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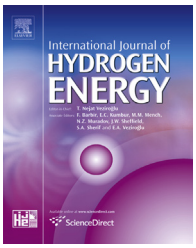

Escherichia coli growth and hydrogen production in batch culture upon formate alone and with glycerol co-fermentation at different pHs

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ABSTRACT

Escherichia coli possesses four [Ni–Fe]-hydrogenase (Hyd) enzymes, encoded by the *hya*, *hyb*, *hyc* and *hyf* operons and which are responsible for H_2 production under different conditions. The influence of formate alone or mixed with glycerol on growth of *E. coli* wild type and Hyd mutants with deletions of key subunits of Hyd 1–4, respectively, and H_2 production in batch culture was analyzed at different pHs (5.5–7.5). The findings identify the conditions when formate alone or with glycerol had stimulatory effects on bacterial growth and H_2 production. The impact of deleting the large subunits of each Hyd (1–4) enzymes on bacterial growth during exponential phase was evaluated. H_2 production was absent in the *hycE* (Hyd-3) mutant during exponential growth phase upon glycerol or formate alone or with glycerol fermentation. H_2 evolution was also not observed at pH 7.5 upon glycerol only fermentation in the *hybC* (Hyd-2) mutant, whereas formate supplementation recovered the H_2 formation. Low and delayed H_2 production was observed at pH 7.5 upon glycerol only fermentation in a *hyfG* (Hyd-4) mutant; again formate recovered and stimulated H_2 production. The results highlight the key role of Hyd-3 at both pH 6.5 and pH 7.5, as well as the role of Hyd-2 and Hyd-4 at pH 7.5 for H_2 production by *E. coli* during glycerol fermentation with formate supplementation. This might have advantages for industrial applications to enhance bio-hydrogen production.

Introduction

Hydrogen (H_2) is considered an ecologically clean, effective and renewable energy source for the future; therefore, nowadays, considerable research focuses on bio-hydrogen production from cheap carbon sources, including various industrial wastes [1–3]. Glycerol is widely recognized as an important industrial waste, which can be used as carbon source by bacteria for H_2 production and the production of other valuable chemical compounds [4–6]. Moreover, both in industry and in the environment, as well as in the human

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intestine, mixed carbon sources are available to microorganisms; hence, investigation of co-utilization of different carbon sources by bacteria is important for many purposes, such as to reveal the role of each carbon in bacterial physiology, and how it enhances bio-hydrogen production.

As has been shown recently, one of the fermentation end products during glycerol fermentation in the bacterium *Escherichia coli* is formate, which is decomposed by the formate hydrogen lyase (FHL) complex leading to H_2 production [7–12]. FHL is formed by formate dehydrogenase H (FDH-H) and hydrogenase (Hyd) 3 or 4 [10,13,14]. It is known that *E. coli* has four reversible [NiFe]-Hyd enzymes, which are responsible for H_2 oxidation and formation. The operational direction of Hyd enzymes is variable; it depends on many factors, such as carbon (glucose or glycerol) source, and medium pH. Recently, it was shown that Hyd-1 and Hyd-2 operate in H_2 oxidation upon growth with glucose and H_2 formation modes upon glycerol fermentation [10,11,15,16]. Moreover, optimal activity of Hyd-1, encoded by the *hya* operon, was observed under anaerobic conditions, at acidic pH [17] and in the presence of formate [18], whereas Hyd-2, encoded by the *hyb* operon, was active under more reducing conditions [19] and at alkaline pH [17]. Hyd-3, encoded by *hyc*, and Hyd-4, encoded by *hyf*, along with FDH-H form FHL-1 and FHL-2 pathways upon glucose fermentation at acidic and alkaline pHs, respectively [20–22].

A relationship between Hyd enzymes and the H^+-translocating FoF_1 ATPase has been shown, which is more specific under glucose fermentation, at alkaline pH: the interaction of Hyd-4 with the primary system the FoF_1 ATPase would supply H^+ and e^- for energy transfer to the secondary transport system [10,21]. A similar interaction was proposed for Hyd-1 and Hyd-2 enzymes with the dependence of carbon source and medium pH [11,23,24]. Recently it has been published that the four Hyd enzymes of *E. coli* together have the potential to form a H_2 cycle across the membrane: Hyd enzyme activity has a role in balancing the proton motive force (Δp) and in regulation of $[pH]_{in}$ [25–27].

