

1 **Indole modifies the central carbon flux in the anaerobic metabolism of *Escherichia***  
2 ***coli*: application to the production of hydrogen and other metabolites**

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23

24 **Abstract**

25 Indole is a bicyclic signaling molecule with effects on both eukaryotic and prokaryotic  
26 cells. The majority of studies of indole action have been performed with bacteria  
27 cultured under aerobic conditions and little information is available about its effects  
28 under anaerobic conditions. Here the effect of the indole on anaerobic metabolism of  
29 *Escherichia coli* WDHL was studied. Indole in the range 0.5 to 8 mM was added to the  
30 culture medium and cell growth, hydrogen and metabolite production were compared to  
31 cultures lacking indole. Results showed that while 8 mM indole abolished growth  
32 completely, 4 mM indole had a partial bacteriostatic effect and the maximum optical  
33 density of the culture decreased by 44% compared to the control cultures. In addition, 4  
34 mM indole had an important effect on anaerobic metabolism. Hydrogen production  
35 increased from  $650 \pm 115$  to  $1,137 \pm 343$  mL H<sub>2</sub>/L, and hydrogen yield increased from  
36  $0.45 \pm 0.1$  to  $0.94 \pm 0.34$  mol H<sub>2</sub>/mol glucose, compared to the control culture. Carbon  
37 flux was also affected and the composition of the final by-products changed. Lactate (41  
38 mM) was the main metabolite in the control cultures, whereas ethanol (56.2 mM) and  
39 acetate (41.2 mM) were the main metabolites in the cultures with 2 mM indole. We  
40 conclude that the supplementation of *E. coli* cultures with exogenous indole is a simple  
41 and novel strategy to improve the production of hydrogen as well as other metabolites  
42 such as ethanol used as biofuels.

43

44 **Keywords:** bacteriostatic, *Escherichia coli*, hydrogen, ethanol, indole, ionophore,  
45 lactate.

46

47

## 48 **Introduction**

49 Under anaerobic conditions *E. coli* naturally produces a mixture of organic acids,  
50 ethanol and hydrogen [1] (Fig. 1). Biotechnology exploits this to produce succinate [2]  
51 and lactate [3], and recently ethanol and hydrogen as potential biofuels [4-8]. Molecular  
52 hydrogen (H<sub>2</sub>) is an attractive energy carrier because it has high energy density and is  
53 environmentally friendly, since its combustion produces only water. Hydrogen is also  
54 an important feedstock for chemical, food, pharmaceutical and some other industries  
55 [9]. Among various production processes, fermentative hydrogen production has the  
56 advantages of high production rate and simple operation [7-10]. Hydrogen production  
57 by dark fermentation is a promising method since it has a higher production rate, it does  
58 not need light and it utilizes a wide range of carbon sources such as glucose and other  
59 simple substrates [6, 8, 11, 12]. *E. coli* is currently used as a model for production  
60 studies of hydrogen, ethanol and other metabolites, since its anaerobic metabolism is  
61 well documented and genetic manipulations are possible. Both genetic modifications  
62 and modified fermentation strategies have been implemented to improve the production  
63 processes [7, 13, 14]. Since the production of metabolites normally correlates with cell  
64 growth, an important fraction of the substrate is inevitably channeled toward unwanted  
65 biomass rather than the desired product. Hence an attractive strategy to improve  
66 metabolite production would be to induce the producer cells to enter a quiescent state,  
67 where they are metabolically active but not growing or dividing.

68

69 Quiescence has been defined as a reversible absence of cell proliferation [15]. In  
70 contrast to multi-cellular organisms where quiescence is the most common cellular  
71 state, bacteria exist mainly in a proliferative state. Quiescent *E. coli* cells have been  
72 obtained through the controlled overexpression of a small RNA called Rcd (regulator of

73 cell division), a ColE1-encoded regulatory transcript [16]. To achieve complete  
74 cessation of growth it was necessary to over-express Rcd in an *hns205* mutant of *E. coli*.  
75 More recently it has been reported that the addition of approx. 3mM indole to the  
76 culture supernatant induces a quiescence state in *E. coli hns205* under aerobic  
77 conditions [17]. The bacteriostatic effect of indole has been attributed to its action as a  
78 proton ionophore, affecting the membrane polarity and respiratory ATP generation [18-  
79 20]. Studies of the effect of indole on the physiology of *E. coli* have been performed  
80 under aerobic conditions [18, 21]. However to our knowledge, there are no reports on  
81 the effect of indole on the anaerobic metabolism of *E. coli*. Thus the goal of this work  
82 was to evaluate the effect of exogenous indole on the anaerobic metabolism of *E. coli*  
83 with a particular focus on its effect on the production of hydrogen.

