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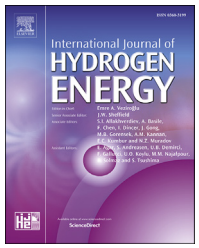

Evidence for hydrogenase-4 catalyzed biohydrogen production in *Escherichia coli*

Satenik Mirzoyan [a], *Pablo Maria Romero-Pareja* [a,b], *Maria Dolores Coello* [b], *Armen Trchounian* [a], *Karen Trchounian* [a,*]

[a] *Department of Biochemistry, Microbiology and Biotechnology, Faculty of Biology, Yerevan State University, 0025, Yerevan, Armenia*

[b] *Department of Environmental Technology, Universidad de Cádiz, Poligono Rio San Pedro S/n, 11510, Puerto Real, Cadiz, Spain*

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ABSTRACT

Biohydrogen production by *Escherichia coli* during fermentation of the mixture of glycerol, glucose and formate at different pH values was studied. Employing mutants lacking large subunits of different hydrogenases (Hyd), it was reported that, at pH 7.5, H_2 production was produced except in a *hyaB hybC hycE* triple mutant, thus suggesting compensatory H_2-producing functions of the Hyd enzymes. Activity of Hyd-4 was revealed in glucose assays at pH 7.5 in the triple mutant whereby 62% of the wild type level of H_2 production was derived from Hyd-4. In formate assays, it was shown, that, first, the *hyaB hybC* double mutant had a H_2 production ~3 fold higher than wild type, indicating that Hyd-1 and Hyd-2 oxidize H_2, and second, that at pH 5.5, Hyd-4 and Hyd-3 were responsible for H_2 production. These findings are significant when applying various carbon sources such as sugars, alcohol and organic acids for biohydrogen production.

Introduction

The current climate of shrinking fossil fuel resources and continually increasing energy demand requires the identification of efficient, renewable and ecologically friendly new sources of energy. Molecular hydrogen, or dihydrogen (H_2), fits these criteria, with its high energy density – 142 kJ/g, and having water as only product of combustion [1,2]. H_2 is produced during dark fermentation by bacteria through biological conversion of organic substrates such as alcohols (glycerol), organic carboxylic acids (e.g. acetic, formic, succinic acids), different sugars (e.g. glucose, lactose, xylose), which are present in organic wastes originating from agricultural and industrial processes [3,4]. Glycerol is massively produced as a by-product during biodiesel generation (1 kg of glycerol is produced for every 10 kg of biodiesel synthesized) [5]. Different sugars and organic acids (e.g. acetic, formic, succinic) are present in agricultural and industrial wastes, making these potentially valuable energy sources [6–9].

Gonzalez et al. [10] reported a decade ago that polyols, such as glycerol, can be metabolized under anaerobic fermentative

* *Corresponding author*. Department of Biochemistry, Microbiology and Biotechnology, Yerevan State University, 1 A. Manoogian Str., 0025, Yerevan, Armenia.
E-mail address: k.trchounian@ysu.am (K. Trchounian).

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conditions by *Escherichia coli* at pH 6.3 and further, at pH 7.5 [11]. Products of the process depend on external pH but biohydrogen was always detected. As a consequence of such a significant finding, different mixtures of inexpensive and readily available carbon sources such as sugars (lactose, xylose etc.) and glycerol have been tested to improve H_2 production by *Escherichia coli* [12–15]. Moreover, different mixtures of carbonaceous substrates have been tested by various research groups in co-cultivation experiments with the aim of transformation these organic substrates into valuable chemicals [16–18].

During mixed acid fermentation *E. coli* produces H_2 at different pHs. H_2 is produced via membrane-bound [Ni–Fe] hydrogenase (Hyd) enzymes. Their activation depends on various external factors such as the type of fermentation substrate, external pH etc. [19–22]. The mechanisms underlying the expression of genes encoding the Hyd enzymes and, furthermore, their posttranslational maturation, assembly and activity, as well as reciprocal interaction with other membrane proteins are an intense focus of study. An important feature of Hyd enzymes in *E coli* is their reversibility with regard to H_2 production and oxidation [9,19]. The main H_2 evolving formate hydrogen lyase (FHL-1) complex consists of Hyd-3 and formate dehydrogenase (FDH-H), is active at low pH [19]. The other formate hydrogen lyase complex – FHL-2 formed by Hyd-4 and FDH-H is proposed to be active at high pH [23]. Although, it is well documented [24–26] that synthesis of both FHL complexes is under transcriptional control, their differential synthesis is not clearly defined.