During glycerol or glucose fermentation formate is derived primarily from pyruvate by pyruvate formate lyase (PFL) [13,28]. The oxidation and reduction potential of the formate:H_2 couple is highly reduced (−420 mV), hence, formate is considered an energy-rich compound [25], which can be an additional energy source during energy-limited, fermentative conditions [14,29]. *E. coli* produces formate up to a concentration of 20 mM and secretes it as a waste product. Investigation of formate concentration in fermenting *E. coli* cultures showed that formate is initially extruded out of the cells to prevent acidification of the cytoplasm [13], and upon a drop of growth pH, during glucose fermentation formate is re-imported into the cell through the formate channel FocA. This channel translocates the formic anion bi-directionally and, as was recently shown, PFL directly interacts with FocA to regulate formate translocation [28]. It was suggested that the *hyc* operon is regulated solely in response to formate concentration at low pH via the FhlA protein [30]. In the cytoplasm formate is metabolized by FHL leading to H_2 and CO_2 generation. Formate accumulation may result in the destruction of Δp, therefore the control of formate metabolism is a crucial checkpoint where the potentially toxic effects of formate surplus must be balanced against the loss of an important source of reducing power and optimization of energy production [31]. Only recently, the FHL complex has been isolated and its activity determined *in vitro* [14]: it was demonstrated that Hyd-3 has a unique ability among [Ni–Fe]-Hyd enzymes to catalyze production of H_2 even at high partial pressures of H_2. This property of Hyd-3 should allow the accumulation of significant concentrations of H_2. It has been established that Hyd-3 but not Hyd-4 activity was increased in *E. coli* grown under glucose fermentation in the presence of external formate (30 mM) at alkaline pH; effects of different concentrations of formate were studied [22,32]. Moreover, formate at high concentration (50 mM) was used to improve H_2 production activity by modifying the expression of the relevant genes [33]. Recently, it has been shown that during glycerol fermentation formate can be imported into the *E. coli* cell through the second formate channel – FocB [34]. Therefore, two formate channels might be involved in the effects of formate on H_2 production under different conditions and at different pHs.

Interestingly, inactivation of uncharacterized genes in *E. coli* has been shown recently to be beneficial for H_2 production from glycerol [35] but functions of those genes are unknown.

The studies of physiology of H_2 metabolism in *E. coli*, as well as the organization and transcriptional regulation of relevant genes involved in the metabolism are in good progress; however, there are many aspects of this system that remain poorly understood to allow us to understand the function of Hyd enzymes and regulation of H_2 metabolism during anaerobic growth in batch culture with peptone medium under fermentative conditions and at different pH. In particular, the effect on H_2 production of externally supplied formate alone or mixed with glycerol must be better understood.

Therefore, the purpose of this study was to investigate the influence of 10–50 mM formate and 10 g L^{-1} glycerol on growth, oxidation-reduction potential (ORP) kinetics and H_2 production of *E. coli* wild type and Δ*hyaB*, Δ*hybC*, Δ*hycE*, Δ*hyfG* mutants lacking the large, catalytic subunits of Hyd-1 to 4, respectively, in batch culture at different pHs. The findings point out the significant role of formate for Hyd-3 activity upon glycerol fermentation. It was shown that in all cases with H_2 producing activities co-supplementation of formate and glycerol had a stimulatory effect on H_2 formation.

Materials and methods

Bacterial strains and their growth conditions

The *E. coli* wild type and different mutant strains used in this study are listed in Table 1. Bacteria from overnight culture were grown anaerobically in batch culture (glass vessels with plastic press-caps) in the liquid growth medium with the composition of 20 g L^{-1} peptone, 2 g L^{-1} K_2HPO_4, 5 g L^{-1} NaCl, 10 g L^{-1} glycerol and/or 10–50 mM formate at pH range of 5.5–7.5, and 37 °C [9,12,16,21,22,34]. The pH was measured by a pH-meter with selective pH-electrode (HJ1131B, Hanna Instruments, Portugal) and adjusted with 0.1 M NaOH or 0.1 N HCl. The pH decrease was not more than 0.4 pH units and 0.25 pH units during the exponential growth phase of *E. coli* (until 7 h) upon glycerol or glycerol with formate fermentation at pH 7.5 and pH 6.5, respectively.

Table 1 – Characteristics of the *E. coli* strains used in the study.

Strains	Genotype	Absent subunit of protein	Source and reference
BW 25113	*lacI*^{q} *rrnB*$_{T14}$ *ΔlacZ*$_{W116}$ *hsdR514* *ΔaraBAD*$_{AH33}$ *Δrha BAD*$_{LD78}$	Wild type	Wood T. K [15]
JW 0955[a]	BW 25113 *ΔhyaB*	Large subunit of Hyd-1	Wood T. K [15]
JW 2962[a]	BW 25113 *ΔhybC*	Large subunit of Hyd-2	Wood T. K [15]
JW 2917[a]	BW 25113 *ΔhycE*	Large subunit of Hyd-3	Wood T. K [15]
JW 2472[a]	BW 25113 *ΔhyfG*	Large subunit of Hyd-4	Wood T. K [15]

[a] Resistant to kanamycin.

Measurement of redox potential and determination of H_2

ORP of bacterial culture was measured with the help of the couple oxidation-reduction platinum (Pt; EPB-1, GSEEE; or PT42BNC, HANNA Instruments, Portugal) and titanium-silicate (Ti–Si); EO-02, GSEEE, Gomel, Belarus) electrodes. In contrast to Pl, Ti–Si electrode readings are not affected by the presence of H_2 (or oxygen) in the medium, which allows discrimination of H_2 during anaerobic growth of bacteria [21,22,36,37]. Measurement of H_2 using the pair of oxidation-reduction electrodes gives more accurate measurements [12,21,36]. All controls (checking electrode readings in control solutions) were done and the effects of different factors (pH, formate or glycerol concentration change, organic content or bacterial cells count variation) on electrode readings were routinely determined, as described [21,22,36,37].

H_2 production during the growth of *E. coli* was qualitatively visualized by the appearance of gas bubbles in the test tubes over the bacterial suspension by the Durham tube method [21,38], H_2 production was verified by the chemical assay based on the bleaching of $KMnO_4$ solution in H_2SO_4 with H_2 [21,22,39].

