletion of *frdD* and *ldhA* genes positively impact the co-production of H_2 and EtOH by *E. coli*, since production, production rate and yield of both biofuels were improved. The engineered WDH-LF strain may utilize hemicellulosic hydrolysates as substrates to produce simultaneously and efficiently H_2 and EtOH, under optimal temperature and pH conditions. The change in the reactor size have not impact on the co-production of H_2 and EtOH by *E. coli* WDH-LF using wheat straw as feedstock, which is inferred because the H_2 and EtOH yields were similar despite the working volumes employed. Still, further genetic modifications, as well as engineering techniques must be employed to improve H_2 and EtOH production rates and makes them competitive in the actual biofuels framework.

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References

[1] Cherubini F. The biorefinery concept : Using biomass instead of oil for producing energy and chemicals. Energy Convers Manag 2010;51:1412–21. doi:10.1016/j.enconman.2010.01.015.

[2] Kumar R, Singh S, Singh O V. Bioconversion of lignocellulosic biomass: Biochemical and molecular perspectives. J Ind Microbiol Biotechnol 2008;35:377–91. doi:10.1007/s10295-008-0327-8.

[3] Kapoor M, Panwar D, Kaira GS. Bioprocesses for Enzyme Production Using Agro-Industrial Wastes: Technical Challenges and Commercialization Potential. In: Dhillon GS, Kaur S, editors. Agro-Industrial Wastes as Feed. Enzym. Prod. Apply Exploit Emerg. Valuab. Use Options Waste Biomass, Elsevier Inc.; 2016, p. 61–93. doi:10.1016/B978-0-12-802392-1.00003-4.

[4] Borja-Bravo M, Reyes-Muro L, Espinosa-Garcia JA, Vélez-Izquierdo A. Producción y consumo de rastrojos en México. In: Reyes-Muro L, Camacho-Villa TC, Guevara-Hernández F, editors. Rastrojos manejo, uso y Merc. en el Cent. y sur México. First edit, Ciudad de México: Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias; 2013, p. 256.

[5] Servicio de Información Agroalimentaria y Pesquera. Avance de Siembras y Cosechas. Resumen nacional por cultivo. Ciudad de Mexico: 2019.

[6] Sivagurunathan P, Kumar G, Mudhoo A, Rene ER, Saratale GD, Kobayashi T, et al. Fermentative hydrogen production using lignocellulose biomass: An overview of pre-treatment methods, inhibitor effects and detoxification experiences. Renew Sustain Energy Rev 2017;77:28–42. doi:10.1016/j.rser.2017.03.091.

[7] Kumar G, Shobana S, Nagarajan D, Lee DJ, Lee KS, Lin CY, et al. Biomass based

hydrogen production by dark fermentation — recent trends and opportunities for greener processes. Curr Opin Biotechnol 2018;50:136–45. doi:10.1016/j.copbio.2017.12.024.

[8] Liao JC, Mi L, Pontrelli S, Luo S. Fuelling the future: Microbial engineering for the production of sustainable biofuels. Nat Rev Microbiol 2016;14:288–304. doi:10.1038/nrmicro.2016.32.

[9] Shaw AJ, Lam FH, Hamilton M, Consiglio A, MacEwen K, Brevnova EE, et al. Metabolic engineering of microbial competitive advantage for industrial fermentation processes. Science (80-) 2016;353:583–6. doi:10.1126/science.aaf6159.

[10] Koskinen PEP, Beck SR, Örlygsson J, Puhakka JA. Ethanol and hydrogen production by two thermophilic, anaerobic bacteria isolated from Icelandic geothermal areas. Biotechnol Bioeng 2008;101:679–90. doi:10.1002/bit.21942.

[11] Balderas-Hernandez VE, Landeros Maldonado KP, Sánchez A, Smoliński A, De Leon Rodriguez A. Improvement of hydrogen production by metabolic engineering of *Escherichia coli*: Modification on both the PTS system and central carbon metabolism. Int J Hydrogen Energy 2019:1–10. doi:10.1016/j.ijhydene.2019.01.162.

[12] Förster AH, Gescher J. Metabolic engineering of *Escherichia coli* for production of mixed-acid fermentation end products. Front Bioeng Biotechnol 2014;2:1–12. doi:10.3389/fbioe.2014.00016.

[13] Maeda T, Sanchez-Torres V, Wood TK. Hydrogen production by recombinant *Escherichia coli* strains. Microb Biotechnol 2012;5:214–25. doi:10.1111/j.1751-7915.2011.00282.x.

[14] Valle A, Cantero D, Bolívar J. Metabolic engineering for the optimization of

399 hydrogen production in *Escherichia coli*: A review. Biotechnol Adv 2019;37:616–
400 33. doi:10.1016/j.biotechadv.2019.03.006.
401 [15] Balderas-Hernandez VE, Landeros Maldonado KP, Sánchez A, Smoliński A, De
402 Leon Rodriguez A. Improvement of hydrogen production by metabolic engineering
403 of *Escherichia coli*: Modification on both the PTS system and central carbon
404 metabolism. Int J Hydrogen Energy 2020;45:5687–96.
405 doi:https://doi.org/10.1016/j.ijhydene.2019.01.162.
406 [16] Liang L, Liu R, Freed EF, Eckert CA. Synthetic Biology and Metabolic Engineering
407 Employing *Escherichia coli* for C2–C6 Bioalcohol Production. Front Bioeng
408 Biotechnol 2020;8:710. doi:10.3389/fbioe.2020.00710.
409 [17] Sluiter A, Hames B, Ruiz RO, Scarlata C, Sluiter J, Templeton D. Determination of
410 Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure.
411 2011. doi:NREL/TP-510-42618.
412 [18] Rodríguez F, Amaya-Delgado L, Sanchez A. Xyloligosacharides production from
413 lignocellulosic biomass using a pretreatment continuous tubular reactor. Modelling
414 and experimental validation. Ind Crop Prod 2019;134:62–70.
415 doi:10.1016/j.indcrop.2019.03.058.
416 [19] Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, et al. Construction of
417 *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection.
418 Mol Syst Biol 2006;2:2006.0008. doi:10.1038/msb4100050.
419 [20] Thomason LC, Costantino N, Court DL. E . coli Genome Manipulation by P1
420 Transduction. Curr. Protoc. Mol. Biol., 2007, p. 1.17.1–1.17.8.
421 doi:10.1002/0471142727.mb0117s79.
422 [21] Davila-Vazquez G, de León-Rodríguez A, Alatriste-Mondragón F, Razo-Flores E.

