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H_2 production by *Escherichia coli* batch cultures during utilization of acetate and mixture of glycerol and acetate

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ABSTRACT

Escherichia coli produces molecular hydrogen (H_2) from different carbon substrates like sugars, glycerol and some organic acids during mixed-acid fermentation. H_2 production in batch cultures during acetate utilization of different concentrations (1 g L^{-1}–5 g L^{-1} was used) was investigated at different pHs. H_2 yield in *E. coli* wild type cells was highest at the log growth phase in the presence of 5 g L^{-1} acetate. Either at pH 7.5 or pH 6.5 H_2 yield was 5.07 mmol L^{-1}. At pH 5.5, H_2 production was detected when 1 g L^{-1}acetate was supplemented in the growth medium. Interestingly, in the presence of 1 g L^{-1} acetate H_2 yield was highest at pH 6.5 compared to 2 and 5 g L^{-1} concentrations. As acetic acid and glycerol are unavoidable constituents of various industrial or agricultural wastes, H_2 generation using the mixture of acetate (5 g L^{-1}) and glycerol (10 g L^{-1}) at different pHs was also investigated. The highest H_2 yield of 5.16 mmol L^{-1} was detected at the log growth phase at pH 7.5. H_2 generation was continuously detected at pH 7.5 and pH 5.5 for 96 h of growth. These data can be applied to further enhancement of H_2 energy production biotechnology and to use different industrial or agricultural wastes where acetate and/or glycerol is present to produce H_2.

Introduction

The substantial shrinkage of natural gas and oil anticipates the exploration of suitable alternative and renewable energy sources. One of these sources is dihydrogen (H_2), which can be evoked by diverse microbial or other biomasses and is perspective alternative to petroleum-based processes [1]. Conversion of sugars, glycerol or organic carbon-containing industrial, agricultural or water wastes to H_2 by microbial fermentation has been affirmed, and the description and engineering of these bioprocesses have been already developed. Co-fermentation or co-utilization of different carbon sources by various bacteria have been featured [2–6] but dependence of H_2 formation on different carbon sources is not clear yet, therefore investigation to disclose a cheap and effective one are highly pertinent.

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In 2006 it has been discovered that glycerol can be utilized anaerobically by *Escherichia coli* at acidic [7,8] and further at alkaline [9] pHs. This is essential because crude glycerol or glycerol-containing wastes are very cheap and readily available [10,11]. Furthermore, development of H_2 production technology currently trends towards the use of inexpensive substrates for producing valuable fuels and chemicals from different organic acids such as formate, acetate, lactate or sugars like lactose, xylose which can be found in miscellaneous industrial or agricultural wastes [12,13].

Acetate and formate are major mixed-acid fermentation end products of *E. coli*. The high concentration of these acids inhibits bacterial cells growth [14]. The production of these two acids reaches maximal concentrations during anaerobic conditions which are an essential concern for large-scale industrial production using bioreactors [15]. During glucose fermentation by *E. coli* up to one-third of this carbon can be converted to formate reaching a concentration of ~20 mM [16]. H_2 production by *E. coli* from formate has been shown clearly [17–19].

Acetate or acetic acid is widely present at hydrolysates generated from hemicellulose and lignin. Acetate in concentrations >5 g L^{-1} has inhibitory effect on the growth of *E. coli* and also reduces ethanol production by *E. coli* and *Sacharomyces cerevisiae* [20,21]. It has been shown that in the mixture of different carbon source, especially glucose and xylose, *E. coli* was engineered to remove acetate under aerobic conditions [22]. Moreover, in industrial production acetate is mainly formed, and problem is to remove and use acetate for producing various valuable chemicals and fuels.

H_2 has been well established to be produced by multiple hydrogenases (Hyd), which reversibly oxidize H_2 to $2H^+$. A large membrane-associated complex combining a hydrogenase with a formate dehydrogenase (FDH) is embodied in the formate hydrogenlyase (FHL) complex. *E. coli* forms two FHL complexes which are active depending on pH [17,18,23]. Hyd-3, encoded by the *hyc* operon, together with FDH-H forms the FHL-1 complex while Hyd-4, encoded by *hyf* operon, together with FDH-H is suggested to form the FHL-2 complex [17,18,23]. H_2 is mainly produced by FHL complex from formate [17,18,23] which further oxidizes formate to H_2 and CO_2. The other organic acids namely acetic acid which is generated as a by-product from mixed-acid fermentation has been not investigated for the production of H_2 by *E. coli*.

