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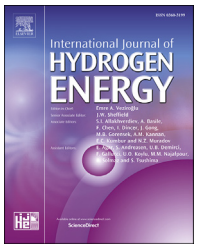

Hydrogen production by *Escherichia coli* growing in different nutrient media with glycerol: Effects of formate, pH, production kinetics and hydrogenases involved

Karen Trchounian [a,b], *Satenik Mirzoyan* [a,b], *Anna Poladyan* [a,b], *Armen Trchounian* [a,b,*]

[a] *Department of Biochemistry, Microbiology and Biotechnology, 1 A. Manoukian Str., 0025 Yerevan, Armenia*
[b] *Research Institute of Biology, Biology Faculty, Yerevan State University, 1 A. Manoukian Str., 0025 Yerevan, Armenia*

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ABSTRACT

The *Escherichia coli* BW25113 or MC4100 wild type parental strains growth and H_2 production kinetics was studied in batch cultures of minimal salt medium (MSM) and peptone medium (PM) at pH of 5.5–7.5 upon glycerol (10 g L^{-1}) fermentation and formate (0.68 g L^{-1}) supplementation. The role of formate alone or with glycerol on growth and H_2 production via hydrogenases (Hyd) was investigated in double *hyaB hybC* (lacking large subunits of Hyd 1 and 2), triple *hyaB hybC hycE* (lacking large subunits of Hyds 1-3) and sole *selC* (lacking formate dehydrogenase H) mutants during 24 h bacterial growth. H_2 production was delayed and observed after 24 h bacterial wild type strains growth on MSM. Moreover, it reached the maximal values after 72 h growth at the pH 6.5 and pH 7.5. Biomass formation of the mutants used was inhibited ~3.5 fold compared with wild type, and H_2 production was absent in *hyaB hybC hycE* and *selC* mutants upon glycerol utilization on MSM at pHs of 5.5–7.5. Formate inhibited bacterial growth on MSM with glycerol, but enhanced and recovered H_2 production by *hybC* mutant at pH 7.5. H_2 evolution was delayed at pH 7.5 in PM, but observed and stimulated at pH 6.5 upon glycerol and formate utilization in *hyaB hybC* mutant. H_2 production was absent in *hyaB hybC hycE* and *selC* mutants upon glycerol, formate alone or with glycerol fermentation at pH 6.5 and pH 7.5; formate supplementation had no effect. The results point out *E. coli* ability to grow and utilize glycerol in MSM with comparably high H_2 yield: as well as they suggest the key role of Hyd-3 at both pH 6.5 and pH 7.5 and the role of Hyd-2 and Hyd-4 at pH 7.5 in H_2 production by *E. coli* during glycerol fermentation with formate supplementation. The results obtained are novel and might be useful in H_2 production biotechnology development using different nutrient media and glycerol and formate as feedstock.

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

Currently considerable interest is focused on molecular hydrogen (H_2), because its energetic value is the highest among all known fuels and, at the same time, it is renewable and clean energy source with no greenhouse gas emission upon combustion [1]. Moreover, biological methods and economic ways of H_2 production from cheap sources such as industrial and agricultural organic wastes are highlighted topics now [2]. So, H_2 can be proposed as an inexpensive solution to the problem of energy demand and environment pollution in near future.

Based on research reports, glycerol, as a carbon source, can be fermented by microbes, particularly by *Escherichia coli*, with the formation of H_2 and other valuable chemical compounds during mixed-acid fermentation [2–4]. Glycerol is a waste product of various industrial and agricultural processes, especially biodiesel production, which made it much more inexpensive in recent years [2–4]. Moreover, mixed carbon sources (mixed carbon) are widely available everywhere – in industrial and agricultural wastes, as well as in household and municipal ones, human intestine etc., and their role in bacterial physiology as well as sole and combined influence on biotechnological applications, particularly on cell growth, biomass formation and H_2 production, are of importance to study.

H_2 is produced upon formate oxidation by *E. coli* during both glycerol and glucose fermentation via operation of membrane-associated formate hydrogen lyase (FHL) complex [5–7]. The latter is composed of formate dehydrogenase H (FDH-H) and hydrogenase (Hyd) 3 or 4, and depending on glucose utilization at acidic or alkaline pHs these enzymes form FHL-1 and FHL-2 pathways, respectively [8,9]. Interestingly, FDH-H has been determined to be important for Hyd activity under glycerol fermentation at different pHs [5,10], however this dependence should be clarified. Four Hyds (1-4) encoded by *hya, hyb, hyc* and *hyf* operons, respectively, contribute to H_2 metabolism in *E. coli* [2,5]. Many factors such as external pH, substrate of fermentation (glucose and/or glycerol), their concentrations, oxidation-reduction potential (ORP) etc. may influence on the activity and operation direction (H_2 formation or oxidation) of Hyds [8–12]. Indeed, Hyd-1 and Hyd-2 operation in H_2 oxidation and H_2 formation modes upon glucose and glycerol fermentation, respectively, was shown [6,11,12]. It should be noted, that Hyd-1 optimal activity was observed at acidic pH, non-reduced conditions and in the presence of formate under anaerobic conditions, whereas for Hyd-2 activity alkaline and more reduced conditions were stated [13,14].