The buffer composition impacts the hydrogen production and the microbial community composition in non-axenic cultures. Biomass and Bioenergy 2011;35:3174–81. doi:10.1016/j.biombioe.2011.04.046.

[22] Rosales-Colunga LM, Razo-Flores E, Ordoñez LG, Alatriste-Mondragón F, De León-Rodríguez A. Hydrogen production by *Escherichia coli* ΔhycA ΔlacI using cheese whey as substrate. Int J Hydrogen Energy 2010;35:491–9. doi:10.1016/j.ijhydene.2009.10.097.

[23] Miller GL. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal Chem 1959;31:426–8. doi:10.1021/ac60147a030.

[24] Rosales-Colunga LM, Razo-Flores E, De León Rodríguez A. Fermentation of lactose and its constituent sugars by *Escherichia coli* WDHL: Impact on hydrogen production. Bioresour Technol 2012;111:180–4. doi:10.1016/j.biortech.2012.01.175.

[25] Sode K, Watanabe M, Makimoto H, Tomiyama M. Construction and Characterization of Fermentative Lactate Dehydrogenase *Escherichia coli* Mutant and Its Potential for Bacterial Hydrogen Production. Appl Biochem Biotechnol 1999;77:317–24. doi:10.1385/ABAB:77:1-3:317.

[26] Yoshida A, Nishimura T, Kawaguchi H, Inui M, Yukawa H. Enhanced hydrogen production from glucose using ldh- and frd-inactivated *Escherichia coli* strains. Appl Microbiol Biotechnol 2006;73:67–72. doi:10.1007/s00253-006-0456-9.

[27] Maeda T, Sanchez-Torres V, Wood TK. Enhanced hydrogen production from glucose by metabolically engineered *Escherichia coli*. Appl Microbiol Biotechnol 2007;77:879–90. doi:10.1007/s00253-007-1217-0.

[28] Majidian P, Tabatabaei M, Zeinolabedini M, Naghshbandi MP, Chisti Y. Metabolic engineering of microorganisms for biofuel production. Renew Sustain Energy Rev

2018;82:3863–85. doi:10.1016/j.rser.2017.10.085.

[29] Lopez-Hidalgo AM, Magaña G, Rodriguez F, De Leon-Rodriguez A, Sanchez A. Co-production of ethanol-hydrogen by genetically engineered *Escherichia coli* in sustainable biorefineries for lignocellulosic ethanol production. Chem Eng J 2021;406:126829. doi:10.1016/j.cej.2020.126829.

[30] Seol E, Ainala SK, Sekar BS, Park S. Metabolic engineering of *Escherichia coli* strains for co-production of hydrogen and ethanol from glucose. Int J Hydrogen Energy 2014;39:19323–30. doi:10.1016/j.ijhydene.2014.06.054.

[31] Łukajtis R, Hołowacz I, Kucharska K, Glinka M, Rybarczyk P, Przyjazny A, et al. Hydrogen production from biomass using dark fermentation. Renew Sustain Energy Rev 2018;91:665–94. doi:10.1016/j.rser.2018.04.043.

[32] Sivagurunathan P, Kuppam C, Mudhoo A, Saratale GD, Kadier A, Zhen G, et al. A comprehensive review on two-stage integrative schemes for the valorization of dark fermentative effluents. Crit Rev Biotechnol 2018;38:868–82. doi:10.1080/07388551.2017.1416578.

[33] Soo CS, Yap WS, Hon WM, Ramli N, Md Shah UK, Phang LY. Co-production of hydrogen and ethanol by *Escherichia coli* SS1 and its recombinant. Electron J Biotechnol 2017;30:64–70. doi:10.1016/j.ejbt.2017.09.002.

[34] Sundara Sekar B, Seol E, Park S. Co-production of hydrogen and ethanol from glucose in *Escherichia coli* by activation of pentose-phosphate pathway through deletion of phosphoglucose isomerase (pgi) and overexpression of glucose-6-phosphate dehydrogenase (zwf) and 6-phosphogluconate dehyd. Biotechnol Biofuels 2017;10:1–12. doi:10.1186/s13068-017-0768-2.

[35] Seol E, Sekar BS, Raj SM, Park S. Co-production of hydrogen and ethanol from

glucose by modification of glycolytic pathways in *Escherichia coli* - from Embden-Meyerhof-Parnas pathway to pentose phosphate pathway. Biotechnol J 2016;11:249–56. doi:10.1002/biot.201400829.

[36] Soo C-S, Yap W-S, Hon W-M, Ramli N, Md Shah UK, Phang L-Y. Improvement of hydrogen yield of ethanol-producing *Escherichia coli* recombinants in acidic conditions. Electron J Biotechnol 2017;26:27–32. doi:10.1016/j.ejbt.2016.12.007.