The main goal of the present work, therefore, was to study H_2 production by *E. coli* during fermentation in the presence of acetate and the mixture of acetate and glycerol at different pHs. This would be important for the removal and usage of acetate for a two- or multi-stage H_2 production biotechnology using glycerol and/or acetate as cheap substrates for valuable fuel production.

Materials and methods

Bacterial strain and growth

The *E. coli* wild type strain BW25113 from the Keio collection was used [24].

Bacteria from an overnight culture were transferred into the buffered peptone liquid medium (20 g L^{-1} peptone, 15 g L^{-1} K_2HPO_4, 1.08 g L^{-1} KH_2PO_4, 5 g L^{-1}NaCl) with sodium acetate added in a range of 1 g L^{-1}–5 g L^{-1} and/or glycerol 10 g L^{-1}, as carbon sources, at different pHs. Bacteria were grown in batch cultures under anaerobic fermentative conditions at 37 °C, as described in literature [4,25]. To achieve anaerobic conditions glass vessels with plastic press caps were used; O_2 and N_2 were bubbled out of the media by autoclaving, after which the vessels were closed by press-caps. The pH was determined by a pH-meter with a selective pH-electrode (ESL-63-07, Gomel State Enterprise of Electrometric Equipment (GSEEE), Gomel, Belarus; or HJ1131B, Hanna Instruments, Portugal) and adjusted using 0.1 M NaOH or HCl.

Measurement of redox potential and hydrogen production assays

Redox potential (ORP) determination and H_2 production assays were done by using a pair of redox, titanium–silicate (Ti–Si) (EO-02, GSEEE) and platinum (Pt) (EPB-1, GSEEE; or PT42BNC, Hanna Instruments, Portugal) electrodes, as described previously [4,17,18,25–27]. H_2 production rate (V_{H2}) was calculated from the difference between the initial decreases in Pt- and Ti–Si-electrodes readings per time and expressed in mol H_2 per min per g dry weight (mol min g dw)$^{-1}$; calibration of ORP decrease with H_2 was done as described recently by Piskarev et al. [28]. The H_2 yield was calculated by the marked decrease of ORP to low negative values in liquid and expressed in mol H_2 per L of growth medium (mol L^{-1}); calibration of ORP decrease with H_2 was done as above. These expressions were presented for V_{H2} and H_2 yield in batch cultures in many papers [29–31].

The H_2 production assays were performed in the assay buffer (150 mM Tris-phosphate (appropriate pH mentioned) containing 0.4 mM $MgSO_4$, 1 mM NaCl and 1 mM KCl) upon glycerol supplementation to bacterial suspension. In the assays, glycerol was supplemented at the same concentrations as added to the growth medium.

Others, reagents and data processing

Preparation of whole cells for H_2 production assays was described before [4,17,18,25–27]. The assays were performed in a thermostatically controlled chamber at 37 °C; bacterial suspension in the closed vessel was mixed gently with a magnetic stirrer bar. Dry weight of bacteria was determined as described previously [4,32].

Agar, glycerol, peptone, sodium acetate, Tris (Carl Roths GmbH, Germany), and the other reagents of analytical grade were used.

Each data point represented is averaged from independent triplicate cultures; the standard deviation, calculated as described [4,26,32], was not more than 3% if they are not represented. The validity of differences between experimental and control data is evaluated by Student's criteria (p) [33]; $p < 0.01$ or less if this is not represented, otherwise $p > 0.5$ if the difference is not valid.

