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Hydrogen production by *Escherichia coli* during glucose fermentation: Effects of oxidative and reductive routes used by the strain lacking hydrogen oxidizing hydrogenases 1 (*hya*) and 2 (*hyb*)

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ABSTRACT

Changes in oxidation–reduction potential (ORP) values during *Escherichia coli* Δ*hyaB* Δ*hybC* double mutant lacking of large subunits of hydrogenases 1 (Hyd-1) and 2 (Hyd-2) and effects of redox reagents were studied upon glucose fermentation at acidic (5.5) and alkaline (7.5) pHs. H_2 production yield was determined by the readings of platinum and titanium-silicate electrodes. Specific growth rate of mutant was inhibited by ~1.3 fold at pH 5.5 in the presence of membrane-permeating reducing agent DL-dithiothreitol (DTT) and impermeable oxidant potassium ferricyanide ($K_3[Fe(CN)_6]$), whereas at pH 7.5 the reagents had no significant effects. H_2 production was stimulated by ~1.4 fold with mutant compared to the wild type at pH 7.5. Moreover, 3 mM DTT stimulated H_2 production yield by ~2 and ~4 fold during bacterial log growth phase at pH 5.5 and pH 7.5, respectively. While at pH 5.5 1 mM $K_3[Fe(CN)_6]$ completely inhibited H_2 formation by mutant as well as wild type resulting in alternation of ORP to positive values (200 ± 10 mV) at the end of log growth phase. At pH 7.5H_2 formation by the wild type was inhibited by ~1.2 fold, while in the case of mutant H_2 formation was completely inhibited, but resulting in decrease of ORP to negative values (−270 ± 12 mV) at the end of log growth phase. These findings point out the effects of lacked Hyd-1 and Hyd-2 both on H_2 production by *E. coli* and indicate the importance of reductive conditions for enhanced H_2 production as well.

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Nomenclature

[ox]	concentration of oxidized compounds
[red]	concentration of reduced compounds
DTT	DL-dithiothreitol
E_0	standard ORP, mV
E_h	readings of Ti–Si electrode
E_h'	readings of Pt electrode
F	Faraday constant, 9.64853399(24) × 10^4C mol^{-1}
Hyd	hydrogenase
$K_3[Fe(CN)_6]$	potassium ferrycyanide
$K_4[Fe(CN)_6]$	potassium ferrocyanide
n	number of transferred electrons
ORP	oxidation–reduction potential
Pt	platinum
R	Universal gas constant, 8.3144621, JK^{-1} mol^{-1}
T	Absolute temperature, 273 K
Ti–Si	titanium – silicate

Introduction

The possible use of microorganisms for production of molecular hydrogen (H_2) as a future ecologically clean, renewable and effective energy source [1,2] makes H_2 metabolism a promising field of research. Many microorganisms can produce H_2 by reactions linked to their energy metabolism: the bacterium *Escherichia coli* is among H_2 producing bacteria performing mixed-acid fermentation of sugars (glucose) or glycerol [3].

H_2 is produced during formic acid oxidation and its production activity depends on operation of special enzymes – hydrogenases (Hyd) which catalyze the reversible oxidation of H_2 into two protons ($2H^+$) and two electrons ($2e^-$): $2H^+ + 2e^-$-> H_2. The latter is coupled by energy-conserving electron transfer chain reactions that allow energy to be obtained either from H_2 or from the oxidation of substrates with lower potential [4]. The existence of multiple Hyd enzymes within a living organism allows the organism to meet its energy need best: *E. coli* possesses four reversible [NiFe]-Hyd enzymes mainly with H_2 oxidizing (Hyd-1 and Hyd-2) and producing (Hyd-3 and Hyd-4) activities [5]. Reversibility of *E. coli* Hyd enzymes depends on carbon source and growth medium pH: Hyd-1 (*hya*) and Hyd-2 (*hyb*) can operate in H_2 oxidizing or producing mode upon glucose or glycerol fermentation, respectively [6,7]. It was demonstrated that maximal expression of *hya* gene appears at acidic pH [8] and in the presence of formate [9] and *hyb* operon expression – at alkaline pH (Table 1) [10]. During glucose fermentation Hyd-3 (*hyc*) and Hyd-4 (*hyf*) operate in H_2 producing mode [11,12], while upon glycerol utilization they work in reverse mode [6,7]. H_2 recycling across the bacterial membrane due to four Hyd enzymes operation is proposed [3,13,14].