Originally, formate was secreted out of the cells in fermenting *E. coli* culture most probably for maintaining cytoplasmic pH [2,15]. When external medium pH drop is achieved, formate is going back into the cells and induces Hyd 3 activity via the FhlA transcriptional activator protein [16,17]. However, being a weak acid, formate might also affect as uncoupling agent and distract the proton motive force (Δp) [18]. According to literature, two FNT (formate-nitrite transporter) membrane channels, FocA and FocB, contribute to formate translocation across the membrane in *E. coli* [16,19]. However, compared with FocB the role and structure of FocA is better characterized. It was demonstrated, that two glycyl-radical enzymes, pyruvate formate-lyase (PflB) and 2-ketobutyrate formate-lyase (TdcE), might catalyze formate formation as a product of fermentation during anaerobic growth of *E. coli* [17]. Moreover, the TdcE and PflB proteins specific interaction with FocA regulates formate transport [16,17]. It is interesting that during glycerol fermentation formate might be imported through FocB, whereas formate is exported preferentially through FocA at pH 7.5 [19]. So, the control of formate transport and metabolism of bacterial cells is very complicated and probably directed to balance the harming effect of formate excess and the loss of an important source of reducing ability.

The relationship between all Hyd enzymes with formation of H_2 cycle in the membrane of *E. coli* was also proposed [18]. Moreover, the activity of some of Hyd enzymes might be dependent on Δp, particularly, on the H^+ translocating F_0F_1-ATPase activity, generating Δp, thus linking of the H^+ cycle to the H_2 cycling during fermentation [10,20]. Consequently, the primary role of these two cycles ($\Delta p/H_2$) in controlling the energetics of the bacterial cell during mixed-acid fermentation, mainly in response to pH was suggested [18]. However, the nature of the link and coordinated operation of Hyd enzymes and the F_0F_1-ATPase and their role in bacterial cell physiology should be still clarified.

Moreover, in our previous study the effect of formate alone or mixed with glycerol on growth of *E. coli* wild type and Hyd single mutants with deletions of key subunits of Hyd 1-4, respectively, and on H_2 production was investigated at different pHs [21–23]. The results suggested the important role of Hyd-3 at 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* upon glycerol only and its fermentation with formate supplementation.

As medium composition, carbon sources, pH, ORP etc., are significant both for bacterial growth and H_2 metabolism [8,10,12,21], the aim of the present work was to continue the study of physiology of *E. coli* in different nutrient media with various substrates utilization and at different pHs. Particularly, bacterial utilization of only glycerol as carbon source in the minimal salt medium, without other nutrient addition, and its comparison with the media rich of nutrients and upon other substrates utilization were of interest. And H_2 production might vary in different nutrient media with glycerol. Thus, bacterial growth of a sufficient amount of biomass, pH decrease, ORP kinetics and H_2 production were investigated upon only glycerol fermentation in poor, minimal salt medium (MSM) at pHs of 5.5–7.5. The results were compared with those obtained with rich, peptone medium (PM).

Taking into the account the relationship of four Hyd enzymes both with each other and in the formation of H_2 cycle, novel approaches (group of Hyd candidates) are considered in the present study to interrupt the H_2 cycling and to investigate H_2 production upon glycerol fermentation and externally supplied formate. Particularly, the role of formate alone or with glycerol on ORP kinetics and H_2 production was investigated in double *hyaB hybC* (lacking large subunits of Hyd 1 and 2; triple *hyaB hybC hycE* (lacking large subunits of Hyds 1-3), and *selC* (lacking FDH-H) mutants [10,24] during growth in bacterial batch culture up to 72 h.

Materials and methods

Bacterial strains, growth conditions and growth characteristics determination

The *E. coli* wild type BW25113 or MC4100 parental and different Hyd mutant strains details are presented in Table 1. For comparison K12(λ) wild type strain was also used (see Table 1). The strains were provided by Prof. R.G. Sawers (Institute of Biology/Microbiology, Martin Luther University of Halle-Wittenberg, Halle, Germany). Note *selC* gene is required for the biosynthesis of FDH-H especially incorporation of selenocysteine into FDH-H [24,25] which interacts with Hyd 3 or Hyd 4 to form FHL pathways under glucose utilization at different pHs, as suggested [5–7,10]. This gene was also important for Hyd activity under glycerol fermentation [10].

Bacteria were grown anaerobically in batch culture at 37 °C. The bacterial culture was attained in 150 mL glass vessels covered by plastic press-caps and composed of 120 mL MSM and PM [21,23,26]. The MSM contained 8.004 g L^{-1} K_2HPO_4, 3.128 g L^{-1} KH_2PO_4, 1.056 g L^{-1} $(NH_4)SO_4$, 0.048 g L^{-1} $MgSO_4$, 0.009 g L^{-1} $FeSO_4$; whereas PM was the mixture of 20 g L^{-1} peptone, 2 g L^{-1} K_2HPO_4, 5 g L^{-1} NaCl; pH 6.5 or pH 7.5. 10 g L^{-1} glycerol or 0.68 g L^{-1} (10 mM) formate were added, when mentioned in the text. The concentrations of glycerol and formate used have been shown before [10–12,19,21–23] to be optimal The pH was measured by pH selective electrode of HJ1131B, Hanna Instruments (Portugal) pH-meter and adjusted by 0.1 M NaOH or KOH or 0.1 N HCl. It should be mentioned that different inoculums (MSM with glucose or glycerol, or PM with glucose or glycerol) were tasted to initiate the growth on MSM. PM with 2 g L^{-1} (0.2%) glucose was the preferable medium to cultivate bacteria under such an energy limited conditions as growth on and glucose was used to have all Hyds expressed and to get well adapted culture for further growth in different media especially with glycerol [12,14].

