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# *Escherichia coli* hydrogen gas production from glycerol: Effects of external formate

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ABSTRACT

Hydrogen gas ($H_2$) production was studied in bacteria cultured at different pH conditions, and on different carbon substrates. $H_2$ production by *Escherichia coli* was first studied during glycerol fermentation when external formate (10 mM) was supplemented. Changes in $H_2$ production rate ($V_{H2}$) were determined when in the assays glycerol and/or formate were supplemented at pH 7.5 and pH 6.5, using single and double mutants coding large subunits of different hydrogenases (Hyd-1, Hyd-2 and Hyd-3) as well as *selC* (coding for formate dehydrogenases), *hyaB hybC* (coding for large subunits of Hyd-1 and Hyd-2, respectively) *selC* or *hyaB hybC hycE* (coding for large subunit of Hyd-3) triple mutants at both pHs. The results point out that Hyd-3 becomes mainly responsible for $H_2$ production by *E. coli* during glycerol fermentation when external formate is added; Hyd-4 can also contribute to $H_2$ production. Besides, in the glycerol supplemented assays, three hydrogenases can work in $H_2$ producing mode and only deletion of three of them decreases the production of $H_2$ which might be due to disturbance of $H_2$ cycling. This is of significance in application of different carbon sources in renewable energy production technology using bacteria.



## 1. Introduction

The current state with reducing fossil fuels has led to find out alternative and renewable energy sources. One of these energy sources is biodiesel which is produced mostly by algae without a big impact on carbon dioxide emission [1,2]. The other energy source can be hydrogen gas ($H_2$) which is ecologically clean, effective and renewable one; $H_2$ can be produced from glycerol during microbe-mediated biological conversion [3]. Glycerol is a main waste (about 10% w/w) of biodiesel production; it has increased amount during the last years [4]. Moreover, glycerol becomes a cheap substrate, since this carbon source gradually changes from a chemical commodity to a chemical waste. Therefore, $H_2$ production from agricultural, industrial, kitchen, water and other kinds of wastes is of great interest for finding out other substrates or cheap sources for energy production which are available in nature and environment [5].

Gonzalez et al. [6] have established absolutely novel phenomenon that, in addition to different sugars, glycerol can be fermented by bacteria, especially *Escherichia coli*, at slightly acidic pH. Interestingly, among the end products of mixed-acid fermentation of glycerol $H_2$ is detected not only at acidic but also at slightly alkaline pH [7,8]. However, no clear data are available about glycerol metabolic pathways and fermentation end products by *E. coli*, their dependence on external pH and different chemicals during co-fermentation of glycerol with other carbon substrates and on other external factors. Nowadays already several studies are ongoing using different mixtures of carbon sources like sugars (glucose, xylose, mannitol etc.) and glycerol to enhance $H_2$ production by increased bacterial biomass and utilization of cheap carbon sources which are present in the mixtures [9–12]. Interestingly, formate is one of the end products of glycerol mixed-acid fermentation in *E. coli* and can be oxidized to $H_2$ and $CO_2$ or exported into external medium. Recently, it has been shown that external formate can pass into the cell and affect $H_2$ production [13]. This depends on pH and other factors and might be important for regulation of $H_2$ production.

It is well-known that $H_2$ is evolved by *E. coli* via special enzymes named hydrogenases (Hyd), which catalyze the reaction of

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$H_2 \leftrightarrow 2H^+ + 2e^-$ [14–16]. The latter is simple redox reaction but responsible enzymes as well as mechanisms of activity and regulation are complex. The distinguished properties of Hyd enzymes are their multiplicity and reversibility [17]. *E. coli* has the capacity to encode four membrane-associated [Ni–Fe]-hydrogenases [17]. Hyd-1 and Hyd-2 are reversible Hyd enzymes: during glycerol or glucose fermentation they operate in $H_2$ evolving or uptake mode, respectively [17–19]. Hyd-3 and Hyd-4 are $H_2$ producing Hyd enzymes under glucose fermentation but these enzymes both are able to work in reverse mode during glycerol fermentation [17–21]. The mode or direction of Hyd enzymes operation depends not only on fermentation substrate but also on external pH and other factors [17–21].