External pH is important for the activity of Hyd enzymes [27,28], which makes a key contribution to the enhancement of H_2 production. Moreover, in the case of Hyd-4 activity, the fermentation substrate is important, as well as its concentration. Thus, Hyd-4 is active within a glucose concentration range from low to moderately high (0.2%) [22]. Several other considerations are highly relevant for enhancing biohydrogen generation, including understanding the physiology of the Hyd enzymes, the involvement of heavy metals and stimulating and inhibiting metabolites [15,29–31]. Moreover, the choice of strains, and whether the genes encoding some of the Hyd enzymes and their regulatory proteins are absent, has a major influence on H_2 production [32,33].

Currently, a key issue in the field of biohydrogen production is the fermentation or utilization of various mixed carbon sources released from wastes of industry or agriculture. Other major goals are the assessment and the improvement of the process of H_2 production and a major aim is to understand what controls the activity of Hyd enzymes.

Fermentation of a combination of three carbon sources (glucose, glycerol and formic acid) by *E. coli*, which constitutes itself a novel approach, with different external pHs, is conducted in the current work, targeting conditions for enhancement of H_2 production and on providing further insights into the Hyd enzymes involved in H_2 generation.

Materials and Methods

Bacterial strains and cultivation

The characteristics of *E. coli* strains used in the study, BW25113 or MC4100 (wild type parents) and mutant strains with defects in the genes coding Hyd enzymes are described in Table 1.

Bacterial cells culture was grown overnight (O/N) under anaerobic fermentative conditions and transferred into buffered growth medium containing peptone (20 g L^{-1}) at pH of 7.5, 6.5 and 5.5, with salt compositions as follow: 15 g L^{-1} K_2HPO_4, 1.08 g L^{-1} KH_2PO_4 and 5 g L^{-1} NaCl (pH 7.5); 7.4 g L^{-1} K_2HPO_4, 8.6 g L^{-1} KH_2PO_4 and 5 g L^{-1} NaCl (pH 6.5), and 1.08 g L^{-1} K_2HPO_4, 15 g L^{-1} KH_2PO_4 and 5 g L^{-1} NaCl (pH 5.5). The medium was simultaneously supplemented with the following carbon sources: glucose (2 g L^{-1}), glycerol (10 g L^{-1}) and sodium formate (0.68 g L^{-1}). Kanamycin (25 μl mL^{-1}) was also added when appropriate (see Table 1).

Bacterial cultures were grown in sealed flasks with closed lids under fermentative conditions for 18–24 h at 37 °C; anaerobic conditions in the medium were achieved by displacing O_2 during autoclaving [11,21,22]. The medium pH was determined using a pH-meter with selective pH-electrode (HJ1131B, Hanna Instruments, Portugal) and adjusted to required values (see above) with 0.1 M NaOH or 0.1 N HCl. Bacterial growth was monitored by means of measuring bacterial culture absorbance by spectrophotometric method (600 nm) (Spectro UV–Vis Auto, Labomed, USA).

Table 1 – Characteristics of *E. coli* wild type parents 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 parent	[11]
MC4100	F-*araD139* Δ (*argF-lac*)*U169* λ-*rpsL150 relA1 deoC1 flhD5301* Δ(*fruK-yeiR*)*725*(*fruA25*) *rbsR22* Δ(*fimB-fimE*) *632* (::IS1)	Wild type parent	[21,34]
JW0955 Km^{Ra}	BW 25113 Δ*hyaB*	Large subunit of Hyd-1	[11,28]
JW2962 Km^{Ra}	BW 25113 Δ*hybC*	Large subunit of Hyd-2	[11,28]
JW 2691Km^{Ra}	BW 25113 Δ*hycE*	Large subunit of Hyd-3	[35]
JW2472 Km^{Ra}	BW25113 Δ*hyfG*	Large subunit of Hyd-4	[9,37]
MW 1000	BW25113 Δ*hyaB* Δ*hybC;*	Large subunits of Hyd-1 and Hyd-2	[9,37]
FTD147	MC4100 Δ*hyaB* Δ*hybC* Δ*hycE*	Large subunits of Hyd-1, Hyd-2 and Hyd-3	[26]
FTD150	MC4100 Δ*hyaB* Δ*hybC* Δ*hycE* Δ*hyfG*	Large subunits of Hyd-1, Hyd-2, Hyd-3 and Hyd-4	[26]