The H_2 yield (concentration) was calculated by the decrease of ORP to low negative values in liquid as described recently by Piskarev et al. [40] and expressed in mol H_2 per L of growth medium (mol L^{-1}). This has been presented in many papers for H_2 yield in batch cultures [16,36,37,41].

Others and data processing

Bacterial culture absorbance at 600 nm was measured by a Spectro UV–vis Auto spectrophotometer (Labomed, USA); the dry cell weight of the bacterial biomass [21], and specific growth rate were determined as described [11,34,36]. All assays were done at 37 °C.

Data were averaged from triplicate independent measurements, for which the standard errors did not exceed 3% (if not indicated).

Results and discussion

Effect of formate on growth of E. coli *wild type and Hyd mutants during glycerol fermentation at different pHs*

To understand the effect of formate on growth in batch culture, H_2 production was studied after growth at different pHs (5.5–7.5) during glycerol fermentation, *E. coli* BW 25113 wild type and Δ*hyaB*, Δ*hybC*, Δ*hycE*, Δ*hyfG* Hyd mutants (Table 1) with deletions of different key subunits of Hyd-1 to 4, respectively. Addition of 10 mM formate reduced growth ~2 fold while 30 or 50 mM formate completely inhibited wild type growth during glycerol fermentation at pH 5.5, whereas at pH 7.5 and pH 6.5 formate in the same concentrations stimulated or had no effect on bacterial growth (Fig. 1). For this reason the study was continued at pH 7.5 and pH 6.5. Note, 10 mM formate was preferred as optimal concentration, so in further experiments when indicated 10 mM formate was supplemented to the growth medium.

At pH 6.5, formate alone or with glycerol slightly stimulated wild type growth at the late stationary phase (growth yield) ~1.1 and 1.30 ± 0.03 fold, respectively, compared with glycerol only fermentation (Fig. 2a). The stimulating effect of formate (~1.2 fold) was also observed in the *hybC* deleted (Hyd-2) mutant. Whereas formate alone reduced growth of mutant strains lacking large subunits of Hyd-1 and Hyd-4 ~1.1 by 1.30 ± 0.03 fold, respectively. But, formate in combination with glycerol showed similar bacterial growth-stimulating effects in *hyaB*, *hybC* and *hyfG* mutants compared with glycerol only supplementation. Despite the fact that the *hycE* mutant had 1.60 ± 0.03 fold higher growth rate than wild type upon glycerol fermentation, formate alone or in combination with glycerol had no effect on bacterial growth (Fig. 2a).

In contrast, at pH 7.5, formate alone suppressed or had no effect on growth of wild type and all tested mutants (Fig. 3a). Mixture of glycerol and formate had no stimulatory effects on

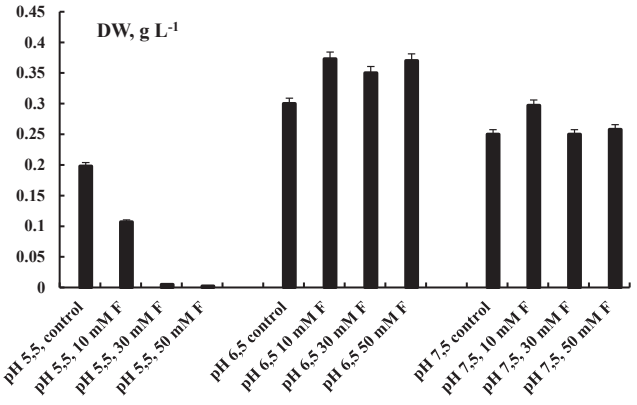

Fig. 1 – The influence of different concentrations of formate on *E. coli* BW 25113 wild type growth in batch culture at pHs 5.5–7.5. Dry cell weight (DW) was determined after 24 h bacterial growth. Control was bacterial growth in the medium with 10 g L^{-1} glycerol (G).