In addition, Hyd enzymes of *E. coli* have complex mechanisms for genetics, maturation and regulation. Hyd-1 is encoded by the *hya* operon; it consists of three different subunits [22]. The *hya* gene is expressed under anaerobic conditions at acidic pH [22] and by formate [24] though *E. coli* hasn't any requirement of Hyd-1 for growth under anaerobic conditions [23]. Hyd-2 is encoded by the *hyb* operon; it consists of four different subunits three of which are similar to Hyd-1 subunits [25]. Unlike *hya*, the *hyb* operon is maximally expressed at alkaline pH [23] in the cultures grown on $H_2$ and fumarate. This is in accordance with pH optimum of this enzyme [26]. Moreover, Hyd-2 has activity in more reducing environment [27]; however it's activity is absent under aerobic conditions [28]. The role of Hyd-1 in bacterial physiology is yet unclear, but it has been propounded to shuttle electrons from formate to fumarate during reduction [29] or to contribute electrons to the quinones pool when oxidizing of $H_2$ [30]. On the other hand, Hyd-2 may reversibly oxidize $H_2$ *in vitro* [28]. But this Hyd enzyme has potential to function as a "valve" to release excess reducing equivalents ($H^+ + e^-$) in the form of $H_2$. Therefore, Hyd-2 might have a role in physiology under anaerobic conditions at alkaline pH. These findings on Hyd-1 and Hyd-2 might have some input in investigating the exact functions of these enzymes which can be applied in $H_2$ production biotechnology. However, their functions and role are still not defined clearly.

Two other Hyd enzymes - Hyd-3 and Hyd-4 are encoded by the *hyc* and *hyf* operons, respectively [31–33]. Hyd-3 consists of different large and small subunits and with formate dehydrogenase (FDH-H) forms the formate hydrogen lyase (FHL-1) complex, producing $H_2$ preferably at acidic pH [20]. On the other hand, Hyd-3 has been shown to work in reverse, $H_2$ oxidizing mode under certain conditions [34]. Hyd-4 also consists of different large and small subunits and together with FDH-H is suggested to form the second FHL-2 complex which must have functional HycB protein, a small subunit of Hyd-3, to evolve $H_2$ at slightly alkaline pH [20,32]. Interestingly, the subunit compositions of these FHL complexes are not confirmed yet and their mechanisms and regulation pathways are under the on-going study. For both FHL-1 and FHL-2 complexes the *fhlA* gene is required: it is coding transcriptional activator for the *hyc* [35,36] and *hyf* [37] operons – FhlA and its expression might be dependent on formate. In addition, Hyp proteins and other factors are required for the maturation and assembly of Hyd enzymes [38]. Some interaction with each other or metabolic cross-talk is also proposed for these enzymes [17,39], however the nature of the link between different Hyd enzymes is not known at all.

Beforehand, it has been established that $H_2$ production by *E. coli* during glucose or glycerol fermentation was inhibited by *N,N′*-dicyclohexylcarbodiimide (DCCD), inhibitor of the proton $F_0F_1$-ATPase [20]. This finding suggests some link of Hyd enzymes or FHL complexes with $F_0F_1$. Especially, under glucose fermentation at pH 7.5 a link between Hyd and $F_0F_1$ could result from Hyd-4 interaction with $F_0F_1$ to supply reducing equivalents ($H^+ + e^-$) for energy transfer to the secondary transport system, especially to potassium uptake TrkA system [40]. The metabolic cross-talk between $H_2$ producing Hyd-1 and Hyd-2 and $F_0F_1$ is also suggested during glycerol fermentation at high and low pHs [41]. Importantly, the relationship between different Hyd enzymes and $F_0F_1$ or proton gradient ($\Delta\mu_H^+$) generated by $F_0F_1$ has been also demonstrated [18,20,42]. Such relationship has been also shown by Kim et al. [43] for the archaeon *Thermococcus onnurineus*, generating $\Delta\mu_H^+$, which is driven by formate disproportionation via FHL complex. Moreover, Sasahara et al. [44] have independently revealed a relationship between FHL complex and $F_0F_1$ during thiosulfate reduction by *Salmonella typhimurium*.

Furthermore, it has been shown in different studies [6,13,17–20,39,45] that *E. coli* Hyd activity is pH dependent. The latter seems to be very important property in regulating Hyd enzymes activity to enhance $H_2$ production efficiency. Currently, growing interest of $H_2$ production by bacteria in different environment and of developing $H_2$ bio-production technology is with mixed carbon sources fermentation or co-fermentation since different carbon substrates, including glycerol and different organic acids (namely formate), could be found in agricultural, industrial, kitchen, water and other wastes but a little is available on the fermentation of mixed carbon sources, metabolic pathways, responsible Hyd enzymes and $H_2$ production as well.

In the present paper *E. coli* $H_2$-producing activity by Hyd enzymes during glycerol fermentation upon formate supplemented (mixed carbon sources fermentation) has been studied at both slightly alkaline and acidic pHs. The possibilities of modifying metabolic pathways, coordinating appropriate genes and regulatory factors to perform some biochemical functions related to the production of $H_2$ were chosen during mixed carbon substrates fermentation. Moreover, novel functions of different Hyd enzymes especially Hyd-3 and Hyd-4 were revealed.