[a] Resistant to kanamycin.

Redox potential determination and hydrogen production assays

Redox potential (E_h) in bacterial biomass was determined using two different redox, titanium-silicate (Ti–Si) (EO-02, Gomel State Enterprise of Electrometric Equipment (GSEEE), Gomel, Belarus) and platinum (Pt) (EPB-1, GSEEE, or PT42BNC, Hanna Instruments, Portugal) glass electrodes [11,21,36,37]. The Ti–Si-electrode measures the overall E_h, whereas the Pt-electrode is responsive to H_2 under anaerobic conditions [38]. This characteristic of the dual electrode system (Ti–Si/Pt) has been used [19,21,36,37] to detect H_2 gas production in bacterial biomass, by measuring the H_2 production rate (V_{H2}) of bacteria calculated as the difference between the initial rates of decrease in Pt- and Ti–Si-electrodes readings and expressed in mV of E_h per min per mg dry weight of bacteria. This electrochemical approach applied for hydrogen determination is similar to the Clark-type electrode used by Fernandez [39] and other researchers [40,41]. As a control experiment were used cells without any addition of carbon source. In this case no any H_2 production has been detected. Importantly, salt content of the solution does not affect the evolution of E_h by H_2 saturation [42], and, moreover, supplementation of H_2 into the solution does not have any impact on external or medium pH [42]. In conclusion, the method with two redox electrodes for determining H_2 production by bacteria is well established and validated [19,21,36,37]. V_{H2} is recalculated and expressed as mmol H_2 min^{-1} (g dry weight)$^{-1}$.

The E_h measurements were performed in the assay buffer solution (150 mM Tris-phosphate, at the indicated pH, including 0.4 mM $MgSO_4$, 1 mM NaCl and 1 mM KCl) to determine H_2 production upon glucose (glucose assay), glycerol (glycerol assay) or formate (formate assay) addition. Glucose, glycerol or formate were added for assays at similar concentrations as used for cell cultivation.

H_2 generation by cells was independently checked by chemical [21,22,36,37,43] and microbiological methods using Durham test tubes [21].

Chemicals, data analysis and cell preparation

Bacterial whole cells and dry weight of cell mass were determined as described previously [8,10,17,19,21,46–48]. All reagents and chemicals used for experiments were of analytical grade (Sigma Aldrich, Carl Roth GmbH, Germany).

All measurements were performed in triplicate. Figures show average values. The standard errors were calculated [19,21,37] and mention was only made for values over 3%. Statistical significance of experimental assays were confirmed if $p < 0.01$; otherwise, the results were considered not valid if $p > 0.5$ [19,21,37].

Results and discussion

H_2 production during utilization of glucose, glycerol and formate mixture at slightly alkaline pH

It has been well reported [11,19–21] that *E. coli* is able to use glucose and formic acid under anaerobic fermentative conditions producing H_2 at different external pHs. More recently [10,18–20], it was also confirmed that *E. coli* is able to ferment glycerol at different pHs generating H_2

To determine the role of different Hyd enzymes in H_2 producing metabolic pathways during utilization of various carbon sources (glucose, glycerol and formate), the H_2 production by different *E. coli* Hyd mutants was conducted and compared with the wild type strain. Note, that all three carbon sources were added simultaneously in the growth medium for obtaining cell biomass. After harvesting the cells, H_2 production assays were performed (see Materials and methods). In