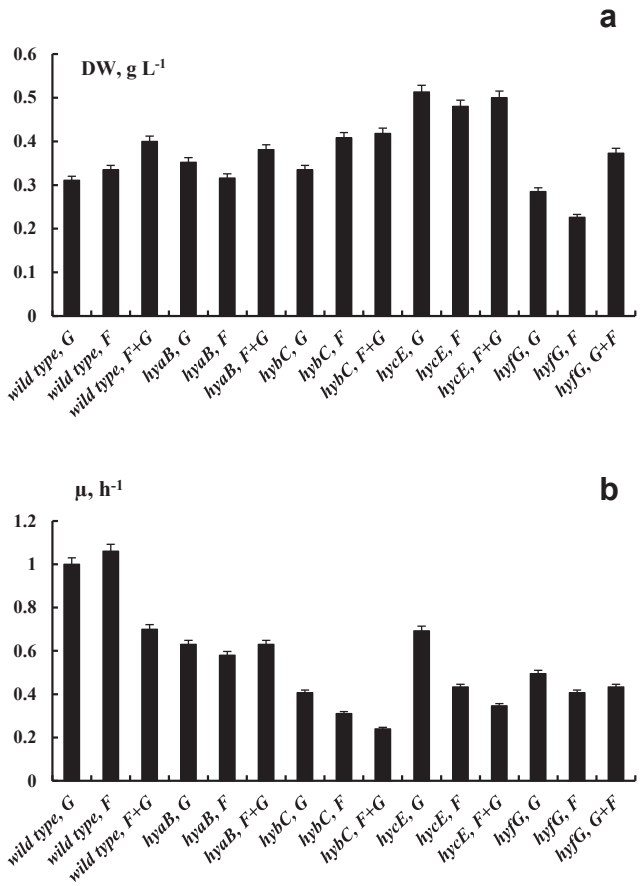

Fig. 2 – The influence of formate on *E. coli* BW 25113 wild type and mutants growth during glycerol fermentation at pH 6.5. Bacteria were grown under glycerol fermentation at pH 6.5, a) Dry cell weight (DW) after 24 h bacterial growth; b) specific growth rate (μ) data are presented. Control was bacterial growth in the medium without formate added. 10 mM formate (F) and 10 g L^{-1} glycerol (G) was supplemented when mentioned. For mutant strains see Table 1 and for details, see Materials and methods.

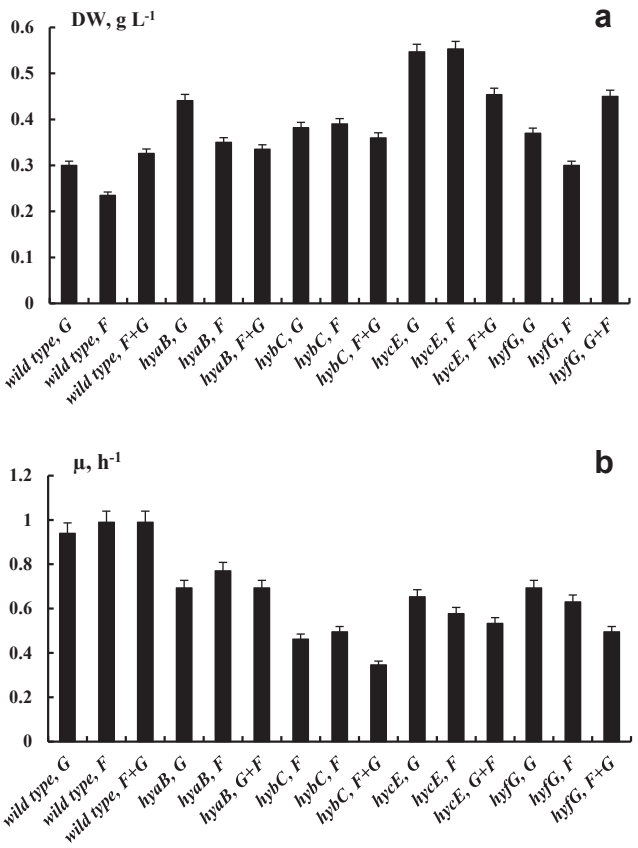

Fig. 3 – The influence of formate on *E. coli* BW 25113 wild type and mutants growth during glycerol fermentation at pH 7.5. a) Dry cell weight (DW) after 24 h bacterial growth; b) specific growth rate (μ) data are presented. For other details, see legend to Fig. 1.

growth compared to pH 6.5, except in the *hyfG* mutant, where growth was stimulated ~1.2 fold as at pH 6.5.

Thus, the bacterial growth-stimulating effect of formate in combination with glycerol fermentation was pH-dependent. In contrast to pH 7.5, the bacterial growth-stimulating effect was observed at pH 6.5 at the late stationary phase for wild type and Hyd-1, Hyd-2 and Hyd-4; this effect was not determined for the Hyd-3-inactivated mutant. These effects can be compared with optimal glycerol fermentation at pH 6.3 [7,8]. Moreover, Hyd-3 was shown to be more active at acidic pH and upon formate supplementation. Hyd-3 activity via FHL may be required to supply CO_2 necessary for bacterial growth [8].

The situation was quite different with the investigation of bacterial specific growth rate (μ, h^{-1}): for wild type it was 1.00 ± 0.05 and higher compared with all mutants; formate alone and in combination of glycerol had no significant effects on μ at both pH 6.5 and pH 7.5 (Figs. 2b and 3b). μ for all mutants was suppressed, compared with wild type at both pHs: μ of *hyaB* and *hycE* mutants was similar 0.69 ± 0.03 h^{-1} upon glycerol fermentation at both pHs, whereas in *hybC* and *hyfG* mutants it was reduced ~1.1 fold and ~1.4 fold, respectively, at pH 6.5 compared to pH 7.5. In *hyaB* mutant, formate alone or with glycerol had inhibitory effect on μ for mutants with defects in Hyd-2 to Hyd-4 at both pHs: the obvious reduction of specific growth rate was detected in *hybC* and *hycE* mutants ~1.4 fold and ~1.7 fold at pH 6.5 and ~1.6 and ~2 fold at pH 7.5, respectively.

Thus, compared with wild type, μ was reduced in all Hyd 1–4 inactivated mutants: this effect on μ highlights the significant role of each Hyd enzyme during exponential growth phase, and it is partly in accordance with the later statement that the Hyd-2 and Hyd-3 activity, but not Hyd-1 or Hyd-4 is required for optimal bacterial growth and the maintenance of redox balance using glycerol as a sole carbon source [41] and seems to contradict data as a result of different growth conditions and assays [42]. Moreover, it was shown that Hyd-2 is important for growth of the bacterium when it grows with H_2 as electron donor and fumarate as electron acceptor [43,44].