## 2. Materials and methods

### 2.1. Bacterial strains, growth and preparation for assays

*E. coli* BW25113 or MC4100 wild type and mutant strains with deletions in the genes coding subunits for different Hyd enzymes were used in the study. The strains used are listed in Table 1.

Bacteria from an overnight (O/N) growth culture were transferred into the fresh buffered liquid peptone medium containing 20 g/l peptone, 15 g/1 $K_2HPO_4$, 1.08 g/l $KH_2PO_4$, 5 g/l NaCl (pH 7.5) or 20 g/l peptone, 7.4 g/1 $K_2HPO_4$, 8.6 g/l $KH_2PO_4$, 5 g/l NaCl (pH 6.5) and supplemented with glycerol (10 g/l) and/or sodium formate (0.68 g/l). O/N medium was supplemented with kanamycin (25 μl/ml) for some kanamycin-resistant mutants when appropriate (see Table 1). Bacteria were grown in batch culture for 18–22 h at 37 °C; anaerobic conditions were described previously [13,18–20]. The medium pH was measured by a pH-meter using a selective pH-electrode (ESL-63-07, Gomel State Enterprise of Electrometric Equipment (GSEEE), Gomel, Belarus; or HJ1131B, Hanna Instruments, Portugal) and adjusted by 0.1 M NaOH or 0.1 N HCl. Bacterial growth was monitored by measuring bacterial culture absorbance at 600 nm with a spectrophotometer (Spectro UV–Vis Auto, Labomed, USA). The growth specific rate was determined as before [13,18–20].

### 2.2. Redox potential determination and hydrogen production assays

Redox potential ($E_h$) in bacterial suspension was assayed using the oxidation-reduction, titanium-silicate (Ti–Si) (EO-02, Gomel State Enterprise of Electrometric Equipment (GSEEE), Gomel, Belarus) and platinum (Pt) (EPB-1, GSEEE, or PT42BNC, Hanna

**Table 1**
Characteristics of *E. coli* wild type and mutant strains used.

| Strains | Genotype | Absent hydrogenase subunit or related protein | References |
|---|---|---|---|
| BW25113 | *lacI*$^{q}$ *rrnB*$_{T14}$Δ*lacZ*$_{W116}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rha BAD*$_{LD78}$ | Wild type | [10] |
| MC4100 | F-*araD139* Δ (*argF-lac*)*U169* λ-*rpsL150 relA1 deoC1 flhD5301*Δ (*fruK-yeiR*)*725*(*fruA25*) *rbsR22*Δ (*fimB-fimE*) *632* (::IS*1*) | Wild type | [20,46] |
| JW0955 KmR[a] | BW 25113 Δ*hyaB* | Large subunit of Hyd-1 | [18,19] |
| JW2962 KmR[a] | BW 25113 Δ*hybC* | Large subunit of Hyd-2 | [18,19] |
| JW 2691KmR[a] | BW 25113 Δ*hycE* | Large subunit of Hyd-3 | [47] |
| JW2472 KmR[a] | BW25113 Δ*hyfG* | Large subunit of Hyd-4 | [18,19] |
| FM 460 | MC4100 Δ*selC400::Kan* | tRNA$^{SEC}$ | [39] |
| MW 1000 | BW25113 Δ*hyaB* Δ*hybC*; | Large subunits of Hyd-1 and Hyd-2 | [18,19] |
| SW 1001 KmR[a] | BW 25113 Δ*fhlA* Δ*hyfG* | FHL activator and large subunit of Hyd-4 | [18,19] |
| HDK 103 KmR[a] | MC4100 Δ*hya* Δ*hycA-H* | Hyd-1 and Hyd-3 | [46] |
| HDK 203 KmR[a] | MC4100 Δ*hybBC*Δ*hycA-H* | Hyd-2 and Hyd-3 | [46] |
| KT2110 | BW25113 Δ*hyaB* Δ*hybC* Δ*selC* | Large subunits of Hyd-1, Hyd-2 and tRNA$^{SEC}$ | [39] |
| FTD147 | MC4100 Δ*hyaB* Δ*hybC* Δ*hycE* | Large subunits of Hyd-1, Hyd-2 and Hyd-3 | [37] |

[a] Resistant to kanamycin.