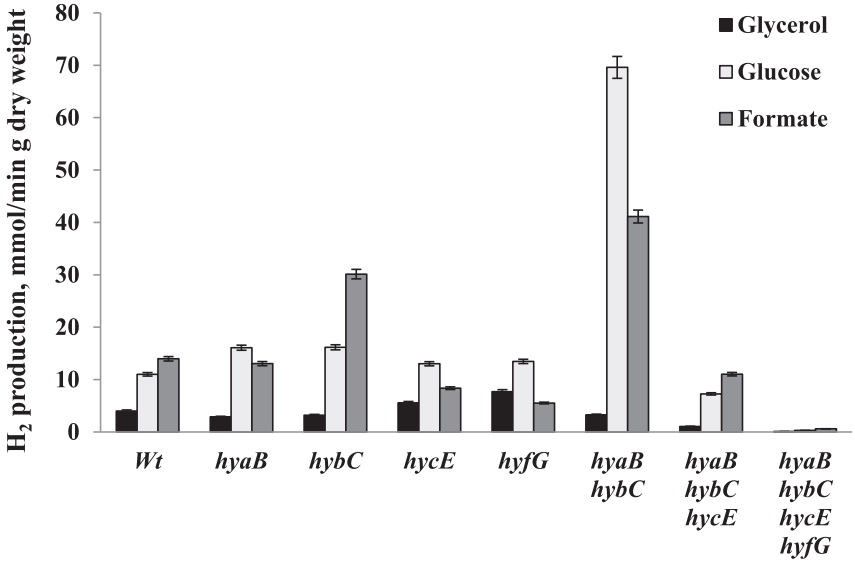

Fig. 1 – H_2 production rate (V_{H2}) by the *E. coli* BW25113 wild type parent and mutant strains with various defects in Hyd-1 to 4 during mixed carbon sources fermentation in the assays supplemented with glycerol, glucose or formate at pH 7.5. For strains used see Table 1; for others, see Materials and Methods.

BW25113 wild type (Fig. 1) or MC4100 (not shown) in glycerol assays at pH 7.5, V_{H2} was ~4 mmol min^{-1} (g dry weight)$^{-1}$. Similar V_{H2} (see Fig. 1) was determined for single and double mutant strains devoid of large subunits of Hyd-1 and/or Hyd-2 (*hyaB*, *hybC* and *hyaB hybC*, see Table 1). As observed in Fig. 1, the assay of the *hyfG* mutant revealed a ~2 fold increase in V_{H2} suggesting that under these conditions (glycerol assay, pH 7.5) Hyd 4 functions in the H_2 uptake direction. As several enzymes are present, a single Hyd enzyme could not be considered to be solely responsible for the H_2 production. For example, Hyd-2 and Hyd-1 have been determined to be responsible for H_2 production and Hyd-3 and Hyd-4 for H_2 oxidation [11,19] during glycerol only fermentation.

To further understand the role of the Hyd-4 enzyme in H_2 metabolism under these conditions, the FTD147 triple and FTD150 quadruple mutants with defects in large subunits of Hyd-1 to Hyd-3, and Hyd-1 to Hyd-4, respectively (see Table 1) were assayed for H_2 production at pH 7.5. In FTD147 the assay with glycerol revealed that V_{H2} was decreased ~4 fold compared with wild type and negligible in FTD150 (see Fig. 1), indicating that H_2 production was hardly affected unless deletions of genes for three or four Hyd enzymes were introduced. Moreover, the results for FTD147 indicate that Hyd-4 must be responsible for the residual H_2 production. The results obtained also suggest that, in the wild type, after mixed carbon-source fermentation and using glycerol in assays, each Hyd enzyme can potentially compensate for the absence or lack of activity of others in the H_2 production direction, and that the different enzymes are reversible during the fermentation under these conditions. Moreover, this shows that only when activity of minimally three of the Hyd enzymes is disturbed can H_2 production be abolished. This compensatory mechanism and inter-dependence between Hyd enzyme activities has been proposed previously for other conditions [3,44], suggesting that H_2 cycling might be a common phenomenon for anaerobic fermentation in many cases. Probably H_2 cycling pathways act towards maintaining cytoplasmic pH and, thus, proton motive force [44].