As was mentioned above, Hyd 1–4 enzymes of *E. coli* form a H_2 cycle across the membrane [25], which together with a H^+ cycle is suggested to have an important role in modulating the cell's energetics. Disturbance of the cycle due to lack of one Hyd enzyme might have an effect on bacterial growth. The

formate inhibitory effect during bacterial exponential growth phase might be explained by that weak acid acting as an uncoupling agent that might destroy Δp leading to the bacterial growth reduction [22,34].

Effect of formate on ORP kinetics and H_2 production of E. coli *wild type and Hyd mutants upon glycerol fermentation at different pHs*

The purpose of this part of the study was to investigate the regulation of H_2 production and ORP kinetics in *E. coli* by external formate during glycerol fermentation. ORP kinetics and H_2 production were measured in bacterial batch cultures by the difference of pair of redox Pt and Ti–Si electrodes readings [12,16,21,36,38,41]. In the cells grown under fermentative conditions on glucose in the presence of 30 mM formate at pH 7.5 it was observed that H_2 production became mostly Hyd-3 dependent [21,22,32,34].

In this study the ORP kinetics, of *E. coli* wild type and Δ*hyaB*, Δ*hybC*, Δ*hycE*, Δ*hyfG* mutants at pH 6.5 and pH 7.5 under glycerol or formate alone or their co-utilization conditions were measured mainly by Pt electrode and are analyzed and illustrated (Figs. 4–7).

From the beginning of the lag growth phase the drop of two redox Pt and Ti–Si electrodes from positive to negative values was detected in wild type upon glycerol or formate alone or their combined conditions at pH 6.5 (Fig. 4). H_2 formation was observed in wild type with the yield of 0.75 ± 0.03 mmol H_2 L^{-1} at the end of exponential growth phase (7 h growth), formate supplementation led to 0.83 ± 0.05 mmol H_2 L^{-1} H_2 generation at early exponential phase (2 h growth), which was stimulated ~1.1 fold upon formate and glycerol co-supplementation at pH 6.5 (Fig. 4). The same situation of a stimulatory effect on H_2 evolution by formate or in combination with glycerol was observed at pH 7.5 (data not shown). This stimulation might be explained if formate produced via PFL is not exported through the formate channels because of supplemented external formate, but it can be further oxidized producing more H_2. It

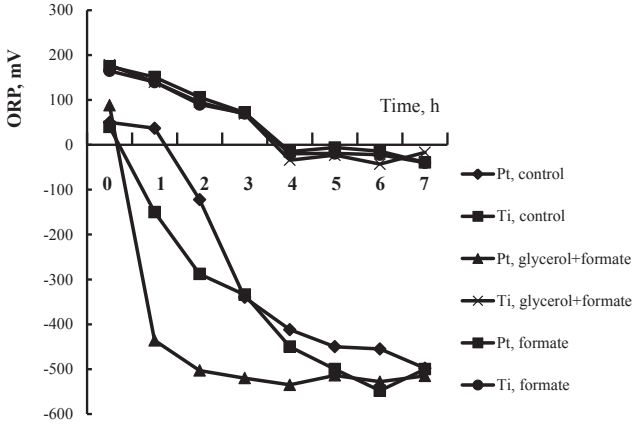

Fig. 4 – The ORP kinetics by *E. coli* BW 25113 wild type during glycerol fermentation at pH 6.5. ORP measured by Pt and Ti–Si (Ti) electrodes was expressed in mV (vs Ag/AgCl (saturated by KCl)). 10 g L^{-1} glycerol (G) and 10 mM formate (F) were added into the growth medium. Control was ORP kinetics in the medium with 10 g L^{-1} glycerol (G).

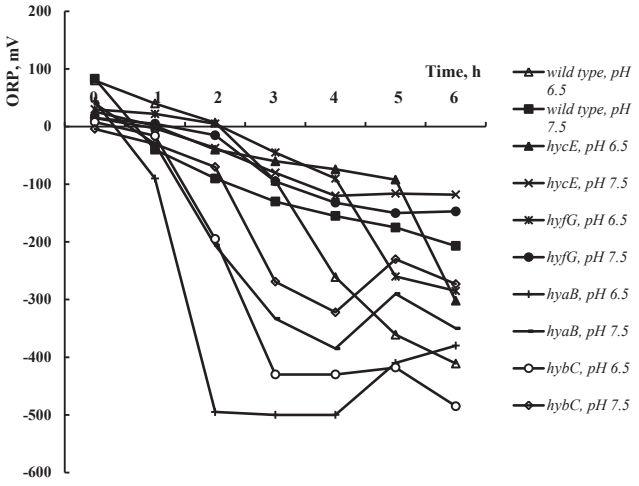

Fig. 5 – The ORP kinetics by *E. coli* wild type and mutants during glycerol fermentation at pH 6.5 and pH 7.5. 10 g L^{-1} glycerol was added into the growth medium. ORP measured by Pt electrode was expressed in mV (vs Ag/AgCl (saturated by KCl)).

should be noted that H_2 yield by *E. coli* wild type during glycerol fermentation at pH 6.5 (see above) was close to that determined using different method, for example, gas chromatography [45].