Instruments, Portugal) glass electrodes [13,18,20,48–50]. Ti–Si-electrode is measuring the overall $E_h$, whereas Pt-electrode is sensitive to $H_2$ under anaerobic conditions (in the absence of $O_2$) [51]. This difference between Ti–Si and Pt electrodes properties is allowing detection of $H_2$ evolution in bacterial suspension. This electrochemical determination of $H_2$ is closed to the method with Clark-type electrode employed by Fernandez [52] and different groups [45,53]. Importantly, $E_h$ and $H_2$ production have been established by Piskarev with co-authors [54] to be correlated with each other well, and $E_h$ decrease by $H_2$ evolution in water solution did not depend on salt content. They have also shown that the addition of $H_2$ into a solution didn't affect medium pH [54]. Different controls were done as detailed in previous papers [13,18,20,48–50,55]. Therefore, $H_2$ production rate ($V_{H2}$) by bacteria was calculated determining the difference between the initial rates of decrease in Pt- and Ti–Si-electrodes readings and using correlation between this difference and $H_2$ as employed before [50,54,55] and expressed as mmol $H_2$ per min per g dry weight.

The $E_h$ measurements were performed in the assay buffer (150 mM Tris-phosphate (appropriate pH mentioned) containing 0.4 mM $MgSO_4$, 1 mM NaCl and 1 mM KCl) upon glycerol (glycerol assay) or formate (formate assay) supplementation to bacterial suspension. Glycerol or formate was supplemented in the concentrations used for the bacterial growth in the culture. The data with bacteria grown on mixed carbon sources (glycerol and formate) were compared with those by the cells grown on glycerol as a sole carbon as previously [13,18,20].

$H_2$ production by bacteria was confirmed also by the chemical method [19,21,48–50,56] and using the Durham test tubes [20].

### 2.3. Others, reagents and data processing

Preparation of whole cells for $H_2$ production assays was done as described elsewhere [8,10,18,20,48–50]. Dry weight of bacteria was measured as before [18,20].

Agar, glycerol, peptone, sodium formate, Tris (Carl Roth GmbH, Germany) were used, the other reagents were of analytical grade.

Each data point represented in Tables and on Figures was averaged from independent duplicate or triplicate cultures at least. The standard errors of average data were calculated as described [18,20,50,55]: they were not more than 3% if not represented. The validity of data differences between experimental and control assays was evaluated by Student's criteria (p) [18,20,50,55], the difference was valid if $p < 0.01$ or less; otherwise, the difference was not valid if $p > 0.5$ (not represented).

## 3. Results and discussion

### 3.1. $H_2$ production by E. coli wild type and mutants with defects in Hyd-1 and Hyd-2 during mixed carbon fermentation in assays supplemented with glycerol or formate at pH 7.5

It is established that *E. coli* is able to ferment glucose and formate at different pHs producing $H_2$ [13,15–17,19,20]. Besides glucose and formate, this bacterium can grow on glycerol under anaerobic conditions at different pHs and also evolve $H_2$ [6,17–19,48]. Actually, *E. coli* wild type cells (see Table 1) grew well during glycerol and mixed carbon (glycerol with formate supplemented) fermentation at pH 6.5 or pH 7.5 having similar $V_{H2}$ (data not shown).

It is worth to mention that the specific growth rate when wild type cells were grown on glycerol and formate supplemented at pH 7.5 was almost the same ($0.92 \pm 0.03$ $h^{-1}$) compared to the cells grown on either glycerol or formate. At pH 6.5 wild type cells specific growth rate grown on mixed carbon was lowered ~1.31 fold compared to pH 7.5. At pH 5.5 when the medium was supplemented with glycerol and formate the growth of wild type cells was strongly inhibited (data not shown). That was the reason why for further investigation pH 5.5 was excluded and not tested for different assays.

To reveal the role of Hyd-1 and Hyd-2 in bacterial growth and $H_2$ metabolism during mixed carbon fermentation *E. coli* JW0955 (*hyaB*) or JW2962 (*hybC*) mutants were investigated and compared with wild type cells. It was shown that either at pH 7.5 or pH 6.5, especially in *hybC* mutant grown on mixed carbon the specific growth rate was ~50% lower compared to the wild type. This points out a role of Hyd enzymes in the *E. coli* growth. Together with wild type cells growth rate differences between pH 7.5 and pH 6.5 (see above), the results suggest different Hyd enzymes activity. Indeed, at pH 7.5 in wild type cells in the assays supplemented with glycerol (see Methods) $V_{H2}$ was ~4.57 mmol $H_2$/min.g dry weight (Table 2). Different combinations of *E. coli* double mutants coding Hyd-1 and Hyd-2 (see Table 1) had the same $V_{H2}$ as in the wild type (see Table 2). Moreover, deletions of *hya* and *hyc* (HDK103) or *hyb* and *hyc* (HDK203) operons had no significant effect on $V_{H2}$ in glycerol supplemented assays (Fig. 3).