84

## 85 **Materials and methods**

### 86 *Strain and culture media*

87 *E. coli* WDHL is a hydrogen over-producer strain obtained by the deletion of *hycA* and  
88 *lacI* in *E. coli* W3110. A complete description of WDHL can be found elsewhere [11].  
89 The strain was stored routinely on Luria Bertani (LB) agar plates at 4°C. The hydrogen  
90 production medium (HP) contains per liter: K<sub>2</sub>HPO<sub>4</sub> 0.125 g, Na<sub>2</sub>HPO<sub>4</sub> 11.86 g,  
91 KH<sub>2</sub>PO<sub>4</sub> 4.5 g, MgSO<sub>4</sub> 1 g, tryptone (Difco) 1 g, yeast extract (Difco) 1 g, glucose 15 g  
92 and 1 mL of trace elements solution [11]. The initial pH of the HP medium was 7.2.

93

### 94 *Evaluation of the effect of indole on E. coli metabolism*

95 To evaluate the effect of indole on hydrogen production, pre-inocula were grown  
96 aerobically overnight in LB medium at 37°C, shaken at 150 rpm. Anaerobic

97 experiments were performed in 120 mL serological bottles containing 110 mL of HP  
98 medium. Bottles were closed with 20 mm butyl septum and an aluminum crimp seal  
99 (Supelco) using an E-Z crimper (Wheaton). The cultures were started at pH 7.2 with an  
100 initial OD<sub>600</sub> of 0.3 and incubated at 37°C in a water bath shaking at 175 rpm.  
101 Concentrations of indole (Sigma) between 0.5 to 8 mM were assessed, for this the  
102 required volume of a 2 M indole stock was added to the serological bottles using a  
103 sterile syringe. Control cultures were grown in HP medium plus 400 µL of ethanol  
104 (ethanol was used as the solvent for the stock indole solution). Samples of 1 mL taken  
105 at various times during fermentation were centrifuged at 13,000 rpm and stored at -20°C  
106 until analyzed as described below.

107

#### 108 *Analytical methods*

109 Hydrogen produced was measured by liquid displacement using 2 N NaOH in an  
110 inverted burette and a gas chromatograph with a thermal conductivity detector (GC-  
111 TCD, Agilent Technologies) as described previously [22]. Optical cell density was  
112 measured at 600 nm (OD<sub>600</sub>) in a GeneQuant 1300 (G&E) spectrophotometer. Indole  
113 was determined by the Kovacs spectrophotometric method at 540 nm using the *p*-  
114 dimethyl amino-benzaldehyde reagent [23]. The concentrations of glucose and the  
115 metabolites succinate, lactate, acetate, formate and ethanol were analyzed by High  
116 Performance Liquid Chromatography coupled to a Refraction Index Detector (HPLC,  
117 Infinity LC 1220, Agilent Technologies, Santa Clara CA, USA), with a column  
118 Phenomenex Rezex ROA (Phenomenex, Torrance, CA, USA) at 60°C, and using  
119 0.0025 M H<sub>2</sub>SO<sub>4</sub> as mobile phase at 0.41 mL/min.

120

#### 121 *Statistics.*

122 The data are presented as the mean  $\pm$  standard deviation. We used Student's t-test  
123 unpaired to compare each treatment with the control group, and  $p$  values  $< 0.05$  were  
124 considered significant. The experiments were carried out at least in triplicate.