[37] Maeda T, Tran KT, Yamasaki R, Wood TK. Current state and perspectives in hydrogen production by *Escherichia coli*: roles of hydrogenases in glucose or glycerol metabolism. Appl Microbiol Biotechnol 2018;102:2041–50. doi:10.1007/s00253-018-8752-8.

[38] Oh S-E, Zuo Y, Zhang H, Guiltinan MJ, Logan BE, Regan JM. Hydrogen production by Clostridium acetobutylicum ATCC 824 and megaplasmid-deficient mutant M5 evaluated using a large headspace volume technique. Int J Hydrogen Energy 2009;34:9347–53. doi:10.1016/j.ijhydene.2009.09.084.

[39] Sekoai PT, Yoro KO, Daramola MO. Effect of nitrogen gas sparging on dark fermentative biohydrogen production using suspended and immobilized cells of anaerobic mixed bacteria from potato waste. Biofuels 2018;9: 595–604. doi:10.1080/17597269.2018.1432275.

[40] Nemestóthy N, Bélafi-Bakó K, Bakonyi P. Enhancement of dark fermentative H2 production by gas separation membranes: A review. Bioresour Technol 2020;302:122828. doi:10.1016/j.biortech.2020.122828.

[41] Chezeau B, Fontaine JP, Vial C. Analysis of liquid-to-gas mass transfer, mixing and hydrogen production in dark fermentation process. Chem Eng J 2019;372:715–27. doi:10.1016/j.cej.2019.04.191.

[42] Chezeau B, Vial C. Combined effects of digestate viscosity and agitation conditions on the fermentative biohydrogen production. Biochem Eng J 2019;142:105–16. doi:10.1016/j.bej.2018.11.016.

[43] Lopez-Hidalgo AM, Sánchez A, De León-Rodríguez A. Simultaneous production of bioethanol and biohydrogen by *Escherichia coli* WDHL using wheat straw hydrolysate as substrate. Fuel 2017;188:19–27. doi:10.1016/j.fuel.2016.10.022.

[44] Cheng CL, Lo YC, Lee KS, Lee DJ, Lin CY, Chang JS. Biohydrogen production from lignocellulosic feedstock. Bioresour Technol 2011;102:8514–23. doi:10.1016/j.biortech.2011.04.059.

[45] Paulova L, Patakova P, Branska B, Rychtera M, Melzoch K. Lignocellulosic ethanol: Technology design and its impact on process efficiency. Biotechnol Adv 2015;33:1091–107. doi:10.1016/j.biotechadv.2014.12.002.

[46] Sivagurunathan P, Kumar G, Bakonyi P, Kim SH, Kobayashi T, Xu KQ, et al. A critical review on issues and overcoming strategies for the enhancement of dark fermentative hydrogen production in continuous systems. Int J Hydrogen Energy 2016;41:3820–36. doi:10.1016/j.ijhydene.2015.12.081.

[47] Jarunglumlert T, Prommuak C, Putmai N, Pavasant P. Scaling-up bio-hydrogen production from food waste: Feasibilities and challenges. Int J Hydrogen Energy 2018;43:634–48. doi:10.1016/j.ijhydene.2017.10.013.

[48] Tapia-Venegas E, Ramirez-Morales JE, Silva-Illanes F, Toledo-Alarcón J, Paillet F, Escudie R, et al. Biohydrogen production by dark fermentation: scaling-up and technologies integration for a sustainable system. Rev Environ Sci Biotechnol 2015;14:761–85. doi:10.1007/s11157-015-9383-5.

[49] Ntaikou I, Antonopoulou G, Lyberatos G. Biohydrogen production from biomass

519 and wastes via dark fermentation: A review. Waste and Biomass Valorization
520 2010;1:21–39. doi:10.1007/s12649-009-9001-2.
521 [50] Maeda T, Sanchez-Torres V, Wood TK. Metabolic engineering to enhance bacterial
522 hydrogen production. Microb Biotechnol 2008;1:30–9. doi:10.1111/j.1751-
523 7915.2007.00003.x.
524 [51] Romero Soto L, Byrne E, van Niel EWJ, Sayed M, Villanueva CC, Hatti-Kaul R.
525 Hydrogen and polyhydroxybutyrate production from wheat straw hydrolysate using
526 Caldicellulosiruptor species and Ralstonia eutropha in a coupled process. Bioresour
527 Technol 2018;272:259–66. doi:10.1016/j.biortech.2018.09.142.
528 [52] Valdez-Vazquez I, Pérez-Rangel M, Tapia A, Buitrón G, Molina C, Hernández G, et
529 al. Hydrogen and butanol production from native wheat straw by synthetic microbial
530 consortia integrated by species of Enterococcus and Clostridium. Fuel
531 2015;159:214–22. doi:10.1016/j.fuel.2015.06.052.
532 [53] Toquero C, Bolado S. Effect of four pretreatments on enzymatic hydrolysis and
533 ethanol fermentation of wheat straw. Influence of inhibitors and washing. Bioresour
534 Technol 2014;157:68–76. doi:10.1016/j.biortech.2014.01.090.
535 [54] Pawar SS, Nkemka VN, Zeidan AA, Murto M, Van Niel EWJ. Biohydrogen
536 production from wheat straw hydrolysate using Caldicellulosiruptor saccharolyticus
537 followed by biogas production in a two-step uncoupled process. Int J Hydrogen
538 Energy 2013;38:9121–30. doi:10.1016/j.ijhydene.2013.05.075.
539

Figure captions

Fig. 1. Carbon flux in *E. coli* during anaerobic metabolism and the strategy used to improve the co-production of H_2 and EtOH. (A) Improving the co-production of H_2 and EtOH by deletion of *ldhA* and *frdD* genes. The *dashed orange lines* are the pathways inactivated, and the *bold yellow lines* are the pathways enhanced. Chemical structures are shown for dark fermentation products and pyruvic acid. (B) *Escherichia coli* genome manipulation by phage P1 transduction. Homologous regions are indicated by red and green boxes; dtg, deletion target gen (*ldhA* and *frdD* genes for this study); res, antibiotic resistance gen (kanamycin for this study). Flp recognition sites are indicated.