The bacterial biomass growth was studied with the help of spectrophotometer Spectro UV–Vis Auto, Labomed (USA) monitoring the optical density (OD) readings of bacterial culture absorbance under 600 nm. The bacterial specific growth rate (μ) stated, as lg2/doubling time, was calculated where the logarithm of OD was growing linearly with time. Eventually, bacterial growth yield was estimated by bacterial culture dry weight (CDW) and was expressed in g L^{-1}, as done before [23].

The ORP and H_2 production determination during bacterial growth

ORP of bacterial culture and H_2 production were determined with two redox electrodes; platinum (Pt; EPB-1, Gomel State Enterprise of Electrometric Equipment (GSEEE), Gomel, Belarus); or PT42BNC, HANNA Instruments, Portugal) and titanium-silicate (Ti–Si; EO-02, GSEEE, Gomel, Belarus) [12,19,21–23]. In contrast to Ti–Si electrode, Pt electrode was sensitive to H_2 or O_2, and its readings drop to negative values (<−400 mV) confirmed the formation H_2 in the medium under anaerobic conditions. The difference between two (Pt and Ti–Si) electrodes readings was used to determine the rate of H_2 produced under certain conditions [21], as well as to calculate H_2 yield expressing in mmol H_2 L^{-1}, as detailed [12,23]. The mentioned method is close to that of different group [24], and it gave more accurate results of cumulative H_2 yield in liquids.

During the growth of *E. coli*, H_2 production was confirmed by the appearance of gas bubbles in the test tubes over the bacterial suspension with the help of Durham tubes, and it was verified by chemical reaction of $KMnO_4$ solution in H_2SO_4 with H_2, as before [12,14,20].

Reagents and data processing

Glycerol, peptone (Carl Roths GmbH, Germany) and other reagents of analytical grade were applied in the study. Three independent experiments were done and the average data were calculated with the standard errors, and Student's *t*-test was used to analyze the statistically significant differences between different series of experiments [11,23]; the difference was valid if Student criteria (P) < 0.05.

Results

E. coli wild type and Hyd mutants growth and H_2 production in minimal salt growth medium during glycerol fermentation at pHs of 5.5–7.5

The *E. coli* BW25113 or MC4100 wild type parental (in addition K12(λ) wild type strain) and different Hyd mutant strains (see Table 1) growth and medium acidification were studied upon 10 g L^{-1} glycerol utilization in MSM at pH of 5.5–7.5 (Fig. 1). The salts of the medium provide basic ionic buffering for the cells, and also provide an environment with comfortable osmotic properties [27]. Moreover, it has the advantage of being cheap.

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

Strains	Genotype	Absent subunits of proteins	References
K12 (λ)	Wild type		Laboratory stock
BW25113	*lacI*^{q} *rrnB*$_{T14}$ *ΔlacZ*$_{W116}$ *hsdR514 ΔaraBAD*$_{AH33}$ *Δrha BAD*$_{LD78}$	Wild type parental strain	[11,23]
JW 0955[a]	BW 25113 Δ*hyaB*::Kan	Large subunit of Hyd-1	[11,23]
JW 2962	BW 25113 Δ*hybC*	Large subunit of Hyd-2	[11,23]
MC4100	*araD139 Δ(argF-lac)U169 ptsF relA1 fib5301 rpsL150*	Wild type parental strain	[8]
FTD147	MC 4100 Δ*hyaB* Δ*hybC* Δ*hycE*	Large subunits of Hyd 1, Hyd 2 and Hyd 3	[10,24]
FM460[a]	MC 4100 Δ*selC400*::Kan	tRNA^{sec}	[10,24]

[a] Resistant to kanamycin (Kan).