## 125 **Results**

126 Before investigating the effect of exogenous indole on *E. coli* WDHL, endogenous  
127 indole production by this strain was measured in HP medium under anaerobic  
128 conditions. Indole was assayed in culture supernatants using the Kovacs method, and  
129 was found to be below 0.18 mM. In subsequent experiments, cultures were  
130 supplemented with indole in the range 0.5 to 8 mM at the time of inoculation. As shown  
131 in Fig. 2, cultures to which 1 mM indole was added behaved indistinguishably from the  
132 control cultures with a specific growth rate of  $0.35 \text{ h}^{-1}$ , whereas the cultures with 2 and  
133 4 mM indole showed slightly reduced specific growth rates of  $0.29$  and  $0.26 \text{ h}^{-1}$ ,  
134 respectively. Cultures with 8 mM indole did not grow.

135 The effect of the indole supplementation on the maximum optical density and hydrogen  
136 production by each culture is shown in Fig. 3. The inhibitory effect of indole on the  
137 growth of *E. coli* WDHL is evident and the maximum  $\text{OD}_{600}$  decreased from  $3.13 \pm 0.54$   
138 to  $0.18 \pm 0.04$  as external indole was increased from 0 to 8 mM (Fig. 3A). Indole up to 4  
139 mM increased hydrogen production ( $1,032 \pm 124 \text{ mLH}_2/\text{L}$  at 4 mM, compared to  
140  $650 \pm 115 \text{ mLH}_2/\text{L}$  in the control). However, no hydrogen production was observed in  
141 the culture with 8 mM indole (Fig. 3B). Both hydrogen production per unit biomass and  
142 hydrogen per unit substrate (Figs. 3C and 3D, respectively) showed the same response  
143 to indole addition. When 4 mM indole was added, the yields increased from  $23.8 \pm 7.9$  to  
144  $66.8 \pm 14.5 \text{ mLH}_2/\text{OD}$  and from  $0.45 \pm 0.1$  to  $0.94 \pm 0.3 \text{ mol H}_2/\text{mol glucose}$ , respectively.

145 Metabolite analysis revealed that succinate was found in the cultures with 1 mM indole  
146 or less, with a maximum concentration of  $6.7\pm 0.5$  mM in the control culture. Lactate  
147 was the main metabolite in the cultures with 1 mM indole and below, reaching a  
148 maximum of  $57.1\pm 3.3$  mM in the cultures with 0.5 mM indole, whereas in the cultures  
149 with 2 and 4 mM indole, ethanol and acetate were the main metabolites, reaching  
150  $56.2\pm 22.7$  and  $41.2\pm 6.2$  mM respectively in the culture supplemented with 2 mM  
151 indole. Formate was not detected in any of the cultures (Fig. 4).

152

## 153 **Discussion**

154 We have explored the effect of indole on the growth and the production of hydrogen  
155 and ethanol by *E. coli* in anaerobic culture, and have found that productivity can be  
156 increased significantly by the addition of 2 to 4 mM to the growth medium. Previous  
157 studies of indole signaling [24-28] and the use of indole to induce entry of *E. coli* into a  
158 quiescent state [17] have been carried out under aerobic culture conditions. Indeed, very  
159 little information is available on the effect of indole in the anaerobic metabolism of *E.*  
160 *coli*. It has been reported that when *E. coli* is grown anaerobically in medium containing  
161 tryptophan and sodium nitrate, it produces toxic 3-nitrosoindole and its derivatives, as a  
162 result of the condensation of nitrite with indole [24]. However, in the experiments  
163 reported here, nitrate or ammonium salts were not used as nitrogen source because they  
164 inhibit the hydrogen production [25, 26]. Since only organic nitrogen sources like yeast  
165 extract and tryptone were used, nitroindole compounds were not produced.

166 Typical indole concentrations in aerobic stationary phase *E. coli* cultures are 0.5-1 mM  
167 [21, 27]. Even our unsupplemented control cultures contained a low concentration of  
168 indole (0.18 mM). This was almost certainly generated from tryptophan, originating

169 from the yeast extract and tryptone in the HP medium. It has been reported that in  
170 aerobic culture indole production up to 5 mM is a direct reflection of the amount of free  
171 tryptophan in the culture medium [28].