In wild type cells in the assays supplemented with formate (see Methods) $V_{H2}$ was high - ~65.6 mmol $H_2$/min.g dry weight (see Table 2). *E. coli hyaB* or *hybC* single mutants showed similar $V_{H2}$ as in wild type cells. Interestingly, in MW1000 (*hyaB hybC*) double mutant $V_{H2}$ was increased ~1.5 fold, compared to the wild type

(Fig. 1A). Based on these data it can be suggested that Hyd-1 or Hyd-2 works in $H_2$ uptake direction and, therefore, these Hyd enzymes have no impact on $H_2$ producing activity by *E. coli*. Similar data were observed with the cells grown during glucose only fermentation [16,19,20]. Importantly, in contrast to glycerol supplemented assays, in HDK103 or HDK203 mutants $V_{H2}$ was decreased significantly (~5.6 fold, $p < 0.001$) compared to the wild type (see Fig. 3). These results clearly show that during mixed carbon sources (glycerol and formate) fermentation at pH 7.5 Hyd-3 is responsible for $H_2$ production by *E. coli* (in the formate assays). This might be due to activation of the *hyc* operon. The finding is in good conformity with the result obtained previously by Mnatsakanyan et al. [49] that at pH 7.5 Hyd-3 was major for $H_2$ production during glucose fermentation when external formate was added. Interestingly, it has been shown that some *hyb* genes are required for efficient processing of Hyd-1 and *hyb* operon expression, hence, is important for synthesis of active Hyd-1 [57]. Therefore, it may be also possible to suggest that there is some effect of gene for one Hyd enzyme on the expression of the genes or activity for the other one; however this might depend on growth conditions and different factors and requires a special study.

Thus, the principal findings at pH 7.5 are with that $V_{H2}$ in the assays with supplemented glycerol has no changes compared to those with glycerol only fermentation. No Hyd-2 or Hyd-1 $H_2$ evolving activity is detected compared to glycerol only fermentation where Hyd-2 works in $H_2$ production mode as reported previously [8]. Moreover, in glycerol assays all four Hyd enzymes might operate in $H_2$ production mode. In addition, the deletions of the operons for Hyd-1 and Hyd-3 or Hyd-2 and Hyd-3 don't change $V_{H2}$. This might indicate that the primary role of all Hyd enzymes is towards $H_2$ production as to neutralize formate and to maintain $H_2$ cycling for the generation of $\Delta\mu_H^+$ as suggested recently by Trchounian and Sawers [58].

### 3.2. $H_2$ production by *E. coli* wild type and mutants with defects in Hyd-1 and Hyd-2 during mixed carbon fermentation in assays supplemented with glycerol or formate at pH 6.5

During mixed carbon fermentation at pH 6.5 in the glycerol assays *E. coli* wild type had similar $V_{H2}$ compared to pH 7.5 (see Table 2). The analysis of single and double mutants with defects in Hyd-1 and Hyd-2 (see Table 1) showed no role of these Hyd enzymes in $H_2$ producing activity by *E. coli* (see Table 2). In HDK103 and HDK203 double mutants $V_{H2}$ was slightly decreased ~1.3 and ~1.7 fold, respectively, compared to the wild type (see Fig. 3). The decrease of $H_2$ production in these mutants might be due to deletions of the operons reflecting on the $H_2$ producing enzymes.

In the formate assays with wild type cells $V_{H2}$ was decreased ~1.5 fold compared to the cells grown at pH 7.5. However the single or double mutants with defects in Hyd-1 and Hyd-2 (see Table 1) had the same $V_{H2}$ as the wild type (Fig. 2A). These data showed that neither Hyd-1 nor Hyd-2 has no role in $H_2$ metabolism (in formate supplemented assays). But in HDK103 and HDK203 double mutants $V_{H2}$ was decreased ~3.3 fold ($p < 0.001$) compared to the wild type (see Fig. 3). At this pH also Hyd-3 is suggested to have the major contribution for $H_2$ production and the rest of $H_2$ is produced either by Hyd-1 or Hyd-2.

### 3.3. $H_2$ production by *E. coli* wild type and mutants with defects in Hyd-3 and Hyd-4 during mixed carbon fermentation in assays supplemented with glycerol or formate at pH 7.5

To understand the role of different Hyd enzymes required for $H_2$ production by *E. coli* during mixed carbon fermentation, mutant strains with defects in Hyd-3 and Hyd-4 were also investigated.

