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

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Abstract

Indole is a bicyclic signaling molecule with effects on both eukaryotic and prokaryotic cells. The majority of studies of indole action have been performed with bacteria cultured under aerobic conditions and little information is available about its effects under anaerobic conditions. Here the effect of the indole on anaerobic metabolism of *Escherichia coli* WDHL was studied. Indole in the range 0.5 to 8 mM was added to the culture medium and cell growth, hydrogen and metabolite production were compared to cultures lacking indole. Results showed that while 8 mM indole abolished growth completely, 4 mM indole had a partial bacteriostatic effect and the maximum optical density of the culture decreased by 44% compared to the control cultures. In addition, 4 mM indole had an important effect on anaerobic metabolism. Hydrogen production increased from 650±115 to 1,137±343 mL H_2/L, and hydrogen yield increased from 0.45±0.1 to 0.94±0.34 mol H_2/mol glucose, compared to the control culture. Carbon flux was also affected and the composition of the final by-products changed. Lactate (41 mM) was the main metabolite in the control cultures, whereas ethanol (56.2 mM) and acetate (41.2 mM) were the main metabolites in the cultures with 2 mM indole. We conclude that the supplementation of *E. coli* cultures with exogenous indole is a simple and novel strategy to improve the production of hydrogen as well as other metabolites such as ethanol used as biofuels.

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

Introduction

Under anaerobic conditions *E. coli* naturally produces a mixture of organic acids, ethanol and hydrogen [1] (Fig. 1). Biotechnology exploits this to produce succinate [2] and lactate [3], and recently ethanol and hydrogen as potential biofuels [4-8]. Molecular hydrogen (H_2) is an attractive energy carrier because it has high energy density and is environmentally friendly, since its combustion produces only water. Hydrogen is also an important feedstock for chemical, food, pharmaceutical and some other industries [9]. Among various production processes, fermentative hydrogen production has the advantages of high production rate and simple operation [7-10]. Hydrogen production by dark fermentation is a promising method since it has a higher production rate, it does not need light and it utilizes a wide range of carbon sources such as glucose and other simple substrates [6, 8, 11, 12]. *E. coli* is currently used as a model for production studies of hydrogen, ethanol and other metabolites, since its anaerobic metabolism is well documented and genetic manipulations are possible. Both genetic modifications and modified fermentation strategies have been implemented to improve the production processes [7, 13, 14]. Since the production of metabolites normally correlates with cell growth, an important fraction of the substrate is inevitably channeled toward unwanted biomass rather than the desired product. Hence an attractive strategy to improve metabolite production would be to induce the producer cells to enter a quiescent state, where they are metabolically active but not growing or dividing.

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

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

Materials and methods

Strain and culture media

E. coli WDHL is a hydrogen over-producer strain obtained by the deletion of *hycA* and *lacI* in *E. coli* W3110. A complete description of WDHL can be found elsewhere [11]. The strain was stored routinely on Luria Bertani (LB) agar plates at 4°C. The hydrogen production medium (HP) contains per liter: K_2HPO_4 0.125 g, Na_2HPO_4 11.86 g, KH_2PO_4 4.5 g, $MgSO_4$ 1 g, tryptone (Difco) 1 g, yeast extract (Difco) 1 g, glucose 15 g and 1 mL of trace elements solution [11]. The initial pH of the HP medium was 7.2.

Evaluation of the effect of indole on E. coli metabolism

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

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

Analytical methods

Hydrogen produced was measured by liquid displacement using 2 N NaOH in an inverted burette and a gas chromatograph with a thermal conductivity detector (GC-TCD, Agilent Technologies) as described previously [22]. Optical cell density was measured at 600 nm (OD_{600}) in a GeneQuant 1300 (G&E) spectrophotometer. Indole was determined by the Kovacs spectrophotometric method at 540 nm using the *p*-dimethyl amino-benzaldehyde reagent [23]. The concentrations of glucose and the metabolites succinate, lactate, acetate, formate and ethanol were analyzed by High Performance Liquid Chromatography coupled to a Refraction Index Detector (HPLC, Infinity LC 1220, Agilent Technologies, Santa Clara CA, USA), with a column Phenomenex Rezex ROA (Phenomenex, Torrance, CA, USA) at 60°C, and using 0.0025 M H_2SO_4 as mobile phase at 0.41 mL/min.