Fig. 2. Effect of *ldhA* gene deletion on the H_2 and metabolites production by WDH and WDHL strains. Kinetic of H_2 production (A) and final concentration of metabolites (B). Data are presented as mean ± standard deviation. Batch culture were done in 0.1 L anaerobic serological bottles using glucose (20 g/L) as substrate at 37°C and initial pH of 7.5.

Fig. 3. Improvement of H_2 and EtOH co-production by metabolic engineering in *E. coli* strains. Kinetic of H_2 production (D), cumulative H_2 production (E) and final concentration of metabolites (F). Data are presented as mean ± standard deviation. * indicates statistically significant differences compared with the wild type (WT) cultures ($p < 0.05$). Batch culture were done in 0.1 L anaerobic serological bottles using glucose (20 g/L) as substrate at 37°C and initial pH of 7.5.

Fig. 4. Effect of the increase of reactor size on co-production of H_2 and EtOH by *E. coli* WDH-LF using WSP as substrate. Batch cultures were done in 0.01, 0.1, 1 and 10 L at 31°C and initial pH of 8.2. Production (A), production rate and yield (B) of H_2. Production and yield of EtOH (C). Data are presented as mean ± standard deviation.

Fig. 5. Kinetics of H_2 production by *E. coli* WDH-LF using WSP hydrolysate as substrate. Batch cultures were done in 0.01, 0.1, 1 and 10 L at 31°C and initial pH of 8.2.

Fig. 6. pH profile during co-production of H_2 and EtOH by *E. coli* WDH-LF using 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	*Escherichia coli* W3110 (lac^+, gal^+, F^-, $^-$ IN (rrnD-rrnE)1, rph-1)	Laboratory stock
W3110 Δ*frdD*	WT Δ*frdD*	Laboratory stock
W3110 Δ*ldhA*	WT Δ*ldhA*	Laboratory stock
WDH	WT Δ*hycA*	[22]
WDHL	WT Δ*hycA* Δ*lacI*	[22]
WDHL–L	WT Δ*hycA* Δ*lacI* Δ*ldhA*	This work
WDH–L	WT Δ*hycA* Δ*ldhA*	This work
WDH–F	WT Δ*hycA* Δ*frdD*	This work
WDH–LF	WT Δ*hycA* Δ*ldhA* Δ*frdD*	This work
Plasmid		
pCP20	FLP recombinase expression plasmid (*bla*, *cat*) p_R FLP^+, $cI857^+$, pSC101 ori TS	[50]
Primers	**Sequence**	
OGF-F	GAGGGGCAGCAAATGTGGAG	This work
OGF-R	TGAACTGGCACCGAAAGCGG	This work
OG-L-F	CGCGGCTACTTTCTTCATTG	This work
OG-L-R	GGTTGCGCCTACACTAAGCAT	This work