172 Indole-induced quiescence in aerobic *E. coli* cultures has been used to improve the  
173 expression of heterologous proteins [17]. The authors reported that the addition of 0-3  
174 mM indole had little effect on the growth of wild-type *E. coli*, while 4 or 5 mM indole  
175 caused significant growth inhibition. Under anaerobic conditions we found that 1-2 mM  
176 indole had little effect while 4 mM indole caused a reduction in both the specific growth  
177 rate (25% of wild-type) and the maximum optical density (44.4% of wild-type). The  
178 growth-inhibited cultures showed an increased yield of both hydrogen and ethanol.

179 In anaerobic glycolysis, *E. coli* accumulates mainly lactate, but succinate, formate,  
180 acetate and a low yield of ethanol are also produced. In the case of glucose fermentation  
181 by strain WDHL, formate was not detected as result of the *hycA* deletion, indicating that  
182 the WDHL strain is very efficient in the conversion of formate into hydrogen and CO<sub>2</sub>.  
183 The *hycA* gene codes for the negative regulator of the formate regulon, which contains  
184 the formate hydrogen lyase complex (FHL), therefore strains with defective *hycA* gene  
185 overproduce hydrogen [29]. The theoretical maximum yield of hydrogen is 2 mol  
186 H<sub>2</sub>/mol glucose, if there are no other cellular requirements and all pyruvate is converted  
187 into formate and acetyl-CoA. However, glucose is also needed for building biomass,  
188 anabolism and ATP production for maintaining energy. In the case of strain WDHL, a  
189 typical value is 0.3 mol H<sub>2</sub>/mol glucose due to catabolic repression by glucose and the  
190 conversion of pyruvate into lactate by lactate dehydrogenase (LDH) to recycle the  
191 NADH cofactor [30]. However, here we attained up to 0.94±0.3 mol H<sub>2</sub>/mol glucose in  
192 the cultures with 4 mM indole. Succinate was a minor by-product with 6.7±0.5 mM in  
193 the control cultures. Succinate is generated by the fumarate reductase (FRD), an enzyme

194 with high similarity to succinate dehydrogenase, but FRD uses FADH instead of  
195 NADH. FRD also has important differences in both regulation and catalytic activity  
196 [31]. As expected, lactate was the main by-product in the control culture and at lower  
197 indole concentrations (e.g.  $57.1 \pm 3.3$  mM in the cultures supplemented with 0.5 mM  
198 indole) because the recycling of NADH by the LDH is the main route to continue the  
199 assimilation of glucose [32]. In the cultures supplemented with 2 or 4 mM indole, the  
200 hydrogen yield increased to  $0.94 \pm 0.34$  mol H<sub>2</sub>/mol glucose, which strongly suggests  
201 that a greater proportion of pyruvate was transformed by the pyruvate formate lyase  
202 (PFL). This is consistent with the accumulation of C<sub>2</sub> by-products (ethanol and acetate)  
203 instead C<sub>3</sub> by-products (lactate) as shown in Fig. 4. Consistent with the results reported  
204 here, Chen *et al.* [17] observed that at higher indole concentrations (up to 2.5 mM in  
205 aerobic cultures of *E. coli*), the specific yield of GFP increased throughout their  
206 experiment. The maximum value (three-fold greater than the no-indole control) was  
207 obtained for the culture that entered quiescence in response to treatment with 2.5 mM  
208 indole.

209 Acetate is typically generated as by-product in the anaerobic metabolism of *E. coli*. It  
210 can be produced from acetyl CoA by acetyl CoA synthetase (ACS) as well by the  
211 phosphotransacetylase followed by acetate kinase A. Acetate can be also produced by  
212 pyruvate oxidase, and a typical inhibitory concentration for *E. coli* is 5 g/L (83mM)  
213 [13]. However, inhibitory acetate concentrations were not reached in in this study where  
214 the maximum concentration was  $41.2 \pm 6.2$  mM. Ethanol is produced in *E. coli* by the  
215 alcohol dehydrogenase (ADH). In the control cultures ethanol production was 6.5 mM  
216 with a yield of 0.11 mol ethanol/mol glucose. Ethanol accumulation increased  
217 substantially in the cultures treated with indole even though WDHL is not an  
218 alcohologenic strain. In cultures supplemented with 2 mM indole, ethanol production

219 increased approximately ten-fold (56.2 mM with a yield of 1.02 mol ethanol/mol  
220 glucose). Thus indole supplementation could be a useful novel strategy to increase  
221 ethanol production in processes designed for this purpose [33-35].