Actually, during mixed carbon fermentation at pH 7.5 in the assays supplemented with glycerol $V_{H2}$ in JW2691 (*hycE*), JW2472 (*hyfG*) single and SW1001 (*hyfG fhlA*) double mutants (see Table 1) was similar to the wild type (Fig. 1B). However, at pH 7.5 or pH 6.5 the specific growth rate for these mutants was ~30% lower than that of the wild type but not more than for *hybC* mutant (see above). This suggests that Hyd-3 with Hyd-4 is involved in $H_2$ cycling by compensating each other when one enzyme is absent. On the contrary, during glucose fermentation Hyd-4 has been shown to be the main enzyme responsible for $H_2$ production [20]. Interestingly, in FM460 (*selC*) single mutant (see Table 1) $H_2$ production by *E. coli* decreased ~2 fold suggesting that FDH-H is involved in $H_2$ production probably supplying reducing equivalents to Hyd-3 or to the other enzymes which are contributing to $H_2$ evolution. However, the deletions of genes for three Hyd enzymes in FTD147 (*hyaB hybC hycE*) or KT2110 (*hyaB hybC selC*) mutants (see Table 1) resulted in the marked decrease of $V_{H2}$ ~2 fold and ~3.5 fold ($p < 0.001$), respectively, compared to the wild type (Fig. 4). The remaining $H_2$ seems to be produced by Hyd-4. These data about $H_2$ producing activity at pH 7.5 are in conformity with the findings obtained previously by Redwood et al. [27]. The results propose that there are compensatory $H_2$ oxidizing or producing functions of Hyd enzymes but one Hyd enzyme cannot compensate the other three ones. This causes disturbance of $H_2$ cycling between different Hyd enzymes where $H_2$ production already is not favored for the cell. It may be also suggested that each Hyd enzyme cannot function by its own: the all Hyd enzymes in *E. coli* are associated each with other to form some assemble and operate together for maintaining $H_2$ cycling. This idea is novel but it should be ruled out for further study.

During growth on mixed carbon at pH 7.5 in formate supplemented assays $V_{H2}$ decreased ~3 fold in *hycE* single mutant and the strong decrease of $V_{H2}$ ~17 fold ($p < 0.001$) was observed in *selC* single mutant (Fig. 1B). These data suggest that, in the presence of external formate, in the absence of Hyd-3 the remaining production of $H_2$ can be performed probably by supplying reducing equivalents from FDH-H to Hyd-4. Moreover, in *hyfG fhlA* double mutant $V_{H2}$ was decreased ~2 fold (see Fig. 1B). The results indicate the role of Hyd-3 in $H_2$ production. In addition, *hyaB hybC selC* triple mutant had similar $V_{H2}$ as *selC* mutant (see Fig. 4).

**Table 2**
$H_2$ production rate ($V_{H2}$) (mmol $H_2$/min·g dry weight) for *E. coli* BW25113 wild type during glycerol and/or formate fermentation in assays supplemented with glycerol or formate at pH 7.5 and 6.5. Bacteria were transferred into a 150 mM Tris-phosphate (appropriate pH) buffer containing 0.4 mM $MgSO_4$, 1 mM NaCl, 1 mM KCl, and glycerol or formate was subsequently added at time zero.

| Strain | pH 7.5 | | | pH 6.5 | | |
|---|---|---|---|---|---|---|
| | Glycerol assay[a] | Glycerol assay[b] | Formate assay[b] | Glycerol assay[a] | Glycerol assay[b] | Formate assay[b] |
| BW25113 | 4.48 | 4.57 | 65.6 | 5.12 | 5.79 | 46.69 |

[a] Cells grown in peptone medium were supplemented with glycerol only as a carbon source.
[b] Cells grown in peptone medium were supplemented with glycerol and formate as carbon sources.