575

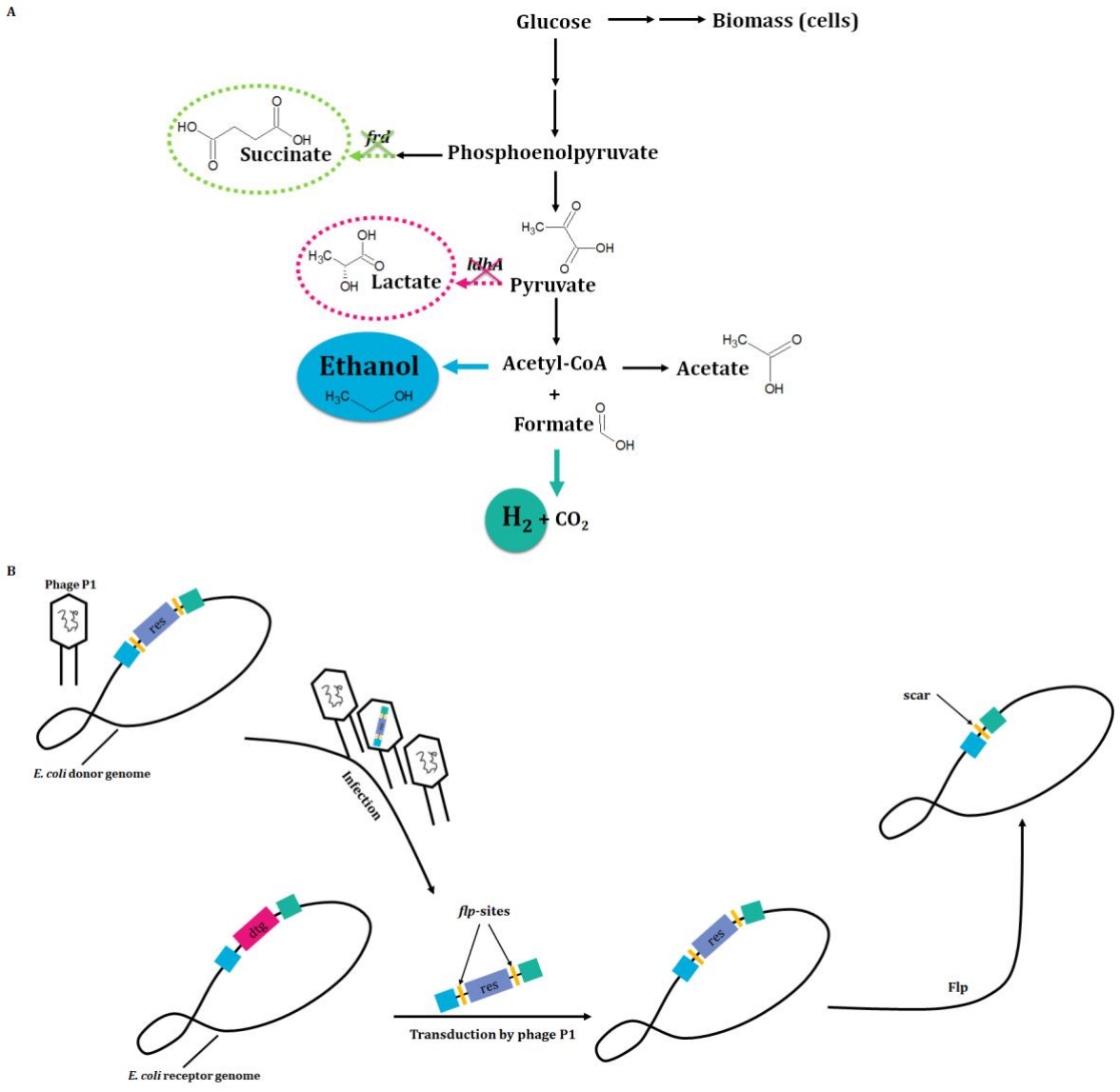
576

Table 2

Comparison of the production and yield of hydrogen and ethanol from wheat straw biomass.

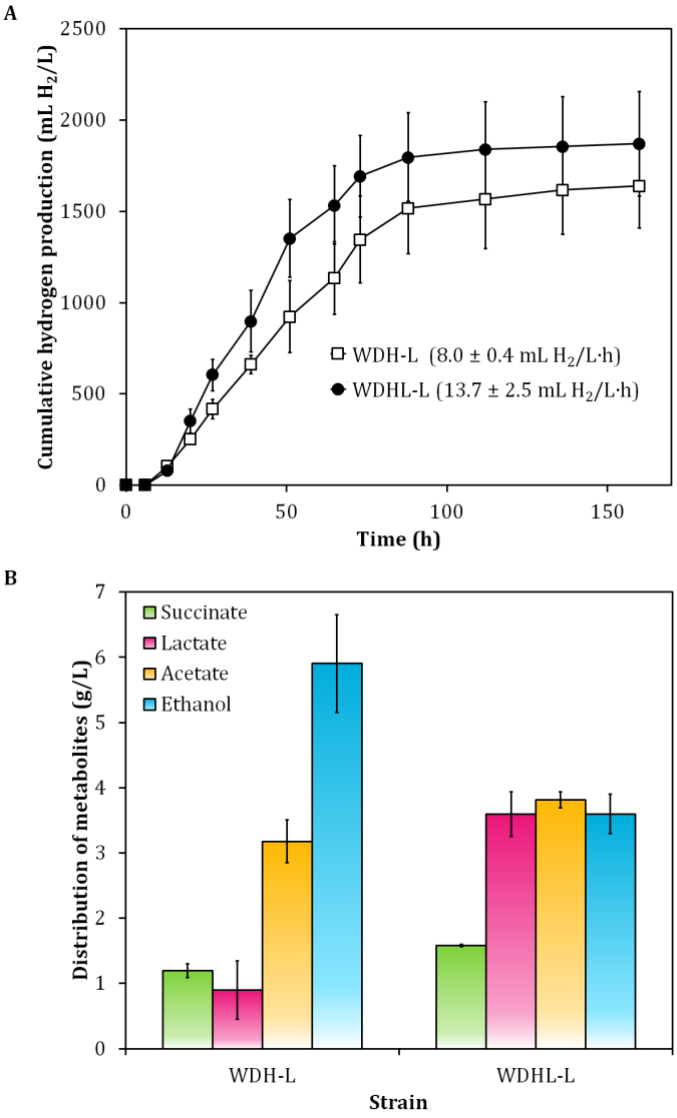
Inoculum	Substrate treatment	Operation mode	Working volume (L)	Hydrogen production (mL H_2/L)	Hydrogen yield (mL H_2/g consumed substrate)	Ethanol production (g EtOH/L)	Ethanol yield (g EtOH/g consumed sugars)	Reference
Caldicellulosiruptor saccharolyticus and *C. owensensis*	Steam explosion (190 °C, 10 min), enzymatic hydrolysis	Continuous pH 6.5, 70ºC	1.5	3350 [a] (134 mmol H_2/L)	-	-	-	[51]
A three-species culture of epiphytic *Enterococcus*	Hydration of fibres	Batch pH 6, 37ºC	0.1	-	386	-	-	[52]
Clostridium beijerinckii and *C. cellulovorans*	Biologically treated WS enriched in cellulose	Batch pH 5.5, 37ºC	0.1	-	-	3.7	-	
Pichia stipites	5% H_2O_2 (pH 11.5, 50ºC, 120 rpm, 60 min)	Batch pH 5, 30ºC	0.025	-	-	17.37	0.44	[53]
Caldicellulosiruptor saccharolyticus	Acid pretreatment (H_3PO_4, 190ºC, 5 min), enzymatic hydrolysis	Continuous 6.5 pH, 70ºC	1	-	413 [a] (3.04 mol H_2/mol sugars)	-	0.05 [a] (0.19 mol EtOH/mol sugars)	[54]
Escherichia coli WDH-LF	Autohydrolysis (180°C, 150 psi, retention time: 18 min) followed by acid hydrolysis (0.25% H_2SO_4 v/v, 121°C, 21 psi, 1h)	Batch pH 8.2, 31ºC	0.01	5259.9 ± 540.2	328.7 ± 33.8	8.9 ± 1.3	0.56 ± 0.08	This work
			0.1	4076.1 ± 468.4	253.2 ± 29.1	7.8 ± 1.7	0.48 ± 0.11	
			1	5574.8 ± 156.6	350.6 ± 9.8	7.9 ± 0.6	0.50 ± 0.04	
			10	5063.0 ± 233.5	342.7 ± 14.3	7.9 ±0.3	0.48 ± 0.01	