The metabolism of bacteria is a complex of oxidation–reduction processes which can be affected and regulated by environment oxidation–reduction potential (ORP). ORP is suggested to be one of the most important physicochemical parameters for microbial community [15]. Sometimes ORP can be one of the most complex indicators of the physiological state of microbial cultures and even its measurement could be a practical means for the qualitative and quantitative determination of the microbial contamination [16], or, to discriminate among species of bacteria [17]. Indeed, it has been shown that Hyd enzymes activity can be regulated by ORP [18]. Hyd-2 activity is determined in a more reduced environment [13,19,20] and it was absent under aerobic conditions [21]. During the bacterial growth, ORP of the medium decreases: it was clearly demonstrated the decline of ORP to negative values (−550 ± 12 mV) and lowering medium pH during fermentation by *E. coli* and the other bacteria [22–27]. In the assay mixture without bacteria ORP (E_h) dependence on pH in two-based system is determined by the equation (1):

$$E_h = E_0 + (RT/nF)\ \ln([ox]/[red]) + (RT/nF)\ \ln[H^+] \quad (1)$$

(E_0 – standard ORP, R-universal gas constant, T-absolute temperature, F-Faraday constant, [ox] and [red] are concentrations of oxidized and reduced compounds and n-number of transferred electrons) following E_h decrease with increasing of pH [15]. Because of complexity of processes under fermentation of different substrates ORP decrease could not be expressed by the equation above. ORP's correlation with bacterial growth medium acidification is very significant and it is not studied well. However ORP may affect membrane-associated enzymes like Hyd and regulate their activity [18,21]; however the mechanisms of this action are not clear.

Thus, in the present study the relationship of ORP with Hyd-1 and Hyd-2 enzymes was studied. During *E. coli* wild type and double mutant with defective Hyd-1 and Hyd-2 growth ORP change and H_2 production regulation were investigated upon glucose fermentation at acidic and alkaline pHs. ORP was manipulated by redox regents such as $K_3[Fe(CN)_6$ and DTT, and determined using two different redox electrodes [26,28]. The double mutant strain compared to the wild type demonstrated higher specific growth rate. Moreover, it was stated that H_2 production decreased in more oxidative condition, while reductive one stimulated the production of H_2 at both acidic and alkaline pHs.

Table 1 – Comparison of *E. coli* Hyd-1 and Hyd-2 enzymes characteristics upon glucose fermentation.

Characteristics	Hyd-1	Hyd-2	References
Genes or operon	*hya*	*hyb*	[3,4]
Structure (subunits)	3	4	[3,4]
Operation mode	H_2 uptake	H_2 uptake	[3,14]
ORP	Reducing conditions	Oxidizing conditions	[19–21]
$[pH]_{out}$	acidic	alkaline	[8,10]

Materials and methods

Bacterial strains and growth conditions

The *E. coli*BW25113 (*lacI*^{q} *rrnB*$_{T14}$ *ΔlacZ*$_{W116}$ *hsdR514 ΔaraBAD*$_{AH33}$ *Δrha BAD*$_{LD78}$) wild type and MW1000 (BW25113*ΔhyaB ΔhybC*) mutant with defective Hyd-1 and Hyd-2 [29] were used. The mutant was provided by Prof. T. Wood (Pennsylvania State University, University Park, PA, USA).

Bacteria were grown overnight in batch culture under anaerobic conditions at 37 °C in peptone rich medium (20 g/L peptone, 2 g/L K_2HPO_4, 5 g/L NaCl) containing 2 g/L glucose at acidic (5.5) and alkaline (7.5) pHs. 2% of inoculums were transferred into the fresh peptone medium (150 ml) with 2 g/L glucose supplemented at appropriate pH.