Statistics.

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

Results

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

The effect of the indole supplementation on the maximum optical density and hydrogen production by each culture is shown in Fig. 3. The inhibitory effect of indole on the growth of *E. coli* WDHL is evident and the maximum OD_{600} decreased from 3.13±0.54 to 0.18±0.04 as external indole was increased from 0 to 8 mM (Fig. 3A). Indole up to 4 mM increased hydrogen production (1,032±124 mLH_2/L at 4 mM, compared to 650±115 mLH_2/L in the control). However, no hydrogen production was observed in the culture with 8 mM indole (Fig. 3B). Both hydrogen production per unit biomass and hydrogen per unit substrate (Figs. 3C and 3D, respectively) showed the same response to indole addition. When 4 mM indole was added, the yields increased from 23.8±7.9 to 66.8±14.5 mLH_2/OD and from 0.45±0.1 to 0.94±0.3 mol H_2/mol glucose, respectively.

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

Discussion

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

Typical indole concentrations in aerobic stationary phase *E. coli* cultures are 0.5-1 mM [21, 27]. Even our unsupplemented control cultures contained a low concentration of 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_2.
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_2/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_2/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_2/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

with high similarity to succinate dehydrogenase, but FRD uses FADH instead of NADH. FRD also has important differences in both regulation and catalytic activity [31]. As expected, lactate was the main by-product in the control culture and at lower indole concentrations (e.g. 57.1±3.3 mM in the cultures supplemented with 0.5 mM indole) because the recycling of NADH by the LDH is the main route to continue the assimilation of glucose [32]. In the cultures supplemented with 2 or 4 mM indole, the hydrogen yield increased to 0.94±0.34 mol H_2/mol glucose, which strongly suggests that a greater proportion of pyruvate was transformed by the pyruvate formate lyase (PFL). This is consistent with the accumulation of C2 by-products (ethanol and acetate) instead C3 by-products (lactate) as shown in Fig. 4. Consistent with the results reported here, Chen *et al.* [17] observed that at higher indole concentrations (up to 2.5 mM in aerobic cultures of *E. coli*), the specific yield of GFP increased throughout their experiment. The maximum value (three-fold greater than the no-indole control) was obtained for the culture that entered quiescence in response to treatment with 2.5 mM indole.

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

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

Conclusions

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

List of abbreviations

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

$FADH_2$	Flavin adenine dinucleotide reduced
FRD	Fumarate reductase
PFL	Pyruvate formate lyase
TnaA	Tryptophanase

Competing interests

No competing financial interests exist.

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Authors' contributions

All authors contributed in the development of this manuscript.