222

## 223 **Conclusions**

224 Our results indicate that indole has a bacteriostatic activity on *E. coli* WDHL in  
225 anaerobic culture, as well as a substantial effect on hydrogen production due to  
226 increased pyruvate flux through PFL rather than LDH. The bacteriostatic effect and  
227 metabolic switching are evident at 2 mM indole, however, the mechanisms  
228 underpinning this metabolic change are not clear. They could be related to changes in  
229 H<sup>+</sup> flux through the cytoplasmic membrane mediated by the proton ionophore effect of  
230 indole [19] or to regulatory changes in the central carbon metabolism [1, 36]. Whatever  
231 the mechanism we conclude that addition of exogenous indole into the anaerobic *E. coli*  
232 cultures can be used as a strategy to improve the production of hydrogen and other  
233 metabolites such as ethanol.

234

## 235 **List of abbreviations**

236	ACS	Acetyl CoA synthetase
237	hycA	Negative transcriptional regulator of the formate regulon
238	lacI	Lac I repressor
239	LDH	Lactate dehydrogenase
240	NADH	Nicotinamide adenine dinucleotide reduced

241 FADH<sub>2</sub> Flavin adenine dinucleotide reduced

242 FRD Fumarate reductase

243 PFL Pyruvate formate lyase

244 TnaA Tryptophanase

245

### 246 **Competing interests**

247 No competing financial interests exist.

248

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252

### 253 **Authors' contributions**

254 All authors contributed in the development of this manuscript.

255

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259

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379

380 **Figure Caption**

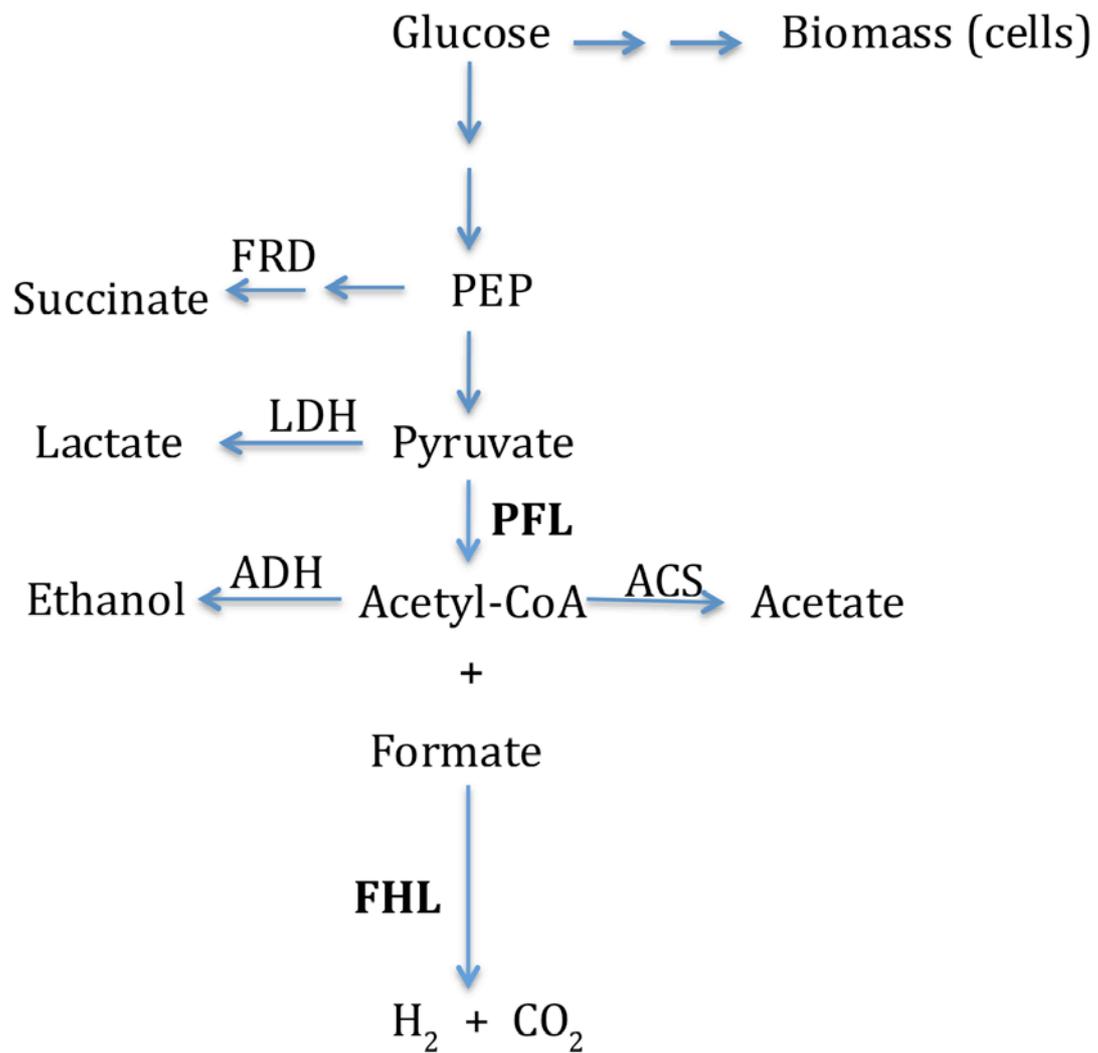
381 Fig. 1. Carbon flux in *E. coli* during anaerobic metabolism. The key enzymes to  
382 produce hydrogen are shown in bold. FRD: Fumarate reductase, LDH: Lactate  
383 dehydrogenase, ADH: Alcohol dehydrogenase, PFL: Pyruvate formate lyase, FHL:  
384 Formate hydrogen lyase, ACS: Acetyl CoA synthetase.