Biohydrogen production rate by wild type cells was doubled at pH 7.5 in the glucose assay in comparison to supplementation of glycerol (see Fig. 1). The observed increase of V_{H2} in glucose assays with either single or double mutant versus production of wild type revealed which Hyd enzymes were working in H_2 oxidation direction. The increase in V_{H2} was especially significant for the *hyaB hybC* double mutant, being of ~6.3 fold higher (see Fig. 1). V_{H2} in the *hyaB hybC hycE* triple mutant decreased only slightly (~1.5 fold), indicating that Hyd-4 was responsible 62% of the H_2 production under these conditions. This was confirmed by analysis of the FTD150 mutant (with absence of large subunits of Hyd-1 to 4, see Table 1) where H_2 production was essentially null. Based on the observation of the large decrease (~10 fold) in V_{H2} when comparing the *hyaB hybC hycE* triple mutant and the *hyaB hybC* double mutant, it can be confirmed also that Hyd-3 also makes a major contribution towards H_2 production. This enzyme was previously assigned to be the main contributor for H_2 production during dark fermentation with only glucose as carbon source, regardless of pH [45]. However, activity in the reverse direction, i.e. H_2 uptake, was also observed during glucose fermentation for Hyd-3 [46]. Moreover, at pH 7.5H_2 uptake activity was observed for Hyd-1 and Hyd-2 in glucose assays, the latter having been previously reported to act in this direction at pH 5.5 [27,28]. The increased H_2 production in the *hyaB hybC* double mutant might be also due to the fact that there is more capacity for the cofactor biosynthetic machinery to mature more H_2 producing Hyd-3 and Hyd-4 enzymes [47,48].

Interestingly, whereas lack of Hyd-1 had a negligible effect in the formate assay on H_2 metabolism and evolution, in the *hybC* mutant (Hyd-2 deficient) V_{H2} was increased ~2.2 fold (Fig. 1). The increase in the V_{H2} for the *hyaB hybC* double mutant with formate and glucose assays were similar (~3 fold), when compared to wild type (see Fig. 1). When the formate assays were performed in *hycE* or *hyfG* single mutants, H_2 production at pH 7.5 was reduced ~1.7 and ~2.5 fold, respectively (see Fig. 1). These results might point to a net H_2 production activity of Hyd-3 and Hyd-4 upon formate supplementation, which may be justified by the fact that formate is a weak acid and functions as an uncoupler. In such an instance, the cells need to export it into external medium for maintaining stable cytoplasmic pH via Hyd enzymes resulting in H_2 production. Again, it was observed that Hyd-4 was also responsible for H_2 production in the formate assay, as V_{H2} only decreased ~1.3 fold in the triple mutant. This observation, similar to that registered in glucose assay, implies a new role for Hyd-4 activity in the absence of the other three Hyd enzymes, which was not detected when cells were grown during glucose only fermentation [26]. Addition of glucose, glycerol or formate in the assays at pH 7.5 revealed the ability for a dual role of the same Hyd enzyme, confirming the reversibility and the activation of all enzymes.

H_2 production during utilization of glucose, glycerol and formate mixture at slightly acidic pH

E. coli wild type showed a similar V_{H2} for the glycerol assay at pH 7.5 and 6.5 (Figs. 1 and 2). H_2 production decreased only in the *hycE* or *hyfG* single mutants (~6.8 and ~2 fold, respectively), compared to wild type (see Fig. 2), suggesting that Hyd-3 and/or Hyd-4 are responsible for H_2 production at pH 6.5. Surprisingly, Hyd-3 was the only enzyme responsible for H_2 production during glycerol only fermentation [49]. Interplay between different enzymes might be different at pH 6.5 and at pH 7.5, given that at pH 6.5 no compensatory uptake or producing functions are present. The finding that a similar H_2 production rate in FTD147 and *hycE* mutants (see Fig. 2) supports the mentioned role of Hyd-3 and Hyd-4 in H_2 production direction. Similar trends to glycerol were observed for the glucose assay, with perhaps a more marked H_2 production role for the Hyd-3 enzyme (see Fig. 2), as shown in the *hycE* mutant where V_{H2} decreased to a greater extent compared to wild type than in *hyfG* mutant, where it decreased by ~2.8 fold (see Fig. 2). In contrast to the glycerol assay, H_2 production increased in *hyaB hybC* double mutant (~1.7 fold, Fig. 2) suggesting that these enzymes are working in the H_2 uptake mode. Although comparable results were obtained for formate and glucose assay in *hyaB* and *hybC* single mutants, an important difference between these assays was the increase in V_{H2} in the *hyfG* mutant compared to wild type (~1.3 fold, Fig. 1) suggesting a new formate dependent H_2 oxidizing