During bacterial growth samples were taken and ORP, pH, and bacterial growth rate were determined. The pH was measured by a pH-meter with 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 and 0.1 N HCl. Bacterial growth was monitored by measuring optical density (OD) with a spectrophotometer at the wavelength of 600 nm and specific growth rate was determined as described [18,30].

ORP measurements and H_2 production assays

ORP was determined by using redox Pt (EPB-1, GSEEE; or PT42BNC, HANNA Instruments, Portugal) and Ti–Si electrodes (EO-02, GSEEE); Ag/AgCl (saturated by KCl) electrode was as reference electrode as described earlier [6,25,28].

Drop of ORP to low negative values measured by Pt electrode occurs mainly by the production of H_2, because ORP of −420 mV is characterized for the H_2 formation reaction. In contrast to Pt electrode which is sensitive to H_2 (or oxygen), Ti–Si electrode measures the overall ORP and is not affected by the presence of H_2 (or oxygen) that allows H_2 determination under anaerobic conditions (in the absence of oxygen). The H_2 yield was calculated by the decrease of ORP to low negative values as described by Piskarev et al. [31] and expressed in mol L^{-1}.

Before assays ORP of two electrodes were calculated in the control solution (the mixture of 0.049 M $K_3[Fe(CN)_6]$ and 0.05 M $K_4[Fe(CN)_6]\cdot 3H_2O$, pH 6.86) according to the manufacturer's instructions. E_h'(readings of Pt electrode) and E_h (readings of Ti–Si electrode) at 25 °C were of 245 ± 10 mV.

Note, no significant differences between E_h' and E_h were detected during H_2 assays in bacterial suspension without carbon source added; bacterial count alteration in the suspension by ~8–10-fold had no marked effect on E_h' and E_h. Moreover, a direct correlation between E_h and H_2 production was shown that was not affected by salt concentration. The addition of H_2 had no effect on the external pH as well [31].

Reagents and data processing

DTT, agar, meat peptone, $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]\cdot 3H_2O$ were from Sigma (USA), other reagents of analytical grade were used.

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

Results and discussion

The acidification of E. coli growth medium and ORP changes

Bacterial growth medium acidification and ORP changes were simultaneously investigated during growth of wild type BW25113 and Δ*hyaB*Δ*hybC* double mutant MW1000 with defective Hyd-1 and Hyd-2 as described (see Materials and methods). Specific growth rate for wild type was higher at alkaline pH (0.88 ± 0.05 h^{-1}), compared to acidic one (0.66 ± 0.05 h^{-1}) (Fig. 1). Interestingly, in contrast to wild type, mutant demonstrated ~1.6 and ~1.4 fold higher specific growth rate at pH 5.5 and pH 7.5, respectively (Fig. 2).

Bacterial growth was investigated upon redox reagents supplementation: specific growth rate for mutant was decreased by ~1.3 fold at acidic pH in the presence of 3 mM reducer DTT and 1 mM oxidizer $K_3[Fe(CN)_6]$ compared to control sample (without supplementation of reagents) whereas at alkaline pH the mentioned reagents had no significant effect.

It was shown that from the beginning of lag growth phase drop of external ORP followed by medium acidification was observed at acidic (5.5) and alkaline (7.5) pHs both in wild type and mutant strains. Acidification occurs due to mixed-acid fermentation of glucose by *E. coli* as suggested [3]. Almost from the beginning of log growth phase H_2 production was detected by the pair of Pt and Ti–Si electrodes (see Materials and methods): in double mutant H_2 production was stimulated by ~1.3 fold compared to wild type (data not shown). These results confirm that Hyd-1 and Hyd-2 operate in H_2 oxidation mode during glucose fermentation at both pHs and