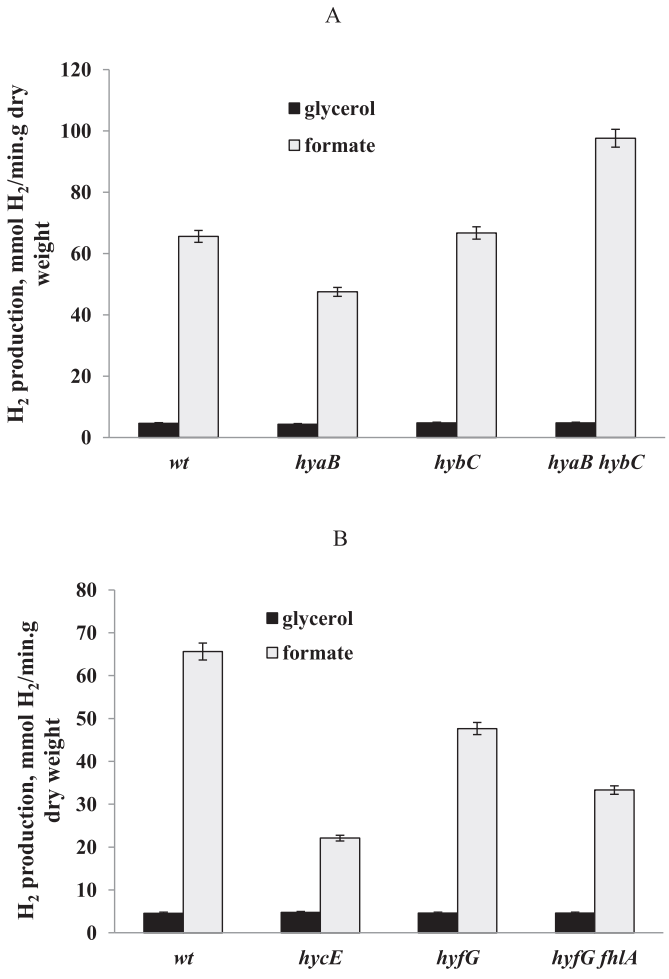


**Fig. 1.** $H_2$ production rate ($V_{H2}$) by *E. coli* BW25113 wild type (wt) and mutants with defects in Hyd-1 and Hyd-2 (A), Hyd-3 and Hyd-4 (B) during mixed carbon fermentation in the assays supplemented with glycerol or formate at pH 7.5. For mutant strains see Table 1; for others, see Materials and methods.

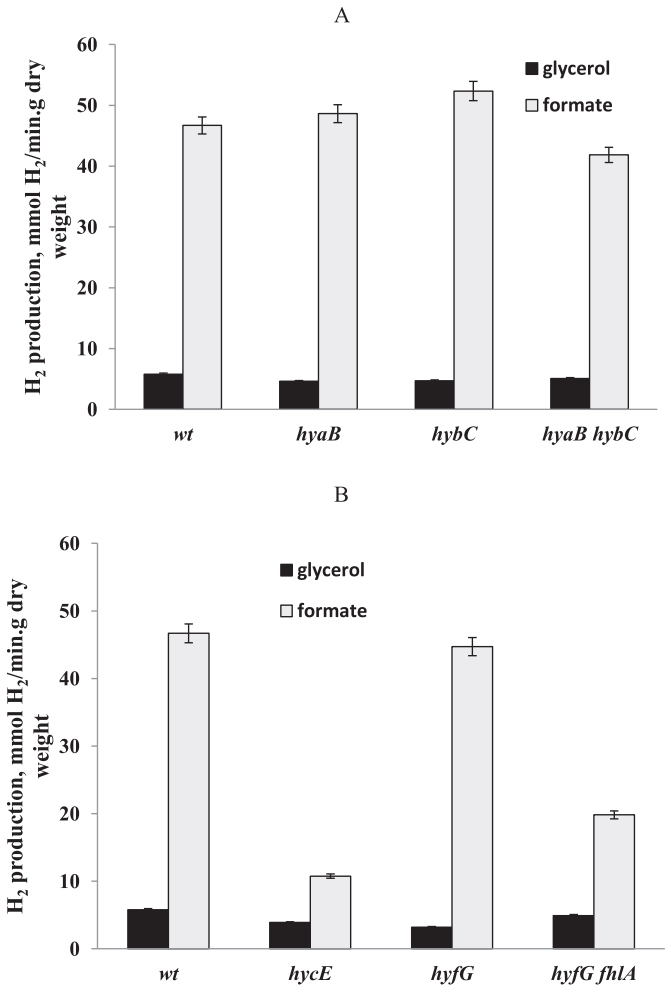


**Fig. 2.** $H_2$ production rate ($V_{H2}$) by *E. coli* BW25113 wild type (wt) and mutants with defects in Hyd-1 and Hyd-2 (A), Hyd-3 and Hyd-4 (B) during mixed carbon fermentation in the assays supplemented with glycerol or formate at pH 6.5. For others see legends to Fig. 1.

### 3.4. $H_2$ production by *E. coli* wild type and mutants with defects in Hyd-3 and Hyd-4 during mixed carbon fermentation in assays supplemented with glycerol or formate at pH 6.5

At pH 6.5 during mixed carbon fermentation by *E. coli* in glycerol assays the overall situation regarding the study of single or double mutants with defects in Hyd-3 and Hyd-4 is similar to that at pH 7.5. But surprisingly in *selC* mutant no decrease of $V_{H2}$ was detected (see Fig. 4). As following, no FDH-H is needed. In *hyaB hybC selC* triple mutant $V_{H2}$ was decreased ~3.6 fold (see Fig. 4). But in contrast to the findings at pH 7.5, at pH 6.5 $H_2$ production was completely absent in *hyaB hybC hycE* triple mutant (see Fig. 4). These data clearly point out that $H_2$ production is lowered but not totally abolished due to partial destabilization of FHL-1 complex in *hyaB hybC selC* mutant, where FDH-H was absent, and Hyd-3 can work in $H_2$ producing mode.