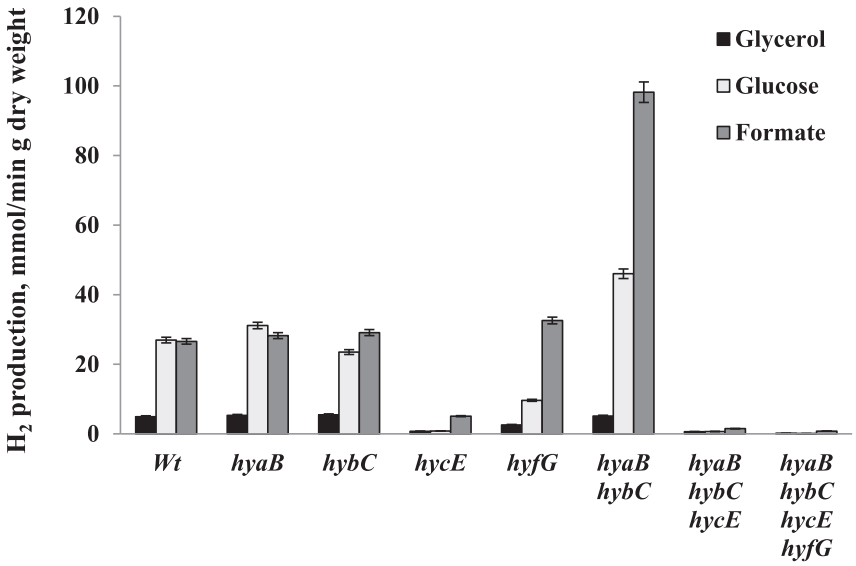

Fig. 2 – H_2 production rate (V_{H2}) by the *E. coli* BW25113 wild type parent and mutant strains with various defects in Hyd-1 to 4 during mixed carbon sources fermentation in the assays supplemented with glycerol, glucose or formate at pH 6.5. For other information see legend to Fig. 1.

Hyd-4 activity. This observation for Hyd-4 is new and potentially adds new insights into understanding the regulation of Hyd enzyme activity and H_2 cycling.

It is concluded that for continuous H_2 production, the activity of two Hyd enzymes might be important, and Hyd-3 and Hyd-4 seem to such a pair of such enzymes establishing a H_2 cycle in both directions: producing and oxidizing H_2, respectively. When the *hyfG* mutant was grown on glycerol and formate mixture, or on glucose only, and employed consequently for formate assays, no activity or contribution of Hyd-4 in H_2 metabolism and evolution was found [1,3]. Clearly, therefore, the carbon source added to the assays has clear effect on total Hyd enzyme activity whose suggested function is to maintain the H_2 cycle and cytoplasmic pH.

H_2 production during utilization of glucose, glycerol and formate mixture at acidic pH

H_2 production by *E. coli* during mixed carbon source fermentation in wild type cells at pH 5.5 upon glycerol supplementation was the same as at pH 6.5 and pH 7.5 (comp. Fig. 3 with Figs. 1 and 2). However, in glucose-supplemented assays it was comparable only to pH 7.5 (see Figs. 1–3). Glycerol assay of *hycE* or *hyfG* single mutants resulted in a similar V_{H2}