385 Fig. 2. The growth kinetics of cultures of *E. coli* WDHL exposed to indole (0-8 mM)..  
386 Data are plotted as the average (n=3) and the bars correspond to the standard deviation.

387 Fig. 3. Effect of the indole concentration on the cell-growth and hydrogen production  
388 measured at 48h of culture. Maximum OD<sub>600</sub> (A), hydrogen production (B), hydrogen  
389 yield by biomass (C) and hydrogen yield by substrate (D). Control corresponds to the  
390 cultures without addition of indole. Data are presented as mean ± SD. \* indicates  
391 significant difference compared with the control cultures ( $p < 0.05$ ).

392 Fig. 4. Final concentration of the metabolites in the cultures supplemented with  
393 different initial concentration of indole measured at 48h of culture. Control corresponds  
394 to the cultures without addition of indole. Data are presented as mean ± SD of three  
395 independent experiments (n=3). \* indicates significant difference compared with the  
396 control cultures ( $p < 0.05$ ).

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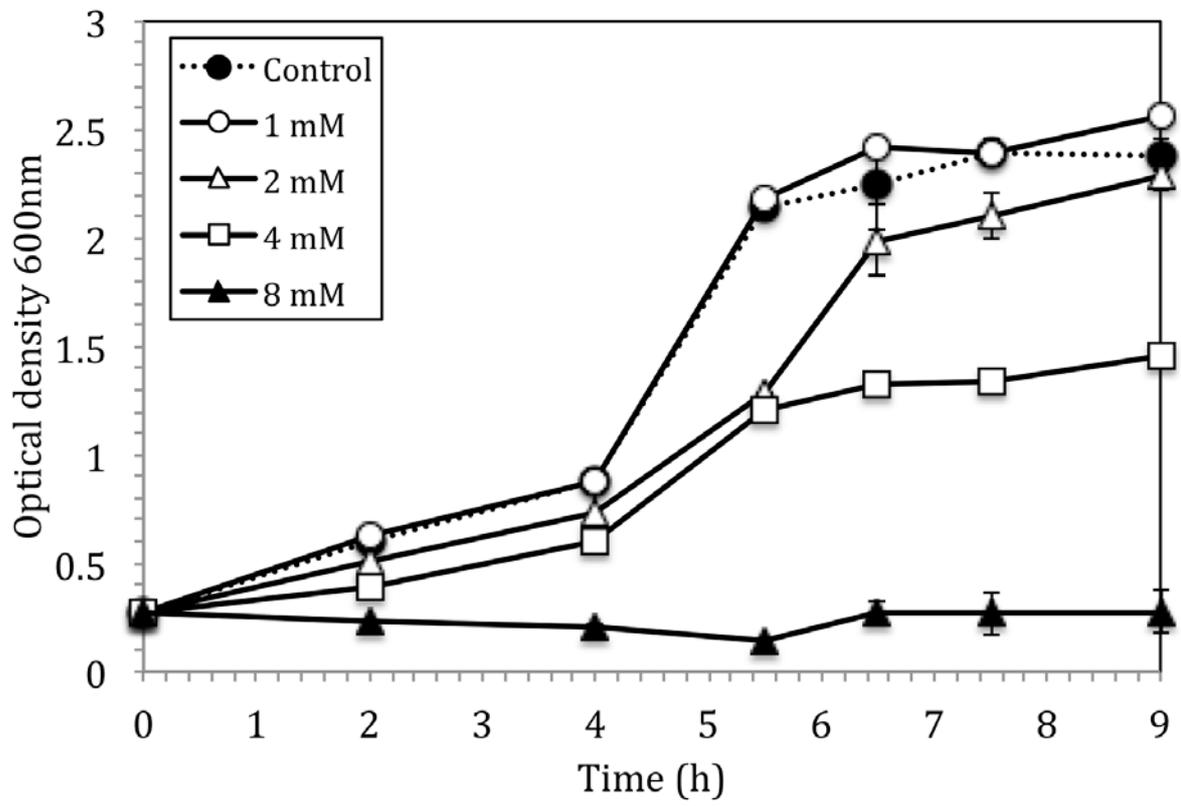
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Fig. 1

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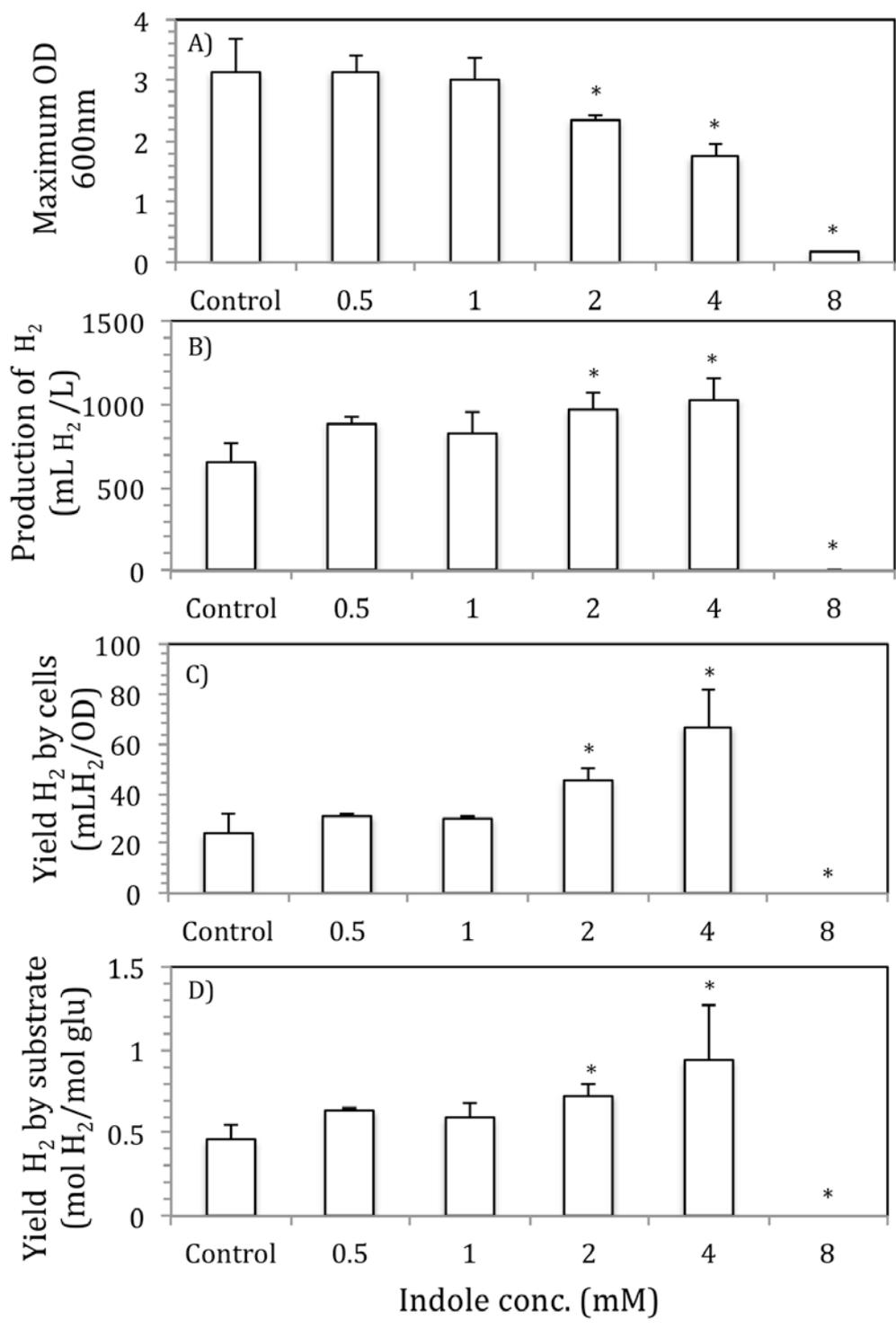
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Fig. 2