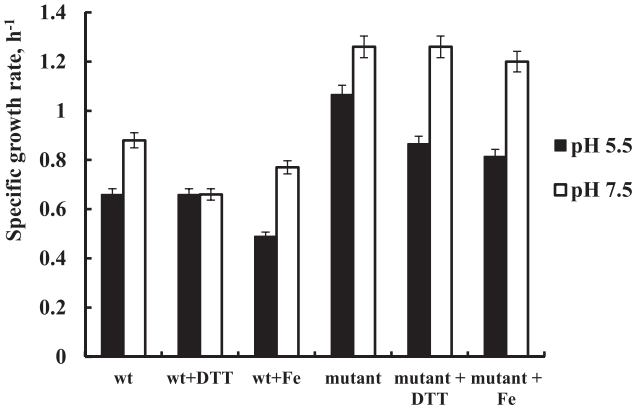

Fig. 1 – Specific growth rates of *E. coli* wild type (BW25113, wt) and MW1000 (BW25113 *ΔhyaB ΔhybC*) double mutant with defective Hyd-1 and Hyd-2 and effects of redox reagents at different pHs. Bacteria were grown under fermentation of glucose (0.2%) at different pHs; 1 mM $K_3[FeCN_6]$ (Fe) or 3 mM DTT was added into the growth medium when shown. For the others, see Materials and methods.

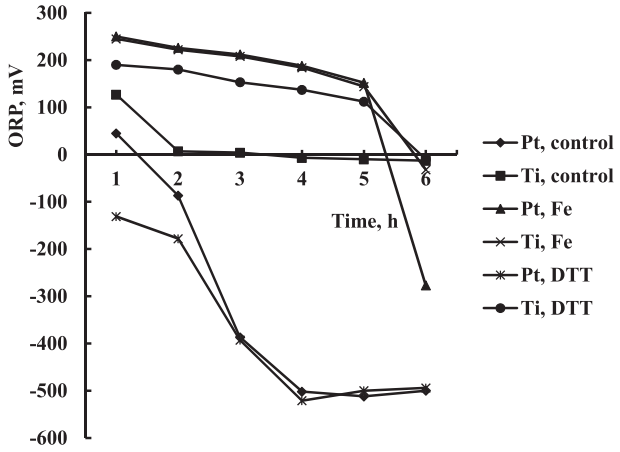

Fig. 2 – ORP changes during *E. coli* MW1000 (BW25113 *ΔhyaB ΔhybC*) double mutant growth upon glucose fermentation and the effects of redox reagents at pH 7.5. 1 mM $K_3[FeCN_6]$ (Fe) or 3 mM DTT supplemented to the growth medium. Control was bacterial growth in the medium without redox reagents. Averages of 3 separate experiments are presented.

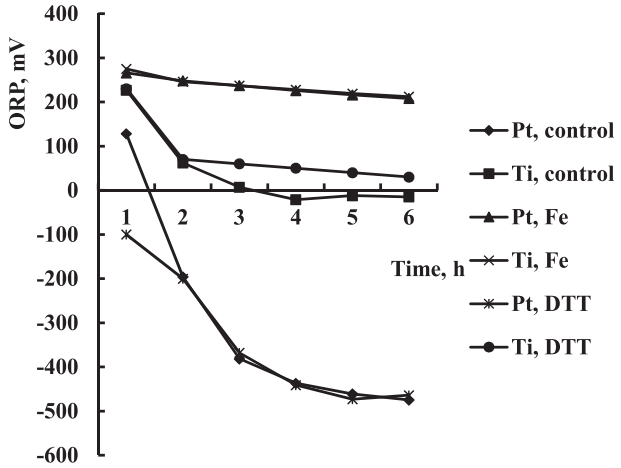

Fig. 3 – ORP changes of *E. coli*MW1000 (BW25113 *ΔhyaB ΔhybC*) double mutant upon fermentation of glucose and effects of redox reagents at pH 5.5. For details see legends to Fig. 2.

their absence can enhance H_2 production by *E. coli* [3,5,7,18–21].

Redox reagents effects on ORP changes by E. coli *mutant lacking both Hyd-1 and Hyd-2*

ORP is suggested to be valuable for monitoring changes in metabolic state of anaerobic bacteria and for optimizing yield of aerobic and anaerobic fermentation end products [15]. Bacterial anaerobic growth and H_2 formation regulation can be investigated by manipulating ORP with oxidizers and reducers: oxidizer $K_3[Fe(CN)_6]$ in low concentration determines positive values of ORP, suppresses *E. coli* growth and decreases acidification of the medium [28]. On the other hand, the environment restored conditions adjusted with DTT, which suppresses the bacterial growth and may result in increased formation of formic acid by *E. coli* [32].