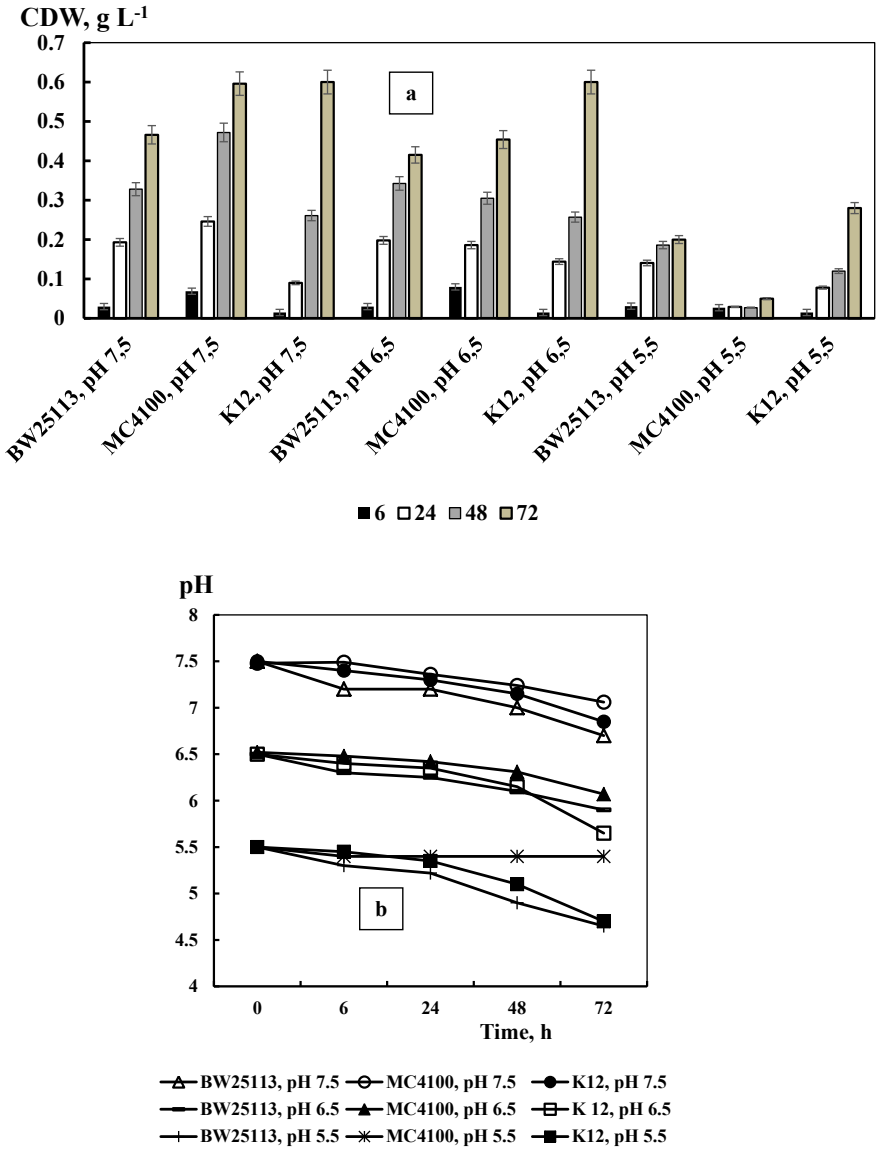

Fig. 1 – *E. coli* BW25113, MC4100 wild type parental and K12 (λ) wild type strains growth in MSM during glycerol fermentation at pHs 5.5 to 7.5. a) Biomass formation, culture dry cell weight (CDW) (g L^{-1}) was determined in 6 h, 24 h and 72 h of bacterial growth; b) pH changes during growth of bacteria. 10 g L^{-1} glycerol was supplemented into MSM The errors bars were within the designations.

There were no important differences among the data of BW25113 and K12(λ) strains, expect MC4100, which had significant difficulties to grow at pH 5.5 (see Fig. 1).

Compared with the data of bacterial growth on PM, physiological adaptation of *E. coli* cells to MSM was prolonged; a longer lag-phase was observed during anaerobic growth on MSM. Bacterial growth was ~10 fold inhibited in MSM at all pHs at the 6th h of growth (see Fig. 1). The BW25113 growth was ~10 fold inhibited in MSM at all pHs at the 6th h of growth (see Fig. 1). Whereas, after 24 h growth bacterial biomass formation was achieved with 0.143 g L^{-1}, 0.204 g L^{-1} and 0.2 g L^{-1} (CDW, see Materials and methods), at pH 5.5, pH 6.5 and pH 7.5, respectively. Moreover, at the 72nd h of bacterial growth, surprisingly, biomass reached up to ~0.26 g L^{-1}, 0.423 g L^{-1} and 0.474 g L^{-1} at different pHs (as above), respectively. The medium pH drop was noticeable, when bacterial growth reached 72 h: it was 4.7, 6.0 and 6.7 upon growth in MSM with initial pH 5.5, pH 6.5 and pH 7.5, respectively (Fig. 1b). It was stated, that starting from the end of the log growth phase, the drop of two redox Pt and Ti–Si electrodes readings from positive to low negative values (−450 ± 5 mV for (Pt)) was detected in *E. coli* wild type upon glycerol fermentation in PM, pointing out the H_2 yield of ~0.75 mmol H_2 L^{-1}, as shown before [21,23]. *E. coli* wild type ORP drop and H_2 production were also observed in MSM at pH 5.5, pH 6.5 and pH 7.5 (Fig. 2, Table 2): but the drop of Pt electrode reading was delayed and reached up to −490 ± 10 mV, −550 ± 15 mV and −580 ± 10 mV with the ~1.4 mmol H_2 L^{-1}, ~2.2 mmol H_2 L^{-1} and ~3.62 mmol H_2 L^{-1} yield of H_2 at different pHs (as above), respectively. These data on H_2 production