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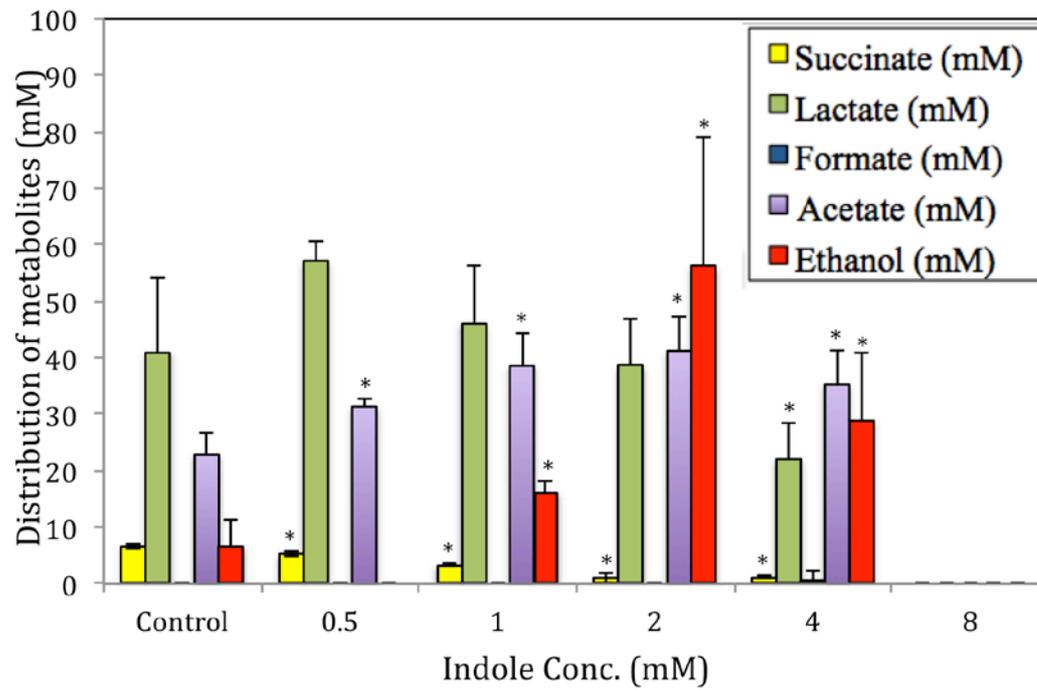
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Fig. 3

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Fig. 4

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