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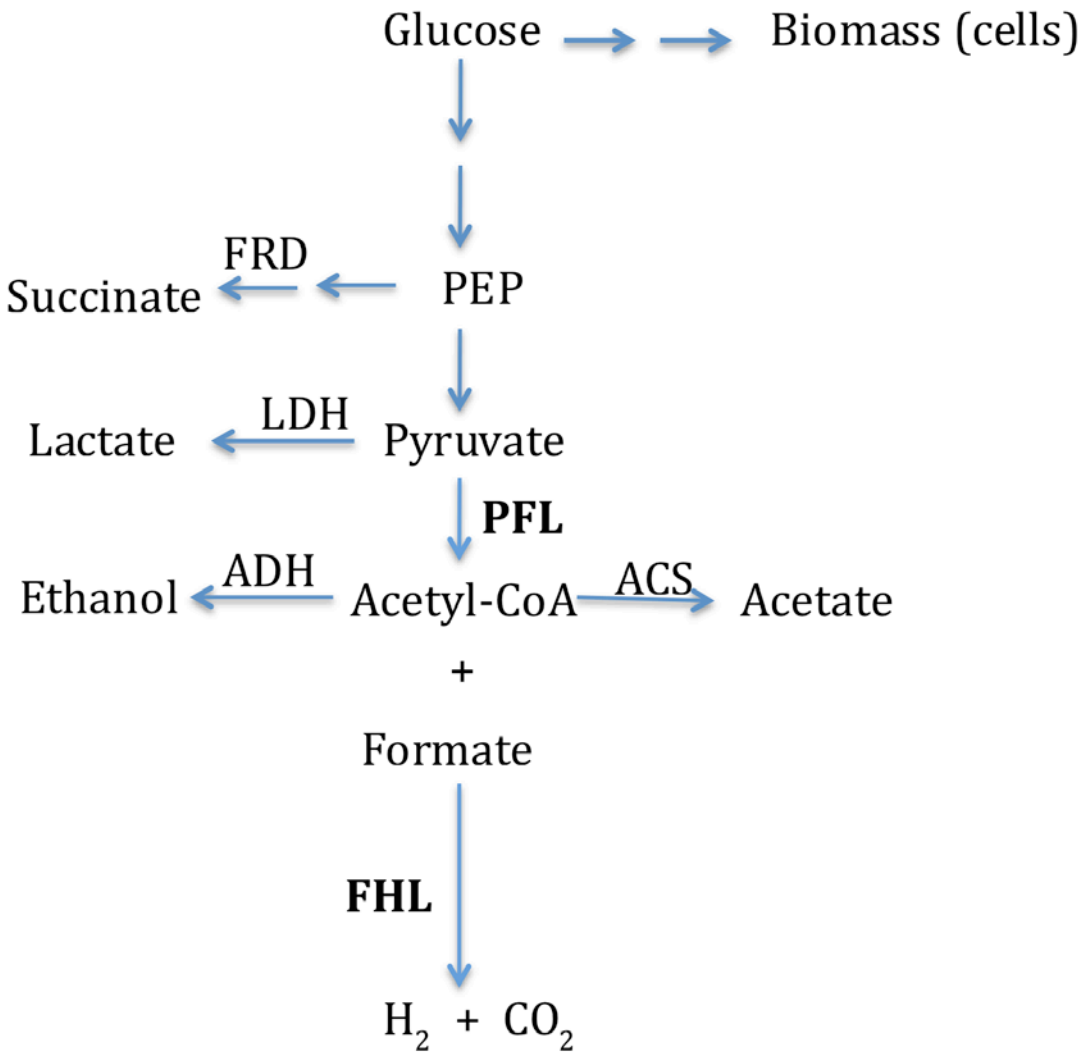
Figure Caption

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

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

Fig. 3. Effect of the indole concentration on the cell-growth and hydrogen production measured at 48h of culture. Maximum OD_{600} (A), hydrogen production (B), hydrogen yield by biomass (C) and hydrogen yield by substrate (D). Control corresponds to the cultures without addition of indole. Data are presented as mean ± SD. ∗ indicates significant difference compared with the control cultures ($p < 0.05$).

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



398

399 Fig. 1

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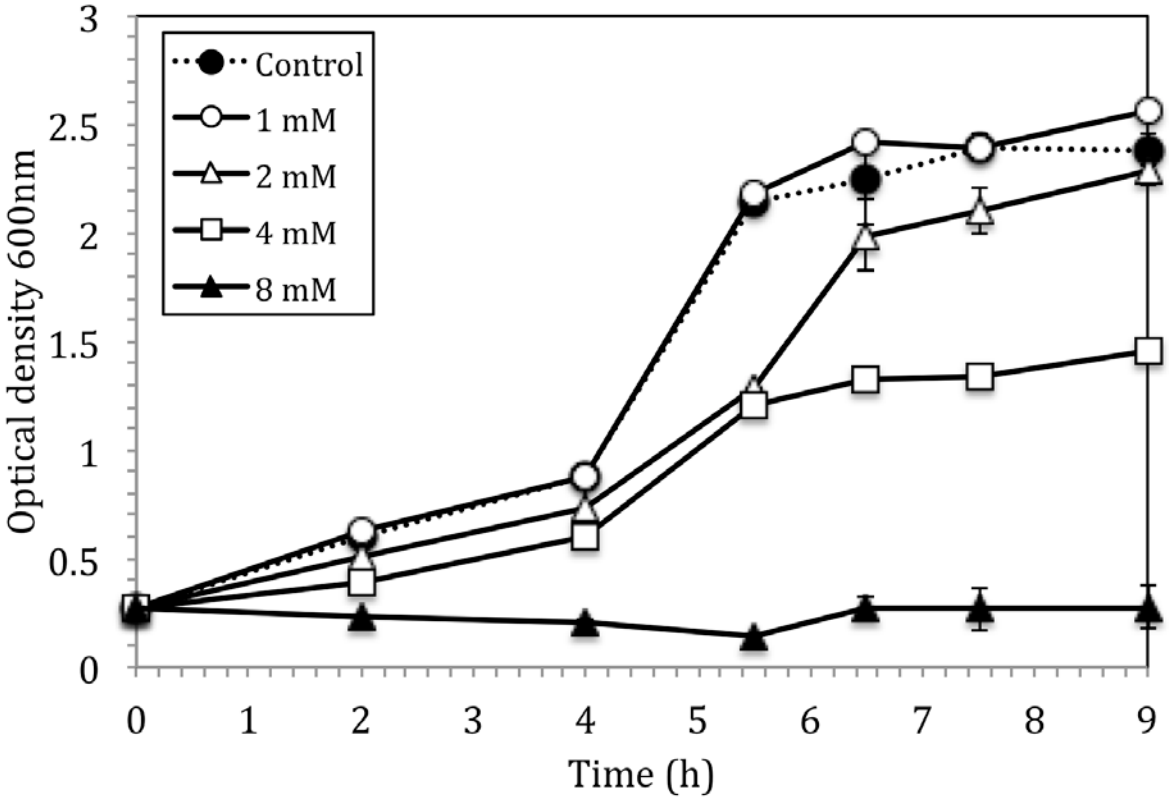