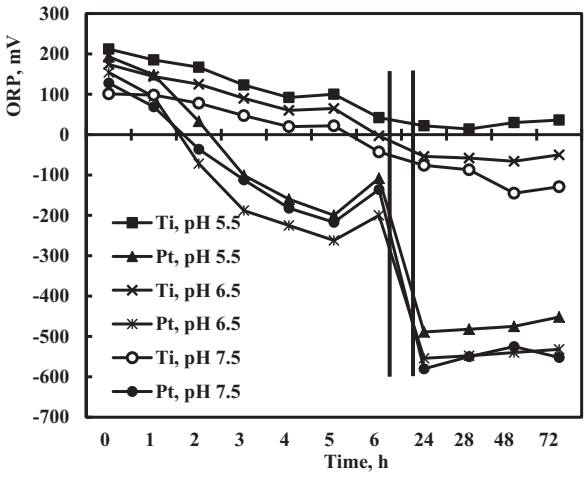

Fig. 2 – The kinetics of ORP by the *E. coli* BW25113 strain during glycerol fermentation at pH 5.5 to pH 7.5 in MSM. ORP measured by Pt and Ti–Si electrodes was expressed in mV (vs Ag/AgCl (saturated by KCl)). 10 g L^{-1} glycerol was supplemented into PM. The errors bars were within the designations.

were confirmed (data not shown) by the other methods used (see Materials and methods). The results are of interest to obtain bacterial biomass, using different media with glycerol and/or formate supplemented.

It was stated, that starting from the end of the log growth phase, the drop of two redox Pt and Ti–Si electrodes readings from positive to low negative values (−450 ± 5 mV for (Pt)) was detected in *E. coli* wild type upon glycerol fermentation in PM, pointing out the H_2 yield of ~0.75 mmol H_2 L^{-1}, as shown before [21,23]. ORP drop and H_2 production by BW25133 were also observed in MSM at pH 5.5, pH 6.5 and pH 7.5 (Fig. 2, Table 2): but the drop of Pt electrode reading was delayed and reached up to −490 ± 10 mV, −550 ± 15 mV and −580 ± 10 mV with the ~1.4 mmol H_2 L^{-1}, ~2.2 mmol H_2 L^{-1} and ~3.62 mmol H_2 L^{-1} yield of H_2 at different pHs (as above), respectively. Again, there were no noticeable differences among wild type strains used, apart from MC 4100, which had ~2 times less H_2 producing activity at pH 5.5 (data not shown). These parental strains has numerous mutational differences with each other (see Table 1) and from K12(λ) [32]. Some of them are probably responsible for adaptation to different pHs, thus lead to pour biomass formation and H_2 producing activity on MSM and at acidic pH 5.5: a further study is required.

Formate alone or formate with glycerol supplementation has been determined to lead to ~2–3 fold less biomass formation by MC4100 compared with only glycerol fermentation (Fig. 3a). This was in contrast to bacterial growth on PM with glycerol, as shown [23], It should be noted that the biomass formation of all mutants used was suppressed upon growth on MSM at pH 5.5 to pH 7.5 compared with wild type parental strains data (Fig. 3a). The results point out the critical role of hydrogen metabolism for bacterial growth. The strong inhibitory effect of formate on bacterial growth at pH 5.5 was shown earlier [26], so pH 5.5 was not considered in further experiments.

Though in the experiment with formate only supplementation of formate led to less biomass (~0.084 CDW g L^{-1}) formation, but enhanced up to ~3.62 mmol H_2 L^{-1} yield of H_2 was already observed at 6th h of growth, the addition of formate stimulated H_2 production in *hyaB* mutant ~2.5 fold (Fig. 3,b). As formate cannot be neutralized by FHL defective mutant strain, formate excess both in and out of the cells might have toxic effect on cell metabolism [22,23]. H_2 production was abolished in *selC* mutant during bacterial growth upon glycerol or/and formate fermentation at pH 7.5: Pt electrode reading drop up to only −130 ± 2 mV or −150 ± 3 mV was observed (see Fig. 3b). Moreover, combined supplementation of formate and glycerol had no effect at all (Fig. 3b). H_2 production was not observed too upon *hybC* mutation (defect in Hyd 2) and in *hyaB hybC hycE* (with functional only Hyd 4) (see Table 1), but upon formate addition in *hybC* H_2 producing activity was observed with the yield of ~2.2 mmol H_2 L^{-1} (see Fig. 3b). Formate might lead to Hyd 3 enzyme activation, as was previously shown [15,22], and thus recover H_2 production. Thus, upon bacterial growth on MSM formate leads to enhanced H_2 production, but, on the other hand, as an weak acid formate may negatively affect bacterial growth [22]. The obtained results at pH 7.5 were not significantly different from those at pH 6.5, expect *hybC* mutation: in contract to pH 7.5, at these conditions H_2 was produced with the yield of ~0.82 mmol H_2 L^{-1} (data not shown). It should be noted that these data on H_2 production were confirmed (data not shown) by the other methods used (see Materials and methods.

Hence, the results confirm, that under certain condition at pH 7.5 Hyd 2, Hyd 3 and Hyd 4 are responsible for H_2 production.

Table 2 – Oxidation-reduction potential (ORP) drops and H_2 production yields of the *E. coli* BW25113 strain upon growth on different growth medium upon 10 g L^{-1} glycerol fermentation.