[a] Converted unit from the original data

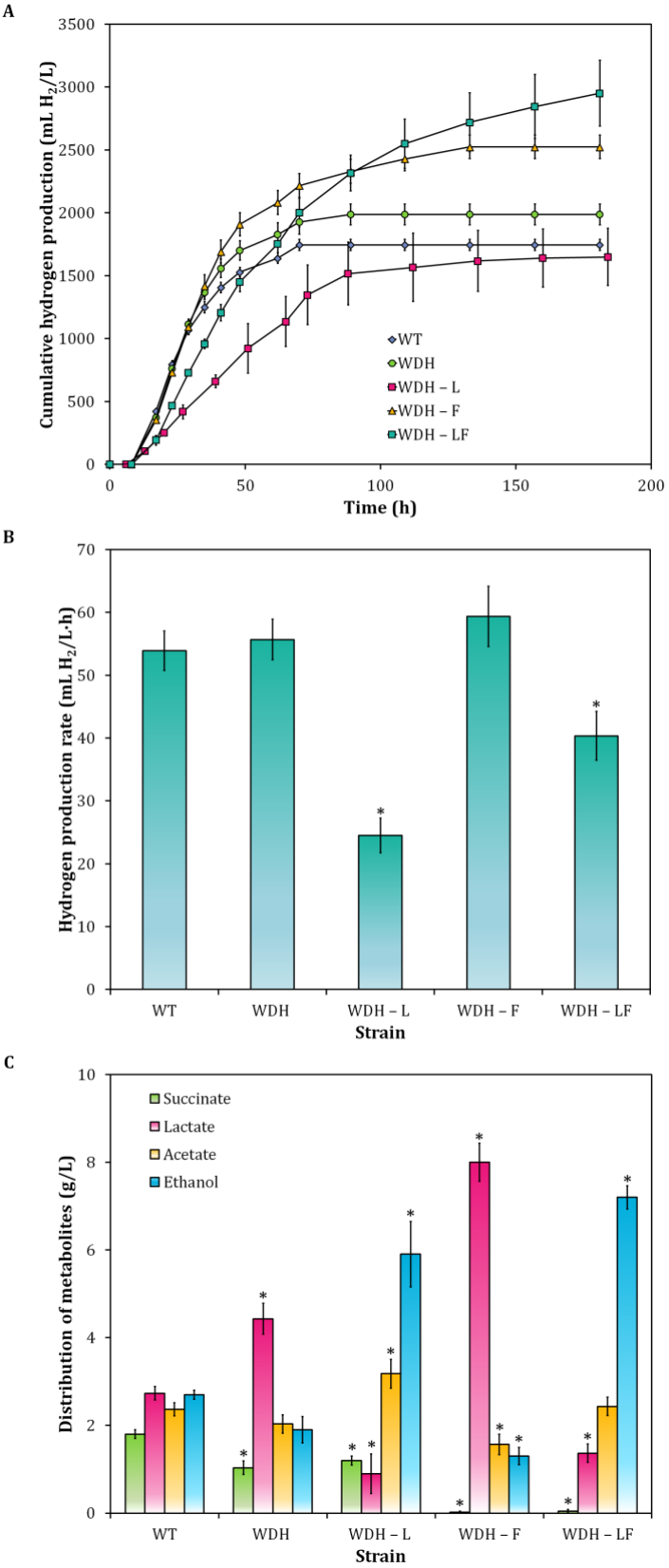

A
Glucose
Biomass (cells)
Succinate
frd
Phosphoenolpyruvate
Lactate
ldhA
Pyruvate
Ethanol
Acetyl-CoA
Acetate
Formate
H_2 + CO_2
B
Phage P1
res
E. coli donor genome
Infection
flp-sites
dhs
res
Transduction by phage P1
res
scar
Flp
E. coli receptor genome