Increased H_2 production (1.45 to 2.20 ± 0.05 mmol H_2 L^{-1}) was detected during growth of the *hyaB* mutant upon all substrate combinations at pH 6.5 and formate alone at pH 7.5, except upon glycerol only fermentation at pH 7.5 at the beginning of exponential growth phase. H_2 was also produced in the *hybC* mutant during glycerol fermentation at pH 6.5 with the yield of 0.80 ± 0.05 mmol H_2 L^{-1}, and again stimulated ~1.2 to ~1.3 fold when formate was supplied alone or in combination with glycerol. H_2 production was absent at pH 7.5 upon glycerol-only fermentation in the *hybC* mutant and

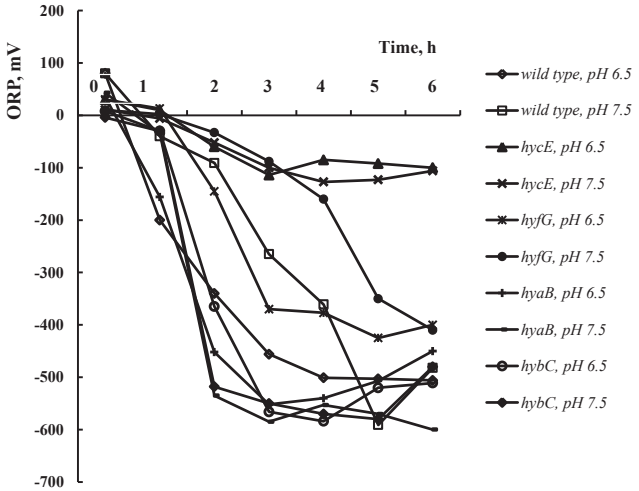

Fig. 6 – The ORP kinetics by *E. coli* wild type and mutants during formate fermentation at pH 6.5 and pH 7.5. 10 mM formate was added into the growth medium. For further details, see legend to Fig. 5.

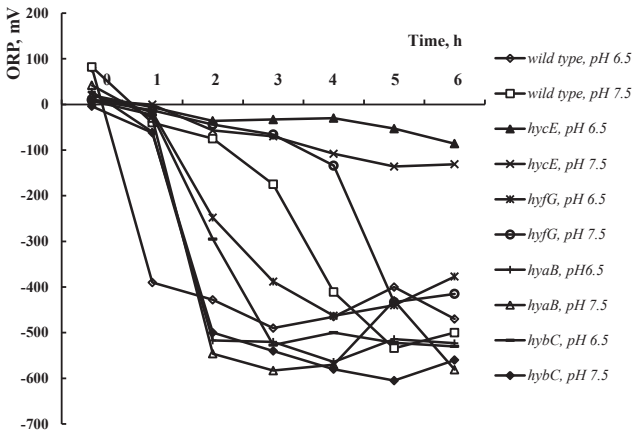

Fig. 7 – The ORP kinetics by *E. coli* wild type and hydrogenase mutants during co-fermentation of glycerol and formate at pH 6.5 and pH 7.5. 10 g L^{-1} glycerol and 10 mM formate were added into the growth medium. For further details, see legend to Fig. 5.

recovered upon formate supplementation. This is in accordance with the recent observation that, in contrast to what occurs during glucose fermentation, Hyd-2 has H_2-producing activity during glycerol fermentation at pH 7.5 [12] and formate increases activity of Hyd 3 [22,32].

In contract, H_2 production was absent in the *hycE* mutant during exponential phase growth with glycerol or formate alone or when both were supplied in combination (Figs. 5–7). The results point out the key role of Hyd 3 in H_2 production during glycerol fermentation and are in accordance with the findings obtained [15,42].

Low and delayed H_2 production was observed at 7.5 pH upon glycerol-only fermentation in the Δ*hyfG* mutant, whereas at pH 6.5 H_2 production was detected at the middle of exponential growth phase with the yield of 0.75 ± 0.05 mmol H_2 L^{-1} (see Fig. 5), which was stimulated ~1.5 fold with formate alone or with glycerol in combination at the beginning of exponential growth phase at 6.5 and the end of exponential growth phase at pH 7.5.

Thus, in the cells grown both with external formate and glycerol H_2 production was stimulated. This stimulation was similar for pH 7.5 and pH 6.5. The effect can be attributed to increased Hyd-3 enzyme activity during bacterial glycerol fermentation.

Conclusions

In the present study, H_2 producing activity and growth properties of *E. coli* during mixed carbon source (formate and glycerol) fermentation in batch culture were investigated using wild type and different mutants with inactivated Hyd enzymes at slightly alkaline and alkaline pHs. The main new findings are that: (i), all Hyd (Hyd 1–4) enzymes have a significant role for bacterial growth on glycerol; (ii), H_2 production and cell growth can be regulated by external formate in *E. coli* during glycerol fermentation; and (iii), Hyd-3 has a key role in H_2 metabolism at both pH 6.5 and pH 7.5 while Hyd-2 and Hyd-4 are important at pH 7.5 during glycerol and formate fermentation.

Mixed carbon fermentation (co-supplementation of formate with glycerol) might have advantages for industrial applications to enhance bio-hydrogen production; there are both economic (cheap wastes) and environmental (wastes utilization) benefits. However, the details of the underlying mechanisms how activity and regulation of Hyd enzyme synthesis and H_2 metabolism is achieved by bacteria during mixed carbon fermentation requires further study.