Results and discussion

Role of mixture of acetate and glycerol or acetate alone in E. coli growth at different pHs

To investigate the growth of *E. coli* on acetate in the buffered peptone medium *E. coli* wild type cells were grown on glycerol and/or acetate. At first, *E. coli* BW25113 were grown on acetate with 1, 2 or 5 g L^{-1} concentrations as a sole carbon source added into the peptone medium at slightly alkaline (pH 7.5) and slightly acidic (pH 6.5) pHs (Fig. 1). According to available reports, acetate exceeding 5 g L^{-1} concentration might be an inhibitor of cell growth and acts as uncoupler [34]. When wild type cells were grown at pH 7.5 under anaerobic conditions on 1 g L^{-1} acetate as sole carbon source at early exponential phase the specific growth rate (μ) was 0.35 ± 0.03 h^{-1}. The same result was obtained at pH 6.5 (see Fig. 1). In comparison, cells grown on formate alone either at pH 7.5 or pH 6.5 had ~3 fold higher μ, compared to the cells grown on acetate only. Moreover, in the cells grown in the same conditions but in the presence of 2 g L^{-1}, acetate did not change the growth rate. In addition, cells grown at acidic (pH 5.5) pH had μ of 0.15 ± 0.01 h^{-1} which was lower ~2.3 fold (see Fig. 1). This might be due to acidic environment and acetic acid, which can suppress the cell growth due to uncoupling effects by dissipation of the transmembrane pH gradient (ΔpH) [34,35], as suggested with formate [35]. At pH 7.5 μ was 0.22 ± 0.01 h^{-1} (see Fig. 1). The same growth rate was achieved with *E. coli* cells under aerobic conditions in the presence of 10 g L^{-1} acetate [22]. After ~10 h of growth cells were able to consume all acetate at μ of 0.23 ± 0.01 h^{-1} [22]. The decrease of pH resulted in less μ, and at pH 6.5 μ was ~1.7 fold less, compared to the cells grown on 1 or 2 g L^{-1} acetate. Even worse cells grew at pH 5.5 with μ of 0.08 ± 0.01 h^{-1} (see Fig. 1). It is important to address that different groups were investigating acetate consumption by bacteria in the presence of various sugars e.g. glucose and xylose [22]. The critical growth inhibition at low pH might be due to combined effect of high concentration of acetate and acidic pH. The addition of external acetate did not affect the medium pH (data not shown), as it is buffered, and, thus, confirming the uncoupling effect.

As in different industrial and agricultural wastes not only acetic acid but also glycerol is present, it is of great interest to explore the effects of mixture of glycerol and acetate on growth of *E. coli* and, thus, use mixture for producing valuable chemicals, biofuels and other products. As it was shown before, optimal concentration of glycerol for growth was 10 g L^{-1} [30], and the highest H_2 yield was determined at 5 g L^{-1} acetate, the mixture of 10 g L^{-1} glycerol and 5 g L^{-1} acetate was taken to study. When cells were grown on acetate or on the mixture of acetate with glycerol, μ for tested pHs was the same (Fig. 2). This indicates that acetate inhibits cell growth even in the presence of other carbon source. These data are in good conformity with the results obtained previously showing that acetate inhibits cell growth at early exponential growth phase in the presence of glucose [36]. It is noteworthy that different groups have employed mainly pH 7.0 for acetate fermentation and optimized acetate removal or utilization by *E. coli* for neutral pH [14,36]. As we were interested in the converting acetate to H_2, wide range of pH 5.5–7.5 was applied (see Fig. 2). Moreover, this pH range is optimal for H_2 production by *E. coli* as Hyd enzyme is active at this pH range, which was described previously [23].

Effect of mixture of acetate and glycerol or acetate alone on H_2 production by E. coli in batch culture at different pHs

It was mentioned that *E. coli* produces H_2 during mixed-acid fermentation, and H_2 is produced by disproportionation of formate by FHL complex [23]. Previously it was shown that formate, produced during mixed-acid fermentation, can then import back into the cell via formate channels [37]. Moreover, externally added formate enhanced H_2 generation [17–19,37]. It is well known that besides formate one of the major fermentation end products is acetic acid. It is considered that acetate, like formate, accumulates in the external medium and then re-assimilates back once glucose is consumed [35]. To investigate if acetate is reused and transmuted to H_2, ORP change and appropriately H_2 evolution kinetics in *E. coli* batch