581

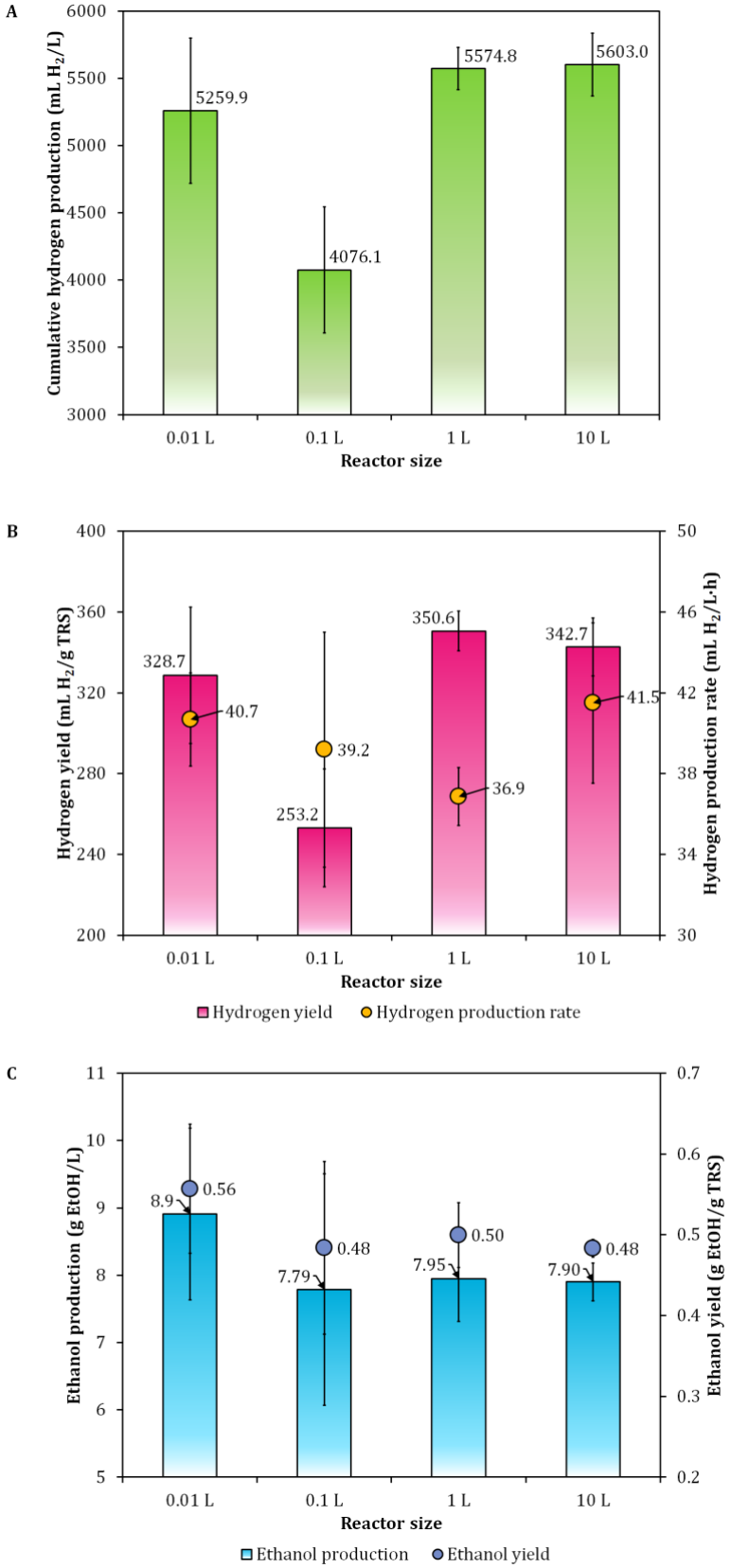
582 **Fig. 7.**


A
Cumulative hydrogen production (mL H2/L)
2500
2000
1500
1000
500
0
0
50
100
150
Time (h)
WDH-L (8.0 ± 0.4 mL H2/L·h)
WDHL-L (13.7 ± 2.5 mL H2/L·h)
B
Distribution of metabolites (g/L)
7
6
5
4
3
2
1
0
Succinate
Lactate
Acetate
Ethanol
WDH-L
WDHL-L
Strain