ORP change was determined during growth of *E. coli* double mutant with defective Hyd-1 and Hyd-2 upon glucose fermentation in the presence of 3 mM reducer DTT and 1 mM oxidizer $K_3[FeCN_6]$ at both acidic and alkaline pHs (Figs. 2 and 3). As was mentioned above, redox regents changed external ORP since oxidizer set positive (+230 ± 10 mV) ORP value, while reducer – negative one (−130 ± 10 mV).

H_2 production was assayed (see Materials and methods): in mutant the difference between Pt and Ti–Si electrodes readings was higher compared to the wild type upon DTT supplementation at both acidic and alkaline pHs as demonstrated (Figs. 2 and 3). Thus, in mutant H_2 production yield was stimulated by ~2 fold and ~4 fold during bacterial log growth phase at pH 5.5 and pH 7.5, respectively (Table 2).

While, at pH 5.5 1 mM impermeable oxidant $K_3[Fe(CN)_6]$ completely inhibited H_2 formation in mutant and wild type resulting in a change of ORP to positive values (+200 ± 10 mV) at the end of log growth phase (see Figs. 2 and 3). At pH 7.5 H_2 formation was inhibited by~1.2 fold in wild type (data not shown), while in mutant H_2 formation was completely inhibited, but resulting in a decrease of ORP to negative values (−270 ± 12 mV) at the end of log growth phase (see Table 2).

Effects of DTT on H_2 production by bacteria may be due to its direct or indirect influences on accessible thiol groups of H_2 metabolizing key enzymes, and thus regulate their activity. On the other hand, it was demonstrated that DTT supplementation may lead to the greater formation of formic acid, one of the end products during glucose fermentation [32] thus is resulting in the higher H_2 yield.

One of the promising fields of future clean energy technologies is the bio-hydrogen production by bacteria. Different groups of scientists have investigated metabolic engineering approaches to improve fermentation and to enhance H_2 production in *E. coli* [3]. Based on these statements made, obviously four Hyd enzymes of *E. coli* together have the potential to form a H_2 cycle across the bacterial membrane [3,21]. Disturbance of the cycle due to lacking of two oxidizing Hyd enzymes instead one might be more informative.

Table 2 – Oxidizer and reducer effects on H_2 yield by *E. coli* *ΔhyaBΔhybC* double mutant during log phase growth upon glucose fermentation at different pHs.

Growth medium pH	H_2 yield, mol L^{-1}		
	Control[a]	DTT[b]	$K_3[FeCN_6]$[b]
5.5	0.80 ± 0.01	1.45 ± 0.04[b]	0.00[c]
7.5	1.50 ± 0.03[d]	5.60 ± 0.05[b]	0.00

[a] Control was without reagents supplementation.
[b] 3 mM DTT and 1 mM$K_3[FeCN_6]$ were added into the growth medium.
[c] H_2 was not produced.
[d] H_2 yield after 3–4 h of growth of bacterial culture.

Conclusions

In this paper we concluded that (i) *E. coli* double mutant lacking both Hyd-1 (*hya*) and Hyd-2 (*hyb*) demonstrated ~1.6 fold and ~1.4 fold higher specific growth rates at pH 5.5 and pH 7.5, respectively; (ii) reducer DTT stimulated H_2 yield by ~2 fold and ~4 fold with the mutant during bacterial log growth phase at pH 5.5 and pH 7.5; (iii) oxidizer $K_3[Fe(CN)_6]$ completely inhibited H_2 production in the mutant. Thus, the effects of *hya* and *hyb* double mutations on bacterial growth and on the onset of H_2 production during growth under reducing conditions were stated.

This study revealed that H_2 production in *E. coli* upon glucose fermentation is regulated by ORP and reductive conditions, which are essential for H_2 production. This approach may have industrial advantageous application to regulate the bio-hydrogen production.

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