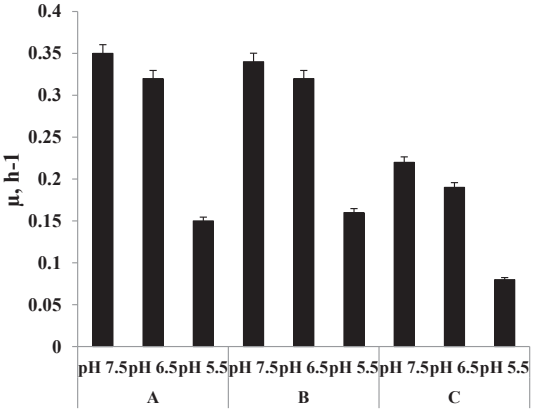

Fig. 1 – Specific growth rate (μ) of *E. coli* BW25113 grown in buffered peptone medium at 37 °C during concentration-dependent acetate utilization at different pHs. The cells were grown in the presence of 1 g L^{-1} (A); 2 g L^{-1} (B) and 5 g L^{-1} (C) acetate. For details see Materials and methods.

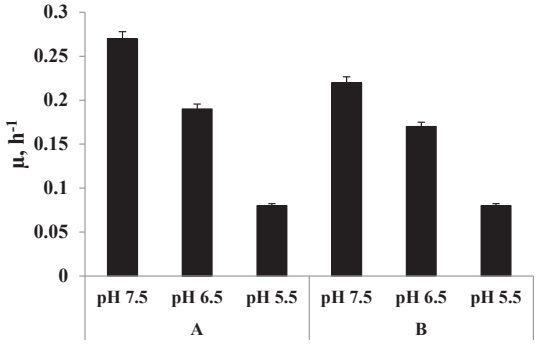

Fig. 2 – Specific growth rate (μ) of *E. coli* BW25113 grown in buffered peptone medium at 37 °C in the presence of 5 g L^{-1} acetate alone (A) or the mixture of 5 g L^{-1} acetate and 10 g L^{-1} glycerol (B) at different pHs. For others see Materials and methods.

cultures at different pHs in the presence of various concentrations of acetate (see Materials and methods) were studied (Fig. 3). The marked decreases of ORP to −450 mV up to −560 mV (see Fig. 3) have been shown to indicate H_2 production during cell growth in fermentative conditions (ORP for the couple $2H^+/H_2$ in standard conditions equals to −420 mV) [4,25–27,30,31]. H_2 production by *E. coli* was tracked for 48 h. At first, at all tested pHs ORP decreased to −140 ± 10 mV when *E. coli* BW25113 cells were growing in peptone medium (see Materials and methods) without acetate or glycerol added as sources of carbon. This indicated that *E. coli* wild type did not evolve H_2 from peptone. The latter might be explained by the absence of substrates for mixed-acid fermentation resulted in the formation of H_2. Indeed, the highest H_2 yield of 5.07 ± 0.15 mmol H_2 L^{-1} was detected at the early log growth phase with acetate concentration of 5 g L^{-1} at pH 7.5 and the late growth phase at pH 6.5 (Fig. 4). At pH 5.5 H_2 yield was residual (0.27 ± 0.01 mmol H_2 L^{-1}). When the cells were grown on 2 g L^{-1} acetate either at pH 6.5 or pH 7.5, the same H_2 yield (2.2 ± 0.07 mmol H_2 L^{-1}) was reached early at the exponential growth phase. This was less ~2.3 fold than that at the same pH but with 5 g L^{-1} acetate (see Figs. 3 and 4). In addition, at pH 5.5, H_2 evolution was detected only at the late stationary growth phase, and H_2 yield was 0.7 ± 0.02 mmol H_2 L^{-1} (see Figs. 3 and 4). *E. coli* grown at pH 7.5 and pH 5.5 in the presence of 1 g L^{-1} acetate resulted in the production of 1.36 ± 0.04 and 1.45 ± 0.04 mmol H_2 L^{-1} at the late exponential growth phase, respectively. This is a quite intriguing finding because at acidic pH, when 1 g L^{-1} acetate was employed, at the exponential growth phase H_2 production was ~3.9-fold more than at the same conditions but in the presence of 2 g L^{-1} acetate (see Figs. 3 and 4). This fact clearly demonstrated that substrate

