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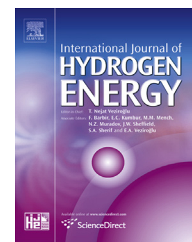

H_2 production by *Escherichia coli* batch cultures during utilization of acetate and mixture of glycerol and acetate

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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.

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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.

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