Growth medium pH	MSM		PM	
	ORP[a], mV	H_2 production yield[b], mmol H_2 L^{-1}	ORP, mV	H_2 production yield[b], mmol H_2 L^{-1}
7.5	−580 ± 10	3.62 ± 0.02	−400 ± 10	0.73 ± 0.02
6.5	−550 ± 15	2.20 ± 0.02	−415 ± 15	0.75 ± 0.02
5.5	−490 ± 10	1.40 ± 0.02	−380 ± 15	0.64 ± 0.03

[a] ORP measured by Pt electrode.
[b] H_2 production yields after 24 h of bacterial growth in MSM and 6 h growth in PM.

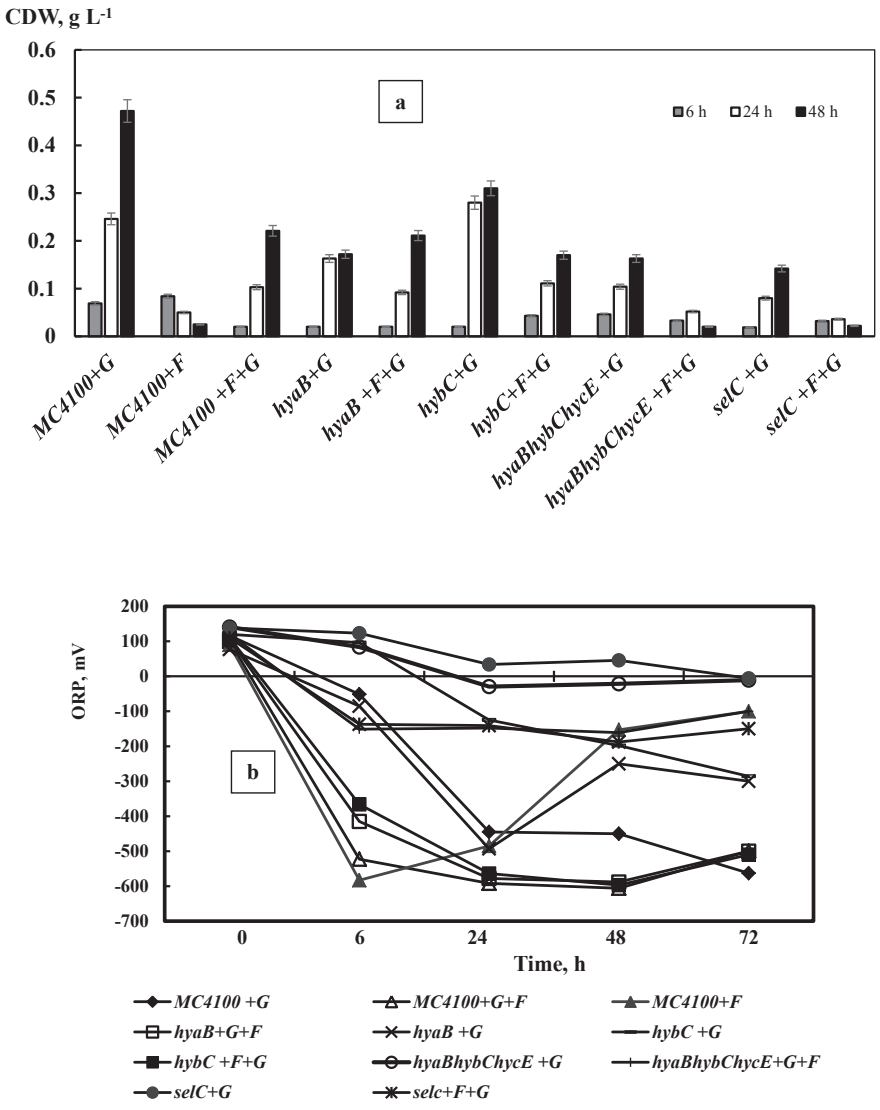

Fig. 3 – The growth (a) and kinetics of ORP (b) of the *E. coli* MC 4110 wild type parental and mutant strains during glycerol fermentation. Bacteria were grown at pH 7.5. 10 g L^{-1} glycerol (G) was supplemented and 0.68 g L^{-1} formate (F) was supplemented into MSM when mentioned Pt electrode readings are presented. For strains used see Table 1; for details, see Materials and methods and legend to Fig. 2.

H_2 production kinetics during E. coli *wild type and Hyd mutants growth on formate and/or glycerol supplementation*

ORP kinetics and H_2 production were investigated in *E. coli* wild type parental strains and double *hyaB hybC*, triple *hyaB hybC hycE* and sole *selC* mutants (see Table 1) for 24 h of bacterial batch culture growth upon 10 g L^{-1} glycerol alone or with 0.68 g L^{-1} formate supplementation at pH 6.5 and pH 7.5 in PM (see Materials and methods). The inhibitory effect of formate on bacterial growth at pH 5.5 was shown earlier [26], so pH 5.5 was not considered in further experiments. Two wild type strains had similar ORP kinetics and H_2 production. Indeed, starting from the end of the log growth phase, the drop of two redox Pt and Ti–Si electrodes readings from positive to low negative values (−450 ± 5 mV for (Pt)) was detected in *E. coli* wild type upon glycerol fermentation, pH 6.5, in PM (Fig. 4), pointing out the H_2 formation with the ~0.75 mmol H_2 L^{-1} yield. In case of only glycerol fermentation, H_2 was evolved in the middle of the log phase while during glycerol and formate fermentation H_2 production was observed and stimulated 1.2 fold during the early log phase (see Fig. 4).