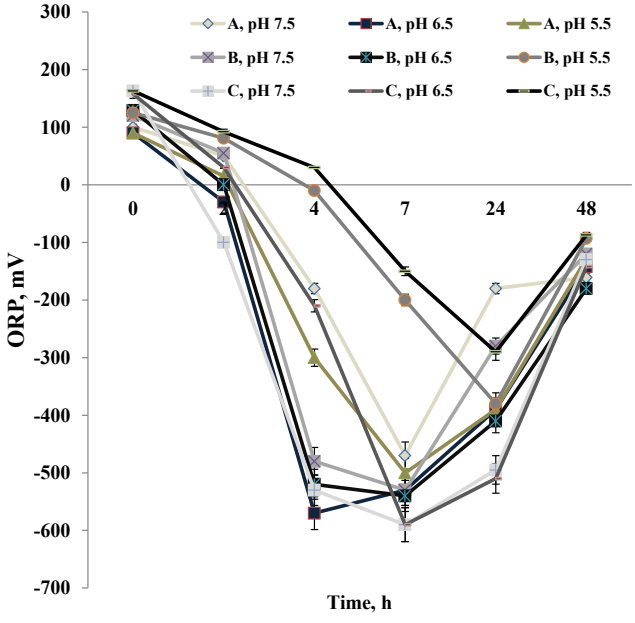

Fig. 3 – The ORP kinetics of *E. coli* BW25113 grown in buffered peptone medium at 37 °C in the presence of acetate at different pHs. ORP was measured by Pt redox electrode and expressed in mV vs Ag/AgCl (saturated by KCl). For others see legends to Fig. 1.

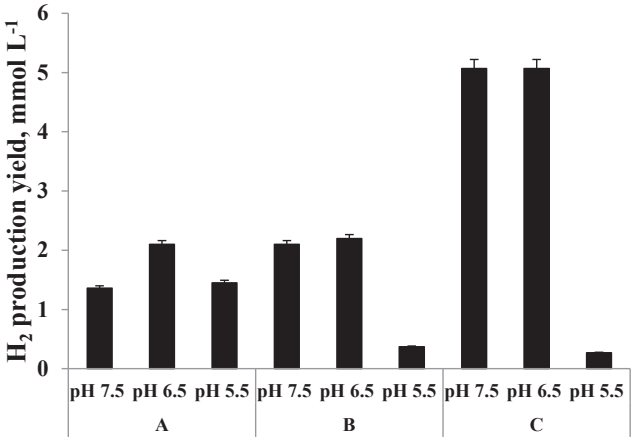

Fig. 4 – H_2 production by *E. coli* BW25113 in the presence of acetate at different pHs. For others see Materials and methods and legends to Fig. 1.

limitation resulted in a higher product formation. These data are similar to the outcome shown before that high concentration of glucose inhibits H_2 production [38]. Moreover, the uncoupling effect of acetic acid at pH 5.5 might result in low and delayed H_2 production. On the contrary, at pH 6.5, H_2 production was disclosed at early exponential growth phase and has similar level, as for the data obtained for 2 g L^{-1}. In overall, optimal pH for H_2 generation is pH 6.5 and optimal concentration of acetate is 5 g L^{-1}. Besides, the absence of H_2 evolution from peptone without acetate or glycerol added and H_2 production from peptone when acetate or glycerol was supplemented indicate assimilation of acetate or glycerol in the fermentative conditions used.

To further investigate H_2 production, the mixture of acetate and glycerol was taken, as in natural environment and in different wastes glycerol and acetate are among major byproducts. H_2 production was monitored for 96 h of growth. ORP decrease and appropriately H_2 production in the mixture of 5 g L^{-1} acetate and 10 g L^{-1} glycerol was delayed and detected at the late exponential growth phase, compared to the cells grown on acetate only (Figs. 5 and 6). This delay might be explained if acetate as uncoupler affects ΔpH [34,35] and, thus, intracellular pH. The addition of glycerol can maintain or stabilize intracellular pH, and delayed H_2 generation would occur, as in the presence of acetate as a sole carbon source *E. coli* are producing H_2 as one of the ways to get H^+ expelled from the cell. This prevents toxic effect of acidic intracellular pH and, therefore, H_2 production at early exponential growth phase is detected. At pH 5.5 H_2 production was first detected at the late stationary phase and yielded with 0.73 ± 0.02 mmol H_2 L^{-1} which was ~2.7-fold more, compared to the cells grown on acetate only (see Fig. 5). In addition, the cells grown in the presence of the mixture of acetate and glycerol were harvested and assayed for H_2 production. Interestingly, at pH 7.5 V_{H2} in glycerol supplemented assays was the same compared to the cells grown on glycerol only but at pH 6.5 V_{H2} was ~1.5-fold higher compared to the cells grown on glycerol (Fig. 6). This might be used in further pre-cultivation of the cells and enhanced H_2 production from glycerol.

