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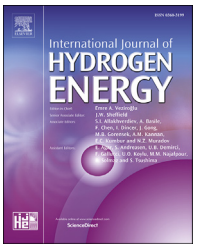

Hydrogen production by *Escherichia coli* growing in different nutrient media with glycerol: Effects of formate, pH, production kinetics and hydrogenases involved

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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.

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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 (Δp/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.

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