As formate cannot be neutralized by FHL defective mutant strain, formate excess both in and out of the cells might have toxic effect on cell metabolism [22,23]. H_2 production was abolished in *selC* mutant during bacterial growth upon glycerol or/and formate fermentation at pH 6.5 and pH 7.5. Moreover, combined supplementation of formate and glycerol had no effect at all (Fig. 4a and b): Pt electrode reading drop up to only −30 ± 2 mV or −50 ± 3 mV was observed (Fig. 4c). The same behavior (abolished H_2 formation) was observed after 144 h bacterial growth (data not shown). It should be noted, that growth of the mutant was ~2 fold suppressed compared with wild type (data not shown). The

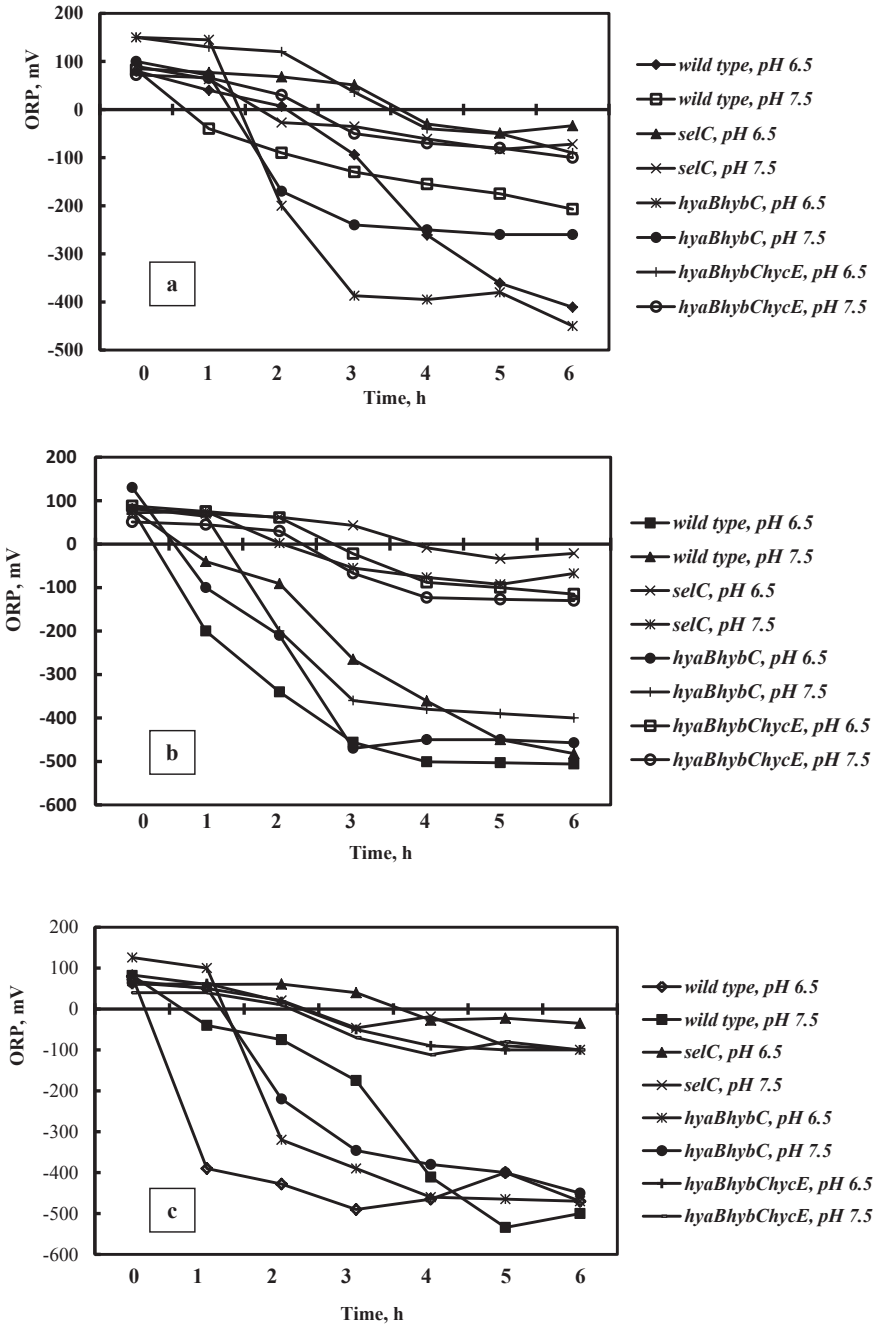

Fig. 4 – The kinetics of ORP by the *E. coli* BW25113 wild type parental and Hyd mutant strains during glycerol fermentation at pH 6.5 and pH 7.5. a) 10 g L^{-1} glycerol was supplemented into PM; b) 0.68 g L^{-1} formate was supplemented into PM; c) 0.68 g L^{-1} formate and 10 g L^{-1} glycerol were added into PM. Pt electrode readings are presented. For strains used see Table 1; for details, see Materials and methods and legend to Fig. 2.

same situation (no H_2 production) was observed in triple *hyaB hybC hycE* mutant, with the lack of Hyds 1-3 and with only one Hyd enzyme (Hyd 4) operating during the log growth phase upon glycerol or/and formate fermentation at pH 6.5 and pH 7.5, and formate supplementation had no effect again (see Fig. 4a and b).