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REFERENCES

[1] Momirlan M, Veziroglu TN. The properties of hydrogen as fuel tomorrow in sustainable energy system for a cleaner planet. Int J Hydrog Energy 2005;33:795–802.
[2] Mudhoo A, Forsater-Carniero T, Sanchez A. Biohydrogen production and bioprocess enhancement: a review. Crit Rev Biotechnol 2011;31:250–63.
[3] Hallenbeck PC. Fermentative hydrogen production: principles, progress, and prognosis. Int J Hydrog Energy 2009;34:7379–89.
[4] Khanna S, Goyal A, Moholkar VS. Microbial conversion of glycerol: present status and future prospects. Crit Rev Biotechnol 2012;32:235–62.
[5] Clomburg JM, Gonzalez R. Anaerobic fermentation of glycerol: a platform for renewable fuels and chemicals. Trends Biotechnol 2013;31:20–8.
[6] Mattam AJ, Clomburg JM, Gonzalez R, Yazdani SS. Fermentation of glycerol and production of valuable chemical and biofuel molecules. Biotechnol Lett 2013;35:831–42.
[7] Gonzalez R, Murarka A, Dharmadi Y, Yasdani SS. A new model for the anaerobic fermentation of glycerol in enteric bacteria: trunk and auxiliary pathways in *Escherichia coli*. Metab Eng 2008;10:234–45.
[8] Dharmadi Y, Murarka A, Gonzalez R. Anaerobic fermentation of glycerol by *Escherichia coli*: a new platform for metabolic engineering. Biotechnol Bioeng 2006;94:821–9.
[9] Trchounian K, Pinske C, Sawers G, Trchounian A. Characterization of *Escherichia coli* [NiFe]-hydrogenase distribution during fermentative growth at different pHs. Cell Biochem Biophys 2012;62:433–40.
[10] Trchounian K, Poladyan A, Vassilian A, Trchounian A. Multiple and reversible hydrogenases for hydrogen production by *Escherichia coli*: dependence on fermentation substrate, pH and the F_0F_1-ATPase. Crit Rev Biochem Mol Biol 2012;47:236–49.
[11] Trchounian K, Trchounian A. *Escherichia coli* hydrogenase 4 (*hyf*) and hydrogenase 2 (*hyb*) contribution in H_2 production mixed carbon (glucose and glycerol) fermentation at pH 7.5 and pH 5.5. Int J Hydrog Energy 2013;38:3921–9.