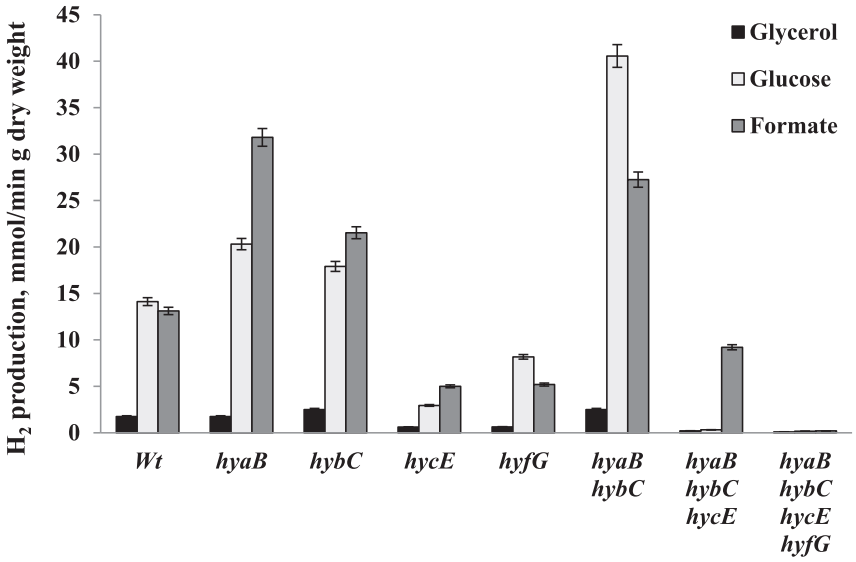

Fig. 3 – H_2 production rate (V_{H2}) by the *E. coli* BW25113 wild type parent and mutant strains with various defects in Hyd-1 to 4 during mixed carbon sources fermentation in the assays supplemented with glycerol, glucose or formate at pH 5.5. For other information see legend to Fig. 1.

decrease of ~3 fold compared to wild type (see Fig. 3). No role for Hyd-1 or Hyd-2 was detected, which is in contrast with the fact that the same Hyd enzymes were reported as responsible for H_2 oxidation when grown on glycerol only [49].

Hydrogen production in glucose assays was strongly Hyd-3 but not Hyd-4 dependent. That is inferred from the following observations: decrease of V_{H2} in *hycE* compared to wild type (~4.8 fold) and negligible H_2 production in *hyaB hybC hycE* mutant (see Fig. 3). The main H_2 uptake activity is contributed by Hyd-1 in glucose assay.

H_2 production in formate assays was similar in wild type cells at pH 5.5 and pH 7.5, but not at pH 6.5 (see Fig. 1). Moreover, in *hycE* or *hyfG* single mutants H_2 production was decreased ~2.6 fold, and in *hyaB hybC* double mutant V_{H2} was increased ~2.1 fold, compared to wild type. Taken together it can be concluded that Hyd-3 and Hyd-4 were responsible for H_2 production upon formate supplementation, probably for neutralization of formate via FHL complex.

As reported for other pHs, during mixed carbon sources fermentation at pH 5.5 the activities of Hyd enzymes differ depending on the substrate added. The clear difference might be explained via physiological conditions and requirements of the cell to act in one or another direction. So, for example when a weak acid such as formate is externally added, the cells act to neutralize it and switch two using Hyd enzymes (Hyd-3 and 4) for neutralization via H_2 production. A relevant consequence is that Hyd-4 is also formate-dependent at pH 5.5.

Conclusions and significance

The physiological direction of Hyd enzymes is affected by the 4fermentation carbon sources. In particular, formate-dependent Hyd-4 activity was demonstrated. At low pH in formate assays mainly Hyd-4 was responsible for H_2 production. Enhanced H_2 production was detected in formate-supplemented assays when Hyd-1 and Hyd-2 activity were absent.

Thus, all Hyd enzymes can either work in H_2 uptake or production directions at a wide range of external pH depending on the carbon source added. They are likely to regulate the pH gradient across the membrane via producing or taking up H_2. It is suggested that Hyd enzymes are proton sensors and act in H_2 production or uptake direction when the pH or proton concentration inside or outside the cell are changed.

The results pointed out compensatory producing functions of Hyd enzymes in the presence of glycerol and thus only disturbance of H_2 cycling decreased H_2 production. These findings provide an essential understanding when applying various bacteria or biomass to generate energy sources during dark fermentation via utilization of different organic waste materials.

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