In our previous study low and delayed H_2 production was shown at pH 7.5 upon glycerol only fermentation in Hyd 4 defective mutant, whereas at pH 6.5 H_2 production was detected in the middle of log growth phase, which was stimulated ~1.5 fold upon formate alone or with glycerol combination at the beginning of log growth phase at pH 6.5 and the end of log growth phase at pH 7.5 [21,23]. Again, H_2 evolution was delayed at pH 7.5, but was observed, even stimulated at pH 6.5 upon glycerol and formate utilization in double *hyaB hybC* mutant. This was in accordance with the recent observation of our group that, in contrast to glucose fermentation, Hyd 2 had H_2 producing activity upon glycerol fermentation at pH 7.5 [12]. This was also confirmed by the other group [28].

Discussion

As was mentioned above, glycerol can be fermented by *E. coli* with the formation of H_2 and other valuable products [3,4]. Among them is molecular hydrogen, which was formed upon formate decomposition by FHL complex [5].

E. coli BW25113 or MC4100 wild type parental strains growth, ORP kinetics and H_2 production by bacterial cultures were investigated upon glycerol utilization in MSM at the pH of 5.5–7.5: during glycerol fermentation on nutrient poor media bacteria managed to change the ORP of the medium from positive to negative values at all pHs mentioned, indicating that reductive conditions were important for bacterial metabolism under anaerobic conditions. Moreover, it was shown in the study that sole glycerol might be sufficient for bacterial biomass formation and H_2 production when other nutrients are not available. Even though H_2 production was delayed upon bacterial growth on MSM, it was stimulated when observed. In addition, compared to pH 5.5 and pH 6.5, both biomass and H_2 yields were maximal at pH 7.5 during anaerobic growth. pH 7.5 probably serves more sufficient (reliable) buffer capacity for bacterial metabolism in MSM, although, earlier was mentioned that pH 6.3 is optimal for *E. coli* glycerol fermentation [4]. Thus, the results point out that formate metabolism is significant for *E. coli*, particularly under energy-restricted conditions, and bacteria possibly cope with nutrient stress by stimulating formate production or FHL activity, which leads to increased H_2 and CO_2 production; excess of CO_2 might be used to enhance the bacterial biomass formation [23].

According to literature, formate initially is extruded from bacterial cells when produced and can be imported back into the cells and affect H_2 metabolism [16,17]. So, the next step of our study was to investigate the formate influence on glycerol metabolism in nutrient rich PM, during wild type and Hyds and FHL defective mutant's growth. Compared to bacterial growth on MSM, bacterial specific growth rate and biomass in PM were obviously much more enhanced (see Fig. 1), as shown before [23]. With the help of FHL defective *E. coli* mutant strain (*selC*) (see Fig. 3), affecting formate decomposition, the essential role of FDH-H in H_2 metabolism was stated pointing out that formate might be the only source of H^+ and e^- for H_2 formation under certain conditions. This is likely to that FDH-H was required for H_2 production during glucose fermentation [25]. Moreover, the metabolism of *E. coli* upon externally supplemented formate was examined in details in experiments with only functional Hyd 4 (triple mutant with defects in Hyds 1 to 3) and functional Hyd 4 and 3 (double mutant defective in Hyd 1 and 2). The results confirmed the important role of Hyd-3 in bacterial growth at both pH 6.5 and pH 7.5; in addition, they pointed 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.

H_2 production from glycerol by bacteria becomes one of the main ways in hydrogen energy strategy [29–31] when its enhancement by co-fermentation of different carbon sources such as formate could be of significance.

Conclusions and significance

The *E. coli* BW25113 or MC4100 wild type parental strain growth, medium acidification, ORP change and H_2 production by bacterial cultures were first examined during up to 72 h bacterial growth on glycerol in MSM at different pHs. Surprisingly, bacteria showed high H_2 producing activity, particularly at pH 7.5. Critical role of formate metabolism, particularly under energy limited conditions (MSM) was suggested. So, formate influence on growth properties and H_2 producing activity of *E. coli* during glycerol fermentation in batch culture in different nutrient media (MSM and PM) were also investigated in details using different mutants with inactivated Hyd enzymes and FHL at pH 6.5 and pH 7.5. The results showed that formate might be the only source of reducing equivalents for H_2 production by *E. coli* during glycerol fermentation. Moreover, the findings of the experiments when H_2 cycling was disrupted by inactivation of Hyd 1 and Hyd 2 (double mutation) or Hyd 1, Hyd 2 and Hyd 3 (triple mutation) pointed out that each Hyd (2, 3, 4) is essential for proper H_2 metabolism, mainly in H_2 formation upon glycerol fermentation particularly at pH 7.5.

The results obtained are novel and might add knowledge about H_2 metabolism upon bacterial co-substrate utilization and, in addition, be useful in biotechnology to control and enhance H_2 bio-production.

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