In formate supplemented assays, in *hycE* single and *hyaB hybC selC* triple mutants $V_{H2}$ was lowered ~4.5 fold and ~13 fold ($p < 0.001$), respectively, compared to the wild type (see Fig. 4). In *hyfG fhlA* double mutant the decrease of $V_{H2}$ in ~2.5 fold was obtained (see Fig. 4). In addition to the results at pH 7.5 (see above), the data for the last mutant at pH 6.5 suggest that the cells regulate $H_2$ production regardless of pH which has no effect on activation of FHL complex by *fhlA* transcriptional activator.

Thus, at pH 6.5 during mixed carbon fermentation, in the assays with added glycerol, in wild type cells $V_{H2}$ is similar to that with the cells grown on glycerol only. Interestingly, no role of FDH-H has been observed for pH 6.5. The deletion of single or double genes or even the whole operons for one or more Hyd enzymes don't reveal

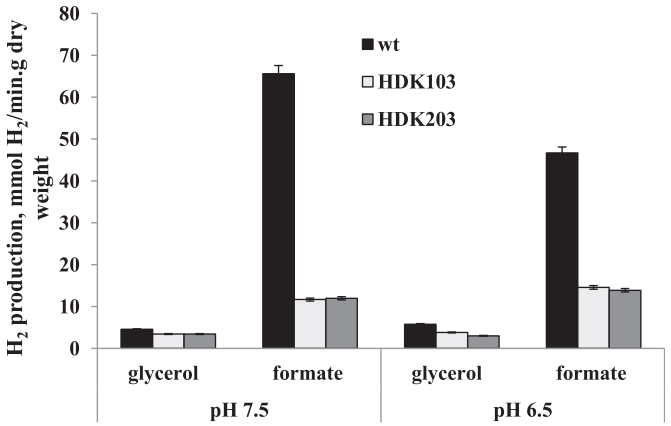


**Fig. 3.** $H_2$ production rate ($V_{H2}$) by *E. coli* MC4100 wild type (wt) and HDK103 and HDK203 mutant strains during mixed carbon fermentation in the assays supplemented with glycerol or formate at pH 7.5 and 6.5. For others see legends to Fig. 1.

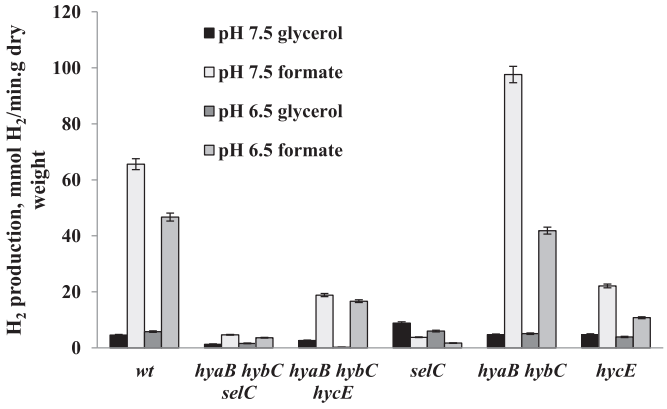


**Fig. 4.** $H_2$ production rate ($V_{H2}$) by *E. coli* wild type (wt) and mutant strains with defects in FDH-H, Hyd-1, Hyd-2 and Hyd-3 during mixed carbon fermentation in the assays supplemented with glycerol or formate at pH 7.5 and 6.5. BW25113 or MC4100 wild type was used as control when appropriate (see Table 1). For others see legends to Fig. 1.

Hyd enzyme/s mainly responsible for $H_2$ production. Obviously, as at pH 7.5, in formate assays, Hyd-3 is major $H_2$ producing Hyd enzyme at pH 6.5.

Taken together, the main results have been obtained with *hyaB hybC selC* and *hyaB hybC hycE* triple mutants. Only deletions of genes for three Hyd enzymes affect $H_2$ production at both pHs in glycerol assays. The data obtained suggest that during mixed carbon fermentation the disturbance of $H_2$ cycling reduces the production of $H_2$ and one of Hyd enzymes cannot work simultaneously either in $H_2$ uptake or production direction.

However, in spite of significant findings, $H_2$ metabolism pathways, mechanisms and regulation of Hyd enzymes activity by *E. coli* during mixed carbon fermentation require further investigation. Characterization of carbon balance and analyzing concentration-dependent $H_2$ production would be of significance for metabolic engineering of *E. coli*.

## 4. Conclusions

The results obtained point out that Hyd-3 becomes mainly responsible for $H_2$ production by *E. coli* during glycerol fermentation when external formate is added; Hyd-4 can also contribute to $H_2$ production. Besides, in the glycerol supplemented assays, three hydrogenases can work in $H_2$ producing mode and only deletion of three of them decreases the production of $H_2$ which might be due to disturbance of $H_2$ cycling.

This is of significance in application of different carbon sources, especially mixed carbons, in $H_2$ production technology, in energy production using bacteria.

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