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Deeply mechanism analysis of hydrogen production enhancement of *Ethanoligenens harbinense* by Fe^{2+} and Mg^{2+}: Monitoring at growth and transcription levels

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ABSTRACT

For deeply understanding the H_2 production promotion mechanism by adding Mg^{2+} and Fe^{2+} in *Ethanoligenens harbinense* fermentation process, the effects on cell growth, liquid byproducts production and H_2 yield at growth level were investigated, meanwhile the expressions of [FeFe]-hydrogenase, acetate kinase, alcohol dehydrogenase and lactate dehydrogenase with quantitative reverse transcription PCR at transcript level were monitored. The experimental results indicated that Mg^{2+} makes more contributions on cell growth, but with Mg^{2+} concentration increasing the expressions of functional genes were obviously suppressed. Fe^{2+} has slightly positive effects on increasing cell growth and functional genes expression, which is inferred the reason of H_2 production enhancement. A maximum hydrogen yield of 2.14 mol-H_2/mol-glucose was obtained in optimal medium supplemented with 600 mg/l $MgCl_2 \cdot 6H_2O$ and 100 mg/l $FeSO_4 \cdot 7H_2O$. The realization of hydrogen production enhancement of *E. harbinense* by adding promoting factors was not only realized through increasing cell biomass growth and hydrogenase activity, but also some promotion effects on functional enzyme genes expressions.

Introduction

Hydrogen, regarded as a kind of clean, efficient and renewable energy carrier, can be produced in various methods, not only chemical-physical methods, but also biological processes [1–3]. Biological hydrogen production by microorganisms provides a natural avenue from renewable organic resources to usable hydrogen, which is much more environmentally friendly and economically [3–6]. Thus, studies were carried out on enhancing hydrogen production efficiency. Previous studies showed that the metal ions have significantly

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influence effects on hydrogen production functional enzyme activities [7]. Adding of ferrous ions enhances H_2 production, hydrogenase activity, substrate utilization and shortens acclimation time in a mixed cultures [8–10]. Ferrous iron has significant enhancing effect on hydrogen production in *Enterobacter* [11,12]. Besides ferrous iron, nickel and magnesium ions have also been found to have a certain role in promoting hydrogen production by *Carboxydothermus*, *Enterobacter* and *Rhodobacter* [13,14]. Although studies on relationships between metal ions and hydrogen production enhancement have been reported, the molecular mechanism on the simulation of H_2 production is unknown yet.

Ethanoligenens harbinense, an efficient hydrogen-producing bacterium at low pH, was the dominant functional population and the reason of ethanol-type fermentation [15–17]. In order to understand the physiology and metabolism of ethanol-H_2 co-production of *E. harbinense*, studies have been carried out at different levels, including studies of ecological factors optimizations, hydrogenase activity determination and functional gene cloning [18–21]. However, the ethanol-type hydrogen production mechanism is still unclear.

In dark fermentative hydrogen production bacteria, [FeFe]-hydrogenases (*hyd*As) are considered to be the key functional enzymes and play critical roles in microbial energy metabolism [22]. They have the best performing hydrogen producing biocatalysts, with turnover frequency of up to 10^4/s [23,24]. Thus, the gene sequences of *hyd*As were developed as biomarkers to analyze the relationships between H_2 production and operational parameters [17,25–27]. However, although acetate kinase (AK), alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH) were also considered as important hydrogen production functional genes, the investigations were mainly at the cellular level, and lacking for in-depth research. To date, there were quite a few studies focus on *hyd*A gene expression in *Clostridium* during hydrogen production processes [28–31]. To our known, the relationships among promoting factors, cell growth and hydrogen production functional gene expression, which will lead to a better knowledge of the mechanisms of hydrogen production enhancement for improving biotechnological applications, are lacking of investigations.

In this study, for a further understanding in ethanol-type hydrogen production mechanism, the effects of metal ions (ferrous and magnesium) on cell growth, hydrogen production and fermentative byproducts of *E. harbinense* were investigated at growth level. The expressions of *hyd*A, AK, ADH and LDH under different conditions were evaluated with qRT-PCR (quantitative reverse transcription PCR) at transcript level.

Materials and methods

Strain and culture

Hydrogen-producing strain YUAN-3, the type strain of *E. harbinense* used in this study, was maintained in SKLUWRE [15]. EH medium was used for routine culture of strain YUAN-3 as previous description [32]. The EH medium was supplemented with 0–1000 mg/l $MgCl_2 \cdot 6H_2O$ or 0–300 mg/l $FeSO_4 \cdot 7H_2O$ for investigation. The EH media without ferrous or magnesium ions was used as negative control (NC). Prior to inoculation (4% V/V), the strain was cultured until logarithmic growth phase in a thermostatic shaker at 35 °C. Batch culture test was performed in 100-ml anaerobic culture bottles with 50 ml of liquid EH medium in parallel [20,31]. Biogas was collected after 48 h cultivation using draining method [31,32].

Analyses methods

Hydrogen content and volatile fatty acids (VFAs) concentration in byproducts were measured using an Agilent gas chromatography (7890A) with capillary DB-FFAP for liquid products and GS-GASPRO for gas products. Cell biomass was measured as dry cell weight (DCW) after drying to constant weight at 105 °C [32]. Glucose utilization was measured with a glucose assay kit (Rongsheng Biotech, Shanghai, China) following the instruction's description.

Nucleic acid extraction

Genomic deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) extraction were carried out using a Bacterial DNA Mini Kit (Solarbio, China) and an RNeasy Protect Bacteria Mini Kit (Qiagen, USA), respectively, following the manufacturer's instructions. Dnase I was used to remove the residual DNA in the RNA extraction process. Concentration and purity of nucleic acids were determined in parallel at 260 nm and 280 nm using a Beckman DU 800 (Beckman, USA). The integrity of DNA or RNA was analyzed by 1% (w/v) agarose electrophoresis [31].

Quantitative reverse transcription PCR analysis

The specific primer pairs (Table 1) for *hyd*A I, *hyd*A II, ADH I, ADH II, AK and LDH of *E. harbinense* were designed based on

Table 1 – Primers for RT-PCR.

Gene	Primer	Sequence
*hyd*A I	RTF232	5′-GGCGAATCACTTGGTCTGC-3′
	RTR382	5′-CGGAGCTTTGCGAGGAACT-3′
*hyd*A II	EhydF383	5′-GCGACCTCCCGCTCGATACCAGC-3′
	EhydR577	5′-CGCAGCCCACGCAACCCACAT-3′
ADH I	Eadh1F664	5′-GGTGAGGTGCTGCCAAACTTGCT-3′
	Eadh1R878	5′-GCGGGCTGGTTGAACTTGAGGTA-3′
ADH II	Eadh2F457	5′-AATCCCGAAAACCACGACAAA-3′
	Eadh2R622	5′-GACCGATCCTGGAAATAAAGGC-3′
AK	EakF111	5′-CGGGCGGTTCAAGCACAAAAC-3′
	EakR319	5′-CGTCGGTCACCAGCACGGAGT-3′
LDH	EldhF533	5′-ACGGCGACAGTGAGTTTGTGC-3′
	EldhR891	5′-ATTTTCTTCCGGTGTGAGCGA-3′

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