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405 Fig. 2

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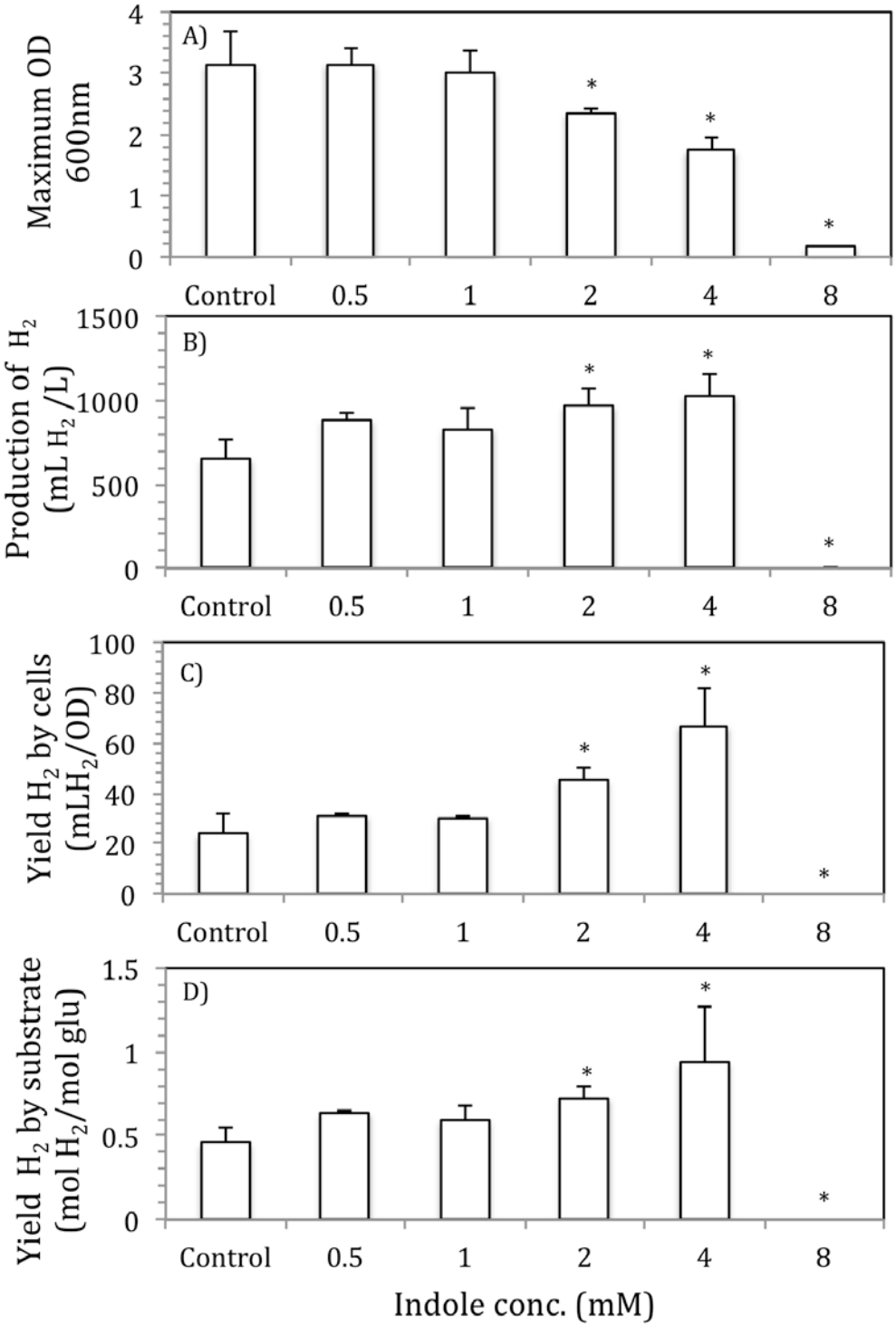
A)
Maximum OD 600nm
4
3
2
1
0
Control
0.5
1
2
4
8
B)
Production of H_2 (mL H_2 /L)
1500
1000
500
0
C)
Yield H_2 by cells (mLH_2/OD)
100
80
60
40
20
0
D)
Yield H_2 by substrate (mol H_2/mol glu)
1.5
1
0.5
0
Indole conc. (mM)