[12] Trchounian K, Soboh B, Sawers G, Trchounian A. Contribution of hydrogenase 2 to stationary phase H_2 production by *Escherichia coli* during fermentation of glycerol. Cell Biochem Biophys 2013;66:103–8.
[13] Sawers G. Formate and its role in hydrogen production *Escherichia coli*. Biochem Soc Trans 2005;33:42–6.
[14] McDowall JS, Murphy BJ, Haumann M, Palmer T, Armstrong AF, Sargent F. Bacterial formate hydrogenlyase complex. Proc Nat Acad Sci U S A 2014;111:E3948–56.
[15] Sanchez-Torres V, Wood TK. *Escherichia coli* hydrogenase 3 is a reversible enzyme possessing hydrogen uptake and synthesis activities. Appl Microbiol Biotechnol 2007;76:1035–42.
[16] Abrahamyan V, Poladyan A, Vassilian A, Trchounian A. Hydrogen production by *Escherichia coli* during glucose fermentation: effects of oxidative and reductive routes using the strain lacked of hydrogen oxidizing hydrogenases 1 (*hya*) and 2 (*hyb*). Int J Hydrog Energy 2015;40:7459–64.
[17] King PW, Przybyla AE. Response of *hya* expression to external pH in *Escherichia coli*. J Bacteriol 1999;181:5250–6.
[18] Richard DJ, Sawers G, Sargent F, McWalter L, Boxer DH. Transcriptional regulation in response to oxygen and nitrate of the operons encoding the [NiFe] hydrogenases 1 and 2 of *Escherichia coli*. Microbiology 1999;145:2903–12.
[19] Redwood MD, Mikheenko IP, Sargent F, Macaskie LE. Dissecting the roles of *Escherichia coli* hydrogenases in biohydrogen production. FEMS Microbiol Lett 2008;278:48–55.
[20] Andrews SC, Berks BC, McClay J, Ambler A, Quail MA, Golby P, et al. A 12-cistron *Escherichia coli* operon (*hyf*) encoding a putative proton-translocating formate-hydrogenlyase system. Microbiology 1997;143:3633–47.
[21] Bagramyan K, Mnatskanyan N, Poladian A, Vassilian A, Trchounian A. The roles of hydrogenases 3 and 4, and the F_0F_1-ATPase, in H_2 production by *Escherichia coli* at alkaline and acidic pH. FEBS Lett 2002;516:172–8.
[22] Mnatsakanyan N, Bagramyan K, Trchounian A. Hydrogenase 3 but not hydrogenase 4 is major in hydrogen gas production by *Escherichia coli* formate hydrogenlyase at acidic pH and in the presence of external formate. Cell Biochem Biophys 2004;41:357–66.
[23] Trchounian K, Pinske C, Sawers G, Trchounian A. Dependence on the F_0F_1-ATP synthase for the activities of the hydrogen-oxidizing hydrogenases 1 and 2 during glucose and glycerol fermentation at high and low pH in *Escherichia coli*. J Bioenerg Biomembr 2011;43:645–50.
[24] Blbulyan S, Avagyan A, Poladyan A, Trchounian A. Role of *Escherichia coli* different hydrogenases in H^+ efflux and the F_OF_1-ATPase activity during glycerol fermentation at different pH. Biosci Rep 2011;31:179–84.
[25] Trchounian A, Sawers RG. Novel insights into the bioenergetics of mixed-acid fermentation: can hydrogen and proton cycles combine to help maintain a proton motive force? Int Union Biochem Mol Biol Life 2014;66:1–7.
[26] Sawers RG, Blokesch M, Bock A. Anaerobic formate and hydrogen metabolism [Chapter 3.5.4]. In: Curtiss III R, editor. *EcoSal–Escherichia coli and Salmonella*. Cellular and molecular biology. Washington, DC: ASM Press; 2004 [Online], http://www.ecosal.org.
[27] Pinske C, Jaroschinsky M, Linek S, Kelly CL, Sargent F, Sawers RG. Physiology and bioenergetics of [NiFe]-hydrogenase 2-catalyzed H_2-consuming and H_2-producing reactions in *Escherichia coli*. J Bacteriol 2015;197:296–306.
[28] Doberenz C, Zorn M, Falke D, Nannemann D, Hunger D, Beyer L, et al. Pyruvate formate-lyase interacts directly with the formate channel FocA to regulate formate translocation. J Mol Biol 2014;426:2827–39.
[29] Hakobyan M, Poladyan A, Bagramyan K. Energy transformation coupled to formate oxidation during anaerobic fermentation. Biophysics 2006;51:418–22.
[30] Rossmann R, Sawers G, Bock A. Mechanism of regulation of the formate-hydrogenlyase pathway by oxygen, nitrate, and pH: definition of the formate regulon. Mol Microbiol 1991;5:2807–14.
[31] Leonhartsberger S, Korsa I, Böck A. The molecular biology of formate metabolism in enterobacteria. J Mol Microbiol Biotechnol 2002;4:269–76.
[32] Mnatsakanyan N, Vassilian A, Navasardyan L, Bagramyan K, Trchounian A. Regulation of *Escherichia coli* formate hydrogenlyase activity by formate at alkaline pH. Curr Microbiol 2002;45:281–6.
[33] Seol E, Jang Y, Kim S, Oh Y-K, Park S. Engineering of formate-hydrogen lyase gene cluster for improved hydrogen production in *Escherichia coli*. Int J Hydrog Energy 2012;37:15045–51.
[34] Trchounian K, Trchounian A. Different role of *focA* and *focB* encoding formate channels for hydrogen production by *Escherichia coli* during glucose or glycerol fermentation. Int J Hydrog Energy 2014;39:20987–91.
[35] Tran KT, Maeda T, Sanchez-Torres V, Wood TK. Beneficial knockouts in *Escherichia coli* for producing hydrogen from glycerol. Appl Microbiol Biotechnol 2015;99:2573–81.
[36] Gabrielyan L, Sargsyan H, Hakobyan L, Trchounian A. Regulation of hydrogen photoproduction in *Rhodobacter sphaeroides* batch culture by external oxidizers and reducers. Appl Energy 2014;131:20–5.
[37] Poladyan A, Avagyan A, Vassilian A, Trchounian A. Oxidative and reductive routes of glycerol and glucose fermentation by *Escherichia coli* batch cultures and their regulation by oxidizing and reducing reagents at different pHs. Curr Microbiol 2013;66:49–55.
[38] Sasahara KC, Heinzinger NK, Barrett E. Hydrogen sulfide production and fermentative gas production by *Salmonella typhimurium* require F_0F_1 ATPase activity. J Bacteriol 1997;179:6736–40.
[39] Maeda T, Wood TK. Formate detection by potassium permanganate for enhanced hydrogen production in *Escherichia coli*. Int J Hydrog Energy 2008;33:2409–12.
[40] Piskarev M, Ushkanov VA, Aristova NA, Likhachev PP, Myslivets TS. Establishment of the redox potential of water saturated with hydrogen. Biophysics 2010;55:19–24.
[41] Sargsyan H, Gabrielyan L, Hakobyan L, Trchounian A. Light–dark duration alternation effects on *Rhodobacter sphaeroides* growth, membrane properties and bio-hydrogen production in batch culture. Int J Hydrog Energy 2015;40:4084–91.
[42] Sanchez-Torres V, Yusoff MZ, Nakano C, Maeda T, Ogawa H, Wood TK. Influence of *Escherichia coli* hydrogenases on hydrogen fermentation from glycerol. Int J Hydrog Energy 2013;38:3905–12.
[43] Sawers RG, Ballantine SP, Boxer DH. Differential expression of hydrogenase isoenzymes in *Escherichia coli*: evidence for a third isoenzyme. J Bacteriol 1985;164:1324–31.
[44] Ballantine S, Boxer DH. Nickel-containing hydrogenase isoenzymes from anaerobically grown *Escherichia coli* K-12. J Bacteriol 1985;163:454–9.
[45] Murarka A, Dharmadi Y, Yazdani SS, Gonzalez R. Fermentative utilization of glycerol by *Escherichia coli* and its implications for the production of fuels and chemicals. Appl Environ Microbiol 2008;74:1124–35.