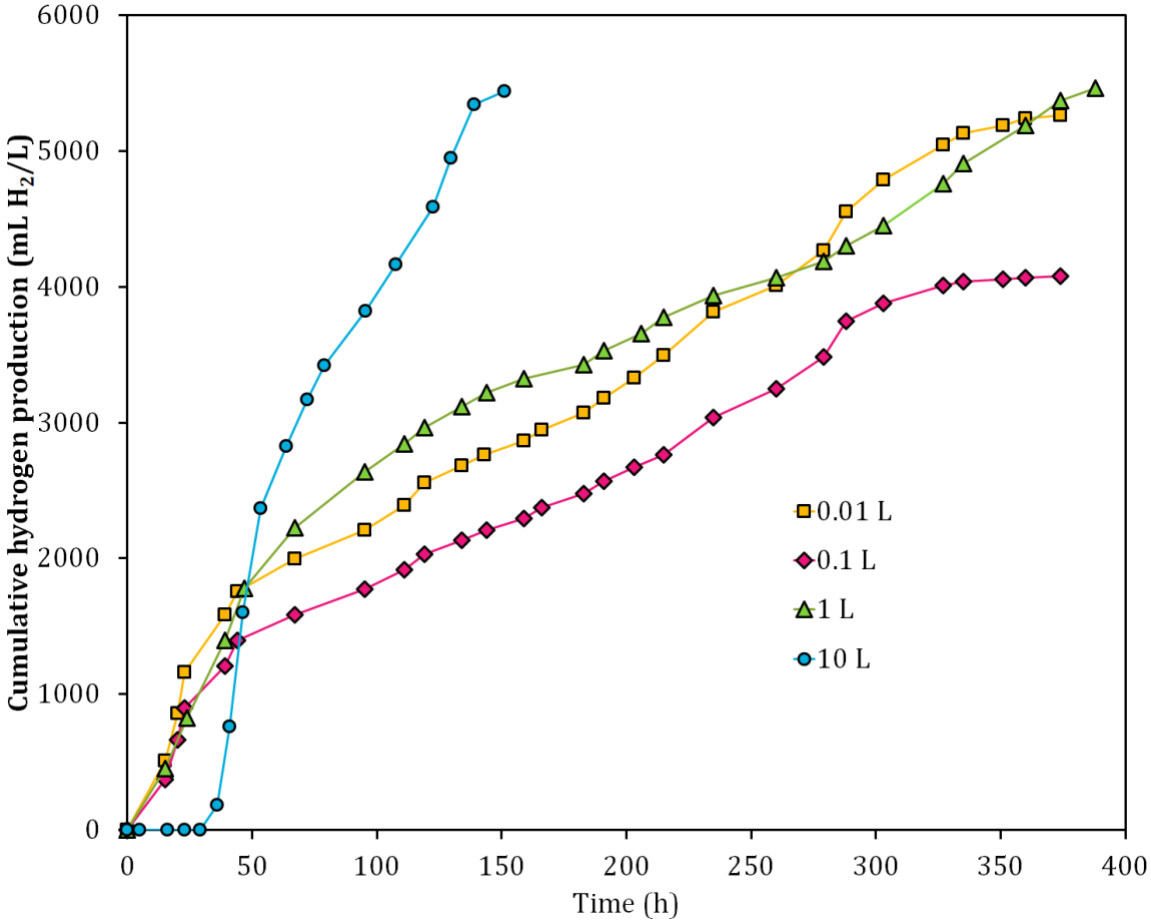
583
584 **Fig. 8.**


A
Cumulative hydrogen production (mL H2/L)
Time (h)
WT
WDH
WDH – L
WDH – F
WDH – LF
B
Hydrogen production rate (mL H2/L·h)
Strain
WT
WDH
WDH – L
WDH – F
WDH – LF
C
Distribution of metabolites (g/L)
Succinate
Lactate
Acetate
Ethanol
Strain
WT
WDH
WDH – L
WDH – F
WDH – LF

585
586 **Fig. 9.**



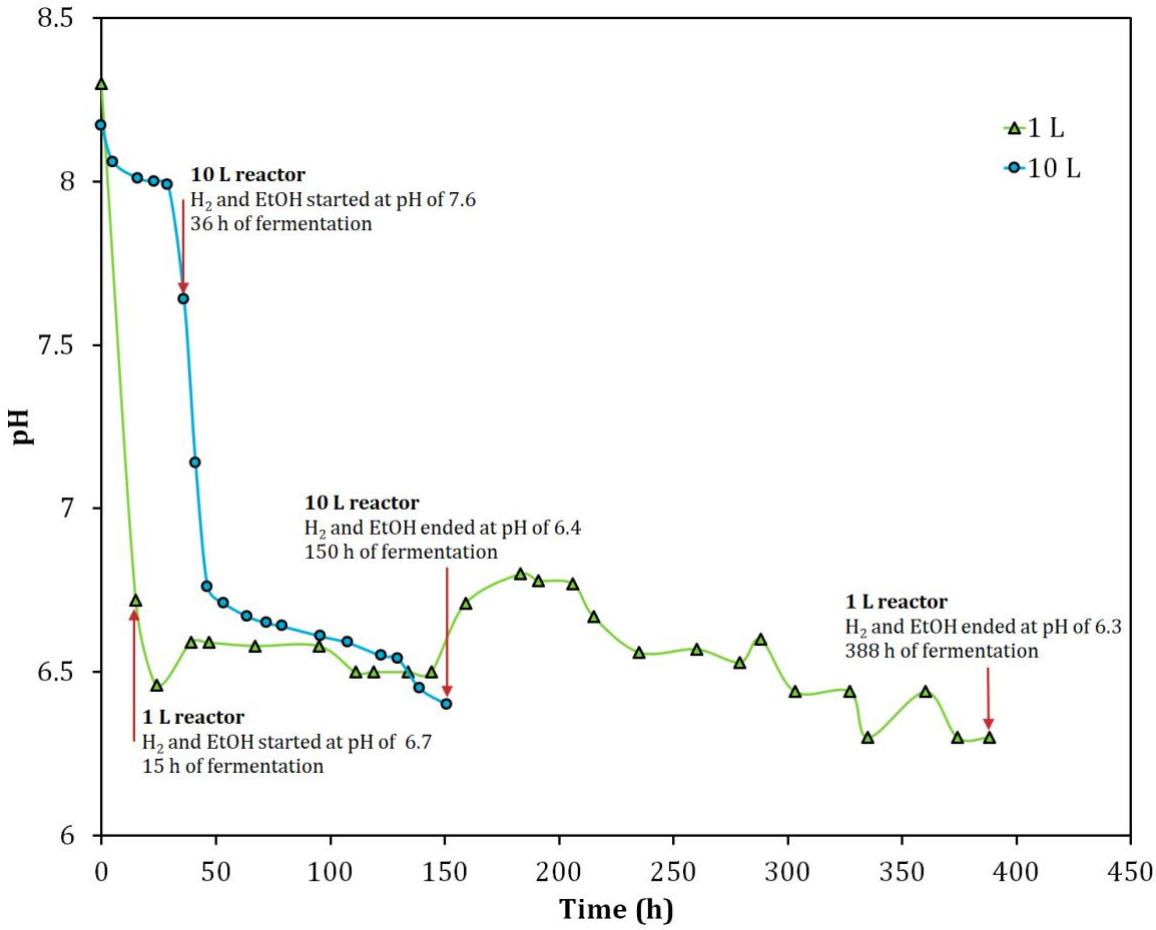
587
588 **Fig. 10.**



589

590 **Fig. 11.**

591



592

593 **Fig. 12.**