Fig. 3

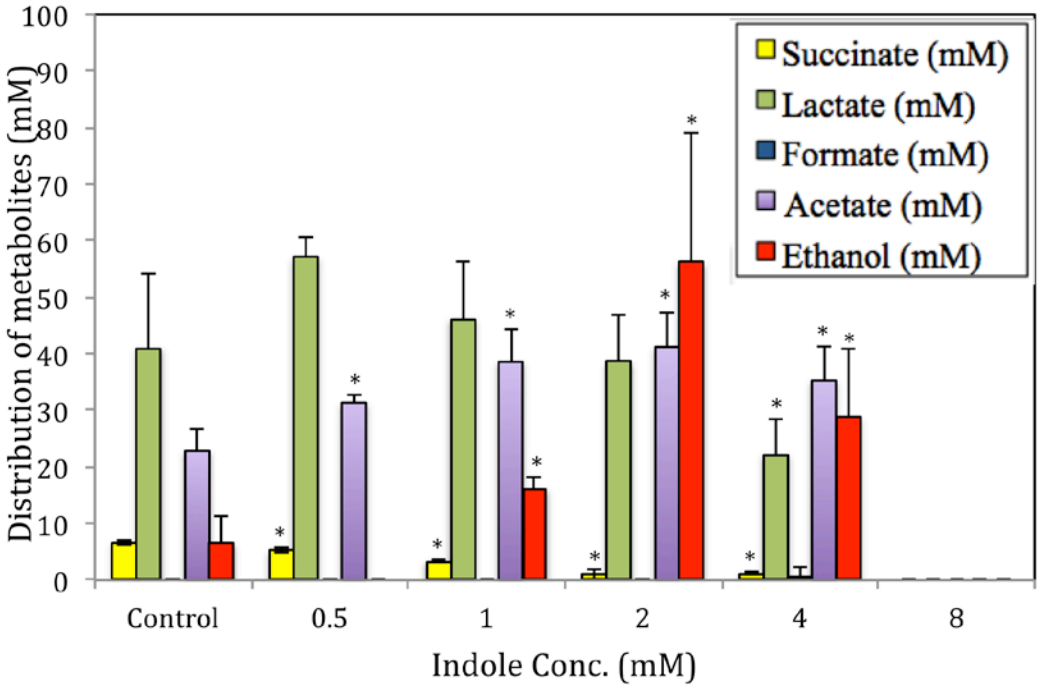
Succinate (mM)
Lactate (mM)
Formate (mM)
Acetate (mM)
Ethanol (mM)
Distribution of metabolites (mM)
100
90
80
70
60
50
40
30
20
10
0
Control
0.5
1
2
4
8
Indole Conc. (mM)

Fig. 4