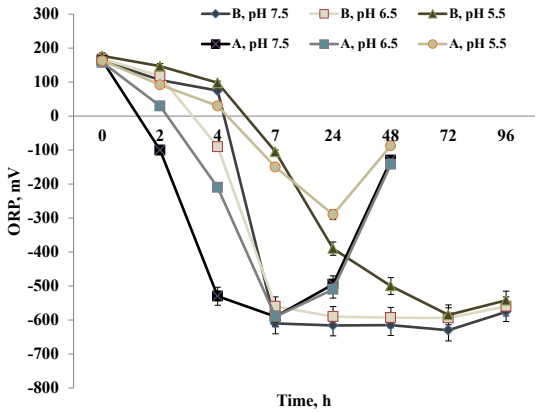

Fig. 5 – The ORP kinetics of *E. coli* BW25113 grown in buffered peptone medium at 37 °C in the presence of acetate or the mixture of acetate with glycerol at different pHs. For others see legends to Fig. 2.

Interestingly, H_2 production rate yield was not correlated with μ whereas both parameters depended on acetate or glycerol concentration (comp. Figs. 1 and 4 or Figs. 2 and 6). A higher H_2 yield at 5 g L^{-1} acetate or upon addition of glycerol might be explained by maintaining intracellular pH via reducing H^+ to H_2. Low intracellular pH has been determined for *E. coli* during glycerol fermentation [39]. Therefore, H_2 production can be a tool in regulating intracellular pH. This seems to be likely to the proposal of Trchounian and Sawers

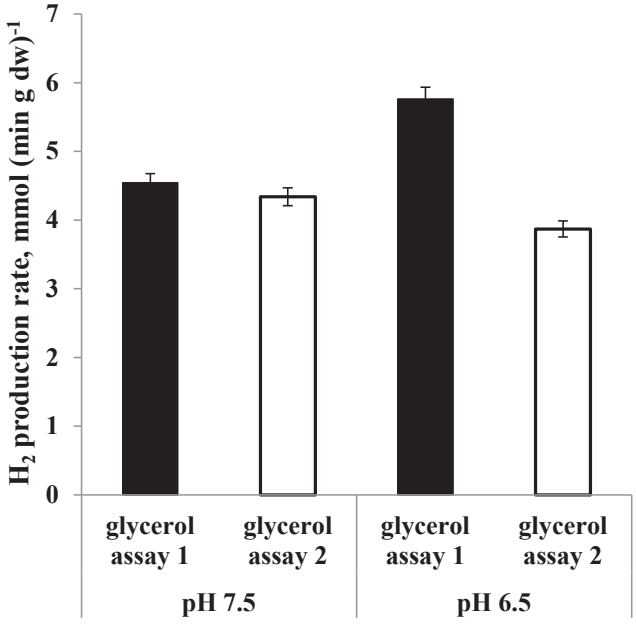

Fig. 6 – H_2 production rate (V_{H2}) in *E. coli* BW25113 upon supplementation of glycerol in the assays. Cells were grown under anaerobic conditions at 37 °C at pH 7.5 and pH 5.5. Glycerol assay 1 corresponds to the cells grown on acetate and glycerol; glycerol assay 2 corresponds to the cells grown on glycerol only. For others see legends to Fig. 2.

[40] that H_2 production and H_2 cycling can help to maintain proton motive force during fermentation depended on intracellular pH. A role of H_2 production in regulation of intracellular pH is a complex problem; a future study is required.

Thus, the production of H_2 by *E. coli* batch culture from acetate and its mixture with glycerol has been shown. This could be useful to produce H_2 from different organic substrates and to enhance H_2 production in multistep dark- and light-fermentative H_2 production biotechnology, as employed in the last years [1,31], or in microbial fuel cell, as developed recently [41,42].

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